

# Liver glutathione concentrations in dogs and cats with naturally occurring liver disease

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**Objective**—To determine total glutathione (GSH) and glutathione disulfide (GSSG) concentrations in liver tissues from dogs and cats with spontaneous liver disease.

**Sample Population**—Liver biopsy specimens from 63 dogs and 20 cats with liver disease and 12 healthy dogs and 15 healthy cats.

**Procedure**—GSH was measured by use of an enzymatic method; GSSG was measured after 2-vinylpyridine extraction of reduced GSH. Concentrations were expressed by use of wet liver weight and concentration of tissue protein and DNA.

**Results**—Disorders included necroinflammatory liver diseases (24 dogs, 10 cats), extrahepatic bile duct obstruction (8 dogs, 3 cats), vacuolar hepatopathy (16 dogs), hepatic lipidosis (4 cats), portosystemic vascular anomalies (15 dogs), and hepatic lymphosarcoma (3 cats). Significantly higher liver GSH and protein concentrations and a lower tissue DNA concentration and ratio of reduced GSH-to-GSSG were found in healthy cats, compared with healthy dogs. Of 63 dogs and 20 cats with liver disease, 22 and 14 had low liver concentrations of GSH ( $\mu\text{mol}$ ) per gram of tissue; 10 and 10 had low liver concentrations of GSH (nmol) per milligram of tissue protein; and 26 and 18 had low liver concentrations of GSH (nmol) per microgram of tissue DNA, respectively. Low liver tissue concentrations of GSH were found in cats with necroinflammatory liver disease and hepatic lipidosis. Low liver concentrations of GSH per microgram of tissue DNA were found in dogs with necroinflammatory liver disease and cats with necroinflammatory liver disease, extrahepatic bile duct occlusion, and hepatic lipidosis.

**Conclusions and Clinical Relevance**—Low GSH values are common in necroinflammatory liver disorders, extrahepatic bile duct occlusion, and feline hepatic lipidosis. Cats may have higher risk than dogs for low liver GSH concentrations. (*Am J Vet Res* 2002;63:1187–1197)

**G**lutathione (GSH, L- $\gamma$ glutamyl-L-cysteinylglycine), a tripeptide thiol synthesized in all mammalian cells, is essential for normal health owing to its role in detoxification reactions, maintenance of reduced thiol status in certain proteins and other molecules, and as a storage mechanism for tissue and plas-

ma cysteine.<sup>1</sup> The important role of GSH in maintenance of thiol-disulfide metabolism and detoxification of products of aerobic metabolism (eg, superoxide, hydrogen peroxide, and toxic oxygen radicals) is to protect tissues from cell and organelle membrane damage. Glutathione detoxifies reactive molecules either by spontaneous conjugation or by a reaction catalyzed by GSH-S-transferase. Intracellular antioxidant activity of GSH involves oxidation to GSH disulfide (GSSG) in a reaction catalyzed by GSH peroxidase. Reduction of GSSG back to GSH, under the influence of GSSG reductase and NADPH, is rapidly accomplished in a closed system redox cycle; otherwise, GSSG is exported from the cell. In liver tissue from healthy animals, most GSH is maintained in its reduced form. In addition to antioxidant protection, GSH participation in intracellular thiol-disulfide equilibrium may regulate certain metabolic pathways by activating or inactivating key enzymes.<sup>2</sup> The molecular configuration of GSH renders it resistant to intracellular degradation, restricting its catabolism to cell membranes imbued with  $\gamma$ -glutamyltranspeptidase.<sup>2</sup> Systemic transport of GSH occurs in plasma and RBC as the intact molecule or substrate constituents. Plasma GSH undergoes dynamic fluctuation as a result of variations in hepatic GSH synthesis and efflux, systemic tissue GSH utilization, oxidant challenge, as well as nutritional and metabolic variables. Erythrocyte GSH content declines with increasing cell age but overall is maintained within a constant range, unless a systemic oxidant challenge is encountered.<sup>2,3</sup> Nevertheless, circulating GSH values do not reliably reflect tissue GSH concentrations. Although the liver, kidney, and lungs are most important in GSH turnover, most of the systemically distributed GSH or its substrate components is derived from the liver.<sup>1,4</sup>

The liver of healthy individuals maintains an elaborate antioxidant system of which GSH is an important component. Hepatocytes are unique, compared with many other cells, because they synthesize a large supply of GSH that can be either used locally or effluxes into the systemic circulation and bile.<sup>1,4</sup> Liver GSH concentrations have special importance considering the pivotal role of the liver in intermediary metabolism and in drug and toxin biotransformation and detoxication. The liver has a sentinel position between the systemic and portal circulatory beds and acts as a donor of systemic GSH. Furthermore, because lipid peroxidation is an important mechanism of tissue injury in most forms of necroinflammatory and cholestatic liver disorders, hepatic GSH adequacy may reflect oxidant injury or organ susceptibility to oxidant damage.<sup>5-7</sup> Liver GSH concentrations decline in a variety of naturally occurring liver diseases in humans and in animals

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with experimentally induced hepatic injury.<sup>8-15a</sup> Liver tissue GSH deficits derive from multiple and complex mechanisms including the following: 1) reduced dietary intake or cell utilization of GSH precursors (eg, methionine or cysteine precursors); 2) inadequate availability of NADPH necessary for redox-recycling of GSSG to GSH; 3) an acquired defect in the trans-sulphuration pathway impairing the transformation of methionine to s-adenosylmethionine (implicated as a major cause of diminished tissue GSH in severe liver disease); 4) reduced transcription of the rate controlling enzyme in GSH formation; and 5) increased efflux of hepatic GSH into plasma or bile, or 6) increased liver tissue oxidant challenge resulting in GSH utilization.<sup>1,2,5,8,14,16,17</sup>

Inadequate hepatocellular GSH concentrations are thought to make the liver more susceptible to oxidant damage associated with necroinflammatory and cholestatic liver disease, including adverse effects on cell and organelle membranes, increased susceptibility to toxic adducts, and increased vulnerability of essential enzymes to inactivation, which impairs synthetic, metabolic, and catabolic activities.<sup>17</sup> Consequently, therapeutic intervention aimed at rectifying acquired hepatic GSH deficiency has been proposed by use of a variety of donor substrates in humans with severe liver disease.<sup>1,5,8,14</sup> The purpose of the study reported here was to investigate liver concentrations of GSH in dogs and cats with naturally occurring liver disease to clarify whether therapeutic augmentation of GSH may be purposeful.

## Methods and Materials

**Animals**—Liver tissue specimens from dogs (n = 63) and cats (20) with naturally occurring liver disease were acquired during routine diagnostic biopsy specimen collection from June 1999 to December 2000. Liver tissue from healthy animals (12 dogs, 15 cats) was acquired at the time of baseline liver biopsy specimen collection before animal inclusion in nonrelated studies. The health of these clinically normal animals was determined on the basis of physical, routine hematologic, serum biochemical, and urine assessments, and liver function evaluation by use of baseline and postprandial serum bile acids concentrations and plasma clearance of indocyanine green. Healthy animals were provided with maintenance dry food and water ad libitum; food and water were withheld overnight before sample collections.

The patient population and disease entities included in this study were characterized (**Appendix**). A diagnosis of cirrhosis was made on the basis of histologically confirmed nodular regeneration, bridging fibrosis, and permanently altered hepatic architecture in addition to clinicopathologic evidence of impaired hepatic function (eg, serum bile acid values, hyperammonemia, coagulation abnormalities, and hypoalbuminemia). Chronic hepatitis was histologically characterized by nonsuppurative inflammation effacing the limiting plate, along with clinicopathologic and physical evidence of illness persisting for at least 8 weeks. **Extrahepatic bile duct obstruction (EHBDO)** was confirmed on the basis of clinicopathologic assessments, abdominal ultrasonography, and gross inspection and palpation of the extrahepatic biliary structures during exploratory laparotomy. Four dogs had cholecystitis and choledochitis associated with obstruction, 2 dogs and 1 cat had choleliths causing obstruction, 2 dogs and 1 cat had obstruction associated with neoplasia, 3 dogs had biliary mucoceles (inspissated bile) causing obstruction, 2 dogs had strictures of the common bile duct

causing obstruction, and 1 cat had cholecystitis and choledochitis and inflammatory bowel disease associated with obstruction. Cholangitis and cholangiohepatitis were characterized by inflammation involving the intrahepatic, and in 2 animals, extrahepatic biliary structures and clinicopathologic features were indicative of inflammatory cholestatic liver disease (high liver enzyme activity, hyperbilirubinemia, high serum bile acid concentrations). A diagnosis of hepatic lipodosis was made on the basis of typical clinicopathologic features (poikilocytosis, high liver enzyme activity, hyperbilirubinemia) in an anorectic cat, including hyperechoic hepatic parenchyma, nonpainful hepatomegaly, and cytologic and histopathologic confirmation of diffuse hepatocellular vacuolation with triglyceride. A diagnosis of canine vacuolar hepatopathy was made on the basis of hepatic cytologic and histologic evaluation detailing panlobular hepatocellular expansion with glycogen (periodic acid Schiff positive and diastase digested vacuole content). Hepatic lymphosarcoma was confirmed on the basis of a monotonous diffuse or multifocal distribution of neoplastic lymphocytes in liver tissue, in the absence of an inflammatory reaction. **Portosystemic vascular anomalies (PSVA)** were confirmed on the basis of abdominal ultrasonography, colorectal scintigraphy, and portovenography. A diagnosis of diffuse hepatic necrosis was made in 1 dog with panlobular hepatic necrosis derived from an adverse drug reaction (trimethoprim-sulfadiazine). The other patients with hepatic necrosis did not have known toxin or drug exposure as a causal factor. The term mild hepatitis characterized low-grade nonsuppurative inflammation confined to the area of the portal triad, but was not associated with vascular or biliary structures and did not involve the zone 1 hepatocytes; animals with this diagnosis had inflammatory bowel disease.

**Specimen collection**—All biopsy specimens were collected after the withholding of food and water for 12 hours, with the animal under general anesthesia during exploratory laparotomy (n = 33; all were wedge biopsy specimens) or laparoscopic diagnostic procedures (77; biopsy specimens were acquired with laparoscopic clam shell cutting forceps and weighed at least 100 mg). Tissue specimens for histologic assessments were immediately fixed in neutral-buffered 10% formalin. After fixation, tissues were set in paraffin, sectioned at 7  $\mu$ m thickness, and stained with H&E, Masson's trichrome, Prussian blue, and Gomori's reticulin to characterize morphologic changes. Tissue for determination of GSH concentrations were immediately frozen and stored at -80 C until thawed at the time of analyses. Patient samples were assayed within 4 months of collection, whereas control samples (reference range) were assayed within 8 months of collection.

**Analyses**—All tissue processing for GSH measurements was conducted in a melting ice bath. Concentrations of total GSH and GSSG were determined by use of a modification of the kinetic method described by Griffith.<sup>18</sup> Briefly, tissue was weighed and immediately homogenized in a 3-ml-volume hand-held Dual glass homogenizer during a 1 minute interval in ice cold 125 mM phosphate buffer (canine liver tissue suspended at 1:10 [wt/vol] and most feline liver tissue suspended at 1:20 [wt/vol]) containing 6.3 mM NaEDTA, pH adjusted to 7.5 with 1 N NaOH. A sample of homogenate was immediately removed and frozen at -80 C for later determination of protein and DNA concentrations to be used as units of expression for GSH equivalent values. The remainder of the homogenate was deproteinized (within 1 minute) in ice cold 5% (wt/vol) trichloroacetic acid (TCA) at 1:6 (vol/vol) of homogenate. The TCA and the supernatant were separated by centrifugation in a prechilled (-20 C) centrifuge. Prior to evaluations of patient samples, the influence of tissue handling on GSH values and validation of the analytic proce-

dures were completed. To determine the best circumstances for tissue homogenization and deproteinization, GSH concentrations were measured in tissue homogenized in buffer and then deproteinized in 5% TCA ( $n = 38$ ) and in tissue homogenized and deproteinized simultaneously in 5% TCA (15). The intra-assay ( $n = 18$ ) and interassay (38) repeatability of GSH and GSSG measurements as well as recovery of GSH added to tissue specimens before homogenization (22) were determined. Intra-assay, interassay, and recovery studies were conducted by use of tissue derived from a single cat with diffuse necroinflammatory liver disease. Liver tissue from a cat with necroinflammatory liver disease was selected for study of assay variation and GSH recovery to determine variability in tissue prone to oxidation versus study of tissue from a healthy animal. Intra-assay repeatability was determined by use of a freshly prepared pooled homogenate made from 3 sections of liver taken from different liver lobes. The accuracy of the homogenization and assay method ultimately used for tissue GSH determinations was verified by determining GSH and GSSG concentrations in tissue from healthy adult mice (3 mice, 15 specimens) and by comparing results to values reported by others using similar and alternative assay methods. The influence of the method of freezing (snap freezing vs freezing by placing freshly acquired tissue in a  $-80$  C freezer) on tissue GSH values was determined by use of fresh mouse liver. The influence of long term storage at  $-80$  C for 18 months was determined by use of liver tissue acquired from an ill dog and cat at the time of their death; separately stored tissue specimens were thawed and analyzed at 6-month storage intervals. The extent of spontaneous generation of GSSG in standard solutions was evaluated by use of standards (98% reduced GSH [RGSH], 2% GSSG) made initially in buffer and then diluted 1:6 in TCA (emulating final sample preparation); spontaneous GSSG formation was studied on 9 separate days in solutions stored on melting ice for 0.5 to 6 hours during periods of patient tissue assays.

The final assay procedure used a 200- $\mu$ l sample of deproteinized supernatant of a tissue homogenate prepared in buffer and TCA. Reactions were initiated as soon as the deproteinized supernatant was separated; supernatant was combined with 700  $\mu$ l of 0.3 mM NADPH and 100  $\mu$ l of 6 mM 5,5'-dithiobis-(2-nitrobenzoic acid) in a light restricted enclosure, resulting in a solution with a final pH ranging between 6.4 and 6.8. The reaction was immediately started by the addition of GSH reductase<sup>b</sup> (10  $\mu$ l, 50 units/ml) and a continuous kinetic recording<sup>c</sup> made in a light restricted enclosure at 412 nm at 25 C until the optical density exceeded 2.0 for GSH and for 45 minutes for GSSG. A kinetic recording of the rate of color change was made. Color change in this assay linearly equates with the quantity of GSH in solution as determined from the recycling reaction, which measures all GSH moieties (RGSH and GSSG, which reacts with NADPH yielding RGSH). Freshly prepared GSH standard solutions ranging from 0.0006 to 0.02 nmol/ $\mu$ l were evaluated in triplicate within each assay. The lower limit of detection with this method approximates 0.00012 nmol/ $\mu$ l; this range was linear ( $R^2 = 0.99$ ) and encompassed the range of values experienced with tissue homogenates that spanned 0.005 to 0.01 nmol/ $\mu$ l. Standards of GSH and GSSG were prepared with buffer and TCA dilutions analogous to samples. Determination of GSH after derivatization of RGSH with 2-vinylpyridine<sup>c</sup> in the presence of triethanolamine<sup>d</sup> (pH of reaction solution maintained between 6.5 and 7.0; incubation in 25 C water bath for 60 minutes) was done to measure GSSG. The ambient temperature in the laboratory during sample preparation and assay conductance was 20 C, but all solutions were handled on melting ice and a temperature controller maintained assay solutions at 25 C during the spectrophotometric detection of the reaction color change.

Homogenate DNA and protein concentrations were measured on each tissue specimen in consideration that low liver GSH values have been associated with increased cell fluid accumulation and ostensibly with changes in tissue weight and cell density per tissue specimen.<sup>19</sup> Furthermore, because vacuolar distention of hepatocytes with glycogen is common in ill dogs and because vacuolar distention of hepatocytes with triglyceride is common in ill cats, we anticipated that these effects might influence measurements of GSH when normalized on the basis of wet tissue weight. We reasoned that measuring tissue protein and DNA concentrations might normalize tissue GSH concentrations to the number of cells sampled. However, because necroinflammatory disorders, extrahepatic bile duct occlusion, and neoplastic disorders are complicated by an influx of cells and because liver disease in general can be associated with changes in the extracellular matrix, these changes may collectively distort tissue GSH, DNA, and protein concentrations in various ways. Thus, wet tissue weight also was used as a basis for GSH expression. Tissue protein concentration was determined by use of the Bradford method on the basis of protein binding to Coomassie Brilliant Blue.<sup>20</sup> Standards were prepared by use of known quantities of bovine serum albumin to correct for binding characteristics of the dye in the buffer used for tissue suspension. Tissue DNA concentrations were determined by use of a modification of the fluorescent method described by Downs and Wilfinger<sup>21</sup> on the basis of the reaction between bisbenzimidazole reagent (Hoechst dye<sup>e</sup>) and DNA. A fluorometer<sup>f</sup> was used with excitation and emission filters appropriately selected. A DNA standard was prepared from highly polymerized calf thymus DNA.<sup>8</sup>

**Statistical analysis**—Data were evaluated for Gaussian distribution by use of histograms and descriptive statistics. Data from assay repeatability and recovery studies were normally distributed and are reported as mean, SD, and coefficient of variation percentage (CV%). The Pearson correlation was used to evaluate the relationship between spontaneous GSSG formation in standard solutions of various concentrations. Because clinical data were not normally distributed, nonparametric comparisons were used; thus, descriptive statistics included the median value and range. Concentrations of tissue homogenate GSH (expressed as  $\mu$ mol/g of wet tissue, nmol/mg of tissue protein, and nmol/ $\mu$ g of tissue DNA) and the GSH-to-GSSG ratio in patient samples were compared with values from healthy dogs and cats by use of the Mann-Whitney 2 sample rank sum test. Similar evaluation was done to test for significant differences in patient age among disease categories and significant differences between values from healthy dogs and cats. Association between age and GSH concentration was examined by use of Spearman rank correlation for all dogs with liver disease. For all evaluations made,  $\alpha < 0.05$  was used to determine significant differences or associations. Patient GSH values also were categorized as within reference range or abnormally low on the basis of the lower 97.5% confidence interval (CI) of values from healthy animals. A value of  $P < 0.05$  was considered significant.

## Results

Recovery of GSH was best when tissues were homogenized in buffer followed by homogenate deproteinization (Table 1). Liver concentrations of GSH and GSSG (nmol) per microgram of tissue DNA and per milligram of tissue protein were similar to liver concentrations of GSH and GSSG ( $\mu$ mol) per gram of tissue. Calculation of the recovery percentage of GSH added to tissue proved that the assay method used

Table 1—Mean, SD, and percent coefficient of variation (CV%) for glutathione (GSH) and glutathione disulfide (GSSG) determinations completed on liver tissue from a cat with necroinflammatory liver disease where tissues were homogenized in system buffer and then deproteinized or were directly homogenized in 5% trichloroacetic acid (TCA)

Measurements	SBTH intra-assay (n = 18)			SBTH interassay (n = 38)			5% TCA TH intra-assay (n = 15)		
	Mean	SD	CV%	Mean	SD	CV%	Mean	SD	CV%
GSH (μmol/g liver)	2.26	0.96	5.3	2.18	0.29	13.2	1.41	0.27	18.3
GSSG (μmol/g liver)	0.08	0.01	11.6	0.1	0.04	37.6	0.05	0.02	65.8
Protein (mg/ g liver)	48.3	6.04	12.5	53.10	13.70	23.3	—	—	—
DNA (μg/g liver)	4574	390	8.5	5036	947	17.7	—	—	—
GSH (nmol/mg protein)	46.80	5.58	11.9	40.0	0.09	21.9	—	—	—
GSSG (nmol/mg protein)	1.70	0.30	19.2	1.70	0.56	30.2	—	—	—
GSH (nmol/μg DNA)	0.49	0.05	9.8	0.42	0.09	21.9	—	—	—
GSSG (nmol/μg DNA)	0.02	0.001	11.9	0.02	0.01	27.5	—	—	—
GSH:GSSG	22.1	11.6	46.2	25.6	9.4	41.7	32.1	14.3	44.8

SBTH intra-assay = System buffer tissue homogenization intra-assay determinations. SBTH interassay = System buffer tissue homogenization interassay determination. 5% TCA TH intra-assay = 5% TCA tissue homogenization intra-assay determination.

Table 2—Recovery of total GSH and GSSG (standards containing 98% reduced GSH and 2% GSSG) directly added to liver tissue from a cat with necroinflammatory liver disease where tissues were homogenized in system buffer and then deproteinized or were directly homogenized and deproteinized in 5% TCA

Measurements	Homogenate method*		
	System buffer (n = 12)	System buffer (n = 10)	5% TCA (n = 10)
Mean ± SD baseline GSH (μmol/g liver)	2.11 +/-0.53	1.93 +/-0.58	1.36 +/-0.33
Mean ± SD baseline GSSG (μmol/g liver)	0.15 +/-0.6	0.13 +/-0.04	0.06 +/-0.03
GSH added (μmol/g liver)	1.3	3.9	1.3
Mean ± SD measured GSH (μmol/g liver)	3.51 +/-0.83	5.09 +/-0.77	2.19 +/-0.38
Mean ± SD measured GSSG (μmol/g liver)	0.11 +/-0.05	0.13 +/-0.03	0.08 +/-0.04
GSH recovered (%)	100.0 +/-13.9	87.1 +/-8.9	82.3 +/-10.2

\*Studies completed with separate tissue homogenates.

herein achieved or exceeded recovery performance of similar or alternative GSH assay methods.<sup>22,23</sup> Repeatability of tissue GSH measurements was comparable to performance described by others using similar or alternative techniques for GSH determination.<sup>23-26</sup> Because different tissue specimens were used for each of the interassay determinations, CV% reflect not only assay variation but also differences between liver specimens from a single individual with necroinflammatory liver disease (Table 2).

Determination of GSH and GSSG concentrations in liver specimens of mice from which food had been withheld yielded a mean (± SD) GSH concentration of 5.65 ± 0.7 μmol/g of wet liver tissue (range, 4.73 to 6.77 μmol/g of wet liver tissue), a mean GSSG concentration of 0.11 ± 0.06 μmol/g of wet liver tissue (range, 0 to 0.20 μmol/g of wet liver tissue), and a mean GSH-to-GSSG ratio of 93.7 ± 33 (range, 54 to 153). Values for mouse liver were within the range most commonly reported by others.<sup>27-35</sup> Mouse liver GSH and GSSG values broadly overlapped in tissue that was freshly homogenized, stored for 3 hours at -80 C, homogenized and stored for 3 hours at -80 C, or snap frozen and stored in liquid nitrogen for 3 hours (data not shown). However, on inspection, the freeze-thawed homogenate yielded the highest GSH values and a greater proportion of GSSG. Higher GSH values likely reflect greater release of cell and organelle GSH (possibly mitochondria) caused by the freeze-thaw cell membrane rupture, whereas the

higher proportion of GSSG likely reflects greater spontaneous GSH oxidation during the longer interval of tissue manipulation before deproteinization.

Liver specimens stored frozen at -80 C for 18 months from an ill dog and cat assayed at 6-month intervals yielded mean GSH concentrations of 1.13 ± 0.19 μmol/g of liver tissue for the dog and 0.85 ± 0.25 μmol/g of liver tissue for the cat, with a CV% of 16.8 and 29.7%, respectively. On inspection, there was no relation between concentration change and storage interval. The mean GSSG concentration and GSH-to-GSSG ratio were 0.14 ± 0.04 μmol/g of liver tissue and 8.7 ± 3.6 for the longitudinal study with canine tissue, respectively, and were 0.10 ± 0.06 μmol/g of liver tissue and 10.0 ± 2.8 for the longitudinal study with feline tissue, respectively. These results suggest that storage of tissue at -80 C for up to 18 months maintained tissue GSH and GSSG concentrations within the variation of interassay repeatability.

A small amount of GSSG spontaneously formed in standards prepared in buffer and then diluted 1:6 with 5% TCA to emulate treatment of tissue specimens. A significant negative correlation was found between the percentage of the GSH-to-GSSG ratio and GSH concentration in the standard solutions (Pearson correlation, -0.69; n = 24; P < 0.001), indicating a fixed amount of spontaneous GSSG formation. Spontaneous GSSG formation in standards stored on ice (over intervals ranging from 30 minutes to 6 hours on 9 separate days) con-

Table 3—Median values and (range) of GSH, total protein, and DNA concentrations, and GSH-to-GSSG ratio in liver tissue from healthy dogs and cats and patients with naturally occurring liver disease

Disorder and species	No.	Measurements					
		GSH (μmol/g liver)	GSH (nmol/mg protein)	GSH (nmol/μg DNA)	GSH:GSSG	Total protein (mg/g liver)	DNA (μg/g liver)
Dogs	75						
Necroinflammatory disorders	24	1.97 (0.36–7.79)	14.9 (3.0–43.6)	0.38* (0.01–1.47)	29.8* (1.2–132.0)	120 (73–252)	5,100* (1,190–38,000)
EHBDO	8	1.63 (0.22–7.43)	14.4 (2.6–41.40)	0.49 (0.10–1.64)	29.8* (1.0–133.1)	156 (82–180)	3,820 (1,600–4,660)
Vacular hepatopathy	16	1.96 (0.81–4.65)	12.6 (8.4–25.1)	0.59 (0.28–1.46)	22.9* (9.7–89.3)	136 (70–203)	3,040 (1,210–7,130)
PSVA	15	2.16 (0.32–6.37)	13.1 (1.9–37.7)	0.70 (0.01–2.11)	34.4* (10.7–153.0)	168 (86–271)	3,700 (1,350–4,400)
Healthy dogs	12	1.97† (1.46–2.90)	13.6† (9.8–19.7)	0.70† (0.50–1.04)	127.8† (20.6–181.2)	154† (101–195)	3,330† (1,420–3,800)
Cats	35						
Necroinflammatory disorders	10	2.46* (0.80–5.94)	18.2 (4.9–44.4)	0.58* (0.04–2.20)	39.8 (1.6–110.4)	137* (70–357)	4,630* (1,040–48,100)
EHBDO	3	2.91 (2.63–3.52)	23.9 (22.1–26.3)	0.76* (0.37–1.28)	73.1 (47.7–79.9)	132* (110–134)	4,630* (2,050–7,870)
Hepatic lipidosis	4	1.39* (0.83–3.41)	10.3 (8.0–29.6)	0.39* (0.29–1.0)	28.8 (12.3–87.7)	109 (88–176)	3,120* (2,020–5,330)
Lymphosarcoma	3	3.74 (2.24–5.21)	21.7 (11.2–40.6)	0.75 (0.40–2.07)	56.7 (25.7–65.9)	235 (77–252)	12,500* (2,690–25,940)
Healthy cats	15	3.73† (1.99–5.45)	23.1† (13.3–37.5)	1.74† (1.00–3.07)	56.9† (17.5–155.0)	160† (152–174)	2,050† (1,730–2,380)

\*Significantly ( $P < 0.05$ ) different compared with healthy animals of the same species. †Significant ( $P < 0.05$ ) difference between healthy dogs and healthy cats.  
PSVA = Portosystemic vascular anomalies. EHBDO = Extrahepatic bile duct obstruction.

taining  $> 0.008$  nM/μl of GSH (similar to GSH concentrations in tissue homogenate dilutions used in our study) was  $< 3.2\%$  (mean,  $0.9 \pm 1.1\%$ ; range, 0 to 3.2%).

Concentrations of GSH and GSSG (μmol/g of wet liver tissue, nmol/mg of tissue protein, and nmol/μg of tissue DNA), tissue total protein, and DNA, and the GSH-to-GSSG ratio for healthy animals and for patients were determined (Tables 3 and 4). Significantly higher GSH, GSSG, and protein concentrations and significantly lower GSH-to-GSSG ratio and DNA concentrations were found in liver tissue from healthy cats, compared with healthy dogs. Dogs with necroinflammatory liver disease had significantly lower liver concentrations of GSH (nmol) per microgram of tissue DNA and higher liver concentrations of DNA (μg/g), compared with healthy animals. Dogs in each disease category had significantly higher GSSG concentrations (all units of expression) and a lower GSH-to-GSSG ratio than healthy dogs. Cats with necroinflammatory liver disease and hepatic lipidosis had significantly lower liver concentrations of GSH (μmol) per gram of tissue than healthy cats; cats with necroinflammatory disease, extrahepatic bile duct occlusion, and hepatic lipidosis had significantly lower liver concentrations of GSH (nmol) per microgram of tissue DNA. Cats with necroinflammatory liver disorders also had a significantly lower liver protein concentration than healthy cats. Overall, feline patients had significantly higher liver DNA concentrations than healthy cats.

On inspection, GSH values for various disorders encompassed broad ranges. The concentration of GSH for individual animals with liver disease, compared with the lower 97.5% CI from healthy dogs and cats, were

Table 4—Median values and (ranges) of GSSG concentrations in liver tissue from healthy dogs and cats and patients with naturally occurring liver disease

Disorder and species	No.	Measurements		
		GSSG (μmol/g liver)	GSSG (nmol/mg protein)	GSSG (nmol/μg DNA)
Dogs	75			
Necroinflammatory disorders	24	0.07* (0.01–0.54)	0.50* (0.10–3.32)	0.01* (0.001–0.15)
EHBDO	8	0.07* (0.02–0.38)	0.49* (0.17–3.32)	0.02* (0.001–0.15)
Vacular hepatopathy	16	0.0* (0.02–0.22)	0.67* (0.21–1.61)	0.02* (0.01–0.03)
PSVA	15	0.04* (0.02–0.32)	0.37* (0.07–1.35)	0.03* (0.001–0.197)
Healthy dogs	12	0.02† (0.01–0.07)	0.11† (0.07–0.68)	0.004† (0.004–0.007)
Cats	35			
Necroinflammatory disorders	10	0.04 (0.03–6.62)	0.36 (0.16–37.1)	0.03 (0.03–0.24)
EHBDO	3	0.04 (0.04–0.06)	0.33 (0.33–0.47)	0.01 (0.01–0.02)
Hepatic lipidosis	4	0.05 (0.02–0.13)	0.52 (0.13–1.10)	0.02 (0.003–0.04)
Lymphosarcoma	3	0.08 (0.07–0.09)	0.44 (0.38–0.62)	0.02 (0.01–0.03)
Healthy cats	15	0.07† (0–0.17)	0.45† (0.27–1.06)	0.04† (0–0.07)

\*Significantly ( $P < 0.05$ ) different compared with healthy animals of the same species. †Significant ( $P < 0.05$ ) difference between species.  
See Table 3 for remainder of key.

determined (Fig 1 and 2). Using the 97.5% CI as a cutoff value for differentiating low values, 10 of 24 dogs with necroinflammatory liver disorders, 4 of 8 dogs with

extrahepatic bile duct occlusion, 5 of 15 dogs with vacuolar hepatopathy, and 4 of 16 dogs with PSVA had low liver concentrations of GSH (nmol) per gram of tissue; 8 of 24 dogs with necroinflammatory disorders, 3 of 8 dogs with extrahepatic bile duct occlusion, 7 of 15 dogs with vacuolar hepatopathy, and 7 of 16 dogs with PSVA had low liver concentrations of GSH (nmol) per milligram of tissue protein; and 15 of 24 dogs with necroinflammatory disorders, 6 of 8 dogs with extrahepatic bile duct occlusion, 6 of 15 dogs with vacuolar hepatopathy, and 4 of 16 dogs with PSVA had low liver concentrations of GSH (nmol) per microgram of tissue DNA. Values for GSH were highly variable among dogs with liver disease, with some patients having values exceeding the 97.5% CI of healthy dogs. Specifically, of 63 dogs, 21 had high liver concentrations of GSH (mmol) per gram of tissue, 25 had high liver concentrations of GSH (nmol) per milligram of tissue protein, and 15 had high liver concentrations of GSH (nmol) per microgram of tissue DNA. Using the lower 97.5% CI of healthy cats, 8 of 10 cats with necroinflammatory disorders, 2 of 3 cats with extrahepatic bile duct occlusion, 3 of 4 cats with hepatic lipidosis, and 1 of 3 cats with hepatic lymphosarcoma had low liver concentrations of GSH (nmol) per gram of tissue; 6 of 10 cats with necroinflammatory disorders, 0 of 3 cats with extrahepatic bile duct occlusion, 3 of 4 cats with hepatic lipidosis, and 1 of 3 cats with hepatic lymphosarcoma had low liver concentrations of GSH (nmol) per milligram of tissue protein; and 9 of 10 cats with necroinflammatory disorders, 3 of 3 cats with extrahepatic bile duct occlusion, 4 of 4 cats with hepatic lipidosis, and 2 of 3 cats with hepatic lymphosarcoma had low liver concentrations of GSH (nmol) per microgram of tissue DNA. Values exceeding the reference range were found in 2 of 20 cats for liver concentrations of GSH (mmol) per gram of tissue, in 4 of 20 cats for liver concentrations of GSH (nmol) per milligram of tissue protein, and in 0 of 20 cats for liver concentrations of GSH (nmol) per microgram of tissue DNA.

An abnormally low GSH-to-GSSG ratio was common in canine patients. Using a GSH-to-GSSG ratio < 65.4 as a cutoff (lower 97.5% CI of healthy dogs), 49 of 63 (78%) ill dogs had low values as follows: 10 of 15 with PSVA, 12 of 15 with chronic active hepatitis or cirrhosis, 6 of 8 with EHBDO, 1 of 2 with cholangiohepatitis, 15 of 16 with vacuolar hepatopathy, 3 of 4 with hepatic necrosis, and 2 of 2 with mild hepatitis. An unusually low median GSH-to-GSSG ratio was found in healthy cats. Although this may represent autoxidation of samples during processing, these tissues were managed and processed identically to those from dogs and mice. In patients, 9 of 20 (45%) cats had a GSH-to-GSSG ratio < 31.0 (lower 97.5% CI of healthy cats) as follows: 3 of 8 with cholangitis and cholangiohepatitis, 3 of 4 with hepatic lipidosis, and 1 each with hepatic necrosis, hepatic lymphosarcoma, and mild hepatitis.

On inspection, dogs with PSVA had higher GSH values and a median age that was significantly younger than dogs in other disease categories (necroinflammatory disease, extrahepatic bile duct occlusion, vacuolar hepatopathy). However, age was not significantly associated with liver GSH concentration within the population of dogs with liver disease.

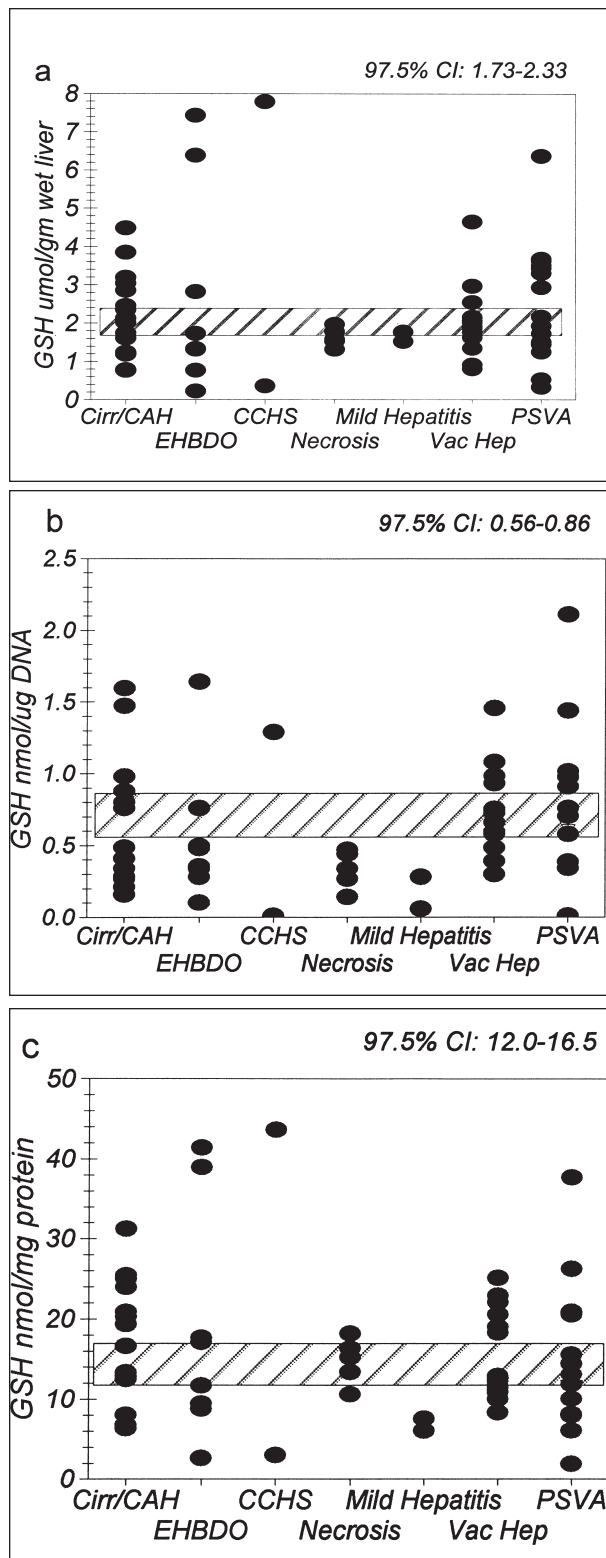


Figure 1—Total GSH concentration expressed on the basis of liver wet weight (a), tissue DNA (b), and tissue protein (c) in dogs with naturally occurring hepatobiliary disease. CI = Confidence interval (97.5%) of values determined on 12 healthy dogs also represented by the gray shaded area. Cirr/CAH = Cirrhosis and/or chronic active hepatitis. EHBDO = Extrahepatic bile duct occlusion. CCHS = Cholangitis/cholangiohepatitis. Vac Hep = Vacuolar hepatopathy. PSVA = Portosystemic vascular anomaly.

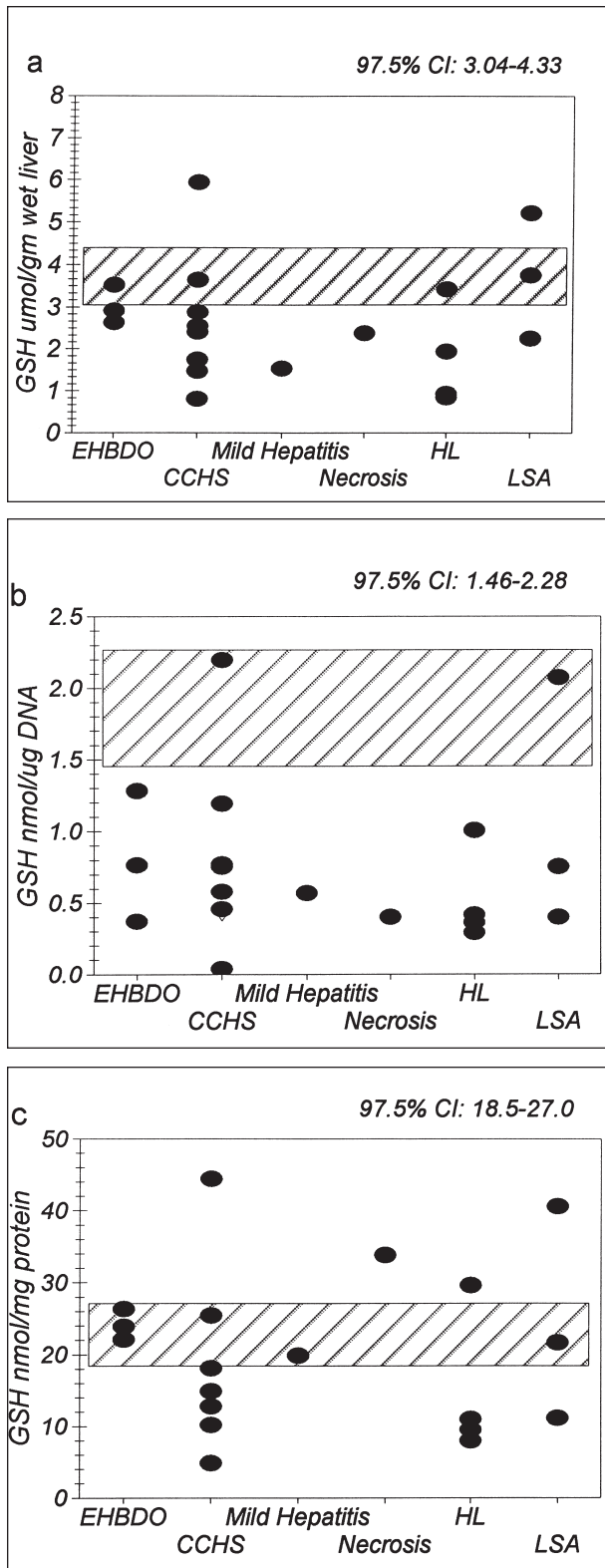


Figure 2—Total GSH concentration expressed on the basis of liver wet weight (a), tissue DNA (b), and tissue protein (c) in cats with naturally occurring hepatobiliary disease. CI = Confidence interval (97.5%) of values determined on 15 healthy cats also represented by the gray shaded area. EHBDO = Extrahepatic bile duct occlusion. CCHS = cholangitis/cholangiohepatitis. HL = Hepatic lipidosis. LSA = Hepatic lymphosarcoma.

## Discussion

Measurement of GSH in tissues from patients is problematic because of the need to handle tissues expediently to minimize interference caused by spontaneous degeneration and oxidation of GSH. The influence of freezing at  $-80^{\circ}\text{C}$  on tissue GSH concentrations for 18 months was minor, in agreement with previous findings for tissues frozen short term.<sup>36</sup> Because there are a multitude of analytic methods and investigator opinions regarding the merits of individual techniques, we determined the capabilities of the assay system used herein, which has previously been applied to tissue homogenates in several species.<sup>28-31</sup> Although this well-documented method is used for determination of GSH, it measures sulfhydryl groups and, therefore, is not specific for GSH. However, it is considered useful as a measure of GSH, as there is limited interference by other nonprotein sulfhydryls estimated to contribute  $< 5\%$  of the total quantity.<sup>28</sup> Because we hypothesized that expression of liver tissue GSH concentrations on the basis of wet tissue weight might introduce errors in data interpretation, tissue protein and DNA concentration also were measured as a basis for GSH expression. This required homogenization of tissue in buffer before acid deproteination. The method of tissue preparation and GSH analysis was able to recover  $> 87\%$  of various amounts of GSH added to liver tissue, and assay repeatability was similar to previous reports of this method as well as several other GSH assay methods. Determining GSH concentration in mouse liver proved the accuracy of our assay system.<sup>28-36</sup> The smaller intra-assay CV%, compared with the inter-assay determinations, reflected differences in assay reagents prepared on different days (enzyme activity, standard solutions) as well as regional differences within liver tissue from the single individual with necroinflammatory liver disease used for these evaluations.

Compared with other species (predominantly work in rodents) where GSH cellular content approximates 5 to 7  $\mu\text{mol/g}$  of liver tissue, GSH concentrations in liver tissue from healthy dogs and cats were unexpectedly low.<sup>37</sup> The significant difference between healthy canine and feline liver GSH and GSSG concentrations and especially the comparatively lower GSH values in healthy dogs also was unexpected. However, a marked difference in the concentration of GSH in liver tissue among species has been previously reported.<sup>28</sup> Despite a broad search of the literature for GSH values reported for canine and feline tissue, we were unable to find values for comparison to our results. Low GSH concentrations in canine liver tissue is consistent with the apparent susceptibility of the dog to acetaminophen hepatotoxicity, which is associated with hepatic GSH depletion in a number of species.<sup>38-42</sup>

In illness, the physiologic importance of low liver GSH concentrations is proposed to reflect the influence of oxidant damage on the liver, nutritional deprivations, as well as an increased vulnerability of the organ to ongoing oxidant events. Although liver injury associated with chronic alcohol ingestion is the best studied hepatic injury associated with low liver GSH concentrations, other chronic necroinflammatory disorders in humans also are associated with diminished liver tissue GSH. Contributing factors include nutritional deficiency of GSH precursor

sors, chronic hepatotoxicosis that increases GSH utilization, increased GSH efflux from hepatocytes, reduced regeneration of GSH from GSSG, and impaired synthesis of s-adenosylmethionine from methionine as a result of reduced activity of the enzyme catalyzing this transformation (s-adenosylmethionine synthase, also known as methionine adenosyltransferase).<sup>8-13,41-46,h</sup>

Low tissue GSH concentrations in our study were not related to differences in intervals of withholding food preceding tissue collection, as all animals were deprived of food for 12 hours before liver specimen collection. However, nutritional considerations in some patients (eg, total inappetence, feeding of an ultra-low protein diet, enteric disease) may have altered the availability of essential GSH substrates in some individuals. The many different diets fed to the patients, as well as the irregularity of diet ingestion in some, made nutritional associations impossible to evaluate. While medical treatments given to individuals may have influenced findings in some animals, the complexity of defining and evaluating individual management variables given the tertiary referral status of the teaching hospital patient population precluded their investigation. Nevertheless, we did verify that no patient had been treated with s-adenosylmethionine, n-acetylcysteine, or silymarin derivatives, known to contribute GSH precursors, before obtaining liver biopsy specimens.

Although the median age of the healthy dogs and cats was younger (on inspection) than animals in most disease categories, it is unlikely that age-related changes influenced our findings, as these have been reported only in extremely young and old individuals in other species.<sup>47,48</sup> However, the significantly younger age in dogs with PSVA may have contributed to our finding of high GSH values in a few patients, as some of these dogs were only 4 months old when tissue specimens were collected.

A low hepatocellular GSH-to-GSSG ratio may develop as a result of impaired cell GSH synthesis, excessive oxidant challenge, impaired GSSG reduction (eg, inadequate NADPH), or reduced GSSG exportation.<sup>1</sup> Although severe oxidant stress may overwhelm the hepatocytes ability to reduce GSSG via GSSG reductase back to GSH, rapid exportation of GSSG by the hepatocyte can attenuate evidence of altered cell redox status when crude tissue extracts are measured.<sup>1</sup> The situation is made even more complex by the spontaneous slow *in vitro* formation of GSSG during assay manipulations, particularly during derivatization procedures. Although spontaneous generation of GSSG in GSH standard solutions with concentrations emulating tissue homogenate GSH concentrations was < 3.2% (mean  $\pm$  2 SD), we did find a significant inverse association between the concentration of GSH and the percentage of spontaneously formed GSSG in standard solutions. This finding cautions against over interpretation of an abnormally low GSH-to-GSSG ratio in patients with low GSH tissue concentrations, because the low GSH-to-GSSG ratio simply may represent a low but slow steady spontaneous GSH oxidation during sample processing as well as an altered *in vivo* redox status. Because the GSH-to-GSSG ratio is usually  $\geq$  100 in tissues from healthy rodents, our finding of values ranging from

54 to 152 in healthy mice suggests that auto-oxidation of GSH during sample analysis affected GSSG measurements in some of these specimens.<sup>49</sup> We presume that this occurred during GSH derivatization when homogenate was incubated with 2-vinylpyridine. Oxidation of GSH during analytic processing continues to be a problem in many different assay systems beyond the scope of this discussion. Consequently, owing to a number of variables, the full pathologic spectrum of subtle but important alterations in hepatocellular GSH-to-GSSG ratio are difficult to ascertain by use of crude tissue homogenization and many different GSH assays.<sup>1,50,51</sup> It is now widely recognized that more definitive evidence of altered cell redox status is reflected by GSH changes at a subcellular level, for example within mitochondria where approximately 10 to 15% of intracellular GSH is located.<sup>1,51-55</sup> As a compartmentalized site where toxic oxygen intermediates form and accumulate, mitochondria are highly vulnerable to changes in GSH availability because they lack enzymes for GSH synthesis, rely on shared cytosolic GSH, and are unable to export GSSG.<sup>1</sup> Despite the complications associated with GSSG determinations, the total GSH values measured in crude tissue extracts are reliable when the recycling enzymatic assay is used and attention is given to achieve rapid homogenate acidification for deproteination. Homogenate acidification reduces the activity of GSH catabolism by endogenous  $\gamma$ -glutamyltranspeptidase and GSH auto-oxidation and removes protein sulfhydryls also measured by the recycling reaction.

Differences in cell GSH concentrations are reported to normally exist between periportal (zone 1) and perivenular (zone 3) hepatocytes.<sup>55-57</sup> Periportal hepatocytes maintain GSH values approximately double that found in perivenular cells, reflecting the prioritization of cystine for taurine as opposed to GSH synthesis in zone 3.<sup>55-57</sup> Although speculative, this zonal influence may have relevance to our finding of GSH values that were within reference range to high in dogs with PSVA that have a relative increase in periportal zones (higher number of juvenile portal triads per hepatic lobule). It is also possible that the zonal difference in hepatocyte GSH concentrations emphasized the decline in tissue GSH concentrations in necroinflammatory disorders and extrahepatic bile duct occlusion, which primarily involved inflammation and damage confined to the periportal region.

At the inception of this study, we hypothesized that finding abnormal tissue GSH values in patients with severe acquired liver disease may reflect reduced hepatocyte numbers per gram of sampled tissue as a consequence of cell replacement (connective tissue), hepatocyte distention (with either glycogen or lipid), or tissue infiltration (inflammatory or neoplastic cells). We also acknowledge, however, that expression of tissue GSH concentration on the basis of any of our normalizing constants (total tissue protein, tissue DNA) can be influenced by complex variables affecting not only cell size but also by enzyme activity influencing protein and DNA synthesis, GSH precursors or NADPH, as well as effects derived from infiltrating cells (inflammatory cells, neoplastic cells). Inspection of the clinical data for relevant influences revealed that low GSH values were most often found when GSH was expressed on the basis of tissue DNA (73% of



patients had low values on the basis of DNA normalization). In addition to low GSH, this may reflect a high DNA density per gram of tissue that could result from a regenerative tissue response (larger number of small young hepatocytes) or DNA present in cell infiltrates. A significantly greater tissue DNA concentration was found in dogs and cats with necroinflammatory disorders, as well as in cats with extrahepatic bile duct obstruction, hepatic lipidosis, and lymphosarcoma. The increase in necroinflammatory disorders and extrahepatic bile duct occlusion likely reflects cell regeneration and inflammatory infiltrates. The increase in cats with hepatic lipidosis may reflect dispersal of cell components into a smaller than normal aqueous phase where the DNA was measured. The marked increase in tissue DNA concentration in cats with hepatic lymphosarcoma reflects invading neoplastic cells. Thus, we cannot conclude that a single expression unit (tissue weight, tissue protein, or tissue DNA) can normalize data for comparison between diseases and argue that inspection of data in several ways assists in confirming authenticity of our findings.

On inspection, differences in GSH concentrations in animals with EHBDO were associated with increasing chronicity and severity of their illness (data not shown). Two dogs with short-term partial EHBDO associated with marked biliary tract inflammation (cholecystitis, choledochitis, cholangitis) and hyperbilirubinemia had GSH per gram of tissue exceeding the upper 97.5% CI of healthy animals, whereas 3 dogs and 2 cats with chronic complete EHBDO (> 2 weeks) had low values. Because bilirubin impairs hepatic sinusoidal GSH egress, it remains possible that high GSH concentrations reflected this effect in short-term complete obstruction whereas the oxidant damage incurred by chronic exposure to membranocytolytic bile acids culminated in low GSH values.<sup>38</sup> Disorders with the fewest abnormal GSH values were vacuolar hepatopathy and PSVA in dogs, and these disorders typically lack histologic evidence of necroinflammatory injury.

That 3 of 4 cats with hepatic lipidosis had low GSH concentrations per gram of liver tissue and per microgram of tissue DNA suggests increased risk for hepatic and systemic oxidant injury related to their impaired hepatic metabolism. The low tissue protein concentration in some of these cats masked low GSH values when tissue protein was used for GSH normalization. Low tissue protein concentrations were not unexpected considering the negative nitrogen balance and catabolism associated with the hepatic lipidosis syndrome. A negative nitrogen balance has previously been shown to facilitate development of low liver GSH concentrations in rodents.<sup>30</sup>

It is important to consider that the relevance of liver GSH concentrations to health extends beyond the implications of liver susceptibility to oxidant injury. The liver is the major source of GSH for the body. In fact, hepatic GSH exported across the sinusoidal membrane accounts for 90% of plasma GSH.<sup>4</sup> Systemic distribution of GSH from the liver is impaired in patients with serious liver disease not only as a result of liver tissue GSH deficits but also because of the influence of other variables (eg, increased extracellular methionine and bilirubin inhibit sinusoidal GSH efflux from the

liver).<sup>59,60</sup> We conclude that the results of our study lay the foundation for further investigation of oxidative injury in dogs and cats with naturally occurring liver disease as well as of GSH in patients with other disease processes. Considering the low GSH values found in liver tissue taken from healthy dogs, compared with other species, a canine predisposition for hepatotoxicity associated with GSH depletion might exist. This is consistent with the canine predisposition for hepatic necrosis subsequent to acetaminophen toxicosis known to be associated with hepatic GSH depletion.<sup>38-42</sup> Because we did not determine liver tissue GSH in patients with extrahepatic systemic disorders lacking liver involvement, it remains undetermined whether hepatic GSH stores are generally depleted by illness and malnutrition associated with inappetence beyond that observed with withholding food. The impact of nutritional status (food fed, anorexia, vomiting, diarrhea) of each patient was not explored and may have contributed to our finding of low liver GSH concentrations in certain individuals.

<sup>38</sup>Lauterberg BH, Velez ME, Mitchell JR. Plasma glutathione (GSH) as an index of intrahepatic GSH in man: response to acetaminophen and chronic ethanol abuse (abstr). *Hepatology* 1984;4:1051(A 180).

<sup>39</sup>Glutathione reductase, Sigma Chemical Co, St Louis, Mo.

<sup>40</sup>2-vinylpyridine, Aldrich Chemical Co, Milwaukee, Wis.

<sup>41</sup>triethanolamine (2,2' 2' nitrotriethanol), Sigma Chemical Co, St Louis, Mo.

<sup>42</sup>Hoecsht 33258, Aldrich Chemical Co, Milwaukee, Wis.

<sup>43</sup>TD-700 Fluorometer, Turner Designs, Sunnyvale, Calif.

<sup>44</sup>Deoxyribonucleic acid (DNA), sodium salt, Type 1, highly polymerized, from calf thymus, Sigma Chemical Co, St Louis, Mo.

<sup>45</sup>Marchesini G, Almasio P, Luca A, et al. Sulfur amino acid (SAA) pattern in compensated chronic liver diseases (Part II) (abstr). *Eur J Clin Invest* 1989;19:(A)94.

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Appendix—Animal signalment and disorders

Disorders	Dogs			Cats		
	No.	Sex	Age (range)*	No.	Sex	Age (range)*
<b>Necroinflammatory</b>						
Cirrhosis and chronic active hepatitis	15	1 F, 2 SF, 3 M, 9 CM	6 (1–12)	—	—	—
Cholangitis and cholangiohepatitis	2	2 SF	8.3 (3.5–13)	8	3 SF, 5 CM	10 (3.5–14)
Extrahepatic bile duct obstruction	8	7 SF, 1 CM	10 (7–12)	3	1 SF, 2 CM	10 (9–10)
Hepatic necrosis	5	3 SF, 1 M, 1 CM	3 (0.69)	1	1 SF	13
Focal mild hepatitis	2	1 SF, 1 CM	8 (7–9)	1	1 SF	8
<b>Non-necroinflammatory disorders</b>						
Hepatic lipidosis	—	—	—	4	3 SF, 1 MC	10 (7–13)
Vacuolar hepatopathy	16	5 F, 5 SF, 3 M, 3 CM	6 (3–10)	—	—	—
Hepatic lymphosarcoma	—	—	—	3	2 SF, 1 MC	13 (9.5–13)
Portosystemic vascular anomaly	15	1 F, 5 SF, 4 M, 5 CM	1 (0.4–6)	—	—	—
<b>Total patients</b>	<b>63</b>	<b>7 F, 26 SF, 11 M, 19 CM</b>	<b>5 (0.3–13)</b>	<b>20</b>	<b>11 SF, 9 MC</b>	<b>10 (3.5–15)</b>

\*Median age in years (range).  
F = Sexually intact female. SF = Spayed female. M = Sexually intact male. CM = Castrated male.