PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 20–27 (1983)

Dose-Related Effects of Phenobarbital on Hepatic Microsomal Enzymes[1] (41698)

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Abstract. To study the relationship between the dose of phenobarbital (PB) and the magnitude of its effects on microsomal enzymes, cytochrome P-450, UDP-glucuronyl transferase (UDPGT), and glucose-6-phosphatase (G6P) activities were determined in liver homogenate and microsome preparations from control rats and rats treated for 6 days with PB at doses ranging from 1 to 125 mg/kg/day. Both P-450 and UDPGT activities were enhanced by PB in a dose-related fashion. However, while the lowest dose of the drug to produce significant induction of both enzymes was the same (3 mg/kg), maximal induction of P-450 (214%) and UDPGT (285%) was obtained with different doses of PB, namely 75 and 125 mg/kg, respectively. UDPGT induction could equally be demonstrated regardless of whether "native" enzyme or enzyme activated by UDP-*N*-acetyl glucosamine, digitonin or deoxycholate was employed. In contrast to these inducing effects of the drug on P-450 and UDPGT, PB treatment resulted in a dose-related inhibition of G6P activity. The inhibitory effect was observed with both "native" and deoxycholate-activated enzymes, and could be demonstrated whether the data were expressed as enzyme specific activity (nanomoles per minute per milligram microsomal protein) or as total G6P activity (micromoles per minute per 100 g body weight). These results indicate that: (i) enzyme induction by PB is dose-related; (ii) induction of both P-450 and UDPGT is obtained in the rat with doses of the drug similar to those given to man; and (iii) observed inhibition of G6P activity by PB does not solely reflect an enzymatic dilution secondary to the proliferated endoplasmic reticulum.

The effect of phenobarbital (PB) on microsomal enzymes has been studied extensively and the inducing properties of the drug have been demonstrated for a variety of enzymatic pathways (1). However, most of these studies have been carried out with a single dose of PB, and the effect of the drug as a function of dose has not been investigated thoroughly. Although Breckenridge *et al.* (2) demonstrated the dose dependency of enzyme induction following administration of several barbiturates to rats, the doses of PB used in their studies were limited and only one enzymatic reaction, *N*-demethylation of ethyl morphine, was measured.

Determination of the dose–effect relationship is essential to define the potency and efficacy of a drug and to establish the variability of its effects, all pharmacodynamic parameters which are of practical importance when animal data are to be extrapolated to man. For obvious reasons microsomal enzyme activities in healthy human subjects can very rarely be measured directly. Hence information about enzyme induction in man following drug therapy or exposure to pollutants can only be obtained either from measurements of other pharmacokinetic parameters (e.g., plasma half-life of other medicaments) or, most often, from studies in laboratory animals.

At least with respect to PB, however, enzyme induction is usually observed in laboratory animals with doses of the drug which are, on a body-weight basis, far higher than those given to human patients or healthy volunteers (3). Thus, the relevance of these animal findings to an inducing effect of the drug in man is not known. Accordingly, in the present report we have studied the *in vitro* activities of hepatic microsomal cytochrome P-450 and bilirubin UDP-glucuronyl transferase (UDPGT) following administration of PB to rats over a wide range of doses, including those usually administered to man. Additionally, we have extended our observations to the effect of PB treatment on glucose-6-phosphatase (D-

[1] This work was supported by Grant AM-26438 from NIADDK, and generous gifts from the Polly Annenberg Levee Charitable Trust and the Jack Martin Fund.

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0037-9727/83 $1.50

glucose-6-phosphate phosphohydrolase) (G6P) activity, as the effect of the drug on this enzymatic pathway is controversial.

Materials and Methods. Male Sprague–Dawley rats were obtained from Perfection Breeders (Douglasville, Pa.) and housed at constant temperature (22°C) with alternating 12-hr light/dark cycles for at least a week prior to use. They were fed Purina Lab Chow *ad libitum* with free access to water, and were not fasted before being sacrificed. Rats were randomly divided in groups and each group was treated once daily with an ip injection of either saline or phenobarbital at doses ranging from 1 to 125 mg/kg. Animals were treated for 6 consecutive days and sacrificed 24 hr after the last injection. Rats were killed by stunning followed by exsanguination, and 25% liver homogenate and microsome suspensions were prepared in 250 m*M* sucrose/1 m*M* ethylenediaminetetraacetate (EDTA) as previously described (4).

Both UDPGT and G6P activities were measured in homogenate and microsome suspensions as both "native" and activated enzymes. UDPGT was activated by UDP-*N*-acetyl glucosamine (UDPNAG) or by the detergents digitonin or deoxycholate, whereas G6P was activated by deoxycholate. The source of "native" enzyme for both UDPGT and G6P was a 12.5% suspension of liver homogenate or microsomes obtained by diluting 1 vol of the original suspension (25%) with 1 vol of sucrose–EDTA. Similarly, the detergent-activated enzymes were prepared by adding 1 vol of sucrose–EDTA containing an appropriate concentration of the desired detergent to 1 vol of the original suspension. The optimal concentrations of deoxycholate in the final suspensions were 0.125% in homogenate and 0.1% in microsomes for UDPGT and 0.15% in both preparations for G6P. Digitonin produced maximal activation of UDPGT at 1.25% in homogenate and 0.65% in microsomes. The activity of UDPNAG-activated UDPGT was determined using untreated homogenate or microsome suspensions, and by adding UDPNAG to the incubation mixture at 3.07 m*M* (4).

The activity of UDPGT was determined in quadruplicate samples which contained "native" enzyme or enzyme activated by UDPNAG, digitonin, or deoxycholate. Duplicate samples were prepared for G6P in which the enzyme activity was measured as "native" or activated by deoxycholate. In the UDPGT assay, incubations were done for 30 min, whereas in the G6P assay samples were incubated for 10 min. The activity of UDPGT was determined by a minor modification of the method of Strebel and Odell (5) as described in detail elsewhere (4). G6P activity was measured by the method of Nordlie and Arion (6).

Cytochrome P-450 activity was determined in microsome suspensions by its diothionite difference spectrum as described by Omura and Sato (7), using an extinction coefficient ($\Delta OD_{450-490}$) of 91 mM^{-1} cm^{-1}. Protein concentration was measured by the Lowry method (8), using bovine serum albumin as standard. Experimental data were analyzed for statistical differences by using Student's *t* test.

Results. *Rat growth and liver weight.* Small but statistically significant differences in rat weights were observed among groups before treatment was initiated. Hence, changes in rat growth pattern associated with PB administration could not be determined directly by comparing the mean body weight of treated and control rats at the end of the treatment period. However, if the mean weight gain was calculated as a percentage of the initial body weight, an increase in its value was obtained in the PB-treated rats which appeared to be related to the dose of the drug over the range 1–75 mg/kg/day (Table I). Thus, while control rats gained less than 9% of their original weight over the 6-day treatment period, an increase of more than 17% was observed in rats treated with 75 mg/kg PB. However, when PB was given at 125 mg/kg rats gained only 5.2% of their original weight, a value lower than that seen in all the other groups including the controls.

As expected, both liver weight and liver/body weight ratio also increased following PB treatment. These effects also appeared dose-related in the range 1–75 mg/kg. At 125 mg/kg, PB did increase liver weight compared to control values, but this effect was less pronounced than that seen with 75 mg/kg (Table I).

Liver protein. As illustrated in Table I, no significant changes in protein content of liver homogenate could be detected in the PB-

TABLE I. EFFECT OF PHENOBARBITAL (PB) TREATMENT ON BODY AND LIVER WEIGHT AND ON LIVER HOMOGENATE AND MICROSOMAL PROTEIN IN THE RAT

Treatment[a]	Rat weight (g)			Liver weight (g)	Liver weight/ body weight[c]	Protein (mg/g liver)	
	Before	After	Gain (%)[b]			Homogenate	Microsomes
Controls (8)[d]	283 ± 14[e]	308 ± 12	8.8	13.26 ± 1.03	4.31 ± 0.37	203.4 ± 12.1	25.6 ± 3.1
PB 1 (7)	275 ± 15	306 ± 18	11.3	13.92 ± 1.51	4.54 ± 0.34	198.9 ± 7.8	27.1 ± 2.9
PB 3 (7)	290 ± 17	317 ± 11	9.3	13.39 ± 0.94	4.32 ± 0.35	200.5 ± 9.7	26.5 ± 2.6
PB 6 (6)	278 ± 18	314 ± 13	12.9	13.95 ± 1.17	4.63 ± 0.42	182.1 ± 10.5	27.4 ± 2.3
PB 12.5 (6)	299 ± 21	333 ± 19	11.4	14.35 ± 1.12	4.71 ± 0.40*	191.5 ± 9.2	33.4 ± 1.5*
PB 25 (6)	273 ± 14	318 ± 16	16.5	14.75 ± 0.99	4.64 ± 0.31*	209.9 ± 7.1	34.4 ± 1.7*
PB 75 (7)	273 ± 16	320 ± 17	17.2	17.25 ± 1.19	5.39 ± 0.43*	200.6 ± 3.9	34.7 ± 2.9*
PB 125 (6)	286 ± 15	301 ± 22	5.2	14.75 ± 1.77	4.88 ± 0.51*	200.4 ± 7.9	35.7 ± 4.8*

[a] PB dose (mg/kg/day ip × 6).
[b] Mean percentage gain compared to the pretreatment value.
[c] Percentage.
[d] Number of animals.
[e] Values are means ± SD.
* Significantly different from controls ($P < 0.05$–0.001).

treated rats at any dose. Recovery of microsomal protein, however, was higher in all PB groups, although differences from controls were significant only with a dose of 12.5 mg/kg or higher.

Cytochrome P-450. PB, at doses ranging from 1 to 75 mg/kg, produced a dose-related induction of hepatic cytochrome P-450 activity (Fig. 1). P-450 activity in control rats was 0.76 ± 11 (SD) nmole/mg microsomal protein and was not enhanced significantly following PB treatment at 1 mg/kg (0.79 ± 0.09 nmole/mg). With 3 mg/kg PB, however, a significant induction was obtained (0.98 ± 0.07 nmole/mg, $P < 0.001$). Maximal P-450 induction was obtained with 75 mg/kg PB (1.63 ± 0.14 nmole/mg), a 114% increase over control levels. Interestingly, P-450 specific activity in livers of rats treated with 125 mg/kg PB (1.42 ± 0.16 nmole/mg) was significantly lower ($P < 0.025$) than that observed with 75 mg/kg PB (Fig. 1) and approximated the activity obtained with 25 mg/kg PB.

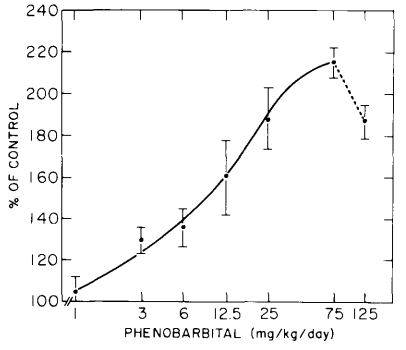

FIG. 1. Effect of phenobarbital treatment on hepatic cytochrome P-450 activity. Doses of the drug are in a log scale. Values are means ± SD and represent the percentage increase over the control activity. (nanomoles per milligram microsomal protein). See legend to Table I for number of animals used for each dose of the drug.

Bilirubin UDP-glucuronyl transferase. To determine whether UDPGT induction by PB was influenced by enzyme activation status, UDPGT activity was measured in liver homogenate or microsomes as "native" enzyme, and following UDPNAG or detergent (digitonin or deoxycholate) activation. At the optimal concentrations found in the present studies, digitonin and deoxycholate enhanced "native" UDPGT activity by 330–400% and 390–450%, respectively, regardless of whether homogenate or microsome suspension was employed. UDPNAG, however, produced a different degree of enzyme activation depending on whether homogenate or microsomes were used. Although maximal activation was obtained in the two preparations with the same UDPNAG concentration (3.07 mM), somewhat greater enzyme activation was observed in homogenate (60–100%) than in microsomes (40–70%).

As observed with P-450, PB produced induction of UDPGT in dose-related fashion.

By contrast with the cytochrome, however, UDPGT induction was related to the PB dose over the entire range 1–125 mg/kg (Fig. 2, Table II). Irrespective of whether the enzyme activity was determined in homogenate or microsomes and expressed as activity per gram of liver or per milligram of microsomal protein, respectively, maximal UDPGT induction was obtained with the highest dose of PB. Additionally, enhancement of UDPGT activity by PB was independent of the enzyme activation condition and similar dose–response curves were obtained when untreated ("native") or UDPNAG-, digitonin-, or deoxycholate-treated homogenate or microsome suspensions were used. Figure 2 illustrates the effect of PB treatment on deoxycholate-activated UDPGT activity in homogenate; Table II summarizes the results obtained when microsome suspensions were employed.

When UDPGT activity was determined in liver homogenate, and the enzyme activity expressed per gram of liver, a significant induction over baseline could be demonstrated with a dose of PB as low as 3 mg/kg provided that deoxycholate-(3.56 ± 0.26 vs controls 2.72 ± 0.39 mg/hr/g liver; $P < 0.001$) or digitonin-activated enzyme (2.92 ± 0.17 vs 2.35 ± 0.32 mg/hr/g liver; $P < 0.001$) was employed, but not with "native" (0.74 ± 0.14 vs 0.64 ± 0.12 mg/hr/g liver; $0.05 < P < 0.1$) or UDPNAG-activated enzyme (1.49 ± 0.22 vs 1.36 ± 0.19 mg/hr/g liver; $0.05 < P < 0.1$). Significant induction of "native" or UDPNAG activated enzyme in homogenate preparations was achieved only with PB doses of 6 mg/kg/day of higher. However, when microsomes were employed and the specific enzyme activity was determined, a significant UDPGT induction was obtained in rats treated with PB at 3 mg/kg, regardless of whether "native" enzyme or enzyme activated by UDPNAG or by detergents was used (Table II). Using either homogenate or microsome suspension, the increases in UDPGT activity observed in rats treated with 1 mg/kg PB did not achieve statistical significance.

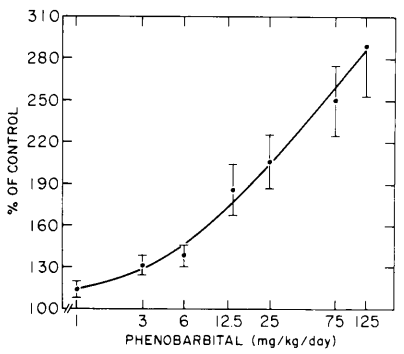

FIG. 2. Effect of phenobarbital treatment on deoxycholate-activated bilirubin UDP-glucuronyl transferase activity of rat liver homogenate. Doses of the drug are in a log scale. Values are means ±SD and represent the percentage increase over the control activity (milligrams per hour per gram liver). See Table II for number of animals used for each dose of phenobarbital.

Glucose-6-phosphatase. Hepatic G6P activity in control rats was 17.88 ± 2.25 and 24.05 ± 2.95 μmole/min/g liver when determined in untreated and deoxycholate-treated homogenate, respectively, and 274.6 ± 38.5 and 361.8 ± 49.4 nmole/min/mg protein when measured in resuspended microsomes ($N = 8$). Recovery of enzyme activity in microsomes ranged from 35 to 50%, regardless of enzyme activation conditions. PB, at the doses used in these studies, produced a dose-related inhibition of G6P activity which could equally be detected when "native" or deoxycholate-activated enzyme was employed. When the specific activity was determined in microsomes, a significant reduction was observed with a dose of PB as low as 3 mg/kg (241.3 ± 29.1 and 317.8 ± 42.3 nmole/min/mg protein for "native" and deoxycholate-activated enzyme, respectively; ($N = 7$), $P < 0.05$ vs respective control values reported above). Maximal inhibition was produced with 125 mg/kg PB, which represented a 49% decline from the control activity (Fig. 3). The reduction of G6P activity associated with PB treatment could not be accounted for solely by the increase in microsomal protein produced by the drug. Thus, as illustrated in Table III, a dose-related inhibition was also observed when G6P activity was determined in homogenate and expressed as either micromoles per minute per gram liver or micromoles per minute 100 g body weight. In both of these situations, however, the inhibitory effect was less pronounced and a significant reduction of the enzyme activity could be demonstrated only with a dose of PB of 6 mg/kg or greater.

Discussion. Breckenridge *et al.* (2) first observed that PB, injected to rats at doses ranging

TABLE II. EFFECT OF PHENOBARBITAL TREATMENT (PB, mg/kg/day) ON BILIRUBIN UDP-GLUCURONYL TRANSFERASE (UDPGT) ACTIVITY OF RAT LIVER MICROSOMES[a]

Treatment	(*N*)	"Native"	UDPNAG	Digitonin	Deoxycholate
Controls	(8)[b]	3.64 ± 0.48	5.96 ± 0.75	13.33 ± 1.71	15.68 ± 2.39
PB 1	(7)	3.85 ± 0.59	6.19 ± 0.64	14.94 ± 2.82	16.38 ± 3.00
PB 3	(7)	4.50 ± 0.55*	6.90 ± 1.06*	16.51 ± 1.35*	18.33 ± 2.37*
PB 6	(6)	4.75 ± 0.80*	7.56 ± 1.28*	18.72 ± 2.53*	19.92 ± 3.02*
PB 12.5	(6)	6.22 ± 0.54*	9.50 ± 0.68*	22.75 ± 2.46*	26.35 ± 2.39*
PB 25	(6)	6.71 ± 1.36*	9.95 ± 1.75*	23.99 ± 1.34*	27.45 ± 3.57*
PB 75	(7)	7.87 ± 1.38*	11.03 ± 1.40*	26.81 ± 2.90*	31.10 ± 2.74*
PB 125	(6)	9.32 ± 0.71*	12.98 ± 1.64*	32.78 ± 3.55*	40.86 ± 5.47*

[a] Values are means ± SD and represent nmole/10 min/mg microsome protein.
[b] Number of animals.
* Regardless of the enzyme activation status, all activities from rats treated with a dose of PB of 3 mg/kg or greater are significantly greater than controls ($P < 0.05$–0.001).

from 1 to 40 mg/kg, produced a dose-related induction of the *in vitro* activity for *N*-demethylation of ethyl morphine. The present results are in agreement with these previous observations and indicate that both P-450 and UDPGT are also induced by PB in a dose-related fashion. These findings are not surprising since they are consistent with the concept that enzyme induction may only occur when tissue levels of the inducing agent exceed the physiological capacity of those enzymes deputed to metabolize it (9). What is surprising, instead, is the finding that while UDPGT was maximally induced with the largest dose of PB employed, P-450 induction was adversely affected as the dose of the drug was increased from 75 to 125 mg/kg. At present, the reasons, for this lower P-450 induction with 125 mg/kg PB are not entirely clear. However, undesirable side effects, as reflected by the reduced growth of body and liver weights, were associated with the administration of PB at this high dose. Thus, it is likely that induction of the enzyme was less pronounced with this high dose of PB because of these untoward effects of the drug.

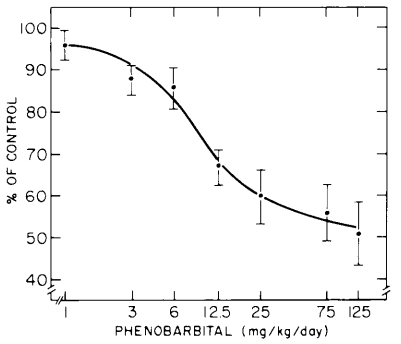

FIG. 3. Effect of phenobarbital treatment on glucose-6-phosphatase activity of rat liver microsomes. Doses of the drug are in a log scale. Values are means ±SD and represent the percentage decrease of the deoxycholate-activated enzyme specific activity (nanomoles per minute per milligram microsomal protein) from controls. See Table III for number of animals used for each dose of phenobarbital.

UDPGT is an enzyme or a group of enzymes tightly bound to membranes of the endoplasmic reticulum. Because of this characteristic, the *in vitro* activity of UDPGT can be measured either in its "native" form or fully activated. Several procedures can be used to activate UDPGT, including sonication (10), or treatment with phospholipases (11), detergents (12, 13) or the sugar nucleotide, UDPNAG (5). All of these activators have previously been studied extensively.

Our principal objectives in the present investigation were twofold: to examine the dose–response relationship for PB and UDPGT, and to determine whether equivalent UDPGT induction by PB could be obtained when "native" or activated forms of the enzyme were used. Some previous studies have, in fact, reported that "native" UDPGT is not responsive to PB treatment and that UDPGT induction can only be detected when detergent-activated enzyme is employed (14). Our studies, however, indicate that in the present assay conditions UDPGT induction by PB is obtained irrespective of the enzyme activation status,

TABLE III. EFFECT OF PHENOBARBITAL TREATMENT (PB, mg/kd/day) ON GLUCOSE-6-PHOSPHATASE (G6P) ACTIVITY OF RAT LIVER HOMOGENATE

Treatment	(N)	G6P activity (μmole/min/g liver)		G6P activity (μmole/min/100 g body wt)
		"Native"[a]	Activated[b]	Activated[b]
Controls	(8)[c]	17.88 ± 2.25[d]	24.05 ± 2.95	105.4 ± 13.4
PB 1	(7)	17.33 ± 1.76	22.87 ± 1.82	103.9 ± 8.7
PB 3	(7)	16.65 ± 1.92	21.93 ± 2.17	95.7 ± 10.2
PB 6	(6)	15.51 ± 1.41*	20.73 ± 1.64*	92.9 ± 7.3*
PB 12.5	(6)	15.23 ± 1.25*	19.47 ± 1.63*	83.4 ± 6.8*
PB 25	(6)	13.75 ± 1.80*	17.52 ± 1.44*	81.8 ± 6.7*
PB 75	(7)	13.05 ± 0.92*	16.86 ± 1.31*	91.7 ± 7.3*
PB 125	(6)	9.78 ± 1.21*	13.23 ± 1.27*	66.2 ± 6.7*

[a] Untreated homogenate.
[b] Homogenate treated with deoxycholate (0.15%).
[c] Number of animals.
[d] Values are means ± SD.
* Enzyme activities from rats treated with a dose of PB of 6 mg/kg or greater are significantly lower than those obtained from controls ($P < 0.05$–0.001), when expressed either as μmole/min/g liver or as μmole/min/100 g body weight.

and that, on a percentage basis, equally enhanced UDPGT activities are observed when "native" enzyme or enzyme activated by UDPNAG or by detergents is used.

The finding that PB produced significant induction of both P-450 and UDPGT at a drug dose as low as 3 mg/kg/day warrants special consideration in view of its relevance to an inducing effect of the drug on these enzymes in man. Both normal volunteers and patients with Gilbert's syndrome or congenital nonhemolytic jaundice type II have, indeed, failed to show consistently enhanced UDPGT activity following PB administration at doses ranging from 0.5 to 7.5 mg/kg/day, despite the widely documented efficacy of the drug in lowering the plasma levels of unconjugated bilirubin over the same dose range (15–17). Similarly, an increase in "early labeled" bilirubin production, which in the rat is principally derived from the turnover of hepatic cytochrome P-450 (18, 19), has not been observed in man following administration of the drug at these doses (20, 21).

Among other possibilities, the large difference in the dose of PB employed in experimental animals as opposed to humans has been evoked to conciliate these negative clinical findings with the inducing effects overwhelmingly demonstrated in laboratory animals (22). Our studies, however, indicate that the PB dose difference may not provide an explanation for these discordant effects of the drug in man and rat. Thus, a daily dose of PB as low as 3 mg/kg, which is similar to that given to man, produces a significant induction of both UDPGT and P-450 activities in the rat. Even though extrapolation of these animal data to man must be done with caution because of species differences in the metabolism of the drug and differences in the route of its administration, pharmacokinetic studies have shown that PB is metabolized in man (23, 24) at a much slower rate than it is in the rat (25, 26). This suggests that the 3-mg/kg dose which produces enzyme induction in the rat is pharmacologically equivalent to an even lower dose in man. Thus, the doses of PB which have failed to produce UDPGT induction (15–17) and to enhance total heme turnover (20, 21) in man are well within the range now shown to produce enzyme induction in the rat. Other factors, including species differences and/or methodological differences in measuring the *in vitro* activity of these enzymes, may therefore underlie the failure to observe these effects during PB administration in man.

Finally, the present studies have demon-

strated that PB administration at doses ranging from 1 to 125 mg/kg/day results in a dose-related inhibition of G6P activity. As numerous other investigators have already reported this effect of PB (27–30), such a finding was not entirely unexpected. However, while some of these previous reports have concluded that the reduced G6P activity simply reflects an enzymatic dilution due to the proliferated endoplasmic reticulum (27, 28) our studies have demonstrated that the absolute activity of the enzyme was also reduced following PB treatment. This indicates that the dilution factor accounts only in part for the diminished G6P activity, a conclusion also made by Wanson *et al.* (30), who found that the depressed enzyme activity following PB administration was associated with a decline in the G6P:DNA ratio.

As to the mechanism of this inhibitory effect of PB on G6P activity, little can be said at the present time. Crampton *et al.* (31) suggested that the diminished enzyme activity associated with PB treatment may be related to increased glucose metabolism via the pentose cycle required to sustain the enhanced activity of a variety of microsomal enzymes. Some support for this hypothesis comes from the finding that PB administration is, indeed, associated with an increased activity of glucose-6-phosphate dehydrogenase (32), which can histochemically be localized at the centrilobular cells where the depressed G6P activity occurs (31). Regardless of the mechanism, however, the present studies clearly indicate that induction of drug-metabolizing enzymes by PB is, indeed, associated with a reduction of G6P activity. Although the significance of G6P inhibition is presently unknown, the finding that it occurs in the rat with doses of PB similar to those given to psychiatric or hyperbilirubinemic patients (3–6 mg/kg/day), suggests that a similar effect might be expected in man.

We sincerely thank Ms. Annette Seaborough for her expert assistance in preparing this manuscript.

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Received January 20, 1983. P.S.E.B.M. 1983, Vol. 174.