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LACTIC ACID INHIBITION OF SALMONELLA TYPHIMURIUM
IN YOGURT

by

Howard Eugene Rubin

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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To the memory of my Grandparents

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ABBREVIATIONS

1. Artificial Milk Whey Yogurt - Milk whey which has been adjusted to represent yogurt's inhibitory conditions.
2. AMY - Artificial Milk Yogurt - Skim-milk which has been adjusted to represent yogurt's inhibitory conditions.
3. Artificial SMM Yogurt - SMM which has been adjusted to represent yogurt's inhibitory conditions.
4. Cationic whey - Acidless whey - Yogurt whey which has had the anions (acids) removed.
5. Die-off Rate - Negative of the reciprocal of the time required for the population to halve; minute^{-1} .
6. Growth rate - Reciprocal of the time required for the population to double; hour^{-1} .
7. ORP - Oxidation-Reduction Potential.
8. Reconstituted Yogurt - Yogurt whey which has had the yogurt casein fraction added back to it.
9. SMM - Synthetic Milk Medium.
10. Yogurt - Skim-milk fermented with the yogurt starter culture.
11. Yogurt Inhibitory Conditions - pH, 3.85; ORP, -80 mV; 1.42 % lactic acid; and 0.022% acetic acid.

12. Yogurt Starter Culture - Active cultures of Streptococcus thermophilus and Lactobacillus bulgaricus.
13. Yogurt Whey - Yogurt from which the yogurt casein fraction has been removed.

INTRODUCTION

A. Epidemiology of Salmonellosis

Salmonellae are ubiquitous zoonotic microorganisms. The reservoir for Salmonellae is usually the intestines of animals. Salmonellosis can occur as a result of intestinal infection by these organisms. Salmonella typhimurium is the species most frequently isolated, and is involved in about 70% of all cases of salmonellosis.

Introduction of these organisms into the alimentary canal can result in a) primary salmonellosis, in which a Salmonella serotype is the only etiological factor and the cause of pathogenesis, b) secondary salmonellosis, in which the pathogenic activity of the bacteria develops because the host is debilitated due to a primary disease or to a stress condition, or c) symptomless salmonellosis, in which the bacteria are located in the alimentary tract with no clinical indication of disease accompanied by either a temporary or permanent carrier state, with Salmonella possibly being shed in the feces. (11,69)

Salmonellae induced gastroenteritis takes from 6 to 48 hours to incubate, with the onset of disease usually between 12 to 24 hours. The infection usually lasts from 3 days to 3 weeks. The clinical manifestations are characterized by

onset of abdominal pain, diarrhea, nausea and vomiting. Dehydration may be severe, and fever is usually present. The infection may develop into an enteric fever or septice-mia, or the infectious agent may localize in one of the body's tissues causing severe inflammation or abscesses. Mortality due to salmonellosis is rare, usually occurring among the very young or very old. (7)

Salmonellae can be spread either through contact with an infected animal or through a vehicle such as food, milk, or water. Transmission of the infectious agent via contaminated food is the prevalent mode by which Salmonellae are spread. However, food is usually contaminated with low concentrations of bacteria. Therefore, the contaminating microorganisms must grow during transmission in order to reach pathogenic concentrations. (11,69)

The number of cases of typhoid fever, between 1946 and 1962, dropped from 3,268 to 608 while the number of cases of other salmonellosis increased from 723 to 9,680 in the United States (11). The trend is still toward an increase with the number of cases of salmonellosis rising from 19,723 in 1967 to 26,110 in 1972 (60,61). The decline in numbers of typhoid cases is probably due to a) better investigation and reporting, b) better diagnostic techniques, c) better sanitation, d) improved hygiene, e) increased use of pasteurized milk and chlorinated water, and f) prophylactic immunization against typhoid (10). The rise in cases of other salmonellosis has been attributed to a) better

reporting methods and diagnosis, b) increased incidence of Salmonellae in animal reservoirs, c) increased incidence of human carriers, d) "build-up" of Salmonellae in livestock as they pass from the farm to the kitchen, e) increasing habit of eating raw or undercooked foods, f) over reliance on food inspection, g) increase in mass food production, and h) increasing international food trade. (11,69)

From January to June, 1970, salmonellosis accounted for 12.5% of all reported outbreaks of food-borne disease and 11.9% of all cases reported in the United States (63). There are about 2 million incidents of human morbidity per year, while mortality due to salmonellosis is less than 1% per annum (63). Some observers believe that only 1 to 5% of the total number of cases of salmonellosis are actually reported (54,69). The economic loss due to salmonellosis is estimated to be approximately 300 million dollars annually (63).

Salmonellosis is the third most common food-borne communicable disease (63) but the most common disease transmitted through milk (54). Salmonella typhimurium has been found to be the most frequent cause of human salmonellosis and is the species most frequently isolated from contaminated milk samples (63). Since Salmonellae are ubiquitous organisms, they should always be considered as a potential threat. While milk-borne outbreaks of salmonellosis in the United States are rare, it is not uncommon in Great Britain where, from 1943 to 1960, 47 outbreaks involving 3,201 cases were reported (11,63). In 1954, there was an incidence of

salmonellosis involving 300 cases where the mode of transmission was through pasteurized milk in England and Wales (63). In England, in 1955, a 17 case outbreak of salmonellosis was again due to pasteurized milk. Both of these outbreaks were traced to S. typhimurium (63). Several other outbreaks of pasteurized milk-borne salmonellosis have been reported since 1955 (54). Pasteurized milk can be recontaminated by contacting inadequately cleaned and sanitized equipment, contaminated water and contact with either unpasteurized milk (26,54) or a human carrier (26,35). These studies (26,38,54) indicate that even milk packaged under the most sanitary industrial conditions can become a vehicle of salmonellosis.

In 1966, Salmonella was discovered in non-fat dry milk (54). Since that time, 11 serotypes have been isolated from non-fat dry milk in nine states causing the United States dairy industry and the Food and Drug Administration to become concerned with the safety of manufactured dairy products (38,59). Dry milk has been found to be contaminated by workers or contaminated equipment or possibly the inlet air (37,54).

B. Salmonellosis from Fermented Dairy Products

Salmonellae have been found to persist in fermented milk products. The organism survived for 3 days in cottage cheese, lasted for 58 days in acidophilous milk, survived for more than 63 days in buttermilk (54), survived for more

than 10 weeks in butter (78) and survived for up to 10 months in cheddar cheese (54). However, the length of survival of Salmonella in yogurt has never been established and has been reported to vary from a few hours (66) to a few weeks (30). At present there are no adequate explanations for this large range in survival time, and the safety of yogurt has never been adequately demonstrated (63).

Yogurt is not as hygienically produced as it should be commercially. In 1972 Arnott (2) sampled 152 commercial Canadian yogurts and found that 25% were contaminated with Staphylococci, 20% were contaminated with psychrotrophic bacteria, 33% with Enterococci and 50% were contaminated with either yeast or mold. Unfortunately, he did not sample for the presence of Salmonellae.

World-wide, yogurt is one of the most common fermented milk products and has been for centuries. It is fast becoming a popular food product in the United States as evidenced by a dramatic rise in sales (50). In 1962 about 45.5 million pounds of yogurt were consumed. This figure reached 288.8 million pounds by 1972 (49).

Along with the dramatic rise in the popularity of yogurt is a parallel rise in the amount of yogurt prepared in the home. Homemade yogurt is produced under much less stringent conditions than commercial yogurt, and in rural areas it can be made from raw, unpasteurized milk. Silberstein and Gerichter (77) found that in Israel the largest number of outbreaks of salmonellosis originated from

infections contracted in the home as a result of home prepared foods. There have been no reported incidences of salmonellosis associated with certain cultured milk products such as buttermilk, acidophilous milk, or yogurt in the United States, but there have been reported incidences of fermented milk salmonellosis in other countries (54). As a consequence, the potential of yogurt as a vehicle for salmonellosis deserves further study.

Milk has been found to possess certain inherent antibacterial properties. Present in milk are lactenin, lactoperoxidase, agglutinins, thioxynate, immune globulins (17, 20, 71, 84, 85), lysozyme and a bactericidal fraction associated with casein proteins (84). Bacteriophages are also present in milk and can control the numbers of specific bacteria (82). Daga, Goepfert and Amundson (27) showed that high concentrations of milk solids were inhibitory to the growth of *Salmonellae*. Busta (14) demonstrated that the inhibitory fraction of milk against Bacillus stearothermophilus is associated with casein. Ashton and Busta (3) then showed that the inhibitory factor in the casein fraction, lactotransferrin, exerts its inhibition by binding iron.

C. Bactericidal Properties of Fermented Dairy Products

Fermented milk products have been shown to be bactericidal to several bacteria. Different cheeses are inhibitory to a variety of bacteria (32, 34, 35, 51, 64, 89). Butter (78), sour cream (32, 57), buttermilk (32, 54), yogurt

(30,32,57,65,66) and other dairy starter cultures (24,64,70,75,80,81,82) have also been found to be inhibitory to both pathogenic and spoilage microorganisms. Thus, fermented milk products possess inherent bactericidal properties. Of all these products yogurt has been found to be the most inhibitory (54,63). Fermented milk products have a longer shelf life than natural milk because of their inherent inhibitory properties.

D. Bactericidal Properties of Yogurt

The inhibitory character of yogurt is probably due to the unfavorable environment produced by the yogurt starter culture which consists of two bacteria, Streptococcus thermophilus and Lactobacillus bulgaricus, present in equal proportions (2,43). These are homofermentative bacteria which ferment lactose and produce lactic acid as the metabolic end-product (43). These microbes have been shown to have a mutualistic relationship. L. bulgaricus liberates amino acids subsequently utilized by S. thermophilus, which is unable to hydrolyze casein. S. thermophilus on the other hand, lowers the pH and Eh of the medium to a more optimal condition for the growth of L. bulgaricus. Early in the fermentation, S. thermophilus is the predominant proliferating organism, however, as the fermentation proceeds, L. bulgaricus becomes increasingly predominant. (43)

Certain products produced by the yogurt starter culture have been shown to be bactericidal. Hydrogen

peroxide (23,24,31,68,82) and bulgarican (63,69,72) have been shown to be inhibitory. Volatile fatty acids (9,31,33,39,40,41,43,45,46,47,48,50,55,56,74), especially acetic acid (6,10,76,80) and lactic acid (10,35,76,79) have also been shown to be inhibitory. However, the role these potential inhibitory agents play in yogurt has not been demonstrated (63).

The following paragraphs summarize the state of knowledge concerning the mode of inhibition of bacteria in yogurt. Reddy and Shahani (72) demonstrated that L. bulgaricus produces an antibiotic, bulgarican, which is active against varied Gram Negative and Gram Positive bacteria. The investigations of Hargrove, McDonough, and Mattingly (36), indicated that L. bulgaricus by itself was not detrimental to *Salmonellae*. However, Dahiya and Speck (23), using a cup-plate assay with Staphylococcus aureus as their test organism, found that L. bulgaricus produced inhibitory amounts of hydrogen peroxide. Park and Marth (66) found experimentally that both S. thermophilus and L. bulgaricus were inhibitory separately. When they were both present, inhibition was much greater against S. typhimurium probably due to their symbiotic relationship.

Park and Marth (65) incubated S. typhimurium in milk for 9 hours with S. thermophilus and L. bulgaricus as well as with L. bulgaricus alone. They found that even though the pH was similar there was a greater die-off of Salmonella in the dual lactic acid starter culture. Park and Marth (65) then found that S. typhimurium could grow in artificial

media acidified to pH 4.4 with lactic acid, but that the organism was inactivated in cultured skim milks with similar pH values. From this evidence they concluded that factors other than acid production alone is involved in the inhibition of S. typhimurium in yogurt. This has also been observed in the action of other lactic Streptococci against Salmonellae. It remains to be demonstrated whether any of the other factors in yogurt act in concert with acid to cause a more efficient inhibition of Salmonellae than could be produced by acid alone (66). Thus, the main inhibitor in yogurt as well as the mode of inhibition is not fully understood.

E. Toxicological Inter-relationships

Minor and Marth (56) demonstrated that in a 1:1 mixture of lactic acid and acetic acid added to pasteurized milk no synergism or antagonism was observed between the two acids against S. aureus. Sorrels and Speck (80) found that Leuconostoc citrovorum, a heterofermentative organism, was active in inhibiting S. gallinarum at a pH of 5.4. The main inhibitory factor was found to be acetic acid (0.025 M); while lactic acid (0.024 M) was found to be minimally inhibitory. When a 0.012 M solution of each acid was added together, they found that inhibition was only slightly greater than that for acetic acid alone, which would indicate an additive effect. Sklan, Rosen, Keller and Gordin reported that in 10% skim milk lactic acid and acetic acid had

complementary inhibitory effects on coliforms (79). However, Bohnhoff, Miller and Martin (10) demonstrated that 0.1 M lactic acid would prevent the in vitro inhibition of S. enteritidis by 0.04 M acetic acid or butyric acid in nutrient broth buffered at pH 6.0. Succinic and citric acids were about as effective in preventing acetic acid inhibition as lactic acid, with pyruvic acid being more effective than lactic acid in counteracting acetic acid's inhibitory activity. Glucose did not antagonize fatty acid inhibition but did increase growth of the test microorganism in the control medium. Pyruvic acid was equal in its counter-inhibition either aerobically or anaerobically, while lactic acid was more counter-inhibitory anaerobically. The interactions of the acid toxicants present in yogurt require further studies because of these diverging results.

There is then, a paucity of knowledge concerning the inhibition of bacteria, especially *Salmonellae*, by the yogurt starter culture. It remains to be shown if low pH is the main inhibitor in yogurt or if inhibition is predominantly a toxicant working alone or in conjunction with low pH.

F. The Role of Acid and pH in Bacterial Inhibition

Acid may be the predominant inhibitory agent in yogurt. Goepfert and Hicks (33) demonstrated that the effectiveness of acid inhibition against S. typhimurium diminished as the pH of the medium was increased; by adding

a given concentration of acid (formic, acetic, propionic, butyric) and then adjusting the pH with either hydrochloric acid or potassium hydroxide. They also showed that a) the longer the volatile fatty acid chain, the lower was the bactericidal effect, b) the lower the concentration of acid present, the lower the bactericidal effect at varying pH's, c) the higher the temperature, the more inhibitory the acid became, d) 20% glucose (sucrose, peptone) was partially protective against acid inhibition, and e) the acids' inhibitory abilities decreased as their pK decreased.

Minor and Marth (56) found that to achieve a 99% reduction in growth of Staphylococcus aureus, a final pH of 5.0 was required for acetic acid, 4.6 for lactic acid, 4.5 for citric acid, 4.1 for phosphoric acid and 4.0 for hydrochloric acid. Subramanian and Marth (83) reported that growth of S. typhimurium in broth was not inhibited by hydrochloric acid until there was sufficient acid to obtain a pH of 4.0. Enough acid was added to skim milk gradually over a period of 16 hours to bring the pH down to 4.0, and they found that citric acid was more inhibitory than lactic acid which was more inhibitory than hydrochloric acid. Westhoff and Engler (89) reported that they observed complete inhibition of S. typhimurium in cottage cheese whey in 9 hours; but by neutralizing the whey, inhibition was at least partially reversed. This indicated that the pH might play an important role by augmenting acid inhibition.

Levison (50) also showed that the lower the pH, the

greater the antibacterial activity against Enterobacteriaceae. Antibacterial activity was also greater at higher fatty acid concentrations. At each pH value inhibition was greatest for the fatty acid having the highest pK. Bohnhoff, Miller and Martin (9) found that the inhibitory activity of acetic and butyric acid was conditioned by pH and favored by anaerobiosis in nutrient broth. Hentges (40) found that anaerobiosis increased formic and acetic acid inhibition of Shigella flexneri, in a synthetic medium, and that pH also exerted an increased inhibitory effect. In investigations with synthetic media Hentges (41) reported that the coliform bacteria inhibit Shigella in mixed cultures by producing volatile fatty acids and concomitantly reducing the media. Hentges (39) also showed that Shigella inhibition was not only due to acidity alone, because when the pH was lowered with hydrochloric acid there was little or no reduction in numbers of Shigella. When a given concentration of formic and acetic acid was added to the synthetic medium and the pH then adjusted with hydrochloric acid, there was a corresponding increase in inhibition as the pH was lowered. Meynell (55) also showed that acetic, butyric and propionic acids were more inhibitory against S. typhimurium at low pH and were also more inhibitory at a low Eh, in vitro. Bohnhoff, Miller and Martin (9) have also reported that a low Eh augments acid inhibition. Therefore, each acid becomes more inhibitory as the concentration of the undissociated species increases.

Baskett and Hentges (6) using S. flexneri and Weiner and Draskoczy (88) using E. coli demonstrated that acid inhibition was due to the undissociated molecule. They also showed that acetic acid (6), mandelic acid (88), and hippuric acid (88) inhibited oxidative metabolism by measuring oxygen uptake in cell free extracts. The pK of an acid must be high, or there must be large concentrations of acid in order for the acid to be inhibitory at a high pH. Therefore, the function of low pH in acid inhibition may be mainly to produce undissociated molecules which is the form in which organic acids are transported into the cell.

Baskett and Hentges (6) demonstrated that in cell-free extracts acetic acid inhibition of oxygen uptake occurred at both pH 6.0 and 7.0. From this they concluded that once inside the cell, the degree of dissociation of acetic acid is unimportant in inhibiting oxidative metabolism indicating that both the undissociated and dissociated species of acetic acid are inhibitory. This is in direct conflict with the results obtained by Weiner and Draskoczy (88), who showed that mandelic and hippuric acid inhibition of oxidative metabolism could be directly correlated with the undissociated acid in intact cells, but not in cell-free extracts. In cell-free extracts inhibition of oxidative metabolism was found to be proportionate to the dissociated acid. From this evidence they stated that it is not the undissociated acid that is inhibitory but rather the anion or dissociated molecule. It would appear then that

the inhibitory species of the acid molecule remains to be elucidated.

Samson, Katz and Harris (74) demonstrated that fatty acids inhibit phosphate uptake in a eukaryote, Saccharomyces cerevisiae by interfering with the formation of a phosphate carrier complex. Fenc1 and Leopold (29) studied the mechanism of inhibition of acetic acid on the germination of Aspergillus niger spores. They found that about 20 mM of undissociated acetate was sufficient to reversibly inhibit utilization of the phosphate anions by interfering with its permeability. This inhibited spore germination because phosphate is an essential germination factor. Therefore, acid inhibition in these organisms is due to an interference with the cell's permeability. However, it has also been shown that fatty acids inhibited fermentation of glucose in both intact S. cerevisiae cells and in cell-free extracts (74). Baskett and Hentges (6) showed that acetic acid was able to inhibit glucose metabolism of S. flexneri. Eisler and Heckly (28) demonstrated that long chain fatty acids were able to prevent glucose metabolism by inhibiting 6-phosphogluconate dehydrogenase and to a lesser extent glucose-6-phosphate dehydrogenase in cell-free extracts of Pasteurella pestis. Neal, Weinstock and Lampen (62) showed that the internal pH of Saccharomyces cerevisiae dropped rapidly when exposed to 0.2 M acetic acid at pH 4.0. They demonstrated an almost instantaneous, although reversible, inhibition of respiration and glycolysis. A 0.5 M concentration of acetic

acid was sufficient to irreversibly inhibit several glycolytic enzyme systems. Azukas, Costilow and Sadoff (5) showed that 0.25 mM sorbic acid inhibited enolase, thereby preventing alcoholic fermentation in S. cerevisiae. They stated that sorbic acid inhibition appeared to be quite complex.

Weiner and Draskoczy (88) reported that the inhibitory effect of acid against Escherichia coli was related to the concentration of undissociated acid in medium, the pK of the acid and the intracellular pH. Inhibition of metabolism is thought to be due to the interaction of acids with metabolic enzymes, rather than due to acidification of the internal cellular environment (74). The mechanism of acid inhibition, however, is not understood (6).

G. Summary

In summary, the number of reported cases of salmonellosis is increasing, with milk and milk products being one of the most common vehicles of dissemination. Yogurt has been shown to be the most inhibitory of the fermented milk products which possess bactericidal properties. There are conflicting reports concerning the length of Salmonella's survival in yogurt. Yogurt has been shown to be often contaminated when commercially produced. While lactic acid is the most abundant inhibitory compound in yogurt, acetic acid has been shown to be more highly bactericidal. Reports on the inhibitory inter-relationship between these two acids are

conflicting, with some researchers claiming that an additive effect is present while another researcher claims that there is an antagonistic effect. Acids have been shown to be most inhibitory at their pK because the undissociated molecule is the form in which organic acids are transported into the cell. Once inside the cell it is not certain how acid inhibition occurs.

H. Statement of the Problem

The emphasis of this study was on elucidating the factor(s) in yogurt responsible for inhibiting the most prevalent of the milk-borne pathogens, Salmonella typhimurium. This study was prompted for several reasons. First, the literature (30,66) has demonstrated that the die-off rate of S. typhimurium in yogurt is variable. There are no adequate explanations for these observed differences (63). Secondly, yogurt is not produced commercially with adequate sanitary conditions (2). Also, there is an increase in the practice of non-commercial production of yogurt in homes (49,53). Therefore, an understanding of the inhibitory abilities of yogurt should both help to explain the discrepancy in the die-off rates of potential pathogens and to help establish the safety of yogurt for consumer health.

Factors (pH, Oxidation-Reduction Potential, lactic acid concentration, and acetic acid concentration) potentially responsible for the death of S. typhimurium in yogurt were studied. The inhibitory effect that each of these

conditions exerted against S. typhimurium was investigated. Also, the interactions of these conditions in the inhibition of Salmonella was investigated.

The main inhibitor of S. typhimurium in yogurt, lactic acid, was studied in order to determine its bactericidal properties. Additionally, the role that the undissociated and dissociated species of lactic acid played in the inhibition of Salmonella was determined.

MATERIALS AND METHODS

A. Organisms

Salmonella typhimurium strain sal was obtained from the culture collection of the Microbiology Department, The University of Michigan, Ann Arbor, Michigan. Enteropathogenic strains of S. typhimurium (B-25, b-30, B-42, B-64, and B-114) were obtained from Dr. Gene Britt, St. Joseph Mercy Hospital, Ann Arbor, Michigan. All strains of S. typhimurium were maintained by weekly transfer on Trypticase Soy Agar slants incubated overnight at 35C. Also, all strains were lyophilized and new cultures were used monthly to minimize genetic mutation.

Streptococcus thermophilus and Lactobacillus bulgaricus were isolated from a commercial yogurt and identification was accomplished by the following tests: 1) the organisms were Gram Positive, 2) grew at 50C but not at 20C, 3) 1.0% lactic acid was produced in milk by S. thermophilus and 1.4% was produced by L. bulgaricus, 4) the final pH was 4.25 for S. thermophilus and 3.8 for L. bulgaricus, 5) both organisms were catalase negative, 6) acid was produced from glucose, lactose, sucrose (L. bulgaricus did not ferment sucrose), and galactose (S. thermophilus did not ferment galactose),

7) gelatin was not liquified, and 8) no growth was observed on mannitol or maltose. S. thermophilus and L. bulgaricus were maintained by daily transfer in 10% reconstituted non-fat dry milk and overnight incubation in a water bath at 42 C. (13,16)

B. Media

1. Phosphate buffer

Phosphate buffer stock was prepared by adding 34.0 g of KH_2PO_4 to 500 ml of distilled water. The pH was adjusted to 7.2 with 1 N NaOH and then the solution was diluted to 1 liter with distilled water. To dilute for use, 1.25 ml of phosphate buffer stock was added to 1 liter of distilled water. (1)

2. Salts-glucose medium

Salts-glucose medium contained the following (73):

Na_2HPO_4	11.97 g
KH_2PO_4	4.0 g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2 g
NaCl	2.0 g
NH_4Cl	2.0 g
water	to 1 liter

The medium was made at double strength (2X salts) for convenience. The salts were autoclaved prior to the addition of 1.0% sterile glucose.

3. Brilliant Green Agar

A commercial brand (Difco) of Brilliant Green Agar

was used in these studies.

4. Galactose agar

Galactose agar consisted of the following:

tryptone	10.0 g
yeast extract	10.0 g
galactose	10.0 g
KH_2PO_4	0.5 g
Na_2HPO_4	0.5 g
Bromcresol purple (BCP)	0.02 g
agar	18.0 g
water	to 1 liter

The pH was adjusted to 7.0 before autoclaving. The BCP was maintained separately as a stock solution and added just prior to use.

5. Lee's agar

Lee's agar (49) was prepared as follows:

tryptone	1.0%
yeast extract	1.0%
lactose	0.5%
sucrose	0.5%
CaCO_3	0.3%
K_2HPO_4	0.05%
BCP	0.002%
agar	1.8%

The pH of the medium was adjusted to 7.0 ± 0.1 before autoclaving. BCP was added to the medium just prior

to plating. To insure uniform distribution of CaCO_3 in the agar, the medium was thoroughly mixed and then poured into pre-cooled petri plates. Gentle mixing prevented excessive incorporation of air in the medium.

6. Synthetic milk medium

The synthetic milk medium (SMM) was prepared as follows:

Salt Stock (87)

<u>A</u>		<u>B</u>	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	6.15 g	$\text{AlK}(\text{SO}_4) \cdot 12\text{H}_2\text{O}$	9.0184 g
NaCl	1.0 g	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.178 g
KH_2PO_4	2.0 g	$\text{Na}_2\text{MO}_4 \cdot 2\text{H}_2\text{O}$	0.0117 g
NH_4Cl	12.0 g	water	to 100 ml
$\text{NaH}_2\text{PO}_4 \cdot 7\text{H}_2\text{O}$	2.0 g		
water	to 100 ml		
<u>C</u>			
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	0.0183 g		
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	1.6 g		
water	to 100 ml		

Carbohydrate Stock (87)

<u>A</u>		<u>B</u>	
glucose	7 g	lactose	48 g
galactose	2 g	water	to 100 ml
water	to 100 ml		

Vitamin Stock (87)

<u>Water Soluble</u>		<u>Fat Soluble</u>	
ascorbic acid	1.6 g	carotene	0.034 g
biotin	0.0035 g	calciferol	1.0 g
choline	13.0 g	tocopherol	0.06 g
folic acid	0.00023 g	quinone	5.0 g
inositol	13.0 g	glycerol	to 100 ml
nicotinic acid	0.085 g		
pantothenic acid	0.350 g		
pyridoxine	0.048 g		
riboflavin	0.157 g		
thiamine	0.042 g		
B ₁₂	0.00056 g		
citric acid	16.0 g		
water	to 1 liter		

Fatty Acid Stock (87)

oleic	11.13 ml
palmitic	7.29 g
myristic	3.73 g
stearic	3.59 g
butyric	0.95 ml
palmitoleic	0.86 ml
lauric	0.68 g
capric	0.49 g
linolelaidic	0.32 ml
arachidic	0.30 g

Fatty Acid Stock (continued)

caprylic	0.27 ml
triton X 100	10.0 ml
water	to 1 liter

Amino Acid Stock (87)

glycine	8.0 g	threonine	15.0 g
alanine	13.0 g	cystine	8.0 g
valine	26.5 g	methionine	5.0 g
leucine	20.0 g	arginine	12.0 g
isoleucine	18.0 g	histidine	10.0 g
proline	23.0 g	lysine	21.0 g
phenylalanine	17.0 g	aspartate	16.0 g
tyrosine	3.5 g	glutamate	16.0 g
tryptophan	8.0 g	water	to 1 liter
serine	5.0 g		

The water soluble vitamins were sterilized by filtration through a filter of 0.45 μ pore size. The other stock solutions were sterilized by autoclaving at 121 C for 15 min. The fat soluble vitamins were heated and then diluted to 500 ml in ethanol. Both vitamin stocks were stored in the dark at 4 C. The fatty acid stock was homogenized before sterilizing and was then stored at a minimum temperature of 35 C with continuous mixing.

The Synthetic Milk Medium was made double strength (2X), for convenience, by adding the following amounts of the stock solutions:

2X SMM

Salts A	0.4 ml
Salts B	0.4 ml
Salts C	0.4 ml
Carbohydrate A	0.4 ml
Carbohydrate B	4.0 ml
Amino acids	4.0 ml
Fatty acids	4.0 ml
Fat soluble vitamins	2.0 ml
Water soluble vitamins	0.4 ml
Triton X 100	4.2 ml
Concentrated phosphate buffer	20.0 ml
Water	to 100 ml

The pH was adjusted to 6.5 and then sterilized by heating at 121 C for 15 min. and then cooled in ice before adding the water soluble vitamin stock. SMM could be made into artificial SMM yogurt by duplicating the pH, Eh, lactic acid and acetic acid concentrations found in 18 hr yogurt (Section E).

C. Preparation of Inocula

L. bulgaricus and S. thermophilus were maintained as described. The cultures were transferred serially ~~seven times~~ before the cultures were considered stable enough in their acid production to get reproducible results. These cultures were then used as the starter culture to produce yogurt. (63)

S. typhimurium was grown statically on salts-glucose medium for 18 hr at 37 C. The cells were harvested and washed twice with sterile phosphate buffer (1). The washed cells were resuspended in phosphate buffer to a turbidity which when added to the test medium, would give the initial concentration of cells desired (2.5×10^3 cells/ml).

D. Preparation of Yogurt

Sixteen ounce screw-cap bottles were filled with 250 ml of 10% reconstituted non-fat dry milk and autoclaved at 121 C for 15 min. The milk was tempered at 42 C and then inoculated with 6.25 ml (2.0×10^7 cells/ml) each of 18 hr active cultures of L. bulgaricus and S. thermophilus. The inoculated milk was then incubated statically for 18 hr at 42 C. S. typhimurium was added either simultaneously with the yogurt starter culture or at the end of 18 hr incubation.

E. Enumeration of the Microorganisms

Galactose agar and Lee's agar spread plates were incubated at 42 C for 24-48 hr in anaerobic candle jars before colonies were counted. L. bulgaricus colonies were enumerated directly on galactose agar spread plates, as can be seen in Table 1 which shows the ability of various microorganisms to grow on the media used. S. thermophilus colonies were counted directly since these two organisms could be differentiated on Lee's agar (49). As a check, the

Table 1
Growth of Various Organisms on Different Media

	<u>S. thermophilus</u>	<u>L. bulgaricus</u>	<u>S. typhimurium</u>
Lee's Agar	a+	+	+
Galactose Agar	b-	+	+
Brilliant Green Agar	-	-	+

a growth

b no growth

number of colonies grown on galactose agar (L. bulgaricus) was equal to the number of colonies of S. thermophilus.

Colonies of S. typhimurium were enumerated on Brilliant Green Agar spread plates at 37 C for 24-48 hr. The initial dilution blank was adjusted with sodium hydroxide so that the pH was approximately 7.0 after addition of the sample. This gave a ten-fold greater recovery than was obtained without adjustment of the pH in the initial dilution blank. (65) Salmonella could grow in both Lee's agar and galactose agar. However, S. typhimurium was not in sufficient concentration to be isolated from the high dilutions required to enumerate S. thermophilus or L. bulgaricus. In order to compare the obtained results with the literature, enumeration was accomplished by the number of organisms per weight for fermenting yogurt and per volume for fermented (18 hr) yogurt. All counts were reported as colony forming units (CFU) for accuracy.

F. Determination of Inhibitory Concentrations of Lactic and Acetic Acid

In order to determine the concentrations of lactic and acetic acid that are inhibitory under controlled conditions, determinations were performed by incubating the organisms statically in salts-glucose medium at 37 C for 8 hr. This was found to be in the late logarithmic phase of growth when no acid was present. S. typhimurium was prepared as described in Section C, and the washed cell pellet

was resuspended to a turbidity of 0.4 at 540 nm using a Bausch and Lomb Spectrophotometer 20. One-tenth ml of this suspension was used to inoculate 10 ml of medium. One ml of phosphate buffer stock (1) was added to the medium to control the pH at 7.15. The medium was equilibrated with O_2 -free nitrogen when the effects of anaerobiosis on lactic and acetic acid inhibition of S. typhimurium were studied.

G. Acid Interaction Model

A model to determine the amount of potentiation between toxicants was modified so it could be used to determine the inter-inhibitory relationship between lactic and acetic acid against Salmonella (22). This is based on two assumptions that define the model. First, the slopes of the dose-response curves for both acids are equal. Secondly, the toxicities of both acids are additive. In each assay the amount of the two acids together was sufficient to give 50% inhibition of the growth rate of S. typhimurium if they were additive. At either end of the spectrum, only one acid was present, and at the median they were in equal inhibitory concentrations. The two acids would be synergistic in their effect if there is an increase in the amount of inhibition and antagonistic if there is a decrease in the amount of inhibition of the growth rate. If the acids are either synergistic or antagonistic, the greater the divergence from the 50% inhibition baseline the closer toward the center of the spectrum a point migrated. Each point on the

spectrum should give a similar response (50% inhibition) if the acids are additive in their effect. The inter-inhibitory possibilities, addition, synergism, and antagonism are illustrated graphically in Figure 1.

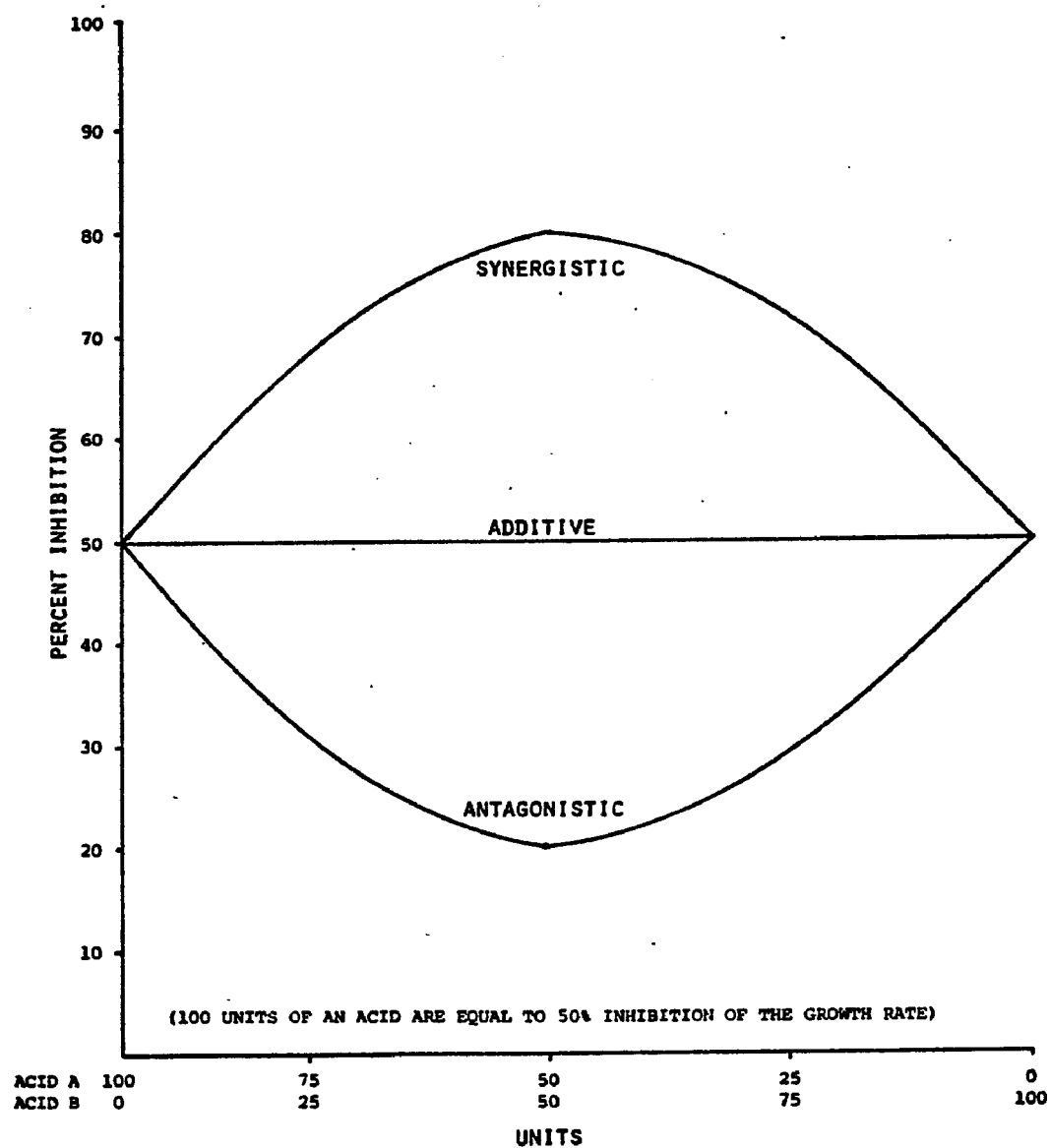
The amount of lactic and acetic acid that gave 50% inhibition of the growth rate of S. typhimurium was determined in the synthetic milk medium. Then, varying concentrations of the acids were combined so that the total concentration of both acids would result in 50% inhibition if their inhibitory effects were additive. The growth rate of the microorganism was followed turbidimetrically for 12 hr at 37C. Inhibition of the growth rate for each acid combination was determined by dividing the growth rate of each assay by the growth rate of S. typhimurium in the absence of acid. One hundred units of an acid was chosen as the amount needed to elicit 50% inhibition of the growth rate of the test organism.

H. Determination of Intracellular Conditions

S. typhimurium was grown in two liters of salts-glucose medium containing each possible combination of the following pH's and lactic acid concentrations: pH 7.0, 6.5, 6.0, and 5.5; lactic acid 1.0%, 0.75%, 0.50% and 0.25%. Resulting cell growth was followed by viable counting on Brilliant Green Agar spread plates. In order to determine the generation time, the following equation was used:

$$G_t = \frac{T}{3.3 \log \frac{B}{b}} \quad (a)$$

Figure 1. Toxicological Model for a Two-Acid System



Where T is the length of time; B is the number of cells at T hr; and b is the initial number of cells.

The cultures were grown until an absorbancy of 0.7 at 540 nm (9.2×10^8 cells/ml) was obtained. The cultures were then split into two aliquots and washed twice with phosphate buffer. One portion was used for the calculation of the internal pH, and the other for the determination of the internal lactic acid concentration.

I. Intracellular pH

Intracellular pH was determined spectrophotometrically by measuring the amount of 2,4-dinitrophenol (DNP) concentrated intracellularly at a particular external pH (44). This is an indirect measure of intracellular pH. It was used in this investigation because there are no direct methods currently available with which to measure intracellular pH.

DNP was added to salts-glucose medium similar to the medium in which the culture was grown. Incubation lasted for 5 min at 37 C before DNP determinations were conducted. This method relied on the use of the Henderson-Hasselbalch equation (b) to compute the concentration of the undissociated (HA) and dissociated (A^-) moieties of DNP.

$$pH - pK_{DNP} = \log \frac{(A^-)}{(HA)} \quad (b)$$

Where pH is the pH of the medium and pK_{DNP} is the pK of DNP (4.1).

Using equation b, the ratio of the dissociated to the

undissociated species of DNP in the medium at the end of the incubation period was calculated. Knowing the ratio of the dissociated to undissociated species of DNP in the medium and the total concentration of DNP in the medium, then it is possible to determine the concentration of each species of DNP. Since the undissociated species within the cell is assumed to be equal to the undissociated species outside the cell at equilibrium, by using equation c, the concentration of the dissociated species of DNP within the cells can be assessed.

$$(A^-)_i = (DNP)_{\text{initial}} - 2(HA)_o - (A^-)_o \quad (c)$$

Where $(A^-)_i$ is equal to the concentration of dissociated DNP within the cells, $(DNP)_{\text{initial}}$ equals the total concentration of DNP at the beginning of incubation, $(HA)_o$ is the undissociated DNP concentration outside of the cells at the end of incubation, and $(A^-)_o$ represents the concentration of the dissociated species of DNP outside of the cells at the end of incubation.

The following equation can be used to determine the intracellular pH (pH_i) of the cells since $(A^-)_o$ and $(A^-)_i$ can be computed.

$$pH_i = pH_o + \log \frac{(A^-)_i}{(A^-)_o} \quad (d)$$

Where pH_o is equal to the pH of the medium.

Equation d is derived by subtracting the Henderson-Hasselbalch equation for the calculation of the external

species of DNP at equilibrium from the equation for the calculation of the internal species of DNP. After rearrangement, this yields equation d as presented above.

J. Intracellular Lactic Acid

The washed cell pellet was added to salts-glucose medium at a pH and lactic acid concentration identical to that in which it was grown. This suspension was incubated for 1 hr at 37 C and then 2 ml was removed to determine the dry weight. Lactic acid was determined by oxidation to glyceraldehyde, which reacted with p-phenylphenol and resulted in a blue color which could be measured spectrophotometrically (25). Samples of the medium were removed before addition of the cell pellet and after incubation. The amount of lactic acid taken up by the cell mass was determined by the difference in the medium before and after incubation.

The cell dry weight was determined by filtering 2 ml of the suspension through a pre-weighed 0.45 μ filter, then evaporating at 105 C for at least 6 hr. The lactic acid concentration could then be calculated by the following formula:

$$\mu\text{g lactic acid/mg cell dry wgt} = \frac{2(\mu\text{g lactic acid/ml})}{\text{mg cell dry wgt}} \quad (e)$$

RESULTS

A. Determination of Yogurt's Inhibitory Conditions

The pH of yogurt was measured with a Beckman pH meter equipped with a glass combination electrode. The pH of 18 hr yogurt was found to be 3.85. The oxidation-reduction potential (ORP) of 18 hr yogurt, -80 mV, was determined with the use of a platinum electrode and a calomel reference electrode. The ORP was adjusted for the low pH of yogurt with the use of a standard curve. The standard curve was constructed by plotting pH versus ORP of milk. The reference electrode was calibrated with the use of a solution containing equal amounts of 0.1 M FeCl_2 and 0.1 M FeCl_3 . ORP was then adjusted in order to relate the obtained value to the hydrogen electron by adding +248 mV. (86)

Lactic acid was measured spectrophotometrically using a Guilford Model 240 Spectrophotometer by the method of Davidson (25) which was developed to determine the lactic acid concentration in milk and milk products. A lactic acid concentration of 1.42% was found in 18 hr yogurt.

An acetic acid concentration of 0.022% was determined in 18 hr yogurt by gas-liquid chromatography using a Packard Model 7401 Gas Chromatograph. Samples were acidified with 3% metaphosphoric acid and then centrifuged before analysis by flame ionization. The injection volume was 3 μl .

Nitrogen served as the carrier gas at a flow rate of 30 ml/min. The hydrogen flow rate was maintained at 30 ml/min and the oxygen rate by 400 ml/min. The inlet and detector temperatures were 120 C, while the column temperature was maintained at 110 C. The volatile fatty acid column consisted of 1.4 g FFAP and 10 g Chromabsorb W (60/80 mesh) conditioned overnight at 180 C. These results fall within the range expected for fermented yogurt (16). A pH of 3.85, ORP of -80 mV, lactic acid concentration of 1.42%, and acetic acid concentration of 0.022% when added to a medium was considered to be representative of the inhibitory yogurt conditions and was, therefore, called artificial yogurt.

B. Comparison of a Known Strain of *S. typhimurium* with Several Pathogenic Strains

The die-off rate ($-\text{minute}^{-1}$) of *S. typhimurium* strain Sal in 18 hr yogurt was compared with the die-off rates of 5 different pathogenic strains isolated from the feces of infected patients. The die-off rates of the 5 enteropathogenic strains in Table 2 were found not to be different at the 0.05 level of significance ($P > 0.47$) using a one sample student t test. This indicates that in 18 hr yogurt strain Sal is indeed a typical representative of *S. typhimurium*.

C. Die-off of *S. typhimurium* in Fermenting Yogurt

The relationship between the growth in milk of *S. typhimurium* and the yogurt starter culture, *S. thermophilus*

Table 2

The Die-off Rates of Different Strains of
Salmonella typhimurium in 18 Hour Yogurt at 37C

Strain	^a Die-off Rate
Sal.	-.100
B-25	-.109
B-42	-.110
B-64	-.099
B-30	-.105
B-114	-.091

^a -Minute⁻¹

and L. bulgaricus, was studied. In Figure 2-A, the growth of S. typhimurium in milk and in fermenting yogurt, i.e., milk undergoing fermentation by the yogurt starter culture, are compared. S. typhimurium was observed to grow to a final concentration of approximately 8.0×10^8 cells/ml by the end of 12 hr of incubation in milk. In contrast, in fermenting yogurt the initial concentration of Salmonella remained constant for the first 4 hr after which a logarithmic decline of numbers was observed. This suggests that initially the conditions produced by the yogurt starter culture are bacteriostatic and then become bactericidal. Figure 2-B follows the pH and lactic acid concentration in fermenting milk. At the end of 4 hr of incubation, when the starter culture became bactericidal, there was a concentration of .034% lactic acid and a pH of 4.54.

D. The Effect of Lactic and Acetic Acid on the Growth of S. typhimurium

S. typhimurium's growth in varying concentrations of lactic and acetic acid was followed in order to determine the effect of these two acids on the microorganism. Figure 3 shows the growth of S. typhimurium, measured spectrophotometrically, in the presence of varying concentrations of lactic acid in salts-glucose medium. A plateau of 0.95 OD was observed in salts-glucose medium without lactic acid. A concentration of 0.0125% lactic acid had a slight stimulatory effect on the final concentration of Salmonella (0.995 OD)

Figure 2B. The pH and Lactic Acid Concentration of Fermenting Yogurt

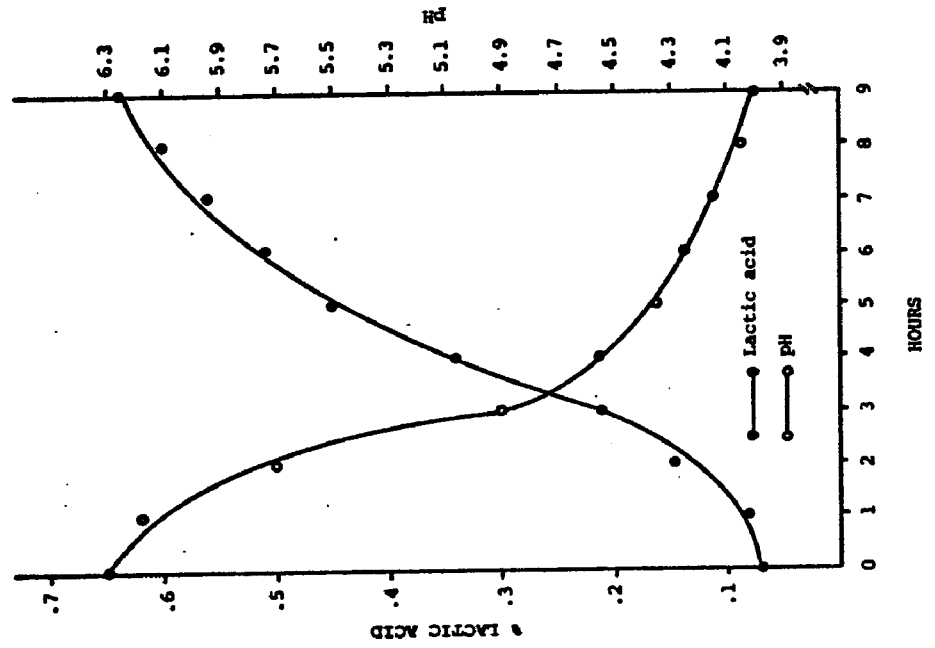


Figure 2A. Growth of *S. typhimurium* in Fermenting Yogurt

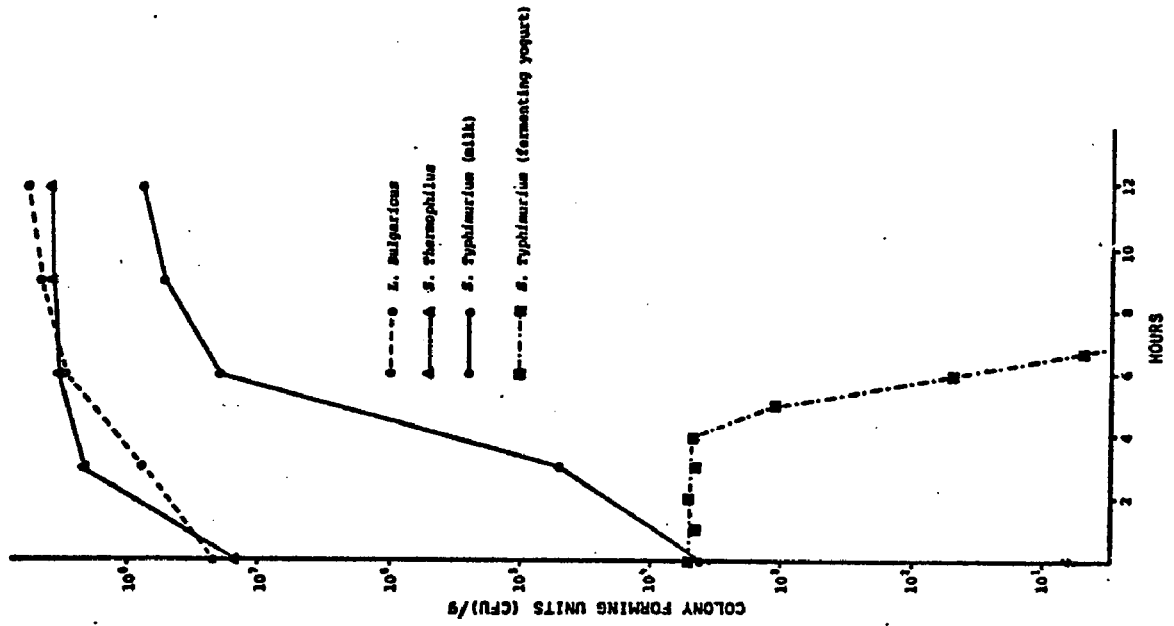
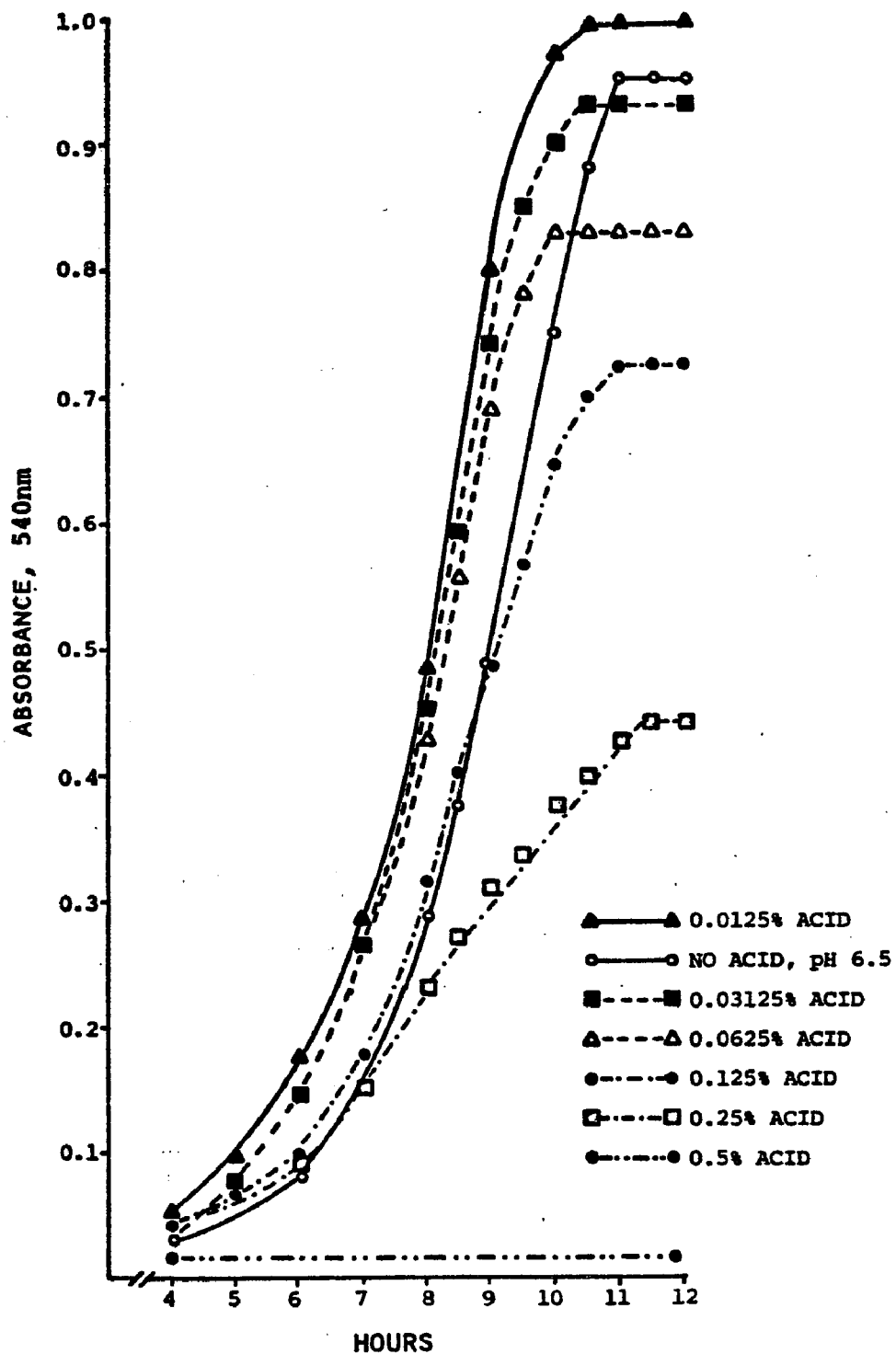


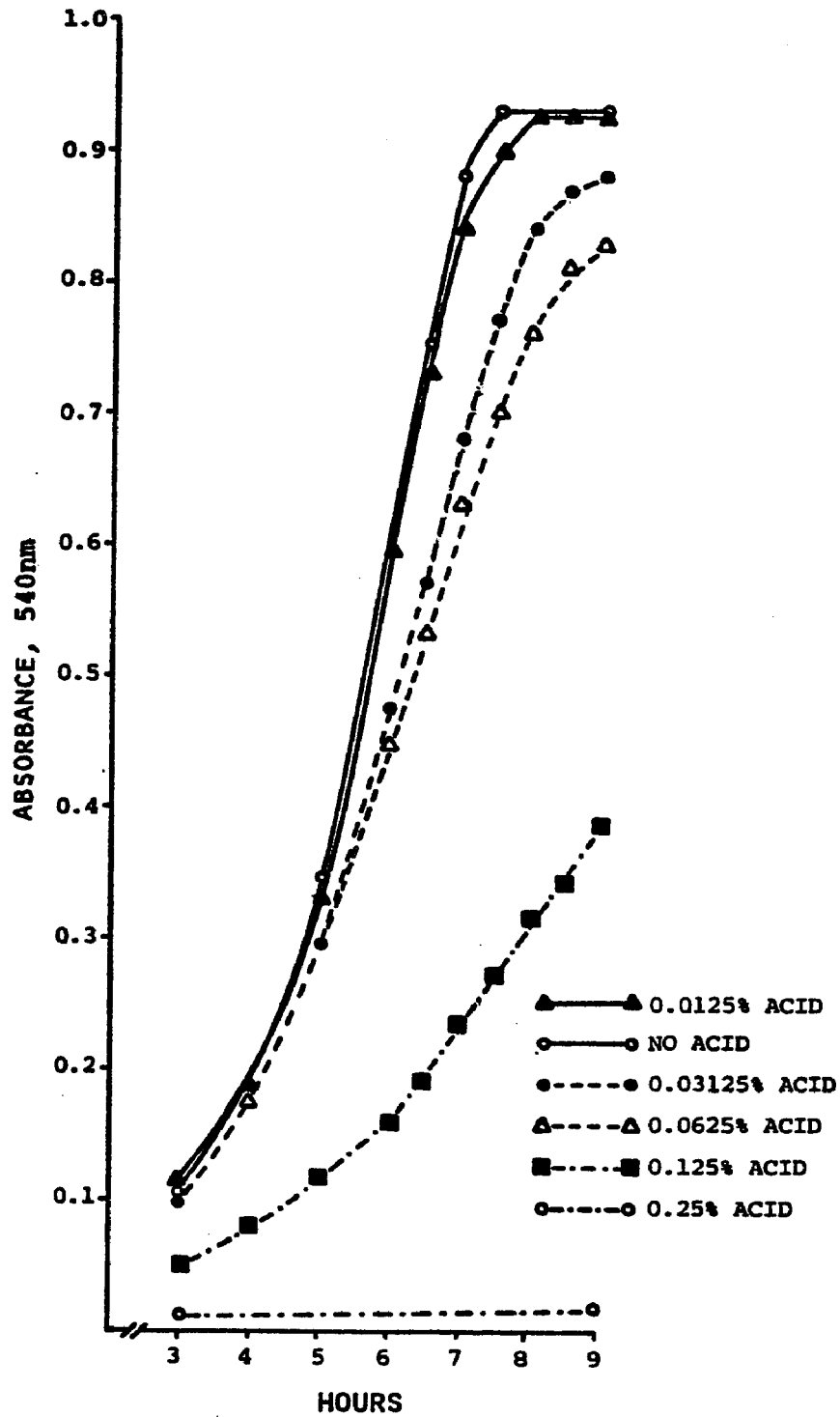
Figure 3. Growth of *S. typhimurium* at Different Concentrations of Lactic Acid



when its growth was compared with the growth of the micro-organism in the medium without lactic acid. A slight decrease in final numbers was observed at a 0.03125% lactic acid concentration. A 0.0625% concentration resulted in a final turbidity of 0.825 OD which represented an actual decrease in the bacterial population even though the growth rate remained unchanged. When the concentration was increased to 0.125% the OD was reduced to 0.725. This was found to give a significant reduction in both final numbers of Salmonella and the growth rate at this acid concentration. When 0.25% lactic acid was added to the medium the absorbancy was measured at 0.44. This was about half the turbidity observed when no acid was added to the medium. At concentrations of 0.5% or greater there was complete inhibition of growth.

The results of exposing Salmonella to various concentrations of acetic acid in salts-glucose medium is shown in Figure 4. A maximum absorbancy of 0.935 was obtained when no acid was added to the salts-glucose medium. A concentration of 0.01563% acetic acid added to the medium gave virtually the same result as when no acid was present. The growth rate and final concentration of Salmonella was decreased when 0.03125% acetic acid was present. This decrease in growth rate and numbers was greater when the acetic acid concentration was increased to 0.0625%. An OD of 0.385 was observed at the end of 9 hr of incubation when the salts-glucose medium contained 0.125% acetic acid, which is about 40% of the final OD when no acid was present in the medium. No

Figure 4. Growth of *S. typhimurium* at Different Concentrations of Acetic Acid



growth of S. typhimurium was observed at acetic acid concentrations of 0.5% or greater. Thus, in salts-glucose medium, acetic acid was found to be about twice as inhibitory towards Salmonella as was lactic acid (by weight). Optical density was used as an indication of cell numbers when a standard curve was produced correlating OD with viable cell numbers.

Determination of the inhibitory concentrations of the 2 acids against S. typhimurium were performed under the following established conditions: 1) initial aerobic growth with no pH control; 2) initial anaerobic growth with no pH control; 3) initial aerobic growth at a constant pH of 7.15; and 4) initial anaerobic growth at a constant pH of 7.15 (Materials and Methods, Section F). In Figure 5, the change in OD of suspensions of S. typhimurium incubated in various concentrations of lactic acid under these conditions can be seen. Lactic acid, at a constant pH of 7.15, was only slightly inhibitory at all concentrations tested. A greater amount of inhibition of S. typhimurium was observed when the pH was held constant under anaerobic conditions. Inhibition of growth increased with a concurrent increase in the concentration of lactic acid when the pH was not held constant. This rate of inhibition was again greater when the medium was reduced.

The change in OD of suspensions of Salmonella in various concentrations of acetic acid can be seen in Figure 6. The least amount of inhibition was obtained when the pH was maintained at 7.15 under aerobic conditions. The

Figure 5. Lactic Acid Inhibition of *S. typhimurium*

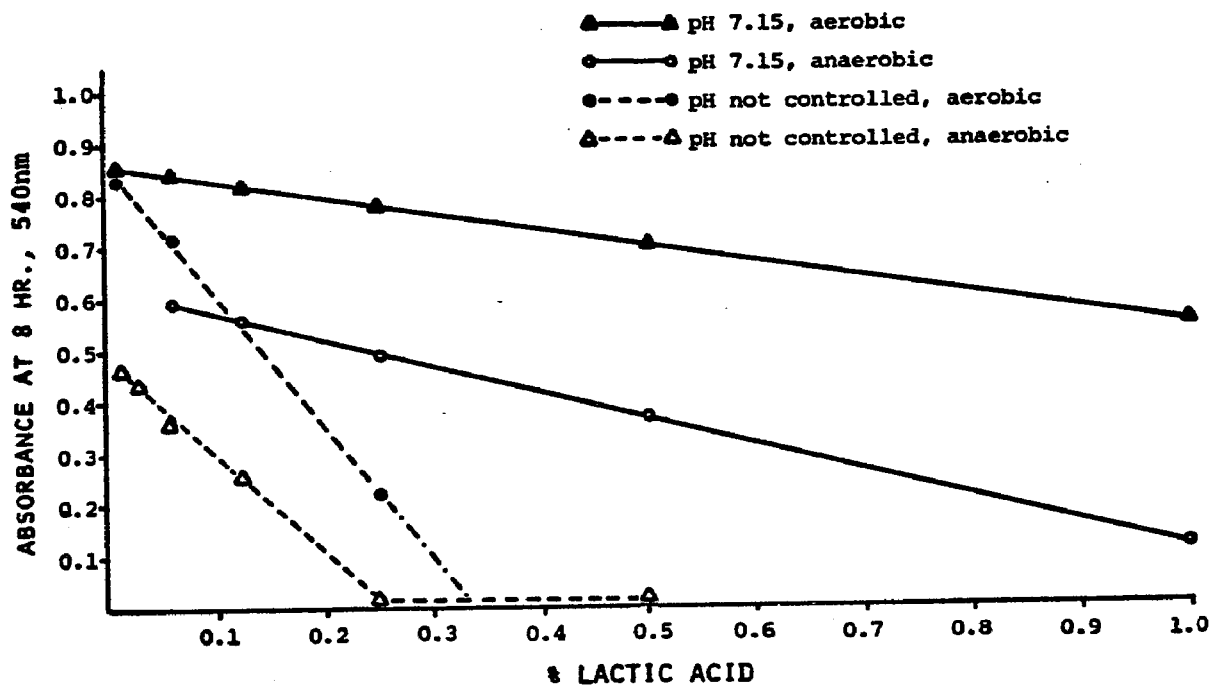
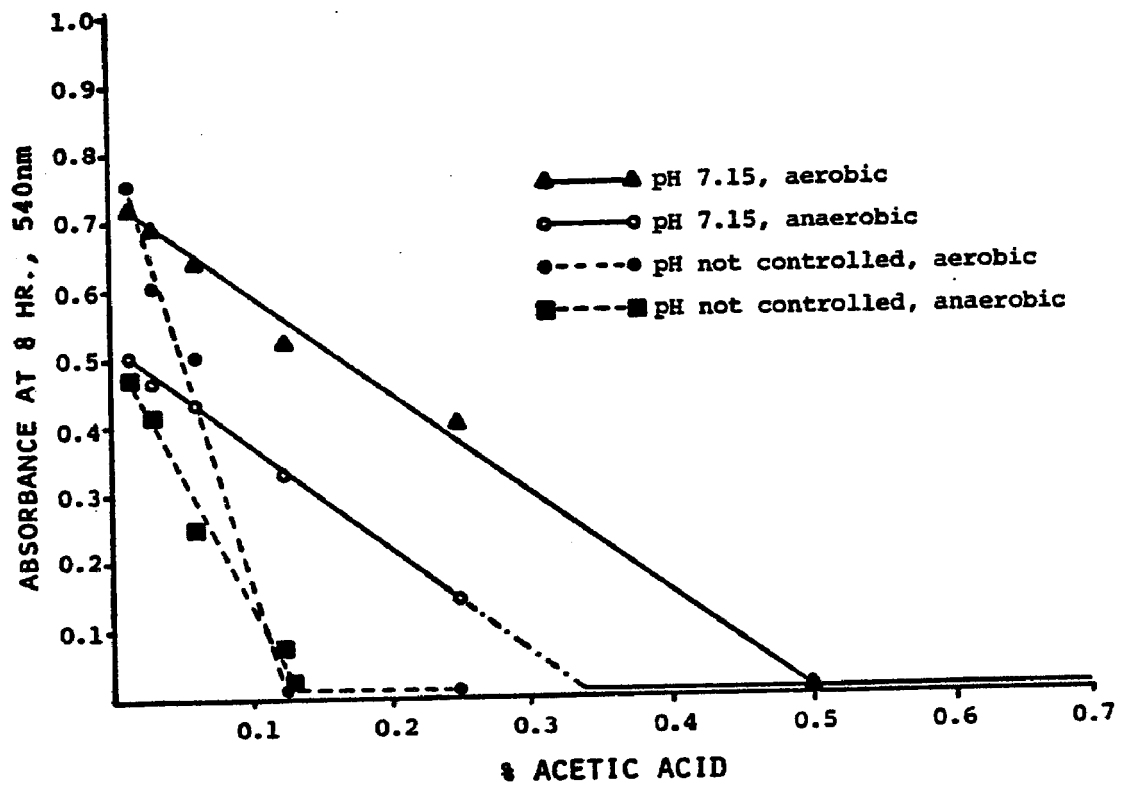


Figure 6. Acetic Acid Inhibition of *S. typhimurium*



inhibition was greater when the pH was held at 7.15 under anaerobic conditions. There was a significant amount of inhibition when the pH was allowed to drop. However, the greatest amount of inhibition was observed when the pH was not held constant under anaerobic conditions.

These results for lactic and acetic acid are summarized in Table 3 in which the concentrations of the acids that are not inhibitory, 50% of inhibitory, or completely inhibitory are listed. When comparing the rates of inhibition under all conditions for lactic and acetic acid, it can be observed that acetic acid is inhibitory to a greater degree than lactic acid. In summary, the rate of lactic and acetic acid inhibition was more rapid when the pH was allowed to decline than when it was held constant, indicating that the acids' inhibitory effect is pH related. Anaerobiosis stimulated the rate of inhibition for both acids whether the pH was held constant or was allowed to decline.

E. Synthetic Milk Medium

Milk and yogurt represent empirical media which cannot be easily manipulated by the addition or subtraction of their constituent components without drastically altering the normal environment. Therefore, a completely defined synthetic milk medium (SMM) was developed. This medium contained the relative percentages of the 60 most common compounds found in skim milk (87), i.e., vitamins, carbohydrates, minerals, amino acids (rather than proteins), and

Table 3
Comparison of the Amounts of Acetic and Lactic Acid
Needed to Inhibit Salmonella

	^a Per Cent of Acid Needed to Inhibit Growth of <u>Salmonella</u>						
	Acetic Acid				Lactic Acid		
	100% Growth	50% Growth	No Growth		100% Growth	50% Growth	No Growth
No Control	.04	.085	.12		.044	.19	.32
ORP Controlled	.01	.06	.135		.01	.12	.22
pH Controlled	.01	.255	.50		.01	1.31	2.0
pH + ORP Controlled	.01	.175	.32		.06	.635	1.15

^aSalts-glucose medium

fatty acids (rather than fats). The characteristics of Salmonella in skim milk and SMM were compared in Table 4. It can be seen that the die-off rate, the amount of acidity produced, and the drop in pH is the same for both media. There was a difference found in the growth rate of S. typhimurium in these two media, even though the final numbers were similar. The growth rate of the microorganism in SMM was 67% of the growth rate found in skim milk. It might be expected that growth rates cannot be duplicated exactly when a synthetic medium is compared with a highly complex natural system.

F. Die-off of S. typhimurium in Artificial Yogurt

In Figure 7 the number of Salmonella surviving after incubation periods in natural yogurt fermented for 18 hr was compared with that in artificial milk yogurt and in artificial SMM yogurt. S. typhimurium was found to have a similar die-off rate in natural yogurt and in artificial milk yogurt. However, in artificial SMM yogurt, S. typhimurium had a greater die-off rate than it did in the yogurts made from skim milk. These results indicate the presence of a factor(s) in skim milk, not present in SMM, that may exert a protective effect toward Salmonella in yogurt. In Figure 7 it can also be seen that bacterial numbers did not notably change after 4 hr of incubation in all 3 yogurts when the pH's were adjusted to 6.5. This suggests that the inhibitory substance(s) in yogurt is dependent on low pH to be

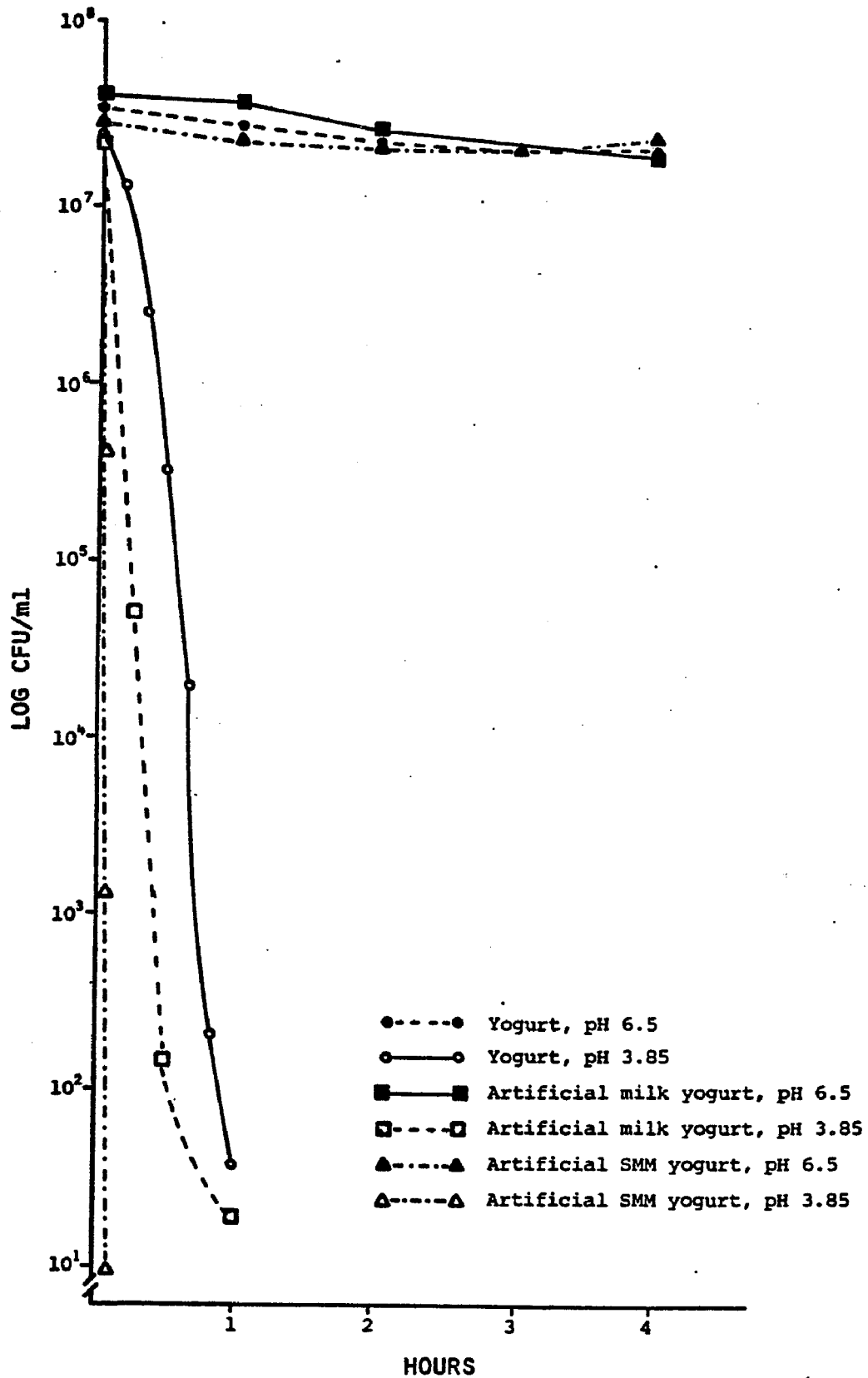
Table 4

Comparison of Skim Milk and Synthetic Milk Medium

Condition	Milk	Synthetic Milk Medium
^a Number of Cells After 15 Hours of Growth	5 x 10 ⁸ cells/ml	3.1 x 10 ⁸ cells/ml
^b Growth Rate	^c 1.41	^c 2.09
^a Drop in pH due to Sal Growth	0.25 units	0.15 units
^a Acidity Produced by Sal	0.2% acid	0.2% acid
^a Die-off Rate	^d 18.87	^d 18.52

^aSalmonella typhimurium^bArtificial yogurt conditions at pH 6.5^cHour⁻¹^d-Hour⁻¹

Figure 7. Comparison of the Inhibitory Ability of Artificial Yogurts With Natural Yogurt

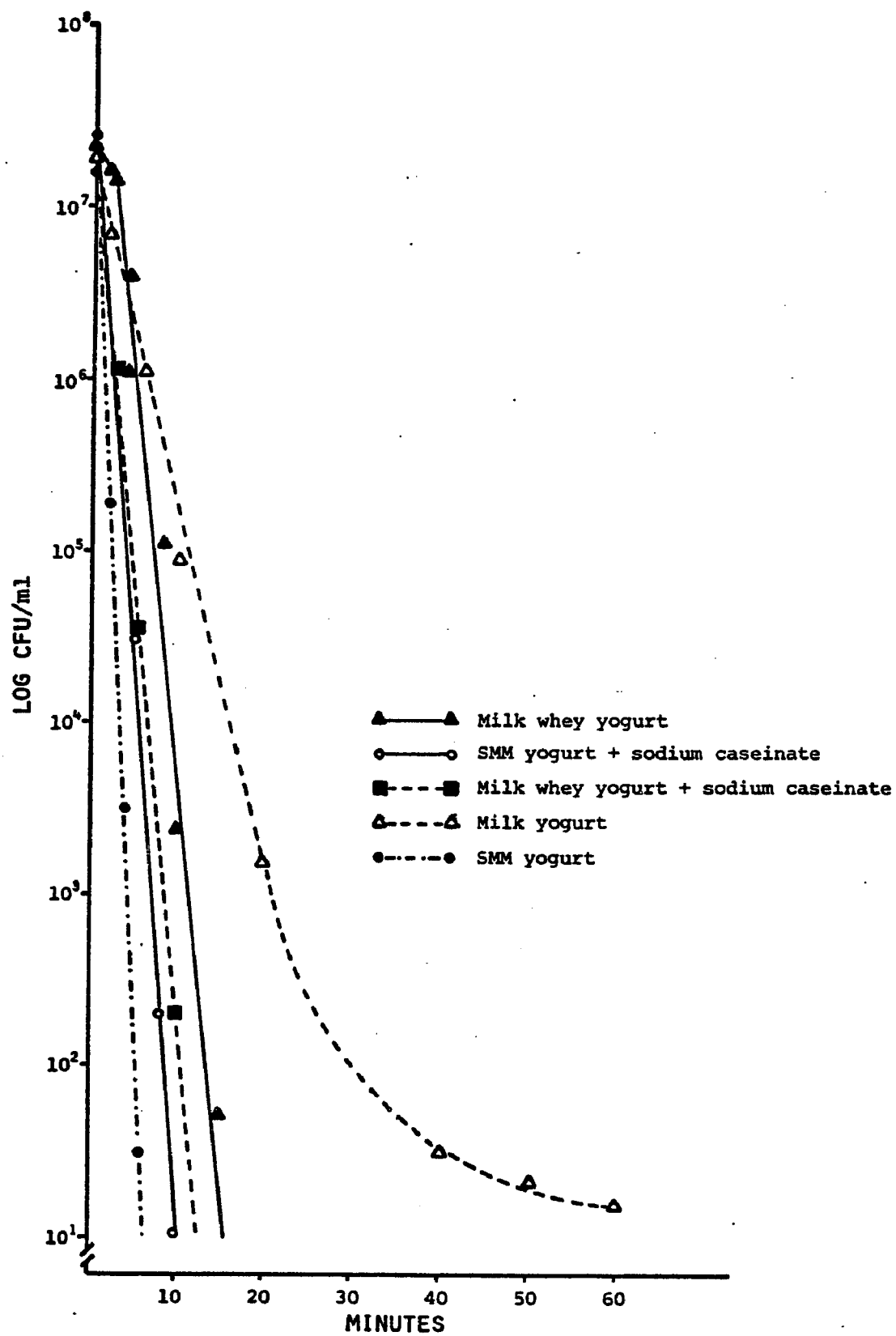


bactericidal but is in sufficient quantity, even at high pH, to be bacteriostatic.

An attempt was made to elucidate the protective factor(s) found in artificial milk yogurt and natural yogurt. The casein fraction of milk was precipitated. The whey was then converted into artificial whey yogurt. In Figure 8 the survival of S. typhimurium in the different artificial yogurts was compared. In artificial whey yogurt, with no casein present, the die-off rate was found to be similar to that found in artificial SMM yogurt. The die-off rates of the microorganism in these artificial yogurts was substantially greater than that found in artificial milk yogurt. This indicated that the casein precipitate or compounds associated with it exert a protective influence toward Salmonella. In order to determine if the casein fraction was the protective factor, 4.8% sodium caseinate was added to artificial whey yogurt and artificial SMM yogurt. S. typhimurium did not survive longer in either artificial yogurt when sodium caseinate was present. Therefore, this indicated that either a protective compound(s) or a compound(s) acting in conjunction with casein, rather than casein itself, exerted a protective effect toward S. typhimurium in yogurt.

In order to determine the amount of bactericidal activity due to lactic and acetic acid, artificial milk yogurt was compared to artificial milk yogurt with only one

Figure 8. Inhibition of *S. typhimurium* in Artificial Yogurts With and Without Sodium Caseinate



of the acids present. These results are found in Figure 9. At a pH of 6.5, the bacteria grew in milk under anaerobic conditions similar to 18 hr yogurt. Acetic acid was barely more bacteriostatic than was the hydrochloric acid (pH) control. This indicated that acetic acid by itself was not the main inhibitor in yogurt. When only lactic acid was present in artificial milk yogurt, Salmonella's die-off rate was similar to the die-off rate when both acids were present.

Figure 10 shows the survival of S. typhimurium in artificial SMM yogurt with acetic and lactic acid added separately. These results are similar to that found in artificial milk yogurt except that the die-off rate was observed to be much greater. Salmonella was able to grow at pH 6.5 even though the medium was anaerobic. In artificial SMM yogurt with just acetic acid present, the die-off rate was found to be only slightly greater than in the hydrochloric acid control. The die-off rate when just lactic acid was present was similar to artificial SMM yogurt which contains both acids. Therefore, the inhibitory activity of yogurt seems to be due mainly to lactic acid.

G. Die-off of S. typhimurium in Cationic Yogurt

The following experiment was conducted to further investigate the role of acid as a factor in the inhibitory nature of 18 hr yogurt. Figure 11 graphically illustrates the survival of Salmonella with time in cationic (acid-free) and untreated yogurt. Yogurt was separated into its whey

Figure 9. Inhibition of *S. typhimurium* by Artificial Yogurts and Selected Acids in Milk

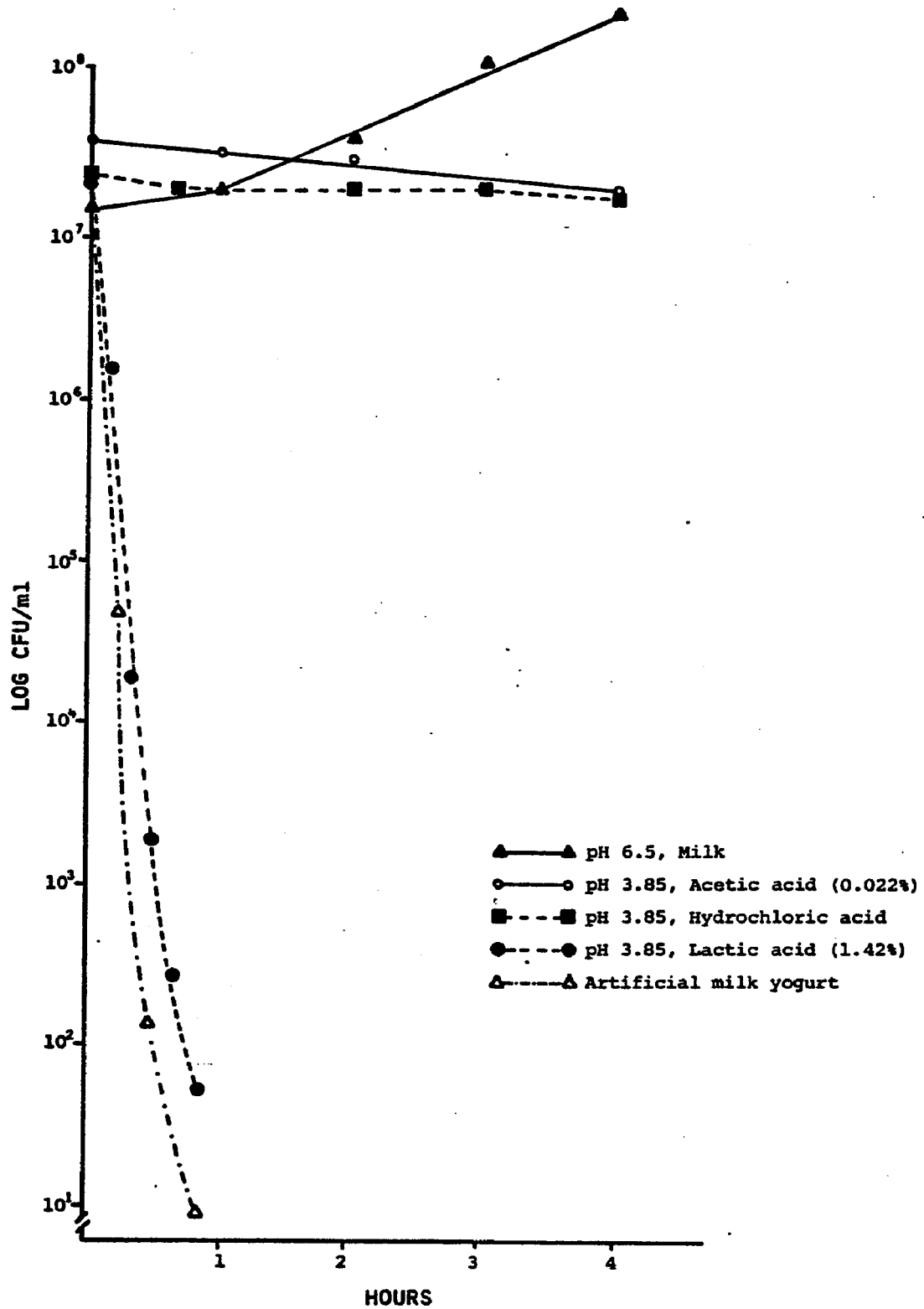


Figure 10. Inhibition of *S. typhimurium* by Artificial Yogurts and Selected Acids in SMM

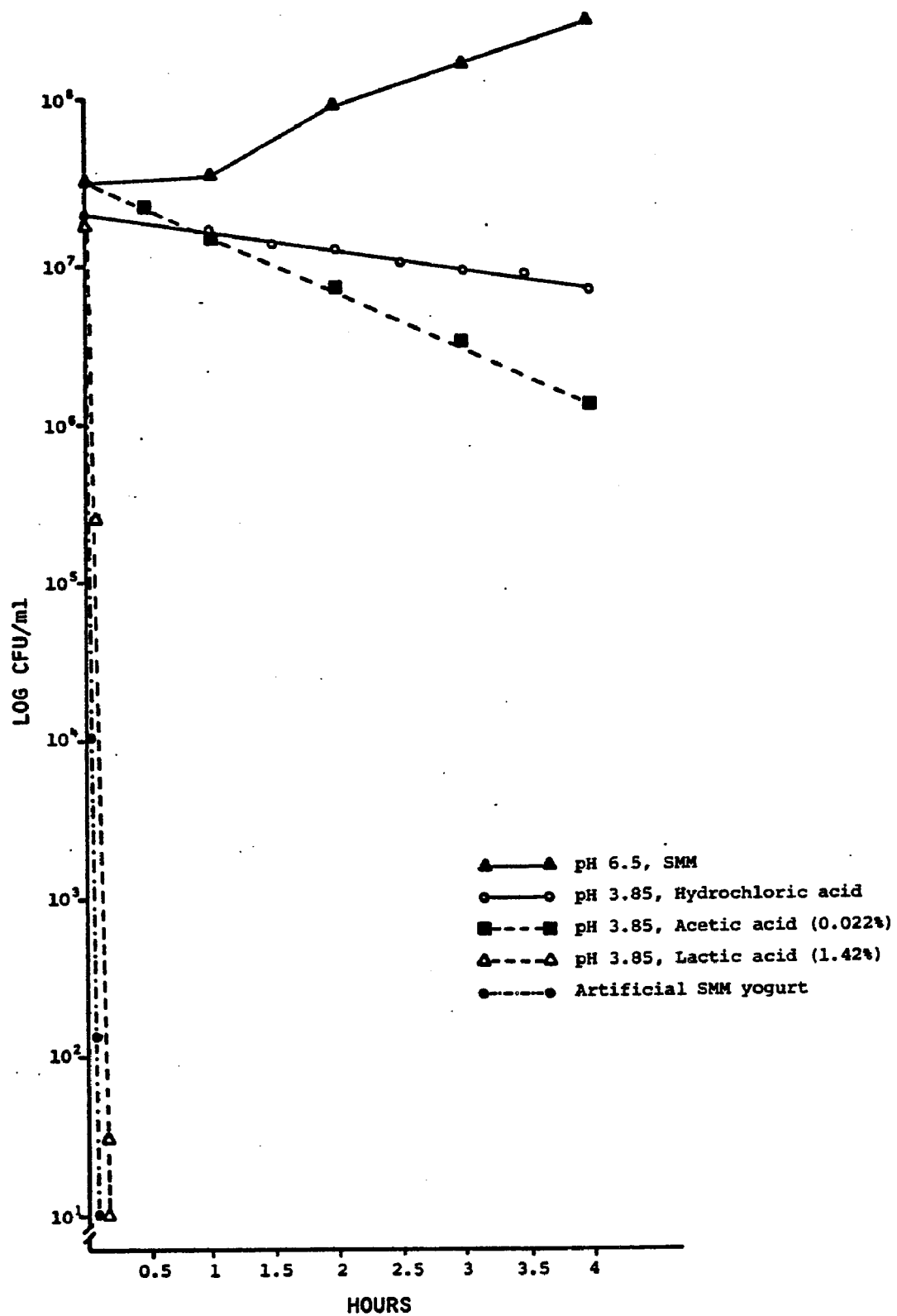
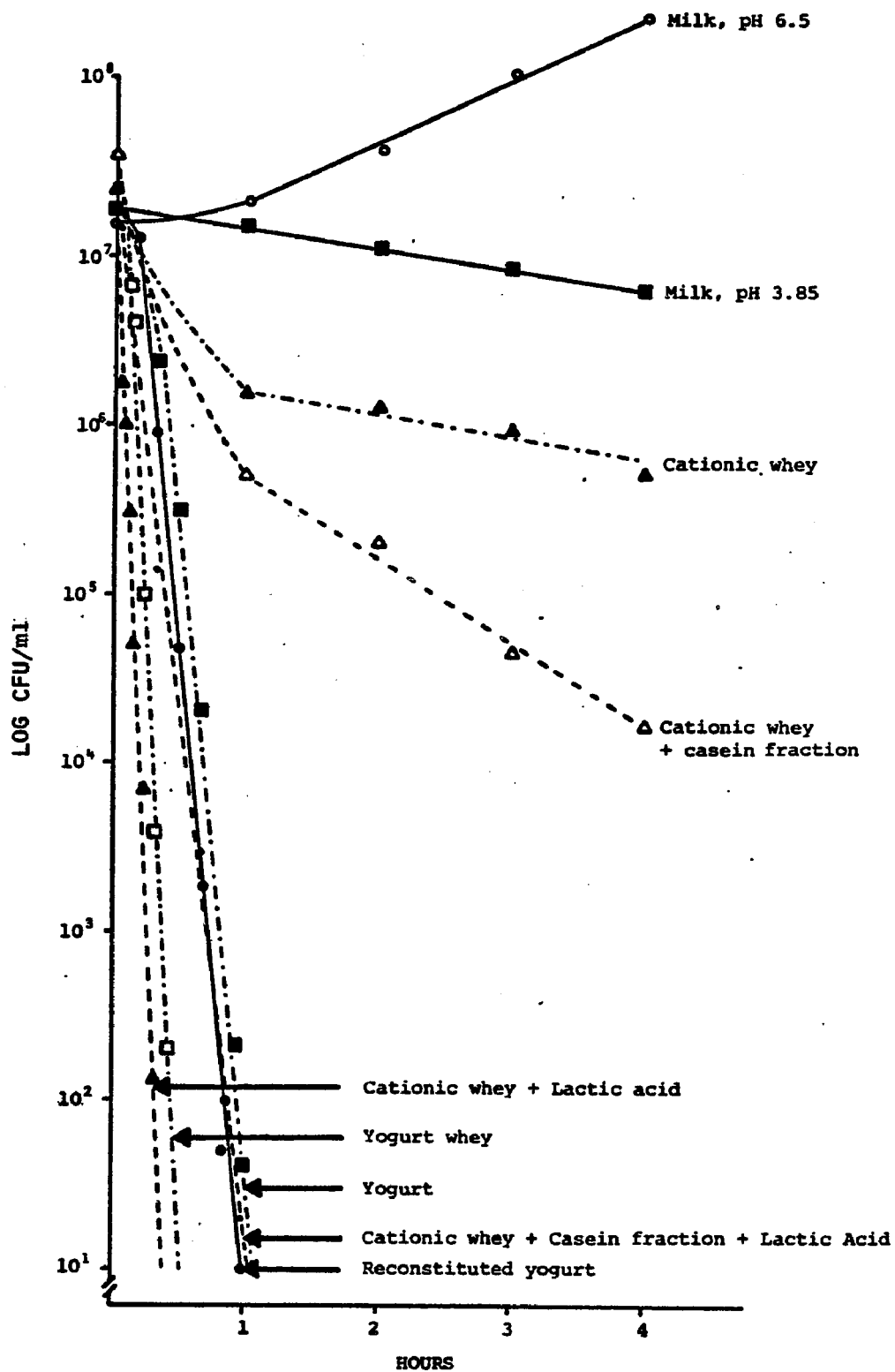


Figure 11. Inhibition of *S. typhimurium* by Cationic Whey Yogurt

and casein fractions. The die-off rate of S. typhimurium in the whey fraction was significantly greater than in complete yogurt. The die-off rate was again similar to that of yogurt when the casein fraction was added to the whey fraction (reconstituted yogurt). This indicated that either casein or a factor(s) associated with the casein fraction exerted a protective effect toward S. typhimurium as was also demonstrated in artificial yogurt.

Next, yogurt whey was passed through a Dowex-1 (Cl^-) anion exchange column to remove the acids present in yogurt. The cationic (acid-free) whey fraction was then collected and found to be only slightly bactericidal against S. typhimurium (Figure 11). Salmonella died more rapidly in cationic whey plus the casein fraction than in cationic whey. However, the die-off rate in the former was still much less than in yogurt. An analysis of covariance showed that the die-off rates with and without the casein fraction are different at the 0.01 level of significance. The increased die-off rate of Salmonella in cationic whey, when the casein fraction was added was probably due to the anions that remained associated with the casein fraction, rather than its being inhibitory. Therefore, it appears that the anionic fraction of yogurt is responsible for the die-off of S. typhimurium in yogurt.

Lactic acid was added to cationic whey and to cationic whey plus the casein fraction. Die-off of Salmonella in cationic whey was similar to the rate of die-off in yogurt

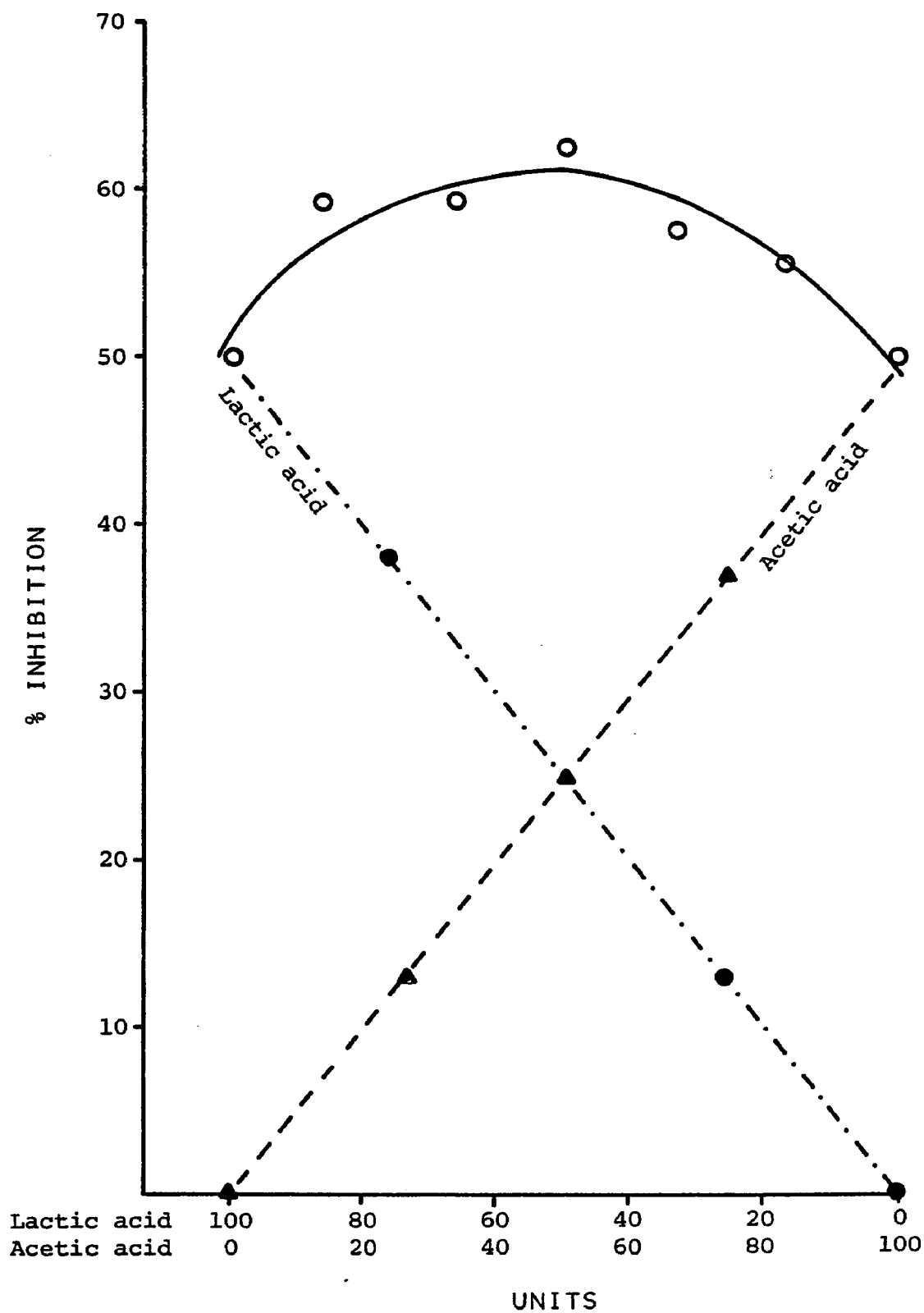
wey (Figure 11). The bacterial die-off rate in cationic wey plus the casein fraction was similar to the die-off rate found in yogurt. Acetic acid, when added to cationic wey plus the casein fraction, resulted in a slightly elevated increase in Salmonella's die-off rate. Salmonella was observed to grow only in the milk control at pH 6.5. The data indicate that lactic acid is the main inhibitor in yogurt.

The die-off rates of S. typhimurium in artificial milk yogurt (Figure 7), yogurt (Figure 11), and cationic wey plus the casein fraction plus lactic acid (Figure 11) were found to be similar. This indicated that artificial milk yogurt provided the essential factors that are responsible for Salmonella die-off in 18 hr yogurt. Lactic acid was found to be responsible for the decline in numbers of the microorganism both when it was removed from 18 hr yogurt (cationic yogurt) and then subsequently added in the same concentration originally found. Similar results were obtained when the inhibitory conditions of 18 hr yogurt were duplicated.

H. Inhibitory Interrelationships Between Lactic and Acetic Acid

Since acetic acid might affect lactic acid inhibition of S. typhimurium, a toxicological model was used to show the type of inhibitory relationship these two acids exhibit. Figure 12 shows the effects of varying concentrations of

Figure 12. Inhibition of *S. typhimurium* at Different Mixtures of Lactic and Acetic Acid



lactic and acetic acid on inhibition of the growth rate of S. typhimurium. Lactic and acetic acid together were found to be slightly synergistic in their inhibitory effect against Salmonella. An analysis of the scatter plot of the data indicated that the relationship is non-linear (not additive). A polynomial regression analysis yielded the following quadratic equation:

$$y = 0.0042x^2 + 0.45x + 49.4 \quad (F)$$

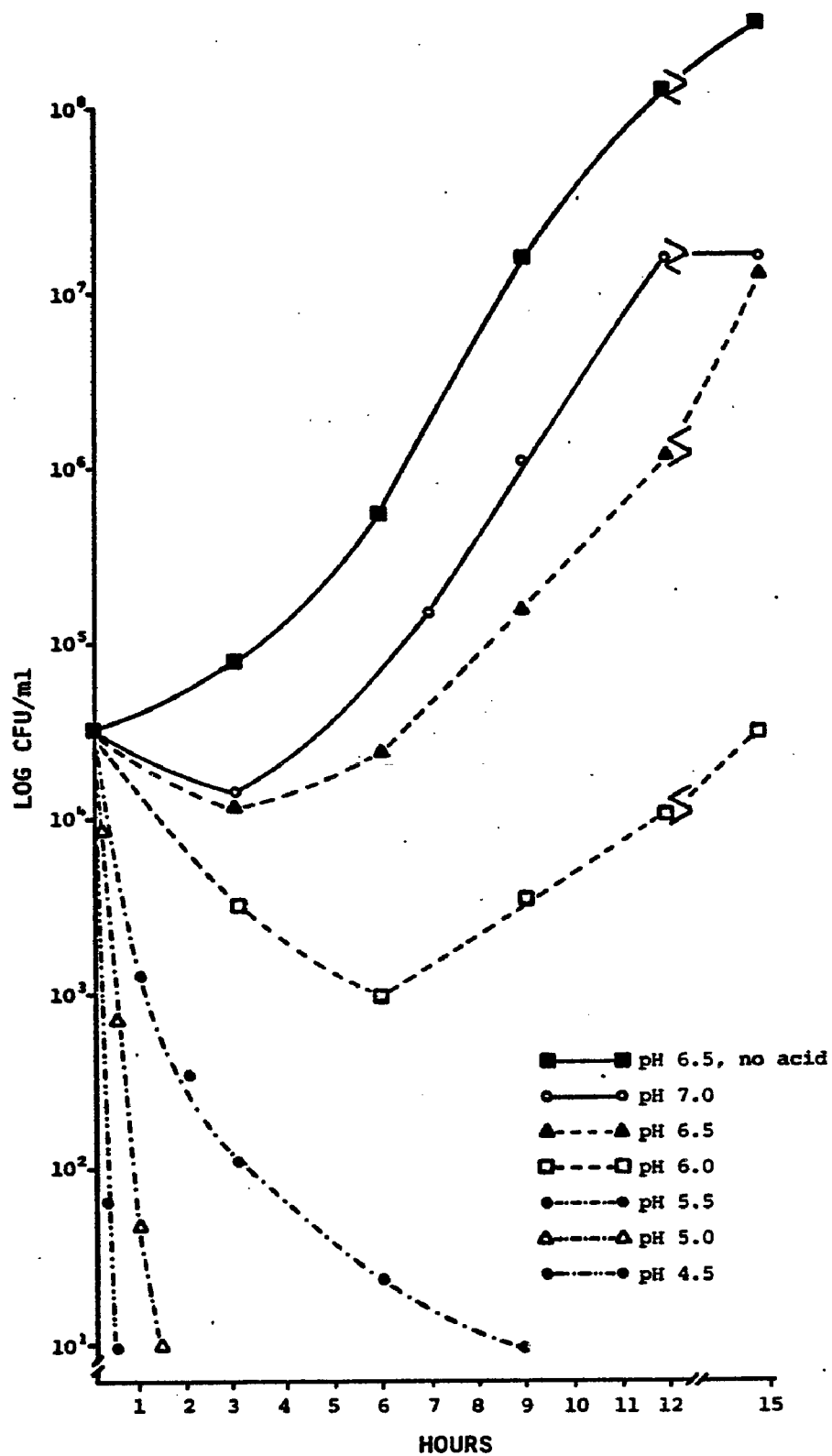
The data fitted this parabola at the 0.01 level of significance.

Probably no synergism takes place between lactic and acetic acid because of the low concentration of acetic acid in yogurt. The relationship between lactic and acetic acid in yogurt is virtually additive. Therefore, the inhibitory role that acetic acid plays in yogurt is minimal.

I. Mechanism of Inhibition of Lactic Acid Against S. typhimurium

The previous experiment demonstrated that lactic acid in yogurt is bactericidal and not bacteriostatic. To further demonstrate this phenomenon, growth of S. typhimurium when added to SMM containing 1.5% lactic acid at varying pH's was followed over time as seen in Figure 13. The growth rate was substantially less at pH 6.5 when lactic acid was present as compared to the positive control (without lactic acid) even though the pH was the same. At a pH of 7.0, the growth rate was significantly greater than at 6.5, but was

Figure 13. Influence of pH and 1.5% Lactic Acid On *S. typhimurium*



still much less than when no acid was present. The growth rate and final numbers of the microorganism were greatly reduced at a pH of 6.0. After 9 hr of incubation no viable Salmonella was detected at a pH of 5.5. As the pH declined from 5.5 to 4.0, there was an increase in the die-off rate. For a pH of 5.0, no microorganisms were recovered after 1.5 hr. At pH 4.5, Salmonella could not be isolated after 0.5 hr. Salmonella died off within 15 min at a pH of 4.0 or at 3.5. This shows that lactic acid inhibition is pH dependent.

A bactericidal effect toward S. typhimurium could be due to either cellular death or injury after exposure to inhibitory conditions. Therefore, the cultures inhibited at pH 3.5, 4.0, and 4.5 were tested. The cultures were 2 times washed with phosphate buffer to remove lactic acid, and then aliquots were used to inoculate Trypticase Soy Broth, Trypticase Soy Agar, Brilliant Green Broth, and Brilliant Green Agar. At the end of 48 hr incubation at 37 C, no increase in either turbidity or acidity and no decrease in pH was observed in either broth. No colony formation was found on either Trypticase Soy Agar or Brilliant Green Agar plates at the end of 72 hr. This indicated that the observed effect was probably cellular death because of the cessation of observable metabolic activity and the cells' inability to reinitiate growth.

Next an attempt was made to determine if the end-result of lactic acid inhibition of Salmonella was bacteriolysis. S. typhimurium was suspended in phosphate buffer

containing 1.42% lactic acid at a pH of 3.85 and incubated for 24 hr at 37 C. The OD, direct count, and viable count are summarized in Table 5. The numbers obtained by direct count had not substantially changed at the end of 24 hr of incubation. No viable counts were observed on Brilliant Green Agar at the end of 72 hr. This indicated that S. typhimurium was killed and that no appreciable amount of lysis took place. When the optical density was measured at 540 nm, the absorbancy increased by 20.8% at the end of 24 hr. According to other reports, there was a shrinkage of the cellular volume associated with an increase in absorbance.

The method employed to measure intracellular pH is based on the assumption that the intracellular and extracellular undissociated concentrations of DNP are equal since DNP acts as a weak acid by having a hydrogen ion that can dissociate. Unfortunately, this assumption can only be partially demonstrated.

It was observed in this study that increasing amounts of DNP were found to accumulate inside the cells of S. typhimurium as the pH became lower. This indicates that the undissociated moiety of DNP is the form in which it entered the cell. Accumulation of DNP intracellularly indicates that the intracellular pH is higher than the pH of the medium. Thus, once the undissociated species enters the cell it dissociates because of the higher pH of the internal environment. DNP should enter the cell in the undissociated state until an

Table 5

The Effect of 1.42% Lactic Acid and pH 3.85 in
Phosphate Buffer on Salmonella typhimurium

Measurement	Time	
	Initial	24 hrs
^a Absorbance	0.57	0.72
Direct Count	^b 2.4×10^9	^b 2.5×10^9
Plate Count	^b 4.2×10^8	^c --

^a540 nm

^bcells/ml

^cNo growth

equilibrium is established where the concentration of the two undissociated species are equal. When the external pH is raised, DNP re-enters the medium. The reason for this is that when the external pH is raised, there is a higher concentration of DNP inside the cell than outside of it.

Conway and Downey (21) compared three methods to determine the internal pH of resting cells of Saccharomyces cerevisiae. They used cellular uptake of acetic acid as one method and the uptake of carbonic acid as another. In the third method they directly measured the pH of a mixture in which the yeast cells had been lysed by rapidly thawing the frozen cells. The first two measurements were conducted using the assumption that the undissociated concentrations of the acids are equal in and out of the cell. In all three methods they found that the intracellular pH of resting S. cerevisiae was about 5.8. These experiments demonstrate that the assumption used in the present investigation is very possibly valid. Other researchers have also determined intracellular pH by the uptake of weak acids in procaryotes (15,62,86) and in eucaryotes (15,88), thereby relying on the probable validity of this assumption.

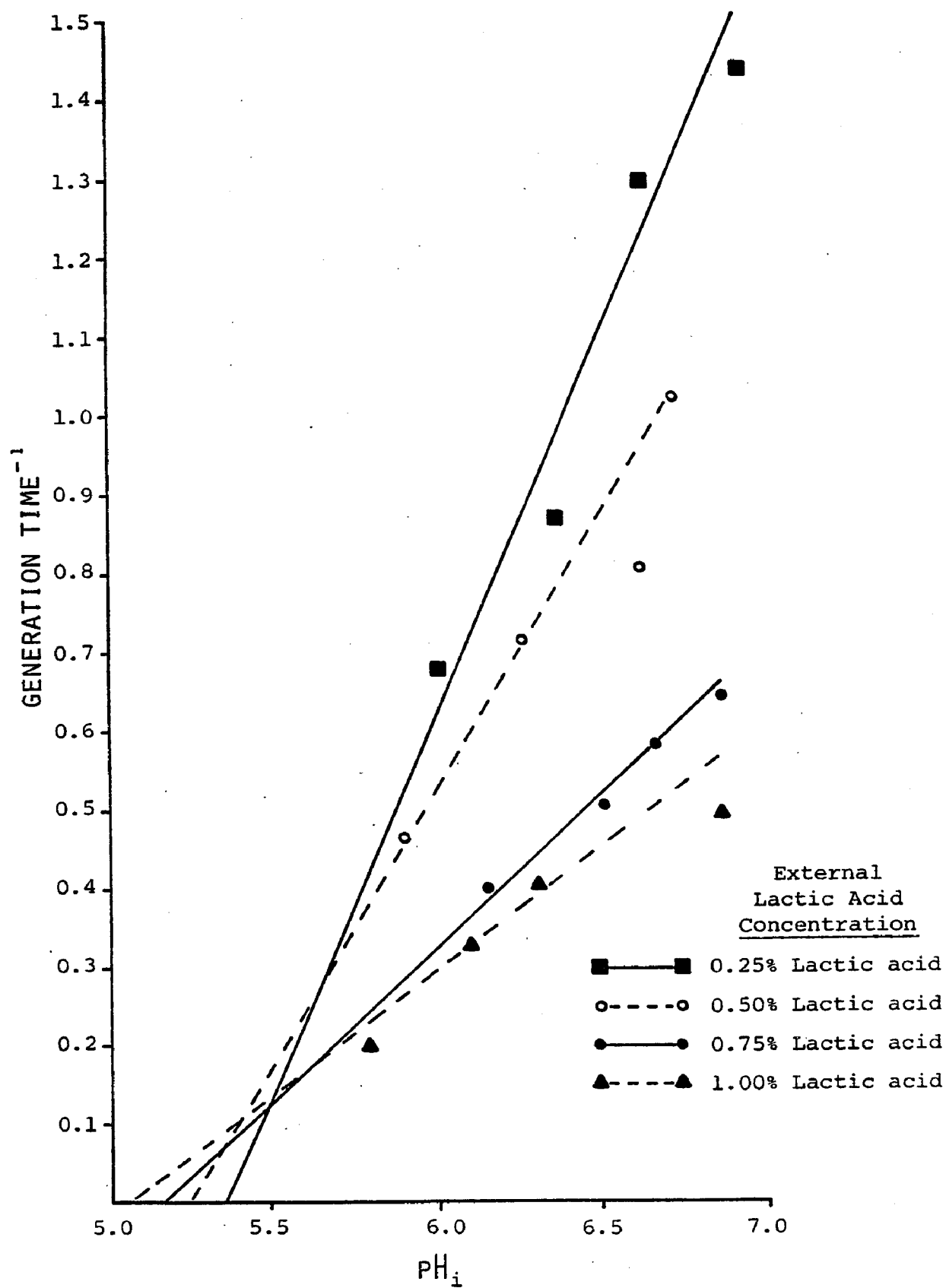
An experiment was performed to test the assumption that the undissociated DNP concentration is equal inside and outside the cell. A washed cell pellet of S. typhimurium was suspended in 6 ml of pH 3.7 minimal salts containing 0.335 mM DNP. After 5 minutes of incubation, the cells were filtered through a 0.45 μ filter. The concentration of DNP

in the filtrate was found to be 0.085 mM. Therefore, 0.25 mM DNP went into the cells. The concentration of DNP within the cells was calculated to be 2.5 mM correcting for the fact that the volume of the cell mass (.6 ml) was one-tenth that of the medium. The intracellular pH was then calculated as 5.3.

The cells retained on the filter were resuspended in minimal salts at pH 3.7 for five minutes longer, and were again filtered through a 0.45 μ filter. The DNP concentration in the filtrate was 0.067 mM. This was the amount of DNP that went out of the cells leaving an intracellular DNP concentration of 2.11 mM at the end of the second incubation. The undissociated intracellular and external species of DNP could be calculated since the intracellular and external pH's and the concentration of DNP inside and outside the cells were known. The undissociated intracellular concentration was found to be 0.018 mM and the undissociated external concentration was 0.048 mM. These values are similar and tend to support the assumption that intracellular and extracellular undissociated DNP concentrations are equal.

As a further check, if the assumption being tested is correct, then the calculated intracellular pH which is based on the extracellular undissociated DNP concentration should be approximately 5.3. An intracellular pH of 5.55 was found when the extracellular undissociated concentration of 0.048 mM was used. Since the intracellular pH's were similar, this indicates that the undissociated species of DNP inside the cells and outside the cells are probably equal.

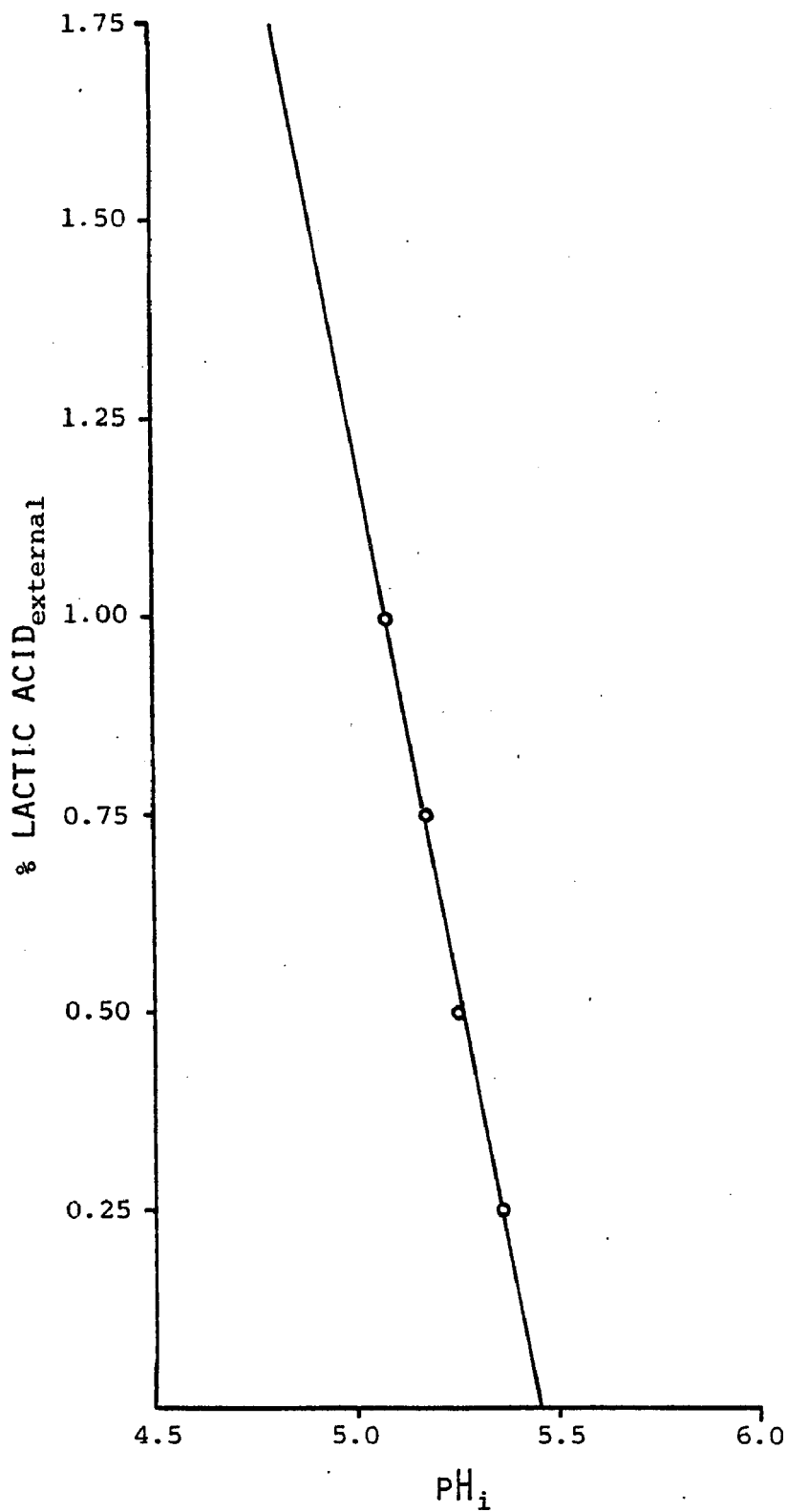
Figure 14. Effect of Intracellular pH on the Growth of *S. typhimurium*



Viable cells were used to determine the intracellular pH at bacteriostasis. Figure 14 shows the relationship between intracellular pH (pH_i) and the reciprocal of the generation time (generation time⁻¹). A concentration of 0.5% lactic acid was more inhibitory than 0.25%, but not as inhibitory as 0.75% lactic acid. One per cent lactic acid was found to be the most inhibitory concentration against S. typhimurium. As the lactic acid concentration increased, the generation time increased for a given pH_i . This indicated that there is no correlation between intracellular pH and the generation time. The intracellular pH at bacteriostasis for each lactic acid concentration was determined by extrapolating each curve to 0 generation time⁻¹ (bacteriostasis). These values were then plotted against the external lactic acid concentration in Figure 15. Since fermenting yogurt had a 0.34% lactic acid concentration at the end of bacteriostasis (4 hr; Figure 2), the intracellular pH at bacteriostasis in fermenting yogurt was determined to be approximately 5.3. Thus, intracellular lactic acid was probably the inhibitory factor rather than pH_i down to an intracellular pH of 5.3. The hydrogen ion concentration might be sufficient at a pH_i lower than 5.3 to be bactericidal.

Intracellular lactic acid was probably not metabolized during the course of the experiment as indicated by the following information. S. typhimurium did not grow in salts medium with lactic acid (in concentrations used in the experiment) as the sole carbon and energy source. This indicated that lactic acid may not be metabolized. Secondly, a

Figure 15. Effect of pH_i on the Extracellular Lactic Acid Concentration of *S. typhimurium*



linear curve was obtained when intracellular pH was plotted against the concentration of lactic acid in the medium (Figure 15). If lactic acid were metabolized by the cells, this relationship would not be expected. Also, lactic acid was measured directly by lysing the cells via sonification. The intracellular lactic acid concentrations measured by direct means were found to be similar to the intracellular lactic acid concentrations computed indirectly as seen in Table 6.

Figure 16 shows the relationship between the reciprocal of the generation time and the intracellular lactic acid concentration. As the lactic acid concentration increased, the generation time also increased. There was an increasing intracellular lactic acid concentration as the pH decreased. pH 7 was the least inhibitory pH for all lactic acid concentrations, while pH 5.5 was the most inhibitory. A pH of 6.5 was more inhibitory than 6.0, both of which were intermediate in their inhibitory abilities. Therefore, there is a direct relationship between the intracellular lactic acid concentration and the generation time. The intracellular lactic acid concentration at bacteriostasis was extrapolated and these values were plotted against external pH as seen in Figure 17. Since fermenting yogurt had a pH of 4.54 at bacteriostasis, the intracellular lactic acid concentration was determined to be 197 ug/mg dry wgt.

The amount of dissociation of intracellular lactic acid at the end of bacteriostasis could be determined using the Henderson-Hasselbalch equation (Equation b), since the intracellular pH is also known. The dissociated lactic acid

Table 6

Comparison of the Intracellular Lactic Acid Concentrations
Measured Indirectly and Directly

External Lactic Acid	Intracellular pH							
	7.0		6.5		6.0		5.5	
	a _{indir}	b _{dir}	a _{indir}	b _{dir}	a _{indir}	b _{dir}	a _{indir}	b _{dir}
1.0%	^c 120	136	162	165	195	176	214	242
0.75%	104	127	116	81	160	175	174	190
0.50%	57	43	91	102	127	132	160	171
^d 0.25%	2	5	14	19	103	141	119	91

^aindirect measurement performed by determination of the difference of lactic acid in the medium before and after incubation of the cell pellet

^bdirect measurement performed by determination of the concentration of lactic acid recovered after lysing the filtered cell pellet by sonification

^cμg lactic acid/mg cell dry wgt

^dno lactic acid observed in cells incubated in salts-glucose medium

Figure 16. Effect of Total Intracellular Lactic Acid on the Growth Rate of *S. typhimurium*

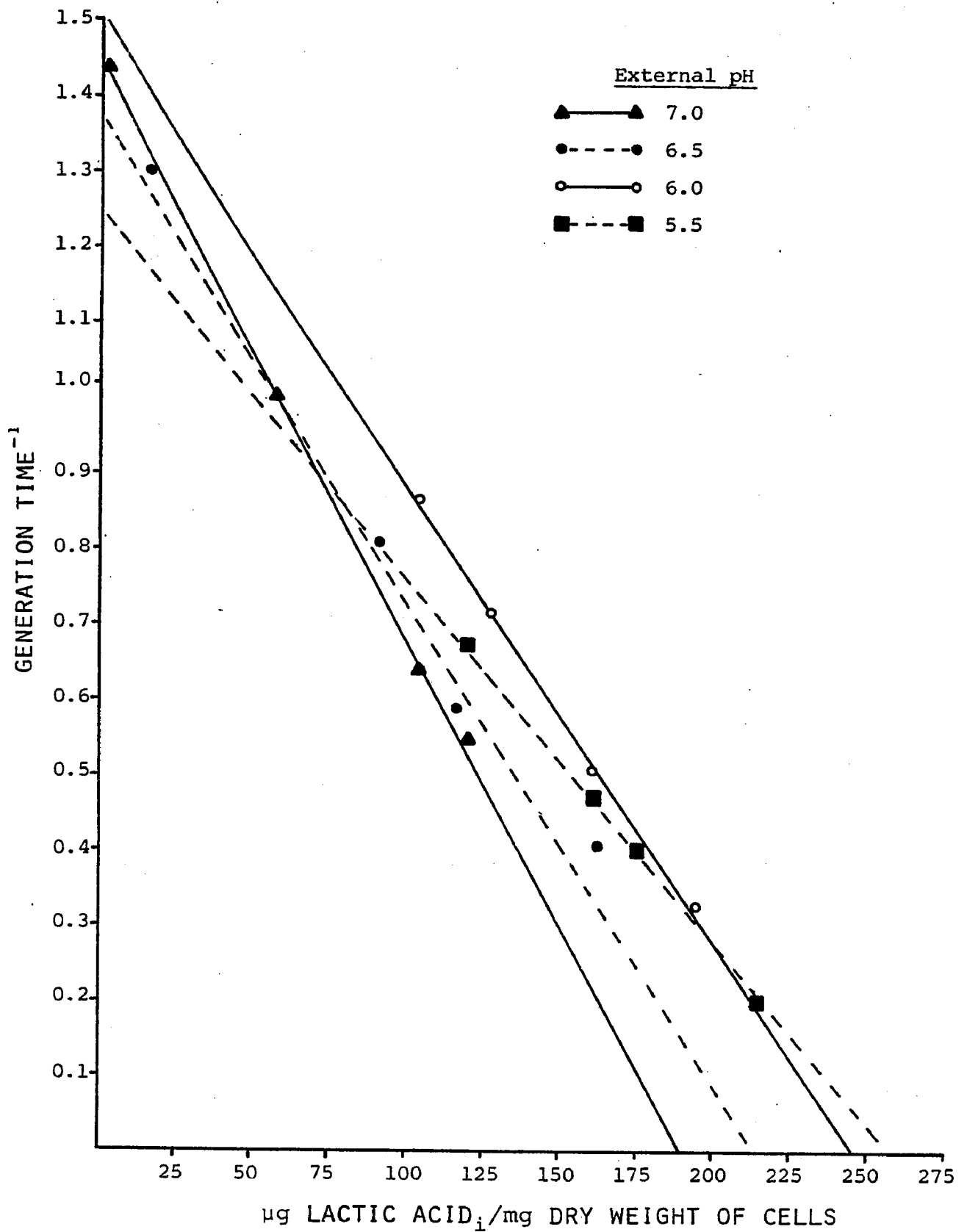
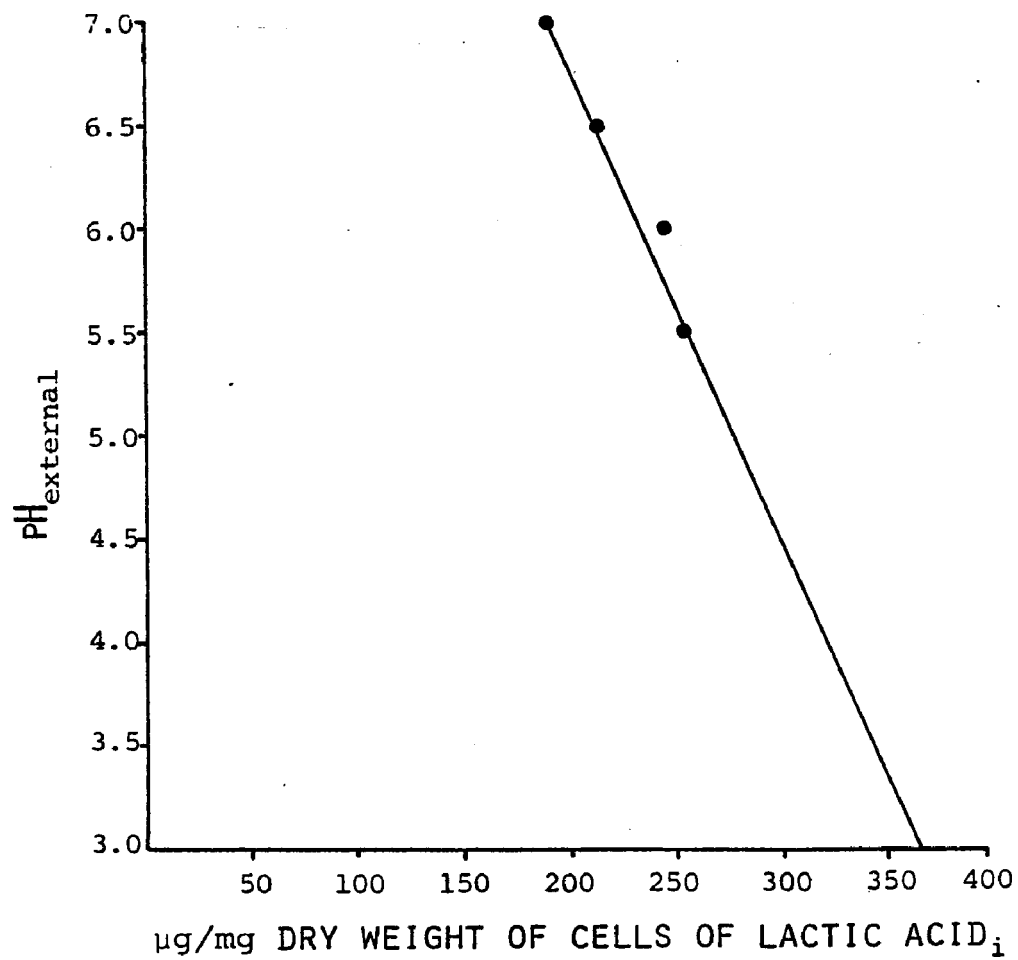


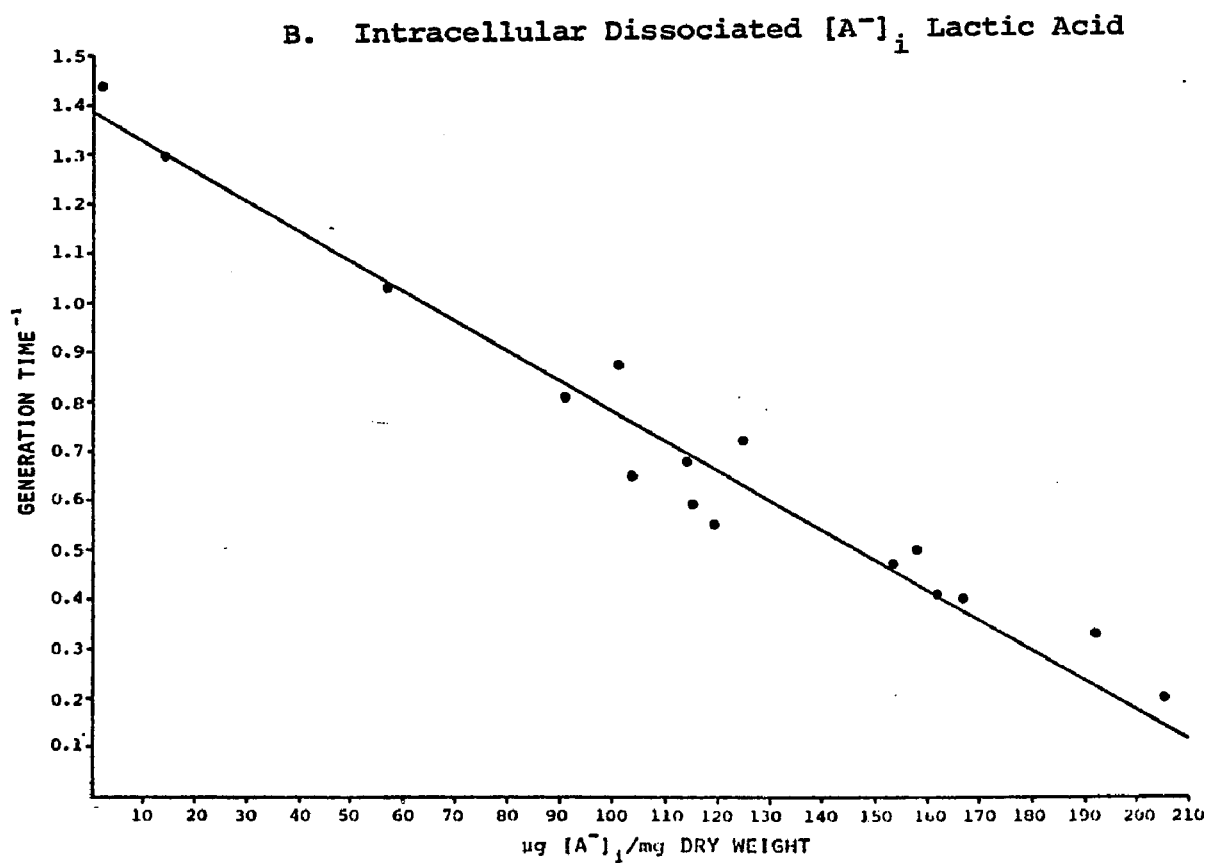
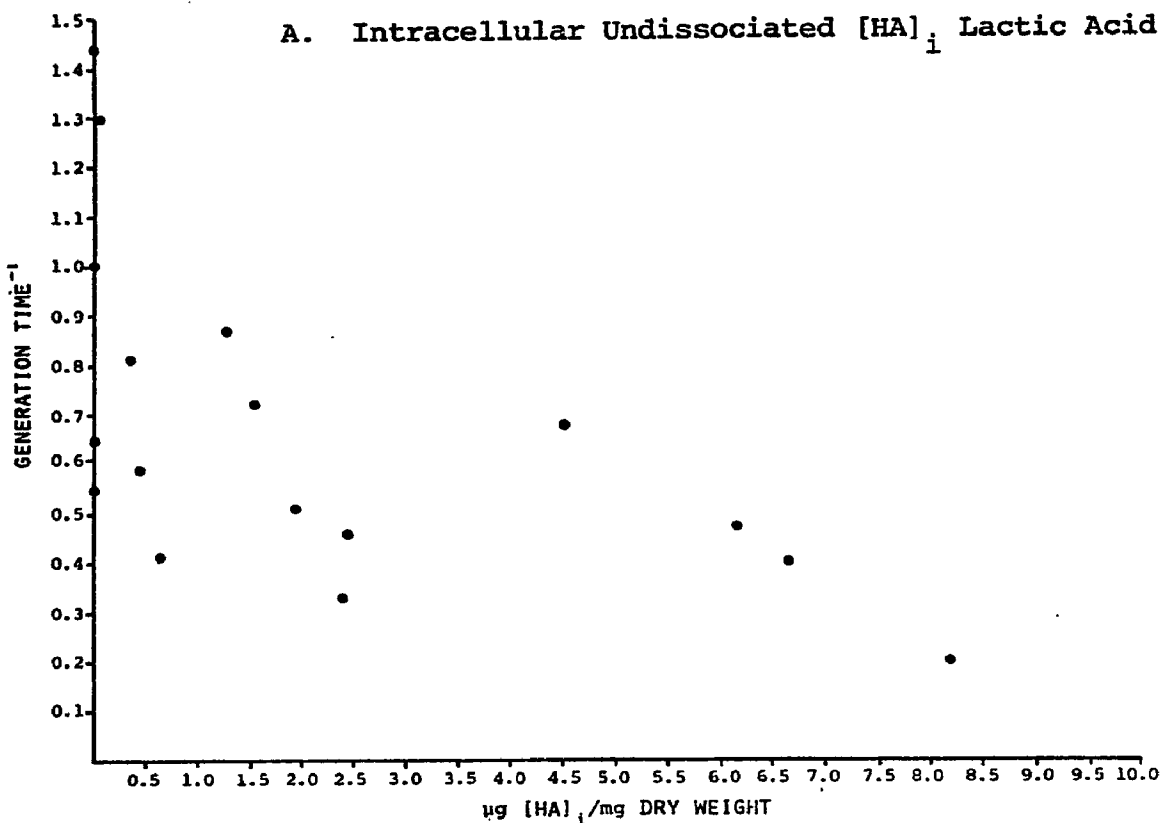
Figure 17. Effect of Intracellular Lactic Acid on the external pH of *S. typhimurium*



concentration was calculated to be 287 $\mu\text{g}/\text{mg}$ dry wgt, and the undissociated lactic acid concentration was found to be 10 $\mu\text{g}/\text{mg}$ dry wgt. Therefore, the dissociated moiety of lactic acid is the predominant intracellular species at the end of bacteriostasis.

The intracellular concentrations of the undissociated and dissociated moieties of lactic acid were calculated for each intracellular lactic acid concentration and pH value. In Figure 18-A the intracellular undissociated moiety of lactic acid is plotted against the reciprocal of the generation time. There is no real correlation between the undissociated species and the generation time of S. typhimurium because the points do not represent a straight line. However, a good correlation between the intracellular dissociated species and the generation time can be observed in Figure 18-B, which compares the intracellular dissociated lactic acid moiety with the reciprocal of the generation time. This indicated that the dissociated intracellular lactic acid moiety is the species responsible for the bactericidal effect of S. typhimurium in yogurt.

Figure 18. Effect of the Intracellular Species of Lactic Acid on the Growth Rate of *S. typhimurium*



DISCUSSION

Acid has been shown by many authors (28,33,44,48,77, 79,87) to be inhibitory against *Salmonellae* in fermented milk products. However, in those investigations the presence of acidity was determined by titration with sodium hydroxide and it was reported as either total acidity or as percent lactic acid. The amount of inhibition of microbial growth due to acid in yogurt also had not been previously determined. Most importantly, it remained to be demonstrated if lactic acid, the most predominant acid in yogurt, is the compound responsible for the die-off of *S. typhimurium*. This was demonstrated by this investigation.

The role of pH in yogurt inhibition of bacteria had not previously been clarified. pH alone is probably not bactericidal in yogurt. *Salmonella* has been observed to grow at a pH as low as 4.0 when the medium was adjusted with hydrochloric acid (18). This study found that *S. typhimurium* was able to survive in milk when the pH was adjusted to 3.85 with hydrochloric acid. However, acidity does play an integral part in lactic acid inhibition of the microorganism by controlling the concentration of the undissociated species present in the medium which is the form taken up by the cell.

Acetic acid was found to be in insufficient concentration to be inhibitory in yogurt. There was no greater amount of inhibition due to acetic acid than was found for pH alone. Therefore, acetic acid does not play an integral part in yogurt's inhibitory abilities against S. typhimurium.

This study demonstrated that a low ORP augmented lactic acid inhibition of S. typhimurium. However, the mechanism of the increased bactericidal effect due to low ORP is not understood. The mechanism of ORP augmentation of acid inhibition should be examined. Low ORP may either have an indirect role by enhancing the bactericidal effect of lactic acid or it may play a more active part.

This study demonstrated that the concentration of lactic acid at a low pH is sufficient to be lethal against S. typhimurium at the end of 4 hr in fermenting yogurt. The factors present that prevent Salmonella from proliferating during the first 4 hr after inoculation have yet to be determined. It is possible that Salmonella never gets out of lag phase due to hydrogen peroxide and/or lactic acid production by the yogurt starter culture.

The results from Figure 2-A agree with those of Park and Marth (65), who showed that S. typhimurium is not detectable after the first 9 hr of incubation in fermenting yogurt. The die-off rate of the bacteria in yogurt is directly related to the incubation temperature. There is

an increasing die-off rate as the temperature increases (66). In a preliminary experiment, S. typhimurium was found to survive for at least 12 hr in a commercial yogurt stored at 4 C. At 22 C, Salmonella was able to survive for approximately 6 hr and at 32 C survival was only 2hr. The large divergence in the length of time that Salmonellae can survive in yogurt (66) can then at least be partially explained by the various storage temperatures used. A second factor could be the varying size of inocula used. The larger the inoculum, the longer the time necessary to kill all the viable bacteria. In another preliminary experiment S. typhimurium, at a concentration of 1×10^7 cells/g, took about 3 hr to die-off in 18 hr fermented yogurt stored at 22C, while it took less than 1 hr to kill 1×10^5 S. typhimurium/g. Therefore, the discrepancies found in published reports (30,65,66) on the length of survival of Salmonella in yogurt are probably due to the different conditions used by various workers.

There is a discrepancy in the literature between the inhibitory inter-relationships between lactic and acetic acid. Several authors (56,79,80) have indicated that the acids exert an additive inhibitory effect while another paper (10) indicated that 0.1 M lactic acid was antagonistic toward 0.04 M acetic acid inhibition of Salmonella. This study found that lactic and acetic acid, when combined, are not antagonistic in their inhibitory effects on S. typhimurium.

Several factors could have influenced the findings of Bohnhoff, Miller, and Martin (10). They measured the degree of inhibition of growth by determining the concentration of S. typhimurium at one point in time, rather than by measuring the amount of inhibition of the growth rate when subjected to lactic and/or acetic acid. At the concentrations of lactic and acetic acid used (10) there would only be 0.7 mM lactic acid and 2.1 mM acetic acid available for cellular uptake, because the undissociated moiety is the form in which organic acids are transported into the cell (6,33,50). Under the experimental conditions used in the present study, some growth was obtained at pH 6.0 in salt-glucose medium with similar lactic and acetic acid concentrations. The results reported by Bohnhoff, Miller, and Martin (10) can not, therefore, be considered conclusive.

This study has shown that there is at least one compound associated with the casein fraction of milk that exerts a protective influence toward S. typhimurium in yogurt. This corroborates the findings of Minor and Marth (57) who noted that Staphylococcus aureus died more rapidly in artificial medium than in yogurt. This indicated that the artificial medium lacked the protective factor that is associated with the casein fraction of milk. This compound is not produced as a fermentation product of the starter culture because it is found in artificial milk yogurt as well as natural yogurt. Thus, it is a normal constituent of bovine milk. The mechanism of this

protective action as well as the compound(s) responsible for it deserve further study.

Lactic acid inhibition of S. typhimurium is only partially understood. The cell remains contiguous after death as indicated by the absence of bacteriolysis, but the cells shrink in size (8,52) perhaps because the cell membrane becomes leaky. There was no restoration of either metabolic activity or growth when the intracellular pH was raised to 6.5 indicating that lactic acid inhibition of S. typhimurium in yogurt is irreversable. There is an accumulation of lactic acid inside the cell which is much greater than the lactic acid concentration in the external environment. This indicates that the membrane is intact and at least partially functioning until death. The amount of lactic acid accumulated intracellularly increases as the pH of the medium is lowered. There is also a proportionate increase in the die-off rate with an increase in the intracellular lactic acid concentration. This evidence indicates that the undissociated species of lactic acid is the form in which it enters the cell and that lactic acid is inhibitory when it accumulates intracellularly. However, the mechanism of lactic acid inhibition of S. typhimurium is not known. Weiner and Draskoczy (88) noted that indophenol blue reduction is inhibited by lactic acid in E. coli. From this they concluded that lactic acid inhibited oxidative metabolism by inhibiting dehydrogenase activity. It is possible, therefore, that lactic acid may inhibit

oxidative metabolism in S. typhimurium.

Weiner and Draskoczy (88) demonstrated that in cell-free extracts of E. coli, inhibitory effects of mandelic acid and hippuric acid could be correlated to the concentration of the dissociated species. However, Basket and Hentges (6) obtained similar amounts of inhibition of oxidative metabolism in cell-free extracts of Shigella flexneri for both moieties of acetic acid. This research demonstrated a direct correlation between inhibition of whole cells of S. typhimurium and the intracellular concentration of dissociated lactic acid, thus agreeing with the conclusions drawn by Weiner and Draskoczy (88).

Either the lactate anions and/or the hydrogen ions could be responsible for the inhibitory effects of the dissociated moiety against S. typhimurium. It was observed (Figure 14) that there was no clear relationship between intracellular pH and inhibition of the growth rate. Bacteriostasis was observed when hydrochloric acid lowered the intracellular pH to 5.5. A rapid die-off rate was noted for Salmonella, at the same intracellular pH, when lactic acid was present. This indicates that the lactate anion, rather than the hydrogen ion, exerts the inhibitory effect against S. typhimurium in yogurt.

Intracellular pH may be bactericidal without lactic acid. In a preliminary experiment the intracellular pH of Salmonella was lowered to 5.5 with hydrochloric acid and growth ensued. When sufficient hydrochloric acid was added

to the medium to obtain an intracellular pH of about 5.2, cellular death occurred. It can be concluded from this evidence that when the intracellular pH becomes lower than 5.3 the hydrogen ion concentration may be sufficient to cause cell death. In summary, inhibition of S. typhimurium seems to be due to the lactate anion when the intracellular pH is 5.3 or above. When the intracellular pH is less than 5.3, inhibition may be due to both the lactate anion and to the hydrogen cation. Thus, it can be seen that lactic acid inhibition is probably a complex mechanism and variable according to the intracellular pH and lactic acid concentration.

In conclusion, lactic acid has been shown to be responsible for the die-off of S. typhimurium in yogurt. This inhibition has been demonstrated to be irreversible and not due to lysis of the cell. Lactic acid was shown to enter the cell in the undissociated form. Once inside the cell, the acid dissociated because the intracellular pH was higher than the external pH. The dissociated moiety could not leave the cell in this form so it accumulated and concurrently lowered the intracellular pH. Inhibition of S. typhimurium has been shown to be due to the intracellular dissociated moiety of lactic acid, rather than the hydrogen ion concentration down to an internal pH of 5.3. If the intracellular pH becomes lower than 5.3, the intracellular hydrogen ion concentration may be sufficient to be inhibitory to S. typhimurium.

Many points need to be clarified. The role that pH and acidity play should be further studied. The effect of lactic acid on transport systems should be investigated. The enzymes inhibited and the metabolic pathways affected by lactic acid should be determined. The sites on the inhibited enzymes should be elucidated. Also, the role that ORP plays in the augmentation of lactic acid inhibition should be assessed.

An important variable in dairy research is the lack of a standard milk. The composition of milk varies from cow to cow as well as varying in the same cow at different times. To be able to get reproducible results, researchers must use the same batch of milk for all experiments included in that research. This may be feasible for small scale experiments but not for extended investigations. Therefore, a synthetic medium similar to milk was developed during the course of this study which compared favorably with natural milk. Using this synthetic milk, experiments can now be reproduced in different laboratories without milk as an uncontrollable variable.

LITERATURE CITED

- (1) American Public Health Association. Standard Methods for the Examination of Water and Wastewater. 1971. 13th ed. APHA, Washington, D.C.
- (2) Arnott, D. R., Duitschaeffer, C. L. and Bullock, D. H. Microbiological Evaluation of Yogurt Produced Commercially in Ontario. 1974. J. Milk Food Technol. 37:11-13.
- (3) Ashton, D. G. and Busta, F. F. Milk Components Inhibitory to Bacillus stearothermophilus. 1968. J. Dairy Sci. 51:842-47.
- (4) Ashton, D. H., Busta, F. F. and Warren, J. A. Relief of Casein Inhibition of Bacillus stearothermophilus by Iron, Calcium and Magnesium. 1968. Appl. Microbiol. 16:628-35.
- (5) Azukas, J. J., Costilow, R. N. and Sadoff, H. L. Inhibition of Alcoholic Fermentation by Sorbic Acid. 1961. J. Bacteriol. 81:189-94.
- (6) Baskett, R. and Hentges, D. Shigella flexneri Inhibition by Acetic Acid. 1973. Infect. Immun. 8:91-97.
- (7) Benson, A. S. Control of Communicable Diseases in Man. 1970. 11th ed. APHA, Washington, D.C.
- (8) Bernheim, F. Factors which Affect the Size of the Organisms and the Optical Density of Suspensions of Pseudomonas aeruginosa and Escherichia coli. 1963. J. Gen. Microbiol. 30:53-58.
- (9) Bohnhoff, M., Miller, C. P. and Martin, W. R. Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection I. Factors which Interfere with the Initiation of Infection by Oral Inoculation. 1964. J. Exper. Med. 120:805-16.
- (10) Bohnhoff, J., Miller, C. P. and Martin, W. R. Resistance of the Mouse's Intestinal Tract to Experimental Salmonella Infection II. Factors Responsible for Its Loss Following Streptomycin Treatment. 1964. J. Exper. Med. 120:817-28.

- (11) Bowmer, E. J. Salmonellae in Food: A Review. 1965. J. Milk Food Technol. 28:74-86.
- (12) Brown, R. W. Compounds Affecting Streptococcus agalactiae Growth in Milk. 1974. J. Dairy Sci. 57:797-802.
- (13) Buchanan, R. E., and Gibbons, N. E. Bergey's Manual of Determinative Bacteriology. 1974. 8th ed. Williams & Wilkins Co., Baltimore.
- (14) Busta, F. F. Milk Component(s) Inhibitory to Bacillus stearothermophilus. 1966. J. Dairy Sci. 49: 751-56.
- (15) Caldwell, Peter C. Intracellular pH. 1956. Int. Rev. Cytol. 5:229-277.
- (16) Carr, J. G., Cutting, C. V. and Whiting, G. C. Lactic Acid Bacteria in Beverages and Food. 1975. Academic Press Inc., New York.
- (17) Carroll, K. H. and Jain, N. C. Bactericidal Activity of Normal Milk, Mastitic Milk and Colostrum Against Aerobacter aerogenes. 1963. Am. J. Vet. Res. 30:1123-32.
- (18) Chung, Keng Chee and Goepfert, J. M. Growth of Salmonella at Low pH. 1970. J. Food Science. 35: 326-28.
- (19) Collins, E. B. Domination Among Strains of Lactic Streptococci with Attention to Antibiotic Production. 1961. Appl. Microbiol. 9:200-5.
- (20) Collins, J. D., Carroll, E. J., Jasper, E. E. and Jain, N. C. Studies on the In Vitro Activity of Bovine Sera Against Selected Udder Pathogens. 1969. Res. Vet. Sci. 10:540-46.
- (21) Conway, E. J. and Downey, Mary. pH Values of the Yeast Cell. 1950. Biochem. J. 47:355-360.
- (22) Crafts, A. S. and Cleary, C. W. Toxicity of Arsenic, Borax, Chlorate, and Their Combinations in Three California Soils. 1936. Hilgardia. 10:401-13.
- (23) Dahiya, R. S. and Speck, M. L. Hydrogen Peroxide Formation by Lactobacilli and Its Effects of Staphylococcus aureus. 1968. J. Dairy Sci. 51: 1568-72.

- (24) Daly, D., Sandine, W. E. and Elliker, P. R. Interactions of Food Starter Cultures and Food-borne Pathogens: Streptococcus diacetylactis Versus Food Pathogens. 1972. J. Milk Food Technol. 35: 349-57.
- (25) Davidson, J. The Colorimetric Determination of Lactic Acid in Milk and Milk Products. 1949. J. Dairy Res. 16:209-16.
- (26) Davis, J. G. A Dictionary of Dairying. 1966. 2nd ed. Leonard Hill, London.
- (27) Dega, C. A., Goepfert, J. M. and Amundson, C. H. Growth of Salmonella typhimurium in Skim Milk Concentrates. 1972. Appl. Microbiol. 23:82-87.
- (28) Eisler, D. M. and Heckly, R. J. Possible Mechanisms of Action of an Anti-Pasteurella Pestis Factor. 1968. J. Bacteriol. 96:1977-81.
- (29) Fencel, Z. and Leopold, J. The Inhibition of Spore Germination of the Mold Aspergillus niger by Acetic Acid. 1959. Folia Microbiol. 4:7-15.
- (30) Ghoniem, N. A. M. The Viability of Some Salmonella Strains and Their pH Values in Egyptian Yogurt During Different Storage Temperatures. 1971. Milchwissenschaft. 26:418-20.
- (31) Gilliland, S. E. and Speck, M. L. Interactions of Food Starter Cultures and Food-borne Pathogens. Lactic Streptococci and Salmonellae. 1972. J. Milk Food Technol. 35:307-10.
- (32) Goelk M. C., Kulschrestha, D. C. and Marth, E. H. Fate of Coliforms in Yogurt, Buttermilk, Sour Cream and Cottage Cheese During Refrigerated Storage. 1971. J. Milk Food Technol. 34:54-58.
- (33) Goepfert, J. M. and Hicks, R. Effect of Volatile Fatty Acids on Salmonella typhimurium. 1969. J. Bacteriol. 97:956-58.
- (34) Goepfert, J. M., Olson, N. F. and Marth, E. H. Behavior of Salmonella typhimurium During Manufacture and Curing of Cheddar Cheese. 1968. Appl. Microbiol. 16:862-66.
- (35) Haines, W. C. and Harmon, L. G. Effect of Selected Lactic Acid Bacteria on Growth of Staphylococcus aureus and Production of Enterotoxin. 1973. Appl. Microbiol. 25:436-41.

- (36) Hargrove, R. E., McDonough, F. E. and Mattingly, W. A. Factors Affecting Survival of Salmonella in Cheddar and Colby Cheese. 1969. J. Milk Food Technol. 32:480-84.
- (37) Hedrick, T. I. Sources of Salmonella Contamination in Dry Milks. 1967. Am. Dairy Rev. 29:30-104.
- (38) Heldman, D. R., Sunga, C. A. and Hendrick, T. I. Microorganism Shedding by Human Beings. 1967. Contamin. Control. 6:28-45.
- (39) Hentges, D. J. Influence of pH on the Inhibitory Activity of Formic and Acetic Acids for Shigella. 1967. J. Bacteriol. 93:2029-30.
- (40) _____. Inhibition of Shigella flexneri by the Normal Intestinal Flora I. Mechanisms of Inhibition by Klebsiella. 1967. J. Bacteriol. 93:1369-73.
- (41) _____. Inhibition of Shigella flexneri by the Normal Intestinal Flora II. Mechanisms of Inhibition by Coliform Organisms. 1969. J. Bacteriol. 97:513-17.
- (42) Huhtanen, C. N. Use of pH Gradient Plates for Increasing the Acid Tolerance of Salmonellae. 1975. Appl. Microbiol. 29:309-12.
- (43) Humphreys, C. L. and Plunkett M. Yogurt: A Review of Its Manufacture. 1969. Dairy Sci. Abstr. 31: 607-22.
- (44) Kotyk, A. Uptake of 2,4-Dinitrophenol by the Yeast Cell. 1962. Folia Microbiol. 7:109-13.
- (45) Kulshrestha, D. C. and Marth, E. H. Inhibition of Lactic Streptococci and Some Pathogenic Bacteria by Certain Milk-Associated Volatile Compounds as Measured by the Disc Assay. 1970. J. Milk Food Technol. 33:305-10.
- (46) Kulshrestha, D. C. and Marth, E. H. Growth and Activity of Streptococcus cremoris in Skim Milk Fortified with Some Volatile Compounds. 1970. J. Milk Food Technol. 33:492-96.
- (47) Kulshrestha, D. C. and Marth, E. H. Inhibition of Bacteria by Some Volatile and Non-volatile Compounds Associated with Milk II. Salmonella typhimurium. 1974. J. Milk Food Technol. 39: 539-44.

- (48) Lee, A. and Gemmell, E. Changes in the Mouse Intestinal Microflora During Weaning: Role of Volatile Fatty Acids. 1972. Infect. Immun. 5:1-7.
- (49) Lee, S. Y., Vedamuthu, E. R., Washam, C. J. and Reinbold, G. W. An Agar Medium for the Differential Enumeration of Yogurt Starter Bacteria. 1974. J. Milk Food Technol. 37:272-76.
- (50) Levison, M. E. Effect of Colon Flora and Short-Chain Acids on Growth In Vitro of Pseudomonas aeruginosa and Enterobacteriaceae. 1973. Infect. Immun. 8:30-35.
- (51) McDonough F. E., Hargrove, R. E. and Tittsler, R. P. The Fate of Salmonellae in the Manufacture of Cottage Cheese. 1967. J. Milk Food Technol. 30:354-56.
- (52) Mager, J., Juczinski, M., Schatzberg, G. and Avi-Dor, Y. Turbidity Changes in Bacterial Suspensions in Relation to Osmotic Pressure. 1956. J. Gen. Microbiol. 14:69-75.
- (53) Manufactured Milk Products Supplement. The Household Yogurt Market. 1974. American Dairy Review. 36:31-34.
- (54) Marth, E. H. Salmonellae and Salmonellosis Associated with Milk and Milk Products. A Review. 1969. J. Dairy Sci. 52:283-315.
- (55) Meynell, G. G. Antibacterial Mechanisms of the Mouse Gut II. The Role of Eh and Volatile Fatty Acids in the Normal Gut. 1963. British J. Exper. Path. 44:209-19.
- (56) Minor, T. E. and Marth, E. H. Growth of Staphylococcus aureus in Acidified Pasteurized Milk. 1970. J. Milk Food Technol. 33:516-20.
- (57) Minor, T. E. and Marth, E. H. Fate of Staphylococcus aureus in Cultured Buttermilk, Sour Cream and Yogurt During Storage. 1972. J. Milk Food Technol. 35:302-6.
- (58) Minor, T. E. and Marth, E. H. Loss of Viability by Staphylococcus aureus in Acidified Media II. Inactivation by Acids in Combination with Sodium Chloride, Freezing and Heat. 1972. J. Milk Food Technol. 35:548-55.

- (59) Moore, J. E., Valois, A. B. and Robertson, C. Salmonellae: Ubiquitous Pathogens of Animals and Man. 1968. Canadian J. Public Health. 59:408-10.
- (60) National Communicable Disease Center. Salmonella Surveillance. Annual Summary, 1967.
- (61) National Communicable Disease Center. Salmonella Surveillance. Annual Summary, 1972.
- (62) Neal, S. L., Weinstock, J. O. and Lampen, J. O. Mechanisms of Fatty Acid Toxicity for Yeast. 1965. J. Bacteriol. 99:125-31.
- (63) Park, Hong Sik. Behavior of Salmonella in Milk and Some Milk Products. 1972. Ph.D. Thesis. University of Wisconsin, Madison, Wisconsin.
- (64) Park, Hong Sik, Marth, E. H., Goepfert, J. M. and Olson, F. F. The Fate of Salmonella typhimurium in the Manufacture and Ripening of Low-Acid Cheddar Cheese. 1970. J. Milk Food Technol. 33:280-84.
- (65) Park, Hong Sik and Marth, E. H. Behavior of Salmonella typhimurium in Skim Milk During Fermentation by Lactic Acid Bacteria. 1972. J. Milk Food Technol. 35:482-88.
- (66) Park, Hong Sik and Marth, E. H. Survival of Salmonella typhimurium in Refrigerated Cultured Milks. 1972. J. Milk Food Technol. 35:489-95.
- (67) Potashnik, L., Sklan, D. Rosen, B. and Gordin, S. Coliforms and Keeping Quality of a Streptococcus lactis Based Cultured Milk Product. 1972. J. Milk Food Technol. 35:651-54.
- (68) Price, R. J. and Lee, J. S. Inhibition of Pseudomonas Species by Hydrogen Peroxide Producing Lactobacilli. 1970. J. Milk Food Technol. 33:13-18.
- (69) Prost, E. and Riemann, H. Food-borne Salmonellosis. 1967. Ann. Review Microbiol. 21:495-528.
- (70) Radulovic, Draginja. The Effect of Lactobacillus bifidus on Escherichia coli 0111B₄. 1969. Mikrobiologica. 6:225-33.
- (71) Randolph, H. E. and Gould, I. A. Characterization of the Natural Inhibitors in Skim Milk Affecting Lactic Acid Bacteria. 1968. J. Dairy Sci. 51: 8-15.

- (72) Reddy, G. V. and Shahani, J. M. Isolation of an Antibiotic from Lactobacillus bulgaricus. 1971. J. Dairy Sci. 54:748.
- (73) Rubin, H. E. and Love, S. H. Production of a Precursor to the Pyrimidine Moiety of Thiamine. 1976. Appl. Environ. Microbiol. 31:936-41.
- (74) Samson, F. E., Katz, A. M. and Harris, D. L. Effects of Acetate and Other Short-Chain Fatty Acids on Yeast Metabolism. 1955. Arch. Biochem. Biophys. 54:406-23.
- (75) Sandine, W. E., Muralidhara, K. S., Elliker, P. R. and England, D. C. Lactic Acid Bacteria in Food and Health: A Review with Special Reference to Enteropathogenic Escherichia coli as well as Certain Enteric Diseases and Their Treatment with Antibiotics and Lactobacilli. 1972. J. Milk Food Technol. 35:691-702.
- (76) Scheusner, D. L., Hood, I. L. and Harmon, L. G. Effect of Temperature and pH on Growth and Enterotoxin Production by Staphylococcus aureus. 1973. J. Milk Food Technol. 36:249-52.
- (77) Silberstein, W. and Gerichter, C. B. Salmonellosis in Israel. 1964. Monogr. Biol. XII: The World Problem of Salmonellosis. 335-52.
- (78) Sims, J. E., Kelley, D. C. and Foltz, V. C. Effects of Time and Temperature on Salmonellae in Inoculated Butter. 1969. J. Milk Food Technol. 32:485-88.
- (79) Sklan, D., Rosen, B., Keller, P. and Gordin, S. Fate of Coliform Bacteria in Buttermilk. 1974. J. Milk Food Technol. 37:99-100.
- (80) Sorrells, K. M. and Speck, M. L. Inhibition of Salmonella gallinarum by Culture Filtrates of Leuconostoc citrovorum. 1970. J. Dairy Sci. 53:239-41.
- (81) Speck, M. L. Interactions of Food Starter Cultures and Food-Borne Pathogens: An Introductory Statement. 1972. J. Milk Food Technol. 35:306.
- (82) _____. Control of Food-Borne Pathogens by Starter Cultures. 1972. J. Dairy Sci. 55:1019-22.
- (83) Subramanian, C. S. and Marth, E. H. Multiplication of Salmonella typhimurium in Skim Milk with and without Added Hydrochloric, Lactic and Citric Acids. 1968. J. Milk Food Technol. 31:232-326.

- (84) Vedamuthu, E. R., Reinbold, G. W. and Hammond, E. G. Inhibitory Activity of Acid and Rennet Whey on Propionibacteria. 1968. J. Dairy Sci. 51:503-10.
- (85) Vedamuthu, E. R., Washam, C. J. and Reinbold, G. W. Isolation of Inhibitory Factor in Raw Milk Whey Active Against Propionibacteria. 1971. Appl. Microbiol. 22:552-56.
- (86) Waddell, W. J. and Butler, T. C. Calculation of Intracellular pH From the Distribution of 5,5-dimethyl-2,4-oxacolidinedione (DMO). Application to Skeletal Muscle of the Dog. 1959. J. Clin. Invest. 38:720-729.
- (87) Webb, Byron H., Johnson, Arnold H. and Alford, John A. Fundamentals of Dairy Chemistry. 1974. 2nd ed. The Avi Publishing Co., Westport, Conn.
- (88) Weiner, N. and Draskoczy, P. The Effects of Organic Acids on the Oxidative Metabolism of Intact and Disrupted *E. coli*. 1961. J. Pharmacol. Exper. Therap. 132:299-305.
- (89) Westoff, D. C. and Engler, T. The Fate of Salmonella typhimurium and Staphylococcus aureus in Cottage Cheese Whey. 1973. J. Milk Food Technol. 36: 19-22.
- (90) Willard, Hobart H., Merritt, Lynn L. Jr., and Dean, John A. Instrumental Methods of Analysis. 1974. 5th ed. D. Van Nostrand Co., New York.