Effect of oral administration of low doses of pentobarbital on the induction of cytochrome P450 isoforms and cytochrome P450-mediated reactions in immature Beagles

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Objective—To determine the effect of oral administration of low doses of pentobarbital on cytochrome P450 (CYP) isoforms and CYP-mediated reactions in immature Beagles.

Animals—42 immature (12-week-old) Beagles.

Procedure—Dogs were grouped and treated orally as follows for 8 weeks: low-dose pentobarbital (50 µg/d; 4 males, 4 females), mid-dose pentobarbital (150 µg/d; 4 males, 4 females), high-dose pentobarbital (500 µg/d; 4 males, 4 females), positive-pentobarbital control (10 mg/kg/d; 2 males, 2 females), positive-phenobarbital control (10 mg/kg/d; 2 males, 2 females), and negative control (saline [0.9% NaCl] solution; 5 males, 5 females). Serum biochemical and hematologic values were monitored. On necropsy examination, organ weights were determined, and histologic evaluation of tissue sections of liver, kidney, small intestine, testes, epididymis, and ovaries was performed. Hepatic and intestinal drug-metabolizing enzyme activities were measured, and relative amounts of CYP isoforms were determined by western blot analysis.

Results—The amount of a hepatic CYP2A-related isoform in dogs from the high-dose pentobarbital treatment group was twice that of dogs from the negative control group. CYP2C was not detectable in small intestinal mucosa of dogs from the negative control group; measurable amounts of CYP2C were found in dogs from the various (low-, mid-, and high-dose) pentobarbital treatment groups and from positive-pentobarbital and positive phenobarbital control groups. Several CYP-mediated reactions increased in a dose-dependent manner. The lowest calculated effective dose of pentobarbital ranged from 200 to 450 µg/d.

Conclusions and Clinical Relevance—Several CYP isoforms and their associated reactions were induced in dogs by oral administration of low amounts of pentobarbital. (*Am J Vet Res* 2003;64:1167–1175)

According to anecdotal reports from veterinarians during the 1990s, pentobarbital appeared to be losing its effectiveness as an anesthetizing agent in dogs. It was speculated that pentobarbital was present as a contaminant in dog food, and that this was altering the physiologic response to pentobarbital-induced anesthesia. Pentobarbital is used as an euthanatizing agent by veterinarians and animal shelter employees throughout the United States.[1] Until recently, carcasses from these euthanatized animals were disposed of by rendering.[2] In a study designed to develop an analytic method for the determination of pentobarbital residues in dog food, several lots of commercial dog food were found to contain confirmable concentrations (10 to 60 ppb) of pentobarbital.[3] On the basis of these and other results,[4] a limited study was initiated to assess the potential effects of low degrees of barbiturate exposure in dogs.

Amacher et al[5] showed that induction of microsomal enzyme activities was associated with exposure to many drug types without concomitant increases in liver weights or histopathologic findings or changes in serum biochemical values that would be indicative of hepatocellular or hepatobiliary damage. In an unrelated study, Nims et al[6] showed that low concentrations (< 1 ppm) of dietary **1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP)** induced phenobarbital-inducible **cytochrome P450 (CYP)** isoforms. On the basis of these reports,[5,6] and because overt toxicologic effects were not expected, our hypothesis was that oral administration of pentobarbital might result in an increase in its own metabolism as an effect of induced drug-metabolizing enzymes. As such, induction of hepatic and intestinal drug-metabolizing enzymes might be a sensitive indicator of a physiologic response to orally administered barbiturates. Therefore, the purpose of the study reported here was to determine the effect of low doses of pentobarbital on CYP isoforms and CYP-mediated reactions in immature Beagles.

Besides the usual items, such as body and organ weights, hematologic and serum biochemical analyses of peripheral blood samples, and histologic evaluation of potentially affected tissues (liver, kidneys, small intestine, ovaries, testes, and epididymis), the induction of drug-metabolizing enzyme activities in liver and small intestinal mucosa were also measured. To this end, metabolism of several high-throughput

Received November 26, 2002.
Accepted March 3, 2003.
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Presented in part at the 11th North American International Society for the Study of Xenobiotics Meeting, Orlando, Fla, October 2002.
The authors thank Dr. Richard Cullison for clinical assistance and David Heller and Vicki Cui for analysis of the dog food.
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screening substrates were included that are associated with barbiturate-inducible CYP isoforms as follows: **coumarin hydroxylase (COH)** for evaluation of CYP2A; **pentoxy resorufin O-dealkylase (PRF)**, **benzyloxy resorufin O-dealkylase (BRF)**, **7-ethoxy-4-methylcoumarin O-deethylase (ETOMC)**, **7-ethoxy-4-trifluoromethylcoumarin O-deethylase (ETOFMC)**, and **aminopyrine N-demethylase (AMP)** for evaluation of CYP2B; **dibenzylfluorescein O-dealkylase (DBF)**, **7-methoxy-4-methylcoumarin O-demethylase (MOMC)**, **7-methoxy-4-trifluoromethylcoumarin O-demethylase (MOFMC)**, and **7-benzyloxy-4-trifluoromethylcoumarin O-dealkylase (BZOFMC)** for evaluation of CYP2C, and **erythromycin N-demethylase (ERY)**, BZOFMC, **benzyloxyquinoline O-dealkylase (BZQ)**, and AMP for evaluation of CYP3A.[7-11] Western blot analysis was also used to measure the relative concentrations of barbiturate-inducible CYP isoforms, namely CYP2A, CYP2B, CYP2C, and CYP3A. In addition to these isoforms, CYP2D and CYP2E were measured, which were not expected to be affected by the barbiturate treatment.

Materials and Methods

Materials—Pentobarbital sodium salt[a] served as a test compound and a positive control compound. Phenobarbital sodium salt[b] also served as a positive control compound. Physiologic saline (0.9% NaCl) solution was used as the negative control agent and diluent for the test and positive control compounds. The following chemicals were of reagent grade and also obtained from 1 supplier:[c] NADPH, NADP, NADH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione, **3,4-dichloronitrobenzene (DCNB)**, **2,4-dinitrochlorobenzene (DNCB)**, resorufin, coumarin, erythromycin, aminopyrine, formaldehyde, 4-methyl-umbelliferone, 7-ethoxy-4-methylcoumarin, 7-methoxy-4-methylcoumarin, and 7-hydroxycoumarin. A second company[d] supplied the dibenzylfluorescein, 7-benzyloxyquinoline, 7-hydroxyquinoline, and 7-benzyloxy-, 7-ethoxy-, and 7-methoxy-4-trifluoromethylcoumarins, in addition to the 4-trifluoromethyl umbelliferone. A third company[e] supplied 7-benzyloxyresorufin and fluorescein. In addition to the antibody preparations and CYP isoform standards that were previously reported in detail,[12] the second company[d] also supplied polyclonal goat antirat 2C6 and rat CYP2C6. Bicinchoninic acid reagents for protein determinations and bovine serum albumin protein standard were obtained from another supplier.[f]

Dogs and animal husbandry—This study was conducted in accordance with the US Food and Drug Administration *Good Laboratory Practice Regulations for Nonclinical Laboratory Studies*,[13] along with the *Guide for the Care and Use of Laboratory Animals*[14] under a protocol approved by the FDA and the Office of Research Institutional Animal Care and Use Committee. Forty-two 8-week-old Beagles (21 males and 21 females) were obtained from a commercial breeding facility.[g] Prior to being shipped, dogs were vaccinated and received prophylactic treatments for intestinal parasites. Dogs were housed in pairs in 1.2 × 3.1 m runs (2 runs contained 3 control dogs of each sex) in a climate controlled (24°C with a relative humidity of 70 to 75%) certified animal facility with 12 hour light-dark cycles. Dogs were identified by ear tattoo and assigned to each treatment group by randomization for body weight by use of a computer software program. Dogs were acclimated and trained to accept the handling and treatment regimen for 4 weeks prior to study initiation. Dogs were fed pelleted dog food.[h] During the course of the study, 2 lots of feed were used, and each lot was tested to ensure that it contained no detectable pentobarbital. Dogs were fed at 6% of the mean body weight of all dogs. The ration was divided into 3 portions with feeding times being at 7:30 AM and 12:00 and 4:00 PM. Water was administered in bowls and changed at each feeding; feed and water consumption was not recorded. Dogs were moved to clean pens for each feeding. During the 12 PM feeding, dogs from several pens were allowed approximately 10 minutes of a free run and playtime in the center aisle; this was continued throughout the course of the study. All dogs were weighed each Friday morning. On the basis of this body weight, feed amounts were adjusted, and the new feeding amounts were started on the following Monday morning. This body weight was also used to adjust the dosage of pentobarbital and phenobarbital.

Treatments—On the basis of estimated daily food consumption and the amounts of reported pentobarbital residues in dog food,[3] a maximal exposure of < 50 µg of pentobarbital/d was calculated. Therefore, 50 µg of pentobarbital/d was chosen as the low-dose pentobarbital treatment with 150 and 500 µg/d as the mid- and high-dose pentobarbital treatments, respectively. Because the doses were low, positive control group dogs were included that received either pentobarbital or phenobarbital at 10 mg/kg/d. The 42 dogs were placed in the various groups as follows: 8 low-dose pentobarbital treatment group dogs (4 males and 4 females), 8 mid-dose pentobarbital treatment group dogs (4 males and 4 females), 8 high-dose pentobarbital treatment group dogs (4 males and 4 females), 4 positive-pentobarbital control group dogs (2 males and 2 females), 4 positive-phenobarbital control group dogs (2 males and 2 females), and 10 negative (saline [0.9% NaCl] solution) control group dogs (5 males and 5 females).

All pentobarbital and phenobarbital solutions were prepared each morning. For the low pentobarbital doses, a 5% solution of pentobarbital was diluted to 0.05, 0.15, and 0.500 mg/mL with physiologic saline (0.9% NaCl) solution; 1 mL of each dose was administered. One milliliter of physiologic saline solution was administered to the negative control group dogs. The positive pentobarbital and phenobarbital control group dogs were treated with 5% solutions; the volumes administered ranged from 1 to 2 mL. All treatments were administered orally by squirting solutions into the mouth by use of 3 mL syringes.

Blood samples for hematologic evaluation and serum biochemical analysis were collected on the day after arrival, after 4 weeks of treatment, and during the eighth week; a contract laboratory[i] performed these analyses. Dogs were necropsied starting on Monday of the ninth week after the start of dose administration. Food was withheld from dogs for at least 15 hours prior to euthanasia; dogs were weighed just before being transported to the necropsy facility. The order of necropsy proceeded from the negative control dogs to the highest pentobarbital treatment group dogs with each group of dogs necropsied on a separate day; the 2 groups of positive control dogs were necropsied on the last day of the week. Dogs were euthanatized by heavy sedation with pentobarbital[j] (45 mg/kg, IV), followed by exsanguination from the carotid arteries and jugular veins. Tissues (liver, kidneys, small intestine, both ovaries, both testes, and epididymis) were removed, weighed, and sections (1 to 2 g of liver; left kidney cut into longitudinal sections and the right kidney cut into lateral sections; approximately 1-cm-thick section of the small intestine; 1 section each of the ovaries, testes, and epididymis) were put into neutral-buffered 10% formalin for histologic evaluation. Tissue specimens were sent to a contract laboratory for histologic processing[k] and evaluation.[l] The remainder of the small intestine was cut open longitudinally and either flushed with tap water or wiped with tissues[m]

to remove any residual ingesta or bile secretions; the mucosal lining was removed by scrapping with a microscope slide. After taking sections for histologic evaluation, the remainder of the liver (cut into a single 10- to 12-g piece and 5 to 6 other separate sections) and mucosal scrapings from an approximately 30-cm-long segment of the small intestine were put on ice until they were transferred to a -80°C freezer. The 10- to 12-g portions of the liver and all of the scrapings of the small intestinal mucosa were used to prepare subcellular fractions for analysis of drug metabolizing enzymes and western blot analysis.

Methods—Microsomes and cytosolic subfractions were prepared by differential centrifugation at 10,000 × *g* and 100,000 × *g* from 20% homogenates of liver tissue specimens or scrapings of the small intestinal mucosa. The supernatant derived from the ultracentrifugation step is the cytosolic fraction. Pellets derived from ultracentrifugation were resuspended and reprecipitated by use of 15% polyethylene glycol precipitation as previously described.[15] Microsomes were stored as pellets and overlaid with 1 mL of 0.1M potassium phosphate buffer (pH, 7.4). All subcellular fractions were stored in small aliquots at -80°C, so only enough material required on any given day was removed from storage.

The hepatic drug-metabolizing enzyme proteins were measured as described previously.[15] The concentration of CYP was determined from the CO-difference spectra of dithionite-reduced samples by use of an extinction coefficient of $91 mM^{-1} cm^{-1}$ between 450 and 490 nm.[16] Cytochrome b_5 was measured after reduction with NADH.[17] Cytochrome P450 reductase activity was determined by the NADPH-dependent reduction of cytochrome c.[18] Cytochrome b_5 reductase was determined by the NADH-dependent reduction of potassium ferricyanide.[19] Glutathione-S-transferase activities, by use of DNCB and DCNB as substrates, were performed as described previously.[15] All spectra and kinetic assays were determined by use of a microtiter plate reader.[n] Protein concentrations of all subcellular fractions were determined by use of the bicinchoninic acid procedure as described in the instructions provided by the manufacturer.[f]

The AMP, ERY, COH, **uridine diphosphoglucuronic acid (UDPGA)**-glucuronyl-transferase, and alkoxymethylcoumarins (ETOMC, MOMC, BZOFMC, ETOFMC, and MOFMC) assays, and the determination of alkoxyresorufin (BRF and PRF) O-dealkylase activities were performed as described previously[15,20] by use of 0.04 to 0.15 mg of microsome fractions from each individual dog in triplicate. Results of previous experiments indicated that these reactions were linear with respect to protein content and time.[15] The BZQ and DBF assays were performed under the same conditions, except that the stopping solutions and other substrate-product specific conditions were the same as that previously described.[10] Fluorometric and spectrophotometric assay measurements were performed with microtiter plate readers.[n,o] Fluorescence and absorbance units were converted to nanomoles or picomoles of product formed by use of concurrently generated external standard curves. Enzymatic activity is expressed as nmol or pmol of product formed/min/mg of microsomal protein, and results are reported as mean (± SD) values.

Western blot analyses—Hepatic and intestinal microsomes (10 and 40 µg of microsomal protein/lane, respectively) were subjected to western blot analysis as previously described[21] by use of modifications described for tissue specimens from swine.[12] Because of the number of samples, they were divided into 2 separate gels with 1 sample being repeated on both gels; this sample was used to normalize the data between the 2 gels. Authentic P450 standards, appropriate for each antibody, and molecular weight standards were included on each western blot. Developed western blots were scanned.[p] Areas were quantified as the volume of intensity of staining, and the **absorbance units (AU)** were reported as AU/0.01 mg of microsomes and AU/0.04 mg of microsomes for liver tissue and small intestinal mucosa, respectively. Values reported for these assays cannot be considered as quantitative numbers, because each antibody preparation provides a different response that can vary from gel to gel. Hence, the values reported are merely an attempt to produce a numeric value that is representative of the staining intensity measured by the scanner.

Statistical analysis—After a preliminary graphic evaluation, a 2-way ANOVA[q,r] was used to identify the effects of sex, treatment group, and treatment group by sex interaction. If the *P* value of the treatment group by sex interaction was < 0.05, the effect of treatment group was evaluated separately by sex. For purposes of tabulating all treatment group means and indicating the significance of differences among them, a Tukey adjustment for a family-wide error rate of 0.05 was used for comparisons among all pairs of treatment groups. For purposes of identifying variables that may have an increasing (or decreasing) response to dose, Dunnett tests were conducted against the negative control with a family-wide error rate of 0.1. These tests were constructed separately by sex as already indicated. Results were pooled across sex if the main effect of treatment group was identified at $\propto = 0.1$ and the treatment group by sex interaction were not significant. If the results of these comparisons suggested an increasing (or decreasing) response to dose, a linear regression method was used to evaluate whether a straight line adequately represented the response in the dose range of 0 to 500 µg/d. The need to include separate slope and intercept parameters for males and females was also evaluated. If an adequate fit to a straight line was observed, then the results were used to interpolate the lowest dose with a 95% confidence interval that did not overlap with the 95% confidence interval at 0 dose. This interpolated dose, if it existed, was termed the lowest effective dose. Significance was assigned when *P* values were < 0.05.

Results

Dogs in all treatment groups gained weight at a relatively constant rate, and consistent differences were found in body weight between males and females within each treatment group (data not shown). Although significant differences in body weights were found between females and males (8.92 ± 0.3 vs 10.40 ± 0.3 kg, respectively), no treatment-related differences were found.

The 3 treatment groups received a fixed amount of pentobarbital (50, 150, and 500 µg in 1 mL of solution/d). As a result of growth, the actual dosage (µg/kg) declined during the course of the treatment phase (data not shown). Differences in dosage across the treatment period for the low-dose pentobarbital treatment group were not significant, whereas that of the mid-dose pentobarbital treatment group for the last 3 weeks were significantly different from the initial calculated dose. For the high-dose pentobarbital treatment group, significant differences in dosage were found across the entire treatment period, although dosages between adjacent weeks were not significantly different from each other.

No indications of any obvious adverse effects were found for dogs in any of the treatment groups. In the positive-pentobarbital control group, females and males had ataxia of the hind limbs within 5 to 10 minutes after dose administration. This effect dissi-

pated within 1 hour. During the last week of treatment, this effect was hardly noticeable. One of the males in the low-dose pentobarbital treatment group developed a cough during the last 4 weeks of treatment, but no abnormalities were observed upon necropsy examination. Serum biochemical analysis and results of hematologic evaluation did not change over the course of the treatment period (data not shown). Several serum biochemical parameters (eg, alkaline phosphatase activity and calcium and phosphorus concentrations) were consistently out of the reference range, but this was attributed to the fact that the dogs in our study were immature, and the reference values are determined from data of adult dogs. On the basis of the serum biochemical analysis and results of hematologic evaluation, no indications of systemic adverse effects of treatment were found.

Organ weights and histologic evaluation—Except for differences in liver and small intestinal weights, no significant differences in organ weights were found (data not shown). No significant differences in the liver weights were found among any groups, but differences in liver weights were found between males and females (data not shown). Although a dose-dependent response in relative liver weight was found, which was confirmed by regression analysis (data not shown), no experimental dose within our study design produced a response that was significantly different from that of the negative control group. Histologic evaluation of tissues did not reveal any treatment-related changes.[5]

Phase II enzyme activities—The DNCB- and DCNB-glutathione transferase activities (nmol/min/mg of cytosolic protein) in the small intestine were considerably lower than that measured in the liver (DNCB, 282 ± 76 vs 1,200 ± 225 nmol/min/mg of cytosolic protein; DCNB, 2.5 ± 1.2 vs 17.5 ± 7.3 nmol/min/mg of cytosolic protein for small intestine and liver, respectively). No significant differences in this enzymatic activity in the liver or small intestine were found among the various (low-, mid-, high-dose) pentobarbital treatment groups or the positive-pentobarbital and positive-phenobarbital control groups (data not shown).

Although no treatment-related effects on the hepatic glucuronyl transferase activities were found, a difference in phenolphthalein-UDPGA glucuronyl transferase activity was found between females and males, with females having an approximately 6-fold greater activity than the males (0.41 ± 0.39 vs 0.07 ± 0.06 nmol of phenolphthalein used/min/mg of microsomal protein, respectively). Conversely, no sex- or treatment-related difference in hepatic activity was found with ***p*-nitrophenol (PNP)** as the substrate (data not shown). Glucuronyl transferase activities in the small intestinal mucosal microsomes were at least equivalent to that in the liver subfractions. *p*-Nitrophenol transferase activity was several fold greater in the small intestine than in the liver (4.12 ± 3.68 vs 0.76 ± 0.27 nmol of PNP used/min/mg of microsomal protein for small intestine and liver, respectively). Except for differences in PNP transferase activity between females and males (2.37 ± 3.55 vs 5.88 ± 3.81 nmol of PNP used/min/mg of microsomal protein, respectively), no treatment-related differences in PNP transferase activity was found in the small intestine.

Microsomal cytochromes and reductases—In hepatic microsomes, CYP in the positive-phenobarbital control group (0.71 ± 0.1 nmol/mg of microsomal protein) was significantly greater than in all the other groups (0.18 to 0.29 nmol/mg of microsomal protein). A similar difference occurred in the cytochrome b_5 content of these treatment groups (0.43 ± 0.03 vs 0.22 to 0.31 nmol/mg of microsomal protein). Results of a 2-way ANOVA indicated that the NADPH- and NADH-dependent microsomal reductases had interactions between sex and treatment groups, which negated the use of pooled data to describe these results (data not shown). With the NADPH-dependent CYP (cytochrome c) reductase, a difference was found between males and females only in the high-dose pentobarbital treatment group; males in the high-dose pentobarbital treatment group and males and females in the positive-pentobarbital control group had the highest P450 reductase activity (146 to 255 nmol of cytochrome c reduced/min/mg of microsomal protein). The NADH-cytochrome b_5 reductase was unchanged in males in the negative control group and in the various (low-, mid-, and high-dose) pentobarbital treatment groups (3.2 to 4.2 µmol of ferricyanide reduced/min/mg of microsomal protein). However, NADH-cytochrome b_5 reductase activity was increased in females of the positive-phenobarbital control group, compared with males (5.4 ± 0.5 vs 2.4 to 4.1 µmol/min/mg of microsomal protein). Results of a 2-way ANOVA indicated that differences in NADH-cytochrome b_5 reductase activity existed between males and females within the high-dose pentobarbital treatment group and the 2 positive control groups. Males that received pentobarbital had slightly higher activities, whereas females in the positive-phenobarbital control group had higher activities than males (data not shown).

In the small intestinal mucosa (data not shown), CYP was not measurable by use of the usual CO-difference spectra procedure. Dilute protein concentrations and insufficient sample precluded reanalysis. Concentrations of measurable cytochrome b_5 were at least a sixth of that measured in the liver microsomes (0.02 to 0.06 nmol/mg of microsomal protein). The NADH-dependent cytochrome b_5 reductase was about a third of that measured in liver microsomes (1.05 to 1.55 µmol of potassium ferricyanide reduced/min/mg of microsomal protein), and no treatment-related differences were found in this activity. The NADPH-dependent P450 (cytochrome c) reductase (20 to 57 nmol of cytochrome c reduced/min/mg of microsomal protein) was at least a sixth of that measured in the liver microsomes.

CYP-mediated enzymatic activities—To associate at least a single reaction with the CYP isoforms, multiple substrates were tested to ensure that substrates were representative of barbiturate-induced CYP isoforms. With liver-mediated reactions, several of the substrates associated with barbiturate induction (ERY [CYP3A, 3.52 to 4.85 nmol/min/mg of microsomal protein], PRF [CYP2B], and DBF [CYP2C, 131 to 173

Table 1—Mean (± SD) values of liver CYP2B-, CYP2C-, and CYP3A-mediated reactions in female and male Beagles in treatment groups

		Cytochrome P450 (CYP)-mediated reactions (pmol/min/mg of microsomal protein)					
Treatment groups	**Sex**	**BRF**	**ETOMC**	**MOMC**	**BZOFMC**	**ETOFMC**	**MOFMC**
Negative (0.9% NaCl) control	F	272 ± 55[a]	597 ± 217[a,b]	207 ± 59[a]	793 ± 190[a]	99 ± 112[a]	938 ± 296[a]
	M	170 ± 37[a]	555 ± 147[a]	154 ± 45[a]	598 ± 148[a]	116 ± 75[a]	798 ± 299[a]
Low-dose (50 µg/d) PtB	F	431 ± 166[a,b]	423 ± 62[a]	222 ± 81[a]	819 ± 238[a]	326 ± 186[a]	947 ± 317[a]
	M	284 ± 69[a,b]	609 ± 134[a,b]	190 ± 43[a]	831 ± 200[a]	368 ± 121[a,b]	1046 ± 263[a,b]
Mid-dose (150 µg/d) PtB	F	364 ± 72[a]	434 ± 193[a]	237 ± 34[a]	936 ± 211[a,*]	495 ± 192[b]	1130 ± 241[b]
	M	449 ± 211[b]	629 ± 173[a,b]	200 ± 26[a]	580 ± 132[a,*]	258 ± 101[a,b]	1089 ± 92[a,b]
High-dose (500 µg/day) PtB	F	363 ± 83[a]	865 ± 205[b]	329 ± 119[a]	987 ± 288[a]	597 ± 115[b,c]	1456 ± 435[a]
	M	524 ± 137[b]	1001 ± 170[b]	242 ± 28[a]	844 ± 101[a]	530 ± 142[c]	1455 ± 79[b]
Positive PtB (10 mg/kg/d) control	F	700 ± 14[b,*]	1076 ± 265[b,c]	504 ± 110[b]	1835 ± 510[b]	956 ± 444[c]	2902 ± 227[b,*]
	M	1167 ± 25[c,*]	1300 ± 68[b,c]	531 ± 20[b]	1493 ± 181[b]	795 ± 13[c]	2183 ± 93[c,*]
Positive PhB (10 mg/kg/d) control	F	1353 ± 167[c]	2177 ± 98[c]	1236 ± 7[b,*]	4016 ± 157[b,*]	2339 ± 460[d,*]	5494 ± 157[b,*]
	M	1333 ± 107[c]	2006 ± 441[c]	904 ± 111[b,*]	2709 ± 209[b,*]	1515 ± 59[d,*]	3477 ± 138[c,*]

[a,b,c,d]Different superscript letters within a column indicate significant differences ($P < 0.05$) in values among treatment groups.
*Values differed significantly ($P < 0.05$) between females and males within a treatment group.
PtB = Pentobarbital. PhB = Phenobarbital. F = Female. M = Male. BRF = Benzyloxyresorufin O-dealkylase. ETOMC = 7-ethoxy-4-methylcoumarin O-deethylase. MOMC = 7-methoxy-4-methylcoumarin O-demethylase. BXOFMC = 7-benzyloxy-4-trifluoromethylcoumarin O-dealkylase. ETOFMC = 7-ethoxy-4-trifluoromethylcoumarin O-deethylase. MOFMC = 7-methoxy-4-trifluoromethylcoumarin O-demethylase.

pmol/min/mg of microsomal protein]) had no significant treatment-related differences (data not shown). Although PRF activity was increased approximately 3-fold in the positive-pentobarbital and positive-phenobarbital control groups (8.16 to 27.68 pmol/min/mg of microsomal protein), the high variances in the various (low-, mid-, and high-dose) pentobarbital treatment groups prevented detection of significant differences. Coumarin hydroxylase (CYP2A, 45.4 to 79.0 pmol/min/mg of microsomal protein) had erratic and nondose-dependent differences among some treatment groups (data not shown). Aminopyrine N-demethylase activity (CYP2B and CYP3A) was significantly different between males and females (data not shown), and some nondose-related differences in aminopyrine N-demethylase activity was found among females in the various pentobarbital treatment groups, whereas no differences in aminopyrine N-demethylase activity was found among males in the various pentobarbital treatment groups. Erratic effects were also observed with BZQ (CYP3A, data not shown); BZQ activity was also significantly different between males and females. Activity of BRF was induced in males and females in the positive-pentobarbital and positive-phenobarbital control groups and in males of the mid-dose and high-dose pentobarbital treatment groups (**Table 1**). Results of a 2-way ANOVA indicated that this substrate had significant sex by treatment group interactions, although the only measurable difference between males and females occurred in the positive-pentobarbital control group.

Except for BZOFMC, the alkoxycoumarin substrates as a group (Table 1) had linear dose-dependent effects on the basis of regression analysis results (data not shown). On the basis of linear associations, the lowest effective doses were calculated; these included ETOMC (CYP2B, 200 µg/d), ETOFMC (CYP2B, 200 µg/d; **Fig 1**), MOFMC (CYP2C, 200 µg/d), and MOMC (CYP2C, 300 µg/d). In addition to the alkoxycoumarins, ERY (CYP3A) also had a linear association from which a lowest effective dose of 450 µg/d was calculated. All of these substrates, except ETOMC, had sex by treatment interactions.

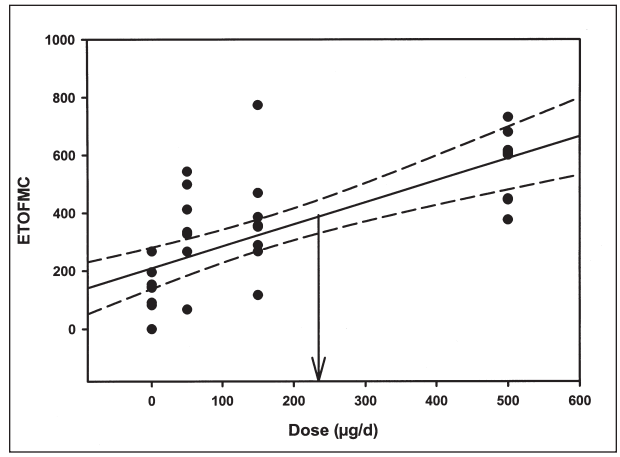

Figure 1—Amount of measured 7-ethoxy-4-trifluoromethylcoumarin O-deethylase (ETOMC) versus pentobarbital dose. Pentobarbital was administered orally to 3-month-old Beagles (4 males and 4 females per pentobarbital treatment group) at the indicated amounts for 8 weeks. Values represent the means of triplicate determinations from individual dogs. The fitted regression lines with 95% confidence limits are shown. The arrow indicates the lowest effective dose (ie, the lowest interpolated dose with a 95% confidence interval that did not overlap with the 95% confidence interval at 0).

The CYP-mediated activities measured in the small intestinal mucosal microsomes (data not shown) were much lower than that found for microsomes of the liver. Results of several of assays were inconsistent within various (low-, mid-, and high-dose) pentobarbital treatment groups and probably reflect the low P450 content and P450 reductase activity. For most measured reactions, significant differences in CYP-mediated activities were not found among the various pentobarbital treatment groups and the positive-pentobarbital and positive-phenobarbital control groups.

Western blot analysis of liver tissue—Isoforms CYP2A, CYP2B, CYP2C (2 bands), CYP2E, and CYP3A

Table 2—Mean (± SD) of hepatic CYP isoforms detected in microsomes from female and male Beagles in treatment groups

Treatment groups	Sex	CYP isoforms (AU/0.01 mg of microsomal protein) CYP2A	CYP2B	CYP2C-T	CYP2C-B	CYP2E	CYP3A
Negative (0.9% NaCl) control	F	43 ± 24[b]	102 ± 34[b]	52 ± 36[a,c]	135 ± 84	429 ± 79*	116 ± 74[b]
	M	45 ± 18[b]	69 ± 19[b]	22 ± 16[a]	97 ± 31[a]	281 ± 73[a,*]	72 ± 12[b]
Low-dose (50 µg/d) PtB	F	75 ± 26[b]	117 ± 46[b]	39 ± 14[a,c]	134 ± 24	387 ± 107	79 ± 22[b]
	M	63 ± 18[b,c]	125 ± 22[a,b]	39 ± 8[a]	171 ± 31[a,c]	418 ± 88[a,b,c]	115 ± 31[c]
Mid-dose (150 µg/d) PtB	F	82 ± 44[b]	151 ± 67[b]	74 ± 16[a,c]	168 ± 44*	424 ± 112*	121 ± 6[b,*]
	M	126 ± 60[c]	205 ± 49[a,c]	132 ± 44[c]	381 ± 119[b]	538 ± 60[b,*]	257 ± 137[a,*]
High-dose (500 µg/day) PtB	F	117 ± 48[b]	122 ± 32[b]	102 ± 26[b,c]	258 ± 87	386 ± 62*	93 ± 24[b,*]
	M	93 ± 28[b,c]	119 ± 12[b]	68 ± 39[a]	256 ± 42[b,c]	531 ± 21[b,*]	254 ± 97[a,c,*]
Positive PtB (10 mg/kg/d) control	F	336 ± 83[c]	199 ± 45[a,b]	81 ± 4[a,b]	260 ± 10[a,b,c]	531 ± 0*	381 ± 86[a,*]
	M	325 ± 69[d]	164 ± 9[b,c]	85 ± 2[a,c]	229 ± 14	283 ± 7[c,*]	217 ± 42[a,b,c,*]
Positive PhB (10 mg/kg/d) control	F	785 ± 24[a]	266 ± 54[a]	153 ± 13[b,c]	261 ± 16	469 ± 41*	374 ± 41[a]
	M	718 ± 40[a]	248 ± 49[c]	143 ± 13[b,c]	324 ± 20[b,c]	276 ± 64[c,*]	396 ± 46[a]

AU = Absorbance units. CYP2C-T = Top or upper band in the western blot that cross-reacted with the anti-CYP2C antibody. CYP2C-B = Bottom or lower band in the western blot that cross-reacted with the anti-CYP2C antibody.
See Table 1 for remainder of legend.

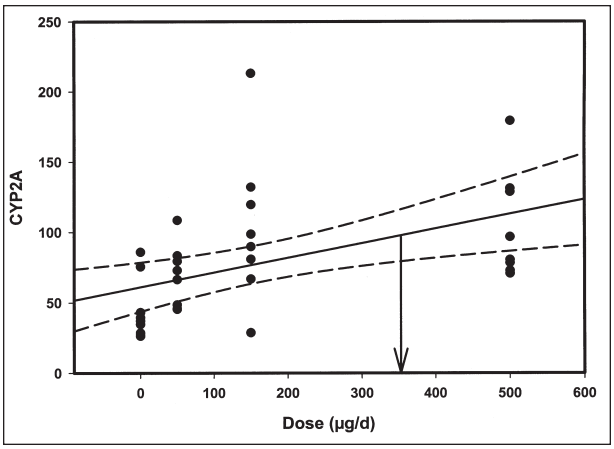

Figure 2—Amount of measured CYP2A versus pentobarbital dose. Pentobarbital was administered orally to 3-month-old Beagles (4 males and 4 females per pentobarbital treatment group) at the indicated amounts for 8 weeks. Values represent the means of 2 replicate determinations from individual dogs. The fitted regression lines with 95% confidence limits are shown. The arrow indicates the lowest effective dose (ie, the lowest interpolated dose with a 95% confidence interval that did not overlap with the 95% confidence interval at 0).

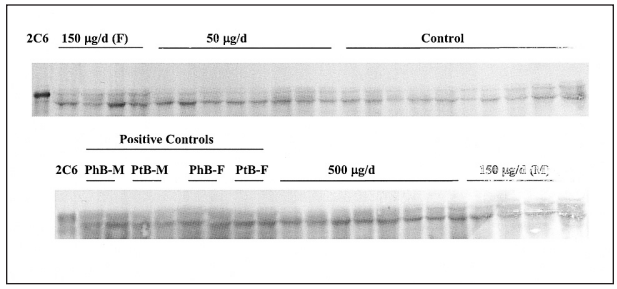

Figure 3—Results of western blot analysis of CYP2C. Pentobarbital was administered orally to 3-month-old Beagles at the indicated amounts for 8 weeks. Hepatic microsomal protein bands were detected immunochemically by use of an antirat CYP2C6 antibody. Results were compared with rat CYP2C6. Two bands are visible; 1 with a molecular weight similar to rat CYP2C6 and 1 with a lower molecular weight. The negative control group had 5 males and 5 females; the various (50, 150, and 500 µg/d) pentobarbital treatment groups each had 4 males and 4 females; and the positive-pentobarbital and positive-phenobarbital control groups each had 2 males and 2 females. For the low- and high-dose pentobarbital treatment (50 and 500 µg/d) groups and the negative control groups, results for females are to the right, and results for males are to the left. 2C6 = Rat CYP2C6. F = females. M = Males. PhB = Phenobarbital. PtB = Pentobarbital.

were detected in hepatic microsomes (**Table 2**). Isoform CYP2A (**Fig 2**) had a dose-dependent increase; on the basis of the results of regression analysis, an apparent pentobarbital dose was calculated (approx 350 µg/d) that was significantly greater than that calculated for the negative control group. The upper band of CYP2C gels (CYP2C-T) migrated with the same apparent molecular weight as the rat CYP2C standard (**Fig 3**). Although CYP2B was present and increased in the positive-phenobarbital control group, no significant difference in the amount of CYP2B was found among any of the dogs that received pentobarbital. Significant differences in the amount of CYP2E were found between males and females within most of the groups, but no treatment-related effects were found. In addition to differences in the amount of CYP3A among the various (low-, mid-, and high-dose) pentobarbital treatment groups, significant sex-related differences were also observed. Isoform CYP2D was not detectable, although the antibody used in our study bound to human recombinant CYP2D6 (data not shown).

Western blot analysis of small intestinal mucosa—Isoforms CYP2B, CYP2C, and CYP3A were the only CYP isoforms detectable in the small intestinal mucosa (**Table 3**). Some binding by use of the CYP2A6 antibody was observed, but the staining patterns were so weak as to be not measurable, and no obvious treatment effects were found. The anti-CYP2B antibody also demonstrated the presence of CYP2B in the small intestinal mucosa; however, none of the treatment groups, including the 2 positive control groups, had an increase in the concentrations of this isoform. No intestinal CYP2C reactivity was found for any of the 10 dogs in the positive control groups. However, the anti-CYP2C antibody did react with the small intestinal mucosa of dogs in the various (low-, mid-, and high-dose) pentobarbital treatment groups in the positive-pentobarbital and positive-phenobarbital control groups, but only 1 CYP2C band was detected for

Table 3—Mean (± SD) of small intestine mucosal CYP isoforms detected in microsomes from female and male Beagles in treatment groups

		CYP isoforms (AU/0.04 mg of microsomal protein)		
Treatment groups	**Sex**	**CYP2B**	**CYP2C**	**CYP3A**
Negative (0.9% NaCl) control	F	45 ± 22	0 ± 0	21 ± 14
	M	53 ± 27	0 ± 0[b]	42 ± 24[b]
Low-dose (50 µg/d) PtB	F	61 ± 11	28 ± 15	70 ± 21
	M	39 ± 19	75 ± 22[a,b]	46 ± 16[b]
Mid-dose (150 µg/d) PtB	F	52 ± 26	38 ± 15	93 ± 56
	M	88 ± 51	31 ± 16[a,b]	109 ± 64[b]
High-dose (500 µg/day) PtB	F	41 ± 15	185 ± 247	61 ± 19
	M	46 ± 19	86 ± 28[a]	156 ± 88[a,b]
Positive PtB (10 mg/kg/d) control	F	31 ± 13	89 ± 57	201 ± 166*
	M	73 ± 41	88 ± 10[a,b]	467 ± 558[a,*]
Positive PhB (10 mg/kg/d) control	F	29 ± 34	59 ± 18	275 ± 128
	M	46 ± 19	91 ± 50[a,b]	313 ± 211[a,b]

See Tables 1 and 2 for legend.

the mucosal tissue. This isoform corresponded with the upper band found for the liver tissue specimens (CYP2C-T) and also had an apparent molecular weight similar to that of rat CYP2C6 (data not shown). Use of anti-CYP3A antibody demonstrated the presence of constitutive amounts of this isoform in the small intestinal mucosa of male and female dogs. Induction of intestinal CYP3A occurred in males and females in the pentobarbital-positive and phenobarbital-positive control groups.

Discussion

Results of our study indicate that low doses of orally administered pentobarbital in dogs are capable of inducing hepatic CYP isoforms (CYP2B, CYP2C, and CYP3A) that are usually associated with induction by phenobarbital. As expected, pentobarbital treatments did not induce CYP2E. We could not demonstrate the constitutive presence of canine CYP2D by use of a monoclonal antibody to human CYP2D6; a canine CYP2D isoform can be detected by use of polyclonal antisera to the purified protein.[22] Our results indicate that the epitope recognized by the antihuman CYP2D6 monoclonal antibody is absent from canine CYP2D. Isoform CYP2A is induced in mice by phenobarbital,[23] and this response seems to be mimicked in dogs.

In our study, differences in the response of the CYP3A isoform were found between males and females; a positive CYP3A response was found for males in the low-dose pentobarbital group and for females in the positive-pentobarbital and positive-phenobarbital control groups. Dogs possess 2 distinct members of the CYP3A family, CYP3A12 and CYP3A26, which are induced by phenobarbital.[24-26] These 2 isoforms have distinct substrate specificities[25] and differ by approximately 1.5 kd. Because our electrophoretic gels were not capable of resolving proteins that have this small of a difference in molecular weight, it is unclear as to which forms were induced in the hepatic microsomes from the male dogs. In addition, we did not measure any of the steroid hydroxylase activities, which could have provided differential results.

Ingestion of food containing phenobarbital analogs results in induction of hepatic CYP2B in rats and mice.[27,28] In another study, low-dose dietary exposure to TCPOBOP resulted in induction of CYP2B and CYP3A in mice.[6] Coincidentally, rats are not as responsive to such low concentrations of TCPOBOP.[29] This differential effect indicates a species difference in response to exposure to low concentrations of CYP2B inducers. On the basis of our results, it is possible that dogs might respond to TCPOBOP in a similar fashion as mice. This conclusion is further supported by induction of a CYP2A isoform in dogs (reported here) and mice[23] by barbiturates. The BRF is a specific substrate for the major phenobarbital inducible dog hepatic CYP, CYP2B11.[30] Although BRF activity was induced in liver microsomes from the positive-phenobarbital control group, it was interesting that the males of the pentobarbital-positive control group had the highest turnover number for BRF activity (4.5 ± 0.4, 1.9 ± 0.1, and 0.8 ± 0.3 min^{-1} for males of the pentobarbital-positive control group, phenobarbital-positive control group, and negative control group, respectively). No difference in turnover number was found for females in the various (low-, mid-, and high-dose) pentobarbital treatment groups. These results indicate that the CYP isoform(s) that are responsible for BRF activity were specifically induced in males after exposure to pentobarbital.

As with mucosal CYP3A, CYP2B was not induced in the small intestine by low-level exposure to pentobarbital. In fact, small intestinal mucosal CYP2B does not appear to be responsive to barbiturate induction at all, because neither of the positive control groups had induction of this isoform or reactions associated with this isoform. This result suggests tissue-specific gene regulation of this CYP isoform. Whether differences in inducibility are the result of time of exposure or some fundamental difference at a molecular level cannot be determined from our study. Our finding of CYP2B in the canine mucosal preparations is in sharp contrast to the absence of this isoform in the human small intestine.[31]

The canine hepatic CYP2A isoform was induced in our study in a dose-dependent manner without a corresponding increase in COH activity. Coumarin hydroxylase is a reaction normally associated with

CYP2A6, the human isoform. If the human and dog isoforms are the same protein, then increases in the amount of CYP2A should result in increased COH activity. Unlike the rat CYP2A isoform, which is induced by polycyclic aromatic hydrocarbons and specific for catalyzing 7α-hydroxylation of testosterone, the murine CYP2A5 is inducible by phenobarbital and pyrazole[23] and is the COH in mice.[32] In addition to COH activity, the purified human isoform can also catalyze 7-ethoxycoumarin O-deethylation.[33] Unfortunately, we did not use 7-ethoxycoumarin as a substrate for our studies. However, because of the structural similarity between 7-ethoxycoumarin and the other alkoxymethylcoumarins studied here (MOMC, ETOMC, ETOFMC, and MOFMC), it may be inferred that the canine CYP2A isoform may preferentially catalyze the O-dealkylation of the alkoxycoumarins instead of the expected aromatic hydroxylation of coumarin. Our results beg the question as to whether the enhanced activities measured with these alkoxycoumarins may be related to the increased concentrations of the CYP2A isoform.

In contrast to the results reported for humans,[31,34] we did not detect CYP2C in mucosal microsomes from dogs in the negative control group, whereas a band (CYP2C-T) that corresponds to the rat CYP2C6 isoform was readily detected in dogs that received barbiturates. This is another example of an apparent tissue-specific regulation of CYP2C. The liver had 2 distinct isoforms that were constitutively expressed in dogs from the positive control groups, whereas the small intestinal mucosa only expressed 1 band, and this band was only apparent after exposure to the barbiturates. Although MOFMC, BZOFMC, and DBF are substrates recommended for high-throughput screening for CYP2C in humans,[10] this does not rule out the possibility that this latter isoform may also be involved in the metabolism of other alkoxycoumarin substrates, such as those tested here.

Ample evidence is found in the literature that supports drug-drug interactions in dogs exposed to barbiturates and other drugs. Pharmacologic effects of the anti-coagulant bishydroxycoumarin are substantially enhanced in dogs when the dosage is adjusted to compensate for concomitant treatment and subsequent withdrawal of phenobarbital (10 mg/kg).[35] Similar effects are observed when either tolbutamide or phenylbutazone is substituted for phenobarbital.[35] It is interesting that the commonly administered anticoagulants warfarin and tolbutamide are metabolized by CYP2C isoforms.[36] It is likely that the effects observed by Welch et al[35] are the result of drug-drug interactions with CYP2C isoforms.

Amacher et al[5,37] demonstrated that rats and dogs responded to exposure to various drug classes by induction of hepatic drug metabolizing enzymes with minimal, if any, adverse histopathologic findings or changes in serum biochemical parameters. Granted, these studies[5, 37] were performed with doses that were considerably greater than those used in our study; however, this does not mean that some measurable response would not have occurred at the lower doses, just as Nims et al[6] reported for TCPOBOP. For some of the activities measured in our study, only minor differences in the induced activities (eg, ETOMC and ETOFMC) were found between dogs in the positive-pentobarbital control group (10 mg/kg/d) and the high-dose pentobarbital treatment group (500 µg/d). This may be interpreted as inductions occurring at considerably lower doses than normally used.

Since the finding of pentobarbital in dog food,[3] results of 2 subsequent surveys indicate that pentobarbital is present in some, but not all, dog food samples.[4,38] The concentrations of pentobarbital in dog food as measured by the gas chromatography- or liquid chromatography-mass spectrometry procedures[3,4,38] are considerably less than those originally reported by O'Connor et al[2] in rendered products. Findings in this early study[2] probably provided a basis for early anecdotal observations concerning the loss of effectiveness of pentobarbital as an anesthetic inducing agent. To our knowledge, there have not been any studies to confirm or refute these observations. Our study was conducted to determine whether any measurable effects could be found in dogs after oral exposure to concentrations of pentobarbital similar to that measured in dog food. We found no discernable effects at a treatment concentration that approached that found in dog food (50 µg/d). This not withstanding, it is apparent that orally administered pentobarbital induces several CYP isoforms and the enzymatic reactions mediated by them.

[a]Pentobarbital sodium (CAS #57-33-0, Sigma P-3761, Lot #59H0612), Sigma Chemical Co, St Louis, Mo.
[b]Phenobarbital sodium (CAS #57-30-7, Sigma P-5178, Lot #88H0023), Sigma Chemical Co, St Louis, Mo.
[c]Sigma Chemical Co, St Louis, Mo.
[d]Gentest, Woburn, Mass.
[e]Molecular Probes Inc, Eugene, Ore.
[f]Pierce Chemicals Inc, Rockford, Ill.
[g]Ridglan Farms, Mount Horeb, Wis.
[h]Purina Certified Canine Lab Chow #5007, Purina Co, St Louis, Mo.
[i]Antech Diagnostics Inc, Lake Success, NY.
[j]Euthasol, Delmarva Laboratories, Midlothian, Va.
[k]American Histo-Labs, Gaithersburg, Md.
[l]Anmed/Biosafe Inc, Rockville, Md.
[m]KimWipes, Kimberly Clark Inc, Neenah, Wisc.
[n]SPECTRAmax Plus, Molecular Devices Inc, Sunnyvale, Calif.
[o]GeminiXS, Molecular Devices Inc, Sunnyvale, Calif.
[p]Personal densitometer SI, Molecular Dynamics, Sunnyvale, Calif.
[q]Minitab, v12, State College, Pa.
[r]SigmaStat, v2.06, SPSS Inc, Chicago, Ill.
[s]Accession No. 39198, evaluated by Steven M. Stiefel, DVM, Anmed/Biosafe Inc, Rockville, Md.

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