Biochemical Pharmacology, Vol. 28, pp. 849–857.

0006-2952/79/0315-0849 $02.00/0

HETEROGENEOUS RESPONSE OF HEPATIC MIXED FUNCTION OXIDASES TO CHRONIC PHENOBARBITAL ADMINISTRATION

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(*Received* 21 *March* 1978; *accepted* 3 *August* 1978)

Abstract—Effects of chronic phenobarbital (PB) administration (75 mg/kg, p.o. for 59 days) on certain hepatic mixed function oxidases (HMFO) were investigated in male Sprague–Dawley rats to determine whether maximal induction is maintained throughout this period or whether further alterations occur in the inductive effect. As demonstrated by measurements of $^{14}CO_2$ excretion rates in breath after a single i.p. injection of [dimethylamine-^{14}C]aminopyrine ([^{14}C]aminopyrine), PB in the above dose for 7 days accelerated hepatic aminopyrine demethylation 92 per cent above control values. However, after 59 days of PB administration, $^{14}CO_2$ excretion was only 5 per cent above control values; thus, initial PB-induced enhancement of aminopyrine *N*-demethylation declined with time. Phenobarbital administration did not affect total (unlabeled) CO_2 output, indicating that decreased $^{14}CO_2$ excretion did not result from potential PB-induced reduction of CO_2 output. In contrast, total cytochrome P450 content and aniline hydroxylase activity remained elevated throughout the 59 days of PB treatment. Ethylmorphine *N*-demethylase activity and aminopyrine *N*-demethylase activity (using a 3.0 mM substrate concentration) remained elevated until 28 days of PB administration, after which time these activities decreased significantly ($P < 0.05$), finally reaching 71 and 83 per cent respectively, of peak activity. Kinetic analyses of aminopyrine *N*-demethylation by hepatic microsomes revealed the K_m values were unchanged from 4 to 59 days of PB administration, but that V_{max} increased to a maximum of 15.5 on day 28, after which it decreased to 6.60 on day 59. This pattern of change for V_{max} of aminopyrine *N*-demethylase was similar to that for enzyme activity obtained using a constant 3.0 mM concentration of aminopyrine. Although aminopyrine *N*-demethylase activity (using a 3.0 mM substrate concentration) peaked after 2 days of PB treatment, 7 days of PB treatment reduced this activity to 14 per cent below peak values. After 59 days of PB treatment, aminopyrine *N*-demethylase activity (at a 0.3 mM substrate concentration) declined to 61 per cent of the peak value attained on day 2. Another chronic PB study was performed and different time points selected to determine the reproducibility of these differential alterations of MFO activity. Results in the second study were similar to those obtained in the first study. Appreciable loss with time of the inductive effects produced by PB on aminopyrine metabolism both *in vitro* and *in vivo* suggests a heterogeneous response of hepatic MFO to PB; metabolism of aniline remained at peak inductive values throughout the 59 days of PB administration, whereas ethylmorphine metabolism declined only slightly.

Phenobarbital (PB) enhances the activities of certain hepatic microsomal enzymes by a direct effect on the liver that involves increased protein synthesis and liver weight [1–3]. The magnitude of these increases in enzyme activity varies with dose, duration and route of PB administration, as well as with the age, sex and species studied [4, 5]. A dose–response relationship has been described for the inductive effects of PB and other barbiturates over a 15-day period [4, 5]. All hepatic enzymes studied increased during this 15-day period of PB administration. However, induction potency was related directly to the biological half-life of the barbiturate, as well as its lipid solubility.

One study on chronic administration of PB (75 mg/kg, i.p.) injected every other day for 52 days in Long–Evans rats revealed enhanced hexobarbital metabolism; peak hepatic hexobarbital oxidase activity attained on day 15 was maintained throughout the 52 days [6]. In dogs, Burns and Conney [7] demonstrated that maximal induction of hepatic microsomal phenylbutazone metabolism was maintained throughout 2 months of PB treatment (16 mg/kg).

The activities of hepatic microsomal drug-metabolizing enzymes during chronic, daily PB administration in the rat have not been investigated extensively either *in vivo* or *in vitro*. The present study evaluates *in vivo* and *in vitro* effects of chronic, daily PB administration on certain hepatic mixed function oxidases (MFO) of rat liver in order to determine whether induction is maintained at peak levels throughout this period or whether further adaptations occur that alter the magnitude of the inductive effect.

MATERIALS AND METHODS

Animals and treatments. Male Sprague–Dawley rats weighing between 90 and 100 g at the onset of the chronic studies were supplied by Charles River Breeding Laboratories Inc. (Wilmington, MA), and kept under observation in quarantine facilities for 1 week prior to study. Throughout the investigation rats were housed in a windowless room in stainless steel wire cages with six animals in each cage. Kimpack bedding from Kimberly Clark Co. (Neenah, WI), placed 3 inches below the floor of the cages, was changed every 3–4 days. The temperature of the room was 22 ± 1°, and the relative humidity varied between 40 and 60 per cent. A 100 per cent exchange of fresh air was accom-

plished 18–22 times every hour. The room was illuminated from 7:00 a.m. to 7:00 p.m. Animals were maintained on Purina Lab Chow and water *ad lib.*

Every day at 8:00–9:00 a.m. all rats received by stomach tube PB (75 mg/kg) dissolved in physiological saline. The PB solution (pH 8.3) was made up in a concentration such that each animal received 1 ml volume/100 g body wt. A fresh solution of PB was made up every day. An equal number of control animals received by stomach tube physiological saline (adjusted to pH 8.3) (also 1 ml/100 g body wt). Rats were killed 24 hr after the last PB dose.

Each rat used for measurement of $^{14}CO_2$ excretion was given an i.p. injection of [dimethylamine-^{14}C]aminopyrine ([^{14}C]aminopyrine) (1 μCi/100 g body wt in solution with unlabeled aminopyrine, 12 mg/100 g body wt) [8].

Drugs and chemicals. The following drugs or chemicals were used: phenobarbital (Merck & Co., Rahway, NJ); aniline hydrochloride (Eastman Organic Chemicals, Rochester, NY); ethylmorphine hydrochloride (Merck & Co.); aminopyrine (Aldrich Chemical Co., Inc., Milwaukee, WI); [dimethylamine-^{14}C]aminopyrine (12 mCi/m-mole) (Amersham & Searle Corp., Arlington Heights, IL); NADP$^+$, D-glucose 6-phosphate and glucose 6-phosphate dehydrogenase (Sigma Chemical Co., Phillipsburg, NJ).

Preparation of microsomes. On days 0, 3, 8, 14, 29 and 60 of the study six PB-treated and six saline-treated rats were decapitated between 8:00 a.m. and 9:00 a.m.; livers were immediately removed, weighed and placed in 2 vol. of ice-cold 1.15% (w/v) KCl solution buffered with 0.02 M Tris–HCl buffer, pH 7.4 (Tris–KCl). Rats used for kinetic analysis of aminopyrine *N*-demethylation in hepatic microsomes were killed after 4, 14, 28 and 59 days of PB administration. The microsomes were isolated as described by Valerino *et al.* [4]. After isolation, the microsomal pellets were resuspended in Tris–KCl, using a glass homogenizer. The final protein concentration after resuspension was approximately 10 mg/ml.

Analytical methods. Cytochrome P450 content was determined by the dithionite difference method of Omura and Sato [9] using an Aminco–Chance dual-beam spectrophotometer, operated in the dual wavelength mode. An extinction coefficient of 91 mM^{-1} cm^{-1} was employed for determination of cytochrome P450 content, and the determination was done immediately after isolation of the microsomes. Microsomal aminopyrine *N*-demethylase activity was assayed at two different substrate concentrations. The total volume of the incubation mixture was 3 ml, consisting of the following components: aminopyrine, 9 μmoles or 0.9 μmole; NADP$^+$, 1.31 μmoles; magnesium chloride, 25 μmoles; glucose 6-phosphate, 32.8 μmoles; glucose 6-phosphate dehydrogenase, 1 unit. The lower substrate concentration was used to approximate more closely non-saturating conditions occuring *in vivo*. Previous studies have revealed that the 0.3 mM substrate concentration correlates most closely with *in vivo* $^{14}CO_2$ excretion data [10]. Aminopyrine concentrations used for kinetic analysis of microsomal aminopyrine *N*-demethylation ranged from 0.1 to 7.5 mM. Protein concentration, determined by the method of Gornall *et al.* [11], was 5 mg/incubation vial. All incubations were performed in air at 37°, using a Dubnoff shaking incubator (120 oscillations/min) for 30 min. Formaldehyde (HCHO) produced during the demethylation process was measured according to the method of Nash [12], as described in detail by Holtzman *et al.* [13]. Corrections were made for apparent formaldehyde produced in the absence of substrate [14]. Enzyme activities were expressed as nmoles HCHO/mg of microsomal protein/min.

Ethylmorphine *N*-demethylase activity was assayed under the same conditions described above for aminopyrine *N*-demethylase activity, except that the substrate was ethylmorphine hydrochloride (15 μmoles/incubation flask), and the incubation time was 12 min.

Aniline hydroxylase activity was assayed in an incubation mixture of 3 ml, consisting of the following: aniline hydrochloride, 6.5 μmoles; NADP, 1.31 μmoles; magnesium chloride, 25 μmoles; glucose 6-phosphate, 32.8 μmoles; glucose 6-phosphate dehydrogenase, 1 unit. The concentration of protein was 10 mg/incubation vial. The *p*-aminophenol formed [15] was measured as described by Chhabra *et al.* [16]. The mixture was incubated at 37° for 22 min (120 oscillations/min).

Under the conditions described above, the rates of *N*-demethylation of aminopyrine and ethylmorphine and of the hydroxylation of aniline were linear with time using microsomes from saline- or PB-treated animals.

Measurement of CO_2 in breath of rats. The rate of aminopyrine *N*-demethylation was assessed *in vivo* by the measurement of the rate of exhaled $^{14}CO_2$ by rats after i.p. administration of [^{14}C]aminopyrine. Recovery of $^{14}CO_2$ in exhaled breath of rats after [^{14}C]aminopyrine administration closely reflects hepatic mixed function oxidase activity [8, 10, 17]. In man, the rates of decline of $^{14}CO_2$ in breath after oral administration of [^{14}C]aminopyrine, the aminopyrine breath test (ABT), is a useful noninvasive tool for assessing some aspects of hepatic drug metabolism both in normal subjects and in patients with hepatocellular disease [18–20].

Measurement of $^{14}CO_2$ excretion rates was performed as described previously by Sultatos *et al.* [10]; the apparatus for the collection of breath $^{14}CO_2$ was adapted from Cowgill and Pardee [21]. This method differs from that previously described by Lauterberg and Bircher [8] in that they utilized restraining cages connected to flasks containing 20 ml of 1:4 ethanolamine–methanol mixture, which they replaced every 8 min.

Data analysis. The microsomal enzyme activities were calculated as nmoles product formed/mg of microsomal protein/min and cytochrome P450 content as nmoles cytochrome P450/mg of microsomal protein.

In order to compare the effects of PB administration on these mixed function oxidase activities at the specified time intervals, the results were expressed as per cent of control. Control values were determined whenever PB-treated animals were killed because of well-recognized, day-to-day variations in hepatic microsomal enzyme activity [4].

An analysis of covariance was performed and adjusted means were calculated according to a method presented by Steel and Torrie [22]. According to this procedure, covariance may be adjusted to adjust treatment means of the dependent variable (enzyme activities after PB treatment) for differences in the independ-

ent variable (enzyme activities after saline treatment).

Kinetic analyses of microsomal aminopyrine *N*-demethylation were performed using Woolf–Augustinsson–Hofstee plots [23]. When biphasic enzyme kinetics were observed, the V_{max} for each segment was determined by extrapolation to the y-intercept, while the slope of each segment was equal to $-K_m$ [23]. Kinetic parameters were determined by linear regression analysis on a Hewlett Packard 9810A calculator.

At the completion of the chronic PB study, the mean weights for the saline-treated and PB-treated animals were plotted against time. The data were analyzed by a Tetronics 30 calculator and a best-fit regression line for the points was determined. The Tetronics 30 calculator indicated that each weight curve was a parabola; a parabolic regression on a Hewlett Packard 9810A calculator was performed to find the equation of each line. Each parabola was transformed into a straight line by taking its first derivative. The slopes of these lines were then tested using the formula

$$t = \frac{b - b^1}{Sy \cdot x \sqrt{(1/SS_x) + (1/SS_x)}}$$

taken from Goldstein [24]. Values of $P < 0.05$ were considered to represent significant differences between means.

Histological techniques. Liver slices were obtained immediately after death and placed in 10% buffered formalin solution. Sections cut from paraffin-embedded blocks were stained with hematoxylin-eosin and examined under a light microscope.

Liver tissue removed for electron micrographs was removed in a cold room at 4°. They were immersed in 4 CFIG (4% commercial formaldehyde, 1% glutaraldehyde, Sorenson buffer, pH 7.2). The block stain was 4% uranyl acetate in H_2O, and the tissues were finally embedded in EPON 812.

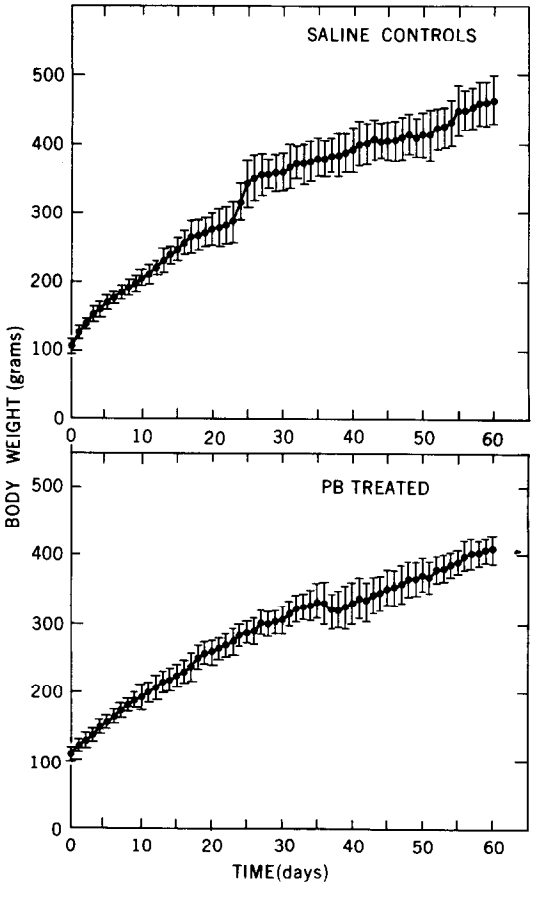

Fig. 1. Effects of oral PB or saline administration daily for 59 days on total body weight.

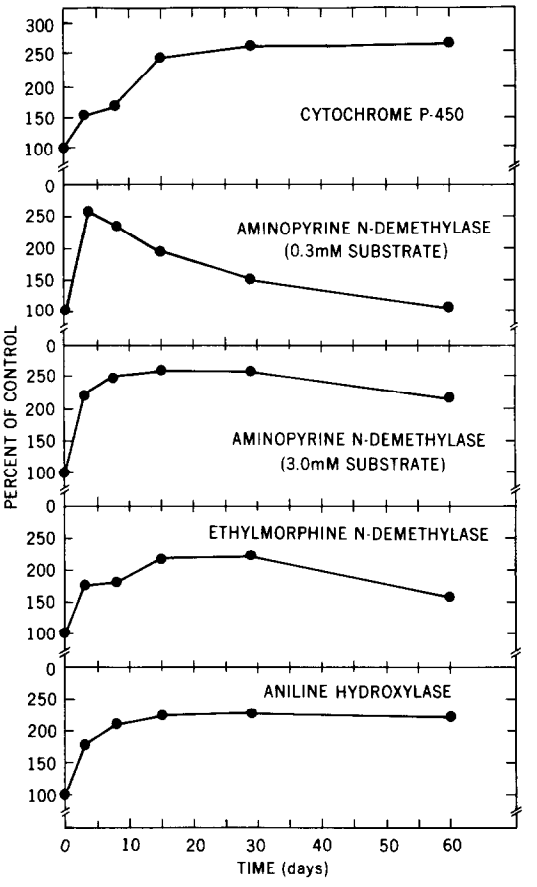

Fig. 2. Effects of PB administration (75 mg/kg, p.o. daily for 59 days) on hepatic microsomal systems expressed as per cent of saline controls.

RESULTS

Statistical analysis by the method of Goldstein [24] revealed no significant differences between rates of growth of saline- and PB-treated groups (Fig. 1). PB-induced liver weight and microsomal protein/g of liver failed to decline after 59 days of PB administration (Table 1).

Table 2 shows hepatic MFO activities of saline- and PB-treated rats. Values from PB-treated rats were then compared to those of the corresponding control group by expressing the data as per cent of control (Fig. 2).

After 2 days of PB administration, cytochrome P450 content and ethylmorphine *N*-demethylase and aniline hydroxylase activities increased 160, 129 and 132 per cent respectively; aminopyrine *N*-demethylase activity at a 3.0 mM substrate concentration increased 214 per cent (Fig. 2). Aminopyrine *N*-demethylase

Table 1. Liver weights and hepatic microsomal protein during chronic saline or PB administration

Day of saline or PB administration	Drug administered	Liver wt* (g)	Microsomal protein content* (mg/g liver)
0	Saline	4.2 ± 0.1	7.9 ± 0.3
	PB	4.2 ± 0.1	8.1 ± 0.2
2	Saline	4.4 ± 0.1	8.0 ± 0.4
	PB	5.2 ± 0.3†	9.7 ± 0.5†
7	Saline	5.4 ± 0.2	8.4 ± 0.6
	PB	7.3 ± 0.2†	11.9 ± 1.1†
14	Saline	7.2 ± 0.5	9.3 ± 0.9
	PB	10.3 ± 0.3†	14.6 ± 2.0†
28	Saline	9.2 ± 0.8	12.9 ± 1.2
	PB	15.4 ± 1.0†	22.5 ± 1.8†
59	Saline	11.5 ± 1.1	11.8 ± 0.9
	PB	18.4 ± 1.6†	23.4 ± 2.0†

* Each value represents the mean ± S.D. of six animals.
† Significantly different from the corresponding saline control by Student's *t*-test ($P < 0.05$).

activity, measured using a 0.3 mM substrate concentration, was enhanced 257 per cent after 2 days of PB administration (Fig. 2).

Throughout the study, cytochrome P450 content and aniline hydroxylase activity displayed similar patterns of response. Both increased steadily until reaching a maximum value of 264 per cent of controls for cytochrome P450, and 227 per cent of controls for aniline hydroxylase activity at 28 days of PB treatment, after which they reached a plateau and remained unchanged for the duration of the study (Fig. 2). Ethylmorphine *N*-demethylase and aminopyrine *N*-demethylase (at 3.0 mM substrate concentration) activities also increased until maximum induction of 246 and 259 per cent, respectively, after 28 days of PB treatment. Ethylmorphine *N*-demethylase activity then remained unchanged until 59 days of PB administration, at which time it decreased significantly ($P < 0.05$) to 159 per cent (Fig. 2). Aminopyrine *N*-demethylase activity at a 3.0 mM substrate concentration decreased significantly ($P < 0.05$) from 259 per cent at day 28 of the study to 216 per cent on day 59 (Fig. 2). Aminopyrine *N*-demethylase activity at a 0.3 mM substrate concentration was stimulated maximally to 257 per cent of control values after 2 days of PB administration. After 2 days, the enzyme activity at the 0.3 mM substrate concentration decreased steadily from 257 to 110 per cent of control values by day 59 (Fig. 2).

Kinetic analysis of hepatic microsomal aminopyrine *N*-demethylase activity revealed two V_{max}'s and two K_m's for all saline-treated rats (Table 3). Biphasic enzyme kinetics of aminopyrine *N*-demethylation have been described previously [25]. However, only one V_{max} and one K_m were observed in PB-treated rats for induced aminopyrine *N*-demethylase activity (Table 3). This phenomenon has also been described previ-

Table 2. Comparison of values for hepatic microsomal systems in rats given saline or PB*

	Day of saline or PB administration	Aminopyrine demethylase† 3.0 mM Aminopyrine	0.3 mM Aminopyrine	Ethylmorphine demethylase†	Aniline hydroxylase†	Cytochrome P450‡
Saline-treated	0	6.1 ± 1.1	2.5 ± 0.5	6.4 ± 1.1	0.63 ± 0.08	1.01 ± 0.10
	2	6.4 ± 1.2	2.3 ± 0.3	6.2 ± 0.9	0.71 ± 0.10	1.24 ± 0.22
	7	5.7 ± 0.9	1.9 ± 0.4	5.9 ± 0.8	0.61 ± 0.09	0.98 ± 0.14
	14	7.2 ± 1.1	2.4 ± 0.5	6.8 ± 1.0	0.82 ± 0.09	0.98 ± 0.14
	28	5.9 ± 1.3	2.2 ± 0.2	7.4 ± 1.2	0.74 ± 0.09	1.13 ± 0.14
	59	5.7 ± 1.1	2.0 ± 0.5	6.6 ± 0.9	0.69 ± 0.08	1.18 ± 0.19
Phenobarbital-treated	0	6.0 ± 1.0	2.3 ± 0.4	6.4 ± 1.3	0.65 ± 0.08	1.00 ± 0.08
	2	13.7 ± 1.2	5.9 ± 0.9	8.0 ± 2.1	0.97 ± 0.10	1.98 ± 0.18
	7	13.7 ± 1.4	4.6 ± 0.8	10.9 ± 1.8	1.28 ± 0.09	1.70 ± 0.21
	14	16.8 ± 1.8	3.7 ± 1.1	16.2 ± 2.0	1.85 ± 0.14	2.82 ± 0.28
	28	15.3 ± 1.5	4.3 ± 1.0	18.1 ± 2.4	1.67 ± 0.18	2.98 ± 0.31
	59	12.3 ± 1.4§	2.2 ± 0.06§	10.4 ± 1.7§	1.55 ± 0.14	3.13 ± 0.32

* Each value represents the mean ± S.D. of six animals.
† Expressed as nmoles product formed/mg of microsomal protein/min.
‡ Expressed as nmoles cytochrome P-450/mg of microsomal protein.
§ Significantly less than peak attained on day 28 of PB treatment ($P < 0.05$) by analysis of covariance followed by Newman Kuel's test.

Table 3. V_{max} and K_m of hepatic microsomal aminopyrine *N*-demethylase during 59 days of daily saline or PB adminstration

Day of saline or PB administration	Drug administered	V_{max} *	V_{max} *	K_{m1} †	K_{m2} †
4	Saline	5.40	4.13	1.17	0.31
4	PB	11.04		0.66	
14	Saline	6.33	4.37	1.73	0.34
14	PB	12.17		0.48	
28	Saline	7.24	5.24	1.39	0.28
28	PB	15.50		0.67	
59	Saline	4.84	3.34	1.44	0.27
59	PB	6.60		0.55	

* Expressed as nmoles HCHO/mg of microsomal protein/min.
† Expressed as mM aminopyrine.

ously [25]. After 4 days of PB administration the V_{max} increased to 11.04 nmoles/mg of microsomal protein/min. PB administration for 14 and 28 days further increased the V_{max} to 12.17 and 15.50 nmoles/mg of microsomal protein/min respectively. After 59 days of PB treatment, the V_{max} decreased to 6.60 nmoles/mg of microsomal protein/min (Table 3). From 4 to 59 days of PB administration, no change occurred in K_m (Table 3).

Half-lives for the elimination of $^{14}CO_2$ in the breath of saline-treated and PB-treated rats administered [^{14}C]aminopyrine are plotted against time in Fig. 3. In control rats the half-life of elimination of $^{14}CO_2$ gradually increased from 0.72 hr on day 0 until day 28, when it appeared to reach a plateau of 1.56 hr, probably due to increasing age. In PB-treated rats the half-life of $^{14}CO_2$ elimination decreased to 0.5 hr at 7 days, indicating accelerated demethylation of aminopyrine. After 7 days the half-life of $^{14}CO_2$ elimination gradually increased to 1.21 hr on day 60 (Fig. 3). Table 4 shows that PB administration did not significantly alter total CO_2 output. Prolonged $^{14}CO_2$ half-lives, therefore, did not result from PB-induced suppression of total CO_2 output.

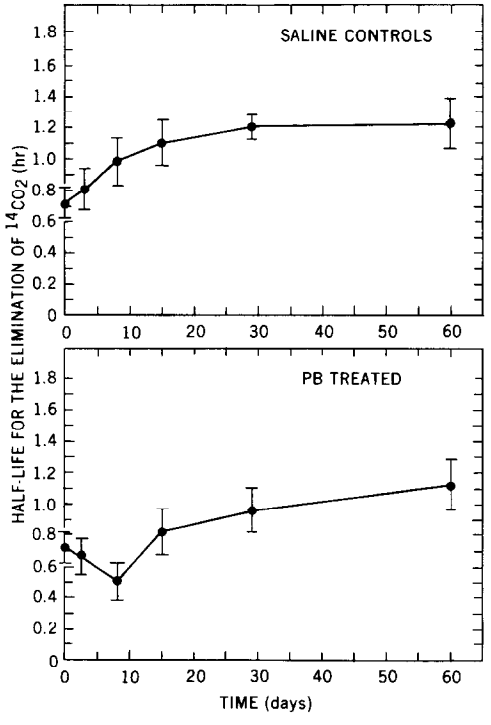

Fig. 3. Half-life for $^{14}CO_2$ elimination in breath by intact rats after intraperitoneal administration of [^{14}C]aminopyrine. Each point represents the mean ± S.D. of six male Sprague-Dawley rats during 59 days of saline or PB administration.

Electron micrographs after 59 days of saline or PB treatment indicated proliferation of hepatic smooth endoplasmic reticulum in PB-treated rats (Figs. 4 and 5). Histological examination of hematoxylin-eosin stained liver sections revealed normal tissue.

The results of a second chronic PB study were similar to those shown in Figs. 2 and 3 and Table 2, indicating high reproducibility of these experiments.

DISCUSSION

Effects of short-term PB administration on induction of hepatic MFO have been investigated thoroughly. However, little is known qualitatively or quantitatively about PB induction for periods of time beyond 14 days. This lack of information on long-term inductive effects of PB or other compounds is both surprising and disappointing because induction of hepatic MFO by various compounds can affect their capacity to produce cancer, mutation, physical defects *in utero* and direct tissue toxicity, all arising presumably after prolonged exposure. Furthermore, PB is a model compound used more frequently than any other to investigate the complex process of MFO induction. For these reasons, it seemed important to determine whether inductive effects on hepatic MFO are maintained at peak levels for the entire period of chronic administration of a model inducing agent such as PB or whether further adaptations occur that modify initial inducing effects. Thus, this investigation was not designed to provide new insights into inductive mechanisms, but to accomplish what seemed to be a prerequisite first step: to obtain a reliable and detailed qualitative and quantitative description of the inductive response during chronic administration of an inducing agent.

The results reveal heterogeneity of hepatic MFO response to 59 days of PB administration in that aniline hydroxylase activity remained elevated without depression throughout this time, whereas ethylmorphine *N*-demethylase and aminopyrine *N*-demethylase activities

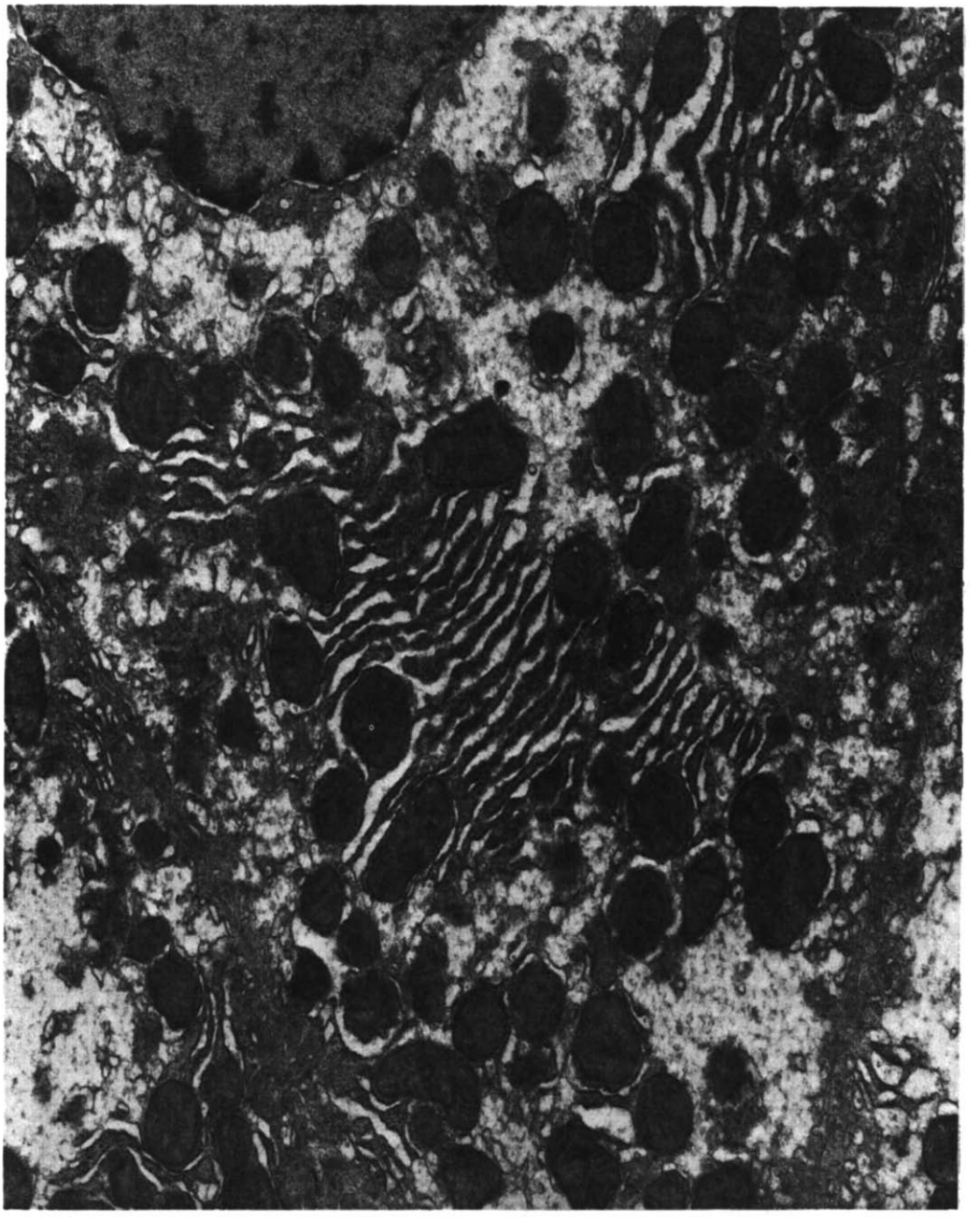

Fig. 4. Electron micrograph of rat liver after saline administration for 59 days. Magnification 21,000 x.

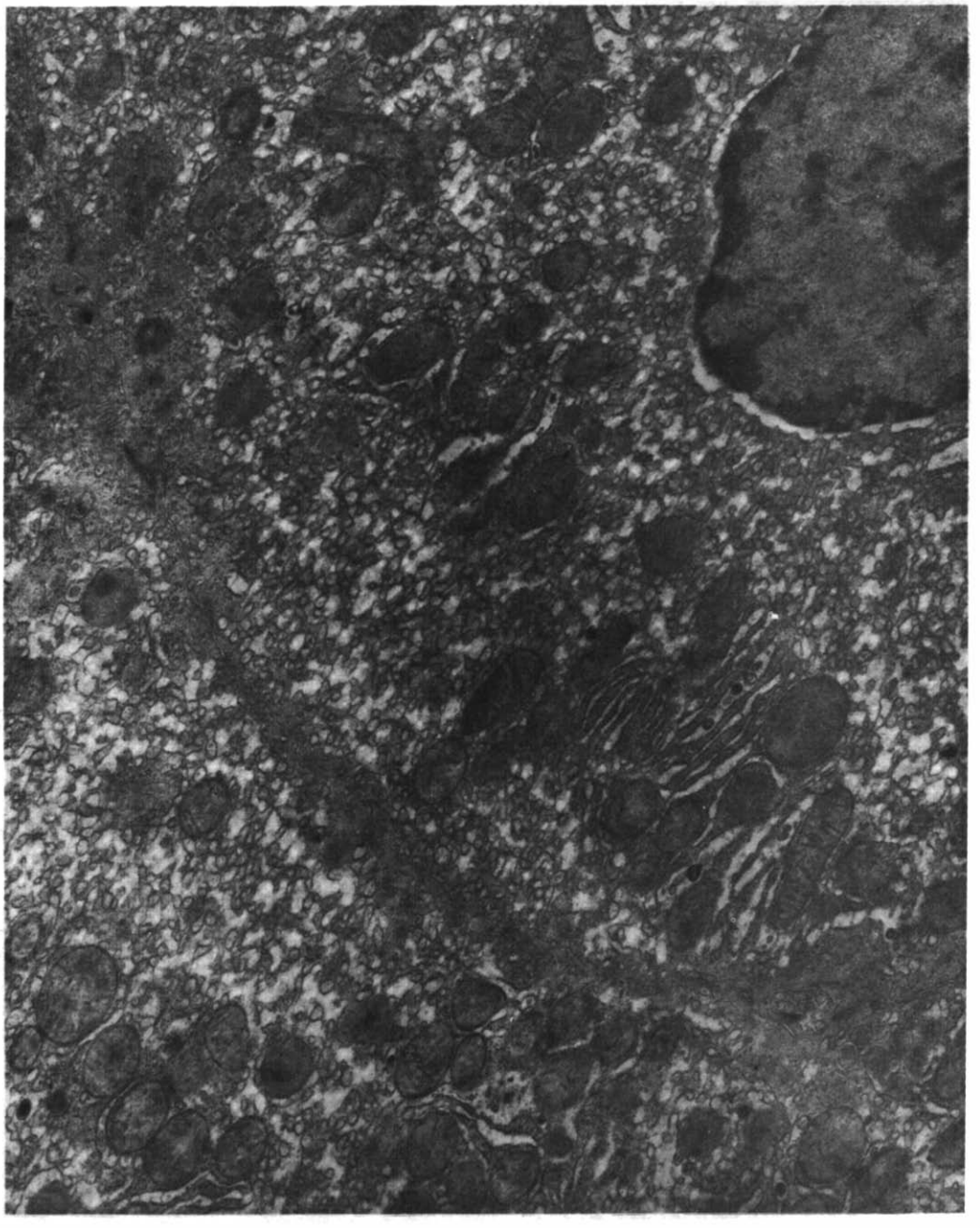

Fig. 5. Electron micrograph of rat liver after 59 days of PB administration. Magnification 21,000 x.

Table 4. Measurement of total CO_2 in breath of intact rats during a 59-day period of daily saline or PB administration

Day of saline or PB administration	Drug administered	CO_2 output (m-moles/hr)
0	Saline	4.99 ± 0.37
	PB	5.02 ± 0.29*
2	Saline	5.10 ± 0.42
	PB	5.03 ± 0.35*
7	Saline	6.02 ± 0.41
	PB	6.15 ± 0.28*
14	Saline	7.84 ± 1.15
	PB	7.77 ± 0.83*
28	Saline	7.92 ± 0.78
	PB	8.16 ± 0.83*
59	Saline	7.77 ± 0.60
	PB	7.41 ± 0.67*

* Not statistically different from the corresponding saline control ($P > 0.05$ by Student's t-test).

(at 3.0 mM substrate concentration) decreased significantly ($P < 0.05$) to 71 and 83 per cent, respectively, of peak induced activities. Even more marked decreases from peak induced activities occurred with aminopyrine N-demthylase at 0.3 mM substrate concentration, which returned to control levels (Table 2, Fig. 2). Thus, inducibility of two type I substrates, aminopyrine and ethylmorphine, decreased with time from peak induced levels, whereas inducibility of the type II substrate, aniline, remained at peak levels throughout the course of study.

Kinetic analyses were performed to determine whether after prolonged PB administration changes in aminopyrine N-demethylation resulted from altered V_{max}, K_m, or both. Two V_{max}'s and two K_m's have been reported for hepatic microsomal aminopyrine N-demethylase. According to LaDu *et al.* [26], aminopyrine is first demethylated to monomethyl-4-aminoantipyrine, which is further demethylated to 4-aminoantipyrine. Since measurement of formaldehyde as an end product does not distinquish between these reactions, biphasic kinetics may arise from different affinities of monomethyl-4-aminoantipyrine, aminopyrine or both for the MFO system. Biphasic kinetics might also result from different binding affinities of aminopyrine for two or more forms of cytochrome P450.

After PB administration, only one V_{max} and one K_m for aminopyrine occur [25], an observation confirmed by our data. Unchanged K_m for aminopyrine from day 4 to 59 of PB administration (Table 3) strongly suggests that reductions from peak aminopyrine N-demethylase activity during this period result from V_{max} alterations. In accord with this expectation, V_{max} decreased by 43 per cent from day 28 to 59 of PB administration (Table 3). Thus, our kinetic data on V_{max} and K_m of aminopyrine n-demethylase activity during chronic PB administration agree with the results of the experiment shown in Fig. 2 and Table 2.

Aminopyrine serves both in rat and man as a model compound to assess hepatic MFO activity [10, 18–20]. In the rat, aminopyrine undergoes hydroxylation and glucuronidation in addition to N-demethylation. However, since 50–70 per cent of radioactive label was recovered as $^{14}CO_2$ in exhaled breath of rats after [^{14}C]aminopyrine administration [8, 10], N-demethylation appears to be the major pathway for aminopyrine metabolism in the rat. Nevertheless, our studies did not provide such recovery data during chronic PB administration, thereby preventing us from concluding that during chronic PB administration N-demethylation remains the major pathway for aminopyrine metabolism.

$^{14}CO_2$ excretion in breath closely correlated with hepatic aminopyrine N-demethylase activities *in vitro* using 0.3 mM, but not 3.0 mM, aminopyrine concentrations (Figs. 2 and 3). Our *in vivo* data on $^{14}CO_2$ excretion in breath during chronic PB administration agree with *in vitro* data on aminopyrine N-demethylase activity in liver microsomes using 0.3 mM aminopyrine. Specifically, during chronic PB administration the course of both *in vitro* and *in vivo* systems is characterized initially by a rise extending up to 1 or 2 weeks followed thereafter by a steady decline to day 59. In normal human subjects approximately 20 per cent of usual therapeutic doses of aminopyrine are bound to albumin and the rest is evenly distributed in total body water [27, 28]. Because rats received aminopyrine in a dose of 120 mg/kg, aminopyrine concentrations below those necessary for saturation of aminopyrine N-demethylase had to be achieved *in vivo*. Under these conditions the highest aminopyrine concentration that this dose could have produced *in vivo* was 0.5 mM; therefore, 0.3 mM was selected as the concentration for the *in vitro* studies of enzyme activity because 0.3 mM is much closer than 3.0 mM to aminopyrine concentrations existing *in vivo* after usual aminopyrine doses.

Since body weight of rats receiving either saline or PB chronically was not significantly different (Fig. 1), decreases from peak induced hepatic MFO activities appear not to have resulted from PB-associated sedation, with consequent suppression of appetite and reduction in food intake and hepatic protein synthesis. In addition, in rats receiving PB chronically, microsomal protein/g of liver weight and liver weights were significantly higher than in saline controls; furthermore, peak induced values were maintained throughout the course of PB administration (Table 1).

Whatever mechanisms are responsible for differential effects of chronic PB administration on hepatic MFO, these observations are of interest for several reasons: they suggest that (1) enhancement of MFO activity does not continue indefinitely, but reaches a level (plateau) not increased even by continued administration of the inducing agent at the same dose; and (2) heterogeneity of response occurs, characterized by decreased MFO activity from peak induced levels for some substrates but by maintenance of peak induced levels for others.

Acknowledgements—We wish to thank Dr. Arthur Abt for kindly preparing the electron micrographs. We also express our gratitude to Dr. Frank E. Greene for his helpful suggestions, and to Mrs. Eileen Drust for typing this manuscript.

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