

Anaerobic degradation of tetrachloromethane by *Acetobacterium woodii*: separation of dechlorinative activities in cell extracts and roles for vitamin B₁₂ and other factors

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Abstract

The transformations of ¹⁴CCl₄ by whole cells of *Acetobacterium woodii* suspended in phosphate buffer containing reducing agents, and by the cobalt corrinoid aquocobalamin in the same solution, were compared. Each catalyst transformed ¹⁴CCl₄ not only to reduced products (CHCl₃ and CH₂Cl₂) but also to CO and CO₂ as well as non-volatile products. The mass balance for radioactive carbon was complete in each case. Thus, the reactions of the pure cobalt corrinoid resemble the reactions in vivo. The proton in CHCl₃, formed from CCl₄ by *A. woodii*, was derived from water. Extracts of *A. woodii* were fractionated into large and small molecules, and each of the two fractions was separated chromatographically. Fractions of proteins demonstrated poor correlation between content of the corrinoid vitamin B₁₂ and rates of transformation of CCl₄. The correlation was somewhat improved if the fractions were autoclaved, but dechlorination in the absence of vitamin B₁₂ was observed. Separation of the small molecules yielded only one fraction containing vitamin B₁₂, and this fraction catalyzed dechlorination, whereas several other fractions were able to dechlorinate CCl₄ in the absence of vitamin B₁₂. We presume there to be unrecognized dechlorinative factors in anaerobic bacteria.

Abbreviations: GC – gas chromatograph(y); GC-MS (or -TCD or -FID) – GC coupled to a mass spectrometer (or a thermal conductivity detector or a flame ionization detector); HPLC – high pressure liquid chromatograph(y); FPLC – high pressure protein chromatograph(y)

Introduction

Transformations of halogenated aliphatic hydrocarbons in strictly anaerobic bacteria are well known (Cook et al. 1988; Vogel et al. 1987; Wackett 1991), but the biochemistry of the reactions in vivo is largely unclear. Only dichloromethane and chloromethane have been shown to be carbon and

energy sources for anaerobic growth (Freedman & Gossett 1991; Stromeyer et al. 1991; Traunecker et al. 1991), and it is unclear whether the same mechanisms of dechlorination are used during growth and biotransformations. The known biotransformation reactions are several variants of reductive (Cook et al. 1988; Gälli & McCarty 1989; Vogel et al. 1987), substitutive (Egli et al. 1988, 1990) or oxidative

(Egli et al. 1990) reactions. These biotransformations usually give complete mass balances for educt and products, which include water-soluble, non-volatile material and CO₂ (Egli et al. 1988, 1990; cf. Criddle et al. 1990a, b).

Pure cobalt, nickel or iron tetrapyrrole cofactors can catalyze reductive dechlorination of chlorinated aliphatic hydrocarbons (Klecka & Gonsior 1984; Gantzer & Wackett 1991; Krone et al. 1989a, b). However, these experiments usually result in incomplete mass balances for the chlorinated compound. These cofactors catalyze reductive dechlorination of CCl₄, and, in one case (Krone et al. 1991), the formation of CO (but not CO₂). The established reactions with pure tetrapyrrole cofactors thus do not adequately represent the biotransformations observed *in vivo*. The suggestion that flavins, as opposed to flavoproteins, also catalyze dechlorinative reactions under anaerobic conditions (Kuhn & Sufliita 1989; Criddle et al. 1990a) has not been examined in detail.

Acetobacterium woodii catalyzes the reductive dechlorination of CCl₄ and its substitutive transformation to CO₂; with CHCl₃, these two reaction types and an oxidation step (to CO₂) are known. These reactions are also observed in cell-free extracts and in autoclaved cells or extracts (Egli et al. 1988, 1990). The organism does not contain coenzyme F₄₃₀ or cytochromes (Dangel et al. 1987; Stupperich et al. 1988) so it presumably lacks both iron porphyrins and nickel porphyrins, whereas the presence of one cobalt corrinoid (vitamin B₁₂) is well established (Stupperich et al. 1988). *A. woodii* is thus a suitable organism to test the hypothesis that vitamin B₁₂ alone is responsible for the dehalogenations observed in this bacterium.

Materials and methods

Materials and apparatus

The sources of chlorinated hydrocarbons, ¹⁴CCl₄ and CH₄, Viton stoppers, scintillation fluid and general chemicals were described elsewhere (Egli et al. 1987, 1988, 1990). Aquocobalamin was from Fluka (Buchs, Switzerland) and D₂O (98%) from

the Paul Scherrer Institut (Villingen, Switzerland). The FPLC anion-exchange column (Mono Q, 100 × 10 mm) and PD-10 columns of Sephadex G-25 were obtained from Pharmacia, Uppsala, Sweden. HPLC was done on reversed-phase columns (Grossenbacher et al. 1986).

The anaerobic glovebox (Egli et al. 1989), HPLC, GC-TCD, liquid scintillation counter and spectrophotometer (Egli et al. 1988), GC-FID equipped with a methanizer (8700; Perkin Elmer, Beaconsfield, Bucks, UK), GC-MS (5890-5870, Hewlett-Packard, Avondale, PA) and atom absorption spectrophotometer equipped with a graphite tube atomizer (Spectra AA400/GTA-96; Varian, Palo Alto, CA) were commercially-available equipment. FPLC was done with LKB apparatus (Pharmacia/LKB) attached to a fraction collector (2110; Bio-Rad, Richmond, CA, USA) which could be passed into the glovebox, and which was adapted to allow collection of samples under anaerobic conditions. The gassing manifold was described by Kiener & Leisinger (1983).

Analyses

Chlorinated hydrocarbons and methane were determined by GC-FID after separation on a Porapak P column (Scholtz et al. 1987). A fused silica capillary column was used for GC-MS. CO and CO₂, produced in experiments initially devoid of CO₂ and H₂S, were determined as methane by GC-FID after separation on Carbosieve B in apparatus equipped with a methanizer. Radiolabelled CO and CO₂ were separated by GC-TCD, trapped, and quantified in a liquid scintillation counter (Egli et al. 1988); ¹⁴CO₂ was also quantified as described by Fuchs et al. (1980). Water-soluble, radioactive products from ¹⁴CCl₄ were assayed as that radioactivity remaining in aqueous solution after acidification (to pH 2.5 with 1 M H₂SO₄) and extraction with one volume of hexane. Vitamin B₁₂ was determined in a radioimmunoassay (Magic No Boil B₁₂-Folate; Ciba-Corning, Medfield, MS, USA). The metal ions cobalt, nickel and iron from biological samples were determined by atomic absorption spectrometry (Welz 1983). Protein in whole cells

was measured in a Lowry-type reaction (Kennedy & Fewson 1968). Protein in cell extracts was measured by the method of Bradford (1976).

The eluents for FPLC contained dithiothreitol and were sparged with He. A blank elution purged the system of oxygen. Proteins in a 2-ml portion of processed cell extract (see below) were separated on the anion exchanger equilibrated with eluent A (25 mM potassium phosphate and 2 mM dithiothreitol, pH 7.4) at 4 ml/min. Proteins were eluted isocratically for 10 min, when a linear gradient of eluent B (eluent A containing 1 M Na₂SO₄) to 15% B over 42 min was started; thereafter, the flow rate was reduced to 2.5 ml/min and the gradient was ramped to 100% B over 13 min, after which the initial conditions were regenerated. 20-ml fractions were collected anaerobically and examined immediately.

Gradient separations by HPLC were done on reversed-phase columns (Grossenbacher et al. 1986) at a flow rate of 1 ml/min with 20- μ l or 3-ml samples, and 1-ml fractions were collected. UV detection (205 nm) was used. The initial eluent, C, was 100 mM-potassium phosphate buffer, pH 4.0; the second eluent, D, was 80% (v/v) methanol in 10 mM-potassium phosphate buffer, pH 4.0. Eluent C was pumped for 5 min, when a linear gradient to 10% D over 15 min was applied followed by a linear gradient to 100% D over 25 min; after one min, the initial conditions were regenerated. The separated fractions were then brought to pH 7.4. When products from dechlorination of ¹⁴CCl₄ by aquocobalamin were examined, portions of fractions were examined for radioactivity.

Organism and growth media

Acetobacterium woodii DSM 1030 was obtained from the culture collection (Deutsche Sammlung von Mikroorganismen, Braunschweig, FRG).

A. woodii was grown under autotrophic and heterotrophic conditions (Balch et al. 1977). Inocula for experiments were grown under autotrophic conditions. Batches of cells from which extracts were to be made were grown in the dark at 30° C in 500-ml portions of fructose (10 mM) medium in 1-1

bottles under an atmosphere of N₂ plus CO₂ (80 : 20, v/v) from an inoculum of 20 ml. The cultures were harvested anaerobically in the late exponential phase of growth (5000 g, 15 min, 4° C) and washed anaerobically (60 mM NaHCO₃, 5 mM potassium phosphate, 20 mM NH₄Cl, 0.4 mM MgSO₄, 3 mM Na₂S, 2.5 mM L-cysteine-HCl and 4 μ M resazurin, pH 7.0). All subsequent steps in the experiment were done anaerobically at 0–4° C in the dark, unless otherwise stated: fresh cells were used, because alterations in properties were observed if cells were stored. Pellets of *A. woodii* were suspended (250 mg wet weight/ml) in extraction buffer (40 mM Tris-HCl, 2 mM NaHCO₃, 3 mM Na₂S, 2.5 mM L-cysteine-HCl, 1 μ M phenylmethane sulfonyl fluoride (PMSF) and 4 μ M resazurin, adjusted to pH 7.0 at room temperature) and ruptured by three passages through a cold French pressure cell at 120 MPa. Whole cells and debris were removed by centrifugation (30 000 g, 60 min, 4° C). The supernatant fluid was treated with protamine sulfate to remove nucleic acids: 20% (v/v) of cold 2% protamine sulfate in eluent buffer A/p (buffer A containing 0.1 mM PMSF) was stirred into crude extract over 10 min at 4° C, after which the precipitate was removed by centrifugation (30 000 g, 30 min, 4° C). This supernatant fluid (portions of 2.5 ml) was first desalted on a PD-10 column to remove molecules of *M_r* < 5000 (e.g. free vitamin B₁₂) and to bring the protein fraction into buffer A/p. Small molecules from the crude extract were considered to be those in the 5.5–8.5 ml fraction eluted from the PD-10 column.

Conditions for the assays of dechlorination

The transformation of CCl₄ in solutions of aquocobalamin was examined at 30° C in 20-ml reaction mixtures contained in 150-ml screw-cap bottles fitted with Viton stoppers; the gas phase was a mixture of N₂ and CO₂ (80 : 20, v/v). The reaction mixture contained 2 mmol potassium phosphate buffer, pH 7.2, 83 μ mol L-cysteine, 128 μ mol Na₂S, 1 μ mol CCl₄ (containing 360 MBq ¹⁴CCl₄), and, after a preincubation period, 7.4 to 74 nmol aquocobalamin, with which the reaction was started.

Some experiments were done with 4 mmol titanium (III)-citrate in place of the sulfur-containing reductants. Samples of headspace were taken at intervals for 20 h and examined for chlorinated solvents, CO and CO₂ (see below). Thereafter, the reaction mixture was acidified, extracted and examined for water-soluble products (see below). In some experiments, the aquocobalamin was replaced by suspensions of native or autoclaved cells of *A. woodii* (4.8 mg of protein).

The source of the proton in CHCl₃ formed from CCl₄ was examined in a suspension of cells of *A. woodii*. *A. woodii* was grown heterotrophically with fructose as the carbon source in medium made up with H₂O or D₂O. Cells were harvested and washed in buffer prepared in H₂O and D₂O, respectively. The transformation of CCl₄ in suspensions of *A. woodii* was done in 25-ml reaction mixtures contained in 150-ml screw-cap bottles fitted with Viton stoppers; the gas phase was a mixture of either N₂ and CO₂ (80 : 20, v/v) or H₂ and CO₂ (80 : 20, v/v). The reaction mixture contained 2 mmol potassium phosphate buffer, pH 7.2, 83 μmol L-cysteine, 128 μmol Na₂S, 5 μmol CCl₄ and 40 mg wet wt of cells, with which the reaction was started after a preincubation period. The reaction was terminated after 9 h by the addition of 5 μmol of NaOH. Samples (1 ml) of headspace were then taken and analyzed by GC coupled to a mass spectrometer. The relative abundance of the fragments of *m/z* 83 (¹²CH³⁵Cl₂⁺) and 84 (¹²CD³⁵Cl₂⁺) was used to determine the relative amounts of CHCl₃ and CDCl₃ formed during the incubation.

The degradation of CCl₄ by fractionated proteins from extracts of *A. woodii* was examined at 30° C in undiluted 20-ml portions in 125-ml screw-cap bottles closed with Viton stoppers: the gas phase was a mixture of N₂ and CO₂ (80 : 20, v/v). Extract and CCl₄ (1 μmol) were incubated for 30 min to allow equilibration of CCl₄ between the gas and aqueous phases, and the reaction was considered to be initiated by the addition of the reductant, titanium (III)-citrate (about 20 μmol); samples of headspace were taken at 0 and 12 h for analysis by GC-FID. Thereafter, samples for determinations of vitamin B₁₂, Ni, Fe and Co were taken. When fractions were due to be examined after autoclav-

ing, each pooled fraction was divided into two equal portions, one of which was autoclaved, and reactions were followed as above.

The degradation of CCl₄ by fractionated small molecules from extracts of *A. woodii* was examined at 30° C in undiluted 5-ml portions in 125-ml screw-cap bottles closed with Viton stoppers: the gas phase was a mixture of N₂ and CO₂ (80 : 20, v/v). Extract and CCl₄ (1 μmol) were incubated for 30 min to allow equilibration of CCl₄ between the gas and aqueous phases, and the reaction was considered to be initiated by the addition of the reductant, titanium(III)-citrate (about 20 μmol); samples of headspace were taken at 0 and 12 h for analysis by GC-FID after which samples were taken for the determination of vitamin B₁₂.

Results

Analysis of products from the reaction of A. woodii with CCl₄

As anticipated (see Introduction), native cells of *A. woodii* transformed CCl₄ to reduced products (CHCl₃ and CH₂Cl₂), CO₂, water-soluble non-volatiles and material associated with the cell (Table 1). The amount of reduced products changed and decreased with time, with a corresponding increase in polar non-volatiles. In addition, CO was observed in the gas phase: it cochromatographed with authentic material (radioactivity) and was converted to CH₄ in the methanizer. CO was detected at levels of about 1% of the educt, whether measured radiochemically or with the methanizer (Table 1). The recovery of radioactivity from CCl₄ was 88 to 99%.

The products formed from CCl₄ by autoclaved cells of *A. woodii* differed from the control with native cells in that negligible amounts of CHCl₃ remained after 20 h (Table 1). As with native cells, the autoclaved material catalyzed the formation of CO. The recovery of radioactivity was consistently lower than from experiments with native cells (Table 1).

Cells of *A. woodii* which had been autoclaved in the presence of air were also able to catalyze the

anaerobic transformation of CCl_4 . The products, and their rates of formation, were essentially identical with those from cells autoclaved under anaerobic conditions. The rate of dechlorination in native cells could be increased by a factor of 2 to 4 by replacing nitrogen in the gas phase with hydrogen, or by retaining the gas phase of nitrogen plus carbon dioxide and adding fructose.

Reaction products from CCl_4 in a solution of aquocobalamin and reducing agents

The rate of disappearance of CCl_4 catalyzed by aquocobalamin was directly proportional to the concentration of aquocobalamin between 7 and 74 nmol/assay, and the turnover was 14 mkat/mol of aquocobalamin. The fate of radioactivity from $^{14}\text{CCl}_4$ in anaerobic solutions of aquocobalamin was examined. The yield of reduced products was low, and most of the products consisted of non-volatile, water-soluble material, CO and CO_2 (Table 1). The non-volatile material was separated by gradient elution from a reversed-phase HPLC column in four distinct peaks (T_r 4.0, 7.5, 19 and 34 min). The fourth peak eluted shortly after aquo-

cobalamin (T_r 24 min). It was red when the material was concentrated, and had a UV spectrum resembling that of aquocobalamin (e.g., λ_{max} values close to 278, 361 and 551 nm). We presume this to be derivatized aquocobalamin.

The transformations of CCl_4 by native aquocobalamin (Table 1) were also observed with autoclaved aquocobalamin. The range of products was altered very little, but the specific rate of transformation was increased by about 30%. Aquocobalamin was also autoclaved in an atmosphere of air and tested for its ability to transform CCl_4 anaerobically. The cofactor was active, with relatively small changes in the distribution of products formed, and the rate was some 50% higher than in the control.

The range of reactions catalyzed by aquocobalamin is wider than previously recognized (see Introduction). Qualitative comparisons in Table 1 show that the same range of products is formed by *A. woodii* and by aquocobalamin. It is thus possible to hypothesize that cellular vitamin B_{12} , an analogue of aquocobalamin, could be responsible for all the reactions with CCl_4 that are catalyzed by the organism in vivo.

Table 1. Products formed from CCl_4 in the presence of reducing agents in suspensions of native or autoclaved cells of *A. woodii* and in solutions of aquocobalamin^a.

Compound	Amount of product (% of initial amount of CCl_4) present after the given incubation period in the presence of:					
	native cells		autoclaved cells		aquocobalamin	
	4.5 h	20 h	4.5 h	20 h	15 nmol, 20 h	74 nmol, 20 h
CCl_4	0	0	0	5	0	0
CHCl_3	47	3	26	< 0.5	0	1
CH_2Cl_2	6	17	9	< 0.5	10	1
CO_2	6	8	4	9	8	29
CO	nd	1	nd	2	15	10
Polar non-volatiles	34	50	36	52	49	54
Associated with cells	6	6	5	9	-	-
Mass balance	99	85	80	78	82	95

^a About 1000 nmol of CCl_4 was incubated in phosphate buffer with aquocobalamin or native or autoclaved cells of *A. woodii*, and each reaction mixture was reduced with H_2S and L-cysteine-HCl. Most of the data were obtained radiochemically, but CO was quantified in a methanizer in an experiment with $^{12}\text{CCl}_4$. CO_2 from CCl_4 could also be detected in the methanizer if the system was scrupulously free of exogenous CO_2 . Similar products were observed when the system was reduced with titanium(III) citrate in place of H_2S and L-cysteine-HCl. Data from representative experiments are shown, but each experiment was done in duplicate at least twice, where appropriate with independently-grown cells.

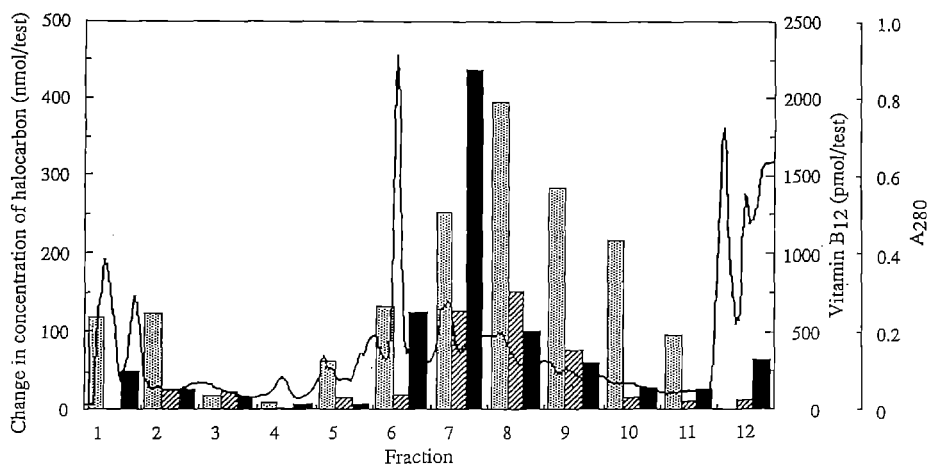


Fig. 1. Separation of proteins from desalted extract of *A. woodii*, and the dechlorinative activities of the resulting fractions. The eluate from the anion-exchange column was divided into twelve 5-min fractions which were examined for dechlorinative activity under native conditions and for vitamin B₁₂, as detailed in the Methods section. —, A₂₈₀; □, disappearance of CCl₄; ▨, formation of CHCl₃; ■, vitamin B₁₂.

Transformation of CCl₄ by protein fractions from *A. woodii*

Desalted soluble proteins from *A. woodii* were separated by ion-exchange chromatography (Fig. 1). Dechlorinative activity (disappearance of CCl₄) was routinely observed in many fractions, whereas others (3, 4 and 12) had negligible activity. The proportion of the reductive reaction (measured as formation of CHCl₃) varied from undetectable (fraction 1) to 50% (fraction 7): no attempt was made to define the other reaction products (cf. Table 1). We presume that both reductive and substitutive reactions were catalyzed by native fractions, but the reaction rate correlated very poorly with the levels of vitamin B₁₂ observed in the fractions. The relative activity attributed to vitamin B₁₂ varied widely (8 and 0.5 mkat/mol for fractions 7 and 10, respectively).

In experiments similar to that in Fig. 1, dechlorination of CCl₄ in native separated proteins and in autoclaved material were compared (Fig. 2). A major alteration in the pattern of dechlorination was caused by autoclaving. In general there was a decreased discrepancy between specific activities of dechlorination (calculated per mol vitamin B₁₂) and levels of vitamin B₁₂ in autoclaved extracts, compared with native enzymes. Further, some

samples now dechlorinated although little or no vitamin B₁₂ was present (fractions 8 and 11). We attempted to correlate the levels of iron, nickel and cobalt with dechlorination (not shown), but there was no direct relationship between dechlorination and the presence of these ions.

Transformation of CCl₄ by fractionated small molecules from *A. woodii*

The small molecules in cell extracts of *A. woodii* were separated on a reversed phase column (Fig. 3). There was dechlorination in at least five of the ten blocks of fractions. One of the least active was fraction 7, in which the vitamin B₁₂ eluted. The catalytic activity calculated for sample 7 is, however, 6 mkat/mol of vitamin B₁₂, similar to that of aquocobalamin (14 mkat/mol), so vitamin B₁₂ separated from growing cells behaves normally in our dechlorinative test in vitro. At least four fractions catalyzed dechlorination in the absence of vitamin B₁₂. The organism must, therefore, contain other (co)factors that are able to catalyze dechlorination.

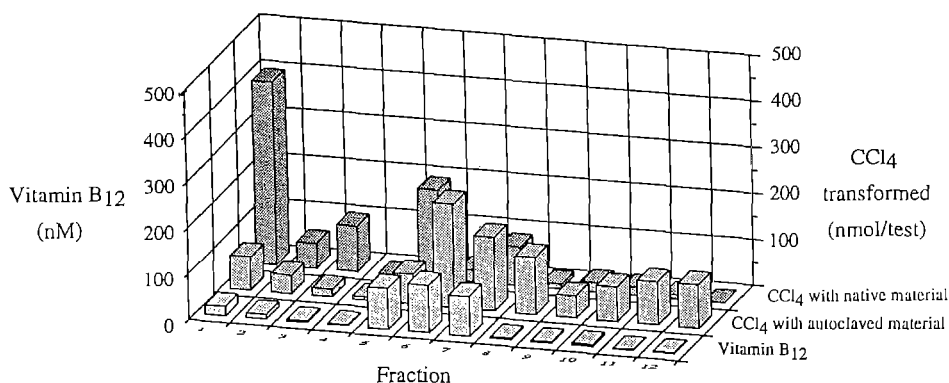


Fig. 2. Correlation of vitamin B₁₂ and disappearance of CCl₄ catalyzed by native and autoclaved fractions from the chromatographic separation of native proteins of *A. woodii*. A separation of native proteins of *A. woodii* was done, analogous to that in Fig. 1. Each fraction was split in two, one of which was autoclaved. The disappearance of CCl₄ from reaction mixtures containing native and autoclaved material was examined, as was the content of vitamin B₁₂. This is a typical experiment, as is Fig. 1, and both were done under identical chromatographic conditions. The block of fractions containing most vitamin B₁₂ (5, 6 and 7) eluted earlier than the same material in Fig. 1 (fractions 6, 7 and 8). The general pattern, however, was consistent.

The nature of CHCl₃ formed from CCl₄ by *A. woodii*

Cells grown in medium based on H₂O and incubated in a reaction mixture based on H₂O produced only protochloroform from CCl₄ (Table 2). When D₂O was used in the reaction mixture, the product was largely deuteriochloroform, whether the cells derived from growth medium prepared in H₂O or D₂O. The presence of H₂ in the gas phase of the reaction mixture had little effect on the relative amount of deuteriochloroform produced.

Discussion

We have confirmed and extended our earlier work to show that native cells of *A. woodii* quantitatively convert ¹⁴CCl₄ not only to C-1 compounds (CHCl₃, CH₂Cl₂, CO and CO₂) but also to non-volatile, water-soluble compounds and to material firmly bound to cells (Table 1); there is high to complete recovery of the labelled carbon. In contrast to earlier work with tetrapyrrole cofactors (see Introduction) we observe a complete mass balance for the degradation of CCl₄ by aquocobalamin (Table 1). Our use of ¹⁴CCl₄ allowed us to trace the products, CHCl₃, CH₂Cl₂, CO and CO₂, and non-volatile, water-soluble compounds. So aquocobalamin does

indeed catalyze a set of reactions analogous to those in native cells of *A. woodii* (cf. Introduction).

Our data with aquocobalamin confirm and extend those of Krone et al. (1989b, 1991). Stromeyer (unpublished) reproduced the core data of Krone et al. (1991), but we chose to work with phosphate buffer at physiological pH, to reproduce conditions in the growth medium. Krone et al. (1989a, b, 1991) detected solely those volatile compounds with a low background by GC-(methanizer)-FID (CHCl₃, CH₂Cl₂, CH₃Cl and CO), the absence of significant amounts of formate and the very incomplete mass balance for carbon (50 to 70%). Our detection of CO₂ and the four separable, water-soluble products completes the mass balance for carbon. There is no mass balance for chlorine, which reflects the difficulty of measuring chloride ion accurately by colourimetric methods in samples containing reducing agents.

Krone et al. (1991) attributed our observation of CO₂ production (Egli et al. 1988, 1990) to the action of CO dehydrogenase on CO produced by e.g., vitamin B₁₂. The assumption may be largely true in growing cells (Egli et al. 1988), but it is unlikely in autoclaved cells (Table 1; Egli et al. 1990). Indeed, CO₂ is formed from CCl₄ in organisms without the acetyl-CoA pathway, *Escherichia coli* and *Pseudomonas* sp., in which some or

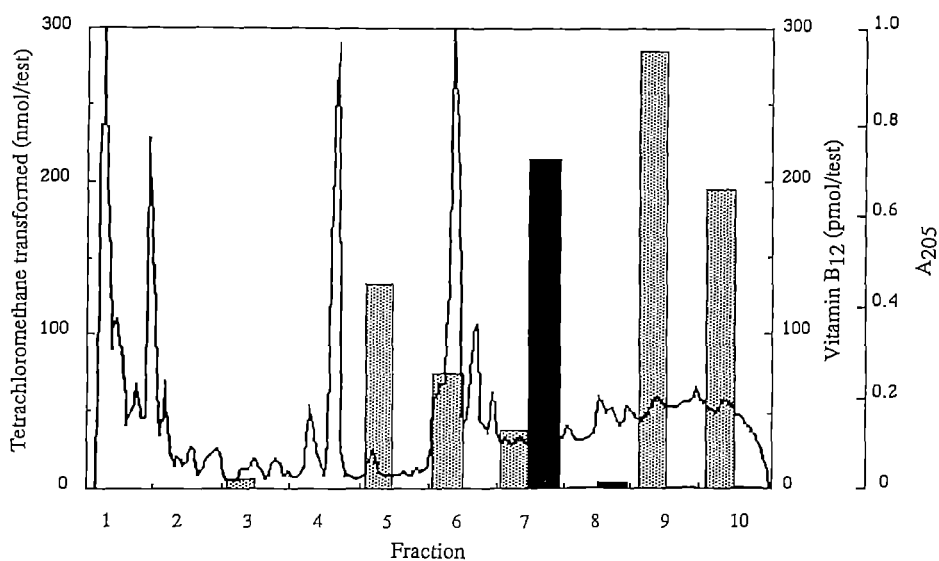


Fig. 3. Separation of small molecules from crude extract of *A. woodii*, and the dechlorinative activities of the resulting fractions. Small molecules ($M_r < 5000$) from crude extract of *A. woodii* were separated on a reversed-phase column, as described in the Methods section. These fractions were examined for dechlorinative activity and for vitamin B₁₂. —, the elution diagram; ■, disappearance of CCl₄; ▨, vitamin B₁₂. The GC-assay for disappearance of CCl₄ was straightforward in the early fractions, but with increasing amounts of methanol, the CCl₄-peak was located on the 'tail' of the methanol peak, and could be measured with only reduced accuracy. In blank runs we confirmed that eluent from the column did not cause disappearance of CCl₄.

all of the products in Table 1 are formed (Criddle et al. 1990a, b).

A major argument for tetrapyrroles as the non-enzymic catalysts is their heat stability (Fridrich 1975). The heat stability of the whole range of reactions involved in dechlorination by whole cells (Table 1) and aquocobalamin (see text) is reconfirmed in this work. If any activity is curtailed by autoclaving, it is the reductive reaction (cf. Egli et al. 1990), but the high levels of reduced intermediates at 4.5 h in the autoclaved cells compared with the low residual levels of reduced intermediates (20 h) may actually indicate that the reductive reaction is faster or more complete or altered under these conditions. The repeatedly low recovery of carbon from autoclaved cells (Table 1; Egli et al. 1990) leads us to suspect the formation of as yet unidentified volatile products under these conditions.

We found free vitamin B₁₂ from *A. woodii* to have a specific activity for dechlorination (6 mkat/mol; Fig. 3) similar to that of aquocobalamin (14 mkat/mol) in our system, which was chosen to represent simplified growth medium for the organism.

The protein-bound vitamin B₁₂ had a maximum specific activity in the same order of magnitude (about 8 mkat/mol in fraction 7 in Fig. 1) and a minimum of about 0.5 mkat/mol (fraction 10 in Fig. 1). We presume that this range of dechlorinative

Table 2. The source of protons in chloroform from CCl₄ in reactions catalyzed by whole cells of *A. woodii* in the presence of H₂O or D₂O.

Incubation conditions	Distribution (%) of products	
	CHCl ₃	CDCl ₃
Cells grown in H₂O		
Reaction buffer	H ₂ O	
Gas phase	N ₂ + CO ₂	> 99.5 < 0.5
Reaction buffer	D ₂ O	
Gas phase	N ₂ + CO ₂	< 0.5 > 99.5
Cells grown in D₂O		
Reaction buffer	D ₂ O	
Gas phase	N ₂ + CO ₂	6 94
Reaction buffer	D ₂ O	
Gas phase	H ₂ + CO ₂	9 91

activities observed for vitamin B₁₂ bound to proteins reflects different molecular environments of the cofactor (cf. Dangel et al. 1987). Indeed, some of the very broad peaks we observe in Fig. 1 may represent degradation products from native enzymes, because this column routinely gives much sharper protein bands (Locher et al. 1991). When the dechlorinative activity of cellular vitamin B₁₂ depends on its molecular environment, it becomes almost impossible to predict the dechlorinative potential of that vitamin B₁₂ in the organism, even if, as with *A. woodii*, only one known tetrapyrrole is involved. Correspondingly, it is almost impossible to test quantitatively the hypothesis that vitamin B₁₂ is the sole factor responsible for dechlorination in *A. woodii*. In preliminary calculations with data from methanogens, Stumpf (1990) concluded that there was no correlation between dechlorinative activity and published levels of cobalt corrinoids.

The poor correlation of amounts of vitamin B₁₂ and the level of dechlorination in native separated proteins (Fig. 1) was repeated in an independent experiment (Fig. 2): the correlation was somewhat improved in that portion of the experiment in which the separated material was autoclaved prior to analysis of dechlorination. Further inconsistencies (dechlorination in fractions 8, 11 and 12 in the absence of vitamin B₁₂), however, indicate that other dechlorinative factors may also be present. This supposition was confirmed when we examined components of low molecular weight from the organism (Fig. 3). This material represented about 50% of the dechlorinative activity in cell extracts and only a small portion of the dechlorination is attributable to vitamin B₁₂. At least four other unidentified fractions are involved. It is thus clear that, however much vitamin B₁₂ contributes to dechlorination in *A. woodii*, other factors in the cell can also dechlorinate CCl₄, though here also we do not know what contribution they make to activity in vivo. The detection of small molecules other than vitamin B₁₂ that dechlorinate CCl₄ is not surprising. On the one hand, several metallo-tetrapyrrole-containing systems, besides vitamin B₁₂, are known to catalyze dechlorination, though none is known to be present in *A. woodii* (see Introduction). On the other hand, Criddle et al. (1990a,

1990b) have confirmed and extended their older observation (Bouwer & McCarty 1983) of dechlorination by organisms lacking the acetyl-CoA pathway (and the corresponding corrinoids), so there must be other dechlorinative factors such as the flavins postulated in the introduction.

The reductive reaction with CCl₄ has been known for a considerable time, firstly in mixed cultures and more recently in pure cultures (Cook et al. 1988; Vogel et al. 1987), but we present the first evidence to show that the proton involved is derived from water (Table 2) rather than from e.g., cellular protons or molecular hydrogen. Our data contrast with results from liver microsomes, where the proton was derived not from water or hydrogen but from cellular protons (Ahr et al. 1980). It is unclear whether water itself is involved in the reaction or whether a proton from water, as proposed by Krone et al. (1991). However this is resolved, one basis of the dechlorinations with the cobalt corrinoid is a series of radical reactions (Krone et al. 1989a, b, 1991). Up till now, only a portion of the products was recognized (CHCl₃, CH₂Cl₂, CO, CO₂). We interpret the radioactivity from ¹⁴CCl₄ in a molecule with a similar UV-visible spectrum to, but lower polarity than, vitamin B₁₂ to be a chloromethyl derivative of aquocobalamin, caused by a chloromethyl radical being quenched on a molecule of the corrinoid. Similarly, the other materials in the chromatogram could also represent quenched radicals. Correspondingly, the radioactivity in cell material (Table 1) would represent radicals which were quenched on cellular structures.

The complete disappearance of CCl₄ in the presence of aquocobalamin, *A. woodii* or methanogens (Krone et al. 1989b) thus does not represent complete degradation but transformation by a non-enzymic catalyst to largely unidentified, but probably chlorinated, products. It is also difficult to see what the organism gains from the reaction. It may be a detoxicative reaction, where the cost of derivatizing cell components is a lower risk than the halocarbon. The reaction was probably not selected for by evolution, being the side reaction of a cofactor, rather than an enzymic reaction. The reaction in native cells can be accelerated by providing reducing equivalents (from H₂ or fructose) but

it seems unlikely that the cell will be able to harvest any physiological gain from e.g., the reduction of CCl_4 , because this electron sink is apparently not coupled to an electron transport chain.

The non-directed nature of the biotransformation of CCl_4 , discussed above, differs markedly from the utilization of haloaliphatic compounds as carbon and energy sources under anaerobic conditions. In the latter cases, there is complete mass balance for carbon and chlorine (Egli et al. 1989; Stromeyer et al. 1991; Freedman & Gossett 1991; Traunecker et al. 1991). The reactions are enzyme-catalyzed and, where known, involve a defined degradative pathway.

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