THE VITAMIN B\textsubscript{12} COENZYME

D. DOLPHIN, A. W. JOHNSON, R. RODRIGO and N. SHAW

Department of Chemistry, University of Nottingham, U.K.

INTRODUCTION

In 1858 Barker and his associates\textsuperscript{1−3} recognized a new coenzyme which controlled the conversion of glutamate into β-methylaspartate by \textit{Clostridium tetanomorphum}. The coenzyme was shown\textsuperscript{4} to be related to ψ-vitamin B\textsubscript{12}, \textit{i.e.} containing an adenine nucleotide grouping in place of the 5,6-dimethylbenzimidazole nucleotide of vitamin B\textsubscript{12}, although similar coenzymes containing benzimidazole or 5,6-dimethylbenzimidazole were produced by growing \textit{C. tetanomorphum} in the presence of the appropriate base\textsuperscript{5}. Other variations of the nucleotide base have been achieved using \textit{Propionibacterium arabinosum} in the presence of other purines and benzimidazoles\textsuperscript{6}. The presence of the coenzymes in a wide variety of micro-organisms such as several species of Actinomycetes including \textit{Streptomyces olivaceus} and \textit{S. griseus} has been demonstrated by the glutamate isomerase assay\textsuperscript{7} or by isolation. It appears that vitamin B\textsubscript{12} and its analogues are always biosynthesized in the form of their coenzymes. Preliminary physical and chemical studies suggested that in the 5,6-dimethylbenzimidazolyl cobamide coenzyme the cyanide group of vitamin B\textsubscript{12}, cyanocobalamin, was replaced by an adenine nucleoside\textsuperscript{1−5,8} and the determination\textsuperscript{9} of the complete structure (I; \( R = 5'\)-deoxyadenosyl) of the coenzyme by X-ray analysis revealed the existence of an essentially covalent bond between the cobalt atom and the 5′-carbon atom of the additional 5′-deoxyadenosine group. The molecule

\[
\text{In the vitamin B}_{12} \text{ coenzyme}
\]

\[
R = 5'\text{-deoxyadenosyl}
\]

![Chemical Structure](image-url)
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is thus an organometallic compound and is the first naturally occurring compound containing a metal–carbon bond.

It is presumed that the adeninyl and benziminazolyl cobamide coenzymes have similar structures apart from the nature of the nucleotide base. In all of these compounds the cobalt–carbon bond is readily broken and several methods, including irradiation of aqueous solutions, exist for replacing the 5'-deoxyadenosyl group by hydroxo (i.e. formation of vitamin B₁₂₈, and the action of cyanide on the coenzymes in the absence of light gives cyanocobalamin (initially in its dicyano form). These reactions will be discussed below in greater detail.

PARTIAL SYNTHESIS

A partial synthesis of the coenzyme was achieved¹⁰⁻¹³ by treating a reduced form of vitamin B₁₂₉ or B₁₂ itself with 5'-tosyl-2', 3'-O-isopropylideneadenosine¹⁴ followed by hydrolytic removal of the isopropylidene grouping. Sodium borohydride, zinc and acetic acid, or chromous acetate were suitable reducing agents and appeared to convert vitamin B₁₂₉ to a grey-green reduction product, the so-called vitamin B₁₂₈, which under acid or alkaline conditions was found to react with a variety of alkylating agents to give good yields of coenzyme analogues. The product from the reaction with 5'-tosyl-2',3'-isopropylidene-adenosine, after hydrolytic removal of the isopropylidene group and purification was identical with the 5,6-dimethylbenziminazolyl cobamide coenzyme (henceforward referred to as the vitamin B₁₂ coenzyme) both in physical, chemical and biological properties (for example, the enzyme assay of Abeles and Lee¹⁵). Analogous experiments using 2',3'-O-isopropylidene-5'-tosylinosine or 2',3'-O-isopropylidene-5'-tosyluridine yielded crystalline products having physical and chemical properties similar to those of the coenzyme (I), but containing hypoxanthine and uracil residues respectively in place of adenine. In later experiments it was found that protection of the 2',3'-hydroxy groups of the sugar was not essential.

A wide range of alkyl coenzyme analogues has been prepared from reduced hydroxocobalamin using alkyl halides, dimethyl sulphate and triethyl phosphate as alkylating agents. Reactions with ethylene bromohydrin, chloroacetic acid, and acetyl chloride or acetic anhydride also gave related products (II; R = CH₂·CH₃OH, CH₃·CO₂H or Ac respectively). It therefore seems that a wide variety of electrophilic reagents will react with the reduction product of hydroxocobalamin, steric conditions permitting, to give compounds of the B₁₂ coenzyme type. Bromobenzene and t-butyl bromide did not react to an appreciable extent, probably for steric reasons. The crystalline alkyl–cobalt derivatives were stable at room temperature in the absence of strong light. They are almost the first representatives of this class of compound to be made, although alkyl-cobalt intermediates probably have a transient existence in the Fischer-Tropsch synthesis¹⁶.

An understanding of the reaction mechanism involved in the above alklylation reactions clearly requires a knowledge of the constitution of the reduction products of vitamin B₁₂₉, hydroxocobalamin. It is agreed generally that the cobalamins are complexes of trivalent cobalt¹⁷⁻²⁰. Intermediate between the cobalamins and vitamin B₁₂₈ is the vitamin B₁₂₉²¹⁻²³ which has
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been variously stated to contain divalent$^{22-24}$ and monovalent$^{25}$ cobalt. We regard vitamin $B_{12r}$ as a derivative of divalent cobalt on the basis of the quantitative hydrogenation of hydroxocobalamin in the presence of platinum when 0.5 mol of hydrogen is absorbed during the formation of vitamin $B_{12r}$:

$$Co^{III} + \frac{1}{2}H_2 \rightleftharpoons Co^{II} + H^+.$$  

Vitamin $B_{12s}$ is believed to be a cobalt hydride$^{11, 13, 25, 26}$ and as such it can be added$^{11, 26}$ to various acetylenes or activated olefins to yield the same cobalt-alkyl compounds as are obtained by substitution reactions from the hydride and alkyl halides e.g.

\[
\begin{align*}
&\text{H} \\
&\text{Co} + \text{BrCH}_2\text{CH}_2\text{CO}_2\text{Me} \quad \text{CH}_2\text{CH}_2\text{CO}_2\text{Me} \\
&\text{H} \quad \text{Co} + \text{CH}_2\text{N}_2 \quad \text{CH}_2 \quad \text{Co} + \text{CH}_2\text{I} \\
&\text{Co} + \text{CH}_2\text{CH}\cdot\text{CO}_2\text{Me} \\
&\text{Co} + \text{CH}_2\text{CH}\cdot\text{CO}_2\text{Me}
\end{align*}
\]

Treatment of vitamin $B_{12s}$ with cyanogen bromide yields$^{27}$ cyanocobalamin, vitamin $B_{12}$. The acidity of the hydride is emphasized by its reaction with diazomethane to give the methyl analogue$^{11, 23}$ and these reactions of vitamin $B_{12}$, suggest that the product can best be formulated as an equilibrium:

$$\text{H}^- \quad \text{H}^+$$

\[\text{Co}^{III} \rightleftharpoons \text{Co}^{I}\]

Moreover, we have found$^{28}$ that quantitative oxidation of vitamin $B_{12s}$ to $B_{12b}$ requires 0.5 mol of oxygen:

\[
\begin{align*}
&H^+ \\
&\text{Co}^{I} + \frac{1}{2}\text{O}_2 \quad \text{OH}_2 \\
&\text{H}_2\text{O} \quad \downarrow \\
&\text{Co}^{III} + \text{OH}^-
\end{align*}
\]

BIOSYNTHESIS

The cobalt-hydride intermediate, vitamin $B_{12s}$, does not appear to be involved in the biological synthesis of the vitamin $B_{12}$ coenzymes. Cell-free extracts of Propionibacterium shermanii or Clostridium tetanomorphum, for example, required to be supplemented with adenosine triphosphate, a sulphhydryl compound, such as glutathione or $\beta$-mercaptoethanol, reduced flavin and manganese ions. By the use of isotope-labelled adenosine triphosphate (ATP) it was shown$^{29, 30}$ that both the adenine and sugar fragments of the 5'-deoxyadenosyl group of the coenzyme were derived from ATP. Although the precise mechanism of this conversion was not established it is clear that a reduction step is involved and in this sense it parallels the purely chemical synthesis although the cobalt-hydride intermediate is probably not concerned in the biosynthesis.

In an examination of the reaction of hydroxocobalamin with various thiols we have recently shown$^{31}$ that violet coloured products are initially formed and that these may be transformed to brown compounds. The
spectra of solutions of the brown products closely resembles that of vitamin B_{12}^{22, 23} and it is believed that they contain cobalt–sulphur bonds and are formed from the violet compounds by reduction. The reaction of hydroxocobalamin with sodium hydrogen sulphide was examined in detail and the brown product, unlike the violet intermediate, was found to react very rapidly with methyl iodide to yield methylcobalamin in the absence of light. When the solution was photolyzed the methylcobalamin was converted back to hydroxocobalamin and in the presence of excess sulphide and methyl iodide the cycle could be repeated many times.

\[
\begin{align*}
\text{OH}_2 & \quad \xrightarrow{h\nu} \quad \text{SR} \\
\text{Co}^{\text{III}} & \quad \xrightarrow{\text{RSH}} \quad \text{Co}^{\text{II}} & \quad \xrightarrow{\text{Mel}} & \quad \text{Me} \\
& & & \quad \xrightarrow{\text{Co}^{\text{II}}} \quad \text{Me}
\end{align*}
\]

The displacement of the sulphur ligand by an alkyl group is more susceptible to steric hindrance than the corresponding hydride displacement although many examples have been provided. With ethyl iodide for example the reaction is markedly slower than with methyl iodide and the formation of the brown intermediate (spectrum) can be clearly observed. These reactions appear to be analogous to the biological syntheses of the coenzymes although the exact parallel of the biosynthesis, i.e. the use of ATP as alkylating agent, does not appear to operate under *in vitro* conditions.

**PROPERTIES AND REACTIONS OF THE COENZYMES AND THEIR ALKYL ANALOGUES**

Although the results of magnetic measurements on the coenzyme reported from different schools are at variance\textsuperscript{22}, the observed chemical reactions of the coenzyme are best interpreted by regarding them as complexes of trivalent cobalt. Quantitative hydrogenation of the methyl analogue in the presence of platinum resulted in the formation of vitamin B_{12r} and an increase in volume of 0.5 mol caused by the liberation of methane\textsuperscript{28}. The methane was identified by gas–liquid chromatography.

\[
\begin{align*}
\text{CH}_3 & \quad \text{OH}_2 \\
\text{Co} + \frac{1}{2}\text{H}_2 & \quad \xrightarrow{\text{H}_2\text{O}} \quad \text{Co} + \text{CH}_4
\end{align*}
\]

The coenzyme itself cannot be hydrogenated in a similar manner probably because the increased size of the nucleoside substituent renders the cobalt atom more inaccessible to the reducing agent.

**PHOTOLYSIS OF THE COENZYMES AND THEIR ALKYL ANALOGUES**

In the absence of oxygen (10^{-6} mm) the alkyl coenzyme analogues are stable in light of moderate intensity; in the presence of small quantities of air however (ca. 10^{-4} mm), photolysis occurs giving vitamin B_{12r} and a mixture of olefins and paraffins. Finally, in presence of excess oxygen,
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vitamin B₁₂ is oxidized to vitamin B₁₂₃ and the radical undergoes some oxidation to the corresponding aldehyde²⁸. The photolytic fission is regarded as a homolytic process although evidence other than the nature of the products is still lacking.

\[
\begin{align*}
    \text{Et} & \quad \text{hv} \quad \text{O}_2 \\
    \text{Co}^{\text{III}} & \quad \rightarrow \quad \text{Co}^{\text{II}} + [\text{Et}'] \quad \rightarrow \quad \text{C}_2\text{H}_4, \quad \text{(but no appreciable amounts of C}_4\text{H}_{10} \text{ or C}_2\text{H}_6) \\
    \text{Excess O}_2 & \quad \rightarrow \quad \text{Co}^{\text{III}} + [\text{Et}'] \quad \rightarrow \quad \text{CH}_3\cdot\text{CHO}
\end{align*}
\]

In the case of the coenzyme itself the reactions are analogous except that photolysis occurs even at \(10^{-6}\) mm when the intermediate free radical undergoes cyclization to the so-called nucleoside³³ A (II), while in presence of oxygen the free radical is oxidized to the corresponding acid³² (III) or aldehyde³⁴ (Figure 1).

![Diagram of reaction](image)

*Figure 1*

When the photolytical decompositions are carried out in presence of a radical acceptor such as a thiol the radical is captured before the transformations described above can occur³⁵ and similar enzymic controlled reactions have also been reported³⁶. Thus photolysis of the methyl coenzyme analogue in presence of either cysteine or homocysteine yields S-methylcysteine or methionine respectively. A similar reaction of the vitamin B₁₂ coenzyme in presence of homocysteine gave S-adenosylhomocysteine (IV), a reaction of significance in the biogenesis of active methionine. It was not found possible to transfer the adenosyl group to methionine itself (Figure 2).
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\[
\begin{align*}
\text{HO} & \quad \text{OH} \\
\text{CH}_2\text{CH} & \quad \text{O} \\
\text{Co}^{\text{III}} & \quad \text{O} \\
\text{CH} & \quad \text{N} \\
\text{N} & \quad \text{NH}_2 \\
\end{align*}
\]

\(+\) \quad \text{HS}\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{CH(NH}_2\cdot\text{CO}_2\text{H}

\[
\begin{align*}
\text{OH} & \quad \text{OH} \\
\text{Co}^{\text{II}} & \quad \text{OH}_2\cdot\text{CH(NH}_2\cdot\text{CH}_2\cdot\text{CH}_2\cdot\text{S}\cdot\text{CH}_2\cdot\text{CH} & \quad \text{O} \\
\text{N} & \quad \text{NH}_2 \\
\end{align*}
\]

\text{(IV)}

\text{Figure 2}

REACTION WITH ACIDS

A striking difference between cyano- and hydroxo-cobalamin and the coenzymes or their alkyl analogues is the relative ease of protonation of the latter compounds. This reaction is characterized by a red to yellow colour shift and a consequent major change in the absorption spectrum$^{25, 37}$. Although dilute mineral acids will suffice to convert the coenzyme series to the corresponding conjugate acids, the cobalamin normally require concentrated sulphuric or perchloric acids to bring about this change. Analysis of the spectra of the yellow protonated forms shows that the bond between the nucleotide and the metal has been broken and that a proton is located on the nucleotide base. However, this displacement of the nucleotide base is in itself not sufficient to account for the marked colour change, and the observed red to yellow colour change at pK 3.3 probably involves an addition of a proton to the chromophoric system. According to Williams

\[
\begin{align*}
\text{R} & \quad \text{Co}^{10} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{N} & \quad \text{N} \\
\text{Me} & \quad \text{Me} \\
\text{Me} & \quad \text{Me} \\
\end{align*}
\]

\(\xrightarrow{2\text{H}^+, \text{H}_2\text{O}}\)

\(\xleftarrow{2\text{OH}^-}\)

\text{(V)}

\text{Figure 3}

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and his co-workers⁵⁵, the meso position, C₁₀ in the chromophore is a likely site for this addition to occur (Figure 3).

The higher electron density at C₁₀ in the coenzymes and their alkyl analogues as compared with the cobalamins correlates with the observed course of reaction with chlorine (see below). An estimation of the pKₐ values of a series of alkyl coenzyme analogues has shown a relation between the observed values and the inductive effect of the alkyl group (Table 1). This suggests that the reactivity at C₁₀ is to some extent controlled by the nature of the substituent R (V; R = alkyl, CN, H₂O etc.) which recalls the phenomenon of perpendicular conjugation³⁸.

<table>
<thead>
<tr>
<th>Nature of cobalt–alkyl group (R in V)</th>
<th>pKₐ</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃</td>
<td>2.7</td>
</tr>
<tr>
<td>n-C₆H₁₄</td>
<td>3.93</td>
</tr>
<tr>
<td>n-C₅H₁₀</td>
<td>4.01</td>
</tr>
<tr>
<td>Coenzyme</td>
<td>3.52</td>
</tr>
<tr>
<td>2',3'-Isopropylidene-coenzyme</td>
<td>2.94</td>
</tr>
<tr>
<td>HO₂C·CH₃</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Free adenine is released from the 5'-deoxyadenosyl group of the coenzymes by the action of 0.1 N hydrochloric acid at 100° for 90 minutes⁵⁵, ⁸, ³². In the same reaction vitamin B₁₂b is formed and the sugar is liberated as d-erythro-2,3-dihydroxy-pent-4-enal (VI), formed by an elimination reaction³² (Figure 4):

![Image of chemical structure](image)

**Figure 4**

The structure of (VI) was determined by Hogenkamp and Barker⁵⁹ and confirmed³² by a synthesis of erythro-pent-4-en-1,2,3-triol, the racemic form of the product of sodium borohydride reduction of the sugar:

![Image of chemical structure](image)

(as monoformate)
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REACTION WITH CYANIDE

Vitamin B$_{12h}$, hydroxocobalamin, as well as its coenzyme, react readily with cyanide when the nucleotide base is detached from the cobalt in an elimination reaction similar to that brought about by hot dilute acid (above). The products formed from the coenzyme with cyanide$^8$, $^{32}$, $^{39}$ are dicyano-cobalamin$^{40}$ (VII), the cyanhydrin of (VI) and free adenine (Figure 5).

\[ \text{Figure 5} \]

On the other hand, the simple alkyl coenzyme analogues do not react with aqueous potassium cyanide when light is excluded. A slow displacement reaction occurs with hydrogen cyanide and it therefore seems that an initial protonation of the nucleotide base facilitates the substitution reaction.

CYCLIZATIONS OF THE RING B ACETAMIDE SUBSTITUENT

Of the other known reactions of the cobalamins the cyclizations of the ring B acetamide substituent$^{41}$ are of special interest. The action of one equivalent of chlorine (as chloramine T) on cyanocobalamin causes the formation of a stable fused lactone (VIII; R = H) and only the further action of chlorine brings about substitution, possibly at $C_{10}$, with the formation of a chlorolactone (VIII; R = Cl) (Figure 6).

\[ \text{Figure 6} \]

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The reaction of the methyl coenzyme analogue took a different course and the first equivalent of chloramine T caused substitution without cyclization and the formation of a 10(?)-monochloro compound. However the reaction of excess chloramine T caused the formation of a monochloro-lactone of the cobalt–methyl derivative, revealed by its characteristic infrared absorption at 1778 cm<sup>-1</sup> and by its electrophoretic behaviour. Photolysis of the product in presence of air caused the formation of the corresponding hydroxocobalamin derivative. This with cyanide gave the same chlorolactone (VIII; R = Cl) as had been obtained previously<sup>41</sup> by treatment of cyanocobalamin with excess chlorine (Figure 7).

![Chemical diagrams showing reactions of cyanocobalamin](https://example.com/figure7)

The cyclization of the ring B acetamide side chain of cyanocobalamin to a lactam<sup>41</sup> is not paralleled in the coenzyme series. Certain of the results of the chlorination experiments were originally interpreted<sup>42</sup> as supporting a theory that the chromophore of the coenzyme contained one double bond less (I with C<sub>9-10</sub> bond saturated) than that of the vitamin. However this theory has been rejected<sup>43, 44</sup> and the coenzymes and their alkyl analogues are regarded as containing the normal cobalamin chromophore.

There thus exists a large series of cobalamins varying from vitamin B<sub>12</sub> (I; R = CN) to the alkylcobalamins (I; R = alkyl) including the coenzyme, and the properties of a particular cobalamin depends markedly on the nature of the substituent R. Other partially synthetic cobalamins are known which show properties intermediate between these extremes. For example, sulphotocobalamin<sup>25, 48</sup>, which is converted to hydroxocobalamin by aerobic photolysis, is readily protonated by dilute acid and behaves like methylcobalamin on chlorination with chloramine T, i.e. substitution into the chromophore occurs before the cyclization of the acetamide side chain.

BIOCHEMICAL FUNCTION

As mentioned above, the vitamin B<sub>12</sub> coenzymes were discovered through their ability to bring about the conversion of glutamate into β-methylaspartate<sup>1, 2, 8</sup>. Apart from this reaction they also appear to be involved in

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the biological interconversion of C-methylmalonate and succinate, and of propane-1,2-diol and propionaldehyde, and a number of related reactions, e.g. the interconversion of glycerol and β-hydroxypropionaldehyde. The precise mechanism of these reactions has not yet been established, although deuterium studies have shown that the rearrangements are intramolecular and stereospecific. An early free radical mechanistic theory for the rearrangement of C-methylmalonate to succinate has now been abandoned. The recent demonstration of the participation of compounds of the vitamin B₁₂ coenzyme type in methane production is also of interest.

It is a pleasure to acknowledge the friendly collaboration we have enjoyed throughout this work with Dr E. Lester Smith, F.R.S. and Dr L. Mervyn of Glaxo Laboratories Ltd.

References

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50 F. Lynen. Private communication.