INDUCTION OF BOTH CYTOCHROMES P-450 AND P-448
BY 2,3',4,4',5-PENTABROMOBIPHENYL,
A COMPONENT OF FIREMASTER

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SUMMARY

        The synthesis and purification of a component of fireMaster BP-6 and
fireMaster FF-1, 2,3',4,4',5-pentabromobiphenyl, is described. The compound
was found to be a potent inducer of liver microsomal drug-metabolizing enzymes
in the rat, enhancing those enzymic activities induced by both phenobarbitone
and 3-methylcholanthrene (i.e. cytochromes P-450 and P-448). The pentabromo-
biphenyl enhanced the activities of benzo[a]pyrene hydroxylase, dimethylamino-
antipyrine N-demethylase and NADPH-cytochrome c reductase. The hepatic cyto-
chromes b_5 and P-450 were increased and the Soret peak maximum of the latter
was shifted to 448.5 nm. The relative peak intensities and spectral shifts
for the ethylisocyanide-binding difference spectra confirmed the mixed induc-
tion characteristics of 2,3',4,4',5-pentabromobiphenyl.

INTRODUCTION

        In 1973 and 1974 large quantities of an industrial flame retardant

(fireMaster), containing among other chemicals a complex mixture of poly-

brominated biphenyls (PBBs) were inadvertantly introduced into Michigan's

food supply. Exposure of farm animals, farm families and the consuming public

to fireMaster followed (1,2). The need to identify the composition, the

biologic effects and the persistence of fireMaster became apparent.

        Induction of the hepatic microsomal drug-metabolizing enzymes has been

widely used to compare the biologic effects of various chemicals. Inducers

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of these enzymes are often categorized as phenobarbitone(PB)-type inducers of

cytochrome P-450- or 3-methylcholanthrene(MC)-type inducers of cytochrome P-448-

dependent monooxygenase activities. In rats and mice fireMaster is a potent

inducer of hepatic microsomal enzymes, producing a pattern of induction con-

sistent with the simultaneous induction of cytochromes P-450 and P-448 (3,4).

For several classes of halogenated aromatic hydrocarbons [chlorinated

biphenyls (5,6), dibenzo-p-dioxins and dibenzofurans (7), azobenzenes and

azoxybenzenes (8)] there appears to be a good correlation between the toxicity

of the chemical and its ability to induce cytochrome P-448. It is of parti-

cular interest, therefore, to identify those brominated biphenyls present in

fireMaster which are capable of inducing cytochrome P-448.

Only one substance in fireMaster, namely the 2,3',4,4',5,5'-hexabromo-

biphenyl, has been found capable of inducing cytochrome P-448 (9).

This study describes the synthesis and purification of a component of

fireMaster which is a potent inducer of cytochrome P-448.

METHODS

(syntheses)

2,4,5-Tribromoaniline was synthesized from 2,5-dibromoaniline (Aldrich)
by bromination with liquid bromine in carbon tetrachloride in the presence of
iron metal filings. Various batches yielded products between 90 and 99.5%
pure after methanol/water recrystalization. The major impurity in each batch
was the starting material. The proton nuclear magnetic resonance (NMR)
spectrum of the product showed 3 singlets at 4.13, 6.98 and 7.57 ppm.

2,3',4,4',5-Pentabromobiphenyl(PBBp) was synthesized by the diazo
coupling (10) of 2,4,5-tribromoaniline and o-dibromobenzene (Aldrich). The
products were purified by alumina/Florisil column chromatography to remove
possible brominated dibenzofuran impurities followed by repeated thin-layer
chromatography on silica gel HF_{254} to separate the two major products,
PBBp and 2,2',3',4,5-pentabromobiphenyl. PBBp was further purified by methan-
ol recrystalization. The NMR proton spectrum confirmed the identification
of PBBp as 2,3',4,4',5-pentabromobiphenyl with singlets at 7.92 and 7.54
ppm, doublets at 7.63 [J = 2.0 Hz] and 7.68 [J = 8.2 Hz] ppm and a quartet
centered at 7.17 ppm. The proton chemical shifts for PBBp are consistent
with published data (11).

By gas chromatographic (GC) response and by comparison with known
standards, PBBp was found to be 96.14% pure and was contaminated with the
following: 2.83% 2,3',4',5-tetrabromobiphenyl (TBBp), 0.82% 2,2',3',5-
tetrabromobiphenyl with 0.21% unidentified.

A sample of the major impurity, TBBp, was kindly supplied by Drs. G. Sundström and O. Hutzinger. The synthetic TBBp was found by GC response to be greater than 99% pure and was used without further cleanup. The synthesis, purification and identification of TBBp have been described (12).

(Biochemicals)

Cytochrome c (horse heart, type III), NADP$^{\oplus}$, NADPH, α-D-glucose-6-phosphate, α-D-glucose-6-phosphate dehydrogenase (Baker's yeast), MC, benzo-[a]pyrene (B[a]P) and ethylisocyanide (EIC) were purchased from Sigma Chemical Co.; 4-dimethylaminoantipyrine (DMAP) from Aldrich Chemical Co.; carbon monoxide (CO) (research purity) from Matheson, and PB from the Ontario Veterinary College, Guelph. [^{3}H]-B[a]P was obtained from New England Nuclear Co. and purified by thin-layer chromatography using hexane as eluant.

(Animal treatment and isolation of microsomes)

One month old male Wistar rats, average weight 100 g, were housed in wire cages and allowed free access to Purina Certified Rodent Chow #5002 and water. PBBp was dissolved in corn oil and doses of 30 μmol Kg^{-1} (low dose) and 150 μmol Kg^{-1} (high dose) were administered to four animals at each dose level by intraperitoneal injection (ca. 0.5 ml) on days 1 and 3. The animals were killed by cervical dislocation on day 6. TBBp was administered in a similar manner only at the 150 μmol Kg^{-1} dose level. PB (400 μmol Kg^{-1}) dissolved in isotonic saline and MC(100 μmol Kg^{-1}) dissolved in corn oil were administered (ca. 0.5 ml) individually as well as coadministered to animals on days 1 and 2 and the animals killed on day 3. Animals injected with corn oil (ca. 0.5 ml) served as controls. All animals were fasted over the last 24 hours to lower liver glycogen levels.

(Assays)

In all assays the final concentration of microsomal protein was 1.0 mg ml^{-1} as determined by the method of Lowry et al. (13). The cytochrome P-450 content was determined by the method of Omura and Sato (14), from the CO-difference spectrum of dithionite-reduced microsomes using an extinction coefficient of 91 cm^{-1} mM^{-1} between A_{max} and A_{490}. The EIC-difference spectrum was determined in a similar manner to the CO-difference spectrum except that EIC was added to the sample cuvette (final concentration 4.5 mM) instead of CO. The concentration of cytochrome b_5 was determined from the difference spectrum between NADH-reduced microsomes and oxidised microsomes (15) using the corrected extinction coefficient of 185 cm^{-1} mM^{-1} (14). All spectra were recorded on a Cary 118C spectrophotometer with a repetitive scan accessory. Holmium oxide was used to calibrate all spectra.

The rate of oxidative N-demethylation of DMAP was measured by quantifying the production of formaldehyde as described (16). The formaldehyde, trapped as the semicarbazone, was developed in double strength Nash reagent (17). The rate of B[a]P hydroxylation was measured by the radiometric assay of DePierre et al. (18) as improved by Nesnow et al. (19), by quantifying the base-soluble metabolites following hexane-extraction of the unreacted B[a]P. Because an NADPH-regenerating system was employed in both metabolic assays, all tubes were preincubated for 15 minutes. The activity of NADPH-cytochrome P-450 reductase was measured by the rate of reduction of cytochrome c (20). To prolong the linearity of the cytochrome c reduction, samples with high NADPH-cytochrome P-450 reductase activity were diluted to a final microsomal protein concentration of 0.2 mg ml^{-1}.

RESULTS

The effects of pretreatment with corn oil, PB, MC, PB plus MC, PBBp

and TBBp on the hepatic drug-metabolizing enzymes are shown in Table 1.

The activity of DMAP N-demethylase and of NADPH-cytochrome c reductase

was increased by pretreatment with PB, PBBp and TBBp but not by MC. PBBp

induced these enzymic activities more than TBBp. Since TBBp was administered

at more than 30-times the concentration injected as a contaminant in PBBp,

the results suggest that PBBp is itself a PB-type inducer of cytochrome

P-450-dependent enzymic activity.

Pretreatment with PB and TBBp increased the activity of B[a]P

hydroxylase by 3.2- and 2.8-fold, respectively. In contrast, the activity

was stimulated 15.2-fold by MC-pretreatment and 17.1-fold by pretreatment

with PBBp at the high dose level. At the lower dose level of 30 μmol Kg^{-1},

PBBp-pretreatment enhanced the activity of B[a]P hydroxylase by 14.7-fold

indicating that PBBp was a potent inducer of cytochrome P-448-dependent

monooxygenase activity.

The concentrations of cytochrome b_5 and cytochrome P-450 were

increased by PBBp-pretreatment. The increase in cytochrome P-450 was

accompanied by a hypsochromic shift in the CO-difference spectrum

from 450.0 nm to 448.5 nm.   Both Soret peaks of the EIC-

difference spectrum were shifted: from 428.0 to 428.6 nm and

from 455.0 to 453.0 nm. The ratio of the two Soret peak heights (455:

428) increased from 0.49 (controls) to 1.1 and 1.5 for the low and high

dose, respectively. The qualitative spectral characteristics displayed by

PBBp-induced microsomes were intermediate between PB- and MC-induced micro-

somes.

The pattern of enzymic activities and the spectral characteristics of

PBBp-induced microsomes were simulated by the coadministration of PB and MC.

This indicates that PBBp-pretreatment resulted in a mixed-type induction of

both cytochromes P-450 and P-448.

TABLE I

THE EFFECTS OF BROMOBIPHENYL ISOMERS AS HEPATIC MICROSOMAL ENZYME INDUCERS IN MALE WISTAR RATS.

Treatment	% Liver Wt. of Body Wt.	mg Protein g Liver⁻¹	Benzo[a]pyrene Hydroxylase (nmol B[a]P metabolized mg protein⁻¹ min⁻¹)	Dimethylamino-antipyrine N-demethylase (nmol HCHO formed mg protein⁻¹ min⁻¹)	NADPH-Cytochrome c reductase (nmol mg protein⁻¹ min⁻¹)	Cytochrome b_5 (pmol mg protein⁻¹)	Cytochrome "P-450" (nmol mg protein⁻¹ (peak maximum))	Ethylisocyanide Difference Spectra — peak maxima (nm)	Ethylisocyanide Difference Spectra — 455nm to 428nm peak heights
Corn Oil (Control)	4.10 ± 0.31	17.6 ± 1.4	155 ± 17	3.45 ± 0.27	58.4 ± 7.2	233 ± 18	0.640 ± 0.032 (450.0)	428.0, 455.0	0.49 ± 0.04
Phenobarbitone (PB)	5.21 ± 0.46	27.4 ± 3.7	490 ± 43	9.89 ± 0.76	165 ± 13	350 ± 30	1.63 ± 0.09 (450.0)	428.0, 455.0	0.60 ± 0.07
3-Methylcholanthrene (MC)	4.55 ± 0.28	19.3 ± 2.1	2350 ± 180	4.00 ± 0.31	61.4 ± 9.1	322 ± 26	1.28 ± 0.10 (448.0)	429.7, 452.0	1.9 ± 0.2
PB + MC	5.46 ± 0.37	25.0 ± 2.9	2450 ± 210	10.1 ± 0.9	172 ± 18	387 ± 31	2.18 ± 0.17 (448.5)	428.5, 452.0	1.2 ± 0.2
2,3',4,4',5-Penta-bromobiphenyl (PBBp)	4.59 ± 0.20†	19.7 ± 3.3	2280 ± 60	5.40 ± 0.44	80.1 ± 6.4	406 ± 49	1.46 ± 0.12 (448.6)	428.6, 453.0	1.1 ± 0.1
	5.73 ± 0.34*	20.6 ± 1.1	2650 ± 180	6.33 ± 1.10	131 ± 12	423 ± 39	2.27 ± 0.51 (448.5)	428.6, 453.0	1.5 ± 0.1
2,3',4',5-Tetra-bromobiphenyl (TBBp)	4.44 ± 0.25*	23.0 ± 0.9	437 ± 130	4.05 ± 0.65	76.8 ± 6.5	290 ± 36	0.890 ± 0.076 (450.0)	428.0, 455.0	0.57 ± 0.11

† Low dose

* High dose

DISCUSSION

FireMaster BP-6 and FF-1 are commercial mixtures of brominated biphenyls possessing both PB and MC inducing characteristics. Two components, comprising more than 80% of fireMaster, namely the 2,2',4,4',5,5'-hexabromobiphenyl and 2,2',3,4,4',5,5'-heptabromobiphenyl, were PB-type inducers (21, 22).

Aust and coworkers reported that 2,3',4,4',5,5'-hexabromobiphenyl, isolated from fireMaster, was a mixed-type inducer and thereby identified the first component of fireMaster which causes MC-type effects (9). In this report PBBp has also been shown to be a mixed-type inducer. Using the activity of B[a]P hydroxylase as an index of cytochrome P-448-dependent monooxygenase activity, it can be concluded that PBBp is a potent inducer of MC-type characteristics with almost maximum effects observed at the relatively low dose of 30 μmole Kg^{-1}. Recently the chloro analog of PBBp, the 2,3',4,4',5-pentachlorobiphenyl, was shown in our laboratory to be a mixed-type inducer. The properties of PBBp, which together with the other pentabromobiphenyl, 2,2',4,5, 5'-pentabromobiphenyl, comprises up to 8% of fireMaster mixtures (23), strongly suggests that PBBp contributes significantly to the MC-type characteristics of the commercial PBB mixtures.

The identification of PBBp as a mixed-type inducer may be significant not only in terms of explaining the inducing properties of fireMaster but also in terms of toxicity. It has been suggested for several classes of halogenated aromatic hydrocarbons, including polychlorinated biphenyls, that a correlation exists between various toxic responses to a chemical and its ability to induce cytochrome P-448. It has yet to be shown whether this generalization can be extended to include the brominated biphenyls.

Pharmacokinetic studies in vivo have revealed that the individual PBBs in fireMaster are eliminated from the rat at different rates (24). Due to this differential rate of elimination, those components more slowly removed from the body appear to become more concentrated relative to the other PBB isomers and congeners. PBBp is among those components which apparently

concentrate in mammals. Metabolic studies in vitro produced similar results in that, due to differential rates of metabolism, PBBp, as well as other poorly metabolized components of fireMaster, appeared to concentrate within the PBB mixture (25). These results show that PBBp is a persistent chemical. This correlates with reports which have demonstrated that 2,3',4,4',5-pentachlorobiphenyl, a component of commercial PCBs, preferentially biocon-centrates in human adipose tissue and breast milk (26).

In conclusion, PBBp has been identified as mixed-type inducer of both cytochromes P-450 and P-448. The present data concerning the toxicity of several classes of halogenated aromatic hydrocarbons suggest that PBBp is potentially toxic. It is interesting to speculate that the persistence of PBBp in mammals together with its potential toxicity may explain in part why PBBs derived from contaminated beef and poultry were more toxic to mink than the original fireMaster (27).

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