ON THE METABOLISM OF COENZYME B₁₂
III. CONVERSION PROCESS AND RATE FROM CYANOCOBALAMIN AND HYDROXOCOBALAMIN INTO COENZYME B₁₂ IN THE BODY¹

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Since the coenzyme B₁₂ was discovered as an active form of vitamin B₁₂ (B₁₂) by Barker *et al.* (1), its biochemical properties including its coenzymic roles, and its metabolism have been studied in many respects. Today it is considered that vitamin B₁₂ in the liver exists in a coenzyme form (2), 5,6-dimethyl benzimidazolyl cobamide coenzyme (DBCC), which plays an important role in converting methylmalonyl CoA into succinyl CoA in mammalian cells (3, 4).

On the other hand, studies on the enzymic biosynthesis of DBCC from B₁₂ derivatives were made by Pawelkiwicz *et al.* (5) and Bernhauer *et al.* (6), who showed that the extract of *Propionibacterium shermanii* could catalyze the conversion from aquocobalamin to coenzyme B₁₂ in the presence of ATP and Mg. Barker and Brody *et al.* (7, 8) obtained the enzyme for biosynthesis using the same bacterium and searched for the substrate and cofactor necessary for biosynthesis. Weissbach and Peterkofsky *et al.* (9-11) succeeded in the biosynthesis of DBCC using the cell-free extract of *Clostridium tetanomorphum*. On the other hand, Gorna (12) and Pawelkiwicz *et al.* (13) reported that the homogenate of liver and kidneys could convert cyanocobalamin (CN-B₁₂) into DBCC *in vitro*. However, only short reports have been made on the conversion process in the body, of which Fenrych (14) used rabbits, and Martson (15) used the sheep.

The present author (16) used animal tissues for the study of fractional determination of CN-B₁₂, OH-B₁₂, and DBCC and further studied the conversion process leading to DBCC in the liver and kidneys from ⁵⁷Co-CN-B₁₂ or ⁵⁷Co-OH-B₁₂ injected intravenously into rats or human subjects. He also examined the conversion process in vitamin B₁₂-deficient state and in the liver injured by carbon tetrachloride and observed how the conversion process was affected by intrinsic factor (IF) known to promote which is the organ uptake of B₁₂.

EXPERIMENTAL

1. Experimental Animals and Subjects.

Rats of Wister strain, weighing 150 to 200 g, were used as the test animal.

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As the human subject, the patients subjected to gastrectomy and splenectomy at the Department of Surgery, Kyoto University were used.

2. Radioactive Compounds.

The radioactive B₁₂ used were ⁵⁷Co-CN-B₁₂ (the specific activity 7.40 μc/μg) and ⁵⁷Co-OH-B₁₂ (the specific activity 2.15 μc/μg.)

3. Administration Methods.

Rats received injections of 100 μg of radioactive B₁₂ in the tail vein with or without IF. Two to 4 μg of radioactive B₁₂ alone was administered to the patients in the cubital vein 12, 24, and 48 hours respectively before operation. The amount of B₁₂ used was enough to make the measurement easier, in spite of the dose higher than the physiological one.

4. Fractional Determination of Vitamin B₁₂ Derivatives.

The liver and kidneys of the rats bled to death after a specified time were used as the samples. The samples of human subjects were obtained from a part of the liver taken during the operation. The method employed was the same as mentioned before (19): acetone powder of the tissue sample was extracted with ethanol.

5. B₁₂-Deficient Rats and Carbon Tetrachloride-Injured Rats.

The same method was used as reported in the previous paper (19).

6. Adjustment of Column.

To phosphate-cellulose were added the same amounts of acetone and 2 N NaCl solution, and the mixture was stirred for 10 to 12 hours. The P-cellulose was taken out of the solution and washed well with distilled water. It was packed in a tube (1×10 cm) and bufferized with 0.1 N acetate buffer, pH 3.5.

7. Fractionation.

The column was placed in the dark room. The extract of liver or kidneys was poured on the column and the flow rate was adjusted to 1 ml per minute. Elution was made by Heinrich's method (20) as shown in Fig. 1. First, the extract were eluted with 20 ml of distilled water, collecting in several different test tubes in 2 ml each. These fractions were called A fractions. Then, elution was made with 30 ml of 0.05 M acetate buffer, pH 3.0, collecting in test tubes as given above. Thus fraction B was obtained. Finally, elution with 20 ml of 0.1 M ammonium acetate, pH 6.9, was made and fraction C was obtained. Flow rate was kept at 1 ml per minute for all the fractionations.

8. Measurement of Radioactivity.

A spectrometer EC-17-C and a well-type scintillation counter with a thallium-activated NaI crystal of 2×2" manufactured by Shimadzu Co. Ltd. were used. The data obtained for each fraction are shown in Fig. 2.

Elution: with aqua. dest.-CN-B₁₂
with 0.05 M CH₃COOH-
OH-B₁₂ (pH 3.0)
with 0.1 M CH₃COONH₄-
DBCC (pH 6.9)

FIG. 1 System for the Chromatographic Separation of CN-B₁₂, OH-B₁₂ and DBCC

FIG. 2 Chromatography of $CN-B_{12}$, $OH-B_{12}$ and DBCC on *P-Cellulose* Column, 1×10 cm; fraction volume, 2 ml.

9. Calculation.

The conversion rate from $CN-B_{12}$ or $OH-B_{12}$ to DBCC was defined to be equal to the percentage of the radioactivity count of the isotopes of the C fraction to the total count, which consists of the counts of all the fractions plus that of the isotopes remaining in the column. The converted quantity can be obtained by multiplying this conversion rate by the mean value of the uptake of liver or kidneys reported in the previous paper (21).

RESULTS

1. Separation and Identification of $CN-B_{12}$, $OH-B_{12}$, and DBCC by Column Chromatography.

Both absorption spectra and counting of the radioactivity of the paper strip electrophoretogram were used to identify the eluted fractions. First of all, the non-labeled $CN-B_{12}$, $OH-B_{12}$, and DBCC of such high concentration as 500,500 and 300 $\mu\text{g/ml}$, respectively, were mixed together, and the fractions A, B, and C were obtained by elution. The test tube of the strongest red tone was chosen from the fractions to observe the absorption spectrum with a spectrophotometer, Cary Model 15, as shown in Fig. 3. The A fraction showed the absorption spectrum of $CN-B_{12}$, the B fraction that of $OH-B_{12}$, and the C fraction that of DBCC.

Then, the test tubes showing the strongest radioactivity were chosen from the fractions, on the basis of the preparative and analytical chromatography as shown in Fig. 2. The chosen extract was concentrated and spread on the paper strip, on which the solutions of crystalline $CN-B_{12}$, $OH-B_{12}$ buffer, and DBCC were also applied. It was submitted to electrophoresis in acetate, pH 2.7, with a field strength 16 V/cm for three and a half hours. Then, the paper was dried and cut

FIG. 3 Absorption Spectra of CN-B₁₂, OH-B₁₂ and DBCC after P-Cellulose Chromatographic Separation Determined with a Spectrophotometer, Cary Model 15

1, CN-B₁₂ eluted with aq. dest. (260, 360, 540 mμ); 2, OH-B₁₂ eluted with 0.05 M acetate (351, 510, 540 mμ); 3, DBCC eluted with 0.1 M ammonium acetate (278, 525 mμ).

into pieces, 0.5 cm wide. The radioactivities of these pieces were measured and the results are shown in Fig. 4. The A, B and C fraction showed the same electrophoretic patterns as those of crystalline CN-B₁₂, OH-B₁₂, and DBCC, respectively.

It is thus proved that among the vitamin B₁₂ derivatives adsorbed on the column, CN-B₁₂ is first eluted with distilled water followed by OH-B₁₂ by elution with 0.05 M acetate buffer, and finally DBCC by elution with 0.1 M ammonium acetate, pH 6.9.

2. Production of DBCC *in vivo*

When the rat received an injection of 100 mμg of DBCC, most of the radioactivity was found in the C fraction and little in the A and B fractions. The conversion rate from CN-B₁₂ and OH-B₁₂ were then measured.

Normal Rats—The conversion rate from CN-B₁₂ and OH-B₁₂ to DBCC in the liver and kidneys of normal rats are shown in Table 1.

Liver: The conversion rate of CN-B₁₂ in the liver were on the average 11.3, 19.4, 26.9, and 36.2 % and those of OH-B₁₂ 16.1, 27.1, 43.5 and 52.1 % 3, 12, 24, and 48 hours after the injection, respectively. In both cases the rate showed an

FIG. 4 Paper Electrophoresis of CN-B₁₂, OH-B₁₂ and DBCC in 0.5 N Acetate, pH 2.7 (16 V/cm, 3.5 hr)

A, OH-B₁₂; B, coenzyme B₁₂; C, CN-B₁₂.

TABLE 1
Conversion Rate of CN-B₁₂ and OH-B₁₂ to DBCC in Normal Rat Liver and Kidneys

	3 hr		12 hr		24 hr		48 hr	
	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂
Liver	11.9	15.3	19.6	24.8	26.3	46.9	35.9	52.7
	10.7	16.9	19.1	29.4	27.6	40.1	36.6	51.5
Mean	11.3	16.1	19.4	27.1	26.9	43.5	36.2	52.1

	12 hr		24 hr		48 hr	
	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂
Kidneys	8.1	11.6	12.6	21.7	17.5	26.3
	7.9	10.8	11.5	17.6	18.4	30.3
Mean	8.0	11.2	12.1	19.7	18.0	28.3

Conversion rate is defined to be equal to the percentage of the radioactivity count of isotopes of the C fraction to the total count.

increase with time. It should be noted that OH-B₁₂ which apparently plays a more important role in the metabolism of B₁₂ than CN-B₁₂ showed a higher conversion rate than CN-B₁₂. The difference in the rate became larger with time.

Kidney: The conversion rate of CN-B₁₂ in the kidneys was on the average 8.0, 12.1 and 18.0 %, while those of OH-B₁₂ 11.2, 19.7 and 28.3 % 12, 24 and 48 hours after the injection, respectively. The rate in this case also showed an increase with time, but it was about the half of that in the liver.

Vitamin B₁₂-deficient Rats—The conversion rate of CN-B₁₂ and OH-B₁₂ in the liver of the vitamin deficient rats are shown in Table 2. The conversion rates of CN-B₁₂ 3 and 12 hours after the injection were on the average 13.4 and 21.1 %, while those of OH-B₁₂ 29.5 and 35.0 %, respectively. In other words, the conversion rate for the vitamin deficient rat is remarkably high compared with that for normal rats. The quantities of CN-B₁₂ and OH-B₁₂ actually converted in such a case must be higher than those calculated from the conversion rate, because the hepatic uptake of the vitamin deficient rat was also higher as reported in the previous paper (21).

Carbon Tetrachloride-Injured Rat—The Rats injured by the intraperitoneal injections of carbon tetrachloride received intravenous injections of 100 mμg

TABLE 2
Conversion Rates of CN-B₁₂ and OH-B₁₂ to DBCC in B₁₂ Deficient Rat Liver

Time after adm.	CN-B ₁₂		OH-B ₁₂	
	Control	Deficient	Control	Deficient
3	12.2	13.8	16.9	29.6
	10.8	13.0	16.1	29.3
Mean	11.5	13.4	16.5	29.5
12	21.3	21.2	27.6	39.7
	20.8	21.0	25.3	30.3
Mean	21.0	21.1	26.7	35.0

TABLE 3
Conversion Rates of CN-B₁₂ and OH-B₁₂ to DBCC in CCl₄-Injured Rat Liver

	6 hr after injury		12 hr after injury	
	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂
Mean	7.9	11.5	6.6	9.5
	8.9	13.3	6.8	11.6
	8.0	12.4	6.7	10.8

of CN-B₁₂ or OH-B₁₂ 6 and 12 hours after the liver damage respectively. The conversion rate for these two groups was measured 3 hours after the injections and the results are shown in Table 3.

The conversion rate of CN-B₁₂ was 8.0 and 6.7 % for the group receiving injections 6 and 12 hours after the liver damage, respectively, while that of OH-B₁₂ 12.4 and 10.8 % for the 6-hour and 12-hour groups respectively. These values were always lower than those of normal rats. There are two interesting points to be noted. One is that no significant difference in the conversion rate of OH-B₁₂ was noted between the normal and injured ones, while a rather larger difference between the two in the conversion rate of CN-B₁₂ was recognized. The other point is that the rats belonging to the 12-hour injection group had a higher uptake, showing a kind of recovery from the liver injury, but a lower conversion rate than those belonging to the 6-hour group.

The Effect of IF—Table 4 shows the conversion rate and the converted quantity of CN-B₁₂ or OH-B₁₂ in normal rats when the IF and the cobamides in question were simultaneously administered. In the case of such administration with IF, CN-B₁₂ and OH-B₁₂ showed lower conversion rates, but they were converted much more strongly into DBCC than in the case of single administration.

Production of DBCC in the Human Liver—The conversion rate in the human liver is summarized in Table 5.

TABLE 4
Conversion Rates of CN-B₁₂ and OH-B₁₂ to DBCC in Liver of Normal Rats Following Administration with or without IF

	CN-B ₁₂		OH-B ₁₂	
	Alone	With IF	Alone	With IF
After 3 hr				
Uptake by liver (mμg)	5.3	44.0	10.2	73.5
Conversion rate (%)	11.3	8.0	16.1	9.1
Absolute value of DBCC (mμg)	0.60	3.52	1.64	6.69
After 12 hr				
Uptake	4.9	21.4	15.0	63.6
Conversion rate	19.4	10.1	27.1	17.2
Absolute value	0.85	2.16	4.06	10.93
After 24 hr				
Uptake	5.0	12.6	16.6	43.8
Conversion rate	26.9	20.3	43.5	30.9
Absolute value	1.34	2.56	7.22	13.53

TABLE 5
Conversion Rate of CN-B₁₂ and OH-B₁₂ to DBCC in Human Liver
 The patients had no hematologic changes and damages of liver functions.

	12 hr		24 hr		48 hr	
	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂	CN-B ₁₂	OH-B ₁₂
	41.1	51.7	49.5	57.5	67.9	72.0
Mean	—	—	—	—	65.8	—
					66.8	

Man with the normal liver function: The conversion rate of CN-B₁₂ was 41.1, 49.5, and 66.8 %, while that of OH-B₁₂ 51.7, 57.5, and 72.0 % 12, 24 and 48 hours after the injection respectively. Both rates showed an increase with time and they were remarkably higher than those of normal rats. It can be said that more than 50 % of CN-B₁₂ or OH-B₁₂ was converted into DBCC 24 hours after administration.

Case in which the liver function was abnormal: This case was obtained from a patient, who was a 42-year-old man, diagnosed as having hemolytic jaundice and subjected to splenectomy. The liver functions and hematological findings are listed in Table 6. The details are as follows: Meulengracht 34, total bilirubin 4.6 mg per 100 ml, direct bilirubin 2.8 mg per 100 ml, van den Bergh's indirect bilirubin was positive, cobalt reaction (Co R) 9, cadmium reaction (Cd R) 4, thymol turbidity test (TTT) 4, and BSP test 15 % in 30 minutes. Thus it was concluded that the liver function was abnormal. He received an intravenous injection of 2 μ g of ⁵⁷Co-CN-B₁₂ about 24 hours before the

TABLE 6
Laboratory Findings and Conversion Rate of CN-B₁₂ to DBCC in Liver of Patient of Hemolytic Jaundice

Case	
42 y. Male	
Laboratory findings:	
Liver function	
MG 34	
Serum bilirubin	{ Total 4.6 mg/100 ml
	{ Direct 2.8 mg/100 ml
H. V. d. Bergh indirect bilirubin	(+)
CoR 9	CdR 4
TTT 4	
BSP :	30 min after Injection 15 %
Hematological findings:	
RCC :	184 $\times 10^4$
Hb :	5.3
WBCC :	4,200
Thrombocyte :	144 $\times 10^4$
Hematocrit :	17.0 %
Conversion rate of CN-B ₁₂ to DBCC in liver	
24 hr after administration :	36.5 %

operation. The DBCC conversion rate in the liver obtained during his operation was 36.5 %, which was fairly lower than that for a man with the normal liver function.

DISCUSSION

It is well-known that B₁₂ existing in the body has the form of coenzyme B₁₂ and that CN-B₁₂ is clinically very effective for B₁₂ deficiency. It is therefore suggested that the given CN-B₁₂ is converted into DBCC in the body. OH-B₁₂ is also expected to be converted into DBCC because it is considered to be the form actually existing in the body and is highly fitted for storage. In this report it is shown

experimentally how DBCC was formed from CN-B₁₂ or OH-B₁₂.

The finding concerning the fractions of coenzyme B₁₂ appearing in the tissue after the administration of radioactive CN-B₁₂ or OH-B₁₂ evidently show that the fractions contain DBCC. In fact, the fractions eluted with distilled water and acetate were shown to contain CN-B₁₂ and OH-B₁₂ respectively. However, this does not exclude the possibility that the fractions contain the derivatives other than CN-B₁₂ and OH-B₁₂, because not all the B₁₂ derivatives are yet known. Fenrych *et al.* (14) and Martson *et al.* (15) reported that the conversion from CN-B₁₂ to DBCC could take place in the liver and kidneys in the body. The author also has now showed the existence of such a conversion process in the rat and human. According to the author's experiment, the conversion takes place rapidly in 3 hours after the injection, increasing further with the lapse of time. Actually 36% of CN-B₁₂ and 52 % of OH-B₁₂ were converted into DBCC in the liver of the normal rat after 48 hours, while in the case of the human 66 % of CN-B₁₂ and 72 % of OH-B₁₂ were converted in the same time. The converted DBCC was also observed in the kidneys, but with a lower rate than in the liver. However, it is still open to discussion whether the observed DBCC was really converted in the kidneys or whether it appeared in the organs through the blood from the liver, where DBCC was actually produced. On this point it should be mentioned that Pawelkiwicz *et al.* (13) showed the conversion process in the kidneys by experiments *in vitro* using rats and human organs. The author's experiment seems to indicate that some of the DBCC found in the kidneys must have been converted in the organs themselves because DBCC production in the kidneys was recognized in a short time following the administration. This finding is consistent with the suggestion by Uchino *et al.* (23) who reported that the kidneys also must have something to do with the metabolism of vitamin B₁₂ because the observed distribution of the vitamin in the kidneys is too great to be explained by a simple excretion only.

Tamao *et al.* (22) tried an experiment to observe the conversion to DBCC by adding CN-B₁₂ or OH-B₁₂ to the suspension of *Propionibacterium shermanii* and concluded that the conversion of OH-B₁₂ to DBCC was remarkably higher than that of CN-B₁₂. The author's experiment also leads to the same conclusion.

However, Weissbach *et al.* (11) showed in a report that the conversion is associated with the reduced form of B₁₂, the reduction of which requires reduced FAD, and suggested that CN-B₁₂ was converted into DBCC by way of OH-B₁₂. According to Tamao's experiment (24) using the suspension of bacteria, OH-B₁₂ was not observed at all in the conversion process from CN-B₁₂ to DBCC. As a result they concluded that either the production of OH-B₁₂ was not required on the way from CN-B₁₂ to DBCC, or if OH-B₁₂ is necessary, it appears only for a very short time so that the rate-limiting step for the reaction in question is just the process from CN-B₁₂ to OH-B₁₂. In either case it is easily seen that the conversion from the reduced form of OH-B₁₂ to DBCC proceeds much faster. OH-B₁₂ is, therefore, preferable to any other vitamin B₁₂ preparations because of the easy conversion to DBCC and its high affinity for tissues.

Fig. 5 shows the conversion rates of CN-B₁₂ and OH-B₁₂ in the livers of normal rats, vitamin B₁₂-deficient ones, and carbon tetrachloride-injured ones 3

FIG. 5 Conversion Rate of CN-B₁₂ and OH-B₁₂ to DBCC in Normal, CCl₄-Injured and B₁₂-Deficient Rats

□, normal; ▨, CCl₄-injury 6 hr; ■, CCl₄-injury 12 hr; ▩, B₁₂-deficient.

hours after the administration. The conversion rate in the case of the deficient rats becomes remarkably high in a short time after administration as compared with that for normal ones. The decrease of coenzyme B₁₂ content and the increase of hepatic uptake in the case of the deficient rats indicate that the amount actually converted to DBCC is much larger than the observed one and that the requirement for coenzyme B₁₂ is greatly increased.

The author's experiment shows that the conversion to DBCC takes place mostly in the liver, suggesting that the conversion rate slows down if the liver is impaired. In fact, the decrease of the conversion rate was observed in the case of carbon tetrachloride-injured rats in the author's experiment. On the other hand, many authors (5-11) showed that ATP and FAD were somehow connected with the conversion process leading to DBCC and also that the release of ATP (25) and the decrease of FAD (26) occur in the carbon tetrachloride-damaged liver. These effects, remaining for a fairly long time, seem to be the main cause of the decrease of the conversion rate to DBCC in the liver injured with carbon tetrachloride.

The carbon tetrachloride injury has more influence on the conversion process from CN-B₁₂ to DBCC than that from OH-B₁₂ to DBCC. Therefore, carbon tetrachloride seems to have a considerable effect on the conversion process from CN-B₁₂ to OH-B₁₂, if the actual process is considered to take place from CN-B₁₂ through OH-B₁₂ to DBCC. This point and the finding of the marked decrease of coenzyme B₁₂ content due to the liver damage as was shown in the previous paper (19), strongly suggest the following possibility. In certain cases of liver damage the trouble in utilizing B₁₂ is likely to appear in such a way that the vitamin stored in the liver is released into the blood stream, and subsequently the vitamin available in the body becomes poor. Moreover, the administered B₁₂ can hardly be changed to the coenzyme. An example is the hyperchromic macrocytic anemia appearing in cases of serious liver damage, which is supposed to result partly from the inhibited utilization of B₁₂ in the body. This possibility

is supported also by Uchino's research concerning the urinary excretion of methylmalonic acid. (27)

As mentioned in the previous paper (21), IF gives rise to a remarkable increase of the hepatic uptake of B₁₂ derivatives. In the case of administration with IF the conversion rate becomes lower, but the quantity converted is considerably higher than in the case of single administration. This implies that IF has a noticeable effect on increasing the hepatic uptake and probably on stimulating the production of DBCC after simultaneous administration.

An interesting phenomenon was observed in the experiments on the kidneys and also on the liver when the experiment extended over a long period, more than 5 days. In these cases the chromatographic behavior was different in that the fraction eluted with distilled water had two peaks as shown in Fig. 6. The additional peak, the latter one, seems to correspond to an unknown B₁₂ derivative, not yet identified. In any case, this finding suggests that the metabolism in the kidneys is somewhat different from that in the liver.

FIG. 6 Chromatography of CN-B₁₂, OH-B₁₂ and DBCC on P-Cellulose for Kidney Extract

SUMMARY

1. The metabolism of vitamin B₁₂ in the body of rats and human subjects was examined.
2. Fractionation of three cobamides, CN-B₁₂, OH-B₁₂ and DBCC, and their identification were carried out.
3. The conversion from cyanocobalamin or hydroxocobalamin to coenzyme B₁₂ was found to take place in the liver and kidneys of rats and humans. Considerable quantities were converted in a short time. In fact, more than half of the administered derivatives were converted into the coenzyme in 48 hours.
4. The conversion from hydroxocobalamin to the coenzyme was faster than that from cyanocobalamin, which means that hydroxocobalamin is favorable for utilization of the vitamin.
5. The conversion rate in question was higher for the vitamin deficient rats

than that for normal rats, showing that the requirement for the coenzyme is increased in the case of the deficient rats.

6. The conversion rate in the liver was lower in the carbon tetrachloride-injured rats and in the damaged human liver than that of normal ones. In these cases the conversion rate from hydroxocobalamin to the coenzyme was not changed appreciably as compared with the normal, showing that hydroxocobalamin is more available in the case of liver damage.

7. The intrinsic factor gives rise to an increase of the hepatic uptake and the quantity converted.

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