ADVERSE EFFECTS OF PHENOBARBITAL THERAPY IN EPILEPTIC DOGS

Thesis submitted to the Graduate Faculty in partial fulfilment of the requirements for the degree of Doctor of Philosophy in the Department of Anatomy and Physiology, Faculty of Veterinary Medicine, Atlantic Veterinary College, University of Prince Edward Island

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ABSTRACT

Two poorly understood effects of phenobarbital, the most common drug used to treat canine epilepsy, are (1) serum thyroid hormone alterations, and (2) serum liver enzyme abnormalities. Alterations in concentrations of serum total thyroxine (T4), the major thyroid hormone, have been anecdotally reported to occur in dogs receiving phenobarbital, but at the time of this investigation, this had not been documented. The cause and clinical significance of alterations in serum thyroid hormone concentrations associated with phenobarbital therapy also had not been determined in dogs. Elevated serum liver-associated enzymes have been associated with hepatotoxicity in a retrospective study of dogs receiving phenobarbital (Dayrell-Hart *et al.* 1991). However, elevated serum liver-associated enzyme activities, especially serum alkaline phosphatase (AP) and serum alanine aminotransferase (ALT), are also commonly found in phenobarbital-treated dogs that do not have clinical signs of liver disease. This has led some to conclude that increased serum AP and ALT activities might also be due to induction of these enzymes, although conclusive evidence of this is lacking. The difficulty faced by veterinarians is differentiating between cases of possible liver enzyme induction and cases of early subclinical liver injury.

The goals of this study were to investigate the cause, clinical significance, and risk factors associated with the development of thyroid and liver abnormalities in phenobarbital-treated epileptic dogs. Cross-sectional and prospective investigations of clinically healthy client-owned epileptic dogs receiving phenobarbital were performed to examine these issues. Additionally, samples obtained from these patients were used to

perform *in vitro* investigations of the effects of phenobarbital on the liver.

In this study, phenobarbital therapy caused a significant decrease in the serum T4 concentration within 3 weeks of the start of therapy, and the decrease continued for at least 1 year. The cause was most likely an increased metabolism and clearance of the hormone from the body. Dogs with low serum T4 concentrations did not have clinical signs of hypothyroidism, and did not have better or worse seizure control than dogs with normal serum T4 concentrations. Dogs receiving phenobarbital had significantly greater histopathological liver abnormalities than controls. Duration of phenobarbital therapy was a possible risk factor. *In vitro* studies indicated that increased serum ALT activity was not due to induction of the enzyme in the liver. This suggested that serum ALT concentration might be used as a non-invasive test to differentiate between early liver injury and enzyme induction in dogs with elevated serum liver-associated enzyme activities. Induction of AP activity was not observed in this study, but it is possible that induction was masked by a simultaneous increase in release of the enzyme from the hepatocyte membrane. Additional information discovered during this study, including pancreatitis associated with use of phenobarbital/potassium bromide combination therapy, will also be presented in this thesis.

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TABLE OF CONTENTS

CHAPTER 1. GENERAL INTRODUCTION

1.1 General overview

Drug therapy is an integral part of modern medicine. Many disease conditions can be effectively and safely treated with therapeutic drugs. However, every drug, no matter how effective, has the potential to cause unwanted or adverse effects. The severity of untreated disease determines what degree of adverse drug effect risk can be tolerated. Full understanding of the adverse effects associated with each drug is vital to the decision process when determining appropriate pharmacological therapeutics.

The goal of this thesis is to investigate two of the most poorly understood adverse effects of phenobarbital therapy in epileptic dogs. Phenobarbital is the anticonvulsant drug most commonly used for maintenance therapy of epilepsy in dogs. Epilepsy is a fairly common seizure disorder in dogs, affecting up to an estimated 3% of the canine population (Podell 1996). Epilepsy generally cannot be cured, but with appropriate medical management with anticonvulsant drugs, satisfactory seizure control can be achieved in an estimated 60-80% of epileptic dogs (Boothe 1998, Thomas 2000).

Due to sometimes dramatic differences between species in drug disposition, metabolism, and excretion, many of the anticonvulsant drugs used in humans are not appropriate for use in dogs. Only two anticonvulsant drugs, phenobarbital and potassium bromide, are currently used to any extent for maintenance therapy of epilepsy in dogs (Boothe 1998, Podell 1996, Thomas 2000). Phenobarbital is currently the first drug of choice for treatment of canine epilepsy, and much information is available concerning its

safety, efficacy, adverse effects, and pharmacokinetics in dogs. Potassium bromide is used much less frequently than phenobarbital, in part because the drug is not available commercially and must be specially formulated for each patient, and in part because relatively little information is available about its use in dogs. Potassium bromide is most commonly used as an addition to phenobarbital therapy in dogs whose seizures are not adequately controlled with phenobarbital alone (Boothe 1998, Schwartz-Porsche and Jurgens 1991). No studies have yet been published documenting the safety or efficacy of potassium bromide as a monotherapy for epilepsy in dogs.

Phenobarbital therapy in dogs is associated with a number of unwanted or adverse effects. Many of these are fairly minor side effects that generally do not constitute life-threatening problems. These include polydipsia, polyuria, polyphagia, sedation, and ataxia (Boothe 1998, Podell 1996, Thomas 2000). Many dogs develop tolerance to these side effects within a few weeks after the start of phenobarbital therapy. However, two additional poorly understood adverse effects are the actions of phenobarbital on the hypothalamic-pituitary-thyroid axis and on the liver. These two adverse effects were chosen as the focus of this thesis for the following reasons. (1) At the start of this investigation, the effects of phenobarbital on the hypothalamic-pituitary-thyroid axis had not been documented, and the clinical significance of the problem was unknown. (2) The effects of phenobarbital on the liver are the most serious and life-threatening of all of the reported adverse effects of phenobarbital therapy in dogs, and yet very little was known about early detection and prevention of this problem.

Because phenobarbital is the mainstay of therapy for epilepsy in dogs, this study focused on the adverse effects of this drug. However, in the course of the study,

potassium bromide was added to phenobarbital for maintenance therapy for a number of dogs. Although the study was not designed to investigate adverse effects of phenobarbital/potassium bromide combination therapy, certain trends were noted concerning this combination. These included the effects on the hypothalamic-pituitary-thyroid axis and the pancreas. This information will also be presented in this thesis, because it helps confirm that phenobarbital should remain the first drug of choice until further studies of the adverse effects of potassium bromide are completed.

Following are several sections designed to give the reader a general background about adverse drug effects (section 1.2), epilepsy (section 1.3), the anticonvulsant drugs phenobarbital and potassium bromide (section 1.4), and the adverse effects of phenobarbital therapy on the hypothalamic-pituitary-thyroid axis, the liver, and the pancreas (section 1.5). More complete introductions on the adverse effects of phenobarbital on the thyroid, liver, and pancreas will be presented at the beginning of the chapters dealing with each of these topics. The general introduction will conclude with an outline of the major hypotheses and questions generated during this thesis, and a guideline indicating where in the thesis the answers to each of these questions can be found (section 1.6).

1.2 Introduction to adverse drug effects

An adverse drug effect is a noxious or unintended response to a drug that occurs at appropriate doses used for prophylaxis, diagnosis, or therapy (Cribb 2001). Adverse drug effects are a very serious problem in human medicine, where adverse effects caused

by prescription drugs rank anywhere from 4th to 6th as the leading cause of death in the United States (Lazarou *et al.* 1998). Of 33 million patients admitted to U.S. hospitals in 1994, more than 100,000 died from adverse drug effects to medications that were administered properly (Lazarou *et al.* 1998). An additional 2 million patients suffering from adverse drug effects survived. The incidence of adverse drug effects in veterinary species has not been well documented, in large part due to inadequate reporting by owners and veterinarians, but also due to the difficulties of showing causation of a particular clinical event by a particular drug exposure. However, adverse drug effects are a very real and very important issue in veterinary medicine, and often limit the usefulness of otherwise efficacious drugs.

Adverse drug effects can manifest as toxicities or damage to any organ or system in the body, and can range from minor self-limiting reactions to severe, life-threatening conditions. Many different classification schemes can be used to catagorize adverse drug effects. These include classifications based upon clinical signs, time of onset of signs, pharmacological and chemical properties of the drug, and mechanism of toxicity. A commonly used classification divides adverse drug effects into those that are dose-related, and those that are idiosyncratic (or non-dose related) (Cribb 2001).

Most adverse drug effects are dose-dependent (Cribb 2001). The larger the dose of the drug, the greater the chance of the adverse effect occurring, and the more severe the reaction. Usually these adverse effects are predictable, can be reproduced experimentally, and are identified before the drug becomes available on the market. Often dose-dependent adverse drug effects are precipitated by factors that change the drug's disposition in the body. These include patient factors such as age, sex, concurrent disease

such as renal, liver, or cardiovascular disease, and use of concurrent drugs that may interfere with normal drug metabolism and clearance. The majority of dose-dependent adverse effects can be avoided by careful selection of drugs and dosages to accommodate the individual patient.

Dose-dependent adverse drug effects can be further subdivided into pharmacological toxicities and intrinsic toxicities (Cribb 2001). Pharmacological toxicities are due to the pharmacological effects of the drug or its metabolites on a specific receptor or target. These may or may not be related to the therapeutic target. Pharmacological toxicities can potentially occur in any patient, and are often precipitated by conditions that cause greater drug exposure such as decreased clearance or increased absorption. Examples of pharmacological toxicities include hypotension from alpha-receptor blocking sedative/analgesic agents, and gastrointestinal ulcerations from inhibition of cyclooxygenase activity by non-steroidal anti-inflammatory drugs (Boothe 2001, Cribb 2001).

Intrinsic toxicities are dose-dependent reactions that are due to the chemical properties of the drug or its metabolites, and not to the pharmacological properties of the drug (Cribb 2001). These toxicities occur when the drug or its metabolites bind non-specifically to cellular proteins or nucleic acid, or disrupt cell membranes. Clinical signs of these types of adverse drug effects often relate to the site of accumulation or bioactivation of the drug or its metabolites. Examples of intrinsic toxicities include nephrotoxicity associated with aminoglycoside antibiotics, and sulfonamide-induced hypothyroidism (Boothe 2001, Cribb 2001).

Idiosyncratic adverse drug effects are reactions due to an inherent abnormality or

characteristic of an individual patient that predisposes that particular patient to the reaction (Cribb 2001, Gibaldi 1992, Pohl *et al.* 1988). These are not dose-dependent in that they occur at therapeutic concentrations of the drug, and do not occur in the majority of patients even if the dosage is increased to otherwise toxic concentrations. Importantly, susceptible patients generally cannot be identified prior to exposure of the drug. Therefore, unlike dose-dependent adverse drug effects, idiosyncratic drug reactions cannot be prevented by careful selection of the drug or the dosage. Idiosyncratic reactions generally cannot be reproduced experimentally or detected in pre-clinical drug testing. Only after marketing to large genetically variant populations containing predisposed individuals will idiosyncratic reactions typically be detected. Idiosyncratic adverse drug reactions usually occur much less frequently that do dose-dependent reactions, but in certain populations the reaction rate can be high (Cribb 2001).

The mechanism of idiosyncratic reactions are dependent upon the chemical properties of the drug, and often involve an immune-mediated component (Cribb 2001, Gibaldi 1992, Pohl *et al.* 1988). In humans, many patients develop antibodies against specific cellular proteins. Clinical manifestations are generally systemic, but localized signs can also occur. Systemic signs include fever, blood dyscrasias, hepatitis, dermatopathies, arthropathies, and nephropathies. Clinical signs of idiosyncratic hypersensitivity reactions typically develop 1-2 weeks after the start of the drug, differentiating these reactions from IgE-mediated immediate hypersensitivity drug reactions. Immediate drug hypersensitivities require a previous drug exposure, whereas idiosyncratic hypersensitivity reactions do not. If a patient experiences an idiosyncratic reaction to a particular drug, that drug should be considered contraindicated for that

patient in the future.

As previously mentioned, the seriousness of a medical condition determines the degree of risk of adverse drug effects that can be tolerated. Epilepsy is a serious medical condition that occurs in dogs, as well as in humans, cats, horses, and other species. Epilepsy generally cannot be cured, but can be controlled with appropriate drug therapy. If left uncontrolled, many epileptics die as a result of the condition, and many suffer permanent mental and physical injuries. Therefore, a fair degree of risk of adverse effects of drug therapy can be acceptable for many epileptic patients. Before the adverse effects of anticonvulsant drugs in dogs are addressed, general background information about epilepsy and anticonvulsant drugs used in dogs will be presented.

1.3 Introduction to seizure conditions in dogs

Epilepsy, a condition characterized by recurring seizures, is a fairly common disorder in dogs. Although the incidence is unknown in the general canine population, seizure disorders account for 2-3% of veterinary teaching hospital referrals (Podell *et al.* 1995). Epilepsy is also a common disorder in humans, and the current seizure classification systems used to categorize and describe seizures in dogs is derived from the classifications used in humans. Seizures can be classified as non-epileptic or epileptic in origin. Non-epileptic seizures are due to extra-cranial causes such as cardiac disease, metabolic disorders (hypogylcemia, hypercalcemia), and toxicities (Berendt 1998, Berendt and Gram 1999, Podell 1996, Thomas 2000). Epileptic seizures have a specific neurological origin, and are due to an imbalance in excitatory and inhibitory

neurotransmission in the cerebral cortex of the brain (Berendt 1998, Berendt and Gram 1999, Podell, 1996, Thomas 2000).

Epileptic conditions can be classified based on the underlying etiology (Berendt 1998, Podell 1996, Thomas 2000), and include symptomatic, cryptogenic, and idiopathic epilepsies. Symptomatic epilepsies are caused by a known central nervous system (CNS) disorder, such as a tumor, site of previous trauma or scar tissue, infection or inflammation, or other structural abnormalitiy. These structural abnormalities can often be detected with appropriate diagnostics such as magnetic resonance imaging (MRI) or computed tomography (CT) imaging. Cryptogenic epilepsies are thought to be symptomatic, but no etiology can be identified with current diagnostic tests available. Idiopathic epilepsy is thought to be due to biochemical imbalances within the brain, but with no underlying structural cause. Idiopathic epilepsy often involves a hereditary predisposition. Heritable epilepsy has now been documented in many breeds, including the Labrador Retriever, Belgian Tervueren Shepherd, and Golden Retriever (Famula *et al.* 1997, Heynold *et al.* 1997, Jaggy and Heynold 1996, Thomas 2000).

Epileptic seizures can also be classified according to location of the seizure focus, degree of alteration in consciousness, and electroencephalographic (EEG) abnormalities (Berendt 1998, Berendt and Gram 1999, Thomas 2000). The two main categories of seizures are generalized seizures and partial seizures. Generalized seizures are due to a generalized abnormal neuronal activity in the cerebral cortex and are characterized by a sudden change in consciousness, sudden epileptiform discharges on EEG, and symmetrical and synchronous motor abnormalities. Generalized seizures can be further divided into convulsive and non-convulsive seizures. Partial seizures originate from

specific focal areas of the cerebral cortex, and the EEG and clinical manifestations of the seizure reflect the focus and function of that area of the brain. Consciousness may or may not be impaired during partial seizures (simple and complex seizures, respectively). Partial seizures may then become secondarily generalized.

Clinically, epileptic seizures manifest many different ways, depending on the type of seizure (Chrisman 1995, Thomas 2000). Motor activity alterations can occur, including involuntary rigidity (tonic) or jerking (clonic) of muscles and limb paddling. Dogs may be unconscious, may have partial impairment of consciousness, or may be fully conscious. Salivation, chomping of the jaws, urination, and defecation may occur. Some dogs will exhibit pacing, circling, snapping at the air, and facial twitches. Sensory alterations, which occur frequently in humans, are difficult to detect in dogs, but might be expressed by behavioral changes and self-traumatizing actions. Generalized seizures with loss of consciousness typically last for less than 5 minutes, but less severe partial seizures may last 30 minutes or longer (Chrisman 1995).

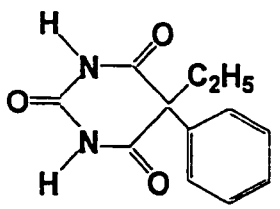
A wide range of anticonvulsant drugs are available to treat epilepsy in humans, with the drug of choice depending on the classification of seizure to be treated. In dogs, however, the selection is much more limited than in humans. Classification of seizures in dogs can be very difficult due to inability of owners to detect subtle pre-and post-ictal changes, and diagnostic tests such as MRI and EEG are frequently not performed due to cost constraints or lack of accessible testing facilities. Therefore, treatment of seizures in dogs based on seizure classification is generally not done. Additionally, many of the anticonvulsant drugs commonly used in humans have very different pharmacokinetic profiles in dogs, resulting in serum drug half-lives that are too rapid to allow reasonable

dosing intervals for pet owners. Also, the costs of many of the anticonvulsants used in humans are prohibitively high for routine use in pet animals. Currently, there are only 2 drugs available that have long enough serum half-lives, that are affordable, and that are effective enough to provide good maintenance seizure control in the majority of epileptic dogs. These are phenobarbital and potassium bromide.

1.4 Introduction to the major anticonvulsant drugs used in dogs

Phenobarbital (**Figure 1**), a barbituric acid derivative, is the most common anticonvulsant used in dogs for maintenance therapy of epilepsy (Boothe 1998, Thomas 2000). It is also used to treat seizure disorders in cats, horses, and humans. With appropriate dosing and patient selection, phenobarbital therapy can provide satisfactory seizure control in an estimated 60 to 80% of epileptic dogs (Boothe 1998, Thomas 2000). Although the mechanism of action of phenobarbital for seizure control is still not fully understood, it has been better delineated in recent years (Allan *et al.* 1992, ffrench-Mullen *et al.* 1993, McNamara 1996, Plumb 1999, Podell 1995, Rho *et al.* 1996, Schwartz-Porsche and Jurgens 1991, Yakushijit *et al.* 1989). The major mechanism by which phenobarbital is thought to cause its pharmacological action is via interaction with gamma-aminobutyric acid (GABA) type A and picrotoxin receptor sites on chloride channels in the postsynaptic neuronal membranes of the central nervous system (CNS). This causes an influx of chloride into the neurons, resulting in hyperpolarization of the membranes and decreased sensitivity to excitatory neurotransmitters. Other postulated mechanisms include inhibition of postsynaptic membrane potentials caused by

Figure 1. Structure of phenobarbital.



presynaptic release of the excitatory neurotransmitter glutamate, and inhibition of voltage-gated calcium channels in nerve terminals that mediate excitatory synaptic neurotransmitter release.

For maintenance therapy of canine epilepsy, phenobarbital is given orally and is generally administered twice daily. The pharmacokinetic properties of phenobarbital in dogs have been well described (Abramson 1988, Boothe 1995, Pedersoli *et al.* 1987, Plumb 1999, Ravis *et al.* 1989, Thurman *et al.* 1990, Watson *et al.* 1995). Phenobarbital is almost completely absorbed after oral administration, with a bioavailability of over 80%. Up to 25% of the absorbed phenobarbital is excreted unchanged in the urine. The rest is metabolized in the liver via cytochrome P450-mediated hydroxylation of the phenyl group in the para position to form para-hydroxyphenobarbital, which has very weak anticonvulsant activity. Para-hydroxyphenobarbital is then conjugated with glucuronide and excreted in the urine.

Potassium bromide (KBr) is the other commonly used anticonvulsant drug for maintenance therapy of canine epilepsy (Boothe 1998, Thomas 2000). Potassium bromide is most commonly used as an addition to phenobarbital therapy in dogs whose seizures are not well controlled with phenobarbital alone. However, it is now being used more commonly as the drug of first choice for treatment of epilepsy in dogs. This is because there have been no reported major organ injuries associated with the use of KBr, whereas phenobarbital therapy has been associated with hepatotoxicity. However, no prospective studies have yet documented the safety or efficacy of KBr monotherapy.

The mechanism of action of KBr appears to involve stabilization of neuronal cell membranes by altering chloride transport across cell membranes (Trepanier 1997, Plumb

1999). This causes a hyperpolarization of the membranes and increases the seizure threshold. The pharmacokinetics of potassium therapy have been briefly described in dogs (Schwartz-Porsche *et al.* 1990, Plumb 1999). Potassium bromide is well absorbed after oral administration, and penetrates readily into the CNS. The average serum half-life of KBr in dogs is approximately 25 days. Elimination is predominantly renal, with no hepatic metabolism occurring.

Both phenobarbital and potassium bromide treatment are associated with a number of adverse effects. However, for the majority of dogs, the benefits of the drugs far outweigh the risks of adverse effects. An introduction to the adverse effects of phenobarbital treatment investigated by this thesis will be described in the next section.

1.5 Introduction to the adverse effects of phenobarbital in dogs

Phenobarbital therapy is associated with numerous adverse effects in dogs. The most common of these include polydipsia, polyuria, polyphagia, sedation, lethargy, ataxia and disorientation (Boothe 1998, Brown 1988, Podell 1996, Schwartz-Porsche *et al.* 1985). Many dogs develop tolerance to these effects within weeks of starting therapy. Other adverse effects that are reported more rarely include hepatotoxicity (Dayrell-Hart *et al.* 1991) and blood dyscrasias (Boothe 1998, Jacobs *et al.* 1998).

Alterations in concentrations of serum total thyroxine (T4), the major thyroid hormone, have been anecdotally reported to occur in dogs receiving phenobarbital (Belshaw 1983, Boothe 1998, Brown 1988, Evinger and Nelson 1984, Feldman and Nelson 1996, Ferguson 1984, Podell 1995). However, at the time of the present

investigation, this had not been documented in published studies. The cause and clinical significance of alterations in serum thyroid hormone concentrations had not been determined in dogs. Whether or not serum T4 concentration and degree of seizure control were associated, or whether or not clinical signs of hypothyroidism existed in phenobarbital-treated dogs with low serum T4 concentrations were also unknown. Many veterinarians initiate thyroid hormone supplementation in phenobarbital-treated dogs with low serum T4 concentrations, despite the fact that no studies have documented the benefits or risks of this practice.

Increased serum liver-associated enzyme activities, in particular serum alkaline phosphatase (AP) and alanine aminotransferase (ALT) activities, are commonly found in dogs receiving phenobarbital (Boothe 1998, Center 1995, Chauvet *et al.* 1995, Sisson 1997). The increased activities are thought to be caused by either hepatic injury or by induction (increased synthesis) of these enzymes within the liver. In a retrospective study of 18 dogs receiving phenobarbital, hepatotoxicity was associated with high serum phenobarbital concentrations (Dayrell-Hart *et al.* 1991). As expected, these dogs had elevated serum liver-associated enzyme activities. Cause and effect could not be definitively concluded due to the retrospective nature of the study and concurrent administration of other hepatotoxic drugs to many of the dogs. Elevated serum liver-associated enzymes have also been found in many phenobarbital-treated dogs that do not have clinical signs of liver disease (Boothe 1998, Center 1995, Chauvet 1995, Foster *et al.* 2000, Gieger *et al.* 2000, Muller *et al.* 2000 (a), Sisson 1997). Many of these researchers concluded that the increased serum liver-associated enzymes in these cases were due to induction of the enzymes within the liver, rather than liver injury. However,

they based these conclusions on the findings that the dogs either were clinically healthy, or had relatively normal liver histology. Phenobarbital has been shown to increase the amount and activities of many enzymes in the liver, including many cytochrome P450 enzymes, glucuronosyl transferases, and glutathione-S-tranferases (Barter and Klaassen 1994, Hendrich *et al.* 1991, Larsen and Jefcoate 1995, Oguri *et al.* 1996, Okey *et al.* 1986). However, no studies have been done to confirm that phenobarbital treatment at therapeutic concentrations increases amounts and activities of AP or ALT enzymes within liver tissue.

If a phenobarbital-treated dog with good seizure control develops increased serum liver-associated enzyme activities, the situation poses a serious dilemma for the veterinarian. If the increased serum enzyme activities are due to induction and not liver injury, discontinuation of the phenobarbital would not be indicated. However, if the increased serum enzyme activities are due to early subclinical liver injury, continued phenobarbital therapy might possibly lead to clinical liver disease and to irreversible cirrhosis of the liver. In such case, switching to an alternative anticonvulsant would be warranted, but might risk compromising seizure control. At present, there is no way for veterinarians to differentiate between cases of enzyme induction and cases of early subclinical liver injury.

Pancreatic abnormalities, including pancreatitis, have been reported in 2 studies of dogs receiving phenobarbital in combination with potassium bromide (Podell and Fenner 1993, Schwartz-Porsche and Jurgens 1991). However, in neither study was the phenobarbital or the potassium bromide implicated as the direct cause of the pancreatic abnormalities. In a third study, epilepsy was found to be a risk factor for development of

acute fatal pancreatitis in dogs, but associations were not made with specific anticonvulsant drugs (Hess *et al.* 1999).

Potassium bromide has also been associated with a number of adverse effects in dogs. Similar to phenobarbital, these include sedation, lethargy, ataxia, polyuria, polydipsia, and polyphagia (Dowling 1994, Boothe 1998, Trepanier 1995). Other adverse affects reported are behavioral changes such as hyperactivity and aggression, dermatopathies, and gastric irritation. Potassium bromide has not been associated with any major organ damage or failure, and so has recently been considered by some as a better first choice for the treatment of epilepsy in dogs (Sisson 1997, Trepanier 1997). However, no studies have been published that document the safety or efficacy of potassium bromide monotherapy in dogs.

1.6 Scope of this thesis

The goals of this study were to investigate the cause, clinical significance, and risk factors associated with the development of thyroid and liver abnormalities in phenobarbital-treated epileptic dogs. Cross-sectional and prospective studies of clinically healthy client-owned pet dogs receiving phenobarbital were used to investigate these issues. Samples obtained from these patients were used to perform *in vitro* investigations of the effects of phenobarbital on the liver. *In vitro* techniques were first perfected using samples from phenobarbital-treated and untreated rats and dogs.

Additionally, an unexpectedly high incidence of pancreatitis associated with phenobarbital/potassium bromide combination therapy was discovered in the prospective

study. Therefore, a retrospective study was performed to investigate the relationship between pancreatitis and phenobarbital and/or potassium bromide treatment.

Following is a list of the major questions we had at the start of this investigation. Additional questions will be outlined in the introduction section to each chapter.

I. Effects of phenobarbital on the hypothalamic-pituitary-thyroid axis (Chapter 2, and Appendices A and B)

(1) Is phenobarbital therapy associated with decreased serum thyroxine (T4) concentrations in dogs?

(2) What is the potential mechanism of the decrease in serum T4 concentration?

(3) What is the clinical significance of the low serum T4 concentration in phenobarbital-treated dogs?

(4) Additional question raised during the course of the study: In phenobarbital-treated dogs, are serum T4 and TSH concentrations altered after the addition of potassium bromide?

II. Effects of phenobarbital on the liver (Chapter 3)

(1) Is phenobarbital therapy associated with histopathological evidence of early liver injury in epileptic dogs that do not have clinical signs of liver disease?

(2) Are increases in serum ALT and AP activities associated with increases in ALT and AP activities in the liver tissue of phenobarbital-treated dogs?

III. Effects of phenobarbital on the pancreas (Chapter 4)

(1) Is the high incidence of pancreatitis noted in this study in dogs receiving phenobarbital/potassium bromide combination therapy due to the combination of the two drugs, or due to one or the other drug?

CHAPTER 2. EFFECTS OF PHENOBARBITAL ON THE HYPOTHALAMIC-PITUITARY-THYROID AXIS

2.1 General introduction to the effects of phenobarbital on the hypothalamic-pituitary-thyroid axis

Phenobarbital is one of the most commonly used anticonvulsant drugs in dogs and is considered by many to be the drug of choice for the treatment of canine epilepsy (Boothe 1998, Brown 1988, Frey 1989, Parent 1988, Podell 1996, Schwartz-Porsche *et al.* 1985). Anecdotal reports have suggested that phenobarbital therapy causes a decrease in serum T4 concentrations in dogs (Belshaw 1983, Boothe 1998, Brown 1988, Evinger and Nelson 1984, Feldman and Nelson 1996, Ferguson 1984, Podell 1995). However, no clinical studies have been published that document the effects of phenobarbital therapy on the hypothalamic-pituitary-thyroid axis in epileptic dogs. It is not known if low serum T4 concentrations in phenobarbital-treated dogs are associated with clinical hypothyroidism or altered seizure control, or if thyroid hormone supplementation is indicated for a potential hypothyroid state or for improved seizure control. It is also not known if epilepsy *per se* is associated with alterations in thyroid hormone status in dogs.

Studies to determine the effects of phenobarbital on the hypothalamic-pituitary-thyroid axis in humans have produced conflicting results, but the majority conclude that serum T4 concentration is decreased in humans receiving phenobarbital therapy (Deda *et al.* 1992, Gomez *et al.* 1989, Ilyes *et al.* 1985, Kimura *et al.* 1995, Liewendahl *et al.* 1980, Rootwelt *et al.* 1978, Tanaka *et al.* 1987, Verma and Haidukewych 1994, Yeo *et al.*

1978, Yuksel *et al.* 1993). Additional studies in humans investigating the clinical significance of the decreased serum T4 concentrations associated with anticonvulsant therapy and the effects of thyroid hormone supplementation in these patients also have conflicting results (Kodama *et al.* 1989, Tiihonen *et al.* 1995). The mechanism of this decreased T4 concentration in humans is not clear.

In rats, phenobarbital therapy decreases serum T4 concentration with an accompanying increase in thyroid-stimulating hormone (TSH) concentration. This is due to a phenobarbital-induced increase in glucuronosyltransferase activity in the liver with a resultant increase in glucuronidation and excretion of T4 (Attia and Aref 1991, Barter and Klaassen 1992, Barter and Klaassen 1994, Curran and DeGroot 1991, De Sandro *et al.* 1991, Liu *et al.* 1995, McClain *et al.* 1989, Surks *et al.* 1969). Species differences exist throughout the hypothalamic-pituitary-thyroid axis (Capen 1994, Feliers and Pavlovic-Hournac1994, Ferguson 1989, Ferguson 1994, Ferguson 1998, Ichikawa *et al.* 1989, Larsson *et al.* 1985, Quin and Thomson 1994, Rapoport 1982, Schoenmakers *et al.* 1992, Toyoda *et al.* 1997), so the mechanism of decreased serum T4 concentration in rats may be different from that in humans or dogs.

Despite some specific differences, the hypothalamic-pituitary-thyroid axis has many general characteristics shared by most mammalian species and has been well described in the literature (Boye and Laurberg 1984, Chastain and Panciera 1995, Farwell *et al.* 1996, Feldman and Nelson 1996, Ferguson 1998, Kaptein *et al.* 1994, Oppenheimer 1972, Quin and Thomson 1994). Thyrotropin-releasing hormone (TRH) produced in the hypothalamus stimulates TSH production and secretion in the anterior pituitary gland; TSH stimulates the thyroid gland to produce predominantly T4, with much smaller

amounts of tri-iodothyronine (T3) and reverse T3 also produced. These thyroid hormones are released into the blood and are carried primarily in protein-bound form to the tissues, with usually less than 0.1% in the free form. Upon reaching tissues, the free hormones diffuse into the cells where much of the T4 is deiodinated to T3, the active thyroid hormone. The T4 and T3 are then further metabolized via progressive deiodinations in tissues to inactive mono-iodinated products, or by glucuronide and sulfate conjugation in the liver to metabolites that are excreted in urine and bile. The free hormones in blood have a negative feedback effect on the pituitary, hypothalamus, and thyroid. Decreases in concentrations of free thyroid hormones cause an increase in TSH synthesis and release, which in turn stimulates the thyroid gland to increase production of thyroid hormones. Conversely, a high concentration of free thyroid hormones causes the pituitary to decrease production of TSH and thus decrease production of thyroid hormones by the thyroid gland.

A number of drugs, including corticosteroids, propranolol, furosemide, sulfonamides, certain non-steroidal anti-inflammatory drugs and several anticonvulsant drugs such as carbamazepine, phenytoin, diphenylhydantoin, and phenobarbital have been reported to affect the hypothalamic-pituitary-thyroid axis in humans (Capen 1994, Cavalieri and Pitt-Rivers 1981, Curran and DeGroot 1991, Davies and Franklyn 1991, Farwell and Braverman 1996, Gupta *et al.* 1992, Lim *et al.* 1988, Lim *et al.* 1996). Many of these drugs may impact the hypothalamic-pituitary-thyroid axis in dogs as well (Chastain and Panciera 1995, Feldman and Nelson 1996, Ferguson 1998, Merchant and Taboada 1997). Some drugs have a direct effect on the thyroid gland; others affect the hypothalamus or pituitary gland, alter protein binding of thyroid hormones, or influence

metabolism and excretion of thyroid hormones; and some drugs cause their effect via a combination of several of the above. Species differences exist in all areas of the thyroid axis (Capen 1994, Feliers and Pavlovic-Hournac1994, Ferguson 1989, Ferguson 1994, Ferguson 1998, Ichikawa *et al.* 1989, Larsson *et al.* 1985, Quin and Thomson 1994, Rapoport *et al.* 1982, Schoenmakers *et al.* 1992, Toyoda *et al.* 1997), so species-specific studies are required to elucidate drug-thyroid axis interactions.

The purposes of this study were to determine if PB therapy is associated with changes in the hypothalamic-pituitary-thyroid axis in epileptic dogs, and if so, to determine the clinical significance and risk factors associated with a low serum T4 concentration in these patients. The following major questions are addressed in the next 3 sections.

(1) Is phenobarbital therapy associated with decreased serum thyroxine (T4) concentrations in dogs? If so, then:

— is the decrease in serum T4 concentration caused by the phenobarbital, or is it due to having the condition of epilepsy?

— is seizure activity associated with a low serum T4 concentration?

— when does the decrease in serum T4 concentration occur after the start of phenobarbital, and is the decrease transient or long-lasting?

(2) What is the potential mechanism of the decrease in serum T4 concentration?

— is the thyroid gland functioning properly in the phenobarbital-treated dogs with low serum T4 concentrations? Does it respond appropriately to stimulation with thyroid -stimulating hormone?

— are the central regions of the hypothalamic-pituitary-thyroid axis functioning properly? Are serum thyroid-stimulating hormone (TSH) concentrations altered in phenobarbital-treated dogs? If so, at what point in time after the start of phenobarbital does the serum TSH concentration change? How long does it remain altered? Is the change in serum TSH concentrations due to phenobarbital treatment or due to epilepsy?

— is the low serum T4 concentration an artifact due to assay interference by the parent drug phenobarbital?

— is the decrease in serum T4 concentration due to an alteration in protein binding of the serum T4 caused by the presence of the phenobarbital?

(3) What is the clinical significance of the low serum T4 concentration in phenobarbital-treated dogs?

— do these dogs with low serum T4 concentrations have clinical signs of hypothyroidism? Do they have better or worse seizure control than the dogs with normal serum T4 concentrations?

— does thyroid hormone supplementation alter clinical signs, degree of seizure control, or serum phenobarbital concentrations in phenobarbital-treated dogs with low serum T4 concentrations?

(4) In phenobarbital-treated dogs, are serum T4 and TSH concentrations altered after the addition of potassium bromide?

2.2 Cross-sectional study: Effects of phenobarbital on serum thyroxine and thyroid-stimulating hormone concentrations in epileptic dogs.

(This chapter is presented as published in J Am Vet Med Assoc 1999: 215: 489-496)

ABSTRACT

In a cross-sectional study, mean T4 concentration was significantly lower in phenobarbital (PB) treated epileptic dogs (n=78) than in untreated epileptic dogs (n=48). Forty percent of PB-treated dogs had T4 concentrations below reference range, compared to 8% of untreated dogs. Recent seizure activity was associated with low T4 concentrations in untreated dogs. Phenobarbital did not interfere with T4 assays. Serum T4 concentration was not related to dose or duration of PB therapy or degree of seizure control. Dogs with low T4 concentrations had no evidence of clinical hypothyroidism. Mean serum TSH concentration was significantly higher in PB-treated dogs than in untreated dogs, but only 7% of PB-treated dogs had TSH concentrations above reference range. Low T4 concentrations in epileptic dogs treated with phenobarbital should not be assumed to reflect true hypothyroidism.

INTRODUCTION

Phenobarbital (PB) is one of the most commonly used anticonvulsant drugs in dogs and is considered by many to be the drug of choice for the treatment of canine

epilepsy (Boothe 1998, Brown 1988, Frey 1989, Parent 1988, Podell 1996, Schwartz-Porsche and Jurgens 1991). Phenobarbital is a relatively safe and effective drug, but can be associated with several adverse effects. The most well documented of these include polydipsia, polyuria, polyphagia, lethargy and sedation (Boothe 1998, Brown 1988, Parent 1988). Hepatotoxicity has also been associated with PB therapy (Dayrell-Hart *et al.* 1991), as have hematological abnormalities and bone marrow dyscrasias (Boothe 1998, Jacobs and Kaufman 1998). In addition, anecdotal reports have suggested that PB therapy causes a decrease in serum thyroxine (T4) concentrations in dogs (Belshaw 1983, Boothe 1998, Brown 1988, Evinger and Nelson 1984, Feldman and Nelson 1996, Ferguson 1984, Podell 1995b). However, no clinical studies have been published that document the effects of PB therapy on the hypothalamic-pituitary-thyroid axis in epileptic dogs. It is not known if low serum T4 concentrations in PB-treated dogs are associated with clinical hypothyroidism or altered seizure control, or if thyroid hormone supplementation is indicated for a potential hypothyroid state or for improved seizure control. It is also not known if epilepsy per se is associated with alterations in the thyroid hormone status in dogs.

Studies to determine the effects of PB on the hypothalamic-pituitary-thyroid axis in humans have produced conflicting results, but the majority conclude that serum T4 concentration is decreased in humans receiving PB therapy (Deda *et al.* 1992, Gomez *et al.* 1989, Ilyes *et al.* 1985, Kimura *et al.* 1995, Liewendahl *et al.* 1980, Rootwelt *et al.* 1978, Tanaka *et al.* 1987, Verma and Haidukewych 1994, Yeo *et al.* 1978, Yuksel *et al.* 1993). Additional studies in humans investigating the clinical significance of the decreased serum T4 concentrations associated with anticonvulsant therapy and the effects

of thyroid hormone supplementation in these patients also have conflicting results (Kodama *et al.* 1989, Tiihonen *et al.* 1995). The mechanism of this decreased T4 concentration in humans is not clear.

In rats, PB therapy decreases serum T4 concentration with an accompanying increase in thyroid-stimulating hormone (TSH). This is due to a PB-induced increase in glucuronosyltransferase activity in the liver with a resultant increase in glucuronidation and excretion of T4 (Barter and Klaassen 1994, Curran and DeGroot 1991, De Sandro *et al.* 1991, Liu *et al.* 1995, McClain *et al.* 1989). Species differences exist throughout the hypothalamic-pituitary-thyroid axis, so the mechanism of decreased serum T4 concentration in rats may be different from that in humans or dogs.

The purposes of this study were to determine if PB therapy is associated with changes in the hypothalamic-pituitary-thyroid axis in epileptic dogs, and if so, to determine the clinical significance and risk factors associated with a low serum T4 concentration in these patients.

MATERIALS AND METHODS

Study population: Between September 1997 and June 1998, 126 epileptic dogs were recruited for the study by 96 veterinarians from 57 practices in Atlantic Canada and at the Atlantic Veterinary College (AVC). Two groups of epileptic dogs were studied. One group consisted of epileptic dogs that were receiving PB therapy (Group 1; n = 78). The second group consisted of epileptic dogs prior to the initiation of anticonvulsant therapy

(Group 2; n = 48). For serum T4 and TSH concentration comparisons, Group 2 dogs were subsequently divided into those dogs who had experienced seizure activity within 24 hours of blood collection (Group 2A; n=31) and those who had not recently seizured (Group 2B; n=16). Recent seizure history was unavailable for one of the original Group 2 dogs. No Group 1 dog had seizured within 24 hours of blood collection.

Dogs of different breeds, ages, sex, and duration of PB therapy were eligible for enrollment in the study, but the following criteria had to be met: (1) the dog must not have any major medical condition other than a presumptive diagnosis of idiopathic epilepsy. Physical examination must be normal and the dog must appear to be healthy. Dogs with very mild superficial skin and ear conditions (12 dogs in Group 1 and 3 dogs in Group 2) were included in the study. Subsequent exclusion of these dogs from statistical analysis did not alter statistical interpretation of the data, so these dogs were included in all statistical analyses. (2) The dog must have had its first seizure between 1 and 6 years of age, to help eliminate the possibility of conditions other than idiopathic epilepsy as the cause of seizures. (3) The dog could not be receiving, or have received within 2 months of the study, any major therapeutic drug (other than PB in the Group 1 dogs) with the following exceptions: dogs receiving monthly heartworm and flea and tick preventatives [Lufenuron (Program ®); milbemycin oxime (Interceptor ®); imidacloprid (Advantage ®); lufenuron plus milbemycin oxime (Sentinel ®)] (7 dogs in Group 1, 4 dogs in Group 2A, 1 dog in Group 2B), and dogs receiving therapy of less than one week duration with certain otic preparations [Canaural ® (diethanolamine fusidate, framycetin sulphate, nystatin, prednisolone 2.5 mg per gram of suspension); Surolan ® (miconazole nitrate, polymyxin B sulphate, prednisolone acetate 5 mg per ml of suspension);

Tresaderm ® (thiabendazole, neomycin sulphate, dexamethazone 1 mg per ml of solution)] (3 dogs in Group 1, 1 dog in Group 2A) were included in the study because these drugs at these dosages or durations of therapy were not expected to significantly affect serum T4 or TSH concentrations. Subsequent statistical analysis was the same regardless of whether these dogs were included or excluded from the analysis. Dogs who had recently been anesthetized for seizure control prior to blood collection were excluded from the study due to the reported effect of anesthesia on serum T4 concentrations (Chastain and Panciera 1995, Ferguson 1998). However, dogs who had received diazepam for seizure control prior to blood collection were included in the study (4 dogs in Group 2A). Subsequent removal of these dogs from statistical analysis did not change the interpretation of the data, so these dogs were included in all statistical analyses.

Data collection and assays: The following data were obtained from Group 1 and Group 2 dogs: signalment, seizure history, information on recent drug administration or concurrent medical conditions, serum total T4 (enzyme immunoassay) [CEDIA T4 homogenous enzyme immunoassay, Boehringer Mannheim Corp, Indianapolis, IN] (Horney *et al.* 1999) and TSH [Coat-A-Count canine TSH immunoradiometric assay, Diagnostic Products Corp, Los Angeles, CA] (Williams *et al.* 1996) concentrations, and serum biochemical analysis [Hitachi 911, Boehringer Mannheim Corp, Indianapolis, IN] (including alkaline phosphase (AP), aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyl transpeptidase (GGT), cholesterol and fasting bile acids). Serum PB [CEDIA phenobarbital homogenous enzyme immunoassay, Boehringer Mannheim Corp, Indianapolis, IN] concentration and dose and duration of PB

therapy were recorded for all Group 1 dogs.

Blood samples were collected and sent by courier to the AVC Diagnostic Laboratory as per routine for each participating veterinarian, with the following stipulations: patients were to be fasted for 8-12 hours prior to blood collection if possible; samples from Group 1 dogs were to be drawn 8-12 post-dosing of phenobarbital; and use of serum separator tubes was prohibited due to the effect of the gel in these tubes on PB concentration in serum (Boothe *et al.* 1996). Severely hemolyzed or lipemic serum samples were rejected.

Additional tests performed by the Michigan State University (MSU) Animal Health Diagnostic Laboratory [Animal Health Diagnostic Laboratory, Michigan State University, East Lancing, MI] included the following tests: T4 (radioimmunoassay) [Total T4, Radioimmunoassay, Chiron Diagnostic Corp (formerly CIBA-Corning), East Walpole, MA], fT4 (equilibrium dialysis) [Free T4 by dialysis, Radioimmunoassay, Nichols Institute Diagnostics, San Juan Capistrano, CA] and T3 [Total T3, Radioimmunoassay, in-house charcoal separation method, Michigan State University, East Lancing, MI] concentrations, and determination of autoantibody concentrations against thyroglobulin [Canine thyroglobulin autoantibody, ELISA, Oxford Biochemical Research, Oxford, MI], T4, and T3 [T4 and T3 autoantibodies, in-house charcoal separation binding assay, Michigan State University, East Lancing, MI].

Reference group: A historical reference group of 100 healthy dogs used to determine the AVC Diagnostic Laboratory serum T4 reference range was used in comparisons when appropriate.

Seizure classification: Seizure history for the three month period immediately prior to study enrollment was used to categorize seizure control in Group 1 dogs. Dogs that had not experienced any seizures in the three months prior to enrollment were categorized as having good seizure control. Dogs that had one or two seizure episodes in the three months previous to enrollment were classified as having fair control; and those that had experienced one or more seizure episodes per month were categorized as having poor control. A second data set depicting seizure control was devised by assigning to each dog one point for each seizure episode in the last 3 months prior to study enrollment, plus one point for generalized seizure activity, and an additional point for cluster type seizure activity. (For example, a dog experiencing one generalized seizure in the 3 month period would receive a seizure score of 2, whereas a dog having one episode of clustered generalized seizures in the 3 month period would have a seizure score of 3).

In vitro experiments: The *in vitro* effects of PB on serum T4 and fT4 concentrations were studied by adding therapeutically relevant concentrations of PB to sera from normal dogs. Serum samples from each of 4 dogs were divided into 2 aliquots. One aliquot from each sample was spiked with PB [Sigma Aldrich Canada Ltd., Oakville Ontario, Canada] dissolved in 0.5% DMSO [Sigma Aldrich Canada Ltd., Oakville Ontario, Canada] to give a mean serum PB concentration of 115 μmol/L. The other aliquot served as a control, with only the equivalent amount of 0.5% DMSO carrier added. All aliquots were analyzed for PB [CEDIA phenobarbital homogenous enzyme immunoassay, Boehringer Mannheim Corp, Indianapolis, IN] and T4 (enzyme immunoassay) [CEDIA T4

homogenous enzyme immunoassay, Boehringer Mannheim Corp, Indianapolis, IN] concentrations at the AVC Diagnostic Laboratory, and fT4 [Free T4 by dialysis, Radioimmunoassay, Nichols Institute Diagnostics, San Juan Capistrano, CA] and T4 (radioimmunoassay) [Total T4, Radioimmunoassay, Chiron Diagnostic Corp (formerly CIBA-Corning), East Walpole, MA] concentrations at the MSU Animal Health Diagnostic Laboratory [Animal Health Diagnostic Laboratory, Michigan State University, East Lancing, MI].

Statistical analysis: Continuous variables were examined for normality and then compared by use of Students T-test [SPSS Inc. Software, Chicago, IL] Mann-Whitney U test [SPSS Inc. Software, Chicago, IL] or one-way ANOVA [GraphPad Software, Inc., Prism2, San Diego, CA]. When significant F values were found by ANOVA, Bonferroni's multiple comparison test was used to compare groups. Results of *in vitro* experiments were compared by paired-samples T-test [SPSS Inc. Software, Chicago, IL]. Categorical variables were examined for association by use of Chi Square analysis [SPSS Inc. Software, Chicago, IL]. Pearson's or Spearman correlation coefficient (r) [SPSS Inc. Software, Chicago, IL] tests were used to determine correlations, depending upon normality of the data. Where appropriate, multiple regression models [SPSS Inc. Software, Chicago, IL] were used to examine possible linear relationships among the variables. The observed level of significance was taken to be 0.05 throughout.

RESULTS

Comparison and description of groups: Group 1 consisted of 46 males (36 of which were neutered) and 32 females (27 spayed). Ages ranged from 1 to 12.5 years (mean ± SD: 5.6 ± 2.8 years). Body weight ranged from 1.3 to 47.0 kg (2.9 to 103.4 lb), median 21.2 kg (46.6 lb). Breeds most commonly represented were: mix breed (n=22), Dalmation (7), Golden Retriever (5), Miniature Poodle (5), Labrador Retriever (4), Yorkshire Terrier (4), Border Collie (3), Husky (3), Beagle (3), Shetland Sheepdog (3), and German Shepherd (3).

Serum PB concentrations in Group 1 dogs ranged from 19 to 304 μmol/L (4 to 70 μg/ml), median 76.5 μmol/L (17.6 μg/ml), and total duration of PB therapy ranged from 1.3 weeks to 8 years (median 12.5 months). Total daily dose of PB ranged from 1.0 to 16.4 mg/kg/day (0.5 to 7.5 mg/lb/day), median 4.0 mg/kg/day (1.8 mg/lb/day). Seventy-one of the 78 Group 1 dogs (91%) had received PB for a sufficient period of time (3 months) to categorize seizure control. Thirty-seven percent had good seizure control, 36% had fair control, and 27% had poor control. The data set of seizure scores ranged from 0 (no seizures in the preceding 3 months) to 13.5 (many generalized cluster-type seizure episodes per month). Median seizure score was 3.0 points.

Group 2 dogs consisted of 26 males (21 neutered) and 22 females (16 spayed). Ages ranged from 1 to 10 years (3.9 ± 2.1 years). Body weight ranged from 2.7 to 68.2 kg (5.9 to 150 lbs), median 22.6 kg (49.7 lbs). Breeds most commonly represented were: mix breed (n=26), Golden Retriever (3), Border Collie (2), Labrador Retriever (2), German Shepherd (2), and Nova Scotia Duck Tolling Retriever (2).

Two significant differences in signalment and seizure history were found between Group 1 and Group 2 dogs. Mean age was significantly higher ($P<0.001$) in Group 1

dogs (5.6 ± 2.8 years) than in Group 2 dogs (3.9 ± 2.1 years). Also, a difference in recent seizure activity was found between Group 1 and Group 2 dogs. No Group 1 dogs had experienced seizure episodes within 24 hours prior to blood collection, but 66% of Group 2 dogs had experienced seizures within that time period.

T4 comparisons: Because of the difference between Group 1 and Group 2 dogs in recent seizure activity, a comparison within Group 2 dogs was made to determine if recent seizure activity affected serum T4 concentrations. Group 2 was subdivided into Group 2A (those dogs that had experienced seizures within 24 hours of blood collection) and Group 2B (those dogs that had not recently seizured). Mean serum T4 concentration was significantly lower ($P<0.05$) in Group 2A dogs (23.5 ± 8.4 nmol/L) than Group 2B dogs (30.4 ± 7.7 nmol/L) (**Figure 1**). Exclusion of Group 2 dogs that had recently received diazepam did not change the results of the comparison. When the 4 dogs who had received diazepam were excluded from analysis, the mean serum T4 concentration was still significantly lower ($P<0.05$) in Group 2A dogs (24.3 ± 8.4 nmol/L) than Group 2B dogs (30.4 ± 7.7 nmol/L). Therefore these 4 dogs were included in all statistical analyses.

Mean serum T4 concentration in Group 2A dogs was also significantly lower ($P<0.001$) than the mean serum T4 concentration of a reference group of 100 healthy dogs (33.5 ± 9.3 nmol/L) used to determine the AVC Diagnostic Laboratory T4 reference range. However, mean serum T4 concentration in Group 2B dogs was not significantly different than this reference group.

Mean serum T4 concentration was significantly lower in Group 1 dogs (18.3 ± 8.4 nmol/L) than in either Group 2A ($P<0.05$) or Group 2B ($P<0.001$) dogs (**Figure 2**).

Figure 1. Effect of recent seizure activity on mean serum T4 concentration in untreated epileptic dogs. Group 2A = untreated epileptic dogs that had experienced seizures within 24 hours prior to blood collection (n=31). Group 2B = untreated epileptic dogs that had not seizured within 24 hours of blood collection (n=16). Reference = AVC Diagnostic Laboratory historical reference group (n=100). Dashed line represents lower limit of AVC normal range. Bars represent mean ± SEM.

(a = Reference vs Group 2A, P<0.001. b = Group 2A vs Group 2B, P<0.05).

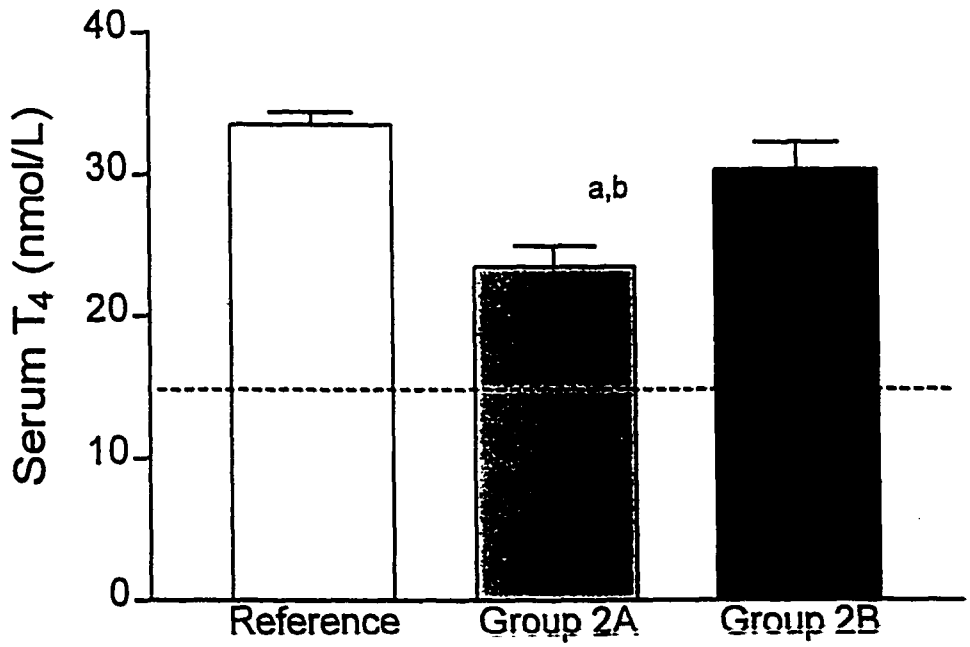
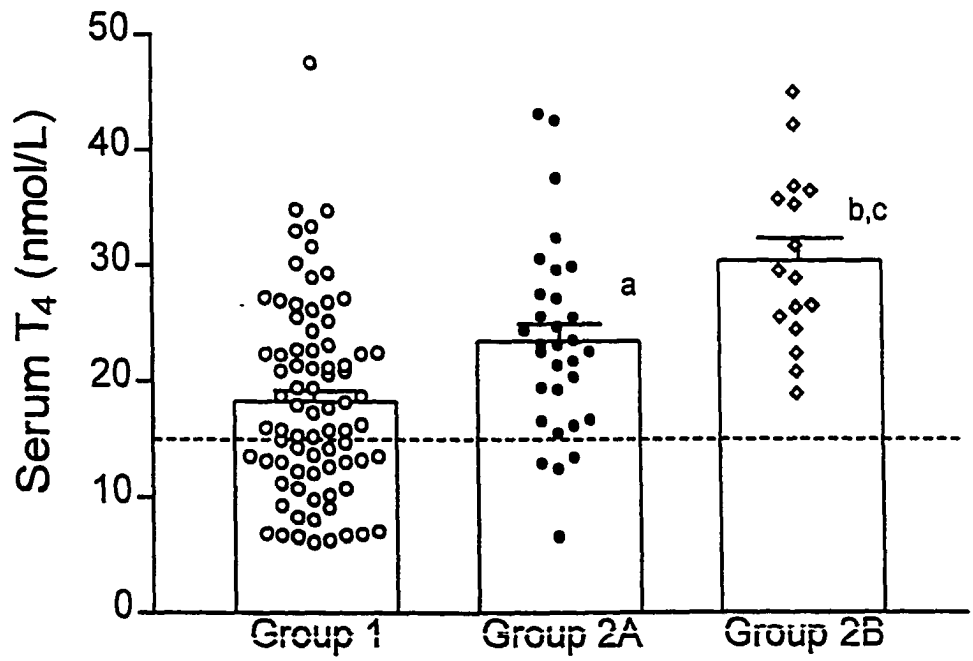


Figure 2. Effect of PB therapy on serum T4 concentration in epileptic dogs. Group 1 = PB-treated epileptic dogs (n = 78). Group 2A = untreated epileptic dogs who had experienced seizures within 24 hours prior to blood collection (n = 31). Group 2B = untreated epileptic dogs who had not seizured within 24 hours of blood collection (n = 16). Dashed line represents lower limit of AVC normal range. Individual data points are presented, and bars represent mean ± SEM. (a = Group 1 vs Group 2A, $P<0.05$. b = Group 1 vs Group 2B, $P<0.001$. c = Group 2A vs Group 2B, $P<0.05$).



Thirty-one of the 78 Group 1 dogs (40%), 4 of the 31 Group 2A dogs (13%), and none of the 16 Group 2B dogs had a serum T4 concentration below the AVC Diagnostic Laboratory reference range (range 15-52 nmol/L).

TSH comparisons: Mean serum TSH was significantly higher ($P<0.01$) in Group 1 dogs (0.22 ± 0.16 ng/ml) than in either Group 2A (0.12 ± 0.04 ng/ml) or Group 2B dogs (0.11 ± 0.05 ng/ml) (**Figure 3**). Mean serum TSH concentration of Group 2A was not significantly different than Group 2B. Five of the 72 Group 1 dogs (7%) and none of the 46 Group 2 dogs for whom TSH data were available had serum TSH concentrations above the reference range (reference range: < 0.50 ng/ml). Three of the five Group 1 dogs with a high TSH concentration had a low T4 concentration; two had normal T4 concentrations. Serum TSH concentrations for six Group 1 dogs and two Group 2 dogs were unavailable due to inadequate serum volume. Serum TSH concentrations of < 0.1 ng/ml were recorded as 0 ng/ml.

Correlations: There was no significant relationship between serum T4 concentration and TSH concentration, age, sex, PB dosage, duration of treatment, or serum PB concentration in the Group 1 dogs (**Figure 4**). There were no clinical signs consistent with overt hypothyroidism reported in those Group 1 dogs with low serum T4 concentrations, with the exception of excessive body weight in occasional dogs. There was no clear relationship between serum T4 concentration and the degree of seizure control or seizure score (**Figure 5**).

Figure 3. Effect of PB therapy on serum TSH concentration in epileptic dogs.

Group 1 = PB-treated epileptic dogs (n = 72). Group 2A = untreated epileptic dogs who had seizured within 24 hours prior to blood collection (n = 29). Group 2B = untreated epileptic dogs who had not seizured within 24 hours of blood collection (n = 16). Dashed line represents upper limit of AVC normal range. Individual data points are presented, and bars represent mean ± SEM.

(a = Group 1 vs Group 2A, $P<0.01$. b = Group 1 vs Group 2B, $P<0.01$).

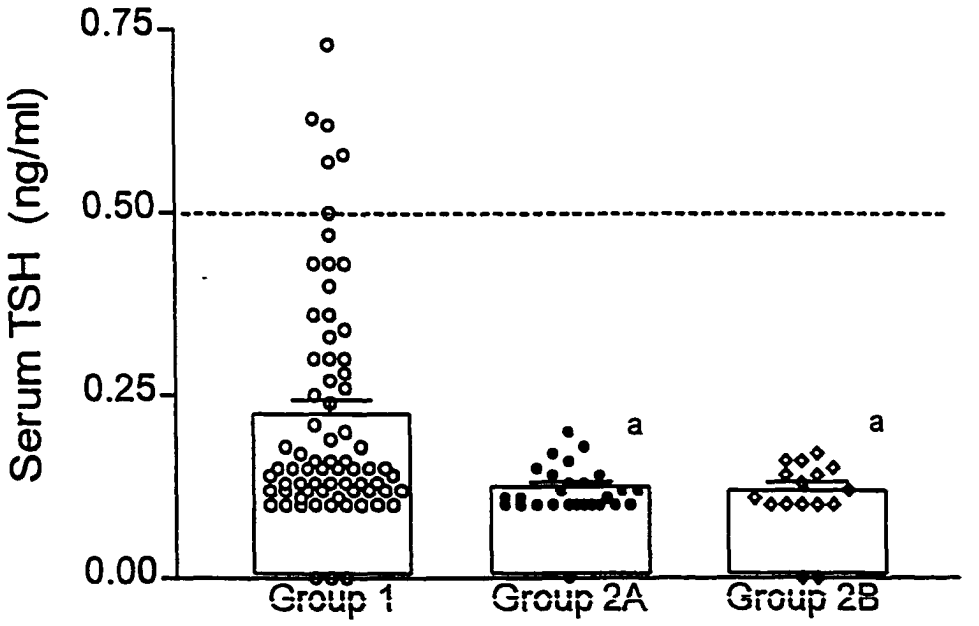


Figure 4. Scatterplots showing lack of significant linear relationship between serum T4 concentrations and TSH concentration, age, PB dosage, duration of treatment, or serum PB concentration in PB-treated epileptic dogs; and only weak positive correlation between serum T4 and cholesterol concentrations.

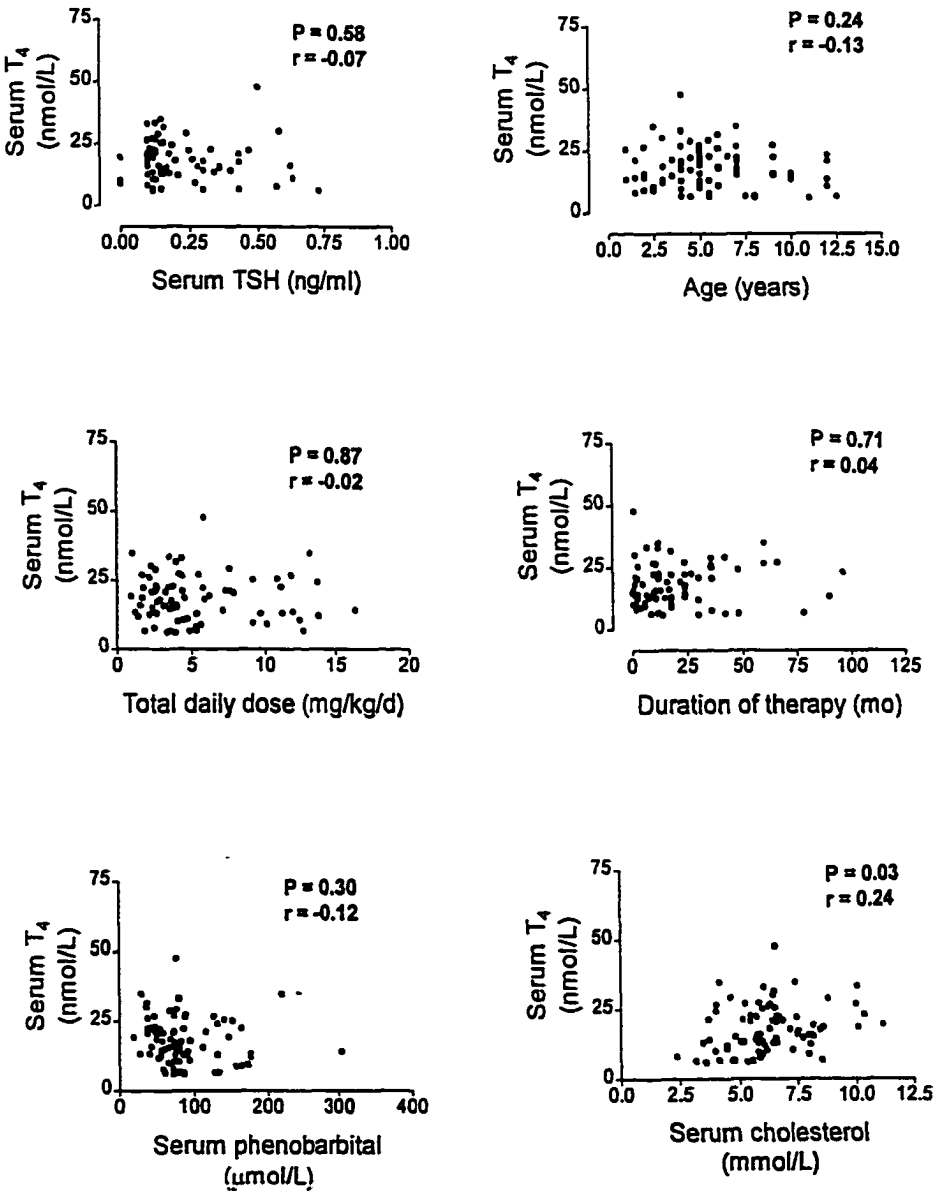
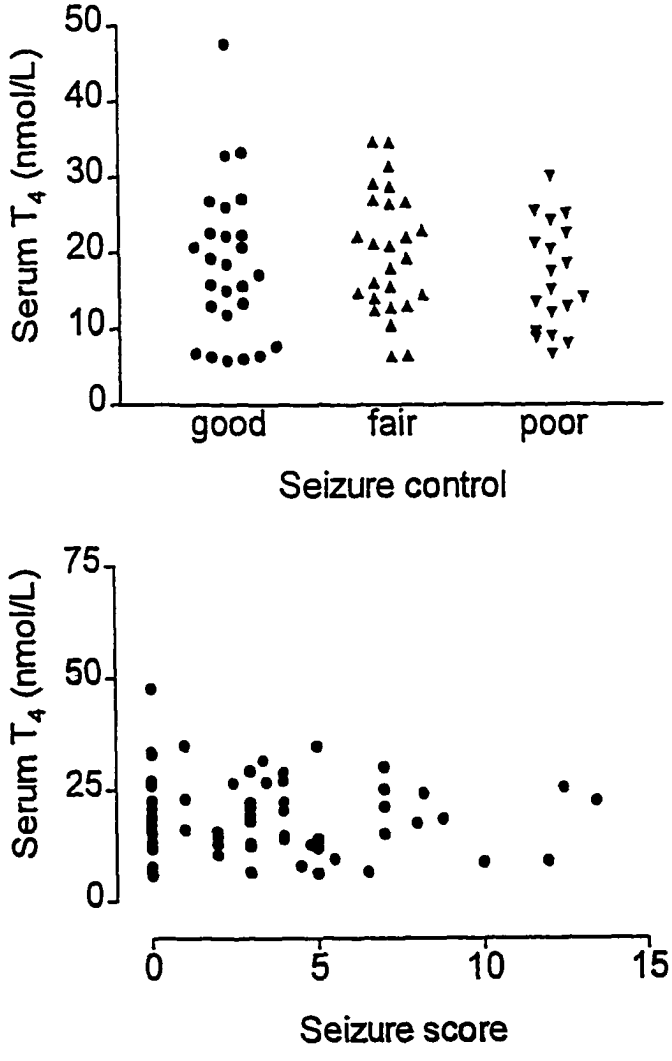


Figure 5. Serum T4 concentration vs seizure control and seizure score in PB-treated epileptic dogs (n = 71). Good control = less than one seizure every 3 months. Fair control = one seizure every 2 months. Poor control = more than one seizure per month. Seizure score for each dog was calculated as follows: one point was assigned for each seizure episode in the last 3 months prior to study enrollment; an additional point was added for generalized seizure activity, plus an additional point if seizures occurred in clusters.



Other tests: Additional serum biochemical test results (including ALT, AP, AST, GGT, cholesterol, and fasting bile acids) were evaluated in Group 1 and Group 2 dogs as part of a concurrent study on effects of PB on the liver in epileptic dogs. As expected, several abnormalities commonly associated with PB therapy (ie, increased alkaline phosphatase activity) were detected in many of the Group 1 dogs. However, for the purposes of the study reported here, only associations between serum T4 concentration and these other biochemical tests were considered. A weak positive correlation between serum T4 and cholesterol concentrations ($r=0.24$) was the only significant relationship found (**Figure 4**).

Standard clinical cut-off points were utilized to develop contingency tables for further statistical analysis. Other than the previously mentioned association between T4 and cholesterol, there were no significant relationships between T4 concentration and other factors (including other biochemical tests, seizure control, serum PB concentration, TSH concentration) in PB-treated dogs. Multiple regression models were also employed, but due to multi-colinearity and underlying correlations between secondary variables, no stable models were obtained.

Additional thyroid tests: Analysis of 9 randomly selected serum samples from Group 1 dogs with low serum T4 concentrations revealed that 6/9 had fT4 (equilibrium dialysis) concentrations below the reference range; 1/9 had low T3 concentration, and none had autoantibodies against thyroglobulin, T4, or T3.

In vitro tests: The addition of PB to serum (final mean concentration of 115 μmol/L) had

no significant effect on serum T4 concentration. Mean T4 concentrations for the control aliquots compared to the spiked aliquots were, respectively, 27.5 ± 10.9 versus 28.1 ± 6.3 nmol/L for total T4 measured by enzyme immunoassay ($P>0.05$); and 24.25 ± 8.3 verus 23.0 ± 6.4 nmol/L for total T4 measured by radioimmunoassay ($P>0.05$). Addition of PB to serum did have a statistically significant effect on serum fT4: mean fT4 concentrations for the control aliquots compared to the spiked aliquots were 17.0 ± 4.7 versus 18.5 ± 4.8 pmol/L, respectively ($P<0.05$).

DISCUSSION

The hypothalamic-pituitary-thyroid axis has general characteristics shared by most mammalian species and has been well described in the literature (Chastain and Panciera 1995, Farwell and Braverman 1996, Feldman and Nelson 1996, Kaptein *et al.* 1994, Quin and Thomson 1994). Thyrotropin-releasing hormone produced in the hypothalamus stimulates TSH production and secretion in the anterior pituitary gland; TSH stimulates the thyroid gland to produce predominantly T4, with much smaller amounts of T3 and reverse T3 also produced. These thyroid hormones are released into the blood and are carried primarily protein-bound to the tissues, with usually less than 0.1% in the free form. Upon reaching tissues, the free hormones diffuse into the cells where much of the T4 is deiodinated to T3, the active thyroid hormone. The T4 and T3 are then further metabolized via progressive deiodinations in tissues to inactive mono-iodinated products, or by glucuronide and sulfate conjugation in the liver to metabolites

that are excreted in urine and bile. The free hormones in blood have a negative feedback effect on the pituitary, hypothalamus, and thyroid. Decreases in concentrations of free thyroid hormones cause an increase in TSH synthesis and release, which in turn stimulates the thyroid gland to increase production of thyroid hormones. Conversely, a high concentration of free thyroid hormones causes the pituitary to decrease production of TSH and thus decrease production of thyroid hormones by the thyroid gland.

A number of drugs including corticosteroids, propranolol, furosemide, sulfonamides, certain non-steroidal anti-inflammatory drugs and several anticonvulsant drugs such as carbamazepine, phenytoin, diphenylhydantoin, and phenobarbital have been reported to affect the hypothalamic-pituitary-thyroid axis in humans (Capen 1994, Curran and DeGroot 1991, Davies and Franklyn1991, Farwell and Braverman 1996, Lim *et al.* 1988). Many of these drugs may impact the hypothalamic-pituitary-thyroid axis in dogs as well (Chastain and Panciera 1995, Feldman and Nelson 1996, Ferguson 1998, Merchant and Taboada 1997). Some drugs have a direct effect on the thyroid gland; others affect the hypothalamus or pituitary gland, alter protein binding of thyroid hormones, or influence metabolism and excretion of thyroid hormones; and some drugs cause their effect via a combination of several of the above (Cavalieri and Pitt-Rivers 1981). Species differences exist in all areas of the thyroid axis (Capen 1994, Feliers and Pavlovic-Hournac 1994, Ferguson 1998, Ichikawa *et al.* 1989, Kaptein *et al.* 1994, Larsson *et al.* 1985, Rapoport *et al.* 1982, Schoenmakers *et al.* 1992) so species-specific studies are required to elucidate drug-thyroid axis interactions.

Reports of the effect of PB on the hypothalamic-pituitary-thyroid axis in epileptic dogs have been unsubstantiated until now. In our study, phenobarbital therapy was

associated with significantly lowered serum T4 concentrations in epileptic dogs. The *in vitro* portion of the study showing that PB in serum does not interfere with T4 assays verifies that the decrease in serum T4 concentration is not an artifact caused by presence of the parent drug in serum. The comparisons of fT4 concentrations before and after the addition of PB to serum showed only minimal displacement (<10%) of T4 from serum protein binding sites, suggesting that a change in protein binding alone cannot account for the decrease in serum T4 concentration in epileptic dogs treated with phenobarbital.

Total T4 rather than fT4 by equilibrium dialysis was chosen as the baseline thyroid test for this study because total T4 is more commonly used as a routine thyroid screening test than is fT4. Analysis of the 9 randomly selected serum samples from Group 1 dogs with low T4 showed that 6 out of the 9 also had low fT4 concentrations. Additional studies would be needed to fully evaluate the effect of phenobarbital on serum fT4 concentrations, but based on these results, fT4 would be expected to be decreased.

Serum autoantibodies against T4 may affect T4 determinations. They may falsely decrease T4 determinations by enzyme immunoassay, or falsely increase T4 determinations by radioimmunoassay (Horney *et al.* 1999). Nine randomly selected serum samples from Group 1 dogs with low T4 concentrations showed that none of the samples had autoantibodies to T4, T3, or thyroglobulin. Because there was no indication that autoantibodies against T4 played a role in the decreased mean T4 concentration found in Group 1 dogs, other serum samples were not submitted for these additional tests.

The finding that seizure activity within 24 hours prior to blood collection may decrease serum T4 concentrations suggests that assessment of T4 concentrations in serum

obtained in that time frame may be misleading. Those dogs with low serum T4 concentrations after seizuring had serum T4 concentrations within the normal range when repeated at a time not associated with seizure activity (data not shown). The finding that serum T4 concentrations were normal in untreated epileptic dogs who had not experienced seizures within 24 hours of blood collection suggests that epilepsy per se is not the cause of the low serum T4 concentration in PB-treated epileptic dogs.

The difference in mean age of Group 1 dogs (5.6 ± 2.8 years) compared to Group 2 dogs (3.9 ± 2.1 years) was found to be statistically significant, but from a physiological perspective this difference is small. Initial seizure activity in all dogs was reported to occur between the ages of 1 and 6 years of age. Some studies have reported that serum T4 concentration is inversely correlated with age (Ferguson 1989, Gonzalez and Quadri 1988, Reimers *et al.* 1990), with the decrease in T4 starting soon after adulthood is reached. This trend reverses and T4 concentration begins to increase again after age 9 or 10. Because of the small difference in age between our groups, and because we observed no relationship between age and T4 in either Group 1 or Group 2 dogs, it is unlikely that the difference in age between our groups accounts for the differences in serum T4 concentration, either as a result of the normal aging process or number of years of seizure activity. Previous studies have shown that other factors such as time of day, length of patient fast, and storage and transport of blood should not have significant effect on the T4 concentrations obtained in our study (Behrend *et al.* 1998, Feldman and Nelson 1996, Ferguson 1989, Kaptein *et al.* 1994, Miller *et al.* 1992, Minten *et al.* 1985).

No clinical signs of overt hypothyroidism were reported in the Group 1 dogs with decreased T4 concentrations in this study, other than an occasional dog with weight gain.

Many of the dogs with weight gain were also polyphagic, another common effect of PB, so the weight gain is more likely due to increased caloric intake rather than decreased metabolic rate. Additional studies are needed to further evaluate the clinical significance of the low serum T4 concentration in PB-treated dogs. Many veterinarians prescribe thyroid hormone supplementation for PB-treated epileptic dogs with low T4 concentrations, but the risks or benefits of this practice are unknown. No clinical trials evaluating the effects of thyroid hormone supplementation in PB-treated dogs with low serum T4 concentrations have been reported. Thyroid hormone supplementation trials have been conducted in humans receiving anticonvulsants that develop low T4 concentrations, but the results of these studies have been conflicting (Kodama *et al.* 1989, Tiihonen *et al.* 1995).

No obvious relationship, either favorable or unfavorable, was found between degree of seizure control and T4 concentration in Group 1 dogs. If more detailed seizure history had been available, an association between T4 concentration and type of seizure or length of each seizure episode might have been observed. However, this appears to be unlikely.

Concurrent illness was not found to play a role in the decrease in serum T4 concentration in the PB-treated dogs in this study. No dogs had clinical signs of disease other than epilepsy, except a small number of animals with minor superficial skin or ear conditions. Exclusion of these animals from the statistical analysis did not change the interpretation of the data. Mean serum T4 concentration of all Group 1 dogs was 18.3 ± 8.4 nmol/L; when the 12 dogs with minor skin or ear conditions were excluded from the analysis, the mean serum T4 concentration of the remaining 66 dogs in Group 1 was 18.6

± 8.5 nmol/L. Several biochemical abnormalities commonly associated with PB therapy (ie, increased alkaline phosphatase activity) were detected in many of the Group 1 dogs. However, no relationships between T4 concentration and alterations in ALT, AST, AP, or GGT activities were found.

Use of monthly heartworm or flea and tick preventatives and the short-term use of otic preparations also were not found to play a role in the decreased T4 concentrations in Group 1 dogs. Mean serum T4 concentration of all Group 1 dogs was 18.3 ± 8.4 nmol/L: mean serum T4 concentration of Group 1 dogs excluding those dogs receiving heartworm or flea and tick preventative was 18.0 ± 8.4 nmol/L, and mean T4 of Group 1 dogs excluding those dogs receiving otic preparations was 18.3 ± 8.3 nmol/L. Statistical P values were not affected by exclusion of these dogs.

The weak positive correlation between cholesterol and T4 concentrations, with cholesterol increasing as T4 increased, was opposite to what would be expected if these dogs were truly hypothyroid (Chastain and Panciera 1995, Feldman and Nelson 1996, Ferguson 1994, Panciera 1994). The cause and the significance of this relationship between cholesterol and T4 concentration was not determined by this study.

Mean serum TSH concentration in Group 1 dogs was approximately double that of Group 2 dogs. This indicates that the hypothalamus and pituitary are responding to the decrease in blood concentrations of T4. Therefore, the mechanism of the decrease in serum T4 concentration in PB-treated dogs is likely due to a more peripheral effect on either the thyroid gland or on T4 disposition rather than a central effect on the hypothalamus or pituitary.

Several recent studies evaluating the usefulness of the canine TSH test have

indicated that a linear relationship between serum TSH and T4 concentrations is not necessarily present in dogs. Studies have shown that anywhere from 8 to 12% of euthyroid dogs may have an elevated TSH concentration, and that 25 to 38% of dogs with hypothyroidism have a normal TSH concentration (Peterson *et al.* 1997, Scott-Moncrieff *et al.* 1998). Also, considerable overlap of TSH concentrations between hypothyroid, euthyroid sick, and euthyroid dogs exists (Jensen 1996, Ransey 1997). Therefore, the lack of a linear correlation between T4 and TSH concentrations in our study was not surprising. Furthermore, the lack of correlation cannot be interpreted to suggest a central effect of PB, because no correlation between T4 and TSH was found in either Group 1 or Group 2 dogs.

Additional studies are needed to determine the mechanism of decreased T4 concentrations in PB-treated dogs. Thyroid function tests such as TSH stimulation tests would be very helpful in determining thyroid gland function. Due to lack of availability of medical grade TSH, TSH stimulation tests were not performed in this study. Because of the potential adverse effects (Feldman and Nelson 1996, Ferguson 1994) of thyrotropin-releasing hormone TRH stimulation tests, we elected not to perform this test in our study. Other tests such as thyroid scintigraphy, thyroid gland biopsies, or radio-labeled hormone excretion studies were also inappropriate for a clinical study of client-owned dogs but could be considered for an experimental study.

Many studies have shown that the decrease in T4 concentrations in rats treated with PB is due to induction of glucuronosyltransferase activity in the liver with a resultant increase in glucuronidation and excretion of T4 (Barter and Klaassen 1994, Curran and DeGroot 1991, De Sandro *et al.* 1991, Liu *et al.* 1995, McClain *et al.* 1989).

Measurement of glucuronosyltransferase activity in liver tissue from PB-treated dogs may help determine if this mechanism is involved in the decreased serum T4 concentration in PB-treated dogs.

The study reported here was a clinical study that depended upon multi-veterinarian cooperation. Presumptive diagnosis of epilepsy was made by each participating veterinarian based on physical examination, history, age of onset of seizures and absence of identifiable extra-cranial causes of seizures. Although most diagnoses were made without benefit of CT scans and cerebrospinal fluid analysis, we do not believe this influences the interpretation of our results.

The cross-sectional nature of the study gave us the ability to evaluate dogs who had been receiving PB therapy for many years. Inherent in this design, however, is the lack of pre-treatment blood analyses for those dogs. A prospective study of the epileptic dogs whose baseline pre-PB blood values are reported here is underway. The prospective study will be able to address many of the issues and questions raised by the present study.

In conclusion, we have shown that PB therapy decreases serum T4 concentrations and increases serum TSH concentrations in epileptic dogs. In our study, the decrease in T4 concentration was not associated with clinical signs of hypothyroidism or degree of seizure control. No evidence was found to support the use of thyroid hormone supplementation in these dogs. Clinicians should use caution when interpreting thyroid test results in dogs receiving PB therapy, and should be aware that the risks or benefits of thyroid hormone supplementation in these cases are still unknown.

2.3 Prospective study: Changes in serum thyroxine and thyroid-stimulating hormone concentrations in epileptic dogs receiving phenobarbital for one year

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ABSTRACT

A multicentric prospective study was conducted to monitor the effect of phenobarbital on serum total thyroxine (T_4) and thyroid-stimulating hormone (TSH) concentrations in epileptic dogs. Serum T_4 concentrations were determined for 22 epileptic dogs prior to initiation of phenobarbital therapy (time 0), and 3 weeks, 6 months, and 12 months after the start of phenobarbital. Median T_4 concentration was significantly lower at 3 weeks and 6 months compared to time 0. Thirty-two percent of dogs had T_4 concentrations below the reference range at 6 and 12 months. Nineteen of the 22 dogs had serum TSH concentrations determined at all sampling times. A significant upward trend in median TSH concentration was found. No associations were found between T_4 concentration, dose of phenobarbital, or serum phenobarbital concentration. No signs of overt hypothyroidism were evident in dogs with low T_4, with one exception. Thyroid-stimulating hormone stimulation tests were performed on 6 of 7 dogs with low T_4 concentrations at 12 months, and all but 1 had normal responses.

In conclusion, phenobarbital therapy decreased serum T_4 concentration but did not appear to cause clinical signs of hypothyroidism. Serum TSH concentrations and TSH stimulation tests suggest that the hypothalamic-pituitary-thyroid axis is functioning

appropriately.

INTRODUCTION

Phenobarbital is one of the most commonly used anticonvulsants in dogs (Podell, 1996; Boothe, 1998). In a recent study (Gaskill *et al.* 1999), mean serum total thyroxine (T_4) concentration of 78 phenobarbital-treated epileptic dogs was significantly lower than that of untreated epileptic dogs. In the same study, mean serum thyroid-stimulating hormone (TSH) concentration was significantly higher in the phenobarbital-treated dogs than in the untreated dogs. Another study (Kantrowitz *et al.* 1999) of 55 phenobarbital-treated epileptic dogs showed serum T_4 concentrations were significantly lower in the phenobarbital-treated epileptic dogs compared to untreated healthy dogs, but no difference in serum TSH concentrations between the groups was found. Pre-treatment serum T_4 and TSH concentrations were not available for the phenobarbital-treated dogs in either study, so causation of the low serum T_4 concentration could not be definitively concluded. In a short-term study (Daminet *et al.* 1999) of 9 non-epileptic Beagle dogs with pre-treatment serum T_4 and TSH concentrations available, serum T_4 and TSH concentrations were not significantly affected during 6 weeks of treatment with phenobarbital.

The purposes of the study reported here were (1) to determine changes in serum T_4 and TSH concentrations during the first year after the initiation of phenobarbital therapy in epileptic dogs; and (2) to determine if low serum T_4 concentrations were associated with other risk factors or with clinical signs of hypothyroidism.

MATERIALS AND METHODS

Study population. The study was conducted according to the guidelines of the Canadian Council on Animal Care and was approved by the Animal Care Committee of the University of Prince Edward Island. Epileptic dogs were recruited for the study by veterinarians in 23 practices in the Atlantic provinces of Canada between September 1997 and June 1998. To be included in the study, epileptic dogs had to be otherwise healthy based on physical examination, history, and results of complete blood count (CBC) and serum biochemical analyses. Dogs with minor skin and ear conditions were allowed in the study. Dogs had to have been between 1 and 6 years of age when seizures first started, to decrease the possibility of extra-cranial causes of seizures. Dogs could not be receiving, or have received within 2 months prior to study enrollment, any major therapeutic drug. Dogs receiving monthly heartworm or flea and tick preventatives (Program®, Novartis; Advantage®, Bayer; Heartgard®, Merial) (3 dogs) and dogs receiving otic preparations (Surolan®, Janssen; Tresaderm®, Merial; Synotic®, Ayerst) (2 dogs) for < 1 week were included in the study, because these drugs at these dosages were not expected to affect serum T_4 concentrations (Gaskill *et al.* 1999). Dogs that had been anesthetized for seizure control immediately prior to blood collection were excluded from the study due to the reported effect of anesthesia on serum T_4 concentration (Chastain and Panciera 1995; Ferguson, 1998). However, 1 dog that had received diazepam for seizure control prior to blood collection was not excluded. Dogs that received long-term treatment with major therapeutic drugs other than phenobarbital during the course of the study were disqualified and removed from the study.

Fifty dogs were originally enrolled in the study so that, with an anticipated attrition rate of 30-40%, at least 30 dogs would remain in the study until completion. This would provide an estimated 90% power of detecting a 20% change in mean serum T_4 concentration (two-tailed significance of 0.05). Twenty-two dogs completed the study, with serum T_4 concentrations determined at all 4 sampling times. This sample size reduced the power to approximately 80%. Twenty-eight dogs were removed from the study. Four dogs were removed because their owners did not continue phenobarbital treatment. Six dogs were removed after potassium bromide was added to their anticonvulsant regimens. One dog was excluded from the study after being anesthetized for seizure control prior to blood collection. One dog died from unrelated trauma, and 2 dogs changed ownership. The history provided on the laboratory submission forms for 1 dog reported that the dog did not consistently receive the phenobarbital, and so the dog was disqualified from the study. Several dogs were euthanized at some point in the study: 3 for poor seizure control, and 2 for personal reasons of the owners. Eight dogs did not return for 1 or more blood collections for unknown reasons.

Data collection and assays. Serum samples were collected by participating veterinarians from all dogs prior to initiation of phenobarbital therapy (time 0), and again at 3 weeks, 6 months, and 12 months after the start of phenobarbital. Serum samples were sent by overnight courier to the Atlantic Veterinary College (AVC) Diagnostic Laboratory. Owners were requested to withhold food from their dogs for 8-12 hours prior to blood collection, and samples were collected 8-12 hours after administration of phenobarbital. Use of serum separator tubes was prohibited due to the effect of the gel in these tubes on

serum phenobarbital concentration (Boothe *et al.* 1996). Severely hemolyzed or lipemic serum samples were rejected and repeat samples were obtained. Data obtained for all dogs included signalment, seizure history, information on recent drug administration or concurrent medical conditions, total daily dose of phenobarbital, serum total T_4 (CEDIA T_4 homogeneous enzyme immunoassay, Bohringer Mannheim Corp, Indianapolis, Ind.) (Horney *et al.* 1999) and TSH (Coat-A-Count canine TSH immunoradiometric assay, Diagnostic Products Corp, Los Angeles, Calif.) (Williams *et al.* 1996) concentrations. Serum TSH concentrations < 0.1 ng/ml were recorded as 0 ng/ml. Additionally, results of serum biochemical analyses (Hitachi 911, Boehringer Mannheim Corp, Indianapolis, Ind.) including alkaline phosphatase, alanine aminotransferase, aspartate aminotransferase, gamma-glutamyltransferase activities and concentrations of cholesterol, albumin, urea, and fasting bile acids were recorded.

Seizure classification. Seizure control for each dog was determined using a previously described seizure classification scheme (Gaskill *et al.* 1999). Briefly, seizure history for a 3 month period prior to blood collection was used to categorize seizure control for the 6 month and 12 month rechecks. Dogs that had no seizures during the 3 month period preceding blood collection were categorized as having good control. Dogs with 1 or 2 seizures during the 3 months were classified as having fair control; and dogs with ≥ 1 seizure per month were categorized as having poor control. A second dataset for assessing seizure control was devised by assigning to each dog 1 point for each seizure episode during the 3 month period, plus 1 point for generalized seizure activity, and a second additional point for cluster-type seizure activity.

TSH stimulation tests. TSH stimulation tests were performed on dogs with low serum T_4 concentrations at 12 months. Unreconstituted desiccated TSH (Thytropar®, Roper Canada, Inc., Ontario) was provided to the participating veterinarians along with standard test protocol instructions (TSH to be administered by intravenous injection at a dose of 0.1 units per kg of body weight, to a maximum of 5 units; serum samples obtained pre- and 6 hour post-injection) (Chastain and Panciera 1995; Feldman and Nelson 1996). A normal response was defined as a post-TSH serum T_4 concentration that increased by at least 25 nmol/L or exceeded 50 nmol/L (normal reference range, 15 to 52 nmol/L). The TSH product was tested using the above protocol on 4 healthy dogs to confirm efficacy.

Statistical analyses. Data from the 22 dogs with serum T_4 concentration determinations for all 4 sampling times were used in the following statistical analyses. Data obtained at time 0, 3 weeks, 6 months, and 12 months were compared by repeated measures ANOVA (non-parametric Friedman test) (GraphPad Software Inc.). When significant F values were detected, a Dunn's multiple comparison post-test was used to compare groups. Parametric repeated measures ANOVA with post-test for linear trend was performed to establish trends. Standard clinical cutoff points were used to develop contingency tables, and categorical variables were examined for associations by use of χ^2 analysis (SPSS Inc. Software). Spearman's correlation coefficient (r) tests (SPSS Inc. Software) were used to determine correlations. Significance was defined as $P \leq 0.05$.

Several of the original 50 dogs enrolled in the study had serum T_4 and TSH concentrations determined at 3 of the 4 sampling times. Data from these dogs were not included in the analyses listed above, but were included in the following analyses. Non-

parametric Wilcoxon Signed Rank Sum Test paired t tests (GraphPad Software Inc.) were used to compare serum T_4 and TSH concentrations in (1) all dogs with these data determined at times 0 and 3 weeks; (2) times 0 and 6 months; and (3) time 0 and 12 months. Significance was defined as $P \leq 0.05$.

RESULTS

Population description. The 22 dogs that completed the study consisted of 10 males (9 neutered) and 12 females (8 spayed). Dogs ranged from 1 to 8 years old (mean ± SD, 3.9 ± 1.7 years) and weighed between 3.0 and 36.4 kg (median, 20.9 kg). Breeds consisted of 14 mixed breed dogs, 2 Golden Retrievers, 2 Labrador Retrievers, 1 Border Collie, 1 Husky, 1 Miniature Dachshund, and 1 Standard Poodle.

Serum phenobarbital concentrations ranged from 33 to 85 μmol/L (mean ± SD, 58.6 ± 15.0 μmol/L) at 3 weeks; 8 to 120 μmol/L (62.5 ± 25.7 μmol/L) at 6 months; and 11 to 116 μmol/L (62.2 ± 23.5 μmol/L) at 12 months. Total daily dose of phenobarbital ranged from 1.3 to 6.0 mg/kg/day (3.6 ± 1.3 mg/kg/day) at 3 weeks, 1.3 to 8.3 mg/kg/day (3.7 ± 1.4 mg/kg/day) at 6 months, and 1.3 to 8.3 mg/kg/day (3.7 ± 1.6 mg/kg/day) at 12 months. At 6 months, 17 dogs (77.3%) had good seizure control, 4 (18.2%) had fair control, and 1 (4.5%) had poor control. At 12 months, 15 dogs (68.2%) had good control, and 7 (31.8%) had fair control. Seizure scores at both time periods ranged from 0 to 4 (median, 0). Twelve dogs had seizures within 24 hours prior to blood collection at time 0. No dogs had seizures within 24 hours prior to any subsequent blood collection.

Serum T_4 concentration. Median serum T_4 concentration of the 22 dogs who completed the study was significantly ($P < 0.05$ and $P < 0.01$, respectively) lower at 3 weeks (19.0 nmol/L) and 6 months (17.6 nmol/L) compared to time 0 (24.5 nmol.L; **Fig. 1**). Median serum T_4 concentration at 12 months (19.4 nmol/L) was not significantly lower than time 0. Serum T_4 concentrations were below the lower limit of the AVC Diagnostic Laboratory reference range (15 to 52 nmol/L) for 3 dogs (13.6%) at time 0, 6 dogs (27.3%) at 3 weeks, and 7 dogs (31.8%) at both 6 and 12 months. All 3 of the dogs with low serum T_4 concentration at time 0 had seizures within 24 hours prior to blood collection. Two of the 3 dogs with low serum T_4 concentration at time 0 had concentrations within the reference range at the 3 week recheck.

Of the 50 dogs originally enrolled in the study, 38 had serum T_4 concentrations determined at time 0 and 3 weeks; 27 dogs at time 0 and 6 months; and 24 dogs at time 0 and 12 months. Serum T_4 concentrations were significantly ($P < 0.01$) lower at each of these sampling times compared to time 0, including the 12 month sample (median serum T_4 concentrations: 25.6 nmol/L and 21.2 nmol/L at times 0 and 12 months, respectively).

Serum TSH concentrations. Serum TSH concentrations were determined for 19 of the 22 dogs at all 4 sampling times. The remaining 3 dogs had insufficient serum sample size for TSH determination at one of the sampling times (2 dogs at 3 weeks, and 1 dog at 6 months). Median serum TSH concentration did not significantly change at any time point, but a significant ($P < 0.05$) positive upward trend was evident (**Fig. 2**). Median serum TSH concentrations were 0.13 ng/ml, 0.13 ng/ml, 0.16 ng/ml, and 0.18 ng/ml at times 0, 3 weeks, 6 months, and 12 months, respectively. Serum TSH concentration was

Figure 1. Effect of phenobarbital therapy on serum T_4 concentration in 22 epileptic dogs over a one year period of time. Serum T_4 concentrations were measured prior to initiation of phenobarbital (time 0), and again at 3 weeks, 6 months and 12 months after the start of phenobarbital. Dotted line represents the lower limit of the reference range. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the lowest to highest datapoints. Individual datapoints represent values for specific dogs. The 3 solid black points represent the 3 dogs with low serum T_4 concentration at time 0, followed over all time points. A = six-year-old Golden Retriever with low serum T_4 concentration at all 4 time points, and mild clinical signs supportive of hypothyroidism prior to the start of phenobarbital therapy. * = median value differs significantly ($P < 0.05$) from that of time 0. ** = median value differs significantly ($P < 0.01$) from that of time 0.

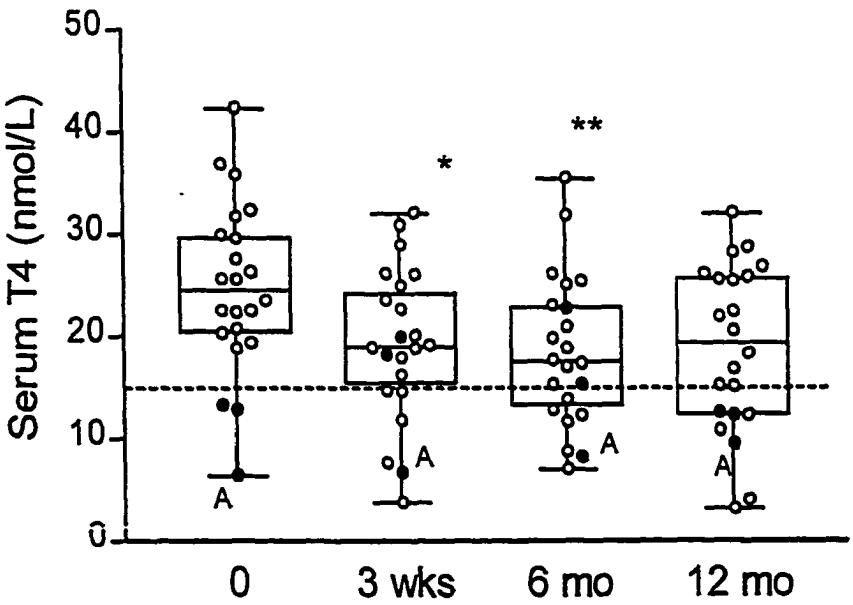
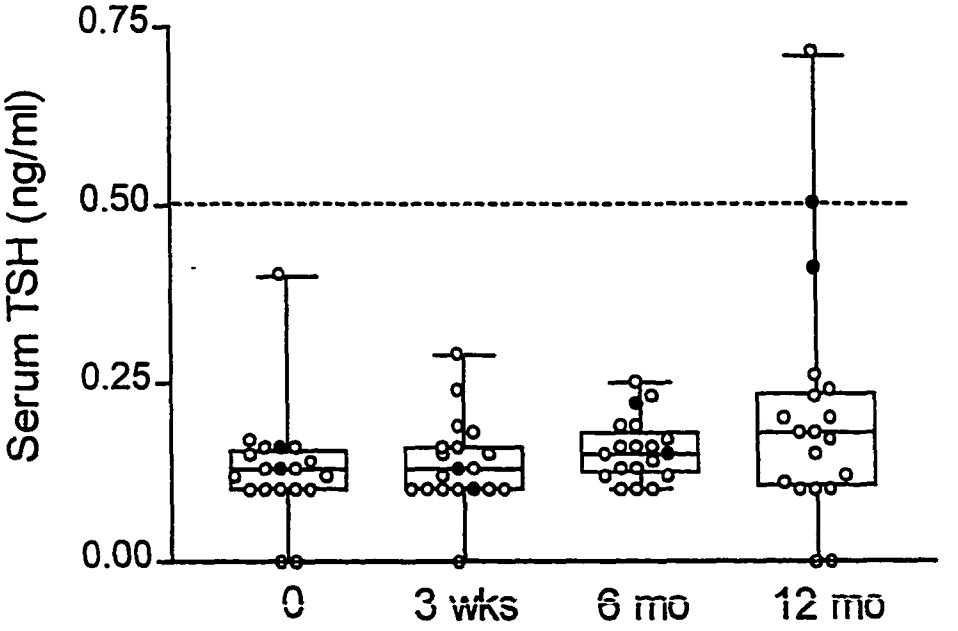


Figure 2. Effect of phenobarbital therapy on serum TSH concentration in 19 epileptic dogs over a one year period of time. Serum TSH concentrations were measured prior to initiation of phenobarbital (time 0), and again at 3 weeks, 6 months and 12 months after the start of phenobarbital. Dotted line represents the upper limit of reference range. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the lowest to highest datapoints. Individual datapoints represent values for specific dogs. The 2 solid black points represent 2 of the 3 dogs with low serum T_4 concentrations at time 0, followed over all time points. (Serum TSH concentration at 6 months was not determined for the remaining dog with low serum T_4 at time 0, so this dog was not included in TSH analyses). There is a significant ($P < 0.05$) positive trend over time.



above the upper limit of the AVC Diagnostic Laboratory reference range (< 0.5 ng/ml) for no dog at times 0, 3 weeks, and 6 months, and for only 1 dog at 12 months.

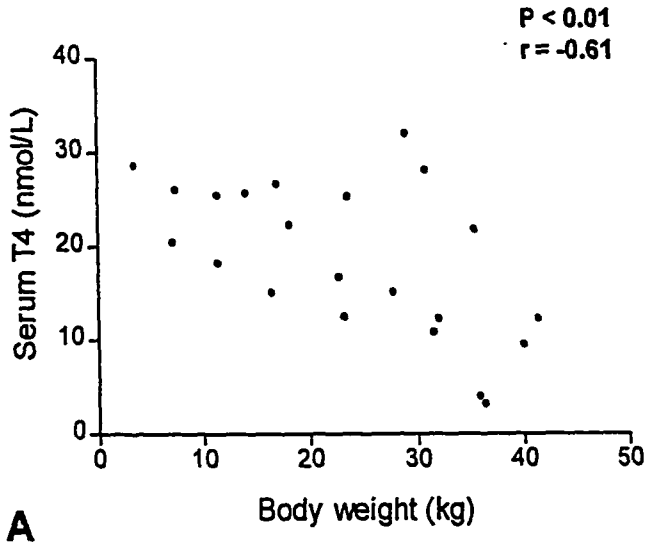
Thirty-five of the original 50 dogs had serum TSH concentrations available at time 0 and 3 weeks; 25 dogs at time 0 and 6 months; and 24 dogs at time 0 and 12 months. Median serum TSH concentration was significantly ($P < 0.01$) higher at 6 months compared to time 0 (0.12 ng/ml and 0.15 ng/ml, respectively). Serum TSH concentration was also significantly ($P < 0.05$) higher at 12 months compared to time 0 (0.12 ng/ml and 0.18 ng/ml, respectively).

Some investigators prefer to record values below the lower limit of assay detection as an average of the lower limit and 0. However, analysis of data using 0.05 ng/ml instead of 0 ng/ml for those dogs with serum TSH concentrations below the lower limit of detection did not alter test results.

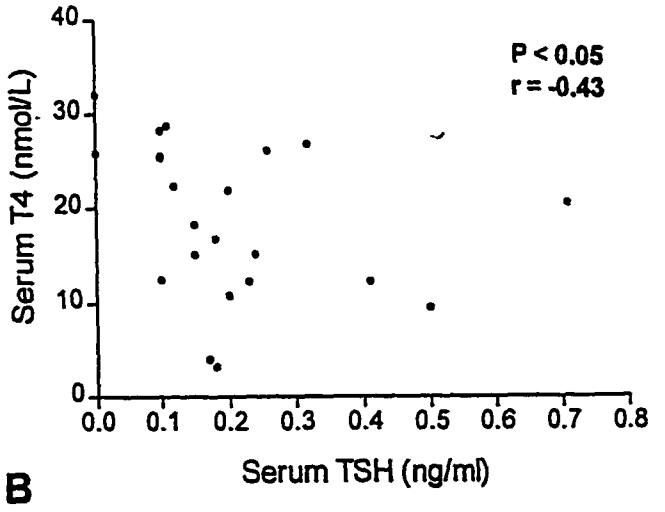
Correlations. No significant correlations between serum T_4 concentration and serum phenobarbital concentration or total daily dose of phenobarbital were found at any of the sampling times. There was no significant association between serum T_4 concentration and age or sex. However, there was a negative correlation between serum T_4 concentration and body weight at the 12 month sampling time ($P < 0.01$; $r = -0.61$), and a negative correlation between serum T_4 concentration and serum TSH concentration at the 12 month sampling time ($P < 0.05$, $r = -0.43$; **Fig. 3**).

Clinical signs. No associations were found between serum T_4 concentration and degree of seizure control or seizure score at either 6 or 12 months. No clinical signs consistent

Figure 3. Scatterplots showing a significant negative correlation at the 12 month recheck between (A) serum T_4 concentration and body weight and (B) serum T_4 and TSH concentrations.



A



B

with overt hypothyroidism were evident in any of the dogs that developed low serum T_4 concentration, other than weight gain in a few dogs. The only dog with clinical signs and history suggestive of hypothyroidism was a 6-year-old Golden Retriever (dog A) that had a serum T_4 concentration below the reference range at time 0 as well as at all other sampling times. This dog was moderately overweight and had chronic otitis externa problems before the initiation of phenobarbital. These signs continued throughout the study. The serum TSH concentration had increased from 0.13 ng/ml (time 0) to 0.5 ng/ml at the 12 month recheck. Based on signalment, low serum T_4 concentration prior to the start of phenobarbital, and a history of mild clinical signs consistent with hypothyroidism prior to the start of phenobarbital, this dog was suspected to have primary hypothyroidism unrelated to phenobarbital therapy.

TSH stimulation tests. TSH stimulation tests were performed on 6 of the 7 dogs with low serum T_4 concentrations at the 12 month sampling time. Test results were normal for all but one dog. The one dog with inadequate response to TSH was the Golden Retriever (dog A) described above that had a low serum T_4 concentration and mild clinical signs consistent with hypothyroidism prior to the start of phenobarbital. The other 5 dogs had no clinical signs of hypothyroidism, and had serum TSH concentrations (ranging from 0.17 to 0.23 ng/ml) within the reference range. The owners of the one untested dog with a low serum T_4 concentration at 12 months elected not to pursue the TSH stimulation test. This dog was healthy, with no clinical signs of hypothyroidism.

Other associations. Results of other serum biochemical analyses, including alkaline

phosphatase, alanine aminotransferase, aspartate aminotransferase, and gamma-glutamyl transferase activities, and fasting bile acids concentrations, were also evaluated. As expected, some abnormalities commonly associated with phenobarbital therapy (e.g., increased alkaline phosphatase activity) were detected. However, for the purposes of the study reported here, only associations between serum T_4 and TSH concentrations and these other biochemical tests were considered. No significant associations were found.

DISCUSSION

In this study, phenobarbital therapy in epileptic dogs caused a significant decrease in serum T_4 concentration within 3 weeks of the start of phenobarbital. Serum T_4 continued to decrease at the 6 month recheck after drug initiation. By 12 months, serum T_4 concentration was no longer decreasing, and was slightly higher than at 6 months. Serum TSH concentrations increased over time, and at 6 and 12 months were significantly higher than at time 0 when data from all dogs were considered.

Our findings are contradictory to those found in a study of 9 healthy beagle dogs treated with phenobarbital for 6 weeks (Daminet *et al.* 1999). In that study, no significant difference in serum T_4 or TSH concentrations occurred during phenobarbital treatment. The most likely cause for the difference in results between our study and that of Daminet, et al. is a difference in sample size (22 dogs vs 9 dogs).

Factors that may influence serum T_4 concentrations include age, breed, and sample handling. Serum T_4 concentration is reported to be inversely correlated with age, with a decrease in T_4 concentration starting soon after adulthood (Gonzalez and Quandri

1988; Reimers *et al.* 1990). However, this is unlikely to be the cause of the decrease in serum T_4 concentration in our study because of the relatively short duration of the study (1 year), and because the serum T_4 concentration was lowest at 6 months, not 12 months. No one breed was over-represented in our study, so breed effect was not a likely factor. Length of patient fast was not long enough to affect T_4 concentrations in our study, and techniques for blood collection, storage and transport did not vary enough between practitioners or between rechecks to affect median serum T_4 concentration.

Concurrent illness did not play a role in the decrease in median serum T_4 concentration, because all dogs that completed our study were healthy at the start of the study and remained healthy, with the exception of the Golden Retriever with suspected primary hypothyroidism (dog A). This dog was included in statistical analyses because although his serum T_4 concentration was low, a definitive diagnosis of hypothyroidism was not made at the time of study enrollment. Removal of this dog did not affect results of the statistical analyses. In a previous study (Gaskill *et al.* 1999), epilepsy itself was not the cause of the low serum T_4 concentration found in phenobarbital-treated dogs. In the same study, recent seizure activity (within 24 hours prior to blood collection) caused a significant decrease in serum T_4 concentration. We did not find this in our present study, most likely due to use of conservative non-parametric statistical analyses and the smaller numbers of dogs. However, as none of the dogs in the current study had seizures immediately prior to any of the blood collections other than time 0, recent seizure activity did not play any role in the decrease in serum T_4 concentration due to phenobarbital therapy.

Use of monthly heartworm or flea and tick preventatives, short-term use of otic

preparations, and short-term use of diazepam for control of active seizures did not play a role in the decrease in serum T_4 concentration in a previous study (Gaskill *et al.* 1999). In our current study, very few dogs received any of these drugs during the study (3 dogs received heartworm or flea and tick preventatives, 2 dogs received otic preparations short-term, and 1 dog received diazepam prior to one blood collection). Therefore, the low serum T_4 concentration should not be attributed to use of other drugs.

The general scheme of the hypothalamic-pituitary-thyroid axis in dogs is similar to that in most mammalian species (Chastain and Panciera 1995; Feldman and Nelson 1996; Ferguson 1998; Kaptein *et al.* 1994). Thyrotropin-releasing hormone is produced and secreted by the hypothalamus. This hormone stimulates the pituitary gland to produce and secrete T_4 and small amounts of triiodothyronine (T_3) and reverse T_3. These thyroid hormones are carried primarily protein bound in the blood to the various tissues of the body. Less than 0.1% of T_4 is in the free form in the blood. Once in the tissues, the free thyroid hormones diffuse into cells, where a large portion of the T_4 is deiodinated to T_3, the active thyroid hormone. The thyroid hormones are further deiodinated to inactive mono-iodinated products, or are metabolized by glucuronide and sulfate conjugation in the liver to metabolites that are excreted in bile and urine. Free thyroid hormones in the blood act in a negative feedback fashion on the hypothalamus, pituitary gland, and thyroid gland. Decreased concentration of free thyroid hormones cause an increase in TSH synthesis and release, which then stimulates the thyroid gland to increase synthesis and release of thyroid hormones. Conversely, increased amounts of free thyroid hormones in serum decrease TSH production and release, and subsequently decrease the synthesis and release of thyroid hormones from the thyroid gland.

Drugs may affect the hypothalamic-pituitary-thyroid axis in many ways (Cavalieri and Pitt-Rivers 1981; Curran and DeGroot 1991; Davies and Franklyn 1991; Lim *et al.* 1988). Some drugs directly affect the thyroid gland, while others can affect the hypothalamus or the pituitary gland. Drugs can also alter protein binding of thyroid hormones, or influence metabolism and excretion of the hormones. Some drugs exert their effect by a combination of several of the above mechanisms. Species differences do occur in all areas of the thyroid axis, so the mechanism of decreased thyroid hormones caused by a drug in one species may be quite different than in other species.

The mechanism of the decreased serum T_4 concentration caused by phenobarbital therapy in dogs is not yet known. However, the list of possible mechanisms has been considerably narrowed by this and other recent studies. We have previously shown that phenobarbital in serum does not interfere with T_4 assays, and so the decrease in serum T_4 concentration is not artifactually caused by the presence of the parent drug in serum (Gaskill *et al.* 1999). Also, the decrease in T_4 is not caused by interference from serum autoantibodies against T_4 (Gaskill *et al.* 1999). We have previously shown that serum free T_4 is also decreased in dogs receiving phenobarbital (Gaskill *et al.* 1999), and this has been confirmed in 2 other recent studies (Kantrowitz *et al.* 1999; Müller *et al.* 2000b). Using our data, we found that serum total T_4 was highly correlated with serum free T_4 in dogs receiving phenobarbital ($r= 0.68$; $P=0.005$) (Gaskill unpublished data). Therefore, we chose to measure only total T_4 in the present study. We have previously found that phenobarbital has minimal effect on protein binding of T_4 *in vitro* (Gaskill *et al.* 1999), with displacement of less than 0.006% of bound T_4. These findings, consistent with our *in vivo* results, indicate that the decrease in total T_4 is not caused by displacement of T_4

from serum protein binding sites with subsequent increase in clearance. Serum total T_3 has been shown to be either unaffected (Kantrowitz *et al.* 1999), or minimally and temporarily increased (Müller *et al.* 2000b), by phenobarbital treatment. This suggests that the decrease in total T_4 is not due to an increased rate of deiodination of T_4 to T_3.

In our previous study (Gaskill *et al.* 1999), serum TSH concentration was significantly increased in 78 phenobarbital-treated epileptic dogs compared to untreated epileptic dogs, suggesting that the hypothalamus and pituitary gland are functional and responding to the decrease in T_4. In our current study, there was a significant positive trend in serum TSH concentration over time, and serum T_4 and TSH concentrations were significantly negatively correlated at the 12 month recheck. When all dogs with serum TSH concentrations available for time 0 and 12 months (24 dogs) were analyzed, serum TSH concentration was significantly higher at 12 months compared to time 0. These findings support the conclusion that the pituitary gland and hypothalamus are responding appropriately to the decrease in serum T_4 concentration. This increase in TSH concentration is likely the reason why the median serum T_4 concentration at the 12 month recheck was not as low as at the 6 month recheck. Median serum T_4 concentration for the 22 dogs who completed the study was still lower at the 12 month recheck than at time 0, but the difference was no longer significant. When all dogs with serum T_4 data available at time 0 and 12 months (24 dogs) were analyzed, serum T_4 was significantly lower at 12 months compared to time 0.

The normal TSH stimulation test results for all but one of the dogs with low serum T_4 concentrations at the 12 month recheck showed that the thyroid glands are functioning properly in these dogs. The one abnormal TSH response was in the Golden

Retriever (dog A) suspected to have primary hypothyroidism unrelated to phenobarbital therapy.

In rats, an increased rate of glucuronidation and excretion of T_4 due to induction of glucuronosyltransferase activity in the liver is the cause of the low serum T_4 concentrations associated with phenobarbital treatment (Barter and Klaassen 1994; De Sandro and DeGroot 1991; Liu *et al.* 1995; McClain *et al.* 1989). There is now evidence that this may be the mechanism in dogs as well. In a recent study (PaiBir *et al.* 1999), the mean fold induction of thyroxine UDP-glucuronosyltransferase activity due to phenobarbital treatment was 1.6-fold in both rats and dogs. However, the study was based on data from a small number of dogs (3 treated dogs and one control dog). More studies are needed to confirm that this is the mechanism in dogs.

No dog with low serum T_4 had clinical signs suggestive of hypothyroidism, with the exception of the Golden Retriever described above (dog A), or had TSH concentrations above the normal range. The negative correlation between serum T_4 concentration and body weight at the 12 month recheck may appear to support a potentially subtle hypothyroid state; ie, as serum T_4 concentration decreased, body weight increased. However, polyphagia is a common complication of phenobarbital therapy, and can lead to weight gain (Brown, 1988; Parent, 1988). Median body weight increased significantly over time (data not shown); median serum T_4 concentration significantly decreased over time. Because of the colinearity of these variables with time, we cannot conclude that the increase in weight was due to the decrease in serum T_4 concentration or vice versa.

In conclusion, we have documented that phenobarbital therapy in epileptic dogs

caused a significant decrease in serum T_4 concentrations within 3 weeks after initiation of treatment. Serum T_4 concentration continued to decline through 6 months after the start of therapy. By 12 months, serum T_4 concentrations were slightly higher than at 6 months, but still lower than at time 0. A significant upward trend in serum TSH concentration was found. The decrease in serum T_4 concentration was not related to any obvious risk factor other than phenobarbital therapy, and was not associated with clinical signs of hypothyroidism or degree of seizure control. The decrease in T_4 is suspected to be caused by an increased rate of clearance and excretion due to induction of hepatic enzymes by phenobarbital.

2.4 General discussion of the effects of phenobarbital on the hypothalamic-pituitary-thyroid axis in dogs

The studies described in the previous two sections have shown that phenobarbital therapy caused a decrease in serum total T4 concentrations in epileptic dogs, and that the decrease was not due to epilepsy itself. The decrease in serum T4 concentration occurred within 3 weeks of the start of phenobarbital, and serum T4 concentrations were still low 12 months after the start of treatment. Serum T4 decreased below the lower limit of the normal reference range in approximately 30-40% of dogs receiving phenobarbital. There were no associations between serum T4 concentration and degree of seizure control. Also, dogs did not have clinical signs consistent with hypothyroidism, with the exception of weight gain. However, the weight gain was most likely related to polyphagia due to the phenobarbital, rather than from decreased metabolic rate due to hypothyroidism. These studies showed that the decrease in serum T4 was not an artifact from protein displacement or interference from the presence of phenobarbital in the serum. Recent seizure activity was associated with a transient decrease in serum T4 concentration, indicating that samples for T4 determinations should be collected 24 hours or more after a seizure episode. Serum thyroid-stimulating hormone (TSH) concentrations were significantly increased in these dogs, although rarely out of the normal range, and TSH stimulation tests were normal. These findings indicated that the central nervous system and the thyroid gland were functioning appropriately in these dogs with decreased serum T4. The delay in TSH response to the drop in serum T4 concentration might suggest some central mechanistic component. However, we propose that the decreased serum T4

was caused predominantly by an increased metabolism and subsequent clearance of the thyroid hormones due to phenobarbital induction of the involved enzymes. This mechanism has been documented in rats, but has not yet been proven in dogs.

Some veterinarians prescribe thyroid hormone supplementation for phenobarbital-treated dogs with low serum T4 concentrations, despite the fact that no studies have documented the risks or benefits of this practice in dogs. Studies evaluating the effects of T4 supplementation on serum concentrations of anticonvulsants in humans (Sarich and Wright 1996, Walker and Levy 1988) have produced conflicting results about the effects of supplementation on serum phenobarbital concentrations. These studies suggests that the serum phenobarbital concentration might either increase or decrease after the start of thyroid hormone supplementation in dogs. Other studies in humans have linked hyperthyroidism with precipitation of seizures (Primavera *et al.* 1990), thereby raising the possibility that excessive supplementation could be dangerous.

Our studies showed no signs of hypothyroidism or poor seizure control in the phenobarbital-treated dogs with low serum T4 concentrations. However, a few of the veterinarians participating in our study wanted to supplement these dogs with thyroid hormone. Therefore, we performed a small study to evaluate the possible benefits or risks of this practice. Because of small sample size (3 dogs), statistical analyses and conclusions were not possible. However, important preliminary information was gained that may be useful in designing future studies. (See Appendix A for study design and results). In these dogs, thyroid hormone supplementation did not appear to have any major effect on seizure control. It also did not appear to have a clinically significant effect on serum phenobarbital concentrations. The serum phenobarbital concentration

slightly decreased in one dog, slightly increased in one dog, and remained unchanged in one dog, despite no changes in dosage, body weight, or time of blood collection post-pill for any of the dogs. There were no apparent ill effects from the thyroid hormone supplementation, or any apparent major beneficial effects. These results provide preliminary evidence that a thyroid supplementation trial of phenobarbital-treated epileptic dogs with low serum T4 concentrations would likely be safe.

During the course of our study, potassium bromide (KBr) was added to the phenobarbital treatment for dogs whose seizures were not well controlled with phenobarbital alone, or who had evidence of liver abnormality. After the addition of KBr, the dogs were removed from the study. However, we continued to monitor serum biochemical profiles, serum T4 concentrations, and serum phenobarbital and KBr concentrations in these dogs for several months after the start of KBr. We found that the majority of the dogs responded favorably to the KBr, and maintained good seizure control. We also noted that several of these dogs developed very low serum thyroxine (T4) concentrations after the addition of KBr, even lower than with phenobarbital. However, the serum T4 concentration remained the same in some dogs, and increased in some dogs after the start of KBr.

Recent studies have shown that rats receiving KBr, at dosages similar to those used for maintenance treatment of epilepsy in dogs, have significantly decreased serum T4 concentrations (Velicky *et al.* 1997a, Velicky *et al.* 1997b, Velicky *et al.* 1998). The bromide appears to competitively inhibit the transport of iodine into the thyroid gland, and interferes with iodination of thyroglobulin. Bromide and iodine are in the same

family in the periodic table, and hence have many similar chemical properties. Because of the decreased ability to produce thyroid hormones by the thyroid gland, rats treated with KBr develop hypothyroidism and goiter. Surprisingly, serum TSH concentrations did not increase in these rats. This was hypothesized to be due to a decreased ability of the pituitary gland to release TSH.

A study was recently published that investigated the effects of KBr on serum T4 and TSH concentrations in epileptic dogs (Kantrowitz *et al.* 1999). The study showed no significant changes in serum T4 and TSH concentrations in 15 dogs receiving KBr/phenobarbital combination therapy and 8 dogs receiving KBr monotherapy, compared to healthy dogs not being treated with phenobarbital. However, no pre-treatment serum T4 or TSH concentrations were available, and serum T4 and TSH concentrations were determined only once for each dog. We were able to collect multiple pre- and post-KBr serum samples for the dogs we monitored. Data we collected from dogs receiving both phenobarbital and KBr are presented in Appendix B. Because of small sample size (n = 10), and because the data were incidental findings during a study designed to investigate another problem (ie, the effects of phenobarbital on the thyroid), results must be interpreted with this in mind. However, important preliminary information was gained that may be useful in designing future studies.

Before the start of KBr, 6/10 phenobarbital-treated dogs had serum T4 concentrations at or below the lower limit of the Atlantic Veterinary College Diagnostic Laboratory normal reference range. However, after KBr was added to their treatment, serum T4 decreased even further in 4 of those dogs. Additionally, after the start of KBr, serum T4 concentrations decreased in the 4 dogs with normal pre-KBr serum T4

concentrations. Serum T4 did not decrease consistently at any particular time point after the start of KBr, nor did it appear to be related to serum KBr concentration or duration of therapy.

The cause of the decrease in serum T4 concentration following KBr therapy found is unknown. The lack of an increase in serum TSH concentration and a normal TSH stimulation test result in the one dog for whom the test was performed suggests that the cause is not a primary thyroid gland problem. The decrease in serum TSH concentration noted in several dogs despite decreased serum T4 suggests possibly a central nervous system problem, involving decreased thyrotropin-releasing hormone by the hypothalamus or decreased TSH production or release by the pituitary. This is contrary to what has been found in rats, where bromide interferes with iodine uptake in the thyroid and interrupts production of thyroglobulin.

It is possible that the decreased serum T4 concentrations are artifacts caused by interference from hemolysis and lipemia of the serum samples. Hemolysis and lipemia can both interfere with the spectrophotometric method used to measure total T4 concentration in this study and cause erroneously low values. Five /14 serum samples with low T4 concentrations after KBr treatment had some degree of hemolysis or lipemia (see Table 1 in Appendix B). However, 9/14 samples with low T4 concentrations did not have hemolysis or lipemia, including the samples from 2/3 dogs with very low serum T4 concentrations (< 5 nmol/L). Additionally, 5/19 samples before the start of KBr had some degree of hemolysis or lipemia. We believe that it is unlikely that interference from hemolysis or lipemia accounts for all of the decrease in serum T4 concentration after the start of KBr.

Our data suggests that KBr treatment in dogs already receiving phenobarbital might cause a decrease in serum T4 concentrations. Clinical signs of hypothyroidism or other disease did not develop in the dogs with decreases serum T4 concentrations associated with KBr therapy, but further studies are warranted to investigate this issue.

CHAPTER 3. EFFECTS OF PHENOBARBITAL ON THE LIVER

3.1 General introduction to the effects of phenobarbital on the liver

Phenobarbital is one of the most commonly used anticonvulsant drugs in dogs, and is considered to be a relatively safe and effective drug (Boothe 1998, Frey 1989, Podell 1996). An infrequent but potentially fatal adverse effect of phenobarbital treatment is hepatotoxicity (Dayrell-Hart *et al.* 1991). Hepatotoxicity has also been associated with other anticonvulsants in dogs, in particular primidone and phenytoin, as well as with combinations of these drugs with each other and with phenobarbital (Bunch *et al.* 1982, Bunch *et al.* 1984, Bunch *et al.* 1985, Bunch *et al.* 1987). However, only one study reports an association between hepatotoxicity and phenobarbital monotherapy (Dayrell-Hart *et al.* 1991). This was a retrospective study of 18 dogs receiving phenobarbital that developed clinical liver disease and signs of phenobarbital intoxication. The majority of the dogs had increased serum alkaline phosphatase (AP) and alanine aminotransferase (ALT) activities, and increased total bilirubin concentration. Seven dogs had serum phenobarbital concentrations > 40 μg/ml (typical therapeutic reference range: 15-40 μg/ml) for 6 months to 4 years before the onset of clinical signs of liver disease. At the time of presentation for liver disease, 12 dogs had serum phenobarbital concentrations higher than the typically recommended therapeutic range (> 40 μg/ml). Ten dogs died or were euthanized, and liver biopsies revealed moderate to severe chronic hepatic fibrosis with nodular regeneration, lesions consistent with cirrhosis. Other histopathological findings included biliary stasis and hyperplastic bile

ducts, inflammatory infiltrate, and hepatocellular necrosis. The investigators hypothesized that there was an association between prolonged high serum phenobarbital concentrations and liver cirrhosis, and established the current recommendation of maintaining serum phenobarbital concentrations below 35 μg/ml (152 μmol/L) for maintenance therapy of canine epilepsy. However, a complicating factor in this study was that 9/18 dogs were concurrently receiving or had received in the past other hepatotoxic drugs such as diethycarbamazine, phenytoin, and primidone.

The pathophysiological mechanism of the hepatic injury associated with phenobarbital treatment in dogs is not known. The mechanisms for hepatotoxicity due to primidone and phenytoin, other anticonvulsants which are no longer recommended in dogs because of high risk of hepatotoxicity, are also unknown. However, the histopathological lesions found with phenobarbital, primidone and phenytoin hepatotoxicity are similar, suggesting similar mechanisms. Possible mechanisms include: (1) an intrinsic, dose-dependent toxicity, and (2) an idiosyncratic toxicity. These mechanisms have been well reviewed by the following authors: Batt and Ferrari 1995, DeLeve and Kaplowitz 1995, Gibaldi 1992, Hinson *et al.* 1994, Pohl *et al.* 1988, Speeg and Bay 1995, Zimmerman and Ishak 1995, and Zimmerman and Lewis 1995. Intrinsic toxicities are generally dependent upon the chemical properties of the drug, are dose dependent, and are usually reproducible in experimental studies. Idiosyncratic toxicities are host dependent, apparently not dose dependent, difficult to reproduce in laboratory studies, and unpredictable. In humans, these are often immune-mediated hypersensitivity type reactions. However, in dogs, immune-mediated hypersensitivity reactions are rarely thought to be the cause of hepatic injury due to drug exposure (Bunch 1993, Center

1995). Instead, most drug-associated hepatic injury in dogs is believed to be due to intrinsic toxicities caused by the generation of toxic metabolites via biotransformation of the parent drug in the liver. These metabolites may be activated oxygen species that directly or indirectly cause membrane lipid peroxidation, or electrophilic free radicals that bind to cellular macromolecules and impair cellular functions. No studies in dogs have determined the mechanism behind phenobarbital-associated hepatotoxicity, and studies in rats and humans are not particularly useful because chronic phenobarbital treatment in these species does not generally cause chronic hepatic disease (Kingsley *et al.* 1980, Spielberg *et al.* 1981). The few reported cases of hepatic disease associated with phenobarbital therapy in humans appeared to have an immune-mediated component (Dreifus and Langer 1987, Gaedigk *et al.* 1994, Leeder *et al.* 1992, Roberts *et al.* 1990).

Early liver injury in phenobarbital-treated dogs can be very difficult to diagnose non-invasively. The most common screening tests consist of measuring liver-associated enzyme activities in the blood. Elevation of these enzymes in the blood can indicate hepatic injury. However, many of these serum liver-associated enzymes are commonly elevated in phenobarbital-treated dogs that have no clinical signs of liver disease (Chauvet *et al.* 1995, Foster *et al.* 2000, Gieger *et al.* 2000, Muller *et al.* 2000a). This has led some investigators to conclude that these enzymes might be induced by phenobarbital. Enzyme induction is the increased synthesis of the enzyme (Goldberg 1970, Okey *et al.* 1986), resulting in increased amounts of the enzyme protein and its related activity. Many drugs and chemicals have been shown to induce a number of hepatic enzymes such as cytochrome P450s, glucuronosyl transferases, and glutathione-S-transferases among others (Barter and Klaassen 1994, Hendrich *et al.* 1991, Larsen and

Jefcoate 1995, Okey *et al.* 1986, Oguri *et al.* 1996). Whether or not the liver enzymes commonly used as markers of liver injury in serum biochemical profiles can be induced by drugs or chemical remains unclear. Marker enzymes include alanine aminotransferase (ALT) and alkaline phosphatase (AP) in particular, as well as others such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and sorbital dehydrogenase (SDH) (Abdelkader and Hauge 1986, Benichou 1990, Center 1995, Cornelius 1989, Evans G. 1996, Evans R. 1988, Woodman 1996).

Two of the most common serum liver-associated enzymes used as markers of hepatic disease are ALT and AP. Alanine aminotransferase is a relatively liver-specific enzyme in the dog (Evans G. 1996, Evans R. 1988), and is involved in intermediate metabolism and gluconeogenesis. Alanine aminotransferase is primarily a cytosolic enzyme in hepatocytes, but is also found in small quantities in the mitochondria (Evans R. 1988, Keller 1981). Increased serum ALT activity is typically associated with hepatocellular membrane damage and leakage of the enzyme from the hepatocytes. Text books and journal articles often state anecdotally that ALT is induced by phenobarbital (Aiges *et al.* 1980, Balasz *et al.* 1978, Boothe 1998, Center 1995, Deutsch *et al.* 1986, Dowling 1999, Gieger *et al.* 2000, Sisson 1997, Trepanier 1999). However, we are aware of only one study (Muller *et al.* 2000a) that investigated more closely the issue of ALT induction by phenobarbital in dogs. In that study, 12 non-epileptic dogs were treated with phenobarbital for 29 weeks. Blood samples and liver biopsies were obtained before and at specified times after the start of the study. The liver biopsies were evaluated histopathologically and scored in a subjective fashion. Serum ALT and AP activities increased significantly over time, but the researchers did not find histological evidence of

liver injury. They concluded that the elevation in serum ALT activity must be due to enzyme induction. However, they did not measure the activity of the enzyme within the liver tissue itself, and therefore could not definitively determine if the enzyme was induced. Studies investigating induction of liver ALT are generally lacking for most drugs. However, one recent study (Edgar *et al.* 1998) showed that fenofibrate, a lipid modifying drug, increased the expression of liver ALT mRNA and cytosolic ALT activity in human cultured hepatocytes independent of cellular injury. This type of study has not been done to show induction of ALT by phenobarbital in any species.

Alkaline phosphatase in dogs is found in a multitude of organs, including liver, bone, intestine, and kidney (Allen *et al.* 1998, Hoffmann 1988, Keller 1981, Kidney and Jackson 1988, Sanecki *et al.* 1987, Sanecki *et al.* 1993, Simon and Sutherland 1977, Syakalima *et al.* 1997, Syakalima and Takiguchi 1998, Unakami *et al.* 1987). However, due to the very short half-lives of the intestinal and kidney isoenzymes, the only isoenzymes found in canine serum are the bone (B-AP) and liver (L-AP) isoenzymes and a unique isoenzyme called the corticosteriod-induced (C-AP) isoenzyme (Kidney and Jackson 1988, Sanecki *et al.* 1993, Syakalima and Takiguchi 1998). Other than the involvement of B-AP in bone development, the function of AP is unknown. The B-AP isoenzyme originates from osteoblasts, and increased activity of this isoenzyme in serum is associated with conditions involving increased osteoblastic activity (Ehrhart *et al.* 1998, Syakalima and Takiguchi 1998). These include active bone growth in young dogs, fracture healing, certain bone cancers, and osteopathies such as rickets and osteomalacia. The L-AP and the C-AP isoenzymes are produced in the liver and are located on the hepatocyte plasma membranes lining the bile canaliculi and sinusoids (Sanecke *et al.*

1987, Sanecki *et al.* 1990, Solter and Hoffmann 1995, Syakalima and Takiguchi 1998). Increased activity of the L-AP isoenzyme in serum is typically associated with liver disease and cholestasis. Increased serum C-AP activity was originally associated with exogenous glucocorticoid treatment, or endogenous corticosteriod excess as occurs with hyperadrenocorticism. However, increases in this isoenzyme have also been shown to be associated with many chronic illnesses (Kidney and Jackson 1988, Syakalima *et al.* 1997, Syakalima and Takiguchi 1998), possibly as a result of stress and the resultant increase in endogenous glucocorticoid secretion. Serum total AP activity is commonly elevated in phenobarbital-treated dogs. The isoenzymes contributing to the increased total activity have been determined for only a small number of phenobarbital-treated dogs (Kidney and Jackson 1988, Sturtevant *et al.* 1977, Unakami *et al.* 1987). No one specific isoenzyme was found to be consistently increased. In humans, elevations of serum AP activity associated with phenobarbital and other anticonvulsant therapy have been suggested to be associated with osteomalacia, thereby inferring that the increase in the serum total AP is due primarily to the B-AP isoenzyme (Ashworth and Horn 1977, Dymling *et al.* 1979, Hunt *et al.* 1986, Pugh and Garnett 1991, Rico *et al.* 1992, Schmitt *et al.* 1984). However, no serum AP isoenzyme analyses in humans treated with phenobarbital have been published.

As with ALT, many textbooks and journal articles state that AP is induced by phenobarbital in dogs (Aiges *et al.* 1980, Balasz *et al.* 1978, Boothe 1998, Center 1995, Deutsch *et al.* 1986, Dowling 1999, Gieger *et al.* 2000, Muller *et al.* 2000, Sisson 1997, Trepanier 1999). However, these statements are often anecdotal, or as discussed above for ALT, induction was inferred because no histopathological hepatic lesions were

present in the dogs with elevated serum total AP. *In vitro* studies have shown that phenobarbital can inhibit AP activity (Tardivel *et al.* 1992). Only two *in vivo* studies have investigated whether increased serum AP activity in phenobarbital-treated dogs is associated with increased activity of the enzyme in the liver (Litchfield and Conning 1972, Unakami *et al.* 1987). These studies used small numbers of phenobarbital-treated dogs (n=3 each), and phenobarbital doses nearly 10X the typical maintenance dose of phenobarbital used to treat canine epilepsy. Also, one of the studies (Litchfield and Conning 1972) evaluated only the microsomal fraction of the tissue homogenates, thereby excluding evaluation of much of the plasma membrane where both the L-AP and C-AP isoenzymes are located. These studies found significantly increased AP activity (8 to 12-fold increases) in liver tissue of treated dogs compared to controls, and the conclusion that phenobarbital induced AP has become firmly ingrained in the literature.

Theoretically, induction of ALT and AP by phenobarbital would increase the amounts of the enzymes in the liver and subsequently cause increased enzyme activity in the blood indistinguishable from that caused by hepatocellular damage and cholestasis. This poses a serious problem for clinicians when a phenobarbital-treated dog with no clinical signs of liver disease develops elevated serum liver-associated enzyme activities. At present, there is no way to determine non-invasively if the elevations are due to enzyme induction, or to early liver injury that has not yet progressed to overt disease. The major dilemma faced by the practitioner is deciding whether or not to discontinue the phenobarbital. If the serum liver enzymes are elevated due to induction, there would be no indication to alter the anticonvulsant regimen. However, if the elevations are due to early liver damage, switching to a different anticonvulsant would be warranted to prevent

continued liver damage that might ultimately result in liver failure.

The purposes of our study were two-fold: (1) To evaluate histologically liver tissue from clinically healthy phenobarbital-treated dogs that had elevations in serum AP and ALT activities, and (2) to determine if liver AP and ALT activities are increased in phenobarbital-treated epileptic dogs with elevations in serum AP and ALT activities compared to healthy untreated control dogs. Our overall goal was to better answer the question of whether or not ALT and AP are induced by phenobarbital at typical maintenance doses. The following major questions are addressed in the next two sections.

(1) Do clinically healthy phenobarbital-treated dogs with elevated serum ALT and/or AP activities have histopathological evidence of liver injury? If so, then:

— are these histopathological abnormalities more pronounced than in untreated control dogs?

— do any of the histopathological changes help differentiate injury due to phenobarbital versus injury from other causes?

— are histopathological abnormalities correlated with serum ALT or AP activities?

— what risk factors are associated with liver injury due to phenobarbital? Dose of drug? Duration of therapy? Serum phenobarbital concentration? Other?

(2) Are increased serum ALT and/or AP activities associated with increased ALT and/or AP activities in the liver tissue?

— Which isoenzyme of AP is elevated in the serum of these phenobarbital-treated

dogs? Is it the liver, bone, or corticosteriod-induced isoforms, or some previously unidentified isoform? Is serum AP isoenzyme determination helpful at differentiating between enzyme induction and liver injury in these dogs?

— What assay conditions are optimal for measuring liver tissue ALT and AP activities?

3.2 Liver homogenate enzyme assay techniques

INTRODUCTION

In order to perfect *in vitro* techniques to be used in our study of the effects of phenobarbital on the liver in epileptic dogs, we first performed all assays on liver tissue from phenobarbital-treated and untreated rats. We then confirmed whether these techniques would also be appropriate for dogs by repeating these steps with liver tissue from one phenobarbital-treated dog and four untreated control dogs. However, because tissue from only one phenobarbital-treated dog was available for this preliminary testing, statistical analyses and conclusions were not possible. Therefore, only the results from the rats will be presented in this section, unless a important difference was detected between the rat and dog samples that altered the assay technique developed for the dogs. In instances where important differences existed between assay development in rats versus dogs, the data from the dogs will also be presented. The final study results from liver tissue from the clinical study dogs will be presented in the following section.

Because we anticipated that liver samples obtained in our clinical study would be too small to separate into cellular fractions, our first goal was to perfect all techniques on the smallest possible amount of crude liver homogenate. Where appropriate, we performed the assays in multiple cell fractions to determine the suitability of using whole homogenate for each of the assays. We then determined the smallest amount of homogenate that would provide reproducible results well within the linear range of each assay (hereafter referred to as "optimal sample size").

Our second goal was to determine if phenobarbital treatment in rats induced liver

alanine aminotransferase (ALT) and/or alkaline phosphatase (AP) activity. Previous studies investigating whether or not liver ALT or AP activities are induced by phenobarbital treatment in rats have provided conflicting results (Clampitt 1978, Pickering 1978, Platt 1967), and so further investigation was warranted.

METHODS AND MATERIALS

Preparation of samples: Ten mature male Spraque Dawley rats, weighing approximately 340 g, were obtained from Charles River [Charles River Laboratories, Quebec, Canada]. Rats were housed as standard and fed and watered ad libitum, following guidelines of the Canadian Council for Animal Care. After 1 week for acclimatization, 5 rats were administered phenobarbital 100 mg/kg (total volume 1ml) once daily by intraperitoneal injection for 7 days. Five control rats were administered equal volumes of sterile saline once daily by intraperitoneal injection for 7 days. At the conclusion of treatment, all rats were weighed and killed by pentobarbital injection. Death was ensured by cervical dislocation. Blood samples were drawn from the caudal vena cava and centrifuged to separate the plasma from the blood cells. Plasma was stored at -20° C. The livers were removed and rinsed in Tris/KCl buffer [6.06 g Tris base, 11.5 g KCl; *quantum sufficiat* (qs) with deionized water for a total volume of 1,000 ml; pH to 7.4 with 6 N HCl]. One portion of each liver was fixed in 10% buffered formalin, and another portion was frozen at -80° C.

One phenobarbital-treated epileptic dog and 4 untreated non-epileptic dogs were anesthetized with acepromazine and pentobarbital. Liver samples were obtained by

percutaneous needle biopsy using a spring-driven biopsy instrument [Biopty, C.R. Bard, Inc, Covington, Georgia] with an 14 gauge needle. Portions of each liver biopsy were fixed in buffered formalin, and the rest were flash frozen in liquid nitrogen. The dogs were then euthanized by pentobarbital overdose, and larger liver samples were taken post-mortem. Liver samples were frozen in liquid nitrogen, broken into smaller pieces, and stored at -80° C.

Frozen liver samples were later thawed and manually homogenized in 9 volumes of Tris/KCl buffer. Portions of each homogenate were subjected to sequential centrifugation at 9,000*g* and 105,000*g* to isolate the S-9, microsomal and cytosolic fractions. The microsomal fractions were resuspended in ½ volume of Tris/KCl buffer. Aliquots of crude homogenate, S-9, cytosol, and microsomes were frozen and stored at -80° C.

Assays: For each of the following assays, the linear range of the assay was determined, and quantities of each of plasma, liver homogenate, and hepatocellular cell fraction were chosen to provide results well within the linear ranges. All assays were repeated at least once to ensure reproducibility.

(1) Protein determination:

Protein contents of crude homogenates, S-9, cytosolic and microsomal fractions were determined using the Biorad DC Microplate protein assay [Biorad Laboratories, Hercules, CA]. This is a colorimetric assay for protein concentration following detergent

solubilization. The reaction is similar to the Lowry assay, but with a much more rapid color development and more stable color change. The assay is based on the reaction of protein with an alkaline copper tartrate solution and Folin reagent. Protein reduction of Folin causes the production of a product which can be measured spectrophotometrically between 404 and 750 nm. Color development is due primarily to the amino acids tyrosine and tryptophan, and secondarily to cystine, cysteine, and histidine.

(2) Alanine aminotransferase (ALT) activity:

Plasma, liver homogenates, and cytosolic and microsomal fractions of liver cells from all animals were assayed for ALT activity using a quantitative, colorimetric assay kit [Procedure #104, Sigma Diagnostics, St. Louis, MO]. The assay is based on the ALT-mediated transfer of α-amino groups from specific amino acids to α-ketoglutaric acid, resulting in glutamic acid and pyruvic acid. These keto acids then react with 2,4-dinitrophenylhydrazine and the resultant colored product is measured colorimetrically between 490 to 520 nm. The color intensity is proportional to the ALT activity. Results for liver fractions are expressed as International Units/mg protein. This technique was chosen after extensive review of previous studies evaluating ALT activity in liver tissue (De Rosa *et al.* 1975, Keller 1981, Marmo *et al.* 1976, Menache 1980, Righetti and Kaplan 1971, Schweiger 1983a and 1983b, Segal and Kim 1963, Segal and Matsuzawa 1968, Swick 1965, Wroblewski and LaDue 1956, Zinkl *et al.* 1971). This assay is based on the same principle as is the automated procedure used by the AVC Diagnostic Laboratory [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind].

(3) Alkaline phosphatase (AP) activity:

Plasma and liver homogenates from all animals were assayed for AP activity using a quantitative, colorimetric assay kit [Procedure #505, Sigma Diagnostics, St. Louis, MO]. The assay is based on the hydrolysis of para-nitrophenyl phosphate by AP, yielding para-nitrophenol and inorganic phosphate. When alkalinized, para-nitrophenol is converted to a yellow complex which can be measured spectrophotometrically at 400-420 nm. The intensity of the color is proportional to the AP activity. Results for liver fractions are expressed as International Units/g protein. This technique was chosen after extensive review of previous studies evaluating AP activity in liver tissue (Frederiks *et al.* 1990, Hoffmann *et al.* 1994, Kaplan *et al.* 1983, Keller 1981, Litchfield and Conning 1972, Nishimura and Teschke 1982, Okegbile *et al.* 1998, Olagunju *et al.* 2000, Righetti and Kaplan 1971, Sarrouilhe *et al.* 1993, Simon and Sutherland 1977, Solter and Hoffmann 1995, Solter *et al.* 1997, Solter and Hoffmann 1999, Thurmon *et al.* 1972, Unakami *et al.* 1987, Yamada *et al.* 1985). This assay is based on the same principle as is the automated procedure used by the AVC Diagnostic Laboratory [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind].

(4) Benzyloxyresorufin-*O*-dealkylase (BROD) activity: Benzyloxyresorufin-*O*-dealkylase activity, a specific assay for the major phenobarbital-induced cytochrome P450 in rats (CYP2B1, CYP2B2) and dogs (CYP2B11) (Burke *et al.* 1985, Klekotka and Halpert 1995), was measured as a positive control for verification of induction of a liver enzyme known to be induced by phenobarbital. The amount of resorufin produced by the

CYP2B-mediated O-dealkylation of benzyloxyresorufin can be measured spectrofluorimetrically and the enzyme activity calculated. The assay was developed by modifying published techniques (Burke *et al.* 1985, Klekotka and Halpert 1995, Lubet *et al.* 1985a and 1985b, Nims *et al.* 1987, Wink *et al.* 1993). The assay was performed on liver homogenates, S-9, and microsomal fractions from all animals. Reactions were carried out in a fluorimeter microcuvette at 37°C using a Shimadzu RF-5301 PC series spectrofluorophotometer [RF5301PC, Shimadzu Scientific Instruments, Inc., Kyoto, Japan]. The reaction mixture contained varying amounts of sample (to determine optimal sample size), 2.5 µl of a 1 mM benzyloxyresorufin substrate in DMSO solution, and 465 µl of PBS buffer (pH 7.4) [4.0 g NaCl, 0.19 g KCl, 0.138 g NaH_2PO_4-H_2O, 1.01 g Na_2HPO_4 anhydrous; qs with deionized water for a total volume of 500 ml; pH to 7.4 with 0.12 M HCl]. Added to this were varying amounts (to determine optimal concentration) of dicumarol dissolved in 0.1 N NaOH, to minimize the NADPH-mediated reduction of resorufin that would otherwise occur after initiation of the reaction in samples containing cytosol (Lubet *et al.* 1985b, Nims and Lubet 1983, Nims *et al.* 1984, Wink *et al.* 1993). The above mixture was equilibrated at 37°C for 1 minute, and the reaction was then initiated by the addition of 25 µl of 5 mM NADPH (500 µl final reaction mixture) and allowed to run for varying amounts of time (to determine optimal reaction time). The rate of production of the end product, resorufin, was determined by measuring the increasing fluorescence of the reaction mixture. The fluorimeter settings were: excitation and emission slits, 5 nm; excitation and emission wavelengths, 530 and 585 nm, respectively. Fluorescence was calibrated using a standard containing 5 µl of

sample, 2.5 µl of 1 mM benzyloxyresorufin, 485 µl of PBS buffer, 2.5 µl of 0.1 mM dicumarol, and 5 µl of 10 µM resorufin in H_2O. Results were normalized to protein concentrations of the samples and expressed as nmol of product/minute/mg of protein.

(5) Immunoblotting of CYP2B protein: Amounts of CYP2B protein in the homogenates, S-9, and microsomal fractions from all animals were determined using standard Western Blotting techniques (Cribb *et al.* 1997, Delaporte *et al.* 1993, Gallagher *et al.* 1997, Towbin *et al.* 1979). Varying amounts of sample (to determine optimal amounts) were diluted 1:4 or 1:5 in Laemmli Buffer [378 mg Tris Base added to 10 ml millipore H_2O, pH 6.8, plus 5 mg bromophenol blue, 5 ml glycerol, and 5 ml 10% SDS; aliquots of 475 µl frozen at -20°C] containing beta-mercaptoethanol and heated at 95°C for 3 minutes. Samples were cooled, and varying amounts were loaded onto 10% SDS-polyacrylamide mini-gels [Mini-PROTEAN 3, Biorad Laboratories, Hercules, CA] (overlayed with a 4% stacking gel) and electrophoresed (200 volts, 60 milliamps, 40 watts) for approximately 45 minutes or until the dye reached the bottom of the gel. The proteins were transferred to a nitrocellulose membrane by a semidry transfer process (2 gels: 25 volts, 200 milliamps, 100 watts, 1 hour), and immunodetection of transferred CYP2B proteins was performed using goat anti-rat anti-CYP2B antibody [Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan] (varying dilutions to determine optimum) and rabbit anti-goat IgG peroxidase conjugate secondary antibody [Sigma, St. Louis, MO] (varying dilutions to determine optimum). Bands were visualized by enhanced chemiluminescence techniques, digitized [Eagle Eye, Stratagene, La Jolla, CA] and

intensities determined [NIH Image for MacIntosh, Scion Corp., Frederick, Maryland].

(6) Histopathology: Sections from the formalin-fixed liver samples from all animals were stained for light microscopy by the AVC Diagnostic Laboratory using standard techniques. Sections were stained with hematoxylin-eosin, rhodanine (copper), prussian blue (iron), periodic acid Schiff (glycogen), and a reticulum stain (fibrosis). Histopathology slides were randomized and evaluated in a blinded fashion by a single pathologist. Histopathological categories evaluated included those indicative of induction (hepatocyte size and cytoplasmic characteristics representative of endoplasmic reticulum), and those indicative of injury (necrosis, inflammation, fibrosis, vascular characteristics, and an overall score indicative of injurious type changes). Degree of staining for copper, iron, and glycogen was also evaluated.

Statistical analysis: After optimizing techniques, final results from phenobarbital-treated rats were compared to untreated rats for all assays. All analyses were non-parametric (Mann Whitney U for comparisons) [GraphPad Software Inc., Prism2, San Diego, CA] 2-tailed. Significance was designated as $P \leq 0.05$ throughout. As previously mentioned, no statistical analyses were performed for dog samples due to small sample size.

RESULTS

(1) Protein assays: Average protein concentrations of crude homogenates, S-9, cytosolic and microsomal fractions from rat liver samples are shown in **Table 1.**

Table 1. Mean protein concentrations (± standard deviation) of crude homogenates, S-9, cytosolic and microsomal fractions from rat liver samples.

	Homogenate protein (mg/ml)	S-9 protein (mg/ml)	Cytosol protein (mg/ml)	Microsomal protein (mg/ml)
PB-treated rats	19.9 ± 1.8	15.6 ± 0.5	12.9 ± 0.6	28.6 ± 5.6
Control rats	18.7 ± 0.4	19.3 ± 0.0	14.1 ± 0.9	18.8 ± 1.7

(2) ALT assays: Total ALT activity in rat liver homogenates was not significantly different than that found in the cytosolic fraction, where the majority of hepatocyte ALT is located. Serum samples did not need dilution. Optimal dilution for liver homogenate was 1:80. Neither plasma ALT activity or liver homogenate ALT activity were significantly different in phenobarbital-treated rats compared to controls (**Figure 1**).

(3) AP assays: Rat plasma did not need to be diluted for the AP assay, but crude homogenates were diluted 1:2. There was no significant difference between phenobarbital-treated or control rats for either serum AP activities or liver homogenate AP activities (**Figure 2**).

(4) BROD assay:

(A) Microsomes: The microsomal fraction of the crude liver homogenate contains the CYP2B enzymes that carry out the *O*-dealkylation of benzyloxyresorufin. Optimal sample size for microsomal BROD activity was from 0.03 to 0.07 mg of microsomal protein per reaction. A reaction time of 1 minute produced results well within the linear range of the assay. Phenobarbital-treated rats had significantly ($P < 0.01$) higher microsomal BROD activity than did controls (median activities of 7.12 and 0.07 nmol/min/mg protein, respectively) (**Figure 3**).

(B) Homogenates vs S-9 fractions: Smaller amounts of BROD activity per mg protein were detectable in liver homogenates than in microsomal fractions, but essentially no activity was found in the S-9 fractions (**Figure 4**) for either rats or dogs. We found

Figure 1. Plasma ALT activities and liver homogenate ALT activities in 5 phenobarbital-treated rats compared to 5 controls. Median ALT activities in plasma or liver homogenates were not significantly different in treated rats compared to controls.

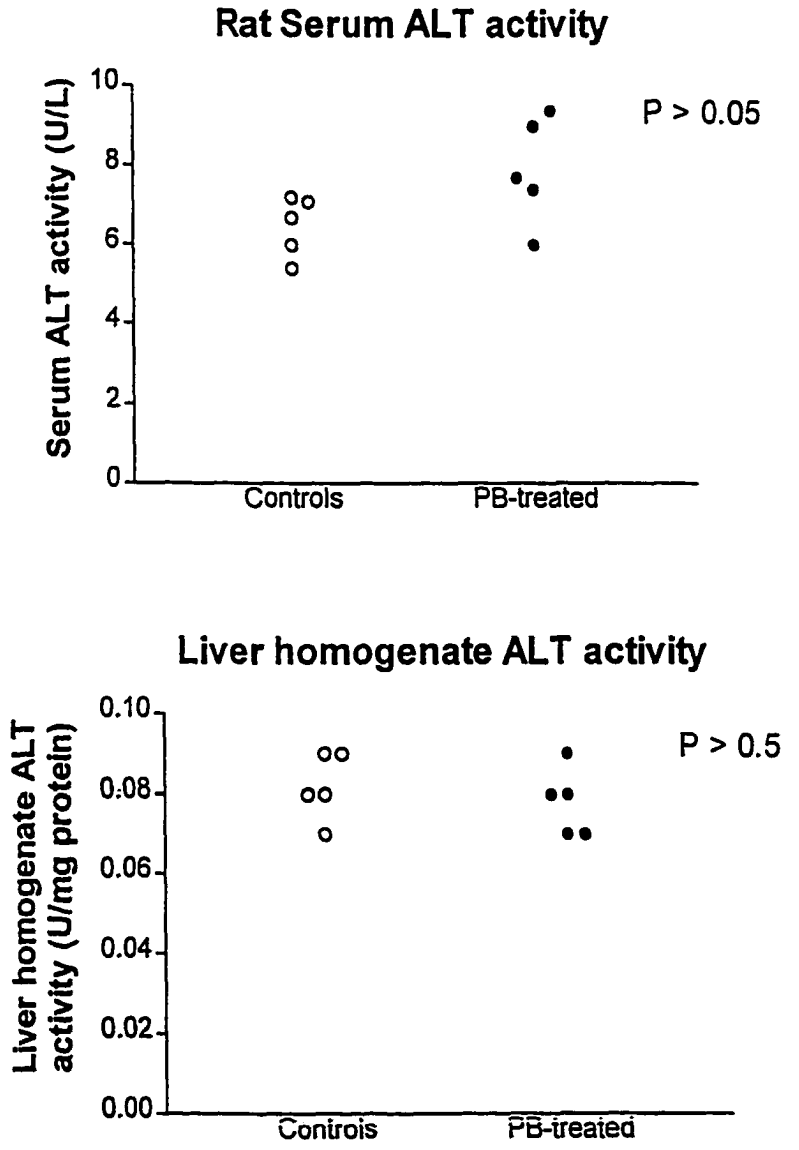


Figure 2. Plasma AP activities and liver homogenate AP activities in 5 phenobarbital-treated rats compared to 5 controls. Median AP activities in plasma or liver homogenates were not significantly different in treated rats compared to controls.

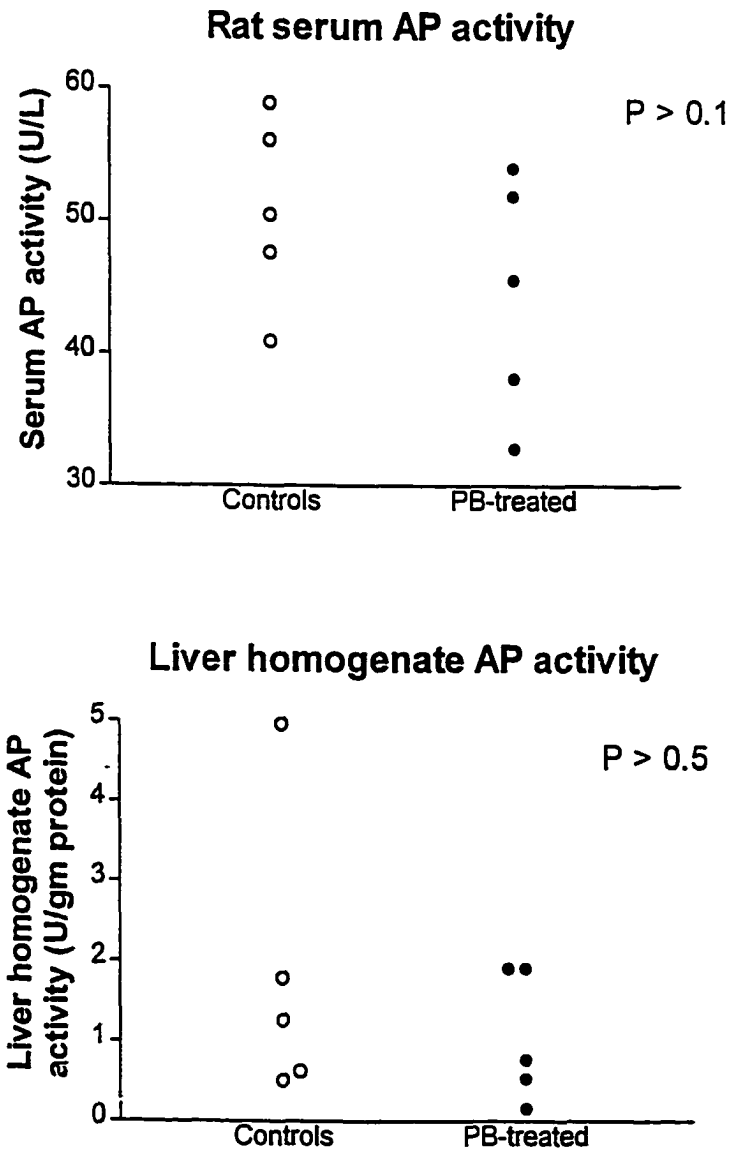


Figure 3. BROD activity in microsomes from 5 phenobarbital-treated rats compared to controls. Note the break in the Y axis scale. ** Mean value differs significantly from controls ($P < 0.01$).

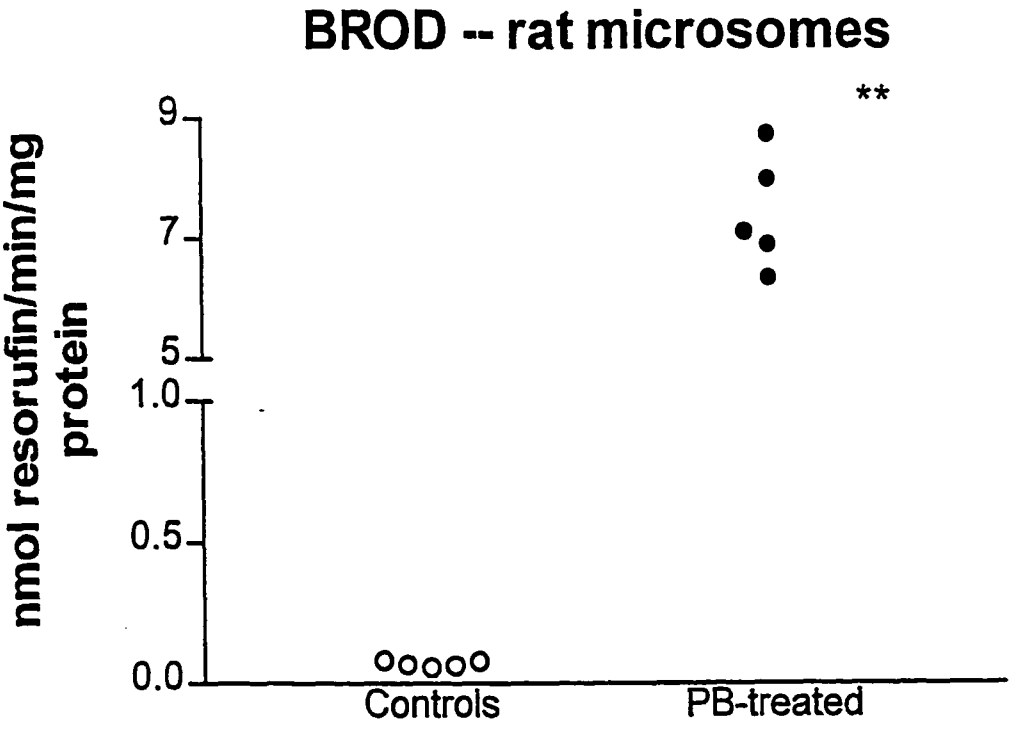
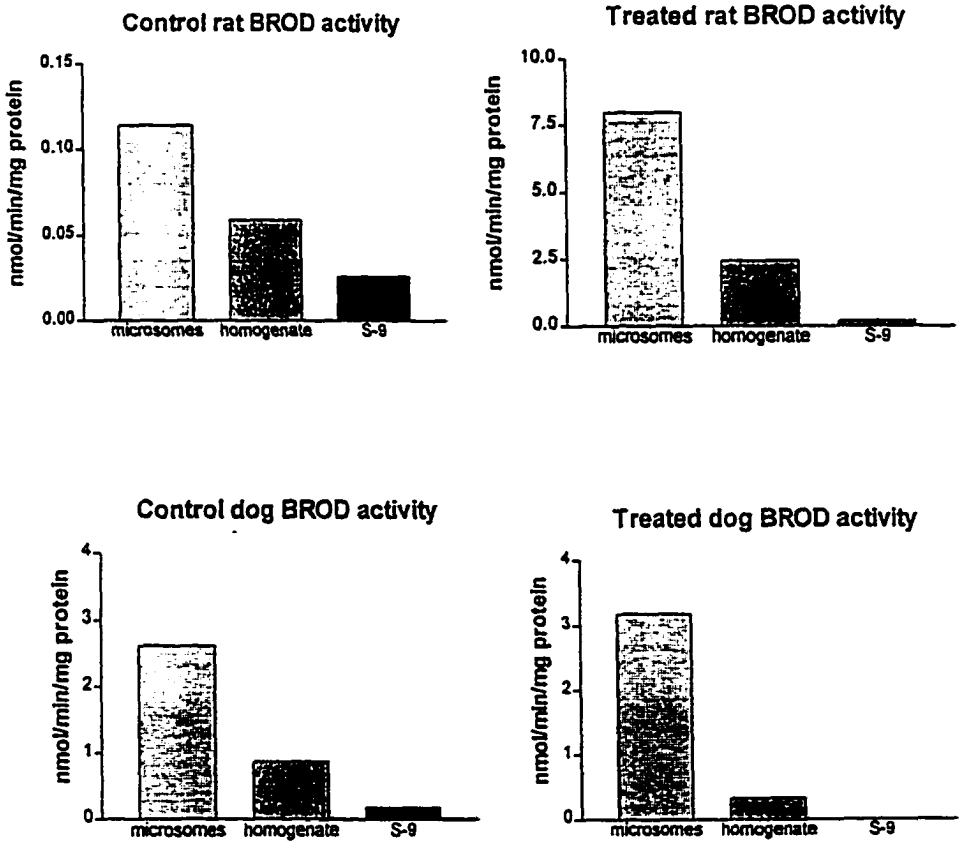


Figure 4. Comparisons of BROD activity in microsomes, liver homogenate, and S-9 fractions in phenobarbital-treated and untreated rats and dogs (n = 4 for treated rats, control rats, and control dogs, and n = 1 for treated dogs).



BROD activity present in the pellets from the 9,000 *g* spins used to prepare the S-9 fraction. Immunoblotting (see next subsection) showed that CYP2B protein was present in the microsomes, S-9, homogenates, and in the pellet from the 9,000 *g* spin. By adding the reaction components one at a time to a solution containing resorufin, we determined that addition of samples containing cytoplasm caused reduction of the resorufin, that this reduction reaction was NADPH-mediated, and that dicumarol inhibited this reduction of resorufin (**Figure 5**).

We next investigated if the dicumarol or its solvent had an effect on microsomal BROD activities in either rats or dogs. The addition of dicumarol to give a final concentration of 10 μM had no effect on BROD activity of rat microsomes. However, we found that both the dicumarol and the NaOH solvent used to dissolve the dicumarol inhibited microsomal BROD activity in the dog microsomes (**Figure 6**). At final concentrations greater than 2 mM, the NaOH solvent inhibited microsomal BROD activity. Therefore, concentrations of stock dicumarol concentration for our dog assay were adjusted so that the final reaction mixture concentration of NaOH was 0.5 mM. The dicumarol could not be properly solubilized using other solvents.

Liver homogenates were chosen over S-9 fractions to use in the BROD assays, as our immunoblotting (see next subsection) showed we would capture all of the CYP2B protein in the homogenate for both rats and dogs. Varying amounts of dicumarol were used to determine the optimal concentration necessary to inhibit resorufin-reducing factors in the cytosol contained in the homogenates. Optimum dicumarol concentration differed in rat compared to dog homogenates. The concentration of dicumarol used in most previous rat studies (10 μM) caused an inhibition of BROD activity in the dog

Figure 5. NADPH-mediated reduction of resorufin by a component of liver homogenate, and dicumarol inhibition of this reduction. Reaction components were added one at a time to a solution containing resorufin. The addition of homogenate and NADPH caused a reduction of resorufin. Dicumarol inhibited this reduction.

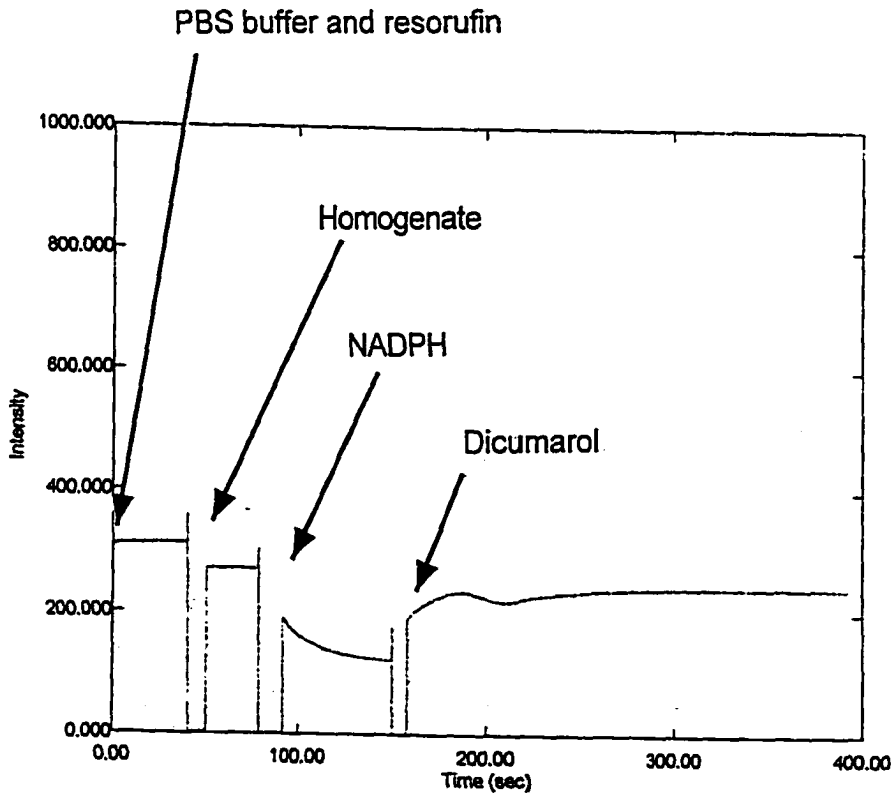
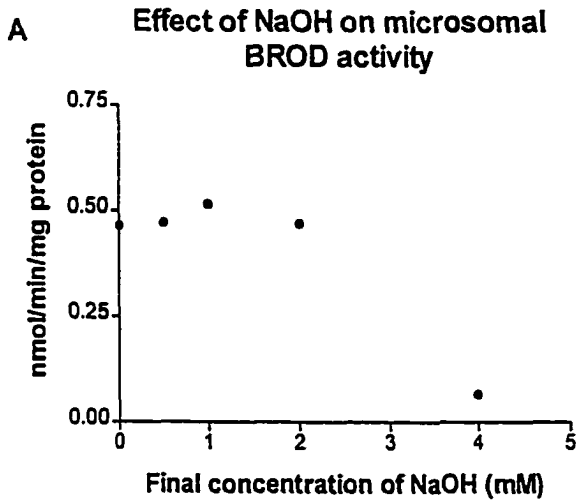
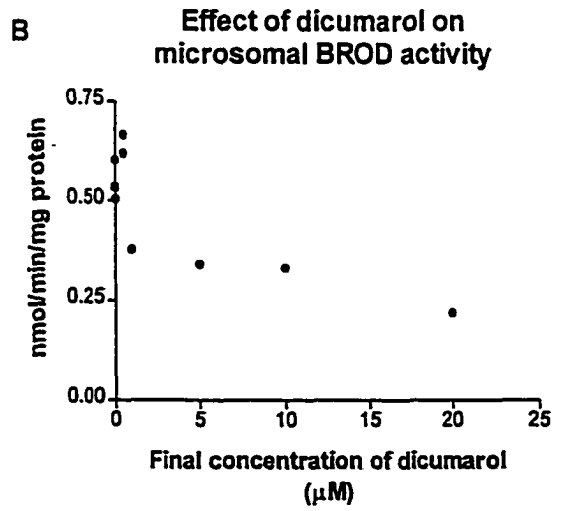


Figure 6. Effect of (A) NaOH and (B) dicumarol on dog microsomal BROD activity.





samples that outweighed the decrease in resorufin reduction (**Figure 7**). As noted with the microsomal fractions, the NaOH solvent inhibited liver homogenate BROD activity at concentrations greater than 2 mM (**Figure 7**). Therefore, concentrations of stock dicumarol concentration were adjusted so that the final reaction mixture concentration of NaOH was 0.5 mM. The optimum final concentration of dicumarol in the reaction mixture for dog liver homogenates was between 0.05 and 0.5 μM (**Figure 8**). A final dicumarol concentration of 0.06 μM was chosen as the concentration that resulted in the optimal balance between inhibition of CYP2B activity in microsomes and inhibition of resorufin-reduction by cytoplasmic factors.

Optimal homogenate sample size was determined to be 0.05 to 0.1 mg of homogenate protein per reaction (**Figure 9**). Larger samples resulted in a decreased rate of resorufin production, most likely due to quenching. Optimum reaction time for liver homogenate samples was approximately 3 minutes (**Figure 10**). Rates of resorufin production were visually linear for this time period.

(5) Immunoblotting of CYP2B protein: CYP2B protein was detected in liver homogenates, S-9 and microsomal fractions, and a very small amount in the pellet from the 9,000 x g spin (**Figure 11**) in both rats and dogs. The molecular weight of the detected protein was approximately 50,000, which is identical to that determined for rat CYP2B1 and 2 protein. Bands could be visualized for both CYP2B1 and 2 in control animals. Because of the density of bands in treated animals, distinction between the 2 bands was not possible for microsomal fractions, but were apparent for homogenate samples (separation of the two bands is not visible in the computer generated

Figure 7. Effect of NaOH (A and B) and dicumarol (C and D) on control and treated dog liver homogenate BROD activity.

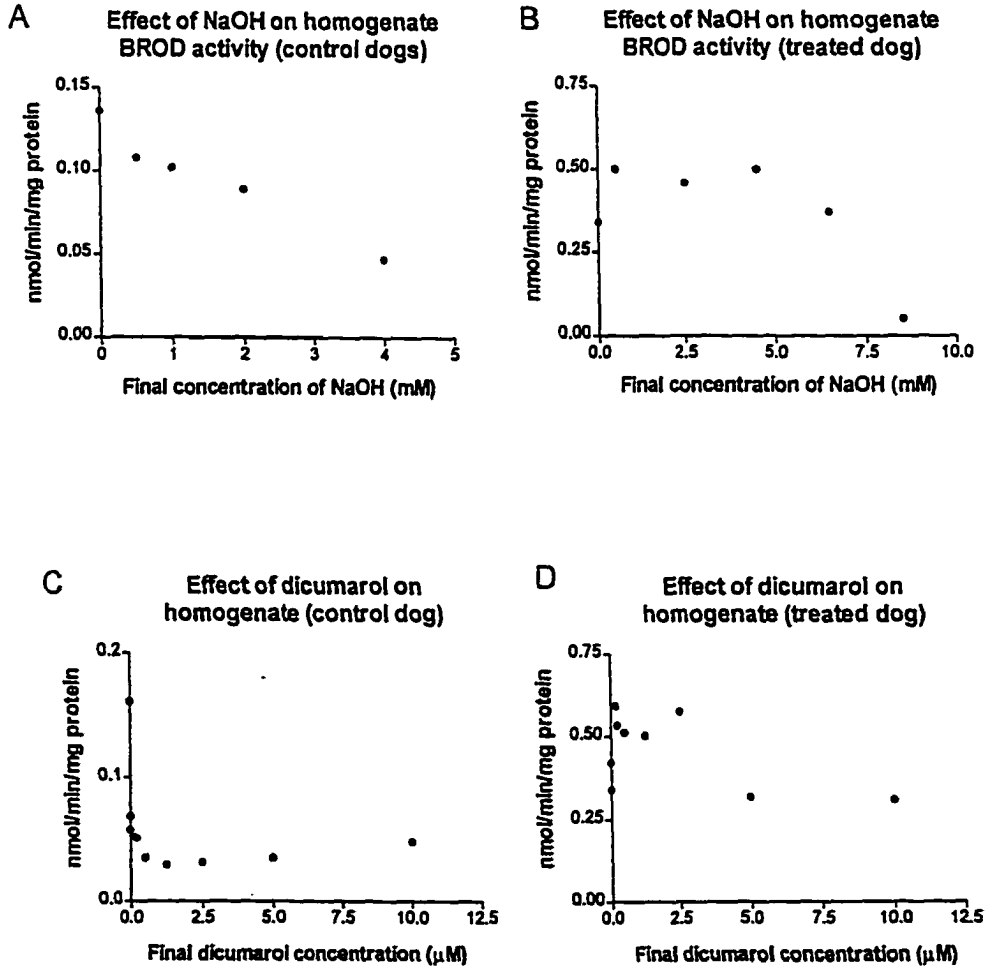
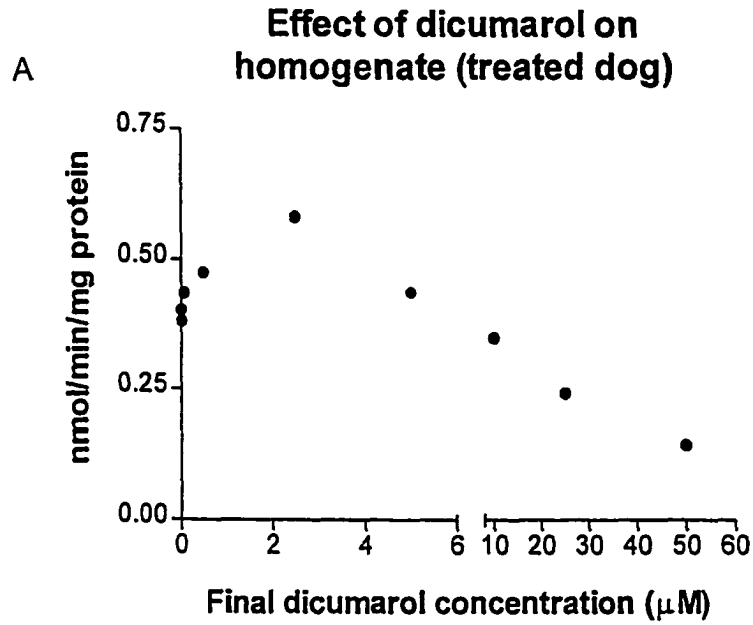


Figure 8. Effect of dicumarol concentration on BROD activity in dog liver homogenate. (A) Wide range of dicumarol concentrations (note the break in the X axis scale). (B) Focus on dicumarol concentrations between 0.05 and 0.5 μM. (Concentration of NaOH kept constant at 0.5 mM).



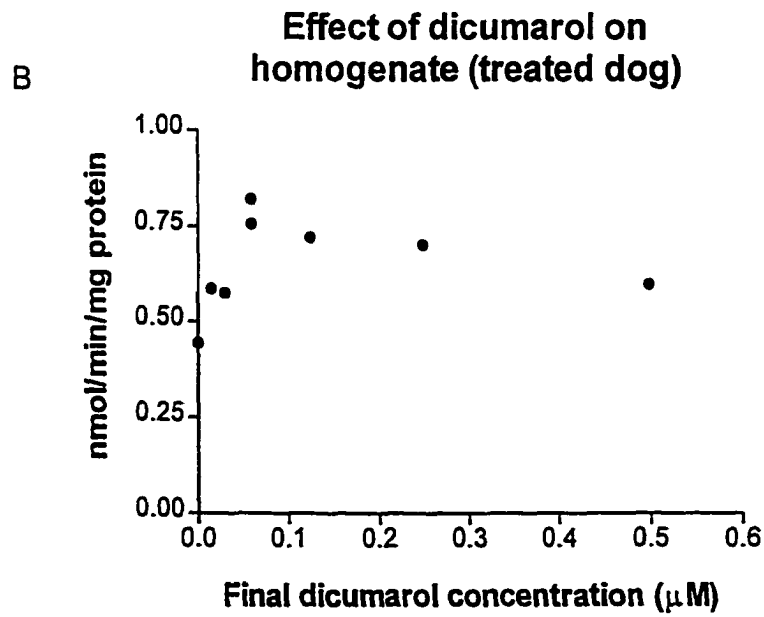


Figure 9. Effect of homogenate sample size on BROD activity, using 0.06 μM dicumarol in the reaction mixture. The drop in the curve at 0.3 mg protein is likely due to quenching.

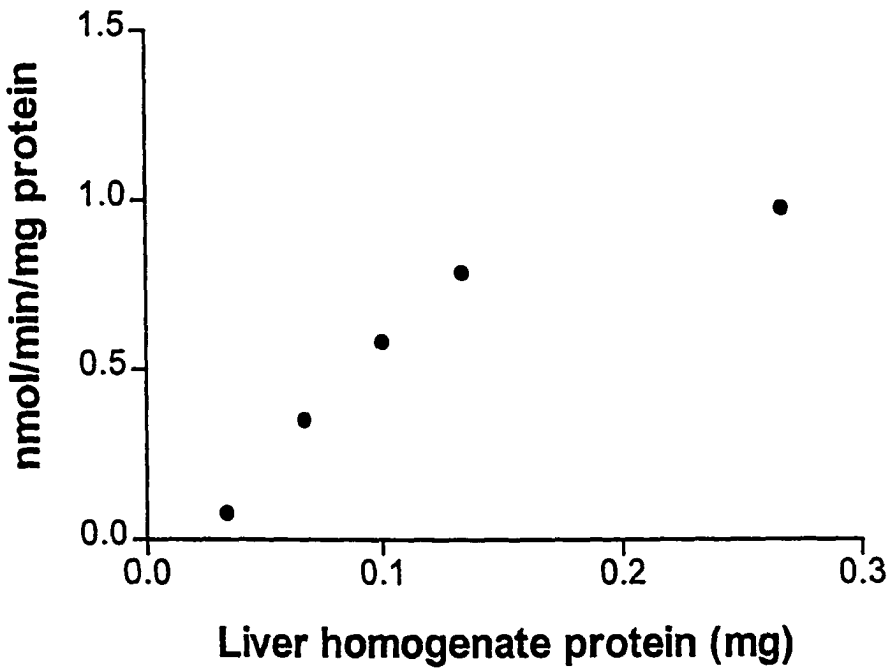


Figure 10. BROD activity in liver homogenates over time. Rates of resorufin production were measured over time for multiple liver homogenate samples. Rates of resorufin production were visually linear for approximately 180 to 200 seconds for these samples.

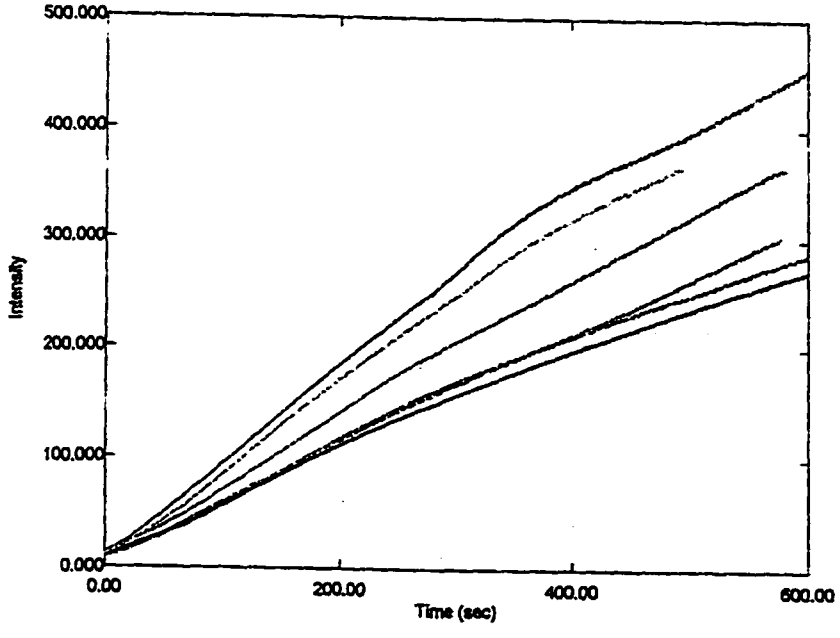
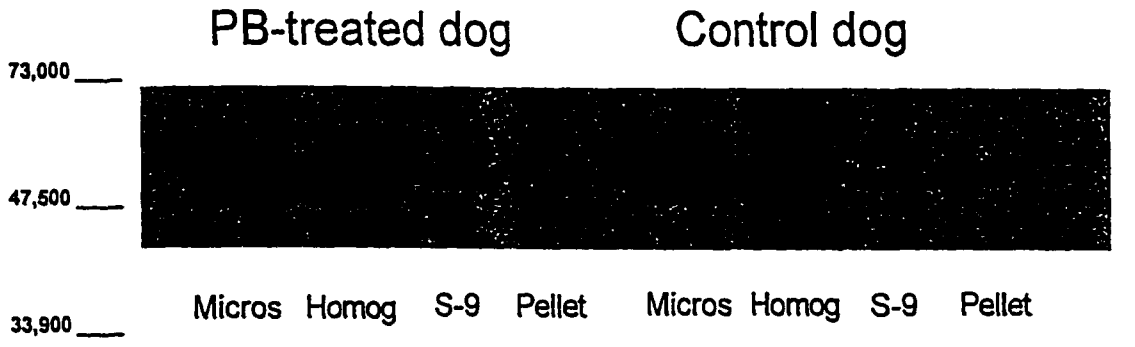
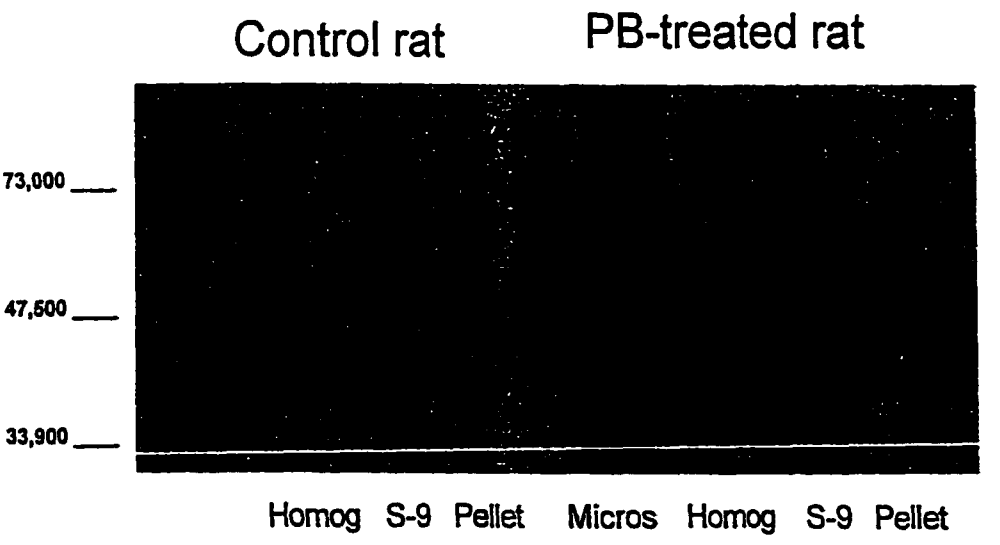


Figure 11. Immunoblots of CYP2B protein in microsomes, liver homogenates, S-9, and pellet from the 9,000 x g spin in rats and dogs. Each lane contains 50 μg of protein. (Note: no microsomal protein was loaded for the control rat).





reproduction for this thesis). Optimal sample size for the microsomal fraction was 2 μg of microsomal protein for control animals, and 0.5 μg for treated animals. Optimal concentrations for the primary and secondary antibodies were 1/10,000 and 1/25,000, respectively. Film exposure times of 5 to 10 minutes were optimal. Samples in the first 2 lanes of the gel did not transfer well and were difficult to visualize. Therefore, results from these two rats (one treated rat and one control rat) were discarded. Median microsomal concentration of CYP2B from the remaining 4 phenobarbital-treated rats was significantly ($P < 0.05$) greater than controls (intensities of 1,787 and 241, respectively) (**Figure 12**).

Homogenates contained more CYP2B protein per unit volume than did the S-9 fractions (possibly due to the loss of small amounts in the 9,000 x g pellet) for both rats and dogs, so homogenates were chosen as the sample to use for our clinical study, and techniques were perfected using homogenate. Optimum homogenate sample size was 50 μg of homogenate protein (treated and control) if 1.5 mm spacers were used, and half this if 0.75 mm spacers were used. Optimal primary and secondary antibody concentrations were 1/10,000 and 1/25,000, respectively. Film exposure times of 10 to 15 minutes were optimal.

(6) Histopathology: The phenobarbital-treated rats showed liver changes typically associated with increased amounts of endoplasmic reticulum caused by induction of enzymes residing within. These changes included swelling, enlargement, and pleomorphism of hepatocytes, and a finely granular appearance to the cytoplasm (**Figure 13**). There was no evidence of hepatic injury in either the treated rats or the controls, and

Figure 12. (A) Microsomal CYP2B protein concentration in phenobarbital-treated rats compared to controls. (B) Immunoblot showing representative samples from each group (n = 4 for each group). Treated lanes contain 0.5 μg of protein; control lanes contain 2 μg of protein. * = Median value differs significantly ($P < 0.05$) from controls.

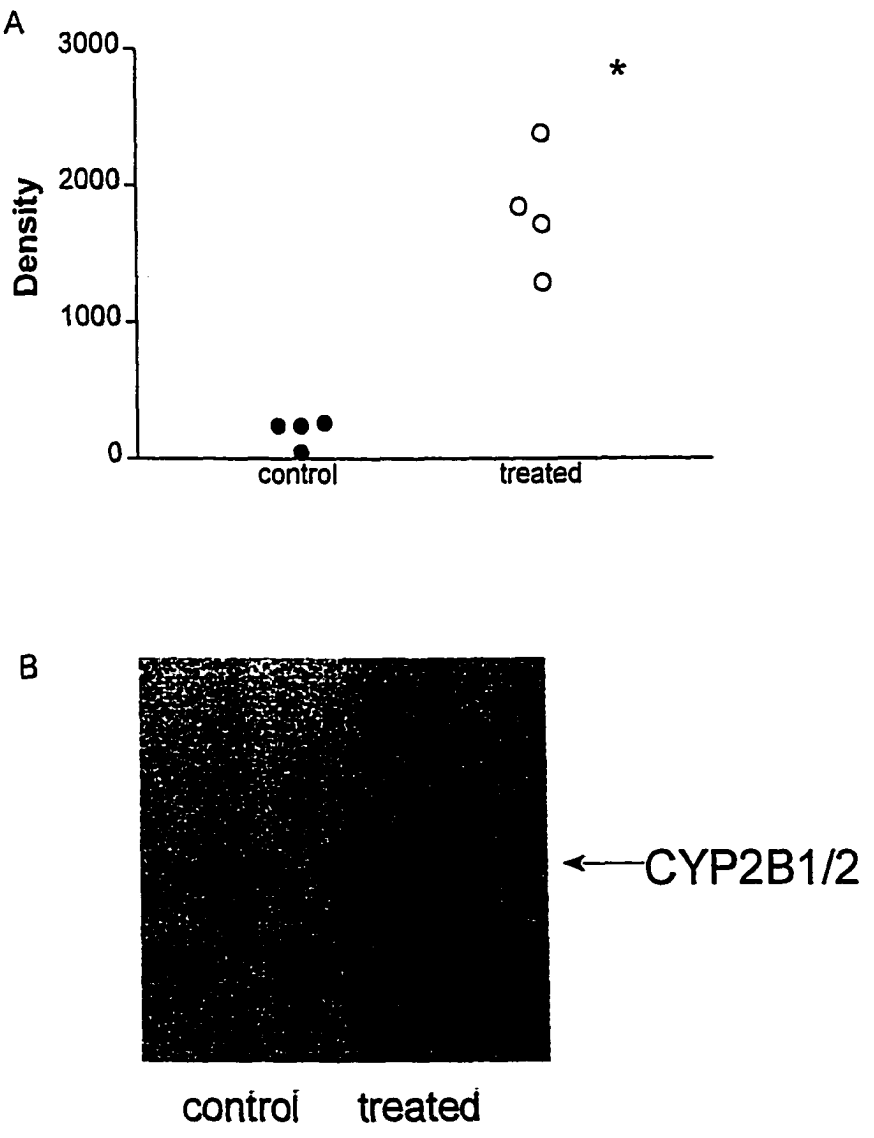
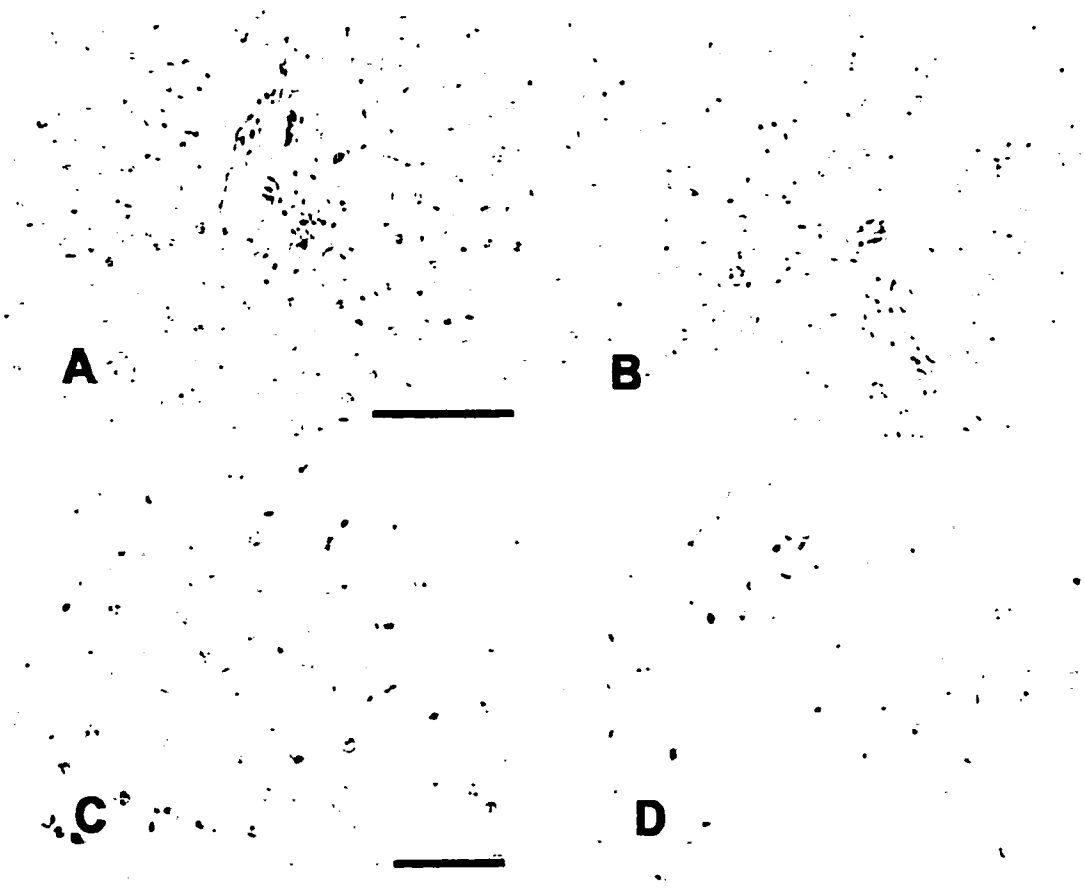


Figure 13. Photomicrographs of liver samples from untreated rats (A, C) and phenobarbital-treated (B, D) rats. Magnification bar for A and B = 100 micrometers. Magnification bar for C and D = 50 micrometers.


A
B
C
D

stains for copper, iron, and fibrosis were negative for both groups.

DISCUSSION

We have determined the optimum conditions for each assay to be used in our clinical study of the effects of phenobarbital on the liver in dogs. We have shown that homogenates were suitable for each of the assays we intend to use in our clinical study, and that very small sample sizes should produce accurate and reproducible results.

For the BROD assay, when using liver homogenates or cell fractions that contain cytoplasm, factors in the cytoplasm reduce the resorufin product as it is being produced. These factors have been hypothesized to be quinone oxidoreductases (Nims and Lubet 1983, Nims *et al.* 1984). Dicumarol inhibits these factors and slows or stops the rate of resorufin reduction. However, we found that the concentration of dicumarol used to inhibit these resorufin-reductase factors in rat cytosol is not appropriate for dog cytosol. This appears to be due to a greater inhibition of dog CYP2B compared to rat CYP2B enzymes. The dicumarol concentration needs to be decreased approximately 100-fold for assays of dog cytosol-containing samples. Liver homogenate was found to be superior to S-9 for the BROD assay. Some of the microsomal content of the S-9 fraction was lost to the pellet from the 9,000 *g* centrifugation, so larger amounts of S-9 were required to obtain reaction rates equal to that of homogenate.

The results from the rats showed that phenobarbital treatment did not increase liver ALT or AP activity, despite dramatically increasing in the activity of CYP2B, an enzyme well known to be induced by phenobarbital in rats. This suggests that liver ALT

and AP activities are not induced by phenobarbital in rats. Comparing ALT and AP protein and mRNA concentrations in phenobarbital-treated rats compared to controls would help complete the investigation of whether or not these enzymes are induced by phenobarbital. However, enzyme activity is the primary clinical endpoint of interest for our investigation. There was no evidence of hepatic injury due to this short phenobarbital treatment. Histopathological examinations showed no evidence of hepatic injury, and plasma ALT and AP activities were not increased in the treated rats compared to controls

3.3 Effects of phenobarbital on the liver in dogs

Abstract

Increased serum alanine aminotransferase (ALT) and alkaline phosphatase (AP) activities are commonly found in phenobarbital-treated dogs. The purpose of this study was to determine if increased serum ALT and AP activities in phenobarbital-treated epileptic dogs are associated with hepatic enzyme induction.

Serum biochemical profiles and serum phenobarbital concentrations were obtained from 128 epileptic dogs receiving phenobarbital. Liver biopsies were obtained from 12 of the dogs with increased serum ALT and/or AP activities but with no clinical signs of liver disease. Liver biopsies were also obtained from 8 healthy control dogs not receiving phenobarbital that had normal serum biochemical profiles. Biopsies were evaluated histopathologically, and liver homogenates were assayed for ALT and AP activities. As a positive control, cytochrome P4502B (CYP2B), an enzyme known to be induced by phenobarbital, was measured by benzyloxyresorufin-*O*-dealkylase activity and immunoblotting. Serum AP isoenzyme analyses were also performed.

Activities of ALT and AP in liver homogenates were not increased but CYP2B was dramatically increased in the phenobarbital-treated dogs compared to controls. Histopathological examination of liver biopsies revealed more severe and frequent abnormalities in treated dogs compared to controls. Serum AP isoenzyme analyses in treated dogs demonstrated significant increases in both the corticosteroid-induced and liver isoenzymes, and minor increases in the bone isoenzyme.

We conclude that increased serum ALT in phenobarbital-treated dogs likely

reflects hepatocellular injury and not induction. Persistently elevated serum ALT might help identify dogs that will ultimately develop clinical liver disease. Although no increases in liver homogenate AP activities were seen, induction might have been masked by a simultaneous increase in release of AP from hepatocyte membranes. Further investigation is required.

INTRODUCTION

Phenobarbital is one of the most commonly used anticonvulsant drugs in dogs, and is considered to be relatively safe and effective (Boothe 1998, Podell 1996, Schwartz-Porsche *et al.* 1985). An infrequent but potentially fatal adverse effect of phenobarbital treatment is hepatotoxicity (Dayrell-Hart *et al.* 1991). Dogs that develop clinical signs of liver disease due to phenobarbital treatment usually have increased serum liver-related enzymes due to liver injury (Dayrell-Hart *et al.* 1991). However, increased serum liver-associated enzymes are also commonly seen in phenobarbital-treated dogs with no clinical signs of liver disease (Center 1995, Chauvet *et al.* 1995, Foster *et al.* 2000, Gieger *et al.* 2000, Muller *et al.* 2000a). This has raised the possibility that these enzymes may be induced in the liver by phenobarbital. Enzyme induction is the increased synthesis of the enzyme (Goldberg 1970, Okey *et al.* 1986), resulting in increased amounts of the enzyme protein and its related activity. Many drugs and chemicals have been shown to induce a number of hepatic enzymes (Barter and Klaassen 1994, Hendrich *et al.* 1991, Larsen and Jefcoate 1995, Okey *et al.* 1986, Oguri *et al.* 1996), such as cytochrome P450s, glucuronosyl transferases, and glutathione-S-

transferases. Whether or not the liver enzymes commonly used as markers of liver injury in serum biochemical profiles can be induced by drugs or chemicals remains unclear. These enzymes include alanine aminotransferase (ALT) and alkaline phosphatase (AP) in particular, as well as others such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and sorbital dehydrogenase (SDH).

Two of the most common serum liver-associated enzymes used as markers of hepatic disease are ALT and AP. Alanine aminotransferase is a relatively liver-specific enzyme in the dog (Evans G. 1996, Evans R. 1988), and is involved in intermediate metabolism and gluconeogenesis. Alanine aminotransferase is primarily a cytosolic enzyme in hepatocytes, but is also found in small quantities in the mitochondria (Evans R. 1988, Keller 1981). Increased serum ALT activity is typically associated with hepatocellular membrane damage or distortion and leakage of the enzyme from the hepatocytes. Text books and journal articles often state anecdotally that ALT is induced by phenobarbital (Aiges *et al.* 1980, Balsz *et al.* 1978, Boothe 1998, Center 1995, Deutsch *et al.* 1986, Dowling 1999, Gieger *et al.* 2000, Sisson 1997, Trepanier 1999). However, we are aware of only one study (Muller *et al.* 2000a) that investigated more closely the issue of ALT induction by phenobarbital in dogs. In that study, 12 non-epileptic dogs were treated with phenobarbital for 29 weeks. Blood samples and liver biopsies were obtained before and at specified times after the start of the study. The liver biopsies were evaluated histopathologically and scored in a subjective fashion. Serum ALT and AP activities increased significantly over time, but the researchers did not find histological evidence of liver injury. They concluded that the elevation in serum ALT activity must be due to enzyme induction. However, they did not measure the activity of

the enzyme within the liver tissue itself, and therefore could not definitively determine if the enzyme was induced. Studies investigating induction of liver ALT are generally lacking for most drugs. However, one recent study (Edgar *et al.* 1998) showed that fenofibrate, a lipid modifying drug, increased the expression of liver ALT mRNA and cytosolic ALT activity in human cultured hepatocytes independent of cellular injury. This type of study has not been done to show induction of ALT by phenobarbital in any species.

Alkaline phosphatase in dogs is found in a multitude of organs, including liver, bone, intestine, and kidney (Allen *et al.* 1998, Keller 1981, Hoffmann 1988, Kidney and Jackson 1988, Saneci *et al.* 1987, Sanecki *et al.* 1993, Simon and Sutherland 1977, Syakalima *et al.* 1987, Syakalima and Takiguchi 1998, Unakami *et al.* 1987). However, due to the very short half-lives of the intestinal and kidney isoenzymes, the only isoenzymes found in canine serum are the bone (B-AP) and liver (L-AP) isoenzymes and a unique isoenzyme called the corticosteriod-induced (C-AP) isoenzyme (Kidney and Jackson 1988, Sanecki *et al* 1993, Syakalima and Takiguchi 1998). Other than the involvement of B-AP in bone development, the function of AP is unknown. The B-AP isoenzyme originates from osteoblasts, and increased activity of this isoenzyme in serum is associated with conditions involving increased osteoblastic activity (Ehrhart *et al.* 1998, Syakalima and Takiguchi 1998). These include active bone growth in young dogs, fracture healing, certain bone cancers, and osteopathies such as rickets and osteomalacia. The L-AP and the C-AP isoenzymes are produced in the liver and are located on the hepatocyte plasma membranes lining the bile canaliculi and sinusoids (Sanecki *et al.* 1987, Sanecki *et al.* 1990, Solter and Hoffmann 1995, Syakalima and Takiguchi 1998).

Increased activity of the L-AP isoenzyme in serum is typically associated with liver disease and cholestasis. Increased serum C-AP activity was originally associated with exogenous glucocorticoid treatment, or endogenous corticosteriod excess such as occurs with hyperadrenocorticism. However, increases in this isoenzyme have also been shown to be associated with many chronic illnesses (Kidney and Jackson 1988, Syakalima *et al.* 1997, Syakalima and Takiguchi 1997), possibly as a result of stress and the resultant increase in endogenous glucocorticoid secretion. Serum total AP activity is commonly elevated in phenobarbital-treated dogs. The isoenzymes contributing to the increased total activity have been determined for only a small number of phenobarbital-treated dogs (Kidney and Jackson 1988, Sturtevant *et al.* 1977, Unakami *et al.* 1987). No one specific isoenzyme was found to be consistently increased. In humans, elevations of serum AP activity associated with phenobarbital and other anticonvulsant therapy have been attributed to osteomalacia, thereby suggesting that the increase in the serum total AP is due primarily to the B-AP isoenzyme (Ashworth and Horn 1977, Dymling *et al.* 1979, Hunt *et al.* 1986, Pugh and Garnett 1991, Schmitt *et al.* 1984). However, no serum AP isoenzyme analyses in humans treated with phenobarbital have been published.

Several authors have suggested that AP is induced by phenobarbital in dogs (Balsz *et al.* 1978, Boothe 1998, Center 1995, Dowling 1999, Gieger *et al.* 2000, Sisson 1997, Trepanier 1999). However, these statements are often anecdotal, or as discussed above for ALT, induction was inferred because no histopathological hepatic lesions were present in the dogs with elevated serum total AP. Only two studies have evaluated if the increased serum AP in phenobarbital-treated dogs is associated with increased amounts and activity of the enzyme in the liver (Litchfield and Conning 1972, Unakami *et al.*

1987). These studies used small numbers of phenobarbital-treated dogs (n=3 each), and phenobarbital doses nearly ten times the typical maintenance dose of phenobarbital used to treat canine epilepsy. Also, one of the studies (Litchfield and Conning 1972) evaluated only the microsomal fraction of the tissue homogenates, thereby excluding evaluation of much of the plasma membrane where both the L-AP and C-AP isoenzymes are located. These studies found significantly increased AP activity (8 to 12-fold increases) in liver tissue of treated dogs compared to controls, and the conclusion that phenobarbital induced AP has become firmly ingrained in the literature.

Theoretically, induction of ALT and AP by phenobarbital would increase the amounts of the enzymes in the liver and subsequently cause increased enzyme activity in the blood indistinguishable from that caused by hepatocellular damage and cholestasis. This poses a serious problem for veterinarians when presented with phenobarbital-treated dogs that have no clinical signs of liver disease but have elevated serum liver-associated enzyme activities. At present, there is no non-invasive way to differentiate between elevations due to enzyme induction and elevations due to early liver injury. The major dilemma faced by the veterinarian is deciding whether or not to discontinue the phenobarbital. If the serum liver enzymes are elevated due to induction, there would be no indication to alter the anticonvulsant regimen. However, if the elevations are due to early liver damage, switching to a different anticonvulsant would be warranted to prevent continued liver damage that might ultimately result in liver failure.

The purposes of our study were (1) to determine if liver ALT and AP activities are increased in phenobarbital-treated epileptic dogs with elevations in serum ALT and AP activities compared to healthy control dogs, and (2) to evaluate liver tissue from these

dogs for histopathological evidence of hepatic injury. Our goal was to better answer the question of whether or not ALT and AP are induced by phenobarbital at typical maintenance doses.

MATERIALS AND METHODS

Study population — Between September 1997 and July 1999, client-owned epileptic dogs were recruited for the study by veterinarians throughout the Atlantic provinces of Canada, as previously published (Gaskill *et al.* 1999, Gaskill *et al.* 2000). Our goal was to identify phenobarbital-treated dogs with elevated serum liver-associated enzymes, in particular serum AP and ALT activities. The study consisted of a cross-sectional study of 78 epileptic dogs receiving phenobarbital, and a prospective study of 50 epileptic dogs before and for 1 year after the start of phenobarbital. Dogs of various breeds, age, sex, and duration of phenobarbital treatment were eligible for enrollment in the study. The dogs were healthy (other than having epilepsy) as determined by physical examination and history, and were not receiving any other major therapeutic drugs. Dogs in the prospective study had normal CBC and serum biochemical profiles prior to the start of phenobarbital therapy. Owners of all animals signed informed client consent documents (see Appendix C).

Control animals consisted of 8 healthy Humane Society dogs slated for euthanasia that were not receiving phenobarbital. These dogs were healthy based on physical examination, normal biochemical profile and CBC results, and had no recent histories indicative of concurrent disease or medication. These dogs were used as controls for the

liver biopsy cases (biopsy cases described later).

Data collected — Data obtained for the 78 phenobarbital-treated dogs in the cross-sectional study included signalment, physical examination results, seizure history, dose and duration of phenobarbital treatment, information on administration of other drugs or concurrent medical conditions, results of CBC and serum biochemical analyses [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind.] (including AP, ALT, GGT, AST, SDH, T4, conjugated bilirubin, urea, albumin, and fasting bile acids), and serum phenobarbital concentrations [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind]. Pre- and post-prandial bile acids testing was performed on those dogs with serum liver-associated biochemical abnormalities.

For the 50 dogs in the prospective study, signalment, physical examination, seizure history, information on administration of other drugs or concurrent medical conditions, and results of CBC and biochemical analyses were obtained before the start of phenobarbital therapy. Physical examinations, seizure histories, serum biochemical profiles, and serum phenobarbital concentrations were also obtained 3 weeks, 6 months, and 12 months after the start of phenobarbital. Twenty-two dogs had data collected at all 4 time points. Eight dogs did not return for 1 or more blood collections for reasons unknown. Twenty dogs were removed from the study at various times. Six dogs were removed after potassium bromide was added to the therapeutic regimen. Four dogs were removed after their owners elected not to continue phenobarbital treatment. One dog was removed after being anesthetized for seizure control prior to blood collection. One dog was removed for inconsistent medication history. One dog died from unrelated trauma.

Two dogs changed ownership and moved away. Five dogs were euthanized due to seizures or other causes.

Degree of seizure control was determined for all phenobarbital-treated dogs (good control = no seizures in the previous 3 months; fair = 1 or 2 seizure episodes in the previous 3 months; poor = 1 or more seizure episodes per month for the previous 3 months). A second data set depicting seizure control was devised by assigning to each dog one point for each seizure episode in the previous 3 months, plus one point for generalized seizure activity, and an additional point for cluster type seizure activity.

Data collected for the 8 control dogs consisted of signalment, physical examination results, and results of CBC and serum biochemical analyses.

Serum AP isoenzyme determination — Serum AP isoenzyme analysis for quantification of L-AP, C-AP, and B-AP isoenzymes were performed for 30 of the dogs in the cross-sectional study, for 25 dogs in the prospective studies, and for the 8 control dogs. Methods were those of Sanecki *et al.* and Hoffman *et al.* (Hoffmann *et al.* 1988, Sanecki *et al.* 1993). Briefly, total AP activity was determined by hydrolysis of para-nitrophenyl phosphate in 1.0 M diethanolamine buffer containing 0.5 mM $MgCl_2$, pH 9.8, on the Hitachi 911 biochemical analyzer [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind]. Bone-AP activity was determined by adding 100 µl of 5 mg/ml wheat germ lectin (WGL) [Triteum Vulgaris lectin, Sigma Aldrich Canada Ltd, Oakville, Ontario] to 100 µl of serum in a microfuge tube and incubating at 37°C for 20 minutes. This causes approximately 97% of B-AP, 76-100% of C-AP, and 18% of L-AP to precipitate. The tube was centrifuged for 2 minutes at 16,000 x g and the supernatant

analyzed for AP activity as described above (multiplied by 2 to correct for dilution). An aliquot of the supernatant and an aliquot of the original serum were simultaneously assayed for C-AP using a levamisole-inhibition technique. With this technique, greater than 98% of L-AP and B-AP are inhibited by 4.2 mM levamisole [Sigma Chemical Company, St. Louis, MO] whereas only 42% of C-AP is inhibited. The levamisole was added to the diethanolamine buffer in the autoanalyzer to give a final concentration of 4.2 mM, and samples were incubated in the buffer for 5 minutes prior to addition of substrate in the autoanalyzer. Positive control samples for each isoenzyme were included in each run. Isoenzyme activities were calculated as follows:

(1) Total AP activity = AP activity of serum without WGL or levamisole added

(2) C-AP activity was calculated using the following equation:

$$\text{C-AP activity} = \frac{\text{AP remaining} - (\text{L-AP fraction} \times \text{total AP})}{\text{C-AP fraction} - \text{L-AP fraction}}$$

Where:

AP remaining = AP activity in serum sample after levamisole inhibition

C-AP fraction = fraction of control C-AP activity remaining after levamisole inhibition

L-AP fraction = fraction of control L-AP remaining after levamisole inhibition

(3) B-AP = Total serum AP — (C-AP + L-AP)

(4) L-AP activity = 2 x (total AP in WGL supernate — C-AP in WGL supernate)

Where: C-AP in WGL supernate determined as described above for C-AP, using the WGL supernate instead of original serum.

Cellulose acetate affinity electrophoresis as described by Kidney and Jackson (1988) was performed to qualitatively evaluate serum AP isoenzymes. Briefly, cellulose acetate membranes [Sepraphore III, Gelman Sciences Inc., Ann Arbor, MI] were presoaked for 10 minutes at room temperature in Tris-barbital buffer (0.06mol/L) [Gelman High Resolution Buffer , 0.06 mole/L ionic strength (32.7% w/w Tris |2 amino-2-(hydroxymethyl)-1,3-propanediol)|; 13.7% w/w barbital; 54.2% sodium barbital; Gelman Sciences, Inc., Ann Arbor, MI] containing WGL at a final concentration of 50 mg/L. This causes the C-AP to remain at the origin and slows the migration of the B-AP, which allows for much clearer separation of bands compared to traditional electrophoresis. Approximately 20 to 40 µl of sample was applied to the membrane, depending on total AP activity (more sample applied if total AP was low). Three control samples containing large amounts of B-AP, L-AP, and C-AP, respectively, and 4 patient samples were loaded per membrane. Electrophoresis was performed using a Gelman Sepratek chamber [Gelman Sciences, Inc., Ann Arbor, MI] (200 volts for 40 minutes) and 0.06 mol/L Tris-barbital buffer. The AP isoenzyme bands were visualized by staining a second membrane with phosphatase substrate [Kirkegaard & Perry Laboratories, Gaithersburg, Maryland] and superimposing the second membrane over the electrophoretic strip on a glass slide. A second glass slide was placed on top, and the membranes were incubated at room temperature for approximately 15-20 minutes until bands could be distinctly visualized. Because of rapid fading of color, bands were evaluated and photographed immediately.

Liver biopsies — Dogs from the cross-sectional or prospective studies that met specific

criteria qualified for referral to the Atlantic Veterinary College (AVC) Small Animal Hospital for additional diagnostic tests and liver biopsy. These criteria included having a serum total AP and/or ALT activity >2X the upper limit of the normal AVC reference range, low serum albumin and urea, or an abnormal pre-and post-prandial bile acids test. Thirty of the original 128 (23%) dogs in the combined cross-sectional and prospective studies met the criteria for additional diagnostic tests and biopsy. However, not all owners elected to pursue additional diagnostic tests. Liver biopsies were obtained from 15 phenobarbital-treated dogs. Two of the 15 dogs were excluded from analyses because of subsequent histories of possible exposure to another chemical or evidence of possible concurrent disease. Of the remaining 13 dogs, adequate amounts of liver tissue were obtained from 12 dogs to allow inclusion in the present study. For dogs that underwent liver biopsy, in addition to the data listed above, coagulation profiles (prothrombin time, activated partial thromboplastin time), and abdominal ultrasound examinations were performed. Eleven of the 12 dogs had serum AP isoenzyme analyses data available.

Liver samples were obtained via ultrasound-guided percutaneous needle biopsy from 11/12 phenobarbital-treated dogs, using a Biopty [C.R. Bard Inc., Covinton, Georgia] spring-driven biopsy instrument with an 18 gauge needle. A wedge liver biopsy sample was obtained during exploratory laparotomy in the remaining dog. Liver biopsies were obtained from the 8 control dogs under anesthesia immediately prior to euthanasia. Liver biopsies in the control dogs were obtained using the Biopty instrument with a 14 gauge needle. One portion of each biopsy was fixed in 10% buffered formalin for histopathology, and another portion was flash frozen in liquid nitrogen and stored at -80° C.

Liver tissue analyses:

Histopathology — Sections from the formalin-fixed liver samples were stained for light microscopy by the AVC Diagnostic Laboratory using standard techniques. Sections were stained with hematoxylin-eosin, rhodanine (copper), prussian blue (iron), periodic acid Schiff (glycogen), and a reticulum stain (fibrosis). Histopathology slides were randomized and scored in a blinded fashion by a single pathologist. Histopathological categories scored included those indicative of induction (hepatocyte size and cytoplasmic characteristics representative of endoplasmic reticulum), and those indicative of injury (necrosis, inflammation, fibrosis, vascular characteristics, and an overall score indicative of injurious type changes). Degree of staining for copper, iron, and glycogen was also scored. The scoring system used in all categories consisted of a scale of 1 through 4 (1 = normal, 2 = mildly abnormal, 3 = moderately abnormal, 4 = markedly abnormal).

Liver homogenate enzyme assays — Frozen liver samples were thawed and manually homogenized in 9 volumes of Tris/KCl buffer (pH 7.4) [6.06 g Tris base, 11.5 g KCl; qs with deionized water for a total volume of 1,000 ml; pH to 7.4 with 6 N HCl] using disposable pestle-and-microfuge homogenization sets [Kontes Glass Company, Vineland, New Jersey]. Protein content of the homogenates was determined using the Biorad DC protein assay [Biorad Laboratories, Hercules, CA]. The linear ranges of all assays were determined, and quantities of liver homogenate were chosen to provide results well within the linear ranges. All assays were repeated once to assure

reproducibility (duplicate data not shown).

(1) Alanine aminotransferase activity — ALT activity in each liver homogenate was determined using a quantitative, colorimetric end-point assay [Procedure #104, Sigma Diagnostics, St. Louis, MO] that used α-ketoglutaric acid as the substrate and detected the production of pyruvic acid. Assays for ALT activity were performed immediately after homogenization, in one batch. Results were normalized to protein concentration and reported in International Units/mg protein. Assays were performed on liver homogenates from the 12 phenobarbital-treated dogs and the 8 controls.

(2) Alkaline phosphatase activity — Total AP activity in liver homogenate was determined using a quantitative, colorimetric end-point assay kit [Procedure #505, Sigma Diagnostics, St. Louis, MO] that used para-nitrophenyl phosphate as the substrate, 2-amino 2-methyl-1propanol buffer, and detection of the end product para-nitrophenol. Assays for AP activity were performed after one freeze-thaw cycle of the homogenates. Results were normalized to protein concentration and reported in International Units/mg protein. The assay was performed on liver homogenates from 6 phenobarbital-treated dogs with adequate amounts of sample remaining, as well as the 8 controls.

(3) Benzyloxyresorufin-*O*-dealkylase activity — Liver homogenate benzyloxyresorufin-*O*-dealkylase (BROD) activity, a specific assay for the major phenobarbital-induced cytochrome P450 in dogs (CYP2B11) (Klekotka and Halpert

1995), was measured as a positive control for verification of induction of a liver enzyme known to be induced by phenobarbital. The assay was developed by modifying published techniques (Burke *et al.* 1985, Klekotka and Halpert 1995, Lubet *et al.* 1985a and 1985b, Nims *et al.* 1987, Wink *et al.* 1993). Reactions were carried out in a fluorimeter microcuvette at 37°C using a Shimadzu RF-5301 PC series spectrofluorophotometer [RF5301PC, Shimadzu Scientific Instruments, Inc., Kyoto, Japan]. The reaction mixture contained 5 µl of crude homogenate (~75-100 µg of protein), 2.5 µl of a 1 mM benzyloxyresorufin substrate solution, and 465 µl of PBS buffer (pH 7.4) [4.0 g NaCl, 0.19 g KCl, 0.138 g NaH_2PO_4-H_2O, 1.01 g Na_2HPO_4 anhydrous; qs with deionized water for a total volume of 500 ml; pH to 7.4 with 0.12 M HCl]. Added to this was 2.5 µl of a 0.1 mM solution of dicumarol in DMSO to minimize the NADPH-mediated reduction of resorufin that would otherwise occur after initiation of the reaction by NADPH (Lubert *et al.* 1985a and 1985b, Nims and Lubet 1983, Nims *et al.* 1984, Wink *et al.* 1993). We determined that higher concentrations of dicumarol caused inhibition of the CYP2B enzymes, and lower concentrations did not maximally diminish the rate of reduction of resorufin. The above mixture was equilibrated at 37°C for 1 minute, and the reaction was then initiated by the addition of 25 µl of 5 mM NADPH (500 µl final reaction mixture) and allowed to run for 6.5 minutes. The rate of production of the end product, resorufin, was determined by measuring the increasing fluorescence of the reaction mixture. The fluorimeter settings were: excitation and emission slits, 5 nm; excitation and emission wavelengths, 530 and 585 nm, respectively. Fluorescence was calibrated using a standard containing 5 µl of sample, 2.5 µl of 1 mM

benzyloxyresorufin, 485 µl of PBS buffer, 2.5 µl of 0.1 mM dicumarol, and 5 µl of 10 µM resorufin. Results were normalized to protein concentrations of the homogenates, and expressed as nmol of product/minute/mg of protein. The assay was performed on liver homogenate from 5 phenobarbital-treated dogs with adequate amounts of sample remaining and from 8 control dogs.

(4) Immunoblotting of CYP2B protein — Amounts of CYP2B protein in the liver homogenates of 5 phenobarbital-treated dogs with adequate amounts of sample remaining and 8 control dogs were determined using standard Western Blotting techniques (Cribb *et al.* 1997, Delaporte *et al.* 1993, Gallagher *et al.* 1997, Towbin *et al.* 1979). Briefly, 100 µg of crude homogenate protein were loaded per lane on a 10% SDS-polyacrylamide gel and electrophoresed under reducing conditions. The proteins were transferred to a nitrocellulose membrane by a semidry transfer process, and immunodetection of transferred CYP2B proteins was performed using goat anti-rat anti-CYP2B antibody (dilution: 1/10,000) [Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan] and rabbit anti-goat IgG peroxidase conjugate secondary antibody (dilution: 1/25,000) [Sigma, St. Louis, MO]. Bands were visualized by enhanced chemiluminescence techniques, digitized [Eagle Eye, Stratagene, La Jolla, CA] and intensities determined [NIH Image for MacIntosh, Scion Corp., Frederick, Maryland].

Statistical analyses — Correlations were determined using non-parametric Spearman's correlation coefficient (r) tests because of small sample size (prospective study) or non-

normal data (cross-sectional study) [SPSS Inc. Software, Chicago, IL]. In the prospective study, for those dogs with data at each time point, comparisons were made using repeated measures analysis of variance (ANOVA) [GraphPad Software Inc., Prism2, San Diego, CA]. Additionally, data collected from all dogs that presented at each time point were compared to the data from other time points using non-parametric Wilcoxon Signed Rank Sum Test paired *t* tests [GraphPad Software Inc., Prism2, San Diego, CA]. For the liver biopsy data, non-parametric methods were used for all statistical analyses (Mann-Whitney test for comparisons, Spearman's correlation coefficient (r) test for correlations [GraphPad Software Inc., Prism2, San Diego, CA]). One-tailed analyses were used in comparisons of serum ALT and AP activities between the phenobarbital-treated dogs and the controls, as increased serum ALT and AP activities were selected for in phenobarbital-treated dogs, and hence the treated dogs were expected to have higher values of these serum enzymes than the controls. Two-tailed analyses were used for all other comparisons.

For histopathological comparisons, the 12 phenobarbital-treated dogs were compared to the 8 controls. For liver homogenate ALT activity comparisons, the 12 phenobarbital-treated dogs were compared to the 8 controls. For liver homogenate AP activity comparisons, 6 phenobarbital-treated dogs with adequate amounts of liver homogenate remaining to perform the assay were compared to the 8 control dogs. For the CYP2B comparisons, 5 phenobarbital-treated dogs with adequate amounts of liver homogenate remaining were compared to the 8 controls. Significance was defined as $P \leq 0.05$ throughout.

RESULTS

Epidemiological descriptions:

Cross-sectional study — The 78 dogs in the cross-sectional study consisted of 46 males (36 of which were neutered) and 32 females (27 spayed). Ages ranged from 1 to 12.5 years (mean ± SD: 5.6 ± 2.8 years). Body weight ranged from 1.3 to 47.0 kg (2.9 to 103.4 lb), median 21.2 kg (46.6 lb). Breeds most commonly represented were: mixed breed (n=22), Dalmation (7), Golden Retriever (5), Miniature Poodle (5), Labrador Retriever (4), Yorkshire Terrier (4), Border Collie (3), Husky (3), Beagle (3), Shetland Sheepdog (3), and German Shepherd (3).

Serum PB concentrations in this group ranged from 19 to 304 μmol/L (4 to 70 μg/ml), median 76.5 μmol/L (17.6 μg/ml), and total duration of PB therapy ranged from 1.3 weeks to 8 years (median 12.5 months). Total daily dose of PB ranged from 1.0 to 16.4 mg/kg/day (0.5 to 7.5 mg/lb/day), median 4.0 mg/kg/day (1.8 mg/lb/day). Seventy-one of the 78 dogs (91%) had received PB for a sufficient period of time (3 months) to categorize seizure control. Thirty-seven percent had good seizure control, 36% had fair control, and 27% had poor control. The data set of seizure scores ranged from 0 (no seizures in the preceding 3 months) to 13.5 (many generalized cluster-type seizure episodes per month). Median seizure score was 3.0 points.

Prospective study — Of the 50 dogs initially enrolled in the prospective study, 40, 30, and 26 dogs returned for data collection at 3 weeks, 6 months, and 12 months, respectively. Twenty-two of the dogs had data collected at all 4 time points. The 22 dogs

that completed the prospective study consisted of 10 males (9 neutered) and 12 females (8 spayed). Dogs ranged from 1 to 8 years old (mean ± SD, 3.9 ± 1.7 years) and weighed between 3.0 and 36.4 kg (median, 20.9 kg). Breeds consisted of 14 mixed breed dogs, 2 Golden Retrievers, 2 Labrador Retrievers, 1 Border Collie, 1 Husky, 1 Miniature Dachshund, and 1 Standard Poodle.

Serum phenobarbital concentrations in the 22 dogs ranged from 33 to 85 μmol/L (mean ± SD, 58.6 ± 15.0 μmol/L) at 3 weeks, 8 to 120 μmol/L (62.5 ± 25.7 μmol/L) at 6 months, and 11 to 116 μmol/L (62.2 ± 23.5 μmol/L) at 12 months. Total daily dose of phenobarbital ranged from 1.3 to 6.0 mg/kg/day (3.6 ± 1.3 mg/kg/day) at 3 weeks, 1.3 to 8.3 mg/kg/day (3.7 ± 1.4 mg/kg/day) at 6 months, and 1.3 to 8.3 mg/kg/day (3.7 ± 1.6 mg/kg/day) at 12 months. At 6 months, 17 dogs (77.3%) had good seizure control, 4 (18.2) had fair control, and 1 (4.5%) had poor control. At 12 months, 15 dogs (68.2%) had good control, and 7 (31.8%) had fair control. Seizure scores at both time periods ranged from 0 to 4 (median, 0). Twelve dogs had seizures within 24 hours prior to blood collection at time 0. No dogs had seizures within 24 hours prior to any subsequent blood collection.

Liver biopsy cases — The 12 phenobarbital-treated dogs from which liver biopsies were obtained consisted of 10 males (8 neutered) and 2 females (1 spayed). Ages ranged from 1.5 to 8 years (median 4.5 years). Breeds represented included 2 Huskies, 1 each of Labrador Retriever, English Springer Spaniel, Collie, Poodle, Shetland Sheepdog, and Pomeranian breeds, and 4 mixed-breed dogs. Serum phenobarbital concentrations ranged from 42 to 192 μmol/L ([9.7 to 44 μg/ml]; median 99 μmol/L

[22.8 μg/ml]), and total duration of phenobarbital treatment ranged from 4 months to 6.5 years (median 1.7 years). Total daily dose of phenobarbital ranged from 2.1 to 12.9 mg/kg of body weight per day (median 5 mg/kg/d). Seizure control was classified as good for 3 dogs, fair for 3 dogs, and poor for 6 dogs.

Control dogs — The 8 control dogs consisted of 3 males (all intact) and 5 females (3 spayed). Estimated ages ranged from 1 to 3 years (median 1.75 years). All 8 were mixed-breed dogs. Median age was significantly ($P < 0.005$) lower for the control dogs than the phenobarbital-treated liver biopsy cases.

Serum Biochemical profiles:

Cross-sectional study — Of the 78 phenobarbital-treated dogs in the cross-sectional study, 9 dogs (12%) had a serum ALT >2X the upper limit of the AVC reference range (reference range, 5 to 69 U/L) (**Figure 1**). Twenty-two dogs (28%) had a serum AP activity >2X the upper limit of the AVC reference range (reference range, 23 to 87 U/L) (**Figure 1**). One dog had serum urea and albumin concentrations less than the lower limit of the reference range. No dogs had increased AST, SDH, or conjugated bilirubin >2X upper limit of normal. Four dogs (5%) had increased GGT >2X upper limit of normal, but none of the 4 had serum AP or ALT >2X upper limit of normal. Serum AP activity was significantly correlated with dose of phenobarbital ($P < 0.01$, $r = 0.36$), and serum phenobarbital concentration ($P < 0.01$, $r = 0.33$) (**Figure 2**). There were also correlations between serum AP activity and duration of therapy ($P < 0.05$, $r = 0.27$), and serum ALT activity ($P < 0.05$, $r = 0.26$) (**Figure 2**). There was a significant

Figure 1. Serum alkaline phosphatase (AP) activities and serum alanine aminotransferase (ALT) activities in 78 phenobarbital-treated epileptic dogs in a cross-sectional study. Shaded area represents reference range. The dashed line indicates 2X the upper limit of the normal reference range. Individual datapoints represent values for specific dogs.

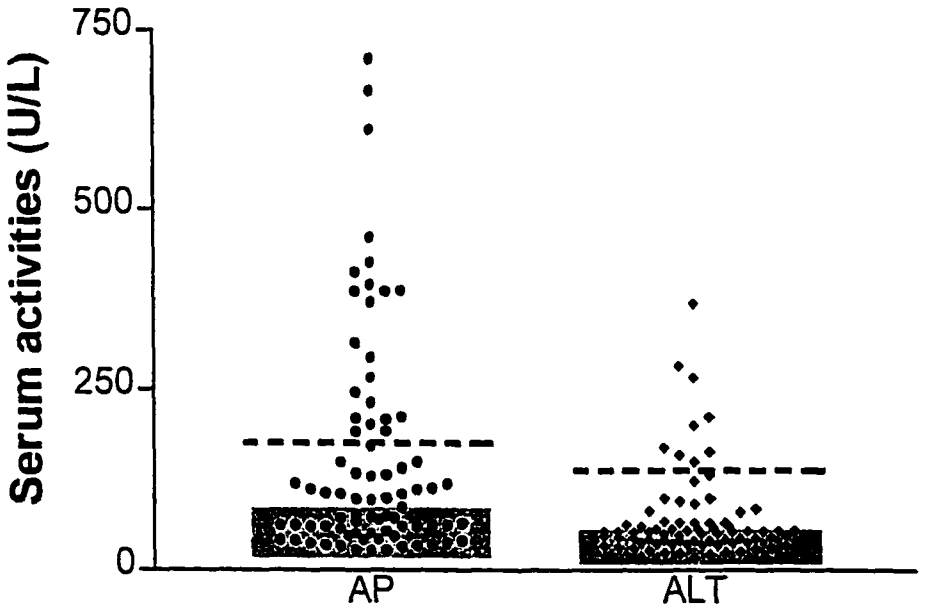
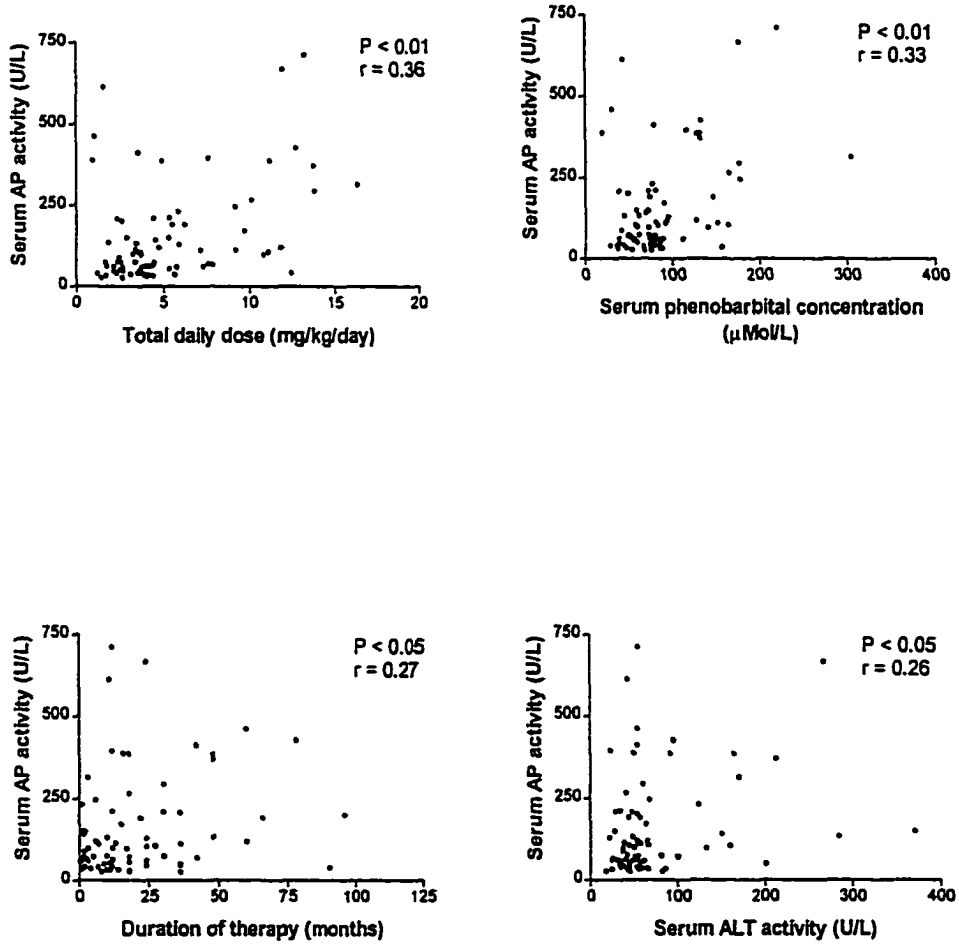


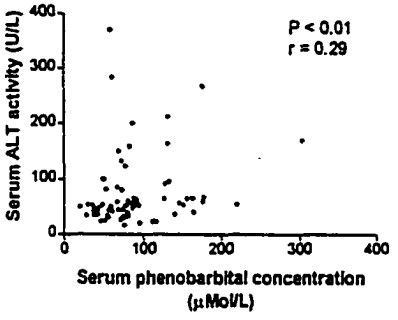
Figure 2. Scatterplots of significant correlations between serum alkaline phosphatase (AP) activity and phenobarbital dosage, serum phenobarbital concentration, duration of therapy, and serum alanine aminotransferase (ALT) activity in 78 phenobarbital-treated epileptic dogs in a cross-sectional study.

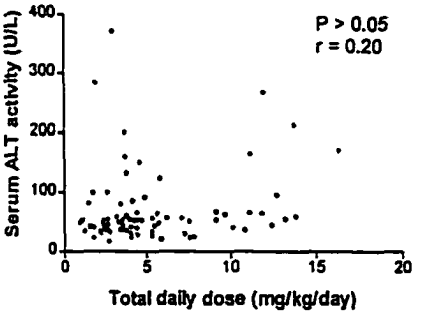


association between serum ALT activity and serum phenobarbital concentration ($P < 0.01$, $r = 0.29$) but no significant correlations between serum ALT activity and dose of phenobarbital or duration of therapy (**Figure 3**).

Prospective study — During the 1-year prospective study period, 4 of the 50 dogs originally enrolled in the prospective study developed serum ALT activities >2X upper limit of normal (**Figure 4a**). All 4 dogs remained in the study for the full 12 months. Two of the 4 dogs developed elevated serum ALT activities at the 12 month recheck; the other 2 developed elevated serum ALT activities at 3 and 6 months, with normal serum ALT activities at all other times. Five dogs developed serum AP activities >2X upper limit of normal (**Figure 4b**). Three/5 dogs demonstrated elevated serum AP activities at the 6 month recheck, and 2/5 demonstrated elevated serum AP activities at the 12 month recheck. One of the 5 dogs with an elevated serum AP activity at 6 months had potassium bromide added to his therapeutic regimen at that point because of poor seizure control, and so was not included in the data reported at the 12 month recheck. The remaining 4 dogs with serum AP activities >2X upper limit of normal continued in the study for the full 12 months, with continually elevated serum AP activities. Three dogs developed very slightly and transiently decreased serum urea concentrations, but no dogs developed serum albumin concentrations below the lower limit of the normal range. No dogs developed increased serum AST or conjugated bilirubin concentrations. Two dogs developed increased serum SDH activity and 3 dogs developed increased serum GGT activities >2X upper limit of normal, but none of these dogs had increased serum AP or ALT activities >2X the upper limit of normal. A significant correlation was found

Figure 3. **Scatterplots** showing a significant correlation between serum alanine aminotransferase (ALT) activity and serum phenobarbital concentration, but not between ALT activity and phenobarbital dosage or duration of therapy, in 78 phenobarbital-treated epileptic dogs in a cross-sectional study.





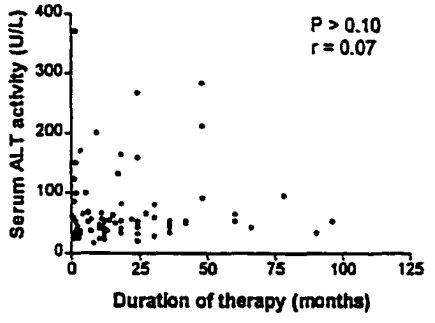
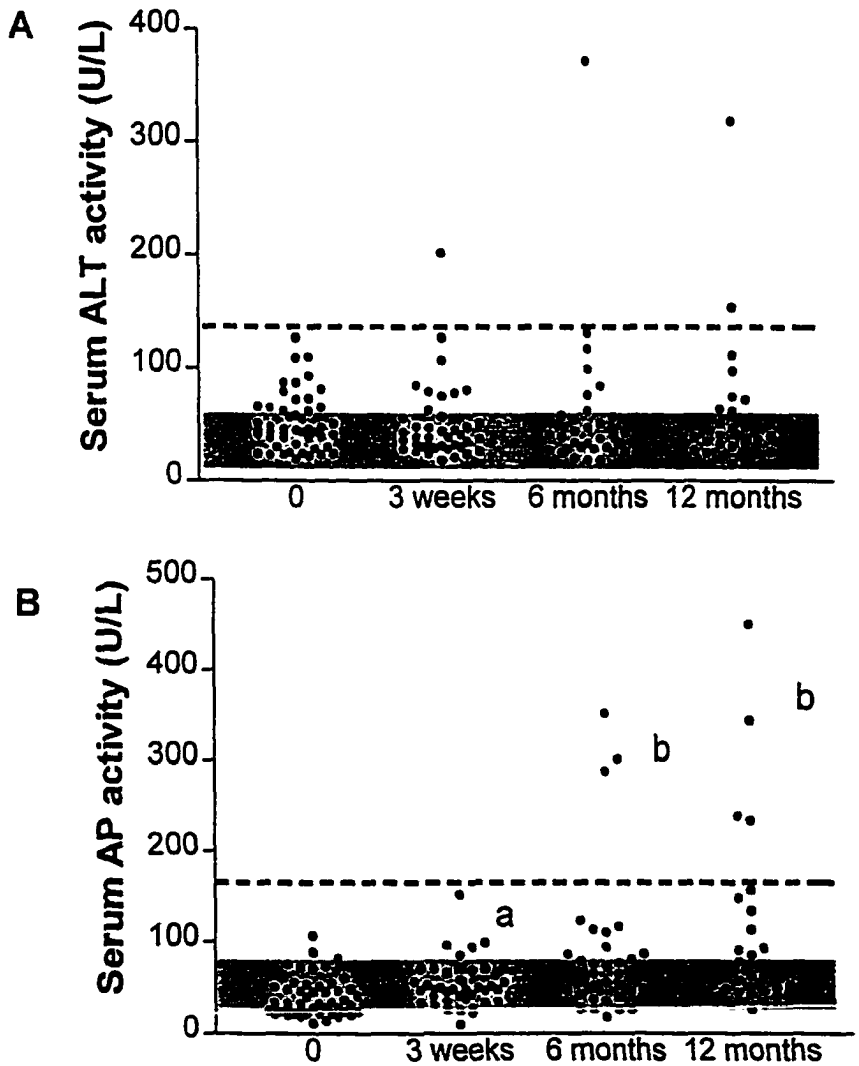


Figure 4. (A) Serum alanine aminotransferase (ALT) activities and (B) serum alkaline phosphatase (AP) activities before the start of phenobarbital therapy (time 0) and 3 weeks, 6 months, and 12 months after phenobarbital initiation. Fifty dogs were initially enrolled in the prospective study; progressively fewer dogs presented at each time point. Individual datapoints represent values for specific dogs. Shaded area represents the AVC Diagnostic Laboratory normal reference range. a = Median value differs significantly ($P<0.01$) from that at time 0. b = Median value differs significantly ($P<0.001$) from that at time 0.



between serum AP activity and duration of therapy ($P < 0.001$, $r = 0.48$), but not between serum AP activity and dose of phenobarbital or serum phenobarbital concentration at any of the time points. Serum ALT activity was not correlated with duration of therapy or with dose of phenobarbital at any time point, but was correlated with serum phenobarbital concentration at 12 months ($P < 0.05$, $r = 0.46$).

Liver biopsy cases — Of the 12 phenobarbital-treated dogs that had liver biopsies performed, 7/12 dogs (58%) had serum ALT activities greater than the upper limit of the normal range, and 3/12 (25%) had a serum ALT >2X the upper limit of the normal range. Eleven dogs (92%) had a serum AP activity >2X the upper limit of the normal range. One dog had serum urea and albumin concentrations less than the lower limit of the reference range. No dogs had increased AST, SDH, GGT, or conjugated bilirubin >2X upper limit of normal. Neither serum ALT or AP activities were significantly correlated with serum phenobarbital concentration, total daily phenobarbital dose, duration of therapy, or age (**Figure 5**).

Control dogs — Serum biochemical analyses were within normal limits for the 8 control dogs. As expected in young dogs, serum total AP activity was significantly negatively correlated with age ($P < 0.01$, $r = -0.89$).

Comparisons — As anticipated, median serum ALT activity in the 12 phenobarbital-treated biopsy dogs was significantly ($P < 0.05$) higher than the median serum ALT activity of the 8 controls (92.5 and 43.5 U/L, respectively) (**Figure 6a**).

Figure 5. Scatterplots showing lack of significant linear correlations between serum ALT activities in 12 phenobarbital-treated epileptic dogs from whom liver biopsies were obtained and (A) serum phenobarbital concentration, (B) total daily dose of phenobarbital, (C) duration of therapy, and (D) age.

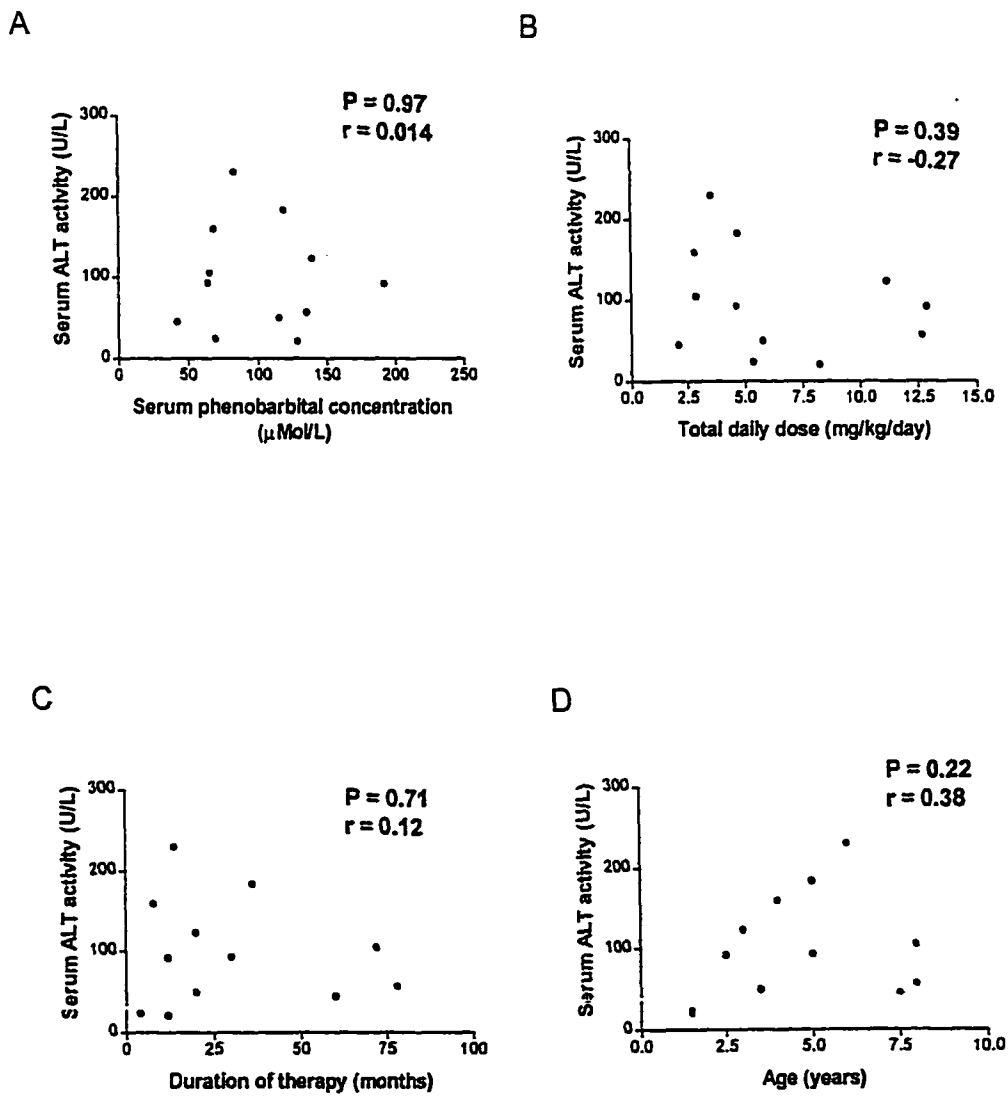
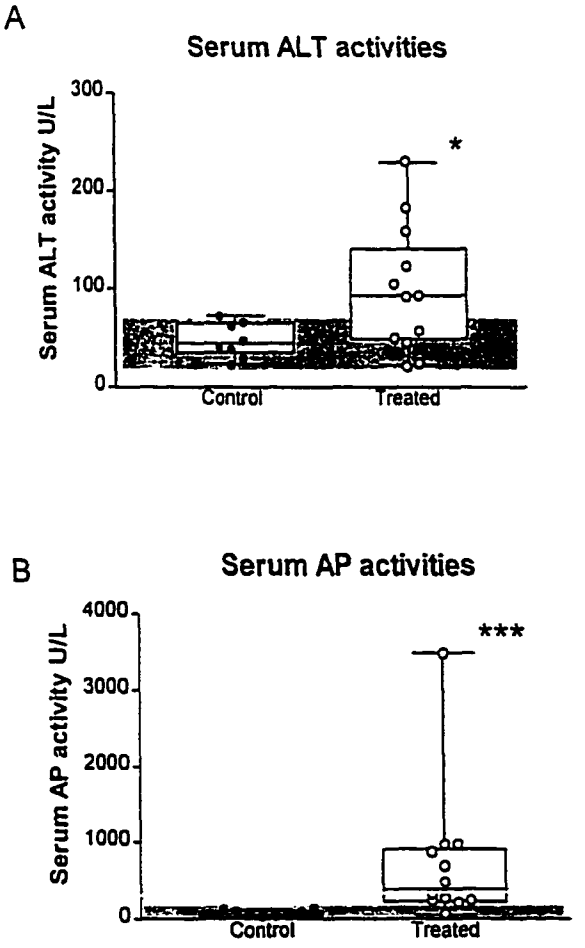


Figure 6. (A) Alanine aminotransferase (ALT) and (B) alkaline phosphatase (AP) activities in serum from 12 phenobarbital-treated epileptic dogs (PB-treated) and 8 normal control dogs. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Data points represent values for specific dogs. Shaded area represents the AVC Diagnostic Laboratory normal reference range.

*Median value differs significantly ($P < 0.05$) from the control dogs.

***Median value differs significantly ($P < 0.001$) from the control dogs.



Median serum total AP activity of the phenobarbital-treated liver biopsy dogs was significantly ($P < 0.001$) higher than the controls (378.5 and 80.5 U/L, respectively; **Figure 6b**).

Serum AP isoenzyme analyses

Cross-sectional study -- Serum AP isoenzyme activities were analyzed for 20 of the 22 dogs that had elevated serum AP activities in the cross-sectional study (**Figure 7**). Twelve/20 dogs had predominantly C-AP, 7/20 had predominantly L-AP, and 1/20 had approximately equal amounts of both. Ratios of C-AP to L-AP varied greatly. Only 1 dog had very slightly elevated B-AP. Serum AP isoenzymes were also analyzed for 10 phenobarbital-treated dogs that had normal serum total AP activities, and that had been receiving phenobarbital for at least 6 months (**Figure 8**). All but 1 of these dogs had predominantly L-AP, with virtually no C-AP. The remaining dog had C-AP slightly higher than L-AP, but still within the typical reference range. Median serum C-AP, L-AP, and B-AP activities were all significantly increased in the dogs with increased serum total AP activities compared to the dogs with normal serum total AP activities. However, the majority of the elevation in serum total AP activity was due to C-AP. Serum total AP was significantly correlated with C-AP ($P < 0.001$, $r = 0.87$), but not with L-AP or B-AP in the 20 dogs with increased serum total AP activity (**Figure 9a**). In the dogs with normal serum total AP activity, total AP activity was significantly correlated with B-AP ($P < 0.05$, $r = 0.72$), but not with L-AP or C-AP (**Figure 9b**). No unusual or previously unidentified bands were observed by cellulose acetate affinity electrophoresis for any of the dogs. Bands were located only in the previously described regions for B-AP, L-AP,

Figure 7. **(A) Serum alkaline phosphatase (AP) isoenzyme activities in 20 phenobarbital-treated epileptic dogs with serum total AP activity above the upper limit of the reference in a cross-sectional study.** Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme. **(B) Bar charts of isoenzymes for individual dogs.**

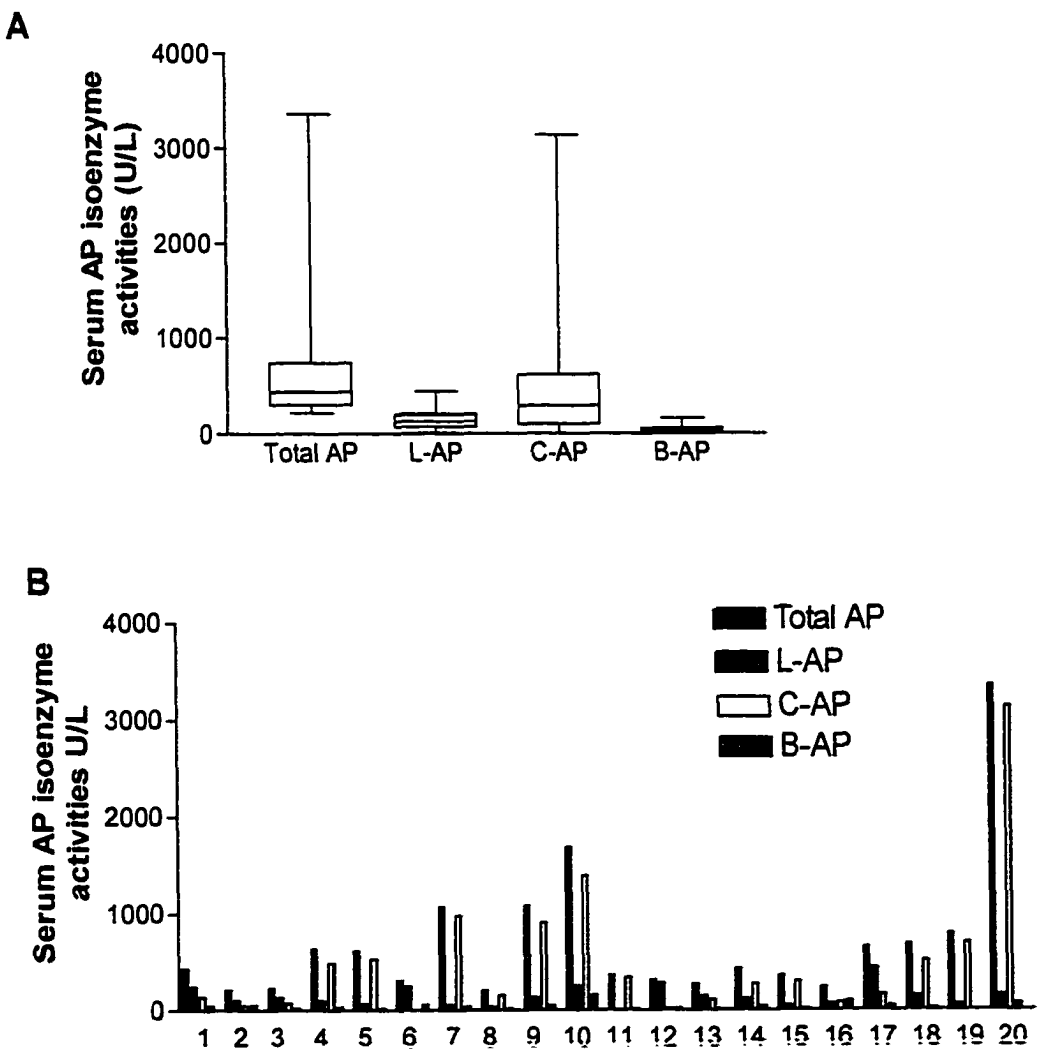


Figure 8. (A) Serum alkaline phosphatase (AP) isoenzyme activities in 10 phenobarbital treated epileptic dogs with normal serum total AP activities in a cross-sectional study. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme. **(B) Bar charts of isoenzymes from individual dogs.** (Note: Y axis ranges from 0 to 100 U/L. Y axis range in Figure 4 ranged from 0 to 4,000 U/L).

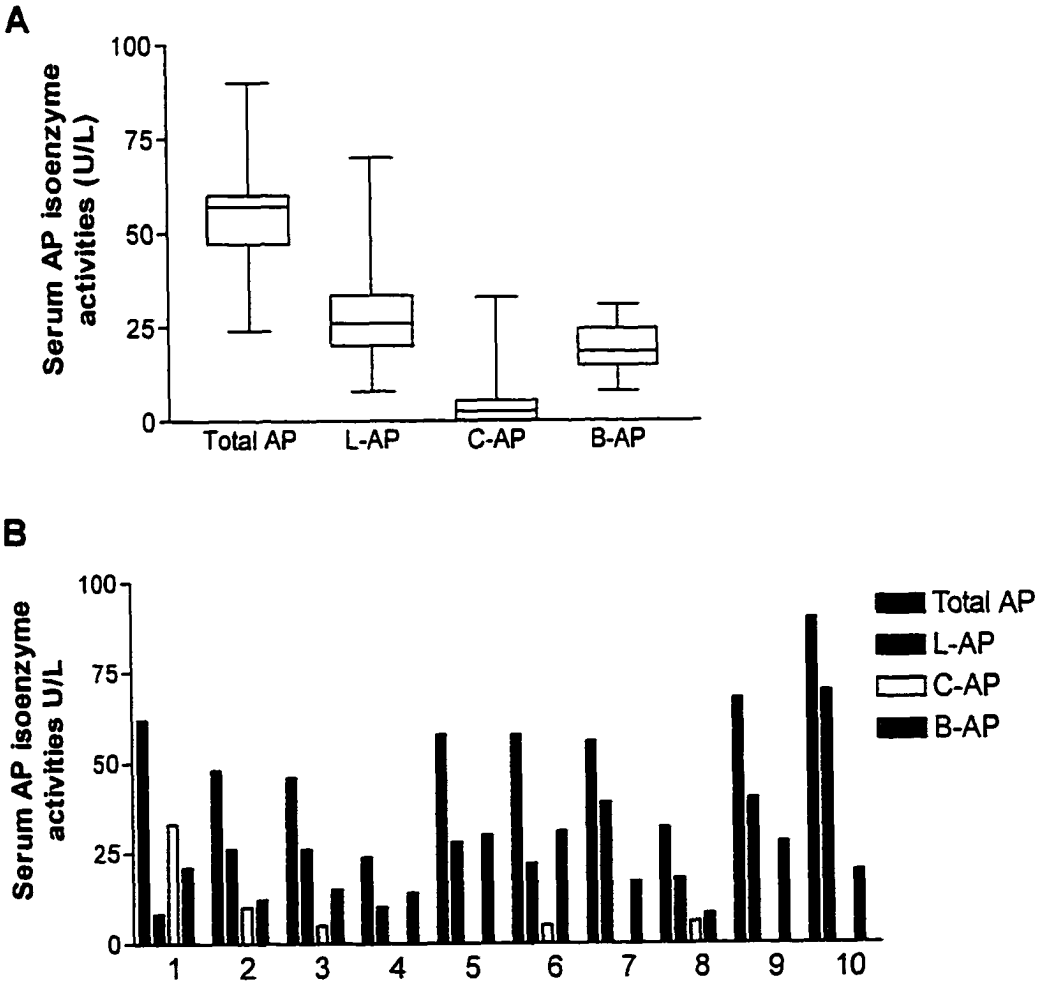
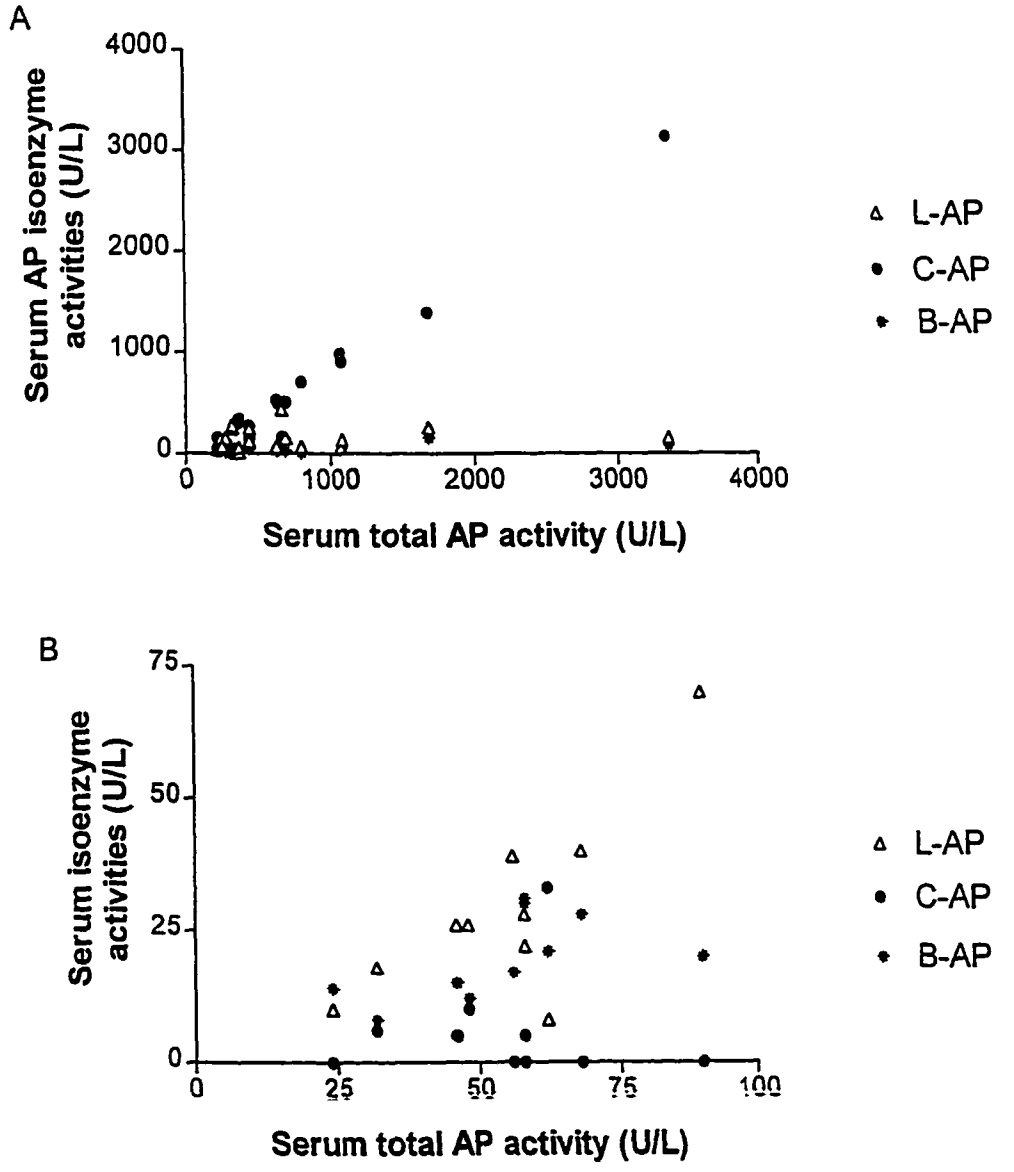


Figure 9. (A) **Scatterplot** showing serum total AP activity was significantly correlated with serum C-AP activity ($P < 0.001$, $r = 0.87$), but not L-AP or B-AP, **in 20 phenobarbital-treated dogs with elevated serum total AP activities.** (B) **Scatterplot** showing serum total AP activity was significantly correlated with B-AP activity ($P < 0.05$, $r = 0.72$), but not L-AP or C-AP, **in 10 phenobarbital-treated dogs with normal serum total AP activities.**



and C-AP (**Figure 10**).

Prospective study — Serum AP isoenzyme activities were analyzed for 25 dogs in the prospective study (**Figure 11**). Isoenzyme analyses were not performed on all dogs at all time points because of insufficient serum sample quantities. Of the 25 dogs, 9 completed the study with data from all time points, 10 had data for 3 of the 4 time points, and 6 had data from 1 or 2 of the time points (the 6 and/or 12 month time points). When data from all dogs presented at each time point were analyzed, median serum total AP, C-AP, L-AP, and B-AP activities were all significantly increased at both 6 months ($P < 0.01$, $P < 0.01$, and $P < 0.05$, respectively) and 12 months ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively) compared to time 0. When data from the 9 dogs with isoenzymes determinations at all 4 time points were compared by repeated measures ANOVA, median serum total AP and C-AP activities were significantly increased ($P < 0.05$ and $P < 0.05$, respectively) at 12 months compared to time 0. Median serum L-AP activity was significantly increased ($P < 0.05$) at both 6 and 12 months compared to time 0. Median serum B-AP was significantly increased ($P < 0.01$) at 6 months, but not at 12 months. No unusual or previously unidentified bands were found for any dogs on cellulose acetate affinity electrophoresis.

Liver biopsy dogs — Serum AP isoenzyme analyses were performed during the cross-sectional or prospective studies for 11 of the 12 phenobarbital-treated dogs that had liver biopsies (**Figure 12**). The remaining dog had insufficient serum quantity remaining for isoenzyme analysis. The majority of the serum total AP activity was due to the C-AP isoenzyme in 6 dogs, 5 of whom had serum total AP activities > 500 U/L. The L-AP

Figure 10. Cellulose acetate affinity electrophoresis separation of canine serum alkaline phosphatase (AP) isoenzymes. The wheat germ lectin incorporated in the buffer causes the corticosteroid isoenzyme (C-AP) to remain at the origin and slows the migration of the bone isoenzyme (B-AP), leaving only the liver (L-AP) isoenzyme to migrate with the solvent front. This results in much better separation of bands compared to traditional electrophoresis. Samples 1 and 2 have primarily C-AP, sample 3 has both B-AP and L-AP, sample 4 has both C-AP and L-AP, and sample 5 has predominantly L-AP.

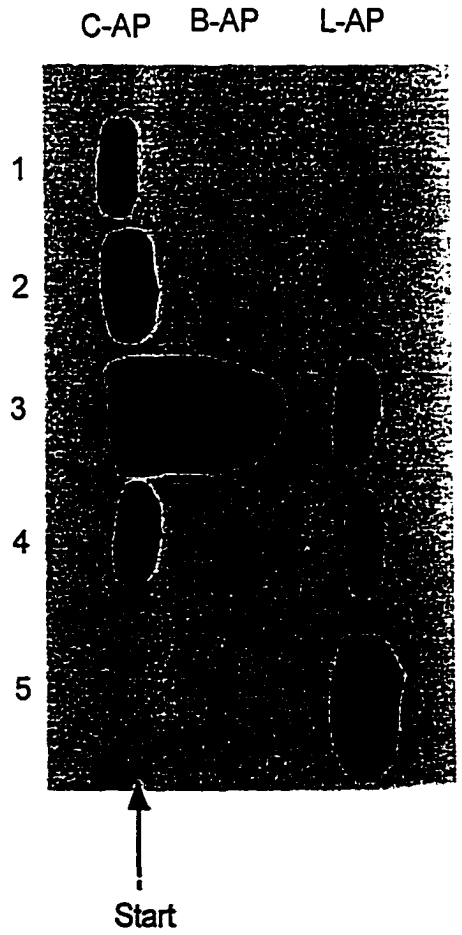


Figure 11. Serum alkaline phosphatase (AP) isoenzyme activities in a prospective study of epileptic dogs before the start of phenobarbital therapy (time 0) and 3 weeks, 6 months, and 12 months after phenobarbital initiation. Fifty dogs were initially enrolled in the prospective study; progressively fewer dogs presented at each time point. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme. Note the break in the Y axis for serum C-AP activity. * = Median value differs significantly ($P < 0.5$) than time 0. ** = Median value differs significantly ($P < 0.01$) than time 0. *** = Median value differs significantly ($P < 0.001$) than time 0.

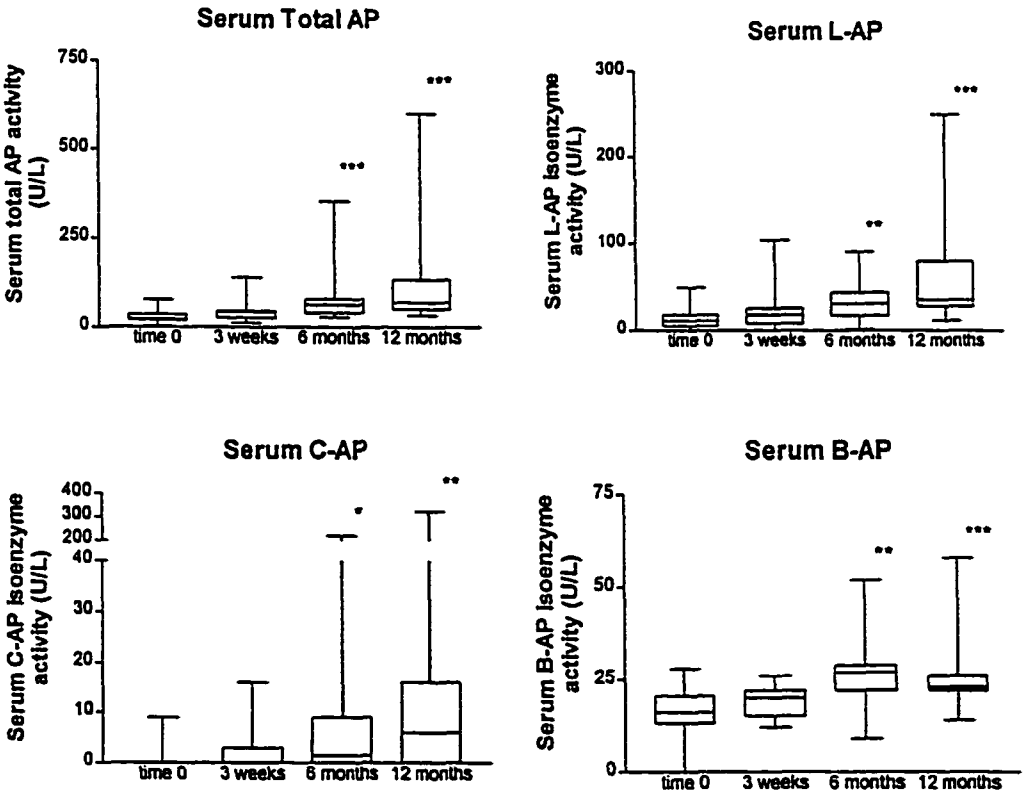
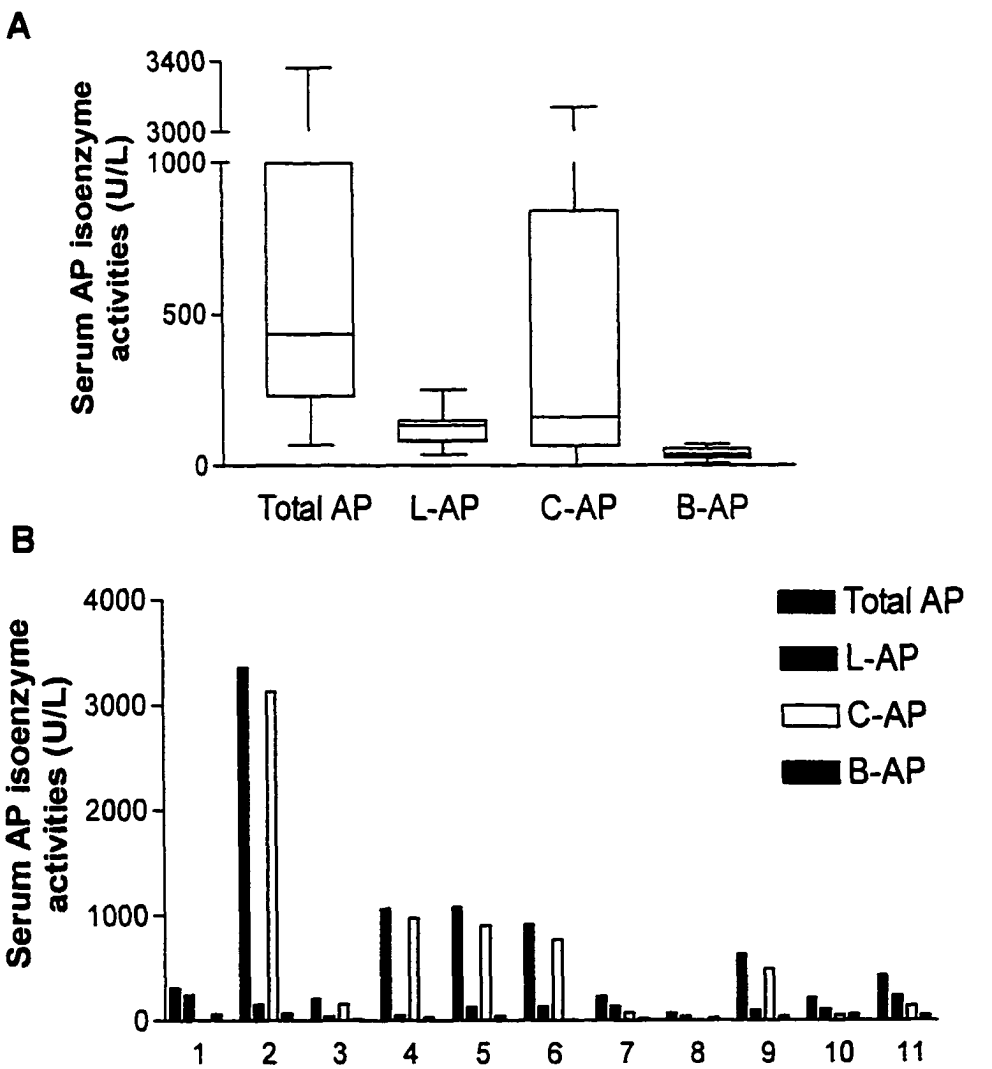


Figure 12. (A) Serum alkaline phosphatase (AP) isoenzyme activities in 11 phenobarbital-treated epileptic dogs (ages ranging from 1.5 to 8 years) from whom liver biopsies were obtained. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme. **(B) Bar charts of isoenzymes from individual dogs.**



isoenzyme was the predominant serum AP isoenzyme for the other 5 dogs, all of whom had serum total AP activities < 500 U/L.

Serum total AP activity was highly correlated with serum C-AP activity ($P < 0.001$, $r = 0.1$). Cellulose acetate affinity electrophoresis did not show any unusual or previously unidentified bands for any of the dogs.

Control dogs — Serum AP isoenzyme analyses were performed for all 8 control dogs (**Figure 13**). As is expected in young dogs, B-AP was the predominant serum AP isoenzyme in 7 of the 8 dogs. Serum L-AP activity was within the normal reference range for all control dogs. Only 1 control dog had C-AP present in the serum. As expected in young dogs, the B-AP activity was significantly negatively correlated with age ($P < 0.05$, $r = -0.83$) and positively correlated with serum total AP ($P < 0.01$, $r = 0.85$) (**Figure 14**).

Liver tissue analyses:

Histopathology — The phenobarbital-treated dogs had enlarged, swollen hepatocytes with finely granular cytoplasmic appearance indicative of increased amounts of endoplasmic reticulum (**Figure 15a**). Inflammatory cell infiltration, single cell and piecemeal necrosis, fibrosis, and vascular abnormalities resembling veno-occlusive changes were found in many of the dogs (**Figure 15b,c,d**). Many of the control dogs showed these same hepatic lesions, but to a lesser degree (ie, lower scores) than the phenobarbital-treated dogs. Sections from both phenobarbital-treated (4/12) and control (5/8) dogs stained positively for copper (**Figure 15e**). Nine of 12 samples from the

Figure 13. (A) Serum alkaline phosphatase (AP) isoenzyme activities in 8 normal control dogs (estimated ages ranging from 1 to 3 years) from whom liver biopsies were obtained. Box extends from the 25th percentile to the 75th percentile, with a horizontal line at the median. Whiskers show the range of the data, extending from the highest to the lowest data points. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme. **(B) Bar charts of isoenzymes from individual dogs.**

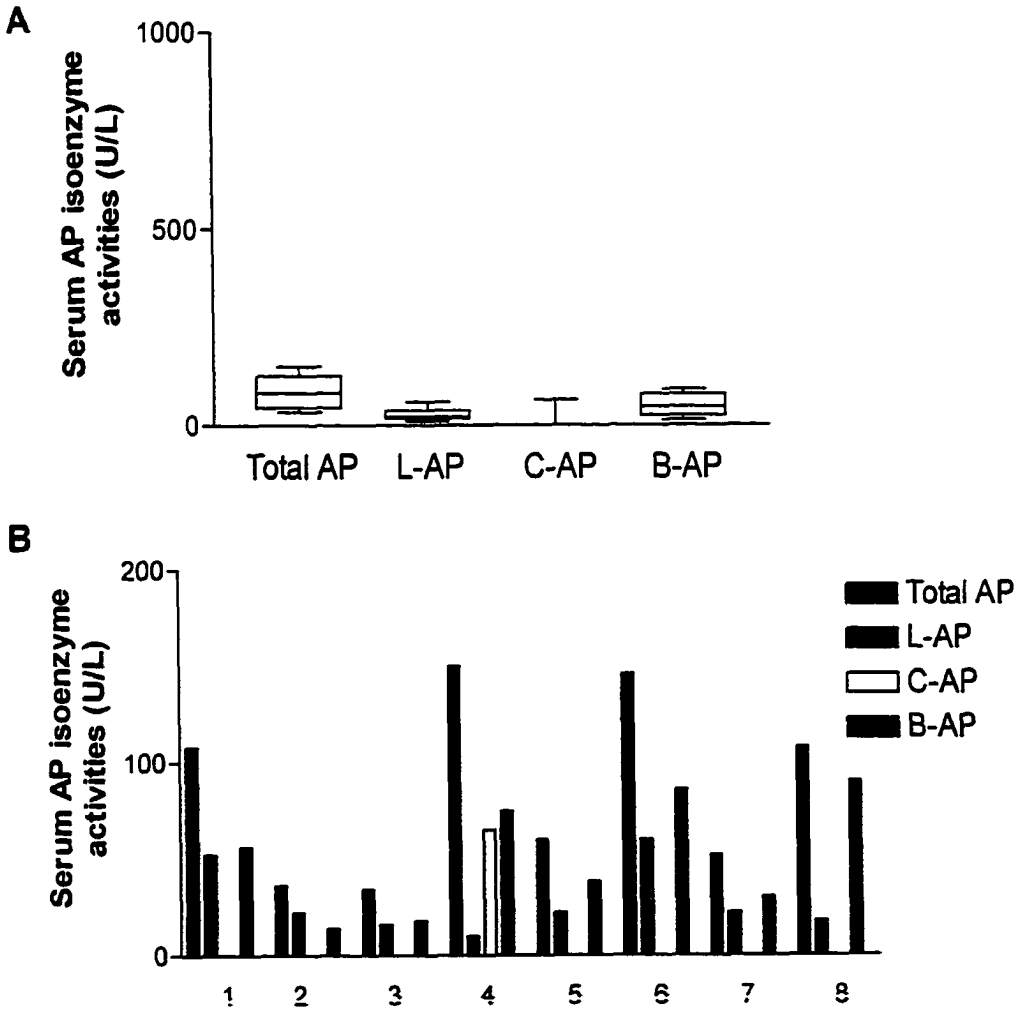


Figure 14. Scatterplots showing the significant negative correlation between B-AP activity and (A) age ($P < 0.05$, $r = -0.83$), and (B) serum total AP ($P < 0.01$, $r = 0.85$) in 8 control dogs. (C) There were no correlations between liver homogenate AP activity and serum B-AP, C-AP or L-AP activity in the control dogs.

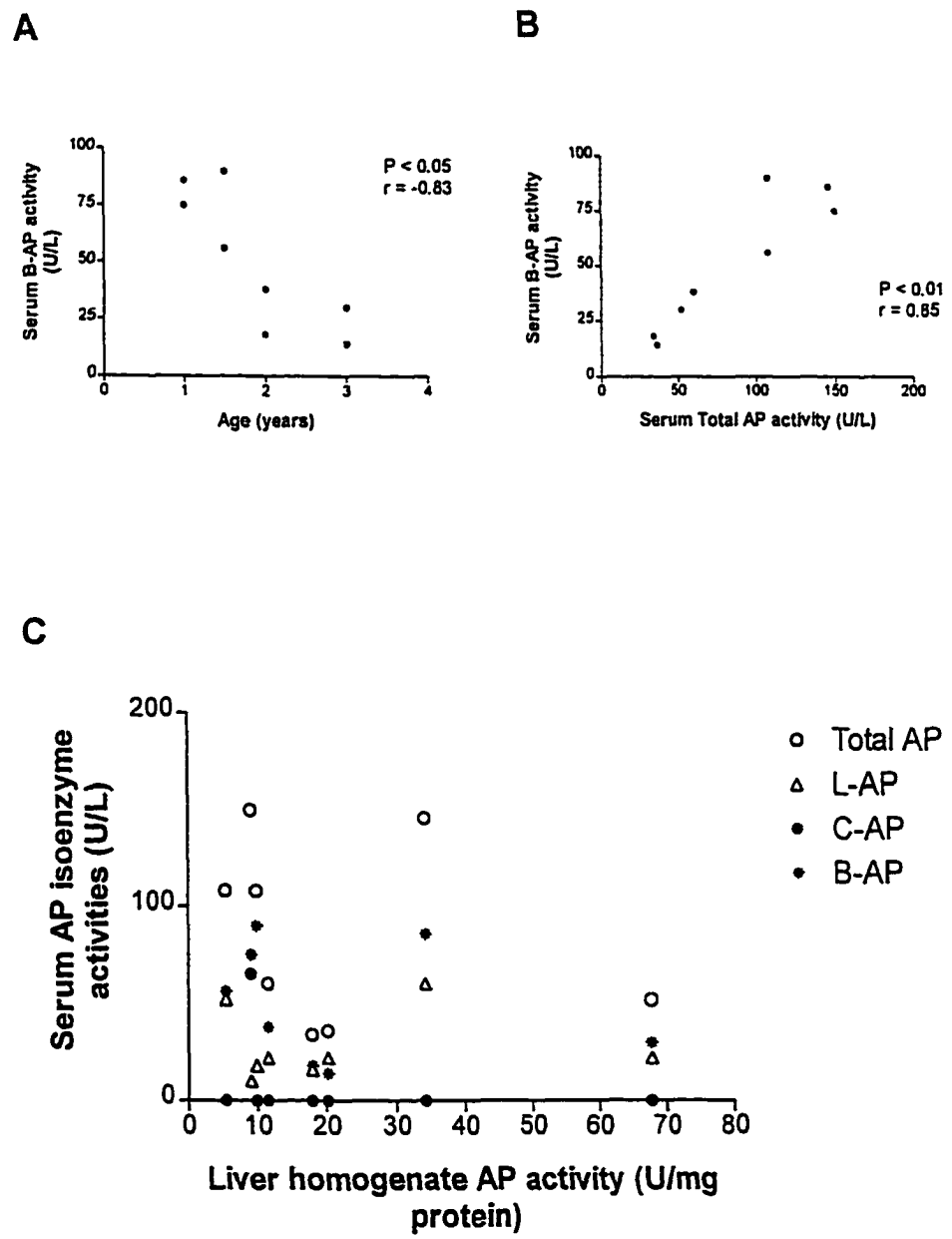
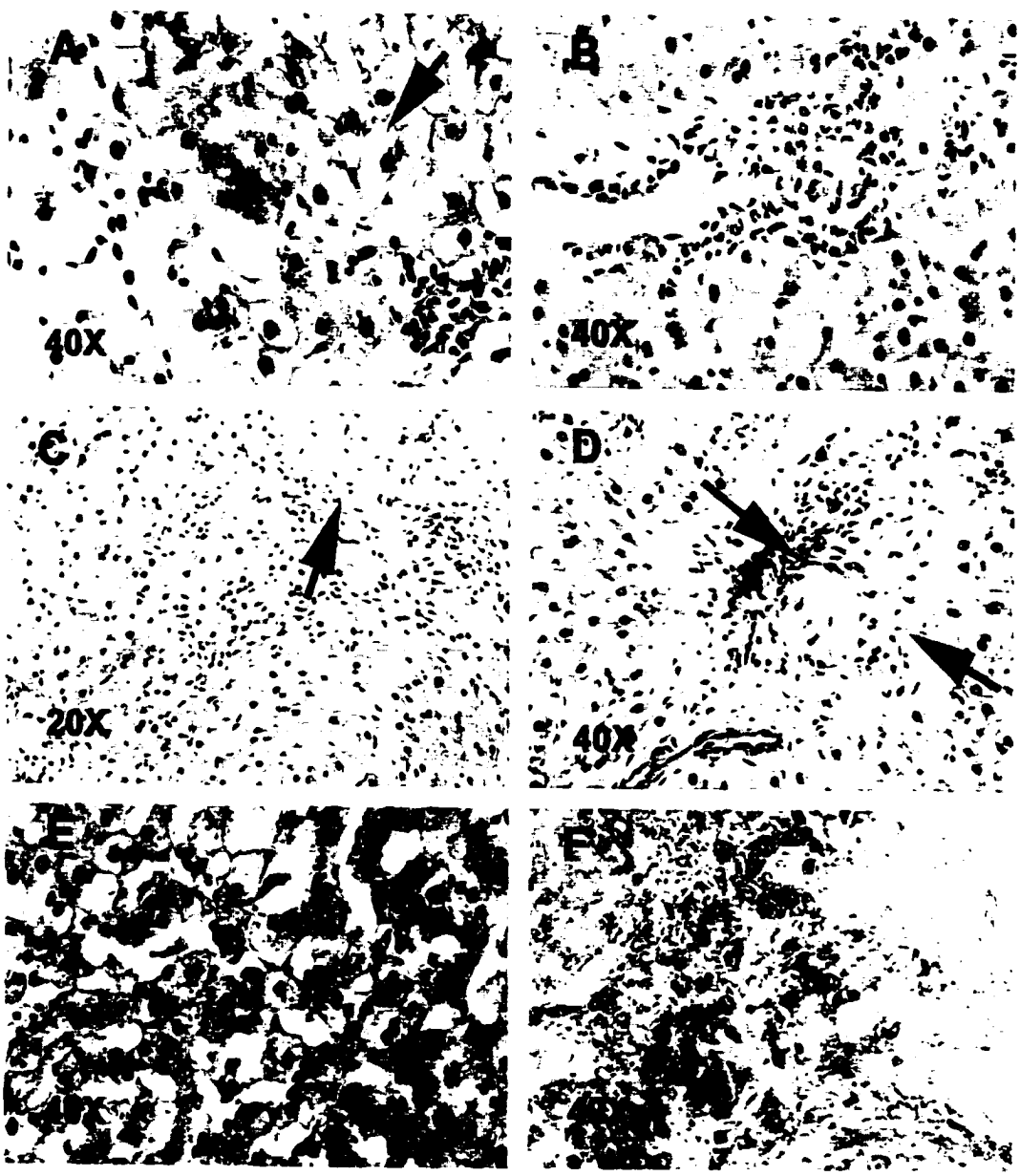


Figure 15. Photomicrographs of liver biopsies obtained from phenobarbital-treated epileptic dogs. (A) Enlarged, swollen hepatocytes with granular cytoplasmic characteristics representative of increased amounts of endoplasmic reticulum. Arrow is pointing to typical example. (B) Infiltration of inflammatory cells and piecemeal necrosis. (C) Collagen deposition (arrow) and inflammation. (D) Vascular changes similar to those found with veno-occlusive diseases. Notice the markedly thickened vessel walls (arrows). (E) Rhodanine stain for copper. Note the pigmented granules of copper throughout the hepatocytes. Copper staining was not restricted to any particular zone. (F) Prussian blue stain for iron showing iron accumulation within macrophages, Kuppfer cells, and hepatocytes. All of the above histological findings were also present in several of the control dogs, but not to the same overall severity.


A
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phenobarbital-treated dogs stained positively for iron, which was found predominantly in the macrophages and Kupffer cells but was also present within hepatocytes in 3 dogs (**Figure 15f**). However, half of the control dogs stained positively for iron as well. Median histopathological scores and ranges of scores for the 12 phenobarbital-treated dogs and 8 controls are shown in **Table 1**. Numbers of dogs with scores ≥ 3 are shown in **Table 2**. No vacuoles were noted in hepatocytes, and there was no evidence of biliary stasis or biliary hyperplasia in any of the samples.

In the phenobarbital-treated dogs, no associations were found between scores of any of the histopathological categories and dose of phenobarbital, serum phenobarbital concentration, serum ALT or AP activities, or liver ALT or AP activities. However, duration of treatment was significantly associated with the overall histopathological score ($P < 0.01$, $r = 0.72$) (**Figure 16**), and with the degree of iron staining ($P < 0.01$, $r = 0.79$). As expected, age was also highly correlated with duration of therapy ($P < 0.01$, $r = 0.83$), so colinearity of these variables confounds interpretation. There was no correlation between overall histopathological score and age in the control dogs.

Liver homogenate enzyme assays:

(1) ALT activities — Median ALT activity in the liver homogenates from the 12 phenobarbital-treated biopsy dogs was significantly lower ($P < 0.01$) than the median ALT activity in the liver homogenates from the control dogs (0.069 and 0.115 U/mg protein, respectively; **Figure 17**). When only the 7 phenobarbital-treated dogs with serum ALT activities above the upper limit of the AVC reference range were compared to the 8 control dogs, serum ALT activity was still significantly higher and liver

Table 1. Histopathological scores for 12 phenobarbital-treated epileptic dogs (PB-treated dogs) and 8 normal control dogs. (Scoring system: 1 = normal; 2 = mildly abnormal; 3 = moderately abnormal; 4 = markedly abnormal). Data is presented as median and (range). Categories include those indicative of induction changes and increased amounts of endoplasmic reticulum (hepatocyte size and cytoplasmic characteristics), and those indicative of injury (inflammation, necrosis, vascular changes, fibrosis, and overall score). Median scores of the 2 groups were compared for all categories, and significant differences were noted.

*Median value differs significantly ($P < 0.05$) from the control dogs.

**Median value differs significantly ($P < 0.01$) from the control dogs.

NS = no significant difference.

Category	PB-treated dogs	Control dogs	Significance
Hepatocyte size	2.75 (2-3)	2 (1-2)	**
Cytoplasmic changes	2.25 (1-3)	1 (1-1.5)	**
Inflammation	2 (1-3)	1 (1-2)	NS
Necrosis	2 (1-2.5)	1.25 (1-2.5)	NS
Vascular changes	2 (1-3.5)	1.25 (1-2.5)	NS
Fibrosis	2 (1-3)	1 (1-2)	*
Copper staining	1 (1-3)	1.75 (1-3)	NS
Iron staining	2 (1-3.5)	1.25 (1-2.5)	NS
Overall score indicative of injury	2 (1.5-3)	1.25 (1-2)	**

Table 2. Number of dogs (out of 12 phenobarbital-treated dogs [PB-treated] and 8 control dogs) with histopathological scores ≥ 3 in various categories. (Scoring system: 1 = normal; 2 = mildly abnormal; 3 = moderately abnormal; 4 = markedly abnormal).

	Number of dogs with scores ≥ 3	
Category	**PB-treated dogs**	**Control dogs**
Hepatocyte size	6	0
Cytoplasmic changes	3	0
Inflammation	2	0
Necrosis	0	0
Vascular changes	3	0
Fibrosis	1	0
Copper staining	2	3
Iron staining	2	0
Overall score indicative of injury	2	0

Figure 16. Scatterplot showing a significant correlation between duration of treatment and overall histopathological score of liver biopsies from 12 phenobarbital-treated epileptic dogs.

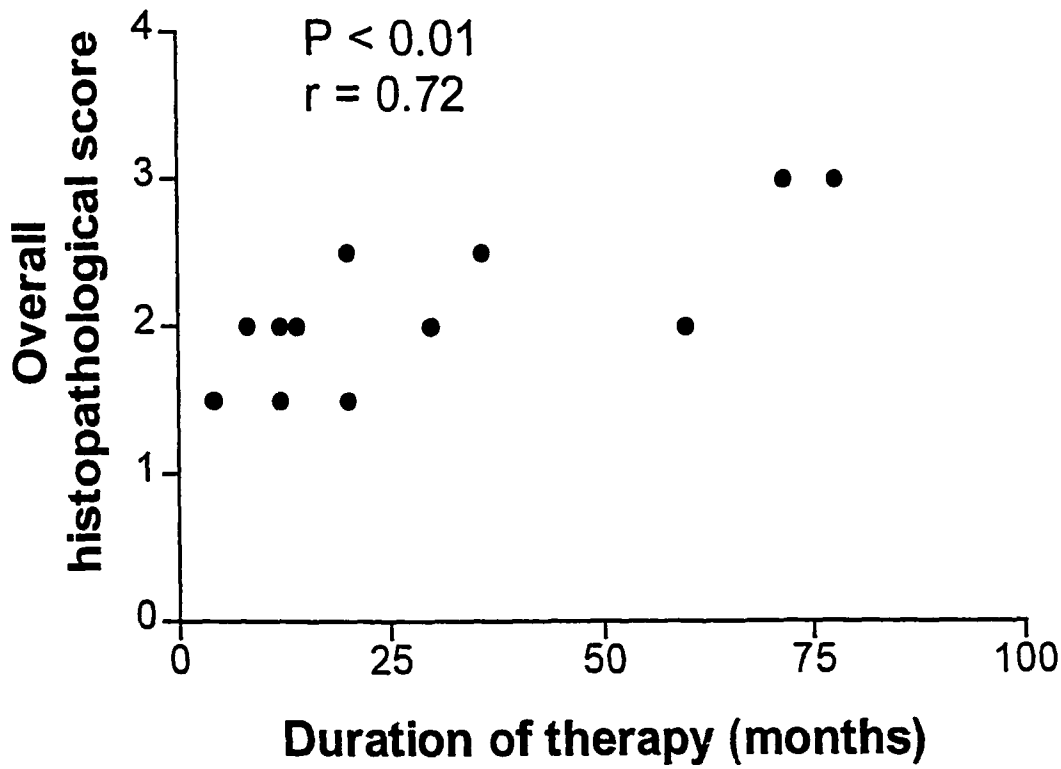
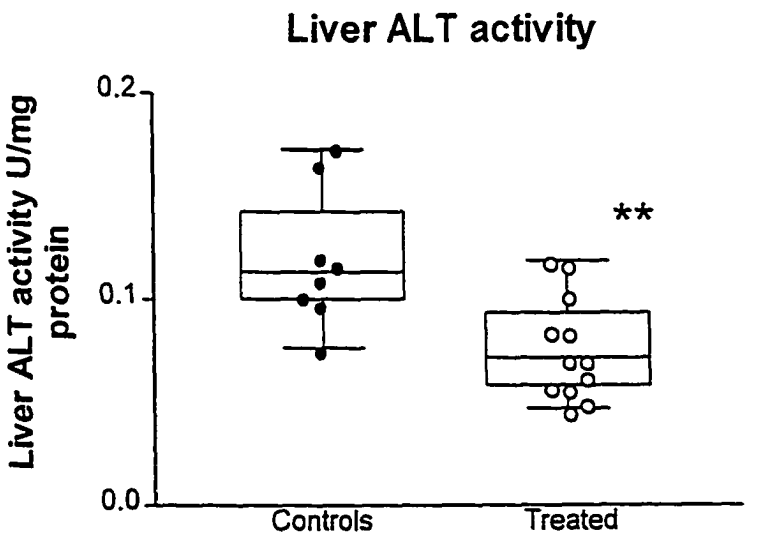


Figure 17. Alanine aminotransferase (ALT) activities in liver homogenates from 12 phenobarbital-treated dogs (Treated) compared to 8 healthy control dogs.

**Median value differs significantly ($P < 0.01$) from the control dogs.



homogenate ALT activity was still significantly lower in the treated dogs compared to the controls.

Serum ALT activity was not significantly correlated with liver homogenate ALT activity in the phenobarbital-treated dogs (**Figure 18**). Liver homogenate ALT activity was not correlated with dose of phenobarbital or serum phenobarbital concentration, but was significantly associated with duration of therapy ($P < 0.01$, $r = 0.72$) and age ($P < 0.01$, $r = 0.81$) (**Figure 18**). However, duration of therapy and age were also significantly correlated ($P < 0.001$, $r = 0.83$), so co-linearity existed between these 3 variables. For the control dogs, no significant correlations were found between serum ALT activities and liver homogenate ALT activities or age (**Figure 19**).

(2) AP activities — Eleven of the 12 phenobarbital-treated dogs had a serum total AP activity above the upper limit of the AVC reference range. However, only 6 of these 11 dogs had adequate amounts of liver homogenate remaining to perform the AP assay on the liver tissue. Median total AP activity in the liver homogenates of the 6 phenobarbital-treated dogs was not significantly different than the controls (10.87 and 14.7 U/mg protein, respectively; **Figure 20**).

Liver homogenate AP activity in the phenobarbital-treated dogs was correlated with the serum total AP activity ($P < 0.05$, $r = 1.0$), but not with serum C-AP, B-AP or L-AP activity (**Figure 21**). However, serum total AP activity was significantly correlated with serum C-AP activity ($P < 0.001$, $r = 1.0$). Liver homogenate AP activity was not correlated with serum phenobarbital concentration, dose of phenobarbital, duration of treatment, age, or either serum or liver ALT activity. In the control dogs, serum total AP

Figure 18. Scatterplots showing lack of significant correlation between liver homogenate ALT activities in 12 phenobarbital-treated epileptic dogs and (A) serum phenobarbital concentration, (B) total daily dose of phenobarbital, and (C) serum ALT activity; but significant correlations with (D) duration of therapy and (E) age. Duration of therapy and age were also highly correlated (F), so colinearity existed between duration of therapy, age, and liver homogenate ALT activity.

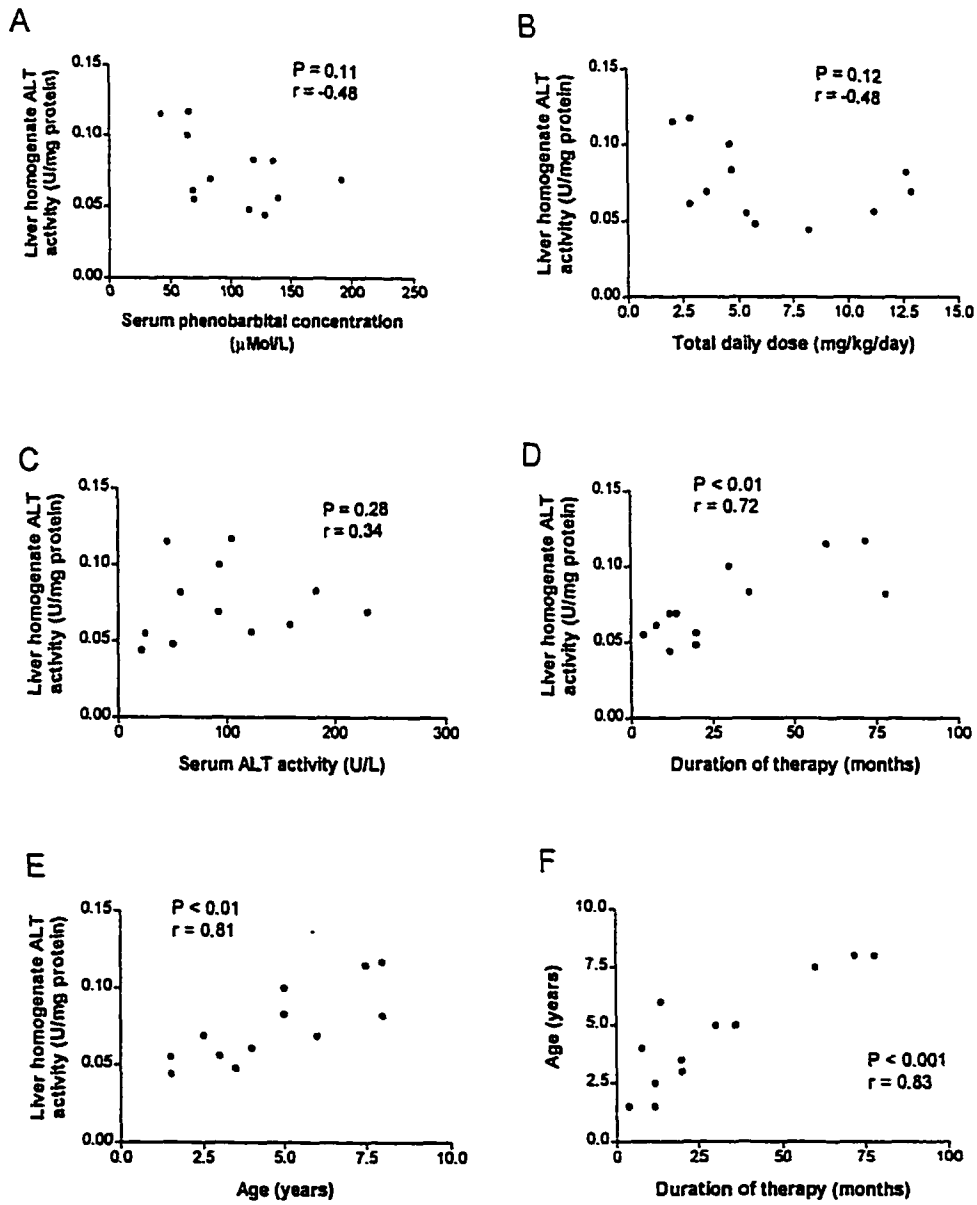


Figure 19. Scatterplots showing lack of significant correlations between serum ALT activities and (A) liver homogenate ALT activities or (B) age; or between liver homogenate ALT activity and age (C), in the 8 control dogs

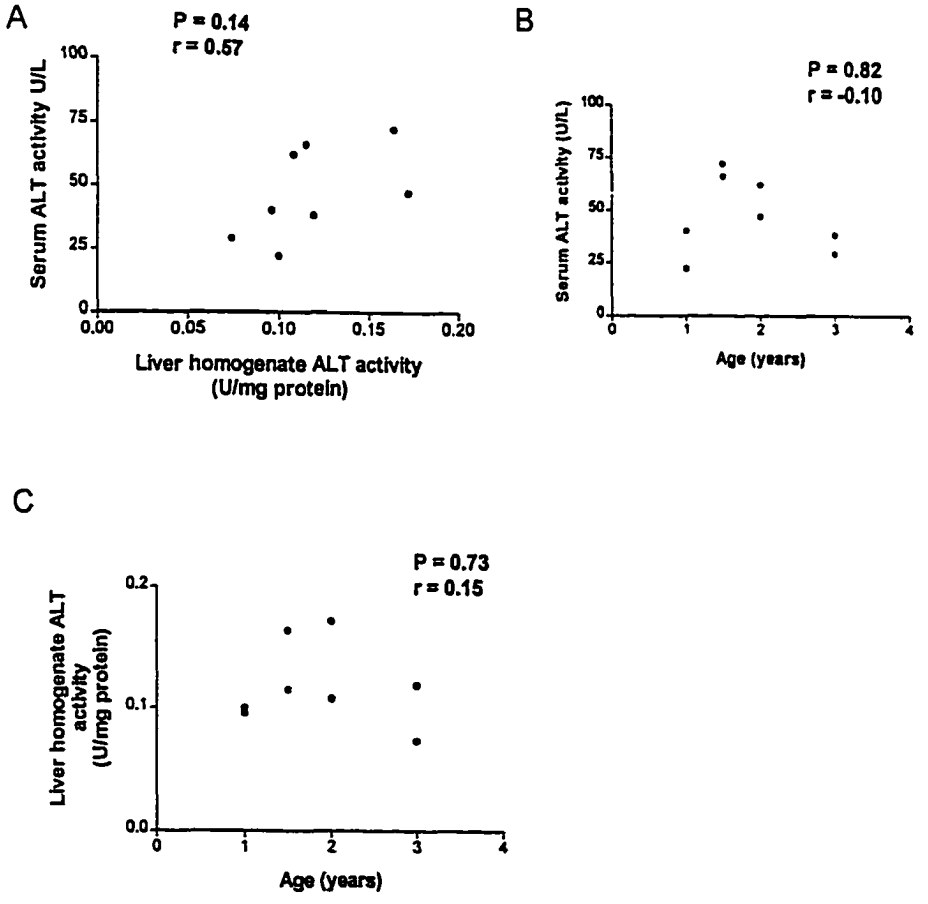


Figure 20. Total alkaline phosphatase (AP) activities in liver homogenates from 6 phenobarbital-treated dogs (Treated) and 8 healthy control dogs.

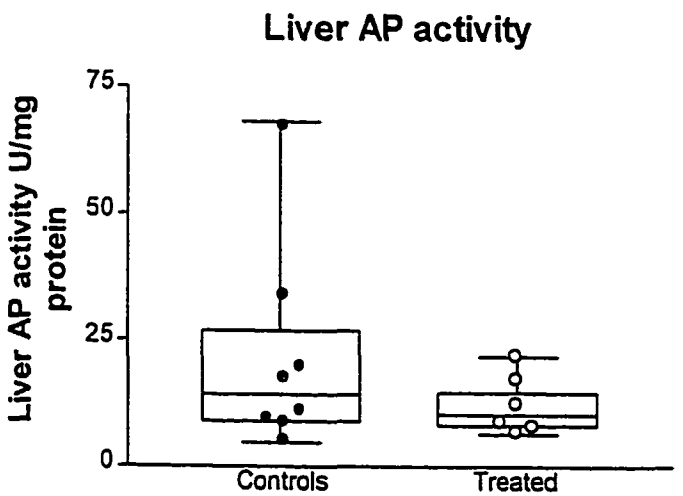
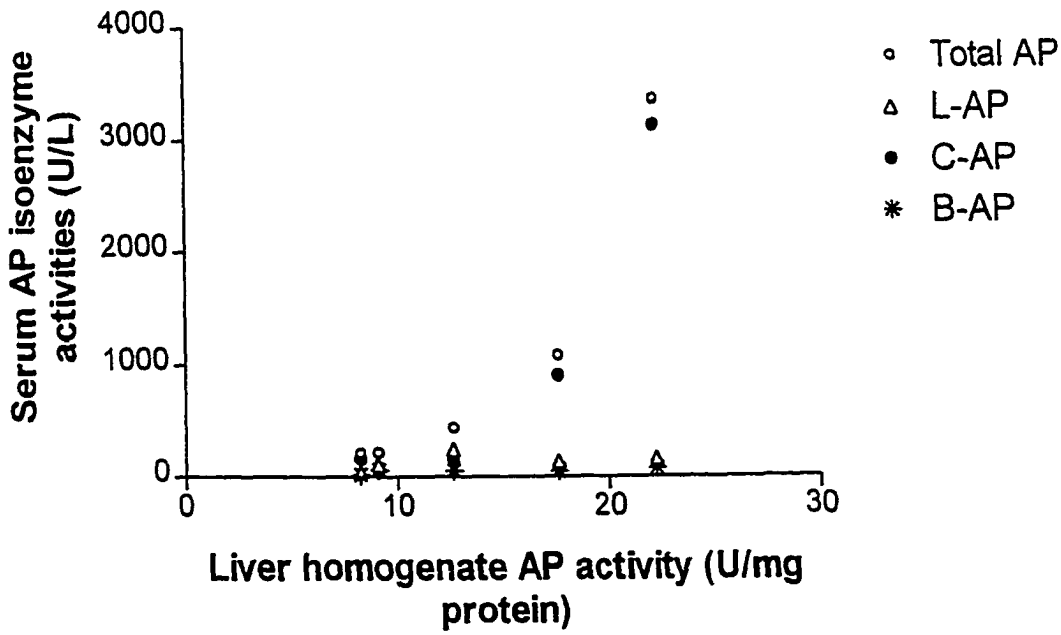


Figure 21. Scatter plot of serum alkaline phosphatase (AP) isoenzyme activities plotted against the liver homogenate AP activities from 5 phenobarbital-treated epileptic dogs. Serum total AP activity was highly correlated with the liver homogenate AP activity ($P < 0.05$, $r = 1.0$) and with serum C-AP activity ($P < 0.001$, $r = 1.0$). Note the lack of correlation between the serum L-AP activity or serum B-AP isoenzyme activity and the liver homogenate AP activity. Total AP = serum total AP. L-AP = serum liver AP isoenzyme. C-AP = serum corticosteroid-induced AP isoenzyme. B-AP = serum bone AP isoenzyme.



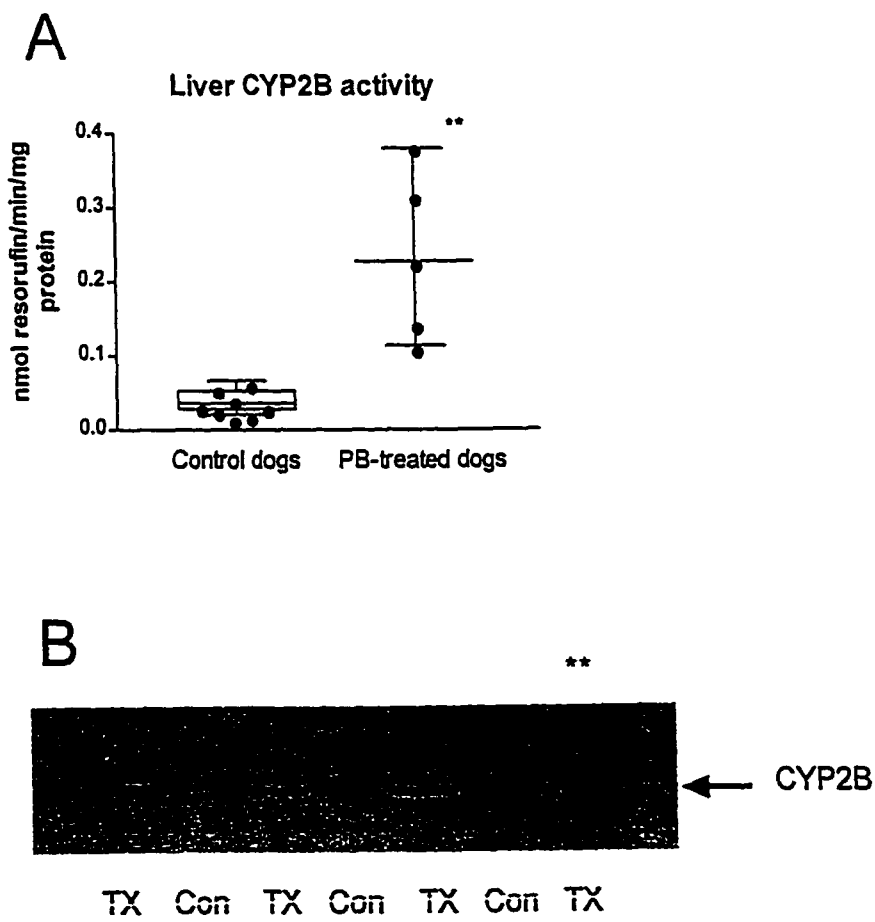
activity was not correlated with liver homogenate AP activity, but was significantly negatively correlated with age ($P < 0.01$, $r = -0.89$).

(3 and 4) Benzyloxyresorufin-*O*-dealkylase activity and CYP2B immunoblotting— As a positive control to verify induction of a hepatic enzyme known to be induced by phenobarbital, we measured the activity and protein quantity of CYP2B in the liver homogenate. Metabolism of benzyloxyresorufin to resorufin (BROD activity, representative of CYP2B activity) was significantly ($P < 0.01$) greater in the phenobarbital-treated dogs than in the controls (median rates of 0.22 and 0.02 nmol/min/mg protein, respectively; **Figure 22a**). Amount of CYP2B protein was significantly ($P < 0.01$) greater in the phenobarbital-treated dogs than in controls (median densities of 487.0 and 61.5, respectively; **Figure 22b**).

DISCUSSION

A primary goal of our study was to determine if increased serum ALT and AP activities in phenobarbital-treated dogs are due to induction of these enzyme activities in the liver. Previous clinical studies concluded that elevated serum ALT and serum total AP activities in phenobarbital-treated dogs are due to induction because the dogs in these studies did not have histopathological evidence of injury (Muller *et al.* 2000a), or because the dogs were clinically healthy at the time (Gieger *et al.* 2000). However, none of these studies evaluated the activity of the enzyme in liver tissue to verify that it was in fact increased. Histological evaluations can be subjective, and early injury may be difficult to

Figure 22. Verification of induction of CYP2B protein and activity in phenobarbital-treated dogs. (A) Median liver homogenate BROD activity in 5 phenobarbital-treated dogs (PB-treated) compared to 8 control dogs. (B) Immunoblot of CYP2B protein in crude homogenates from phenobarbital-treated dogs (TX) compared to control dogs (Con). The blot depicted above is representative of blots from 5 phenobarbital-treated dogs and 8 control dogs. Each lane contains 50 μg of protein. **Median value differs significantly ($P < 0.01$) from the control dogs.



detect histologically (Abdelkader and Hauge 1986, Bunch 1993, Meyer 1998, Zimmerman and Ishak 1995, Zimmerman and Lewis 1995). Also, liver injury can occur before signs of overt clinical disease develop, so clinical signs cannot be used to assess for early subclinical liver damage. A better way to determine if these enzyme activities are induced in the liver is to measure the activities as was done in the present study. If increased serum enzyme activities are due to induction and not to liver damage, we would expect to find: (1) histopathological evidence of induction (ie, enlarged hepatocytes and changes in the cytoplasmic characteristics) but not of liver injury, (2) increased liver homogenate enzyme activities in the phenobarbital-treated dogs compared to controls, and (3) correlations between serum and liver homogenate enzyme activities and dose of phenobarbital, duration of therapy, or serum phenobarbital concentration.

In the phenobarbital-treated liver biopsy cases in our study, histopathologically we found induction changes as well as histopathological abnormalities indicative of liver injury. We also found the same types of histopathological abnormalities in the control dogs. However, the abnormalities were more severe and found more frequently in the phenobarbital-treated dogs compared to controls. In particular, the scores for fibrosis and overall histopathological injury were significantly higher in the phenobarbital-treated dogs compared to controls. Varying degrees of inflammation, necrosis, and vascular changes were noted in both groups, but more phenobarbital-treated dogs showed these changes, and although not significantly different, median scores were higher in the phenobarbital-treated dogs. On a case-by-case basis, there appeared to be no good histological marker of early, pre-clinical liver injury that could be definitively attributed to phenobarbital in our study. During our study, post-mortem liver samples were also

evaluated from 4 additional phenobarbital-treated dogs that died for reasons unrelated to liver disease. All had mildly to moderately elevated serum AP activities, and 2/4 had mildly to moderately elevated serum ALT activities. When the scores from the 4 post-mortem samples are included in statistical analyses, the phenobarbital-treated dogs had significantly higher scores for inflammation as well as for overall injury and fibrosis. Other statistical results remained the same.

Surprisingly, more of the control dogs had positive copper staining than did phenobarbital-treated dogs. Copper accumulation in the liver has typically been thought to be either a consequence of or a cause of liver injury (Cooper *et al.* 1997, Noaker *et al.* 1999, Thornburg 2000). However, the dog differs from other animals in that the range for normal hepatic copper concentrations is very wide and poorly defined in dogs. One study of 623 clinically normal dogs found liver copper concentrations ranging from <100 up to 6,800 ppm dry weight, and other studies have found no histological evidence of hepatitis in dogs with <2,000 ppm copper (Thornburg *et al.* 2000). Rhodanine, the copper stain we used in our study, provides a qualitative measure of copper, and does not detect copper until tissue concentrations are approximately 400 ppm (Fuentealba *et al.* 1997). One investigator has proposed that the very high liver copper concentrations found in many normal dogs is due to a overabundance of copper in the typical canine diet (Thornburg 2000).

We found a significant association between age and some of the histopathological liver abnormalities in our phenobarbital-treated dogs. Median age of the phenobarbital-treated dogs was significantly higher than controls (4.5 and 1.75 years, respectively). Therefore, it is possible that the greater severity of histopathological abnormalities in our

phenobarbital-treated dogs was due to age, rather than the phenobarbital treatment. However, there were no correlations between age and any of the histopathological abnormalities in the control dogs.

In our study, the median liver homogenate ALT activity in the phenobarbital-treated dogs was significantly lower than in the controls. This is opposite to what we would expect to see with induction. This decrease in activity could be due to several possible causes. One possibility is that the phenobarbital-treated dogs may have had fewer functional hepatocytes per gram of liver tissue due to fibrosis and necrosis, which then resulted in fewer hepatocytes containing ALT. Histopathologically, we did find significantly more fibrosis in the phenobarbital-treated dogs in our study compared to the controls. A second possible reason is that hepatocellular damage or membrane distortion causing increased leakage or release of ALT from hepatocytes into the circulation may have resulted in lesser amounts of ALT remaining in the hepatocytes. Thirdly, phenobarbital might have caused down-regulation of ALT expression in the hepatocytes. Down-regulation of some enzymes in conjunction with up-regulation of others has been shown to occur with other drugs and chemicals, including glucocorticoids and polychlorinated biphenyls (Iber *et al.* 1999, White *et al.* 1997). It is possible that this phenomenon occurs with phenobarbital as well.

Median liver homogenate AP activity was also lower in the phenobarbital-treated dogs than in the controls, although the decrease was not statistically significant. As with the liver ALT activity results, this is opposite to what we would expect to see with induction. The lack of increased liver homogenate AP activity in the phenobarbital-treated dogs in our study differs from the findings of 2 previous studies investigating liver

AP activity in phenobarbital-treated dogs (Litchfield and Conning 1972, Unakami *et al.* 1987). These studies found significantly increased AP activity (8 to 12-fold increases) in liver tissue of treated dogs compared to controls. There are several possible reasons why our results differ from the two previous studies. The previous studies used much higher doses of phenobarbital than are typically used therapeutically (40-50 mg/kg/day, as compared to the median dose of 5 mg/kg/day in our study). Also, small numbers of dogs (n = 3 for each study) in the previous studies may have influenced results. One study (Litchfield and Conning 1972) evaluated only the microsomal fraction of the liver homogenates for AP activity, while we evaluated whole liver homogenates. This is a very important difference. We now know that the AP isoenzymes originating from liver tissue (L-AP and C-AP) are found predominantly on the external hepatocyte plasma membranes, primarily in the sinusoidal and canalicular regions (Sanecki *et al.* 1987, Sanecki *et al.* 1990, Solter and Hoffmann 1995, Syakalima and Takiguchi 1998). Theoretically, the best cell fraction to evaluate for AP activity would be the pellet from the first 9,000 X *g* spin, which would contain the hepatocyte plasma membranes. Only a very small portion of the hepatocyte AP would be found in the microsomal fraction. However, enhanced synthesis of the AP enzyme may result in increased amounts of the enzyme in the endoplasmic reticulum and the golgi, both of which sediment in the microsomal fraction after differential centrifugation. This increased AP enzyme concentration in the endoplasmic reticulum and golgi may result in a very large increase in the AP enzyme concentration in the microsomal fraction, due to the concentrated endoplasmic reticulum and golgi, but the increase in the overall AP activity in the entire hepatocyte might still be unmeasurably small.

We hypothesize that induction of AP activity might have occurred in our study, but that the induction was masked by a simultaneous increase in release of the AP enzyme from the hepatocyte cell membrane. This theory is based on recent studies investigating the mechanism of increased serum L-AP activity due to cholestasis, and the mechanism of increased serum C-AP (Deng *et al.* 1996, Solter and Hoffmann 1995, Solter *et al.* 1997, Solter and Hoffmann 1999). These studies suggest that the elevated L-AP is due to an increased cleavage and release of the enzyme from the hepatocyte membrane via bile acid enhancement of glycosylphosphatidylinositol phospholipase D (GPI-PLD) activity. Since L-AP and C-AP were the isoenzymes causing the elevated total serum AP activity in the phenobarbital-treated dogs in our study, it is possible that the mechanism of their increase in serum is also an increased release from the hepatocyte membrane, possibly due to either cholestasis or induction of GPI-PLD by phenobarbital. Although we did not find overt biliary stasis histopathologically in our phenobarbital-treated dogs, we did find significant enlargement and swelling of the hepatocytes, which might contribute to intrahepatic cholestasis. Therefore, we hypothesize that the AP enzyme might have been induced by phenobarbital, but an increased rate of release from the plasma membrane might have caused the total enzyme amount in the liver tissue to remain unchanged or even slightly decrease. While initially appearing to contradict our findings, the Litchfield study (Litchfield and Conning 1972) showing the increased AP activity in the microsomal fraction may actually help support this hypothesis that induction of liver AP enzymes by phenobarbital does occur, but that the induction is masked by a simultaneous increased release of the enzyme from the hepatotcyte membranes. In the Litchfield study, the AP in the microsomal fraction had not yet been

subjected to the GPI-PLD, so the increased AP concentration in the microsomes would not be reduced as it would in homogenate containing hepatocyte plasma membrane. Our study samples were too small to allow us to prepare microsomal fractions to test this theory.

In our cross-sectional study, serum ALT activity was correlated with serum phenobarbital concentration, but not with dose or duration of therapy. Serum AP activity was significantly correlated with dose of phenobarbital, duration of therapy, and serum phenobarbital concentration.

In the liver homogenates, there were no correlations between serum ALT activity and liver homogenate ALT activity, dose of phenobarbital, duration of therapy, or serum phenobarbital concentration in the 12 phenobarbital-treated dogs. There was no association between liver homogenate ALT activity and serum phenobarbital concentration or dose of phenobarbital. There was a significant correlation between liver homogenate ALT activity and duration of therapy, but liver homogenate ALT activity was also significantly correlated with age, and age was highly correlated with duration of therapy. Because of the colinearity of these 3 variables, interpretation of the correlations is difficult. The correlation between liver homogenate ALT activity and duration of therapy may actually be due to the age of the animal. Neither serum or liver AP activity were correlated with serum phenobarbital concentration, dose, or duration of therapy, or with serum or liver ALT activity. However, serum total AP activity and liver homogenate AP activity were highly significantly correlated in the phenobarbital-treated dogs. This correlation supports our theory that induction of AP might have occurred, but been masked by an increased release of AP from the hepatocyte. This correlation also

supports the generally accepted theory that the increased C-AP in the serum likely originates from the liver, and not some other as yet unidentified site (Sanecke *et al.* 1987).

Alkaline phosphatase isoenzyme analysis in our study showed that serum L-AP, C-AP, and B-AP activities were all elevated in the phenobarbital-treated dogs. However, serum B-AP activity was very minimally increased, and only accounted for a very small portion of the increased serum total AP activity. The increase was typically within the typical reference range (17 ± 7 U/L). This helps rule out induction of enzymes involved in bone metabolism and subsequent increased release of the B-AP isoenzyme into circulation as the primary cause of the elevated serum total AP activity. Also, we did not find any unusual or previously unidentified bands with cellulose acetate affinity electrophoresis. This suggests that there is no novel isoform of AP induced by phenobarbital treatment. This possibility cannot be ruled out completely, because a novel band may have been hidden within one of the bands of the known isoforms. However, as affinity electrophoresis causes much more distinctive separation of isoform bands than does traditional electrophoresis, the possibility is diminished. It is interesting that the serum total AP activity was highly correlated with the liver homogenate AP activity, and with serum C-AP, but not with serum L-AP. This might suggest that possibly C-AP is induced, but L-AP is not. If that were the case, measuring serum C-AP and L-AP activities may help differentiate between increased serum total AP activity due to induction (increased C-AP), or due to hepatocellular injury (increased L-AP). However, in our study there were no correlations between either serum C-AP or serum L-AP activities and histopathological scores indicative of liver injury. That is, the

phenobarbital-treated dogs with the highest histopathology scores did not have the highest serum L-AP activities, and the dogs with the least amount of liver abnormalities histopathologically often had higher serum L-AP than C-AP activity. Therefore, measuring serum AP isoenzymes does not appear to be helpful at differentiating between induction and early liver injury. In the control dogs, the correlation between serum total AP activity and B-AP was not surprising, as many of these dogs were young, and B-AP activity decreases with age as bone growth diminishes.

The association between serum phenobarbital concentration or phenobarbital dosage and occurrence of hepatotoxicity remains uncertain. In our study, only one dog had severe histopathological liver lesions diagnostic of chronic hepatic damage, but this dog was excluded from analyses when the owners later reported possible exposure to an unnamed chemical substance. No other dogs with clinical signs of liver disease were identified during our study period. Because phenobarbital is metabolized by the liver, when the liver fails, metabolism and clearance of phenobarbital from the body is affected. This can result in a dramatic increase in serum phenobarbital concentration, despite no increase in drug dosage. Many dogs that present with phenobarbital-associated hepatic disease have high serum phenobarbital concentrations at the time of liver failure. This high concentration is often blamed as the cause of the liver damage, when in actuality the phenobarbital concentration might have been within the typical recommended therapeutic range before the onset of liver failure. Our evidence suggests that hepatic damage can be occurring even when serum phenobarbital concentrations are within the typically accepted therapeutic ranges. A few of the dogs receiving low doses of phenobarbital and with low serum phenobarbital concentrations and very high serum ALT activities (see

pages 132 and 133), which supports the possibility of a genetically predisposed idiosyncratic reaction. However, the correlations between serum ALT activity and serum phenobarbital concentration found in our study may also support the idea of increased risk of hepatic injury with increasing serum phenobarbital concentrations.

Additional studies would help complete the investigation of phenobarbital induction of ALT and AP in dogs. Evaluation of ALT and AP mRNA and protein concentrations in liver homogenates of phenobarbital-treated and untreated dogs would determine if increased transcription and increased production of these enzymes occur. Although increased transcription and protein amounts does not equate with increased activity, these measurements would complement our activity studies. Additional studies to further investigate inducibility of AP might include immunohistochemistry of liver sections to determine location and relative amounts of C-AP and L-AP, and determination of GPI-PLD quantity and activity. Because of the clinical nature of our study and the small sizes of the needle biopsies obtained from the phenobarbital-treated dogs, insufficient material remains to allow us to pursue further investigations with the samples from our study.

We conclude that increased serum ALT activity in phenobarbital-treated dogs without clinical signs of liver disease is likely due to subclinical hepatocellular damage and leakage of the enzyme into the circulation, and not due to induction. Elevated serum ALT in phenobarbital-treated dogs might help identify those dogs predisposed to ultimately develop hepatotoxicity and clinical liver disease due to phenobarbital treatment. Serum AP isoenzyme analysis does not appear to be helpful in differentiating hepatic enzyme induction from early hepatic injury. Although no increase in liver

homogenate AP activity was found in our study, induction might have been masked by a simultaneous increase in release of AP from hepatocyte membranes. Further investigation is required.

3.4 General discussion of the effects of phenobarbital on the liver in dogs

In the previous section, we showed that healthy phenobarbital-treated dogs, with no clinical signs of liver disease, had histopathological evidence of liver injury. These changes included hepatocellular necrosis, fibrosis, inflammation, and vascular changes. However, these abnormalities were not distinctive for injury by phenobarbital, and were also found in some of the control dogs, albeit to a lesser degree in most cases. Therefore, we conclude that these early changes are non-specific, and that mild to moderate changes are not particularly useful in determining whether or not phenobarbital should be discontinued. Copper accumulation also cannot be assumed to be a marker for phenobarbital-associated liver injury, because more control dog livers stained positively for copper, and had higher copper scores, than did the phenobarbital-treated dog livers. None of the histopathological changes were correlated with degree of elevation of serum AP or ALT activities. However, overall histopathological score was significantly positively correlated with duration of therapy, and duration of therapy was positively correlated with age in the phenobarbital treated dogs. Although this raises the possibility that the greater severity of histopathological abnormalities in our phenobarbital-treated dogs was due to their greater age, rather than the phenobarbital treatment, we have evidence that this might not be the case. Another recent study (Muller *et al.* 2000a) evaluated histopathological changes associated with phenobarbital treatment of 12 dogs over a 29 week period of time. Liver biopsies were taken before the start of phenobarbital treatment and at 10 and 27 weeks after phenobarbital initiation. They found no significant histolopathological changes indicative of liver injury at any time

point in the study. The investigators allowed us to evaluate the histopathology slides from their study. The pathologist for our study scored all slides from all time points in a blinded fashion. Scores were determined for the same categories we used in our study (ie, inflammation, necrosis, fibrosis, vascular change, hepatocyte size, cytoplasmic characteristics) with the exception of copper, iron and glycogen staining. We found significantly increased scores over time (repeated measures ANOVA) for necrosis, fibrosis, vascular changes, and overall histopathological score indicative of injurious changes. We concluded that there was evidence of liver injury occurring over time associated with the phenobarbital treatment. This suggests that the higher histological scores found in the phenobarbital-treated dogs in our study might not be due to the increased age of these dogs compared to the controls.

We showed in the previous two sections that increased serum AP and ALT activities are not associated with increased activities of these enzymes in liver tissue from phenobarbital-treated dogs or rats compared to controls. As no previous studies have evaluated liver ALT activity in phenobarbital-treated dogs or rats, these results were not surprising. Our conclusion is that increased serum ALT activity in phenobarbital-treated dogs or rats is likely due to hepatocellular damage and leakage of the enzyme out of the cell, and is not due to induction. We were surprised, however, at the lack of increased liver AP activity in the phenobarbital-treated dogs or rats compared to controls. Induction of liver AP is well ingrained in the literature, and two previous studies showed an increase in liver AP activity in phenobarbital-treated animals compared to controls. As discussed in the previous section, we believe the most likely reasons for the discrepancy in results from our study and the 2 previous studies are the cell fraction for

which AP was measured, and dose of phenobarbital used. However, we also investigated several other possible cause of the discrepancy, as presented next.

Some studies have suggested that magnesium chloride ($MgCl_2$) concentrations influence results of AP activity (Simon and Sutherland 1977). We performed the AP assay using varying concentrations of $MgCl_2$. The addition of $MgCl_2$ did not change the interpretation of our results. We surmise that there was adequate amounts of $MgCl_2$ in the whole tissue homogenate, in contrast to the deficiency that might be found using subcellular fractions or isolated enzyme. In order to rule out differences between the AP assay used in our laboratory and the automated AP assay technique used by the AVC Diagnostic Laboratory, we performed both AP assays on serum samples from dogs with normal AP activities and from dogs with very high serum total AP activities. The AP activities were higher using the automated technique for both the dogs with high serum AP activity and the dogs with normal AP activity. However, the increase was proportionally equal for all samples, so interpretation of results did not change. We also determined AP activities of liver homogenates from phenobarbital-treated dogs and control dogs using both techniques. As with the serum samples, the overall activity of AP in serum and liver tissue was higher for all samples using the automated technique compared to the mechanical technique. However, the increase was proportional equal for all samples, so interpretation of results did not change. Therefore, we believe that the manual assay we used could reliably determine AP activity.

Some investigators have first solubilized the AP enzyme from hepatocyte membranes before measuring activity (Solter and Hoffmann 1995, Solter and Hoffmann 1999). However, studies in several species have measured AP activity in whole

homogenate without solubilization (Kaplan *et al.* 1983, Frederiks *et al.* 1990, Nishimura and Teschke 1982, Unakami *et al.* 1987), and histochemical analysis verifies that the membrane-bound AP enzyme is able to react with its substrate (Sanecki *et al.* 1987, Solter *et al.* 1997). Therefore, use of whole liver homogenates was appropriate in our study.

We believe that although no increase in liver AP activity was found in the phenobarbital-treated dogs in our study, it is possible that induction of AP occurred but was masked by a simultaneous increase in release of the AP enzyme from the hepatocyte membrane. This conclusion is based on studies (Deng *et al.* 1996, Solter and Hoffmann 1995, Solter *et al.* 1997, Solter and Hoffmann 1999) that have delineated the mechanism of increase in serum AP activities from other causes. It is also supported by the discrepancy of results between our study using whole liver homogenates, versus studies using microsomal fractions. Therefore, we believe that increases in serum AP activity can be due to either induction or liver abnormality.

Measuring serum AP isoenzymes did not appear to be helpful at differentiating liver injury from induction. We found that the isoforms elevated in the serum of phenobarbital-treated dogs were the liver and the corticosteriod-induced forms, and not the bone isoform, but there were no associations between histopathological abnormalities and the different isoenzymes. Also, no usual or previously identified "phenobarbital-associated" isoforms were detected by cellulose acetate affinity electrophoresis.

The association between serum phenobarbital concentration or phenobarbital dosage and occurrence of hepatotoxicity remains uncertain. In our study, only one dog had severe histopathological liver lesions diagnostic of chronic hepatic damage, but this

dog was excluded from analyses when the owners later reported possible exposure to an unnamed chemical substance. No other dogs with clinical signs of liver disease were identified during our study period. However, in the 2 year period after the study ended, the AVC Diagnostic Laboratory processed liver samples from 4 dogs with clinical liver failure attributed to phenobarbital usage. One of these dogs, a 5 year old male Dalmation, had originally been in our cross-sectional study. At the time of the study, the dog had increased serum ALT and AP activities >2X the upper limit of normal (serum AP activity of 373 U/L, serum ALT activity of 213 U/L), and a serum phenobarbital concentration of 132 μmol/L (typical therapeutic reference range: 54-190 μmol/L). The owner elected no additional diagnostics at that point, and decided to continue with phenobarbital. Two years later, the dog developed clinical liver failure and dramatically elevated serum AP and ALT activities (serum AP 5,022 U/L, serum ALT 671 U/L), as well as increased serum GGT and AST activities, and decreased serum albumin concentration. The serum phenobarbital concentration at that time was 231 μmol/L, despite no change to the phenobarbital dosage over the years.

Three other dogs that were not in our study developed clinical liver failure attributed to phenobarbital and had liver samples processed by the AVC Diagnostic Laboratory. A serum biochemical profile had been performed for one of the dogs at the AVC Diagnostic Laboratory 1 year before the onset of clinical liver failure. At that time, the serum AP and ALT activities were above the upper limit of the normal reference range (1,656 and 87 U/L, respectively), and a serum phenobarbital concentration of 129 μmol/L was measured 2 years before presentation for clinical liver disease. The dog was maintained on the same dosage of phenobarbital until presentation for clinical liver

disease, at which time the serum phenobarbital concentration was 395 μmol/L and the serum AP and ALT activities were 1,312 and 268 U/L, respectively. At that point, the dog also had developed an increase in serum GGT and AST activities, and decreased serum albumin and urea concentrations. Another of the 3 dogs did not have any biochemical analyses or serum phenobarbital concentration determinations before presentation for clinical liver failure. At the time of presentation for clinical liver disease, the serum AP and ALT activities were very elevated (serum AP 10,300U/L, serum ALT 477 U/L). The trough serum phenobarbital concentration was 162 μmol/L, which is within the typical recommended therapeutic range, and the peak concentration was 199 μmol/L. The third dog was receiving potassium bromide in addition to phenobarbital, to allow for a lower dose of phenobarbital and decrease the risk of hepatotoxicity. However, even with a serum phenobarbital concentration of only 75 μmol/L, the dog still developed severe hepatic fibrosis and liver failure.

Liver histopathology of all 4 dogs with liver failure revealed moderate to severe fibrosis, nodular hyperplasia, distended bile cannaliculi and bile duct hyperplasia, and chronic inflammation. This is consistent with findings from the study that first described phenobarbital-associated hepatotoxicity (Dayrell-Hart *et al.* 1991). In addition, 1 biopsy contained large amounts of intrahepatocyte lipid, and several showed vascular abnormalities. All of the dogs had serum phenobarbital concentrations within the typical recommended therapeutic range before the onset of liver failure; 2/4 had serum phenobarbital concentrations above the typically recommended therapeutic range at the time of clinical illness, despite no change in phenobarbital dosage. These data support our belief that in some dogs with a high serum phenobarbital concentration measured at

the time of presentation for liver disease, the high concentration is due to decreased metabolism and clearance of the drug.

As mentioned earlier, the association between duration of phenobarbital therapy and risk of hepatic injury is also unclear. Because duration was also correlated with age of the dog, it is difficult to make any conclusions. However, as phenobarbital-associated hepatotoxicity tends to be a disease of chronicity, and is rarely seen acutely, it seems reasonable that the longer the duration of therapy, the higher the risk of liver disease.

Lastly, but most importantly, all of our major conclusions are based on the results of our liver enzyme activity assays. Therefore, it is vital that these results accurately reflect the true enzyme activity in the liver tissue. As shown in section 3.2, a great deal of time and effort was expended verifying that the assays used were appropriate for our samples. Optimum conditions were determined for each assay, and assays were repeated multiple times to ensure reproducibility. Extensive literature searches were done to ensure that no flaws to our assay techniques have been previously described. We believe the results of our studies are accurate and precise. However, additional investigations would complement our work. These would include measuring ALT and AP mRNA and protein concentrations in liver homogenates, immunohistochemistry for localization of C-AP and L-AP in liver tissue, and further investigation of enzymes involved in release of AP from hepatocyte membranes. These additional tests would require larger tissue samples, and might best be obtained from laboratory animals rather than clinical cases.

CHAPTER 4. EFFECTS OF PHENOBARBITAL ON THE PANCREAS

4.1 General introduction to the effects of phenobarbital and potassium bromide on the pancreas

We became interested in the effects of phenobarbital on the canine pancreas when we found in our studies an unexpected high incidence of pancreatitis in dogs receiving both phenobarbital and potassium bromide (KBr). In our prospective study of 50 epileptic dogs (previously described in Chapters 2 and 3), KBr was added to the phenobarbital treatment regimen in 6 dogs at some point during the 1-year study period. Two of the 6 dogs receiving phenobarbital and KBr subsequently developed marked elevations of serum amylase and lipase activities and corresponding clinical signs of pancreatitis. A 3rd dog developed acute clinical signs of pancreatitis and died before presentation to the veterinarian. Hemorrhagic necrotizing pancreatitis was diagnosed in this dog at postmortem examination. Serum biochemical profiles performed prior to the addition of KBr showed normal serum amylase and lipase activities for all 3 dogs. We did not see any cases of pancreatitis in the dogs receiving only phenobarbital.

Acute pancreatitis is a serious condition of inflammation and autodigestion of the pancreas, an organ vital for proper food utilization and for insulin secretion. Pancreatitis is thought to result from an intracellular activation of digestive enzymes within the pancreas, with subsequent auto-digestion of the pancreatic parenchyma (Gorelick and Otani 1999, Leib 1997, Steer 1999a and 1999b, Williams 1995). This induces an

inflammatory reaction, which can amplify into a generalized, systemic response and lead to multiple organ failure. Dogs with pancreatitis suffer abdominal pain, vomiting, and dehydration. In cases with secondary complications such as sepsis, bleeding disorders, and multiple organ failure, mortality rates can be high (Hess *et al.* 1999, Ruaux *et al.* 1999, Schaer 1998, Williams 1995). Dogs that survive the initial stage of acute pancreatitis can have recurring bouts of pancreatitis and eventually develop chronic pancreatitis and permanent organ damage. The trigger of pancreatitis is often unknown, but suspected etiologies include hyperlipidemia, high fat meals, obstruction of the pancreatic ducts, trauma of the pancreas, infection, and certain drugs such as thiazide diuretics, azathioprine, sulfonamides, tetracyclines, corticosteroids, and anticholinesterase inhibitors (Cook *et al.* 1993, Hess *et al.* 1998, Leib 1997, Williams 1995).

Anticonvulsant drugs have only rarely been associated with pancreatitis in dogs. Pancreatic abnormalities, including pancreatitis, were reported in 2 studies of epileptic dogs receiving phenobarbital (Podell and Fenner 1993, Schwartz-Porsche and Jurgens 1991). In both of those studies, the dogs were also receiving potassium bromide (KBr) in addition to phenobarbital. The first study involved 22 dogs receiving phenobarbital/KBr combination therapy (Schwartz-Porsche and Jurgens 1991). An unspecified number of dogs in the study had evidence of pancreatic problems, such as pancreatitis, pancreatic atrophy and insufficiency, and pancreatic fibrosis. In the second study, pancreatitis attributed to polyphagia and garbage ingestion developed in 2 of 23 dogs (8.6%) receiving both phenobarbital and KBr (Podell and Fenner 1993). In neither study were the pancreatic problems attributed solely to either the phenobarbital or the KBr. Another

study showed epilepsy to be a risk factor for acute fatal pancreatitis, but associations with individual antiepileptic drugs were not made (Hess *et al.* 1999).

Acute pancreatitis is a serious disease in humans as well (Brady *et al.* 1999, Eland *et al.* 2000, Isenmann and Beger 1999, Raraty *et al.* 1999). The most common etiologies known to trigger acute pancreatitis in humans include ethanol abuse, biliary tract obstructive disease, pancreatic trauma or ischemia, hypercalcemia, hyperlipidemia, and hereditary familial predisposition (Eland *et al.* 2000, Steer 1999a and 1999b, Whitcomb and Ulrich 1999). Pancreatitis has also been associated with drugs, including thiazides, estrogens, azathioprine, codeine, olanzapine, and metronidazole (Doucette *et al.* 2000, Elisaf *et al.* 2000, Hastier *et al.* 2000, Sura *et al.* 2000). Anticonvulsants have rarely been associated with pancreatitis in humans (Diener *et al.* 1993, Diener *et al.* 1998, Pezzilli *et al.* 1992).

The pathophysiological mechanisms by which pancreatitis occurs are poorly understood. Hypothesized mechanisms include disruption in intracellular calcium signaling pathways, co-localization of digestive zymogen and lysosomal hydrolases in intracellular vacuoles, mutations in the trysinogen gene resulting in aberrant zymogen processing, and disruption of pancreatic circulation due to endogenous opioids (Brady *et al.* 1999, Gorelick and Otani 1999, Mayer *et al.* 2000, Naruse *et al.* 1999, Raraty *et al.* 1999, Steer 1999a and 1999b). One possible mechanism for pancreatitis associated with anticonvulsant drugs involves an interference with ion channels that regulate chloride transport, similar to that found with cystic fibrosis (Boucher 2001, Naruse *et al.* 1999, Tarran *et al.* 2001). Cystic fibrosis is a lethal autosomal recessive disease characterized by chronic lung disease, pancreatic fibrosis, and an abnormally high sweat chloride

concentration. The disease is caused by genetic mutations that affect normal chloride transport into epithelial tissues, including the pancreas (Naruse *et al.* 1999) and the lung (Boucher 2001, Tarran *et al.* 2001). Abnormally high sweat chloride concentrations have been observed in patients with chronic pancreatits caused by ethanol abuse, which supports the idea that chloride channel malfunction may be involved in the development of pancreatitis (Naruse *et al.* 1999). Ethanol, one of the most common causes of pancreatitis in humans, has its major effect on ion channels in the central nervous system. Both phenobarbital and potassium bromide are thought to exert their anticonvulsant effects in part through alteration of chloride transport in neuronal cells in the central nervous system (Allan *et al.* 1992, Boothe 1998, ffrench-Mullen *et al.* 1993, Plumb 1999, Rho *et al.* 1996, Schwarz-Porsche and Jurgens 1991, Trepanier 1995, Yakushiji *et al.* 1989). It stands to reason that they may also have an effect on chloride transport in other tissues as well. It is possible that the combined effects of phenobarbital and KBr on chloride channels predisposes the patient to pancreatitis.

Because of the apparent high incidence of pancreatitis associated with phenobarbital/KBr therapy in our prospective study, we embarked on the following retrospective study to determine if there was an increased risk of pancreatitis associated with phenobarbital monotherapy, KBr monotherapy, or only with the combination of phenobarbital and KBr.

4.2 Pancreatitis associated with phenobarbital/potassium bromide combination therapy in epileptic dogs

(Note: this chapter is presented as published in Can Vet J 2000: 41: 555-558)

ABSTRACT

In a retrospective study, at least 10% of dogs receiving potassium bromide/ phenobarbital combination therapy, compared with 0.3% of dogs receiving phenobarbital monotherapy, had probable pancreatitis. Pancreatitis may be a more frequent and more serious adverse effect of potassium bromide/phenobarbital combination therapy than has been reported previously.

BRIEF COMMUNICATION

Potassium bromide (KBr) is gaining popularity as an antiepileptic drug in dogs. Potassium bromide is most commonly used as an addition to phenobarbital therapy in dogs whose seizures are not well controlled with phenobarbital alone. However, because potentially fatal hepatotoxicity can be associated with phenobarbital therapy, KBr has also been recommended as the drug of first choice for canine epilepsy (Sisson 1997, Trepanier, 1997). Historically, KBr has been considered a safe drug for dogs, with less risk of organ toxicity than has been associated with the use of other antiepileptic drugs. The most commonly reported adverse effects of KBr therapy are polydipsia, polyuria,

polyphagia, gastric irritation, sedation, ataxia, and behavioral changes (Boothe 1998, Dowling 1994, Trepanier 1995). Pancreatitis is only occasionally listed in the literature as a potential adverse effect, and the occurrence is reported to be rare (Chrisman 1995). To our knowledge, only 2 studies have reported evidence of pancreatic disorders possibly associated with KBr therapy (Podell and Fenner 1993, Schwartz-Porsche and Jurgens 1991). The first involved 22 dogs receiving KBr in addition to phenobarbital therapy for poorly controlled epilepsy (Schwartz-Porsche and Jurgens 1991). An unspecified number of dogs in the study had evidence of pancreatic problems, such as pancreatitis, pancreatic atrophy and insufficiency, and pancreatic fibrosis. In the second study, pancreatitis attributed to polyphagia and garbage ingestion developed in 2 of 23 dogs (8.6%) receiving both KBr and phenobarbital (Podell and Fenner 1993). In neither study were the pancreatic problems attributed solely to the KBr. One recent study showed epilepsy to be a risk factor for acute fatal pancreatitis, but associations with individual antiepileptic drugs were not made (Hess *et al.* 1999).

In a prospective study we are presently conducting of 51 epileptic dogs receiving phenobarbital therapy, KBr was added to the therapeutic regimen in 6 of the dogs at some point during the 1-year study period. Two of the 6 dogs receiving KBr subsequently developed marked elevations of serum amylase and lipase activities and corresponding clinical signs of pancreatitis (see cases 1 and 2 in Table 1). One of these dogs was euthanized as a consequence of the pancreatitis. A 3rd dog developed acute clinical signs of pancreatitis and died before presentation to the veterinarian. Hemorrhagic necrotizing pancreatitis was diagnosed in this dog at postmortem performed at the Atlantic Veterinary College (AVC) (see case 13 in Table 1). Serum biochemical profiles performed prior to

Table 1. Case descriptions of dogs with elevations in serum amylase and/or lipase activities while receiving either potassium bromide/phenobarbital combination therapy, or phenobarbital monotherapy.

Case	Patient	History	Amylase (300-1,400 U/L)[f]	Lipase (30-560 U/L)[f]	Serum KBr (12.5-37.5 mmol/L)[f]	Serum PB (54-190 µmol/L)[f]
1[a]	Springer spaniel, 4.5 y N/M	Vomiting for 24 h.[b]	6,474	2,965	10.4	44
2[a]	Terrier, 3.5 y N/M	Vomiting for 24 h, lethargic, dehydrated. Ingested fatty meal.	9,466	3,384	16.0	87
3[a]	Terrier cross, 6 y S/F	Vomiting 24 h. Got into garbage 3 d ago.	8,009	2,062	24.5	81
4[a]	Miniature poodle, 10 y S/F	Diarrhea, vomiting, depression.	3,821	1,994	26.4	84
5[a]	Poodle cross, 9 y N/M	Anorexic, lethargic for 2 d. History of pica.	18,108	6,444	3.9	142
6[a]	Staffordshire terrier, 2 y N/M	Vomiting, shaking.[b,d]	3,897	986	23.8	80
7	Golden retriever, 14 y F	No history of illness.	1,456	510	19.4	92
8	Greyhound, 7.5 y N/M	No history given.	1,655	99	21.8	138
9	Welsh springer spaniel, 9 y M	Routine check-up.	1,581	178	19.4	96
10	Spaniel cross, 5.5 y N/M	Ataxic, bumping into walls.	1,527	220	36.8	126
11	Rhodesian ridgeback, 7 y N/M	Seizures getting worse. Also has Cushing's disease.	1,824	514	18.6	85
12	Boxer cross, 5 y F	No history given.[d,e]	1,552	225	12.3	101
13[a]	Shetland sheepdog, 3 y N/M	Acute onset of vomiting, depression, death. AVC postmortem diagnosis of hemorrhagic necrotic pancreatitis.	–	–	13.1	82
A[a]	Border collie, 5 y N/M	Vomiting for 24 h.[f]	3,052	2,852		49
B[a]	Standard schnauzer, 9 y N/M	Painful abdomen.[b,d]	2,079	197		64
C	Dalmation, 11 y N/M	Painful limb. (History of pancreatitis prior to PB).[d]	1,877	196		39
D	Bulldog, 5 y M	Weight loss over 3 mo. Activity and appetite normal.[e]	1,558	160		80

Case	Patient	History	Amylase (300-1,400 U/L)[a]	Lipase (30-560 U/L)[a]	Serum KBr (12.5-37.5 mmol/L)[a]	Serum PB (54-190 µmol/L)[a]
E	Labrador retriever, 14 y S/F	No history of illness.	2,279	294		49
F	Shetland sheepdog, 10 y N/M	No history of illness.	2,000	474		67
G	Labrador retriever, 5.5 y S/F	Healthy; pre-anesthetic examination. (Elevated amylase/lipase prior to starting PB)	4,021	679		72
H	Labrador retriever cross, 6 y N/M	Vomiting, diarrhea, hepatomegaly, splenomegaly, atypical lymphoid cells noted on CBC. AVC postmortem diagnosis of lymphosarcoma.	1,566	95		130

KBr = potassium bromide; PB = phenobarbital. Cases 1-13 were receiving KBr/PB combination therapy; cases A-H were receiving PB monotherapy. Serum KBr and PB concentrations recorded here were those determined closest to the episode of pancreatitis.

[a] Cases with clinical signs consistent with pancreatitis that were used to determine prevalence rates of suspected pancreatitis.

[b] Mild hemolysis of serum sample

[c] AVC Diagnostic Laboratory reference range

[d] Moderate lipemia of serum sample

[e] Moderate hemolysis of serum sample

[f] Marked lipemia of serum sample

the addition of KBr showed normal serum amylase and lipase activities for all 3 dogs.

Because of the apparent high incidence of pancreatitis associated with the addition of KBr to the therapy in our prospective study (3/6 dogs), we did a retrospective survey of submissions to the AVC Diagnostic Laboratory from December 1997 to March 1999 for serum KBr and phenobarbital concentrations. The goal of the survey was to determine what percentage of dogs receiving KBr and/or phenobarbital had biochemical analyses and clinical histories supportive of pancreatitis. Sixty-eight dogs were identified as receiving KBr or KBr/phenobarbital combination therapy. One dog was receiving KBr monotherapy, and 64 dogs were receiving both KBr and phenobarbital. Information concerning inclusion of phenobarbital in the therapeutic regimen was unavailable for 3 dogs receiving KBr. Of the 68 dogs receiving KBr, 18 (26%) had serum biochemical profiles, including serum amylase and lipase activities, performed at the AVC Diagnostic Laboratory during the same 16 months (and after the start of KBr therapy). Twelve dogs had increased serum amylase and/or lipase activities (see cases 1-12 in Table 1), with 6 of the 12 having a history supportive of a diagnosis of clinical pancreatitis. Clinical signs considered supportive of a diagnosis of pancreatitis included vomiting, diarrhea, anorexia, and abdominal pain. One additional dog receiving KBr and phenobarbital had a clinical history and AVC postmortem diagnosis of acute pancreatitis (case 13). The remaining 6 dogs with elevations in serum amylase and/or lipase activities had only mild elevations of amylase, with either no history on the laboratory submission form or a history that was not suggestive of clinical pancreatitis. If only those dogs with available biochemical analyses or postmortem diagnosis were used as the denominator in our calculations, 37% (7/19) of dogs receiving KBr had evidence of suspected pancreatitis.

Even if serum biochemical analyses had been available and found to be normal for all of the remaining KBr-treated dogs, the prevalence of suspected pancreatitis associated with KBr therapy would still have been at minimum 7/68 (10%). All 7 dogs with suspected pancreatitis were receiving both KBr and phenobarbital, and all had serum KBr and phenobarbital concentrations within or below the typically recommended therapeutic ranges (KBr 12.5-37.5 mmol/L; phenobarbital 54-190 μmol/L).

The retrospective survey identified 698 dogs receiving phenobarbital monotherapy. Eighty-eight (13%) of these dogs had serum biochemical profiles, including amylase and lipase activities, performed during the same 16-month period of time (and after the start of phenobarbital therapy). Eight dogs had increased serum amylase and/or lipase activities (see cases A-H in Table 1). Two of the 8 dogs had histories supportive of clinical pancreatitis. Both of these dogs had very lipemic serum samples, which may cause falsely increased serum amylase and lipase activities. Five of the remaining dogs with increases in amylase and/or lipase activities had only mild elevations in amylase, and histories not suggestive of pancreatitis. The 6th dog had more marked increases in serum amylase and lipase activities but was healthy on physical examination performed at the time of blood collection, with no history of illness. This dog also had a history of increased serum amylase and lipase activities prior to phenobarbital therapy. If only those dogs with biochemical analyses available were used as the denominator in our calculations, 2% (2/88) of dogs receiving phenobarbital monotherapy had evidence of possible pancreatitis. If biochemical analyses had been available and were normal for all other dogs receiving phenobarbital monotherapy, the prevalence of suspected pancreatitis in dogs receiving phenobarbital monotherapy would

have been 2/698 (0.3%). An odds ratio based only on those dogs with biochemical analyses available showed that the risk of pancreatitis was 29 times greater in dogs receiving KBr/phenobarbital combination therapy than in dogs receiving phenobarbital monotherapy (95% confidence interval: [6.9 to 118.8]). If the odds ratio was based on all dogs (least-case scenario), the risk would be 37 times greater in dogs receiving KBr/phenobarbital combination therapy (95% confidence interval: [10.5 to 130.5])

A number of factors need to be considered when interpreting the results of the retrospective study presented here. First, pancreatitis was confirmed by postmortem in only 1 dog. A definitive diagnosis could not be made for the other dogs with suspected pancreatitis. Clinical signs of pancreatitis are nonspecific, and can be seen with other conditions. Similarly, elevations in serum amylase and lipase activities are not specific for pancreatitis. Azotemia, renal disease, and other gastrointestinal conditions may also cause elevations of these enzymes. None of the dogs with suspected pancreatitis in our study had biochemical evidence of azotemia or renal disease, and many had inflammatory hemograms that supported the possibility of pancreatitis. Also, not all dogs with pancreatitis have increased serum amylase and lipase activities. Second, we could only estimate the prevalence of pancreatitis in this study. Serum biochemical analyses were not available for all dogs. Many veterinary clinics now perform their biochemical analyses in-house, so some cases of pancreatitis in both groups of dogs (dogs receiving KBr/phenobarbital combination and dogs receiving phenobarbital monotherapy) may not have been identified by our study. Also, digestive upset and vomiting are frequently seen with KBr therapy. Often biochemical analyses are not performed at the time of presentation for vomiting, because the signs are attributed to gastric irritation from the

KBr. Therefore, some cases of self-resolving or mild pancreatitis may be missed. Third, information concerning drug dosages, duration of treatment, and severity of seizure activity was not available for many of the dogs in this study. All of these factors may play a role in the risk of pancreatitis. This information was available for 3 of the 7 KBr-treated dogs that developed pancreatitis. All 3 had been receiving phenobarbital ≤ 2 y (2 y, 1 y, 10 mo), and KBr ≤ 1 y (1 y, 8 mo, 1 mo). All 3 had seizures that were poorly controlled with phenobarbital alone. Phenobarbital and KBr dosages were within typical maintenance ranges.

An important question is whether the risk of pancreatitis is associated with KBr in general, or only with KBr in combination with phenobarbital. All of the KBr-treated dogs with suspected pancreatitis in our retrospective study were also receiving phenobarbital. This was also the case with the 2 previously mentioned studies that reported pancreatic abnormalities in KBr-treated dogs (Podell and Fenner 1993, Schwartz-Porsche and Jurgens 1991). It is possible that KBr monotherapy does not carry the same risk. Only 1 dog in our study was known to be receiving KBr monotherapy, so assessment of the risk of pancreatitis associated with KBr monotherapy was not possible.

In our prospective study of 51 epileptic dogs, no dog receiving phenobarbital died from phenobarbital-associated hepatotoxicity (Gaskill, unpublished data). However, 2 of the 6 dogs receiving KBr in combination with phenobarbital died due to pancreatitis. This suggests that the risk of fatal pancreatitis associated with KBr/phenobarbital combination therapy may be as or more serious than the risk of fatal hepatotoxicity associated with phenobarbital therapy, at least during the 1st year of therapy.

We conclude that KBr therapy, in combination with phenobarbital, is associated

with an increased risk of pancreatitis in epileptic dogs. The prevalence of suspected pancreatitis associated with KBr/phenobarbital combination therapy in our retrospective survey was at least 10%, compared with 0.3% with phenobarbital monotherapy. Because KBr is often very effective in controlling seizures in dogs that have not been well controlled with phenobarbital alone, this drug has an important place in antiepileptic therapy. However, before KBr replaces phenobarbital as the drug of choice for treatment of canine epilepsy, studies are needed to further investigate the association between KBr and pancreatitis, and to determine if the risk of pancreatitis is increased with KBr monotherapy. Additionally, owners of dogs receiving KBr and phenobarbital should be informed of the potential risk of pancreatitis.

4.3 General discussion of the effects of phenobarbital/potassium bromide on the pancreas in epileptic dogs.

In the previous section, we showed that phenobarbital/potassium bromide combination therapy is associated with a high prevalence of pancreatitis. Phenobarbital alone did not appear to cause this high risk of pancreatitis. Unfortunately, not enough dogs receiving potassium bromide monotherapy were identified to determine the prevalence of pancreatitis with potassium bromide alone. Before our study, we had no qualms of recommending that potassium bromide be added to phenobarbital treatment, or that potassium bromide should replace phenobarbital for monotherapy in certain cases. However, now that we have seen this increased risk of sometimes fatal pancreatitis associated with the addition of potassium bromide to phenobarbital, we are more cautious in our recommendations. We believe that a prospective study is needed to determine if the increased risk of pancreatitis is high with potassium bromide monotherapy as well as with combination therapy. Until then, we do not recommend that potassium bromide replace phenobarbital as the first drug of choice for treatment of canine epilepsy.

Additional investigations of mechanism are warranted. In particular, investigation of the effects of potassium bromide on chloride transport would be helpful. This possible mechanism involving abnormal chloride transportation is supported by a recent discovery of an association between lung disease and bromide therapy in cats. In a retrospective study (Wagner 2001) of 26 cats started on potassium bromide therapy for seizures, 11 developed coughs. Thoracic radiographs in all cats showed peribronchial infiltrated, and bronchoalveolar lavage done in 2 cats showed eosinophilic inflammation. Two cats died

from this airway disease. The potassium bromide was discontinued in the remaining cats, and all but one of the cats had complete resolution of signs. However, resolution of signs took up to 16 months in some cases. As lung disease and pancreatitis are the major clinical conditions associated with cystic fibrosis in humans, and the disease in humans is due a genetic defect in chloride transport, it seems logical that the lung disease and pancreatitis noted in cats and dogs receiving bromide may also involve a similar mechanism. It is possible that potassium bromide-treated cats and dogs might produce a valuable model for cystic fibrosis in humans.

Our study showed that potassium bromide might not be as safe as previously thought. This confirmed that phenobarbital is still the major anticonvulsant drug in dogs, and that our work on the adverse effects of phenobarbital is relevant and important.

CHAPTER 5. SUMMARY AND FUTURE DIRECTION

(1) Effects of phenobarbital on the hypothalamic-pituitary-thyroid axis:

Phenobarbital treatment caused serum total thyroxine (T4) concentrations to decrease in epileptic dogs. Serum T4 concentrations were significantly decreased within 3 weeks of the start of phenobarbital treatment, and continued to decrease over the next 6 months. By one year after the start of therapy, serum T4 concentrations were still significantly decreased, although not quite as low as at 6 months. The decrease in serum T4 concentration appeared to persist as long as the dog was receiving phenobarbital, even in those dogs who had been receiving the drug for many years.

Recent seizure activity (within 24 hours of blood collection) decreased serum T4 concentrations, so T4 concentration determinations during this time period may be misleading. The decrease in serum T4 concentration found in our study was not an artifact due to interference of the assay used by phenobarbital or by autoantibodies to T4 in the serum, and was not due to decreased protein binding of T4 in the serum.

Serum thyroid-stimulating hormone (TSH) concentrations significantly increased with phenobarbital treatment, although not out of the normal range in most cases. Also, TSH stimulation tests were normal in dogs with low serum T4 concentrations. These findings suggest that the central nervous system areas are functioning appropriately. The mechanism of the decreased T4 concentration is most likely due to increased metabolism and clearance of the hormone from the body, although this mechanism was not investigated in our study.

Phenobarbital-treated dogs with low serum T4 concentrations did not have

clinical signs of hypothyroidism, and serum T4 concentrations were not correlated with degree of seizure control. This suggests that thyroid hormone supplementation is not indicated in these dogs. Our small study of thyroid hormone supplementation supports this theory. However, if a dog does have clinical signs of hypothyroidism, it is possible that the dog has concurrent thyroid disease of other causes, and supplementation in such case would be appropriate.

Future studies to complete the picture include investigations into the metabolism and clearance of thyroid hormones in phenobarbital-treated dogs. Also, a larger scale thyroid supplementation trial would help confirm if supplementation is helpful, harmful, or neither.

(2) Effects of phenobarbital on the liver:

Phenobarbital treatment caused an increase in serum alanine aminotransferase (ALT) activity in approximately 25% of dogs, and an increase in serum alkaline phosphatase (AP) activity in approximately 50% of the dogs in our study. These dogs were clinically healthy dogs, without clinical signs of liver disease.

Histopathological liver abnormalities typically associated with liver injury were more severe and found more frequently in the phenobarbital-treated dogs than control dogs. However, there were no histopathological lesions in the liver samples from the phenobarbital-treated dogs that were pathognomonic for early injury caused by phenobarbital. Similar changes and abnormalities were found in both treated and control dogs.

The ALT activity in liver tissue from phenobarbital-treated dogs was lower than

controls. In our cross-sectional, prospective, and liver biopsy studies, there were no consistent correlations between serum or liver ALT activity and serum phenobarbital concentration, dose of phenobarbital, and duration of therapy. These findings indicate that liver ALT is not induced by phenobarbital, and that increased serum ALT activity even in clinically healthy dogs is likely due to hepatocellular injury and leakage of the enzyme into the circulation.

The AP activity in liver tissue from phenobarbital-treated dogs was lower, although not significantly lower, than controls. Other recent studies suggest that increased total serum AP from other causes is due to an increased cleavage and release of the enzyme from the hepatocyte membrane. We hypothesize that liver AP activity might have been induced by phenobarbital, but that the induction could not be detected in liver homogenates because of an increased release of the enzyme from the hepatocyte membrane. This theory is supported by the correlations between serum and liver AP activities and serum phenobarbital concentration, dose of phenobarbital, and duration of therapy.

Serum alkaline phosphatase isoenzyme analysis were not helpful as a predictive test for early liver injury caused by phenobarbital. The isoenzyme activity that was predominantly increased by phenobarbital treatment was the corticosteroid-induced isoenzyme. However, in several cases the liver isoenzyme formed the majority of the total AP activity in the serum. Also, there were no correlations between histopathological scores and serum AP isoenzyme activities. However, if AP is induced, the most likely isoenzyme is the corticosteroid-induced isoenzyme, because this isoenzyme activity was highly correlated with total serum AP activity. Serum bone isoenzyme activity was only

marginally increased, and did not appear to be clinically significant.

We concluded that elevated serum ALT activity in clinically healthy phenobarbital-treated dogs might be used as a marker of early hepatic injury, and might help identify those dogs that will ultimately go on to develop clinical liver disease. In contrast, serum AP activity, serum AP isoenzyme analyses, and liver biopsy histopathology do not appear as useful at identifying dogs with early subclinical liver injury due to phenobarbital treatment.

Future studies to evaluate liver ALT and AP protein concentrations via immunoblotting will help provide additional information to either support or refute our current findings. Investigations into whether or not phenobarbital causes induction of enzymes involved in releasing AP enzyme from the hepatocyte membranes will also help provide a more complete picture.

(3) Effects of phenobarbital on the pancreas:

We found an increased risk of pancreatitis in dogs receiving phenobarbital in combination with potassium bromide. The risk of pancreatitis in dogs receiving phenobarbital alone was low, and did not appear to be different than that for the general canine population. Whether the increased risk of pancreatitis is due to the potassium bromide, or to the combination of phenobarbital and potassium bromide, is unknown at this time. Also, the mechanism of the pancreatitis is unknown. However, this information indicates that further studies are indicated to investigating the risk of pancreatitis with potassium bromide monotherapy. More dogs died in our study as a result of pancreatitis associated with phenobarbital/potassium bromide than from liver

disease associated with phenobarbital. This underscores the importance of retaining phenobarbital as the drug of choice for treatment of canine epilepsy until all risks and benefits of potassium bromide monotherapy are delineated. Until then, we recommend that owners of dogs receiving phenobarbital/potassium bromide therapy be warned of the potential risk of pancreatitis.

APPENDIX A. INFORMATION ON THE EFFECTS OF THYROID HORMONE SUPPLEMENTATION IN PHENOBARBITAL-TREATED DOGS

METHODS AND MATERIALS

Phenobarbital-treated epileptic dogs with serum thyroxin (T4) concentrations below the lower limit of the normal reference range were identified in the prospective and cross-sectional portions of our study (Gaskill *et al.* 1999, Gaskill *et al.* 2000). If the attending veterinarians chose to initiate thyroid hormone supplementation, and the owner consented, the dog was enrolled in the thyroid hormone supplementation trial. Blood samples were taken before the start of thyroid hormone supplementation, and biochemical profiles [Hitachi 911, Boehringer Mannheim Corp., Indianapolis, Ind] serum T4 [CEDIA T4 homogeneous enzyme immunoassay, Boehringer Mannheim Corp., Indianapolis, Ind] and TSH [Coat-A-Count canine TSH immunoradiometric assay, Diagnostic Products Corp., Los Angeles, CA] concentrations, and serum phenobarbital [CEDIA phenobarbital homogeneous enzyme immunoassay, Boehringer Mannheim Corp., Indianapolis, Ind] concentrations were determined. Blood samples were taken 8-12 hours after the last dose of phenobarbital (trough sample), after a 12-24 hour fast. Serum separator tubes were not used due to their possible effect on serum phenobarbital concentrations. Owners completed a detailed questionnaire concerning the dog's seizure history, behavior, and appearance. Attending veterinarians performed physical and neurological examinations and recorded the results on the submission forms accompanying the blood samples. We provided the thyroid supplement [Soloxine, Jones Medical Ind./Daniels Pharmaceuticals,

St. Louis, Missouri] to be dosed at 22 μg/kg PO BID. Dogs returned for blood collection and physical examinations at 1, 3, 4, and 6 months after the start of thyroid supplementation; owners completed questionnaires as above at 1, 2, 3, 4, and 6 months. Biochemical profiles, serum T4 and TSH concentrations, and serum phenobarbital concentrations were determined for each sample at each recheck.

RESULTS

Three of the phenobarbital-treated epileptic dogs identified in our previous studies were enrolled in the thyroid hormone supplementation trial. One dog was maintained on thyroid hormone supplement and returned for all rechecks during the 6 month trial. The other 2 dogs returned for the 1 month recheck. However, before the 3 month recheck, one dog died after being hit by a car, and the owners of the second dog discontinued the thyroid supplementation because they believed that the dog's seizures had worsened since the start of the thyroid supplementation.

Before the start of supplementation, all dogs had normal biochemical profiles with the exception of a very mild increase in serum alkaline phosphatase in one dog (serum AP: 93 U/L; normal AVC reference range, 23 to 87 U/L). Serum T4 concentrations for the 3 dogs were 9.5, 5.9, and 13.0 μmol/L before the start of thyroid hormone (normal AVC reference range: 15 to 52 μmol/L). Serum TSH concentrations were 0.79, 0.12, and 0.16 ng/ml, respectively (normal reference range, < 0.50 ng/ml). Serum phenobarbital concentrations were 80, 86, and 76 μmol/L, respectively (normal therapeutic reference range, 54 to 190 μmol/L). The dogs were in good physical condition, without clinical

signs of overt hypothyroidism. Two dogs had not had seizures in the month previous to the start of thyroid hormone supplementation; one dog had had one seizure.

At the 1 month recheck, one dog had developed a very mild elevation in serum alanine aminotransferase activity, and one dog continued to have a very mild elevation in serum alkaline phosphatase activity. Otherwise, the biochemical profiles remained normal. Serum T4 concentrations were all within or slightly above the normal reference range. Serum TSH concentrations were all 0.1 ng/ml. All dogs had remained on the same dosage of phenobarbital they were receiving before the start of thyroid hormone supplementation. Serum phenobarbital concentrations were 62, 100, 74 μmol/L, respectively. The dogs remained in good physical condition, with normal mentation and behavior. Two dogs were more active since starting the thyroid supplementation. Two dogs had no seizures in the month since starting thyroid hormone supplementation. The dog that had one seizure in the month prior to initiation of thyroid hormone supplementation had 2 seizures during the first month after the start of supplementation.

One dog continued on thyroid hormone supplementation and returned for rechecks at 3, 4, and 6 months. The serum biochemical profile and serum T4 and TSH concentration remained normal for this dog. The serum phenobarbital concentration varied at each recheck (74, 83, 57, and 67 μmol/L, respectively, for the 1, 3, 4, and 6 month rechecks), despite no change in dosage, body weight, or collection times post-pill. The dog had no seizures until the 6 month point, when she had 2 seizures that were of typical severity and duration for this dog.

APPENDIX B. INFORMATION ON THE EFFECTS OF POTASSIUM BROMIDE ON THE HYPOTHALAMIC-PITUITARY-THYROID AXIS IN DOGS

METHODS AND MATERIALS

Population: During our previous studies (see Chapter 2, sections 2 and 3), 12 phenobarbital-treated dogs were identified that had serum thyroxine (T4) concentrations determined before and after the addition of potassium bromide (KBr) to their anticonvulsant therapies. The dogs presented for liver biopsies because of elevations in serum alanine aminotransferase and/or alkaline phosphatase, but the dogs had no clinical signs of liver or other disease. Potassium bromide was added to the phenobarbital therapy in these dogs after biopsies showed mild to moderate liver abnormalities. Two dogs developed signs of other diseases soon after starting KBr (one dog developed pancreatitis, the other developed pneumonia) and so were excluded from this study. The phenobarbital dosage was gradually decreased in the remaining 10 dogs during the following year. Phenobarbital therapy was completely discontinued in 4/10 dogs during this time.

Data: Serum T4 concentrations were determined before and after the addition on KBr. In most cases, serum T4 concentrations were determined several times before and after the start of KBr. Serum TSH concentrations were determined for 8/10 dogs before and after the start of KBr. A TSH stimulation test (Appendix C for description) was performed for one of the dogs that developed a low serum T4 concentration after the initiation of KBr.

Serum phenobarbital concentrations and serum biochemical profiles (see Chapter

1, section 2 for details) were determined for all dogs before and after the start of KBr. Additionally, serum KBr concentrations[a] [PHM 240 pH/Ion Meter, Radiometer Analytical S.A., Lyon, France] were determined for all dogs after the start of KBr.

Statistical analyses: Spearman's correlation coefficient (r) tests (SPSS Inc. Software) were used to determine correlations. Significance was defined as $P \leq 0.05$.

RESULTS

At some point during the first year after the addition of KBr, the serum T4 concentration decreased in 8 of the 10 dogs (**Table 1**). The decrease in serum T4 concentration was persistent in 5/8 dogs. The serum T4 concentration dropped very low (< 5 nmol/L) in 3 dogs. Serum TSH concentration decreased in 2 dogs, and remained the same in 6 dogs.

Serum KBr concentrations ranged from 91 to 260 ng/ml (typical therapeutic reference range, 100-250 ng/ml). There was no correlation between serum T4 concentration and serum KBr concentration or duration of KBr therapy (**Fig 1**).

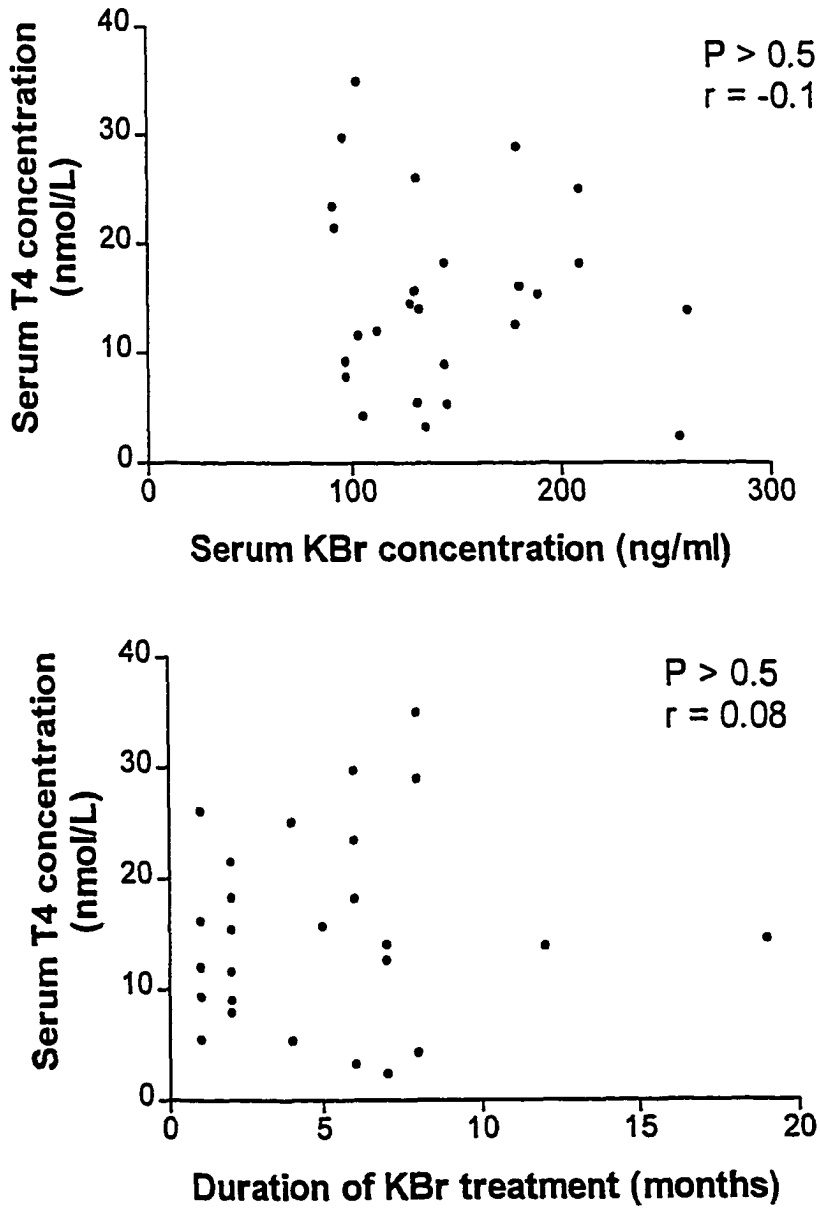
One dog (case X) had a TSH stimulation test performed when the serum T4 concentration decreased after the addition of KBr. The TSH stimulation test results were normal.

Table 1. Serum thyroxine (T4) and thyroid-stimulating hormone (TSH) concentrations in 10 phenobarbital-treated dogs before and after the addition of potassium bromide (KBr) to the therapeutic regimen. In several cases, serum T4 concentrations were measured several times before the start of KBr.

Case	Serum T4 prior to KBr (15-52 nmol/L)[a]	Serum TSH prior to KBr (<0.5 ng/ml)[b]	Months after start of KBr	Serum T4 afer KBr (15-52 nmol/L)[a]	Serum TSH after KBr (<0.5 ng/ml)[b]
1	8.1[++]	0.10	2	18.3	--
	9.9[+\]	0.11	6	23.5[+]	
2	6.4		1	12.0	
	11.2		7*[c]	2.4	0.28
	12.1	0.32	12[c]	13.9	0.32
	11.1	0.25			
3	13.1[++]		2*	9.0[+\]	
	12.0	0.12	5	15.7	
			7	14.0	0.15
			19	14.5	
4	17.6	0.42	1	16.1	0.15
	9.7	0.15	2	15.4	0.13
			6	18.2[\]	
5	15.9	0.14	2*	7.9[+\]	
			4	25.1	0.13
			8[c]	29.0	0.15
6	34.8[++]	--	2*	21.5	--
			8*	4.3[\\]	
7	34.9	0.15	1*	26.1[+]	
	31.9[+++]		7*[c]	12.6	0.12
8	21.3	0.13	2*	11.6[++]	
			6*	3.3	0.10
9	47.7	0.50	1*	9.3[+\\\]	0.30
	40.9	0.63	6*[c]	29.8	
			8*[c]	35.0	0.15
10	13.9	0.10	1*	5.5	
	16.3		4*	5.4	0.14

[a] AVC Diagnostic Laboratory reference range
[b] AVC Diagnostic Laboratory reference range
[c] Phenobarbital discontinued
[+] Degree of hemolysis of serum sample (+ = mild, ++ = moderate, +++ = marked)
[\] Degree of lipemia of serum sample (\ = mild, \\ = moderate, \\\ = marked)
*Time after start of KBr when serum T4 concentration was lower than the average before KBr.

Figure 1. Scatterplots showing lack of correlations between serum thyroxine (T4) concentration and (A) serum potassium bromide (KBr) concentrations, or (B) duration of KBr therapy in 10 epileptic dogs receiving phenobarbital and potassium bromide.



APPENDIX C. STUDY FORMS

ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island, Canada C1A 4P3

Clinical Pharmacology
Tel. 902-566-0804
Fax 902-566-0832

September 9, 1997

Dear Doctor,

Doctors Cribb, Burton, Ihle, Miller, and Shaw of the Atlantic Veterinary College (AVC) have received a grant from the Animal Welfare Unit to study the safety and efficacy of phenobarbital therapy in dogs. Dr. Gaskill will be involved in the study as part of her Master's degree program. By providing thorough diagnostic and clinical evaluations to approximately 150 dogs in Atlantic Canada receiving phenobarbital, we hope to gather sufficient data to understand the occurrence and significance of liver and thyroid dysfunction caused by the drug. The AVC Therapeutic Drug Monitoring (TDM) service receives over 500 submissions per year for serum phenobarbital concentrations. Many patients receiving phenobarbital are also monitored for changes in liver enzymes and thyroid hormone concentrations, and many show abnormalities in these tests. Interpreting these biochemical abnormalities is a common dilemma faced by veterinarians. When do liver biochemical changes indicate true hepatotoxicity is taking place, and when do they simply reflect enzyme induction? What is the significance of lowered T_4 levels and how should we manage these cases? Existing information on phenobarbital toxicity in dogs and current recommendations on avoidance of toxicity are based on studies of very small numbers of dogs (18 animals or less), and on data extrapolated from other drugs or other species. This study will draw on information from over 150 dogs. The results will provide better guidelines to help detect true hepatotoxicity and thyroid dysfunction earlier in the course of disease, and provide guidelines on how to avoid or manage these conditions.

Recruitment of cases for the study will be from patients seen here at the AVC as well as patients from private practices like yours throughout Atlantic Canada. We encourage you to enlist epileptic dogs that you are already treating with phenobarbital (Phase I patients) as well as dogs you newly diagnose with epilepsy and have not yet started on anticonvulsant therapy (Phase II patients). Phase II dogs should be between the ages of 1 year and 6 years at the time of the first known seizure, and both Phase I and Phase II dogs must not be currently receiving any other therapeutic drugs. Some drugs such as flea sprays and wormers may be acceptable and will be evaluated on a case-by-case basis. If you have patients that you wish to enroll in the study that do not meet these requirements, please call us for more information.

In Phase I, data will be collected on 100 dogs currently receiving phenobarbital, including dogs who have been receiving the drug for years. Baseline data will include patient information such as breed, sex, age, weight, dose of drug, duration of therapy, clinical history, response to therapy, and any concurrent medications; and biochemical data including phenobarbital, T_4, TSH, glucose, cholesterol, total protein, albumin, total and direct bilirubin, BUN, fasting bile acids, and liver-related enzymes (AP, ALT, AST, GGT, SDH). The biochemical data will be collected from the blood samples you routinely send to the TDM service for serum phenobarbital concentrations, so no additional blood sampling is necessary. For dogs enrolled in Phase I, the grant will cover the cost of the phenobarbital analysis and the additional biochemical tests by direct payment to the diagnostic laboratory.

In Phase II, we will collect data on 50 dogs newly diagnosed with epilepsy. The initial diagnosis should be based on history, physical exam, and neurologic exam. Serum will be obtained prior to administration of phenobarbital to provide baseline data; all of the above patient data will be obtained, and

a CBC and biochemical tests including a total body chemistry panel, direct bilirubin, T_4, fasting bile acids and TSH will be run. The grant will cover the cost of all laboratory fees by direct payment to the diagnostic laboratory. For patients whose initial blood work indicates that epilepsy is indeed the most likely cause of the seizures and who are begun on phenobarbital therapy, blood samples also will be collected at approximately 3 weeks, 6 months, and 12 months after the start of phenobarbital therapy, and all of the data as listed for Phase I patients will be obtained each time. The grant will reimburse practitioners for professional fees associated with each office visit recheck and blood collection and interpretation, and will cover the cost of the laboratory tests and blood shipment. The diagnostic laboratory will be paid directly from the grant for all laboratory services. Reimbursement to practitioners will be in accordance with provincial veterinary medical fee guidelines. For reimbursement for professional services, please submit a bill to Dr. Alastair Cribb, Department of Anatomy and Physiology, at the address given on the letterhead.

If a patient is euthanized or dies from any cause while enrolled in the study, please perform a post-mortem if at all possible and obtain a section of the liver and the thyroid gland to send to the AVC diagnostic laboratory for histopathology. Even if the owner does not wish to have a post-mortem performed, they may allow you to obtain a percutaneous biopsy. The grant will reimburse the practitioner for professional fees associated with biopsy or post-mortem as well as cover the cost of the histopathology.

Dogs from Phase I or Phase II that have evidence of liver and/or thyroid dysfunction are eligible for enrollment in Phase III. These patients will be provided additional biochemical and clinical diagnostic evaluations to help determine the nature and degree of organ dysfunction. The tests that will be included in the study will be determined by the internal medicine specialists at the AVC and may include phenobarbital half-life determinations, pre- and post-prandial bile acids, radiographs, ultrasonography, coagulation profiles, hepatic biopsy, and follow-up testing after therapeutic intervention. Some of these diagnostics may be performed by the private practitioner, and some will require referral to the AVC. The grant will reimburse the practitioner for diagnostic tests pre-approved by the study directors, and will pay for all diagnostic tests done at the AVC. Reimbursement will be in accordance with provincial guidelines. Please contact Drs. Shaw, Ihle, or Miller for additional information. If additional office visits are required for other conditions, status epilepticus, or poor regulation, these will not normally be covered by the grant. An owner may decline any test they wish, and may withdraw their pet from the study at any time.

The results of Phase III will provide the data to link the results obtained in Phases I and II with clinical organ dysfunction, and lead to the development of better guidelines for avoiding and managing phenobarbital induced hepatotoxicity and thyroid dysfunction. This is an opportunity for you to make a real contribution to our understanding of phenobarbital and its potential toxicities, and to provide your patients with the very best in veterinary care. Patient recruitment will begin immediately. We encourage you to ascertain if any of your epileptic patients are eligible for Phases I or II, and consider enrolling these patients in the study. Please find enclosed copies of the laboratory submission form to use when sending samples, a client information sheet, a quick reference guide, and a client consent form to be filled out and returned to us with the blood samples. We will provide you with as many of these forms as you need, or you may photocopy the enclosed forms. If you have any questions or comments, please contact Dr. Cribb or Dr. Gaskill, Clinical Pharmacology, at 902-566-0804; Dr. Burton, Clinical Pathology, at 902-566-0541; or Drs. Ihle, Miller, or Shaw, Internal Medicine, at 902-566-0950.

Phenobarbital Safety and Efficacy Study
Veterinarians' Quick Reference

Phase I

-dogs currently receiving phenobarbital and no other drugs (call if you have questions concerning other drugs).

(1) Send the completed consent form with the blood sample
(2) Completely fill out the Phenobarbital Study Request Form and mark the analysis request box for "Patients on Phenobarbital"
(3) Send an 8-12 hr post-pill, fasted serum sample (2cc)

Phase II

-dogs presenting for seizures with history, physical exam and neurological exam consistant with idiopathic epilepsy; dog must be between 1 yr and 6 yr of age, and not yet on any anticonvulsant therapy (call if you have dogs outside these parameters that you wish to enroll).

(A) Initial examination (pre-therapy):
1) Send completed consent form with the blood sample
2) Completely fill out the Phenobarbital Study Request Form and mark the analysis request box for "New Patient prior to Phenobarbital Therapy"
3) Send an 8-12 hr fasted serum sample (2cc), a purple top tube of blood, and two air-dried, unstained blood smears

(B) Dogs that have normal CBC and biochemical tests will return at 3 wk, 6 mo, and 12 mo after the start of phenobarbital therapy for additional blood work
1) Completely fill out the Phenobarbital Study Request Form and mark the analysis box for "Patients on Phenobarital"
2) Send an 8-12 hr post-pill, fasted serum sample (2cc)

Phase III

-dogs from Phases I and II that have biochemical evidence of liver and/or thyroid dysfunction. Enrollment contingent upon approval by Drs. Shaw, Ihle, and Miller.

(A) Call Dr. Cribb or Dr. Gaskill at 902-566-0804, or Dr. Shaw, Dr. Ihle, or Dr. Miller at 902-566-0950 for information and enrollment approval.

Phenobarbital Study Request Form

ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island C1A 4P3

CLINICAL PHARMACOLOGY
902-566-0804

OWNER SURNAME | FIRST NAME

NAME OF CLINIC | CLINIC BILLING NO.

PATIENT'S NAME

SPECIES | BREED

DATE OF BIRTH
DAY MO. YR.

PHENOBARBITAL STUDY #553
Check one: ☐ Phase I
☐ Phase II (circle one: 0 wk, 3 wk, 6 mo, 12 mo)

COLLECTION DATE	CLINICIAN
DATE RECD	LAB NO.

Sample requested: 2cc (1cc minimum) of frozen or refrigerated serum. Recommend 8-12 hr fasted sample. Do not use serum separator tubes (red/grey swirl tops). For analysis that includes CBC, also send purple-top tube of blood and 2 air-dried, unstained blood smears.

Patient Data:

Weight:_____ (kg / lb) Actual or estimated (circle one)
Sex:____; Neutered?_____; Pregnant?_____
Current drug dose and frequency:_____
How long has dog been on current dose?_____
How long has dog been on phenobarbital?_____
Manufacturer of drug:_____
Current seizure activity (last 3 months) – frequency and severity:_____
_____
_____
Seizure activity prior to initiation of phenobarbital – frequency and severity:_____
_____
_____
Concurrent medical conditions (use back side of form if necessary)_____
_____
_____
_____
Concurrent medications or recent administration of other drugs (please specifically inquire about over-the counter products):_____
_____
_____

Analysis requested: Time of sample collection_____(AM/PM)
Time of last pill_____(AM/PM); Time of last meal_____(AM/PM)

☐ Patients on Phenobarbital (Phase I and rechecks of Phase II patients):
–phenobarbital, liver panel 904, AST, T_4, fasting bile acids, TSH

☐ New Patients prior to Phenobarbital Therapy (Phase II initial exam):
–CBC, total body panel 901, direct bilirubin, T_4, fasting bile acids, TSH

ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island, Canada C1A 4P3

Clinical Pharmacology
Tel. 902-566-0804
Fax 902-566-0832

Safety and Efficacy of Phenobarbital Therapy in Dogs

Client Information

Epilepsy, one of the most common heritable disorders in dogs, is a condition characterized by repeated seizures (convulsions). Seizures can be dangerous for the animal if they occur in clusters or for prolonged periods of time. Epilepsy cannot be cured, but anticonvulsant drugs may minimize seizures in many dogs. Phenobarbital is currently the drug of choice for anticonvulsant therapy in dogs, and can be very effective. However, on occasion it may cause liver damage that either necessitates discontinuation of the drug or leads to severe complications, even death, in some patients. Unfortunately, the early diagnosis of liver damage is difficult because phenobarbitals' normal effects on the liver can interfere with the routine blood tests used to detect liver damage. Phenobarbital also affects normal thyroid hormone regulation, which may affect seizure control.

The objective of this project is to improve the safety and effectiveness of anticonvulsant therapy in dogs. By providing thorough diagnostic and clinical evaluations to epileptic dogs throughout Atlantic Canada receiving phenobarbital, we will gather the information needed to better detect any organ damage earlier in the course of disease, and to provide guidelines to help veterinarians avoid and manage these conditions. This project will directly benefit the patients who participate through enhanced patient care, and the final results will benefit all dogs with epilepsy.

If your dog is already being treated for epilepsy with phenobarbital (Phase I patients), the study protocol is very simple. Additional tests will be run on the same blood sample your veterinarian routinely sends to the lab to measure phenobarbital levels. While you will pay your veterinarian as usual for the office visit, the study will cover the costs of all of the laboratory tests. If your dog is newly diagnosed with epilepsy and not yet on any drugs (Phase II patients), in addition to this first blood sample, blood samples will be collected three more times over the course of a year. The study will cover the costs for the follow-up office visits, the blood collection and shipping, and all of the laboratory tests for each visit. The only cost to you will be the initial office call and exam.

If your pet shows evidence of liver or thyroid abnormalities on these blood tests, he or she may be eligible for enrollment in Phase III of the study. If your pet is enrolled in this phase, the internal medicine specialists at the Atlantic Veterinary College will recommend tests to help determine the extent of organ damage. These tests may include additional blood tests, xrays, ultrasound exam, and liver biopsy. These are the normal, usual tests recommended for any patient with evidence of liver or thyroid disorders, and so are in no way experimental or unnecessary. Some of these tests may be done by your regular veterinarian, and some may need to be done through referral to the Atlantic Veterinary College. The study will cover all of the costs associated with these additional examinations and tests.

You may decline any test you wish, and you may withdraw your pet from the study at any time. All test results will be reported back to your veterinarian immediately so he or she can initiate any necessary changes in your pets' therapy. If you have any questions, please contact your veterinarian, or call either Dr. Alastair Cribb or Dr Cynthia Gaskill, Clinical Pharmacology, Atlantic Veterinary College, 902-566-0804.

ATLANTIC VETERINARY COLLEGE
UPEI University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island, Canada C1A 4P3

Clinical Pharmacology
Tel. 902-566-0804
Fax 902-566-0832

Safety and Efficacy of Phenobarbital Therapy in Dogs

Consent Form

I,_____, consent to the enrollment of my dog _____ in the study conducted by the Atlantic Veterinary College (AVC) to help improve the safety and efficacy of phenobarbital use in dogs. I understand that the first two phases (Phase I and II) of this study involve conducting additional laboratory (biochemical) tests using the blood sample that my veterinarian routinely obtains to measure phenobarbital levels. No unusual or experimental tests will be done and the results of the testing will be available to my veterinarian to help in the care of my dog. I give my permission for the results of these tests to be included in a large study to help all dogs. I understand that neither I nor my dog will be identified in any of the study results.

If my dog is currently receiving phenobarbital to treat epilepsy, he/she will be enrolled in Phase I of the study. I understand that there will be no charge for the diagnostic tests run by the AVC Diagnostic laboratory on the blood samples my veterinarian sends them. The only costs incurred will be the routine office visit related fees my veterinarian would normally charge. If my dog is newly diagnosed with epilepsy and is going to start treatment with phenobarbital, he/she will be enrolled in Phase II of the study. Once again, the study will cover the cost of all the blood tests, so the only expense to me is the normal office visit related fee at the time of the initial exam Subsequent office visits, blood collections, and laboratory tests at 3 weeks, 6 months, and 12 months after starting phenobarbital will be provided at no charge as the Study will reimburse my veterinarian for associated costs.

If any of the blood results indicate medical problems related to the thyroid gland or the liver, the Study will cover the costs of additional diagnostic tests recommended by the internal medicine specialists at the AVC (Phase III animals). These tests may be performed by either the AVC or by my regular veterinarian, depending on the tests and my wishes. I understand that I may accept or refuse any of the diagnostic tests recommended. These tests may include additional blood tests, xrays, ultrasound, and biopsy.

I understand the purpose of the Study. I understand that there is no risk to my pet other than that related to the normal medical procedures that have been explained by my veterinarian, and that I may withdraw my pet from the study at any time. My veterinarian has explained the study to me and I have had my questions answered. I may contact Dr. Alastair Cribb at the above number for further information.

I understand that my dog will be enrolled in Phase _____ of the study.

Signed: _____ Date: _____

Witness: _____ Date: _____

Phenobarbital Safety and Efficacy Study
Patient Enrollment Criterion

Criterion for entry into Phase I:

— Dog must be receiving phenobarbital therapy for epilepsy; dog must not be on any other therapeutic drug.

Criterion for entry into Phase II:

— History, physical exam, and neurologic exam must not suggest any cause for seizures other than idiopathic epilepsy.

— Dogs must have had their first seizure after the age of 1 year and before the age of 6 years.

Criterion for entry into Phase III:

(HN = upper limit of normal; LN = lower limit of normal)
— ALT > 2X HN. (ALT > 138 IU/L)
— Conjugated bili > HN. (Dbili > 4 μmol/L)
— AP > 2X HN. (AP > 174 IU/L)
— Fasting bile acids > HN. (Bile acids > 10 μmol/L)
— T4 < LN. (T4 < 15 nmol/L)
— BUN < LN. (BUN < 3 mmol/L)
— Alb < LN. (Alb < 22 g/L)
— Clinical signs suggestive of liver or thyroid disorder, even if biochemical results are normal.
— Any unexpected or unusual changes in phenobarbital concentrations or in dose requirements of phenobarbital.

UPEI ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island, Canada C1A 4P3

Clinical Pharmacology
Tel. 902-566-0804
Fax 902-566-0832

04/01/98

Dear Doctor,

As part of our current Phenobarbital Study, we are providing monitoring for those dogs receiving phenobarbital whose veterinarians believe may benefit from thyroid supplementation. The decision to initiate thyroid supplementation must be yours and the client's. To be eligible for monitoring, the dog must be enrolled in Phase I of our study and have a low T_4 concentration. The supplementation should consist of Levothyroxine (Soloxine) at 22 μg/kg (0.02mg/kg) PO twice a day. The owners must fill out a brief questionnaire prior to initiation of supplementation and at monthly intervals to be eligible for continued monitoring.

We will pay for serum biochemical profiles including liver panel, phenobarbital and T_4 concentrations prior to the start of supplementation and 1 month, 3 months, and perhaps 6 months after initiation of supplementation. We will pay the laboratory directly for all of the laboratory tests and will reimburse you for all professional fees associated with the office rechecks and the blood draws (send a bill directly to Dr. Cribb, Clinical Pharmacology, Department Of Anatomy and Physiology, Atlantic Veterinary College, University of Prince Edward Island, 550 University Ave., Charlottetown, PEI, Canada C1A 4P3). We will provide the Levothyroxine (Soloxine, Daniels Pharmaceuticals) free of charge for 3 months. This first supply of Soloxine will be enough for 1 month of supplementation. We will courier a second supply to you before the one month recheck.

Please find attached: (1) a quick reference sheet with instructions for each recheck; (2) submission/questionnaire forms for you and the owner to complete at each recheck; (3) an owner consent form; and (4) a copy of the drug insert for the Soloxine.

If you have any questions or if your patient develops any problems, please call Dr. Gaskill or Dr Cribb at 902-566-0804, fax 902-566-0832. Thank you very much for your participation in our study.

Sincerely,

Cynthia Gaskill, DVM

Phenobarbital Study Request Form

ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island C1A 4P3

CLINICAL PHARMACOLOGY
902-566-0804

OWNER SURNAME FIRST NAME

NAME OF CLINIC CLINIC BILLING NO.

PATIENT NAME

PHENOBARBITAL STUDY #553

SPECIES BREED

Thyroid Supplementation Monitoring

AGE SEX

Check one: ☐ Time 0 ☐ 1 month
☐ 2 months ☐ 3 months

COLLECTION DATE CLINICIAN

DATE REC'D LAB NO.

SECTION 1: VETERINARIAN'S SECTION

Weight:_____(kg / lb)..... Heart rate:_____(beats per min)

Respiratory rate:_____(per min)..... Temperature:_____(F / C)

Phenobarbital: Current dose and frequency: _____

Duration on current dose:_____Total duration on phenobarbital:_____

Soloxine: Current dose and frequency of Soloxine:_____

Duration on current dose:_____Total duration on Soloxine:_____

Body weight.....☐ thin ☐ normal ☐ overweight ☐ obese

Hair coat condition:.....☐ very abnormal ☐ mildly abnormal ☐ normal ☐ excellent

Alopecia.....☐ none ☐ mild ☐ severe

Hyperpigmentation;.....☐ none ☐ mild ☐ severe

Dull or dry coat.....☐ none ☐ mild ☐ severe

Seborrhea.....☐ none ☐ mild ☐ severe

Behavior at time of exam:.....☐ lethargic ☐ normal ☐ hyperactive

General condition..... ☐ Very poor ☐ mildly poor ☐ good ☐ very good

Additional descriptions:_____

SECTION 2: LABORATORY SECTION: ANALYSIS REQUESTED

Sample requested: 2-3 cc of non-hemolyzed serum; 8-12 hours post-phenobarbital; 12-24 hour fast. Do not use serum separator tubes (red/grey swirl tops).

Time of: Sample collection:_____(AM / PM) Last meal:_____(AM / PM)

Last dose phenobarb:_____(AM / PM) Last dose Soloxine:_____(AM / PM)

☐ Time 0: No blood work

☐ 1 month: Phenobarbital, liver panel 904, AST, fasting bile acids, T4, TSH

☐ 3 month: Phenobarbital, liver panel 904, AST, fasting bile acids, T4, TSH

SECTION 3: CLIENT'S SECTION

SEIZURE HISTORY:

Age of pet when seizure activity first noted:_____

Number of seizures in the last year:_____

Frequency in the last month:..... ☐ no seizures ☐ 1 episode ☐ 2 episodes ☐ 3 or more

Duration of each episode:..... ☐ < 1 min ☐ 1-3 min ☐ 3-5 min ☐ > 5 min

Severity of seizures:..... ☐ mild ☐ moderate ☐ severe

Multiple seizures during each episode?...☐ yes ☐ no

Loss of consciousness during seizure?.....☐ yes ☐ no

Loss of bowel or urine control during seizure?.....☐ yes ☐ no

Unusual behavior prior to seizure?.....☐ yes ☐ no

If yes, please describe:_____

Unusual behavior after seizure?..... ☐ yes ☐ no

If yes, please describe:_____

PHYSICAL CONDITION:

Body weight:..... ☐ thin ☐ normal ☐ overweight ☐ obese

Hair coat condition:..... ☐ very poor ☐ poor ☐ normal ☐ excellent

Thin or bald areas in hair coat?.....☐ yes ☐ no

If yes, describe:_____

Dry or dull coat or skin?.....☐ yes ☐ no

Excessive shedding?.....☐ yes ☐ no

Greasy coat or skin?.....☐ yes ☐ no

If body weight or hair coat condition have changed, please describe and note when the change occurred:_____

BEHAVIOR:

Appetite:..... ☐ poor ☐ normal ☐ excessive

Type of food:... ☐ canned ☐ dry ☐ both

Treats or snacks?..... ☐ yes ☐ no

Estimate how many measuring cups of food (total) your pet eats per day:_____

Water consumption:..... ☐ less than normal ☐ normal ☐ more than normal

Estimate how many measuring cups of water your pet drinks per day:_____

If appetite or water consumption have changed, please describe and note when change occurred:

_____

Activity level:.... ☐ less active than normal ☐ normal ☐ more active than normal

If activity level has changed, please describe and note when it changed:_____

_____

Personality:..... ☐ not normal ☐ normal

If personality has changed, please describe and note when it changed:_____

_____

ADDITIONAL COMMENTS:_____

_____

_____

_____

Phenobarbital Safety and Efficacy Study
Veterinarians' Quick Reference
THYROID SUPPLEMENTATION

Time: Initial exam, time 0

(1) Have the owner sign the client consent form for the thyroid supplementation.
(2) Have the owner complete the client information section of the questionnaire.
(3) The veterinarian should complete a physical examination of the pet and fill out the veterinarian's section of the questionnaire.
(4) No blood draw is necessary on this office visit unless otherwise requested by the study.
(5) Fax the completed questionnaire and consent form to Dr. Gaskill at 902-566-0832.
(6) Dispense the first month's worth of Soloxine to the client.

Time: One month recheck

(1) Have the owner fill out the client information section of the questionnaire.
(2) The veterinarian should re-examine the pet and fill out the veterinarian's section of the questionnaire.
(3) Obtain a fasted serum sample (12-24 hour fast, 24 hours if previous hyperlipidemia problems), 8-12 hours post-phenobarbital. We need 2-3 cc of non-hemolyzed serum (1.5 cc may be enough if the dog is less than 8 lbs.). Do not use serum separator tubes. If the sample is grossly lipemic, please redraw the blood after a 24 hour fast.
(4) Return the completed questionnaire/submission form along with the serum sample to the AVC diagnostic laboratory.
(5) Dispense a 1-2 month refill of Soloxine to the client.

Time: Two months

(1) Have the owner complete the client information section of the questionnaire.
(2) No examination by the veterinarian and no completion of veterinarian section of questionnaire is necessary unless the pet is having problems.
(3) No blood draw is necessary unless the pet is having problems. If the veterinarian believes a blood recheck is necessary, please call Dr. Gaskill at 902-566-0804.
(4) The client can bring the completed questionnaire to you to fax to Dr. Gaskill at 902-566-0832, or they can mail it directly to Dr. Gaskill in the attached envelope.
(5) Dispense additional Soloxine to the client if needed.

Time: Three month recheck

(1) Have the owner fill out the client information section of the questionnaire.

(2) The veterinarian should re-examine the pet and fill out the veterinarian's section of the questionnaire.

(3) Obtain a fasted serum sample (12-24 hour fast, 24 hours if previous hyperlipidemia problems), 8-12 hours post-phenobarbital. We need 2-3 cc of non-hemolyzed serum (1.5 cc may be enough if the dog is less than 8 lbs.). Do not use serum separator tubes. If the sample is lipemic, please redraw the blood after a 24 hour fast.

(4) Return the completed questionnaire/submission form along with the serum sample to the AVC diagnostic laboratory.

(5) Dispense additional Soloxine (at the owners expense at this point) if indicated.

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Clinical Pharmacology
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Fax 902-566-0832

Safety and Efficacy of Phenobarbital Therapy in Dogs

Consent Form

I,_____, consent to the initiation of thyroid hormone supplementation therapy for my dog_____. I understand that my veterinarian believes that supplementation may be beneficial to my dog, but that much is yet unknown about the relationship between phenobarbital therapy and thyroid hormone regulation. I agree to bring my pet to my veterinarian for an examination and blood draw at the end of 1 and 3 months, and to complete a short questionnaire once a month for three months after initiation of thyroid hormone supplementation. I give my permission for the results of the blood tests to be included in a large study to help all dogs. I understand that neither I nor my dog will be identified in any of the study results.

I understand that the Phenobarbital Study will pay the diagnostic laboratory directly for all of laboratory tests, will reimburse my veterinarian for associated office visit and blood draw fees, and provide the thyroid supplement for 3 months.

I understand that the purpose of this monitoring is to detect any beneficial or adverse effects of thyroid supplementation in epileptic dogs receiving phenobarbital therapy. If any unwanted effects occur, my veterinarian may discontinue thyroid supplementation at any time. My veterinarian has explained the study to me and I have had my questions answered. I may contact Dr. Alastair Cribb at the above number for further information.

Signed: _____ Date: _____

Witness: _____ Date: _____

Phenobarbital Study Request Form

ATLANTIC VETERINARY COLLEGE
University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island C1A 4P3

CLINICAL PHARMACOLOGY
902-566-0804

OWNER SURNAME | FIRST NAME

NAME OF CLINIC | CLINIC BILLING NO.

PATIENT'S NAME

SPECIES | BREED

DATE OF BIRTH
DAY MO. YR.

COLLECTION DATE	CLINICIAN
DATE RECD	LAB NO.

PHENOBARBITAL STUDY #553

Check one: ☐ Phase III (post-biopsy recheck)
☐ Other follow-up

Patient Information:

Current weight of dog:_____(lb / kg)

What is the current dose of phenobarbital, and how long has the dog been on this dose?_____

_____

_____

Is the dog any other drugs at this time (Kbr, etc)? If so, what is the dosage, and how long has dog been on the drug(s)?_____

_____

_____

_____

Any problems clinically? Is the dog healthy and normal on physical exam?

_____

_____

_____

Describe seizure activity since the change in drugs: _____

_____

Analysis requested: Time of sample collection_____(AM/PM)
Time of last pill_____(AM/PM); Time of last meal_____(AM/PM)

☐ ___cc of serum; 8-12 hour fasted sample, non-hemolyzed. (Do not use serum separator tubes for phenobarbital concentrations).

- ☐ Total body panel 901
- ☐ Liver panel 904
- ☐ Pre-prandial bile acids
- ☐ Phenobarbital: 4- 6 hr post-pill
- ☐ Phenobarbital: 8-12 hr post-pill
- ☐ T_4
- ☐ TSH
- ☐ Serum sample for Dr. Gaskill (Please notify her of arrival)
- ☐ Other:_____

☐ Purple-topped tube of blood and two air-dried, unstained blood smears.

- ☐ CBC
- ☐ Other:_____

☐ ___cc of serum, 2 hours post-prandial. Non-hemolyzed.

- ☐ Post-prandial bile acids
- ☐ Other:_____

☐ Blue-topped tube of blood (in-house cases only)

- ☐ Coagulation profile

Phenobarbital Study Request Form

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CLINICAL PHARMACOLOGY
902-566-0804

OWNER SURNAME | FIRST NAME

NAME OF CLINIC | CLINIC BILLING NO.

PATIENT NAME

PHENOBARBITAL STUDY #553

SPECIES | BREED

Check one: ☐ Post-biopsy (___ mo)
☐ Other follow-up

AGE | SEX

Current weight:_____ (kg / lb)

COLLECTION DATE | CLINICIAN

DATE REC'D | LAB NO.

Patient Information:

Current phenobarbital dose, and how long on this dose:_____
What was the previous phenobarbital dose?_____
Is the dog on potassium bromide? If yes, what dose and for how long?_____
What was the previous dose of potassium bromide?_____
Please describe seizure activity in last few months_____

Is there any evidence of liver or thyroid disorder on physical exam or history? If yes, please explain:_____

Does the patient have any apparent health problems other than the seizures, or has the owner complained of the dog having any other problems? If yes, please explain:_____

Analysis requested: Time of sample collection_____(AM/PM)
Time of last pill_____(AM/PM); Time of last meal_____(AM/PM)

☐ ___cc of serum; 8-12 hour fasted sample, non-hemolyzed. (Do not use serum separator tubes for phenobarbital concentrations).

- ☐ Total body panel 901
- ☐ Liver panel 904
- ☐ Pre-prandial bile acids
- ☐ Phenobarbital: 4- 6 hr post-pill
- ☐ Phenobarbital: 8-12 hr post-pill
- ☐ T_4
- ☐ TSH
- ☐ Serum sample for Dr. Gaskill (Please notify her of arrival)
- ☐ Potassium bromide

☐ Purple-topped tube of blood and two air-dried, unstained blood smears.

- ☐ CBC
- ☐ Other:_____

☐ ___cc of serum, 2 hours post-prandial. Non-hemolyzed.

- ☐ Post-prandial bile acids
- ☐ Other:_____

☐ Blue-topped tube of blood (in-house cases only)

- ☐ Coagulation profile
- ☐ Other:_____

Phenobarbital Study Request Form

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PHENOBARBITAL STUDY #553

Check one: ☐ Phase III
☐ Other follow-up

Current weight:_____ (kg / lb)

OWNER SURNAME FIRST NAME

NAME OF CLINIC CLINIC BILLING NO.

PATIENT NAME

SPECIES BREED

AGE SEX

COLLECTION DATE CLINICIAN

DATE REC'D LAB NO.

Patient Information:

Current phenobarbital dosage, and how long on this dose:_____

If recent change in dose, what was the previous phenobarbital dose?_____
Please describe seizure activity in last few months_____

Is there any evidence of liver or thyroid disorder on physical exam or history? If yes, please explain:_____

Does the patient have any apparent health problems other than the seizures, or has the owner complained of the dog having any other problems? If yes, please explain:_____

Analysis requested: Time of sample collection_____(AM/PM)
Time of last pill_____(AM/PM); Time of last meal_____(AM/PM)

☐ ___cc of serum; 8-12 hour fasted sample, non-hemolyzed. (Do not use serum separator tubes for phenobarbital concentrations).
- ☐ Total body panel 901
- ☐ Liver panel 904
- ☐ Pre-prandial bile acids
- ☐ Phenobarbital: 4- 6 hr post-pill
- ☐ Phenobarbital: 8-12 hr post-pill
- ☐ T_4
- ☐ TSH
- ☐ Serum sample for Dr. Gaskill (Please notify her of arrival)
- ☐ Other:_____

☐ Purple-topped tube of blood and two air-dried, unstained blood smears.
- ☐ CBC
- ☐ Other:_____

☐ ___cc of serum, 2 hours post-prandial. Non-hemolyzed.
- ☐ Post-prandial bile acids
- ☐ Other:_____

☐ Blue-topped tube of blood (in-house cases only)
- ☐ Coagulation profile
- ☐ Other:_____

Phenobarbital Study Histopathology Form

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Prince Edward Island C1A 4P3

CLINICAL PHARMACOLOGY
902-566-0804

OWNER _____

ADDRESS _____

TELEPHONE NO. _____ CLINIC NO. _____

PATIENT _____ BILLING NO. 553

SPECIES CANINE BREED _____

DATE OF BIRTH _____ SEX _____

CLINICIAN

LAB NO. | DATE RECEIVED

NOTE: Please send a copy of the histopathology results to Dr. Cynthia Gaskill, Dept. of Anatomy and Physiology, AVC.

SPECIMEN(S) SUBMITTED:

☐ Biopsy specimen (Check one: ☐ Surgical; ☐ Blind; ☐ Ultrasound-guided)

☐ Necropsy specimen

TISSUE SUBMITTED:

☐ Liver tissue ☐ Thyroid tissue ☐ Other: _____

TEST(S) REQUIRED:

☐ Histopathology ☐ Other: _____

CLINICAL HISTORY: (duration of illness, clinical signs, drug therapies, etc.)

_____

_____

_____

_____

_____

PERTINENT CLIN-PATH DATA:

_____

_____

_____

ULTRASONOGRAPHIC FINDINGS, OR FINDINGS ON GROSS EXAMINATION:

_____

_____

_____

_____

_____

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Clinical Pharmacology
Tel. 902-566-0804
Fax 902-566-0832

INSTRUCTIONS FOR TSH STIMULATION TEST

1. Fast dog for a minimum of 12 hours prior to test.

2. Weigh dog in kilograms.

3. Obtain a blood sample for baseline T4 determination. We need at least 1 cc of serum. The patient may be offered water, but no food, until the completion of the test. Lipemia or hemolysis of the sample may interfere with the T4 assay and cause falsely low results.

4. Administer TSH intravenously at a dose of 0.1 units per kg (to a maximum of 5 units). The Thytropar is provided as 10 units of desiccated TSH , plus 2 ml of diluent. Hence, the reconstituted TSH contains 5 units of TSH per ml. (Example: a 25 kg dog would receive 0.5 ml of the reconstituted TSH IV).

5. Obtain a post-TSH blood sample 6 hours after IV injection of the TSH.

6. Be sure to label tubes (ie, pre-TSH and 6 hours post-TSH), and fill out the provided submission form. Refrigerate samples until time of shipment (or freeze if shipment will be delayed for more than 24 hours).

7. The unused portion of TSH can be divided into 2.5 or 5 unit aliquots and refrigerated for up to 3 weeks, or frozen for up to at least 3 months (some references suggest 6 months). HOWEVER: since this product is outdated, it may not maintain efficacy in the same manor as a non-outdated product. You may wish to test a normal healthy dog as a control at the same time you test a patient when using the frozen or refrigerated reconstituted product.

8. Owners should be informed that the product is outdated, and of the small chance of adverse effects of a TSH stimulation test (ie, hypersensitivity reaction, etc. as with any injection of foreign protein). Informed consent should be obtained for your records. Outdating of the product should not cause any increase in incidence of adverse effects.

Phenobarbital Study Request Form

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University of Prince Edward Island
550 University Avenue, Charlottetown
Prince Edward Island C1A 4P3
CLINICAL PHARMACOLOGY
902-566-0804

OWNER SURNAME | FIRST NAME

NAME OF CLINIC | CLINIC BILLING NO.

PATIENT NAME

PHENOBARBITAL STUDY #553

SPECIES | BREED

Check one: ☐ TSH stim test
☐ Other

AGE | SEX

Current weight:_____ (kg / lb)

COLLECTION DATE | CLINICIAN

DATE REC'D | LAB NO.

Patient Information:

Current phenobarbital dosage, and how long on phenobarbital:_____

Please describe seizure activity in last few months_____

Is there any evidence of a thyroid disorder on physical exam or history (ie, overweight, skin problems, chronic infections, behavioral changes etc)? If yes, please explain:_____

Did the patient have any of these signs prior to the initiation of phenobarbital therapy?_____

Does the patient have any apparent health problems other than the seizures, or has the owner complained of the dog having any other problems? If yes, please explain:_____

Is the patient receiving any drugs other than phenobarbital?_____

Analysis requested: Time of last pill_____(AM/PM); Time of last meal_____(AM/PM)

☐ ___ Baseline, pre-TSH serum sample. Time of blood collection:_____
Time of TSH injection:_____

- ☐ T_4
- ☐ Other:_____

☐ ___ 6 hours post-TSH serum sample. Time of blood collection:_____

- ☐ T_4
- ☐ Other :_____

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