VITAMIN ASSAY

Rapid Spectrophotometric Determination Of Vitamin B_{12} in Microbial Material

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Perhaps no other vitamin has received, in such a short time, the concentrated effort that has been expended on vitamin B_{12}. Yet since vitamin B_{12} was isolated in 1948 (9), the analytical determination of the vitamin in natural substances has remained a difficult problem. The rapid spectrophotometric method described here was developed for the determination of vitamin B_{12} in fresh and dried bacteria cells. Benzyl alcohol or *n*-propyl alcohol is used to extract vitamin B_{12} selectively from dry or moist bacteria cells. The extracted vitamin is then determined by differential spectrophotometric measurement. Assay results are presented in comparison with microbiological results for bacteria cells, and for feed supplements. The new method is simple to operate, and is much faster and more accurate than bioassay. It will be useful for control of vitamin B_{12} production by fermentation, and for the standardization of finished products.

Microbiological assay of vitamin B_{12} in fresh and dried bacteria cells in the author's laboratory was time-consuming and unreliable. A chemical method designed for the determination of vitamin B_{12} in crude material (*10*) gave lower results than bioassay. Colorimetric (*4, 5*) photolytic (*2*), hydrolytic (*1*), polarographic (*3*), and chromatographic (*8,13*) techniques were not adaptable to rapid accurate analysis of microbial material.

These problems led to the development of a new spectrophotometric method that is specific for vitamin B_{12} activity in microbial materials. The method is designed for dry cellular material containing at least 5 γ of vitamin B_{12} per gram, or moist cellular material containing at least 10 γ of B_{12} per gram of solids.

Vitamin B_{12} is extracted from dry material by contact with hot benzyl alcohol containing cyanide ions and water. This solvent system was selected because it quantitatively extracts vitamin B_{12} from cellular material with a minimum of impurities. The extracting solvent is held between pH 7 and 8 to convert vitamin B_{12} variants into the more stable cyanocobalamin form. The vitamin may then be measured directly in the benzyl alcohol extract, or after transference to water, by inducing the formation of cyanocobalamin in one aliquot, and dicyanocobalamin in another aliquot, and determining the difference in the absorbance of these compounds at a wave length of 582 mμ in a spectrophotometer. The extinction coefficient for this difference is 54, as determined by Rudkin and Taylor (*10*), who showed that vitamin B_{12} may be measured by this technique in the presence of impurities.

In applying the method to moist cellular material, *n*-propyl alcohol is used as the extracting solvent in place of benzyl alcohol, which cannot completely extract the vitamin from moist cells. Vitamin B_{12} is measured in the propyl alcohol extract in the manner described above.

The working time of the method is 60 minutes using direct spectrophotometric measurement in the solvents, or 120 minutes using measurement after transference to water. The standard deviation of replicate determinations in dry or moist cellular material is 3%.

Experimental Work

Reagents. Reagent grade chemicals are recommended:

Benzyl alcohol
n-Propyl alcohol
Ethyl alcohol, 95%
Chloroform
Sodium cyanide, 0.5, 1, and 10% solutions
Potassium dihydrogen phosphate, 12.5% solution
Citric acid, 5 and 10% solutions
Crystalline vitamin B_{12} standard solution, approximately 100 γ per ml., standardized at 361 mμ, $E^{1\%}_{1\text{ cm.}} = 207$ (*12*)

Apparatus. In addition to standard laboratory equipment:

Spectrophotometer. This instrument should be capable of measurements between 361 and 582 mμ using 1-cm. absorption cells. It is advisable to calibrate the instrument against crystalline vitamin B_{12} solutions.

Boiling water bath
Centrifuge
Separatory funnels, Squibb type, capacity 125 ml.

Sample. The sample should contain a total of at least 100 γ of vitamin B_{12}. If it contains less than 10 γ of vitamin B_{12} per gram, no more than 10 grams should be used, and this quantity should be supplemented with vitamin B_{12} standard solution. The sample is transferred to a 100-ml. borosilicate glass centrifuge tube.

Extraction. Step 1. Thoroughly mix 100 ml. of benzyl alcohol and 5 ml. of 1% sodium cyanide solution. If the sample is alkaline or poorly buffered, neutralize the sodium cyanide solution with 5 *N* hydrochloric acid in a well ventilated hood before adding to the benzyl alcohol.

Step 2. Pipet 50 ml. of this benzyl alcohol–cyanide solution into the weighed sample. If necessary, add to the suspension a quantity of vitamin B_{12} standard solution to make the total vitamin B_{12} content at least 100 γ. Make

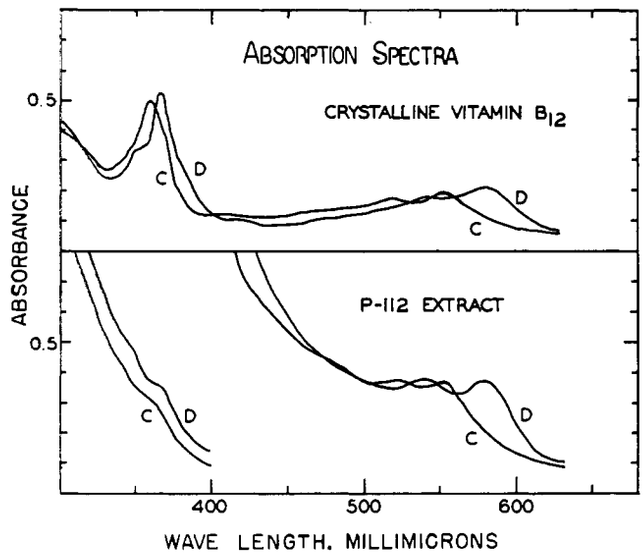

Figure 1. Absorption spectra for comparision ot crystalline vitamin B_{12} and extract of dry cellular material. The extract of dry cellular material was diluted 1 to 10 to obtain the curves shown in the range 300 to 400 mμ.

C. Cyanocobalamin curves
D. Dicyanocobalamin curves

a mark on the tube to indicate total volume.

Step. 3. Heat the sample suspension in a boiling water bath, so that the temperature of the suspension exceeds 92° C. for exactly 15 minutes. Stir the suspension frequently, using a rubber policeman to scrape down the sides of the centrifuge tube.

Cool the suspension to room temperature and restore the volume to the mark with benzyl alcohol–cyanide solution. If the suspension gels on cooling, reheat and centrifuge hot (Step 4). Use the standard spectrophotometric measurement, adding the chloroform while the supernatant liquid is still warm.

Step 4. Centrifuge the suspension 5 to 10 minutes, decant, and measure the volume of the supernatant liquid.

Spectrophotometric Measurement

Direct Reading for Rapid Determination of Vitamin B_{12}. Many samples may be analyzed directly in the benzylalcohol extract by this technique. If occasional samples continue to develop haze after filtration, they should be analyzed by the technique described in the standard spectrophotometric measurement.

Add to a 10-ml. aliquot (Step 4) 5 ml. of ethyl alcohol solution containing 6 ml. of 10% sodium cyanide solution per 100 ml. To another 10-ml. aliquot add 5 ml. of an ethyl alcohol solution containing 6 ml. of 10% citric acid solution per 100 ml. to set the pH at 6 or below. Mixt hese solutions, filter if necessary, and measure the difference in absorbance, ΔA, of the filtrates at 582 mμ.

$$\text{Vitamin } B_{12}, \gamma \text{ per gram of sample} = \frac{\Delta A \times 1.5 \times 50}{0.0054 \times \text{sample wt.}}$$

If vitamin B_{12} was added to the sample, add the volume used to the factor 50 and subtract vitamin B_{12} added per gram of sample from the final result.

Standard Spectrophotometric Measurement. Transfer the supernatant liquid (Step 4) to a separatory funnel and add 0.5 volume of chloroform. Extract this mixture with 8-, 7-, and 7-ml. portions of water, allowing 10 minutes for each of the three extractions. Dilute the combined water extracts to 25 ml. with water, and let stand for 5 minutes to allow settling of undissolved solvent droplets.

Add to 10 ml. of the water extract 2 ml. of 10% sodium cyanide solution. Add to another 10-ml. aliquot 2 ml. of 12.5% potassium dihydrogen phosphate solution to set the pH at 6 or below. Mix, and allow a 30-minute reaction time. Then measure the difference in absorbance, ΔA, of the solutions at 582 mμ.

$$\text{Vitamin } B_{12} \gamma \text{ per gram of sample} = \frac{\Delta A \times 6/5 \times 25 \times 1.03 \times 50}{0.0054 \times \text{Step 4} \times \text{sample wt.}}$$

If vitamin B_{12} was added to the sample, add the volume used to the factor 50, and subtract vitamin B_{12} added per gram of sample from the final result.

The Rudkin extraction factor 1.03 (*10*) has been retained as a necessary quantity, even though only one liquid-liquid extraction is used.

Procedure for Moist Cellular Material

Sample Centrifuge in a borosilicate glass tube a quantity of liquid containing up to 3 grams of solids in the form of moist cellular material, and discard the supernatant liquid.

Extraction Step 1. Mix 100 ml. of *n*-propyl alcohol with 10 ml. of 0.5% sodium cyanide solution.

Step 2. Pipet 40 ml. of this *n*-propyl alcohol–cyanide solution into the centrifuge tube containing the sample. If the sample contains less than 10 γ of vitamin B_{12} per gram of solids, add a quantity of vitamin B_{12} standard solution to the centrifuge tube to make the total vitamin B_{12} content at least 100 γ. Make a mark on the tube to indicate total volume.

Step 3. Heat the centrifuge tube in a boiling water bath for 30 minutes, so that the sample suspension boils gently for at least 15 minutes. Stir frequently. Cool the tube to room temperature and restore the volume to the mark with *n*-propyl alcohol–cyanide solution.

Step 4. Centrifuge the suspension for 10 minutes.

Spectrophotometric Measurement Add to a 10-ml. aliquot of the supernatant liquid (Step 4) 2 ml. of 10% sodium cyanide solution. To another 10-ml. aliquot add 2 ml. of 5% citric acid solution to set the pH at 6 or below. Mix the solutions gently and allow a 30-minute reaction time. Then filter both solutions if necessary and measure the difference in the absorbance, ΔA, of the two solutions at 582 mμ.

$$\text{Vitamin } B_{12}, \gamma \text{ in original liquor} = \frac{\Delta A \times 11.9/10 \times \text{total vol. Step 2}}{0.0054}$$

Results Obtained

The method has been applied to routine control analysis of dried and moist bacteria cells in the author's laboratory. Typical results are presented in Table I.

Table I. Reproducibility of Results

Sample	Vitamn B_{12}, γ/Gram	Standard Deviation, %
Dried bacteria cells		
112	201	3.0 (8)[a]
114	89.5	2.3 (3)
115	210	1.5 (3)
120	259	1.5 (3)
Moist bacteria cells		
117	308 (on solids)	3.0 (3)
129	403 (on solids)	1.3 (2)

[a] Numbers in parenthesis indicate number of replicate determinations.

The method has also been applied to the analysis of material other than the author's own product. The results shown in Table II are reasonably good,

Table II. Analysis of Commercial Products

Product Tested	Vitamin B_{12} Activity, γ/Gram: Claimed potency (LL Assay)	Vitamin B_{12} Activity, γ/Gram: Pacific Yeast (Spectrophotometric method)
Commercial feed supplement A	6.6	7.9
Commercial feed supplement B	13.2	14.8
B. megatherium feed supplement	8.4	6.6[a]

[a] Supplemented during assay with crystalline vitamin B_{12}. Spectrophotometric results are averages of duplicate assays.

considering that all products tested are in the extreme low range of the method's sensitivity to vitamin B_{12}.

The data in Table III were obtained from an independent laboratory's first trial of the new spectrophotometric method. Comparison of their results with the author's tests on the same sample indicates that interlaboratory agreement will be good.

Table III. Comparison of Analyses from Two Laboratories

(Sample 01346)

	Vitamin B_{12} Activity, γ/Gram	
	Pacific Yeast Laboratory	Laboratory B
1st assay	92.5	92.8
2nd assay	90.0	92.8
3rd assay	100.6	..
Average	94.4	92.8
Standard deviation, %	3.8	0.0

The specificity of the new method for vitamin B_{12} activity has been confirmed by chemical and microbiological tests.

Test A. Crystalline vitamin B_{12} added to dry or moist bacteria cells was recovered 100%. Typical results are presented in Table IV.

Table IV. Recovery of Crystalline Cyanocobalamin

Sample (Dried Bacteria)	Crystalline Cyanocobalamin Added, γ	Vitamin B_{12} Found, γ	Crystalline Cyanocobalamin Recovered, γ
No. 112	None	201	...
No. 112	115	319	118 (102.6%)
No. 114	None	89.5	...
No. 114	90	179	89.5 (99.4%)
...	230	223[a]	(97.0%)
...	230	205[b]	(89.1%)

[a] Extracting solvent neutralized.
[b] Extracting solvent alkaline.

Under the conditions of assay, the buffering action of cellular material normally holds the extraction pH between 7 and 8. If crystalline vitamin B_{12} alone is added to the benzyl alcohol–cyanide solution, however, the solvent must first be neutralized. When this is done, the recovery of crystalline vitamin B_{12} is 97%.

For the analysis of alkaline or poorly buffered materials, therefore, the sodium cyanide solution used in the extracting solvent should be neutralized with hydrochloric acid in a fume hood, so that the pH of the extraction will be held between 7 and 8.

Test B. Figure 1 presents spectrophotometric proof that the dicyanide complex of cyanocobalamin is formed in extracts of dry cellular material at the point where these extracts are prepared for spectrophotometric measurement.

The identity of the dicyanide complex was determined by means of Rudkin's criterion (*10*) that cyanocobalamin and dicyanocobalamin absorption curves cross at 530, 545, and 558 mμ. The two sets of curves in Figure 1 fulfill this condition. According to Rudkin (*10*), spectrophotometric response to substances other than vitamin B_{12} would tend to interfere with the crossing of the absorption curves at the specified points.

As a matter of interest, curves made in the 300- to 400-mμ region with a 1 to 10 additional dilution of the crude extract show evidence of the major absorption peaks of both cyanocobalamin and dicyanocobalamin.

Vitamin B_{12} is determined at 582 mμ, where the difference in each pair of absorption curves is at a maximum (Figure 1).

Test C. Benzyl alcohol extracts of dry cellular material were subjected to heat at 121° C. for 30 minutes at pH 9.6, a condition known to destroy vitamin B_{12} (*6*). Vitamin B_{12} activity at a wave length of 582 mμ was reduced to zero by this treatment.

Test D. The identification of the dicyanide complex in Test B showed that the spectrophotometric method was not responding to impurities in crude extracts. Further proof of this important point was obtained by ion exchange removal of impurities from water extracts of benzyl alcohol.

Columns were constructed of Amberlite ion exchange resins IRA 400 and IR 120, according to the recommendations of Marsh and Kuzel (*7*). To eliminate hydrolytic effects, the water extracts were reacted with an excess of cyanide for 30 minutes before passage through the columns.

The red-purple column effluents contained 87 to 91% of the original B_{12} activity. Furthermore, microbiological assay of these column effluents accounted for 91% of the spectrophotometric vitamin B_{12} activity. Finally, crystalline vitamin B_{12} recovery from the ion exchange columns ranged from 87 to 94%. These data show that the spectrophotometric determination represents actual vitamin B_{12} activity in dry cellular material.

Table V. Vitamin B_{12} Activity by Spectrophotometric and Microbiological Assay

(Dried bacteria sample 112)

Pacific Yeast Spectrophotometric Method, γ/Gram	Skeggs *L. leichmannii*[a] Microbiological Assay (*11*), γ/Gram
206	199
196	188
209	200
189	204
205	189
201	
203	
202	
Av. 201	196
Standard deviation, % 3.0	2.8

[a] Modified to include 4 mg. of ascorbic acid and 5γ of sodium cyanide per assay tube.

Test E. The spectrophotometric method consistently gave results equal to those obtained with the Skeggs *Lactobacillus leichmannii* microbiological assay (*11*). These results add further confirmation of the chemical specificity of the new method. The average results of comparative assays were found to differ by only 2.5%, as shown in Table V.

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