Vitamin B₁₂ Uptake by Intestinal Microorganisms:

Mechanism and Relevance to Syndromes of

Intestinal Bacterial Overgrowth

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A B S T R A C T The mechanism of bacterial uptake of vitamin B₁₂, the spectrum of microorganisms capable of such uptake, and the factors involved were the subject of this study. Bacterial uptake of vitamin B₁₂ was found to be at least a two stage process. A primary uptake phase which was rapid (1 min or less), pH dependent, nontemperature dependent, did not require viable organisms and was insensitive to either the metabolic inhibitor dinitrophenol or to the sulfhydryl inhibitor N-ethyl-maleimide. Protein denaturation (formalin treatment or autoclaving) abolished all B₁₂ uptake. This primary uptake phase is thought to represent adsorption to binding or "receptor" sites on the cell wall. Second stage uptake was slower, pH and temperature dependent, required living bacteria, and was abolished by either dinitrophenol or N-ethyl-maleimide. This phase is dependent upon metabolic processes and may reflect transfer of B₁₂ from surface "receptor" sites into the bacterial cell. Although differences among organisms were observed in total 1 hr uptake, number of surface "receptor" sites, and relative avidities for B₁₂, all organisms except *Streptococcus fecalis* shared the two stage mechanism. Two Gram-positive organisms, *Bacillus subtilis* and Group A streptococcus, demonstrated the highest 1 hr vitamin B₁₂ uptake values; Gram-negative bacteria required 2,000–10,000 the number of organisms for comparable uptake. Binding constants (K_m) varied from 5.05 ±1.67 × 10^{-10}M for *B. subtilis* to 6.18 ±3.08 × 10^{-8}M for *Klebsiella pneumoniae* which approximate the K_m for human intrinsic factor (0.38 × 10^{-10}M). Competition between bacteria and intrinsic factor for vitamin B₁₂ may be inferred from the similarity of these constants.

These observations suggest that a variety of enteric and nonenteric organisms, not requiring exogenous B₁₂, may play a role in the pathogenesis of the vitamin B₁₂ malabsorption found in the intestinal bacterial overgrowth syndromes.

INTRODUCTION

In recent years, attention has been focused upon the relationship of bacterial uptake of vitamin B₁₂ to the vitamin B₁₂ malabsorption present in patients with intestinal bacterial overgrowth (1–5). Donaldson (1) and Donaldson, Corrigan, and Natsios (2), demonstrated that bacteria isolated from surgically created blind loops in rats could take up significant amounts of vitamin B₁₂ which had been previously bound to I.F. (Intrinsic Factor). It is not clear, however, whether the entire I.F.–B₁₂ complex was taken up or whether the B₁₂ was detached from I.F. by the bacteria before or during the uptake process. While these studies have contributed to our understanding of the interrelationships between bacteria and vitamin B₁₂, the mechanism of B₁₂ uptake by bacteria, the spectrum of bacteria capable of B₁₂ uptake, and factors determining such uptake remain unclear. The present studies are concerned with these points and demonstrate that a wide variety of nonvitamin B₁₂ requiring enteric and nonenteric bacteria take up vitamin B₁₂ by a two stage process.

METHODS

Escherichia coli K₁₂ (wild type) was the prototype organism, (hereafter referred to as *E. coli*). Other organisms

used were obtained from the stock culture collection of the Department of Microbiology, Boston University School of Medicine.[1] Cyanocobalamine[2] labeled with ⁵⁷Co (specific activity 13–18 mCi/mg) was used in all experiments and was diluted with nonradioactive cyanocobalamine[2] to give the desired B_{12} concentrations.

All procedures were carried out with aseptic technique. The various solutions used were sterilized by filtration through a Millipore filter with a pore size of 0.45 μ.[3]

Growth and preparation of cells. Organisms were stored at 5°C on trypticase soy agar[4] slants until used. An 18 hr growth (in trypticase soy broth[4] with a vitamin B_{12} concentration of less than 0.005 ng/ml) of each organism was used throughout. This consistently gave cultures in early stationary growth phase as demonstrated by sequential culturing. The coefficient of variation (6) on day to day determinations was 9%. Organisms were quantitated in duplicate by the serial dilution and drop plate technique (7). Replicate plating of a single culture of various organisms yielded a coefficient of variation of 15–17%. When plate counts were compared with direct microscopic counts, the results were the same. Thus, nonviable organisms did not comprise a significant portion of the bacterial population.

Organisms were harvested by centrifugation for 30 min at 3500 rpm, the bacterial sediment washed with sterile saline, recentrifuged, and suspended in a volume of distilled water to obtain the desired number of bacteria as determined turbidimetrically. This bacterial suspension was used immediately and the number or bacteria quantitated for each experiment.

Unless otherwise specified, each tube contained a total of 4 ml (pH 6.9–7.1) consisting of 1 ml of each of the following: (*a*) bacterial suspension, (*b*) 0.6 ng ⁵⁷Co vitamin B_{12}/ml, and 2 ml of isotonic saline. In studies concerned with the effects of the inhibitors or pH, 2 ml of the appropriate aqueous solution of inhibitor of buffer was substituted for isotonic saline. Each determination was done in duplicate.

Measurement of bacterial uptake of vitamin B_{12}. A modification of the microbial adsorption inhibition method of Grasbeck (8) was used as follows: bacteria were incubated at 37°C for various periods of time with vitamin B_{12}-⁵⁷Co and were separated from the rest of the reaction mixture by centrifugation. The radioactivity of each tube was counted before organisms were added and the total radioactivity remaining in the supernatant determined by counting a 1 ml portion. Per cent vitamin B_{12} uptake was calculated by dividing the amount of radioactivity removed from the supernatant by the initial total radioactivity. In some studies the bacterial pellet was washed, digested overnight with concentrated H_2SO_4, and the radioactivity bound to the bacteria thus directly measured. Both procedures gave comparable results. A Packard gamma isotope counter (Packard Instrument Co., Downers Grove, Ill.) with a counting error of 0.5–1.0% was used. Recovery of added radioactivity ranged between 95 and 105%.

At the conclusion of each experiment the pH of the supernatant was measured with a glass electrode, the bacterial pellet was resuspended in distilled water to a volume of 4 ml and the number of surviving bacteria counted by the serial dilution and drop plate technique (7). pH never

varied more than 0.2 pH units and the number of bacteria recovered was identical with the number originally added.

Results are expressed as mean ±1 SE and were analyzed for statistical significance by the Student t test (6).

RESULTS

Accuracy of the method

The accuracy and reproducibility of this method was determined in two ways. In eight replicate samples the 1 hr vitamin B_{12} uptake by (mean ±SE) *E. coli* (1 × 10⁹ organisms) was 96.9 ±0.03%; and of 52 samples done on 26 different days 92.0 ±0.05%.

Kinetics of bacterial uptake of vitamin B_{12}; demonstration of two stage process

Bacterial uptake of vitamin B_{12} with time. The velocity of the reaction between *E. coli* and vitamin B_{12} is shown in Fig. 1. Two distinct components were noted in the uptake curve: an initial rapid stage (1 min or less) and a slower second stage. The 1 min uptake value is an approximation of the initial rate of uptake since it was not feasible to measure B_{12} uptake in less than 1 min with the methods used. Both the first stage uptake and total 1 hr uptake (which includes both first and second stage) increased with increasing numbers of *E. coli.*

Effect of vitamin B_{12} concentration on first stage uptake. When a fixed number of bacteria was incubated with increasing concentrations of vitamin B_{12}, 1 min (first stage) bacterial uptake of vitamin B_{12} demonstrated saturation kinetics. These data were analyzed by the method of Lineweaver and Burk (9) and a *Km* for first stage B_{12} uptake was calculated (Table I). For *E. coli,* the *Km* was 6.03 ±0.62 × 10⁻¹⁰M (mean ±1 SE). Additional *Km* values for various aerobic bacteria, also listed in Table I, illustrate the similarity among 10 dif-
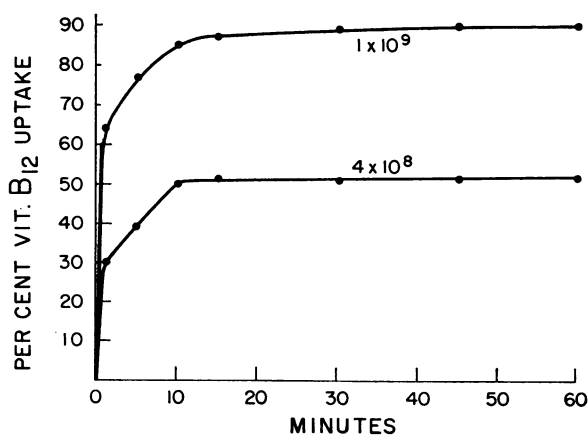

FIGURE 1 Vitamin B_{12} uptake by various numbers of *E. coli.* Typical plot representative of eight experiments. Bacteria were incubated with 0.6 ng vitamin B_{12} at 37°C and at pH 7.0 as described in text.

TABLE I

K_m (Binding Constant) Values of Aerobic Bacteria*

‡A.	Bacillus subtilis	$5.05 \pm 1.67 \times 10^{-10}$ M
	§Escherichia coli K_{12} (mutant)	$5.38 \pm 1.63 \times 10^{-10}$ M
	Escherichia coli K_{12} (wild type)	$6.03 \pm 0.62 \times 10^{-10}$ M
B.	Proteus mirabilis	$8.34 \pm 1.77 \times 10^{-10}$ M
	Escherichia coli B	$1.08 \pm 0.26 \times 10^{-9}$ M
	Salmonella paratyphi	$2.00 \pm 0.74 \times 10^{-9}$ M
C.	Pseudomonas aeruginosa	$2.00 \pm 0.48 \times 10^{-9}$ M
	Staphylococcus aureus	$3.15 \pm 0.33 \times 10^{-9}$ M
	Group A streptococcus (β-hemolytic)	$3.16 \pm 0.18 \times 10^{-9}$ M
	Klebsiella pneumoniae	$6.18 \pm 3.08 \times 10^{-9}$ M

* Values expressed as mean $\pm$SE.

‡ Bacteria within each group not significantly different from each other $P = > 0.05$. Groups A and C significantly different from each other $P = < 0.05$, but neither group significantly different from group B. $P = > 0.05$.

§ Methionine requiring mutant.

TABLE II

Saturation of First Stage Bacterial Uptake of Vitamin B_{12}*

Bacteria‡	Saturating amount ng/10^9 organisms§	Molecules vit. B_{12} bound/organism
1. Group A. streptococcus (4)	799.8 ± 117.5	355,000‖
2. B. subtilis (5)	402.4 ± 69.8	179,000
3. Kleb. pneumoniae (4)	15.0 ± 4.6	6,680
4. Ps. aeruginosa (5)	5.0 ± 0.4	2,230
5. Sal. paratyphi (6)	4.7 ± 1.1	2,090
6. P. mirabilis (4)	3.5 ± 1.2	1,550
7. E. coli K_{12} (wild type) (6)	2.9 ± 0.4	1.280
8. Staph. aureus (4)	2.6 ± 0.7	1,140
9. E. coli B (4)	2.2 ± 0.3	950
¶10. E. coli K_{12} (mutant) (6)	2.2 ± 0.5	950
11. Strep. fecalis (5)	0	0

* Group A streptococcus and B. subtilis significantly different from each other and from all others $P = < 0.05$. Kleb. pneumoniae and Ps. aeruginosa not significantly different from each other, $P = > 0.05$, but each is significantly different from organisms 7–11, $P = < 0.05$. Organisms 5–10 are not significantly different, $P = > 0.05$.

‡ Numbers in parentheses represent number of trials.

§ Expressed as mean $\pm$SE.

‖ Calculated from saturating amount

(No. moles $B_{12} \times$ Avogadro's No.)/(No. bacteria)

When data calculated by the method of Scatchard (10), values obtained were essentially the same as those listed.

¶ Methionine requiring mutant.

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ferent bacteria, with *Bacillus subtilis* having the smallest Km and *Kleb. pneumoniae* the largest. There were no significant differences in the Km values among three different strains of *E. coli*.

First stage (1 min) B_{12} uptake by *E. coli* (1×10^9 organisms) was saturated by 2.9 ± 0.4 ng of vitamin B_{12}. The quantity of B_{12} necessary for first stage saturation of a variety of organisms is listed in Table II. The last column of Table II shows the number of molecules of B_{12} necessary for first stage saturation. The wide range in the number of B_{12} molecules bound at saturation of first stage is apparent (355,000 for Group A streptococcus to 950 for *E. coli* K_{12} [mutant]). *Strep. fecalis* was the sole exception demonstrating no significant B_{12} uptake. There was no correlation between the amount of B_{12} bound at saturation and Km value.

1 hr bacterial uptake of vitamin B_{12}. As shown in Table III, B_{12} uptake by *E. coli* (1×10^9) and *Salmonella paratyphi* (1×10^9) after 1 hr was 7.0 and 19.7 ng respectively. Gram-negative enteric organisms varied between 6.6 ± 1.0 and 255 ± 35.9 ng/hour and Gram-positive organisms, between 3.2 ± 0.9 and 21,700 ± 2200 ng/hour. All organisms demonstrated saturation kinetics. *Strep. fecalis*, however, demonstrated no significant B_{12} uptake in 1 hr.

TABLE III

1 Hr Vitamin B_{12} Uptake of Aerobic Bacteria*

Bacteria‡	1 hr uptake§
	ng
1) B. subtilis (6)	21,700 $\pm 2,200$
2) Group A streptococcus (7)	1,810 ± 258
3) P. mirabilis (5)	255 ± 35.9
4) E. coli B (6)	30.6 ± 6.7
5) Sal. paratyphi (5)	19.7 ± 4.1
6) Kleb. pneumoniae (5)	17.3 ± 7.8
7) Ps. aeruginosa (4)	12.5 ± 2.9
8) E. coli K_{12} (wild type) (5)	7.0 ± 1.5
‖9) E. coli K_{12} (mutant) (12)	6.6 ± 1.0
10) Staph. aureus (5)	3.2 ± 0.9
11) Strep. fecalis (5)	0

* Number of bacteria in each case 1×10^9.

‡ Numbers in parentheses represent number of trials.

§ Values expressed as mean ± 1 SE. The only significant differences, $P = < 0.05$, as follows: B. subtilis, Group A. streptococcus, and P. mirabilis from each other and all others; E. coli B from organisms 7–11; Sal. paratyphi from organisms 9–11; Kleb. pneumoniae from Strep. fecalis; Ps. aeruginosa from Staph. aureus and Strep. fecalis; E. coli K_{12} (wild type) from Strep. fecalis; E. coli K_{12} (mutant) from Staph. aureus and Strep. fecalis; and Staph. aureus from Strep. fecalis.
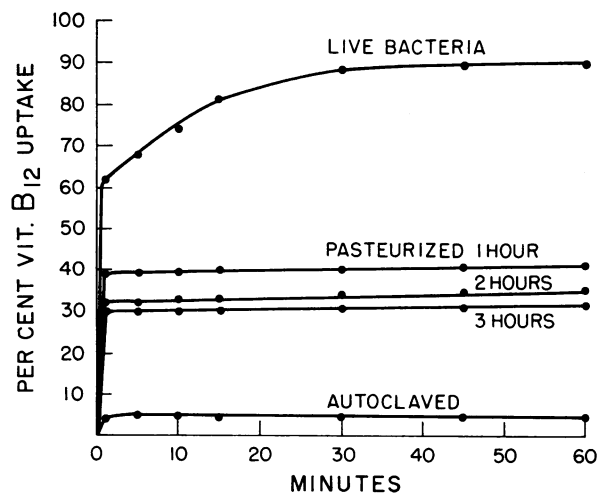
‖ Methionine requiring mutant.

FIGURE 2   Effect of bacterial viability on vitamin B_{12} uptake. *E. coli*, live and killed as indicated, were incubated with 0.6 ng vitamin B_{12} at 37°C and at pH 7.0 as described in text. Bacteria were pasteurized by incubation in a water bath at 60°C for the times indicated. Bacteria were autoclaved at 120°C for 20 min. Number of bacteria initially 1×10^9 in each instance. Plate counts of pasteurized and autoclaved bacteria revealed no surviving bacteria. Fig. 2 is typical of three experiments.

Effect of viability of bacteria on vitamin B_{12} uptake

E. coli autoclaved at 120°C for 20 min showed no vitamin B_{12} uptake (Fig. 2). Coincident with cellular disruption and protein denaturation, Gram-staining reaction was also lost. *E. coli*, pasteurized at 56°C for 1–3 hr, retained approximately one-half the control first stage vitamin B_{12} uptake but little second stage uptake. Although viability was lost, bacteria appeared intact microscopically, and retained their Gram-staining properties.

Effect of prior vitamin B_{12} deprivation or saturation on subsequent bacterial uptake of vitamin B_{12}

In these experiments, *E. coli* was grown in trypticase soy broth either with or without added nonradioactive vitamin B_{12} (20 ng/ml). After 24 hr the bacteria were counted (no difference was noted in number between those grown in the presence or absence of B_{12}), harvested by centrifugation, washed three times with 0.15 M saline and an aqueous suspension prepared as described in Methods.

The B_{12} uptake rate of 1×10^9 of each *E. coli* was then measured. As shown in Fig. 3, *E. coli* grown in media enriched with nonradioactive vitamin B_{12} lost its ability to take up radioactive vitamin B_{12}, as compared to organisms grown in media lacking vitamin B_{12}.
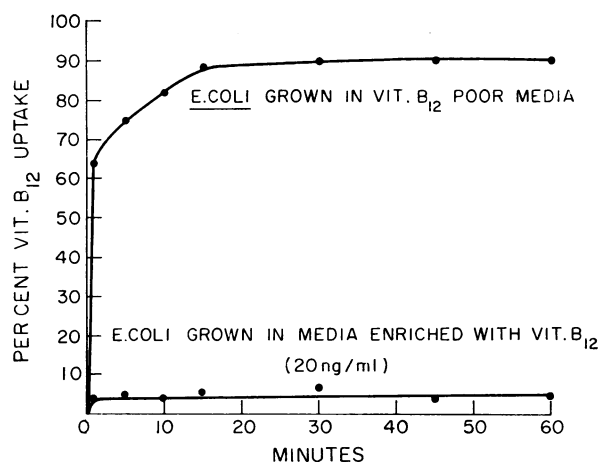

FIGURE 3   Effect of prior vitamin B_{12} exposure on vitamin B_{12} uptake by *E. coli*. Bacteria were grown in vitamin B_{12} poor media or in media enriched with nonradioactive vitamin B_{12} (20 ng/ml). Bacteria were harvested and prepared as described in text and incubated with 0.6 ng B_{12}-^{57}Co at 37°C and at pH 6.9. Number of bacteria in each instance 1×10^9. This fig. is typical of three trials.

Effect of temperature on vitamin B_{12} uptake

First stage uptake (Fig. 4) was largely independent of temperature while second stage uptake was clearly temperature dependent. At 5°C the second stage uptake rate was substantially reduced such that the total 1 hr uptake was only 13% greater than 1 min uptake. At 37°C second stage uptake was 50% greater.
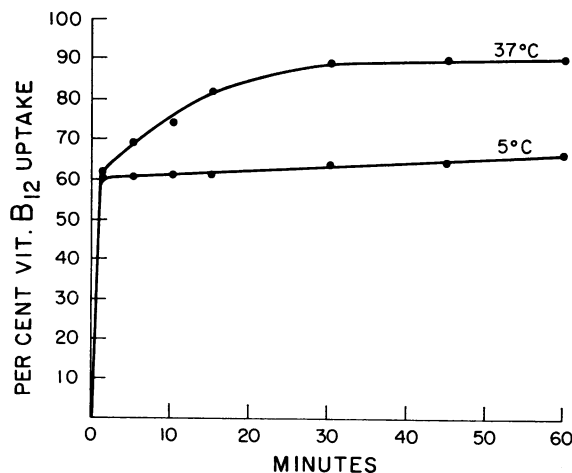

FIGURE 4   Effect of temperature on bacterial uptake of vitamin B_{12}. *E. coli* (1×10^9) incubated with 0.6 ng vitamin B_{12} at either 5°C or 37°C at pH 7.1 as described in text. Fig. 4 is typical of five trials.
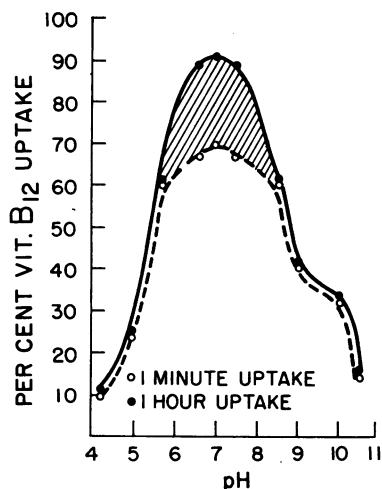
FIGURE 5 Effect of pH on vitamin B_{12} uptake by *E. coli.* Per cent vitamin B_{12} uptake at 1 min and 1 hr plotted against pH. The difference between 1 min and 1 hr (shaded area) represents second stage uptake. Bacteria (1×10^9) were incubated with 0.6 ng vitamin B_{12} at 37°C at the indicated pH as described in the text. pH 4.2, 5.0, and 5.7 maintained with 2 ml 0.1 M citrate buffer; pH 6.6, 7.0, and 7.5 with 2 ml 0.2 M phosphate buffer; and pH 8.5, 9.0, 10.0, and 10.5 with 2 ml 0.2 M bicarbonate buffer. Various buffers of same pH gave comparable values. The number of bacteria surviving at the conclusion of each experiment was unchanged from the number initially added. Fig. 5 is typical of three experiments.

TABLE IV

*Effect of Inhibitors on E. coli Vitamin B_{12} Uptake**

		1 min uptake %	60 min uptake %
Control		60	90
Formalin	0.30%	58	74
	2.5%	36	52
	10.0%	2	12
Dinitrophenol	0.025 mM	60	80
	0.20 mM	58	61
	0.50 mM	59	60
	1.0 mM	60	59
N-ethyl-maleimide	10^{-7} M	60	72
	10^{-4} M	60	71
	10^{-2} M	56	65
NaF	2.5 mM	60	90
	25.0 mM	59	91
	100.0 mM	61	89
Acetylstrophanthin	10^{-8} M	60	90
	10^{-5} M	59	89
	10^{-2} M	61	89

* Figures represent mean values of three experiments per inhibitor.

Effect of pH on bacterial uptake of vitamin B_{12}

For both first and second stage vitamin B_{12} uptake, optimum was between 6.6 and 7.5. As shown in Fig. 5, both stages of B_{12} uptake were pH sensitive, with second stage uptake being more sensitive to pH change. As the pH was varied from the optimum range first stage uptake progressively decreased, but was never completely abolished. Second stage uptake, however, was completely abolished as the pH was either lowered or elevated out of the optimum range. The number of bacteria surviving at the conclusion of each experiment was unchanged from the number initially added.

Effect of various inhibitors on bacterial uptake of vitamin B_{12}

Increasing concentrations of formalin progressively inhibited both first and second stage *E. coli* vitamin B_{12} uptake (Table IV). Dinitrophenol and *N*-ethyl-maleimide selectively inhibited only second stage B_{12} uptake, while sodium fluoride and acetylstrophanthin were without effect on either stage.

Number of bacteria necessary for vitamin B_{12} uptake

Approximately 2×10^7 *E. coli* were necessary before vitamin B_{12} uptake could be detected and 1×10^9 organisms were necessary to give 90% uptake constantly. Other Gram-negative rods, i.e. *Proteus mirabilis, Salmonella paratyphi, Pseudomonas aeruginosa,* and *Kleb. pneumoniae,* were similar to *E. coli,* but Gram-positive organisms demonstrated a greater spectrum. Group A streptococcus and *B. subtilis* showed appreciable B_{12} uptake with as few as 5×10^4 and 6×10^5 organisms respectively. *Staphylococcus aureus* was similar to the Gram-negatives while *Strep. fecalis* was unable to take up significant amounts of B_{12} even with numbers as high as 1×10^{11} organisms.

Other bacteria demonstrating vitamin B_{12} uptake

Other Gram-positive and negative bacteria tested which demonstrated B_{12} uptake included *Aerobacter aerogenes, Shigella sonnei,* and *Shigella flexneri, Salmonella typhimurium* and *Salmonella schottmuelleri, Corynebacterium diphtheriae,* three strains of Group A streptococci, and two strains each of Group C and G streptococci. All organisms studied, with the exception of *Strep. fecalis* (see above), demonstrated B_{12} uptake and had a similar two stage vitamin B_{12} uptake process.

DISCUSSION

These studies, concerned with the mechanism of vitamin B_{12} uptake by bacteria, indicate that B_{12} is taken up by a variety of microorganisms, which do not require ex-

ogenous B_{12}. This process which occurs in at least two stages, is similar to that in the vitamin B_{12} requiring parasite *Ochromonas malhamensis* (11) and various B_{12} requiring species of lactobacillus (12).

While a precise delineation of the mechanisms involved is not possible at present, certain conclusions may be drawn from the available data. Simple diffusion through cell membranes is not likely owing to the physical size and hydrophilic properties of vitamin B_{12}. That binding sites function either as the first step of a carrier system or as a step before transport into the cell appears more likely. This interpretation is compatible with the following data:

First stage uptake. First stage uptake (1 min or less) was nontemperature dependent, insensitive to metabolic inhibitors, and occurred (though to a lesser degree) even when bacteria were killed by pasteurization. Thus, viability of bacteria per se, is not a necessary prerequisite for first stage B_{12} uptake. However, since pasteurization decreased first stage uptake by 50%, it is likely that some of the surface B_{12} binding sites were denatured by pasteurization (13). With more drastic heat treatment, i.e. autoclaving, the bacterial cells were disrupted, and all B_{12} uptake and Gram-staining reaction abolished. Formalin inhibition of B_{12} uptake may also be related to protein denaturation. Formalin, which reacts with amino, imino, hydroxyl, and many other groups to form methylene bridges between proteins (14), presumably alters the surface B_{12}-binding sites.

Data in support of the concept of cell wall B_{12} binding sites have been presented by Sasaki and Kitahara (15) who fractionated *Lactobacillus leichmannii* after 30 min of radioactive vitamin B_{12} uptake and found 55% of the radioactivity in the cell wall fraction. Clues to the nature of the surface binding sites are provided by the observations that the vitamin B_{12} could not be washed off, nor could it be removed when cell walls were subjected to digestion by trypsin, RNAase, or pepsin (15). Failure to inhibit first stage uptake by the sulfhydryl inhibitor N-ethyl-maleimide indicates that —SH groups are not involved in this binding. pH, however, is of major import. At pH less than 5.7 or greater than 7.5, B_{12} uptake was progressively decreased, a finding noted also by Davis and Chow (16). This suggests the possibility of dissociation of binding sites on the bacterial cell wall, similar to that postulated for the parasite *Ochromonas* (11). Since bacterial surfaces possess a net negative charge (most likely due to dissociated acid groups [17]), sensitivity to both high and low pH is compatible with a binding phenomenon of electrochemical nature.

First stage uptake was directly proportional to the number of bacteria. Preloading with vitamin B_{12} abolished further B_{12} uptake, presumably by saturating both the surface binding sites and intracellular B_{12} storage

capacity. While this interpretation is reasonable, low radioactive B_{12} uptake values could, in part, reflect dilution of the radioactive B_{12} by cold B_{12} exiting from the bacteria (12). Nevertheless, the fact that first stage uptake exhibited saturation kinetics is evidence for the existence of binding or "receptor" sites. The wide range observed in the number of B_{12} molecules bound per organisms (950–355,000) at saturating concentrations, also point out the wide range in the number of binding sites among the organisms studied. Similar data for various B_{12} requiring organisms have been reported, i.e., *Lactobacillus leichmannii* 10,500 molecules/organism (12), *E. coli* 1,150/organism (18), and *Ochromonas malhamensis* 524,000/organism (19).

Application of Michaelis-Menton enzyme-substrate kinetic analysis to bacterial transport systems has been made with a variety of substrates (20, 21). Such analysis permits calculation of Km, which for the purpose of this study is an affinity or binding constant, for first stage B_{12} uptake. As with enzyme-substrate interactions, the smaller the Km value the greater the avidity of bacteria for vitamin B_{12}. Km values in the present studies ($6.03 \times 10^{-10} - 6.18 \times 10^{-9}$M) show great avidity of bacteria for B_{12} and are similar to the Km reported for *Lactobacillus leichmannii* (2.8×10^{-9}M) by Kashket, Kaufman, and Beck (12) and approximates the Km reported for human intrinsic factor (0.38×10^{-10}) by McGuigan (22).

It is concluded that the first stage B_{12} uptake by bacteria is an adsorptive process related to vitamin B_{12} binding or "receptor" sites on the cell wall.

Second stage uptake. Second stage bacterial uptake of vitamin B_{12} was pH and temperature dependent, required living bacteria, and was inhibited by dinitrophenol and N-ethylmaleimide. While this seems to be a metabolic process, second stage uptake was insensitive to NaF or acetylstrophanthin, suggesting that neither glycolysis nor Na-K activated membrane bound adenosine triphosphatase (ATPase) appeared to be involved. Supporting the concept that second stage B_{12} uptake may represent transport of vitamin B_{12} from the cell wall receptor sites into the cell interior, are the studies of Kashket, Tave, and Beck (23). After incubating *Lactobacillus leichmannii* and *E. coli* for 10–16 hr in Co^60 vitamin B_{12} enriched media and fractionating the bacteria they found in excess of 90% of the vitamin B_{12} in the ribosomal fraction.

Similar kinetics of vitamin B_{12} uptake by various bacterial species implies similar mechanisms of B_{12} uptake. Grasbeck (8) however, suggested that the pH differences in the B_{12} uptake between *Lactobacillus leichmannii* and *E. coli* reflected differences in the mechanism of uptake.

1 hr B_{12} uptake values for gram-negative bacteria observed in this study are somewhat higher than those reported by Donaldson et al. (2) but similar to those reported by Sherwood, Goldstein, Haurani, and Wirts

(5). It is interesting that two Gram-positive organisms, *B. subtilis* and Group A streptococcus, demonstrated 1 hr B_{12} uptake values (21,700 ± 2200 and 1810 ± 258 ng respectively) far in excess of those observed for Gram-negative bacteria (6.6 $\pm 1.0 - 255 \pm 35.9$ ng). Both *B. subtilis* and Group A streptococcus were also capable of measurable vitamin B_{12} uptake with as few as 6×10^5 and 5×10^4 organisms respectively, 2000–10,000 times fewer than the number required for the Gram-negative enteric organisms. Thus, it is apparent that organisms other than the so-called "enteric organisms" were capable of substantial vitamin B_{12} uptake. The inability of *Strep. fecalis* to take up vitamin B_{12} in 1 hr is unexplained and paradoxical in view of the fact that it shares many characteristics in common with other streptococci demonstrating B_{12} uptake. Perhaps its vitamin B_{12} uptake is merely much slower than that of the other organisms studied, or alternatively, it may not possess surface receptor sites capable of immediate vitamin B_{12} uptake. Similar behavior of *Strep. fecalis* has been observed by others (2, 3, 5, 24).

The wide range in both first and second stage B_{12} uptake values among the various organisms studied is unexplained. It does not appear to be related to either a requirement for exogenous B_{12} or to ability to synthesize B_{12}. All the organisms examined have been shown not to require exogenous B_{12} (18, 25–28). This study, as well as that of others (12, 18, 25), confirms the fact that a wide variety of bacteria, not requiring exogenous B_{12} for growth, take up as much B_{12} as quickly and by a similar mechanism, as B_{12} requiring organisms. Differing bacterial avidities and capacities for B_{12} must remain unexplained at present.

Avidities of bacteria for B_{12} comparable to that of intrinsic factor for B_{12}, implies that bacteria resident in "blind-loops" may be able to compete with intrinsic factor for ingested B_{12}. Such a conclusion is supported by Donaldson's studies of rats with blind-loops (1, 2). Furthermore, while a variety of bacteria are capable of B_{12} uptake, the differences noted in both avidity and capacity for B_{12} uptake, suggest that overgrowth with certain organisms may more frequently lead to vitamin B_{12} malabsorption. While anaerobes have been shown to be a prominent part of the flora of blind-loops (29–32) their avidity and capacity for B_{12} uptake and their role on the B_{12} malabsorption remains to be elucidated. Thus, in patients with intestinal bacterial overgrowth, a variety of organisms may compete with one another and the host mucosa for available vitamin B_{12}.

ACKNOWLEDGMENTS

REFERENCES

1. Donaldson, R. M., Jr. 1962. Malabsorption of Co^{60}-labelled cyanocobalamin in rats with intestinal diverticula. I. Evaluation of possible mechanisms. *Gastroenterology.* 43: 271.
2. Donaldson, R. M., Jr., H. Corrigan, and G. Natsios. 1962. Malabsorption of Co^{60}-labelled cyanocobalamin in rats with intestinal diverticula. II. Studies on contents of the diverticula. *Gastroenterology.* 43: 282.
3. Doig, A., and R. H. Girdwood. 1960. The absorption of folic acid and labelled cyanocobalamin in intestinal malabsorption, with observations on the faecal excretion of fat and nitrogen and the absorption of glucose and xylose. *Quart. J. Med.* 29: 333.
4. Booth, C. C., and J. Heath. 1962. The effect of *E. coli* on the absorption of vitamin B_{12}. *Gut.* 3: 70.
5. Sherwood, W. C., F. Goldstein, F. Haurani, and C. W. Wirts. 1964. Studies of the small-intestinal bacterial flora and of intestinal absorption in pernicious anemia. *Amer. J. Dig. Dis.* 9: 416.
6. Snedecor, G. W., and W. G. Cochran. 1967. Statistical Methods. Iowa State University Press, Ames, Iowa. 6th edition. 59.
7. Mallmann, W. L., and S. A. Broitman. 1956. A surface plating technique for determining bacterial population of milk. *Amer. J. Public Health Nat. Health.* 46: 1018.
8. Gräsbeck, R. 1957. The vitamin B_{12}-binding principle of human gastric juice: influence of pH on the bacterial adsorption of free and bound vitamin B_{12}. *Scand. J. Clin. Lab. Invest.* 9: 50.
9. Lineweaver, H., and D. Burk. 1934. The determination of enzyme dissociation constants. *J. Amer. Chem. Soc.* 56: 658.
10. Scatchard, G. 1949. The attractions of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* 51: 660.
11. Reeves, R. B., and F. S. Fay. 1966. Cyanocobalamin (vitamin B_{12}) uptake by *Ochromonas malhamensis*. *Amer. J. Physiol.* 210: 1273.
12. Kashket, S., J. T. Kaufman, and W. S. Beck. 1962. The metabolic functions of vitamin B_{12} III. Vitamin B_{12} binding in *Lactobacillus leichmannii* and other lactobacilli. *Biochim. Biophys. Acta.* 64: 447.
13. Reddish, G. F., editor. 1954. In Antiseptics, Disinfectants, Fungicides, and Chemical and Physical Sterilization. Lea & Febiger, Philadelphia, Pa. 751.
14. Pearse, A. G. 1960. In Histochemistry, Theoretical and Applied. Little, Brown and Co., Boston, Mass. 2nd edition. 53.
15. Sasaki, T., and K. Kitahara. 1963. A role of the cell wall as a "primary reservoir" of vitamin B_{12} in a B_{12}-requiring Lactobacillus. *Biochim. Biophys. Acta.* 74: 170.
16. Davis, R. L., and B. F. Chow. 1952. Some applications of the rapid uptake of vitamin B_{12} by resting *Lactobacillus leichmannii* organisms. *Science (Washington).* 115: 351.
17. Smith, D. T., and N. F. Conant. 1960. *In* Zinsser, Microbiology. Appleton-Century-Crofts, New York. 12th edition. 1026.

18. Oginsky, E. L. 1952. Uptake of vitamin B₁₂ by *Escherichia coli. Arch. Biochem.* **36**: 71.
19. Marchesi, S. L., and L. G. Lajtha. 1962. Vitamin B₁₂ and 14C utilization by *Ochromonas malhamensis. In* Vitamin B₁₂ and Intrinsic Factor. Second European Symposium. H. C. Heinrich, editor. Ferdinand Enke, Stuttgart. 175.
20. Cirillo, V. P. 1961. Sugar transport in microorganisms. *Annu. Rev. Microbiol.* **15**: 197.
21. Cohen, G. N., and J. Monod. 1957. Bacterial permeases. *Bacteriol. Rev.* **21**: 169.
22. McGuigan, J. E. 1967. Measurement of the affinity of human gastric intrinsic factor for cyanocobalamin. *J. Lab. Clin. Med.* **70**: 666.
23. Kashket, S., J. L. Tave, and W. S. Beck. 1960. Vitamin B₁₂ binding in bacterial ribosomes and observation of a ribosomal vitamin B₁₂ binding glycoprotein. *Biochem. Biophys. Res. Commun.* **3**: 435.
24. Dellipiani, A. W., and R. H. Girdwood. 1964. Bacterial changes in the small intestine in malabsorptive states and in pernicious anemia. *Clin. Sci. (London).* **26**: 359.
25. Davis, R. L., L. L. Layton, and B. F. Chow. 1952. Uptake of radioactive vitamin B₁₂ by various microorganisms. *Proc. Soc. Exp. Biol. Med.* **79**: 273.
26. Perlman, D. 1959. Microbial synthesis of cobamides. *Advan. Appl. Microbiol.* **1**: 87.
27. Halbrook, E. R., F. Cords, A. R. Winter, and T. S. Sutton. 1950. Vitamin B₁₂ production by microorganisms isolated from poultry house litter and droppings. *J. Nutr.* **41**: 555.
28. Rickes, E. L., N. G. Brink, F. R. Koniuszy, T. R. Wood, and K. Folkers. 1948. Comparative data on vitamin B₁₂ from liver and from a new source, *Streptoyces griseus. Science (Washington).* **108**: 634.
29. Donaldson, R. M., Jr. 1967. Role of enteric microorganisms in malabsorption. *Fed. Proc.* **26**: 1426.
30. Polter, D. E., J. D. Boyle, L. G. Miller, and S. M. Finegold. 1968. Anaerobic bacteria as a cause of the blind-loop syndrome. A case report with observations on response to antibacterial agents. *Gastroenterology.* **54**: 1148.
31. Goldstein, F. C., W. Wirts, G. Salen, and R. J. Mandle. 1969. Diverticulosis of the small intestine: clinical, bacteriologic, and metabolic observations in a group of 7 patients. *Amer. J. Dig. Dis.* **14**: 170.
32. Gorbach, S. L., and S. Tabaqchali. 1969. Bacteria, bile, and the small bowel. *Gut.* **10**: 963.