Biochemical Pharmacology, Vol. 45, No. 2, pp. 521–526, 1993.
Printed in Great Britain.

0006-2952/93 $6.00 + 0.00
Pergamon Press Ltd

Comparative pharmacodynamics of CYP2B induction by phenobarbital in the male and female F344/NCr rat

(*Received* 13 *July* 1992; *accepted* 2 *October* 1992)

Abstract—The phenobarbital dose–CYP2B induction response relationships and pharmacodynamics of CYP2B induction have been characterized in female and male F344/NCr rats. The ED_{50} and EC_{50} values for the induction, by phenobarbital, of hepatic CYP2B1 or 2B1/2B2 protein or associated catalytic activities (benzyloxy- or pentoxyresorufin O-dealkylation or testosterone 16β-hydroxylation) were 2- to 7-fold higher in the female than in the male rat, indicating a somewhat decreased potency for this effect in the female rat. In contrast, the maximal induction, expressed as the ratio of induced activity to control activity, was as great or greater in the female rat than in the male. Thus, any difference in the responsiveness of female rats to hepatic CYP2B induction by phenobarbital, compared to male rats, is reflected in potency but not degree of induction of catalytic activity or immunoreactive protein.

There exist marked gender-related differences in constitutive P450-mediated catalytic activity in the rat, with the male exhibiting 2.5- to 21-fold higher basal activity than the female for many substrates [1–10]. These differences in basal activity are reflected by differences in both V_{max} and K_m for such substrates as hexobarbital, aminopyrine and ethylmorphine [3–6], while kinetic parameters for aniline hydroxylation apparently are more similar between the sexes [3, 4]. It is now recognized that these differences in constitutive activities reflect both differences in sex-specific, non-inducible forms of P450 [1, 11], as well as altered constitutive levels of the major phenobarbital (PB*)- and steroid-inducible P450s (isozymes of the CYP2B and CYP3A subfamilies, respectively) [11]. For instance, the basal rates of CYP2B-mediated 16β-hydroxylation of testosterone or O-dealkylation of benzyloxyresorufin (BZR) or pentoxyresorufin (PTR) are 1.6- to 5-fold greater in the male than in the female rat [9, 10]. Such differences in constitutive P450 levels among the two sexes appear to be unique to the rat, and with the exception of the albino ferret [12], are rarely observed among the rodents and other mammalian orders [1, 11]. In addition to this well-characterized difference in constitutive CYP2B activity, certain previous studies have also suggested that the male rat displays a greater responsiveness to PB in the *induction* of CYP2B [6, 9, 13]. In the present study, we have employed a number of relatively sensitive endpoints in an examination of the relative responsiveness of female and male F344/NCr rats to PB, and have described for the first time the pharmacodynamics of this effect.

Materials and Methods

Drugs and chemicals. Phenobarbital and testosterone were purchased from the Sigma Chemical Co., St. Louis, MO, and 7-pentoxy- and 7-benzyloxyresorufin were purchased from Molecular Probes, Inc., Eugene, OR. Dicumarol and resorufin were purchased from the Aldrich Chemical Co., Milwaukee, WI, and fluorescamine was from the Fluka Chemical Corp., Ronkonkoma, NY. *p*-Nitro blue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate, *p*-toluidine salt were obtained from Bio-Rad Laboratories, Richmond, CA.

Animal treatment and preparation of samples. Male and female F344/NCr rats were obtained at 6 weeks of age from the Animal Production Area, Frederick Cancer Research and Development Center, and were randomized by body weight at 8 weeks of age into groups of three rats each. The rats were maintained on hardwood chips at 68–72° and ~50% humidity in an American Association for Laboratory Animal Care-certified laboratory. The various groups received either control diet (Purina Lab Chow No. 5010) or diet containing PB at 6.17, 18.5, 55.6, 167, 500, or 1500 ppm. After 14 days on test diet, individual body weights were recorded, and blood samples were obtained from individual rats by cardiac puncture for the determination of serum PB concentration. The rats were then killed by CO_2 asphyxiation, and their livers were removed *in toto*, trimmed free of extraneous tissue, and weighed. The livers were rinsed repeatedly in 0.15 M potassium chloride/0.2 M sucrose (4°) until the rinse fluids were devoid of blood, and then were homogenized in ~3 mL/g wet liver weight of the same solution with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, NY). Post-mitochondrial supernatants (S-9s), resulting from sequential 2000 *g* and 9000 *g* centrifugations, were aliquoted into 1/2 dram glass vials and were stored at −70° prior to use in enzyme assays. Microsomal pellets were obtained following centrifugation of individual S-9s (105,000 *g* for 75 min) and were resuspended in 0.05 M phosphate buffer, pH 7.5, containing 0.15 M KCl/0.2 M sucrose and recentrifuged. Protein content in the S-9 and microsomal samples was determined using fluorescamine [14] or the method of Bradford [15], respectively, with bovine serum albumin as the standard.

PB analysis in serum samples. Prior to analysis, serum samples (300 μL) were extracted with 4 mL ethyl acetate, shaken for 10 min, and then centrifuged (10 min, 2500 rpm). The ethyl acetate layer was transferred to 15 or 25 mL volume round bottom flasks. A second extraction with ethyl acetate was performed, and the ethyl acetate layers were combined in the round bottom flasks and evaporated to dryness under nitrogen. The serum extracts were reconstituted in 300 μL methanol and subjected to reversed-phase high performance liquid chromatographic analysis. A C_{18} column (25 cm × 4.6 mm, 5 μm, Burdick & Jackson, Muskegon, MI) was eluted under isocratic conditions with 20% acetonitrile/80% dibasic sodium phosphate buffer (0.01 M, pH 8.0) at a flow rate of 1 mL/min. The integrated absorbance (240 nm) peak areas were compared to standards prepared by the addition of known amounts of PB to rat serum. Standards were extracted in parallel with the test sera.

Assay of catalytic activity for alkoxyresorufins and testosterone. The O-dealkylation of pentoxy- and ben-

* Abbreviations: BZR, benzyloxyresorufin; CYP2B1, cytochrome P450 isozyme 2B1; CYP3A1, cytochrome P450 isozyme 3A1; PB, phenobarbital; PTR, pentoxyresorufin; S-9, post-mitochondrial supernatant; and TCPOBOP, 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene.

zyloxyresorufin by hepatic S-9 samples was measured as described previously [16]. The final concentration used for each substrate was 5 μM, the S-9 concentrations used ranged from 0.25 to 1.3 mg protein/mL reaction mixture, and reaction rates were determined at ~25° using a Perkin–Elmer LS-50 spectrophotofluorimeter (excitation λ = 522 nm, emission λ = 586 nm). Testosterone 16β-hydroxylase activity was determined in microsomal subfractions with the HPLC method of Sonderfan *et al.* [17] as described in detail previously [16]. In the present studies, the substrate concentration employed was 250 μM, and the incubation time was 4 min.

Immunodetection of CYP2B protein. Microsomal protein (1.0, 0.33 or 0.11 μg/20 μL) from the various treatment groups was blotted directly onto nitrocellulose (0.45 μm pore size), using the Minifold II apparatus (Schleicher & Schuell, Inc., Keene, NH). The slot blots were immersed in a blocking solution consisting of a 10% (w/v) solution of Carnation® non-fat dry milk in Tris-buffered saline (20 mM Tris, 500 mM sodium chloride, pH 7.5; TBS) for 1 hr at ambient temperature. Following this, the blots were rinsed three times in TBS containing 0.05% Tween-20 (T/TBS), 10 min/rinse. Membranes were then incubated with specific primary antibody (B50 or BE28, mouse monoclonal antibodies which detect rat CYP2B1 or CYP2B1/2B2, respectively [18]) diluted 1:125 in T/TBS containing 1% gelatin. After overnight incubation at ambient temperature, unbound primary antibody was removed by rinsing the membranes three times in T/TBS, 10 min/rinse. The membranes were then placed in buffer containing the secondary antibody, goat anti-mouse IgG conjugated to alkaline phosphatase (GIBCO BRL, Gaithersburg, MD; 1:1000 dilution). After a 1-hr incubation at ambient temperature, unbound secondary antibody was removed by rinsing the membranes three times in T/TBS, 10 min/rinse, followed by a single rinse in TBS to remove excess Tween-20. The membranes were then immersed in alkaline phosphatase color development solution (*p*-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate *p*-toluidine salt in sodium bicarbonate buffer, pH 9.8). The blots were scanned with an LKB Ultroscan XL densitometer and the band intensities were compared between PB-treated and control groups.

Analysis of data. Group mean liver/body weight ratios, pentoxy- and benzyloxyresorufin O-dealkylation values, testosterone 16β-hydroxylation values, and densitometric readings from protein slot-blot analyses were transformed to percentage of maximal response by calculating the absolute increase over the control (no drug) value, then expressing the increases obtained as a percentage of the maximum increases obtained in the series of PB doses. These values were then plotted versus nominal dietary PB concentration or versus the group mean serum total PB concentration and the resulting data were fitted to the sigmoid E_{max} equation [19]:

$$E = \frac{E_{max} \cdot C^N}{EC_{50}^N + C^N}$$

with curve-fitting software (TableCurve®, Jandel Scientific, Corte Madera, CA). In this equation, E is in units of percentage of maximal effect, E_{max} is the maximal response obtained, C is the concentration of drug in diet or serum, EC_{50} (ED_{50}) represents the drug concentration in serum (or in the diet) associated with a half-maximal response, and N is a curve shape factor. In cases for which complete dose or concentration–response curves were obtained, the ED_{50} and EC_{50} values were calculated, along with the SE, by the computer program. In cases where the response curves were truncated (missing the top plateau region), the ED_{50} and EC_{50} values were expressed as "≥ a given dose–concentration", obtained by evaluating the E_{max} equation for the value of C at E = 50%.

Results and Discussion

Basal activities. Basal levels of BZR- and PTR O-dealkylation activities, which have been shown to be mediated preferentially by isozymes of the CYP2B subfamily in the rat [20, 21], were 3- to 5-fold higher in the male than in the female rat (Table 1). This gender difference in constitutive O-dealkylation activity was similar in magnitude to that previously detected [9, 10]. The difference in constitutive testosterone 16β-hydroxylation activity (also mediated preferentially by isozymes of the CYP2B subfamily [16, 22]) observed between the male and female rats was more profound (~10-fold). Other investigators have observed that the male/female differences in the basal level of this activity range from ~4-fold [17] to <2-fold [9]. The discrepancy in the observed degree of difference between the basal testosterone 16β-hydroxylation activities in male and female rats may be due in part to the relatively low basal levels of testosterone 16β-hydroxylase, especially in the female rat.

Induction of CYP2B catalytic activities. Following treatment with various dietary concentrations of PB, marked induction of each of the hepatic CYP2B-mediated catalytic activities was exhibited in both male and female rats (Table 1). The maximal alkoxyresorufin metabolism rates achieved in male rats were 8–9% greater than in female rats, while the maximal value for testosterone 16β-hydroxylase was ~38% greater in the male than in the female. However, when calculated as a ratio of induced/basal activity, the maximal responses exhibited in the female rat were greater for each substrate examined than in the male rat. This finding, which was in large part a reflection of the lower constitutive activities present in the female rat, has been observed previously for BZR O-dealkylation activity [10], for testosterone 16β-hydroxylation activity [11, 17, 23] and for other activities as well (e.g. alkoxycoumarin O-dealkylation [2], ethylmorphine N-demethylation [7], hexobarbital hydroxylation [7], androstenedione 16β-hydroxylation [11] and aminopyrine N-demethylation [23]). Other investigators have found the increases in ethylmorphine N-demethylation activity following administration of PB to be nearly equal among the two sexes in various strains of rats [3]. In addition, at least two reports have described a greater relative induction by PB of alkoxyresorufin or testosterone metabolism in male rats compared to female rats [9, 24]. These studies have, for the most part, involved administration of a single dose level of PB. In the present study, the use of a series of dietary concentrations (6.17 to 1500 ppm) enabled the determination of no-effect levels, the maximal responses, and a determination of the potency of PB for CYP2B induction in each sex. The ED_{50} values for induction of BZR and PTR O-dealkylation and testosterone 16β-hydroxylation activities were calculated, using curve-fitting software, to be ≥240, ≥470 and ≥350 ppm, respectively, for female rats, and 140, 140 and 100 ppm, respectively, for male rats (Table 2). Thus, the difference in apparent responsiveness of the two sexes of rats was manifested by a 2- to 3-fold shift to the right of the dose–induction response curves (not shown), while the maximal induction responses were actually somewhat greater in the female than in the male rat (Table 1). In a previous dose–response study by Tavoloni *et al.* [25] involving male Sprague–Dawley rats, the ED_{50} for induction of total cytochrome P450 was ~11 mg PB/kg body wt/day (given as daily i.p. injections for 6 days). This daily dose level is approximately equivalent to the dose received by rats exposed to a diet containing 110 ppm PB. In a dose–response study involving female Sprague–Dawley rats in which the sodium salt of PB was administered as daily i.p. injections for 3 days, Poland *et al.* [26] reported that the ED_{50} for the induction of aminopyrine N-demethylation activity (generally considered to be mediated preferentially by isozymes of the CYP2B subfamily) was 0.67×10^{-4} mol/kg body wt/day

Table 1. Effect of various dietary concentrations of phenobarbital on CYP2B-mediated catalytic activities in the female and male F344/NCr rat

Dietary PB concentration* (ppm)	BZR O-dealkylation activity (pmol/min/mg S-9 protein)		PTR O-dealkylation activity (pmol/min/mg S-9 protein)		Testosterone 16β-hydroxylation activity (pmol/min/mg microsomal protein)	
	Female rats	Male rats	Female rats	Male rats	Female rats	Male rats
0	4.5 ± 1.3†	24 ± 5	3.0 ± 0.4	11 ± 2	2.0 ± 1.4	31 ± 1
6.17	6.4 ± 1.9 (1.4)	28 ± 2 (1.2)	4.7 ± 0.4‡ (1.6)	13 ± 1 (1.2)	4.3 ± 2.1‡ (2.2)	54 ± 18‡ (1.7)
18.5	13 ± 3‡ (2.9)	65 ± 33‡ (2.7)	9.0 ± 2.7‡ (3.0)	31 ± 10‡ (2.8)	39 ± 9‡ (20)	177 ± 66‡ (5.7)
55.6	156 ± 119‡ (35)	345 ± 34‡ (14)	58 ± 34‡ (19)	112 ± 8‡ (10)	257 ± 20‡ (129)	1176 ± 162‡ (38)
167	559 ± 52‡ (124)	745 ± 126‡ (31)	185 ± 31‡ (62)	373 ± 46‡ (34)	943 ± 114‡ (472)	2445 ± 393‡ (79)
500	788 ± 139‡ (175)	1264 ± 24‡ (53)	288 ± 12‡ (96)	661 ± 69‡ (60)	1549 ± 382‡ (775)	2894 ± 916‡ (93)
1500	1193 ± 179‡ (265)	1289 ± 63‡ (54)	590 ± 130‡ (197)	641 ± 50‡ (58)	2641 ± 111‡ (1321)	3661 ± 710‡ (118)

* Rats were exposed to phenobarbital or control diet for 14 days.
† Values are means ± SD for three F344/NCr rats per treatment (values in parentheses represent the ratio of activity in treated rats to that in the controls).
‡ Significantly greater than value for rats receiving control diet, $P < 0.05$, Mann–Whitney *U*-test.

(17 mg/kg/day), which corresponds roughly to a dietary dose of 170 ppm.

Induction of immunoreactive CYP2B protein. The induction of immunoreactive CYP2B1 protein (detected with monoclonal antibody B50 [18], Fig. 1) in female and male rats fed various concentrations of PB was dose-responsive, displaying ED_{50} values of 79 and 53 ppm, respectively (Table 2). Similar ED_{50} values (84 and 46 ppm, for female and male rats, respectively) were obtained for immunoreactive CYP2B1/2B2 (detected with monoclonal antibody BE28 [18], Table 2). The ED_{50} values for the induction of immunoreactive protein in male rats were 2- to 3-fold lower than those obtained with the catalytic endpoints, while in female rats this difference in ED_{50} values ranged from 3- to 6-fold (Table 2). This may be a reflection of the higher degree of specificity of the immunochemical detection technique relative to the catalytic endpoints, or may imply the induction of immunoreactive, albeit catalytically inactive CYP2B protein by PB, especially in the female rats. The induction of CYP2B1 or CYP2B1/2B2 protein was evident at lower PB doses in male rats than in females, and the dose–response curves for the females were shifted ~1.5-fold to the right relative to the response in the males. In terms of the maximal increases observed (calculated based on densitometry), the responses displayed by female rats were as great or greater than those in males (CYP2B1, 28-fold vs 26-fold; CYP2B1/2B2, 14-fold vs 8-fold).

Increase in liver/body weight ratio. Based upon the study involving male Sprague–Dawley rats described by Tavoloni *et al.* [25], the ED_{50} for the increase in liver/body weight ratio (a component of the PB-type pleiotropic response in the rat [10]) was estimated to be ≥30 mg PB/kg body wt/day. This daily dose level is approximately equivalent to the dose received by rats exposed to a diet containing 300 ppm PB. In the present study, the ED_{50} values for the increase in liver/body weight ratio were ≥610 and 340 ppm, respectively, for female and male rats (Table 2). Thus, while the potency of PB in inducing this effect is lower than for the induction of CYP2B protein or associated catalytic activities, the dose–response curve for the increases observed in liver/body weight ratio also displayed an ~2-fold shift to the right in the case of the female rats. However, in this case the maximal responses (~1.35-fold increase in liver/body weight ratio) were approximately equivalent in the two sexes of rat.

Pharmacodynamics of CYP2B induction. Steady-state serum total (free plus protein bound) PB concentration displayed a linear concordance with nominal dietary PB concentration in female and male rats, although the serum levels attained at any given dietary PB concentration were greater in the female rat than in the male (Fig. 2). Due to this, when CYP2B-mediated catalytic activities were related to serum total PB concentration, the resulting concentration–induction response curves for the female rat were shifted even further (5- to 7-fold) to the right. Thus, the EC_{50} values for induction of BZR and PTR O-dealkylation activities and for testosterone 16β-hydroxylation activity were calculated to be 46, ≥77 and ≥58 μM, respectively, for female rats, compared to 9.8, 10 and 8.8 μM, respectively, for male rats (Table 2). The EC_{50} values for the induction of immunoreactive protein in the female and male rats were 16 and 6.9 μM PB, respectively, for CYP2B1, and 17 and 5.6 μM PB, respectively, for CYP2B1/2B2. The corresponding values for the increase in liver/body weight ratio in the female and male rats were ≥97 and 27 μM PB, respectively (Table 2).

The EC_{50} values for the *in vivo* induction of rat hepatic CYP2B isozymes by PB have not been published previously. In fact, Pei and coworkers [27] reported that there was no significant correlation between the percent increase in antipyrine clearance and steady-state plasma PB concentration in male Sprague–Dawley rats. Kocarek *et al.*

Table 2. Pharmacodynamics of phenobarbital (PB)-type induction in the female and male F344/NCr rat

Endpoint	ED_{50} (dietary ppm PB)		EC_{50} (μM PB in serum)	
	Female rats	Male rats	Female rats	Male rats
Benzyloxyresorufin O-dealkylation	≥240*	140 ± 17	46 ± 10	9.8 ± 0.1
Pentoxyresorufin O-dealkylation	≥470	140 ± 13	≥77	10 ± 0.2
Testosterone 16β-hydroxylation	≥350	100 ± 21	≥58	8.8 ± 0.2
Immunoreactive CYP2B1 protein	79 ± 6	53 ± 2	16 ± 1	6.9 ± 0.4
Immunoreactive CYP2B1/2B2 protein	84 ± 2	46 ± 2	17 ± 0	5.6 ± 0.7
Liver/body weight ratio increase	≥610	340 ± 33	≥97	27 ± 1.7

* Values given were obtained by means of TableCurve® software and are shown ± SE. Values given as "≥" were taken from truncated concentration–response curves (without a complete plateau region), and were obtained by solving the sigmoid E_{max} equation for an E of 50% (50% of maximal response). All values have been rounded to two significant figures.

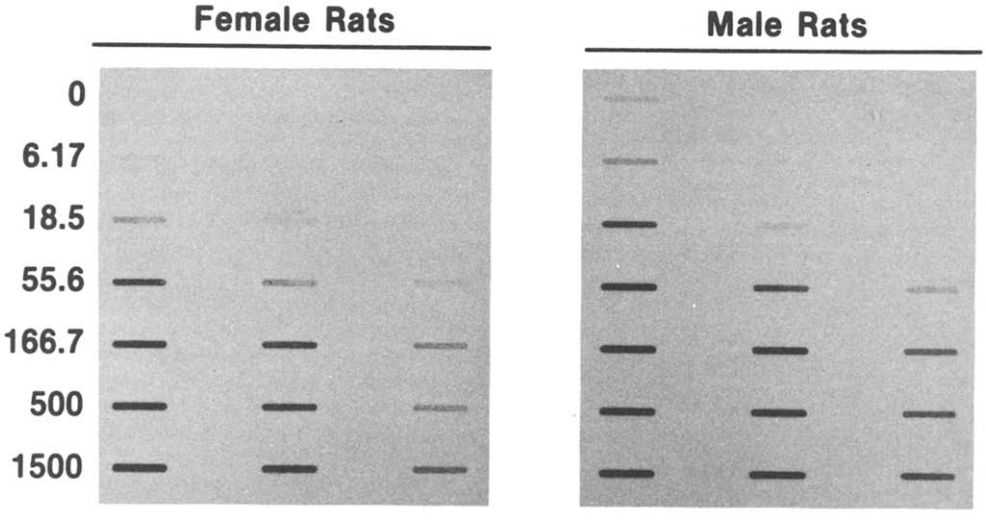

Fig. 1. Levels of immunoreactive CYP2B1 protein in female and male F344/NCr rats exposed for 14 days to the indicated dietary concentrations (in ppm) of phenobarbital. Slots in the three lanes of each panel were loaded with 1.0, 0.33 or 0.11 μg (left to right) hepatic microsomal protein pooled from three rats/treatment. The primary antibody employed was B50, a mouse monoclonal which detects CYP2B1 but does not cross-react with CYP2B2 [18].

[28] have reported recently that the ED_{50} values for the induction of CYP2B1 and CYP2B2-specific mRNAs in hepatocyte cultures derived from adult male Sprague–Dawley rats are 15 and 5.7 μM PB, respectively. These values are in good agreement with the EC_{50} values obtained in the present study for induction of CYP2B-mediated catalytic activity or immunoreactive CYP2B1 or CYP2B1/2B2 protein. It appears, therefore, that serum total PB concentration is a valid approximation of the PB concentration in the extracellular fluid surrounding the hepatocyte *in vivo*.

In summary, any differential which exists in the hepatic CYP2B induction response following PB administration to male and female rats would appear to be limited to potency, rather than efficacy, of the drug in eliciting this response. The decreased potency of PB in the female rat is demonstrated by a 2- to 3-fold shift to the right in the ED_{50} values and a 5- to 7-fold shift to the right in EC_{50} values. The magnitude of this difference in potency between the two sexes, like the previously reported species differences in the induction response caused by 1,4-bis[2-(3,5-dichloropyridyloxy)]-benzene (TCPOBOP) [26], would appear to argue against a non-specific mechanism for CYP2B induction. Such differences might be explained by subtle alterations in the binding site of a putative induction receptor, or by changes in the number of available receptor sites between the different sexes or species. However, such differences are not proof of the existence of receptor mediation in CYP2B induction. The efficacy of the drug in terms of CYP2B induction, as reflected in the maximal induction ratios obtained, would appear to be at least as great or greater in the female rat than in the male. The *in vivo* pharmacodynamic values obtained in the present studies with the male rat are in good agreement with previously published values for the induction of CYP2B-specific mRNAs by PB in cultured adult male rat hepatocytes.

Acknowledgements—The authors would like to acknowl-

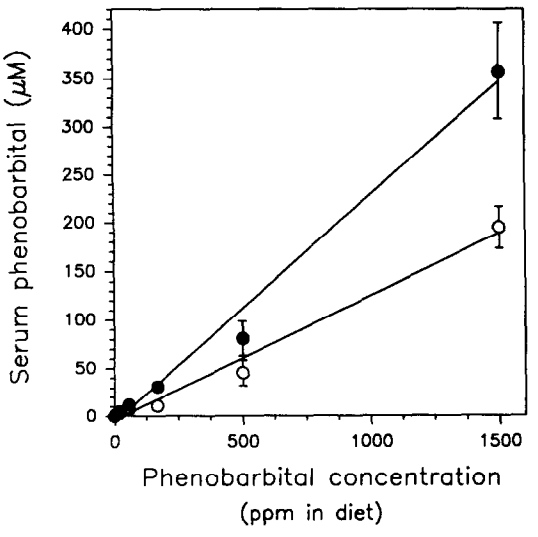

Fig. 2. Relationship between dietary phenobarbital concentration and steady-state serum total (free plus bound) phenobarbital concentration in female (●) and male (○) F344/NCr rats. Each point represents the mean value for three rats, and the error bars indicate the SD.

edge the skilled technical assistance of Dan Logsdon, Craig Driver, Lisa Riffle and Gwen Magruder. This project has been funded at least in part with Federal funds from the Department of Health and Human Services under Contract Number N01-CO-74102. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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