Biochemical Pharmacology, Vol. 32, No. 22, pp. 3335–3344, 1983.
Printed in Great Britain.

0006-2952/83 $3.00 + 0.00

MODIFICATIONS OF CARCINOGEN METABOLISM IN HEPATIC MICROSOMES OF SUCKLING YOUNG BY 3-METHYLCHOLANTHRENE OR β-NAPHTHOFLAVONE ADMINISTERED TO LACTATING RATS*†

DANUTA MALEJKA-GIGANTI,‡ RICHARD W. DECKER and CLARE L. RITTER

Laboratory for Cancer Research, Veterans Administration Medical Center, and Department of Laboratory Medicine and Pathology, University of Minnesota, Minneapolis, MN 55417, U.S.A.

(*Received* 19 *November* 1982; *accepted* 8 *April* 1983)

Abstract—The effects of treating lactating rats with 3-methylcholanthrene (3-MC) or β-naphthoflavone (β-NF) (three i.p. injections of 20 or 40 mg compound/kg of body weight) on hepatic microsomal enzymes of their suckling young were examined. This treatment had no apparent effect on the contents of cytochromes P-450 and b_5 or on the activities of NADH- and NADPH–cytochrome *c* reductases in hepatic microsomes of the pups. However, these microsomes had 8- and 6-fold increased capacities for hydroxylations of benzo[*a*]pyrene (B[*a*]P) and *N*-2-fluorenylacetamide (2-FAA) respectively. These increases were about 5-fold greater in the hepatic microsomes of the dams, in which they were inhibited by 0.1 mM α-naphthoflavone (α-NF) *in vitro* 72–81 and 89–95% and by 0.1 mM β-NF *in vitro* 12–41 and 60–76% respectively. In the pups, the induced activities were also inhibited, whereas the basal hydroxylations of B[*a*]P and 2-FAA were stimulated by α-NF 2.7- and 5.0-fold and by β-NF 1.4- and 2.4-fold respectively. The inhibition of the induced hydroxylations by α-NF and β-NF may be explained by their higher affinities (K_s, 0.14 and 0.28 μM, respectively) than those of B[*a*]P and 2-FAA (K_s, 4.4 to 8.8 and 2.4 to 3.1 μM, respectively) for cytochrome P-450. Whereas β-NF gave a type I binding spectrum, α-NF gave a spectrum composed of type I and reverse-type I elements. Analysis of metabolites of 2-FAA showed differences in their type and amounts formed by hepatic microsomes of β-NF-treated lactating rats and their pups. Thus, in the dams the formation of 1-, 3-, 5-, 7-, 9- and N-hydroxy-2-FAA was increased by 9-, 30-, 40-, 5-, 20- and 5-fold respectively. In the pups, the formation of 1-, 3-, 5-, 7- and N-hydroxy-2-FAA was increased by 2-, 30-, 18-, 4- and 27-fold respectively. All these increased hydroxylations were inhibited by 0.1 mM α-NF *in vitro*. In the hepatic microsomes of pups from the corn oil-treated dams, α-NF stimulated all ring-hydroxylations, but not N-hydroxylation of 2-FAA. The results support earlier findings that microsomal enzymes differ in immature and mature rat liver and suggest that N-hydroxylation of 2-FAA, the activation required for carcinogenesis, and specific ring-hydroxylations are catalyzed by different cytochrome P-450 isozymes. Our studies showed that 3-MC and β-NF and/or their metabolites were transferred with milk of dams to their suckling pups in which they modified metabolism of carcinogens.

Lactational transmittal of pharmacologically active compounds and the resultant changes in xenobiotic metabolism in suckling young have generated considerable interest [1–6]. We reported recently that treatment of lactating rats with 3-MC§ or β-NF increased hydroxylations of the carcinogens B[*a*]P and 2-FAA in hepatic and mammary gland microsomes [7]. This suggested that the inducers and/or their metabolites may be excreted with milk and possibly modify metabolism of the carcinogens in the suckling young. Since the primary metabolism of carcinogens involves their oxidation by a cytochrome P-450‖ containing MFO with possible participation of the cytochrome b_5 system [8, 9], we examined the effects of treatment of dams with 3-MC or β-NF on these enzyme systems and on hydroxylations of B[*a*]P and 2-FAA in hepatic microsomes of their pups. It was shown that addition of α-NF to hepatic microsomes stimulated hydroxylation of B[*a*]P in newborn rats, had little effect on it in mature females, and inhibited it in rats pretreated with 3-MC [10]. Since α-NF differentiated between the basal and induced MFO activities, we used this compound to show the lactational induction of hydroxylations of B[*a*]P and 2-FAA in the livers of pups of 3-MC- or β-NF-treated lactating rats. We

* This investigation was supported by the U.S. Veterans Administration and by PHS Grant CA-28000 awarded by the National Cancer Institute, DHHS.

† Presented in part at the Sixty-sixth Annual Meeting of the Federation of American Societies for Experimental Biology (FASEB) in New Orleans, LA, April 15–23, 1982.

‡ Address all correspondence to: Dr. D. Malejka-Giganti, Laboratory for Cancer Research, Veterans Administration Medical Center, 54th and 48th South, Minneapolis, MN 55417.

§ Abbreviations: 3-MC, 3-methylcholanthrene; β-NF, β-naphthoflavone or 5,6-benzoflavone; B[*a*]P, benzo[*a*]pyrene; 2-FAA, *N*-2-fluorenylacetamide; MFO, mixed function oxidase; α-NF, α-naphthoflavone or 7,8-benzoflavone; DTT, dithiothreitol; PB, phenobarbital; DMSO, dimethyl sulfoxide; HPLC; high performance liquid chromatography; 1-, 3-, 5-, 7-, 9- or N-OH-2-FAA, 1-, 3-, 5-, 7-, 9- or *N*-hydroxy-*N*-2-fluorenylacetamide; 2-FA, *N*-2-fluorenamine; and CO, carbon monoxide.

‖ In this paper, the term cytochrome P-450 refers to multiple isozymes present in hepatic microsomes of corn oil-, 3-MC-, β-NF- or PB-treated lactating rats and their suckling young as specified in the text.

also compared the *in vitro* effects of α-NF to those of β-NF on the hydroxylations of the two carcinogens. In addition, we examined the *in vitro* effects of α-NF on the metabolites of 2-FAA formed by hepatic microsomes of the uninduced and induced dams and their pups.

The production of a difference spectrum on the addition of substrates to hepatic microsomes indicates binding of the substrate to cytochrome P-450, the terminal oxidase of the microsomal electron transport chain [11, 12]. The spectral dissociation constant, K_s, is regarded as a measure of the affinity of a substrate for cytochrome P-450 [13]. To better understand interactions with cytochrome P-450 of α-NF, β-NF and the carcinogenic substrates, we compared their binding and affinities in the hepatic microsomes from lactating rats and their pups.

MATERIALS AND METHODS

Labeled and unlabeled compounds. B[*a*]P, 2-FAA and 4-hydroxybiphenyl were obtained from the Aldrich Chemical Co., Milwaukee, WI. B[*a*]P, m.p. 182–183°, was mixed with [7,10-^{14}C]B[*a*]P (16 mCi/mmole) (California Bionuclear Corp, Sun Valley, CA) to obtain the desired activity of the labeled substrate for the enzyme assays. 2-FAA and 4-hydroxybiphenyl were recrystallized from ethanol–water (7:3) and had m.p. of 196–198° and 163° respectively. 2-[1′-^{14}C]FAA (1.30 mCi/mmole), m.p. 194–196°, was prepared as described previously [14]. 3-MC, m.p. 181–182°, was obtained from the Eastman Kodak Co., Rochester, NY. DTT was from the Calbiochem-Behring Corp., LaJolla, CA. α-NF, m.p. 154–156°, β-NF, m.p. 163–165°, hexobarbital, cytochrome *c* from horse heart (type III), NADH, NADPH and dithionite were from the Sigma Chemical Co., St. Louis, MO. PB, sodium salt, USP grade, was from the Ganes Chemical Co., Carlstadt, NJ. DMSO, glass distilled, was from Matheson Coleman & Bell Manufacturing Chemists, Inc., Cincinnati, OH, and glycerol was from the Fisher Scientific Co., Pittsburg, PA.

Animals, maintenance and treatment. Pregnant Sprague–Dawley rats (Holtzman Co., Madison, WI), 3 to 4-months-old, were maintained on regular Purina Chow pellets and water *ad lib.* in individual breeding boxes. Rats from the same source were also mated in this facility, and pregnant dams were kept in the breeding boxes. In the majority of dams, the number of offspring was adjusted to twelve after delivery. The lactating rats were injected i.p. for 2 or 3 consecutive days before sacrifice with 3-MC or β-NF dissolved in corn oil at concentrations of 8 or 16 mg/ml to achieve dose levels of 20 or 40 mg/kg of body weight. Control rats were injected i.p. with corn oil at 2.5 ml/kg of body weight. One lactating rat received 0.1% PB, sodium salt, in drinking water for 5 days. Food was withdrawn 12 hr before decapitation. Pups were also decapitated.

Preparation of microsomal fractions. All buffers were cooled in ice, and all procedures were carried out at 4°. The livers from lactating rats and pups were perfused with 154 mM KCl, 50 mM Tris–HCl buffer, pH 7.4, excised, and immersed in the same buffer. Livers of pups from individual lactating rats were pooled. After removal of connective tissue, the livers were minced in four parts (w/w) of the same buffer and homogenized with a Polytron homogenizer (type PT 10, Kinematica GMBH, Lucerne, Switzerland) for three 3-sec intervals at speed setting 3.0. The homogenates were centrifuged at 600 *g* for 10 min, and the supernatant fractions were centrifuged at 16,000 *g* for 20 min to separate mitochondria. The supernatant fractions were then centrifuged at 105,000 *g* for 60 min to separate the microsomes. To further remove hemoglobin, microsomal pellets were suspended in 1 mM Tris–HCl buffer, pH 7.4, and homogenized gently in a Teflon pestle homogenizer [15]. The suspensions were centrifuged at 105,000 *g* for 60 min. The washed pellets were then suspended to a protein concentration of about 30 mg/ml in 10 mM potassium phosphate, 2 mM $MgCl_2$, 2 mM DTT buffer, pH 7.5, containing 20% glycerol.

Determination of protein, cytochromes b_5 *and P-450, and cytochrome* c *reductase activities.* Protein was determined by the procedure of Lowry *et al.* [16] with a bovine serum albumin standard. For spectrophotometric determinations of cytochromes, the microsomal suspensions were diluted with 300 mM sodium phosphate buffer, pH 7.4, containing 50% glycerol to protein concentrations of 1 mg/ml. Cytochrome b_5 was determilned from the NADH-reduced spectrum (ε, 185 mM^{-1} cm^{-1}) and cytochrome P-450 from the reduced hemoprotein–CO complex (ε, 91 mM^{-1} cm^{-1}) [17]. NADH- and NADPH-cytochrome *c* reductase activities were measured essentially as described [18], using 300 mM potassium phosphate buffer, pH 7.5, containing 0.10 mM EDTA, 0.07 mM cytochrome *c* and 0.12 mM NADH or 0.10 mM NADPH in a 1.0 ml volume. A Hitachi 110 computerized double beam ratio recording spectrophotometer was used for all determinations.

Determination of hydroxylations of [7,10-^{14}*C]B*[a]*P and* 2-[1′-^{14}*C]FAA by hepatic microsomes and of the effects of α-NF and β-NF on these reactions.* The composition of the incubation mixtures and the radioactive assays for hydroxylations of [7,10-^{14}C]B[*a*]P and 2-[1′-^{14}C]FAA were described previously [7]. The microsomal suspensions were preincubated with 0.1 mM α-NF or β-NF in 0.01 ml of 95% ethanol or the solvent alone for 5 min at 37° before the additions of the ^{14}C-labeled substrates and NADPH. Incubations to measure hydroxylating activities of hepatic microsomes from lactating rats and their pups were carried out for 10 and 20 min respectively. The hexane and ethyl ether used for extraction of the incubation mixtures were separated from aqueous phases by freezing the latter [19]. Radioactivity of NaOH solutions containing the hydroxylated metabolites of [7,10-^{14}C]B[*a*]P and 2-[1′-^{14}C]FAA was determined as described previously [7].

Determination of metabolites of 2*-FAA formed by hepatic microsomes of lactating rats and their suckling young.* Incubation mixtures consisted of 6.0 mg microsomal protein, preincubated with 0.1 mM α-NF in 0.05 ml of 95% ethanol or the solvent alone for 5 min at 37°, 200 nmoles 2-FAA in 0.1 ml methanol, and 4 μmoles NADPH in a total volume of

Table 1. Effects of treatment of lactating rats with 3-MC or β-NF on the body and liver weights and the hepatic microsomal enzymes of their suckling young

Treatment of lactating rats			Age of pups at sacrifice (day)	Avg. body weight/pup (g ± S.D.)	Avg. liver weight/pup (mg ± S.D.)	Cytochrome†		NADH-cytochrome *c* reductase† (nmoles/mg protein/min)	NADPH-cytochrome *c* reductase† (nmoles/mg protein/min)
No. of rats	Compound	Dose* (mg/kg body wt)				b_5 (pmoles/mg protein)	P-450 (pmoles/mg protein)		
2	Corn oil	2 ×	3–4	10.4 ± 2.9 (26)‡	331 ± 59	112 ± 48	458 ± 88	166 ± 61	98 ± 11
2	β-NF	2 × 40	3–4	7.8 ± 0.28 (23)	226 ± 78	110 ± 14	355 ± 92	91 ± 20	92 ± 7
3	Corn oil	3 ×	5	13.0 ± 0.30 (37)	410 ± 45	134 ± 59	499 ± 143	253 ± 12	90 ± 27
2	3-MC	3 × 20	4–5	9.45 ± 2.2§ (28)	248 ± 78§	168 ± 26	416 ± 12	272 ± 11	108 ± 3
2	3-MC	3 × 40	5	10.0 ± 1.6‖ (24)	296 ± 30§	120 ± 20	540 ± 60	254 ± 26	101 ± 15
3	Corn oil	2 ×	6	15.5 ± 0.36 (32)	472 ± 44	201 ± 86	578 ± 214	273 ± 45	91 ± 37
2	3-MC	2 × 20	6	11.4 ± 1.0¶ (22)	308 ± 46‖	136 ± 9	500 ± 126	150 ± 40	93 ± 13
3	Corn oil	3 ×	8–9	17.5 ± 1.4 (28)	460 ± 66	220 ± 111	533 ± 221	244 ± 107	100 ± 60
2	3-MC	3 × 20	8–9	13.8 ± 0.10‖ (23)	369 ± 8	284 ± 48	640 ± 5	363 ± 29	96 ± 21
3	3-MC	3 × 40	8	12.8 ± 0.95¶ (36)	365 ± 30	285 ± 130	698 ± 200	440 ± 89	93 ± 16
11	β-NF	3 × 40	8–9	14.9 ± 1.9‖ (132)	370 ± 66‖	264 ± 131	585 ± 220	298 ± 152	69 ± 34
3	Corn oil	3 ×	11–12	21.0 ± 3.3 (36)	537 ± 154	303 ± 72	554 ± 226	401 ± 95	96 ± 21
3	3-MC	3 × 40	11	15.2 ± 1.3‖ (33)	391 ± 103	309 ± 100	356 ± 105	358 ± 113	76 ± 20
3	β-NF	3 × 40	11	19.6 ± 0.30 (37)	545 ± 86	250 ± 17	512 ± 43	343 ± 61	69 ± 20
1	Corn oil	3 ×	14	21.3 (12)	475	332	664	459	94
4	β-NF	3 × 40	13–14	21.9 ± 1.4 (48)	556 ± 61	190 ± 66	372 ± 91	324 ± 84	71 ± 12
2	β-NF	3 × 40	15	26.9 ± 2.3 (23)	718 ± 15	181 ± 27	307 ± 33	328 ± 96	101 ± 21

* Injected i.p. 3 times (72, 48 and 24 hr) or 2 times (48 and 24 hr) before sacrifice.
† Determined as described in Materials and Methods. Values are the means ± S.D.
‡ Numbers in parentheses are the number of pups.
§ $0.1 < P < 0.05$.
‖ $P < 0.05$.
¶ $P < 0.01$.

5.0 ml of 0.1 M potassium phosphate buffer, pH 7.4. Incubations were in flasks open to air at 37° for 10 and 20 min, respectively, for hepatic microsomes of lactating rats and their suckling young. Control incubations consisted of the mixtures above containing α-NF, but not 2-FAA, or heat-inactivated microsomal protein. The reactions were terminated by the additions of 5 ml of 1 M sodium acetate, pH 6.0, and 1.0 μg of the internal standard, 4-hydroxybiphenyl, in 0.05 ml methanol. The incubation mixtures were extracted twice with 5 ml diethyl ether. The combined ethereal extracts were washed twice with 3 ml water, dried with sodium sulfate, and evaporated to dryness under a stream of nitrogen. The residues were dissolved in 0.025 ml methanol, 0.01 ml of which was analyzed by the HPLC method described previously [20]. Peak detection was by u.v. absorbance, and metabolite quantitation was by peak area determination relative to standard curves. Correction factors for losses of metabolites encountered during extraction and for u.v. light-absorbing compounds due to α-NF metabolism were determined from the appropriate controls.

Determination of binding of various substrates to hepatic microsomes. Microsomes were suspended in 50 mM potassium phosphate buffer, pH 7.5, or in 300 mM sodium phosphate buffer, pH 7.5, containing 30% glycerol at a concentration of 1.0 to 2.0 mg or 2.0 to 4.0 mg protein/ml respectively. Substrates for binding were dissolved in DMSO in the range of concentrations 0.1 μM to 1.0 mM, and aliquots of 5 μl were added to the sample cuvette containing 1.2 ml of the microsomal suspension. Three to five additions of DMSO solutions of a given compound were made to the same cuvette. The reference cuvette contained the microsomal suspension to which aliquots of 5 μl DMSO were added. After each addition, the mixtures were allowed to stand at room temperature for 15 min, and the spectra were then recorded from 450 to 360 nm. A Hitachi 110 computerized double beam ratio recording spectrophotometer was used. The maximal absorbance change (ΔA) produced by a given concentration of the substrate was used to develop Lineweaver–Burk and Eadie–Scatchard plots. The slopes of these plots were used for calculation of the spectral dissociation constant, K_s (μM) [13, 21].

RESULTS

Effects of treatment of lactating rats with 3-MC or β-NF on the body and liver weights and on hepatic microsomal enzymes of their suckling young. Treatment of lactating rats with 3-MC prevented normal increases in the body weights of their suckling young (Table 1). Its effect on the liver weight was significant only in the 6-day-old pups. Treatment of lactating rats with β-NF affected the pups less than with 3-MC; significant decreases in the body and liver weights were noted only in 8- to 9-day-old pups.

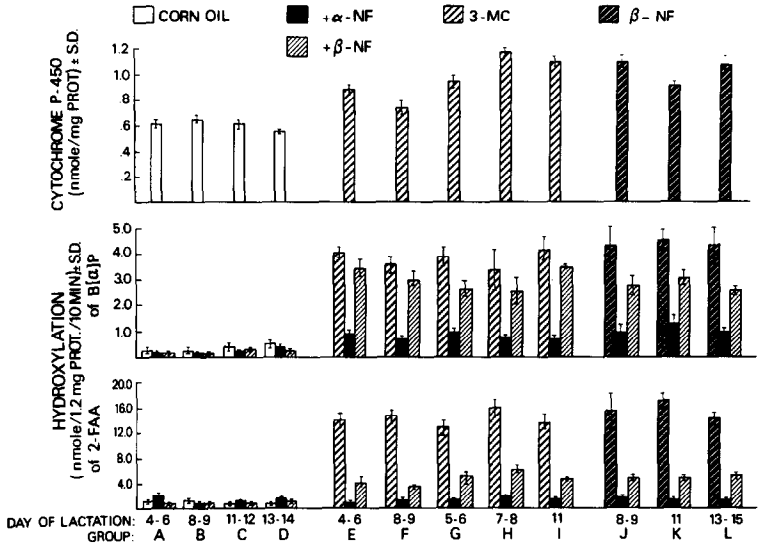

Fig. 1. Effects of α-NF and β-NF *in vitro* on hydroxylations of B[*a*]P and 2-FAA by hepatic microsomes of lactating rats. Each group consisted of two to eight lactating rats. Groups E and F, and G through L received 20 and 40 mg/kg of body weight, respectively, of 3-MC or β-NF in corn oil, i.p., for 3 consecutive days before sacrifice. Assays are described in Materials and Methods. Differences in cytochrome P-450 content (top section) between 3-MC- or β-NF-treated groups and the respective controls were significant at $P < 0.05$ except for groups F and K. The increases in hydroxylations of B[*a*]P (middle section) and 2-FAA (bottom section) in groups E through L were significant at $P < 0.001$ vs respective controls, groups A through D. The effect of α-NF (+α-NF) on hydroxylations of B[*a*]P in groups A, B and C, and of 2-FAA in groups A and D was significant at $P < 0.025$. The effect of β-NF (+β-NF) on hydroxylations of B[*a*]P in groups A, B and D, and of 2-FAA in groups C and D was significant at $P < 0.005$ and $P < 0.05$ respectively. The effects of α-NF on hydroxylations of B[*a*]P and 2-FAA, and of β-NF on hydroxylation of 2-FAA in groups E through L were significant at $P < 0.001$. The effect of β-NF on hydroxylations of B[*a*]P in groups E and J ($P < 0.005$), F and I ($P < 0.025$) and G, K and L ($P < 0.001$) was significant.

Changes in the contents of cytochrome P-450 and b_5 in the livers of the pups suckling 3-MC- or β-NF-treated dams were insignificant compared to their wide ranges in pups from control dams (Table 1). The absorption maxima of the reduced cytochrome P-450–CO complex of the hepatic microsomes was the same (449 nm) in all sets of pups. The content of cytochrome b_5 tended to increase with age and approached a level similar to that of corn oil-treated dams [7]. The average content of cytochrome P-450 in hepatic microsomes of the corn oil-treated dams (Fig. 1) was similar to that of their pups (Table 1). As reported earlier and confirmed here, treatment of lactating rats with 3-MC or β-NF almost always significantly induced cytochrome P-450 (Fig. 1) which had an absorption maximum of the CO complex of 447 nm. The activities of NADH- and NADPH-cytochrome *c* reductases in the pups, 4- and 2-fold lower than that in the dams [7], respectively, were unaffected by the treatment of dams with 3-MC or β-NF (Table 1).

Hydroxylations of B[a]*P and 2-FAA in hepatic microsomes of lactating rats and their suckling young and effects of α-NF and β-NF* in vitro *on these reactions.* The induction of cytochrome P-450 by 3-MC or β-NF in hepatic microsomes of lactating rats was coincident with an up to 20-fold increase in hydroxylations of B[*a*]P and 2-FAA (Fig. 1). We compared the effects of α-NF and β-NF *in vitro* on these reactions. α-NF inhibited the increased hydroxylations of B[*a*]P and 2-FAA by 72–81 and 89–95%, and β-NF inhibited these reactions by 12–41 and 60–76% respectively. Thus, α-NF was a more potent inhibitor of the induced enzyme(s) activities than β-NF, and both inhibited hydroxylation of 2-FAA more than of B[*a*]P. Inhibitions of basal hydroxylating activities were small or insignificant. Hydroxylations of B[*a*]P and 2-FAA increased up to 8- and 6-fold, respectively, in the hepatic microsomes of the suckling young of 3-MC or β-NF-treated dams even though no significant increases of cytochrome P-450 content were found (Fig. 2). Thus, the measurement of hydroxylating enzyme(s) activities was a more sensitive indicator of the induction of a specific cytochrome P-450 than the spectral determination of the total cytochrome P-450 content. In the presence of 0.1 mM α-NF, the extents of hydroxylations were equal in hepatic microsomes of pups suckling control and induced dams (Fig. 2). With 0.1 mM β-NF *in vitro*, the extents of hydroxylations in pups suckling control and 3-MC-treated dams were also similar (data not shown). These

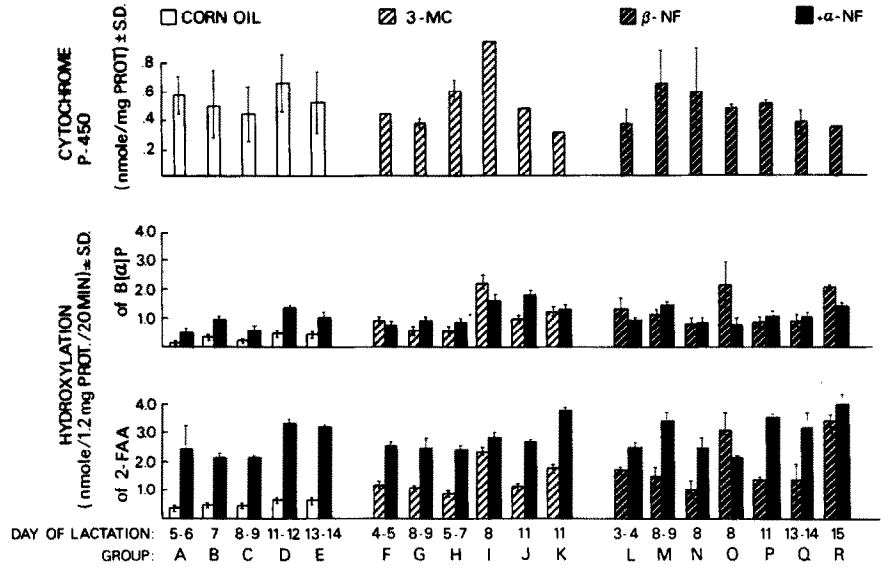

Fig. 2. Induction of hydroxylations of B[*a*]P and 2-FAA in hepatic microsomes of suckling young after treatment of lactating rats with 3-MC or β-NF and the effect of α-NF *in vitro* on the hydroxylations. Each group consisted of one to five lactating rats with a set of eleven or twelve pups each. Groups F and G, and H through K received 3-MC in corn oil, i.p., at a dose level of 20 and 40 mg/kg of body weight, respectively, for 3 consecutive days before sacrifice. Groups L and M through R received β-NF in corn oil, i.p., at a dose level of 40 mg/kg of body weight, for 2 and 3 consecutive days respectively. Pooled livers of each set of pups were used for preparation of microsomes. The assays are described in Materials and Methods. The amounts of cytochrome P-450 (top section) in groups F through R were not significantly different from those in groups A through E. The increases in hydroxylations of B[*a*]P (middle section) in hepatic microsomes of suckling rats from groups F, G, I, L, M, N, R ($P < 0.001$), J, K, O ($P < 0.005$), H, P ($P < 0.01$) and Q ($P < 0.025$), and of 2-FAA (bottom section) in groups F, G, H, I, K, M, O, P, R ($P < 0.001$) and J, L, N, Q ($P < 0.005$) were significant vs respective controls, groups A through E. The effect of α-NF (+α-NF) on hydroxylations of B[*a*]P in groups B, C, D, G, M ($P < 0.001$), H, R ($P < 0.005$), A, E ($P < 0.01$), J, O ($P < 0.025$) and L, P ($P < 0.05$) and of 2-FAA in groups B through H and J, L, M, N, P, Q ($P < 0.001$), K ($P < 0.005$), A ($P < 0.01$) and I ($P < 0.05$) was significant.

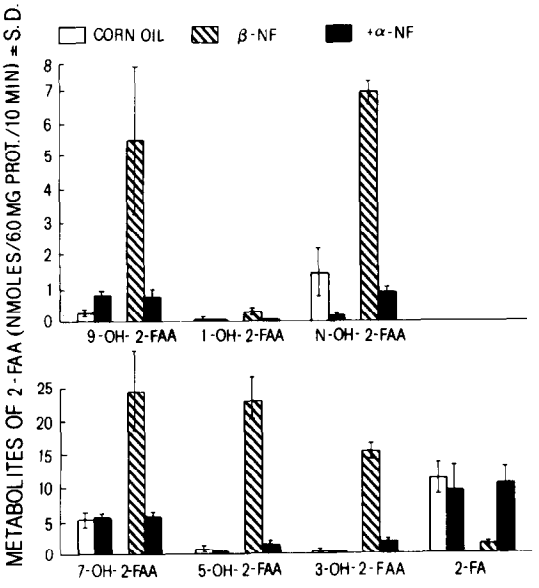

Fig. 3. Effect of α-NF *in vitro* on the formation of metabolites of 2-FAA by hepatic microsomes of corn oil- or β-NF-treated lactating rats. Each group consisted of four lactating rats which received three i.p. injections of β-NF in corn oil (40 mg/kg of body weight) or the vehicle alone on days 9 through 11 of lactation. Preparation of hepatic microsomes, composition of the incubation mixtures, and assays of metabolites of 2-FAA are described in Materials and Methods. Differences in the amounts of 7-, 5-, 3-, N-OH-2-FAA and 2-FA ($P < 0.001$) and of 9- and 1-OH-2-FAA ($P < 0.005$) formed by hepatic microsomes of corn oil- and β-NF-treated lactating rats were significant. The effect of 0.1 mM α-NF *in vitro* (+α-NF) on the formation of 9-OH-2-FAA ($P < 0.001$), 5-, 3- and N-OH-2-FAA ($P < 0.005$) and 1-OH-2-FAA ($P < 0.05$) by hepatic microsomes of corn oil-treated dams was significant. The effect of α-NF on the formation of 9-, 7-, 5-, 3- and N-OH-2-FAA ($P < 0.001$) and 1-OH-2-FAA and 2-FA ($P < 0.005$) by hepatic microsomes of β-NF-treated dams was significant.

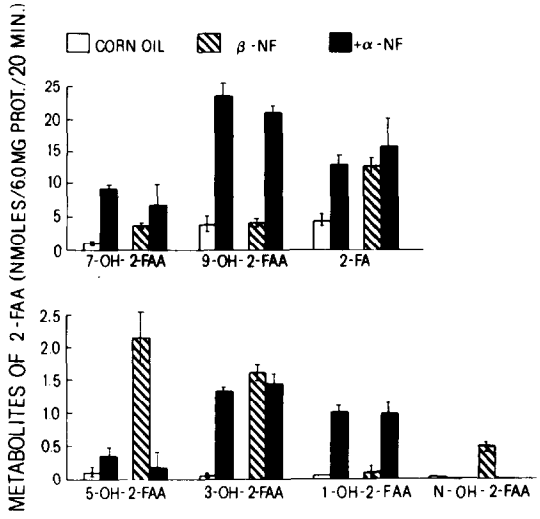

Fig. 4. Effect of α-NF *in vitro* on the formation of metabolites of 2-FAA by hepatic microsomes of suckling young from corn oil- or β-NF-treated lactating rats. Treatment of lactating rats is dexribed in the legend to Fig. 3. Each dam had a set of twelve pups, the livers of which were pooled for preparation of microsomes. The assays of metabolites of 2-FAA are described in Materials and Methods. The differences in the amounts of 7-, 5-, 3-, N-OH-2-FAA and 2-FA ($P < 0.001$) and 1-OH-2-FAA ($P < 0.005$) formed by hepatic microsomes of pups from corn oil- and β-NF-treated dams were significant. The effect of 0.1 mM α-NF *in vitro* (+α-NF) or the formation of 9-, 7-, 3-, 1-OH-2-FAA and 2-FA ($P < 0.001$) and 5-OH-2-FAA ($P < 0.025$) by hepatic microsomes of pups from corn oil-treated dams was significant. The effect of α-NF on the formation of 9-OH-2-FAA ($P < 0.001$) and 5- and 1-OH-2-FAA ($P < 0.005$) by hepatic microsomes of pups from β-NF-treated dams was significant. Inhibition of the formation of N-OH-2-FAA by α-NF was complete.

results indicate that both α-NF and β-NF *in vitro* stimulated the basal, but inhibited the induced, hydroxylating enzyme(s) activities. Stimulation of the basal enzyme(s) activities by α-NF was 2-fold greater than that by β-NF. Thus, the responses to α-NF and β-NF in the hepatic microsomes of the dams and suckling pups were similar in that the induced activities were inhibited, but were dissimilar in that the basal activities were stimulated in the pups and unaffected in the dams.

Effect of α-NF in vitro *on metabolites of 2-FAA formed by hepatic microsomes of lactating rats and their suckling young.* We compared the effect of α-NF *in vitro* on individual metabolites of 2-FAA formed by hepatic microsomes of corn oil- and β-NF-treated lactating rats (Fig. 3) and of their suckling young (Fig. 4). Since both α-NF [22–24] and 2-FAA [14, 25, 26] are metabolized by the cytochrome P-450 system of rat liver microsomes, we first determined if α-NF or its metabolites would interfere in the HPLC assays of metabolites of 2-FAA. We found that α-NF itself did not interfere since it did not elute from a Zorbax C_8 column with the mobile phase employed by us in the separation of 2-FAA metabolites [20]. The elution of 2-FAA and its metabolites was completed within 26 min. The major metabolite of α-NF eluted with a retention time of 40.3 min and a second major metabolite coeluted with 9-oxo-2-FAA at 11.8 min. However, 9-oxo-2-FAA was formed only in trace amounts by hepatic microsomes of lactating rats and, therefore, was not quantified in our assays. The remaining two metabolites of α-NF were formed in trace amounts and coeluted with 3-OH-2-FAA and 2-FAA at 16.3 and 25.9 min respectively. Quantitation of 3-OH-2-FAA in our assays was corrected for this trace contamination. Pretreatment of lactating rats with β-NF increased the phenolic metabolites of 2-FAA, 7-, 1-, 3- and 5-OH-2-FAA, 4.6-, 9.4-, 30- and 40-fold, respectively, as well as 9- and *N*-OH-2-FAA, 20- and 4.5-fold respectively (Fig. 3). Preincubations of the microsomes with 0.1 mM α-NF resulted in the inhibition of the induced hydroxylations. In the hepatic microsomes of the corn oil-treated dams, α-NF *in vitro* stimulated 9-hydroxylation of 2-FAA about 3-fold, had no effect on 1- and 7-hydroxylation, and

inhibited 3-, 5- and, in particular, N-hydroxylation of 2-FAA. These variations in rates of specific hydroxylations in response to α-NF suggested that specific ring carbon- and N-hydroxylation of 2-FAA are catalyzed by different cytochrome P-450 isozymes. These differences were not apparent from measurements of total hydroxylation of 2-FAA (Fig. 1).

Hepatic microsomes of pups suckling β-NF-treated dams showed 1.9-, 3.5-, 18-, 30- and 27-fold increases of 1-, 7-, 5-, 3- and N-hydroxylation of 2-FAA respectively (Fig. 4). By contrast, 9-hydroxylation of 2-FAA was unaffected. When α-NF was added to microsomes of pups suckling the uninduced and β-NF-induced dams, similar amounts of each C-hydroxylated metabolite of 2-FAA were found. This indicated that α-NF inhibited the induced and stimulated the basal hydroxylations to yield 2.9-, 5.8-, 8.7-, 25- and 40-fold increases of 5-, 9-, 7-, 3- and 1-OH-2-FAA respectively. By contrast, the basal N-hydroxylation of 2-FAA was not increased. These differences in the induction by β-NF *in vivo* and inhibition or stimulation by α-NF *in vitro* of the phenolic, 9- and N-hydroxy metabolites of 2-FAA suggested involvement of different cytochrome P-450 isozymes in these oxidation reactions.

The deacetylation of 2-FAA to 2-FA followed different patterns in the microsomes of the pups from that of their dams (Figs. 3 and 4). The amounts of 2-FA formed by hepatic microsomes of the pups suckling corn oil-treated dams were about 2.5-fold smaller than those of the dams. α-NF *in vitro* increased this amount about 2.8-fold as did β-NF pretreatment of dams. The latter was not altered significantly by α-NF *in vitro*. It thus appears that in the pups the basal deacetylase activity was stimulated whereas the induced activity was inhibited by α-NF *in vitro*. This contrasted with the response in the hepatic microsomes of the β-NF-treated dams in which the amount of deacetylase product decreased and α-NF *in vitro* restored it (Fig. 3). The data suggested that, because of the lower levels of total metabolism in the pups, the amounts of 2-FAA were sufficient to avoid the effects of competition between the deacetylation and hydroxylation observed in the dams. These results are consistent with those of other investigators who reported that in rat liver microsomes the amount of deacetylated product depended on the concentration of 2-FAA and that inhibition of the deacetylase by fluoride increased C- and N-hydroxylation of 2-FAA [27, 28].

Characteristics of substrate/inhibitor binding to microsomal cytochrome P-450. Various xenobiotics bind to cytochrome P-450 in hepatic microsomes to give characteristic absorbance changes known as difference spectra [11, 29]. Empirically, these spectra

Table 2. Types of spectral changes and dissociation constants (K_s) obtained on addition of various substrates to hepatic microsomes of lactating rats

Substrate	Microsomes from rat treated with*:	Type of spectral change	Wavelength (nm)			$K_s \times 10^{-6}$ M† (mean ± S.D.)
			Peak (maximum)	Intercept	Trough (minimum)	
B[*a*]P	3-MC	I	387	406–408	414–416	4.39 ± 0.76‡
	β-NF	I	387	395–398	414–416	8.83 ± 2.09‡
	PB	I	387	395–396	414–415	26.4
2-FAA	3-MC	I	384–388	404–409	413–417	2.44 ± 0.16§
	β-NF	I	383–388	402–404	412–416	3.08 ± 0.87§
	PB	RI	420–422	404–408	384–390	1.69 ± 0.69‖
β-NF	3-MC	I	385–389	405–408	415–420	0.16 ± 0.03§
	β-NF	I	385–390	405–410	418–423	0.12 ± 0.03¶
	PB	RI	417–420	410–413	387–392	1.76 ± 0.29‖
α-NF	3-MC	Mixed	407–412	418–422 395–402	428–432 386–391	0.33 ± 0.13¶
	β-NF	Mixed	410–415	419–426 396–405	430–435 384–390	0.24 ± 0.11§
	PB	I	385–390	407–408	420–424	0.98
Hexobarbital	3-MC	Mixed	408–413	420–424 396–402	427–430 390–392	57.0 ± 17.0‡
	β-NF	Mixed	410–412	424–426 391–396	432–434 388–390	84.7
	PB	I	385–388	403–408	417–421	77.0 ± 5.01‖
	Corn oil	I	386–389	406–411	422–428	428 ± 48.8‡

* 3-MC or β-NF in corn oil was injected i.p. at 40 mg/kg of body weight 72, 48 and 24 hr before sacrifice. PB at 0.1% was given in drinking water for 5 days.

† Determined from Lineweaver–Burk plot. Calculations of K_s from Eadie–Scatchard plot gave identical values.

‡ Determined with microsomal preparations from livers of two lactating rats.

§ Determined with microsomal preparations from livers of three lactating rats.

‖ Mean of three separate determinations.

¶ Determined with microsomal preparations from livers of five lactating rats.

are of three basic types: type I, type II and reverse-type I. We determined types of spectra given by the compounds used in this study and their spectral dissociation constants (K_s) in an attempt to explain the effects of inhibitors on metabolism of carcinogens in lactating rats and their suckling young (Table 2). In agreement with previous reports [7, 14, 25, 30], the two carcinogens, B[*a*]P and 2-FAA, produced type I binding spectra on addition to microsomes from 3-MC- or β-NF-treated lactating rats. With hepatic microsomes of a PB-treated lactating rat, B[*a*]P gave also a type I binding spectrum. The spectral dissociation constants showed that the affinity of B[*a*]P for the cytochrome P-450 varied with the inducing agent: 3-MC (K_s, 4.39 μM) > β-NF (K_s, 8.83 μM) > PB (K_s, 26.4 μM). The affinities of 2-FAA, which gave type I spectra with 3-MC- and β-NF- and a reverse-type I spectrum with PB-induced cytochromes P-450, were similar (K_s, ~1.7 to 3.1 μM). β-NF also gave type I spectra with microsomes of 3-MC- or β-NF-treated rats and had a 15- to 70-fold higher affinity (K_s, ~0.14 μM) than 2-FAA or B[*a*]P. With microsomes of a PB-treated lactating rat, β-NF, like 2-FAA, gave a reverse-type I spectrum and had the same affinity as 2-FAA. With these microsomes α-NF gave a type I spectrum, and with microsomes of the 3-MC- or β-NF-treated rats it gave a spectral change that we termed "mixed" (Fig. 5). This spectrum had two intercepts with the baseline, two absorbance minima (troughs) and one absorbance maximum (peak) with the wavelength ranges indicated in Table 2. Thus, this spectrum was different from the classical type I spectrum reported for α-NF with hepatic microsomes of 3-MC-induced mice [31] and β-NF-induced rats [22]. We compared the spectra and binding affinities of α-NF with those of hexobarbital, a classical type I binding substrate for cytochrome P-450 from control or PB-induced rats [30]. With hepatic microsomes from the PB-induced lactating rat, α-NF had much higher binding affinity (K_s, 1 μM) than hexobarbital (K_s, 77 μM). The affinity of hexobarbital for microsomes of control rats was less (K_s, 430 μM). With hepatic microsomes of the 3-MC- or β-NF-treated lactating rats, hexobarbital gave a mixed spectrum (K_s, 57 and 85 μM, respectively) resembling that given by α-NF (K_s, ~0.28 μM). Thus, α-NF and hexobarbital produced qualitatively similar spectra with microsomes of the PB-, 3-MC- or β-NF-induced lactating rats, but affinities of α-NF were considerably greater. The data showed that, even though both α- and β-NF inhibited the 3-MC- or β-NF-induced hydroxylations of B[*a*]P and 2-FAA and had similar affinities, their interactions with the cytochrome P-450 systems differed. Whereas β-NF probably competed with B[*a*]P and 2-FAA for the same binding site(s) on cytochrome P-450 and acted as a competitive inhibitor, the mixed binding spectrum of α-NF indicated that it interacted with cytochrome P-450 in another manner.

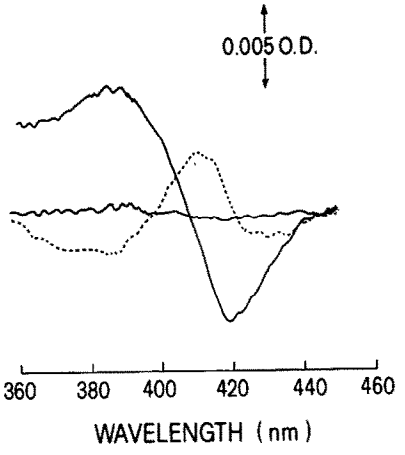

Fig. 5. Binding spectra of β-NF and α-NF to hepatic microsomes of 3-MC-treated lactating rats. Microsomes were suspended in 300 mM sodium phosphate buffer, pH 7.5, containing 30% glycerol. The compounds were added in 5 μl DMSO at final concentrations of 0.8 μM β-NF (——) and 0.01 mM α-NF (·····) to suspensions of 1.0 mg and 2.0 mg protein/ml respectively. DMSO was added to the reference cuvette. The spectra for α-NF were recorded with the use of split cells to correct for the absorbance due to α-NF [29]. The spectra were recorded after 15 min at room temperature with a Hitachi 110 computerized double beam ratio recording spectrophotometer with automatic baseline correction (2.0 nm band width).

Spectral changes due to binding were rarely detected in the hepatic microsomes of pups. We found that the difference spectra could be obtained with some microsomal preparations from the pups only if very low concentrations of the binding substrates were used. Since the microsomes from the pups had a cytochrome P-450 level comparable to, or even higher than, that of their dams, we felt that cytochrome P-450 present in the suckling young had low capacity for binding, which was reflected by the low levels of metabolism of the substrates. In several instances, the additions of α- or β-NF to hepatic microsomes of pups suckling β-NF-induced dams produced spectral changes similar to type I, of very low amplitudes, suggesting that the binding was due to the induced cytochrome P-450. More definite spectra were obtained when the dams were given PB-containing water *ad lib.* for 5 days. Then the hepatic microsomes of the pups had a high content of cytochrome P-450 (1150 pmoles/mg protein), and hexobarbital clearly gave a type I spectral change (K_s, 4.7 μM).

DISCUSSION

We presented evidence indicating that 3-MC and β-NF and/or their metabolites are transmitted to the pups with the milk after treatment of nursing dams with these two compounds. Both prevented normal increases in body and liver weights of the pups in certain age groups. In the hepatic microsomes of pups suckling 3-MC- or β-NF-treated rats, induction of cytochrome P-450 measured spectrally by complexation with CO or binding was insignificant. However, the hydroxylations of B[*a*]P and 2-FAA were increased significantly in the pups. This suggested that cytochrome P-450 catalyzing these hydroxylations was indeed induced in hepatic microsomes of pups via ingestion of milk of the 3-MC- or β-NF-treated rats. In hepatic microsomes of the pups, the basal hydroxylating activities were stimulated by

α-NF and, to a lesser extent, by β-NF *in vitro*. Since the levels of hydroxylations of B[*a*]P and 2-FAA were similar when α- or β-NF was added to the microsomes of the pups suckling 3-MC-, β-NF- or corn oil-treated dams, we concluded that α- and β-NF *in vitro* inhibited the induced hydroxylations in the pups just as they did in the dams. Hence, we confirmed the earlier report of Wiebel and Gelboin [10] showing that α-NF *in vitro* stimulated the basal, but inhibited the 3-MC-induced, hydroxylating enzyme activities in newborn rats.

In the pups suckling 3-MC- or β-NF-treated dams, the induction of cytochrome P-450 could only be detected by the increased hydroxylating capacities of microsomes and their responses to α- and β-NF. On the other hand, after treatment of a lactating rat with PB in drinking water for 5 days, the increase in the content of cytochrome P-450 in the hepatic microsomes of the suckling pups could be measured spectrally. Likewise, marked effects of treatment of lactating rats with PB for several days [1, 6] or with polybrominated biphenyls in diet [2] on hepatic cytochromes and enzymes of their pups have been reported. In addition to possible intrinsic factors of these compounds favoring excretion in milk, the duration of treatment and continuous mode of administration of the compounds may have led to greater induction in the suckling young than did i.p. injections given to the dams for 2 or 3 consecutive days.

Compounds that are metabolized by the cytochrome P-450 system of rat liver, such as B[*a*]P, 2-FAA, α- and β-NF, bind to this cytochrome yielding a type I spectral change [14, 22, 23, 25, 30]. Accordingly, we recorded type I binding spectra on addition of B[*a*]P, 2-FAA and β-NF to hepatic microsomes of 3-MC- or β-NF-treated lactating rats. Since type I binding affinity of β-NF was higher than that of B[*a*]P and 2-FAA, we suggest that competitive inhibition of metabolism of these two carcinogens by β-NF underlies its mechanism of action. However, α-NF behaved like hexobarbital in that it gave a type I and a mixed type binding spectra with microsomes of PB- and 3-MC- or β-NF-treated lactating rats respectively. Variable types of spectral changes produced by hexobarbital have been shown to depend on its concentrations [32]. Kumaki *et al.* [33] suggested that hexobarbital is a mixed type molecule exhibiting a type I and/or reverse-type I (or type II) character, depending on the spin state of P-450 iron at the start of the experiment. A similar interpretation may explain the variable behavior of α-NF. Thus, with the low spin P-450 iron predominantly induced *in vivo* with PB, α-NF interacted and produced a type I spectral change; it also interacted with high spin P-450 iron predominantly induced *in vivo* with 3-MC or β-NF to give a mixed type change that appeared to be composed of the type I and reverse-type I elements.

The stimulation of basal hydroxylation of B[*a*]P in hepatic microsomes of pups by α-NF *in vitro* measured in this work confirmed earlier findings in newborn rats [10]. A similar stimulation of the hydroxylation of B[*a*]P by α-NF or flavone *in vitro* has been reported in hepatic microsomes from rabbit, hamster and man [34]. The data suggested that the stimulatory effects of flavonoids are due, at least in part, to enhancement of the interaction between cytochrome P-450 and NADPH–cytochrome P-450 reductase, which facilitates the flow of electrons to cytochrome P-450. Studies with several highly purified cytochrome P-450 isozymes from rabbit liver microsomes showed that, depending on the flavonoid itself and on the specific form of cytochrome P-450, inhibition or stimulation of metabolism of B[*a*]P was measured [35].

The stimulation of basal hydroxylation of 2-FAA in hepatic microsomes of pups by α-NF *in vitro* is reported for the first time in this work. To better understand the *in vitro* effect of α-NF, we compared the amounts of the ring- and N-hydroxylated metabolites of 2-FAA generated by hepatic microsomes of dams and pups in its presence. In the dams, α-NF *in vitro* did not affect formation of phenolic metabolites of 2-FAA; it stimulated 9-hydroxylation but inhibited N-hydroxylation of 2-FAA. In the pups, formation of the phenols and an alcohol from 2-FAA was stimulated many-fold, whereas N-hydroxylation, which was at a very low level, was not stimulated. These variable responses to α-NF suggest that specific C- and N-hydroxylations of 2-FAA are catalyzed by different cytochrome P-450 isozymes which differ in the mature (dam) and immature (pup) rat liver. This suggestion is consistent with our results from *in vivo* induction studies. Both present indirect evidence for multiple forms of cytochrome P-450 in the dams and suckling pups which may have overlapping but clearly not identical activities.

The inhibitory action of α-NF on the formation of N-hydroxy-2-FAA by hepatic microsomes of both uninduced and induced lactating rats and their pups was rather selective and may cause it to be a potential inhibitor of carcinogenesis by 2-FAA. To our knowledge, such inhibition has not thus far been explored, although α-NF was reported to inhibit polycyclic aromatic hydrocarbon-induced skin tumors in mice (reviewed in Refs. 36 and 37).

The results of our studies indicate that the inducers of hepatic MFO in the dams were transmitted with milk to the suckling young in which they modified hepatic metabolism of the carcinogens. Such modifications may be permanent as shown by the increased capacities at 37 weeks of age for formation of DNA adducts with aflatoxin B_1 after lactational exposure to PB [6]. Our recent work showed that lactational exposure of the suckling young to 2-FAA and its metabolites resulted in tumor induction later in life [38].

Acknowledgement—We thank Mr. Blake Elmquist for his valuable technical assistance.

REFERENCES

1. J. U. Bell, M. M. Hansell and D. J. Ecobichon, *Can. J. Physiol. Pharmac.* **53**, 1147 (1975).
2. R. W. Moore, G. A. Dannan and S. D. Aust, *Environ. Hlth Perspect.* **23**, 159 (1978).
3. W. J. Rogan, A. Bagniewska and T. Damstra, *New Engl. J. Med.* **302**, 1450 (1980).
4. A. Parkinson, L. W. Robertson and S. Safe, *Biochem. biophys. Res. Commun.* **96**, 882 (1980).

5. J. L. Eiseman and A. P. Alvares, *J. Pharmac. exp. Ther.* **214**, 250 (1980).
6. R. A. Faris and T. C. Campbell, *Science* **211**, 719 (1981).
7. C. L. Ritter and D. Malejka-Giganti, *Biochem. Pharmac.* **31**, 239 (1982).
8. J. Baron, A. G. Hildebrandt, J. A. Peterson and R. W. Estabrook, *Drug Metab. Dispos.* **1**, 129 (1973).
9. A. Y. H. Lu, S. B. West, M. Vore, D. Ryan and W. Levin, *J. biol. Chem.* **249**, 6701 (1974).
10. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
11. H. Remmer, J. B. Schenkman, R. W. Estabrook, H. Sasame, J. R. Gillette, S. Narasimhulu, D. Y. Cooper and O. Rosenthal, *Molec. Pharmac.* **2**, 187 (1966).
12. J. B. Schenkman and R. Sato, *Molec. Pharmac.* **4**, 613 (1968).
13. W. Lenk, *Biochem Pharmac.* **25**, 997 (1976).
14. D. Malejka-Giganti, R. C. McIver, A. L. Glasebrook and H. R. Gutmann, *Biochem. Pharmac.* **27**, 61 (1978).
15. T. Matsubara, R. A. Prough, M. D. Burke and R. W. Estabrook, *Cancer Res.* **34**, 2196 (1974).
16. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
17. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
18. A. H. Phillips and R. G. Langdon, *J. biol. Chem.* **237**, 2652 (1962).
19. E. Hietanen, *Pharmacology* **21**, 233 (1980).
20. C. N. Ryzewski and D. Malejka-Giganti, *J. Chromat.* **237**, 447 (1982).
21. I. H. Segel, *Enzyme Kinetics*, p. 214. John Wiley, New York (1975).
22. S. Nesnow and H. Bergman, *Cancer Res.* **41**, 2621 (1981).
23. M. M. Coombs, T. S. Bhatt and C. W. Vase, *Carcinogenesis* **2**, 135 (1981).
24. M. M. Coombs, *Carcinogenesis* **3**, 229 (1982).
25. H. R. Gutmann and P. Bell, *Biochim. biophys. Acta* **498**, 229 (1977).
26. E. Kaplan, H. R. Gutmann and T. H. Emory, *Biochem. Pharmac.* **27**, 1581 (1978).
27. U. S. Seal and H. R. Gutmann, *J. biol. Chem.* **234**, 648 (1959).
28. C. Razzouk, M. Mercier and M. Roberfroid, *Cancer Res.* **40**, 3540 (1980).
29. C. R. Jefcoate, in *Methods in Enzymology* (Eds. S. Fleischer and L. Packer), Vol. LII, part C, p. 258. Academic Press, New York (1978).
30. J. B. Schenkman, S. G. Sligar and D. L. Cinti, *Pharmac. Ther.* **12**, 43 (1981).
31. F. M. Goujon, D. W. Nebert and J. E. Gielen, *Molec. Pharmac.* **8**, 667 (1972).
32. I. Jansson, S. Orrenius, L. Ernster and J. B. Schenkman, *Archs Biochem. Biophys.* **151**, 391 (1972).
33. K. Kumaki, M. Sato, H. Kon and D. W. Nebert, *J. biol. Chem.* **253**, 1048 (1978).
34. M-T. Huang, R. L. Chang, J. G. Fortner and A. H. Conney, *J. biol. Chem.* **256**, 6829 (1981).
35. M-T. Huang, E. F. Johnson, U. Muller-Eberhard, D. R. Koop, M. J. Coon and A. H. Conney, *J. biol. Chem.* **256**, 10897 (1981).
36. L. W. Wattenberg, *Adv. Cancer Res.* **26**, 197 (1977).
37. F. J. Wiebel, in *Carcinogenesis, A Comprehensive Survey* (Ed. T. J. Slaga), Vol. 5, p. 57. Raven Press, New York (1980).
38. D. Malejka-Giganti, R. W. Decker and R. E. Rydell, *Proc. Am. Ass. Cancer Res.* **24**, (1983).