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The Influence of Environment on the Antagonistic Relationship between Shigella and Aerobacter: An Ecological Study

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THE INFLUENCE OF ENVIRONMENT ON THE ANTAGONISTIC RELATIONSHIP BETWEEN SHIGELLA AND AEROBACTER; AN ECOLOGICAL STUDY

by

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LIFE

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CHAPTER I

INTRODUCTION AND HISTORY

Ecology is defined as the interrelationship among living organisms and the interaction between them and their environment. The concept of ecology is fundamental to an understanding of the interrelationships among microorganisms in their natural habitats. It is also fundamental to an understanding of the effect of environmental changes on microbial activities. An extensive literature which deals with the relationships between microorganisms has been reviewed by Waksman (1945). Little or no work, however, has been done on the influence of the environment on microbial interrelationships.

The organisms chosen for this investigation, Shigella and Aerobacter, exhibit an antagonistic interrelationship. In ecology, antagonism between species is called interspecific competition. It refers to any interaction between two or more species populations which adversely affect their multiplication or survival. The competitive interaction may involve common space, nutrients, waste materials and many other types of mutual interactions. Competition can result in equilibrium adjustments between two species or it can result in the replacement of one species by another. It is often observed that closely related

organisms having similar habits of life do not occur in the same places. If they do occur in the same places, they use different food, are active at different times or are otherwise occupying different niches. No two species can have exactly the same niche and still be different but closely related species are often so similar as to have virtually the same niche requirements. Competition between species occurs whenever their niches overlap even to a partial extent. It is not known how great the overlap must be before the successful species forces the other out. In any event, experimental and observational research has shown that the rule of one species to a niche is true in a high proportion of cases (Odum, 1959).

For a given species pair, the type of interrelationship between them often changes when the environment is altered. Thus, two species which exhibit antagonism under certain conditions might be completely neutral toward one another under other conditions. The ecological niche of each of the species changes as the environmental conditions change. If the niches no longer overlap, competition between them, theoretically, is no longer expressed.

Since antagonism has been demonstrated between Aerobacter and Shigella both in vitro and in vivo (Hentges, 1958), it seems probable that these organisms occupy similar ecological niches and therefore compete under most environmental conditions. If this is true, do environmental conditions exist in which the niches are

sufficiently different so that the competitive interaction is reduced? Under what environmental conditions do the niches overlap to an even greater extent so that competition is keener? It is the purpose of this investigation to answer these questions. This is accomplished by studying the effects of various alterations in the environment on the competitive interrelationship existing between Aerobacter and Shigella in mixed culture. Studies dealing with the influence of environmental conditions on bacterial populations unfortunately have been confined to pure cultures of bacteria. Nevertheless, these studies have a direct bearing on this investigation and will be reviewed here.

When bacteria are multiplying exponentially, the multiplication rate is determined by certain rate-limiting processes responsible for synthesis of new cellular substance. These rate-limiting processes have a property common to all reaction rates - that of temperature dependence. (Mitchell, 1951)

The influence of temperature on bacterial multiplication has been widely investigated. The rate of bacterial multiplication increases with temperature up to a certain point after which it begins to decrease. This effect was demonstrated by Barber (1908). In his classic study, he isolated single cells of Escherichia coli and placed them in hanging drops at different temperatures. After incubation, the cells were stained and counted directly to determine the rate of multiplication. Barber found that the multiplication rate of Escherichia coli gradually increased with

increased temperature to about 42°C. Lane-Claypon (1909) confirmed this observation. Allen (1953) working with the thermophilic organisms, Bacillus circulans, demonstrated a maximum multiplication rate at approximately 55°C. In comparison, the maximum multiplication rate for Aerobacter aerogenes occurred at 30°C according to the data of Greene and Jaseski (1954).

Lane-Claypon (1909), Johnson (1946) and Allen (1953) pointed out that the dependence of the multiplication rate on temperature can be expressed by the Arrhenius equation:

$$k = s\, e^{\frac{-\Delta Ha}{RT}}$$

where k is the growth rate constant, s a constant, Ha the heat of activation, R the gas constant and T absolute temperature

Over a broad temperature range there appears to be a straight line relationship between the logarithm of the multiplication rate constants and the reciprocals of the absolute temperature. Above this temperature range, the rate of multiplication increases less rapidly than theory demands and the Arrhenius equation no longer applies. An optimum temperature for multiplication rate is reached and as temperature is increased further, the rate of multiplication drops abruptly.

The gradual decline in multiplication rate at temperatures approaching the optimum was also expressed mathematically. Observations were made of the proportionate

increase in multiplication rate for a 10 C rise in temperature over the temperature ranges in which cell multiplication occurred. Barber (1908) showed that the temperature coefficient for Escherichia coli fell from a value of 4 over the range of 5°C to 15°C to a value of 2 over the 27°C to a 37°C range. Slater (1916) and Greene and Jeseski (1954) demonstrated similar phenomena with strains of Lactobacillus and Aerobacter. Jennison (1935) found, with several genera of bacteria, an inverse relationship existing between temperature and temperature coefficients up to the optimum temperature for multiplication. Temperature coefficients were consistently larger in the lower temperature ranges than in the ranges approaching the optimum. Thus, the effect of a temperature increase on the multiplication rate appeared to be less pronounced near the optimum temperature for multiplication than in the lower temperature ranges.

Jennison (1935) also studied the effect of temperature on the length of the exponential phase during bacterial multiplication. His work along with that of Lane-Claypon (1909) indicated that an inverse relationship exists between temperature and time necessary for completion of the exponential phase. Jennison demonstrated that a fairly constant ratio existed (5:1) between the length of the exponential phase in hours and the generation time in minutes at any temperature up to the optimum for multiplication. Despite variations in the length of the exponential phase with temperature changes, the total number of generations remained the same. It

would appear from these results that the size of the total cell crop remains fixed regardless of incubation temperature. Experiments fail to bear this out. Dorn and Rahn (1939) pointed out that maximum total cell populations are often achieved at temperatures below the optimum for multiplication. The yield of cells per unit of substrate decreases near the optimum temperature. This occurs either as a result of an increased rate of side reactions which remove essential metabolites or as a result of destruction of cellular components which necessitates rebuilding. Jordon and Jacobs (1947) attributed the decrease in the percentage of cells which are viable near the optimum temperature to an increase in the production of non-viable cells. In their studies with Escherichia coli, they demonstrated that the multiplication rate continued to increase to the optimum temperature yet a decreased total viable cell population at higher temperatures occurred. The studies of Greene and Jezeski (1954) with Aerobacter substantiated Jordan and Jacobs' findings. These workers found a gradual decrease in the total viable population as temperature was increased. There appeared to be an inverse relationship between temperature and total viable cell crop.

Data on the effect of temperature on the lag phase of bacterial multiplication are few. Barber (1908) reported that a decrease in temperature below the optimum increased the duration of the lag phase of Escherichia coli. Chick (1912) and Penfold (1914) confirmed this observation. In a study by Anderson and Meanwell

(1936) streptococci cultured in milk showed a lag of half an hour at 42°C, 2 hours at 30°C, and 3 hours at 20°C. Recently, Braekkan (1960) demonstrated the occurrence of a lag phase when Lactobacillus arabinosus was incubated at 37°C. When the incubation temperature was lowered to 30°C, the lag phase disappeared. This apparent contradiction was possibly due to differences in optimal temperatures for multiplication. The optimal temperature for Lactobacillus arabinosus is below 37°C. Thus the duration of the lag phase increased above the optimum as well as below it.

The pH of the environment also affects bacterial multiplication. For normal metabolism and reproduction to occur in the bacterial cell, the pH level must be suitable for the effective functioning of cellular enzyme systems. Not only must the important energy producing and synthetic enzyme systems function but various coordinate systems must continue so that the chain of reactions is not broken. For a complete understanding of the effects of pH on bacterial behavior, it is necessary to study the effects of pH on each enzyme system involved in synthetic and metabolic activities (Hewitt, 1957). An unravelling of the tangled skein of pH effects has only begun. Attention has necessarily been confined to the gross effects of pH on bacterial behavior.

A majority of the early studies was concerned with the effect of H - ion concentration in the medium on bacterial multiplication. Shohl and Janney (1907) studied the effects of pH

on the multiplication of Escherichia coli. Their results showed that as the pH of the medium deviated from the optimum of about 6.5, marked changes in bacterial multiplication occurred. The organisms were sensitive to changes of 0.2 on the pH scale. Multiplication was completely inhibited at a pH of 4.6 to 5.0 and at a pH of 9.2 to 9.6. Dernby and Avery (1918) showed that the pH range for multiplication of pneumococci was high on the pH scale and narrow when compared to the range for Escherichia coli. The optimum hydrogen ion concentration of the medium was at a pH of about 7.8. It appeared to be the same for a number of pneumococcal types. Multiplication did not occur at all in media in which the pH was lower than 7.0 or greater than 8.3. Cohen and Clark (1919) observed a broad range of pH for the multiplication of Shigella and Aerobacter. They emphasized that slight changes of pH at the extreme of this range produced the most pronounced decreases in the multiplication rates. Since the effect was so sharp, the method was used for the definition of the limiting pH ranges for several bacterial species.

Sherman and Holm (1922) pointed out, however, that the composition of the medium affects the pH range for bacterial multiplication. The range for Escherichia coli in a peptone medium was narrow. It increased when 0.2 molar NaCl was added to the medium. The effect of NaCl was tested at pH values representing the approximate limit of multiplication in the acid region. At a pH of 4.8, Escherichia coli rarely multiplied without NaCl. It

multiplied in the same medium upon the addition of NaCl. The effect was not general for all bacteria studied, but it was even more pronounced with Alkaligenes. Similar results were obtained using other inorganic slats but the degree of range-broadening effect varied with the nature of the salt. Conversely, an organic salt such as sodium citrate narrowed the limits.

The compensatory mechanisms exhibited by some strains of bacteria complicate determination of the pH limits for bacterial multiplication. As early as 1917, Fred and Loomis, investigating the effects of soil pH on the multiplication of alfalfa bacteria, noticed that at extremely high or low pH the organisms move the pH toward neutrality. While studying enzymatic mechanisms in Escherichia coli, Gale and Epps (1942) showed that the shift in the pH of the medium is accomplished by a dual enzyme mechanism. There is a group of amino acid decarboxylases with optimum activity at a pH of about 4 and almost no activity above pH 5.5. In a medium of low pH, the decarboxylases cause a rise in the pH of the media. There is also a group of amino acid deaminases which have optimum activity at about pH 8 and cause a lowering of pH.

Gale and Epps also studied the effect of pH on the formation and activity of a number of other enzymes of Escherichia coli. The enzymes were found to fall into two distinct groups. In the first group, including formic dehydrogenase, formic hydrogenase, urease, and catalase, the formation of the enzyme by the multiplying organisms increased as the pH of the medium was varied from the

optimum for enzyme activity. Thus the loss of activity due to unfavorable pH was compensated by an increase in the amount of enzyme produced. In the second group, including the amino acid decarboxylases and deaminases, enzyme production was most rapid near the optimum pH for enzyme activity. Gale and Epps concluded that enzymes of the first group have a protective role in the cell and function over a wide pH range. Enzymes in the second group act as neutralization mechanisms functioning only when the pH of the environment is extreme.

Shohl and Janney (1917) demonstrated an increased lag phase as the pH of the medium deviated from the optimum for multiplication. These results were confirmed by Cohen and Clark (1919). According to their findings, the lag phase for Escherichia coli was prolonged in alkaline media.

In a bacterial culture, the oxidation-reduction systems of the cellular enzymes, the carriers and the substrates form a complex network of potentials which are interrelated by rigidly controlled enzyme pathways. These systems are not in equilibrium with each other. The potential measured in a culture is that of nutrients and waste products, not of the cells themselves. Nevertheless, the activities of bacterial cultures are related to the oxidation-reduction potentials of the media (Mitchell, 1951). Thus aeration of medium effects bacterial behavior.

The studies of Winslow, Walker, and Sutermeister (1932) and Rahn and Richardson (1942) demonstrated that aeration prolonged

the lag phase. These workers attributed this effect to either the removal of intermediate substances necessary for enzyme synthesis or the removal of CO_2 essential for initiation of bacterial multiplication (Valley and Rettger, 1927).

Different bacteria appear to react differently to aeration. Winslow, Walker and Sutermeister (1932) showed that the multiplication rate of Escherichia coli was increased with aeration. In peptone broth the multiplication rate increased 32%, and increased by 15% in lactose broth. As a result, the viable cell count at about seven hours was nine times as great in the aerated as in the non-aerated peptone medium and three times as great in standard lactose medium. Barnes and Dewey (1947) demonstrated a tremendous increase in the multiplication rate of Shigella flexneri in a beef-extract medium when a large surface area was exposed to air. On the other hand, Wilson (1930) working with what he classified as Bacterium aertrycke, found that the multiplication rate was much the same under conditions of aeration and anaerobiosis. The results of Rahn and Richardson (1942) with Bacillus mesenterious support Wilson's findings.

Other workers demonstrated an inhibitory effect of oxygen. Winslow, Walker and Sutermeister (1932) found that the multiplication rate of Escherichia coli increased only when aerated in a rich medium. When a dilute medium was substituted, the aerated culture failed to multiply at all. Cohn-Bronner (1940) attributed this phenomenon to an inadequate supply of carbon

compounds. He cultured Escherichia coli in a synthetic medium containing only mineral salts, a single carbon source (lactic acid) and a single source of nitrogen (NH_4Cl). As the quantities of these constituents were decreased in aerated cultures, the line of bacterial growth became deeper below the surface of the medium. When the source of carbon was kept constant but the nitrogen source decreased, the phenomenon did not occur. When the carbon source was decreased, and the nitrogen source kept constant, then the characteristic layer formation occurred in aerated cultures. Thus, increased sensitivity to oxygen is due to an insufficient supply of carbon compounds, i.e., a lack of an easily oxidizable substance or an adequate oxygen acceptor. The less carbon in the medium, the more inhibitory is the action of the oxygen or the action of a substance formed by bacteria in the presence of oxygen.

In a favorable medium, under favorable conditions, a bacterium, as a rule, will quickly adapt itself to the new environment (Porter, 1947). Hence, the lag phase will be short. The lag phase may be prolonged for some time in a medium which is not optimal for multiplication. Heheworth, (1901) reported that transfer of a culture of Escherichia coli or Salmonella typhosa to a fresh tube of the same medium in which the organism was multiplying was accompanied by a shorter lag than transfer to a different medium. Winslow, Walker and Sutermeister (1932) noted with Escherichia coli a difference of six hours in the lag phase in peptone broth and in a synthetic medium, the synthetic medium

showing the longer lag.

Sometimes the length of the lag period differs in two media even though the parent culture is multiplying in each. As an illustration, Coplans (1910) and Penfold (1914) transferred a dulcitol-peptone broth culture of Escherichia coli into dulcitol-peptone broth and a plain peptone broth culture into plain peptone broth. The dulcitol-peptone broth consistently showed a longer lag than the peptone broth even though the parent cultures were the same age.

As a general rule, the more favorable the concentration of nutrients in the medium, the more rapid is the rate of multiplication (Porter, 1947). This is true whether dealing with the energy source, an essential amino acid or with essential ions. An example of the influence of substrate concentration on bacteria is the effect of inorganic phosphate concentration on the multiplication rate of Aerobacter aerogenes (Dagley and Hinschelwood, 1938). The optimum phosphate concentration for maximum multiplication was extremely small - approximately 3×10^{-5} moles per liter. At this concentration the mean generation time was 44 minutes. At .025 optimal concentration the generation time increased to 330 minutes. Penfold and Norris (1912) carried out experiments with Salmonella typhosa to determine the effect of peptone and glucose concentrations on the multiplication rate. The peptone concentrations were varied between .0125% and 1.25%. Tubes containing these various

concentrations of peptone were inoculated with from 200 to 500 organisms per ml. of medium. The cultures were incubated at 37°C and plate counts were made at regular intervals. Results indicated that there was a rapid decrease in the multiplication rate as the concentration of peptone fell below 1%. At .0125%, very little multiplication occurred. As the peptone concentration was decreased, the generation time increased from a minimum of 40 minutes in 1% peptone to about 800 minutes in .0125%. When a small quantity of glucose (0.175%) was added to the peptone medium, the multiplication rate increased in cultures containing low concentrations of peptone. In the case of 1% peptone, the multiplication rate was not greatly increased. At lower concentrations, the generation time fell from 111 minutes in the absence of glucose to about 50 minutes when glucose was added.

Other workers also demonstrated the beneficial effect of glucose. Heap and Cadness (1924) found that the addition of 2% glucose to peptone medium increased the multiplication rate of Salmonella typhimurium. Monod (1949) studied the rate limiting effect of glucose on both Escherichia coli and Mycobacterium tuberculosis. The rate of multiplication was related to the concentration of glucose only at very low levels. Escherichia coli responded proportionately when the glucose concentrations were less than 0.001 moles. For Mycobacterium tuberculosis the glucose concentration could be increased to about 0.05 moles.

Monod (1949) found that the carbohydrate concentration

also affected the size of the stationary cell population. When Escherichia coli was cultured in a synthetic medium with mannitol as the sole carbon source, the population size was found to be proportional to the mannitol concentration. This relationship held only with low concentrations of mannitol. Similar results were reported with other compounds. Van Niel (1944) demonstrated that the stationary cell population of the non-sulfur purple bacteria was rigorously proportional to the amount of acetate present in the synthetic medium as long as the acid is added in small quantities. Dagley, Dawes, and Morrison (1951) working with Aerobacter aerogenes, found a similar relationship when the nitrogen source was the limiting factor. Balls and Brown (1925) cultivated Saccharomyces cerevisiae in a medium containing ammonium salts and beet sugar molasses. At intervals during multiplication, the cell population and the concentration of sugar in the medium were measured. Cessation of multiplication occurred after approximately 8 hours incubation when the sugar in the medium was almost exhausted. The addition of more sugar at this time postponed for about 3 hours the onset of the stationary phase. The authors concluded from the results that the onset of the stationary phase was due to exhaustion of essential nutrients in the medium.

If nutrient depletion is responsible for the onset of the stationary phase, it would seem that the size of the stationary cell population should be proportional to the concentration of available foodstuffs. Monod (1949) and Van Neil (1944) pointed out

that this linearity held only with low concentrations of nutrients. Dagley, Dawes and Morrison (1951) showed that the linearity broke down with glucose concentrations above 8.6×10^{-3} molar or nitrogen ($(NH_4)_2SO_4$) concentrations above 1.3×10^{-3} molar. Where the glucose concentration was limiting (below 8.6×10^{-3} molar) none could be detected in the stationary phase supernatants but in concentrations above the range of linear increase, excess glucose was present. Similarly, excess ammonia was demonstrated in cultures containing ($(NH_4)_2SO_4$) concentrations above the range of linear increase. Thus cell division ceased in the presence of excessive substrates.

Other evidence tended to refute the theory that the stationary phase is solely the result of foodstuff exhaustion. Penfold (1914) centrifuged a 24 hour broth culture of Escherichia coli. The few bacteria remaining in the supernatant fluid rapidly multiplied when reincubated. Graham-Smith (1920) sterilised cultures of Escherichia coli and Staphylococcus aureus which had attained a maximal stationary population. Reinoculation with the original species resulted in demonstrable growth. From these experiments it appeared that other factors might play a role in the initiation of the stationary phase.

The onset of the stationary phase was attributed to the depletion of oxygen in the culture medium by some workers. Rahn and Richardson (1942) cultured Bacillus mesentericus in peptone broth under continuous aeration. Multiplication continued rapidly

through the twelth hour resulting in a large stationary cell population. When the culture was kept under a layer of oil, multiplication ceased after about five and one half hours. The resultant stationary population was greatly reduced. This phenomenon was also demonstrated with Salmonella typhimurium by Wilson (1930). In cultures incubated anaerobically multiplication ceased comparatively early and the total stationary population was determined at 5×10^8 cells per ml. In 5% oxygen, multiplication continued for a longer period and the total population reached 2×10^9 cells per ml. In 100% oxygen, multiplication continued still further and the total stationary population was estimated to be 8×10^9 cells per ml. Based on these observations, Barnes and Dewey (1947) developed a method for obtaining large numbers of organisms in high concentrations for laboratory use. Shigella flexneri organisms were cultured in vessels which permitted aeration of a large surface area of the medium. By this method, cell concentrations were obtained 8 to 10 times greater than usual. Lodge and Hinshelwood (1943) aerated a culture of Aerobacter aerogenes which had already attained its stationary population size under non-aerated conditions. Aeration reinitiated cell multiplication and the total stationary population reached a size comparable to that of a continuously aerated culture. These investigators considered the favorable effect of aeration to be a result of removal by oxygen of an inhibitor formed during the exponential phase. The inhibitor was probably not volatile because

the maximum stationary population reached was independent of the rate of aeration but was dependent on the concentration of oxygen in the gas mixture (Wilson, 1930). From these experiments it appeared that the factor responsible for onset of the stationary phase might be oxygen supply. But this hypothesis did not explain the fact that a stationary phase finally occurred despite vigorous aeration of the culture. Nor did it apply to such organisms as streptococci or lactobacilli which were not affected by aeration (Rahn and Richardson, 1942).

Some investigators maintain that cessation of cell multiplication is governed by accumulation of toxic products in the medium. The size of the total stationary population is inversely proportional to the rapidity with which inhibiting agents are produced by the organisms. For example, Chesney (1916) demonstrated that filtrates from 24 hour pneumococcal cultures inhibited further multiplication of pneumococci. Aliquots of the culture were removed at the onset of the exponentail phase and also at the onset of the stationary phase. The aliquots were centrifuged so that only a few organisms remained behind in the supernatant fluids. On further incubation, the supernatant fluid taken at the onset of the exponential phase showed multiplication. The bacteria in the supernatant fluid obtained at the end of the exponential phase did not continue to multiply but decreased in number. The fact that a close parallism existed between the behavior of bacteria in the supernatant fluid and the behavior of

bacteria subjected to the action of disinfectants, suggested to Chesney that the supernatant fluid contained injurious substances capable of destroying large numbers of bacteria.

The exact nature of such injurious substances was the subject of a great deal of speculation. According to McLeod and Gordon (1922) the inhibitory substance which developed in pneumococcal cultures was hydrogen peroxide. Their conclusion was based on chemical tests for the presence of peroxides in the culture medium. The authors extended the studies with pneumococci to include other gram positive organisms. They (Gordon and McLeod, 1926) found that various amino acids present in the culture medium inhibited bacterial multiplication. Tryptophane was most toxic and inhibited the greatest variety of bacteria. The authors speculated that the toxic substance results from the deamination of certain amino acids.

Several workers speculated that cessation of multiplication is simply the result of acid accumulation in the medium. Callen and Chesney (1918) observed that pneumococci multiplied in beef infusion broth until the pH of the medium dropped to approximately 7. At this pH a stationary phase was initiated. The same year (1918) Dernby and Avery showed that pneumococci failed to multiply at all in medium of pH 7 or lower. From these results the authors speculated that bacterial multiplication ceases when the pH of the medium reaches a definite value which varies with different species. This contention was

supported by Cohen and Clarke (1919) who worked with Escherichia coli. In a glucose-peptone medium, multiplication continued only until the pH of the medium approached the region found to inhibit bacterial multiplication.

Other results, however, showed that the pH cannot be considered the sole factor limiting multiplication. Although the onset of the stationary phase often coincided with the establishment of an adverse pH in the medium, Cohen and Clark (1919) showed that this was not always the case. In a medium lacking carbohydrate, the pH remained constant throughout the exponential phase for Escherichia coli and during the onset of the stationary phase. Lodge and Hinshelwood (1939) inoculated Aerobacter aerogenes into media of various pH values. The pH was measured initially and at the onset of the stationary phase. Results showed that the pH measurements made at the onset of the stationary phase varied greatly depending on the initial pH of the medium. Cultures multiplied in media with an initial pH considerably lower than many pH values recorded at the onset of the stationary phase. Further they found, in dilute media, where exhaustion of nutrients might be a limiting factor, the size of the stationary population was independent of pH. The establishment of the stationary population was influenced primarily by nutrient exhaustion rather than pH effects. In more concentrated medium, however, the accumulation of metabolic products and drop in pH brought on by the acid forming reactions of the cells played a more important part. On the basis

of quantitative observations. Hinshelwood (1946) speculated that that mode of initiation of the stationary phase depends on the composition of the medium.

Bail (1929) studied the problem in some detail and finally decided that there is a maximum viable population for each bacterial speciedwhich is never surpassed regardless of environmental factors. To this maximum viable stationary population he applied the term "M-concentration". The "M-concentration" value varied with different species. For example, the "M-concentration" reached by Shigella species was never as great as that reached by Aerobacter species.

Fukuda (1929) investigated the concept of "M-concentration" and gave some support to Bail's claims. He believed that the concept of "M-concentration" is only applicable to certain bacterial species. To prove his contention, he demonstrated that if broth cultures of Pseudomonas aeruginosa, sterilized by heat, were reinoculated with fresh organisms, multiplication occurred again until the "M-concentration" was attained. With Salmonella gallinarum, on the other hand, only one quarter of the "M-concentration" was reached after heating the original culture.

The theory proposed by Bail was adopted by von Wikullil (1932) in an effort to explain antagonism on the basis of "M-concentration". When two organisms having extremely different "M-concentrations" such as 1.6×10^9 and 3.0×10^8 per ml. were

inoculated together, the "M-concentration" reached 1.6 x 10^9 cells per ml. In this experiment the total viable population was made up almost entirely of the species with the larger "M-Concentration". Thus the organisms with the larger "M-concentration" value successfully competed for available biological space. Antagonism was attributed solely to a difference in "M-concentration" values.

Other theories, advanced to explain the stationary phase of the population cycle, were subsequently adopted and revised to explain antagonistic mechanisms. Several investigators advanced the theory that antagonism between two bacterial species simply represents the outcome of a competition for available nutrients or oxygen (Pasteur, 1878, Freudenreich, 1888, Broom, 1929). One of the organisms competes successfully for essential nutrients. Other workers attributed antagonism to the establishment of adverse physicochemical conditions in the medium. (Tissier and Martelly, 1902). The outcome of the competition depends to a great extent on the relative degree of resistance of each of the species to the environmental conditions.

Studies were undertaken by Wynne and Norman (1953) to determine the mechanism by which an Aerobacter strain exerts its antagonistic effect. A flask of nutrient broth was inoculated with Aerobacter aerogenes. Cellophane dialysis bags containing sterile nutrient broth were immersed in the flask and the entire apparatus was incubated at 37°C. After 48 hours incubation, the cellophane bags were inoculated with various organisms including

Shigella strains. On further incubation no multiplication inside the cellophane bags was evident. When the broth containing test species was removed from the cellophane bags and reincubated, there was only transient inhibition after which test organisms multiplied at a rate equal to untreated controls. It did not appear that depletion of nutrients accounted for the antagonism. Wynne and Norman speculated that antagonism was probably due to production of an unstable toxic substance to which the test strains (including *Shigella*) were sensitive.

In summary, a review of the literature shows temperature, pH, oxygen supply, and nutrient supply all have an effect on both the multiplication rates and on maximum sizes of microbial populations. Several of these conditions of environment have been implicated as factors responsible for bacterial antagonism.

In mixtures of *Aerobacter* and *Shigella*, an antagonistic effect of *Aerobacter* on *Shigella* has been clearly demonstrated both *in vivo* and *in vitro* (Hentges, 1958). The mechanism of this antagonism has not been explored except for the rather inconclusive study by Wynne and Norman (1953) which was just described. No comprehensive study has been made of a specific association of bacteria in which environmental factors are taken into account. Such information might lead to an understanding of the mechanisms by which bacteria exert an antagonistic effect. A study was therefore planned to examine the effect of controlled alterations in the environment on the population equilibrium which exists

between Shigella and Aerobacter. Cultures were studied under various conditions of temperature, hydrogen ion concentration, oxygen supply and nutrient concentration. In conjunction with each experiment, growth curves were plotted for each species in association and also for pure culture controls. Pure culture controls were included so that a comparison can be made of the behavior of each of the organisms in mixed cultures with behavior in pure cultures. This provided data for determination of the extent to which the behavior in the mixture could be predicted from the behavior in pure culture.

Ecological problems are usually undertaken first by identifying the organisms in the habitat and then by studying the effects of such broad characteristics of environment as temperature, oxygen supply or food supply on the organisms (Odum, 1959). Microbial ecology begins with the isolation of pure cultures of organisms and the identification of the organisms by well known bacteriological methods. In what might be considered experimental microbial ecology, the cultures are mixed in order to study their interrelationships (Waksman, 1945). Investigators have not extended these studies to include the effects of environmental factors on the mixtures as the conventional ecologist might do. Consequently, no studies exist of bacterial associations under a variety of environmental conditions.

CHAPTER II

PROCEDURES AND RESULTS

A. Microorganisms

The organisms used for this study were taken from stock cultures maintained at the Stritch School of Medicine. They were transfered every 2 months on veal infusion agar slants and were stored under refrigeration.

The *Aerobacter* and *Shigella* strains were identified with the aid of morphological, biochemical and serological tests. The gram stain and the flagella stain (Leifson, 1951) revealed that both organisms were gram negative, non-flagellated rods. Results of biochemical tests are presented in Table I. These tests were performed at the beginning of the investigation and at its termination. From the results, the organisms were identified as *Shigella flexneri* and as *Aerobacter aerogenes*. Tests done at the State of Illinois Department of Public Health confirmed both of these identifications. The *Shigella flexneri* strain was identified serologically as type II. The serotype of the *Aerobacter* strain was not determined.

B. Plating Methods

1. Flood plate method

A plating method was developed for enumeration of

pure cultures of Shigella and Aerobacter and for differential counting of the two species multiplying in mixed culture. (Hentges and Fulton, 1960) Petri dishes having metal tops lined with absorbent disks were used. After the plates were poured and the agar had hardened, they were placed in a 37°C incubator for 48 hours to dry the surface of the agar. When the plates had dried, 1 ml of liquid, consisting of a culture suspension or a saline dilution, was pipetted onto each plate. The plate was tilted back and forth until the liquid was distributed over the entire agar surface. The covered plate was then placed, top up, on a level surface until the liquid was absorbed. This occurred within 20 minutes.

Plates inoculated by flooding in this manner were compared with poured plates. The total number of colonies counted proved to be the same in both poured and flooded plates. The flooded plates were easier to count because the surface colonies grew to a larger size in a shorter incubation time. It was practically impossible to differentiate subsurface colonies of Shigella and Aerobacter when the standard pour plate method was used. In contrast, surface colonies of Shigella and Aerobacter on the flooded plates were readily distinguishable because of differences in size and convexity. Colonies were uniformly distributed over the surface at random, as would be expected for a Poisson distribution. On a plate with a mixture of 300 Aerobacter and Shigella colonies, no difficulty was experienced in assigning

each colony to its proper catagory.

2. VIV Agar

Preliminary experiments were carried out with mixed cultures of Shigella and Aerobacter incubated for 24 hours at 37°C in brain heart infusion broth. When cultures were streaked on desoxycholate agar (Difco), and the agar plates were incubated, Aerobacter colonies appeared but no Shigella colonies were observed. If Shigella organisms were present in the mixed culture, they could not be detected by this method.

Thus, a plating method was required for detection of a small number of Shigella organisms coexisting with a large number of Aerobacter organisms. The method was based on an observation that the Aerobacter strain was more sensitive to Viocin (Viomycin sulfate, Pfizer) than the Shigella strain. When 0.05 mg/ml Viocin was incorporated in veal infusion agar medium (BBL), Aerobacter colonies developed poorly on the agar plates but Shigella colonies appeared to develop in the usual manner. (Table II-A) For each plate a rough estimate was made of the extent of colonial development. If 1% glucose was added to the agar medium and the mixture was autoclaved twice (115 pounds pressure for 15 minutes) 0.4 mg/ml Viocin could be incorporated in the agar medium without greatly affecting development of Shigella colonies. Aerobacter colonies failed to develop at all under these conditions (Table II-B). Veal infusion agar prepared with the addition of 1% glucose and autoclaved twice before incorporation of 0.4% Viocin

was referred to as VIV agar. It was necessary to add glucose to the agar medium to obtain the desired results. When glucose was omitted, a statistically significant decrease occurred in the number of Shigella colonies appearing on the plates (Table III). This was demonstrated by comparing the mean colony counts on agar plates containing glucose with counts on plates without glucose.

VIV agar was adopted for detection of Shigella organisms in pure and mixed culture. Veal infusion agar was used for detection of Aerobacter organisms. When VIV agar was employed in conjunction with the flood plate method, it was possible to detect the presence of a very small number of Shigella organisms in mixture with as many as 10^8 Aerobacter organisms per ml. medium. There was no statistically significant difference in the number of Shigella colonies which developed on VIV agar whether Shigella was diluted in saline or in a heavy suspension of Aerobacter (Table IV). A comparison of colony counts showed that the presence of Aerobacter cells appeared to have no effect on the development of Shigella colonies. Aerobacter colonies rarely appeared on VIV agar.

A comparison was made of the relative effectiveness of various agar media for the development of Shigella colonies. When both veal infusion agar plates and VIV agar plates were flooded with a Shigella inoculum derived from veal infusion agar, significantly fewer colonies developed on VIV agar plates than on veal infusion agar plates (Table V-A). When the inoculum was

derived from VIV agar, there was no statistically significant difference in the number of colonies which developed on the two types of agar (Table V-B). Nor was there a significant difference between the number of colonies of blood agar and VIV agar (Table VI). There was a statistically significant decrease in the colony count, however, when the inoculum derived from VIV agar was flooded on desoxycholate agar (Table VI). Similar results were obtained with Aerobacter. Colonies appeared to develop equally well on either veal infusion agar or blood agar. Colony counts decreased significantly on desoxycholate agar (Table VII).

C. Synthetic Media

12-S medium supported multiplication of both the Shigella and Aerobacter strains. It was composed of:

12-S Medium:

L-Glutamic acid	0.5%
Glucose	1.0%
Na_2HPO_4	0.6%
KH_2PO_4	0.2%
$MgSO_4$	0.01%
NaCl	0.1%
NH_4Cl	0.1%
Niacin	0.001%

The pH of the medium was approximately 7.0 without further adjustment.

Experimentation revealed that the nutrient requirements for the Aerobacter species were not complex. A medium consisting

only of 0.1% glutamic or aspartic acid and 0.5% NaCl was capable of supporting multiplication of the Aerobacter strain. Development of turbidity in the medium after 24 hours incubation of the culture at 37°C was taken as evidence of multiplication. The amino acid-NaCl medium sustained multiplication of Aerobacter through at least 3 successive serial transfers. Amino acids served as the sole source of carbon and nitrogen. When glucose and inorganic nitrogen were substituted for the amino acid, the medium was incapable of consistently supporting multiplication (Table VIII). This was demonstrated by inoculating 10 tubes of a medium containing glucose and ammonium chloride and 10 tubes of a medium containing glutamic acid with approximately 10^2 Aerobacter cells. A comparison was made of the number of tubes showing turbidity after 48 hours incubation.

It is of interest to note here that several workers have demonstrated that the nutrient requirements for Shigella species are quite exacting. Koser et. al. (1938) for example, showed that niacin was essential for multiplication of Shigella. Although individual strains varied in their requirement for this vitamin (Weil and Black, 1944) the majority of Flexneri strains required niacin to multiply in synthetic media (Dorfman et. al. 1939). Erlander and Mackey (1958) showed that an organic nitrogen source was necessary for multiplication of Shigella flexneri. Ammonium salts could not be substituted for an amino acid. Aspartic acid was the most effective amino acid. A carbon source in addition to

aspartic acid was necessary for multiplication of the Flexneri strains. Erlander and Mackey (1958) developed a simple synthetic medium for cultivation of Shigella flexneri strains. The medium, consisting of mineral salts, glucose, aspartic acid, thiamin and niacin, supported multiplication of all 6 serotypes of Shigella flexneri.

The medium of Erlander and Mackey (1958) was incapable of supporting multiplication of the Shigella strain used in this investigation, however. The strain specifically required glutamic acid as the nitrogen source - no other amino acid could be substituted. Table IX shows results of attempts to substitute other amino acids for glutamic acid. The Shigella strain multiplied favorably with glutamic acid only. A medium consisting of glutamic acid, glucose or glucosamine, niacin and a phosphate buffer sustained multiplication through at least 5 successive serial transfers. The concentration of carbohydrate and amino acid could be reduced to 0.1% without seriously impairing multiplication (Table X). The total cell population appeared to be greater with glucosamine, although either glucose or glucosamine could serve as the carbohydrate source (Table XI). Tubes containing various concentrations of glucose and glutamic acid were inoculated with Shigella and inspected after 24 hours incubation. The degree of turbidity was judged and recorded as ++++ indicating heavy turbidity and + slight turbidity. Only at 0.05% concentration was no turbidity apparent. Incorporation of

NaCl into the medium increased its effectiveness for support of Shigella multiplication (Table XII). A large number of tubes with and without 0.5% NaCl were inoculated with Shigella and observed for turbidity. Multiplication occurred in 69 of 70 tubes with NaCl but in only 61 of 82 tubes without NaCl, a statistically significant difference.

These observations resulted in the formulation of Medium-S. It consisted of:

S-Medium:		
	L-Glutamic acid	0.1%
	Glucosamine hydrochloride	0.1%
	Na_2HPO_4	0.6%
	KH_2PO_4	0.15%
	NaCl	0.5%
	Niacin	0.001%

No special precautions were taken in preparing the medium except that the glucosamine was autoclaved separately. The pH of the medium was approximately 7.0. S-medium effectively supported multiplication of both the Shigella and Aerobacter strains through at least 5 successive serial transfers. Viable cells of both species could be recovered from the medium after 40 days incubation at 37°C.

Experiments with S-medium confirmed some earlier observations on the nutritional requirements of Shigella species. Niacin was required by the Shigella strain (Table XIII). No multiplication occurred when it was deleted from the medium.

Ammonium salts were incapable of replacing glutamic acid as the nitrogen source for Shigella multiplication (Table XIII). Glutamic acid apparently could not function both as a nitrogen and a carbon source, since the Shigella strain failed to multiply if a carbohydrate was ommitted from the medium (Table XIII).

D. Inocula

A rapid method was developed for quantitative estimation of Shigella and Aerobacter organisms for use as inocula. The method was based on observations that the Shigella strain attained a viable population of approximately 1.0×10^7 organisms per ml when cultured in S-medium 18 hours at 37°C. The Aerobacter strain attained a population of approximately 3.0×10^8 organisms per ml when cultured under the same conditions.

1. Shigella Inoculum

A VIV agar plate was streaked with a stock culture of Shigella. The plate was incubated for 28 hours at 37°C. Approximately 10 isolated colonies were picked from the plate and suspended in 10 ml S-medium. The suspension was incubated for exactly 18 hours in a 37°C water bath. After incubation a 10-fold and then a 100-fold dilution was made of the culture in the S-medium. The resultant suspension contained approximately 10^4 organisms per ml medium.

2. Aerobacter Inoculum

A stock culture of Aerobacter was streaked onto a veal infusion agar plate. The plate was incubated for 22 hours at

37°C. Portions of about 5 isolated colonies were picked from the plate and suspended in 10 ml S-medium. The suspension was incubated for 18 hours in a 37°C water bath. After incubation, a 3-fold dilution, then a 100-fold dilution, then again a 100-fold dilution was made of the culture in S-medium. The resultant suspension contained approximately 10^4 organisms per ml medium.

E. Standard Conditions for Cultivation

Under standard conditions the organisms were cultivated in S-medium. Two ml of a dilution of approximately 10^4 Shigella organisms was added to 98 ml sterile S-medium. Two ml of a dilution of approximately 10^4 viable Aerobacter organisms was added to 98 ml S-medium. For pure cultures, 5 ml of either the S-medium inoculated with Shigella or the medium inoculated with Aerobacter was combined with 5 ml sterile S-medium in a 15 x 1.75 cm tube. This resulted in a final concentration of approximately 1.0×10^2 organisms per ml medium. The small inoculum insured an exponential phase of adequate duration for easy definition. For mixed cultures, 5 ml of the S-medium inoculated with Shigella was combined with 5 ml of the S-medium inoculated with Aerobacter. The resultant mixture contained approximately 1.0×10^2 organisms of each species per ml of medium. The S-medium (pH 7.0) culture tubes were plugged with cotton, covered with metal caps and placed in a 37°C water bath for incubation.

F. Determination of Population Size

At intervals during incubation, 0.1 ml or 1 ml aliquots

were removed from the culture tubes. 10-fold and 100-fold dilutions were made with saline when necessary. One ml of Shigella culture or an appropriate saline dilution was flooded on the surface of a previously dried VIV agar plate. Aerobacter cultures were flooded on the surface of veal infusion agar plates. Both VIV and veal infusion agar plates were used for mixed cultures. Inoculated veal infusion agar plates were incubated for 24 hours in a 37°C air incubator before enumeration of Aerobacter colonies. VIV agar plates were allowed to incubate 48 hours to ensure sufficient development of Shigella colonies. An electronic colony counter (New Brunswick Scientific Co., Model C-100) was used for enumeration of Shigella and Aerobacter colonies.

G. Alterations in Environmental Conditions

1. Temperature

a. Departure from standard conditions

The culture tubes were placed in water baths adjusted to various temperatures. Water bath temperature remained relatively stable during the experiments. The temperature did not fluctuate more than + 1°C.

b. Temperature ranges

Observation of both Aerobacter and Shigella cultures at different temperatures permitted a rough estimate of the temperature ranges over which the organisms multiply (Table XIV). Cultures were incubated a total of 48 hours. At 24 hours and at 48 hours tubes were observed for turbidity, which was

taken as evidence of multiplication. Results showed that the temperature range for multiplication of the Aerobacter strain was much broader than the range for the Shigella strain. There was no evidence of turbidity in the Shigella cultures at 25°C or at 44°C. Multiplication of Aerobacter occurred at the lower temperatures but there was no turbidity at 44°C.

The optimum temperature for the multiplication of the Shigella strain appeared to be in the vicinity of 37°C. An optimum for the Aerobacter strain was not apparent. Using more refined methods, Greene and Jazeski (1954) set the optimum temperature for the multiplication of Aerobacter aerogenes at 30°C.

c. Growth curves; pure and mixed cultures

The number of viable cells in cultures were determined at intervals during incubation at 37°C (Figure 4). Although no determinations were made before 5 hours incubation, it appeared that both the Aerobacter and the Shigella strains exhibited a lag phase. The lag phase for the Shigella strain was clearly evident; it was less evident with the Aerobacter strain. The Aerobacter strain appeared to multiply faster than the Shigella strain. Its total viable population was greater than Shigella after 48 hours incubation.

The multiplication rate for the Aerobacter strain, the length of its exponential phase and the size of its total viable population were approximately the same in mixed culture and in pure culture. With Shigella, the multiplication rate was the same

in mixed and in pure culture, but in mixed culture, the length of the exponential phase was greatly shortened. This resulted in a 1000-fold reduction in the size of the viable population after 48 hours incubation. Extrapolation of growth curves (Figures 1 thru 13, dotted lines) in a manner based on the interpretation of growth curve forms by Bushanan (1918) demonstrated that the negative acceleration phase for the Aerobacter strain was initiated probably after about 12 hours incubation. In pure cultures of Shigella, this phase was not initiated until sometime after 27 hours. In mixed cultures, the negative acceleration phase occurred after about 11 hours incubation - nearly the same time it occurred with the Aerobacter strain.

Comparison of growth curves (Figures 4 and 5) revealed that the duration of the lag phase was greater at 30°C than at 37°C. An increased lag phase at lower temperatures was reported earlier by Barber (1908), Chick (1912), Penfold (1914) and Anderson and Meanwell (1936) who studied the effects of temperature on bacteria. The multiplication rate for the Shigella strain was considerably lower at 30°C than at 37°C. It appeared to be about the same for the Aerobacter strain both at 30°C and at 37°C.

At 30°C, as at 37°C, the multiplication rate for the Aerobacter strain, the length of its exponential phase and the size of its total viable population, were about the same both in pure and in mixed culture. The negative acceleration phase occurred in each case probably after about 14 hours incubation at

30°C. This phase was initiated at about the same time for Shigella in mixed culture, when the culture appeared to be in the early stages of the exponential phase. The premature initiation of the negative acceleration phase resulted in a greatly reduced viable population. The ratio of viable Aerobacter cells to viable Shigella cells in mixed culture was at least 10 times greater after 24 hours incubation at 30°C (5.6×10^5 to 1) than at 37°C (2.3×10^4 to 1).

At 44°C, little or no multiplication occurred with Shigella. In the course of the experiments, the organisms died off (Figure 6). After 48 hours incubation no viable cells could be recovered. At 44°C, the Aerobacter strain multiplied at a very slow rate. Turbidity was not apparent in any of the culture tubes after 48 hours incubation although a viable population of nearly 10^7 cells per ml was recorded at this time in some instances. Thus, in mixed culture, only Aerobacter cells were recoverable after 48 hours incubation.

2. pH

a. Departure from standard conditions

Standard control cultures were run in S-medium at pH 7. pH adjustments were made simply by adding NaOH or HCl to S-medium until the desired pH was attained. All pH measurements were made with a Coleman Metrion pH Meter. The pH of the culture medium was determined at the beginning of each experiment. With growth curve studies, it was determined at the termination of each

experiment as well, permitting a check of the efficiency of the phosphate buffer.

b. pH ranges

Observations of both Aerobacter and Shigella cultures in media of different pH values permitted a rough estimate of the pH ranges over which the organisms multiplied (Table XV). Culture tubes were incubated at 37°C for 48 hours. At 24 hours and at 48 hours tubes were observed for turbidity which was taken as evidence of multiplication. Results showed that the pH range for multiplication of the Aerobacter strain was much broader than the range for the Shigella strain. There was no evidence of Shigella multiplication at pH 6 or at pH 8.8. The Aerobacter strain appeared to multiply equally well over the pH range which was investigated.

It should be noted here that these results are in accord with the observations of Cohen and Clarke (1919) and Erlandsen and Mackey (1958) who defined the pH limits for the multiplication of Aerobacter and Shigella respectively. Cohen and Clarke pointed out that the pH range for the multiplication of Aerobacter species generally extends from about 5.0 to 9.5. A strain of Shigella flexneri studied by Erlandsen and Mackey failed to multiply at either of these pH values. Thus the Aerobacter species were able to tolerate considerable extremes in pH while the Shigella species were more sensitive.

Table XV shows that the optimum pH for multiplication of

the Shigella strain was probably in the neighborhood of 7.0. The methods employed were not sufficiently sensitive to detect an optimum pH for multiplication of the Aerobacter strain.

c. Growth curves; pure and mixed cultures

Conditions of control cultures run in S-medium were similar to the conditions of temperature experiments run at 37°C except that the final determinations were made after 27 and 53 hours incubation. Results from these 2 sets of experiments were nearly identical (Figures 1 and 4). Corresponding lag phases were of about the same duration, multiplication rates of the same magnitude, and the viable population sizes were nearly equal. In mixed culture, the concurrent initiation of the negative acceleration phases for Aerobacter and Shigella occurred after about 11 or 12 hours incubation.

A comparison of growth curves obtained with the control medium (pH7) and the medium adjusted to pH 6 (Figures 1 and 2) showed that the pH change and little effect on the multiplication of Aerobacter. The rate of multiplication, the length of its exponential phase and the size of its viable population remained about the same in both cases. The multiplication rates for Shigella was markedly reduced when the organisms were cultured in medium at pH 6 (Figures 1 and 2). The rate appeared to level off considerably after about 9 hours incubation. This resulted in a 10,000-fold difference between the number of viable cells recovered at 53 hours in medium at pH 7 and pH 6.

At pH 6, growth curves for Aerobacter in pure culture were nearly identical with the curves in mixed culture (Figure 2). The negative acceleration phase was initiated probably after about 12 hours incubation. In mixed culture, the exponential phase for Shigella was interrupted in its very early stages. A death phase, which probably was initiated after about 9 hours incubation continued through 53 hours incubation. Thus, after 27 hours, the ratio of viable Aerobacter to Shigella cells was approximately 1.6×10^6 to 1 while the ration increased to 5.4×10^6 to 1 after 53 hours incubation. The total viable Aerobacter count remained about the same.

A comparison of Figures 1 and 3 show that corresponding growth curves were nearly identical. An increase in the pH of the medium from a value of 7.0 to a value of about 8.0 had little effect on pure culture multiplication or on the relationship between organisms in mixed culture. The ratio of viable Aerobacter cells to viable Shigella cells in mixed culture remained the same in media of both pH values.

Table XVI shows that multiplication of Shigella cultures for 53 hours had little or no effect on the final pH of the medium regardless of the initial pH of the medium. The multiplication of Aerobacter cultures had a more pronounced effect. Changes in reaction in the media with mixed cultures were comparable to the changes observed with pure cultures. In every case, the buffering capacities of the media were sufficient to prevent shifts of more

than 0.5 of a pH unit during cultivation.

3. Oxygen Supply

a. Departure from standard conditions

Standard control culture tubes were incubated under stationary conditions in an air incubator set at 37°C. The temperature of the incubator fluctuated between 34°C and 37°C in the course of the experiments.

Tubes were vigorously aerated by placing them in a "Midgitator" (Elmoc Engineering Co.) which rotated the tubes in a horizontal position at 25 r.p.m. inscribing a circle with a 3½ inch diameter. Mild aeration was accomplished with a tissue culture roller tube apparatus which rotated the tubes at 1/5 r.p.m. and inscribed a circle with a 13 inch diameter. All tubes and equipment were incubated in an air incubator at 37°C.

For experiments on multiplication under conditions of reduced oxygen supply, tubes containing 10 ml S-medium were placed in a boiling water bath for 10 minutes to remove dissolved oxygen. Control tubes containing 0.0002% methylene blue became colorless after this treatment indicating reduced conditions in the medium. Tubes were then placed in an ice bath for a minute or so to cool. They were then inoculated with 0.1 ml of a suspension containing either 10^4 Shigella organisms per ml, 10^4 Aerobacter organisms per ml or a mixture of 10^4 cells of each species per ml. This resulted in a final concentration of approximately 1.0×10^2 cells of either species per ml S-medium. Immediately after inoculation,

a 1 cm. layer of melted petrolatum was poured on the surface of the liquid medium. Control tubes containing methylene blue which were colorless before inoculation developed a pale blue color as a result of the inoculation procedure. This indicated that a slight reoxygenation of the medium had occurred. The color disappeared, however, after a few hours incubation in the 37°C air incubator. For each determination of viable cell counts, samplings were made from different culture tubes.

b. Growth curves; pure and mixed cultures

Control determinations were made under stationary conditions in the air incubator. Conditions of these experiments were similar to those of controls with the temperature experiments run at 37°C and the pH experiments in medium at pH 7 except that the air incubator was substituted for the 37°C water bath. A comparison of the Aerobacter growth curves showed that they very nearly corresponded in all 3 controls (Figures 1,4,7). Corresponding lag phases appeared to be of about the same duration, multiplication rates were of approximately the same magnitude and population sizes were nearly equal. With the oxygen experiments, however, the curves for Shigella failed to correspond with the equivalent curves obtained with the other controls. In pure culture, the multiplication rate was reduced somewhat. In mixed culture, death occurred probably after about 9 hours incubation. It continued through 53 hours incubation. The only difference between cultural conditions with the oxygen control and

the other controls was the use of the incubator instead of the water bath.

A comparison was made of growth curves obtained under stationary control conditions (Figure 7), with curves obtained when the oxygen supply was limited (Figure 8). Oxygen limitation reduced to some degree both the rate of multiplication of Aerobacter and the size of its total population. A similar effect was apparent with Shigella and to an even greater degree.

Under conditions of reduced oxygen supply, Aerobacter growth curves in mixed culture were nearly identical with pure culture curves (Figure 8). The negative acceleration phase in both cases was initiated probably after about 16 hours incubation. The exponential phase for Shigella in mixed culture was interrupted in its very early stages. A death phase was initiated after about 7 hours incubation. This continued through 53 hours incubation. After 27 hours incubation, the ratio of viable Aerobacter to viable Shigella cells in mixed culture was approximately 1.6×10^6 to 1. This represents a slight decrease compared to the ratio with stationary control cultures.

Mild aeration (rotation of culture tubes at 1/5 r.p.m.) had some influence on the growth curves (Figure 9). Comparison of Figures 7 and 9 shows that mild aeration doubled the total viable Aerobacter population, and with Shigella in mixed culture, postponed the interruption of the exponential phase. Thus, the Aerobacter to Shigella ratio in mixed culture was decreased when

compared with stationary conditions. Multiplication rates for pure cultures of Shigella and Aerobacter were about the same under both stationary conditions and conditions of mild aeration.

Vigorous aeration (rotation of culture tubes at 25 r.p.m.) had a marked influence on the multiplication of Aerobacter and Shigella (Figure 10). Comparison of Figure 6 with Figure 10 showed that the multiplication rate for Aerobacter in both pure mixed culture was considerably reduced under conditions of vigorous aeration. Under no other environmental conditions was the multiplication of Aerobacter so proundly affected. After 53 hours incubation, the viable population was smaller than the population attained in less than 27 hours incubation under stationary conditions. Vigorous aeration also markedly reduced the rate of multiplication for Shigella. In addition, it diminished the size of the total viable population. These results are in accord with the findings of Winslow, Walker and Sutermeister (1932), who demonstrated that aeration increased the multiplication rate for Escherichia coli in concentrated medium only. In a dilute synthetic medium, the aerated culture failed to show turbidity.

In mixed culture, under conditions of vigorous aeration, the Shigella strain appeared to multiply without interference from the Aerobacter strain. Only under these conditions was Shigella multiplication unaffected by the presence of multiplying Aerobacter cells. As a result, growth curves for Shigella in mixed culture and in pure culture were nearly identical. The ratio of viable

Aerobacter to Shigella cells was profoundly reduced (50 to 1) after 27 hours incubation when compared with the ratios under other environmental conditions.

4. Nutrient Concentration

a. Deviation from standard conditions

Standard control cultures were designated as 1x, i.e., 1 times normal concentration. For the dilute and concentrated media, the organic constituents of S-medium were altered. In dilute medium, designated as 0.1x, the concentrations of glutamic acid and glucosamine hydrochloride were reduced to 0.01% and the concentration of niacin was reduced to 0.0001%. In concentrated medium, designated as 10x, the concentrations of glutamic acid and glucosamine hydrochloride were increased to 1% and the concentration of niacin was increased to 0.01%. In 10x medium, NaCl was deleted to minimize the increased molarity over the standard control. The different media were of the following compositions:

	1x(S-med., control)	o.1x	10x
Glutamic acid	0.1%	0.01%	1%
Glucosamine HCl	0.1%	0.01%	1%
Na_2HPO_4	0.6%	0.6%	0.6%
KH_2PO	0.15%	0.15%	0.15%
NaCl	0.5%	0.5%	---
Niacin	0.001%	0.0001%	0.01%

It was necessary to adjust the pH of 0.1x-medium and 10x-medium to

approximately 7 with NaOH or HCl.

b. Growth curves; pure and mixed cultures

Conditions of the control experiments in S-medium (1x) were similar to the temperature experiments run at 37°C and identical with the pH control experiments in S-medium. Results were nearly the same in all cases. Corresponding lag phases appeared to be of the same duration, corresponding multiplication rates were of about the same magnitude and viable population sizes were nearly equal. In mixed cultures, the concurrent initiation of the negative acceleration phases for Aerobacter and Shigella occurred after about 11 or 12 hours incubation in all cases.

A comparison of growth curves in the control medium with growth curves in dilute medium (0.1x) showed that the decrease in nutrient concentration had a more pronounced effect on the multiplication of Shigella than on the multiplication of Aerobacter (Figures 11 and 12). The rate of multiplication for the Aerobacter strain was reduced only slightly in dilute medium, but the multiplication rate for Shigella was markedly reduced. The total viable Aerobacter population appeared to be about the same in the control medium and in the dilute medium. For Shigella, in dilute medium, a stationary phase was initiated after about 27 hours incubation. As a result, the viable cell count was greatly reduced when compared with control cultures. The duration of the lag phase was prolonged for both species when the organisms were cultivated in dilute medium.

Growth curves for Aerobacter in mixed cultures were nearly identical with curves in pure culture in dilute medium (Figure 12). The negative acceleration phase was initiated probably after about 16 hours incubation. This occurred about 4 hours earlier in the control cultures. In mixed culture, the exponential phase for Shigella was interrupted in its very early stages. A stationary phase was initiated after about 9 hours incubation followed by a death phase. Thus, after 27 hours incubation, the ratio of viable Aerobacter to Shigella cells was approximately 9.0×10^5 to 1 but after 53 hours in most instances only Aerobacter cells could be recovered.

An increased concentration of organic constituents had a beneficial effect on the multiplication of both Aerobacter and Shigella. A comparison of Figure 13 with Figure 11 indicated that the duration of the lag phases for both species was decreased when the organisms were cultivated in concentrated medium (10x). Although the increased nutrient concentration appeared to have little effect on the multiplication rate for Aerobacter, it greatly increased the multiplication rate for Shigella. The viable population sizes for both were approximately the same in concentrated medium and in the control medium. With Shigella, therefore, the negative acceleration phase was initiated much earlier in concentrated medium. This is suggested by the difference in the shapes of the growth curves for Shigella in concentrated medium and in control medium (Figures 11 and 12).

The results of these studies with pure cultures in concentrated and in dilute media are in accord with the results of earlier studies on the effects of nutrient concentration on bacterial multiplication. Porter (1947) stated that the lag phase for bacteria may be prolonged in a medium that is not optimal for multiplication. This effect had been demonstrated with Escherichia coli by Winslow, Walker and Sutermeister (1932). A comparison of Figures 11, 12 and 13 shows that this effect was also apparent with Shigella and with Aerobacter to a lesser extent. Porter (1947) also stated that the multiplication rate for bacteria increases as the concentration of nutrients becomes more favorable. This was demonstrated for Salmonella typhosa by Penfold and Norris (1912) and Heap and Cadness (1924) for Aerobacter by Dagley and Hinshelwood (1938) and for Escherichia coli by Monod (1949). The multiplication rate for the Shigella strain used in these experiments increased as the concentration of the organic constituents of the medium was increased. Under these conditions, there was, however, no apparent increase in the multiplication rate for Aerobacter.

In concentrated medium, growth curves for Aerobacter in mixed culture were nearly identical with curves in pure culture (Figure 13). The negative acceleration phase was initiated after approximately 11 hours incubation, about the same time that it occurred in control cultures. In mixed culture, the negative acceleration phase for Shigella occurred after about 10 hours

incubation. The exponential phase was interrupted as it was incontrol cultures. Because of an increased multiplication rate, the Shigella strain attained a greater population size during the limited period of multiplication in concentrated medium than it did during multiplication in control medium. As a result, the ratio of viable Aerobacter organisms to viable Shigella organisms decreased at 27 hours from approximately 1.5×10^4 to 1 in control medium to approximately 2.6×10^3 to 1 in concentrated medium.

This concludes the presentation of experimental results. Temperature and oxygen experiments, run in duplicate, were repeated a total of 4 times. Nutrient and pH experiments, also run in duplicate were repeated 3 times.

TABLE I

Cultural Reactions of Test Organisms (48 hours, 37°C)

	Shigella		Aerobacter	
	7-1-58	1-20-61	7-1-58	1-20-61
Adonitol	-	-	+	+
Aesculin	-	-	+	+
Dulcitol		-	+	+
Glucose	+	+	+	+
Gas (glucose)	-	-	+	+
10% Lactose	-	-	+	+
0.5% Lactose	-	-	+	+
Maltose	-	-	+	+
Mannitol	+	+	+	+
Salicin	-	-	+	+
0.5% Sucrose	-	-	+	+
Xylose	-	-	+	+
Urea	-	-	-	+ *
Indole	-	-	-	-
M R	+	-	-	-
V P	-	-	+	+
Citrate	-	-	+	+
Gelatin	-	-	-	-
Motility	-	-	-	-
Sulfide	-	-	-	-
ph-alanine	-	-	-	-
Nitrate	+	+	+	+
Gas (nitr)		-		-
Malonate		-		+
H L oxid	+	+	+	+
H L ferm	+	+	+	+
Flagella	-		-	

* after 48 hours only

TABLE II

Viocin Sensitivity on Agar Plates

48 hour results

Viocin Concentration	Shigella	Aerobacter
A. Veal Infusion Agar		
0.4 mg/ml	trace growth	no growth
0.3 mg/ml	trace growth	no growth
0.2 mg/ml	very light growth	no growth
0.1 mg/ml	light growth	no growth
0.05 mg/ml	growth	very light growth
B. Addition of 1% glucose		
0.4 mg/ml	growth	no growth
0.3 mg/ml	growth	no growth
0.2 mg/ml	growth	trace growth
0.1 mg/ml	growth	light growth
0.05 mg/ml	growth	growth

TABLE III

Necessity of Glucose - VIV Agar

Glucose Omitted		Glucose Added	
Plate[1]	No. Shigella Colonies[2]	Plate[1]	No. Shigella Colonies[2]
1	59	1	97
2	40	2	93
3	36	3	100
4	68	4	113
5	0	5	88
6	31	6	128
7	5	7	106
8	0	8	103
9	90	9	90
10	43	10	119
Mean	37.2 ± 30	Mean	103.7 ± 13

$t_{(18)} = 6.5$, Significant at the 0.1% level

1. Each plate flooded with 1 ml Shigella suspension
2. Same source of inoculum

TABLE IV

Dilution of Shigella Inoculum in Aerobacter Suspension

In Aerobacter Suspension		In Saline	
Plate[1]	No. Shigella Colonies[2]	Plate[1]	No. Shigella Colonies[2]
1	107	1	99
2	124	2	118
3	128	3	112
4	127	4	106
5	101	5	98
6	96	6	112
7	126	7	107
8	111	8	138
9	111	9	101
10	136	10	96
11	112	11	124
12	118	12	105
13	113	13	100
14	109	14	100
15	106	15	107
16	118	16	138
17	102	17	107
18	112	18	88
19	85	19	86
Mean	112.7 ± 12	Mean	107.4 ± 14

$t_{(36)} = 1.23$, not significant at the 5% level

1. Each plate flooded with 1 ml Shigella suspension
2. Same source of inoculum

TABLE V

Comparison of Veal Infusion and VIV Agar - Cultivation of Shigella

A. Inoculum Dereived from Veal Infusion Agar

Veal Infusion Agar Plate[1]	No. Shigella Colonies[2]	VIV Agar Plate[1]	No. Shigella Colonies[2]
1	76	1	49
2	88	2	76
3	74	3	83
4	89	4	79
5	80	5	76
6	105	6	20
7	74	7	24
8	79	8	13
Mean	83 ± 11	Mean	52.5 ± 30

$t_{(14)} = 2.75$; significant at the 5% level

B. Inoculum Derived from VIV Agar

Veal Infusion Agar Plate[1]	No. Shigella Colonies[3]	VIV Agar Plate[1]	No. Shigella Colonies[3]
1	179	1	135
2	147	2	155
3	171	3	154
4	158	4	158
5	176	5	164
6	182	6	171
7	141	7	174
8	180	8	172
9	149	9	161
10	180	10	173
Mean	163.3 ± 16	Mean	161.7 + 12

$t_{(18)} = 0.25$; not significant at the 5% level

1. Each plate flooded with 1 ml Shigella suspension
2. Some source of inoculum
3. Same source of inoculum

TABLE VI

Comparison of Various Agar Media -

Cultivation of Shigella

Blood Agar	
Plate[1]	No. Shigella Colonies[2]
1	71
2	94
3	106
4	95
5	95
6	87
7	106
8	89
9	90
10	89
Mean	92.2 ± 10

VIV Agar	
Plate[1]	No. Shigella Colonies[2]
1	90
2	103
3	78
4	112
5	82
6	69
7	93
8	83
9	76
10	86
Mean	87.2 ± 13

Desoxycholate Agar	
Plate[1]	No. Shigella Colonies[2]
1	73
2	66
3	58
4	63
5	74
6	80
7	75
8	75
9	57
10	67
Mean	68.8 ± 8

Statistical Analysis

1. Comparison of counts on VIV Agar with counts on Blood Agar

   $t_{(18)}$ = .99; not significant at the 5% level

2. Comparison of counts on VIV Agar with counts on Desoxycholate Agar

   $t_{(18)}$ = 3.9; significant at the 1% level

1. Each plate flooded with 1 ml Shigella suspension
2. Same source of inoculum

TABLE VII

Comparison of Various Agar Media - Cultivation of Aerobacter

Plate[1]	Blood Agar No. Aerobacter Colonies[2]
1	106
2	101
3	95
4	85
5	98
6	95
7	100
8	91
9	104
10	99
Mean	97.4 + 6

Plate[1]	Veal Infusion Agar No. Aerobacter Colonies[2]
1	80
2	115
3	101
4	111
5	109
6	94
7	105
8	128
9	122
10	102
Mean	106.7 + 14

Plate[1]	Desoxycholate Agar No. Aerobacter Colonies[2]
1	75
2	82
3	56
4	70
5	82
6	80
7	74
8	85
9	66
10	65
Mean	74.5 ± 8

Statistical Analysis

1. Comparison of counts on veal infusion agar with counts on blood agar

   $t_{(18)}$ = 1.93; not significant at the 5% level

2. Comparison of counts on veal infusion agar with counts on desoxycholate agar

   $t_{(18)}$ = 6.4; significant at the 0.1% level

1. Each plate flooded with 1 ml Aerobacter suspension
2. Same source of inoculum

TABLE VIII

Glucose - Ammonium Chloride Medium and S-Medium -

Multiplication of Aerobacter

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Saline dilution of an 18 hour glutamic acid medium culture of Aerobacter to give approximately 10^7 organisms per ml medium

Media:

Control Medium	G-AC Medium	S-Medium
L-Glutamic acid - 0.1%	Glucose - 0.1%	(see Section C)
NaCl - 0.85%	NH_4Cl - 0.1%	
	NaCl - 0.85%	

Results 48 hours incubation

Control Medium - 10/10*
G-AC Medium - 5/10
S-Medium - 10/10*

A chi-square value of 6.66 was obtained when the results with G-AC Medium and the Control Medium were analysed statistically. This value is significant at the 1% level.

* **Fractions represent the number of tubes which were turbid over the total number of tubes inoculated. Turbidity was apparent when the culture attained a population of approximately 10^7 viable cells per one medium.**

TABLE IX

Substitution of Various Amino Acids - Cultivation of Shigella

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Saline suspension of a 24 hour 12 S-medium culture of Shigella. Organisms were washed twice in saline before suspension.

Size of Inoculum:

One drop (approximately 0.05 ml) of a turbid saline suspension of Shigella

Media:

12 S-Medium, Control (see Section C)	Altered Medium
	Same as control except for substitution of various amino acids for glutamic acid, each in a concentration of 0.5%

Results 24 hours incubation

1.	dl Alanine	No turbidity
2.	l Arginine monohydrochloride	" "
3.	dl Aspartic acid	" "
4.	l Cystine	" "
5.	l Glutamic acid (12 S-medium, control)	Turbidity
6.	Glycine	No turbidity
7.	l Histidine monohydrochloride	" "
8.	dl Isoleucine	" "
9.	dl Leucine	" "
10.	dl Lysine monohydrochloride	" "
11.	dl Methionine	" "
12.	dl Phenylalanine	" "
13.	dl Serine	" "
14.	dl Threonine	" "
15.	l Tyrosine	" "
16.	dl Valine	" "

TABLE X

Concentrations of Carbohydrate and Amino Acid - Multiplication of Shigella

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Saline suspension of a 24 hour 12 S-medium culture of Shigella. Organisms were washed twice in saline before suspension, to give approximately 10^2 organisms per ml medium.

Media:

12 S-Medium, Control (see Section C)	Altered Medium Same as control except for variation in concentrations of glucose and glutamic acid

Results 24 hours incubation

12 S-medium (control	-	-	-	-	-	-	++++*
0.5 % glucose; 0.5 % glutamic acid-						-	++
0.2 % glucose; 0.2 % glutamic acid-						-	++
0.1 % glucose; 0.1 % glutamic acid-						-	++
0.05% glucose; 0.05% glutamic acid-						-	-

++++*	very turbid, sediment
+++	very turbid
++	turbid
+	slight turbidity
-	no turbidity

TABLE XI

Substitution of Glucose for Glucosamine - Multiplication of Shigella

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Saline dilution of an 18 hour S-medium culture of Shigella to give approximately 10^2 organisms per ml medium.

Media:

S-medium, Control (see Section E)

Glucose Medium

L-Glutamic acid - 0.1 %
Glucose - 0.1 %
Na_2HPO_4 - 0.6 %
KH_2PO_4 - 0.15%
NaCl - 0.5 %
Niacin - 0.001%

Results 48 hours incubation

S-Medium, (Control) - 20/20*
Glucose Medium - 17/20

Control cultures were clearly more turbid than glucose medium cultures. A chi-square value of 3.16 was obtained when the results were analysed statistically. This value is not significant at the 5% level.

* Fractions represent the number of tubes which were turbid over the total number of tubes inoculated. Turbidity was apparent when the culture attained a population of approximately 10^6 viable cells per ml medium.

TABLE XII

Addition of NaCl to Medium -

Multiplication of Shigella

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Shigella colonies washed from the surface of a VIV agar plate with saline. Dilution of the saline suspension, to give approximately 10^2 organisms per ml medium.

Media:

Control Medium	-	S-Medium (NaCl addition)
L-Glutamic acid	- 0.1%	(see Section E)
Glucosamine	- 0.1%	
Na_2HPO_4	- 0.6%	
KH_2PO_4	- 0.15%	
Niacin	- 0.001%	

Results 48 hours incubation

Control Medium	- 61/82*
S-Medium (NaCl addition)	- 69/70

A chi-square value of 17.72 was obtained when the results were analysed statistically. This value is significant at the 0.1% level.

* Fractions represent the number of tubes which were turbid over the total number of tubes inoculated. Turbidity was apparent when the culture attained a population of approximately 10^6 viable cells per ml medium.

TABLE XIII

Alterations in medium-S -

Multiplication of Shigella

Departure from Standard Conditions (Section E)

Origin of Inoculum:

Saline dilution of an 18 hour S-medium culture of Shigella to give approximately 10^2 organisms per ml medium.

Media:

S-Medium, Control (see Section C)	Altered Media Same as control except for alterations listed below

Results 48 hours incubation

S-Medium, Control - - - -	10/10*
Niacin deletion - - - -	0/10
Substitution of NH_4Cl for L-Glutamic acid - - -	0/10
Glucosamine deletion - - -	0/10

* Fractions represent the number of tubes which were turbid over the total number of tubes inoculated. Turbidity was apparent when the organisms attained a population of approximately 10^6 viable cells per ml medium.

TABLE XIV

Effect of Variation in Incubation Temperature - Multiplication of Shigella and Aerobacter

Results

24 hours incubation

Incubation temperature	Shigella	Aerobacter
25°C	-	++++*
30°C	-	++++
37°C	++	++++
40°C	+	++++
44°C	-	-

48 hours incubation

Incubation temperature	Shigella	Aerobacter
25°C	-*	++++*
30°C	+	++++
37°C	+++	++++
40°C	++	++++
44°C	-	-

++++* very turbid, sediment
+++ very turbid
++ turbid
+ slight turbidity
- no turbidity

TABLE XV

Effect of Variation in the pH of the Medium - Multiplication of Shigella and Aerobacter

Results

24 hours incubation

Initial pH of Medium	Shigella	Aerobacter
6	-*	++++*
7	++	++++
8	++	++++
8.8	-	++++

48 hours incubation

Initial pH of Medium	Shigella	Aerobacter
6	-*	++++*
7	+++	++++
8	++	++++
8.8	-	++++

++++* Very turbid, sediment
+++ very turbid
++ turbid
+ slightly turbid
- no turbidity

TABLE XVI

pH Readings - Culture Media

A. Beginning of Experiments

	S6	S7	S8
	6.05	7.00	7.80
	6.05	7.00	7.90
	6.00	7.00	8.00
Mean	6.03	7.00	7.90

B. Termination of Experiments

Shigella,	5.95	6.95	7.85
pure culture	6.10	7.00	7.95
	6.00	7.05	8.05
Mean	6.02	7.00	7.95
Aerobacter	5.60	6.75	7.50
pure culture	5.60	6.85	7.55
	5.50	6.70	7.65
Mean	5.57	6.77	7.57
Mixed	5.80	6.80	7.50
Culture	5.65	6.85	7.55
	5.55	6.80	7.50
Mean	5.67	6.82	7.52

LEGEND - Figures 1 - 14

●——————● - *Aerobacter*, pure and mixed culture

●— — — —● - *Shigella*, pure culture

●— · —· —● - *Shigella*, mixed culture

..... - extrapolation of growth curve

Population sizes given as log viable organisms per ml culture.

FIGURE 1

pH 7

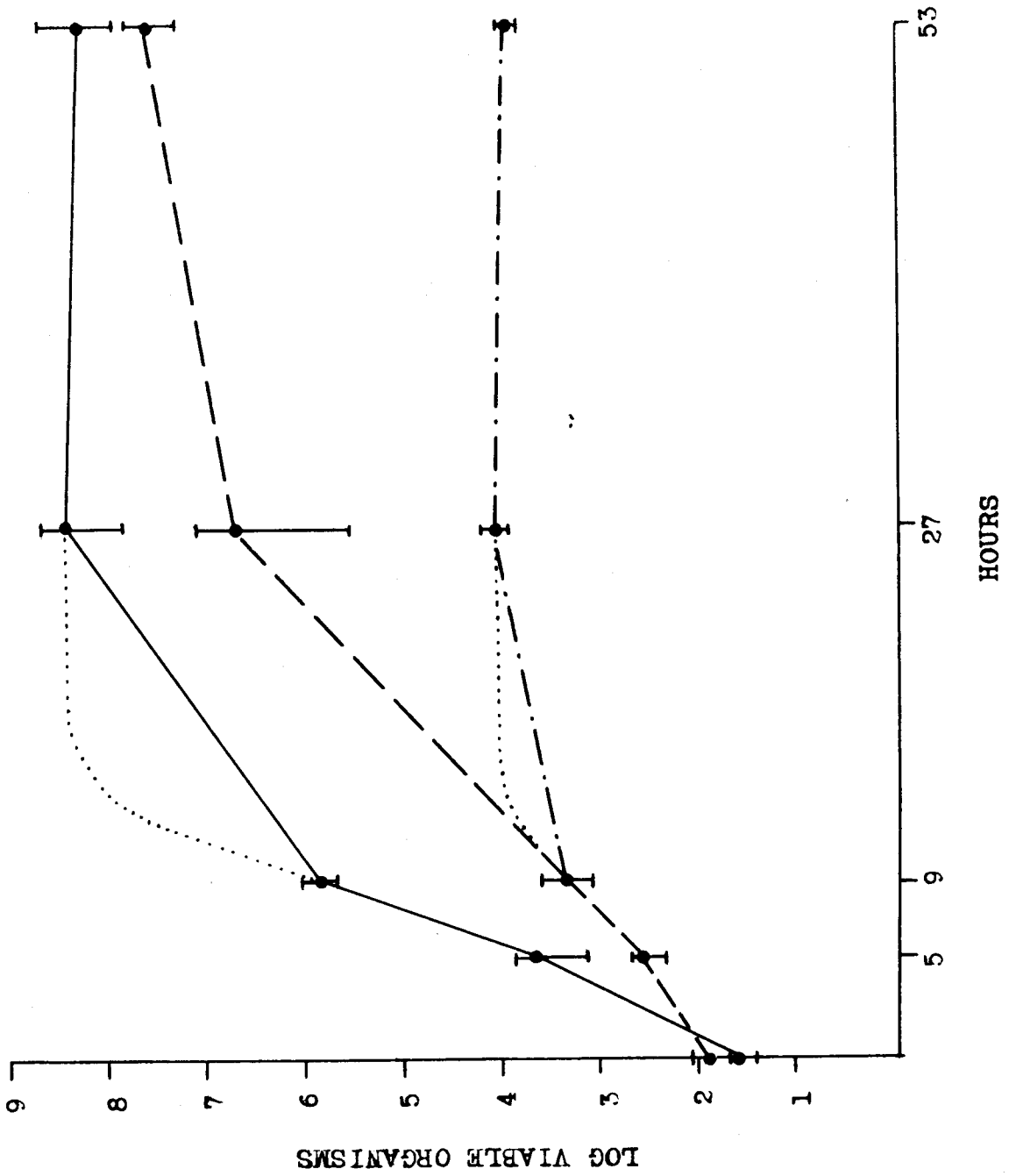

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FIGURE 2

pH 6

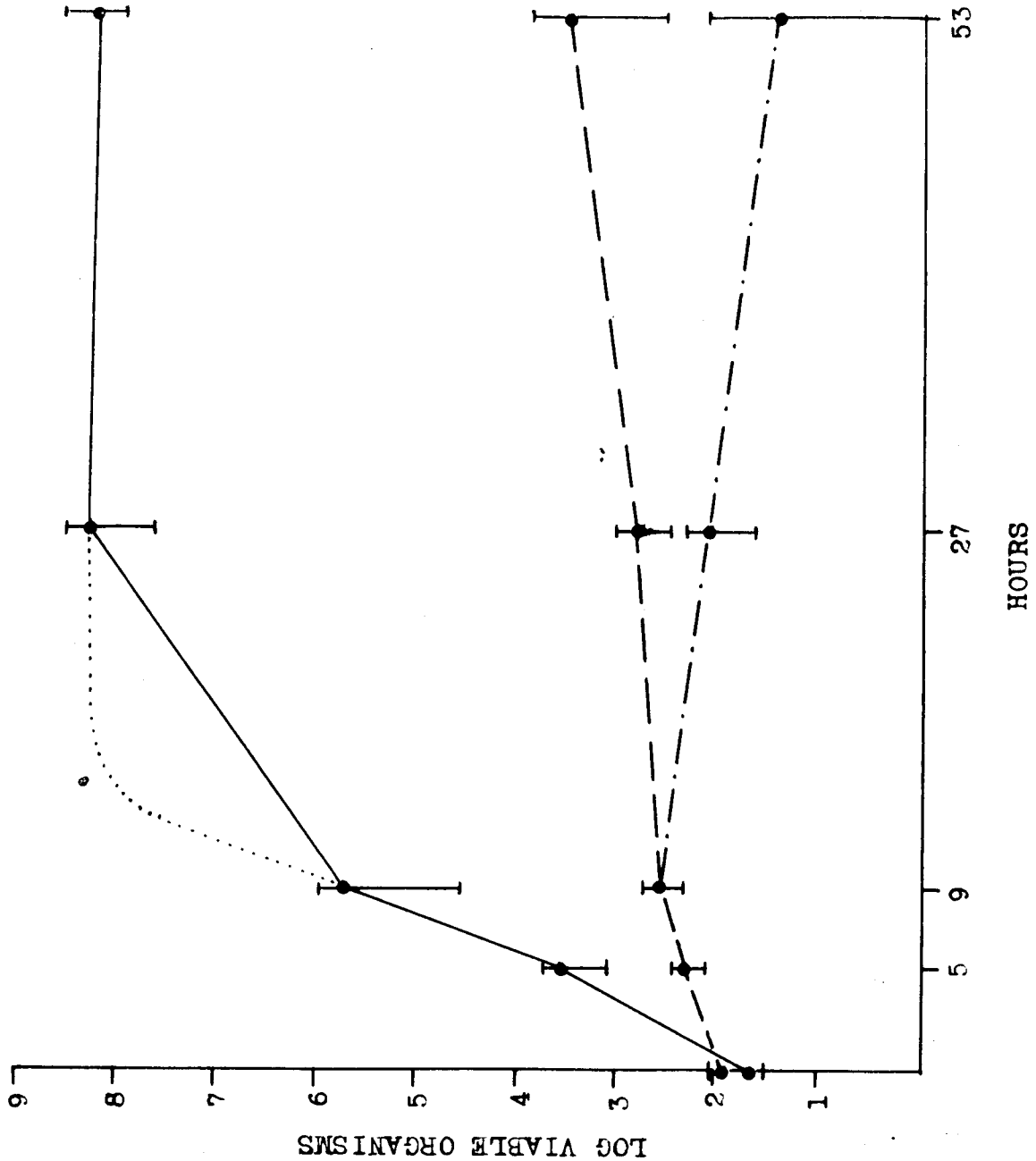

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FIGURE 3

pH 8

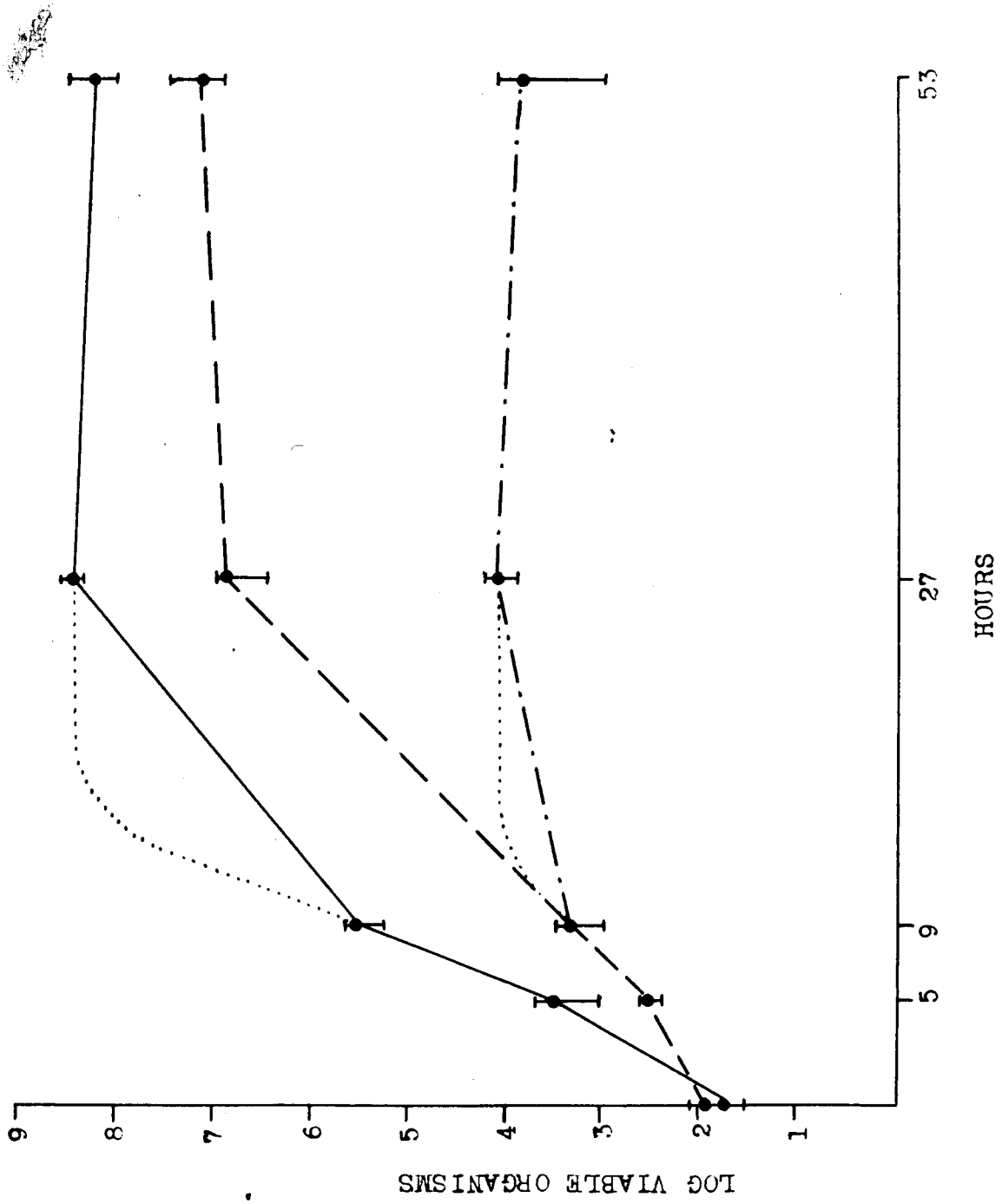

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FIGURE 4

37° C

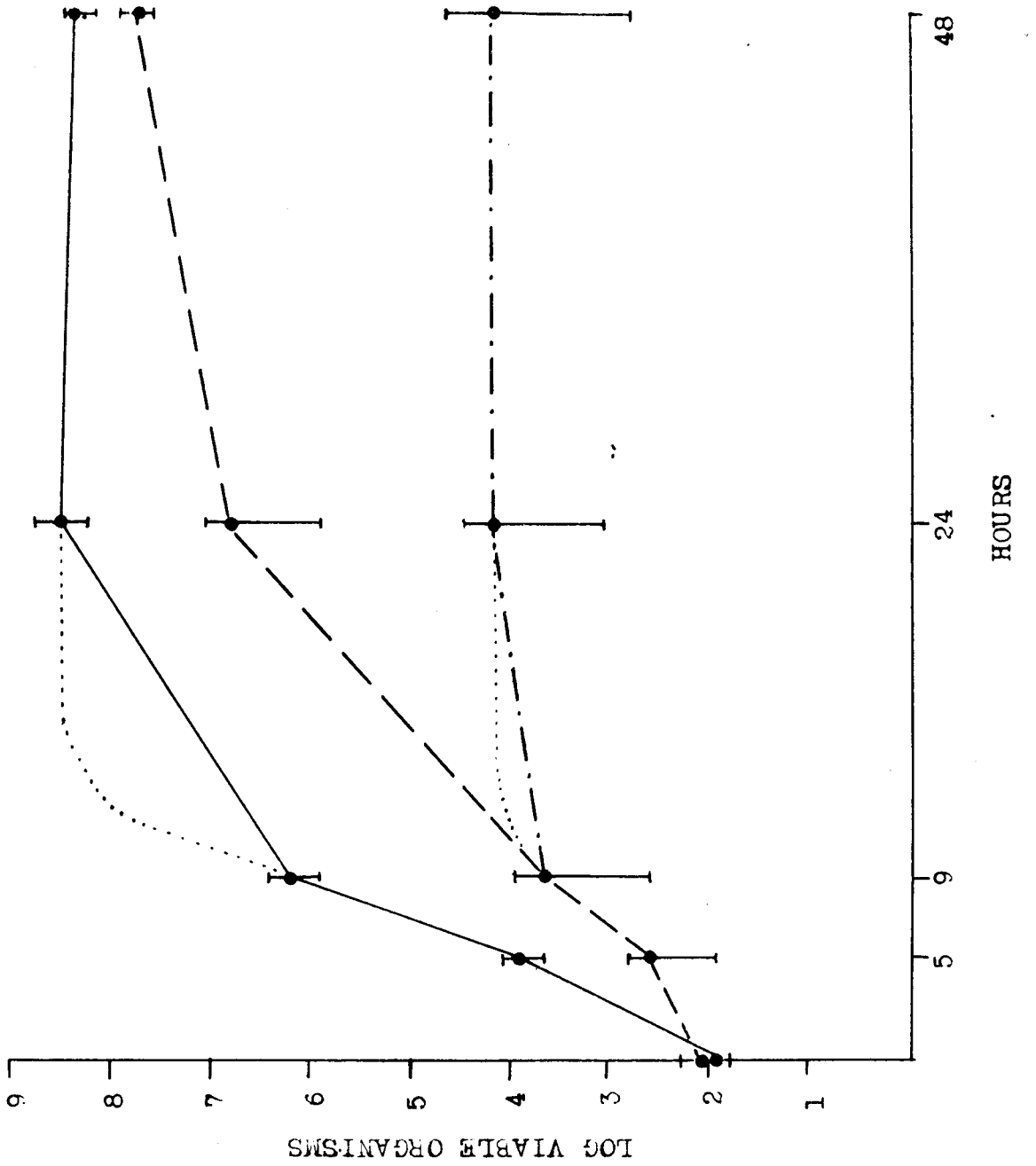

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FIGURE 5

30° C

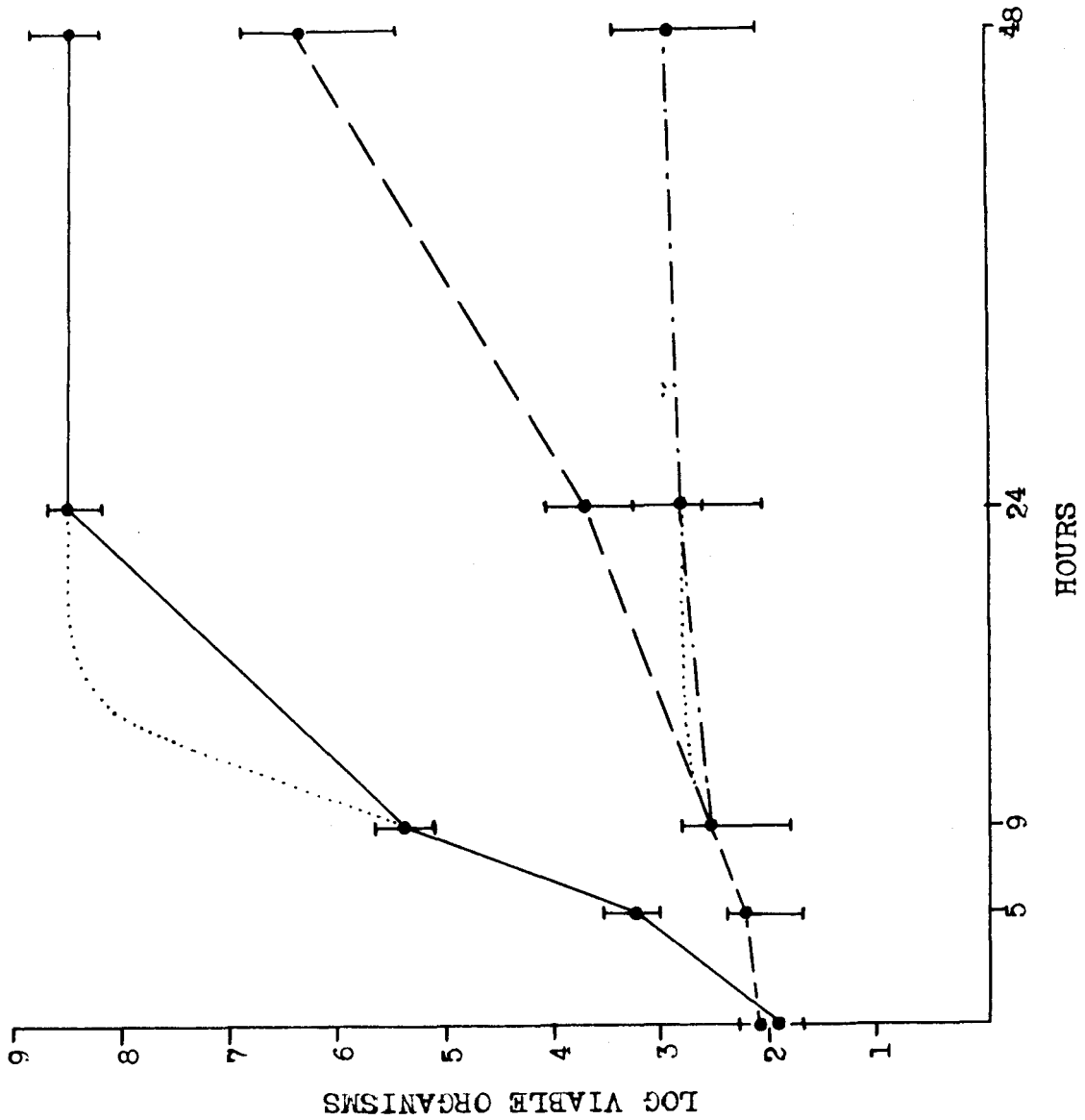

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FIGURE 6

44 C

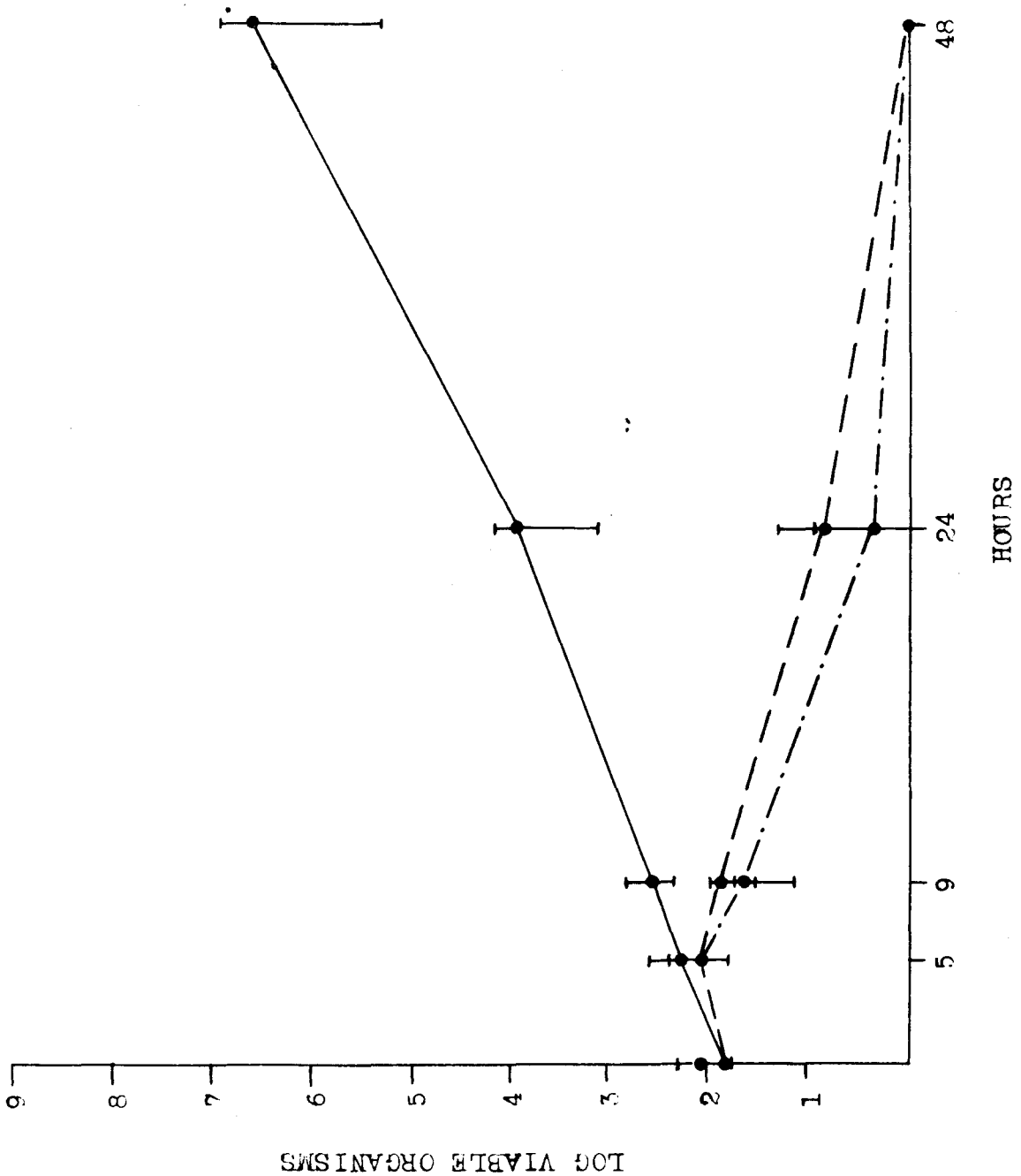

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FIGURE 7

OXYGEN SUPPLY
Stationary Conditions

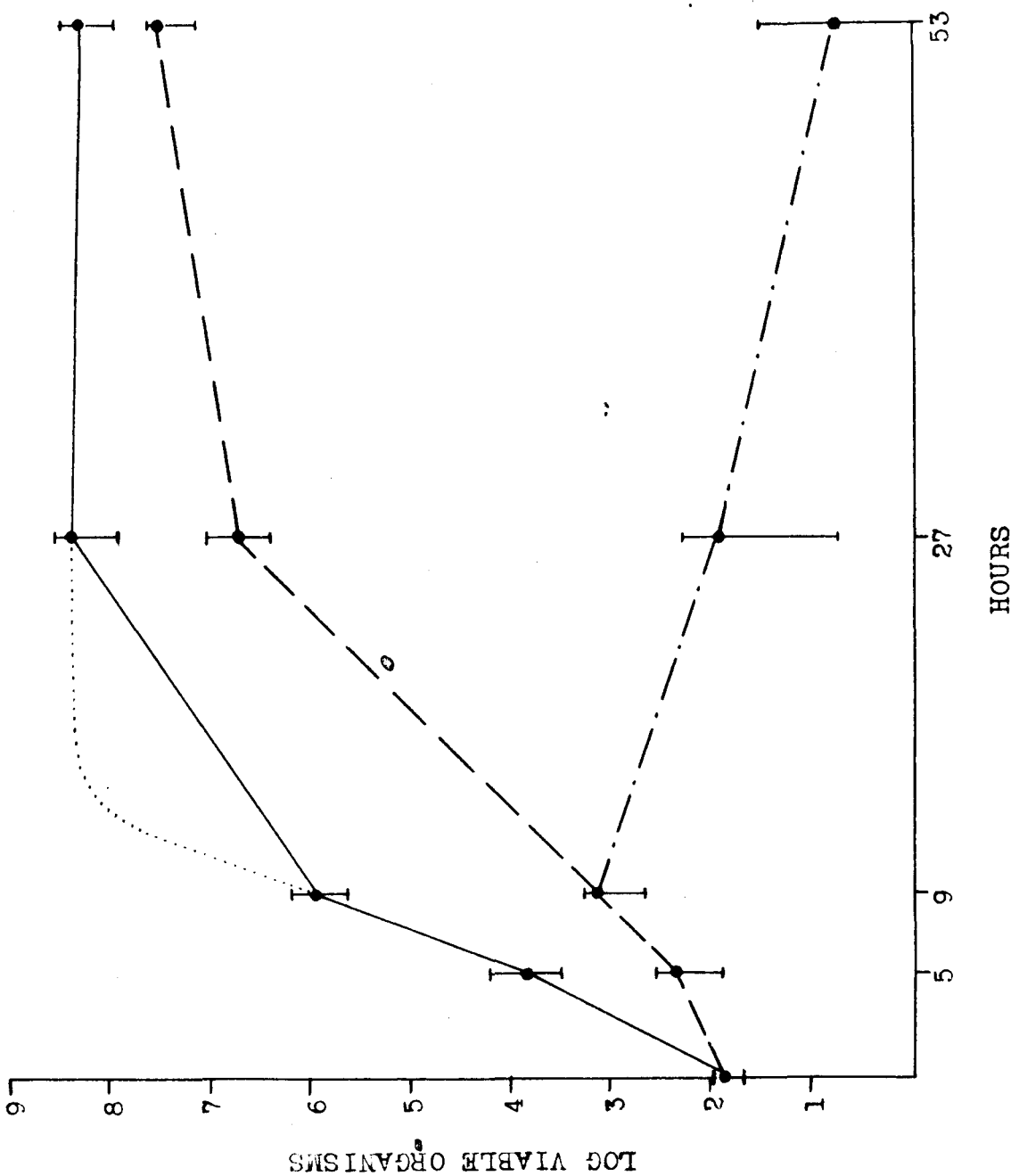

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FIGURE 8

OXYGEN SUPPLY
Reduced

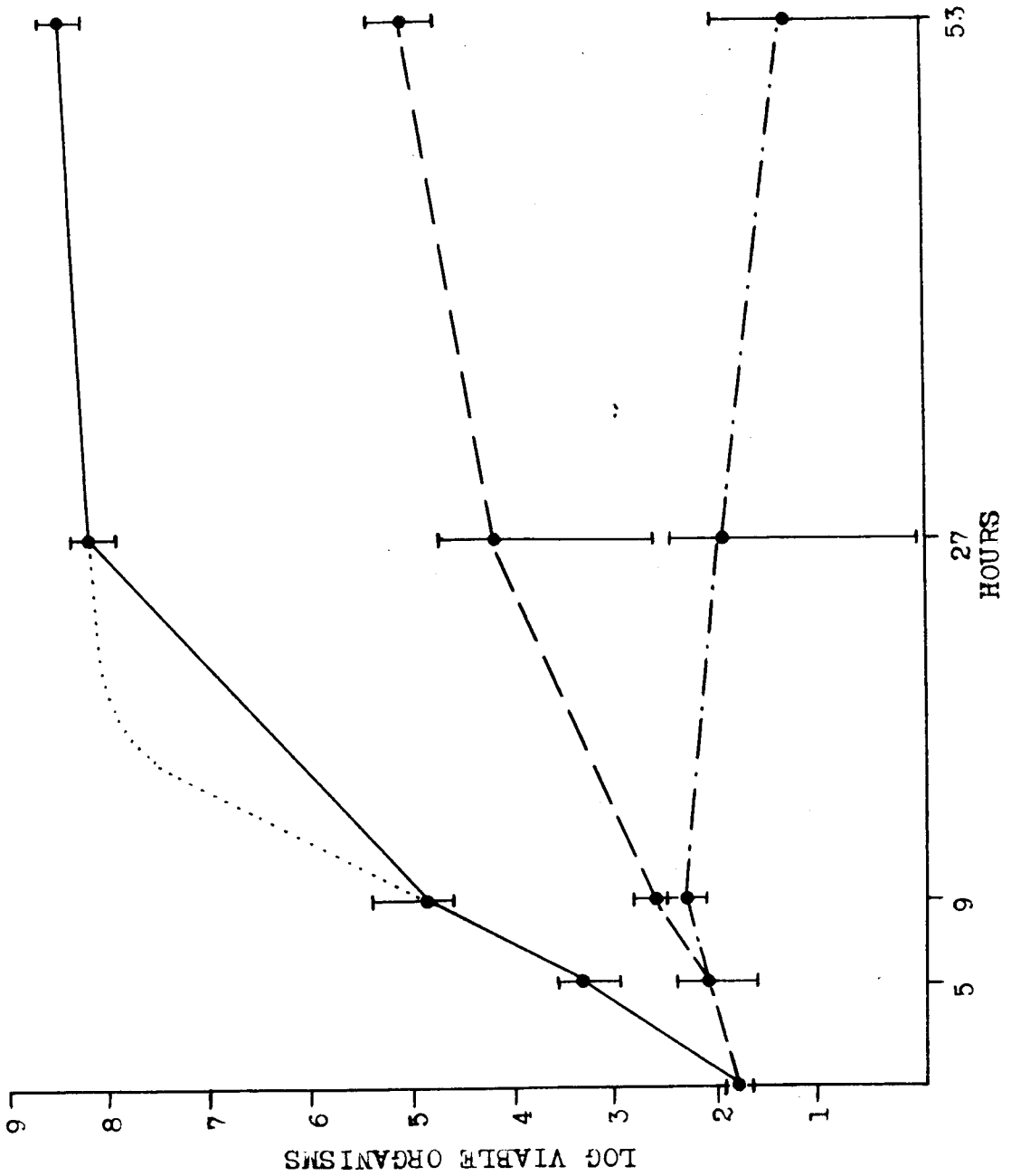

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FIGURE 9

OXYGEN SUPPLY
Mild Aeration

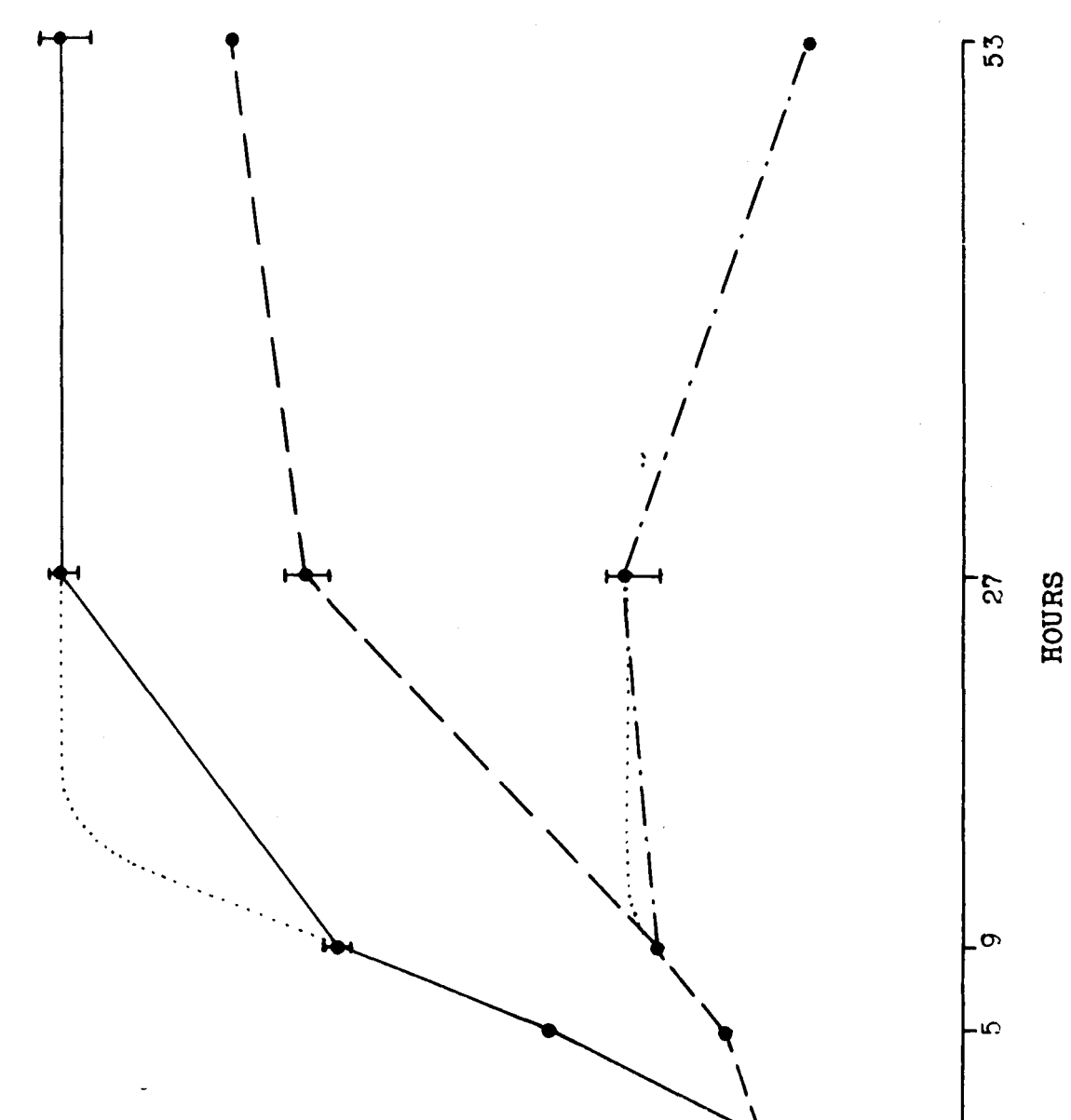

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FIGURE 10

OXYGEN SUPPLY
VIGOROUS AERATION

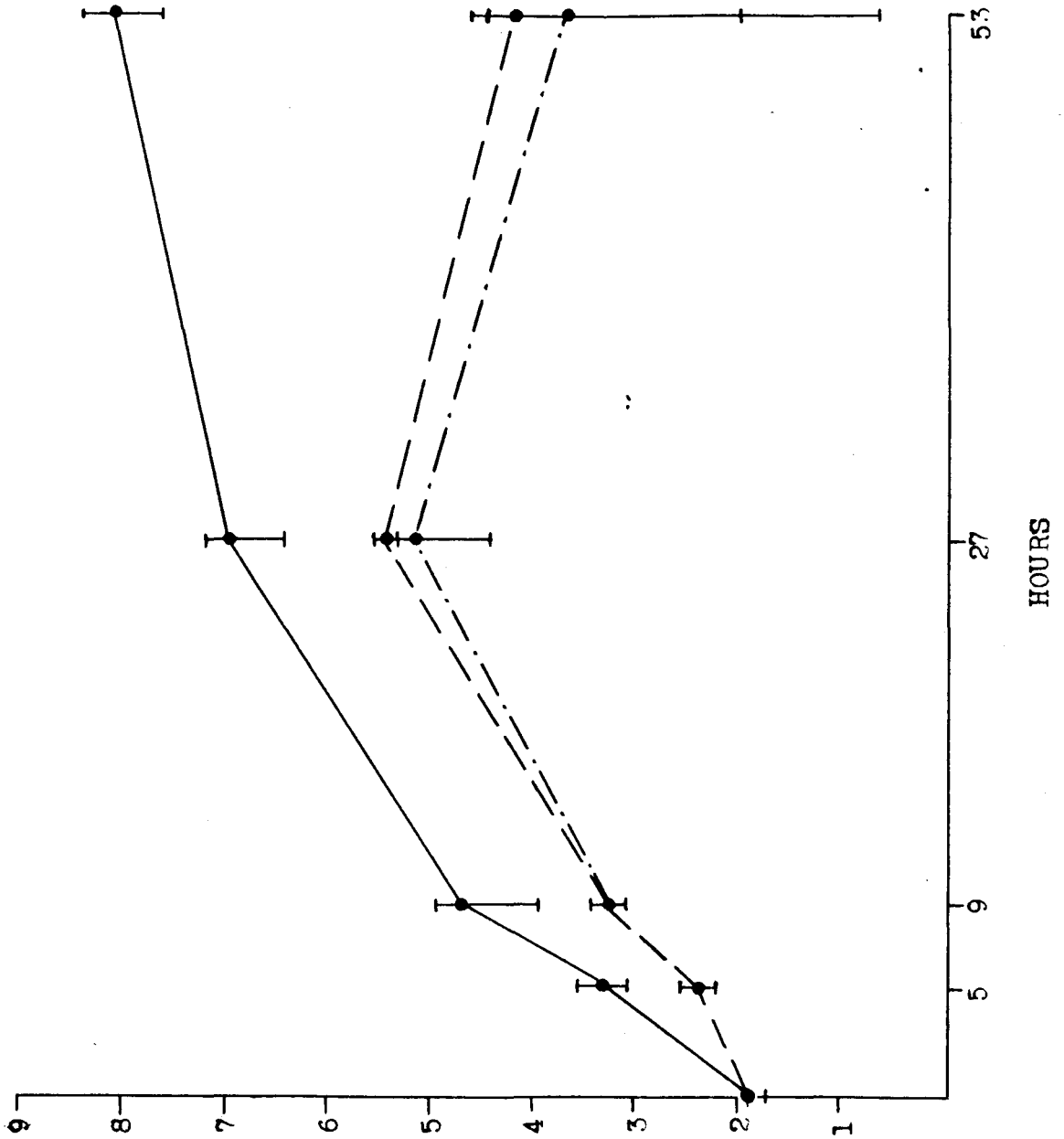

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FIGURE 11

NUTRIENT CONCENTRATION - 1X

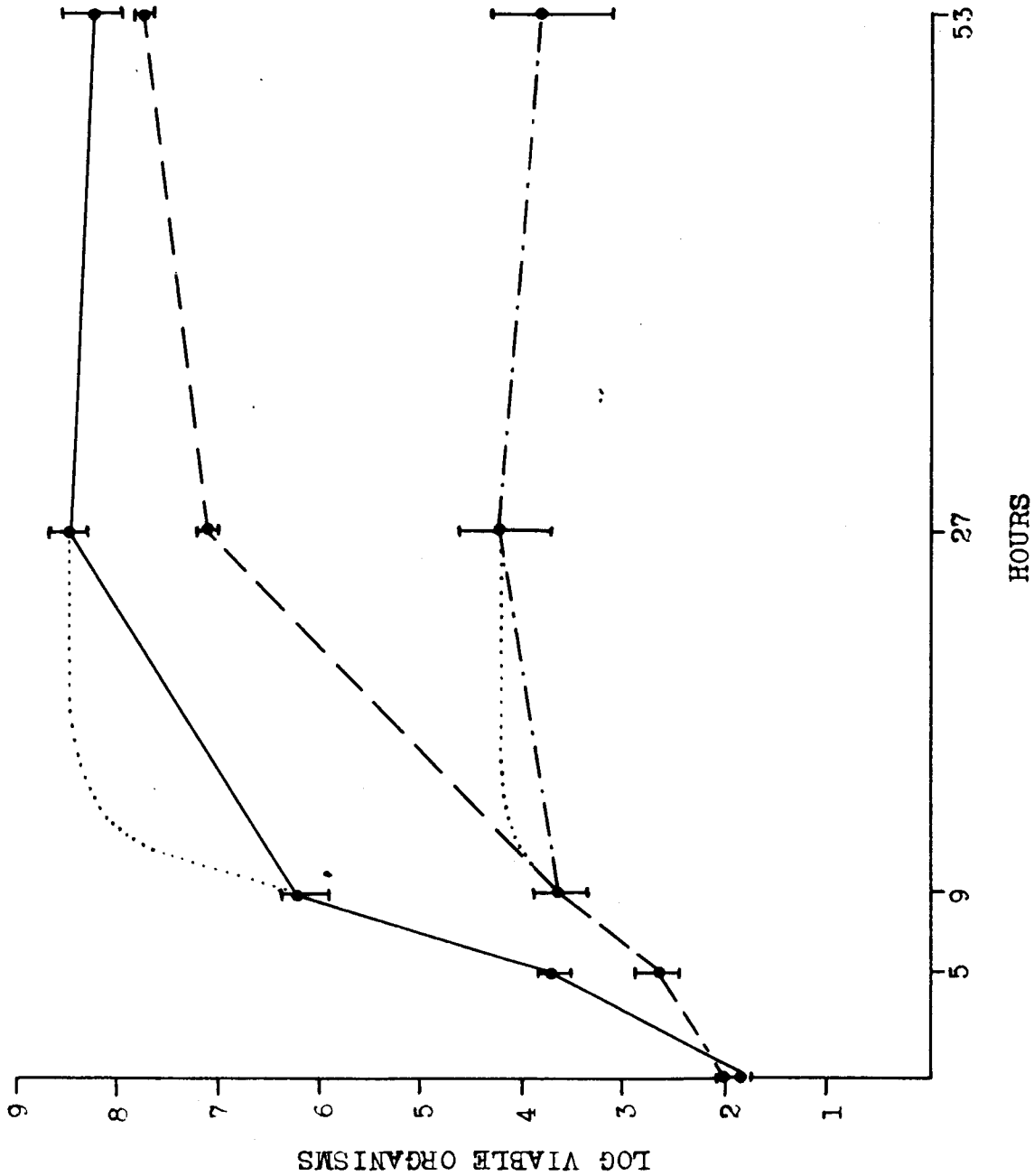

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FIGURE 12

NUTRIENT CONCENTRATION - 0.1X

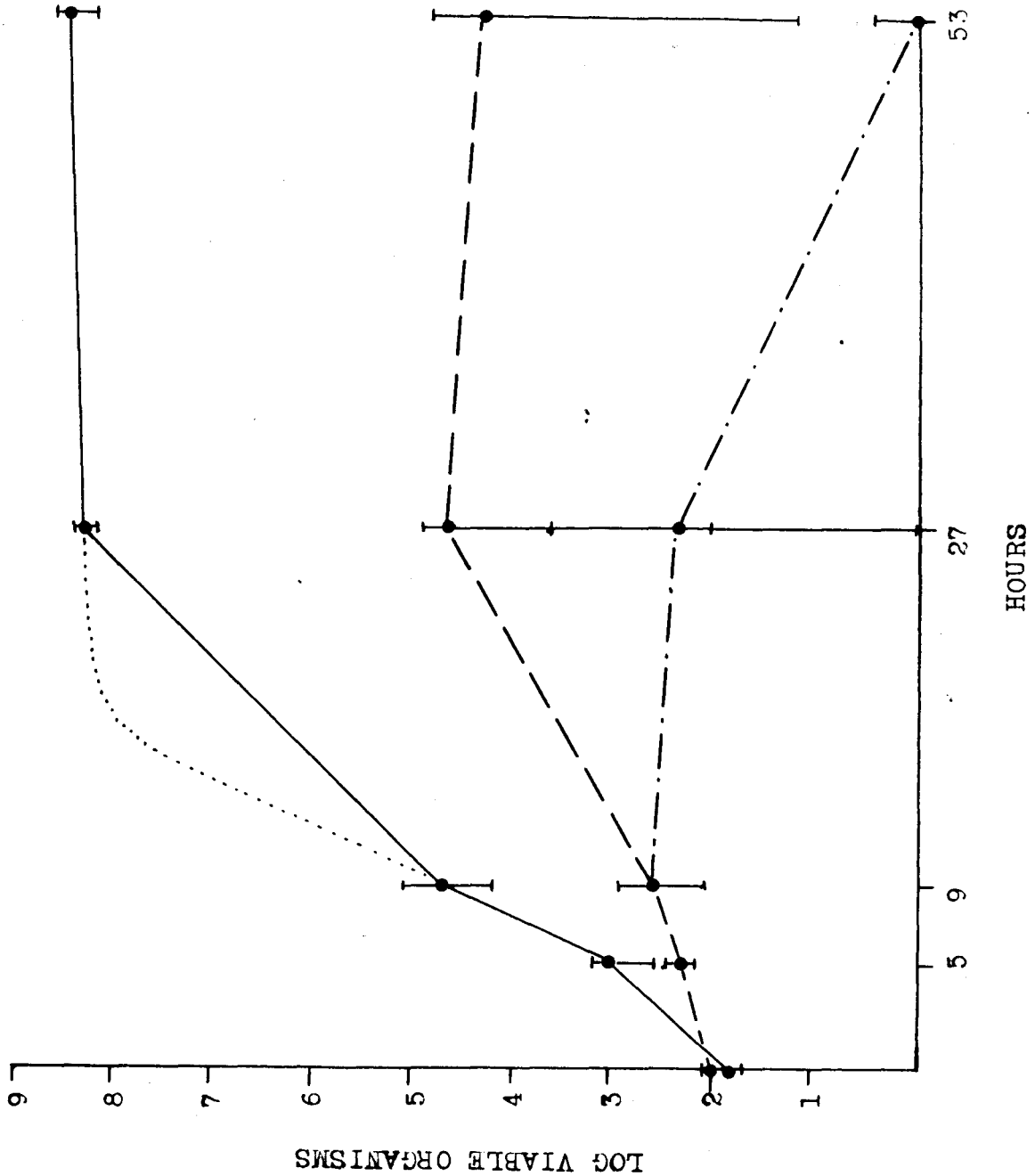

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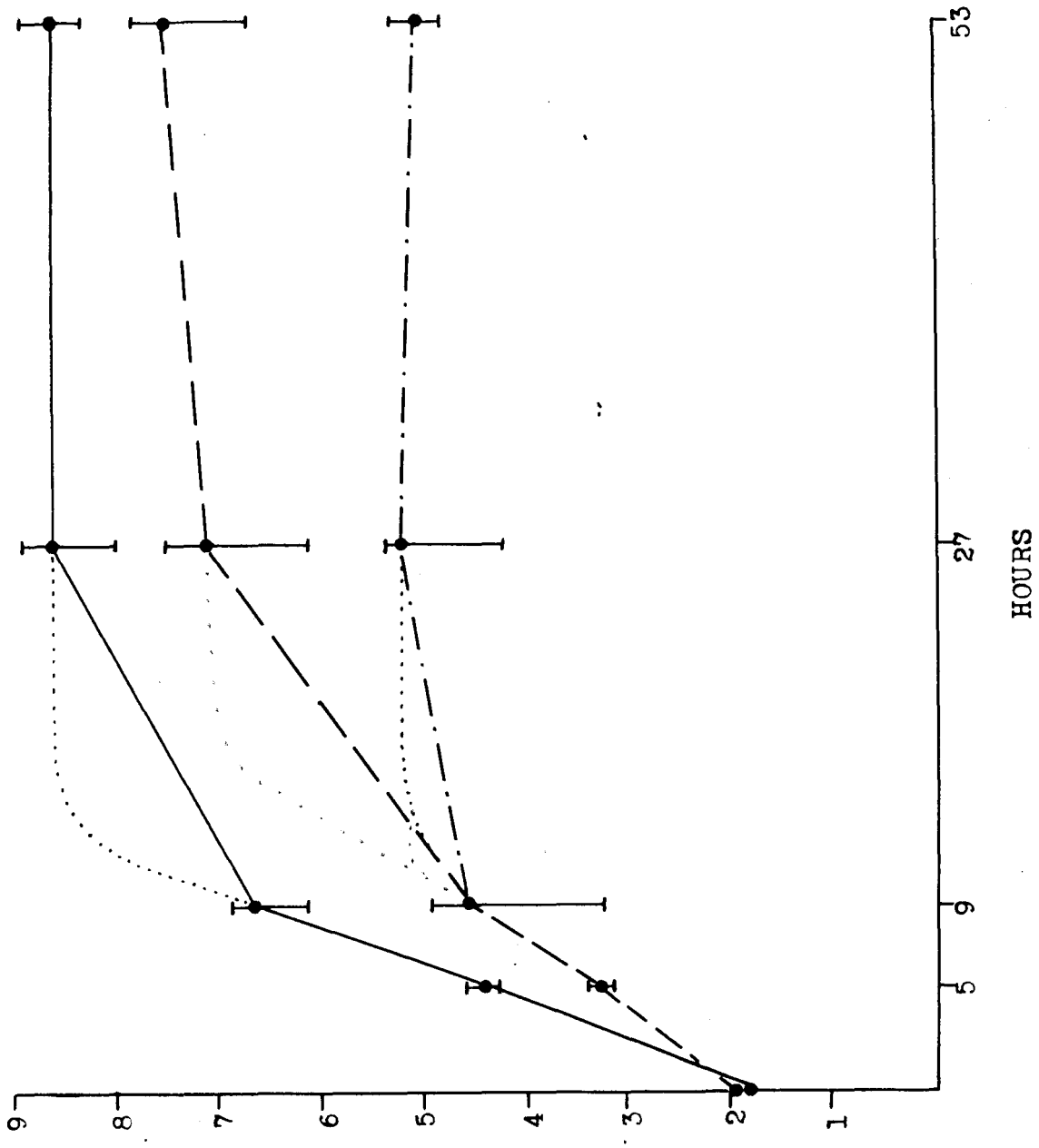
FIGURE 13

NUTRIENT CONCENTRATION - 10X



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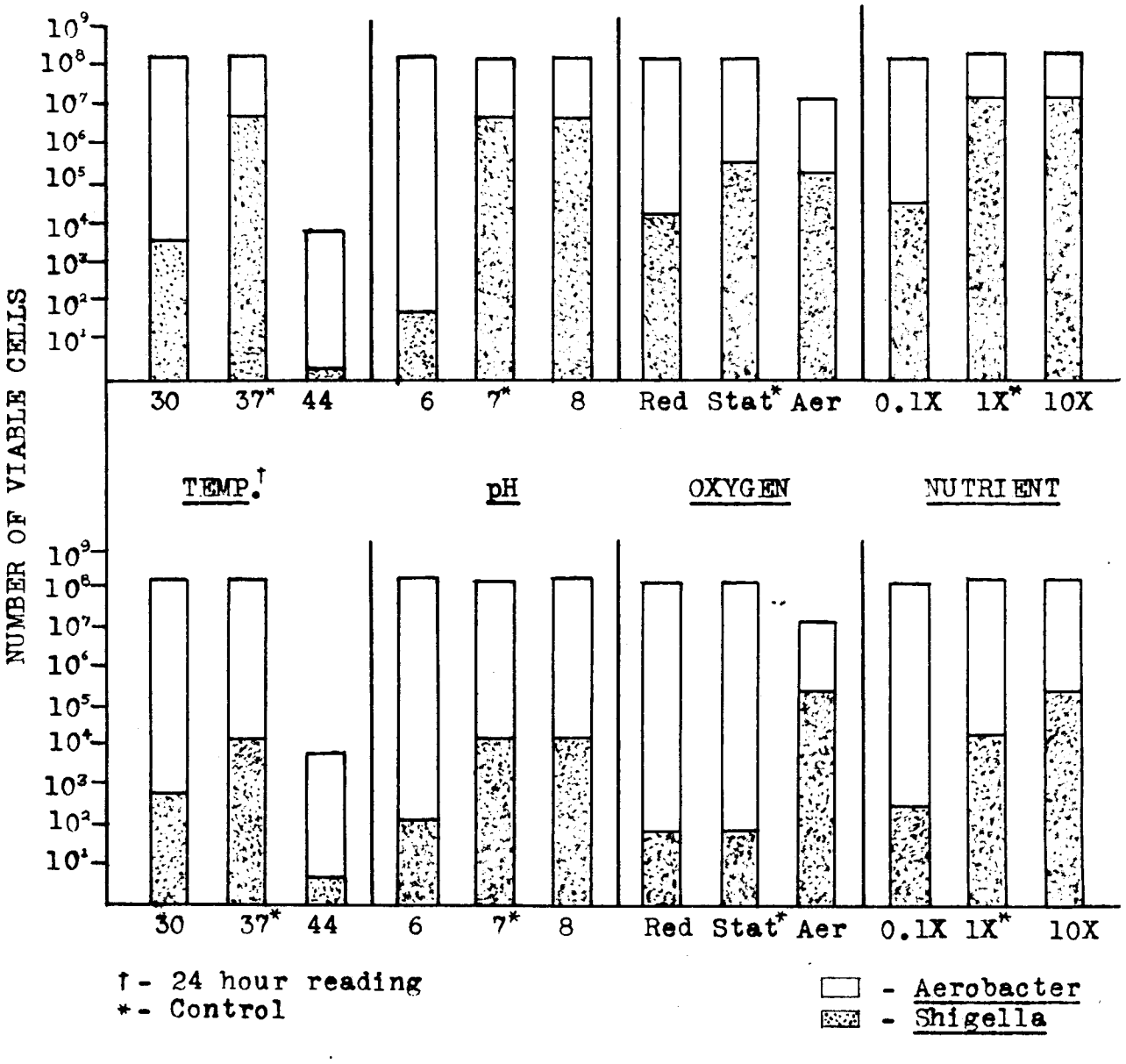

FIGURE 14

VIABLE CELLS AFTER 27 HOURS INCUBATION

CHAPTER III

DISCUSSION AND CONCLUSION

A. Discussion

1. Methods

An investigation of bacterial population growth from an ecological standpoint constitutes a new approach to the study of population dynamics in microbiology. When an area of investigation is approached from a different point of view, old methods for study must be refined and new methods must be established to cope with new problems. The development of effective methods for the study of mixed bacterial populations constitutes an important contribution to the field of microbial ecology. Several of the methods used in the course of this investigation are discussed here.

It was not difficult to choose a technique for the estimation of bacterial population sizes. Population determinations are performed either by direct counts (total counts) or by the indirect counting of viable cells. Only the counting of viable cells permits differential analysis of mixed bacterial populations. Hence, a viable cell counting technique was adopted for these studies.

Plate counting is the usual technique employed to

determine the number of viable cells. This method is based on the assumption that every living cell is capable of producing a colony on agar medium (Wilson and Kullman, 1931). Yet, cultural conditions on agar media probably differ considerably from conditions prevailing in liquid media. Viable cells in a liquid medium may fail in some instances to give rise to colonies on agar medium. An additional source of error often encountered with the plate counting technique results from the tendency of some organisms to form clumps. When clumping occurs, the discrepancy between the colony count and the actual viable cell count may further increase (Jennison, 1937). An aggregate of several viable cells gives rise to only one colony. In spite of these difficulties, plate counting is the most sensitive method for enumeration of bacterial populations (Monod, 1949). Furthermore, with mixed cultures, relative numbers are generally the significant feature. Whether or not the counts give an accurate estimation of absolute cell numbers is less important.

The plate counting method used in this investigation, the floodplate method, (Hentges and Fulton, 1960) proved to be convenient for the differential counting of mixed populations. It appears that this method can be used for analysis of any mixture of bacteria when the agar plates which are employed permit differentiation of the species present in the mixture.

If the ratio between the organisms in the mixture exceeds about 100 to 1 something must de done to inhibit formation of

colonies of the predominant species. Otherwise, it is difficult to detect the small number of colonies formed by the minority species. Furthermore the dilution of the mixed culture to obtain a countable number of colonies per plate might dilute out the minority species. In this investigation, the Aerobacter species always predominated - usually at a rationin excess of 100 to 1.

The device used to inhibit the colonial development of Aerobacter was based on a differential sensitivity of Aerobacter and Shigella to the effects of Viocin (Table II). The addition of Viocin to the agar medium effectively inhibited the formation of Aerobacter colonies without greatly interfering with Shigella colonial development. It is probable that a similar scheme, based on the differential sensitivity of microorganisms to antibiotics may be used for the analysis of other mixtures when the ratio between the organisms is great.

Incorporation of glucose in the agar medium containing Viocin produced unusual results. The sugar addition modified the effects of Viocin on both species as can be seen in Table II. The effect was more pronounced with Shigella. Thus, Viocin could be added to the medium in a concentration clearly inhibitory to Aerobacter colonial development without affecting Shigella colony formation to any great degree. The mechanisms of this effect are not understood. When glucose was added to the agar medium, a darkening occurred on autoclaving. The sugar apparently underwent caramelization. Perhaps this process modified the antibiotic in

some way or protected the Shigella organisms from its effects. It would be interesting to find out if this effect is general for other antibiotics and for other microorganisms as well. If so, the caramelization process might be generally applied as an adjunct to the methods described for the analysis of mixed cultures.

The medium devised for these studies, S-medium, is of simpler composition than the synthetic media described in the literature for Shigella multiplication. Most media contain a considerable array of inorganic salts and often thiamin, in addition to amino acids, a carbohydrate and niacin. The Shigella strain used for this investigation apparently had no thiamin requirement. The trace elements, such as magnesium, were probably supplied in sufficient quantity with other constituents of the medium, in the water or on the glassware. The studies were not designed to ascertain in detail the nutritional requirements of the organisms. It was only desirable to define a medium which would consistently support multiplication of both species. Hence, no special precautions were taken in preparing the medium.

A simple technique was employed to estimate the sizes of the Shigella and Aerobacter inocula used for the experiments. It was based on an observation that both species attain a fixed population size after 18 hours incubation in S-medium. The count for Shigella was approximately 1.0×10^7 viable cells per ml culture; for Aerobacter it was approximately 3.0×10^8 cells per

ml. These population sizes varied little from time to time although no attempts were made to carefully regulate the number of organisms added to the medium before incubation. Thus, it was only necessary to suspend several Aerobacter or Shigella colonies in S-medium, incubate the suspension for 18 hours and dilute the grown cultures to the desired concentration of cells. Apparently both species attain a maximum population size by this time. The technique proved sufficiently accurate for estimation of Aerobacter and Shigella inocula. It provides a convenient method for the rapid estimation of inocula for population studies.

2. Growth Curves: Pure and Mixed Cultures

In assessing the sources of error in a population study, one must take into account errors made in the dilution of cultures and errors made in plate counting. In this study, the points from which the growth curves were plotted represent average results derived from experiments run at different times. Inability to exactly duplicate conditions of experiments from tome to time constituted an additional source of error. The variations encountered in these experiments were expressed as ranges. The ranges for the readings from which each mean result was calculated are indicated on the graphs of growth curves. Experiments run in duplicate were repeated either 3 or 4 times. Thus, there were, in general, 6 or 8 readings from which each mean result was calculated. When the number of observations is less than 10, the range is being used to a great extent as a substitute

for standard deviation and variance (Dixon and Massey, 1957).

The evidence indicates that the Aerobacter species is by nature a hardier organism than the Shigella species in several ways. Its rate of multiplication appears to be inherently greater than the rate for Shigella. Inspection of growth curves, Figures 1 through 13, shows that the slopes of the steepest parts of the curves are greater for Aerobacter than for Shigella. These slopes do not accurately measure the maximum multiplication rates in the exponential phase but they are largely determined by the maximum multiplication rates. It is probable that calculation of multiplication rate constants, k, would bear out the contention that Aerobacter multiplies more rapidly than Shigella under the various environmental conditions.

Aerobacter multiplies favorably at temperatures or pH values or in media in which Shigella multiplies poorly. For example, Aerobacter multiplies at 44°C whereas Shigella does not multiply at all (Figure 6). Aerobacter appears to multiply about as well at pH 6 as at pH 7 (Figures 1 and 2). The rate of Shigella multiplication declines considerably at pH 6 compared to pH 7. At 53 hours, the population size at pH 6 is 0.001 of the population size at pH 7. The total population size for Aerobacter remains nearly the same despite nutrient concentration (Figures 11, 12 and 13). The population size for Shigella is greatly decreased when a comparison is made of the growth curves for the organisms in dilute medium (Figure 12) with the curves for the organisms in

control medium (Figure 11). Without exception, Aerobacter multiplies without interference from Shigella in mixed culture. When competition occurs between Aerobacter and Shigella, the Aerobacter species is always the successful competitor.

The degree to which Shigella multiplication is inhibited depends on environmental conditions. When conditions are favorable for Shigella multiplication, inhibition is less marked than when conditions are adverse. In terms of ecology, the ecological niches of the spies overlap to a greater or lesser extent as the environment changes. In an adverse environment, conditions are more competitive and the niches for Aerobacter and Shigella overlap considerably. The Aerobacter species adapts readily to adverse environmental changes and competes with nearly the same facility under adverse conditions as it does under more favorable conditions. Under adverse conditions, the competitive effectiveness of the Shigella strain is decreased. Hence, its inhibition is more pronounced (Figures 2, 5, 8, and 12). Conversely, inhibition is less pronounced when conditions are favorable for Shigella multiplication (Figures 1, 4, 6 and 13). It is clear, therefore, that the ratio between the 2 organisms in mixed culture varies considerably depending on conditions of environment.

When mixed cultures are vigorously aerated, the presence of Aerobacter appears to have little or no effect on Shigella multiplication (Figure 10). Under these conditions only, the

ecological niches for the Aerobacter and Shigella species are sufficiently different so that competition between them does not exist. Although the multiplication rate for Shigella is reduced somewhat under vigorous aeration, the rate for Aerobacter multiplication is markedly reduced. This effects the population size for Aerobacter. The Aerobacter species fail to attain a maximum population size within the time limit of the experiments. Shigella multiplication is not suppressed in mixed culture. Thus, it appears that the exponential phase for Shigella is interrupted in mixed culture only when the Aerobacter strain multiplies favorably. A survey of the growth curves illustrates this point.

Interruption of exponential growth occurs with Shigella in mixed culture before or at the same time the negative acceleration phase is initiated for Aerobacter. It depends on environmental conditions. When the initial pH of the medium is low or when the supply of oxygen or nutrients is limited, the exponential phase for Shigella apparently is interrupted before the onset of the negative acceleration phase for Aerobacter (Figures 2, 8 and 12). Under other competitive conditions, the exponential phase for Shigella is interrupted at approximately the same time the negative acceleration phase is initiated for Aerobacter (Figures 1, 4, 5, 6, 9, 11 and 13). The oxygen control is the exception (Figure 7).

It is difficult to account for the results obtained with the oxygen control. The exponential phase for Shigella is

interrupted in mixed culture before initiation of the negative acceleration phase for Aerobacter. With other controls, it is interrupted at about the same time. When the oxygen control is compared to other controls, the single known variable in cultural conditions is the mode of incubation. The oxygen control was incubated in an air incubator; the other controls in a water bath. The temperature in the air incubator fluctuated 3°C to 4°C. The temperature in the water bath remained quite stable. Levine et. al. (1934) pointed out that in an air incubator, the temperature of the culture medium is often 2°C lower than the recorded temperature of the incubator. This was not so with a water bath. Thus, if the temperature of the air incubator dropped to 33°C, the temperature in the culture medium could possibly fall as low as 31°C. A low temperature might partially account for the incongruous results obtained with the oxygen control culture.

Does all this information reveal anything about the nature of the antagonism existing between Shigella and Aerobacter? Perhaps it suggests that different mechanisms of antagonism are operative - depending on environmental conditions.

Depletion of nutrients may constitute one such mechanism. When the concentration of organic constituents is reduced to 0.1 of standard, the medium becomes limiting for Shigella multiplication. After 27 hours incubation, the population size for the control and for Shigella in concentrated test medium if more than 100 times greater than its population size in medium of

reduced nutrient concentration (Figures 11, 12 and 13). Growth curves for pure culture Shigella are approximately equivalent in control and concentrated media. In medium of reduced nutrient concentration, the Shigella strain apparently attains a maximum population size after about 27 hours incubation. Under control conditions or when the medium is concentrated there is no evidence of a maximum population size even after 53 hours incubation. The control or concentrated cultures appear to initiate a prolonged negative acceleration phase about 27 hours incubation. Although it is impossible to actually compare maximum population sizes under the different conditions of nutrient concentration, control and concentrated cultures at 27 hours achieved a population size greater than the maximum population size for cultures in medium of reduced nutrient concentration. The total population size for Aerobacter remains nearly the same despite nutrient concentration.

According to Monod (1949) the total viable population will be reduced when nutrients are a limiting factor. Based on this fact, it appears that one or more nutrients are limiting for Shigella in medium of reduced nutrient concentration but nutrients are not limiting for Aerobacter under these conditions.

Since the exponential phase for Shigella in mixed culture is interrupted earlier when nutrients are limiting than when they are not, it seems plausible that the medium is exhausted of some nutrient or nutrients essential for Shigella multiplication but not essential for Aerobacter multiplication. Thus, Aerobacter

multiplication continues until a maximum population size is attained while Shigella multiplication is interrupted early in the exponential phase. Experiments showed that the nutrient requirements for Shigella are more exacting than the requirements for Aerobacter (Tables VIII and XIII). For example, Shigella requires that niacin be added to the medium. Aerobacter can apparently synthesize naicin or the compounds which contain it. If niacin is present in the medium, Aerobacter may utilize it, as such, until the supply is exhausted. It then synthesizes the compound and continues to multiply. Shigella, on the other hand, cannot synthesize niacin and its multiplication ceases when the niacin supply is exhausted. Hence, under conditions of nutrient limitation, antagonism might be attributed to exhaustion of a nutrient or nutrients essential to the multiplication of one of the species in the mixed culture.

Antagonism may also be attributed to accumulation of acid in the culture medium. When the pH of the medium is adjusted to a value of 6, the Shigella strain multiplies at an exceedingly slow rate (Figure 2). This might suggest that a pH of 6 is close to the acid limit for the multiplication of Shigella. The multiplication rate for Aerobacter at pH 6 is approximately the equivalent of its rate at pH 7 or 8 (Figures 1, 2 and 3). With Shigella in pure culture, the pH of the medium remains fixed at a value of 6 during multiplication while in mixed culture, the pH of the medium drops to a value averaging 5.66 (Table XVI). Apparently

this drop in pH is the result of acid accumulation due to metabolic activities of the Aerobacter strain. It is reasonable to speculate that in mixed culture the comparatively early interruption of the exponential phase for Shigella is the result of the accumulation of acid. Shigella multiplication continues only until the pH of the medium approaches the region found to be inhibitory. Aerobacter multiplication is unaffected at this pH. In this case antagonism might be attributed to acid accumulation resulting from the activities of the Aerobacter strain.

When the oxygen supply is limited, the growth curves resemble the curves from experiments in which the nutrient concentration is limiting (Figures 12 and 13). Oxygen limitation reduces the multiplication rate for Shigella in pure culture considerably but reduces the multiplication rate for Aerobacter only slightly. The Aerobacter strain is apparently better able to adapt itself to conditions of low oxygen tension than the Shigella strain in the synthetic medium. In mixed culture, the comparatively early interruption of the exponential phase for Shigella may be the result of the depletion of dissolved oxygen or exhaustion of compounds which may be used as hydrogen acceptors by the Shigella species. The rapidly multiplying Aerobacter species utilizes the available oxygen and then adapts more efficiently to the conditions of anaerobiasis. When oxygen supply is limiting, antagonism might thus be attributed to depletion of available oxygen by the faster multiplying Aerobacter species.

Hence, antagonism might be due to depletion of essential nutrients, exhaustion of oxygen supply or accumulation of acid products of metabolism - depending on environmental conditions. Yet antagonism occurrs under what appears to be conditions of nutrient excess (Figure 13) continuous oxygenation (Figure 9) or favorable pH for Shigella multiplication (Figure 3). At pH 8, for example, Shigella multiplication in pure culture is about identical with its multiplication in medium adjusted to pH 7. (Figures 4 and 6) In mixed culture at pH 8, acid accumulates but the final pH of the medium drops to a value no lower than about 7.5. Although Shigella multiplies favorably at 7.5, its exponential phase is interrupted in mixed culture. This occurs about the same time the negative acceleration phase is initiated for Aerobacter. From this it appears that yet another mechanism is responsible for antagonism when environmental conditions remain favorable for Shigella multiplication. What then causes the antagonism? Apparently it is not the depletion of oxygen, the exhaustion of nutrients or the accumulation of acid. Since Shigella multiplication is interrupted at about the time the Aerobacter species attains a maximum stationary population, what appears to be antagonism may be in reality the attainment of an M-concentration for the mixed culture.

Von Wikullil (1932) stated that the sum of the population sizes of the species in mixed culture never exceeds the greatest population size attained by either of the species in pure culture.

In other words, the sum total of the M-concentration in mixed culture is never greater than the largest M-concentration for the species in pure culture. This theory is difficult to prove or disprove. The techniques employed in these experiments were not refined enough to detect exact population sizes. It is therefore impossible to evaluate the relationships between Aerobacter and Shigella in terms of M-concentration.

Another theory proposed to account for antagonism is the production of toxic substances in the medium by one of the species. As Aerobacter approaches a maximum population size, its products of metabolism may accumulate to a degree where multiplication of both species is inhibited. Or perhaps, the dead cells which begin to accumulate as the negative acceleration phase is initiated in some way inhibit multiplication. Bonventre and Kempe (1960) showed with Clostridium botulinum that the liberation of toxin into the medium occurred extensively only after cessation of the exponential growth phase. After cessation of the exponential growth, autoysis of cells occurred. The liberation of toxin was attributed to this activity.

By analogy, a substance which inhibits Shigella multiplication may be released on autolysis of Aerobacter cells. Simple methods for demonstrating the presence of a toxic substance were proposed by Halbert (1948) and Frederieq (1947). In unpublished experiments, the Aerobacter and Shigella strains were tested by these methods. No toxic substance could be demonstrated.

Furthermore, in the course of these experiments, Shigella colonies were often found growing adjacent to Aerobacter colonies on agar plates. Thus, there is no direct evidence to implicate the accumulation of toxic products as the mechanism responsible for antagonism between Aerobacter and Shigella. Yet the evidence against the production of a toxic substance by Aerobacter is not conclusive enough to be ruled out as a possible mechanism.

The evidence does suggest that the antagonistic mechanisms may vary with environmental conditions. It is possible, for example, that inhibition of Shigella multiplication is due to exhaustion of nutrients under conditions of limited nutrient supply, acid accumulation when the pH of the medium is low, and accumulation of toxic products or some other mechanism when conditions are favorable for multiplication.

B. Conclusion

It was desireable to condense all the observations of bacterial multiplication into a form which may be easily grasped. Of several possible ways to do this, it appeared that the total population sizes of the organisms after 27 hours multiplication served as a key to the effects of the environment on bacterial multiplication. 27 hour readings from the various experiments were therefore collected and are represented in Figure 14.

The mean 27 hour populations of the organisms multiplying under various environmental conditions in pure culture are represented by the bar graphs in the left hand portion of Figure 14

A comparison of pure culture population sizes shows that environmental change had little effect on the Aerobacter population sizes after 27 hours incubation. The viable cell count was in the neighborhood of 2.0 to 3.0 x 10^8 cells per ml culture medium under varied conditions of temperature, pH, oxygen supply or nutrient concentration. There were 2 notable exceptions - when Aerobacter cultures were incubated at 44°C and when cultures were vigorously aerated. At 44°C, the mean total viable population after 27 hours incubation was 7.27 x 10^3 cells per ml culture medium; under vigorous aeration, the average count was 9.23 x 10^6 cells per ml culture medium. An examination of the growth curves for Aerobacter under the above conditions of environment (Figures 6 and 10) suggests that the multiplication rate for Aerobacter was decreased when compared with control cultures (Figures 4 and 7). This may account for the smaller populations at 44°C or under conditions of vigorous aeration after 27 hours incubation. Figure 14 shows that the population sizes for Shigella in pure culture varied considerably depending on environmental conditions. For example, at 44°C the average viable population was recorded as 2 cells per ml culture medium, at 37°C the population was 6.7 x 10^6 cells per ml medium, and at 30°C the average count was 4.25 x 10^3 cells per ml culture medium. Examination of Figures 4, 5 and 6 shows that Shigella failed to multiply at all at 44°C and that its multiplication rate at 30°C appeared to be considerably less than its rate at 37°C. The differences in population sizes after 24

hours multiplication may be attributed to differences in multiplication rates at the different temperatures.

The population sizes of the organisms multiplying in mixed culture are represented by the bar graph in the right hand portion of Figure 14. A comparison of Aerobacter population sizes in mixed culture with corresponding population sizes in pure culture shows that total populations were nearly the same in both cases. In medium at pH 8, for example, the mean viable population for Aerobacter in pure culture was 2.90×10^8 cells per ml medium after 27 hours incubation and was 2.42×10^8 cells per ml medium in mixed culture. Thus in mixed culture, the presence of the Shigella species appeared to have little or no effect on the Aerobacter population size. On the other hand, the presence of the Aerobacter species affected the population size for Shigella in mixed culture. This can be seen by comparing the population sizes for Shigella in mixed culture with corresponding population sizes in pure culture. Figure 14 shows that the total Shigella populations were, in general, smaller in mixed culture. For example, after 27 hours incubation in nutrient medium at 10x concentrated, the average population size for Shigella in pure culture was 1.24×10^7 viable cells per ml medium but was only 1.45×10^5 viable cells per ml medium in mixed culture. When the nutrient concentration was reduced to 0.1x standard concentration the mean viable count was only 4.58×10^4 viable cells per ml medium in pure culture but dropped even lower to 2.19×10^2 viable

cells per ml medium in mixed culture. Apparently the presence of Aerobacter in the mixture caused a reduction in the Shigella population sizes. Examination of growth curves under the various environmental conditions (Figures 1,2,4,5,6,7,8,9,11,12 and 13) confirms this. In mixed culture, the exponential phase for Shigella was interrupted before or about the time the negative acceleration phase was initiated for Aerobacter. A reduction in the population size for Shigella in mixed culture can be attributed to this interference with Shigella multiplication.

Figure 14 shows that when mixed cultures were vigorously aerated or when cultures were incubated at 44°C the population sizes for Shigella were not reduced when compared with pure culture controls. At 44°C, this apparent discrepency can be accounted for. Shigella failed to multiply either in pure culture or in mixed culture at 44°C (Figure 6). Under conditions of vigorous aeration (Figure 10), multiplication rates for both the Aerobacter and the Shigella species appear to have been reduced but the effect was more pronounced with Aerobacter. Because of its greatly reduced multiplication rate, the Aerobacter species failed to attain a maximum population size within the time limit for the experiments. The presence of Aerobacter in the mixture appeared to have little or no effect on the multiplication of Shigella. This can be seen by comparing the growth curves for Shigella (Figure 10) in mixed culture with the curves in pure culture. The curves are similar.

From these results it appears that in mixed culture

Shigella multiplication is interrupted when Aerobacter attains a maximum population size. If environmental conditions are such that this is attained then antagonism occurs; if Aerobacter fails to attain a maximum population size then antagonism does not occur.

This investigation illustrates the application of ecological principles to studies of bacterial population dynamics. In nature bacteria usually exist as mixed populations. A knowledge of bacterial behavior in pure cultures is a prerequisite to the understanding of the activities of the species in association. Yet the outcome of mixed culture associations cannot always be predicted by observing pure cultures alone. For example, pure culture observations show that the Aerobacter species inherently multiplies at a greater rate than the Shigella species, that it adapts itself to adverse conditions of environment with greater facility and that it tolerates greater extremes of temperature or pH than the Shigella species. Pure culture observations could not predict that Shigella multiplication is interrupted in mixed culture before or at about the same time the Aerobacter species initiates a negative acceleration phase. Fulton (1937) observed the succession of species in mixed culture. Initially Escherichia coli inhibited multiplication of Salmonella typhosa but as the Escherichia coli population declined, the Salmonella multiplication rate began to increase until its population surpassed that of Escherichia coli. There is no way that this could have been predicted by observing the organisms in

pure culture. To clearly understand the dynamics of mixed populations, mixed culture studies appear to be essential. Environment affects the relationship between the organisms in mixed culture. Thus bacterial population studies can be approached effectively from an ecological viewpoint - an examination of the relationships amoung microorganisms with an appreciation of the effects of environment on these relationships.

CHAPTER IV

SUMMARY

This investigation illustrates the application of ecological principles to studies of bacterial population dynamics. The effects of alterations in the environment on pure culture and mixed culture population growth of Shigella and Aerobacter were studied. Viable cell counts were made during growth under predetermined conditions of temperature, pH, oxygen supply and nutrient concentration.

Environmental changes had little effect on the size of Aerobacter populations after 27 hours incubation. Exceptions occurred when the Aerobacter cultures were incubated at 44°C or when cultures were vigorously aerated. Under these conditions, the populations were smaller in size.

Environmental changes had marked effects on the Shigella population sizes. For example, after 27 hours incubation in medium adjusted to pH 6, the population size was smaller than when the medium was adjusted to pH 7. It was smaller at 30°C than at 37°C. When the supply of oxygen or concentration of nutrients was reduced, the population size for Shigella was reduced.

In mixed culture, Aerobacter interferred with Shigella multiplication under most environmental conditions. The

exponential growth phase for Shigella was interrupted at about the time Aerobacter attained a maximum population size. When mixed cultures were aerated, Aerobacter failed to attain a maximum population size in the time limit for the experiment. Under these conditions, the presence of Aerobacter in the mixture appeared to have little or no effect on the multiplication of Shigella. The relative numbers of Shigella in mixed culture varied with changes in environment. When conditions were favorable for Shigella multiplication, the proportion of Shigella in the mixture was, as a rule, greater than when conditions were unfavorable.

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APPENDIX

TEMPERATURE

Study 1
Inocula: Shigella 6.4 x 10^1, 6.7 x 10^1 Aerobacter 9.1 x 10^1, 8.9 x 10^1

		37°C	30°C	44°C
		5 Hours		
Shigella	Pure	1.0 x 10^2	7.5 x 10^1	7.5 x 10^1
		8.0 x 10^1	7.3 x 10^1	6.1 x 10^1
	Mixed	9.0 x 10^1	6.8 x 10^1	8.6 x 10^1
		1.1 x 10^2	6.9 x 10^1	8.0 x 10^1
Aerobacter	Pure	6.5 x 10^3	2.21 x 10^3	1.5 x 10^2
		4.7 x 10^3	2.19 x 10^3	2.5 x 10^2
	Mixed	5.9 x 10^3	1.97 x 10^3	2.22 x 10^2
		4.6 x 10^3	2.92 x 10^3	2.2 x 10^2
		9 Hours		
Shigella	Pure	9.0 x 10^2	8.0 x 10^1	4.0 x 10^1
		1.0 x 10^3	8.1 x 10^1	4.4 x 10^1
	Mixed	4.2 x 10^2	9.0 x 10^1	5.8 x 10^1
		9.0 x 10^2	1.1 x 10^2	6.3 x 10^1
Aerobacter	Pure	7.5 x 10^5	2.1 x 10^5	6.1 x 10^2
		8.4 x 10^5	3.5 x 10^5	4.6 x 10^2
	Mixed	1.23 x 10^6	4.1 x 10^5	3.2 x 10^2
		1.2 x 10^6	2.7 x 10^5	5.2 x 10^2
		24 Hours		
Shigella	Pure	8.0 x 10^5	1.2 x 10^3	0
		7.0 x 10^5	1.1 x 10^3	0
	Mixed	1.3 x 10^3	1.84 x 10^2	0
		1.1 x 10^3	1.12 x 10^2	0
Aerobacter	Pure	3.5 x 10^9	3.3 x 10^9	1.17 x 10^4
		3.7 x 10^8	4.1 x 10^9	9.7 x 10^3
	Mixed	2.4 x 10^8	3.8 x 10^8	1.49 x 10^4
		2.7 x 10^8	2.0 x 10^9	1.21 x 10^4

Pop. sizes recorded as no. of organisms / ml culture.

Study 1		TEMPERATURE (cont'd) 37°C	30°C	44°C
		48 Hours		
Shigella	Pure	4.1×10^7	2.1×10^5	0
		3.2×10^7	2.4×10^5	0
	Mixed	8.0×10^2	2.0×10^1	0
		1.39×10^3	7.0×10^1	0
Aerobacter	Pure	2.6×10^8	2.6×10^8	6.3×10^6
		1.93×10^8	3.4×10^8	5.1×10^6
	Mixed	2.2×10^7	6.3×10^7	7.4×10^6
		1.81×10^6	3.9×10^6	4.1×10^6

Study 2

Inocula:	**Shigella**	**Aerobacter**
	7.4×10^1	7.0×10^1
	8.2×10^1	6.8×10^1

		5 Hours		
Shigella	Pure	6.61×10^2	2.04×10^2	7.7×10^1
		6.61×10^2	2.02×10^2	7.6×10^1
	Mixed	6.44×10^2	2.26×10^2	1.12×10^2
		6.53×10^2	2.26×10^2	8.6×10^1
Aerobacter	Pure	1.04×10^4	1.95×10^3	1.24×10^2
		1.12×10^4	2.38×10^3	1.57×10^2
	Mixed	9.4×10^3	2.05×10^3	1.61×10^2
		1.24×10^4	2.45×10^3	1.93×10^2
		9 Hours		
Shigella	Pure	4.7×10^3	5.3×10^2	1.4×10^1
		5.6×10^3	6.1×10^2	3.3×10^1
	Mixed	4.6×10^3	5.6×10^2	6.1×10^1
		4.0×10^3	5.5×10^2	3.3×10^1
Aerobacter	Pure	1.28×10^6	1.1×10^5	2.5×10^2
		1.44×10^6	2.5×10^5	3.0×10^2
	Mixed	1.26×10^6	1.62×10^5	2.7×10^2
		1.85×10^6	2.0×10^5	3.2×10^2

Study 2		TEMPERATURE (cont'd) 37 C	30 C	44 C
		24 Hours		
Shigella	Pure	9.6×10^6	1.14×10^4	0
		6.4×10^6	1.01×10^4	0
	Mixed	2.76×10^4	8.3×10^2	1.8×10^1
		2.18×10^4	6.1×10^2	1.1×10^1
Aerobacter	Pure	2.7×10^8	4.5×10^8	9.7×10^3
		3.3×10^8	3.3×10^8	9.9×10^3
	Mixed	4.1×10^8	3.1×10^8	1.07×10^4
		2.3×10^7	3.7×10^7	8.1×10^3
		48 Hours		
Shigella	Pure	5.7×10^7	1.11×10^6	0
		3.4×10^7	1.83×10^6	0
	Mixed	1.67×10^4	7.0×10^1	0
		4.26×10^4	6.0×10^2	0
Aerobacter	Pure	2.4×10^8	3.6×10^8	5.0×10^6
		1.51×10^8	2.8×10^8	4.1×10^6
	Mixed	3.1×10^8	3.2×10^8	4.8×10^6
		2.4×10^8	3.7×10^8	3.9×10^6

Study 3
Inocula:

Shigella	**Aerobacter**
1.5×10^2	6.9×10^1
1.88×10^2	7.6×10^1

		5 Hours		
Shigella	Pure	6.2×10^2	2.04×10^2	1.87×10^2
		5.1×10^2	2.01×10^2	1.81×10^2
	Mixed	6.8×10^2	2.05×10^2	2.24×10^2
		5.4×10^2	1.82×10^2	2.08×10^2
Aerobacter	Pure	7.6×10^3	1.14×10^3	1.1×10^2
		4.5×10^3	1.23×10^3	1.6×10^2
	Mixed	6.2×10^3	1.13×10^3	1.1×10^2
		8.5×10^3	1.04×10^3	1.3×10^2

Study 3

TEMPERATURE (cont'd)

		37 C	30 C	44 C
		9 Hours		
Shigella	Pure	9.6×10^3 6.5×10^3	3.3×10^2 2.0×10^2	5.7×10^1 5.8×10^1
	Mixed	7.3×10^3 9.0×10^3	2.1×10^2 2.7×10^2	8.8×10^1 8.8×10^1
Aerobacter	Pure	2.2×10^6 8.2×10^5	2.2×10^5 1.55×10^5	2.4×10^2 2.3×10^2
	Mixed	2.68×10^6 2.39×10^6	1.92×10^5 1.1×10^5	2.8×10^2 3.4×10^2
		24 Hours		
Shigella	Pure	7.5×10^6 7.1×10^6	7.7×10^2 4.1×10^2	8 3
	Mixed	1.39×10^4 1.86×10^4	3.6×10^2 2.9×10^2	0 1.6×10^1
Aerobacter	Pure	1.7×10^8 3.1×10^7	2.4×10^7 3.0×10^7	1.29×10^3 1.47×10^3
	Mixed	4.4×10^8 3.5×10^8	3.6×10^8 2.2×10^7	NR 2.6×10^3
		48 Hours		
Shigella	Pure	3.7×10^7 6.1×10^7	NR NR	0 0
	Mixed	NR NR	NR NR	0 0
Aerobacter	Pure	2.1×10^9 1.9×10^9	1.82×10^9 3.0×10^9	2.0×10^5 5.0×10^5
	Mixed	1.63×10^8 1.71×10^8	2.9×10^8 1.71×10^8	2.0×10^5 1.56×10^6

TEMPERATURES (cont'd)

Study 4

Inocula: Shigella 1.28×10^2, 1.41×10^2; Aerobacter 9.9×10^1, 9.5×10^1

5 Hours

		37°C	30°C
Shigella	Pure	3.89×10^2 3.9×10^2	1.81×10^2 1.67×10^2
	Mixed	2.85×10^2 3.8×10^2	2.28×10^2 2.25×10^2
Aerobacter	Pure	8.5×10^3 8.2×10^3	2.16×10^3 1.95×10^3
	Mixed	4.3×10^3 7.9×10^3	1.39×10^3 1.41×10^3

24 Hours

Shigella	Pure	1.02×10^7 1.12×10^7	5.0×10^3 6.0×10^3
	Mixed	1.3×10^4 1.8×10^4	8.55×10^2 1.7×10^3
Aerobacter	Pure	4.0×10^8 5.7×10^8	2.62×10^8 3.3×10^8
	Mixed	3.3×10^8 2.58×10^8	3.37×10^8 3.01×10^8

48 Hours

Shigella	Pure	7.1×10^7 7.9×10^7	5.3×10^6 8.4×10^6
	Mixed	9.4×10^3 1.18×10^4	2.42×10^3 8.6×10^2
Aerobacter	Pure	2.11×10^8 2.59×10^8	3.8×10^8 2.59×10^8
	Mixed	1.88×10^8 2.08×10^8	2.04×10^8 4.3×10^8

pH (cont'd)

Study 1		pH 6	pH 7	pH 8
		27 Hours		
Aerobacter	Pure	1.61×10^8	2.2×10^8	3.2×10^8
		1.56×10^8	2.3×10^8	3.2×10^8
	Mixed	1.9×10^8	3.1×10^8	2.2×10^8
		2.2×10^8	2.5×10^8	2.4×10^8
		53 Hours		
Shigella	Pure	4.8×10^7	6.0×10^2	7.0×10^6
		NR	3.0×10^2	1.31×10^7
	Mixed	NR	0	1.08×10^4
		NR	0	9.0×10^2
Aerobacter	Pure	1.32×10^8	1.07×10^8	1.9×10^8
		9.0×10^7	1.9×10^8	2.9×10^8
	Mixed	1.51×10^8	1.42×10^8	1.24×10^8
		1.04×10^8	1.81×10^8	1.2×10^8

Study 2

Inocula: Shigella 9.6×10^1, 9.1×10^1 — Aerobacter 3.1×10^1, 3.3×10^1

Initial pH: 6 - 6.05; 7 - 7.0; 8 - 7.8

Final pH:

	6	7	8
Shigella	5.95	6.95	7.85
Mixture	5.8	6.8	7.5
Aerobacter	5.6	6.75	7.5

		5 Hours		
Shigella	Pure	4.3×10^2	2.4×10^2	2.8×10^2
		3.5×10^2	2.3×10^2	3.7×10^2
	Mixed	2.8×10^2	2.0×10^2	3.5×10^2
		4.3×10^2	1.93×10^2	3.1×10^2
Aerobacter	Pure	2.9×10^3	1.9×10^3	1.01×10^3
		2.6×10^3	1.7×10^3	3.6×10^3
	Mixed	2.9×10^3	1.43×10^3	2.5×10^3
		1.52×10^3	1.13×10^3	1.9×10^3

pH

Study 1

Inocula: Shigella 6.6 x 10^{1}, 5.5 x 10^{1} — Aerobacter 4.1 x 10^{1}, 5.1 x 10^{1}

Initial pH: 6 - 6.05; 7 - 7.0; 8 - 7.9

Final pH:

	6	7	8
Shigella	6.1	7.0	7.95
Mixture	5.65	6.85	7.55
Aerobacter	5.6	6.85	7.55

		pH 6	pH 7	pH 8
		5 Hours		
Shigella	Pure	3.5 x 10^{2} 2.3 x 10^{2}	1.93 x 10^{2} 1.82 x 10^{2}	2.6 x 10^{2} 3.8 x 10^{2}
Shigella	Mixed	3.4 x 10^{2} 2.1 x 10^{2}	2.19 x 10^{2} 2.3 x 10^{2}	3.9 x 10^{2} 3.4 x 10^{2}
Aerobacter	Pure	4.6 x 10^{3} 5.2 x 10^{3}	4.2 x 10^{3} 4.5 x 10^{3}	3.3 x 10^{3} 5.0 x 10^{3}
Aerobacter	Mixed	4.4 x 10^{3} 7.1 x 10^{3}	4.8 x 10^{3} 4.3 x 10^{3}	3.4 x 10^{3} 3.9 x 10^{3}
		9 Hours		
Shigella	Pure	1.31 x 10^{3} 1.1 x 10^{3}	5.5 x 10^{2} 4.5 x 10^{2}	2.3 x 10^{3} 2.3 x 10^{3}
Shigella	Mixed	1.7 x 10^{3} 1.6 x 10^{3}	5.5 x 10^{2} 5.2 x 10^{2}	3.0 x 10^{3} 2.3 x 10^{3}
Aerobacter	Pure	7.3 x 10^{5} 6.9 x 10^{5}	7.8 x 10^{5} 8.5 x 10^{5}	3.7 x 10^{5} 4.3 x 10^{5}
Aerobacter	Mixed	6.4 x 10^{5} 5.8 x 10^{5}	6.7 x 10^{5} 8.1 x 10^{5}	3.7 x 10^{5} 4.2 x 10^{5}
		27 Hours		
Shigella	Pure	3.4 x 10^{4} 3.0 x 10^{5}	1.05 x 10^{3} 3.0 x 10^{2}	7.9 x 10^{4} 8.9 x 10^{4}
Shigella	Mixed	NR NR	2.1 x 10^{2} 5.7 x 10^{1}	1.24 x 10^{4} 7.6 x 10^{3}

Study 2		pH 6	pH 7	pH8
		8 Hours		
Shigella	Pure	3.4×10^3	5.0×10^2	1.56×10^3
		2.1×10^3	4.0×10^2	9.0×10^2
	Mixed	2.7×10^3	3.0×10^2	1.9×10^3
		1.76×10^3	2.0×10^2	1.56×10^3
Aerobacter	Pure	6.0×10^5	2.1×10^5	2.0×10^5
		8.7×10^5	4.6×10^5	4.0×10^5
	Mixed	4.9×10^5	4.0×10^4	3.2×10^5
		6.0×10^5	6.7×10^5	1.84×10^5
		27 Hours		
Shigella	Pure	1.26×10^7	NR	8.6×10^6
		5.3×10^6	NR	2.7×10^6
	Mixed	1.38×10^4	1.09×10^2	1.42×10^4
		1.09×10^4	2.0×10^2	NR
Aerobacter	Pure	2.9×10^8	2.8×10^8	2.3×10^8
		7.0×10^7	2.8×10^8	3.2×10^8
	Mixed	2.9×10^8	9.0×10^7	3.0×10^8
		1.9×10^8	3.9×10^7	2.2×10^8
		53 Hours		
Shigella	Pure	4.8×10^7	5.0×10^3	7.0×10^6
		4.0×10^7	7.0×10^3	2.6×10^7
	Mixed	7.4×10^3	NR	9.1×10^3
		8.3×10^3	1.3×10^2	1.05×10^4
Aerobacter	Pure	1.08×10^8	3.3×10^8	1.1×10^8
		8.0×10^7	2.5×10^8	1.2×10^8
	Mixed	1.73×10^8	1.32×10^8	1.81×10^8
		1.81×10^8	2.2×10^8	1.36×10^8

Study 3

Inocula: Shigella: 9.8×10^1, 1.04×10^2 — Aerobacter: 4.6×10^1, 4.5×10^1

Initial pH: 6 - 6.0; 7 - 7.0; 8 - 8.0

Final pH:

	6	7	8
Shigella	6.0	7.05	8.05
Mixture	5.55	6.80	7.50
Aerobacter	5.50	6.70	7.65

5 Hours

		pH6	pH 7	pH 8
Shigella	Pure	3.9×10^2 3.3×10^2	1.53×10^2 1.28×10^2	3.2×10^2 3.2×10^2
	Mixed	3.4×10^2 3.0×10^2	1.79×10^2 1.86×10^2	2.8×10^2 3.1×10^2
Aerobacter	Pure	4.8×10^3 6.2×10^3	4.1×10^3 4.1×10^3	3.7×10^3 3.4×10^3
	Mixed	7.0×10^3 5.5×10^3	3.8×10^3 4.8×10^3	3.2×10^3 3.3×10^3

9 Hours

Shigella	Pure	2.1×10^3 1.1×10^3	2.0×10^2 2.7×10^2	3.1×10^3 2.2×10^3
	Mixed	2.0×10^3 2.5×10^3	4.5×10^2 4.6×10^2	1.3×10^3 2.1×10^3
Aerobacter	Pure	1.0×10^6 5.4×10^5	6.7×10^5 4.2×10^5	2.1×10^5 2.0×10^5
	Mixed	7.0×10^5 6.0×10^5	3.6×10^5 3.5×10^5	2.8×10^5 2.6×10^5

27 Hours

Shigella	Pure	3.8×10^6 8.7×10^6	9.0×10^2 4.0×10^2	5.2×10^6 6.2×10^6
	Mixed	1.0×10^4 9.0×10^3	4.1×10^1 1.81×10^2	1.06×10^4 7.7×10^3

Study 3

pH (cont'd)

		pH 6	pH 7	pH 8
		27 Hours		
Aerobacter	Pure	2.2×10^{9} 1.9×10^{8}	3.2×10^{8} 3.0×10^{7}	2.6×10^{8} 2.9×10^{8}
	Mixed	3.3×10^{9} 3.6×10^{8}	2.5×10^{9} 3.0×10^{8}	2.4×10^{8} 2.3×10^{8}
		53 Hours		
Shigella	Pure	5.8×10^{7} 2.6×10^{7}	7.0×10^{2} NR	1.01×10^{7} 1.24×10^{7}
	Mixed	8.3×10^{3} 7.1×10^{3}	0 0	4.4×10^{3} 1.9×10^{3}
Aerobacter	Pure	2.3×10^{8} 2.1×10^{7}	1.73×10^{8} 1.63×10^{8}	1.34×10^{8} 1.9×10^{8}
	Mixed	4.3×10^{8} 3.4×10^{8}	8.0×10^{7} 1.04×10^{8}	1.9×10^{8} 9.0×10^{7}

OXYGEN

Study 1
Inocula: Shigella 7.6 x 10^1, 6.9 x 10^1; Aerobacter 7.1 x 10^1, 6.9 x 10^1

8 Hours

		Stat.	Sealed	Aerated
Shigella	Pure	3.2 x 10^2	2.06 x 10^2	1.82 x 10^2
		2.6 x 10^2	8.3 x 10^1	2.48 x 10^2
	Mixed	2.0 x 10^2	1.76 x 10^2	2.64 x 10^2
		1.9 x 10^2	4.2 x 10^1	2.0 x 10^2
Aerobacter	Pure	4.2 x 10^3	2.16 x 10^3	1.44 x 10^3
		4.5 x 10^3	1.18 x 10^3	1.18 x 10^3
	Mixed	3.3 x 10^3	1.66 x 10^3	1.52 x 10^3
		4.8 x 10^3	1.25 x 10^3	1.67 x 10^3

9 Hours

Shigella	Pure	1.3 x 10^3	1.34 x 10^2	1.28 x 10^3
		1.01 x 10^3	4.8 x 10^2	1.58 x 10^3
	Mixed	1.71 x 10^3	2.3 x 10^2	2.02 x 10^3
		1.6 x 10^3	2.9 x 10^2	1.07 x 10^3
Aerobacter	Pure	5.5 x 10^5	2.48 x 10^5	1.13 x 10^4
		5.9 x 10^5	7.5 x 10^4	7.6 x 10^3
	Mixed	4.3 x 10^5	8.4 x 10^4	1.04 x 10^4
		6.6 x 10^5	4.8 x 10^4	1.36 x 10^4

27 Hours

Shigella	Pure	5.5 x 10^4	4.0 x 10^2	NR
		4.2 x 10^4	4.7 x 10^3	NR
	Mixed	6.0 x 10^1	2.9 x 10^2	NR
		8.0 x 10^1	1.43 x 10^2	NR
Aerobacter	Pure	1.71 x 10^8	1.7 x 10^8	4.7 x 10^4
		2.1 x 10^8	1.8 x 10^8	4.2 x 10^4
	Mixed	1.21 x 10^8	1.62 x 10^8	3.0 x 10^4
		2.4 x 10^8	1.6 x 10^8	2.9 x 10^4

Pop. sizes recorded as no. of organisms / ml culture.

OXYGEN (cont'd)

Stat.		Sealed		Aerated
		53 Hours		
Shigella	Pure	1.5×10^{7}	2.32×10^{5}	4.16×10^{4}
		3.1×10^{7}	NR	2.29×10^{4}
	Mixed	3.1×10^{1}	9.6×10^{1}	2.79×10^{4}
		0	3	1.2×10^{3}
Aerobacter	Pure	2.0×10^{8}	3.3×10^{7}	4.0×10^{7}
		1.9×10^{8}	3.9×10^{7}	1.47×10^{8}
	Mixed	1.54×10^{8}	3.4×10^{7}	1.32×10^{8}
		2.0×10^{8}	5.1×10^{7}	9.0×10^{7}

Study 2 Inocula:

Shigella	Aerobacter
5.0×10^{1}	8.3×10^{1}
5.3×10^{1}	6.9×10^{1}

		5 Hours		
Shigella	Pure	2.0×10^{2}	4.6×10^{1}	2.2×10^{2}
		2.0×10^{2}	6.7×10^{1}	1.64×10^{2}
	Mixed	1.83×10^{2}	5.0×10^{1}	2.0×10^{2}
		1.81×10^{2}	2.13×10^{2}	2.3×10^{2}
Aerobacter	Pure	5.7×10^{3}	1.83×10^{3}	2.2×10^{3}
		7.5×10^{3}	4.3×10^{3}	3.5×10^{3}
	Mixed	5.8×10^{3}	5.2×10^{3}	2.4×10^{3}
		5.1×10^{3}	2.6×10^{3}	4.0×10^{3}
		9 Hours		
Shigella	Pure	1.07×10^{3}	NR	1.31×10^{3}
		5.0×10^{2}	4.3×10^{2}	2.5×10^{3}
	Mixed	8.0×10^{2}	3.0×10^{2}	1.21×10^{3}
		1.42×10^{3}	1.6×10^{2}	1.13×10^{3}
Aerobacter	Pure	8.4×10^{5}	3.8×10^{4}	5.6×10^{4}
		1.33×10^{6}	3.8×10^{4}	7.61×10^{4}
	Mixed	9.6×10^{5}	6.1×10^{4}	4.4×10^{4}
		7.9×10^{5}	NR	9.6×10^{4}

OXYGEN (cont'd)

Study 2

27 Hours

		Stat.	Sealed	Aerated
		3.6×10^6	4.5×10^2	2.26×10^5
	Pure	2.7×10^6	5.06×10^4	2.89×10^5
Shigella				
	Mixed	1.45×10^2	0	1.37×10^5
		2.09×10^2	4.1×10^1	2.5×10^4
		1.32×10^8	1.41×10^8	1.54×10^7
	Pure	8.0×10^7	1.3×10^8	1.14×10^7
Aerobacter				
	Mixed	2.6×10^5	8.0×10^7	1.01×10^7
		2.1×10^7	1.11×10^8	9.4×10^6

53 Hours

		2.9×10^7	1.63×10^5	1.8×10^4
	Pure	2.9×10^7	7.4×10^4	1.01×10^2
Shigella				
	Mixed	0	0	1.01×10^2
		?	0	4.0×10^2
		1.82×10^9	3.1×10^7	2.36×10^8
	Pure	1.26×10^9	4.2×10^7	9.5×10^7
Aerobacter				
	Mixed	1.71×10^9	1.9×10^7	9.4×10^7
		2.4×10^8	2.0×10^7	1.78×10^8

Study 3

Inocula:

Shigella	Aerobacter
5.5×10^1	7.5×10^1
6.3×10^1	9.5×10^1

5 Hours

		1.8×10^2	2.3×10^2
	Pure	2.2×10^2	4.3×10^2
Shigella			
	Mixed	8.0×10^1	3.4×10^2
		1.4×10^2	4.2×10^2
		3.0×10^3	1.33×10^3
	Pure	6.8×10^3	1.64×10^3
Aerobacter			
	Mixed	5.0×10^3	1.49×10^3
		3.1×10^3	1.45×10^3

OXYGEN (cont'd)

Study 3

		Stat.	Sealed	Aerated
		9 Hours		
Shigella	Pure	1.1×10^3		2.6×10^3
		1.2×10^3		1.9×10^3
	Mixed	8.0×10^2		3.6×10^3
		1.6×10^3		3.8×10^3
Aerobacter	Pure	7.7×10^5		4.4×10^4
		1.15×10^6		5.6×10^4
	Mixed	8.3×10^5		4.5×10^4
		7.4×10^5		6.3×10^4
		27 Hours		
Shigella	Pure	6.3×10^4		2.4×10^5
		5.1×10^6		3.5×10^5
	Mixed	illegible		2.3×10^5
		1.4×10^1		2.2×10^5
Aerobacter	Pure	2.5×10^8		1.05×10^7
		1.9×10^9		9.2×10^6
	Mixed	2.7×10^8		8.9×10^6
		3.5×10^8		1.17×10^7
		53 Hours		
Shigella	Pure	3.8×10^7		3.0×10^5
		3.4×10^7		6.5×10^3
	Mixed	0		3.8×10^1
		0		4
Aerobacter	Pure	2.3×10^8		9.6×10^7
		3.3×10^8		1.37×10^8
	Mixed	1.8×10^8		1.68×10^8
		2.2×10^8		1.1×10^8

Dwarfed colonies, some with irregular outlines, appeared with the normal colonies.

Study 4

Inocula: **Shigella** 7.3×10^1, 7.3×10^1 — **Aerobacter** 6.1×10^1, 7.2×10^1

		Stat.	Sealed	Aerated*
		5 Hours		
Shigella	Pure	2.8×10^2	6.8×10^1	1.4×10^2
		2.6×10^2	2.33×10^2	1.4×10^2
	Mixed	2.3×10^2	8.8×10^1	1.6×10^2
		3.5×10^2	9.2×10^1	1.5×10^2
Aerobacter	Pure	1.53×10^4	2.48×10^3	7.5×10^3
		1.48×10^4	1.44×10^3	8.1×10^3
	Mixed	1.69×10^4	9.3×10^2	9.4×10^3
		1.43×10^4	3.65×10^3	6.7×10^3
		9 Hours		
Shigella	Pure	1.71×10^3	6.1×10^2	8.7×10^2
		1.71×10^3	2.4×10^2	8.2×10^2
	Mixed	1.07×10^3	1.54×10^2	7.1×10^2
		1.36×10^3	1.33×10^2	7.9×10^2
Aerobacter	Pure	1.42×10^6	5.87×10^4	9.6×10^5
		1.41×10^6	8.02×10^4	8.3×10^5
	Mixed	1.89×10^6	8.74×10^4	1.01×10^6
		1.12×10^6	5.11×10^4	6.4×10^5
		27 Hours		
Shigella	Pure	1.07×10^7	3.9×10^3	2.96×10^6
		6.6×10^6	2.91×10^4	1.05×10^6
	Mixed	NR	2.0×10^1	2.7×10^3
		NR	2.0×10^1	7.0×10^2
Aerobacter	Pure	2.1×10^8	1.57×10^7	4.3×10^8
		2.2×10^8	1.21×10^8	2.5×10^8
	Mixed	2.2×10^8	1.16×10^8	5.4×10^8
		2.4×10^8	2.1×10^8	5.5×10^8

*Mildly aerated- 1/5 rpm

OXYGEN (cont'd)

Study 4

53 Hours

Shigella	Pure	2.8×10^7	5.2×10^4	2.4×10^7*
		3.7×10^7	8.5×10^4	3.23×10^7
	Mixed	NR	2	3.0×10^1
		NR	0	2.4×10^1
Aerobacter	Pure	2.4×10^8	3.0×10^7	7.3×10^8
		2.0×10^8	2.0×10^7	2.3×10^8
	Mixed	2.1×10^8	2.0×10^7	4.3×10^8
		9.0×10^7	3.0×10^7	5.6×10^8

*Mildly aerated - 1/5 rpm

NUTRIENT CONCENTRATION

Study 1
Inocula: Shigella 6.7×10^1, 8.2×10^1 Aerobacter 6.4×10^1, 6.3×10^1

		1X	0.1X	10X
		5 Hours		
Shigella	Pure	4.4×10^2 3.6×10^2	1.39×10^2 1.53×10^2	1.31×10^3 1.47×10^3
Shigella	Mixed	4.1×10^2 3.3×10^2	1.29×10^2 1.28×10^2	1.35×10^3 1.26×10^3
Aerobacter	Pure	7.6×10^3 6.2×10^3	1.42×10^3 1.36×10^3	3.8×10^4 2.4×10^4
Aerobacter	Mixed	7.1×10^3 8.1×10^3	1.4×10^3 1.51×10^3	1.8×10^4 2.9×10^4
		9 Hours		
Shigella	Pure	3.0×10^3 2.6×10^3	3.55×10^2 2.9×10^2	1.43×10^3 1.45×10^3
Shigella	Mixed	2.5×10^3 2.3×10^3	3.54×10^2 3.08×10^2	1.64×10^3 1.73×10^3
Aerobacter	Pure	1.39×10^6 9.6×10^5	6.0×10^4 6.0×10^4	3.6×10^6 3.5×10^6
Aerobacter	Mixed	1.21×10^6 1.88×10^6	3.0×10^4 1.31×10^5	2.1×10^6 2.8×10^6
		27 Hours		
Shigella	Pure	1.5×10^7 9.8×10^6	NR NR	NR 1.13×10^5
Shigella	Mixed	1.16×10^4 9.4×10^3	6 0	5.1×10^4 7.7×10^4
Aerobacter	Pure	3.1×10^8 2.6×10^8	2.2×10^7 2.2×10^8	7.6×10^8 5.7×10^8
Aerobacter	Mixed	2.3×10^8 2.1×10^8	2.2×10^4 2.6×10^8	7.3×10^8 7.8×10^8

Pop. sizes recorded as no. of organisms / ml culture.

NUTRIENT CONCENTRATION (cont'd)

Study 1		1X	0.1X	10X
		53 Hours		
Shigella	Pure	4.0×10^7	1.29×10^3	NR
		5.0×10^7	1.75×10^2	NR
	Mixed	6.1×10^3	3	6.6×10^4
		7.9×10^3	0	8.8×10^4
Aerobacter	Pure	1.1×10^8	2.7×10^8	6.2×10^8
		1.5×10^8	2.6×10^8	7.0×10^8
	Mixed	2.2×10^8	3.3×10^8	5.8×10^8
		1.7×10^9	3.9×10^8	6.0×10^8

Study 2
Inocula:

Shigella	Aerobacter
1.0×10^2	9.7×10^1
1.06×10^2	1.11×10^2

		5 Hours		
Shigella	Pure	5.9×10^2	2.14×10^2	1.91×10^3
		6.8×10^2	2.08×10^2	1.46×10^3
	Mixed	8.2×10^2	2.28×10^2	1.92×10^3
		5.6×10^2	2.41×10^2	2.32×10^3
Aerobacter	Pure	5.6×10^3	4.0×10^2	1.78×10^4
		6.4×10^3	3.5×10^2	2.26×10^4
	Mixed	4.3×10^3	2.4×10^2	1.74×10^4
		3.4×10^3	3.0×10^2	1.52×10^4
		9 Hours		
Shigella	Pure	8.4×10^3	9.0×10^2	8.3×10^4
		7.3×10^3	8.1×10^2	6.2×10^4
	Mixed	8.4×10^3	6.5×10^2	5.7×10^4
		7.6×10^3	8.3×10^2	3.8×10^4
Aerobacter	Pure	2.32×10^6	2.5×10^4	7.6×10^6
		1.99×10^6	1.8×10^4	8.1×10^6
	Mixed	1.75×10^6	2.0×10^4	4.7×10^6
		1.93×10^6	1.6×10^4	4.4×10^6

NUTRIENT CONCENTRATION (cont'd)

Study 2		1X	0.1X	10X
		27 Hours		
Shigella	Pure	1.32×10^{7}	4.87×10^{4}	3.17×10^{7}
		1.55×10^{7}	8.86×10^{4}	2.25×10^{7}
	Mixed	3.6×10^{4}	2.1×10^{1}	1.78×10^{5}
		3.91×10^{4}	1.27×10^{3}	2.0×10^{5}
Aerobacter	Pure	4.8×10^{8}	2.0×10^{8}	4.3×10^{8}
		4.5×10^{8}	2.4×10^{8}	6.7×10^{8}
	Mixed	4.3×10^{8}	1.51×10^{8}	9.0×10^{7}
		3.1×10^{8}	1.9×10^{8}	2.5×10^{8}
		53 Hours		
Shigella	Pure	4.6×10^{7}	6.9×10^{4}*	7.1×10^{7}
		5.0×10^{7}	4.2×10^{4}*	3.8×10^{7}
	Mixed	1.96×10^{4}	0	5.8×10^{4}
		9.3×10^{5}	3	1.0×10^{5}
Aerobacter	Pure	3.6×10^{8}	3.6×10^{8}	2.6×10^{8}
		1.61×10^{8}	3.6×10^{8}	6.7×10^{8}
	Mixed	2.7×10^{8}	1.9×10^{8}	2.5×10^{8}
		2.8×10^{8}	3.7×10^{8}	2.3×10^{8}

Study 3

Inocula: Shigella	Aerobacter
9.0×10^{1}	5.8×10^{1}
9.7×10^{1}	5.2×10^{1}

		5 Hours		
Shigella	Pure	4.2×10^{2}	1.45×10^{2}	1.86×10^{3}
		4.4×10^{2}	1.38×10^{2}	1.63×10^{3}
	Mixed	4.9×10^{2}	1.51×10^{2}	2.15×10^{3}
		3.8×10^{2}	1.39×10^{2}	2.0×10^{3}
Aerobacter	Pure	6.9×10^{3}	1.42×10^{3}	1.98×10^{4}
		6.0×10^{3}	1.08×10^{3}	1.94×10^{4}
	Mixed	5.8×10^{3}	1.44×10^{3}	1.76×10^{4}
		7.0×10^{3}	1.43×10^{3}	1.46×10^{4}

*Dwarfed colonies

NUTRIENT CONCENTRATION (cont'd)

Study 3		1X	0.1X	10X
		9 Hours		
Shigella	Pure	4.0×10^3 3.4×10^3	1.6×10^2 1.9×10^2	2.3×10^4 3.4×10^4
	Mixed	4.2×10^3 5.4×10^3	2.1×10^2 1.2×10^2	4.7×10^4 4.8×10^4
Aerobacter	Pure	1.03×10^6 1.03×10^6	6.1×10^4 7.3×10^4	3.5×10^6 2.2×10^6
	Mixed	8.5×10^5 1.09×10^6	7.7×10^4 6.1×10^4	4.8×10^6 1.4×10^6
		27 Hours		
Shigella	Pure	1.26×10^7 1.11×10^7	1.0×10^5 NR	3.9×10^6 3.7×10^6
	Mixed	5.1×10^3 9.6×10^3	1.2×10^1 5	1.73×10^5 1.91×10^5
Aerobacter	Pure	4.3×10^8 2.4×10^8	2.1×10^7 1.76×10^7	3.1×10^8 2.3×10^8
	Mixed	2.3×10^8 2.3×10^8	1.5×10^7 2.0×10^7	1.4×10^8 2.6×10^8
		53 Hours		
Shigella	Pure	4.6×10^7 6.2×10^7	9.5×10^1 2.0×10^1	9.6×10^6 5.3×10^6
	Mixed	1.61×10^3 2.4×10^3	0 0	2.03×10^5 1.4×10^5
Aerobacter	Pure	3.0×10^8 9.0×10^7	2.5×10^8 1.4×10^7	3.8×10^8 2.0×10^7
	Mixed	1.8×10^8 1.2×10^8	3.3×10^8 2.7×10^7	2.3×10^8 2.5×10^8

APPROVAL SHEET

The dissertation submitted by David J. Hentges has been read and approved by five members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the dissertation is now given final approval with reference to content, form and mechanical accuracy.

The dissertation is therefore accepted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

May 19 1961
Date

MacDonald Fulton
Signature of Adviser