VITAMIN ASSAY

Determination of Vitamin B_{12} Content of Feed Supplements and Effect of Pseudo-Vitamin B_{12}

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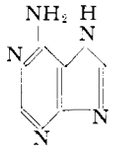
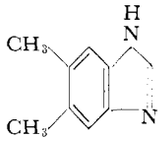
A number of microbially active compounds which are similar to vitamin B_{12} but have no effect on animal growth have been reported. Because such compounds may be present in crude vitamin B_{12} products used for animal feeds, a number of commercial APF products were examined for vitamin B_{12} content by various assay methods. The results obtained indicate that the microbial assay and the spectrophotometric assay methods do not differentiate between vitamin B_{12} and pseudo-vitamin B_{12}. One commercial product was found to contain no vitamin B_{12}. The microbially active ingredient was isolated from this product in pure crystalline form and identified as pseudo-vitamin B_{12}. The average vitamin B_{12} content of the feed supplements was found to be somewhat lower by an isotope dilution assay than by the U.S.P. microbial assay. These findings indicate that full reliance on the U.S.P. microbial assay method or the spectrophotometric assay method for determination of the vitamin B_{12} content of crude concentrates may lead to grossly erroneous results.

SINCE THE ISOLATION of vitamin B_{12} and its analogs, other compounds have been reported which are chemically similar to the cobalamins but which exhibit little or no growth-promoting activity in animals. Most of these compounds have been isolated from animal feces or anaerobic fermentations (*7, 8, 13, 17*).

Pfiffner (*15*) has reported a compound, pseudo-vitamin B_{12}, isolated from an anaerobic fermentation which has many of the properties of vitamin B_{12} including microbial growth activity with several test organisms and an absorption spectrum almost identical with that of vitamin B_{12}. The compound has the ability to bind additional cyanide to form a dicyanide complex and to release bound cyanide on exposure to light in the same manner as that of vitamin B_{12}. However, the psuedo-vitamin B_{12} is inactive in vitamin B_{12} tests of animal growth and can be differentiated from vitamin B_{12} by paper strip chromatography, ionophoresis (*11*), and countercurrent extraction with benzyl alcohol–water. Dion reported (*4*) that on acid cleavage pseudo B_{12} yields adenine rather than 5,6-dimethylbenzimidazole which is obtained with vitamin B_{12}.



Adenine



5,6-Dimethylbenzimidazole

The importance of these findings in the determination of the vitamin B_{12} content of crude concentrates, especially animal feed supplements which normally contain 3 to 20 mg. of vitamin B_{12} per pound (0.0007 to 0.005%), is obvious. Certainly, microbial growth activity with the test organisms now in prevalent use (*L. leichmannii*, *Lactobacillus lactis* Dorner, and *Escherichia coli*) cannot be the sole criterion for establishing the true vitamin B_{12} content of such concentrates. A microbial assay method (*2, 6*), highly specific for cyanocobalamin (or vitamin B_{12} adjuncts which are readily converted to cyanocobalamin) has recently been reported which utilizes the *Ochromonas* organism. The *Ochromonas* assay, no doubt, will find ready application in the crude vitamin B_{12} concentrate field.

A chemical method for determination of vitamin B_{12} in fermentation broths has been described by Rudkin (*16*) and is based on the difference between the absorption spectrum of vitamin B_{12} and the absorption spectrum of the dicyanide complex. These laboratories have found several red pigmented compounds similar to vitamin B_{12} which also form the dicyanide complex, thus indicating that this chemical method can be used as a quantitative procedure only after a determination has been made to ensure that the dicyanide complex formed is solely due to vitamin B_{12}. The data presented indicate that complete reliance on the spectrophotometric assay may lead to grossly erroneous results.

With the isolation of radioactive Cobalt-60 vitamin B_{12} (cyanocobalamin) in crystalline form (*3*), the isotope dilution method became applicable to the determination of vitamin B_{12} potency and has proved to be a valuable tool in these laboratories for evaluating and controlling the vitamin B_{12} content of crude concentrates and commercial feed supplements. The isotope dilution assay must satisfy two criteria:

1. The cobalamins present in the concentrate or feed supplement must be converted to cyanocobalamin (the form

of the radioactive vitamin B_{12}) prior to the addition of the tracer.

2. The cyanocobalamin must be isolated and differentiated from similar compounds so that a measurement of the isolated material is essentially that of cyanocobalamin alone.

Conversion may be accomplished by treatment of the concentrate solution with cyanide at a slightly alkaline pH for a number of hours, or preferably, by boiling the aqueous concentrate for a few minutes in the presence of nitrite and cyanide salts. The radioactive tracer yield is applied to the isolated cyanocobalamin to give the vitamin B_{12} content of the concentrate. Details of an isotope dilution method which meets the two criteria mentioned have been described (*1*).

Samples of various commercial vitamin B_{12} products (presumably from fermentation sources) as well as a vitamin B_{12} concentrate derived from sewage have been evaluated for vitamin B_{12} content by the isotope dilution method described by Bacher *et al.* (*1*) and compared to that obtained by the U.S.P. *L. leichmannii* titrimetric and turbidimetric assay methods. The samples were also assayed for vitamin B_{12} by the *Lactobacillus lactis* Dorner (*9*) and *Escherichia coli* (*10*) microbial cup assay methods. A few samples have been evaluated for vitamin B_{12} activity using a chick growth test method (*14*). The comparative results given in Table I indicate that the average vitamin B_{12} microbial growth activity assayed by the U.S.P. microbial method is 16% higher than that found by the isotope dilution assay method. One of the commercial products (Product B in Table I) was found to contain essentially no vitamin B_{12} by the isotope dilution assay. This lack of vitamin B_{12} was substantiated by chick growth tests. The active principle in the product was isolated in crystalline form and was identified as pseudo-vitamin B_{12} (Method III). The vitamin B_{12} content of the sewage product as measured by the *L. leichmannii* assay was in good agreement with the isotope dilution assay but was two and one-half times this value when measured by the *E. coli* cup assay method.

The commercial (animal protein factor) products were evaluated for vitamin B_{12} content by the Rudkin and Taylor (*16*) spectrophotometric method and also by the Fisher (*5*) modification of the spectrophotometric method. Both methods gave questionable results due to mechanical difficulties such as emulsions which lower the efficiency of vitamin B_{12} extractions, and errors in measuring small differences in absorption due to vitamin B_{12} in the presence of excessive colored impurities. The results obtained by the spectrophotometric methods are shown in Table I. The spectrophotometric method does not differentiate between vitamin B_{12} and pseudo-vitamin B_{12} (product B).

Methods

Method I The dry feed supplements were prepared for assay by slurrying a sample containing the equivalent of 1 to 2 mg. of vitamin B_{12} activity in 500 ml. of water, and boiling for 5 minutes in the presence of 5 grams of sodium nitrite and 2 grams of potassium cyanide at pH 4.0. The vitamin B_{12} analogs thus converted to cyanocobalamin were assayed microbially, and by the isotope dilution method. The dry commercial product was used in the chick assays.

Method II In the Rudkin and Taylor assay method, the dry feed supplement products were slurried in water, and boiled in the presence of nitrous acid and cyanide ions for the dual purpose of releasing vitamin B_{12} activity from the dried solid, and converting all the vitamin B_{12} analogs to cyanocobalamin. It was necessary to filter the slurry after the heat treatment to ease the emulsion difficulties encountered with benzyl alcohol extraction. In those cases where emulsion difficulties persisted after filtration, defecation of the filtrate with zinc hydroxide prior to extraction aided considerably. With product B, the microbial activity was only partially extracted by the benzyl alcohol.

The mechanical difficulty encountered in the Fisher modification of the Rudkin and Taylor method was the turbidity of the extracts obtained for spectrophotometric reading, especially the pH 6.0 buffered solution. The turbidity could not always be removed by filtration and led to erroneous absorption readings. Some samples gave intensely colored extracts even after transfer of the vitamin B_{12} activity from benzyl alcohol into water with chloroform; thus the difference in absorption between the vitamin B_{12} and the dicyanide complex of vitamin B_{12} at 580 mμ was small in comparison to the total absorption of the solutions. In two cases, negative values for vitamin B_{12} content were obtained—i.e., the solution containing the dicyanide complex gave a lower absorbance reading than did the pH 6.0 vitamin B_{12} solution.

In order to check the extraction efficiency figure of 97% used in the calculation by both the Fisher and the Rudkin assay methods, radioactive vitamin B_{12} tracer was added to some of the

Table I. Comparison of Assay Methods for Vitamin B_{12} in Commercial APF Products

		Vitamin B_{12} Assay Method, Mg. Vitamin B_{12}/Lb.							
			L. leichmannii, U.S.P.		Microbial Cup Assay		Spectrophotometric		
Manufacturer	Label, Mg. B_{12}/Lb.	Isotope dilution	Titrimetric	Turbidimetric	LLD	E. coli	Rudkin and Taylor	Fisher	Chick Growth Assay
A	13.5	11.6	13.5	13.7	13.3	10.8	8.0	0.0	...
B	113.0	<0.3	77.0	58.5	98.0	220.0	11.6	113, 71, 94	<4.0
B_1	25.0	0	...	...	...	...	3.3	25	...
C	12.5	8.7	10.0	10.0	11.0	9.8	6.7	3.4	...
D	12.5	8.5	11.0	9.0	8.0	8.5	7.7	10.3	...
E	12.5	11.4	12.5	11.0	11.5	10.5	7.3	14.5	...
F	10.0	8.8	9.5	11.5	9.5	9.4	12.5, 9.6, 6.0	91.0	...
G	10.0	8.6	8.5	10.0	9.0	6.9	4.2	0.0	...
H	6.0	6.3	8.2	7.0	7.3	6.4	4.2	0.0	...
I	6.0	5.5	6.5	6.0	4.5	9.0	3.2	0.0	...
J	6.0	4.0	3.8	4.0	4.8	4.5	1.3	2.9	...
K	6.0	5.6	9.6	8.5	13.6	8.3	4.3	2.0	...
L	3.0	3.0	4.2	3.9	4.7[a]	3.7	1.8	3.7	2.6 ± 0.5
Sewage product	...	1.6	1.7	...	...	4.1	...	...	1.1 ± 0.2
Sewage product extract	...	11.3	12	...	...	...	...	...	...
Average[b]	8.9	7.5	8.3	8.6	8.8	8.4	5.3	3.7[c]	...

[a] The presence of penicillin in this preparation interfered with the LLD assay. The use of penicillinase eliminated interference.
[b] Average includes products A through L and excludes products B and B_1.
[c] This average does not include product F which is abnormally high.

commercial concentrates prior to extraction with benzyl alcohol. The results obtained indicate a tracer yield ranging from 84 to 90%.

Method III A red compound isolated from product B in crystalline form, in about a 40% yield of the labeled content, was characterized as pseudovitamin B_{12} by the following studies.

The absorption spectrum of an aqueous solution of the crystals is nearly identical with that of vitamin B_{12} (see Figure 1), the exception being a missing shoulder at 284 to 285 mμ and higher absorption below 280 mμ. The absorbance at the 361 mμ and 550 mμ peaks of the isolated compound is essentially the same as that of vitamin B_{12}. The isolated material gave up one cyanide molecule in acid solutions when exposed to light and formed a purple dicyanide complex in the presence of excess cyanide ions at alkaline pH.

Differences from Vitamin B_{12}. The compound has essentially no growth activity in chicks (0.13% of the efficacy of vitamin B_{12}).

The compound has a high distribution constant in the system water–benzyl alcohol (*Cw/Cs* approximately 8 compared to 1.2 for vitamin B_{12}). The dicyanide complex also has a high extraction ratio for water.

The nitrogen content (16.7%) is higher than that of vitamin B_{12} (14%).

An ionophoretic paper strip of the crystalline isolate was compared to that of vitamin B_{12} and chlorocobalamin. The ionophoresis was run for 16 hours at 300 volts using 0.5*N* acetic acid as the electrolyte with 0.5*N* acetic acid and 0.01% potassium cyanide in the bridge. The comparative ionophoretic results (Figure 2) indicate the isolate to be one substance and to have a mobility of 2.25×10^{-5} sq. cm. $volt^{-1}$ $sec.^{-1}$ as calculated by a method described by Kunkel and Tiselius (*12*). The mobility of the chlorocobalamin was found to be 5.03×10^{-5} sq. cm. $volt^{-1}$ $sec.^{-1}$. The mobility of vitamin B_{12} was taken as zero although some movement was observed probably due to electro-osmosis.

Paper strip work with acid hydrolysis products of the crystals gave a definite indication of the presence of adenine but not 5,6-dimethylbenzimidazole. The crystals were treated with 0.5*N* hydrochloric acid and the hydrolyzate subjected to paper strip chromatography in an ascending system using a 5% aqueous disodium phosphate–isoamyl alcohol developing system (two phases). The fluorescent spots of adenine and adenylic acid were located at an R_f 0.44 and 0.7, respectively, using ultraviolet light.

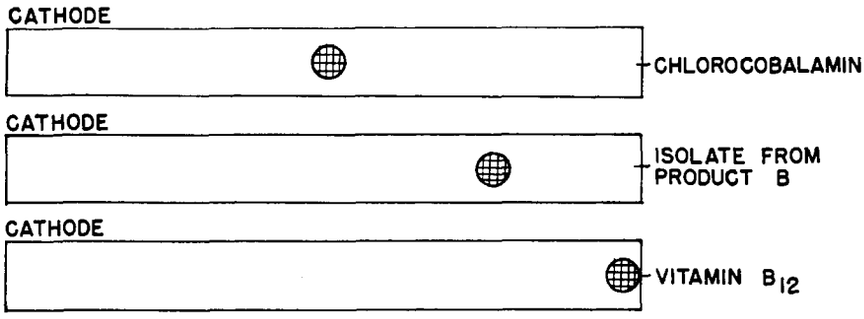


Figure 2. Ionophoresis of vitamin B_{12}, chlorocobalamin, and the crystalline isolate
From product B with 0.5 *N* acetic acid for 16 hours at 300 volts

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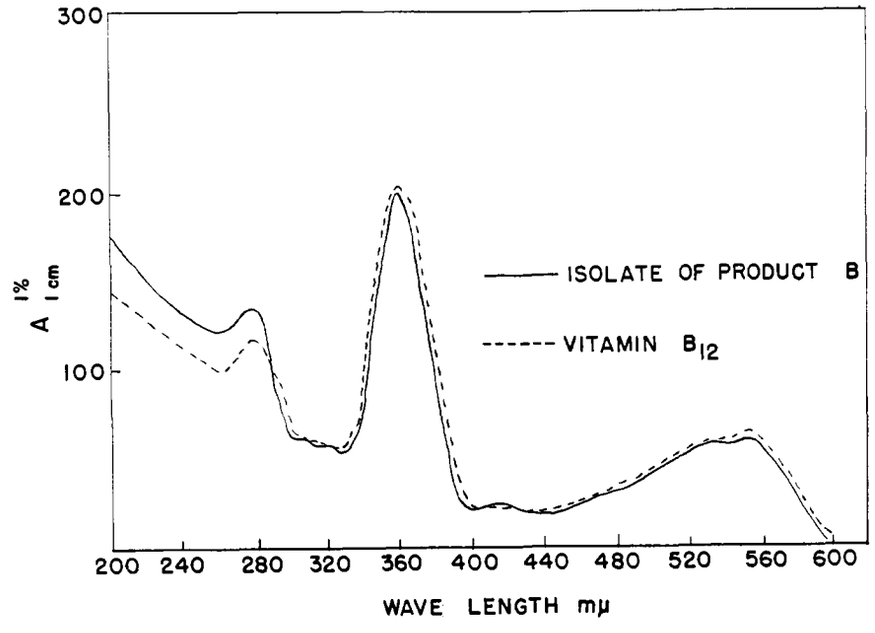
Figure 1. Absorption spectrum of crystalline isolate from product B



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