Pergamon

Biochemical Pharmacology, Vol. 50, No. 8, pp. 1265–1272, 1995.

0006-2952/95 $9.50 + 0.00

0006-2952(95)-02012-2

DEBENZOYLATING AND DEACETYLATING ACTIVITIES OF RAT LIVER AND MAMMARY GLAND MICROSOMES

EFFECT OF OVARIECTOMY

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(*Received* 8 *February* 1995; *accepted* 27 *April* 1995)

Abstract—This study compared the rates of N-deacylations of *N*-hydroxy-*N*-2-fluorenylbenzamide (N-OH-2-FBA) with those of its analogue, *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-2-FAA), by the mammary gland (tumor target for both compounds) and the liver of female Sprague–Dawley rats and examined the effect of ovariectomy on these activities. N-Debenzoylation of N-OH-2-FBA was catalyzed by the mammary and liver microsomes of 50-day-old female rats at similar rates (~24 nmol/min/mg). The activity of both tissues increased (up to 1.8 times) after ovariectomy at 42, 32 and, especially, 22 days of age. The rapid hydrolysis appeared to be unique for the benzoyl group since N-OH-2-FAA was deacylated only ~0.05 and 0.004 times as fast by the liver and mammary microsomes, respectively, and these low rates were unaffected by ovariectomy. Since such substrate specificity would be of significance in the metabolism of xenobiotics and drug design, esterase activity and its sensitivity to ovariectomy at 22 days of age were examined with several acetylated and benzoylated substrates in the liver and mammary microsomes and compared with those of male liver. Tissues of rats of both sexes had a greater capacity to hydrolyze carboxyl esters than amides. Except for *N*-2-fluorenylacetamide (2-FAA) and *o*-nitrophenylacetate (o-NPA), all substrates were hydrolyzed by liver microsomes of the male up to 3.9 times faster than by those of the female. Microsomes of female liver hydrolyzed acetylated substrates 1.2 to 25 times faster than benzoylated analogues except for N-OH-2-FBA and benzamide. By contrast, mammary gland microsomes hydrolyzed benzoylated compounds 1.4 to 333 times faster except for 2-naphthyl benzoate. Respective rates of hydrolysis of o-NPA by microsomes of liver and mammary gland were 1.7 and 0.6 times those of p-NPA. After ovariectomy, deacylating activities increased (up to 1.6 times) except for those of 2-FAA and acetanilide. All deacylations were >98% inhibited by 0.1 mM paraoxon, indicating catalysis by serine hydrolases. The results suggest involvement of multiple carboxylesterases and indicate that certain benzoylated xenobiotics may have a greater effect on the mammary gland than acetylated xenobiotics because of their greater vulnerability to hydrolysis by esterases of mammary gland.

Key words: N- and O-debenzoylation; N- and O-deacetylation; rat liver; mammary gland; microsomal esterases; xenobiotic (carcinogen) metabolism

Tissue-specific effects of xenobiotics depend on many factors including the route of exposure, vehicle, and the presence of hepatic and extrahepatic activating or detoxifying enzyme systems. *N*-Arylhydroxamic acids (e.g. *N*-hydroxyfluorenamides) are systemic and local carcinogens in the rat [reviewed in Ref. 1]. After i.p. injections of aqueous suspensions of N-OH-2-FAA§ and especially its structural analogue, N-OH-2-FBA (Fig. 1), to male or female SD rats, highly invasive peritoneal tumors (pleomorphic sarcomas) developed, which indicated peritoneal serosa to be a target tissue [2, 3]. Systemic effects were manifested by tumorigenicities of both compounds for the mammary gland of female rats and of N-OH-2-FAA for the liver of male rats. Our recent studies showed that enzymatic activities of peritoneal serosa and liver of male SD rats and adducts isolated from serosal and hepatic DNA after treatment of rats with the *N*-hydroxyfluorenamides were consistent with activation of N-OH-2-FAA via *N,O*-acyltransferase-catalyzed formation of N-acetoxy-2-FA and activation of N-OH-2-FBA via a two-step sequence of carboxylesterase-catalyzed N-debenzoylation to N-OH-2-FA and acyltransferase-catalyzed O-acetylation of the latter to N-acetoxy-2-FA [4, 5]. The hydrolysis of N-OH-2-FBA catalyzed by carboxylesterases of male liver and peritoneal serosa microsomes was especially rapid and was 70 to 80 times faster than that of N-OH-2-FAA [4]. Thus, N-OH-2-FBA emerged as an excellent source of *in situ* generated N-OH-2-FA, which may play a role in its tumorigenesis. The data suggested that N-deacylation of N-OH-2-FBA may also occur in the rat mammary gland, another tumor target. This would contrast with no or low N-deacylating activities found with N-OH-2-FAA or its formyl and propionyl analogues in the mammary gland [6–10]. However, tRNA adduct formation *in vitro* with the formyl analogue, but not N-OH-2-FAA, was sensitive to paraoxon, suggesting involvement of deacylation by a carboxylesterase [8]. Thus, the first purpose of this investigation was to examine the N-deacylating activities in the mammary gland and liver of female SD rats, using N-OH-2-FBA, and compare them with N-OH-2-FAA. Carboxylesterases, which hydrolyze amide and ester bonds of a wide variety of compounds, may play a role in activation or detoxification of xenobiotics [reviewed

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§ Abbreviations: OH, hydroxy; 2-FAA, *N*-2-fluorenylacetamide; 2-FBA, *N*-2-fluorenylbenzamide; 2-FA, *N*-2-fluorenamine; AA, acetanilide; BA, benzanilide; o- or p-NPA, *o*- or *p*-nitrophenylacetate; o-NPB, *o*-nitrophenylbenzoate; 2-NA, 2-naphthyl acetate; 2-NB, 2-naphthyl benzoate; SD, Sprague–Dawley; and F344, Fischer 344.

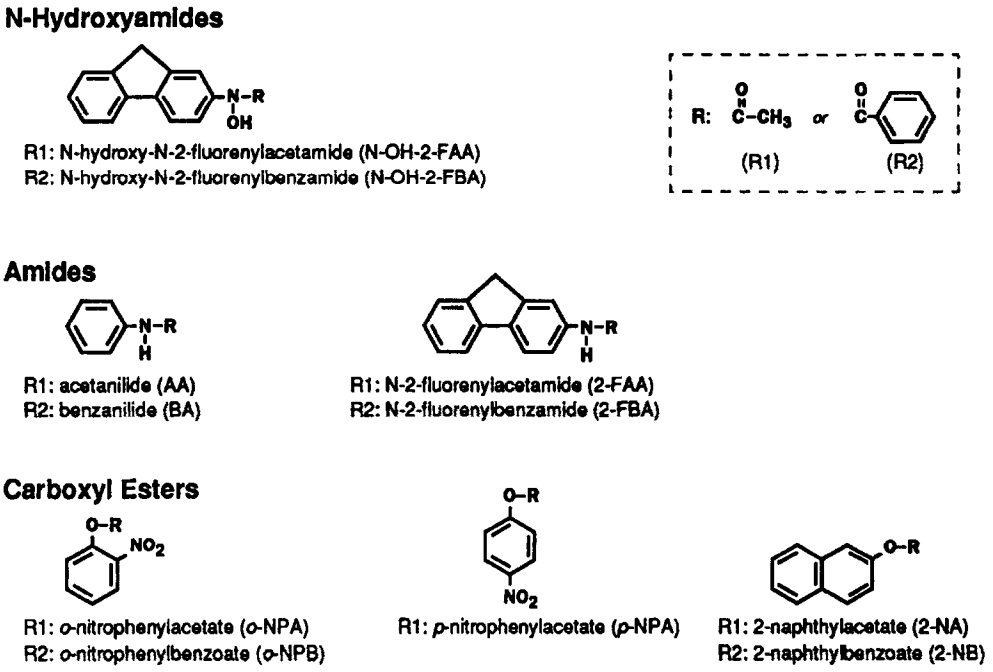

Fig. 1. Structural formulae of the test compounds.

in Refs. 11 and 12]. Esterase activity may influence the duration of action and toxicity of compounds with susceptible bonds depending on whether hydrolysis releases or destroys the active species. A great multiplicity of carboxylesterases has been found in mammalian species [11–13]. Carboxylesterase activity has been studied extensively in the rat liver, and several forms (isozymes) have been characterized [11–17]. However, very little is known of the activity in the mammary gland. Therefore, the second purpose of this study was to compare the specificities of microsomal esterases of the mammary gland and liver of female and the liver of male rats for several acetylated and benzoylated substrates (Fig. 1).

The levels and distribution of carboxylesterase isozymes have been found to change with age and hormonal status of the rat [15, 16, 18–22]. Thus, hormonal manipulations may alter the metabolism of xenobiotics, including carcinogens. Hence, the third purpose of this study was to examine the effects of ovariectomy performed at different stages of rat maturity on the deacylating activities of mammary gland and liver of 50-day-old SD rats, i.e. at the highly susceptible age to mammary gland tumorigenesis [23].

MATERIALS AND METHODS

Chemicals

The following chemicals and supplies were purchased from the sources indicated: o-NPB from the Sigma-Aldrich Co., Inc. (Milwaukee, WI); o-NPA, *o*-nitrophenol, 2-FAA, AA, BA, *N*-1-naphthylethylenediamine dihydrochloride, isoamyl alcohol, 2-NA and 4-aminobiphenyl from the Aldrich Chemical Co., Inc. (Milwaukee, WI); 2-NB from Pfaltz & Bauer, Inc. (Waterbury, CT); pentacyanoaminoferrate, methanol, sodium hydroxide, toluene, methylene chloride, 2-ethoxyethanol, isopropanol and Tween-20 from the Fisher Scientific Co. (Fairlawn, NJ); paraoxon, Tris–HCl, aniline, p-NPA, 2-naphthol, fast violet B salt, dithiothreitol and *p*-nitrophenol from the Sigma Chemical Co. (St. Louis, MO); sodium pyrophosphate and trichloroacetic acid from EM Science (Gibbstown, NJ); ethanol from Pharmco Products, Inc. (Bayonne, NJ); sodium nitrite from Mallinckrodt Chemical Works (St. Louis, MO); ammonium sulfamate from the Amend Drug and Chemical Co., Inc. (New York, NY); anhydrous sodium acetate from the Merck Chemical Co., Inc. (Rahway, NJ); C_{18} extraction columns (Baker-10SPE) from the J. T. Baker Chemical Co. (Phillipsburg, NJ); and Zorbax C_8 columns from MacMod Analytical, Inc. (Chadds Ford, PA). N_2 was passed through an Oxytrap (Alltech Associates, Inc., Deerfield, IL). H_2O was glass-distilled. 2-FAA, 4-aminobiphenyl, AA, and BA were recrystallized until pure by HPLC. 2-FBA [24], N-OH-2-FAA [2], and N-OH-2-FBA [4] were prepared according to published procedures.

Equipment

The Polytron homogenizer, HPLC equipment, and UV-VIS spectrophotometers were described previously [4].

Animals

SD rats (specific pathogen free), purchased from Harlan Sprague Dawley (Indianapolis, IN), were maintained on Teklad Certified Rodent Diet (Harlan Teklad, Madison, WI) and water *ad lib.* Female rats were ovariectomized or sham-treated under Metofane anesthesia 8, 18 or 28 days before killing (decapitation after CO_2 asphyxiation) at 50 days of age. Male rats were killed at 64 days of age.

Preparation of microsomal fractions of rat liver and mammary gland

All buffers were cooled on ice and all procedures were carried out at 4° unless otherwise specified. Livers were

perfused with 50 mM Tris–HCl, 154 mM KCl buffer, pH 7.4. Microsomal fraction was prepared as previously described [4]. Mammary glands from six rats were pooled and homogenized with a Polytron homogenizer with five bursts of 5 sec each at setting 6 in 1 part (w/w) of 50 mM sodium pyrophosphate buffer, pH 7.0, with 1 mM dithiothreitol. After centrifugation at 900 *g* for 10 min, the pellet was rehomogenized with three bursts of 5 sec each. After another centrifugation, the supernatants were combined and centrifuged at 14,000 *g* for 20 min. The supernatant fraction was centrifuged at 105,000 *g* for 60 min. The microsomal pellet was washed once and then resuspended in the pyrophosphate buffer. The protein content of liver and mammary gland microsomal fractions was determined by a modified Lowry assay [25]. The fractions were stored at –80° for up to 2 months.

Determination of deacylating activities of rat liver and mammary gland

Substrate concentrations and solvents depended on the substrate solubility and assay sensitivity. To measure inhibition, paraoxon was added at 0.1 mM in 1% methanol. Velocities of ester hydrolysis were averages from four livers or pools of mammary gland incubated at least in duplicate and were corrected for controls containing heat-inactivated protein. Conditions were chosen at which product formation was linear with time and protein concentration.

Determinations of rates of hydrolysis of N-OH-2-FBA and N-OH-2-FAA (0.2 mM in 2% ethanol) to N-OH-2-FA were according to a previously published method [4]. Incubations were at 37° in 50 mM pyrophosphate buffer, pH 7.0.

For determination of rates of hydrolysis of 2-FBA and 2-FAA, microsomal protein (2 mg/mL) was incubated at 42° for up to 2 hr in 50 mM Tris–HCl, pH 7.7, with 2-FBA (0.075 mM) or 2-FAA (0.075 or 0.375 mM) dissolved in 2-ethoxyethanol (4%). The total volume was 0.5 mL. Controls contained heat-inactivated protein. At the end of the reaction time, 4 nmol of 4-aminobiphenyl was added as internal standard and the mixture extracted with a Baker C_{18} extraction column activated with 1 mL each of isopropanol:methanol (2:1) and buffer. The column was washed with 0.5 mL buffer, and the compounds were eluted with two 0.25-mL aliquots of isopropanol:methanol (2:1). The extract, dried under N_2, was dissolved in 0.04 mL isopropanol:methanol (2:1), and 0.01 mL was analyzed by the HPLC systems described previously [4]. Compounds, identified by retention time and absorbance spectra (420–220 nm), were quantified from peak areas and corrected for extraction efficiencies.

Measurement of hydrolysis of AA at 10 mM was in 100 mM Tris–HCl, pH 8.6, according to a published procedure [26]. To compare rates of hydrolysis of AA and the less soluble BA, 50 mM Tris–HCl buffer, pH 8.6, was added with vortexing to 0.25 mL of 1 mM substrate dissolved in 2-ethoxyethanol (10%) to give a final volume of 0.5 mL. Reaction was started with the addition of protein (1–2 mg/mL), and the mixture was incubated at 42° for 30 min. Standard incubations contained aniline and active enzyme. The reaction was terminated with the addition of 0.2 mL of ice-cold 1 M HCl and chilled in ice for 6 min. Consecutive additions of 0.2 mL of sodium nitrite (2.5% in water) and 0.2 mL of ammonium sulfamate (0.5% in water) were each followed by 3-min incubations in ice. After additions of 0.5 mL of sodium acetate and 1 mL of *N*-1-naphthylethylenediamine dihydrochloride (0.055% in water), color was developed for 20 min at 25°. The mixture was made alkaline with the addition of 0.15 mL of 5 M sodium hydroxide and then extracted with 2 mL of toluene containing 1.5% isoamyl alcohol. One milliliter of the toluene layer, clarified by centrifugation at 1180 *g* for 5 min at 19°, was acidified with 0.125 mL of 25% (w/v) trichloroacetic acid in methylene chloride. The absorbance at 578 nm was recorded. Product formation (nmol/min/mg) was calculated based on the standard curve for aniline.

Determinations of rates of hydrolysis of the o- and p-NPA and o-NPB were based on the methods of Krisch [27]. The esters of o-nitrophenol (0.2 mM, 5% ethanol) and microsomal protein (20–90 μg/mL) were incubated in 0.1 M Tris–HCl, pH 8.0, at 37° in a total volume of 1 mL. Formation of the *o*-nitrophenol was monitored for 90 sec at the λ_{max} of 414 nm with a reference wavelength of 800 nm (E = 3817.6 M^{-1} cm^{-1}). The velocity of product formation was calculated from the linear absorbance changes and corrected for controls containing heat-inactivated protein. Similar conditions were used to compare o-NPA and p-NPA except that the final substrate concentration was 1.5 mM in 5% methanol with 5–17 μg protein/mL. Formation of *p*-nitrophenol was monitored at λ_{max} of 404 nm with a reference wavelength of 800 nm (E = 16,240 M^{-1} cm^{-1}).

To determine rates of hydrolysis of 2-NA and 2-NB, 50 mM pyrophosphate buffer, pH 7.0, at 37° was added with vortexing to 5 μL of 10 mM naphthyl ester in 2-ethoxyethanol. After a 3-min preincubation at 37°, microsomal protein to give 1–2 μg/mL was added. The final volume was 0.5 mL. The mixtures were incubated for 1.3 and 4 min for 2-NA and 2 and 6 min for 2-NB. Controls contained heat-inactivated enzyme. Standard incubations contained active protein and 2-naphthol in 2-ethoxyethanol. The reaction was terminated with the addition of 5 μL of 10 mM paraoxon in methanol and chilling in an ice bath. Determination of the 2-naphthol product was based on the published procedure [28]. After addition of 0.25 mL Tris–HCl, pH 9.0, and 0.25 mL fast violet B (1 mg/mL in water), the mixtures were incubated at 25° for 20 min. Five minutes after the addition of 0.5 mL of 10% Tween-20 in 1 M sodium acetate buffer, pH 4.2, the absorbance at λ_{max} of 520 nm with a reference wavelength of 780 nm was recorded. Absorbance, corrected for that in the controls, was converted to nanomoles of product based on a 2-naphthol standard curve. Initial velocities of product formation were determined from linear regression analysis of the time-dependent product formation in duplicate incubations at each time point.

Statistical analysis

Statistical analyses were conducted with Student's two-sample *t*-test.

RESULTS

The initial rates of N-deacylations of the *N*-hydroxyfluorenamides, N-OH-2-FBA and N-OH-2-FAA, by microsomes of liver and N-OH-2-FBA by microsomes of mammary gland from 50-day-old rats sham-treated or ovariectomized at 22, 32 and 42 days of age are pre-

sented in Fig. 2. The rates of N-debenzoylation of N-OH-2-FBA by microsomes of liver and mammary gland were similar and were much greater than those of N-deacetylation of N-OH-2-FAA. The low rates of N-deacetylation by mammary gland (~0.1 nmol/min/mg) are not shown. The rates of N-debenzoylation of N-OH-2-FBA by both liver and mammary gland were increased significantly after ovariectomy at 22 and 32 days of age and also by the mammary gland at 42 days of age. Ovariectomy performed shortly after weaning, i.e. at 22 days of age, resulted in the greatest increase in N-debenzoylation in both tissues. By contrast, ovariectomy had no significant effect on the rates of N-deacetylation of N-OH-2-FAA by the liver and mammary gland (Fig. 2; Table 1). To examine whether other benzoyl esters and amides (Fig. 1) were likewise hydrolyzed faster than the acetyl analogues and if their hydrolysis was selectively affected by ovariectomy, rates of hydrolysis of several benzoyl and acetyl esters and amides by microsomes from liver and mammary gland of rats sham-treated or ovariectomized at 22 days of age were determined and compared with those of the *N*-hydroxyfluorenamides (Table 1).

The acetylated and benzoylated substrates were compared at the concentration levels of maximum solubility of the latter. 2-FAA and AA were also tested at higher concentrations to increase product and thus magnify any effects of ovariectomy. As described in Materials and Methods, increased temperatures for the hydrolysis of 2-FAA, 2-FBA, AA and BA and optimal pHs for all substrates were used to enhance product formation. All deacylations were >98% inhibited by 0.1 mM paraoxon, indicating involvement of a serine hydrolase (data not shown) [11]. Although the rates of hydrolysis of the substrates used cannot be strictly compared because of the differences in aryl moieties and incubation conditions, the data in Table 1 indicate more facile hydrolysis of the carboxyl esters than the amides. Hydrolysis of the O-acetyl esters by microsomes of liver from sham-treated rats was 4.3 to 9.2 times the rate by mammary gland microsomes, while hydrolysis of O-benzoyl esters was 1.0 to 3.2 times as great. Both O-deacetylation and O-debenzoylation were increased to similar extents (≤1.55 times) after ovariectomy. The rates of N-deacetylation of N-OH-2-FAA, 2-FAA and AA by hepatic microsomes were 11 to 82 times greater than

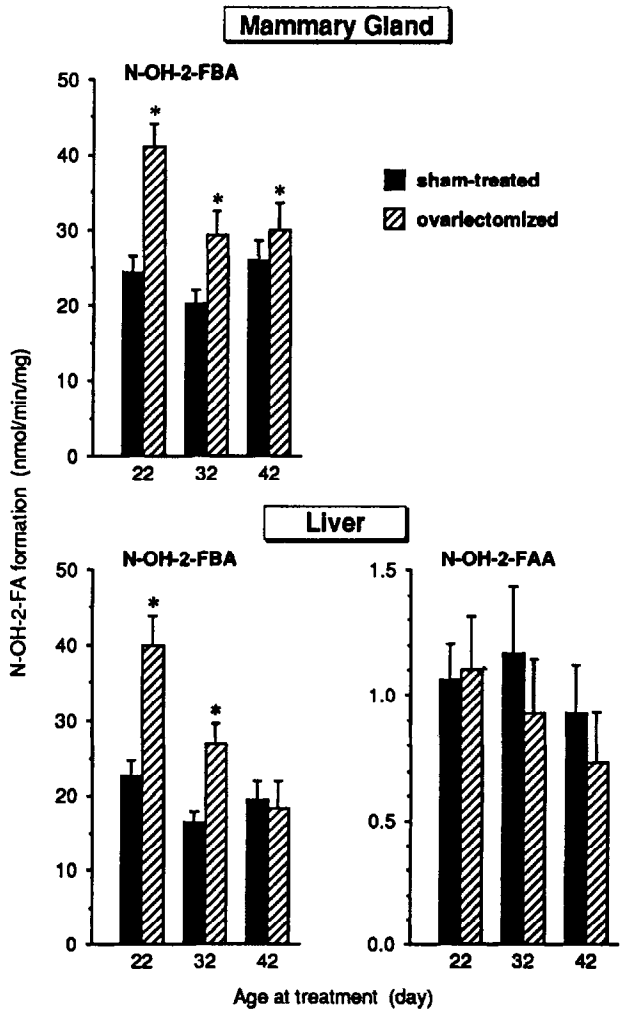

Fig. 2. Effect of age at ovariectomy on the rates of N-deacylations of N-OH-2-FBA and N-OH-2-FAA by the rat liver or mammary gland microsomes. Treatment of rats, composition of incubations and assays are described in Materials and Methods. Values are the means ± SD of the initial rates from four individual livers or four pools of mammary gland from six rats each. An asterisk (*) denotes a statistically significant difference ($P \leq 0.05$) between sham-treated and ovariectomized rats.

Table 1. N- and O-Deacylating activities of female rat liver and mammary gland microsomes: Effect of ovariectomy (OVX)

Substrate	Concentration (mM)	Liver Deacylation (nmol/min/mg) SHAM	Liver Deacylation (nmol/min/mg) OVX	Activity ratio OVX/SHAM
N-Deacylations				
N-OH-2-FAA	0.2	1.06 ± 0.142	1.10 ± 0.21	1.04
N-OH-2-FBA	0.2	22.7 ± 2.17	40.1 ± 4.0	1.77*
2-FAA	0.075	0.136 ± 0.016	0.135 ± 0.018	0.99
	0.375	0.534 ± 0.083	0.532 ± 0.07	1.00
2-FBA	0.075	0.005 ± 0.001	0.007 ± 0.004	1.24
AA	0.5	0.06 ± 0.04	0.06 ± 0.02	1.00
	10	31.3 ± 3.9	33.0 ± 2.4	1.05
BA	0.5	0.63 ± 0.29	0.81 ± 0.40	1.28
O-Deacylations				
o-NPA	0.2	2537 ± 144	2455 ± 95	0.97
o-NPB	0.2	1553 ± 281	2068 ± 288	1.33*
o-NPA	1.5	7274 ± 712	9147 ± 1762	1.26*
p-NPA	1.5	4158 ± 432	5144 ± 222	1.24*
2-NA	0.1	3643 ± 710	5235 ± 906	1.44*
2-NB	0.1	1052 ± 233	1626 ± 509	1.55*

Substrate	Concentration (mM)	Mammary gland Deacylation (nmol/min/mg) SHAM	Mammary gland Deacylation (nmol/min/mg) OVX	Activity ratio OVX/SHAM
N-Deacylations				
N-OH-2-FAA	0.2	0.10 ± 0.005	0.12 ± 0.00	1.16
N-OH-2-FBA	0.2	24.5 ± 2.1	41.1 ± 3.0	1.68*
2-FAA	0.075	0.005 ± 0.0	0.003 ± 0.003	0.61
	0.375	0.028 ± 0.01	0.029 ± 0.005	1.06
2-FBA	0.075	0.008 ± 0.0	0.013 ± 0.004	1.56*
AA	0.5	0.04 ± 0.01	0.05 ± 0.01	1.25
	10	0.38 ± 0.06	0.43 ± 0.03	1.13
BA	0.5	0.66 ± 0.21	0.89 ± 0.24	1.35
O-Deacylations				
o-NPA	0.2	394 ± 50	571 ± 55	1.45*
o-NPB	0.2	1196 ± 305	1518 ± 267	1.27*
o-NPA	1.5	1673 ± 366	2218 ± 309	1.33*
p-NPA	1.5	2719 ± 948	3197 ± 1537	1.18
2-NA	0.1	395 ± 76	479 ± 127	1.21
2-NB	0.1	332 ± 89	390 ± 122	1.17

Treatment of rats, preparation of microsomes, and enzyme assays are described in Materials and Methods. Values are means ± SD of the initial rates from four individual livers or four pools of mammary gland from six rats, each sham-treated or ovariectomized at 22 days of age.

* $P < 0.05$.

those by the mammary gland microsomes. In contrast, the rates of N-debenzoylation of N-OH-2-FBA, 2-FBA and BA were similar or slightly faster by the mammary gland microsomes than by liver microsomes. Moreover, the rates of N-debenzoylation of all three compounds by microsomes of mammary gland and liver were increased after ovariectomy, with statistically significant differences for N-OH-2-FBA by both tissues and for 2-FBA by mammary gland microsomes.

The rates of hydrolysis of the hydroxamic acids, amides and O-esters by liver microsomes of the male rats are presented in Table 2. The effect of gender was most apparent on the hydrolysis of the N-OH-2-FBA, which was hydrolyzed ~3.9 times faster by liver microsomes of the males than of the females. Hydrolysis of N-OH-2-FAA was only 1.2 times faster by male liver microsomes. Gender differences were also apparent in the hydrolysis of the fluorenamides since the rate of deacetylation of 2-FAA by male liver microsomes was ~0.8 times that by female liver microsomes, while debenzoylation of 2-FBA was 1.7 times as great. The carboxyl esters were hydrolyzed at greater rates by the male than by the female hepatic microsomes, but the males showed a large range of activities in hydrolysis of o-NPB at 0.2 mM and p-NPA at 1.5 mM.

The effects of the acetyl and benzoyl groups on the rates of hydrolysis by liver microsomes of male rats and by liver and mammary gland microsomes of sham-treated and ovariectomized females are presented in Table 3. The presence of the N-hydroxy group greatly affected the relative rates of hydrolysis of acetyl and benzoyl fluorenamide analogues. While 2-FAA was hydrolyzed 11, 25 and 20 times faster than 2-FBA by liver microsomes of male, sham-treated female and ovariectomized rats, respectively, the respective rates of hydrolysis of N-OH-2-FBA were 71, 21 and 37 times those of

Table 2. N- and O-Deacylating activities of liver microsomes of male rats

Substrate	Concentration (mM)	Deacylation (nmol/min/mg)
N-OH-2-FAA	0.2	1.27 ± 0.30
N-OH-2-FBA	0.2	88 ± 15
2-FAA	0.075	0.106 ± 0.027
2-FBA	0.075	0.009 ± 0.002
o-NPA	0.2	2589 ± 682
o-NPB	0.2	2925 ± 1290
o-NPA	1.5	9277 ± 1653
p-NPA	1.5	7945 ± 3957
2-NA	0.1	5015 ± 944
2-NB	0.1	1490 ± 516

Preparation of microsomes and enzyme assays are described in Materials and Methods. Values are means ± SD of the initial rates from four individual livers.

N-OH-2-FAA. With the mammary gland microsomes, the rate of hydrolysis of 2-FBA was 1.5 to 3.8 times that of 2-FAA, and N-OH-2-FBA was hydrolyzed 250 to 330 times faster than N-OH-2-FAA. Although liver microsomes of the male (Table 2) hydrolyzed the *N*-hydroxyfluorenamides 1.2 to 3.9 times faster than did those of the female (Table 1), both showed a preference for the benzoyl rather than the acetyl derivative. In contrast to a much faster rate (up to 25 times) of hydrolysis of 2-FAA than 2-FBA by liver of female rats, the rate of hydrolysis of AA at 0.5 mM was much slower (~0.1 times) than that of BA. The rates of hydrolysis of both benzamides, 2-FBA and especially BA, by mammary gland were faster than that of the acetamides. The acetylated carboxyl esters o-NPA and 2-NA were hydrolyzed faster than the benzoylated compounds by female liver microsomes, but the male liver microsomes only showed a preference for the acetyl group with 2-NA. In contrast, microsomes of mammary gland, which also tended to prefer 2-NA, hydrolyzed the o-NPB faster than the o-NPA. Liver and mammary gland also differed in substrate stereoselectivity in that liver microsomes hydrolyzed the o-NPA faster than the p-NPA, whereas the mammary gland microsomes hydrolyzed the p-NPA at a faster rate. Male liver microsomes showed less stereoselectivity than those of female with these nitrophenyl carboxyl esters. The data in Table 3 also depict differences between liver and mammary gland in preference for benzoyl esters. Whereas acetamides and acetates, except for N-OH-2-FAA and AA, were hydrolyzed by the liver more rapidly than their benzoyl analogues, the benzoyl esters except for 2-NB were hydrolyzed more rapidly by the mammary gland.

DISCUSSION

Our previous studies [4, 5] indicated involvement of esterase-catalyzed hydrolysis of N-OH-2-FBA in its activation in the peritoneal serosa, a chief tumor target tissue for this compound. Since N-OH-2-FBA is also tumorigenic for the mammary gland of female SD rats [3], we examined herein esterase activities in this tissue. We used mammary gland of 50-day-old SD rats because at this age they exhibited optimal susceptibility to polycyclic aromatic hydrocarbon-induced mammary tumorigenesis, which was prevented by ovariectomy at 22 days of age [23]. The rats of similar age were also susceptible to mammary tumor induction by N-OH-2-FAA and ovariectomy 1 week before the carcinogen application had a preventive effect [29]. The levels of esterase activities depend on gender, hormonal status and age of the rat [15, 16, 18–22]. Since hepatic microsomal carboxylesterase activity toward p-NPA was shown to be relatively stable after 49 and 56 days of age in the female and male rats, respectively [22], we examined the hepatic esterase activities of 50-day-old females and 64-day-old males, i.e. at comparable ages of esterase stability.

This study showed that microsomes of mammary gland of female rats hydrolyzed N-OH-2-FBA at a remarkably rapid rate (24 nmol/min/mg), which was as great as that by the liver microsomes (Table 1; Fig. 2). This level of esterase activity in mammary gland with N-OH-2-FBA contrasts with the low or absent activities detected herein and in earlier studies with N-OH-2-FAA or its formyl and propionyl analogues as substrates [6–10]. Our study also showed that although N-deacetylation of N-OH-2-FAA by microsomes of liver and mammary gland was unaffected by ovariectomy, N-debenzoylation of N-OH-2-FBA by both tissues was increased (Table 1; Fig. 2). Ovariectomy of rats at 22 days of age (shortly after weaning) resulted in an increase of ~1.7 times in hydrolysis of N-OH-2-FBA by the microsomes of liver and mammary gland of 50-day-old rats, which was greater than after ovariectomy at 32 or 42 days of age, indicating that ovariectomy performed before the onset of ovarian hormone production had the most significant effect.

It has been recognized that rat liver microsomal carboxylesterases are also active on aromatic amides although at generally lower rates [26]. Likewise, the much greater rates of hydrolysis by rat livers of both sexes of the O-esters than the amides used herein (Tables 1 and 2) are consistent with this observation. Our study also showed a similar trend with the mammary gland microsomes. Inhibition by paraoxon of hydrolysis of N- and O-acylated substrates indicated involvement of serine hydrolases in both tissues [11].

The rat liver microsomal carboxylesterase has been resolved into multiple forms [11–17]. Most substrates are hydrolyzed by more than one form (isozyme). Differences in distribution of the isozymes are tissue specific [17, 18]. It is also possible that deacylase composition in the rat, similarly to the mouse [30], is strain dependent. Since the rats used herein were of an outbred strain, the individuals might differ in relative amounts of isozymes, which would reflect upon the standard deviation of the average hydrolysis rates. However, the size of the population studied was insufficient to determine whether polymorphic esterase phenotypes were present.

Our data showed that the microsomes of the female liver hydrolyzed AA, 2-FAA and N-OH-2-FAA 82, 19 to 27 and 11 times faster, respectively, than the mammary gland microsomes. On the other hand, the latter hydrolyzed benzamides as fast or slightly faster than did the liver microsomes. Ovariectomy produced similar increases in the benzamide hydrolysis by microsomes of both tissues. The results suggest involvement of different carboxylesterase(s) with the form(s) cleaving the benzamides present at similar levels in liver and mammary gland and the form(s) mainly responsible for hydrolysis

Table 3. Effect of substrate structure on microsomal N- or O-deacylating activities

	Activity ratios				
	Liver			Mammary gland	
	Male	Female		Female	
Substrate*		SHAM	OVX	SHAM	OVX
N-OH-2-FAA:N-OH-2-FBA	0.014†	0.047†	0.027†	0.004†	0.003†
2-FAA:2-FBA	11.45†	25.17†	20.15†	0.66†	0.26†
AA:BA	ND‡	0.10†	0.08†	0.06†	0.06†
o-NPA:o-NPB	0.89	1.63†	1.19	0.33†	0.38†
o-NPA:p-NPA	1.17	1.75†	1.78†	0.62†	0.69
2-NA:2-NB	3.36†	3.46†	3.22†	1.19	1.23

Ratio values were calculated from the means of the initial rates from four individual livers or four pools of mammary gland from six rats, each sham-treated or ovariectomized at 22 days of age.

* Substrate analogs were compared at equal concentrations.

† $P < 0.05$.

‡ ND = not determined.

of acetamides being more abundant in the liver microsomes. These tissue-specific differences may be relevant in the metabolism of xenobiotics and drug design. Increases in the rates of hydrolysis of N-OH-2-FBA after ovariectomy, which did not affect hydrolysis of N-OH-2-FAA, suggest involvement of different isozymes. The isozymes that increased after ovariectomy in liver of SD rats were those more abundant in males than females (esterases with pI 6.0 to 6.4) [16], suggesting that these are responsible for the increases of N-OH-2-FBA hydrolysis by the liver and also mammary gland of ovariectomized rats (Fig. 2). On the other hand, N-OH-2-FAA hydrolysis was catalyzed by esterase with pI 5.0 at an ~10 times faster rate than by esterases with pI 6.0 and 6.5 from the liver of the female F344 rat [31]. We found a slightly greater rate of hydrolysis of N-OH-2-FAA by liver microsomes of male than female SD rats. The discrepancy between our results and the reported slightly faster rate of hydrolysis of N-OH-2-FAA by livers of female than male SD rats [7] may be due to use of microsomes rather than whole tissue homogenates and rats of different ages. Cytosolic esterases would contribute to the activity of the homogenates. Cytosolic esterases have not been investigated extensively, but evidence has been presented to indicate that they may differ from microsomal esterases [17, 21]. In agreement with our previous report [4] describing deacylase activity with N-OH-2-FAA, N-OH-2-FBA and p-NPA in the hepatic cytosol of male rats and with the latter two compounds in the peritoneal serosa cytosol, we found considerable esterase activity with N-OH-2-FBA and p-NPA in the hepatic and mammary gland cytosols of female rats (data not shown).

Our observation that ovariectomy did not affect AA hydrolysis by liver microsomes (Table 1) differed from that of a previous study showing that hepatic hydrolysis of AA was decreased in 56-day-old, corn oil-dosed SD rats by ovariectomy at 35 days of age [19]. This variability may be due to the corn oil and/or the different ages of rats at ovariectomy and termination. Since an isozyme with pI ~5.5 was found to decrease in female Wistar rats after 24 days of age [15] and AA was found to be rather specific for an isozyme with pI 5.5 in female SD rats [16], ovariectomies at 22 (Table 1) and 35 days of age [19] might have had different effects.

Our finding that 2-FAA was hydrolyzed by liver microsomes much more rapidly than 2-FBA was consistent with evidence from previous investigations using liver slices [32]. This result contrasts with the more rapid hydrolysis of the benzoyl than acetyl derivatives of anilide and *N*-hydroxyfluorenamide (Table 3). The extremely poor rate of hydrolysis of 2-FBA may reflect its hydrophobicity and/or involvement of different isozyme(s). Chemical hydrolysis of 2-FBA was much less facile than of 2-FAA [33]. The rates of deacylations of O-acetyl and O-benzoyl esters, although faster by the liver than mammary gland microsomes, were increased similarly in both tissues after ovariectomy. This suggests that the contribution of hormone-sensitive isozyme(s) to the rate of hydrolysis of these compounds is similar in both tissues.

The rates of hepatic hydrolysis of several substrates determined after ovariectomy (Table 1) approached those in the males (Table 2). Thus, ovariectomy may imbue the female rat liver with a level of activation and/or detoxification of xenobiotics similar to that of the male. The extent of this modulation may be dependent on the age at ovariectomy and may affect the level of the parent compound and/or its metabolite, which reaches the mammary gland. Of particular interest is the fate of N-OH-2-FAA and N-OH-2-FBA, which are mammary gland carcinogens [2, 3, 29]. The low level of N-deacetylation of N-OH-2-FAA by the mammary gland and the lack of effect of ovariectomy on mammary and hepatic deacetylation (Table 1) suggest that deacetylation of N-OH-2-FAA is not involved in its tumorigenicity. On the other hand, the high rates of N-debenzoylation of N-OH-2-FBA, which were increased after ovariectomy (Fig. 2), indicate potential formation of large amounts of N-OH-2-FA in both the mammary gland and liver. The results of our recent studies with liver and peritoneal serosa of male rats indicated that N-OH-2-FBA is activated via N-debenzoylation to N-OH-2-FA [4]. Subsequent acetyl CoA-dependent O-acetylation yields N-acetoxy-2-FA, which forms a DNA adduct implicated in initiation of carcinogenesis [5]. The effect of ovariectomy on carcinogenicity of N-OH-2-FBA has not yet been examined, but its effect on activation of N-OH-2-FBA in the mammary gland and liver of female rats *in vivo* is under investigation.

Acknowledgements—We thank Mejah Ben-Yonatan and Kristen Bennett for technical assistance. This work was supported by Grant CA-28000 from the National Cancer Institute, USPHS, and USVA Biomedical Research Funds.

REFERENCES

1. Malejka-Giganti D and Ritter CL, Peroxidase-mediated metabolism of *N*-arylhydroxamic acids in relation to tumorigenesis. In: *Carcinogenic and Mutagenic Responses to Aromatic Amines and Nitroarenes* (Eds. King CM, Romano LJ and Schuetzle D), pp. 199–209. Elsevier Science Publishing, New York, 1988.
2. Miller EC, Miller JA and Hartmann HA, *N*-Hydroxy-2-acetylaminofluorene: A metabolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. *Cancer Res* **21:** 815–824, 1961.
3. Gutmann HR, Galitski SB and Foley WA, The conversion of noncarcinogenic aromatic amides to carcinogenic arylhydroxamic acids by synthetic N-hydroxylation. *Cancer Res* **27:** 1443–1455, 1967.
4. Ritter CL and Malejka-Giganti D, Activation of the carcinogens *N*-hydroxy-*N*-2-fluorenylbenzamide and *N*-hydroxy-*N*-2-fluorenylacetamide via deacylations and acetyl transfers by rat peritoneal serosa and liver. *Carcinogenesis* **15:** 163–170, 1994.
5. Malejka-Giganti D, Ritter CL, Fullerton NF and Beland FA, Detection of *N*-(deoxyguanosin-8-yl)-2-fluorenamine in DNA of peritoneal serosa and liver after intraperitoneal exposure of rats to *N*-hydroxy-*N*-2-fluorenylbenzamide or *N*-hydroxy-*N*-2-fluorenylacetamide. *Carcinogenesis* **15:** 2883–2890, 1994.
6. Malejka-Giganti D, Gutmann HR and Rydell RE, Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids. *Cancer Res* **33:** 2489–2497, 1973.
7. Irving CC, Species and tissue variations in the metabolic activation of aromatic amines. In: *Carcinogens: Identification and Mechanisms of Action* (Eds. Griffin AC and Shaw CR), pp. 211–227. Raven Press, New York, 1979.
8. Shirai T, Fysh JM, Lee M-S, Vaught JB and King CM, Relationship of metabolic activation of *N*-hydroxy-*N*-acylarylamines to biological response in the liver and mammary gland of the female CD rat. *Cancer Res* **41:** 4346–4353, 1981.
9. Allaben WT, Weeks CE, Weiss CC, Burger GT and King CM, Rat mammary gland carcinogenesis after local injection of *N*-hydroxy-*N*-acyl-2-aminofluorenes: Relationship to metabolic activation. *Carcinogenesis* **3:** 233–240, 1982.
10. Wang CY, Yamada H, Morton KC, Zukowski K, Lee M-S and King CM, Induction of repair synthesis of DNA in mammary and urinary bladder epithelial cells by *N*-hydroxy derivatives of carcinogenic arylamines. *Cancer Res* **48:** 4227–4232, 1988.
11. Heymann E, Carboxylesterases and amidases. In: *Enzymatic Basis of Detoxification, II* (Ed. Jakoby WB), pp. 291–323. Academic Press, New York, 1980.
12. Wang CY, Microsomal amidases and carboxylesterases. In: *Handbook of Experimental Pharmacology* (Ed. Kauffman FC), Vol. 112, pp. 161–187. Springer, Berlin, 1994.
13. Hosokawa M, Maki T and Satoh T, Characterization of molecular species of liver microsomal carboxylesterases of several animal species and humans. *Arch Biochem Biophys* **277:** 219–227, 1990.
14. Mentlein R, Heiland S and Heymann E, Simultaneous purification and comparative characterization of six serine hydrolases from rat liver microsomes. *Arch Biochem Biophys* **200:** 547–559, 1980.
15. Beaufay R and Beaufay H, Purification and characterization of various esterases from rat liver. *Eur J Biochem* **137:** 293–301, 1983.
16. Hosokawa M, Maki T and Satoh T, Multiplicity and regulation of hepatic microsomal carboxylesterases in rats. *Mol Pharmacol* **31:** 579–584, 1987.
17. McCracken NW, Blain PG and Williams FM, Nature and role of xenobiotic metabolizing esterases in rat liver, lung, skin and blood. *Biochem Pharmacol* **45:** 31–36, 1993.
18. Holmes RS and Masters CJ, The developmental multiplicity and isoenzyme status of rat esterases. *Biochim Biophys Acta* **146:** 138–150, 1967.
19. Hosokawa M, Satoh T, Ohkawara S, Ohmori S, Igarashi T, Ueno K and Kitagawa H, Gonadal hormone-induced changes in hepatic microsomal carboxylesterase in rats. *Res Commun Chem Pathol Pharmacol* **46:** 245–258, 1984.
20. Hosokawa M and Satoh T, Effects of hypophysectomy and pituitary hormones on hepatic microsomal carboxylesterase isozymes in male rats. *Res Commun Chem Pathol Pharmacol* **62:** 279–288, 1988.
21. Hosokawa M, Maki T and Satoh T, Differences in the induction of carboxylesterase isozymes in rat liver microsomes by xenobiotics. *Biochem Pharmacol* **37:** 2708–2711, 1988.
22. Morgan EW, Yan B, Greenway D and Parkinson A, Regulation of two rat liver microsomal carboxylesterase isozymes: Species differences, tissue distribution, and the effects of age, sex, and xenobiotic treatment of rats. *Arch Biochem Biophys* **315:** 513–526, 1994.
23. Huggins C, Grand LC and Brillantes FP, Mammary cancer induced by a single feeding of polynuclear hydrocarbons, and its suppression. *Nature* **189:** 204–207, 1961.
24. Gutmann HR and Peters JH, Studies on the metabolism of 2-benzoylamino-fluorene-9-C^{14} and 2-acetylaminofluorene-9-C^{14} in the rat. *Cancer Res* **13:** 415–421, 1953.
25. Hess HH, Lees MB and Derr JE, A linear Lowry–Folin assay for both water-soluble and sodium dodecyl sulfate-solubilized proteins. *Anal Biochem* **85:** 295–300, 1978.
26. Heymann E, Mentlein R and Rix H, Hydrolysis of aromatic amides as assay for carboxylesterases-amidases. In: *Methods in Enzymology* (Ed. Jakoby WB), Vol. 77, pp. 405–409. Academic Press, New York, 1981.
27. Krisch K, Reaction of a microsomal esterase from hog-liver with diethyl *p*-nitrophenyl phosphate. *Biochim Biophys Acta* **122:** 265–280, 1966.
28. Hopsu VK and Glenner GG, Characterization of enzymes hydrolyzing acyl naphthylamides: I. Mono- and dihalogen derivatives. *J Histochem Cytochem* **12:** 674–685, 1964.
29. Malejka-Giganti D, Rydell RE and Gutmann HR, Mammary carcinogenesis in the rat by topical application of fluorenylhydroxamic acids and their acetates. *Cancer Res* **37:** 111–117, 1977.
30. Hultin TA and Weber WW, Genetic variability in deacetylation of 2-acetylaminofluorene and *N*-hydroxy-2-acetylaminofluorene in inbred strains of mice. *Carcinogenesis* **8:** 1939–1941, 1987.
31. Wang CY, Zukowski K, Lee M-S and Sone T, Purification and characterization of rat hepatic microsomal *N,O*-acyltransferases. *Carcinogenesis* **13:** 2017–2020, 1992.
32. Nagasawa HT and Gutmann HR, A note on the deacylation of the carcinogen 2-acetamidofluorene and related compounds by rat liver and intestine. *Biochim Biophys Acta* **25:** 186–189, 1957.
33. Argus MF and Ray FE, Possible relation between carcinogenicity and ease of hydrolysis *in vitro* of derivatives of 2-aminofluorene. *Nature* **184:** 2018–2019, 1959.