A Differential Microbiological Assay for Vitamin B_{12} and Pseudovitamin B_{12}[1,2]

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Received for publication September 22, 1955

Although vitamin B_{12} can be assayed biologically with mice, chicks or rats, the microbiological method of assay is preferred since it is more rapid and economical. One serious shortcoming of many microbiological B_{12} assay procedures is the lack of specificity when crude materials are assayed. Crude materials can contain inhibitory or stimulatory factors which cause serious errors in assay results.

One of the interfering substances in the microbiological assay of vitamin B_{12} with *Lactobacillus leichmannii* is pseudovitamin B_{12}. Pfiffner *et al.* (1951) isolated an organism from bovine rumen contents which produced a new form of vitamin B_{12} which was completely inactive for chicks, rats, and humans, but was active for *L. leichmannii* and *Lactobacillus lactis* var. Dorner. These workers crystallized two major red pigments from an anaerobic fermentation broth containing this rumen organism and named the pigments pseudovitamin B_{12} and pseudovitamin B_{12}b. The chemical differences between pseudovitamin B_{12} and vitamin B_{12} have been described (Dion *et al.*, 1952; Pfiffner *et al.*, 1952). Lewis *et al.* (1952) reported that a vitamin B_{12} analog which they designated vitamin B_{12}f appeared to be identical with pseudovitamin B_{12}. Using paper chromatography in conjunction with a bioautographic technique, these workers found that vitamin B_{12}f was present in the fecal matter of the rat, cow, sheep, pig, horse, chicken, guinea pig, and man. Sheep rumen contents were also a source of vitamin B_{12}f. No vitamin B_{12}f was detected in rat or beef liver or in a sample of injectable liver extract.

The experimental work described in this paper was conducted in an attempt to develop a simple microbiological procedure for detecting the presence of pseudovitamin B_{12} in crude samples. In addition, the application of the method in the differential quantitative assay of vitamin B_{12} and pseudovitamin B_{12} was also investigated.

The observation by O. D. Bird (personal communication, 1950) that pseudovitamin B_{12} was almost as potent as vitamin B_{12} for *L. leichmannii* 7830, but was much more active than vitamin B_{12} for *Lactobacillus acidophilus* 832, was the basis for this work.

Materials and Methods

The test organisms, *Lactobacillus leichmannii* strain ATCC 7830 and *L. acidophilus* strain ATCC 832 were carried in stock cultures using a tomato juice-yeast extract agar culture medium (A.O.A.C., 1953). The same medium minus agar was used for broth subcultures of the organisms. In preparing the inoculum for assays, 24-hour subcultures of the organisms were centrifuged and the cells washed three times with saline. The turbidity of the suspended cells was adjusted to 70 per cent light transmission (Coleman Model 14 Spectrophotometer) for *L. leichmannii* and 50 per cent transmission for *L. acidophilus*. One loop of the cell suspension was used to inoculate each assay tube.

The assay medium for both organisms was a commercial vitamin B_{12} assay broth[4] (Pharmacopeia of the United States, 1951) supplemented with 400 μg of pantethine[5] per liter of double strength medium. Five ml of the double strength medium were added to each assay tube. The assay tubes were 16 x 20 mm and were graduated at 10 ml. The volume of each tube was adjusted to 10 ml with water after the samples were added. The tubes were covered with glass caps and sterilized at 15 pounds pressure for 5 minutes. After cooling, the tubes were inoculated with a wire loop and then incubated for 20 hours at 37 C. Growth was determined turbidimetrically using a Coleman Spectrophotometer containing a PC-4 filter at a wave-length setting of 650 mμ.

Samples were extracted with 0.1 N acetate buffer containing 1 per cent sodium bisulfite. In most experiments a trace of cyanide was also added to prevent the conversion of vitamin B_{12} to B_{12}a. The assay results on crude samples were the same in the presence or absence of cyanide. After autoclaving at 15 pounds pressure for 20 min, the sample was adjusted to pH 6, brought to

[1] Supported in part from funds granted to The Ohio State University by the Research Foundation for aid in fundamental research.

[2] From a thesis submitted by D. Berman to the Graduate School of The Ohio State University in partial fulfillment of the requirements for the degree of Master of Science.

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[4] Difco Bacto-B_{12} Assay Medium USP, dehydrated.

[5] Kindly supplied by Dr. O. D. Bird, Parke, Davis and Co., Detroit, Michigan.

TABLE 1. *The growth of Lactobacillus leichmannii and Lactobacillus acidophilus in Difco medium with and without added pantethine**

Organism	Vitamin B_{12} per Tube	No Pantethine	Plus Pantethine*
		Light transmission	Light transmission
	mμg	*per cent*	*per cent*
L. leichmannii	0.0	100.0	100.0
	0.04	67.0	67.0
	0.08	45.5	42.5
	0.12	35.5	36.5
	0.20	27.5	25.0
L. acidophilus	0.0	100.0	100.0
	0.4	93.5	69.5
	0.8	100.0	54.0
	1.2	100.0	41.5
	1.6	98.0	34.5

* Four hundred μg of pantethine added per liter of double strength medium.

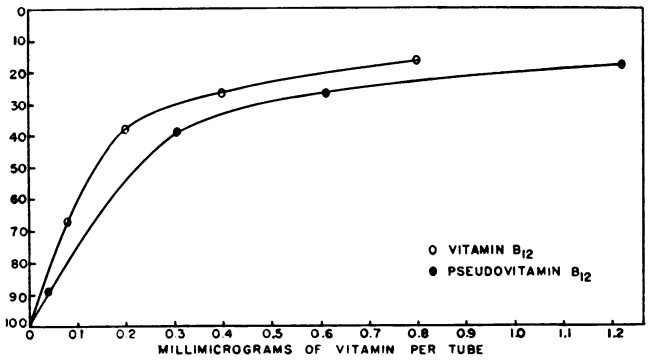

FIG. 1. The growth response of *Lactobacillus leichmannii* ATCC 7830 to vitamin B_{12} and pseudovitamin B_{12}.

a convenient volume, filtered and the filtrates assayed. Four or more levels of each sample were used in an assay. Duplicate tubes were used for each level of sample. None of the samples assayed contained appreciable alkali-stable activity, thus indicating the absence of desoxyribosides.

Crystalline vitamin B_{12}[6] was used as a standard in the range of 0.01 to 0.8 millimicrograms per tube for *L. leichmannii*, and from 0.05 to 2.0 millimicrograms for *L. acidophilus*. Crystalline pseudovitamin B_{12}[7] was used in the range of 0.04 to 1.2 and 0.02 to 0.2 millimicrograms per tube for *L. leichmannii* and *L. acidophilus* respectively.

RESULTS AND DISCUSSION

In order to employ *L. leichmannii* and *L. acidophilus* in a differential assay to detect pseudovitamin B_{12}, it was deemed advisable to first find a convenient assay

[6] Kindly supplied by Dr. E. L. R. Stokstad, Lederle Labs. Pearl River, New York

[7] Kindly supplied by Dr. J. J. Pfiffner, Parke, Davis and Co., Detroit, Michigan

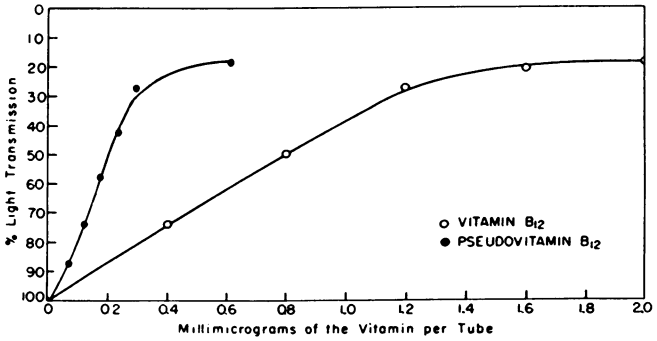

FIG. 2. The growth response of *Lactobacillus acidophilus* ATCC 832 to vitamin B_{12} and pseudovitamin B_{12}.

medium which would support growth of both organisms.

The Difco medium was found to be a satisfactory assay medium for *L. leichmannii*; however, *L. acidophilus* would not grow in this medium unless pantethine was added. Table 1 shows that the Difco medium supported good growth of *L. acidophilus* when supplemented with pantethine. The addition of pantethine had no effect on the response of *L. leichmannii* to vitamin B_{12}.

Repeated assays were conducted in which the relative potency of vitamin B_{12} and pseudovitamin B_{12} was determined for both test organisms. The results of two typical assays are shown in figures 1 and 2. The average of a number of assays showed that pseudovitamin B_{12} was 0.66 as active as vitamin B_{12} for *L. leichmannii*. This agrees with the potency of 0.7 observed by Pfiffner *et al.* (1954). In the *L. acidophilus* assay, pseudovitamin B_{12} was 3.3 times as active as vitamin B_{12}.

Using crystalline vitamin B_{12} as a standard, mixtures of crystalline vitamin B_{12} and crystalline pseudovitamin B_{12} were assayed with both organisms to learn if there was any interaction between the two analogs. The results of these assays are shown in table 2. The experimental activity of the mixtures was in good agreement with the calculated theoretical activity. The method of calculation using the factors 0.66 and 3.3 for the potency of pseudovitamin B_{12} is shown at the bottom of table 2.

Based on these results, it is obvious that a sample containing only vitamin B_{12} would yield identical assay results with either *L. leichmannii* or *L. acidophilus*. If the sample contained pseudovitamin B_{12} as well as vitamin B_{12}, the *L. acidophilus* assay would give a higher value since pseudovitamin B_{12} is 3.3 times as active as B_{12} for *L. acidophilus*. If we let x equal the vitamin B_{12} content, then $x + 0.66y$ would equal the assay value obtained using *L. leichmannii* and $x + 3.3y$ would equal the assay values obtained using *L. acidophilus*. Thus we have two equations and two unknowns which can be solved simultaneously to determine the vitamin B_{12} and pseudovitamin B_{12} content of the sample.

TABLE 2. *Microbiological assay of mixtures containing crystalline vitamin B_{12} and pseudovitamin B_{12}*

Assay Organism	Composition of Mixture		Theoretical Activity*	Experimental Activity†	Per Cent of Theoretical
	Vitamin B_{12}	Pseudovitamin B_{12}			
	mμg	mμg	mμg	mμg	
Lactobacillus leichmannii (Typical assay)..	0.05	0.030	0.070	0.066	95
	Avg of 4 experiments involving 20 mixtures, 90. Range, 85–97.				
Lactobacillus acidophilus (Typical assay)...	0.10	0.029	0.20	0.21	105
	Avg of 4 experiments involving 28 mixtures, 98. Range, 92–104.				

* For *L. leichmannii* theoretical activity was calculated using the factor of 0.66 as the potency of pseudovitamin B_{12}. That is, B_{12} in mixture + (0.66) × (pseudovitamin B_{12} in mixture) = theoretical B_{12} activity. For *L. acidophilus* theoretical activity was calculated using the factor 3.3 as the potency of pseudovitamin B_{12}. That is, B_{12} in mixture + (3.3) × (pseudovitamin B_{12} in mixture) = theoretical B_{12} activity.

† Experimental B_{12} activity was determined by assaying the mixture using crystalline vitamin B_{12} as the standard.

To test the accuracy of the above calculations, an unknown mixture of the crystalline vitamins was prepared and assayed with both microorganisms. The unknown mixture contained 3.0 millimicrograms of vitamin B_{12} and 0.47 millimicrograms of pseudovitamin B_{12} per ml. The average results of 4 assays calculated by use of simultaneous equations showed that the unknown mixture contained 3.12 millimicrograms of vitamin B_{12} and 0.423 millimicrograms of pseudovitamin B_{12} per ml. The experimental data was 104 per cent of theoretical for vitamin B_{12} and 90 per cent of theoretical for pseudovitamin B_{12}.

The results obtained from the assay of various samples are presented in table 3. Liver and kidney samples and vitamin B_{12} supplement #2 appear to contain only vitamin B_{12}; fish solubles, rabbit feces and chick droppings contain appreciable amounts of non-B_{12} activity. Vitamin B_{12} supplement #1 may contain a small quantity of non-B_{12} activity. These results are in good agreement with the distribution of B_{12} analogs reported by other workers (Lewis *et al.*, 1952; Coates *et al.*, 1953).

Assuming that vitamin B_{12} and pseudovitamin B_{12} are the only two growth stimulatory factors present in fish solubles, rabbit feces and chick droppings, the concentration of both of these vitamins can be calculated using the simultaneous equations mentioned previously. The results of such theoretical calculations showed the following vitamin content per g: fish solubles #1, 0.26 μg of B_{12} and 0.17 μg of pseudovitamin B_{12}; fish solubles #2, 0.23 μg of B_{12} and 0.095 μg of pseudovitamin B_{12}; chick droppings, 0.013 μg of B_{12} and 0.087 μg of pseudovitamin B_{12}.

TABLE 3. *Microbiological assay of various materials with Lactobacillus leichmannii and Lactobacillus acidophilus*

Sample	Avg. B_{12} Activity		Pseudovitamin B_{12} or Other Analogs Present
	L. leichmannii	*L. acidophilus*	
	μg/g	μg/g	
Lyophilized beef liver.....	2.14	2.22	No
Lyophilized beef kidney.....	0.85	0.89	No
Fresh chick liver.....	0.13	0.14	No
Fresh chick kidney.....	0.14	0.14	No
Fish solubles #1*.....	0.37	0.83	Yes
Fish solubles #2*.....	0.29	0.54	Yes
Vitamin B_{12} feed supplement #1.....	11.50	14.20	?
Vitamin B_{12} feed supplement #2.....	2.53	2.40	No
Rabbit feces.....	0.31	1.40	Yes
Fresh chick droppings†.....	0.07	0.30	Yes

* These samples were stored at room temperature for several months before they were assayed.

† Obtained from vitamin B_{12} deficient chicks which were 5 weeks old.

The reports of Holdsworth (1953) and Ford and Porter (1953) show that crude products such as feces contain B_{12} analogs called Factors A, B, and C in addition to pseudovitamin B_{12}. The presence of numerous analogs of B_{12} in natural products and the difference in microbiological potency of these analogs makes it very unlikely that the differential assay procedure described in this paper is applicable to quantitative assays of crude samples.

DISCUSSION

The detection of non-B_{12} activity in crude samples appears to be possible by means of assaying the sample with the two test organisms using the method described here. This method involves one medium and that medium can be obtained commercially. The method is therefore more simple than the chromatographic and bioautographic procedures of Lewis *et al.* (1952) or the microbiological procedures used by Coates *et al.* (1953).

The use of the quantitative differential assay procedure is satisfactory when using pure mixtures of vitamin B_{12} and pseudovitamin B_{12}, but is probably not valid for crude products since they may contain additional B_{12} analogs.

Ford (1953) developed a microbiological B_{12} assay which he reported to be specific for vitamin B_{12}. The procedure employes the organism *Ochromonas malhamensis* in a 72-hour assay at 29 C with constant shaking during the incubation period. The procedure outlined in this paper for the detection of non-B_{12} activity in crude samples might have application in the rapid screening of a large number of samples. Those

samples which show higher values with *L. acidophilus*, thus indicating the presence of non-B_{12} activity, could then be reassayed using the more tedious method employing *Ochromonas malhamensis*.

The presence of non-B_{12} activity in samples of fish solubles is of interest since fish solubles are used commercially as a source of vitamin B_{12} in animal feeds. Fish solubles are stored in large tanks without refrigeration and it is quite possible that the B_{12} analogs are synthesized by microorganisms which contaminate this product.

ACKNOWLEDGMENT

We wish to thank Dr. O. D. Bird of Parke, Davis and Company for furnishing information regarding the pantethine requirements of *L. acidophilus*.

SUMMARY

A simple microbiological procedure is described for the detection of pseudovitamin B_{12} and other non-B_{12} activity in crude samples. The method employs a differential assay using *Lactobacillus leichmannii* 7830 and *Lactobacillus acidophilus* 832 as the test organisms.

Using this method it appeared that lyophilized beef liver, lyophilized beef kidney, fresh chick liver, and fresh chick kidney contained only vitamin B_{12}. Fish solubles, rabbit feces and fresh chick droppings contained appreciable amounts of non-B_{12} activity.

The differential assay procedure was quantitative when mixtures of pure vitamin B_{12} and pseudovitamin B_{12} were assayed, but was probably not quantitative for crude samples due to the presence of other vitamin B_{12} analogs.

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