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KINETIC AND MECHANISTIC STUDY OF REACTION OF CYANOCOBALAMIN WITH SULFITE

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Kinetics of reaction between cyanocobalamin and sulfite was studied in acidic medium. It was shown that sulfite is capable of replacing cyanide with the formation of sulfitocobalamin. Acids were found to catalyse the reaction. Associative mechanism of reaction was proposed. Method of preparation of sulfito- and aquacobalamin from cyanocobalamin was developed.

Key words: cobalamin, sulfite, cyanide, kinetics.

INTRODUCTION

Adenosyl- (AdoCbl) and methylcobalamin (MeCbl) are biologically active forms of vitamin B_{12}. In mammals, MeCbl takes part in the synthesis of methionine from homocysteine and AdoCbl provides isomerisation of methylmalonyl-CoA to succinyl-CoA (Brown, 2005; Gruber et al., 2011). The deficiency of vitamin B_{12} in humans causes various diseases such as megaloblastic anemia and neurological malfunctions (Ruiz-Sánchez et al., 2008). For the treatment of these states, cyanocobalamin (CNCbl) is usually used.

Formation of enzymaticaly active Cbl species requires removal of bound CN^-, exhibiting extremely challenging task due to very high cyanide affinity to Cbl(III) ($K \sim 10^{12}$ M^{-1} (George et al., 1960)). Cyanide removal can occur by several ways. Reductive pathway is implemented in cycling of CblC Cbl-trafficking protein (Kim et al., 2008) consuming glutathione (GSH) as a source of electrons to give Cbl(II) and CN^- (Cbl(II) binds CN^- very loosely (Lexa et al., 1980)). Moreover, it was shown that reaction of CNCbl with sulfurcontaining reductants (viz., dithionite, hydroxymethanesulfinate) gives Co(II)-species and free cyanide (Salnikov et al., 2013). CN^- can be substituted by GSH giving glutathionylcobalamin (GSCbl(III)) (Pezacka et al., 1990). Jensen and Halpern (Jensen, Halpern, 1999) used SO_2 for the cyanide removal. Cobalamin species (viz., Cbl(III) (Firth et al., 1969) and Cbl(I) (Dereven'kov et al., 2013)) are known to be reactive toward sulfite. However,

mechanism of the reaction between cyanocobalamin and SO_2 has not been fully investigated. To elucidate mechanism of this interaction, kinetics of reaction between CNCbl and sulfite was studied in this work.

MATERIALS AND METHODS

Materials

Cyanocobalamin and sodium sulfite were purchased from Sigma-Aldrich and used as received. Other chemicals used throughout this study were of reagent grade. All solutions were prepared from distilled water. Concentrations of Cbl were found by means of conversion to dicyano form (ε_{367} = 30400 M^{-1} cm^{-1}) (Barker et al., 1960).

Kinetic measurements

Conventional kinetic experiments were performed on a Cary 50 UV-vis spectrophotometer under aerobic conditions. Concentrated buffer solutions were used to control pH of the solutions. The data were analyzed using Origin 7.5. Kinetic traces were usually monitored at 361 and 550 nm. Most experiments were done in duplicate and reported rate constants are the average of at least four kinetic runs recorded at different wavelengths. The reported errors represent the standard deviation of the results.

RESULTS AND DISCUSSION

UV-vis studies of reaction between CNCbl and S(IV)

It is known (van Eldik, Harris, 1980; Brandt, van Eldik, 1995; Schmidt, 1972) that the following equilibria exist in aqueous solutions of sulfite (abbreviated as "S(IV)-species"; reactions 1-3).

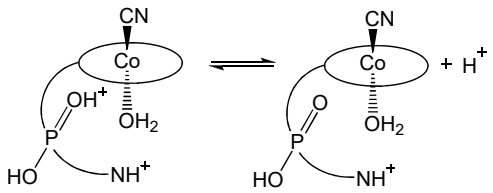
$$SO_2 \cdot H_2O \leftrightarrow HSO_3^- + H^+,\ pK_a = 1{,}90 \quad (1)$$

$$HSO_3^- \leftrightarrow SO_3^{2-} + H^+,\ pK_a = 6{,}3 \quad (2)$$

$$2HSO_3^- \leftrightarrow S_2O_5^{2-} + H_2O \quad (3)$$

Therefore, depending on pH, predominant forms of S(IV) are hydrated sulfur dioxide ($SO_2 \cdot H_2O$; pH < 1.5), mixture of bisulfite ions (HSO_3^- and SO_3H^-; pH = 1.5 – 6.5), sulfite (SO_3^{2-}; pH > 6.5). Bisulfite can be reversibly dimerized to pyrosulfite ($S_2O_5^{2-}$; reaction 3).

It was found that acid-base properties of CNCbl are characterized by three macroscopic constants: pK_{a1} = -1.57; pK_{a2} = -0.04 and $pK_{a\ base\text{-}off}$ = 0.11 (reactions 6-8) (Brown, Hakimi, 1984).



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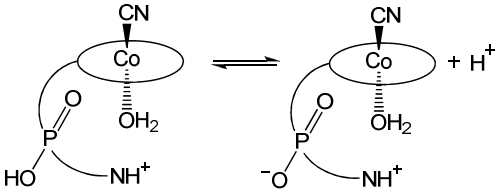

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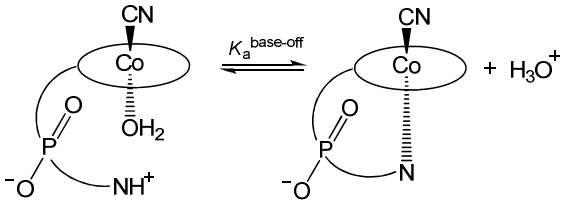

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However, there is no data on the UV-vis spectra of different forms of CNCbl as well as on the effect of anions generated during acid dissociation on cyanocobalamin. By this reason we investigated the influence of acids (HCl, $HClO_4$, H_3PO_4) and their concentrations on UV-vis spectrum of CNCbl (Fig. 1).

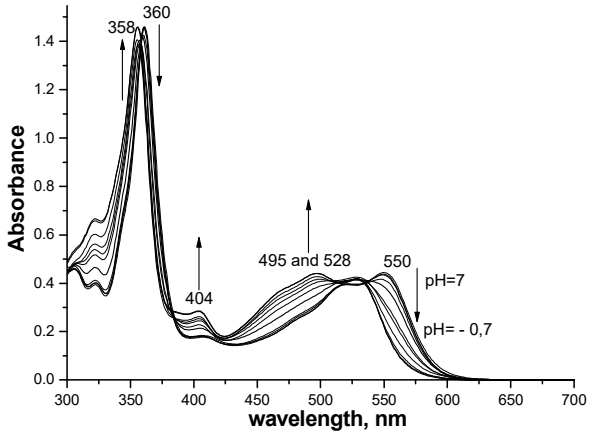


Fig. 1. UV-vis spectra of CNCbl vs [HCl]. [CNCbl] = 5×10^{-5} M; [HCl] = 0.01 - 5.0 M; 20°C.

During the titration of CNCbl solution by HCl, absorption decrease at 360 and 550 nm is observed as well as an intensity of bands at 358, 404 and 528 nm is increased. It is important to note that there are no isosbestic points in UV-vis spectra. This fact could be explained by the presence of several protolytic equilibria in acidic solutions of CNCbl (equations 6-8) that can be overlapped.

Dependencies of absorbance at 550 nm on Hammett acidity function (H_0) for different types of acids (Fig. 2) were plotted. The recalculation of acids concentration in Hammett acidity function was made using data given in paper (Paul, Long, 1957).

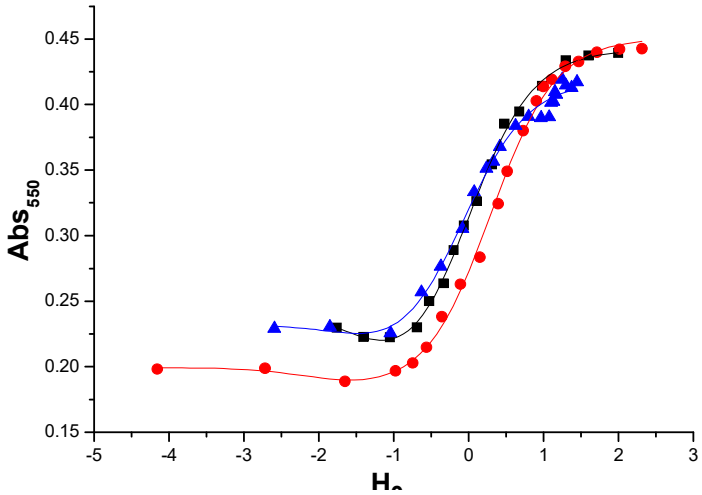


Fig. 2. Dependence of the absorbance of CNCbl at 550 nm on H_0. [CNCbl] = 5×10^{-5} M; HCl (■), $HClO_4$ (●), H_3PO_4 (▲); 20°C.

From equations 6-8 one can see that CNCbl is a tribasic acid. However, the equation for dibasic acid with close ionization constants (Bernstein, Kaminskii, 1986) allows carrying out the most accurate analysis of the obtained dependences. Using it the protolytic equilibrium constants of CNCbl are calculated (Table 1). The use of the equation for tribasic acid dissociation does not enable to calculate three pK_a.

Based on data given in Table 1 and Fig. 2 the following conclusions can be made. First, the type of the acid and anions does not affect acid-base properties of CNCbl. Second, the macroscopic ionisation constants for the reaction (6) can be found from changes in UV-vis spectra only. Besides, on the basis of the spectra it is impossible to distinguish processes (7) and (8) as well as find their pK_a.

Table 1

The protolytic equilibrium constants of CNCbl

Acid	pK_{a1}	pK_{a2}
HCl	-1.35 ± 0.41	0.01 ± 0.04
$HClO_4$	-2.02 ± 0.79	0.30 ± 0.03
H_3PO_4	-1.45 ± 0.89	-0.06 ± 0.06
Mean value of pK_{ai}	-1.61 ± 0.90	0.082 ± 0.47

Preliminary experiments showed that the interaction of CNCbl with S(IV) proceeds at pH < 7. Addition of S(IV) to CNCbl solution results in slow change in UV-vis spectrum. However, spectral changes depend on pH chosen. At pH 4.5 a decrease in absorbance occurs at the 360 and 550 nm, as well as its increase at 311, 418, 517 and 541 nm was found. Isosbestic points are observed at 346 and 545 nm. A colour of the solution changes from red to pale red. The final spectrum of the reaction product (Fig. 3, spectrum 2) is fully identical to that of sulfitocobalamin (Chemaly, 2008). At pH 0.5 decrease of absorbance at 360 and 550 nm is also observed, but UV-vis spectrum includes new maximum at 420 nm; colour of the solution is yellow (Fig. 3, spectrum 3). Latter spectrum is also identical to that of sulfitocobalamin at this pH (Firth et al., 1969).

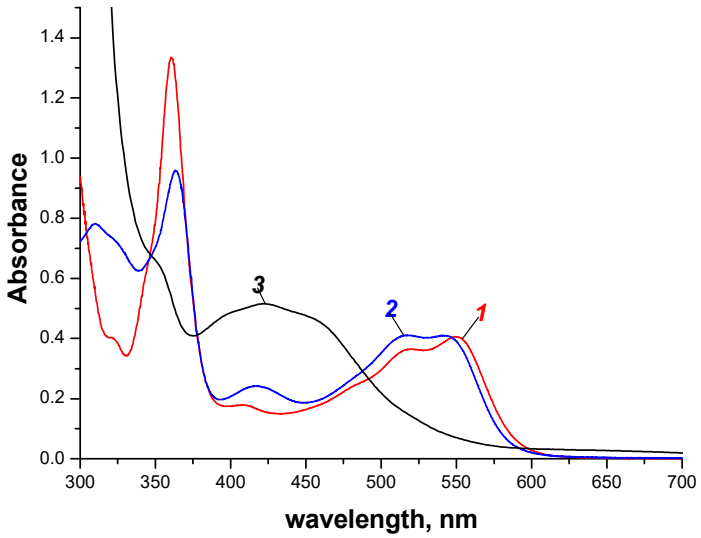
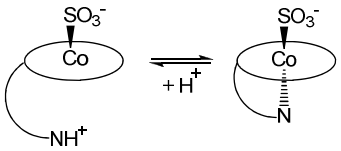


Fig. 3. UV-vis spectra of Cbls: (1) – CNCbl at pH 7; (2, 3) – products of the reaction CNCbl with S(IV) at pH 4.5 and pH 2.0, correspondingly; [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.01 M; [Buffer] = 0.1 M; 20°C.

Difference in sulfitocobalamin UV-vis spectra at various pH values can be explained by the presence of the acid-base equilibrium between "base-on" and "base-off" forms (reaction 9) with $pK_a = 2$ (Firth et al., 1969).



(9)

In our case the dependence of absorbance at 543 nm of the final product on pH is a typical S-shaped curve (Fig. 4). This fact indicates the presence of the acid-base equilibrium between the reaction products. Analysis of the dependence using equation for monobasic acid gives $pK_a = 2.0$.

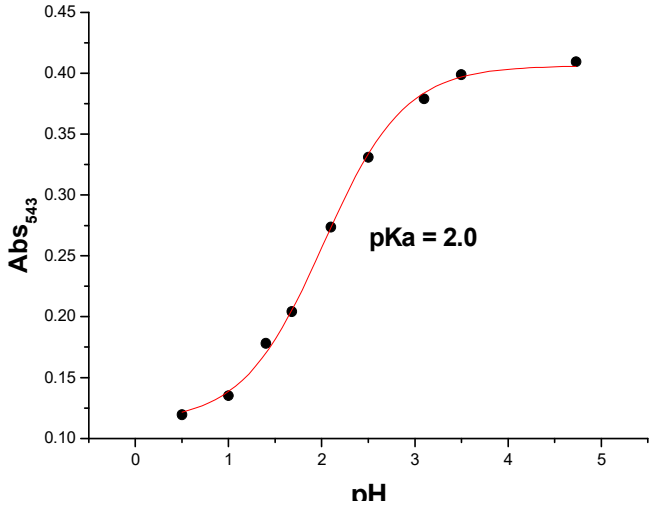


Fig. 4. Dependence of absorbance at 543 nm on pH. [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.01 M; 20°C.

Latter shows that the interaction of CNCbl with sulfite proceeds throughout the substitution of cyanide by S(IV) with the formation of sulfitocobalamin, which exists in "base-off" and "base-on" forms depending on pH.

In order to investigate the mechanism of the reaction, kinetic study was carried out.

Kinetic Study

The kinetics of the interaction between CNCbl and S(IV) was studied under the conditions of pseudo-first order at the excess of S(IV) and wavelengths of 360 and 550 nm. In Fig. 5 the typical kinetic curve for this reaction is shown. Latter can be linearized in coordinates ln(A-A∞) – time (Fig. 6). On the basis of obtained data, the observed rate constants ($k_{obs.}$) for the first-order reactions were calculated.

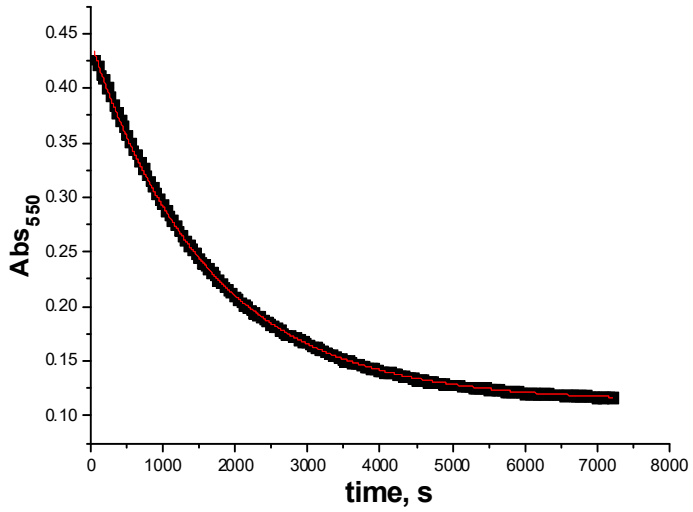


Fig. 5. Plot of absorbance at 356 nm vs time fitted to a first-order rate equation (k_{obs}=(6.14 ± 0.01)×10^{-4} s^{-1}). [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.035 M; [HCl] = 0.125 M; 20°C.

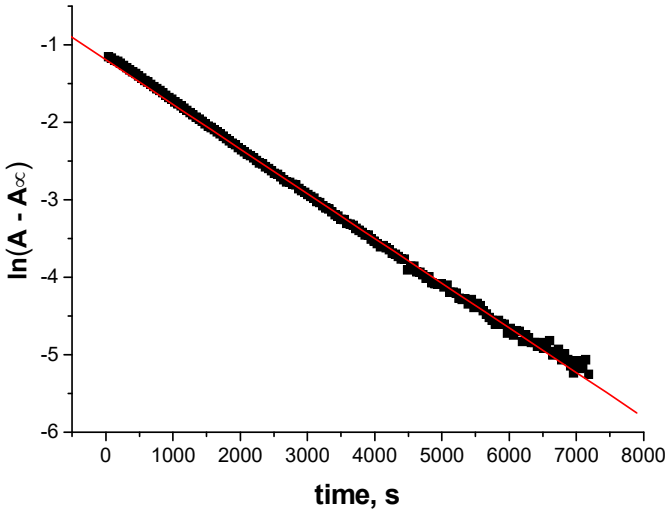


Fig. 6. Plot of ln(A-A∞) vs time for the reaction between CNCbl and S(IV). [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.035 M; [HCl] = 0.125 M; 20°C.

The dependence of observed rate constants on [S(IV)] is in Fig. 7. The plot is linear and passes the origin showing that protonated forms of sulfite takes part in reaction but not pyrosulfite (see reaction 3).

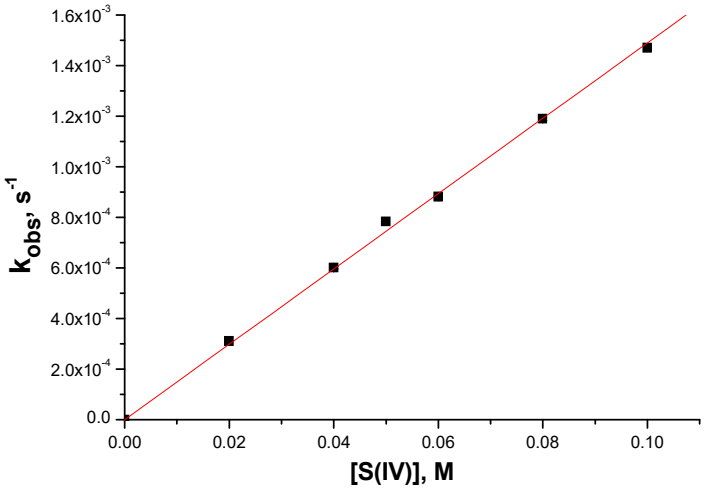


Fig. 7. Dependence of $k_{obs.}$ for the reaction of CNCbl with S(IV) on [S(IV)]. [CNCbl] = 5×10^{-5} M; [HCl] = 0.2 M; 20°C.

The influence of acids concentration on $k_{obs.}$ was studied. It was found that the dependence of the reaction rate is quite complicated. In slightly acidic medium $k_{obs.}$ linearly depends on buffer concentration (Fig. 8).

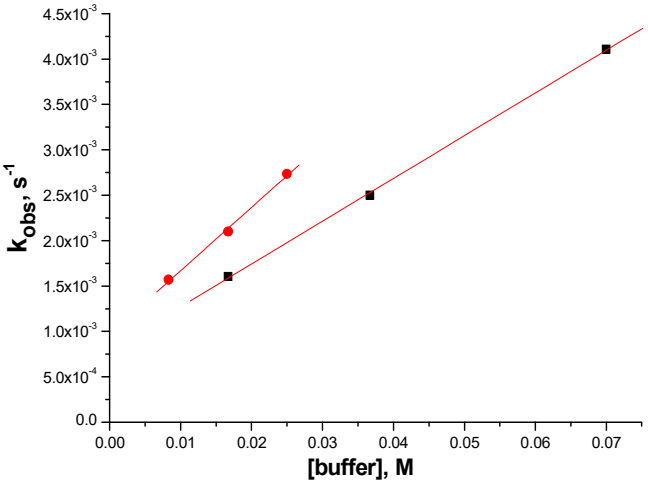


Fig. 8. Dependence of $k_{obs.}$ on buffer concentration. [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.1 M; citric buffer (■); acetic buffer (●); 20°C; pH 3.

With the increase in concentration of strong acids the reaction rate also is increased in linear manner (Fig. 9).

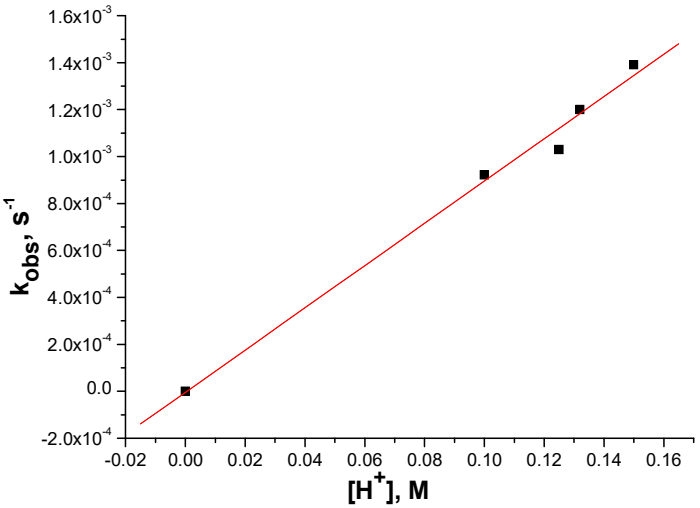


Fig. 9. Dependence of the observed rate constant on $[H^+]$. [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.1 M; 20°C.

However, with further increase in concentrations of strong acids the reaction rate decreases and reaches zero (Fig. 10).

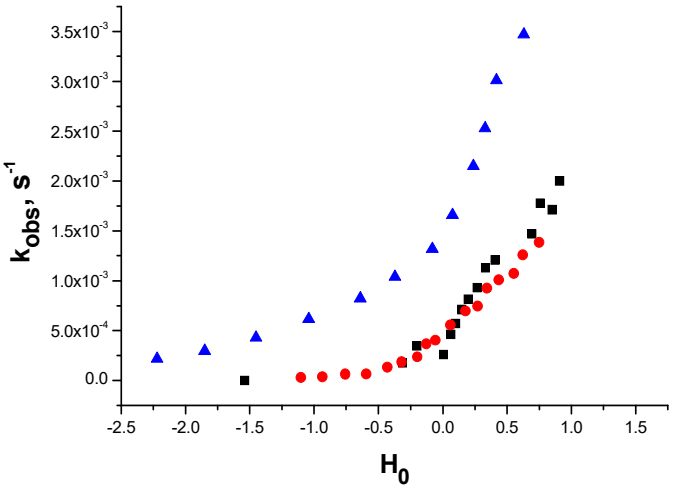


Fig. 10. Dependence of the observed rate constant on H_0. [CNCbl] = 5×10^{-5} M; [S(IV)] = 0.01 M; HCl (■), $HClO_4$ (●), H_3PO_4 (▲); 20°C.

The linear dependence of observed rate constants on buffer concentration in weakly acidic medium shows that the protonation of one of the reagents is a quite slow process. Sulfite reacts with proton very rapidly (reactions 1-3) (van Eldik,

Harris, 1980; Brandt, van Eldik, 1995). So protonation of CNCbl can be the rate determing step of the reaction. In this case, there are two possibilities of proton binding to cyanocobalamin. The first one is protonation of dimethylbenzimidazole with base-off form generation, the second one is protonation of coordinated cyanide (reaction 11).The first one is not possible, since "base-off" ↔ "base-on" interconvertion occurs readily. Cobinamide is known to bind cyanide better then cobalamin (Ma et al., 2012). Consequently one may expect that dimethylbenzimidazole removal increases constant of binding cyanide by cobalamin. Results presented in Fig. 10 supports this conclusion. One can see that the decrease in rate of the reaction between cyanocobalamin and S(IV) is observed in and around the values of pK_{a1}, pK_{a2} and $pK_{abase\text{-}off}$ of CNCbl. On the other hand, proton addition to cyanide would cause the bond weakening with cobalamin as it was with thiolate complexes (Schumacher et al., 2011). So the second case is the most possible one. Thus, the active form of metal complex in the reaction is base-on CNCbl.

From the data presented in Figs. 8 and 9 the third order constants were calculated: $k(H^+) = 0.09\ M^{-2}\cdot s^{-1}$ and $k(HA) = 0.7\ M^{-2}\cdot s^{-1}$ (for acetic buffer with pH 4.5). This fact shows that cyanocobalamin protonation by weak acids (reactions 12 and 13) proceeds better than by proton.

The activation parameters of the reaction were calculated from Eyring plot. They show that the reaction is characterized by associative mechanism (at pH 4.5 $\Delta H^{\neq} = +73 \pm 1\ kJ\cdot mol^{-1}$, $\Delta S^{\neq} = -71 \pm 1\ J\cdot K^{-1}\cdot mol^{-1}$ and at pH = 1 $\Delta H^{\neq} = +31 \pm 1\ kJ\cdot mol^{-1}$, $\Delta S^{\neq} = -212 \pm 9\ J\cdot K^{-1}\cdot mol^{-1}$).

On the basis of the published and obtained in experiments data the following scheme of sulfitocobalamin formation involving bisulfite anion (reaction with hydrated sulphur dioxide is not shown) can be proposed (reactions 10-14):

$$CNCbl + HSO_3^- \rightleftharpoons [SO_3HCNCbl]^- \quad (10)$$

$$CNCbl + H^+ \rightleftharpoons [HCNCbl]^+ \quad (11)$$

$$CNCbl + HA \rightleftharpoons [HCNCbl]^+ + A^- \quad (12)$$

$$[HCNCbl]^+ + HSO_3^- \rightleftharpoons [SO_3HCNCbl]^- + H^+ \quad (13)$$

$$[SO_3HCNCbl]^- \longrightarrow [SO_3Cbl]^- + HCN \quad (14)$$

Synthesis of aquacobalamin

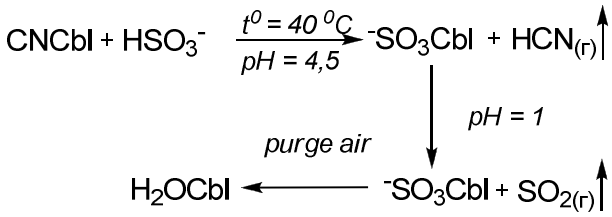
According to experimental data a new method of aquacobalamin (H_2OCbl) synthesis was suggested. It consists of following stages (scheme 1):

1. Hydrochloric acid was added to the aqueous solution of CNCbl to adjust pH ~4.5, then the excess of S(IV) (in the form of Na_2SO_3) was introduced. The

mixture was heated at 40°C for 2-3 h. Bisulfite ion generated in the solution replaces cyanide to give sulfitocobalamin (solution changes colour from red to dull-red). Hydrogen cyanide, which also formed, as a volatile liquid evaporated from the solution (t_b = 26-30°C).

2. Solution of HCl solution was added to sulfitocobalamin produced previously to reach pH ~1 in order to carry out the transformation of free bisulfite ion to SO_2 (see reaction 1).

3. Then air flow was passed through obtained solution in order to remove SO_2. At the end of SO_2 removal the colour of the solution changed from yellow to red. At the end of the process UV-vis spectrum of the product is to be recorded that should coincide with that of H_2OCbl. The solution was neutralized to pH 7. Cyanocobalamin formation was not observed; this fact meant total removal of cyanide from the solution. Further purification of cobalamin can be carried out according the procedure (Jensen, Halpern, 1999).



Scheme 1. Synthesis of aquacobalamin

CONCLUSIONS

Present work showed that in acidic media (pH 1-5) cyanocobalamin reacts with S(IV) leading to the formation of sulfitocobalamin. The active form of metal complex in reaction is base-on cyanocobalamin. The protonation of dimethylbenzimidazole results in inhibition of reaction. Weak acids serve as catalysts of the reaction. The reaction proceeds via associative mechanism without changing of oxidation state of cobalt ion in cobalamin molecule.

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