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The tissue profile of metabolically active coenzyme forms of vitamin B_{12} differs in vitamin B_{12}-depleted rats treated with hydroxo-B_{12} or cyano-B_{12}

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Abstract

Recent rat studies show different tissue distributions of vitamin B_{12} (B_{12}), administered orally as hydroxo-B_{12} (HO-B_{12}) (predominant in food) and cyano-B_{12} (CN-B_{12}) (common in supplements). Here we examine male Wistar rats kept on a low-B_{12} diet for 4 weeks followed by a 2-week period on diets with HO-B_{12} (*n* 9) or CN-B_{12} (*n* 9), or maintained on a low-B_{12} diet (*n* 9). Plasma B_{12} was analysed before, during and after the study. The content of B_{12} and its variants (HO-B_{12}, glutathionyl-B_{12}, CN-B_{12}, 5'-deoxyadenosyl-B_{12} (ADO-B_{12}), and methyl-B_{12} (CH_3-B_{12})) were assessed in the tissues at the end of the study. A period of 4 weeks on the low-B_{12} diet reduced plasma B_{12} by 58 % (from median 1323 (range 602–1791) to 562 (range 267–865) pmol/l, *n* 27). After 2 weeks on a high-B_{12} diet (week 6 *v.* week 4), plasma B_{12} increased by 68 % (HO-B_{12}) and 131 % (CN-B_{12}). Total B_{12} in the tissues accumulated differently: HO-B_{12} > CN-B_{12} (liver, spleen), HO-B_{12} < CN-B_{12} (kidneys), and HO-B_{12}≈CN-B_{12} (brain, heart). Notably, more than half of the administered CN-B_{12} remained in this form in the kidneys, whereas HO-B_{12} was largely converted to the bioactive ADO-B_{12}. Only <10 % of the other cofactor, CH_3-B_{12}, were found in the tissues. In conclusion, dietary CN-B_{12} caused a higher increase in plasma and total kidney B_{12} but provided less than half of the active coenzymes in comparison to dietary HO-B_{12}. These data argue that HO-B_{12} may provide a better tissue supply of B_{12} than CN-B_{12}, thereby underscoring the lack of a direct relation between plasma B_{12} and tissue B_{12}.

Key words: Dietary vitamin B_{12}: Hydroxocobalamin: Cyanocobalamin: Tissue distribution: Vitamin B_{12}-depleted rats: Active coenzymes

Vitamin B_{12} (B_{12}, cobalamin) is a water-soluble compound important for neural function and erythropoiesis. Humans and other animals, including rats, cannot synthesise B_{12} and obtain it from foods of animal source. Inadequate intake or impaired absorption may lead to B_{12} deficiency accompanied by clinical signs of neurological impairment and/or anaemia[1,2]. Deficiency is treated with B_{12} injections or oral supplementation depending on severity and cause of the disease.

B_{12} exists in different forms that share the same core structure but differ in the upper exchangeable ligand[3]. Supplements for oral use ordinarily contain cyano-B_{12} (CN-B_{12}), the stable synthetic form of the vitamin, whereas food contains natural forms such as hydroxo-B_{12} (HO-B_{12})/aquo-B_{12} (H_2O-B_{12}) or the metabolically active coenzyme forms 5'-deoxyadenosyl-B_{12} (ADO-B_{12}) and methyl-B_{12} (CH_3-B_{12})[4]. HO-B_{12} and H_2O-B_{12} are interchangeable (depending on pH), and the term HO-B_{12} will be used to designate both forms. As the coenzymes are light sensitive and easily converted into HO-B_{12} upon brief light exposure[5], HO-B_{12} is the ubiquitous form of B_{12} in food. HO-B_{12} can be further converted into sulphito-B_{12}, glutationyl-B_{12} (GS-B_{12}), CN-B_{12}, and a number of other variants in the presence of the $B_{12}[Co^{3+}]$-coordinating anions. These reactions occur irrespectively of light[6].

All forms of B_{12} are metabolically transformed into ADO-B_{12} and CH_3-B_{12} in the cell[3]. Here, ADO-B_{12} acts as a cofactor for methylmalonyl-CoA mutase in the conversion of methylmalonyl-CoA to succinyl-CoA in the mitochondria. CH_3-B_{12} acts as a cofactor for methionine synthase in the folate-dependent methylation of homocysteine to methionine in the cytoplasm[1,2].

Both human and animal studies have demonstrated that synthetic and natural forms of B_{12} are absorbed equally[7–11]. However, our recent data show that HO-B_{12} accumulates in the liver to a higher degree than CN-B_{12}, but that the patterns are opposite in the brain and plasma. These observations were based on the administration of acute doses of radiolabelled HO-B_{12} and CN-B_{12} to rats[10,11]. In accordance with this, acute human studies showed CN-B_{12} to cause a 2–3-fold higher increase in the active circulating B_{12}, holotranscobalamin, relative to HO-B_{12} upon oral administration[12]. These findings

Abbreviations: ADO-B_{12}, 5'-deoxyadenosyl-B_{12}; B_{12}, vitamin B_{12}; CH_3-B_{12}, methyl-B_{12}; CN-B_{12}, cyano-B_{12}; GS-B_{12}, glutathionyl-B_{12}; HO-B_{12}, hydroxo-B_{12}.

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question whether HO-B_{12} and CN-B_{12} are equally efficient for supplying the tissues with B_{12}.

The current study was undertaken to investigate the tissue distribution of B_{12} after 2 weeks of dietary intake of the two vitamin forms, either HO-B_{12} or CN-B_{12}, in B_{12}-depleted rats. We also provide new information on the prevalence of various B_{12} forms encountered in the key organs such as liver and brain.

Methods

Animals

Male Wistar rats (RjHan:WI) (n 30) from Janvier Labs, France, were used for the experiment (7 weeks old; weighing approximately 200 g upon arrival to the animal facility). This strain of rats was chosen as it is commonly used for animal studies in B_{12} research[10,11,13,14]. The study was authorised by the Danish Animal Experimental Inspectorate in agreement with the EU directive 2010/63/EU on animal experiments (approval no.: 2016-15-0201-00984) and conducted at the animal facility at Health Faculty, Aarhus University, Denmark. The institutional and national guidelines for care and use of animals were followed, and the rats were checked daily for any health or welfare problems. No signs of pain, suffering or distress was observed before or during the study.

The rats were housed in pairs in standard cages (Makrolon 1291 H type III H, 800 cm^2; Techniplast) in a controlled environment (20·0 ± 0·5°C; 60 % humidity) with a 12 h light–12 h dark cycle. Bedding material (asp chips; Tapvei) and soft paper wool (LBS Biotech) were changed daily. The rats were allowed 2 weeks of acclimatisation in the animal facility before the experiment was initiated. During this time, the rats were kept on a standard stock rat fodder (Altromin 1324; Brogaarden) containing 24 µg/kg diet CN-B_{12} (according to the manufacturer) and had free access to tap water. The rats weighed 237 (range 204–258) g at the beginning of the study.

Study design

The experiment was conducted over a period of 6 weeks (study design is outlined in Fig. 1). Thirty male Wistar rats were fed a low-B_{12} diet containing 11 µg/kg diet B_{12} (Altromin C-1024; Brogaarden) for 4 weeks in order to obtain a suboptimal B_{12} status. At this point, three rats were killed to serve as 4-week controls, and the remaining twenty-seven rats were divided into three groups (HO-B_{12}, CN-B_{12} and low-B_{12}) and fed with three different custom-made diets for 2 additional weeks. The HO-B_{12} group (n 9) received a diet with added HO-B_{12}, the CN-B_{12} group (n = 9) received a diet with added CN-B_{12}, and the low-B_{12} group (n 9) continued on the low-B_{12} diet (Altromin C-1024). The diets with HO-B_{12} and CN-B_{12} were custom-made by Brogaarden by addition of free (protein-unbound) HO-B_{12} (24 µg/kg diet) (cat. no. H1428000; Sigma-Aldrich) or CN-B_{12} (24 µg/kg diet) (cat. no. 68-19-9; Sigma-Aldrich) to the same stock of low-B_{12} Altromin C-1024 diet. Consequently, the three diets differed only in the content and the form of the B_{12} added. For quality check, we analysed the forms and content of B_{12} in the three diets received from Brogaarden before starting the study (see the 'Evaluation of custom-made rat diets' section). All rats had free access to food and water throughout the study. The amount of food consumed was calculated by subtracting the weight of left-overs from the ration provided each day.

Three times during the experiment (baseline, week 4 and week 6), the rats were weighed and blood samples were drawn by puncture of the sublingual vein with a 23-gauge needle. After the end of the study (week 6), the rats were anaesthetised with isoflurane gas and killed by cervical dislocation. Liver, kidneys, brain (cerebrum), heart and spleen were collected, weighed and snap-frozen in liquid N_2 before stored at −80°C until further processing (see the 'Determination of vitamin B_{12} in rat plasma and tissue' and 'Determination of vitamin B_{12} forms in rat tissue' sections).

Evaluation of custom-made rat diets

To verify that the content of B_{12} in the custom-made diets (HO-B_{12} diet, CN-B_{12} diet and low-B_{12} diet) was consistent with the specifications provided, 0·3 g of solids were dissolved in 1·5 ml demineralised water and centrifuged for 40 min at 20 000 ***g*** and 4°C. The supernatant was diluted 1:4 and analysed for total B_{12} content on the Advia Centaur CP Immunoassay System (Siemens). During the analysis, all B_{12} was converted to CN-B_{12}; thus, the B_{12} content was calculated using the molecular weight of CN-B_{12} (MW: 1355·7). To assess the forms of B_{12} in the diets, 0·3 g of solids were dissolved in 500 µl of 0·4 M acetic acid and 1000 µl of 50 % methanol, whereupon the forms of B_{12} were determined as explained for the tissues (see the 'Determination of vitamin B_{12} in rat plasma and tissue' section).

Determination of vitamin B_{12} in rat plasma and tissue

Blood samples were collected into 4 ml lithium heparin tubes (BD Vacutainer), and plasma was removed after centrifugation at room temperature for 9 min at 1850 ***g*** and stored at −20°C until analysis. Plasma was measured for B_{12} content on the Advia Centaur CP Immunoassay System.

The tissues were thawed on ice and endogenous B_{12} was extracted from liver, kidneys, brain, heart and spleen by homogenising 0·2 g of tissue in 750 ml of Na-acetate buffer (0·4 mol/l, pH 4·4) using the Precellys 24 (Bertin Technologies) with three centrifugation cycles of 20 s at 6800 rpm with 30 s pauses between cycles. After homogenisation, 20 µl of KCN solution (30 mmol/l) was added to convert all B_{12} in the samples to CN-B_{12}. Then, the mixtures were boiled for 10 minutes and centrifuged for 40 min at 20 000 ***g*** and 4°C, and the supernatants were collected and stored at −20°C until analysed. The supernatants were measured for total B_{12} content on the Advia Centaur CP Immunoassay System after dilution with 0·9 % solution of NaCl. Supernatants from the B_{12}-depleted rats were diluted 1:5 (spleen, brain), 1:10 (liver, heart) or 1:100 (kidneys). Supernatants from the B_{12}-replete rats (HO-B_{12} group and CN-B_{12} group) were diluted 1:5 (brain), 1:10 (liver, spleen), 1:20 (heart) or 1:500 (kidneys). The dilutions were chosen to ensure that the B_{12} concentrations would be within the range of measurements (100–1476 pmol/l) of the Advia Centaur CP

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Immunoassay system. The results were expressed as pmol B_{12}/g of tissue unless otherwise indicated.

Determination of vitamin B_{12} forms in rat tissue

We determined the forms of B_{12} in liver, kidneys and brain from two rats selected from each of the three groups (HO-B_{12}, CN-B_{12} and low-B_{12}). For all groups, we chose the two rats with liver B_{12} closest to group mean. To determine the forms of B_{12}, the following procedure was carried out in dim red light to prevent photolytic loss of the coenzymes ADO-B_{12} and CH_3-B_{12}. Tissues were thawed on ice and 0·2 g was mixed with 750 µl of homogenisation buffer containing 10 mM Pipes, pH 7·4 (Sigma-Aldrich), 1 mM EDTA (Sigma-Aldrich), 3 mM $MgCl_2$, $6H_2O$ (Merck), 400 mM NaCl, and two tablets of Complete Protease Inhibitor Cocktail (Roche Diagnostics) added per 50 ml of the buffer. The mixtures were homogenised using the Precellys 24 with three centrifugation cycles of 20 s at 6800 rpm with 30 s pauses between cycles. After homogenisation, the mixtures were centrifuged for 40 min at 20 000 ***g*** and 4°C and 500 µl of the supernatants were incubated with 500 µl of 0·4 M acetic acid and 1000 µl 50 % methanol (final pH 3·8) for 15 min at 65°C before being centrifuged again for 10 min at 2600 ***g*** at room temperature. The mixtures were then centrifuged for 3 min at 11 000 ***g*** through a 0·22-µm Durepor PVDF filter (Merck Millipore Ltd), and the B_{12} forms present in the supernatant were separated by HPLC essentially as described previously[15,16]. In brief, a filtered sample was injected into an Agilent 1260 Infinity HPLC (Agilent Technologies) attached to a reverse-phase column (Luna 3 u reverse-phase C18(2) 150 × 4·6 mm; Phenomenex) and run with a flow rate of 1 ml/min. To apply the approximately similar amounts of B_{12}, different volumes of filtered sample were injected onto the column depending on the tissue (90 µl of the brain sample; 45 µl of the liver sample; 9 µl of the kidney sample). A gradient of acetonitrile (HPLC S-grade; Rathburn Chemicals) increasing from 5 to 30 % over 28 min in 0·010 mol/l phosphoric acid (H_3PO_4, pH 3) was applied 4 min after injection. Due to the low amount of B_{12} present in the samples, we could not measure the B_{12} profile by recording absorbance. Therefore, we collected forty-six post-column fractions every 20 s (320 µl/fraction) between 9 and 25 min after injection. The samples were lyophilised and dissolved in 240 µl of 0·1 % PBS with 0·1 % bovine serum albumin (PBA) before measurement of B_{12}, employing in-house haptocorrin ELISA[15,17] with a detection limit of 8 pmol/l B_{12}. In brief, 100 µl (sample or calibrator) was incubated with apo-haptocorrin, and excessive apo-haptocorrin was removed with B_{12}-coated magnetic beads. The amount of B_{12}-saturated haptocorrin in the sample was measured by the haptocorrin ELISA, and the results from the samples were read on a calibration curve (0–218 pmol/l). The assay signal was linear for B_{12} concentrations between 0 and 218 pmol/l and the total imprecision (CV) was ≤10 % (measured for B_{12} concentration between 45 and 200 pmol/l). The results from the ELISA were used to construct HPLC elution profiles for each sample, and to calculate the fractional distribution of the various forms of B_{12} out of the total amount of B_{12} measured in each run. The amount of each form (pmol/g tissue) was calculated by multiplying the mean fractional content (from the two rats per group) with the mean B_{12} content per gram (from the two rats per group).

Standards with pure HO-B_{12} (GEA), CN-B_{12} (Sigma-Aldrich), ADO-B_{12} (Sigma-Aldrich) and CH_3-B_{12} (Sigma-Aldrich) were used to identify the elution time of HO-B_{12} (10·8 min), CN-B_{12} (14·8 min), ADO-B_{12} (16·8 min) and CH_3-B_{12} (19·8 min) from the HPLC column. The elution time of GS-B_{12} (12·4 minutes) was identified by incubating trace elements of labelled [^{57}Co]HO-B_{12}≈0·4 nM (synthesised as described in Kornerup *et al.*[10]) with 2 mM GSH in 0·1 M phosphate buffer (pH 3) for 1 h at room temperature. The mixture was applied to the HPLC column, and the elution time was identified by measuring the amount of cpm in the post-column fractions.

Recovery of the HPLC method was estimated by using radiolabelled [^{57}Co]HO-B_{12} and [^{57}Co]CN-B_{12} (catalogue no. 06B-430000; MP Biomedicals). The labelled B_{12} forms were added to rat liver extracts and run on the HPLC. The radioactivity counts in the injected samples and the collected elution fractions were used to calculate recovery of the two B_{12} forms. By this method, recovery was found to be 93 % for HO-B_{12} and 98 % for CN-B_{12}.

Statistical analysis

The number of animals in each group was based on power calculations using a multiple linear regression model showing a statistical power of 90 % and confidence level of 95 %. The calculations were based on an earlier study showing a mean plasma B_{12} of 1330 pmol/l in rats kept on a standard diet with CN-B_{12}[11], and an anticipated mean decrease of 25 % for rats fed a diet with HO-B_{12}.

The D'Agostino-Pearson omnibus test was used to determine if the data followed the Gaussian distribution. Logarithmic transformation was used to obtain a normal distribution. Differences between the groups at the given time points were estimated by one-way ANOVA with Tukey's *post hoc* corrections for multiple comparisons. Differences between the time points within the same group were estimated by repeated-measures one-way ANOVA with Tukey's *post hoc* corrections for multiple comparisons. For some data (daily intake of diet (all groups), liver B_{12} (pmol/g) and heart B_{12} (pmol/g) (CN-B_{12} group)), normality could not be achieved by logarithmic transformation. In these cases, comparisons were made using the Kruskal–Wallis test with Dunn's corrections. Values of $P \leq 0{\cdot}05$ were accepted as statistical significant. The data analysis was performed using the statistical software available in GraphPad Prism version 7.03.

Results

We present data on rats kept on a low-B_{12} diet for 4 weeks before 2 weeks supplementation with HO-B_{12}, CN-B_{12}, or continuation on the low-B_{12} diet. The design is shown in Fig. 1.

Diet and vitamin B_{12} intake

The rats had a daily dietary intake of (median 23·4 (range 21·6–32·9) g/rat per d (HO-B_{12} group), 24·8 (range 20·4–33·8) g/rat

per d (CN-B_{12} group), and 24·4 (range 21·5–31·6) g/rat per d (low-B_{12} group). There was no difference in the amount of food consumed per day between the groups ($P = 0{\cdot}18$). Also, there was no significant weight difference among the rats in the three groups at the end of the study (see Table 1) or at any time point during the study (data not shown).

The B_{12} content of the diet assigned by the supplier (see the 'Methods' section) differed somewhat from our estimates. Here we indicate the values measured by us. Before the start of our study, all rats were kept on a standard rat diet containing 33 μg of CN-B_{12}/kg thereby supplying 0·79 μg (583 pmol) of CN-B_{12}/d. The low-B_{12} diet, used for 4 weeks to obtain a suboptimal B_{12} status, contained 5 μg B_{12}/kg equivalent to 0·12 μg (87 pmol) of B_{12}/d (>90 % HO-B_{12}). The custom-made CN-B_{12} diet contained 24 μg/kg supplying 0·58 μg (428 pmol) of CN-B_{12}/d. The HO-B_{12} diet contained 21 μg/kg supplying 0·50 μg (362 pmol) of HO-B_{12}/d.

The amount of B_{12} (approximately 0·5 μg/d) supplied to the rats from the custom-made diets corresponds to a daily intake of approximately 60 μg/d B_{12} in a human setting (weight 60 kg). This dose is within the normal range (9 μg/d to 1 mg/d) of commercially available oral supplements used for prevention and treatment of B_{12} deficiency.

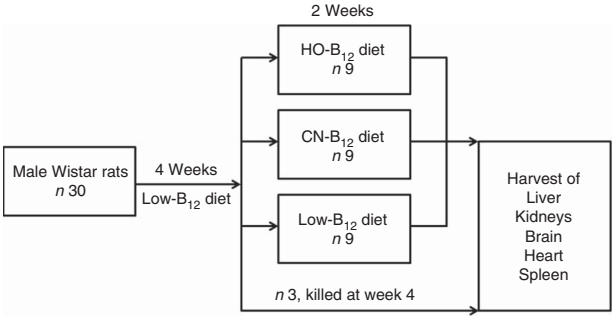

Fig. 1. Study design. Male Wistar rats (*n* 30) received a low-vitamin B_{12} diet for 4 weeks. At this time, three rats were killed, and the remaining twenty-seven rats were divided into three groups, who received custom-made diets for two additional weeks with added hydroxo-B_{12} (HO-B_{12}) or cyano-B_{12} (CN-B_{12}), or continued on the low-B_{12} diet. Hereafter, all rats were killed and tissues were harvested.

Plasma and tissue vitamin B_{12} content in vitamin B_{12}-depleted rats

We measured B_{12} in plasma at baseline and after 4 and 6 weeks on a low-B_{12} diet. After 4 weeks, the plasma B_{12} level had dropped from a mean of 1279 to 558 pmol/l (*n* 30, all rats). A further decline to 531 pmol/l was observed after 2 additional weeks on the low-B_{12} diet (*n* 9, low-B_{12} group at week 6) ($P < 0{\cdot}006$) (see Fig. 2). Three rats were killed after 4 weeks as controls. No statistically significant difference between the contents of B_{12} in liver and kidney was noticed after 4 and 6 weeks on the low-B_{12} diet (data not shown). These results suggest that the rats had reached a steady state in the B_{12} turnover on the low-B_{12} diet after 4 weeks, where the different custom-made diets were introduced.

Plasma and tissue distribution of vitamin B_{12} after dietary intake of hydroxo-B_{12}, cyano-B_{12} or low-B_{12} supplement

After the 4 weeks of the low-B_{12} diet, the HO-B_{12} group and the CN-B_{12} group received 2 weeks of special diets with HO-B_{12} and CN-B_{12}, respectively. Following this treatment (week 6), plasma B_{12} had returned to the baseline levels in the CN-B_{12} group, but this was not the case for the HO-B_{12} group (see Fig. 2).

Liver, kidneys, brain, heart and spleen were harvested from the two repleted groups and the depleted group of rats and analysed for content of B_{12}. The results are reported as pmol of B_{12}/g tissue and total organ B_{12} (pmol) in Fig. 3 and Table 1, respectively.

Compared with the low-B_{12} group, the following overall surplus of B_{12} had accumulated in the organs studied during the 2 weeks of dietary supplementation (+600 pmol in the HO-B_{12} group and +2000 pmol in the CN-B_{12} group). The high accumulation of CN-B_{12} was almost totally driven by an increase in the kidney content of B_{12}. In contrast, significantly

Table 1. Total organ contents of vitamin B_{12} (B_{12}) in rats on diets with hydroxo-B_{12} (HO-B_{12}) or cyano-B_{12} (CN-B_{12}) or on a low-B_{12} diet* (Mean values and ranges)

	HO-B_{12} (*n* 9)				CN-B_{12} (*n* 9)				Low-B_{12} (*n* 9)			
	Organ weight (g)		Whole-organ B_{12} (pmol)		Organ weight (g)		Whole-organ B_{12} (pmol)		Organ weight (g)		Whole-organ B_{12} (pmol)	
	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Organs												
Liver	16·8	14·6–18·4	760	603–1212	18·0	13·6–19·3	618	538–1089	17·0	14·7–18·6	649	556–746
Kidneys	3·5	2·7–4·0	1100	610–1345	3·6	3·1–4·4	2620	1814–3115	3·4	3·0–3·8	567	412–1141
Brain	1·5	1·5–1·8	23·5	22·4–25·3	1·6	1·6–1·7	25·5	21·9–28·8	1·4	1·1–1·7	18·5	14·9–27·3
Heart	1·8	1·5–2·1	88·2	72·2–166	1·8	1·6–2·3	89·4	68·9–193	1·8	1·4–1·9	78·0	67·9–91·6
Spleen	1·3	1·1–1·8	34·4	26·6–41·9	1·3	1·1–1·9	28·4	20·1–38·2	1·4	1·1–1·6	22·0	17·6–27·4
Total B_{12} recovered (pmol)			2006	1334–2790			3381	2463–4464			1335	1068–2033
Total body weight (g)			450	405–522			450	367–486			454	415–486

* Rats were kept for 4 weeks on a low-B_{12} diet followed by 2 weeks on diets with HO-B_{12} or CN-B_{12}, or continuing on the low-B_{12} diet for 2 weeks before killed. The total amount (sum) of B_{12} (total B_{12} recovered) in the harvested rat organs is shown. The three groups of rats consumed the same amount of rat diet during the study (24 g/d). There was no difference in organ weights or total rat weights between the three groups as judged by the one-way ANOVA. The tissue B_{12} contents between the three groups (B_{12}/g tissue) are compared in Fig. 3.

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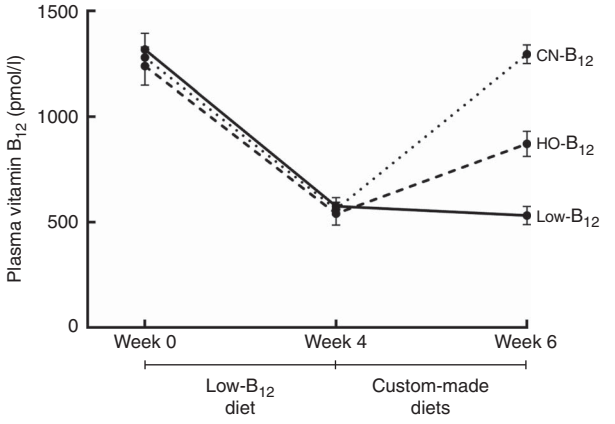

Fig. 2. Plasma vitamin B_{12} in rats on diets with hydroxo-B_{12} (HO-B_{12}) or cyano-B_{12} (CN-B_{12}) or a low-B_{12} diet. Three groups of rats (*n* 9 in each group) were kept for 4 weeks on a low-B_{12} diet followed by 2 weeks on custom-made diets with HO-B_{12} or CN-B_{12}, or maintained on a low-B_{12} diet. Values are means with their standard errors. No difference in plasma B_{12} was found between the three groups at baseline or after 4 weeks (week 0, week 4). As expected, 4 weeks on the low-B_{12} diet reduced plasma B_{12} for all three groups (week 4) ($P<0{\cdot}0001$). After the 2 weeks on custom-made diets (week 6), plasma B_{12} was increased in both supplemented groups. The increase in plasma B_{12} in the CN-B_{12} group was more than 2-fold higher than in the HO-B_{12} group ($P<0{\cdot}0001$). Plasma B_{12} in the low-B_{12} group declined further from week 4 to week 6 ($P=0{\cdot}015$). Differences between the groups at given time points (or between the time points of the same group) were estimated by the one-way (repeated-measures) ANOVA with Tukey's *post hoc* corrections for multiple comparisons.

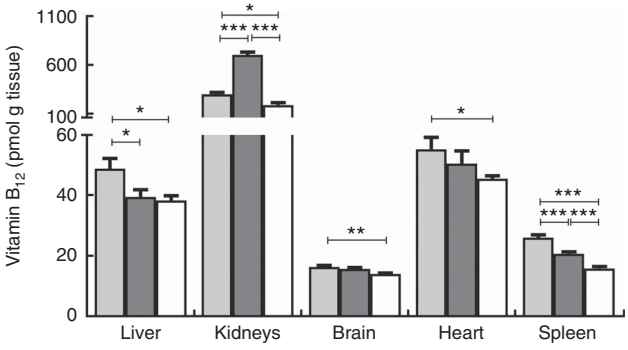

Fig. 3. Tissue distribution of vitamin B_{12} in rats on diets with hydroxo-B_{12} (HO-B_{12}, ▫) or cyano-B_{12} (CN-B_{12}, ▪) or on a low-B_{12} (□) diet. Liver, kidneys, brain, heart and spleen were harvested from rats after 4 weeks on a low-B_{12} diet followed by 2 additional weeks on custom-made diets containing HO-B_{12} (*n* 9) or CN-B_{12} (*n* 9) or continuing on the low-B_{12} diet (*n* 9). Tissues were analysed for contents of total B_{12}, and the results are given as pmol/g tissue. Values are means with their standard errors. Level of statistical significance (* $P<0{\cdot}05$, ** $P<0{\cdot}005$, *** $P<0{\cdot}0005$) for group comparisons using the one-way ANOVA with Tukey's *post hoc* corrections for multiple comparisons (kidney, brain, spleen; normalised data) or the Kruskal–Wallis test with Dunn's corrections (kidney, heart; not normalised data). Notably, a higher amount of B_{12} was found in the liver and spleen of the HO-B_{12} group compared with the CN-B_{12} group; but in the kidney the B_{12} amount was highest in the CN-B_{12} group. There was no difference in liver B_{12} between the low-B_{12} group and the CN-B_{12} group.

more HO-B_{12} than CN-B_{12} accumulated in the liver ($P=0{\cdot}027$), and a similar tendency (HO-B_{12} > CN-B_{12}) was found in the spleen and the heart (Table 1). All in all, 1400 pmol more CN-B_{12} was recovered in the harvested organs compared with HO-B_{12}. As we previously have shown, the two forms of B_{12} are absorbed equally well[10,11], which implies that a surplus of approximately 1400 pmol HO-B_{12} relative to CN-B_{12} is distributed in the remaining rat tissues.

Table 2. Distribution of the five vitamin B_{12} (B_{12}) forms in tissues from rats on diets with hydroxo-B_{12} (HO-B_{12}) or cyano-B_{12} (CN-B_{12}) or on a low-B_{12} diet*

	Form				
Tissues	HO-B_{12} (%)	GS-B_{12} (%)	CN-B_{12} (%)	ADO-B_{12} (%)	CH_3-B_{12} (%)
Liver					
HO-B_{12}	5	9	0	86	0
CN-B_{12}	7	9	3	81	0
Low-B_{12}	27	14	1	55	3
Kidneys					
HO-B_{12}	12	6	1	76	5
CN-B_{12}	17	14	51	17	1
Low-B_{12}	21	15	2	56	6
Brain					
HO-B_{12}	11	35	1	52	1
CN-B_{12}	12	19	20	48	1
Low-B_{12}	22	16	3	57	2

GS-B_{12}, glutathionyl-B_{12}; ADO-B_{12}, 5'-deoxyadenosyl-B_{12}; CH_3-B_{12}, methyl-B_{12}.
* Forms of B_{12} were determined by HPLC followed by ELISA. Fractional contents given in % were calculated based on the total amount of B_{12} measured in each run. Five different B_{12} peaks were identified, which eluted as HO-B_{12} (10·8 min), GS-B_{12} (12·4 min), CN-B_{12} (14·8 min), ADO-B_{12} (16·8 min) and CH_3-B_{12} (19·8 min). Results are given as the calculated fractional distribution (mean% from two rats per group). The fractional distribution from each rat (not merged data) is shown in the online Supplementary data S1. The calculated amounts of each B_{12} form are shown in Fig. 4. The HPLC elution profiles are shown in the online Supplementary data S2.

Forms of vitamin B_{12} in rat tissue after dietary intake of hydroxo-B_{12}, cyano-B_{12} or low-B_{12} supplement

The forms of B_{12} in liver-, kidney- and brain homogenates prepared from organs of two rats from each group were measured following separation of the various forms by HPLC. The HPLC profiles showed peaks corresponding to the elution times for HO-B_{12} (10·8 min), GS-B_{12} (12·4 min), CN-B_{12} (14·8 min), ADO-B_{12} (16·8 min) and CH_3-B_{12} (19·8 min). The fractional distributions of the five different B_{12} forms found in the tissues are shown in Table 2, and their estimated quantities are shown in Fig. 4.

In general, ADO-B_{12} was the predominant form in the rat tissues, followed by GS-B_{12} and HO-B_{12}, whereas CH_3-B_{12} and CN-B_{12} (for groups not treated with CN-B_{12}) were almost undetectable (see Table 2 and Fig. 4). Compared with the low-B_{12} group, the HO-B_{12} group showed a marked increase in ADO-B_{12} in the liver and the kidneys and an increase in GS-B_{12} in the brain. The CN-B_{12} group showed a less pronounced increase in liver ADO-B_{12} and even a minor drop in brain ADO-B_{12}, compared with depleted rats. Changes in the kidney of the CN-B_{12} group was characterised by a dominant accumulation of CN-B_{12}, which was also partially encountered in the brain (Table 2 and Fig. 4).

Discussion

We explored the uptake of B_{12} and its accumulation and conversion to the coenzymes in rats kept on a low-B_{12} diet for

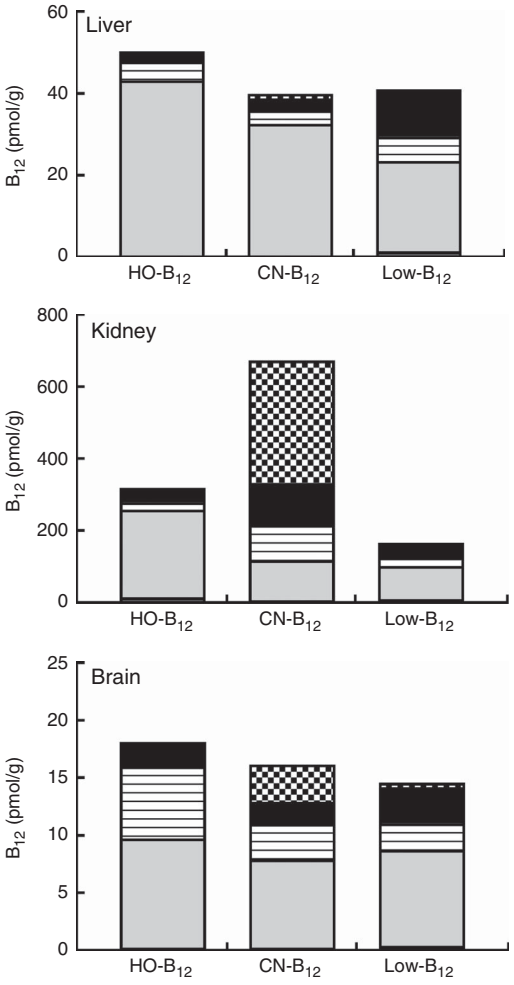

Fig. 4. Vitamin B_{12} (B_{12}) forms in rat tissue. Liver, kidneys and brain were harvested from rats after 4 weeks on a low-B_{12} diet followed by 2 additional weeks on custom-made diets containing hydroxo-B_{12} (HO-B_{12}) (*n* 9) or cyano-B_{12} (CN-B_{12}) (*n* 9) or continuing on the low-B_{12} diet (*n* 9). Tissues from two rats per group were analysed for forms of B_{12} (CN-B_{12}, HO-B_{12}, glutathionyl-B12 (GS-B_{12}), 5'-deoxyadenosyl-B_{12} (ADO-B_{12}) and methyl-B_{12} (CH_3-B_{12})) by HPLC followed by ELISA. Results are given as the amount (pmol/g tissue) of each of the five B_{12} forms in the particular tissue. The amount was calculated by multiplying the fractional amount of each B_{12} form (mean% from the two rats per group) with the B_{12} amount (mean pmol/g tissue from the two rats per group). ▩, CN-B_{12}; ■, HO-B_{12}; ▤, GS-B_{12}; □, ADO-B_{12}; ▩, CH_3-B_{12}.

4 weeks followed by a 2-week diet containing HO-B_{12} or CN-B_{12} or a 2-week continuum on a low-B_{12} diet.

We found major differences in both the distribution and the degree of conversion to the B_{12} coenzymes for HO-B_{12} and CN-B_{12} supplementations. Notably, more than half of the administered CN-B_{12} remained in this form in the kidneys, whereas HO-B_{12} was largely converted to the bioactive ADO-B_{12} providing more than the doubled amount of active coenzymes to the kidney tissues than CN-B_{12}. The degree of conversion becomes even larger if subtracting ADO-B_{12} in the low-B_{12} organs and counting only its increments, accumulated in the cause of HO-B_{12} and CN-B_{12} supplementation. Our study also highlights the uncertainty of using plasma B_{12} as a sole marker of B_{12} tissue content.

The work has some limitations. Even though no B_{12} had been added, the low-B_{12} diet still contained 5 μg/kg B_{12}, possibly due to an unforeseen contamination with B_{12}-producing micro-organisms in the production line. As a result, our low-B_{12} group showed a milder B_{12} depletion in the tissues, compared with the previous study employing a different batch of the same low-B_{12} diet[11]. However, as all three groups of rats were equally depleted, we do not believe that the 'less pronounced' level of depletion detracts from the value of our findings. We measured the B_{12} content and forms of the B_{12} present in the custom-made diets before administration but did not repeat these measures at the end of the study. The diets were kept in the cold (4°C) in the air- and light-tight containers until administration to the rats, and we consider B_{12} to be stable under these conditions throughout the 2 weeks study period. The CN-B_{12} diet contained 14 % more B_{12} than the HO-B_{12} diet. We do not expect this difference in supplemented quantities of CN-B_{12} and HO-B_{12} to be of importance, because the uptake of B_{12} reaches a plateau at high doses of the vitamin. Despite these weaknesses, we believe our study has merit and provides new interesting information on the metabolism of CN-B_{12} and HO-B_{12} in the tissues.

Acute uptake studies of CN-B_{12} and HO-B_{12} showed a higher accumulation of CN-B_{12} than HO-B_{12} in the kidneys and brain, but a lower accumulation in the liver and spleen[10,11]. Contrary to our expectations, the initial difference between the two B_{12} forms did not level out after 2 weeks of their dietary intake, and we observed both quantitative and qualitative differences in the distribution and conversion of CN-B_{12} and HO-B_{12}.

A much higher accumulation of B_{12} was discovered in the liver of HO-B_{12} animals, whereas the CN-B_{12} group and the low-B_{12} group exhibited the same total B_{12} in this organ. Our findings support the view that the hepatocytes preferentially accumulate the natural forms of B_{12}, whereas the synthetic vitamin has a reduced uptake (or increased export).

An interesting finding pertains to the threefold surplus of B_{12} accumulated in the examined organs of rats on a CN-B_{12} diet compared with an HO-B_{12} diet. This difference is driven by the vast accumulation of CN-B_{12} in the kidney, which has no bearing on the B_{12} coenzymes synthesised in this organ (nearly equal to that in the low-B_{12} group). Acute studies showed an equal uptake of the two forms of the vitamin[10,11], and thus we anticipate that the animals have approximately the same amounts of B_{12} in the body, irrespectively of the B_{12}-form supplied. This in turn suggests that other tissues would contain somewhat more B_{12}, when the vitamin is provided as HO-B_{12} as compared with CN-B_{12}. This interpretation is clearly supported by the results for the liver and the spleen, whereas the difference is insignificant in the brain and heart (Fig. 2).

The highest B_{12} concentrations and amounts were found in the kidneys of all animals, irrespectively of their diet. This agrees with our previous studies[10,11] and also with the work of Quadros *et al.*[13], where rats were given a mixed diet of ADO-B_{12} (60 %), HO-B_{12} (25 %) and CN-B_{12} (15 %). The total kidney B_{12} increased by factors of 1·6 and 4 on the HO-B_{12} and CN-B_{12} diets, respectively, when compared with the rats remaining on the low-B_{12} diet. It is well known that the kidneys serve as a storage organ for B_{12} in rats[14,18,19], but such difference in

kidney accumulation of the two forms of B_{12} was unexpected. The effect can be ascribed to a relatively low accumulation of CN-B_{12} in the liver (and possibly other organs), which leaves high quantities of the circulating CN-B_{12} (eventually filtered and stored in the kidneys). Another explanation might rely on a preferential accumulation of CN-B_{12} in the kidney. It should be noticed in this regard, that B_{12} in kidney remains physiologically 'inert', being stored mainly as a free ligand, not bound to any protein or enzyme[18,19].

We also examined the coenzyme patterns of B_{12} in two animals from each group studied. The results for all in-group rats compared very well, and we take the data to be representative. In line with Quadros et al, we find ADO-B_{12} and HO-B_{12} (part of it converted to GS-B_{12}) to account for the majority of the total B_{12}, whereas CH_3-B_{12} was almost undetectable. This might suggest that CH_3-B_{12} is of little importance for the methylation of homocysteine in the rat. Quadros et al speculated that a B_{12}-independent methylation mechanism of homocysteine (e.g. via the betaine pathway) could be involved. An alternative explanation might be that CH_3-B_{12} in the rat cells mainly exists as the catalytic intermediate [Co^{1+}]B_{12} spontaneously oxidised to HO-B_{12} under extraction[13]. We would like to point out in this regard that tissue HO-B_{12} (as well as its derivative GS-B_{12}) is not necessarily an inert compound (like CN-B_{12}). The enzymatic cycles of ADO-B_{12} and CH_3-B_{12} go through the reduced intermediates ([Co^{2+}]B_{12} and [Co^{1+}]B_{12}) with the 'disconnected' active groups (ADO and CH_3, respectively[20]). Both reduced cofactors are easily oxidised to HO-B_{12}, making the latter merely a reflection of the catalytic steady state balance, for example CH3-B_{12} ↔ [Co^{1+}]B_{12}.

We are not aware of other studies relating dietary forms of B_{12} to the pattern of B_{12} forms present in the key organs. Interestingly, these patterns are different in brain, liver, and kidney. Irrespectively of the diet, the brains showed comparable amounts of ADO-B_{12} in all groups, but the CN-B_{12} group also contained 20 % of CN-B_{12}. We do not know whether this compound is an innocent bystander or a competitive antagonist of B_{12}-dependent enzymes (the latter case presenting a clear physiological problem). The liver eventually converted both HO-B_{12} and CN-B_{12} to ADO-B_{12}, albeit the conversion was most prominent in the HO-B_{12} group. Perhaps the most surprising result was observed in the kidney. Dietary intake of CN-B_{12} did not increase ADO-B_{12}, and most of the extra B_{12} accumulated in the organ as CN-B_{12}. In contrast, the supplement of HO-B_{12} diet almost doubled the amount of ADO-B_{12} in the kidney due to increments of ADO-B_{12} = +145 pmol/g (HO-B_{12} group) *v.* +30 pmol/g (CN-B_{12} group), added to the 'background' level of 93 pmol/g (low-B_{12} group). Similar pictures were also observed in other organs. It seems that HO-B_{12} got converted to ADO-B_{12} at a higher rate than CN-B_{12} did. The result agrees with previous data suggesting that the conversion of CN-B_{12} to ADO-B_{12} is slower and more demanding than the conversion of HO-B_{12}[21,22]. For example, Uchino et al described a 3-fold higher conversion to ADO-B_{12} in rat liver 24 h after intravenous injection of HO-B_{12} in comparison to CN-B_{12}[21]. This difference has a biochemical basis. Formation of ADO-B_{12} and CH_3-B_{12} in the cell requires the initial reduction of CN-B_{12} or HO-B_{12}, and this process is mediated by the specific chaperon CblC, assisted in its function by methionine synthase reductase (MSR) and NAPDH. The rate of reduction is considerable faster for HO-B_{12}, which is also reduced by MSR and NAPDH without CblC, as well as by glutathione ± MSR[23].

The finding, that dietary HO-B_{12} provides more active coenzymes to the tissues compared with CN-B_{12} (despite of a lower plasma B_{12} concentration), is of clinical and diagnostic relevance. B_{12} is traditionally evaluated from measurements of total plasma B_{12}. Yet, our data suggest that plasma B_{12} concentrations do not truly reflect the tissue status of B_{12}. For this reason, we recommend that plasma B_{12} measurements for diagnostic purposes are accompanied by, for example assessment of plasma methylmalonic acid, a marker of intracellular B_{12} status. Our findings also suggest that natural food items, such as milk and meat (containing HO-B_{12}) provide a better source of B_{12} for the tissues than an equal amount of synthetic B_{12} from a vitamin pill. Studies are needed to investigate this subject closer.

In conclusion, tissue distribution of HO-B_{12} and CN-B_{12} after 2 weeks of dietary intake shows a great resemblance to our previous results observed 24 h after the acute uptake. CN-B_{12} is better at restoring plasma B_{12} than HO-B_{12}. Yet, the latter provides a better supply of ADO-B_{12} to the tissues. The data raise a question whether HO-B_{12} provides a better supply of metabolically active B_{12} than CN-B_{12} does.

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Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S000711451800123X

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