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Presence of cobalamin analogues in animal tissues

(vitamin B-12/intrinsic factor/R protein/cobalt)

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ABSTRACT **Cobalamin (Cbl, vitamin B-12) has been extracted and isolated from a number of animal tissues by using (*i*) reverse-affinity chromatography on R protein-Sepharose followed by adsorption to and elution from charcoal-coated agarose and (*ii*) paper chromatography. Radioisotope dilution assays showed that only 75–97% of the Cbl chromatographed in the position of crystalline Cbl. The remaining 3–25% was present in a number of slower and faster moving fractions. This suggested that Cbl analogues are present in animal tissues because appropriate controls ruled out the possibility that this material was artifactually derived from Cbl during the extraction and purification procedures. With a large-scale isolation from rabbit kidney, the material in five such fractions contained cobalt and had absorption spectra that were similar to but different from the spectrum of Cbl, indicating that they were Cbl analogues. Compared to Cbl, these Cbl analogues had decreased but definite affinities for Cbl-binding proteins with the following order of strength of binding: R protein > transcobalamin II > intrinsic factor. Compared to Cbl, they also had decreased but definite growth-promoting activity for two microorganisms, *Euglena gracilis* and *Lactobacillus leichmannii,* which require Cbl for growth. These Cbl analogues differed from each other and from 18 synthetic Cbl analogues, including the most common Cbl analogues synthesized by microorganisms, in at least one of the above features. These studies indicate that animal tissues contain a number of Cbl analogues whose origins, structures, and biologic activities remain to be determined.**

Cobalamin (Cbl, vitamin B-12) and various Cbl analogues are synthesized only by microorganisms which utilize them as coenzymes for a number of enzymatic reactions (1). Man and other animals require Cbl as a coenzyme for methionine synthase (tetrahydropteroylglutamate methyltransferase; 5-methyltetrahydropteroyl-L-glutamate : L-homocysteine-*S*-methyltransferase, EC 2.1.1.13) (2) and methylmalonyl-CoA mutase (methylmalonyl-CoA CoA-carbonyl mutase, EC 5.4.99.2) (3). Man and other animals cannot synthesize Cbl and have developed intricate mechanisms in which Cbl-binding proteins facilitate the gastrointestinal absorption and plasma transport of this trace substance (4, 5).

Gastric intrinsic factor (IF) binds Cbl and facilitates its absorption from the terminal ileum. Plasma transcobalamin II binds Cbl and facilitates its uptake by cells throughout the body. R protein* is present in granulocytes and secretions. Although its exact function is unknown, R protein does bind Cbl and prevent its uptake by microorganisms, some of which require Cbl for growth. The granulocyte R protein is also released into plasma and delivers Cbl exclusively to hepatocytes (6, 7) by utilizing the receptor-mediated mechanism elucidated by Ashwell and Morell (8) that recognizes terminal galactose residues on glycoproteins.

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Several of these mechanisms also function to prevent the gastrointestinal absorption and tissue dissemination of inactive and potentially toxic Cbl analogues. Most naturally occurring Cbl analogues are not bound by IF with high affinity and thus do not gain entry into ileal mucosal cells (7, 9, 10). In many cases, those Cbl analogues that do gain entry do not enter the portal plasma (7). Transcobalamin II binds and facilitates the cellular uptake of a number of Cbl analogues (7), but the prevention of their dissemination in tissue appears to be served by the granulocyte R protein which binds a large number of Cbl analogues and delivers them exclusively to hepatocytes, which in turn retain them and eventually effect their excretion in the urine and feces (7). Because of these mechanisms, and because Cbl analogues were not observed when Cbl was initially purified from animal tissues (11) by a long series of chromatographic procedures and microbiologic assays, it has been assumed that Cbl analogues are not present in animal tissues.

We have covalently attached R protein to Sepharose and have shown that this affinity adsorbent can be utilized to isolate Cbl and Cbl analogues from bacterial lysates in a single step (12). We have also utilized this technique to isolate Cbl from pooled normal human plasma (13). When this Cbl was fractionated by paper chromatography and assayed for Cbl with a radioisotope dilution assay (RIDA) using R protein, a number of slow and fast moving peaks were observed in addition to the major peak which coincided with that of crystalline Cbl. Each of the additional peaks was relatively small but, taken together, they represented approximately 40% of the total Cbl present in human plasma. These minor peaks were either barely or not detectable with a RIDA for Cbl that used IF or with microbiologic assays using *Euglena gracilis* and *Lactobacillus leichmannii.* These observations suggested that human plasma contains a number of Cbl analogues, but the low Cbl concentration (0.3 nM) in plasma made it impossible to demonstrate this directly. We now report the results of similar studies performed with various animal tissues from which it has been possible to obtain larger quantities of Cbl.

MATERIALS AND METHODS

[^{57}Co]Cbl (100–300 μCi/μg; 1 Ci = 3.7 × 10^{10} becquerels) and [^{58}Co]Cbl (3 μCi/μg) were obtained from Amersham. Crystalline Cbl from Sigma was assayed spectrophotometrically (7). Human IF (13), salivary R protein (13), and transcobalamin II were obtained as described (7). The preparation of most of the

Abbreviations: Cbl, cobalamin; IF, intrinsic factor; RIDA, radioisotope dilution assay.

* The term "R protein" was originally devised to denote a Cbl-binding protein in human gastric juice that was devoid of IF activity and that showed rapid mobility on electrophoresis. Subsequently, immunologically related proteins have been observed in various human tissues and body fluids and have been referred to as the R proteins.

synthetic Cbl analogues has been described (7). Additional Cbl analogues with alterations in the nucleotide portion were prepared by a modification (12) of the biosynthetic technique of Perlman and Barrett (14) using *Propionibacterium arabinosum* (ATCC 4965). The following bases were added to cultures of this bacterium to obtain the indicated Cbl analogues: 5(6)-chlorobenzimidazole, [5(6)-ClBZA]CN-Cba; 5(6)-methoxybenzimidazole, [5(6)-OCH_3BZA]CN-Cba; 3,4-dinitrophenol, [5(6)-OHBZA]CN-Cba; and 5(6)-carboxybenzimidazole, [5(6)-COOHBZA]CN-Cba. Serum, muscle, heart, and ileum were obtained from male New Zealand White rabbits. Liver, kidney, brain, bile, and spinal cord were obtained frozen from Pel-Freez. Human placenta was obtained from healthy subjects immediately after vaginal delivery. Beef and pork muscle, oysters, and sole fillets were obtained from a local meat market. Rabbit chow (Wayne 15% rabbit ration) was obtained from Allied Mills (Chicago, IL).

Endogenous Cbl was extracted from tissues (10–300 g) and other sources by a modification (13) of the method of Frenkel *et al.* (15). To each 1 g of material, 1 ml of 0.5 M sodium acetate/HCl at pH 4.5 and 2 ml of 0.15 M NaCl containing 50 μg of KCN per ml were added followed by homogenization in a Waring Blendor at 4°C for 3 min. Approximately 0.1 pmol of [^{57}Co]CN-Cbl was added and the entire homogenate was heated for 45 min at 100°C. After centrifugation at 20,000 × *g* for 30 min at 4°C, the supernatant was removed and subjected to reverse-affinity chromatography on hog R protein-Sepharose as described (12) except that Cbl was eluted with 60% pyridine. The eluates were dried with a Speed-Vac concentrator (Savant) and further purified by adsorption to and elution from charcoal-coated agarose (13). After drying as described above, approximately 4 pmol of [^{58}Co]CN-Cbl was added and the samples were subjected to descending paper chromatography on Whatman no. 3MM paper at room temperature for 30 hr; the developing solvent (solvent I) was 880 ml of *sec*-butanol, 8.2 ml of glacial acetic acid, 6.2 nmol of HCN, and a saturating amount (approximately 425 ml) of H_2O. The paper was dried in a fume hood and cut into 38 equal fractions, each of which was eluted with H_2O. The recovery of labeled Cbl was 50–80% for [^{57}Co]Cbl and 92–98% for [^{58}Co]Cbl. The heat step was omitted during the large-scale (1.5 kg) kidney preparation and the major fractions obtained by paper chromatography in solvent I were further purified by paper chromatography with a solvent (solvent II) in which 1.8 ml of NH_4OH was present in place of the 8.2 ml of acetic acid. For [^{57}Co]Cbl coenzyme determination, tissues were extracted by using Amberlite XAD-2 (16) and assayed by chromatography on SP-Sephadex (17).

RIDAs for Cbl were performed as described (13). Microbiologic assays for Cbl using *E. gracilis* (18) and *L. leichmannii* (19) were performed by V. Michael Whitehead and William S. Beck, respectively. The relative affinities of Cbl analogues for human Cbl-binding proteins were determined as described (7) except that salivary R protein was used instead of granulocyte R protein. Cobalt content was measured by Rodney Smythe by using x-ray fluorescence (20). Absorption spectra were obtained with a Gilford spectrophotometer equipped with a wavelength scanner.

RESULTS

Cbl Isolated from Animal Tissues and Other Sources. Fig. 1 shows the paper chromatography profiles of endogenous Cbl purified from various sources. With crystalline Cbl, RIDAs using R protein and IF gave essentially equivalent results: a single peak that contained approximately 98% of the total Cbl. This peak coincided with the major peak of [^{57}Co]Cbl that was added at the start of the extraction procedure and with the major peak of [^{58}Co]Cbl that was added just before paper chromatography. No changes in the chromatographic profiles of [^{57}Co]Cbl or [^{58}Co]Cbl were observed.†

With Cbl isolated from various animal tissues and from rabbit chow, RIDAs using R protein showed that only 75–97% of the total material was present in the major Cbl peak with the remaining 3–25% of the material being represented by a number of slow- and fast-moving peaks. This suggested that Cbl analogues are present. This concept is supported by the fact that the slow- and fast-moving peaks were smaller when measured by RIDA using IF; IF is much more specific for Cbl than is R protein (7). Because even R protein binds many Cbl analogues with affinities that are slightly or moderately lower than the affinity for Cbl (see below), it is likely that Cbl analogues are present in animal tissues in even greater amounts than indicated in Fig. 1 and Table 1.

In all of the sources shown in Fig. 1, the paper chromatography profiles of [^{57}Co]Cbl and [^{58}Co]Cbl, which were added prior to the initial extraction and prior to paper chromatography, respectively, were unchanged. This indicates that the Cbl analogues in Fig. 1 were not produced artifactually from Cbl during the extraction, purification, or paper chromatographic procedures. Additional evidence to exclude this possibility was obtained from an experiment in which 10 pmol of [^{57}Co]CN-Cbl was injected intravenously into a rabbit. Two days later, at which time most of the [^{57}Co]Cbl is bound to Cbl-dependent enzymes and is close to being in equilibrium with endogenous Cbl (21), the rabbit was sacrificed and the liver and kidneys were extracted. Much of the [^{57}Co]CN-Cbl had been converted to Cbl coenzymes with the following being found for kidney and liver, respectively: CN-Cbl or SO_3^--Cbl, 30 and 32%; CH_3-Cbl, 27 and 26%; Ado-Cbl, 11 and 32%; and OH-Cbl, 32 and 10%. When the [^{57}Co]Cbl was purified and examined by paper chromatography, its chromatographic profile was unchanged from that of the [^{57}Co]Cbl that was injected. The fact that the formation of [^{57}Co]-Cbl analogues was not observed provides strong evidence that Cbl analogues are not produced from endogenous Cbl during the extraction, purification, or paper chromatographic steps and also indicates that, if they are synthesized from Cbl within the body, such synthesis must proceed at a very slow rate.

The fact that Cbl analogues were observed in rabbit chow is of particular interest because we have shown previously (7) that some Cbl analogues with moderately decreased affinities for IF are absorbed by rabbits. The origin of the Cbl analogues in rabbit chow is unknown, although direct analysis of the Cbl supplement utilized in the preparation of the rabbit chow did not reveal the presence of Cbl analogues. Because all of the components of the rabbit chow except for "light-activated animal sterols" are of plant origin, this suggests that the Cbl analogues may be generated from Cbl during the preparation or storage of the rabbit chow. It is also possible that plants contain Cbl analogues even though they do not contain Cbl itself.

Large-Scale Purification of Cbl Analogues from Rabbit Kidney. In order to provide further evidence for the presence of Cbl analogues in animal tissues, endogenous Cbl was isolated from 1.5 kg of rabbit kidney by using a modified procedure in which the heat step was omitted—i.e., only non-protein-bound

† With commercial preparations of [^{57}Co]Cbl and [^{58}Co]Cbl, only 93–97% of the radioactivity cochromatographed with crystalline Cbl during paper chromatography. The remaining 3–7% consisted of multiple slow- and fast-moving peaks that varied from lot to lot. This observation has been reported (13).

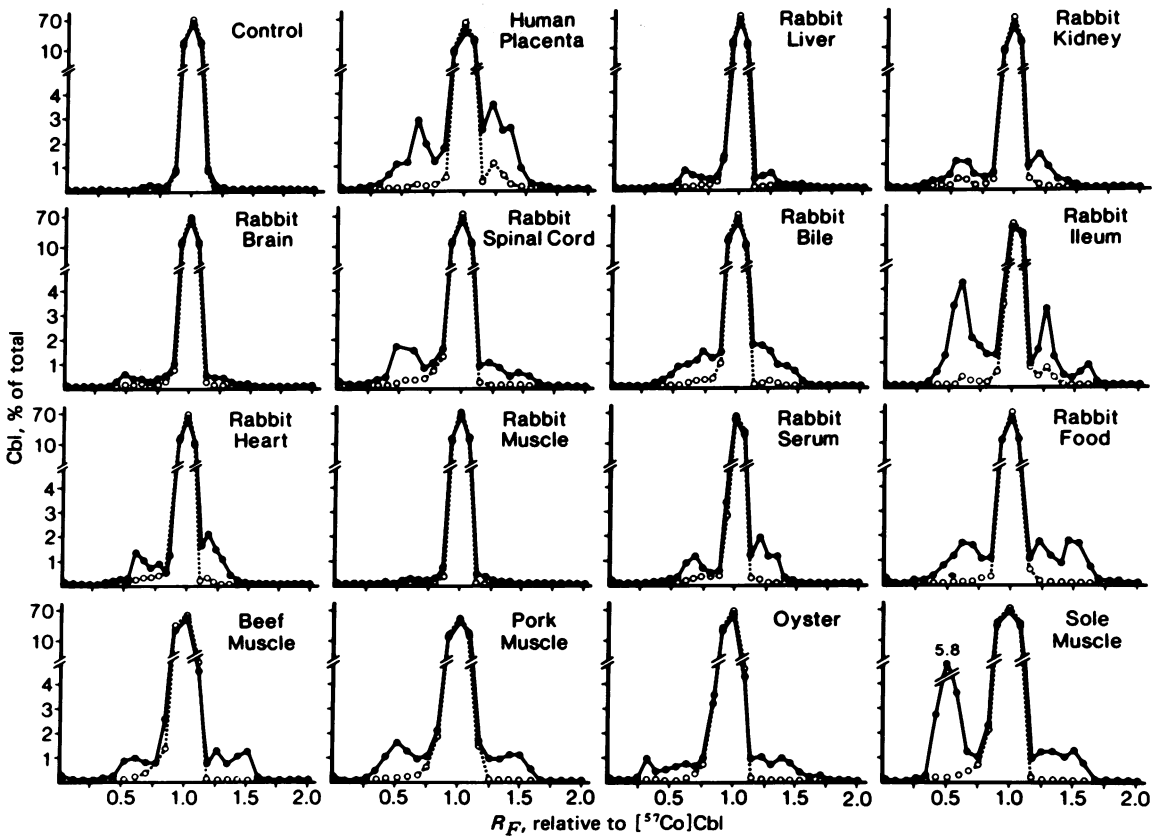

FIG. 1. Paper chromatography profiles of crystalline Cbl (control) and Cbl isolated from animal tissues and other sources. Cbl was extracted, purified, and fractionated by paper chromatography in solvent I. Each paper chromatogram was cut into 38 equal fractions; each fraction was eluted with H_2O. Individual fractions were assayed for Cbl by RIDA using R protein (●—●) and IF (O·····O). Recoveries are given in Table 1.

Cbl was isolated‡, and a second paper chromatography step was added. During paper chromatography in solvent I, a major red spot (fraction D) was observed in the position of Cbl together with three slower moving red spots (fractions A, B, and C). Faster moving orange (fraction E) and yellow (fraction F) spots were also observed. During paper chromatography in solvent II, fractions A, B, C, and D gave single major purple§ spots although several additional minor purple spots were also observed with fractions A, B, and C. Fractions E and F retained their respective orange and yellow colors in solvent II. X-ray fluorescence analysis of the major spots isolated by paper chromatography in solvent II showed that all of them contained cobalt. The amounts, expressed as nmol of cobalt, were: A, 3.9; B, 13.8; C, 2.5; D, 52.3; E, 3.6; and F, 3.1.

Additional data concerning rabbit kidney Cbl fractions A–F are presented in Table 2. Fraction D was indistinguishable from crystalline Cbl on the basis of its mobility during paper chromatography in solvents I and II, its affinity for human R protein, transcobalamin II, and IF, and its ability to promote the growth of *E. gracilis* and *L. leichmannii*. Fractions A, B, C, E, and F differed from crystalline Cbl, from each other, and from each of 18 synthetic Cbl analogues, in at least one of these variables. Compared to Cbl, these fractions had slightly to moderately decreased affinities for R protein and moderately to markedly decreased affinities for IF. Intermediate affinities were observed for transcobalamin II. Compared to Cbl, these fractions had slightly to markedly decreased abilities to promote the growth of *E. gracilis* and *L. leichmannii*. The fact that their affinities for R protein were decreased suggests that the Cbl analogue content of various tissues (see Table 1) will be underestimated by varying degrees when measured with RIDAs using R protein. The data also indicate that tissue Cbl analogues will be measured to a smaller extent with microbiologic assays

Table 1. Recoveries of Cbl and Cbl analogue (Anal)

Source	Cbl* pmol/g	Cbl* %	Anal† pmol/g	Anal† %
Control‡	196	98	4	2
Human placenta	0.3	75	0.1	25
Rabbit liver	297	94	18	6
Rabbit kidney	437	89	54	11
Rabbit brain	40	95	2	5
Rabbit spinal cord	19	86	3	14
Rabbit bile	0.8	80	0.2	20
Rabbit ileum	104	76	32	24
Rabbit heart	46	87	7	13
Rabbit muscle	37	97	1	3
Rabbit serum	17	89	2	11
Rabbit food	1.2	80	0.3	20
Beef muscle	2.8	88	0.4	12
Pork muscle	1.5	88	0.2	12
Oyster	108	89	13	11
Sole muscle	0.9	82	0.2	18

* Amount determined by RIDA (with R protein) of fraction with R_F = 1 (Fig. 1) and the two fractions on either side.

† Amount determined by RIDA (with R protein) of remaining 33 fractions.

‡ Starting material was 225 pmol of crystalline Cbl.

‡ Preliminary studies indicate that much but not all of the endogenous Cbl analogue in rabbit kidney homogenates is either loosely bound or is not bound to protein.

§ Cbl and many Cbl analogues bind a second CN molecule at alkaline pH. The dicyanide forms of Cbl and many Cbl analogues are purple.

Table 2. Comparison of Cbl fractions isolated from rabbit kidney to crystalline Cbl and 18 synthetic Cbl analogues

Cbl analogue	Rel. mobility on paper chromatography*		Rel. affinity for human Cbl-binding proteins†			Rel. biol. activity‡	
	Solvent I	Solvent II	R protein	Transcobalamin II	IF	*E. gracilis*	*L. leichmannii*
Crystalline CN-Cbl	1.0	1.0	1.1	0.9	1.0	0.9	1.0
From rabbit kidney							
Fractions:§							
A	0.4	0.4	0.4	0.1	0.06	0.1	0.1
B	0.5	0.4	0.4	0.09	0.003	0.02	0.2
C	0.7	0.3	0.8	0.2	0.04	0.3	0.7
D	1.0	1.0	1.2	0.9	0.9	1.2	0.8
E	1.3	1.1	0.6	0.08	0.02	0.4	0.07
F	1.6	1.2	0.05	0.002	0.001	0.3	0.004
Synthetic Cbl analogues							
Alterations adjacent to corrin ring:							
CN-Cbl[b-OH]	1.9		0.3	0.006	0.0006	0.9	0.3
CN-Cbl[d-OH]	1.5		0.9	0.3	0.3	0.9	0.2
CN-Cbl[e-OH]	1.9		0.8	0.02	0.004	0.9	0.3
CN-Cbl[bde-OH]	2.4		0.1	0.00002	0.000007	0.1	<0.04
CN-Cbl[13-epi]	0.7		0.6	0.7	0.8	< 0.03	0.04
Alterations in nucleotide:							
[5,6-Cl_2BZA]CN-Cba	1.4		1.2	1.1	1.0	0.3	0.7
[NZA]CN-Cba	1.2		1.3	0.3	0.06	<0.03	0.6
[5,(6)-MeBZA]CN-Cba	0.8		0.9	0.9	0.8	0.6	0.7
[5,(6)-ClBZA]CN-Cba	1.0		0.7	0.7	0.2	0.2	0.4
[5,(6)-NO_2BZA]CN-Cba	0.7		1.0	0.3	0.07	0.07	0.7
[5,(6)-OCH_3BZA]CN-Cba	0.7		1.0	0.7	0.05	0.2	0.7
[5,(6)-COOHBZA]CN-Cba	0.6		0.7	0.3	0.0004	<0.03	1.0
[5,(6)-OHBZA]CN-Cba	0.6		0.5	0.3	0.003	<0.03	0.6
[BZA]CN-Cba	0.6		1.0	0.8	0.2	0.9	0.9
[3,5,6-Me_3BZA](CN,OH)Cba	1.2		1.1	0.0002	0.0002	<0.03	<0.04
[CN,OH]Cbi	1.6		1.1	0.007	<0.000001	<0.03	<0.04
[2-MeAde]CN-Cba	0.4		1.2	0.05	0.0005	<0.03	0.4
[Ade]CN-Cba	0.5		0.8	0.1	0.00007	<0.005	0.7

* Shown as analogue/CN-Cbl.
† Shown as K_a analogue/K_a CN-Cbl.
‡ Growth-promoting activity for microorganisms, shown as analogue/CN-Cbl.
§ Concentrations were determined by x-ray fluorescence analysis for cobalt and are based on the assumption that 1 mol of cobalt is present per mol of Cbl analogue.

using *E. gracilis* and *L. leichmannii*, and to the smallest extent with RIDAs using IF.

The absorption spectra of crystalline Cbl and rabbit kidney fractions A–F at the same cobalt concentration are shown in Fig. 2. Fraction D was essentially indistinguishable from crystalline Cbl. The absorption spectra of fractions A, B, and C resembled the spectrum of crystalline Cbl although the absolute absorption was increased at all wavelengths. The possibility that these increases are due to differences in light scattering or to undetected impurities in these fractions has not been excluded. The absorption spectra of fractions E and F are the most distinct and are clearly different from the spectrum of crystalline Cbl and from each other.

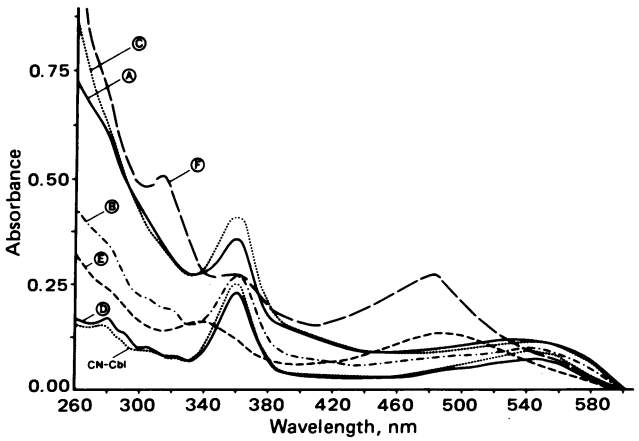

FIG. 2. Absorption spectra of crystalline CN-Cbl and rabbit kidney Cbl fractions A, B, C, D, E, and F. All spectra were obtained in water at a cobalt concentration of 10 nmol/ml.

DISCUSSION

We have reported indirect evidence (13) indicating that human plasma contains a number of Cbl analogues that have much lower relative affinities for IF than for R protein. The present study provides similar evidence that a number of Cbl analogues are present in various animal tissues. It also provides direct evidence for the presence of Cbl analogues in animal tissues because we have shown that the Cbl analogue fractions isolated from rabbit kidney contain cobalt, have decreased but definite affinities for human R protein, transcobalamin II, and IF compared to Cbl, and have decreased but definite growth-promoting activities for *E. gracilis* and *L. leichmannii*. We have also shown that these fractions have absorption spectra that are similar to, but different from, the spectrum of Cbl.

The origins and structures of these newly recognized Cbl analogues are unknown, but these analogues are clearly dif-

ferent from the most common Cbl analogues synthesized by microorganisms—i.e., [Ade]-Cba, [2-MeAde]-Cba, Cbi, [5(6)-MeBZA]-Cba, [BZA]-Cba, and [5(6)-OCH_3BZA]-Cba (22). They could represent either degradation products of these Cbl analogues or other Cbl analogues that are synthesized by microorganisms in trace amounts (22). It is also possible that they are derived from Cbl itself, either before or after absorption, although the latter would have to take place slowly because no changes in [^{57}Co]Cbl were observed during the 48 hr after its parenteral administration to a rabbit. The chemical conversion of Cbl into Cbl analogues prior to absorption is a definite possibility because Cbl analogues were observed in rabbit chow and because we have recently observed (unpublished data) that Cbl analogues are present in varying amounts in multivitamin preparations and in breakfast cereals and diet foods that are supplemented with Cbl and other vitamins. The alteration of Cbl by other vitamins has been observed previously (23). This could explain the presence of Cbl analogues in human, rabbit, bovine, and porcine tissues and could also explain their presence in oysters and sole fillets because large amounts of animal wastes are discharged into the ocean. It is also possible that these Cbl analogues are the result of bacterial degradation of Cbl, which has been reported (24, 25).

The activities or inhibitory properties of these newly recognized Cbl analogues for the two animal Cbl-dependent enzymes and the enzymes that convert Cbl to its coenzyme forms are also unknown. It is unlikely that they possess full Cbl activity, however, because they all have decreased growth-promoting activity for *E. gracilis* or *L. leichmannii* and because we have observed (13) that plasma levels of Cbl analogues often are not decreased in patients with clinical manifestations of Cbl deficiency and low plasma levels of Cbl. Differences in the tissue distribution of Cbl analogues or in their activities and inhibitory properties toward the individual Cbl-dependent enzymes and the individual enzymes involved in Cbl coenzyme synthesis, together with differences in the contents of various Cbl analogues among patients with Cbl deficiency, could explain why some patients with Cbl deficiency develop either hematologic or neurologic abnormalities whereas others develop both (19).

We are indebted to Drs. V. Michael Whitehead (McGill University) and William S. Beck (Harvard University) for performing the Cbl assays using *Euglena gracilis* and *Lactobacillus leichmannii*, respectively, to Dr. Rodney Smythe (University of Colorado at Boulder) for performing the cobalt assays using x-ray fluorescence, and to Mrs. Maria Ignacio for technical assistance. This work was supported by Grant CA22976 from the National Cancer Institute and Grant AM21365 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

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