MICROBIOLOGICAL SYNTHESIS OF RIBOFLAVIN TO ENRICH SWINE VISCERA USED FOR POULTRY AND LIVESTOCK FEED

by

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INTRODUCTION

Wilgus, Norris and Heuser (43) have stated that the variable growth promoting properties of protein supplements used in poultry rations are due to the quantity and quality of the protein components and to the riboflavin content. On this basis dried skim milk and sardine fishmeal are classed as two of the better common supplements. Tankage, a packing house by-product - sometimes referred to as digester tankage and meat meal - has a protein value nearly equal to that of the two above mentioned supplements, but in a comparative sense tankage is inferior in vitamin B_2 potency. As reported by Wilgus and his coworkers, the relative vitamin B_2 potency values per gram of supplement material are 19 mcg. (micrograms per gram) for dried skim milk, 10 mcg. for sardine fishmeal and 6 mcg. for meat scraps (43). When assayed microbiologically, the particular batch of tankage used by the authors gave the approximate value of 3.5 mcg. of riboflavin per gram of material (dry weight).

Although tankage satisfies the minimum vitamin B_2 requirement of 2.5 mcg. per gram of feed for poultry (13), it is desirable to enhance the nutritional value of this product for livestock and poultry so as to raise its competitive standing as a feed supplement. It was the purpose of this work to find a microorganism which, when grown on a tankage broth medium, would synthesize an amount of riboflavin sufficient to raise the B_2 potency of the tankage above 19 micrograms per gram dry weight. This would afford a low cost B_2 enrichment of tankage and markedly enhance its value as a poultry and livestock supplement.

A microbiological approach to the solution of this problem was undertaken because of the information that has accrued in the literature concerning *in vitro* and *in vivo* synthesis of vitamins by microorganisms (1, 4, 28, 40). The microorganisms investigated for vitamin B_2 synthesis on tankage medium were of two groups, namely; the organisms obtained from stock-cultures and those isolated from hog viscera.

Included in the stock-cultured group are some of the microorganisms reported to be riboflavin producers (5, 16, 10, 22, 27, 33, 38, 40, 42). Their ability to synthesize riboflavin on various media suggested the possibility that these organisms might be capable of B_2 synthesis on a tankage medium. On the other hand, the unknown isolates consisted of the microorganisms isolated in pure culture from the flora of a representative sample of hog viscera - excluding the heart, liver and kidneys. The choice of these microorganisms as potential vitamin B_2 producers was influenced by the reports of many investigators that the intestinal flora of various animals synthesized certain of the B vitamins (1, 4, 28).

Using the microbiological assay method to determine the amount of riboflavin synthesized, these two groups of organisms were investigated to determine those capable of the highest productivity and the conditions most favorable to synthesis.

LITERATURE REVIEW

The ability of various animals to synthesize B vitamins in their alimentary tracts has been an object of investigation for many years. It is reported that this synthesis is a mechanism of the microbiological flora of the digestive tract (1). Bechdel and coworkers in 1928 (4) reported the production of vitamin B-complex in the rumen of the cow. They further established that a Flavobacterium sp. constituted ninety per cent of the micro-flora responsible for this synthesis. It has been found that other ruminants possess this faculty. McElroy and Goss in 1939 found that thiamine, riboflavin, pantothenic acid, and B_6 are synthesized in the rumen of the sheep (24). B vitamins are also produced in the alimentary tract of the horse (9).

It was later found that the bacterial flora of monogastric animals are also capable of producing vitamins. Members of the B-complex are synthesized in the cecum of the rat (26, 15). Riboflavin, pantothenic acid, folic acid, and biotin are synthesized in the intestines of the rabbit. The amounts of riboflavin and pantothenic acid produced are sufficient to satisfy the dietary needs of the rabbit (28).

The microbiological synthesis of vitamins is not limited to members of the B group. Certain intestinal organisms are found to be capable of producing vitamins E (17) and the antihemorrhagic K (2).

From this evidence it would appear that the majority of animals have no need for riboflavin supplement in their diet. However, livestock that are capable of supporting the synthesis of B_2, require supplementary riboflavin to maintain normal growth and development (9, 18).

The knowledge of *in vivo* synthesis has stimulated many investigations concerning the *in vitro* production of riboflavin by microorganisms. *Proteus vulgaris*, *Escherichia coli*, *Aerobacter aerogenes*, *Alcaligenes faecalis*, *Bacillus mesentericus*, and *Bacillus vulgatus* have been found to produce riboflavin on various synthetic media (5, 10, 34, 38). Members of the following genera also have exhibited B_2 synthesis on various synthetic media: *Micrococcus* (27), *Clostridium* (42), *Mycobacterium* (23), *Azotobacter* (20, 38), *Actinomyces* (38). Many yeasts, principally *Candida* and *Torula*, synthesize sizeable amounts of riboflavin on synthetic media (6, 7, 21, 22, 38). Some B_2 production by molds has also been reported (31, 38).

Microbiological synthesis of riboflavin has progressed to a large-scale production of B_2 on a commercial basis. This development came about through investigations of the two fungi, *Eremothecium ashbyii* and *Ashbya gossypii*. Foreign and domestic patents have been issued on B_2 production by *Eremothecium ashbyii* (19, 40). Tanner and his associates have been largely responsible for successful production of riboflavin by *Ashbya gossypii*. Their investigations of pH, medium constituents, aeration, and other environmental factors have facilitated increased production by this organism (32, 40).

The medium used for the microbiological production of B_2, ranges from corn steep liquor, yeast extract, meat scraps or tankage, and disaccharides (32, 33, 40), to the simple peptone, glucose, and ammonium sulfate required by *Candida* yeasts (7, 21). It is generally agreed, however, that a source of nitrogen and a utilizable carbohydrate are essential for the microbiological synthesis of riboflavin (7, 34, 40).

Various methods of assay are employed in the analysis of riboflavin synthesis. Some investigators use the relative growth of test animals, such as rats (38) and chicks (9). When the time requirement is essential, most investigators use the more rapid method of fluorometric analysis (12, 20). The majority of workers prefer the microbiological method of Snell and Strong (41), which has been found to be the more accurate assay (12). The test organism, Lactobacillus casei, measures only B_2, even in the presence of other water-soluble vitamins (12).

Riboflavin requirements of poultry (13, 29) and livestock (9, 18, 25) have been reasonably well established. An inexpensive source of this growth factor for feeding poultry and livestock has become a pertinent problem. Synthesis by chemical methods such as the condensation process of Farkas and Flexser (14), produce a pure but expensive vitamin. Riboflavin obtainable by the commercial microbiological processes (19, 32) is not yet economically practical for stock consumption. The inexpensive incorporation of B_2 into an excellent protein source such as tankage (25), would seem to be of considerable value in livestock and poultry feeding.

EXPERIMENTAL

It was desired that an organism be found which was capable of synthesizing riboflavin, on tankage medium, in a quantity sufficient to raise the vitamin B_2 potency of the tankage above the values recorded for similar protein supplements. The succeeding experimental phases discuss the methods used and the results obtained in seeking this objective.

Materials and Cultures

1. The tankage used was obtained by the usual processing method and consisted of scraps occurring as by-products of swine slaughter (25). The sample used in this assay was composed of tankage made from the slaughtering of thirty-one healthy hogs weighing between 200 and 250 lbs. From the time of processing at a slaughter house in Staunton, Virginia until used in this investigation, the tankage was maintained in the frozen state.

2. Isolates were obtained from the flora present in a representative sample of hog viscera from which the heart, kidney and liver had been excluded. The hog viscera was maintained in the frozen state until ready for sampling.

3. Of the many microorganisms proven capable of riboflavin synthesis, a few were chosen to be used in this work. The choice of the organisms was governed primarily by their availability and their suspected adaptability to the tankage medium.

The following organisms, obtained from stock cultures carried in the Virginia Polytechnic Institute Bacteriology Laboratory, are listed

with reference to the reported ability of respective species to synthesize riboflavin:

a. Aerobacter aerogenes (5, 16)

b. Alcaligenes faecalis (5)

c. Escherichia coli (5, 38)

d. Escherichia coli var. communior (5, 38)

e. Clostridium acetobutylicum (40, 42)

f. Micrococcus flavus (27)

g. Proteus vulgaris (5, 10)

h. Torula lactosa (22, 33)

i. Torula spaerica (22, 33)

Proteus vulgaris #355 and Bacillus subtilis #480 were secured through the cooperation of Dr. Raymond E. Crandall, University of North Carolina (10).

Clostridium acetobutylicum was cultured on a medium consisting of: 250 ml. - distilled water; 6.25 gm. - Heart Infusion Broth (Difco); 2.7 gm. - Brain Veal Agar (Difco). All other organisms were cultured on Nutrient Agar (Difco).

4. The assay test organism, Lactobacillus casei - American Type Culture Collection #7469, is reported by Snell and Strong (35) to be probably identical with the Bacillus casei of Freudenreich. This organism, requiring a medium containing riboflavin for growth, was cultured in Micro-Assay Agar (Difco). The eighteen-hour inoculum used in the assay procedure was cultured in Micro Inoculum Broth (Difco) supplemented with riboflavin (41).

5. A stock solution of 10 mcg./ml. of U.S.P. riboflavin, with toluene added to prevent mold growth, was maintained in the dark at 4°C. To obtain the assay standard, this stock solution was diluted with distilled water in Riboflavin Assay Medium (Difco) (41).

6. In order to estimate the percentage accuracy of the assay technique, samples of known riboflavin content were obtained from the Association of Vitamin Chemists (41). These samples consisted of liver extract containing an estimated 19.6 mcg. of riboflavin per gram.

Experimental Procedure

In the first phase of the investigation the authors concerned themselves with the familiarization of the microbiological assay for riboflavin. Before endeavoring to assay samples of unknown vitamin B_2 potency, it was necessary to test the assay microorganism, Lactobacillus casei, for its requirement of riboflavin for growth. The test, based on the principle that Lactobacillus casei produces acid in amount proportional to the amount of riboflavin present in the medium, was accomplished by determining the amount of acid produced by the test organism after a three-day incubation period in a series of tubes containing Riboflavin Assay Medium (Difco) and gradient amounts of a standard riboflavin solution (0.1 mcg. B_2/ml. solution). A typical standard curve was obtained by plotting the riboflavin concentration against the volume of weak alkali solution required to neutralize the acid. The results were in agreement with those reported in Methods of Vitamin Assay (41), and exemplified the assay principle that growth and the resulting acid formation are a function of riboflavin concentration.

The assay procedure, described in greater detail in Experiment I, was taken from Methods of Vitamin Assay (41). When employed on oven-dried tankage samples, the approximate value of 3.5 mcg. B_2 per gram of material was obtained. The validity of this value was established by the assay of liver extract check samples sent to the authors by The Committee on Collaborative Assay, The Association of Vitamin Chemists, 808 South Wood Street, Chicago, Illinois. One gram of check sample, as determined by the authors, contained approximately 19.2 mcg. riboflavin (based on three separate determinations) as compared with the average value of 19.6 mcg. per gram reported to the Committee on Collaborative Assay by other investigators.

The second phase of experimentation involved the isolation in pure culture of those microorganisms that constitute the normal flora of hog viscera. Aerobic and anaerobic isolations were made from non-putrefied and slightly putrefied visceral samples. Pure cultures of eighteen, apparently different, microorganisms were obtained. Since a given microorganism cultivated on different media may display varied characteristics, two or more microorganisms included in the group might have been of the same strain. Due to the luxuriant growth obtained under aerobic conditions, these organisms were cultured on agar slants in all subsequent work.

Using pure cultures of the eighteen isolates and eleven stock-culture organisms, each microorganism was investigated to determine its ability to synthesize riboflavin. To accomplish this purpose, each organism was cultivated in tankage broth over a period of six days. Separate sample bottles of tankage medium, one for assay after each succeeding day of incubation, were inoculated with each organism to be investigated. This

procedure was used to determine the rate of riboflavin synthesis and the day on which maximum production could be expected.

Although some difficulty arose in the interpretation of data obtained using the separate sample method, it was possible to select the highest vitamin B_2 producing microorganism for further study. In an effort to explain the discrepancies encountered in this phase of investigation, it was necessary to exclude the environmental variable introduced by the separate sample method. A batch method of sampling, withdrawing assay samples from a quart jar of inoculated tankage medium, was designed to show riboflavin synthesis resulting from the progressive growth of the highest vitamin B_2 producing organism in a single cultural environment.

Certain physical and chemical factors were investigated as to their effect on the riboflavin synthesis of the highest yielding bacterium. The effects of pH, surface to volume ratio of the medium, and agitation were observed.

Finally, studies were made on the morphological, cultural and biochemical properties of the highest riboflavin-producing microorganism.

Experiment I - Preliminary Study of Technique and Other Factors Involved in the Assay of Riboflavin

All steps in the microbiological assay of riboflavin, except enzymatic hydrolysis and ether extraction, were followed as outlined in Methods of Vitamin Assay (41). Essentially, the assay consisted of the following steps: The preparation of unknown samples followed by serial dilution of the unknown and serial dilution of the riboflavin standard series; the inoculation of all tubes with the assay test organism, Lactobacillus casei; titration of the acid following 72 hours of incubation; the construction of the riboflavin standard curve from titration values of the standard series; and the calculation of vitamin B_2 content of the unknown samples by interpolation from titration values.

Several factors related to the assay and the problem at hand were studied:

1. Construction of a Riboflavin Standard Curve

All riboflavin standard curves used in this investigation were prepared in the following manner:

To a duplicate set of eight tubes containing 5.0, 4.5, 4.0, 3.5, 3.0, 2.5, 2.0, and 1.5 ml. of Riboflavin Assay Medium (Difco), 0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 3.5 ml. of riboflavin standard solution (0.1 mcg. B_2/ml. of solution) were added respectively. All tubes were brought to a final volume of 10 ml. with distilled water and sterilized in the autoclave (121°C. for 15 min.). After inoculation with L. casei and 72 hours of incubation at 37°C. the acid content of each tube was titrated to a pH

of 6.8 with approximately 0.1 N NaOH solution. In this case, the titration end point was ascertained with brom-thymol-blue indicator, but in all subsequent work the Beckman pH meter was used.

Curve A of Figure 1 illustrates the nature of the standard curve obtained when the titration values (Table 1) were plotted against known riboflavin concentration. To obtain a comparative study, a second set of riboflavin tubes was prepared and treated in the same manner as the previous set. The titration values are shown in Table 2. Curve B (Figure 1) was plotted from these values.

From the curves shown in Figure 1, it was concluded that the amount of acid formed during the growth of Lactobacillus casei was proportional to the amount of riboflavin present in the medium. Agreement is noted in the general slope of both curves (Figure 1). The difference observed in their magnitudes results from the different concentrations of alkali used in the separate assay titration for Curve A and Curve B. This difference in alkali concentration was of no consequence to the validity of the assay, since each set of unknown samples derived its values from a standard series which was titrated with the same alkali solution as that used in the titration of the unknown samples. Thus an allowance is made in alkali concentration between assays but not within the assay.

The general slope of the curves shown in Figure 1 is correlated with the nature of the standard curves reported in the literature (41).

Table 1

Titration Values for Riboflavin Standard Curve A*

mcg. B_2 per 10 ml. of Assay Medium	Titration to pH 6.8	
	ml. NaOH (0.1 N)	Average titration value
0.00	0.60	
		0.73
0.00	0.85	
0.05	5.65	
		5.63
0.05	5.60	
0.10	8.70	
		8.50
0.10	8.30	
0.15	10.00	
		10.10
0.15	10.20	
0.20	12.50	
		12.25
0.20	12.00	
0.25	12.40	
		12.40
0.25	12.40	
0.30	13.00	
		12.95
0.30	12.90	
0.35	13.80	
		13.80
0.35	-	

*These values are plotted in Figure 1, Curve A.

Table 2

Titration Values for Riboflavin Standard Curve B*

mcg. B_2 per 10^2 ml. of Assay Medium	Titration to pH 7.0	
	ml. NaOH (0.1 N)	Average titration value
0.00	0.4	
		0.55
0.00	0.7	
0.05	2.8	
		3.05
0.05	3.3	
0.10	5.9	
		5.90
0.10	5.9	
0.15	8.3	
		7.90
0.15	7.5	
0.20	8.4	
		8.65
0.20	8.9	
0.25	9.2	
		9.25
0.25	9.3	
0.30	9.5	
		9.50
0.30	9.5	
0.35	9.9	
		9.85
0.35	9.8	

*These values are plotted in Figure 1, Curve B.

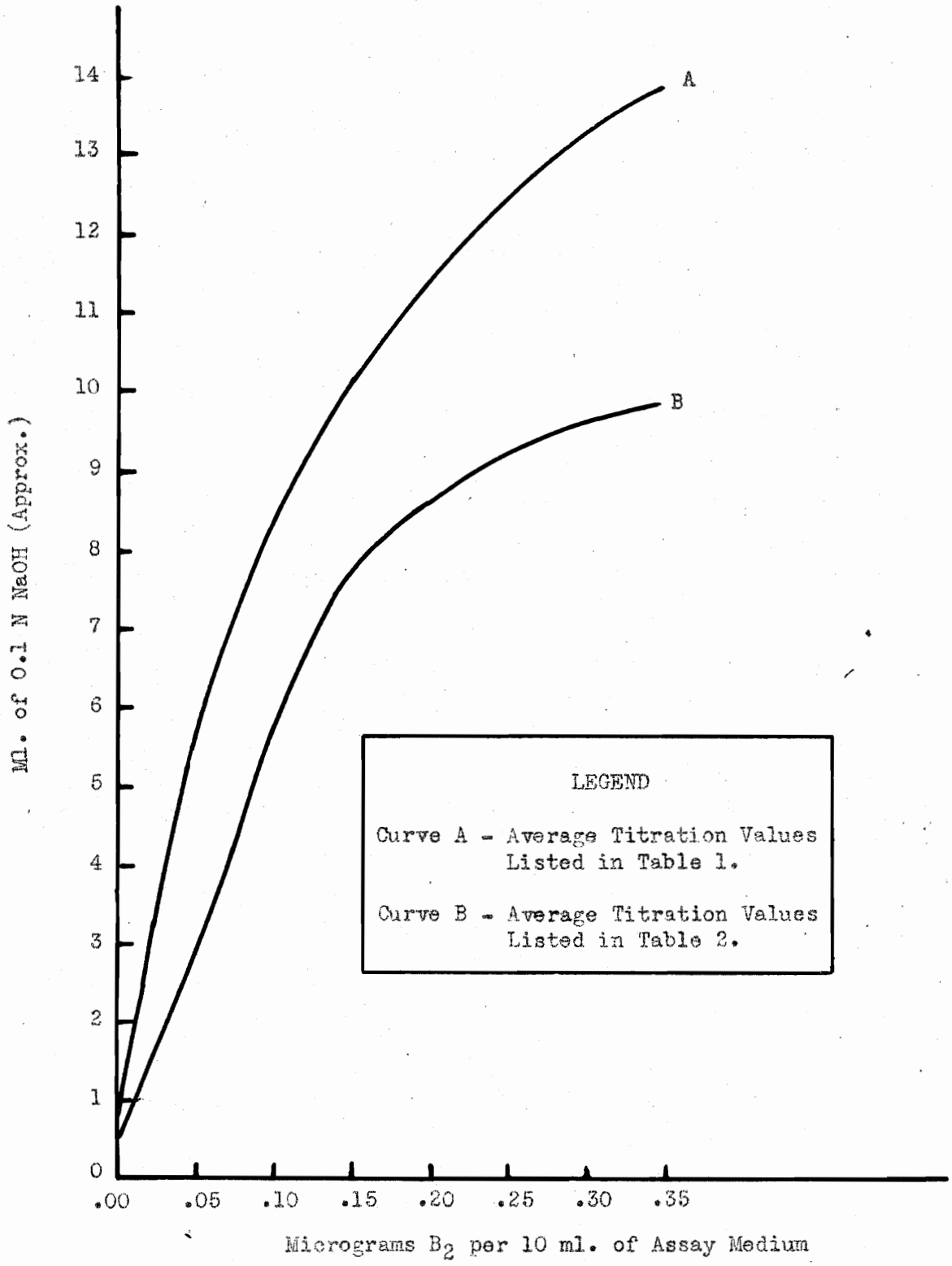


Figure 1 - Comparison of Titration Values Obtained from Two Series of Determinations on Known Amounts of Riboflavin

2. Vitamin B_2 Content of Tankage and the Effect of Incubation Temperature on Its Determination

Since all subsequent vitamin B_2 values were to represent the vitamin B_2 content of tankage plus that amount synthesized by the microorganisms, it was desirable to know beforehand the riboflavin potency of tankage and any change in this quantity due to the incubation temperature. The probable error associated with the assay was also determined.

Three sets of seven bottles (4 ounce), each containing 3 grams of tankage (oven-dried for 12 hrs. at 105°C.) and 10 ml. of distilled water, were autoclaved for 15 minutes at 121°C. Since the authors were interested in increasing the riboflavin content of the tankage by microbial synthesis, it was considered unnecessary to determine the effect of heat (oven drying and autoclaving) on the riboflavin content of the uninoculated tankage. These autoclaved samples were placed in a 37°C. incubator. At the end of each 24 hr. period, one set of seven bottles was removed and the contents assayed for riboflavin. The assay procedure, essentially the same method as that described by The American Association of Vitamin Chemists (41), was as follows:

50 ml. of 0.1 N HCl were added to the tankage medium contained in each bottle and autoclaved for 15-20 minutes at 121°C. At this point, until the time of titration, the assay was conducted under darkened conditions. The extract mixture containing free riboflavin was permitted to cool; then adjusted to a pH of 4.5 (brom-cresol-green indicator) with a weak NaOH solution to remove starch, fatty acids and phospholipids from the extract mixture (40). The adjusted mixture was brought to 100 ml. volume with distilled water and filtered twice - once through a Buchner

funnel with the aid of a vacuum pump, and then through Whatman No. 40 filter paper without a vacuum. A 50 ml. aliquot was adjusted to a pH of 6.8 (brom-thymol-blue indicator), brought to 100 ml. volume with distilled water and again filtered through Whatman No. 40 filter paper. 0.5 ml. and 1.5 ml. volumes were taken from the clear filtrate and added respectively to tubes containing 9.5 and 8.5 ml. of Riboflavin Assay Medium (Difco). A set of tubes for the standard riboflavin curve was simultaneously prepared in the manner previously described. All tubes were autoclaved, inoculated with an 18-hour culture of L. casei, and incubated at 37°C. for 72 hrs. Acid titrations were performed on the contents of each tube following incubation. By interpolation, the amount of riboflavin per ml. of unknown sample was determined. Calculation of the riboflavin content per gram of tankage (dry basis) was made by means of the formula:

$$\text{mcg. } B_2/\text{gram sample} = \frac{\text{mcg. } B_2/\text{ml. extract} \times \text{volume}}{\text{weight of sample material assayed}} \times \text{dilution}$$

Table 3 shows the results obtained over the three day incubation period. The riboflavin content per gram of tankage fluctuates between the extremes of 2.4 and 4.4 mcg. (except in the case of 7.2 shown for sample number 1 on the second day), and gives an arithmetic mean of 3.5 mcg. No convincing evidence was obtained to show that a temperature of 37°C. appreciably affects the stability of riboflavin during the three day period. On the average, the smaller volume (0.5 ml.) of tankage extract assayed at a lower vitamin B_2 value than the larger volume (1.5 ml.) of extract. This was exemplified in the average value reported on the first day where the 0.5 ml. samples assayed at 3.0 mcg., as compared with 3.7 mcg. obtained from the 1.5 ml. samples. A better index to the actual vitamin B_2 potency

Table 3

Temperature Effect on the B_2 Content of Seven Identical Samples of Tankage Broth

Sample	Tankage Extract ml.	Days Incubated 37°C.		
		Vitamin B_2 (mcg./gm.)	Vitamin B_2 (mcg./gm.)	Vitamin B_2 (mcg./gm.)
1	0.5	2.7	3.3	3.4
	1.5	4.0	7.2	4.0
2	0.5	3.3	2.6	3.3
	1.5	3.3	2.8	4.0
3	0.5	3.3	3.3	3.3
	1.5	4.4	4.0	4.0
4	0.5	3.3	3.3	3.3
	1.5	3.4	2.8	4.0
5	0.5	3.3	3.3	3.3
	1.5	4.0	3.5	4.0
6	0.5	2.7	4.0	3.3
	1.5	3.4	2.4	3.3
7	0.5	2.7	2.6	4.0
	1.5	3.3	3.2	4.0
Average of 0.5 ml Tankage Extract		3.0	3.2	3.4
Average of 1.5 ml Tankage Extract		3.7	3.7	3.9
Average of 0.5 ml and 1.5 ml Tankage Extract		3.4	3.5	3.7

of an unknown sample could be acquired by taking the average of two or more determinations. All subsequent assay values were based on the average of four separate evaluations of the same sample using 1.0, 1.5, 2.0 and 2.5 ml. volumes of sample extract.

An effort was made to determine the volume of tankage extract which would assay with the least probable error. The mean $\pm$ 3 $P.E_m$, was calculated from the data reported in Table 3. The chances that the true value lies within $\pm$ 3 $P.E_m$. are 22:1 (3). The formulae used in the calculations are:

$$\sigma = \pm\sqrt{\frac{(x-m)^2}{n-1}}$$

and

$$P.E_{m.} = \pm \frac{0.6745\,\sigma}{\sqrt{n}}$$

σ = standard deviation
Σ = sum of
x = actual value of each determination
m = arithmetic mean
n = number of determinations
$P.E_{m.}$ = probable error of the mean

The probable errors reported in Table 4 are in most cases low and indicate the reliability of the assay method.

As a whole, no great difference is noted in the magnitude of the probable error calculated from the assay values of 0.5 and 1.5 ml. samples.

3. Tankage Fat as a Possible Inhibitory or Stimulatory Agent on the Growth of L. casei

One of the steps in the assay was the adjustment of the extraction mixture to a pH of 4.5 prior to filtration. This was done to precipitate those residual substances from the extract mixture that are reportedly capable of affecting the growth of the assay test organism (3). Strong

Table 4

Mean ± 3 $P.E_m$. for Riboflavin Determinations on Seven Identical Tankage Broth Samples

Days Incubated at 37°C.	Volume of Extract Assayed		
	0.5 ml.	1.5 ml.	Aver. of 0.5 and 1.5 ml.
1	3.04 ± 0.03	3.69 ± 0.33	3.37 ± 0.21
2	3.20 ± 0.37	3.70 ± 1.26	3.45 ± 0.63
3	3.20 ± 0.48	3.90 ± 0.19	3.65 ± 0.25

and Carpenter (39) gave conclusive evidence for the inhibitory action of palmitic and linoleic acids and stimulatory action of stearic and oleic acids on the growth of L. casei. Since tankage has a fat content ranging between 8-10 per cent (25), and was subjected to acid hydrolysis in the assay procedure, it was desirable to know whether or not the fat component or its hydrolytic products had an effect on the growth of the test organism. The effect was measured in terms of change in the vitamin B_2 value of tankage material obtained previously.

Fat and fat-like substances were removed from the tankage extract mixture with ether prior to inoculation with L. casei. An unextracted sample was assayed simultaneously as a control. The results obtained are shown in Table 5.

As shown in Table 5, lower and less consistent values were obtained from the assay of three different amounts of extract taken from a single sample of tankage exposed to ether extraction. No explanation is offered for this decreased value and lessened accuracy. In fact, the extraction step with ether was to prevent such discrepancies by the removal of those agents affecting the growth of L. casei (39). Due to the more constant values obtained from the assay of the unextracted sample, it was concluded that ether extraction is of no value in improving the validity of the assay results.

4. Evaluation of Assay Technique with Liver Extract Samples

To check the accuracy of the assay, liver extract of known vitamin B_2 potency was obtained from the Association of Vitamin Chemists, Inc. Three, one-gram samples were assayed, each on separate days and with

Table 5

The Effect of Ether Extraction on Riboflavin Assay Results

Tankage Extract Assayed (ml.)	Tankage Sample	
	Unextracted mcg.B_2/gm.	Ether Extracted mcg.B_2/gm.
0.5	4.0	1.3
1.0	3.2	2.0
1.5	3.6	3.1

Table 6

Comparative Assay Values for Riboflavin Content of Liver Extract

Assayer	Mcg. B_2/gm. of Liver Extract	
	Per Sample	Average
Authors	20.8	19.2
	18.3	
	18.4	
Others	18.2	19.6
	22.1	
	18.9	
	21.5	
	18.4	
	20.4	
	19.2	
	18.0	

separate standard riboflavin curves. The results of the assay are recorded in Table 6.

These values were sent to the Association of Vitamin Chemists, Inc., and they, in turn, forwarded the results obtained by other investigators which are recorded in Table 6.

On the basis of the values reported by other investigators, the authors were assured that their assay technique was accurate and the values obtained were valid.

Summary of Experiment I

The development of an assay technique and investigation of several factors affecting the microbiological assay of riboflavin, produced the following conclusions:

(1) Working with known amounts of riboflavin, it was determined that the amount of acid formed during the growth of Lactobacillus casei was proportional to the amount of riboflavin present in the medium.

(2) The riboflavin content of the tankage without enrichment was found to be 3.5 mcg./gm.

(3) An incubation temperature of 37°C. has no appreciable effect on the stability of the riboflavin contained in the tankage.

(4) It was determined by experimentation that an average of four volumes of extract, 1.0, 1.5, 2.0, and 2.5 ml., would give an accurate determination of the riboflavin potency of unknown samples.

(5) Calculation of the mean $\pm$ 3 $P.E_m$. showed the reliability of the assay determinations.

(6) The removal of fat and fat-like substances by ether extraction was of no value in improving the validity of the assay results.

(7) The assay of liver extract containing a definite quantity of riboflavin and a comparison of the results with values obtained by other investigators assured the accuracy of the assay technique and the validity of the assay values.

Experiment II - Riboflavin Synthesis by Hog Viscera Isolates

This phase of the investigation involved the isolation in pure culture of microorganisms present in hog viscera - and the determination of their ability to synthesize vitamin B_2 when grown on tankage medium.

Procedure

Isolation of Microorganisms in Pure Culture from Hog Viscera. The viscera contained in a large lard tin was removed from the deep freeze unit and permitted to thaw for three hours in a warm water bath. Two representative samples were then collected aseptically from the visceral organs. One of the samples was subjected to putrefaction for 24 hrs. in a 37°C. incubator. After macerating the samples with a mortar and pestle and suspending the tissue material in physiological saline, aerobic and anaerobic isolations were attempted by means of the pour-plate method involving the principle of serial dilution. Four types of media, Nutrient Agar (Difco), Glucose-Tryptone-Extract Agar (Difco), Potato-Dextrose Agar (Difco) and Brain-Veal Agar (Difco), were used in the aerobic isolations to obtain nutritionally different microorganisms. Anaerobic isolations using the pyrogallol method described in the *Manual of Methods for Pure Culture Study of Bacteria* were obtained on Nutrient Agar (Difco) and Brain-Veal-Agar (Difco) media (36). Following 48 hrs. incubation at 37°C. and 30°C., isolations were made from discrete colonies displaying marked cultural differences. Primary subcultures were made on the same medium that supported the growth of the parent colony. Since the microorganisms that were isolated from anaerobic cultures grew better under

aerobic conditions, they were treated as aerobes in all further work.

The purity of each primary subculture was ascertained by the Gram-stain (Hucker's Modification). Since it was found that all of the isolated microorganisms grew well in nutrient broth at 37°C., stock cultures were maintained on nutrient agar slants.

Table 7 contains general information about the microorganisms isolated from hog viscera. A total of eighteen microorganisms were isolated - five from the non-putrefied visceral sample and thirteen from the slightly putrefied sample.

Riboflavin Synthesis by Isolates from Hog Viscera. Each of the eighteen microorganisms isolated from the flora of hog viscera was investigated for vitamin B_2 synthesis on tankage broth over a period of six days. Tankage broth consisted of 3 grams of tankage material (oven-dried for 12 hrs. at 105°C. and mixed in a Waring blender) plus 10 ml. distilled water to each bottle (4 ounces). Following sterilization by autoclaving, the broth was adjusted aseptically to a pH of 6.8 with sterile 0.1 N NaOH solution. Since this pH adjustment was an approximation, further comment on the adjustment procedure is given.

Following sterilization, five bottles, containing the amounts of tankage broth mentioned above, were taken for pH adjustment. The Beckmann pH meter was used to determine the desired pH. The number of drops of 0.1 N NaOH solution required to adjust each broth sample was noted, and the average of these five values was taken as the amount of alkali needed to adjust the remaining broth samples.

The term "broth" when used in conjunction with tankage does not denote a true solution of tankage material, but rather a water preparation of this substance in which the tankage settles out and collects on the bottom of the vessel.

Table 7

Microorganisms Isolated from Hog Viscera

Isolation from				Primary Isolation Medium*	Gram Reaction	Morphology
Non-Putrefied Visceral Sample		Putrefied Visceral Sample				
Aerobic	Anaerobic	Aerobic	Anaerobic			
1				BV	+	large rod
2				GTE	+	rod
3				GTE	+	small rod large
4				PD	+	thick rod large
5				PD	+	thick rod
		6		GTE	-	small rod
		7		GTE	+	small rod
		8		GTE	-	--------
		9		GTE	+	coccus
		10		BV	+	medium rod
		11		GTE	+	coccus
		12		BV	+	small rod
		13		GTE	-	small rod
		14		GTE	-	small rod
			15	BV	-	small rod
			16	N	+	coccus
			17	BV	-	small rod
			18	N	-	small rod

*BV - Brain-Veal Agar
GTE - Glucose-Tryptone-Extract Agar
PD - Potato-Dextrose Agar
N - Nutrient Agar

Each of the isolated organisms was treated as follows: A set of six bottles, each containing an equal quantity of sterile tankage broth, was inoculated with one drop of the microorganism suspension and incubated at 37°C. over a period of six days. In certain instances, the incubation period was extended over nine days. An uninoculated set was carried as a control. After each day of incubation, a unit of each set was removed for riboflavin assay.

Results of Experiment II

The results of this experiment are recorded in Tables I, II, III and IV (see Appendix) and plotted in Figures 2, 3, 4 and 5.

Table 8 lists a group of microorganisms which demonstrated no appreciable increase in the riboflavin content of the tankage (3.5 mcg. of B_2/gm.).

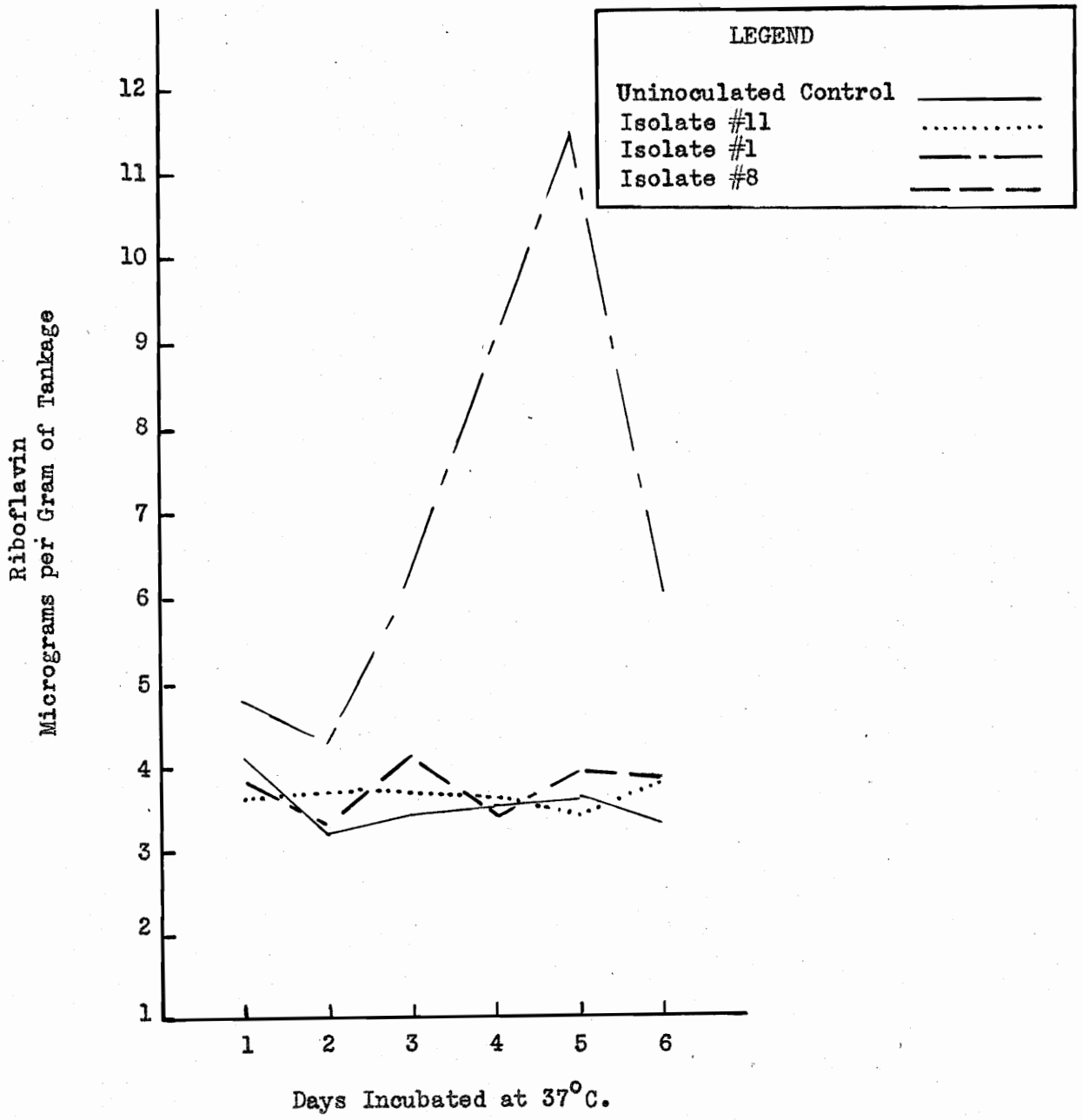


Figure 2 - Synthesis of Riboflavin in Tankage Medium by Isolates #1, #8 and #11

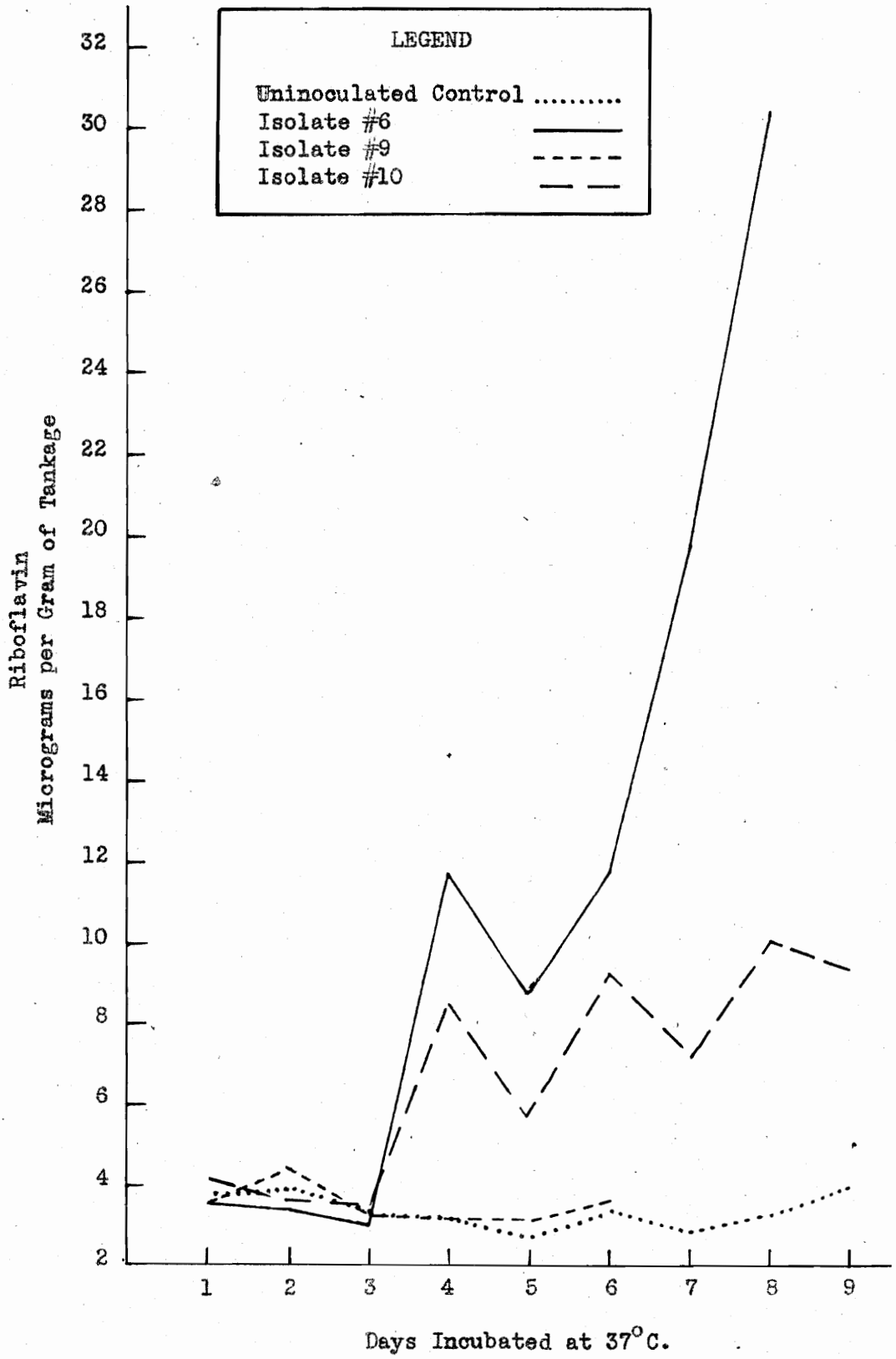


Figure 3 - Synthesis of Riboflavin in Tankage Medium by Isolates #6, #9 and #10

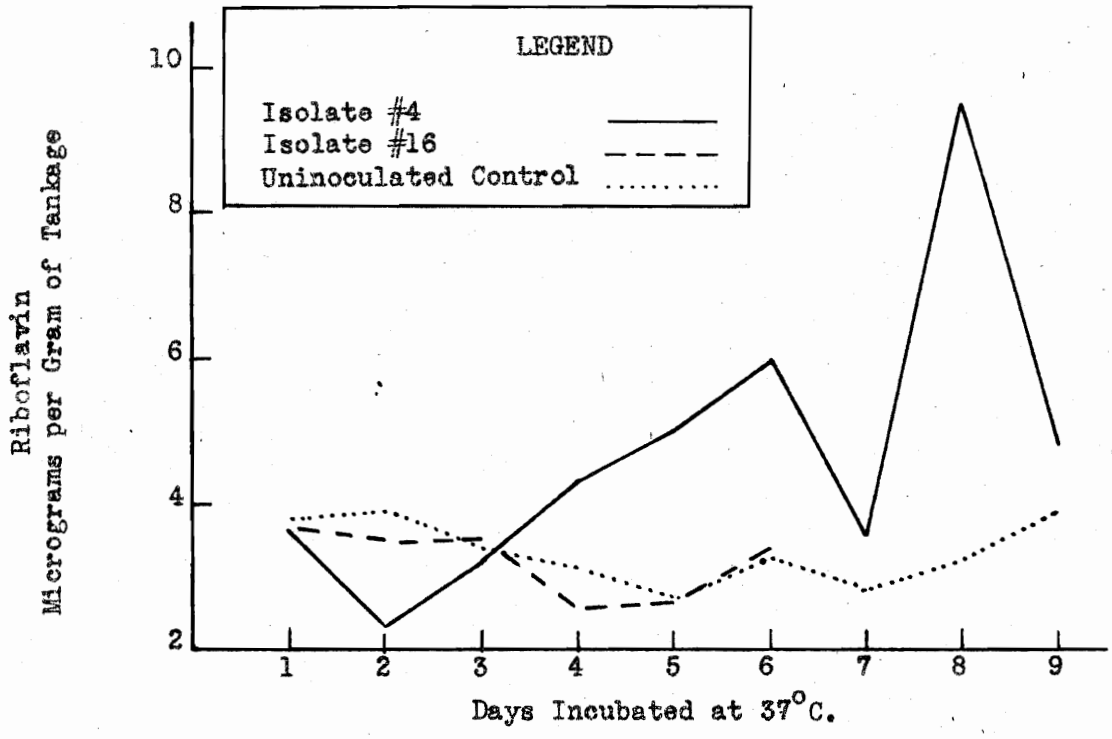


Figure 4 - Synthesis of Riboflavin in Tankage Medium by Isolates #4 and #16

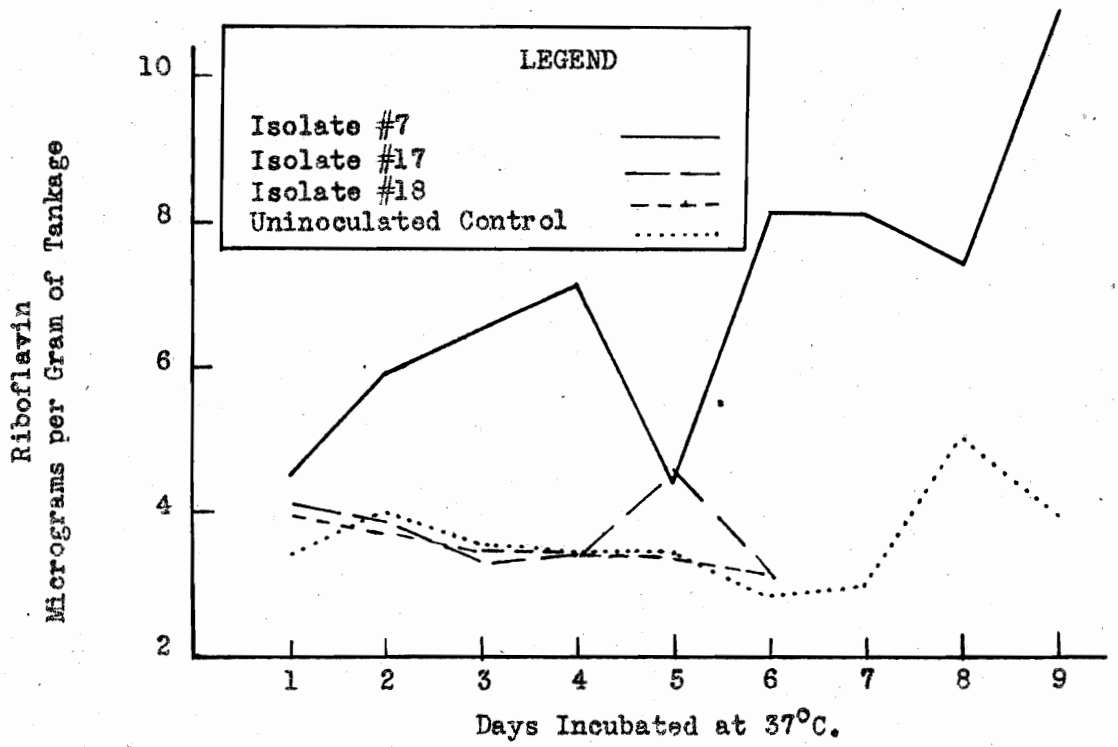


Figure 5 - Synthesis of Riboflavin in Tankage Medium by Isolates #7, #17 and #18

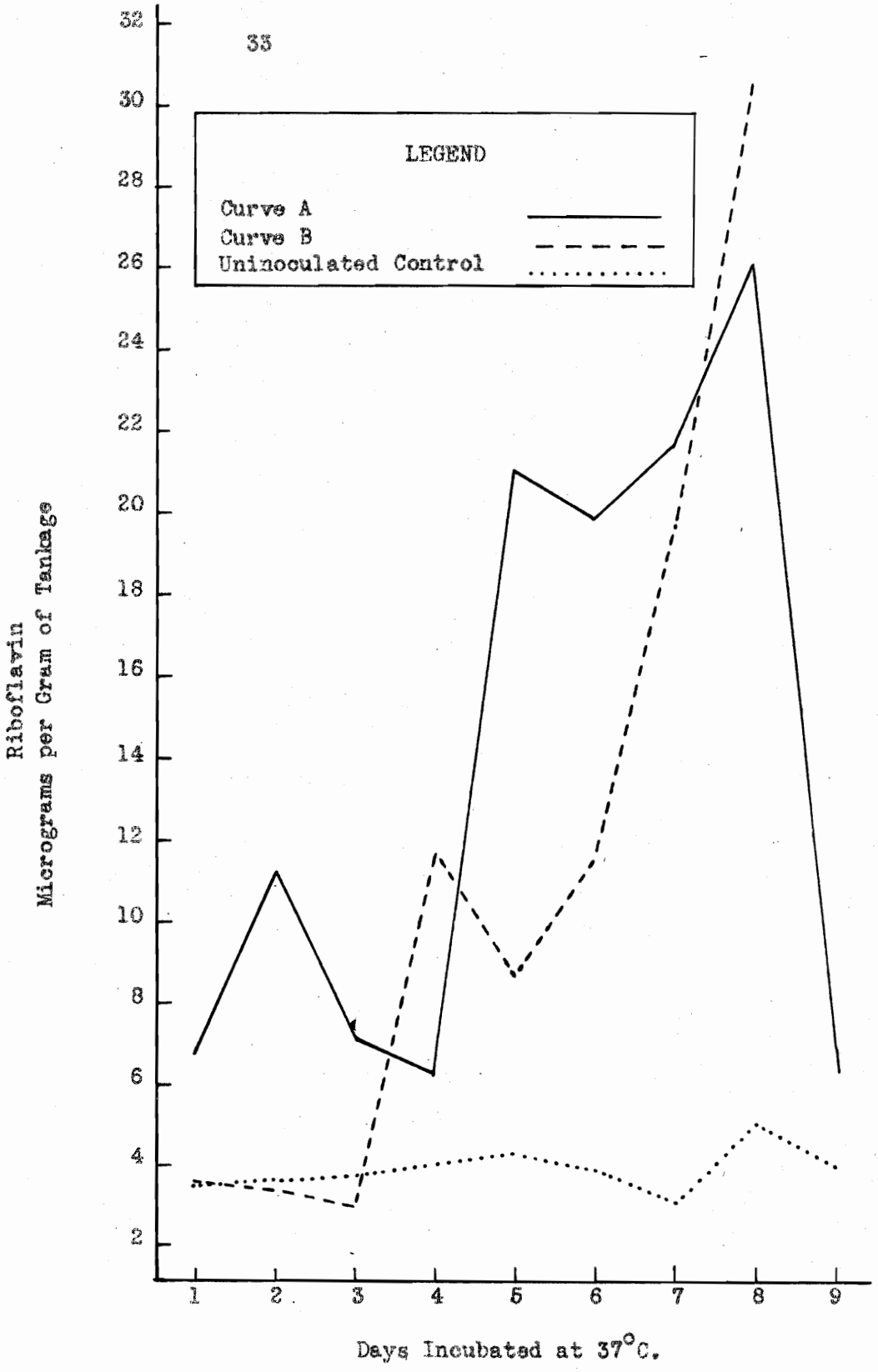


Figure 6 - Comparison of Duplicate Determinations of Riboflavin Synthesis in Tankage Medium by Microorganism #6

Table 8

Comparison of Vitamin B_2 Synthesis by Isolates From Hog Viscera

Tankage Medium Inoculated with	Riboflavin Content in mcg./gm. of Tankage for each Day of Incubation					
	1	2	3	4	5	6
Organism #2	3.0	4.6	2.2	5.1	3.9	2.7
Organism #3	4.0	4.4	2.3	5.0	3.9	2.9
Organism #5	2.8	5.9	2.9	5.6	4.6	5.2
Organism #12	3.0	3.5	2.2	5.2	3.7	2.7
Organism #13	3.4	3.6	3.0	4.5	4.4	2.5
Organism #14	3.3	4.2	2.2	5.1	4.0	2.8
Organism #15	2.5	5.0	2.5	5.0	4.3	3.0
Uninoculated Control	3.7	6.0	2.6	5.4	3.3	3.7

Discussion of Results of Experiment II

The object of this experiment was to determine which isolates were capable of synthesizing riboflavin, and the incubation day on which the highest vitamin B_2 yields could be expected. In view of this object and the results obtained, the isolates were divided into three groups:

(1) Microorganisms #8, #9, #11, #16, #17 and #18 and those listed in Table 8 showed little or no increase in vitamin B_2 content after six days of incubation.

(2) Microorganism #1 exhibited a maximum yield of riboflavin after five days of incubation with a noticeable decrease after six days of incubation.

(3) Microorganisms #6, #10, #4 and #7 showed increased vitamin B_2 yield after six days of incubation.

Since the vitamin B_2 yield of the third group increased on the last day of incubation, the length of incubation was extended from six to nine days for microorganisms #6 and #10 (Figure 3), #4 (Figure 4) and #7 (Figure 5).

Microorganism #6 (Figure 3) continued to enrich the vitamin B_2 content of the tankage medium and exhibited a strikingly high yield of 30.5 mcg. per gram of tankage on the eighth day. No result was recorded for the ninth day because the sample unit was accidentally dropped on the floor during one of the steps of the assay. The other organisms, which were incubated for the three additional days, did not exhibit sufficient increase in riboflavin content to warrant further investigation.

A duplicate experiment was performed with Microorganism #6 under conditions identical to those previously imposed. The results from the original and duplicate nine-day periods of synthesis are recorded in Table IV

(see Appendix) and plotted in Figure 6. It was of interest to note that Microorganism #6 showed maximum vitamin B_2 synthesis in both experiments after the eighth day of incubation. In Figure 6, Curve A, the duplicate synthesis shows a maximum yield of 26 mcg./gm. of tankage after the eighth day, and in Curve B, the original synthesis shows a maximum yield of 30.5 mcg./gm. of tankage after the eighth day.

The assay results did not maintain a steady increase during the period of incubation for any of the isolates tested. Only those results recorded in Table 8, which so nearly simulate results of the uninoculated control, exhibit a rather consistent trend. The decreases occurring over 24-hour periods of incubation are too great to attribute the loss of riboflavin to metabolic degradation. A difference in establishment of growth seems to be the most plausible explanation of these irregularities. The environmental factors influencing growth were essentially the same for all samples. However, it was previously explained that the tankage medium was not a true broth, but rather a mixture of constant amounts of tankage and distilled water. It is therefore possible that the amount of nutrients available at a given level of the medium would vary from sample to sample. This nonhomogeneous quality of the medium together with the aerophilic nature of the microorganisms might be of sufficient effect to cause a difference in the degree of growth establishment among the samples.

Summary of Experiment II

Experiments were performed to determine which of the microorganisms isolated from hog viscera would synthesize the greatest amount of vitamin B_2 grown in tankage medium.

Under the particular set of experimental conditions, microorganisms #1, #6, #10, #4 and #7 were capable of vitamin B_2 synthesis. Microorganism #6 was the highest producer, producing an enrichment of the tankage above 25 mcg. of B_2 per gram of tankage. Confirmation of this high production was obtained by a duplicate experiment.

Experiment III - Riboflavin Synthesis on Tankage Broth by Eleven Organisms Reported to be Capable of Vitamin B_2 Production

Procedure

In order that a comparison could be made of the vitamin B_2 synthesizing ability of all test organisms, the growth conditions used in working with the isolates from hog viscera were employed in testing the eleven stock organisms. These organisms were obtained from V.P.I. stock-cultures, and are of the same species but not of the same strain as those reported to be capable of riboflavin synthesis. Using tankage medium as described in the previous experiment, 37°C. as the incubation temperature, and the separate sample method of analysis (method explained under Experiment II, Procedure), each organism was incubated for a period of six days with a riboflavin assay being made every 24 hours. A 30°C. incubation temperature was used in testing four of the eleven organisms, Micrococcus flavus, Torula sphaerica, Torula lactosa and Clostridium acetobutylicum.

Clostridium acetobutylicum was cultured at 30°C. under anaerobic as well as aerobic conditions. The following procedure was used to establish anaerobiosis. Six sample bottles containing tankage medium inoculated with Cl. acetobutylicum were sealed with rubber stoppers. Each stopper had a small piece of glass tubing inserted through the center. A mixture of petrolatum and paraffin was used as a seal for the joints. The glass tubing from each of the six bottles was connected to a manifold which in turn was connected to a vacuum pump. These bottles were evacuated until a partial pressure of 200 microns was obtained on a McLeod Pressure Gauge.

The glass tubing connected to the manifold was then separated from the manifold and sealed by an oxygen torch. The sealed bottle was then checked with an electric spark to ascertain the existence of a partial vacuum. To insure that no leakage occurred during the days of incubation, a bottle containing a pre-heated solution of methylene blue was subjected to the same treatment (36). The partial vacuum produced in the check bottle was maintained, as indicated by the colorless appearance of the methylene blue, throughout the six day incubation period.

Results of Experiment III

The data is recorded graphically in Figures 7, 8, 9, 10, 11, 12, and 13. For a tabulation of the data of these graphed results refer to Tables V, VI, VII, VIII, IX, and X found in the Appendix.

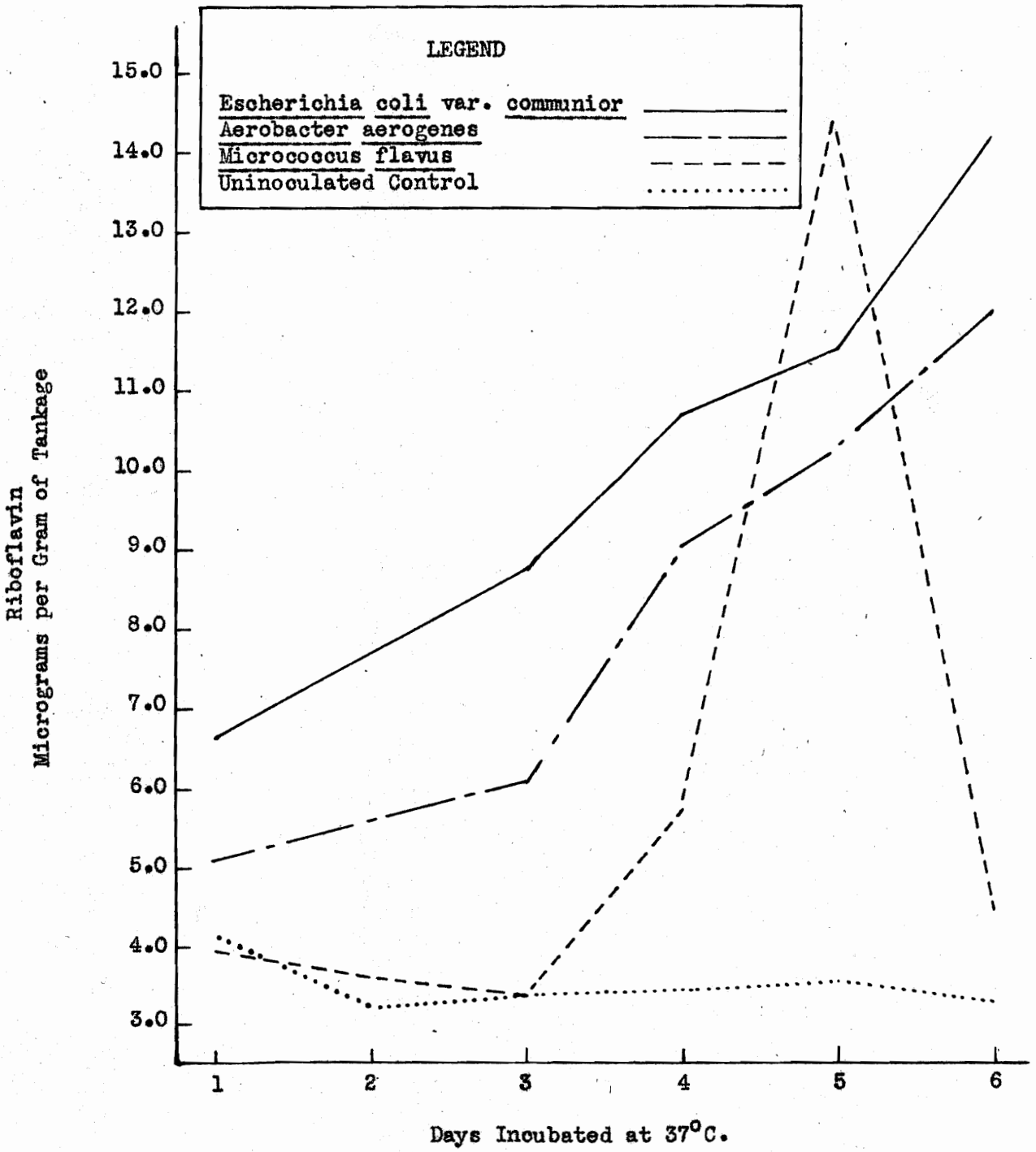


Figure 7 - Synthesis of Riboflavin in Tankage Medium by Escherichia coli var. communior, Aerobacter aerogenes, and Micrococcus flavus

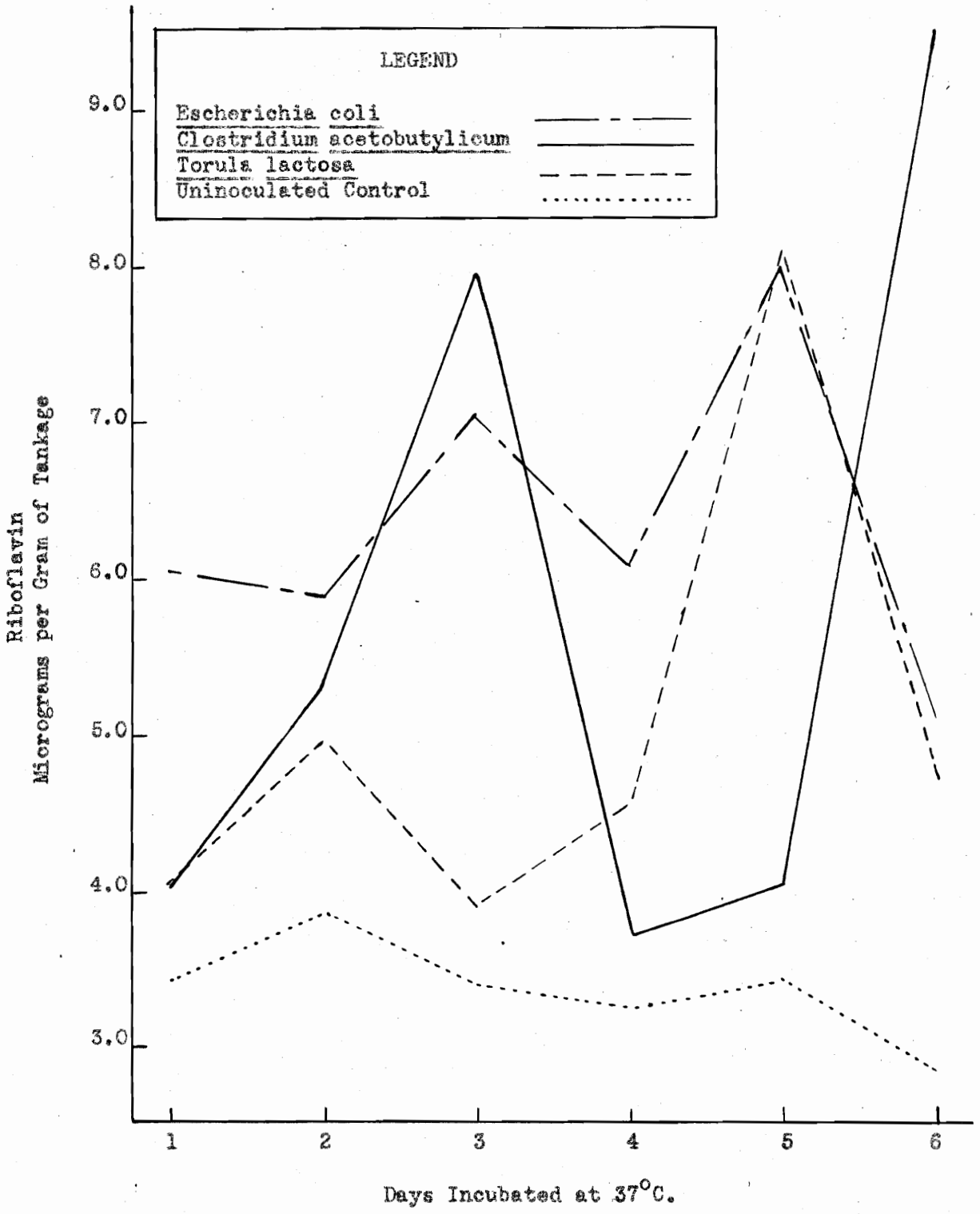


Figure 8 - Synthesis of Riboflavin in Tankage Medium by Escherichia coli, Clostridium acetobutylicum and Torula lactosa

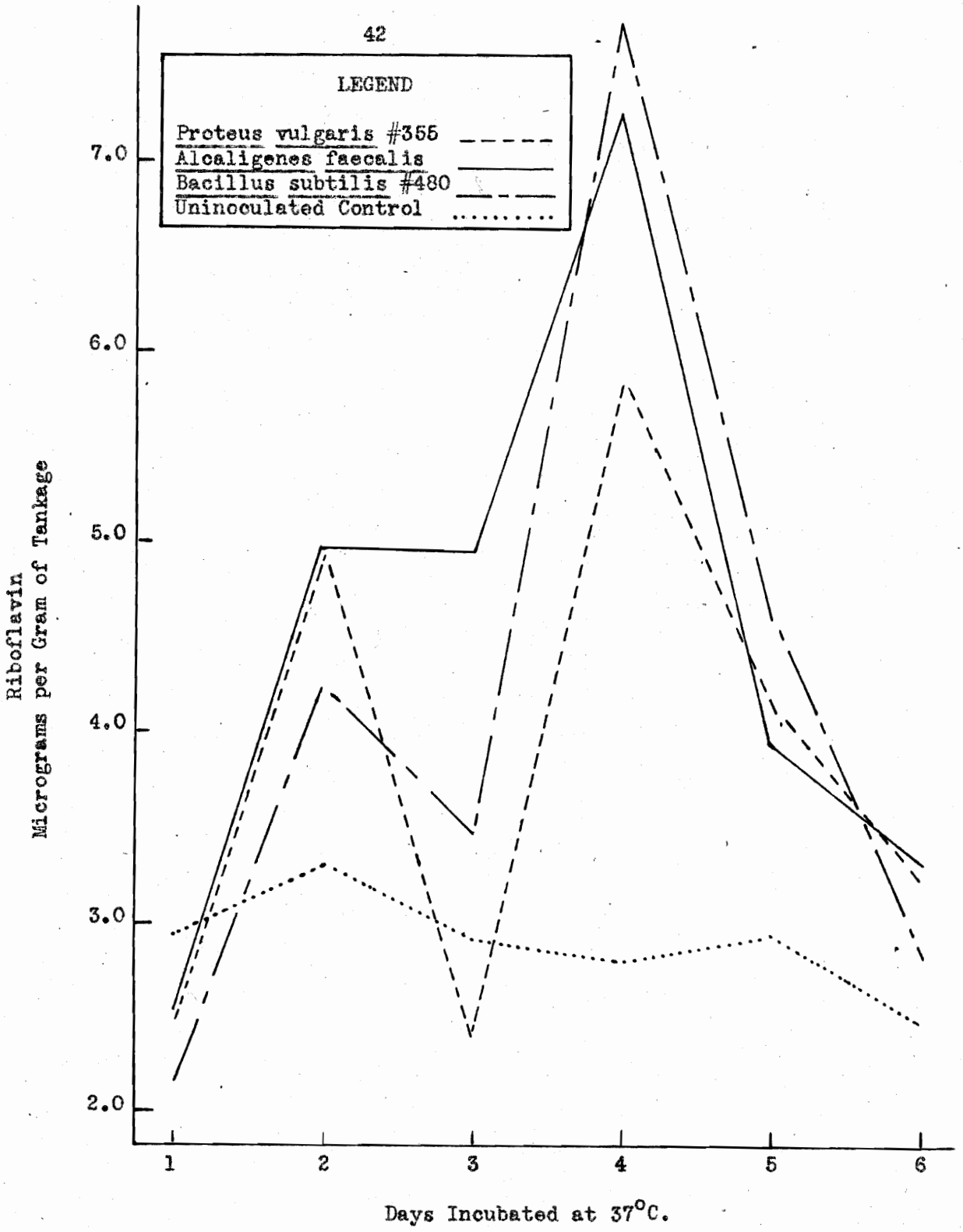


Figure 9 - Synthesis of Riboflavin in Tankage Medium by Proteus vulgaris, Alcaligenes faecalis and Bacillus subtilis

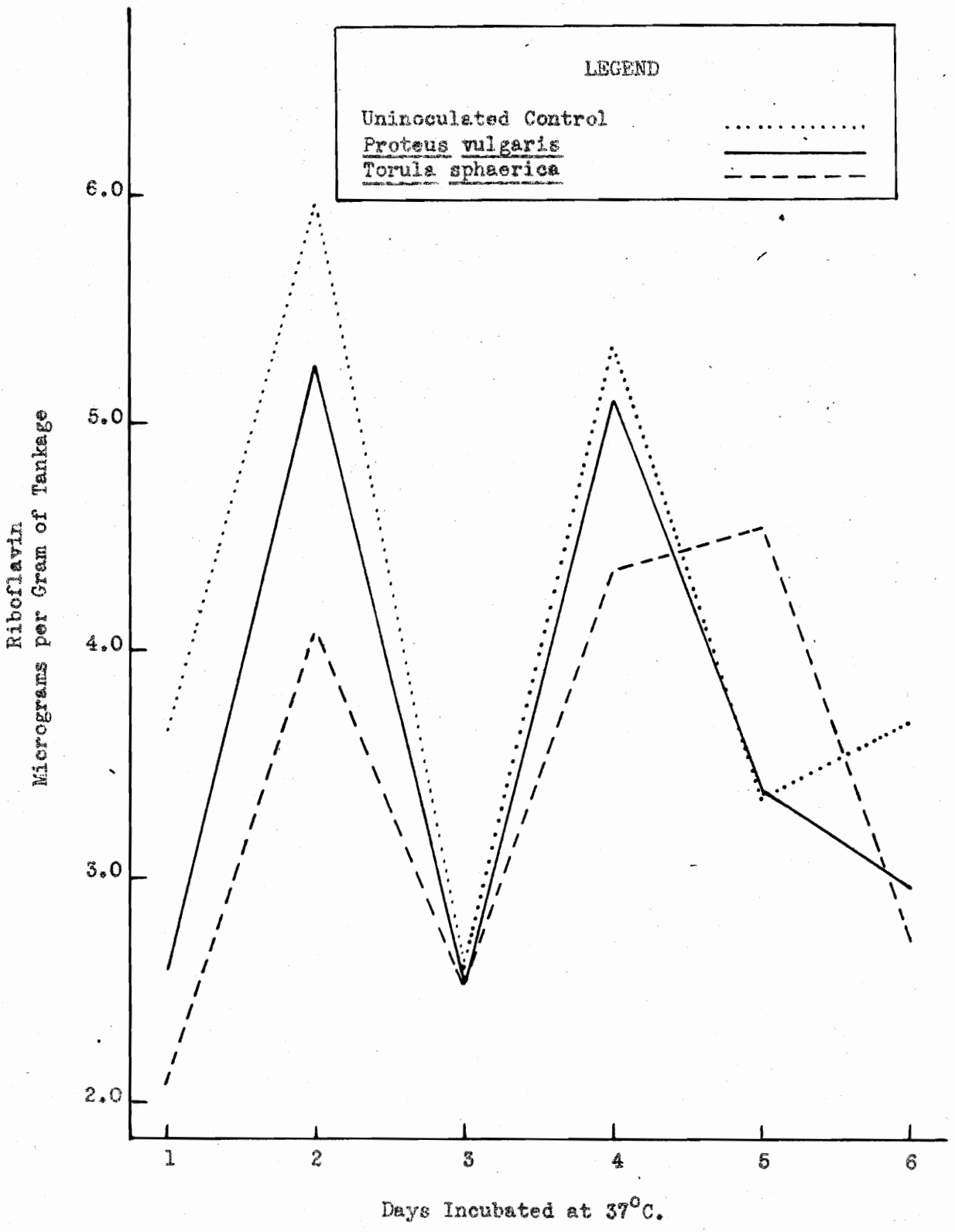


Figure 10 - Synthesis of Riboflavin in Tankage Medium by Proteus vulgaris and Torula sphaerica

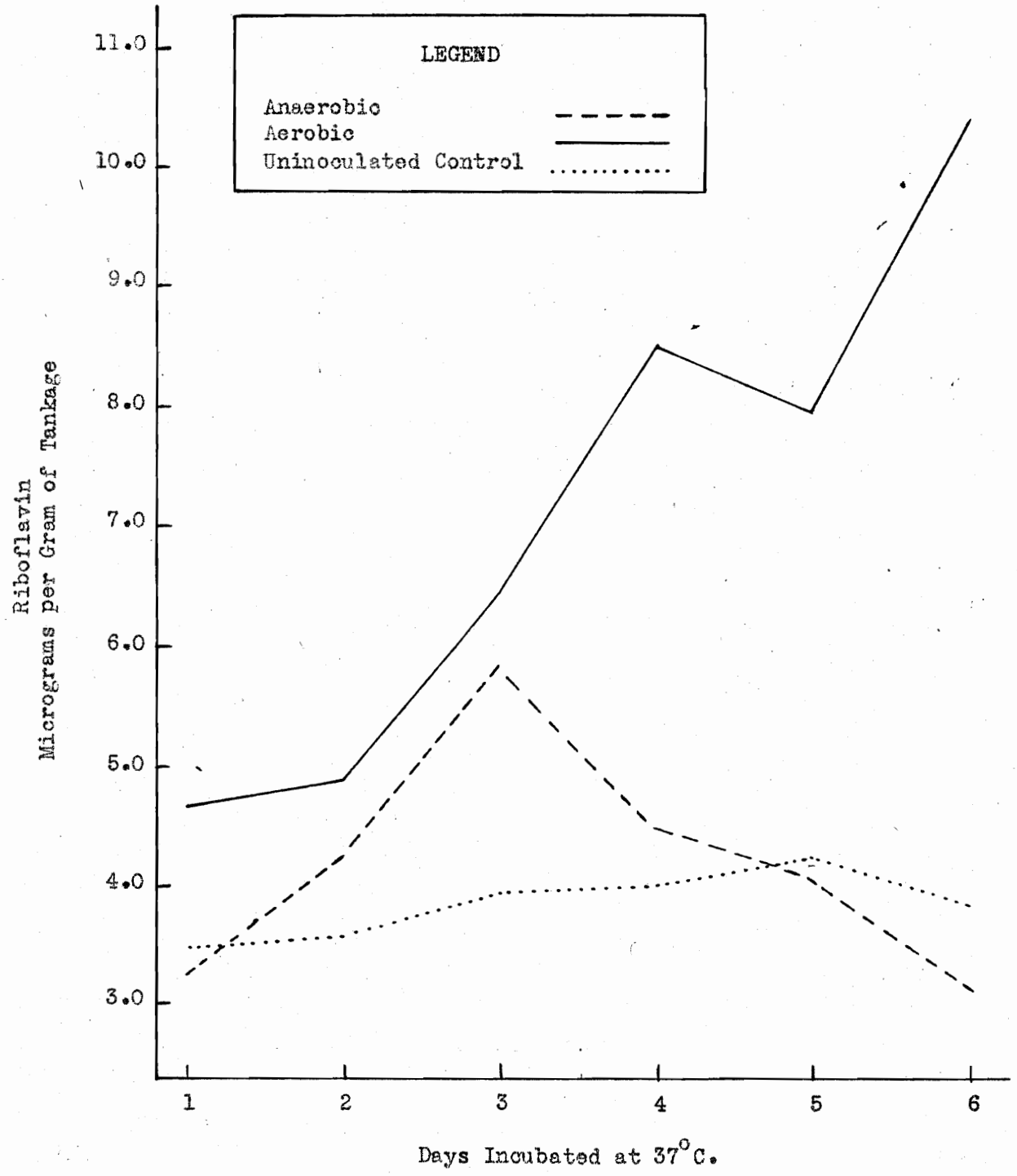


Figure 11 - Comparison of B_2 Synthesis in Tankage Medium by Clostridium acetobutylicum Under Aerobic and Anaerobic Conditions

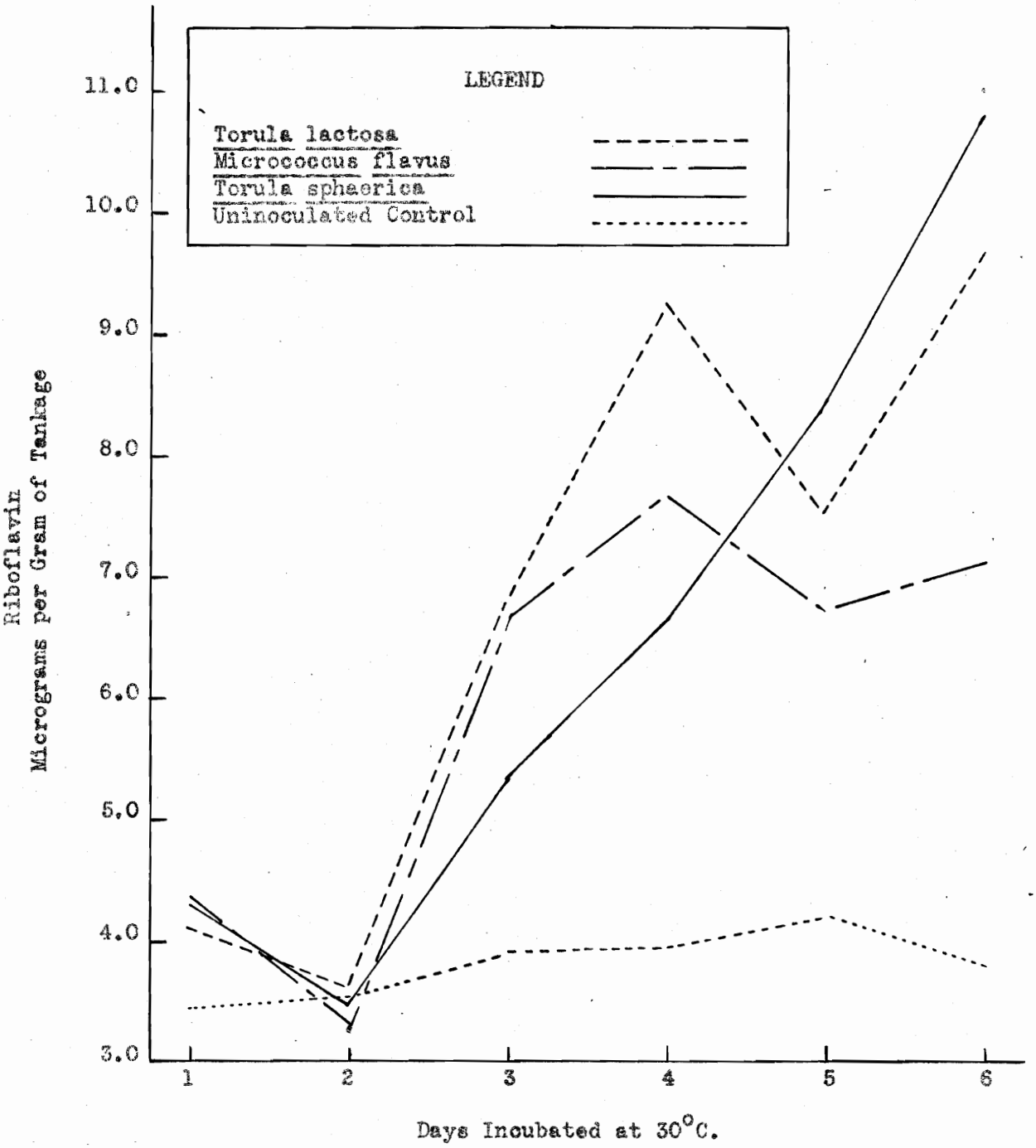


Figure 12 - Synthesis of Riboflavin in Tankage Medium by Torula lactosa, Micrococcus flavus and Torula sphaerica at 30°C.

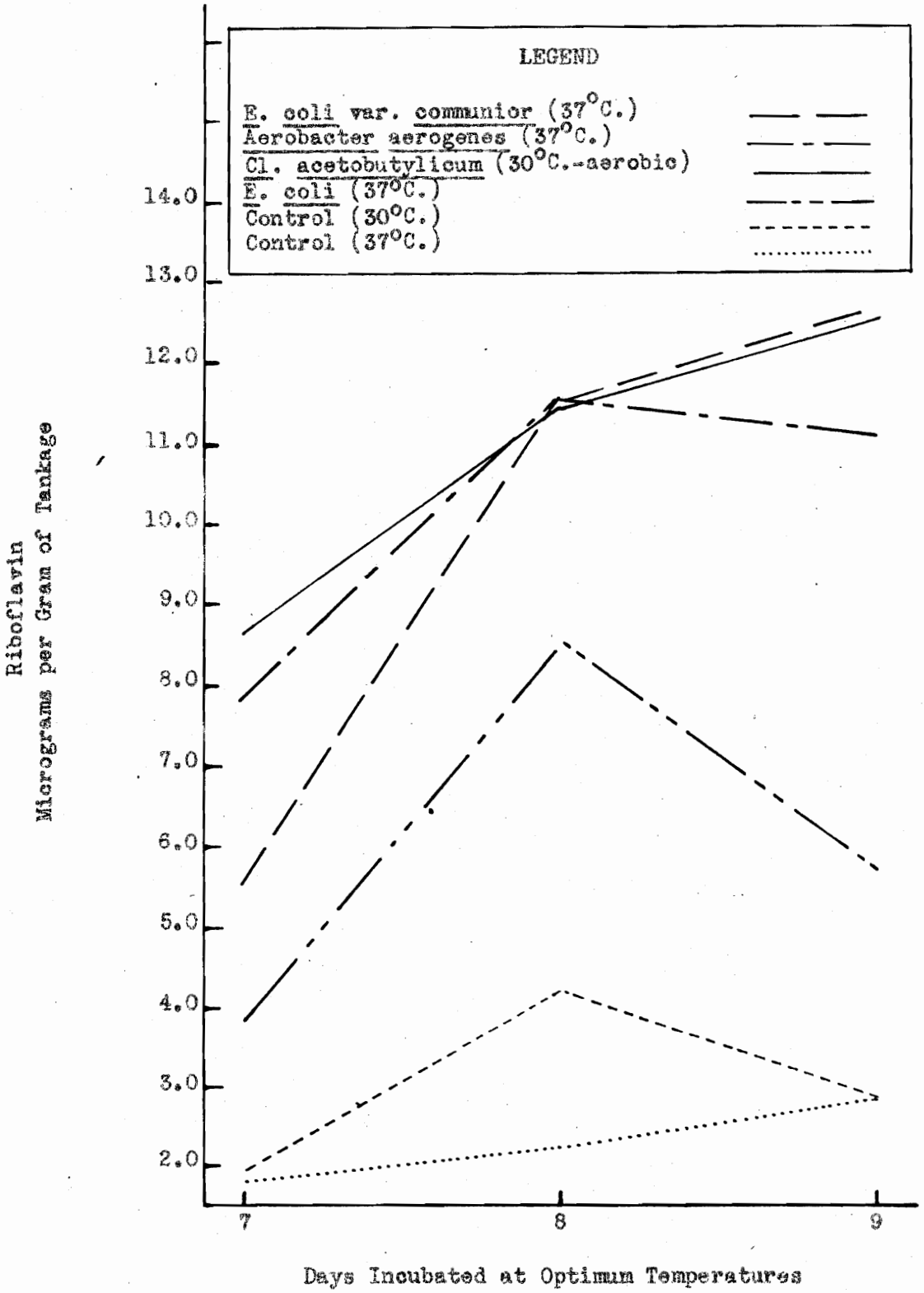


Figure 13 - Synthesis of Riboflavin in Tankage Medium by Escherichia coli var. communior, Aerobacter aerogenes, Clostridium acetobutylicum and Escherichia coli During Additional Period of Incubation

Discussion of Results of Experiment III

Aerobacter aerogenes and Escherichia coli (Figure 7) and Clostridium acetobutylicum (Figure 8) showed increased riboflavin synthesis from the fifth to the sixth day of incubation. The strain of Cl. acetobutylicum used in this experiment exhibited greater ability to synthesize riboflavin under aerobic rather than anaerobic conditions (Figure 11).

The irregular decreases noted in the production curves of the hog viscera isolates (Results of Experiment II) occurred in the synthesis of riboflavin by the eleven stock organisms. This factor is noticeable in Figures 7-10 and is most evident in the drastic difference between the decreases and increases of vitamin B_2 content exhibited by Escherichia coli (Figure 8). This phenomenon is unexplainable but indicates that a difference in degree of growth establishment occurred among the separate samples.

The organisms that showed an increase in riboflavin synthesis from the fifth to sixth day assays were incubated for nine days with assays being made on the seventh, eighth and ninth days (Figure 13). Three of the organisms showed increase in riboflavin content to a maximum value after nine days of incubation: Esch. coli var. communior - 13.68 mcg. B_2/gm. of tankage; Cl. acetobutylicum - 13.54 mcg.B_2/gm. of tankage; Aero. aerogenes - 12.10 mcg.B_2/gm. of tankage.

Summary of Experiment III

The object of this experiment was to determine which of the eleven reported vitamin B_2 producing organisms could synthesize the maximum amount of riboflavin on tankage broth, and to compare this value with the maximum value obtained with the hog viscera isolates (Summary of Experiment II). The results obtained indicate the following conclusions:

(1) The strain of Clostridium acetobutylicum used in this work demonstrated a greater ability to synthesize riboflavin when grown under aerobic conditions rather than anaerobic conditions.

(2) The highest vitamin B_2 producers were:

(a) Escherichia coli var. communior - 13.68 mcg.B_2/gm. of tankage.

(b) Clostridium acetobutylicum - 13.54 mcg.B_2/gm. of tankage.

(c) Aerobacter aerogenes - 12.10 mcg.B_2/gm. of tankage.

(3) The maximum value of 13.68 mcg. riboflavin/gm. of tankage obtained with Esch. coli var. communior was much less than the 30.5 mcg. riboflavin/gm. of tankage produced by hog viscera isolate #6 (Results of Experiment II, Figure 6).

Experiment IV - Control of Some Environmental Factors in an Attempt to Increase Riboflavin Synthesis by Hog Viscera Isolate #6

Microorganism #6 produced 30.5 mcg. riboflavin/gm. of tankage after eight days of incubation at 37°C. (Figure 6, Experiment II). This was the greatest amount of vitamin B_2 synthesis exhibited by any of the hog viscera isolates or stock organisms. Thus, Isolate #6 was chosen for further investigation. Environmental factors concerned in the previous testing of this organism were investigated in an effort to find conditions which would be favorable to further increase in riboflavin synthesis.

Procedure

Adjustment of pH for Separate Sample Technique. The same method of separate sample analysis discussed in Experiment II, Procedure was used for investigating the effect of different hydrogen-ion concentrations on riboflavin synthesis. It was desired to obtain tankage broth samples for each 0.5 increment over the pH range 5.5 - 8.0. The tankage medium had an unadjusted pH of 5.5. To determine the amount of base required to adjust the medium to the five remaining pH levels, five samples of broth were adjusted to pH measurements of 6.0, 6.5, 7.0, 7.5 and 8.0 with 0.2 N NaOH. These pH readings were made with a Beckman Laboratory Model pH Meter. The adjustment of these trial samples determined the number of milliliters of 0.2 N NaOH required for the adjustment of the test samples prior to inoculation with Microorganism #6. Assays were made each day of a ten-day incubation period to determine the amount of riboflavin synthesis.

Adjustment of pH for Batch Method. In an effort to eliminate the irregularities of vitamin B_2 production obtained by the separate sample method, it was desired to grow Organism #6 in a large batch of tankage medium from which samples for daily assay could be withdrawn. Quart jars were used and the volume of broth was increased sufficiently to allow for sampling each day over a ten-day incubation period. To allow for sampling with a large bore pipette, the water and tankage were thoroughly mixed in a Waring Blender. The pH was adjusted in the manner described in the previous paragraph. In an effort to obtain a non-variable pH prior to inoculation, six jars containing uninoculated broth with adjusted pH were incubated at 37°C. for several days. These broth samples whose pH readings were initially adjusted to 7.0, 8.0, 9.0, 10.0, 11.0, and 12.0, were checked at intervals to establish the time necessary for the pH to become constant. It was determined that the pH normally reached stability after eighteen hours of incubation. Six batch samples were adjusted by the described method, incubated at 37°C. for eighteen hours, and then measured for their pH values. The readings were 6.7, 6.85, 7.0, 7.2, 7.4, and 7.95. Leaving the 7.2 batch as the uninoculated control, the remaining five were inoculated with Microorganism #6 and all six jars were incubated at 37°C. for a ten-day period. The jars were agitated on a mechanical shaker each day prior to sampling. This five minute agitation was sufficient to facilitate the sample transfer by pipette.

Effect of Other Factors When pH is Held Constant. It was necessary to plan a phase whereby factors that had previously varied could be controlled for a comparison of their effect on the synthetic process. To assure a

constant mixture of medium, the tankage was prepared in large volume. The ratio of 3 gms. of tankage per 10 ml. of water was maintained by using 192 gms. of dry tankage to a total volume of 640 ml. This total volume included the milliliters of NaOH necessary for adjusting the pH. Prior to mixing, the frozen tankage was ground in a meat grinder and dehydrated in a drying oven. The tankage and water was thoroughly mixed in a Waring Blender and adjusted to a pH of 8.7. Then the broth was distributed among the various sample jars and autoclaved. After the broth had cooled to the incubation temperature (37°C.) for eighteen hours, the pH was found to be 8.2. A pH of 8.0 was desired for maximum growth and increased synthesis (Discussion of Results of Experiment IV). The distribution of the broth and control of the various factors were carried out according to the following procedure:

1. Adjustment of Surface Area to Volume Ratio

Using rectangular quart jars, rectangular four-ounce sample bottles, and standard size test tubes, the samples were prepared for incubation in the manner outlined in Table 9.

Table 9

Description of Samples According to Surface Area and Volume of Tankage Medium

Inoculated with	Container		Grams of Tankage	Surface Area to Volume	
	Type	Position		sq.cm./ml.	Ratio (cm^2)
1 ml. #6	Quart Jar	Vertical	19.5	51.5/90	0.57
1 ml. #6	Quart Jar	Horizontal	19.5	119/90	1.32
.1 ml. #6	Sample Bottle	Vertical	3.9	14.5/18	0.81
.1 ml. #6	Sample Bottle	Horizontal	3.9	45.1/18	2.51
.1 ml. #6	Test Tube	Vertical	3.9	2.07/18	0.12
Uninoculated Control	Sample Bottle	Vertical	3.9	14.5/18	0.81

2. Agitation

Two sets of samples were prepared as in Table 9. One set was agitated for fifteen minutes every 24 hours. The other set was agitated for five minutes prior to sampling. Since the separate sample bottles were only sampled for bacterial counts prior to assay, this five minute agitation could not affect the riboflavin synthesis. The test tube was not agitated at any time. This five minute agitation could only affect the two batch jar samples. With this in mind, for the sake of clarity, the set agitated for fifteen minutes will be referred to as the agitated samples, and the set agitated for five minutes prior to sampling will be referred to as the unagitated samples. The agitation of these samples was accomplished by shaking on a 50 to 60 cycle Fisher Gyrosolver.

In conjunction with the daily sampling for riboflavin assay, samples were taken for plate counts. The plating was accomplished by the pour-plate method (33) using Glucose-Tryptone-Extract Agar (Difco). These bacterial counts were made not only to check the possibility of contamination, but also in an attempt to correlate bacterial growth with riboflavin synthesis.

As mentioned previously, this phase of experimentation was conducted over a ten-day incubation period. Previous results obtained with Microorganism #6 indicated that no significant increase in riboflavin should be expected until after the third day of incubation. Samples were assayed, therefore, every forty-eight hours starting with the fourth day of incubation. In certain instances the incubation period was extended over twelve days with assays being made after eleven and twelve days of incubation.

Results of Experiment IV

The separate samples, which were adjusted originally to each 0.5 increment over the pH range of 5.5 to 8.0, were assayed each day of the ten-day incubation period with pH measurements being made at the time of assay. Table 10 lists the pH determinations over the first nine days of the incubation period with accompanying observations concerning the visible growth present in the samples. The assay results for each twenty-four hour interval during the ten days of incubation are presented in Figures 14 - 19 and are tabulated in Table XI, Appendix.

Results obtained with the batch method, when varying the pH from 6.7 to 7.95, are plotted in Figures 20 and 21 and recorded in Table XII, Appendix.

The synthesis of riboflavin by Microorganism #6 was tested at a pH of 8.2 with varying conditions of surface to volume ratio of the medium and time of agitation. Figures 22 and 23 show riboflavin production over the ten-day incubation period with additional results after eleven and twelve days of incubation for those samples showing production increases on the tenth day. Bacterial counts obtained at the time of each assay determination are plotted in Figures 24 and 25. These assay and growth results are tabulated in Table XIII, Appendix.

Table 10

Varying pH Values Observed in Tankage Medium Inoculated with Microorganism #6
With Accompanying Values for Uninoculated Control

Original pH of Medium	Days of Incubation																	
	1		2		3		4		5		6		7		8		9	
	#6	Control	#6	Control	#6	Control	#6	Control	#6	Control	#6	Control	#6	Control	#6	Control	#6	Control
5.5	5.7	5.6	5.9	5.55	5.85	5.53	6.0	5.6	6.35	5.58	6.20	5.50	6.3	5.55	6.2	5.6	6.15	5.55
6.0	6.25	5.7	6.15	5.50	6.30*	5.75	6.35	5.73	6.25	5.75	6.40	5.65	6.3	5.75	6.4	5.75	6.1	5.6
6.5	6.50	5.95	6.5	5.7	6.35*	5.91	6.55	5.95	6.4	5.90	6.45	5.85	6.75	5.75	6.6	5.90	6.1	5.65
7.0	6.50	6.2	6.25	6.0	6.6**	6.75	6.6	6.25	6.6	6.2	6.***55	6.15	6.92	6.05	6.**90	6.2	6.**6	6.0
7.5	6.65	6.32	6.55	6.1	6.***80	6.5	6.6	6.4	6.55	6.35	6.***55	6.30	6.45	6.22	6.**78	6.25	6.***6	6.05
8.0	6.55	6.50	6.***8	6.65	6.***80	6.68	6.***80	6.65	6.***85	6.6	6.***7	6.6	7.**15	6.45	6.6	6.55	6.***65	6.3

* - Fair Growth
** - Good Growth
*** - Heavy Growth
No asterisk denotes absence of visible growth

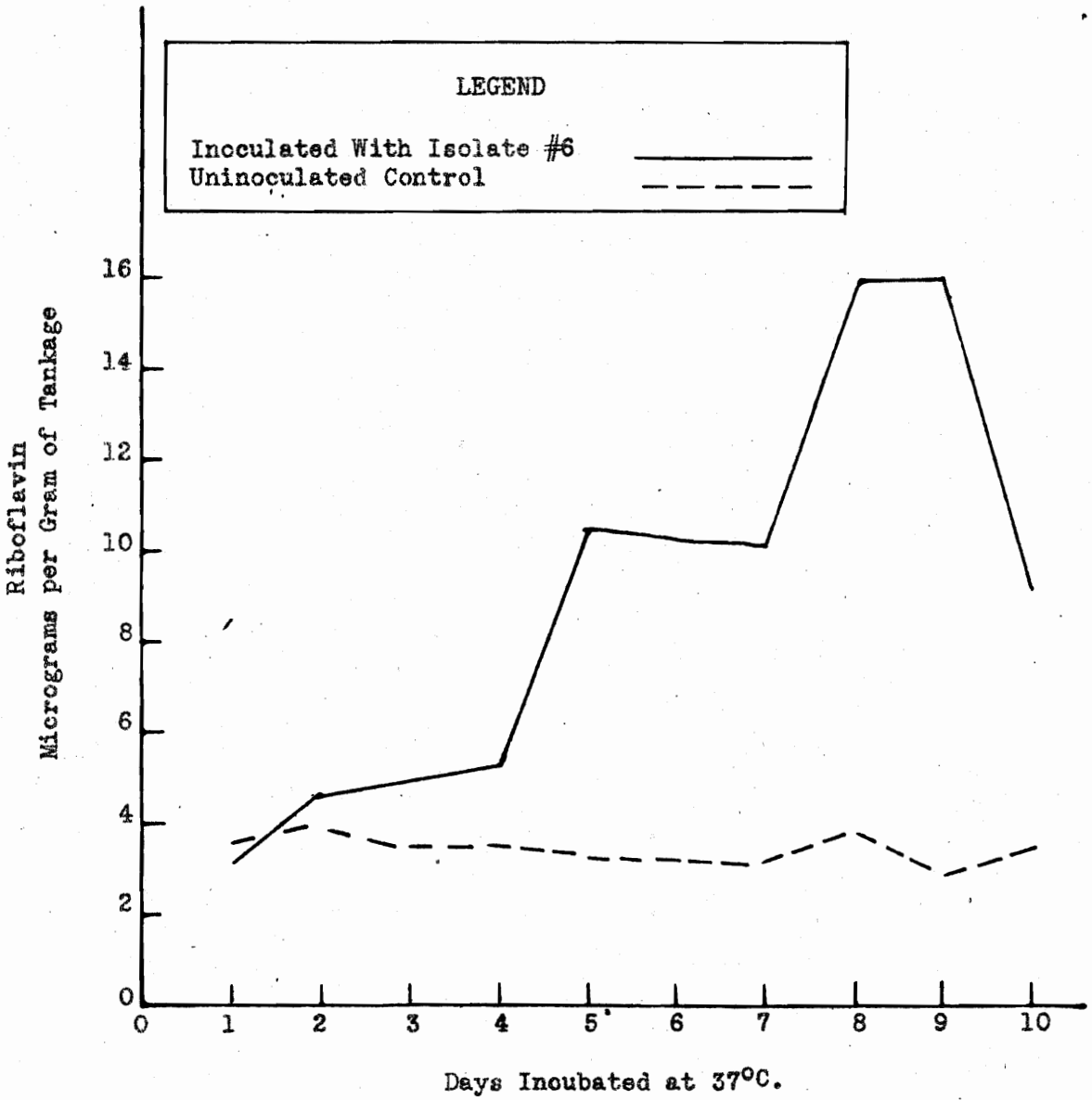


Figure 14 - Riboflavin Synthesis by Microorganism #6 in a Tankage Medium of Initial pH 5.5 Using a Separate Sample for Each Analysis

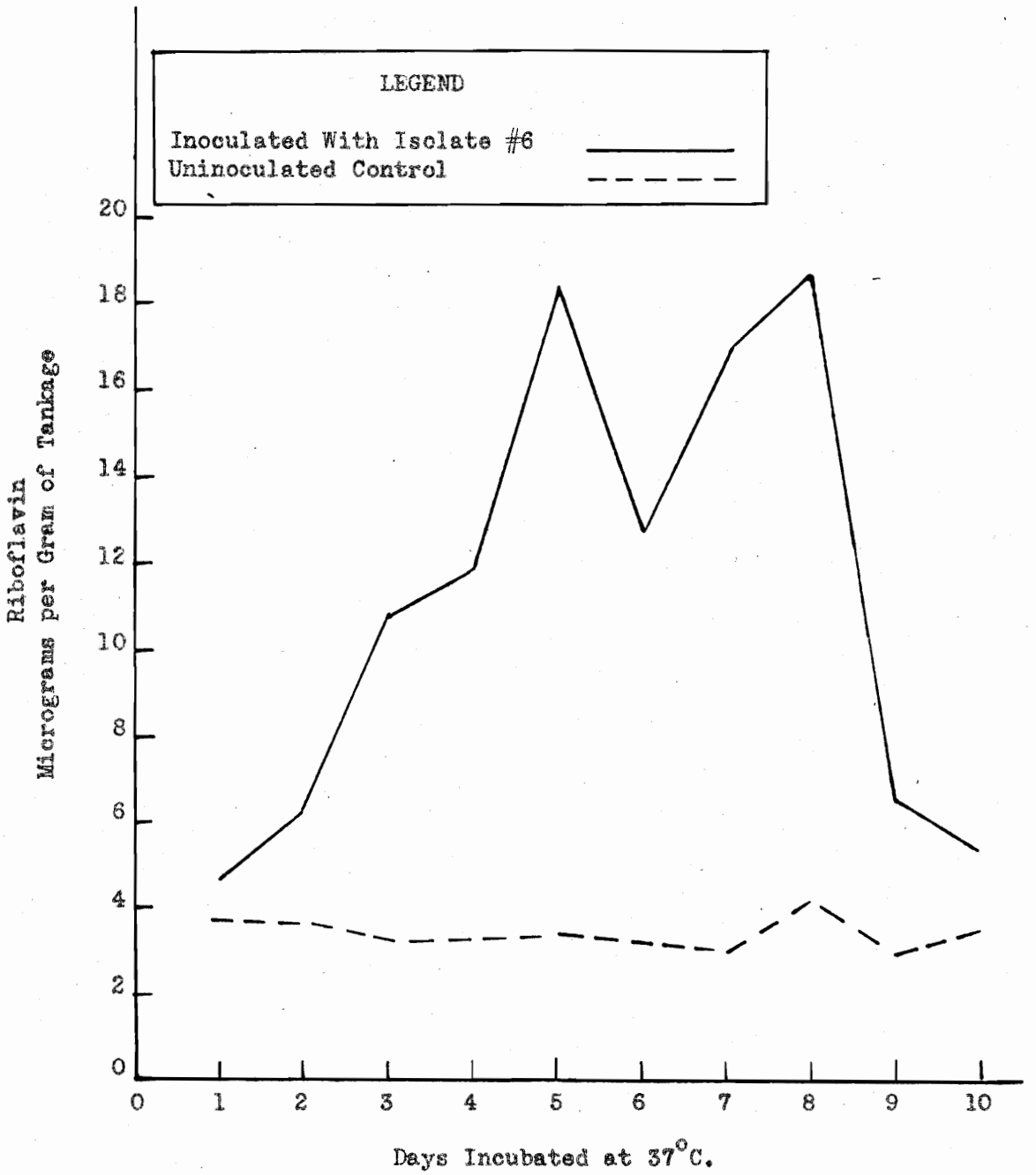


Figure 15 - Riboflavin Synthesis by Microorganism #6 in a Tankage Medium of Initial pH 6.0 Using a Separate Sample for Each Analysis

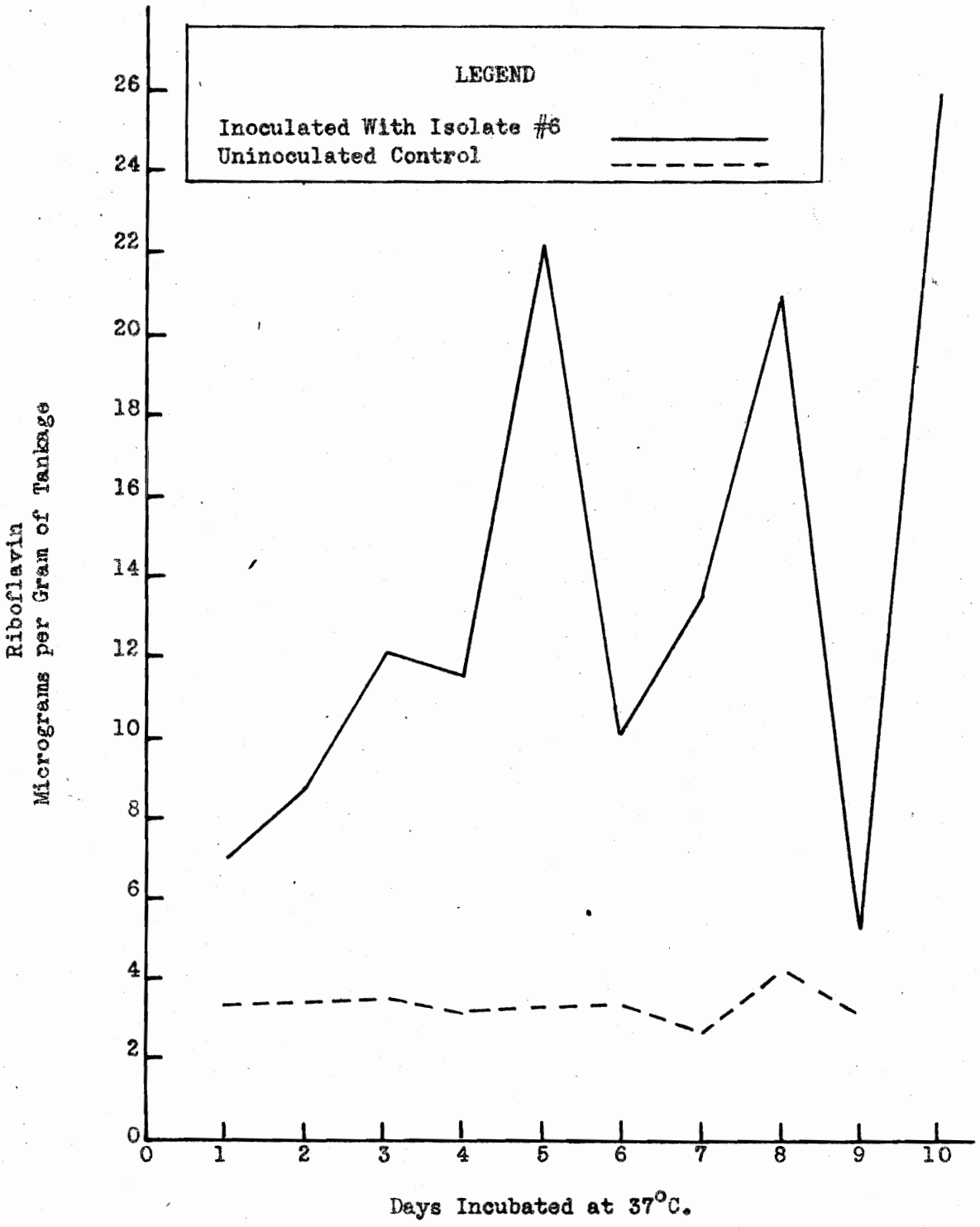


Figure 16 - Riboflavin Synthesis by Microorganism #6 in a Tankage Medium of Initial pH 6.5 Using a Separate Sample for Each Analysis

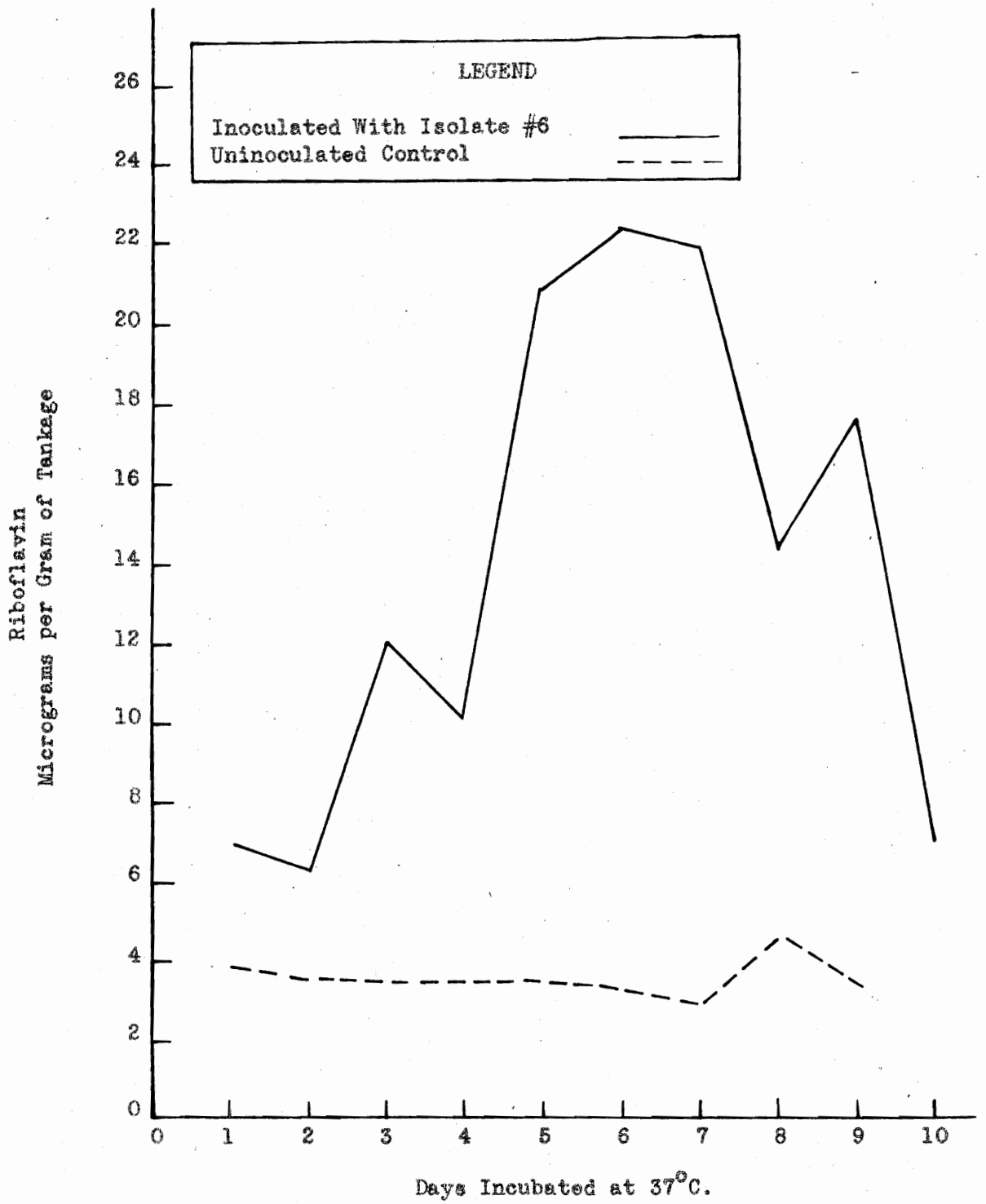


Figure 17 - Riboflavin Synthesis by Microorganism #6 in a Tankage Medium of Initial pH 7.0 Using a Separate Sample for Each Analysis

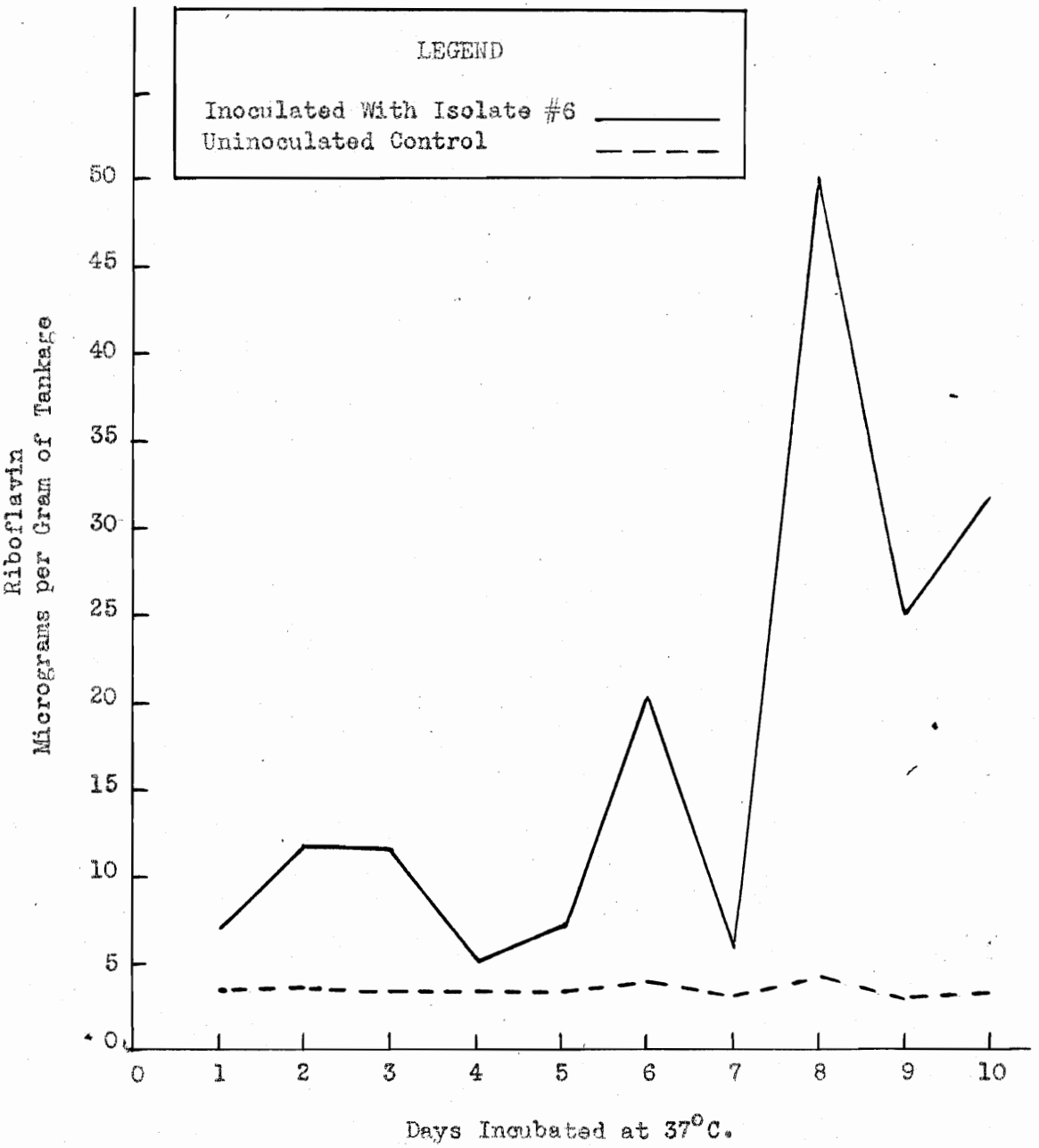


Figure 18 - Riboflavin Synthesis by Microorganism #6 in a Tankage Medium of Initial pH 7.5 Using a Separate Sample for Each Analysis

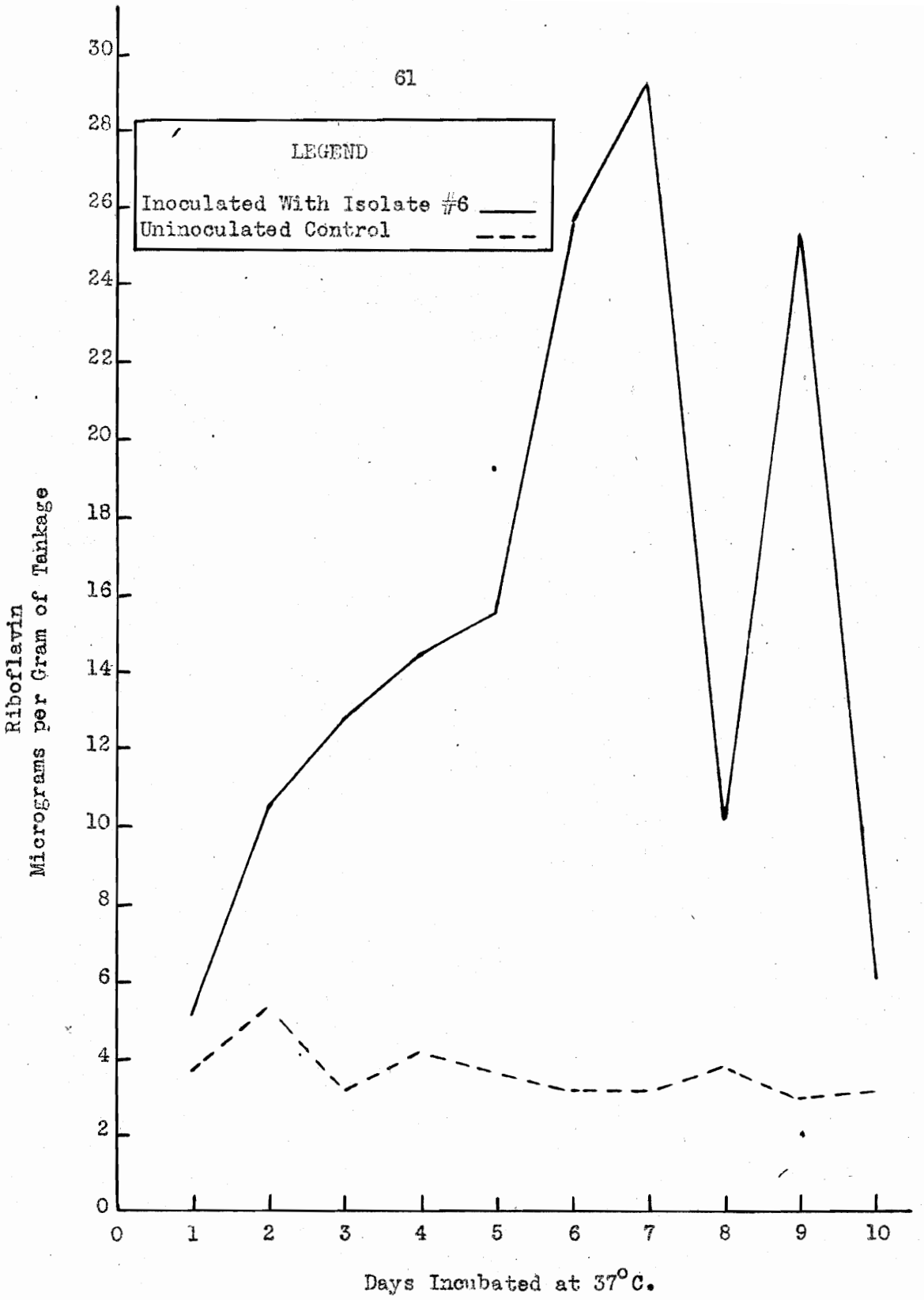


Figure 19 - Riboflavin Synthesis by Microorganism #6 in Tankage Medium of Initial pH 8.0 Using a Separate Sample for Each Analysis

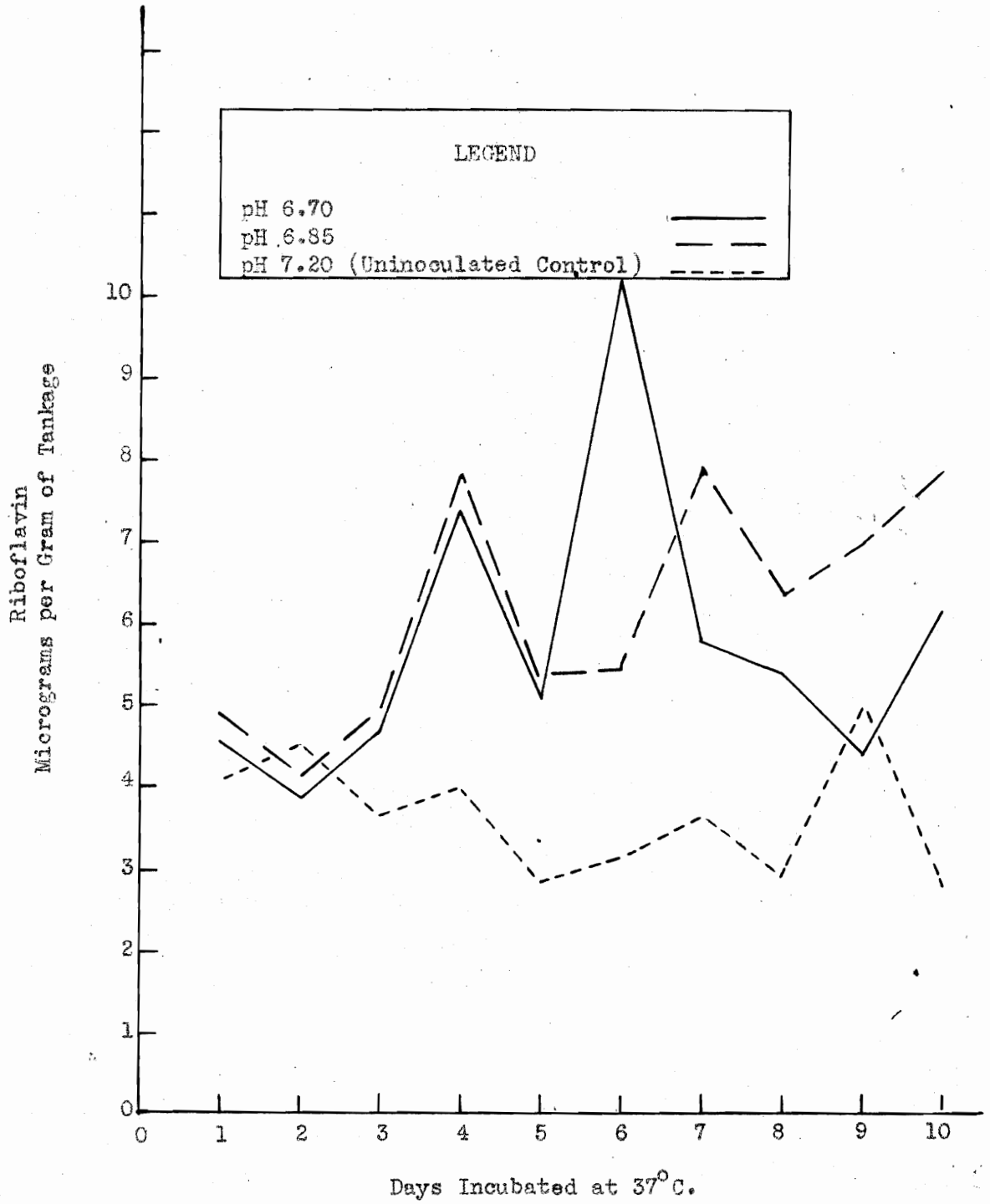


Figure 20 - Riboflavin Synthesis by Microorganism #6 in Tankage Media at Initial pH Measurements of 6.70, 6.85 and 7.20 With All Samples in Series Taken from a Single Batch

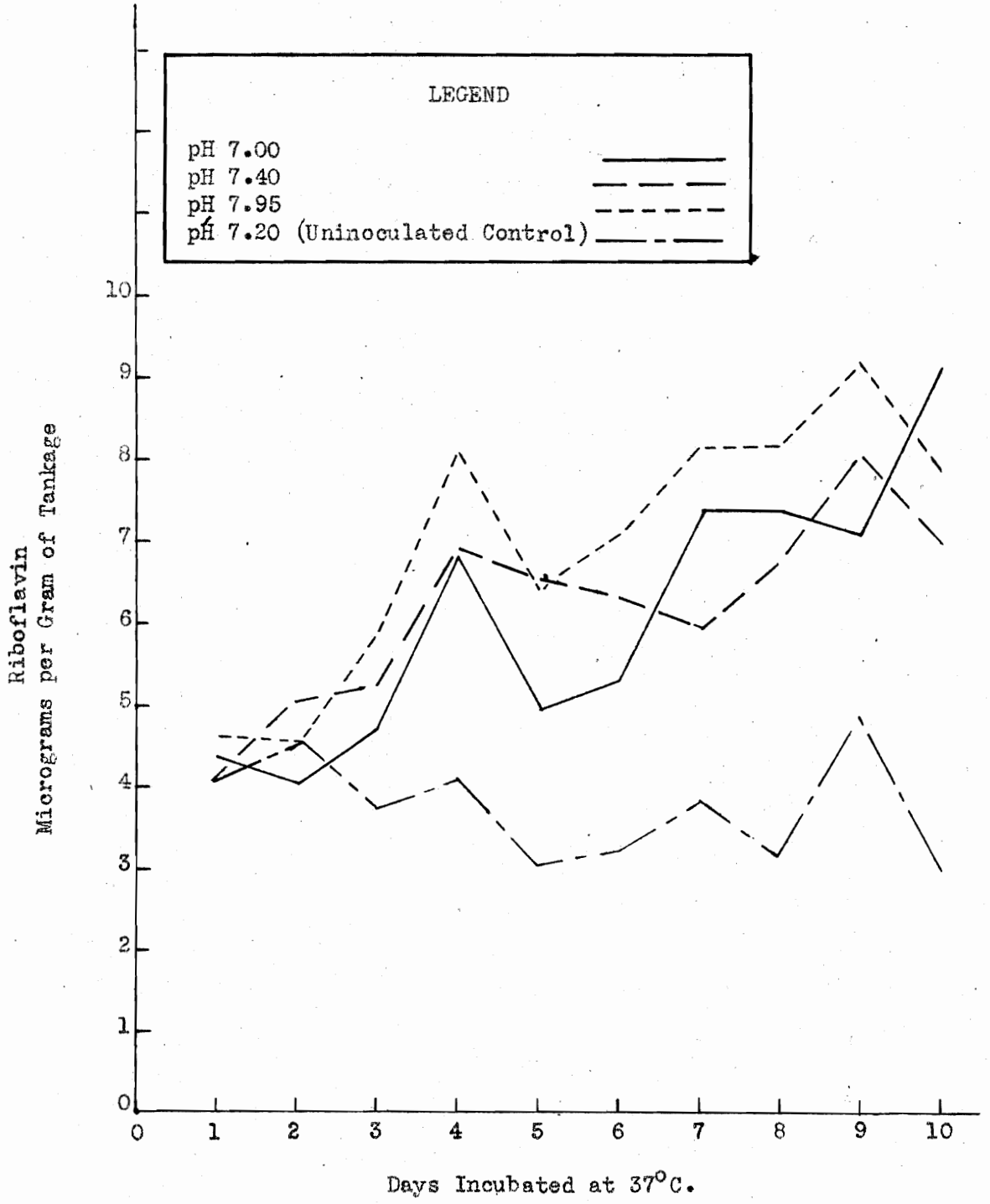


Figure 21 - Riboflavin Synthesis by Microorganism #6 in Tankage Media at Initial pH Measurements 7.00, 7.40 and 7.95 With All Samples in Series Taken From a Single Batch

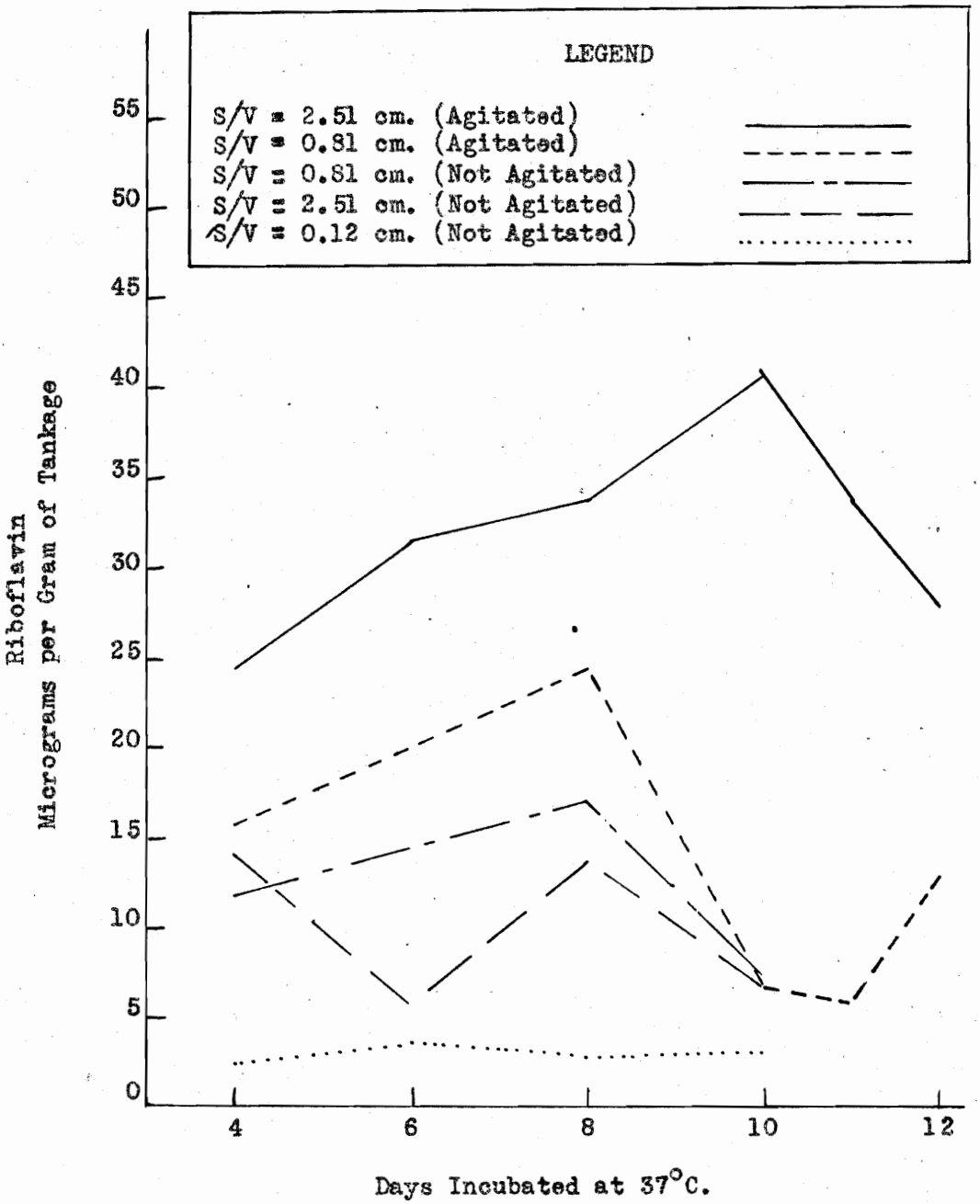


Figure 22 - Riboflavin Synthesis by Microorganism #6 in Tankage Medium of Initial pH 8.2, Varying Conditions of Agitation and Surface to Volume Ratio and Using a Separate Sample for Each Analysis

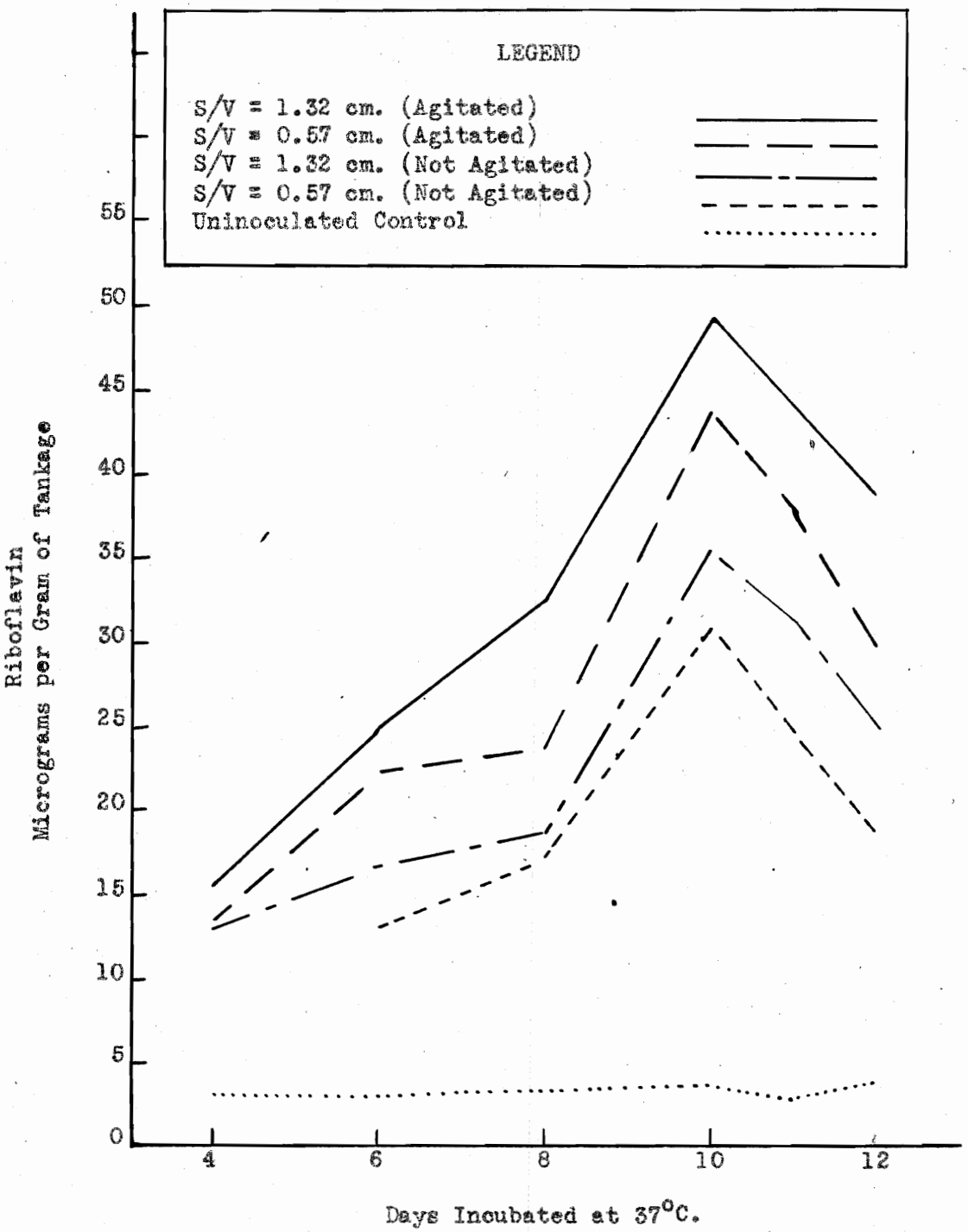


Figure 23 - Riboflavin Synthesis by Microorganism #6 in Tankage Medium of Initial pH 8.2, Varying Conditions of Agitation and Surface to Volume Ratio, With All Samples in Series Taken from a Single Batch

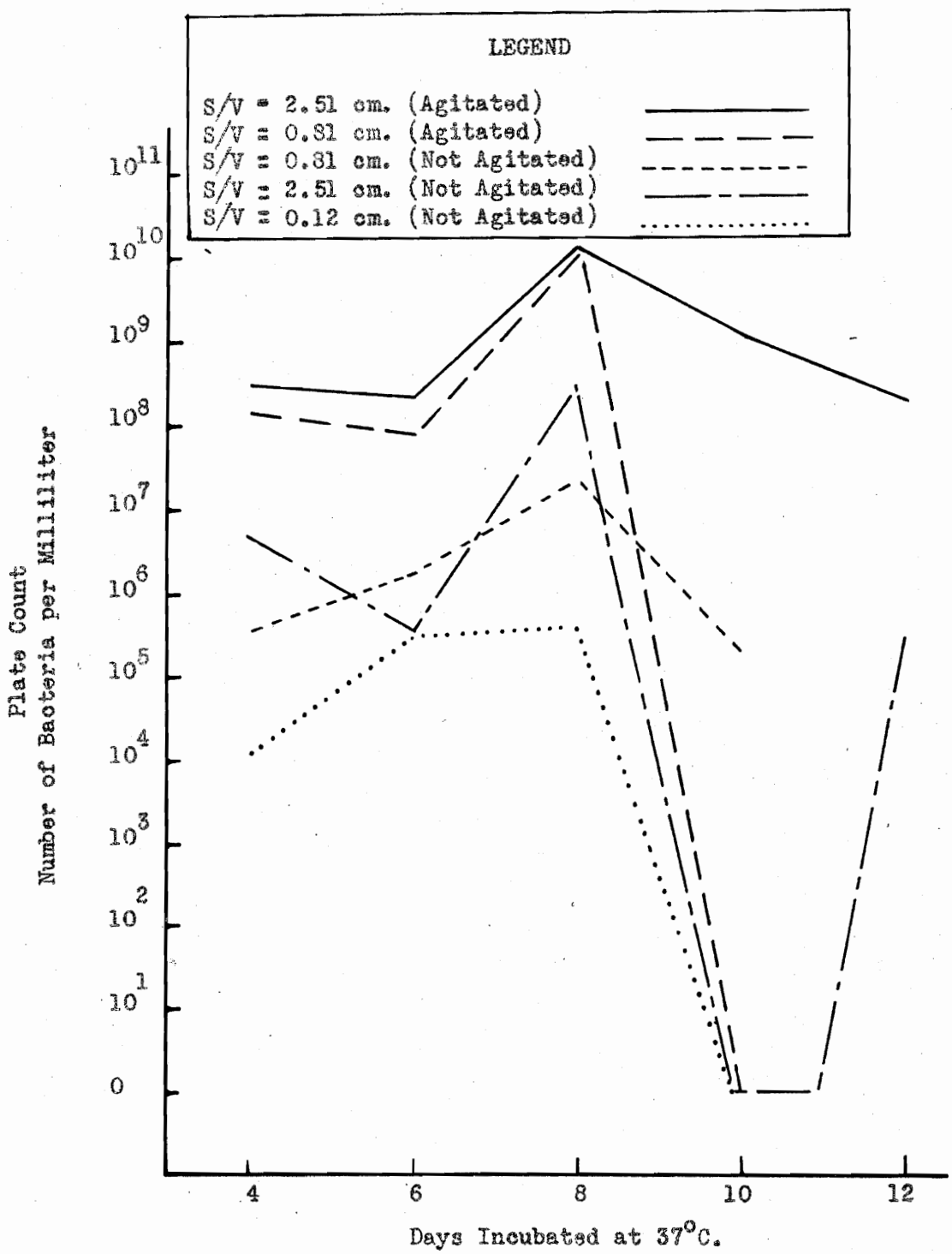


Figure 24 - Plate Counts of Microorganism #6 Growing on Tankage Medium of Initial pH 8.2, Varying Conditions of Agitation and Surface to Volume Ratio, With a Separate Sample Used for Each Count

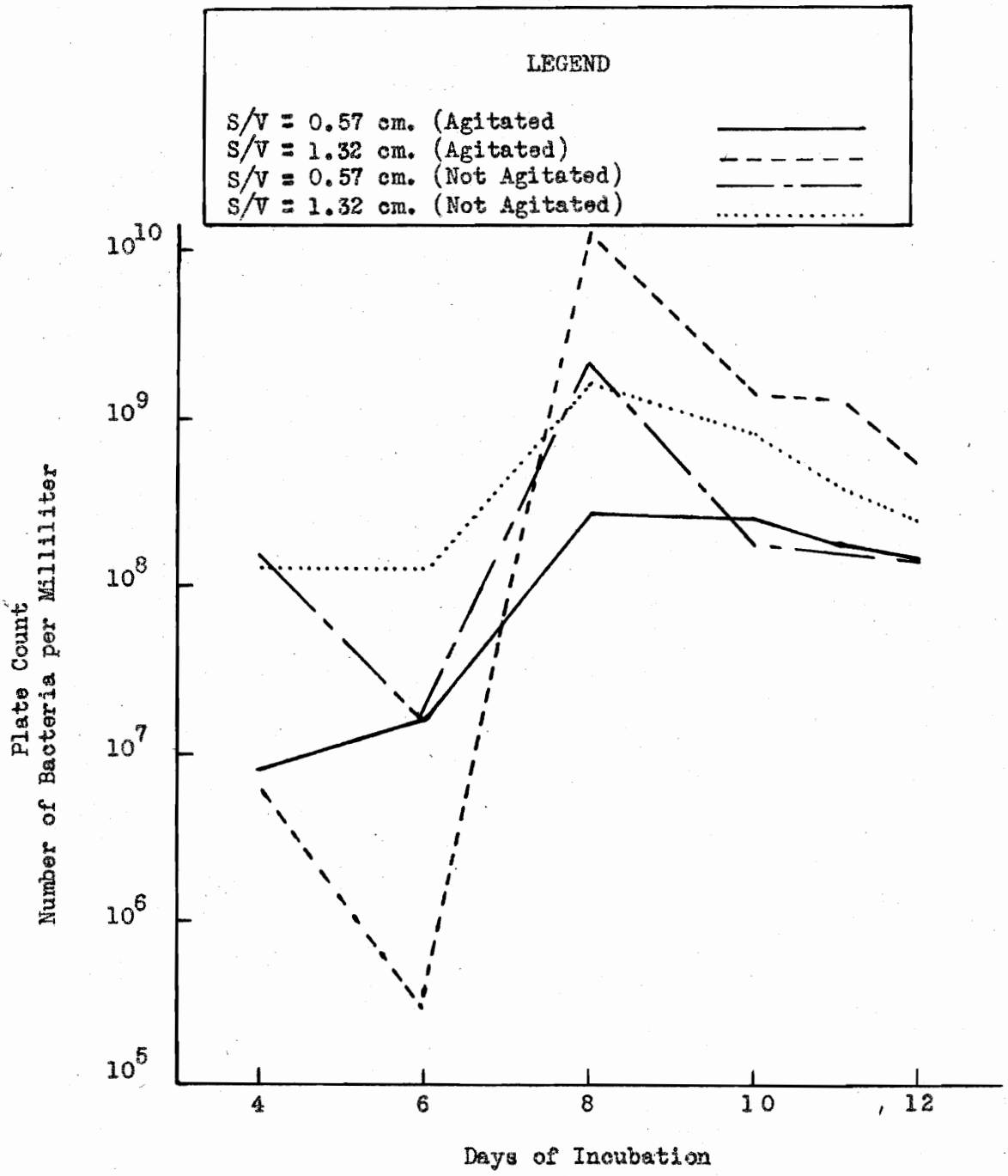


Figure 25 - Plate Counts of Microorganism #6 Growing on Tankage Medium of Initial pH 8.2, Varying Conditions of Agitation and Surface to Volume Ratio, With Each Curve Showing Periodic Count in a Single Batch

Discussion of Results of Experiment IV

On the basis of the maximum amount of riboflavin produced at each 0.5 pH increment from 5.5 through 8.0, increased riboflavin levels were observed with increased alkalinity of the medium. Vitamin B_2 production levels of 15.9, 18.6, 25.8, 22.4, 50.1 and 29.3 mcg./gm. tankage were obtained with initial pH adjustments of 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 respectively (Figures 14 through 19). Although the 50.1 mcg. of B_2/gm. of tankage was considerably higher than that obtained with other pH values, the over-all productivity curve (Figure 18) was extremely irregular. On the other hand, the curve in Figure 19, for a pH of 8.0 shows a maximum productivity of 29.3 mcg./gm. of tankage. This curve shows constant increases in vitamin B_2 production through seven days of incubation and fewer irregularities than most of the other curves discussed in this phase of the experiment. These irregularities, in certain instances, were correlated with variable amounts of growth occurring from sample to sample. The growth results recorded in Table 10 substantiate this statement, especially synthesis of riboflavin by Organism #6 at pH 8.0 (Figure 18). From the results of this phase of the work it was concluded that a pH range between 7.5 and 8.0 was more suited for growth and riboflavin productivity by Organism #6.

The pH of the medium in the sample bottles varied over the nine-day incubation period with irregularities similar to those observed in the assay results (Table 10). The pH of the control varied but little. The unusual change noted for the first-day incubation samples resulted primarily from autoclaving the adjusted medium prior to inoculation.

Since the curves in Figures 14 through 19 show the same irregular assay values that the authors had observed throughout their work with the separate sample technique, a single cultural environmental method that permitted periodic sampling over the entire period of incubation was adopted for experimentation. Figures 20 and 21 show the results obtained from a single cultural environment. A slight decrease in the irregularities of production was noted, however, a definite decrease was also observed in the amounts of riboflavin synthesized. A pour plate of each batch sample after ten days of incubation showed that the control sample and the samples with an adjusted pH of 6.7 and 7.9 were each contaminated. The contaminated samples do not show a striking difference in riboflavin content as compared with the uncontaminated samples. Due to the many variables involved in sampling from a single cultural environment, namely, pH, surface area of medium, volume of medium, agitation and contamination, the results were inconclusive. A comparison of the separate sample method with the batch method was necessary to determine the effect of each of these variables on the productivity of riboflavin.

Since the results obtained from varying pH measurements with the separate sample method of analysis indicated a pH range of 7.5 to 8.0 as most suitable for growth and riboflavin productivity by microorganism #6, a homogeneous batch of tankage medium was adjusted to a pH of 8.7. On the basis of previous pH adjustments it was predicted that autoclaving followed by incubation at 37°C. for eighteen hours would reduce the pH to within the range of 7.5 to 8.0. However, the pH of the medium after this period of incubation was found to be 8.2. A shortage of tankage material made it necessary to utilize the medium at this pH. This batch

of medium was divided among the various samples as described in the preceding procedure (Effect of Other Factors When pH is Held Constant). The riboflavin synthesis resulting from this experimentation is shown in Figures 22 and 23.

The production curves plotted in Figure 22 are a result of the separate sample method of analysis. The samples incubated in a horizontal position, 2.51 cm^2, surface area to volume ratio, with fifteen minutes agitation daily, supported a steady increase in riboflavin with a maximum yield of 40.8 mcg. B_2 per gram recorded for a ten-day incubation period. It is also noted that Microorganism #6 was unable to synthesize riboflavin when the medium was confined to a test tube. The other three production curves included in Figure 22 show the irregularities in production which were typical of previous results using the separate sample method. The control values for this phase of experimentation are plotted in Figure 23.

The results obtained using the batch method of analysis are plotted in Figure 23. It is observed that a steady increase in production occurred through the tenth day of incubation in the case of all samples. It must be recalled that all four jars of inoculated media were agitated prior to sampling. Thus the difference in treatment of the agitated and non-agitated samples is fifteen minutes daily agitation for twelve days with the agitated sample and five minutes daily agitation from the fourth through the twelfth day for the non-agitated batch. This difference in treatment is not as great as might be interpreted from use of the terms "agitated" and "not-agitated". However, it is significant that the curves in Figure 23 show that the amount of agitation is directly

proportional to riboflavin production. This proportionality also exists between surface area to volume ratio and riboflavin production (Figure 23).

A comparison of Figure 22 with Figure 23 indicates the more consistent nature of the batch sampling as compared with the separate sample technique. The probability that agitation is partly responsible for this condition must also be considered. An analysis of the curves in these two figures does not afford a correlation of surface area to volume ratio with riboflavin production. It is apparent from each of the production curves that, under conditions prevailing in this experiment, the maximum vitamin B_2 production is attained during the first ten days of incubation (Figures 22 and 23).

Figures 24 and 25 contain graphed results of the viable cell count taken at the time of each riboflavin determination. These curves indicate the growth trend over the incubation period, but do not allow any specific correlation between viable cell count and rate of riboflavin production. In comparing these growth figures with the irregularities occurring in the production curves in Figure 22, it is observed that in some, not all, abnormal decreases, Microorganism #6 has failed to grow in the particular sample. By such comparison it must be concluded that failure to establish growth is not solely responsible for the production decreases occurring in the separate sample method of analysis. Since the exact number of cells used in the initial inoculum is not known, these curves cannot be considered as actual growth curves.

Table 11

The Effect of Surface Area, Surface Area to Volume Ratio, and Agitation on Riboflavin Production by Microorganism #6

Surface Area of Sample (cm^2)	Ratio of Surface Area to Volume (cm)	Maximum Amount of Riboflavin mcg/gm of Tankage	
		Agitated	Non-agitated
2.1	0.12	---	3.5
14.5	0.81	24.5	17.0
45.1	2.51	40.8	13.7
51.5	0.67	43.7	30.8
119.0	1.32	49.3	35.4

In order to determine the factors involved in the maximum production of riboflavin by Microorganism #6, a summary of the maximum amounts of riboflavin produced and the status of the environmental factors involved is given in Table 11. These maxima are taken from Figures 22 and 23. Table 11 shows that agitation increases riboflavin synthesis. There appears to be no correlation between surface area to volume ratio and riboflavin production. However, surface area, alone, is directly proportional to the amount of riboflavin produced, providing the medium is agitated for fifteen minutes daily (Table 11). This Table shows that a sample of tankage medium with 119.0 cm^2 surface area and a ratio of surface area to volume of 1.32 cm, when agitated fifteen minutes daily, can support a maximum production by Microorganism #6 of 49.3 mcg. B_2 per gram of tankage.

Summary of Experiment IV

Results obtained through investigation of the effect of growth and some environmental factors on the synthesis of riboflavin by Microorganism #6 lead to the following conclusions:

(1) The pH range 7.5--8.0 is the more desirable hydrogen-ion concentration for the establishment of growth and maximum B_2 synthesis by Microorganism #6 on tankage medium.

(2) The number of viable cells determined by bacterial plate count on any given day of incubation is not directly correlated with the amount of B_2 present in the medium.

(3) Agitation as a single factor is capable of enhancing riboflavin synthesis by #6.

(4) The ratio of surface area to volume is not directly correlated with B_2 production.

(5) The surface area is directly proportional to the amount of riboflavin present in the medium, when the samples are agitated for fifteen minutes every twenty-four hours.

(6) Failure to establish growth is one of the factors responsible for irregularities in B_2 production when the separate sample method is employed.

(7) Microorganism #6 is capable of producing 49.5 micrograms of B_2 per gram of dry tankage in a batch-type sample of tankage medium with pH 8.2, surface area of 119 $cm.^2$, fifteen minutes agitation per day, and an incubation period of ten days.

Experiment V - Characteristics of Microorganism #6

Since Microorganism #6 was capable of greater riboflavin production than any organism tested, it was desirable to investigate the morphological, cultural and physiological characteristics of Microorganism #6 so that an attempt at classification could be made. The following information based on repeated observations was obtained by the authors.

1. Morphological Characteristics

shape	coccobacillus
size	0.45 to 0.7 by 1.10 microns
cell arrangement	singly and in clumps
spore formation on nutrient agar and glucose-tryptone-extract agar in 48 and 72 hrs.	none as determined by heating 10 minutes at 85°C
capsule formation on nutrient agar and glucose-tryptone-extract agar in 3-4 days	none using Anthony Tyler's modification; Leifson's staining procedure
motility at 25°C in nutrient agar and nutrient broth	none in 18 hr cultures (hanging drop)
motility at 30°C in nutrient agar and nutrient broth	none in 18 hr cultures (hanging drop)
motility at 37°C in nutrient agar and nutrient broth	none in 18 hr cultures (hanging drop and soft agar)
gram reaction from 18 hr nutrient agar culture	negative (Hucker's modification)
simple stain (gentian violet)	stain not uniform; darker in region of cell periphery
acid fast stain from 18 hr nutrient broth culture	negative

2. Cultural Characteristics

(a) A fetid odor was produced in all the media employed by the authors. Ammonia was identified in nutrient broth using Nessler's reagent.

(b) Pigmentation is not produced in nutrient broth (with or without the addition of fermentable carbohydrates), on nutrient agar or gelatin. In the presence of brom-thymol-blue, a distinct yellow color occurs within the cells regardless of the nature of carbohydrate (fermentable or non-fermentable) contained in the broth.

(c) The optimum temperature for growth was 37°C., but good growth occurred at 30°C. and moderate growth at 25°C. No growth at 45.5°C. or 55°C.

(d) Growth type on nutrient agar slant: luxuriant, filiform, greyish-white, glistening and soft.

(e) Growth in nutrient broth: blue-gray membranous surface growth with slime layer forming along inner wall of the test-tube above the surface of the medium. Membrane and slime layer were somewhat irredescent. Flaky sediment and some diffuse growth.

(f) Relation to free oxygen: growth predominately on surface of broth and agar shake cultures, but some subsurface growth also occurs.

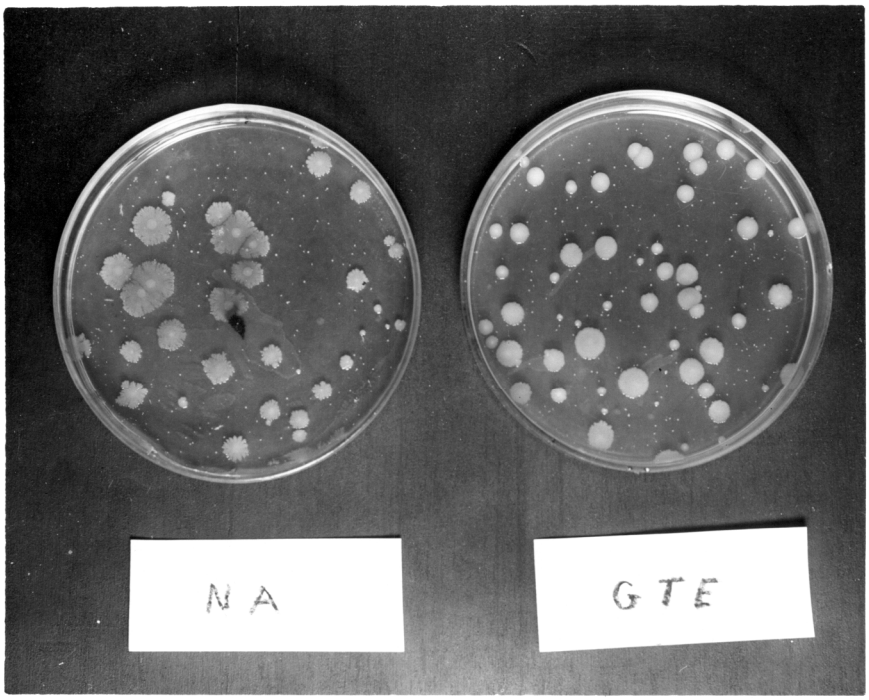
(g) Colony characteristics after 24-48 hrs. at 37°C on nutrient agar (see photograph):

margin ---------- lobate

form ---------- irregular

elevation ---------- slightly raised

internal structure -- granular



Photograph Showing the Colonial Appearance of Microorganism #6 on Pour Plates of Nutrient Agar (Difco) and Glucose-Tryptone-Extract Agar (Difco)

A definite tendency towards spreading was noted on this medium. The small pin-point colonies observed in the plates (see photograph) were subsurface colonies.

On glucose-tryptone-extract agar (see photograph):

margin ---------- entire

form ---------- circular

elevation ---------- somewhat raised

(h) Gelatin stab: profuse surface growth and slight subsurface growth in line of stab. After three weeks incubation at 37°C. slight stratiform liquefaction was evident.

(i) Potato slant: moderate growth, light yellow, non-spreading, soft and glistening.

3. Physiological Characteristics

(a) Reaction on carbohydrates: No acid or gas from sucrose, maltose, mannitol, dulcitol, d-levulose, raffinose, l-rhamnose, inositol and d-arabinose. Acid but no gas from glucose, galactose, l-arabinose and d-xylose. Fermented lactose slowly with acid production but no gas in 6-10 days. The above characteristics were observed in nutrient broth fortified with 1% carbohydrate. Acid but no gas formation was observed in nutrient agar plus 1% dextrose medium (brom-cresol-purple indicator).

(b) Gelatin stab: very slow stratiform liquefaction suspected after 21 days of incubation at 37°C. It was difficult to determine whether this was true bacterial liquefaction or the result of endo-enzyme released from the cell after death and lysis of the cells.

(c) Urea broth: no growth after 5 days.

(d) No nitrite produced from 1% KNO_3 medium (agar slants and broth) over a period of 3 days.

(e) Profuse growth in citrate broth after 18 hrs.

(f) Slight H_2S production in 48 hrs. on iron-petone agar medium.

(g) Methyl Red test negative (tested daily over 4 day period).

(h) Acetyl methyl carbinol test negative (tested daily over a period of 4 days).

(i) A positive test for indole formation could not be obtained (Kovac's reagent added directly into medium).

(j) Action in milk:

Litmus milk: alkaline reduction in 24 hrs; curd in 5 days.

Ulrich's milk: alkaline reaction plus reduction in 24 hrs.; rennet curd formed after 11 days.

(k) No indication of acid formation from starch agar slant cultures.

(l) Catalase reaction on a 24 hr nutrient agar plate culture was positive.

(m) No growth in yeast water containing up to 10% ethyl alcohol.

4. Discussion of Characteristics

From the characteristics observed by the authors, the bacterium was believed to be either an Achromobacter sp. or a Shigella sp. Classification, however, could not be reduced to a definite genus or to any of the species of the above genera as reported in Bergey's Manual (8). Skerman's mechanical key for the generic identification of bacteria was of value in the selection of the two probable genera mentioned (37).

SUMMARY AND CONCLUSIONS

The growth promoting properties of protein supplements used in poultry and livestock rations depend upon the riboflavin content as well as the quantity and quality of the protein in the supplement (43). As reported by Wilgus, Norris, and Heuser (43), the relative B_2 potency values expressed in micrograms per gram of supplement are 19 for dried skim milk, 10 for sardine fishmeal, and 6 for meat scraps. The particular batch of tankage or processed swine viscera used in this work was determined by microbiological assay to contain 3.5 micrograms of riboflavin per gram dry weight.

It was the purpose of this work to find a microorganism which, when grown on a medium of pre-cooked swine viscera (with the exception of heart, kidney and liver), would synthesize an amount of riboflavin sufficient to raise the B_2 potency of the tankage above 19 micrograms per gram dry weight. This increase in B_2 would be sufficient to raise the competitive standing of the tankage as a supplement for poultry and livestock rations.

By literary reference and experimentation the microbiological assay of riboflavin, as outlined by the American Association of Vitamin Chemists (41), was chosen as the most accurate type of assay procedure. The culture of the assay test organism, Lactobacillus casei, A.T.C.C. #7469, was checked as to its exacting nutritional requirements for riboflavin. The effect of the 8 to 10 per cent fat content of the tankage on L. casei was determined to be neither noticeably inhibitory nor stimulatory.

Twenty-nine microorganisms were investigated as to their ability to synthesize riboflavin. Eighteen of these organisms were unknowns isolated

from the natural flora of non-putrefied and putrefied viscera. The remaining eleven were organisms of known species which had previously shown a marked degree of ability for B_2 synthesis as recorded in literature by other workers. These organisms were cultured in an identical manner on a simple, broth-like medium having a ratio of 3 gms. of dehydrated tankage to 10 cc. of distilled water.

To determine the relative synthetic ability of all the organisms a technique was employed, whereby, separate sample bottles of tankage medium, one for assay after each succeeding day of incubation, were inoculated with each organism to be investigated. All tankage medium samples were adjusted to an initial pH of 6.8 and each organism tested was incubated at its predetermined optimum growth temperature. The period of incubation was limited to a maximum of nine days with a riboflavin assay determination being made every twenty-four hours. Thus, by the process of elimination, it was possible to select the organism which exhibited the most ability for synthesizing riboflavin on this medium.

Microorganism #6, a visceral isolate, produced the highest yield, in excess of 25 mcg. per gram dry weight of tankage, after eight days of incubation. This result was substantiated by a duplicate experiment. Thus, Microorganism #6 was chosen for further investigation. Environmental factors concerned in the previous testing of this organism were investigated in an effort to find conditions which would be favorable to further increase in riboflavin synthesis.

Using exact pH measurements over a range of 5.5 to 8.5, the optimum pH (7.5 to 8.0) was determined for production of riboflavin by isolate #6.

The assay results obtained with the separate sample method of analysis

consistently produced irregular increases and decreases in the rate of riboflavin synthesis during the incubation period. In order to obtain a more uniform assay of riboflavin, a method of sampling from a batch of medium was developed. This method consisted of growing the test organism in a single container of medium and extracting samples, by pipette, for assay at desired intervals of incubation.

A comparison of the separate sample and batch methods was used in an investigation of the effect of ratio of surface area to volume of the medium, mechanical agitation of the culture vessel and amount of bacterial growth on riboflavin synthesis by Microorganism #6. This phase of experimentation produced the following conclusions.

(1) The amount of riboflavin produced is directly proportional to the surface area of the medium when the surface area is varied from 2.07 cm^2 to 119.0 cm^2.

(2) The surface area to volume ratio does not exhibit any direct correlation with riboflavin production.

(3) Mechanical agitation of the culture vessel, for fifteen minutes daily, aids in the synthesis of riboflavin by Microorganism #6.

(4) Bacterial counts, determined by plating at the time of assay, cannot be directly correlated with riboflavin assay results. In most cases these counts do serve to explain the decreases occurring in riboflavin production when the separate sample method of assay is employed.

(5) A steady increase to a maximum riboflavin production was obtained with the batch method of sampling.

(6) Microorganism #6 exhibited a maximum yield of 49.30 mcg. per gram of dry tankage under the following conditions:

(a) Surface area of medium - 119 cm^2

(b) Volume of medium - 90 ml.

(c) Contents of medium - a ratio of 3 gm. of tankage (dehydrated and ground) to 10 ml. of water.

(d) pH of medium - 8.2, initially

(e) Incubation temperature - 37°C.

(f) Period of incubation - 10 days

(g) Mechanical agitation - 15 min., daily

In an attempt to identify Microorganism #6 an examination was made of all characteristics which might lend themselves to a classification according to Bergey's Manual (8). The organism is a gram-negative, non-motile, non-spore-forming, aerobic, coccobacillus, producing acid but no gas from glucose, galactose, l-arabinose, d-xylose, and (in 6-10 days) lactose. It is believed to be an Achromobacter sp. or a Shigella sp. according to Skerman's mechanical key for the generic identification of bacteria (37). On the basis of morphological, physiological and cultural characteristics it was not feasible to reduce the classification to any specific genus or any species of the aforementioned genera.

The production of 49.30 mcg. of riboflavin per gram dry weight of tankage suggests that the synthesis of riboflavin by Microorganism #6 is a possible means of materially enriching the vitamin B_2 content of tankage supplement.

APPENDIX*

Table I - Synthesis of Riboflavin by Isolates #1, #8 and #11

Unknown Organism	Days Incubated at 37°C					
	1	2	3	4	5	6
1	4.8	4.3	6.1	8.9	11.4	6.1
8	3.8	3.3	4.1	3.4	3.9	3.8
11	3.6	3.7	3.7	3.6	3.4	3.8
Uninoculated Control	4.1	3.2	3.4	3.5	3.6	3.3

Table II - Synthesis of Riboflavin by Isolates #4, #6, #9, #10 and #16

Unknown Organism	Days Incubated at 37°C								
	1	2	3	4	5	6	7	8	9
4	3.6	2.3	3.2	4.4	5.0	5.9	3.5	9.5	4.8
6	3.6	3.4	3.0	11.7	8.7	11.5	19.4	30.5	20 to
9	3.6	4.3	3.2	3.2	3.1	3.6			
10	4.2	3.6	3.5	8.4	5.8	9.1	7.2	10.0	9.3
16	3.7	3.5	3.5	2.6	2.7	3.4			
Uninoculated Control	3.8	3.9	3.4	3.1	2.7	3.3	2.8	3.2	3.9

*The Twelve Tables in this section contain assay values, for each day of incubation, expressed in micrograms of riboflavin per gram of tankage.

Table III - Synthesis of Riboflavin by Isolates #7, #17 and #18

Unknown Organism	Days Incubated at 37°C								
	1	2	3	4	5	6	7	8	9
7	4.5	5.9	6.6	7.1	4.3	8.1	8.1	7.4	10.9
17	4.1	3.8	3.3	3.4	4.4	3.0			
18	3.9	3.7	3.4	lost	3.3	3.1			
Uninoculated Control	3.4	3.9	3.5	3.3	3.4	2.8	3.0	5.0	3.9

Table IV - Comparison of Duplicate Determinations of Riboflavin Synthesis Isolated by Microorganism #6

Run	Unknown Organism	Days Incubated at 37°C								
		1	2	3	4	5	6	7	8	9
1	6	3.6	3.4	3.0	11.7	8.7	11.5	19.4	30.5	
	Uninoculated Control	3.8	3.9	3.4	3.1	2.7	3.3	2.8	3.2	
2	6	6.8	11.3	7.1	6.2	21.0	19.8	21.6	26.0	6.5
	Uninoculated Control	3.5	3.6	3.9	4.0	4.2	3.8	3.0	5.0	3.9

Table V - Riboflavin Synthesis by *A. aerogenes*, *E. coli* var. *communior* and *M. flavus*

Organism	Micrograms of B_2/gram					
	1	2	3	4	5	6
Aerobacter aerogenes	5.12	5.61	6.13	9.05	10.28	11.99
Escherichia coli var *communior*	6.65	7.66	8.74	10.67	11.48	14.13
Micrococcus flavus	3.92	3.62	3.38	5.76	14.4	4.54
Uninoculated Control	4.11	3.22	3.38	3.49	3.55	3.29

Dry wt. of tankage = 2.9 gms.
Incubated at 37°C
pH of medium = 6.4

Table VI - Riboflavin Synthesis by *Cl. acetobutylicum*, *E. coli* and *T. lactosa*

Organism	Micrograms of B_2/gram					
	1	2	3	4	5	6
Clostridium aceto-butylicum	4.04	5.315	7.94	3.72	4.06	9.53
Escherichia coli	6.04	5.905	7.04	6.08	7.96	5.1
Torula lactosa	4.06	4.995	3.94	4.57	8.08	4.73
Uninoculated Control	3.43	3.89	3.49	3.25	3.42	2.81

Dry wt. of tankage = 2.9 gms
pH = 6.4
Temperature = 37°C

Table VII - Riboflavin Synthesis by Al. faecalis, B. subtilis #480, P. vulgaris #355, P. vulgaris and T. sphaerica

Organism	Micrograms of B_2/gram					
	1	2	3	4	5	6
Alcaligenes faecalis	2.48	4.99	4.94	7.2	3.95	3.23
Bacillus subtilis #480	2.11	4.2	3.4	7.6	4.5	2.83
Proteus vulgaris #355	2.44	4.95	2.34	5.82	4.11	3.19
Proteus vulgaris	2.54	5.26	2.53	5.01	3.37	2.97
Torula sphaerica	2.09	4.09	2.53	4.32	4.51	2.76
Uninoculated Control	3.64	5.97	2.61	5.35	3.34	3.69

Dry wt. of tankage = 2.9 gms.
pH = 6.4
Temperature = 37°C

Table VIII - Comparison of Riboflavin Synthesis by Cl. acetobutylicum Under Aerobic and Anaerobic Conditions

Culture Conditions of Cl. acetobutylicum	Micrograms of B_2/gram					
	1	2	3	4	5	6
Aerobic (30°C)	4.64	4.87	6.44	8.48	7.94	10.37
Anaerobic (30°C)	3.27	4.24	5.82	4.44	4.03	3.10
Uninoculated Aerobic Control (30°C)	3.45	3.58	3.93	3.98	4.21	3.80

Dry wt. of tankage = 2.9 grams
pH = 6.4

Table IX - Riboflavin Synthesis by M. flavus, T. lactosa and T. sphaerica incubated at 30°C.

Organism	Micrograms of B_2/gram					
	1	2	3	4	5	6
M. flavus	4.39	3.36	6.69	7.67	6.73	7.12
T. lactosa	4.11	3.62	6.87	9.21	7.54	9.63
T. sphaerica	4.32	3.50	5.39	6.68	8.43	10.83
Uninoculated Control	3.45	3.58	3.93	3.98	4.21	3.80

Dry wt. of tankage = 2.9 grams
pH = 6.4
Temperature = 30°C

Table X - Riboflavin Synthesis by A. aerogenes, E. coli, E. coli var. communior and Cl. acetobutylicum Over an Extended Incubation Period

Organism and Culture Conditions	Micrograms of B_2/gram		
	7	8	9
A. aerogenes (37°C)	8.80	12.55	12.1
E. coli (37°C)	4.88	9.51	6.75
E. coli var. communior (37°C)	6.59	12.53	13.68
Cl. acetobutylicum (30°C)	9.67	12.5	13.54
Uninoculated Control (37°C)	2.81	3.23	3.88
Uninoculated Control (30°C)	2.97	5.02	3.85

Dry wt. of tankage = 2.9 grams
pH = 6.4

Table XI - Effect of pH on Riboflavin Synthesis by Isolated Microorganism #6, Using the Separate Sample Method of Analysis

Original pH of Medium	Inoculated with	Mcg. of B_2/gm. Dry Wt. of Tankage									
		1	2	3	4	5	6	7	8	9	10
5.5	#6	3.19	4.66	5.06	5.39	10.64	--	10.10	15.85	15.94	9.17
	Uninoculated Control	3.55	3.94	3.43	3.51	3.31	--	3.14	3.87	2.86	3.42
6.0	#6	4.74	6.31	10.75	11.81	18.36	12.72	16.9	18.66	6.71	5.37
	Uninoculated Control	3.72	3.65	3.32	3.33	3.35	--	3.03	4.22	3.05	3.48
6.5	#6	7.04	8.82	12.12	11.53	22.19	10.16	13.48	20.87	5.24	25.86
	Uninoculated Control	3.41	3.48	3.55	3.18	3.26	3.36	2.80	4.29	3.11	--
7.0	#6	7.02	6.29	11.98	10.01	20.92	22.42	21.94	14.29	17.48	7.05
	Uninoculated Control	3.85	3.55	3.45	3.47	3.47	3.32	2.93	4.64	3.38	--
7.5	#6	7.13	11.82	11.45	5.03	6.56	20.44	5.35	50.08	25.05	31.80
	Uninoculated Control	3.37	3.48	3.25	3.40	3.29	3.92	3.10	4.18	2.90	3.32
8.0	#6	5.32	10.6	12.80	14.5	15.68	25.59	29.30	9.65	25.4	6.24
	Uninoculated Control	3.68	5.27	3.22	4.17	3.61	3.07	3.12	3.83	2.94	3.08

Table XII - Effect of pH on Riboflavin Synthesis by Isolated Microorganism #6 Using the Batch Method of Analysis

pH of Medium	Inoculated with	Mcg. B_2/gm. Dry Wt. of Tankage									
		1	2	3	4	5	6	7	8	9	10
7.2	Uninoculated Control	4.09	4.51	3.64	3.98	2.84	3.13	3.64	2.92	5.00	2.83
6.7	#6	4.56	3.88	4.71	7.4	5.2	10.19	5.79	5.39	4.43	6.17
6.85	#6	4.85	4.17	4.95	7.85	5.38	5.45	7.9	6.36	6.99	7.8
7.0	#6	4.36	4.02	4.75	6.73	4.97	5.38	7.37	7.37	7.09	9.11
7.4	#6	4.11	5.05	5.25	6.93	6.56	6.30	5.95	6.78	8.02	6.97
7.95	#6	4.61	4.52	5.85	8.11	6.46	7.05	8.11	8.19	9.19	7.90

Table XIII - The Synthesis of Riboflavin and Corresponding Bacterial Counts of Isolated Microorganism #6 on Tankage Medium With pH 8.2 and With Variation of Agitation and Surface to Volume Ratio

Type Sample	Dry Weight of Tankage in Medium (Gms)	Surface to Volume Ratio (cm^2)	Inoculated with	4 Days of Incubation				6 Days of Incubation				8 Days of Incubation		10 Days of Incubation				11 Days of Incubation				12 Days of Incubation			
				Rest		Agitation		Rest		Agitation		Rest		Agitation		Rest		Agitation		Rest		Agitation		Rest		Agitation	
				Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram	Bacterial Count per ml.	Mcg. of B_2 per Gram
Quart Jar	19.5	0.57	1 ml of #6	1.9×10^{8}	----	9.0×10^{6}	13.5	2.0×10^{7}	13.1	2.0×10^{7}	22.3	3.0×10^{9}	17.0	4.2×10^{8}	23.8	2.5×10^{8}	30.8	4.0×10^{8}	43.7	1.8×10^{8}	24.5	2.5×10^{8}	37.6	1.7×10^{8}	18.0	1.2×10^{8}	29.8
Quart Jar	19.5	1.32	1 ml of #6	1.0×10^{8}	13.0	8.0×10^{6}	15.4	1.0×10^{8}	16.7	5.0×10^{5}	24.8	2.0×10^{9}	18.6	1.0×10^{10}	32.4	9.0×10^{8}	35.4	1.5×10^{9}	49.3	6.0×10^{8}	31.3	1.0×10^{9}	43.5	3.8×10^{8}	24.9	7.0×10^{8}	38.6
Sample Bottle (4 oz)	3.9	0.81	0.1 ml of #6	5.9×10^{5}	11.6	1.8×10^{8}	15.7	2.5×10^{6}	14.5	9.0×10^{7}	20.0	3.8×10^{7}	17.0	1.0×10^{10}	24.5	3.0×10^{5}	7.3	sterile	6.7								
Sample Bottle (4 oz)	3.9	2.51	0.1 ml of #6	7.0×10^{6}	14.0	5.0×10^{8}	24.5	5.5×10^{6}	5.8	4.0×10^{8}	31.5	5.0×10^{8}	13.7	1.5×10^{10}	33.7	sterile	6.6	1.0×10^{9}	40.8	sterile	5.7	5.0×10^{9}	33.8	5.0×10^{5}	12.7	3.2×10^{8}	27.7
Test Tube	3.9	0.12	0.1 ml of #6	1.0×10^{4}	2.2			5.0×10^{5}	3.5			6.0×10^{5}	2.7			sterile	3.0										
Sample Bottle (4 oz)	3.9	0.81	Uninoculated Control		3.0				2.8				3.3				3.6				2.7				3.8		

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