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Comparison of β-Naphthoflavone and 3-Methylcholanthrene as Inducers of Hepatic Cytochrome(s) P-448 and Aryl Hydrocarbon (Benzo[a]pyrene) Hydroxylase Activity

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SUMMARY

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Intraperitoneal 3-methylcholanthrene induces hepatic aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC 1.14.14.2) in the C57BL/6N mouse about 5-7-fold; a dose of 80 mg/kg of body weight maintains maximal levels on the second through the seventh day after a single injection. The same dose of β-naphthoflavone induces the hydroxylase about 4-fold, the maximal level occurring 40 hr, and the activity returning to basal levels 72 hr, following a single injection. Dose-response studies, time-response studies, simultaneous treatment with maximal and submaximal amounts of both inducers, and multiple-injection experiments all suggested that β-naphthoflavone is absorbed from the peritoneal cavity much more rapidly than 3-methylcholanthrene. With radiolabeled inducers, it was confirmed that β-naphthoflavone is absorbed from the peritoneal cavity into the liver and blood and is excreted (presumably as metabolites) in the feces and urine about 10 times more rapidly than 3-methylcholanthrene during the first 12-18 hr after a single injection of 80 mg/kg of body weight. Concomitant with the rise and fall, respectively, in β-naphthoflavone- or 3-methylcholanthrene-induced aryl hydrocarbon hydroxylase activity are (a) increases and decreases in microsomal total cytochrome P-450 content; (b) a spectral shift to the blue, and then back toward the red, in the Soret peak of the reduced cytochrome-CO complex; and (c) and decreases in two bands (having molecular weights of about 55,500 and 54,500) on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Which of the two bands is associated with the induced hydroxylase activity remains to be determined (or perhaps both bands are). Similar results were found with β-naphthoflavone- and 3-methylcholanthrene-treated sexually immature male Sprague-Dawley rats. These data indicate a common mechanism of induction of hepatic cytochrome(s) P-448 by β-naphthoflavone and 3-methylcholanthrene.

INTRODUCTION

The microsomal monooxygenase aryl hydrocarbon (benzo[a]pyrene) hydroxylase (EC 1.14.14.2) is found in almost all tissues of the body. This enzyme system metabolizes benzo[a]pyrene and other polycyclic hydrocarbons to more polar intermediates (2-7). In view of its known and suspected role in the toxicity and carcinogenicity of many xenobiotics, studies on the inducibil-

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ity of this enzyme are of obvious importance (for reviews, see refs. 2–7). MC[1] has been the most widely used test compound to evoke aryl hydrocarbon hydroxylase induction.[2] Unfortunately, this compound is carcinogenic in animals and probably in humans (9, 10), and is thus a potential hazard to the investigator. The use of a noncarcinogenic inducer of aryl hydrocarbon hydroxylase with properties similar to those of MC is therefore desirable. A group of compounds that appear to meet this requirement are the flavones, whose inducing properties were first described by Wattenberg; the most potent inducer of this group is BNF, a synthetic derivative (11). In studies to date, aryl hydrocarbon hydroxylase induction by this compound has been shown, at least in the rat and mouse (4, 8, 11–16), to resemble that by MC.

We sought to determine whether the mechanism of induction by BNF *in vivo* is indeed the same as that by MC. The purpose of this report is also to characterize in detail the rise and fall in induction of aryl hydrocarbon hydroxylase by BNF and by MC in the genetically responsive C57BL/6N mouse and in the rat. We found that differences between intraperitoneally administered BNF and MC can be explained on the pharmacokinetic basis of differences in absorption from the peritoneal cavity.

MATERIALS AND METHODS

The materials used and their sources were as follows: NADPH (Calbiochem); bovine serum albumin (fraction V), NADH (yeast, grade III), Coomassie brilliant blue R, quinine sulfate, rabbit muscle aldolase, ovalbumin, beef liver glutamate dehydrogenase, bovine liver catalase, and benzo[*a*]pyrene (Sigma); MC (Eastman); BNF (Aldrich); sodium dodecyl sulfate, specially pure (BDH Chemicals, Ltd.); corn oil (Mazola brand, Best Foods); and acrylamide (Bio-Rad Laboratories). Generally tritiated [^{3}H]BNF (91 μCi/μmole) was synthesized for us by New England Nuclear. Generally tritiated [^{3}H]MC (9.7 mCi/μmole) was purchased from Amersham/Searle.

Treatment of animals. All animals used were 4–6-week-old, sexually immature (C57BL/6N mice of either sex and sexually immature male Sprague-Dawley rats. Animals were obtained from the Veterinary Resources Branch, National Institutes of Health, at least 1 week before treatment. The mice were housed in plastic cages, up to six mice per cage, on hardwood chip bedding in a controlled-environment animal room with controlled temperature and lighting (14 hr of light, 10 hr of dark cycles. The mice were permitted free access to food (Purina NIH open formula rat and mouse ration 5018) and tap water. Mice were injected intraperitoneally with inducer dissolved in corn oil. The concentration of inducer was adjusted so that mice received the same volume of corn oil at all doses of inducer (20 ml/kg of body weight). Control mice received an equal volume of corn oil alone.

Enzyme assay. Preparation of liver microsomes and the assay for aryl hydrocarbon hydroxylase activity were carried out as previously described (17), except that the livers were homogenized with a Polytron tissue homogenizer for 30 sec at setting No. 5. One unit of aryl hydrocarbon hydroxylase activity is defined (17) as that amount of enzyme catalyzing, per minute at 37°, the formation of hydroxylated product causing fluorescence equivalent to that of 1 pmole of recrystallized 3-hydroxybenzo[*a*]pyrene standard (see refs. 4, 17, and 18 for further discussion of the enzyme assay). Specific activity denotes units per milligram of microsomal protein.

Spectrophotometry. The CO difference spectral method (19) was used for determining the concentrations of cytochromes P-450. The extinction coefficient of 91 mM^{-1} cm^{-1} was used for the difference in

[1] The abbreviations used are: MC, 3-methylcholanthrene; BNF, β-naphthoflavone.

[2] The term induction is used throughout this report to denote an increase in enzyme activity, or content, above control values after treatment of mice with MC or BNF. Recent evidence (8) suggests that the increase in hepatic aryl hydrocarbon hydroxylase activity (and cytochrome P-448 content) observed after treatment of mice with polycyclic aromatic compounds reflects principally protein synthesis *de novo*.

absorbance between the Soret maximum and the 490-nm baseline for the cytochrome-CO complex following reduction with sodium dithionite (19). Wavelength measurements were standardized with the use of a holmium oxide crystal (Fisher Scientific Company).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Microsomes were resuspended to a concentration of 5–10 mg of protein per milliliter in 0.05 M Tris-Cl buffer, pH 7.25, containing 0.1 mM EDTA and 30% glycerol; potassium-containing buffer could not be used because it precipitated the sodium dodecyl sulfate. The slab gel electrophoresis was carried out by the method of Laemmli (20). The gels used were 1.5 mm thick, and the same amount of protein (usually 12 μg in 15 μl) was applied to each well. The standards (0.2–0.3 mg of each), with their subunit molecular weights, included aldolase from rabbit muscle (40,000), ovalbumin (43,000), glutamate dehydrogenase from beef liver (53,000), bovine liver catalase (58,000), and bovine serum albumin fraction V (68,000). The gels were stained with Coomassie brilliant blue R, 0.2% solution, for 1 hr and then destained overnight in methanol-acetic acid-water (250:75:675 by volume) (21). The strip of gel corresponding to each microsomal sample was carefully cut out and scanned, using a linear transport at 550 nm (22) on a Gilford 240 spectrophotometer fitted with an x-y recorder. The areas under the peaks corresponding to the different forms of cytochrome P-450 (mol wt region 48,000–55,000) (8, 22) were estimated with a Dietzgen planimeter (23). Any minor fluctuations in the actual amount of protein placed in the electrophoretic well were adjusted by determining the area under a peak at about 58,000 mol wt, which does not change (24) after treatment with microsomal enzyme inducers.

Uptake and fate of intraperitoneal [^{3}H]BNF and [^{3}H]MC. Either radioisotope (10 μCi/20 g of body weight) was administered intraperitoneally at the usual 80 mg/kg dose in corn oil. For each inducer the radioactivity in tissue samples or excreta from each of two mice was examined separately at each time point; remarkably good agreement between duplicates at any time point was found.

To obtain intraperitoneal fat, epididymal and mesenteric tissue was removed, blotted with filter paper, minced, and rinsed twice in phosphate-buffered 0.9% NaCl, pH 7.2. The material was homogenized with the Polytron in 10 volumes of buffered NaCl. Aliquots of 100 μl were digested overnight at 50° with 1 ml of "NCS" (Nuclear-Chicago); 10 ml of Aquasol (New England Nuclear) were added, and the radioactivity was determined in a Packard Tri-Carb scintillation counter. Whole livers were rinsed, minced, and washed three times in buffered 0.9% NaCl. After homogenization with the Polytron in 10 volumes of buffered NaCl, radioactivity in 100-μl aliquots was determined as described above. As much heparinized whole blood as possible (usually 0.2–0.7 ml/mouse) was collected from the severed jugular veins at the time of killing. The radioactivity in 20-μl aliquots was determined as described above, except that 0.20 ml of 30% H_2O_2 was added for several hours prior to the "NCS" and subsequently the Aquasol (15 ml). For feces, the entire contents of the colon, sigmoid, and rectum were homogenized with a Teflon-glass Potter-Elvehjem homogenizer in 10 volumes of buffered 0.9% NaCl. Radioactivity in 100-μl aliquots was determined as described above for whole blood. Urine was collected at the time of killing. Aliquots of 20–100 μl were added directly to 10 ml of Aquasol for scintillation spectroscopy. All values were calculated on the basis of nanomoles per gram of tissue, per milliliter of whole blood, and per gram or milliliter of excreta; no attempt was made to separate the parent inducing chemical from the more polar metabolites.

RESULTS

Aryl hydrocarbon hydroxylase induction by BNF or MC as a function of time and dose. Because previous work (25) had shown that maximum induction of hepatic aryl hydrocarbon hydroxylase in genetically responsive mice by BNF or MC occurred with a dose of about 80 mg/kg, this dose was selected for the initial kinetic

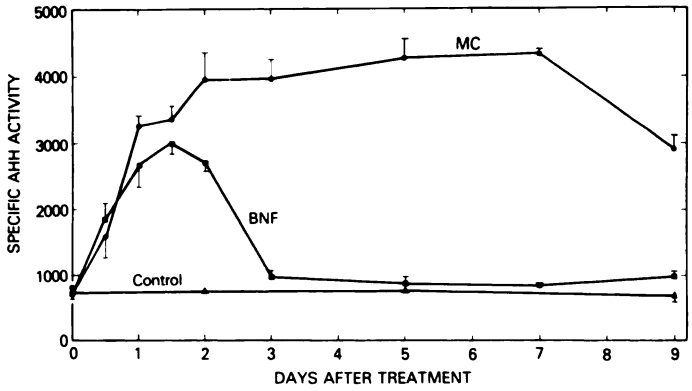

FIG. 1. *Time-response curves of aryl hydrocarbon hydroxylase (AHH) activity in hepatic microsomes following treatment with intraperitoneal MC or BNF*

C57BL/6N mice were treated with 80 mg/kg of BNF (■) or MC (●) in corn oil. Controls (▲) received corn oil alone. In this figure and all subsequent figures, the symbols and brackets represent the means and standard errors, respectively, for at least four animals, each assayed in duplicate.

studies with the two inducers. Figure 1 shows that both MC and BNF caused statistically significant induction of the enzyme within 12 hr. Inducible activity remained maximal 7 days after MC treatment and was still 3–4 times higher than control values at 9 days. In contrast, BNF-induced activity returned to control values within 72 hr.

From these data, 40 hr was selected as the optimum time at which to study dose responses of aryl hydrocarbon hydroxylase induction by MC and BNF (Fig. 2A). Induction by both MC and BNF reached a maximum at a dose of 80 mg/kg. At 40 hr after treatment, there was no significant difference between induction by MC and BNF at any dose examined, with the exception of 10 mg/kg, at which dose BNF caused significantly ($p < 0.01$) more induction than did MC. Figure 2B illustrates the dose responses for MC and BNF 24, 48, and 72 hr after administration of the inducer. At 24 hr no significant differences were apparent, at any dose, between the induction caused by MC and that by BNF. At 48 hr MC produced slightly greater aryl hydrocarbon hydroxylase induction than BNF at all doses except 500 mg/kg, the highest dose investigated. At 72 hr, although the pattern of induction by MC was similar to that observed at 48 hr, no induction was apparent with BNF at doses lower than 160 mg/kg; at doses of BNF greater than 160 mg/kg there was a dose-dependent increase in the enzyme activity, and the level of induction by BNF at 500 mg/kg was similar to that found with MC (also shown in Fig. 3). At low doses of MC (Fig. 3, top), the hydroxylase activity increased submaximally but remained at that level for several days rather than declining rapidly to control values. This was in contrast to BNF: at all doses examined below 500 mg/kg, enzyme activity declined toward control values before 72 hr (Fig. 3).

Submaximal doses of MC and BNF administered concomitantly resulted in additive induction (Table 1). In contrast, the concomitant administration of maximally inducing doses of both MC and BNF did not cause any greater induction than that found with 80 mg/kg of either compound alone.

Aryl hydrocarbon hydroxylase induction by BNF or MC as a function of multiple injections. Five injections of MC, administered at 24-hr intervals, did not induce aryl hydrocarbon hydroxylase activity above the level observed following a single injection administered either 24 hr or 5 days prior to death (Fig. 4). After repeated injections of BNF, maximal levels of enzyme activity were maintained for up to 5 days, in contrast to the decline in activity after 2 days following a single treatment (cf. Fig. 1).

Cytochrome(s) P-448 induction by BNF

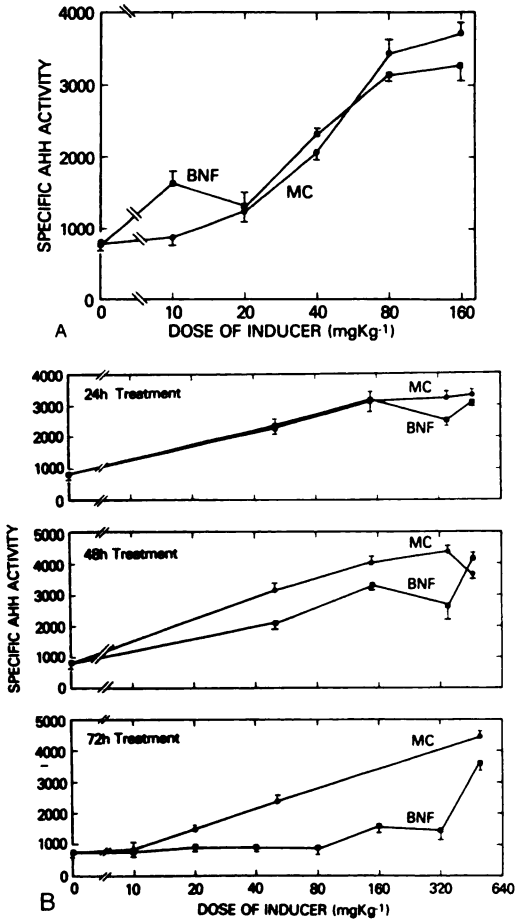

FIG. 2. *Log_2 dose-response curves of hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activity*

A. Mice were injected with BNF (■) or MC (●) in corn oil 40 hr prior to death. B. Mice were injected 24 hr (top), 48 hr (middle), or 72 hr (bottom) prior to death.

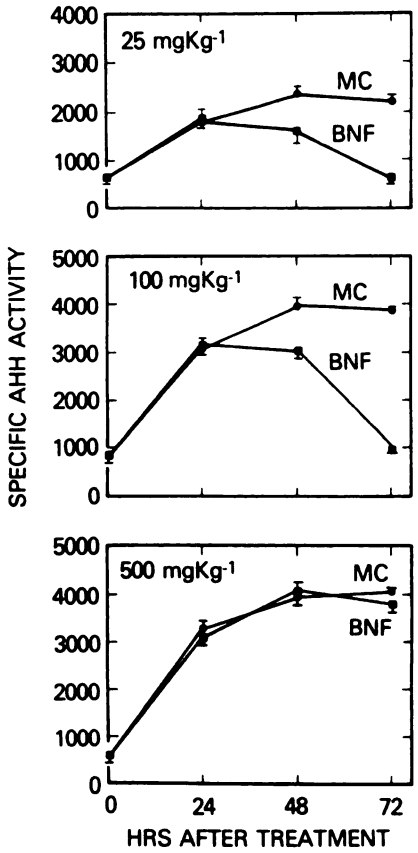

FIG. 3. *Time-response curves of hepatic microsomal aryl hydrocarbon hydroxylase (AHH) activity after treatment with either BNF* (■) *or MC* (●)

The doses were 25 mg/kg (top), 100 mg/kg (middle), and 500 mg/kg (bottom).

or MC as a function of time. Within 16 hr after treatment with BNF or MC (Fig. 5), there was a significant increase in microsomal total cytochrome P-450 content.[3] Following MC treatment, total cytochrome P-450 content continued to increase during the 72-hr experiment, whereas total P-450 content was maximal 40 hr after BNF treatment and declined to control values within 72 hr. These changes in P-450 content were paralleled by changes in the Soret maximum (Fig. 5), indicating protein synthesis *de novo* (8) of inducible cytochrome P-448.

Electrophoretic changes in microsomes evoked by BNF or MC as a function of time. After MC or BNF treatment (Fig. 6), two bands in the P-450 region of the electrophoretic gels increased in intensity (data following BNF treatment not illustrated here). As determined from the molecular weight standards, bands 6 and 5 had molecular weights of about 55,500 and about 54,500, respectively. In Fig. 7 are plotted the areas under the corresponding absorbance peaks during aryl hydrocarbon hydroxylase induction. The patterns of change in both bands were very similar;

[3] This increase is known (4, 8, 18) to represent principally increases in the polycyclic hydrocarbon-inducible form(s) of cytochrome P-448, also called P_1-450.

TABLE 1

Induction of hepatic microsomal aryl hydrocarbon hydroxylase activity by MC and BNF, alone or in combination

Animals were treated with the dose of inducer shown in a volume of corn oil adjusted so that a constant volume of vehicle was injected per kilogram of body weight for the whole experiment. Animals were killed 40 hr after treatment. Values are expressed as means ± standard errors for four mice, each assayed in duplicate.

Treatment[a]	Specific aryl hydrocarbon hydroxylase activity
	units/mg protein
Corn oil	810 ± 69
BNF (40)	2400 ± 110
MC (40)	2200 ± 95
BNF (80)	3100 ± 84
MC (80)	3600 ± 130
BNF (40) + MC (40)	3200 ± 120
BNF (80) + MC (80)	3200 ± 72

[a] Doses, in parentheses, are in milligrams per kilogram of body weight.

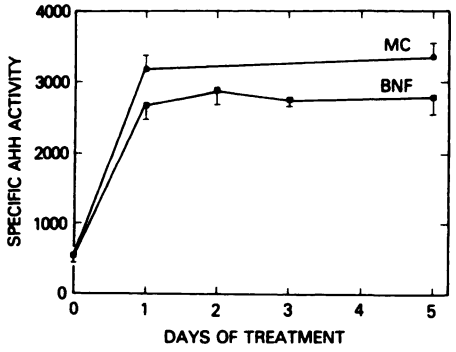

FIG. 4. *Effect of repeated daily injections of BNF (■) or MC (●), at a dose of 80 mg/kg, on hepatic microsomal aryl hydroxylase (AHH) activity*

Animals were killed 24 hr after the last treatment.

however, no change in band 6 was detectable 16 hr after the administration of either inducer, although both MC and BNF caused a significant increase in band 5 at that time. Band 5 was induced maximally and to the same extent by both inducers after 40 hr, whereas band 6 was increased less than 2-fold by BNF but was increased about 4-fold by MC. MC caused both bands to remain elevated for 72 hr; 72 hr after BNF treatment, on the other hand, both bands had decreased to control levels.

Similar studies in the rat. We also performed time-response studies on aryl hydrocarbon hydroxylase induction by MC or BNF in sexually immature male Sprague-Dawley rats. Results similar to those reported here for the mouse were observed (data not illustrated). MC treatment produced an increase in enzyme activity that was maintained much longer than the increase observed after BNF treatment. As in the mouse, these changes were paralleled by changes in spectrally determined total cytochrome P-450 content, in the Soret maximum of the reduced-CO difference spectrum of microsomal P-450, and in two electrophoretically determined protein bands.

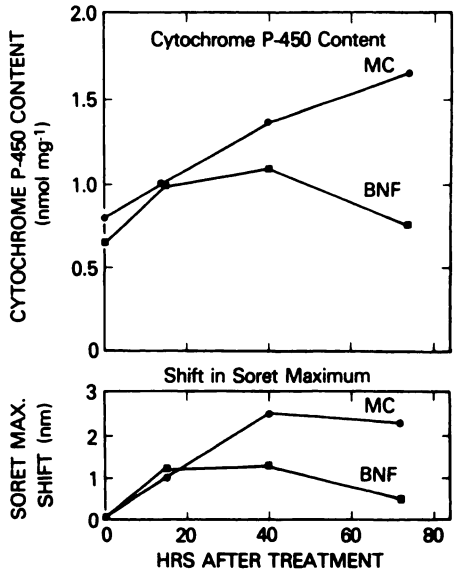

FIG. 5. *Hepatic microsomal total P-450 content (top) and shift in the Soret maximum of reduced hemoprotein-CO complex (bottom) following treatment with 80 mg/kg of BNF (■) or MC (●) at various times before death*

The experiment was performed twice, with similar results on each occasion.

Uptake and fate of intraperitoneal BNF or MC. Aitio has shown (26) that, under conditions when MC is rapidly absorbed, it is also rapidly cleared, and that aryl hydrocarbon hydroxylase induction under these conditions is short-lived. We found (Figs. 2 and 3) that when the dose of MC was lowered, enzyme induction was submaximal but was maintained at that level for several days. This finding was most

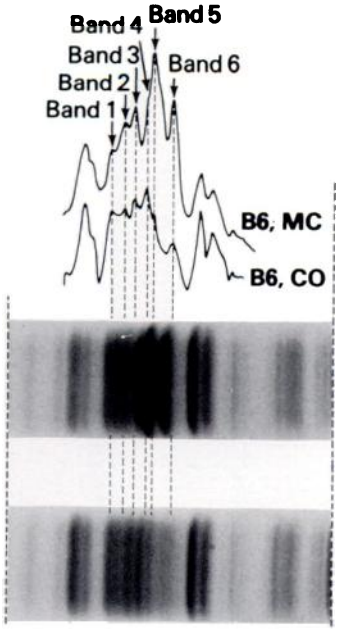

FIG. 6. *Sodium dodecyl sulfate-polyacrylamide slab gel electrophoretogram (bottom) of hepatic microsomes and densitometric tracing of the gel (top)*

C57BL/6N mice (B6) were treated with 80 mg/kg of MC (B6, MC) or an equivalent volume of corn oil alone (B6, CO) 40 hr prior to death. The dotted lines indicate the positions of the six bands, which are thought to represent subunits of different P-450 forms because of their relative changes (8, 24) following treatment of mice with various inducers of P-450. The origin is to the right (not shown), and the electrophoretic front is to the left (not shown).

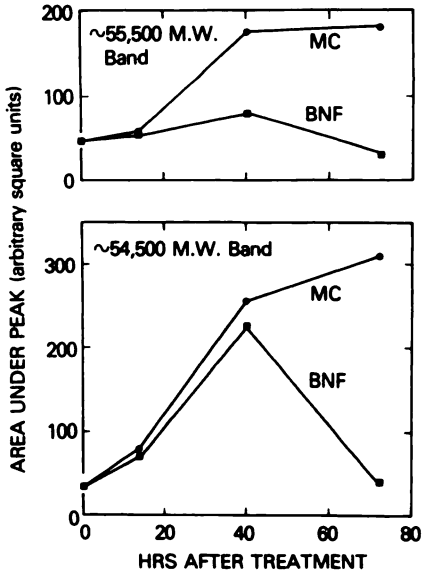

FIG. 7. *Changes in electrophoretically determined protein bands from hepatic microsomes as a function of time following BNF (■) or MC (●) treatment (80 mg/kg)*

Similar results were obtained when the experiment was carried out on two separate occasions.

likely due to concentration-dependent absorption of MC from the peritoneal cavity. Our hypothesis is confirmed in Fig. 8.

Eighteen hours following intraperitoneal injection, we found that about 10 times more MC than BNF remained bound to intraperitoneal fat. Intraperitoneal MC content began to decrease only after 5 days. A comparison of the rates of disappearance of BNF and MC from the liver and whole blood is consistent with our conclusion that MC is absorbed slowly and BNF is absorbed much more rapidly from the peritoneal cavity. During the first 18 hr of the experiment, approximately 10 times more BNF than MC was found in the feces and urine (presumably metabolites). After 24–36 hr more MC than BNF (metabolites) was excreted in the feces andurine, most likely because the total body content of BNF was now much lower than that of MC. Some of the later peaks of radioactivity in the intraperitoneal fat, liver, and blood of BNF-treated mice (e.g., BNF, 7 days) represent either individual variation among the mice killed at each time point or enterohepatic recycling of BNF metabolites. Similar rise and falls in radioactivity after the 1-day time point were observed in a second, similar experiment with BNF.

DISCUSSION

In this study we have shown that time- and dose-dependent increases and subsequent decreases in BNF- and MC-inducible aryl hydrocarbon hydroxylase activity in the livers of mice or rats are closely paralleled by increases and subsequent decreases in the magnitude of the hypsochromic shift in the Soret maximum of the reduced hemoprotein-CO complex, in the microsomal total P-450 content as determined spectrophotometrically, and in two electrophoretically distinct protein bands. To our knowledge this is the first report showing that increases in all these factors

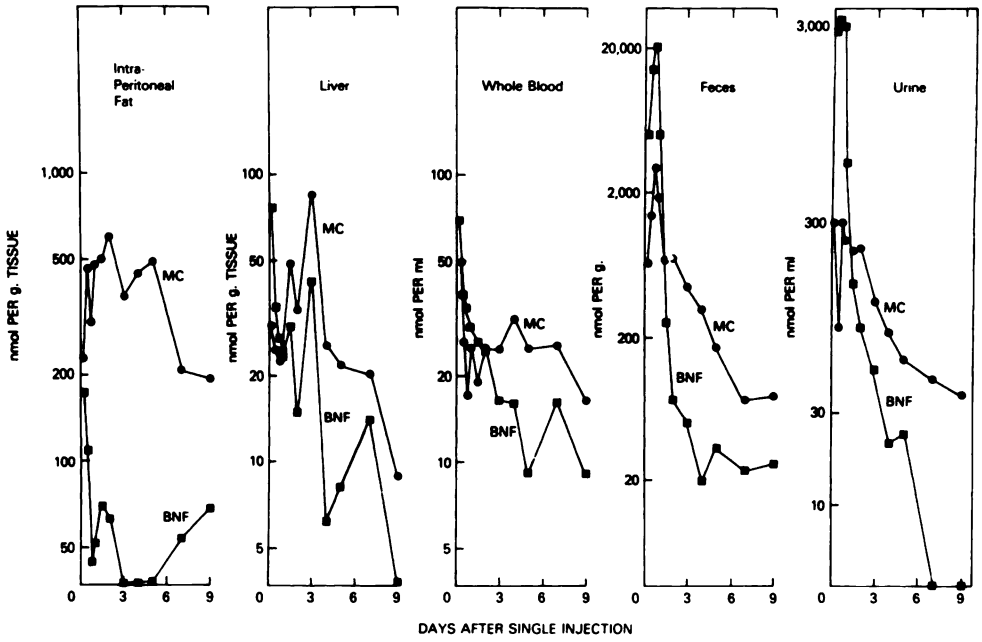

FIG. 8. *Kinetics of uptake, distribution, and excretion of BNF (■) or MC (●) in intraperitoneal fat, liver, whole blood, feces, and urine of C57BL/6N mice following a single intraperitoneal (80 mg/kg) dose*

The experimental protocol is described in detail under MATERIALS AND METHODS. Each point represents the average value for two mice; no two mice at any time point varied more than 16% from each other.

are related in a time-dependent manner and, moreover, that decreases in aryl hydrocarbon hydroxylase activity subsequent to induction are also accompanied by concomitant decreases in all these parameters. These data further support the suggestion (8, 24) that aryl hydrocarbon hydroxylase induction by either MC or BNF is due to increased levels of an electrophoretically distinct form(s) of P-450, the reduced cytochrome-CO complex of which has a Soret maximum shifted 2 nm or more to the blue compared with that of control P-450. Although concern has previously been voiced (27-29) that the magnitude of the increase in cytochrome P-450 does not parallel exactly that of the microsomal monooxygenases after induction, it is clear that a close parallel does exist with aryl hydrocarbon hydroxylase induction when increases in the specific form(s) of cytochrome P-450 rather than microsomal total P-450 content are examined. Because the rise and fall of the enzyme activity correlate so well with the appearance and disappearance of an electrophoretically distinct subunit of P-450, the present study suggests that, in the case of aryl hydrocarbon hydroxylase, cytochrome P-448 content itself controls the induced activity of this enzyme. This suggestion is increasingly likely in view of the finding that cytochrome P-450 reductase activity was not significantly altered by treatment of mice with polycyclic aromatic compounds (16).

Two electrophoretically distinct bands, differing in weight by about 1000, increase and decrease in concert during the rise and fall in aryl hydrocarbon hydroxylase induction by BNF or MC (Fig. 7). Each electrophoretic band probably represents numerous distinct protein subunits (30) that may eventually be resolved by more imaginative separatory techniques. Six forms of rat liver cytochrome P-450 were recently identified immunochemically (31). It is therefore likely that one of these bands (Figs. 6 and 7) is associated with the blue spectral shift in the Soret peak of the reduced hemoprotein-CO complex while the other band is not. Which of the two bands

is associated with the induced hydroxylase activity remains to be determined[4] (or perhaps both bands are).

An intraperitoneal injection of inducer in corn oil provides a depot of the compound, whose rate of clearance will depend on absorption, mostly into the hepatic portal system. The rate of absorption will depend on the lipophilicity of the compound and its concentration gradient. In all likelihood the increased polarity of BNF, compared with MC, will result in more rapid metabolism and, in turn, more rapid absorption because of a more favorable concentration gradient. The result (Fig. 8) is a continuing favorable concentration gradient for BNF during the first 12–18 hr after injection, and about 10 times more rapid depletion of BNF from the peritoneal cavity, compared with MC, during this time. Increasing the amount of BNF originally present (Figs. 2 and 3) or maintaining the peritoneal content of BNF by repeated daily injections (Fig. 4) prolongs induced aryl hydrocarbon hydroxylase activity by prolonging higher levels of BNF in the liver.

From the data in this report it is possible to make an approximation of the rate of induced P-448 turnover. Because maximal induction with BNF exists at 40 hr and returns to control levels by 72 hr (by measuring aryl hydrocarbon hydroxylase activity, total P-450 content, the blue spectral shift in the Soret maximum of the reduced hemoprotein–CO complex, or the intensity of the two electrophoretic bands), essentially all induced P-448 must be degraded within about 30 hr. With a maximally induced hydroxylase activity of 3000 and a basal activity of 800, a half-life of 10 hr would result during 30 hr in a fall in induced activity to 1075, which is about two standard deviations from the mean basal activity. We therefore estimate that the half-life of BNF-induced P-448 is not greater than 10 hr *in vivo*, without the use of any metabolic poisons (e.g., actinomycin D, cycloheximide, or puromycin). This value is very similar to the half-life of 10.5 ± 3.6 hr for benz[*a*]anthracene- or phenobarbital-induced aryl hydrocarbon hydroxylase activity in fetal rat liver primary cultures (33) after washout of the inducer and without use of any metabolic poisons. The doubling time for aryl hydrocarbon hydroxylase induction during the early exposure to BNF or MC (Fig. 1) is also approximately 10 hr.

[4] The MC-inducible aryl hydrocarbon hydroxylase activity can be divorced from the electrophoretic band responsible for the hypsochromic shift in the Soret maximum of the reduced cytochrome-CO complex in the livers of New Zealand white rabbits as a function of age (32) (manuscript submitted for publication).

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