Forms of vitamin B₁₂ in radioisotope dilution assays

JA BEGLEY, CA HALL

From the Hematology Research Laboratory, Veterans Administration Medical Center and the Department of Medicine, Albany Medical College, Albany, New York, 12208

SUMMARY Since the presence of analogues of vitamin B₁₂ (B₁₂, cobalamin, Cbl) has been postulated as the basis for the high values obtained by some radioisotope dilution assays (RIDA) of serum Cbl, we examined serum for analogues. None could be demonstrated in the extracts of serum prepared for RIDA as sought by both direct and indirect techniques. The natural forms of serum Cbl were converted to cyanocobalamin (CN Cbl) by this process of extraction which included cyanide (CN). The correctly performed RIDA for Cbl based on R binder gave higher values than a RIDA based on intrinsic factor or than by bioassay. By exclusion, the difference appeared to be due to unidentified factors rather than the presence of analogues.

Although the responsiveness of several microbiological assays to the various forms and analogues of vitamin B₁₂ (B₁₂, cobalamin, Cbl) are well known, the relation between the form of Cbl and measurement by RIDA are not. Moreover, the form or forms of Cbl present under the conditions of the actual assay are also unknown. The issue came to a head with the occurrence of diagnostic errors because of high serum values as measured with some RIDAs.^{1,2} Kolhouse *et al.*³ then showed that a RIDA based on an R binder of Cbl gave higher values than one based on isolated intrinsic factor (IF) and emphasised the long-forgotten fact that many so-called IF concentrates contain much R binder as well as IF. Since the same investigators had reported earlier a much greater affinity of some analogues of Cbl for R binder than for IF,⁴ they postulated that the RIDA based on R binder was measuring analogues as well as "true" Cbl and that the differences between the two forms of RIDA was a measure of serum analogues.

We report an evaluation of the forms of Cbl observed in the extracts of serum as prepared for RIDA and an unsuccessful search for analogues of Cbl in these extracts. Some of this work has been published in abstract form.⁵

Material and methods

MEASUREMENT OF Cbl

RIDA with saliva R binder (RIDA-R) was performed as described.⁶ The principles of the assay were: extraction of the Cbl at 100 °C for 20 min in

cyanide-acetate buffer, pH 4.8; mixing the supernatant with CN (⁵⁷Co) Cbl; removal of the free Cbl with albumin-coated charcoal (Ch/Alb); reading "bound: free" ratios of unknowns against those of a standard curve. The pH of the final assay, slightly over 5.0, was designed to give good extraction of Cbl with stable and optimal binding to the salivary R binder. Gastric juice from a patient with congenital lack of R-type binders⁷ was used as the binding reagent for the RIDA employing IF as Cbl binder (RIDA-IF). The assays were identical with the exception of the type of binder (R or IF). The dilutions of the saliva and gastric juice necessary to bind approximately 60% of the CN (⁵⁷Co) Cbl were 1/75 and 1/50 respectively, giving a final protein content of 10.8 and 14.7 µg added to the assay. The same R and IF were used in all parts of the study requiring these substances. Bioassay of Cbl was by *E. gracilis*.⁶

FORMS OF Cbl IN SERUM EXTRACTS FOR RIDA

In order to evaluate the ability of the cyanide (CN) in RIDA digestion buffers to convert the endogenous Cbl to one form, we performed the following experiments. Two ml aliquots of normal sera were extracted at 100 °C for 20 min in the presence of 6 ml of sodium-acetate buffer, pH 4.8, containing 20 µg NaCN/ml. For one experiment the concentration of CN was varied. The processed extracts were then passed through columns of Amberlite XAD-2.⁸ The Cbls were eluted with methanol:water (50:50 vol/vol) and evaporated to dryness. The extracts were reconstituted with 25 µl glass-distilled water and subjected to thin layer chromatography (TLC).⁹

Accepted for publication 22 October 1980

Cobalamins were identified by bioautography on agar-based media¹⁰ seeded with *E coli* 113-3.¹¹

PREPARATION OF A POOL OF SERUM EXTRACTS

A pool was made of extracted serum samples collected from hospitalised patients for routine measurement of serum Cbl by RIDA; individual Cbl values were 200-900 ng/l by the RIDA-R. This pool was used in a search for analogues. The pool (approximately 160 ml) was trace labelled with 10 pg CN (⁵⁷Co) Cbl and the corrins absorbed on to an 0.8 × 10 cm column of Amberlite XAD-2 prepared as described.⁸ This column retains corrins allowing considerable purification from natural materials. The column was washed with 20 ml of glass distilled water, and the corrins eluted with 20 ml of methanol: water (50:50 vol/vol). The eluate was evaporated to dryness, redissolved in 2 ml of glass distilled water, and frozen in 0.2 ml aliquot at -20°C. The above procedure was performed in a dark room illuminated by a red light. The amount of Cbl was determined by RIDA-R, RIDA-IF and bioassay with *E gracilis* at intervals during the above procedure (Table 1).

ANALYSIS OF THE ABOVE POOL OF EXTRACTS FOR ANALOGUES

Aliquots (0.2 ml) of the final purified extract (2.50 ng total by RIDA-R and 1.08 ng by RIDA-IF) were lyophilised and redissolved in 100 mmol/l acetic acid containing 1.535 mmol/l KCN. Aliquots (25 µl) were then subjected to two-dimensional electrochromatography on Whatman 3MM paper followed by bioautography using *E coli* 113-3.^{11 12}

Analysis of the above pool of extracts for analogues which do not bind to IF

An aliquot (0.6 ml) of the final purified extract (7.65 ng total by RIDA-R; 3.24 ng by RIDA-IF) was lyophilised and redissolved in 2 ml of the RIDA buffer. 0.25 ml of gastric juice (50 ng Cbl binding capacity) from a patient with congenital deficiency of

R binders⁷ was added, the sample incubated for 1 hr at room temperature (conditions of incubation in the RIDA), and then applied to a column of Sephadex G-50 equilibrated with the RIDA buffer. Fractions were then assayed for Cbl by RIDA-R. Any analogue which would not bind to IF would be found in the included volume of the column. Cobalamins which bind to IF would be found in the excluded volume (MW > 30 000).

Results

FORMS OF Cbl IN SERUM PREPARED FOR RIDA

Fig. 1 (bottom) shows the simultaneous bioautography of three normal sera and a mixture of four known reagent cobalamins following extraction with cyanide-acetate buffer. After mixing sample (2 ml) and buffer (6 ml), the final NaCN concentration was 15.0 µg/ml. Almost all of the serum Cbls were converted to a form which migrated as CN-Cbl. There was no evidence of any unexpected analogue. The bioautography at the top of Fig. 1 shows the growth promotion of different levels of CN-Cbl and of Cbi. Thus, the *E coli* responded to Me, Ado, OH, and CN-Cbl (Cbl mixture—Fig. 1) and Cbi (top right—Fig. 1). The organism was therefore capable of detecting the forms of Cbl biologically active in man and a rather rudimentary analogue without a nucleotide.

In another set of studies (not illustrated), the form of Cbl in the extract of one serum, exposed to final concentrations of NaCN of 0, 3.7, and 15.0 µg/ml were identified by TLC and bioautography. Without CN in the extraction buffer, Me-Cbl, Ado-Cbl and probably OH-Cbl were present. When 3.7 and 15.0 µg/ml NaCN were present, one spot was identified as CN-Cbl. In addition, visual inspection of those samples extracted with CN suggested an increase in the actual amount of Cbl in the growth spots as compared to the sample without CN, supporting previous reports of enhanced extractability of serum

Table 1 Total pool cobalamin

	Cbl (ng)			CN (⁵⁷ Co) Cbl tracer (cpm)
	RIDA*-R	RIDA*-IF	Bioassay <i>E gracilis</i>	
Pooled extracts	14.4	6.38	17.39	6054
Pass through†	0.46	0	Indeterminate	0
20 ml H ₂ O eluate‡	0.16	0	Indeterminate	32
20 ml methanol: water§	25.50	10.80	17.20	5979

*No polyvinylpyrrolidone or other additives to standards.

†Material not retained by purifying XAD-2 column.

‡Wash of column.

§Elution of corrins.

RIDA-R = radioisotope dilution assay based on R binder of cobalamin.

RIDA-IF = radioisotope dilution assay based on intrinsic factor.

Fig. 1 Lower part of fig: Bioautograms of the forms of B_{12} in 3 normal sera (1-3) following extraction for RIDA and then separation by thin layer chromatography (TLC). The bioautography of a mixture of 4 Cbls (OH Cbl, Ado Cbl, CN Cbl and Me Cbl) with and without extraction is also shown. Cbi in the lower part of the figure denotes an amount of [(CN, OH) Cbi] below the sensitivity of the organism.

Top: Growth response of the test organism (*E. coli* 113-3) to various concentrations of CN Cbl and of the cobinamide [(CN, OH) Cbi].

Cbl when the extraction solution contains CN.¹³

CN (0, 5, 10, 20, 50, 100, and 200 μg) had no effect on the binding of aqueous CN Cbl to R binder (data not shown).

ANALYSIS OF POOLED SERUM EXTRACTS FOR ANALOGUES

The amount of "Cbl" in the pooled extracts was greater using R as binder than with IF (Table 1). However, after purification by the Amberlite XAD-2 column, more Cbl was measured by RIDA, while the amount detected by bioassay with *E. gracilis* did not change. The material passing through the column was too low in concentration and not in a

suitable diluent for reliable bioassay. We believe that the increase was due to the presence of residual protein in the initial pool of extracts causing a falsely depressed value. Passage of the extract through the column essentially eliminated this residual protein, so that the sample and standard curve were now equal in protein content. The percentage increase in Cbl after elution from the column was the same irrespective of the binder used.

An initial analysis of an aliquot of the pooled extracts (2.55 ng total by RIDA-R; 1.08 ng by RID-AIF) by two-dimensional electrochromatography and bioautography revealed only one growth spot for *E. coli* 113-3 located where CN-Cbl was expected. In

was equivalent to >2.5 ng of CN-Cbl. Reanalysis of a small aliquot (255 pg by RIDA-R, 108 pg by RIDA-IF) again showed one growth spot equivalent to 184 pg of CN-Cbl (Fig. 2). We could detect no analogues of Cbl which were not converted to CN-Cbl.

Having failed to detect analogues by a direct approach we attempted an indirect one based on a search for those corrins *not* binding to IF, analogues by definition. 0.6 ml of pooled extract containing 7.65 ng of Cbl by RIDA-R (defined as "analogues plus true Cbl") and 3.24 ng of Cbl by RIDA-IF (defined as "true Cbl") was incubated with a 6.5 fold excess of IF under the conditions of the assay. Any "true Cbl" would be IF bound and "analogue" still free. The material was then separated into free and bound components by gel filtration on Sephadex G-50. The

fractions were examined by RIDA-R which would detect either type of Cbl. All of the Cbl was recovered in the bound component, none of the potential 4.41 ng of analogue remaining free of the IF (Fig. 3).

STUDIES ON THE "PROTEIN EFFECT"

The need for protein or similar substances in the standards of some RIDA's was studied in a set of experiments separate from the above but, as will be shown below, related. The data will not be given in great detail here but parts have been presented.¹⁴ One ml of an extract of serum prepared for RIDA but rendered free of Cbl by either of two methods shifted the standard curve of the RIDA to the right, indicating an enhanced apparent binding of the CN (⁵⁷Co) Cbl by the R binder. This was not due to residual Cbl binder from the extract since there was no

Fig. 2 Two dimensional electrochromatography of the corrins in a pool of serum extracts containing 255 pg as measured by RIDA-R and 108 pg by RIDA-IF. The separated corrins were then subjected to bioautography with *E coli* 113-3. The strip at the top is the growth response to increasing amounts of CN Cbl. The migration of the single, detectable component in the extract (184 pg) was that of CN Cbl.

Fig. 3 Gel filtration on Sephadex G-50 of pooled extract (3.24 ng "true Cbl" and 4.41 ng of "analogues") after incubation with a 6.5-fold excess of IF. The fractions were then assayed for Cbl by RIDA-R. Any analogue present which did not bind to IF would be in the included volume of the column.

detectable binding capacity when analysed by incubating 1 ml of extract with 125 pg of CN (⁵⁷Co) Cbl, and removing the free Cbl by either Ch/Alb or by gel filtration on Sephadex G-50. The amount of protein in 1 ml of extract before dialysis was 0.22 mg by the Lowry method¹⁵ and 1.54 mg by absorption at 280 and 260 nm.¹⁶ The net consequence of the addition of the extract to the standard solutions was an increase in Cbl concentrations of a sample of unknown content as read from that curve, conversely omission of the extract would cause an underestimation of the sample. Additions of 100 µg either of bovine serum albumin (BSA), bovine haemoglobin, human gamma-globulin, polyvinylpyrrolidone (PVP), or Plasmagel had the same effect. Dextran was inactive. None of the substances alone bound Cbl nor inhibited the removal of the free Cbl by Ch/Alb. We then studied the effects of increasing concentrations of serum extract (0.1-0 ml), and BSA or PVP (0.1 mg) on R and IF binding. As little as 10 µl of serum extract or 10 µg of BSA or PVP increased the apparent binding of the CN (⁵⁷Co) Cbl to a maximum. This plateau effect was present whether R binder or IF was the binding agent. The enhanced binding when macromolecules were present was time-dependent, since addition of protein just before removal of free Cbl with Ch/Alb had no effect.

COMBINED EFFECTS OF CHOICE OF BINDING AGENT AND ADDITION OF MACROMOLECULES TO THE RIDA

Fig. 4 shows the serum Cbl concentrations of 11 healthy persons measured by RIDA-R, RIDA-IF and bioassay. The RIDAs were performed with the binders diluted in CN-acetate buffer and separately in the buffer containing 200 mg/l PVP. The values were higher with both RIDA-R and RIDA-IF when PVP was added to the buffer. Each individual serum gave a higher value when R was used as the binder instead of IF, with or without PVP. When crystalline CN-Cbl was dissolved in the CN-acetate buffer, heat extracted and assayed by RIDA-R and RIDA-IF without PVP, the values obtained were identical (276 v 272 ng/l and 432 v 400 ng/l) for two separate concentrations. The pattern of comparable values for a serum Cbl concentration as obtained by bioassay and by RIDA-IF (but higher values as measured by RIDA-R) were again observed. In addition, the data of Fig. 4 illustrate that factors other than the choice of binding agent influence the operation of the assay and that manipulation may produce values which can be considered "acceptable" while obscuring real defects in the technique.

Fig. 4 RIDA of the Cbl of 11 normal sera using either R or IF as the binding agent and with or without polyvinylpyrrolidone (PVP) in the sample and standard curve tubes. Assay by *E. gracilis* for comparison.

Discussion

Cyanide converted the serum cobalamins to CN Cbl under the conditions of preparation for RIDA. This conversion may be important to the operation of the assays because it puts the Cbl of the unknown sample into that of the standards, CN Cbl. Should a Cbl of

serum—for example, Me Cbl, remain as such and differ from CN Cbl in affinity for the binding reagent, the assay would not perform correctly. During these experiments we noted that fresh solutions of NaCN were necessary for good conversion.

There was no evidence of the presence or creation of Cbl analogues as detectable by either TLC or by two-dimensional electrochromatography. For the latter study 1.47 ng of “analogues” were theoretically available for study, analogues being defined for the moment as corrins detected by RIDA-R but not by RIDA-IF. Thus there was abundant material to be detected by the chromatographic techniques unless there were a very large number of analogues totalling 1.47 ng but each in too low a concentration to be detected individually. If analogues should enter the circulation of healthy man, the intestine would seem to be the most likely source. The most abundant and ubiquitous intestinal analogues are the first three of Table 2 which have been found in the rumen and faeces of several species.^{11 17} Brandt *et al.*¹¹ observed all four of Table 2 to be both present and synthesised in the small intestine of persons with bacterial overgrowth in that organ. All of the analogues of Table 2 promote the growth of *E coli* 113-3,^{18 19} the least fastidious of assay organisms in its need for B₁₂. *E coli* 113-3 responds to the cobinamide (CN)₂Cbi which contains no nucleotide^{18 19} (Fig. 1, top) and the responsiveness is not altered by any of the known variants of nucleotide that may be added. It is still possible that analogues so remote from Cbl and from the analogues of Table 2 as to be undetectable by *E coli* 113-3 could exist in serum. The indirect experiment of Fig. 3 is strong evidence that in the present study there was none. Further evidence for the absence of analogue was the recovery of all known corrins present as CN Cbl. Our failure to demonstrate analogues either directly or indirectly is supported by a recent study incorporating similar experimental approaches.²⁰

The RIDA-R of our study gave higher values for serum Cbl than a RIDA based on IF as the binder.^{3 21–23} One recent investigator, however,

failed to show a difference and suggested that badly-designed techniques have been the cause of past difficulties.²⁴ Multiple aspects of techniques can influence the results of these assays and one of them, the well-known²³ “protein effect” was evaluated in our study. The protein effect should not be confused with the artefacts induced by a failure to denature all binders of Cbl in a source containing high concentrations of binders.^{6 25 26} Fig. 4, which reports the combined effect of manipulations of binder and macromolecule content, illustrates a principle in some ways more important than the actual data. The RIDA of serum Cbl is sensitive to many factors, each of which must be addressed separately. It is possible, as shown in Fig. 4, to create assays that give “satisfactory” results while still being faulty in design. Moreover, lower values are not necessarily truer values.²⁶

This work was supported by the Medical Research Service of the Veterans Administration.

References

- 1 Hall CA. Reliability of radioisotope dilution kits for vitamin B₁₂. *Lancet* 1977;ii:1255-6.
- 2 Cooper BA, Whitehead VM. Evidence that some patients with pernicious anemia are not recognized by radio dilution assay for cobalamin in serum. *N Engl J Med* 1978;299:816-8.
- 3 Kolhouse JF, Kondo H, Allen NC, Podell E, Allen RH. Cobalamin analogues are present in human plasma and can mask cobalamin deficiency because current radioisotope dilution assays are not specific for true cobalamin. *N Engl J Med* 1978;299:785-92.
- 4 Kolhouse JF, Allen RH. Absorption, plasma transport and cellular retention of cobalamin analogues in the rabbit. *J Clin Invest* 1977;60:1381-92.
- 5 Begley JA, Hall CA. Vitamin B₁₂ (B₁₂) assay and analogues of B₁₂. *Blood* 1979;54, suppl 1:36a.
- 6 Hall CA. The carriers of native vitamin B₁₂ in normal human serum. *Clin Sci Mol Med* 1977;53:453-7.
- 7 Hall CA, Begley JA. Congenital deficiency of human R-type binding proteins of cobalamin. *Am J Hum Genet* 1977;29:619-26.
- 8 Fenton WA, Rosenberg LE. Improved techniques for the extraction and chromatography of cobalamins. *Anal Biochem* 1978;90:119-25.
- 9 Bilkus DI, Mervyn L. Thin-layer chromatography of

Table 2 Common natural analogues of cyanocobalamin

Analogue Formula	Trivial name	In mammalian faeces ¹⁷			In small intestinal bacterial overgrowth ¹¹		Promotes growth of ^{18 19}		Affinity* for human binders ⁴	
		Present	Synthesised		Present	Synthesised	<i>E coli</i> 113-3	<i>E gracilis</i>	IF	R
(Ade) CN Cba	Pseudo B ₁₂	+	+	+	+	+	+	+	7 × 10 ⁻⁵	0.1
(2 Me Ade) CN Cba	Factor A	+	+	+	+	+	+	+	5 × 10 ⁻⁴	0.7
(CN) ₂ Cbi	Factor B	+	+	+	+	+	+	+	<1 × 10 ⁻⁴	0.1**
Unknown	Factor E	+	+	+	+	+	+	?	?	?

*Relative to CN Cbl = ±1.0.

**Form tested was (CN, OH) Cbi.

- vitamin B₁₂ and its coenzymes. In: Arnstein HRV, Wrighton RJ, eds. *Cobalamins*. London: Churchill Livingstone, 1971:17-21.
- ¹⁰ Burkholder PR. Determination of vitamin B₁₂ with a mutant strain of *Escherichia coli*. *Science* 1951;114:459-60.
 - ¹¹ Brandt LJ, Bernstein LH, Wagle A. Production of vitamin B₁₂ analogues in patients with small-bowel bacterial overgrowth. *Ann Intern Med* 1977;87:546-51.
 - ¹² Kon SK. Other factors related to vitamin B₁₂. *Biochem Soc Symp* 1955;13:17-35.
 - ¹³ Friedner S, Josephson B, Levin K. Vitamin B₁₂ determination by means of radioisotope dilution and ultrafiltration. *Clin Chim Acta* 1969;24:171-9.
 - ¹⁴ Begley JA, Hall CA. Effect of residual extract products and the type of binders (R or IF) on serum vitamin B₁₂ levels by radioisotope dilution assay. In: Zagalak B, Friedrich W, eds. *Vitamin B₁₂. Proceedings of the third European symposium on vitamin B₁₂ and intrinsic factor*. Berlin: Walter deGruyter, 1979:655-6.
 - ¹⁵ Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the folin phenol reagent. *J Biol Chem* 1951;193:265-75.
 - ¹⁶ Layne E. Spectrophotometric & turbidimetric methods for measuring proteins. In: Colowick SP, Kaplan NO, eds. *Methods in enzymology*. Vol 3. New York: Academic Press, 1957:447-54.
 - ¹⁷ Porter JGW. Occurrence and biosynthesis of analogues of vitamin B₁₂. In: Heinrich HC, ed. *Vitamin B₁₂ and intrinsic factor*. Germany: Ferdinand Enke, 1957:43-55.
 - ¹⁸ Coates ME. Requirements of animals and microbes. In: Sebrell WH Jr, Harris RS, eds. *The vitamins. Chemistry, physiology, pathology, methods*. Vol II. New York: Academic Press, 1968:243-57.
 - ¹⁹ Coates ME, Kon SK. Biological and microbiological activities of purine and benzimidazole analogues of vitamin B₁₂. In: Heinrich HC, ed. *Vitamin B₁₂ and intrinsic factor*. Germany: Ferdinand Enke, 1957:72-82.
 - ²⁰ Gimsing P, Nexø E. Forms of corrins in human serum during extraction prior to routine quantitation. Joint meeting of the 18th Congress of the International Society of Hematology and 16th Congress of the International Society of Blood Transfusion, Montreal, August 16-22, 1980, p 148.
 - ²¹ Rothenberg SP, Lawson J. Effect of cyanide on radioassay for serum cobalamin. *Clin Chem* 1979;25:639-40.
 - ²² Mollin DL, Hoffbrand AV, Ward PG, Lewis SM. Inter-laboratory comparison of serum vitamin B₁₂ assay. *J Clin Pathol* 1980;33:243-8.
 - ²³ Mollin DL. Microbiological assay for the measurement of serum B₁₂. Joint meeting of the 18th Congress of the International Society of Hematology and 16th Congress of the International Society of Blood Transfusion, Montreal, August 16-22, 1980. Abstract, p 44.
 - ²⁴ Chanarin I. Measurement of cobalamins. Joint meeting of the 18th Congress of the International Society of Hematology and 16th Congress of the International Society of Blood Transfusion, Montreal, August 16-22, 1980. Abstract, p 45.
 - ²⁵ Lee-Own V, Bolton AE, Carr PJ. Formation of a vitamin B-12-serum complexes on heating at alkaline pH. *Clin Chim Acta* 1979;93:239-44.
 - ²⁶ LeFebvre RJ, Virji AS, Mertens BF. Erroneously low results due to high nonspecific binding encountered with a radioassay kit that measures "true" serum vitamin B₁₂. *Am J Clin Pathol* 1980;74:209-13.

Requests for reprints to: Dr CA Hall, Hematology Research (151E) Veterans Administration Medical Center, Albany, New York 12208, USA.