# Is it Isomerization of Cobalamins or Simple Hydrolysis of a Propionamide Side Chain?

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## Abstract

Literature is replete with references to b-, d-, and e-monocarboxylic acids of cobalamin, supposedly resulting from hydrolysis of a propionamide side chain. Our work indicates that these species are not monocarboxylic acids but isomers of cobalamins. The ease of isomerization under mild conditions, its reversibility, the formation of intermediate(s), large interconversions between the new isomers, b-, d-, and e-$B_{12}'$ and the observation using high resolution mass spectrometry that the molecular mass of protonated d-$B_{12}'$ is identical to $B_{12}$, all attest to genuine isomerization. Significant changes, in the puckering of the corrin ring, and in other parts of the molecule are clearly revealed by our earlier Mössbauer, proton and phosphorus nuclear magnetic resonance investigations, and as well as by the more recent carbon-13 and crystal structure studies reported in the literature.

## Introduction

We have reported earlier a new naturally occurring form of cobalamins and designated it the 'prime' form [1–3]. We observed subtle relative changes in NMR chemical shifts of protons on the corrin ring, aminopropanol side chain, and the nucleotide base and significant differences in the emission Mössbauer parameters for the regular and the new form. Changes in the chemical shifts of some protons and their spin-lattice relaxation times for the two isomeric forms of dicyanocobalamin and dicyanocobinamide were also observed [4]. These studies suggest that in the new form a slight variation in the puckering of the corrin ring occurs and that brings about a change in the conformation of the nucleotide base through the amino–propanol linkage. The phosphorus-31 distance to Co(II) was estimated to be larger for the new form by comparing the nuclear relaxation times ($T_1$) in the two isomeric forms [5]. The isomerization was observed to be reversible. For instance, at higher temperatures (~70 °C) deoxyadenosylcobalamin tends to convert into deoxyadenosylcobalamin′ in acidic aqueous solutions (pH ~ 2) in the 'base-off' state, while deoxyadenosylcobalamin′ tends to back convert into deoxyadenosylcobalamin at room temperature [1, 2]. The interconversion can be 'frozen' by raising the pH and putting the base on. Isomerization of hydroxocobalamin at room or lower temperatures, in near neutral solution, shows similar reversibility [6]. Thin layer chromatography revealed the presence of three closely related components in cyanocobalamin′. The three components of cyanocobalamin′ are negatively charged in neutral and alkaline solutions and can be separated from regular $B_{12}$ using anion-exchange chromatography.

Recently, Marzilli *et al.* [7], have shown with the help of TLC and HPLC that the isomers studied by us [1–6] are identical to what have been reported in the literature [8–10] as b-, d-, and e-monocarboxylic acids of cyanocobalamin. They have been prepared by not so mild treatment, *viz.* by warming $B_{12}$ solutions in ~1 M acid or alkali. It is believed that these monocarboxylic acids are derived from the propionamide side chains b, d, and e, which are more susceptible to hydrolysis than the 'protected' amide groups on the acetamide side chains a, c, and g [11]. Considerable efforts have been expended in the assignments of the 'b-, d-, and e-monocarboxylic acids' with the help of chemical studies [12], carbon-13 nuclear magnetic resonance studies [13–16] and X-ray and neutron diffraction studies [17–19].

We present evidence which strongly indicates that these species are not monocarboxylic acids but probably genuine isomers of cobalamins. We designate the three isomers as b-, d-, and e-cyanocobalamin′.

## Experimental

### *Preparation of Cyanocobalamins′*

Cyanocobalamins′ were prepared by the method reported by Anton *et al.* [14], which gives higher yields than the one developed by Katada *et al.* [1, 2]. 600 ml of 1 M HCl solution containing 6 g of

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cyanocobalamin was maintained at 37 °C for 4 h. The solution was then neutralized with NaOH to pH 6 and desalted by phenol extraction and applied to a column of Dowex 1 X 2 (acetate form, 100–200 mesh, 2.5 X 25 cm). The column was thoroughly washed with water to remove unreacted cyanocobalamin and eluted with 0.04 M sodium acetate buffer at pH 4.67. The doubly charged species remained adsorbed on the column. The buffer solution eluate was neutralized to pH 6 and desalted by phenol extraction. For further purification of cyanocobalamins′, the adsorption on Dowex 1 X 2 and elution with 0.04 M sodium acetate buffer may be repeated. However, for separation of d-$B_{12}$′ from b- and e-$B_{12}$′, the solution was applied to a column of Dowex 1 X 2 (acetate form, 200–400 mesh, 5 X 80 cm) and eluted with 0.05% acetic acid. The first zone consists of d-cyanocobalamin′ and the second zone consists of a mixture of b- and e-cyanocobalamins′. The solutions were neutralized and desalted, and dried on a rotary evaporator.

*Thermal Treatment of Cyanocobalamins′ in KCN Solutions*

An aliquot of cyanocobalamin′ was dissolved in solution containing about 20 equivalents of KCN. After various thermal treatments, the solutions were acidified to pH 6 and desalted by phenol extraction or on the Amberlite XAD-2. The neutral species, generally consisting of cyanocobalamin and cyanocobalamin″ [6], were separated from the negatively charged cyanocobalamin′ on a Dowex 1 X 2 (100–200 mesh, acetate form, 2 X 15 cm) or on a Sephadex QAE A-25 column. The adsorbed cyanocobalamins′ were eluted with 0.04 M sodium acetate buffer (pH 4.67) and desalted. The doubly charged species which remained adsorbed on the column was eluted with 2 M sodium acetate solution. The relative amounts of the different fractions were estimated spectrophotometrically.

*Thermal Treatment of Cyanocobalamins′ in 0.5 M NaOH, 1 M HCl or in 1 M Acetic Acid Solution*

After different thermal treatments, the solutions were neutralized to pH 6, desalted and passed over a Dowex 1 X 2 column. The rest of the procedure was the same as in the above section.

*Thermal Treatment of Cyanocobalamin and Cyanocobalamins′ in Glacial Acetic Acid*

After the appropriate thermal treatment, the solution was rapidly neutralized with chilled NaOH solution and the pH adjusted to 10.5, and maintained at room temperature for an hour. The solution was desalted after adjusting the pH to 7. The rest of the procedure was the same as discussed above.

*Thin Layer Chromatographic Separations*

(a) Precoated TLC silica gel 60 silanized RP-2 plates (E. Merck) were used in conjunction with a developer consisting of methanol, acetic acid and water in the proportion 65:1:120. This methanol based system was found to be very reproducible, and convenient for distinguishing between cyanocobalamin, cyanocobalamins′ and cyanocobalamin″ [6]. However, the b- and e-cyanocobalamins′ are not resolved and only a single spot is obtained for them, with higher $R_f$ value than that for d-cyanocobalamin′ (Fig. 1).

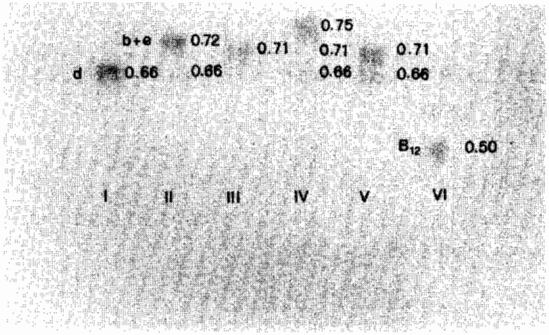



Fig. 1. (I) Reference d-$B_{12}$′. (II) b- and e-$B_{12}$′ formed by glacial acetic acid treatment (2 h at room temperature) of I. (III) Reference b- and e-$B_{12}$′. (IV) d-$B_{12}$′ and the unidentified species, formed by glacial acetic acid treatment (2 h at room temperature) of III. (V) Reference $B_{12}$′ consisting of a mixture of b-, and d-, and e-$B_{12}$′. (VI) Reference $B_{12}$.

E. Merck precoated TLC silica gel 60 silanized RP-2 plates were used with a developer consisting of methanol, acetic acid, and water in the proportion 65:1:120.

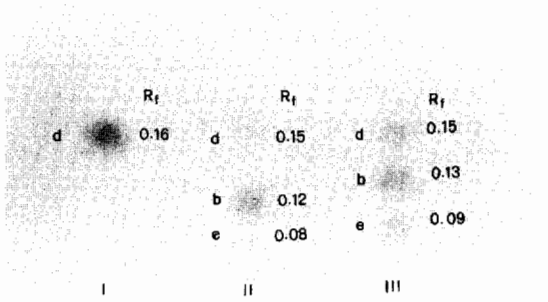



Fig. 2. (I) Reference d-$B_{12}$′. (II) b- and e-$B_{12}$′ formed by glacial acid treatment (2 h at room temperature) of d-$B_{12}$′. (III) Reference $B_{12}$′ consisting of a mixture of b-, d-, and e-$B_{12}$′. Analtech precoated TLC silica gel G plates with butanol based developer [20, 21].

(b) Precoated silica gel G plates (Analtech) with butanol based developer [20, 21] were used to distinguish between the three cyanocobalamins′. A developer consisting of 2-butanol, acetic acid and water in the proportion 127:1:50 was used. The three clearly resolved spots, belong to d-, b-, and e-cyanocobalamins′ in decreasing order of mobility (Fig. 2).

(c) Precoated HPTLC silica gel 60 plates (E. Merck) were used with a developer consisting of acetone, acetonitrile, isopropanol, diethylamine, 5% ammonia in the proportion 30:30:10:3:27 [22]. This system was used primarily to double check the identity of cyanocobalamin formed by treatment of cyanocobalamins'.

## Results and Discussion

### *Isomerization of Cobalamins under very Mild Conditions*

Our earlier observations regarding isomerization of hydroxocobalamin in neutral solutions at ~5 °C [6], were verified again and are summarized in Scheme 1 with the benefit of revisions. The neutral fraction generated from the positively charged hydroxocobalamin (which is separated from hydroxocobalamin on a cation-exchange column) initially consists predominantly of the intermediate hydroxocobalamin''. However, prolonged storage of the neutral fraction at ~5 °C or short thermal treatment at say 50 °C results in the growth of hydroxocobalamin and hydroxocobalamin' at the expense of hydroxocobalamin''. For instance, the storage of the neutral fraction for a few weeks at room temperature produces more than 10% of a positively charged species which is predominantly hydroxocobalamin. These observations highlight the fact that the isomerization can occur under very mild conditions and that it is reversible. Even refrigerated samples of crystalline hydroxocobalamin undergo appreciable isomerization over a period of a few years [1, 2].

A neutral aqueous solution of cyanocobalamin was stored at room temperature for over 4 years. Analysis showed presence of 8% cyanocobalamins' and 1% cyanocobalamin''. The relative intensities of the different components of cyanocobalamins' formed under these conditions (as observed by TLC) differed significantly from that of standard preparations of cyanocobalamins'.

### *Back-conversion of Cyanocobalamin' to Cyanocobalamin*

The likely conditions for the back-conversion are the ones which induce isomerization of cyanocobalamin to cyanocobalamin'; thermal treatment of $B_{12}'$ in aqueous solutions of KCN [1, 2], NaOH [8], HCl [8, 13] and in glacial and dilute acetic acid were tried.

Aqueous solutions of $B_{12}'$ containing excess of KCN were treated at different temperatures, ranging from room temperature to 100 °C, for various intervals of time. A few percent of neutral species were formed consisting primarily of $B_{12}$ and $B_{12}''$. For instance, 4 h at 80 °C yielded 5% of $B_{12}''$ and 2% of $B_{12}$; 1 week at 50 °C yielded 0.8% of each.

Treatment of cyanocobalamins' with 0.5 M NaOH for 3 h at 37 °C generated about 2% $B_{12}''$ and 1% $B_{12}$.

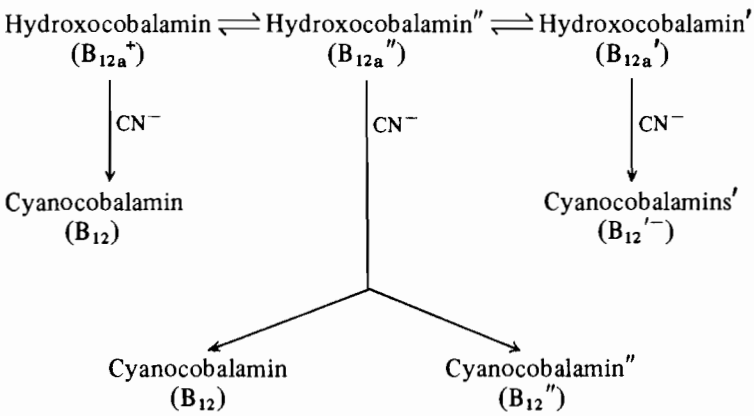
Treatment of cyanocobalamins' in glacial acetic acid at room temperature yielded about 1% of $B_{12}$ after 3–4 days. In contrast, the yield in dilute acetic acid was much higher. For instance, a sample of cyanocobalamin' when heated for 16 h at 80 °C in 1 M acetic acid yielded 4% $B_{12}$.

An aqueous solution of $B_{12}'$ refluxed for 4 days resulted in the formation of 4.5% $B_{12}''$ and 0.5% $B_{12}$.

In all the above conversions, formation of some doubly charged negative species (presumably a monocarboxylic acid) was observed. It tends to increase with longer treatments. No correlation between the yields of cyanocobalamin and doubly charged species is apparent.

### *Interconversion between b-, d-, and e-Cyanocobalamins'*

The interconversions between different cyanocobalamins' occur quite readily (though not exclusively) in glacial acetic acid. When an aliquot of d-cyanocobalamin' was treated in glacial acetic acid for 2 h at room temperature, more than 60% was converted into b-cyanocobalamin' (Figs. 1 and 2). Similarly, when a mixture of b- and e-cyanocobal-




Scheme 1.

TABLE I. Mass Spectrometric Data via Peak Matching

| | Calculated mass | Ratio | |
|---|---|---|---|
| Peak matching in vitamin $B_{12}$ | | | |
| $C_{63}H_{89}CoN_{14}O_{14}P$ | 1355.5752 | Reference | $MH^+$ |
| $C_{62}H_{89}CoN_{13}O_{14}P$ | 1329.5722 | 1.0195574 | $-CN$ |
| | | 1.0195573 | measured |
| Peak matching in d-vitamin $B_{12}'$ or d-monocarboxylic acid | | | |
| $C_{63}H_{88}CoN_{14}O_{14}PNa_2$ | 1400.5470 | 0.9678898 | $+H$ |
| $C_{63}H_{86}CoN_{13}O_{15}PNa_2$ | 1400.5232 | 0.9679062 | $OH/NH_2$ |
| | | 0.9678850 | measured |
| $C_{63}H_{89}CoN_{14}O_{14}PNa$ | 1378.5650 | 0.983323 | $+H$ |
| $C_{63}H_{87}CoN_{13}O_{15}PNa$ | 1378.5412 | 0.983340 | $OH/NH_2$ |
| | | 0.983320 | measured |

amins' was left at room temperature in glacial acid for 2 h, about ~15% was converted into d-cyanocobalamin' and predominantly into another species with $R_f$ 0.75 in reverse phase TLC (Fig. 1). The latter species is generated in several other treatments, and is also present when cyanocobalamins' are prepared by the cyanide method [2]. At higher temperature in glacial acetic acid (65 °C), a smaller fraction of d-$B_{12}'$ converts to b-$B_{12}'$ (~25%). Presumably, the equilibrium shifts in favor of d-$B_{12}'$ at higher temperatures. It is essential that the neutralization is carried out rapidly and that the pH is adjusted above 10, otherwise the results are irreproducible.

The interconversions between the components of $B_{12}'$ were roughly estimated via TLC. In all the cases discussed above, about 15% of multiple charged species were formed which were separated on the Dowex 1×2 column from $B_{12}'$ components by eluting the latter with 0.04 M sodium acetate buffer (pH 4.67) [7, 14]. Therefore, the multiple charged species were not seen on the TLC plates.

*High-resolution Mass-spectrometric Studies via Peak Matching [23]*

The low resolution mass spectrometry of d-vitamin $B_{12}'$ showed that the mass of neutral (protonated) d-$B_{12}'$ is one unit higher than that of regular $B_{12}$. This apparently would support substitution of $NH_2$ group by OH. On the other hand, d-$B_{12}'$ could preferentially pick up a hydrogen atom from the protic matrix of 3-nitrobenzyl alcohol, during fast atom bombardment. A hydrogen atom pick up during fast atom bombardment has been reported for some compounds [24].

High resolution mass spectrometry was used to distinguish between the following two possibilities:

(a) Addition of H to d-$B_{12}'$ during fast atom bombardment.

(b) OH replacement of $NH_2$ and forming d-monocarboxylic acid of $B_{12}$.

The voltage divider was calibrated by measuring $MH^+$ and $MH^+ - CN$ peaks in a sample of regular vitamin $B_{12}$. Then measurements were made with an equimolar mixture of vitamin $B_{12}$ and d-vitamin $B_{12}'$ (sodium salt) on the probe. Couple of peaks observed in d-$B_{12}'$ were matched with the reference molecular ion $MH^+$ of $B_{12}$ (Table I).

The peaks matching experiments show rather unambiguously that the molecular weight of neutral (protonated) d-$B_{12}'$ is identical to that of $B_{12}$. It is not very clear at present as to how a conformational isomer of vitamin $B_{12}$ can acquire a negative charge. It seems that when vitamin $B_{12}$ isomerizes to $B_{12}'$, one of the protons in the molecule becomes labile.

Let us recapitulate our salient observations. Isomerization of cobalamins can occur under very mild conditions and it is reversible. The reversibility is easily observed for the isomerization of hydroxocobalamin and deoxyadenosylcobalamin. The formation of intermediates, particularly cobalamin'', have been observed. The intermediate, cyanocobalamin'' is also formed in the back-conversion of cyanocobalamin' to cyanocobalamin. The high resolution mass-spectrometric data strongly indicates that d-$B_{12}'$ is not a monocarboxylic acid and supports isomerization. The interconversions between b-, d-, and e-cyanocobalamins' are observed under a variety of conditions. However, the interconversions can occur with great ease in glacial and 4 M acetic acid. These observations strongly suggest that cobalamins' are genuine isomers rather than just monocarboxylic acids, resulting from hydrolysis of one of the three propionamide side chains. For instance, if we assume hydrolysis of a propionamide side chain, then the interconversions between b-, d-, and e-monocarboxylic acids can be interpreted in terms of intramolecular exchange of $-NH_2$ and $-OH$ groups between the side chains. This is highly unlikely as the side chains are separated by relatively large distances and

have a benzimidazole ring in-between [18]. The alternative mechanism involving bimolecular collisions may be considered. According to it, the reversibility, the back-conversions of cyanocobalamin′ to cyanocobalamin, and the interconversions between b-, d-, and e-cyanocobalamins′ may be interpreted in terms of a direct exchange between $-NH_2$ and $-OH$ groups during a bimolecular collision (as the $-NH_2$ radical cannot survive in acidic solutions). If the interconversions between b-, d-, and e-cyanocobalamins′ were to occur through such highly improbable bimolecular collisions with specific orientations, then one would expect formation of large amounts of the intermediates, cyanocobalamin and dicarboxylic acid of $B_{12}$. In our experiments, less than 1% of $B_{12}$ was observed. Moreover, one cannot expect such large extent of conversions (~60%) on the basis of this mechanism. Neither does the amount of multiple charged species (presumably resulting from hydrolysis of propionamide side chain(s) of $B_{12}$′) formed (~15%) bear any relation to the yield of the desired product. Therefore, the bimolecular exchange mechanism is definitely untenable.

The X-ray and neutron diffraction studies of the b-cyanocobalamin′ (designated as 'b-monocarboxylic acid' of cyanocobalamin in the literature) do not permit definitive conclusions one way or the other [17–19]. For such large molecules, carboxyl and amide groups are not readily distinguished by X-ray and neutron diffraction methods. Moreover, there is some disorder in some of the propionide side chains. However, these studies as well as our earlier Mössbauer, proton and phosphorus-31 NMR studies [2–5] clearly indicate changes in the puckering of the corrin ring, which in turn brings about conformational changes in other parts of the molecule. The carbon-13 NMR studies [13–16] of b-, d-, and e-cyanocobalamins′ show marked changes in the corresponding propionamide side chains, as well as in other parts of the molecule including the corrin ring. Further work is required to elucidate the nature of the changes occurring on the side chains. Toraya *et al.* [21] have proposed a role for the peripheral side chains of cobalamins in enzymatic reactions.

It is reported in the literature [20, 21] that methyl–amide and other groups can be attached to the side chain believed to be carboxylic acid. On the other hand, our work strongly indicates that there is no carboxylic acid group present; then the question arises as to the site of the actual attachment. Further investigation is called for.

The biological ramifications of isomerization of cobalamins are rather baffling. Hydroxocobalamin is present in fairly large proportions both in foods and in the human body and it happens to be the $B_{12}$ analog which isomerizes most readily.

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