Reductions of Nitro and 9-Oxo Groups of Environmental Nitrofluorenes by the Rat Mammary Gland in Vitro

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Nitrofluorenes and C-9-oxidized nitrofluorenes are widespread environmental genotoxins which may be relevant for breast cancer on the basis of their carcinogenicities, particularly of 2,7-dinitrofluorene (2,7-diNF), for the rat mammary gland. Since their metabolism to active carcinogens may involve nitroreduction, this study examined the reduction of 2-nitrofluorene (2-NF) and 2,7-diNF and their 9-oxo- and 9-hydroxy (OH) derivatives by the rat mammary gland. Cytosolic fractions catalyze NADH- and NADPH-dependent reductions of the 2-nitro and 9-oxo to the respective 2-amino and 9-OH compounds at rates 4- and ≥10-fold greater than those with microsomes. Rates of amine formation catalyzed by cytosol from 2,7-diNF are greater than the rate from 2-NF and increase for C-9-oxidized derivatives: 9-oxo-2-NF > 9-OH-2-NF > 2-NF and 9-OH-2,7-diNF ≫ 9-oxo-2,7-diNF > 2,7-diNF. Nitroreduction is inhibited by O_2 or allopurinol (20 μM), dicoumarol (100 μM), and rutin (50 μM). 9-Oxoreduction is inhibited by rutin, dicoumarol, and indomethacin (100 μM), but not by O_2 or allopurinol. Pyrazole or menadione does not inhibit nitro or 9-oxoreduction. Xanthine, hypoxanthine, 2-hydroxypyrimidine, and *N*′-methylnicotinamide support cytosol-catalyzed nitro, but not 9-oxo, reduction. The data suggest that the nitroreduction is catalyzed largely by a xanthine oxidase and partially by a diaphorase and 9-oxoreduction by a carbonyl reductase. The extents of the nitro and carbonyl reductions of the nitrofluorenes may determine their reactivities with DNA, and thus genotoxicities for the mammary gland.

Introduction

The nitrated polycyclic aromatic hydrocarbons (nitro-PAHs),[1] including nitrofluorenes, originate from incomplete combustion of fossil fuels and atmospheric nitration and are present in exhausts of coal, kerosene, diesel, and gasoline heaters and engines (*1*−*3*). Environmental exposure to genotoxic and carcinogenic nitro-PAHs through inhalation, ingestion, and skin presents a health risk to humans (*3*). Among the nitro-PAHs listed as "possibly carcinogenic to humans" by the International Agency for Research on Cancer (*3*), 2-nitrofluorene (2-NF) is formed at high levels in petroleum exhausts and is found in both the particulate and gas phase with especially high levels in air in winter (*4*, *5*). In the air of Tokyo, the level of 2,7-dinitrofluorene (2,7-diNF) is the highest among those of the characterized nitro-PAHs and is ∼30-fold greater than that of 2-NF and 10-fold greater than that of 1-nitropyrene (*4*). Other nitrofluorenes in diesel exhaust include 2,5-dinitrofluorene (2,5-diNF) and 9-oxo derivatives of 2-NF, 2,7-diNF, and 2,4,7-trinitrofluorene (2,4,7-triNF). 2-NF and 9-oxo-2-NF are also formed by photooxidation of 2-aminofluorene (2-AF), which is used in synthetic petroleums and dye and rubber industries (*6*, *7*). In addition, 9-oxo-2,4,7-triNF is a photoconducting agent, a fungicide, and an industrial chemical reagent (*4*).

The mammary gland of female Sprague-Dawley (SD) rats is the site most susceptible to tumor induction with incidences of 100, 44, and 12.5% after ingestion of 2,7-diNF, 2-NF, and 2,5-diNF, respectively, at 1.62 mmol/kg of diet for 8 months (*8*, *9*). 2,7-DiNF induced mammary tumor incidence and multiplicity equal to that of 2-acetylaminofluorene and also yielded mammary tumors (12.5%) in male rats (*9*). One oral dose of 0.32 mmol of 9-oxo-2,4,7-triNF induced a 35% mammary tumor incidence in female SD rats (*10*). In our recent study involving intramammary injections of 2.04 μmol of a nitrofluorene in 50 μL of dimethyl sulfoxide (DMSO) into each of eight mammary glands per rat, 2,7-diNF exhibited the greatest carcinogenic potency, yielding a 100% mammary tumor incidence with a latent period of 18−48 weeks compared to the 33−87% incidences and latent periods of 22−82 weeks for 2-NF, 9-OH-2-NF, 9-oxo-2-NF, 9-oxo-2,7-diNF, 2,5-diNF, 9-oxo-2,4,7-triNF, or DMSO (*11*). Both 2,7-diNF and 9-oxo-2,7-diNF yielded a greater tumor multiplicity than did DMSO. The carcinogenicity of 2,7-diNF was very similar to that of 6-nitrochrysene and greater than that of 4-nitropyrene tested in DMSO at the same dose level per gland (*12*, *13*). The data suggest that contamination of the environment with certain nitro-PAHs, even at low levels, poses substantial carcinogenic risk which may be relevant for breast cancer (*14*).

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[1] Abbreviations: nitro-PAHs, nitrated polycyclic aromatic hydrocarbons; 2-NF, 2-nitrofluorene; 2,7-diNF, 2,7-dinitrofluorene; 2,5-diNF, 2,5-dinitrofluorene; 2,4,7-triNF, 2,4,7-trinitrofluorene; 2-AF, 2-aminofluorene; 2,7-diAF, 2,7-diaminofluorene; SD, Sprague-Dawley; DMSO, dimethyl sulfoxide; OH, hydroxy; NH_2, amino; IS, internal standard; XO, xanthine oxidase.

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The carcinogenicity of 2,7-diNF by the intramammary route (*11*) indicated that the mammary gland is capable of metabolic activation of this compound and possibly other nitrofluorenes to DNA-reactive species involved in the initiation of tumorigenesis. Nitroreduction appears to play a key role in the activation of nitrofluorenes to mutagens (*15*, *16*) and of 2-NF to an ultimate carcinogen (*4*, *5*, *17*, *18*). In our previous studies, the rates of nitroreduction of nitrofluorenes and C-9-oxidized nitrofluorenes catalyzed by bovine milk xanthine oxidase (XO) and bacterial diaphorase paralleled their reported mutagenicities in *Salmonella typhimurium* (*19*), and nitroreductions of nitrofluorenes catalyzed by mammary gland cytosol suggested one- and/or two-electron reactions (*20*). Preliminary HPLC analyses of the products of nitroreduction revealed that mammary gland cytosol, unlike XO or diaphorase, catalyzes a carbonyl reduction of the 9-oxo group to a 9-OH group as well as nitroreduction. The tumorigenicity or metabolism of 9-OH-2,7-diNF (a new compound synthesized for this study) has not been examined. However, the carcinogenic potential of 9-OH nitrofluorenes is strongly suggested by the findings that 9-OH-2-NF is the most mutagenic metabolite excreted in urine and feces of rats treated with 2-NF (*21*) and treatment of rats with 9-OH-2-NF yields substantial levels of DNA adducts and focal lesions in the liver (*22*). Thus, the aim of the study presented here was to examine the reductive metabolism of 2-NF and 2,7-diNF and their C-9-oxidized, i.e., 9-oxo and 9-OH, derivatives by the rat mammary gland. The effects of nitro and carbonyl reductions on the product formation were determined by HPLC. The cofactor dependence and effects of known inhibitors of reduction were also examined.

Experimental Procedures

Caution: *The nitrofluorenes should be handled according to the NIH Guidelines for the Laboratory Use of Chemical Carcinogens (Publication 81-2385, U.S. Government Printing Office, Washington, DC).*

Reagents. Glass-distilled water was used throughout. Solvents and reagents were of the highest available purity. Aluminum isopropoxide, 2-NF, 2-AF, 2,7-diNF, 2-NH_2-7-NF, 9-oxo-2-NF, 9-oxo-2-AF, 2,7-diaminofluorene (2,7-diAF), and 4-phenylphenol were from Aldrich (Milwaukee, WI), and 9-oxo-2,7-diNF was from Pfaltz and Bauer, Inc. (Waterbury, CT). 9-OH-2-NF, 9-OH-2-AF, and 9-oxo-2-NH_2-7-NF were synthesized as described previously (*19*). All fluorenyl compounds and 4-phenylphenol were recrystallized until pure by HPLC.

Syntheses of 9-OH-2,7-diNF and 9-OH-2-NH_2-7-NF. The 9-oxo compounds were reduced to 9-OH compounds with aluminum isopropoxide (*22*, *23*). All solvents were flushed with N_2 and reduction procedures conducted under N_2. 9-Oxo-2,7-diNF (250 mg, 0.92 mmol) in 90 mL of toluene was refluxed for 2 h with aluminum isopropoxide (550 mg, 2.7 mmol) to give ~90% reduction. The mixture was taken to dryness under reduced pressure in a rotary evaporator. The residue was stirred for 10 min in 20 mL of 1 N H_2SO_4 in 20% 2-propanol. The insoluble material (195 mg) was collected, washed with cold water, and dried over P_2O_5. It was dissolved (1.8 mg/mL) in a benzene/toluene mixture (8:2) by refluxing and cleared by centrifugation. The supernatant was refluxed for ~3 min with a small amount of charcoal, filtered, condensed, and cooled to 4 °C and then to −20 °C. The precipitate was collected and purified by repeated recrystallizations. 9-OH-2,7-diNF (14% yield, mp >300 °C dec) was 98% pure as determined by HPLC: UV/vis λ_{max} 335, 318 (shoulder), 254 nm [ϵ(ethanol) = 23 554, 20 294, and 8247 M^{-1} cm^{-1}, respectively]; FAB-MS 272.1 (M^-), 425.1 [(M + matrix = 3-nitrobenzyl alcohol, 153)$^-$]; HR FAB *m*/*z* 272.0432 (observed), 272.0433 (calcd); ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.41−8.24 (m, 6H, aromatic H), 6.47 (d, H, OH, J = 6.5 Hz), 5.76 (d, H, CH, J = 6.5 Hz). Exchangeable protons were identified by addition of D_2O; the resonance at 6.47 ppm disappeared, and the resonance at 5.76 ppm collapsed to a singlet: ^{13}C NMR (125 MHz, DMSO-d_6) δ 149.7 (C), 148.0 (C), 143.5 (C), 124.9 (CH), 122.8 (CH), 120.3 (CH), 73.0 (CH-OH).

9-Oxo-2-NH_2-7-NF (40 mg, 0.167 mmol) (*19*) was dissolved in 22 mL of toluene under reflux and cleared by centrifugation. The supernatant was mixed with up to 2 portions of aluminum isopropoxide (56 mg, 0.275 mmol) under reflux for 1−2 h to complete reduction, which was monitored by a change of color from red to yellow and TLC on Analtech Uniplate Silica Gel G (Analtech Inc., Newark, DE) developed in an ethyl acetate/*tert*-butyl methyl ether mixture (98:2) with R_f values of 0.64 and 0.72 for 9-OH-2-NH_2-7-NF and 9-oxo-2-NH_2-7-NF, respectively. The mixture was taken to dryness under reduced pressure in a rotary evaporator and extracted in acetone (4.4 mg/mL) under reflux. The solution was evaporated to dryness and the residue redissolved in acetone and subjected to preparative TLC in the system described above. The band with an R_f of 0.64 was extracted in acetone under reflux and the extract evaporated to dryness with N_2 and dried over P_2O_5. 9-OH-2-NH_2-7-NF (16% yield, mp >300 °C dec) was 97% pure as determined by HPLC: UV/vis λ_{max} 400, 257 nm [ϵ(methanol) = 13 250 and 8503 M^{-1} cm^{-1}, respectively]; FAB-MS 242.1 (M^+), 241.1 [(M − H)$^-$]; ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.19 (d, H, C-8, J = 2.0 Hz), 8.17 (dd, H, C-6, J = 8.5, 2.0 Hz), 7.69 (d, H, C-5, J = 8.5 Hz), 7.57 (d, H, C-4, J = 8.5 Hz), 6.82 (d, H, C-1, J = 2.0 Hz), 6.07 (dd, H, C-3, J = 8.5, 2.0 Hz), 5.96 (d, H, OH, J = 7.5 Hz), 5.74 (s, 2H, NH_2), 5.41 (d, H, CH, J = 7.5 Hz). Exchangeable protons were identified by addition of D_2O; resonances at 5.96 and 5.74 ppm disappeared, and the resonance at 5.41 ppm collapsed to a singlet.

Mammary Gland. Female SD rats (Specific Pathogen Free; Harlan Sprague Dawley, Madison, WI) were acclimated for 1 week in a controlled environment with a 12 h light/dark cycle and maintained on a Teklad Certified Rodent Diet (Harlan Teklad, Madison, WI) and water ad libitum. When they were 50−60 days old, rats were decapitated after CO_2 asphyxiation. Mammary glands from individual rats were trimmed of extraneous tissue on an ice block, minced, and rinsed with cold 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl. The mammary tissue was frozen in liquid N_2 and stored at −80 °C for up to 6 months. For tissue fractionation, all buffers were ice-cold and all procedures carried out at 4 °C unless specified otherwise. Frozen mammary gland was pulverized with a Bessman pulverizer, and homogenized in 2 volumes (w/w) of 50 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 1 mM dithiothreitol (DTT) with a Polytron homogenizer type PT10/35 (Kinematica, Gmbh, Lucerne, Switzerland). The homogenate was centrifuged at 14000*g* for 20 min at 4 °C and the solidified fat removed. Cytosolic and microsomal fractions were prepared from the supernatant as described (*24*) immediately before use. Microsomes were stored overnight on ice in homogenization buffer. The amount of protein was determined with Coomassie Plus Protein assay reagent (Pierce, Rockford, IL).

Determination by UV/Vis Spectrometry of the Effect of Modifiers on Reduction of 9-Oxo-2-AF. Cytosolic protein (0.2 mg/mL) was added to cuvettes containing 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT at 37 °C (final volume of 1 mL). To specified incubations was added pyrazole (1 mM), rutin (20 μM), indomethacin (100 μM), or menadione (10 μM) in methanol (final concentration of 1.8%). 5β-Androstan-17β-ol-3-one or 5α-androstane-3,17-dione (100 μM) was added in ethanol (final concentration of 1%). Dicoumarol (100 μM) was added in 0.025 N NaOH (100 μL/mL), and allopurinol (20 μM) was added in buffer. NADH (0.5 mM) was added and the UV/vis absorbance baseline recorded from 260 to 600 nm. 9-Oxo-2-AF (28 μM in 20 μL of DMSO) was added and the absorbance spectrum recorded every 20 s for up to 10 min. The loss of A_{283} of 9-oxo-2-AF and the increase in A_{302} due to 9-OH-2-AF gave

isosbestic points at 290 and 316 nm. The initial rates of increase in A_{302} relative to A_{316} were linear with time and protein concentration and were corrected for changes in the controls which contained inactive cytosol. The rates of change were compared in incubations containing modifier or its solvent. Product formation was confirmed by HPLC analysis of extracts of selected incubations.

Determination of Products from Nitrofluorenes Incubated with Mammary Gland Fractions. Mixtures (1 mL) of 0.2−0.5 mg/mL cytosolic protein or 0.5 mg/mL microsomal protein, 28 μM nitrofluorene compound in 40 μL of DMSO, and 0.5 mM cofactor in 50 mM Tris-HCl buffer (pH 7.4) containing 1 mM DTT were incubated at 37 °C in 2 mL amber vials. Anaerobic incubations contained an O_2-scavenging system consisting of 19 μg/mL catalase, 16 μg/mL glucose oxidase, and 75 μM glucose and were subjected to a gentle stream of argon for 10 min and then 5 min pulses every 15 min (*19*). Controls either contained inactive protein or lacked the cofactor or nitro compound. At the end of timed intervals, 10 nmol of 4-phenylphenol was added as an internal standard (IS) in 10 μL of a 2-propanol/methanol mixture (2:1), and the mixture was immediately applied to a Baker C_{18} extraction column preconditioned with argon-flushed solvent and buffer. After washing with buffer was carried out, the nitrofluorenes and their metabolites were eluted with 5% DMSO in CH_2Cl_2. The eluates were dried over Na_2SO_4, and the CH_2Cl_2 was evaporated at 25 °C under a stream of N_2 passed through an Oxy-Trap (Alltech Associates, Deerfield, IL). Recoveries of all available standard compounds were ≥95% except for 2,7-diNF (~87%). Whereas 9-OH-2,7-diNF was susceptible to oxidation (≤3.5% was detected by HPLC as 9-oxo-2,7-diNF after extraction), 9-OH-2-NF and 9-OH-2-NH_2-7-NF were not recovered as such. Mixtures of mononitrofluorenes and their metabolites were separated on modified HPLC system A (*22*) containing methanol, 2-propanol, and 0.2 M acetic acid (pH 3.8) with gradients of 20 to 40% methanol and 80 to 60% acid within 5 min, 40 to 66% methanol, 0 to 4% 2-propanol, and 60 to 30% acid within 2 min, 66 to 75% methanol, 4 to 5% 2-propanol, and 30 to 20% acid within 3 min, and 75 to 65% methanol, 5 to 10% 2-propanol, and 20 to 25% acid within 6 min. Mixtures of dinitrofluorenes and their metabolites were separated on HPLC system C (*22*) containing methanol, 2-propanol, and 0.2 M acetic acid (pH 4.2) with gradients of 30 to 70% methanol and 70 to 30% acid within 10 min, 70 to 74% methanol, 0 to 6% 2-propanol, and 30 to 20% acid within 3 min, and 74 to 80% methanol, 6 to 10% 2-propanol, and 20 to 10% acid within 3 min. Identification of compounds was based on t_R and UV/vis spectra superimposed with those of their standards and was verified in select incubations by LC/MS or by MS with direct infusion of compounds collected after HPLC separation. The peak areas were integrated in the modified gradient A at 260 nm for IS (t_R = 10.83 min), 280 nm for 9-oxo-2-NF (t_R = 11.66 min), 9-oxo-2-AF (t_R = 9.94 min), 2-AF (t_R = 10.50 min), 300 nm for 9-OH-2-AF (t_R = 9.11 min), 330 nm for 2-NF (t_R = 13.59 min), 9-OH-2-NF (t_R = 10.44 min), and 360 nm for 2-nitrosofluorene (t_R = 13.86 min). In gradient C, peak integration was at 260 nm for 9-oxo-2-NH_2-7-NF (t_R = 12.01 min) and IS (t_R = 12.86 min), 280 nm for 2,7-diAF (t_R = 4.62 min) and 9-oxo-2,7-diNF (t_R = 14.1 min), 340 nm for 9-OH-2,7-diNF (t_R = 11.80 min) (*m/z* 271) and 2,7-diNF (t_R = 14.80 min), and 390 nm for 9-OH-2-NH_2-7-NF (t_R = 9.62 min) (*m/z* 243) and 2-NH_2-7-NF (t_R = 12.48 min). Compounds were quantitated from the peak area relative to standard curves and corrected for recovery of the IS. The HPLC profiles of dinitrofluorenes and their amino derivatives (Figure 1) and the UV/vis spectra recorded during the chromatography (Figure 2) are shown. Initial rates were calculated from time intervals in which product detection was linear with time and protein concentration.

Instrumentation. HPLC data were obtained with a Hewlett-Packard 1090 liquid chromatograph equipped as described previously (*25*). UV/vis data were obtained with Hewlett-Packard 8452A diode array and Beckman UV-70 spectrophotometers. ^{1}H and ^{13}C NMR spectra were obtained at ambient temperature on a 500 MHz Varian Inova spectrometer, and FAB-MS analyses were performed with a VG 7070E-HF mass spectrometer at the Department of Chemistry, University of Minnesota. LC/MS analyses were performed at the University of Minnesota Cancer Center with a Finnigan TSQ 7000 triple-stage quadropole mass spectrometer.

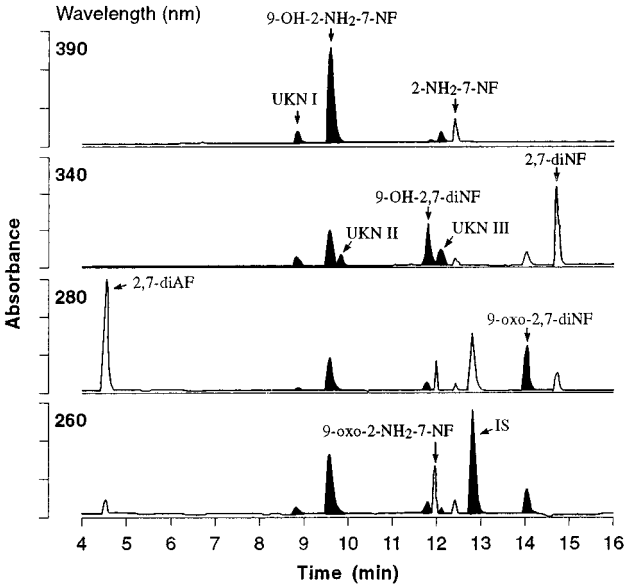

Figure 1. HPLC (system C) profile of the standard dinitrofluorene compounds and their amino derivatives superimposed on the profile (filled peaks) of the extract of an incubation of 9-OH-2,7-diNF with mammary gland cytosol and NADH. Designations of the peaks (UKN is unkown) are at the wavelengths used for integration of the areas.

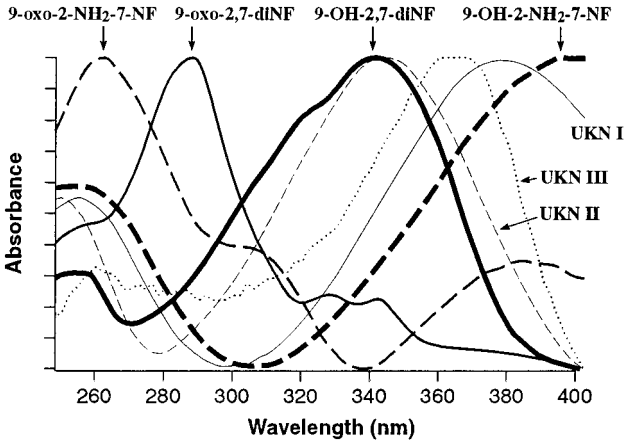

Figure 2. UV/vis absorbance spectra of 9-OH-2,7-diNF and 9-oxo-2,7-diNF, their respective 2-amino derivatives, and unknowns UKN I−III recorded with the diode array detector during HPLC elution.

Statistical Analysis. For comparison of two groups or no fewer than three groups, a two-tailed *t* test or one way ANOVA with Neuman-Keuls post test, respectively, was performed with GraphPad Prism version 2.0 for Macintosh (GraphPad Software, San Diego, CA).

Results

Nitro and 9-Oxoreductions of Nitrofluorenes and 9-Oxonitrofluorenes Catalyzed by Mammary Gland Cytosol. The products of the reduction of nitrofluorenes catalyzed by mammary gland cytosol or microsomes were determined by HPLC analyses. The products from the incubation of 2-NF, 2,7-diNF, 9-oxo-2-NF, 9-OH-2-NF, 9-OH-2,7-diNF, 9-oxo-2,7-diNF, or 9-oxo-2-NH_2-7-NF with

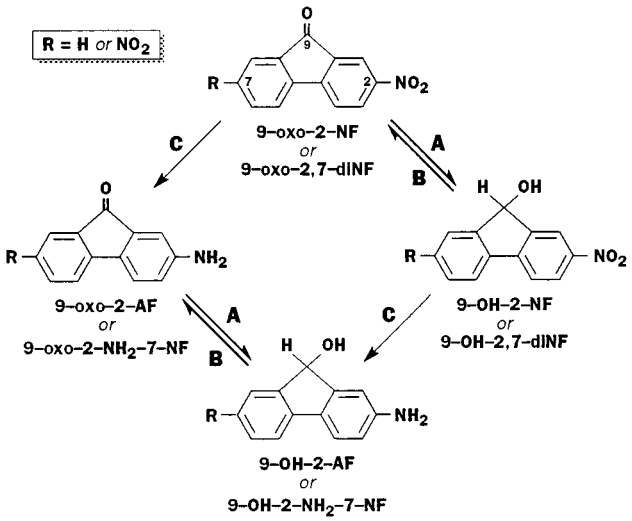

Figure 3. Fate of 9-oxo-2-NF or 9-oxo-2,7-diNF incubated with mammary gland cytosol. Pathway A, carbonyl reductase-dependent, catalyzed by cytosol with NADH or NADPH, but not hypoxanthine. Pathway B, nonenzymic. Pathway C, nitroreductase-dependent, catalyzed by cytosol with NADH, NADPH, or hypoxanthine.

Table 1. Effects of Nitro Groups and the State of Oxidation at C-9 on Reduction of Nitrofluorenes and C-9-Oxidized Nitrofluorenes by Mammary Gland Cytosol

nitrofluorene	product	apparent rate of formation (nmol min^{-1} mg^{-1})[a]
2-NF	2-AF	0.04 ± 0.01
9-OH-2-NF	9-OH-2-AF	0.09 ± 0.02
9-oxo-2-NF	9-OH-2-NF	14.37 ± 4.53
	9-oxo-2-AF	0.21 ± 0.07
	9-OH-2-AF	0.12 ± 0.06
9-oxo-2-AF[b]	9-OH-2-AF	12.83 ± 4.10
2,7-diNF	2-NH_2-7-NF	0.12 ± 0.04
9-OH-2,7-diNF	9-OH-2-NH_2-7-NF	2.99 ± 0.59[c]
9-oxo-2,7-diNF	9-OH-2,7-diNF	0.15 ± 0.25[d]
	9-oxo-2-NH_2-7-NF	0.15 ± 0.09
	9-OH-2-NH_2-7-NF	0.62 ± 0.26[e]
9-oxo-2-NH_2-7-NF	9-OH-2-NH_2-7-NF	≥60

[a] Reductions were determined from incubations of mammary gland cytosol with 28 μM nitrofluorene (4% DMSO) and 0.5 mM NADH at timed intervals as described in Experimental Procedures. Values are means ± SD from duplicate incubations with at least three different cytosols. [b] The product of nitroreduction of 9-oxo-2-NF. [c] Nitroreduction significantly ($P < 0.001$) greater than that of other nitrofluorenes. [d] May be underestimated due to product oxidation. [e] Significantly ($P = 0.0015$) greater than the rate of formation of 9-OH-2-AF from 9-oxo-2-NF.

mammary gland cytosol and NADH indicate reduction of the 9-oxo to 9-OH as well as the reduction of the nitro group to the amine (the structures of the C-9-oxidized substrates and products are shown in Figure 3). Both reductions are dependent on the active enzyme. The nitroreductase activity is partially unstable with losses of ≥30% in cytosols stored overnight in ice or frozen at −80 °C, whereas the 9-oxo reducing activity appears to be stable at -80 °C in the presence of 1 mM DTT. Thus, cytosols were prepared in 1 mM DTT immediately before use. The XO and diaphorase activities in mammary gland cytosols from no fewer than four rats each assayed in duplicate with xanthine (*26*) and menadione (*27*) are 4.98 ± 1.42 and 88.3 ± 15.5 nmol min^{-1} mg^{-1}, respectively.

The rates of both the 9-oxo and nitro reductions are dependent on the state of oxidation at C-9 and the number of nitro groups of the nitrofluorene (Table 1). The rate of NADH-dependent reduction of 9-oxo greatly exceeds that of the nitro group of the mononitro compound, i.e., the formation of 9-OH-2-NF is ∼68-fold faster than that of 9-oxo-2-AF from 9-oxo-2-NF. The effects of oxidation state at C-9 of mono- and dinitrofluorenes differ since total amine formation from 9-oxo-2-NF > 9-OH-2-NF > 2-NF and from 9-OH-2,7-diNF ≫ 9-oxo-2,7-diNF > 2,7-diNF. The addition of a second electron-withdrawing nitro group at the C-7 of 2-NF increases the rate of reduction of the 2-nitro to the 2-amino with the rates of amine formation from 2,7-diNF, 9-OH-2,7-diNF, and 9-oxo-2,7-diNF being greater than those from 2-NF, 9-OH-2-NF, and 9-oxo-2-NF, respectively. The rate of 9-oxoreduction of the 9-oxo-2-AF is similar to that of 9-oxo-2-NF. Although 9-OH-2,7-diNF formation may be underestimated due to its nonenzymatic oxidation and enzymatic conversion to unknowns, the level of 9-OH-2-NH_2-7-NF determined from 9-oxo-2,7-diNF relative to those from 9-OH-2,7-diNF and 9-oxo-2-NH_2-7-NF suggests that the 9-oxoreduction is enhanced by the presence of the 2-amino group. 2-NF or 2-AF is not detected in incubations of 9-oxo-2-NF or 9-OH-2-NF with mammary gland cytosol, nor is 2,7-diNF or 2-NH_2-7-NF detected from 9-oxo-2,7-diNF, 9-OH-2,7-diNF, or 9-oxo-2-NH_2-7-NF. No 2,7-diAF is detected from the dinitro compounds.

Table 2. Cofactors Utilized by Mammary Gland Cytosol or Microsomes for Reductions of 9-Oxonitrofluorenes[a]

	reduction of NO_2 to NH_2		reduction of 9-oxo to 9-OH	
cofactor (0.5 mM)	9-oxo-2-NF	9-oxo-2,7-diNF	9-oxo-2-NF	9-oxo-2,7-diNF
cytosol				
NADH	1	1	1	1[b]
NADPH	0.62[c]	0.74	0.55	1.11[b]
hypoxanthine	1.76	0.54	0	0
2-hydroxypyrimidine	−[d]	0.53	−	0
xanthine	−	0.35	−	0
N′-methylnicotinamide	−	0.25	−	0
microsomes				
NADH	1	−	1	−
NADPH	0.56	−	4.8	−
hypoxanthine	0.1	−	0	−

[a] Mammary gland cytosol (0.2 mg/mL) or microsomes (0.5 mg/mL) from at least two separate rats were incubated in duplicate under argon for 30 or 60 min, respectively, and products were identified and quantitated as described in Experimental Procedures. [b] 9-OH-2,7-diNF may be underestimated due to oxidation. [c] Values represent the ratios of the amounts of total (9-OH + 9-oxo) amine or total (NO_2 + NH_2) 9-OH products relative to those with NADH. [d] Not determined.

Cofactor Requirements for Nitro and 9-Oxo-reductions of 9-Oxonitrofluorenes Catalyzed by Mammary Gland Cytosol and Microsomes. Like NADH, NADPH supports cytosol-catalyzed reductions of nitro to amino and 9-oxo to 9-OH groups (Table 2). Compared to NADH, NADPH is a poorer electron donor for both nitro and 9-oxoreductions with 9-oxo-2-NF. Hypoxanthine, xanthine, *N′*-methylnicotinamide, and 2-hydroxypyrimidine support cytosol-catalyzed nitro but not 9-oxoreductions. The microsomal fraction of mammary gland contains relatively low levels of reductase activity, i.e., NADH- and NADPH-supported rates of conversion of 9-oxo-2-NF to amine and 9-OH products are ∼24 and 1%, respectively, of those of cytosol (data not shown). Hypoxanthine supports only minimal microsome-catalyzed amine formation (∼10% of the NADH-supported rate) from 9-oxo-2-NF.

Table 3. Cofactor Dependence of Reduction Products Formed in Incubations of C-9-Oxidized Nitrofluorenes with Mammary Gland Cytosol[a]

substrate	time (min)	product	nmol of product[b]		
			NADH	NADPH	hypoxanthine
9-oxo-2-NF	30	9-OH-2-NF	25.08 ± 4.02	14.02 ± 1.01	0
		9-oxo-2-AF	0.145 ± 0.095	0.37 ± 0.13	1.80 ± 0.94[c]
		9-OH-2-AF	0.88 ± 0.473	0.263 ± 0.018[d]	0
9-OH-2,7-diNF	30	9-oxo-2-NH_2-7-NF	0.223 ± 0.310	−[e]	1.58 ± 0.72[f]
		9-OH-2-NH_2-7-NF	12.65 ± 1.52	−	8.20 ± 3.29[d]
9-oxo-2,7-diNF	30	9-OH-2,7-diNF	0.127 ± 0.284[g]	2.32 ± 0.69[g]	0
		9-oxo-2-NH_2-7-NF	0.377 ± 0.300	0	3.77 ± 2.46[d]
		9-OH-2-NH_2-7-NF	6.60 ± 3.68	5.18 ± 0.91	0
9-oxo-2-NH_2-7-NF	10	9-OH-2-NH_2-7-NF	17.75 ± 2.60	−	0

[a] Mammary gland cytosol (0.2 mg/mL) was incubated under anaerobic conditions with 28 μM substrate (4% DMSO) and 0.5 mM cofactor; the products were analyzed by HPLC as described in Experimental Procedures. [b] Values are means ± SD with cytosols from at least two separate rats, each incubated in duplicate. [c] Statistically different ($P < 0.01$) from the product with NADH or NADPH. [d] Statistically different ($P \leq 0.025$) from the product with NADH. [e] Not determined. [f] Statistically different ($P \leq 0.005$) from the product with NADH. [g] May be underestimated due to product oxidation.

Table 4. Effects of Modifiers on Reductions of Nitro and 9-Oxo Groups of Nitro and Amino Fluorenes by Mammary Gland Cytosol[a]

modifier (μM)	reduction of NO_2 to NH_2		reduction of 9-oxo to 9-OH	
	substrate	inibition (%)[b]	substrate	inhibition (%)[b]
O_2	9-OH-2-NF, 9-oxo-2-NF,	93 ± 14	9-oxo-2-AF, 9-oxo-2-NF	0
dicoumarol (100)	9-OH-2-NF, 9-oxo-2-NF, 2,7-diNF, 9-OH-2,7-diNF	64 ± 17	9-oxo-2-AF, 9-oxo-2-NF, 9-oxo-2-NH_2-7-NF	39 ± 26
menadione (10)	9-oxo-2,7-diNF[c]	0	9-oxo-2-AF	0
allopurinol (20)	2,7-diNF	88 ± 3	9-oxo-2-AF	0
rutin (50)	9-OH-2-NF, 2,7-diNF	27 ± 6	9-oxo-2-AF, 9-oxo-2-NF, 9-oxo-2-NH_2-7-NF, 9-oxo-2,7-diNF	68 ± 17
pyrazole (1000)	9-oxo-2-NF, 2,7-diNF	0	9-oxo-2-AF, 9-oxo-2-NF	0
indomethacin (100)	−[d]		9-oxo-2-AF	24 ± 11
5β-androstan-17β-ol-3-one (100)	−[d]		9-oxo-2-AF	15 ± 14
5α-androstane-3,17-dione (100)	−[d]		9-oxo-2-AF	7 ± 7

[a] Reductions of nitro and 9-oxo groups were assessed by UV/vis and/or HPLC analyses in incubations containing 28 μM substrate and 0.5 mM NADH, unless specified otherwise, with mammary gland cytosol as described in Experimental Procedures. [b] Inhibition of initial rates of cytosol-dependent changes in reduction with two to seven cytosols, each incubated in duplicate. [c] Determined with 0.5 mM *N*′-methylnicotinamide as a cofactor. [d] Not determined.

The effect of the electron donor on the amounts of products depends on the substrate as shown with 9-oxo-2-NF, 9-OH-2,7-diNF, 9-oxo-2,7-diNF, and 9-oxo-2-NH_2-7-NF with NADH, NADPH, or hypoxanthine as a cofactor for the cytosol-catalyzed reductions (Table 3). After 30 min, the total amount of amine from 9-oxo-2-NF with hypoxanthine is ∼1.8- or 2.8-fold greater than with NADH or NADPH, respectively, and the amount of 9-OH products is greater with NADH than with NADPH. From 9-OH-2,7-diNF, the amounts of amine are slightly greater with NADH than with hypoxanthine (12.87 vs 9.78 nmol). The extent of reduction of 9-oxo-2,7-diNF to amine is 2-fold greater with NADH than with hypoxanthine (6.98 vs 3.77 nmol) and similar to that with NADPH. Hypoxanthine does not support any 9-oxoreduction. Levels of 9-oxo-2-NH_2-7-NF from 9-OH-2,7-diNF are higher with hypoxanthine as a cofactor than with NADH and do not correlate with the levels of 9-OH-2-NH_2-7-NF. At shorter incubation times, no 9-oxo-2-NH_2-7-NF is detected from 9-OH-2,7-diNF even though large amounts of the 9-OH-2-NH_2-7-NF are formed, suggesting that little or no oxidation of the latter occurs during incubation or extraction (Tables 1 and 3). Thus, it appears that the 9-oxo-2-NH_2-7-NF detected in incubations of cytosol with 9-OH-2,7-diNF is derived from 9-oxo-2,7-diNF formed during the incubation.

In addition to the amine, 9-OH-2,7-diNF incubated with active cytosol and NADH or hypoxanthine yields unknown products that can be detected with HPLC system C: UKN I (t_R = 8.8 min; λ_{max} = 375 nm), UKN II (t_R = 9.8 min; λ_{max} = 345 nm), and UKN III (t_R = 12.1 min; λ_{max} = 370 nm) (Figures 1 and 2). The peak areas of UKN I−III in the fresh extract from a 30 min anaerobic incubation of 9-OH-2,7-diNF with 0.2 mg/mL cytosolic protein and 0.5 mM NADH are ∼4, 15, and 15%, respectively, of that of 9-OH-2-NH_2-7-NF. With hypoxanthine as a cofactor, the respective areas are 18-, 0.67-, and 3.5-fold as great as with NADH. The same unidentified peaks are also found from incubations of cytosol with 9-oxo-2,7-diNF and NADH, but not hypoxanthine. However, they are not found in incubations of 9-oxo-2-NH_2-7-NF with cytosol and either cofactor or in controls with inactive cytosol. The data suggest that these unknowns represent or are derived from intermediates of nitroreduction of 9-OH-2,7-diNF, e.g., 9-OH-2-nitroso-7-NF and/or 9-OH-2-NHOH-7-NF. Because of the small amounts, the parent ions could not be unequivocally identified from the LC/MS spectra, and the peaks from HPLC were unstable to collection and lyophilization.

Effects of Modifiers on Mammary Gland Cytosol-Catalyzed Nitro and 9-Oxoreductions of Fluorenyl Compounds. Since the use of different cofactors in the reductions of nitrofluorenes and C-9 oxidized nitrofluorenes suggests involvement of several enzymes in the reductions of the nitro and 9-oxo groups, the effects of known modifiers of enzymic nitro and carbonyl reductions on the initial rates of these reductions catalyzed by mammary gland cytosol were examined with NADH as a cofactor (Table 4). Nitroreduction is inhibited by O_2,

dicoumarol (100 μM), allopurinol (20 μM), and rutin (50 μM) by 93, 64, 88, and 27%, respectively. Menadione (10 μM) has no effect on nitroreduction when *N′*-methylnicotinamide replaces NADH. Reduction of the 9-oxo group is most sensitive to rutin (50 μM) (68% inhibition) and is also inhibited 39% by dicoumarol (100 μM) and 24% by indomethacin (100 μM). In contrast to nitroreduction, reduction of 9-oxo is insensitive to O_2 and allopurinol (20 μM). Reduction of the 9-oxo group is also unaffected by menadione (10 μM). 5α-Androstane-3,17-dione (100 μM) and 5β-androstan-17β-ol-3-one (100 μM), substrates for carbonyl reducing steroid dehydrogenases, did not act as competitive substrates for the reduction of 9-oxo-2-AF. Pyrazole (1000 μM) has no effect on reduction of the nitro or 9-oxo group.

Discussion

This study shows that the cytosolic and microsomal fractions of the rat mammary gland catalyze NADH- and NADPH-dependent reductions of the nitrofluorenes to aminofluorenes and 9-oxonitrofluorenes to the 9-OH-nitro- and/or 9-OH-aminofluorenes. The cytosolic fraction contains greater carbonyl and nitro reducing activities for the C-9-oxidized nitrofluorenes than the microsomal fraction. The effects of the structure of the nitrofluorenes are similar for nitroreduction catalyzed by mammary gland cytosol (Table 1), XO, or diaphorase (*19*) and for the nitrofluorene-stimulated reduction of acetylated cytochrome *c* catalyzed by mammary gland cytosol (*20*). Thus, the rate of reduction of nitrofluorene to amine catalyzed by the cytosol of the mammary gland is greater for the 2,7-diNF than for the 2-NF compounds and is greater with increasing oxidation state at C-9. However, 9-OH-2,7-diNF (not available for the previous studies) is converted to amine faster than is 9-oxo-2,7-diNF, even though their rates of one-electron nitroreduction catalyzed by XO or cytosol and monitored with acetylated cytochrome *c* are similar (data not shown), suggesting that the one-electron nitroreduction is not rate-limiting for amine formation. No evidence for reduction of both nitro groups of 2,7-diNF was found. Several mammalian enzymes catalyze nitroreductions, including aldehyde oxidase, cytochrome *c* reductase, NADPH cytochrome P450 reductase, XO, and diaphorase (*28*). The effects of electron donors and reduction modifiers on product profiles catalyzed by the mammary gland cytosol suggest that more than one enzyme contributes to the nitroreduction of the nitrofluorenes. The mammary gland cytosol-catalyzed nitroreduction of 9-oxo-2,7-diNF is supported with NADH, NADPH, *N′*-methylnicotinamide, 2-hydroxypyrimidine, xanthine, or hypoxanthine as the electron donor (Table 2). XO also catalyzes 9-oxo-2,7-diNF reduction with NADH, NADPH, hypoxanthine, and 2-hydroxypyrimidine, but not with *N′*-methylnicotinamide (our unpublished data). The extents of utilization of xanthine, hypoxanthine, and 2-hydroxypyrimidine (Table 2) and inhibition by allopurinol (Table 4) (*29*) as well as the demonstration herein of XO activity in the mammary gland cytosol suggest its substantial contribution to the cytosolic NADH-dependent nitroreduction. Although aldehyde oxidase, unlike XO, utilizes *N′*-methylnicotinamide, the lack of inhibiton by menadione of the *N′*-methylnicotinamide-dependent reduction (*29*) contraindicates its involvement in the cytosol-catalyzed nitroreduction of the nitrofluorenes (Table 4). This latter activity may be due to a diaphorase isozyme since a purified DT diaphorase [NAD(P)H:quinone oxidoreductase] from rat tumor was found to be nonspecific in its requirement of reduced pyridinium electron donors utilizing 1-methylnicotinamide as efficiently as NADH (*N′*-methylnicotinamide was not evaluated) (*30*). The nitroreduction of the nitrofluorene compounds by bacterial diaphorase (*19*), the demonstration herein of diaphorase activity in the mammary gland cytosols, and the dicoumarol inhibition of cytosol-catalyzed amine formation (Table 4) and of the nitrofluorene-dependent reduction of acetylated cytochrome *c* (*20*) suggest that diaphorase may contribute to the cytosol-catalyzed nitroreduction of the nitrofluorenes. However, dicoumarol also inhibits other enzymes, including XO (ref *31* and our unpublished data). Whether the weak inhibition of nitroreduction by the antioxidant rutin (50 μM), a flavone glucuronide, is a function of enzyme inhibition is unclear. The lack of inhibiton by pyrazole (Table 4) indicates that alcohol dehydrogenase is not involved in nitroreduction (*32*). On the basis of O_2 uptake during NADH-dependent 9-oxo-2,4,7-triNF reduction catalyzed by mammary gland cytosol and microsomes (*20*) and on the basis of the inhibition by O_2 of amine formation from nitrofluorenes catalyzed by diaphorase and XO (*19*), the inhibition by O_2 herein (Table 4) is attributed to its interaction with nitro anion radical and other O_2-sensitive intermediates. Microsomal nitroreduction may be linked to the NADPH cytochrome P450 and NADH cytochrome *c* reductases found in rat mammary gland (*33*). Although microsomal nitroreduction of 9-oxo-2-NF seems to prefer NADH, it may be underestimated with NADPH because of the rapid carbonyl reduction.

Reduction of the 9-oxo group differs from that of the nitro group in the effects of electron donors, O_2, and inhibitors. Only NADH and NADPH support 9-oxoreduction (Table 3), indicating that neither XO nor aldehyde oxidase is a catalyst (*34*). The lack of involvement of XO in 9-oxoreduction is also supported by the insensitivity to allopurinol of cytosol-catalyzed reduction and the lack of 9-oxoreduction of the nitrofluorenes by milk XO (*19*). Likewise, the lack of inhibition by pyrazole indicates that alcohol dehydrogenase is not involved in 9-oxoreduction. Among the many enzymes associated with carbonyl reduction in human and rat liver (*35*), dicoumarol-sensitive DT diaphorase and flavone-sensitive carbonyl reductase have received special attention because of their potential involvement in xenobiotic metabolism. DT diaphorase has been identified in rat and human mammary tissue (*36*, *37*) and carbonyl reductase in human breast tissue (*37*). The mammary gland cytosolic carbonyl reducing activity with the 9-oxonitrofluorenes is partially inhibited by 100 μM dicoumarol and strongly inhibited by flavones (Table 4). Recently, two enzymes were purified from rat liver cytosol that catalyzed reduction of quinones, aldehydes, and ketones, including stereoselective carbonyl reduction (*38*). One enzyme utilized NADH and NADPH, while the other preferred NADPH. Both enzymes were inhibited by quercitrin; neither was inhibited by pyrazole and only the former by dicoumarol. The dicoumarol-insensitive, NADPH-specific enzyme catalyzed reduction of 5α- and 5β-3-keto steroids and resembled 3α-hydroxysteroid dehydrogenase in catalysis and inhibition. Although 3α-hydroxysteroid dehydrogenase is present in the rat mammary gland (*39*), the low level of inhibition by

indomethacin and the ineffectiveness of the 3-keto androstanes as competitive substrates of 9-oxo-2-AF (Table 4) (*38*, *40*) suggest little contribution from this reductase to the NADH-dependent cytosol-catalyzed carbonyl reduction of 9-oxo-2-AF. Thus, the activity of the mammary gland catalyzing the NADH-dependent reduction of the 9-oxo group resembles the liver activity that is partially sensitive to dicoumarol, is sensitive to flavone, and utilizes both NADH and NADPH. This activity appears to be different from the quinone reducing diaphorase activity purified from rat liver, which is inhibited by 1 μM dicoumarol, but not by 10 μM rutin (*41*). An exception to the cytosolic location of most carbonyl reducing activities is 11β-hydroxysteroid dehydrogenase-1, which is located in the endoplasmic reticulum and catalyzes carbonyl reduction of steroids and nonsteroids (*42*). Like 11β-hydroxysteroid dehydrogenase, the microsomal carbonyl reductase activity from mammary gland utilizes NADPH and NADH with a preference for NADPH (Table 2). Thus, the data indicate that the mammary gland of the rat has a powerful capacity to catalyze the carbonyl- and nitroreduction of 9-oxonitrofluorenes via different reductases. The relative extents of the reductions may depend on tissue O_2 levels, cofactor availability, and the substrate with possible consequences for carcinogenicity.

Activation of nitrofluorenes to DNA-reactive species may be determined by both carbonyl- and nitroreduction. DNA adducts consistent with nitroreduction were isolated from the mammary gland of rats treated with 1-nitropyrene, 4-nitropyrene, or 1,6-dinitropyrene (*18*, *43*−*47*). However, specific hydroxylations of 1-nitropyrene (*48*) increased or decreased the extent of reactivity with DNA, and nitroreduction of ring-oxidized 1-nitropyrene was suggested to account for the mixture of adducts found in the mammary gland of 1-nitropyrene-treated rats (*44*, *45*). Oxidation of nitrofluorenes at C-9 could also alter the levels and characteristics of the DNA adducts. Oral administration of 9-OH-2-NF yielded a unique, as yet unidentified, hepatic DNA adduct (*22*). In addition, adducts which appeared similar on HPLC and TLC separation to those from liver of rats treated with 2-NF, including the dG-C8-AF, were found, suggesting reduction of 9-oxo to $-CH_2-$ before or after adduct formation. Although reduction of 9-oxo or 9-OH to $-CH_2-$ is not observed under the conditions of the in vitro incubations with mammary gland herein, such reduction may occur in vivo and be revealed by analyses of the metabolites and/or DNA adducts formed in the rat. Upon intramammary application, 2,7-diNF and 9-oxo-2,7-diNF were carcinogenic at the site of application (*11*). Although two, as yet unidentified, DNA adducts were detected in liver of male rats treated with 2,7-diNF (*49*), adducts in mammary gland from nitrofluorenes have not been analyzed. Determination of DNA adducts of 2,7-diNF, 9-OH-2,7-diNF, and 9-oxo-2,7-diNF formed in the mammary gland is needed to elucidate the roles of C-9 oxidation and carbonyl and nitro reductions in activation of the nitrofluorenes.

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