Metabolism of amino acids in cats with severe cobalamin deficiency

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Objective—To validate an automated chemiluminescent immunoassay for measuring serum cobalamin concentration in cats, to establish and validate gas chromatography-mass spectrometry techniques for use in quantification of methylmalonic acid, homocysteine, cysteine, cystathionine, and methionine in sera from cats, and to investigate serum concentrations of methylmalonic acid, methionine, homocysteine, cystathionine, and cysteine as indicators of biochemical abnormalities accompanying severe cobalamin (vitamin B_{12}) deficiency in cats.

Sample Population—Serum samples of 40 cats with severe cobalamin deficiency (serum cobalamin concentration < 100 ng/L) and 24 control cats with serum cobalamin concentration within the reference range.

Procedure—Serum concentrations of cobalamin were measured, using a commercial automated chemiluminescent immunoassay. Serum concentrations of methylmalonic acid, methionine, homocysteine, cystathionine, and cysteine were measured, using gas chromatography-mass spectrometry, selected ion monitoring, stable-isotope dilution assays.

Results—Cats with cobalamin deficiency had significant increases in mean serum concentrations of methylmalonic acid (9,607 nmol/L), compared with healthy cats (448 nmol/L). Affected cats also had substantial disturbances in amino acid metabolism, compared with healthy cats, with significantly increased serum concentrations of methionine (133.8 vs 101.1 µmol/L) and significantly decreased serum concentrations of cystathionine (449.6 vs 573.2 nmol/L) and cysteine (142.3 vs 163.9 µmol/L). There was not a significant difference in serum concentrations of homocysteine between the 2 groups.

Conclusions and Clinical Relevance—Cats with gastrointestinal tract disease may have abnormalities in amino acid metabolism consistent with cobalamin deficiency. Parenteral administration of cobalamin may be necessary to correct these biochemical abnormalities. (*Am J Vet Res* 2001;62:1852–1858)

Cobalamin (vitamin B_{12}) is a known cofactor for 3 enzyme systems in mammals (ie, methylmalonyl-CoA mutase,[1] methionine synthase,[2] and leucine 2,3-aminomutase[3]). The first 2 enzymes are involved in the metabolism of methionine. Methionine synthase produces methionine through methylation of homocysteine, whereas methylmalonyl-CoA mutase converts methylmalonyl-CoA to succinyl-CoA, which may subsequently enter the tricarboxylic acid cycle (**Fig 1**). Deficiency of cobalamin is associated with reduced activity of both of these enzyme systems and resulting alterations in tissue and serum concentrations of the products of these enzyme systems. Of particular interest in humans is the effect that subnormal availability of cobalamin to tissues has on the methylation of homocysteine, because increases in serum homocysteine concentrations are associated with an increased risk of cardiovascular disease in humans.[4,5] Diets deficient in cobalamin or folate or the effects of enzyme defects (particularly defects in methylene-tetrahydrofolate reductase) are associated with mild hyperhomocysteinemia in fasting human patients.[5] Cobalamin deficiency also has been implicated as a causal agent in numerous neurologic disorders, including subacute combined degenerative myelopathy of humans.[6]

Dietary cobalamin is absorbed in the ileum of cats[a] as a complex with intrinsic factor, a carrier protein of pancreatic origin in cats. Uptake of the cobalamin-intrinsic factor complex is a receptor-mediated process,[3] which may be disturbed in conditions of mucosal disease. Thus, disease of the ileum or disease that affects the small intestinal mucosa in cats may be associated with reduced cobalamin uptake and subsequent deficiency of cobalamin stores. Cats with exocrine pancreatic insufficiency[b] or inflammatory bowel disease may become depleted of cobalamin, with serum cobalamin concentrations markedly lower than those for clinically normal cats. Recently, investigators reported[7] a state of low circulating cobalamin in some cats with chronic signs of gastrointestinal tract disease; however, that study did not specifically investigate the metabolic consequences of the low concentration of circulating cobalamin.

We hypothesized that cats with subnormal serum cobalamin concentrations would have disturbances in the metabolism of sulfur-containing amino acids as a consequence of reduced activity of methionine synthase and methylmalonyl-CoA mutase that would subsequently alter serum concentrations of methylmalonic acid, methionine, homocysteine, and downstream metabolites of homocysteine, compared with a group of control cats that did not have evidence of gastrointestinal tract disease and that had serum concentrations of cobalamin within the reference range.

Materials and Methods

Serum samples—Sera from 40 cats with hypocobalaminemia were selected from samples submitted to the clinical service of the Gastrointestinal Laboratory at Texas A&M University. Selection criterion for inclusion in the study was a serum cobalamin concentration < 100 ng/L. This value represents the lower limit of detection of the chemiluminescent assay system used in our laboratory.

All serum samples were from cats from which food was

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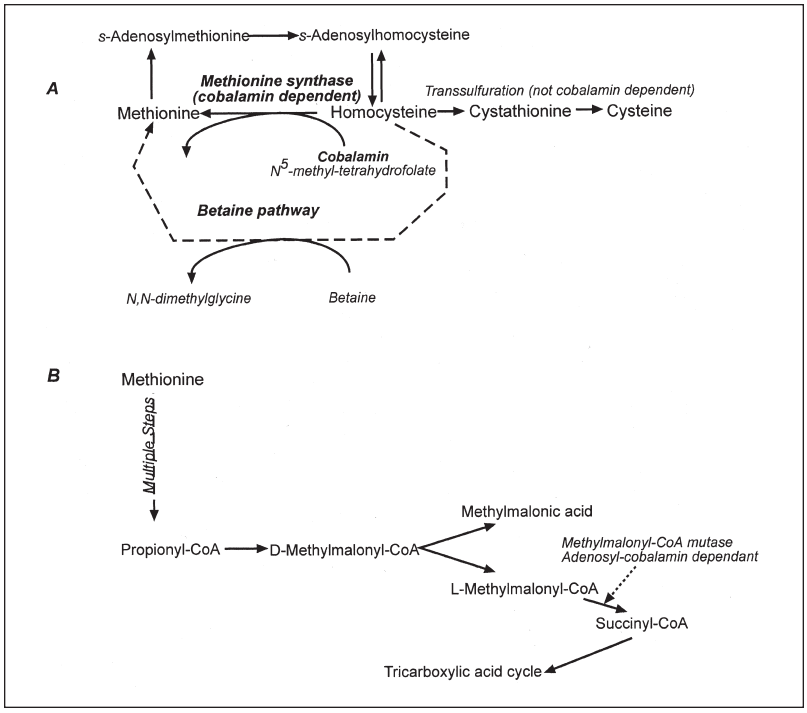

Figure 1—Diagrammatic representation of pathways of amino acid metabolism requiring cobalamin as an essential cofactor. In panel A, resynthesis of methionine from homocysteine by methionine synthase is dependent on methyl-cobalamin and methyl-tetrahydrofolate, whereas transsulfuration to cystathionine and cysteine is not a cobalamin-dependent pathway. An alternative pathway allowing resynthesis of methionine from homocysteine in the non–cobalamin-dependent betaine pathway is indicated. In panel B, formation of succinyl-CoA from L-methylmalonyl-CoA is dependent on adenosyl-cobalamin. Deficiency of cobalamin is associated with alterations in tissue and serum concentrations of the products of these pathways.

withheld for 8 to 12 hours before the samples were obtained. Veterinary practitioners had submitted the samples to our laboratory for measurement of **feline trypsin-like immunoreactivity (fTLI)** and cobalamin and folate concentrations. Serum fTLI was measured with a sandwich ELISA, as described elsewhere,[8] whereas cobalamin and folate concentrations were measured with a commercially available chemiluminescent assay system. Calibrated quality-control standards of low, normal, and high cobalamin and folate concentrations were measured with every batch of the cobalamin and folate assays.

Sera from 24 control cats were selected from banked sera of healthy pet cats. All these control sera had previously been assayed for fTLI and cobalamin and folate concentrations, and all were within our laboratory reference ranges for all analytes.

Cobalamin assay—Serum cobalamin concentration was measured, using a commercially available automated competitive immunoassay.[c] Briefly, 200 µl of serum was heat-denatured by insertion in a boiling water bath for 30 minutes after addition of dithiothreitol and potassium cyanide; this procedure denatured cobalamin-binding proteins and any antibodies to intrinsic factor. The heat-denatured sample and purified porcine intrinsic factor were added to a reaction vessel containing polystyrene beads coated with an immobilized cobalamin analogue. Cobalamin in the serum sample competed with the solid-phase cobalamin analogue for the porcine intrinsic factor. Following incubation and washing, alkaline phosphatase–labeled anti-porcine intrinsic factor antibody was introduced, which bound to the porcine intrinsic factor that had formed a complex with the solid phase. Following a final incubation and washing procedure, chemiluminescent substrate was added, and light production was measured. Higher concentrations of cobalamin in the serum sample competed for and removed larger amounts of porcine intrinsic factor; therefore, light production was inversely related to serum cobalamin concentration. After the heat-denaturation step, all steps of this assay were performed automatically, using formulated buffers supplied by the manufacturer of the assay kit. Although the manufacturer has extensively validated this assay kit for use in human serum, our validation included measurement of intra- and interassay variation to assess precision within our laboratory and recovery of spiked cobalamin to rule out nondenaturable cobalamin-binding proteins in feline serum.

Methylmalonic acid assay—Concentration of methylmalonic acid in serum was measured, using a stable isotope dilution **gas chromatography-mass spectrometry (GC-MS)** assay with selected ion monitoring. The assay was performed as described for determination of serum methylmalonic acid concentration in sera of humans and rats,[9] with some modifications. All reagents were of high-performance liquid chromatography grade or the highest purity commercially available.

Briefly, a stable isotope-labeled form of methylmalonic acid containing 3 deuterium atoms/molecule[d] was added in known quantity (25 µl of a 10 µ*M* solution, representing a final concentration of 500 nmol/L) to 500 µl of feline serum in a 13 × 100-mm disposable borosilicate glass tube.[e] The serum was acidified and proteins precipitated by addition of 500 µl of 3*M* hydrochloric acid. Serum was vortexed for 1 minute and then centrifuged at 1,000 × g for 5 minutes. Volumes of spiking solution and hydrochloric acid were adjusted as necessary when < 500 µl of serum was available.

Disposable liquid-liquid extraction columns[f] (1-ml sample capacity) were conditioned by washing sequentially with 2 ml of 3*M* hydrochloric acid saturated with NaCl, 10 ml of distilled deionized water, 5 ml of methanol, and 5 ml of ethyl acetate. Columns then were dried for 30 minutes under continuous vacuum on a 10-port vacuum elution manifold.[g] After drying, the acidified serum supernatant was applied to the column and allowed to absorb for 2 minutes. Methylmalonic acid and other serum dicarboxylic acids then were eluted, using 4.5 ml of ethyl acetate, into additional 13 × 100-mm borosilicate glass tubes.

Eluents were dried at 64 C under a stream of nitrogen. The *tert*-butyldimethylsilyl ester derivatives of the dicarboxylic acids were generated through addition of 50 µl of *N*-methyl-*N*-(-*tert*-butyl-dimethylsilyl)-trifluoroacetamide[h] and 50 µl of acetonitrile[i] to each tube. Tubes were sealed, vortexed for 10 seconds, and incubated at 64 C for 15 minutes. The derivatized sample mixture was placed into sealed autosampler vials, and 1 µl of each sample was injected into the GC-MS.

A gas chromatograph[j] coupled to a mass spectrometer[k] was used for chromatographic separation and quantification of the derivatized methylmalonic acid. Separation was achieved, using a fused silica gas-chromatography column with 100% dimethylpolysiloxane stationary phase[l] (30 m × 0.25 mm × 0.25 µm) fitted with a 2-m retention gap. Flow

rate of carrier gas (ultra-pure helium) was maintained at 2.0 ml/min. The column was equilibrated at 75 C and maintained at this temperature for 2 minutes following injection. The column then was heated from 75 to 215 C at a rate of 12 C/min. Flow of carrier gas in the column was at a split ratio of 7.5:1; however, the injection was made in splitless mode for 30 seconds. The mass spectrometer was operated in electron-impact positive-ion mode with selective ion monitoring at **mass-to-charge ratios (M/Z)** of 289 and 292 for endogenous methylmalonic acid and the trideuterated internal standard, respectively. Data were collected from the mass spectrometer between 10 and 13.5 minutes after injection, and the methylmalonic acid peaks eluted at approximately 11.49 minutes for the trideuterated standard and 11.51 minutes for endogenous methylmalonic acid. Quantification was based on the ratio of the area under the curve of the trideuterated internal standard and the endogenous methylmalonic acid.

Standard solutions of nonlabeled methylmalonic acid[m] at concentrations ranging from 31.25 to 64,000 nmol/L were prepared, using serial dilution in methanol. Five hundred microliters of each standard solution was dried in a borosilicate tube and then reconstituted with 500 µl of deionized water, and the methylmalonic acid was extracted as described previously to establish assay linearity and dynamic range. Recovery of known quantities of methylmalonic acid added to serum samples, interassay variation, and intra-assay variation of measurement of methylmalonic acid from a pooled feline serum sample also were assessed.

Amino acid assays—Methionine, homocysteine, cystathionine, and cysteine concentrations were measured in feline serum samples, using a stable isotope dilution GC-MS assay with selected ion monitoring, as described and validated for human sera.[2,10] The method of quantification was as previously described for methylmalonic acid, utilizing the ratios of labeled versus unlabeled forms of the amino acids, which eluted at characteristic times from the gas chromatograph. The amino acids were extracted from sera, using disposable chromatography columns[n] packed with an ion-exchange resin.[o] Method of preparation of samples and extraction, using anion-exchange chromatography, has been described elsewhere.[2] The GC-MS retention times in our study differed slightly from those reported in that other study because of differing column length and a 2-m retention gap. The actual retention times were confirmed through daily analysis of an aqueous mixture of pure amino acids. The assay was validated for feline serum samples by investigation of inter- and intra-assay variation (reproducibility and precision, respectively) and accuracy of recovery of added amino acids.

Statistical analysis—Data were tested for normality, using the Kolmogorov-Smirnov test. When necessary, data were normalized by $\log_{10}$ conversion before analysis. Differences between the 2 groups in mean serum concentration of methylmalonic acid and the various amino acids were assessed, using a 2-tailed Student *t*-test and Welch correction for unequal variances, when necessary.[p] Values of $P < 0.05$ were considered significant.

Results

Animals—Data on age, breed, and sex for the 24 control and 40 affected cats were summarized. Complete data were not available for all affected cats. However, age of affected cats (11.8 years; n = 35) was significantly ($P < 0.001$) higher than mean age for the 24 control cats (4.7 years). Affected cats included 23 domestic short hair, 5 domestic long hair, 3 Siamese, 1 Persian, and 1 Abyssinian cat; data on breed was not available for 7 affected cats. Control cats included 14 domestic short hair, 5 domestic long hair, 2 Persian, and 1 each of Siamese, Abyssinian, and Maine Coon cats. Affected cats included 20 castrated males, 3 sexually intact males, 11 spayed females, and 4 sexually intact females; data on sex was not available for 2 affected cats. Control cats included 9 castrated males, 1 sexually intact male, 12 spayed females, and 2 sexually intact females.

Data on clinical signs, other clinicopathologic findings, and definitive diagnoses, if any, were not available for most cats. Information on diet was not available for most of the affected cats. Control samples were from healthy cats owned by students of the College of Veterinary Medicine, Texas A&M University. Those cats were fed various diets, consisting predominantly of dry premium foods provided ad libitum.

Feline serum samples—All samples from affected cats had serum cobalamin concentrations ≤ 100 ng/L (ie, lower limit of detection of the assay system). Mean ± SEM serum fTLI concentrations in the affected cats (165.1 ± 15.38 µg/L; range, 28 to 438 µg/L) was increased relative to our laboratory reference range (12 to 82 µg/L). Thirty of 40 (75%) cats had serum fTLI concentrations greater than the reference range, whereas none had concentrations less than the reference range. Five of 40 (12.5%) affected cats had serum folate concentrations that were less than our laboratory reference range (9.7 to 21.6 µg/L); the remainder of the affected cats had serum folate concentrations within or greater than the laboratory reference range.

Cobalamin assay—Intra-assay coefficient of variation (precision) of the automated chemiluminescent assay system for cobalamin was 2.92% (10 aliquots of 1 sample assayed in a single batch). Interassay coefficient of variation, a measure of repeatability, was 15.25% (10 aliquots of a single feline serum sample processed in 10 successive assays). Mean recovery of cobalamin from spiked sera was 96.05, 113.80, and 121.93% following addition of 100, 200, and 300 pg/L of pure cobalamin, respectively.

Methylmalonic acid assay—Total ion counts from standard solutions extracted and derivatized in the same manner as that described for serum samples revealed excellent linearity and repeatability for concentrations of methylmalonic acid between 62.5 and 16,000 nmol/L ($y = 0.341x^{0.7608}$, $r^2 = 0.9865$). The linearity of the ion response was decreased for standard concentrations greater than 16,000 nmol/L because of saturation of the mass-spectrometer at these concentrations. Methylmalonic acid was readily identified in chromatograms from feline sera at M/Z 292 and 289 (**Fig 2**). Endogenous methylmalonic acid eluted as a single peak at 11.51 minutes, whereas the trideuterated form eluted at 11.49 minutes. Other unidentified dicarboxylic acids were apparent in the M/Z 289 trace. Intra-assay coefficient of variation (6 aliquots of 1 sample evaluated concurrently in 1 assay) was 12.56%, whereas the interassay coefficient of variation of the same sample evaluated 7 times on consecutive days

was 4.99%. Recovery of exogenous methylmalonic acid spiked into feline sera at various concentrations was acceptable, with mean recovery from 3 replicates of 119, 128, and 92%, respectively, for aliquots of 50, 500, and 2,000 nmol/L added to aliquots of pooled feline serum.

Results of stability studies indicated that serum concentrations of methylmalonic acid were not substantially altered during storage at 4 C for up to 10 days (data not shown). There was a slight increase in methylmalonic acid concentrations in serum samples stored at room temperature over the same time period; however, this difference was not greater than the interassay coefficient of variation for the assay.

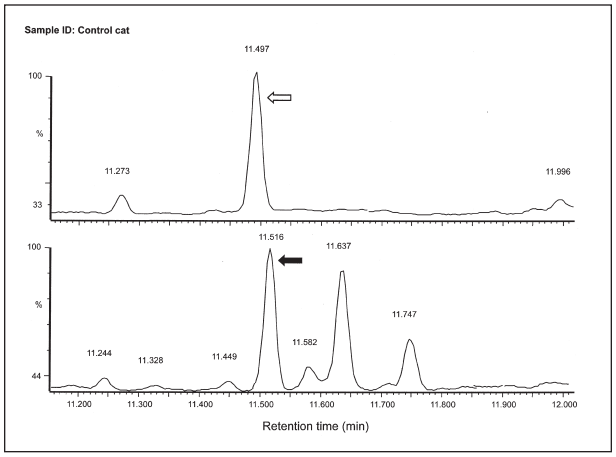

Figure 2—Representative chromatograms revealing elution of methylmalonic acid from feline serum samples following solid-phase extraction and formation of the *tert*-butyldimethylsilyl ester derivatives of serum dicarboxylic acids. Deuterium-labeled methylmalonic acid elutes at 11.49 minutes (open arrow), whereas endogenous nonlabeled methylmalonic acid elutes at 11.51 minutes (solid arrow). Quantification of serum methylmalonic acid concentration is based on the ratio of the areas under the curves of the 2 isotopes, with the deuterium-labeled form added in known quantity. The sample illustrated contained methylmalonic acid at a concentration of 286.9 nmol/L and was from a control cat with serum concentrations of cobalamin, folate, and feline trypsin-like immunoreactivity within the respective reference ranges.

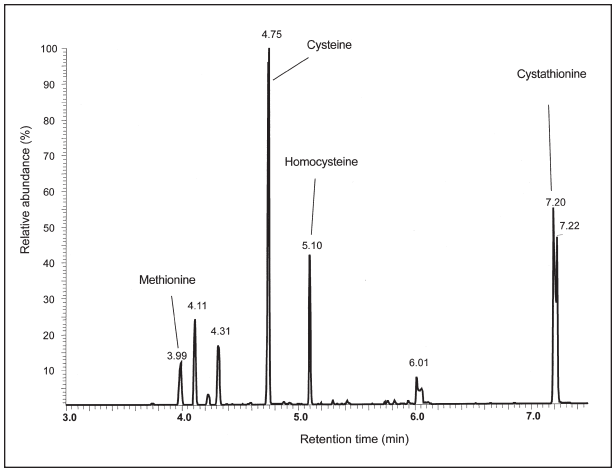

Figure 3—Representative chromatogram revealing total ion counts of the elution of sulfur-containing amino acids methionine, cysteine, homocysteine, and cystathionine in a feline serum sample. Quantification of serum concentrations of these amino acids is based on the ratios of the areas under the curves of the endogenous amino acid and their corresponding isotopically labeled forms, added in known quantity.

Amino acid assay—The 4 amino acids were readily detectable in feline serum in gas chromatograms (**Fig 3**). The amino acid assay had a high degree of precision, with mean intra-assay coefficients of variation (mean of 3 samples assayed 10 times each) ranging from 1.91% for cysteine to 9.03% for cystathionine. Accuracy of the amino acid assay, as assessed from recovery of added nonlabeled amino acids, varied from 92% at physiologic concentrations of homocysteine to 55% at extremely high concentrations of spiked cysteine. Cysteine had the least accurate recovery of the measured amino acids because of saturation of the mass spectrometer at concentrations > 200 µmol/L. Dilution of serum samples or use of a smaller starting volume of serum may improve the accuracy of measurement of cysteine, but it also will reduce sensitivity and the ability to quantify amino acids such as homocysteine and cystathionine that are at lower concentrations.

Methylmalonic acid concentrations in sera of cobalamin-deficient cats—Severely cobalamin-deficient cats had a significant ($P < 0.001$) increase in mean serum methylmalonic acid concentration, compared with mean concentration for the control cats (**Fig 4**). Mean ± SEM serum methylmalonic acid concentration of the control cats was 447.9 ± 42.78 nmol/L, whereas the corresponding value for the affected cats was 9,607 ± 1,478 nmol/L.

Amino acid concentrations in sera of cobalamin-deficient cats—Severely cobalamin-deficient cats had significant differences in serum concentrations of several amino acids. Mean serum methionine concentration was significantly increased in affected cats (133.8

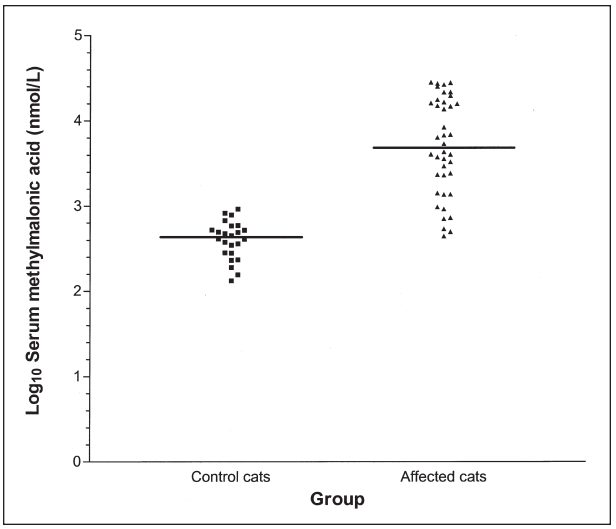

Figure 4—Scatter plot of serum methylmalonic acid concentrations (log_{10} transformed) in samples obtained from 24 control cats and 40 cats with severe cobalamin deficiency. Values were obtained by use of a stable isotope dilution gas chromatography-mass spectrometry assay. Horizontal bar represents mean of each distribution. Mean serum concentration of methylmalonic acid differs significantly ($P < 0.001$; 2-tailed Student *t*-test) between the 2 groups.

± 9.75 μmol/L), compared with the mean value for control cats (101.1 ± 8.85 μmol/L), whereas serum cystathionine (449.6 ± 82.6 nmol/L; $P < 0.001$) and cysteine (142.3 ± 10.28 μmol/L) concentrations in affected cats were both significantly lower, compared with values for control cats (573.2 ± 72.8 nmol/L and 163.9 ± 8.0 μmol/L, respectively). There was not a significant difference in serum homocysteine concentrations between the 2 groups of cats (affected cats, 3.67 ± 0.50 μmol/L; control cats, 3.97 ± 0.45 μmol/L).

Discussion

Data reported here provided evidence of multiple severe biochemical and metabolic abnormalities in cats with severe cobalamin deficiency. The increase in mean serum methylmalonic acid concentration in cats with cobalamin deficiency is consistent with loss or decrease of activity in methylmalonyl-CoA mutase, which is absolutely dependent on adenosyl-cobalamin as a cofactor.[11] Several amino acids (isoleucine, methionine, threonine, valine) as well as cholesterol and odd-chain fatty acids enter the tricarboxylic acid cycle via the common intermediate of propionyl-CoA.[11] D-Methylmalonyl-CoA is at a branching point from which it may be converted to L-methylmalonyl-CoA or methylmalonic acid by either of 2 competing enzyme systems. Accumulation of L-methylmalonyl-CoA as a result of cobalamin deficiency promotes formation and accumulation of methylmalonic acid in serum.

Alterations in serum concentrations of methionine and related amino acids in cats with severe cobalamin deficiency indicate major alterations in metabolism of sulfur-containing amino acids in cobalamin-deficient cats. Particularly noticeable, however, is the lack of a significant difference in serum concentrations of homocysteine in affected cats. Greater than 95% of humans with cobalamin or folate deficiency will have a substantial increase in serum homocysteine concentration.[11] Serum methionine and cysteine concentrations are maintained within their reference ranges in the vast majority of hypocobalaminemic human patients, and cystathionine is commonly increased in humans with cobalamin or folate deficiency.[2,11]

Regulation of methionine and homocysteine metabolism is complex, involving the coordinated regulation of at least 5 enzyme systems that catalyze various reactions (Fig 1). Homocysteine concentrations are tightly regulated through differential entry into the remethylation or transsulfuration pathways,[5] depending on the amount of dietary input of methionine and the requirement for synthesis of *s*-adenosylmethionine for use in physiologic methylation reactions and DNA synthesis.

In addition to the cobalamin-dependent methionine synthase pathway used for remethylation of homocysteine to methionine, there is an alternative pathway that utilizes betaine as a methyl-group donor and yields methionine and N,N-dimethylglycine (Fig 1).[11] The betaine-homocysteine methyltransferase pathway is not dependent on cobalamin or folate and offers 1 possible explanation for the lack of increase in serum homocysteine concentrations in the affected cats. Humans with folate deficiency typically have increased serum concentrations of N,N-dimethylglycine,[11] which would be expected with increased use of the betaine-homocysteine methyltransferase pathway. Only a limited number of humans with cobalamin deficiency, however, have increased serum concentrations of N,N-dimethylglycine,[11] suggesting that, in humans at least, the betaine-homocysteine methyltransferase pathway is not used in conditions of cobalamin deficiency to maintain homocysteine concentrations within the reference range. Because fasting cobalamin-deficient humans have substantial increases in serum homocysteine concentrations, and the data reported here clearly suggest that cats do not, it is indeed feasible that there may be species differences in the importance of the betaine-homocysteine methyltransferase pathway. Elucidation of the role of the betaine-homocysteine methyltransferase pathway in cobalamin-deficient cats will require measurement of serum betaine and N,N-dimethylglycine concentrations in affected cats, which was beyond the scope of the study reported here.

The equilibrium of the reaction generating homocysteine and adenosine from *s*-adenosylhomocysteine favors the synthesis of *s*-adenosylhomocysteine.[12] *s*-Adenosylhomocysteine is a potent inhibitor of many of the physiologic methylation reactions in which *s*-adenosylmethionine acts as a methyl donor[13]; thus, accumulation of *s*-adenosylhomocysteine may result in accumulation of *s*-adenosylmethionine, which is of central importance in coordinating regulation of the remethylation and transsulfuration pathways.[5]

High concentrations of *s*-adenosylmethionine favor transsulfuration of homocysteine.[5] Therefore, in conditions of cobalamin deficiency, increased production of cystathionine and cysteine would be expected. The fact that we found the opposite (significantly decreased serum concentrations of cystathionine and cysteine in cobalamin-deficient cats) suggests that entry of homocysteine into the transsulfuration pathway may be compromised in these cats. Enzymes involved in condensation of homocysteine with serine to produce cystathionine and the subsequent cleavage of cystathionine to cysteine and α-ketobutyrate are dependent on pyridoxine (Vitamin B_6).[11] Because the diets of most domestic cats are rich in cobalamin, and the sole source of circulating cobalamin is by absorption from the distal portion of the small intestine, it is reasonable to assume that hypocobalaminemia in the cats reported here was attributable to dysfunction of the gastrointestinal tract. In accordance with this, affected cats may have a decrease in dietary intake of other vitamins because of decreased voluntary food intake or decreased uptake from the gastrointestinal tract. If this is the case, other vitamin deficiencies may have interfered with metabolic functions of the affected cats reported here.

Detection of hyperhomocysteinemia in a group of human patients with premature atherosclerosis and venous thrombosis required oral administration of a dose of methionine in 78 of 141 (55%) patients.[14] Serum homocysteine concentrations had a 35-fold increase within 2 hours following methionine administration in pyridoxine-deficient rats, yet concentrations declined rapidly and were not distinguishable from

control rats at 8 hours after challenge-exposure.[5] Because we requested that samples be obtained from animals from which food has been withheld to enable us to measure fTLI, cobalamin, and folate concentrations, it is possible that hyperhomocysteinemia and other alterations in serum amino acid concentrations may have developed in these cats after eating, but they resolved by the time the blood samples were collected.

An important additional difference between humans and cats arises from the obligate carnivore nature of cats. Adult cats require a high amount of dietary protein with at least 19% of the calories derived from protein sources.[1] The importance of remethylation of homocysteine to methionine lies in the supply of *s*-adenosylmethionine for physiologic methylation reactions. Methionine is an essential amino acid and cannot be synthesized de novo by mammals; it is obtained only from dietary input. Methionine is specifically added as a urinary acidifier to numerous proprietary foods formulated for cats, and, therefore, it is possible that the control and affected cats in the study reported here had a high dietary input of methionine. A high dietary input of methionine would be expected to reduce the importance of the cobalamin-dependent remethylation pathway in maintenance of adequate tissue concentrations of methionine.

Because cats have a relatively high protein intake as part of their typical diet, it is reasonable to hypothesize that clinically normal cats have a high rate of amino acid degradation[1] with high rates of amino acid flux along degradative pathways. Because several amino acids are degraded through the pathway from propionyl-CoA to succinyl-CoA, the importance of the cobalamin-dependent pathway from L-methylmalonyl-CoA to succinyl-CoA is increased. This may explain the observation that cobalamin-deficient cats in the study reported here had such dramatic increases in serum methylmalonic acid concentrations, whereas the serum concentrations of sulfur-containing amino acids were maintained close to the concentrations observed in the control cats.

Serum homocysteine concentrations are negatively correlated with serum folate-folic acid concentrations in fasting humans,[14] and serum folic acid concentrations were lower in a group of fasting humans with hyperhomocysteinemia.[14] In the study described here, only 5 of 40 cats had a serum folate concentration less than our laboratory reference range. Two of these 5 cats had increased concentrations of homocysteine and cystathionine relative to the control cats, with values more than 3 SD higher than the mean of clinically normal cats. The remaining 3 cats with low serum folate concentrations were not distinguishable from the other affected cats with respect to serum concentrations of amino acids or methylmalonic acid.

The cobalamin-deficient cats were significantly older than the control cats. This is consistent with observations that most chronic diseases of the gastrointestinal tract of cats such as inflammatory bowel disease are in middle-aged to elderly cats.[15] Simpson et al[7] described subnormal serum cobalamin concentrations in 49 cats with a median age of 11 years. Another study[16] in elderly humans revealed changes in 1 or more metabolites related to the tissue concentration of cobalamin in 41 of 64 (64%) healthy and 237 of 286 (83%) hospitalized elderly subjects. The possibility exists that age-related changes in cellular metabolism may lead to alterations in cobalamin-related metabolites in elderly cats, similar to those seen in the cats described in our study. However, the extreme cobalamin deficiency in the cats described here suggests that the observed modifications in serum concentrations of methylmalonic acid and sulfur-containing amino acids are a direct result of pathologic cobalamin deficiency rather than simply changes associated with aging.

The observation that 30 of 40 cobalamin-deficient cats had serum fTLI values greater than our laboratory reference range is intriguing. In the study described here, data were not available regarding the state of the pancreas in any of the affected cats. The mechanism behind the increased serum fTLI in cobalamin-deficient cats is not clear and may involve underlying pancreatic disease.

We previously reported[b] that many cats with exocrine pancreatic insufficiency (fTLI < 8 μg/L) are deficient in cobalamin, which may be attributable to a lack of pancreatic intrinsic factor in these cats. Although an increase in fTLI is certainly suggestive of pathologic changes in the pancreas, experience with this assay in our laboratory (ie, several thousand samples have been analyzed for fTLI in clinically normal cats and cats with signs of intestinal tract disease) suggests that increases of fTLI may be associated with clinically more important disease of the intestinal tract, rather than solely being attributable to pancreatic acinar cellular disease. Other investigators have reported similar findings. Swift et al[17] documented an increase in serum fTLI values in 5 of 6 cats with histopathologically diagnosed small intestinal disease that was not accompanied by detectable pancreatic inflammation. Similarly, Simpson et al[7] documented an increase in fTLI in 14 of 22 cats with gastrointestinal tract disease, 3 of which did not have ultrasonographic or histopathologic evidence of pancreatitis.

Other factors, including the possibility of increased intestinal permeability in cats with gastrointestinal tract disease, may influence serum fTLI in these cats. Additional studies of intestinal and pancreatic function in cats with cobalamin deficiency are necessary to clarify the mechanism behind and possible importance of the finding of increased serum fTLI concentrations in cats with cobalamin deficiency.

The available literature is sparse regarding clinical signs and deficiency states in cats that result from hypocobalaminemia, with only a single case report of cobalamin deficiency in a young cat[18] and a limited investigation by Simpson et al.[7] The importance of cobalamin deficiency in gastrointestinal tract disease of cats has received limited investigation. However, empirical experiences of the authors and results of samples submitted to the clinical service of the Gastrointestinal Laboratory at Texas A&M University suggest that cobalamin deficiency is a common abnormality in cats with clinical signs of gastrointestinal tract disease. In 1999, 650 of 2,377 (27.4%) feline

serum submissions to our laboratory had a serum concentration of cobalamin < 290 ng/L, the lower limit of the reference range. In that same year, 252 of 2,377 (10.6%) feline serum submissions had a measured serum cobalamin concentration < 100 ng/L.

[a]Fyfe JC. Feline intrinsic factor (IF) is pancreatic in origin and mediates ileal cobalamin (CBL) absorption (abstr). *J Vet Intern Med* 1993;7:133.
[b]Steiner JM, Williams DA. Validation of a radioimmunoassay for feline trypsin-like immunoreactivity (fTLI) and serum cobalamin and folate concentrations in cats with exocrine pancreatic insufficiency (abstr). *J Vet Intern Med* 1995;9:193.
[c]DPC IMMULITE Vitamin B_{12}, Diagnostic Products Corp, Randolph, NJ.
[d]Methyl-d_3-malonic acid, CDN Isotopes, Pointe-Claire, QC, Canada.
[e]Disposable culture tubes, Fisher Scientific, Pittsburgh, Pa.
[f]ChemElute columns, Varian, Harbor City, Calif.
[g]Vac-Elut 10 manifold, Varian, Harbor City, Calif.
[h]MTBSTFA, Pierce, Rockford, Ill.
[i]Acetonitrile, Allied Signal, Muskegon, Mich.
[j]Finnigan GC8000, Thermoquest, Schaumburg, Ill.
[k]Voyager, Thermoquest, Schaumburg, Ill.
[l]DB-1, J&W Scientific, Folsom, Calif.
[m]Methylmalonic acid, Sigma Chemical Co, St Louis, Mo.
[n]Poly-Prep Columns, BioRad Laboratories, Hercules, Calif.
[o]AG MP-1, BioRad Laboratories, Hercules, Calif.
[p]GraphPad Prism 3.0 for Windows, GraphPad Software, San Diego, Calif.

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