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MECHANISM OF VITAMIN B_{12} MALABSORPTION IN BLIND LOOP SYNDROME: BACTERIAL UPTAKE OF VITAMIN B_{12} AND STABILITY OF BINDING OF VITAMIN B_{12} TO GASTRIC INTRINSIC FACTOR*1

BY

Takashi NAKAJIMA, Tsukasa ABE and Hiroyoshi OKAMURA*2

ABSTRACT

Both free vitamin B_{12} (B_{12}) and B_{12} bound to the intrinsic factor (IF) were taken up by the microorganisms. The amount of B_{12} taken up in the presence of IF was fairly smaller than in the absence of IF.

The stability of the B_{12}-IF complex was influenced by the temperature as well as by pH. The bacterial uptake of B_{12} appeared to be favourable under the condition where the vitamin dissociated easily from IF. Moreover, when the B_{12}-IF complex was incubated with the microorganisms, the free IF activity in the supernatant increased according to the bacterial uptake of B_{12}.

It seemed that the microorganisms took up B_{12} after the liberation of B_{12} from IF. The amount of B_{12} available for bacterial uptake was regulated by the rate of dissociation of the B_{12}-IF complex as well as by the reciprocal changes in the size of the bacterial population and IF available.

It is suggested that B_{12} malabsorption in the blind loop syndrome could be partly explained by the bacterial uptake of B_{12} in the presence of abundant microorganisms in the loop and by the predominant dissociation of the vitamin from IF.

INTRODUCTION

The mechanism of B_{12} malabsorption in the patients with the blind loop syndrome has been obscure. Microorganisms in the loop are considered to play an important role in this malabsorption. (i) Malabsorption of B_{12} in patients with blind loop was improved immediately after the administration of antibiotics and reappeared by the cessation of the antibiotics[1–11]. (ii) The microorganisms in the loop took up the vitamin in vitro[5,12–20]. (iii) Malabsorption of B_{12} was also observed in the experimental animals with diverticula[21–23]. (iv) It was reported that the mor-

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*2 中島 隆, 阿部 帥, 岡村裕喜: The First Department of Internal Medicine (Chief: Prof. M. KOMIYA), Tokyo Medical and Dental University (Tokyo Ika Shika Daigaku). Received for publication, August 1, 1972.

phological changes of the intestinal mucosa with a blind loop in the patients[5,8,9,24] and in the animals[21–23] were not so severe as those by which B_{12} malabsorption was brought about. Furthermore, B_{12} uptake by the isolated, purfused segment of the intestine was the same in the rat with diverticula as was observed in the control animals[23].

It has been reported that the bacterial uptake of B_{12} initially bound to the gastric intrinsic factor was negligible or remarkably reduced[12–20]. Dellipiani et al.[17] reported that human gastric juice had a marked inhibitory effect on the B_{12} uptake by E. coli, but it was an incomplete inhibition. Although significant amounts of bound B_{12} are taken up by the intestinal microorganisms in vitro, the regulatory mechanism of B_{12} uptake has not been elucidated, especially when the activity of the intrinsic factor remained unchanged. It is essential to understand the role of the microorganisms in B_{12} uptake for the investigation of B_{12} malabsorption. Present investigation was designed to solve this mechanism.

Materials and Methods

Microorganisms Typical B_{12} malabsorption was observed in two patients with a blind loop which was made by anastomosing the middle portion of the small intestine to the transverse colon. Microorganisms including E. coli, Klebsiella and Citrobacter were obtained from the contents of the blind loop, when the operation to liberate the anastomosis was performed for radical treatment of the malabsorption without pre-administration of antibiotics. Some of the standard strains of E. coli and several strains of Klebsiella and Citrobacter from other origins in other patients were also used. These microorganisms were preserved in heart infusion agar media. For examinations, the microorganisms were inoculated twice in heart infusion broth, washed twice with distilled water or saline, and resuspended in these aquae. Usually a certain amount of microorganisms was measured by their turbidity in the final heart infusion broth. The turbidity was determined by multiplying the photometric reading at a wave length of 600 mμ by 1,000. The bacterial suspension with a turbidity from 300 to 400 contained approximately 10^7 to 10^8 microorganisms per ml.

When bacterial suspensions were incubated at temperatures between 0°C and 45°C for 4 hours, the B_{12}-binding capacity of these supernatants was below 0.002 u per tube. The fresh broth contained approximately 2.5 mμg/ml of B_{12}, while B_{12} was detected sparsely in the supernatant after incubation with the microorganisms.

Cyanocobalamin (B_{12}) ^{57}Co-labelled cyanocobalamin (^{57}Co-B_{12}), 10 μCi

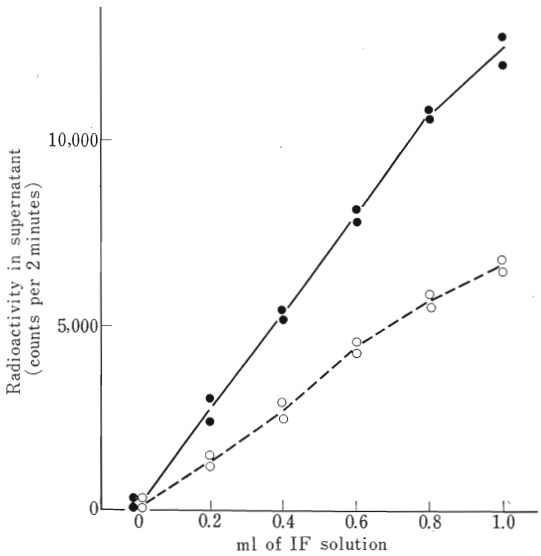

Fig. 1. Separation of bound B_{12} from free B_{12}. After incubating B_{12} (0.089 mμg) and IF (0.087 u/ml) with bovine albumin and adding coated charcoal, the radioactivity in the supernatant was measured by 2-minute counting.
Closed circles: original solution of ^{57}Co-B_{12}
Open circles: mixture of equal amount of ^{57}Co-B_{12} and cold B_{12}

per 82 to 89 mμg per ml, was obtained from the Radiochemical Centre, Amersham, England and diluted with nonradioactive B_{12} to the desired concentration. The radioactivity was counted in duplicated samples for 2 minutes by the well-type scintillation counter (Spectro Scaler RDM-1, Tokyo Shibaura Electric Co., Japan).

Bovine albumin-coated charcoal (*coated charcoal*) The activity of the intrinsic factor (IF) and antibody to IF (IFA) were measured by the "coated charcoal" method of Gottlieb et al.[25] In the present investigation, 1 unit (u) of IF represented the B_{12}-binding capacity of IF which bound 1 mμg of B_{12}, and 1 u of IFA (blocking antibody) was equivalent to 1 u of IF.

Using the coated charcoal, the bound B_{12} was separated from the free B_{12}, and the solutions of "B_{12}-saturated IF" were prepared. The separation of bound B_{12} from free B_{12} was successful, even when 0.089 mμg of B_{12} and small doses of IF were incubated with approximately 0.01 ml of 1% bovine albumin (Fig. 1). The recovery of IF by the above procedure was from 87 to 91% of the expected values. B_{12}-binding capacity of the sera with IFA, of the supernatants of bacterial suspensions and of the bovine albumin

was also determined with 0.089 mμg of B_{12} and 1 ml of coated charcoal. B_{12}-binding capacity of 1% bovine albumin was below 0.01 u/ml.

Gastric intrinsic factor (IF) Gastric juice was obtained from hypersecretors after an administration of 50 mg of betazole hydrochloride. The obtained juice contained IF which was responsible for more than 98% of the total unsaturated B_{12}-binding capacity. It was used as the IF solution which was preserved at −20°C until required.

Antibody to intrinsic factor (IFA) IFA was obtained from the sera of two patients with pernicious anemia. Titers of the "blocking antibody" were 575 and 786 u/ml. These sera also had a "binding antibody" and a B_{12}-binding capacity of approximately 0.1 u/ml.

B_{12}-saturated IF After mixing 1 volume of IF and approximately 3 volumes of B_{12}, coated charcoal was added and followed by centrifugation. The supernatant was employed as the "B_{12}-saturated IF".

Bacterial uptake of B_{12} Bacterial suspensions were incubated with either free or bound B_{12}, and the total volumes of the mixtures were from 3 to 4 ml. After the incubated mixtures were centrifuged, the sediments were washed twcie with distilled water or saline. The amount of the B_{12} taken up by the microorganisms ("bacterial B_{12}") was measured by counting the radioactivity of the sediment or the supernatant. The "B_{12}-binding capacity of the microorganisms" usually indicated the "bacterial B_{12}" when the microorganisms (turbidity 1,000, 1 ml) were incubated at 37°C for 1 hour with 100 mμg of free B_{12}.

Nature of B_{12} taken up by microorganisms In order to examine the possibility that microorganisms directly took up bound B_{12}, IF was incubated at 37°C for 4 hours with various amounts of microorganisms preincubated in the presence or absence of B_{12}. The decrease in the IF activity in the supernatant suggests that the microorganisms take up (or bind with) bound B_{12}. Cold B_{12}-saturated IF (0.125 u) was incubated with E. coli (turbidity 270, 5 ml). After this, the supernatant fraction was incubated with and without IFA (1.5 u) and labelled free B_{12} (0.125 mμg) was added, followed by the administration of 1 ml of coated charcoal. The labelled B_{12} in the final supernatant was measured, and the free IF activity was expressed by the difference between the amount of the labelled B_{12} with and without IFA. Simultaneously, the "bacterial B_{12}" was measured with labelled B_{12}-saturated IF (0.125 u). The increase in free IF in the supernatant according to the increase in the "bacterial B_{12}" suggests that bound B_{12} is taken up by the microorganisms after the liberation of B_{12} from IF.

Stability of B_{12}-IF complex The exchange between free B_{12} and bound B_{12}, or the dissociation of B_{12} from IF, was examined by the following

methods with a single isotope: *Experiment A*: Labelled B_{12}-saturated IF (0.05 u) and cold B_{12} (100 mμg) were incubated at varying temperatures between 0°C and 45°C for the period up to 5 hours. Each mixture was cooled immediately after the incubation and then 1 ml of coated charcoal was added. The amount of B_{12} dissociated from the bound B_{12} was calculated by the radioactivity of the labelled B_{12} which was adsorbed on the coated charcoal. *Experiment B*: Mixtures employed were cold B_{12}-saturated IF (0.05 u) and labelled free B_{12} (0.12 mμg). Procedures of the experiment were the same as in experiment A, except that the radioactivity was counted in the supernatants. Theoretically, 29.4% radioactivity in the supernatant indicated the rate of complete exchange of the labelled B_{12} between free and bound B_{12}. *Experiment C*: Mixtures of labelled B_{12}-saturated IF (0.05 u) and IFA (1.50 u) were employed. The radioactivtiy adsorbed on to the charcoal was determined to be equivalent to the amount of the labelled B_{12} dissociated from IF.

Effects of pH The stability of the B_{12}-IF complex was examined by changing the pH. The incubation medium was adjusted to various pH from 2.4 to 10.3 with phosphate-buffered saline. The mixtures of labelled B_{12}-saturated IF (1.4 u) and cold B_{12} (100 mμg), and the mixture of cold B_{12}-saturated IF (1.4 u) and labelled B_{12} (1.0 mμg) were incubated with this medium at 37°C for 90 minutes. The rate of dissociation of B_{12}-IF complex was calculated as mentioned above. The bacterial uptake of B_{12} was also examined in relation to pH. In these examinations, the pH in the whole incubation mixture was not different from the pH in the stock solution of the phosphate-buffered saline as shown by the pH test paper.

Destruction of B_{12}-saturated IF and IF The B_{12} adsorbed on to the coated charcoal was estimated by the amount of the B_{12} liberated from the B_{12}-saturated IF or the amount destroyed, when the B_{12}-saturated IF and coated charcoal were mixed. The destruction did not exceed 10% in the experiment herein described. The loss of free IF activity was examined by measuring the B_{12}-binding capacity of IF. The rate of IF destruction was the same or somewhat greater than that of the B_{12}-saturated IF.

The results presented in this report were not calibrated by the amount of destruction of IF or the B_{12}-saturated IF.

Results

Part 1. Uptake of free B_{12} by microorganisms

(a) E. coli and Klebsiella, the turbidity of these being adjusted to 1,000, were suspended in 1 ml of distilled water. The "B_{12}-binding capacity

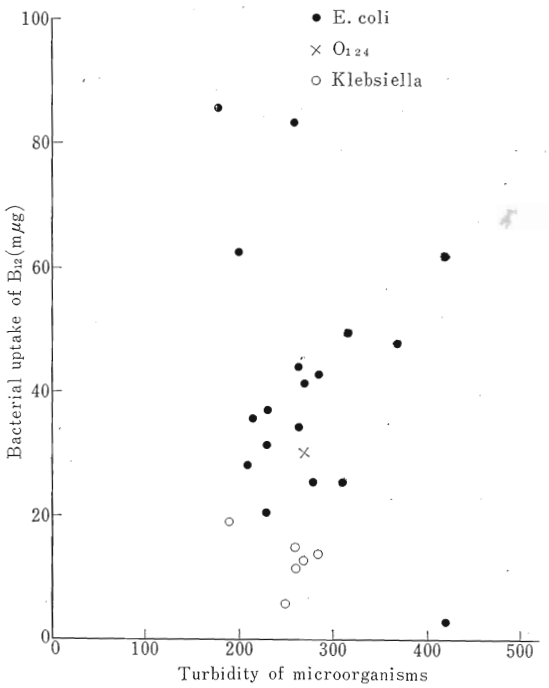

Fig. 2. Bacterial uptake of free B_{12}. E. coli and Klebsiella (turbidity 1,000, 1 ml) were incubated with 100 mμg of B_{12} at 37°C for 1 hour and their B_{12}-binding capacity was measured. The abscissa shows the turbidity of the microorganisms in the final broth.

of microorganisms" is shown in Fig. 2. The amount of B_{12} taken up by E. coli was to some extent greater than that by Klebsiella. One of the standard strains of E. coli (O_{124}), which had no cillium, had the B_{12}-binding capacity of 30 mμg. This was rather low among the E. coli examined. The vitamin taken up by the microorganisms was not removed by repeated washings, incubating at 37°C for 26 hours or mixing with cold B_{12} or with large quantities of IF as shown in Fig. 4.

(b) The "bacterial B_{12}" slightly increased along with the increased amount of B_{12} in the incubation medium, when incubated at 37°C for 1 and 4 hours with 100 to 10,000 mμg of labelled B_{12} with the same radioactivity. It was not observed that the ^{57}Co was prone to be taken up by the microorganisms when incubating with 100 mμg of labelled B_{12} with different radioactivity. When the microorganisms (turbidity 660, 1 ml) were incubated in 1, 2 and 4 ml of distilled water, the B_{12}-binding capacity of these increased slightly as the volume of the incubation medium increased.

Table 1. Bacterial uptake of free B_{12}. Klebsiella (turbidity 330) was incubated with 100 and 0.76 mμg of B_{12}. Percent uptake of B_{12} was expressed as the mean of duplicated data.

Incubation temperature	Bacterial suspension (ml)	Incubation time (minutes)						
		(100 mμg of B_{12})				(0.76 mμg of B_{12})		
		10	60	120	240	10	60	120
37°C	1	5.0	7.3	9.5	13.7	72.9	89.0	89.4
	2	7.8	9.6	14.2	18.7	87.7	89.5	91.5
	4	11.7	14.4	18.8	25.4	91.9	93.3	93.9
25°C	1	3.1	5.1	7.0	10.0	61.8	82.7	90.3
	2	5.3	7.7	9.2	12.9	80.2	88.4	90.4
	4	8.7	11.0	14.1	17.8	88.3	89.3	90.2
2°C	1	0.5	0.4	0.5	0.5			
	2	0.6	0.7	0.7	0.7			
	4	1.2	1.2	1.2	1.3			

(c) The B_{12} uptake by Klebsiella was measured by changing the concentration of the B_{12} and microorganisms or by changing the incubation time and temperature (Table 1). The "bacterial B_{12}" increased as the incubation temperature was elevated. The "bacterial B_{12}" increased gradually as the incubation time was prolonged, not only by incubation with 100 mμg of B_{12} but also with a very small dose of B_{12} (0.076 mμg). The B_{12} uptake by E. coli was also observed by incubating with 100 mμg of B_{12} at 37°C for 1, 5, 16 and 26 hours. They were 14.2, 27.9, 57.3 and 65.1 mμg, respectively. The "bacterial B_{12}" increased along with the increase in bacterial population. The increase in "bacterial B_{12}" was slightly lower than the value expected from the increase in bacterial population, even when a large dose of B_{12} (100 mμg) was used for a short time (10 min.). The same property was found in the "bacterial B_{12}" at time zero, which was suspected on the basis of the "bacterial B_{12}" at 10, 60 and 120 minutes.

(d) The ^{57}Co-B_{12}-binding capacity of E. coli (turbidity 210, 3 ml) was measured, after pre-incubation with 0, 10, 50 and 100 mμg of cold B_{12} at 37°C for 1 hour and washing immediately. It was 26.2, 22.7, 22.5 and 19.5 mμg, respectively.

(e) The microorganisms were inoculated three times in the minimal medium without B_{12}. After each inoculation, the microorganisms were again inoculated in the minimal medium with and without B_{12} (5 mμg/ml). The growth of these was not influenced by the addition of B_{12}.

(f) There was no significant difference in the B_{12}-binding capacity between the microorganisms obtained in the early logarithmic phase of growth and those obtained at the later stage. The B_{12}-binding capacity of

Table 2. Bacterial uptake of free and bound B_{12}. After mixing B_{12} and IF for 10 minutes at room temperature, the mixtures were incubated with microorganisms at 37°C for 30 minutes.

Experiment	B_{12} (mμg)	IF (u)	Microorganism (ml)	Bacterial uptake of B_{12} (%)	
				Klebsiella	E. coli
1	1	0	2	90.5	85.4
2	1	5	2	1.3	0.9
3	1	5	8	4.3	2.4
4	1	5	40	17.3	13.6
5	1	25	40	8.6	4.9
Turbidity of microorganisms				870	620

the microorganisms was reduced to approximately 70 to 80% of the initial values, when the bacterial suspension in distilled water or saline was kept at 37°C or at room temperature for 3 hours. Those microorganisms had little or no B_{12}-binding capacity after they were soaked in acetone, 8% formalin or 70% ethanol, or when heated at 80°C for 10 minutes.

(g) In the present observation, there were approximately 10% of ^{57}Co which could not be taken up by the microorganisms or by the IF, being adsorbed on to the coated charcoal. No further study was made on this point.

Part 2. Uptake of bound B_{12} by microorganisms

(a) Bound B_{12} was also taken up by the microorganisms, although the "bacterial B_{12}" was diminished remarkably as compared with those without IF (Table 2). The "bacterial B_{12}" increased as the bacterial population increased and as the amount of IF decreased, though the "bacterial B_{12}" could not be determined easily by the changes in the size of the bacterial population and IF.

(b) There was no significant difference in the "bacterial B_{12}" among the microorganisms examined (Fig. 3). The percent uptake of B_{12} seemed to reach the plateau of approximately 50 to 60% regardless of the increase in the bacterial population.

(c) The "bacterial B_{12}" was measured when incubated in distilled water and in the heart infusion broth which was prepared freshly. The "bacterial B_{12}" with fresh broth was greater than that incubated in distilled water (Table 3), while the turbidity of the former also increased from 480 to 720 during the 4 hour-incubation. On the contrary, there was no increase in the turbidity of the latter. In regard to the difference in the "bacterial B_{12}", the increase in the bacterial population appeared not to be an important factor, because the ratios of B to A in Table 3 were practically constant.

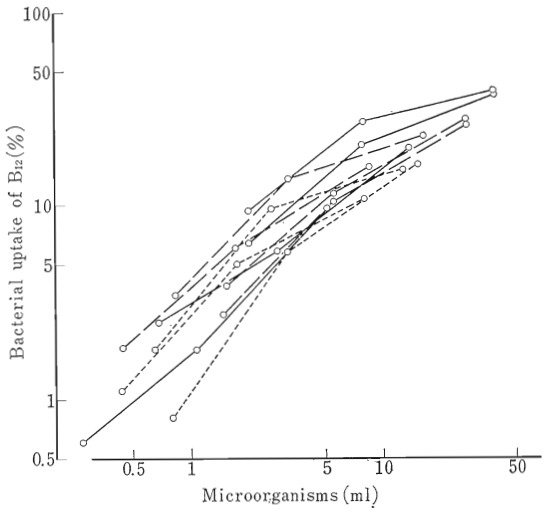

Fig. 3. Bacterial uptake of bound B_{12}. Mixtures of 1 mμg of B_{12} and 3 u of IF were incubated at 37°C for 60 minutes with E. coli (solid lines), Klebsiella (broken lines) and Citrobacter (dotted lines). Microorganisms were obtained in the early logarithmic phase of growth and in the later stage. The amount of microorganisms is expressed in ml of bacterial suspension with turbidity of 950.

(d) By changing the incubation temperature, the B_{12} uptake by Klebsiella was observed (Table 4). The "bacterial B_{12}" increased as the incubation temperature was elevated and as the incubation time was prolonged. At 0°C the bacterial uptake was minimal and no increase in the uptake was observed with the prolongation of the incubation time. The ratios of the "bacterial B_{12}" at 37°C to the amount at 25°C were between 3.6 and 5.0.

Table 3. Bacterial uptake of bound B_{12}. Klebsiella (turbidity 480, 8 ml) and B_{12}-saturated IF (0.02 u) were incubated at 37°C in distilled water and heat infusion broth.

Incubation time (hours)	Bacterial uptake of B_{12} (%)		B/A
	In distilled water (A)	In heart infusion broth (B)	
1	13.0	17.8	1.37
2	25.1	33.3	1.33
3	32.1	46.5	1.45
4	39.9	56.0	1.40

Table 4. Bacterial uptake of bound B_{12}. Klebsiella (turbidity 270, 8 ml) was incubated with 0.02 u of B_{12}-saturated IF.

Incubation time (minutes)	Bacterial uptake of B_{12} (%)		
	Incubation temperature		
	0°C	25°C	37°C
30	4.5	4.7	16.9
60	4.6	6.4	32.2
120	4.2	9.9	44.2

On the other hand, these ratios ranged from 1.2 to 1.6 in the observation with 100 mμg of free B_{12} as shown in Table 1.

(e) The effects of the IF concentrations on the bacterial uptake of B_{12} were observed by changing the order of the mixing of the three components (Fig. 4). The materials employed were E. coli (turbidity 230, 8 ml), 0.016 mμg of B_{12} and 0.03 to 30.0 u of IF. The incubation was done at 37°C.

Experiment A: After incubation of E. coli with B_{12} for 1 hour, IF was added to the mixture and incubated again for another 1 hour. The "bacterial B_{12}" was practically constant regardless of the change in the amount of IF. It appeared that the B_{12} taken up by E. coli was not removed

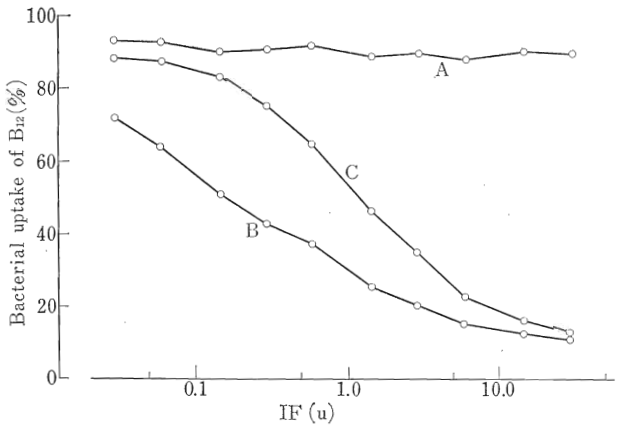

Fig. 4. Bacterial uptake of B_{12}. The bacterial uptake of B_{12} was observed by changing the order of mixing the three components as described in the text. The materials employed were E. coli (turbidity 230, 8 ml), 0.016 mμg of B_{12} and 0.03 to 30.0 u of IF.
A: (E. coli + B_{12}) + IF, B: (B_{12} + IF) + E. coli, C: (E. coli + IF) + B_{12}.

by mixing a large amount of IF. Similar results were obtained with a larger amount of B_{12} or with other microorganisms.

Experiment B: B_{12} and IF were mixed for 10 minutes at room temperature and incubated for 2 hours with E. coli. The increase in the "bacterial B_{12}" was observed with the decrease in the amount of IF.

Experiment C: E. coli and IF were mixed for 5 minutes at room temperature and incubated with B_{12} for 2 hours. The increase in the "bacterial B_{12}" was also observed with the decrease in the amount of IF. The "bacterial B_{12}" in Experiment C was always greater than that obtained in Experiment B. The microorganisms and the IF seemed to take up B_{12} competitively in Experiment C, although there were few exceptions. One of the exceptions was that some calibrations might be necessary on these data, because the percent uptake of B_{12} reached the plateau at approximately 10% and 90% instead of being flat at about 0% and 100%. Another exception was that the B_{12}-binding capacities of the microorganisms might be diminished to a 10th to 30th part, judging from the following findings: (i) The free B_{12}-binding capacity of these at time zero was suspected to be approximately 20 mμg, because the B_{12}-binding capacity at 1 hour was 30 mμg. (ii) In contrast, the B_{12} was taken up by these microorganisms and IF equally, when the microorganisms and 1 to 2 u of IF were incubated in Experiment C.

(f) The bacterial uptake of bound B_{12} was observed on two series of bound B_{12} which had the same ratios of B_{12} to IF. Bound B_{12} (1:3) and bound B_{12} (1:30) were prepared by mixing 1 volume of B_{12} in 3 and 30 volumes of IF, respectively. The "bacterial B_{12}" increased along with the increase in B_{12}, and the percent uptake of B_{12} decreased along with the increase in the bound B_{12} (Fig. 5a). The patterns of the B_{12} uptake with the different series of bound B_{12} were not parallel.

The bacterial uptake of B_{12} was expressed by the changes in the amount of free or total IF (Fig. 5b). Two-thirds to 100% of IF in bound B_{12} (1:3) were estimated to be the amount of free IF in bound B_{12} (1:3). Free IF in bound B_{12} (1:30) was almost the same as the total IF in bound B_{12} (1:30). The percent uptake of B_{12} during incubation with bound (1:3) appeared to be identical in rate with bound B_{12} (1:30). This finding suggested that the "bacterial B_{12}" was determined by the reciprocal changes in the size of the bacterial population and IF available.

Part 3. Nature of B_{12} taken up by microorganisms

(a) The activity of IF was measured after the incubation of IF with microorganisms pre-incubated in the presence or absence of B_{12}. The B_{12}-

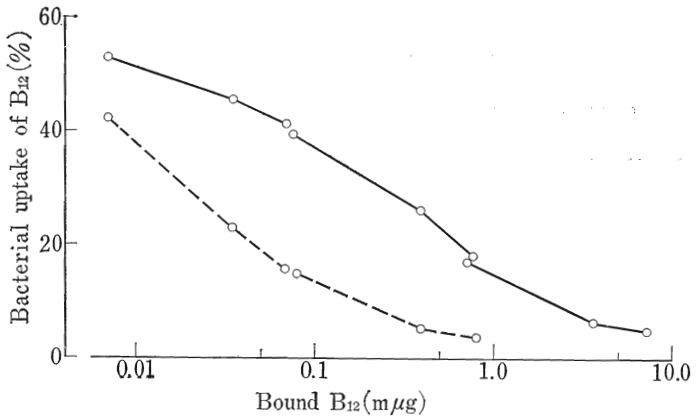

Fig. 5a. Bacterial uptake of bound B_{12}. E. coli (turbidity 240, 8 ml) was incubated with two series of bound B_{12} at 37°C for 2 hours. Bound B_{12} (1:3) and bound B_{12} (1:30) were prepared by mixing 1 volume of B_{12} in 3 and 30 volumes of IF, respectively.

———— series of bound B_{12} (1:3)

— — series of bound B_{12} (1:30)

binding capacity of IF occasionally decreased slightly, but this decrease was not in accordance with the increase in the bacterial population or the duration of incubation period. Therefore, it was not likely that the binding pattern of bound B_{12} to microorganisms was B_{12}-IF-microorganism or IF-B_{12}-microorganism.

(b) B_{12}-saturated IF and E. coli were incubated at 37°C, 40°C and 45°C for 3 hours. The bacterial uptake of B_{12} increased as the incubation temperature was elevated and as the incubation period was prolonged. Free IF activity in the supernatant increased along with the increase in the "bacterial B_{12}". It was also true when Klebsiella was incubated at 37°C and 45°C for 2 hours.

(c) Some of the above observations are shown in Table 5. The radioactivity of free IF in the supernatant increased in accordance with the increased "bacterial B_{12}". The B_{12}-binding capacity of free IF accounted for 96% to 98% of the total unsaturated B_{12}-binding capacity. The ratios of free IF (u) in the supernatant to the "bacterial B_{12}" (mμg) ranged between 82% and 89%.

At time zero, the radioactivity was fairly small and the ratios obtained were not constant. The destruction of B_{12}-saturated IF was 6.6 to 9.5% during this observation and did not increase with the prolongation of incubation time. The B_{12}-binding capacity in the supernatant of the micro-

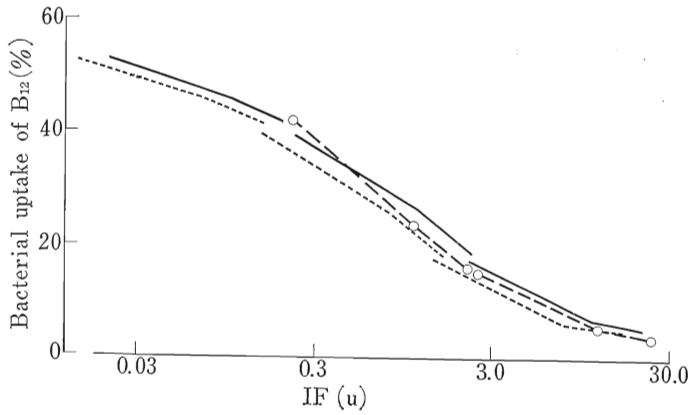

Fig. 5b. The results in Fig. 5a are calibrated by changing the abscissal scale from B_{12} (mμg) to IF (u).
——— total IF in bound B_{12} (1:3)
- - - - - - - - 2/3 of IF in bound B_{12} (1:3)
—— —— total IF in bound B_{12} (1:30)

Table 5. Correlation between the "bacterial B_{12}" and amount of free IF in the supernatant. B_{12}-saturated IF (0.125 u) was incubated with E. coli (turbidity 270, 5 ml) at 40°C for 3 hours. The radioactivity of 19,572 counts per 2 minutes was equivalent to 0.125 mμg of B_{12}. The radioactivity of the background was approximately 100 counts and this value was already subtracted from the counts of the samples.

Incubation time (minutes)	B_{12}-binding capacity in supernatant of micro-organisms	Destruction of B_{12}-IF	Bacterial uptake of B_{12}(A)	B_{12}-binding capacity in supernatant			B—A (%)	(B/C)/A (%)
				Without IFA (B)[1]	With IFA (C)	B–C[2]		
0	228	1,357	347	421	154	208	146	67
	238	1,233	274	483	134			
30		1,762	5,194	4,828	212	4,372	85	82
		1,936	5,514	4,322	192			
60	214	1,806	6,768	5,834	132	5,728	84	83
	141	1,689	6,927	5,917	162			
120		1,740	9,841	9,110	186	8,814	90	89
		1,795	9,943	8,868	163			
180	160	1,698	11,841	10,083	326	9,768	84	82
	144	1,709	11,929	9,898	219			

1) Radioactivity of total unsaturated B_{12}-binding capacity
2) Radioactivity of B_{12}-binding capacity of free IF

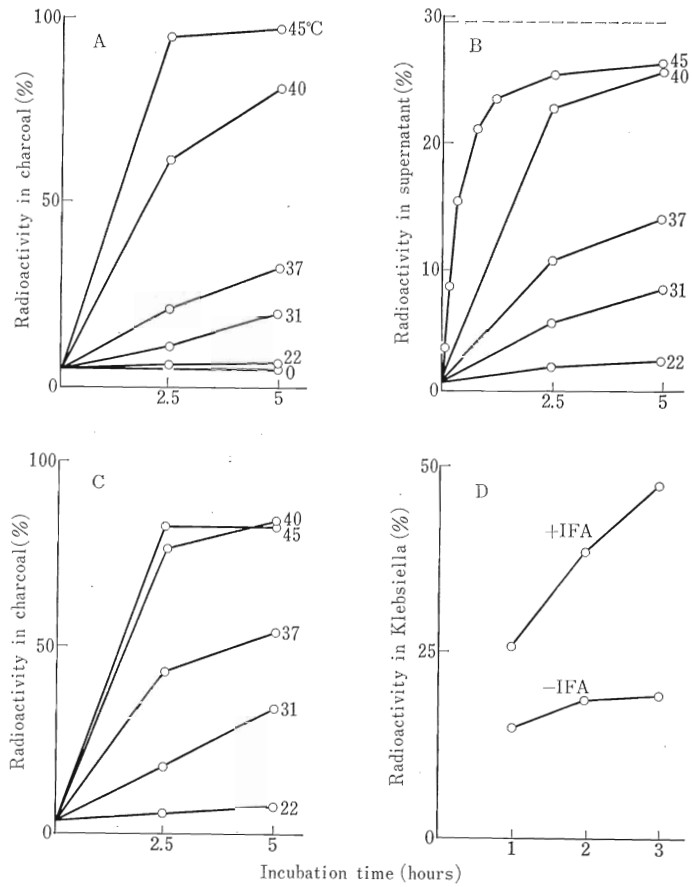

Fig. 6. Stability of B_2-IF complex. The rate of the dissociation of B_{12} from IF was observed by different procedures as described in the text.

Experiment A: labelled B_{12}-saturated IF+cold B_{12}
Experiment B: cold B_{12}-saturated IF+labelled B_{12}
Experiment C: labelled B_{12}-saturated IF+IFA
Experiment D: labelled B_{12}-saturated IF+Klebsiella

organisms, after incubation in the absence of B_{12}-saturated IF, was also negligible.

Part 4. Stability of B_{12}-IF complex

In Experiment A, the radioactivity adsorbed on to the coated charcoal always increased as the incubation temperature was elevated and as the incubation time was prolonged (Fig. 6A). Similar findings were obtained in the supernatant of Experiment B. In these experiments, the destruction of IF appeared to be minimal, because the radioactivity in the supernatant

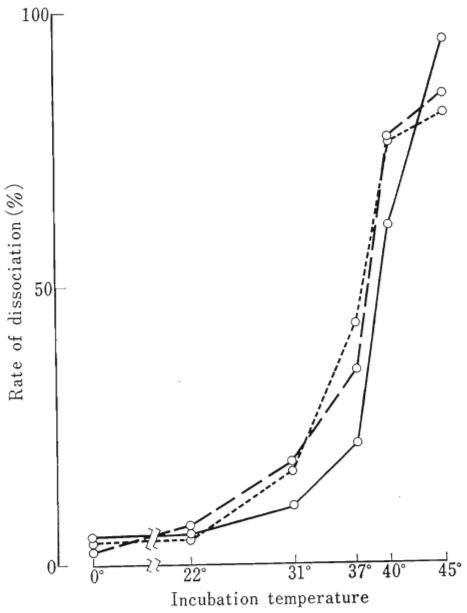

Fig. 7. Dissociation of B_{12}-IF complex. The rate of the dissociation by 150-minute incubation in Fig. 6 is summarized.
Experiment A ———
Experiment B — —
Experiment C - - - - - - - -

increased up to 25.7% by incubation at 45°C (Fig. 6B).

In Experiment C, the radioactivity also increased in the coated charcoal (Fig. 6C). It was suggested that the IF initially bound with labelled B_{12} was liberated and lost its B_{12}-binding capacity after binding with IFA and that the labelled free B_{12} derived from labelled B_{12}-saturated IF remained free of IF as the result of the decrease in the amount of IF.

Klebsiella (turbidity 630, 8 ml) and B_{12}-saturated IF (0.05 u) were incubated at 37°C for 3 hours with and without IFA (0.40 u). The "bacterial B_{12}" by incubation with IFA was greater than that without IFA (Fig. 6D). In this experiment, the "bacterial B_{12}" in the mixture without IFA reached the plateau after a 3 hour-incubation at which time the B_{12}-binding capacity of these microorganisms diminished to 70% of the initial value.

The rate of dissociation after 150-minute-incubation is summarized in Fig. 7. This rate sharply increased when the incubation temperature exceeded 37°C and the binding of B_{12}-IF was relatively stable below 31°C. This was also true when 1.4 u of B_{12}-saturated IF was employed.

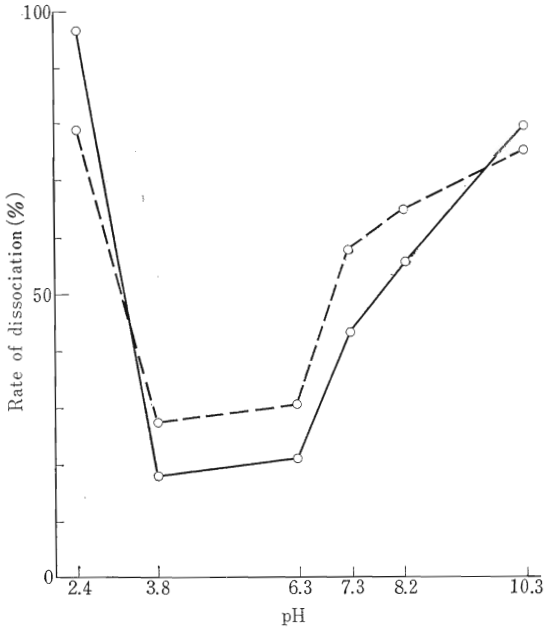

Fig. 8. Effects of pH on the dissociation of B_{12}-IF complex.
Solid lines: labelled B_{12}-saturated IF+cold B_{12}
Broken lines: cold B_{12}-saturated IF+labelled B_{12}

Part 5. Effects of pH

The dissociation of B_{12} from IF was influenced by pH (Fig. 8). The binding of B_{12}-IF was most stable at around pH 3.8. In the range of pH higher than 6.3 the rate of dissociation increased. The rate of dissociation at pH 2.4 was greater than that at pH 3.8. The destruction of B_{12}-saturated IF (0.5 u) was also observed in a higher rate (21.3 %) at this pH. The destruction was minimal (3.6 to 5.7%) at pH above 3.8.

The microorganisms (turbidity 400, 3 ml) were suspended in the same incubation media with various pH and incubated with free B_{12} (100 mμg) and B_{12}-saturated IF (1.4 u). The B_{12}-binding capacity of the microorganisms was lost almost completely at pH 2.4. There was no recognizable difference in the bacterial uptake of B_{12} between pH 3.8 and 10.3 (Fig. 9a), though small decreases in B_{12}-binding capacity of the microorganisms were observed at pH 10.3 in the present experiment and in the other experiments. On the other hand, the bacterial uptake of B_{12} initially bound to IF increased with the elevation of pH. This difference might be attributed to the increased dissociation of bound B_{12} along with the elevation of pH (Fig. 9b).

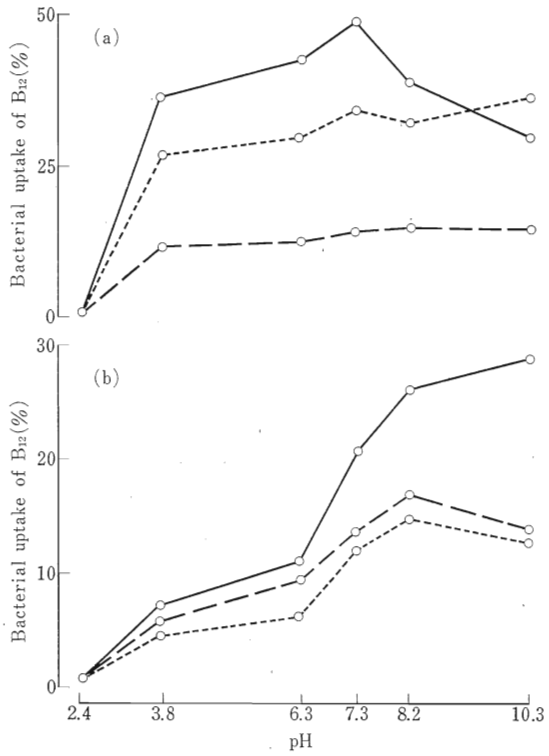

Fig. 9. Effects of pH on the bacterial uptake of B_{12}. Microorganisms (turbidity 400, 3 ml) were incubated at 37°C for 90 minutes with (a) 100 mμg of B_{12} and (b) 1.4 u of B_{12}-saturated IF.
E. coli ————, Klebsiella —— ——, Citrobacter - - - - - - - -

Discussion

The intestinal flora took up free B_{12} remarkably. Recently, it was reported that there is at least a two-stage process in the bacterial uptake of free B_{12}[18]. In the present observations, the bacterial uptake of B_{12} occurred rapidly within 10 minutes and continued slowly up to 26 hours. It was also observed that the B_{12} taken up by the microorganisms could not be removed, and the bacterial uptake of B_{12} was partially inhibited by the pre-administration of another B_{12}. These results also suggested the two phases, slow adsorption after rapid adsorption, in the process of bacterial uptake of free B_{12}. A significant amount of B_{12} was found in the microorganisms when bound B_{12} and microorganisms were incubated. The

amount of B_{12} taken up by the microorganisms in the presence of IF was fairly smaller than that in the absence of IF.

As the next step, several attempts were made to examine the possibility that the microorganisms directly took up bound B_{12} (Part 3). No evidence to support this possibility was obtained, and it was questionable that the microorganisms disturbed IF when incubating in distilled water and saline. The reverse results were reported by Schjönsby and Tabaqchali[19] by incubation in the culture medium, although it is very difficult to measure the activity of IF precisely in the supernatant of the bacterial culture. This difference may come from the incubation medium.

In contrast, the increase in free IF activity in the supernatant was observed in accordance with the increase in the bacterial uptake of B_{12}, when B_{12}-saturated IF was incubated with the microorganisms (Table 5). It seemed that the free B_{12} could be taken up by the microorganisms after the liberation of B_{12} from IF, and that the IF was dissociated from B_{12} without a significant loss in its B_{12}-binding capacity.

There is an interesting report by Donaldson and Katz[26] who demonstrated the exchanges between free and bound B_{12}. The stability of B_{12}-IF complex was influenced by the pH as well as by the temperature (Fig. 6 and 8). The ratios of the "bacterial B_{12}" at 37°C to the amount at 25°C when incubating with bound B_{12} were greater than those ratios when incubating with free B_{12} (Table 1 and 4). This phenomenon could be explained by the fact that the elevation of incubation temperature enchanced the dissociation of B_{12} from IF. The percent uptake of B_{12} reached the plateau regardless of the increase in the bacterial population (Fig. 3). The bacterial uptake of B_{12} was affected by pH (Fig. 9) as was observed by Gräsbeck[27], though his results did not coincide with the authors' results. These observations suggested that the bacterial uptake of bound B_{12} is influenced by the dissociation of B_{12} from IF. On the other hand, it appeared that the B_{12} once taken up by the microorganisms could not be removed from the microorganisms and that the exchange between free B_{12} and the B_{12} taken up by the microorganisms could not occur.

The B_{12}-IF complex could not be taken up directly by the microorganisms, and the IF did not bind with the microorganisms nor with the B_{12} taken up by the microorganisms. These observations suggested that the microorganisms competed with the IF for the same point of B_{12}. This possibility was also supported by the results obtained in Fig. 4-C and 5.

In the present study, there was no suitable observation for the possibility that the microorganisms influenced the stability of the B_{12}-IF complex. However, this possibility could not be denied because the "bacterial

B_{12}" appeared occasionally to be greater than what was expected from the rate of dissociation of the B_{12}-IF complex.

The "bacterial B_{12}" in Experiment 5 was greater than that obtained in Experiment 3 in Table 2. However, when the ratio of free IF to the number of microorganisms was constant, the B_{12}-uptake per microorganisms was constant (Fig. 5). This discrepancy was not explained clearly in the present study.

It might be quite reasonable according to the above-mentioned hypothesis that malabsorption of B_{12} was occasionally improved by the administration of IF in the patients[1,4,8] and in the experimental animals[23]. In these cases, by the supplementation of IF, it appeared that the concentration of IF became sufficient to compete with the microorganisms. On the other hand, there were many cases of B_{12} malabsorption which appeared not to be improved by the supplementation of IF but improved after the administration of antibiotics. In such cases, it seemed that there was so large a number of microorganisms in the blind loop that the supplementation of IF appeared to be of no effect. In the present investigation, a large quantity of additional IF was necessary to overwhelm the activity of the microorganisms to take up B_{12} (Fig. 4 and 5).

There were approximately 10^4 of microorganisms per ml in the contents of the small intestine in the normal subjects[28–31], although it has been considered that the lumen was practically sterile. On the other hand, no suitable explanation has been given to the fact that there were significant amounts of B_{12} (from 20 to 40%) which were not absorbed, when small doses of B_{12} were administrated to the normal subjects. If the unabsorbed B_{12} is attributed to the microorganisms as the resident in the lumen, B_{12} malabsorption in the blind loop syndrome will be explainable by itself.

All microorganisms, which possessed the B_{12}-binding capacity, showed almost the same properties as shown in the present investigation. Therefore, B_{12} malabsorption can be induced by the microorganisms which possess a B_{12}-binding capacity.

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