VITAMIN B_{12}

I. THE RELATION BETWEEN VITAMINS B_{12} AND B_{12b}

by

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INTRODUCTION

Vitamin B_{12b}, a biologically active substance, closely related to vitamin B_{12}, has been isolated from both liver[1, 2] and cultures of *Streptomyces aureofaciens*[2]. Besides, it has been produced by hydrogenation of vitamin B_{12}[3] under conditions which, according to KACZKA *et al.*[4], give rise to the formation of vitamin B_{12a}. Recently, FOLKERS[5] announced that the absorption spectra of vitamins B_{12a} and B_{12b} were indistinguishable and that samples of both vitamins showed no appreciable difference in microbiological activities with respect to *Lactobacillus casei* and *L. Leichmannii*. BROCKMAN'S *et al.*[6] statement that after mild acid hydrolysis of vitamin B_{12} the solution shows the spectrum of vitamin B_{12b}, prompts us to report on our own experiments.

It has been observed that, whereas the spectrum of vitamin B_{12} remains unchanged when kept under acid conditions at room temperature for several days *in the dark*, even diffuse daylight—under otherwise identical conditions—rapidly induces the spectrum of vitamin B_{12b}[2, 3, 7] to appear. When a solution thus obtained or one of pure vitamin B_{12b} is treated with KCN the spectrum of vitamin B_{12} is observed again.

METHODS AND MATERIALS

Crystalline vitamin B_{12} from own sources, prepared from liver was purified by chromatography over neutral alumina using methanol as an elution solvent, followed by three recrystallizations from water-acetone mixtures. The spectrum showed maxima at 548–550, 410, 361, 323, 306 and 278 mμ. The E (1%, 1 cm) values amounted to 64, resp. 28, 207, 59, 71 and 118.

The reagents used were chemically pure. Methyl cyanide was purified by means of the method described by TODA[8] for the purification of butyl cyanide. Methyl *iso*cyanide was prepared according to the method of GAUTIER as modified by HARTLEY[9]. It was freed from HCN by washing an ether solution with a KOH solution and subsequently with water, evaporation of the solvent, and fractional distillation.

Spectroscopy

A Beckman quartz spectrophotometer model DU was used. Use was made of an effective band width of $\pm$ 0.5–1 mμ.

RESULTS

The spectrum of a solution of vitamin B_{12} in 0.1 N acetic acid containing 37 μg of the vitamin per ml, remained unchanged when kept at room temperature in the dark

References p. 228.

for two days. When the same solution was subsequently exposed to diffuse daylight, for 1.5 hours (laboratory window) the spectrum changed to that of vitamin B_{12b}. The same effect was observed in 0.01 *N* and 0.001 *N* acetic and hydrochloric acid, or in an acetic acid-acetate buffer of pH 4.

That actually under these conditions vitamin B_{12b} was formed has been shown by exposing a solution of 44 mg of vitamin B_{12} in 1000 ml of 0.001 *N* hydrochloric acid to diffuse daylight. The termination of the reaction was checked by the shift of the spectrum. The solution was extracted with freshly distilled phenol, saturated with 0.001 *N* hydrochloric acid after which the vitamin was transferred from the phenol solution into water by the addition of 10 volumes of ether. The aqueous solution, after evaporation to a small volume in vacuo at room temperature yielded rod-shaped dark red crystals on the addition of acetone. They were purified by recrystallization from water-acetone and showed the spectrum of vitamin B_{12b}. On the addition of sodium hydroxide to a concentration of 0.01 *N* the absorption spectrum was found to undergo shifts of the main bands to 357 and 536 mμ, as described for vitamin B_{12b}[3].

On paper partition chromatography, using Whatman filter paper No. 1 treated with KH_2PO_4[10], and butanol, the preparation showed the same R_F value as a sample of authentic vitamin B_{12b} kindly supplied by Dr T. H. JUKES, Lederle Laboratories.

At values between pH 4 and 6, intermediate spectra were observed. So, after a three hours' exposure to diffuse daylight the solutions showed a maximum at 358–359 mμ (vitamin B_{12} maximum 361 mμ, vitamin B_{12b} 351 mμ). Even prolonged exposure of a solution at pH 5.4 for altogether 16 hours did not result in a shift of the band to a lower wave-length than 353–357 mμ, while the spectrum of a solution of pH 6 after an initial slight shift remained unchanged on further exposure to light. It may be of interest to note that the spectrum, for all its being an intermediate one, did not show the secondary "peaks" at 306 and 323 mμ of vitamin B_{12}, absent in the vitamin B_{12b} spectrum. In addition, a shift of the maximum from 550 to 525 mμ was observed in all cases.

The spectrum of vitamin B_{12b} was also formed when an acid vitamin B_{12} solution of *e.g.* pH 4 was irradiated with a medium pressure mercury lamp (Philips HPW 300). The high absorption of vitamin B_{12} at 361 mμ and the emission by the mercury lamp of radiation in the range of from 320 to 400 mμ suggested that the 365 mμ Hg-line was responsible for the light-induced conversion of vitamin B_{12} into B_{12b}. In order to check this, two experiments were performed in which the radiation from the mercury lamp was passed through a cell filled with water, respectively a 1% folic acid solution in 0.1 *N* sodium hydroxide. A vitamin B_{12} solution in buffer of pH 4 containing 30 μg of the vitamin per ml was used. The distance between the mercury lamp and the cell containing the vitamin B_{12} solution amounted to 8 cm. The reaction was followed by determining the extinctions at 361, resp. 351 mμ. When water was used as a filter medium a minimum extinction at 361 mμ and a maximum one at 351 mμ were observed after two hours of radiation. The corresponding experiment with the folic acid solution as a filter showed that after the same lapse of time the extinctions of the vitamin B_{12} solution at 361 mμ and 351 mμ had not changed. Determination of the spectra after each of the two experiments revealed that the irradiated solutions showed the spectra of vitamin B_{12b}, resp. vitamin B_{12}.

Folic acid is known to absorb at 365 mμ[11]. On the basis of the above experiments the 365 mμ-range can be assumed as responsible for the photo-conversion.

When an irradiated acid vitamin B_{12} solution showing the spectrum of vitamin

References p. 228.

B_{12b} or some intermediate one is kept in the dark, a gradual, albeit slow reversal to the vitamin B_{12} spectrum is observed. The degree of this reversal is dependent on the pH of the medium, at pH values of 4 or higher a complete reversal to the spectrum of vitamin B_{12}—including the secondary "peaks"—being obtained within 1–2 weeks. At lower pH values in addition to the reversal an increased absorption at 290 mμ and smaller wave lengths is observed, possibly due to a partial degradation of the vitamin leading amongst other things to benziminazoles[12].

When KCN is added to a solution of vitamin B_{12b}—obtained by either dissolving the crystalline substance or irradiating an acid vitamin B_{12} solution—or to a solution showing an intermediate spectrum, almost instantaneously a colour change from brownish-red to purplish is observed. The spectrum is—at any rate within a few hours after the addition of KCN—completely indistinguishable from that of vitamin B_{12}[13]. This effect can be achieved by relatively small quantities of KCN, albeit an excess with regard to the quantity of vitamin B_{12}. So, the addition of *e.g.* 0.5 mg of KCN to 10 ml of a vitamin B_{12b} solution in pH 6 buffer containing 40 μg per ml resulted in a complete formation of the vitamin B_{12} spectrum*.

This, or any similar effect, is not shown by KCNO, KCNS, hydroxylamine, formamide, CH_3CN, CH_3NC, NH_4Cl, urea, ethylene diamine.

DISCUSSION

On the basis of the above mentioned results we are led to believe that the addition of KCN to vitamin B_{12b}, yields either vitamin B_{12} itself or a very closely related compound with the same spectrum. In other words vitamin B_{12} would be either a cyan complex or a complex with a spectrum, identical with the artificially obtained vitamin B_{12b}-cyan complex. (Experiments with ^{14}C-labelled KCN are in progress.)

The light-induced conversion of vitamin B_{12} into vitamin B_{12b} might, accordingly, be considered as a reversible, pH-dependent, dissociation of the complex, diagrammatically to be represented as follows:

$$\left[B_{12b}CN\right] \underset{\text{dark, } CN'}{\overset{\text{light, } H^{\cdot}}{\rightleftharpoons}} B_{12b} + CN'$$

or

$$B_{12} = \left[B_{12b}X\right] \rightleftharpoons B_{12b} + X$$

In our opinion, neither the demonstration of pyrrolic substances among the alkali-degradation products of vitamin B_{12}[14], nor the above mentioned results provide sufficient evidence for the conception that vitamin B_{12} should have a haeminoid structure. On the other hand, however, it is attractive to recall the reversible photo-dissociation of CO-haemoglobin.

Another feature in common with the haemins is the greater stability of the complex as compared with the "parent-compound". Solutions of methaemoglobin *e.g.* are known to be rather unstable. Complexes, however, such as CNO-met-Hb are much more stable[15]. Whereas vitamin B_{12} itself is a stable compound, vitamin B_{12b} is much more susceptible to degradation[16].

* Extensive data on various experiments including paper partition chromatography will shortly be submitted for publication in this journal.

References p. 228.

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SUMMARY

1. In mildly acid solutions, kept in the dark, the spectrum of vitamin B_{12} remains unchanged; on exposure to light, vitamin B_{12b} is rapidly formed.

2. The spectrum of an irradiated solution ($p_H \geqq 4$), subsequently kept in the dark, shows a slow reversal to that of vitamin B_{12}.

3. When KCN is added to a solution of vitamin B_{12b} a rapid shift in the spectrum, from vitamin B_{12b} to vitamin B_{12}, can be observed.

RÉSUMÉ

1. En solution acide le spectre de la vitamine B_{12} ne subit aucun changement tant que la solution est conservée dans l'obscurité; à la lumière cependant, la vitamine B_{12b} se forme rapidement.

2. Le spectre d'une solution ($p_H \geqq 4$) irradiée et mise ensuite dans l'obscurité montre un changement qui la ramène à son stade initial.

3. Si l'on ajoute du CNK à une solution de vitamine B_{12b}, on peut observer une rapide transformation du spectre en celui de la vitamine B_{12}.

ZUSAMMENFASSUNG

1. In einer schwach sauren, dunkel aufbewahrten Vitamin B_{12}-Lösung bleibt das Spektrum unverändert; im Licht dagegen bildet sich schnell das Vitamin B_{12b}.

2. Das Spektrum einer belichteten, nachher dunkel aufbewahrten Lösung ($p_H \geqq 4$) zeigt langsam Rückkehr zu dem des Vitamin B_{12}.

3. Bei Zusatz von KCN zu einer Vitamin B_{12b}-Lösung macht sich schnell das Vitamin B_{12}-Spektrum bemerkbar.

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