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Comparative growth studies of the lactose and non-lactose fermenting strains of Shigella sonnei.

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COMPARATIVE GROWTH STUDIES OF THE LACTOSE AND NON-LACTOSE FERMENTING STRAINS OF SHIGELLA SONNEI

by

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INTRODUCTION

In 1904, Duval and Shorer (6) reported the isolation of a late-lactose fermenting micro-organism from the feces of infants with diarrhea. Later the same year, Duval (5) again reported the isolation of these organisms from the contents and scrapings of the gut in a fatal case of dysentery occurring in an adult. These organisms were short bacilli which grew well on ordinary culture media and corresponded closely with Bacillus dysenteriae* except for their ability to ferment lactose and to produce a secondary acid change in neutral litmus milk. The patient's serum agglutinated the homologous organism at a dilution of 1:400, the Flexner-Harris bacillus at 1:200, B. typhosus* at 1:80, and did not agglutinate Shiga's bacillus. In addition, serum of typhoid fever patients agglutinated the new organisms to high titer, and B. typhosus* was agglutinated by anti-sera of rabbits immunized with the newly isolated bacillus.

The production of common agglutinins showed a close relationship between B. typhosus* and Duval's bacillus. However, Duval considered it as another member of the dysentery group since it had other properties in common with this group.

Baerthlein (1) and others also reported the isolation of late-lactose fermenting enteric bacilli, but they considered them as an unagglutinable type of Flexner's bacillus and did

* Authors terminology

not study them further.

In 1915, Sonne (16), while investigating an outbreak of dysentery in Copenhagen, found the causative agent to be a late-lactose fermenting bacillus which he showed to be serologically specific and distinct from Type I and Type II pseudodysentery organisms previously described by Kruse (13). Sonne proposed creating a new group, Group III to include this organism in the dysentery scheme.

D'Herelle (4), in 1916, also found this atypical organism associated with dysentery in France.

Thjøtta (18), in 1919, while investigating an outbreak of dysentery in Norway, confirmed and extended the work of Sonne. Thjøtta stated that the grouping of the dysentery organisms should be based upon serological rather than fermentative differences since this older method was subject to wide variation.

Since Kruse's Groups I and II were so closely related serologically, Thjøtta proposed placing them in one main group - Group II. He proposed Shiga's bacillus as Group I, although there was slight agglutination with Group II. Sonne's Group III showed no cross-agglutination with either Groups I or II and was maintained as a separate group.

Thjøtta noticed the presence of two characteristic types of colony while studing the cultural characteristics of the Group III organisms. One was small, round and dense, the other, larger with irregular edges, and exhibited irregular-

ities of agglutination, either being unagglutinable or showing spontaneous agglutination in saline.

Later, Koser and Styron (12) found that some of the cultures of Shigella sonnei[1] had a tendency to undergo changes not included under the term dissociation. These investigators thought that changes from Smooth (S) to Rough (R) were a gradual, continuous process and did not occur in distinct abrupt stages.

Glynn and Starkey (7) recognized two serological types that were independent of colony variation. These were Type I and Type II. The latter was found to be serologically broader than Type I. Antisera prepared against Type II cultures agglutinated both Type I and II whereas Type I antisera gave only a slight reaction with Type II. Since both serological types could be found on the original plate, the authors suggested the possibility of diphasic variation.

Wheeler and Mickle (19) extended the work of Glynn and Starkey. They described three morphological and antigenically distinct forms of the organism. They termed these Phase I (smooth), Phase II and Rough.

Later Baker, Goebel and Perlman (2), studied the antigenic structure of these variants of Sh. sonnei. They found that Phase I mutated spontaneously to Phase II (s) and Phase II (r). These phase variants were found to be stable and serologically identical but morphologically distinct. The Rough variant was found to be similar to Phase II (r).

[1] This name was later adopted for the organism described by Sonne.

Delayed fermentation of lactose was noted by early investigations to be one of the distinguishing features of Sh. sonnei. Reynolds and his associates (14) and later Cook, Knox and Tomlinson (3) demonstrated the appearance of secondary papillae after several days' incubation on differential media. Inoculation of lactose broth with these acid papillae resulted in fermentation of the sugar within forty-eight hours.

Glynn and Starkey (7) also demonstrated the appearance of secondary papillae which caused the medium immediately beneath them to be acid. Attempts by these investigators to produce secondary papillae that bred true were unsuccessful. Each generation always began as a lactose-negative colony with secondary papillae occurring at about the same time interval as the initally isolated colonies.

Sears and Schoolnick (15) stated that the late fermentation of lactose was due not to slow utilization of the sugar by the original organisms, but to the invariable appearance in the lactose medium of variants which then attacked this medium vigorously. They found that these variants were stable and that the fermentative reactions remained unchanged even after smooth - rough dissociation had occurred. These investigators were able to produce strains of Sh. sonnei which fermented lactose, sucrose, and raffinose. They were also able to isolate strains capable of prompt fermentation of any or all of these sugars.

In 1951, Cook, Knox and Tomlinson (3) confirmed these results and showed that the variants retained these properties even after several months of occasional transfer into media free of the specific substrate. Since the variants apparently were able to transmit the new characteristics indefinitely to their descendants, these workers referred to them as mutant cells. They considered these mutants to be spontaneous and to occur at a rate of one mutant per 10^5 normal organisms. However, other investigators, including Hinshelwood and his group (8), consider late-lactose fermentation to be due to the presence of a substrate which stimulates the cells to produce the enzyme or enzymes necessary to attack the substrate.

Kacoyanis and Baker (11) and Kacoyanis (9) studied the time of appearance of the lactose fermentation using a single strain of Sh. sonnei. These investigators, using large numbers of lactose broth cultures of Sh. sonnei, found that some cultures turned acid after four to five days' incubation with the number of cultures turning acid per day increasing until the fifteenth day of incubation. They also found lactose positive mutants present in cultures which had turned acid between the tenth and fifteenth days but could isolate these mutants only infrequently after this time. The mutants were found to be stable once isolated and able to ferment lactose broth promptly. Kacoyanis (9), in 1956, employed the term prompt fermentation to indicate the appearance of acid within forty-eight hours and delayed or late fermentation

to indicate the appearance of acid after a period longer than forty-eight hours. He also found that as few as one to ten lactose-fermenting organisms could grow in the presence of a large number of normal organisms and produce prompt lactose fermentation. He therefore concluded that delayed fermentation was due to a lack of lactose positive (Lac+) mutants in the initial inoculum rather than the inhibitory action of the normal cells.

Kacoyanis (10) states further that aeration of the normal strain of Sh. sonnei in lactose broth favored the appearance of Lac+ mutants. However, normal organisms grown under aerated conditions in carbohydrate-free broth did not favor the appearance of Lac+ mutants although Lac-mutants could be isolated from the same broth seeded with a pure culture of Lac+ mutants.

This investigator also found that acid was produced in lactose broth cultures inoculated with Sh. sonnei and incubated anaerobically. Since no Lac+ mutants could be isolated from these acidified cultures, it was postulated that some unknown mechanism which differed from that used by the Lac+ mutants was employed when the organisms were grown under anaerobic conditions.

Shigella sonnei is know to exist in a lactose and non-lactose fermenting form. However, only the non-lactose fermenter is found in nature. The purpose of these investigations is to determine the factors which result in the more successful competition of the non-lactose fermenting form of Sh. sonnei.

MATERIALS AND METHODS

I. Strains of Shigella sonnei

The strain of Sh. sonnei employed in these experiments was originally isolated from a patient at the Boston City Hospital and was coded #1545.

The Lac+ mutant of Sh. sonnei was isolated by Dr. George J. Kacoyanis at the Boston University School of Medicine and was coded #T_2.

These strains were maintained on nutrient agar and stored at 5 C. Additional cultures were maintained in the frozen state.

II. Media

A. Carbohydrate-free Broth

Carbohydrate-free broth was composed of 5.0 g of Bacto Peptone and 3.0 g of Meat Extract in one liter of distilled water. It was brought to a final pH of 7.25. As required, the medium was reinforced with 0.5 per cent of glucose or lactose.

BCP broth was prepared by adding one ml of 1.0 per cent Brom Cresol Purple indicator per liter of broth (with or without carbohydrate). A purple color of the BCP broth was considered to indicate neutral to alkaline conditions and a yellow color was indicative of acid conditions.

B. Differential Media

To differentiate between Lac + mutants and normal Sh. sonnei, Eosin-Methylene-Blue (EMB) agar, Difco, was

used.

Dry, sterile agar plates were prepared by pouring the agar into clay top petri dishes and were incubated at 37 C for twenty-four hours. After incubation, sterile glass tops replaced the clay tops and the plates were stored for no longer than three days before using.

III. Methods

A. Preparation of Cultures

A fresh, young culture was prepared by inoculating a single colony from a nutrient agar plate into 6 ml of nutrient broth and incubating for from seventeen to eighteen hours. One half ml of the fresh nutrient broth culture was inoculated into lactose, glucose, and carbohydrate-free broths and incubated for from seventeen to eighteen hours on two successive days.

A mixed culture was prepared by mixing equal amounts of the Lac+ mutants and normal Sh. sonnei that had been prepared in the same broth medium.

B. Bacterial Counts

The method approved by the American Public Health Association (17) for water analysis was employed to estimate the numbers of Lac+ mutants and normal organisms present in the culture. Ten-fold serial dilutions of the cultures were made using sterile distilled water as the diluent. 0.2 ml of the appropriate dilutions were streaked onto duplicate EMB agar plates and incubated at 37 C for forty-eight hours. The results were expressed as numbers of

organisms per milliliter of sample.

C. Aeration

When required, aeration of cultures in tubes measuring 28 x 195 mm was accomplished on an oscillating shaker which rotated at about 160 rpm. The depth of liquid in the tubes was 20 mm. Thus, relatively good aeration was obtained.

TERMINOLOGY

In the main body of the paper, the following terminology will be used:

Normal organism.....	non-lactose fermenter
Lac+ mutant.....	lactose fermenter
Lac- mutant.....	non-lactose fermenter appearing in cultures seeded with Lac+ variants
Aerated conditions.....	cultures that were incubated under aerated conditions produced by shaking.
Still conditions.....	cultures incubated under non-aerated conditions

EXPERIMENTAL RESULTS

Comparative growth advantage of the lactose and non-Lactose fermenting forms of Shigella sonnei.

To determine the comparative growth advantage of the normal and Lac+ mutant of Sh. sonnei, fresh, young cultures were prepared in the manner describe under Materials and Methods. 0.05 ml of pure and mixed cultures grown in lactose, glucose and carbohydrate-free broth were transferred to 250 ml flasks containing 100-ml amounts of lactose, glucose and carbohydrate-free broths respectively. These cultures were incubated at 37 C under both aerated and still conditions. Immediately following inoculation and daily, or as indicated in Table I, bacterial counts were made on each culture by the methods described under Materials and Methods.

Behavior of the non-lactose fermenting (normal) organisms in various liquid media under still conditions.

Behavior of the normal organisms in lactose broth: After the initial rise to the density of 10^7 organisms per milliliter (see Figure 1.), the normal organisms maintained an essentially constant population in lactose broth for nine days. On the ninth day of incubation, the Lac+ mutant appeared in numbers equal to those of the normal organism. After the appearance of the Lac+ mutant, the population of the culture declined rapidly so that by the fifteenth day no

viable organisms were present. The Lac+ mutants appeared simultaneously with a change from alkaline to acid conditions. The culture remained acid for the remainder of the experiment.

Behavior of the normal organisms in glucose broth: The normal organisms grew rapidly in glucose broth during the first twenty-four hours of incubation (see Figure 3) and the culture maintained itself during the following twenty-four hour period. However, the population dropped precipitously after seventy-two hours' incubation and subcultures were no longer viable after this time. The medium became acid during the first twenty-four hours and remained acid for the duration of the experiment.

Behavior of the normal organisms in carbohydrate-free broth: As seen in Figure 5, the growth of the normal organism in carbohydrate-free medium was comparable to the growth of the same organism under the same conditions in lactose broth. (see Figure 1). The population remained at this level for nine days, gradually declining during the subsequent two-week period. The culture was still viable at a level of 10^7 organisms per milliliter when the experiment was terminated. No Lac+ mutants appeared on the EMB plates used for counting.

The continued growth and viability of the normal

organisms throughout the experiment may be explained by maintenance of a favorable pH. The medium remained alkaline to neutral during the entire course of the experiment.

Behavior of non-lactose fermenting (normal) organisms in various liquid media under aerated conditions.

Behavior of the normal organisms in lactose broth: During the first half of the experiment, (see Figure 7), the only notable difference between the growth of the normal organisms in the still and aerated lactose broth cultures was the more rapid increase of the population to a slightly higher level when aeration was employed. However, different growth patterns developed after eleven days of incubation. During the latter half of the experiment, the population of the normal organisms decreased only gradually and when the experiment was terminated 10^6 organisms per milliliter were present in the culture.

The increase resulting from aeration was reflected in the earlier appearance of Lac+ mutants (after forty-eight hours' incubation). The growth of the Lac+ mutants paralleled the growth of the normal organisms up to the eleventh day but the population declined rapidly after the fifteenth day of incubation and was not detectable thereafter.

As in the case of the still lactose cultures seeded with normal organisms, the appearance of the Lac+ mutants

occurred simultaneously with a change from alkaline to acid conditions. However, unlike the still cultures, a return to alkalinity occurred during the following twenty-four hour period.

Behavior of normal organisms in glucose broth: The results of the growth of normal organisms in aerated glucose broth were no different from those obtained under still conditions except for a somewhat accelerated death phase.

As seen in Figure 9, the population of the culture after twenty-four hours' incubation was somewhat higher than that of the same organisms in glucose broth under still conditions. The population declined sharply after forty-eight hours and no viable organisms were detectable thereafter. As with the still glucose cultures, the medium became acid during the first twenty-four hours' incubation, and remained acid for the duration of the experiment.

Behavior of normal organisms in carbohydrate-free broth: Except for a slightly higher initial population, the growth of the normal organisms in aerated carbohydrate-free was not essentially different from the growth of the same organisms in the still carbohydrate-free medium (see Figures 11 and 5). The population remained at a high level throughout the experiment, with 10^7 organisms per milliliter present at the termination. The medium remained neutral to alkaline for the duration of the experiment.

Behavior of the lactose-fermenting (Lac+) mutants in various liquid media under still conditions.

Behavior of the Lac+ mutants in lactose broth: Maximum growth of the Lac+ mutants occurred after twenty-four hours' incubation (see Figure 1) and was equivalent to the growth of the normal organisms in the same medium under the same conditions. However, unlike the normal organisms, the population decreased steadily and no viable organisms were present in the culture after seven days' incubation.

The BCP lactose medium became acid during the first twenty-four hours' incubation and remained acid for the duration of the experiment. No Lac- mutants (back mutation) were detected in the culture.

Behavior of the Lac+ mutants in glucose broth: In Figure 2, it can be seen that the growth of the Lac+ mutants in glucose broth did not differ from the growth of the normal organisms in glucose broth under the same conditions. Acid conditions were the same in these cultures as seen before with all the other glucose broth cultures.

Behavior of the Lac+ mutants in carbohydrate-free broth: For twenty-one days, the growth of the Lac+ mutants essentially paralleled that of the normal organisms grown in the same medium under still conditions (see Figure 3). When the experiment was terminated, the viable population had decreased from 10^7 to 10^4 organisms per milliliter.

On the eleventh day of incubation, Lac- organisms (back mutation) were first noted in numbers approximately equal to those of the Lac+ mutants (10^5 organisms per milliliter). The population of the Lac- mutants increased gradually and when the experiment was terminated, 10^6 organisms per milliliter were present in the culture. These Lac- mutants were tested biochemically and serologically and were shown to be non-lactose-fermenting mutants of Sh. sonnei which were indistinguishable from the normal organisms. As in the case of all other carbohydrate-free broth cultures, the pH of the medium remained in a neutral to alkaline range throughout the experiment.

Behavior of the lactose-fermenting (Lac+) mutants in various liquid media under aerated conditions.

Behavior of Lac+ mutants in lactose broth: After twenty-four hours' incubation, the Lac+ mutant reached its' maxiumum population of 10^8 organisms per milliliter (see Figure 7) which was maintained for seven days. After this time, there was a gradual decline in the population and when the experiment was terminated the population was at a level of between 10^3 and 10^4 organisms per milliliter.

After five days' incubation, Lac- organisms (back mutations) appeared and when first noticed had reached numbers

between 10^6 and 10^7 per milliliter. This population remained at this level for ten days. Subsequently, it gradually declined and after twenty-four days 10^4 organisms per milliliter were present.

The BCP lactose medium became acid during the first twenty-four hours' incubation and a return to alkaline condition occurred during the following twenty-four hour period. The culture remained alkaline for the remainder of the experiment.

Behavior of Lac+ mutants in glucose broth: The growth of the Lac+ mutants (see Figure 9) did not differ from the growth of the normal organisms under the same conditions. Maximum growth was obtained after twenty-four hours. The population declined rapidly after forty-eight hours' incubation and the culture was no longer viable after this time. The medium became acid during the first twenty-four hours' incubation and remained acid for the duration of the experiment.

Behavior of the Lac+ mutants in carbohydrate-free broth: Essentially, the growth of the aerated culture again resembled the growth of the same organisms in the same medium under still conditions. However, aerated conditions, as seen before, enhanced the growth of the culture (see Figure 11).

A gradual decline in the population of the culture occurred after three days' incubation; however, the population

Stabilized after seven days. A definite decline in the population occurred after seventeen days and when the experiment was terminated the viable count was 10^4 organisms per milliliter.

Once again, the accelerative effect of aeration on the formation of mutants was attested to by the appearance of Lac- organisms (back mutation) after seven days' incubation. In contrast, the Lac- mutants did not appear in the still, carbohydrate-free cultures until the eleventh day of incubation when they were found to number 10^5 organisms per milliliter. The population increased slightly and then began to decline and after nine days (the fifteenth day of the culture) the Lac- mutant no longer was detected. This differed from the still cultures in that the population of the Lac- organisms under still conditions appeared to be rising at the end of the experiment rather than declining. The medium remained neutral to alkaline throughout the experiment.

Behavior of mixed cultures grown in various liquid media under still conditions.

Behavior of mixed cultures in lactose broth: The Lac+ mutant and the normal organisms behaved similarily and resembled the growth of the Lac+ mutants alone under the same conditions. That is, maximum growth was obtained after twenty-four hours, with the population declining during the

following six-day period. After seven days, no viable organisms were detected. In this respect, the growth curve of the mixed population could be superimposed on the growth curve of the Lac+ mutants when grown alone under the same conditions (see Figures 2 and 1). The medium became acid during the initial twenty-four hours' incubation and remained acid for the duration of the experiment.

Behavior of the mixed cultures in glucose broth: The growth of the mixed cultures was indistinguishable from the growth of each individual pure culture. Again, the medium became acid during the initial twenty-four-hour growth period and remained acid for the duration of the experiment. (see Figures 4 and 3).

Behavior of mixed cultures in carbohydrate-free broth: In carbohydrate-free broth, the two components of the mixed cultures reacted differently (see Figure 6). The growth and viability of the normal organisms were essentially no different from the growth of that strain in pure culture under the same conditions. Maxium growth (10^7 organisms per milliliter) was reached after twenty-fours hours' and the growth was maintained for nine days. The population gradually decreased and after twenty-four days were 10^6 organisms per milliliter present in the culture.

Initially the Lac+ mutants exhibited growth that was comparable to both organisms in pure culture. However, a rapid decline in the numbers of the Lac+ mutant began on the third day of incubation and after five days it was no longer detectable in the culture. The culture remained in a neutral to alkaline pH range during the course of the experiment.

Behavior of mixed cultures grown in various liquid media under aerated conditions.

Behavior of mixed cultures in lactose broth: The growth of the normal organism in the mixed population resembled the growth of the same organism in pure culture under the same conditions for the first nine days. After this time, there was first a gradual and later a rapid decline in the population of the normal organism and when the experiment was terminated only 10^2 normal organisms per milliliter were found (see Figure 8).

For the first nine days, the Lac+ mutant in the mixed culture similarly paralleled the growth of that strain grown in pure culture. However, after this time, the decline in the Lac+ mutant was much more rapid than it was in pure culture or than the normal organisms in the mixed culture, and no viable Lac+ organisms were detected at a dilution of 10^3 or 10^4 organisms per milliliter.

Behavior of mixed cultures in glucose broth: There was no significant difference between the growth pattern of the normal organisms and the Lac+ mutants in mixed glucose broth cultures and their growth in similar pure culture (see Figure 10). In all cases, an initial, rapid increase in population was followed by a very rapid decline with no viable organisms detectable after seventy-two hours. As in the other glucose broth cultures, the medium became very acid during the initial twenty-four hours' incubation and remained acid for the duration of the experiment.

Behavior of mixed cultures grown in carbohydrate-free broth: The normal organism in the mixed cultures showed no essential difference from its growth in pure culture for the first seventeen days of incubation. (see figures 12 and 11). After this time, there was a gradual decline in population. When the experiment was terminated, the concentration of normal organisms was 10^5 organisms per milliliter, which was somewhat lower than that of the pure culture.

The Lac+ mutant followed the growth pattern of the same organism grown in a mixed culture under still conditions. Following an initial increase of the population to 10^8 organisms per milliliter after twenty-four hours' incubation,

a rapid decline in the population occurred. After five days' incubation, the organism was no longer detected at a 10^5 dilution for the remainder of the experiment and was not present at a 10^3 dilution when the experiment was terminated. The medium remained neutral to alkaline throughout the experiment.

Elimination of lactose from lactose broth by lactose fermenting (Lac+) mutants of Shigella sonnei

It was noted constantly that the aerated lactose broth cultures containing Lac+ mutants returned to alkaline conditions within twenty-four hours after acid formation had occurred. Since the aerated glucose broth cultures did not behave in a similar manner, an experiment was designed to determine if this return to alkaline conditions was in any way due to utilization of lactose by the Lac+ mutants.

In order to determine the degree of utilization of lactose by Lac+ mutants of Sh. sonnei, 0.05 ml of a fresh, young culture were inoculated into 28 x 195 mm test tubes containing 10-ml amounts of lactose broth. Two concentrations of lactose were utilized in this experiment, a 0.1 per cent and a 0.5 per cent concentration respectively. The cultures were incubated at 37 C under both aerated and still conditions.

Immediately following inoculation and thereafter as shown in Table II, a culture grown under each set of conditions was sterilized by filtration through a Seitz EK filter. When necessary, the medium was readjusted to its' original pH (7.25) before reinoculation with a fresh culture of the Lac+ mutants in order to determine whether or not the lactose had been completely utilized during the initial incubation, these cultures were reincubated and the presence of lactose determined by the formation of acid.

It was found that under aerated conditions, both the 0.1 per cent and 0.5 per cent concentrations of lactose were completely utilized after twenty-four hours' incubation. When the cultures were grown under still conditions, lactose was still present in the medium after seven days' incubation at the 0.5 per cent level. However, when these organisms were grown in the 0.1 per cent concentration of lactose, no lactose could be detected after two days.

As stated previously, the rapid depletion of lactose when the lactose broth cultures were incubated under aerated conditions appears to be related to the greater growth of the organisms in the presence of molecular oxygen. Growth of the cultures under still conditions is less as shown by the bacterial population (as shown by experiments discussed

previously) as well as the incomplete utilization of lactose.

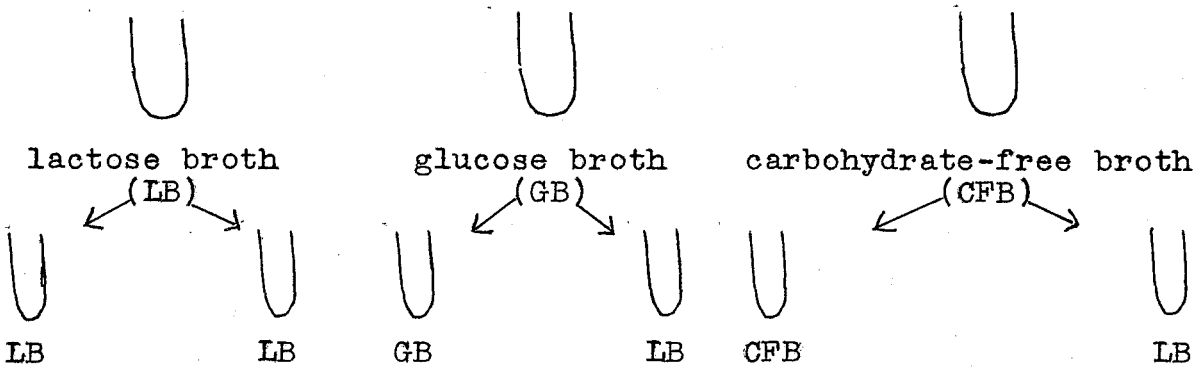
A return to alkaline conditions occurred when Lac+ mutants were grown in lactose broth under aerated conditions. The depletion of lactose from the medium and the subsequent utilization of the acid under aerobic conditions may explain this return to alkaline conditions. The presence of lactose in cultures of Lac+ organisms after seven days' incubation under still conditions is consistent with the persistence of acid conditions in these cultures for the duration of the experiment.

Qualitative determinations of the comparative growth of Lac+ mutants and normal Shigella sonnei.

To determine qualitatively the competitive advantage of the normal and the Lac+ mutants of Sh. sonnei, fresh, young mixed cultures were prepared in the manner described under Materials and Methods.

0.05 ml of the cultures prepared in lactose, glucose, and carbohydrate-free broths were transferred to 28 x 195 mm tubes containing 10-ml amounts of lactose, glucose and carbohydrate-free broth respectively. These cultures were incubated at 37 C under aerated and still conditions. Immediately following inoculation, 0.05 ml of these cultures were transferred to 16 x 125 mm tubes containing 6- ml

amounts of lactose broth to determine the presence of Lac+ mutants in the mixed culture. Daily, or as indicated in Table III, each lactose, glucose, and carbohydrate-free broth culture was transferred to fresh broth containing the same constituents as the parent culture. Transfers also were made into 6- ml amounts of lactose broth daily. Diagrammatically the daily pattern for transfers would appear as follows:



Of twenty mixed cultures in aerated carbohydrate-free broth, all produced acid in the presence of lactose for four days. Thereafter failure to ferment lactose on subculture occurred as follows: one in five days, one in ten days, two in eleven days, fourteen in eighteen days, and one each on the ninteenth and twentieth days.

In contrast, subcultures from aerated glucose or lactose broths revealed Lac+ mutants throughout the duration of the experiment.

In the daily serial still cultures, Lac+ mutants persisted throughout in the carbohydrate-free broth as well as in the lactose and glucose broths.

DISCUSSION

The major differences in behavior of the various cultures were associated with the use or avoidance of aeration. Mechanical agitation supplied cultures with a greater quantity of molecular oxygen as well as a more available supply of nutrients. The effect of these factors was reflected by a faster growth rate and an increase in the total population. The earlier appearance of the mutants in the aerated cultures may be attributed to this acceleration of, and absolute increase in growth.

The behavior of the still lactose broth cultures was similar to that of the glucose broth cultures. The presence of Lac+ mutants in lactose reinforced broth incubated under still conditions resulted in an increase in the acidity of the medium. However, the acidity of these cultures was less than that observed in the glucose cultures. Unlike the aerated lactose cultures, this acidity persisted and resulted in the death of the cultures.

The differences in the times of death of the still lactose cultures and the glucose cultures appear to be related to the degree of acidity of these media. Although the acidity of the lactose broth was not sufficient to cause immediate death of the cultures, it was sufficient to decrease the viability over a period of a week. (Hence the degree of acidity appears to account for the difference in the death rate).

Another condition influenced by aeration was the production of acid from glucose and lactose. Although acid was produced in the presence of either carbohydrate, the acidity was less than that of the still cultures, and in the case of lactose, a return to alkaline conditions occurred. These factors appeared to influence the viability of the cultures.

These conditions, however, do not entirely apply in the case of cultures grown in the presence of glucose. All glucose broth cultures, regardless of the cultural conditions, died after a short period of incubation. The loss of viability appeared to be the result of the high acidity of the medium following the fermentation of glucose by both the normal organism and the Lac+ mutant. The acidity of these cultures increased from pH 7.0 - 7.1 at the start, to pH 4.6 - 4.8 after twenty-four hours' incubation. It was shown further, by a preliminary experiment not recorded in detail with carbohydrate-free broth that had been adjusted for pH before incubation, that organisms did not survive for twenty-four hours in a medium at pH 4.5 or lower.

The continued viability of the aerated lactose cultures in the presence of Lac+ mutants can be explained by the return to alkaline conditions. This return to alkalinity appears to be related to the exhaustion of lactose from the medium during the first twenty-four hours of growth.

Lac+ mutants were detected only in lactose broth that

had originally been inoculated with normal organisms. However, in 1951, Cook, Knox and Tomlinson (3) reported that Lac+ mutants arise spontaneously at the rate of one mutant in 10^5 normal organisms, independent of the substrate. If this be the case, then Lac+ mutants should also be detected in the glucose and carbohydrate-free media. The lack of mutants in the glucose broth is easily explained by the prompt loss of viability of the cultures. In the case of the carbohydrate-free medium the situation is less clear cut. However, experimental evidence suggests that the normal organisms have a selective advantage in this medium. Also, since the sampling technique employed to determine bacterial counts was limited, the fact that the Lac+ mutants were not detected does not necessarily mean that they were not present.

The detection of Lac+ mutants after two days' incubation in the aerated lactose broth which had been inoculated with normal organisms raised the question of the presence of these mutants in the original inoculum. Kacoyanis (9) showed that lactose fermentation (the criterion taken for the presence of mutants) occurred when this medium was seeded with one Lac+ mutant in 10^8 normal organisms and incubated under still conditions. Since Lac+ mutants were not detected for nine days, in still lactose cultures seeded with normal organisms, it is apparent that the early appearance of the mutants in the aerated lactose cultures was due to the

effect of acclerated growth rather than to the presence of the mutant in the original inoculum.

The disappearance of the Lac+ mutant appeared to be related to the competition for the nutrients available in the medium. In the presence of lactose, the growth of the Lac+ mutants was maintained; but once the lactose was exhausted, this advantage was lost. It was seen in both lactose broth and carbohydrate-free broth that the normal organisms have a growth advantage. This is seen immediately in the carbohydrate-free medium with a delay in this condition occurring in lactose broth. This delay, as stated before, appears to be related to the presence of lactose in the medium, the exhaustion of which reduces the growth advantage of the Lac+ mutant. It also may be possible that in gaining the ability to ferment lactose, the Lac+ mutant loses some mechanism (enzyme system?) which would otherwise allow it to compete successfully with the normal organism.

The appearance of Lac+ mutants (back-mutation) in cultures seeded with Lac+ mutants also may be related to the decrease in the viability of the Lac+ mutants, since the Lac+ mutants cannot compete successfully in the absence of lactose. The Lac- mutants would have a selective growth advantage in the absence of lactose and will ultimately predominate.

The experimental evidence obtained when qualitative determinations were made to detect the presence of Lac+ mutants supported the view that normal organisms have a selective advantage over the Lac+ mutants when grown in mixed cultures.

The discrepancy found regarding the viability of the glucose cultures and the still lactose cultures can be explained by the technique of daily subculturing. Since these cultures were transferred to fresh media at frequent intervals, the acid environment was not sustained and therefore was not sufficient to cause a loss of viability in these cultures.

The loss of viability of the Lac+ mutants in the aerated carbohydrate-free broth that was subcultured daily supported the findings of the other experiments. This further supports the supposition that it is the Lac+ mutants' lack of some mechanism that reduces their ability to compete successfully with the normal organisms when no carbohydrate is present.

SUMMARY

1. Aeration of normal organisms in lactose broth favored the appearance of Lac+ mutants.
2. Eventually the Lac+ mutants disappeared and only Lac- organisms could be isolated.
3. Lac+ mutants appeared in cultures of normal organisms grown under unaerated conditions but were delayed as compared with their appearance under aerated conditions.
4. Under aerated conditions, neutral to alkaline conditions returned within twenty-four hours after the first appearance of acid.
5. Lac+ mutants were not observed in glucose or carbohydrate-free broth cultures of normal organisms.
6. Lac- mutants (Back-mutation) appeared in cultures seeded with Lac+ mutants.
7. In mixed cultures the non-lactose fermenter had a selective advantage in the absence of lactose.
8. Qualitative observation made to determine the presence of Lac+ mutants supported the view that normal organisms have a selective advantage over Lac+ mutants when grown in mixed culture in the absence of lactose.

Lac+ Mutants and
rious Broths

ng organisms per milliliter

9			11		15		17		21		24	
L+	L-	L+	L-	L+	L-	L+	L-	L+	L-	L+	L-	L+
$16x10^8$	$68x10^7$	$19x10^8$	$40x10^7$	$16x10^8$	$20x10^7$	$10x10^6$	$10x10^7$	--	$60x10^5$	--	$43x10^5$	--
	$73x10^6$	$13x10^7$	$24x10^5$	$13x10^6$	no growth at 10^4		no growth in undil. samp.					
th in ed sample												
th in ed sample	$24x10^6$		$22x10^6$		$20x10^6$		$28x10^6$		$22x10^6$		$16x10^7$	
	$12x10^7$		$20x10^6$		$65x10^5$		$11x10^6$		$35x10^5$		$33x10^6$	
$78x10^7$	$38x10^6$	$20x10^7$	$13x10^6$	$15x10^7$	$85x10^6$	$23x10^6$	$8x10^5$	$10x10^5$	$14x10^5$	$14x10^5$	$18x10^4$	$25x10^3$
no growth at 10^4	no growth in undil. sample											
no growth in undil.samp.												
1	no growth in undil. sample		$48x10^5$	$35x10^6$	$80x10^4$	$70x10^6$	--	$15x10^6$	--	$52x10^3$	--	$75x10^3$
$28x10^6$	$13x10^6$	$40x10^6$	$28x10^5$	$65x10^5$	$18x10^5$	$78x10^5$	$55x10^5$	$55x10^5$	$27x10^5$	$13x10^5$	$71x10^5$	$43x10^4$
$14x10^7$		$73x10^6$										
$9x10^8$	$44x10^7$	$21x10^7$	$88x10^7$	$80x10^5$	$93x10^6$	--	$50x10^4$	--	$50x10^3$	--	$55x10^2$	--
$30x10^3$	no growth at 10^3		no growth in undil. samp.									
th in samp.												
--	$31x10^6$	--	$16x10^6$	--	$12x10^7$	--	$12x10^6$	--	$20x10^4$	--	$12x10^5$	--
--	$13x10^7$	--	$50x10^5$	--	$18x10^5$	--	$22x10^6$	--	$13x10^6$	--	$97x10^5$	--

o organisms present at
ilution noted

TABLE I

Comparative Growth Studies of the Lac+ Mutants of Normal *Shigella sonnei* Grown in Various Broths

			Number of lactose-fermenting and non-lactose-fermenting organisms								
		Day	0		1		2		3		4
Organism	Medium		L-	L+	L-	L+	L-	L+	L-	L+	L-
Normal	Lactose	A*	13×10^4		23×10^7		11×10^8	15×10^8	93×10^7	14×10^8	88×10^7
		S*	24×10^4		25×10^7		28×10^7		37×10^7		27×10^7
	Glucose	A	98×10^3		40×10^7		16×10^5		no growth at 10^3		
		S	83×10^3		15×10^7		83×10^6		83×10^4		170
	Sugar-Free	A	98×10^3		14×10^8		16×10^8		98×10^7		15×10^7
		S	13×10^4		18×10^7		22×10^7		16×10^7		18×10^7
Lac+	Lactose	A		17×10^4		15×10^8		16×10^8		18×10^8	
		S		18×10^4		22×10^7		12×10^7		29×10^6	
	Glucose	A		12×10^4		63×10^7		35×10^5	no growth at 10^3		1
		S		11×10^4		90×10^6		23×10^6		30×10^4	178
	Sugar-Free	A		10×10^4		83×10^7		34×10^7		12×10^8	
		S		14×10^4		16×10^7		11×10^7		11×10^7	
Mixed	Lactose	A	63×10^3	63×10^3	10×10^8	13×10^8	93×10^7	40×10^7	58×10^7	85×10^7	19×10^8
		S	88×10^3	12×10^4	10×10^7	85×10^6	80×10^6	28×10^6	35×10^6	18×10^6	25×10^6
	Glucose	A	65×10^3	38×10^3	28×10^7	25×10^7	45×10^4	38×10^4	no growth at 10^3		no growth in undil. samp.
		S	50×10^3	80×10^3	80×10^6	83×10^6	23×10^6	80×10^5	18×10^4	13×10^4	97
	Sugar-Free	A	49×10^3	53×10^3	53×10^7	58×10^7	14×10^8	18×10^7	25×10^7	22×10^7	16×10^7
		S	70×10^3	60×10^3	80×10^6	65×10^6	12×10^7	58×10^6	93×10^6	48×10^6	18×10^7

			Number of lactose-fermenting and non-lactose-fermenting organisms								
		Day	4		5		7				
Organism	Medium		L+	L-	L+	L-	L+	L-	L+	L-	
Normal	Lactose	A*	88×10^7	73×10^7	11×10^8	75×10^7	16×10^8	68×10^7			
		S*		14×10^7		16×10^7		73×10^7			
	Glucose	A				no growth in undiluted sample					
		S		89		no growth in undiluted sample					
	Sugar-Free	A		42×10^6		58×10^6		24×10^7			
		S	18×10^7	77×10^6		14×10^7		12×10^7			
Lac+	Lactose	A	18×10^8	30×10^6	14×10^8	30×10^6	78×10^7	38×10^7			
		S	29×10^6		43×10^5		no growth at 10^4				
	Glucose	A	17×10^6				no growth in undil.samp.				
		S					1				
	Sugar-Free	A									
		S									
Mixed	Lactose	A	85×10^7	68×10^7	45×10^7	13×10^8	9×10^8	44×10^7			
		S	98×10^5	67×10^5	24×10^5	78×10^4	80×10^3	no growth			
	Glucose	A	32	5	0	no growth in undil. samp.					
		S	15×10^7	40×10^6	74×10^5	65×10^6	--	31×10^7			
	Sugar-Free	A	18×10^6	16×10^7	28×10^5	18×10^7	--	13×10^7			
		S									

* A denotes aerated conditions
S denotes non-aerated conditions
L+ stands for lactose-fermenting organisms
L- stands for non-lactose-fermenting organisms

-- no organisms dilution noted

TABLE II

Determination of Lactose Utilization by Lac+ Mutants

Initial lactose conc.	Days after filtration and reinoculation	
	Initial growth with aeration	Initial growth without aeration
	0 1 2 3 4 5 6 7	0 1 2 3 4 5 6 7
0.1%	+ - - - - - - -	+ Ø - - - - - -
0.5%	+ - - - - - - -	+ + + + 0 Ø

+ Cultures that were acid after twenty-four hours.
- Cultures that were alkaline after twenty-four hours.
0 Cultures that were acid after forty-eight hours.
Ø Cultures that were weakly acid after forty-eight hours.

TABLE III

Competitive Growth of Mixed Lac+ Mutants and Normal Shigella sonnei in Aerated Carbohydrate-Free Broth.

Number of culture	Day of subculture into fresh lactose broth																				
	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	+	+	+	+	+	+	+	+	+	+	-	-									
2	+	+	+	+	+	+	-	-													
3	+	+	+		+	+	+	ø	ø	ø		-	-								
4	+	+	+		+	+	ø		ø	ø		-	-								
5	+	+	+		+	+	+	+	+	ø	+	+	+	+	+	*	*		-	-	
6	+	+	+		ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	*	*		-	-	
7	+	+	+		ø	+	+	+	+	+	+	+	+	+	+	*	*		-	-	
8	+	+	+		ø	+	+	+	+	+	+	+	+	+	ø	*	*		-	-	
9	+	+	+		+	+	ø	+	ø	ø	ø	ø	ø	ø	ø	*	*		-	-	
10	+	+	+		ø	+	+	+	+	+	+	+	+	+	+	*	*		-	-	
11	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*		-	-	
12	+	+	+		ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	*	*		-	-	
13	+	+	+		ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	ø	*	*		-	-	
14	+	+	+		ø	+	+	+	ø	ø	ø	+	ø	+	+	*	*		-	-	
15	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*				
16	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*		-	-	
17	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*		-	-	
18	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*		ø	ø	-
19	+	+	+		+	+	+	+	+	+	+	+	+	+	+	*	*		ø	ø	-
20	+	+	+		ø	+	+	+	ø	+	+	+	+	+	+	*	*			-	-

+ Cultures which became acid after twenty-four hours.
ø Cultures which were alkaline after twenty-four hours but became acid after forty-eight hours.
* Cultures which became acid after forty-eight hours.
- Cultures which were alkaline after forty-eight hours.

TABLE IV

The Acidity of Mixed Cultures of Lac+ Mutants and Normal Shigella sonnei Grown in Various Broths

Days of incubation	Lactose broth		Glucose broth		Carbohydrate-free broth	
	Aerated cond.	Still cond.	Aerated cond.	Still cond.	Aerated cond.	Still cond.
0	7.2	7.3	7.1	7.0	7.4	7.4
1	6.5	5.3	4.6	4.8	8.0	7.5
2	7.1	5.5	4.6	4.8	8.0	7.5
3						
4	7.7	5.1	4.7	4.8	8.6	8.2
5	8.0	5.4	4.7ø	5.1ø	8.7	8.7

ø Culture no longer viable

Figure 1

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Lactose Broth Under Non-Aerated Conditions.

□ Normal organisms grown in pure culture
■ Lac+ mutants appearing in cultures of normal organisms
● Lac+ mutants grown in pure culture

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms in a 10^4 dilution
2. No viable organisms in an undiluted sample
3. No viable organisms in a 10^4 dilution
4. No viable organisms in an undiluted sample

Figure 2

Behavior of Mixed Cultures of Sh. sonnei in Lactose Broth Under Non-Aerated Conditions

□ Normal organisms
● Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms in a 10^3 dilution

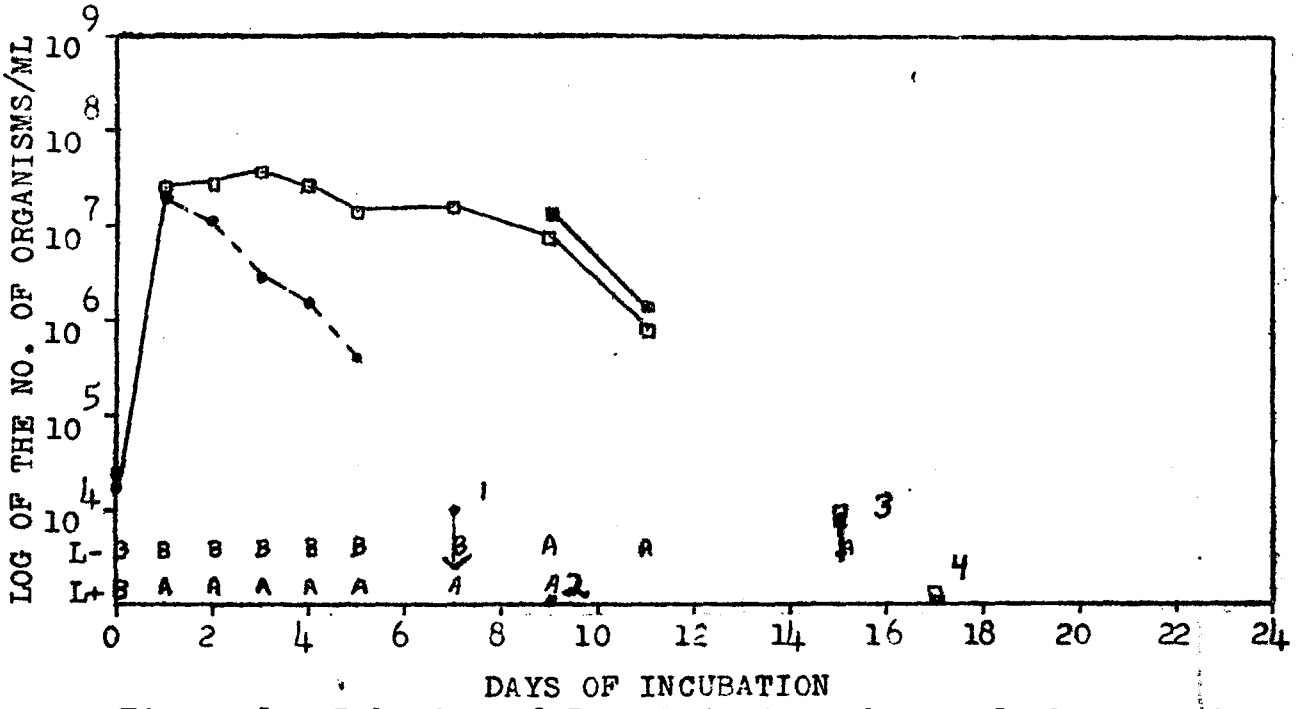


Figure 1. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Lactose Broth Under Non-Aerated Conditions

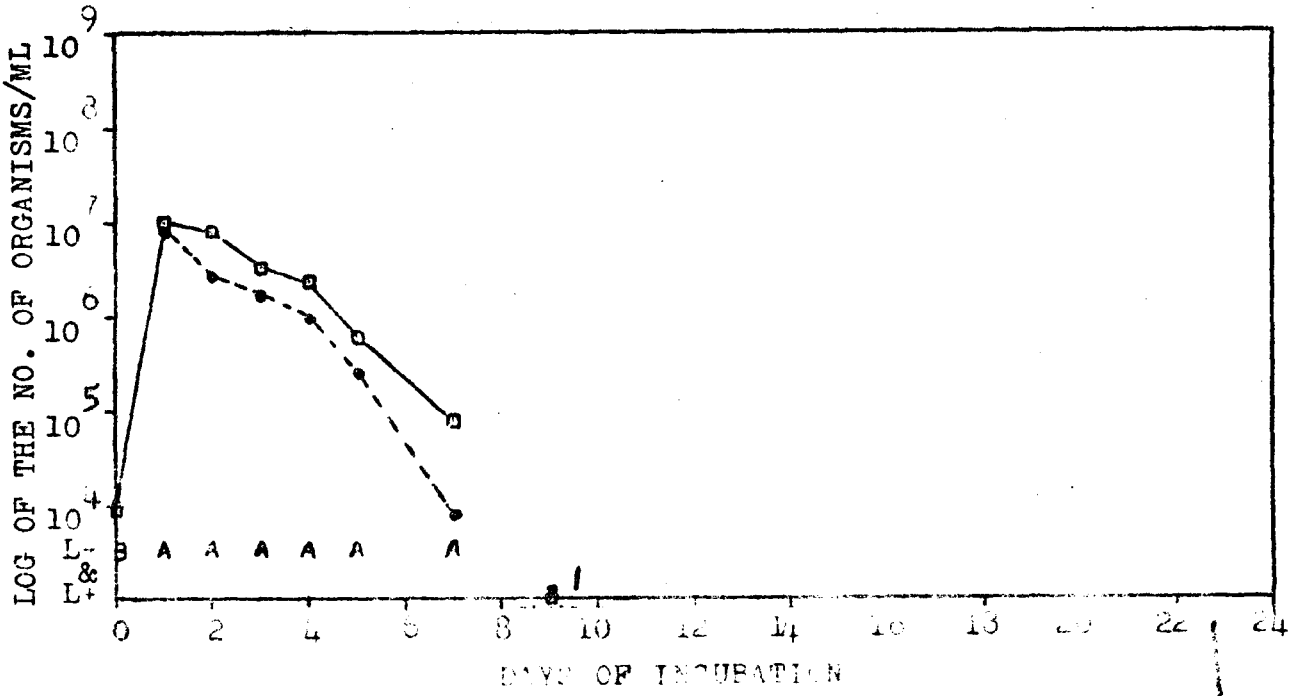


Figure 2. Behavior of Mixed Cultures of Sh. sonnei in Lactose Broth Under Non-Aerated Conditions

Figure 3

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Glucose Broth Under Non-Aerated Conditions

□ Normal organisms grown in pure culture
● Lac+ mutants grown in pure culture

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms present in the cultures after three days incubation

Figure 4

Behavior of Mixed Cultures of Sh. sonnei in Glucose Broth Under Non-Aerated Conditions

□ Normal organisms
● Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms present in the culture after three days incubation

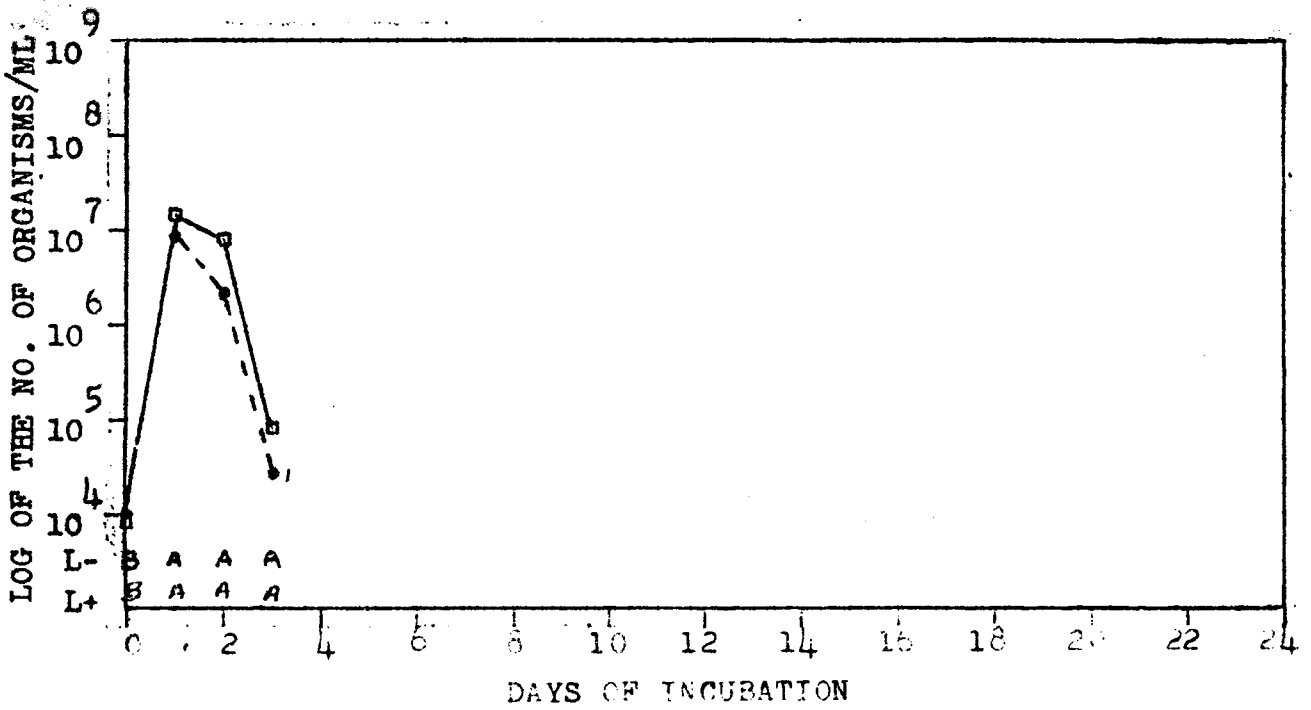


Figure 3. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Glucose Broth Under Non-Aerated Conditions

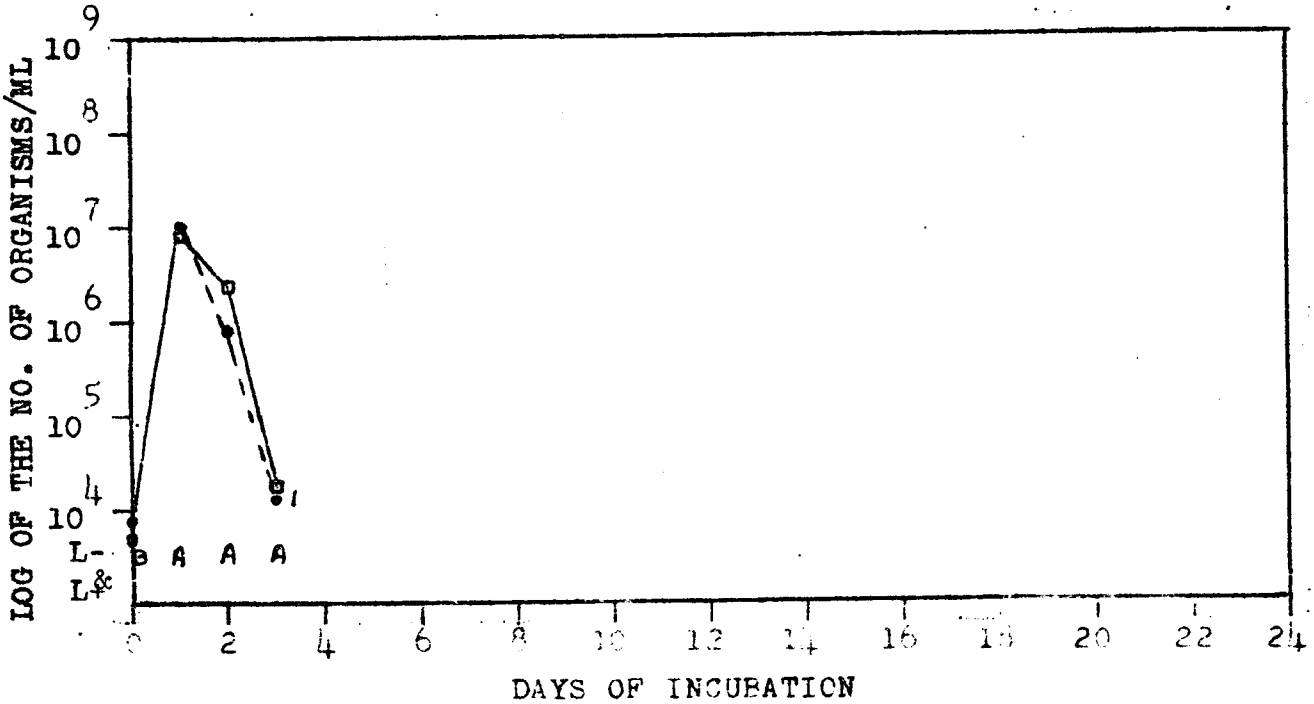


Figure 4. Behavior of Mixed Cultures of Sh. sonnei in Glucose Broth Under Non-Aerated Conditions

Figure 5

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Carbohydrate-Free Broth Under Non-Aerated Conditions

□ Normal organisms grown in pure culture
● Lac+ mutants grown in pure culture
○ Lac- mutants appearing in cultures of Lac+ mutants

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
B Alkaline conditions of the medium

Figure 6

Behavior of Mixed Cultures of Sh. sonnei in Carbohydrate-Free Broth Under Non-Aerated Conditions

□ Normal organisms
● Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
B Alkaline conditions of the medium

1. No organisms present in a 10^6 dilution

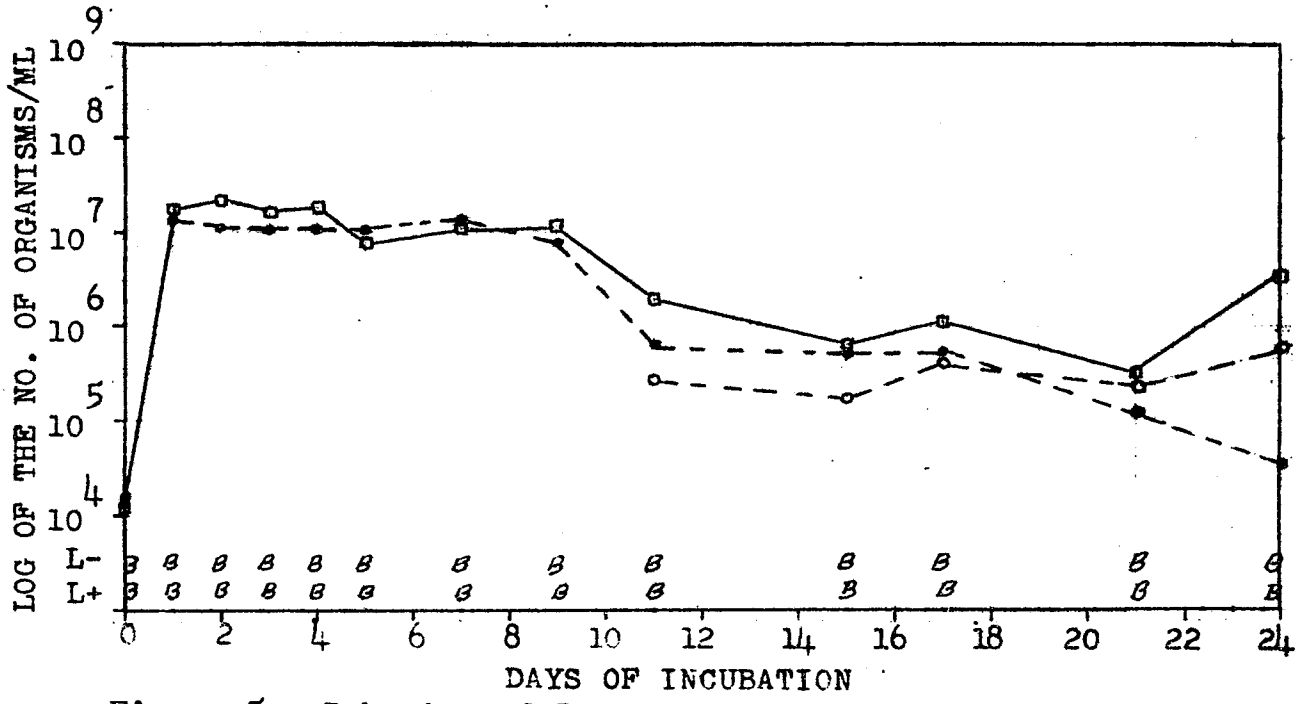


Figure 5. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Carbohydrate-Free Broth Under Non-Aerated Conditions

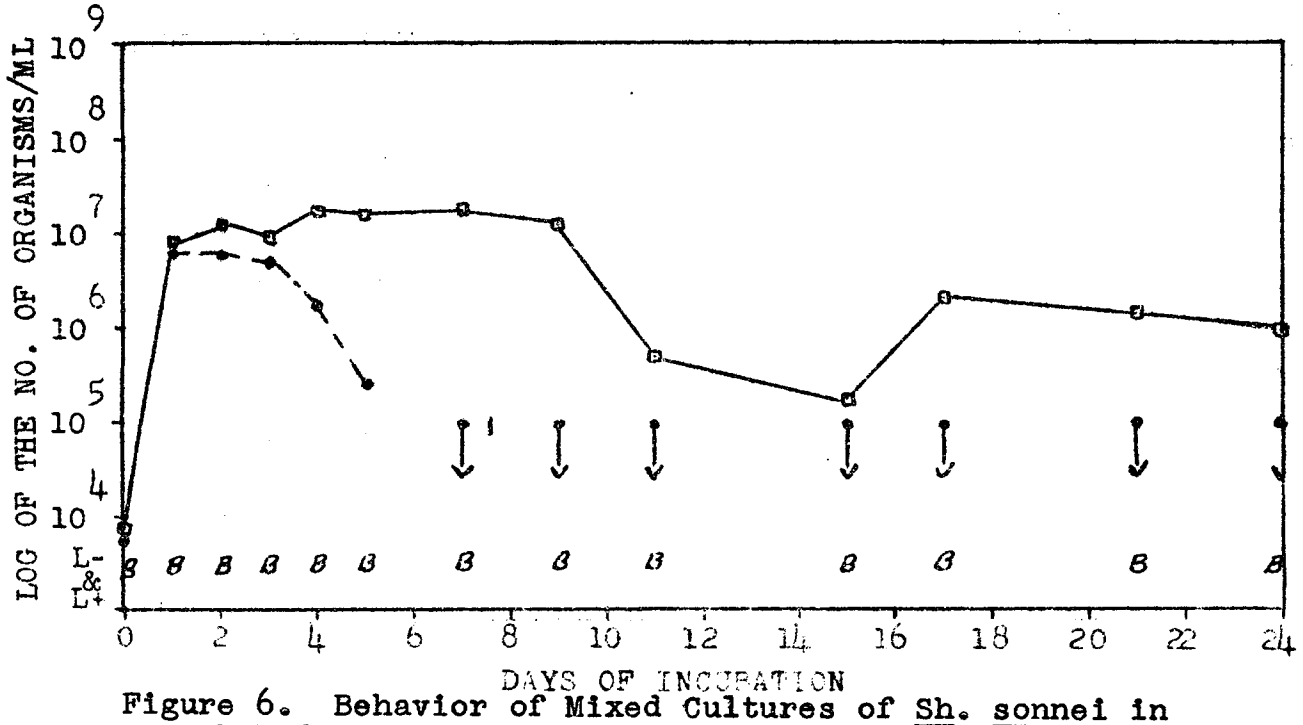


Figure 6. Behavior of Mixed Cultures of Sh. sonnei in Carbohydrate-Free Broth Under Non-Aerated Conditions

Figure 7

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Lactose Broth Under Aerated Conditions

Normal organisms grown in pure culture
Lac+ mutants appearing in cultures of normal organisms
Lac+ mutants grown in pure culture
Lac- mutants appearing in cultures of Lac+ mutants

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
A Acid conditions of the medium
B Alkaline conditions of the medium

Figure 8

Behavior of Mixed Cultures of Sh. sonnei in Lactose Broth Under Aerated Conditions

Normal organisms
Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No Lac+ mutants present in a 10^{6}_{4} dilution
2. No Lac+ mutants present in a 10^{4}_{3} dilution
3. No Lac+ mutants present in a 10^{3} dilution

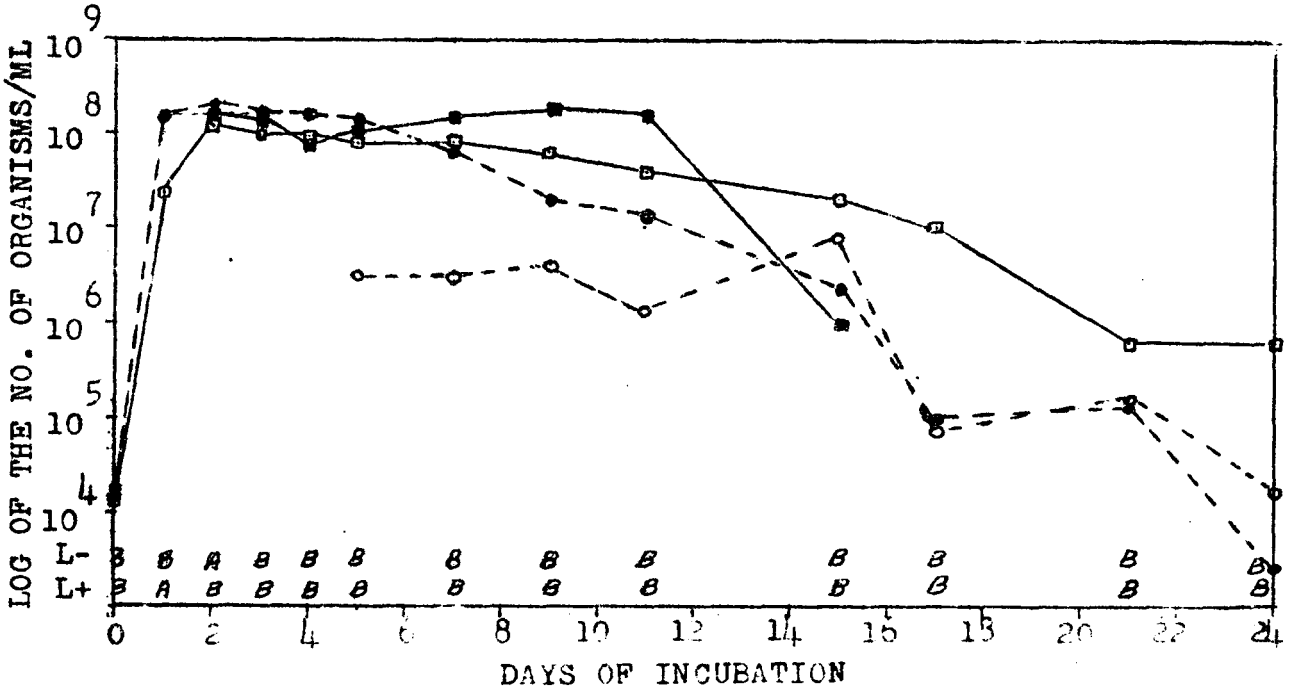


Figure 7. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Lactose Broth Under Aerated Conditions

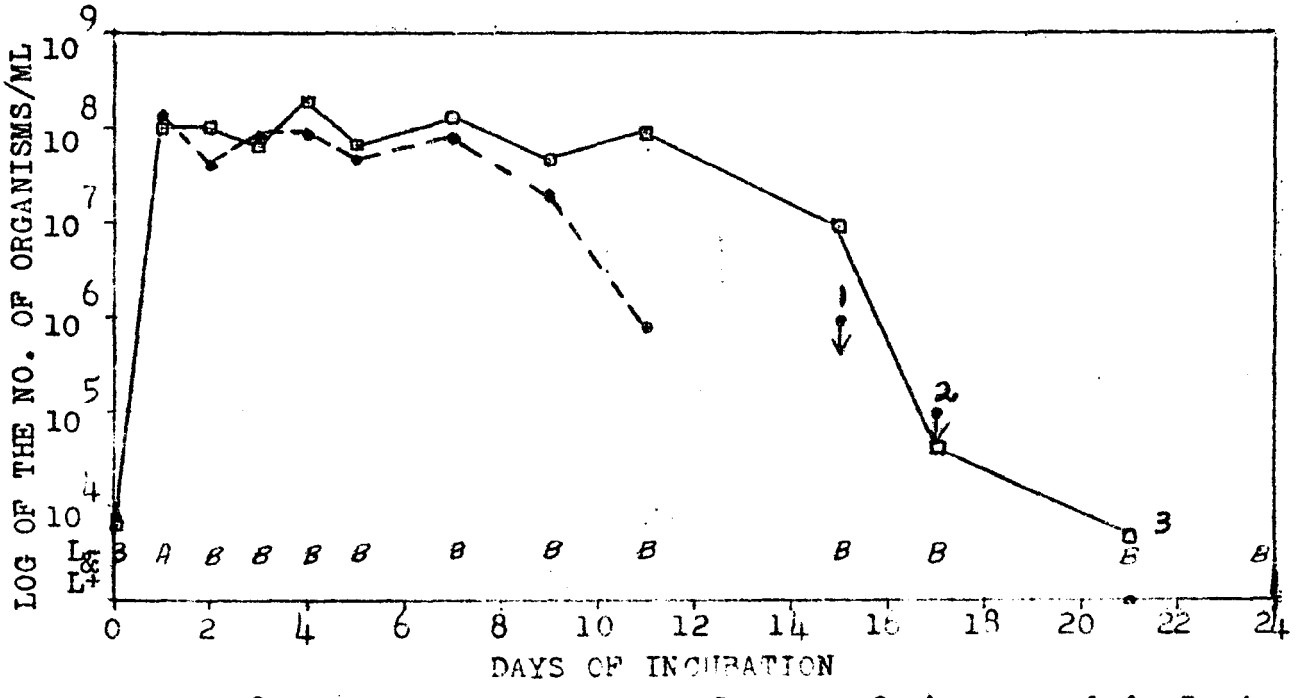


Figure 8. Behavior of Mixed Cultures of Sh. sonnei in Lactose Broth Under Aerated Conditions

Figure 9

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Glucose Broth Under Aerated Conditions

□ Normal organisms grown in pure culture
● Lac+ mutants grown in pure culture

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms present in the culture after three days incubation

Figure 10

Behavior of Mixed Cultures of Sh. sonnei in Glucose Broth Under Aerated Conditions

□ Normal organisms
● Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
A Acid conditions of the medium
B Alkaline conditions of the medium

1. No viable organisms present in the culture after three days incubation

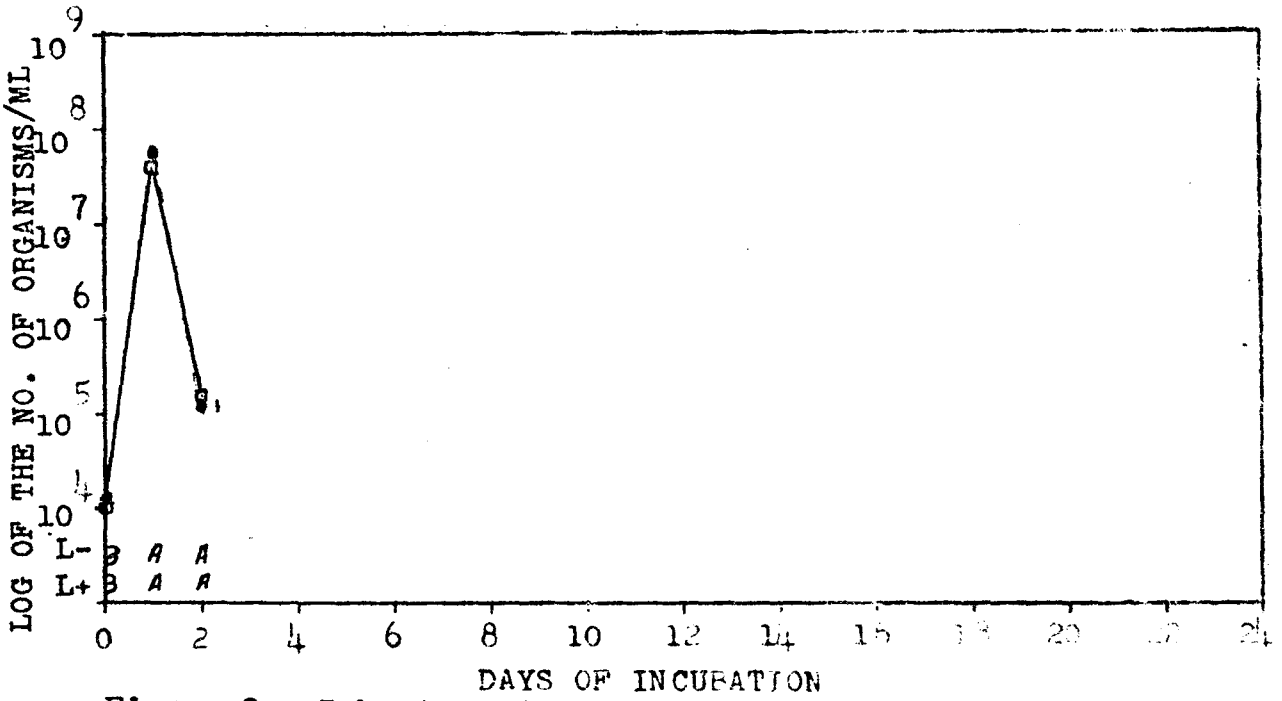


Figure 9. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Glucose Broth Under Aerated Conditions

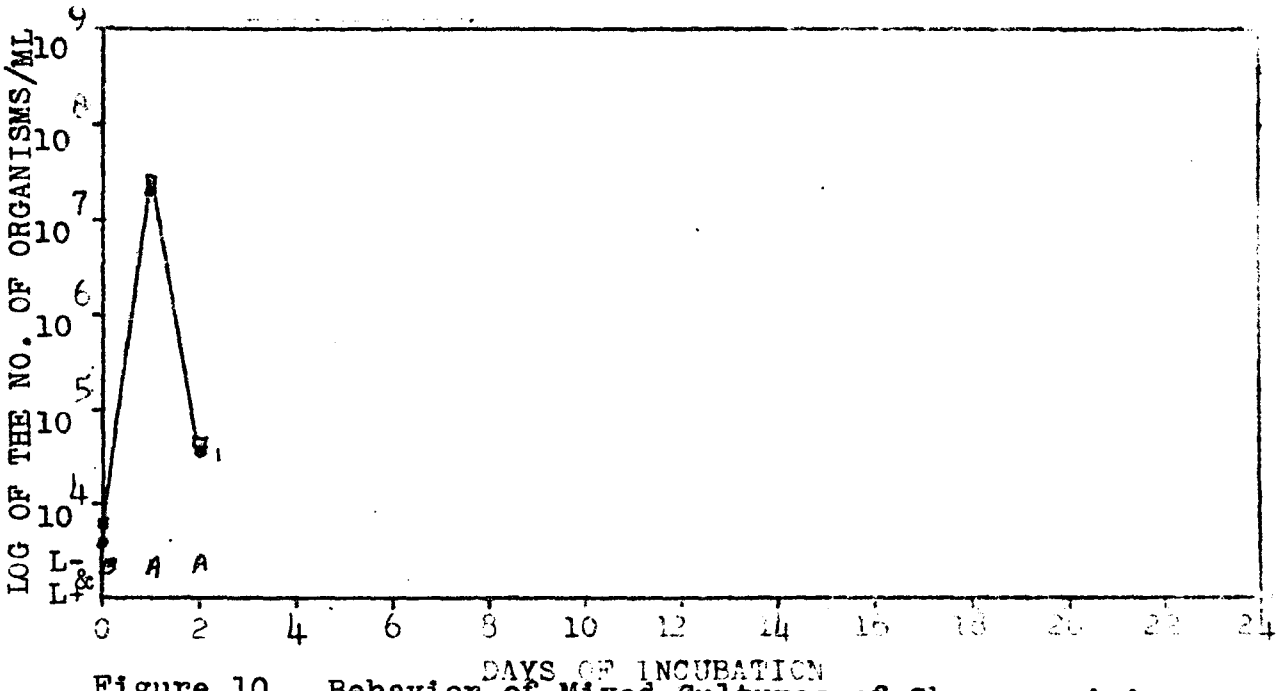


Figure 10. Behavior of Mixed Cultures of Sh. sonnei in Glucose Broth Under Aerated Conditions

Figure 11

Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Carbohydrate-Free Broth Under Aerated Conditions

□ Normal organisms grown in pure culture
● Lac+ mutants grown in pure culture
○ Lac- mutants appearing in cultures of Lac+ mutants

L+ Fermentation readings of cultures of Lac+ mutants
L- Fermentation readings of cultures of normal organisms
B Alkaline conditions of the medium

1. Lac- mutants no longer detected

Figure 12

Behavior of Mixed Cultures of Sh. sonnei in Carbohydrate-Free Broth Under Aerated Conditions

□ Normal organisms
● Lac+ mutants

L+
& Fermentation readings of mixed cultures
L-
B Alkaline conditions of the medium

1. Lac+ mutants no longer detected

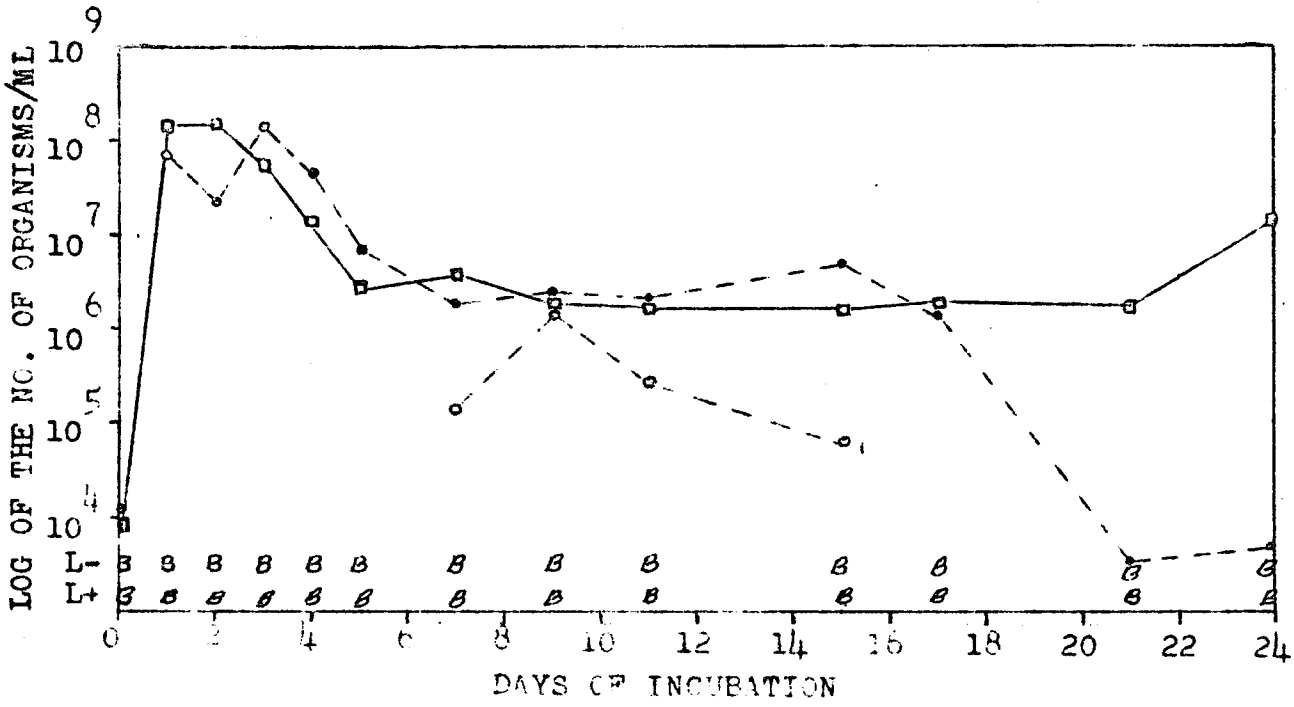


Figure 11. Behavior of Lac+ Mutants and Normal Sh. sonnei Grown in Pure Culture in Carbohydrate-Free Broth Under Aerated Conditions

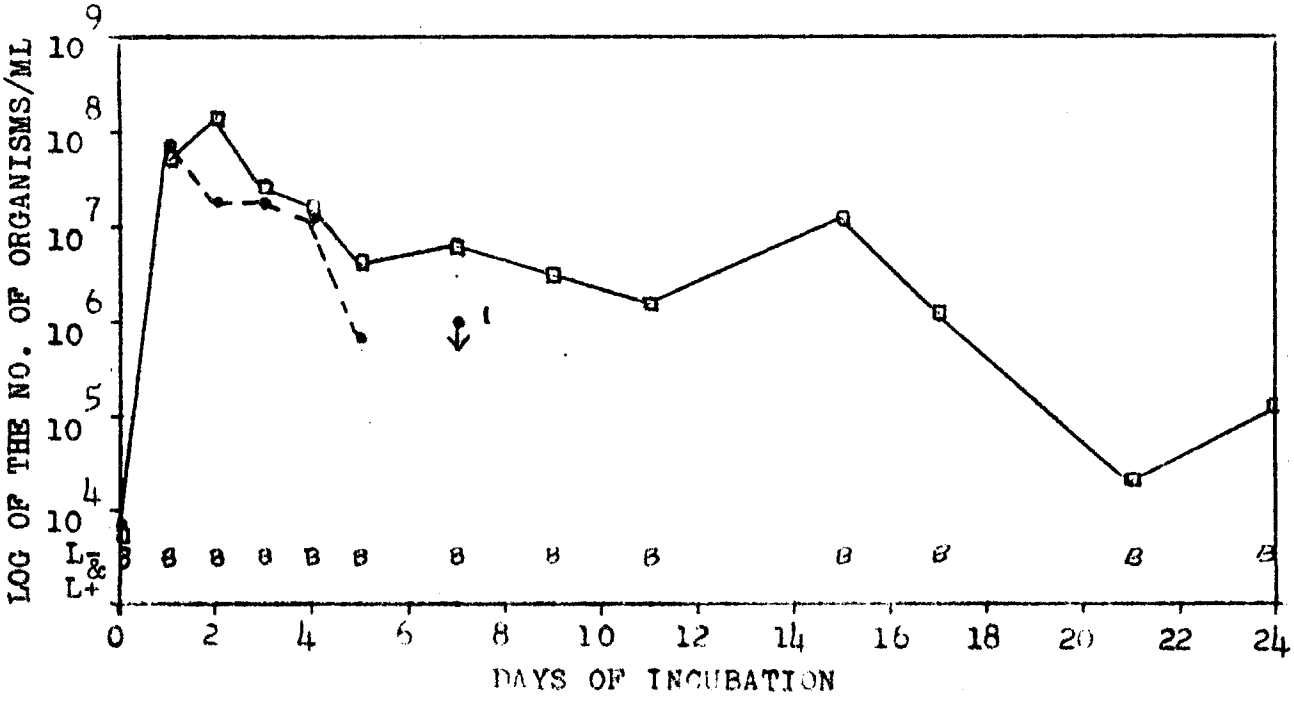


Figure 12. Behavior of Mixed Cultures of Sh. sonnei in Carbohydrate-Free Broth Under Aerated Conditions

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COMPARATIVE GROWTH STUDIES OF THE LACTOSE AND NON-LACTOSE FERMENTING STRAINS OF SHIGELLA SONNEI

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Duval and Schorer (Bact. and Clin. studies from the Rockefeller Inst. for Med. Res., 1904) first isolated the organism now known as Shigella sonnei from cases of infantile diarrhea. Other investigators reported the isolation of organisms closely resembling Duval's bacillus in outbreaks of dysentery. However, it was Sonne (Cent. Bakt. labt. org., 75: 408, 1915) who identified the organism as specific and distinct from the pseudodysentery organisms previously described by Kruse. Thjøtta emphasized the variable fermentation of lactose by this organism. Sears and Schoolnik (J. Bact., 31: 309, 1936), Reynolds, etal. (J. Infect. Dis., 55: 207, 1934) and later Cook, Knox and Tomlinson (Brit. J. Exper. Path., 32: 203, 1951) isolated lactose-fermenting (Lac+) mutants from cultures of normal organisms. Kacoyanis (Disserations, Boston University Graduate School, 1955, 1957) found as few as from one to ten Lac+ mutants grew in the presence of large numbers of normal organisms and produced prompt fermentation of lactose and concluded that delayed fermentation of lactose was due to the absence of lactose rather than to the inhibitory action of the normal cells. He stated further that aeration in the presence of lactose favored the appearance of Lac+ mutants.

Although Lac+ mutants and normal Sh. sonnei are known to exist, only normal organisms are found in nature. The purpose of the present investigation is to determine the

factors that result in the more successful competition of the normal organism in mixed cultures. The comparative growths of the Lac+ mutant and the normal organism were studied by growing them in various liquid media in both pure and mixed culture under aerated and non-aerated (still) conditions.

When the normal organism was grown in lactose broth under non-aerated conditions, the numbers remained high until Lac+ mutants appeared; then their numbers decreased rapidly. In carbohydrate-free broth the normal organisms grew in a similar way except that no Lac+ mutants appeared and the population remained high longer. In aerated lactose broth, the population changes occurred more rapidly and the Lac+ mutants decreased in numbers whereas the population of normal organisms was maintained. The normal organisms in aerated carbohydrate-free broth behaved essentially in the same manner as in the non-aerated medium.

Initially, the growth of the Lac+ mutant in non-aerated lactose broth paralleled the growth of the normal organism under the same conditions except that no viable organisms were detectable after seven days' incubation. This decline in population appears to be related to the acidity of the medium. Under the same conditions, in carbohydrate-free broth, growth was similar but the culture did not die out. After eleven days Lac- mutants (back-mutation) were first noted in numbers approximating those of the Lac+ mutants in carbohydrate-free broth under non-aerated conditions. The

population gradually declined after seven days but organisms were still present at the end of the experiment. The Lac+ mutant in aerated carbohydrate-free broth behaved in essentially the same manner as in the non-aerated medium except that aeration enhanced the initial growth of the culture as well as the appearance of Lac- mutants.

In mixed culture, the growth of normal and Lac+ organisms in non-aerated lactose broth paralleled that of the Lac+ mutant in pure culture. In non-aerated carbohydrate-free broth, the two components of the mixed culture reacted differently. The growth of the normal organism was essentially no different from its growth in pure culture under the same conditions. The growth of the Lac+ mutant was initially comparable to that of each organism in pure culture, but a rapid decline in the population occurred and after five days it was no longer detected.

In mixed cultures in aerated lactose broth, the normal organism behaved as it did in pure culture under the same conditions during the first nine days. Subsequently there was a decline in the population, but normal organisms presisted to the end of the experiment. The Lac+ mutant in mixed culture similarly paralleled its behavior in pure culture for nine days, but declined rapidly and was not detectable when the experiment was terminated. Again, as in all aerated lactose broth cultures, the presence of Lac+ mutants resulted in acid conditions, but alkaline conditions returned within

twenty-four hours. In aerated carbohydrate-free broth the growth of the normal component of mixed cultures resembled that of the same organism in pure culture. The Lac+ mutant disappeared just as it did in the non-aerated cultures.

Loss of viability occurred in all glucose broth cultures regardless as to whether the organisms were grown in pure or mixed culture. The high acidity of these cultures appears to be an important factor in their death.

In the presence of Lac+ mutants, aerated lactose broth cultures became acid but reverted to alkaline conditions rapidly. The utilization of lactose by the Lac+ mutants was determined by growing them in 0.1 and 0.5 per cent lactose under aerated and non-aerated conditions. At frequent intervals, an aerated and a non-aerated culture were filtered through a Seitz EK filter and the broths reinoculated with Lac+ mutants to determine the presence of lactose. Under aerated conditions, both concentrations of lactose were utilized rapidly. However, lactose was present in the still cultures after seven days' incubation. The return to alkalinity of the aerated lactose broth cultures even in the presence of Lac+ mutants may be the result of this utilization of lactose.

Qualitative determinations were also made of mixed cultures of Lac+ mutants and normal organisms. These organisms were grown in lactose, glucose and carbohydrate-

free broth respectively, under aerated and non-aerated conditions. At intervals, these cultures were subcultured into broths containing the same constituents and reincubated under the same conditions. Each culture was also inoculated into lactose broth to determine the presence of Lac+ mutants. Failure to ferment lactose in subculture occurred only in the aerated carbohydrate-free broth cultures. The loss of viability of Lac+ mutants first occurred after five days' incubation and thereafter occurred in other cultures up to twenty days.

The major differences in behavior of the various cultures were associated with aerated and non-aerated conditions. The continued viability of the aerated lactose broth cultures in the presence of Lac+ mutants can be explained by a return to alkaline conditions which appears to be related to the elimination of lactose from the medium.

Lac+ mutants in non-aerated lactose broth cultures and in glucose cultures behaved similarly. In both cases high acidity resulted in the death of the organisms.

The disappearance of Lac+ mutants from carbohydrate-free medium appeared to be related to the competition for nutrients. Lac+ mutants are maintained in the presence of lactose, but once lactose is exhausted the growth advantage is lost. It may be that the Lac+ mutants loses some mechanism (enzyme system?) when it gains the ability to ferment lactose.

Experimental evidence obtained from the qualitative determinations supports this conjecture.