Effect of Diet on the Hepatotoxicity of Polybrominated Biphenyls (FireMaster PB-6)

by J. G. Babish,* G. S. Stoewsand,† and D. J. Lisk‡

The consumption of diets formulated with *Cruciferae* vegetables, e.g., cauliflower, cabbage, and Brussels sprouts, has been shown to result in a stimulation of the intestinal and hepatic microsomal enzyme systems in rats. This study was designed to determine if this increase in intestinal and hepatic microsomal enzyme activity affected the hepatic response to polybrominated biphenyls (PBB).

After three weeks of consuming either a semipurified or 25% cauliflower leaf-supplemented diet (CLD), male Sprague-Dawley rats were maintained for an additional 20 days on their respective diets containing either 0, 1, or 50 ppm PBB. A significant decrease in body weights, but not feed efficiency, was observed over all levels of PBB in animals consuming CLD compared to semipurified diets; consumption of up to 50 ppm of PBB had no effect on body weights with either diet.

Relative liver weights (RLW), hepatic aryl hydrocarbon hydroxylase (AHH), *N*- and *O*-demethylase, as well as intestinal AHH were all increased in CLD-consuming animals before the addition of PBB. While PBB supplementation alone resulted in increased RLW, hepatic AHH, *N*- and *O*-demethylase, microsomal protein, and cytochrome P-450, rats consuming cauliflower diets + PBB had even higher RLW and *N*- and *O*-demethylase activity and microsomal protein concentrations. Hepatic PBB residue and total hepatic lipids were significantly reduced in CLD groups receiving 50 ppm PBB. These results suggest that the antitoxic effects of certain vegetables are related to more rapid metabolism and excretion of xenobiotic compounds.

Excretion of exogenous, lipid-soluble compounds is generally initiated by the microsomal mixed-function oxidase (MFO) system operating in many tissues throughout the body. The MFO system certainly existed long before the advent of modern drugs and environmental pollution, yet their natural role has received little attention. It is generally believed that evolutionary development of the MFO system occurred to counteract the chemical defenses of many plant species (*1*). Plants accumulate many secondary substances—chemicals that do not participate in the basic metabolism of the plant. Among these are many chemicals that serve to repel or discourage the use of the plant by insects, microorganisms, grazing animals and man.

That the consumption of certain vegetables can affect the level of MFO activity has been demonstrated by several researchers (*2, 3*). The most potent vegetables for inducing MFO activity are members of the *Brassicaceae* family, including Brussels sprouts, cabbage, turnips, broccoli, and cauliflower. Inducing activity has also been observed in spinach, dill, and celery (*2, 4*).

This study was designed to determine if the quantitative changes in MFO activity observed when certain MFO inducing vegetables are present in the diet affect qualitative changes in the hepatic response to polybrominated biphenyls (PBB).

Materials and Methods

Animals and Diets

Individually caged, weanling, male Sprague-Dawley rats weighing 42–44 g were assigned to two groups. One group consumed a semipurified diet (*3*) and the second group was fed a diet containing 25%

* Food and Drug Research Labs, Inc., P. O. Box 107, Waverly, New York 14892.
† Department of Food Science, Cornell University, Geneva, New York 14456.
‡ Department of Food Science, Cornell University, Ithaca, New York 14853.

freeze-dried cauliflower leaves (3). After three weeks on their respective diets, PBB in the form of FireMaster PB-6 was introduced into the feed at 0, 1, or 50 ppm for 3 weeks.

All diets and distilled water were available *ad libitum*. Lighting in the animal quarters was set to provide 12 hr of light from 6 AM to 6 PM daily. Temperature and relative humidity were maintained at 23.5°C and 55%, respectively.

During the 3-week period in which the animals were receiving PBB, the following parameters were monitored weekly: body weights, relative liver weights, hepatic microsomal enzyme activity, hepatic cytochrome P-450 levels, hepatic fat content, and hepatic PBB residues.

Preparation of Microsomes

In order to minimize circadian effects on microsomal enzyme activity, all animals were killed between 6 and 8 AM after an 8-hr fast. The livers were perfused with a cold 0.9% NaCl solution, weighed, and homogenized in 4 volumes of ice-cold 1.15% KCl containing $20mM$ Tris–HCl buffer, pH 7.4, using a Potter-Elvehjem Teflon–glass homogenizer fitted to a mechanical drill.

All microsomal enzyme analyses were performed on the 12,000g supernatant (postmitochondrial fraction, PMS) obtained after two successive centrifugal steps: 2000g for 10 min and 12,000g for 20 min. Centrifugation of the PMS fraction at 105,000g for 60 min produced a pellet that was washed in $40mM$ Tris–HCl buffer, pH 7.4, and recentrifuged at 105,000g for 20 min. The washed microsomal pellet was resuspended in the $40mM$ Tris–HCl buffer, pH 7.4, and used for cytochrome P-450 quantitation. All solutions used in preparing both the PMS and microsomal fractions were maintained at 0-4°C. Microsomal enzyme activities were determined within 4 hr of decapitation.

The procedure for preparing kidney and other tissue homogenates involved simply weighing, slicing, and homogenizing the tissue in 9 volumes of the KCl–Tris buffer to obtain a 10% (w/v) suspension for centrifugation.

Determination of Microsomal Enzyme Activity and Cytochrome P-450 Values

The 2-ml incubation mixture contained 1 ml of the 12,000g supernatant, 0.15M KCl, $20mM$ Tris–HCl buffer, pH 7.4, at 37°C, 1.2mM NADP, 9.8mM isocitrate, 6.0mM MgCl$_2$, 7.5mM semicarbazide-HCl when assaying N-demethylation, 0.18 unit of isocitrate dehydrogenase (L$_s$-isocitrate: NADP oxidoreductase, EC 1.1.1.42) and substrate in one

of the following concentrations: aminopyrine, 7.6mM; p-nitroanisole, 0.2mM; benzo[a]pyrene, 100 μM. This mixture was incubated under ambient air in 30 ml beakers by using a Dubnoff shaking incubator at 37°C.

All observations were made during a time interval when reaction rates were linear. Reactions were initiated by addition of the substrate and terminated with either 1 ml of a 20% trichloroacetic acid (TCA) solution or 2 ml of ice-cold acetone.

The activity of N-demethylase was measured by following the production of formaldehyde from aminopyrine as described by Nash (5).

Microsomal p-nitroanisole O-demethylase activity was determined by measuring the product of the reaction, p-nitrophenol (6).

The reaction catalyzed by the aryl hydrocarbon hydroxylase system with benzo[a]pyrene as the substrate was assayed by determining the amount of 3-hydroxybenzo[a]pyrene formed (7).

The method of Omura and Sato (8) was used to quantitate the hepatic microsomal cytochrome P-450. Under the conditions of the assay, the apparent molar extinction coefficient of the reduced P-450-CO complex is 91$mM^{-1}cm^{-1}$.

Total hepatic lipids were determined by using a procedure described by Folch (9), and hepatic PBB levels were assayed using the method described in the Pesticide Analytical Manual (10).

Protein was determined by the modification of the Lowry method described by Sutherland et al. (11). Crystalline bovine serum albumin, fraction V, was used as the standard.

Statistical analyses were performed by using formulas as described in Snedecor and Cochran (12). The factorial design used in analysis of variance included type of diet, level of PBB, and number of days consuming PBB as independent factors.

Tukey's Multiple Range Test was used to determine significant differences among treatments. Differences were deemed significant when the probability of a type I error was less than 5%.

Apparent Michaelis constants and maximum velocities were determined using Hofstee plots (13); by this method the slope of the line is equal to $-1/K_m$ and the x-intercept equals V_m. The equation for the line was determined by linear regression analysis by using the method of least squares (12).

Results and Discussion

Growth and PBB Concentration

Table 1 lists the final body weights of rats consuming either a semipurified or cauliflower leaf diet (CLD) containing one of three levels of PBB. When

Table 1. Effect of polybrominated biphenyl supplementation of semipurified and cauliflower leaf diets on final body weights.

| PBB, ppm | Final body weight, g | | |
	Semipurified diet[a]	Cauliflower leaf diet[a]	Combined diets[b]
0	225.4 ± 13.4[de]	217.3 ± 12.4[de]	221.3 ± 8.9[h]
1	232.4 ± 13.6[de]	216.9 ± 12.2[de]	224.6 ± 9.1[h]
50	238.1 ± 11.4[e]	215.0 ± 13.6[d]	226.5 ± 9.0[h]
All levels combined[c]	231.9 ± 7.2[y]	216.4 ± 7.1[z]	

[a] Values are means ± SEM of 10 observations.
[b] Values are means ± SEM of 20 observations.
[c] Values are means ± SEM of 30 observations; common superscripts denote nonsignificant differences ($p > 0.05$) within contrasts (consecutive letters).

Table 2. Effect of polybrominated biphenyl supplementation of semipurified and cauliflower leaf diets on relative liver weights.

| PBB, ppm | Relative liver weights, % body weights | | |
	Semipurified diet[a]	Cauliflower leaf diet[a]	Combined diets[b]
0	4.36 ± 0.17[d]	5.00 ± 0.21[e]	4.68 ± 0.15[h]
1	4.44 ± 0.11[d]	5.11 ± 0.21[e]	4.78 ± 0.14[h]
50	5.97 ± 0.30[f]	6.59 ± 0.15[g]	6.28 ± 0.18[i]
All levels combined[c]	4.92 ± 0.18[x]	5.57 ± 0.17[y]	

[a] Values are means ± SEM of 10 observations.
[b] Values are means ± SEM of 20 observations.
[c] Values are means ± SEM of 30 observations; common superscripts denote nonsignificant differences ($p > 0.05$) within contrasts (consecutive letters).

the two diets were compared within each of the three levels of PBB supplementation, the slight depression in body weights of animals consuming the CLD was not significant. However, when all levels of PBB were analyzed to test only the effect of the diet, the observed decrease in body weights in the CLD groups became significant.

PBB consumption at 1 and 50 ppm levels had no effect on final body weights during the three weeks it was present in the diet.

Feed efficiency, defined as grams of feed consumed per gram increase in body weight, was not affected by the CLD or the presence of PBB. The feed efficiencies for the semipurified diet and the CLD were 4.10 ± 0.09 and 4.20 ± 0.10, respectively. The three levels of PBB supplementation resulted in feed efficiencies of 4.17 ± 0.11, 4.21 ± 0.12, and 4.07 ± 0.13 for 0, 1, and 50 ppm, respectively.

The PBB concentration found from analysis of the diets differed by less than 2% from the theoretical PBB concentrations at each level.

A comparison of PBB consumption between diets revealed that at 1 ppm animals consuming semipurified diets received 0.95 ± 0.15 μg PBB/kg body weight during the experimental period, while CLD rats received 0.96 ± 0.14 μg PBB/kg body weight. At 50 ppm the figures were 48.79 ± 7.36 and 47.62 ± 7.14 μg PBB/kg body weight for the semipurified and CLD, respectively.

Relative Liver Weights, Hepatic Microsomal Protein, and P-450

As seen in Table 2, the CLD increased relative liver weights (RLW) over all levels of PBB supplementation. At 50 ppm of the diet, PBB was responsible for an increase in RLW in both dietary groups. Since the observed increase in relative liver weights between the 0 and 50 ppm levels of PBB

was nearly identical, 1.61 and 1.59 for the semipurified and CLD, respectively, the effects of PBB and CLD on RLW appear to be additive.

Table 3 shows that the increase in relative liver weights observed over all levels of PBB supplementation was accompanied by a similar increase in microsomal protein content. However, cytochrome P-450 concentration, on a protein basis, was not affected by the CLD.

Table 3. Effect of diet on hepatic microsomal protein and cytochrome P-450 content of polybrominated biphenyl treatments.[a]

Diet	Microsomal protein, mg protein/g liver	Cytochrome P-450, nmole/mg microsomal protein
Semipurified	16.12 ± 0.85[b]	1.401 ± 0.117[d]
Cauliflower leaf	17.78 ± 0.89[c]	1.488 ± 0.150[d]

[a] Includes results of all levels of PBB supplementation.
[b] Values are means ± SEM of 24 observations; common superscripts denote nonsignificant differences ($p > 0.05$).

Diet and Hepatic Microsomal Enzyme Induction by PBB

Table 4 outlines the effect of diet on hepatic microsomal enzyme induction by PBB. CLD increased N-demethylase activity at all levels of PBB supplementation. PBB had no effect on enzyme activity at 1 ppm, but significantly increased enzyme activity at the 50 ppm level. As with RLW, the combined effect of PBB and CLD was additive.

The hepatic microsomal O-demethylase activity exhibited exactly the same response to PBB supplementation in both diets as previously described for the N-demethylase system. CLD increased O-demethylation over all levels of PBB; PBB had no effect on enzyme activity at 1 ppm but significantly elevated O-demethylation when consumed at the 50 ppm level. As before, the combined effect of PBB and CLD was additive.

Table 4. Effect of diet on hepatic microsomal enzyme induction by polybrominated biphenyls.

Diet	Dietary PBB, ppm	Enzyme	Enzyme activity, nmole product mg PMS protein-hr[a]
Semipurified	0	Aminopyrine N-demethylase	37.37 ± 3.71^b
	1	''	40.73 ± 3.43^b
	50	''	127.09 ± 11.38^c
Cauliflower leaf	0	Aminopyrine N-demethylase	62.41 ± 6.70^d
	1	''	72.60 ± 8.11^d
	50	''	152.15 ± 5.76^e
Semipurified	0	p-Nitroanisole O-demethylase	2.97 ± 0.17^f
	1	''	2.95 ± 0.18^f
	50	''	9.27 ± 0.55^g
Cauliflower leaf	0	p-Nitroanisole O-demethylase	4.13 ± 0.43^h
	1	''	4.92 ± 0.38^h
	50	''	10.60 ± 0.44^i
Semipurified	0	Aryl hydrocarbon hydroxylase	5.16 ± 0.48^j
	1	''	6.02 ± 0.52^j
	50	''	13.29 ± 1.07^k
Cauliflower leaf	0	Aryl hydrocarbon hydroxylase	6.21 ± 0.50^j
	1	''	6.55 ± 0.55^j
	50	''	11.51 ± 1.02^k

[a] Values are means $\pm$ SEM of eight observations with common superscripts denoting nonsignificant differences ($p > 0.05$).

Aryl hydrocarbon hydroxylase activity was not affected by CLD, while PBB increased activity only at 50 ppm.

Table 5 shows the hepatic PBB residues in rats consuming both the semipurified and cauliflower leaf diets. As can be seen, no difference existed between diets at the 1 ppm level, indicating no significant differences in metabolism or intestinal absorption of PBB between diets. However, at 50 ppm dietary PBB, the PBB content of the liver was half that of the animals consuming the semipurified diet.

Table 5. Hepatic PBB residues and fatty infiltration of livers in rats consuming semipurified and cauliflower leaf-supplemented diets.

Diet	PBB in diet, ppm	PBB in tissue residue, ppm dry weight[a]	Hepatic lipids, % wet weight[b]
Semipurified	0	0	4.04 ± 0.14^c
Cauliflower leaf	0	0	3.42 ± 0.28^c
Semipurified	1	2.72 ± 0.19^c	$3.67 \pm 0.15^{c,d}$
Cauliflower leaf	1	2.21 ± 0.11^c	3.20 ± 0.28^c
Semipurified	50	340.90 ± 39.71^d	6.84 ± 0.30^f
Cauliflower leaf	50	170.42 ± 12.38^e	4.62 ± 0.25^e

[a] Values are means $\pm$ SEM of six animals.

[b] Values are means $\pm$ SEM of seven animals; common superscripts within columns indicate nonsignificant differences ($p > 0.05$).

Differences in detoxication were also clearly seen in the degree of fatty infiltration observed. At 50 ppm dietary PBB, animals consuming CLD had 32% less lipid infiltration than the semipurified diets.

Second to microsomal enzyme activity as an estimation of *in vivo* detoxication, and perhaps more biologically meaningful, is the apparent Michaelis

constant, or the concentration of substrate necessary to produce one-half the maximal velocity. As seen in Table 6, apparent Michaelis constants de-

Table 6. Effect of cauliflower leaf consumption on apparent Michaelis constants and maximum velocities.

Tissue	Diet	Aryl hydrocarbon hydroxylase[a]	
		$K_m, \mu M$	V_{max}
Liver	Semipurified	88 ± 9^b	109 ± 17^c
	Cauliflower leaf	55 ± 5^d	84 ± 6^c
Intestine	Semipurified	81 ± 10^b	182 ± 22^e
	Cauliflower leaf	44 ± 7^d	367 ± 71^f
Kidney	Semipurified	105 ± 19^b	680 ± 68^g
	Cauliflower leaf	48 ± 10^d	445 ± 39^h

[a] Values are means $\pm$ SEM of six observations; common superscripts denote nonsignificant differences ($p > 0.05$).

creased by 50% for the aryl hydrocarbon hydroxylase system in all tissues examined. This indicates a greater affinity for hydrocarbon hydroxylation in animals consuming CLD. Apparent Michaelis values for N-demethylation were not affected by diet in these experiments.

Overall, this study demonstrates that quantitative effects of simulated "natural product" diets on microsomal MFO can produce qualitative effects in the degree of toxicity demonstrated. It is obvious, therefore, that a closer consideration of diet should be made when examining the metabolism and microsomal enzyme-inducing potential of drugs and environmental contaminants.

REFERENCES

1. Krieger, R. I., Feeny, P. P., and Wilkinson, C. F. Detoxication enzymes in the guts of caterpillars: an evolutionary an-

swer to plant defenses? Science 172: 579 (1971).

2. Wattenberg, L. W. Studies of polycyclic hydrocarbon hydroxylases of the intestine possibly related to cancer. Cancer 28: 99 (1971).

3. Babish, J. G., and Stoewsand, G. S. Hepatic microsomal enzyme induction in rats fed varietal cauliflower leaves. J. Nutr. 105: 1592 (1975).

4. Wattenberg, L. W. Enzymatic reactions and carcinogenesis. Paper presented at Twenty-Fourth Annual Symposium on Fundamental Cancer Research, M.D. Anderson Hospital and Tumor Institute, Williams and Wilkins, Baltimore, Md., 1972.

5. Nash, T. The colorimetric estimation of formaldehyde by means of the Hantzsch Reaction. Biochem. J. 55: 416 (1963).

6. Kato, R., and Gillette, J. R. Effect of starvation on NADPH dependent enzymes in liver microsomes of male and female rats. J. Pharmacol. Exptl. Therap. 150: 279 (1965).

7. Nebert, D. W., and Gelboin, H. V. Substrate-inducible microsomal aryl hydroxylase in mammalian cell culture. J. Biol. Chem. 243: 6342 (1968).

8. Omura, T., and Sato, R. The carbon-monoxide binding pigment of liver microsomes. II. Solubilization, purification, and properties. J. Biol. Chem. 239: 2370 (1964).

9. Folch, J., et al. Preparation of lipid extracts from brain tissue. J. Biol. Chem. 191: 833 (1951).

10. FDA. Pesticide Analytical Manual, Vol. 1, U. S. Department of Health, Education, and Welfare, Food and Drug Administration, Washington, D. C. Revised, 1971, Sections 211.13h, 211.14a, and 211.14d.

11. Sutherland, E. W., et al. Purification of the hyperclycemic-glycogenolytic factor from insulin and gastric mucosa. J. Biol. Chem. 180: 825 (1949).

12. Snedecor, G. W., and Cochran, W. G. Statistical Methods, 6th ed., The Iowa State University Press, Ames, Iowa, 1971.

13. Hofstee, B. H. J. Non-inverted versus inverted plots in enzyme kinetics. Nature, 184: 1296 (1959).