AN EVALUATION OF A DUAL-ISOTOPE METHOD FOR THE MEASUREMENT OF VITAMIN B_{12} ABSORPTION

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The diagnosis of pernicious anemia (PA) by radioisotope tracer techniques depends upon the demonstration of malabsorption of radioactive vitamin B_{12} which is improved by intrinsic factor (IF). The present study was undertaken to evaluate further the dual-isotope method for the diagnosis of PA. Cobalt-58-B_{12} was given in the free form and ^{57}Co-B_{12} bound to normal human gastric juice (NHGJ). Patients with PA showed significant difference between urinary excretion of the two isotopes; controls did not. PA patients receiving the dual-isotope test had significantly higher urinary excretion of B_{12} unaccompanied by IF and lower excretion of B_{12} accompanied by IF than those who received the IF on a different date, probably due to some exchange of the two isotopic forms of B_{12} on IF. PA patients who were retested with ^{57}Co-B_{12} with NHGJ 2 hr after the ^{58}Co-B_{12} showed increase in the difference between the urine excretions of ^{57}Co and ^{58}Co. Plasma level measurement resulted in an overlap of 40% of the PA group with the control group.

The absorption of physiological amounts of vitamin B_{12} in man is an active process mediated by intrinsic factor (IF) occurring only in the terminal ileum (*1,2*). Before 1950 only indirect methods were available to measure the adequacy of vitamin B_{12} absorption in a person suspected of having pernicious anemia (PA): the reticulocyte response assay system of Minot and Castle (*3*) and in vivo testing of the patient's gastric juice for IF activity. In 1950 Chaiet, Rosenblum, and Woodbury (*4*) introduced radioactive cobalt-labeled vitamin B_{12} (^{60}Co-B_{12}) and made possible the first direct testing of B_{12} absorption using radioactive tracer techniques. Since that time several methods for testing B_{12} absorption using radioactive B_{12} have been introduced:

1. Heinle, et al (*5*) determined fecal radioactivity after oral administration of ^{60}Co-B_{12}. This is a true quantitative measure of B_{12} absorption but requires 7–10 days of complete stool collection or the use of purging cathartics. Incomplete fecal collections cause false normal results.
2. The urinary excretion test of Schilling (*6*) has been the most widely used measurement of B_{12} absorption in this country. Reliable urine collections and normal renal and urinary tract function are necessary for accurate results. The large quantities of B_{12} given parenterally as "flushing doses" to saturate the plasma transport proteins may result in nonspecific hematopoietic responses in folate-deficient subjects and prevent an accurate therapeutic trial after injection of more physiological doses of the vitamin.
3. Glass (*7*) described a hepatic uptake test. This is accurate for patients whose urine or feces are difficult to collect and does not require a large parenteral "flushing dose". Observation of the patient 7 days after the oral dose or the use of purging cathartics to remove interfering intestinal radioactivity is necessary, and coexisting liver disease may affect the uptake (*8*).
4. Reizenstein (*9*) employed whole-body scintillation counters in the study of B_{12} absorption. Although reliable and sensitive, this technique requires a 7-day wait or the use of cathartics, and the necessary equipment is not generally available to the clinician.
5. A plasma counting absorption test was de-

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scribed by Booth and Mollin (*10*) in 1956 and has gained a degree of acceptance and use because of its ease and quickness (*11–27*). Plasma counting requires only a minimum of patient cooperation, is complete in less than 1 day, and requires no therapeutic dose of B_{12}. It is independent of renal status, urine or fecal loss, and intestinal radioactivity. The level of radioactivity in the plasma is low and difficult to count with reliability. McIntyre and Wagner (*19*) pointed out in 1966 that significant overlap exists between normal and abnormal populations, and this conclusion has been supported by the work of other investigators (*17,20,26,27*).

A difficulty inherent in any of these tests is the fact that a definitive diagnosis of Addisonian pernicious anemia requires not only that malabsorption of B_{12} be established but also that significant improvement be shown during the simultaneous oral administration of IF. In the Schilling test, the most reliable of the commonly used tests, this involves a wait of at least 3 days between the two consecutive steps of the test: the first without IF and second with IF. This requires a minimum of 1 week for completion of the radioisotopic studies. In the usual hospital or outpatient situation, this often takes longer, and the second part is not infrequently abandoned altogether. Several attempts have been made to use a double-isotope technique to combine the two steps of testing vitamin B_{12} absorption into one (*28–34*). Reizenstein (*9*) administered free ^{60}Co-B_{12} simultaneously with IF-bound $^{58}CoB_{12}$ and used differential whole-body counting. A dual-isotope urinary excretion test was reported by Katz, et al (*32*) in 1963 using free $^{57}CoB_{12}$ administered simultaneously with $^{60}CoB_{12}$ bound to normal human gastric juice (NHGJ) and then differentially counting the isotopes in the urine. Bell, et al (*33,34*) reduced the dose of radioactivity administered to the patient by substituting $^{58}CoB_{12}$ for the $^{60}CoB_{12}$.

Use of a dual-isotope technique involves simultaneous oral administration of two forms of vitamin B_{12}, each labeled with a different cobalt isotope: vitamin B_{12} in free form and vitamin B_{12} prebound to NHGJ (or other intrinsic factor preparation). Subsequent differential counting of the two isotopes in the urine or plasma is possible. The technique provides the immediate availability of a ratio between the bound and the free form of the labeled vitamin and therefore an index of the improvement of vitamin B_{12} absorption brought about by the addition of intrinsic factor. The present study was undertaken to test the accuracy of the dual-isotope Schilling test and to evaluate the usefulness of expressing the ratio of bound-to-free vitamin B_{12} in increasing the accuracy of the plasma counting test.

MATERIALS AND METHODS

One hundred sixty-one patients referred from the hospital wards or clinics to the diagnostic radioisotope laboratory for vitamin B_{12} absorption studies and 37 patients with pernicious anemia were tested after informed consent with the dual-isotope single-step absorption test. All patients were fasted overnight. Four capsules were administered orally in the early morning. Two of these contained a total of 0.5 μg of ^{58}Co-labeled cyanocobalamin with a total nominal activity at activity date of 1.6 μCi. The other two capsules contained a total of 0.5 μg of ^{57}Co-cyanocobalamin prebound to NHGJ with a total nominal activity at activity date of 1.0 μCi. Two 8-ml vials of standard solution, ^{58}Co and ^{57}Co, respectively, were provided by the maker*, each vial with concentration such that each milliliter of solution contained 2% of the activity of the respective oral capsule. No food was allowed until 2 hr later when an intramuscular injection of 1,000 μg unlabeled B_{12} was administered and two consecutive 24-hr urine collections were begun. Urine volumes and specific gravities were noted. Eight hours after administration of the oral dose, 22–25-ml blood samples were drawn in heparinized syringes. The plasma was separated by centrifugation and 10-ml was transferred to a tube for counting. All samples were counted in an automatic gamma-counting scintillation system with a 3-in. NaI(Tl) crystal. After the initial series of patients had been tested, a small number were repeated with a change in procedure such that only the two capsules containing ^{57}Co-B_{12} were given initially, the two capsules containing ^{58}Co-B_{12} being given 2 hr later simultaneously with the administration of the intramuscular unlabeled B_{12}.

RESULTS

The patients were separated into the following groups: (A) Thirty-seven patients with pernicious anemia with a clinical picture consistent with the diagnosis supported by the presence of the following abnormalities: a macrocytic peripheral blood picture, a megaloblastic bone marrow, histalog-fast achlorhydria, a satisfactory response to vitamin B_{12} therapy, and vitamin B_{12} malabsorption corrected by intrinsic factor; (B) Seventy-four patients without pernicious anemia used as controls and having none of the abnormalities listed for the pernicious anemia

* The radioactive vitamin B_{12} capsules and standards were generously supplied by the Radiochemical Center, Amersham.

TABLE 1. FREE AND NHGJ-BOUND VITAMIN B_{12} IN 24-HR URINES AND 8-HR PLASMA SAMPLES

Subjects	% of dose excreted in 24-hr urine: Free B_{12}	% of dose excreted in 24-hr urine: Bound B_{12} or B_{12} with IF	Ratio $^{57}Co/^{58}Co$
Nonpernicious anemia	Range 9.0–40.0	10.0–26.0	0.65–1.45
Dual-isotope (74)	Mean ± s.d. (17.7 ± 6.7)	(17.6 ± 6.1)	——
Normal controls	Range 10.2–39.2	——	
Conventional test (26)	Mean ± s.d. (21.5 ± 8.0)		
Pernicious anemia	Range 0.75–9.0	3.5–23.5	1.75–6.30
Dual-isotope (37)	Mean ± s.d. (3.7 ± 1.7)	(9.9 ± 3.7)	——
Pernicious anemia	Range 0.02–6.0	3.0–30.0	
Conventional test (55)	Mean ± s.d. (1.4 ± 1.4)	(12.2 ± 7.0)	
	8-hr plasma levels of free and NHGJ-bound B_{12}		
	% dose/liter plasma		
Nonpernicious anemia	Range 0.36–3.52	0.44–4.12	0.67–2.26
Dual-isotope (74)	Mean ± s.d. (1.19 ± 0.65)	(1.28 ± 0.73)	——
Normal controls	Range ——	——	
Conventional test (26)	Mean ± s.d.		
Pernicious anemia	Range 0.00–0.70	0.25–2.72	1.30–12.5
Dual-isotope (37)	Mean ± s.d. (0.35 ± 0.29)	(0.93 ± 0.65)	——
Pernicious anemia	Range 0.00–1.90	0.50–2.40	
Conventional test (55)	Mean ± s.d. (0.24 ± 0.30)	(1.35 ± 0.52)	

group or having them adequately explained by proven alternate etiologies such as folate deficiency; (C) Twenty-five patients with total or subtotal gastrectomy; (D) Fifteen patients with previous intestinal surgery involving the terminal ileum; (E) Nineteen patients with at least one other test indicating malabsorption other than B_{12} absorption, but who had had no abdominal surgery; (F) Fifteen patients with suspected urine loss during collection; and (G) Nine patients with renal insufficiency or other urinary tract abnormality resulting in delayed B_{12} excretion. The range of results for PA and non-PA groups are in Table 1.

Urine test: nonpernicious anemia group. Seventy-four patients without pernicious anemia did not show statistical difference between their mean excretion of free and NHGJ-bound B_{12} in 24 hr (Fig. 1). The mean B_{12} excretion in this group, however, was significantly lower ($p < 0.001$) than that of a group of normal control subjects tested with a conventional Schilling test dose of free B_{12} in an earlier study.

Urine test: pernicious anemia group. Thirty-seven patients with pernicious anemia all had free ^{58}Co-B_{12} excretions less than 9% in the first 24 hr. All showed significantly greater excretion of NHGJ-bound ^{57}Co-B_{12} but ten did not attain normal levels (9% or greater) even of the NHGJ-bound vitamin. All had ratios in the first 24 hr urine of NHGJ-bound-to-free B_{12} greater than 1.5 whereas the non-PA subjects had lower ratios of bound-to-free (Fig. 2). While the line drawn at 9% in Fig. 1 separates the free vitamin B_{12} excretions of the pernicious anemia and nonpernicious anemia groups, extreme values approach one another very closely. Similarly, the line drawn at 1.5 in Fig. 2 separates the urinary excretion isotope ratios of these two groups, but extreme values approach one another very closely. This is not what is seen in the conventional single-step Schilling test. A comparison of the present dual-isotope test results with those in large number of single-isotope conventional Schilling tests done at this hospital in cases of pernicious anemia is shown in Fig. 3.

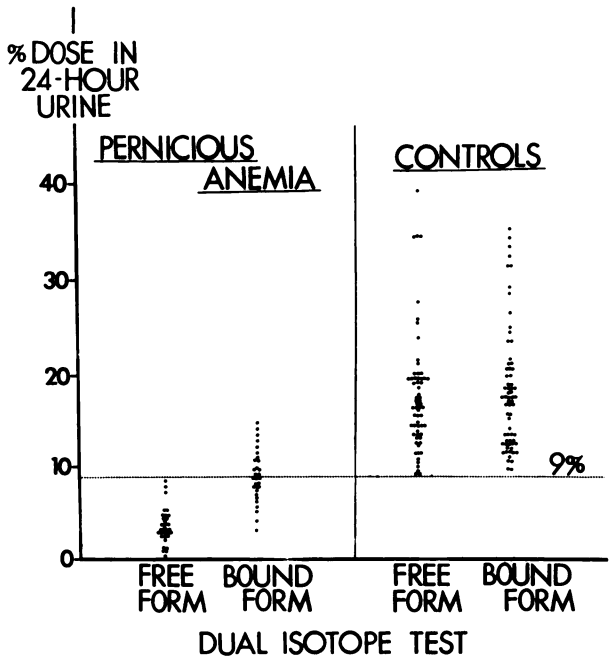

FIG. 1. Dual-isotope urinary excretion results for pernicious anemia and nonpernicious anemia groups.

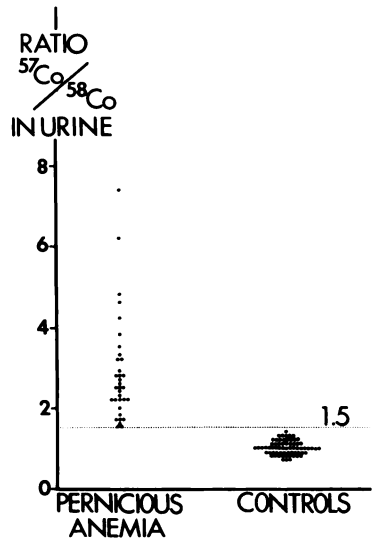

FIG. 2. Urinary bound-to-free excretion ratios in 37 patients with pernicious anemia.

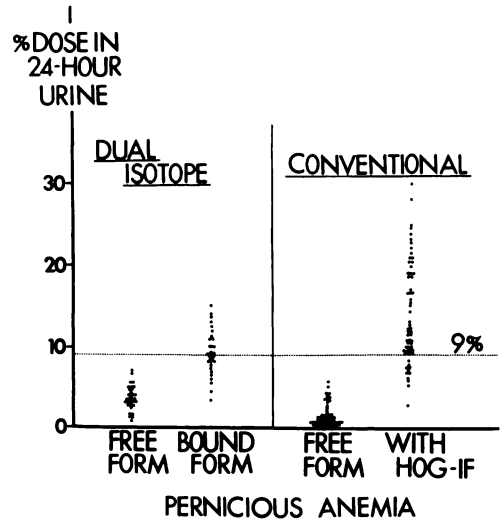

FIG. 3. Comparison of urinary excretion results in pernicious anemia between dual-isotope and conventional tests.

Mean free B_{12} excretion in the dual-isotope test is higher ($p < 0.01$) and significantly lower ($p < 0.001$) excretion with intrinsic factor in the dual-isotope test than in the conventional Schilling test (Fig. 4). One patient with pernicious anemia who had been repeatedly resistant to orally administered heterologous hog intrinsic factor showed a significant response to the homologous normal human gastric juice used in this study (Fig. 4, Patient 11). Following the initial dual-isotope test, five patients with pernicious anemia had the test repeated with only the free ^{58}Co-B_{12}. The excretion of ^{58}Co dropped from a mean of 5% when the administered free B_{12} was accompanied by the NHGJ-bound vitamin to a mean of 2.3%. Each of the five individuals had a decrease in ^{58}Co ranging from 6% to 1.5%. When the original series of patients was complete, tests on five patients with pernicious anemia were repeated with the bound form of the vitamin being given 2 hr after the free form. In four of the five cases, the effect of this 2-hr separation of doses was to increase the difference between the excretions of free and bound forms of the vitamin, i.e., to increase the bound-to-free ratio (Fig. 5).

Twelve patients with malabsorption of vitamin B_{12} secondary to intestinal disease had urinary excretions in the first 24 hr of urine collection which were less than 1% of the administered dose. In two of these cases calculation of the bound-to-free excretion ratio resulted in sufficiently high results (>2.0) to suggest intrinsic factor dependent malabsorption if the ratios alone had been considered.

As was expected, determination of the bound-to-free excretion ratio was useless in discriminating between urine loss and malabsorption secondary to

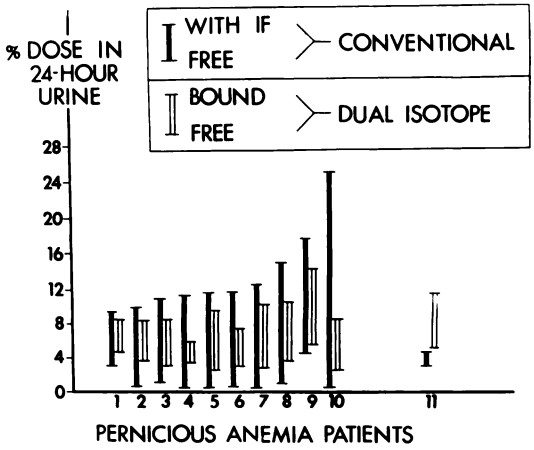

FIG. 4. Consecutive conventional and dual-isotope urinary excretion results for 11 patients with pernicious anemia.

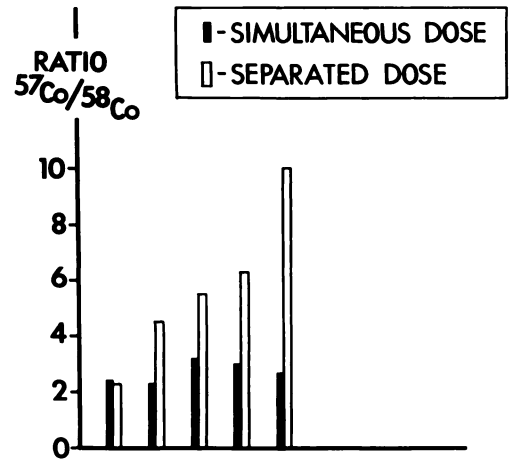

FIG. 5. Consecutive dual-isotope tests for vitamin B_{12} absorption in five patients with pernicious anemia; in first test both nuclides were given simultaneously; in second ^{57}Co-B_{12} bound to gastric juice was given 2 hr after free ^{58}Co-B_{12}.

intestinal disease. In the present study employing two consecutive 24-hr urine collections with monitoring of urine volume and specific gravity, discrepancies in urine volume between the 2 days led to suspicion of urine loss in 15 cases. Nine of these patients were retested and had normal vitamin B_{12} excretion.

Normal individuals usually have excretion of less than 1% of the oral dose in their second 24-hr urine. Eight patients had greater than 2% excretion in their second 24-hr urine collection secondary to renal disease or other urinary tract abnormality. If only the first 24-hr excretion values had been available, these patients would have been considered to have vitamin B_{12} malabsorption.

Plasma test. Figure 6 shows that the free-form vitamin B_{12} plasma levels for the pernicious anemia and nonpernicious anemia groups show significant overlap; 40% of the pernicious anemia group extend into the normal range. The bound-to-free ratios for these two groups show similar overlap (Fig. 7).

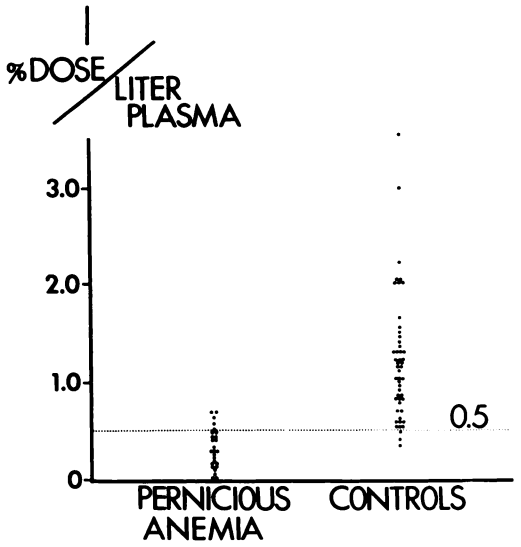

FIG. 6. Dual-isotope test: 8-hr plasma level determinations.

DISCUSSION

The similarity between the excretions of free and NHGJ-bound radioactive B_{12} found in the non-PA group of patients is not unexpected since these people are not dependent on exogenous IF. The fact that their results are lower than those of normal controls can perhaps be explained by the fact that the patients in the current study were a hospital population and a higher risk group for minor degrees of renal impairment as well as general debility. Unpublished experience of one of the authors (McIntyre) with successive Schilling tests on a normal subject before, during, and after a severe bout of pneumonia indicated that a moderate, temporary decrease of the Schilling test results occurred during the acute illness.

The narrowing of the difference between excretion of free and NHGJ-bound B_{12} which was seen in the PA group (Fig. 4) in conjunction with the absolute increase in baseline values for excretion of free B_{12} when accompanied by NHGJ-bound vitamin indicates that the dual-isotope test is less sensitive in discriminating subjects with pernicious anemia from normals. Although the dual-isotope test differs from the conventional Schilling test in source of IF (NHGJ vs. hog-IF) and in quantity of IF administered (only sufficient NHGJ to bind the administered dose of ^{57}Co-B_{12}), the most probable explanation for the observed results lies in the fact that simultaneous administration of the two forms of labeled B_{12} allows exchange between the two forms to occur before absorption can take place. Although no appreciable dissociation of B_{12}-purified hog-IF complexes was found to occur in vitro at 37°C (*35*), significant exchange has been found to occur between free and NHGJ-bound B_{12} when incubated in vitro at 37°C (*36*). Twenty percent of each form was found to have exchanged in 2 hr under these conditions and 50% (signifying complete exchange) in 24 hr.

Although absorption of B_{12} in physiological quantities is a slow process during which the vitamin remains at the surface of or within the intestine for several hours (*37,38*), it was thought that some of the exchange between free and NHGJ-bound forms of vitamin might be avoided by administering the two isotopes 2 hr apart, considering normal gastric emptying times. A 2-hr difference in administration times of the two forms should not significantly alter the total recovered in the first 24-hr urine collection because of the shape of the excretion curve (*39*). The results in the small group of PA patients on whom this was tried, while not conclusive, suggest that this 2-hr separation may be a significant improvement.

Species differences definitely exist in IF (*40*). Scandinavian workers have found that hog-IF became ineffective in some patients with PA treated with oral B_{12} and hog-IF while human gastric juice was still effective in promoting intestinal B_{12} absorption (*41,42*). Abels, et al (*38*) found a patient in whom a hog-IF preparation became ineffective after only three doses. Toporek, et al (*43*) found that increased amounts of hog-IF did not increase absorption to the same extent as did increased amounts of NHGJ.

In the cases of intestinal disease with B_{12} malabsorption, which was sufficiently severe to produce 24-hr urinary excretions of less than 1% of the administered dose, the radioactivity was being counted at a ratio of gross counting rate-to-background rate

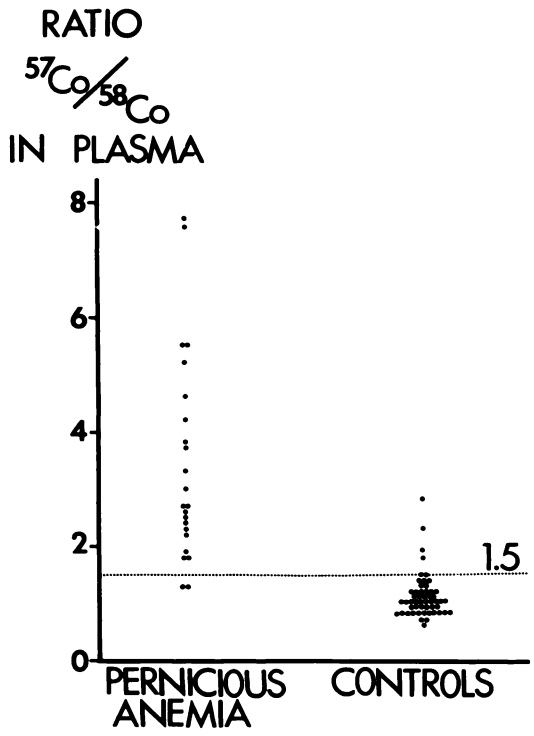

FIG. 7. Eight-hour plasma level bound-to-free ratios.

of less than 1.1 to 1 resulting in relative standard deviations of greater than 50%. This does not affect the value of the result when only the absolute level is being considered since a 50% error would still leave them comfortably within the abnormal range, but the calculation of the NHGJ-bound-to-free B_{12} excretion ratio becomes meaningless under these circumstances.

The plasma counting absorption test is again seen to have significant problems. The previously reported lack of separation between normal and abnormal populations is made worse by the dual-isotope simultaneous administration test and is present even when the NHGJ-bound-to-free B_{12} excretion ratio is considered rather than the absolute level of either. Separated doses would probably not be helpful in this situation since a 2-hr difference in administration of the two forms of the vitamin would affect a one-time determination of a rapidly changing plasma level much more significantly than it would the cumulative 24-hr excretion test.

The recent report by Cottrall, et al (*44*) that in a series of all patients (both PA and non-PA) who received two consecutive plasma-counting B_{12} absorption tests, first with and then without a flushing parenteral dose of nonlabeled B_{12}, the plasma levels were twice as high when the flushing dose was given seems to suggest that the plasma test might be improved simply by excluding the flushing dose of B_{12}. This is not, however, borne out by other investigators. Armstrong and Woodliff (*26*) did plasma counting on 73 PA patients who received flushing doses and 23 who did not and found no significant differences between the means. McIntyre and Wagner (*19*) tested plasma levels in 29 PA patients who received flushing doses and 26 who did not and found the means not to be significantly different (<0.85).

The dual-isotope simultaneous-administration Schilling test is significantly more convenient to perform than the two steps of the conventional single-isotope separate-day dose Schilling test. When used as a urinary excretion test for malabsorption of vitamin B_{12}, it differentiates normal from abnormal and succeeds in aiding diagnosis when it is intelligently considered in relation to other clinical and laboratory data available. The dual-isotope technique is not as sensitive as the conventional single-isotope two-step method. The results of the small number of patients who received 2-hr separated doses of the two isotopes suggest that this modification of the procedure aimed at reducing exchange between the free and NHGJ-bound forms of B_{12} may be a significant improvement. The use of NHGJ avoids the problems of variable potency of hog-IF preparations and avoids mistakes in diagnosis which might be made in cases of PA which are not responsive to the hog preparation. Two 24-hr urine collections are useful in determining whether a low value in the first 24 hr is due to urine loss or renal insufficiency while the ratio of bound-to-free vitamin B_{12} cannot distinguish between urine loss and intestinal malabsorption.

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