SENSITIVITY OF *CORYNEBACTERIUM CAROTENOGENUM* TO VITAMIN B_{12}

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Abstract

The feasibility of using *Corynebacterium carotenogenum* for the rapid assay of vitamin B_{12} in liver and other extracts has been suggested. The response of this organism to the vitamin, though less sensitive, has been shown to be specific and reliable.

Introduction

That *Corynebacterium carotenogenum* holds promise of its use not only in the evaluation of heart proteins and heart extracts, but also for the assay of vitamin B_{12} has been indicated before (Pradhan, 1958; Pradhan and Bhat, 1960 A; 1960 B; 1960 C). In this paper are described the experiments carried out with a view to determine the sensitivity as well as the reliability of using this organism as compared to that of *Lactobacillus leichmannii* for the assay of vitamin B_{12}. It may be mentioned here that the microbiological procedure is the one recommended for the evaluation of this important vitamin in pharmaceutical products.

Materials, Methods and Results

A commercially available liver extract (Cipalon) was examined for its B_{12} content by employing the standard (U. S. P.) method involving the use of *L. leichmannii* and the assay gave the preparation a value of 2.2 μg/ml as compared to 2.5 μg declared on its label. When, however, the very same sample was assayed using *C. carotenogenum*, (maintenance medium : liver extract agar), its vitamin B_{12} content was found to be 2.4 μg; this figure was closer to the value declared in its behalf. It must here be mentioned that the medium recommended for assay with the *Lactobacillus* was found to be unsuitabe for assay with the *Corynebacterium* in that the latter species failed to elicit a quantitative response to graded doses of the vitamin when incorporated into that medium. Attempts had therefore to be made to modify the medium and after several trials the following composition (Basal medium 1) was found to serve the purpose well.

The experimental readings recorded on a Hilger Colorimeter, after growing the organism for 10 days at room temperature ($25-28^{\circ}$C) in medium 1 supplemented with graded quantities of B_{12}, are reproduced in Table I. It became clear from this as well as from other similar experiments that the

organism under test elicited its best response (maximum growth) when the concentration of B_{12} in this medium was of the order of 3.5 μg/10 ml. It was also observed that the characteristic red pigment of the organism would appear

BASAL MEDIUM 1

Glucose	.....	1 g.
Peptone	.....	1 g.
K_2HPO_4	.....	0.4 g.
Citric acid	.....	0.2 g.
Lactic acid	.....	0.1 g.
NaCl	.....	0.2 g.
$MgSO_4.7H_2O$	.....	0.1 g.
Glycine	.....	20 mg.
Cystine	.....	20 mg.
Isoleucine	.....	20 mg.
Valine	.....	20 mg.
Glutamic acid	.....	20 mg.
Threonine	.....	20 mg.
Methionine	.....	20 mg.
Tyrosine	.....	20 mg.
Leucine	.....	20 mg.
Tryptophane	.....	20 mg.
Phenylalanine	.....	20 mg.
Histidine HCl	.....	20 mg.
Lysine	.....	20 mg.
Adenine	.....	1 mg.
Xanthine	.....	1 mg.
Guanine	.....	1 mg.
Uracil	.....	1 mg.
Vitamin B_1	.....	5.0 mg.
Niacinamide	.....	10.0 mg.
Calcium pantothenate	.....	10.0 mg.
Vitamin B_2	.....	100.0 mg.
Vitamin B_6	.....	10.0 mg.
Folic acid	.....	1.0 mg.
Inositol	.....	0.1 mg.
Biotin	.....	0.1 mg.

Double distilled water to make 100 ml.
pH of the medium was adjusted to 7.0 with ammonia

only when adequate vitamin was present therein. However, it must be stated, that this organism required as long a period as 10 or more days for producing its full growth and as such its use, though reliable, was not warranted for the

TABLE I

Growth response of *C. carotenogenum* to vitamin B_{12} in medium 1

Conc. B_{12} in μg/10 ml	Hilger Reading (Absorbancy)
Basal medium (uninoculated)	1.0
0.0	0.960
0.1	0.958
0.3	0.903
0.5	0.870
1.0	0.824
2.0	0.801
2.5	0.796
3.0	0.793
3.5	0.790
4.0	0.810
5.0	0.810
10.0	0.810
15.0	0.810

BASAL MEDIUM 2

Glucose	.....	1 g.
Peptone	.....	0.5 g.
K_2HPO_4	.....	0.4 g.
$MgSO_4.7H_2O$	.....	0.1 g.
NaCl	.....	0.2 g.
$FeSO_4.7H_2O$	.....	0.003 g.
Adenine	.....	1.0 mg.
Guanine	.....	1.0 mg.
Xanthine	.....	1.0 mg.
Uracil	.....	1.0 mg.
Vitamin B_1	.....	0.1 mg.
Vitamin B_2	.....	0.1 mg.
Niacinamide	.....	0.1 mg.
Folic acid	.....	0.1 mg.
Vitamin B_6	.....	1.0 mg.
Paba	.....	10.0 mg.
Calcium pantothenate	.....	0.1 mg.
Biotin	.....	0.1 mg.
Coconut water	.....	5 ml.
Tween 80	.....	0.4 g.

Double distilled water to make 100 ml ; pH was adjusted to 7.0

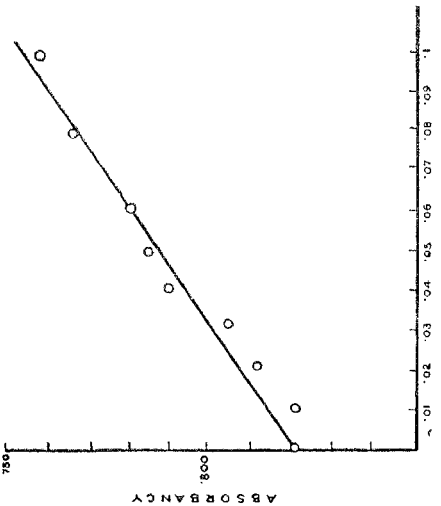

FIG. I *a*

Growth response of *L. leichmannii* to Vitamin B_{12}

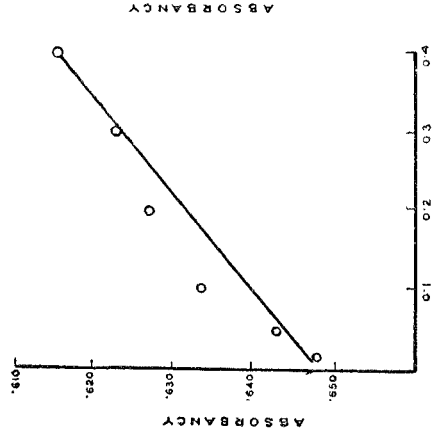

FIG. I

Growth response of *C. carotenogenum* to Vitamin B_{12}

routine assay of B_{12}. Many more trials were subsequently made with a view to determine those constituents in the medium which would promote its rapid growth and bring the assay to completion within a reasonable period and finally the following medium (2) was evolved and found to be the most suitable for the purpose.

For the assay, the tubes were filled with the medium and sterilized at 100°C for 30 minutes each day for three days. The coconut water incorporated into the medium, it may be mentioned, was derived from a freshly opened tender fruit. Ramakrishnan (1957), and Indira *et al.* (1958) had not only shown the use of this water for hastening the growth of *Mycobacterium tuberculosis* but had subsequently succeeded in isolating therefrom a new growth factor which accounts for this phenomenon. In the present case also, it was repeatedly observed, coconut water served to accelerate the growth to such an extent as to reduce the incubation period from as many as 10-21 days to as few as 2–3 days at room temperature. The growth response obtained by adding graded doses of B_{12} to basal medium 2 is recorded in Table II and depicted in Figure I.

TABLE II

Growth response of *C. carotenogenum* to vitamin B_{12} in medium 2

Conc. of vitamin B_{12} in the medium μg/10 ml	Hilger Reading (Absorbancy)
Blank (uninoculated)	0.699
0.0	0.656
0.01	0.648
0.05	0.638
0.1	0,633
0.2	0.627
0.3	0.623
0.4	0.616
0.5	0.620
0.6	0.623
0.8	0.625
1.0	0.623

For comparison, the response of *L. leichmannii* is also presented in Figure I*a* and it is clear that, although the new organism under test responds in a manner similar to the standard culture, it does not display the same sensitivity to the vitamin. At the same time, its response to B_{12} appears to be not only specific but also somewhat critical in that the maximum response is evidenced when the dosage of the vitamin is at a particular level. A smaller or a larger dose

does not yield the peak growth. It is interesting to observe that in medium 2, as in medium 1, optimal growth occurred when the concentration of B_{12} was between 3-4 μg/10 ml. of the media.

The practical usefulness of the assay, employing *C. carotenogenum* as the test organism, was then examined by assaying a few commercially available liver extracts. For comparative purposes the same samples were also tested with *L. leichmannii* by adherence to the standard U.S.P. procedures. The results are presented in Table III. They show that the new organism is as reliable, if not

TABLE III

Concentration of vitamin B_{12} in different samples of liver extracts as determined by *C. carotenogenum* and *L. leichmannii*

Name of liver extract	B_{12} in μg		
	As declared on labels	As measured by	
		C. carotenogenum	*L. leichmannii*
Cipalon B. No. 04426	2.5	2.4	2.2
Oxoid B. No. 14156	2.0	1.5	1.41
Cipalon B. No. 04426	2.5	2.5	2.32
Vitafolex	25*	2.2	2.15

* Estimated three years after the date of manufacture.

more so, for determinig the concentration of B_{12} in liver extracts, though it may not be, as pointed out before, as sensitive as the standard culture recommended by U.S.P.

TABLE IV

Vitamin B_{12} contents of tissue extracts as measured with *C. carotenogenum* as the test organism

Tissue extract			B_{12} values μg/ml
Heart	.....	.....	0.06
Brain	.....	.....	0.032
Meat	.....	.....	0.031
Kidney	.....	.....	0.065
Stomach	.....	.....	0.068
Pancreas	.....	.....	0.055

With a view to ascertain the distribution of B_{12} in some of the tissues of the rat, the following tissue extracts were evaluated by adopting the technique herein described. The various extracts were prepared by using 10 g of minced tissue and extracting each with 10 ml of distilled water at 100°C for half an hour. Each extract was filtered and incorporated, in measured quantities, into the medium 2. The following results (Table IV) were recorded.

The results obtained thus indicate the practical usefulness of the microbiological assay method for a quick evaluation of B_{12} in tissue extracts particularly liver extracts, by the use of *C. carotenogenum*, an organism which demands liver extract for growth and which has been maintained thereon for the past 2 decades without any impairment in its performance.

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