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# Human vitamin $B_{12}$ absorption and metabolism are measured by accelerator mass spectrometry using specifically labeled $^{14}$C-cobalamin.

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There is need for an improved test of human ability to assimilate dietary vitamin $B_{12}$. Assaying and understanding absorption and uptake of $B_{12}$ is important because defects can lead to hematological and neurological complications. Accelerator mass spectrometry (AMS) is uniquely suited for assessing absorption and kinetics of $^{14}C$-labeled substances after oral ingestion because it is more sensitive than decay counting and can measure levels of carbon-14 ($^{14}C$) in microliter volumes of biological samples, with negligible exposure of subjects to radioactivity. The test we describe employs amounts of $B_{12}$ in the range of normal dietary intake. The $B_{12}$ used was quantitatively labeled with $^{14}C$ at one particular atom of the DMB moiety by exploiting idiosyncrasies of *Salmonella* metabolism. In order to grow aerobically on ethanolamine, *S. enterica* must be provided with either pre-formed $B_{12}$ or two of its precursors: cobinamide and dimethylbenzimidazole (DMB). When provided with $^{14}C$-DMB specifically labeled in the C2 position, cells produced $^{14}C$-$B_{12}$ of high specific activity (2.1 GBq/mmol, 58 mCi/mmol) and no detectable dilution of label from endogenous DMB synthesis. In a human kinetic study, a physiological dose (1.5 μg, 2.2 KBq/59 nCi) of purified $^{14}C$-$B_{12}$ was administered and showed plasma appearance and clearance curves consistent with the predicted behavior of the pure vitamin. This method opens new avenues for study of $B_{12}$ assimilation.

Vitamin $B_{12}$ ($B_{12}$) is a compound of significant nutritional and clinical importance (1). The classical manifestations of $B_{12}$ deficiency include pernicious anemia, a type of megaloblastic anemia, and neurological dysfunction (2). The Schilling urinary excretion test (3, 4) indirectly measures $B_{12}$ absorption and has been applied when $B_{12}$ insufficiency is identified and malabsorption is the suspected cause. The test involves ingestion of a physiological quantity of $B_{12}$ labeled with gamma-emitting cobalt, followed by administration of a pharmacological parenteral flushing dose of unlabeled $B_{12}$ to force urinary excretion of radioactivity, which is measured over a twenty-four hour period. The Schilling test is currently the only accepted method for assessing $B_{12}$ absorption. Despite its utility, the method is semi-quantitative and has methodological and practical problems; it is now rarely prescribed, despite the prevalence of $B_{12}$ malabsorption in older adults (2, 5). We describe a new method that has potential to reinvigorate interest in diagnosis of the underlying causes of vitamin $B_{12}$ deficiency. The test has several advantages over the Schilling test --it poses a negligible radiation exposure to the subjects and medical workers and can be performed from a capillary-sized blood sample, without the requirement for a flushing dose of $B_{12}$ or for collection of radioactive urine for an extended period.

The absorption test described uses carbon 14-labeled vitamin $B_{12}$ ($^{14}C$-$B_{12}$) coupled to sensitive detection of the $^{14}C$-$B_{12}$ by accelerator mass spectrometry (AMS). AMS was originally developed for carbon dating in archaeological or earth science samples, however in the past decade or so its sensitivity has been exploited for tracing of biological systems (6). In contrast to liquid scintillation counting, which records decay events of a radioisotope, AMS is a direct atom-counter that was developed for

quantifying long-lived isotopes such as $^{14}C$ (half-life 5,370 yr). It is a tandem isotope ratio mass spectrometer that provides the relative abundance of the $^{14}C$ atom with respect to total carbon ($^{14}C/C$) down to parts per quadrillion (1 in $10^{15}$) (6-8). Thus it is possible to quantify attomole ($10^{-18}$ mol) amounts of $^{14}C$ in a milligram-sized biological sample at high precision (typically < 2% imprecision). The remarkable combination of sensitivity and precision of AMS allows quantitation of $^{14}C$-labeled $B_{12}$ from small biological samples and reduces exposure of subjects to a negligible radiation risk.

The key to the success of the $B_{12}$ absorption test we describe is the synthesis of $^{14}C$-$B_{12}$ by an efficient, micro-scale method which produces $^{14}C$-$B_{12}$ specifically labeled at the carbon 2 position of the dimethylbenzimidazole (DMB) moiety of $B_{12}$. This $B_{12}$ is produced by *Salmonella enterica*, a bacterium that normally produces $B_{12}$ *de novo* only under anaerobic conditions and uses it to support growth on ethanolamine. Cells cannot grow aerobically on ethanolamine because they fail to synthesize two $B_{12}$ precursors, cobinamide and DMB, but they retain ability to assemble $B_{12}$ from these precursors supplied exogenously. When isotopically labeled DMB is supplied, $B_{12}$ is produced with no detectable isotope dilution. The processes leading to the biosynthesis of $^{14}C$-$B_{12}$ by *S. enterica* are shown in Figure 1. The $^{14}C$-$B_{12}$ synthesized by this method was used for quantitative AMS-based assay of human $B_{12}$ absorption.

## Results

### Metabolic engineering of *S. enterica* for biosynthesis of $^{14}$C-labeled $B_{12}$

The method used to produce labeled $B_{12}$ relies on two idiosyncrasies of $B_{12}$ metabolism in *Salmonella enterica*. Under aerobic conditions on minimal medium with ethanolamine as sole carbon source, *S. enterica* produces neither cobinamide (the corrinoid precursor) nor DMB (the lower ligand of $B_{12}$), but retains ability to assemble $B_{12}$ when these precursors are provided exogenously.

The 17-gene ethanolamine (*eut*) operon (9) of *S. enterica* is shown in Figure 2a. Induction of *eut* and the growth conditions pertinent to this work are shown in Figure 2b. Induction of the *eut* operon was monitored by assay of β-galactosidase produced by a *lacZ* gene inserted within a transcribed region of the eut operon that is distal to all genes of the operon. The basal level of *eut* transcription produces 10 Miller units of β-galactosidase activity from an operon fusion and increases about 50-fold when the operon is induced by the combination of $B_{12}$ plus ethanolamine. Surprisingly, the $B_{12}$ precursor cobinamide can replace $B_{12}$ as an inducer but does not permit growth on ethanolamine (Figure 2b). This failure to produce $B_{12}$ is due to lack of DMB since $B_{12}$ production and growth on ethanolamine is restored if DMB is provided in addition to cobinamide. Thus, during aerobic growth on ethanolamine, *S. enterica* fails to make both cobinamide and DMB, but can synthesize $B_{12}$ if these precursors are provided. The CobA, U, S, T, C and EutT enzymes, which catalyze conversion of cobinamide plus DMB to $B_{12}$ are produced at levels sufficient to assemble $B_{12}$ and permit aerobic cell growth on ethanolamine. This provides a situation for efficient conversion of labeled precursors into $B_{12}$ without label dilution. The growth conditions used appear to avoid feedback

repression of $B_{12}$ synthesis, presumably because $B_{12}$ is sequestered and bound by the EutBC enzyme.

**Synthesis and purification of $^{14}$C-labeled DMB and incorporation into $B_{12}$**

High specific activity $^{14}$C-labeled DMB was synthesized by condensation of $^{14}$C formic acid with dimethylphenylenediamine (10) (Figure 1). The reaction product, [2-$^{14}$C]-dimethylbenzimidazole, was purified by HPLC to eliminate unwanted reaction products. When this labeled DMB and unlabeled cobinamide were provided to *S. enterica*, the cells produced $B_{12}$ and grew aerobically on ethanolamine.

**Purification and mass spectral analysis of $^{14}$C-$B_{12}$**

Adenosylcobalamin was extracted from bacteria in the presence of cyanide so that highly stable cyanocobalamin (vitamin $B_{12}$) would be formed. These extracts were purified by HPLC and analyzed by two methods of mass spectrometry to establish chemical identity. The $^{14}$C radiolabel co-eluted precisely with the single chromatographic peak of the vitamin $B_{12}$ standard (Figure 3a). The UV/visible absorption spectrum of the purified $^{14}$C-compound was consistent with that of the $B_{12}$ standard spectrum (Figure 3b). The putative $^{14}$C-labeled cyanocobalamin ($B_{12}$) was also analyzed by two forms of mass spectrometry; the MS/MS product spectra of the vitamin $B_{12}$ standard, $(M+H)^+$ at m/z 1355.8, and the $^{14}$C-labeled vitamin $B_{12}$, $(M+H)^+$ at m/z 1357.8, are shown in Figure 4. There is a 2 Da mass difference between the molecular ions of standard and the $^{14}$C-labeled cobalamins; the fragmentation pattern confirms that this is due to a single $^{14}$C-label on the 5,6-dimethylbenzimidazole moiety. The molecular ion ($M+H^+$) of the unlabeled $B_{12}$ was compared to the $^{14}$C-labeled compound also by high resolution MS.

The measured values for both the unlabeled (m/z 1355.5741) and $^{14}$C-labeled cobalamin (m/z 1357.5787) agreed in molecular mass with the predicted values (m/z 1355.5752 and m/z 1357.5782, respectively). Based on the identified decomposition products of the MS/MS experiments, the accurate mass measurement data, and the HPLC data, we conclude that the product of the directed biosynthesis is ([2-$^{14}$C]5,6-dimethylbenzimidazole) cyanocobalamin.

**Specific Activity of $^{14}$C-$B_{12}$**

The theoretical specific activity for a compound with 1 atom of $^{14}$C at 100% incorporation is 2.308 GBq/mmol. Mass spectral analysis of the DMB isotopomers synthesized as described above indicated that 91% of the DMB product contained the $^{14}$C label, thus the specific activity was 2.1 GBq/mmol (data not shown). MS analysis of the $^{14}$C-$B_{12}$ indicated that the DMB had been incorporated into the $^{14}$C-$B_{12}$ without detectable dilution by endogenous DMB synthesis. The $^{14}$C-$B_{12}$ therefore had approximately the same specific activity, 2.1 GBq/mmol. The total mass yield of vitamin $B_{12}$ produced from the incubation was 25 µg (18.6 nmoles as cyanocobalamin).

**Human $B_{12}$ absorption**

The time course for the appearance and disappearance of $^{14}$C in plasma over seven days post-dose is presented in Figure 5. Data are given as femtomoles of $^{14}$C-vitamin $B_{12}$ per mL plasma. After seven hours, the circulating $^{14}$C-$B_{12}$ reached a peak, which corresponded to less than three percent of the administered dose of $^{14}$C-$B_{12}$. The amount of $B_{12}$ detected at the peak would produce less than one disintegration per minute if assayed by scintillation counting, and thus would be unmeasurable by decay

counting. There is a two to three hour delay in the appearance of the label in plasma, consistent with the time taken for gastric emptying and facilitated absorption in the ileum. After the maximum level of $^{14}C$-$B_{12}$ was achieved, the concentration of the label decreased at a single rate, with evidence for a small reappearance in plasma four days post-dose. A radiochromatogram of cyanidated plasma, taken from the $C_{max}$ sample, revealed that the majority of the $^{14}C$ co-migrated with cyanocobalamin on a reversed-phase HPLC system (data not shown).

Figure 6 illustrates recovery of the label in urine and stool specimens seven days post-dose. The largest single concentration of label appeared in the twenty-four hour urine collection (6.8% of administered dose). The apparent excretion of 6.8% of the $B_{12}$ dose in the 24 hour urine does not agree with previously reported values of 0.10-0.41% urinary $B_{12}$ excretion for normal subjects given radiocobalt $B_{12}$ with no flushing dose of $B_{12}$ (11). However, a radiochromatogram of cyanidated urine indicates that only approximately 1.47% of the $^{14}C$ urinary analytes was $^{14}C$-$B_{12}$ (data not shown). Thus the total 24 hour urine $^{14}C$-$B_{12}$ is approximately 0.1% of the total $^{14}C$-$B_{12}$ dose given, in agreement with previous measurements. The chromatographic analysis of the urine showed that the bulk of the label is distributed among several peaks with mobility distinct from that of $B_{12}$ or free DMB. These may be breakdown products of $B_{12}$ that were not detected for the cobalt labeled compound. After seven days, a total of 15.8% of the oral dose was recovered in urine and feces. Ninety-nine percent of the total quantified urinary and fecal output was accounted for seventy-two hours post-dose.

## Discussion

The absorption of $B_{12}$ in humans is a complex process compared with absorption of other water-soluble vitamins, requiring a specific intestinal transport protein produced in the stomach (intrinsic factor) for active uptake. This process is quickly saturated and doses greater than the current Recommended Dietary Allowance (2.4 μg/day US; 2.0 μg/day Europe) are passed unabsorbed into stool. Therefore, sensitive absorption tests are necessary to assess absorption of $B_{12}$ at normal dietary levels. Recently a new, nonradioactive method for assessing vitamin $B_{12}$ absorption has been reported (17). The method seeks to correlate $B_{12}$ absorption with an increase in holo-transcobalamin using ELISA. While this method holds promise, in its current form it lacks the ability to deliver an unequivocal $B_{12}$ absorption/malabsorption result and cannot convey quantitative or kinetic information. The data described in the present study suggest that a more robust method for assaying $B_{12}$ absorption and turnover can be developed that relies on use of AMS to detect physiological levels of suitably labeled $B_{12}$ with high precision. Production of $^{14}C$-$B_{12}$ substrate is critical for this improved method.

$B_{12}$ biosynthesis starts either from succinyl-CoA and glycine (the Shemin or C4 pathway) or, alternatively, from glutamyl-tRNA (the C5 pathway) (12). These primary metabolites have manifold roles and are not solely committed to $B_{12}$ biosynthesis; their use as a source of radiolabel for $B_{12}$ would result in significant label dilution. In the described method, a specific atom of $B_{12}$ is labeled with $^{14}C$ using a committed, late intermediate, DMB.

Dimethylbenzimidazole is an ideal molecule for labeling $B_{12}$ because it appears to have only a single metabolic fate: incorporation into the α-ligand of $B_{12}$. Fortuitously, it is both stable

and inexpensive to radiolabel by a one-step reaction from $^{14}$C-formic acid and dimethylphenylenediamine. This precursor is efficiently introduced into $B_{12}$ by cells of *S. enterica* because of several metabolic idiosyncrasies of this bacterium. *S. enterica* has a well characterized *de novo* biosynthetic pathway for $B_{12}$ (13) that functions only in the absence of oxygen (14). When grown aerobically on glucose with cobinamide, *S. enterica* makes $B_{12}$ using endogenously synthesized DMB (15), which would dilute the label of any added DMB. However, when ethanolamine is the sole carbon source, aerobic *S. enterica* cells synthesize neither cobinamide nor DMB and both must be added to allow $B_{12}$ production and growth (Figure 1). Thus, during growth of Salmonella on ethanolamine, $^{14}$C-labeled dimethylbenzimidazole provided with cobinamide allows for efficient and specific labeling of $B_{12}$.

The use of this labeled $B_{12}$ and AMS to assay $B_{12}$ absorption was demonstrated in a single, healthy, normal volunteer. A single 1.5 μg dose of the $^{14}$C-$B_{12}$ was administered orally and detected by AMS as it appeared in the bloodstream. Normally, the release of $B_{12}$ from intestinal mucosa cells into the portal vein occurs approximately two hours after the oral consumption of the vitamin, while release into systemic circulation takes an additional hour (16). Consistent with this, the $^{14}$C from the labeled $B_{12}$ appeared in the plasma of the human subject three hours post-dose. A concentration peak of 5 – 6 fmol $^{14}$C-$B_{12}$/ml was observed between five and twelve hours on either side of the $C_{max}$, which occurred at seven hours (Figure 5). This time window would make it possible to assess $B_{12}$ absorption from a single capillary blood sample. The current Schilling urinary excretion test, which has been the standard method of assessing $B_{12}$ absorption since its introduction in 1953, is now rarely prescribed as it requires administration of radiocobalt $B_{12}$, followed by an

intramuscular flushing dose and twenty-four hour total urine collection. By this method, patients with normal $B_{12}$ absorption excrete eight to forty percent of the labeled $B_{12}$ in the urine, compared with patients who have malabsorption, from whom there is little or no recovery of labeled $B_{12}$ from urine (4). By contrast, the method we describe makes it possible to follow the fate of the vitamin, at near ambient levels of radiation exposure, using microliter-sized blood specimens and without a flushing dose. Relatively little of the dose was recovered in either urine or stool; eighty four percent of the administered dose was retained in body tissues after seven days. This observation is consistent with the very slow body elimination of $B_{12}$ (0.1% loss per day after initial excretion of the unabsorbed dose) due to efficient enterohepatic recycling. The 6.8% loss in the 24-hour urine was unexpected in the absence of a parenteral flushing dose, based on previous experiments carried out using radiocobalt $B_{12}$. Examination of the chromatographic behavior of $^{14}C$ labeled compounds in the urine revealed that the predominant urinary products were not vitamin $B_{12}$. The accumulation of $^{14}C$-$B_{12}$ degradation products in the urine may be due to acid hydrolysis of some of the $^{14}C$-$B_{12}$ dose in the stomach or due to bacterial degradation in the gut with consequent release of DMB. Absorption of free DMB and its metabolism, such as through hepatic conjugation to glucuronide, may result in urinary excretion of the product. However, further analysis is necessary to determine the exact nature of the products detected in the urine.

In summary, we describe the biosynthesis of $^{14}C$-labeled $B_{12}$ and human absorption kinetics using near-ambient levels of radiation that pose little or no risk from exposure. The sensitivity of AMS reduces the needed sample size to only tens of microliters of blood and minimizes exposure to radiation. We believe that combined use of $^{14}C$-labeled $B_{12}$ and AMS detection has the potential to be a powerful clinical diagnostic tool and an improved

method for studying the underlying causes of $B_{12}$ uptake disorders, including the development of a sensitive and quantitative test for $B_{12}$ absorption in humans.

## Methods

### Synthesis and purification of $^{14}$C-dimethylbenzimidazole

Radiolabeled DMB was synthesized using a procedure modified from that of Phillips (10). Into a 10 mL boiling flask containing 500µL of sodium phosphate buffer (pH=7.4, 100 mM) was added $^{14}$C-formic acid (1 mCi; 0.0182mmoles, 1.85-2.22 GBq/mmol; Moravek Biochemicals, Brea, CA). The material was dried under reduced pressure and the solids were dissolved in 1 mL of 4M HCl. To initiate the reaction, 15.15 mg (111 µmoles) of *o*-dimethylphenylenediamine was added and the contents taken to a vigorous boil using a reflux system with the condenser maintained at -10°C using a recirculating chiller. After 2 hours of heating the reaction solution was neutralized by dropwise addition of concentrated ammonium hydroxide to approximately pH 7. The reaction product was then loaded onto a solid-phase extraction cartridge (1 g Bond Elut C18, Varian), which had been primed with 3 mL of methanol and 3 mL deionized water. The column was washed with 2 column volumes of deionized water and the bound $^{14}$C-DMB eluted with methanol (3 mL).

The solvent was removed under streaming nitrogen and the product dissolved in 0.5 mL of absolute ethanol. The product was then purified by multiple injections onto an isocratic reverse-phase HPLC using an Agilent 1100 chromatograph fitted with an Alltech Adsorbosphere HS C18 column (150mm x 4.6mm). The isocratic mobile phase, 34:33:33 water:methanol:acetonitrile, was pumped at a flow rate of 0.80 mL/min; the absorbance of the outflow was monitored at 284 nm. The peaks, which had similar retention and spectral characteristics to a purchased DMB standard (Sigma), were

pooled and evaporated to dryness under reduced pressure, dissolved in absolute ethanol and stored at –70 °C. Radioactivity was determined by liquid scintillation counting.

**Microorganism cultivation**

The strain used for reporting β-galactosidase activity was TT10674, genotype *eut38::mudA*. The strain used for $^{14}C$-$B_{12}$ biosynthesis was *Salmonella enterica* (serovar Typhimurium) strain TT24733, genotype *cbiD*24::MudJ. The labeling medium consisted of no carbon E (NCE) medium (17) supplemented with 40mM ethanolamine, 250nM dicyanocobinamide and 500nM $^{14}C$-DMB synthesized as described above. Approximately 130 mL of labeling medium was added to a sterile 500mL conical flask. The medium (130mL in a 500ml flask) was inoculated by a 100 dilution of a *S. enterica* culture grown in NCE medium supplemented with 20mM glycerol. Cultures were incubated in the dark for 24 hours at 30° C with shaking at 250 rpm.

**Extraction and purification of $^{14}C$-cyanocobalamin from cells**

Bacterial cells were pelleted by centrifugation for 20 minutes at 6,000 x g. Supernatants were removed and cell pellets washed three times with NCE medium (17). The pellets were resuspended in 5 mL methanol and 500 μL of 50 mg/mL sodium cyanide, vortex mixed, then placed in a 60°C water bath for 12 hours with intermittent vortex mixing. The samples were then centrifuged at 20,000 x g for one hour. The supernatants were removed from the pellet and evaporated to dryness. Dried samples were resuspended in water and filtered (0.22μm) to remove any insoluble material. A first step in purification of the $^{14}C$-$B_{12}$ was performed by extraction on C18:0 solid phase extraction cartridges (5g bond Elut C18, Varian). The corrinoids were eluted with 50:50

water:methanol and the solvent evaporated to dryness. Purification to homogeneity was carried out by HPLC on an Agilent series 1100 HPLC equipped with a diode array detector and fitted with an Agilent Zorbax Eclipse XDB C18 (3.5µm) column (150mm x 3.0mm). Solvent A was 90/10 water/methanol and solvent B was methanol with initial conditions of 82/18 A/B. At 12 minutes a linear gradient was started which reached 25/75 A/B after 16 min. The flow rate was held constant at 0.360 mL/min. Extracts were run in multiple injections and the peak corresponding to $^{14}$C-cyanocobalamin was collected and pooled for each run. The solvent was evaporated to dryness and samples resuspended in water for storage at -70ºC.

**Mass Spectrometry**

**High Resolution Mass spectrometry-Accurate Mass Measurement**

Exact mass measurement experiments were performed, in positive mode, on Micromass LCT orthogonal acceleration-Time-of-Flight mass spectrometer (Waters-Micromass, Manchester, UK). The cone gas and desolvation gas were set to 50 and 650 L/hour, respectively. Resolution was 8000, measured at 803 Th mass, based on the definition of FWHM (full width at half maximum). Sample source conditions were as follows: capillary voltage 3250 V, sample cone voltage 30 V, extraction cone voltage 6 V, source temperature 100 °C and desolvation temperature 250 °C. Transfer optics settings were as follows: rf lens 250 V, rf dc offset-1 4.0 V, rf dc offset-2 6.0 V, aperture 2.0 V, acceleration 200.0 V, focus 1.0 V and steering -0.3 V. Analyzer settings were as follows: MCP (multi channel plate) detector 2430 V, ion energy 32.0 V, tube lens 4.0 V, grid-2 20.0 V, TOF flight tube 4599 V and reflectron 1713 V. The pusher cycle time was 50 µs, data files were acquired in continuum mode and spectra were stored from m/z 100 to

1600 with a 1.1 second scanning cycle consisting of a 1.0 second scan and a 0.1 second inter-scan time. Typically 20-30 individual spectra were summarized. TOF Calibration: Lteff (effective length of the flight tube) value was set to 1122.7250 in positive mode using molecular ions of leucine-enkephalin (L9133, Sigma, MO, USA) at 556.2771 Th. System calibration was performed using poly-D-L-alanine (P9003, Sigma, MO, USA) which was also used as an internal standard for accurate mass measurement. To obtain accurate masses the following procedure was performed: Savitsky-Golay smoothing using ± 4 channel window, repeated twice and centering, using the center value at the 50% height of the peak. Samples were introduced to the mass spectrometer via direct flow injection using Waters Alliance 2795 (Bedford, MA, USA) HPLC system was used for solvent delivery at the flow rate of 250 µL/min, mobile phase $CH_3CN/H_2O$ (1/1) was used. MassLynx 4.0 sp 3 software (Waters-Micromass, Manchester, UK) was used for instrument control, data acquisition and data evaluation.

**MS/MS experiments**

Positive ion MS/MS experiments were performed in product mode on a Quattro Premier (Waters-Micromass, Manchester, UK) triple quadrapole mass spectrometer with a configuration of QTQ equipped with an interface sprayer (API interface). The instrument was operated with the following instrumental conditions: source temperature, 120 °C; desolvation temperature, 200 °C; capillary voltage, 3.1 kV; cone voltage, 50 V; extraction cone, 5 V, RF lens 0.5 V. Drying gas as well as cone gas was nitrogen, the cone gas flow was set to 50 L/h and the desolvation gas flow to 700 L/h. Quadrapole-1 parameters were as follows: Low mass (LM) resolution 14.0, high mass (HM) resolution 14.0, ion energy 0.4-0.6 V, entrance 7 V, exit 16 V; Quadrapole-2 parameters were LM

resolution 15.5, HM resolution 15.5 V, ion energy 2.0- 2.5 V. Multipliers were set at 550 V. Collision gas was Argon (99.9999%, Airgas, Inc., Radnor, PA, USA) with a pressure of 3.6-4.6×$10^{-3}$ mbar in the collision cell. MS/MS experiments were performed at collision energy of 50-70 eV (in the case of single charged ions) and 30-40 eV energy (in the case of double charged ions). MS/MS data were acquired in continuous mode, scanning speed was in all cases 0.025 sec/decade with 0.1 second inter-scan time, sampling density was set at 16/Da. Infusion experiments were performed on an integral syringe pump controlled from MassLynx, with a flow rate of 20 µL/ minute, directly connected to the interface. Data acquisition, instrument control was performed using MassLynx 4.0 sp 3 software (Waters-Micromass, Manchester, UK).

**Subjects and Human Experimental Design**

The subject was a healthy male aged 40 years with a body mass index (BMI) of 27.5. The subject began complete fecal and urine collection 24 h in advance of the C14-B12 dose and continued complete 24 h collections continued until Day 7. On the day of dose administration, the subject was fitted with an intravenous catheter in a forearm vein. Blood was drawn into 7 mL tubes containing EDTA. A baseline blood sample was drawn (7 AM) and the C14-B12 dose consumed that corresponded to 2.2 KBq of radioactivity (1.5 µg) was administered in 50 mL of drinking water in a paper cup. The volunteer was allowed to have water *ad libitum* thereafter, with a light meal taken 2 hr postdose. Blood samples (5mL) were drawn at frequent intervals for the first 15 hr post-dose and daily thereafter. Other meals were controlled for time and content on the dose administration day. The study was conducted in accordance with the ethical guidelines of the 1975 Declaration of Helsinki and approved by the Institutional Review Boards at

UC Davis and Lawrence Livermore National Laboratory. Informed consent was obtained from the subject.

**AMS analysis**

Aliquots of plasma (30μL), urine (80μL) and a stool slurry (80μL) were dried, combusted to $CO_2$, and reduced to filamentous carbon using previously described procedures (18),(19). No other processing preceded the graphitization step, thus quantitative recovery was ensured. The $^{14}C$ measurements were performed at the Center for Accelerator Mass Spectrometry at Lawrence Livermore National Laboratory (20). Measurements were conducted to < 3% instrument imprecision and, in general, signal acquisition was complete to the desired statistical precision in 3-5 min per sample. The radiation exposure of the subject, due to the $^{14}C$-$B_{12}$ dose, was equivalent to or less than exposure due to 16 hours of intercontinental plane flight (7, 21).

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Figures

Figure 1

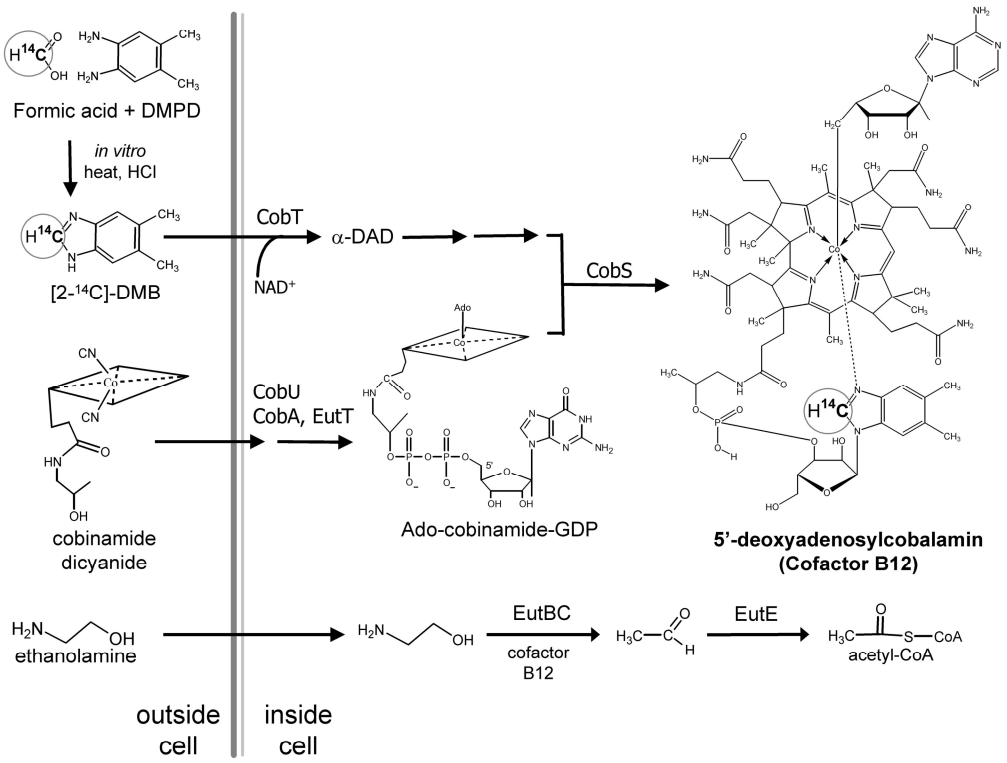
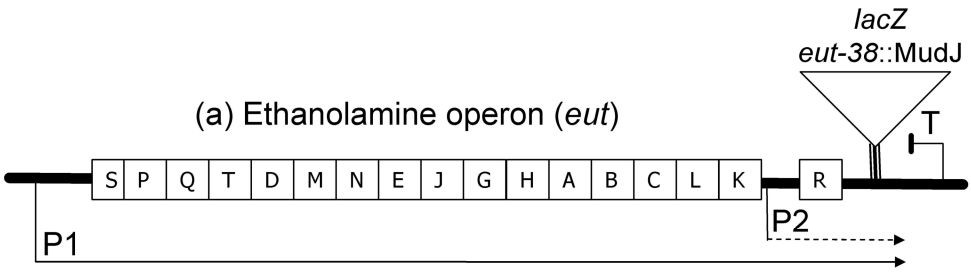


Figure 2




(b) Induction of *eut* and growth on ethanolamine

| Inducer | Mean ± SD | Growth |
|---|---|---|
| 1. None | 10 ± 1 | – |
| 2. EA | 9 ± 3 | – |
| 3. DMB | 14 ± 6 | – |
| 4. CBI | 7 ± 1 | – |
| 5. EA + DMB | 12 ± 3 | – |
| 6. EA + CBI | 447 ± 30 | – |
| 7. EA + CBI+DMB | 313 ± 24 | + |
| 8. EA + B12 | 486 ± 16 | + |

Figure 3

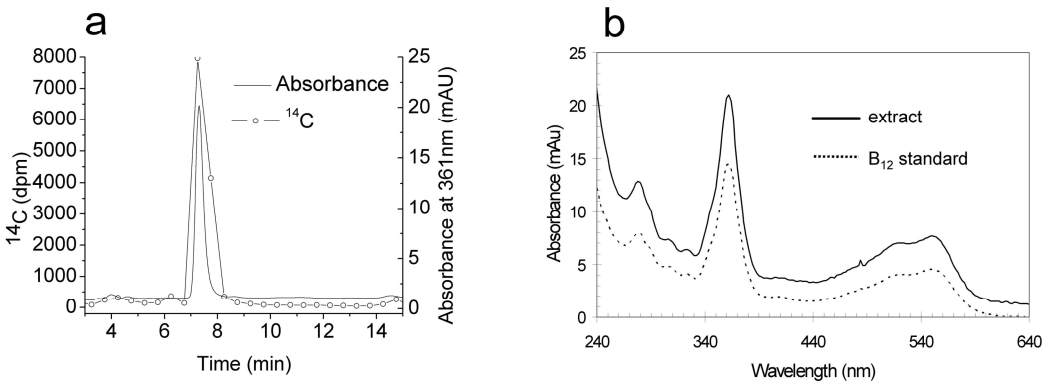

a
Absorbance
14C
14C (dpm)
Absorbance at 361nm (mAU)
Time (min)
b
extract
B12 standard
Absorbance (mAu)
Wavelength (nm)

Figure 4

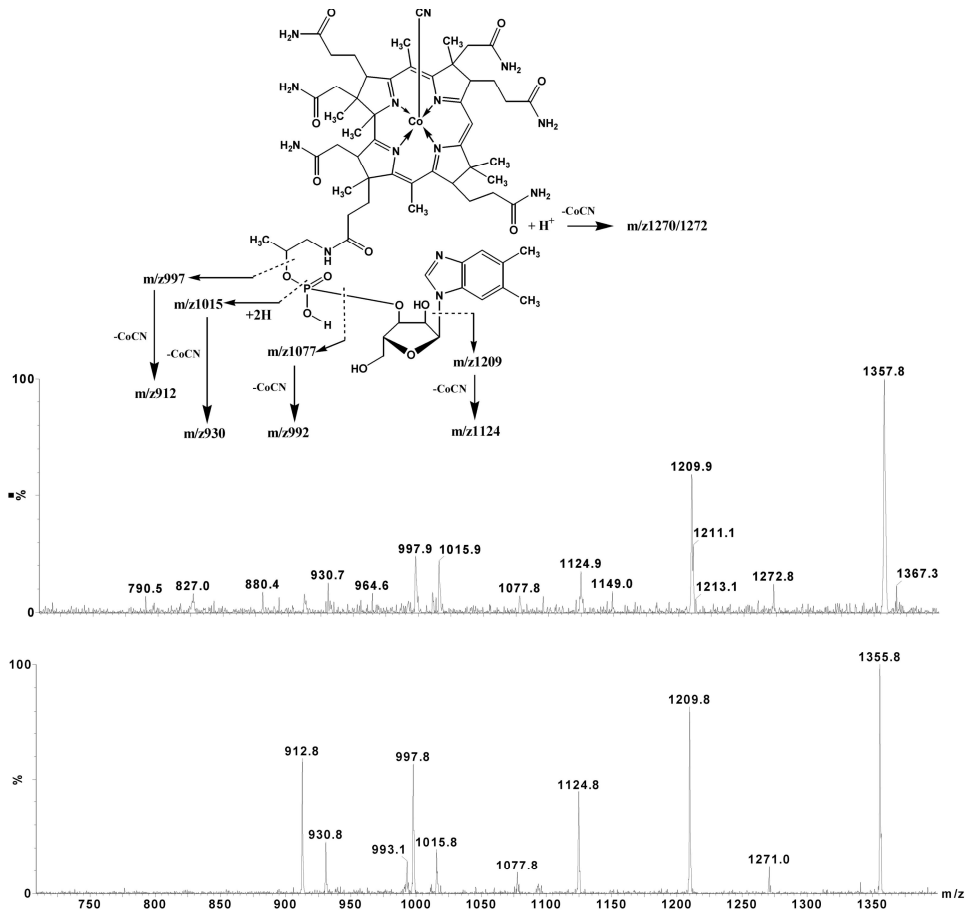

m/z1270/1272
-CoCN
+ H+
m/z997
m/z1015
+2H
m/z1077
m/z1209
m/z912
m/z930
m/z992
m/z1124
1357.8
1209.9
1211.1
997.9
1015.9
1124.9
1149.0
930.7
964.6
880.4
827.0
790.5
1077.8
1213.1
1272.8
1367.3
1355.8
1209.8
912.8
997.8
1124.8
930.8
993.1
1015.8
1077.8
1271.0
m/z

Figure 5

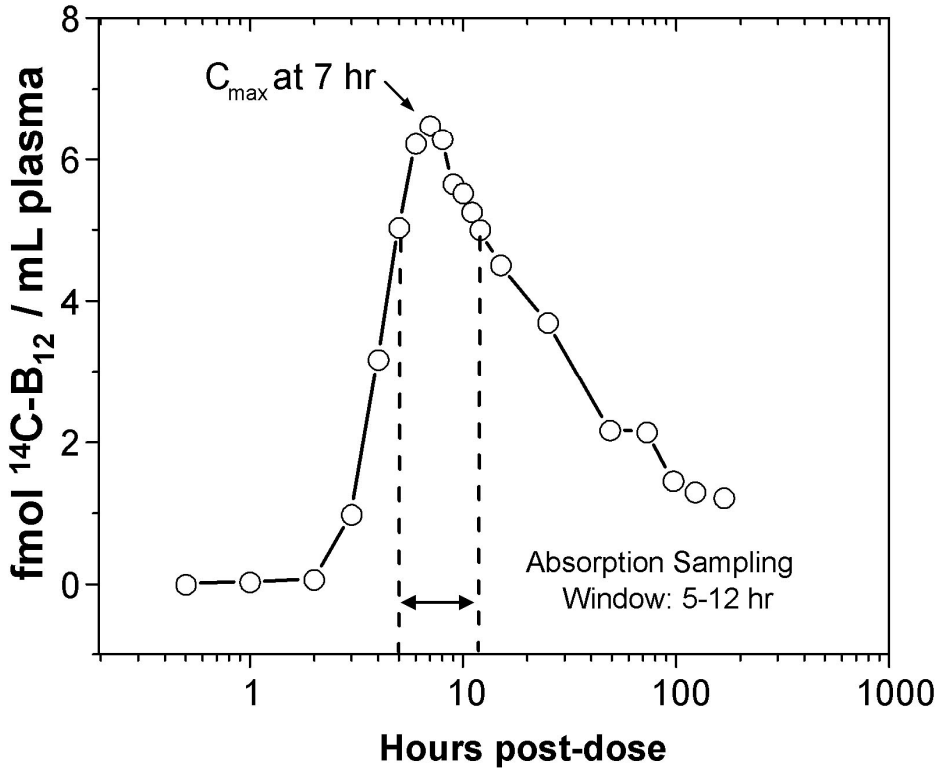
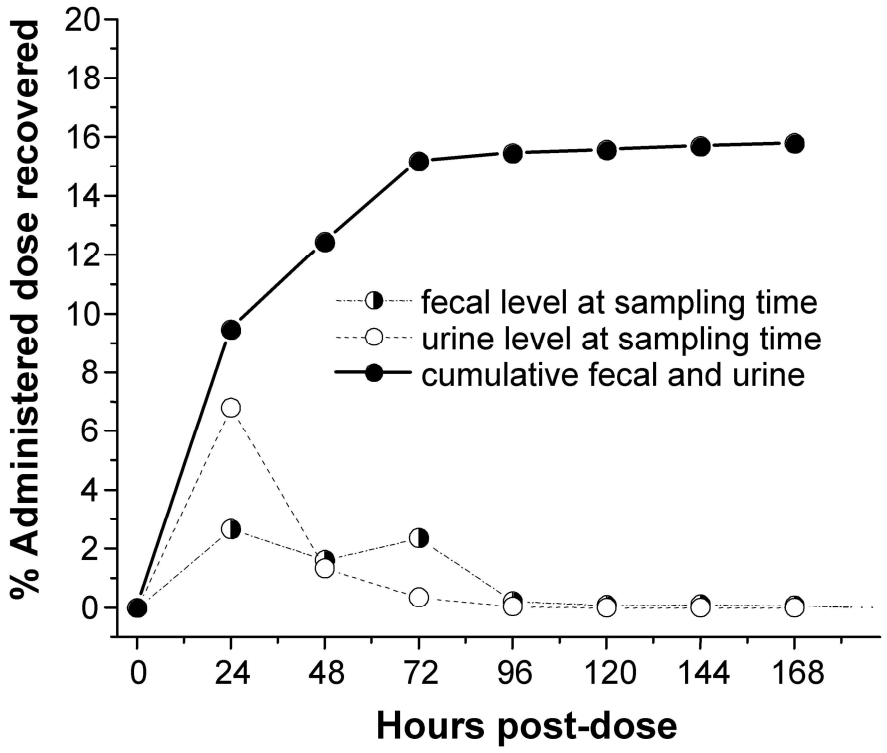

8
6
4
2
0
fmol $^{14}$C-B$_{12}$ / mL plasma
C$_{max}$ at 7 hr
Absorption Sampling
Window: 5-12 hr
1
10
100
1000
Hours post-dose

Figure 6


% Administered dose recovered
20
18
16
14
12
10
8
6
4
2
0
0
24
48
72
96
120
144
168
Hours post-dose
fecal level at sampling time
urine level at sampling time
cumulative fecal and urine

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The authors would like to thank John Thorngate for help with mass spectrometry, as well as Michael Lamé and Andrew Clifford for technical support and use of Laboratory Facilities. This was performed in part under the auspices of the US Department of Energy by University of California, Lawrence Livermore National Laboratory under contract W-7405-Eng-48. Support was provided by NIH grant DK064302 and NIH/NCRR grant RR 13461, NIEHS Superfund Basic Research Program P42 ES04699, NIEHS Center for Environmental Health Sciences P42 ES05707, NIEHS Center for Children's Environmental Health & Diseases Prevention NIEHS P01 ES11269.

Figure Legends

Figure 1: Synthesis of cofactor $B_{12}$ from cobinamide and $^{14}C$-dimethylbenzimidazole during growth on ethanolamine. *S. enterica* enzymes CobTUSC and EutT catalyze the synthesis of adenosylcobalamin (22, 23). Growth on ethanolamine proceeds by ethanolamine ammonia lyase EutBC which requires adenosylcobalamin to catalyze the formation of acetaldehyde from ethanolamine.

Figure 2: (a) Organization of the 17 gene *eut* operon. Genes are transcribed from left to right from the primary promoter P1. P2 is a weak constitutive promoter. (b) Induction of the *eut* operon in the presence of ethanolamine and glycerol is compared to growth on ethanolamine as sole carbon source.

Figure 3: (a) Precise coelution of the $^{14}C$ radiolabel with $B_{12}$. (b) Comparison of standard vitamin $B_{12}$ with that of the putative $^{14}C$-$B_{12}$ extract.

Figure 4: MS/MS spectra in product mode showing the decomposition products of the molecular ions $(M+H)^+$ of $B_{12}$ and $^{14}C$-$B_{12}$ vitamin.

Figure 5: Accelerator mass spectrometry detection of $^{14}C$ in human plasma. Units are expressed as femtomolar $^{14}C$-$B_{12}$. Measurements were performed on 30 μl of plasma, the entire sample set consumed less than 1 mL of whole blood.

Figure 6: Recovery of $^{14}C$ in urine and fecal specimens, and cumulative recovery of $^{14}C$. After seven days, 15.9% of the dose was recovered in the urine and stool.