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INDUCTION OF BENZPYRENE HYDROXYLASE IN FETAL LIVER EXPLANTS BY FLAVONES AND PHENOBARBITAL*

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Abstract—The addition of β-naphthoflavone (BNF) to fetal rat liver explants was accompanied by an increase in benzpyrene (BP) hydroxylase activity. The concentration of BNF which produced a one-half maximal induction was approximately 3×10^{-7} M. (The term "induction" is used in a general sense to describe an elevation in enzyme activity, without specifying a genetic mechanism.) A lag of induction of 12 hr was observed when BNF was added to fresh cultures. This lag of induction was mitigated when BNF was added to 22- and 44-hr preincubated explants. Maximal induction was observed at 48–74 hr after the addition of BNF at 10^{-5} M to 44-hr preincubated cultures. The early but not the later phase of BNF-mediated induction of BP hydroxylase was blocked by mercapto-(pyridethyl)-benzimidazole, an inhibitor of RNA synthesis, while both phases of induction were blocked by cycloheximide, an inhibitor of protein synthesis. The results are in accord with the hypothesis that BNF increases the *de novo* synthesis of BP hydroxylase in the fetal rat liver explant system. Pretreatment of fetal liver explants with pentamethoxyflavone (PMF) resulted in a decreased induction of BP hydroxylase by either BNF or 4′-bromoflavone (BrF), the latter being a much more potent inducer in this system. Since it was recently reported that the flavone derivative, PMF, was unable to induce BP hydroxylase in this system over a wide dose range, the results suggest that a common receptor interaction is required prior to the induction of BP hydroxylase by flavones.

ADMINISTRATION of various polycyclic hydrocarbons to rats is attended by an increase in the activity of several liver microsomal mixed function oxidases,[1–3] one of which is BP† hydroxylase. The elevation of enzyme activity appears to result from *de novo* enzyme synthesis[4–8] and requires a prior genomic activation.[9–12]

BP hydroxylase catalyzes the conversion of BP to a number of hydroxy- and quinone derivatives.[13] This microsomal mixed function oxidase is not only inducible‡ by polycyclic hydrocarbons *in vivo*,[2,6,14,15] but a similar effect can be demonstrated in rat lung organ culture,[16] in hamster cell culture,[17] and in fetal liver explants.[18] Furthermore, BP hydroxylase activity is elevated in the placentas of women who had a history of cigarette smoking.[14,19,20]

The flavones, a class of polycyclic structures many of which occur naturally in a wide variety of plants, possess the same spectrum of inducing activity as 3-MC.[16,21]

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† The abbreviations used are: BNF, β-napthflavone; 3-MC, 3-methylcholanthrene; BrF, 4′-bromoflavone; PMF (tangeretin), 5,6,7,8′-pentamethoxyflavone; BP, 3,4-benz[a]pyrene; MPB, 2-mercapto-1-(β-4-pyridethyl) benzimidazole; Act. D, actinomycin D; RNA, ribonucleic acid; DMSO, dimethylsulfoxide; TCA, trichloroacetic acid; A_{320} or A_{260}, absorbance at 320 or 260 nm, respectively; TdR, thymidine.

‡ The term "induction" is used in a general sense to describe an elevation in enzyme activity, without specifying a genetic mechanism.

Administration of BNF to rats,[21] the addition to lung organ cultures[16] or to fetal rat liver explants[22] resulted in an elevation of BP hydroxylase activity. The effect of BNF and other flavones has been examined further; these results are reported in this manuscript.

MATERIALS AND METHODS

Materials. Pregnant Sprague–Dawley rats, 18–20 days of gestation, were obtained from the Holtzman Rat Company, Wis. BNF and MPB were obtained from Aldrich Chemical Company, Milwaukee, Wis. The purity of BNF was assessed by thin-layer chromatography on Silica gel-coated plastic foils (with fluorescent indicator) that had been purchased from Eastman Organic Chemicals, using toluene–ethyl formate–formic acid, 50:40:10 (v/v/v) and by mass spectroscopy. Cycloheximide was purchased from CalBiochem; hydroxyurea and puromycin dihydrochloride were obtained from Nutritional Biochemicals Corp. Act. D was a generous gift of Merck, Sharp & Dohme, Rahway, N.J. [6-^{14}C]Orotic acid (36·5 to 43·5 mCi/m-mole) was purchased from Schwarz Biochem; [2-^{14}C]thymidine (59 mCi/m-mole) and [U-^{14}C]leucine (238 mCi/m-mole) were purchased from New England Nuclear Corp.

Fetal liver organ culture. A modification of the explant technique of Wicks[23] was employed. One or two pregnant rats were sacrificed by decapitation and the two uterine horns with the intact fetuses were removed. The fetuses were removed from the amniotic sacs and the livers were extirpated. The fetal livers were cut into 2 mm cubes with a scapel blade and placed immediately into sterile plastic Petri dishes containing sterile Eagle's minimal essential media (MEM) with Hanks's balanced salt solution (Grand Island Biological Company), pH 7·4, twice the concentration of glucose and $NaHCO_3$,[18] 0·05 M tricine, penicillin (100 units/ml) and streptomycin (100 μg/ml). The cubes were washed twice with medium and randomized. Seven cubes (approximately 2 mg of protein) were placed on stainless steel grids (prepared from mesh of No. 16 wire diameter) in plastic culture dishes. The dishes were then vapor sealed and the cultures were incubated at 37° in an atmosphere of 95% air: 5% CO_2 in a humidified water-jacketed incubator. The pH of the media was maintained at 7·4 with 0·5 N NaOH. The fetal liver explants were sometimes preincubated for 24 or 44 hr prior to the addition of drugs. BNF, Act. D, 3 MC, BrF, PMF and cycloheximide, dissolved in DMSO were added to the media in a final DMSO concentration of 0·1 or 0·2% (v/v). Control cultures were treated with the same volume of DMSO. These concentrations of DMSO produced no demonstrable effects on BP hydroxylase activity. After incubation, the liver tissue was rinsed in 0·9% NaCl (w/v) and homogenized in 1·1 ml of cold 0·25 M sucrose by use of a plastic plexiglass homogenizer with a Teflon-coated pestle.

Enzyme assay. BP hydroxylase activity was assayed by a modification of the method of Nebert and Gelboin.[17] The reaction mixture in a total volume of 1 ml contained 50 μmole of Tris–HCl buffer (pH 7·5), 0·54 μmole of NADPH, 3 μmole of $MgCl_2$, 0·32 μmole of glucose-6-PO_4, 0·6 unit of glucose 6-phosphate dehydrogenase, 80 m-mole of BP in 50 μl of DMSO and 0·4 ml of fetal liver homogenate (0·7 to 1·2 mg of protein). Control and induced enzyme activity was shown to be linear up to 3·0 mg of protein. Although DMSO has been reported to effect BP hydroxylase activity when added *in vitro*,[24] the addition of DMSO up to 100 μl to our incubation mixture

exerted no demonstrable effect on enzyme activity. The samples were incubated in the dark for 10 min at 37° (enzyme activity was linearly related to time for at least 10 min). After incubation, the tubes were placed in an ice-bath, and the reaction was stopped by the addition of 1 ml of acetone and 3·4 ml of hexane. The samples were shaken for 20 min at room temperature and centrifuged for 10 min. The hexane phase (2 ml) was placed in 3·0 ml of 1 N NaOH, shaken for 1 min and centrifuged for 5 min. The alkaline phase was read in an Aminco spectrophotofluorometer at activation and fluorescence wavelengths, of 400 and 522 nm, respectively. Quinine sulfate in 0·1 N H_2SO_4 was used as a reference standard and read at activation and fluorescence wavelengths of 352 and 452 nm respectively. One unit of enzyme activity was arbitrarily defined as the amount of hydroxylated product with a fluorescence equivalent of 1 ng of 3-hydroxy-BP formed/min at 37°. Authentic 3-hydroxy-BP for calibration of our assay was kindly supplied by Dr. H. V. Gelboin of the National Cancer Institute.

Determination of "soluble" BNF. BNF in DMSO was added to 4 ml of media. The mixture was then filtered through a 0·45 μ Millipore, the filter was washed with two equal volumes of deionized water, the filter was air-dried, dissolved in 4 ml of ethyl acetate, centrifuged at room temperature and the absorbancy was read at 320 nm. A blank consisting of 4 ml of media alone was treated similarly; the A_{320} of the blank was 0·246. The reproducibility of this method was within 10 per cent over a broad range of concentrations of BNF. The amount of "soluble" BNF was determined by subtracting the amount of BNF on the filter from the amount originally added to the media (as determined from a standard curve of BNF in DMSO). At $1{\cdot}3 \times 10^{-5}$ M final concentration, 51 per cent of the inducer initially added to the medium was "soluble". The amount of inducer initially added to the medium was directly related to the absolute amount of "soluble" inducer and was inversely related to the per cent of inducer which was "soluble" above $2{\cdot}8 \times 10^{-5}$ M.[22]

[^{14}C]Orotic acid incorporation into RNA. Thirty min prior to homogenization, [^{14}C]orotic acid (2 μCi/dish) was added to the explants. Orotic acid incorporation was linear for at least 12 hr after the addition of labelled precursor to the cultures. Tissue homogenates were prepared as described previously and the RNA was extracted by the method of Schmidt and Thannhauser[25] as modified by Schneider.[26] The A_{260} of the RNA hydrolysate was determined and an aliquot (0·5 ml) was counted in 10 ml of Aquasol (New England Nuclear Corp.) at an efficiency of 75 per cent.

[^{14}C]Thymidine incorporation into DNA. Five hr prior to sacrifice, [^{14}C]thymidine (1 μc/dish) was added to the explants. Incorporation into DNA was found to be linear for at least 8 hr after the addition of labelled thymidine to the cultures. One ml of cold 10% TCA was added to 1 ml of tissue homogenate, the precipitate was washed three times with 3 ml of cold 5% TCA, and the nucleic acids were extracted from the pellet twice with 5% TCA at 90°. The extracts were combined and a 0·5 ml aliquot was counted in 10 ml of Aquasol at an efficiency of 78 per cent.

[^{14}C]Leucine incorporation into protein. [U-^{14}C]Leucine was added to the explants 30 min prior to sacrifice; leucine incorporation into protein was linear for at least 8 hr. Incorporation into protein was determined by the paper disc method of Griffin *et al.*[27] as modified by Nishimura and Novelli.[28] The dried discs were counted at 50 per cent efficiency in a toluene fluor containing 0·55% 2,5-diphenyloxazole (w/v) and 0·03% 1,4-*bis*-2-(4-methyl-5-phenyloxazolyl) benzene.

Acid-soluble composition. One ml of cold 10% TCA was added to the tissue homogenates (1 ml). The precipitate was resuspended in 3 ml of cold 5% TCA, stirred vigorously and recentrifuged. The combined supernatant fractions represented the acid-soluble pool. The acid-soluble fraction was placed in a 90° bath for 1 hr to convert purine nucleotides to purines and pyrimidine nucleoside di- and triphosphates to monophosphates. The sample was then cooled, neutralized with 4 N KOH to a phenol red end-point and 2 ml was then passed through a 1 × 6 cm column of Dowex-1 formate (× 8, 200–400 mesh). UMP and orotic acid were eluted from the ion exchange resin according to the procedure of Herbert *et al.*[29] with 0·2 and 0·4 N ammonium formate, respectively. UMP and orotic acid were identified by comparison of their elution patterns from the resin with authentic standards. The samples were counted in 10 ml of Aquasol at 75 per cent efficiency.

Analytical determinations. DNA concentration was determined by the method of Burton[30] using 2′-deoxyadenosine as the reference standard (1 μg of 2′-deoxyadenosine was equivalent to 2·5 μg of DNA). Protein concentration of fetal liver homogenates was determined by the method of Lowry *et al.*[31] using bovine serum albumin as the reference standard.

RESULTS

Induction of BP hydroxylase by BNF. BNF at 10^{-5} M resulted in a 4- to 5-fold increase in enzyme activity within 24 hr. The concentration of BNF which produced a one-half maximal increase in enzyme activity (ED_{50}) was approximately 3×10^{-7} M (Fig. 1). It had previously been reported[18] that maximal induction was achieved with 10^{-5} M of 3 MC.

Flavone derivatives and polycyclic hydrocarbons have been reported to inhibit benzpyrene hydroxylase activity when added to the cell-free enzyme system.[32] No

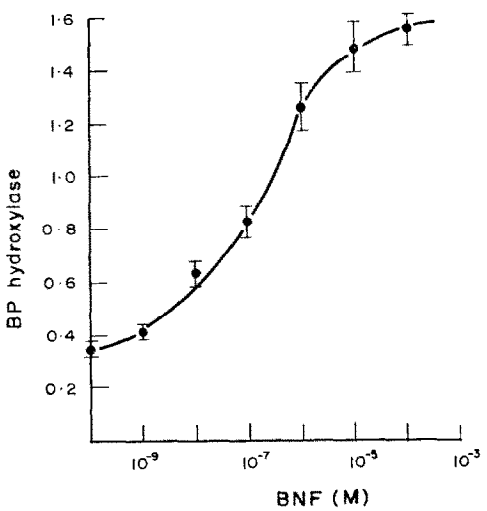

FIG. 1. Dose–response of induction of BP hydroxylase by BNF. Fetal liver explants were preincubated for 24 hr and incubated with either DMSO at a final concentration of 0·1% (v/v) or BNF at the molar concentrations specified. Each point represents the mean ± standard error of enzyme activities of three cultures. Enzyme activity is expressed as units per milligram of protein per minute.

interference was observed in the enzyme assay system *in vitro* under our conditions. When equal amounts of induced and control enzyme were assayed together no less than additive enzyme activity was observed.[22]

Induction of BP hydroxylase in fresh and preincubated cultures. A lag of induction of 12 hr was observed when BNF was added to fresh cultures (Fig. 2a). This lag of induction was decreased when BNF was added to cultures that had been preincubated for 22 hr (Fig. 2b) or 44 hr (Fig. 2c). A similar lag of induction of BP hydroxylase by 3-MC[18] and of tyrosine transaminase by hydrocortisone[23] has been reported with this fetal rat liver explant system. In both fresh and preincubated explants, once the process was initiated, the rate of induction proved to be the same. Maximal induction of approximately 10-fold, was observed at 48–74 hr after the addition of BNF at 10^{-5} M to 44-hr preincubated organ cultures (Fig. 2c).

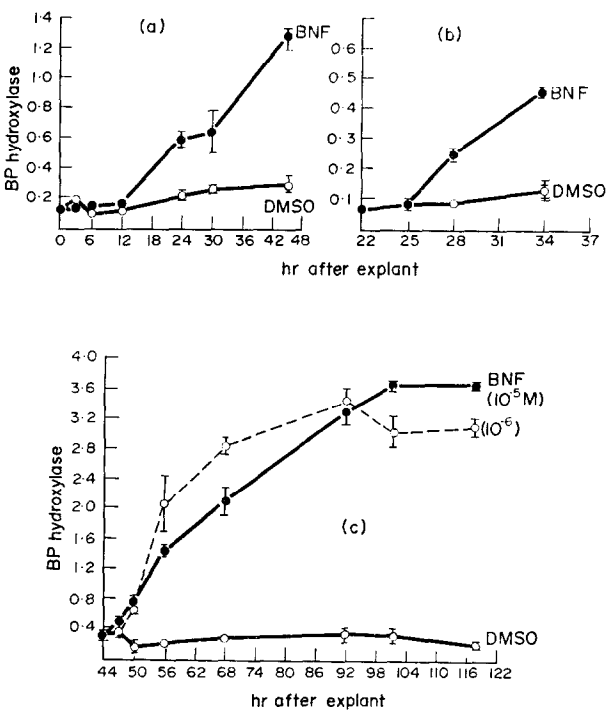

FIG. 2. Kinetics of induction of BP hydroxylase by BNF. Either DMSO (○——○) at a final concentration of 0·1% (v/v) or BNF, 10^{-5} M (●——●) was added to fresh explants (a) of liver or to explants that had been preincubated for 22 hr (b) or 44 hr (c). Each point represents the mean ± standard error of enzyme activities of three cultures. Enzyme activity is expressed as units per milligram of protein per minute. Where standard error bars are omitted the point represents mean ± standard error.

Inhibitors of RNA, protein and DNA synthesis. The effect of BNF as reported in this manuscript is not mediated through an activation of existing BP hydroxylase since addition of this flavone *in vitro* to the assay system actually inhibited enzyme activity.[22,32] Studies with inhibitors of RNA, protein and DNA syntheses were carried out to determine the nature of the mechanism of BNF-mediated induction of enzyme activity.

MPB has been reported to reversibly inhibit nucleic acid synthesis in a variety of cells while only minimally affecting protein synthesis.[33–35] Accordingly in this study, MPB and Act. D were employed as tools in studying the involvement of transcriptional mechanisms in the induction of BP hydroxylase. The effect of these agents on the induction process is shown in Table 1. BNF at 10^{-5} M when added to 44-hr

TABLE 1. EFFECTS OF MPB AND ACTINOMYCIN D ON INDUCTION OF BP HYDROXYLASE BY BNF IN FETAL LIVER EXPLANTS*

Addition	BP hydroxylase (units/mg)	[^{14}C]Orotic acid incorporated (dis/min/A_{260})
None	0·23 ± 0·01	87·1
BNF (10^{-5} M)	0·52 ± 0·06	
BNF + MPB (5×10^{-6} M)	0·67 ± 0·02	
BNF + MPB (5×10^{-5} M)	0·43 ± 0·07	
BNF + MPB (1×10^{-4} M)	0·21 ± 0	
MPB (1×10^{-4} M)		32·3
BNF + Act. D (1×10^{-6} M)	0·35 ± 0	
BNF + Act. D ($7{\cdot}4 \times 10^{-6}$ M)	0·17 ± 0·01	
BNF + Act. D ($1{\cdot}8 \times 10^{-5}$ M)	0·70 ± 0·01	
Act. D ($1{\cdot}8 \times 10^{-5}$ M)		22·1

* Fetal liver explants were preincubated for 44 hr and incubated for 6 hr with either DMSO at a final concentration of 0·1% (v/v) or BNF alone and/or with inhibitor or with inhibitor alone. Values for enzyme activity represent the mean ± standard error of enzyme activities of three cultures. Inhibitors alone had no demonstrable effect on enzyme activity. Thirty min prior to sacrifice, [^{14}C]orotic acid (2 μCi/culture, 43·5 mCi/m-mole) was added. Incorporation values represent the mean from two cultures. DMSO at 0·3% (v/v) had no demonstrable effect on [^{14}C]orotic acid incorporation.

preincubated cultures elicited a 2·5-fold increase in BP hydroxylase activity after 6 hr, but had no effect upon [^{14}C]orotate incorporation into nucleic acids. When MPB at 10^{-4} M was added simultaneously with BNF, the induction was prevented. At this level of MPB, [^{14}C]orotic acid incorporation into RNA was decreased by 65 per cent while [^{14}C]leucine incorporation into total protein was decreased by only 23 per cent. MPB had no effect upon the uptake of BNF into the tissue since the treatment of 44-hr preincubated explants with BNF for 3 hr followed by MPB treatment did not result in any blockade of BP hydroxylase in the next 3 hr.

Some doubt has been cast on MPB as an inhibitor of RNA synthesis *per se* since MPB had been shown to significantly reduce nucleoside uptake into "normal" and tumor cells.[36] Furthermore, since neither the incorporation of [^{32}P]phosphate into RNA and DNA nor the pattern of labelling of RNA species was affected by MPB during short-term incubations, Nakata and Bader[36] concluded that the primary action of this agent was not upon RNA synthesis. Accordingly, the effects of MPB on the uptake of [^{14}C]orotic acid by fetal liver explants and on the inhibition of the incorporation of the precursor into the TCA-insoluble, alkali-labile material were determined. MPB rapidly inhibited orotic acid incorporation into RNA in rat fetal liver explants (Table 2). Within the first 30 min after the addition of MPB to the explants,

labelled orotic acid incorporation into the TCA-insoluble, alkali-labile material was inhibited by 59 per cent. The radioactivity of the orotic acid component of the TCA-insoluble material was not appreciably affected. Thus, MPB inhibits RNA synthesis with [^{14}C]orotic acid as a precursor of RNA synthesis without substantially altering pyrimidine permeability in confirmation of our previous report.[37]

TABLE 2. EFFECT OF MPB TREATMENT UPON THE ACID-SOLUBLE PYRIMIDINE POOL AND UPON RNA SYNTHESIS*

Treatment	TCA-soluble pool (dis/min/A_{260} acid-insoluble)	UMP pool	Orotic acid pool	Inhibition of RNA synthesis (%)
None	2886	971	1915	
MPB for 30 min	2998	715	2283	59
MPB for 60 min	2224	712	1512	77
MPB for 90 min	1930	376	1554	85

* MPB (10^{-4} M) was added for 30, 60 or 90 min to two explants/group preincubated for 44 hr. Thirty min prior to sacrifice, [^{14}C]orotic acid (36·5 mCi/m-mole, 2 μCi/dish) was added. The incorporation of [^{14}C]orotic acid into total RNA was determined as described in Materials and Methods. The TCA-soluble fraction from each group was pooled and the radioactivity in UMP and orotic acid was determined. Since the amount of explant RNA per dish can vary, the incorporation of isotope into the acid-soluble pool was expressed as dis/min in the fraction A_{260} acid-insoluble material. The per cent of inhibition of RNA synthesis was calculated by dividing the amount of radioactivity in RNA at various times after MPB treatment by the control value and subtracting this value from 100.

Act. D when added simultaneously with inducer at $1{\cdot}8 \times 10^{-5}$ M blocked the incorporation of [^{14}C]orotic acid into RNA by 75 per cent (Table 1). The ability of actinomycin D to enhance the induction of various enzymes after the removal of the inducer has been referred to as the "superinduction phenomenon".[38,39] Under conditions of our assay, no superinduction was apparent in BNF-mediated increase in BP hydroxylase activity in fetal rat liver explants. The lack of such a phenomenon has also been reported in the case of hydrocortisone-mediated induction of tyrosine transaminase in fetal liver explants.[40]

The effect of inhibitors of protein synthesis on the induction process was investigated and the data are presented in Table 3. BNF elicited a 2·5-fold increase in enzyme activity without altering the incorporation of leucine into total proteins. Puromycin at either 6×10^{-5} or 6×10^{-4} M when added simultaneously with BNF completely blocked induction. At the latter concentration, puromycin reduced the incorporation of [^{14}C]leucine into total protein by 97 per cent. Cycloheximide at 3×10^{-5} M, added simultaneously with BNF, completely blocked induction and depressed protein synthesis by 96 per cent. The reversibility of the inhibition of protein synthesis by cycloheximide was determined by replacing the medium containing the antibiotic with fresh media containing only BNF. After an additional 24 hr with BNF, induction of enzyme to a normal level was observed.

Table 4 presents the effect of hydroxyurea, an inhibitor of DNA synthesis, on BNF-mediated induction of BP hydroxylase. Addition of BNF alone elicited a 2·5-fold increase in enzyme activity without affecting thymidine incorporation into DNA.

TABLE 3. EFFECTS OF PUROMYCIN AND CYCLOHEXIMIDE ON INDUCTION OF BP HYDROXYLASE BY BNF IN FETAL LIVER EXPLANTS*

Addition	BP hydroxylase (units/mg)	Incorporation (dis/min/mg protein)
None	0·23 ± 0·06	1724
BNF (10^{-5} M)	0·52 ± 0·06	1850
BNF + puromycin (6×10^{-5} M)	0·13 ± 0·02	
BNF + puromycin (6×10^{-4} M)	0·17 ± 0·02	
Puromycin (6×10^{-4} M)		65
BNF + cycloheximide (3×10^{-5} M)	0·16 ± 0·02	
Cycloheximide (3×10^{-5} M)		71

* To fetal liver explants which had been preincubated for 44 hr, either DMSO at a final concentration of 0·1% (v/v), BNF, BNF plus inhibitor, or inhibitor alone, was added for a period of 6 hr. Values for enzyme activity represent the mean ± standard error of enzyme activity of three cultures. Inhibitors alone had no demonstrable effect on enzyme activity. Thirty min prior to sacrifice, [^{14}C]leucine (2 μCi/dish, 238 mCi/m-mole) was added. Incorporation values represent the mean from two cultures. DMSO at 0·1% had no demonstrable effect on [^{14}C]leucine incorporation into protein. Incorporation of label into total protein was determined as described in Materials and Methods.

Hydroxyurea at a dose of 50 mM, a level which reduced [^{14}C]thymidine incorporation into DNA by 99 per cent, was without effect on the induction of BP hydroxylase when added simultaneously with inducer. These data (Tables 1, 3 and 4) indicate that, while active RNA and protein synthesis are required, DNA synthesis is not involved in the induction phenomenon when the inhibitor is added simultaneously with inducer.

TABLE 4. EFFECT OF HYDROXYUREA ON INDUCTION OF BP HYDROXYLASE BY BNF IN FETAL LIVER EXPLANTS*

Addition	BP hydroxylase (units/mg)	[^{14}C]TdR/μg DNA
None	0·23 ± 0·01	25·4
BNF (10^{-5} M)	0·52 ± 0·06	
BNF + hydroxyurea (10 mM)	0·63 ± 0·02	
BNF + hydroxyurea (50 mM)	0·52 ± 0·02	
Hydroxyurea (50 mM)		0·23

* Fetal liver explants were preincubated for 44 hr and incubated for 6 hr under the conditions specified in the Table. Values for enzyme activity represent the mean ± standard error of enzyme activity in three cultures. Inhibitors alone had no demonstrable effect on enzyme activity. Five hr prior to sacrifice, [^{14}C]thymidine (1 μCi/culture, 59 mCi/m-mole) was added. Incorporation values represent the mean of values from two cultures. Incorporation into total DNA was determined as described in Materials and Methods.

The relationship of RNA and protein synthesis to the induction of BP hydroxylase was studied further by inhibiting these processes at select times after the administration of BNF. These results are presented in Fig. 3. MPB or cycloheximide was added simultaneously or at various times after the addition of BNF to 44-hr preincubated cultures, and enzyme activity was determined 6 hr after the inducer. At this time, BNF

elicited a 2·5-fold increase in enzyme activity. Cycloheximide and MPB when added simultaneously or at 1 hr after the addition of inducer completely blocked this increase. At 2 and 3 hr after BNF treatment, MPB only partially blocked induction. At these latter times, [^{14}C]leucine incorporation into total protein was only reduced by 23 per cent when measured in the last half hour of MPB treatment. When cycloheximide was added 2 or 3 hr after the addition of BNF, the induction process was totally blocked. At 4 hr after BNF treatment, however, neither metabolic inhibitor completely blocked induction. As in the case of benzanthracene-mediated induction of BP hydroxylase in fetal hamster cell cultures,[17] the early phase but not the later phase of BNF-mediated induction was blocked by MPB, while both phases were affected by cycloheximide.

That the levels of inhibitors, i.e. MPB and cycloheximide, chosen for this study do indeed block RNA and protein synthesis, respectively, is shown in Table 5. Within 30 min after the addition of the inhibitor to the explants, a significant reduction of the incorporation of the precursor into the macromolecule is apparent. The cycloheximide-mediated blockade occurs more rapidly than the inhibition of RNA synthesis by MPB.

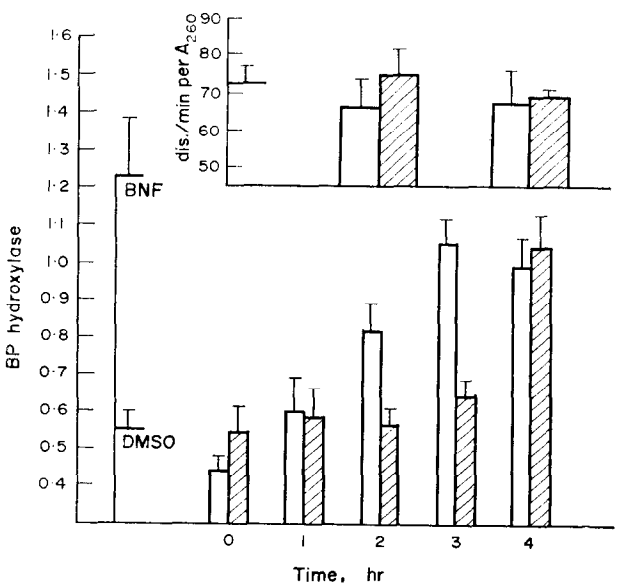

FIG. 3. Inhibition of BNF-mediated induction of BP hydroxylase activity by MPB and cycloheximide. Either MPB (1×10^{-4} M), □; or cycloheximide (3×10^{-5} M), ▨; was added at various times after addition of BNF to explants of liver from 18 to 20-day fetuses that had been preincubated for 44 hr. Enzyme activity was determined 6 hr after treatment with BNF. The abscissa represents the time of addition of inhibitor after BNF treatment. The levels of BP hydroxylase activity obtained in the absence of inhibitors after incubation with either DMSO or BNF are represented by the bars adjacent to the ordinate. Each value represents the mean ± standard error of enzyme activities of three cultures. Enzyme activity is expressed as units per milligram of protein per minute. The effect of BNF on total RNA synthesis is indicated in the insert. Either DMSO, □, at a final concentration of 0·1% (v/v) or BNF, ▨, 10^{-5} M was added to fetal liver explants that had been preincubated for 44 hr previously. The abscissa represents the time at which orotic acid incorporation was measured after the addition of either DMSO or BNF. [^{14}C]Orotic acid incorporation into RNA was measured after 30 min as described in the text and is given as dis/min/A_{260}. The level of orotic acid incorporation in the absence of DMSO or BNF is represented by the bar on the ordinate. Each value represents the mean ± standard error of [^{14}C]orotic acid incorporation of three cultures.

Neither RNA nor protein synthesis was substantially altered by BNF at 2 and 4 hr after addition of the latter to the explants (Fig. 3).

TABLE 5. BLOCKADE OF RNA AND PROTEIN SYNTHESIS IN EXPLANTS*

Treatment	RNA synthesis (dis/min/A_{260})	Inhibition (%)	Treatment	Protein synthesis (dis/min/mg protein)	Inhibition (%)
None	189		None	1181	
MPB for 30 min	91	52	CHX for 30 min	142	88
MPB for 60 min	77	59	CHX for 60 min	121	90
MPB for 90 min	47	75	CHX for 90 min	173	85

* Either MPB (1×10^{-4} M) or cycloheximide (CHX) (3×10^{-5} M) was added to 44-hr preincubated explants. RNA synthesis and protein synthesis were determined by incubating the explants with [^{14}C]orotic acid (2 μCi/dish) and [^{14}C]leucine (2 μCi/dish), respectively, for 30 min after treatment of the explants with the inhibitors for times indicated in the Table. The values represent the means of data from two to three cultures.

Blockade of induction by PMF. Preincubation of fetal liver explants with PMF, a non-inducer of BP hydroxylase,[22] resulted in inhibition of induction by BNF or BrF (Table 6), the latter compound being a much more potent inducer[22] in this system. Simultaneous addition of PMF and BNF to 44-hr preincubated explants produced a similar blockade in the inductive response (data not shown).

TABLE 6. EFFECT OF PMF PRETREATMENT ON THE INDUCTION OF BP HYDROXYLASE BY BNF AND BrF*

Treatment	BP hydroxylase (units/mg)	Control (%)
DMSO	0·29 ± 0·07	
PMF	0·34 ± 0	117
BNF	0·75 ± 0·17	258
PMF + BNF	0·40 ± 0·04	138
DMSO	0·43 ± 0·09	
PMF	0·32 ± 0·04	75
BrF	1·20 ± 0·11	280
PMF + BrF	0·71 ± 0·04	165

* Cultures were preincubated for 24 hr, followed by addition of PMF (2×10^{-5} M) for the next 24 hr. Either BNF (1×10^{-5} M) or BrF (1×10^{-5} M) was added 48 hr after explantation and all cultures were homogenized 72 hr after explanation. The results represent the means ± standard error for three cultures. Controls represented cultures to which DMSO was added instead of the flavones.

Induction of BP hydroxylase by phenobarbital. The addition of phenobarbital at 5 mM to fetal rat liver explants elicited a 3-fold induction of BP hydroxylase (Table 7). A similar 3- to 4-fold induction was observed with BNF (Fig. 1) at a much lower

concentration, i.e. 10^{-5} M. A similar effect of phenobarbital has been reported in a rat fetal liver cell culture system.[41] When the concentration of phenobarbital is raised even higher, significant inhibition of enzyme activity becomes apparent. The latter effect may be due to cytotoxicity as reported previously for cell culture.[41]

TABLE 7. INDUCTION OF BP HYDROXYLASE BY PHENOBARBITAL AND BNF IN FETAL RAT LIVER EXPLANTS*

Treatment	BP hydroxylase (units/mg protein)
None	0·87 ± 0·15
Phenobarbital	
2×10^{-3} M	1·44 ± 0·24
5×10^{-3} M	2·90 ± 0·50
1×10^{-2} M	0·51 ± 0·04

* Cultures were preincubated for 24 hr and treated with either DMSO (0·1%), BNF or sodium phenobarbital in deionized water. The values represent the mean ± standard error of enzyme activities of three cultures.

The effect of administration of inhibitors of RNA and protein synthesis on the induction of BP hydroxylase by phenobarbital is presented in Table 8. Simultaneous administration of either type inhibitor with phenobarbital to 24-hr preincubated cultures resulted in blockade of induction. The data suggest that active RNA and protein synthesis are also required for induction by phenobarbital in this system as shown by Gielen and Nebert[42] in cell culture.

TABLE 8. EFFECTS OF INHIBITION OF RNA AND PROTEIN SYNTHESIS ON INDUCTION OF BP HYDROXYLASE BY PB IN FETAL RAT LIVER EXPLANTS*

Treatment	BP hydroxylase (units/mg protein)	Control (%)
None	0·27 ± 0·04	
PB	0·56 ± 0·07	207
PB + Act. D (4×10^{-6} M)	0·30 ± 0·03	111
PB + MPB (1×10^{-4} M)	0·25 ± 0·02	92
PB + cycloheximide (3×10^{-5} M)	0·16 ± 0·03	60
PB + puromycin (6×10^{-4} M)	0·22 ± 0·04	88

* Fetal liver explants were preincubated for 24 hr and incubated for 6 hr with phenobarbital (PB) and inhibitors; the concentration of phenobarbital employed in these studies was 5×10^{-3} M. Values for enzyme activity represent the mean ± standard error of enzyme activity of three cultures.

DISCUSSION

It had previously been demonstrated that treatment of fetal rat liver explants with 3-MC and its derivatives resulted in induction of BP hydroxylase.[18] In the present report, we have shown that BNF, a non-carcinogenic polycyclic substance, is also a potent inducer of BP hydroxylase in this system.

The kinetics of induction in the latter case showed a similar lag as described after 3-MC administration;[18] the duration of the lag was inversely related to the time of preincubation of the explants. This lag of induction was not due to a general depression of protein synthesis since Wicks[23] was able to demonstrate that [^{3}H]leucine or [^{14}C]valine incorporation into protein of the fetal rat liver explants was as great in the early hours of culture as it was at 42 hr after incubation. He proposed that the synthesis of certain enzymes may be repressed *in utero* and that the factors responsible for this repression may be washed out or degraded early in culture. Alternatively, however, the onset of induction may result from increasing permeability of the tissue after explanation. In concert with the latter explanation, Bürki and Bresnick (unpublished) have shown that the entry of labeled 3-MC into fresh explants is slower and more limited than into 44-hr preincubated cultures.

The inhibition of BNF-mediated induction by blockers of RNA and protein synthesis is consistent with *de novo* synthesis of the enzyme itself or of a protein or RNA species required for the induction process. The inducer may also act by decreasing the degradation of BP hydroxylase, an equally important regulatory mechanism,[43,44] or decreasing the degradation of a protein and/or RNA specie(s) required for the induction phenomenon.

Recently it was shown[22] that simultaneous addition of several flavone derivatives at unsaturating levels resulted in additive induction while, at saturating levels, enzyme induction was no greater than that of the most potent agent alone. These data were interpreted to suggest a similar receptor site for induction. In the present study, it was demonstrated that either pretreatment of fetal liver explants with PMF, a non-inducer of BP hydroxylase,[22] or simultaneous addition of this flavone derivative with BNF resulted in a blockade of induction by active inducer. These data support the hypothesis that a common receptor interaction is required prior to the induction of BP hydroxylase by flavones in the fetal rat liver explant system.

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