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Photolysis of cyanocobalamin in aqueous solution

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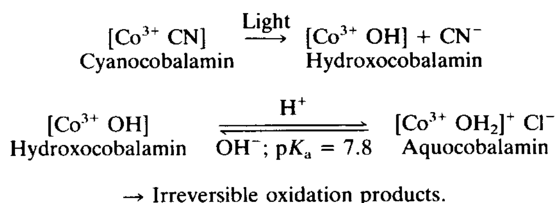
Abstract: Cyanocobalamin is photolysed in aqueous solution to produce hydroxocobalamin. The kinetics of photolysis has been studied at pH 1–12 using a newly developed spectrophotometric method for the simultaneous determination of the two compounds at 550 and 525 nm or 361 and 351 nm. Cyanocobalamin follows zero-order kinetics at the pH values studied and the rate is catalysed by both hydrogen and hydroxyl ions. The log k–pH profile indicates that cyanocobalamin has maximum stability near pH 7. The cationic species appears to be more susceptible to photolysis than the neutral form. The rate constant for the reaction at pH 1 is 1.32×10^{-7} mol l^{-1} min^{-1} compared with 0.050×10^{-7} mol l^{-1} min^{-1} at pH 7.

Keywords: *Cyanocobalamin; hydroxocobalamin; two-component spectrophotometric assay; photolysis; pH effect.*

Introduction

Cyanocobalamin (vitamin B_{12}) is a haemopoietic vitamin [1] and is extensively used in the form of injectable preparations either alone or as a component of vitamin B-complex. Aqueous solutions of cyanocobalamin lose microbial activity on exposure to light [2]. Decomposition is mainly caused by light of short wavelengths [3]. The photochemical conversion of cyanocobalamin to hydroxocobalamin (B_{12b}) takes place readily in acidic solutions [4–6] and may be represented [7–9] as follows:



In the photolysis process CN^- is expelled with its full complement of electrons and is replaced by a H_2O molecule without any change in the valency of the cobalt. This reaction is probably initiated by the absorption of light causing π–π^* transition within the corrin ring [10]. The quantum yield for the photolysis of B_{12} is approximately 10^{-4} [11]. The light-induced conversion of B_{12} has been considered as a pH-dependent reaction [4]; however, there is a lack of information on the effect of pH over a wide range on the rate of photolysis.

One of the major problems in the study of the photolysis of vitamin B_{12} has been the non-availability of specific assay procedures. The official B_{12} assay methods in the BP [12] and USP [13] are based on the measurement of absorbance at 361 nm and are thus incapable of distinction between B_{12} and B_{12b} in the raw material and in degraded solutions. Multicomponent spectrophotometric methods have been applied to the assay of B_{12} in vitamin mixtures [14], other vitamins [15, 16] and degradation products [17–21]. Vitamin B_{12}, in the presence of B_{12b}, has been assayed by various spectrophotometric methods [5, 7, 22–24], which are time consuming and give only approximate results. The use of 361 nm to follow the kinetics of photolysis [11] may lead to unreliable data due to interference from B_{12b}.

In the present work a two-component spectrophotometric method has been developed for the simultaneous assay of B_{12} and B_{12b} in aqueous solutions. The method is specific and reliable for photodegradation studies and has been used to investigate the effect of pH on the rate of photolysis of B_{12} [25].

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Simultaneous spectrophotometric determinations rest on the assumption that the substances concerned contribute additively to the total absorbance at an analytical wavelength. In the assay of a two-component system [26, 27], absorbance (A) measurements are made at two suitably selected wavelengths, λ_1 and λ_2 and if the light path remains constant, two simultaneous equations may be written:

$$A\lambda_1 = k_1^{\lambda_1} C_1 + k_2^{\lambda_1} C_2 \quad (1)$$

$$A\lambda_2 = k_1^{\lambda_2} C_1 + k_2^{\lambda_2} C_2, \quad (2)$$

where $k_1^{\lambda_1}$ and $k_2^{\lambda_1}$ are the molar absorptivities of components 1 and 2 at λ_1; $k_1^{\lambda_2}$ and $k_2^{\lambda_2}$ are the molar absorptivities of components 1 and 2 at λ_2; and C_1 and C_2 are the concentrations of components 1 and 2. The equations are solved to obtain the concentration of each component

$$C_1 = \frac{k_2^{\lambda_2} A_{\lambda_1} - k_2^{\lambda_1} A_{\lambda_2}}{k_1^{\lambda_1} k_2^{\lambda_2} - k_2^{\lambda_1} k_1^{\lambda_2}} \quad (3)$$

$$C_2 = \frac{k_1^{\lambda_1} A_{\lambda_2} - k_1^{\lambda_2} A_{\lambda_1}}{k_1^{\lambda_1} k_2^{\lambda_2} - k_2^{\lambda_1} k_1^{\lambda_2}} \quad (4)$$

Experimental

Materials

Cyanocobalamin and hydroxocobalamin (Eur. P.) were obtained from Fluka (Switzerland) and their purity was confirmed by thin-layer chromatography (TLC). All solvents and reagents were analytical grade or of the purest form available from BDH/Merck. The following buffer systems were employed: potassium chloride–hydrochloric acid, pH 1.0–2.0; citric acid–disodium hydrogen phosphate, pH 2.5–8.0; citric acid–disodium hydrogen phosphate–boric acid, pH 8.5–12.0; the ionic strength was 0.05 M in each case.

Photolysis

A 7×10^{-5} M aqueous solution of cyanocobalamin (200 ml) was prepared at the required pH (1.0–12.0) and put into a 250-ml Pyrex flask. The flask was placed in a radiation chamber and irradiated with Philips HPLN 125 W high-pressure mercury-vapour fluorescent lamp (emission at 405, 436, 545 and 578 nm). The distance between the light source and the centre of the flask was 30 cm. The temperature of the solution during irradiation was maintained at 25 ± 1°C. Samples were withdrawn at appropriate intervals for TLC and spectrophotometric assay. Control solutions wrapped in aluminium foil were placed in the dark and assayed for cyanocobalamin content at the end of photolysis.

Light intensity measurement

The intensity of the 125 W fluorescent lamp was determined by potassium ferrioxalate actinometry [28] as $1.14 \pm 0.10 \times 10^{17}$ quanta s^{-1}.

Thin-layer chromatography

TLC of the photolysed solutions was carried out on 250-μm silica gel GF_{254} plates using the solvent systems: A, 1-butanol–acetic acid–0.066 M potassium dihydrogen phosphate–methanol (36:18:36:10, v/v/v/v) [29]; and B, methanol–water (95:5, v/v) [30]. The spots were located visually (red colour) or under UV light.

Spectral measurements

The UV and visible absorption spectra of cyanocobalamin solutions during irradiation were measured with a Shimadzu UV-240 recording spectrophotometer using matched cells of 10-mm pathlength.

Assay method

A 5-ml aliquot of the photolysed solution was placed in a 10-ml volumetric flask, diluted to volume with acetate buffer (pH 4.0) and the absorbance measured at 361 and 351 nm or 550 and 525 nm. The concentrations of cyanocobalamin and hydroxocobalamin were determined by a two-component assay by solving simultaneous equations (see Introduction).

The assay procedure was carried out in a dark room under subdued light.

Results and Discussion

Assay of cyanocobalamin

Chromatographic examination of the photolysed solutions of cyanocobalamin indicated the presence of hydroxocobalamin as the only photoproduct which is formed by the liberation of cyanide ion and contains the corrin nucleus. In solvent systems (A) and (B), B_{12} and B_{12b} have R_f values of 0.46 and 0.26, and 0.42 and 0.05, respectively. The spectral characteristics of B_{12b} in the UV and visible regions are similar to those of B_{12} and, therefore, inter-

ference in the BP assay of B_{12} [12] may be expected. This can be overcome by the use of a specific method that accounts for the presence of B_{12b} in degraded solutions.

A comparison of the absorption spectra of B_{12} and B_{12b} at pH 4.0 (or 7.0) (Fig. 1) indicated that there is a sufficient distinction between the absorption maxima of the two compounds (i.e. 361 and 351 nm or 550 and 525 nm) for analytical purposes. These wavelengths could provide maximum specificity and sensitivity to the proposed method and a two-component mixture of B_{12} and B_{12b} can be conveniently assayed. The accuracy of the analysis depends in part upon the choice of the two wavelengths. Factors influencing the choice of wavelengths and methods of optimizing wavelength selection in a two-component assay have been discussed in detail [31–33]. The molar absorptivities of B_{12} and B_{12b} determined at pH 4.0 (acetate buffer) are given in Table 1, and the values are in agreement with those previously reported [11, 12, 34–37]. The validity of Beer's law for B_{12} and B_{12b}, alone and in mixtures, was confirmed at the analytical wavelengths. Statistical data for the calibration curves are presented in Table 2. The correlation coefficient values (r = 0.9999)

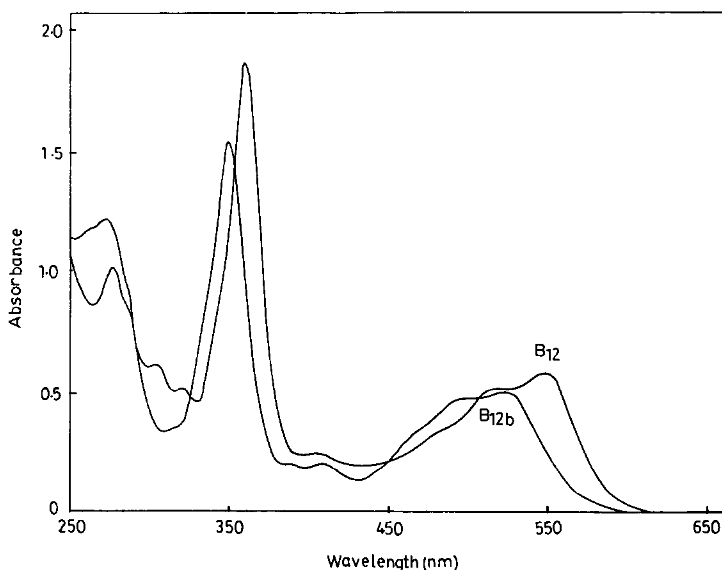

Figure 1
Absorption spectra of cyanocobalamin (B_{12}, 6.7 × 10^{-5} M) and hydroxocobalamin (B_{12b}, 6.0 × 10^{-5} M) at pH 4.0 (acetate buffer).

Table 1
Molar absorptivities (M^{-1} cm^{-1} × 10^{-3})* of cyanocobalamin and hydroxocobalamin at pH 4.0 (acetate buffer)

Compound	351 nm	361 nm	525 nm	550 nm
Cyanocobalamin	17.3	28.0	7.6	8.7
Hydroxocobalamin	25.6	17.4	8.5	4.9

*Each value is a mean of three to five determinations.

Table 2
Calibration data for cyanocobalamin and hydroxocobalamin showing linear regression analysis*

	Cyanocobalamin		Hydroxocobalamin	
λ_{max} (nm)	361	550	351	525
Concentration range (M × 10^5)	1.0–5.0	1.0–5.0	1.0–5.0	1.0–5.0
Slope	28048.74	8663.96	25578.57	8463.45
SE (±) of slope	2.039	1.408	2.147	1.602
Intercept	0.0051	0.00026	0.0047	0.00015
Correlation coefficient (r)	0.9999	0.9999	0.9999	0.9999

*Values represent five determinations of each compound.

indicate a good linear relationship over the concentration range employed.

Since concentration level is not a serious problem in the assay of B_{12} in vitamin preparations, either set of wavelengths could be used to achieve reliable results with almost the same degree of reproducibility. However, sharp peaks exist at 361 nm (B_{12}) and 351 nm (B_{12b}) and there is a relatively small difference (10 nm) between these two absorption maxima. Moreover, the higher sensitivity at those wavelengths to UV absorbing impurities and interfering substances, including other vitamins, and the relatively greater standard error (SE) in measurements (Table 2) suggest that 550 and 525 nm are preferable for the analytical work.

The absorption spectra of B_{12} and B_{12b} are affected by pH fluctuations [6, 24] and, therefore, it is necessary to choose a pH value appropriate for the assay of the mixture. B_{12b} is readily formed between pH 3.5 and 6.5 [5] and the pure substance is assayed at about pH 4 according to the BP method [12]. Thus pH 4.0 (acetate buffer) was finally chosen for the two-component assay and all photolysed solutions were adjusted to this pH before absorption measurements to minimize analytical errors.

In order to test the reproducibility of the assay method, several synthetic mixtures containing different proportions of B_{12} and B_{12b} were prepared and assayed for the two components under the proposed conditions. The results of B_{12} and B_{12b} assay in mixtures at 550 and 525 nm over the concentration range 1–5 × 10^{-5} M are given in Table 3. Judged from the recovery of the two compounds, the reproducibility of the method appears to be within ±3% indicating that the method is reliable for the assay of B_{12} as well as B_{12b} in vitamin preparations or photolysed solutions. It is specific, rapid and convenient for following the kinetics of photolysis reactions. The method applied to the assay of B_{12} and B_{12b} in photolysed solutions produced an almost constant molar balance, with time, with respect to the initial concentration of cyanocobalamin, indicating the validity of the analytical data.

Photolysis of cyanocobalamin

Aqueous solutions of B_{12} exhibit absorption maxima at 361 and 550 nm. Exposure of B_{12} solutions to light results in a decrease of absorbance at both the absorption maxima. It is accompanied by a gradual hypsochromic shift and an ultimate increase in absorbance near 351 and 525 nm, which correspond to the absorption maxima of B_{12b} (Fig. 1). These spectral changes include the appearance of isosbestic points near 294, 326, 355, 449 and 533 nm, which confirm the presence of only two absorbing species in the solution, i.e. B_{12} and B_{12b}, as shown by the TLC results.

These spectral variations indicate the photolytic transformation of the B_{12} molecule to B_{12b}. The magnitude of this change, depicted by the degree of spectral variations, depends upon factors such as pH and light intensity. A typical set of absorption spectra determined for the photolysis of B_{12} at pH 1.0 is shown in Fig. 2. The spectrum of the photolysed solution after irradiation for 6 h almost corresponds to the absorption spectrum of B_{12b} under the same conditions. At pH 4.5, variations in the spectra indicate the relatively slow conversion of B_{12} to B_{12b}. The spectral change at pH 7.0 is much slower and the loss in absorbance at

Table 3
Analyses of synthetic mixtures of cyanocobalamin and hydroxocobalamin*

Cyanocobalamin				Hydroxocobalamin			
Added (M × 10^5)	Found (M × 10^5)	Recovery (%)	RSD (%)	Added (M × 10^5)	Found (M × 10^5)	Recovery (%)	RSD (%)
5.316	5.289	99.5	0.46	1.202	1.183	98.4	0.69
4.652	4.633	99.6	0.23	1.803	1.779	98.7	0.17
4.320	4.335	100.3	0.48	2.103	2.064	98.1	2.18
3.987	4.029	101.1	0.56	2.403	2.380	99.0	0.20
3.655	3.634	99.4	0.46	2.704	2.682	99.2	0.80
3.323	3.277	98.6	0.35	3.004	2.960	98.5	1.21
2.658	2.653	99.8	1.21	3.605	3.648	101.2	0.89
1.994	2.019	101.2	2.35	4.206	4.241	100.8	0.78
1.661	1.660	100.1	0.77	4.507	4.545	100.8	0.10
1.329	1.305	98.2	1.20	4.807	4.882	101.6	0.64

*Values expressed as a mean of three to five determinations.

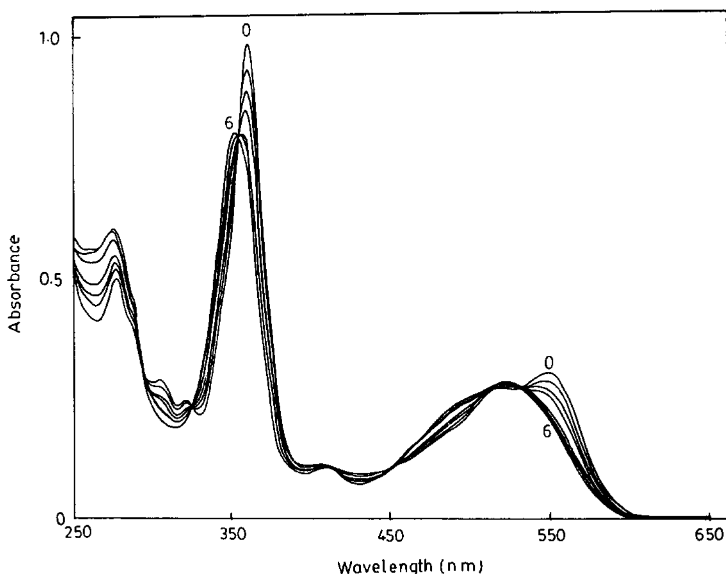

Figure 2
Spectral changes during the photolysis of 7 × 10^{-5} M cyanocobalamin at pH 1.0 (measurement at 1:1 dilution, pH 4.0 acetate buffer). Irradiation time: 0, 1, 2, 3, 4, 5, 6 h.

361 nm is about one-eighth that at pH 1.0 and nearly half that at pH 4.5 for the same period.

In order to determine the rate of photolysis at various pH values, the analytical data obtained in terms of the molar concentrations of B_{12} were treated for compliance with zero-, first- and second-order reactions. It was found that the data are best fitted to zero-order kinetics, in agreement with the observations of Vogler *et al.* [11] for the photolysis of B_{12} in aqueous solution. The concentration versus time graphs for some typical reactions are shown in Fig. 3, and the zero-order rate constants are reported in Table 4. The magnitude of the standard error of rate constants indicates the reliability of the kinetic data. The control solutions stored in the dark showed negligible change in B_{12} content.

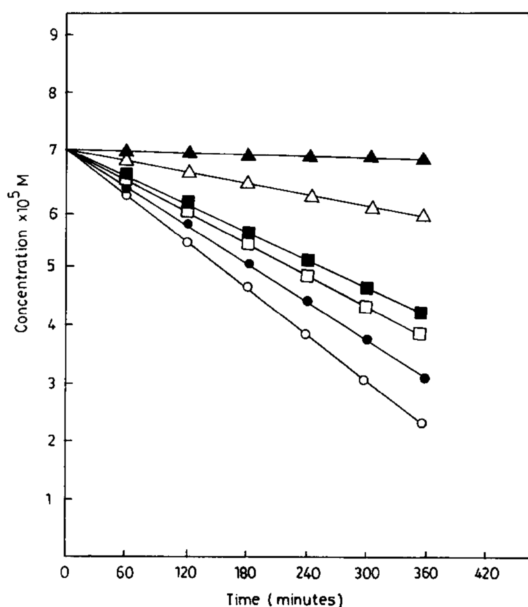

Figure 3
Zero-order plots for the photolysis of cyanocobalamin: pH 1.0 (○), pH 2.0 (●), pH 3.0 (□), pH 4.0 (■), pH 5.0 (△), pH 6.0 (▲).

Effect of pH on photolysis

The photolysis of B_{12} was carried out in the pH range 1.0–12.0 using buffered solutions of

Table 4
Zero-order rate constants for the photolysis of cyanocobalamin at pH 1.0–12.0

pH	$k \times 10^7$* (mol l^{-1} min^{-1})	pH	$k \times 10^7$* (mol l^{-1} min^{-1})
1.0	1.316 ± 0.00567	5.75	0.100 ± 0.00139
1.5	1.193 ± 0.00446	6.0	0.071 ± 0.00134
2.0	1.093 ± 0.00620	6.5	0.055 ± 0.00116
2.5	1.017 ± 0.00357	7.0	0.050 ± 0.00109
3.0	0.946 ± 0.00703	7.5	0.044 ± 0.00114
3.5	0.884 ± 0.00765	8.0	0.042 ± 0.00107
3.75	0.833 ± 0.00695	8.5	0.039 ± 0.00102
4.0	0.775 ± 0.00581	9.0	0.048 ± 0.00121
4.25	0.697 ± 0.00561	9.5	0.063 ± 0.00137
4.5	0.550 ± 0.00556	10.0	0.073 ± 0.00123
4.75	0.395 ± 0.00484	10.5	0.104 ± 0.00153
5.0	0.299 ± 0.00414	11.0	0.131 ± 0.00176
5.25	0.229 ± 0.00338	11.5	0.205 ± 0.00285
5.5	0.158 ± 0.00212	12.0	0.338 ± 0.00421

*Mean ± SE, *n* = 3–4.

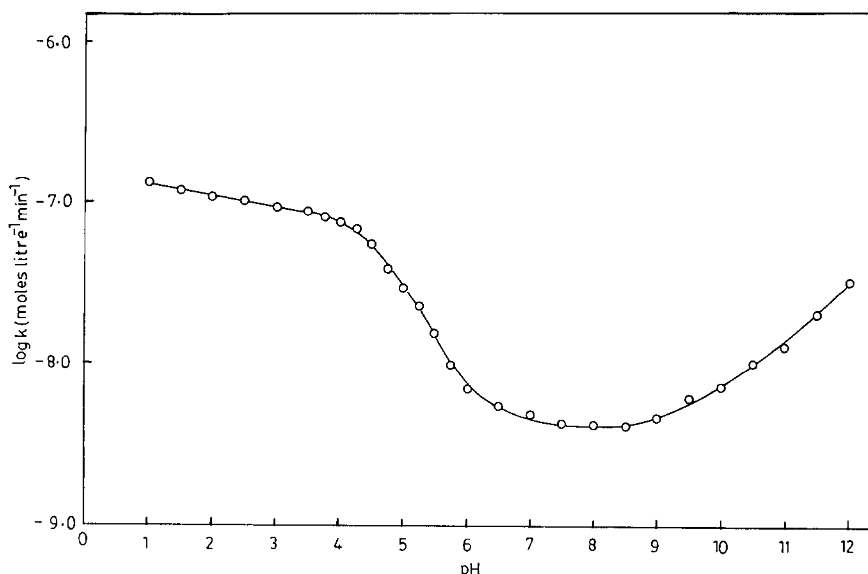

Figure 4
Log k–pH profile for the photolysis of cyanocobalamin.

constant ionic strength (0.05 M) to minimize any salt effects. The log k–pH profile (Fig. 4) indicates a slow change in rate at pH 1–4 followed by a fast decrease in the pH range 4.5–6.5, an almost constant rate in the region 6.5–8.5 and a gradual increase from pH 9 to 12. Thus the rate of photolysis appears to be catalysed by both hydrogen and hydroxyl ions.

B_{12} is a polyacidic base with six weakly basic amide groups and a pK_a of 3.3 [38]. In the acid region the molecule exists as a cation. It is evident from the ionization behaviour of B_{12} (i.e. the 5,6-dimethylbenzimidazole moiety) that the molecule exists 99.5% in the protonated form at pH 1.0 and 99.9% in the neutral form at pH 7.0. The values of the zero-order rate constants follow the same order and decrease from 1.32×10^{-7} mol l^{-1} min^{-1} at pH 1.0 to 0.050×10^{-7} mol l^{-1} min^{-1} at pH 7.0. Thus, the ionized species of B_{12} appears to be more susceptible to photoactivation than the non-ionized species. The rate of reaction is almost independent of pH over the range 6–9, at which B_{12} exhibits maximum stability. Above pH 9 an increase in the rate of photolysis may result from a contribution due to the hydrolysis of amide groups [39]. The major deactivating reaction of B_{12} under basic conditions is the cyclization of the c-acetamide function to give a fused lactam on ring B [8, 40]. Amide cyclization and amide hydrolysis in acidic and basic media also contribute to the overall stability of B_{12} solutions. The effect of pH on the heat-catalysed degradation of B_{12} has been reported [41] and cyanocobalamin appears to be stabilized by phosphate ions in the pH range 3–7.

The present study is in agreement with the observations of Veer *et al.* [4] that a pH lower than 6 favours the photodegradation of B_{12} to B_{12b} and that the destruction of B_{12} at pH 4.0 on light exposure is much greater than that at pH 6.9 [5]. The official requirements for the pH of B_{12} injections are between 3.8 and 5.5 [12] and 4.5–7.0 [13]. Since B_{12} is highly sensitive to light in the pH region lower than 6 (Fig. 4), it is advisable to formulate the injections at pH 6–7 to improve the photostability of cyanocobalamin and to preserve its activity.

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