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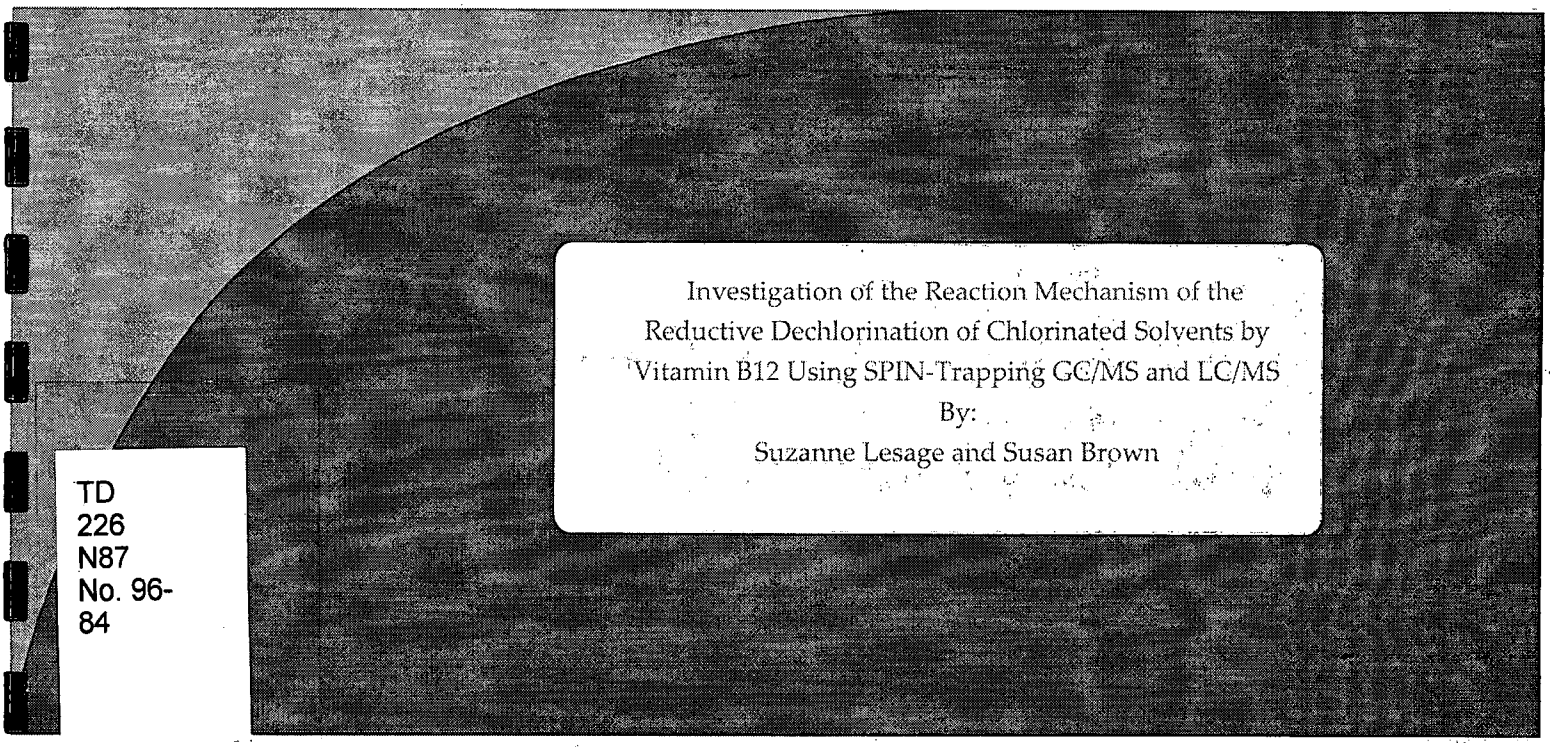

Investigation of the Reaction Mechanism of the Reductive Dechlorination of Chlorinated Solvents by Vitamin B12 Using SPIN-Trapping GC/MS and LC/MS

By:

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# INVESTIGATION OF THE REACTION MECHANISM OF THE REDUCTIVE DECHLORINATION OF CHLORINATED SOLVENTS BY VITAMIN B12 USING SPIN-TRAPPING GC/MS AND LC/MS.

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## ABSTRACT

Vitamin B12 has been suggested as a biochemical catalyst for the reductive dechlorination of chlorinated solvent such as carbon tetrachloride and tetrachloroethylene which are common groundwater pollutants. However, much remained to be understood as to the mechanism of the reaction and the environmental conditions that are likely to be able to sustain the reaction. In this project, the reactivity of vitamin B12 with a variety a reducing agents was examined and the resulting intermediates studied by LC-MS. It was found that sodium dithionite disproportionates to bisulfite which adds itself to the 2- benzimidazole ring of vitamin B12, producing an inactive molecule. It was found that the reversibility of the reduction was one of the most critical factors in the suitability of a reducing agent and that amongst all those readily available, titanium citrate was the reagent of choice for environmental applications. Spin-trapping GC/MS, using phenyl-tert-butyl nitrone as a spin trap, was used as an investigative tool to confirm the formation of several free radicals during the reductive dechlorination of carbon tetrachloride and of tetrachloroethylene.

## MANAGEMENT PERSPECTIVE

Because of the inherent limitations of current methods such as pump-and-treat, innovative technologies are currently being sought for the *in-situ* remediation of groundwater. Vitamin B12 has been suggested as a biochemical catalyst for the reductive dechlorination of chlorinated solvent such as carbon tetrachloride and tetrachloroethylene which are common groundwater pollutants. However, much remained to be understood as to the mechanism of the reaction and the environmental conditions that are likely to be able to sustain the process. In this project, the reactivity of vitamin B12 with a variety a reducing agents was examined and the resulting intermediates studied by LC-MS. Spin-trapping GC/MS, using phenyl-tert-butyl nitrone as a spin trap, was used as an investigative tool to confirm the formation of several free radicals during the reductive dechlorination of carbon tetrachloride and of tetrachloroethylene. Titanium citrate was found to be the reducing agent of choice and some sulfur-based reducing agents were found to inactivate the catalyst. This knowledge is essential in being able to eventually apply the reaction to remediation in the field.

## INTRODUCTION

Vitamin B12 (cyanocobalamin, Figure 1) is the most complicated naturally occurring catalyst known. The array of reactions which it can catalyse is astounding and most of the rearrangements cannot be reproduced using chemical reagents (1). It is an essential nutrient to most living organisms and its occurrence on earth is thought to have preceded the appearance of complex living organisms (2). This molecule, which consists of a tetrapyrrole system, known as a corrinoid, coordinating a central cobalt atom, can catalyse rearrangements as well as oxidation or reduction reactions.The reaction mechanisms of coenzyme B12 (adenosylcobalamin) and vitamin B12 mediated reactions have been the subject of numerous studies that have shown the formation of a carbon-cobalt bond which undergoes homolytic scission (3, 4). Most evidence seem to support the formation of free radicals, although they have never been isolated (5). In the reductive dechlorination of carbon tetrachloride, the formation of carbenes also seems probable (6, 7).

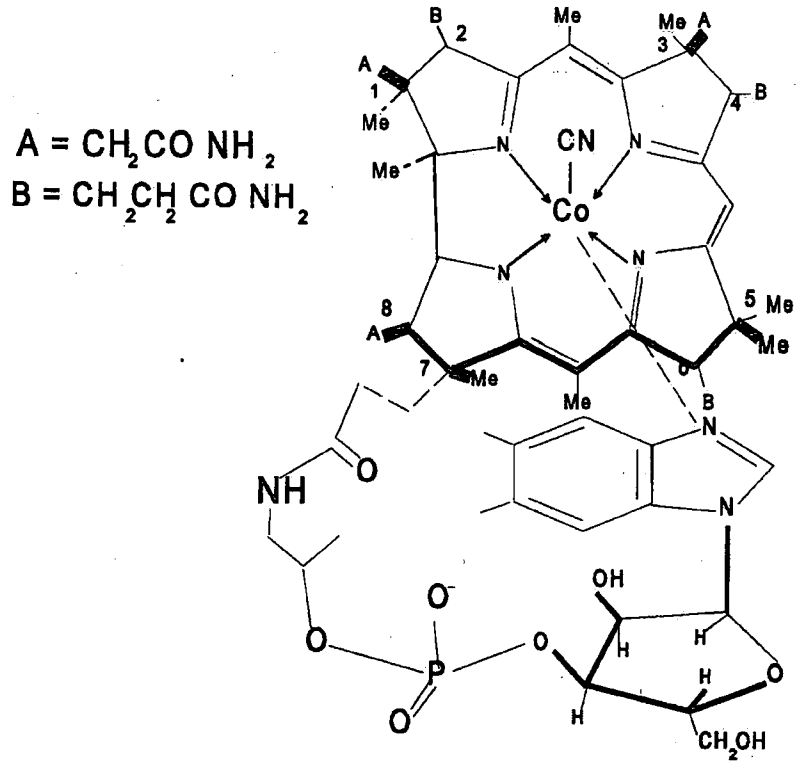


**Figure 1** Cyanocobalamin (Vitamin B12)

One of the interesting features of this compound is that it may function as a coenzyme (as adenosylcobalamin) or as its isolated form of cyanocobalamin. This latter feature is of great interest to the field of environmental chemistry and remediation because the reductive dechlorination

reactions can occur in the absence of living organisms and of oxygen. The problems of substrate toxicity at high concentrations and of delivery of nutrients to specific bacterial populations, which are crucial for the *in-situ* remediation of groundwater, are therefore alleviated. The absence of the protein structure results in some loss of substrate specificity, which broadens the scope of the reaction. This is another desirable feature for environmental remediation where mixtures of several different compounds are generally encountered. Vitamin B12 is capable of reductively dechlorinating chlorofluorohydrocarbons (8), chlorinated aliphatic (9,10) and aromatic hydrocarbons (11,12), which are anthropogenic compounds that are generally persistent in the environment. It has been used for the in-situ dechlorination of tetrachloroethylene (PERC) present as residual dense non-aqueous solvent (DNAPL) in model columns (13) and in the field (14). The application to *in-situ* remediation of contaminated groundwater fostered the need to understand the interaction with different reducing agents as potential alternatives to titanium citrate for field use.

Most of the mechanistic studies published have used one carbon substrates as models for the reductive dechlorination reaction (6,7) whereas kinetic studies have shown the potential interest of the reaction for other molecules of environmental interest (15). One of the major advantage of vitamin B12 is its ability to catalyse the dehalogenation of a broad range of compounds including alkanes, alkenes and aromatic hydrocarbons. This paper is focussed on studying the mechanism of the reductive dechlorination of chlorinated alkanes and alkenes by vitamin B12 using different reducing agents and on the isolation of free-radical intermediates formed during the reaction. The reaction of vitamin B12 with sodium dithionite, sodium borohydride, dithiothreitol and titanium citrate were compared using UV/visible spectroscopy and electrospray LC/MS. Free-radical intermediates were identified using spin-trapping GC/MS.

Assaf-Anid et al. (6) have studied the reductive dechlorination of $CCl_4$ catalysed by vitamin B12 using dithiothreitol as a reducing agent and proposed the formation of carbene intermediates and a Cobalt II cobalamin species ($B_{12r}$) as the catalyst responsible for the reaction. Their evidence was mostly based on visible spectral data or product distribution. They reported the spectral changes associated with the formation of chloroalkyl cobalamins (12). It is apparent that the observed

differences in reactions rates between different reducing agents could be related to their ability to reduce the central cobalt atom. The formation of the alkyl cobalamin complex would only be possible with a reduced species. Many authors have proposed that the reaction occurs with Cobalt I cobalamin ($B_{12s}$) whereas others contend that $B_{12r}$ is sufficiently reduced for the reaction to occur. In the case of the reductive dechlorination of pentachlorophenol, only the fully reduced complex is capable of effecting the reaction (9). The authors also note the difference in the regiospecificity of the reaction compared to what in observed in bacteria. Chiu and Rheinhard proposed a reaction mechanism for the reduction of carbon tetrachloride in titanium citrate (7).

An attempt to understand the effect of the different reducing agents on vitamin B12 and the reason for the difference in reactivity was made by analysing the reaction mixture by LC/MS. Inactivation of porphyrins has been reported for hematin (7) and the lack of reaction of vitamin B12 with stannous chloride and sodium borohydride, sodium dithionite and carbon monoxide noted (16) but without explanations as to why this occurred. The purpose of this research was to verify the postulate that, apart from the differing ability to reduce the cobalt centre to the I or II oxidation state, some reagents might bind irreversibly to the cobalt, or effect some reductions elsewhere in the molecule and produce an intermediate that is less reactive. Electrospray LC/MS was chosen as a soft ionization method with good resolution that would allow the detection of very small changes in molecular weight in a large non- volatile molecule (cyanocobalamin: 1355 a.m.u.).

The second goal of this project was to identify the intermediates formed in the sequential reductive dechlorination of polychlorinated solvents. Much indirect evidence has been used to infer that the vitamin B12 catalysed reactions proceed via the formation of free radicals (17), but no direct measurement of a free radical has ever been reported for this reaction. The addition of a spin-trapping agent and the identification of the adducts by GC/MS was used as a means of identifying the free radicals formed as intermediates.

A spin trap is an unsaturated compound to which a free radical can be added, forming a new compound, the spin adduct, which is a more stable free radical than the original, thus allowing its observation. Spin trapping has been used extensively in organic chemistry, but mostly in conjunction

with e.s.r. spectroscopy (18). More recently spin-traps have been used in investigation of biological reactions in animals (19). The use of GC/MS for identification of free radicals was proposed by Krygsman et al. (20). While e.s.r. can provide structural information on the free radical formed, the technique can only work if there is only one species present, because it does not have any separation ability. GC/MS is superior in this context, because the simultaneous presence of several free radicals can be identified. One of the limitations of the spin-trapping technique has been that most spin-traps, usually C-nitroso compounds or nitrones, are not soluble in water (18). Therefore, they were thought to be unsuitable to monitor aqueous phase reactions. This problem was circumvented by using a two-phase system where the trapping agent was dissolved in the substrate, which also served as the solvent, and shaken with the aqueous vitamin B12 in titanium citrate buffer. The free radical products were continuously extracted back into the solvent/substrate, which was analysed by GC/MS at different time intervals.

## EXPERIMENTAL

### 1. UV/vis

The UV/visible absorbance spectra of solutions of three reducing agents with vitamin B12 was measured using a Varian Cary 3E spectrophotometer. PERC and carbon tetrachloride ($CCl_4$) were added individually to solutions of sodium dithionite, sodium bisulfite, sodium sulfide, sodium borohydride, dithiothreitol (DTT) and titanium citrate in tris buffer at pH 8 and the spectral changes recorded at 10 min, 30 min and 18 hours.

### 2. Electrospray LC/MS

Samples of cyanocobalamin and cyanocobalamin reduced in titanium citrate, dithiothreitol, sodium dithionite and sodium borohydride, buffered at pH 8 in TRIS were adsorbed on a C18 cartridge (Sep-Pak, Waters/Millipore) and then eluted in methanol. The methanol extract was mixed with a 1:1 mixture of acetonitrile/water to which 0.1% trifluoroacetic acid was added to encourage

ionization. The samples were introduced by direct liquid injection at 0.2 mL/min into a VG Electrospray LC/MS system at 35 eV ionization potential (Department of Chemistry, McMaster University, Hamilton Ontario).

### 3. Spin trapping

The spin trapping agent (5 mg) phenyl-tert-butyl nitrone (PBN) (Sigma Chemicals) was dissolved in 200 mL of PERC. The solution was then added to vitamin B12 (100 mg/L) dissolved in aqueous titanium citrate (0.06M) adjusted to pH 8 with sodium carbonate in a sealed 5mL vial. Because cyanocobalamin complexes are photosensitive, the vials were wrapped in aluminium foil for the duration of the incubation. In order to allow more intermediates to form before being trapped, in some experiments, only 100µL of the solvent was placed initially in the vial and the 5mg of PBN was dissolved in another 100µL of the solvent and added after one hour. After 18 hours, 1µL of the organic phase was analysed on a Hewlett-Packard model 5790 GC/MSD equipped with a 30 m, 0.25 mm i.d. DB-5 fused silica column (J&W). The split/splitless injector was kept at 280°C and the transfer line at 275°C. The oven temperature was ramped from 80 to 250°C at 10° /min. The mass spectrometer was scanned from 45 to 450 a.m.u.

## RESULTS

### UV/Vis

1. Influence of reducing agents

The spectra of reduced B12 in the various reducing agents were disctinctly different as shown in Figure 2. The spectrum in DTT has been associated with a Cobalt II species (6). The spectrum of B12 in titanium(III) citrate is that of the cobalt (I) species. The spectrum of Co(I)cobalamin was recorded by Chemaly and Pratt (21) by reduction by zinc dust in acetic acid and is more similar to what was observed with titanium citrate, although recorded at much higher

concentrations and at a lower pH. The spectrum in dithionite differs from the two previous ones, especially from the lack of absorbance at 550nm.

It is interesting to note that the fastest rate of dechlorination is observed in titanium citrate, followed by DTT, and that no reaction is observed in dithionite (for 1,2-dichloroethane, 10). Unlike what has been suggested (12), this difference cannot be strictly due to the Eh produced, because of the three, dithionite produces the lowest Eh (-600 vs -480 for titanium citrate and -330 for DTT;ref 22 ). Sodium borohydride, stannous chloride and carbon monoxide were found to be ineffective for reductive dehalogenation (16) but sodium borohydride has been used to reduce B12 to the Co(II) cobalamin and produced a spectrum which was very similar to that observed from reduction by DTT ($\lambda$max at 312, 361, 404 and 474 nm)(Fig. 2, spectrum 3 and ref.3). The same spectrum was observed upon catalytic hydrogenation of B12, in a study of the crystallographic structure of the Co(II) cobalamin (23). Sodium borohydride has also been used in the synthesis of alkyl cobalamins, which means that it is capable of reducing the cobalt atom sufficiently to form a carbon-cobalt bond (24,25). One possible difficulty with sodium borohydride is that its reductive ability is due to the generation of hydrogen which disappears rapidly from the reaction medium and thus could not continue to regenerate the reduced cobalt centre. It has been involved in the reductive dechlorination of trifluoromethylcobaloxime to methylcobaloxime, but in that reaction, the carbon-cobalt bond seemed to remain intact (26).

2. Interaction with the substrate

The visible effects of the reactions of vitamin B12 with different reducing agents and with PERC and $CCl_4$, are summarized in Table 1. With dithionite as reducing agent, although the solution of vitamin B12 changed from pink to orange, as evidence of reduction, the addition of either chlorinated solvent to the mixture did not produce visible spectral changes, even after 18 hours. No degradation products of PERC were observed. With DTT, the results were similar to those of Assaf-Anid et al. for $CCl_4$ (6) whereas spectral changes with PERC were minimal (increase at 260 and 280nm only). With titanium citrate, the reduced B12 produced a very broad, low

intensity peak at 550nm. The addition of PERC and $CCl_4$ were accompanied by a shift to 530nm and 525 nm respesctively. The broad adsorption maxima could be indicative of the superimposition of several intermediates being present in solution.

It is interesting to observe the reversibility of the reduction as seen by changes in colour of the solution after exposure to air. The solution containing sodium dithionite was very difficult to reoxidize and never returned to the pink colour of the original vitamin B12 solution, but rather to a very faint yellow colour. With DTT, the solution slowly returned to a peach rather than a pink colour, but more rapidly than with dithionite. In sodium sulfide a grayish solution was produced, different to that of any other reaction mixtures. The addition of carbon tetrachloride immediately produced visible changes in the spectrum most prominently at 400nm. The reaction with PERC did not produce significant spectral changes. After days of incubation, the solution which had been exposed to $CCl_4$ turned to an intense mustard yellow colour while the solution exposed to PERC turned a peach colour.

Table 1. Summary of UV/VIS data

| **Reagent** | **Colour** | **UV/Vis of reduced B12 λ (nm)** | **Reaction with $CCl_4$** | **Reaction with PERC** |
|---|---|---|---|---|
| **Bisulfite** | Pink/peach, Irreversible | 265, 320, 420, 445 | No products No spectral changes | No products No spectral changes |
| **Borohydride** | Orange, Reversible to pink | 315, 360, 410, 480, 550 | No products Spectrum similar to oxidized B12 | No products Spectrum similar to oxidized B12 |
| **Dithionite** | Orange Irreversible | | No products No spectral changes | No products No spectral changes |
| **Dithiothreitol (DTT)** | Orange | | Yes | No, no spectral change |

| | | | | |
|---|---|---|---|---|
| **Sulfide** | Dark brown Oxidation to orange | 250, 400 broad low intensity | Yes, stable, very intense yellow colour, 340, 400, | Very little product after 18 hours, no visible or spectral change |
| **Titanium (III)** | Very dark brown Reversible to pink on oxidation | | Yes, rapid Change in UV/vis Same as with PERC - but different than with DTT-B12 | Yes, rapid Change in UV/vis Same as with $CCl_4$ |

The $CCl_4$ mixture was impossible to reoxidize or to re-reduce by the addition of titanium citrate. It is therefore most probable that one of the products of $CCl_4$ reduction irreversibly binds to cobalamin. Carbon monoxide seems to be the most likely candidate for this irreversible binding by analogy to its behaviour with hemoglobin. The sodium sulfide reduced B12 that was not exposed to a chlorinated solvent and that exposed to PERC were reoxidized to a peach coloured solution giving identical spectra.The reduced B12 solution exposed to PERC was still reactive towards $CCl_4$. This means that the reaction towards PERC is minimal. Gas chromatography showed the presence of chloroform and dichloromethane in the carbon tetrachloride treated vial and of very small amount of TCE in the PERC treated vial. In summary then sodium sulfide will work to some extent, but is best generated *in-situ* because of its odour and the possibility of producing the highly toxic $H_2S$ if the pH is not carefully controlled.

In contrast to all the other reducing agents tested, the reoxidation of vitamin B12 treated with titanium citrate was rapid and returned to the original hue of pink. These differences could be the clue to the differences in rates of reation. Indeed, for optimal catalytic function vitamin B12 must undergo a series of reductions and oxidations. If the reducing agent not only alters the redox status of the central cobalt atom, but reacts with the cobalamin molecule either at the metal center or some other part of the cobalamin molecule, the resulting reduced molecule may not be able to function as a catalyst. Similarly any of the products formed also have the potential to bind irreversibly to the catalyst and to deactivate it. In the case of carbon tetrachloride, the production of carbon monoxide is well documented and could lead to the irreversible formation of an adduct.

It is very important to understand the reason for the lack of reaction in dithionite and the cause of deactivation in sodium sulfide because many reduced sulfur species are found in aquifers contaminated with organic solvents. If inactivation of vitamin B12 occurs, it would preclude its application as an *in-situ* treatment for groundwater in areas where sulfur is prevalent.

## LC/MS

In order to understand the nature of the interaction of vitamin B12 with different reducing agents, they were analysed by electrospray LC-MS. Vitamin B12 has been measured by a variety of mass spectral techniques such as Fast Atom Bombardment (28), Laser Desorption (29) Plasma Desorption (30) and more recently MALDI (Matrix Assisted Laser Desorption Ionization) (31). However these techniques were all used on the pure isolated compound and would not be suitable to measure air sensitive reaction products present in aqueous solutions. Direct Liquid Injection negative ion LC/MS (32) and the more recently developed Electrospray (ES) LC/MS had better potential because in these techniques, the reduced solution can be introduced directly into the mass spectrometer by a syringe, without further manipulations, and therefore reduce the chance of exposure to the atmosphere. ES is a very soft ionization technique offering good resolution, which can detect very subtle changes in molecular weight. It has been applied to chlorins which bear some structural similarity to corrins (33). ES produces multiply charged ions which is useful in the analysis of large biomolecules because smaller, more easily measured fragments are produced (34).

The ES LC/MS spectra resulting from the addition of the various reducing agents to cyanocobalamin are summarized in Table 2. Cyanocobalamin shows a molecular ion at 1355.5, which is consistent with its protonated molecular ion. The base peak at 678.3 can be attributed to $M^{++}/2$ . The base peak for all spectra of reduced species is 665 can be assigned to $M+2^{++}/2$, which comes from the addition of two protons to the vitamin B12 less the -CN ligand, i.e. cobalamin. All spectra also have the mass of 673.5 in common which can be attributed to the half mass of hydroxycobalamin.

It is interesting to note that, in the mass spectrum of cyanocobalamin reduced in dithionite, a peak at 705.9 is observed, at 41 mass units higher than the base peak representing cobalamin. Because this is from a doubly charged ion, it represents a species of a molecular weight 82 mass units higher than that of reduced cobalamin. It has an abundance of 37% of the base peak, indicating that it is a major product. It is presumably due to an adduct of dithionite to cobalamin, the formation of which will be discussed below. Similarly, in the mass spectrum of cyanocobalamin reduced by sodium borohydride, a peak at m/e 681.7 is observed, which would correspond to cobalamin + 33 a.m.u. The latter peak was also present in DTT reduced B12, but in none of the other reduced products. A fairly prominent peak at 686.5 was also found in the sodium borohydride reduced spectrum, which is equivalent to cobalamin + 43 a.m.u. Small amount of this are also seen in cyanocobalamin, but in none of the other reduced products. The parent peak is only visible in cyanocobalamin and in the titanium citrate reduced complex.

The assignment of structures to these adducts are not obvious. Apart from the reducing agents themselves, the samples came in contact with trifluoroacetic acid (m.wt. 114), acetonitrile (m.wt. 41) and methanol (m.wt. 32). The addition of these solvents did not produce visible spectral changes. It is presumed that some adducts may be formed in the mass spectrometer only. This would not be the case for the reducing agents because they were removed from the mixture prior to the introduction of the samples into the spectrometer.

Table 2. Electrospray LC/MS of cyanocobalamin with reducing agents.

| Reducing agent | Parent ion (% of base peak) | Base peak (100%) | | | |
|---|---|---|---|---|---|
| None | 1355.5 (10) | 678.3 | 665 (5) | 686.5 (6) | |
| Ti citrate | 1328 (2) | 665 | 672.9 (8) | | |
| DTT | n.d. | 665 | 673.5 (20) | 681.5 (4) | |
| Na dithionite | n.d. | 665 | 673.2 (5) | **705.9** (37) | |
| Na $BH_4$ | n.d. | 665 | 673.5 (25) | 686.5(28) | 681.7 (10) |

In summary then, the LC/MS spectra showed that significantly different intermediates are formed from a variety of reducing agents. The addition of bisulfite (formed from dithionite) produced an adduct which totally inactivates the B12 molecule.

## 2. Spin trapping

After studying the effect of different reducing agents on the catalyst, spin trapping was used to examine the intermediates formed in the reductive dechlorination reaction.

A total ion chromatogram of the PBN adducts of PERC is shown on Figure 3. After one day, the products of reductive dechlorination were mostly trichoroethene and cis-dichloroethene. A large excess of PERC was obviously also present. Two peaks in the chromatogram had mass spectra respectively consistent with a trichloroethenyl (ret. time 15.438) and a dichloroethenyl free radical (ret. time 14.465) adducts to PBN. The spectra and the proposed structures of the intermediates are shown on Figure 4. The molecular ion for the PBN spin adducts are not visible, but the characteristic isotopic clusters of chlorine make the identification of the compounds relatively simple. Only trichloroethenyl and dichloroethenyl adducts were observed, the precursors to trichloroethene and dichloroethene respectively. It is not possible from the mass spectrum to identify whether a single dichloroethenyl radical is formed and then isomerized to either cis- or trans-dichloroethene during the addition of H, or two distinct radicals are formed and coelute. The first possibility is the most plausible because only one sharp peak is observed in the chromatogram and the *cis* and *trans* products themselves are easily resolved on capillary columns.

In laboratory soil columns, *trans*-dichloroethene was found to appear first, but the *cis* isomer eventually predominates. This is could be explained by the fact that a *trans* conformation would be preferred for the dichloroethenyl radical mostly based on steric considerations. However the *trans* isomer would also display higher reactivity towards cobalt. In the *trans* isomer, the chlorine's electron-withdrawing effect create opposing dipoles which tend to reduce the electron density and

elongate the carbon-carbon bond, thus making it more susceptible to attack by the electron-rich cobalt. In the *cis* isomer, the synergy of the two dipoles is not as effective, and, in addition, the steric crowding of the two chlorines tend to compress the carbon-carbon bond, producing a higher electron density than with the *trans* isomer.

Although many other chlorinated compounds, as indicated by the characteristic chlorine clusters, could be identified in the chromatograms, few of them could be matched with spectral library entries or PBN adducts. Tetrachlorobutadiene (m.wt. 190) and trichlorostyrene (m. wt. 206) were identified by analogy with the corresponding library spectra. Tetrachlorobutadiene is formed by the recombination of two dichloroethenyl radicals. It is difficult to rationalize the formation trichlorostyrene from PBN and trichloroethene however, because the addition of the radical usually occurs on the $\alpha$-carbon. Both propenyl ( m.wt. 118) and propyl (m.wt. 120) benzene are formed, presumably as ethene and ethane adducts. The only adduct that could not be found in the degradation path is the monochloroethene. Many other peaks occur in the chromatogram, but all of them were also present in a control. They are either hydrolysis or reduction products of the reagent: benzaldehyde, benzonitrile and benzaldehyde oxime are the most prominent.

In the GC/MS for $CCl_4$/PBN (Figure 5), three adducts were observed, consistent with the formation of $^{\bullet}CCl_3$, $^{\bullet}CHCl_2$ and $^{\bullet}CH_2Cl$ radicals (Figure 6). In addition to the products of sequential reductive dechlorination, large amounts of methane were formed. Two common products (138 a.m.u, single chlorine), identified as *cis*- and *trans*-chlorostyrene, were observed with $CCl_4$, $CHCl_3$ or $CH_2Cl_2$ as starting materials. This provides some evidence of the presence of a chloromethyl radical. The monochlorostyrenes would be formed by the breakage of the carbon-nitrogen bond of the PBN adduct. While this does not totally preclude the formation of carbenes, it is a clear evidence of the sequential formation of free radicals in the reaction.

## DISCUSSION

The results of this investigation complement the knowledge that has been gathered with the goal of elucidating the reaction mechanism of vitamin B12-catalysed reductive dechlorination. It

is apparent that depending on the reducing agent used, a cobalt I or II species may be involved. The partially reduced B12r is only capable of reacting with the more reactive substrate such as $CCl_4$. When considering the environmental applications of vitamin B12, the choice of reducing agent is very important. It must be readily available, non-toxic, water soluble and must be effective for the largest possible number of compounds, because mixed wastes are more the norm than the exception. Compounds such as DTT, which have been used extensively in the laboratory, would be too expensive for field use and do not reduce vitamin B12 sufficiently.

Of the compounds that can produce a cobalt (I) species, not all of them are suitable. Indeed, one of the crucial factors determining the rate of reaction seems to be the reversibility of the reduction, and for this reason, titanium citrate is definitely the preferred reagent. Some reducing agents, which would otherwise fit all of the other criteria, produced irreversible changes in the vitamin B12 molecule. The lack of reactivity of the vitamin B12 molecule reduced with sodium dithionite prompted an investigation of the intermediate product using LC/MS. Initially it was thought that sodium dithionite could reduce the benzimidazole ring and increase the molecular weight by 2 Daltons. The peak at 706 which is very intense (37% of base peak) corresponds to an increase of 82 which is equivalent to the addition of $H_2SO_3$. One possibility is that a nucleophilic addition of the dithionite to the β–position of the dimethylbenzimidazole occurs (Figure 7). This position is one of the most susceptible to nucleophilic attack in the cobalamin molecule. Addition to nitrogenous bases has been observed in other biomolecules. In the case of $NAD^+$, addition occurs with bisulfite whereas reduction occurs

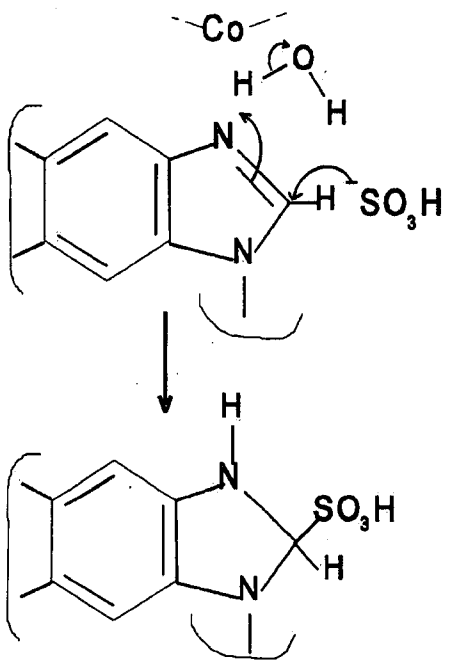


**Figure 7** Addition of bisulfite to the benzimidazole moiety of vitamin B12

with dithionite (1). Both are in fact possible with dithionite since it is known to disproportionate in water to $HSO_3$ and $S_2O_3^=$ (27). This reaction is not reversible and hence the modified B12 cannot be reoxidized. Sodium bisulfite was also used to reduce vitamin B12 to see if the reaction would be similar to that with dithionite. The colour obtained was slightly different than that with dithionite, a reddish peach colour, but the same lack of reactivity towards chlorinated solvents, as measured by the visible spectrum and gas chromatography, was observed.

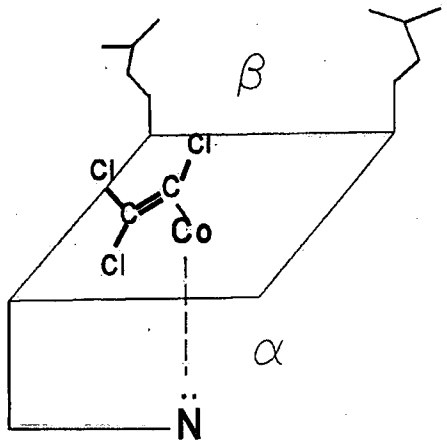


**Figure 8** Schematic view of trichloroethenylcobalamin intermediate

The fact that the UV spectra obtained by reduction with DTT and sodium borohydride are the same, but that their reactivity differs, also pointed towards a possible reduction of the ribofuranosyl-dimethylbenzimidazole moiety by sodium borohydride. They also share the unidentified peak at 681.5 in their mass spectrum. As a hydride donor, $NaBH_4$ it is not as strong as $LiAlH_4$, but could, in theory, attack the 2-carbon of imidazole, resulting in a reduced ring. The expected peak at 666, or at least broadening of the peak at 665 were not observed. Also, the reduction in $NaBH_4$ was reversible by exposure to air. The rapid removal of $H_2$ from the medium is therefore a more likely explanation for the lack of reaction.

The substrates also influence the rate of the reaction. The overall observed rate is dependent on two factors: the rate of formation of the alkyl halide- B12 complex, and the scission of the C-Co bond. The re-reduction of the B12 closes the cycle. It has been shown that the rate decreases with the degree of chlorination (15) which is consistent with the nucleophilic character of B12s.

However, the work presented here supports a mainly radical-mediated reaction mechanism. Homolytic scission of the carbon-cobalt bond has been demonstrated by others, but several free radical intermediates were identified in this work. One electron transfers probably occur and the benzimidazole ligand seems to be key in the reaction. The formation of the carbon-cobalt bond is of foremost importance. In the case of unreactive reduced B12, no visible spectral changes were observed upon addition of the substrates. The lack of reaction is therefore not due to irreversible binding of the substrate. The role of the benzimidazole ring seem to be crucial, probably by adding to the electron density of the cobalt central atom and facilitating the homolytic fission.

The fact that the double bond of the chlorinated ethenes is retained and that a high proportion of dimers are formed, would tend to indicate a highly organized intermediate. The geometry of the vitamin B12 molecule as defined by X-ray cristallography indicates that several amide ligands which are attached to the corrin ring are perpendicular to the plane of the corrin ring and could hinder the binding to the substrate. Fortunately, only two of them are perpendicular on the β-face of the molecule and they are on the a and b ring (2), leaving a relatively accessible center. Also, the molecular size of the chlorinated solvents is small enough that the presence of the chains do not dictate the configuration of the intermediate (Figure 8). The carbon-cobalt bond forms with the concurrent loss of a chlorine free radical. The reducing agent then provides the electron necessary for the formation of the leaving chloroalkyl or alkenyl radicals which are quenched by the medium or dimerize.

The formation of dimers has been the subject of discussion over the potential structure of the alkyl cobalamin intermediates (2). An intermediate where two complexes are arranged β face to β face would certainly be conducive to the preferential formation of dimers. The formation of dimers occurs in solution and is rarely seen in vivo because the presence of a surrounding protein precludes the formation of an intermediate involving two B12 molecules.

## CONCLUSION

This work was aimed at better understanding the mechanism of reductive dechlorination catalysed by vitamin B12. The effectiveness of various reducing agents in promoting the reduction of cyanocobalamin and the subsequent reductive dechlorination of chlorinated solvents such as perchloroethylene and carbon tetrachloride was assessed using UV/vis spectroscopy and gas chromatography. The structure of reduced B12 was measured using electrospray LC/MS.

The use of visible spectra in assessing the reaction intermediates was of limited use because they provide little structural information and several species can be present simultaneously in solution. Free radical trapping agents were used to identify several free radical intermediates formed from PERC and $CCl_4$. The spin-trap adducts of several intermediates were identified by GC/MS, which is direct evidence that the most likely reaction pathway for vitamin B12-catalysed reductive dechlorination involves free radicals.

The influence of different reducing agents on the reaction was also studied. Many sulfur-based reducing agents do not generate a sufficiently low Eh to produce the most reactive cobalt I species. Of those that do, dithionite and bisulfite also reacts with the benzimidazole ring producing an inactive form of B12. The production of a bisulfite adduct as shown by LC/MS attest to this occurrence. If the benzimidazole ring is itself reduced, it can no longer interact effectively with the cobalt center and the vitamin B12 molecule is inactive. Indeed, the critical factors in the suitability of a given reducing agent are not only the reactivity towards cobalt or the Eh produced, but the nucleophilic character towards benzimidazole and the reversibility of the reaction. Many sulfur-containing reducing agents are therefore precluded from use. This severely limits the number of possible reducing agents suitable for the reaction in an aqueous phase and as a groundwater remediation technology.

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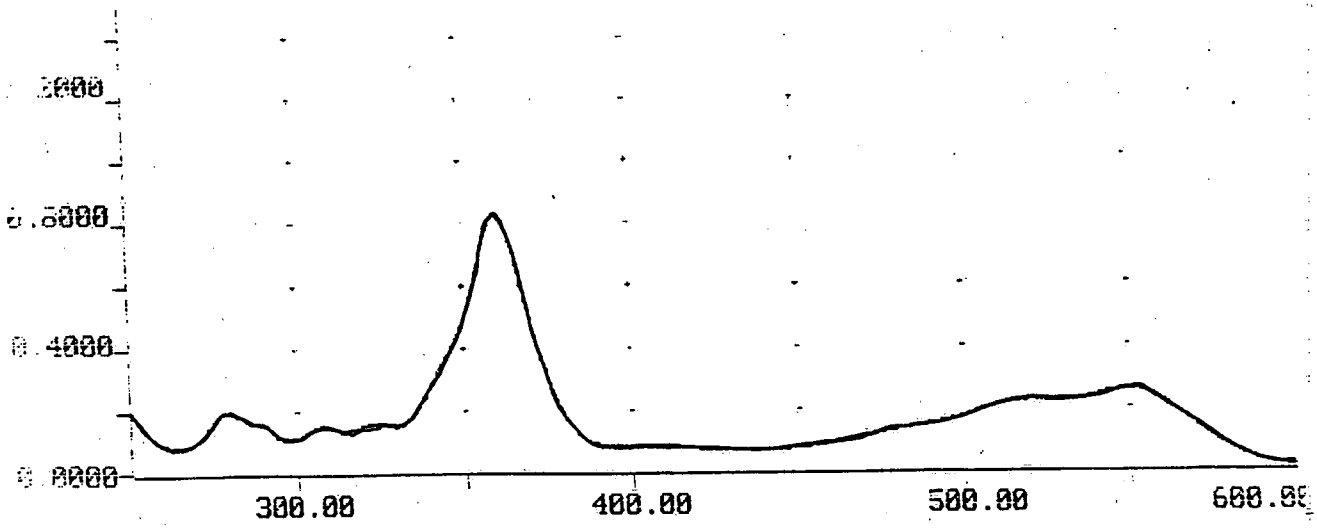
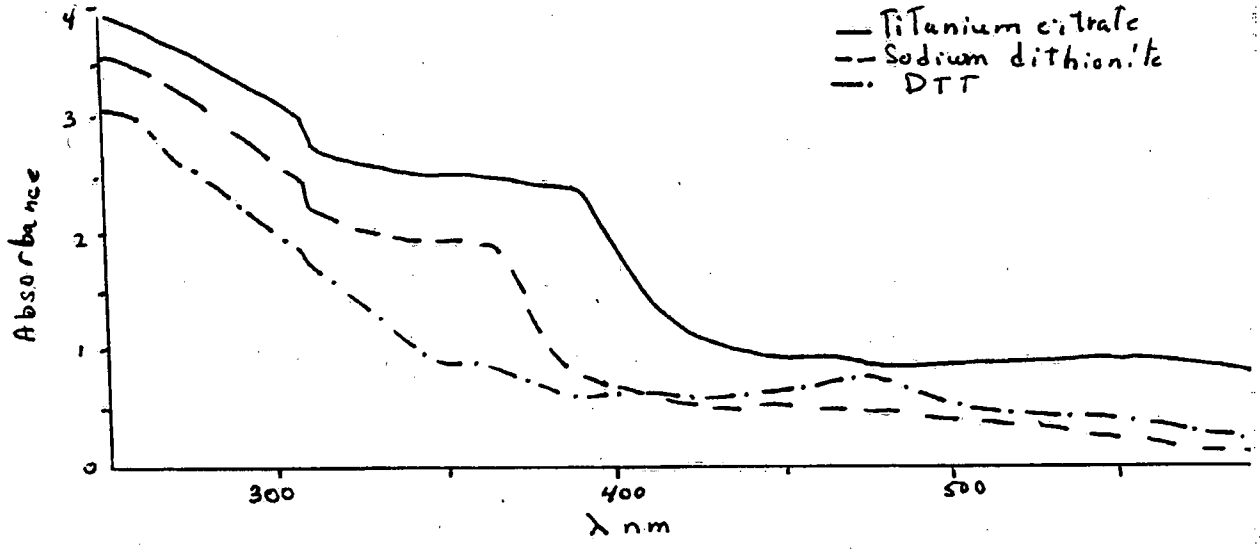
Figure 2

Vitamin B12



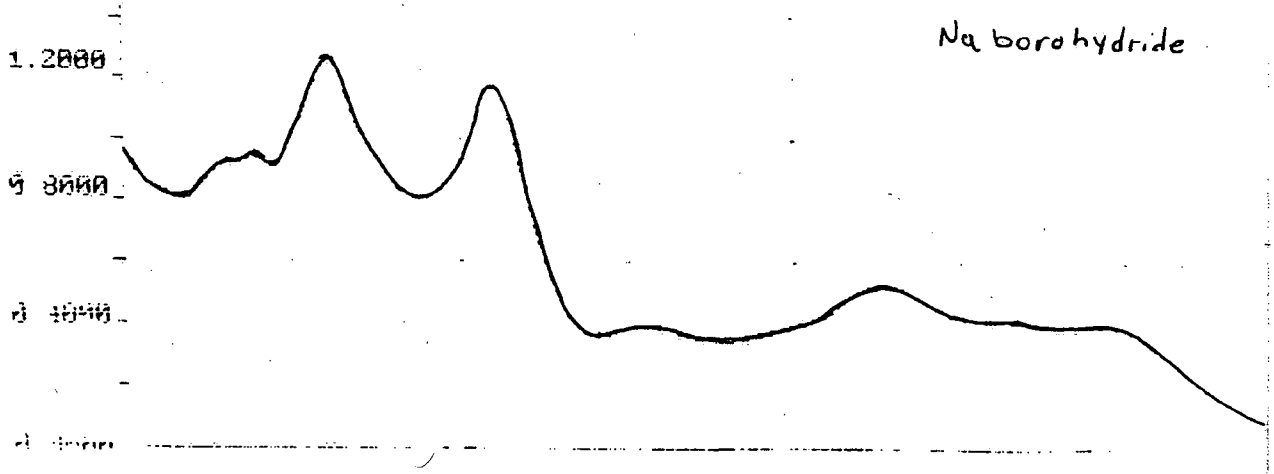
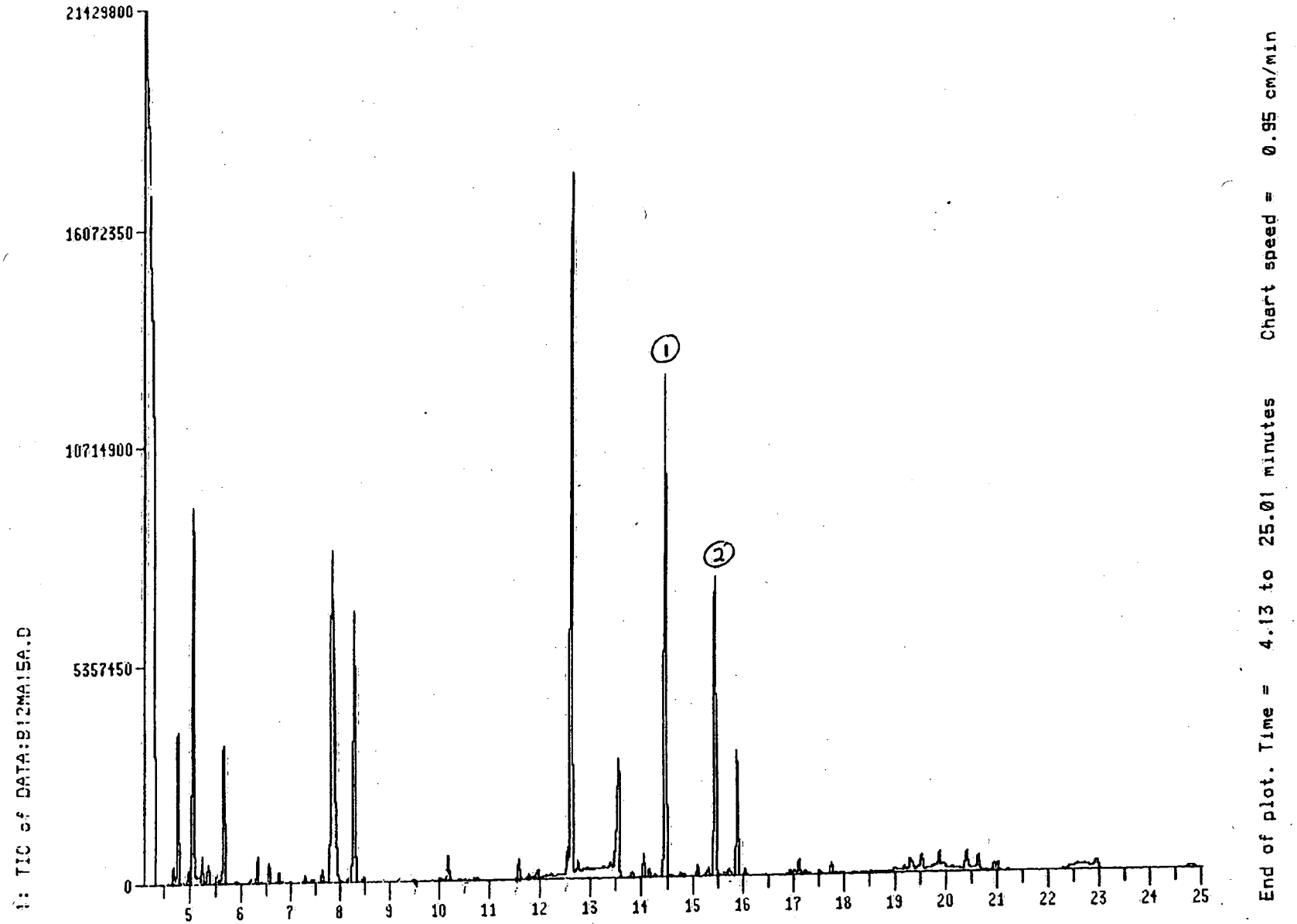
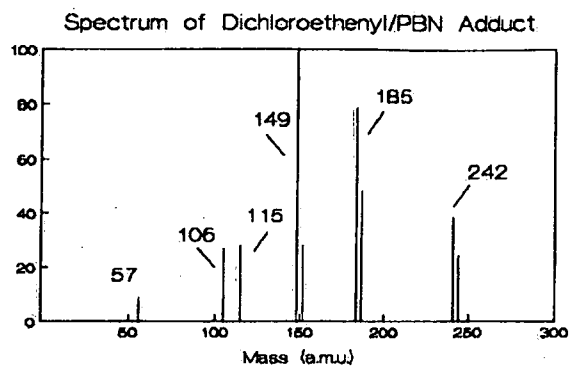
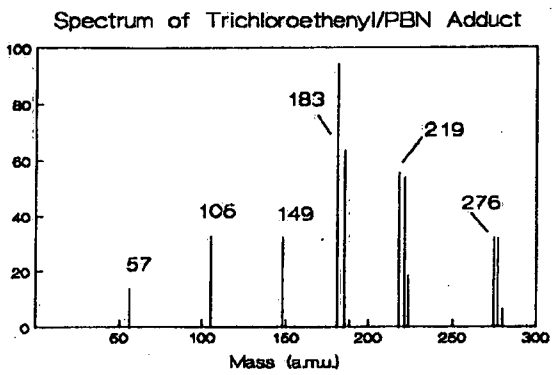



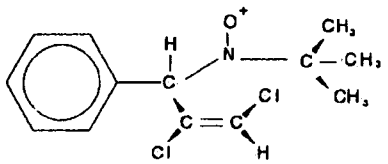
Figure 3

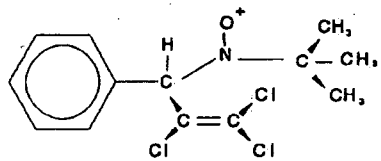
PERC-PBN

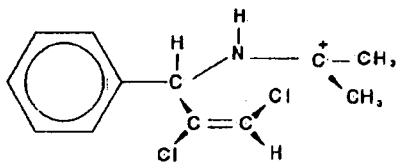








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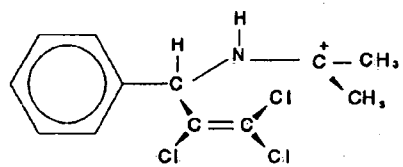

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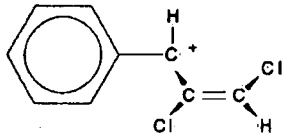

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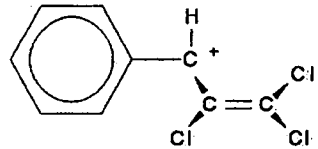

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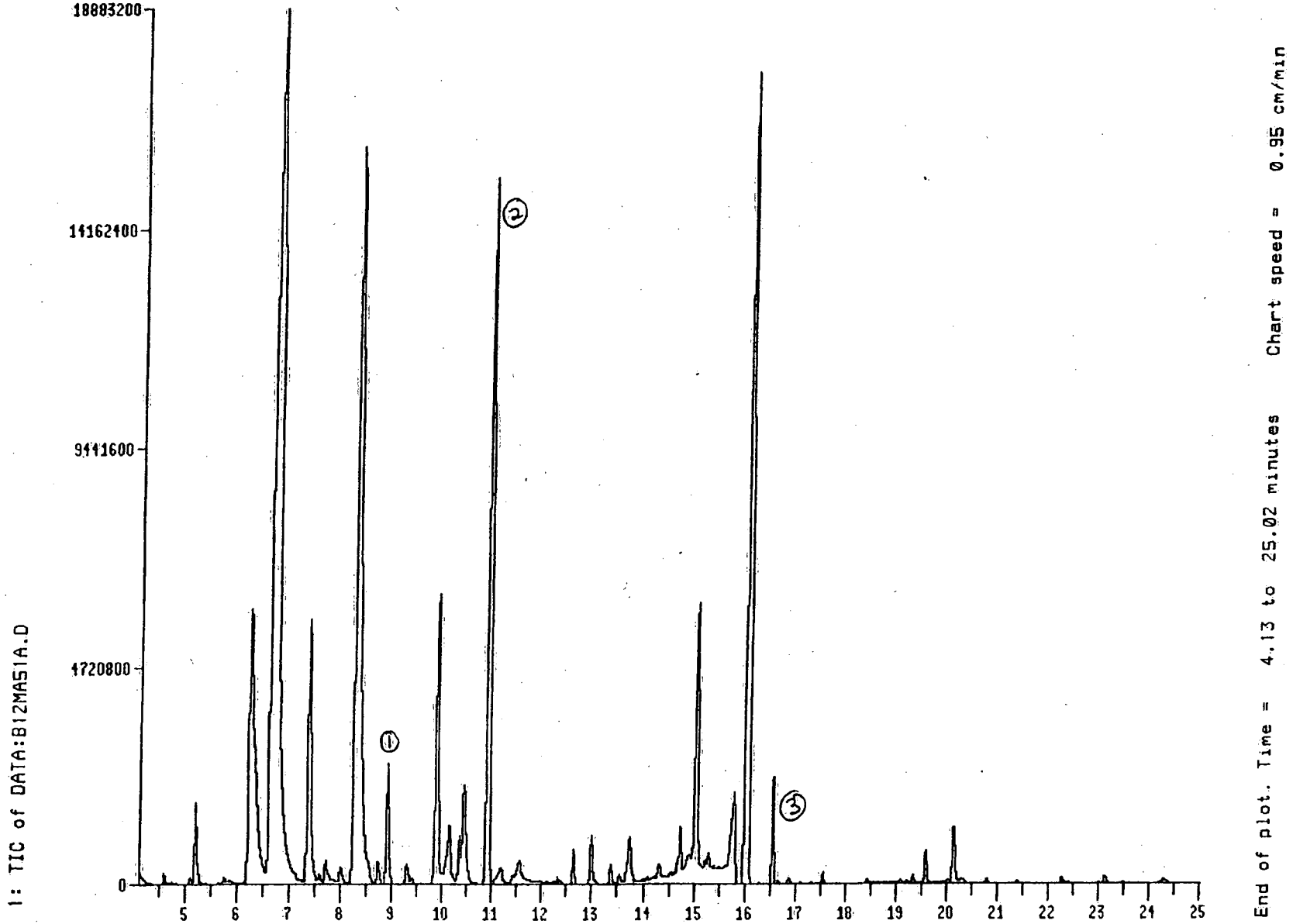
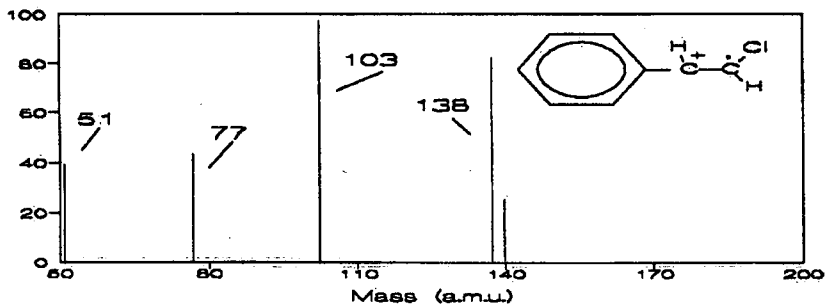
Figure 4

CCl4 - PBN

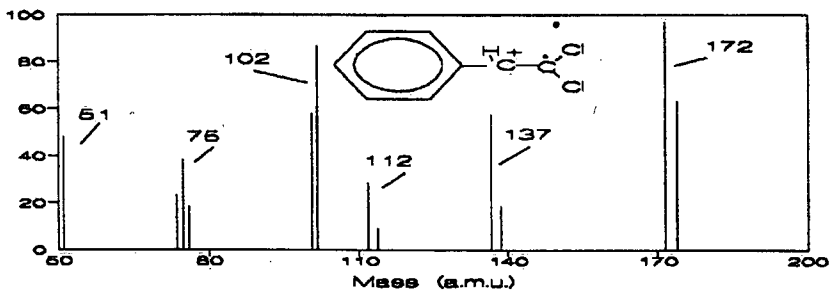
Figure 5



Spectrum of Chloromethyl/PBN Adduct (1)




Spectrum of Dichloromethyl/PBN Adduct (2)




Spectrum of Trichloromethyl/PBN Adduct (3)

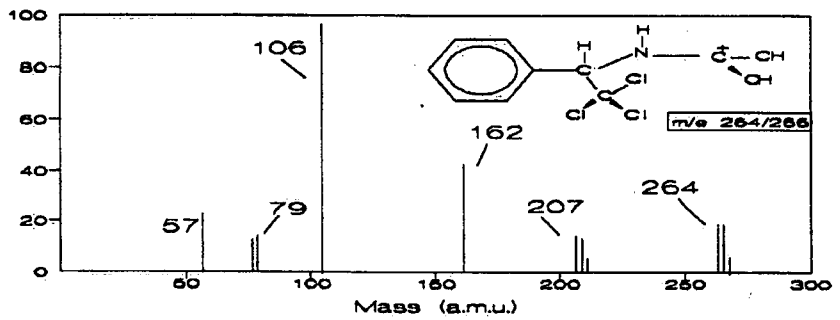



Figure 6

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