# CYTOLOGICAL STUDIES ON LACTOBACILLUS LEICHMANNII IN THE ASSAY OF VITAMIN $B_{12}$

By Sigurdur H. Petursson*

ABSTRACT

IN THE MICROBIOLOGICAL ASSAY OF VITAMIN $B_{12}$ WITH LACTOBACILLUS LEICHMANNII, THE VITAMIN HAS A SIGNIFICANT INFLUENCE ON THE LENGTH OF THE CELLS. IN THE LOWER CONCENTRATIONS OF THE VITAMIN, THE CELLS TEND TO GROW LONGER AND THE DIVISION OF THE CELLS IS RESTRICTED. FORMATION OF VOLUTIN GRANULES IS ALSO DISTURBED.

## INTRODUCTION

In the microbiological assay of vitamin $B_{12}$, the response of Lactobacillus leichmannii to different concentrations of the vitamin can be estimated either by the turbidity of the culture or by the amount of acid produced. In high concentrations of the vitamin the metabolism of the cells is normal, but in very low concentrations the growth is poor and the acid production is near zero.

It was desired to know whether these differences in metabolism could be demonstrated in the bacterial cells themselves. A preliminary microscopical investigation showed that the length of the cells varied in different concentrations of the vitamin. A comparison was then made of the variation in cell length with the turbidity of the broth culture and the amount of acid produced, both in standard tubes and in tubes containing samples. The results obtained are reported here.

## METHODS

The medium used for the assay of vitamin $B_{12}$ was modified from that recommended by Hoffman et al. (1949), and its composition is given in Table 1. The strain of bacteria employed was Lactobacillus leichmannii 313 (ATCC 7830). The standard was run at a concentration that varied from 0.005 to 0.8 millimicrograms of crystalline vitamin $B_{12}$ per tube. Microscopic preparations were made from the tubes after incubation at 37° C. for three days and just prior to titration. In other experiments, microscopic observations and titrations were carried out after incubation periods of one and two days.

The slide films used for measuring the size of the cells were stained with methylene blue. Twenty-five cells were measured on each slide. To exclude subjective selection, every cell found in a given field was measured. When the number of cells in a single field was less than 25, a second or third field was observed to bring the total cells to this number. When the field was crowded, all cells occurring in a 25-cell portion of the field were measured. For the microphotography, the slides were fixed in Bouin's solution and were stained with crystal violet (method of Robinow).

* ECA FELLOWSHIP STUDENT, FISHERY TECHNOLOGICAL LABORATORY, BRANCH OF COMMERCIAL FISHERIES, U.S. FISH AND WILDLIFE SERVICE, SEATTLE, WASHINGTON; PERMANENT ADDRESS: UNIVERSITY RESEARCH INSTITUTE, REYKJAVIK, ICELAND.

## RESULTS

Microscopic observations on the bacteria in the standard tubes were made repeatedly and always gave the same results. That is, in the highest concentration of vitamin $B_{12}$, the rods were of normal length; but, as the concentration of the vitamin decreased, the length of the rods increased. Filaments without septa, 100 to 200 microns in length, were regularly found at the lowest concentrations (0.005 and 0.02 millimicrograms vitamin $B_{12}$ per tube). This inverse correlation of the length of the cells with the concentration of the vitamin and, accordingly, with the final acidity in the medium was so regular that the results of the titrations could be predicted from the length of the cells. Data from a typical experiment in which microscopic observations and titrations were carried out after one, two, and three days are given in Table 2 (see also Figures 1 to 5). Although most of the cells at the lower concentrations of vitamin $B_{12}$ were of long form, as can be seen from Figures 4 and 5, some short cells, 2 to 4 microns in length, were also found. The width of the cells was nearly constant but, except in length and curvature, no variations in the form of the cells could be seen.

Table 1 - Composition of Double-Strength Basal Medium 1/

| Ingredient | Grams |
|---|---|
| Glucose | 40.0 |
| Sodium citrate | 20.0 |
| Sodium acetate, anhydrous | 20.0 |
| Casein - acid hydrolyzed | 10.0 |
| $K_2HPO_4$ | 6.0 |
| $KH_2PO_4$ | 6.0 |
| $MgSO_4.7H_2O$ | 7.0 |
| $MnSO_4.4H_2O$ | 1.5 |
| $FeSO_4.7H_2O$ | 0.42 |
| Asparagine | 0.2 |
| DL-tryptophane | 0.2 |
| L-cystine | 0.4 |
| Ascorbic acid | 2.0 |
| | Milliliters |
| Tween 80 | 2.0 |
| Distilled water | to 1,000 |
| | Milligrams |
| Adenine sulfate | 20.0 |
| Guanine hydrochloride | 20.0 |
| Uracil | 20.0 |
| Xanthine | 20.0 |
| Riboflavin | 2.0 |
| Niacin | 2.0 |
| Thiamine | 2.0 |
| Calcium pantothenate | 2.0 |
| p-Aminobenzoic acid | 0.08 |
| Pyridoxine | 4.0 |
| Pyridoxal | 4.0 |
| Pyridoxamine | 0.8 |
| Biotin | 0.2 |
| Folic acid | 0.4 |

1/ pH IS ADJUSTED TO 5.5.

Observations were made on the length of the bacterial cells in the assay tubes from several samples of fishery products. The samples were fish, fish meal, and stickwater. The length of the cells varied in the same manner as in the standard tubes. That is, at the higher dilutions of the sample, with resulting lower concentrations of vitamin $B_{12}$, the cells grew longer.

In a mixture of beef liver, hog liver, and salmon viscera that had been autoclaved with concentrated NaOH to destroy the vitamin $B_{12}$, a slight vitamin $B_{12}$-like activity was still demonstrated in the microbiological assay. Such activity is usually attributed to thymidine (Wright et al., 1948) or other desoxyribosides. In this particular assay a correlation was found between acid production and cell length. At the higher dilutions of the sample, the activity was lower and the cells were longer.

The appearance of the volutin granules was influenced by the concentration of the vitamin. At the higher concentrations, several spherical granules made visible by methylene-blue staining were found in every cell. In the long cells formed at the lower concentrations of the vitamin, no regular granules could be demonstrated in this way. However, there were often two or three broad belts in the cell that stained darker than the protoplasm. These belts were usually several times broader than the diameter of

Figures 1 to 5 are Microphotographs of the Organism Lactobacillus leichmannii Grown in Media Containing Different Concentrations of Vitamin $B_{12}$

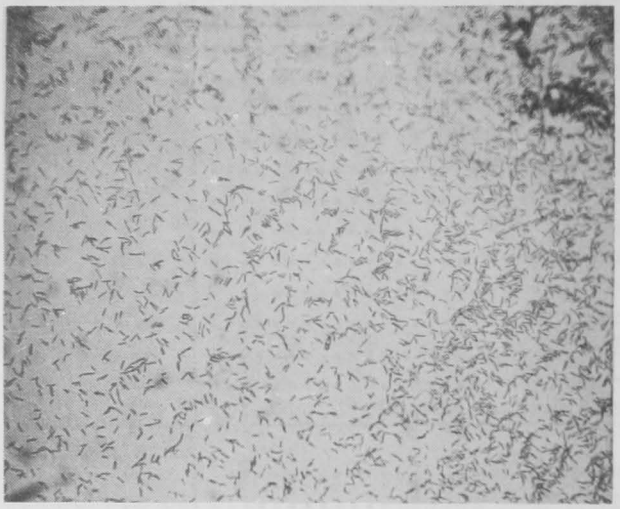

FIG. 1 - 0.8 MILLIMICROGRAMS OF VITAMIN $B_{12}$ PER TUBE.

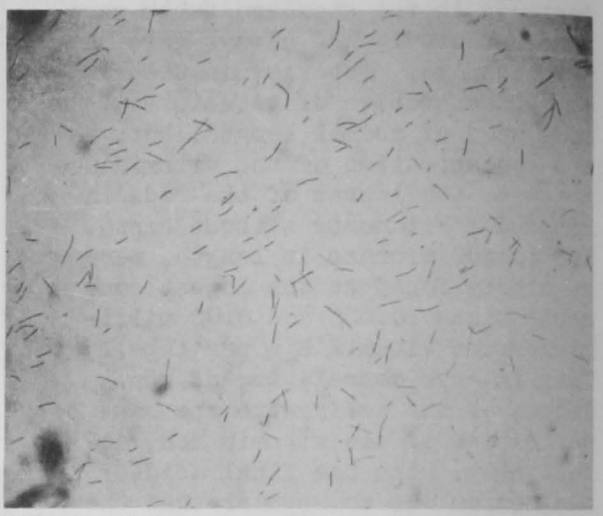

FIG. 2 - 0.2 MILLIMICROGRAMS OF VITAMIN $B_{12}$ PER TUBE.

FIG. 3 - 0.08 MILLIMICROGRAMS OF VITAMIN $B_{12}$ PER TUBE.

FIG. 4 - 0.02 MILLIMICROGRAMS OF VITAMIN $B_{12}$ PER TUBE.

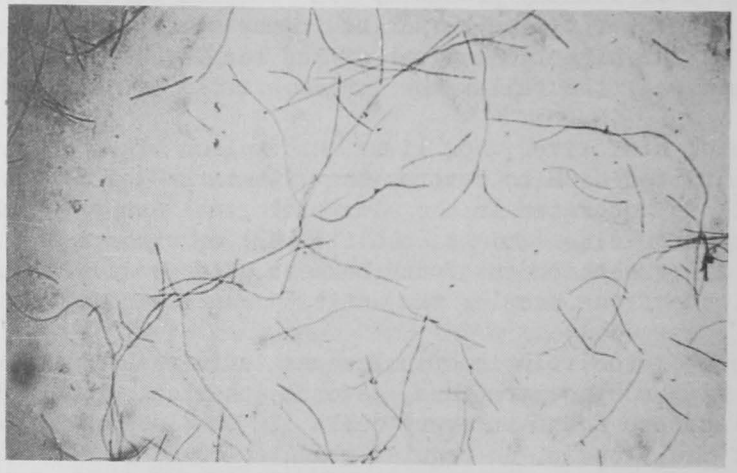

FIG. 5 - 0.005 MILLIMICROGRAMS OF VITAMIN $B_{12}$ PER TUBE.

## Figures 6 and 7 are Electron Micrographs of Lactobacillus leichmannii Grown in Media Containing Different Concentrations of Vitamin $B_{12}$ and Show the Structure of the Individual Cells

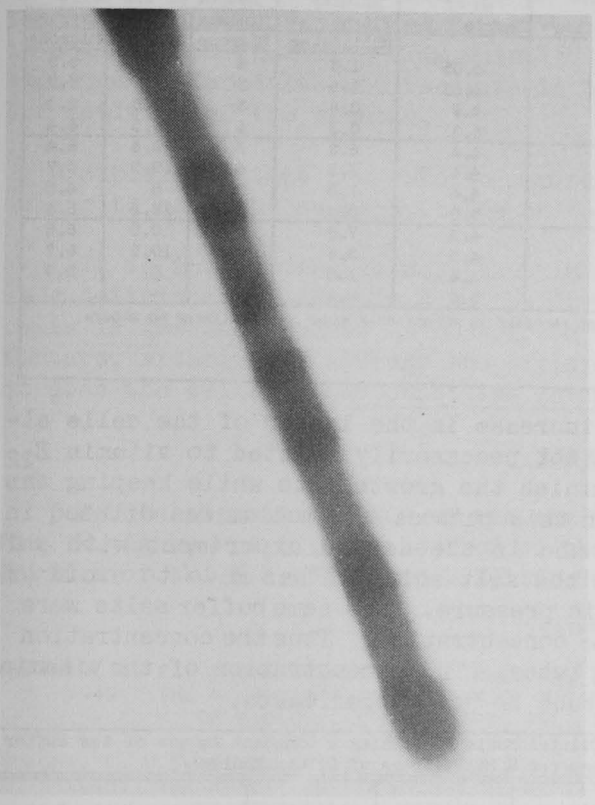

FIG. 6 - 0.8 MILLIMICROGRAMS OF $B_{12}$ PER TUBE. 18,500 X.

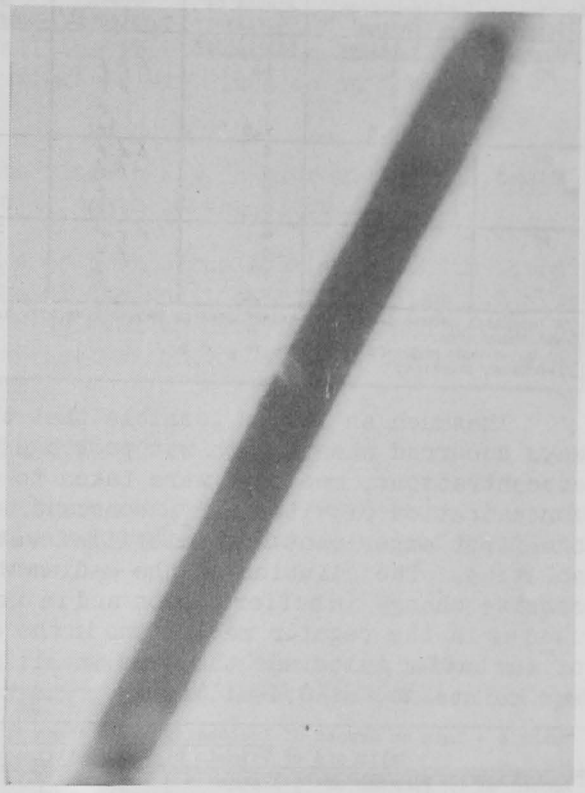

FIG. 7 - 0.005 MILLIMICROGRAMS OF $B_{12}$ PER TUBE. 16,000 X.

Table 2 - Data on Growth of Lactobacillus leichmannii at Different Concentrations of Vitamin $B_{12}$

| Incubation Period | Quantity of Vitamin $B_{12}$ Per Tube | Growth Turbidity[1] | pH | Acidity Titration[2] | Length of the Cells | | |
|---|---|---|---|---|---|---|---|
| | | | | | Minimum | Maximum | Average |
| Hours | Millimicrograms | | | Milliliters | Microns | Microns | Microns |
| 20 | 0.8 | ≠ ≠ | 5.1 | 1.4 | 2 | 5 | 3.0 |
| | 0.2 | ≠ ≠ | 5.1 | 1.4 | 2.5 | 5 | 3.8 |
| | 0.08 | ≠ | 5.35 | 0.5 | 4 | 9.5 | 5.7 |
| | 0.02 | - | | | | | |
| | 0.005 | - | | | | | |
| 44 | 0.8 | ≠ ≠ ≠ | 4.55 | 5.4 | 4 | 8 | 6.1 |
| | 0.2 | ≠ ≠ ≠ | 4.6 | 5.2 | 2.5 | 18 | 10.8 |
| | 0.08 | ≠ ≠ | 4.7 | 4.0 | 7 | 40 | 19.2 |
| | 0.02 | ≠ | 5.0 | 2.0 | 2.5 | 110 | 29.2 |
| | 0.005 | - | | | | | |
| 68 | 0.8 | ≠ ≠ ≠ | 4.2 | 8.1 | 2.5 | 11 | 5.6 |
| | 0.2 | ≠ ≠ ≠ | 4.25 | 7.7 | 4 | 19 | 11.6 |
| | 0.08 | ≠ ≠ ≠ | 4.4 | 6.8 | 5.5 | 40 | 20.6 |
| | 0.02 | ≠ ≠ | 4.7 | 3.4 | 4 | 110 | 35.0 |
| | 0.005 | ≠ | 5.2 | 0.9 | 2.5 | 190 | 55.0 |

1/ ≠ INDICATES GROWTH OF THE ORGANISM. GREATER GROWTH TURBIDITY SHOWN BY INCREASE IN NUMBER OF ≠ MARKS. - INDICATES NO GROWTH.

2/ Ml 0.1-N NaOH REQUIRED TO TITRATE TO pH 5.5.

the rod. Structures corresponding to granules and belts can be distinguished in the electron micrographs, Figures 6 and 7.

Table 3 - Data on Growth of Lactobacillus leichmannii in Diluted Media Containing a Constant Amount of Vitamin $B_{12}$ (0.4 Millimicrograms per 8-Milliliters of Diluted Medium)

| Incubation Period | Amount of Double-Strength Medium | Amount of Water | Growth Turbidity[1] | pH of Control Tubes[2] | pH of Sample Tubes | Acidity Titration[3] | Length of the Cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Minimum | Maximum | Average |
| Hours | Milliliter | Milliliter | | | | Milliliter | Microns | Microns | Microns |
| 20 | 4 | 4[4] | ƒ ƒ ƒ | | 5.05 | 1.6 | 4 | 8 | 5.5 |
| | 2 | 6 | ƒ ƒ | | 4.85 | 1.6 | 3 | 8 | 5.7 |
| | 1 | 7 | ƒ | | 4.9 | 0.6 | 3 | 9.5 | 5.4 |
| | 0.5 | 7.5 | (ƒ) | | 5.2 | 0.2 | 4 | 16.5 | 6.4 |
| 44 | 4 | 4 | ƒ ƒ ƒ | | 4.4 | 6.2 | 3 | 9.5 | 6.4 |
| | 2 | 6 | ƒ ƒ | | 4.4 | 3.5 | 4 | 9.5 | 5.7 |
| | 1 | 7 | ƒ | | 4.4 | 1.8 | 3 | 8 | 4.8 |
| | 0.5 | 7.5 | (ƒ) | | 5.0 | 0.4 | 3 | 13.5 | 6.4 |
| 68 | 4 | 4 | ƒ ƒ ƒ | 5.5 | 4.3 | 7.6 | 3 | 10.5 | 6.8 |
| | 2 | 6 | ƒ ƒ | 5.6 | 4.3 | 3.6 | 2.5 | 10.5 | 6.7 |
| | 1 | 7 | ƒ | 5.7 | 4.4 | 1.8 | 2.5 | 8 | 5.9 |
| | 0.5 | 7.5 | - | 5.8 | 5.0 | 0.2 | - | - | - |

1/ ƒ INDICATES GROWTH OF THE ORGANISM. GREATER GROWTH TURBIDITY SHOWN BY INCREASE IN NUMBER OF ƒ MARKS. - INDICATES NO GROWTH.
2/ NOT INOCULATED.
3/ Ml 0.1-N NaOH REQUIRED TO TITRATE TO pH 5.5.
4/ THE USUAL DILUTION.

Inasmuch as it was possible that the increase in the length of the cells always occurred when growth was poor and was not necessarily related to vitamin $B_{12}$ concentrations, measures were taken to diminish the growth rate while keeping the concentration of vitamin $B_{12}$ constant. For this purpose the medium was diluted in the first experiment with distilled water and in the second experiment with salt solution. The dilution of the medium with the salt solution was made to avoid excessive change in buffer action and in osmotic pressure. The same buffer salts were used as in the regular medium and in the same concentration. Thus the concentration of the buffer salts was the same in all the tubes. The concentration of the vitamin was maintained at 0.4 millimicrograms per tube in both experiments.

Table 4 - Data on Growth of Lactobacillus leichmannii in Diluted Media Containing a Constant Amount of the Buffer Salts and of Vitamin $B_{12}$ (0.4 Millimicrograms per 8 Milliliters of Dilute Medium[1])

| Amount of Double-Strength Medium | Amount of Salt Solution | Amount of Water | Growth Turbidity[2] | pH of Control Tubes[3] | pH of Sample Tubes | Acidity Titration[4] | Length of the Cells | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Minimum | Maximum | Average |
| Milliliters | Milliliters | Milliliters | | | | Milliliters | Microns | Microns | Microns |
| 4 | 0 | 4[5] | ƒ ƒ ƒ | 5.5 | 4.5 | 7.7 | 4.5 | 15 | 8.7 |
| 2 | 2 | 4 | ƒ ƒ | 6.0 | 5.0 | 2.7 | 6 | 15 | 10.5 |
| 1 | 3 | 4 | ƒ | 6.3 | 5.5 | | 8 | 55 | 28.0 |
| 0.5 | 3.5 | 4 | (ƒ) | 6.5 | 6.1 | | 4.5 | 45 | 19.1 |

1/ INCUBATION PERIOD WAS 68 HOURS.
2/ ƒ INDICATES GROWTH OF ORGANISM. GREATER GROWTH TURBIDITY SHOWN BY INCREASE IN NUMBER OF ƒ MARKS. - INDICATES NO GROWTH.
3/ NOT INOCULATED.
4/ Ml 0.1-N NaOH REQUIRED TO TITRATE TO pH 5.5.

Dilution of the medium with distilled water did not appreciably affect cell length (Table 3). The length was nearly the same as would be expected from the concentration of the vitamin, even where poor growth was obtained.

The results of the experiment in which salt solution was used differed from those obtained by dilution of the medium with distilled water. The length of the cells increased greatly in the higher dilutions where the growth was also poor (Table 4).

Addition of 0.0025 to 0.1 millimicrograms of cobalt in the form of $CoCl_2$ to the tubes in the standard having the lowest concentration of vitamin $B_{12}$ (0.005 and 0.02 millimicrograms) did not influence the length of the cells.

## DISCUSSION AND SUMMARY

In the microbiological assay of vitamin $B_{12}$ with Lactobacillus leichmannii, the vitamin has a significant influence on the length of the cells. In the lower con-

centrations of the vitamin, the cells tend to grow longer and the division of the cells is restricted. Formation of the volutin granules is also disturbed. By diluting all the nutrients in the medium except vitamin $B_{12}$, growth rate of the cells was diminished, but the influence on the cell length was insignificant. Therefore, increased length of the cell is not a characteristic of poor growth. The increase in cell length resulting from dilution of all the ingredients in the medium, except the buffer salts and the vitamin $B_{12}$, might be explained as an inhibition of the activity of the vitamin.

Substances that are able to replace the vitamin $B_{12}$ in microbiological tests appear to have the same influence on the cell length as the vitamin itself.

It is interesting to note that in cases of pernicious anemia, in which there is a deficiency of vitamin $B_{12}$, the division of the earliest forms of the red blood cells in the bone marrow is restricted, causing them to grow larger than normal. Perhaps, vitamin $B_{12}$ affects the division of the red blood cells in the same way as it does the cells of Lactobacillus leichmannii.

## ACKNOWLEDGMENT

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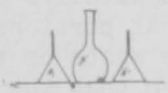

## CURING OF FISHERY PRODUCTS

Fish curing is an important method of preservation in the fishing industry and in the trade generally, but information on the principles involved in the salting and smoking of fish commercially is widely scattered. This report is a reference handbook on the problems of fish curing. It includes information from recent technical studies of the principles on which fish curing is based, discusses improvements in methods and equipment, and describes the standard methods.

By Norman D. Jarvis, Research Report No. 18. Fish and Wildlife Service, Washington 25, D. C. (1950), 270 pages. For sale by the Superintendent of Documents, U. S. Government Printing Office, Washington 25, D. C. Price 75 cents.