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Metabolism of α-Naphthoflavone by Rat Liver Microsomes[1]

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ABSTRACT

α-Naphthoflavone (ANF) or 7,8-benzoflavone, a synthetic flavanoid, has been widely used in biochemical and biological studies concerning the mechanisms of action of chemical carcinogens. It has been shown previously that ANF inhibits benzo(*a*)pyrene metabolism by β-naphthoflavone (BNF)-induced rat liver microsomes but has no inhibitory effects on benzo(*a*)pyrene metabolism in phenobarbital (PB)-induced rat liver microsomes. This study shows that ANF gives type 1 binding spectra with and is metabolized by both BNF- and PB-induced rat liver microsomes. Specific metabolites identified by ultraviolet and mass spectra and in some cases by cochromatography with authentic standards were: 6-hydroxy-α-naphthoflavone, 9-hydroxy-α-naphthoflavone, α-naphthoflavone-5,6-oxide, and 5,6-dihydro-5,6-dihydroxy-α-naphthoflavone. Metabolism at the 5,6 bond of ANF accounted for 73 and 86% of the total organic soluble metabolites produced by PB- and BNF-induced microsomes, respectively. This result is in concert with previous observations on the role of 6 substitution and the loss of inhibitory activity of ANF in BNF-induced rat liver microsomes.

Metabolism of ANF is mediated by the cytochrome P-450 mixed-function oxidases, because it is dependent on NADPH and inhibited by carbon monoxide and other cytochrome P-450 inhibitors. BNF-induced microsomes metabolize ANF to 5,6-dihydro-5,6-dihydroxy-α-naphthoflavone to a much greater extent than do PB-induced microsomes.

INTRODUCTION

ANF[3] (Chart 1), a synthetic flavanoid, has been widely used in biochemical and biological studies concerning the mechanisms of action of chemical carcinogens. ANF inhibits the metabolism, binding to cellular macromolecules, and carcinogenesis of 7,12-dimethylbenz(*a*)anthracene and 3-methylcholanthrene in mouse skin and hamster embryo cells in culture (Refs. 18 and 19 and references therein). In mouse skin, ANF also inhibits benzo(*a*)pyrene metabolism and binding to RNA and protein but has a lesser effect on binding to DNA (4). Conflicting data have been reported on the ability of ANF to inhibit benzo(*a*)pyrene mouse skin tumorigenesis (4, 16). ANF is an inhibitor of rat liver cytochrome P-448-mediated mixed-function oxidation activities, including the metabolism of benzo(*a*)pyrene. However, ANF does not inhibit rat liver cytochrome P-450-mediated mixed-function oxidase activities (8, 19). At present, the mechanism of inhibition of mixed-function oxidase activities by ANF is unknown (19); however, a number of studies, including crystal structure and structure activity analysis, have been reported (15). We have observed that specific structural features of the ANF molecule are necessary for inhibitory activity of induced rat liver microsomes (8). One of these features is a 6 position, which is (*a*) unsubstituted (*i.e.*, 6-bromo-ANF and 6-nitro-ANF are inactive) or (*b*) substituted with an oxidizable moiety (*e.g.*, 6-amino-α-naphthoflavone is active). We therefore postulated that ANF might require metabolism to exert its inhibitory action and that the specificity of inhibition observed with cytochrome P-448 and cytochrome P-450 mixed-function oxidase activities might be related to the ability of those microsomes to metabolize at position 6. Therefore, it was of interest to study the metabolism of ANF by both cytochrome types to further understand the differences in its inhibitory activity in rat liver microsomes.

MATERIALS AND METHODS

Chemicals and Radiochemicals. [^{3}H]ANF was prepared by Amersham/Searle Corp. (Arlington Heights, Ill.), using ANF obtained from Aldrich Chemical Co. (Milwaukee, Wis.), and was purified by HPLC using a DuPont Instruments (Wilmington, Del.) Model 848 chromatograph fitted with a 4.6-mm (inside diameter) x 25-cm Zorbax-ODS column. An isocratic solvent system of methanol was used at a flow rate of 2.0 ml/min, and under these conditions, ANF exhibited a retention time of 5.0 min. Radiochemical purity was estimated to be >99% using this system. The authentic standards of 6-hydroxy-ANF and 9-hydroxy-ANF were synthesized by Dr. Robert Roth of Midwest Research Institute by modifications of the method of Mahal and Venkataraman (6) using the appropriately substituted methoxy-2-acetyl-1-naphthols. The analytic and spectroscopic data obtained from these synthetic flavone metabolites were fully consistent with their structure, and these details will be reported elsewhere.

Preparation of Microsomes. Male Charles River CD rats (80 to 100 g) were maintained on laboratory chow and water *ad libitum*. Groups of at least 4 rats were induced with either PB (Mallinckrodt Chemical Co., St. Louis, Mo.), 80 mg/kg in 0.5 ml NaCl solution (0.85%), or BNF (Aldrich Chemical Co.), 20 mg/kg in 0.5 ml corn oil, injected i.p. on each of 4 successive days. Rats were starved for 24 hr after the last treatment, and microsomes were prepared according to the method of van der Hoeven *et al.* (17). Protein concentrations were determined by the method of Lowry *et al.* (5), using bovine serum albumin as the protein standard (Sigma Chemical Co., St. Louis, Mo.).

Metabolism Studies. [^{3}H]ANF was incubated with micro-

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[3] The abbreviations used are: ANF, α-naphthoflavone or 7,8-benzoflavone; 6-bromo-ANF, 6-bromo-α-naphthoflavone; 6-nitro-ANF, 6-nitro-α-naphthoflavone; HPLC, high-pressure liquid chromatography; 6-hydroxy-ANF, 6-hydroxy-α-naphthoflavone; 9-hydroxy-ANF, 9-hydroxy-α-naphthoflavone; PB, phenobarbital; BNF, β-naphthoflavone; 4′-methoxy-ANF, 4′-methoxy-α-naphthoflavone; ANF-5,6-oxide, α-naphthoflavone-5,6-oxide; 5,6-dihydro-5,6-dihydroxy-ANF, 5,6-dihydro-5,6-dihydroxy-α-naphthoflavone.

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somes and an NADPH-generating system according to the following procedure. A 5.0-ml incubation mixture contained 5 μmol $NADP^+$, 22.5 μmol glucose 6-phosphate, 9 units glucose-6-phosphate dehydrogenase (Sigma Chemical Co., St. Louis, Mo.); 15 μmol $MgCl_2$, 250 μmol potassium phosphate buffer (pH 7.50), 25 μM [^{3}H]ANF (specific activity, 1 to 2 μCi/μmol), and 2.00 mg microsomal protein. Reaction was begun at 37° with agitation and the addition of substrate. The reaction was terminated upon the addition of 15 ml of ethyl acetate:acetone (2:1). After thorough mixing and centrifugation at 800 × g, a 12-ml aliquot of the organic layer was removed and evaporated under dry nitrogen. The samples were reconstituted in 75 μl of methanol and chromatographed by HPLC on a 6.2-mm (inside diameter) x 22.5-cm Zorbax-ODS column using a mixture of methanol:water (85:15) at a flow rate of 1.8 ml/min. Fractions (12 sec) were collected directly into liquid scintillation vials for subsequent radiochemical analysis.

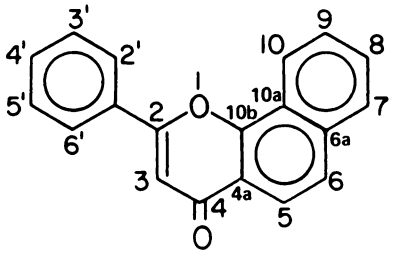

Chart 1. The chemical structure, IUPAC nomenclature, and numbering system of ANF.

Epoxide Hydrase Studies. Purified Metabolite 3 was obtained from multiple incubations, extractions, and HPLC of nonradiolabeled ANF according to the methods described above. BNF-induced rat liver microsomes (5.0 mg protein in 4.95 ml 0.05 M sodium phosphate buffer, pH 8.0) and purified Metabolite 3 in 0.050 ml methanol were incubated for 1.0 hr at 37° with shaking. The reaction mixture was extracted with 5.0 ml ethyl acetate:acetone (2:1), vortexed for 5 min, and centrifuged. The organic layer was evaporated to dryness with dry filtered nitrogen, dissolved in 0.200 ml methanol, and chromatographed by HPLC as described above. Equivalent

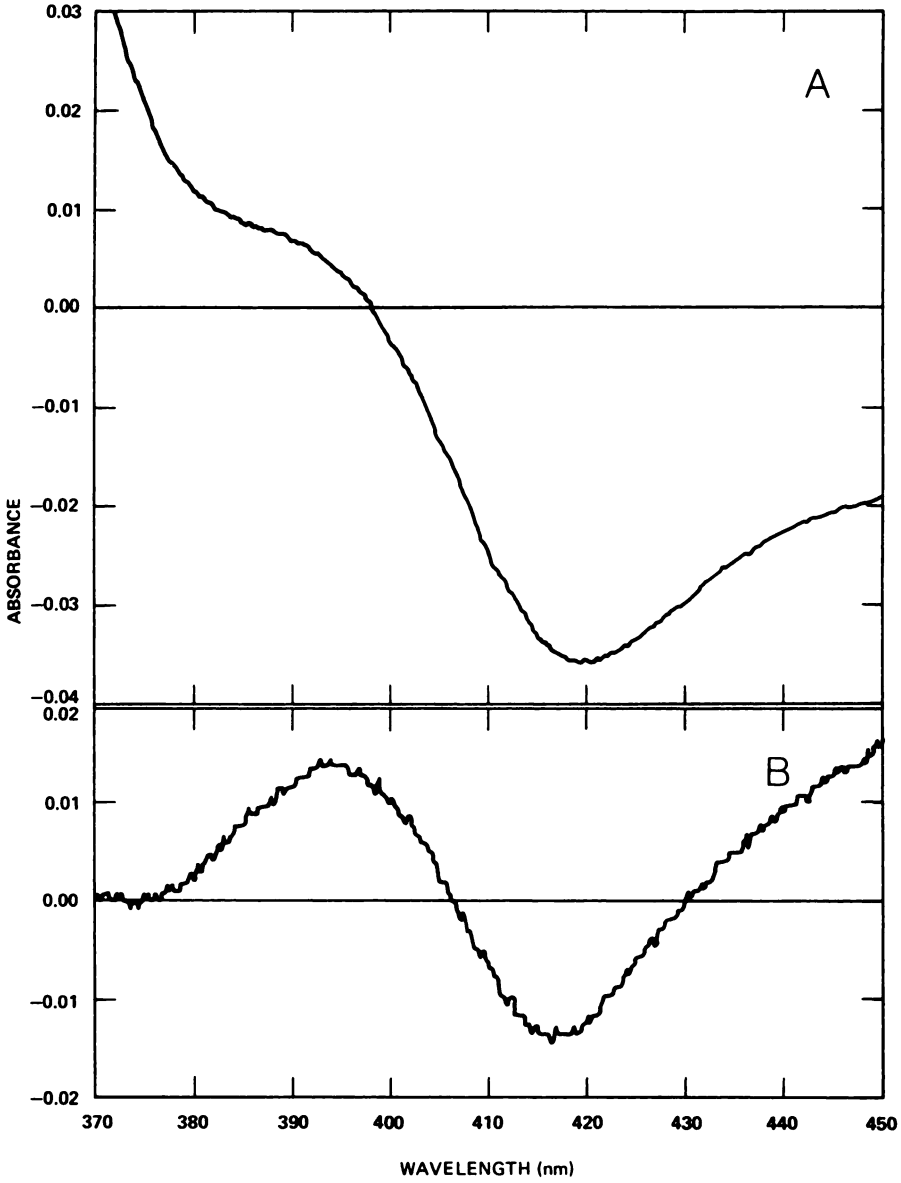

Chart 2. The ANF-induced difference spectra in rat liver microsomes. Hepatic microsomes from PB-treated rats were suspended in 0.05 M Tris buffer, pH 7.5 (1 mg/ml), and placed into 2 cuvets. One cuvet was treated with ANF (67 μM), and the other was treated with solvent. *A*, scan of the difference spectra from 370 to 450 nm (uncorrected spectrum); *B*, spectrum corrected for absorbance due to ANF. The difference spectra of ANF with hepatic microsomes from BNF-induced rats was essentially identical to that shown above.

amounts of purified Metabolite 3, incubated with boiled enzymes, extracted, and chromatographed, served as sham controls. Approximately 30% of Metabolite 3 was converted to Metabolite 1b.

Spectroscopic Studies. UV absorption spectra were recorded on a Beckman Model 25 spectrophotometer. Mass spectra were recorded on a Hewlett-Packard mass spectrometer system or a Micromass mass spectrometer using a direct insert probe. Binding studies were performed on an Aminco-Bowman DW-2, fitted with a Midan T storage and data analysis system, and using the method of Orrenius *et al.* (14).

RESULTS

The interaction of ANF with liver microsomes from rats induced with PB (cytochrome P-450) and liver microsomes from rats induced with BNF (cytochrome P-448) was examined by their difference binding spectra. ANF produces end absorption in the 370- to 420-nm range, and the initial binding spectra observed are a composite of both ANF end absorption and ANF microsome spectral interaction (Chart 2*A*). However, by subtracting the end absorption from the difference spectra, a classical type 1 difference spectrum is obtained (Chart 2*B*) (14). Both PB- and BNF-induced microsomes gave the same type 1 spectra. ANF also gives a type 1 spectrum with mouse liver microsomes (2).

The ability of cytochrome P-450- and cytochrome P-448-containing microsomes to metabolize [^{3}H]ANF was studied using microsomes fortified with an NADPH-generating system. The HPLC chromatograms of profiles obtained after 15 min of incubation (Chart 3, *A* and *B*) revealed that both microsome types metabolize [^{3}H]ANF to at least 5 distinct products, iden-

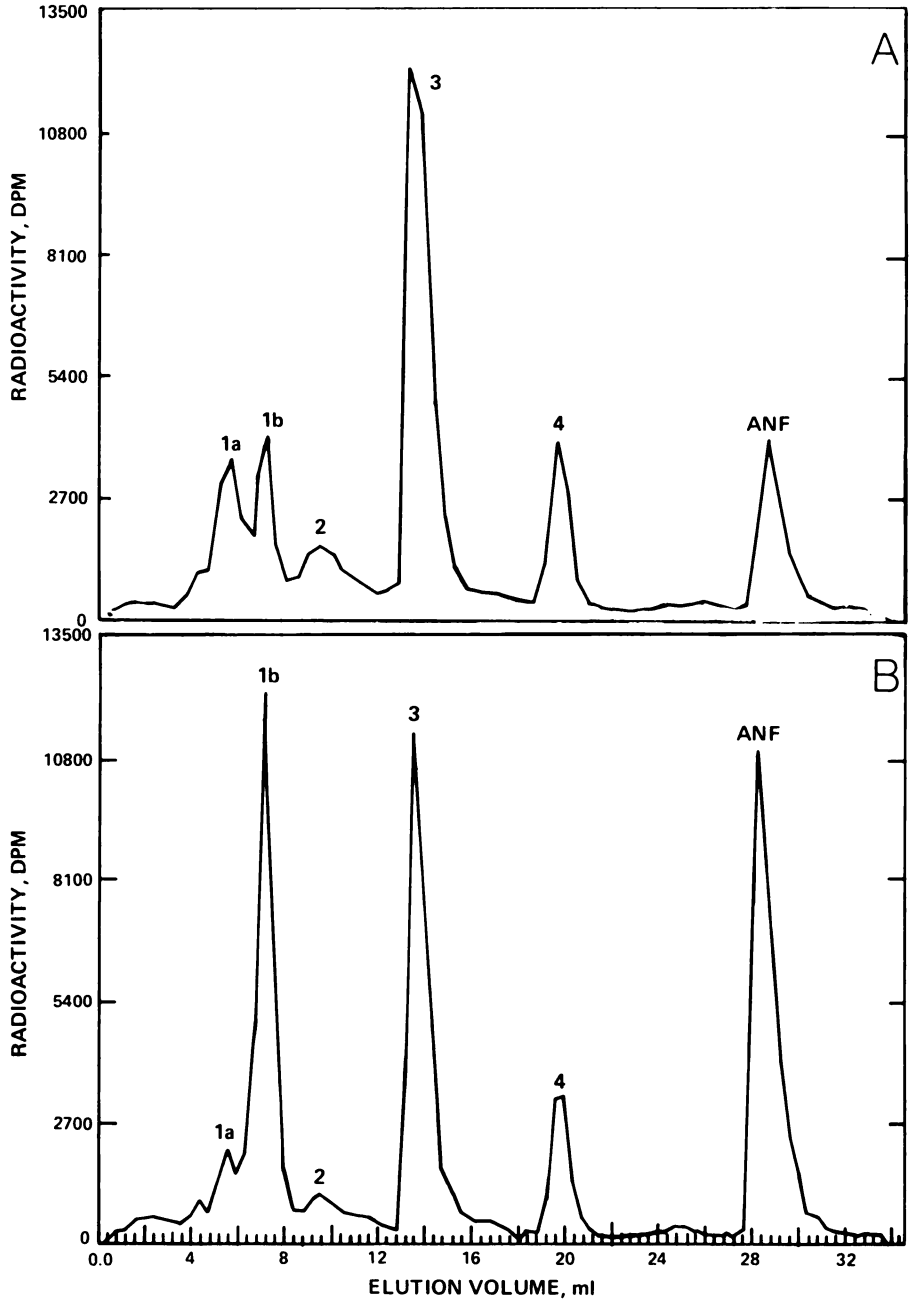

Chart 3. HPLC chromatograms of ANF metabolites produced after 15-min incubations of [^{3}H]ANF (25 μM) with PB-induced hepatic microsomes (*A*) and BNF-induced hepatic microsomes (*B*). *Peak 1a*, mixture of unknown metabolites; *Peak 1b*, 5,6-dihydro-5,6-dihydroxy-ANF; *Peak 2*, 9-hydroxy-ANF; *Peak 3*, ANF-5,6-oxide; *Peak 4*, 6-hydroxy-ANF. See text for details.

tified as Peaks 1a, 1b, 2, 3, and 4. The one major difference between the 2 profiles was in the greater production of 1b by BNF-induced microsomes.

Structural Identification of Metabolites

Four of the 5 metabolites, Peaks 1b, 2, 3, and 4, were isolated by preparative HPLC using the same chromatographic conditions described in "Metabolism Studies," above, from incubations of ANF with induced rat liver microsomes. Each metabolite was isolated and analyzed by a combination of mass and UV spectrometry. Mass spectrometry provided a great deal of information on the structure of all the metabolites. These metabolites, as well as ANF, exhibited a major mass fragmentation loss of *m/e* 102 or C_8H_6. This fragment of phenyl acetylene could arise from the fragmentation of ANF via 2 pathways: (*a*) fragmentation at the O-1,C-2 and C-3,C-4 bonds; or (*b*) fragmentation at the C-4a,C-5 and C-10a,C-10b bonds (Chart 1). Mass spectra of authentic standards 4′-methoxy-ANF, 6-nitro-ANF, and 6-bromo-ANF gave the following results at 70 eV. 4′-methoxy-ANF: *m/e* 302 (M^+, 50), *m/e* 170 (85); 6-nitro-ANF: *m/e* 317 (M^+, 100), *m/e* 287 (22), *m/e* 215 (41); 6-bromo-ANF: *m/e* 352/350 (M^+, 65/65), *m/e* 250/248 (100/100). Link scan analysis (3, 7) of the metastable transitions indicated the loss of *m/e* 102 in 6-nitro-ANF and 6-bromo-ANF and the loss of *m/e* 132 in 4′-methoxy-ANF were a direct fragmentation of the molecular ion. Since 6 substitution yielded loss of *m/e* 102 and 4′ substitution yielded loss of *m/e* 132, it was concluded that Fragmentation Pathway *a* predominated. These results indicate that the generalized site of metabolism of ANF could be identified by analysis of the major mass fragmentation patterns.

Metabolite 2. Metabolite 2 has been identified as 9-hydroxy-ANF. Mass spectrometry (70 eV) yielded: *m/e* 288 (M^+, 60), *m/e* 186 (100). Results of UV spectrometry were: λ_{max}, pH 1, 295 nm; pH 7, 295 nm; pH 14, 295(shoulder), 305 nm. Mass spectrometry indicated a monooxygenated ANF with substitution on the naphthalene ring. Link scan mass spectrometry of the metastable ions confirmed *m/e* 186 as a fragment of *m/e* 288. Cochromatography by HPLC of Metabolite 2 with an authentic standard of 9-hydroxy-ANF and identical UV and mass spectra of both Metabolite 2 and 9-hydroxy-ANF provided conclusive structural proof.

Metabolite 4. Metabolite 4 has been identified as 6-hydroxy-ANF. Mass spectrometry (70 eV) results were: *m/e* 288 (M^+, 70), *m/e* 186 (100); precise mass determination, 288.0879 ($C_{19}H_{12}O_3$ requires 288.086). UV spectrometry results were: λ_{max}, pH 1, 255, 292 nm; pH 7, 255, 290 nm; pH 14, 300 nm. Mass spectrometry indicated a monohydroxylated ANF substituted on the naphthalene ring. This was confirmed by link scan analysis of the metastable ions. Cochromatography by HPLC of Metabolite 4 with an authentic standard of 6-hydroxy-ANF and identical UV and mass spectra of Metabolite 4 and 6-hydroxy-ANF provided conclusive structural proof.

Metabolite 3. Metabolite 3 has been identified as ANF-5,6-oxide. Mass spectrometry (70 eV) results were: *m/e* 288 (M^+, 60), *m/e* 186 (100). Results of UV spectrometry were: λ_{max}, pH 1, 255, 290 nm; pH 7, 270, 300 nm; pH 14, 270, 300 nm. Mass spectrometry indicated a monooxygenated ANF substituted on the naphthalene ring. The irreversible change of the UV spectra upon addition of acid to a neutral solution of Metabolite 3, producing the UV spectra of Metabolite 4, denoted a labile functional group. HPLC analysis of the acidified solution of Metabolite 3 demonstrated 100% conversion to Metabolite 4, 6-hydroxy-ANF. The conversion of an epoxide to a phenol catalyzed by acid has been well documented (21) and proves the structure of Metabolite 3 as ANF-5,6-oxide.

Metabolite 1b. Metabolite 1b has been identified as 5,6-dihydro-5,6-dihydroxy-ANF. Results of mass spectrometry at 70 eV were: *m/e* 306 (M^+, 16), *m/e* 288 (100), *m/e* 275 (21), *m/e* 260 (80), *m/e* 186 (50). Precise mass determination yielded 306.0892 ($C_{19}H_{14}O_4$ requires 306.0892). Mass spectrometry indicated a dihydrodiol derivative of ANF with the substituents located on the naphthalene ring. This was con-

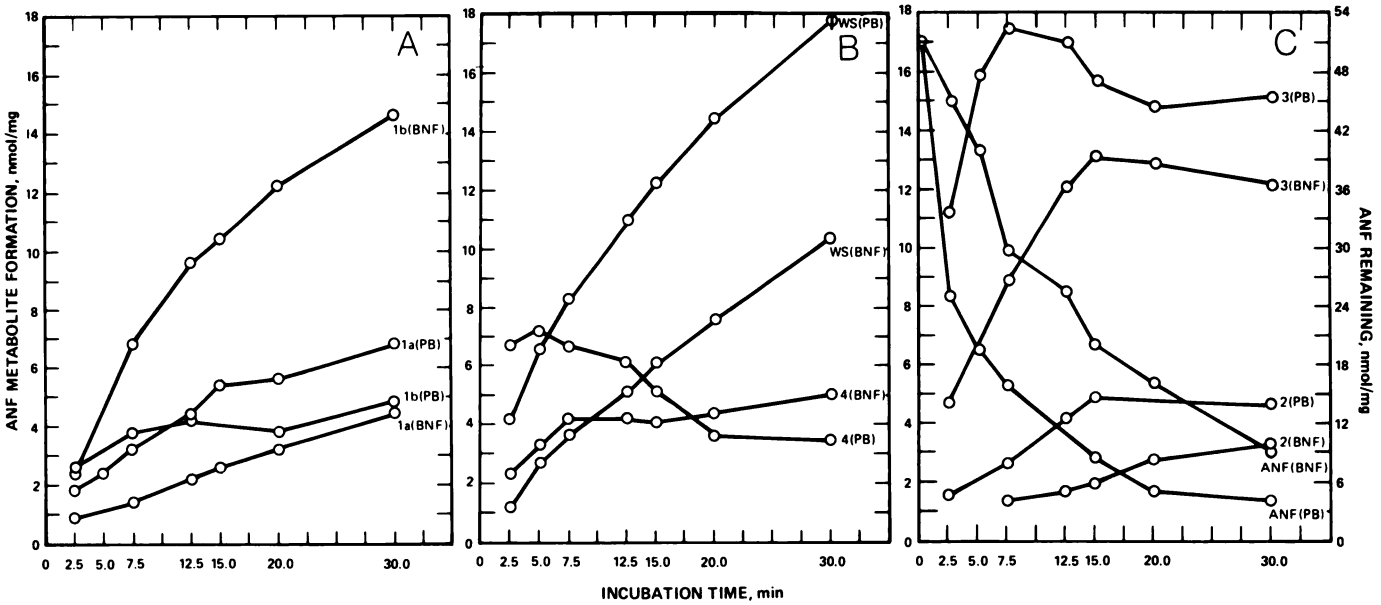

Chart 4. Metabolism of ANF: comparison of inducers. Incubation of [^{3}H]ANF (25 μM) with rat liver microsomes induced by PB or BNF were performed at various times, and metabolites were isolated and quantitated as described in "Materials and Methods." *WS*, radioactivity remaining in the aqueous layer after organic solvent extraction. The coefficient of variation is 20%. See Chart 3 for metabolite identification.

firmed by link scan mass spectrometry of the metastable transitions. Incubation of Metabolite 3 (ANF-5,6-oxide) with microsomal epoxide hydrase (13) and analysis by HPLC produced Metabolite 1b. The enzymatic conversion of ANF-5,6-oxide to the dihydrodiol 1b confirmed its structure as 5,6-dihydro-5,6-dihydroxy-ANF (21).

Comparative Metabolism Studies

Preliminary studies on the metabolism of ANF gave 25 μM as saturating for the production of all metabolites by both BNF- and PB-induced microsomes (data not shown). The time course of ANF metabolite formation was compared between PB- and BNF-induced rat hepatic microsomes. Within experimental error, both induced microsomes metabolized ANF to 9-hydroxy-ANF, 6-hydroxy-ANF, and ANF-5,6-oxide at similar rates (Chart 4). ANF was metabolized at a slightly greater rate and was converted to larger amounts of non-organic soluble forms (polyhydroxylated metabolites) by PB-induced microsomes (Chart 4, *B* and *C*). However, BNF-induced microsomes were superior in metabolizing ANF to its 5,6-dihydrodiol (Chart 4*A*). At 15 min of incubation, the overall metabolic rate was approximately the same for both microsome types, 2.3 to 2.5 nmol ANF metabolized to organic soluble products per min per mg microsomal protein. This represented an overall conversion of approximately 60% of the substrate.

DISCUSSION

As observed with a structurally related polycyclic aromatic hydrocarbon such as phenanthrene (1), ANF is metabolized via the arene oxide-dihydrodiol-phenol pathways by rat liver microsomes (Chart 5). These metabolic pathways have been well documented with studies of the metabolism of benzo(*a*)pyrene (21). From the observed, identified metabolites, it is proposed that ANF is metabolized at the 5,6 and 9,10 bonds, forming ANF-5,6-oxide and α-naphthoflavone-9,10-oxide (not observed). Subsequent rearrangement gives 6-hydroxy-ANF and 9-hydroxy-ANF. Electron resonance structures with positive charges on O-1 support the formation of these phenols rather than the corresponding 5- and 10-hydroxy-α-naphthoflavone. Hydration of ANF-5,6-oxide by epoxide hydrase gives 5,6-dihydro-5,6-dihydroxy-ANF.

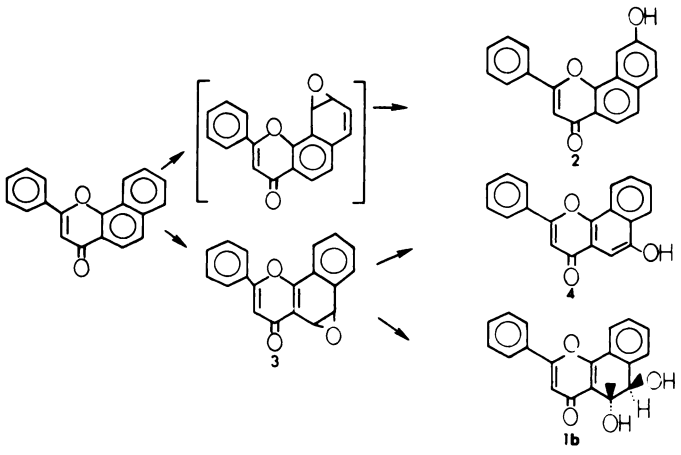

Chart 5. Scheme of metabolism of ANF by rat liver microsomes. *Brackets*, proposed intermediates.

Table 1

Effect of cofactors and inhibitors on the metabolism of [^{3}H]ANF

Incubation mixtures of [^{3}H]ANF (50 μM) with microsomes (0.4 mg/ml) were carried out for 15 min under the various conditions listed below. See "Materials and Methods."

Incubation condition	PB-induced microsomes		BNF-induced microsomes	
Complete mixture	35.3[a]	(100)[b]	36.9[a]	(100)
− NADPH	0	(0)	0	(0)
+ carbon monoxide	2.84	(8)	2.75	(7)
+ SKF 525-A (500 μM)	13.7	(39)	ND[c]	
+ phenanthrene-9,10-quinone (25 μM)	ND		2.06	(6)

[a] Values are mean total organic soluble ANF metabolites formed per mg microsomal protein. The coefficient of variation was 15%.
[b] Numbers in parentheses, percentage of activity compared to the complete mixture.
[c] ND, not determined.

The mixed-function oxidases in PB- and BNF-induced microsomes that oxidize ANF are cytochrome P-450 mediated, because the metabolism of ANF is dependent on NADPH and inhibited by carbon monoxide and by the cytochrome P-450 inhibitors SKF-525A (12) and phenanthrene-9,10-quinone (10) (Table 1).

There are more similarities than differences in the metabolism of ANF by PB- and BNF-induced rat liver microsomes. ANF gives type 1 binding spectra with both cytochrome types. Enzymatic metabolism by both types of induced microsomes gave essentially the same overall rates of conversion of ANF based on microsomal protein. The majority of the ANF metabolism occurs at the 5,6 bond, between 73 and 86%, depending on whether the inducer is PB or BNF. This is comparable to that found under similar conditions for the metabolism of phenanthrene, where 67 to 85% occurs at the structurally analogous 9,10 bond (1). In that case, however, PB microsomes were more efficient at 9,10 bond metabolism. The significant amount of metabolism of ANF at the 5,6 bond confirms our initial observations on the relationship of 6 position substitution with nonoxidizable substituents and the loss of inhibitory activity of ANF in cytochrome P-448-containing microsomes.

It has been reported that pretreatment of rats with PB increases epoxide hydrase activities 3-fold, while polycyclics like 3-methylcholanthrene have essentially little effect on the induction of this enzyme (13). PB-induced microsomes produced less 5,6-dihydro-5,6-dihydroxy-ANF than did BNF-induced microsomes, suggesting that there was an inverse relationship between epoxide hydrase activities and diol formation. However, PB-induced microsomes metabolized more ANF to polyhydroxylated metabolites (water solubles) than did BNF-induced microsomes. In addition, the metabolism of ANF to both 5,6-dihydro-5,6-dihydroxy-ANF and polyhydroxylated metabolites totals 16.5 nmol/15 min/mg protein in BNF-induced microsomes, while PB-induced microsomes produce a total of 16.3 nmol/15 min/mg protein. The similarity between the sum of the 5,6-dihydro-5,6-dihydroxy-ANF and polyhydroxylated ANF in the 2 induced microsome types suggests that 5,6-dihydro-5,6-dihydroxy-ANF could be further metabolized to polyhydroxylated ANF and that this additional metabolic step is more efficient in PB-induced microsomes. The further metabolism of diols to polyhydroxylated metabolites has been reported for benzo(*a*)pyrene (21).

The differential effects of ANF inhibition in BNF- and PB-induced rat liver microsomes are not readily explained by the metabolism data. One major difference, which has been ob-

served and might account for the differences in susceptibility of PB- and BNF-induced rat liver microsomes toward ANF inhibition of oxidative metabolism, is that BNF-induced microsomes which are inhibited by ANF produce more 5,6-dihydro-5,6-dihydroxy-ANF. This metabolite might be the active inhibitor species or may be further metabolized to an active form.

Metabolism of ANF to a more active species has been observed with 9-hydroxy-ANF, which is 7 to 8 times more active than ANF in inhibiting benzo(*a*)pyrene oxidation in BNF-induced microsomes (9). However, 9-hydroxy-ANF is inactive in inhibiting PB-induced microsomes (9) and is not formed in greater amounts in incubations of ANF with BNF-induced microsomes (Chart 4). Therefore, 9-hydroxy-ANF formation cannot explain the differential inhibition of ANF in PB- and BNF-induced rat liver microsomes.

The exact mechanism by which ANF inhibits BNF-induced hepatic microsomal mixed-function oxidase activities and the reasons for the inability of ANF to inhibit microsomal enzymes from PB-induced rats are still unknown. Wiebel and Gelboin (20) suggest from kinetic data that ANF at low concentrations might be a competitive inhibitor while at higher concentrations may inhibit by a more complex mechanism. The data on ANF metabolism seem to support their conclusion, for at high ANF concentrations both ANF and 9-hydroxy-ANF can inhibit mixed-function oxidase activity; while at low concentrations 9-hydroxy-ANF formation would be expected to be barely detectable, and ANF would therefore be the major competitive inhibitor.

Elucidation of the complete pathways of metabolism of ANF in tissues or organisms where ANF affects microsomal processes will provide additional clues to the mechanisms which underlie these processes.

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