Biochemical Studies on Vitamin B_{12}. I~II[1]

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Introduction

Since the year 1948 the chemical and biological studies on vitamin B_{12} have made a very rapid progress due to the effort of E. L. Rickes, E. L. Smith and co-workers who isolated it in crystalline state and established its biochemical researches. Afterwards, many investigations have appeared on the physiologically active principle of vitamin B_{12} and the behavior of Animal Protein Factor (A.P.F.) has also been successfully confirmed.

The recent advance of the vitamin B_{12} chemistry caused the present authors to attempt further studies on a simple preparation of vitamin B_{12} concentrate (Part I) and biochemical synthesis of vitamin B_{12} from 5, 6-dimethyl-

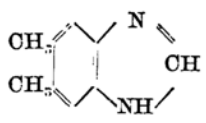
(1) This report was first read, on July 15, 1950, in the 40th meeting of the Vitamin B Research Committee in Japan.

benzimidazole (Part II), because there is little supply of A. P. F in Japan.

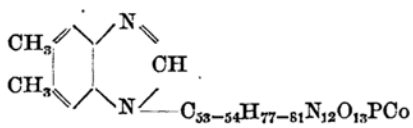
In part I, the isolation of vitamin B_{12} concentrate was carried out with the J. M. Pensack's [2] modified method, and physiological tests were thoroughly studied by Dr. S. Ito (bone marrow culture test), Dr. K. Inoue (clinical test) and Dr. K. Sakaguchi (L. L. D. active test).

In part II, the present authors attempted a few studies on the biochemical synthesis of vitamin B_{12} from 5, 6-dimethyl-benzimidazole.

In 1949, N. G. Brink *et al.*[3] isolated a degradation product from crystalline vitamin B_{12} by acid hydrolysis which has been identified by synthesis as 5, 6-dimethylbenzimidazole (A), and consequently proposed that a provisional formula for vitamin B_{12} may be represented as follows (B):—



(A)



(B)

In the same year V. Kocher *et al.*[4] reported that desoxyribosides of thymine, guanine, cytosine and hypoxanthine can replace vitamine B_{12} as a nutrient of *Lactobacilli*. In March 1950, T. Tomiyama[5] also found in the fish liver a new anti-anemia factor containing no cobalt.

Afterwards many investigations have been reported about the further characterization of vitamin B_{12}, but until quite recently (July 15th, 1950) no publication of biochemical studies on the implications of the vitamin B_{12} and 5, 6-dimethylbenzimidazole has been appeared.

In the physiological investigation of vitamin, it is already well known that the fresh animal tissue contains a substance which activates vitamin B_6 to pseudopyridoxine,[6] and, on the other hand, microorganisms such as yeast possess a power of synthesis of vitamin B_1 from pyrimidine portion and thiazole moiety.[7]

Guided by the above literatures, the present authors at first carried out synthetical experiment of (A), and the pure crystal (A) thus obtained was used in the following biosynthetical experiments.

Experimental

A Simple Preparation of Vitamin B_{12} Concentrate.

Vitamin B_{12} Concentrate.—A solution of 80% ethanol was added to the chopped fresh cow liver, and after refluxing for 5 hours, the mixture was filtered. Refluxing process was repeated three times and the extract was consolidated, evaporated and filtered. The filtrate was treated with activated charcoal, and the charcoal adsorbate was eluted with 5% NH_4OH in 50% ethanol.

On evaporating the filtrate, vitamin B_{12} concentrate was obtained in syrupy state. The vitamin B_{12} concentrate thus obtained was dissolved in saline solution and diluted to 9~10 γ of pure vitamin B_{12} per cc. according to our supposition.

Cobalt determination was tested by Dr. I. Tachi with the polarographic method.

Physiological Tests—Bone marrow culture test was carried out by Dr. S. Ito. 0.1×10^{-3}~0.1×10^{-4} diluted solution of the vitamin B_{12} concentrate gave the satisfactory responses in red blood cell (R.B.C.), white blood cell (W.B.C.) and reticulocyte. 0.1×10^{-2} diluted solution of the concentrate gave a W.B.C. value similar to that given by 0.1 cc. of normal blood serum and 0.1×10^{-1} diluted solution of concentrate gave a R.B.C. count similar to that given by 0.1 cc. of normal blood serum. Moreover, the bone marrow response given by 0.1 cc. of normal blood serum corresponded to 10 γ of folic acid.

Antagonistic activity test was negative against aminopterin and so the concentrate was proved to be free from folic acid. Furthermore, comparative studies of the bone marrow culture with standard solution of vitamin B_{12} (Merck's Cobione) were carried out by Dr. S. Ito. One cc. of the vitamin B_{12} concentrate was accurately estimated to correspond to 10 γ of pure vitamin B_{12}.

Clinical test was carried out by the cooperation of Dr. K. Inoue. For macrocytic anemia patients, favorable results were obtained by subcutaneous injection of 1~2 cc. per week. Details were reported in the Proceedings of the Vitamin B Research Committee (Vol. 36~40, 1950).

L.L.D. active test was also carried out with the microbiological assay by Dr. K. Sakaguchi.

Biochemical Synthesis of Vitamin B_{12} Active substance.

4, 5-Dimethylphenylenediamine (1, 2)-

(2) J. M. Peusack, R. M. Betheke and D. C. Kennard, *J. Nutrition*, **37**, 353 (1949).

(3) N. G. Brink and K. Folkers, *J. Am. Chem. Soc.*, **71**, 2951 (1949).

(4) V. Kocher and O. Schindler, *Inter. Z. Vitaminforsch.*, **20**, 441 (1949).

(5) T. Tomiyama, Reported in the 38th meeting of Vitamin B Research Comittee (1950).

(6) F. Schlenk and E. E. Snell, *J. Bio. Chem.*, **157**, 425 (1945).

(7) J. Ashida, *Bull. Agr. Chem. Soc. Japan*, **18**, 732 (1942); **19**, 719 (1943).

-dihydrochloride (C).—4, 5-Dimethylphenylenediamine was prepared from 3, 4-dimethyl-6-nitroaniline which was previously reserved for the experiments of synthetic flavin[8] from *p*-nitrotoluene and *s*-dichloromethylether. 3, 4-Dimethyl-6-nitroanilin was dissolved in glacial acetic acid and converted to 4, 5-dimethylphenylenediamine (1, 2) by catalytic reduction with platinum oxide, and to the resulting diamine concentrated HCl was added. On evaporating, the hydrochloride (C) was obtained in colorless needles, melting at above 280° (uncorr.). Found: N, 13.10. Calculated for $C_8H_{14}N_2Cl_2$: N, 13.39 %.

5, 6-Dimethylbenzimidazole (A).—4, 5-Dimethylphenylenediamine (1, 2)-dihydrochloride (C) was treated with formic acid in a boiling water-bath, and while still hot the mixture was filtered. The filtrate was neutralized with sodium hydroxide to slightly alkaline state, and on standing over night crystalline precipitates separated out. After recrystallizing the precipitates from hot water, (A) was obtained, fine needles melting at 200° (uncorr.). Found: C, 72.69, 72.66; H, 7.62, 7.23; N, 19.44, 19.82; Calculated for $C_9H_{10}N_2$: C, 73.97; H, 6.84; N, 19.01 %.

Biochemical Synthesis of (B) from (A) with the Fresh Chick Liver Paste.—Ten mg. of (A) was added to 20 g. of the fresh chick liver paste. The mixture was treated with a few drops of toluene, and autolyzed at 37° in a thermostat. After 60 hours, the reaction product was extracted with hot water and the clear filtrate was collected. On evaporating under the diminished pressure, the residual matter was dissolved in 10 cc. of water. The vitamin B_{12} potency was estimated by the microbiological assay using *Lactobacillus lactis* Dorner. The blank test was also carried out with an ordinary chick liver paste in the same condition. The comparative data are shown in Table 1.

Table 1

Vitamin B_{12} Contents in the Fresh Chick Liver Paste Treated with 5, 6-Dimethylbenzimidazole Added.

Contents of vitamin B_{12}	Vitamin B_{12} potency in 2 g. of fresh chick liver, γ	Vitamin B_{12} potency in 100 g. of chick liver, γ
Fresh liver paste (control)	0.22	11.0
Fresh liver paste treated with 5, 6-dimethylbenzimidazole	0.27	13.5

The above result indicated about 20~25 % increase of vitamin B_{12} potency in the chick liver paste by the treatment of (A).

Synthetical Experiments of (B) with Streptomyces from (A).—To the Dulaney broth for Streptomyces x-28, (A) was added and after six days' incubation the broth was assayed microbiologically by *Lactobacillus lactis* Dorner. The results obtained are shown in Table 2.

Table 2

Vitamin B_{12} Potency in the Broth of Streptomyces by Adding 5, 6-Dimethylbenzimidazole.

Broth	Vitamin B_{12} potency in the broth, u/cc.* Exp. 1.	Exp. 2.	Control exp.
Dulaney broth	37.5	50.0	0
Dulaney broth + $CoCl_2$ 0.06 mg./50cc.	50.0	51.0	0
Dulaney broth + 5, 6-dimethylbenzimidazole 1 mg./50 cc.	90.0	98.0	0

* u = L. L. D. unit (1 γ = 11000 L. L. D. units)

The above experiments demonstrated about 100~150 % increase of vitamin B_{12} potency in the broth of streptomyces by adding (A), while the addition of $CoCl_2$ seemed little effective. On the other hand, the effect of pure (A) and inorganic cobalt ions upon the growth of L. L. D. was tested, but no response for the particular increase of vitamin B_{12} potency was observed.

Summary

In part I, a simple method for isolating vitamin B_{12} concentrate was studied.

In part II, (A) was decided to be a prosthetic group in natural (B) molecule biochemically, and the biosynthesis of (B) from synthetic (A) could be attained successfully.

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(8) Y. Sahashi, *Proc. Imp. Acad.*, **21**, 14 (1945).