Activities of Benzphetamine *N*-Demethylase and Aryl Hydrocarbon Hydroxylase in Cells Isolated From γ-Glutamyl Transpeptidase-Positive Foci and Surrounding Liver [1,2]

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ABSTRACT—The levels of two cytochrome P-450-linked enzymes of xenobiotic metabolism, benzphetamine *N*-demethylase and aryl hydrocarbon hydroxylase, were determined in cells isolated from γ-glutamyl transpeptidase (GGT)-positive foci and in cells from surrounding liver obtained from carcinogen-treated inbred F344 rats. Rat liver foci were initiated with diethylnitrosamine (CAS: 55-18-5) and promoted with sodium phenobarbital [(PB) CAS: 64038-21-7] for 4½ or 12 months. The levels of both enzymes were relatively low in the GGT-positive hepatocytes, while the GGT-negative hepatocytes from the surrounding liver had elevated levels of both enzymes comparable to levels seen in rats treated with PB alone. After 12 months of promotion the PB was removed from the diet and the activities of both enzymes fell below the control levels in the GGT-positive hepatocytes and returned to the control levels in the surrounding GGT-negative hepatocytes. Therefore, the cells in the GGT-positive foci contained low levels of these two cytochrome P-450 enzymes in relation to the levels in GGT-negative cells. These levels were responsive to phenobarbital induction, although the induced levels in the GGT-positive cells were much lower than the induced levels in GGT-negative hepatocytes. The liver surrounding the foci responded to phenobarbital induction to the same degree as did the liver of noninitiated rats.—JNCI 1985; 75:1107-1112.

The appearance of chemically induced hepatocellular carcinomas in rats is preceded by the development of focal areas of hepatocytes that show numerous changes similar to those seen in frank carcinomas. These areas, which have been referred to as enzyme-altered foci, have a higher rate of DNA synthesis than that of the surrounding tissue (*1*), and with most hepatocarcinogenic protocols the foci show alterations in one or more of the following traits: increased levels of GGT and epoxide hydratase, decreased levels of ATPase and glucose-6-phosphatase, and reduced storage of iron [for complete review *see* (*2*)]. In the past these enzyme-altered foci could not be isolated from the surrounding tissue, and analysis of the foci was restricted to qualitative assessment of enzymes and cellular constituents by means of histochemical stains. However, the development of a method for the isolation of and/or enrichment for GGT-positive hepatocytes (*3*) has allowed the quantitative measurement of enzyme levels within the hepatocytes from the foci.

In this study P-450 levels were analyzed in liver cell suspensions enriched for GGT-positive hepatocytes from rats whose liver foci were initiated with DEN (CAS: 55-18-5) and promoted with PB (CAS: 64038-21-7). Two enzymes from among the various P-450 activities were chosen for analysis. Benzphetamine *N*-demethylase, which is a representative of the class of PB-inducible enzymes, and aryl hydrocarbon hydroxylase, which is a 3-methylcholanthrene (CAS: 56-49-5)-inducible enzyme (*4*), were chosen. Although aryl hydrocarbon hydroxylase is most responsive to induction by 3-methylcholanthrene, PB will also induce an increase in the activity of the enzyme (*5*).

MATERIALS AND METHODS

Animals.—Male inbred F344 rats, 160 g, were obtained from Harlan Sprague-Dawley, Inc. (Madison, WI). Two separate experiments were performed. In experiment I the animals received one of four treatment protocols. The animals in group A were control animals maintained on Purina Laboratory Chow and were used as weight-matched controls for groups C and D. The animals in group B were maintained on Purina Laboratory Chow containing 0.05% PB and used as weight-matched controls for groups C and D. The animals in group C were treated according to the procedure originally described by Pitot and co-workers (*6*): The rats were subjected to a 70% partial hepatectomy; at 18-24 hours later they were given a single intragastric dose of 30 mg DEN/kg; one week after the DEN treatment, 0.05% PB was added to the diet. The rats were maintained on the PB diet for 11½ months, at which time they were sacrificed. The animals in group D were treated according to the procedure described for group C; they were maintained on the PB diet for 11½ months and then fed Purina Laboratory Chow for 6 weeks.

ABBREVIATIONS USED: DEN = diethylnitrosamine; GGT = γ-glutamyl transpeptidase; PB = sodium phenobarbital.

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In experiment II the animals received one of three treatment protocols. The animals in group A were control animals maintained on Purina Laboratory Chow. The animals in group B were fed a diet containing 0.05% PB. The animals in group C were treated according to the procedure described by Pitot et al. (*6*). The animals were sacrificed after 4½ months of PB feeding.

Preparation of liver cells.—The rat livers were perfused by the collagenase method of Berry and Friend (*7*) as modified by Bonney et al. (*8*). GGT-positive hepatocytes were isolated from liver cell suspensions by selective attachment of the cells to GGT antibody-coated Petri dishes, according to the procedure described by Hanigan and Pitot (*3*). After isolation, the cells were rinsed by pelleting two times at 50×*g* for 5 minutes in phosphate-buffered saline. After the final rinse, they were resuspended in 15 m*M* KCl at a concentration of 5×10^6 hepatocytes/ml. Small aliquots were smeared on a glass slide, air dried, and stained for GGT. The remaining cells were immediately frozen on Dry Ice in 0.1-ml aliquots and stored at −70°C.

GGT determination.—Cells dried on glass slides were stained histochemically for GGT by the method of Rutenburg et al. (*9*). Cells that contain high levels of GGT on their cell surface stain red. These cells are referred to as GGT-positive cells. The hepatocytes that do not have induced levels of GGT remain a light tan and are referred to as GGT negative. The percentage of hepatocytes that were GGT positive was determined by counting the number of GGT-positive and GGT-negative hepatocytes. For each condition, at least 1,000 cells per sample were counted. Biochemical determination of enzyme activity was performed according to Tateishi et al. (*10*). One unit of GGT activity is defined as the amount of enzyme that releases 1 μmol *p*-nitroaniline per minute at 25°C.

Protein determination.—The amount of protein in the samples was determined according to the procedure of Lowry et al. (*11*).

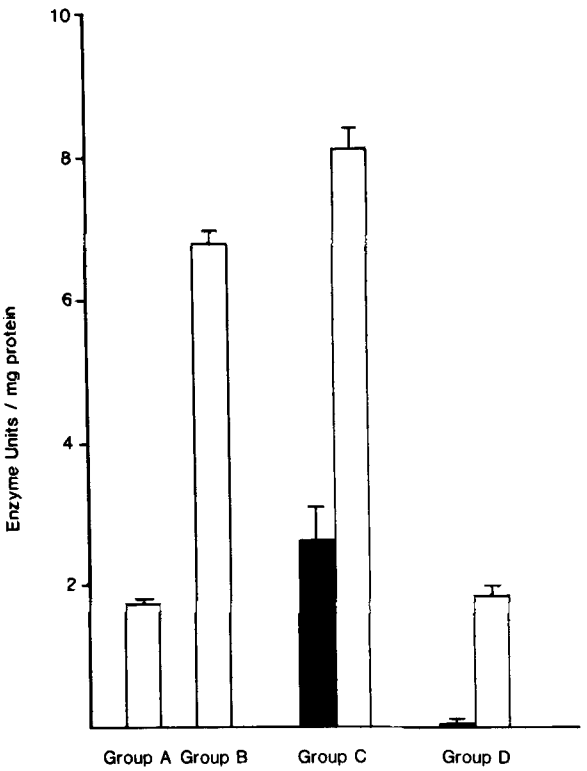
Benzphetamine N-demethylase assay.—Benzphetamine *N*-demethylase activity was determined by a radiometric assay as described by Poland et al. (*12*). This assay procedure is essentially the same as the assay for aminopyrine-*N*-demethylase (*13*) and measures the same demethylase activity. The benzphetamine *N*-demethylase assay was used because the substrate for the benzphetamine assay is more stable than the substrate for the aminopyrene assay. [^{3}H]benzphetamine-HCl and [^{14}C]-formaldehyde were generously provided by Dr. Alan Poland of the McArdle Laboratory for Cancer Research. One unit of activity is defined as the amount of enzyme that releases 1 nmol of product per minute at 37°C. Aliquots of frozen cells were freeze-thawed three times and then used in the assay.

Aryl hydrocarbon hydroxylase assay.—Aryl hydrocarbon hydroxylase activity was assayed according to the procedure described by Poland and Glover (*14*) and modified by Knutson and Poland (*15*). The standard for the assay was 3-hydroxybenzo[*a*]pyrene, which was obtained from the Chemical Carcinogen Reference Standard Repository of the National Cancer Institute (National Institutes of Health, Bethesda, MD). One unit of aryl hydrocarbon hydroxylase activity is defined as the amount of enzyme catalyzing the formation of 1 pmol 3-hydroxybenzo[*a*]pyrene per minute at 37°C. Aliquots of frozen cells were freeze-thawed three times and then used directly in the assay.

Determination of enzyme activities in GGT-positive and GGT-negative cell populations.—The procedure for the isolation of GGT-positive cells yields a population of cells enriched twentyfold to thirtyfold for GGT-positive cells; however, this enriched population often contains a large percentage of GGT-negative cells. For calculation of the enzyme activity in a pure population of GGT-positive and GGT-negative cells, simultaneous equations were used as follows:

Enzyme activity in the nonenriched population = [(*a*) × (% GGT-positive cells in the nonenriched population)] + [(*b*) × (% GGT-negative cells in the nonenriched population)]

Enzyme activity in the enriched population = [(*a*) × (% GGT positive cells in the enriched population)] + [(*b*) × (% GGT-negative cells in the enriched population)]



TEXT-FIGURE 1.—Benzphetamine *N*-demethylase activity in isolated hepatocytes from expt I. Group A, untreated animals; group B, PB-fed animals; group C, DEN-PB-treated animals; and group D, DEN-PB-treated animals with PB removed from the diet 6 wk prior to sacrifice: activity in GGT-positive hepatocytes (*solid bar*) and activity in GGT-negative hepatocytes (*open bar*). Values given are the means of three samples ± SE.

TABLE 1.—*Experiment I, benzphetamine N-demethylase activity*[a]

Rat group	Percent GGT-positive hepatocytes		Enzyme activity[b] assayed		Enzyme activity[b] calculated	
	Nonenriched cell suspension	Suspension enriched for GGT-positive cells	Nonenriched cell suspension	Suspension enriched for GGT-positive cells	GGT-negative hepatocytes	GGT-positive hepatocytes
Group A						
Rat 1	0	—	1.67	—	1.67	—
Rat 2	0	—	1.83	—	1.83	—
Rat 3	0	—	1.79	—	1.79	—
Group B						
Rat 1	0	—	6.88	—	6.88	—
Rat 2	0	—	7.05	—	7.05	—
Rat 3	0	—	6.49	—	6.49	—
Group C						
Rat 1	5.1	45.9	7.73	5.72	7.98	3.06
Rat 2	5.6	70.8	8.38	4.76	8.69	3.14
Rat 3	3.7	69.8	7.52	3.52	7.75	1.69
Group D						
Rat 1	2.1	54.8	1.80	0.75	1.84	0
Rat 2	2.1	57.5	2.06	0.98	2.10	0.15
Rat 3	3.1	59.3	1.60	0.69	1.65	0.03

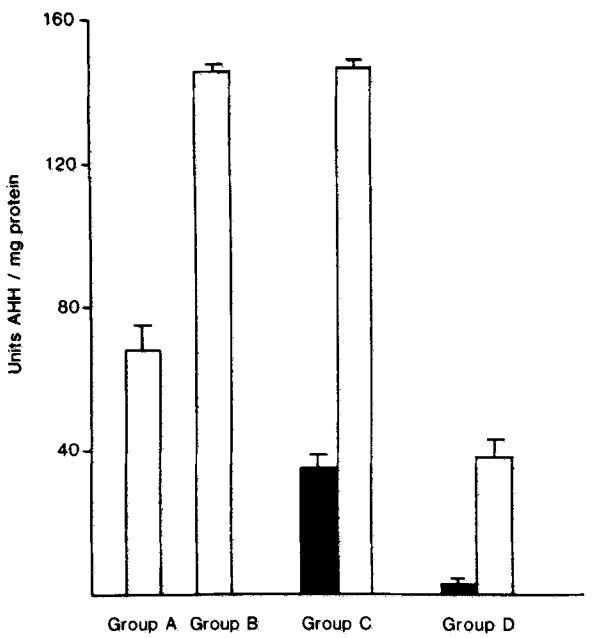
[a] — = not applicable.
[b] Units of benzphetamine *N*-demethylase activity/mg protein.

where *a* is the enzyme activity in a pure population of GGT-positive cells and *b* is the enzyme activity in a pure population of GGT-negative cells. The equations were solved for *a* and *b*.

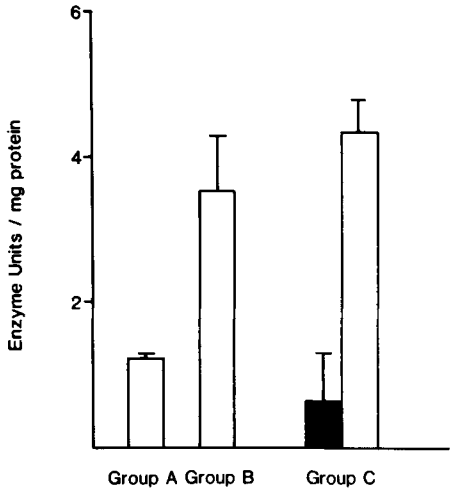
RESULTS

In experiment I benzphetamine *N*-demethylase activity was induced more than fourfold by PB feeding (table 1, groups A and B; text-fig. 1, groups A and B). Liver cell suspensions from animals treated with DEN-PB were enriched for GGT-positive hepatocytes. The enzyme activity was assayed in the enriched and nonenriched cell populations, and from these assay results the enzyme activity was calculated for purified GGT-positive and GGT-negative hepatocytes, as described in "Materials and Methods" and table 1. The GGT-positive hepatocytes from the DEN-PB-treated animals (text-fig. 1, group C) were induced less than 50% over the control level, while the GGT-negative cells in the liver were induced more than fourfold to a slightly higher level than that induced by PB in controls (text-fig. 1, groups A, B, and C). When the DEN-PB-treated animals were removed from PB, the level of benzphetamine *N*-demethylase activity in the GGT-positive cells decreased to almost zero, while the level in the GGT-negative cells returned to control levels (text-fig. 1, group D).

In experiment I aryl hydrocarbon hydroxylase activity was induced more than twofold in animals fed PB (text-fig. 2, groups A and B). As with benzphetamine *N*-demethylase, the aryl hydrocarbon hydroxylase activity in livers of DEN-PB-treated animals was assayed in liver cell suspensions enriched for GGT-positive hepatocytes and in nonenriched cell suspensions. From these data the enzyme activities in purified GGT-positive and GGT-negative hepatocytes were calculated. GGT-positive hepatocytes had only half the level of aryl hydrocarbon hydroxylase activity seen in untreated control animals, while the GGT-negative hepatocytes from the



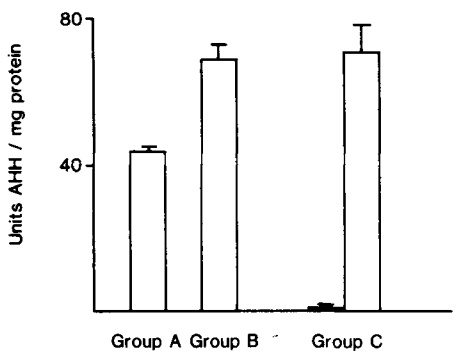
TEXT-FIGURE 2.—Aryl hydrocarbon hydroxylase (AHH) activity in isolated hepatocytes from expt I. Group A, untreated animals; group B, PB-fed animals; group C, DEN-PB-treated animals; group D, DEN-PB-treated animals with PB removed from the diet 6 wk prior to sacrifice: activity in GGT-positive hepatocytes (*solid bar*) and activity in GGT-negative hepatocytes (*open bar*). Values given are the means of three samples ± SE.



TEXT-FIGURE 3.—Benzphetamine *N*-demethylase activity in isolated hepatocytes from expt II. Group A, untreated animals; group B, PB-fed animals; and group C, DEN-PB-treated animals: activity in GGT-positive hepatocytes (*solid bar*) and activity in GGT-negative hepatocytes (*open bar*). Values given are the means of three samples ± SE.

DEN-PB-treated liver had the same level of aryl hydrocarbon hydroxylase activity as that from the control rats fed PB (text-fig. 2, groups A, B, and C). When the DEN-PB-treated animals were taken off the PB diet and fed the control diet for 6 weeks (group D), the aryl hydrocarbon hydroxylase activity in the GGT-positive cells decreased to less than 5% of the activity found in untreated control liver. The aryl hydrocarbon hydroxylase activity in the GGT-negative cells returned to the control level (text-fig. 2, group D).

In experiment II, DEN-PB-treated animals were sacrificed after 4½ months of PB feeding so that the activity of the xenobiotic-metabolizing enzymes could be measured in the cells of the GGT-positive foci that had developed at a relatively early time in this multistage regimen of hepatocarcinogenesis. The enrichment of the cell suspension for GGT-positive cells was comparable to that achieved in experiment I.



TEXT-FIGURE 4.—Aryl hydrocarbon hydroxylase (AHH) activity in isolated hepatocytes from expt II. Group A, untreated animals; group B, PB-fed animals; and group C, DEN-PB-treated animals: activity in GGT-positive hepatocytes (*solid bar*) and activity in GGT-negative hepatocytes (*open bar*). Values given are the means of three samples ± SE.

In experiment II, the benzphetamine *N*-demethylase activity in the GGT-positive cells from DEN-PB-treated animals was the same as the level in uninduced control cells, whereas the level of the enzyme in the GGT-negative cells was equivalent to the enzyme activity in cells from uninitiated PB-treated animals (text-fig. 3). The level of aryl hydrocarbon hydroxylase activity in the GGT-positive cells from DEN-PB-treated animals was not detectable, while the GGT-negative cells had the same level of aryl hydrocarbon hydroxylase activity as the PB-induced controls (*see* text-fig. 4).

The level of benzphetamine *N*-demethylase and aryl hydrocarbon hydroxylase induction by PB was less in experiment II than in experiment I. The difference in absolute levels of enzyme induction between the two experiments may be due to partial degradation of the PB that was added to the diet in experiment II. The diets used for experiment II were stored longer than those used for experiment I.

The enrichment procedure for GGT-positive hepatocytes routinely yields less than 25% of the GGT-positive hepatocytes from the starting cell suspension. This low yield has led to speculation that the isolation procedure selects a subpopulation of GGT-positive cells with a higher level of GGT. The enrichment of the cell suspensions for GGT-positive hepatocytes was calculated from both the cell counts and the GGT activity determined biochemically for each animal. Using the histochemical stain, we found that the enrichment for GGT-positive hepatocytes varied from ninefold to 27-fold. Both the qualitative histochemical stain and the quantitative biochemical stain showed the same level of enrichment. The ratio of the enrichment measured histochemically to the enrichment measured biochemically was 1.00± 0.15. The agreement in the results between these two methods demonstrated that the isolation method did not select for a subpopulation of histochemically GGT-positive cells with a higher level of GGT.

The levels of GGT activity in a pure population of GGT-positive and GGT-negative cells were calculated from the quantitative biochemical data. The GGT-positive cells had 0.0374 U enzyme/mg protein; the level of GGT activity in the negative cells was less than 0.0001 U/mg protein.

DISCUSSION

In both the early and late foci the levels of the aryl hydrocarbon hydroxylase and benzphetamine *N*-demethylase were significantly lower in the GGT-positive hepatocytes than in the GGT-negative hepatocytes from the same liver. After 12 months of promotion, withdrawal of the animals from the PB feeding causes the levels of the enzymes to decrease in both the GGT-positive and GGT-negative cells. Therefore, despite the lower level of the enzyme in the GGT-positive hepatocytes, the level of these xenobiotic metabolizing enzymes in the late foci is responsive to regulation by PB. The level of aryl hydrocarbon hydroxylase activity in the

GGT-positive hepatocytes promoted for 4½ months is equivalent to the level found in the late foci after the animals are withdrawn from the PB diet. This finding suggests that the aryl hydrocarbon hydroxylase activity in the early foci is less responsive to PB induction than the activity in the late foci.

Several investigators have observed qualitative differences in the levels of P-450 enzymes in foci and surrounding liver. Buchmann and co-workers (*16*) treated rats by addition of DEN to the drinking water for 10 days. They analyzed the livers at 2, 4, 6, and 8 months. Using antibodies directed against specific forms of PB- and 3-methylcholanthrene-inducible forms of cytochrome P-450, they found that the GGT-positive and ATPase-negative foci had reduced levels of two 3-methylcholanthrene-inducible forms and one PB-inducible form but increased levels of a second PB-inducible form. Foci and nodules induced by dimethylnitrosamine had decreased levels of all four isozymes.

Using an antibody directed against the major cytochrome P-450 present in PB-treated rat liver, Schulte-Hermann and co-workers (*17*) found that in rats given a single dose of *N*-nitrosomorpholine, followed 12 weeks later by 2 weeks of PB feeding, 95% of the GGT-positive foci had elevated levels of this cytochrome P-450. Nodules and tumors obtained by this regimen also stained strongly for this P-450 isozyme. Measuring the levels of several cytochrome P-450 enzymes biochemically in the tumors, these authors (*17*) found that the enzymes in the tumors were responsive to PB induction.

Many investigators have mechanically dissected hyperplastic nodules and tumors and measured enzymes biochemically in the tissue (*18–22*). Hyperplastic nodules induced by 2-acetylaminofluorene have decreased levels of cytochrome P-450 and aryl hydrocarbon hydroxylase (*19, 22*). Ethionine-induced nodules also show decreased cytochrome P-450 and aminopyrine demethylase activity (*18*). Farber and co-workers (*23*) have demonstrated that the hyperplastic nodules are resistant to the cytotoxic effects of several carcinogens. Farber (*24*) has proposed that the occurrence of reduced levels of cytochrome P-450 is part of a complex phenotype that confers resistance to cytotoxicity of the carcinogen itself and other foreign compounds on the selected group of cells that form the hyperplastic nodule. Analysis of various hepatomas showed reduced levels of cytochrome P-450, with the decreased activity roughly correlated with increasing growth rates (*25*).

The GGT activity in the early and late GGT-positive hepatocytes was several hundredfold higher than the level in the surrounding liver. This high level of induction is comparable to the induction seen in primary rat hepatocellular carcinomas (*26, 27*).

The data presented here have demonstrated the quantitative changes in two P-450 enzymes that occur in GGT-positive foci and the surrounding liver during PB feeding. These data confirm previous qualitative findings and demonstrate the potential of the isolation method for quantitating enzyme levels in GGT-positive foci and surrounding liver.

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