Absorption of Liver-bound Vitamin $B_{12}$
in Relation to Intrinsic Factor

By Kunio Okuda, Isao Takara and Terumi Fujii

The oral liver therapy for pernicious anemia by Minot and Murphy\(^1\) was a historic contribution which has led to a number of important discoveries. However, the rationale of their liver therapy still remains obscure, in spite of the increasingly clear understanding of the pathophysiology of the disease. Their original regimen prescribed 120–240 Gm. of cooked calf's or beef liver which, considering the loss by cooking, probably represents an equivalent of 20–100 $\mu$g. of bound vitamin $B_{12}$\(^2\) as estimated from the reported figures on beef liver $B_{12}$.\(^3\) With the same amount of free $B_{12}$, one has to use an intrinsic factor concentrate (IFC) to secure consistent and comparable effects.\(^6\)

Nyberg and Reizenstein\(^7\) reported in 1958 that absorption of pig liver-bound $^{60}$Co-$B_{12}$ was much greater than that of cyanocobalamin (CN-$B_{12}$) without using IFC in pernicious anemia patients. Radioactivity in such liver represents a biologically active, co-enzyme form of $B_{12}$.\(^8\) Gräsbeck et al.\(^9\) carried out similar experiments with a small dose of liver in gastrectomized rats and found no absorption, but such result may not be comparable because of the total lack of gastric digestion. Later, working with Sullivan and Herbert with a somewhat smaller dose, Reizenstein failed to reproduce the previous results.\(^11\) No clear explanation has been available, except for a possible delay in the fecal excretion. Shortly after the discovery of the co-enzyme form, suggestion was made that absorption of this form of $B_{12}$ was less dependent on intrinsic factor (IF),\(^12\) but later studies proved otherwise.\(^13,14\)

Some investigators believe that $B_{12}$-peptide complexes are better absorbed than free $B_{12}$ and that the lack of $B_{12}$ absorption in pernicious anemia is due to a defective proteolysis in the stomach.\(^15\) $B_{12}$ is known to exist in tissues in polypeptide bound forms,\(^16,17\) yet there is no proof today that food $B_{12}$ is liberated in the digestive tract free from peptides. It is possible also that complete breakdown of food is not necessary for $B_{12}$ absorption, and various intermediate digestion products containing $B_{12}$ may be formed and absorbed as such. It is to be noted that the present knowledge on IF and $B_{12}$ absorption has been gained, in the main, from studies employing crystalline $B_{12}$.

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In this study, attempts were made to assess the extent of intestinal digestion of liver and absorption of liver-bound B₁₂ in relation to IF in man, and to determine whether or not any active, readily absorbable B₁₂ complex is produced in the process of digestion.

**MATERIALS AND METHODS**

Young adult rats of the Wistar strain were used in the preparation of liver containing radioactive B₁₂ in its native form. ⁵⁷Co-hydroxocobalamin (OH-B₁₂)* with a specific activity of 8 μc./μg. was injected subcutaneously to rats, 20 μg. daily, for a period of two to three weeks. Three days after the last dose, the liver was removed, the parenchyma freed of fibrous tissue was minced, dried in vacuo over P₂O₅ at 4 C., and subsequently pulverized; or the liver was immediately homogenized with distilled water in a glass homogenizer. The isotopic dilution of ⁵⁷Co-B₁₂ activity in such materials was fifty- to ninetyfold as determined by the microbiologic assay using the Skeggs' medium and Lactobacillus leichmanii 4797,18 and by the radiometric measurement with a gamma scintillation counter. The same ⁵⁷Co-OH-B₁₂ as well as ⁵⁷Co-cyanocobalamin (CN-B₁₂)* with a specific activity of 12 μc./μg. were used in man for absorption studies, together with a hog IF₃ which is active in pernicious anemia patients at a dose of 5 mg. or less.

Five pernicious anemia patients, 11 total- and 2 subtotal-gastrectomized subjects, and 23 subjects with no major illness or gastrointestinal disorder were used in human studies. The pernicious anemia patients had exhibited typical laboratory findings, and in the gastrectomized the minimal interval after operation was 20 days.

To study the digestibility of liver in vitro, rat gastric juice as well as three proteolytic enzyme preparations, i.e., crystalline pepsin and trypsin (Schwarz Bioresearch, Inc.) and Pronase-P* were used. Gastric juice as collected from the stomach of rats 5 hr. after operative ligation of the pylorus. Liver powder was suspended at a concentration of 50 mg./ml. in gastric juice, in 1/20 N HCl with pepsin, or in 1/15 M phosphate buffer of pH 7.2 with one of the latter two enzymes, and incubated. The amount of enzyme used was 1/100 of the liver by weight, or 4 mg. for 8 ml. of 10 per cent homogenate. The extent of digestion was estimated from the increases of radioactivity in the supernatant after centrifugation at 3,000 rpm for 15 min., and in the dialyzable portion. Dialysis of the supernatant was carried out in Visking cellophane tubings against large quantities of water in the cold room for 48 hr. with frequent changes of outside water.

For an in vivo study on the digestibility of liver, nonradioactive liver was prepared by heating fresh cow liver at 60 C. for 30 min. in an oven, drying and grinding it. To 10 Gm. of this powder was added 0.2 μg. of ⁵⁷Co-CN-B₁₂ in 1 ml., the powder was dried, repulverized, and fed by mouth to test subjects. The control subjects received the same amount of ⁵⁷Co-CN-B₁₂ with or without graded doses of nonradioactive OH-B₁₂. It was assumed that the radioactive B₁₂ was not bound, and the reduction in its absorption by Schilling test in the test subjects compared with the control receiving radioactive B₁₂ alone, was due to the release of B₁₂ from liver, the graded doses of OH-B₁₂ serving as the reference.

For the analysis of digestion products, 4 ml. of fresh 20 per cent homogenate of radioactive liver was digested at 37 C. with 4 mg. of Pronase-P for 1 and 3 hr. in a cellophane tube while being dialyzed against 10 ml. of distilled water in a test tube under constant agitation. Dialysis was continued in the cold for 12 hr. after digestion. The outside dialysate was lyophilized and subjected to biochemical analyses after being dissolved in a small quantity of distilled water. As the control material, the same procedure was carried out

*Kindly supplied by Dr. K. C. Mezey, Merck Co., Rahway, N. J.
†WES #942, kindly supplied by Dr. L. Ellenbogen, Lederle Laboratories, Pearl River, N. Y.
*With activity of 45,000 PUK/Gm., purchased from Kalsen Chemical Co., Ltd., Bunkyo-Ku, Tokyo.
Fig. 1.—Digestion of rat liver containing $^{57}$Co-B$_{12}$ with Pronase and trypsin. Four mg. of the enzyme was mixed with 8 ml. of 10 per cent homogenate and incubated. The solid lines represent radioactivity in the supernatant, and the broken lines, dialyzable radioactivity.

except that the liver was not radioactive and $^{57}$Co-OH-B$_{12}$ in comparable counts was added to the dialysate before lyophilization.

Paper electrophoresis was carried out by the horizontal method for 3 hr. using 40 by 3 cm. strips of Toyo filter paper #51 with a potential gradient of 12 cm., two-dimensional chromatography on a 60 by 60 cm. sheet of the same quality paper by the descending method using 4:2:1 mixture of H$_2$O, butanol and acetic acid for development, and gel filtration with Sephadex G-15 in a 15 by 15 by 65 mm. column using 1/10 M acetate buffer of pH 4.75 as eluant. The localization of peptides was determined by the ninhydrin reaction and of radioactivity by gamma measurement. Paper was cut in 0.5 cm. wide pieces from the electrophoreogram, or in 1 cm. squares from the chromatogram for gamma counting, and the effluent from the column was collected, 5 ml. per tube, for ninhydrin reaction and
Fig. 2.—Paper electrophoresis of the dialyzable digestion products of rat liver containing $^{57}$Co-B$_{12}$. Four ml. of 20 per cent homogenate was digested with 4 mg. of Pronase for 1 hr. The first and third strips are controls, and third and fourth show only radioactivity distribution.

radioactivity measurement. These procedures were carried out with minimal natural light, but no strict measure was taken to avoid light completely.

Absorption of $^{57}$Co-labeled liver, OH-B$_{12}$ and CN-B$_{12}$ was measured either by the fecal excretion test or the Schilling test. In the former, feces were collected for 5 days, each sample was thoroughly homogenized with water and weighed aliquots in the volume of 100 ml. in duplicate were transferred to bottles for counting. Since no subjects with constipation were used, the last sample had only negligible counts to warrant cessation of fecal collection. In the latter test, urine was collected for 24 hours, one-half volume was condensed down to about 100 ml., and 5 ml. aliquots in triplicate were counted.

RESULTS

In Vitro Digestion of Liver

Three hundred mg. of liver powder containing $^{57}$Co-B$_{12}$ was divided into two equal portions. One was mixed with 3 ml. of rat gastric juice, and the other with 3 ml. of water, incubated for 3 hr., and centrifuged. The supernatant after digestion contained about 90 per cent of the original radioactivity as compared to 60 per cent in the control, of which about 85 per cent was nondialyzable. The exact amount of radioactivity rendered dialyzable by digestion could not be measured by dialysis because of the binding power of gastric juice, but it was estimated at 70 per cent taking into account the latter in terms of m$\mu$g. The same amount of liver powder was then digested with pepsin in 6 ml. of 1/20
Fig. 3.—Two-dimensional chromatogram of digested and dialyzed rat liver containing $^{57}$Co-B$_{12}$. The conditions for digestion are the same as Figure 2.

N HCl for 30 min. and 3 hr., centrifuged, the supernatant was neutralized and subjected to radioactivity measurement and dialysis. It was found that the supernatant contained 85 per cent and 90 per cent each, of the total radioactivity, of which about 75 per cent (3/4) and 90 per cent, respectively, were ultrafilterable. Digestion with trypsin gave a similar result except that the extent of digestion was about 10 per cent less than that with pepsin.

Pronase was then used on liver homogenate in comparison with trypsin. This enzyme was used, because of its high potency, to simulate natural intestinal digestion which involves many proteases and peptidases. Eight ml. of fresh 10 per cent liver homogenate containing radioactive B$_{12}$ was incubated with 4 mg. of trypsin or Pronase for 0, 1, 2, 3, and 5 hr., an aliquot was removed each time, immediately centrifuged, and the supernatant was measured for radioactivity directly before and after dialysis. The result (Fig. 1) indicated that fresh liver homogenate was readily but somewhat less efficiently digested than liver powder by trypsin, and that Pronase was more potent, releasing in 5 hr., about 91 per cent of radioactivity in the supernatant, most of which was dialyzable.

Chemical Identification of Radioactivity in Digested Liver

The dialyzable digestion product condensed by lyophilization was applied to two strips and electrophoresed together with two control strips, one with a concentrated aqueous solution of nonradioactive OH-B$_{12}$ (500 μg./ml.) and the other with the control dialysate with added $^{57}$Co-OH-B$_{12}$. Figure 2 illustrates such an electrophoreogram of the dialysate of 1 hr. digestion product. The locality of the major portion of radioactivity of the test material corresponds well with OH-B$_{12}$, and no separate band of radioactivity was detected. Furthermore,
the peak of radioactivity was slightly off the closest visible ninhydrin band. With 3 hr. digestion product, similar patterns were obtained except that the anodic mobility of two major ninhydrin bands was greater, and again, the radioactivity was located at exactly the same distance as the control OH-B₁₂, but off ninhydrin bands.

Figure 3 represents the two-dimensional chromatogram of the test material after digestion for 1 hr. The control material containing added $^{57}$Co-OH-B₁₂ gave exactly the same pattern. The location of radioactivity was off ninhydrin spots and the same as that of free OH-B₁₂ in the control run. The chromatography with a Sephadex G-15 column is shown in Figure 4 which indicated early filtration of radioactivity in one peak followed by ninhydrin-reacting fractions. The control material with added $^{57}$Co-OH-B₁₂ gave exactly the same pattern.

*Human Absorption Study*

Radioactive rat liver powder in the dose of 1.2 Gm. or 0.5 μg. of B₁₂ by the microbiological assay was placed in a capsule and fed to fasting test subjects, 3 with pernicious anemia, 6 total gastrectomy, and 2 subtotal gastrectomy. Absorption of liver radioactivity was compared in each subject with that of 0.5 μg. of $^{57}$Co-OH-B₁₂ given by mouth after an interval of at least 10 days, as determined by the fecal test. The result (Table 1) showed that absorption of the test material was just as poor as that of OH-B₁₂ in these subjects except in the two subtotal and one total gastrectomized who exhibited subnormal absorption with both materials. The average absorption of the liver B₁₂ and OH-B₁₂ were about the same. In some of these and other subjects, the test for absorption of liver
Table 1.—Absorption of Liver-Bound $^{67}$-B$_{12}$ and Aqueous $^{57}$Co-OH-B$_{12}$ by Pernicious Anemia Patients and Gastrectomized Subjects

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Interval After Operation</th>
<th>Absorption (% by Fecal Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver B$_{12}$*</td>
</tr>
<tr>
<td>N.M.</td>
<td>70 yr. F</td>
<td>20 days</td>
<td>21.6</td>
</tr>
<tr>
<td>T.W.</td>
<td>66 yr. M</td>
<td>23 days</td>
<td>17.3</td>
</tr>
<tr>
<td>Y.M.</td>
<td>65 yr. F</td>
<td>—</td>
<td>20.6</td>
</tr>
<tr>
<td>H.H.</td>
<td>55 yr. M</td>
<td>4.5 yr.</td>
<td>21.3</td>
</tr>
<tr>
<td>C.W.</td>
<td>44 yr. F</td>
<td>10 mo.</td>
<td>15.0</td>
</tr>
<tr>
<td>S.O.</td>
<td>44 yr. M</td>
<td>1.5 yr.</td>
<td>20.8</td>
</tr>
<tr>
<td>Y.I.</td>
<td>68 yr. M</td>
<td>1.5 yr.</td>
<td>46.8</td>
</tr>
<tr>
<td>Y.N.</td>
<td>66 yr. F</td>
<td>1.5 yr.</td>
<td>16.7</td>
</tr>
<tr>
<td>A.T.</td>
<td>67 yr. F</td>
<td>—</td>
<td>5.3</td>
</tr>
<tr>
<td>K.W.</td>
<td>40 yr. M</td>
<td>10 yr.</td>
<td>57.2</td>
</tr>
<tr>
<td>S.E.</td>
<td>61 yr. M</td>
<td>8 yr.</td>
<td>35.3</td>
</tr>
<tr>
<td>Average</td>
<td></td>
<td></td>
<td>29.7 ± 5.1†</td>
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</tbody>
</table>

*0.5 µg. or equivalent.
†Standard error.

Table 2.—Effect of Oral Administration of Hog Intrinsic Factor Concentrate on Liver-Bound $^{57}$Co-B$_{12}$ by Pernicious Anemia and Agastric Patients

<table>
<thead>
<tr>
<th>Patient</th>
<th>Disease</th>
<th>Interval After Operation</th>
<th>Absorption (% by Fecal Test)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Liver B$_{12}$*</td>
</tr>
<tr>
<td>K.I.</td>
<td>68 yr. M</td>
<td>20 days</td>
<td>12.6</td>
</tr>
<tr>
<td>Y.S.</td>
<td>26 yr. M</td>
<td>25 days</td>
<td>17.9</td>
</tr>
<tr>
<td>H.M.</td>
<td>55 yr. M</td>
<td>4.5 yr.</td>
<td>21.3</td>
</tr>
<tr>
<td>C.W.</td>
<td>44 yr. F</td>
<td>10 mo.</td>
<td>15.0</td>
</tr>
<tr>
<td>M.N.</td>
<td>66 yr. M</td>
<td>2.5 yr.</td>
<td>18.8</td>
</tr>
<tr>
<td>A.T.</td>
<td>67 yr. F</td>
<td>—</td>
<td>5.3</td>
</tr>
<tr>
<td>S.M.</td>
<td>66 yr. M</td>
<td>3 yr.</td>
<td>8.6</td>
</tr>
<tr>
<td>T.T.</td>
<td>60 yr. F</td>
<td>20 days</td>
<td>6.8</td>
</tr>
<tr>
<td>H.O.</td>
<td>69 yr. M</td>
<td>—</td>
<td>8.0</td>
</tr>
</tbody>
</table>

*10 mg. of WES #942.

B$_{12}$ was repeated with 10 mg. of IFC; IFC was given together with the test material in two subjects, given in a capsule 1 hr. prior to the test dose in five, and 2 hr. prior to the test dose in two. Absorption of liver B$_{12}$ was markedly increased in all except in one who received IFC 2 hr. prior to the dose (Table 2).

Estimation of In Vivo Digestion of Liver

To estimate the digestion of liver in human digestive tract, the characteristic dose-absorption relationship in B$_{12}$ absorption was utilized. Ten Gm. of cow liver powder containing 10.3 µg. of native B$_{12}$ and 0.2 µg. of added free $^{57}$Co-CN-B$_{12}$ was fed to 6 normal subjects in capsules, and absorption measured by the Schilling test was compared with that obtained with the same amount of $^{57}$Co-CN-B$_{12}$ or the same diluted with 2, 8, and 25 µg. of nonradioactive OH-B$_{12}$ in aqueous solutions. Five to 6 normal subjects were used in each control group.
Fig. 5.—Twenty-four hour urinary excretions for varying oral doses by the Schilling test. The oral doses on the abscissa are 0.2 μg. of $^{57}$Co-CN-B$_{12}$ and the same diluted with 2, 8, and 25 μg. of nonradioactive OH-B$_{12}$. The cross denotes the average in the test group which received the same dose of $^{57}$Co-CN-B$_{12}$ mixed with 10 Gm. of cow liver; the vertical bars, standard deviations.

The urinary excretion curve for the control groups at these dilution levels is shown in Figure 5. It is clear that dilution of $^{57}$Co-CN-B$_{12}$ with nonradioactive OH-B$_{12}$ resulted in a sharp decline in absorption in per cent. The average urinary excretion in the test group was 5.1 ± 0.9 per cent (S. D.), and if the dose corresponding to this figure was read off the curve, it was somewhere between 8 and 9 μg. It was assumed that absorption of $^{57}$Co-CN-B$_{12}$ which was about 28 per cent (in urine) without liver was reduced to 5.1 per cent by free OH-B$_{12}$ liberated from liver during digestion, and that its amount was about 8 μg.

**Discussion**

Absorption of a small amount of rat liver was poor and not superior to that of OH-B$_{12}$ of a comparable dose, as measured in pernicious anemia patients and total-gastrectomized subjects, and addition of IFC markedly enhanced absorption. This finding is consistent with the report of Sullivan et al.,$^{11}$ and our separate study in rats likewise showed the lack of evidence for greater absorbability of liver B$_{12}$.$^{21}$ It is of interest to note that IFC given by mouth 1 hr. prior to the dose in these subjects also increased absorption of liver B$_{12}$. Castle and Ham$^{22}$ earlier observed that the administration of gastric juice and beef muscle could be separated as long as 6 hr. for hematologic response. Apparently, IFC retained its activity in the intestine for at least 1 hr. and aided absorption of oncoming liver B$_{12}$; our previous study in man suggested the presence of IF activity in the lumen of the small bowel.$^{23}$
The attempt to detect in the dialyzable portion, active B\textsubscript{12}-peptide complexes produced in the process of liver digestion failed, and all three methods for chemical separation revealed a single component of radioactivity which corresponded to OH-B\textsubscript{12} in mobility and molecular size. It was not unexpected because of the instability of free co-enzyme B\textsubscript{12} which is readily converted to OH-B\textsubscript{12}\textsuperscript{24} and our unpublished data also suggested that a large portion of the former is converted to OH-B\textsubscript{12} in the intestinal lumen. These findings do not preclude the presence of other B\textsubscript{12}-containing products in the test material. However, if present, they were so minute in quantity as to elude detection and could by no means alter the over-all absorption pattern. The discrepancies between the earlier observations by Nyberg and Reizenstein\textsuperscript{7,25} and our result are difficult to explain, and it seems rather unlikely that they were simply due to the differences in dosage or speed of intestinal passage.

The easy digestibility of liver has already been demonstrated\textsuperscript{26}, and one could argue that Pronase digestion was too intense hydrolyzing all the natural B\textsubscript{12}-peptide complexes. In human physiology, ingested food is subjected first to peptic and then to intestinal digestion involving many hydrolytic enzymes, and such natural digestion may be more efficient than test tube conditions employing a single enzyme preparation. Under our conditions, the in vitro digestion with Pronase was not complete in 1 hr., and the same time interval was used for the preparation of the test material. It might be that for B\textsubscript{12} or its analogs to be bound by a protein, the protein molecule has to have certain size to hold this relatively large vitamin in place and, as soon as the size of protein is reduced beyond certain point by proteolysis, it can no longer hold B\textsubscript{12} and releases it. In line with this concept is our earlier observation that the digestion product of IF-B\textsubscript{12} complex contained only free B\textsubscript{12}\textsuperscript{27}.

The in vivo assessment in man of digestion of cow liver in terms of liberation of B\textsubscript{12} suggested that most of the extractable B\textsubscript{12} activity was released free in the digestive tract. This kind of estimation is only feasible when per cent absorption declines sharply as the dose increases. The reason for the use of \textsuperscript{57}Co-CN-B\textsubscript{12} diluted with nonradioactive OH-B\textsubscript{12} was that the urinary excretion of the former is greater and better documented, and that there is practically no difference or preference between the two with respect to absorption\textsuperscript{28,29}.

Considering the poor digestion in achlorhydria and poor absorption of B\textsubscript{12} in the lack of IF in pernicious anemia, it may be that the immediate hematologic response to the liver therapy observed by Minot and Murphy\textsuperscript{4} was accounted for by absorbed liver folate or by a combined effect of liver B\textsubscript{12} and folate. It is interesting to note in their original communication that in the 45 patients they studied, only one had advanced myelopathy and "he remained the least well" after 3 months of treatment.

**Summary**

Rat liver containing radioactive native B\textsubscript{12} was prepared by repeated injections of \textsuperscript{57}Co-OH-B\textsubscript{12}, and absorption of liver B\textsubscript{12} was measured in patients with pernicious anemia and in subjects without stomach, using physiologic doses. It was found that absorption of liver B\textsubscript{12} was very poor, not superior to
that of free OH-B12, and coadministration of IFC markedly enhanced absorption.

In vitro digestion of rat liver with several enzymes, as determined from liberation of dialyzable radioactivity, suggested its easy digestibility. Biochemical studies of the dialyzable products of liver containing 57Co-B12 failed to demonstrate any detectable quantities of radioactivity other than free 57Co-OH-B12. A study in which cow liver powder mixed with a small quantity of 57Co-CN-B12 was fed to humans and digestion of liver was estimated from the reduction in absorption of radioactivity, indicated that most of the extractable liver B12 was liberated free in the intestine. Thus, no evidence has been obtained for the production of B12-peptide complexes from liver by digestion that require no IFC for absorption.

SUMMARIO IN INTERLINGUA
Hepate de ratto a contento de radioactive vitamina B12 native esseva preparate per le injection repetite de 57Co-OH-B12. Le absorption de B12 hepatic esseva mesurate in patientes con anemia perniciose e in subjectos sin stomacho, con le utilisation de doses physiologic. Esseva constatate que le absorption de B12 hepatic esseva multo basse, certo non superior a illo de libere OH-B12. Le coadministration de IFC meliorava le absorption marcatemente.

Le digestion in vitro de hepate de ratto per varie enzymas—determinate a base del liberate radioactivitate dialysabile—suggestionava le prompte digestibilitate del preparato. Studios biochimic del dialysabile productos de hepate a contento de 57Co-B12 non demonstrava ulle detegibile quantitates de radioactivitate a parte le libere 57Co-OH-B12. Un studio in que pulvere de hepate bovin, mixte con un micre quantitate de 57Co-CN-B12, esseva administrate a subjectos human e in que le digestion de hepate esseva estimate a base del reduction in le absorption de radioactivitate indicava que le plus grande parte del extrahibile B12 hepatic esseva liberate ad in le intestino. Assi, nulle evidentia esseva obtenite in supporto del these de un production de complexos de B12 e peptidas ab hepate per un digestion que non require IFC pro le absorption.

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