STUDIES OF THE EFFECTS OF OXYGEN ON MULTIPLICATION AND METABOLISM OF *BRUCELLA*

By

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STUDIES OF THE EFFECTS OF OXYGEN ON MULTIPLICATION AND METABOLISM OF *BRUCELLA*

The emphasis which has been placed on constituents of culture media has overshadowed the importance of certain other factors which influence multiplication and metabolism of microorganisms. Nutritional studies of the *Brucella* organisms have not solved the problem of production of large numbers of cells in a liquid medium. An investigation of this problem resulted in a study of the effect of atmospheric gases on the growth of *Brucella* in tryptose peptone liquid media. It has been found that the oxygen demand of these organisms is extremely high and has been a limiting factor in growth in liquid media. *Brucella* cells have been grown in large numbers in a short period of time, in a simple and practical liquid medium in an atmosphere of pure oxygen.

Studies of the nutritional requirements of the *Brucella* group in various base media have indicated that thiamine, nicotinic acid, biotin and pantothenic acid are either essential or stimulatory (17, 21, 22, 27, 28, 29, 30). Roby (36) included leucine or lysine and tryptophane. McCullough *et al.* (30) reported that cystine, histidine, tyrosine, phenylalanine and tryptophane were essential amino acids and glycine, lysine, arginine, methionine, glutamic acid, isoleucine, aspartic acid, serine and threonine were stimulatory. These authors also found Mg salts essential, Mn and Fe stimulatory, and one per cent glucose required for maximum growth.

Glucose has often been added to various media to enhance the growth of *Brucella*. McAlpine (26) reported utilization of glucose by

some strains of Brucella but not all. Soule (37) found that two per cent glucose in veal infusion agar favored growth of all strains tested. McNutt and Purwin (31, 32) reported that acid production from glucose by Brucella depended on the quality of the base medium. That glucose utilization was not appreciably different in strains was noted by Zobell and Meyer (40).

Coleman et al. (6) studied the fermentation of monosaccharides by 39 strains of the abortus-melitensis group. Acid production indicated that arabinose was fermented by all strains tested, xylose by all except one, galactose by 36, glucose by 19 and levulose by 13. Acid production from rhamnose and lactose was not observed.

Winslow et al. (39) were among the first to show a great increase by aeration in the population of facultative anaerobes. With Escherichia coli the final population was increased five to ten times by aeration. The increase was attributed to removal of toxic waste products of growth and to increased oxygenation. Roby (36) noted a considerable increase in the growth of Brucella cultures when aerated by agitation.

Following preliminary studies of nutritional requirements, the effects of various atmospheric gases on multiplication of Brucella in a practical and satisfactory liquid medium was determined. The metabolism of the organisms when grown in an atmosphere of pure oxygen was briefly studied.

EXPERIMENTS AND RESULTS

A. Preliminary studies of media.

Preliminary studies of nutritional requirements were conducted in 8" x 1" test tubes containing 15 ml. of medium. The twenty-four hour growth of a smooth strain of Brucella from liver agar slants was used for inoculum. The cells were suspended in a diluting fluid containing 0.5 per cent sodium chloride and 0.05 per cent tryptose peptone. The cell count was determined by turbidity using a Libby photonreflectometer which was standardized by plate counts. To each tube of medium were added 5×10^4 viable cells/ml. All cultures were incubated at 37°C. on a push-pull type of shaker machine operating at a rate of 75 strokes per minute. Viable count was determined after 72 hours incubation by plating suitable dilutions of the cultures on tryptose agar.

Since practicality rather than definition was a prime requisite of the medium sought, Difco "Tryptose" peptone was selected as the most satisfactory basal medium (16). By adding various concentrations of sodium chloride it was determined that 0.5 per cent was optimum and this concentration was used in all media studied. All media were adjusted to pH 6.7 with phosphoric acid. As illustrated by data in Table I, viable counts after 72 hours incubation in agitated cultures were

Table I. Growth of Brucella in Stationary and in Agitated Cultures

	Viable Cell Count 10^9/ml.	
	Shelf	Shaker
Br. abortus	0.4	5.0
Br. suis	0.4	8.5
Br. melitensis	0.5	6.8

Medium: 2% tryptose, 0.5% NaCl, pH 6.7; 15 ml. in 8" x 1" test tubes.
Inoculum: 5×10^4 cells/ml. Incubation: 72 hrs.

ten to twenty times greater than in stationary cultures.

When glucose was added to 1 per cent tryptose, the viable count of Brucella suis was significantly increased (Table II). Stanier (38) noted that breakdown products formed in autoclaved solutions of glucose were toxic for the organisms in his study. Autoclaved tryptose medium containing glucose (115°C., 15 min.) exhibited no toxicity for Brucella organisms when compared to medium sterilized by filtration.

Table II. Stimulation of Growth of Br. suis by Glucose and Thiamine

Medium			Viable cell count
Tryptose %	Glucose %	Thiamine mg. %	10^9/ml.
1.0	0.0	0.0	6
1.0	0.5	0.0	12
1.0	1.0	0.0	18
1.0	0.5	0.5	33
1.0	1.0	0.5	40
1.0	2.0	0.5	40
2.0	1.0	0.5	35
1.0	1.0	2.0	31
1.0	1.0	5.0	36

Base: 0.5% NaCl; pH 6.7
Inoculum: 5 x 10^4 cells/ml.
Incubation: 72 hrs. on shaker.

When the tryptose-glucose medium was supplemented by thiamine hydrochloride, a stimulatory effect was noted with all species of Brucella. Representative data for Br. suis in Table II show the stimulation of growth by various concentrations of glucose and thiamine hydrochloride.

Thiamine hydrochloride is more stable at a low pH (8). It was established that thiamine hydrochloride autoclaved in concentrated solution at pH 3 or sterilized by filtration and added aseptically to a medium resulted in an increase in growth of Brucella equal to that in the

autoclaved tryptose medium (pH 6.7) containing thiamine. The last procedure for sterilization was used for all media.

The tryptose-glucose-thiamine medium was supplemented with various other factors. As shown in Table III, multiplication was not increased by any factor tried. Counts of 35 ± 5 x 10^9 viable cells/ml. were consistently obtained with Br. suis in medium containing one per cent tryptose, one per cent glucose, 0.5 mg. per cent thiamine hydrochloride, and 0.5 per cent sodium chloride; higher concentrations of any of the nutrients did not increase growth. An increase in multiplication in this medium was demonstrated with eight strains of Br. suis, ten of Br. abortus and seven of Br. melitensis.

From seven to nine mgs. glucose/ml. were utilized by all strains and there was not a consistent difference in glucose utilization between strains or species of Brucella.

Various carbohydrates were incorporated in one per cent tryptose medium to determine if there was utilization of the sugar and if there was stimulation of growth as was effected with glucose. Cultures in the carbohydrate media were compared to those in plain tryptose medium. An increase in cell count and a decrease in pH were criteria for carbohydrate utilization.

Br. suis utilized arabinose, xylose, galactose, and fructose (Table IV). The stimulatory effect of any one of these carbohydrates was not strikingly different from that of glucose. These results confirm those of Coleman (6).

Table III. Effect on the Growth of Br. suis of the Addition of Various Factors to the Base Medium.

Substance Added	Conc. mg. %	Viable Cell Count 10^9/ml.
None		35 ± 5
Nicotinic Acid	0.5	32
Tryptophane	1.0	33
Tryptophane	2.0	33
Lysine	20.0	32
Ca. pantothenate	0.5	32
Alanine	20.0	33
Alanine	40.0	39
Riboflavin	0.5	33
Leucine	10.0	44
Leucine	20.0	31
Leucine	50.0	28
Cystine	15.0	34
Glucosamine	5.0	32
Glucosamine	10.0	32
Glucosamine	20.0	27
Autolyzed Yeast	250.0	40
Autolyzed Yeast	500.0	42
Liver extract		31
$SnCl_2$	1.0	22
$SnCl_2$	10.0	24
$FeSO_4$	1.0	24
$FeSO_4$	10.0	22
KCl	1.0	23
KCl	10.0	26
KCl	100.0	22
$MgSO_4$	1.0	25
$MgSO_4$	5.0	24
$Mg(C_2H_3O_2)_2$	10.0	25

Base medium: 1% tryptose, 0.5% NaCl,
1% glucose, 0.5 mg. % thiamine.
Inoculum: 5 x 10^4 cells/ml. Br. suis.
Incubation: 72 hours on shaker.

Table IV. Effects of Carbohydrates on Growth of Br. Suis.

Carbohydrate added	pH	Viable Cell Count 10^8/ml.
None	8.1	5
Glucose	6.1	35
Arabinose	6.5	31
Xylose	5.9	33
Galactose	7.2	32
Fructose	7.5	21
Rhamnose	8.1	4
Cellobiose	8.1	5
Lactose	8.3	5
Sucrose	8.4	5
Raffinose	8.2	7
Starch	8.2	7
Inulin	8.2	6
Sorbitol	8.2	6
Mannitol	8.1	5
Dulcitol	8.2	4

Base medium: 1% tryptose, 0.5 mg. % thiamine HCl, 0.5% NaCl; pH 6.7
Carbohydrates: 0.5%
Incubation: 4 days on shaker.

B. Effects of atmospheric gases.

The growth of Brucella cultures in large volumes of the tryptose-glucose-thiamine medium was not comparable to that in the same medium in test tubes. Attempts to attain more efficient aeration in large culture containers resulted in a study of the effects of atmospheric gases on multiplication and metabolism of Brucella.

Subsequent investigation proved that growth of Brucella in this liquid medium was limited by the supply of oxygen. When the demand for oxygen was met, the medium which was more than adequate under former culture conditions did not contain sufficient nutrients to produce a maximum population.

There was simultaneously in progress at another laboratory studies on the nutritional requirements of Brucella and growth in aerated broth cultures. Reports of this group indicate their success in producing large numbers of cells in a short period of time (11, 12, 13, 30). In a medium similar to that discussed here, maximum yields of Br. suis in large volumes were 55 x 10^8 cells/ml. and in small amounts 100 x 10^8 cells/ml.

Rahn and Richardson (34) reported that the oxygen demand of various species of organisms varied from 0.5 to 2 x 10^{-10} mg. oxygen/cell/hour for Streptococci to 10 to 80 x 10^{-8} mg. oxygen/cell/hour for Bacilli and that the demand did not depend only on the size of the cell. He determined that one per cent Bacto-peptone medium was depleted of oxygen when bacteria had multiplied to about 2 to 10 x 10^8 cells/ml. The supply of oxygen by diffusion and by convection currents in stationary cultures was not sufficient to meet the demands of facultative anaerobes. They observed that the rate of multiplication of all anaer-

obes. They observed that the rate of multiplication of all aerobes studied in one per cent Bacto-peptone was the same until the oxygen was almost completely exhausted but that tolerance for anaerobic conditions varied with different groups of aerobic organisms.

Rahn and Richardson (35) studied the population growth of aerobes with an optimal oxygen supply. Streptococci and Lactobacilli, which did not consume large amounts of oxygen, were not affected by aeration and growth was decreased by pure oxygen. The logarithmic portion of the multiplication curves of Pseudomonas flouresoens extended from the second to the sixth hour of incubation in a stationary culture and from the fourth to the twelfth hour in an aerated one. The total growth of Bacillus subtilis was increased by aeration.

Levine (24) observed that 100 per cent oxygen was neither toxic nor inhibitory to spores or vegetative cells of B. subtilis and that 20 per cent oxygen was near the minimum that would support normal growth.

The effects of various rates of flow of air, oxygen, carbon dioxide and mixtures of these gases on multiplication of Brucella were determined and the metabolism of the organisms in optimum conditions for growth studied. It was necessary to reconsider the minimum requirements for constituents of the medium. 250 ml. of each medium were sterilized in one liter bottles fitted with a two-hole rubber stopper and glass tubing. The inlet tube for gas extended to within an inch of the surface of the medium. The rate of flow of each gas into a culture container was measured by means of a "Flowrator" (Fischer and Porter). All cultures were grown on a push-pull type of shaker and glass float tubes were placed in the bottles to intensify the movement of the liquid and distribution of gas into the medium. Cultures grown in a

normal atmosphere in bottles which were fitted with cotton plugs served as controls.

A single smooth strain of each species of Brucella was used in all further studies. Twenty-four hour growth from liver agar slants was used for inoculum. In most of the experiments the media were seeded with 1 x 10^8 cells/ml. of medium. Samples were removed at 24 hour intervals. Viable count was determined by plating suitable dilutions of the cultures on tryptose agar and total count measured turbidimetrically from a standard curve established by direct cell counts. Glucose was determined colorimetrically by a modified Benedict's procedure (4) and pH measured electrometrically.

1. Multiplication.

Growth of the organisms was compared in three media which differed only in the concentration of tryptose. Figure 1 shows the multiplication of Br. suis in a normal culture atmosphere in which the bottles were fitted with cotton plugs, in a flow of air (85 ml./minute) and in an atmosphere of pure oxygen in which the rate of flow was 45 ml./minute. There was only a slight stimulation of growth by oxygen in one per cent tryptose, but there was a marked increase in total cell count in two per cent and three per cent tryptose. A comparable increase was obtained in the latter two media when the rate of flow of oxygen was only 15 ml./minute.

The increase obtained in the multiplication rate of Br. melitensis in an atmosphere of oxygen is illustrated in Figure 2.

The effect of oxygen on growth of Br. abortus (Figure 3) is somewhat different from that observed with Br. suis and Br. melitensis. When one per cent tryptose was inoculated with only 1 x 10^6 cells/ml.

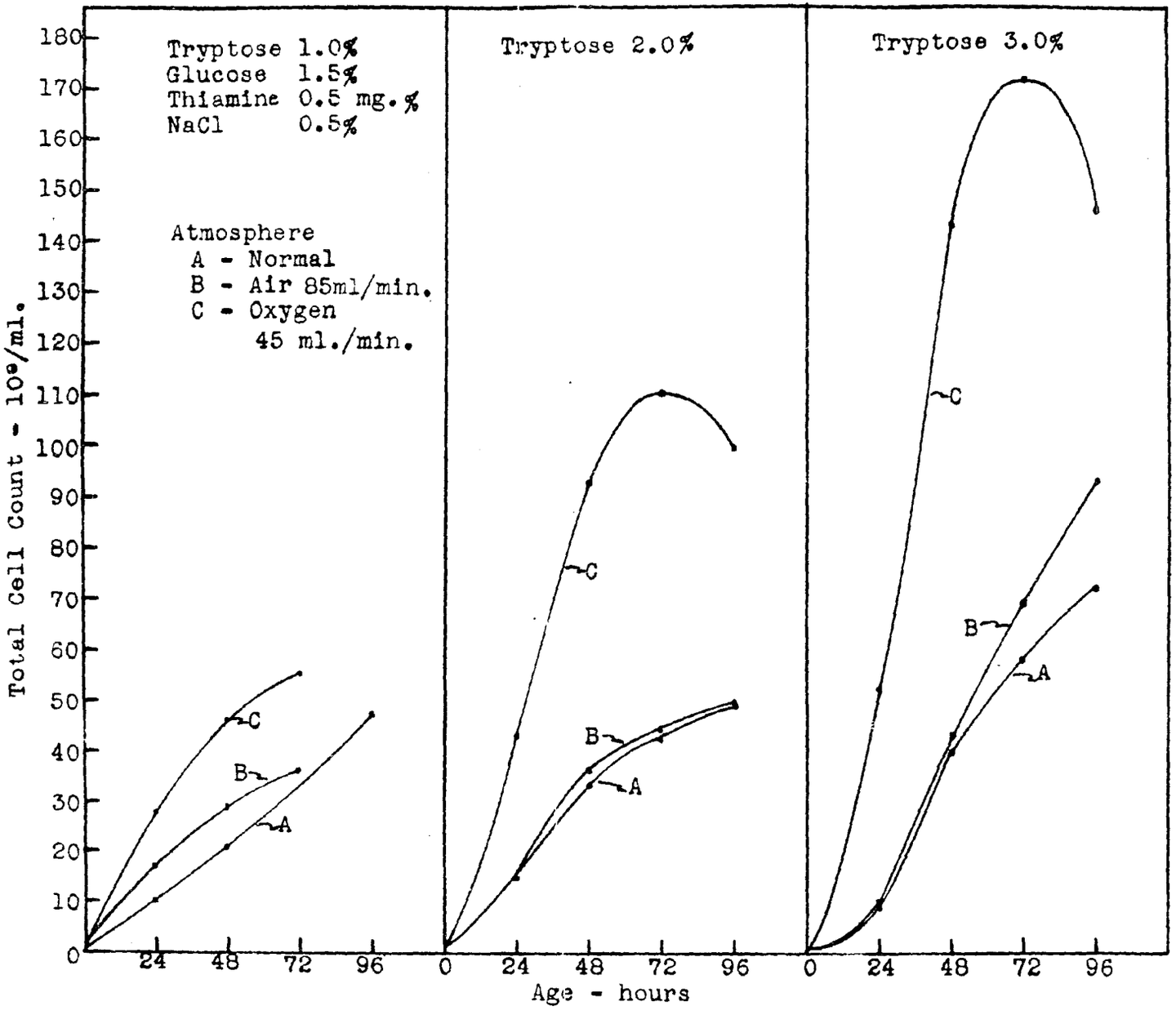

Fig. 1. Multiplication of Br. suis in Three Concentrations of Tryptose

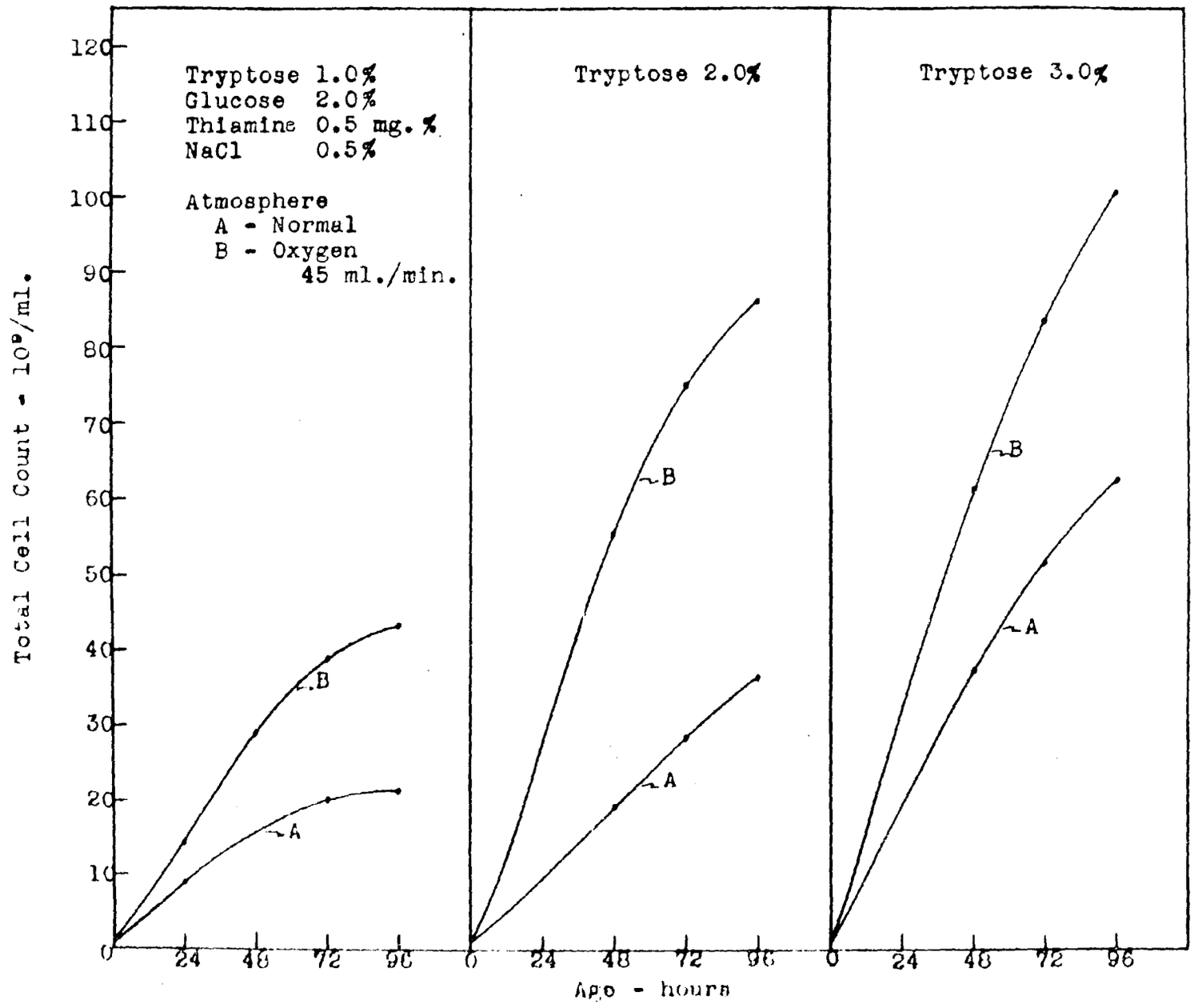

Fig. 2. Multiplication of Br. melitensis in Three Concentrations of Tryptose

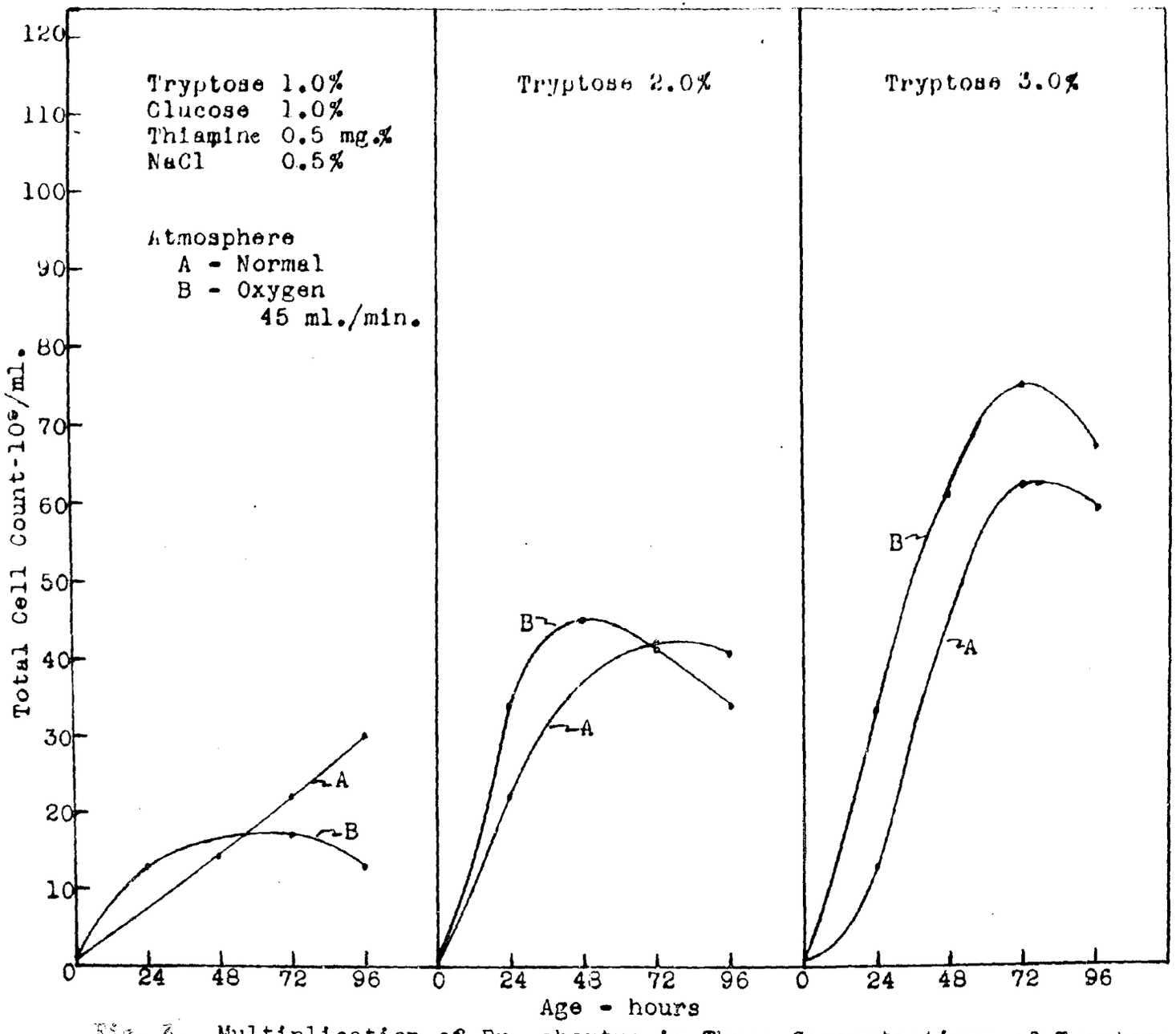

Fig. 3. Multiplication of Br. abortus in Three Concentrations of Tryptose

and oxygenated, there was no visible growth in 72 hours. In two per cent and three per cent tryptose media, stimulation of growth by oxygen was slight.

In Figure 4 the rates of growth of Br. suis in a normal atmosphere, in a stream of air and in oxygen are compared. The air flow was of sufficient volume so that the oxygen content was the same as the quantity of pure oxygen. To one culture, pure oxygen was furnished at a rate of 15 ml./minute and in the other, air flowed at a rate of 85 ml./minute. The increase that occurred in the total count in the atmosphere of pure oxygen was not paralleled in air with any species of Brucella.

Determinations of residual glucose in the media proved that an initial concentration of one per cent was an adequate supply if one per cent tryptose was used. However, in a medium containing three per cent tryptose, multiplication and metabolism increased so much that an initial concentration of glucose of 10 mgs./ml. was virtually exhausted in 72 hours. In Figure 5 data are summarized for Br. suis grown in three media which differed only in the concentration of glucose. The total cell count was slightly higher in two per cent glucose than in one per cent or three per cent. The maximum amount of glucose decomposed was 17.5 mgs./ml.

Similar comparisons are shown for Br. abortus in Figure 6 and for Br. melitensis in Figure 7. These two species decomposed more glucose in medium of three per cent concentration, but maximum total counts were attained in the medium containing two per cent glucose.

When either glucose or thiamine was omitted from the medium, the maximum total cell count in cultures of Br. suis never rose above 29 x 10^9 cells/ml. (Figure 8). Comparable counts were observed with both

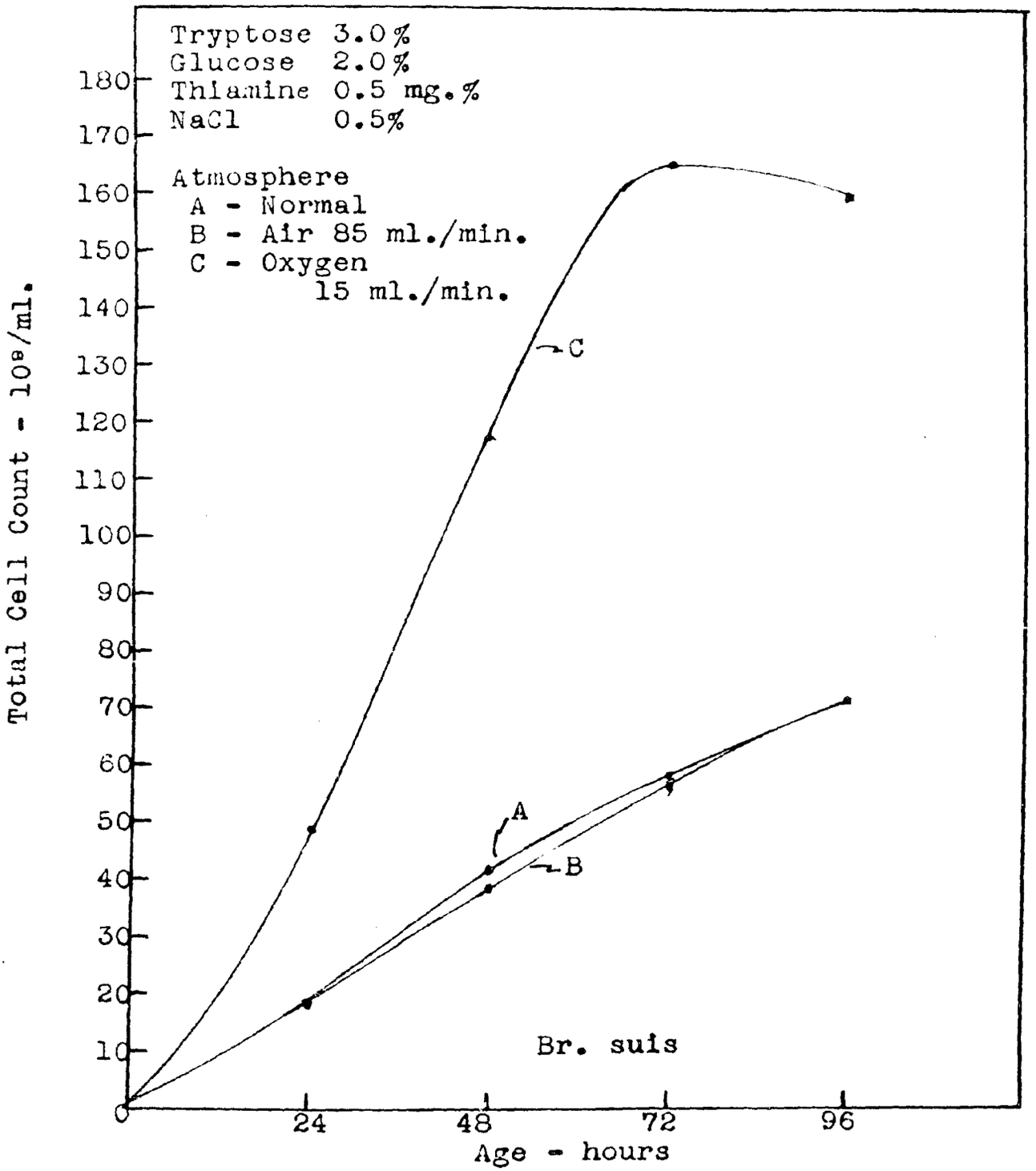

Fig. 4. Comparison of Rates of Multiplication in Atmospheres of Pure Oxygen and Air

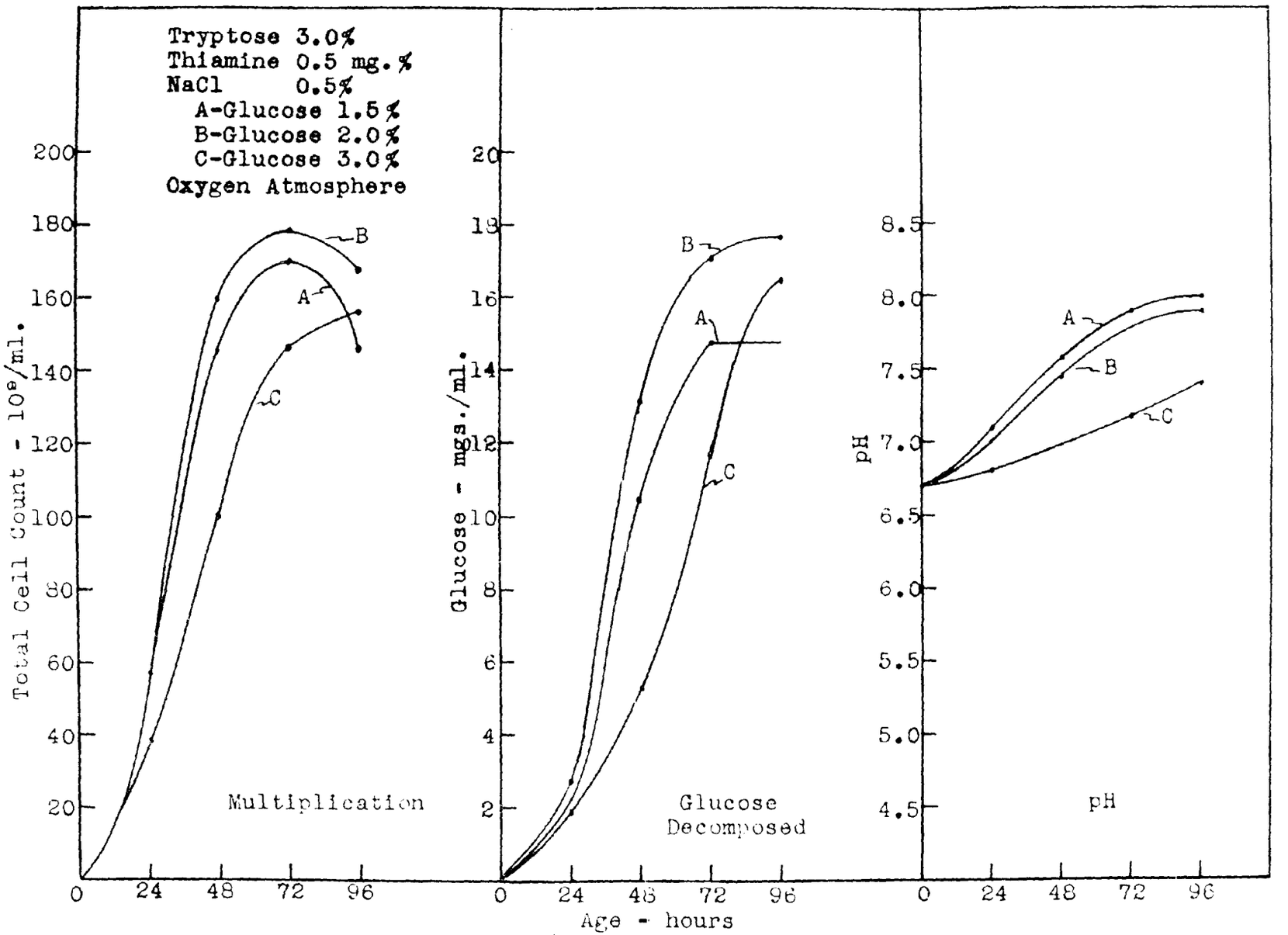

Fig. 3. Multiplication, Glucose Decomposition and pH Changes in Cultures of Br. suis in Three Concentrations of Glucose

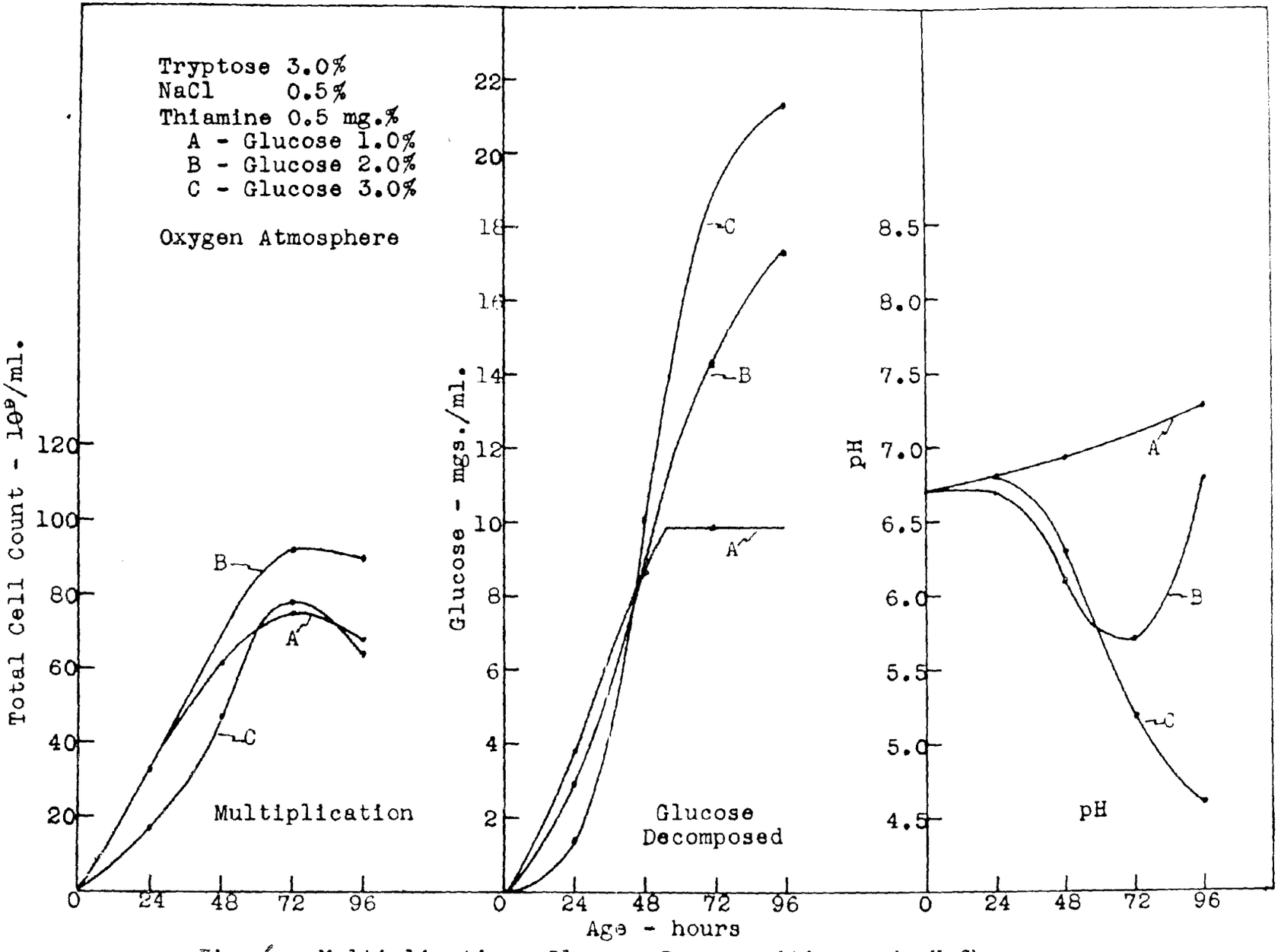

Fig. 6. Multiplication, Glucose Decomposition and pH Changes in Cultures of Br. abortus in Three Concentrations of Glucose

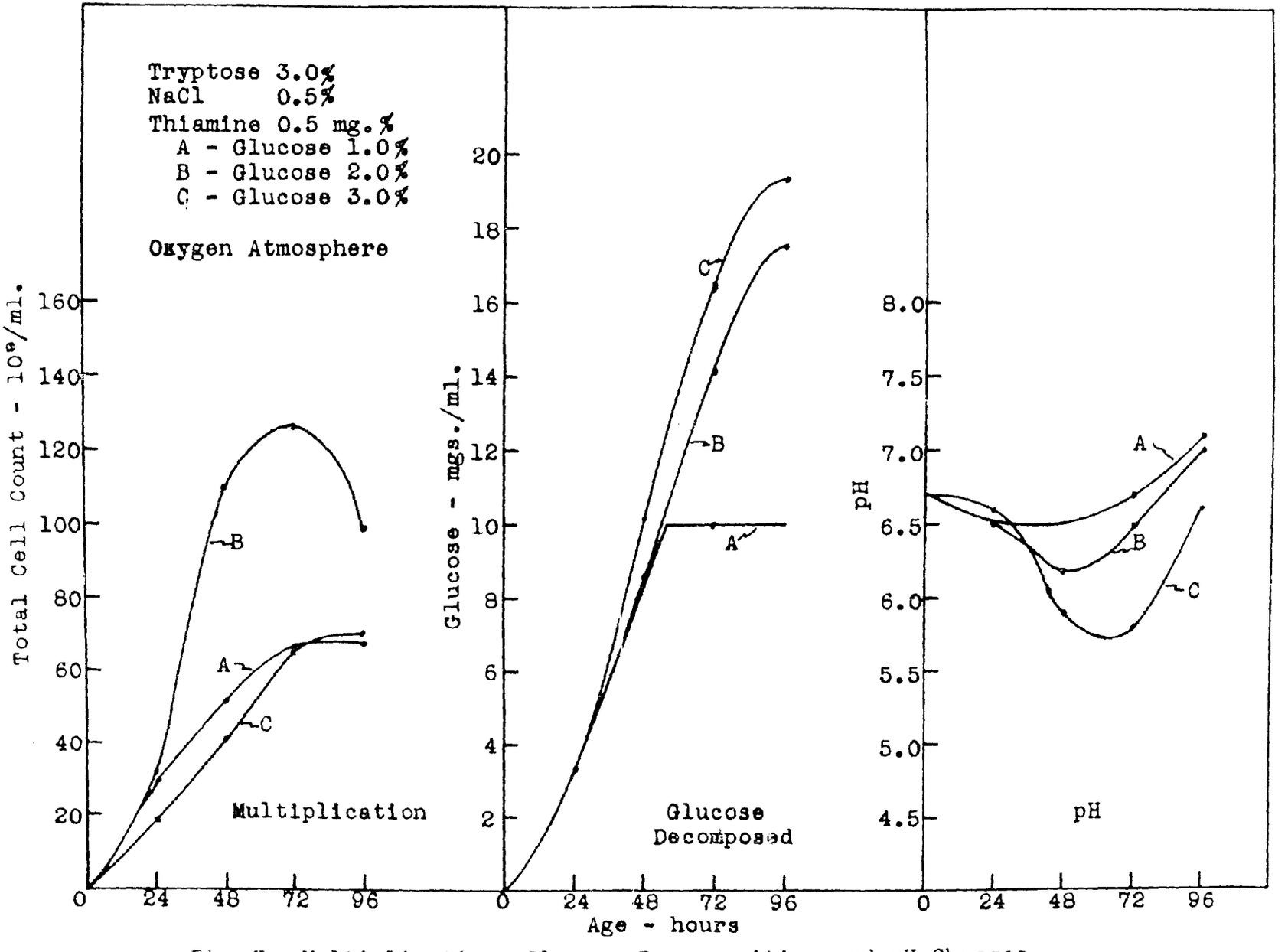

Fig. 7. Multiplication, Glucose Decomposition and pH Changes in Cultures of Br. melitensis in Three Concentrations of Glucose

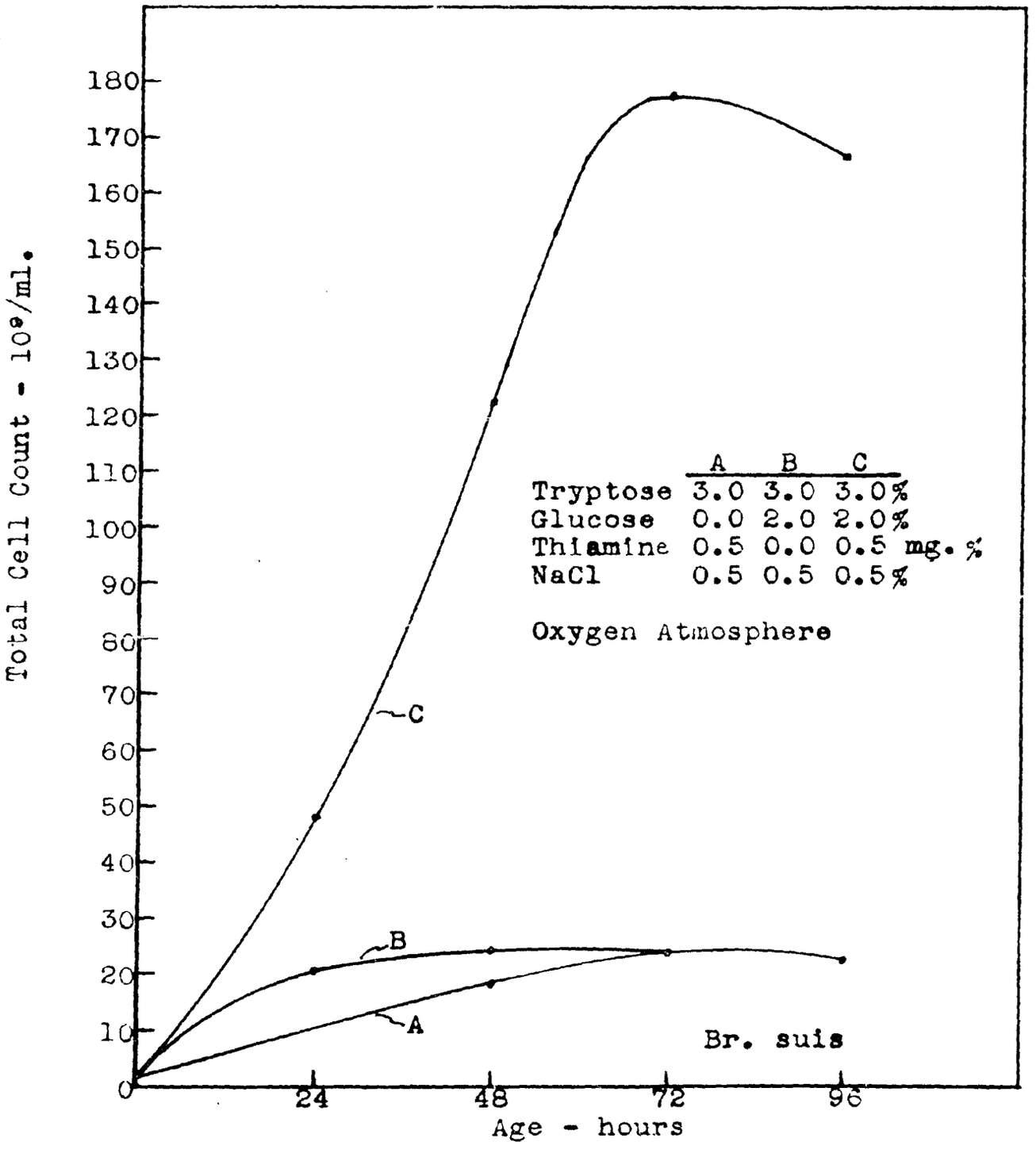

Fig. 8. Effect of Omitting Glucose or Thiamine on Rate of Multiplication

Br. abortus and Br. melitensis. Thiamine hydrochloride added to the medium in amounts greater than 0.5 mg. per cent did not increase the cell count.

Thus, the medium which was the most satisfactory for all three species of Brucella when they were grown in an atmosphere of pure oxygen contained the following constituents:

Tryptose	3.0 gm.
Glucose	2.0 gm.
Thiamine·HCl	0.5 mg.
NaCl	0.5 gm.
Distilled Water	100.0 ml.

Total and viable cell counts for the three species in this medium are shown in Figure 9 and maxima from the curves are given in Table V.

Table V. Maximum Counts under Optimum Conditions.

	Cell Counts 10^9/ml.	
	Total	Viable
Br. suis	178	102
Br. abortus	92	45
Br. melitensis	126	62

Growth was observed in atmospheres of pure carbon dioxide and in mixtures of oxygen and carbon dioxide. The multiplication of all three species was suppressed by pure carbon dioxide, which was supplied at a rate of nine ml./minute. In mixtures of the gases, the amount of carbon dioxide was maintained constant and the supply of oxygen was varied so the ratios of concentrations were 2:1, 9:1 and 18:1. The data in Figure 10 show that the total cell count of Br. suis in pure oxygen was higher than in any atmosphere containing carbon dioxide. In the mixture of two parts of oxygen to one of carbon dioxide, the multiplication of Br. suis was approximately the same as in the normal atmosphere. With mixtures of these gases in ratios of 2:1, or greater, the multi-

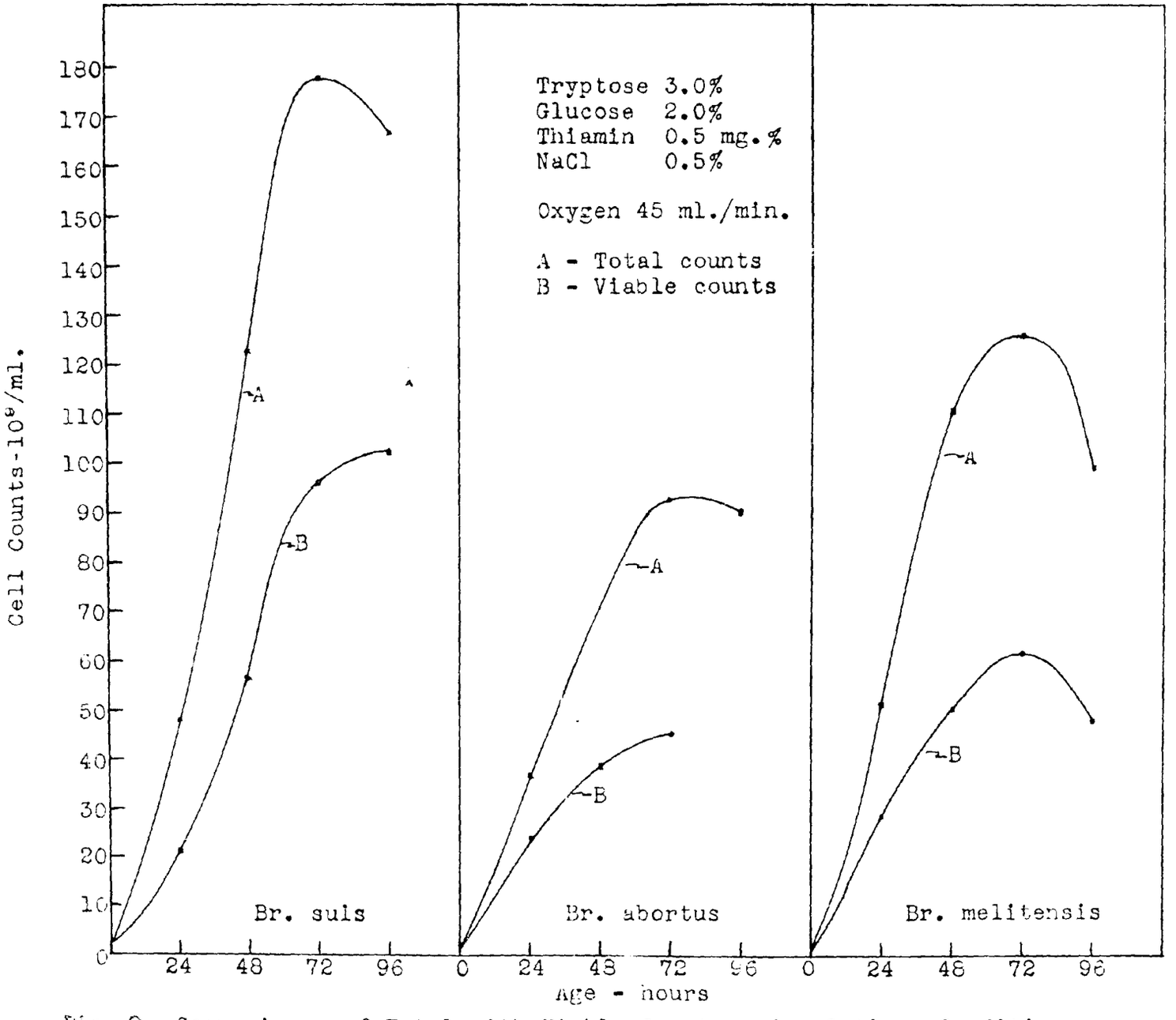

Fig. 9. Comparisons of Total with Viable Counts under Optimum Conditions

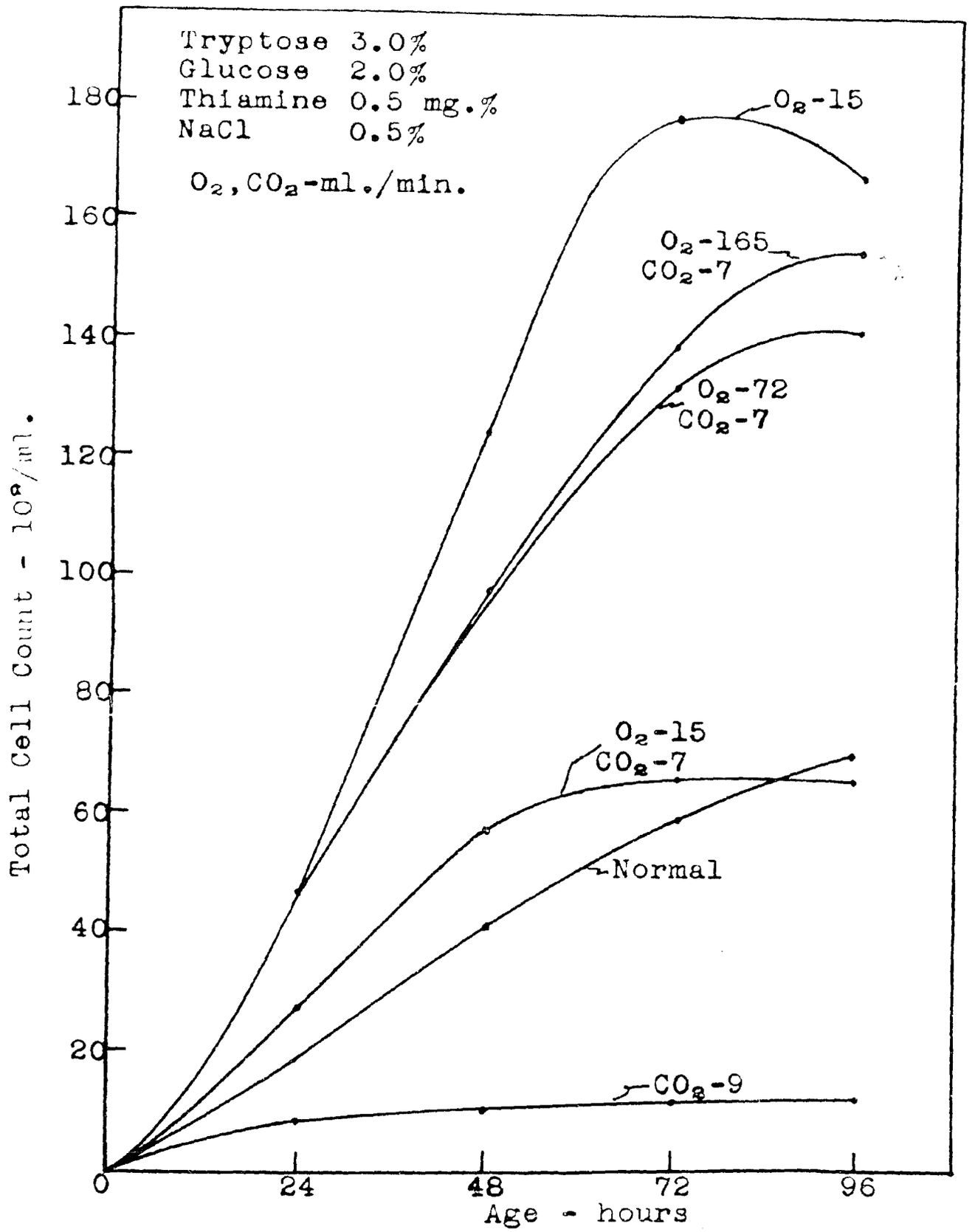

Fig. 10. Multiplication of Br. suis in Atmospheres of Oxygen and Carbon Dioxide

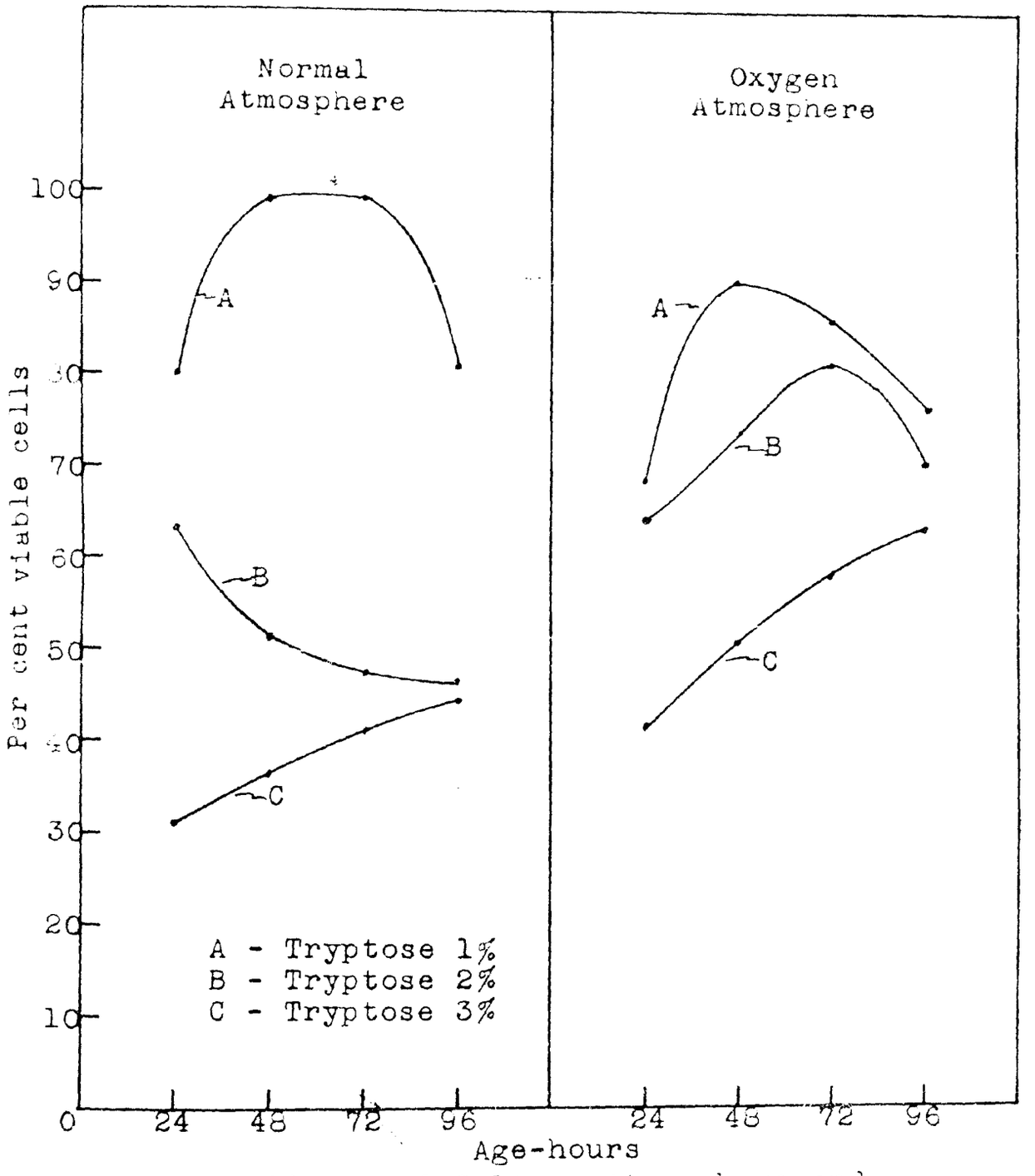

Fig. 11. Effects of Culture Atmosphere and Tryptose Concentration on Viability

plication of Br. abortus was equal to that in pure oxygen; a ratio of 18:1 was as satisfactory for Br. melitensis as pure oxygen.

The per cent of viable cells was calculated for each culture in all media tried. Representative data for Br. suis are shown in Figure 11 for a culture in normal atmosphere and one in pure oxygen, in media containing one, two, and three per cent tryptose. The per cent of viable cells decreased as the concentration of tryptose in the medium was increased. Cultures grown in an atmosphere of oxygen showed in general a slightly higher per cent of viable cells in the two per cent and three per cent tryptose concentration than those in a normal atmosphere.

2. pH Changes.

The pH changes in the media during the growth of Br. abortus, Br. melitensis and Br. suis are plotted in Figures 12, 13 and 14. These are compared in a normal atmosphere and in pure oxygen in media containing one, two and three per cent tryptose, without glucose and with one and two per cent glucose. The arrows on the graphs indicate the time at which the supply of glucose was virtually exhausted.

In media without glucose all cultures became alkaline. In general, an increase in the concentration of tryptose in the medium resulted in a higher pH, while an increase in the concentration of glucose had the opposite effect. When the supply of glucose was exhausted, there was a distinct rise in pH from nitrogen decomposition products.

The decrease in pH in cultures of Br. melitensis was generally not as low as that of Br. abortus. Cultures of Br. suis remained neutral or became slightly alkaline even though large amounts of glucose were decomposed in all media. The pH changes in media during growth of Brucella cultures emphasize the uncertainty of ascertaining carbo-

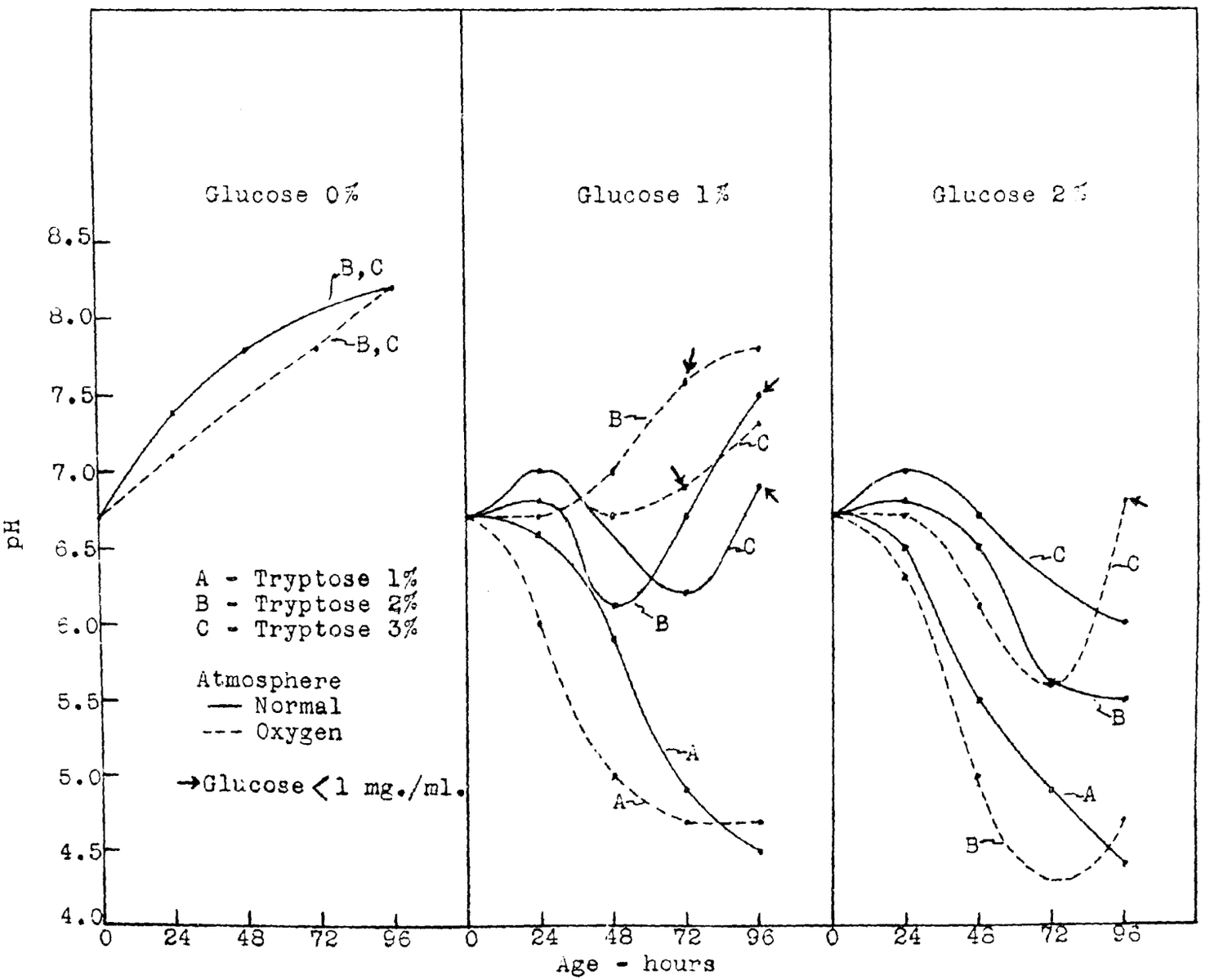

Fig. 12. pH Changes in Cultures of Br. abortus in Three Concentrations of Tryptose, with and without Glucose

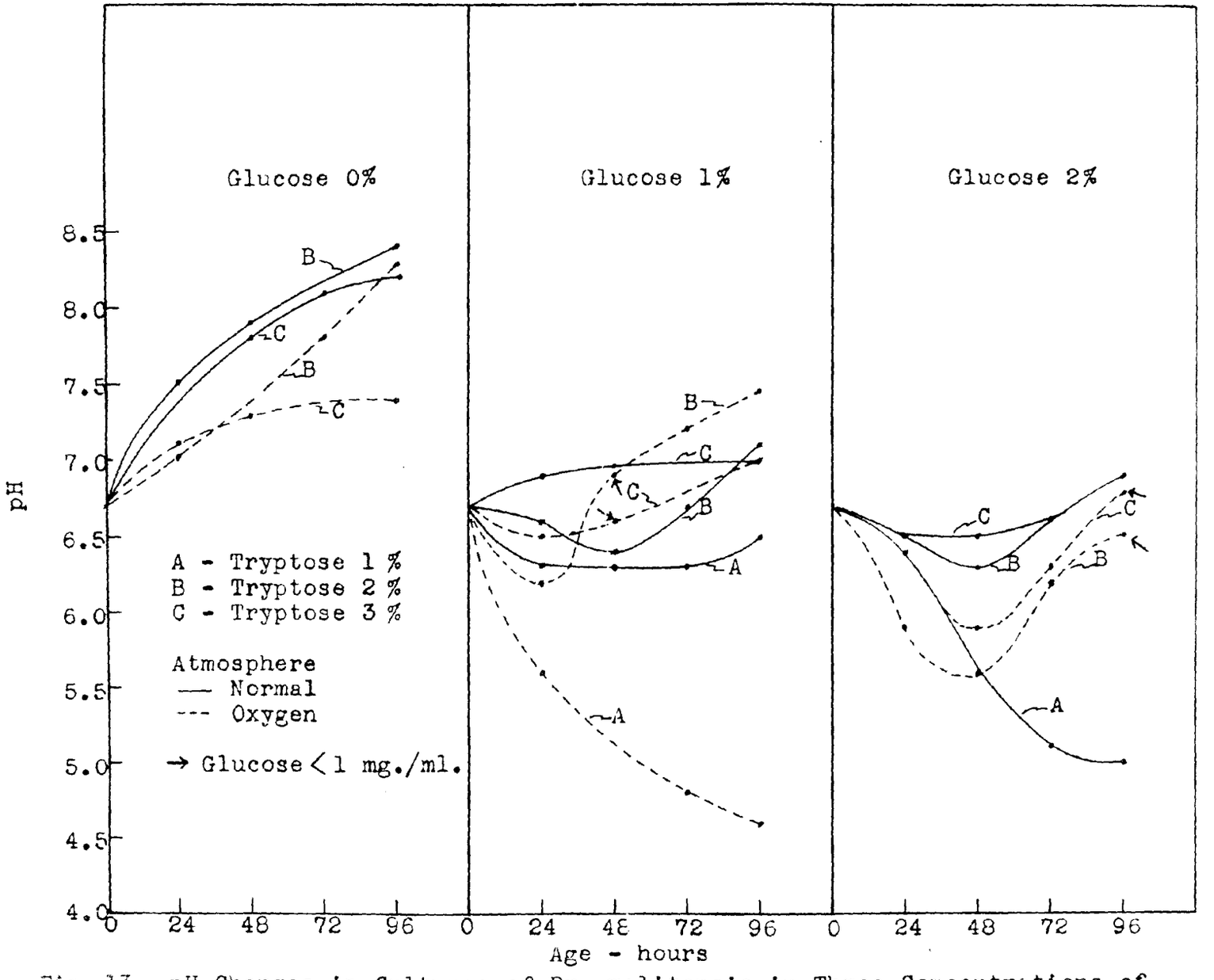

Fig. 13. pH Changes in Cultures of Br. melitensis in Three Concentrations of Tryptose, with and without Glucose

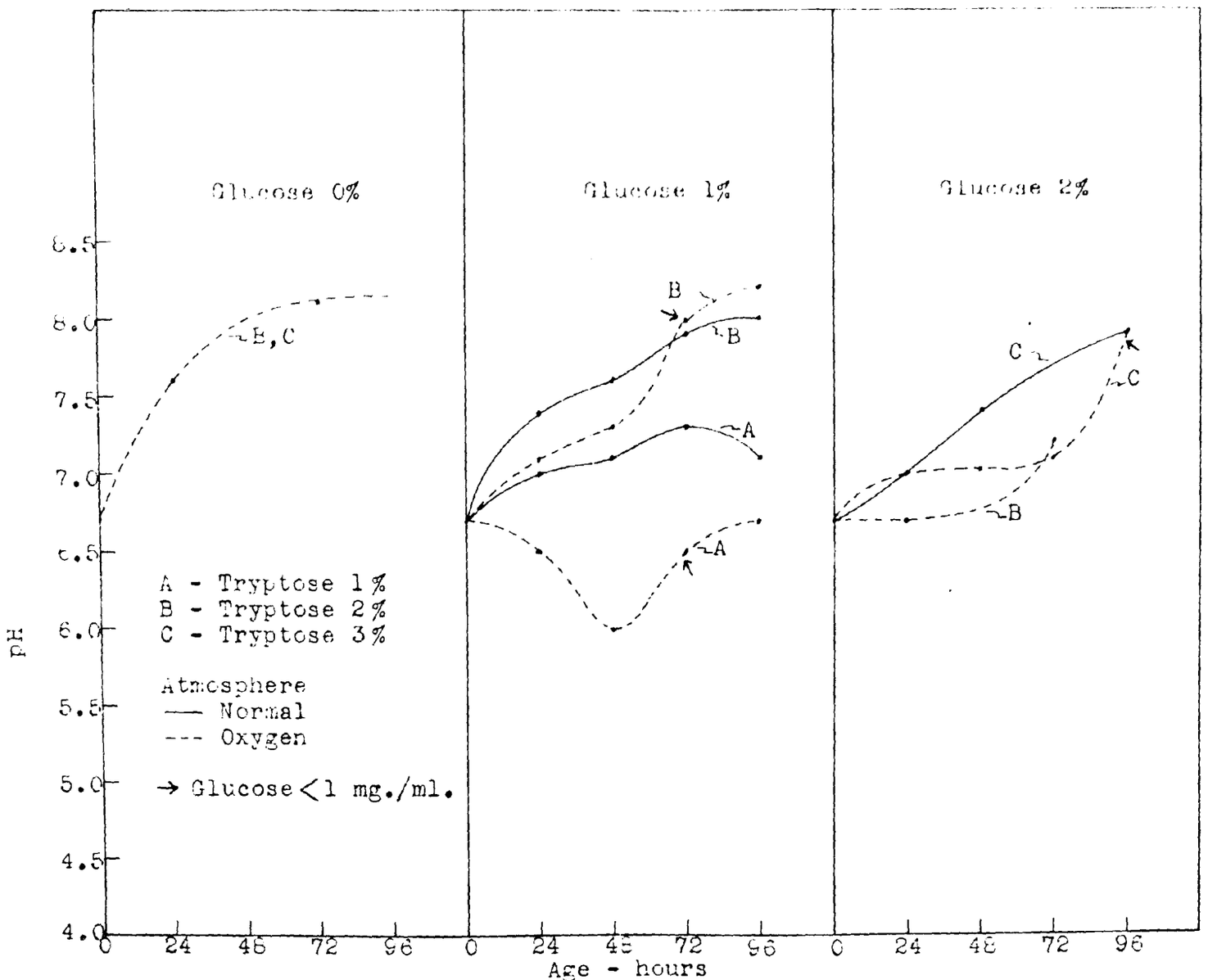

Fig. 4. pH Changes in Cultures of Br. suis in Three Concentrations of Tryptose, with and without Glucose

hydrate utilization by detection of acid production with the usual acid-base indicators. The correlation of pH with products of metabolism will be discussed later.

3. Glucose decomposition.

It is shown by data in Figures 5, 6 and 7 that if an ample supply of glucose was available, the Brucella organisms decomposed 18 to 21 mgs./ml. when grown in an atmosphere of pure oxygen in a medium of three per cent tryptose, 0.5 per cent sodium chloride and 0.5 mg. per cent thiamine hydrochloride. There was not a significant difference in the amounts of glucose decomposed by the three species even though the total populations ranged from 92 x 10^9 cells/ml. for Br. abortus to 178 x 10^9 cells/ml. for Br. suis. In a medium of one per cent tryptose in oxygen, however, Br. suis decomposed a significantly larger amount of glucose than did Br. abortus or Br. melitensis, and the three cultures did not vary widely in total cell count. To facilitate these comparisons, calculations were made of the glucose decomposed per unit number of cells. Data in Table VI are average values for Br. suis in in media which contained thiamine and an ample supply of glucose. As the concentration of tryptose was increased, less glucose was needed for each billion cells produced. The total mgs. of glucose decomposed

Table VI. The Effect of Tryptose Concentration and an Oxygen Atmosphere on Glucose Decomposition by Br. suis.

Tryptose %	Glucose decomposed-mgs./ml./10^9 cells	
	Normal Atmosphere or Flow of Air	Pure Oxygen
1	0.12	0.22
2	0.08	0.12
3	0.05	0.07

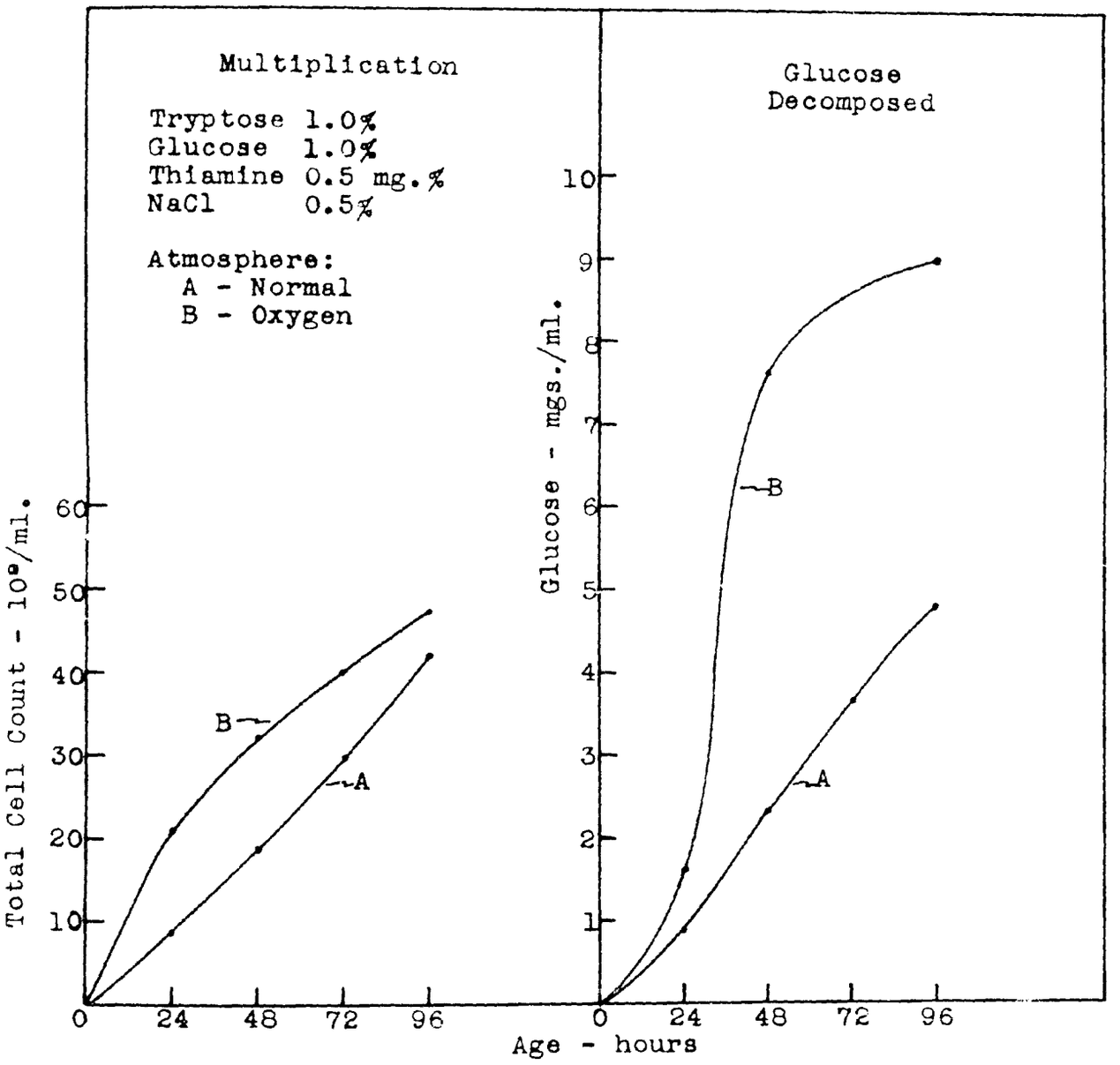

Fig. 15. Effect of Oxygen on Decomposition of Glucose by Br. suis

in three per cent tryptose was much greater, as was the total count, but the amount of glucose utilized by each cell was less. More glucose was decomposed per unit number of cells in an atmosphere of pure oxygen than in a normal atmosphere or in a flow of air. This difference was most marked in one per cent tryptose and is clearly presented in Figure 15. In general, Br. abortus and Br. melitensis decomposed 50 to 100 per cent more glucose per unit number of cells than did Br. suis. The influences of the concentration of tryptose and of the culture atmosphere were evident, though less marked with these two species.

C. Metabolism.

Experiments were designed to study briefly the metabolism of glucose under aerobic conditions. Attempts were made to detect intermediate products and end-products which might be expected from the oxidation of glucose and which would account for the pH curves characteristic of Brucella cultures.

In a study of oxidations produced by gonococci, Barron and Miller (1) summarized that in the presence of atmospheric oxygen, glucose was fermented to lactic acid which was oxidized to pyruvic acid, which in turn was oxidized to acetic acid and carbon dioxide. Krebs (23) reported that the preferential reaction of gonococci and staphylococci was an anaerobic dismutation of pyruvic acid to lactic acid which was then oxidized. Barron and Lyman (2) showed that the dismutation of pyruvic acid observed by Krebs was independent of the oxidative process, that under optimum conditions for oxidation pyruvic acid was directly oxidized to acetic acid and carbon dioxide, and under optimum conditions for reduction it might be reduced to lactic acid or split by dismutation into acetic acid and formic acid.

In later studies Barron and Friedemann (3) showed that glucose was oxidized by a number of bacteria without previous fermentation and that in cases in which the glucose molecule was not oxidized it became oxidizable as soon as the molecule was phosphorylated.

Kliger and Grossowicz (18, 19, 20) studied the role of niacin and thiamine in metabolism of glucose. In medium containing both substances there was quantitative production of carbon dioxide from glucose or lactate by Salmonella. In cultures of Staphylococcus aureus two and a half times as much glucose was utilized as when niacin alone was present and the end-products consisted of about 40 per cent acetic acid, 20 per cent lactic acid and slight amounts of pyruvic acid. If thiamine was lacking there was active growth of Staph. aureus under aerobic conditions and partial utilization of glucose. The reaction was essentially glycolytic; pyruvic acid and lactic acid were produced.

Grossowicz (14) determined that pyruvic acid was an intermediate in metabolism of glucose by Neisseria intracellularis. Thiamine catalyzed the metabolism of pyruvic acid but did not increase growth.

In respiratory studies of propionic-acid-bacteria Quastel and Webley (33) found an accumulation of pyruvic acid during oxidation of glucose, lactic acid, glycerol, and propionic acid by thiamine-deficient bacteria. The amount of pyruvic acid diminished in the presence of thiamine, which also increased the oxidation rate of acetic acid by these bacteria.

In an attempt to determine the direction of the breakdown of glucose, quantitative determinations were made of pyruvic acid, lactic acid, acetic acid and carbon dioxide. The effect of thiamine on the amount of glucose decomposed and the role of thiamine in the utiliza-

tion of pyruvic acid were studied. Glucose was replaced by pyruvate and lactate to determine if the organisms could utilize these substrates.

In these experiments the organisms were grown in 250 ml. of medium in one liter bottles in an atmosphere of pure oxygen as previously described. Samples were removed at 24 hour intervals for total cell count, pH and chemical analyses. All data are representative of tests in at least two preparations of each medium. Pyruvic acid was determined by the method of Clift and Cook (5) as modified by Elliot et al. (7). The procedure of Friedemann and Graeser (9) was followed for the measurements of lactic acid and the method of Friedemann (10) for volatile acids.

1. Glucose and thiamine.

Figures 16, 17 and 18 are graphic representations of results from experiments designed to study the effect of thiamine on multiplication, glucose decomposition and pH changes. The addition of thiamine to tryptose did not alter the total cell counts or pH changes. When glucose was added, but not thiamine, there was a significant increase in rate of multiplication of Br. abortus and Br. melitensis and in these cultures six to seven mgs. glucose/ml. were decomposed. In the culture of Br. suis 14 mgs. glucose/ml. were decomposed but there was not a significant change in the total cell count. When the medium contained both glucose and thiamine, maximum populations were obtained. The thiamine effected an increase of the decomposition of glucose which was most marked with Br. abortus and Br. melitensis.

2. Pyruvate and lactate.

There was evidence of a slight accumulation of pyruvic acid from

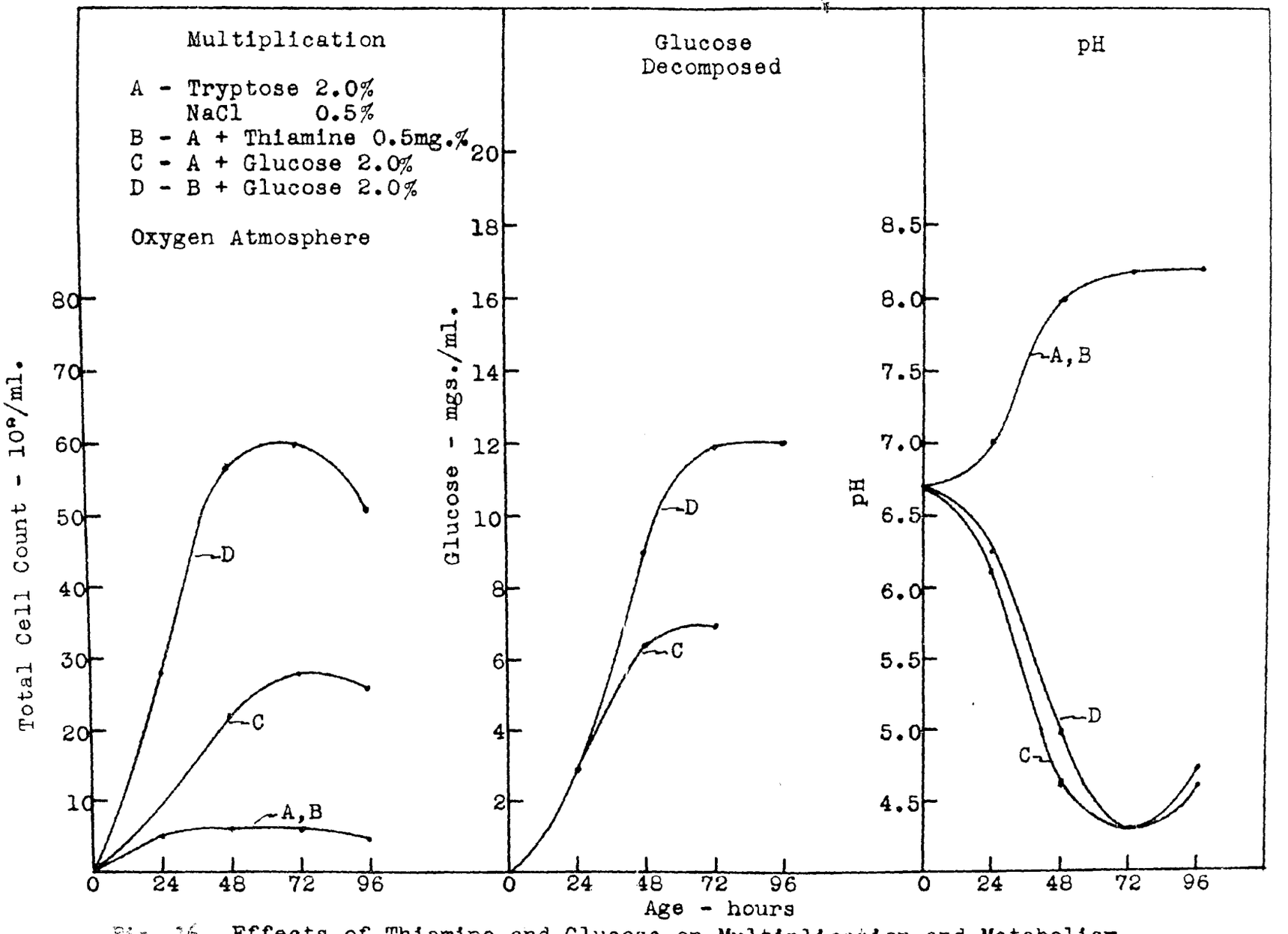

Fig. 16. Effects of Thiamine and Glucose on Multiplication and Metabolism of Br. abortus

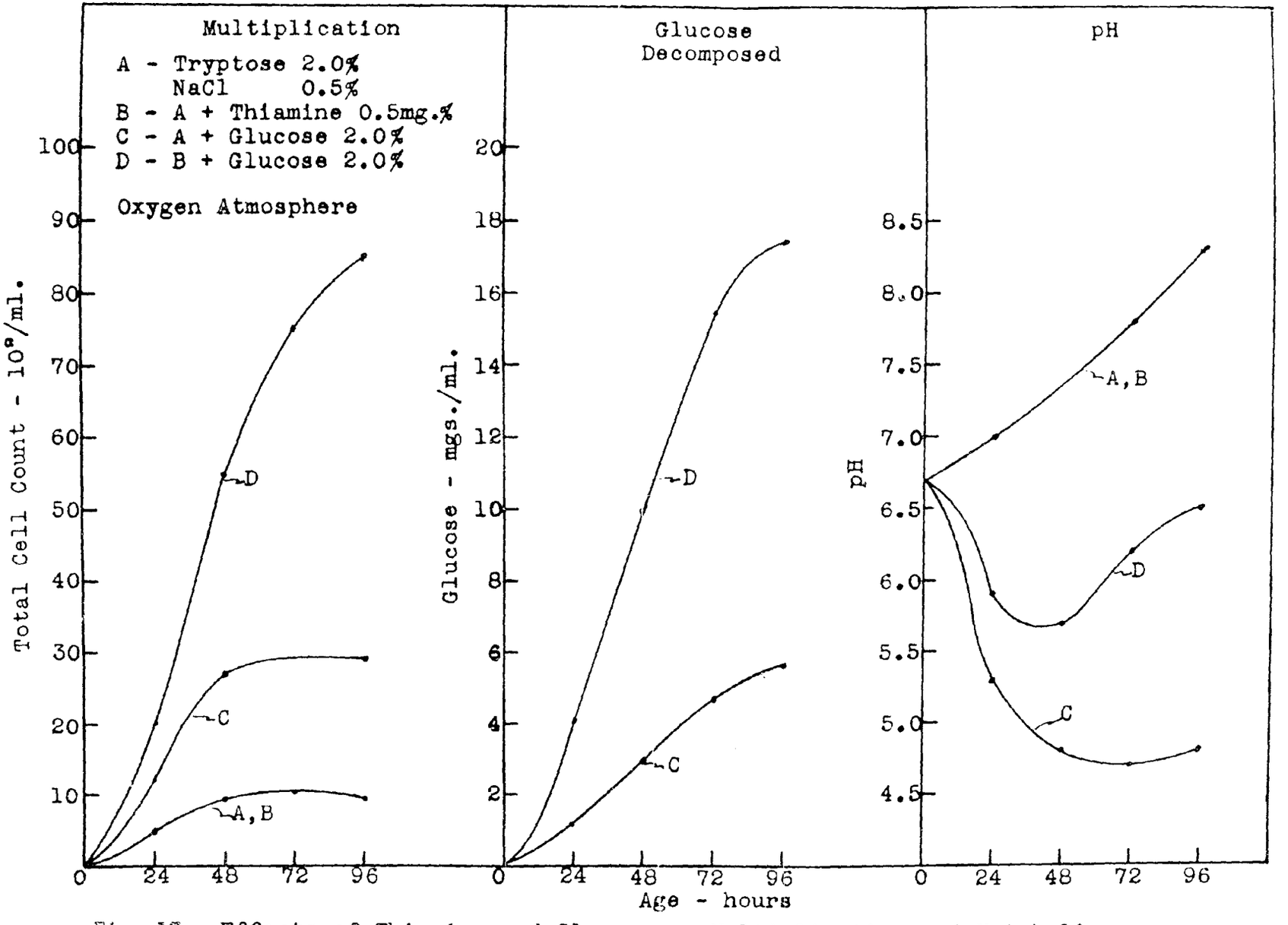

Fig. 17. Effects of Thiamine and Glucose on Multiplication and Metabolism of Br. melitensis

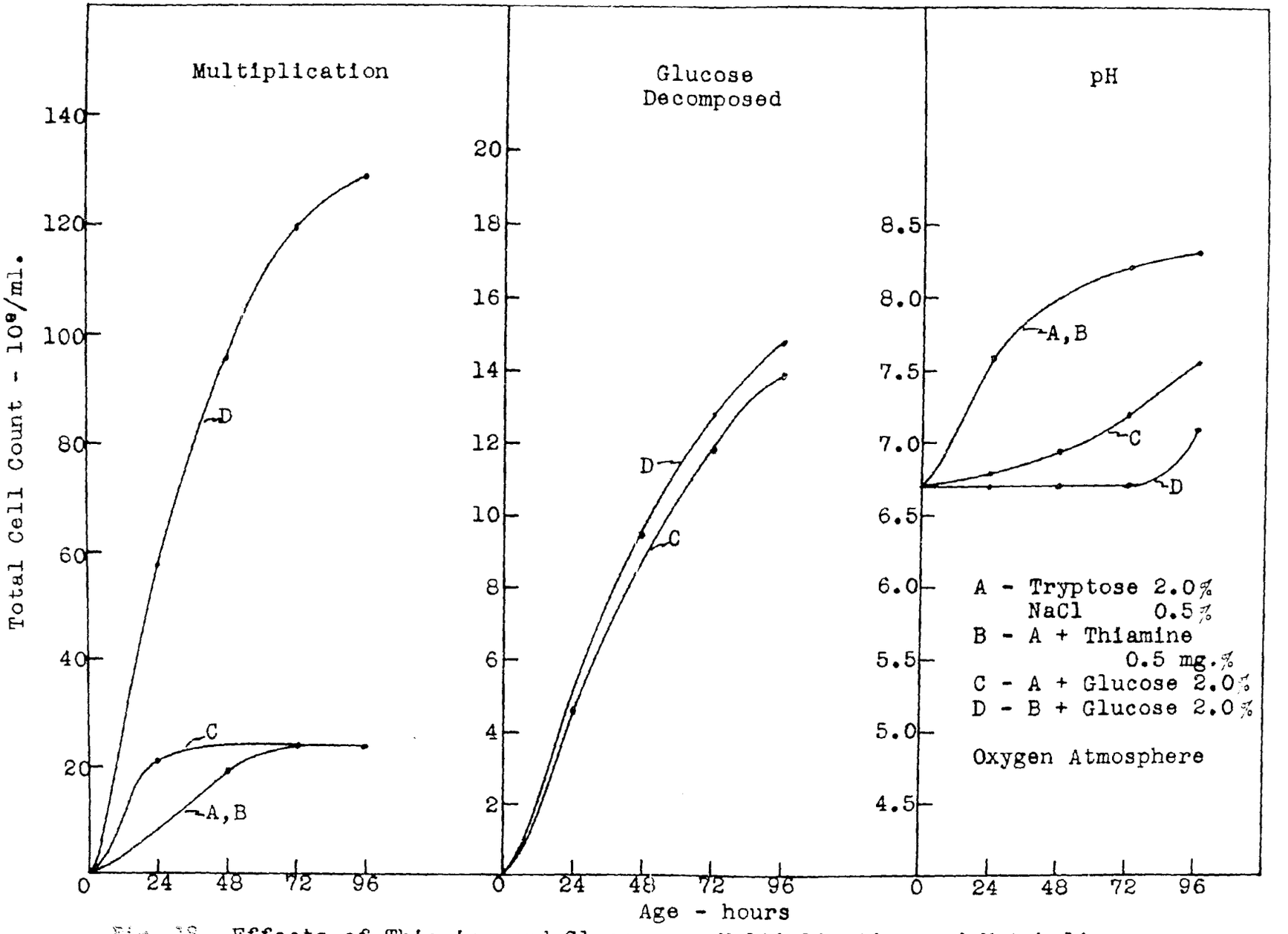

Fig. 18. Effects of Thiamine and Glucose on Multiplication and Metabolism of Br. suis

glucose when the organisms were grown in a medium without thiamine (Table VII). The amounts measured in cultures of Br. suis, however, were within the range of experimental error of the determination. Though only small amounts of pyruvic acid were measured in a medium without thiamine, none could be detected in cultures in medium containing thiamine.

Table VII. Pyruvic Acid Detected in Cultures of Brucella.

Species	Medium	Days 1	2	3	4
		Pyruvic Acid mgs./ml.			
Br. suis	Glucose	0.0	0.0	0.1	0.1
	" + Thiamine	0.0	0.0	0.0	0.0
Br. abortus	Glucose	0.1	0.4	0.5	0.7
	" + Thiamine	0.0	0.0	0.0	0.0
Br. melitensis	Glucose	0.6	1.3	1.4	1.5
	" + Thiamine	0.0	0.0	0.0	0.0

Base medium: 3% tryptose, 0.5% NaCl; pH 6.7.
Glucose: 2%; Thiamine: 0.5 mg.%.
Atmosphere: Oxygen.

Analyses for lactic acid showed that none was present in cultures of any of the three species of Brucella when examined daily during six days of incubation.

The decomposition of pyruvate, with and without thiamine, and decomposition of lactate were determined in media in which these substrates replaced glucose. The acids were neutralized with sodium hydroxide and added to tryptose broth before autoclaving. The relatively low cell counts in pyruvate media ranged from 15 x 10^8 cells/ml. for Br. abortus to 50 x 10^8 cells/ml. for Br. suis. The addition of thiamine did alter multiplication except with Br. melitensis and with that

species the stimulatory effect was slight. The data in Table VIII show that pyruvate was decomposed by each species and its decomposition was most rapid in cultures of Br. suis. The slower rate of utilization by Br. abortus and Br. melitensis was implied by the accumulation of pyruvate from glucose in the absence of thiamine. The pH in all cultures in pyruvate media increased to 8.2 to 8.5.

In the lactate substrate there was virtually no multiplication of Br. melitensis, growth of Br. abortus was slight until the third day, but growth of Br. suis approached that in a glucose medium. The quantity of lactate utilized correlates with the population in the cultures. Results in Table VIII show that considerable amounts of lactic acid were decomposed by Br. suis but none by Br. melitensis. Br. abortus began to decompose lactic acid after the second day.

3. Volatile acids.

To verify the application of Friedemann's procedure for identification of volatile acids to the conditions of the experiment, small amounts of acetic and propionic acids, added to uninoculated medium, were distilled and titrated.

Each of the three species of Brucella was grown in a medium containing three per cent tryptose, two per cent glucose and thiamine. Numerous analyses did not indicate the presence in any culture of acetic acid during four days of incubation in an atmosphere of pure oxygen.

4. Carbon dioxide and ammonia.

In early studies of metabolism of Brucella, McAlpine and Slanetz (26) measured large amounts of free ammonia produced by all strains in plain broth. In glucose medium, strains which used glucose pro-

Table VIII. Decomposition of Pyruvate and Lactate by Brucella.

Medium	Br. suis				Br. abortus				Br. melitensis			
	Days											
	1	2	3	4	1	2	3	4	1	2	3	4
	Pyruvic acid decomposed mgs./ml.											
Pyruvic Acid	2.3	2.8	2.9	2.9	0.3	0.6	0.7	0.7	0.9	1.5	1.5	1.6
" + Thiamine	2.7	2.9	2.9	3.0	0.3	0.8	0.8	1.0	0.9	1.3	1.9	2.0
	Lactic acid decomposed mgs./ml.											
Lactic Acid	2.1	8.7	14.4	18.4	0.6	0.9	7.3	12.2	0.0	0.0	0.0	0.0

Base medium: 3% tryptose, 0.5% NaCl; pH 6.7.
Pyruvic acid: 3.7 mgs./ml.
Lactic acid: 19.4 mgs./ml.
Atmosphere: Oxygen.

duced very slight amounts of ammonia. He was unable to show that Br. abortus, which produced large amounts of ammonia, could utilize glucose in a peptone medium. Since it has been shown in this study that all strains of Brucella are capable of extensive utilization of glucose in optimum culture conditions, it was of interest to observe the effect of this utilization on peptone metabolism. Quantitative measurements of carbon dioxide and ammonia were employed as rough estimates of carbohydrate and peptone decomposition. Metabolism was compared in a tryptose medium without glucose and one containing two per cent glucose.

Cultures were grown in 250 ml. of medium in one liter bottles on a shaker machine in an atmosphere of pure oxygen. The gas was directed from the culture container first through a bottle containing one liter of 0.01 N sulphuric acid and second, through one liter of 0.3 N potassium hydroxide. A 25 ml. aliquot was taken from the standard acid daily and titrated with standard sodium hydroxide. A 50 ml. aliquot of the potassium hydroxide was removed each day and after precipitation with barium chloride, the excess titrated with hydrochloric acid using phenolphthalein as the indicator.

Ammonia in the culture was determined by steam distillation of a sample into dilute boric acid and titration with 0.01 N sulphuric acid. A culture sample was aerated by a stream of air into 0.03 N potassium hydroxide to measure dissolved carbon dioxide.

The cultures were examined daily for four days and final tests were made on the seventh day of incubation. Quantities of ammonia and carbon dioxide were computed as the amount produced in each 24 hour period; the values for the seventh day were calculated as one-third of that accumulated in the three previous days. Calculations included determina-

tions on uninoculated media. The data shown in Figure 19 as rates of ammonia and carbon dioxide production emphasize the preferential breakdown of carbohydrate over peptone decomposition.

Without glucose in the medium the rates of metabolism were highest during the first 24 hours of incubation and followed a similar pattern in all three species. Ammonia production was greatest in the culture of Br. suis; this was the only species of Brucella in which the pH of the culture medium increased sufficiently to release ammonia in measurable amounts in the collection bottle of standard acid.

In medium containing glucose, the carbohydrate breakdown, as measured by carbon dioxide produced was greatest during the second and third days of incubation in all species. From Br. suis 3.4 gms. carbon dioxide were collected in a seven day period, from Br. abortus 5.0 gms., and from Br. melitensis 4.8 gms. The carbon dioxide dissolved in each culture was only 0.1 to 0.2 gms. The ammonia production indicated that Br. suis attacked peptone during the first day and reached a peak on the second day. However, no ammonia was detected until the third day in cultures of Br. melitensis and until the fourth day in Br. abortus. Thus glucose exerts a 'sparing action' on peptides which is most obvious with Br. abortus and Br. melitensis.

The presence of ammonia in cultures correlates with pH in all media. Figure 19 shows that from the time of initiation of growth of Br. suis there was production of ammonia and an increase in alkalinity. The rise in pH produced by Br. abortus and Br. melitensis occurred simultaneously with the appearance of ammonia in the culture media.

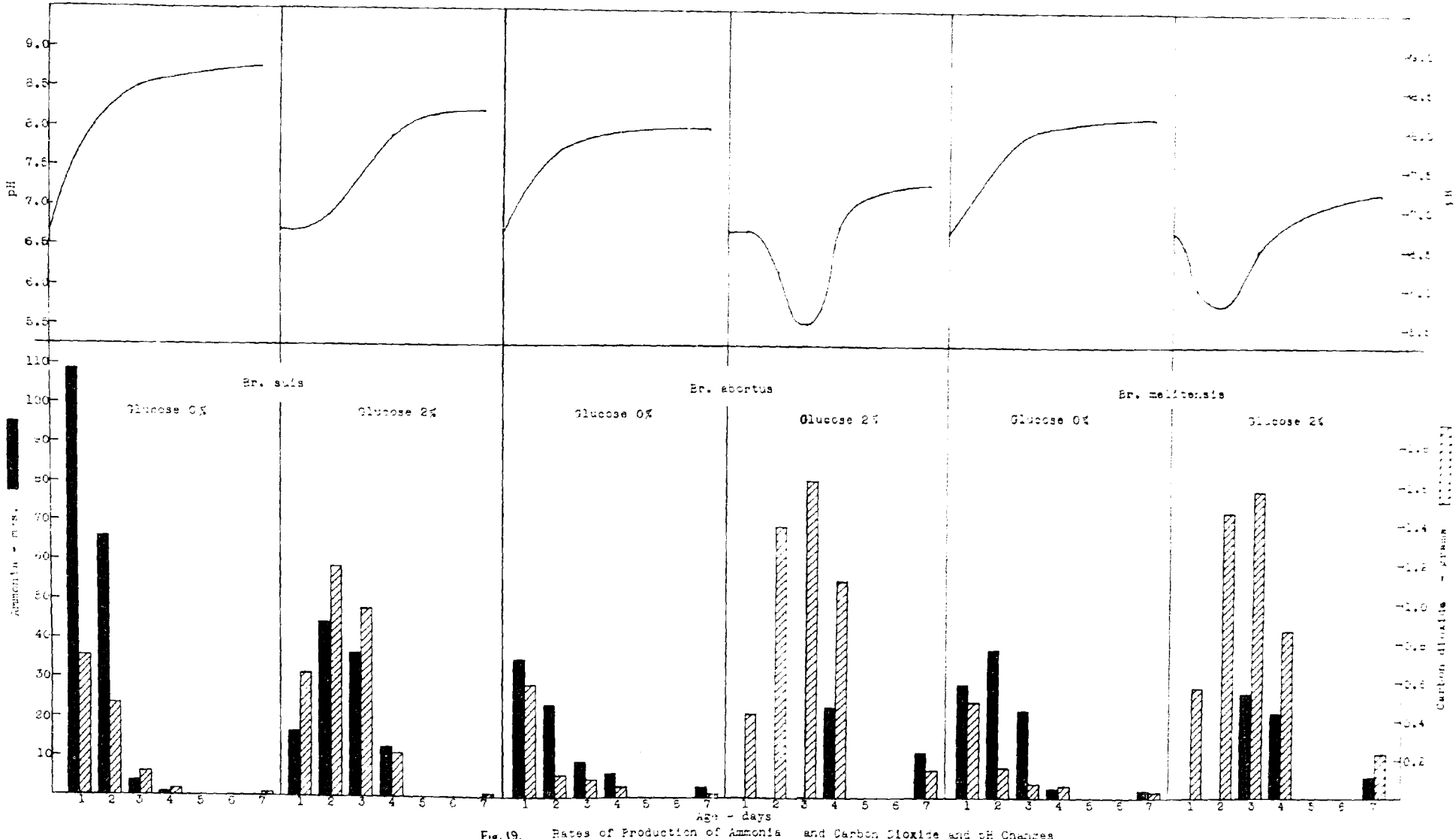

Fig. 19. Rates of Production of Ammonia and Carbon Dioxide and pH Changes during Growth of the Three Species of Brucella

DISCUSSION

Studies of the effects of atmospheric gases on multiplication of the three species of Brucella emphasize that controlled culture atmospheres are equally as important as medium constituents. A continous supply of oxygen to a culture in an adequate medium had a pronounced stimulatory effect on multiplication, but the mechanism by which this was accomplished was not explained. When oxygen was supplied to a culture in a flow of air, the total cell count was not greater than when air was available through a cotton plug. It was established that carbon dioxide, in the concentration which exists in air, was not inhibitory. A stimulation of multiplication by a mixture of carbon dioxide and oxygen in a ratio of 18:1 was equal to or approached that of pure oxygen. However, Br. abortus exhibited a tolerance for carbon dioxide much greater than did Br. suis or Br. melitensis. In view of the requirements for carbon dioxide of this species for isolation from pathological material (15) this finding was not unexpected.

In an oxygen atmosphere maximum total cell counts of each of the species of Brucella were obtained in a medium containing three per cent tryptose, two per cent glucose, thiamine and sodium chloride. In media containing less than these amounts, multiplication was limited by an inadequate supply of nutrients. It seems possible that largeramounts of medium constituents did not support further multiplication because of the physical factor of the high concentration of molecules in the immédiate surroundings of each cell in the early period of growth. It is feasible that populations higher than those reported here for Br. suis might be obtained by replenishing nutrients in the culture medium at intervals during incubation.

Multiplication of Br. abortus did not equal that of Br. melitensis or Br. suis under any conditions studied. It is possible that a greater multiplication of Br. abortus could be obtained by some additional nutritional factor and a different atmosphere. That a small inoculum of Br. abortus did not initiate growth in one per cent tryptose media in pure oxygen indicates that the atmospheric requirements of this species differ from those of Br. suis and Br. melitensis.

There were many observations of differences in the three species in multiplication, glucose decomposition, and pH changes in different media and atmospheres. The amount of glucose decomposed per unit number of cells was less for Br. suis than for the other two species. This indicates less efficient utilization of energy from glucose by Br. abortus and Br. melitensis. An oxygen atmosphere was optimum for performance of the oxidative mechanisms as indicated by a decrease in the glucose required per unit number of cells. The concentration of tryptose in the medium was also a factor in glucose decomposition. As the concentration of tryptose was increased, the glucose decomposed per unit number of cells was less. This effect, greatest with Br. suis, may be correlated with the rate of production of ammonia which was an estimate of the breakdown of nitrogenous substances.

The metabolism of glucose by Brucella when grown in an atmosphere of pure oxygen is a rapid and complete oxidation of the carbohydrate. Carbon dioxide is the major end-product. Addition of thiamine was not required for decomposition of glucose, although, when thiamine was present, the rate of decomposition of glucose and the extent of utilization of the energy, as measured by cell production, was accelerated. In the absence of thiamine, however, only minute amounts of pyruvic acid were

detected in the culture media. There was no evidence of the formation of lactic acid or acetic acid from glucose.

Basic variations in the oxidative enzyme systems in the three species of Brucella were observed when the organisms were cultured in pyruvate and lactate substrates. The rate of utilization of pyruvate by Br. suis was significantly higher than by Br. abortus or Br. melitensis; its utilization by all strains was only slightly accelerated by thiamine.

The decomposition of lactate by Br. suis was rapid and 18 mgs./ml. were utilized to produce more than 100 x 10^8 cells/ml. There was no decomposition of lactate by Br. melitensis and virtually no multiplication. The growth of Br. abortus in lactate medium was initiated after 48 hours incubation. During the third and fourth days of incubation, 12 mgs. lactate/ml. were decomposed.

It is evident that the enzyme systems of Br. suis responsible for peptone metabolism perform more efficiently in the media studied than do those of Br. abortus and Br. melitensis. The accumulation of ammonia from decomposition of nitrogenous substances in media without glucose results in a rise in pH to 8.0 to 8.5. The pH of the media containing glucose in which Br. suis was multiplying remained neutral or slightly alkaline as a result of continued peptone breakdown simultaneously with carbohydrate utilization. A low pH was measured in cultures of Br. abortus and Br. melitensis and even though large amounts of glucose were decomposed, carbon dioxide was the only end-product of metabolism which could be detected. Br. abortus and Br. melitensis produced an initial decrease in pH in the medium during the time that more carbohydrate was decomposed and no ammonia was formed. After two to three days of incubation, utilization of carbohydrate was slower, pep-

tone metabolism was initiated and the pH in the culture media increased as ammonia accumulated.

SUMMARY

The multiplication and metabolism of the three species of Brucella have been studied in a practical liquid medium which will, in an optimum atmosphere, produce large numbers of viable cells in a short period of time. The rate of multiplication, total cell count and glucose metabolism were greatly increased when these organisms were grown in an atmosphere of pure oxygen. The medium which was most satisfactory for all three species contained three per cent tryptose, two per cent glucose, 0.5 per cent sodium chloride, and 0.5 mg. per cent thiamine hydrochloride. Maximum viable cell counts were observed on the third or fourth day of incubation.

The tolerance for carbon dioxide in the culture atmosphere was greater with Br. abortus than with Br. melitensis and Br. suis.

The pH changes during growth were followed in various media and were influenced by the concentrations of tryptose and glucose.

Large amounts of glucose were decomposed by all species of Brucella and utilization was accelerated by thiamine. The oxidation of glucose was rapid and complete, as indicated by a brief study of end-products of metabolism. Pyruvic acid was detected in culture medium lacking thiamine, but a significant accumulation of acids, in the presence or absence of thiamine, was not demonstrated.

The difference in enzyme systems of the three species of Brucella was indicated by a variation in multiplication and utilization of pyruvate and lactate substrate. Peptone decomposition in cultures of Br. suis proceeded at a significantly higher rate than in cultures of Br. abortus and Br. melitensis in which there was a marked preferential breakdown of glucose.

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