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CONTRIBUTIONS TO THE ANALYTICAL CHEMISTRY OF VITAMIN B_{12}. THE THERMAL STABILITY OF CYANOCOBALAMIN, HYDROXOCOBALAMIN AND COBINAMIDE IN THE SOLID STATE

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ABSTRACT

The thermal stability of cyanocobalamin, hydroxocobalamin and cobinamide has been examined using for the first time thermogravimetric (TG), derivative thermogravimetric (DTG), and differential thermal (DTA) analyses carried out simultaneously with a derivatograph. While water is removed from cyanocobalamin and hydroxocobalamin up to 140°C, and up to 120°C for cobinamide, the cyanide ligand is thermally removed from cyanocobalamin at 140–145°C, and at about 120°C in the case of cobinamide. The IR spectra were used to monitor the extent of cyanocobalamin degradation.

Mathematical analysis of the TG curve, using the derived equation of Freeman and Carroll, to evaluate the kinetic parameters of the reaction showed that the removal of water from cyanocobalamin (20–140°) is a zero-order reaction with an activation energy of 26.7 ± 1.9 kcal mole^{-1}, both determined using a computer-programmed least squares regression analysis. These findings are useful for the estimation of the shelf-life of a vitamin B_{12} preparation.

INTRODUCTION

The biosynthesis of cyanocobalamin (vitamin B_{12}) and new cobalt complexes related to vitamin B_{12} by the use of organic precursors and a microorganism is a recognized field for research. It was found that heat treatment of *Propionibacterium shermanii* increased the concentration of vitamin B_{12}-5′-phosphate [1], the immediate precursor of vitamin B_{12} [1,2]. Moreover, the isolation of vitamin B_{12}, produced mainly by *Propionibacterium* sp., includes the extraction of the intracellular bound vitamin B_{12} with aqueous solutions at a temperature of about 120°C, in the presence of cyanide [3].

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In the course of our bioengineering and analytical chemistry studies of vitamin B_{12} by controlled biosynthesis, a logical extension of the above findings was to examine the thermal stability of vitamin B_{12} and some related compounds. In this connection, we have published on the possibility of determining small amounts of cyanide in vitamin B_{12} and some analogues by means of a cyanide ion-selective electrode without distillation of hydrogen cyanide [4], and in solid material and in benzyl alcohol by IR absorption spectrophotometry [5,6] and thermal decomposition [7].

To the authors' knowledge very little has been published on the thermal stability of vitamin B_{12} group substances. Thus, it is reported [8,9] that vitamin B_{12} evolves 1 mole of ammonia when heated to 180°C. At higher temperatures ($\simeq$ 240°C) more ammonia is released and extensive changes occur. Vigorous heating produces vapours which give the pine splinter test, and total combustion produces a lavender residue of cobalt phosphate [10].

This paper reports the preliminary findings on the thermal stability of cyanocobalamin, hydroxocobalamin and cobinamide in the solid state, as the temperature is raised at a constant rate. Infrared spectrophotometry was used to monitor the extent of the degradation and to provide supporting information.

EXPERIMENTAL

Apparatus

Thermogravimetric (TG), derivative thermogravimetric (DTG) and differential thermal (DTA) analyses were carried out simultaneously using a Paulik–Paulik–Erdey type derivatograph (model OD-102, Budapest, Hungary), which has an accuracy of $\pm$0.05 mg and a temperature range up to 900°C.

An IR spectrophotometer (UNICAM-SP 200, Gt. Britain) was used for the samples in the potassium bromide pelleting technique.

Reagents and standards

All reagents were prepared from analytical grade substances and doubly distilled water. Cyanocobalamin and cobinamide were prepared and tested as described previously [4]. Hydroxocobalamin was obtained by treating a solution of cyanocobalamin with sulphur dioxide. The sulphitocobalamin obtained was converted into hydroxocobalamin by UV radiation, purifying on ion-exchangers and recrystallizing from acetone. It was tested as described previously [4], showed no IR band for cyanide, and the absorption maxima were in accordance with the literature values [10].

Procedure

The simultaneous TG, DTG and DTA measurements were performed up to 900°C in a stationary air atmosphere at ambient pressure; weight of sample, 40–100 mg, loosely kept in the crucible; reference substance, α-Al_2O_3; heating rate, 10°C min^{-1}; crucible, platinum; temperature detectors, Pt/Pt–Rh thermocouples.

The DTG galvanometer measured the current induced in the coil suspended from the beam of the analytical balance moving in the magnetic field. The current was proportional to the speed of the movement of the beam.

The DTA galvanometer measured the difference in EMF between the two thermocouples, one from the sample and the other from the reference material. The motion of the light beam coming through the illuminated optical slit fixed to the balance beam gave the TG curve which represented the mass changes during the heating of the sample. The T-galvanometer measured the temperature of the sample alone.

The IR spectra were obtained using the potassium bromide pelleting technique as described previously [5].

RESULTS AND DISCUSSION

Cyanocobalamin

The TG curve (Fig. 1) shows that a change in weight is observed up to 140°C, and analysis of the compounds in the 20–140°C region indicates the removal of water. This was established by: (i) elemental analysis, (ii) UV and IR spectrophotometry and (iii) thin layer chromatography, compared with the cyanocobalamin initially submitted for thermal analysis. The DTA curve shows a strong endothermal process at 135–140°C, and this appears to correlate well with the loss of water observed on the TG curve.

After the loss of water content, a first plateau is observed on the TG curve between 140° and 230°C, which indicates the existence of a stable compound. Using a lower heating rate (3°C min^{-1}) samples taken between 140 and 230°C showed that, in fact, cyanocobalamin is associated with a process involving a mass loss of $\simeq 2\%$ between 140 and 145°C. The results of chemical analysis, UV and IR data, TLC on aluminium oxide using isobutanol–isopropanol–water (1 : 1 : 1) as the solvent system and by quantitative determination of cyanide using an ion-selective electrode [4], confirmed that the loss in mass at 140–145°C is due to the removal of the cyanide group from the cyanocobalamin structure (theoretically, the cyano group content in cyanocobalamin is 1.92%).

The samples of the product taken at 140–145°C exhibit the following: (i)

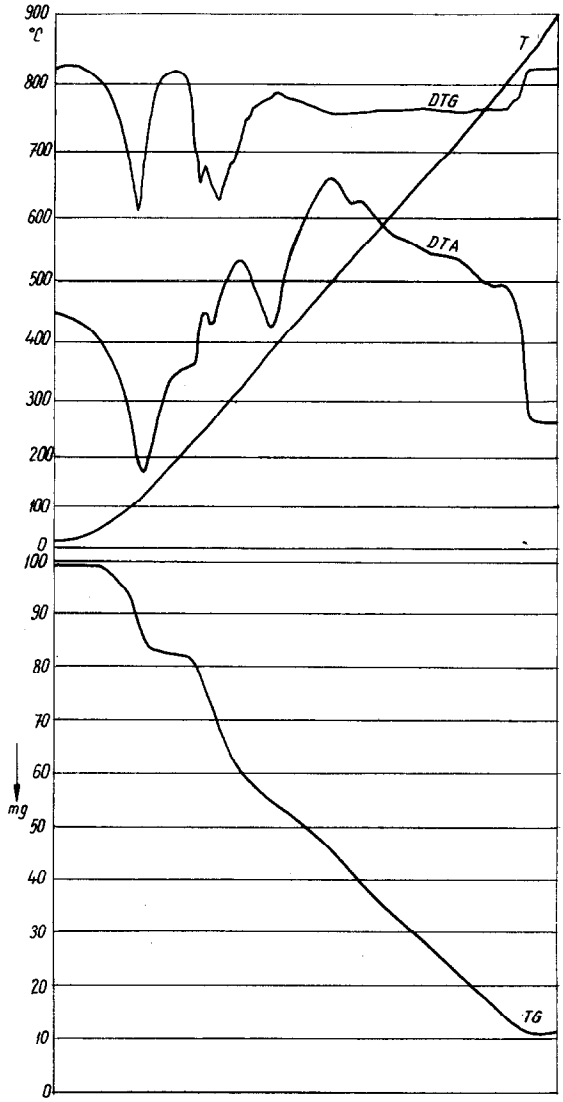

Fig. 1. Thermoanalytical curve for cyanocobalamin.

the absence of the weak but sharp absorption band at 2137 cm^{-1} (specific for the stretching vibration of the cyano group) indicating the absence of the coordinated cyanide, the remaining spectrum (Fig. 2) being unchanged; (ii) the UV and visible spectra show maximum absorption at 278 nm, 361 nm and 550 nm in water and at 360 nm and 550 nm in benzyl alcohol (however, the intensity of the bands in benzyl alcohol is greatly reduced compared to those in water); (iii) they no longer contain the cyano group (compared to a reference standard of hydroxocobalamin).

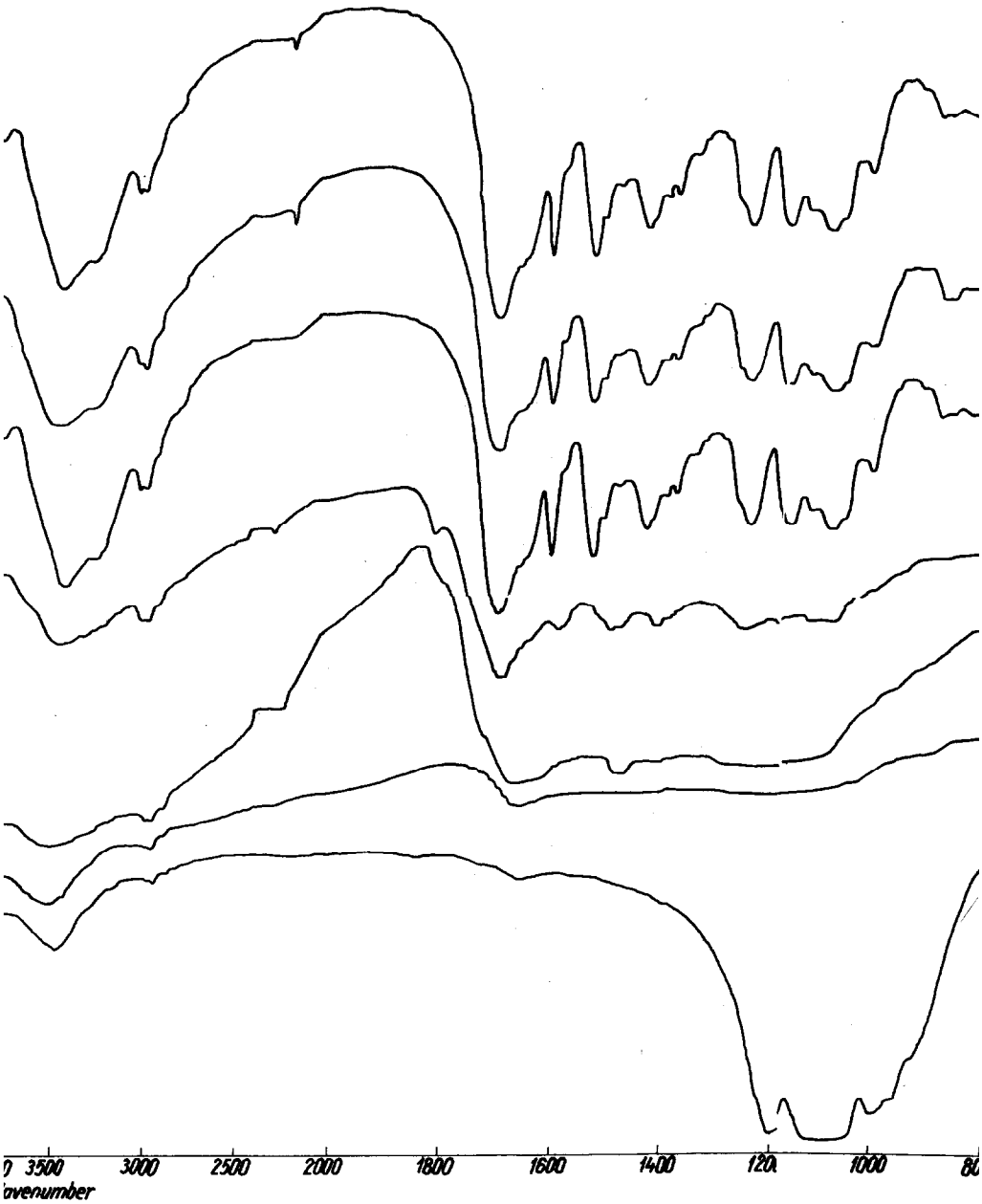

Fig. 2. Infrared spectrum of cyanocobalamin: (a) initial, (b) 120°C, (c) 140°C, (d) 235°C, (e) 285°C, (f) 500°C, (g) 900°C.

Samples of "vitamin B_{12}" taken within the temperature range 140–145° to 230°C have shown that the solubility in water is decreased and the become practically insoluble at 230°C.

The maximum on the DTG curve at 180°C has been ascribed to the fa that vitamin B_{12} evolves 1 mole of ammonia, in agreement with previou reports [8,9].

The product formed up to 230°C seems to be very susceptible to the temperature rise and, consequently, beyond this temperature it begins to decompose and extensive changes occur, in good agreement with previous findings [8,9]. The IR spectrum at 230°C reveals important structural changes: the bands up to 1200 cm^{-1} that imply the bond between 5,6-dimethylbenzimidazole and the corrinoid system [10,11] disappear. Thus, the product becomes insoluble in water, confirming that the nucleotide side-chain plays a very important role in, for example, determining the solubility properties of the corrinoids. The cyano group band is absent, as expected. expected.

Four endothermic effects with peak maxima at about 230, 280, 390 and 520°C (the last one with 3°C min^{-1}) are followed by a second plateau of constant composition which is visible on the TG curve at 800°C. The compound on this second plateau was found to be a mixture of cobalt and phosphorus oxides which, calculated as a sum of $P_2O_5 + Co_2O_3$, represents 11.3% of the initial cyanocobalamin submitted for thermal analysis (theoretical content, 11.1%). At this stage the degradation product is blue-violet, in contrast with the initial red colour (up to 135–140°C) which darkened at about 200°C. Further elevation in temperature up to 900°C showed no other visible effects than those described above.

At 285 and 500°C the IR spectra show that all the bands have practically disappeared, while at 900°C the bands in the region up to 1200 cm^{-1}, in which it is difficult to assign frequencies with any certainty, reveal a strong peak at 740 cm^{-1} absent in the initial spectrum of cyanocobalamin (Fig. 2).

Hydroxocobalamin

Hydroxocobalamin loses its water content up to 140°C (Fig. 3); the first plateau of constant composition takes place at 140–230°C, and the second one at about 760°C. The degradation pattern does not differentiate this product from cyanocobalamin, clearly indicating that the nature of the axial ligand (OH^- instead of CN^-) does not have a marked effect on the thermal stability. Future work will show how far this is valid when applied to a wider range of, and with increasing size, ligands.

Cobinamide

Cobinamide is stable up to 120°C. From 60 to 120°C the removal of water is accomplished (8–9%) (Fig. 4); this fact has been established as described above under cyanocobalamin. At about 120°C the cyan removal takes place (found, 2.3%; theoretical value, 2.5%), and from 220 to 600°C the TG curve clearly indicates successive decompositions, with endothermic activity at 410, 490, 530 and 610°C. At 600°C the TG curve shows the second plateau which corresponds to about 5% of the initial cobinamide

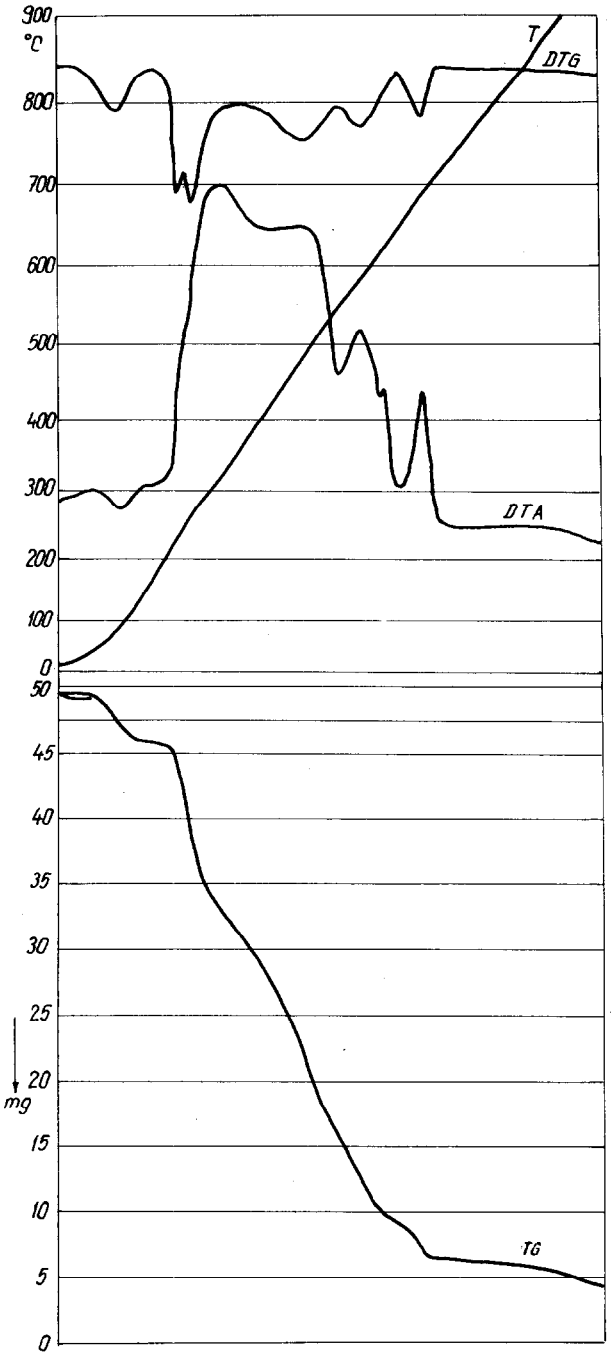

Fig. 3. Thermoanalytical curve for hydroxocobalamin.

weight. At 900°C the product of degradation is violet, characteristic of cobalt oxides.

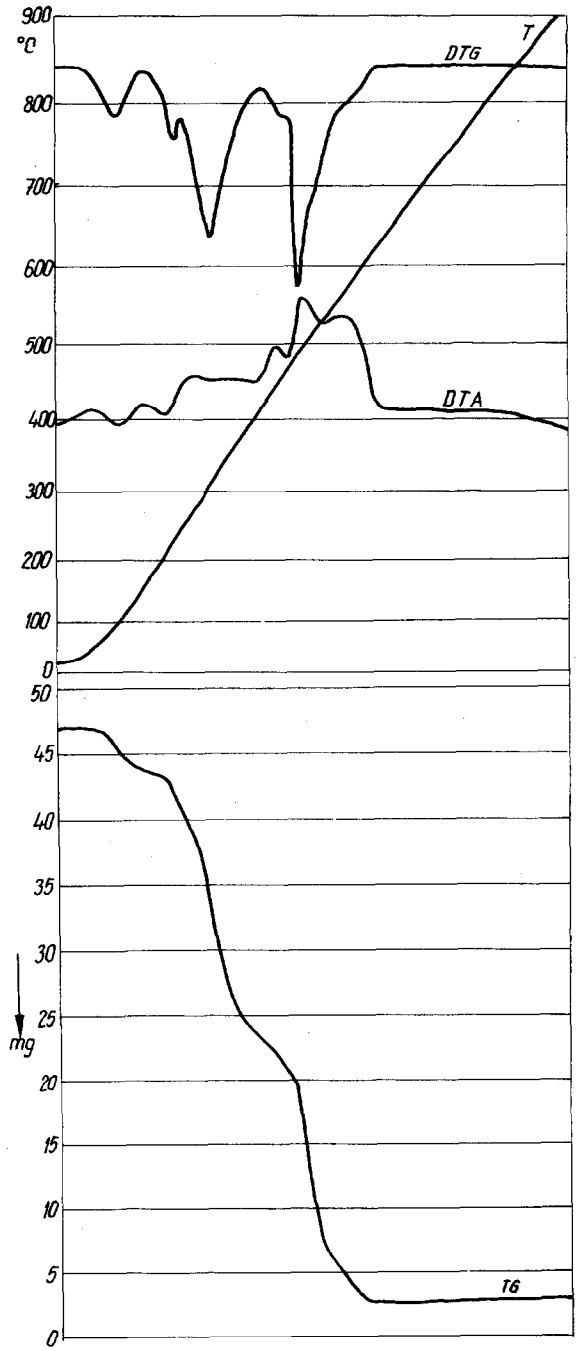

Fig. 4. Thermoanalytical curve for cobinamide.

Mathematical analysis of the TG curve

Thermogravimetric data can be used to evaluate the kinetic parameters of reactions involving weight loss (or gain). One of the advantages of determining kinetic parameters by thermogravimetric methods rather than by conventional isothermal studies is considered to be the possibility of using one simple sample in the study [12,13].

We have considered that vitamin B_{12} follows the general scheme of the heterogeneous reactions of the type

$$A_{(s)} \rightarrow B_{(s)} + C_{(g)}$$

The activation energy and the order of reaction were evaluated [14,15] from analysis of the TG curve in the region 20–140°C, using the derived equation

$$-\frac{E/2.3R\Delta T^{-1}}{\Delta \log W_r} = -n + \frac{\Delta \log(dW/dt)}{\Delta \log W_r} \quad (1)$$

where E is the activation energy, R is the general gas constant, T is the absolute temperature, n is the order of reaction, and $W_r = W_c - W$, where W is the weight loss at time t, W_c is the weight loss at completion of the reaction, and Δ is the differential operator.

This equation was derived by assuming a rate expression

$$-\frac{dx}{dt} = Kx^n$$

where x is the amount of vitamin B_{12} at time t, and the rate constant, K, is given by the simple Arrhenius expression

$$K = Ze^{-E/RT}$$

where Z is the frequency factor.

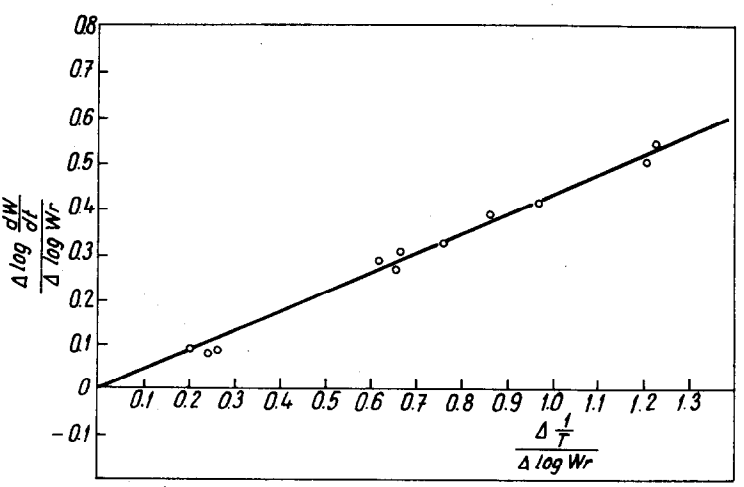

Fig. 5. Freeman–Carroll's mathematical analysis of the TG curve.

Either by plotting a graph of $[\Delta \log(dW/dt)]/(\Delta \log W_r)$ vs. $(\Delta T^{-1})/(\Delta \log W_r)$ or by other suitable rearrangement of eqn. (1) it is possible to derive values for both E and n (Fig. 5). The graph gives a straight line from which the activation energy (26.7 ± 1.9 kcal mole^{-1}) and the order of reaction (zero) were found, both calculated from the slope and intercept, respectively, by means of a computer-programmed least squares regression analysis.

These results, in good agreement with the literature values [16,17], could be used for reasonable estimates of the effect of temperature on the shelf-life of a vitamin B_{12} preparation. The usual range for zero- and first-order reactions, most frequently encountered in the pharmaceutical industry, is about 12–24 kcal mole^{-1}.

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