VITAMIN B_{12}

II. THE INFLUENCE OF HCN ON SOME FACTORS OF THE VITAMIN B_{12} GROUP

by

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INTRODUCTION

In the first paper of this series[1], the conversion of vitamin B_{12} to vitamin B_{12b} under the influence of light in weakly acid solution has been described. This reaction could be reversed by the addition of a small amount of KCN to the solution, as far as could be judged from the change in the absorption spectrum.

As according to FOLKERS[2] vitamin B_{12b} might be identical with vitamin B_{12a}, both substances having been prepared by hydrogenation of vitamin B_{12}[3, 4], the reaction under the influence of the strongly reducing KCN seems quite remarkable.

Therefore, our first object was a further confirmation of the possible identity of the vitamins B_{12a} and B_{12b}.

The second was a further investigation of the reaction product obtained under the influence of KCN under varied conditions. Not directly related to these two, but with the use of the same techniques, the state of vitamin B_{12} in liver extract has been investigated.

METHODS AND MATERIALS

Crystalline vitamin B_{12}, according to the specifications described in a previous communication[1], and prepared in these laboratories, was used in all experiments.

The vitamin B_{12b} was prepared by submitting vitamin B_{12} to daylight in acid solution, as described in the same paper. A reference sample was kindly placed at our disposal by Dr T. H. JUKES (Lederle Laboratories). Through the courtesy of Dr K. FOLKERS (Merck and Co., Inc.) we were able to compare these preparations with an authentic sample of vitamin B_{12a}.

The paper partition chromatography experiments were performed using the descending boundary method first described by CONSDEN, GORDON, AND MARTIN[5]. The apparatus was essentially the same as described by these authors. The substances under investigation were applied as visible red spots, a method already used by LESTER SMITH *et al.*[6, 7]. The intervening space between two spots on the same sheet of paper (11 × 35 cm^2) was 1.8 cm or more. As proposed by WOODRUFF AND FOSTER[8], Whatman filter paper No. 1, previously treated with 0.66 *M* KH_2PO_4 solution, was used with *n*-butanol, saturated with water as the solvent.

Absorption spectra were measured with a Beckmann quartz spectrophotometer, model DU.

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EXPERIMENTAL

a. *Comparison of vitamins B_{12}, B_{12a} and B_{12b}*

The determination of the spectra of the authentic samples of vitamins B_{12a} and B_{12b} confirmed the observations of FOLKERS[2] as to the suspected identity of these two. Both samples gave the same type of absorption curve with the peaks (524–525 mμ, 351–352 mμ, and 273–275 mμ) at the same wave lengths within the experimental error. In Table I, a summary is given of the ratio of the extinctions at the absorption peaks together with the corresponding values for vitamin B_{12b} obtained from vitamin B_{12} under the influence of light and acid, as previously described.

TABLE I

RATIO OF THE EXTINCTIONS AT THE ABSORPTION MAXIMA OF THE VITAMINS B_{12a} AND B_{12b}

	Vit. B_{12a} (FOLKERS)	Vit. B_{12b} (JUKES)	Vit. B_{12b} (VEER *et al.*)
E_{351}/E_{525}	2.86	2.98	2.84
E_{351}/E_{274}	1.14	1.01	1.17

In the curves for each of the three preparations, the peaks at 323 and 306 mμ, characteristic of vitamin B_{12}, were absent whereas on the other hand in this range a minimum of the extinction was observed at approximately 315 mμ. Accordingly, the presence of a small peak in the vitamin B_{12a} spectrum, registered by KACZKA *et al.*[3], could not be confirmed.

In paper chromatography experiments according to WOODRUFF AND FOSTER[8], vitamin B_{12a} as well as the two samples of vitamin B_{12b} showed the same behaviour. In one experiment, all three showed a displacement of 31 mm, whereas a spot of vitamin B_{12} on the same sheet moved 55 mm. In a second experiment these distances were 27, subsequently 62 mm.

b. *Influence of HCN at p_H 5.5 on the spectrum*

The main results of this part of the investigation were already outlined in the first series of this paper. As the vitamins B_{12a} and B_{12b} also in *this* respect behaved in the same way, it may suffice to describe a number of experiments with vitamin B_{12b} prepared by ourselves. For all experiments, one stock solution of vitamin B_{12b} in water was used. With the aid of the extinction coefficients given by PIERCE *et al.*[9] a concentration of 59.7 μg of vitamin B_{12b} per ml was calculated from the absorption spectrum. The p_H was adjusted to the desired value by the addition of a 0.2 *M* acetic acid—Na-acetate buffer.

The spectra of the following solutions were compared, C serving as the blank:

	A	B	C
B_{12b}	3 ml	3 ml	0 ml
Buffer	1 ml	1 ml	1 ml
0.025% KCN	1 ml	0 ml	0 ml
Water	0 ml	1 ml	4 ml

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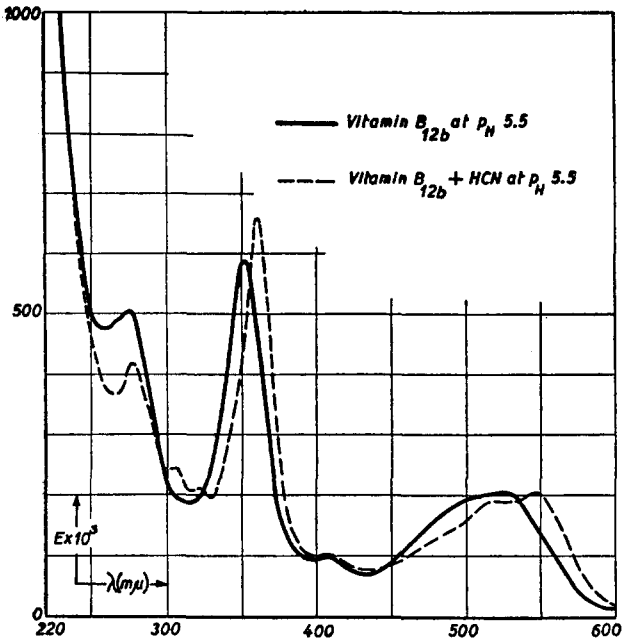

Fig. 1. Absorption spectra, after standing at room temperature in the dark for 3 hours, of
——— Vitamin B_{12b} at p_H 5.5
----- Vitamin B_{12b} + HCN at p_H 5.5

Whereas the spectrum of the solution B did not change in the course of 24 hours, a colour shift in the KCN containing solution A was at once visible to the naked eye. In the spectrophotometer, instead of the two peaks of 525 mμ and 351 mμ of the original spectrum, two others at 544 mμ and 359 mμ were measured immediately after the KCN addition. The absorption curves of the solutions after three hours are recorded in Fig. 1. There is a striking similarity between the spectrum of the solutions of vitamin B_{12b} with KCN and that of vitamin B_{12}, with exactly coinciding maxima.

The typical bands at 323 and 306 mμ are clearly and well developed. The ratio of the extinctions E_{361}/E_{548} equals that of vitamin B_{12}, but the corresponding ratios for the other peaks are somewhat lower than those of vitamin B_{12}. This effect is still more pronounced after 29 hours (Table II).

TABLE II

RATIO OF THE EXTINCTIONS AT THE ABSORTION MAXIMA OF VITAMIN B_{12}, AND OF VITAMIN B_{12b} AFTER 3 HOURS AND 29 HOURS TREATMENT WITH HCN AT p_H 5.5

		E_{361}/E_{548}	E_{361}/E_{410}	E_{361}/E_{323}	E_{361}/E_{306}	E_{361}/E_{278}
Crystalline vit. B_{12}		3.23	7.40	3.50	2.92	1.75
Crystalline vit. B_{12b} and HCN	after 3 hours	3.24	6.78	3.12	2.68	1.57
	after 29 hours	3.30	6.94	2.84	2.39	1.45

c. *Influence of HCN treatment on the partition chromatograms*

On one and the same sheet of filter paper, solutions of the following samples were applied:

1. vitamin B_{12a}

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2. a concentrated solution of vitamin B_{12a} which had been stored for 19 hours with several drops of a concentrated KCN solution of p_H 5.5

3. vitamin B_{12b}

4. same as 2., but now prepared with vitamin B_{12b}

5. a concentrated solution of vitamin B_{12} containing a slight amount of vitamin B_{12b}, formed on storage for several months in a desiccator over sulphuric acid.

The chromatogram of the fifth spot served as a double control. After drying, the spots were developed with *n*-butanol.

The pure vitamins B_{12a} and B_{12b} were allowed to travel a distance of 27 mm. They moved equally fast. The KCN-treated preparations moved as fast as vitamin B_{12}, and were well ahead at a distance of 59 mm.

The conversion of the vitamins B_{12a} and B_{12b} by HCN can also be realized directly on the paper if the air in the cabinet is made to contain some HCN vapour. The dry paper, with the spots already applied, is left for two hours in this atmosphere before the beginning of the experiment to ascertain equilibrium.

After development, the spots of the vitamins B_{12a}, B_{12b} and B_{12} were found to have travelled equally fast over a distance of 63 mm in 19.5 hours.

d. *Preparation of a crystalline product after HCN treatment of vitamin* B_{12b}

To a solution of 10 mg of vitamin B_{12b} in 5 ml of water, 1 ml of a freshly prepared 0.5% KCN solution, adjusted to p_H 5–6 with dilute HCl (using Universal indicator paper), was added. The mixture was left overnight at room temperature and subsequently extracted several times with phenol. The combined phenolic extracts were washed three times with distilled water, avoiding light as much as possible. By the addition of ether to the phenol the red substance was driven into water. From the combined aqueous layers, the phenol was removed by repeated washings with ether. The remaining ether was removed *in vacuo*. To the remaining red aqueous solution, ten volumes of acetone were added. After standing for three days in a desiccator over anhydrous acetone, crystals were formed. After three crystallisations from aqueous acetone the yield was 6 mg.

The absorption spectrum of this material coincided with that of vitamin B_{12} within the error of the experiment.

The ratio of the extinctions of the peaks: $E_{361}/E_{550} = 3.26$ and $E_{361}/E_{278} = 1.81$ matched those of pure vitamin B_{12}, where these same ratios are 3.23 and 1.75.

In the paper chromatography the same displacements for this product and for vitamin B_{12} were registered.

The microbiological activity of the several products will be the subject of another publication.

e. *The influence of KCN at* p_H 9

At alkaline reaction of the solution, the formation of a product different from vitamin B_{12} is observed.

The spectra of the following solutions were measured after having been left in the dark for 5 hours at room temperature:

1. 3 ml of vitamin B_{12b} solution + 1 ml of 0.2 *M* borate buffer p_H 9 + 1 ml of water

2. 3 ml of vitamin B_{12b} solution + 1 ml of 0.2 *M* borate buffer p_H 9 + 1 ml of 0.05% KCN.

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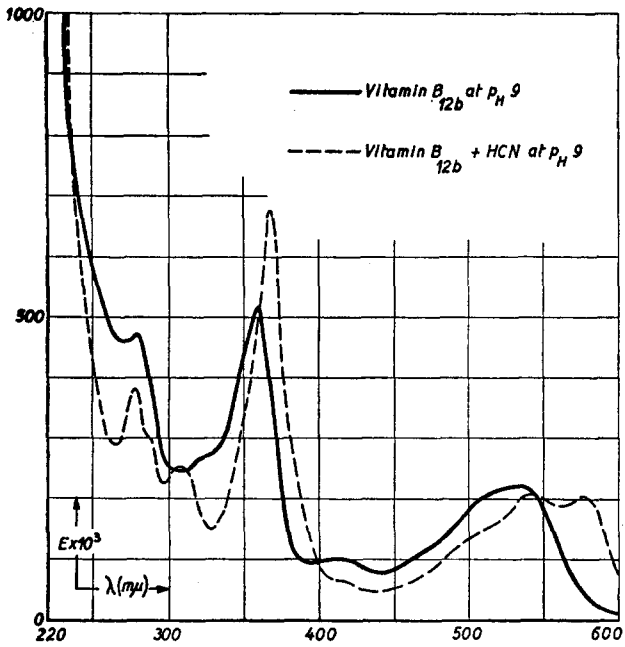

Fig. 2. Absorption spectra after standing at room temperature in the dark for 5 hours, of
——— Vitamin B_{12b} at p_H 9
----- Vitamin B_{12b} + HCN at p_H 9

A 0.04 *M* borate buffer served as the blank.

Whereas the first solution did not show any visible colour change, the second (with KCN) showed a conspicuous change, resulting in a bluish purple coloration in the course of the first 10 minutes. In Fig. 2 the absorption curves measured after 5 hours are recorded.

The control, without KCN, shows a shift already described by BROCKMAN *et al.*[4] for vitamin B_{12b} under the influence of NaOH. Even after a further 17 hours this spectrum remained substantially unaltered. A totally different spectrum was measured for the KCN containing solution, with maxima at 576, 542, 367, 307 and 278 mμ and with inflections at 420 and 285–290 mμ. After 22 hours, the peak at 576 mμ had considerably decreased, but the remainder of the curve, from 560 mμ to the lower wave lengths, still coincided with that obtained after 5 hours.

After acidification with HCl to p_H 6, an instantaneous colour change from bluish violet to purple occurred, and the spectrum, measured after 10 minutes, showed peaks at 545–552, 360–362 and 265–270 mμ. The solution was further acidified to p_H 3.5, whereupon, after one hour, the spectrum was determined and found to be identical with that of vitamin B_{12}. The shift from this alkaline reaction product to vitamin B_{12} is reversible, for if a vitamin B_{12} solution is kept overnight at p_H 9 with a trace of KCN, the spectrum of the alkaline reaction product results. An alkaline vitamin B_{12} solution without KCN shows an almost imperceptible change under otherwise identical conditions.

f. *The influence of HCN on a refined liver extract*

The absorption curves of a number of clinically and microbiologically active liver extracts, almost devoid of strongly interfering, highly coloured impurities, always had the same form with characteristic peaks in the range of from 530 to 540 mμ, and from 353 to 358 mμ, though the curves for different batches sometimes showed irregularities at other wave lengths. However, on the addition of KCN, all samples reacted as described in the preceding sections for vitamin B_{12b}. In an example taken at random a red extract, with maxima at 535 and 358 mμ, was treated with KCN at p_H 5.5 for 3 hours at 52° C, giving a solution now having maxima at 550 and 361 mμ.

As vitamin B_{12b} as well as vitamin B_{12} has been isolated from liver, the possibilities

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of the presence of a mixture of the vitamins B_{12b} and B_{12} in these extracts were explored. The technique, proposed by ELLIS, PETROW, AND SNOOK[10], *viz.* extraction of vitamin B_{12} with *n*-butanol from aqueous solutions, containing fairly high concentrations of ammonium sulphate, proved to give convincing evidence that the anti-anaemic principle is not present in either of these two forms, but in still another. Even on repeated extractions with butanol, a negligible amount of red colour could be withdrawn from a solution of the liver extract, saturated with $(NH_4)_2SO_4$. Any vitamin B_{12} or B_{12b} as such which might have been present, would have been detected in this way.

From the aqueous salt solution, together with the precipitate caused by the salt, the active substance was recovered, by a phenol passage. Part of the thus obtained solution was incubated with KCN at p_H 6 at 52° C, part served as control, to repeat the butanol extraction.

In the control, again no red colour could be extracted, thus proving that the recovery process had not materially altered the behaviour of the extract. From the KCN treated solution, in which the colour had markedly deepened, a red coloured butanol extract was obtained, after the $(NH_4)_2SO_4$ concentration had reached about 7%. By increasing the salt concentration all the red colour could be recovered from the butanol by repeated extractions.

From the butanol, the red colour was shaken back into water. The substance was isolated by shaking the aqueous layers with phenol, and from the phenol back into water by the addition of ether.

The resulting aqueous solution on measurement gave absorption peaks at 548 and 361 mμ. In the paper chromatogram, its behaviour was indistinguishable from that of vitamin B_{12}. On the other hand, the original liver extract before treatment with KCN, on chromatography moved very much slower, even more so than vitamin B_{12b}.

DISCUSSION

From these experiments, the conclusion can be drawn, that the vitamins B_{12a} and B_{12b} cannot be distinguished either by their absorption spectra or by paper chromatography with butanol.

By the addition of CN'-ions to their weakly acid solutions they are converted to a substance which could not be distinguished from vitamin B_{12} with these same criteria. This lead us already to the formulation of the relation between these factors, laid down in the first paper of this series:

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

On the basis of these data only, the restriction would have to be made that the crystalline vitamin B_{12} isolated from natural products might contain another group instead of cyanide. In addition, however, convincing evidence has been obtained that vitamin B_{12} actually is a cyan complex. This could *i.a.* be shown by making use of the reaction described by SNELL AND SNELL[11] for the detection of hydrocyanic acid.*

* Note added October 24th, 1950. These date are fully confirmed by two papers in *Science*, 112 (1950) 354, just becoming available, describing vitamin B_{12} as a cyano-cobalt-complex.

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In alkaline solution, another reaction product results under the influence of KCN. The reaction from this product to vitamin B_{12} appears to be reversible:

$$B_{12} \underset{H^+}{\overset{CN', OH'}{\rightleftarrows}} \text{alkaline reaction product.}$$

The question arises, which of these several modifications of vitamin B_{12} actually is present in the liver.

Though this question cannot yet be answered definitely and categorically, it can be said that in some liver extracts at least, vitamin B_{12} does *not* occur in any of the modifications described above. The latter can be extracted by butanol from an aqueous solution after the addition of $(NH_4)_2SO_4$, whereas in the investigated extracts, all the red colour remained in the aqueous phase. Under the influence of HCN at slightly acid reaction, these extracts alter their properties quite conspicuously. A spectral shift is observed, resulting in an absorption curve with the peaks of the vitamin B_{12} spectrum, and the red colour becomes soluble in butanol under the above mentioned conditions.

The fact that ELLIS *et al.*[10] could use the butanol extraction for their vitamin B_{12} concentrates from liver indicates that these authors used a process involving at some stage a liberation of the vitamin B_{12} from the compound in which it originally occurred. From our own experience we know that even after mild autolysis of the liver as much as 40% of the vitamin B_{12} may be found in the free state as judged from the solubility in butanol and from the spectrum. Even storage of the liver at —10° for some months will cause such an effect.

These results appear to be in agreement with those of LESTER SMITH *et al.*[12, 13] in their experiments on the chromatography of liver extracts over starch columns. Of the two bands observed, the slower could be transformed into the quicker moving band by papain digestion. In proteolysed liver the fast moving band predominated.

To our knowledge, no process has as yet been described for the isolation of vitamin B_{12} from liver, in which, at some stage or other, *no* proteolytic process is applied. KCN appears to be able to replace this proteolysis as it apparently removes a protein or peptide group, originally linked to the vitamin B_{12} molecule.

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SUMMARY

1. The vitamins B_{12a} and B_{12b} show identical absorption spectra in the visible and U.V. region. By paper chromatography with *n*-butanol as a solvent they cannot be distinguished.

2. Treatment of the vitamins B_{12a} and B_{12b} with HCN at p_H 5–6 yields a product with the same absorption spectrum and the same R_F value in paper chromatography with *n*-butanol, as vitamin B_{12}.

3. Under the influence of KCN at p_H 9 a reaction product with a purple blue colour is obtained from the vitamins B_{12a}, B_{12b} and B_{12}, with a characteristic absorption spectrum.

4. Evidence is given, suggesting that in some liver extracts all or part of the vitamin B_{12} may be present in a combined form. The linkage can be broken by KCN.

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RÉSUMÉ

1. Les vitamines B_{12a} et B_{12b} montrent des spectres d'absorption identiques dans la région visible aussi bien que dans celle d'U.V. La chromatographie de partage sur papier, avec le *n*-butanol comme solvant, ne montre, elle aussi, qu'une parfaite ressemblance des deux vitamines.

2. Le traitement des vitamines B_{12a} et B_{12b} par le CNH à p_H 5–6 donne un produit dont le spectre d'absorption et la valeur R_F ne diffèrent pas du tout de ceux de la vitamine B_{12}, quand on chromatographie tous les deux au moyen de papier avec le *n*-butanol comme solvant.

3. Sous l'influence du CNK à p_H 9, un produit de réaction est obtenu, à partir de chacune des trois vitamines B_{12a}, B_{12b} et B_{12}, d'un bleu violet et ayant un spectre d'absorption caractéristique.

4. Il y a lieu de présumer que dans certains extraits de foie soit la totalité soit une partie de la vitamine B_{12} est présente sous une forme combinée. C'est sous l'influence du CNK que cette combinaison peut être rompue.

ZUSAMMENFASSUNG

1. Die Vitamine B_{12a} und B_{12b} zeigen identische Absorptionsspektra im sichtbaren sowie auch im U.V.-Bereich. Bei Papierchromatographie mit *n*-Butanol als Lösungsmittel unterscheiden sie sich nicht von einander.

2. Behandlung der Vitamine B_{12a} und B_{12b} mit HCN bei p_H 5–6 ergibt ein Produkt, dessen Absorptionsspektrum und R_F-Wert bei Papierchromatographie mit *n*-Butanol mit denen des Vitamin B_{12} identisch sind.

3. Bei Einwirkung von KCN bei p_H 9 erhält man aus den Vitaminen B_{12a}, B_{12b} und B_{12} ein violettblaues Reaktionsprodukt mit charakteristischem Absorptionsspektrum.

4. Es liegen gewisse Gründe vor, um anzunehmen, dass in einigen Leberextrakten das Vitamin B_{12} — sei es völlig oder teilweise — in kombinierter Form anwesend ist. Die Bindung ist durch KCN spaltbar.

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