"Saturation" Studies with Vitamin B_{12} in Human Subjects

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DETERMINATION of the amount of vitamin B_{12} excreted in the urine after parenteral administration of a single test dose of this substance has not proved to be of value in distinguishing between normal subjects and patients with pernicious anemia or related megaloblastic anemias. After repeated doses of vitamin B_{12}, given either parenterally or orally, the percentage of the amount administered that is excreted in the urine increases, as do serum concentrations of both free and bound vitamin, suggesting that saturation of body stores may occur.[1-5]

It seemed possible that in the course of saturation of tissue stores of vitamin B_{12} by repeated parenteral injections of this substance, measurement of urinary excretion and of serum levels of free and bound vitamin might indicate abnormalities of metabolism in vitamin B_{12} deficiency states. In addition, the length of time required to attain maximum serum concentrations and maximum urinary excretion of the vitamin might reflect the degree of depletion of tissue stores. The maximum binding capacity of serum proteins for vitamin B_{12} might be estimated *in vivo* by provision of an excess of free vitamin after tissue stores had been saturated. The relationship of free and bound vitamin B_{12} in the serum to urinary excretion of this substance could be studied at various stages of the saturation procedure.

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MATERIALS AND METHODS

Six normal subjects, five patients with pernicious anemia in relapse, five patients with other types of megaloblastic anemia responsive to vitamin B_{12} therapy, and five patients with diabetes mellitus were selected for this study. The patients with diabetes were included because of reports in the literature[6] which suggested abnormalities of vitamin B_{12} requirement or utilization in this condition. All subjects were hospitalized in a metabolism ward throughout the period of observation. The patients with diabetes were maintained in a satisfactory state of control by administration of suitable diets and insulin. All other subjects were given standard hospital diets.

After one to three days of control observations, each subject was given 50 μg of crystalline vitamin B_{12} intramuscularly daily for ten days. This amount was selected in order to facilitate comparison of data with those previously obtained using this dosage. Consecutive 24-hour urine samples were collected during this period for measurement of total vitamin B_{12} content. Blood samples were obtained prior to the initial injection, while subjects were in the post-absorptive state, and at intervals of 1, 4, 8, and 24 hours thereafter. Additional blood samples were obtained at intervals of one to four days.

At the conclusion of the initial ten-day period, the dose of vitamin B_{12} was increased to 1,000 μg daily, intramuscularly, for a second period of ten days. No urine collections were made during this time, but blood samples were collected as in the initial period.

No vitamin B_{12} was administered on the 21st, 22nd, or 23rd days of the experimental period to permit urinary concentrations of the vitamin to return to their pre-injection levels. On the 24th day, blood was obtained for determination of vitamin B_{12} concentrations and a single injection of 50 μg of the vitamin was given intramuscularly. Urine was collected during the subsequent 24-hour period, and additional blood samples were taken at 1, 4, 8, and 24 hours after administration of the test dose. One patient with non-Addisonian megaloblastic anemia received 3,000 μg of crystalline vitamin B_{12} orally in lieu of the final intramuscular injection.

Serum was separated promptly and if not analyzed for total and bound vitamin B_{12} immediately was kept frozen at 4°C until analysis could be carried out. Aliquots of the twenty-four hour urine samples were adjusted to pH 6.8 and kept frozen until analyzed.

Microbiologic assay of vitamin B_{12} activity in urine, employing *Lactobacillus leichmannii* (*ATCC 4797*), was carried out according to the method of Thompson,[7] as modified by Register and Sarett.[8] Total vitamin B_{12} activity in serum was measured by the method of Rosenthal and Sarett,[9] and bound vitamin B_{12} activity by the method of Miller.[10] Free vitamin B_{12} activity was calculated as the difference between total and bound vitamin B_{12} activity.

RESULTS

All patients with anemia responded satisfactorily to the administration of vitamin B_{12} as determined by increases in reticulocytes[11] and in erythrocyte counts.[12]

The patterns of urinary excretion of vitamin B_{12} in normal subjects and in patients with pernicious anemia during the first ten days of the "saturation" regimen and in response to the final intramuscular test dose on the 24th day are shown in Fig. 1. Average urinary excretion of the vitamin following the first injection of 50μg was essentially the same for the two groups, approximating 12 to 15 per cent of the dose administered. Wide variation in excretion within each group is apparent from the range of values found, although it was less pronounced in the patients with pernicious anemia than in the normal subjects.

In both groups, the amount of vitamin B_{12} excreted in the urine increased progressively for the first three days of the regime. By the fourth day, urinary excretion of the vitamin

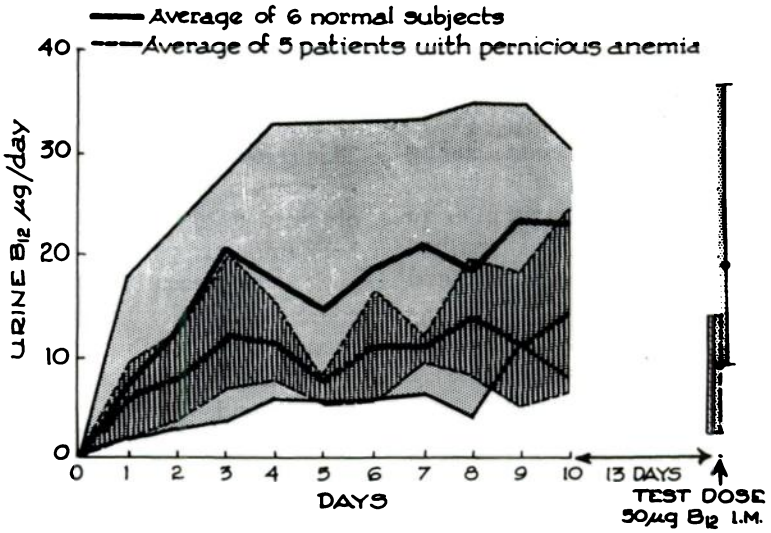

Fig. 1. Urinary excretion of vitamin B_{12} by normal subjects and by patients with pernicious anemia. Range of individual values for normal subjects indicated by stippled area, for pernicious anemia patients by cross-hatched area.

reached average values of approximately 40 per cent of the dose administered in the normal subjects and 20 per cent in the patients with pernicious anemia. From the fourth to the tenth days the average amount of the vitamin excreted in the urine showed no further systematic increase in either group. Wide variations in consecutive daily values were observed in individuals of both groups. Although average urinary excretion of vitamin B_{12} after the third day was definitely lower in the patients with pernicious anemia than average excretion in the normal subjects, values below the normal range were observed on only two occasions.

Following the final test dose of 50 μg on the 24th day of the regimen, average urinary excretion of vitamin B_{12} was essentially the same as that found for each group after the third day of the injections. The amount of vitamin B_{12} found in the urine of one patient with pernicious anemia was well below the range of values found in normal subjects.

The pattern of urinary excretion of vitamin B_{12} during the saturation period of five patients with diabetes mellitus did not differ significantly from that of the normal subjects (Fig. 2). The average amount of the vitamin excreted by this group after the final 50 μg test dose was approximately 50 per cent greater than that observed in the normal subjects, although all individual values fell within the normal range.

Urinary excretion studies were completed in four of the five patients with megaloblastic anemia other than pernicious anemia. In two of these, the excretion of vitamin B_{12} during the first ten days of the saturation period approximated average values of normal subjects. In the third patient, values resembled those of the pernicious anemia group, while those of the fourth subject fell midway between the average values for the normal subjects and the pernicious anemia patients. In the three subjects who received an intramuscular test dose of 50 μg of vitamin B_{12} on the 24th day, the amount of the vitamin excreted in the subsequent 24-hours was not significantly different from the average amount excreted by these individuals during the third to the tenth day of the regimen. The patient who received the oral test dose of 3,000 μg of the vitamin excreted less than 0.4 μg of vitamin B_{12} in the next 24 hours.

A significant increase in the concentration of both free and bound vitamin B_{12} in serum was observed in all subjects during the saturation period. Increases in bound vitamin B_{12} were most rapid and pronounced in the normal subjects and in the patients with diabetes (Fig. 3). The average maximum concentration of bound vitamin B_{12} in patients with megaloblastic anemia was significantly lower than that of normal or of diabetic subjects. Patients H. C. and A. B., however, attained levels of bound vitamin B_{12} comparable to those observed in the normal group.

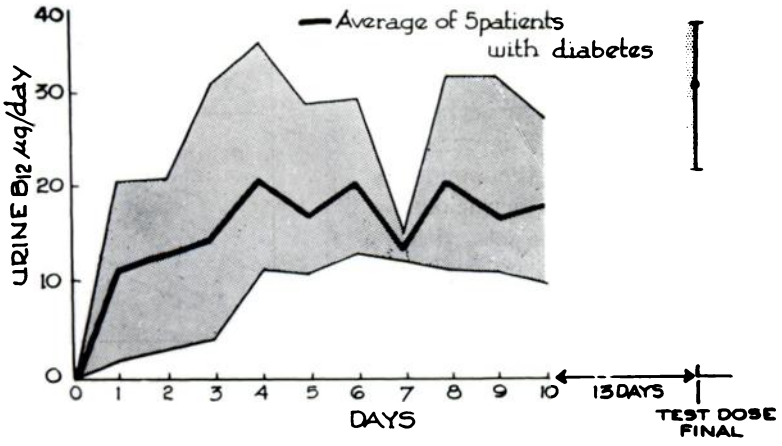

Fig. 2. Urinary excretion of vitamin B_{12} by patients with diabetes mellitus. Range of individual values indicated by stippled area.

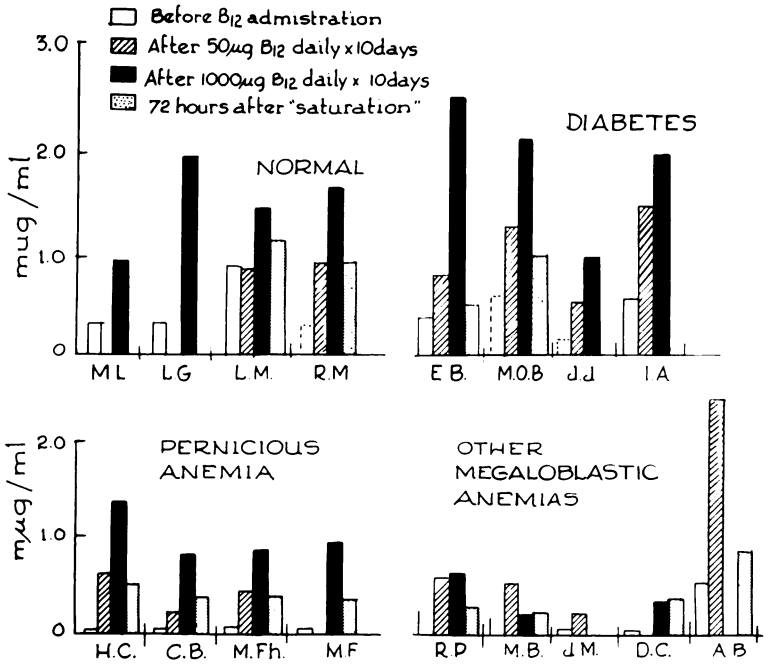

Fig. 3. Changes in concentration of bound vitamin B_{12} in serum during "saturation" regimen.

Total vitamin B_{12} concentrations in the serum of patients with megaloblastic anemia were lower at the end of the saturation period than those of the normal or the diabetic subjects. Since initial concentrations of total and bound vitamin B_{12} in the serum of the anemia patients were lower than those of the other groups, it was necessary to determine whether the amount of vitamin B_{12} bound to serum protein was dependent upon the total amount of the vitamin present in the serum. The relationship between the concentration of bound and total vitamin B_{12} in all samples of serum of normal subjects and patients with pernicious anemia is illustrated in Figure 4. Less vitamin B_{12} was present in the bound form in the serum of patients with pernicious anemia than in that of normal subjects with equivalent total concentrations of the vitamin. The difference in regression coefficients of the two groups is significant at the 1 per cent level. Much more variability in the relationship between total and bound vitamin B_{12} concentrations is apparent in the pernicious anemia patients than in the normal subjects. When data obtained from the patients with non-Addisonian megaloblastic anemia are analyzed in like fashion, a regression line similar to that of the pernicious anemia group is

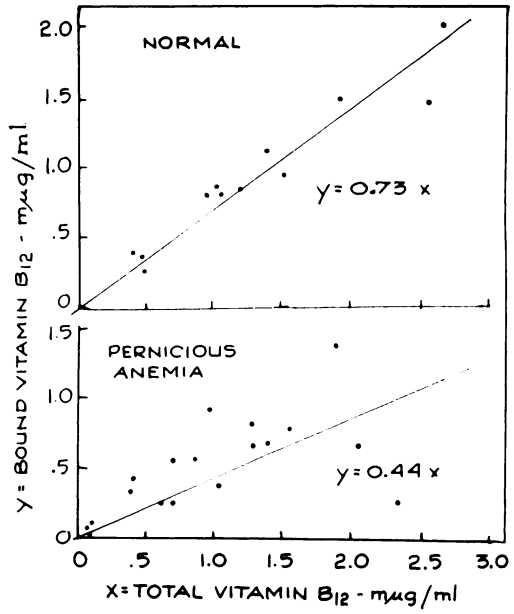

Fig. 4. Relationship between total and bound vitamin B_{12} in the serum of normal subjects and of patients with pernicious anemia during "saturation" regimen.

obtained ($y = 0.51x$). This also differs significantly at the 1 per cent level from that of the normal subjects.

No correlation could be demonstrated between the amount of free, bound, or total vitamin B_{12} present in the serum during the 24-hour periods following administration of either the initial or the final test dose of the vitamin and the amount of this substance excreted in the urine during the same periods. This was determined by plotting concentrations of each of these forms of the vitamin at 1, 4, 8, and 24 hours following the injections, connecting the points by straight lines, and measuring planimetrically the areas so defined. The values thus derived were then plotted against the amount of vitamin B_{12} excreted in the urine during the same period. In the case of M. B., who received 3,000 μg of vitamin B_{12} orally as the final test dose, only 0.38 μg of the vitamin was found in the subsequent twenty-four hour urine sample, despite an increase of free and bound vitamin B_{12} in the serum which exceeded that of some patients who excreted from 2.3 to 14.7 μg following the intramuscular test dose.

DISCUSSION

The marked variability of urinary excretion of vitamin B_{12} among the groups of subjects studied, as well as in individual subjects from day to day, during a constant regimen of vitamin B_{12} injections was noteworthy. These findings would appear to preclude the use of urinary excretion after single or repeated injections of vitamin B_{12} as a measure of body stores of this substance. Although average values for urinary excretion of vitamin B_{12} in patients with megaloblastic anemia were lower than those of the normal and diabetic subjects individual values remained in the low normal range in most instances.

Such results might be due to continued storage of the vitamin. If this is the case, our observations indicate that administration of 10,500 μg of vitamin B_{12} in a period of 23 days is not sufficient to replenish body stores of this substance. If it is assumed that 50 per cent of the vitamin is retained during the ten days of injections of 50 μg daily, and 10 per cent is retained during the period of injections of 1,000 μg daily, this regimen would furnish 1,275 μg of vitamin B_{12} for replenishment of body stores. This is considerably less than the amount normally present in the tissues of nonanemic subjects, as estimated from analyses of Girdwood.[13]

Other possible explanations for the low average urinary excretion of vitamin B_{12} by patients with pernicious anemia during this regimen are (1) excretion of the vitamin by extra-renal routes, (2) excretion in the urine in a form not detectable by the microbiologic assay used and (3) increased destruction of vitamin B_{12} in the body. Studies with vitamin B_{12} labeled with radioactive Co^{60}, to be published elsewhere, do not support the first two of these possibilities and indicate that if increased destruction of the vitamin does occur, the cobalt of the molecule is retained in the body.[14]

An interesting finding of this study is the difference between concentrations of bound vitamin B_{12} attained in the serum of normal subjects during the regimen and those attained in serum of patients with megaloblastic anemia. The failure of serum of the anemia group to bind the vitamin to as great an extent as did that of the normal or of the diabetic subjects might be due to deficiency of the factor in serum which combines with the vitamin. Studies in our laboratory in which this serum factor was estimated by an *in vitro* technic[15] lend support to this concept. Another possible explanation, which has been suggested previously by other investigators,[16,17] is that intrinsic factor may possess activity beyond that of enhancing absorption of vitamin B_{12} from the gastrointestinal tract or that there may be an "extragastric intrinsic factor." Miller[15,18] has found that intrinsic factor promotes the combining of vitamin B_{12} to certain serum proteins and increases the uptake of vitamin B_{12} by tissues *in vitro*. Intrinsic factor may have similar functions *in vivo*. This explanation would appear to be more applicable to findings in patients with pernicious anemia than in those with non-Addisonian megaloblastic anemia. However, we have reported studies[19] made with an *in vitro* technic that suggest that some patients with non-Addisonian megaloblastic

anemia, as diagnosed by the Schilling test,[20] may have varying degrees of deficiency of intrinsic factor in their gastric secretions.

Mollin and Ross[2] reported that urinary excretion of vitamin B_{12} after parenteral administration of this substance is proportional to the concentration of the free vitamin in the serum. Our studies have shown no relationship between the concentration of free vitamin B_{12} in serum and urinary excretion of the vitamin, either in normal subjects or in those with megaloblastic anemia or diabetes. This discrepancy in findings presumably is related to methodology. Mollin and Ross[21] measured free vitamin B_{12} directly after heating serum to 56° C for 30 minutes and total vitamin B_{12} after heating to 100° for 15 minutes. *Euglena gracilis, varo bacillaris* being used as the test organism. Bound vitamin B_{12} was determined by difference. In the technic used by us, bound vitamin B_{12} is determined directly after adsorption of the free vitamin on charcoal, free vitamin B_{12} being determined by the difference between total and bound B_{12}. *L. leichmannii* is used as the test organism. The difference in findings with the two technics could be explained also by postulating the presence of a third form of vitamin B_{12} in serum which is loosely bound to protein, is adsorbable on charcoal and is not excreted by the kidneys.

SUMMARY AND CONCLUSIONS

Normal subjects and patients with megaloblastic anemia or diabetes mellitus were studied during a "saturation" regimen of parenterally administered vitamin B_{12}. Urinary excretion and concentrations of free and bound vitamin B_{12} in serum were determined. Average values for urinary excretion of the vitamin in patients with megaloblastic anemia were lower than those of normal or diabetic subjects, although individual values were within the normal range in most instances. It seems likely that the dosage of vitamin B_{12} employed during this regimen, although large, was not sufficient to produce saturation of body stores in the severely depleted subject. The marked variability in urinary excretion of the vitamin during a constant regimen appears to preclude the use of urinary excretion tests after single or repeated injections of vitamin B_{12} as a measure of body stores. No correlation could be demonstrated between the amount of free vitamin B_{12} in the serum during a 24 hour period following a test dose of this substance and the amount of the vitamin excreted in the urine during the same period. The discrepancy between these results and those reported by other investigators is presumably related to differences in methodology.

Levels of bound vitamin B_{12} attained in the serum of patients with megaloblastic anemia during the "saturation" regimen were lower than those observed in the serum of normal or diabetic subjects. In serum of normal subjects, 73 per cent of the total vitamin B_{12} was present in the bound form, while only 44 to 51 per cent was bound in the serum of patients with megaloblastic anemia. These observations may indicate deficiency of the protein factor in serum which combines with vitamin B_{12} or deficiency of some other substance, such as intrinsic factor, which promotes the combining of the vitamin with serum protein in patients with megaloblastic anemia.

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