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Research Article

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Presence and Formation of Cobalamin Analogues in Multivitamin-Mineral Pills

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ABSTRACT Because the origin of cobalamin (vitamin B_{12}) analogues in animal chows and animal and human blood and tissues is unknown, we investigated the possibility that multivitamin interactions might convert cobalamin to cobalamin analogues. We homogenized three popular multivitamin-mineral pills in water, incubated them at 37°C for 2 h, and isolated the cobalamin. Using paper chromatography we observed that 20–90% of the cobalamin was present as cobalamin analogues. Studies using CN-[^{57}Co]cobalamin showed that these analogues were formed due to the concerted action of vitamin C, thiamine, and copper on CN-cobalamin. These cobalamin analogues are absorbed from the gastrointestinal tract of mice and either fail to stimulate or actually inhibit cobalamin-dependent enzymes when injected parenterally.

We conclude that CN-cobalamin can be converted to potentially harmful cobalamin analogues by multivitamin-mineral interactions and that these interactions may be responsible for the presence of cobalamin analogues in animal chows and animal and human blood and tissues.

INTRODUCTION

In addition to cobalamin (Cbl),[1] microorganisms synthesize large amounts of a variety of Cbl analogues (1) that have not been observed in normal animal tissues because of multiple mechanisms that prevent their absorption and tissue dissemination (2–5). The presence of these mechanisms supports the concept that Cbl analogues of microorganismal origin are inert (1, 6, 7) or even toxic (7) for animals.

We have shown recently that a different class of Cbl analogues is present in human plasma (8), human placenta (9), and variety of animal tissues (9). Although the structures of these analogues are unknown, they are clearly different from the analogues produced most commonly by microorganisms based on studies comparing their absorption spectra, mobilities during paper chromatography, affinities for Cbl binding proteins, and abilities to promote the growth of microorganisms (9).

The origin of these Cbl analogues is also unknown. It appears unlikely that they are formed from Cbl within the body because we have injected [^{57}Co]Cbl into rats and rabbits and have not observed [^{57}Co]Cbl analogues when we killed these animals 2 d (9) or 2 mo[2] later, isolated their total Cbl, and fractionated it by paper chromatography. We have observed Cbl analogues, however, in a commercial rabbit chow (9) that contained no components of animal origin except for "light-activated animal sterols." Because the Cbl that was added to the rabbit chow was free of Cbl analogues (9), we wondered if other components of the chow might have interacted with the added Cbl and converted some of it to Cbl analogues during the preparation or storage of the chow. A number of studies in the literature (10–26) have reported that various vitamins and minerals can "destroy" Cbl under certain conditions. Because of this, we investigated the possibility that Cbl analogues might be present in multivitamin and multivitamin-mineral pills.

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[1] *Abbreviation used in this paper:* Cbl, cobalamin.

[2] Kondo, H., J. F. Kolhouse, and R. H. Allen. Unpublished observations.

METHODS

CN-[^{57}Co]Cbl (100–200 μCi/nmol) was obtained from Amersham Corporation (Arlington Heights, IL). The CN-[^{58}Co]Cbl (4 μCi/nmol) was obtained from the same source in the form of capsules that are utilized in the Dicopac Schilling test kit. Each capsule was opened, and the contents were dissolved in 5 ml of H_2O. After centrifugation at 10,000 *g* for 15 min at 4°C, the supernatant containing the CN-[^{58}Co]Cbl was stored at −20°C. Crystalline CN-Cbl, crystalline OH-Cbl, and thiamine-HCl were obtained from Sigma Chemical Co. (St. Louis, MO). The CN-Cbl was purified by paper chromatography in solvent 1 (see below) and assayed spectrophotometrically (8). Sodium ascorbate was obtained from Hoffman-La Roche, Inc. (Nutley, NJ), copper sulfate from Mallinckrodt, Inc., Science Products Div. (St. Louis, MO), and ferrous fumarate from Lederle Laboratories (Pearl River, NY). Theragran, Theragran-M containing ferrous carbonate or ferrous fumarate (E. R. Squibb & Sons, Inc., Princeton, NJ), One-A-Day and One-A-Day plus minerals (Miles Laboratories, Inc., Elkhart, IN) and Optilets-500 and Optilets-M-500 (Abbott Diagnostic, Diagnostic Products, North Chicago, IL) were obtained from local drugstores and stored at room temperature (20°–25°C). All of these preparations were utilized at least 3 mo before their stated expiration dates. Hog R protein (27), hog intrinsic factor (27), human R protein (28), and human intrinsic factor (28) were obtained as described.

Cobalt analyses were performed using X-ray fluorescence (29). Microbiologic assays for Cbl using *Euglena gracilis* (30) were performed by Dr. V. Michael Whitehead (McGill University, Montreal, Quebec, Canada). Microbiologic assays for Cbl using *Lactobacillus leichmannii* (31) were performed by Dr. William S. Beck (Harvard Medical School, Boston, MA). Radioisotope dilution assays (8) for Cbl and for the total of Cbl and Cbl analogues were performed using human intrinsic factor and human R protein, respectively, as the Cbl-binding proteins. Methylmalonyl-CoA mutase (32, 33) and methionine synthetase (34, 35) were assayed as described except that in the latter the total amount of N^5-methyltetrahydrofolate was reduced from 75 to 3 nmol/assay. Preparation of OH-[^{57}Co]Cbl (34), phenol extraction (2), paper chromatography (9), and reverse affinity chromatography on hog R protein-Sepharose (36) were performed as described except that Cbl was eluted from hog R protein-Sepharose with 60% pyridine rather than 85% phenol. The solvents used for paper chromatography were as follows: solvent I, 880 ml of sec-butanol, 8.2 ml of glacial acetic acid, 6.2 μmol of HCN, and a saturating amount (~425 ml) of H_2O; solvent II, the same except that 1.8 ml of concentrated NH_4OH was used in place of the 8.2 ml of acetic acid.

Cbl was extracted from various multivitamin pills by homogenizing them in H_2O, 10 ml/pill, at 4°C for five 1-min intervals in a Waring blender (Waring Products Div., Dynamics Corp. of America, New Hartford, CT). The samples were cooled in an ice-water bath between the homogenization intervals to ensure that the temperature did not exceed 8°C at any time during the homogenization procedure. After homogenization, the samples were stirred at 4°C for 1 h and centrifuged at 20,000 *g* for 30 min. A trace amount of CN-[^{57}Co]Cbl (0.1 to 5.0 pmol) was added to the supernatant, and the samples were incubated at 37°C for 2 h. The samples were then cooled to 4°C in an ice-water bath, and the Cbl was purified by reverse affinity chromatography on hog R protein-Sepharose followed by phenol extraction. A trace amount of CN-[^{58}Co]Cbl, 10 pmol, was added to the purified samples which were then fractionated by paper chromatography on Whatman 3MM paper (Whatman Inc., Paper Div., Clifton, NJ) in solvent I. Paper chromatograms were dried in a fume hood at 22°C for 1 h, cut into 38 equal fractions, and assayed for ^{57}Co and ^{58}Co. The percentage of ^{57}Co present as [^{57}Co]Cbl analogues was calculated by dividing the percentage of total ^{58}Co in the peak fraction into the percentage of total ^{57}Co present in the same fraction, subtracting this value from 1.00 and multiplying by 100.

Incubations with individual vitamins and minerals were performed in H_2O at 37°C for 2 h. The amount of each item per 10 ml was the same as the stated amount present in one Theragran pill (see below).

The gastrointestinal absorption of [^{57}Co]Cbl analogues was studied by adding CN-[^{57}Co]Cbl to the supernatant (see above) from a Theragran-M (ferrous carbonate form) homogenate, incubating the sample for 2 h at 37°C and immediately giving portions of the sample orally to mice (5 μl containing ~2 pmol of total [^{57}Co]Cbl per mouse) that had been fasted overnight. After 24 h, the mice were killed, the esophagus, stomach, intestine, and colon were removed, and the total ^{57}Co content of the remainder of each animal was determined. This value was divided by the amount of ^{57}Co given orally, multiplied by 100 and taken as the percent ^{57}Co that was absorbed. The [^{57}Co]Cbl was then extracted from these animals (9) and purified by reverse affinity chromatography and phenol extraction. A trace amount of CN-[^{58}Co]Cbl was added and the sample was fractionated by paper chromatography in solvent I as described above.

The activity of Cbl analogues for Cbl-dependent enzymes in liver was studied by combining 20 nmol of crystalline CN-Cbl or various Cbl analogues with 30 nmol of hog R protein in 0.5 ml of 0.01 potassium phosphate pH 7.5, 0.14 M NaCl and injecting the samples intraperitoneally into mice. 5 d after injection, the mice were killed with ether, and the livers were homogenized (34) and assayed for holo-methylmalonyl-CoA mutase and methionine synthetase as described above.

RESULTS

Formation of Cbl analogues from Cbl in a multivitamin-mineral pill. A chromatogram of Cbl obtained from a Theragran-M (ferrous carbonate form) incubation is shown in Fig. 1A together with a control chromatogram obtained with crystalline CN-Cbl, which is shown in Fig. 1B. In the control chromatogram, a single red spot was observed that cochromatographed with the single major peaks of [^{57}Co]Cbl and [^{58}Co]Cbl. A red spot that cochromatographed with the single major peak of [^{58}Co]Cbl (added just before paper chromatography) was also observed in the experimental chromatogram (Fig. 1A), but slow-moving yellow and pink spots and fast-moving orange, pink, and yellow spots were also observed, suggesting that Cbl analogues were present. This possibility was supported by the fact that the additional colored spots cochromatographed with additional peaks of [^{57}Co]Cbl (present during the incubation). Based on the distribution of [^{57}Co]Cbl on the paper chromatogram, at least 65.1% of the CN-[^{57}Co]Cbl had been converted to [^{57}Co]Cbl analogues. This occurred predominantly during the 2-h incubation at 37°C, and not during the subsequent

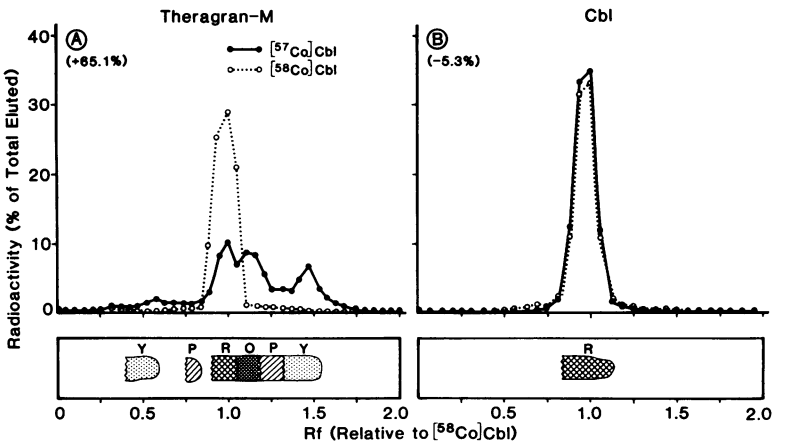

FIGURE 1 Paper chromatograms of Ⓐ Cbl isolated from a multivitamin-mineral pill (Theragran-M, ferrous carbonate form) and Ⓑ crystalline CN-Cbl. Ⓐ, the sample was obtained from the supernatant of an homogenate (Methods) of 100 Theragran-M pills, stated to contain a total of 367 nmol of CN-Cbl, which was incubated with 2 pmol of CN-[^{57}Co]Cbl for 2 h at 37°C. The Cbl was then isolated using reverse affinity chromatography on R protein-Sepharose followed by phenol extraction as described under Methods. The recovery of [^{57}Co]Cbl was 61.3%. CN-[^{58}Co]Cbl, 10 pmol, was added to the purified Cbl immediately before paper chromatography. Ⓑ, the sample contained 200 nmol of crystalline CN-Cbl, 1 pmol of CN-[^{57}Co]Cbl and 10 pmol CN-[^{58}Co]Cbl, all of which were combined immediately before paper chromatography. Paper chromatography was performed in solvent I as described under Methods. Paper chromatograms were examined for the presence of colored spots (Y, yellow; P, pink; R, red; O, orange) and then cut into 38 equal fractions, which were assayed for ^{57}Co and ^{58}Co. The numbers in parentheses indicate the amounts of [^{57}Co]Cbl present as Cbl analogues, relative to that of [^{58}Co]Cbl, and were calculated as described under Methods.

purification steps, because in a separate experiment only 5.3% of CN-[^{57}Co]Cbl was converted to [^{57}Co]Cbl analogues when the CN-[^{57}Co]Cbl was added at the end of the 2-h incubation (chromatogram not shown).

The value of 65.1% for [^{57}Co]Cbl analogues shown in Fig. 1A is a minimal value because additional [^{57}Co]Cbl analogues may have been present that were not recovered during the purification or were not separated from [^{57}Co]Cbl during paper chromatography.

Properties of Cbl and Cbl analogues isolated from a multivitamin-mineral pill. The red spot and the fast-moving orange and yellow spots from the chromatogram shown in Fig. 1A were eluted with H_2O, assayed for cobalt content using X-ray fluorescence, and then assayed for Cbl using a variety of different assays. The results are presented in Table I and demonstrate that the red spot was indistinguishable from crystalline CN-Cbl. The various assays gave definite but reduced values for the fast-moving orange and yellow spots when compared with the values obtained by cobalt analysis, and these results support the concept that they are Cbl analogues.[3] This concept is also supported by the order in which they were detected with these assays, i.e., cobalt analysis > radioisotope dilution assay using R protein > microbiologic assays > radioisotope dilution assay using intrinsic factor.

The red spot and the fast-moving orange and yellow spots from Fig. 1A were purified further by paper

TABLE I
Properties of Cbl and Cbl Analogue Fractions Isolated from Theragran-M and Fractionated by Paper Chromatography as Shown in Fig. 1°

Relative mobility during paper chromatography‡	Relative affinity for human Cbl-binding proteins‡		Relative growth-promoting activity for microorganisms‡	
	R protein	IF	*E. gracilis*	*L. leichmanii*
R_f				
0.9–1.1	1.0	0.8	1.1	0.9
1.1–1.2	0.4	0.01	0.3	0.05
1.3–1.6	0.1	0.008	0.1	0.03

° The molar amounts of the individual fractions were obtained by Cbl analysis using X-ray fluorescence and are based on the assumption that 1 mol of cobalt is present per mol of cbl or Cbl analogue.

‡ All values are relative to those obtained for CN-Cbl.

[3] The unequivocal determination of the structures of Cbl analogues requires the use of X-ray crystallography and was beyond the scope of the current study.

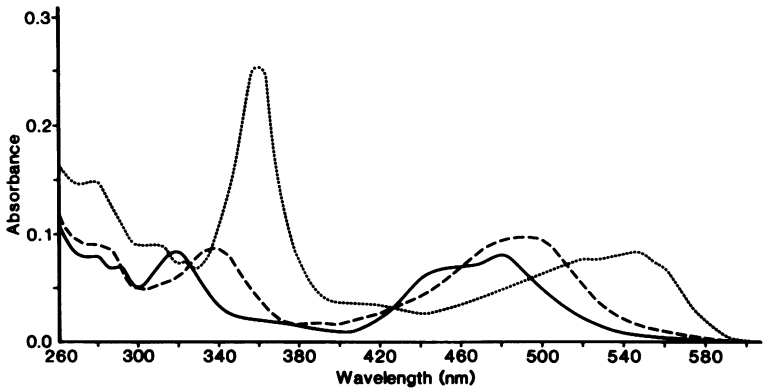

FIGURE 2 Absorption spectra of Cbl and two Cbl analogues isolated from a multivitamin-mineral pill (Theragran-M, ferrous carbonate form). The red, · · ·, fast moving orange, - - -, and yellow, ——, spots from the chromatogram shown in Fig. 1A were eluted with H_2O, purified further by paper chromatography in solvent II, and their absorption spectra were measured in H_2O at pH 7 at a cobalt concentration of 10 nmol/ml. The absorption spectrum of the red spot is indistinguishable from that obtained with crystalline CN-Cbl (not shown). The absorption spectra of the orange and yellow spots resemble that of the red spot but are clearly different. These results support the concept that the various colored spots observed in Fig. 1A are Cbl analogues.

chromatography in solvent II (Methods) and Fig. 2 illustrates their absorption spectra. The absorption spectra of the red spot was indistinguishable from that of crystalline CN-Cbl (not shown). The absorption spectra of the orange and yellow spots resembled that of CN-Cbl, although their absorption maxima were shifted to lower wavelengths and a marked reduction was observed for the absorption maximum that occurs at 361 nm with CN-Cbl. These results support the concept that the orange and yellow spots shown in Fig. 1A are Cbl analogues and suggest that they contain alterations in the corrin ring.

Comparison of Cbl analogue formation in various multivitamin and multivitamin-mineral pills.. Three different multivitamin pills and three corresponding multivitamin-mineral pills whose stated compositions are presented in Table II were also studied with regard to Cbl analogue formation. The results are shown in Fig. 3 and indicate that CN-[^{57}Co]Cbl was not converted to [^{57}Co]Cbl analogues with the three multivitamin pills. The conversion of CN-[^{57}Co]Cbl to [^{57}Co]Cbl analogues was observed with the corresponding pills that contain minerals in addition to essentially the same kinds and amounts of multivitamins.

The formation of [^{57}Co]Cbl analogues with Theragran-M (ferrous fumarate form) was 19.6% as shown in Fig. 3B and this was less than the value of 65.1% observed with Theragran-M (ferrous carbonate form) as shown in Fig. 1A. Differences of this magnitude were observed repeatedly in paired experiments in which the formation of [^{57}Co]Cbl analogues ranged from 60 to 90% with Theragran-M (ferrous carbonate form) and from 16 to 34% with Theragran-M (ferrous fumarate form).

Conversion of CN-Cbl into Cbl analogues by vitamin C, thiamine, and copper. The experiments in the preceding section indicated that one or more minerals played an essential role in the conversion of CN-Cbl to Cbl analogues and that the form of iron present might be of some importance. Therefore, a number of experiments were performed in which individual minerals, in the amounts present in Theragran-M (Table II), were added to supernatants of Theregran homogenates, 10 ml H_2O per pill, and the conversion of CN-[^{57}Co]Cbl to [^{57}Co]Cbl analogues was determined. [^{57}Co]Cbl analogues were not formed (<5%) when magnesium, manganese, zinc, iodide, or iron (ferrous fumarate) were added individually but the addition of copper sulfate alone did result in the formation of 31.6% [^{57}Co]Cbl analogues as shown in Fig. 4A.

The addition of copper sulfate to solutions containing CN-[^{57}Co]Cbl did not result in the formation of [^{57}Co]Cbl analogues (<1%) and this indicated that one or more vitamins also played an essential role in Cbl analogue formation. Additional experiments were performed with individual vitamins and combinations of vitamins in the amounts present in Theragran. As shown in Fig. 4B, we observed that the combined presence of vitamin C, thiamine, and copper caused the formation of [^{57}Co]Cbl analogues (61.3%) from CN-[^{57}Co]Cbl. Less than 1% [^{57}Co]Cbl analogue formation was observed with the individual components or with all combinations of two of the three components.

TABLE II
*Stated Compositions of Multivitamin and Multivitamin-Mineral Pills**

Item	Theragran	One-A-Day	Optilets-500
Vitamins:			
A	10,000 IU	5,000 IU	10,000 IU
C (Na-ascorbate)	200 mg	60 mg‡	500 mg
D	400 IU	400 IU	400 IU
E	15 IU	15 IU	30 IU
B_1 (Thiamine)	10 mg	1.5 mg	15 mg
B_2	10 mg	1.7 mg	10 mg
B_6	5 mg	2 mg	5 mg
B_{12} (CN-Cbl)	5 μg	6 μg	12 μg
Niacin	—	20 mg	—
Niacinamide	100 mg	—	100 mg
Folic acid	—	0.4 mg	—
Pantothenic acid	20 mg	—§	20 mg
Minerals:			
Copper (sulfate)	2 mg	2 mg	2 mg
Iron‖	12 mg	18 mg	20 mg
Calcium	—	100 mg	—
Iodine	150 μg	150 μg	150 μg
Magnesium	65 mg	100 mg	80 mg
Manganese	1 mg	—	1 mg
Phosphorus	—	100 mg	—
Zinc	1.5 mg	15 mg	1.5 mg

* Theragran, One-A-Day, and Optilets-500 contain only the vitamins while Theragran-M, One-A-Day plus Minerals, and Optilets-M-500 contain both the vitamins and the minerals.
‡ Present as ascorbic acid.
§ 10 mg are present in One-A-Day plus minerals.
‖ Present as ferrous carbonate or ferrous fumerate in Theragran-M, ferrous fumarate in One-A-Day plus minerals, and ferrous sulfate in Optilets-M-500.

The presence of iron appeared to modulate Cbl analogue formation because when ferrous fumarate was added to solutions containing vitamin C, thiamine, and copper sulfate, the formation of [^{57}Co]Cbl analogues decreased from 61.3 (Fig. 4B) to 31.8% (Fig. 4C). Ferrous carbonate was not studied because this material is not commercially available. It might be expected to be less effective than ferrous fumarate, however, because ferrous carbonate is very insoluble in H_2O. This difference appears to explain the greater formation of Cbl analogues with Theragran-M (ferrous carbonate form) than with Theragran-M (ferrous fumarate form).

The molecular mechanism by which vitamin C, thiamine, and copper convert CN-Cbl to a number of Cbl analogues is unknown. At the concentrations used in the preceding experiments, vitamin C, thiamine, and copper clearly interact among themselves, however, because when a copper sulfate solution, which has a pale blue color, was added to a solution containing thiamine and vitamin C, which is colorless, a dark brown color developed within seconds. The dark brown color changed to an opalescent yellow color within the first minute and increased in intensity over the following 2 h.

Effect of time, pH, and Cbl-binding proteins. The conversion of CN-[^{57}Co]Cbl to [^{57}Co]Cbl analogues occurred rapidly and was not linear with respect to time, because a 15-min incubation of CN-[^{57}Co]Cbl with vitamin C, thiamine, and copper at 37°C gave a value of 39.9% for the formation of [^{57}Co]Cbl analogues whereas a simultaneously performed 2-h incubation gave a value of 66.1% (chromatograms not shown). When vitamin C, thiamine, and copper were incubated together for 15 min at 37°C before the addition of CN-[^{57}Co]Cbl followed by an additional 2-h incubation, the formation of [^{57}Co]Cbl analogues was only 18.3% (chromatograms not shown). This suggests that CN-[^{57}Co]Cbl must be present from the initial combining of vitamin C, thiamine, and copper if maximal [^{57}Co]Cbl analogue formation is to occur.

The pH was measured for the incubation solutions using Theragran-M, Theragran and copper, and the combination of vitamin C, thiamine, and copper, and values in the range of 6.5 to 7.5 were observed. The

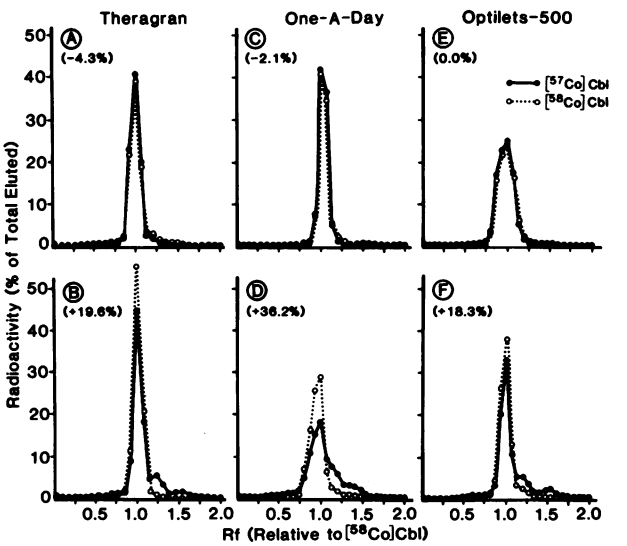

FIGURE 3 Paper chromatograms of CN-[^{57}Co]Cbl that was incubated with aqueous extracts of various multivitamin and multivitamin-mineral pills for 2 h at 37°C. These experiments were performed as described in the legend to Fig. 1A except that 10 pills were used in each case. The multivitamin pills studied were: Ⓐ, Theragran; Ⓒ, One-A-Day; and Ⓔ, Optilets-500. The corresponding multivitamin-mineral pills studied were: Ⓑ, Theragran-M (ferrous fumerate form); Ⓓ, One-A-Day plus minerals; and Ⓕ, Optilets-M-500. The numbers in parentheses indicate the percent conversion of CN-[^{57}Co]Cbl to [^{57}Co]Cbl analogues.

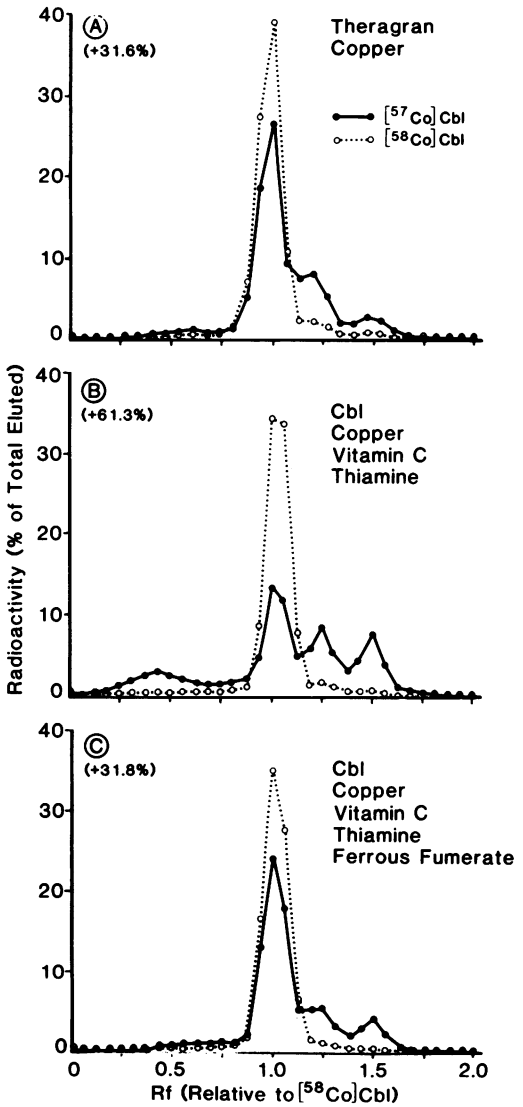

FIGURE 4 Conversion of CN-Cbl to Cbl analogues by the concerted action of copper, vitamin C, and thiamine, and modulation of Cbl analogue formation by iron. These experiments were performed as described in the legends to Figs. 1 and 3. The amounts of the individual vitamins and copper were the same as those stated to be present in Theragran-M (Table II and Methods).

formation of [^{57}Co]Cbl analogues decreased to 10–20% when the pH was lowered to 2.0 with HCl or acetic acid before the standard 2-h incubation (chromatograms not shown). The effect of Cbl-binding proteins on Cbl analogue formation was studied by incubating CN-[^{57}Co]Cbl with a 1.2-fold molar excess of hog R protein, hog intrinsic factor, or bovine serum albumin for 30 min before vitamin C, thiamine, and copper were added under the standard 2-h incubation conditions.[4] The formation of [^{57}Co]Cbl analogues was 7.4, 9.4, and 25.9% for the incubations using hog R protein, hog intrinsic factor, and bovine serum albumin, respectively (chromatograms not shown). These results suggest that the formation of Cbl analogues in the stomach and intestine after the ingestion of multivitamin-mineral pills may be less than that observed when they are incubated in H_2O in vitro. The partial protection afforded by low pH and Cbl-binding proteins may not be realized with pills, however, because extremely high concentrations of various components are likely to occur as small amounts of liquid seep into the interstices of pills in the stomach. Extensive destruction of CN-Cbl (>80%) was observed when either form of Theragran-M was shaken in freshly aspirated human gastric juice (pH 2.0, 30 ml per intact pill) at 37°C for 45 min. The formation of Cbl analogues could not be quantitated precisely in these experiments because these Cbl analogues are underestimated even with the radioisotope dilution assay using R protein (Table I), because insufficient quantities were available for cobalt analyses, and because it was not possible for us to incorporate radioactive CN-Cbl into Theragran-M.

Comparison of CN-Cbl and OH-Cbl. OH-Cbl differs from CN-Cbl in that it is readily converted to Cbl analogues when incubated in H_2O at 37°C with vitamin C alone. At pH values of 1.8, 4.0, 6.0, and 8.0, we observed that 21, 89, 86, and 90% of OH-[^{57}Co]Cbl was converted to [^{57}Co]Cbl analogues, respectively, with a 30-min incubation at 37°C. Under these conditions, <2% of CN-[^{57}Co]Cbl was converted to [^{57}Co]Cbl analogues. The formation of Cbl analogues from OH-[^{57}Co]Cbl by vitamin C was the same as just noted when bovine serum albumin was added to incubation mixtures or when the OH-Cbl was incubated bound to either hog R protein or hog intrinsic factor. In the latter two cases there was a moderate increase in the formation of slow-moving [^{57}Co]Cbl analogues with a corresponding moderate decrease in the formation of fast-moving [^{57}Co]Cbl analogues (chromatograms not shown). Whether Cbl analogues derived from OH-Cbl are the same as those derived from CN-Cbl remains to be determined.

Amount of Cbl and Cbl analogues in multivitamin-mineral pills. The labels on the various Theragran-M and Theragran pills state that each contains 5 μg (3.69 nmol) of CN-Cbl per pill. This value is incorrect in each case, because when we purified Cbl from these

[4] The isolation of Cbl was changed in experiments using Cbl-binding proteins such that the phenol extraction procedure was used before reverse affinity chromatography because Cbl is released from Cbl-binding proteins by phenol (27).

pills in the presence of a known amount of CN-[^{57}Co]Cbl and performed cobalt analyses on the purified material and recovery corrections, we calculated values of 2.8, 6.6, and 12.8 nmol of total Cbl per pill for Theragran-M (ferrous carbonate form), Theragran-M (ferrous fumarate form), and Theragran, respectively. These results suggest the possibility that the manufacturer may have noticed that CN-Cbl is being "lost" during the manufacture or storage of Theragran-M and is adding additional CN-Cbl to compensate for this loss. If 12.8 nmol of CN-Cbl per pill is actually being added to each of the two Theragran-M preparations, this would indicate that extensive destruction of CN-Cbl occurs during their manufacture and storage with possible extrusion of cobalt from the corrin ring of Cbl.

Gastrointestinal absorption of Cbl analogues from a multivitamin-mineral preparation. When CN-[^{57}Co]Cbl was added to the supernatant from a Theragran-M (ferrous carbonate form) homogenate, incubated for 2 h at 37°C, and immediately given to five mice orally (~2 pmol of total [^{57}Co]Cbl per mouse), they absorbed (Methods) only 4.5–8.3% of the ^{57}Co with a mean value of 6.1%. The absorption of CN-[^{57}Co]Cbl that had been incubated with H_2O alone ranged from 26.1 to 42.6% with a mean value of 35.6%. Absorption values ranging from 19.1 to 36.5% with a mean value of 29.9% were observed for CN-[^{57}Co]Cbl that had been incubated with the supernatant from an homogenate of Theragran. The paper chromatography profiles obtained with Theragran-M before and after absorption are presented in Fig. 5 and show that [^{57}Co]Cbl analogues represented 89.6% of the total [^{57}Co]Cbl present in the material that was given orally and 49.8% of the total [^{57}Co]Cbl that was absorbed. [^{57}Co]Cbl analogues were not observed (<3%) before or after absorption with the CN-[^{57}Co]Cbl that had been incubated with H_2O or Theragran (chromatograms not shown). These results indicate that Cbl analogues formed from multivitamin-mineral pills are absorbed by mice. They do not appear to be absorbed as well as Cbl, itself, however, although we cannot rule out the possibility that they are converted back to Cbl in vivo. A comparison of Fig. 5A and B indicates that some Cbl analogues are absorbed with higher efficiencies than others.

Inhibition and lack of stimulation of Cbl-dependent enzymes by Cbl analogues from a multivitamin-mineral pill. The data in Table III show that the intraperitoneal injection of 20 nmol of crystalline CN-[^{57}Co]Cbl bound to hog R protein, which is taken up exclusively by hepatocytes (2, 36), stimulated the activities of both mammalian Cbl-dependent enzymes in mouse liver when they were assayed 5 d after injection. Holo-methylmalonyl-CoA mutase and methionine synthetase activity increased to 318 and 147% of control values, respectively. Similar respective increases to 301 and 166% of control values were observed with 20 nmol of [^{57}Co]Cbl isolated from a Theragran-M (ferrous carbonate form) incubation. Significant stimulation of either enzyme was not observed with 20 nmol of four [^{57}Co]Cbl analogue fractions isolated from the Theragran-M incubation. Two of these fractions actually caused a significant ($P < 0.05$) reduction of methionine synthetase activity to 76 and 65% of control values. These results indicate that Cbl analogues formed in multivitamin-mineral pills have markedly reduced or absent Cbl activities for both Cbl-dependent enzymes in mouse liver and that some of these analogues actually inhibit one of these enzymes.

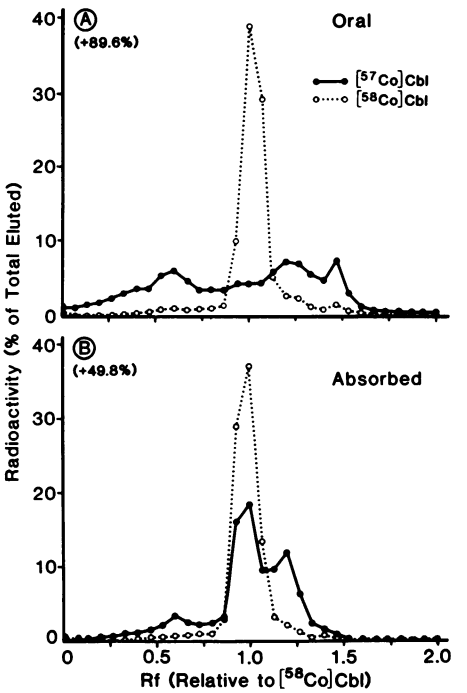

FIGURE 5 Gastrointestinal absorption of [^{57}Co]Cbl analogues from a multivitamin-mineral pill (Theragran-M, ferrous carbonate form). Ⓐ paper chromatogram of CN-[^{57}Co]Cbl that was incubated with an aqueous extract of Theragran-M for 2 h at 37°C and immediately given orally to mice, (~2 pmol of total Cbl per mouse). Ⓑ, paper chromatogram of the same [^{57}Co]Cbl that was extracted from mouse tissues 24 h after its oral administration. Additional details concerning the preparation of the Cbl analogues and the performance of paper chromatography are described in the legend to Fig. 1A and under Methods.

DISCUSSION

Before 1950, humans obtained their Cbl from animal tissues or from animal products such as milk and cheese

TABLE III

Activities of Cbl-dependent Enzymes in Mouse Liver 5 d after the Intraperitoneal Injection of 30 nmol of Hog R Protein Containing 20 nmol of CN-Cbl or Cbl or Cbl Analogues Isolated from a Multivitamin-Mineral Pill

Items injected			Methylmalonyl-CoA mutase		Methionine synthetase	
Cbl	Hog R protein	No. of animals	Mean	Range	Mean	Range
			% control			
—	−	4	(100)*	(90–107)	(100)‡	(86–109)
—	+	4	97	81–121	101	97–104
CN-Cbl	+	4	318§	313–322	147§	144–149
R_f of paper chromatogram fractions from Theragran-M, ferrous carbonate form						
0.0–0.9	+	2	113	111–115	103	102–104
0.9–1.1	+	2	301§	283–320	166§	142–191
1.1–1.2	+	2	112	110–115	76§	76–77
1.2–1.3	+	2	84	81–88	65§	63–66
1.3–1.6	+	2	91	89–94	103	94–112

* Control animals converted 9.7 nmol of DL-2-[*methyl*-^{14}C]methylmalonyl-CoA to [^{14}C]succinyl-CoA per minute per gram of liver.
‡ Control animals converted 47 pmol of N^5-[*methyl*-^{14}C]tetrahydrofolic acid to [^{14}C]methionine per minute per gram of liver.
§ These values were different from the control values ($P < 0.05$) by Student's two-tailed t test.

(1). Animals that are herbivorous obtained their Cbl directly from microorganisms. This was accomplished readily by ruminants, and by the ingestion of feces by nonruminants. Microorganisms in rumens and feces synthesize Cbl and even larger amounts of a variety of Cbl analogues (1) that contain bases other than 5,6-dimethylbenzimidazole, which is the base present in Cbl. These particular Cbl analogues are not found in normal animal tissues, however, because of the existence of multiple mechanisms that prevent their absorption and tissue dissemination (2).

The origin of the Cbl that humans in developed countries ingest has changed dramatically since 1950 and this change is increasing at a rapid rate. Cbl is now purified from bacterial cultures, converted to CN-Cbl, and added to multivitamin pills and foods such as breakfast cereals, diet foods, and infant formulas. The origin of much of the Cbl in foods of animal origin is the same as that just described, because in developed countries poultry and hogs are now raised almost exclusively on synthetic chows. Even ruminants such as cattle are usually kept in feed lots during the last 3–6 mo of life where frequently they are fed synthetic chows and multivitamin-mineral supplements.

In our study, we have shown that the interaction of vitamin C, thiamine, and copper converts CN-Cbl to a large number of Cbl analogues that appear to contain alterations in the corrin ring, partially escape the protective mechanisms that exist for Cbl analogues of microorganismal origin, and closely resemble the Cbl analogues that we have isolated from animal tissues (9). These Cbl analogues are not absorbed as well as Cbl, but eventually they could represent a high percentage of total body Cbl if they are excreted from the body at a slower rate than that of Cbl. This possibility is suggested by the fact that the mean plasma Cbl analogue level was 75% of normal in a group of patients with clinical evidence of Cbl deficiency whose mean plasma Cbl level was only 10% of normal (8). It is interesting in this regard, that the Cbl analogues formed from multivitamin-mineral interactions have markedly reduced or absent Cbl activity for methylmalonyl-CoA mutase and methionine synthetase in mouse liver and may be inhibitory in some cases.

Vitamins and minerals are obviously present in natural foods, although their forms and concentrations are frequently different and usually lower than those in animal chows, supplemented foods, and multivitamin-mineral pills. Thus, they could interact under natural conditions to form Cbl analogues, although this does not appear to happen in animal tissues in vivo (Introduction). The fact that rabbits grow well on chows that contain Cbl analogues might suggest that they are not detrimental. Extrapolations from laboratory animals to humans must be made with caution, however, because humans live much longer than these animals. Humans also differ from animals in some major undefined aspect of Cbl metabolism because Cbl-deficient humans develop megaloblastic anemia, neurologic abnormalities, or both, while megaloblastic ane-

mia has never been observed in Cbl-deficient animals, and neurologic abnormalities have been observed in only a few primates (31).

It is clear that multivitamin-mineral interactions may result in the conversion of other vitamins to analogues with unknown biologic consequences for the general population and for subsets[5] of the population. Considerations should be given to basing the use of vitamins and minerals in pill form on specific therapeutic indications in the same way that we base the use of prescription drugs. The situation with respect to supplemented foods and animal chows is more complex, however, because it is probably not economically feasible to reduce their use. Both of these and vitamin pills should be analyzed more carefully, however, because analogues of various vitamins may not be recognized unless they are looked for directly. The practice of merely adding additional amounts of a vitamin when recovery assays indicate a loss, should be avoided unless it can be shown that the loss does not actually represent the conversion to vitamin analogues, or that these analogues are not harmful. Studies of this kind are clearly worth pursuing as we continue to alter the preparation and composition of our diet.

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[5] Because of genetic heterogeneity, certain individuals could be particularly susceptible to vitamin analogues in the same way that individuals that have one or two genes for hemochromatosis may be susceptible to fortification of foods with iron.

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