Comparison of Toadfish-Serum Competitive Binding and Microbiologic Assays of Vitamin B_{12}

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Toadfish serum (TFS) offers several advantages over other proteins as the binder in a competitive-binding assay for vitamin B_{12}. It is unaffected by pH changes in the range 5.6–9.4 or by the addition of human serum albumin. Prolonged incubation with charcoal does not disrupt the TFS–cyanocobalamin bond, and the addition of albumin as a protein source in the standard tubes was proven unnecessary. The binding capacity of TFS does not increase significantly with increasing concentrations of cyanocobalamin as does the binding capacity of intrinsic factor, normal serum, or transcobalamin I. A single extract was prepared from each of 44 sera and measured for vitamin B_{12} content simultaneously by the TFS assay and the conventional microbiologic method using* Lactobacillus leichmannii. *The values obtained with TFS were in each instance higher than those obtained by the microbiologic assay ($p < 0.001$).

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In recent years radioassays have largely replaced microbiologic assays for the measurement of serum vitamin B_{12}. The advent of commercial kits based on the principle of competitive protein binding has increased the distribution and popularity of this test by shortening the assay time and supplying all reagents in one package. The radioassays eliminate the need to maintain a microorganism and to carry out the procedure under rigidly sterile conditions. Assay using radioactive tracers and sensitive gamma counters is more accurate than a biologic system where growth is quantitated by turbidity (*1,2*). The advantage of radioassays is that they are not influenced by excessive turbidity in the serum or by the presence of antibiotics, antimetabolites, or other drugs, which often invalidate the microbiologic test (*3,4*).

We have previously reported the characteristics of serum from the oyster toadfish, *Opsanus tau*, as a binder for cyanocobalamin (CN-Cbl) (*5,6*). Unlike intrinsic factor or human serum, this binder closely follows the principle of isotope dilution over a broad range of vitamin B_{12} concentrations; it is stable when stored frozen, and it has an extremely high binding capacity for CN-Cbl. We have since examined various parameters of the assay procedure and their effects on the TFS binder. The same serum extract was assayed by both the microbiologic and the TFS competitive-binding methods. Several minor modifications of our previous method were made so that the chemical conditions of the two procedures would be similar.

MATERIALS AND METHODS

After informed consent, venous blood was collected from normal volunteers and patients referred for vitamin B_{12} absorption studies. The serum was immediately separated and stored frozen in vitamin-B_{12}-free glassware. Vitamin B_{12} was extracted from the serum proteins by combining 2.5 ml of serum, 2.5 ml of acetate buffer (0.2 *M*, pH 4.6) containing 18 mg KCN/liter and 10 ml of water (*7*). A concentrated solution of KCN was prepared monthly, refrigerated in a dark bottle, and added to the acetate buffer on the test day. The tubes were placed in a boiling water bath for 30 min, cooled to room temperature, and then centrifuged to separate the precipitated proteins. Nine milliliters of supernа-

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tant was removed and the pH adjusted to 7.0 with 0.8 ml of 0.1 *N* NaOH. Water was added to bring the solution to a final volume of 15 ml (a 1:10 dilution of the original serum). When less serum was available, 1.5 ml was used and all reagents were adjusted proportionally. If the competitive protein-binding radioassay and the conventional microbiologic assay could not be run on the same day, the extract was frozen.

A 20-μg/ml stock solution of crystalline CN-Cbl* was prepared monthly and stored at 4°C in the dark. Standards containing 20–200 pg/ml were prepared the day of the assay.

Toadfish binding assay procedure. Serum from four toadfish† was pooled and diluted 1:2,500 with 0.1% human serum albumin. Aliquots sufficient for one assay were stored frozen. When a new lot of toadfish serum was prepared, it was necessary to determine the dilution that bound approximately 50% of a trace amount of ^{57}Co-CN-Cbl. One milliliter of either the serum extract or the standard solutions was put in duplicate or quadruplicate tubes and combined with 1 ml (approximately 50 pg) of ^{57}Co-CN-Cbl‡ (130–213 Ci/gm). Two milliliters of phosphate buffer (0.26 *M*, pH 7.0) and then the diluted TFS was added. The tubes were mixed mechanically for 60 min at room temperature. Counting tubes containing only the ^{57}Co-CN-Cbl were also prepared and the volume adjusted to 4.7 ml.

Crystallized bovine albumin|| was tested and found to be essentially free of contamination with vitamin B_{12} (2.7 pg/mg of albumin). Albumin-coated charcoal was prepared by combining 5 gm of neutral Norit A charcoal with 1 gm of bovine albumin in 200 ml of water (*8*); it was stored no longer than 1 month in the refrigerator and stirred with a magnetic stirrer during use. The human serum albumin was a 30% solution.¶

To separate the bound and free cyanocobalamin, 0.5 ml of bovine-albumin-coated charcoal was added. The tubes were placed on the mixer for 5 min, then centrifuged for 10 min at 1,700 g. The supernatants were decanted into tubes for counting in an automatic scintillation counter. The ratio of total counts to bound counts (supernatant) was calculated for both standards and samples, plotted on a linear scale, and a straight line drawn through the points. Serum values were read from the line. Extrapolation of this line to the horizontal axis provided an internal check of the amount (pg) of ^{57}Co-CN-Cbl added.

Microbiologic turbidimetric method. The procedure of Matthews (*7*) was followed, using *Lactobacillus leichmannii*.§ The media were Bacto Micro Assay Culture Agar, Micro Inoculum Broth, and B_{12} Assay Medium, *USP*.**

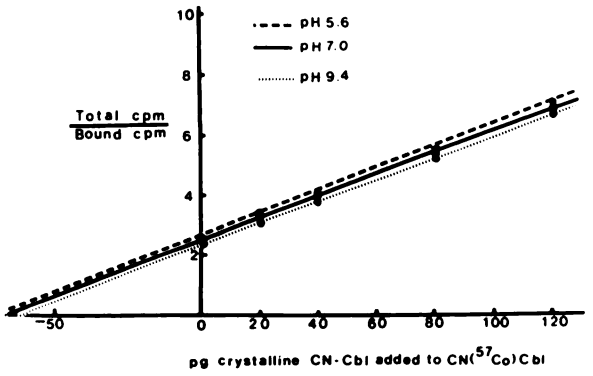

FIG. 1. Standard curves obtained in 0.2 *M* acetate buffer (pH 5.6), in 0.2 *M* phosphate buffer (pH 7.0), and in 0.2 *M* carbonate-bicarbonate buffer, (pH 9.4). Approximately 50 pg/ml of ^{57}Co-CN-Cbl was combined with increasing concentrations of crystalline CN-Cbl, 2 ml buffer, and 0.2 ml of 1:2,500 dilution of toadfish serum. Incubation was for 1 hr at room temperature, and bound and free were separated by charcoal coated with bovine albumin.

RESULTS

Standardization of conditions of TFS assay. Standard curves were all very similar, whether obtained at pH 5.6 in 0.2 *M* acetate buffer, at pH 7.0 in 0.26 *M* phosphate buffer, or at pH 9.4 in 0.2 *M* carbonate–bicarbonate buffer (Fig. 1). An extract of pooled normal serum gave values of 780 pg/ml at pH 5.6, 835 pg/ml at pH 7.0, and 800 pg/ml at pH 9.4. On the basis of these results, a pH of 7.0 and an ionic strength of 0.26 *M* were selected because the assay media used in the microbiologic assay had similar values.

The supernatant of control tubes containing all reagents except the toadfish serum always contained less than 3% of the total radioactivity after separation with bovine-albumin-coated charcoal. A "blank" using a 1:25 dilution of TFS, instead of the usual 1:2,500 dilution, bound only 92–98% of the added ^{57}Co-CN-Cbl, rather than 100%.

When intrinsic factor is the binding protein (as in most of the commercially available kits for vitamin B_{12} assay), careful attention must be given to the time the charcoal remains in contact with the sample to avoid removing excessive amounts of the bound complex (*9*). Table 1 shows that this is not the case with TFS. Within time periods varying from 5 min to 2 hr of mixing with bovine-albumin-coated charcoal, the percent bound remained constant in all standards, blanks, and a sample of serum extract.

Intrinsic factor binds more CN-Cbl in the presence of protein (*10–13*). The mean of 17 standard curves performed with TFS without the addition of protein is shown in Fig. 2. When no additional CN-Cbl was added, a mean of 46.4% of the approximately 50 pg of ^{57}Co-CN-Cbl was bound. It is ap-

TABLE 1. PERCENT OF ^{57}Co-CN-Cbl BOUND AFTER MIXING WITH BOVINE-ALBUMIN-COATED CHARCOAL*

50 pg ^{57}Co-CN-Cbl	plus	5 min	10 min	15 min	30 min	60 min	120 min
	No added CN-Cbl	42.5	37.6	43.0	33.6	35.7	40.6
	40 pg CN-Cbl	26.0	26.8	26.5	26.5	26.3	26.5
	120 pg CN-Cbl	15.3	15.4	15.0	14.9	15.4	15.1
	1 ml serum extract containing 855 pg vitamin B_{12}	18.4	18.7	18.3	18.1	18.7	17.9
"O" blank	No TFS	1.35	1.09	1.00	1.53	2.59	1.24
"100%" blank	1:25 dilution of TFS	92.2	93.8	95.0	94.3	91.2	92.5

* Means of duplicate samples. The binder was 0.2 ml of a 1:2,500 dilution of TFS.

parent from Fig. 2 that the binding by the diluted toadfish serum was unaffected by the addition of human serum albumin (dotted line).

The Rothenberg's method (*14*) was used to determine whether the binding capacity of TFS increased with increasing concentration of CN-Cbl. No significant increase was found (Table 2).

Reproducibility of TFS assay. There was little day-to-day variability in standard curves obtained with the TFS procedure. The mean of 17 standard curves ± one standard deviation is shown in Fig. 2. A serum sample assayed on seven separate occasions gave a mean value of 855 ± 28 pg/ml. Reproducibility within one run was also good. The coefficient of variation of quadruplicate measurements of the same serum extract was 4.53% (n = 63).

Comparison of TFS assay with microbiologic assay. Forty-four sera were assayed by both the TFS and the microbiologic assay (Fig. 3). The same serum extract was used in both procedures. The TFS assay values (mean, 839 pg/ml) were significantly and consistently higher than the values obtained with the microbiologic assay (mean, 59 pg/ml). The p value, calculated by the paired difference method (*15*), was below 0.001.

DISCUSSION

We have previously shown that TFS follows the principle of isotope dilution in the range of vitamin B_{12} concentrations found in human serum (*5*). In the present study the binding capacity of the diluted TFS was shown not to increase as increasing amounts of CN-Cbl were added. In contrast, the binding capacities of intrinsic factor, human serum with increased transcobalamin I, and normal human sera do increase with the addition of more CN-Cbl (*14,16*).

The apparent deviation from expected binding of the trace amount of ^{57}Co-CN-Cbl, which should have been complete with the 1:25 dilution of TFS, could have been due to either "trapping" of some of the protein-bound ^{57}Co-CN-Cbl in the charcoal or to nonspecific adsorption onto the glass tubes. Some competitive protein-binding assays for vitamin B_{12} are corrected for the free CN-Cbl not removed by the charcoal, but few take into account the bound complex left behind in the tube. We found that the opposing differences of these two observations essentially cancelled each other out, making such corrections unnecessary.

Numerous modifications of competitive binding assays for vitamin B_{12} have appeared in the literature (*2,14,17–26*). Some binding assays give serum values that are higher than those of microbiologic assays (*2,13,17,27–30*). We felt it important to make a direct comparison between the serum vitamin B_{12} levels determined with the TFS assay and those of conventional microbiologic assay. Raven (*30*) recently reported that the higher values obtained with his binding assay using intrinsic factor could be explained by the different extraction procedures used in the intrinsic-factor binding and microbiologic assays. Accordingly, we elected to use a single extract of each serum for our comparison of

TABLE 2. ABSOLUTE AMOUNT (PG) OF VITAMIN B_{12} BOUND BY 0.2 ML OF A 1:2,500 DILUTION OF TFS AFTER THE ADDITION OF VARIED AMOUNTS OF NONRADIOACTIVE VITAMIN B_{12}*

CN-Cbl added to ^{57}Co-CN-Cbl (pg)	Increase in binding capacity (%)
20	1.01
40	0.99
80	0.98
120	0.98
200	1.02

* The amount of ^{57}Co-CN-Cbl bound by this amount of TFS was 46% of the 65 pg. The values are expressed as percent increase in binding at each concentration of added nonradioactive vitamin B_{12}.

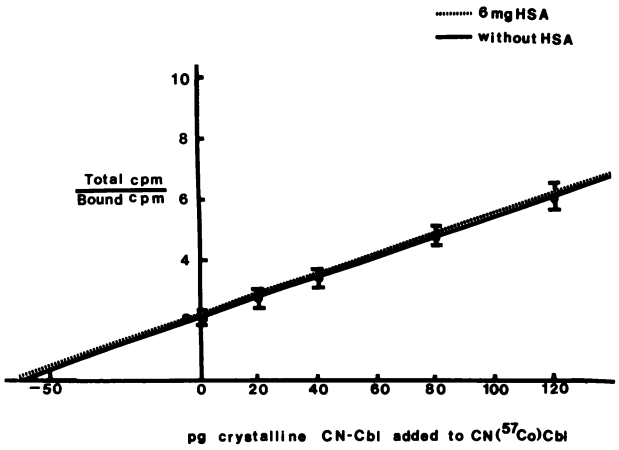

FIG. 2. Solid line depicts mean of 17 standard curves performed without addition of proteins (vertical bars indicate ±1 s.d.). Dashed line is mean of duplicate standard curves done with addition of 6 mg of human serum albumin at each concentration of CN–Cbl.

the TFS and microbiologic methods. Despite this modification, the values were significantly higher with the TFS-binding assay than with the conventional microbiologic method.

Green (*17,28*) speculates that the higher values in his chicken-serum binding assay may be due to a third serum vitamin B_{12} binder which is more resistant to extraction. It remains to be proven whether this third binder exists or whether the difference is an artifact of the separation system (*31*). In any case this would not explain the higher results in our comparison of the TFS and microbiologic systems using a single extraction procedure. A more likely explanation is that the *L. leichmannii* cannot utilize as a growth factor some form of the cobalamin molecule that the toadfish serum is able to bind.

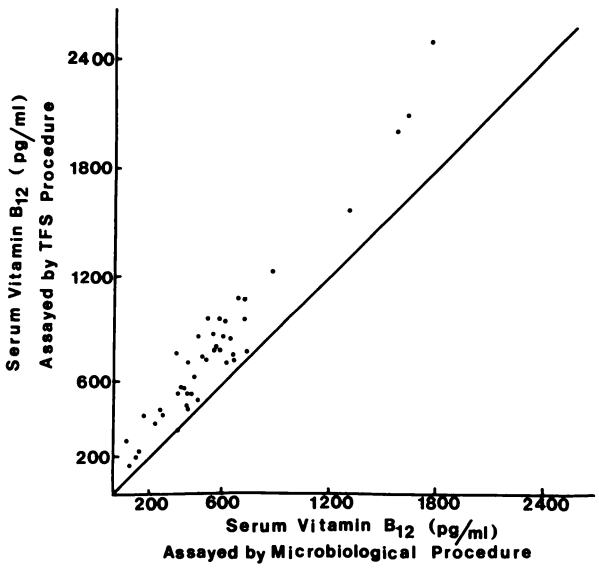

FIG. 3. Same serum extracts assayed by TFS procedure and microbiologic assay using *Lactobacillus leichmannii*. Line of identity is indicated; ($p < 0.001$, $n = 44$, based on paired differences).

Concurrent with the development of this binding assay, we have developed a simplified microbiologic assay based on radiometric techniques (*32*). The results of a more detailed evaluation of this radiometric method for assay of vitamin B_{12} are presented in the following article.

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FOOTNOTES

* *USP* Reference Standards, Rockville, Md.

† Toadfish serum supplied by Ray P. Morgan, Chesapeake Biological Laboratory, Solomons, Md.

‡ Amersham/Searle, Arlington Heights, Ill.

|| Armour Pharmaceutical Co., Chicago, Ill. (Lot # M72603).

¶ ICN Pharmaceuticals, Life Sciences Group, Cleveland, Ohio (Lot # 0680).

§ ATCC7830, obtained from the American Type Culture Collection (Rockville, Md.).

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