Biological Activity of Hydroxo-vitamin B_{12} Degradation Product Formed during Microwave Heating

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When hydroxo-vitamin B_{12}, which predominates in foods, is treated by microwave heating, two degradation products with R_f of 0.16 and 0.27 are found and characterized (Watanabe et al. *J. Agric. Food Chem.* **1998**, *46*, 206−210). A novel hydroxo-vitamin B_{12} degradation product with a R_f of 0.12 was also found by the use of silica gel 60 column chromatography and was the major compound among the degradation products. The degradation product with a R_f of 0.12 was purified to homogeneity and partially characterized. The purified degradation product had about 13% and 23% biological activity of authentic vitamin B_{12} in hog intrinsic factor (a mammalian vitamin B_{12}-binding protein) and *Lactobacillus leichmannii* ATCC 7830, respectively. Administration of the purified degradation product (0.5 μg/week) to rats indicates that the compound does not have significant activity in mammals.

Keywords: *Vitamin B_{12}; degradation; microwave heating; intrinsic factor; Lactobacillus leichmannii; mammalian cells; rat*

INTRODUCTION

Vitamin B_{12} (B_{12}) present in food is derived from animal tissues, milks, and eggs, which contain various B_{12} analogues with different β-ligands. Methyl and adenosyl B_{12}'s function as coenzymes of methionine synthase (EC 2.1.1.13) and methylmalonyl-CoA mutase (EC 5.4.99.2), respectively (Watanabe and Nakano, 1991). Cyano-vitamin B_{12} (CN-B_{12}) does not occur in raw food, which contains predominately hydroxo-vitamin B_{12} (OH-B_{12}) (Schneider, 1987a).

The microwave is widely used for cooking and food processing. Extensive studies (Cross and Fung, 1982; Hoffman and Zabik, 1985) have shown equal or better retention of some vitamins for microwave heating, compared with conventional heating. Appreciable loss (about 40%) of B_{12} occurs in the foods due to degradation of the B_{12} molecule by microwave heating (Watanabe et al., 1998). When OH-B_{12}, which predominates in foods, is treated by microwave heating and then analyzed by silica gel 60 thin-layer chromatography (TLC), two degradation products with R_f of 0.16 (about 18%) and 0.27 (about 4%) are formed (Watanabe et al., 1998). The relative amount (about 22%) of the OH-B_{12} degradation products caused by microwave heating is considerably lower than the loss (about 40%) of B_{12} in food, suggesting that there are other OH-B_{12} degradation products which are not separable by the TLC system.

Using silica gel 60 column chromatography, we found a novel OH-B_{12} degradation product with a R_f of 0.12. Here, we describe the purification and characterization of the novel degradation product and also demonstrate the toxicity and biological activity of the compound in mammals.

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MATERIALS AND METHODS

Materials. OH-B_{12} and CN-B_{12} were obtained from Sigma Chemical Co. (St. Louis, MO). Silica gel 60 TLC plates were obtained from Merck (Darmstadt, Germany). A reversed-phase HPLC column (Wakosil-II 5C18RS, ∅ 4.6 × 150 mm, particle size 5 μm) was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). A Hitachi (Tokyo, Japan) spectrophotometer (U-1000) and a Shimadzu (Kyoto, Japan) spectrophotometer (UV-1600) were used for measuring the turbidity of *Lactobacillus leichmannii* test culture and absorption spectra of the OH-B_{12} degradation product, respectively. The microwave oven used was a Funai (Tokyo, Japan) microwave oven, model MO57-6A. The full power was 500 W, and the frequency of radiation was 2450 MHz.

Isolation of OH-B_{12} Degradation Product by Microwave Heating. Authentic OH-B_{12} used in the experiments was purified with silica gel 60 TLC with 1-butanol/2-propanol/water (10:7:10) as a solvent as described previously (Watanabe et al., 1998), since OH-B_{12} reagent (approximately 98% purity) contained a small amount of an impurity (R_f value = 0.27); 50 mL of the purified OH-B_{12} solution (5 mmol/L) was treated by microwave heating for 6 min in the dark; the 6-min microwave heating is a likely time period for food processing and cooking. The treated solution was evaporated to dryness and dissolved in a small amount of the solvent. The concentrated solution was put on a column (1.4 × 15.0 cm) of silica gel 60 equilibrated with the same solvent and eluted with the same solvent in the dark at room temperature. The red-colored fractions between fraction numbers 60 and 80 (fraction V in Figure 1) were combined, evaporated to dryness, and dissolved in a small amount of distilled water. The solution was put on the silica gel 60 TLC plates and developed with 1-butanol/2-propanol/water (10:7:10) as a solvent in the dark. The plates

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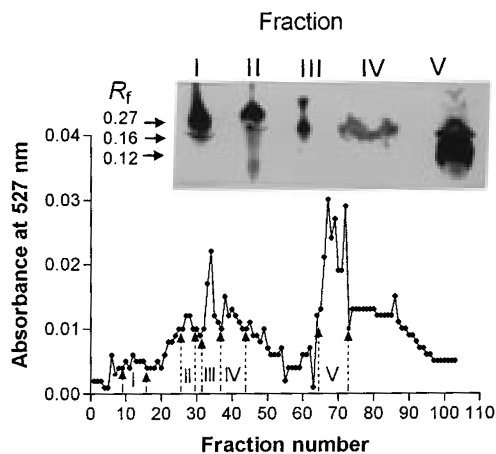

Figure 1. Elution profile of OH-B_{12} treated by microwave heating for 6 min during silica gel 60 column chromatography. Fifty milliliters of the treated OH-B_{12} solution (5 mmol/L) was evaporated to dryness and dissolved in a small amount of 1-butanol/2-propanol/water (10:7:10) as a solvent. The concentrated solution was put on a column (1.4 × 15.0 cm) of silica gel 60 equilibrated with the same solvent and eluted with the same solvent in the dark. The eluate was collected at 4.0 mL with a fraction collector. Fractions I−V were pooled, evaporated to dryness, dissolved with a small amount of distilled water, and analyzed with silica gel TLC. Inset represents the mobile pattern of the OH-B_{12} degradation products of fractions I−V on the TLC plate. Data are a typical one of five experiments.

were dried at room temperature, and the red-colored spot with R_f of 0.12 was collected in the dark. The collected TLC silica gels were dissolved in an appropriate amount of the solvent containing 0.2 g/L KCN to convert the OH form to the CN form in the β-coordination position of OH-B_{12}, because the CN form could be extracted completely from the silica gels. The OH-B_{12} degradation product was extracted from the silica gels three times. The combined extracts were evaporated to dryness and dissolved in a small amount of distilled water. The OH-B_{12} degradation product was further purified by high-pressure liquid chromatography (HPLC) using a Shimadzu HPLC apparatus (LC-6A pump, SPD-6A spectrophotometer, C-R6A chromatopack). The sample was put on a reversed-phase HPLC column (Wakosil-II 5C18RS, ∅ 4.6 × 150 mm, particle size 5 μm) equilibrated with 5% (v/v) methanol solution containing 1% (v/v) acetic acid at 35 °C. The flow rate was 1 mL/min. The OH-B_{12} degradation product was eluted at 40 mL of a linear gradient (5−70%, v/v) of methanol in the same solution, monitored by measuring absorbance at 527 nm, and collected at 1.0 mL with a Bio-Rad Laboratories (Hercules, CA) fraction collector (model 2110). The OH-B_{12} degradation product was separated in a single peak with retention time of 22.0 min (the retention time of authentic CN-B_{12} was 26.0 min). The peak with retention time of 22.0 min was pooled, evaporated to dryness, and dissolved in a small amount of distilled water. The concentration of the purified compound was determined on the basis of a molecular extinction coefficient for CN-B_{12} of 8.74 × 10^3 $(mol/L)^{-1}\cdot cm^{-1}$ at 551 nm in water (Schneider, 1987b).

Ultraviolet−Visible Absorption Spectra. The spectra were measured with a Shimadzu spectrophotometer (UV-1600) at room temperature. Quartz cuvettes (d = 1 cm) were used. Authentic CN-B_{12} and the OH-B_{12} degradation product were dissolved with 2.0 mL of distilled water at the final concentration of 7.3 μg/mL. Ultraviolet−visible spectra of the OH-B_{12} degradation product in H_2O with λ_{max}/nm were at 554 (0.14), 359 (0.23), and 279 (0.20) and those of authentic CN-B_{12} in H_2O with λ_{max}/nm were at 554 (0.14), 362 (0.18), and 279 (0.17).

Biological Activity of the Purified OH-B_{12} Degradation Product in *L. leichmannii* ATCC 7830 and Hog Intrinsic Factor. B_{12} was assayed by the microbiological method with *L. leichmannii* ATCC 7830 and a B_{12} assay medium (Nissui, Tokyo, Japan) and by the fully automated chemiluminescence B_{12} analyzer ACS 180 (using hog intrinsic factor) (Chiron Diagnostics, East Walpole, MA) according to the manufacturer's instructions. The above B_{12} extracts were directly applied to the chemiluminescence B_{12} analyzer. They were diluted with distilled water up to a B_{12} concentration range of 0.01−0.2 μg/L and used as samples for the microbiological method. The turbidity (% T) of *L. leichmannii* test culture was measured at 600 nm with a Hitachi spectrophotometer (U-1000).

Intravenous Administration of the Purified OH-B_{12} Degradation Product in Rats. Eighteen male Wistar rats (3 weeks old) were obtained from Kiwa Laboratory Animals Co. Ltd., (Wakayama, Japan). CN-B_{12}-deprived diet contained 400 g (per kg of diet) of soybean protein (Fuji Oil Ltd., Osaka, Japan), 438 g of glucose anhydrous (Nacalai Tesque, Ltd., Kyoto, Japan), 100 g of soybean oil (Nacalai), 50 g of mineral mixture, 5 g of *dl*-methionine (Nacalai), 5 g of CN-B_{12}-free vitamin mixture, and 2 g of choline chloride (Nacalai) as described previously (Watanabe et al., 1991). They were fed the CN-B_{12}-deprived diet 2 weeks before experiments. The 5-week-old rats were housed in individual metabolic cages at 24 °C in a room with a 12-h light−dark cycle. They had free access to the CN-B_{12}-deprived diet and tap water during experiments. The 5-week-old rats (152.8 ± 10.9 g) were injected intravenously with 0.5 mL (0.5 μg) of the OH-B_{12} degradation product−saline or authentic CN-B_{12}−saline every week for 5 weeks. The rats injected with 0.5 mL of saline under the same conditions were used as a control. Six rats/group were used in the experiments.

The urine of the 10-week-old control, CN-B_{12}-treated and OH-B_{12} degradation product-treated rats in individual metabolic cages was sampled for 24 h. Liver and blood were obtained from the rats killed by decapitation under diethyl ether anesthesia. Livers were washed with saline and weighted. A portion (wet weight, 1 g) of the livers was immediately homogenized (using a glass homogenizer with a Teflon pestle) in about four volumes of 10 mmol/L acetate buffer, pH 4.8. Extraction of total B_{12} from the liver homogenate and blood was done as described previously (Watanabe et al., 1998). B_{12} was bioassayed with *L. leichmannii* ATCC 7830 as described above.

Urinary methylmalonic acid was assayed by HPLC as described previously (Toyoshima et al., 1994).

Cell Culture Experiments. Bovine carotid artery endothelial cells were purchased from Human Science Research Resources Bank (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, heparin (15 U/mL), endothelial cell growth supplement (25 mg/L medium; Sigma Chemical), and antibiotics (100 U/mL penicillin G sodium and 100 U/mL streptomycin) at 37 °C in a 5% CO_2/95% air atmosphere at 100% humidity as described by McQuillan et al. (1994). The cells were seeded into dishes coated with collagen (type I) and subcultured by trypsinization using phosphate-buffered saline (8 g/L NaCl, 0.2 g/L KCl, 1.15 g/L Na_2HPO_4, and 0.2 g/L KH_2PO_4), pH 7.4, containing 0.25% trypsin and 0.02% EDTA. B_{12} concentration (30 pg/mL medium) of the medium was assayed with the chemiluminescence B_{12} analyzer.

Confluent monolayers of the endothelial cells were suspended in the medium at a density of 1.0 × 10^5 cells/mL. The cell suspension (1.0 mL) was seeded into 6-well tissue culture plates coated with collagen. After incubation for 12 h, CN-B_{12} and OH-B_{12} degradation product were added to the medium at the final concentration of 3 ng/mL. The cells were further incubated for 5 days and trypsinized. The viable cell number was determined by staining cells with 0.4% Trypan blue and counting on a hemocytometer. Five experiments were done independently.

Statistics. Statistical analysis was performed using GB-STAT 5.4 (Dynamic Microsystems, Inc., Silver Spring, MD). One-way ANOVA was used with the post-hoc Tukey/Kramer procedure. In the case of effect of the OH-B_{12} degradation product on body weight of the experimental rats, two-way

Table 1. Effects of OH-B_{12} Degradation Product on Binding to Hog Intrinsic Factor and on Growth of *L. leichmannii* ATCC 7830

	relative B_{12} concentration[a] (%)	
	intrinsic factor	*L. leichmannii*
CN-B_{12}	100	100
OH-B_{12} degradation product	12.9 ± 0.7	22.5 ± 2.7

[a] Authentic CN-B_{12} and OH-B_{12} degradation product (same concentration of each) were assayed by the chemiluminescence B_{12} assay system using hog intrinsic factor and *L. leichmannii* bioassay as described in Materials and Methods. The OH-B_{12} degradation product is converted to the CN form by KCN treatment during purification. All values represent means ± SD (n = 3).

ANOVA was used. Differences were considered significant if $P < 0.05$. Values in the text are mean ± SD.

RESULTS AND DISCUSSION

After OH-B_{12} which predominates in foods was treated by microwave heating for 6 min, OH-B_{12} degradation products formed were separated by a silica gel 60 column chromatography. The column eluate was fractionated into fractions I−V as indicated in Figure 1. OH-B_{12} was tightly bound to the top of the gels in this solvent system. When fractions I−V were analyzed by silica gel 60 TLC, the OH-B_{12} degradation products with R_f of 0.27 and 0.16 predominated in fractions I−II and III−IV, respectively; they have been characterized previously (Watanabe et al., 1998). A novel OH-B_{12} degradation product with a R_f of 0.12 was recovered in fraction V and has not been separable from intact OH-B_{12} by the TLC system. These results indicate that the OH-B_{12} degradation product with a R_f of 0.12 is readily separated from intact OH-B_{12} and other OH-B_{12} degradation products by use of silica gel column chromatography and is the major compound among the OH-B_{12} degradation products. The OH-B_{12} degradation product with a R_f of 0.12 was further purified to homogeneity and characterized. The purified OH-B_{12} degradation product (with conversion of the OH form to the CN form by KCN treatment) gave a single peak with a retention time of 22.0 min by reversed-phase HPLC (a retention time of authentic CN-B_{12} is 26.0 min). Ultraviolet−visible absorption spectrum of the OH-B_{12} degradation product was very similar to that of authentic CN-B_{12}.

To determine the biological activity of the purified OH-B_{12} degradation product in a *L. leichmannii* ATCC 7830 (a B_{12}-requiring microorganism) culture, which is used for the bioassay for B_{12} in food (RCSTA 1995), authentic CN-B_{12} and the OH-B_{12} degradation product (an identical amount, 600 pg/mL) were determined by the *L. leichmannii* microbiological method (Table 1); the amount of the degradation product was estimated to be about 22.5 ± 2.7% of that of CN-B_{12}. The results indicate that the OH-B_{12} degradation product does not have significant activity in *L. leichmannii* ATCC 7830 and is not assayable by the *L. leichmannii* method.

Intrinsic factor, which is involved in intestinal absorption of B_{12} (Seetharam and Alpers, 1982), recognizes the structure of the B_{12} molecule. When authentic CN-B_{12} and the purified OH-B_{12} degradation product (an identical amount, 8 ng/mL) were assayed by the chemiluminescence B_{12} assay system using hog intrinsic factor (Table 1), the amount of the degradation product was estimated to be about 12.9 ± 0.7% of that of CN-B_{12}. The results suggest that the OH-B_{12} degradation product is hardly absorbed in mammalian intestine by the intrinsic factor-mediated system. These results in Table 1 indicate that the OH-B_{12} degradation product has a biological activity from about 13% to 23% of that of the authentic CN-B_{12}.

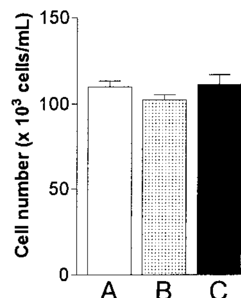

Figure 2. Effect of the purified OH-B_{12} degradation product on growth of bovine carotid artery endothelial cells. Bovine carotid artery endothelial cells were cultured in Dulbecco's modified Eagle's medium (A) at 37 °C in a 5% CO_2/95% air atmosphere at 100% humidity. Authentic CN-B_{12} (B) and the OH-B_{12} degradation product (C) (each 3 ng/mL of medium) were added to the cell culture. Detailed procedures are described in Materials and Methods. The values represent mean ± SD (n = 5).

To determine whether the OH-B_{12} degradation product has an acute toxicity in mammalian cells, effect of the degradation product on growth of bovine carotid artery endothelial cells was studied (Figure 2). Authentic CN-B_{12} and the purified OH-B_{12} degradation product (each at the final concentration of 3 ng/mL) were added to the cells, which were cultured for 5 days. There is no significant difference in the cell numbers between the 5-day-old CN-B_{12}-treated and OH-B_{12} degradation product-treated endothelial cells. Identical results were also shown when each cell was subcultured three times under the same conditions (data not shown). These results indicate that the OH-B_{12} degradation product neither has toxicity nor acts as a B_{12} antagonist in the mammalian cells under the experimental conditions.

To clarify biological activity of the OH-B_{12} degradation product in mammals, effects of intravenous administration of the degradation product on body weight gain, plasma and hepatic B_{12} concentrations, and urinary methylmalonic acid excretion as a index of B_{12} deficiency were studied in rats (Table 2). The 5-week-old rats were injected with 0.5 μg of the purified OH-B_{12} degradation product every week for 5 weeks. The administration of the degradation product did not affect body and liver weights and plasma B_{12} concentration in the 10-week-old rats. The urinary methylmalonic acid excretion was about 2 times greater in the CN-B_{12}-deficient rats (32.8 ± 12.9 mg/day) than in the CN-B_{12}-sufficient rats (17.2 ± 10.8 mg/day). The liver B_{12} concentration decreased significantly in the CN-B_{12}-deficient rats (18.4 ± 3.7 μg/100 g of wet weight) relative to the CN-B_{12}-sufficicent rats (33.6 ± 10.9 μg/100 g of wet weight). These results indicate that the 10-week-old CN-B_{12}-deficient rats develop a B_{12} deficiency.

Although the liver B_{12} concentration decreased significantly in the OH-B_{12} degradation product-treated rats (20.1 ± 8.5 μg/100 g of wet weight) relative to the CN-B_{12}-sufficicent rats (33.6 ± 10.9 μg/100 g of wet weight), the level of urinary methylmalonic acid excretion decreased slightly in the OH-B_{12} degradation product-treated rats relative to the CN-B_{12}-deficient rats; there is not a significant difference between the OH-B_{12} degradation product-treated and the CN-B_{12}-

Table 2. Effects of Intravenous Administration of OH-B_{12} Degradation Product on Body Weight, Urinary Methylmalonic Acid Excretion, and Plasma and Hepatic B_{12} Concentrations in Rats[a]

	body weight (g)	liver weight (g)	urinary methylmalonic acid excretion (mg/day)	B_{12} concentration	
				plasma (pg/mL)	liver (μg/100 g of wet wt)
CN-B_{12}-sufficient	384.4 ± 28.9a	14.5 ± 2.2a	17.2 ± 10.8a	681.7 ± 31.9a	33.6 ± 10.9a
CN-B_{12}-deficient	399.2 ± 40.2a	16.3 ± 2.1a	32.8 ± 12.9b	631.7 ± 46.7a	18.4 ± 3.7b
OH-B_{12} degradation product	395.2 ± 25.7a	14.9 ± 2.1a	26.5 ± 5.8a,b	633.3 ± 48.4a	20.1 ± 8.5b

[a] All values represent means ± SD ($n = 6$). Different letters denote significant differences ($P < 0.05$). The OH-B_{12} degradation product is converted to the CN form by KCN treatment during purification.

deficient (or -sufficient) rats. The low liver B_{12} concentration of the OH-B_{12} degradation product-treated rats may be due to the low biological activity of the compound in *L. leichimannii* used for the B_{12} assay. These results suggest that the OH-B_{12} degradation product does not have significant activity in rats within the experimental time course examined.

Although *L. leichmannii* ATCC 7830 and mammalian cells cannot utilize cobinamide [α-ligand (cobalt-coordinated nucleotide) free corrinoids], the fact that the OH-B_{12} degradation product did not have significant activity in both microbial and mammalian cells as shown in Tables 1 and 2 and that ultraviolet−visible absorption spectrum of the OH-B_{12} degradation product was very similar to that of authentic CN-B_{12} suggest that the OH-B_{12} degradation product contains the α-ligand moiety, however, which would be partially degraded by microwave heating.

In our preliminary experiments, the ^{1}H NMR spectrum of the OH-B_{12} degradation product was measured on a JEOL GX-270 at 270 MHz in D_2O ($\delta = 4.71$ ppm; internal reference). The ^{1}H NMR spectrum of the OH-B_{12} degradation product shows that the degradation product is a B_{12} compound with the α-ligand structure changed slightly. The result supports that the degradation product does not have significant activity in *L. leichmannii* and mammals. The detailed chemical structure of the OH-B_{12} degradation product cannot be determined because a substantial amount of the purified sample was not obtained for NMR study.

We have previously reported that the OH-B_{12} degradation product with a R_f of 0.16 may be due to both elimination of the base portion and change of sugar moiety in the α-ligand by microwave heating and is inactive in *Euglena gracilis* Z (a B_{12}-requiring microorganism) and mammals (Watanabe et al., 1998). In our unpublished studies, the degradation product with a R_f of 0.27 had the identical properties to that with a R_f of 0.16. The OH-B_{12} degradation product with a R_f of 0.12 may be only due to the minor change of the α-ligand structure by microwave heating. There was total loss (about 45%) of OH-B_{12} activity due to all of these degradation products under the experimental conditions.

This and previous (Watanabe et al., 1998) studies suggest that various OH-B_{12} degradation products are formed in foods by microwave heating, and they do not have significant activity in both B_{12}-requiring microorganisms and mammals. These OH-B_{12} degradation products were not toxic under the experimental conditions (considerably lower amounts of the products), but if higher levels were used there might be toxicity problems.

ABBREVIATIONS USED

B_{12}, vitamin B_{12}; CN-B_{12}, cyano-vitamin B_{12}; HPLC, high-pressure liquid chromatography; OH-B_{12}, hydroxo-vitamin B_{12}; TLC, thin-layer chromatography.

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