Microbiological Assay of Vitamin B_{12} with a Mutant Strain of *Escherichia coli*[1]

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Two strains of *Lactobacillus leichmanii*, ATCC 7830 and ATCC 4797, are generally used for vitamin B_{12} assay. However, the complex growth requirements and the lack of specificity to vitamin B_{12} of these lactic acid bacteria make the determination of vitamin B_{12} difficult (Hoffmann *et al.*, 1948; Skeggs *et al.*, 1948; Snell *et al.*, 1948; Capps *et al.*, 1949; Kitay *et al.*, 1949; Peeler *et al.*, 1949; Winsten and Eigen, 1949; Thompson *et al.*, 1950; Emery *et al.*, 1951). *Euglena* responds to the vitamin more specifically and to a much lower concentration, but the prolonged time of incubation (4 to 7 days) is a handicap in routine assay (Hutner *et al.*, 1949).

Davis and Mingioli (1950) reported the isolation of a number of mutants of *Escherichia coli* which require either vitamin B_{12} or methionine for growth in mineral medium. In contrast to the lactic acid bacteria, the B_{12}-requiring mutants did not respond to thymidine. Because of the simple medium required, ease of growing the organism, and the specificity of its B_{12} requirement, a study was made to ascertain the suitability of such a mutant as assay organism for vitamin B_{12}. Bessell *et al.* (1950) used mutant *E. coli* 113-3 and Harrison, Lees, and Wood (1951) used a mutant of *E. coli* for a plate assay of B_{12}. Johansson (1951) reported briefly a turbidimetric procedure. At the time of completion of this work, Burkholder (1951) reported the study of the same mutant for B_{12} assay in a modified medium.

Experimental Methods

The organism used was *E. coli* 113-3, a mutant kindly supplied by Dr. B. D. Davis of Cornell University. The medium was that of Davis and Mingioli (in g per liter: K_2HPO_4, 7.0; KH_2PO_4, 3.0; Na-citrate·$3H_2O$, 0.5; $MgSO_4 \cdot 7H_2O$, 0.1; $(NH_4)_2SO_4$, 1.0; glucose, 2.0) with pH adjusted to 6.8. For routine assay, the tubes (18 x 150 mm) containing the medium without glucose, were autoclaved at 115 C for 10 min. The glucose was autoclaved separately and added aseptically. The inoculum was grown in the medium supplied with 0.2 per cent of acid hydrolyzed casein and 0.5 mμg of B_{12} per 10 ml at 30 C for 8 to 12 hr, centrifuged, washed once and resuspended in 0.9 per cent sterile saline to give a transmission of about 70 per cent with a 540 mμ filter in an Evelyn colorimeter. One drop of the washed and diluted inoculum was added to each of the assay tubes containing a total 10 ml of medium. The tubes were placed in a rack in a tilted position on a reciprocating shaker (84 strokes of 10 cm length per min) for 16 hr in a 30 C incubator. The growth was measured turbidimetrically in the colorimeter with a 540 filter.

The method of extracting B_{12} from samples is that used by the late Dr. H. W. Cromwell (1950) to whom we are indebted for the following procedure. One g of sample was added to 100 ml of water, or to 100 ml of 0.1 M citrate (pH 4.5) containing 0.1 per cent of sodium bisulfite added just before use. The mixture was autoclaved at 115 C for 10 min, adjusted to pH 5.0, filtered and diluted to volume. The B_{12} content of these extracts and of two samples of U. S. P. liver preparations without any treatment was determined with *L. leichmannii* (ATCC 4797) and *E. coli* 113-3. The medium used for the former organism was that of Thompson *et al.* (1950). The methionine content of these extracts and liver preparations was determined with *Leuconostoc citrovorum* 8081 (Steele *et al.*, 1949).

The cyanide treatment for converting all forms of the vitamin to B_{12} developed by Skeggs *et al.* (1951) was applied to citrate extracts and liver preparations as follows: The sample containing 0.2–2.0 μg of B_{12} was mixed with 8 mg of KCN in about 100 ml of 1 per cent phosphate buffer (pH 5.8) at 60 C for 1 hr, diluted and assayed for total activity. The excess of the cyanide was removed by aeration with air for 30 min. The mutant was sensitive to cyanide.

Results

Growth response of the mutant to crystalline vitamin B_{12}. Crystalline vitamin B_{12} from Merck and Co., Inc. was used as the standard. The growth response in shaken tubes is given in figure 1. The standard curve was reproducible, as illustrated in table 1. The usable range was from 0.05 to 1.5 mμg per 10 ml. With the small inoculum used, 16 hr were required for maximal growth. Longer incubation increased the growth slightly but cut down the usable range of the standard curve.

In trying to avoid the necessity of aeration, assays in test-tubes and Erlenmeyer flasks without shaking were

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tested. The growth responses are given in figure 1. The assay range became smaller, with an upper limit of about 0.7 mμg per 10 ml, and the growth was much less than that in the shaken tubes.

The medium sterilized with all its ingredients together gave less growth than that with glucose autoclaved separately. By adding 0.0075 per cent of sodium bisulfite, glucose could be sterilized with the other constituents and the resulting medium supported a growth equal to that with glucose sterilized separately. The growth curve given by the medium containing bisulfite is shown in figure 1.

Bessell *et al.* (1950) used the synthetic medium of Tatum and Lederberg (1947) enriched with the trace elements of Beadle and Tatum (1945), and also a simpler one (in g per liter: NH_4H_2PO4, 1.0; KCl, 0.2; $MgSO_4 \cdot 7H_2O$, 0.2; glucose, 10; pH 7.0) in a plate assay for vitamin B_{12}. These two media were tested with the mutant under the conditions of incubation used. The first medium gave a minimum transmission of about 45 per cent and covered the same range as Davis and Mingioli's medium. The second medium supported less growth because of the low buffer capacity.

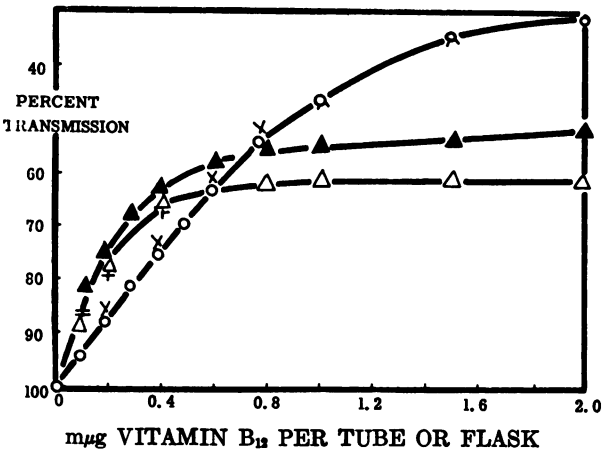

FIG. 1. Growth response of *E. coli* 113-3 to vitamin B_{12} at 30 C for 16 hr. (○) shaken tubes (18 x 150 mm), (×) shaken tubes (18 x 150 mm) glucose and bisulfite autoclaved with medium, (▲) 50-ml Erlenmeyer flasks, 10 ml medium each, (△) 25-ml Erlenmeyer flasks, 10 ml medium each, (∓) stationary tubes (18 x 150 mm).

Burkholder (1951) added asparagine, arginine, glutamic acid, glycine histidine, tryptophan, and sodium thioglycollate to Davis and Mingioli's medium and also increased the glucose to 1 per cent. The growth of the mutant in Burkholder's medium under the conditions of incubation used was essentially the same as that in the original medium. The addition of thioglycollate did not prevent the formation of a light yellow color in the medium during sterilization.

Recovery of added vitamin B_{12}. Crystalline vitamin B_{12} was added to several samples and the total B_{12} was then determined. Examples of such recovery experiments are given in table 2. From a large number of these experiments, the average recovery of total B_{12} was found to range from 95 to 108 per cent.

The assay results did not show any drifting at different levels of sample as is illustrated in table 3.

TABLE 1. *Reproducibility of standard curve*

VITAMIN B_{12}	EVELYN READING WITH 540 mμ FILTER	
	Range for 20 runs	Mean
mμg/tube		
0.0	98–100	99.4 ± 0.5*
0.1	92–94	93.6 ± 0.9
0.2	85–88	87.2 ± 0.9
0.3	80–84	81.5 ± 1.2
0.4	73–83	75.1 ± 1.3
0.5	68–72	69.0 ± 1.7
0.6	62–66	64.2 ± 1.9
0.7	56–61	57.9 ± 1.7
0.8	50–56	53.4 ± 2.0
1.0	43–48	46.1 ± 1.4
1.5	31–38	34.6 ± 2.2
2.0	28–33	31.6 ± 1.8

* Standard error of the mean.

TABLE 2. *Recovery of added vitamin B_{12}*

SAMPLE	VITAMIN B_{12} ADDED	TOTAL VITAMIN B_{12} FOUND	RECOVERY OF TOTAL B_{12}
	mμg	*mμg*	*per cent*
Hog liver prep. 2	0.00	0.16	
	0.20	0.39	108
	0.00	0.27	
	0.20	0.45	96
	0.00	0.48	
	0.20	0.68	100
Beef liver prep. 3	0.00	0.18	
	0.20	0.36	95
	0.00	0.30	
	0.20	0.52	104
	0.00	0.44	
	0.20	0.65	102
Reticulogen,* diluted	0.00	0.12	
	0.20	0.32	100
	0.00	0.26	
	0.20	0.45	98
	0.00	0.44	
	0.20	0.63	98
	0.00	0.31	
	0.40	0.69	97
	0.00	0.56	
	0.40	0.94	98

* Liver concentrate of Eli Lilly and Co.

Interference of methionine. The mutant requires either vitamin B_{12} or methionine for growth in the medium. Because of the possible presence of methionine in B_{12} containing materials and especially in liver preparations, the growth response of the mutant to methionine and the interference of the latter in the assay was

studied. In the medium without B_{12}, 100 μg of methionine gave a maximum growth of 35 after 16 hr incubation under the conditions used. For testing the interference of methionine, varying amounts of L-methionine were added to assay tubes containing different amounts

TABLE 3. *Vitamin B_{12} content of reference samples assayed at different levels*

SAMPLE	VITAMIN B_{12} FOUND	VITAMIN B_{12}
	mμg/tube	*μg/g*
APF No. 2 W*	0.16	3.2
	0.31	3.1
	0.47	3.1
	0.62	3.1
	0.83	3.3
APF No. 2 C†	0.24	4.8
	0.37	5.0
	0.46	4.6
	0.76	5.0
Beef liver	0.20	0.27‡
	0.39	0.26
	0.55	0.25
	0.78	0.26

* W denotes water extract of APF.
† C denotes citrate buffer extracts of APF.
‡ Wet basis.

TABLE 4. *Recovery of vitamin B_{12} in the presence of methionine*

VITAMIN B_{12} ADDED	L-METHIONINE	RATIO OF METHIONINE/B_{12}	APPARENT B_{12}
mμg	*μg*		*mμg*
0.2	2.0	10,000	0.205
	4.0	20,000	0.196
	6.0	30,000	0.196
	8.0	40,000	0.200
	10.0	50,000	0.202
	12.0	60,000	0.200
	16.0	80,000	0.246
	20.0	100,000	0.320
0.4	4.0	10,000	0.408
	8.0	20,000	0.388
	16.0	40,000	0.380
	20.0	50,000	0.400
	24.0	60,000	0.412
	32.0	80,000	0.452
	40.0	100,000	0.692
0.5	5.0	10,000	0.490
	10.0	20,000	0.510
	25.0	50,000	0.495
	50.0	100,000	1.000

of B_{12} and the recovery of the latter was determined. Table 4 summarizes the results.

When the ratio of methionine to vitamin B_{12} on a weight basis was 50,000 or less, the presence of the former did not introduce any significant error in the assay. The methionine content of liver preparations and APF extracts was determined and found to be far below the limiting ratio (see table 5). Johansson (1951) reported a ratio of 300,000 based on the detectable amounts of methionine (150 mμg per ml) and of B_{12} (0.0005 mμg per ml) when tested separately. However, there may be a difference in the growth response of the mutant to methionine alone and to methionine in the presence of B_{12}. The more reliable method for the determination of the interference is the recovery experiment.

TABLE 5. *Vitamin B_{12} and methionine contents of commercial APF and liver preparations*

SAMPLE	VITAMIN B_{12} BY *L. leichmannii*		VITAMIN B_{12} BY *E. coli* 113-3		METHIONINE
	Without cyanide	With cyanide	Without cyanide	With cyanide	
	μg/g	*μg/g*	*μg/g*	*μg/g*	*μg/g*
APF No. 1 W*	7.2	—	6.3	—	680
C†	7.6	8.7	7.0	8.1	800
APF No. 2 W*	4.2	—	3.4	—	600
C†	5.4	5.3	4.8	4.9	1040
APF No. 3W*	4.5	—	3.3	—	1070
C†	8.5	8.6	8.5	8.6	1200
APF No. 4 W*	4.3	—	3.8	—	540
C†	4.7	5.5	4.7	5.3	1000
APF No. 5 W*	1.7	—	1.2	—	10
C†	3.3	3.2	2.7	2.8	10
APF No. 6 W*	3.4	—	3.0	—	160
C†	9.3	10.0	8.1	8.7	360
APF No. 7 W*	3.8	—	3.0	—	360
C†	4.7	4.7	4.3	4.6	360
	μg/ml	*μg/ml*	*μg/ml*	*μg/ml*	*μg/ml*
Liver prep. No. 3	8.1	10.8	7.5	10.0	160
Liver prep. No. 8	30.0	30.6	25.6	32.0	10

* W denotes water extracts.
† C denotes citrate buffer extracts.

The effect of other nutrients which may be present in B_{12} containing materials on the assay was also tested. Nine vitamins: thiamine, pyridoxal, pyridoxine, pantothenic acid, riboflavin, nicotinic acid, p-aminobenzoic acid, biotin and folic acid added together at the concentrations used for *Leuconostoc citrovorum* 8081 in amino acid assay did not show any effect on the B_{12} assay. Guanine, uracil, adenine and xanthine added together at a concentration 8 times that used for the lactic acid bacteria also gave no stimulation to the mutant. Fifteen amino acids, exclusive of methionine, added singly at levels up to 0.5 mg per tube did not affect the assay.

Vitamin B_{12} content of commercial APF and liver preparations. The B_{12} and methionine content of these

samples are given in table 5. The citrate buffer extracted more B_{12} from the APF samples than water alone. The bisulfite contained in the citrate buffer did not affect the growth of the organism at the dilutions used. The APF extracts and liver preparations were also treated with alkali to destroy the vitamin as described by Hoffmann *et al.* (1949) and then assayed. At the same dilutions used before treatment, the samples were inactive. This indicated that the entire activity was due to B_{12}, and not to the desoxyribosides which are stable to alkali.

The *E. coli* results for the water extracts of APF samples ranged from 71 to 88 per cent (average 81) and for the citrate extracts, 82 to 98 per cent (average 91) of the *L. leichmannii* values. The mutant also gave low figures for the two liver preparations, 85 and 93 per cent of the *L. leichmannii* results.

A cyanide enhancement effect on B_{12} assay has been reported (Skeggs *et al.*, 1951; Cooperman *et al.*, 1951). Results of this treatment on the citrate extracts and liver preparations are given in table 5. The activity of the citrate extracts for the mutant ranged from 87 to 99 per cent (average 95) of the *L. leichmannii* results. The *E. coli* figures for the liver samples were 93 and 105 per cent of the corresponding *L. leichmannii* values. The greater increase in activity of the samples after the cyanide treatment when assayed with the mutant may be due to the lower response of the organism to other forms of B_{12} present in the samples before the cyanide treatment. In general the mutant gave results not higher than those given by *L. leichmannii*.

Activity of different forms of vitamin B_{12}. Different forms of vitamin B_{12} occur in nature and have different potencies for certain assay organisms. The activity of four natural forms of vitamin B_{12} to the mutant was tested. Effect of cyanide on each of these forms was also determined.

Vitamin B_{12b} was found to have 75 per cent of the activity of B_{12}. After cyanide treatment and aeration, B_{12b} showed the same potency as B_{12}, but only 88 per cent activity without aeration. B_{12} gave quantitative recovery after the same treatment and aeration, but a much lower recovery, 93 per cent, without aeration. These results indicated the sensitivity of the mutant to the excess of cyanide, and that in using this organism for the assay of natural material treated with cyanide, the excess of the latter must be removed.

Crystalline pseudovitamin B_{12} (Pfiffner *et al.*, 1951), obtained from the anaerobic fermentation of an as yet unidentified organism from bovine rumen content was kindly furnished by Dr. J. J. Pfiffner and was found to have a low activity to the mutant. Two μg of this material was equivalent to 0.77 μg of vitamin B_{12} and the relative activity about 0.4. In a private communication, Dr. Pfiffner gave the following comparison of activity (activity of vitamin B_{12} = 1) to three organisms: *L. leichmannii* 7830, autoclaved 0.9, added aseptically 1.9; *Lactobacillus acidophilus* 8322, autoclaved 4.5, added aspetically 5.8; *Lactobacillus lactis* Dorner, 0.8. The *E. coli* mutant is less responsive than any of these bacteria.

Vitamin B_{12f}, a material from rat feces, was found to have the same R_f value as the pseudovitamin B_{12} by Dr. U. J. Lewis, of this Department. A purified preparation of this material with an activity of 1.5 μg per ml to *L. leichmannii* ATCC 7830, determined by Dr. Lewis, was equivalent to 1.27 μg per ml to the mutant.

No cyanide enhancement was observed with either pseudovitamin B_{12}, or with vitamin B_{12f}.

Summary

A turbidimetric assay of vitamin B_{12} with a mutant strain of *Escherichia coli* is described. The standard curve was reproducible in shaken tubes and recovery of added vitamin B_{12} was satisfactory. Assay with stationary tubes and flasks gave smaller usable range of the growth curve. Methionine interfered only when present in amounts 50,000 times that of B_{12}. Samples analyzed to date showed a ratio of methionine to B_{12} on a weight basis of less than 400.

Citrate buffer containing a little bisulfite released more B_{12} from the commercial APF samples than water alone. Seven commercial APF samples and two liver preparations were analyzed with the mutant and also with *Lactobacillus leichmannii* (ATCC 4797). The first gave figures ranging from 71 to 105 per cent of that given by the second. Six of the samples showed an increased B_{12} content of from 7 to 33 per cent after cyanide treatment. Vitamin B_{12b} with the same treatment and removal of the excess of cyanide showed the same activity as B_{12}.

Pseudovitamin B_{12} and vitamin B_{12f} were found to have a lower activity than vitamin B_{12}. No cyanide enhancement was obtained with these two forms of vitamin B_{12}.

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