American Journal of Hematology 34:90–98 (1990)

Diagnosis of Cobalamin Deficiency I: Usefulness of Serum Methylmalonic Acid and Total Homocysteine Concentrations

Robert H. Allen, Sally P. Stabler, David G. Savage, and John Lindenbaum

Department of Medicine and Department of Biochemistry, Biophysics and Genetics, University of Colorado Health Sciences Center, Denver (R.H.A., S.P.S.); Department of Medicine, College of Physicians and Surgeons, Columbia University and Department of Medicine, Columbia-Presbyterian and Harlem Hospital Centers, New York (D.G.S., J.L.)

The serum cobalamin assay is the primary diagnostic test for cobalamin deficiency. It appears to be an excellent screening test since most patients with clinically confirmed cobalamin deficiency have low levels. Recent studies indicate that the clinical picture of cobalamin deficiency is much more diverse than previously believed. It is also apparent that many patients with low serum cobalamin concentrations are not cobalamin deficient. Thus, there is a need for additional diagnostic tests to further distinguish patients with low serum cobalamin levels who are actually cobalamin deficient and will benefit from lifetime treatment from those who are not deficient and will not benefit. Serum levels of methylmalonic acid and total homocysteine have been shown to be markedly elevated in most patients with cobalamin deficiency, and total homocysteine concentrations are markedly elevated in most patients with folate deficiency. The levels of these metabolites fall to normal if these patients are treated with the appropriate vitamin but remain essentially unchanged if the wrong vitamin is administered. These observations demonstrate that serum methylmalonic acid and total homocysteine levels are useful in diagnosing patients with cobalamin and folate deficiency and in distinguishing between these two vitamin deficiencies.

Key words: methylmalonic acid, homocysteine, cobalamin, vitamin B_{12}, folate

INTRODUCTION

Although most noted for the cobalamin (vitamin B_{12}, Cbl) absorption test that bears his name, Robert F. Schilling, M.D. has made other major contributions related to the diagnosis of Cbl deficiency. His prominence in this area is illustrated by the fact that in 1979 when the Food and Drug Administration asked the National Committee for Clinical Laboratory Standards (NCCLS) to appoint a panel to investigate major problems in serum Cbl assays [1–3], Dr. Schilling was chosen to chair this panel. One of these problems related to the observation that previously unknown Cbl analogues were present in human blood. Serum Cbl assays in use at that time contained nonspecific Cbl-binding proteins that bound and measured the Cbl analogues together with Cbl, and thus gave erroneously high results. This finding was of diagnostic importance because approximately 20% of patients with clinically confirmed severe Cbl deficiency had values for the total of Cbl and Cbl analogues that were within the normal range, as shown in Figure 1. When the nonspecific R protein was replaced with the specific intrinsic factor, only Cbl was measured, and lower values were obtained. In addition, and most importantly, a complete separation between the normal subjects and those with severe clinically confirmed Cbl deficiency was obtained (Fig. 1).

It is of interest that Dr. Schilling was the first to publish about differences in specificity of Cbl binding proteins when in 1956 he wrote [4],

Address reprint requests to Robert H. Allen, M.D., Division of Hematology, Campus Box B170, University of Colorado Health Sciences Center, 4200 E. Ninth Avenue, Denver, CO 80262.

This work was supported by Department of Health and Human Services Research Grants (DK31765 and DK21365) from the National Institute of Diabetes and Digestive and Kidney Diseases.

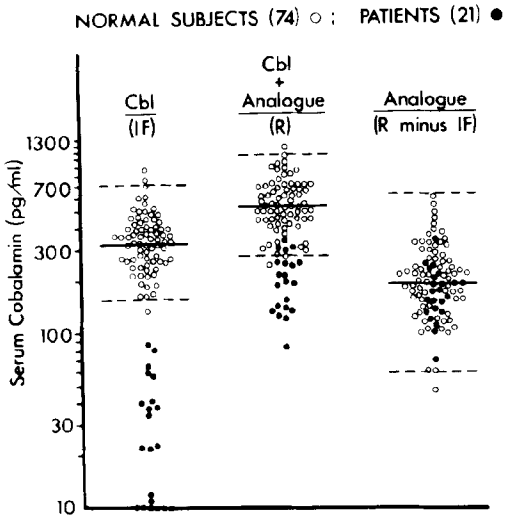

Fig. 1. Serum Cbl values for 74 normal subjects (○) and 21 patients with clinical evidence of Cbl deficiency (●) determined by radioisotope dilution assays using various Cbl-binding proteins. Solid and dashed lines represent the mean ± 2 S.D. for the 74 normals. Cbl analogue levels were obtained by subtraction of values obtained with human intrinsic factor (IF) from those obtained with human salivary R protein (R) [adapted from reference 2 with permission].

The *in vitro* binding of cyanocobalamin by gastric juice is a selective process showing a distinctive preference for cyanocobalamin over pseudo vitamin B_{12} [a Cbl analogue that contains adenine in place of 5,6-dimethylbenzimidazole] . . .

The process of cobalamin binding by serum, unlike that in gastric juice, does not manifest a selectivity for cyanocobalamin in the presence of excess pseudo vitamin B_{12}.

A second problem with the Cbl assays in use in the mid-1970s involved imprecision with respect to establishing normal ranges. The two largest manufacturers of Cbl assay kits stated that 200 pg/ml was the appropriate lower limit of normal for their nonspecific Cbl assays [2]. Based on the data in Figure 1, together with data from other manufacturers [2] and subsequent investigators [5,6], it is clear that the appropriate lower limit of normal for the nonspecific Cbl assays is close to 300 pg/ml. The use of a lower limit of normal of 200 pg/ml for the nonspecific Cbl assays resulted in "normal values" being obtained for about 50% of severe clinically confirmed Cbl-deficient patients (see Fig. 1).

The NCCLS panel chaired by Dr. Schilling made a number of recommendations that included the use of specific binding proteins in Cbl assays and the recommendation that every Cbl assay be verified using test results obtained with normal subjects *and* clinically confirmed Cbl-deficient patients. These recommendations have now been met for essentially all Cbl assays. As a result, package inserts for Cbl assays contain more clinical documentation than is found with virtually any other clinical assay.

Although the diagnostic sensitivity of Cbl assays was markedly improved by the adoption of the NCCLS recommendations, problems were soon noted with respect to their diagnostic specificity, especially if they were used to screen apparently healthy individuals for Cbl deficiency. This problem was explicitly stated by Dr. Schilling in 1982 when he wrote [7],

> As a result of the change in observed levels of the vitamin with the newer radioligand kits, it is highly probable that physicians will soon be required to explain increasing numbers of unexpectedly low serum vitamin B_{12} concentrations in patients with no other signs of deficiency.

The potential enormity of this problem is apparent when one realizes that by definition 2.5% of normal healthy subjects will have low serum Cbl levels. Based on a population of 250,000,000, one can calculate that there are approximately 6,250,000 people in the United States with low serum Cbl values. It has been estimated that between 0.1% and 1.0% of the population will become Cbl deficient at some time in their life [8]. If one assumes an average lifetime of 75 years and that Cbl deficiency will exist for an average of 5 years before it is treated or the patient dies, one can calculate that at any point in time there are 15,000 to 150,000 Cbl-deficient patients in the United States. Even if all of these patients have low serum Cbl values, which seems unlikely, they will represent only 0.24% to 2.4% of individuals with low serum Cbl values.

The magnitude of the difference between the incidence of low serum Cbl values and that of Cbl deficiency suggests that problems will still remain even if one limits Cbl testing to those individuals who have hematologic or neuropsychiatric abnormalities of the kind caused by Cbl deficiency [9]. This is particularly likely because none of these abnormalities is specific for Cbl deficiency and because recent studies [10–13] have shown that the clinical picture of Cbl deficiency is much more diverse than previously believed. Thus, many Cbl-deficient patients are not anemic, have normal mean cell volumes, normal white blood cell and platelet counts, normal peripheral smears as reported by routine laboratories, and normal lactate dehydrogenase and bilirubin levels. In addition, many patients with neuropsychiatric abnormalities that respond to Cbl therapy lack most, or even all, of the

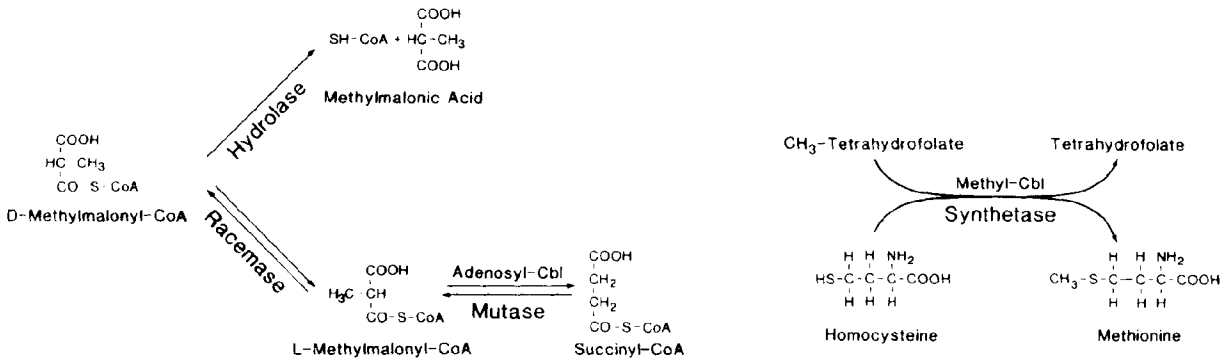

Fig. 2. L-methylmalonyl-CoA mutase (left) and methionine synthetase (right) are the two mammalian Cbl-dependent enzymes. In Cbl deficiency, L-methylmalonyl-CoA accumulates and is converted to D-methylmalonyl-CoA, which is hydrolyzed to methylmalonic acid. Homocysteine accumulates in either Cbl or folate deficiency.

hematologic abnormalities [13]. One cannot solve this problem by using a value of 100 pg/ml as the cut-off between Cbl deficiency and normality since the same recent studies [10–13] have shown that approximately 40% of clinically confirmed Cbl-deficient patients have serum Cbl values in the 100 to 200 pg/ml range.

In the early 1980s, the additional diagnostic tests for Cbl deficiency that were widely available were limited to treating patients with Cbl and looking for objective responses to such therapy or to use of the Schilling test. Therapeutic trials may require months before responses can be evaluated and can be difficult to interpret in patients with neuropsychiatric abnormalities since these do not always respond to Cbl therapy even if Cbl deficiency is the cause of the abnormalities. The use of the Schilling test as a diagnostic test for Cbl deficiency has been criticized by Dr. Schilling who wrote in 1982 [7],

> A technique often mistakenly utilized as a means for establishing or refuting vitamin B_{12} deficiency is measurement of the patient's ability to absorb an orally administered dose of the vitamin. Such estimation of the ability to absorb vitamin B_{12} may be helpful in delineating the mechanism by which a patient has become B_{12} deficient or in predicting which patients might become deficient in the vitamin while on a normal diet, but testing the patient's ability to absorb the vitamin will not give the diagnosis of deficiency per se.

Nevertheless, as a practical point, few would argue with the widely accepted practice of instituting lifetime Cbl therapy in patients with abnormal Schilling tests. Although some patients are unable to collect proper 24 hour urine specimens as required in the Schilling test [14], the major problem with using it as the sole additional diagnostic test in patients with low serum Cbl values is that the classic Schilling test uses crystalline Cbl, whereas Cbl in food is tightly bound to protein and this protein-bound Cbl is what must be absorbed under physiologic conditions. Studies by Doscherholmn and Swaim [15] and others [16,17] have documented that patients with hypochlorhydria are unable to absorb food Cbl, even though their ability to absorb crystalline Cbl remains intact. Furthermore, recent studies by Carmel et al. [18] have shown that food Cbl malabsorption occurs commonly in patients with low serum Cbl levels.

It is of interest that studies [19] by Dr. Schilling in 1967 anticipated the importance of food Cbl malabsorption. Dr. Schilling injected radioactive Cbl into chickens and obtained eggs that contained radioactive Cbl. He showed that patients with acid gastric juice containing pepsin absorbed more of the egg Cbl than did pernicious anemia patients supplemented with oral intrinsic factor. He suggested that ". . . peptic activity is important in food B_{12} absorption."

In the early 1980s, we set out to develop new diagnostic tests for Cbl deficiency per se with an emphasis on assays that could be performed with serum since this is what is left in the laboratory after serum Cbl is measured. Because Cbl serves as a co-factor for only two mammalian enzymes, we concentrated on compounds related to these two pathways, which are shown in Figure 2. Although many compounds were tested using gas chromatography-mass spectrometry [20,21], methylmalonic acid and total homocysteine were the only ones with diagnostic utility [22,23]. The utility of measuring serum methylmalonic acid was not surprising since many studies dating from the 1950s have shown that many Cbl-deficient patients excrete increased amounts of methylmalonic acid in their urine [24,25].

The initial studies [22,23] of normal subjects and patients with moderate and severe clinically confirmed Cbl

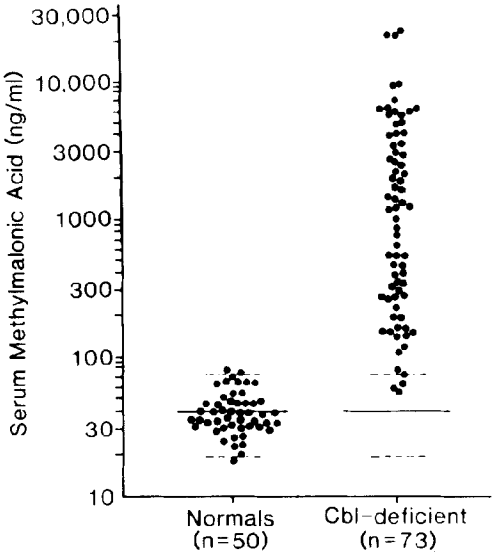

Fig. 3. Values for serum methylmalonic acid obtained with normal subjects and patients with clinically confirmed Cbl deficiency. Solid and dashed lines represent the mean ± 2 S.D. for the normal subjects [adapted from reference 22 with permission].

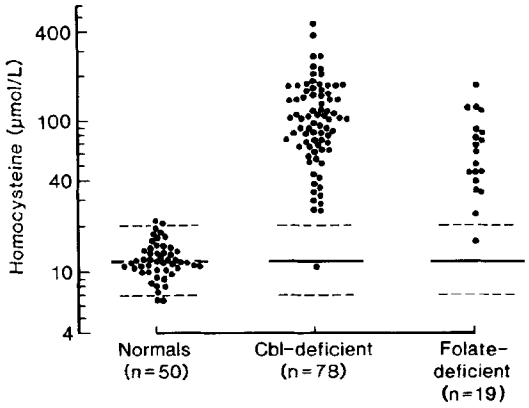

Fig. 4. Values for serum total homocysteine obtained with normal subjects, patients with clinically confirmed Cbl deficiency, and patients with clinically confirmed folate deficiency. Solid and dashed lines represent the mean ± 2 S.D. for the normal subjects [adapted from reference 23 with permission].

deficiency are shown in Figures 3 and 4. Approximately 95% of these Cbl-deficient patients had elevations of serum methylmalonic acid, and the same was true for serum total homocysteine. In recent studies [11] with 86 consecutive Cbl-deficient patients with serum Cbl levels <200 pg/ml, many of whom had mild Cbl deficiency although all had objective responses to Cbl therapy, 77% had marked elevations (>3 S.D. above the mean for normal subjects) of both serum methylmalonic acid and homocysteine. Another 9% had a marked elevation of methylmalonic acid alone, and 8% had a marked elevation of total homocysteine alone. Only 6% failed to have a marked elevation of either metabolite. Based on these results, we believe that measurement of both serum methylmalonic acid and total homocysteine is often required for the optimal diagnosis of Cbl deficiency.

Serum levels of methylmalonic acid and total homocysteine are particularly useful in patients with neuropsychiatric abnormalities due to Cbl deficiency who lack anemia or macrocytosis, since all such patients evaluated in a recent study had a marked elevation of at least one metabolite [13].

About 90% of patients with clinically confirmed folate deficiency have markedly elevated levels of serum total homocysteine [23]. Our initial studies indicated that about 20% of folate-deficient patients also had increases in serum methylmalonic acid, but re-evaluation of these patients indicates that most of these elevations were due to renal insufficiency or intravascular volume contraction. Less than 2% of folate-deficient patients with normal renal function and volume status have elevated levels of serum methylmalonic acid, and these elevations are modest (<500 nmoles per liter). These results indicate that measurement of both serum methylmalonic acid and total homocysteine can differentiate between Cbl and folate deficiency in most cases.

Serum methylmalonic acid and total homocysteine levels also provide useful information when they are obtained both before and after therapy with Cbl or folate [22,23]. As shown in Figure 5, serum methylmalonic acid and total homocysteine values fell toward normal within several days of treatment with Cbl in a patient with pernicious anemia. Levels of serum total homocysteine fell in a similar manner (Fig. 6) after folate treatment in a patient with folate deficiency.

The availability of assays for serum methylmalonic acid and total homocysteine has made it possible to perform a number of additional investigations in patients with Cbl and folate deficiency. These studies include the following: 1) The response of hematologic findings and serum levels of methylmalonic acid and total homocysteine in patients with Cbl or folate deficiency who were treated with the incorrect vitamin; 2) estimation of the prevalence of patients with normal Cbl levels and elevated methylmalonic acid and total homocysteine levels who had subsequent objective responses to Cbl therapy; 3) estimation of the relative sensitivities of serum Cbl, methylmalonic acid, and total homocysteine levels in previously diagnosed Cbl-deficient patients who have been without parenteral Cbl therapy for intervals of 2 to 66 months; and 4) the influence of the gut flora on levels

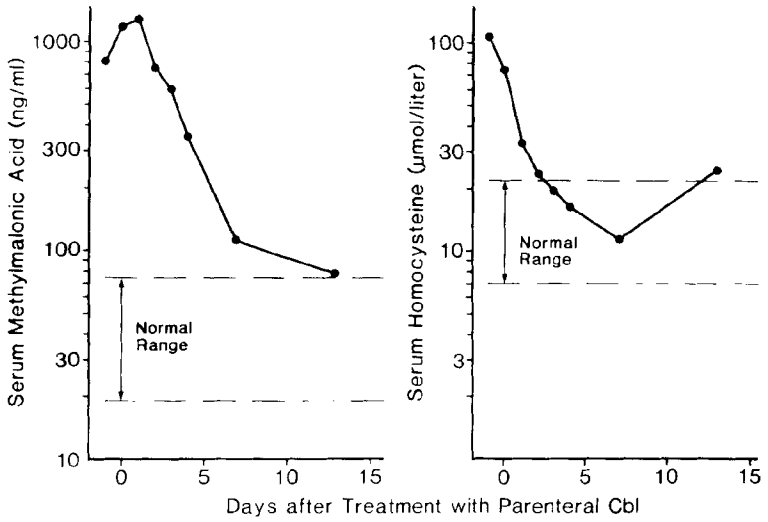

Fig. 5. Levels of serum methylmalonic acid and total homocysteine in a patient with Cbl deficiency due to pernicious anemia before and after treatment with parenteral Cbl. Dashed lines represent 2 S.D. above and below the mean for normal subjects [adapted from references 22 and 23 with permission].

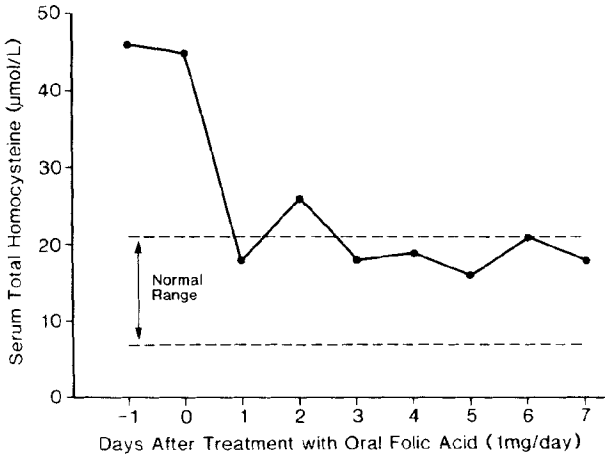

Fig. 6. Levels of serum total homocysteine in a patient with folate deficiency due to alcoholism before and after treatment with oral folic acid. Dashed lines represent 2 S.D. above and below the mean for normal subjects [adapted from reference 23 with permission].

of methylmalonic acid and total homocysteine, which was evaluated by studying Cbl-deficient patients treated with antibiotics. The remainder of this report contains the results of the first set of studies. The latter three are contained in the accompanying paper in this series [26].

MATERIALS AND METHODS

Serum Cbl and folate levels were determined by radioassay using purified intrinsic factor and purified folate binding protein (Quantiphase, BioRad Laboratories, Richmond, CA). Methylmalonic acid and total homocysteine concentrations were measured by modifications of recently developed techniques [21,22,27] using capillary-gas chromatography and mass spectrometry.[1] The major modifications, which will be described in detail elsewhere, consist of the following: 1) the HPLC step in the methylmalonic acid assay was replaced by a step that involved the adsorption and elution from a disposable silica column or a single anion-exchange column; 2) homocysteine was reduced with dithiothreitol, treated with iodoacetamide, and further purified using a single cation exchange column; and 3) the rate of temperature increase during gas chromatography was increased from 8°C per minute to 30°C per minute for both assays.

The within-run precision (CV) was approximately 2%, and the between-run precision was approximately 6% for both modified assays. Revised normal ranges were obtained with the modified assays using 50 normal human blood donors, 25 males and 25 females, who ranged in age from 18 to 65 years. Normal ranges were calculated as the mean ± 2 S.D. after log normalization to correct for the skewness of data toward higher values. The revised normal range for serum methylmalonic acid was 73–271 nmoles/liter and for serum total homocysteine was 5.4–16.2 μmoles/liter. The corresponding ranges for the mean ± 3 S.D. were 53–376 nmoles/liter and 4.1–21.3 μmoles/liter.

[1]Aspects of these assays are the subject of patent applications filed on behalf of the University of Colorado and Columbia University.

DOCKET ALARM

Explore Litigation Insights

Docket Alarm provides insights to develop a more informed litigation strategy and the peace of mind of knowing you're on top of things.

Real-Time Litigation Alerts

Keep your litigation team up-to-date with **real-time alerts** and advanced team management tools built for the enterprise, all while greatly reducing PACER spend.

Our comprehensive service means we can handle Federal, State, and Administrative courts across the country.

Advanced Docket Research

With over 230 million records, Docket Alarm's cloud-native docket research platform finds what other services can't. Coverage includes Federal, State, plus PTAB, TTAB, ITC and NLRB decisions, all in one place.

Identify arguments that have been successful in the past with full text, pinpoint searching. Link to case law cited within any court document via Fastcase.

Analytics At Your Fingertips

Learn what happened the last time a particular judge, opposing counsel or company faced cases similar to yours.

Advanced out-of-the-box PTAB and TTAB analytics are always at your fingertips.

API

Docket Alarm offers a powerful API (application programming interface) to developers that want to integrate case filings into their apps.

LAW FIRMS
Build custom dashboards for your attorneys and clients with live data direct from the court.

Automate many repetitive legal tasks like conflict checks, document management, and marketing.

FINANCIAL INSTITUTIONS
Litigation and bankruptcy checks for companies and debtors.

E-DISCOVERY AND LEGAL VENDORS
Sync your system to PACER to automate legal marketing.