A STUDY OF THE GROWTH REQUIREMENTS OF THE GENUS BRUCELLA

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE

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1944

THESIS

A STUDY OF THE GROWTH REQUIREMENTS OF THE GENUS *BRUCELLA*

by

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A THESIS

Submitted to the Graduate School of Michigan State College of Agriculture and Applied Science in partial fulfilment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

April, 1944

THESIS

This study was made possible by a grant from the Difco Laboratories, Detroit, Michigan.

TABLE OF CONTENTS

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Introduction

The problem of determining the necessary growth requirements for bacteria is a complex one. In their evolution bacteria may become adapted to an environment which differs widely from the original. Not only are there differences in requirements between families, but also among members of the same genera. There is also the possible occurrence of "adaptation" of a particular micro-organism; it may acquire the ability to gradually overcome an unfavorable environment when grown in a certain medium and still perpetuate itself.

Knowledge of the nutritional requirements of bacteria serves many purposes. Among these are, (1) the perfection of a suitable culture medium, (2) a better means of studying metabolic activity, (3) the determination of differences in various species, (4) a means of determining the factors that promote dissociation, (5) a method for obtaining soluble cell constituents for biochemical studies.

This study was directed toward finding the exact nutritional requirements of the species of Brucella and the development of a highly satisfactory synthetic medium. A medium that fulfills this requirement should possess the following properties: (1) promote rapid growth from a small inoculum of bacterial cells, (2) permit multiplication of cells and a slow death rate over a maximum period of time, (3) freedom from factors that promote dissociation, (4) cause no reduction in the virulence or other change in the characteristics of the organism.

Although the literature contains several reports of studies pertaining to the food requirements of Brucella, none of them have been conducted with sufficient exactness to warrant final conclusions as to what the requirements are.

Review of Literature

The early attempts to grow Brucella organisms in synthetic media indicate that the media then used were deficient in many respects, because maximum growth, equaling that in a peptone medium, was never obtained. Zobell and Myer (1) reported that they were unable to find a liquid synthetic medium in which Brucella organisms would produce an appreciable turbidity. However, they found that the carbon or energy requirements of Brucella were best met with lactates, citrates and cystine; the nitrogen demands were fulfilled with 0.2 per cent cystine and 0.5 per cent asparagine; the optimum phosphorus concentration was that supplied by phosphates at 0.02 to 0.15 per cent. Sulphur was best furnished by cystine at 0.02 per cent. The mineral requirements were completed by the addition of sodium, potassium, iron and magnesium ions.

Zobell and Myer (2) in another report on the physico-chemical requirements of Brucella in synthetic media stated: "the optimum osmotic pressure for Brucella is slightly lower than an isotonic solution; by depressing the surface tension of the medium to 50 dynes with sodium taurocholate multiplication is expedited, the maximum multiplication occurs at a pH between 6.6 and 7.4, and cultures of Brucella grow best at a slightly reduced oxygen tension which may be accomplished by the addition of 0.2-0.3 per cent washed agar, retarding the diffusion of gases and giving a medium of semi-solid consistency."

The composition of the synthetic medium which evolved from their work follows:

Sodium ammonium hydrogen phosphate ----------2.0 g.

Cystine ----------0.2 g.

Potassium acid phosphate ----------1.0 g.

Sodium chloride ----------2.0 g.

Magnesium sulphate ----------0.1 g.

Asparagine ----------3.0 g.

Glycerol ----------20.0 ml.

Ammonium lactate ----------3.0 ml.

Water to make ----------1,000.0 ml.

After the fifth transfer in this medium growth began to improve, indicating an adaptation to the medium. Multiplication was slow but the death rate was less than in a peptone medium. An inoculum of less than 10,000 viable Brucella cells per cubic centimeter of medium failed to grow.

Kerby (3) found that the addition of 30 mg. of nicotinic acid and 25 mg. of thiamin hydrochloride to each liter of Bact-Tryptose Agar resulted in a marked increase in the growth of Brucella abortus. This was indicated by an increase in the initial rate of growth and size of the colonies.

The first detailed report on the accessory growth factors required by Brucella was made by Koser, Breslove and Dorfman (4). They employed a basal synthetic medium of amino-acids, glucose and inorganic salts. The following substances were studied for their growth promoting effect on Brucella: nicotinamide, Coenzyme 1., thiamin hydrochloride, diphosphothiamin, beta-alanine, calcium pantothenate, vitamin B_6 hydrochloride, riboflavin, inositol, glutamine, adenine, sodium pyrophosphate and biotin. The amounts used were 0.2 to 0.5 micrograms

per ml. of basal medium. From 8,000 to 20,000 bacterial cells were added to each ml. of basal medium. Their results indicated that the significant accessory growth factors involved in the growth of the three species of Brucella were thiamin, pantothenic acid (calcium salt), nicotinamide and probably biotin. They also found the optimum concentration of sodium chloride to be 0.6 to 1.0 per cent.

The synthetic basal medium Koser and associates (4) used in the above work consisted of the following:

Basal Synthetic Medium No. 4	per liter
Glycine	0.2 g.
dl-alpha-alanine	0.5 g.
dl-valine	0.1 g.
dl-leucine	0.1 g.
(+) l-lysine dihydrochloride	0.1 g.
(+) l-arginine hydrochloride	0.1 g.
dl-serine	0.015 g.
dl-threonine	0.015 g.
(+) d-glutamic acid	0.5 g.
(-) l-cystine	0.15 g.
dl-methionine	0.1 g.
(-) l-histidine hydrochloride	0.2 g.
(-) l-tyrosine	0.05 g.
dl-phenylalanine	0.1 g.
(-) l-proline	0.1 g.
(-) l-hydroxyproline	0.1 g.
(-) l-tryptophane	0.2 g.
K_2HPO_4	1.0 g.

$MgSO_4$ ----------0.1 g.

NaCl ----------6.0 g.

Glucose ----------3.0 g.

The pH of the medium was brought between 6.8 and 7.0 with N/1 NaOH. It was sterilized by autoclaving at 15 pounds pressure for 15 minutes.

Plate counts made after three days incubation at 37°C. yielded 600 million to 800 million bacteria per ml. when the basal medium contained the four accessory growth factors.

In a continuation of the studies, Koser and Knight (5) reported on the effect of biotin as a growth promoting factor for Brucella. The medium they used consisted of the following:

Basal Synthetic Medium No. 4 ----------one liter.

Nicotinamide (0.2 microgram/ml.) ----------0.2 mg./liter.

Thiamin (0.2 microgram/ml.) ----------0.2 mg./liter.

Calcium pantothenate (0.2 microgram/ml.) ---0.2 mg./liter.

Thioglycolic acid (50-100 microgram/ml.) ---50-100 mg./liter.

They compared the effect of varying the concentration of pure biotin, biotin concentrate and biotin methyl ester. Either of the three biotin preparations was effective in promoting growth of three strains of Br. abortus. A concentration of 0.0001 microgram of biotin per ml. gave 90 per cent maximum growth. They also found that the pyrimidine, but not the thiazole component of thiamin was required by seven Brucella cultures.

McCullough and Dick (6) using the basal synthetic medium No. 4 and the growth factors employed by Koser and associates, investigated the differences in the requirements of the three species of Brucella. Also,

they determined the optimum concentration of each substance required for growth. They found that all three species of Brucella required thiamin for growth, that Br. abortus also required biotin, and that Br. suis and Br. melitensis also required nicotinic acid. It was found that while Br. suis did not require calcium pantothenate for growth, the presence of this substance initiated earlier growth. The amount of growth was estimated by visual examination of the turbidity of the cultures.

McCullough and Dick (6) suggested that the amounts of the accessory growth factors necessary for growth of Brucella in the basal medium were:

Thiamin ----------0.2 microgram per ml.

Nicotinic acid ----------0.2 microgram per ml.

Calcium pantothenate -----0.04 microgram per ml.

Biotin ----------0.001 microgram per ml.

The addition of these substances failed to initiate growth when only 200 bacterial cells were added to each ml. of medium.

In a second paper, McCullough and Dick (7) analyzed the critical growth factor requirements of recently isolated strains of Br. abortus. The cultures that required carbon dioxide failed to grow in the complete medium even with increased carbon dioxide tension. After becoming adapted to grow aerobically, 30 of the 41 original strains grew in the basal medium plus the four accessory growth factors. None of the strains grew if either thiamin or biotin were absent. Of the 30 strains which grew, 23 had an absolute requirement for thiamin and biotin. However, 10 of these strains grew more quickly if nicotinic acid was present, and 11 strains grew more quickly if calcium pantothenate was present. The final growth, however, was no greater than in a medium without nicotinic acid and calcium pantothenate. It was noted that when the

inoculum was less than 25,000 cells per ml. of medium, growth did not occur.

McCullough and Dick (6) and (7) based the quantitative measurement of growth on the turbidity of the culture medium as determined by visual examination. No plate counts were made to determine the number of viable cells present at the time measurements were taken. The viable cell count must be taken into consideration in the development of an ideal medium for any bacterium. For, while the turbidity of the medium is a comparative index of growth it does not give any information as to the proportion of live to dead bacteria during the growth phase.

Materials and Methods

A. Preparation of Media.

The basal material used in this study was Casamino acids, a commercial product prepared by DIFCO Laboratories. It consists of the amino-acids resulting from the strong acid hydrolysis of commercial casein. The amino-acid analysis of Casamino acids as submitted to us by the manufacturer is given below:

	Average percentage of several lots
Cystine	less than 0.1
Methionine	2.0
Arginine	2.1
Histidine	1.6
Lysine	4.4
Tyrosine	0.8
Tryptophane	absent
Phenylalanine	1.2
Threonine	2.7
Valine	5.0
Leucine	4.2
Isoleucine	2.2
Glycine	0.35
Serine	3.5
Proline	5.4
OH-proline	none found
Alanine	2.5
Glutamic acid	5.5
Aspartic acid	2.3

The glassware in which the media was dispensed was cleaned in acid-chromate cleaning solution, washed several times with distilled water, and then placed in the autoclave in distilled water and autoclaved for one-half hour at fifteen pounds pressure. This last step was done to assure complete removal of foreign material from the glass surfaces. It was found in preliminary trials that growth of the bacteria was not uniform in different tubes of the same medium if this step was omitted. After the cleaning process, the glassware was dried in a gas oven at 65°C., then plugged with absorbent cotton and sterilized by dry heat.

The preparation of the media was accomplished by making a solution of Casamino acids in distilled water. The other ingredients were added subsequently and all mixed thoroughly. Throughout most of the study 3/4 by 6 inch test tubes (pyrex) were used. Fifteen milliliters of media was placed in each tube.

Sterilization was accomplished by autoclaving for ten minutes at twelve pounds pressure.

B. Inoculation of the Media.

The strains of Brucella used were representative cultures which had been maintained on liver infusion agar. They were periodically checked for dissociation and only smooth cultures were employed in the preparation of the inoculum. The major part of the study was done with Brucella abortus No. 1257 (aerobic); Brucella suis No. 1722 (aerobic); and Brucella melitensis No. 2469 (aerobic). These were stock cultures maintained in the collection at the Central Brucella Laboratory.

In order that the data obtained throughout the investigation might be comparable, an attempt was made to inoculate the same number of organisms into constant volumes of media. Each tube of medium (15 ml.) was inoculated with approximately 100,000 viable organisms. This was done by first making a suspension of Brucella cells in sterile distilled water. Enough cells were added to produce a turbidity of 28 as measured by the Photronreflectometer described by Libby (8), when the initial setting of the galvanometer was at fifteen. Before the turbidity was measured in the reflectometer the suspension was thoroughly shaken on a shaking machine to achieve complete dispersion of the cells. Forty-eight hour liver agar slants of a smooth culture of Brucella were used as the source of organisms.

When the suspension was adjusted to a turbidity of 28 by the reflectometer method, each ml. contained approximately 15×10^8 organisms. Hence one-half a ml. of a 1-5000 dilution of the original suspension contained approximately 15×10^4.

In making the dilution to obtain the inoculum, one ml. of the original suspension (turbidity-28) was placed in 99 milliliters of sterile distilled water and thoroughly shaken. Then one ml. of this solution (1-100) was added to 49 ml. of sterile distilled water, resulting in a 1-5000 final dilution. One half of a ml. of this suspension was used to inoculate each tube of medium. After the inoculum had been added, the tubes were rotated by hand to obtain complete mixing.

The inoculated media were incubated at 37°C.

C. Methods of Measuring Growth of Cultures.

1. Turbidity Evaluation: Using the Photronreflectometer as described by Libby (8) the turbidity of each set of inoculated liquid medium was determined on the 3rd, 7th, and 15th day following the date of inoculation. The scale of the reflectometer was first set at 50 and then the culture medium, after thorough shaking, was placed in the glass cell and the turbidity value read directly from the galvanometer scale. The final turbidity value was that of an uninoculated tube subtracted from that of the inoculated tube.

2. Plate Count Method: Using the dilution method, plate counts were made to give an accurate index of the number of viable organisms contained in the medium at 3, 7, and 15 days. The diluting fluid was composed of 0.05 per cent Bacto-Tryptose and 0.5 per cent sodium chloride in distilled water. Bottles containing 100 ml. of diluting fluid were prepared and sterilized by autoclaving. Using 3 of these bottles and transfering one ml. each time, a 1:1,000,000,000 dilution of the culture was made. One ml. of the final dilution was placed in a sterile petri dish and 30 ml. of melted Bacto-Tryptose agar added. After 4 days incubation the colonies were counted. Counts were made in this manner in duplicate.

Data and Discussion

The initial part of the study on the growth requirements of Brucella consisted of adding each of the available known bacterial accessory growth factors in turn to the basal ingredient, Casamino acids. In addition to daily observation of the cultures, quantitative turbidimetric readings were made on the 3rd, 7th, and 15th day after inoculation. Bacto-Tryptose broth was used throughout the study as the control medium. The Brucella organism grew readily in it. Comparison of growth in the control medium afforded a means of evaluating the growth promoting property of the experimental synthetic media.

Preliminary Growth Factor Tests

Although the accessory growth factor requirements for the genus Brucella had been determined for several strains by Koser and his associates (4, 5) and that knowledge had been expanded by McCullough and Dick (6, 7) , it was thought advisable to make some preliminary studies with Casamino acids as the basal medium. As Casamino acids was a recently developed commercial product, its use in a bacterial medium has not been extensively investigated. The work of Koser and Wright (9) had shown that the growth response of bacteria to vitamins depends considerably upon the basal material used.

In the early trials it was found that in 3 days no growth occurred in a medium consisting of Casamino acids, sodium chloride, and Na_2HPO_4. The initial pH of the medium was 6.8, which is considered optimum for the growth of Brucella (2). After 7 days incubation a very slight turbidity developed, indicating definite but greatly retarded growth.

The next step was the addition in turn, of each of the available accessory growth factors to the basal medium. The resulting growth in each case was slight. In fact no greater than in the Casamino acids and salt alone. The accessory growth factors used in these trials were nicotinic acid, nicotinamide, calcium pantothenate, thiamin hydrochloride, riboflavin, biotin, para-amino benzoic acid, ascorbic acid, inositol, pimelic acid, pyridoxine hydrochloride, menadione, methionine, glutamic acid, cystine and choline. Growth in the Tryptose control medium was rapid and reached a maximum in about 5 days.

According to Mueller and Johnson (10), the tryptophane content of casein is destroyed by the strong acid hydrolysis used in the preparation of the hydrolysate. Hence, it was expedient to make a supplementary addition of tryptophane and to repeat the experimental trials. With this addition it was seen that growth was appreciably improved. Greatest growth acceleration occurred when both tryptophane and nicotinic acid were added.

The fat-soluble vitamins A and E were also added to the basal medium of Casamino acids. Glycerin was used as a vehicle to incorporate these vitamins into the medium. The resulting turbidity in 3 days was equal to that in the control Tryptose broth. However, the addition of glycerin alone was followed by an equally stimulating effect. In his work on dissociation, Henry (11) used a 2 per cent glycerin-dextrose broth for growing bovine and porcine strains of Brucella.

The preliminary studies indicated that when using Casamino acids as a basal material, the growth of Br. abortus was enhanced by the addition of tryptophane, nicotinic acid, and glycerin, as is shown in Table I.

Table 1. Accessory growth factors for Br. abortus

Constituents per 100 ml. of medium						Turbidity		
Casamino acids	NaCl	Na_2HPO_4	Tryptophane	Nicotinic acid	Glycerin	3-days	7-days	15-days
1.0 g.	0.6 g.	0.27 g.				0	7	11
1.0 g.	0.6 g.	0.27 g.	1.0 mg.			5.5	26	32
1.0 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.		6.0	25	33
1.0 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.	0.5 ml.	12	29	43
Control-Tryptose broth.						13	27	43

Optimum Quantities of Growth Stimulating Factors

To determine the most effective amount of each of the above ingredients, a series of tubes was set up containing constant amounts of two materials and varying concentrations of the other one. This was done with tryptophane, nicotinic acid and glycerin. It was observed that tryptophane in a concentration of one mg. per 100 ml. of medium gave maximum growth. Glycerin produced as much growth at a concentration of 0.5 ml. per 100 ml. of medium as did larger amounts. Nicontinic acid in quantities of 0.2 mg. per 100 ml. of medium was the most effective concentration. The protocols for these observations are shown in Table 2.

Optimum Quantity of Casamino Acids

Most synthetic media used by previous workers contained from one to 1.5 per cent casein hydrolysate. Hence, it was considered most likely that the effective concentration of Casamino acids would also be within that range. By using Casamino acids in concentrations of one to 2.5 per cent, it was found that a one per cent solution of this material when combined with glycerin, nicotinic acid and tryptophane gave optimum growth. Table 3 illustrates this finding.

The Most Suitable Type of Casamino Acids

The composition of a casein hydrolysate will vary slightly with the procedure employed in its manufacture. However, it can be standardized for making a uniform basal medium by adjusting its nitrogen content.

Three different lots of Casamino acids were obtained from the Difco Laboratories with their analyses. A medium made from each Lot was

Table 2. Optimum quantities of accessory factors for Br. abortus

Constituents per 100 ml. of medium						Turbidity		
Casamino acids	NaCl	Na_2HPO_4	Tryptophane	Nicotinic acid	Glycerin	3 days	7 days	15 days
a.								
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.05 mg.	0.5 ml.	6	16	16
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.10 mg.	0.5 ml.	5	13	11
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.20 mg.	0.5 ml.	5	10	11
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.30 mg.	0.5 ml.	5	14	12
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.40 mg.	0.5 ml.	3	16	14
1.0 g.	0.6 g.	0.23 g.	0.25 mg.	0.50 mg.	0.5 ml.	2	14	21
b.								
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.05 mg.	0.5 ml.	9	19	23
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.10 mg.	0.5 ml.	8	19	24
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.20 mg.	0.5 ml.	7	19	22
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.30 mg.	0.5 ml.	8	19	22
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.40 mg.	0.5 ml.	9	22	24
1.0 g.	0.6 g.	0.23 g.	0.50 mg.	0.50 mg.	0.5 ml.	8	19	22
c.								
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.05 mg.	0.5 ml.	15	27	33
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.10 mg.	0.5 ml.	14	27	35
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.20 mg.	0.5 ml.	14	30	31
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.30 mg.	0.5 ml.	14	29	31
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.40 mg.	0.5 ml.	14	26	28
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.50 mg.	0.5 ml.	14	26	30
d.								
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.05 mg.	0.5 ml.	9	23	22
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.10 mg.	0.5 ml.	14	26	31
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.20 mg.	0.5 ml.	9	23	25
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.30 mg.	0.5 ml.	14	27	28
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.40 mg.	0.5 ml.	14	23	26
1.0 g.	0.6 g.	0.23 g.	2.0 mg.	0.50 mg.	0.5 ml.	15	29	35
e.								
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.05 mg.	0.5 ml.	15	28	31
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.10 mg.	0.5 ml.	14	28	32
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.20 mg.	0.5 ml.	15	28	27
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.30 mg.	0.5 ml.	14	24	24
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.40 mg.	0.5 ml.	15	25	27
1.0 g.	0.6 g.	0.23 g.	3.0 mg.	0.50 mg.	0.5 ml.	15	27	27
f.								
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.05 mg.	0.5 ml.	10	21	21
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.10 mg.	0.5 ml.	11	21	19
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.20 mg.	0.5 ml.	10	20	20
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.30 mg.	0.5 ml.	10	20	20
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.40 mg.	0.5 ml.	10	22	22
1.0 g.	0.6 g.	0.23 g.	4.0 mg.	0.50 mg.	0.5 ml.	8	19	20
g.								
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	0.05 ml.	12	25	29
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	0.10 ml.	12	24	31
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	0.30 ml.	13	29	36
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	0.50 ml.	12	29	43
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	1.00 ml.	12	30	42
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	2.00 ml.	13	29	41
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	3.00 ml.	12	29	36
1.0 g.	0.6 g.	0.23 g.	1.0 mg.	0.2 mg.	4.00 ml.	13	28	40
Control-Tryptose broth						13	27	43

Note: a. through f. show the effect of varying the concentration of nicotinic acid and tryptophane. Group g. shows the effect of varying the amount of glycerin with optimum amounts of the other two materials.

Table 3. Optimum quantity of Casamino acids for growth of Br. abortus.

Constituents per 100 ml. of medium						Turbidity		
Casamino acids	NaCl	Na_2HPO_4	Tryptophane	Nicotinic acid	Glycerin	3-days	7-days	15 days
1.0 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.	0.5 ml.	12	22	40
1.5 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.	0.5 ml.	11	25	35
2.0 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.	0.5 ml.	10	21	28
2.5 g.	0.6 g.	0.27 g.	1.0 mg.	0.2 mg.	0.5 ml.	9	21	28
Control-Tryptose broth						10	19	38

Table 4. The most suitable type of Casamino acids for the growth of Brucella.

	Constituents per 100 ml. of medium						Turbidity		
	Casamino acids	NaCl	Na_2HPO_4	Tryptophane	Nicotinic acid	Glycerin	3 days	7 days	15 days
Br. abortus	(42521) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	15	26	33
	(43219) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	14	28	42
	(44220) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	11.5	25	32
	Control-Tryptose broth						9	21.5	32
Br. suis	(42521) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	14	30	29
	(43219) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	12	16	20
	(44220) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	12	27	28 (15)
	Control-Tryptose broth						12	28	22
Br. melitensis	(42521) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	12	28	29
	(43219) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	10	9	9
	(44220) 1.0 g.	0.6 g.	0.2 g.	1.0 mg.	0.2 mg.	0.5 ml.	8	19	24
	Control-Tryptose broth						11	27	36

Note: When (15) is placed next to the turbidity reading the reflectometer was first adjusted to a galvanometer reading of fifteen before the tube of suspension was added. Thus, indicating a higher turbidity then when the machine was set initially at fifty.

Table 5. Growth of Br. abortus from small inocula.

Number of cells inoculated per tube	Medium	Turbidity		
		3-days	7-days	15-days
10	Casamino acids	0	18	39
1000	Casamino acids	0	21	40
100,000	Casamino acids	14	26	41
10	Tryptose broth	0	25	20 (15)
1000	Tryptose broth	5	26	20 (15)
100,000	Tryptose broth	12	27	20 (15)

Note: The fifteenth day turbidity reading on the Tryptose tubes was done with an initial setting on the reflectometer of (15) as the turbidity was so great that the reading would have been off the scale if the machine was initially set at fifty (50).

Table 6. Utilization of glucose by *Brucella*.

Strain	Milliliter of medium per bottle	Glucose mg. per 100 ml.	Available glucose used	Turbidity
1257 (*Br. abortus*)	30	282	1 per cent	18
Control (Not inoculated)	30	285	------	0
1722 (*Br. suis*)	30	275	8.3 per cent	22
Control (Not inoculated)	30	300	------	0

Table 7. Growth response to additional amino acids.

Medium	Species	Turbidity			Million bacteria per ml.		
		3-days	7-days	15-days	3-days	7-days	15-days
Casamino acids medium No. 1 plus- Lysine-0.02 g. per 100 ml.	abortus	19	26	44	324	570	260
	suis	23	32	47	423	390	305
	melitensis	20	35	49	503	730	542
Casamino acids medium No. 1 plus- Leucine-0.01 g. per 100 ml.	abortus	17	26	45	337	360	355
	suis	24	35	51	502	400	116
	melitensis	20	33	53	540	580	661
Casamino acids medium No. 1 plus- Phenylalanine-0.01g. per 100 ml.	abortus	19	26	43	351	350	225
	suis	23	34	42	376	360	98
	melitensis	21	34	46	396	50[0]	429
Casamino acids medium No. 1 plus- Glutamic acid-0.05g. per 100 ml.	abortus	19	23	32	355	300	1
	suis	20	26	30	354	350	1
	melitensis	18	24	31	424	660	1
Casamino acids medium No. 1 (Control medium)	abortus	16	25	34	295	470	2
	suis	18	31	33	368	100	1
	melitensis	18	29	34	481	730	11

Table 8. Effect of other vitamins.

Added vitamin	Species	Turbidity			Million bacteria per ml.		
		3-days	7-days	15-days	3-days	7-days	15-days
None	abortus	14	27	32	343	530	1
	suis	14	31	35	389	400	1
	melitensis	14	28	29	445	770	1
Biotin 0.03 gamma per 100 ml.	abortus	14	29	40	369	550	120
	suis	16	40	50	412	750	86
	melitensis	14	34	40	426	920	296
Calcium pantothenate 0.02 mg. per 100 ml.	abortus	13	27	32	326	460	1
	suis	16	33	44	405	670	35
	melitensis	15	29	33	427	720	147
Thiamin hydrochloride 0.02 mg. per 100 ml.	abortus	18	39 (15)	16 (15)	319	350	198
	suis	22	22 (15)	30 (15)	313	390	318
	melitensis	19	22	26	307	310	42
Biotin 0.03 gamma per 100 ml. Ca-pantothenate 0.02 g. per 100 ml. Thiamin HCl 0.02 g. per 100 ml.	abortus	19	40 (15)	16 (15)	294	350	227
	suis	27	23 (15)	29 (15)	320	460	278
	melitensis	25	30	42	410	450	87
Tryptose broth control medium	abortus	18	27	44 (15)	294	310	7
	suis	17	37	19	284	250	42
	melitensis	18	39	42	274	150	1

Note: When the turbidity reading has (15) above it, the turbidity of the culture was so great that the reflectometer had to be set from fifty down to fifteen in order to obtain a reading on the scale.

Table 9. Acceleration of growth by agitation.

Amino acid added per 100 ml. medium	Vitamin added per 100 ml. medium	Species	Turbidity (reflectometer set at 15) 3-days	7-days	Million bacteria per ml. 3-days	7-days
Leucine 0.01 g.	Thiamin 0.02 mg.	abortus	25	32	2,660	1,440
"	"	suis	28	45	4,070	4,500
"	"	melitensis	29	40	2,660	1,850
Leucine 0.01 g.	Ca-pantothenate 0.02 mg.	abortus	15	16	1,810	10
"	"	suis	15	15	1,170	20
"	"	melitensis	20	20	2,400	140
Leucine 0.01 g.	Biotin 0.05 gamma	abortus	15	17	1,730	20
"	"	suis	14	15	1,440	40
"	"	melitensis	19	15	2,030	180
Lysine 0.02 g.	Thiamin 0.02 mg.	abortus	26	33	2,610	1,610
"	"	suis	40	45	4,950	3,100
"	"	melitensis	28	40	2,920	1,940
Lysine 0.02 g.	Ca-pantothenate 0.02mg.	abortus	11	11	1,330	10
"	"	suis	8	9	1,130	30
"	"	melitensis	12	14	1,890	150
Lysine 0.02 g.	Biotin 0.05 gamma	abortus	15	14	1,980	10
"	"	suis	9	7	1,350	70
"	"	melitensis	14	12	1,780	150
Leucine 0.01 g. Lysine 0.02 g.	Thiamin 0.02 mg.	abortus	17	30	1,840	1,320
"	"	suis	35	46	5,970	4,230
"	"	melitensis	30	46	2,740	3,420
Leucine 0.01 g. Lysine 0.02 g.	Ca-pantothenate 0.02mg.	abortus	15	20	1,850	10
"	"	suis	23	25	2,690	260
"	"	melitensis	22	27	2,590	250
Leucine 0.01 g. Lysine 0.02 g.	Biotin 0.05 gamma	abortus	17	20	1,780	10
"	"	suis	19	22	2,510	190
"	"	melitensis	22	19	2,410	120

Table 10. Growth of Brucella at room temperature.

Species	Turbidity days							Growth millions per ml. of medium days						
	3	7	11	15	19	23	30	3	7	11	15	19	23	30
abortus	(50) 0	(50) 35	18	22	48	54	54	9	1440	1100	1240	1220	1190	340
suis	(50) 3	20	28	31	36*	39*	45*	80	1390	1730	2200	2630	3200	2890
melitensis	3	50	24	31	33*	35*	36*	104	1640	1750	2650	2600	1690	1770

Note: Unless labeled (50), the reflectometer was initially set at a scale of fifteen (15).

* On the ninteenth, twenty-third and thirtieth days the 1722 and 2469 cultures were diluted equal parts before the turbidity measurement was made. Hence, the actual value was twice that shown on the chart.

Table 11 The influence of several agents on the growth of the species of Brucella

Species Brucella	Constituents per 100 ml. medium							Turbidity (a) per cent light ext.			Colony count million per ml.		
								Days incubation					
	Casamino ac. gr.	Glycerin ml.	Leucine mg.	Tryptophane mg.	Thiamin mg.	Nicotinic ac. mg.	NaCl gr.	3	7	15	3	7	15
abortus	0	0.5	0.01	1	0.02	0.2	0.6	0	0	0	0	0	0
	1	"	"	"	"	"	"	67	73	78	2380	1700	32
	"	"	"	"	"	0	"	62	65	63	2680	860	32
	"	"	"	"	0	0.2	"	51	52	58	1320	1	2
	"	"	"	0	0.02	"	"	52	61	63	1530	90	25
	"	"	0	1	"	"	"	66	69	72	2620	1460	1
	"	0	0.01	"	"	"	"	67	69	86	2620	1890	0
suis	0	0.5	0.01	1	0.02	0.2	0.6	0	0	0	0	0	0
	1	"	"	"	"	"	"	70	82	90	3270	4220	1300
	"	"	"	"	"	0	"	66	68	72	1610	1900	10
	"	"	"	"	0	0.2	"	39	39	42	190	20	2
	"	"	"	0	0.02	"	"	74	84	88	3870	4080	1070
	"	"	0	1	"	"	"	64	77	84	3390	4600	310
	"	0	0.01	"	"	"	"	75	85	89	3970	4700	240
melitensis	0	0.5	0.01	1	0.02	0.2	0.6	0	0	0	0	0	0
	1	"	"	"	"	"	"	61	77	81	2160	2440	20
	1	"	"	"	"	0	"	60	71	74	1870	2390	10
	1	"	"	"	0	0.2	"	42	49	45	1530	40	2
	1	"	"	0	0.02	"	"	64	78	81	2300	2270	10
	1	"	0	1	"	"	"	52	78	79	1810	200	2
	1	0	0.01	"	"	"	"	62	73	76	2210	2430	10

(a) Light extinction measured by Cenco-Shear photelometer.
Cenco filter #2 (green)
Medium adjusted to pH 6.8 with K_2HPO_4
Sterilization-autoclave 115°C for 12 min.

Table 12 Showing daily colony counts of the species of Brucella during growth in two liquid media.

Days incubation		Br. abortus		Br. suis		Br. melitensis	
		Medium					
		Synthetic	Tryptose	Synthetic	Tryptose	Synthetic	Tryptose
		Colony count, millions per ml.					
1	Shaken	1.6	7.8	5	24	1	3.6
	Not Shaken	2.8	24	8	81	2.5	15
2	Shaken	455	1010	2150	381	951	1920
	Not Shaken	330	255	247	259	260	273
3	Shaken	4900	3720	5440	5440	4140	4350
	Not Shaken	403	350	338	299	450	360
4	Shaken	3550	5000	7200	8120	7210	4360
	Not Shaken	360	402	518	336	510	498
5	Shaken	2630	3270	5860	7240	1890	5940
	Not Shaken	334	324	524	227	626	390
6	Shaken	1470	3960	5860	3900	770	2050
	Not Shaken	450	352	360	324	780	560
7	Shaken	830	1780	4810	3080	248	470
	Not Shaken	280	250	430	250	481	312
8	Shaken	548	710	4310	1590	19	229
	Not Shaken	312	225	625	238	525	294
9	Shaken	220	192	2750	870	4	10
	Not Shaken	300	164	586	219	424	262
10	Shaken	35	275	1500	1860	2	1
	Not Shaken	234	108	112	287	450	87
11	Shaken	35	192	343	468	1	1
	Not Shaken	187	125	565	150	386	0
12	Shaken	24	119	188	572	1	0
	Not Shaken	180	62	560	125	412	75

Table 15. Showing daily colony counts of the species of *Brucella* during growth in two liquid media.

Table 13. Showing daily colony counts of the species of Brucella during growth in two liquid media.

Days incubation		Br. abortus		Br. suis		Br. melitensis	
		Medium					
		Synthetic	Tryptose	Synthetic	Tryptose	Synthetic	Tryptose
		Colony count, millions per ml.					
1	Shaken	1.6	7.8	5	24	1	3.6
	Not Shaken	2.8	24	8	81	2.5	15
2	Shaken	455	1010	2150	381	951	1920
	Not Shaken	330	255	247	259	260	273
3	Shaken	4900	3720	5440	5440	4140	4350
	Not Shaken	403	350	338	299	450	360
4	Shaken	3550	5000	7200	8120	7210	4360
	Not Shaken	360	402	518	336	510	498
5	Shaken	2630	3270	5860	7240	1890	5940
	Not Shaken	334	324	524	227	626	390
6	Shaken	1470	3980	5860	3900	770	2050
	Not Shaken	450	352	360	324	780	560
7	Shaken	850	1780	4810	3080	248	470
	Not Shaken	280	250	430	250	481	312
8	Shaken	548	710	4310	1590	19	229
	Not Shaken	312	225	625	238	525	294
9	Shaken	220	192	2750	870	4	10
	Not Shaken	300	164	586	219	424	262
10	Shaken	35	275	1500	1860	2	1
	Not Shaken	234	108	112	287	450	87
11	Shaken	35	192	343	468	1	1
	Not Shaken	187	125	565	150	386	0
12	Shaken	24	119	188	572	1	9
	Not Shaken	180	62	560	125	412	75

used for each of the three species of Brucella. The composition of the three Lots is as follows:

Casamino acids (Regular)	Rx-42521		
	Moisture	-	3.12 per cent
	Ash	-	16.36 per cent
	Sodium chloride	-	13.1 per cent
	Nitrogen	-	10.6 per cent
	P as PO_4	-	1.64 per cent
	Copper	-	100 parts per million.
Casamino acids (Regular)	Rx-44220		
	Moisture	-	5.98 per cent
	Ash	-	18.40 per cent
	Sodium chloride	-	11.9 per cent
	Nitrogen	-	9.88 per cent
	P as PO_4	-	3.90 per cent
	Copper	-	18 parts per million.
Casamino acids (Technical)	Rx-43219		
	Moisture	-	5.04 per cent
	Ash	-	40.92 per cent
	Sodium chloride	-	36.70 per cent
	Nitrogen	-	6.94 per cent

The effect of each of these lots upon the growth of members of the genus Brucella is shown in Table 4. Casamino acids Rx-43219 was used in a 1.5 per cent solution because of its lower nitrogen content in relation with the regular grades. Also, less sodium chloride was added to the medium prepared from Rx-43219 as its original sodium chloride content was very high.

Growth in media made from the different lots of Casamino acids indicate that the Technical grade, Rx-43219 did not have the growth promoting property of the other two regular grades. Also, of the two regular grades, the one which was lower in its copper content, Rx-44220, gave no improvement in growth over the other regular grade, Rx-42521.

Growth of *Brucella* in Casamino Acids Medium From Small Inocula

Previous work was done with an inoculum of approximately $15x10^6$ organisms in 15 ml. of medium. If the medium devised was capable of promoting rapid and good growth from an inoculum of a smaller number of organisms, its value would be greatly increased. To test this a Casamino acids medium of the following composition was made:

	per 100 ml.
Casamino acids	- 1.0 gm.
Sodium chloride	- 0.6 gm.
Disodium hydrogen phosphate	- 0.2 gm.
Nicotinic acid	- 0.2 mg.
Tryptophane	- 1.0 mg.
Glycerin	- 0.5 ml.
Distilled water, to make	100.0 ml.

Tryptose broth was used as the control medium. The size of the inoculum was varied so that tubes of each medium received approximately $15x10^4$, $15x10^2$ cells and 15 cells per tube. Turbidity measurements were made to indicate the amount of bacterial growth. The results are recorded in Table 5. Growth occurred in both media from small inocula. Although the initial growth was less in tubes inoculated with fewer cells, the final growth, as indicated by the turbidity measurements, was equivalent after 15 days incubation. The final bacterial concentration in the Tryptose broth medium exceeded that obtained in the Casamino acids medium.

Glucose Utilization in Casamino Acids Medium

In their studies on *Brucella* metabolism, Zobell and Myer (1) found that there was very little difference in the dextrose utilization of the three species of *Brucella*. A glucose utilization test was devised by Plastridge (12) for the differentiation of the species of *Brucella*. To determine if glucose was utilized when added to the Casamino acids medium a similar test was made with *Br. abortus* and *Br. suis*.

It had already been observed that the growth of Brucella was less in the Casamino acids medium when 0.5 per cent glucose was added. A medium was prepared consisting of Casamino acids, salts, tryptophane, nicotinic acid, glycerin and glucose. Duplicate bottles of medium were prepared. One was inoculated and the other left as an uninoculated control. After two weeks incubation, glucose analyses were made on both bottles according to Benedicts method (13). The original concentration was not determined as a decrease was expected to occur upon autoclaving and during the two weeks incubation period.

The resulting glucose concentration after a 2 weeks period showed that in the case of Brucella abortus there was one per cent utilized and that Brucella suis used 8.3 per cent of the available glucose. The results are set forth in Table 6. However, the turbidity of the cultures were approximately 50 per cent less than that which had been obtained with the same medium minus glucose when inoculated with the same number of organisms.

Effect of Other Materials on Medium

On several occasions the turbidity in Tryptose broth had greatly exceeded that in the synthetic medium when the turbidity reading was made on the 15th day. This necessitated a lower initial setting of the reflectometer when the controls were tested in order to have the galvanometer reading within the scale limits. It was obvious that maximum growth was not occurring in the synthetic medium. Dilution plate counts made at the time of the turbidity measurements, indicated that there was no correlation between the total count of viable cells and the corresponding turbidity. Although the turbidity was greater at 15 days, the number of viable cells per ml. progressively decreased after the first few days. It was considered possible that the Brucella

organisms might be gradually developing anaerobic tendencies, or becoming adapted to the synthetic medium and not growing on Tryptose-agar when plate counts were made, or else they were dying off rapidly after the first several days of incubation. If the latter explanation is correct, the death rate would be greater than the growth rate. Hence, while the turbidity was gradually increasing, the total viable cell content was decreasing.

Upon incubation under anaerobic conditions in 10 per cent carbon dioxide atmosphere there was no increase in the growth as indicated by turbidity or plate count.

The use of the Casamino acids medium in solid form for making plate counts gave no increase in the total cell count over the usual Tryptose-agar plates. Hence, it appeared that failure to obtain maximum turbidity and constant cell counts over the 2 week period was caused by a high death rate of the bacteria in the synthetic medium.

Various materials which might have growth promoting qualities were added to the ingredients already in use.

In a medium of amino acids, salts and vitamins, Koser (4) had found that some strains of Brucella required the addition of thioglycollic acid before growth would occur in the synthetic medium. This material and other substances with reducing properties were added to the Casamino acids medium. Thioglycollic acid, sodium thiosulphate, thiourea and glutathione were used individually at various levels of concentration. In no instance was the growth of Brucella improved.

The amino acid content of Casamino acids afforded the bacteria an available supply of carbon to meet their energy requirement. However, in case there might be other organic compounds containing a

source of carbon more readily utilized, other materials were tried. Glucose had already been eliminated as having this quality. Zobell and Myer (1) found that the lactates and citrates supplied carbon most effectively. The ammonium form of these compounds were added to the Casamino acids medium. Neither ammonium citrate, ammonium lactate, nor ammonium malate produced any increased growth response.

The Influence of Additional Amino-Acids

The presence of one amino acid has a direct influence on the action of others in promoting or preventing bacterial growth. This was demonstrated by Gladstone (14). Using B. anthracis, Gladstone observed that "the toxic effect of one may be counteracted by one or more of others." At the time these studies were initiated, very little information was available on the quantitative amino-acid requirement of Brucella. Although the constituents of the casein hydrolysate had been determined, the optimum quantity of each amino-acid for Brucella was unknown.

An addition of several amino-acids, except tryptophane which was already in the medium, was made to the Casamino acids medium. Each of the species of Brucella was grown in each combination of the ingredients. The purpose was to see if the Casamino acids lacked any particular amino-acid necessary for the maximum growth of Brucella. The quantities used were those employed by Koser (4) in his synthetic medium.

The amino-acids used were glycine, dl-valine, dl-alpha-alanine, dl-leucine, d-lysine-HCl, d-arginine, dl-serine, dl-threonine, dl-glutamic acid, l-cystine, dl-methionine, l-histidine-di-HCl, l-tyrosine, dl-phenyl-alanine, l-proline, l-cystine and

l-hydroxy-proline. These were added singly to the following combination of ingredients:

(Casamino Acids Medium No. 1)

Casamino Acids	1.0 gm.
Sodium chloride	0.6 gm.
Disodium hydrogen phosphate	0.20 gm.
l-tryptophane	1.0 mg.
Nicotinic acid	0.2 mg.
Glycerin	0.5 ml.

There was no striking growth response in any of the cultures. However, there was a slight increase in turbidity on the 15th day in the tubes containing dl-leucine, d-lysine-HCl, d-glutamic acid and dl-phenyl-alanine. The greater turbidity was consistent in each strain under study, Br. abortus (1257), Br. suis (1722), and Br. melitensis (2469).

The next step in evaluating the efficiency of the amino-acid supplement was to repeat the experiment and to make plate counts along with turbidity readings. The results are shown in Table 7.

The addition of glutamic acid failed to produce an increase in the growth rate. The other three amino-acids did cause a greater growth response. This is most apparent after the first week of incubation. With phenyl-alanine, lysine, and leucine, the turbidity at 15 days was greater than in the control medium. Most significant was the number of viable organisms in each group at the termination of the incubation period. The control medium dropped sharply to a very few million living bacteria per ml. of medium. In the media containing the 3 stimulating amino-acids the plate counts indicated a high proportion of living bacteria.

All possible combinations of these 4 amino-acids were added to the Casamino acids medium No. 1. The group containing both

lysine and leucine held the viable cell count at a high peak more consistently than did any other combination. These 2 compounds alone, as seen in Table 7 were also quite efficient in maintaining viability over a 2 week period in addition to producing maximum turbidity.

Thiamin Hydrochloride As A Growth Stimulating Factor

The growth resulting from the addition of either leucine or lysine was better than any previously obtained. However, the turbidity in the Tryptose broth tubes occasionally exceeded that in the synthetic medium. A s the nutritive requirements were apparently being fulbilled, it seemed that some stimulative substance was still lacking. Other investigators (3, 4, 6) had found thiamin to be essential to the growth of Brucella in synthetic media. Bacterial growth in the Casamino acids medium was proceeding without the addition of this vitamin. It appeared possible that a trace of thiamin might be in the casein hydrolysate. Biotin and panothenic acid were also members of the vitamin B-complex which had been reported (5, 6) as accessory growth factors for Brucella. These vitamins were added to the Casamino acids medium No. 1 which was then inoculated with Brucella to see if any stimulation of growth would occur. The results are shown in Table 8.

The turbidity and plate count methods of recording bacterial growth offer an interesting contrast in their results. The turbidities of the cultures containing thiamin at the 7th and 15th day exceeded any which had been obtained previously in any medium. The turbidities resulting after adding biotin and calcium pantothenate were not much greater than those obtained without these materials .

With these 3 substances together, turbidities were no greater than in the medium containing thiamin. Judging from the turbidities alone, thiamin appeared to be the factor necessary for maximum growth of Brucella.

However, the plate counts, showing the number of viable cells in the media gave more pertinent information. The counts at 7 days were higher in the medium without thiamin than in that containing it. Although there were more living organisms at 15 days in the medium containing thiamin, the number of living bacteria was not at all proportional to the turbidity. In fact, the biotin medium had nearly as many living cells on the 15th day as did the thiamin medium. But in the cultures with biotin turbidities were far less than those containing thiamin. This would seem to indicate that the death rate in the medium with thiamin was very high and although growth was abundant the bacteria lived only a short time.

In the Casamino acids media without the vitamins there were very few viable cells present after 15 days incubation. The addition of biotin alone increased the final count for each of the 3 strains. Calcium pantothenate failed to maintain a high level of viable cells for strain 1257, (Br. abortus), but did increase the 15th day count for strain 1722 (Br. suis), and for strain 2469 (Br. melitensis). The combination of all 3 vitamins was no more satisfactory than thiamin alone in maintaining viability.

Acceleration of Growth by Agitation

Two years ago Favorite and Hammon (15) found that slow rotation of growing cultures of staphlococci produced a higher toxin titre on casein hydrolysate medium. A similar procedure was applied to the growth experiments of Brucella in Casamino acids medium.

The media were prepared so as to contain all possible combinations of lysine, leucine, thiamin, biotin and calcium pantothenate in addition to the Casamino acids medium No. 1. After inoculation the tubes were kept in continuous motion during the period of incubation. Measurements of turbidity and plate counts were made on the 3rd and 7th days. The results are set forth in Table 9.

In every series of agitated cultures, the growth in 3 days was far greater than previously observed without agitation. More than 10×10^6 living cells per ml. of medium were produced in each combination of the 2 amino-acids and the 3 vitamins under study. The turbidity was visible at 36 hours and increased rapidly thereafter.

The medium containing both leucine and lysine, when thiamin was present, produced a somewhat higher total viable cell count than did either alone with thiamin. However, the addition of leucine and thiamin, or of lysine and thiamin produced not only a very high turbidity and cell count but also succeeded in maintaining the elevated state of growth through the 7 day period.

The turbidities of the cultures containing biotin and calcium pantothenate with the amino-acids never were as great as in those with thiamin plus lysine or leucine. The number of living bacteria was also low. This is shown by the lowered plate colony count made at 7 days. Several of the turbidity readings of the biotin cultures were lower at 7 days than at 3 days. This would appear to indicate lysis of the bacteria.

The greatly increased growth of all 3 strains of Brucella was obviously due to the constant agitation plus the growth promoting effect of leucine, lysine and thiamin. It was not due to thiamin alone. For when thiamin was added to the medium and either leucine

or lysine omitted, even with agitation the viable cell count dropped sharply after 3 days incubation.

When each growth measurement was made, a loopful of the culture was streaked on a Tryptose-agar plate. The plate was incubated for 4 days. The appearance of colony variation was studied under low power magnification with reflected oblique light according to the method prescribed by Henry (16). The colonies of Brucella were typically smooth in all of the cultures. In no instance was dissociation seen.

Growth at Room Temperature

The optimum incubating temperature for Brucella is approximately 37°C according to Topley and Wilson (17). At this temperature, reproduction proceeds rapidly. After maximum growth is reached the death rate is proportional to the multiplication rate until growth begins to diminish. It was not known whether Brucella would grow measurably in liquid cultures at room temperature.

A medium was prepared which had the following composition:

Casamino Acids	1.0 gm.
Sodium chloride	0.6 gm.
Dipotassium hydrogen phosphate	0.14 gm.
Tryptophane	1.0 mg.
Nicotinic Acid	0.2 mg.
Glycerin	0.5 ml.
Leucine	0.01 gm.
Lysine	0.02 gm.
Thiamin	0.2 mg.
Calcium pantothenate	0.02 mg.
Biotin	0.05 gamma.
Distilled water to make	100.0 ml.

This medium was bottled in 20 ml. quantities in 50 ml. bottles. It was inoculated with Br. abortus (1257), Br. suis (1722), and Br. melitensis (2469). Each bottle received approximately 150,000 cells, or 7,500 cells per ml. of medium. They were then held at room

temperature for 30 days. The bottles were rotated by hand for 10 to 15 seconds several times daily. Growth recordings were made at regular intervals during the 30 day period. The average of the daily low and high temperatures for the period was 23°C. Table 10 shows the results of this experiment.

Between the 4th and 5th day an appreciable turbidity developed. After maximum growth was established, it proceeded on a constant level for almost 30 days. While the cell count of the Br. abortus strain was lower at 30 days, that of Br. suis and Br. melitensis had not started to decline. Growth at room temperature with occasional agitation was slower than at 37°C. However, growth was persistent and continuous. There was no dissociation. All 3 cultures remained smooth.

Influence of Casamino Acids Medium on Pathogenicity

A medium containing Casamino acids plus additional growth promoting substances was inoculated with Br. suis (1722). The composition of the medium was:

Casamino Acids	1.0 gm.
NaCl	0.6 gm.
K_2HPO_4	0.14 gm.
Leucine	0.01 gm.
Lysine	0.02 gm.
Tryptophane	1.0 mg.
Nicotinic acid	0.2 mg.
Thiamin	0.2 mg.
Glycerin	0.5 ml.
Distilled water to make	100 ml.

The culture was then incubated at 37.5°C., with continuous agitation for 6 days. A 1:1,000,000 dilution was made of a portion of the culture. Each ml. of this dilution contained approximately 4,000 living organisms. Two adult guinea pigs were inoculated intraperitoneally with one ml. of the diluted culture. Thirty days later they were both sacrificed and an autopsy performed. Brucella suis was isolated from typical lesions

throughout the body. Both of the animals showed extensive characteristic gross lesions of brucellosis, as described by Huddleson (12).

Absolute Essentials for Brucella

To determine if any of the ingredients now used in the synthetic medium could be omitted, studies were made omitting each material. The procedure followed heretofore had been one of accumulating essential materials that promoted a heavier growth of the organism than had been obtained with any previous combination. However, it might be possible that the presence of certain factors now used would eliminate the beneficial effect of others.

The media were prepared with all of the materials, and also a series omitting one ingredient in each set. The pH was adjusted to 6.8 with K_2HPO_4. It was sterilized at 115°C. for 12 minutes. The turbidity measurements were made with a Cenco-Shear photolometer, using a Cenco filter #2 (green), and are expressed as percentage of light extension.

The results are shown in Table 11. It is seen that glycerin is not necessary for growth. The omission of thiamin greatly retards the amount of growth and also results in a rapid death rate of the bacteria. The amino-acid, leucine is not needed for the growth of Br. abortus or Br. suis, but is required by Br. melitensis. Tryptophane is essential for Br. abortus, but is not required by Br. suis or Br. melitensis.

Daily Colony Counts Of Brucella In Casamino Acids and Tryptose Medium

Using the complete synthetic medium with the three species of Brucella, daily counts were made of the number of living organisms.

One set of tubes was incubated under continuous agitation and another identical set was incubated on the shelf and not shaken. Plate counts were made for 12 consecutive days after inoculation. Tryptose broth medium was examined in the same manner to compare the results in the Casamino acids broth.

As seen from the tabulated data in Table 12, more rapid multiplication took place in the unshaken tubes than in those that were shaken during the first 24 hours. The multiplication rate was very rapid up to 4 days in those tubes which were agitated. All species of Brucella began to decline in number of living organisms after 6 days. With Br. suis the decline is more rapid in the Tryptose medium.

Growth curves of each species are shown on the following pages in Figures 1, 2, and 3.

Summary and Conclusions

A. Br. abortus (1257) developed practically no turbidity in broth medium consisting of Casamino acids and necessary salts. The addition of tryptophane, nicotinic acid and glycerin enhanced the growth.

B. The optimum quantities of each of these three growth promoting substances per 100 ml. of medium were: tryptophane, one mg., nicotinic acid, 0.2 mg., and glycerin, 0.5 ml.

C. In combination with tryptophane, nicotinic acid and glycerin, Casamino acids equalling one per cent of the medium produced the best growth.

D. The technical grade of Casamino acids, which was high in sodium chloride, did not produce as much growth as did the regular grade. The growth promoting ability of Casamino acids was not increased by lowering its copper content.

E. When the Casamino acids medium was inoculated with a very small number of organisms abundant growth occurred.

F. The addition of glucose to the Casamino acids medium had a depressant effect on the growth of Brucella.

G. Various reducing substances did not facilitate a greater growth of the organisms. The addition of organic carbon-furnishing compounds were also without beneficial effect.

H. The addition of dl-leucine and d-lysine-HCl either singly or together, resulted in prolonging the viability of the organisms.

I. When thiamin hydrochloride was added to the Casamino acids medium it initiated very rapid multiplication of the bacteria. However, the death rate in these cultures was also high.

J. Casamino acids medium containing leucine, lysine and thiamin in addition to tryptophane, nicotinic acid and glycerin when subjected to constant agitation produced a very good growth of Brucella.

K. Growth of Brucella in Casamino Acids medium at room temperature, with occasional shaking, was good and was maintained over a 30 day period.

L. Br. suis (1722) remained pathogenic for guinea pigs after growth in Casamino Acids medium.

M. Brucella organisms grown in Casamino Acids medium developed no increased tendency toward dissociation.

N. As seen throughout this study, there is not necessarily any direct correlation between the turbidity of a culture and the number of living organisms it contains. For the maintenance of a high proportion of viable organisms, the growth requirements of the particular bacterium must be fully met. An ideal medium should contain those substances which will cause a continued multiplication without decline in the total number of living cells. Therefore, in determining the exact accessory growth factors the total number of viable cells after growth has been initiated is a more important criterion than comparison of turbidities.

O. Under the conditions employed in this study, a synthetic medium capable of originating and maintaining growth of strains, Br. abortus (1257), Br. suis (1722) and Br. melitensis (2469) was found to consist of the following ingredients:

Casamino Acids (Difco)	1.0 gm.
Sodium chloride	0.6 gm.
Dipotassium hydrogen phosphate	0.14 gm.
dl-leucine	0.01 gm.
d-lysine-HCl	0.02 gm.
Tryptophane	1.0 mg.
Nicotinic acid	0.2 mg.
Thiamin-HCl	0.2 mg.
Glycerin	0.5 ml.
Distilled water to make	100.0 ml.

P. Glycerin may be omitted without reducing the growth promoting qualities of the medium. Tryptophane is essential for Br. abortus and leucine is essential for Br. melitensis.

Q. Growth of all species of Brucella in Casamino Acids medium equaled that in Tryptose broth. The bacteria multiply very rapidly after the first day of incubation when the cultures are shaken. A decline in growth occurs after 6 days in both media.

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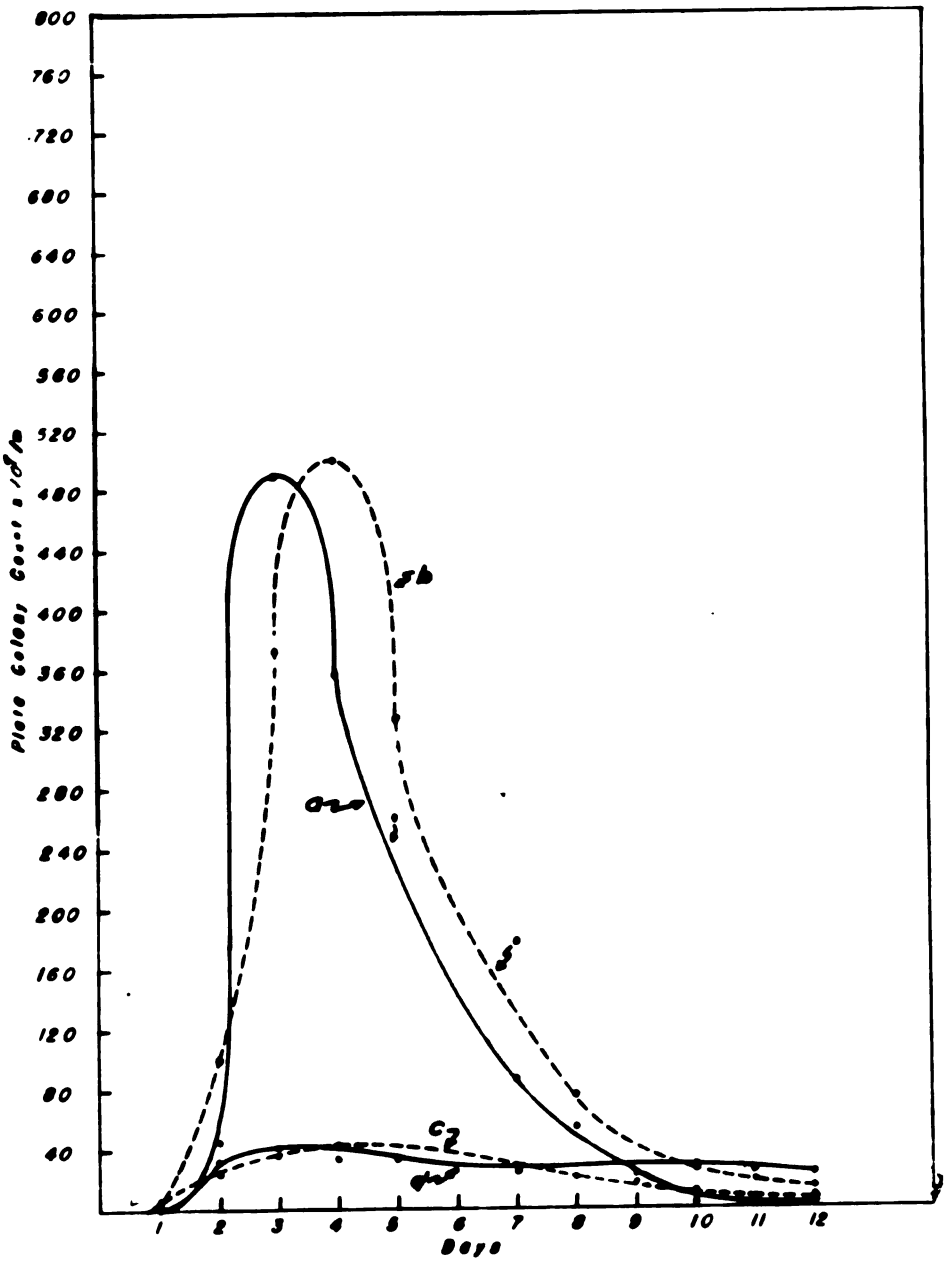

Fig. 1. Growth curves of Br. abortus.
—Synthetic medium. a;b—cultures incubated on shaker.
--Tryptose medium. c;d—cultures incubated on shelf.

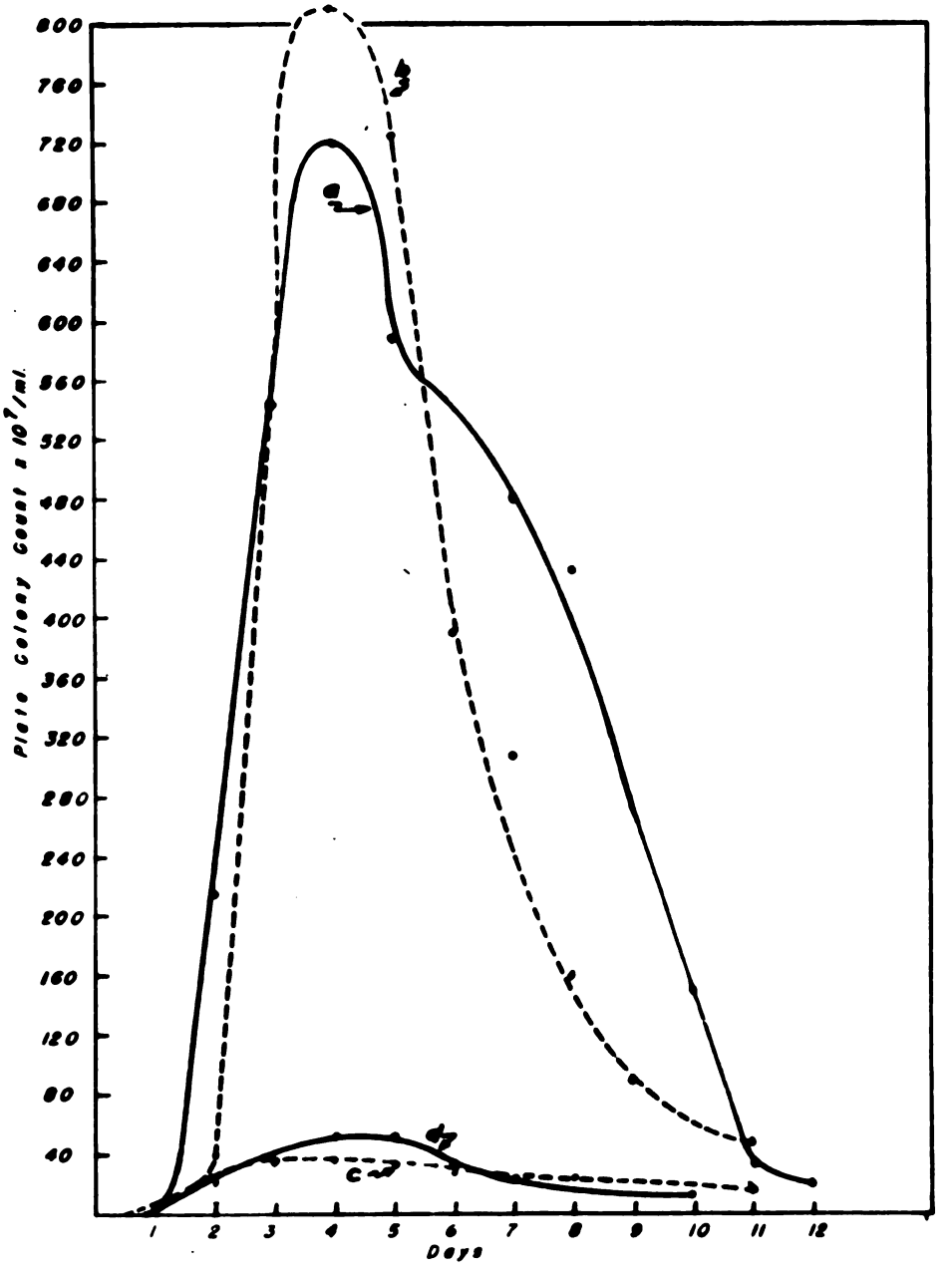

Fig. 2. Growth curves of Br. suis.
—Synthetic medium. a;b—cultures incubated on shaker.
--Tryptose medium. c;d—cultures incubated on shelf.

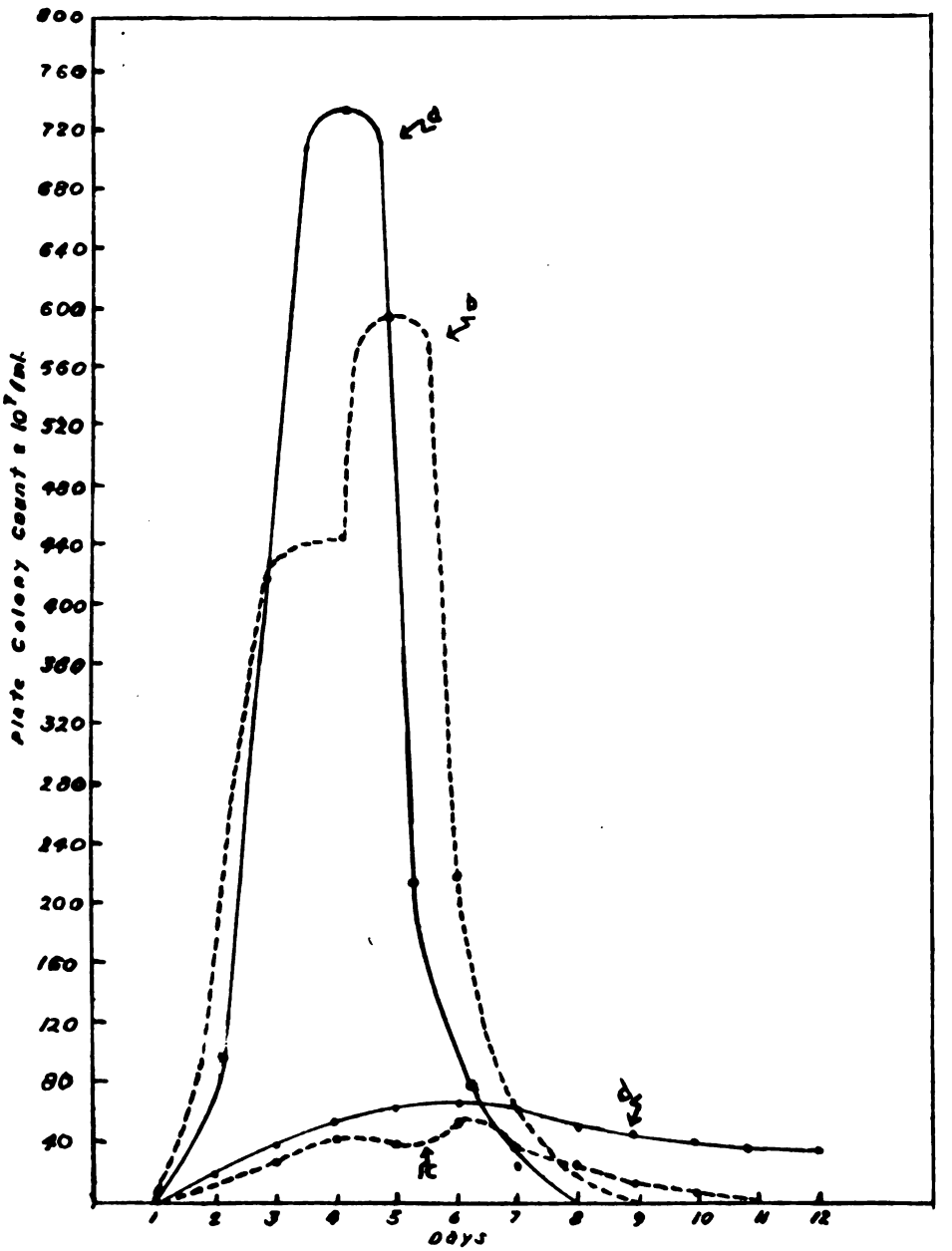

Fig. 3. Growth curves of Br. melitensis.
--Synthetic medium. a;b--cultures incubated on shaker.
--Tryptose medium. c;d--cultures incubated on shelf.

ACKNOWLEDGMENT

The author wishes to express his appreciation to Dr. I. F. Huddleson, Research Professor of Bacteriology, Michigan State College, for his wise advice and patient counsel which made this work possible; to Dean Ward Giltner and the workers at the Central Brucella Laboratory for their many considerations; and to the Difco Laboratories for their cooperation.