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THE METABOLISM OF α-NAPHTHOFLAVONE (7,8-BENZOFLAVONE) BY HEPATIC MICROSOMES FROM THE MARINE FISH *STENOTOMUS VERSICOLOR*

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SUMMARY: Incubation of α-naphthoflavone with fish (scup; *Stenotomus versicolor*) liver microsomes and NADPH resulted in the production of a major component and several minor components analyzed by high pressure liquid chromatography. The appearance of these components was dependent on time, native protein, NADPH, and O_2 and was strongly inhibited by carbon monoxide. The appearance of the major component was abolished by addition of the epoxide hydrase inhibitor trichloropropene oxide. Mass spectral analysis of the major component yielded a molecular weight of 306. The results strongly indicate that α-naphthoflavone is metabolized by scup hepatic microsomal mixed-function oxygenases and epoxide hydrase and that the major product is a dihydrodiol.

The flavone α-naphthoflavone (7,8-benzoflavone; ANF) is a compound that is known to have pronounced effects on the activation of some chemical procarcinogens in vitro (1,2), on the action of certain drugs in vivo (3) and on the induction of tumors by polycyclic aromatic hydrocarbons (PAH) (4). These effects may be related to the interaction of ANF with some forms of cytochrome P-450. ANF, like its isomer 5,6-benzoflavone, is able to induce certain forms of cytochrome P-450 (5,6). ANF is also known to inhibit in vitro activity of cytochromes P-450 induced by 3-methylcholanthrene (3-MC) and other PAH in mammals, while it can stimulate the activity of cytochromes P-450 in control or phenobarbital-treated animals (7,8,9). In some fish species, however, in vitro inhibition of benzo[a]pyrene hydroxylase (BPH) is seen both in control and PAH-treated animals (10,11), raising questions about the nature of the cytochromes P-450 in those control animals.

While the mechanism of stimulation of some cytochromes P-450 is not known, the action of ANF on 3-MC-induced cytochromes P-450 appears to be one of competitive inhibition (12). Such inhibition suggests that ANF associates with the active site of these cytochromes P-450 and in fact ANF produces a

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type I binding spectrum with 3-MC-induced cytochromes P-450 in rats (5). While this implies that ANF might serve as a substrate for some cytochromes P-450, there have been no reports describing the metabolism of ANF. We here report on the metabolism of this compound by hepatic microsomes of a marine fish (scup) that when untreated has BPH activity inhibited by ANF (13).

MATERIALS AND METHODS: α-Naphthoflavone and 1,1,1-trichloropropene oxide (TCPO) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Other substrates including NADPH, salts, and buffers were all the best grades available, obtained from Sigma Chemical Co. (St. Louis, MO). Solvents used in extractions and high pressure liquid chromatography (HPLC) were obtained from Burdick and Jackson Laboratories (Muskegon, MI). Water used in HPLC preparations was glass distilled and filtered through a 0.22 μm Millipore GS filter (Bedford, MA) prior to use.

Hepatic microsomes of the fish scup (*S. versicolor*) were prepared as described previously (13) except that microsomal pellets were resuspended, washed, and repelleted in a buffer consisting of 10 mM ethylene dinitrilotetraacetic acid (EDTA) in 0.1 M sodium pyrophosphate (pH 7.4). Final microsomal pellets were resuspended as described previously (13).

Reaction mixtures used for the preparation of ANF metabolites consisted of 0.375 mg/ml liver microsomes with 0.35 mg/ml NADPH in 50 mM Tris-HCl (pH 7.5). Final concentrations of ANF (added in 10 μl methanol) were 100 μM and 200 μM for reactions examining metabolic requirements and the effects of TCPO, respectively. Reactions were incubated at 30°C for either 15 minutes or 30 minutes. Zero-time blanks in each case consisted of aliquots of reaction mixtures stopped and extracted immediately after addition of substrate. Reaction mixtures used to determine the effects of hypoxia ($-O_2$) and carbon monoxide (CO) were gently bubbled for 10 minutes with either N_2 or CO, substrate was added, and incubation carried out sealed under N_2 or in the case of CO, open. All reactions were stopped by the addition of 1.0 ml cold acetone per ml of reaction mixture. ANF and its metabolites were extracted from stopped reactions with 2.0 ml ethyl acetate per ml of reaction mixture. The ethyl acetate extract layer (upper layer) was transferred to a tube containing $MgSO_4$ to remove any residual water and then transferred to a final tube and dried under a stream of high purity nitrogen. Extracted material was resuspended in 100 μl of acetonitrile per ml of reaction mixture.

Resuspended samples were analyzed by high pressure liquid chromatography using a DuPont LC 850 HPLC fitted with a 25 cm Zorbax ODS column and equipped with a filter photometer UV detector operating at 254 nm. Samples were eluted by running a gradient of 40-70% acetonitrile in water at 30°C. The areas under the absorbance peaks appearing in the elution profile were measured planimetrically for comparative purposes. The major component eluting from the column was collected, extracted with benzene and subjected by probe distillation to electron ionization mass spectrometry on a Finnegan model 1015 mass spectrometer.

RESULTS: HPLC analysis performed on extracts of 30 minute incubations of complete reaction mixtures revealed a large peak of UV absorbance eluting

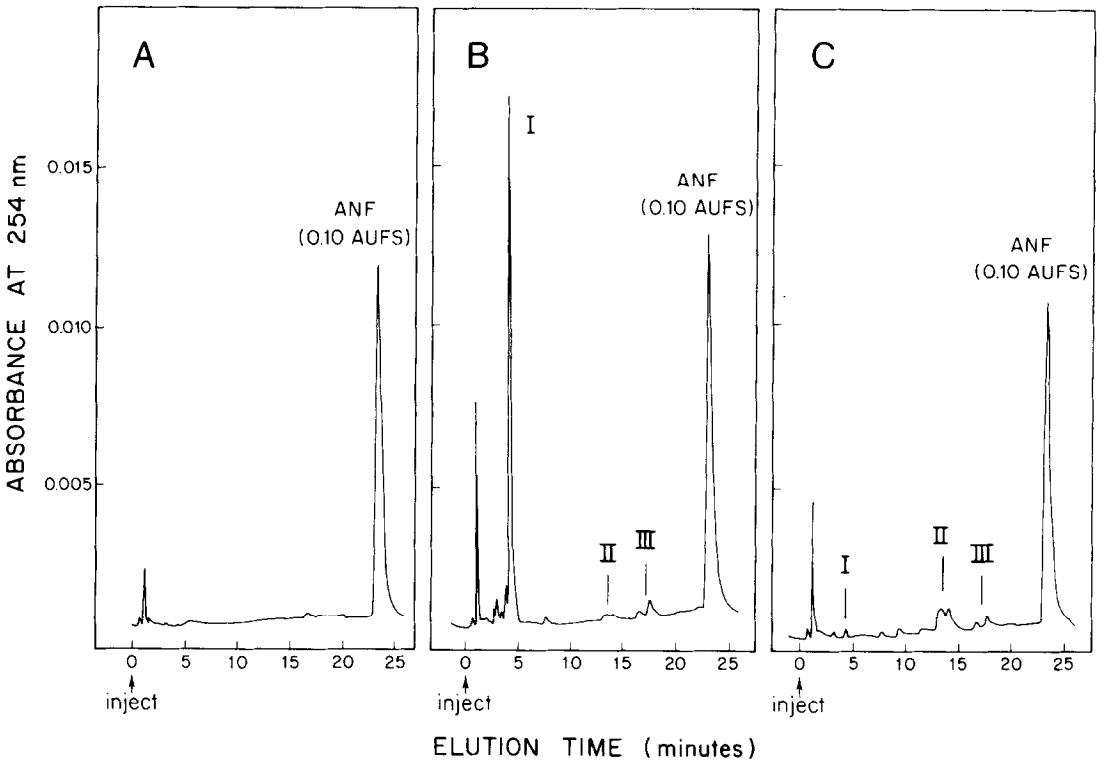

FIGURE 1. High pressure liquid chromatography of α-naphthoflavone and putative metabolites. Extraction and HPLC analyses are indicated in Materials and Methods section. A: Analysis of extracts obtained at time 0. B: Analysis of extracts obtained after 30 minutes incubation. C: Analysis of extracts after 30 minutes incubation but with TCPO added to the reaction mixture. ANF, α-naphthoflavone. AUFS, absorbance units full scale. AUFS was changed from 0.02 to 0.1 just prior to elution of ANF.

from the column well ahead of ANF (Figure 1B; I). This component was not present in aliquots of the same reaction mixtures that were extracted at time 0 (Figure 1A). In addition to the major components, there were smaller peaks whose appearance was also time dependent. These components eluted in regions between the major component and ANF (Figure 1B; II and III). The appearance of the principal component at 15 minutes was also dependent on the presence of native protein, and NADPH (Table 1). Its appearance was strongly inhibited by incubation under a nitrogen atmosphere, and was also strongly inhibited by carbon monoxide. The appearance of the smaller components at regions II and III had similar requirements.

TABLE 1. REQUIREMENTS FOR METABOLISM OF 7,8-BENZOFLAVONE BY SCUP LIVER MICROSOMES

Reaction Condition	Metabolite Formation	Percent
Complete (plus NADPH)	3.72 ± 0.13*	100
Minus NADPH	N.D.	< 1
Minus O_2†	0.62 ± 0.04	17
Plus CO**	0.55 ± 0.03	15
Boiled microsomes	N.D.	< 1

*Values are arbitrary area units for the HPLC peak representing the principal metabolite. Data represent means ± standard deviation of measurement error. N.D., not detected.

†Reaction mixtures were bubbled with N_2, given an N_2 atmosphere, then incubated sealed.

**Reaction mixtures were bubbled with CO and incubated unsealed.

These requirements clearly imply that ANF is metabolized by a mixed-function oxygenase (MFO) reaction. Hydroxylation of aromatic structures by microsomal MFO reactions can proceed via arene oxide intermediates (14), which may subsequently be hydrated by microsomal epoxide hydrase. The addition of TCPO, a potent epoxide hydrase inhibitor (15), completely abolished the appearance of the principal HPLC peak (Figure 1C). On the other hand, peaks at region II but not region III increased in magnitude when TCPO was added. The fact that the major component was thus dependent on epoxide hydrase activity, strongly suggests that this component is probably a dihydrodiol.

Mass spectrometry of the major component revealed a molecular weight of 306. This addition of 34 mass units relative to the parent compound molecular weight of 272 is consistent with the formation of a dihydrodiol metabolite of ANF by scup liver microsomes. Moreover, an analysis of mass spectra of ANF and the metabolite suggested that the site of metabolism was on the fused ring portion of the molecule rather than on the phenyl group.

DISCUSSION: The data presented here offer convincing evidence that ANF can be metabolized by hepatic microsomal mixed-function oxygenases and epoxide hydrase in scup to a major derivative that is a dihydrodiol. This report constitutes the first published description of such metabolism in a fish species, and to our knowledge in any species. However, in a recent review (16), Nebert and Jensen indicated that an α-naphthoflavone monooxygenase was induced in a TCDD-responsive strain of mice, but they did not describe this activity. Nevertheless, if ANF metabolism is a feature of PAH-induced cytochromes P-450 in mammals, then the data here offer additional support to the suggestion (13) that untreated scup possess cytochromes P-450 resembling those induced by PAH in mammals.

ANF and other flavones are important either as inducers of certain forms of cytochrome P-450 (5), as in vitro modulators that discriminate between different forms of cytochrome P-450 (7), or as agents that modify tumorigenesis (4). Certain other flavonoids are natural products with mutagenic potential, for example, quercitin (17). The influence of the metabolism of ANF and other flavones on these capacities is unknown. The extent of such metabolism in various systems requires further investigation.

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