Vitamin B₁₂ absorption — a study of intraluminal events in control subjects and patients with tropical sprue¹

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SUMMARY The intraluminal fate of orally administered radioactive vitamin B₁₂ has been studied in control subjects with normal vitamin B₁₂ absorption and those with vitamin B₁₂ malabsorption due to tropical sprue. In control subjects 1 to 21% of the dose was bound to sedimentable material and 37 to 75% was bound to immunoreactive intrinsic factor. In subjects with vitamin B₁₂ malabsorption due to tropical sprue, the results were identical with the control subjects. Bacteriological studies showed a statistically significant correlation between both the number of flora in the jejunum and the number of bacteroides in both the jejunum and ileum and vitamin B₁₂ malabsorption. In patients with tropical sprue who have normal intrinsic factor secretion, the vitamin B₁₂ absorptive defect is not due to binding of the vitamin to bacteria or to alteration to the intrinsic factor vitamin B₁₂ complex in the intestinal lumen. The lesion appears to be one of the mucosal cell receptors or of the cells themselves, possibly caused by bacterial toxins.

Surprisingly little is known of the fate of the intrinsic factor-vitamin B₁₂ complex during its transit down the small intestine to its site of absorption in the ileum. What proportion of an oral physiological dose of vitamin B₁₂ is present in the lumen of the small intestine as free vitamin, how much of it is bound to intrinsic factor, and how much to other binders? Such knowledge is necessary before possible abnormalities in intraluminal factors can be studied in vitamin B₁₂ malabsorptive states. The purpose of the present study was to investigate the fate of orally administered vitamin B₁₂ in its transit down the small intestine; firstly, in subjects with normal vitamin B₁₂ absorption and, secondly, in patients with vitamin B₁₂ malabsorption due to tropical sprue.

Methods

SUBJECTS STUDIED

Eight patients with tropical sprue who had malabsorption of vitamin B₁₂ not corrected by administration of exogenous intrinsic factor and six control

subjects with normal absorption of vitamin B₁₂ were studied. Previous informed consent was obtained from all subjects.

All subjects were hospitalized in a metabolic ward. They received a diet containing 50 g of fat per day and the faecal fat excretion was measured daily. Xylose absorption tests were done using a 5 g dose of d-xylose and measuring the urinary excretion of xylose at five hours. Tests of vitamin B₁₂ absorption were done by administering a dose of 1 µg vitamin Co⁵⁷ labelled vitamin B₁₂³ (0.5 µc) and then measuring plasma radioactivity at eight hours (Mathan *et al.*, 1973). In those cases where the absorption of the vitamin was low, the tests were repeated, giving the labelled vitamin together with hog intrinsic factor. An augmented histamine test, jejunal biopsy, and barium meal examination were done on all subjects.

INTUBATION

After an overnight fast the subjects were intubated with a sterile radiopaque polyvinyl tube (internal diameter, 2 mm) weighted with a mercury bag. The passage of the tube was followed by fluoroscopy using an x-ray machine fitted with an image intensifier and television chain. When the tube was in the first loop of the jejunum, about 10 cm to 20 cm beyond the ligament of Treitz, it was fastened to the cheek to inhibit further transit and the subjects were

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given an oral dose of ⁵⁷Co labelled vitamin B₁₂ (1 µg, 0.5 µc). Jejunal aspirates were then collected on ice and 1 ml aliquots periodically checked in a Packard well type scintillation counter for radioactivity. All specimens obtained before the detection of radioactivity were discarded. Aspiration was continued until aliquots showed a fall in the radioactivity to less than about 25% of the maximum radioactivity detected. Some of the aspirate was collected in a sterile container and transferred immediately to the microbiology laboratory for analysis. In the first two subjects studied, no tests were carried out at the jejunal level.

The tube was then freed and the subjects continued to swallow it. A diet consisting of fluids and flavoured rice gruel was given during the transit of the tube.

When the tube was in the upper ileum (220 to 250 cm from the incisor teeth) another dose of ⁵⁷Co labelled vitamin B₁₂ was given by mouth and aspirates collected as described above.

As soon as the collection of the aspirates from a given site was completed, all those containing radioactivity were pooled, the pH was measured on a Beckman Zeromatic pH meter, and processing continued immediately as described below.

SEDIMENTABLE RADIOACTIVITY

The pooled aspirate was centrifuged in a Sorvall RC 2-B centrifuge at 12,100 g, at 4°C for one hour. The sediment was washed thrice with 0.15 M saline, recentrifuging each time for 15 minutes under similar conditions. The washed sediment was suspended in 3 ml saline and the radioactivity determined.

The radioactivity in a 3 ml aliquot of the initial supernatant and in 3 ml aliquots of the washings was also measured and the results added to give the total non-sedimentable radioactivity in the initial sample. This was then expressed as a percentage of the total radioactivity in the pooled aspirate and is referred to hereafter as radioactivity in the supernatant.

A drop from the supernatant and washings were taken on slides and gram stains made to monitor the effectiveness of the centrifugation to sediment the bacteria. No bacteria were seen in any of the washings, but occasionally in the original supernatant a very few scattered bacteria were seen.

COATED CHARCOAL ASSAY

An aliquot of the initial supernatant was treated with haemoglobin coated Norit A charcoal (Gottlieb *et al.*, 1965) and the radioactivity adsorbed to the charcoal and that remaining in the supernatant was determined.

INTRINSIC FACTOR BOUND RADIOACTIVITY

The percentage of radioactivity present in the initial supernatant as intrinsic factor-vitamin B₁₂ complex was determined by the method of Jacob and O'Brien (1972) using zirconium phosphate gel at pH 6.25. Binding type intrinsic factor antibody was obtained from a patient with pernicious anaemia. Appropriate dilutions of the supernatants of intestinal aspirates were used to work in the range of binding antibody excess. The radioactivity bound to the zirconium phosphate gel when antibody serum was added, minus any radioactivity bound to the gel when control serum was added, gave the amount of ⁵⁷Co vitamin B₁₂-intrinsic factor complex present in the sample. This assay was carried out on all the subjects in the control group and in six of the eight in the group with vitamin B₁₂ malabsorption.

RADIOACTIVITY BOUND TO NON-INTRINSIC FACTOR HIGH MOLECULAR WEIGHT BINDERS

The amount of intrinsic-factor-bound radioactivity subtracted from the amount of radioactivity in the supernatant after treatment with charcoal, represented the amount bound to other high molecular weight binders—that is, those not adsorbed by charcoal.

FREE RADIOACTIVE VITAMIN B₁₂

With each intrinsic factor assay, additional tubes were set up, in which normal human gastric juice of known intrinsic factor content, was added before the addition of the binding antibody. In the event of any free ⁵⁷Co vitamin B₁₂ being present, this would bind to the excess intrinsic factor. The amount of intrinsic factor-vitamin B₁₂ complex measured by the assay would then be increased by the amount of free radioactive vitamin B₁₂ present.

MICROBIOLOGICAL STUDIES

The microbiological methods used were those detailed previously (Bhat *et al.*, 1972). Aspirates were cultured for aerobic, anaerobic, and microaerophilic organisms. Surface viable counts were made by the spreading method (Cruickshank, 1965) and the results expressed as the number of viable organisms per millilitre of intestinal juice.

ANTIBIOTIC THERAPY

In three of the eight patients with tropical sprue who had vitamin B₁₂ malabsorption, after the initial study, oral tetracycline (250 mg six hourly) was given for two to three weeks. Vitamin B₁₂ absorption tests were carried out several times after the initiation of therapy, and an intubation for microbiological studies repeated after the vitamin B₁₂ absorption had returned to normal.

Results

METABOLIC STUDIES

In the eight patients with tropical sprue and vitamin B₁₂ malabsorption the serum content of Co⁵⁷ after the test dose of radioactive vitamin B₁₂ ranged from 0 to 0.17% of the dose per litre of plasma. All of the patients had steatorrhoea, d-xylose malabsorption, and typical jejunal biopsy and barium meal findings (Baker and Mathan, 1971). The test was repeated with concomitant administration of hog gastric mucosal extract and in none of them did it return to normal. The results of the augmented histamine test were in the normal range in all subjects (maximal acid output 4.6 to 20.3 mmol/h).

In the six control subjects with normal vitamin B₁₂ absorption the serum content of Co⁵⁷ after the test dose of radioactive vitamin B₁₂, ranged from 0.24 to 2.1% of the dose per litre of plasma. Four of the controls had intestinal structure and function within the normal range, one had a mild degree of steatorrhoea of undetermined aetiology and could be classified as having tropical enteropathy (Baker and Mathan, 1972) and one had mild tropical sprue with steatorrhoea, xylose malabsorption, and jejunal biopsy and radiographic changes, but consistently normal vitamin B₁₂ absorption.

PH OF INTESTINAL ASPIRATES

The pH of the jejunal and ileal aspirates in the control group and in patients with tropical sprue with vitamin B₁₂ malabsorption was similar (Table 1).

DISTRIBUTION OF RADIOACTIVITY IN ASPIRATES

The results of analysis of the distribution of radioactivity in jejunal and ileal aspirates are summarized in Table 1 and the Figure.

SEDIMENTABLE RADIOACTIVITY

In one control subject 21% of the radioactivity in the ileal aspirate was sedimentable. In all other specimens, less than 10% of the radioactivity was

Fig Radioactivity of different fractions expressed as a percent of the radioactivity of intestinal aspirates from the jejunum (solid symbols) and ileum (open symbols). C: controls (circles); S: subjects with tropical sprue and vitamin B₁₂ malabsorption (squares).

sedimentable, and results in controls and patients were the same.

COATED CHARCOAL ASSAY

The results of the coated charcoal assay were similar in all subjects. The amount of radioactivity in the supernatants of the jejunal aspirates ranged from 81 to 92% and in the ileal aspirates from 64 to 92%. The wider range in the ileal aspirates was due to the one control subjects who had a high percentage of sedimentable radioactivity and therefore a lower percentage in the charcoal supernatant.

INTRINSIC FACTOR BOUND RADIOACTIVITY

In normal subjects 37 to 75% of radioactivity was

Site	Subjects	Intestinal pH (range)	Initial centrifugation		Coated charcoal assay		Z gel assay		
			Sediment (% in aspirate)	Supernatant (% in aspirate)	Charcoal Adsorbed (% in aspirate)	Supernatant (% in aspirate)	Immunoreactive IF (% in aspirate)	Free B ₁₂	High mol. wt. binders (% in aspirate)
Jejunum	Control	4.7-7.6	0.7 (0.9)	99.3 (0.9)	12 (3.4)	88 (3.4)	63 (11.2)	0	24 (11.1)
	Sprue	5.9-7.2	1.6 (1.0)	98.4 (1.0)	14 (3.6)	85 (2.7)	64 (12.3)	0	21 (14.8)
Ileum	Control	6.2-7.7	4.7 (8.1)	95.3 (8.1)	13 (3.2)	81 (9.3)	55 (11.6)	0	26 (5.3)
	Sprue	6.7-7.4	3.0 (1.7)	97.0 (1.7)	13 (4.1)	83 (4.6)	62 (12.1)	0	20 (12.1)

Table 1 Intestinal pH and mean and standard deviation (in parentheses) of radioactivity, expressed as percentage of initial intestinal aspirate, of various fractions, in jejunum and ileum of control group and patients with tropical sprue who had vitamin B₁₂ malabsorption (sprue)

present as immunoreactive intrinsic factor-vitamin B₁₂ complex. A similar range was found in those with vitamin B₁₂ malabsorption due to tropical sprue (46 to 79%).

RADIOACTIVITY BOUND TO NON-INTRINSIC FACTOR HIGH MOLECULAR WEIGHT BINDERS
Eleven to 43% of radioactivity was bound to non-intrinsic factor-high molecular weight binders in the control subjects and 1 to 40% in those with vitamin B₁₂ malabsorption due to tropical sprue.

The patient with tropical sprue who had 40% of the radioactivity in the jejunal aspirate bound to non-intrinsic factor high molecular weight binders was studied further. When another dose of radioactive vitamin B₁₂ was given and the distribution of radioactivity in the gastric juice studied immediately, 40% of the radioactivity was again found attached to non-intrinsic factor high molecular weight binders in spite of the presence of a large amount of free intrinsic factor. This indicates that in this individual the findings in the jejunum were determined by events in the stomach.

FREE RADIOACTIVE VITAMIN B₁₂

No free radioactive vitamin B₁₂ was detected in any specimen from any subject.

MICROBIOLOGICAL STUDIES

The results of bacteriological studies are outlined in Table 2. In four of the six control subjects the aspirates from the upper jejunum did not grow any organisms and in the remaining two, bacteroides were not isolated. In the group with vitamin B₁₂ malabsorption all of the jejunal aspirates obtained had viable organisms and bacteroides were isolated from five of the eight subjects, though in only two of them did the viable bacteroides count exceed 10⁴ organisms per ml. The difference between the two groups with regard to bacteroides, total aerobic flora, and total flora were statistically significant.

All of the ileal aspirates from both controls and subjects with vitamin B₁₂ malabsorption had viable

organisms. The only significant difference between the two groups was in the number of bacteroides which were isolated in only two out of the six control subjects in low numbers (10 and 10³ organisms/ml), whereas eight of the nine patients with vitamin B₁₂ malabsorption had these organisms and in all eight the counts were greater than 10⁴/ml.

EFFECTS OF ANTIBIOTIC THERAPY

The effects of antibiotic therapy given to three patients with tropical sprue and vitamin B₁₂ malabsorption are shown in Table 3. In all three vitamin B₁₂ absorption returned to normal within 11 days and concomitantly there was a decline in the total ileal flora.

	Case 1		Case 2		Case 3	
	Before	After	Before	After	Before	After
Vitamin B ₁₂ absorption %	0.03	0.48	0.08	0.62	0.08	1.6
dose/l plasma						
Total ileal flora log count/ml	6.3	4.0	8.8	2.6	6.7	Ni
Bacteroides log count/ml	5.3	3.8	4.7	Nil	4.9	Nil

Table 3 Results of tests on three subjects before and after therapy with tetracycline

Discussion

CONTROL SUBJECTS

This study has confirmed the utility of the intrinsic factor-vitamin B₁₂ complex assay of Jacob and O'Brien (1972) in the investigation of intraluminal events connected with vitamin B₁₂ absorption in the small intestine, and has extended the observations they made in two normal subjects.

After the oral administration of radioactive vitamin B₁₂ most of the radioactivity in the intestinal lumen was in the initial supernatant and very little was bound to bacteria even in the ileum. This confirms the earlier centrifugation studies of Schjónsby *et al.* (1973).

	Enterobacteria	Enterococci	Other streptococci	Total aerobes	Lactobacilli	Bifidobacteria	Fusobacteria	Bacteroides	Veillonella	Total anaerobes	Total flora
Jejunum											
Controls	0.9 (1.8)	1.1 (1.6)	0.9 (1.7)	1.3 (2.1)*	0.4 (1.1)	0	0.5 (1.2)	0	0.3 (0.8)	0.5 (1.2)	1.3 (2.1)*
Sprue	3.2 (2.7)	0.7 (2.0)	3.3 (2.4)	4.6 (1.8)	1.6 (2.2)	1.8 (2.0)	2.1 (2.2)	2.7 (2.4)†	2.3 (2.7)	3.0 (2.7)	4.8 (1.7)‡
Ileum											
Controls	5.3 (3.1)	2.6 (2.9)	2.9 (2.5)	6.5 (1.3)	2.8 (3.2)	1.7 (2.7)	1.1 (1.7)	0.9 (1.6)‡	4.1 (1.8)	4.6 (1.8)	6.8 (0.9)
Sprue	5.7 (3.2)	3.6 (2.7)	3.3 (3.6)	7.0 (1.8)	3.8 (2.6)	2.8 (2.6)	0.4 (1.1)	4.3 (2.0)†	4.7 (2.3)	5.5 (1.4)	7.1 (1.8)

Table 2 Major groups of organisms isolated from jejunal and ileal aspirates expressed as mean log count and standard deviation of viable organisms per ml of intestinal aspirate (*P < 0.05; † P < 0.01; ‡ P < 0.005)

In both the jejunum and the ileum there was a proportion of the luminal radioactivity (7 to 22%) which was adsorbed by haemoglobin coated charcoal but which was not free vitamin B₁₂ since it did not bind to intrinsic factor. Presumably, this was either some breakdown product of vitamin B₁₂ or vitamin B₁₂ bound to some small molecular weight material which could be adsorbed by the charcoal.

Although only 37 to 75% of the original activity was present in the lumen bound to immunoreactive intrinsic factor, even the lowest amount was clearly adequate to provide vitamin B₁₂ absorption within the normal range. The remainder of the radioactivity present in the supernatant after treatment with coated charcoal (11 to 43% of the original) represented radioactivity bound to non-intrinsic factor high molecular weight binders which would presumably have been unabsorbed.

SPRUE PATIENTS WITH VITAMIN B₁₂ MALABSORPTION

Vitamin B₁₂ malabsorption in patients with tropical sprue may be due to a variety of factors (Baker, 1972). In a few, there is absence of intrinsic factor (Baker and Mathan, 1971), in others, such as those reported here, intrinsic factor secretion is normal and the defect must therefore be in the intestine.

In the present study the intraluminal fate of the orally administered vitamin B₁₂ in the patients with tropical sprue was found to be identical with that in the control subjects. The vitamin B₁₂ malabsorption in tropical sprue cannot therefore be attributed either to binding by intraluminal bacteria, or to degradation of the intrinsic factor-vitamin B₁₂ complex as it passes down the intestine. Further, since the intrinsic factor-vitamin B₁₂ complex reaches the ileum in adequate amounts, the lesion responsible for the vitamin B₁₂ malabsorption must be either at the ileal vitamin B₁₂ receptors or in the ileal mucosal cells themselves.

In spite of the finding that bacteria do not take up the vitamin B₁₂, or alter the intrinsic factor vitamin B₁₂ complex, there is circumstantial evidence that, at least in some patients with tropical sprue, the vitamin B₁₂ absorptive defect may be related in some way to bacteria. Thus, in a previous study of 38 patients who had vitamin B₁₂ malabsorption, antibiotic therapy resulted in a fairly rapid return of vitamin B₁₂ absorption to normal in 50% of the patients (Baker and Mathan, 1971). In the present investigation, in the three subjects who were treated with tetracycline, the vitamin B₁₂ absorption returned to normal and there was a marked decrease in total luminal flora. Furthermore, there was some correlation between the bacteriological findings in both jejunum and ileum and vitamin B₁₂ malabsorption. In the jejunal

aspirates of patients with vitamin B₁₂ malabsorption the mean log counts of aerobic flora, total flora, and bacteroides were significantly greater than the counts in the controls, and in the ileal aspirates, all except one of the vitamin B₁₂ malabsorbers had bacteroides present in counts greater than 10⁴ organisms per ml, whereas in those with normal vitamin B₁₂ absorption, only two out of eight had bacteroides and none had more than 10³ organisms per ml.

Although the numbers of organisms found in these patients are fewer than usually occur in the stagnant loop syndrome, the possibility that the bacteroides may have played a role in the pathogenesis of the vitamin B₁₂ malabsorption cannot be excluded. If this be so, then perhaps they acted by producing a toxin which damaged the ileal cells. Such a mechanism has been postulated for *E. coli* in a case of diverticulosis (Paulk and Farrar, 1964). However, the finding of a relationship between bacteria and vitamin B₁₂ malabsorption in patients with tropical sprue in this study is in contradiction to earlier work from this laboratory (Bhat *et al.*, 1973) and require confirmation.

Further studies to delineate the precise nature of the cellular lesion responsible for the vitamin B₁₂ absorptive defect in tropical sprue and the possible role of bacteria in its pathogenesis are in progress.

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