Articles

Influence of Metal Substitution on Vitamin B_{12} Binding to Human Intrinsic Factor and Transcobalamins I and II†

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ABSTRACT: Metal-free, zinc, copper, and rhodium analogues of vitamin B_{12} were synthesized to further characterize structural requirements for the binding to human intrinsic factor, transcobalamin I, and transcobalamin II. Binding affinities of the various analogues were studied by competition against cyano[^{57}Co]cobalamin. When albumin-coated charcoal was used for the separation of free and bound corrinoids, the relative 50% inhibition indexes were determined. The influence of metal substitution was similar among the three binding proteins. For analogues with a strong coordinative linkage between the heterocyclic base and the central metal ion, similar to that with cobalt (e.g., zincobalamin and cyanorhodibalamin), the indexes range from 0.65 to 2.35 for all three binding proteins. Analogues in which coordination is impossible (hydrogenobalamin and dicyanorhodibalamin) exhibit markedly reduced binding with indexes between 10 and 160. Cupribalamin shows 50% inhibition indexes ranging from 2.3 to 5.0, thus suggesting a weak coordinative bond between the copper ion and the 5,6-dimethylbenzimidazole moiety. These results emphasize the importance of the coordinative linkage between the central metal ion and the nucleotide moiety for optimal recognition by vitamin B_{12} binding proteins.

The structural requirements for the binding of vitamin B_{12} to intrinsic factor and serum binders were previously determined with cobalt-containing corrinoids (Ellenbogen, 1975). From these results an unmodified nucleotide group with 5,6-dimethylbenzimidazole as the heterocyclic base below the plane of a native corrin ring is considered sufficient for optimal cobalamin–protein binding. However, the role of the central metal ion in the coordination with the heterocyclic base of the nucleotide and thus in the recognition of cobalamins by their binding proteins was not investigated further due to the lack of appropriate cobalamin derivatives.

After the discovery of the metal-free corrinoids[1] (Toohey, 1965) synthesis of a new class of metal-substituted vitamin B_{12} analogues (Koppenhagen & Pfiffner, 1970, 1971; Koppenhagen et al., 1973, 1974) became possible. This investigation was designed to show the influence of metal substitution in cobalamins on the binding affinities for human vitamin B_{12} binding proteins.

Materials and Methods

Cyano[^{57}C]cobalamin (high specific activity) was purchased from Amersham Searle Corp., Arlington Heights, IL. Unlabeled cyanocobalamin and 5,6-dimethylbenzimidazole were obtained from Sigma Chemical Co., Inc., St. Louis, MO. Rhodium carbonyl chloride was purchased from Ventron Alfa Products, Beverly, MA, 5′-iodo-5′-deoxyadenosine from Aldrich Chemical Co., Inc., Milwaukee, WI, and Amberlite, sized by Serva and obtainable as Servachrom XAD-2 (50–100 μm), from Accurate Chemical and Scientific Corp., Hicksville, NY. Other materials were obtained from standard sources.

Culture Methods. Chromatium vinosum (ATCC 17899) was grown photosynthetically on a carbonate medium (Arnon et al., 1963); 5,6-dimethylbenzimidazole (10 mg/L) was added prior to inoculation. After a growth of 72 h cells were harvested by centrifugation, washed with saline, and stored at −20 °C until use.

Preparation of Corrinoids. Hydrogenobalamin was isolated from *C. vinosum* cells and purified on Amberlite XAD-2 as described previously (Koppenhagen et al., 1973). The copper and zinc analogues of vitamin B_{12} were prepared by insertion of the corresponding ions in aqueous solution (Koppenhagen & Pfiffner, 1971). Chlororhodibalamin, methylrhodibalamin, and adenosylrhodibalamin were synthesized according to previously published methods (Koppenhagen et al., 1974), and methylcobalamin and adenosylcobalamin were prepared by the same procedure.

Adenosylrhodibalamin (0.8 mg) was dissolved in 1 mL of 1 M potassium cyanide, kept at room temperature for 6 h, then diluted with 10 mL of water, and introduced on a column (1.5 × 4 cm) of Amberlite XAD-2 (50–100 μm). After the column was washed with water (50 mL) and 2% aqueous *tert*-butyl alcohol (10 mL), corrinoids were eluted with 20% aqueous *tert*-butyl alcohol. This fraction was concentrated to dryness, dissolved in 2 mL of water, passed through a small CM-cellulose column (0.5 × 1.5 cm), and introduced on a small column (0.5 × 1.0 cm) of DEAE-cellulose in the acetate form. The column was washed with water (10 mL) and dicyanorhodibalamin eluted by 0.25 M ammonia water. Rechromatography on Amberlite XAD-2 and elution with 10% aqueous *tert*-butyl alcohol yielded 0.45 mg of dicyanorhodibalamin, which was pure by Whatman No. 1 paper chromatography using water-saturated *sec*-butyl alcohol as the solvent system ($R_{CN\text{-}Cbl}$ = 1.23) or by paper electrophoresis at pH 2.5, 6.5, and 11.

Monocyanorhodibalamin was prepared from dicyanorhodibalamin by treatment with silver nitrate (Koppenhagen et al.,

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[1] Names and abbreviations for corrinoids are used according to the IUPAC–IUB Commission on Biochemical Nomenclature (1975). The term [M]Cbl represents a 5,6-dimethylbenzimidazolylcobamide in which cobalt is replaced by a metal ion (generalized by M).

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1973). Cyanoaquacobinamide was synthesized by cerous hydroxide hydrolysis (Renz, 1971).

Preparation of Binding Proteins. Intrinsic factor was prepared by the previously described method (Wagstaff et al., 1973) except that a Sephadex G-150 column (2.5 × 100 cm) was used for gel filtration and Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, NJ) for concentration of gastric juice. Aliquots of the preparation were saturated with CN-[^{57}Co]Cbl and analyzed by DEAE-cellulose chromatography (Retief et al., 1967), showing less than 1% contamination by R binder.

The serum binding proteins transcobalamin I and transcobalamin II were isolated from pooled human serum (1000 mL) by ammonium sulfate fractionation (Begley & Hall, 1975) followed by gel filtration on Bio-Rad P-150 for transcobalamin II and DEAE-cellulose chromatography (Zittoun et al., 1975) for transcobalamin I.

All binding proteins were prepared without adding cyanocobalamin. To monitor the preparations the unsaturated B_{12} binding capacities were measured by a charcoal assay previously published (Gottlieb et al., 1965) with the modification that 0.01 M sodium phosphate (pH 7.3) containing 0.154 M sodium chloride was used to prepare the 2.5% bovine albumin coated charcoal suspension.

Determination of Binding Affinities of the Analogues. Cyano[^{57}Co]cobalamin was diluted with cold cyanocobalamin to a specific activity of 20–50 μCi/nmol. To a solution of 0.274 pmol of cyano[^{57}Co]cobalamin (approximately 3 times the amount of cyanocobalamin needed for complete saturation of the binder) in 0.95 mL of 0.01 M sodium phosphate (pH 7.3) containing 0.154 M sodium chloride, usually from 0.05 to 20 pmol of analogue (for low-affinity analogues up to 80 pmol) was added. After addition of 50 μL of intrinsic factor (TC-I or TC-II was used in amounts of 0.5–1.0 mL so that less buffer was used to make up a volume of 1 mL), the mixture was allowed to stand for 30 min at room temperature, then 0.2 mL of coated charcoal suspension was added, and the mixture was shaken occasionally for 2 min and centrifuged at 4000*g* for 20 min. One milliliter of supernatant was removed and counted in a Packard auto gamma spectrometer (Packard Instruments Co., Inc., Downers Grove, IL).

For each analogue two blanks (i.e., without binding protein) were employed, one with the lowest and one with the highest analogue concentration used in the assay. This was to ensure the complete uptake of free cyano[^{57}Co]cobalamin by the coated charcoal in the presence of the analogues.

For the measurements of the binding affinities of the vitamin B_{12} metal analogues, human intrinsic factor, transcobalamin I, and transcobalamin II were separated from other vitamin B_{12} binding proteins in human gastric juice and serum, respectively.

With these preparations the competitive assay system was tested by using a fixed amount of cyano[^{57}Co]cobalamin, increasing amounts of unlabeled cyanocobalamin as "inhibitor", and bovine serum albumin coated charcoal for the separation of bound and free corrinoid. In order to achieve real competition between substrate and inhibitor for the binding sites, preincubation (Gottlieb et al., 1967; Hippe et al., 1971; Lien et al., 1974) with the analogue and binding protein was omitted and cyano[^{57}Co]cobalamin was added to increasing amounts of unlabeled corrinoid prior to the addition of the binding protein.

As presented in Figure 1, similar sigmoidal curves were obtained for all three protein preparations. Theoretically, at equal concentrations of nonradioactive and ^{57}Co-labeled cyanocobalamin, the protein-bound radioactivity should have been reduced by 50%. This was observed with intrinsic factor and, with a slight deviation, for the transcobalamins, thus showing the validity of the assay system. The observed deviations with the transcobalamins were probably due to nonspecific binding of cyanocobalamin.

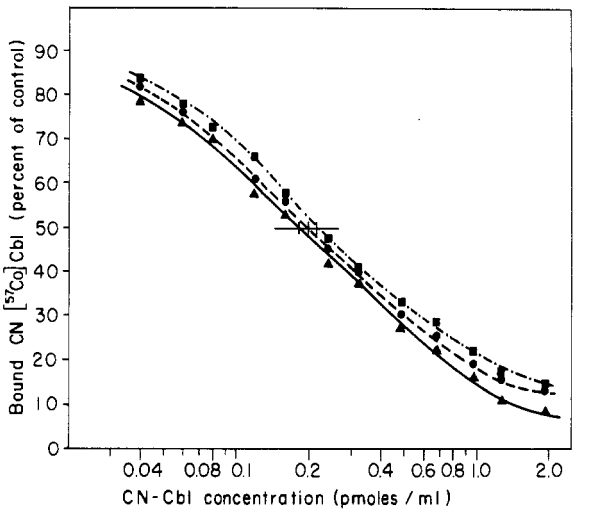

FIGURE 1: Behavior of the binding proteins in the competitive assay. Cyano[^{57}Co]cobalamin (0.185 pmol/mL) as substrate and increasing concentrations of "inhibitor" (unlabeled cyanocobalamin) were employed prior to the addition of the binding protein. Incubation was carried out at room temperature for 30 min. Bovine serum albumin coated charcoal was used to separate free from bound cyano-[^{57}Co]cobalamin. The concentrations found to give 50% inhibition are 0.185 pmol/mL CN-Cbl for intrinsic factor (▲), 0.215 pmol/mL CN-Cbl for transcobalamin I (■), and 0.20 pmol/mL CN-Cbl for transcobalamin II (●).

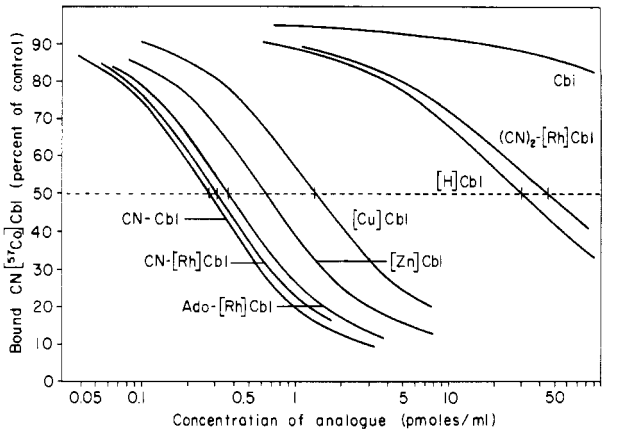

FIGURE 2: Inhibition of cyano[^{57}Co]cobalamin binding to human intrinsic factor by various vitamin B_{12} metal analogues. CN-[^{57}Co]Cbl was present at 0.275 pmol/mL and the concentration of analogues was varied from 0.05 up to 80 pmol/mL. Conditions of incubation were the same as described in the legend of Figure 1. Results are expressed as percentage of control, i.e., the amount of CN-[^{57}Co]Cbl bound in the absence of any analogue.

By the same competitive assay, binding affinities of the various analogues were determined. A typical plot of the results obtained with human intrinsic factor is given in Figure 2. For better quantitation, the 50% inhibition index of each analogue was determined graphically from these data. The index is the ratio of the concentration of analogue to the concentration of radioactively labeled cyanocobalamin at which binding of the labeled cyanocobalamin is reduced to 50% of control, i.e., the amount of protein-bound cyano[^{57}Co]cobalamin without any analogue. This analogue concentration is obtained from the intercept of the particular inhibition curve with the 50% line. Thus, ratios near 1 indicate strong inhibitors

Table I: 50% Inhibition Indexes of Vitamin B_{12} Metal Analogues for Human Intrinsic Factor (IF), Transcobalamin I (TC-I), and Transcobalamin II (TC-II) Compared with the Values Obtained for Some Cobalt-Containing Vitamin B_{12} Derivatives

	binding protein		
corrinoid	IF	TC-I	TC-II
[H]Cbl	110	13.5	25.0
[Cu]Cbl	5.0	2.3	3.5
[Zn]Cbl	2.4	1.3	1.8
CN-[Rh]Cbl	1.1	1.25	1.4
Cl-[Rh]Cbl	1.1	1.05	1.2
Me-[Rh]Cbl	1.05	0.65	0.75
Ado-[Rh]Cbl	1.3	1.7	1.25
$(CN)_2$-[Rh]Cbl	165	9.5	19.0
CN-Cbl	1.0	1.0	1.0
Me-Cbl	1.0	0.8	0.65
Ado-Cbl	1.15	1.8	1.05
(CN,aq)Cbi	>2000	28.5	75

with affinities similar to that of cyanocobalamin, whereas higher ratios are obtained with weaker inhibitors. Ratios even smaller than 1 are conceivable for analogues with higher binding affinities than cyanocobalamin. The binding affinities of the analogues for transcobalamin I and transcobalamin II were determined in the same manner.

The 50% inhibition indexes of the investigated metal analogues are shown in Table I and compared with those for methylcobalamin, adenosylcobalamin, and cyanoaquacobinamide. The highest values, representing analogues with low affinity for all three binding proteins, were obtained with hydrogenobalamin and dicyanorhodibalamin. Whereas in the case of the transcobalamins these indexes are comparable to those of cyanoaquacobinamide (a corrinoid lacking the nucleotide part of cyanocobalamin), with intrinsic factor as the binding protein the indexes are quite different from that of cyanoaquacobinamide. Cupribalamin exhibits an intermediate affinity for the three binding proteins, showing that this analogue is partially recognized as a vitamin B_{12} like compound. The inhibition indexes for zincobalamin and in particular for the rhodium analogues are close to 1, indicating that the replacement of cobalt by its homologue rhodium or by zinc retains the molecular shape, so that recognition by the binding proteins remains essentially the same. This is also reflected by the observation that cyano-, methyl-, and adenosylrhodibalamins exhibit an affinity pattern close to that of the corresponding cobalt-containing compounds. The close relationship between rhodibalamins and cobalamins is also emphasized by the behavior of their methyl derivatives. For both transcobalamins the preference for the methyl derivative of rhodibalamin could be demonstrated. This was quite similar to the preference for methylcobalamin over cyanocobalamin by the serum binding proteins, and the results are in good agreement with previously reported values (Gräsbeck & Puutula, 1971).

Results and Discussion

At present, metal analogues of vitamin B_{12} are only available by insertion of the appropriate metal ion into the metal-free compound. A short survey of the synthesis of the analogues used in this study and their structural relationship to cyanocobalamin is schematically given in Figure 3. Hydrogenobalamin, produced by photosynthetic bacteria, reacts readily with copper and zinc ions in aqueous solution to form cupribalamin and zincobalamin, respectively (Koppenhagen & Pfiffner, 1971). Rhodium is inserted with rhodium carbonyl chloride (Koppenhagen et al., 1974); the first reaction product, chlororhodibalamin, was subsequently converted into the other

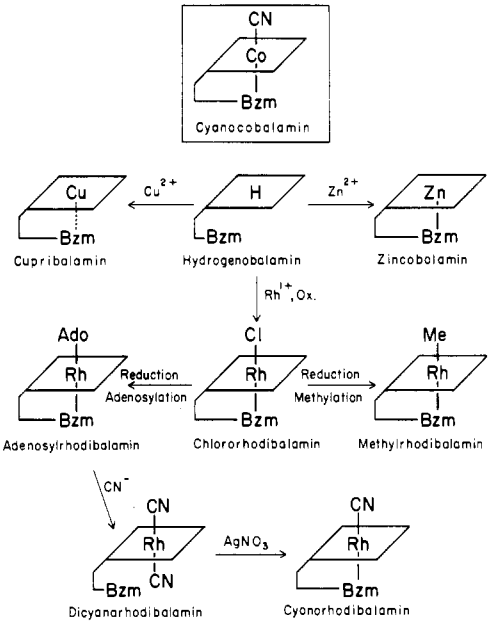

FIGURE 3: Scheme of chemical and structural relationship between the synthesized vitamin B_{12} metal analogues and cyanocobalamin. The weaker coordination between copper and the 5,6-dimethylbenzimidazole moiety (Bzm) in cupribalamin is indicated by the dotted line.

rhodium derivatives used in this study.

Up to date detailed structural data are only available for dicyanorhodibyric acid (Koppenhagen, 1981), which may be representative for rhodibamides too. Increases in the central metal–nitrogen distances as well as a concomitant widening of the methine bridge angles are the results of the accommodation of the larger rhodium cation without marked further changes of the planarity of the corrin ligand. For the other analogues any effect of metal substitution on the conformation of the corrin ligand was disregarded. How metal insertion alters the reactivity of the corrin ligand and its peripheral groups and how this may influence the binding affinities of vitamin B_{12} analogues remain open. Definitely, the inserted metal ion affects the two axial ligands: the heterocyclic base *below* (α-position) and variable alkyl, anionic, or neutral ligands *above* (β-position) the plane of the corrin ring. Thus, different binding affinities of vitamin B_{12} metal analogues may be determined mainly by the presence and nature of the axial ligands. This view is suggested by looking at the sequence [H]Cbl, [Cu]Cbl, [Zn]Cbl, and [Rh]Cbl. (For simplicity [H]Cbl might be considered a special "metal" analogue.) As the coordination properties of the inserted metal ion allow a coordination bond between the central metal ion and the nitrogen N-3 of 5,6-dimethylbenzimidazole, there is a better recognition of the corresponding analogue by all three binding proteins.

Despite the fact that intrinsic factor is much more specific for cobalamins than are the transcobalamins (Ellenbogen, 1975), all three binding proteins exhibit a definite preference for those metal analogues in which 5,6-dimethylbenzimidazole is strongly coordinated. The higher but similar inhibition indexes for hydrogenobalamin and dicyanorhodibalamin indicate similar effects on binding when coordination of the heterocyclic base is prevented either by lack of a coordination center or by blockade by another practically unexchangeable ligand in the α-position. Here, it should be mentioned that ligand exchange reactions of dicyanorhodium(III) corrinoids are much slower and require more drastic conditions than those of their corresponding Co^{III} complexes (Koppenhagen et al.,

1973; Koppenhagen et al., 1974).

Some further insight into the interaction of the binding proteins with cobalamins may be derived by comparing the monosubstituted rhodibalamins with their corresponding cobalt derivatives. As found by others (Ellenbogen, 1975), binding to intrinsic factor was not affected by changes in the β-position. The transcobalamins, however, exhibited at least some selectivity for alterations at this axial position. Thus, methylrhodibalamin and methylcobalamin were preferentially bound by both transcobalamins. This might be due to an increase in the hydrophobic interaction between cobalamin and binding protein (Hippe & Olesen, 1971) or to the trans effect of the alkyl group (Pratt, 1972), an effect resulting in an easier exchange of the heterocyclic base in methylcobalamin (Pratt, 1972) as well as methylrhodibalamin (Koppenhagen et al., 1974).

Since binding to the transcobalamins is less affected by the strength of the coordinative linkage between the heterocyclic base and the central metal ion, it may be presumed that with binding to transcobalamins the heterocyclic base is replaced by a basic amino acid residue of the protein, a mechanism proposed for hog intrinsic factor (Lien et al., 1973). In fact, this kind of mechanism should have been facilitated by the presence of an alkyl group in the β-position. But, in contrast to the findings with transcobalamins I and II, no preference of human intrinsic factor for methylcobalamin or methylrhodibalamin was found. Thus, for mechanisms that require the exchange of the ligand in the α-position for intrinsic factor, binding is unlikely.

Registry No. Zn, 7440-66-6; Cu, 7440-50-8; Rh, 7440-16-6; Co, 7440-48-4; TC-I, 12651-27-3; TC-II, 12651-28-4; [H]Cbl, 41632-95-5; [Cu]Cbl, 65231-06-3; [Zn]Cbl, 88326-63-0; CN-[Rh]Cbl, 51950-32-4; Cl-[Rh]Cbl, 53848-95-6; Me-[Rh]Cbl, 53848-96-7; Ado-[Rh]Cbl, 53880-55-0; $(CN)_2$-[Rh]Cbl, 51899-09-3; Me-Cbl, 13422-55-4; Ado-Cbl, 13870-90-1; (CN,aq)Cbi, 13963-62-7.

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