Vitamin B₁₂ Uptake by Intestinal Microorganisms:
Mechanism and Relevance to Syndromes of
Intestinal Bacterial Overgrowth

R. A. GIANNELLA, S. A. BROITMAN, and N. ZAMCHECK

From the Mallory Gastroenterology Laboratory, Harvard Medical Unit and the
Thorndike Memorial Laboratory, Boston City Hospital; the Department of
Medicine, Harvard Medical School; and the Departments of Microbiology
and Pathology, Boston University School of Medicine,
Boston, Massachusetts 02118

ABSTRACT 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 inhibi-
tor 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 ad-
sorption 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, Bacil-
lus 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 (Kₐ) varied from 5.05 ±1.67 × 10⁻⁶M for B. subtilis to 6.18 ±3.08 ×
10⁻⁶M for Klebsiella pneumoniae which approximate the

Kₘ for human intrinsic factor (0.38 × 10⁻⁶M). Com-
petition 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 vitamin B₁₂
malabsorption found in the intestinal bacterial over-
growth syndromes.

INTRODUCTION

In recent years, attention has been focused upon the rela-
tionship of bacterial uptake of vitamin B₁₂ to the vita-
min B₁₂ malabsorption present in patients with intestinal
testinal bacterial overgrowth (1–5). Donaldson (1) and Don-
aldson, 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 Fac-
tor). 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 fac-
tors determining such uptake remain unclear. The present
studies are concerned with these points and demon-
strate 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 or-

Other organisms
used were obtained from the stock culture collection of the Department of Microbiology, Boston University School of Medicine. Cyanocobalamin \(^4\) labeled with \(^{88}\)Co (specific activity 13-18 mCi/mg) was used in all experiments and was diluted with nonradioactive cyanocobalamin \(^8\) to give the desired \(B_2\) 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 \(\mu\)m.

Growth and preparation of cells. Organisms were stored at 5°C on tryptic soy agar \(^4\) slants until used. An 18 hr growth (in tryptic soy broth \(^*\) with a vitamin \(B_2\) 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 contribute 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 \(^{88}\)Co vitamin \(B_2\)/ml, and 2 ml of isotonic saline. In some 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 \(B_2\). A modification of the microsomal adsorption inhibition method of Grasbeck (8) was used as follows: bacteria were incubated at 37°C for various periods of time with vitamin \(B_2\) \(^{88}\)Co 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 \(B_2\) 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 HCL, and the radioactivity bound to the bacteria was determined by precipitation of a Packard 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 \(\pm SE\) 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 \(B_2\) uptake by (mean \(\pm SE\)) \(E.\) coli (1 \(x\) \(10^6\) organisms) was 96.9 \(\pm 0.03\%\); and of 52 samples done on 26 different days 92.0 \(\pm 0.05\%\).

Kinetics of bacterial uptake of vitamin \(B_2\); demonstration of two stage process

Bacterial uptake of vitamin \(B_2\) with time. The velocity of the reaction between \(E.\) coli and vitamin \(B_2\) 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 \(B_2\) 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 \(B_2\) concentration on first stage uptake. When a fixed number of bacteria was incubated with increasing concentrations of vitamin \(B_2\), 1 min (first stage) bacterial uptake of vitamin \(B_2\) demonstrated saturation kinetics. These data were analyzed by the method of Lineweaver and Burk (9) and a \(K_m\) for first stage \(B_2\) uptake was calculated (Table I). For \(E.\) coli, the \(K_m\) was \(6.03 \pm 0.62 \times 10^{-4}\)M (mean \(\pm SE\)). Additional \(K_m\) values for various aerobic bacteria, also listed in Table I, illustrate the similarity among 10 dif-

\[
\text{Figure 1 Vitamin } B_2 \text{ uptake by various numbers of } E. \text{ coli. Typical plot representative of eight experiments. Bacteria were incubated with 0.6 ng vitamin } B_2 \text{ at 37°C and at pH 7.0 as described in text.}
\]

Mechanism and Relevance of Vitamin B-12 Uptake by Intestinal Bacteria 1101

\(\text{\footnotesize \cite{1}}\) Kindly supplied by Dr. E. E. Baker and Dr. L. M. Corwin.


\(\text{\footnotesize \cite{3}}\) Millipore Corp., Bedford, Mass.

\(\text{\footnotesize \cite{4}}\) Baltimore Biological Laboratory, Baltimore, Md.
different bacteria, with *Bacillus subtilis* having the smallest $K_m$ and *Klebsiella pneumoniae* the largest. There were no significant differences in the $K_m$ values among three different strains of *E. coli*.

First stage (1 min) $B_m$ uptake by *E. coli* ($1 \times 10^9$ organisms) was saturated by 2.9 ± 0.4 ng of vitamin $B_m$. The quantity of $B_m$ 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 $B_m$ necessary for first stage saturation. The wide range in the number of $B_m$ molecules bound at saturation of first stage is apparent (355,000 for Group A streptococcus to 950 for *E. coli K12* [mutant]). *Strep. fecalis* was the sole exception demonstrating no significant $B_m$ uptake. There was no correlation between the amount of $B_m$ bound at saturation and $K_m$ value.

1 hr bacterial uptake of vitamin $B_m$. As shown in Table III, $B_m$ uptake by *E. coli* ($1 \times 10^9$) and *Salmonella paratyphi* (1 × 10⁹) 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 $B_m$ uptake in 1 hr.

## Table I

$K_m$ (Binding Constant) Values of Aerobic Bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>$K_m$ (Binding Constant) Value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em></td>
<td>5.05 ± 1.67 × 10⁻¹⁰ M</td>
</tr>
<tr>
<td><em>E. coli</em> K12 (mutant)</td>
<td>5.38 ± 1.63 × 10⁻¹⁰ M</td>
</tr>
<tr>
<td><em>E. coli</em> K12 (wild type)</td>
<td>6.03 ± 0.62 × 10⁻¹⁰ M</td>
</tr>
<tr>
<td><em>P. mirabilis</em></td>
<td>8.34 ± 1.77 × 10⁻¹⁰ M</td>
</tr>
<tr>
<td><em>Escherichia coli B</em></td>
<td>1.08 ± 0.26 × 10⁻⁹ M</td>
</tr>
<tr>
<td><em>Salmonella paratyphi</em></td>
<td>2.00 ± 0.74 × 10⁻⁹ M</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>2.00 ± 0.48 × 10⁻⁹ M</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>3.15 ± 0.33 × 10⁻⁹ M</td>
</tr>
<tr>
<td>Group A streptococcus (β-hemolytic)</td>
<td>3.16 ± 0.18 × 10⁻⁹ M</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>6.18 ± 3.08 × 10⁻⁹ M</td>
</tr>
</tbody>
</table>

* Values expressed as mean ± 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 $B_m$

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Saturation amount ng/10⁹ organisms</th>
<th>Molecules vit. $B_m$ bound/organism</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Group A streptococcus (4)</td>
<td>799.8 ± 117.5 (3)</td>
<td>355,000 (1)</td>
</tr>
<tr>
<td>2. <em>B. subtilis</em> (5)</td>
<td>402.4 ± 69.8 (2)</td>
<td>179,000 (1)</td>
</tr>
<tr>
<td>3. <em>Klebsiella pneumoniae</em> (4)</td>
<td>15.0 ± 4.6 (2)</td>
<td>6,680 (1)</td>
</tr>
<tr>
<td>4. <em>P. aeruginosa</em> (5)</td>
<td>5.0 ± 0.4 (2)</td>
<td>2,230 (1)</td>
</tr>
<tr>
<td>5. <em>Sal. paratyphi</em> (6)</td>
<td>4.7 ± 1.1 (2)</td>
<td>2,090 (1)</td>
</tr>
<tr>
<td>6. <em>P. mirabilis</em> (6)</td>
<td>3.5 ± 1.2 (2)</td>
<td>1,550 (1)</td>
</tr>
<tr>
<td>7. <em>E. coli</em> K12 (wild type) (6)</td>
<td>2.9 ± 0.4 (2)</td>
<td>1,280 (1)</td>
</tr>
<tr>
<td>8. <em>Staph. aureus</em> (4)</td>
<td>2.6 ± 0.7 (2)</td>
<td>1,140 (1)</td>
</tr>
<tr>
<td>9. <em>E. coli</em> B (4)</td>
<td>2.2 ± 0.3 (2)</td>
<td>950 (1)</td>
</tr>
<tr>
<td>10. <em>E. coli</em> K12 (mutant) (6)</td>
<td>2.2 ± 0.5 (2)</td>
<td>950 (1)</td>
</tr>
<tr>
<td>11. <em>Strep. fecalis</em> (5)</td>
<td>0 (2)</td>
<td>0 (1)</td>
</tr>
</tbody>
</table>

* Group A streptococcus and *B. subtilis* significantly different from each other and from all others $P = < 0.05$. *Klebsiella pneumoniae* and *P. 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.
§ Values expressed as mean ± 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; *Klebsiella pneumoniae* from *Strep. fecalis*; *P. aeruginosa* from *Staph. aureus* and *Strep. fecalis*; *E. coli* K12 (wild type) from *Strep. fecalis*; *E. coli* K12 (mutant) from *Staph. aureus* and *Strep. fecalis*; and *Staph. aureus* from *Strep. fecalis*.
¶ Methionine requiring mutant.

## Table III

1 hr Vitamin $B_m$ Uptake of Aerobic Bacteria

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>1 hr uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>1) <em>B. subtilis</em> (6)</td>
<td>21,700 ± 2,200</td>
</tr>
<tr>
<td>2) Group A streptococcus (7)</td>
<td>1,810 ± 258</td>
</tr>
<tr>
<td>3) <em>P. mirabilis</em> (5)</td>
<td>255 ± 35.9</td>
</tr>
<tr>
<td>4) <em>E. coli</em> B (6)</td>
<td>30.6 ± 6.1</td>
</tr>
<tr>
<td>5) <em>Sal. paratyphi</em> (5)</td>
<td>19.7 ± 4.1</td>
</tr>
<tr>
<td>6) <em>Klebsiella pneumoniae</em> (5)</td>
<td>17.3 ± 7.8</td>
</tr>
<tr>
<td>7) <em>P. aeruginosa</em> (4)</td>
<td>12.5 ± 2.9</td>
</tr>
<tr>
<td>8) <em>E. coli</em> K12 (wild type) (5)</td>
<td>7.0 ± 1.5</td>
</tr>
<tr>
<td>9) <em>E. coli</em> K12 (mutant) (12)</td>
<td>6.6 ± 1.0</td>
</tr>
<tr>
<td>10) <em>Staph. aureus</em> (5)</td>
<td>3.2 ± 0.9</td>
</tr>
<tr>
<td>11) <em>Strep. fecalis</em> (5)</td>
<td>0</td>
</tr>
</tbody>
</table>

* Number of bacteria in each case $1 \times 10^9$.
† 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; *Klebsiella pneumoniae* from *Strep. fecalis*; *P. aeruginosa* from *Staph. aureus* and *Strep. fecalis*; *E. coli* K12 (wild type) from *Strep. fecalis*; *E. coli* K12 (mutant) from *Staph. aureus* and *Strep. fecalis*; and *Staph. aureus* from *Strep. fecalis*.
¶ Methionine requiring mutant.

R. A. Giannella, S. A. Broitman, and N. Zamcheck
Effect of viability of bacteria on vitamin B₁₂ uptake

E. coli autoclaved at 120°C for 20 min showed no vitamin B₁₂ 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 B₁₂ uptake but little second stage uptake. Although viability was lost, bacteria appeared intact microscopically, and retained their Gram-staining properties.

Effect of prior vitamin B₁₂ deprivation or saturation on subsequent bacterial uptake of vitamin B₁₂

In these experiments, E. coli was grown in trypticase soy broth either with or without added nonradioactive vitamin B₁₂ (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 B₁₂), harvested by centrifugation, washed three times with 0.15 M saline and an aqueous suspension prepared as described in Methods.

The B₁₂ uptake rate of 1 x 10⁹ of each E. coli was then measured. As shown in Fig. 3, E. coli grown in media enriched with nonradioactive vitamin B₁₂ lost its ability to take up radioactive vitamin B₁₂, as compared to organisms grown in media lacking vitamin B₁₂.

Effect of temperature on vitamin B₁₂ 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.

Mechanism and Relevance of Vitamin B-12 Uptake by Intestinal Bacteria
**Effect of pH on bacterial uptake of vitamin B<sub>12</sub>**

For both first and second stage vitamin B<sub>12</sub> uptake, optimum was between 6.6 and 7.5. As shown in Fig. 5, both stages of B<sub>12</sub> 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<sub>12</sub>**

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

**Number of bacteria necessary for vitamin B<sub>12</sub> uptake**

Approximately $2 \times 10^9$ E. coli were necessary before vitamin B<sub>12</sub> uptake could be detected and $1 \times 10^6$ 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<sub>12</sub> uptake with as few as $5 \times 10^6$ and $6 \times 10^6$ organisms respectively. Staphylococcus aureus was similar to the Gram-negatives while Strep. fecalis was unable to take up significant amounts of B<sub>12</sub> even with numbers as high as $1 \times 10^6$ organisms.

**Other bacteria demonstrating vitamin B<sub>12</sub> uptake**

Other Gram-positive and negative bacteria tested which demonstrated B<sub>12</sub> 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<sub>12</sub> uptake and had a similar two stage vitamin B<sub>12</sub> uptake process.

**DISCUSSION**

These studies, concerned with the mechanism of vitamin B<sub>12</sub> uptake by bacteria, indicate that B<sub>12</sub> is taken up by a variety of microorganisms, which do not require ex-
ogenous B<sub>12</sub>. This process which occurs in at least two stages, is similar to that in the vitamin B<sub>12</sub> requiring parasite *Ochromonas malhamensis* (11) and various B<sub>12</sub> 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<sub>12</sub>. 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<sub>12</sub> uptake. However, since pasteurization decreased first stage uptake by 50%, it is likely that some of the surface B<sub>12</sub> binding sites were denatured by pasteurization (13). With more drastic heat treatment, i.e. autoclaving, the bacterial cells were disrupted, and all B<sub>12</sub> uptake and Gram-staining reaction abolished. Formalin inhibition of B<sub>12</sub> 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<sub>12</sub>-binding sites.

Data in support of the concept of cell wall B<sub>12</sub> binding sites have been presented by Sasaki and Kitahara (15) who fractionated *Lactobacillus leichmannii* after 30 min of radioactive vitamin B<sub>12</sub> 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<sub>12</sub> 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-ethylmaleimide 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<sub>12</sub> 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<sub>12</sub> abolished further B<sub>12</sub> uptake, presumably by saturating both the surface binding sites and intracellular B<sub>12</sub> storage capacity. While this interpretation is reasonable, low radioactive B<sub>12</sub> uptake values could, in part, reflect dilution of the radioactive B<sub>12</sub> by cold B<sub>12</sub> 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<sub>12</sub> 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<sub>12</sub> 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 K<sub>m</sub>, which for the purpose of this study is an affinity or binding constant, for first stage B<sub>12</sub> uptake. As with enzyme-substrate interactions, the smaller the K<sub>m</sub> value the greater the avidity of bacteria for vitamin B<sub>12</sub>. K<sub>m</sub> values in the present studies (6.03 x 10<sup>-4</sup> - 6.18 x 10<sup>-3</sup>) show great avidity of bacteria for B<sub>12</sub> and are similar to the K<sub>m</sub> reported for *Lactobacillus leichmannii* (2.8 x 10<sup>-3</sup>) by Kashket, Kaufman, and Beck (12) and approximates the K<sub>m</sub> reported for human intrinsic factor (0.38 x 10<sup>-3</sup>) by McGuigan (22).

It is concluded that the first stage B<sub>12</sub> uptake by bacteria is an adsorptive process related to vitamin B<sub>12</sub> binding or "receptor" sites on the cell wall.

**Second stage uptake.** Second stage bacterial uptake of vitamin B<sub>12</sub> 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 acetylstrepoxanthin, suggesting that neither glycolysis nor Na-K activated membrane bound adenosine triphosphatase (ATPase) appeared to be involved. Supporting the concept that second stage B<sub>12</sub> uptake may represent transport of vitamin B<sub>12</sub> 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<sup>125</sup> vitamin B<sub>12</sub> enriched media and fractionating the bacteria they found in excess of 90% of the vitamin B<sub>12</sub> in the ribosomal fraction.

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

1 hr B<sub>12</sub> 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 B₉ absorption values (21,700 ± 2200 and 1810 ± 258 ng respectively) that were higher than those observed for Gram-negative bacteria (6.6 ± 1.0 – 255 ± 35.9 ng). Both *B. subtilis* and Group A streptococcus were capable of measurable vitamin B₉ uptake with a few exceptions.

While absorption.

While a variety of bacteria are capable of uptake, it may not possess surface receptor sites capable of immediate vitamin B₉ uptake. Similar behavior of *Strep. fecalis* has been observed by others (2, 3, 5, 24).

The wide range in both first and second stage B₉ uptake values among the various organisms studied is unexplained. It does not appear to be related to either a requirement for exogenous B₉ or to ability to synthesize B₉. All the organisms examined have been shown not to require exogenous B₉ (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 B₉ for growth, take up as much B₉ as quickly and by a similar mechanism, as B₉ requiring organisms. Differing bacterial avidities and capacities for B₉ must remain unexplained at present.

Avidities of bacteria for B₉ comparable to that of intrinsic factor for B₉, implies that bacteria resident in "blind-loops" may be able to compete with intrinsic factor for ingested B₉. 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 B₉ uptake, the differences noted in both avidity and capacity for B₉ uptake, suggest that overgrowth with certain organisms may more frequently lead to vitamin B₉ malabsorption. While anaerobes have been shown to be a prominent part of the flora of blind-loops (29–32) their avidity and capacity for B₉ uptake and their role on the B₉ 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 B₉.

**ACKNOWLEDGMENTS**

We are indebted to Dr. R. M. Donaldson, Jr. for his invaluable help and many suggestions and to Dr. P. A. Kantrowitz, and Dr. B. M. Babior, for their critical review of this manuscript.

This investigation was supported by grants from the National Cancer Institute CA 04486 and CA 02090; the National Institute of Arthritis and Metabolic Disease TI AM 5320, and the National Institute of Allergy and Infectious Diseases AI 07913.

**REFERENCES**


Mechanism and Relevance of Vitamin B-12 Uptake by Intestinal Bacteria 1107