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PHENOBARBITAL-MEDIATED INCREASE IN RING- AND *N*-HYDROXYLATION OF THE CARCINOGEN *N*-2-FLUORENYLACETAMIDE, AND DECREASE IN AMOUNTS BOUND TO LIVER DEOXYRIBONUCLEIC ACID

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Abstract—The effect of phenobarbital (PB) pretreatment of young male rats on the metabolism *in vivo* and *in vitro* of the carcinogen *N*-2-fluorenylacetamide (FAA) was studied. PB increased the urinary excretion of ^{14}C from labeled FAA, mainly as metabolites conjugated with glucuronic acid. There was a small drop in excretion of sulfuric acid conjugates. In the glucosiduronic acid fraction, there were increases in the *N*- and 7-hydroxy derivatives of FAA and a decrease in the 5-hydroxy compound, while the 3-hydroxy metabolite was virtually unchanged. The concentration of metabolites of FAA in the liver was considerably lower, as was the amount of isotope bound to DNA. This important finding, correlated with a reduced carcinogenicity of FAA in rats given PB, is ascribed to increased conjugation with glucuronic acid and lesser formation of sulfate esters. The microsomal fraction *in vitro* from the livers of young rats yielded metabolites of FAA, in decreasing order, hydroxylated at 7-, 5-, *N*- and 3-. Carbon monoxide significantly inhibited formation of the 7-hydroxy metabolite but elevated somewhat that of the *N*-hydroxy compound. Pretreatment with PB increased cytochrome P-450 and hydroxylation at all positions 2- to 4-fold, the greatest effect being with *N*-hydroxy-FAA. With microsomes from PB-treated rat livers, carbon monoxide depressed hydroxylation at the 3-position, while *N*-hydroxylation was least affected. Because hydroxylation of FAA occurs at several well-defined ring positions and on the amido nitrogen, FAA is a good substrate to explore the mechanisms of hydroxylation reactions. The data obtained suggest that these metabolic reactions are performed by a family of related enzyme systems.

THE METABOLISM of aromatic compounds through hydroxylation reactions by the endoplasmic reticulum, principally in liver but also in other tissues of many species, is well known.[1–3] There are numerous modifying factors.[4–8] The induction of enzymes by polynuclear aromatic hydrocarbons, phenobarbital, chlorinated hydrocarbons or naphthoflavones, which increase their own metabolism or that of other chemicals, usually to a hydroxylated metabolite, has been extensively investigated. With acetanilide, the presence of *p*-hydroxyacetanilide is often the parameter examined. Some investigators have studied the production of nitroso derivatives, reflecting *N*-hydroxylation, from select mononuclear arylamines.[9–11]

With the carcinogenic polynuclear aromatic amine derivative *N*-2-fluorenylacetamide (FAA), 3-methylcholanthrene led to an increase in ring-hydroxylated meta-

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bolites in rats after a single dose and under subacute conditions, but the amount of *N*-hydroxy derivative underwent only minor changes.[12,13] On the other hand, in hamsters there were increases not only in ring-hydroxylated metabolites, but also in the *N*-hydroxylated product.[14]

In rats phenobarbital appeared to increase the *N*-hydroxylation of monocyclic aromatic amines more than ring-hydroxylation. It was, therefore, of interest to examine the effect of phenobarbital on the metabolism of FAA, an excellent tool to study the mechanisms of hydroxylation. Techniques are available to determine hydroxylation both at several of the key ring positions of the polynuclear fluorene nucleus and also on the nitrogen, which yields the biologically and toxicologically important *N*-hydroxy derivative.[11, 15–18] On the basis of species differences in ring substitution, it was postulated that several similar yet specific hydroxylating systems existed.[19] With phenobarbital induction, additional data on this point were obtained.

Furthermore, there were suggestions that *N*-hydroxylation with rat liver microsomes was less sensitive to carbon monoxide.[20–22] Thus, this reaction may involve an enzyme system distinct from that yielding C-hydroxylation. This aspect was also studied.

MATERIALS AND METHODS

Chemicals. FAA was purchased from Aldrich Chemical Co., Milwaukee, Wis. *N*-2-fluoren-9-^{14}C-ylacetamide was from Tracerlab, Waltham, Mass. *N*-hydroxy-*N*-2-fluorenylacetamide and the ring-hydroxylated *N*-(3-, 5-, and 7-hydroxy-2-fluorenyl) acetamides were synthesized by known methods or were available from earlier work in this laboratory. Bacterial β-glucuronidase, type II, Sigma Chemical Co., St. Louis, Mo., and glusulase were from Endo Laboratories, Inc., Garden City, N.Y. Other reagent grade or USP-grade chemicals were obtained from local laboratory supply houses.

Animals. Rats were 6-week-old Holtzman strain from the NIH Rodent and Rabbit production section.

Experiments in vivo *on the effect of phenobarbital on urinary metabolites of* N-2-*fluorenylacetamide*

Six male weanling rats were injected i.p. daily for 7 days with 80 mg/kg of phenobarbital; the corresponding control animals were injected with 0·9% sodium chloride solution. Twenty-four hr after the last phenobarbital dose, all 12 rats were injected i.p. with 50 mg/kg of *N*-2-fluoren-9-^{14}C-ylacetamide (specific activity, $4{\cdot}17 \times 10^6$ cpm/mg). The rats were placed in stainless steel metabolism cages (Acme Metal Co., Cincinnati, Ohio 45238) permitting collection of urine, separate from feces, in ice-cold receivers. The 24-hr urine samples were analyzed for total radioactivity. Metabolites were separated into a free fraction, a glucosiduronic acid fraction, and a sulfuric acid fraction by sequential series of solvent extraction, specific hydrolysis with β-glucuronidase (500 units/ml of urine), and further enzymic hydrolysis with glusulase, which splits select sulfuric acid esters, or alternatively by refluxing for 15 min at pH 1, which hydrolyzes all sulfuric acid esters.[18,23]

The ether extracts obtained after the three specific fractionation steps were further analyzed for hydroxylated metabolites by thin-layer chromatography on Silica gel utilizing the solvents: 97% chloroform, 3% methanol, or 90% chloroform, 10%

methanol. During the chromatography steps, standard unlabeled metabolites were run concurrently to locate specific metabolites. After chromatographic resolution the corresponding spots were removed, eluted and counted to obtain quantitative data on the relative amounts of individual metabolites.

Effect of phenobarbital on metabolite binding in vivo *to liver DNA*

Rats were pretreated with phenobarbital or control saline solution as described above. The following procedures were patterned after those described.[24–26] The rats were killed 51 hr after the intraperitoneal injection of ^{14}C-FAA by withdrawing blood from the heart with a heparinized syringe. The liver was perfused *in situ* via the portal vein with chilled saline and homogenized in 4 vol. of cold 0·32 M sucrose solution, containing 2 mM $MgCl_2$ and 1 mM KH_2PO_4, pH 6·8, with a Teflon-pestle Potter–Elvehjem homogenizer. The homogenates were centrifuged at 850 *g* for 10 min; the pellet was taken up in the original volume of medium and centrifuged again. This procedure was repeated three times. The final pellet was suspended in 25 ml of 2·1 M sucrose solution containing 1 mM $MgCl_2$ and 1 mM KH_2PO_4, pH 6·8, and centrifuged at 35,000 *g* for 2 hr.

The pelleted purified nuclei were suspended in 1·8 ml of homogenizing medium and lysed by adding 9·45 ml of 6·5 M CsCl solution containing 5 mM EDTA, pH 6·8. The nuclear lysate was subjected to isopicnic centrifugation at 183,000 *g* for 64 hr. The DNA fractions were pooled, the amount was estimated by the absorption at 260 nm, precipitated with 10% trichloroacetic acid (TCA) and washed with 5% TCA. DNA was dissolved in Hyamine and radioactivity was measured using a mixture of POPOP*–PPO–toluene (50 mg:5 g:1000 ml).

Effect of phenobarbital pretreatment on the metabolism in vitro *of* N-2-*fluorenylacetamide by the microsomal fraction of rat liver, including determination of the effect of carbon monoxide*

Rats pretreated with phenobarbital or saline, as above, were killed by decapitation and the liver was perfused *in situ* from the portal vein with chilled saline. The liver was homogenized in 4 vol. of ice-cold 1·15% KCl solution in a Teflon-pestle Potter–Elvehjem apparatus. The homogenates were centrifuged at 12,000 *g* for 25 min. The supernatant was spun at 105,000 *g* for 60 min; the resulting pellet was suspended in the original volume of 1·15% KCl, and recentrifuged as described above. The microsomal pellet was resuspended in a volume of 1·15% KCl solution equal to twice the wet weight of starting liver.

The incubation mixture (5·0 ml) in a rubber-stoppered 25-ml Erlenmeyer flask contained 20 mM phosphate buffer, pH 7·4, 75 mM KCl, 100 mM NaF, 2 mM NADPH, 0·2 mM ^{14}C-FAA and microsomes (equivalent to 500 mg liver for normal control rats and equivalent to 100 mg liver for phenobarbital-treated rats). Each sample was assayed in two to four flasks which were incubated in air at 37° for 30 min, during which time the reactions measured were linear. The carbon monoxide series was carried out by bubbling carbon monoxide through the phosphate buffer, NaF

* POPOP = 1,4-bis-2-(4-methyl 5-phenyloxazolyl)benzene; PPO = 2,5-diphenyloxazole.

and KCl solution, and replacing the air phase in the flask by blowing a carbon monoxide–air mixture (1:1, flow rate 500 ml/min) for 2 min over the solution and performing the incubation under this carbon monoxide–air mixture in a stoppered flask.

After the incubation, 1 ml of 1 M acetate buffer, pH 6·0 was added to each flask and the contents of the appropriate flasks were combined. To the pooled reaction mixture there was added 0·1 μmole each of N-OHFAA and 7-OHFAA as carriers, followed by extraction four times with ether. The ether extracts were partitioned against 0·25 N NaOH three times to separate FAA in the ether phase and the hydroxylated metabolites in the aqueous NaOH phase. After washing with ether once, 5 ml of 1 M acetate buffer, pH 6·0, was added to the alkaline extract and the pH was adjusted to 5–6 with HCl, followed by extraction with ether. This ether extract was taken to dryness in a stream of nitrogen and the residue was dissolved in ethanol.

Hydroxylated FAA metabolites in the ethanol solution were separated by paper chromatography using the solvent system: cyclohexane–*t*-butanol–acetic acid–water (16:4:2:1 vol. or 16:2:2:1 vol.).

Each radioactive spot corresponding to a metabolite was detected by autoradiography on film. Radioactivity in each spot and also other samples was determined to secure quantitative data, using a mixture of POPOP–PPO–methanol–toluene (100 mg: 3 g:300 ml:700 ml), in a liquid scintillation spectrometer.

Protein was measured by a modification of the method of Lowry *et al.*[27] and cytochrome P-450 by the procedure of Omura and Sato.[28]

RESULTS

Experiments in vivo *on the effect of phenobarbital on urinary, liver and DNA-bound metabolites of* N-2-*fluorenylacetamide*

After 24 hr the urine of the phenobarbital-pretreated rats contained more radioactivity than was found for controls. The effect was clearly due to an increase in the glucosiduronic acid fraction and a slight reduction in the sulfate ester fraction (Table 1).

Examination of the individual hydroxylated metabolites excreted in urine as glucosiduronic acids showed that phenobarbital pretreatment more than doubled the

TABLE 1. EFFECT OF REPEATED PHENOBARBITAL INJECTION ON URINARY METABOLITES OF *N*-2-FLUORENYLACETAMIDE IN MALE WEANLING RATS*

Group	Urinary metabolites (% of dose)			
	Total ^{14}C	Free	Glucuronides	Sulfates
Control (5)†	56·7 ± 1·8‡	2·2 ± 0·4	27·1 ± 2·1	9·1 ± 1·1
Phenobarbital (6)†	63·2 ± 3·2‡	3·0 ± 0·5	34·6 ± 2·1	7·1 ± 0·5

* Male weanling Holtzman rats were injected i.p. for 7 days with 80 mg/kg of phenobarbital or with saline (controls). One day after the last dose, all rats received an i.p. dose of 50 mg/kg of *N*-2-fluoren-9-^{14}C-ylacetamide (specific radioactivity, $4{\cdot}17 \times 10^6$ cpm/mg). Urines were collected for 24 hr from rats individually housed in metabolism cages. The urines were analyzed for metabolites by our standard methods. The data are expressed as per cent of dose ± standard error.

† Number of rats; all determinations were done in duplicate.

‡ The balance of the metabolites not further identified and now under investigation are as yet unknown, water-soluble compounds with some properties of mercapturic acids.

amount of *N*-hydroxy derivative and almost doubled the level of 7-hydroxy compound, yet reduced the relative amounts of the 5-hydroxy derivative and did not affect the urinary level of the 3-hydroxy metabolite (Table 2).

TABLE 2. EFFECT OF PHENOBARBITAL INJECTION ON HYDROXYLATED DERIVATIVES OF *N*-2-FLUORENYLACETAMIDE IN THE GLUCURONIDE FRACTION OF URINARY METABOLITES*

Group	Hydroxylated metabolites of *N*-2-fluorenylacetamide† (% of dose)			
	7-OHFAA	5-OHFAA	3-OHFAA	N-OHFAA
Control (5)‡	8·3 ± 0·6	8·5 ± 0·3	3·2 ± 0·4	1·4 ± 0·3
Phenobarbital (6)‡	15·7 ± 1·6	4·9 ± 0·5	2·8 ± 0·3	3·0 ± 0·2

* Treatment of animals is described in text and in Table 1.

† The hydroxylated metabolites at positions 7, 5, 3 (7-OHFAA etc.) were *N*-(7-hydroxy-2-fluorenyl)acetamide, etc., and *N*-2-fluorenylacetohydroxamic acid (N-OHFAA).

‡ Refers to the number of animals for which 24-hr urine samples were analyzed in duplicate.

The body weights of the phenobarbital-treated and control rats were similar, but the liver weights were increased somewhat in the phenobarbital group. However, the amount of metabolites as measured by radioactivity in the liver was decreased severely, and roughly the same lower proportion was also found with respect to isotope bound to DNA (Table 3).

TABLE 3. EFFECT OF PHENOBARBITAL INJECTION ON METABOLITES FROM *N*-2-FLUORENYLACETAMIDE IN LIVER AND BOUND TO LIVER DNA*

Group	Body wt. (g)	Liver wt. (g)	^{14}C from FAA			
			Liver		Bound to DNA	
			(nmoles/g)	(% of dose)	(pmoles/mg DNA)	(pmoles/g liver)
Control (3)	143 ± 10	5·7 ± 0·4	35·5 ± 1·0	0·58 ± 0·01	53·2 ± 2·4	121 ± 5·8
Phenobarbital (4)	147 ± 4	6·8 ± 0·4	17·2 ± 0·6	0·33 ± 0·01	22·2 ± 2·2	47·7 ± 5·1
% of Control†	103	119	48·5	56·9	41·7	39·5
P value†	< 0·5	< 0·05	< 0·001	< 0·001	< 0·001	< 0·001

* Animals treated as described in Table 1 were killed 51 hr after the injection of labeled *N*-2-fluorenylacetamide (FAA). It was shown earlier that binding was fairly stable between 1 and 3 days.[29] The liver was treated as described in the text for the isolation of pure nuclei and of DNA. The data are given in units shown ± standard error.

† These two lines show the results in groups treated with phenobarbital as per cent of controls and the P value of the significant comparisons.

Experiments in vitro *on the effect of phenobarbital on metabolites of* N-2-*fluorenylacetamide*

As was true for the rats used in the tests *in vivo*, the animals which were given phenobarbital and utilized for the isolation of the microsomal fraction exhibited

increased liver weights compared to controls. Microsomal liver protein was up 165 per cent and the amount of cytochrome P-450 was augmented about 5-fold (Table 4).

TABLE 4. EFFECT OF REPEATED PHENOBARBITAL PRETREATMENT ON RING- AND *N*-HYDROXYLATION OF *N*-2-FLUORENYLACETAMIDE BY MICROSOMAL FRACTION OF LIVER OF INJECTED RATS USING AIR OR AIR–CARBON MONOXIDE GAS PHASES

	Control	Phenobarbital-treated	% Change
Body wt. (g)	152 ± 10	155 ± 9	
Liver/body (%)	4·50 ± 0·06	5·95 ± 0·25	132
Microsomal protein (mg/g)	14·8 ± 0·4	24·4 ± 0·5	165
Cytochrome P-450 (nmoles/g)	11·2 ± 0·5	61·6 ± 2·0	550

Metabolites of FAA	Air (nmoles/30 min/ mg protein)	Effect of CO* (%)	Air (nmoles/30 min/ mg protein)	Phenobarbital induction (% increase)	Effect of CO* (%)
7-OHFAA	5·52 ± 0·94	46†	12·5 ± 0·70	226	84
5-OHFAA	3·17 ± 0·02	91	8·27 ± 0·36	261	85
3-OHFAA	0·94 ± 0·12	120	3·49 ± 0·17	370	70†
N-OHFAA	1·85 ± 0·40	148	8·47 ± 0·81	458	93

* Inhibition or increase of metabolite by carbon monoxide atmosphere compared to air.
† $P < 0·05$.

Microsomal preparations from livers of control animals yielded the 7-, 5-, 3- and *N*-hydroxylated FAA metabolites in roughly the same relative proportions as those eventually excreted in urine under conditions *in vivo*. However, the 3-hydroxy metabolite was lower. Another exception was the *N*-hydroxy derivative, which was higher *in vitro*; the difference may have been accounted for by the known reductive reactions in the gut.[18]

With microsomal preparations from phenobarbital-pretreated animals, there was appreciable enhancement of the production of all hydroxylated metabolites, with the largest increase involving the *N*-hydroxy and the 3-hydroxy derivatives.

Effect of carbon monoxide on the yield of hydroxylated metabolites of N-2-*fluorenylacetamide* in vitro

Introduction of a 1:1 carbon monoxide–air mixture during the incubation severely reduced the yield of the 7-hydroxylated metabolite and had relatively little effect on the 5- and 3-hydroxy products (Table 4), but increased the *N*-hydroxy derivative. On the other hand, in the presence of carbon monoxide–air mixtures, microsomal preparations from phenobarbital-pretreated animals produced slightly less of only the 3-hydroxy metabolite. There was virtually no effect with the other ring- or *N*-hydroxy derivatives, the latter being influenced the least.

DISCUSSION

As was found with other arylamines and arylamine derivatives,[30] phenobarbital was a potent modifier of biochemical hydroxylation reactions, *in vitro* and *in vivo*, of the carcinogen FAA. Because its overall metabolism is well known, FAA offers

advantages to examine the biochemical transformation mechanisms. Present views consider that hydroxylation reactions are performed either: (1) by an enzyme system of mixed-function oxidases where the conformation of the substrate–enzyme complex controls the amount of specific metabolites;[2,6,9,10,31] or (2) by a family of several closely related yet distinct enzyme systems.[4,5,11,32,33]

In agreement with earlier studies involving arylamines and their derivatives, we noted a larger increase of the *N*-hydroxylated product, relative to ring-hydroxy derivatives with phenobarbital as inducer. In contrast, methylcholanthrene seemed to enhance more the ring-hydroxy derivatives, at least in rat liver.[13,14] The effect of chlorinated hydrocarbons is less impressive. Large doses of DDT appeared to enhance *N*-hydroxylation of aniline, but with lower levels and *p*-chloroaniline as substrate, DDT had little effect.[21,22] Simultaneous administration of DDT or carbon tetrachloride appeared to potentiate the carcinogenic effect to the liver of *N*-2-fluorenylacetamide,[34] and perhaps related was a doubling of *N*-hydroxy-FAA excreted in urine.[35] Phenobarbital influences the secretion of bile and the relative clearance of metabolites in bile.[36–38] Resorption and further metabolism via the enterohepatic cycle would affect less the stable 7- and 5-hydroxy derivatives of *N*-2-fluorenylacetamide than the *N*-hydroxy compound, which is reduced back to the parent compound by bacterial enzymes in the gut,[18] and the *ortho*-hydroxy derivatives such as 3-hydroxy-FAA, which undergo ready deacylation and polymerization to materials excreted in the feces.[39] Hence, even though the liver may initially yield more of the *N*-hydroxy and *ortho*-hydroxy metabolites in phenobarbital-pretreated rats, the urinary excretion levels may be relatively lower because of such side reactions.

With phenobarbital, the amount of cytochrome P-450 increased much more than that of liver or microsomal protein. The enhancement of the liver in P-450 paralleled that of the *N*-hydroxy product more than that of the ring-hydroxylated materials, but it is difficult to draw cause and effect relationships.[40]

The effect of a carbon monoxide atmosphere on the oxidation of FAA with liver microsomes from normal weanling rats extended previous findings with simpler hydrocarbons or arylamines in which *p*-hydroxylation was highly sensitive to carbon monoxide. The 7-carbon position of the fluorene ring has been considered equivalent to a *p*-position, being so located with respect to the phenyl ring, and also in an extended *p*-position to the 2-substituted arylamino group. On the other hand, the 5-position is *ortho* to the biphenyl type link and, of course, the 3-position is *ortho* to the acylamino substituent. Thus, while 7-hydroxylation was decreased, the 5- and 3-hydroxylation reactions were little affected by carbon monoxide. Even more, *N*-hydroxylation was not affected or was slightly increased by carbon monoxide. Kiese,[10] Lange,[21] and also Uehleke[22,40] have noted that *N*-hydroxylation of smaller arylamines appears insensitive to carbon monoxide. The much higher *C*- and *N*-hydroxylating capability of microsomes from phenobarbital-pretreated animals was relatively insensitive to carbon monoxide at all positions, except 3-hydroxylation, which was depressed. These comparative data provide further evidence that even phenobarbital-induced enzyme systems differ from basal level systems.[4,33,41] Significantly, *N*-hydroxylation seemed to be affected the least. Yet, Uehleke *et al.*[9,42] have obtained results suggesting that *N*-hydroxylation and cytochrome P-450 levels are related under a variety of inhibitory or enhancing situations. In some respects, our findings are similar, namely the pronounced increase in *N*-hydroxylation and cytochrome P-450 *in vivo* after phenobarbital

pretreatment. Nonetheless, an explanation is required of the carbon monoxide effect under our conditions *in vitro*, which leaves the basal *N*-hydroxylation capacity untouched and reduces only slightly the increased level in stimulated microsome preparations. The mechanism of *ortho*-hydroxylation is not well understood. It could involve direct substitution via the cytochrome P-450 system, or else may be the result of an isomerization from the *N*-hydroxy compound.[43,44] Both schemes may be operative, and to different degrees, in control and induced systems.

Our data and those already obtained by others[42,45] would indicate that microsomal oxidation reactions of arylamines involve several distinct but closely related enzymes.

Finally, of interest is the sharply lower total level of radioactivity from the labeled carcinogen present in the liver and that bound specifically to isolated DNA. Two laboratories have reported that the simultaneous chronic administration of phenobarbital and FAA yielded fewer liver and extrahepatic tumors compared to groups given the carcinogen alone.[46,47] Thus, the biologic effect corresponds to our finding of lower labeling of a crucial receptor molecule, DNA, and of other components of the liver.

Present views on the metabolic activation of carcinogens such as *N*-2-fluorenylacetamide indicate that two steps are required, at least for liver tumor induction and binding of agent to molecular receptors. (1) The carcinogen must undergo *N*-hydroxylation.[11,17,48] Current data show that this reaction is enhanced by phenobarbital; thus, this activation process is not limiting in this instance. (2) The *N*-hydroxy derivative is esterified, most likely by formation of the sulfate ester.[49,50] The necessary sulfotransferase found in liver cytosol does not seem to be inducible by many of the known enzyme inducers, although the endocrine situation affects the level of the enzyme appreciably.[49] Our results indicate that formation of glucosiduronic acids, mediated by inducible enzymes on the endoplasmic reticulum, is influenced by phenobarbital. *cf.*[50,51,52] It seems likely that in the phenobarbital-pretreated rats the enhanced formation of the glucuronic acid conjugate of *N*-hydroxy-*N*-2-fluorenylacetamide withdraws this important intermediate from the competition for sulfate ester formation, which thus is lower. In turn, there is less binding to cell macromolecules and less cancer. Thus, these experiments further support the concept that the formation of sulfate esters of *N*-hydroxy compounds is relevant to the carcinogenic process by these compounds.

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