Vitamin B\textsubscript{12}: Are Serum Radioassay Measurements Reliable?

Although it had been recognized earlier, Thomas Addison in 1855 called attention to a disorder with systemic, gastrointestinal, neurologic, and hematologic manifestations that was ultimately fatal. The discovery by Minot and Murphy (1) in 1926 that these manifestations could be reversed by liver therapy, and the studies clarifying the role of the stomach in the pathogenesis of pernicious anemia by Castle (2) in 1926, led to the discovery of vitamin B\textsubscript{12} (cyanocobalamin) in 1948 by Merck Laboratories. Today, appropriate therapy based on early, accurate diagnosis is curative, preventing permanent neurologic sequelae in most patients. Screening for pernicious anemia depends largely on measurements of cobalamin concentrations in the blood. Because of the implications of a missed diagnosis, there should be very few, if any, falsely normal results. That this necessity for sensitivity has not always been realized with competitive protein binding vitamin B\textsubscript{12} assays has been cause for considerable concern.

Although the cause of Addisonian pernicious anemia is an absence of gastric intrinsic factor manifest by low serum vitamin B\textsubscript{12} concentrations, similar findings are encountered in intestinal malabsorption after certain intestinal surgery (gastric resection, blind loops, ileal resection), occasionally by reduction in intestinal B\textsubscript{12} concentration by bacterial or other parasites, and by decreased serum binding protein concentrations. Measurement of the serum cobalamin concentration, therefore, may constitute a screen for B\textsubscript{12} deficiency of whatever cause, but it is not specific for pernicious anemia.

Cobalamins are freed from ingested animal proteins by acid digestion in the stomach and are bound by intrinsic factor before transit through the bowel. Absorption occurs mainly in the distal ileum, after which cobalamins are bound in the blood by high-affinity protein binders of various specificity. Transcobalamin II, a polypeptide transport protein with the greatest specificity binds nearly 30\% of the serum total cobalamins (3). Other, more ubiquitous binders, the so-called R proteins, bind the remainder of the serum cobalamins.

Of the three major protein binders, intrinsic factor is highly specific for cobalamin. R proteins, initially discovered in gastric juice devoid of intrinsic factor obtained from patients with pernicious anemia, and so named for their rapid electrophoretic mobility, bind cobalamin with a high affinity but also bind a wide variety of cobalamin analogs. These proteins are widely present in tissue and other body fluids as well as in the serum. Transcobalamin II is intermediate in its specificity between intrinsic factor and R proteins, binding some but not all analogs in addition to biologically active cobalamins.

Vitamin B\textsubscript{12} (cyanocobalamin) consists of a planar porphyrin ring and nucleotide moiety surrounding a cobalt atom along with an adjacent cyanide radical. Cyanocobalamin comprises only a small fraction of the biologically active cobalamins present in the blood. Methylcobalamin is present in higher concentrations, and there are lesser amounts of adenosylcobalamin and hydroxocobalamin. In addition, other analogs, believed biologically inactive but bound by R proteins, are apparently also present in serum. Thus, assays designed to measure the biologically potent compounds will quantitate several forms of cobalamin but ideally would not detect the presence of inactive analogs.

The observations that cobalamins support the growth of various bacteria form the basis for the microbiological assays in use for many years. Assays utilizing Euglena gracilis or Lactobacillus leichmanii have been used clinically but are cumbersome, requiring otherwise sterile conditions, inclusion of recovery samples to detect the presence of inhibitors (i.e., antibiotics), and several days to complete. In the early 1960s, the application of the principles of saturation analysis led to simple, easily performed, competitive binding assays for cobalamin. These assays used a serum extraction step generally involving boiling at either acid or alkaline pH in the presence or absence of
cyanide followed by the addition of radiocobalt-labeled cobalamin and a binder. The free fractions (nonantibody bound) were most commonly separated by charcoal. Initially, the binder was crude intrinsic factor, although serum (transcobalamin) and other binders have been used. Although occasionally a patient with pernicious anemia was found to have an apparently normal serum B\textsubscript{12} concentration when one of these assays was used, results generally appeared to correlate with clinical impressions. After competitive protein binding assays became commercially available, they were widely applied.

Although Schilling had noted nonspecificity of the binding properties of crude intrinsic factor preparations as early as 1958 (4), it remained for Cooper and Whitehead (5) and Kolhouse and coworkers (6) in 1977 and 1978 to report that this nonspecificity resulted in apparently normal serum cobalamin concentrations in 10–20% of patients with proven pernicious anemia. In 1980 Cohen and Donaldson expanded these observations to a larger patient population and confirmed the alarming lack of clinical sensitivity (7).

In a careful analysis of this problem, Kolhouse and coworkers reported that endogenous cobalamin inhibited binding of Co-57 cobalamin to human R protein to a greater extent than it inhibited pure intrinsic factor binding, thus implying the presence of B\textsubscript{12} analogs bound by R protein but not by pure intrinsic factor. Although suggested by Rothenberg and Lawson (8), it appears that the cyanide used in the extraction step is an unlikely explanation for these observations, since quantitative recoveries of cyanocobalamin, hydroxocobalamin, methylcobalamin, and 5'-deoxyadenosylcobalamin could be demonstrated for assays using either R protein or intrinsic factor as the binder. In the Kolhouse experiments human serum was extracted and subjected to paper chromatography. The intrinsic factor assay measured only a cobalamin migrating equivalently with Co-57 cobalamin. The R protein assay measured a number of other substances migrating both faster and slower than true cobalamin so that the total apparent cobalamin concentration measured by the R protein assay was nearly 70% greater than that measured by the intrinsic factor assay. Microbiologic assays (E. gracilis and L. leishmanii) measured essentially the same activity in each chromatography fraction as did the intrinsic factor assay, indicating that the other fractions measured by the R protein assay are biologically inactive.

Further, Kolhouse investigated ten commercially available radioassay kits and discovered that the binders, stated to be intrinsic factor, in fact consisted of 51–85% R protein, and only 10–49% intrinsic factor was found. Further, the pH of the assay critically determined which binder was the more important. R proteins retain their binding properties over a pH range from 9 to 2. Intrinsic factor exhibits maximal binding at pH 9, loses 10% of its maximal binding at pH 4, and loses greater than 98% at pH 2. Thus, in assays using an acid extraction only R protein binding was effective even if intrinsic factor was nominally present.

The obvious implication from these demonstrations was that no commercially available assay could reliably measure the low cobalamin serum concentrations present in patients with pernicious anemia. In perhaps 20% of such patients inappropriate, apparently normal serum cobalamin concentrations would be measured.

Shortly after the findings of Cooper and Whitehead and Kolhouse et al. were published in October 1978, the Food and Drug Administration convened the Clinical Chemistry and Hematology Devices Panel to discuss these reports. On the basis of the Panel's recommendations, the FDA required that manufacturers reevaluate their products for potential erroneous results and, in addition, notify their customers of potential problems and subsequent modifications. The FDA also requested that the National Committee for Clinical Laboratory Standards (NCCLS) establish a task force to develop a voluntary standard for vitamin B\textsubscript{12} radiolodilution assays. The NCCLS Task Force for the Assay of Vitamin B\textsubscript{12}, chaired by Robert F. Schilling, has prepared a proposed Standard, published in March 1980 (9). The committee recognized three approaches to the problem of assay binder specificity and defined methods for validation. These are as follows.

1. Purified Intrinsic Factor. Methods utilizing pure intrinsic factor should not measure cobaminide (an analog) up to levels of 10,000 pg/ml in serum when the appropriate amount of serum for the kit is used. Under these conditions, 10,000 pg/ml of cobaminide in serum should read less than 75 pg/ml when read against a standard curve constructed from cobalamin standards. Vitamin B\textsubscript{12} binding should be greater than 95% inhibited by specific anti-IF blocking antibody, and/or the IF-B\textsubscript{12} complex should be greater than 95% precipitated by specific IF precipitating antibody.
2. Intrinsic Factor plus other binders. Other binders (i.e., R proteins) can be blocked by the addition to the binder of a large mass of B$_{12}$ analog, such as cobinamide. Since intrinsic factor does not appreciably bind cobalamin analogs intrinsic factor should, under these conditions, be the only operative binder. Such binder preparations must meet the same validation criteria set forth for the pure intrinsic factor preparations.

3. Operative binders other than Intrinsic Factor. Binders other than intrinsic factor have been utilized in B$_{12}$ radioassays (i.e., fish sera, human transcobalamin II, dog stomach binders, and anti-B$_{12}$ antibodies) (10–12). Since assays using binders other than intrinsic factor cannot be evaluated with anti-intrinsic factor antibody, they must be validated by the direct demonstration that these binders do not measure analogs in human serum and/or plasma.

Manufacturers of B$_{12}$ assay kits currently utilize one of these approaches to produce a specific binder. Kubasik and colleagues (13) earlier compared one manufacturer's nonpurified intrinsic factor-R protein binder, "cobinamide"-blocked R protein binder, and "purified" intrinsic factor kits* with results from a microbiological assay (L. leishmannii). They reported that results with both modifications, R protein — intrinsic factor binder blocked by an analog, and purified intrinsic factor, were comparable to those obtained with the microbiological assay and were consistent with the clinical impressions. Elsewhere in the Journal Chen and coworkers (14) have extended these observations, utilizing crude intrinsic factor — blocked R protein† and purified intrinsic factor* binders. Although generally confirming the findings of Kubasik, Chen identified a significant nonspecific binding difference between standard and patient sera extracts in the blocked R protein binder assay. Unless corrected for with nonspecific binding measurements, this difference results in apparent inappreciably low serum cobalamin concentrations.

Where does all this leave users of radioassay kits designed to measure serum vitamin B$_{12}$ concentrations? Clearly, binder nonspecificity leads to falsely normal results in perhaps 20% of patients who do have abnormally low serum cobalamin concentrations. Whether the majority of these patients will be diagnosed correctly anyway depends upon the clinical manifestations of disease and the index of suspicion of the referring clinician. Nevertheless, an apparently normal serum B$_{12}$ concentration may be reassuring but misleading in a patient with minimal neurologic manifestations and no anemia, leading to a potentially disastrous delay in diagnosis and treatment. It seems obvious, therefore, that a radioassay kit modified so that binder specificity is achieved should be used for serum B$_{12}$ measurements. Although radioimmunoassays for serum B$_{12}$ have not been widely developed or applied, the development of anticobalamin antibodies may help solve the specificity problem. Careful validation studies, however, are needed to ascertain that antibody cross-reactivity with nonbiologically active structural analogs do not exist. Microbiological assays involve specific problems and are not reasonable alternatives to competitive protein binding assays in most hospitals.

The correlations between results with early competitive protein binding serum B$_{12}$ assays and microbiological reference assays were excellent (15,16). Such demonstrations now in limited numbers of selected patients are not necessarily reassuring. The findings of Chen demonstrate again the need for users to validate carefully methods used in their own laboratories. The nonspecific binding difference discovered in the R protein blocked binder assay was present despite the manufacturer's claim that they met the NCCLS recommendations.

Notwithstanding the meticulous work of Kolhouse and the subsequent demonstration that modified B$_{12}$ radioassay kits do possess the requisite specificity for cobalamins, many clinicians view these measurements with renewed skepticism. Although they may be helpful in the evaluation of liver disease and myeloproliferative diseases, in the confirmation of the impression of B$_{12}$ deficiency, and to monitor treatment, a normal serum B$_{12}$ measurement should not be used as the sole criterion for excluding the diagnosis of pernicious anemia. The more judicious application of Schilling tests and careful followup of patients with suspected but not proved B$_{12}$ deficiency is warranted.

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FOOTNOTES

* Diagnostic Products Corporation, Los Angeles, CA.
† Becton Dickinson Immunodiagnostics, Orangeburg, NY.
REFERENCES

1. MINOT GR, MURPHY WP: Treatment of pernicious anemia by special diet. JAMA 87:470–476, 1926

MISSOURI VALLEY CHAPTER
ANNUAL FALL MEETING
SOCIETY OF NUCLEAR MEDICINE

September 25–27, 1981
Radisson Muehleback Hotel
Kansas City, Missouri

Announcement and Call for Abstracts

The Missouri Valley Chapter will hold its annual Fall meeting in Kansas City, Missouri, September 25–27, 1981.

Drs. E. William Allen and Robert Henkin and Mr. William O'Neill have been invited to speak on "The Practical Use of Nuclear Medicine Computers in Patient Care," which is the theme of Saturday's program. Co-program chairmen are David F. Preston, M.D., Kansas University Medical Center and James Fletcher, M.D., VA Hospital, St. Louis.

Contributed papers on any nuclear medicine subject will be presented Sunday morning. Submit abstracts to:

James Fletcher
Director of Nuclear Medicine
VA Hospital
John Cochran Division 115JC
St. Louis, MO 63125

Deadline for submitted abstracts is August 1, 1981.

Young Investigator and/or Technologist Awards will be presented for the best scientific papers.

Application has been made for AMA Category 1 and VOICE CEU credit.

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