Inhibition of Cobalamin-dependent Enzymes by Cobalamin Analogues in Rats

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Abstract

To determine which parts of the cobalamin (cbl) molecule are required for enzyme activity and which parts, if altered, might inhibit cbl-dependent enzyme activity, we synthesized 16 cbl analogues and administered them to nutritionally normal rats. The cbl analogues, with either modifications of the propionimide side chains of the A-, B-, and C-rings, the acetamide side chain of the B-ring, or the nucleotide moiety, were administered to rats by continuous 14-d subcutaneous infusion. Infusion of cbl-stimulated, cbl-dependent activity. Changes in any part of the cbl molecule always abolished stimulation and, in some cases, caused potent inhibition of both cbl-dependent enzymes. The most inhibitory analogues, OH-cbl[c-lactam], a B-ring analogue, and OH-cbl[e-dimethylamide] and OH-cbl[e-methylamide], two C-ring analogues, decreased mean liver holo-L-methylmalonyl-coenzyme A mutase activity to 65% of control values and increased serum methylmalonic acid concentrations to as high as 3,200% of the control values. Liver methionine synthetase activity was decreased to ~ 20% of the control and mean serum total homocysteine concentrations were increased to 340% of control. A similar level of inhibition was demonstrated in rats who were exposed to 28 d of inhaled nitrous oxide or a prolonged period of dietary cbl deficiency. The inhibitory cbl analogues, nitrous oxide, and diet deficiency all depleted liver cbl. The naturally occurring cbl analogues with modifications of the nucleotide moiety had no effects. We conclude that all parts of the cbl molecule are necessary for in vivo cbl-dependent enzyme activity and that modifications of the side chains of the B and C rings are associated with potent in vivo inhibition of cbl-dependent enzyme activity. (*J. Clin. Invest.* 1991. 87:1422–1430.) Key words: vitamin B_{12} • methylmalonic acid • homocysteine • folic acid • nitrous oxide

Introduction

Cobalamin (cbl)[1] deficiency is a major cause of reversible megaloblastic anemia and neurobehavioral abnormalities, such as myelopathy and dementia, in humans (1). The pathophysiology underlying these demyelinating central nervous system lesions resulting from deficiency is not understood and has been hampered by the lack of convenient animal models. Lengthy periods (up to 3 yr) of dietary deprivation with prevention of copraphagia (2), or inhaled nitrous oxide (N_2O) have been used in the past to produce the deficient state (3).

Cbl is required by man and other animals as the co-factor for two enzymes: L-methylmalonyl-coenzyme A (CoA) mutase (EC-5.4.99.2) and methionine synthetase (tetrahydropteroylglutamate; L-homocysteine-*S*-methyltransferase EC 2.1.1.13). Valine, isoleucine, methionine, odd chain fatty acids and other compounds are metabolized to propionyl-CoA which is carboxylated to form D-methylmalonyl-CoA (Fig. 1 *A*), which is either hydrolyzed by D-methylmalonyl-CoA hydrolase to form methylmalonic acid and CoA (4) or racemized to L-methylmalonyl-CoA (5) which is the substrate for the adenosyl-cbl (ado-cbl) requiring enzyme L-methylmalonyl-CoA mutase (6). The other cbl-dependent enzyme, shown in Fig. 1 *B*, is methionine synthetase which demethylates methyltetrahydrofolate as it methylates homocysteine to form methionine (7).

Using newly developed sensitive and specific capillary gas chromatographic/mass spectrometric assays (8, 9), we have shown that serum levels of methylmalonic acid and total homocysteine are markedly elevated in ~ 95% of patients with clinically confirmed cbl deficiency and that the elevated levels fall promptly after cbl therapy is begun in deficient patients (10–12). Thus, elevated levels of serum methylmalonic acid and total homocysteine can be used to confirm metabolic conditions of cbl deficiency or cbl-dependent enzyme inhibition.

Because cbl is synthesized only by bacteria and other prokaryotic organisms (13), higher animals have developed mechanisms for the gastrointestinal absorption and plasma transport of this substance (14, 15). In addition to synthesizing cbl, microorganisms synthesize cbl analogues which have bases as their nucleotide moieties other than 5,6 dimethylbenzimidazole which is present in cbl (see Fig. 2, Nos. 12–16). Previous investigations have shown that there are a variety of mechanisms which appear to prevent the absorption and tissue dissemination of certain of these cbl analogues (16–18). Previous studies have also shown that there are cbl analogues of unknown structure in human serum (19), animal tissues (20), and foodstuffs and vitamin supplements (21), though their effects on cbl metabolism are largely unknown.

In addition to the naturally occurring cbl analogues, many cbl analogues have been synthesized with side chain modifications (22). Many of these analogues have shown inhibitory effects against bacteria (23). However, the studies performed in the past of various analogues administered to higher animals have been disappointing (24).

To determine which sections of the cbl molecule are important for in vivo activity, we synthesized 16 cbl analogues and have demonstrated that none had cbl activity and that some were potent inhibitors of cbl-dependent enzyme activity. Inhi-

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1. *Abbreviations used in this paper:* ado-cbl, adenosyl cobalamin; cbl, cobalamin; CN-cbl, cyano-cbl; CoA, coenzyme A; Me-cbl, methyl-cbl; N_2O, nitrous oxide; OH-cbl, hydroxy-cbl.

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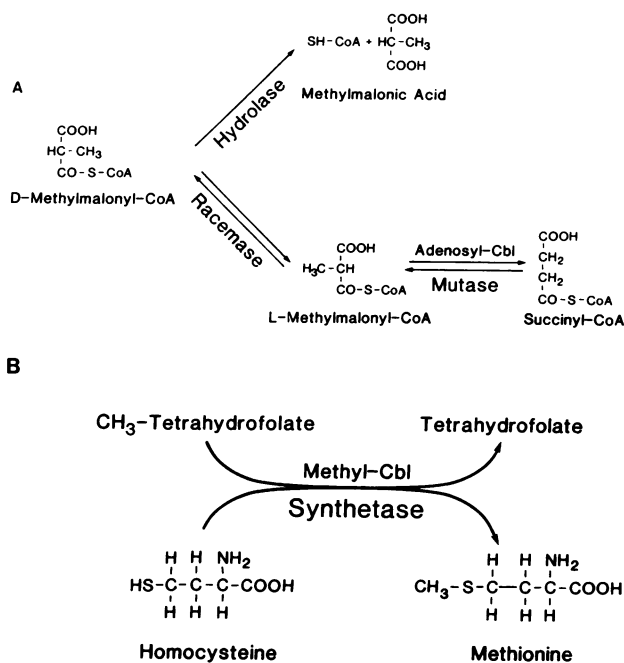

Figure 1. The two known mammalian cbl-dependent enzymes are shown. D-Methylmalonyl-CoA can be either specifically hydrolized to methylmalonic acid and CoA (4) or racemized to L-methylmalonyl-CoA (5) which can then be converted to succinyl-CoA by the adenosyl cobalamin-requiring enzyme, L-methylmalonyl-CoA mutase (6) (*A*). The other cbl-dependent enzyme, methionine synthetase, methylates homocysteine to form methionine while demethylating methyltetrahydrofolate to form tetrahydrofolate (7) (*B*).

bition has been demonstrated not only by in vitro enzyme assay of liver homogenates, but also by marked increases in serum metabolites (methylmalonic acid and homocysteine) that reflect in vivo enzyme inhibition.

Methods

D,L-2[Methyl-^{14}C]methylmalonyl-CoA (51.6 mCi/mmol) was obtained from New England Nuclear (Boston, MA). D,L-5[Methyl-^{14}C]-tetrahydrofolic acid barium salt, (56 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). D,L-5-Methyltetrahydrofolic acid barium salt, 1,ethyl-3-(3-dimethylamino propyl)carbodiimide, D,L-methylmalonyl-CoA, succinic thiokinase, dimethylamide HCl, *S*-adenosylmethionine, flavin adenine dinucleotide, L-homocysteine thiolactone, adenosylcobalamin, cyanocobalamin, cobinomide, Norit activated charcoal, and L-methionine were obtained from Sigma Chemical Co. (St. Louis, MO). *N-N*-Dimethylformimide was obtained from Fisher Scientific Co. (Fair Lawn, NJ), triethylamine from Pierce Chemical Co. (Rockford, IL), and ethylchloroformate from Eastman-Kodak Co. (Rochester, NY). Model 2002 osmotic minipumps were obtained from Alza Corp. (Palo Alto, CA). Sprague-Dawley and Fisher rats were obtained from Sasco, Inc. (Omaha, NE). The cbl-deficient and control diets were obtained from Teklad (Madison, WI). Reagents and solvents for gas chromatography/mass spectrometry and HPLC were obtained as previously reported (8, 9). Anion exchange resin AG1-X2 200-400 mesh acetate form was obtained from Bio-Rad Laboratories, Inc. (Richmond, CA).

Synthesis of analogues. CN-cbl[c-lactam], a B-ring modification, was prepared by a modification of the method of Bonnett et al. (22) by heating CN-cbl (5 mg/ml) in 0.1 M NaOH at 100°C for 10 min. Then after the solution was neutralized with monobasic KPO_4 the CN-cbl[c-lactam] was separated from impurities by paper chromatography as previously described (16). CN-cbl[c-lactone], another B-ring modification, was prepared with chloramine T and purified as previously described (25). The monocarboxylic acids of cbl, CN-cbl[b-OH], CN-cbl[d-OH], and CN-cbl[e-OH] (modifications of A-, B- and C-rings, respectively) were synthesized by incubating CN-cbl in 0.4 N HCl and were separated by anion exchange chromatography and paper chromatography as previously described (16, 25). The structural assignments of the monocarboxylic acids was followed as recommended by Anton et al. (26).

Analogues with substitutions at the e-propionamide side chain of the C-ring were made by a modification of the methods of Armitage (27) and Smith (28). CN-cbl[e-OH] was dissolved in dimethylformamide with triethylamine and ethylchloroformate, and the amide was formed by treating with the appropriate amine. CN-cbl[e-dimethylamide] was also synthesized by the alternate method described below, adapted from Smith (23).

CN-cbl[b-dimethylamide], CN-cbl[d-dimethylamide], and CN-cbl[e-dimethylamide] were synthesized from CN-cbl[b-OH], CN-cbl[d-OH], and CN-cbl[e-OH] by incubating 50 mg of the respective analogue in 50 ml of H_2O at 22°C with 300 mg dimethylamine HCl. After adjusting the pH to 4.0 with NaOH, 10 mg of 1-ethyl-3(3-dimethylamino-propyl) carbodimide was added at 8-h intervals three times. The analogue solutions were separated from starting material by high-speed counter current chromatography on a Ito multi-coil separator-extractor model No. 1 (P.C. Inc., Potomac, MD) with a capacity of 310 ml. CN-cbl[b-dimethylamide] was purified using H_2O, 44.2%: sec-butanol, 44.2%: phenol, 11.5% (vol:vol:vol) as a solvent system. The fractions were analyzed by HPLC, as previously described (25). CN-cbl[d-dimethylamide and e-dimethylamide] were purified using a buffer system of H_2O 60%; 1-butanol 33%; phenol 7% (vol:vol:vol). The CN-cbl[e-dimethylamide] which was synthesized by either method had an identical peak when analyzed by HPLC and had similar inhibitory effects in rats. CN-cbl[13-epi] was synthesized and purified as previously described (25).

The naturally occurring cbl analogues with nucleotide moieties different than that in cbl were produced by bacterial fermentation by the method of Perlman and Barrett (29). The following respective bases were added to cultures of *Propionibacterium arabinosum* to obtain the following cbl analogues: benzimidazole, [BZA]CN-cobamide (Cba); 2-methyladenine, [2-MeAde]CN-Cba; adenine, [Ade]CN-Cba; and 5,6-carboxybenzimidazole, [5(6)-COOH BZA]CN-Cba. The analogues were purified from the bacteria by boiling in KCN containing buffer followed by affinity chromatography on hog non-IF Sepharose and separation by paper chromatography as described previously (30).

All of the analogues were synthesized in their CN-form and were converted into their OH-(aquo)-form before administration to the rats by the method of Dolphin (31). The concentration of cbl and cbl analogues was determined spectrophotometrically at 367.5 nm in 0.1 M KCN. A molar extinction coefficient of 30,800 M^{-1} cm^{-1} was used for each analogue except for CN-cbl[13-epi] for which a value of 20,600 $M^{-1}cm^{-1}$ (16) was used. The final analogue solutions gave a single peak when they were analyzed for purity by HPLC utilizing separation methods previously described (25).

Cbl analogue administration. To test the metabolic effects of the administration of cbl analogues to rats, eight experimental male Sprague-Dawley rats (300-400 g size) and eight simultaneous control rats were implanted subcutaneously with model 2002 osmotic minipumps (Alza Corp., Palo Alto, CA) which contained ~ 1 mg OH-cbl analogue in H_2O or H_2O alone, respectively. The pumps released 2 μg/h for 14 d (672 μg total). In one experiment, pumps were replaced at 21 d and the animals were sacrificed after 42 d. Rats were sacrificed with ether, blood was immediately obtained by cardiac puncture, and the livers were immediately frozen in a dry ice acetone bath. For all of the analogue studies, the rats were housed in the University of Colorado Animal Resource Center and fed a standard cbl-containing lab chow obtained from various sources. Coprophagia was not prevented. For the nitrous oxide (N_2O) experiments, eight experimental rats were

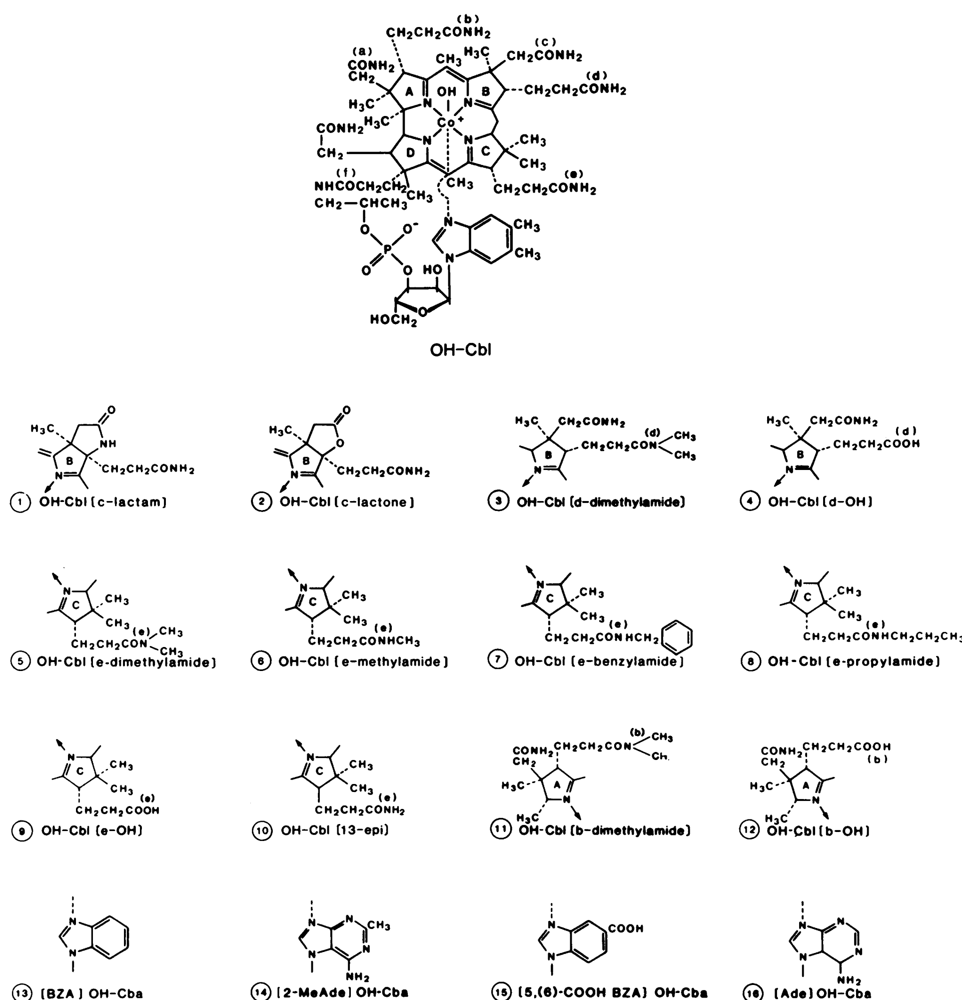

Figure 2. Structure of CN-cbl (*top*) and the partial structure of 16 cbl analogues. The numbers under the structure refer to their order in Table I.

placed in an air-tight Plexiglass box and exposed to 50% N_2O/50% O_2 for 28 d as previously described (32). Control rats were housed as above in the Animal Resource Center. Another control experiment with eight rats exposed to 50% N_2/50% O_2 was also performed. Fischer strain male weanling (15 g) rats were used for the cbl-deficient diet studies. The animals were placed in individual wire bottom metabolic cages and were fed an amino acid–based cbl-deficient diet or control diet, which was identical to the deficient diet except that it contained cbl (33). To determine the effects of OH-cbl[c-lactam] in the Fischer rat strain, the same method was used as described above in the Sprague-Dawley rats except that the control synthetic diet was fed to both experimental rats and controls, and the control pumps contained 0.15 M saline.

Enzyme and metabolite assay methods. Frozen liver was homogenized in 2.5 vol of 0.028 M $NaPO_4$, pH 7, and centrifuged at 30,000 *g* for 1 h. The supernatants were assayed for L-methylmalonyl-CoA mutase as previously described (34). Assays were done without added ado-cbl (holo-L-methylmalonyl-CoA mutase) and with ado-cbl in the dark (total L-methylmalonyl-CoA mutase). Methionine synthetase was assayed in liver supernatants as previously described (7, 35), both with and without CN-cbl in the assay. It is not clear whether the assays with and without cbl present represent the total and holo forms of this enzyme (7). D-Methylmalonyl-CoA hydrolase and D,L-methylmalonyl-CoA racemase were assayed on liver supernatants as previously described (4, 5). An enzyme unit (EU) refers to 1 μmol of substrate converted per minute.

Serum methylmalonic acid, succinic acid, total homocysteine, methionine, and total cysteine were assayed by capillary gas chromatography/mass spectrometry (GC/MS) utilizing tert-butyldimethylsilyl derivatives as previously described (8, 9), using a stable isotope internal standard for each metabolite quantified. Dimer formation does not interfere with quantitation of homocysteine and cysteine with these methods because stable isotope containing internal standards are added to the samples before sample reduction and thus are randomized with endogenous homocysteine and cysteine throughout sample preparation and quantitation.

Serum and liver cbl and folate levels were determined using the Ciba-Corning Immophase Vitamin B_{12}[^{57}Co] Folate[^{125}I] Radioassay kit. The kit utilizes purified hog intrinsic factor (IF) and bovine milk folate binding protein as binders. The relative affinity of the various cbl analogues for binding to hog IF was determined by assaying various concentrations of the cbl analogues with the IF binding kit and comparing the results to those obtained with cbl in an amount that gave 50% inhibition of binding of the CN-[^{57}Co]cbl binding by hog IF.

Data was analyzed for statistical significance using Student's *t* test comparing each experimental group with its own simultaneous control group with $P < 0.05$ considered significant.

Results

Inhibition of Cbl-dependent metabolism by OH-Cbl[c-lactam]. When eight male Sprague-Dawley rats were treated with an OH-cbl[c-lactam] infusion (1.34 mg) for 42 d, their mean liver holo-L-methylmalonyl-CoA mutase activity (assayed without ado-cbl) fell to 65% of the mean level in eight control rats treated simultaneously with H_2O (Fig. 3). This decrease in

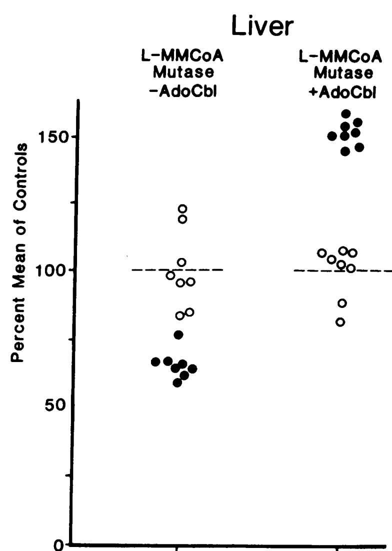

Figure 3. L-Methylmalonyl-CoA mutase activity assayed in liver supernatants without and with ado-cbl from eight experimental rats (*solid circles*) as compared to eight control rats (*open circles*). Experimental rats were infused with OH-cbl[c-lactam] for 42 d. The data for each animal is expressed as a percent of the mean of the control rats. The assay without ado-cbl corresponds to holo-L-methylmalonyl-CoA mutase activity and when assayed with ado-cbl is total mutase activity. Mean holo-L-methylmalonyl-CoA mutase activity for experimentals was 86±7.1 SD and for controls 133±19 SD E.U./g wet weight. Total L-methylmalonyl-CoA mutase activity for experimentals was 2,160±66 controls and for controls 1,430±140 E.U./g wet weight, respectively.

holo-methylmalonyl-CoA mutase was physiologically significant as the mean serum levels of methylmalonic acid in the experimental animals (Fig. 4) were markedly increased to 3,200% of the values in control rats. The serum succinic acid levels were unchanged. The total liver L-methylmalonyl-CoA mutase activity (assayed with ado-cbl) increased to a mean of 152% as compared to the control (Fig. 3). Fig. 5 shows that liver methionine synthetase activity was also decreased in the experimental animals who received OH-cbl[c-lactam]. When assayed without CN-cbl, the mean methionine synthetase activity was 43% of the mean control activity and when assayed with CN-cbl, it was 18%. This suppression in methionine synthetase activity appeared to be physiologically important because the mean serum total homocysteine level of the experimental rats was elevated to 341% of the mean control levels (Fig. 6). Serum methionine levels were not decreased and, in fact, were 118% of the mean control levels. Serum total cysteine levels were also unchanged (data not shown). At the end of the 42-d infusion, cbl levels in the liver were markedly decreased to 17% of control values, as shown in Fig. 7. Liver folate levels were moderately decreased to 69% of the controls (Fig. 7).

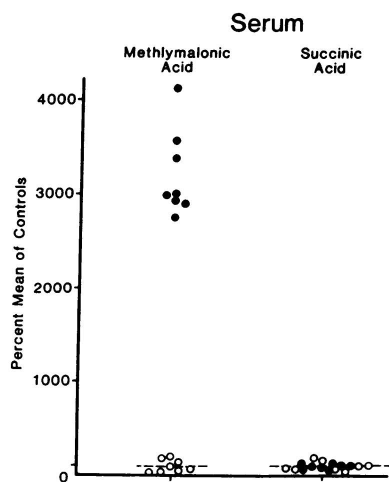

Figure 4. Serum methylmalonic acid and succinic acid levels are shown for the eight experimental rats (*solid circles*) that were infused as in Fig. 3 as compared to eight control rats (*open circles*). The data are expressed as in Fig. 3. Mean serum methylmalonic acid levels for experimentals were 138,000±20,100 nmol/liter and controls 4,300±2,850 nmol/liter and succinic acid were 50,400±23,000 nmol/liter for experimentals and for controls 47,400±12,100 nmol/liter.

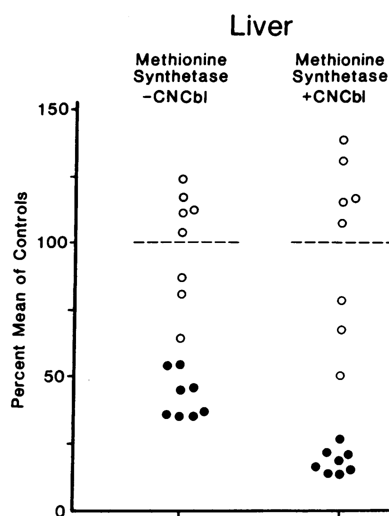

Figure 5. Methionine synthetase activity assayed in liver supernatants without CN-cbl or with CN-cbl is shown for eight experimental rats (*solid circles*) as compared to eight control rats (*open circles*) infused as in Fig. 3. The data is expressed as in Fig. 3. When assayed without CN-cbl, mean methionine synthetase activity was 1.2±0.2 E.U./g wet weight in the experimentals and 2.8±0.6 enzyme units per gram in the controls. Assayed with CN-cbl, the mean methionine synthetase activity decreased to 0.9±0.2 in the experimental animals and increased to 5.2±1.6 E.U./g wet weight in the control animals.

Comparison of Cbl and 17 Cbl analogues. Fig. 2 shows the structure of cbl and of the 16 cbl analogues which were synthesized and then purified from cbl before administration to the rats. The number of each analogue in the figure corresponds to the number of each analogue in Table I which shows the results on cbl metabolism of the continuous infusion of cbl or the cbl analogues. In each experiment, eight experimental and eight control rats were studied and 672 μg of the cbl or cbl analogue was continuously infused by osmotic minipump for 14 d. OH-

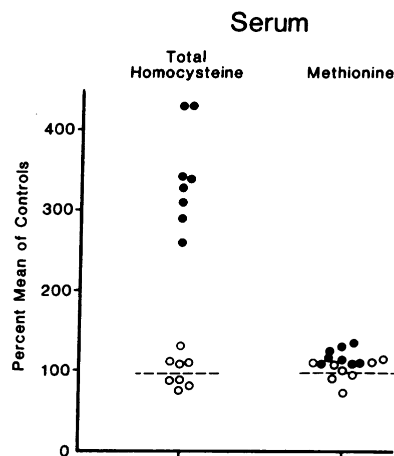

Figure 6. Serum total homocysteine and methionine levels are shown for the eight experimental rats (*solid circles*) that were infused as in Fig. 3 as compared to eight control rats (*open circles*). The data is expressed as in Fig. 3. Mean serum total homocysteine for experimentals was 16.6±2.9 μmol/liter and for controls 4.9±1.0 μmol/liter. Mean serum methionine for the experimentals was 66.9±6.0 μmol/liter and 56.6±7.9 μmol/liter for the controls.

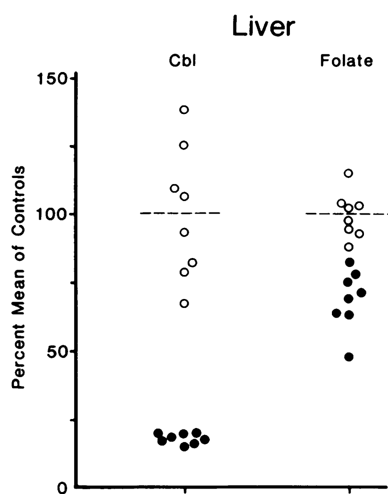

Figure 7. Liver cbl and folate levels are shown for the experimental rats and control rats as described in Fig. 3. The mean liver cbl level for the experimentals was 28±3.0 and for the controls 161±27 ng/g wet weight. The mean liver folate levels for experimentals were 4.8±0.8 and for controls 7.0±0.6 μg/g wet weight.

cbl[c-lactam] was also studied for 42 d (see above). The numbers in Table I are the mean of the eight experimental rats, expressed as the percent of the mean of the eight simultaneous control rats (mean experimental rat value/mean control rat value × 100).

OH-cbl infusion resulted in increased holo-L-methylmalonyl-CoA mutase (177% of the control values) and methionine synthetase activity (132%) and corresponding decreases in serum methylmalonic acid to 58% of control and serum total homocysteine to 74% of control. Serum succinic acid and methionine levels were not changed significantly.

The most effective analogues in inhibiting liver holo-L-methylmalonyl-CoA mutase activity were the B-ring analogue, OH-cbl[c-lactam] (69% of the mean control value) and the analogues with the substitutions at the e-propionamide side chain of the C-ring especially OH-cbl[e-dimethylamide] (66% of control) and OH-cbl[e-methylamide] (66% of control). Another B-ring analogue, OH-cbl[c-lactone] also caused modest suppression (74% of the control). The serum methylmalonic acid levels correlated inversely with the holo-L-methylmalonyl-CoA mutase activity with the highest levels being achieved by OH-cbl[c-lactam] for 42 and 14 d (3,200% and 2,310% of the mean control value, respectively) followed by OH-cbl[e-dimethylamide] (871% of control), OH-cbl[e-methylamide] (646% of control), OH-cbl[e-benzylamide] (357% of control), OH-cbl[e-propylamide] (346% of control), OH-cbl[e-OH] (339% of control), and OH-cbl[c-lactone] (230% of control). Serum succinate levels were not significantly altered with cbl or any of the cbl analogues (data not presented).

The liver methionine synthetase activity was also suppressed by the cbl analogues which suppressed holo-L-methylmalonyl-CoA mutase activity, with the maximum suppression being achieved with OH-cbl[c-lactam] which was suppressed to 18% of the mean control value after 42 d and 19% of mean control value after 14 d, OH-cbl[e-dimethylamide] (24% of control), OH-cbl[e-methylamide] (47% of control), OH-cbl[e-benzylamide] (53% of control), and OH-cbl[e-propylamide] (46% of control). OH-cbl[c-lactone] also decreased liver methionine synthetase activity. In every experiment with inhibitory analogues, methionine synthetase activity was decreased further when assayed in the presence of CN-cbl (Table I). In contrast, the addition of CN-cbl increased methionine synthetase activity in controls, rats infused with OH-cbl or inert analogues (Table I). Similar findings (not presented in Table I) were found with CN-cbl[c-lactam] when it was added to liver homogenates from rats exposed to OH-cbl[c-lactam] for 14 d or controls. The CN-cbl[c-lactam] inhibited methionine synthetase an additional 16% in the analogue-treated rats and increased methionine synthetase 37% in the controls.

The mean liver methionine synthetase activity correlated inversely with serum total homocysteine levels. Serum total homocysteine levels were increased with administration of the following analogues: OH-cbl[c-lactam] for 42 d (341% of control), for 14 d (229% of control), OH-cbl[e-dimethylamide] (344% of control), OH-cbl[e-methylamide] (248% of control), OH-cbl[e-benzylamide] (237% of control), OH-cbl[e-propylamide] (201% of control), OH-cbl[e-OH] (169% of control), and OH-cbl[c-lactone] (141% of control). Of the three monocarboxylic acids of OH-cbl (e-OH, d-OH, and b-OH) (see Fig. 2), the C-ring modification, e-OH, demonstrated the most inhibition of cbl metabolism and was therefore used to make the series of substituted amides. The corresponding dimethylamine substitutions of OH-cbl[d-OH] and OH-cbl[b-OH] resulted in little inhibition of either enzyme (compounds 3 and 11 in Table I). Serum methionine levels were not significantly altered by cbl or any of the cbl analogues (data not presented).

The naturally occurring cbl analogues with nucleotide moieties different than that in cbl (compounds 13–16 in Fig. 2) showed little effect on either of the cbl-dependent enzymes with the exception of [BZA]OH-Cba which caused modest inhibition of both enzymes.

OH-cbl[13-epi] is an isomer of OH-cbl with the only change being the position of the e-propionamide side chain in relation to the C-ring (Fig. 2, compound 10) but when infused into rats by osmotic minipump, it neither inhibited or stimulated cbl-dependent enzyme activity. No analogue was found that increased the activity of liver holo-methylmalonyl-CoA mutase or methionine synthetase when infused. Liver total L-methylmalonyl-CoA mutase activity increased in the rats receiving OH-cbl[c-lactam]. Serum succinic acid and methionine were not significantly decreased in any of the experiments. The activity of liver D-methylmalonyl-CoA hydrolase and D,L-methylmalonyl-CoA racemase were unchanged by OH-cbl[c-lactam] infusion (data not shown) and were not tested with the other analogues. The cbl analogues had no apparent toxic effects on growth or activity of the rats.

Cbl and folate levels in analogue-treated rats. As shown in Table I, the binding of the analogues to hog IF varied widely among the different analogues tested as compared to cbl. The poorest binder was OH-cbl[c-lactam] and the substituted monocarboxylic acids (compounds 3, 5–8, 11 in Table I) were equivalent to cbl. Of the naturally occurring analogues, [BZA]OH-Cba was the only analogue with significant binding to hog IF.

When OH-cbl was infused by osmotic minipumps into eight rats, the mean liver cbl levels significantly increased to 246% of the mean control values and serum levels to 531% of control. Levels of liver cbl were decreased by some of the analogue infusions even when the analogue infused was detected by the IF radiodilution assay used to assay liver cbl. The animals who received the analogues that were the most effective in suppressing cbl metabolism, OH-cbl[c-lactam] and OH-cbl[e-dimethylamide] had the lowest liver cbl levels (19% of mean control values and 56%, respectively). OH-cbl[c-lactam] which may have been present in the liver of the experimental rats

Table I. Effects in Rats of the Continuous Infusion of 672 μg of 17 Different cbl Analogues, or cbl (2 μg/h) for 14 or 42 d, 50% Nitrous Oxide Exposure, or cbl Dietary Deficiency on Various Parameters of cbl Metabolism

Treatment	Days	Liver L-methylmalonyl CoA mutase: ado-cbl (−)	Liver L-methylmalonyl CoA mutase: ado-cbl- (+)	Serum methylmalonic acid	Liver methionine synthetase: CN-cbl (−)	Liver methionine synthetase: CN-cbl (+)	Serum total homocysteine	Liver: cbl¶	Liver: Folate	Serum: cbl¶	Serum: Folate	IF affinity**
		%		%	%		%	%		%		%
Sprague-Dawley rats												
B Ring												
1 OH-cbl[c-lactam]	42	65*	152*	3,200*	43*	18*	341*	17*	69*	120*	112*	0.1
1 OH-cbl[c-lactam]	14	69*	128*	2,310*	53*	19*	229*	19*	75*	114*	107*	0.1
2 OH-cbl[c-lactone]	14	74*	99	230*	65*	50*	141*	48*	94	116*	105	0.2
3 OH-cbl[d-dimethylamide]	14	99	109	202*	80*	71*	141*	66*	96	131*	103	3
4 OH-cbl[d-OH]	14	94	102	130	87	81*	111	85*	102	102	104	2
C Ring												
5 OH-cbl[e-dimethylamide]	14	66*	126*	871*	52*	24*	344*	56*	87*	401*	103	90
6 OH-cbl[e-methylamide]	14	66*	112*	646*	57*	47*	248*	79*	88*	620*	104	90
7 OH-cbl[e-benzylamide]	14	76*	108	357*	79*	53*	237*	67	89*	515*	111	100
8 OH-cbl[e-propylamide]	14	78*	112*	346*	61*	46*	201*	66*	95	665*	113	100
9 OH-cbl[e-OH]	14	86	108	339*	77	61*	169*	101	100	243*	109*	30
10 OH-cbl[13-epi]	14	99	96	18	96	90	107	106	97	366*	105	100
A Ring												
11 OH-cbl[b-dimethylamide]	14	81	96	111	94	89	93	82*	96	149*	107*	30
12 OH-cbl[b-OH]	14	89	91	97	77*	74*	89	89	101	104	92	1
Nucleotide substitution												
13 [BZA]OH-Cba	14	71*	97	157*	83	71*	129	186*	96	498*	108	60
14 [2-MeAde]OH-Cba	14	105	113*	144	114	85	137*	87	113*	164*	112*	2
15 [5, (6)COOHBZA]OH-Cba	14	86	93	120	97	87	134	98	96	124*	104	2
16 [Ade]OH-Cba	14	89	91	105	114	97	98	105	95*	122*	90*	0.5
Other												
17 OH-Cobinimide	14	88	96	87	97	89	125	80	99	112	102	0.1
OH-cbl	14	177*	98	58*	132*	156*	74*	246*	95	531*	108	100
50% Nitrous Oxide	1.5	107	117	101	62*	16*	549*	87	80	94	128*	
50% Nitrous Oxide‡	28	55*	132*	2,186*	55*	13*	432*	36*	43*	49*	99	
Fischer rats												
Cbl-deficient diet§	168	79*	274*	18,100‖*	99	53*	257‖*	26*	112	8*‖	113‖	
OH-cbl[c-lactam]	42	73*	148*	28,500‖*	68*	26*	216‖*	11*	71*	107‖	138*‖	0.1

The data in the table is the mean of the experimental values expressed as a percent of the simultaneous mean control values (n = 8 control vs. 8 experimental rats except as footnoted.) * $P < 0.05$. ‡ Eight control rats vs. seven experimental rats. § Six control rats vs. five experimental rats. ‖ Plasma assayed instead of serum. ¶ These measurements in some cases include both cbl and cbl analogue. The degree to which the analogue may contribute to the value can be seen by inspecting IF affinity. ** Relative to CN-cbl as described under "Methods".

would have been measured in the radiodilution assay as an extremely low "cbl" level due to its low affinity to the IF, but all of the OH-cbl[e-dimethylamide] would have been measured and in these rats the level was still decreased to 56% of the controls. The rats who received [BZA]OH-Cba had an increase in measured liver cbl to 186% possibly reflecting the deposition of [BZA]OH-Cba in the liver because it was detected by the radiodilution assay. Serum cbl levels were increased slightly in the OH-cbl[c-lactam] and [c-lactone-]–treated animals, and were markedly increased in the animals infused with the analogues with a high affinity to IF, i.e., compounds 3, 5, 6, 7, 8, 11, and 13 in Table I.

Liver folate levels were moderately decreased in the animals who received the most potent cbl analogues to 69% of the mean control values with 42 d of OH-cbl[c-lactam] and to 87% with OH-cbl[e-dimethylamide]. Serum folate levels were increased slightly in the OH-cbl[c-lactam]–treated animals and not significantly changed in the other experiments.

N_2O-induced inhibition of Cbl metabolism. Table I shows the data from rats who were exposed to 50% N_2O/50% O_2 for either 1.5 or 28 d as compared to control rats. One experimental rat in the long-term exposure died of unknown causes during the second week. As has been reported previously (32), liver L-methylmalonyl-CoA mutase activity was not decreased after

short exposure to N_2O, but mean liver methionine synthetase activity when assayed with CN-cbl showed a marked decrease to 16% of the mean control values with a corresponding increase in the serum total homocysteine to 549% of control. Serum methionine concentration decreased significantly to 78% of control levels. After 28 d of N_2O exposure, liver holo-L-methylmalonyl-CoA mutase activity decreased to 55% of control with a corresponding increase in serum methylmalonic acid concentration to 2,190% of control levels. The liver methionine synthetase activity was decreased with an increase in serum total homocysteine concentration, similar to the data obtained after 1.5 d. The liver cbl level was markedly depleted to 36% of the mean control values in the long-term N_2O-treated rats. Liver folate levels were depleted to 43% of control after long-term exposure. Serum cbl was unchanged in the short-term exposed animals and decreased to 49% of control in the long-term–treated animals. D-Methylmalonyl-CoA hydrolase activity was significantly increased to 147% of the mean control values in the 50% O_2/50% N_2 treated rats. An additional experiment with 50% O_2/50% N_2 exposure of rats for 28 d resulted in no changes in liver L-methylmalonyl-CoA mutase or methionine synthetase activity or serum methylmalonic acid and total homocysteine levels. Serum cbl and folate levels were significantly decreased to 55 and 63%, respectively, of the mean control values in the 50% O_2/50% N_2–treated rats.

Effects of dietary Cbl deficiency. Table I also shows the results obtained in Fischer rats subjected to either a synthetic cbl-deficient diet with prevention of coprophagia (described in Methods) as compared to Fischer rats who were infused with OH-cbl[c-lactam] as described above for Sprague-Dawley rats. After 168 d on the deficient diet, mean liver holo-L-methylmalonyl-CoA mutase in the experimental rats had decreased to 79% of control with a marked increase in serum methylmalonic acid concentration to 18,100% of control levels. Liver methionine synthetase when assayed with CN-cbl decreased to 53% and serum total homocysteine concentration was increased to 257%. Liver and serum cbl levels were decreased to 26 and 8% of control values, respectively. Liver folate and serum folate concentrations were increased slightly to 112 and 113%, respectively. When Fischer rats, eight experimental and eight controls, were placed on the synthetic control diet and infused with OH-cbl[c-lactam] or H_2O, respectively, results qualitatively similar to the deficient diet were obtained after only 42 d. It is not known to what extent the synthetic diet or the strain of rat utilized contributed to the greater increase in serum methylmalonic acid in response to OH-cbl[c-lactam] which was seen in the Fischer rats as compared to the experiments performed in Sprague-Dawley rats on a standard natural diet.

Discussion

We have synthesized and purified 16 cbl analogues and determined the resulting effects on cbl-dependent enzyme activity when these analogues, or commercially available cobinamide and cbl, were continuously infused by osmotic minipumps into rats. The most effective inhibitors of both cbl-dependent enzymes, L-methylmalonyl-CoA mutase and methionine synthetase, were the chemically synthesized analogues, OH-cbl[c-lactam], a B-ring analogue, and two C-ring analogues, OH-cbl[e-dimethylamide] and [e-methylamide]. For the C-ring analogues with modifications of the e-propionimide side chain, the size of the side chain modification did not seem to be a factor in the inhibition. Unlike OH-cbl[e-OH], substitution of the two other monocarboxylic acids, OH-cbl[d-OH] (A-ring) and [b-OH] (B-ring) did not result in inhibitors of cbl-dependent metabolism. The analogues with base modifications, i.e., the naturally occurring analogues, generally did not cause significant inhibition of the two enzymes with the possible exception of [BZA]OH-Cba. No analogue was found that increased the activity of either of the two enzymes when it was infused in rats, suggesting that the requirements for cbl as an active cofactor appear to be quite specific, and that rats cannot convert these analogues back to cbl. Even OH-cbl[13-epi], an isomer of cbl, was inert.

Though the level of inhibition of holo-L-methylmalonyl-CoA mutase appeared modest when assayed in vitro in the liver supernatants (a maximum of 40% inhibition for OH-cbl[c-lactam]), the serum methylmalonic acid levels were markedly increased, ranging from 3- to ~ 280-fold in the various experiments, demonstrating that the level of inhibition achieved caused a significant metabolic change in vivo. These serum levels of methylmalonic acid are comparable to levels seen in humans with symptomatic cbl deficiency (10–12). The metabolic role or fate of methylmalonic acid is unknown at present, though we have shown in preliminary experiments that most (~ 85%) of a dose of ($^{-14}$C-methyl) methylmalonic acid is metabolized and only 15% is excreted in the urine (4) after subcutaneous injection in rats. Levels of liver D,L-methylmalonyl-CoA racemase and D-methylmalonyl-CoA hydrolase were not increased in the rats receiving OH-cbl[c-lactam], thus the block in L-methylmalonyl-CoA mutase activity must have been the primary reason for the marked elevations in serum methylmalonic acid.

We have also shown that several cbl analogues with modifications of the B and C rings inhibit methionine synthetase activity to a degree similar to that found with N_2O, a previously known inhibitor of methionine synthetase activity (32, 36). Nitrous oxide has been shown to interact with reduced forms of cbl, resulting in rapid inactivation of methionine synthetase (36). On prolonged exposure, nitrous oxide has been shown to cause the formation of cbl analogues and to deplete cbl (32). When liver supernatants were assayed for methionine synthetase with CN-cbl in the incubation assay, a lower level of activity was detected than when the same supernatants were assayed without CN-cbl under the following conditions: (*a*) exposure to N_2O; (*b*) infusion with the inhibitory analogues (Fig. 5 and Table I); and (*c*) dietary deficiency. In contrast, in assays of liver supernatants from control rats or rats receiving inert analogues, the presence of CN-cbl in the incubation increased methionine synthetase activity. Similar findings were seen when CN-cbl[c-lactam] was added to liver homogenates from OH-cbl[c-lactam] exposed rats and controls. It is not known whether CN-cbl or CN-cbl[c-lactam] is actually converting apo enzyme to holo enzyme in the control rats or is simply increasing the reducing system of the assay (7). The explanation for the inhibition caused by CN-cbl or CN-cbl[c-lactam] in the depleted animals is also unknown. Regardless of the explanation for these phenomenon, the common practice of using the increase in methionine synthetase activity resulting from adding CN-cbl to enzyme assays as a measure of apo-enzyme cannot be accepted without further studies of the mechanism involved.

The mechanism of inhibition of the cbl analogues is not known. However, it is possible that some of the analogues may displace cbl from the liver. Table I shows the percent of binding to hog IF as compared to cbl for the various analogues. Thus, for some analogues, the amount of cbl measured in liver supernatants represents the sum of endogenous cbl and analogue if present. OH-cbl[c-lactam], which is measured only 0.1% as well as cbl, resulted in the marked displacement of cbl from the liver because the measured mean total cbl in the experimentals was only 19% of the control value. Analogues which were measured in the IF assay (i.e., OH-cbl[e-dimethylamide]) also did not appear to accumulate in the liver because, in this case, the total cbl level had decreased to 56% of the control values. In contrast, when similar amounts of OH-cbl were infused, the mean liver cbl levels increased to 246% of the control animals. The inhibitory analogues also appeared to deplete the liver of folate. This is consistent with the hypothesis of the "methyl folate trap" (37), wherein, because of the blocked activity of methionine synthetase, N_5-methyltetrahydrofolate levels increase. It is then released from the cells and excreted in the urine resulting in eventual folate depletion of the organism.

There are many steps involved in cbl uptake, metabolism, and release from cells, and cbl analogues could act by inhibiting any of these steps. The analogues could act by actively inhibiting an enzyme or receptor or, if inert, by displacing cbl from the enzyme or receptor, or by stimulating the release of cbl from cells. Our data show that some of the inhibitory analogues displaced cbl and did not progressively accumulate in the liver. Because 80–90% of liver cbl is bound to either methionine synthetase or L-methylmalonyl-CoA mutase, and there is no cbl storage form (38), it appears that cbl had been therefore depleted from these two enzymes. However, the inhibition of steps in cbl metabolism before and after binding of the coenzyme forms to the two enzymes would decrease their cbl content as would direct competition of cbl and cbl analogue for actual binding to L-methylmalonyl-CoA mutase and methionine synthetase. In experiments with purified human apo methionine synthetase, every cbl analogue studied has been found to stimulate enzyme activity (Kolhouse, J. F., personal communication) rather than inhibit activity, in contrast to what was seen when the same analogues were infused in rats as described in the present experiments. Analogous experiments with coenzyme forms of the analogues have not yet been done with purified L-methylmalonyl-CoA mutase.

It is interesting that the naturally occurring analogues synthesized by bacteria are basically inert when infused in rats, demonstrating that which nucleotide moiety is present in the molecule is extremely important for the in vivo activity of cbl. Many of the analogues are present in large quantities in animal, and also presumably, human feces (39).

Though, the levels of serum methylmalonic acid and homocysteine achieved in the rats by the inhibitory analogues were similar to those in cbl-deficient humans the rats did not develop hematologic or neurologic abnormalities. This is not surprising because dietary deficiency (40) and exposure to N_2O (41) produces megaloblastic anemia only in humans and neurologic abnormalities only in humans, monkeys, fruit bats, and possibly swine. The inhibitory analogues can now be used in the animal species susceptible to cbl deficiency to produce models of cbl deficiency. The analogues can also be used to induce models of methylmalonicaciduria (42). The changes induced by the analogues were similar to those obtained with extensive periods of diet deficiency with prevention of copraphagia and N_2O administration. The analogue infusions were more convenient, more economical, and in the case of N_2O, much safer for laboratory personnel.

The analogues that have been shown to inhibit methionine synthetase activity in the present experiments might have potential as antiproliferative agents against human malignant cells. N_2O, which also decreases methionine synthetase activity, has already been shown to suppress the proliferation of malignant human hematopoietic cells in culture (43) and has been used to treat a few cases of human leukemia (44, 45). It is also known to cause severe bone marrow suppression when humans are continuously exposed for more than a few days (46). In vitro studies with cbl analogues and various cells grown in tissue culture are in progress.

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