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Elevations of serum cystathionine and total homocysteine in pyridoxine-, folate-, and cobalamin-deficient rats

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Because both basal and postmethionine load hyperhomocysteinemia are correlated with vascular disease, there is a need to determine the underlying causes. Although deficiencies of folate, cobalamin, and pyridoxine have all been implicated there are difficulties in distinguishing among them.

The objective was to determine the changes in total homocysteine, cystathionine, and other methionine, one carbon- and tryptophan-related metabolites in rats with dietary induced folate or pyridoxine deficiency and cobalamin analogue induced deficiency.

Pyridoxine-deficient diet (0 mg/kg) increased basal serum cystathionine levels (14 fold) after 51 days with a smaller increase in basal total homocysteine. Diets with 0 to 1.5 mg/kg pyridoxine also caused graded increases in serum cystathionine. Total homocysteine and cystathionine were markedly increased postmethionine load in the pyridoxine deficient rats as compared to the control, folate-, or cobalamin-deficient rats. Folate- and cobalamin-deficient rats had marked increases in basal total homocysteine and moderate increases in basal cystathionine, but the increments in postmethionine load values were not different than control. Pyridoxine deficiency decreased liver serine transhydroxymethylase activity to 6%. Post-tryptophan load serum xanthurenic acid was equal in cobalamin- and pyridoxine-deficient rats. Serum quinolinic acid was increased only in the cobalamin-deficient rats. Plasma pyridoxal-5'-phosphate levels were not as sensitive as serum cystathionine in pyridoxine deficiency.

Basal and postmethionine load serum cystathionine levels are very sensitive in detecting pyridoxine deficiency and will be useful when determining the cause of clinical hyperhomocysteinemia. (J. Nutr. Biochem. 8: 279–289, 1997) © *Elsevier Science Inc. 1997*

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Introduction

Elevations in serum total homocysteine (tHcy) either in baseline or post-methionine load samples are frequently found in subjects with all types of vascular disease.[1–4] Although recent studies in humans show that vitamin deficiencies play an important causative role in this hyperhomocysteinemia, it is not clear at present which of the vitamins associated with methionine metabolism are the most important (*Figures 1,* and *2*).[5–7] In past investigations we have shown that human subjects with cobalamin, folate, or pyridoxine deficiency have different patterns of abnormality of methionine-related metabolites, although they may all have elevations to varying degrees of serum tHcy.[8–10] In cobalamin deficiency, subjects also have elevations of methylmalonic acid (MMA)[11] and many have elevations of cystathionine[12] and 2-methylcitric acid,[13] and *N*,N-dimethylglycine.[14] MMA and 2-methylcitric acid levels are normal in subjects with folate deficiency.[13] Serum cystathionine is often elevated[12] in folate deficiency and many subjects have elevations of both *N*,-N-dimethylglycine and *N*-methylglycine.[14]

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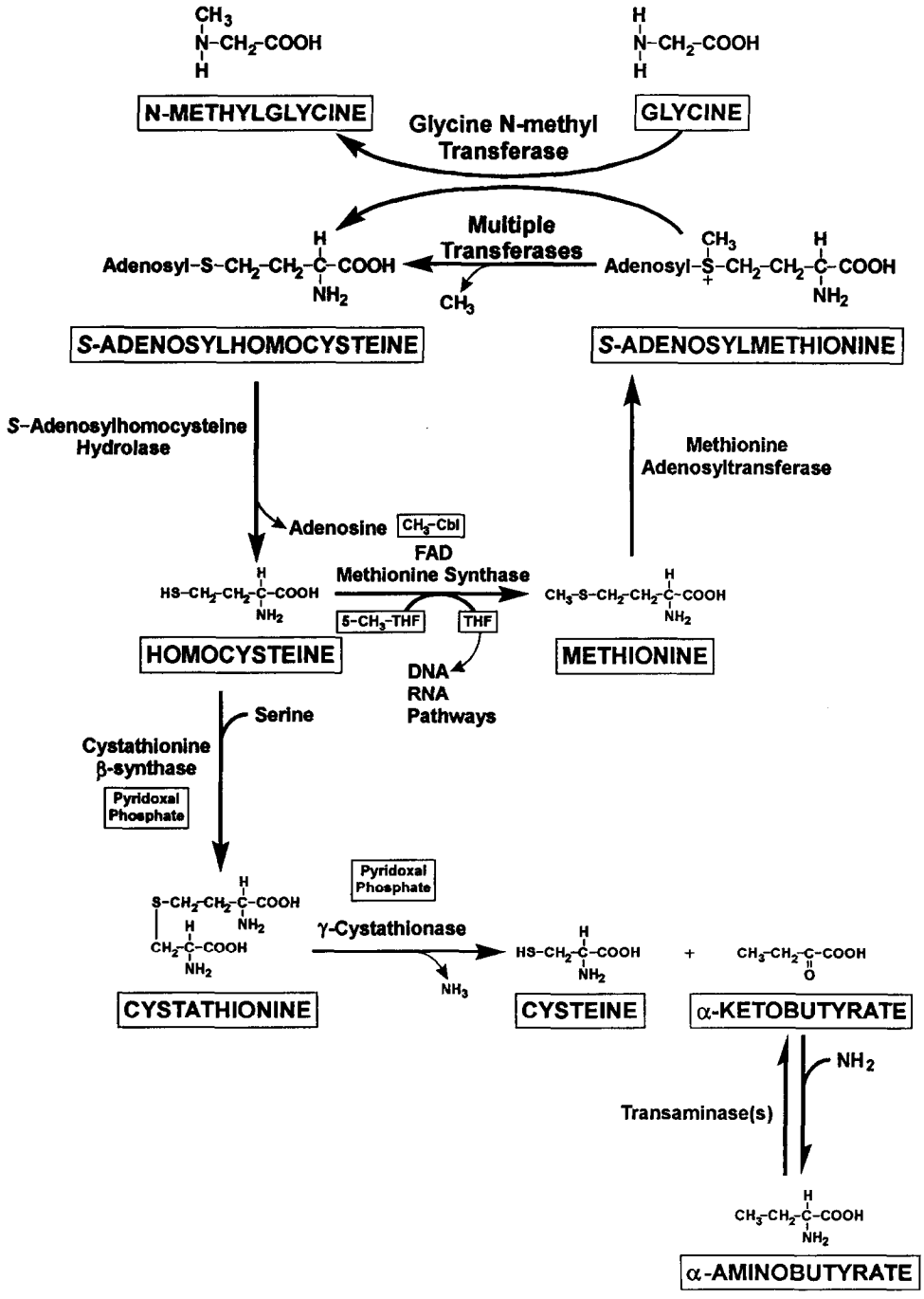

Figure 1 Methionine metabolism—the metabolic role of homocysteine is shown.[6] It can be methylated by N,5-methyltetrahydrofolate to form methionine in a methylcobalamin requiring reaction by methionine synthase. Alternatively, it can be condensed with serine in a pyridoxal-5'-phosphate dependent reaction by cystathionine-β-synthase to form cystathionine. Cystathionine can be cleaved in another pyridoxal-5'-phosphate dependent reaction to form cysteine, α-ketobutyrate, and ammonia. α-ketobutyrate can be transaminated to form α-aminobutyrate. Methionine can be activated to *S*-adenosylmethionine, which is the cofactor for many methylation reactions, including the methylation of glycine by glycine N-methyltransferase.[7] With high methionine intake, the excess *s*-adenosylmethionine is removed by production of N-methylglycine and increased *s*-adenosylmethionine activates cystathionine β-synthase to remove excess homocysteine.

In contrast to folate and Cbl deficiency, it is not known how important pyridoxine (B_6) deficiency might be in causing basal hyperhomocysteinemia. Serum levels of vitamin B_6 have been found to be low in some subjects with hyperhomocysteinemia.[5,15,16] A study of elderly subjects on a vitamin B_6-deficient diet, however, revealed only one who had a marked increase in tHcy.[17] Animal studies have shown that although basal homocysteine is often not increased in B_6 deficiency, postmethionine load homocysteine levels are quite elevated.[18] Thus, it is possible that some subjects with postload hyperhomocysteinemia in studies of vascular disease had B_6 deficiency.[1,3] Unlike Cbl and folate

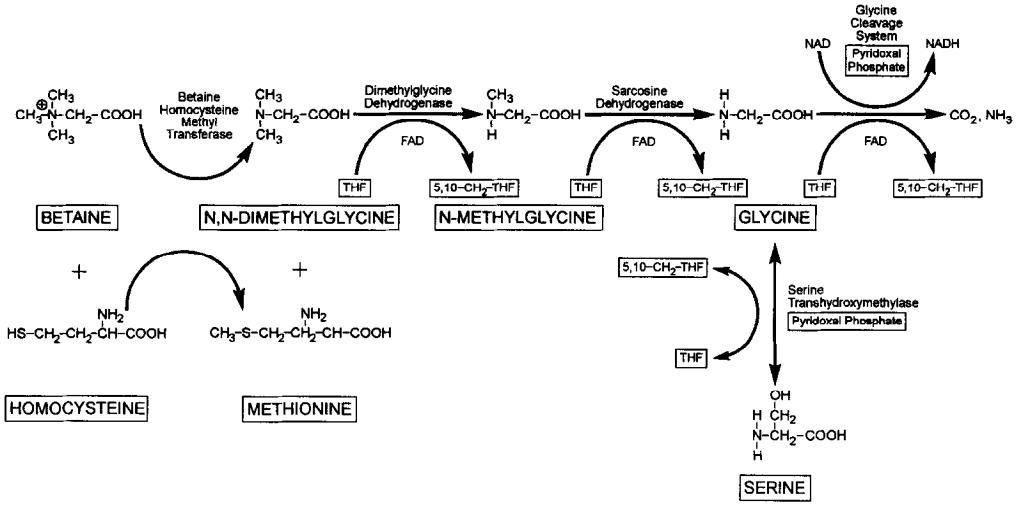

Figure 2 The cobalamin and folate independent methylation of homocysteine is shown.[7] Betaine can donate a methyl group to homocysteine to form methionine by betaine homocysteine methyltransferase. In reactions dependent on folate, *N*, N-dimethylglycine can be demethylated to N-methylglycine and glycine. Serine and glycine can be interconverted by a folate and pyridoxal-5'-phosphate dependent reaction by serine transhydroxymethylase. In addition, glycine can undergo the glycine cleavage reactions.

deficiency syndromes, which are clinically characterized and fairly common,[8] no common clinical syndrome of vitamin B_6 deficiency has been described. Vitamin B_6 is protein-bound in food and it is plausible, but speculative, that patients with various types of malabsorption might have difficulty in extraction and B_6 absorption analogous to the situation with Cbl. In addition, there are some drugs in clinical usage that interfere with vitamin B_6 metabolism.[19] We have recently shown that asthma subjects taking the pyridoxal kinase inhibitor, theophylline, have increased basal and postload cystathionine levels and increased post-methionine load tHcy levels that can be improved with pyridoxine treatment.[9] There had been previous studies of cystathionine levels in experimental models of B_6 deficiency in both animals and humans; however, the lack of a sensitive assay for cystathionine had precluded its use previously as a clinical diagnostic tool. Other methods of diagnosing B_6 status have been reviewed[19] and have included pyridoxal phosphate assays, urinary pyridoxic acid excretion, erythrocyte alanine aminotransferase percentage stimulation, and changes in post-tryptophan load excretion of tryptophan metabolites such as xanthurenic acid and kynurenic acid. There have been many studies showing abnormalities in tryptophan metabolites in B_6-deficient rats and humans,[20–24] but few have studied these simultaneously with the methionine metabolites to determine their relative diagnostic utility.

Thus, to determine whether changes in serum cystathionine and other methionine-related metabolites would be useful markers of B_6 deficiency, we have induced vitamin deficiency in rats with defined diets deficient in folic acid or B_6 and have induced Cbl deficiency with subcutaneous infusion of a Cbl analogue. We have shown that B_6 deficiency causes a markedly different pattern of methionine, one-carbon and tryptophan metabolites than either Cbl or folate deficiency. This will prove useful in studying the causes of hyperhomocysteinemia, diagnosing vitamin B_6-deficient patients and guiding replacement therapy.

Methods and materials

Induction of vitamin deficiency

Adult male Sprague-Dawley rats with a starting weight of 200 to 300g were maintained in standard cages with continuous access to food. The diets were an L-amino acid-defined pelleted rat diet, similar to a diet described previously[25] obtained from Dyets, Inc., Bethlehem, PA, USA, containing methionine 6g/kg, choline bitartrate 2 g/kg, folic acid 8 mg/kg, pyridoxine 7 mg/kg, cyanocobalamin 10 μg/kg, and succinyl sulfathiazole 1 g/kg. The folic acid-deficient diet was identical, except that folic acid was omitted. In the B_6-deficient diets, pyridoxine was deficient or provided in graded amounts. The Cbl-deficient rats consumed the control diet and had a continuous infusion of a Cbl analogue OH-Cbl (c-lactam) 1.0 μg/hr by osmotic minipump (Alza Corp., Palo Alto, CA USA). This Cbl analogue has been shown previously to produce marked elevations in serum MMA and tHcy and decreases in liver methionine synthase and L-methylmalonyl-CoA mutase activity in rats within 2 weeks.[26] The osmotic minipumps were placed under Metofane anesthesia in the subcutaneous tissue of the interscapular area and replaced every 2 weeks. Some animals were made deficient in two vitamins or in one group all three vitamins simultaneously. A pair-feeding design was not used, because we wished to duplicate the human clinical situation more closely.

Three experiments were performed. In the first experiment groups of 8 or 9 rats were fed the control, folate, or B_6 (0 mg/kg)-deficient diet or infused with the cobalamin analogue for 48 days, at which time they were subjected to a subcutaneous methionine load (see below). Three days later they were killed and blood was obtained for assays as described below. Blood samples obtained by percutaneous cardiac puncture under Metofane anesthesia were obtained also at 2 and 4 weeks. Because of the marked changes in serum cystathionine seen in the animals on the 0 mg/kg B_6 diet, an experiment was performed with diets graded in pyridoxine content. In this experiment (experiment 2) groups of 8 rats were fed diets containing 0, 0.25, 0.5, 0.75, 1.0, 1.5, and 7 mg/kg pyridoxine for 39 days, at which time they underwent the methionine load and were sacrificed on day 42 as below. Blood samples were obtained at 2 weeks in this experiment also. Because human subjects commonly present with combined vitamin defi-

ciency, a third experiment studying combined vitamin deficiency in the rats was performed. In experiment 3, groups of 8 rats each were made deficient in both folate and B_6, folate and Cbl, B_6 and Cbl, or all three.

The rats were weighed every 2 weeks for the first 6 weeks, and in Experiment 3, were weighed weekly thereafter. In the long-term experiments of combination vitamin deficiency when the mean weight had fallen to 85% of the previous weight, the animals were killed. In addition to weight loss, the animals were observed for loss of righting ability, grooming activity, or other signs of distress. A number of anesthesia deaths decreased the final group sizes. Euthanasia was accomplished by exsanguination under deep Metofane anesthesia. All experiments were performed under protocols approved by the University of Colorado Institutional Animal Care and Use Committee following the guidelines of the Animal Welfare Act.

Methionine loading

Both methionine and combined methionine-tryptophan loading studies were performed to determine their effect on serum metabolites. Either L-methionine (100 mg/kg) or a combination of L-methionine (100 mg/kg) and L-tryptophan (50 mg/kg) were given by subcutaneous injection of the dorsal surface of the rat and blood was obtained by cardiac puncture 40 min later. This time point was chosen because of pilot studies showing the peak tHcy level at that time. The levels of metabolites after the methionine load were compared with A.M. blood levels obtained 3 days after the load at the time of euthanasia. It was not technically possible to obtain two 1 mL blood samples in the same rat on the same day by cardiac puncture.

Metabolite assays

Blood was obtained by cardiac puncture under Metofane anesthesia and allowed to clot for 1 hr. After centrifugation, serum was stored at −20°C. In some experiments, blood was obtained in heparinized tubes and stored at −70°C for vitamin B_6 assays. Serum was assayed for the following metabolites by stable isotope dilution capillary gas chromatography/mass spectrometry in which an individual internal standard is added for each metabolite quantified; MMA, 2-methylcitric acid, total Hcy, methionine, cystathionine, total cysteine, N-methylglycine, *N*,N-dimethylglycine, glycine, and serine as described previously.[11–14]

L-tryptophan-indole-d_5 was obtained from CIL (Woburn, MA USA) D,L-kynurenine 3,3,3′,5′ d_4 xanthurenic acid 3,5,6,7 d_4, and kynurenic acid 3,5,6,7,8 d_5 were obtained from MSD (Montreal, Canada). D,L-α-aminobutyric d_6 acid was obtained from CDN Isotopes (Pointe Claire, Canada and 2,3 pyridoxine 4,5,6 d_3 dicarboxylic acid (quinolinic) was obtained from Isotec, Inc., Miamisburg, OH USA). Tryptophan and kynurenine were assayed by modification of our assay for tHcy. The initial column temperature was 75°C and then was increased at 10°C/min to 113°C, then by 30°C/min to 320°C. The retention times and monitored ions for tryptophan and kynurenine were 10.88 min and M/Z 249 and 244 and 9.92 min and M/Z 365 and 362, respectively. The temperature program for α-aminobutyric acid was an initial temperature of 80°C and increased by 30°C/min to 320°C and the monitored ions M/Z 280 and 274 eluted at 4.20 minutes. Kynurenic acid, xanthurenic acid, and quinolinic acid were assayed by modification of our methods for organic acids,[13] except that the final temperature was 320°C instead of 300°C and had retention times of 7.90, 9.08, and 6.69 min, respectively. The ions monitored were as follows: kynurenic acid M/Z 365, 360 and xanthurenic acid M/Z 493, 490, and quinolinic acid M/Z 341, 338.

Serum Cbl and folate levels were measured by radiodilution assay with a commercial kit, Magic B_{12}/Folate, Ciba-Corning, Medfield, MA USA. Heparinized plasma stored at −70°C was shipped on dry ice to Colorado State University, where it was assayed by a HPLC method described previously quantifying pyridoxal-5′-phosphate, pyridoxine, and pyridoxal.[27]

Enzyme assay

Serine transhydroxymethylase (SHMT) was assayed on liver homogenates by the method of Chen and Schirch, which assays activity by demonstrating an exchange of the alpha hydrogen of glycine with protons of the solvent.[28] At the time of killing, rat liver was frozen immediately on dry ice and stored at −20°C. The frozen rat liver was homogenized in 2.5 volumes of 0.028 M NaPO4 pH7 and centrifuged at 37,000 × G for 20 min. The supernatants were assayed both with and without the cofactors, tetrahydrofolate 0.2 nM, and pyridoxal-5′-phosphate 4 nM. This assay on a total liver homogenate will not distinguish between cytoplasmic and mitochondrial SHMT activity, but measures the combined activity.

Statistics

The mean ± standard deviation was calculated for the various metabolites. Statistical significance was determined by analysis of variance (ANOVA). Ranked means were analyzed with a Kruskal–Wallis one-way ANOVA for nonparametric values. The significance level was 0.05.

Results

Changes in cystathionine and tHcy levels

In the first experiment comparing the different vitamin deficiency syndromes, a B_6-deficient diet (0 mg/kg) caused a significant 14 fold increase in basal serum cystathionine levels 13.7 ± 2.6 μmol/L versus 0.9 ± 0.21 μmol/L in the controls, after day 51 as shown in *Table 1*. Induction of Cbl deficiency by an analogue and dietary folate deficiency also significantly increased the basal cystathionine levels to 1.6 ± 0.1 and 2.0 ± 0.9, respectively. The rise in cystathionine in the B_6-deficient rats was significantly greater than that in Cbl- and folate-deficient rats. In contrast to the changes in basal cystathionine, B_6 deficiency increased the basal tHcy levels only modestly over the control as shown in *Table 1*. As expected, the basal tHcy levels in the folate- and Cbl-deficient rats were increased markedly and significantly greater than the controls and the B_6-deficient rats. In addition, the rise in basal tHcy because of folate deficiency was significantly greater than that seen with the Cbl analogue. Elevations of basal serum cystathionine were very sensitive to the amount of B_6 in the diet as shown in *Table 1*. In Experiment 2, when groups of 8 rats were fed with diets graded in B_6 content ranging from 0 to 7 mg/kg for 42 days, the level of cystathionine increased as the B_6 was decreased in the diet. Basal cystathionine levels from rats consuming 0 to 1.0 mg/kg, were significantly higher than levels in animals consuming 1.5 mg/kg and 7 mg/kg (control diet). In addition, the basal cystathionine level was significantly greater in those consuming 0 mg/kg than those consuming 0.5 to 1.5 mg/kg. The group consuming 0.25 mg/kg was significantly greater than 0.75 to 1.5 mg/kg and 0.5 was significantly greater than 1.5 mg/kg. The basal serum tHcy levels obtained simultaneously on these animals did not show the same graded changes, with only the levels

Table 1 Basal serum methionine-related metabolites and vitamin levels in vitamin-deficient rats

Condition	Cystathionine (μmol/L)	Hcy (μmol/L)	Aminobutyric Acid (μmol/L)	Folate (nmol/L)	Cobalamin (pmol/L)	Total B_6‡ (μmol/L)	PLP‡ (μmol/L)
Experiment 1—Day 51							
Control (N = 8)	0.9 ± 0.2	6.3 ± .97	N/A	95 ± 8	1030 ± 110	N/A	N/A
Cbl Analogue (N = 8)	1.6 ± 0.1*	18.9 ± 2.7*[a,f]	N/A	100 ± 11[f]	1400 ± 100*	N/A	N/A
Folate Deficiency (N = 6)	2.0 ± 0.9*	41.0 ± 20.1*[a,g]	N/A	3 ± 0.7*[g]	890 ± 80*[g]	N/A	N/A
Pyridoxine Deficient 0 mg/kg diet (n = 9)	13.7 ± 2.6*[f,g]	10.7 ± 5.3*[f,g]	N/A	89 ± 12[f,g]	810 ± 100*[g]	N/A	N/A
Experiment 2—Day 42							
Pyridoxine Deficient							
0 mg/kg diet (N = 7)	6.5 ± 1.7*	6.3 ± 1.5	3.8 ± 0.3*	59 ± 10*	960 ± 80*	.019 ± .009*	.017 ± .009*
0.25 mg/kg diet (N = 8)	3.6 ± 1.3*	5.0 ± 1.0	4.1 ± 0.4*	64 ± 4*	960 ± 120*	.045 ± .033*	.021 ± .008*
0.5 mg/kg diet (N = 8)	2.0 ± .07*[a]	4.7 ± 0.9[a]	4.5 ± 0.6	66 ± 7*	1030 ± 130	.038 ± .022*	.049 ± .057*
0.75 mg/kg diet (N = 8)	1.4 ± 0.3*[a,b]	4.1 ± 0.6[a]	5.3 ± 1.4[a,b]	77 ± 9[a,b]	1100 ± 120	.061 ± .015*	.045 ± .010*
1.0 mg/kg diet (N = 8)	1.3 ± 0.3*[a,b]	5.0 ± 0.6	5.7 ± 0.5[a,b]	75 ± 8[a]	1200 ± 120[a,b]	.076 ± .026*	.047 ± .016*
1.5 mg/kg diet (N = 8)	0.9 ± 0.1[a,b,c,d,e]	4.8 ± 0.9	5.6 ± 0.6[a,b]	75 ± 9[a]	1200 ± 60[a,b]	.183 ± .056*	.073 ± .022*
Control 7.0 mg/kg diet (N = 7)	0.8 ± 0.1	5.2 ± 0.7	8.1 ± 1.5	82 ± 7	1200 ± 100	.880 ± .293	.497 ± .268

Different than: *control $P < 0.05$, [a]0 mg/kg pyridoxine diet $p < 0.05$, [b]0.25 mg/kg pyridoxine diet $P < 0.05$, [c]0.5 mg/kg pyridoxine diet $P < 0.05$, [d]0.75 mg/kg pyridoxine diet $P < 0.05$, [e]1.0 mg/kg pyridoxine diet $P < 0.05$, [f]folate deficient $P < 0.05$, [g]Cbl analogue $P < 0.05$. ‡Total B_6 refers to the sum of pyridoxal and pyridoxal-5′-phosphate (PLP) except in one rat in the 0.75 mg/kg pyridoxine diet group that also had measureable amounts of pyridoxine that was included in the total B_6. N/A, not available.

in the animals consuming 0.5 mg/kg and 0.75 mg/kg being less than the 0 mg/kg, but none of the levels being significantly greater than in the control animals. Basal α-aminobutyric acid levels were significantly decreased in the graded diet experiment at the lowest levels 0 and 0.25 mg/kg B_6, as compared to the control or 0.75 to 1.5 mg/kg levels (*Table 1*). α-aminobutyrate was not measured in the first experiment comparing the different vitamin deficiency syndromes.

In *Experiment 1*, changes in basal cystathionine levels occurred early in the 0 mg/kg B_6 deficient diet and had significantly increased over the controls (6 fold) and the Cbl- and folate-deficient animals by 2 weeks. In the graded diet study (Experiment 2), the basal cystathionine levels in the 0 mg/kg consuming animals were significantly higher also than those consuming 0.5 mg/kg and the 0.25 group was significantly higher than those consuming 0.75, and the 0.5 mg/kg and the 0.75 mg/kg were also higher than the controls by 2 weeks. The basal cystathionine levels in folate- and Cbl-deficient animals were also significantly higher at 2 weeks than the controls in Experiment 1. Increases in basal tHcy occurred early at 2 weeks in the folate- and Cbl-deficient animals, but not in any of the B_6-deficient animals (data not shown).

Methionine loading in vitamin-deficient rats

Figure 3 shows the basal and postmethionine load tHcy levels for the various vitamin deficient groups in *Experiment 1*. Although the vitamin B_6-deficient group (0 mg/kg diet) had only a slightly elevated serum tHcy level in the basal condition, after loading with methionine there was a marked rise in the serum tHcy level. The difference between the postmethionine load and basal level was significantly greater in the rats consuming 0 mg/kg B_6 than in the control or folate- or Cbl-deficient rats. Although the tHcy levels in the basal condition were significantly higher than control in the folate- and Cbl-deficient rats, the increment in tHcy postload was not significantly different than the control. In Experiment 2 (graded B_6-containing diet), only the group consuming 0 mg/kg B_6 had a significantly greater rise in tHcy after loading than that in the control group. The 0 mg/kg group mean increment in tHcy was also higher than the rise seen in the rats consuming 0.5 to 1.5 mg/kg (*Table 2*).

In Experiment 1, the rise in serum cystathionine post-methionine load was significantly greater (approximately 10 fold) in the B_6-deficient animals (0 mg/kg) than in the control, folate-, or Cbl-deficient animals, and was very

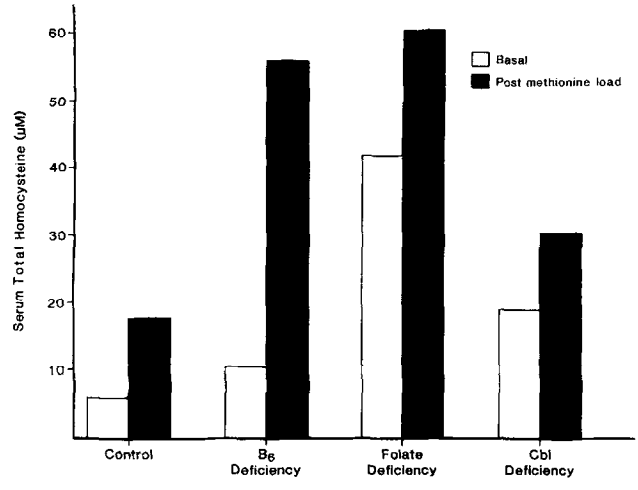

Figure 3 Total homocysteine levels pre- and post-methionine load (100 mg/kg subcutaneously) in groups of control, pyridoxine (B_6), folate and cobalamin deficient rats as described in Methods and materials. The rise in homocysteine postmethionine load was significantly greater in pyridoxine deficient rats ($P < 0.05$) as compared to the control, folate-, or cobalamin-deficient rats.

Table 2 The change in serum metabolites 40 minutes after subcutaneous injection of methionine 100 mg/kg and tryptophan 50 mg/kg

Condition	**Δ Hcy	Δ Cystathionine	Δ Aminobutyric acid	Δ Xanthurenic acid
Experiment 1—Day 51				
Control (N = 8)	11.0	0.3	N/A	94
Cbl analogue (N = 8)	10.9[a]	0.3[a]	N/A	202*
Folate deficiency (N = 6)	15.5[a]	1.3[a]	N/A	153
Pyridoxine Deficiency (N = 9) (0 mg/kg diet)	45*	3.9*	N/A	236*
Experiment 2—Day 42				
Pyridoxine Deficiency				
0 mg/kg diet (N = 7)	26.6*	3.1*	3.7*	368*
0.25 mg/kg diet (N = 8)	19.3	1.2*	7.1*	173
0.5 mg/kg diet (N = 8)	17.8[a]	1.0*	8.8*	202
0.75 mg/kg diet (N = 8)	15.4[a]	0.8*	12.5*	142[a]
1.0 mg/kg diet (N = 8)	15.4[a]	0.5[a,b,c]	10.9*	139[a]
1.5 mg/kg diet (N = 8)	15.2[a]	0.4[a,b,c,d]	14.0*[,a]	141[a]
7.0 mg/kg diet control (N = 7)	15.9	0.3	24.7	111

*Different from control $p < 0.05$. **ΔDifference between the basal and postload value.
Different from [a]0 mg/kg pyridoxine deficient $P < 0.05$, [b]0.25 mg/kg pyridoxine deficient $P < 0.05$, [c]0.5 mg/kg pyridoxine deficient $P < 0.05$, [d]0.75 mg/kg pyridoxine deficient $P < 0.05$. N/A, assay not performed on these samples.

sensitive to the amount of B_6 in the graded diet (experiment 2). The increase in cystathionine was significantly greater for the 0 mg/kg as compared to control. In addition, 0 was greater than 1.0 and 1.5 mg/kg, 0.25 was greater than 1.0 and 1.5 mg/kg, 0.5 greater than 1.0 and 1.5 mg/kg, and 0.75 was greater than 1.5 mg/kg.

In Experiment 2, the rise in α-aminobutyric acid after methionine loading was significantly decreased in the rats consuming 0 to 1.5 mg/kg B_6 as compared to the control group and the 0 mg/kg group was significantly lower than the 1.5 mg/kg group also.

Mixed vitamin deficiency

In the third experiment, groups of 8 rats each were placed on combination vitamin-deficient regimens. By day 14, the mean basal cystathionine level was increased significantly in the groups subject to combinations including B_6 deficiency, but not more increased than the B_6 deficiency group alone. By day 28, the mean basal tHcy had increased since the last time point in all the combined groups and the triple-deficient and combined Cbl- and folate-deficient group were higher than any other group with the exception of the folate deficiency group alone. From these data, it appeared that the effect of B_6 deficiency was mostly in increasing the basal cystathionine level and the effect of folate deficiency was predominant in increasing the basal tHcy level. The combined deficiency groups were maintained until they lost 15% of weight between two weighing periods. Therefore, the different groups were killed after different time intervals. As expected, the triple-deficient group deteriorated the quickest and were killed at day 84 followed by the B_6 and folate group and Cbl- and folate-deficient group also at day 96. The combination of B_6 deficiency and Cbl analogue infusion was tolerated the best with the animals not achieving the target weight loss until day 254.

Serine, glycine, and other metabolites involved in one-carbon metabolism

The mean levels of glycine, serine, *N*-N-dimethylglycine, N-methylglycine and liver SHMT activity are shown in *Table 3* for the vitamin deficient rats in Experiment 1 at day 51. B_6 and folate deficiency increased glycine levels significantly (>2 fold) whereas, Cbl deficiency did not. Also, the lowest (0 to 0.5 mg/kg) of B_6 dietary content in Experiment 2 caused significantly higher levels of glycine than 0.75 to 7 mg/kg (data not shown). Serine levels were significantly

Table 3 Basal serum metabolites and liver enzyme activity related to one-carbon metabolism

Condition	Glycine (μmol/L)	Serine (μmol/L)	*N*,N-Dimethylglycine (μmol/L)	N-Methylglycine (μmol/L)	Holo SHMT (e.u.)‡	Total SHMT (e.u.)‡
Experiment 1—Day 51						
Control (N = 8)	708 ± 110	369 ± 28	3.4 ± .39	3.0 ± .60	1.6 ± .41	1.8 ± .40
Cbl analogue (N = 8)	834 ± 46	450 ± 51*	3.7 ± .45	2.8 ± .38	1.5 ± .20	1.6 ± .25
Folate deficiency (N = 6)	1890 ± 1660*[,b]	430 ± 16*	4.1 ± .76[a]	4.6 ± 1.4*[,a,b]	2.0 ± .26[b]	2.0 ± .25
Pyridoxine deficiency 0 mg/kg diet (N = 9)	1630 ± 230*[,b]	313 ± 29*[,b,c]	3.1 ± .30	2.6 ± .19	0.1 ± .06*[,b,c]	0.4 ± .10*[,b,c]

Different than: *control $P < 0.05$, [a]0 mg/kg pyridoxine diet $p < 0.05$, [b]Cbl analogue $P < 0.05$, [c]folate deficiency $P < 0.05$. ‡μmol/min/g liver wet weight.

increased by Cbl analogue infusion and folate deficiency and significantly decreased by B_6 deficiency at day 51 in experiment 1 (*Table 3*). After 42 days in Experiment 2, serine was not changed, however (data not shown). Liver SHMT was assayed both without added tetrahydrofolate and PLP (holo) and with added cofactors (total) on liver homogenates from the groups in Experiment 1. Vitamin B_6 deficiency (0 mg/kg) decreased holo and total liver SHMT to 6% and 22% of control activities. Folate deficiency increased the holo activity slightly over the control and Cbl analogue groups. For the control, folate- and Cbl-deficient groups, there was only a small augmentation in activity when the enzyme was assayed with added cofactors. In the case of the B_6-deficient rats, however, the level of activity increased 4 fold with added PLP and tetrahydrofolate as shown in *Table 3*.

The only significant change in *N*,N-dimethylglycine levels at day 51 in Experiment 1 was that the level in the B_6-deficient rats was significantly less than that in the folate- and Cbl-deficient groups. N-methylglycine was significantly increased in the folate deficient rats as compared to the control, Cbl-, or B_6-deficient animals (*Table 3*). Methionine was not significantly different in the vitamin-deficient groups as compared to control (data not shown). The total cysteine level was increased in the B_6-deficient animals at day 51 (Experiment 1) as compared to the control and the folate-deficient animals. The significance of this change in total cysteine is called into question; however, because in Experiment 2 (graded B_6 diet) the levels at day 42 were not significantly different from the control and the mean cysteine levels were actually lower rather than higher than the control (data not shown).

Changes in vitamin levels

The levels of serum Cbl, folate, and total plasma B_6 (the sum of pyridoxal and PLP) and the PLP are shown in *Table 1* from Experiment 1. The serum Cbl levels decreased significantly as compared to control in both the B_6- and folate-deficient animals after day 51 of deficiency. The serum Cbl levels were increased in the Cbl analogue infused animals because of displacement of Cbl from tissue and/or decreased plasma clearance. In addition, the analogue is also measured to a small degree by the Cbl binding assay. In the graded B_6-deficient diet, Experiment 2, at day 42, the two lowest groups 0 and 0.25 mg/kg had significantly lower Cbl levels than the controls and the 1.0 and 1.5 mg/kg groups. Severe Cbl deficiency was confirmed in the Cbl analogue treated rats (despite the elevated Cbl levels) because the MMA levels were markedly increased over the control (160 fold) 58,000 versus 362 nmol/L. It is unlikely that the decrease in serum Cbl in the folate- and B_6-deficient animals represented Cbl deficiency because the serum MMA was actually *lower* than control in these groups (data not shown). The total 2-methylcitric acid levels were significantly increased in the Cbl-deficient rats (confirming deficiency also) as compared to the control and unchanged in the folate- or B_6-deficient rats.

The serum folate level was significantly decreased in the folate-deficient group as compared with control, Cbl- and B_6-deficient groups in Experiment 1. The folate level in the B_6-deficient group was less than control at day 51 but did not reach statistical significance. In Experiment 2, however, the groups of rats consuming the lowest amounts of B_6 (0 to .5 mg/kg) had a significantly decreased serum folate as compared to the control group. The total plasma B_6 (sum of pyridoxal and PLP) and the PLP levels are shown in *Table 1* for only Experiment 2. Only one animal (from the 0.5 mg/kg group) out of the entire experiment had detectable levels of pyridoxine and that amount was included in the total B_6 value for that animal. Both the total B_6 and PLP levels were decreased in all of the diet groups 0 to 1.5 mg/kg as compared to the control group, however, the levels were not graded. B_6 levels were not performed on the animals in Experiment 1.

Tryptophan metabolites

There was no difference in basal serum tryptophan levels in the vitamin deficient animals as compared to control in Experiments 1 or 2 (data not shown). Basal kynurenine levels were increased in the control as compared to the B_6- or folate-deficient animals at day 51 from Experiment 1 (*Table 4*). In Experiment 2 (graded B_6 diet), however, at day 42 there was no significant difference in the kynurenine levels as compared to controls. In experiment 1, after day 51, the B_6-deficient animals (0 mg/kg) had significantly lower basal kynurenic acid levels as compared to the other vitamin deficiencies and the controls (*Table 4*). The folate-deficient animals also had a decrease in basal kynurenic acid as compared to the control and the Cbl-deficient group. Similar findings were found in Experiment 2 in that the basal kynurenic acid was significantly decreased in the animals consuming 0 to 0.5 mg/kg. The levels in those consuming 0.5 mg/kg were significantly less than those eating 1.5 mg/kg, and the lowest level was obtained in those eating 0.25, which was significantly less than the 0.75 mg/kg and 1.0 mg/kg groups also. In Experiment 1 at day 51 there was no difference in basal serum xanthurenic acid in the vitamin-deficient groups. In the graded diet at day 42, the highest level was seen in the group eating 0 mg/kg, but this was only significantly greater than the group eating 0.25 and not different than the controls or the others. Basal serum xanthurenic acid, however, was significantly increased in the B_6-deficient animals (8 fold) at 14 days over the other groups in Experiment 1. In Experiment 2, the 0 and 0.5 mg/kg consuming groups were significantly increased over control also at day 14. In the subgroup of rats from Experiment 1 (n = 3, 4, or 5) who received a combined methionine/tryptophan load, the rise in xanthurenic acid was significantly increased and was approximately equal in the Cbl-deficient and B_6-deficient animals. In Experiment 2, only the 0 mg/kg group had a significantly greater rise in xanthurenic acid than the control and also higher than the 0.75 to 1.5 mg/kg groups. In Experiment 1, after day 51, the basal quinolinic acid was increased in the Cbl-deficient rats and was significantly greater than the control, B_6-, or folate-deficient rats. The level in the B_6 deficient rats was significantly less than the controls. After day 42 of deficiency in the graded diet group (Experiment 2) only the group consuming 0 mg/kg and the group consuming 0.75

Table 4 Basal serum metabolites related to tryptophan metabolism

Condition	Kynurenine (μmol/L)	Kynurenic acid (μmol/L)	Xanthurenic acid (μmol/L)	Quinolinic acid (nmol/L)
Experiment 1--Day 51				
Control (N = 8)	2.2 ± .34	27 ± 5.1	15 ± 2.8	340 ± 61.7
Cbl analogue (N = 8)	2.0 ± .40	26 ± 3.6[a]	14 ± 3.6	530 ± 88.7*
Folate deficiency (N = 6)	1.5 ± .33*	19 ± 2.4*,[a,c]	12 ± 2.5	290 ± 97.1[c]
Pyridoxine deficient 0 mg/kg diet (N = 9)	1.3 ± .15*,[c]	13 ± .45*	14 ± 5.2	180 ± 47.5*
Experiment 2--Day 42				
Pyridoxine deficient				
0 mg/kg diet (N = 7)	2.7 ± .87	23 ± 8.5*	38.4 ± 19.0	215 ± 53.7
0.25 mg/kg diet (N = 8)	2.0 ± .54	16.9 ± 3.1*	12.7 ± 7.5[a]	231 ± 42.6
0.5 mg/kg diet (N = 8)	2.2 ± .81	21.8 ± 3.3*	19.1 ± 16.2	236 ± 42.2
0.75 mg/kg diet (N = 8)	2.3 ± .66	27.8 ± 8.2[b]	17.0 ± 8.0	215 ± 77.1
1.0 mg/kg diet (N = 8)	2.8 ± .63	32.5 ± 12.6[b]	15.2 ± 2.5	331 ± 99.0[a,d]
1.5 mg/kg diet (N = 8)	2.9 ± .80	33.2 ± 7.6[b,c]	14.0 ± 3.9	286 ± 74.5
Control 7.0 mg/kg diet (N = 7)	2.5 ± .78	51.7 ± 34.1	16.0 ± 4.1	278 ± 62.0

Different than: *control $P < 0.05$, [a]0 mg/kg pyridoxine diet $P < 0.05$, [b]0.25 mg/kg pyridoxine diet $P < 0.05$, [c]0.5 mg/kg pyridoxine diet $P < 0.05$, [d]0.75 mg/kg pyridoxine diet $P < 0.05$, [e]Cbl analogue $P < 0.05$, [f]folate deficiency $P < 0.05$.

mg had lower quinolinic acid than the group consuming 1 mg/kg and not significantly less than the control group.

Health effects

In Experiment 1, by day 51, the B_6 deficient (0 mg/kg) and the folate-deficient rats had gained significantly less weight than the controls (31% and 53% versus 62% of their starting weight, respectively). In addition, the B_6-deficient rats had gained significantly less than the folate and Cbl analogue groups. In Experiment 2, only the group consuming 0.25 mg/kg gained less weight than the controls by day 42. No changes in activity level, grooming, or coat condition were noted in the vitamin-deficient groups. In the long-term combined vitamin deficiency groups (Experiment 3), no apparent neurologic disabilities were observed. After day 75 of the combined vitamin deficiencies the mean hemoglobin, white blood cell count, or mean cell volume (MCV) of the different groups were not significantly different from control or each other. However, at the time of sacrifice (day 96) the combined B_6- and folate-deficient group and Cbl and folate deficient group had developed significant anemia and leukopenia. For the combined B_6- and folate-deficient group, the mean hemoglobin dropped from 15.8 to 12.7 g/dL and the white blood count from 6,200 to 1,800 mm^3 for day 75 versus day 96, respectively. The values for the combined Cbl- and folate-deficient group were 17.7 to 13.8 g/dL and 7,400 to 2,600 respectively. The MCV had also significantly fallen from the values at day 75, 58.2 to 51.6 Fl and 55.7 to 53 Fl for the two groups. The group that survived the longest (combined B_6 and Cbl deficiency) was studied at day 187 and had developed neutropenia (2,400 mm^3) and a decreased hemoglobin (14.8 g/dL) without a change in MCV (53.8 Fl).

Correlations between variables

In Experiment 2 (graded B_6 diet) the total plasma B_6, pyridoxal and PLP were all inversely correlated to the 42 day basal cystathionine level (−0.354, −0.364, −0.327 for Pearson correlation coefficients, respectively). In contrast, the basal tHcy level was not correlated with any B_6 value.

In experiment 1, at day 51 in the B_6 deficient group, the basal cystathionine was correlated with basal tHcy and glycine (Pearson correlation coefficients 0.820 and 0.788, respectively) and glycine and serine were correlated (0.728). In the folate-deficient group, basal cystathionine correlated with basal tHcy and glycine also (0.971 and 0.995) and tHcy with glycine (0.972). The only correlation in the Cbl analogue group was a weaker inverse relationship of serum Cbl and serine (−0.778).

Discussion

We have determined that elevations of basal and post-methionine load serum cystathionine levels are an early and sensitive marker of B_6 intake in rats. The rise in serum cystathionine level is so sensitive that it is possible to distinguish diets containing 0 to 1 mg/kg from the control diet of 7 mg/kg. The stepwise rise in serum cystathionine demonstrated that diet concentrations such as 0.25 could be distinguished from 0.75 mg/kg. In contrast, increases in basal tHcy levels were much less impressive in the B_6-deficient rats. The folate- and Cbl-deficient rats had much greater increases in tHcy and much lower increases in cystathionine than the B_6-deficient rats.

We did not use pair feeding to better duplicate the clinical situation in human vitamin deficiency. In Experiment 1, where the animals were maintained for the longer period, the vitamin B_6-deficient and the folate-deficient animals gained less weight than the controls. It is unlikely, however, that the resulting marked increases in cystathionine resulted only from a probable decrease in food intake because similar increases were found in Experiment 2 in which a significant decrease in weight gain was only found in one of the groups after 42 days. We have previously used a pair feeding design in studying Cbl deficiency.[29] The levels of MMA found in the present investigation in the controls and experimentals were virtually identical to the

values found previously.[29] The fact that serum methionine levels were not lower in the different vitamin-deficient groups as compared with control is also against major differences in protein and food intake between the groups. It seems likely that the decreased growth in Experiment 1 in the B_6-deficient rats may not have been totally because of decreased food intake, because previous investigations in young women[30] and rats[31] have shown that more food is necessary to maintain weight or support growth in B_6-deficient individuals.

The changes in serum cystathionine that we found were not unexpected in that early studies in B_6 deficient humans and rats have also found increases[21,32–36] usually in urine samples, but also in plasma in two studies.[33,34] Despite these promising early studies, there has not been much investigation of cystathionine for use in the diagnosis of B_6 deficiency. Because humans have much lower levels of serum cystathionine than rats (0.2 versus 0.9 μmol/L, respectively),[12] it is not surprising that accurate measurements of serum cystathionine and its potential use in the diagnosis of human and animal B_6 deficiency were not practical until more sensitive and specific methodology for quantitation was developed.[12] The isotope dilution gas chromatography/mass spectrometry method used in this investigation made it possible for us to show for the first time that the level of serum cystathionine (basal or postmethionine load) increased with small decrements of B_6 content in the diet.

It has been postulated that B_6 deficiency would cause hyperhomocysteinemia and there is some clinical data inversely correlating lower plasma PLP levels with serum tHcy in humans.[15,16] In contrast to the sensitivity of serum cystathionine in detecting B_6 deficiency, however, the basal serum tHcy in these rats was barely elevated over the control. The folate-deficient and Cbl analogue-treated rats developed the expected hyperhomocysteinemia, as well as modest increases in serum cystathionine, similar to humans with clinical Cbl and folate deficiency.[12] In contrast to our data, a previous study using weanling rats showed that protein-bound homocysteine increased during 5 weeks of a B_6-deficient diet.[37] In a more recent study, with diet induced-vitamin B_6 deficiency, Miller et al. found that 23-month-old rats did not have an increase in fasting tHcy, although 3-month-old rats did have an increase after 9 weeks.[17] In a follow-up study using an amino acid-based diet similar to the one in the present investigation, the same investigators confirmed that fasting tHcy was not elevated after 4 weeks in vitamin B_6-deficient rats as compared to controls. A simultaneous group consuming a folate-deficient diet had the expected 10-fold rise in basal plasma tHcy.[18] It is possible that rats are not a good model for B_6-deficient humans; however, as recently reported, only 1 of 11 elderly human subjects developed hyperhomocysteinemia on a B_6-deficient diet.[17]

In contrast to the insensitivity of basal tHcy levels in B_6 deficiency, we confirmed the findings of Miller et al.,[18] showing that the increment in postload tHcy is markedly elevated in B_6-deficient animals as compared to folate- and Cbl-deficient rats. Even more potentially diagnostically useful than the changes in tHcy were our observations on the postmethionine load cystathionine level. Our observations and those of others should have some effect on the debate about the best ways to detect hyperhomocysteinemia; either in basal samples or postmethionine load, and help diagnose the major underlying causes of hyperhomocysteinemia. For instance, it had been thought previously that a marked rise in tHcy after methionine loading was specific for heterozygosity for cystathionine β-synthase deficiency,[1,3] but it is necessary to include B_6 deficiency in the differential diagnosis. Elevations of basal or postmethionine load cystathionine may distinguish between cystathionine β-synthase deficiency and vitamin B_6 deficiency. We have found that two obligate heterozygotes for cystathionine β-synthase deficiency had normal postmethionine load serum cystathionine levels (S.P. Stabler and R.H. Allen, unpublished observations), but theophylline-treated asthma patients with B_6 deficiency had higher cystathionine levels postload, than controls.[9] The results of our investigations and others[18] suggests that future studies in humans should determine both basal and postload tHcy, and the difference in pre and post values should be reported in addition, because subjects with folate and Cbl deficiency may have a small tHcy increment, but a high post-load value because the preload value was elevated.

Because both cystathionine-β-synthase and γ-cystathionase are PLP dependent, it is interesting that cystathionine, but not tHcy, increases in B_6 deficiency. This may be explained by studies showing that liver γ-cystathionase activity in B_6-deficient rats is decreased markedly as compared with liver cystathionine β-synthase activity, which is either normal or only modestly decreased in B_6-deficient rats.[33,38] The fact that basal and postload α-aminobutyric acid levels were significantly decreased in the B_6-deficient animals strongly suggests that γ-cystathionase activity was decreased. We found that α-aminobutyric acid levels were low in B_6-deficient asthma patients also.[9] Although α-aminobutyric acid levels were reported to increase postmethionine load in a study reported in 1947, there do not seem to be any other recent studies of this amino acid in B_6 or other vitamin deficiencies.[39]

In this investigation we have confirmed the work of previous investigators who have found increases in glycine and decreases in serine in B_6-deficient rats.[40,41] Although glycine increased also in the folate-deficient rats, serine was increased rather than decreased. These changes in glycine and serine may be because the activity of serine transhydroxymethylase was markedly decreased in the B_6-deficient rats. It was surprising that inhibition in SHMT was not seen in the folate-deficient animals despite their elevated glycine levels. Levels of N-methylglycine were elevated in folate deficient rats similar to our findings in folate-deficient humans.[14] Recent studies by Balaghi et al. can provide an explanation for this finding.[42] Using a similar diet to induce folate deficiency in weanling rats, they determined that the activity of glycine N-methyltransferase increased markedly, which they linked to the fact that there was a decrease in N,5-methyltetrahydrofolate, which inhibits glycine N-methyltransferase. This rise in N-methylglycine seems to be quite specific to folate deficiency as compared to Cbl[14] or B_6 deficiency.

The increased excretion in xanthurenic acid after a tryptophan load has been said to be one of the most sensitive

measures of B_6 deficiency.[19] We found that serum xanthurenic acid increased significantly and to the same degree in both B_6- and Cbl-deficient animals' post-tryptophan load. Unlike the postmethionine load cystathionine level, the xanthurenic acid could not detect differences in the content of the graded diet. Responses to tryptophan loading have not been described before in Cbl-deficient humans or other animals. In the present investigation we did not determine the urinary xanthurenic acid, thus, it is possible there would be a different ratio of serum to urine xanthurenic acid in Cbl-deficient rats as compared to B_6-deficient rats. Because the Cbl-deficient animals had marked rises in serum MMA and 2-methylcitric acid, there might have been interference with renal clearance of other organic acids; however, our present investigation did not address this hypothesis. In any case, a rise in *serum* xanthurenic acid post-tryptophan load is not specific to B_6 deficiency and should not be used clinically. We conclude that serum xanthurenic acid levels are not as useful as basal or postload serum cystathionine in detecting B_6 deficiency, and/or decreased oral B_6 intake. The total B_6, pyridoxal and PLP levels were all decreased in the B_6-deficient rats to values consistent with those reported previously. Unlike the cystathionine levels, however, there were not significant differences between the graded diet levels, although the values increased as diet content increased. Measurements of plasma PLP in humans have been found to be affected by conditions other than vitamin B_6 status,[19] thus it seems likely that a functional measure of B_6 status such as the basal or postload cystathionine level may be more useful in diagnosing B_6-deficient humans. We have found for the first time that basal serum quinolinic acid was increased in Cbl-deficient rats and confirmed an old report that quinolinic acid is decreased in B_6-deficient rats.[43]

For the first time a full range of metabolites involved with methionine, one-carbon and tryptophan metabolism have all been measured simultaneously in rats with well documented vitamin B_6, folate and Cbl deficiency. Our description of the specific patterns of metabolite abnormalities induced by the three different deficiency syndromes may very well be clinically useful when diagnosing these deficiency syndromes in humans. Future studies should be directed at determining whether there are common vitamin B_6 deficiency syndromes in clinical medicine. It has already been determined that the elevations in basal and postload cystathionine in B_6-deficient asthma subjects can be corrected with a vitamin B_6 supplement.[19] Cystathionine levels should be studied in subjects found to have postmethionine load hyperhomocysteinemia in vascular disease studies. We have already shown that elevations of serum cystathionine decrease in elderly subjects treated with a combined folate, Cbl, and pyridoxine injection.[44] It is not known, however, whether there are a significant number of elderly subjects with isolated elevated cystathionine levels who would benefit with B_6 treatment. Another cause of elevated serum cystathionine levels is chronic renal failure attributed at least partly to decreased renal excretion.[12] Renal failure, however, may be associated with B_6 deficiency.[45] By measuring cystathionine levels before and after B_6 treatment, in such patients confirmation of this hypothesis may be obtained.

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