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Some factors influencing lactose fermentation by *Shigella Sonnei*

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BOSTON UNIVERSITY

GRADUATE SCHOOL

Dissertation

SOME FACTORS INFLUENCING LACTOSE FERMENTATION BY SHIGELLA SONNEI

by

George John Kacoyanis

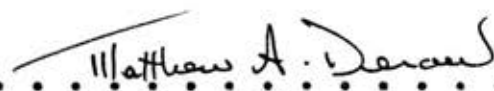
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Approved
by

First Reader 
Professor of Microbiology

Second Reader 
Assistant Professor of Microbiology

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iv

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Table of Contents

	<u>Page</u>
Introduction	1
Material and Methods	21
I. Strain of <u>Shigella sonnei</u> employed	21
II. Media	21
a. Complete	21
b. Minimal Medium	21
III. Methods	22
a. Bacterial Counts	22
b. Aeration of Broth Cultures	22
Experimental Results	23
I. Factors other than lac ⁻ mutants responsible for acid production by <u>Shigella sonnei</u> in lactose broth	23
II. The effect of increased concentration of components of lactose broth medium on the rate of lac ⁻ mutant production	25
III. The effect of aging of cultures on the rate of production of lac ⁻ mutants	26
IV. The effect of filtrates on the rate of lac ⁻ mutant production	28
V. The effect of aerobic conditions on the rate of lac ⁻ mutant production	29
VI. The effect of anaerobic conditions on the rate of lac ⁻ mutant production	33
VII. Back-mutation	36
VIII. Relative growth rates of normal and lac ⁻ cells	37
IX. Mutagenic effect of physical agents on <u>Shigella sonnei</u>	39
X. Mutagenic effect of chemical agents on <u>Shigella sonnei</u>	42
Discussion	44
Summary	54
Illustrations	56

Illustrations

	<u>Page</u>
TABLE I The effect of acid in lactose broth cultures of <u>Shigella sonnei</u> on lac ⁻ mutants	56
TABLE II Daily sampling of aerated cultures of <u>Shigella sonnei</u> grown in lactose broth for the detection of lac ⁻ mutants	57
TABLE III Daily sampling of "still" cultures of <u>Shigella sonnei</u> grown in lactose broth for the detection of lac ⁻ mutants	58
TABLE IV The effect of aeration in lac ⁻ cultures of <u>Shigella sonnei</u> investigated by daily sampling	59
TABLE V Daily sampling of aerated cultures of Lac ⁻ cells of <u>Shigella sonnei</u> grown in plain nutrient broth for the detection of lac ⁻ mutants	60
TABLE VI Relative growth rates of lac ⁻ mutants and normal <u>Shigella sonnei</u>	61
TABLE VII Effect of chemical agents on <u>Shigella sonnei</u>	62

Figure 1 Number of Cultures turned acid and time of occurrence of acid production by Lac ⁻ mutants and normal <u>Shigella sonnei</u> grown under anaerobic conditions in lactose broth	63
Figure 2 Relative growth rates of lac ⁻ mutants and normal <u>Shigella sonnei</u>	64
Figure 3 Effect of ultraviolet irradiation on normal <u>Shigella sonnei</u>	65

Introduction

Ever since the early days of bacteriology, differences in fermentative reactions have been used to identify bacterial species. This identification was based on the ability of a given organism either to ferment or not to ferment various carbohydrates. In order for any fermentative reaction to be of value for the differentiation of a species, it must be constant. However, it was soon established that there were carbohydrates that some species of bacteria fermented at times but that were not ordinarily utilized by them. This irregular behavior was found associated with various species, occurring more frequently in some than in others.

The first recorded example of fermentative variation was described by Neisser in 1906 (1) and Massini in 1907 (2). Both authors investigated a coliform organism which on first isolation failed to ferment lactose. When this organism was seeded on lactose agar, colonies were produced that at first showed no evidence of lactose fermentation. However, from the third day of incubation onward, red papillae or secondary colonies appeared on the surface of the primary colonies. Subcultures of these secondary colonies in lactose broth fermented lactose immediately and on lactose agar growth appeared as non-papillated red colonies. Subculture of the lactose-fermenting variant was continued for successive generations in lactose-free broth without loss of the newly acquired characteristic. Massini gave the name Bacterium coli mutabile* to this

(1) Neisser, M. Ein Fall von Mutation nach DeVries bei bakterien und andere demonstrationen. Centr. f. Bakt. I. Ref., 38: 98, 1906.

(2) Massini, R. Ueber einen in biologische Bezeichnung interessanten Kolistamm. Arch. Hyg., 61: 250-292, 1907.

* In presenting the work of other investigators the nomenclature used by them will be employed even though it is not consistent with current usage.

coliform organism. On the other hand, Burri (3) studied a subculture of Neisser's original B. coli-mutabile and found both lactose-fermenting and non-lactose fermenting colonies of mutants when plain agar cultures were plated on Endo's agar. He concluded that Neisser and Massini dealt with a culture which consisted of a mixture of two types of cells. He isolated from soil a coliform organism which he later named B. imperfectum. Upon successive subculture in sucrose broth this organism acquired the ability to ferment sucrose. All cells of this organism were said to be alike in their inability to ferment sucrose and could acquire this capacity only through the stimulus of sucrose. Burri explained such variation as due to the activation of a latent ability to secrete a specific enzyme through stimulation derived from the sugar. At the same time, Benecke (4) and Kowalenko (5) independently published reports which greatly amplified the findings of Neisser and Massini. Even after starting a culture with a single cell, they obtained fermentation of lactose and thereby eliminated the possibility that an original admixture of two types of cell was responsible for the acquisition of this new lactose-fermenting property.

It was soon established that the production of fermenting variants was not an exclusive characteristic of B. coli mutabile. Twort (6) showed that Eberthella typhosa after two years of growth

(3) Burri, R. Zur Frage der Mutationen bei Bakterien der Coligruppe. cited by Lewis (15).

(4) Benecke, W. Ueber neuere bakteriologische Befunde bei Ruhrerkrankungen. Z. induct. Abstamm. u. vererbLehr., 2: 215-218, 1908.

(5) Kowalenko, A. Studien über sogenannte Mutation-Erscheinungen bei Bakterien unter besonderer Berücksichtigung der Einzellen Kultur. Ztschr. f. Hyg. u. Infektionskrankh., Leipz., 66: 277-290, 1910.

(6) Twort, F. W. The fermentation of glucosides by bacteria of the typhoid-coli group and the acquisition of new fermenting powers by Bacillus dysenteriae and other microorganisms. Proc. Roy. Soc. London, 79: 329-336, 1907.

in lactose peptone water produced lactose-fermenting variants. His method consisted of subculturing at rather long intervals. However, this additional fermentative power was quickly lost upon growth in lactose free media. Twort reasoned that a sufficient period must elapse during which the protein constituents of the medium become depleted and any organisms with the capacity to ferment lactose would then attack this sugar. Penfold (7-9) experimented with the strain of E. typhosa used by Twort. After several months' continual subculture of this organism on lactose agar, two types of colonies appeared: lactose-fermenting and non-lactose fermenting. On subculture of lactose-fermenting colonies, mixtures of the two types were noted and a stable pure culture of the lactose-fermenting variant could not be obtained. However, Penfold found that this strain of E. typhosa had acquired the ability to ferment a greater variety of carbohydrates than was to be expected of the usual strains of E. typhosa. Variants capable of fermenting dulcitol, arabinose, and rhamnose were isolated from a series of broth cultures containing these sugars. The ability to ferment a given sugar was acquired independently. He reported that parent colonies on dulcitol agar produced papillae or secondary colonies in five days. However, subcultures of these papillae in dulcitol broth showed a delay in acid production of from two to ten days. Only after a period of continued subculture of the variants in dulcitol for from three to nine months did the variants appear to be stable. This behavior

(7) Penfold, W. J. Variations of the fermentation properties of B. typhosus. Brit. M. J., London, 2: 1672, 1910.

(8) Penfold, W. J. Bacterial variation. Brit. M. J., London, Suppl., 2: 363, 1911.

(9) Penfold, W. J. Studies in bacterial variation, with special reference to the chemical functions of the members of the typhoid-coli group. J. Hyg., 11: 30-67, 1911.

he found to be equally true of arabinose- and rhamnose-fermenting variants.

Many examples of variants in fermentative reactions have been reported in the literature. To mention a few, Bernhart and Markoff (10) obtained maltose-fermenting variants of Bacterium dysenteriae flexner from cultures grown in maltose agar; Müller (11) obtained rhamnose-fermenting variants of E. typhosa and raffinose-fermenting variants of Bacterium paratyphi B; Sears and Rohner (12) obtained sucrose-, inositol-, and raffinose-fermenting variants of Proteus vulgaris.

In 1931, Nungester (13) proceeded a step further in studies of B. coli mutabile. This investigator reported the production of variants in sucrose, maltose, and lactose broths. He was able to isolate, under suitable conditions, strains able to ferment rapidly any or all of these substances. In this manner, it was possible to produce seven different variants. In the same report, Nungester described a B. coli-like organism isolated from a gall bladder empyema, which after six days of continuous incubation in broth, produced two forms: a rough variant which fermented lactose and a smooth variant which did not attack this sugar. It was possible to change the non-lactose-fermenting smooth form into a lactose-fermenting variant but the reverse change with the rough culture was not effected. He

(10) Bernhart, R. and Markoff, W. N. Studien über die Variabilität der Bakterien. Zugleich ein Beitrag zur Morphologie und Biologie der Milzbrandbazillus. Ztschr. f. Infektionskr., 12: 137-158, 1912.

(11) Müller, R. Mutation bei Typhus- und Ruhsbakterien. Mutation als spezifisches Kulturmerkmal. Centralbl. f. Bakt. 1. Abt., Jena, 58: 97-106, 1911.

(12) Sears, H. J. and Rohner, M. A variable Proteus-like organism fermenting carbohydrate without gas formation. J. Bact., 25: 50-51 1933.

(13) Nungester, W. J. Independent variation of bacterial properties. J. Bact., 25: 49, 1933.

reasoned that lactose fermentation correlated only with rough colonies and that these colonies were stable, never reverting to the non-lactose-fermenting "S" form. Earlier Dulaney (14) made a similar observation.

Lewis (15) attempted to repeat the experiments of Nungester using several strains of Aerobacter cloacae-mutabile. He found that all Aerobacter strains were capable of producing both rough and smooth colonies by plating from liquid or agar cultures. These would arise on either plain or lactose agar. Further, it was possible to produce "S" type lactose-fermenting colonies by the transfer of "R" type lactose-fermenting colonies on to fresh lactose agar. He concluded from these results that variation (in form) from smooth to rough and from rough to smooth is not correlated with variation in the ability to ferment lactose. Lewis agreed with Nungester that lactose-fermenting variants are stable. This observation has been confirmed and extended to other organisms by Kriebel (16), Sears and Rohner (12) and Müller (17).

Hall (18) reported similar observations with lactose-fermenting variants of E. coli mutabile, Bacterium cloacae, Bacterium dysenteriae dispar and Bacterium dysenteriae sonnei. In all aspects these mutants behaved similar to the parent type except with respect to the acquired

(14) Dulaney, A. D. Microbic dissociation of B. coli communis. J. Infect. Dis., 42: 575-588, 1928.

(15) Lewis, C. M. Variation with special reference to behavior of some mutabile strains of colon bacteria in synthetic media. J. Bact., 28: 619-638, 1934.

(16) Kriebel, R. A comparative bacteriological study of a group of non-lactose-fermenting bacteria from stools of healthy food handlers. J. Bact., 27: 357-372, 1937.

(17) Müller, R. Ueber mutationsartige Vorgänge bei Typhus- Paratyphus- und verivandten Bakterien. Zentralbl. f. Bakt., Ref., 1 Abt., 42: 57-59, 1908.

(18) Hall, I. C. Metabolic mutation and colonial dissociation in the genus Bacterium. J. Bact., 29: 13, 1935.

lactose-fermenting characteristics. Hall established criteria which a mutant must manifest.

"The ultimate criterion of lactose mutation is the repeated isolation of rapid lactose fermenters from a parent type which perpetuates both itself and the mutant indefinitely, but this criterion is usually preceded by observations of slow fermentation of lactose and of multiple papillae or secondary fermenting colonies on lactose agar media, such for example, as on eosin methylene blue lactose agar. The mutant type rarely, if ever when pure, reverts to the parent type, and, as a rule, is indistinguishable from it morphologically, culturally, and serologically, except by its more rapid fermentation of lactose, and by its inability to form characteristic multiple papillae or secondary colonies. Secondary colonies of a different type from those seen in the parent culture on lactose media may occur, however, both in the parent type on a non-lactose media, and in the mutant on various solid media on long incubation.

The same criteria of metabolic mutation are known to apply to certain forms in relation to other carbohydrates and are probably capable of general application."

However, a contradictory report was published by Hersey and Bronfenbrenner (19) in 1936. A lactose-fermenting variant of E. coli was readily reversible to the parent type upon serial cultivation in succinate synthetic medium. A normal E. coli strain was used as control. Both cultures were carried through a series of six successive subcultures at two- or three-day intervals. Lactose-fermenting power was measured periodically by inoculating the culture into tubes containing lactose broth. The relative lactose-fermenting capacity was indicated by the amount of gas produced and the presence or absence of acid. Their results showed that lactose-fermenting variants were not stable but lost their lactose-fermentative power in succinate medium. On the other hand, none of the "normal" E. coli cultures failed to ferment

(19) Hersey, A. D. and Bronfenbrenner, J. Dissociation and lactose activity in slow lactose-fermenting bacteria of intestinal origin. J. Bact., 31: 453-464, 1936.

lactose. These investigators emphasized the fact that the cultures which "regained" the capacity to ferment lactose actively are not identical to typical E. coli cultures.

Other investigators have reported reversion of variants other than E. coli mutabile. Sage and Spaulding (20) isolated cultures of E. typhosa from which they could isolate lactose- and sucrose-fermenting variants. These variants appeared in lactose and sucrose broths after three days of continuous incubation. By serial cultivation on extract agar at monthly intervals, these variants reverted to the original types after eight months of growth. Variants from both strains reverted in about the same length of time. However, once these variants reverted to the typical biochemical pattern, even nineteen months of bi-weekly subculturing in lactose broth failed to reinduce lactose fermentation. In follow-up studies of a carrier, E. typhosa could be readily isolated from his urine and feces. For a period of one year, isolations obtained were identical in biochemical behavior to that of the original culture. One year later both typical and atypical types were present. By the third year after onset, only typical organisms could be isolated. Believing that the utilization of lactose may originally have been associated with virulence, serial passage through mice was resorted to. However, no relation between virulence and ability to ferment lactose could be established. Poston (21), 1938, observed the same

(20) Sage, D. and Spaulding, E. H. A study of two atypical strains of E. typhosa. J. Bact., 44: 647-651, 1942.

(21) Poston, M. A. Atypical typhoid fever caused by atypical strains of Eberthella. J. Infect. Dis., 35: 56-57, 1938.

instability of a sucrose-fermenting variant of S. typhi on primary isolation. Following plating on plain nutrient agar the power of fermentation of sucrose was lost. In 1944, Stuart and Rustigian (22), while investigating a number of strains of Shigella alkaescens, observed that two freshly isolated strains having the antigenic structure of Sh. alkaescens fermented sucrose (normally non-sucrose-fermenting organism) within twelve to eighteen hours. However, when replated this acquired characteristic was rapidly lost.

The Effect of Different Conditions of Cultivation

The slow or delayed fermentations which are encountered now and then in bacteriological work have received more attention and study recently. It was Massini's (2) original observation that the "training" of B. coli mutabile to ferment lactose could be accomplished in liquid media as well as on solid. He reported that B. coli seeded into several tubes containing lactose broth and incubated continuously did not at first attack lactose but did so after a lapse of days or even weeks. Once fermentation had occurred, subculture of organisms to fresh medium resulted in immediate fermentation of the sugar. Hersey and Bronfenbrenner (19) also employed a liquid medium for the isolation of variants of E. coli mutabile. However, they observed a more rapid development of variants than was reported by Massini. These investigators obtained lactose-fermenting variants after ten daily transfers of cultures into lactose peptone medium. It was reasoned by them that the serial transfer of cultures into

(22) Stuart, C. A. and Rustigian, R. Sucrose fermentation by Shigella alkaescens. J. Bact., 48: 497, 1944.

fresh lactose enhanced the fermentative powers of the organism.

Kriebel (16) reported contrasting results after testing twenty-five non-lactose-fermenting Gram-negative bacteria isolated from healthy food handlers. Her observations were that these organisms grown without subculture fermented lactose within from two to fourteen days. Once fermentation appeared lactose-fermenting mutants could be readily isolated. On the other hand, the daily transplant of the same culture into lactose broth, resulted in a longer delay before the occurrence of lactose fermentation. Nungester and Anderson (23) in 1931 carried out a similar experiment using E. coli on solid medium. It was found possible to carry E. coli mutabile on lactose agar plates by daily transfers with but little tendency for variation either in structure of colony or in fermentation of lactose. However, when the interval of transfer was extended to more than a day, it was often observed that secondary colonies began to make their appearance. The subplants of these showed a generous number of non-papillated lactose-fermenting colonies. They concluded that the original culture must be allowed to age for a matter of days on lactose before the appearance of lactose-fermenting variants.

Lewis (15) made a detailed study of this phenomenon with a slow lactose-fermenting Aerobacter strain which usually fermented lactose in seventy-two hours. His results were similar to those reported by Kriebel (16). However, in addition to daily transfers into fresh lactose broth, a portion from each of these cultures was

(23) Nungester, W. J. and Anderson, S. A. Variation of a bacillus coli-like organism. J. Infect. Dis., 49: 455-472, 1931.

plated on lactose agar. Plating from the tubes, at this time, revealed the fact that variant cells were so few as to escape detection by conventional methods. The number of variant colonies appearing in lactose agar with each daily subculture varied but little. It was concluded by Lewis that the number of lactose-fermenting variants appearing within the first twenty-four hours of growth in lactose broth is so small that transplanting from tube to tube with a straight needle would lead to erroneous conclusions.

Bronfenbrenner and Davis (24) reported the use of higher percentages of lactose to induce the formation of lactose-fermenting variants in cultures of slow lactose-fermenting strains of E. coli. Though their cultures did not ferment lactose in one per cent lactose-peptone broth, they did ferment in concentration of two to three per cent lactose in ten days. Kriebel (16) extended this work by inoculating several strains of late lactose-fermenting organisms of the colon-paratyphoid series into one, two, three, five, and ten per cent lactose broth tubes. Whereas, these cultures fermented in a one per cent lactose broth in from two to fourteen days, cultures in five per cent lactose fermented in from two to five days. Lower percentages of lactose generally were fermented more slowly and ten per cent concentrations produced irregular results. Once any given culture fermented the lactose, on subculture it also fermented one per cent lactose rapidly.

The effect of concentration of d-arabinose on delayed fermentation of this sugar by eighteen members of the colon group and

(24) Bronfenbrenner, J. and Davis, C. R. On methods of isolation and identification of the members of the colon-typhoid group of bacteria. Late fermentation of lactose. J. Med. Res., 32: 33-37, 1918.

allied types was studied by Koser and Vaughan (25). D-arabinose was added to meat extract and peptone broth in concentrations ranging from 0.5 to 5.0 per cent and then inoculated. It was concluded that there was very little difference in the time required for fermentation in the several concentrations. In fact, fermentation was observed to appear earlier in tubes of two per cent and lower concentrations. Several strains of A. aerogenes, however, were consistently different in that acid first appeared in the five per cent concentration and later in the lower concentrations.

Several reports have been published which indicate that slow fermentation can be accelerated by increased aeration. In 1932, Kennedy, Cummings, and Morrow (26) observed a shortening of the time necessary to produce acidity in lactose broth when a larger surface area was used in relation to the volume of culture medium. Several Gram-negative, non-spore-bearing bacteria were used in this experiment. Similar results have been reported by Dulaney and Michelson (27), who observed that the delayed fermentation of lactose by E. coli mutabile was accelerated and that lactose-fermenting variants appeared earlier in large flasks containing shallow layers of medium.

Contrasting data were obtained by Koser and Vaughan (25) with representative members of the colon, paratyphoid and dysentery groups. D-arabinose broth in 0.5 ml amounts was added into: (a) 50-ml flasks, (b) ordinary test tubes of 14 mm inside diameter, and (c) narrow

(25) Koser, S. A. and Vaughan, E. F. A study of d-arabinose fermentation. *J. Bact.*, 33: 587-602, 1937.

(26) Kennedy, J. A., Cummings, P. L. and Morrow, N. M. Atypical lactose-fermenters belonging to the genus *Bacterium* (Bergey). *J. Infect. Dis.*, 50: 333-343, 1923.

(27) Dulaney, A. D. and Michelson, I. D. A study of B. coli mutabile from an outbreak of diarrhea in the new born. *Am. J. Pub. Health*, 25: 1241-1251, 1935.

test tubes of 10 mm diameter, and then inoculated. The results obtained with narrow tubes and with the flasks were not strikingly different from those obtained in the ordinary test tubes.

In 1936, Hersey and Bronfenbrenner (19) deduced from an earlier experiment that the change in fermenting power of slow lactose-fermenting strains of E. coli occurring after a prolonged cultivation in lactose medium must be due to the appearance of variants whose production was stimulated by anaerobic conditions. To test this hypothesis, these investigators created anaerobic conditions in several types of lactose medium contained in closed fermentation tubes. Open tubes were used for controls. Both types were inoculated with a non-fermenting strain. Delayed fermentation occurred in all media used in both open and closed tubes. However, the addition of one per cent succinic acid to closed tubes resulted in more rapid fermentation. They concluded that conditions of limited oxygen supply are favorable to the selection of fermenting variants, but only when the lactose is accompanied by an additional source of carbon. On the other hand, Koser and Vaughan (25) tested the same hypothesis with their strains of the colon, paratyphoid, and dysentery groups in d-arabinose broth under anaerobic conditions. Two series of anaerobic cultures were prepared, one secured by the action of pyrogalllic acid and sodium carbonate solution; the other under vaseline seal. Under these conditions the delayed fermentation of d-arabinose occurred in the usual manner and the results did not differ from those obtained with aerobic incubation in ordinary test tubes. Similar data were obtained by Sage and Spaulding (20)

in 1942 with E. typhosa both aerobically and anaerobically.

Origin of Variants:

It had been suggested by Smith (28) that variation in ability of microorganisms to ferment certain sugars occurs spontaneously in the absence of the specific sugar. However, should the sugar be present it acts as a selective agent. The variant cells are benefited by it and are thus enabled to multiply more rapidly than the original type. In 1931, Nungester and Anderson (23) studied a coli-form bacillus which varied with respect to lactose and obtained a lactose-fermenting variant by plating a sucrose broth culture on lactose agar. In 1932, Havens and Irwin (29) were able to isolate both sucrose-positive and negative strains from plain agar cultures of Salmonella morgani. In 1940, Kristensen (30, 31) described in a coli-like organism two instances where a mixture of fermenting and non-fermenting colonies appeared on lactose and sucrose plates. The organism had never come in contact with the particular sugar before. In 1944, Stuart and Rustigian (22) described a similar occurrence on primary isolation of Sh. alkalescens on sucrose agar. Lewis (15) pointed out that several similar observations pertaining to many species and several carbohydrates have been recorded in the literature. At this time some investigators concluded that mutations arise in

(28) Smith, J. H. On the organisms of the typhoid-colon group and their differentiation. Centralbl. f. Bakt. 1 Abt. Orig., 68: 151-165, 1913.

(29) Havens, L. C. and Irwin, A. G. A correlated fermentative and antigenic variation in certain strains of Morgan's bacillus. J. Infect. Dis., 50: 550-554, 1932.

(30) Kristensen, M. Recherches sur la Fermentation mutative des bacterias. Acta path. et Microbiol. Scand., 17: 193-231, 1940.

(31) Kristensen, M. Mutative bacterial fermentation. Acta path. et microbiol. Scand., 25: 244-248, 1948.

the absence of any specific stimulating substances and occur spontaneously.

On the other hand, Burri (3) believed that all the cells of "B. imperfectum" were alike in the non-fermentation of sucrose and could acquire the fermentative power gradually with "training" in sucrose. He explained variation as ". . . . due to an activation of a latent ability to secrete enzyme through stimulation derived from the sugar. The capacity once activated for any cell is transmitted to its progeny." More recently, this school of thought has received the support of Hinshelwood and his followers (32, 33).

However, no adequate experimental evidence supported either hypothesis until in 1934 Lewis (15) conducted a detailed study of this problem. It was his opinion that the truth of the "training" hypothesis is difficult if not impossible to obtain, but if it could be shown that such variation occurs in the absence of the specific sugar, then the theory of a definite inciting stimulus will have lost its support. Therefore, an experiment was conducted by Lewis with E. coli mutabile to determine whether cultures were capable of growing in a synthetic medium containing lactose as the sole source of carbon and ammonia as the source for the supply of nitrogen.

"A twenty-four hour agar slant culture was washed down with ten cc of water and further diluted by steps of ten to include the complete range from one-tenth to one-billionth part of the culture. All dilutions were plated in glucose as well as lactose synthetic agar in order to determine with

(32) Hinshelwood, C. N. The chemical kinetics of the Bacterial Cell. Oxford, Clarendon Press, 1946.

(33) Dean, A. C. R. and Hinshelwood, C. N. Adaptation in Micro-organisms. The Syndics of the Cambridge University Press, p. 21-38, 1953.

certainty that failure to obtain growth in the preceding experiment could not have been due to the nitrogen source. The highest dilutions were plated in peptone-beef-extract agar to determine the number of cells introduced into the plates of lower dilutions. Computing from the plain agar series, it was estimated that the lowest dilution contained about three billion cells per cubic centimeter. Growth occurred throughout the range in glucose synthetic agar. The colonies in plain agar and glucose agar were practically equal in the high dilutions. Lactose synthetic agar supported growth in low dilutions only. Plates above the 100,000 dilution were sterile. The number of colonies in plates which supported growth was proportional to the dilutions. It was determined that growth was proportional to the dilutions. It was determined that not more than one cell per 100,000 could grow in this medium."

These variants in transfer produced acid on eosin-methylene blue lactose beef extract agar. Lactose broth was fermented with the production of acid and gas in twelve hours. These cells reacted identically with variants isolated by plating from secondary colonies. Therefore, Lewis concluded that the lactose-fermenting variants were present in the original culture.

In 1952, Ryan (34) submitted further evidence that lactose-fermenting variants arose spontaneously in the absence of lactose. This evidence was based on the fluctuation test of Luria and Delbrück (35). A series of culture tubes containing plain broth were inoculated from a single parent culture of E. coli mutabile. They were incubated until a certain population was reached. The number of lactose-fermenting mutants was determined in each tube by streaking it on lactose agar plates. After a statistical analysis of his

(34) Ryan, F. J. Adaptation to use lactose in E. coli. J. Gen. Microbiol., 7: 69-88, 1952.

(35) Luria, S. E. and Delbrück, M. Mutation of bacteria from virus sensitivity to virus resistance. Genetics, 28: 491-511, 1943.

data, Ryan concluded that the lactose-fermenting mutants arose spontaneously.

Fermentative Reactions of *Shigella sonnei*:

An organism, later named Sh. sonnei, associated with infantile diarrhea, was first isolated in 1904 by Duval and Schorer (36). Duval (37) in his description reported this organism to be a late lactose fermenter. In 1915 Sonne (38), during an outbreak of dysentery in Copenhagen, re-discovered this organism and again found it to be a late lactose-fermenting organism. Shortly afterwards, in a detailed study of its properties, Thyotta (39) described its action on lactose agar. After a delay of several days, parent colonies of Sh. sonnei developed lactose-fermenting secondary colonies or papillae on their surface which when plated on suitable medium fermented lactose promptly. Sears, Bilderback, Ashley and Rohner (40) noted a similar behavior in sucrose medium and that the abilities to ferment lactose and sucrose are acquired entirely independently. On different occasions, Glynn and Starkey (41); Rhodes and Reid (42); Reynolds, MacClesky and Werkman (43) under the same conditions succeeded in obtaining cultures

(36) Duval, C. W. and Schorer, E. H. Studies of the diarrheal disease of infancy. Studies from the Rockefeller Institute of Medical Research, 2: 42-45, 1904.

(37) Duval, C. W. Another member of the dysentery group. J. A. M. A., 43: 381-383, 1904.

(38) Sonne, C. Ueber die Bakteriologie des giftarmen Dysenteriebacillen (Paradysenteriebacillen). Centralbl. f. Bakt. 1 Abt. Org., 75: 408-456, 1915.

(39) Thyotta, M. On the bacteriology of dysentery in Norway. J. Bact., 4: 355-377, 1919.

(40) Sears, H. J., Bilderback, B. L., Ashley, C. and Rohner, M. Sucrose-fermentation by Shigella sonnei. J. Bact., 25: 50-51, 1933.

(41) Glynn, J. H. and Starkey, D. H. Properties of Shigella sonnei. J. Bact., 37: 315-331, 1939.

(42) Rhodes, A. J. and Reid, C. A study of forty strains with particular reference to the appearance of the colonies. J. Hyg., 41: 105-110, 1941.

(43) Reynolds, H., MacClesky, C. S. and Werkman, C. H. Dissimilation of sucrose by Shigella dysenteriae var. sonnei. J. Infect. Dis., 55: 207-219, 1934.

capable of prompt fermentation of lactose in liquid media. The first of these investigators, Glynn and Starkey (41), and later, Sears and Schoolnik (44), in 1936, found the parent organisms and the lactose-fermenting variants to possess identical serological properties. The latter investigators demonstrated that these variants are stable and do not differ otherwise from the parent culture. They also showed that variation in fermentation remained unaffected during smooth to rough dissociation. Although the ability of Sh. sonnei to ferment lactose, sucrose, or raffinose is acquired independently, the above mentioned investigators were able to isolate, under suitable conditions, strains capable of prompt fermentation of any or all of these substances. In this manner it was possible to produce seven different variants. This work was confirmed by Cook, Knox and Tomlinson (45) in 1951, who produced a double variant which fermented both lactose and sucrose within twenty-four hours. Furthermore, it was noted that this variant retained these properties even after several months of occasional transfer in media free of the specific substances. They found it possible to isolate lactose-fermenting variants from cultures of normal organisms which had been streaked on synthetic agar containing lactose as the only source of carbon. These investigators considered these mutants to be spontaneous and occurring at a rate of one mutant in 10^5 normal organisms. Thibault and Rubenstein (46) using a similar technic found that the spontaneous

(44) Sears, H. J. and Schoolnik, M. Fermentative variability of Shigella paradysenteriae sonnei. J. Bact., 31: 309-312, 1936.

(45) Cook, G. T., Knox, R. and Tomlinson, A. H. Production of fermentative variants by Shigella sonnei and other "late fermenting" organisms Brit. J. Exper. Path., 32: 203-211, 1951.

(46) Thibault, P. and Rubenstein, S. Frequence des Mutants de Shigella sonnei Fermentant rapidement le Lactose. Ann. Inst. Pasteur, 82: 106-108, 1952.

rate of mutation varied for different strains of Sh. sonnei. Of seven strains investigated, they reported the frequency of lactose-fermenting mutants to be one mutant in from 10^6 to 10^8 normal cells.

Kacoyanis and Baker (47) and Kacoyanis (48) conducted detailed studies of the time of appearance of lactose fermentation of a single strain of Sh. sonnei. Large numbers of lactose broth cultures of Sh. sonnei first began to become acid after about four to five days of incubation. The number of cultures turning acid per day increased through about the fifteenth day (thereafter there was a decrease in number of cultures becoming acid per day until about the twenty-fifth day of incubation). By this time almost all cultures had turned acid. Although a few cultures never became acid, lactose-fermenting mutants could always be isolated from cultures which had fermented within from ten to fifteen days of incubation but could be isolated less frequently from cultures that had turned acid after fifteen days of incubation. Lactose-fermenting mutants represented at least one-third of the organisms present in cultures fermenting lactose within ten days. Once obtained in pure culture, these lactose-fermenting microorganisms were established to be stable mutants. This was accomplished by showing that these organisms retained their ability to ferment lactose after thirty daily subcultures in a medium containing glucose.

Lactose-fermenting mutants, isolated from cultures in which

(47) Kacoyanis, G. J. and Baker, E. E. A study of lactose fermentation by Shigella sonnei. Bact. Proc., 1955, p. 103.

(48) Kacoyanis, G. J. Lactose fermentation by Shigella sonnei. Dissertation. Boston University Graduate School, 1955.

lactose fermentation was delayed, were capable of fermenting lactose promptly. Therefore, the term prompt fermentation was used to indicate the appearance of acid within forty-eight hours; and delayed or late fermentation, an acid reaction in the medium only after a period of time greater than forty-eight hours.

The question arose as to whether the lactose-fermenting mutants isolated from acid cultures occurred spontaneously from normal Sh. sonnei or were induced by the substrate, lactose. First, it became necessary to determine whether a few lactose-fermenting mutants in the presence of a large number of normal Sh. sonnei would be inhibited and therefore the appearance of acid in a lactose broth culture be delayed for several days. However, it was determined that as few as from one to ten lactose-fermenting organisms could grow in the presence of a large number of normal organisms and produce prompt lactose fermentation. This is evidence that the delay with which fermentation usually occurs is due not to the inhibiting action of normal organisms on lactose-fermenting mutants but rather to the absence of lactose-fermenting mutants in the inoculum.

Experiments carried out indicated that lactose-fermenting mutants possibly may arise spontaneously from normal cultures of Sh. sonnei which had been grown in a medium free of lactose. This appeared to be so when it was noted that of 320 lactose broth cultures that had been inoculated with a normal culture, two turned acid within forty-eight hours. By definition this time taken for acidification represented prompt fermentation of lactose. It seemed

that mutants were present in the original inocula of the two cultures fermenting within forty-eight hours. However, these results could not be repeated when subsequent experiments were carried out. In testing several hundred lactose broth cultures, lactose-fermenting mutants could not be detected within forty-eight hours. Therefore, it has not been conclusively established whether or not lactose-fermenting mutants arise spontaneously or are stimulated by the substrate, lactose.

When a large number of normal organisms was screened for mutants, using lactose synthetic agar plates, lactose-fermenting mutants were not detected. Therefore, the sensitivity of this method for the detection of lactose-fermenting mutants was investigated and it was shown that when artificial mixtures of lactose-fermenting and normal organisms were plated on lactose synthetic agar only twelve per cent of the lactose-fermenting organisms produced colonies within forty-eight hours. This poor recovery was found to be due to both the inability of the synthetic agar to support growth of all lactose-fermenting mutants seeded and possibly to some inhibition of the lactose-fermenting organisms by the normal organisms. Thus, this method was not sensitive enough to demonstrate the presence of only a few lactose-fermenting mutants.

Materials and Methods

I. Strain of Shigella sonnei employed

Sh. sonnei strain #1545, used for these experiments, was isolated from a patient at the Boston City Hospital. This strain was maintained in nutrient agar at 5 C and periodically plated in nutrient agar to preserve the smooth form of the organism. Additional stock cultures were maintained in the frozen state.

Cultures used for experiments were prepared by inoculating single colonies from nutrient agar plates into 3 ml of nutrient broth. These were incubated for from twelve to sixteen hours at 37 C.

II. Media

A. Complete Media

Nutrient broth was employed as a complete medium. The composition of this medium was 3 g of meat extract and 5 g of Bacto peptone in 1 liter of water. It was brought to a final pH of 7.25. According to the requirements of each experiment, this medium was re-enforced with 0.5 per cent carbohydrate. Twenty grams of agar were added when solid medium was required.

BCP nutrient broth was made by adding Brom Cresol Purple (BCP) to give a concentration of 0.001 per cent in nutrient broth (with or without 0.5 per cent carbohydrate).

Endo agar (Difco) was used to differentiate between lactose-fermenting and normal bacteria.

B. Minimal Medium

Cook's et al. medium (45) was used as the minimal medium. The composition of this is as follows: $(\text{NH}_4)_2 \text{HPO}_4$, 0.4 per cent;

NaCl, 0.1 per cent; K_2HPO_4 , 0.1 per cent; agar, 2.0 per cent; nicotinic acid, 0.5 microgram per ml in tap water. The medium was adjusted to a pH of 7.25 and sterilized at 120 C for 15 minutes.

III. Methods

A. Bacterial Counts

For estimating numbers of organisms in a culture, the counting technic approved by the American Public Health Association (49) for water analysis was employed. This was accomplished by making ten-fold serial dilutions of cultures using sterile distilled water as the diluent. One ml of the appropriate dilution was pipetted into plates. Approximately 30 ml of nutrient agar were added to each of these plates which were then incubated at 37 C for 48 hours. Colony counts were made using a Quebec Colony Chamber.

B. Aeration of Broth Cultures

Aeration of broth cultures was carried out on an Arthur H. Thomas apparatus. Cultures were mounted and shaken vigorously with the apparatus operated at 150 to 200 strokes per minute rotating in a $\frac{1}{2}$ inch radius.

(49) Standard Methods for the Examination of Water and Sewage, 9th edition, A. P. H. A., New York, 1946.

Experimental Results

I. Factors other than Lac⁻ Mutants* Responsible for Acid Production by *Sh. sonnei* in Lactose Broth

The occasional failure to isolate lac⁻ mutants from cultures which became acid only after long incubation led to an investigation of this phenomenon. In previous examinations of acid cultures for lac⁻ mutants, only small samples (one loopful) were tested. It was thought that the sampling of small amounts might lead to failure to detect the presence of only a few lac⁻ mutants. Another possibility was that the acidity produced by the cultures might have been sufficient to kill any lac⁻ mutants that were present.

In order to examine both possibilities, a fresh culture of normal organisms** in nutrient broth was agitated vigorously to break up clumps. One drop (0.05 ml) of this culture was added to each of 72 tubes (16 x 125 mm in size) containing 6 ml of BCP lactose broth. The cultures were then incubated at 37 C and observed for the earliest appearance of acid production. When a culture turned acid a loopful was streaked on plates of Endo agar for isolation of lac⁻ mutant colonies. The culture was then centrifuged at 3,000 rpm for 30 minutes. The supernatant fluid was aseptically decanted from the culture tube into a 10-ml beaker and the pH determined with a Beckman pH meter. To the culture tube containing the sedimented bacteria, 6 ml of fresh BCP lactose broth were added. The bacteria were resuspended and the culture reincubated for 48 hours. It was

* Lac⁻ mutants denotes lactose-fermenting variants of *Shigella sonnei*.

** Normal organisms denotes the parent organism of *Shigella sonnei*.

believed that if only a few lac^- mutants were present, the presence of lactose should provide a selective advantage for these mutants and permit lactose fermentation to occur within 48 hours. Some, but not all of these reincubated cultures became acid within 48 hours and were plated on Endo agar for the detection of lac^- mutants. The results of this experiment are summarized in Table I.

All of the 72 cultures fermented lactose (became acid) within 21 days of incubation. The isolation of lac^- mutants on Endo agar could be made only from cultures that turned acid within the first ten days of incubation. The colonies that developed on the plates were about one-third lac^- mutants and two-thirds normal organisms. On the other hand, lac^- mutants could not be isolated after more than ten days of incubation. Moreover, the addition of fresh lactose broth to sedimented bacteria from the cultures from which lac^- mutants could not be isolated did not reveal the presence of lac^- mutants, as indicated by the absence of acidification within 48 hours. The pH of cultures from which lac^- mutants could not be isolated did not differ markedly from that of cultures from which lac^- mutants could be isolated. These data indicate that failure to isolate lac^- mutants from Sh. sonnei cultures showing lactose fermentation only after long incubation (ten days in the present experiment) is associated with the absence of lac^- mutants in the culture at the time of subculture. The reason for the absence of mutants in these cultures is not clear. Although it was thought possible that the absence of lactose-fermenting organisms might be due to death of the mutants as a result of the acidity produced by

lactose fermentation. The data, however, do not support this mechanism.

Since it appeared that *lac*⁻ mutants of *Sh. sonnei* were not responsible for the appearance of acid in the majority of the lactose broth cultures, it seemed desirable to test the stability of lactose under the experimental conditions used. BCP lactose broth was prepared in the manner which has been described in the section on Materials and Methods. The medium, in 6-ml amounts, was added into each of 80 tubes (16 x 125 mm) and sterilized at 120 C for fifteen minutes. These were then incubated for thirty days at 37 C, at which time each tube of medium was inoculated with 0.05 ml of a young culture of normal organisms and reincubated. At the same time, a control experiment was carried out with fresh BCP lactose broth.

In both experiments, prompt lactose fermentation failed to occur. The interval required for fermentation of lactose in both the control and the test series was similar and consistent with previous results. Thus, it appears that under these conditions lactose is a stable disaccharide and that hydrolysis had not occurred. If hydrolysis of lactose had occurred, the hydrolysis products, glucose and galactose, would have been fermented promptly by normal organisms.

II. The Effect of Increased Concentration of the Components of Lactose Broth Medium on the Rate of *Lac*⁻ Mutant Production

Several days of incubation were required for cultures to become acid. With prolonged incubation, some evaporation of culture media had occurred. A study was made on the effect of increased

concentrations of the components of lactose broth on the rate of appearance of *lac*⁻ mutants of Sh. sonnei.

Three different concentrations of lactose broth were prepared. Lactose was added to give final concentrations of 1.0, 1.5 and 2.0 per cent respectively. The peptone and meat extract of the broth were also increased proportionally. Thus, the concentration of the components of the lactose nutrient medium was doubled, tripled, and quadrupled in strength as compared to the formula described in Materials and Methods. In addition, a flask was prepared as described in the formula of Materials and Methods and used as a control.

The contents of each flask was dispensed in 6-ml amounts into each of 80 tubes resulting in four series of 80 tubes each. These were then sterilized by autoclaving. All tubes were inoculated with 0.05 ml each of a young culture of normal organisms, incubated at 37 C and observed throughout the entire experiment.

There was very little difference in the time required for fermentation of sugar in the several concentrations when compared to the control. It seems that the increased concentration of lactose, peptone, and meat extract have no effect on the rate of formation of *lac*⁻ mutants by Sh. sonnei.

III. The Effect of Aging of Cultures on the Rate of Production of *Lac*⁻ Mutants

The question arose as to whether there is any change in the cells while aging in lactose-free broth which would enable them to make use of the lactose promptly when it is supplied to them or whether the cells must be in contact with the lactose for a period

of time before being capable of utilizing it.

Eight hundred tubes each containing 6 ml of BCP nutrient broth without lactose were inoculated with 0.05 ml of a young culture of normal cells of Sh. sonnei. These cultures were incubated for periods ranging from two to twenty days. At the same time, a control group of 80 tubes containing lactose broth was inoculated with the same culture.

Beginning with the second day of incubation and every other day thereafter, sufficient lactose to give a final concentration of 0.5 per cent was added to groups of 80 cultures. Each group was then reincubated and examined daily for the presence of acidity in the cultures.

An additional experiment was carried out as a control. A group of 80 tubes each containing 6 ml of BCP lactose broth was inoculated with 0.05 ml of a young normal culture of Sh. sonnei and incubated for thirty days. It was observed that fermentation of the broth first appeared on the sixth day and thereafter increasing numbers of cultures showed acid formation. This increase continued through the fourteenth day of incubation. There was then a decrease in the number of cultures turning acid until the nineteenth day of incubation, by which time, all cultures had turned acid.

In several tubes of the groups pre-incubated for six and twelve days respectively, fermentation of lactose occurred promptly upon its addition. Lac⁻ mutants were isolated from each of these cultures. These were tested and found to be mutants of Sh. sonnei. On the other hand, all the other cultures showed acidification of

lactose only after a delay of over 48 hours. The results of these individual group experiments resembled the results obtained in the control experiments.

Several attempts have been made to confirm the results obtained with cultures incubated for six and twelve days. However, the results were not reproducible. The times of appearance of acid in cultures were consistent with results of control experiments. Thus, it appears that mere aging of cells in broth will not induce prompt fermentation of lactose.

IV. The Effect of Filtrates on the Rate of Lac⁻ Mutant Production.

Certain strains of bacteria grown in the presence of culture filtrates from related strains may acquire properties of the related strains. This phenomenon has been termed transformation by Avery, McLeod and McCarty (50), and has been defined as a hereditary alteration in a susceptible cell which is acquired from its environment and not by cellular fusion. More recently, it has been found that DNA is the factor responsible for such reactions. A phenomenon resembling transformation is transduction, described by Zinder and Lederberg (51), which involves the transfer of hereditary traits of a related strain to susceptible cells by means of a filterable agent.

It was of interest to determine whether filtrates of lac⁻ cultures would induce normal organisms to ferment lactose promptly.

(50) Avery, O. T., McLeod, C. M. and McCarty, M. Studies of the chemical nature of the substance inducing transformation of pneumococcal types. *J. Exper. Med.*, 79: 137-158, 1944.

(51) Zinder, N. O. and Lederberg, J. Genetic exchange in Salmonella. *J. Bact.*, 64: 679-699, 1952.

0.05 ml of a fresh lac⁻ culture was used to inoculate each of 160 tubes containing 6 ml of BCP lactose nutrient broth. After twenty-four hours of incubation at 37 C, all cultures had turned acid. These cultures were then centrifuged at 3000 rpm for thirty minutes. The supernatants were collected and the original volume was reconstituted by the addition of sterile distilled water. Additional lactose was added to a concentration of 0.5 per cent. The material was then neutralized with 1 N sodium hydroxide solution and sterilized by passing through a Selas filter (porosity 02). The sterile filtrate was dispensed in 6-ml amounts into each of 160 sterile test tubes. These were divided into two groups. Each tube of one group received a 0.05-ml inoculum from a young culture of normal Sh. sonnei; the other group received an equal volume of inoculum from a young lac⁻ culture. Both groups were incubated at 37 C.

After twenty-four hours, all cultures inoculated with lac⁻ cells fermented lactose. However, there was no evidence of lactose fermentation in cultures inoculated with normal organisms. The interval required for fermentation in this group was the same as that of the control tubes without the filtrate. Thus, the presence of an "activator" for rapid fermentation in the filtrates was not demonstrated.

V. The Effect of Aerobic Conditions on the Rate of Lac⁻ Mutant Production

Preliminary studies of oxygenation as a factor affecting the rate of mutation indicated that if acid production were taken as an indicator of the presence of mutants, aeration inhibited the pro-

duction of lac^- mutants. To study the effect of oxygenation, several experiments were conducted.

In a typical experiment, each tube of a group of 33 (19 x 200 mm) containing 10 ml of BCP lactose broth was inoculated with 0.05 ml of a fresh culture of Sh. sonnei. These cultures were incubated at 37 C and aerated by shaking on an oscillating shaker at 170 rpm. Beginning with the second day of incubation, each culture was subcultured daily by the inoculation of one loopful into 6 ml of fresh BCP lactose broth and observed for prompt lactose fermentation. Daily subculturing of aerated cultures was continued for twenty days.

Data obtained in this experiment are presented in Table II. Daily subcultures of aerated cultures revealed the presence of lac^- mutants before sufficient acid had been produced to change the indicator (pH above 6.8). Most of these cultures turned acid the following day. With continued incubation and aeration, several of the acidified cultures became alkaline, usually within twenty-four hours. Upon continued subculturing it was found that lac^- mutants were present in these alkaline cultures for several days but eventually disappeared and only normal organisms could be isolated. However, in some cultures lac^- mutants reappeared. This phenomenon occurred at irregular intervals throughout the experiment. It was observed that eight cultures did not turn acid at any time during the experiment. Nevertheless, daily subculturing revealed that lac^- mutants were present in these cultures.

The data acquired in the preceding experiment are in contrast with the results obtained from experiments involving cultures incubated

without shaking (hereafter termed "still" cultures). Thirty-three tubes containing 6 ml of BCP lactose broth were inoculated with 0.05 ml each of a fresh culture of normal Sh. sonnei and incubated without agitation for twenty days. At daily intervals, a sample loopful from each culture was inoculated into a tube containing 6 ml of fresh BCP lactose broth and incubated to detect lac⁻ mutants.

The data are presented in Table III. Acidification of broth was first observed on the sixth day and thereafter increasing numbers of cultures showed acid formation. This increase continued until the fourteenth day at which time all but two cultures had turned acid. These remaining two cultures failed to produce acid and remained unchanged for the duration of the experiment. Lac⁻ mutants could not be isolated from these cultures. The daily subculture of "still" cultures into lactose broth revealed the presence of lac⁻ mutants in cultures which had turned acid within the first ten days of incubation and upon continued subculture lac⁻ mutants were identified throughout the entire experiment. On the other hand, from "still" cultures which had turned acid after ten days, lac⁻ mutants were shown not to be present on subculture into lactose broth.

In view of the fact that aeration increased the rate of appearance of acid in cultures, a control experiment was conducted with 80 tubes, each containing 6 ml of BCP lactose broth. Each tube was inoculated with 0.05 ml of a young culture of normal organisms and incubated for thirty days.

The first culture to become acid appeared on the fifth day of incubation. Increasing numbers of cultures fermented lactose until

the thirteenth day of incubation. Following this, a decreasing number of cultures turned acid until the eighteenth day of incubation, by which time, all cultures had become acid. The results of this experiment are similar and consistent with those of Table III.

From these data, the behavior of aerated cultures seems unlike that of cultures which are not aerated. Rather than inhibiting mutations, aeration appears to favor the production of mutants. In aerated cultures, acid production cannot be used as a sole indicator for the presence of *lac*⁻ mutants.

In order to determine whether aeration alone was responsible for the increased rate of *lac*⁻ mutant production, the following experiment was conducted. A series of ten tubes (19 x 200 mm) each containing 10 ml of nutrient broth free of lactose was inoculated with 0.05 ml of a young culture of normal *Sh. sonnei*. These were then aerated for twenty days.

By means of daily subculture into lactose broth, *lac*⁻ mutants could not be detected in these aerated cultures. In addition, several attempts to plate the contents of aerated cultures on Endo agar failed to reveal the presence of mutants. These could not be detected in any of the aerated cultures throughout the twenty days of experimentation. It seems that the stimulatory effect of aeration on the production of *lac*⁻ mutants is apparent only when lactose is present. Aeration alone will not cause the production of *lac*⁻ mutants.

It remained to be determined whether the disappearance of *lac*⁻ mutants from aerated cultures of normal *Sh. sonnei* in lactose broth was due to an effect brought about by normal organisms present in

these cultures. The above mentioned experiment was repeated with the exception that lac⁻ cells were aerated in BCP lactose nutrient broth. It was observed (Table IV) that all cultures became acidified within twenty-four hours. These cultures did not remain acid but upon continued aeration became alkaline; the greater number turning alkaline in from two to four days after the appearance of acid. One culture remained acid. However, from this culture, no viable organisms could be isolated from the third day onward. On daily subculture of the aerated cultures, into lactose broth, fermentation occurred promptly and lac⁻ cells could be isolated on Endo agar throughout the entire experiment.

The results of this experiment are in contrast to the results of the aeration experiment carried out with normal organisms in lactose broth. In that experiment, lac⁻ mutants were detected eventually in all cultures. However, these mutants disappeared after a time. In the present experiment, once lac⁻ mutants were detected, they could be isolated throughout the entire course of the experiment. It appears that aeration is not the cause of the disappearance of lac⁻ mutants. It seems probable that once lactose becomes exhausted, the normal organisms present in cultures under aerobic conditions might have an increased growth rate thereby replacing the lac⁻ mutants.

VI. The Effect of Anaerobic Conditions on the Rate of Lac⁻ Mutant Production

It was thought that anaerobic conditions might have an effect opposite to that of aerobic conditions. To test this hypothesis, 80

tubes, each containing 6 ml of freshly prepared BCP lactose broth, were each inoculated with 0.05 ml of a young culture of normal Sh. sonnei and sealed with 2 to 3 ml of melted vaspar*. In addition, 0.05 ml of the same culture was pipetted into each of 80 tubes containing 6 ml of BCP lactose broth and these were not sealed. Both groups of cultures were incubated at 37 C and observed for thirty days.

The results of both experiments are represented in Figure 1. In the anaerobic experiment, all cultures turned acid on the sixth day of incubation. The pH of these cultures, compared colorimetrically with Brom Cresol Purple standards, was found to range from 5.8 to 6.1. With each succeeding day of incubation, the acidity of each culture increased until on the tenth day all cultures had a pH of 5.2. At this time, 20 tubes were selected at random and opened. Loopful samples were both streaked on plates of Endo agar and inoculated into fresh lactose broth. However, no lac⁻ mutants were found to be present in the sealed cultures even though they became acidified during the observation period.

The results of the experiment with open cultures are similar to the data obtained in previous tests. Lactose fermentation first appeared on the eighth day of incubation and all cultures had become acidified by the twentieth day. As in previous experiments, the largest number of cultures to turn acid occurred on the fourteenth and fifteenth day of incubation. Lac⁻ mutants could be isolated

* Vaspar was composed of a mixture of equal parts of paraffin and vaseline

from all cultures that turned acid on the first nine days of incubation, a total of eleven cultures (of 80). However, no lac⁻ mutants could be isolated from cultures which acidified lactose broth later than the ninth day of incubation.

It appears from the data obtained in these experiments that anaerobic conditions inhibit the production of lac⁻ mutants. Therefore, acid production observed under these conditions must be due to some other mechanism.

The observation that acid production in lactose-containing medium occurred in the absence of lac⁻ mutants led to the question of whether or not decomposition of some component of the medium other than lactose might be responsible for acidification. Eighty tubes, each tube containing 6 ml of BCP nutrient broth without lactose, were inoculated with Sh. sonnei and sealed with vaspar. No acidification occurred within thirty days of incubation. This indicated that Sh. sonnei is incapable of producing acid from such substances as peptones and amino acids under anaerobic or partial aerobic cultural conditions. Thus, the production of acid by Sh. sonnei under anaerobic conditions in the absence of mutants must be in some way associated with the presence of lactose.

Although, the data supported the view that lac⁻ mutants are responsible for the acid production in anaerobic cultures, their absence in acid cultures remained unanswered. A possible explanation for the absence of lac⁻ mutants was that the cultural conditions are not favorable for their existence. A similar experiment was carried out as described above. Eighty tubes, each tube containing

6 ml lactose broth, were inoculated with lac⁻ microbes and sealed. These were then incubated.

From the data shown in Figure 1, it was found that all anaerobic cultures fermented lactose within eighteen hours. Also, it was equally possible to isolate lac⁻ mutants from such acid cultures.

Sh. flexneri was tested to determine whether the ability of Sh. sonnei to produce acid when grown in lactose broth under anaerobic conditions was a property of all organisms. In an experiment similar to that conducted above, eighty tubes containing BCP lactose broth were inoculated with Sh. flexneri. These cultures were sealed and incubated for thirty days. It was observed that growth occurred in all cultures but that acid did not form. Thus, it seems that the ability to produce acid from lactose under anaerobic conditions might well be a unique property of Sh. sonnei, since Sh. flexneri was incapable of producing acid under identical conditions.

VII. Back-Mutation

In preliminary experiments, ten tubes containing nutrient broth without lactose were inoculated with a pure culture of lac⁻ organisms and aerated for several days. At daily intervals samples were inoculated onto Endo Agar plates. Lac-* colonies appeared on the surface of these plates. In this preliminary work, it appeared that aerobic conditions had induced the lac- mutants to arise from lac⁻ mutants.

In a detailed experiment, each of thirty three tubes (19 x 200 mm) containing 10 ml of nutrient broth without lactose was inocu-

* Lac- mutants denotes non-lactose-fermenting organisms appearing in lac⁻ cultures.

lated with 0.05 ml of a young culture of *lac*⁻ cells. These cultures were incubated and aerated by shaking for twenty days in the manner previously described. Each day a sample loopful of each aerated culture was inoculated into 6 ml of fresh BCP lactose broth. The time of appearance of acid formation was recorded.

From the data presented in Table V, it was found that the first eight daily subcultures into lactose broth fermented lactose promptly. From these subcultures which had turned acid, *lac*⁻ mutants could be confirmed on Endo agar plates. However, starting on the ninth day of aeration and subsequently through the sixteenth day a total of six subcultures showed a delay in lactose fermentation. From these subcultures only *lac*⁻ mutants could be detected on Endo agar. Moreover, subcultures from the sources of these *lac*⁻ strains remained consistently negative through the last (20th) day of the trial. From these data, it appears that *lac*⁻ mutants arise from *lac*⁻ cells and that these *lac*⁻ mutants seem to be favored under the conditions of the experiment.

VIII. Relative Growth Rates of Normal and *Lac*⁻ Cells

The work of Monod (52, 53) has contributed greatly to the phenomenon of "enzymatic adaptation". Working on the fermentation of lactose by *E. coli mutabile* and *Bacillus subtilis*, Monod distinguished between the role played by "mutation", as a hereditary factor and "adaptation" as an environmental factor. The environmental factor

(52) Monod, J. The phenomenon of enzymatic adaptation. Growth Symposium, 11: 223-289, 1947.

(53) Monod, J. La Croissance des Cultures Bacteriennes. Herman & Cie, Paris, 1942.

is the response of microorganisms to new surroundings. This response is evoked only as a result of environmental stimulus and is transmitted indefinitely in subculture if the stimulus remains the same. This adapted state is reversible, being rapidly lost in subculture when the stimulus is removed. The capacity to respond "adaptively" is part of the genetic pattern of each cell, being lost or gained if a mutation occurs.

Normal cells and lac^- cells grown in nutrient broth and in lactose synthetic medium were compared in their ability to ferment lactose and glucose. Comparisons were made on the basis of relative growth rates measured turbidimetrically in a Lumitron Colorimeter (Model 402-E). Fresh cultures of lac^- and normal cells were diluted so that 2.5×10^4 organisms were present in 0.05 ml. This volume constituted the inoculum for each of ten 12 x 100 mm cuvettes which contained 5 ml of a 0.5 per cent carbohydrate in synthetic medium. The cuvettes were then incubated at 37 C and, at intervals, the turbidity of each culture was read in the colorimeter using a 660 mμ red filter. The relative growth rates of normal and lac^- cells were measured in the various media and are shown in Figure 2 and summarized in Table VI.

Both types of organism regardless of source grew at the same rapid rate in the presence of glucose but grew very poorly in carbohydrate-free synthetic medium. On the other hand, normal cells regardless of source in lactose synthetic medium grew slightly better than in synthetic medium without lactose. The lac^- cells grew at a somewhat slower rate in lactose synthetic medium than they did in

glucose. Although initial growth occurred at the same time as in glucose medium, the rate lagged for several hours in the lactose broth before growth approximated that in glucose broth. It is probable that the lag represents a period of "enzymatic adaptation" required before *lac*⁻ mutants are able to ferment lactose rapidly.

The question arose as to whether or not normal cultures would increase their rate of growth if allowed to "adapt" in glucose. Therefore, in addition to the above experiments, cuvettes containing glucose synthetic medium were inoculated with normal organisms grown in glucose synthetic broth. In Figure 2, curve 3, it is found that the rate of growth could not be distinguished from that of other cultures grown in glucose synthetic medium.

Similarly normal organisms grown in lactose synthetic medium did not differ appreciably from those grown in nutrient broth when inoculated in plain synthetic medium, synthetic medium plus lactose or plus glucose (Table V).

IX. Mutagenic Effect of Physical Agents on *Sh. sonnei*

Witkin (54), employing *E. coli* strain Br, demonstrated that ultraviolet irradiation of cultures increased the rate of appearance of *lac*⁻ mutants. Since *lac*⁻ mutants arise from normal *Sh. sonnei* it was thought that ultraviolet irradiation might increase the rate of *lac*⁻ mutation. Demerec and Latajet (55) reported that increased mutation of *E. coli* varied inversely with the survival rate of *lac*⁻

(54) Witkin, E. M. Nuclear segregation and the delayed appearance of induced mutants in *Escherichia coli*. Cold Spring Harbor Symp. Quant. Biol., 16: 357-372, 1947.

(55) Demerec, M. and Latajet, R. Mutations in bacteria induced by radiations. Cold Spring Harbor Symp. Quant. Biol., 11: 38-50, 1946.

bacteria. Therefore, a curve of survival for Sh. sonnei as a function of ultraviolet dosage was plotted. From this curve a dose of irradiation that might cause an increased mutation rate could be chosen.

In order to establish the ultraviolet death curve for Sh. sonnei the following experiment was conducted. A 1-ml sample was taken from a twenty-four-hour culture of normal Sh. sonnei and inoculated into a tube (19 x 200 mm) containing 75 ml of nutrient broth. This culture was incubated for four hours. At this time the growth phase of the culture was logarithmic. A total of 3.5×10^7 organisms was contained in 7 ml of this culture which was dispensed into a petri dish forming a layer 1.5 mm thick. The culture was then irradiated with a Westinghouse T-15 Germicidal Lamp held at a distance of 15 cm. During the period of irradiation the culture was gently agitated to prevent shielding of organisms. Six test cultures were prepared in duplicate (a total of twelve) and exposed to ultraviolet irradiation for periods ranging from one-half minutes to five minutes. The treated cultures were concealed from light, therefore, allowing for only a minimal amount of photoreactivation. Bacterial counts were made of each treated sample and the logarithm of survival numbers plotted in Figure 3. Each point represented an average value of duplicate samples. The survival curve was sigmoid in shape. No attempt will be made to explain the departure of the survival curve from the expected straight line. An irradiation exposure of five minutes allowed for the survival of 0.2 per cent of the treated cultures. It was believed that the five-minute irradiation exposure was adequate for increasing the rate of lac⁻ mutant production.

In the manner described above, 7 ml of a Sh. sonnei culture containing 5×10^7 organisms per ml were irradiated with ultraviolet for five minutes at a distance of 15 cm. Once treated, bacterial counts were made and found to contain 2.0×10^2 surviving organisms per ml. From this treated culture, 3 ml were diluted ten-fold in 27 ml of distilled water. From this culture, 0.1 ml was pipetted into 100 tubes, each containing 6 ml of BCP lactose broth. All cultures were incubated at 37 C. From the same diluted culture, 0.2 ml were streaked onto the surfaces of each of 50 plates of Endo agar and incubated. Both broth and agar cultures were examined at twenty-four- and forty-eight-hour intervals. However, neither prompt lactose fermentation occurred in the broth nor were lac⁻ colonies detected on the plates.

In a similar experiment, a culture of normal organisms containing 6×10^6 organisms per ml was exposed to the same dose of ultraviolet irradiation. Bacterial counts made of the treated culture indicated 2×10^2 surviving organisms per ml. Samples of the treated cultures were inoculated into tubes containing BCP lactose broth and streaked onto plates of Endo agar. Lac⁻ mutants could be detected neither by prompt lactose fermentation of broth nor by acid colony formation on Endo agar within forty-eight hours of incubation.

The experiments described above have been repeated several times with the same result. Thus, it would appear that ultraviolet irradiation used under these conditions does not increase the rate of formation of lac⁻ mutants from Sh. sonnei.

X. Mutagenic Effect of Chemical Agents on *Sh. sonnei*

Since chemical mutagenic agents are known to increase the rate of mutation, it was thought that perhaps lac⁻ mutants of *Sh. sonnei* might arise at an increased rate as a result of treatment with such agents. Therefore, manganous chloride, formaldehyde, phenol, and sodium desoxycholate were studied for their mutagenic action.

A tube (19 x 200 mm) containing 75 ml of freshly prepared nutrient broth was inoculated with a culture of normal *Sh. sonnei* and incubated for sixteen hours. At the end of this time, the culture was washed three times with sterile distilled water in order to free the cells from medium. Bacterial counts were made on this suspensions. Then, 2 ml of this washed culture were used to inoculate each of several tubes containing solutions of chemical agents varying in concentrations from 0.02 per cent to 5.0 per cent in distilled water. These cultures were then incubated and at various intervals (from one to five hours) 5-ml samples were removed. Bacterial counts were made to determine the per cent survival and groups of 50 tubes containing 6 ml of BCP lactose broth were inoculated with 0.05 ml of the treated culture and incubated for thirty days.

From the data in Table VII it is apparent that all chemical concentrations to which the organisms were exposed had a lethal effect. Both phenol and formaldehyde were used in concentrations of from 0.2 to 0.3 per cent. The cultures were exposed to these chemical agents for from two to four hours. The organisms treated with phenol had from 50 to 5.7 per cent survival, a per cent survival

larger than organisms treated with any of the other chemicals. The organisms treated with formaldehyde had from 38.4 to 0.6 per cent survival. Sodium desoxycholate used at 5.0 per cent concentration for periods of from two to three hours had allowed from 13.1 to 2.0 per cent of the organisms to survive. Under the conditions of these experiments manganous chloride appeared to be the chemical most lethal to Sh. sonnei. Cultures were exposed to concentrations of from 0.02 to 0.1 per cent manganous chloride for from one to five hours. The survival of organisms was from 5.5 to 0.0001 per cent of the culture.

It was found that under the experimental conditions studied that these chemicals neither increased nor decreased the rate of appearance of lac⁻ mutants. Therefore, it seems that under the conditions of these experiments, these chemical agents had a lethal but not a mutagenic action on Sh. sonnei.

Discussion

In the past, the fermentation of lactose by Sh. sonnei has been associated only with the presence of variants. It was our observation that lac⁻ mutants of Sh. sonnei could be isolated from all cultures which fermented lactose within about ten days of incubation. These data confirm the observations of Rubenstein and Thibault (46). In addition, lac⁻ mutants frequently could not be isolated from cultures which had turned acid after this period. It was thought possible that acid production resulting from the occurrence of lactose fermentation might be responsible for death of the mutants. In our experiment, the acidity of each culture which became acid was measured. However, no appreciable differences in the hydrogen ion concentration were found between cultures from which lac⁻ mutants could or could not be detected. These data do not support the hypothesis of Rubenstein and Thibault that the absence of mutants might be due to death by increased acid production.

Inasmuch as lac⁻ mutants could not be detected in cultures that fermented lactose only after long periods of incubation, it was thought that acid production might have been caused by a few lac⁻ mutants present in cultures, which were not detectable by ordinary methods of isolation. With this in mind, fresh lactose broth was added to sedimented bacteria from cultures which had fermented lactose, but from which lac⁻ mutants could not be isolated. These cultures were then reincubated to allow any lac⁻ mutants to grow and ferment lactose. In the experiments described this did not occur. Kacoyanis and Baker (47) previously had shown that as few as from

one to ten lac⁻ mutants mixed with large populations of normal organisms fermented lactose within forty-eight hours. From these mixed cultures, lac⁻ mutants invariably could be isolated when subcultured onto Endo agar plates. It was believed that this would have been the case if a few lac⁻ mutants were present in the reincubated cultures. From our data it would seem that lac⁻ mutants were not responsible for the acid production in these cultures. These observations are not in agreement with Cook's et al. (45) suggestion that the absence of mutants in some cultures was due to a few mutants present in cultures which had turned acid and which were not ordinarily detectable by streaking one loopful onto Endo agar.

Even though lac⁻ mutants could not be isolated from some cultures, it was believed that acid production in these cultures was due to utilization of lactose. This was established (48) in earlier experiments by the fact that Sh. sonnei did not produce acid in the same medium containing no lactose. Since Sh. sonnei is incapable of producing acid from such substances as peptones and amino acids, it was thought possible that under the experimental conditions used lactose might hydrolyze to produce sufficient glucose and galactose to allow normal Sh. sonnei to acidify the medium in the absence of lac⁻ mutants. However, sterile lactose broth which had been incubated for periods exceeding the time usually required for the appearance of acid in cultures of Sh. sonnei, were inoculated with normal organisms but acid was not produced until after the usual lapse of several days. Thus, lactose is apparently stable and able to withstand the experimental conditions without hydrolysis. In the hope of explaining the

apparent production of acid by normal Sh. sonnei, the effect of various cultural conditions on both the production of lac⁻ mutants and the apparent production of acid by normal organisms was examined.

One of the effects of long incubation of cultures is the concentration of the medium constituents because of evaporation. It was observed that increased concentrations of lactose, meat extracts or peptone in the medium had no influence on the rate of lac⁻ mutant production. Thus, it is apparent that evaporation of medium plays no role in the production of mutants. It was thought that accumulation of metabolic products might have an effect on the production of mutants. However, growth of normal organisms in neutralized filtrates of acid cultures of lac⁻ cells did not result in an increase in the rate of lac⁻ mutant production. Preliminary studies seem to indicate that aging of cultures in nutrient broth to which lactose was then added was related to production of lac⁻ mutants. However, repeated experiments did not confirm preliminary studies.

When normal cultures in lactose broth were aerated, it was observed that aeration favored the appearance of lac⁻ mutants. These were isolated from all aerated cultures prior to the appearance of acid. Nearly all aerated cultures became acidified within five days and then became alkaline in another forty-eight hours. From these tubes lac⁻ mutants could be isolated for several days. From several aerated cultures lac⁻ mutants were isolated even though at no time did acidification occur. This apparent lack of acid production may be due to the highly aerobic conditions and the consequent lack of accumulation of acid metabolic products. Eventually the lac⁻ mutants

disappeared in the aerated cultures and only lac- cells could be isolated. In some cultures lac- mutants reappeared again. This phenomenon occurred irregularly.

Since aeration seemed to have a stimulatory effect on the formation of lac- mutants, it was thought that aeration in the absence of lactose might stimulate mutant production. Cultures of normal organisms were aerated in nutrient broth in the absence of lactose. However, lac- mutants could not be isolated. The apparent stimulatory effect of aeration on the production of lac- mutants is seen only when lactose is present. This may be the result of some slight advantage that the lac- organisms have in the absence of lactose. It seems to be corroborated by the results of aerating lac- strains in the absence of lactose. Under these conditions lac- mutants disappeared completely in six out of thirty three different cultures leaving only lac- organisms. Aeration alone will not cause the production of lac- mutants.

In contrast to other organisms as reported by Hersey and Bronfenbrenner (19) and Koser and Vaughn (25), when lactose broth cultures of Sh. sonnei were grown under anaerobic conditions, all cultures appeared acid on the sixth day of incubation. Although these cultures appeared acid, lac- mutants were not detected in any of these cultures. The possibility arose that this phenomenon was due to reduction of the indicator, brom-cresol-purple, brought about by the growth of normal organisms under anaerobic conditions. However, when cultures were vigorously aerated to reoxidize the medium no change occurred in the indicator. Another possibility to explain

the acidification of cultures under anaerobic conditions without the presence of lac⁻ mutants was the accumulation of carbon dioxide. However, when cultures were heated to remove any carbon dioxide present no change in the indicator occurred, indicating the absence of carbon dioxide.

Another possibility is that acid production under anaerobic conditions is the result of the action of Sh. sonnei on some components of the medium other than lactose. However, acid did not appear in cultures grown anaerobically in nutrient broth without lactose. Thus, Sh. sonnei is incapable of producing acid from such substances as peptones and amino acids under anaerobic conditions. This indicated that the presence of lactose was required for the production of acid in these cultures. An experiment was conducted using lac⁻ cells as the inoculum. Fermentation of lactose occurred promptly, within forty-eight hours. Furthermore, lac⁻ mutants could be isolated from these cultures. The acidity of these cultures as measured colorimetrically was from pH 5.5 to 5.1. In cultures grown anaerobically from which lac⁻ mutants could not be isolated, the pH ranged from 6.1 to 5.8. From these data it is apparent that anaerobic conditions inhibit the production of lac⁻ mutants. Furthermore, it seems probable that the production of acid by normal Sh. sonnei grown under anaerobic conditions occurs by some unknown mechanism which is different from that used by lactose-fermenting mutants. This ability to produce acid from lactose under anaerobic conditions might well be a unique property of Sh. sonnei, since Sh. flexneri was incapable of producing acid under identical conditions.

When aerated cultures of Sh. sonnei in lactose broth were compared with non-aerated cultures, it was found that in the non-aerated cultures lac⁻ mutants could be isolated only from cultures which had turned acid during the first ten days of incubation. In contrast to the results obtained with aerated cultures, mutants once present could be isolated at any time during the balance of the experiment. However, lac⁻ mutants could not be isolated from cultures which became acid only after ten days of incubation.

As was discussed above, acid was produced in some non-aerated but open lactose broth cultures of Sh. sonnei from which lac⁻ mutants could not be isolated. Even though lac⁻ mutants were not isolated, lactose was required for the production of acid by these cultures. As was pointed out, an unknown mechanism of Sh. sonnei operating under anaerobic conditions resulted in the production of acid in cultures in the absence of mutants. This mechanism may account for the acid production in open cultures from which lac⁻ mutants could not be isolated. In many instances, it was observed that acid first appeared in such cultures at the base of the tubes. This observation lends support to the idea that this hypothetical mechanism may be responsible for the appearance of acid. However, another possibility is that acid is produced by weak, lactose-fermenting mutants only capable of fermenting at such a slow rate that they are not detectable on Endo agar or lactose broth. As weak fermenters, these mutants may require several days of incubation before acid production is sufficient to change the indicator used.

Still another possibility exists that acid production in some cultures is caused by mutants capable of fermenting lactose but unable to continue to exist in the presence of normal organisms. The aeration experiments lend support to this hypothesis. As was discussed previously, lac^- mutants tend to die out when aerated in mixtures with normal organisms. Although these conditions are different from those encountered in non-aerated cultures, it is quite possible that lac^- mutants with an even poor ability to compete with normal organisms may exist and be responsible for the acidification of the cultures in which lac^- mutants could not be isolated. However, at the present time there is insufficient data available to make it possible to determine which, if any, of these possibilities are responsible for the lack of lac^- mutants in non-aerated open cultures of Sh. sonnei in which acid was produced.

It was noted that in a few cultures of lac^- cells after several days of aeration, the lac^- mutants were replaced with lac^+ organisms. The same experiment was repeated several times to eliminate the possibility of technical errors. Each time the same results were obtained and therefore this phenomenon must have been due to back mutation. Whether or not back mutation can be used to explain the lack of mutants in acidified, non-aerated cultures from which mutants could not be isolated, is not clear. Also, it is difficult to evaluate the significance of back mutation for the explanation of the acidified cultures from which lac^- mutants could not be isolated until or unless the presence of such mutants can be established.

It was thought that the absence of mutants in cultures which

had become acid may have been due to poor growth of the *lac*⁻ mutants in competition with normal organisms. Therefore, relative growth rates of *lac*⁻ mutants and normal organisms were studied. It was found that mutants and normal cells subcultured from cultures grown in nutrient broth grew at different rates in lactose and in glucose synthetic broth. In lactose, the *lac*⁻ mutant did not grow as well as in glucose synthetic broth. A continuous lag occurred in the rate of growth. Normal organisms grew poorly in lactose. The hypothesis of Monod states that a *lac*⁻ mutant cell is not a cell with a fully developed enzyme system but that this enzyme reaches capacity by adapting in the presence of the stimulus, lactose. It was found that *lac*⁻ mutants subcultured from lactose broth medium into fresh lactose synthetic broth grew at a rate slightly but not significantly better than was observed with *lac*⁻ organisms which were subcultured from nutrient broth. Therefore, it appeared that enzymatic adaptation did not occur in this mutant. In contrast, Cook demonstrated that *lac*⁻ mutants from his strain of *Sh. sonnei* subcultured in lactose synthetic agar grew at a rate equal to cells in glucose grown in synthetic broth. It appears that this *lac*⁻ mutant may not be similar to mutants described by other investigators.

Both physical and chemical agents capable of increasing the rate of mutation have been reported. As physical mutagenic agents, ultraviolet and x-ray have been used by Witkin (54) to increase the rate of *lac*⁻ mutant production of *E. coli mutabile*. In our experiments, normal *Sh. sonnei* treated with doses of ultraviolet,

found to be optimum for other microorganisms, neither increased nor decreased the rate of production of lac⁻ mutants. In addition, chemical mutagenic agents were used in an effort to induce the rate of production of lac⁻ mutants in Sh. sonnei. The agents included manganous chloride, formaldehyde, phenol and sodium desoxycholate which were used at various concentrations. However, none of these chemical agents as employed in these experiments had a mutagenic effect on Sh. sonnei. Under the conditions of these experiments, the physical and chemical agents used had a lethal but not a mutagenic action on Sh. sonnei.

One of the questions that arose during the course of this work was whether lac⁻ mutants arose spontaneously or were induced by the presence of lactose, or both. In previous experiments (48) it was difficult to establish the pre-existence of lac⁻ mutants in cultures of Sh. sonnei. It was shown that of 1520 tubes containing lactose broth inoculated with normal organisms, only two cultures fermented lactose within forty-eight hours; the remainder required a period of several days before fermentation occurred. These 1520 tubes were not seeded at the same time, but were the aggregate of several series of 80 tubes of medium inoculated during the span of two years. In the present studies, when normal organisms were grown in nutrient broth for various lengths of time up to 20 days, lactose fermenting mutants were detected in only twelve of a total of 1120 of these cultures when lactose was added. As previously described, if more of these cultures had contained mutants, it is probable that they would have been detected because when a few lac⁻

mutants were present in the inoculum, lactose fermentation occurred within forty-eight hours.

In view of the advantage for growth of normal organisms in nutrient broth in the absence of lactose, there is a relative disadvantage of lac^- mutants. In competition with normal cells these mutants tend to die out in the nutrient broth. This was observed when pure cultures of lac^- cells were aerated in nutrient broth in the absence of lactose. In several cultures, lac^- cells disappeared and eventually only lac^- mutants could be detected. This seemed to indicate that lac^- mutants arising spontaneously in cultures of normal organisms in nutrient broth tend to die out thereby lessening the chance of finding them. Therefore, the presence of lactose seems to be selective for lac^- mutants. However, from the data presented the possibility of a stimulatory effect of lactose cannot be eliminated.

Summary

In studies of the rate of appearance of lac⁻ mutants of Shigella sonnei grown in lactose broth, it was observed that lac⁻ mutants could be isolated from cultures which became acid within ten days of incubation. However, lac⁻ mutants could not be isolated from cultures which became acid after this time. In all cultures, lactose was found to be necessary for acid production.

The inability to detect lac⁻ mutants in cultures which became acid after ten days of incubation was found not to be due to death of the mutants because of increased acid produced in the cultures during incubation. The possibility that acid production might be due to lac⁻ mutants present in numbers too few to be detected by loopful sampling was examined. No lac⁻ mutants were detected. The cultural conditions such as increased concentrations of lactose, meat extract and peptone in the medium; neutralized filtrates of acid cultures of lac⁻ cells; and aging of cultures in nutrient broth to which lactose was added were not responsible for the absence of lac⁻ mutants in cultures which became acid.

Aeration of normal organisms in lactose broth favored the production of lac⁻ mutants in all cultures. Eventually, the lac⁻ mutants disappeared in the aerated cultures and only lac⁻ cells could be isolated. The production of lac⁻ mutants was apparent only when lactose was present in the aerated cultures. Lac⁻ cells aerated in nutrient broth without lactose gave rise to lac⁻ mutants.

When lactose broth cultures of Sh. sonnei were grown anaero-

bically, acid appeared on the sixth day of incubation but lac⁻ mutants could not be detected. These cultures required lactose for the production of acid. It seemed probable that the production of acid by normal Sh. sonnei grown under anaerobic conditions occurred by some unknown mechanism different from that used by lactose-fermenting mutants.

Relative growth rates of lac⁻ mutants and normal organisms were studied. Lac⁻ mutants grew at a better rate in lactose synthetic broth than normal organisms. The fermentation of lactose by lac⁻ mutants did not seem to adapt when previously grown in lactose medium.

Physical and chemical agents were found to have a lethal effect but not a mutagenic effect on an increased rate of production of lac⁻ mutants.

The presence of lactose seems to be selective for lac⁻ mutants. However, from the data presented the possibility of a stimulatory effect of lactose cannot be eliminated.

TABLE I

The Effect of Acid Produced in Lactose Broth Cultures of Shigella sonnei on Lac- Mutants

Days of Incubation	Initial Incubation			Reincubation*	
	Number of cultures turned acid	Range of pH	Subcultured on Endo agar	Number of cultures turned acid in 48 hours**	Subcultured on Endo agar
6	1	5.6	lac- & lac-	1	lac- & lac-
7	3	5.0-5.6	lac- & lac-	3	lac- & lac-
8	5	6.2-6.7	lac- & lac-	5	lac- & lac-
9	3	6.0-6.7	lac- & lac-	3	lac- & lac-
10	1	6.0	lac- & lac-	1	lac- & lac-
11	2	6.0-6.7	lac-	0	lac-
12	33	5.5-6.2	lac-	0	lac-
15	10	6.0-6.4	lac-	0	lac-
18	12	5.5-6.2	lac-	0	lac-
21	2	6.1	lac-	0	lac-

* Refers to incubation of the sedimented organisms after the addition of fresh lactose broth.

** Most of these cultures became acid within 18 hours, although all were observed for 48 hours.

TABLE II

Daily Sampling of Aerated Cultures of Shigella sonnei Grown in Lactose Broth for the Detection of Lac \neg Mutants

Number of cultures	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	-	-	-	-	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
3	-	-	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
4	-	-	-	+	Ø	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
6	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
7	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
8	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
9	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
10	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
11	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-
12	-	-	-	+	Ø	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-
13	-	-	-	+	Ø	+	+	+	+	+	+	+	-	-	+	+	-	-	-	-
14	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	+	+	-	-	-
15	-	-	-	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-
16	-	-	-	-	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	-
17	-	-	-	-	+	+	Ø	+	+	+	-	-	+	-	-	-	-	-	-	-
18	-	-	-	+	Ø	+	+	+	+	-	-	-	-	-	-	+	+	-	-	-
19	-	-	-	+	Ø	+	+	+	+	-	-	+	-	-	-	+	+	-	-	-
20	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
21	-	-	-	-	+	Ø	+	+	+	-	-	-	-	-	-	+	-	+	-	-
22	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
23	-	-	-	-	+	+	Ø	+	+	-	-	-	-	-	-	-	+	-	-	-
24	-	-	-	+	+	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
25	-	-	-	+	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-
26	-	-	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-	-	-
27	-	+	+	+	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
28	-	-	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
29	-	-	-	+	Ø	Ø	+	+	+	+	+	-	-	-	-	-	-	-	-	-
30	-	-	-	-	-	Ø	+	+	+	+	+	-	-	-	-	-	-	-	-	-
31	-	-	-	+	+	Ø	+	+	+	+	+	+	-	-	-	-	-	-	-	-
32	-	-	-	-	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	+	+	+	+	+	-	+	-	-	-	-	-	-	-	-

- Cultures which appeared alkaline and from which lac \neg mutants could not be detected.
- +
- Ø Cultures which appeared acid and from which lac \neg mutants could be detected.
- 0 Cultures which appeared acid and from which no viable organisms could be detected.

TABLE III

Daily Sampling of "Still" Cultures of Shigella sonnei Grown in Lactose Broth for the Detection of Lac⁻ Mutants

Number of Cultures	Days of subculture into fresh lactose broth																			
	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	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
4	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
5	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
6	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
7	-	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
8	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
9	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
10	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*
11	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
12	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
13	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*
14	-	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
15	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*
16	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
17	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
18	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*	*
19	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
20	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
21	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*
22	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
23	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
24	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
25	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
26	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*
27	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
28	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*
30	-	-	-	-	-	-	-	-	-	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø	Ø
31	-	-	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*
32	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	-	-	*	*	*	*	*	*	*	*	*

- Cultures which were alkaline and from which lac⁻ mutants could not be detected on subculture into lactose broth.
 * Cultures which were acid and from which lac⁻ mutants could not be detected.
 Ø Cultures which were acid and from which lac⁻ mutants could be detected.

TABLE IV

The Effect of Aeration on Lac- Cultures of Shigella sonnei
Investigated by Daily Sampling

Number of culture	1 ^o	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1		Ø	Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2		Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3		Ø	Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4		Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5		Ø	Ø	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6		Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7		Ø	Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8		Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9		Ø	Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10		Ø	Ø	Ø	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

- ^o All cultures became acid within twenty-four hours.
 Ø Aerated cultures which appeared acid and from which lac- mutants could be detected.
 + Cultures which appeared alkaline and from which lac- mutants could be detected.
 0 Aerated cultures which appeared acid and from which no viable organisms could be detected.

TABLE V

Daily Sampling of Aerated Cultures of Lac- Cells of Shigella sonnei Grown in Plain Nutrient Broth for the Detection of Lac- Mutants

Number of culture	Day of subculture into fresh lactose broth																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	-
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
26	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
28	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
29	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-
30	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
32	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
33	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

+/ Cultures in which lac+ cells were detected.

- Cultures in which lac- mutants were detected.

TABLE VI

Relative Growth Rates of Lac⁻ Mutants and Normal *Saligella sonnei*

			Inoculated into Synthetic Medium		
			No carbohydrate	With glucose	With lactose
Source of Inoculum	Nutrient	broth	negligible 12 hours	100% 9 hours	12% (max) 6 hours
		lac ⁻ normal	negligible 12 hours	100% 9 hours	80% 12 hours
	Synthetic	lactose	negligible 12 hours	100% 9 hours	20% (max) 7 hours
		lac ⁻ normal	negligible 12 hours	100% 9 hours	90% 12 hours

Normal organisms grown in glucose synthetic medium and inoculated into glucose synthetic medium (Fig. 2, curve 3) were considered the 100% level. Other results are expressed as percentages of this arbitrary standard. The times show when each series reached the percentage indicated.

TABLE VII

Effect of Chemical Agents on Shigella sonnei

Chemical agent	% conc.	Time of exposure in hours					
		0	1	2	3	4	5
Manganous chloride	0	9.0×10^7	8.9×10^7	8.9×10^7	8.5×10^7	-	8.3×10^7
			99%	99%	94%		92%
	0.02	9.0×10^7	5.0×10^6	4.5×10^6	2.6×10^5	-	-
			5.5%	5.0%	0.3%		
	0.04	9.0×10^7	1.0×10^6	5.0×10^4	-	-	-
			1.1%	0.06%			
	0.05	9.0×10^7	8.6×10^4	1.0×10^3	-	-	-
			0.1%	0.001%			
	0.06	9.0×10^7	5.6×10^3	-	-	-	-
			0.006%				
	0	1.5×10^8	1.4×10^8	-	-	-	-
			93%				
	0.1	1.5×10^8	1.1×10^3	-	-	-	2.0×10^2
			0.0007%				0.0001%
Phenol	0	4.0×10^7	-	-	3.6×10^7	3.3×10^7	-
					90%	83%	
	0.20	4.0×10^7	-	-	2.0×10^7	1.5×10^7	-
					50%	37%	
	0	1.0×10^8	-	-	9.1×10^7	-	-
					91%		
	0.30	1.0×10^8	-	-	3.8×10^7	5.7×10^6	-
					38%	57%	
Formaldehyde	0	5.2×10^7	-	5.0×10^7	5.0×10^7	4.8×10^7	-
				96%	96%	90%	
	0.20	5.2×10^7	-	2.0×10^7	1.5×10^7	9.5×10^6	-
				38.4%	28.8%	18.2%	
	0.30	5.2×10^7	-	3.1×10^5	-	-	-
				0.6%			
Sodium desoxycholate	0	3.8×10^7	-	-	3.6×10^7	-	-
					93%		
	5	3.8×10^7	-	5.0×10^6	7.5×10^5	-	-
				13.1%	2.0%		
	0	3.3×10^7	-	3.1×10^7	-	-	-
				94%			
	5	3.3×10^7	-	2.2×10^6	-	-	-
				6.6%			

Survivors expressed in numbers and percentages.

Figure 1

Number of Cultures Turned Acid and Time of Occurrence of Acid Production by Lac⁻ Mutants and Normal Shigella sonnei Grown under Anaerobic Conditions in Lactose Broth

1. Lac⁻ mutants grown under anaerobic conditions in lactose broth.
2. Normal organisms grown under anaerobic conditions in lactose broth.
3. Normal organisms grown in open tubes containing lactose broth.
4. Normal organisms grown under anaerobic conditions in nutrient broth.

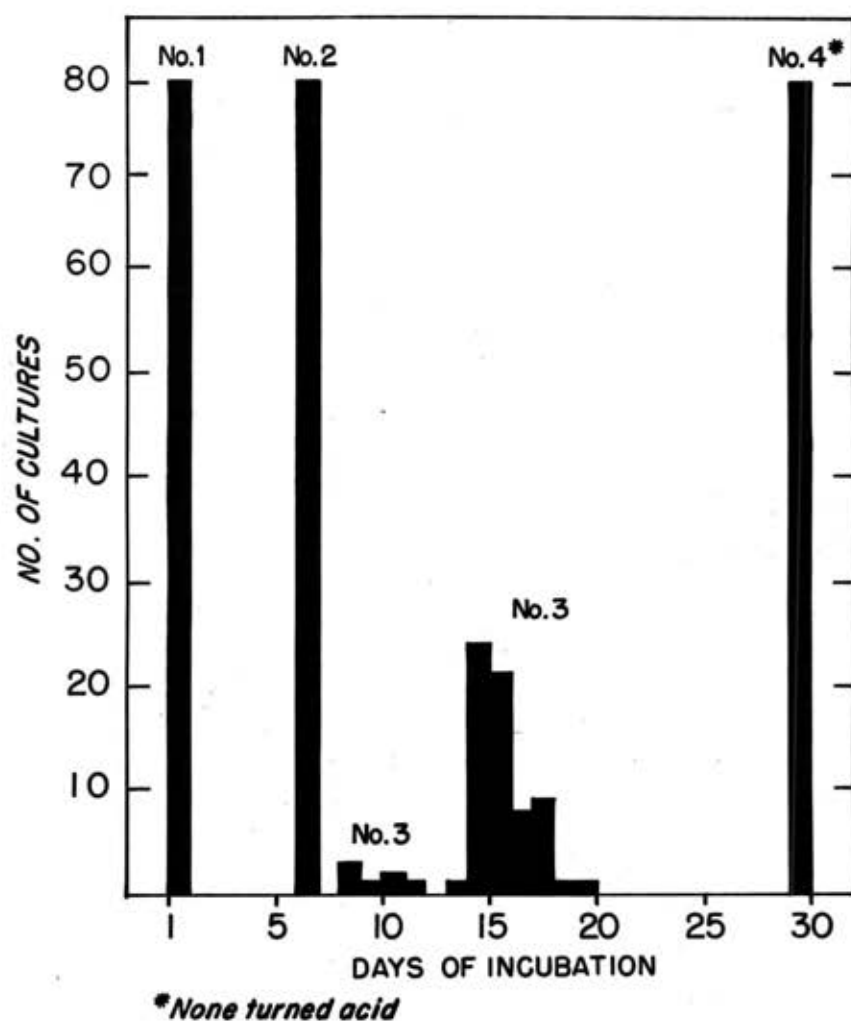


Figure 1. Number of Cultures Turned Acid and Time of Occurrence of Acid Production by Lac⁻ Mutants and Normal *Shigella sonnei* Grown under Anaerobic Conditions in Lactose Broth

Figure 2

Relative Growth Rates of Lac⁻ Mutants and Normal Shigella sonnei

Lac⁻ mutants and normal Shigella sonnei subcultured from nutrient broth and grown in the following media during growth rate studies:

1. Normal organisms grown in glucose synthetic medium
2. Normal organisms grown in lactose synthetic medium
3. Normal organisms grown in plain synthetic medium
4. Lac⁻ mutants grown in glucose synthetic medium
5. Lac⁻ mutants grown in lactose synthetic medium
6. Lac⁻ mutants grown in plain synthetic medium

Lac⁻ mutants of Shigella sonnei subcultured from lactose synthetic medium and grown in the following media during growth rate studies:

7. Lac⁻ mutants grown in glucose synthetic medium
8. Lac⁻ mutants grown in lactose synthetic medium
9. Lac⁻ mutants grown in plain synthetic medium

Normal Shigella sonnei subcultured from glucose synthetic medium and grown in the following medium during growth rate studies:

10. Normal organisms grown in glucose synthetic medium

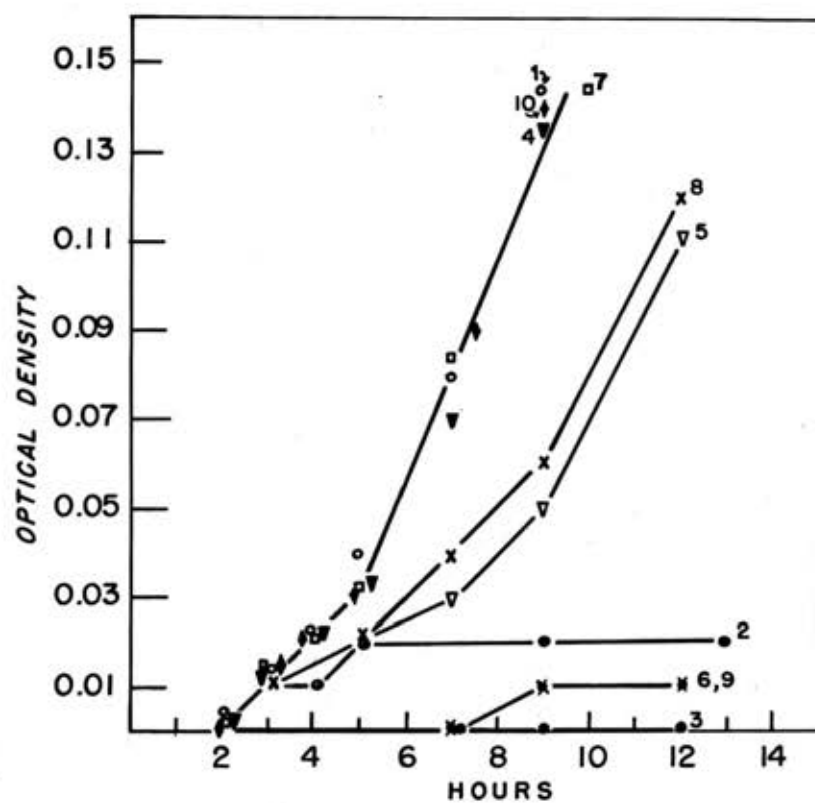


Figure 2. Relative Growth Rates of Lac⁻ Mutants and Normal Shigella sonnei

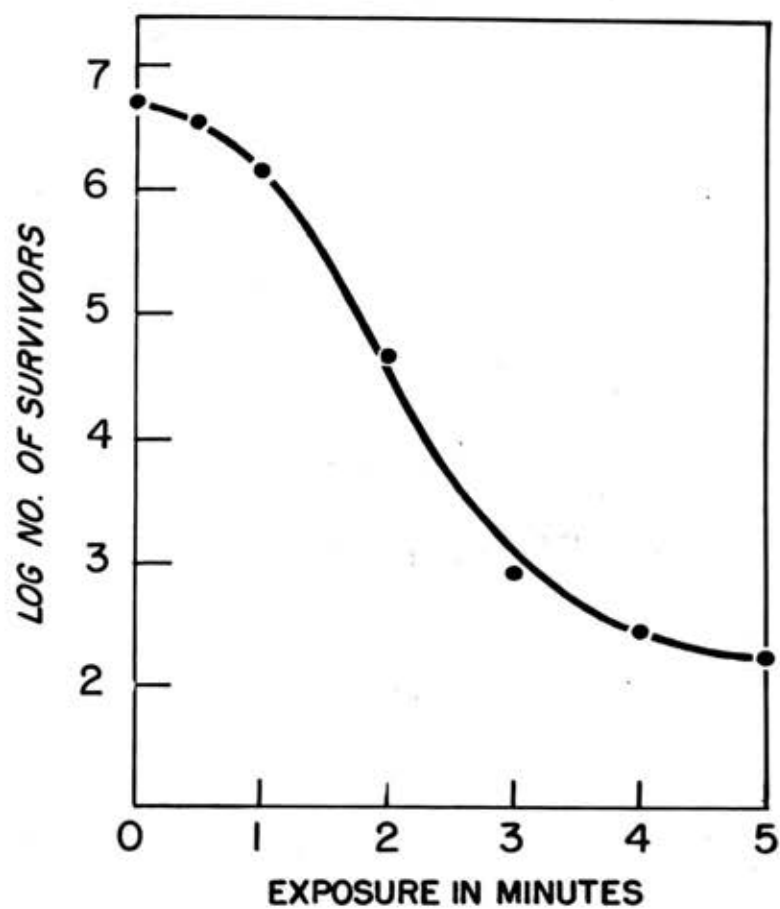


Figure 3. Effect of Ultra-Violet Irradiation on Normal Shigella sonnei

SOME FACTORS INFLUENCING LACTOSE FERMENTATION BY SHIGELLA SONNEI
(Publication No.)

George John Kacoyanis, Ph.D.

Boston University Graduate School, 1957

Major Professor: Professor Edgar E. Baker

The fermentation of lactose by Shigella sonnei has been associated with the presence of mutants. We observed that lactose-fermenting (lac^+) mutants of Sh. sonnei could be isolated from all lactose broth cultures which had become acid within about ten days of incubation. However, lac^- mutants could not be isolated from cultures which had become acid after this time. It was thought that the absence of lactose-fermenting organisms in these cultures might be due to the death of the mutants because of increased acidity produced by lactose fermentation. The pH of all acid cultures was measured but no apparent difference was found between those from which lac^+ mutants were isolated and those in which lac^- mutants could not be detected. The possibility existed that acid production might have been caused by lac^- mutants present in numbers too few to be detected by loopful sampling. By the method described by Kacoyanis and Baker (Proc. Bact., 1955, p. 103) entire bacterial cultures were screened for lac^+ mutants. However, lac^- mutants could not be detected. The fact that Sh. sonnei did not produce acid in the same medium containing no lactose established that acid production was due to utilization of lactose.

In the hope of explaining the apparent production of acid by normal Sh. sonnei, the effect of various cultural conditions on both the production of lac⁻ mutants and the apparent production of acid by normal organisms was examined. Increased concentrations of lactose, meat extract or peptone in the medium; neutralized filtrates of acid cultures of lac⁻ cells; and aging of cultures in nutrient broth to which lactose was added did not increase the rate of lac⁻ mutant production. Aeration of normal organisms in lactose broth favored the production of lac⁻ mutants. Nearly all cultures became acidified within five days and then became alkaline again. Lac⁻ mutants could be isolated for several days afterward. Eventually, the lac⁻ mutants disappeared in the aerated cultures and only normal cells could be isolated. Lac⁻ mutants could not be isolated from cultures of normal organisms when aerated in lactose-free nutrient broth. The stimulatory effect of aeration on the production of lac⁻ mutants is apparent only when lactose is present. When lactose broth cultures of Sh. sonnei were grown under anaerobic conditions, all cultures became acid on the sixth day of incubation, but lac⁻ mutants were not detected. The possibility that this phenomenon might be due to reduction of the indicator or to accumulation of carbon dioxide was examined and ruled out experimentally. Acid did not occur in cultures grown anaerobically in nutrient broth without lactose indicating that the presence of lactose was required for the production of acid in these cultures. When lac⁻ cells were inoculated into lactose broth and grown anaerobically, acid appeared within forty-eight hours. Under these conditions the lac⁻ mutants produced con-

siderably more acid than did normal organisms. Furthermore, lac⁻ mutants could be isolated from these cultures. It is apparent that anaerobic conditions inhibit the production of lac⁻ mutants. Furthermore, it seems probable that the production of acid by normal Sh. sonnei grown under anaerobic conditions occurs by some unknown mechanism different from that used by lactose-fermenting mutants.

Relative growth rates of lac⁻ mutants and normal organisms were studied. Lac⁻ mutants grew at a better rate in lactose synthetic broth than normal organisms when subcultured from cultures grown in nutrient broth. However, the lac⁻ mutants did not grow as well in lactose as in glucose synthetic broth, a lag occurring in the rate of growth. Lac⁻ mutants subcultured from lactose broth medium into fresh lactose synthetic broth did not grow significantly better than lac⁻ organisms which were subcultured from nutrient broth. Therefore, it appeared that enzymatic adaptation did not occur in this mutant. It seems that this lac⁻ mutant may differ from mutants described by other investigators.

Ultraviolet irradiation of normal Sh. sonnei neither increased nor decreased the rate of production of lac⁻ mutants. In addition, chemical mutagenic agents (manganous chloride, formaldehyde, phenol and sodium desoxycholate) were used in an effort to induce an increase in the rate of production of lac⁻ mutants. Under these experimental conditions, the physical and chemical agents used had a lethal but not a mutagenic action on Sh. sonnei.

Preliminary work suggested that aeration of lac⁻ mutants in nutrient broth induced the rise of lac⁻ mutants. This was confirmed

in detailed studies which also indicated that these lac- mutants are favored by aeration in the absence of lactose. The results seem to point to the selective action of lactose in the rise of lac⁺ mutants. However, the possibility of a stimulatory effect of lactose cannot be eliminated.

Autobiography

NAME: George John Kacoyanis

DATE OF BIRTH: January 8, 1925

PLACE OF BIRTH: Somerville, Massachusetts

PERMANENT ADDRESS: 94 Ten Hills Road, Somerville, Massachusetts

COLLEGES ATTENDED	GRADUATION	DEGREE
Northeastern University	1950	B.S.
Boston University	1955	A.M.
Boston University	1957	Ph.D.

ACADEMIC APPOINTMENT: Research Fellow, Boston University School of Medicine, 1954-1957.

SOCIETY MEMBERSHIPS: Boston University Chapter, Society of the Sigma Xi
Northeast Branch, Society of American Bacteriologists
Society of American Bacteriologists

