MEASUREMENT OF CYANOCOBALAMIN IN SERUM BY A SPECIFIC RADIOIMMUNOASSAY

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Reprinted from
THE JOURNAL OF LABORATORY AND CLINICAL MEDICINE
St. Louis

Vol. 103, No. 6, pp. 959-972, June,1984

Measurement of cyanocobalamin in serum by a specific radioimmunoassay

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Antiserum to cobalamin was raised in rabbits by immunization with the monocarboxyl derivative of cyanocobalamin coupled to albumin. The antiserum was treated to remove transcobalamin II and transcobalamin I. The partially purified antibody bound free cyano[^{57}Co]cobalamin, but not the vitamin precoupled to the transcobalamins. Cyano[^{57}Co]cobalamin bound by the antiserum eluted from Sephadex G-200 as a single peak with a mol wt of 160,000 and was precipitated by goat anti-rabbit gamma globulin, indicating that the vitamin was bound to an IgG immunoglobulin. Unlabeled cyanocobalamin and hydroxocobalamin competitively inhibited the binding of cyano[^{57}Co]cobalamin to this antibody. Neither adenosylcobalamin, in a similar concentration range, nor cyanocobinamide at a concentration 30-fold greater than the tracer cobalamin competed appreciably with the binding of cyano[^{57}Co]cobalamin. The association constant for the interaction of the antibody with cyanocobalamin and cyanocobinamide was estimated to be 8.6×10^9 and 9.6×10^6 L/mol, respectively. The association constant for adenosylcobalamin, methylcobalamin, and hydroxocobalamin was indirectly determined, and values of 2.5×10^5, 1.7×10^9, and 2.3×10^9 L/mol, respectively, were obtained. Photolysis in the presence of potassium cyanide rendered each of the three cobalamins equivalent to cyanocobalamin in immunoreactivity. The mean concentration of cobalamin in normal human sera and cobalamin-deficient sera measured as cyanocobalamin by radioimmunoassay using this anticobalamin antibody was significantly lower than the concentration measured in the same extracts by competitive ligand–binding radioassays using intrinsic factor and transcobalamin I. These findings, although indirect, support the proposition that there may be factor(s) in normal and cobalamin-deficient sera that falsely elevate the concentration of true cobalamin if the radioassay uses R protein as the binder. However, the lower concentration of serum cobalamin measured by radioimmunoassay compared with the intrinsic factor radioassay also indicates that this "purported" factor(s) reacts to some extent with intrinsic factor but not with the cobalamin antibody. (J LAB CLIN MED 103:959-972, 1984.)

Abbreviations: association constant (K_a), bovine serum albumin (BSA), cobalamin (Cbl), cyanocobalamin (CN-Cbl), human serum albumin (HSA), immunoglobulin G (IgG), intrinsic factor (IF), radioimmunoassay (RIA), transcobalamin I (TC-I), transcobalamin II (TC-II)

Serum Cbl concentration is a sensitive index of Cbl homeostatis and is of value in the clinical diagnosis of Cbl deficiency. Although microbiologic assays[1,2] were initially used to

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Supported by Grant AM 28561 from the National Institutes of Health.
Submitted for publication April 21, 1983; accepted Feb. 21, 1984.
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measure this vitamin in biologic fluids, these procedures have been replaced in many laboratories by the technically easier competitive ligand–binding radioassays.[3] The Cbl binding proteins for these radioassays have been IF,[4] salivary R protein,[5] TC-I,[6] and human serum, which contains mostly TC-II.[7]

Although each one of these proteins has high affinity for Cbl, the R protein binders (which include TC-I) also bind other corrinoids.[8] The use of this binder in the radioassay, therefore, may be a source of error, because nonphysiologic Cbl analogues in serum extracts will compete for binding sites and falsely elevate the concentration of the physiologic Cbl.[9] IF, on the other hand, appears to bind only physiologic forms of Cbl, and the radioassay using this binding protein is purported to provide a measure of "true" Cbl.[9] There are, however, a number of disadvantages in using IF. One important disadvantage is that it is not generally available free of contaminating R protein, which must then be selectively saturated with cobinamide, a corrinoid that has low affinity for IF. In addition and for as yet unexplained reasons, the Cbl concentration in some normal serum samples appears to be falsely low when IF is used as the binding protein.[10]

To circumvent these difficulties that have been encountered using the physiologic Cbl binding proteins for the radioassay of Cbl, we have raised a highly specific antiserum to CN-Cbl. This antiserum has very low affinity for both the nonphysiologic cobinamide derivative of Cbl and for the physiologic cofactor adenosyl-Cbl. We report the properties of this antiserum and its application to measurement of Cbl in human serum by RIA.

Methods

Cyano[^{57}Co]Cbl (220 Ci/gm), sodium ^{125}I-iodide (100 mCi/ml), and $^{3}H_2O$ (5 Ci/ml) were purchased from Amersham/Searle Corp., Arlington Heights, Ill. HSA and IgG was purchased from Schwarz/Mann, Division of Becton, Dickinson & Co., Orangeburg, N.Y. Bovine IgG, BSA, crystalline Cbl, adenosyl-Cbl, methyl-Cbl, hydroxo-Cbl, and Norit-A neutral charcoal were purchased from Sigma Chemical Co., St. Louis, Mo. Goat anti-rabbit gamma globulin were purchased from Miles Research Products, Elkhart, Ind. Silica gel (QUSO G-32) was obtained from Philadelphia Quartz Co., Valley Forge, Pa. Blue dextran 2000, dextran T10, and Sephadex G-200 were purchased from Pharmacia Laboratories, Piscataway, N.J. Normal HSA (Albuminar-25) was obtained from Armour Pharmaceutical Co., Phoenix, Ariz. This preparation does not bind Cbl, and was used as a carrier protein for the RIAs described below.

Cobalamin derivatives. The monocarboxyl derivative of Cbl was prepared by the method of Allen and Majerus,[11] and the purity of the derivative was established by high-voltage paper electrophoresis.[11]

Mono-, tri- and tetracarboxyl forms of cobinamide were prepared by the method of Allen et al.[12] The mixture of derivatives was lyophilized from 3 volumes of distilled water four times.

CN[^{57}Co]cobinamide was prepared similarly from CN[^{57}Co]Cbl, and the final lyophilized material was redissolved in water and mixed with an amount of purified IF equivalent to the initial molar concentration of CN[^{57}Co]Cl, which was subjected to hydrolysis. After 20 minutes of incubation, the preparation was mixed with 1 volume of 1% charcoal in 0.5% dextran T10 and centrifuged. Ninety-eight percent of the radioactivity was recovered in the charcoal pellet, which was rinsed with 5% phenol and water and then eluted with 65% acetone at 50° C. The eluate was mixed with 4 volumes of water and lyophilized.

Standardization of Cbl and cobinamide. The Cbl derivatives and cobinamide were dissolved in 0.1 mol/L phosphate buffer, pH 7.4. Adenosyl-Cbl and methyl-Cbl were dissolved and stored in this buffer in the dark. An aliquot of each preparation was mixed with this buffer, containing potassium cyanide (25 μg/ml), and brought into the light. Adenosyl-Cbl and methyl-Cbl were thus measured after conversion to $(CN)_2$Cbl (E^{1}_{368} = 30,000). The concentrations of hydroxo-Cbl and cobinamide were determined using their molar absorptivity coefficients (E^{1}_{358} = 20,600 and E^{1}_{367} = 30,680, respectively).[13]

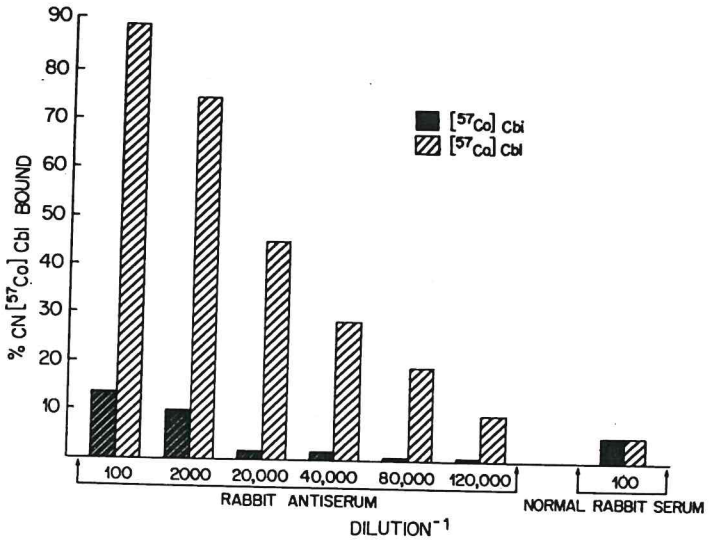

Fig. 1. Binding of CN[^{57}Co]Cbl and CN[^{57}Co]cobinamide by serial dilutions of purified rabbit anti-Cbl antibody and by purified nonimmune gamma globulin. *Cbi,* cobinamide; *Cbl,* cobalamin.

Synthesis of haptenic conjugates of cobalamin. Monocarboxyl-Cbl was covalently coupled to BSA as described by Ahrenstedt and Thorell.[14] After the coupling procedure, unreacted Cbl was removed by ultrafiltration through dialysis tubing under negative pressure, the conjugate contained within the membrane tubing was diluted with 1 L of 0.1 mol/L sodium phosphate buffer, pH 7.4, and the ultrafiltration was repeated. The total Cbl coupled to albumin was determined using its absorptivity coefficient (see above). Assuming no loss of albumin during the preparation of the conjugate, the molar ratio of Cbl to protein was calculated to be 3.4.

Immunizations. The albumin-Cbl conjugate was adjusted to contain 96 nmol/ml of hapten. Each preparation was emulsified with an equal volume of complete Freund's adjuvant, and 0.5 ml aliquots of the emulsion were injected into each foot pad of rabbits initially, and then subcutaneously at 7- to 10-day intervals for three additional injections. Blood was collected from the marginal ear vein 12 days after the last injection.

One rabbit received a subcutaneous injection of the albumin-Cbl complex coupled to agarose[15] weekly for 4 weeks prior to immunization with the soluble albumin-Cbl complex. The plasma of this rabbit did not contain any antibodies to Cbl before immunization with the soluble complex.

Molecular sieve gel chromatography. A Sephadex G-200 column (2.5 cm × 100 cm) was packed at 6° C at a constant flow rate of 11 to 13 ml/hr. The column was equilibrated with 0.05 mol/L Tris HCl buffer, pH 7.4, containing 0.02% NaN_3 and aprotinin, 5000 U/L. This solution was used as the elution buffer, and 1.0 to 2.0 ml fractions were collected. Purified human IgG and HSA were labeled with ^{125}I using chloramine T.[16] Aliquots of these labeled proteins (0.3 to 0.6 μCi), blue dextran 2000 (1 to 2 mg), and ^3H_2O (0.1 mCi) were added as column markers to the chromatographed samples.

Purification of the antiserum. The endogenous Cbl-binding proteins were removed from the immune and nonimmune rabbit plasma as follows: 1 volume of plasma was mixed with 2 volumes of 0.1 mol/L phosphate buffer, pH 7.4, and then 3 volumes of 30% sodium sulfate (wt/vol). The precipitated proteins were pelleted by centrifugation and then dissolved in 6 volumes of the phosphate buffer. This solution was then mixed with an additional 4 volumes of the phosphate buffer containing 1% (wt/vol) silica gel (QUSO G32). After thorough vortex mixing, the silica gel, which adsorbs transcobalamin II,[17] was pelleted by centrifugation, and the supernatant solution containing the immunoglobulins was used for the RIA. A 1% (vol/vol) solution of IgG from normal plasma was similarly prepared in the phosphate buffer and used as the diluent for the IgG from the plasma of the immunized rabbit.

Dose-response competitive inhibition RIA. The reaction mixtures were prepared by consecutive mixing of 0.2 ml of 0.1 mol/L phosphate buffer (pH 7.4) containing 0.033% HSA (Albuminar-25), 10 pg CN[^{57}Co]Cbl contained in 0.1 ml buffer, 0.1 ml of either the unlabeled Cbl, Cbl derivative, or cobinamide (0.1 to 1.5 ng), and 0.1 ml of the purified rabbit antiserum, which was diluted to bind approximately 40% to 50% of the CN[^{57}Co]Cbl in the absence of the unlabeled ligand. All the assays using the photosensitive methyl-Cbl and adenosyl-Cbl were carried out in the dark. After incubation for specific time periods, the free and bound ligands were separated by one or both of the following methods: (1) 1 ml of 2.5% (wt/vol) suspension of Norit-A neutral charcoal in a solution of 0.125% hemoglobin to remove the free ligand,[18] or (2) 0.01 ml of goat anti–rabbit gamma globulin followed in 60 minutes by 0.1 ml of 10% cold polyethylene glycol to precipitate antibody-bound ligand.[19] The precipitate was pelleted by centrifugation, and the radioactive CN[^{57}Co]Cbl was measured using a gamma counter.

Measurement of serum Cbl. The following method was used to extract Cbl from serum and denature the endogenous Cbl binding proteins. One volume of serum was mixed with 1 volume of a 0.06 mol/L citrate-disodium phosphate buffer, pH 2.6, and 1 volume of water containing cyanide (100 μg/ml) in a screw-cap tube, and the mixture was boiled for 30 minutes with occasional shaking. The precipitate was then pelleted by centrifugation, a volume of the clear supernate was mixed with one third its volume of 0.2 mol/L sodium phosphate buffer, pH 8.8, and this neutralized extract was assayed for Cbl as described below.

The standard CN-Cbl dose-response curve for the RIA was prepared in a solution consisting of equal volumes of 0.06 mol/L citrate-phosphate buffer, pH 2.6, the potassium cyanide solution, 0.2 mol/L sodium phosphate, pH 8.8, and Ringer's bicarbonate solution; final pH of the mixture was 7.4. The total reaction mixture contained [^{57}Co]Cbl, 10 pg/ml; unlabeled Cbl, 10 to 160 pg/ml; and diluted antiserum, 0.1 ml/ml, always added last. The reactions were incubated overnight at 4° C, and the free and antibody-bound ligand were separated using 0.5 ml of the coated charcoal suspension (see above). The radioactivity in the supernate, representing the bound [^{57}Co]Cbl, was then measured. The standard dose-response curve was constructed by plotting the ratio of [^{57}Co]Cbl bound in the presence of competing unlabeled Cbl or serum extract (B_s) to tracer bound in the absence of competing Cbl (B_o), against the unlabeled Cbl in the reaction mixtures.

The Cbl in the neutralized serum extracts prepared as described above was determined by substituting 0.4 ml and 0.8 ml of the extracts for the equivalent volumes of the standard curve buffer. These mixtures were similarly incubated with the [^{57}Co]Cbl and diluted antiserum, and the B_s/B_o ratio was referred to the standard curve to obtain the corresponding value of Cbl. This value was multiplied by the dilution factors 10 and 5 for 0.4 ml and 0.8 ml of extract assayed, respectively, to obtain the Cbl concentration per milliliter of serum.

The Cbl concentration of the same extracts was also measured using TC-I and human IF in the competitive ligand–binding radioassay. The source of IF was human gastric juice in which all R protein was saturated by the addition of cobinamide (3 ng/μl). The binders were diluted to bind approximately 50% of the CN[^{57}Co]Cbl, which for these assays was 25 pg. The total volume of these reactions was 2 ml, and the incubation time was 30 minutes. One milliliter of the coated charcoal suspension was then added to separate the free from bound ligand.

Results

Identification and characterization of anticobalamin antibodies. Fig. 1 shows the binding of CN[^{57}Co]Cbl and CN[^{57}Co]cobinamide by the partially purified immunoglobulin fraction prepared from normal and immune rabbit plasma. Normal control rabbit immunoglobulin bound ≤5% of either the CN[^{57}Co]Cbl or the CN[^{57}Co]cobinamide. The anti-Cbl immunoglobulin bound CN[^{57}Co]Cbl even at a dilution of 1 : 80,000. This anti-Cbl preparation did bind some CN[^{57}Co]cobinamide, but substantially less than observed for the binding of Cbl.

Fig. 2, *A*, shows the filtration through Sephadex G-200 of 1 ng CN[^{57}Co]Cbl bound to the anti-Cbl IgG. All the radioactivity eluted as a well-defined peak just before the elution of the ^{125}I-labeled human IgG marker protein. This immune complex, after incubation with

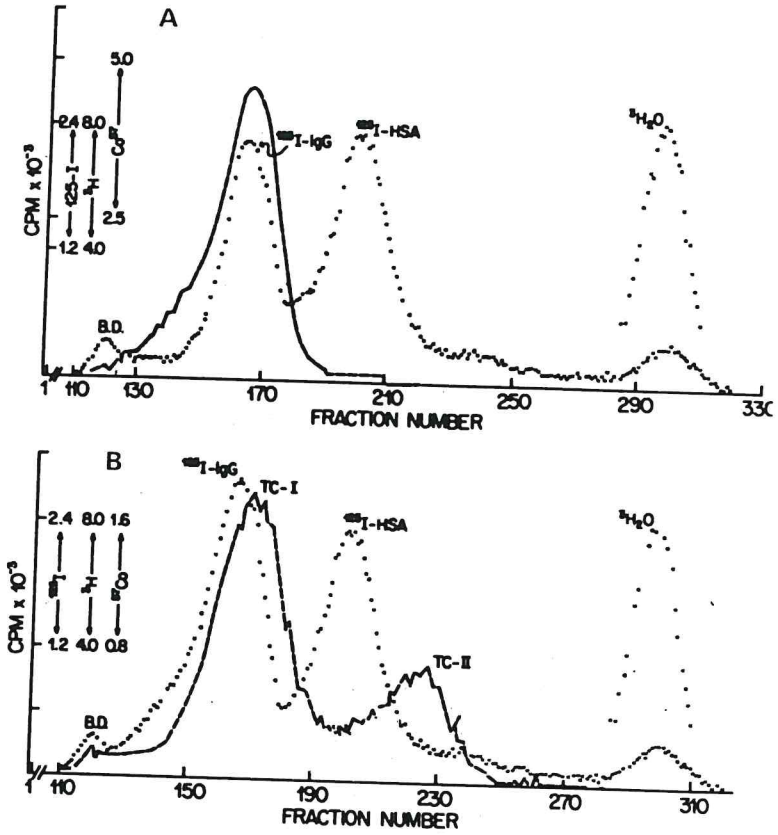

Fig. 2. Gel filtration of antibody-bound CN[^{57}Co]Cbl. *A*, Sephadex G-200 filtration of 1 ng CN[^{57}Co]Cbl (——) coupled to anti-Cbl antibody. *B*, Sephadex G-200 filtration of 1 ng CN[^{57}Co] bound to 1.5 ml of normal human serum and incubated with anti-Cbl antibody as in *A*. CN[^{57}Co]Cbl did not elute in the fractions corresponding to the elution of antibody bound tracer in *A*.

0.1 ml of goat anti–rabbit gamma globulin, shifted into the void volume of the column (chromatogram not shown), indicating that the antibody was of the IgG class. When TC-I and TC-II contained in normal human serum (1.5 ml) were saturated with 1.0 ng of CN[^{57}Co]Cbl, incubated for 2 hours with 1.0 ml of a 1 : 100 dilution of the anti-Cbl IgG, and filtered through the same column, the labeled vitamin remained bound to TC-I and TC-II (Fig. 2, *B*), indicating that the binding of Cbl to the transcobalamins blocks the immunoreactive site of Cbl. The relative affinity of Cbl for the antibody, compared with its affinity for TC-I and TC-II, cannot be determined from this experiment, because the incubation of the antibody with the Cbl prebound to TC-I and TC-II was not long enough to reach equilibrium.

Dose-response curves. Two competitive inhibition dose-response curves using unlabeled Cbl as competing ligand are shown in Fig. 3. We used both the coated charcoal and the double antibody methods for separating the free from antibody-bound Cbl, and the curves appear comparable. Therefore, in the subsequent experiments, only the coated

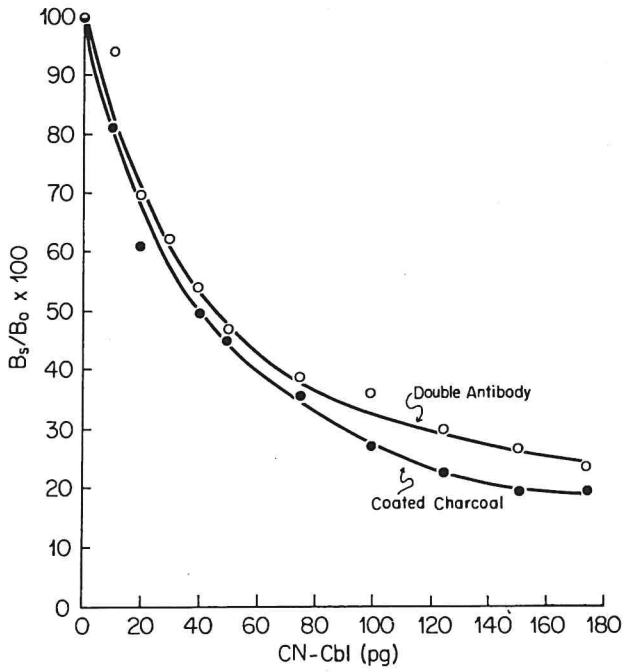

Fig. 3. Comparison of dose-response curves of two competitive radiometric assays for unlabeled CN-Cbl using hemoglobin-coated charcoal and the double antibody to separate free from bound ligand. B_s/B_o is the ratio of CN[^{57}Co]Cbl bound to the antibody in the presence of competing unlabeled ligand (B_s) to the CN[^{57}Co]Cbl bound to the antibody in the absence of competing unlabeled ligand (B_o).

charcoal method was used to study the immunoreactivity of other Cbl(s) and measure Cbl in serum extracts.

We also found that, with these antiserums to Cbl, the optimal incubation conditions for maximal sensitivity was 24 hours at 4° C. For example, 90 pg of competing unlabeled Cbl resulted in a B_s/B_o fraction of 0.5 with a 2-hour incubation at room temperature, whereas the same B_s/B_o fraction was obtained with 34 pg of Cbl using an overnight incubation at 4° C.

Specificity of the anti-cobalamin antibody. The dose-response curves showing the competitive effect of adenosyl-Cbl, methyl-Cbl, hydroxo-Cbl, and the nonphysiologic analogue cobinamide are illustrated in Fig. 4. The assays of the two photosensitive Cbl cofactors were carried out in the dark. Both methyl-Cbl and hydroxo-Cbl are immunoreactive, but have lower affinity for the antiserum than CN-Cbl. Adenosyl-Cbl, in contrast, was not immunoreactive even at a concentration 150-fold greater than the CN[^{57}Co]Cbl. In a similar concentration range, cobinamide was slightly more competitive than adenosyl-Cbl, but its affinity for the antibody was substantially lower than methyl-Cbl and hydroxo-Cbl.

Adenosyl-Cbl and methyl-Cbl undergo photolysis in the presence of cyanide to form $(CN)_2Cbl$.[13] Hydroxo-Cbl is also converted to CN-Cbl in the presence of excess CN.[13] When the two photosensitive Cbl derivatives were subjected to such photolysis in the presence of potassium cyanide, the dose-response curves became superimposed with the

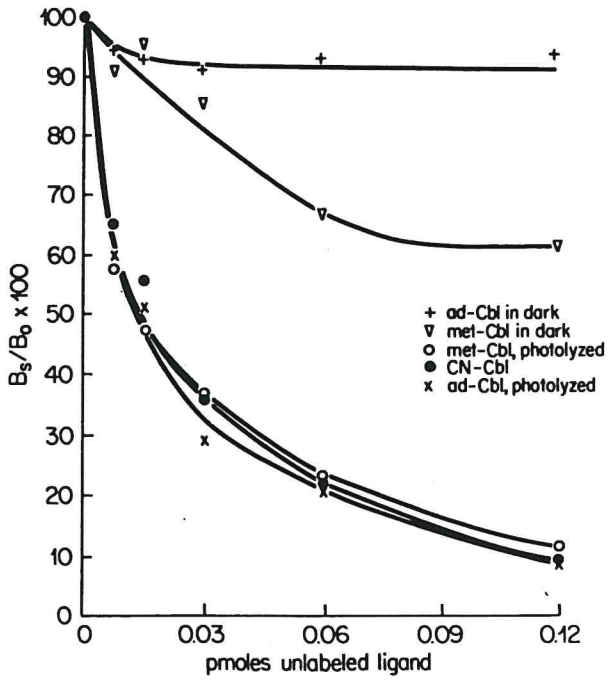

Fig. 5. Competitive inhibition of the binding of CN[^{57}Co]Cbl to the anti-Cbl antibody by adenosyl-Cbl and methyl-Cbl in the dark and after photolysis in the presence of potassium cyanide.

Table I. Recovery of cobalamin added to an extract of serum and measured by RIA

Cbl added (pg)	*Cbl concentration measured in extract (pg/ml)*		*Recovery* (%)*
	Expected†	*Assayed†*	
0	—	122	—
20	142	142	100
40	162	165	102
80	202	194	96

*Computed as: Assayed/Expected × 100.
†Each value is the mean of five replicate determinations.

proteins for the radioassay. The concentrations of Cbl in normal serum measured by the RIA and by the radioassays with IF and TC-I as the binders are shown in Table III. The lower mean value obtained by the RIA (364 pg/ml) compared with the IF radioassay (417 pg/ml) was small but significant. The serum Cbl measured using TC-I (517 pg/ml) was substantially and significantly higher than the values obtained by RIA or with the IF radioassay.

A group of serum samples that contained a low Cbl concentration (≤200 pg/ml) assessed at a laboratory that uses a commercial kit to measure "true" Cbl were reassayed using our RIA and the IF and TC-I radioassays; the results are shown in Table IV. The

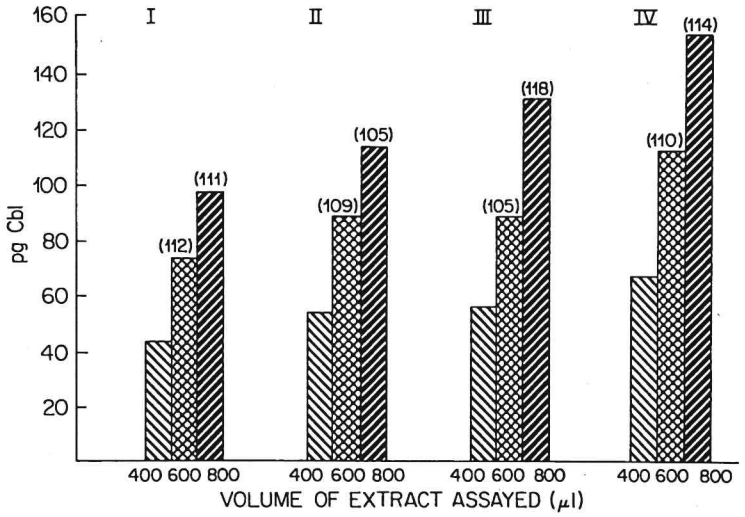

Fig. 6. Stoichiometry of the cobalamin concentration with the volume of extract assayed by RIA.

Table II. Precision of the radioimmunoassay*

Extract	*Cbl concentration (pg/ml, mean ± SD)*	*Coefficient of variation (1%)*
I	442 ± 16	3.5
II	677 ± 57†	8.4
III	412 ± 33	7.9

*Three pools of five human serum samples were extracted, and the Cbl concentration was measured in quintuplicate in each extract.
†One tube was subject to experimental loss, and this value was computed from quadruplicate assays of the extract.

mean concentration obtained with the RIA and IF radioassay was substantially below normal; yet, like the concentration of Cbl in normal serums, the RIA gave a significantly lower value than the IF radioassay. The mean Cbl concentration in these same extracts measured with the TC-I radioassay was in the normal range, indicating that the value in some patients with Cbl deficiency would have been considered normal by this assay method as previously shown by Cooper and Whitehead[21] and Kolhouse et al.[9] It should be noted that these serum samples were extracted using a high concentration of cyanide, which has been previously shown to augment the apparent Cbl concentration measured by TC-I radioassay.[10]

Discussion

These studies, like those from a number of other laboratories,[22–24] demonstrate that Cbl coupled to a carrier protein has haptenic properties. Gershman et al.[22] obtained antiserum to carboxypropyl-Cbl that reacted equally well with CN-Cbl, methyl-Cbl, adenosyl-Cbl, and hdyroxo-Cbl, but with much lower affinity for CN-cobinamide. Van de Wiel et al.[23] raised antiserums to Cbl by immunizing rabbits with CN-Cbl bound to hog IF and

Table III. Concentration of cobalamin in normal sera measured by this RIA, and the intrinsic factor and transcobalamin I competitive ligand–binding radioassays*

Assay	Concentration (pg/ml)		
	Range	Mean ± SEM	p†
IF (n = 29)	252 to 584	417 ± 17	—
RIA (n = 29)	188 to 592	364 ± 17	<0.001
TCI (n = 29)	344 to 788	578 ± 23	<0.001

*Serum samples were obtained from healthy blood donors.
†The p value is the significance of the difference from the values obtained using the IF radioassay, and was determined using the method of one-way analysis of variance for repeated measurements.

Table IV. Concentration of cobalamin measured by RIA and the radioassays using IF and TCI in sera that contained a low cobalamin concentration by a commercial kit radioassay*

Assay	Concentration (pg/ml)		
	Range	Mean ± SEM	p†
IF (n = 23)	24 to 272	143 ± 15	—
RIA (n = 23)	0 to 200	83 ± 15	<0.001
TCI (n = 23)	64 to 424	278 ± 22	<0.001

*The serum samples (n = 20) (provided by Dr. E. Rieber and Mr. John Lawson, Metropolitan Hospital Medical Center, New York) had a mean cobalamin concentration (±SEM) of 106 ± 14 pg/ml by the commercial kit used in their laboratory.
†The p value is the significance of the difference from the IF radioassay, and was determined using Student's paired t test.

coupled to BSA. The cross-reactivity of Cbl analogues and cobinamide with these antiserums was not determined. Endres et al.[24] obtained antiserum to monocarboxyl-Cbl conjugated to BSA, and although the cross-reactivity of other Cbl analogues was not determined, the antiserum was useful for a RIA; the concentration of Cbl in the serum from normal volunteers ranged from 130 to 700 pg/ml.

The complexity of the cobalamin molecule, with an axial ligand above (CN, methyl, adenosyl, and hydroxo) and below (dimethylbenzimidazole) the planar corrin ring, contributes to the antigenic properties of this hapten. It is apparent that each axial conformation is independently immunogenic because the antiserum raised to CN-Cbl had lower affinity for methyl-Cbl and hydroxo-Cbl, and still lesser affinity for adenosyl-Cbl; however, the same antiserum had very low affinity for cyanocobinamide, which is missing only the lower axial ligand.

The methods used to measure serum Cbl have been the subject of considerable discussion recently, because serum from some patients with clinical Cbl deficiency may appear to contain a normal Cbl concentration when measured using R protein in the competitive ligand–binding radioassay.[9,21] Kolhouse et al.[9] could separate by column chromatography a substance in the serum extract that reacted in the radioassay using R protein, but not in the radioassay using IF or in the microbiologic assay for Cbl. These important observations have led to the suggestion that the serum after extraction contains

a "nonphysiologic" Cbl that neither reacts with IF nor supports growth of the assay organisms in the microbiologic procedure. The corollary to that conclusion is that only the radioassay using IF as the binder measures "true" Cbl in serum[9] or tissues.[25]

It is important to note in this regard that this antiserum to CN-Cbl has very low affinity for cobinamide. Cobinamide contains the corrin ring but lacks the nucleotide, and because it reacts with R protein, it may be the prototype structure of the nonphysiologic Cbl analogue in serum.[9] If this is true, then this compound, or one structurally similar to it, will not interfere with this RIA for Cbl.

The nature of the nonphysiologic Cbl in serum is still puzzling and open to question. Presumably, the K_a for the binding of the "nonphysiologic" Cbl analogue(s) to R protein is substantially lower than the K_a for the binding of Cbl. Therefore, the effect of this analogue(s) would be very small, unless present in a concentration several orders of magnitude greater than the CN[^{57}Co]Cbl used in the radioassay. It would seem likely that an analogue present in such high concentration would be easily detected, yet Begley and Hall[26] have not been able to identify any "nonphysiologic" Cbl in serum by column chromatography. Finally, the concentration of cyanide used for the extraction of Cbl from serum may play some role in this phenomenon, because the difference between values obtained using R protein and IF in the radioassay decreases as the amount of cyanide used in the extraction is lowered.[10]

Our studies support the observation that in serum that has been extracted with a high concentration of cyanide, there is a substance(s) in addition to Cbl that appears to react selectively with R protein.[9,21] However, our studies also indicate that this substance has some affinity for IF, because the Cbl concentrations in normal and Cbl-deficient serum measured by this RIA were significantly lower than values obtained for the same extracts with the IF radioassay. Chanarin and Muir[27] have recently shown that sufficient IF covalently coupled to acrylamide could remove from solution this "analogue" that reacts with TC-I, and that this Cbl analogue has some affinity for IF.

The objective of most assay procedures in clinical medicine is to provide the quantitative information for only a single substance in the biologic sample under analysis. One of the major advantages of RIA is that it can, with the proper antiserum, provide such specificity. The antiserum that we raised against CN-Cbl can distinguish nonphysiologic CN-Cbl derivatives and cobinamide forms of cobamide. This RIA, therefore, should eliminate the two major problems observed with currently available radioassays for serum Cbl: falsely elevated values obtained using the R protein radioassays, and the falsely low values for Cbl obtained using the IF radioassay.[10,28]

Note added in press

To prepare the antiserum for the RIA, we now omit the precipitation with 30% sodium sulfate and add only silica gel to the antiserum, because the unsaturated Cbl–binding capacity of rabbit serum is virtually all transcobalamin II.

We thank Mr. Mathew Avitable of the Scientific Computing Center of SUNY-Downstate Medical Center for assistance in the statistical analysis.

Appendix

Determination of association constants (K_a) for the binding of CN-Cbl and derivatives to the antibody raised to CN-Cbl

The K_a for the binding of CN-Cbl and CN-cobinamide to the antibody (Ab) was computed from the equation of the law of mass action as follows:

$$K_a = [\text{CN-Cbl}]_b/[\text{Ab}]_f \times [\text{CN-Cbl}]_f \quad (1)$$

where $[CN - Cbl]_b$ and $[Ab]_f$ are, respectively, the molar concentration of CN-Cbl coupled to the antibody and the molar concentration of unsaturated antibody at equilibrium. Because $[Ab]_f = [Ab]_t(\text{total}) - [\text{CN-Cbl}]_b$, it follows from equation 1 that:

$$([Ab]_t/[\text{CN-Cbl}]) - 1 = 1/K_a[\text{CN-Cbl}]_f \quad (2)$$

and $K_a = 1/[\text{CN-Cbl}]_f$, when $1/[\text{CN-Cbl}]_b = 0$. The reciprocal of the concentration of $[\text{CN-Cbl}]_b$ (plotted on the ordinate) is a linear function of the reciprocal of the concentration of $[\text{CN-Cbl}]_f$ (plotted on the abscissa), with the intercept on the abscissa equal to $-K_a$. The experimental points were obtained by incubating an increasing concentration of CN[^{57}Co]Cbl with a constant concentration of antibody and determining the bound and free ligand as described in the Methods section.

The K_a for the binding of CN-cobinamide was determined by substituting CN [^{57}Co]cobinamide for the CN[^{57}Co]Cbl, and unlabeled cobinamide for CN-Cbl.

The K_a for adenosyl-Cbl, methyl-Cbl, and hydroxo-Cbl could not be measured directly because the radioisotopic forms of these compounds are not available for direct competitive inhibition experiments. However, the corresponding association constants were measured indirectly from the formula of Edsall and Wyman[20] illustrated below:

$$K_b = (1/[B])(K_a/K'_a - 1) \quad (3)$$

which gives the mathematical relationship of the association constant of two ligands (K_a and K_b) that react against a common antibody or binding protein.

This formula was derived to measure the association constant (K_b) of a second ligand (B) competing with a primary ligand (A) having a known association constant (K_a) for the common binding protein. K_a is the true K_a of the primary ligand A for the binding protein in the absence of any competing ligand.

The results of our experiments to determine the competitive inhibition of each analogue on the binding of CN[^{57}Co]Cbl by the antiserum (Fig. 4) were used to compute the association constant of the antibody for each analogue by modifying the terms of equation 3 as follows:

$$K_a = (1/[A])\,(K_b/K'_b - 1) \quad (4)$$

where K_a = the computed association constant for CN-Cbl (8.6×10^9 L/mol), [A] = the molar concentration of CN[^{57}Co]Cbl in the incubation mixture (14.76×10^{-12} mol/L), K_b = the association constant of the competing Cbl analogue, and K'_b = the apparent association constant of the competing analogue in the presence of the CN[^{57}Co]Cbl.

Equation 4 was rearranged as follows:

$$K_b = (K'_b)\,(K_a\,[A] + 1) \quad (5)$$

Because K_a and [A] are known, only the value of K'_b has to be determined to solve equation 4 for K_b. K'_b, therefore, was determined as follows.

Using the experimental data obtained from the CN-Cbl dose-response curve (Fig. 4), the fraction of CN[^{57}Co]Cbl bound to the antibody was plotted graphically against the

corresponding amount of unlabeled Cbl that was bound. From similar dose-response inhibition experiments with each Cbl derivative competing against the CN[^{57}Co]Cbl tracer, the percent of CN[^{57}Co]Cbl bound was referred to this graph, and the value of bound derivative was taken as equal to the corresponding value of bound unlabeled CN-Cbl. The fraction of free or unbound Cbl derivative could then be simply computed from the total concentration of derivative in the reaction mixture. These data were then graphed by the double reciprocal method to compute the apparent association constant, which in this instance represents K'_b. This value of K'_b was then substituted in equation 5 to compute the true association constant (K_b) for each derivative.

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