THE USE OF *LACTOBACILLUS VIRIDESCENS* AS A THIAMINE ASSAY ORGANISM

BY

THOMAS DAVID PITTS

THE USE OF *LACTOBACILLUS VIRIDESCENS* AS A THIAMINE ASSAY ORGANISM

A Research Paper
Presented to
the Graduate Council of
Austin Peay State University

In Partial Fulfillment
of the Requirements for the Degree
Master of Science

---

by
Thomas David Pitts
August 1970

To the Graduate Council:

I am submitting herewith a Research Paper written by Thomas David Pitts entitled "The Use of Lactobacillus viridescens as a Thiamine Assay Organism." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biology.

Charles S. Boruff
Major Professor

Accepted for the Council:

Wayne C. Stamper
Dean of the Graduate School

ACKNOWLEDGEMENTS

The author wishes to express appreciation to Mr. Charles Boehms, Dean of Students, Austin Peay State University, for his aid during the course of this study; to Dr. Ben P. Stone, Department of Biology, and Dr. Durward S. Harris, Department of Chemistry, for their suggestions and constructive criticisms of the manuscript.

Appreciation is also extended to Dr. Jimmy N. Trentham, Biology Department, University of Tennessee at Martin, who suggested the problem and furnished materials and assistance throughout the study.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Practically all organisms, whether aerobic or anaerobic, require thiamine (Vitamin B_1). Many bacteria, fungi, and protozoans, and most, if not all, multicellular green plants can synthesize the pyrimidine and thiazole rings and from these the thiamine molecule (Prosser and Brown, 1962). Many bacteria (Burkholder, in Natarajan, 1965), some algae (Provasoli, 1958), and some protozoans (Prosser and Brown, 1962) are able to synthesize thiamine if they are provided with a source of the pyrimidine and/or thiazole rings. Some of these organisms are able to synthesize one moiety, while others can synthesize neither moiety. Many protozoans, some fungi, some algae, and all investigated multicellular animals require the complete thiamine molecule from an outside source (Prosser and Brown, 1962).

Since thiamine is produced by a number of aquatic organisms and is water soluble, its occurrence would be expected in fresh natural water. Hutchinson (1943) found concentrations of thiamine ranging from 0.03 millimicrograms per milliliter of water (= mug/ml) to 1.2 mug/ml in fresh water ponds of Connecticut. Hutchinson (1943) also indicated the possibility of seasonal changes in the thiamine concentrations in fresh water. However,

his measurements were not evenly distributed throughout the year, and no other limnologists have continued his work. Measurements in salt water indicated variation in the seasonal distribution of thiamine. More work is needed to verify the pattern and source due to low thiamine concentrations and insufficient sampling (Natarajan, 1965).

Several techniques have been used to assay the thiamine content of a variety of materials. The test most widely used by chemists is the thiochrome technique which requires instruments not readily available to most limnologists. Burkholder and McVeigh (1940) described conditions under which the fungus Phycomyces blakesleeanus could be used for thiamine assays. Hutchinson (1943) used this technique in the assays of pond water noted above. The P. blakesleeanus technique has a number of undesirable features: ten or more days are required to complete an assay; the fungus will respond to the moieties of thiamine, as well as the complete molecule; and, water must be concentrated to bring the thiamine levels within a measurable range.

A recently described bacterium, Lactobacillus viridescens has been suggested as a thiamine assay organism (Diebel, et al., 1957). The present study attempts to adopt the L. viridescens technique of Skeggs

(1963) for fresh water thiamine assays due to its response only to the intact thiamine molecule, the small amount of time required for an assay, the response to amounts of thiamine as low as 1 mug/ml, and the comparative ease with which the assay could be run. The purposes of this study were to describe the L. viridescens assay technique, suggest modifications of the technique, and verify the technique for thiamine assays in fresh natural water.

CHAPTER II

MATERIALS AND METHODS

Description of Lactobacillus viridescens

The bacterium Lactobacillus viridescens possesses the following characteristics according to Niven and Evans (1957). The cells are gram positive, nonmotile, nonspore-forming, small rods occurring singly or in pairs. They are faculatively anaerobic and produce considerable quantities of carbon dioxide and lactic acid when growing vigorously. Frequently these organisms are the cause of greening of cured meat pigments and are often found in cured meat.

Assay Design

In suggesting L. viridescens as a thiamine assay organism, Skeggs (1963) proposed the following procedure. The growth of L. viridescens in a medium containing known amounts of thiamine was established by using six concentrations of thiamine. Tubes containing 0, 5, 10, 15, 20, 30, and 50 mug of thiamine, respectively, were used. Thiamine hydrochloride was used as the standard and was prepared at a concentration of 10 mug/ml. Five milliliters of assay medium (See Materials, below, for description of assay medium.) were added to each tube,

and the volume of liquid in each tube was brought to 10 ml with distilled water.

A sample of the material to be assayed was dissolved, or diluted, in distilled water and added to tubes in the amounts of 1, 2, 3, 4, and 5 ml, respectively. Five milliliters of assay medium were added to each tube plus sufficient distilled water to bring the volume of liquid in each tube to 10 ml.

The tubes containing known amounts of thiamine and tubes with unknown amounts were autoclaved at 121°C for five minutes. After the tubes were cooled one drop of a previously prepared inoculum was added to each tube. The inoculum was prepared by growing cells of L. viridescens at 30°C for 16 to 20 hours in a medium enriched with thiamine. The cells were centrifuged and washed three times in 0.9% saline and were then suspended and frozen in assay medium until needed. Storage was not recommended for periods exceeding 30 days.

After inoculation an incubation period of 20 hours at 30°C was recommended. Results were determined by measuring turbidity, expressed as absorbance units, with colorimeter using a 660 mu filter. A standard curve may be drawn by plotting the absorbance readings of tubes with known amounts of thiamine against the amount of thiamine present. Results of the assay may then be

determined by comparing the absorbance readings of tubes containing an unknown amount of thiamine with the standard curve.

Except as noted, the above procedure was used in the current work with a standard curve being run for each experiment.

Materials

In the present study the following materials were used as indicated in the above procedure. L. viridescens Strain S38A was obtained from the American Type Culture Collection, Rockville, Maryland. Inoculum was prepared by growing cells in Difco Micro Inoculum Broth, 0320-02 (Difco Laboratories, Detroit, Michigan) and was then frozen at -5°C. Assay medium used was Thiamine Assay Medium LV, 0808-15 (Difco Laboratories, Detroit, Michigan). Standard solutions were prepared using thiamine hydrochloride (Nutritional Biochemicals Corp., Cleveland, Ohio). In experiments using filtered solutions filtering was accomplished by using 0.45 micron Millipore filters (Millipore Corp., Bedford, Mass.). Measurements of growth were determined with a Spectronic 20 (Bausch and Lomb, Rochester, N. Y.).

CHAPTER III

EXPERIMENTS AND RESULTS

Inoculum Preparation

In an attempt to verify the suggested 16 to 20 hours of incubation for inoculum (Skeggs, 1963), a growth curve was plotted for L. viridescens. One tube of stored inoculum was used to inoculate two tubes of inoculum broth. The two tubes of inoculum, which contained thiamine, were incubated until a visibly high turbidity was obtained at 24 hours. Material from these tubes was then used to inoculate another pair of inoculum broth tubes. Absorbance readings were taken from the second pair of tubes at intervals of one or two hours. A tube of sterile inoculum broth served as the zero reference point. The resulting growth curve is shown in Figure 1. In this experiment exponential growth began about five hours after inoculation and continued for approximately thirteen hours.

Comparison of Mass Inoculation And Individual Tube Inoculation

Due to variations in results when individual tube inoculation was used as suggested by Skeggs (1963), an alternative procedure was considered. Sterile assay medium was mass inoculated and distributed to the tubes

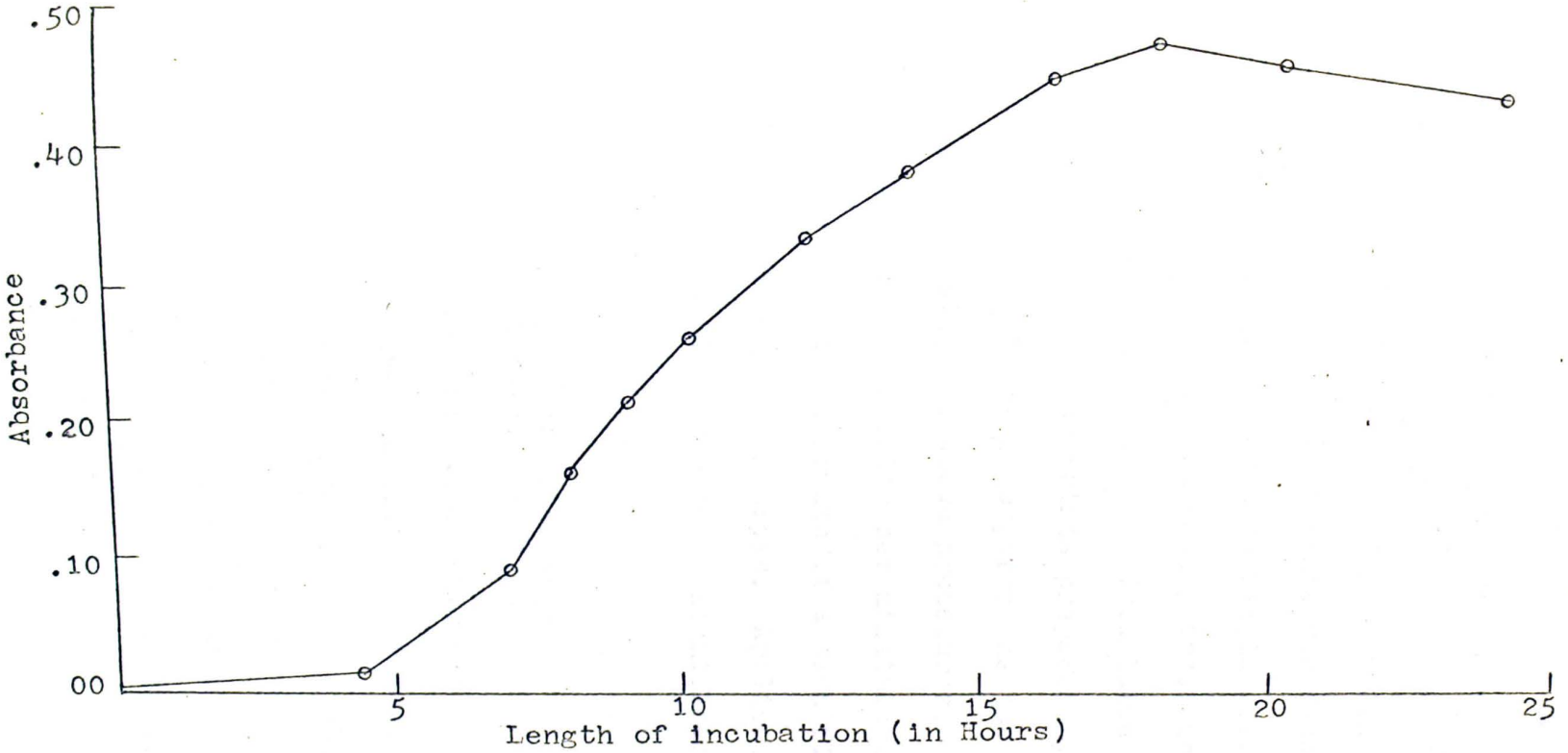

Figure 1. Growth curve for L. viridescens in inoculum broth.

followed by the addition of sterile water and standard solution in the appropriate amounts.

Table I shows the ranges of results at each point on the standard curve using mass inoculation and individual tube inoculation. Each range was determined by subtracting the lowest absorbance reading from the highest absorbance reading at each point. Ten replications were made at each point. As seen from Table I, the average range was less for points prepared by individual tube inoculation. Figure 2 compares the two standard curves obtained using the above procedures. The yields, calculated in absorbance units per millimicrogram of thiamine, were used to determine if a significant difference exists between the two curves. Application of the student's t-test to data showed no significant difference between the curves.

Comparison of Autoclaved And Filtered Standards

Natarajan (1965) indicated destruction of some thiamine when autoclaved in distilled water. To determine if thiamine was destroyed by autoclaving, a comparison was made between standard curves using autoclaved and filtered standard solutions. The autoclaved tubes containing thiamine solution were heated at 250°F and 15 pounds of pressure for 15 minutes. Filtered

TABLE I

RANGES OF RESPONSES USING MASS INOCULATION OF MEDIUM AND INDIVIDUAL TUBE INOCULATION

Point on Curve (in mug/ml)	Range (in absorbance units)	
	Mass Inoculation	Individual Tube Inoculation
0	.055	.035
5	.025	.02
10	.11	.045
20	.055	.06
30	.07	.08
50	.15	.06
	Average= .087	Average= .05

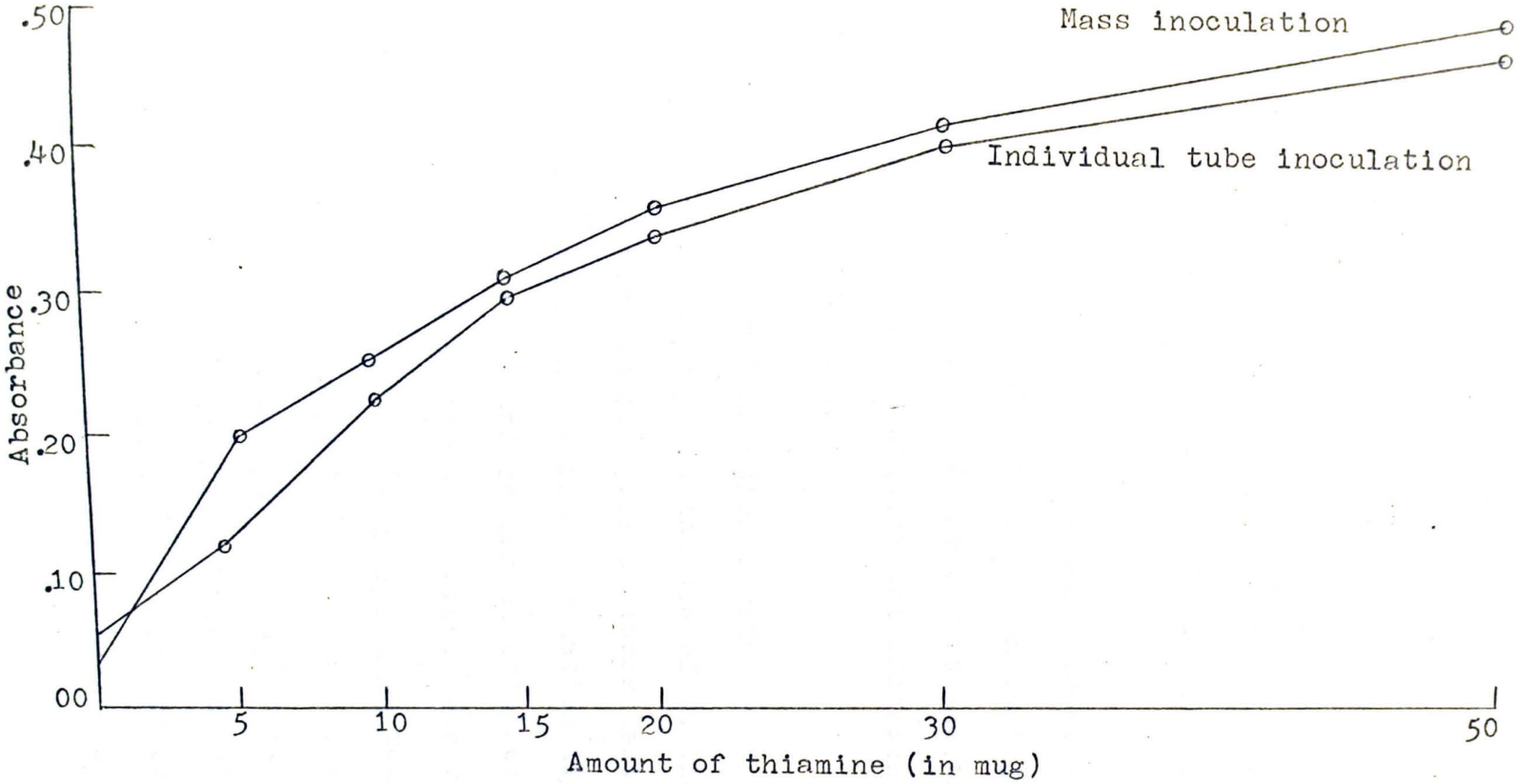

Figure 2. Standard curves obtained by using mass inoculation and individual tube inoculation.

standard solution was prepared by passing freshly mixed thiamine solution through a sterile 0.45 micron Millipore filter. Each standard curve was determined using 7 concentrations with each point representing the mean of 10 tubes. All factors, other than the method of standard solution sterilization, were identical for the two groups of tubes.

The results of the comparison between autoclaved and filtered standard solutions are shown in Figure 3. Yields were calculated for each of the curves and the student's t-test was applied. No significant difference was found between the two curves.

Optimum Incubation Period

Skeggs (1963) proposed the use of an incubation period of 20 hours at 30°C for cultures less than 30 days old. To determine the optimum incubation time, a comparison was made of the standard curves obtained at varying incubation times with three cultures. One of the cultures was 3 days old (i.e., the L. viridescens cells had been stored frozen in assay medium for 3 days), another culture was 34 days old, and the third culture was 50 days old. Each point on each curve represents the mean absorbance value of three tubes. Absorbance values were taken at intervals of two or four hours. Standard

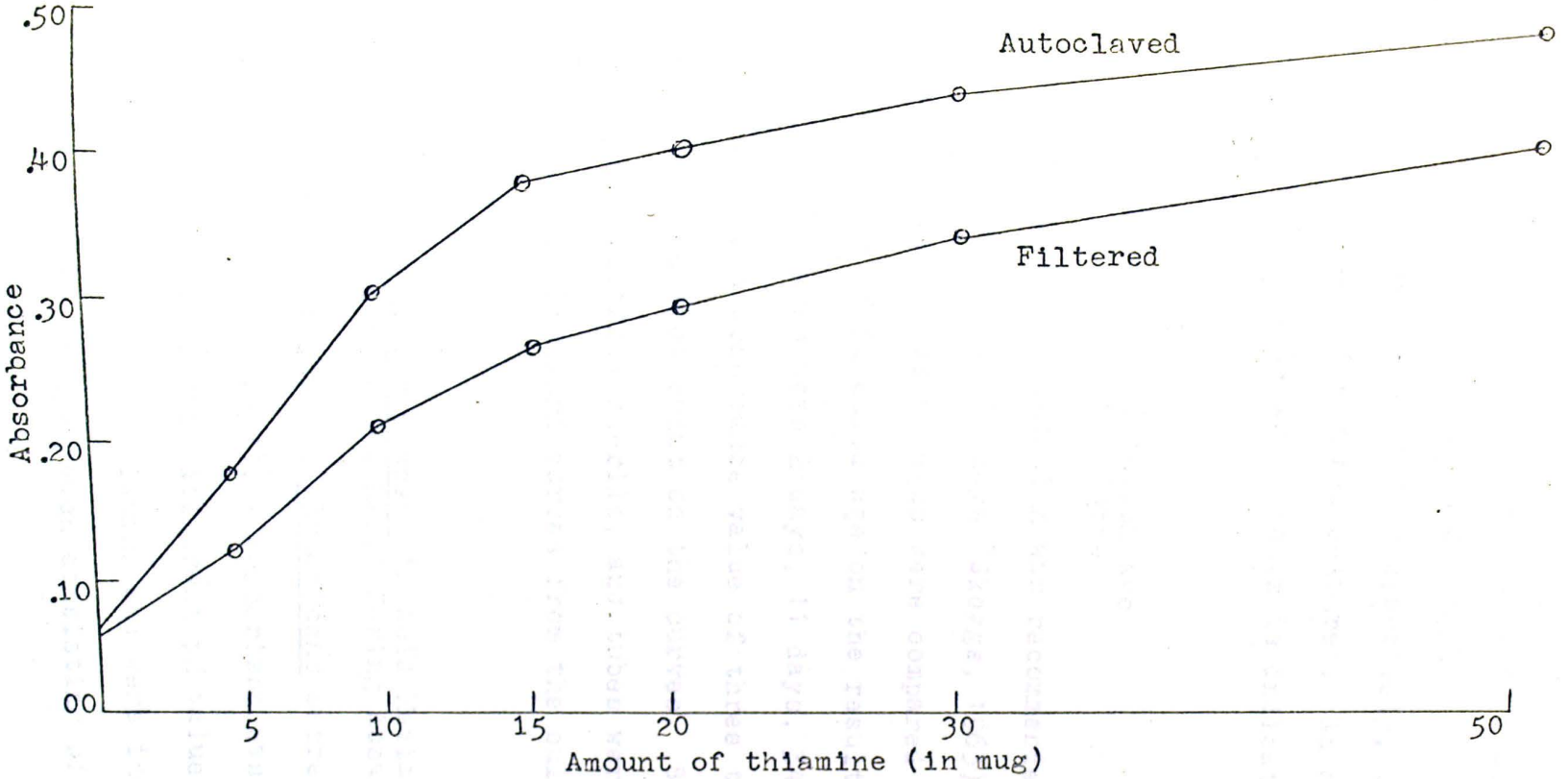

Figure 3. Standard curves obtained by using filtered and autoclaved standard solutions.

solution for each curve was sterilized by filtering and all tubes were individually inoculated.

Figures 4, 5, and 6 show, respectively, the standard curves obtained from the three cultures. An optimum incubation period of 24 to 28 hours is indicated for each of the cultures.

Effects of Inoculum Age On Standard Curve

Storage of frozen inoculum was recommended for periods not longer than 30 days (Skeggs, 1963). Four inoculum cultures of known ages were compared to determine the effects of inoculum age on the resulting standard curve. Inoculum ages were 2 days, 11 days, 34 days, and 50 days. The mean absorbance value of three tubes was used to determine each point on the curves. Standard solutions were filtered sterile, and tubes were individually inoculated. Standard curves from the cultures are shown in Figure 7.

Growth of L. viridescens in Acid Media, And Changes in pH of Media During Assay

Due to the fact that L. viridescens secretes an acid (Niven and Evans, 1957), a comparison was made of growth in assay medium with different pH values. All tubes contained 30 mug of thiamine and were incubated for 20 hours. Four groups, each consisting of three

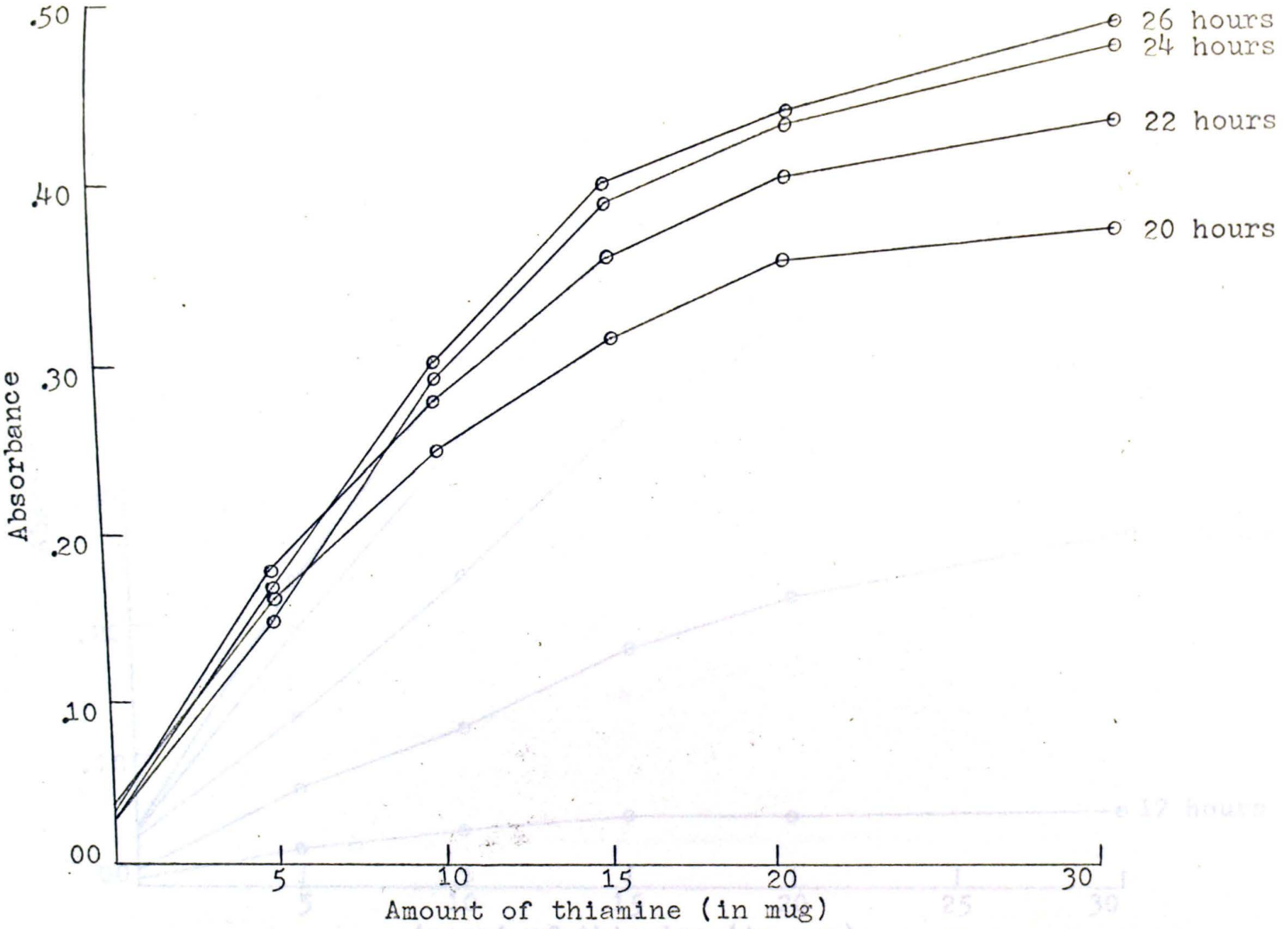

Figure 4. Standard curves for a 3 day old culture.

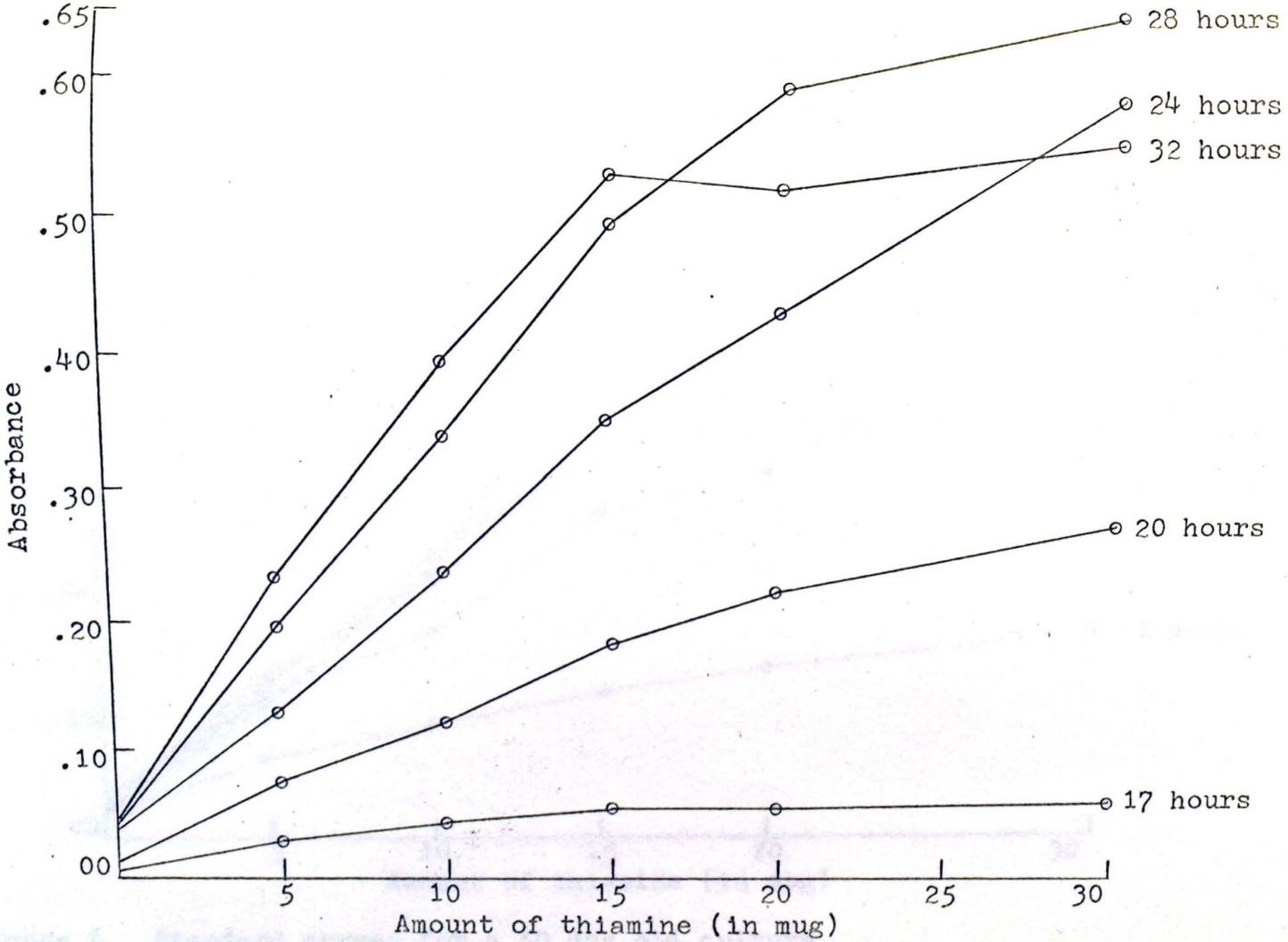

Figure 5. Standard curves for a 34 day old culture.

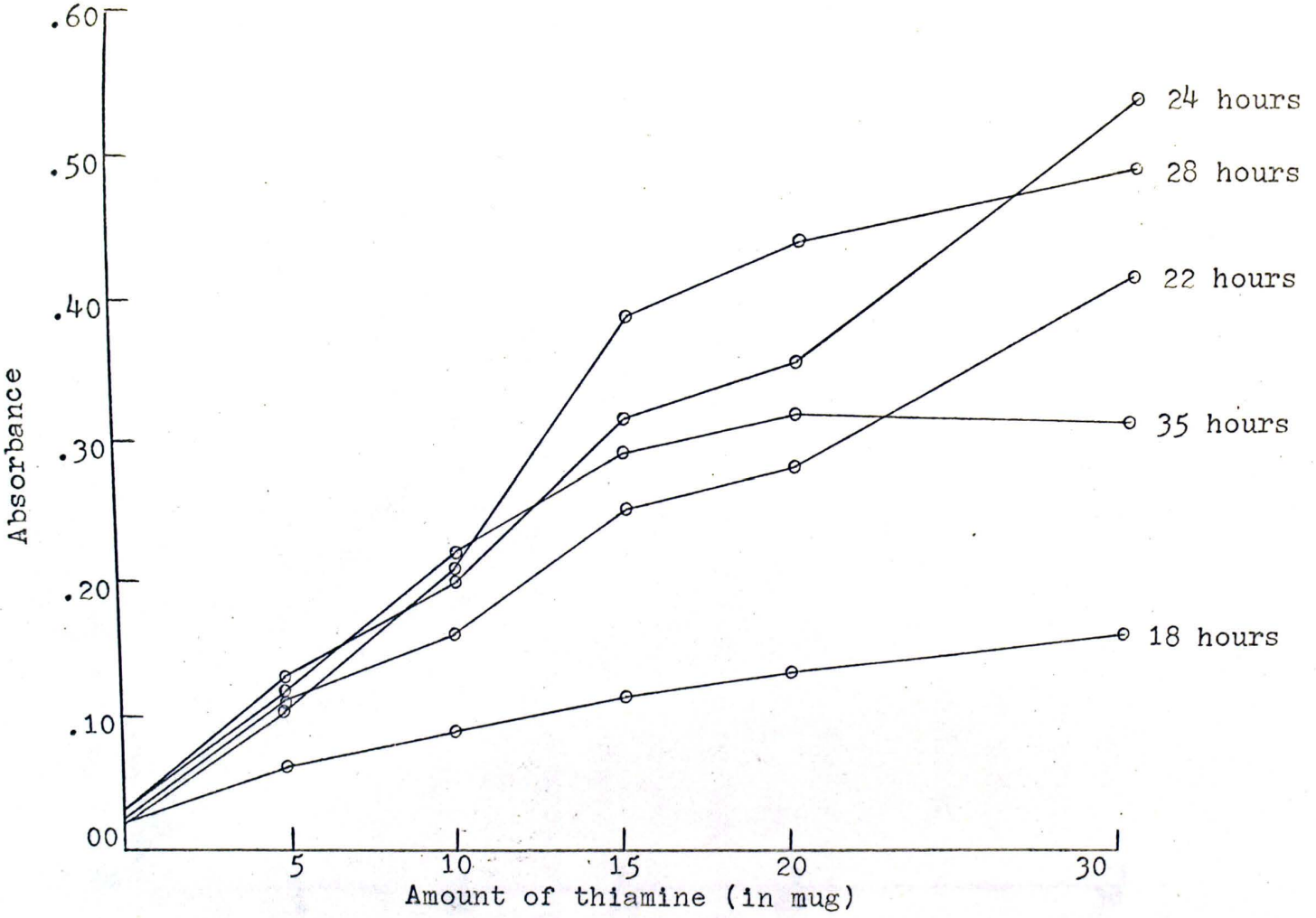

Figure 6. Standard curves for a 50 day old culture.

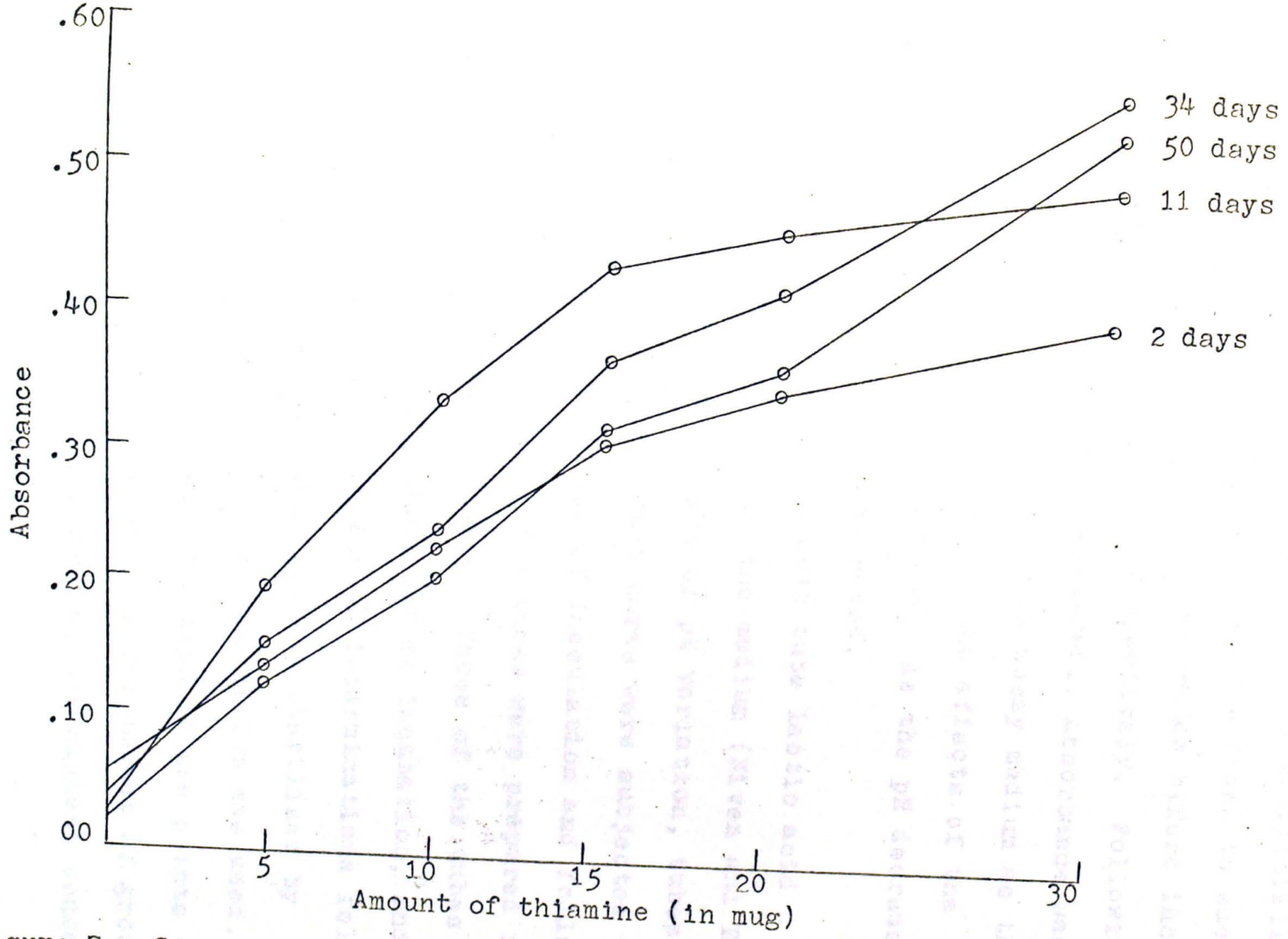

Figure 7. Comparison of standard curves of different age cultures.

tubes, were prepared. One group served as the control in which the pH of the assay medium was not artificially altered. Hydrochloric acid (0.1 N) was added to each of the remaining groups to produce pH values before incubation of 5.20, 4.10, and 3.00, respectively. Following incubation the pH was again measured. Absorbance was determined using a tube of sterile assay medium as the reference point. Table II shows the effects of the medium pH on the amount of growth. As the pH decreased, the amount of growth also decreased.

As growth progresses in each tube lactic acid accumulation alters the pH of the medium (Niven and Evans, 1957). To determine the range of pH variation, tubes from each point on the standard curve were subjected to pH determinations at the time of inoculation and following 20 hours of incubation. Six tubes were prepared for each point on the standard curve. Three of the tubes were used for pH determinations before incubation, and three of the tubes were used for pH determinations following incubation. Standard solution was sterilized by filtering and mass inoculation of the medium was used. Table III shows the pH changes for each of the points on the standard curve. Correlated with the amount of growth, the greatest change in pH occurred at the highest concentration of thiamine.

TABLE II

EFFECTS OF MEDIUM pH ON THE GROWTH OF L. VIRIDESCENS

pH of Medium		Absorbance
Beginning	End	
6.20	5.95	0.22
5.20	5.00	0.07
4.10	3.90	0.04
3.00	2.75	0.01

TABLE III

pH VARIATION IN MEDIA OF STANDARD CURVE

mug B_1	pH of Medium	
	Beginning	End
0	6.20	6.20
5	6.20	6.10
10	6.20	6.05
15	6.20	6.00
20	6.20	6.00
30	6.20	5.95
50	6.20	5.85

Verification of L. viridescens Assay

To verify the use of L. viridescens assay technique, fresh natural water and distilled water samples, each containing known amounts of thiamine, were assayed. Three thiamine-distilled water solutions were prepared. Each solution consisted of 200 ml of water plus 2, 4, or 6 mug of thiamine per ml of water. Three 5 ml samples of distilled water were taken before thiamine was added, and three 5 ml samples were taken from each solution after thiamine was added. Natural water from a University of Tennessee at Martin farm pond was also assayed. Three 5 ml samples were taken to determine the amount of natural thiamine present. Two 50 ml thiamine-natural water solutions were prepared. One solution contained 2 mug of artificial thiamine per ml of water, and the other solution contained 4 mug/ml. Table IV presents the results of the assays. No natural thiamine (0 mug/ml) was detected in the distilled water, and 0.72 mug/ml were detected in the pond water. Results in Table IV refer only to the amount of thiamine artificially added.

TABLE IV

VERIFICATION OF ASSAY USING WATER WITH KNOWN AMOUNTS OF THIAMINE

Type Sample	Amount of Thiamine Added (in mug/ml)	Amount of Thiamine as Determined by Assay (in mug/ml)	Percentage of Error
Distilled water	2	2.6	30
Distilled water	4	4.4	10
Distilled water	6	5.7	5
Pond water	2	2.08	4
Pond water	4	4.48	12

CHAPTER IV

DISCUSSION

Verification was made of Skeggs'(1963) recommendation of inoculum preparation from a 16 to 20 hour culture. The growth curve obtained for L. viridescens (Figure 1) indicates the period of exponential growth extends from 5 to 22 hours of incubation. Due to variation in lag phase duration, this curve would probably not be repeated exactly for every culture, but cultures treated as above should give comparable results.

Several changes are suggested in the assay technique of Skeggs (1963). Optimum incubation time would appear to be 24 to 28 hours (Figures 4, 5, and 6). The 20 hour incubation time recommended by Skeggs (1963) was partially determined by use of only 5 minutes of autoclaving. This short period of autoclaving would not sterilize the media and, consequently, a short incubation period was used to avoid contamination. Results of the present study indicate no significant difference between thiamine solutions autoclaved for 15 minutes and those filtered sterile. A longer incubation time (24 to 28 hours) and a longer period of autoclaving would be recommended. The method of sterilization should be consistent with that used for samples to be assayed even

though no significant difference was detected between filtered and autoclaved standards. If samples are filtered sterile, the standards should be filtered, and if samples are autoclaved, the standards should be autoclaved.

The recommended maximum storage time of 30 days for inoculum is questioned. Standard curves from cultures of ages 2 days to 50 days are not significantly different. Possibly even older cultures may give usable standard curves.

Efforts to reduce the range of responses at the points on the standard curve were futile. Table I shows the range of responses using mass inoculation of media to be slightly greater than the range using individual tube inoculation. Neither method can be given preference. In some cases the individual tube inoculation technique may be preferred even though it requires more time to perform. In other cases the mass inoculation technique may be preferred for its ease of preparation.

Growth of L. viridescens is limited in media of low pH (Table II). However, the changes in medium pH during incubation are small for all points on the standard curve (Table III) and probably have little limiting effect on the growth of L. viridescens.

To verify the detection ability of the L. viridescens technique, water samples of known thiamine content were assayed (Table IV). The results are within the expected ranges, and, consequently, the L. viridescens assay technique is recommended for thiamine assays of fresh water.

LITERATURE CITED

Burkholder, P. R. 1963. Some nutritional relationships among microbes of the sea sediments and waters. p. 133-150. In Sym. Mar. Microbiol., C. H. Oppenheimer, (ed.), Charles C. Thomas, Publisher, Springfield, Illinois, cited by Natarajan, K. V. 1965. Bioassay and distribution of thiamine in the sea. Ph. D. Thesis. Univ. of Alaska. 102 p. Univ. Microfilms. Ann Arbor, Mich.

Burkholder, P. R., and Ilda McVeigh. 1940. Studies on thiamine in green plants with the Phycomyces assay method. Amer. Jour. Bot. 27: 853-861.

Diebel, R. H., J. B. Evans, and C. F. Niven. 1957. Microbiological assay for thiamine using Lactobacillus viridescens. J. Bacteriol. 74: 818-821.

Hutchinson, G. T. 1943. Thiamine in Lake Waters and Aquatic Organisms. Arch. Biochem. 2: 143-150.

Natarajan, K. V. 1965. Bioassay and distribution of thiamine in the sea. Ph. D. Thesis. Univ. of Alaska. 102 p. Univ. Microfilms. Ann Arbor, Mich.

Niven, C. F., and J. B. Evans. 1957. Lactobacillus viridescens nov. spec., a heteroferementative species that produces a green discoloration of cured meat pigments. J. Bacteriol. 73: 758-759.

Prosser, C. L., and F. A. Brown. 1961. Comparative Animal Physiology. W. B. Saunders Co., Philadelphia. 688 p.

Provasoli, L. 1958. Nutrition and ecology of protozoa and algae. p. 279-308. In C. E. Clifton, S. Raffel, and M. P. Starr, (eds.). Annual Review of Microbiology. Vol. 12 Annual Reviews, Inc., Palo Alto, Calif.

Skeggs, H. R. 1963. Turbidimetric Assay for Thiamine. p. 521-525. In F. Kavanagh, (ed.), Analytical Microbiology. Academic Press, New York.