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THE UNIVERSITY OF ALBERTA

RELIABILITY OF A SELECTIVE MEDIUM FOR THE ENUMERATION OF *ESCHERICHIA COLI* EXPOSED TO CHEMICAL PRESERVATIVES

by

© SIWAPORN SIWAWEJ

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

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EDMONTON, ALBERTA

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled "Reliability of a selective medium for the enumeration of *Escherichia coli* exposed to chemical preservatives", submitted by Siwaporn Siwawej in partial fulfilment of the requirements for the degree of Master of Science, in Foods.

Michael E. Stiles
.....
Supervisor

.....

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Date April 27, 1973

ABSTRACT

In this study, two strains of *E. coli* were exposed to allowable food preservatives, in different growth media, and were enumerated on selective and nonselective plating media. The preservatives used in the study were intended primarily for the inhibition of yeasts and molds, yet preservative, growth medium and preservative-growth medium interaction effects, influenced the reliability of the counts on violet red bile agar. The addition of NaCl and skimmilk to the growth media, with and without preservatives, affected the reliability of the counts. However the addition of up to 3% agar had no effect on the counts. Although methyl paraben influenced the growth curves, colony counts on violet red bile agar grown in media containing methyl paraben, appeared to be more reliable, when compared to counts on nutrient agar. The study emphasizes the need for improved media for the selective growth of coliform bacteria, because of the need to enumerate coliform bacteria for some food standards.

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INTRODUCTION

Detection and enumeration of pathogenic, indicator and spoilage types of microorganisms is important to the safety and keeping quality of food supplies. Selective media have been developed to distinguish and enumerate certain microorganisms. Ideally, a selective medium should give counts equivalent to a non-selective medium. However, results from studies of the reliability of many selective media have indicated that some selective media partially inhibit the growth of the selected microorganisms. Injury induced by freezing, heating, irradiation and chemical treatments commonly used in the processing and preservation of foods is of particular concern for the food microbiologist. Such injury is evidenced by a prolonged lag phase, a narrower pH and temperature range permitting growth, more fastidious nutrient requirements and a greater sensitivity of injured cells to inhibitors and selective agents. Thus tests for microoganisms are generally less reliable after processing or chemical treatments have been applied to a food. Food-borne pathogens retain their pathogenicity after they recover from injury, so that the reduced ability of selective media to support the growth of injured bacteria reduces the effectiveness of the tests and could have serious public health consequences.

The main selective agent used in media for the detection and enumeration of coliforms is bile salts, or the purified bile salt, sodium desoxycholate. Various workers have shown that exposure of coliform organisms to adverse conditions, causes a large proportion of the cells to become sensitive to selective media containing bile salts. Tests for coliform organisms are widely used for assessing the sanitary quality of foods and water. Public health standards require the detection of small numbers of these organisms, since the presence of coliforms in heat processed foods suggests post-processing contamination and poor sanitary control in the food plant. Certain species may be of faecal origin which suggests the possibility that enteric pathogens may also be present.

The problem of evaluating samples with low numbers of coliform organisms is confounded by the cells having been exposed to various kinds of injury. Some types of injury have been extensively studied, but information on the injury of coliforms by chemical preservatives is limited.

Chemical preservatives may be used where their use is permitted by the food regulations. Chemical preservatives are generally considered to be those chemicals which, when added to food, prevent or retard its deterioration. In this study, such preservatives are not

considered to include common salt, sugars, vinegars, spices and their oils or those substances incorporated into food by direct exposure to the product, even though natural preservatives may have the same general action as chemical preservatives. The Canadian Food and Drug regulations (National Health and Welfare, 1972) lists the concentration of preservatives that may be added to specified foods. These include the acids and salts of benzoate, propionate, sorbate, the parabens, and sulfurous acid; also sodium dithionate and sodium diacetate.

The foods generally preserved with chemical preservatives include fruit juices, carbonated beverages, pickles, relishes, jams, preserves, etc. In Alberta there has been concern about the keeping quality of cottage cheese. A study by Huang (1969) suggested that chemical preservatives such as potassium sorbate and sodium propionate might be used to prolong the shelf life of cottage cheese. Although there is controversy about the advisability of using preservatives in many foods, the possible effect that preservatives might have on the detection of indicator microorganisms warrants study.

OBJECTIVE

It is well recognized that physical and chemical treatments intended to inhibit or kill specific microorganisms frequently cause injury of surviving cells. The object of this study was to study injury by chemical preservatives, using the counts on nonselective and selective media after exposure of *Escherichia coli* to chemical preservatives as the measure of injury.

The preservatives selected for the study were not necessarily intended for the inhibition of *Escherichia coli*, but were selected from those allowable in terms of the Canadian food and drug regulations. The growth media containing the preservatives will also be modified with the object of simulating conditions that might exist in some foods.

REVIEW OF THE LITERATURE

Injury caused by heat treatment

Heat injury may be manifested by changes that result in more fastidious growth requirements of the heated cells. These have been demonstrated as:-

(i) pH and temperature of recovery medium. Heated cells have been shown to recover within a narrower pH range (Nelson, 1956; Heather and Vanderzant, 1957a; Allwood and Russell, 1966; Iandolo and Ordal, 1966; Clark, Witter and Ordal, 1968). Similarly, growth was limited to a narrower temperature range after heat treatment of bacterial cells (Heather and Vanderzant, 1957a; Macauley, Hawirko and James, 1963; Clark, Witter and Ordal, 1968).

Ordal (1970) reviewed the effect of NaCl and other permitted food preservatives on the growth of thermally stressed cells. He reported that the temperature range in which the stressed cells were able to repair themselves, and then multiply in the presence of permitted food preservatives, was narrower for heat damaged than unheated cells.

(ii) Reduced oxygen demand. Hershey (1939) found that the oxygen consumption of heat treated cells was decreased by about 90%. Other workers (Baird-Parker and Davenport, 1965; Harries and Russell, 1966) showed that heat treated cells required less oxygen for growth,

since higher recoveries were obtained by the pour plate technique, than by the surface plate technique. This could indicate the loss of an enzyme such as catalase.

(iii) Prolonged lag phase. Heated bacteria exhibited an extended lag phase of growth in various recovery media. The lengthening of the lag phase depended on the temperature to which the cells were heated (Hershey, 1939; Lawton and Nelson, 1955; Kaufmann, Harmon, Pailthorp and Pflug, 1959; Jackson and Woodbine, 1963; Strange and Shon, 1964; Iandolo and Ordal, 1966; Allwood and Russell, 1969).

(iv) More fastidous nutrient requirements for growth. Microorganisms which survived heat treatment frequently exhibited greater demands for nutrients than unheated cells. Beef infusion agar gave higher counts of heat treated bacteria than a synthetic medium (Nelson, 1943). Counts of heat treated cells were greatly increased when tryptone was added to the synthetic medium (Nelson, 1944). Heather and Vanderzant (1957b) showed that the incorporation of yeast extract in the growth medium increased the counts of heat treated *Pseudomonas fluorescens*. Similar results were obtained by Russell and Harris (1968) for heat treated *Escherichia coli*. Allwood and Russell (1966) noted increased counts when either glucose or phosphate was added to nutrient agar, used for the enumeration of heated *Staphylococcus aureus*.

They also reported that added glucose or yeast extract decreased the length of the lag phase, indicating that these compounds stimulated growth of heated cells (Allwood and Russell, 1969). Heat treated *Streptococcus faecalis*, in a synthetic medium, recovered at a much slower rate than in a complex medium (Clark, Witter and Ordal, 1968). Subsequently, they reported that 90% of heated *Salmonella typhimurium* cells were unable to reproduce on Levine eosin methylene blue agar containing 2% NaCl, but that they grew at a rate equivalent to that of normal cells when incubated in tryptic soy broth (Clark, Witter and Ordal, 1969). A similar finding was observed, with heated *Salmonella typhimurium* incubated in a fresh citrate medium (Tomlins and Ordal, 1971).

(v) Increased sensitivity to selective agents. Sublethal heat treatment of *Staphylococcus aureus* was shown to inhibit the ability of heated cells to form colonies on Staphylococcus medium No. 110 (Busta and Jezeski 1963; Dabbah, Moats and Edwards, 1971). Increased concentrations of salt resulted in decreased counts (Busta and Jezeski, 1963; Stiles and Witter, 1965). Similar results were reported for *Streptococcus faecalis* (Beuchat and Lechowich, 1968) and for *Escherichia coli* (Maxcy, 1969, 1970; Dabbah, Moats and Edwards, 1971).

Injury caused by freezing

Freeze injured bacteria also showed a pronounced

increase in the lag phase time for growth, (Squires and Hartsell, 1955; Sinskey and Silverman, 1970). Sinskey and Silverman also found that peptides and casamino acids added to the recovery medium shortened the lag time. The pH range for growth was also limited after bacteria were subjected to low temperature treatment (Matches and Liston, 1972).

Improved growth of bacteria after freezing and thawing was found when cells were plated on enriched media compared to minimal media (Gunderson and Rose, 1948; Hartsell, 1951). The inability of the bacteria to form colonies on simple media was described as metabolic injury (Straka and Stokes, 1959; Arpai, 1962, 1964; Nakamura and Dawson, 1962; Moss and Speck, 1963, 1966a; Sorrell, Speck and Warren, 1970). The metabolic injury of the cells was indicated by the increasing nutritional requirements for growth. Injured cells appeared unable to synthesize some essential metabolites, and supplemental growth substances had to be added to support growth of the injured cells. The addition of trypticase to the growth medium supported the recovery of injured cells (Straka and Stokes, 1959; Moss and Speck, 1966b). The addition of yeast extract to the growth medium was shown to have a similar effect (Arpai, 1964; Ray, Janssen and Busta, 1972; Ray and Speck, 1972). Similarly, the incorporation of meat extract, peptone and casamino acids

was shown to greatly improve the growth of injured cells on the growth medium (Nakamura and Dawson, 1962).

Injury caused by irradiation

There are conflicting reports about the growth requirements of irradiation damaged bacteria. Some workers have concluded that the growth of irradiated cells was better on a chemically defined medium than on complex media, such as nutrient agar (Roberts and Aldous, 1948; Alper and Gillies, 1958 a, b; Nakamura and Ramage, 1963). However, other workers indicated that the number growing on complex media was greater than on defined media (Stapleton, Sbarra and Hollaender, 1955; Freeman and Bridges, 1960; Cook and Widdowson, 1967). These workers attributed the better growth on complex media to the presence of certain factors in the media which support the repair of the injured cells.

The survival of *Escherichia coli* following ultraviolet irradiation was observed to be strongly dependent on the pH of the plating medium (Weatherwax, 1956). The number of colonies decreased as the pH of the medium increased (Alper and Gillies, 1958b). The sensitivity of certain bacteria to NaCl was increased by radiation treatment (Okazawa, Namiki, Yamashita and Matsuyama, 1960). There was a marked effect with *Escherichia coli* but not with *Bacillus subtilis* spores.

Injury caused by chemical treatment

Similar to other types of injury, bacteria injured by chemicals also exhibited an extended lag period before growth. Chloramphenicol, sodium benzoate and potassium sorbate caused an extension of the lag period of *Pseudomonas fragi* before growth in lactose yeast extract broth (Moustafa and Collins, 1969). Unlike heat injury, chemical injury increased the oxygen requirement for growth. Harris and Whitefield (1963) concluded that *Escherichia coli* damaged by phenol, required a higher oxygen content for growth, because counts were substantially lower by the roll tube technique than by the surface plating technique. They also noted that phenol damaged *Escherichia coli* became very sensitive to the temperature of the molten agar.

More exacting nutrient requirements for growth were also observed for chemical injured bacteria. Kaplan, Reilly and Stock (1957) indicated that the viable count for survivors of azaserine treated cells varied with the medium used. Counts were approximately 30 fold greater on M-9 synthetic medium (glucose-NH_4Cl-salts agar) than on heart infusion agar. The addition of heart infusion to M-9 resulted in the expected decrease in numbers of survivors, which was shown to be due to at least two agents in the heart infusion, a neutral molecule and an anionic compound.

on rich media. Up to 1000 times more colonies grew on nutrient agar than on the minimal agar. Bacteria grown in rich media were much more resistant to chlorine compared to bacteria grown in minimal media (Milbauer and Grossowicz, 1959b).

A comprehensive study of the effect of the environmental conditions on the recovery of phenol damaged *Escherichia coli* and *Staphylococcus aureus* was undertaken by Jacobs and Harris (1960). They observed that at temperatures above 37°C the viability of *Escherichia coli* and *Staphylococcus aureus* damaged by phenol declined markedly; and that nutrient broth containing Norit (activated charcoal) reduced both the rate and extent of death of treated cells. Flett, Haring, Guiteras and Shapiro (1945) reported that the presence of ferric chloride or activated charcoal in the subculture medium led to increased extinction times for phenol treated bacteria. Jacobs and Harris (1960) also noted that the addition of cations to the recovery broth (Mg^{++}) favored the survival of phenol damaged *Escherichia coli*, but did not influence the survival of phenol damaged *Staphylococcus aureus*. The type of agar may also be critical (Jacobs and Harris, 1961). Japanese agar was shown to be better for the recovery of phenol treated *Escherichia coli*.

A further study (Harris, 1963) indicated that untreated suspensions were not affected by the nature of the

plating medium, while phenol damaged cells were markedly influenced. High counts of treated bacteria were found on supplemented media, indicating that replacement of leaked materials by nutrients from the medium may be important (Gale, and Taylor, 1947; Salton and Alexander, 1950). Amino acids, nucleic acid residues and growth factors liberated from damaged cells supported this replacement, therefore meat and yeast extracts might also be expected to be more favorable for revival than peptones.

Nutrient agar or media containing yeast extract are usually used in routine testing for the evaluation of the antibacterial activity of surface active compounds (Davis, 1960). These media permitted luxuriant growth, while selective media tended to be inhibitory to growth of damaged cells. Acid injured *Escherichia coli* also exhibited an increased sensitivity to selective media, death rates determined on violet red bile agar were much faster than those established on nutrient agar (Roth and Keenan, 1971).

Scheusner, Busta and Speck (1971 a, b) investigated several strains of *Escherichia coli* injured by sanitizers. Injury could be detected by the differences in counts on a complete and a selective medium. They noted that sanitizer concentration was the main factor in death and injury. Tests on violet red bile agar, indicated

that sodium desoxycholate was the bile salt most inhibitory to damaged *Escherichia coli*. The bile salts mixture alone prevented as many injured cells from growing, as did any combination of the selective agents. The same results were obtained when these dyes and salts were added to minimal agar.

The injury of cells for growth on plating media is caused by many chemical and physical treatments intended for the control of microorganisms. Roth and Keenan (1971) studying the effect of pH on the reliability of cell counts indicated that other conditions or treatments might also cause injury and affect the reliability of cell counts. Hence it seemed justified to study the effect of preservatives intended for the control of yeasts and molds on the reliability of coliform counts.

MATERIALS AND METHODS

Test organisms

Escherichia coli SA211 (*E. coli* B thr⁻leu⁻ilu⁻lac⁻, str-r) and *Escherichia coli* SA603 (*E. coli* K12 F⁻his⁻ T1 res trp⁻str-r) were supplied by Dr. K. Sanderson, Department of Biology, University of Calgary.

Propagation

For the studies on strain 603, except the preservative-salt studies, the cultures were transferred daily into 100 ml sterile Difco tryptic soy broth (TSB) in 250 ml erlenmeyer flasks and incubated in a New Brunswick Gyrotory shaking incubator, shaking at 200 rpm through a 1 inch circular stroke at 37°C. Cultures were checked for purity by microscopic examination of a gram stained smear. The streptomycin resistance of the test organisms was tested periodically by plating the culture on nutrient agar containing 300 µg of streptomycin sulfate (Pfizer) per ml.

The method used by Roth (1969) for propagating the culture, resulted in strain 211 developing two distinct colony sizes when grown on violet red bile agar. Although the amino acid requirements and streptomycin resistance of the cells of both colony types were identical, the method of propagation was altered to avoid these size changes.

For studies with strain 211 and the preservative -

salt studies on strain 603, a freeze dried culture was inoculated into TSB and incubated at 37°C in the shaking incubator for 24 hr. A 0.1 ml aliquot of this 24 hr culture was inoculated into 5 ml sterile TSB, frozen and stored in a freezer at -10°C, according to the method used by Stiles (1963).

For use in experiments, a frozen culture was thawed and incubated at 37°C for 24 hr. A 0.1 ml aliquot was inoculated into 100 ml sterile TSB and incubated in the shaking incubator at 37°C for 16 hr before use in the experiments. This procedure avoided the development of different colony sizes for strain 211.

Growth Experiments

1. Preservative studies

Sodium benzoate (Fisher Scientific Co.) potassium sorbate, methyl *p*-hydroxybenzoate (methyl paraben) and ethyl *p*-hydroxybenzoate (ethyl paraben) (Matheson, Coleman and Bell, Los Angeles, California) were selected as the preservatives for use in this study.

Initial studies in 250 ml erlenmeyer flasks resulted in contamination of the cultures, because of the frequent opening of the flasks. To avoid contamination, 10% (w/v) solutions of sodium benzoate and potassium sorbate were prepared in distilled water and filter sterilized (Millipore bacterial filter, 0.20 μ pore size). The sterile preservative solution was added aseptically to TSB to give

100 ml of 0.1% preservative solution. Because of their low solubility, the paraben solutions were prepared by adding 0.1 gm to 100 ml TSB and sterilizing at 121°C for 15 min. Ten ml aliquots of these preservative solutions were dispensed aseptically into sterile test tubes, and incubated for 48 hr at 37°C in the shaking incubator, before use.

These tubes were then inoculated with 0.1 ml of a 0.01 dilution (in sterile 0.1% w/v peptone water blank) of a 16 hr actively growing culture of the test organism. The inoculated tubes were incubated at 37°C in the shaking incubator.

In addition to the growth experiments in TSB, experiments to simulate conditions that might be experienced in foods were designed. In skimmilk studies, concentrations of 10 and 20% skimmilk solids could be used without excessive caramelization or coagulation as a result of autoclaving. Agar concentrations which did not solidify under the conditions of the experiments or cause excessive foaming, and salt concentrations which allowed growth of the test organisms without the added preservatives, were selected for use.

2. Preservative - skimmilk studies

Potassium sorbate and methyl paraben were used as the preservatives for these studies. The preservatives were added to 100 ml of 10% and 20% (w/v) Carnation

skimmilk powder, reconstituted in TSB to give final concentrations of 0.1% of the preservative in the skimmilk solutions. The skimmilk powder was previously tested for absence of antibiotic effect.

The skimmilk solutions were sterilized by autoclaving at 10 lb pressure for 20 min. The methyl paraben was added to the skimmilk solution prior to autoclaving; the potassium sorbate was prepared as a 10% filter-sterilized solution and added to the skimmilk, aseptically, after autoclaving.

Because of the viscosity of these solutions, the experiments were carried out in 250 ml erlenmeyer flasks. The flasks were inoculated with 0.1 ml of a 16 hr culture of the test organism, and incubated at 37°C in the shaking incubator.

3. Preservative - agar studies

A procedure similar to the preservative - skimmilk studies was followed. Difco agar was added to give final concentrations of 1, 2 and 3% in TSB. Sterilization was carried out by autoclaving at 15 lb pressure for 15 min. After autoclaving, solutions were held at 37°C in the shaking incubator to prevent solidification.

4. Preservative - salt studies

The procedure was the same as the preservative - agar studies, except that NaCl (J.T. Baker Chemical Co.) was added to give final concentrations of 2.0 and 4.05% NaCl,

to give reduced water activities, as described by Scott (1957).

Controls for the growth studies

For each study, TSB with and without the preservatives used for that study, was included as an inter-experiment control. In addition, the appropriate growth media, without preservatives, were used as reference controls to measure the effect of the preservatives on the reliability of the counts.

Sampling

Samples were withdrawn aseptically at times planned to give results for the lag, log, stationary and death phases of growth.

A 1 ml aliquot of the culture was withdrawn and diluted in 99 ml 0.1% (w/v) peptone water blanks to the desired dilution.

Plating

Difco nutrient (NA) and violet red bile agars (VRBA) were used as the plating media for all studies, using the pour plate technique. For VRBA a 3-4 ml overlay was poured on each plate.

For one experiment, using 4 preservatives, Difco tryptic soy (TSA) and desoxycholate agars (DA) were also used for plating the samples.

Appropriate dilutions of the samples were plated in triplicate onto sterile plastic petri dishes (100 x 15 mm),

poured with agar tempered to 45°C, and incubated at 37°C. DA and VRBA plates for strain 603 were counted after 24 hr incubation, all other plates were counted after 48 hr incubation.

Analyses

The means of the viable counts were calculated from the 3 plates for the selected dilution (containing 30 to 300 colonies per plate).

(i) Growth curves. Growth curves were drawn on semilogarithmic graph paper to give a plot of the log of the viable count against time of incubation.

(ii) Growth phases. The growth curves were divided into growth phases by visual inspection of the data. In most cases the sampling procedure did not give more than one reading in each of the lag and log phases of growth. The stationary phase was represented by a series of readings, except where the culture was grown in TSB containing methyl paraben. In this case, there was no stationary phase by this sampling procedure, and the maximum cell count was taken to represent the stationary phase.

The death phase in the preservative studies (section C of results) was divided into two phases for analysis, based on differences in the death rate. This was not applicable to subsequent studies (section D of results).

(iii) Transformation of data. The data was transformed

to natural logarithms (ln) by computer program (Weingardt, 1973 a).

(iv) Linear regression analyses. Linear regression analyses were carried out on the ln transformed data for the counts on each plate (3 plates for each sampling), using the computer program *MULTREGR-Stepwise multiple regression program (Buttels, 1973).

(v) Data tabulation. Means for the stationary phase and the maximum cell count for the cells grown in TSB with methyl paraben were tabulated for comparative analysis. Similarly, the intercepts and slopes were tabulated separately for the death phases.

(vi) Analysis of variance. The data fitted a factorial design and was subjected to analysis by the computer program *ANOFPR (Weingardt,1973 b).

(vii) Duncan's Multiple range test. The Duncan's multiple range test at the 5% level of significance was carried out to determine the source of statistical differences in the data, using the computer program, Function Duncan in STATLIB (a collection of APL functions, from the University of Guelph, Guelph, Ontario).

RESULTS AND DISCUSSION

A. Verification of the test organisms

The propagation procedure for strain 211 was changed during the experimentation, because of a change that occurred in colony size with continued propagation. Before making this change, the streptomycin resistance, amino acid and vitamin requirements and the growth curves of the test strains 211 and 603, as well as the large colony mutant of strain 211, were determined.

Large and small colonies of strain 211 were isolated from a VRBA plate, and grown separately in TSB, under the conditions specified in methods (p. 15). Strain 603 was grown under similar conditions. After 24 hr growth, the cultures were streaked on NA containing filter sterilized streptomycin sulfate (Pfizer, Inc., New York) at a concentration of 300 μg/ml. These plates were incubated at 37°C for 24 hr.

The test strains, including the 211 mutant, grew in the presence of 300 μg streptomycin per ml. Since all strains were streptomycin resistant, their amino acid and vitamin requirements were checked (see test organism strain nutritional requirements, p. 15). To confirm these requirements, solutions of the required amino acids and vitamin were prepared separately, and in combination, as shown in Table 1. They were added to minimal broth (Difco) to give a concentration of 20 μg/ml of each factor. These solutions

Table 1. The amino acid and vitamin requirements for the test strains of *E. coli*, and the requirements of the test strains and the mutant of strain 211.

Amino acid and vitamin requirements[1]:	Strain 211 threonine (thr) leucine (leu) isoleucine (ilu)	Strain 603 folic acid (F) histidine (his) tryptophan (trp)

Amino acid - vitamin media	Strain 211		Strain 603
	small colonies[2]	large colonies[3]	
thr, leu, ilu	+++	+++	
thr, ilu	-	-	
thr, leu	+	+	
leu, ilu	+	+	
F, his, trp			+++
F, his			-
F, trp			+
his, trp			+
Minimal broth	-	-	-

[1]K. Sanderson, University of Calgary, Alberta

[2]Original test strain of *E. coli* SA211

[3]strain 211 mutant

were filter sterilized (millipore filter, 0.20 μ pore size) and dispensed aseptically in 10 ml amounts, and incubated at 37°C for 24 hr before use in the studies.

The test organisms were grown in TSB at 37°C for 24 hr, streaked on NA and reincubated 24 hr. A small portion of the cells was removed with a flamed needle, and diluted in sterile distilled water. The diluted culture was inoculated in duplicate (1 drop, 0.05 ml per tube) into the nutrient solutions.

The amino acid and vitamin requirements of these strains are shown in Table 1, and the results indicated that the strains were those originally obtained for the *E. coli* studies. The large colony forming mutant of strain 211 was genetically similar to the original culture, in its requirement for threonine, leucine and isoleucine, and in its streptomycin resistance.

The growth curves for both colony types of strain 211 were also similar. The large colonies were observed on VRBA plates within 10 days of subculturing in TSB, hence it was decided to alter the system for propagating strain 211, as described in methods (pp. 15 and 16).

B. Inhibitory effect of preservatives for *E. coli*

The inhibitory effect of the selected preservatives (Na benzoate, K sorbate, methyl and ethyl parabens) on the test organisms was studied in TSB containing, separately, 0.1% of each of these preservatives. Only ethyl paraben

had a marked inhibitory effect on the test organisms. Ethyl paraben caused the cultures to die off rapidly, from an initial inoculum of 10^5 organisms per ml to less than 10 organisms per ml, on both NA and VRBA, in approximately 24 hr incubation at 37°C.

The bactericidal effect of ethyl paraben on the test organisms resulted in it being eliminated from subsequent studies. The object of the overall study was to determine if commonly used food preservatives, not intended for the inhibition of *E. coli* would affect the reliability of the count on selective media. In the following study it was decided to incorporate another nonselective medium (TSA) and a selective medium for coliforms (DA). DA was selected because it contains sodium desoxycholate as the inhibitory agent in place of bile salts No. 3, and no crystal violet.

C. Preservative studies

1. *E. coli* strain SA603.

The test organism was inoculated into the growth media: TSB, TSB + 0.1% Na benzoate, TSB + 0.1% K sorbate, and TSB + 0.1% methyl paraben. Samples were withdrawn according to the procedures described, and viable counts determined on NA, TSA, VRBA and DA. The growth curves for this strain in TSB and TSB with benzoate and sorbate were similar, and are represented by the growth curves in Fig. 1. However, in the presence of 0.1% methyl paraben, the growth curves were markedly different to those observed for TSB. Typical growth

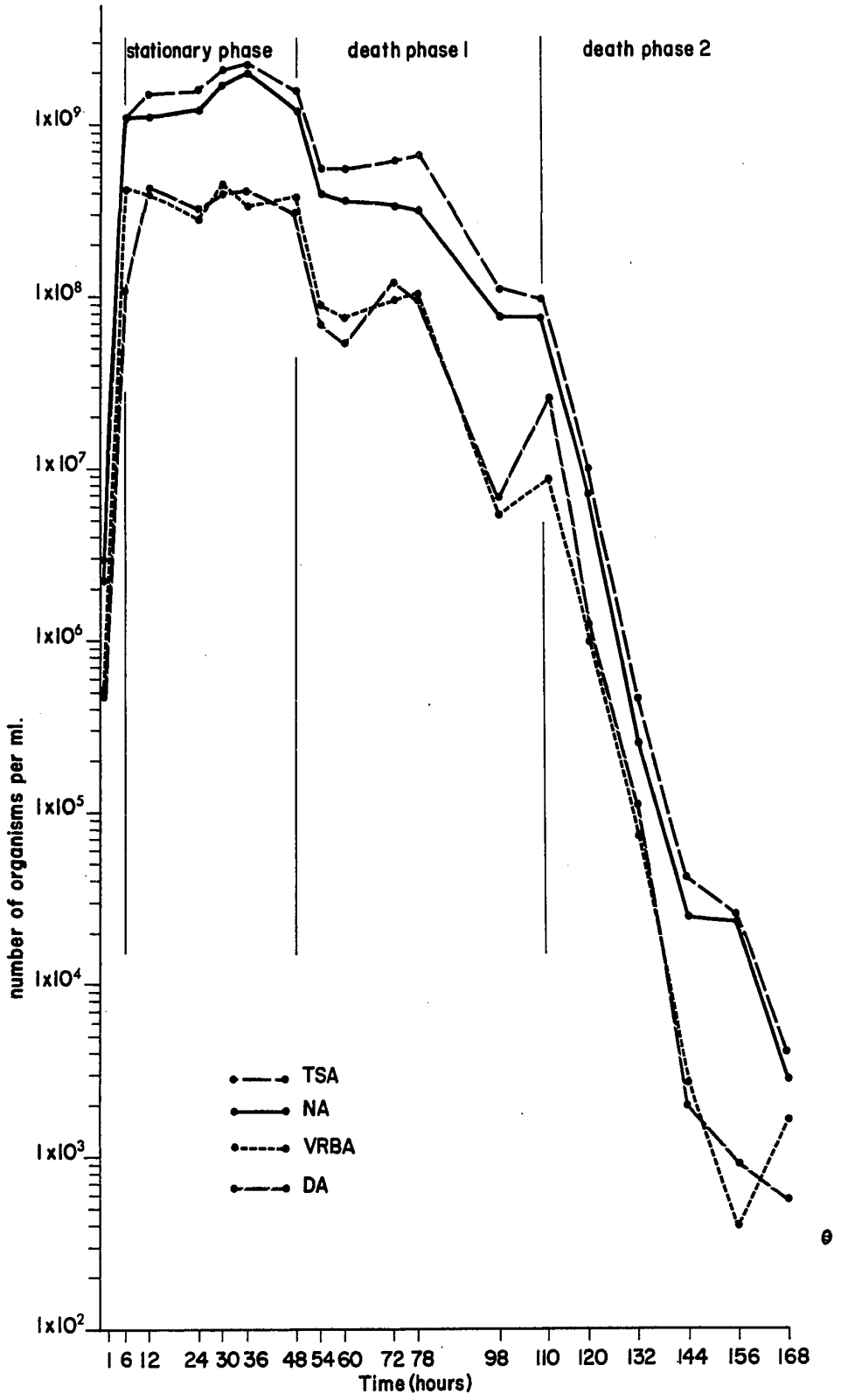
stationary phase
death phase I
death phase 2
1x10⁹
1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
1x10²
number of organisms per ml.
TSA
NA
VRBA
DA
1 6 12 24 30 36 48 54 60 72 78 98 110 120 132 144 156 168
Time (hours)

Fig. 1 Growth curves for *E. coli* SA603 grown in TSB at 37°C and plated onto 4 plating media, and showing the growth phases used for analysis of the data. (Based on duplicate results)

curves in the presence of 0.1% methyl paraben are shown in Fig. 2.

The sampling procedure did not give multiple samples in the lag or log phases of growth. However, differences in the counts of these samples from these growth phases, plated on the selective and nonselective media, were generally equivalent to those observed during the stationary growth phase.

In Fig. 1 it can be seen that the cells were in the stationary growth phase for 30 to 36 hr. However, with this sampling procedure, the cells grown in TSB with methyl paraben did not exhibit a stationary growth phase. Data for the stationary phase were obtained by recording the maximum cell count observed, as illustrated in Fig. 2.

In all cases, the death phase could be divided into (i) an initial slow death phase, and (ii) a subsequent fast death phase. These phases are illustrated in Figs. 1 and 2, and were generally clearly evident by visual inspection of the data.

The means for the data from two replicate trials were subjected to statistical analysis to determine the effect of plating medium and preservatives on the reliability of the colony counts. For the stationary phase, the mean counts and maximum cell count (for methyl paraben), were used in the analysis. For the death phases, both the slopes and the intercepts of the data obtained from the regression analyses were used in the analyses. The data are shown

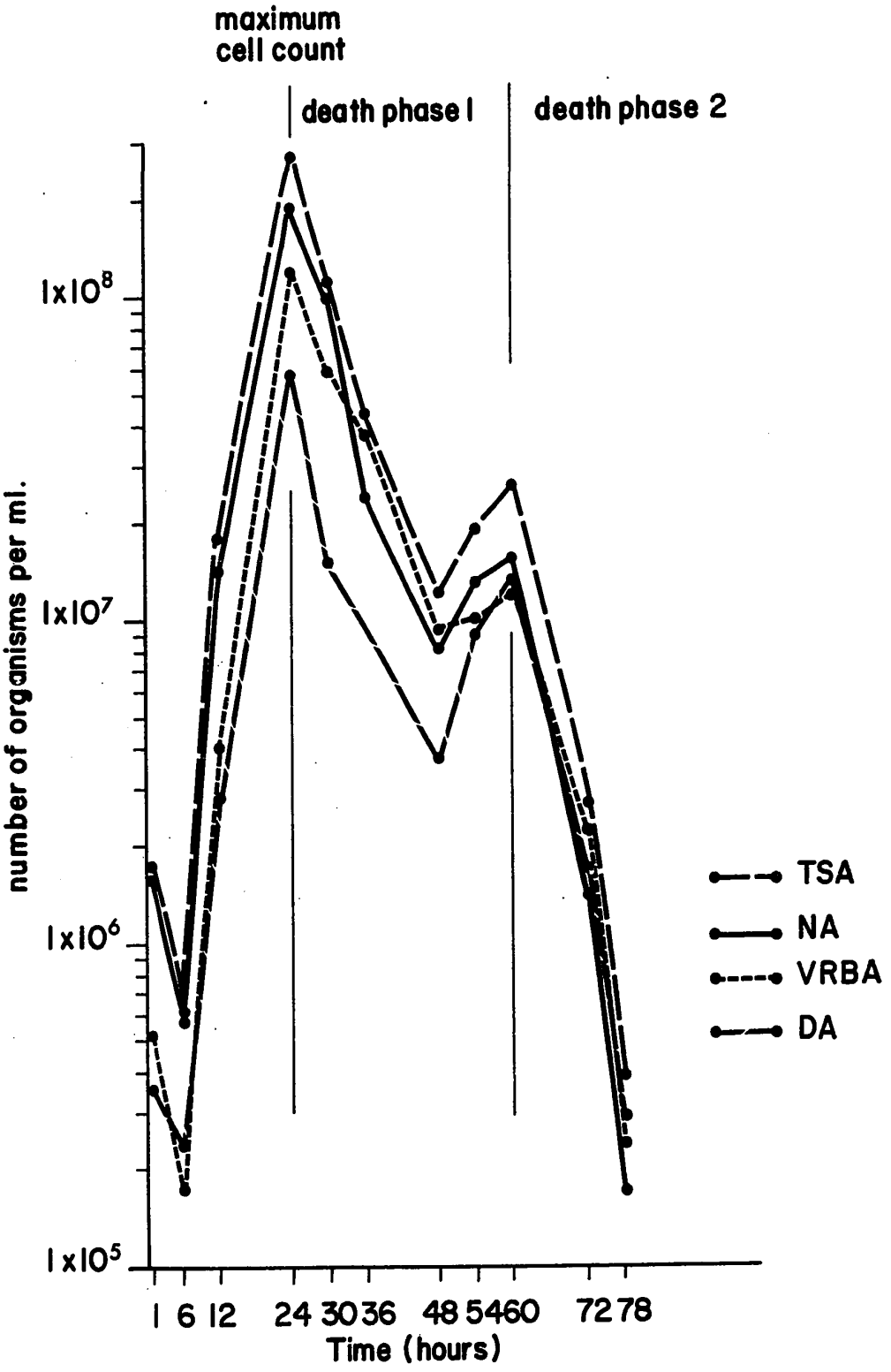
maximum
cell count
death phase I
death phase 2
1x10^8
1x10^7
1x10^6
1x10^5
number of organisms per ml.
1 6 12 24 30 36 48 54 60 72 78
Time (hours)
TSA
NA
VRBA
DA

Fig. 2. Growth curves for *E. coli* SA603 grown in TSB with 0.1% methyl paraben at 37°C, and showing the maximum cell count and death phases used for analysis of the data. (Based on duplicate results)

in Appendix A, Tables 1 to 5.

The summary of the analyses of variance is given in Table 2. Media and preservatives generally had a marked effect on the counts of the cells, but did not have a significant effect on the death rates. The media x preservatives interaction effects were non-significant. These effects were further studied by applying Duncan's multiple range test at the 5% level of probability. The results of the Duncan's test are shown in Table 3. The results are also illustrated as histograms in Fig. 3. Data for the stationary phase means and maximum cell counts (for methyl paraben), and the intercept data for the death phases were transformed to $\log_{10}$ for use in the histograms.

There was no statistical difference between NA and TSA or between VRBA and DA. The only exception to this observation was for the control (TSB) in death phase 2, in which NA, TSA and DA were equivalent and different from VRBA. This unexpected result could possibly be due to the large difference between replicates, which gave a large error mean square.

There was a statistically significant difference between counts on the nonselective media (NA and TSA) and counts on the selective media (VRBA and DA). In general, there was a difference of one log cycle (90%) between the counts of the selective and nonselective media. However,

Table 2. Results of analysis of variance for *E. coli* SA603 grown in TSB and TSB with preservatives.

Source of variation	D.F.	F-value	Probability
Stationary phase			
Media	3	92.38	0.000***
Preservatives	3	125.16	0.000***
Media x Preservatives	9	2.44	0.057
Death: Phase 1 (intercepts)			
Media	3	14.23	0.000***
Preservatives	3	23.91	0.000***
Media x Preservatives	9	0.64	0.745
Death: Phase 1 (slopes)			
Media	3	3.90	0.029*
Preservatives	3	0.27	0.845
Media x Preservatives	9	0.93	0.526
Death: Phase 2 (intercepts)			
Media	3	11.80	0.000***
Preservatives	3	0.93	0.450
Media x Preservatives	9	1.05	0.444
Death: Phase 2 (slopes)			
Media	3	0.15	0.927
Preservatives	3	1.56	0.239
Media x Preservatives	9	0.24	0.982

* $0.05 > p > 0.01$

*** $p < 0.001$

Table 3. Results[1] of the Duncan's multiple range test for differences between plating media, preservatives, and their interaction effects.

		(a) Stationary phase (means)	(b) Death phase 1 (intercept data)	(c) Death phase 2 (intercept data)
Media		V D N T	V D N T	D V N T
Preservatives		P_4 P_3 P_2 C	P_4 P_3 P_2 C	C P_2 P_3 P_4
Media x Preservatives	C	D V N T	D V N T	V D N T
	P_2	D V N T	D V N T	V D N T
	P_3	V D N T	V D N T	D V N T
	P_4	D V N T	D V N T	V D N T

N-nutrient agar
T-tryptic soy agar
V-violet red bile agar
D-desoxycholate agar

C-Control, TSB
P_2-TSB + 0.1% Na benzoate
P_3-TSB + 0.1% K sorbate
P_4-TSB + 0.1% methyl paraben

[1]Values were ranked from the lowest to the highest, and underlined values indicate no significant difference at the 5% level of probability.

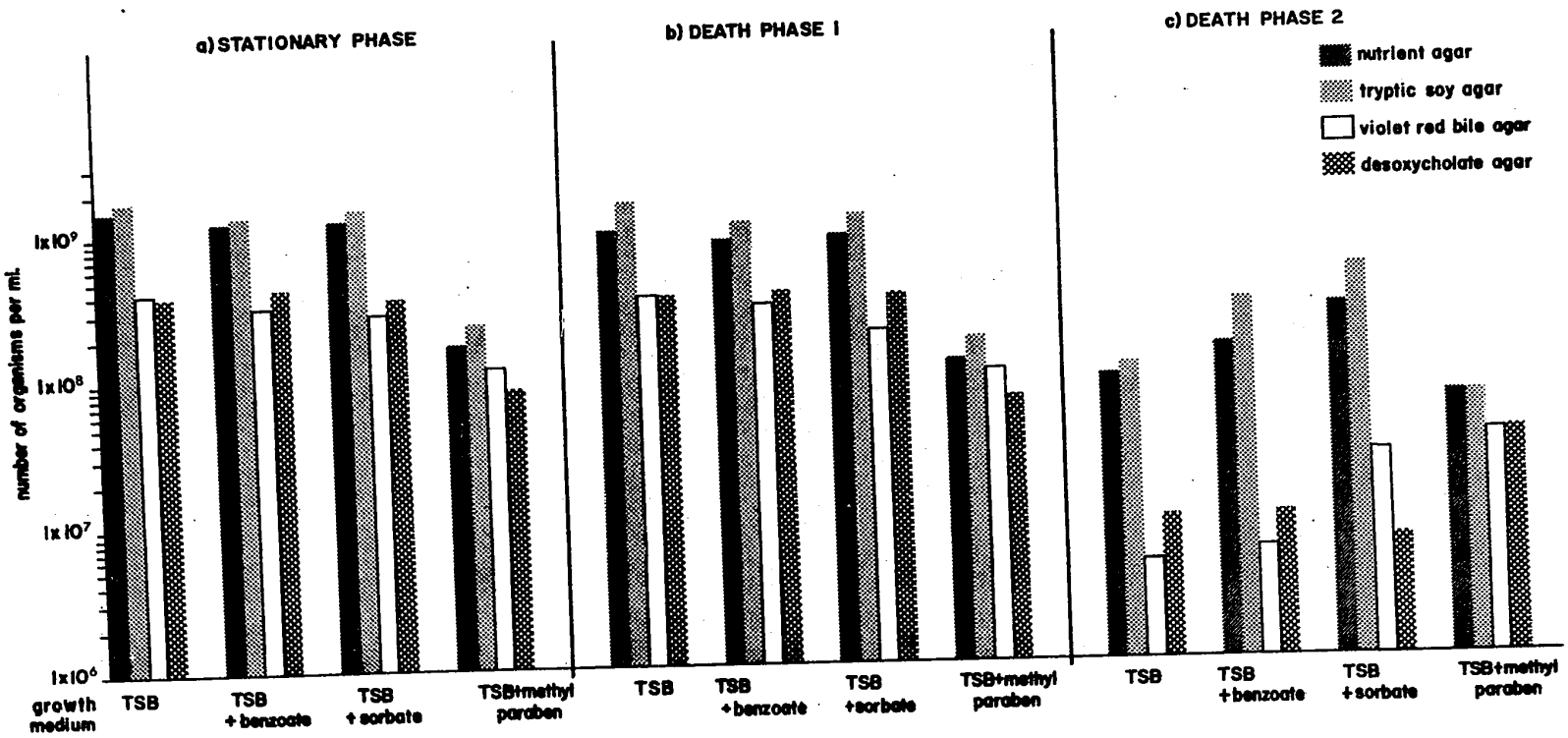
a) STATIONARY PHASE
b) DEATH PHASE 1
c) DEATH PHASE 2
nutrient agar
tryptic soy agar
violet red bile agar
desoxycholate agar
number of organisms per mL
1x10⁹
1x10⁸
1x10⁷
1x10⁶
growth medium
TSB
TSB + benzoate
TSB + sorbate
TSB+methyl paraben
TSB
TSB +benzoate
TSB +sorbate
TSB+methyl paraben
TSB
TSB +benzoate
TSB +sorbate
TSB+methyl paraben

Fig. 3. Log differences in the cell counts for the 3 growth phases studied, showing preservative effects on the reliability of the counts.

cells grown in TSB with methyl paraben gave counts on the selective and nonselective media that differed by less than ½ a log cycle.

The presence of benzoate or sorbate in the growth medium did not influence the growth curves or the reliability of the plating media. In TSB with methyl paraben, however, a marked difference was observed. This is illustrated in Fig. 2. An initial death phase, followed by log growth up to 24 hours, with no stationary phase, distinguished this growth curve from the others. There were 2 distinguishable death phases, but the culture had decreased to the numbers initially inoculated within 80 hr, compared to 120 to 140 hr for the other growth media.

The death rates observed on all media showed little difference as a result of plating medium or preservative. However, from the Duncan's test results on death phase 1 (see Table 4), there was an apparent difference in the death rate on VRBA compared to the other 3 media. Organisms plated on VRBA, grown in TSB and TSB with Na benzoate appeared to be dying at a faster rate than those from other preservative-plating media combinations.

The similarities between the results for NA and TSA and the results for VRBA and DA, indicated that only one nonselective and one selective medium was needed out of the 4 studied. NA was selected as the nonselective medium, despite the fact that slightly higher counts were generally

Table 4. Results of the Duncan's multiple range test for differences between plating media, preservatives and their interaction effects, based on the death rates[1].

		(a) Death phase 1	(b) Death phase 2
Media		V D N T	N V T D
Preservatives		P_2 C P_4 P_3	C P_3 P_4 P_2
Media x Preservatives	(C)	V D T N	N T D V
	(P_2)	V D N T	D V T N
	(P_3)	D V N T	V T N D
	(P_4)	D V N T	N V T D

[1]See Table 3 (p. 31) for the key to, and arrangement of, data in this table.

recorded on TSA. Stiles, Roth and Clegg (1973) had selected NA in preference to TSA because the highest counts were recorded on NA for these strains. This observation conflicted with the observation in this study, and Roth and Keenan (1971) reported no difference between NA and TSA. The selective medium VRBA was selected because of its use in other studies on these strains of *E. coli* (Roth and Keenan, 1971; Stiles *et al.*, 1973).

It was also decided that there was not sufficient difference in the results for both Na benzoate and K sorbate to be included as preservatives. As a result Na benzoate was no longer used as a preservative in these studies.

2. *E. coli* strain SA211

A similar study was carried out for strain 211. However, this strain was only grown in TSB, TSB + 0.1% K sorbate and TSB + 0.1% methyl paraben, and samples were only plated onto two media: NA and VRBA.

The growth curves for strain 211 showed marked differences between replicates. In the first experiment on strain 211, two frozen cultures were taken and propagated separately for use in the growth experiment. The growth curves observed for both TSB and TSB + 0.1% sorbate differed markedly between replicates, however, the replicate growth curves for the organisms grown in TSB + 0.1% methyl paraben were similar.

The experiment was repeated to determine which growth curve was correct for strain 211, and similar differences between replicates were observed. This limits the validity of the results for this strain. The two types of growth curves are shown in Figs. 4 and 5; and the growth curve in TSB + methyl paraben, in Fig. 6.

Despite the differences between replicate trials, this strain gave counts on NA and VRBA in the stationary phase which were relatively close. During the death phase, large differences between the NA and VRBA counts occurred, giving differences of up to 2 log cycles. This appeared to be even greater when the cells were grown in the presence of 0.1% K sorbate. This only occurred between the third and fifth days of growth, thereafter the counts on VRBA were similar to those on NA. After the fifth day, a further growth phase occurred, which in replicate type 1 (see Fig. 4) was followed by another stationary phase and finally death occurred. Throughout the latter phases, counts on NA and VRBA remained similar.

Cells grown in TSB with 0.1% methyl paraben gave counts (see Fig. 6) on NA and VRBA which were relatively close, but deviated as the death phase progressed. The counts differed by up to 1 log cycle, but between the second and third day, a change occurred in which the count on VRBA was greater than the counts on NA. The latter phenomenon was repeated in all 4 replicates grown

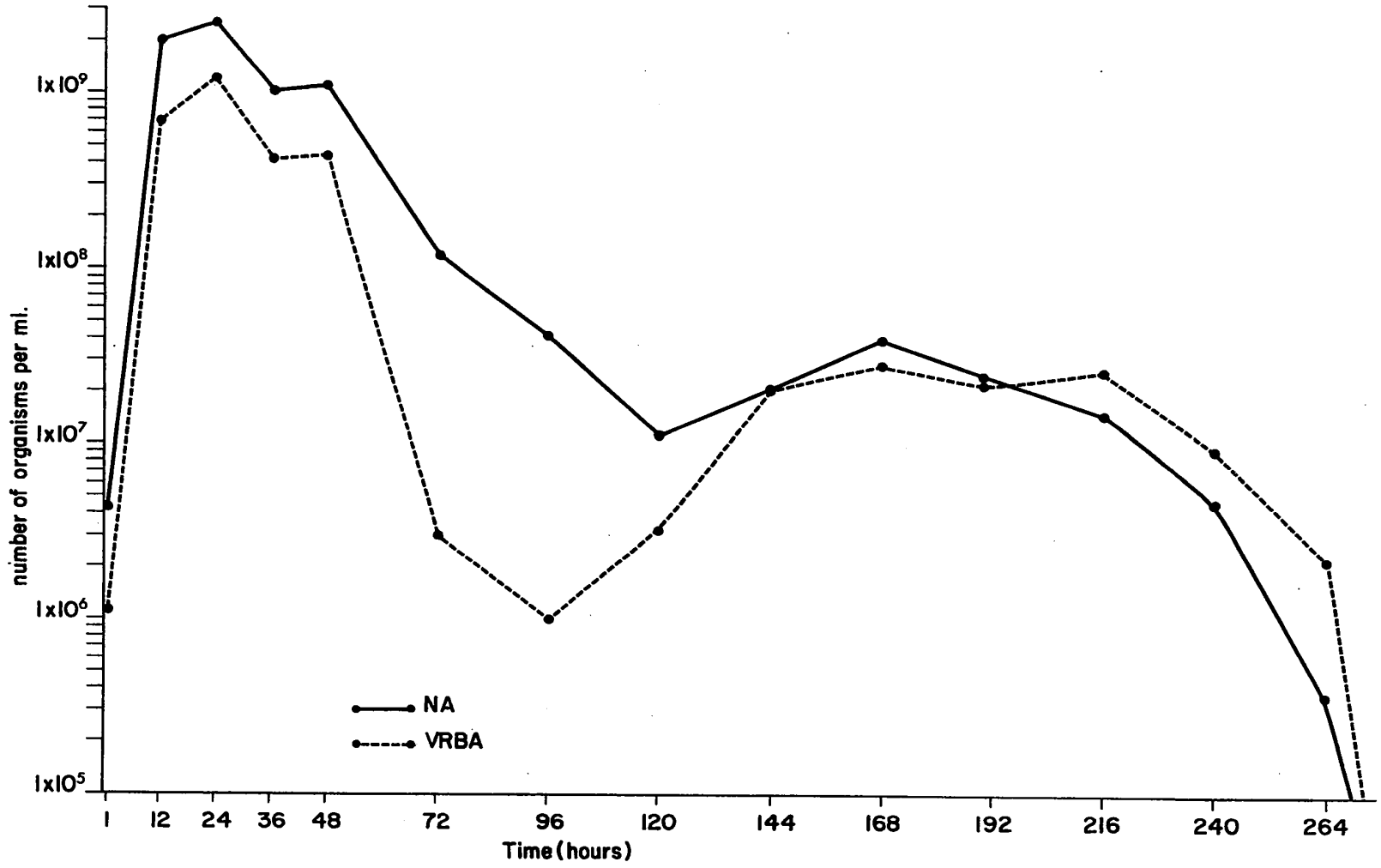
1x10⁹
1x10⁸
1x10⁷
1x10⁶
1x10⁵
number of organisms per ml.
NA
VRBA
1
12
24
36
48
72
96
120
144
168
192
216
240
264
Time (hours)

Fig. 4. Growth curves for *E. coli* SA211 grown in TSB at 37°C, plated on NA and VRBA. Replicate type 1. (Based on results from 4 determinations)

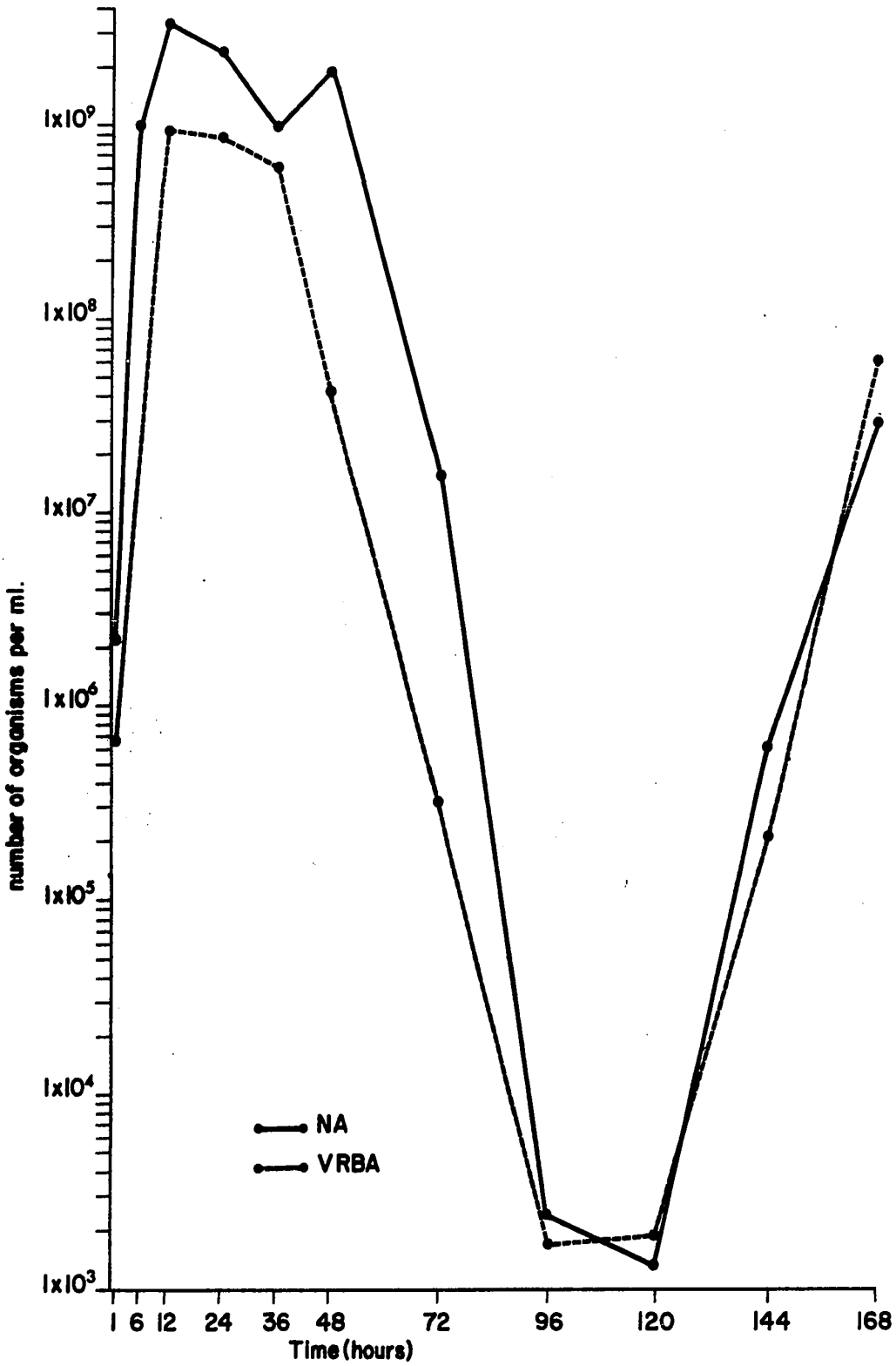
1x10⁹
1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
number of organisms per ml.
NA
VRBA
1 6 12 24 36 48 72 96 120 144 168
Time (hours)

Fig. 5. Growth curves for *E. coli* SA211 grown in TSB at 37°C, plated on NA and VRBA. Replicate type 2. (Based on results from 4 determinations)

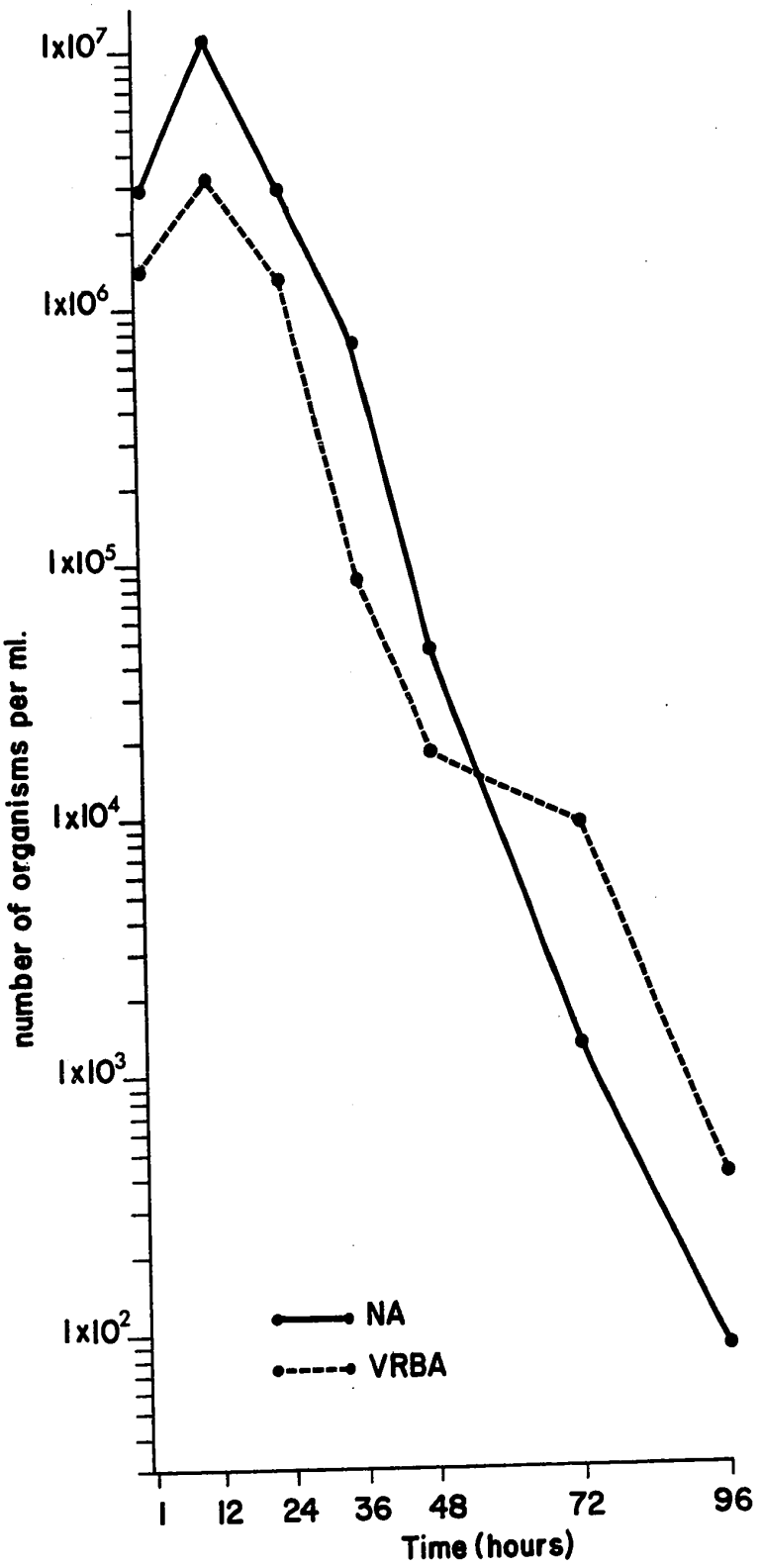
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
1x10^2
number of organisms per ml.
NA
VRBA
1
12
24
36
48
72
96
Time (hours)

Fig. 6. Growth curves for *E. coli* SA211 grown in TSB with 0.1% methyl paraben at 37°C, and plated on NA and VRBA. (Based on results from 4 determinations)

in TSB with methyl paraben.

D. Effect of growth media

Growth medium in this study refers to the liquid medium in which the culture was grown, for example: TSB, TSB + skimmilk, agar or salt. This experiment was designed to determine the effect of the growth media and the effect of preservatives added to these growth media, on the reliability of VRBA for the enumeration of *E. coli*.

The experiment was divided into the 3 sections described in methods (pp. 17-18): skimmilk, agar and salt studies. Each trial was done in duplicate and the results subjected to statistical analysis. The analysis of variance for each phase of growth, based on counts (means and intercepts) and slopes of the death phase were calculated. Significant differences were further studied by the Duncan's multiple range test. Because of the volume of the data, the results will be considered on the basis of growth medium and growth phase.

(a) Preservative - skimmilk studies

1. *E. coli* strain SA603

The culture was grown in TSB with 10 and 20% skimmilk, powder, and in these growth media with 0.1% K sorbate and 0.1% methyl paraben added. Controls in TSB and TSB with the respective preservatives differed from those presented in Fig. 1 (p. 26). Slightly larger differences were observed between the counts on NA and VRBA, and only one

death phase was apparent for TSB and TSB with K sorbate. This change in the growth curves could not be explained, however the experiments to determine the effect of growth media were done in erlenmeyer flasks, compared to test tubes for the preservative studies. The different level of aeration might account for the differences, but it does not account for the similarity in the methyl paraben results.

When skimmilk was used as the growth medium, a much greater difference in the counts on NA and VRBA was observed (generally 2 log cycles difference). Typical data for growth in skimmilk are shown in Fig. 7. The growth curves in skimmilk with 0.1% K sorbate are shown in Fig. 8, and similarly skimmilk with 0.1% methyl paraben are shown in Fig. 9. The similarity between the methyl paraben and the K sorbate results is reflected in the Duncan's multiple range test, as shown in Table 5. This is contrary to observations for the effect of K sorbate in TSB (p.31).

It appears that the growth medium can markedly influence the effect of preservatives on the bacterial cells, so that different recovery responses are noted on the selective medium, VRBA.

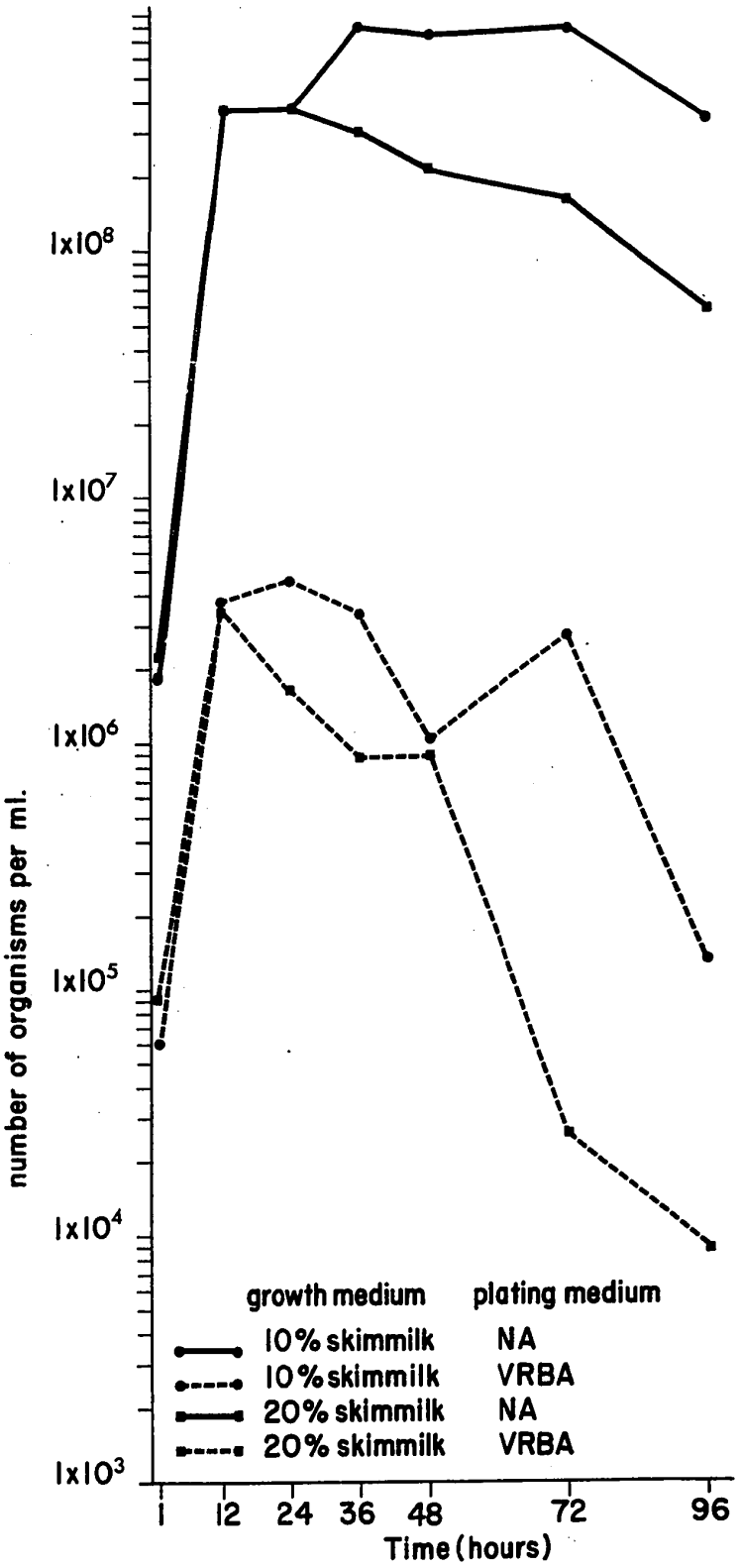
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
number of organisms per ml.
growth medium
plating medium
10% skimmilk
NA
10% skimmilk
VRBA
20% skimmilk
NA
20% skimmilk
VRBA
1
12
24
36
48
72
96
Time (hours)

Fig. 7. Growth curves for *E. coli* SA603 grown in TSB containing 10 and 20% skimmilk powder, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

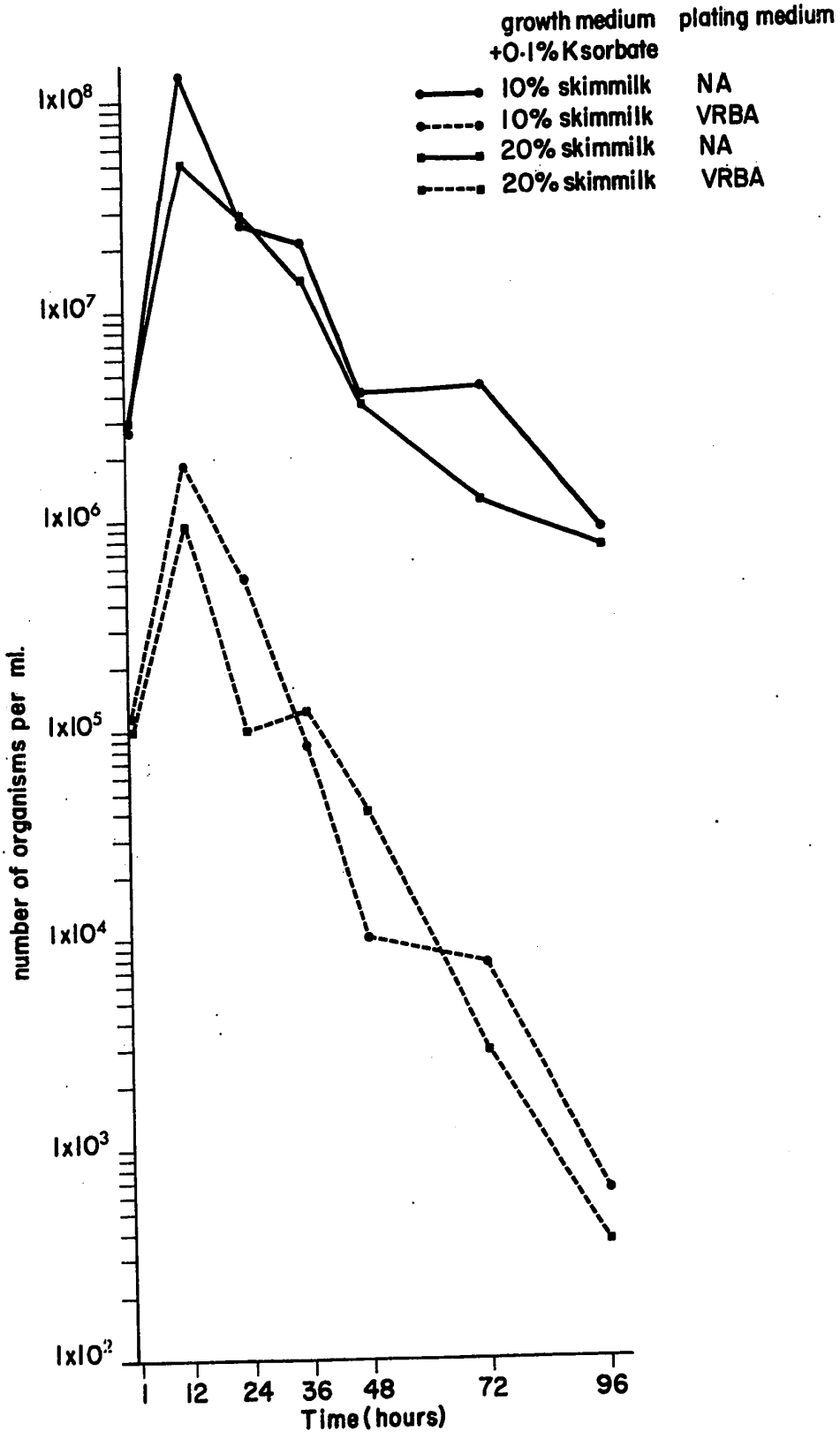
growth medium
+0·1% K sorbate
plating medium
10% skimmilk NA
10% skimmilk VRBA
20% skimmilk NA
20% skimmilk VRBA
1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
1x10²
number of organisms per ml.
1
12
24
36
48
72
96
Time (hours)

Fig. 8. Growth curves for *E. coli* SA603 grown in TSB containing 10 and 20% skimmilk powder and 0.1% K sorbate, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

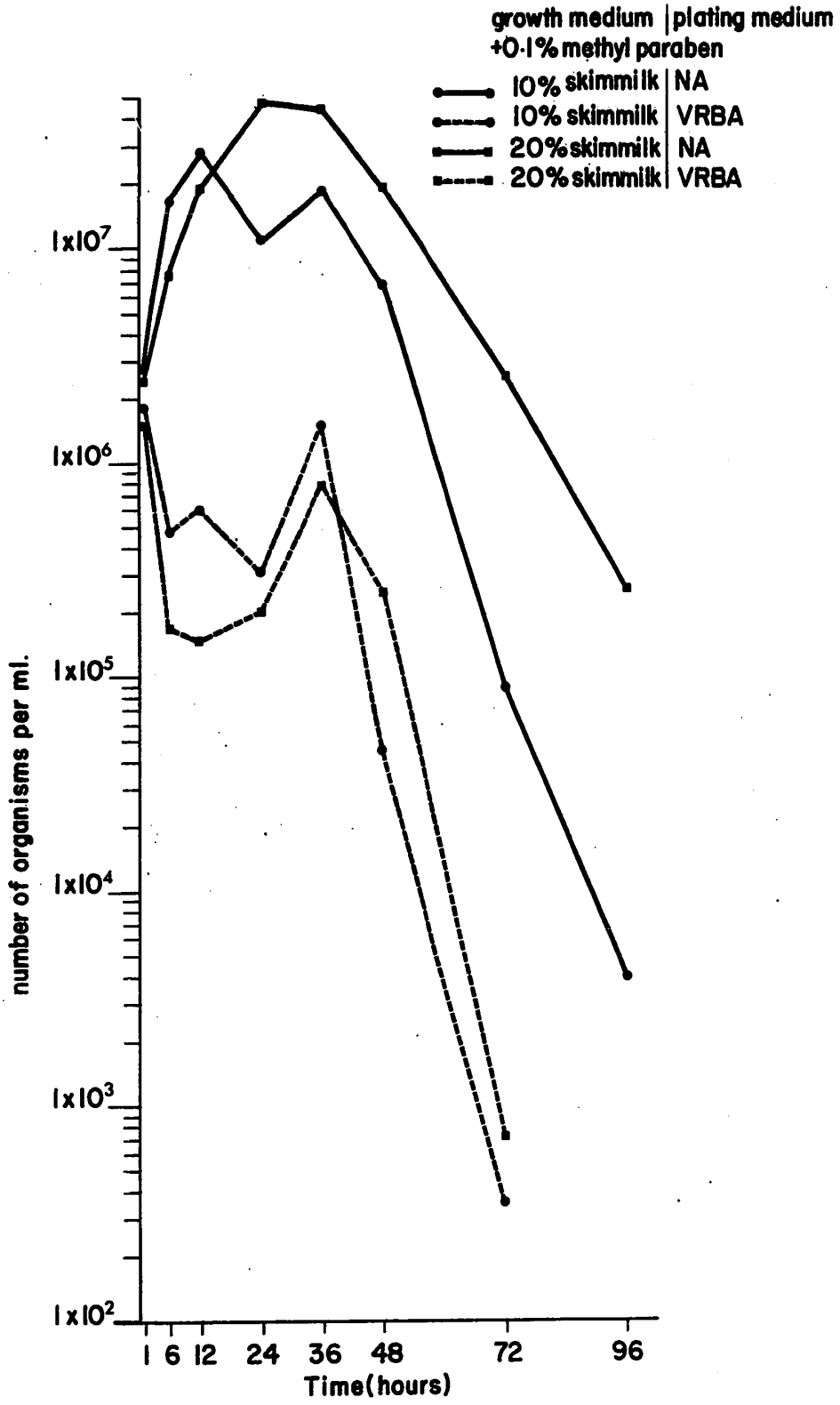
growth medium | plating medium
+0·1% methyl paraben
10% skimmilk | NA
10% skimmilk | VRBA
20% skimmilk | NA
20% skimmilk | VRBA
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
1x10²
number of organisms per ml.
1 6 12 24 36 48 72 96
Time(hours)

Fig. 9. Growth curves for *E. coli* SA603 grown in TSB containing 10 and 20% skimmilk powder and 0.1% methyl paraben, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

Table 5. Results[1] of the Duncan's multiple range test for differences between plating media, preservatives and growth medium.

	(a) Stationary phase	(b) Death phase (intercepts)	(c) Death phase (slopes)
Plating media	V N	V N	V N
Preservatives	P_4 P_3 C	P_4 P_3 C	P_4 P_3 C
Growth media	SM1 SM2 TSB	SM1 SM2 TSB	SM1 SM2 TSB

N-nutrient agar
V-violet red bile agar
SM1-10% skimmilk
SM2-20% skimmilk
C-Control, TSB
P_3-TSB + 0.1% K sorbate
P_4-TSB + 0.1% methyl paraben

[1]Values were ranked from the lowest to the highest, and underlined values indicate no significant difference at the 5% level of probability.

2. *E. coli* strain SA211. A similar experiment to that described for strain 603 was carried out with strain 211. Unlike the results for strain 603 grown in skimmilk, counts on NA and VRBA differed by less than ½ a log cycle, and the stationary phase continued over a period of 26 days, with little overall change in the viable counts or differences between NA and VRBA counts.

For the cells grown in the skimmilk with 0.1% K sorbate, similar results to the skimmilk controls (without preservatives) were observed. During the prolonged stationary phase, the counts on NA and VRBA remained relatively close. However, after the 6th day and for the following 10 days, the count on VRBA exceeded that on NA. In the death phase the count on NA exceeded the count on VRBA again, and some larger deviations between NA and VRBA counts occurred.

The skimmilk growth medium with 0.1% methyl paraben supported more growth of strain 211 than strain 603 (as shown in Fig. 9). The typical growth curve for strain 211 in skimmilk with methyl paraben is shown in Fig. 10. In the death phase, large differences between the NA and VRBA counts (up to 4 log cycles) occurred, indicating marked injury of the dying cells. However, indications were that this experiment was not complete, because the culture might have revived from this death phase and continued growth, as indicated by the upward trend in the final reading on VRBA, on the 5th day in 10% skimmilk (Fig. 10).

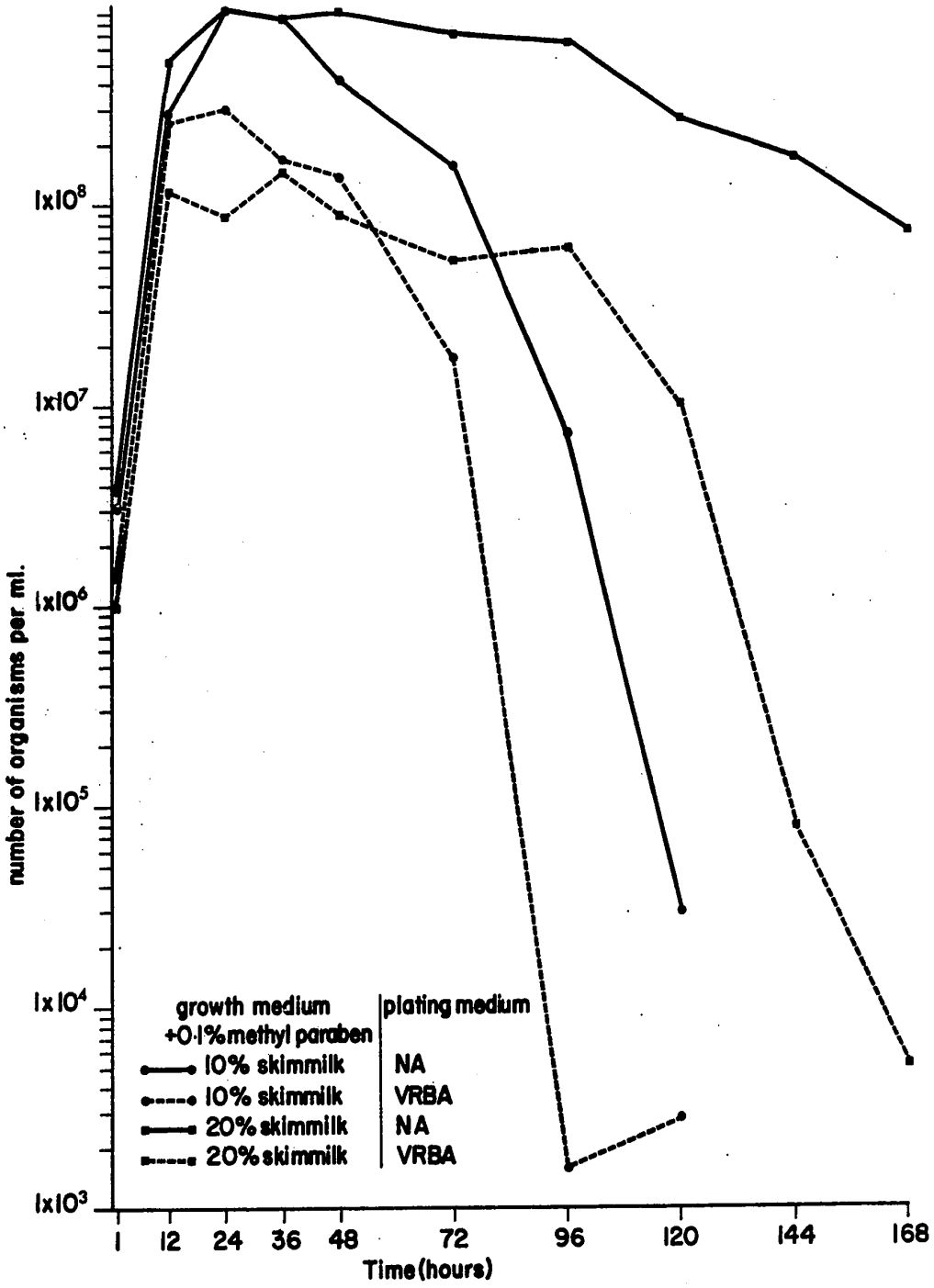
number of organisms per ml.
1x10^8
1x10^7
1x10^6
1x10^5
1x10^4
1x10^3
growth medium
+0·1% methyl paraben
plating medium
10% skimmilk
NA
10% skimmilk
VRBA
20% skimmilk
NA
20% skimmilk
VRBA
1
12
24
36
48
72
96
120
144
168
Time (hours)

Fig. 10. Growth curves of *E. coli* SA211 grown in TSB containing 10 and 20% skimmilk powder and 0.1% methyl paraben, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

This experiment illustrated between strain, as well as between growth medium, and growth medium x preservative interaction effects which markedly influence the reliability of the counts on VRBA. The statistical significance of these effects is indicated in Table 6.

(b) Preservative-agar studies

The culture was grown in TSB with 1, 2 and 3% levels of agar added to the growth medium. The effect of the added preservatives in the presence of these agar concentrations was studied. The agar was added to the growth medium to reduce the water activity and to study the effect of the preservatives under conditions that might be simulated in some foods.

1. *E. coli* strain SA603

The results for the Duncan's multiple range test carried out on the data for this study are shown in Table 7. The expected difference in the counts on NA and VRBA was observed; the presence of sorbate in the growth medium did not influence the results, but methyl paraben caused a significant effect; and there was no significant difference between results obtained for organisms grown in TSB with 0, 1, 2 and 3% agar.

The only change noted as a result of adding agar to the growth medium was in the effect of methyl paraben on the test culture. The results in Table 7 indicate that for the preservative x growth medium interaction effect,

Table 6. Results of analysis of variance for *E. coli* SA211, grown in skimmilk and skimmilk with preservatives.

Source of variation	DF	F-value	Probability
Stationary phase			
Media	1	108.65	0.000***
Preservatives	2	245.73	0.000***
Media x Preservatives	2	2.08	0.154
Growth media	2	81.50	0.000***
Media x Growth media	2	2.47	0.113
Preservatives x Growth media	4	68.20	0.000***
Death phase (intercepts)			
Media	1	33.81	0.000***
Preservatives	2	64.79	0.000***
Media x Preservatives	2	8.47	0.003**
Growth media	2	76.80	0.000***
Media x Growth media	2	2.70	0.094
Preservatives x Growth media	4	26.79	0.000***
Death phase (slopes)			
Media	1	5.38	0.032*
Preservatives	2	7.03	0.006**
Media x Preservatives	2	1.26	0.306
Growth media	2	7.52	0.004**
Media x Growth media	2	0.62	0.549
Preservatives x Growth media	4	3.11	0.041*

* $0.05 > p > 0.01$

** $0.01 > p > 0.001$

*** $p < 0.001$

Table 7. Results of the Duncan's multiple range test for differences between plating media, preservatives and their interaction effects for *E. coli* SA603 grown in 1, 2 and 3% agar and with preservatives added. (Summary of data for Phase 1, and Phase 2 intercepts and slopes)

Media	V	N		
Preservatives	P_4	P_3	C	
Growth medium	3%	TSB	2%	1%
Preservative-growth medium interaction effect				
TSB	P_4	P_3	C	
TSB + 1% agar	P_4	P_3	C	
TSB + 2% agar	P_4	C	P_3	
TSB + 3% agar	P_4	C	P_3[1]	
	C	P_4	P_3[2]	

N - nutrient agar
C - Control, TSB
P_3 - K sorbate
P_4 - Methyl paraben
V - violet red bile agar
1% - TSB + 1% agar
2% - TSB + 2% agar
3% - TSB + 3% agar

[1]Phase 1 and Phase 2 intercept data

[2]Phase 2 slope data

methyl paraben did not have the expected effect in the presence of agar. Even with 3% agar added, the water activity appeared to be insufficiently affected to influence the results.

2. *E. coli* strain SA211.

Because of the lack of an effect on strain 603, agar studies were not done for strain 211.

(c) Preservative-salt studies

Initially the culture was grown in TSB with 3.55 and 7.02% salt added to the growth medium, to approximate water activities of 0.98 and 0.96, reported by Scott (1957). However, at the higher NaCl concentration (7.02%), in TSB, the *E. coli* died rapidly and marked injury (4 log cycles difference) between counts on NA and VRBA occurred. A similar result was observed with sorbate in the 7.02% salt containing growth medium. In the 7.02% salt containing growth medium with methyl paraben added, when a count of 6×10^5 was observed on NA, there was no count on VRBA at 10^{-1} dilution (i.e. with 6×10^4 organisms inoculated per plate).

As a result, the 7.02% NaCl concentration was not studied, and a 1.5% NaCl containing growth medium was included, to indicate an intermediate effect. Total NaCl concentrations in these growth media were 0.50% in the control, 2.00 and 4.05%. These growth media were studied for their effect on the test organisms, with and without

the addition of the 2 preservatives, K sorbate and methyl paraben.

1. *E. coli* strain SA603

The organisms inoculated into TSB with 1.5% added NaCl, and TSB with 1.5% NaCl and 0.1% K sorbate grew normally and gave growth curves comparable to the TSB control for this experiment. The data for growth in TSB and TSB with NaCl are shown in Fig. 11. The growth curves for the salt containing growth media, with 0.1% methyl paraben added, are shown in Fig. 12. Results for growth in the presence of salt and K sorbate were sufficiently similar to the results for the salt controls, that they are not shown in the data.

The effect of increasing salt concentration in TSB can be seen from the growth curves in Fig. 11. Between the control and 1.5% NaCl, the only effect noted was in the death phase. In the presence of 1.5% NaCl, there was a faster death rate, and increased injury of the cells grown on VRBA was observed.

At 3.55% added NaCl a marked difference in the growth curve was observed. There was an initial death phase, in which the inoculated organisms decreased from 1.6×10^6 and 5.0×10^5 organisms per ml on NA and VRBA, respectively, 1 hr after inoculation, to 3.0×10^3 after 6 hr incubation at 37°C. Thereafter, growth occurred with increasing levels of injury were observed on VRBA, up to 2 log cycles

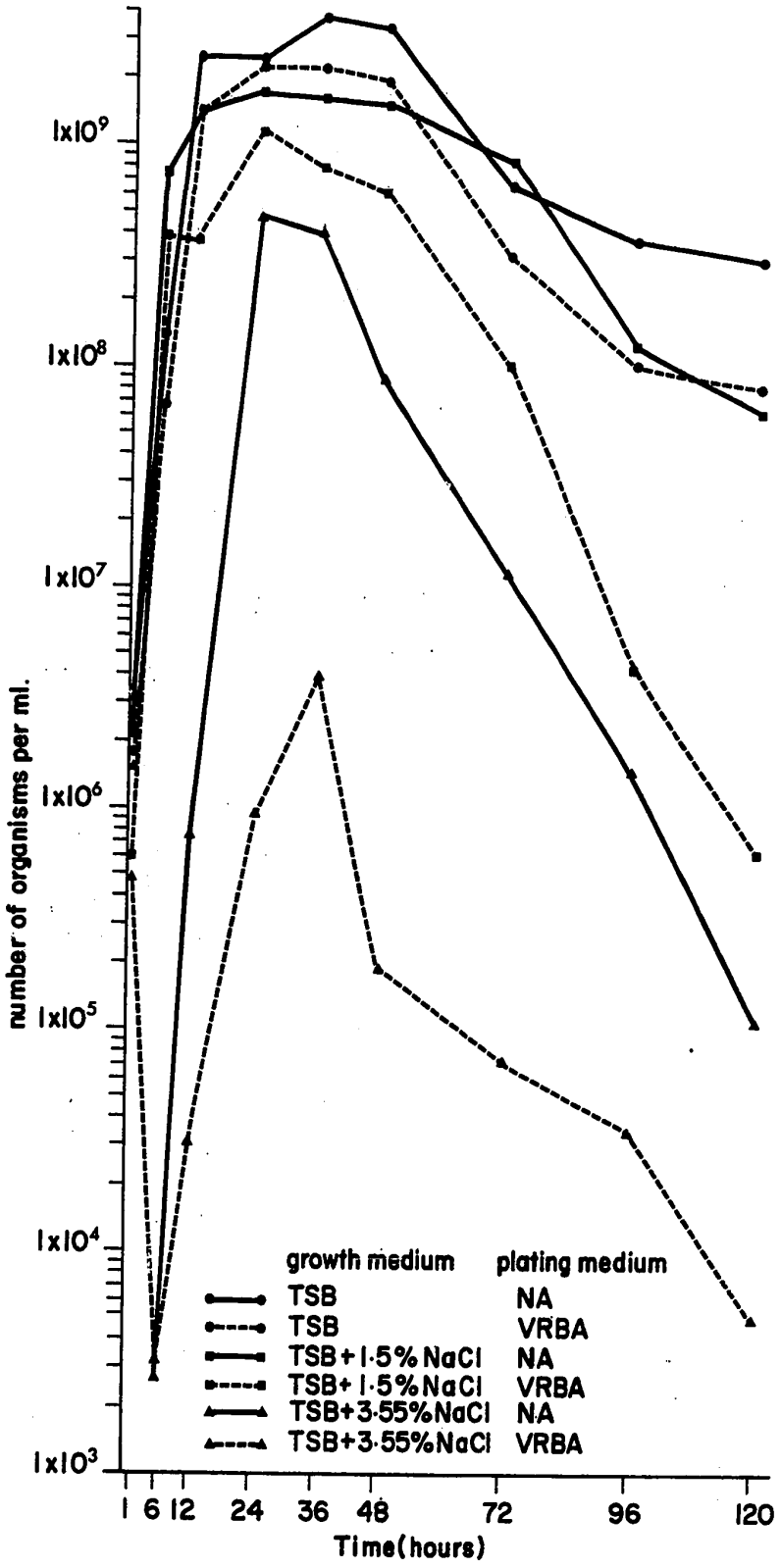
1x10⁹
1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
number of organisms per ml.
growth medium
plating medium
TSB
NA
TSB
VRBA
TSB+1·5%NaCl
NA
TSB+1·5%NaCl
VRBA
TSB+3·55%NaCl
NA
TSB+3·55%NaCl
VRBA
1 6 12 24 36 48 72 96 120
Time(hours)

Fig. 11 Growth curves for *E. coli* SA603 grown in TSB, TSB with 1.5% NaCl and TSB with 3.55% NaCl, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

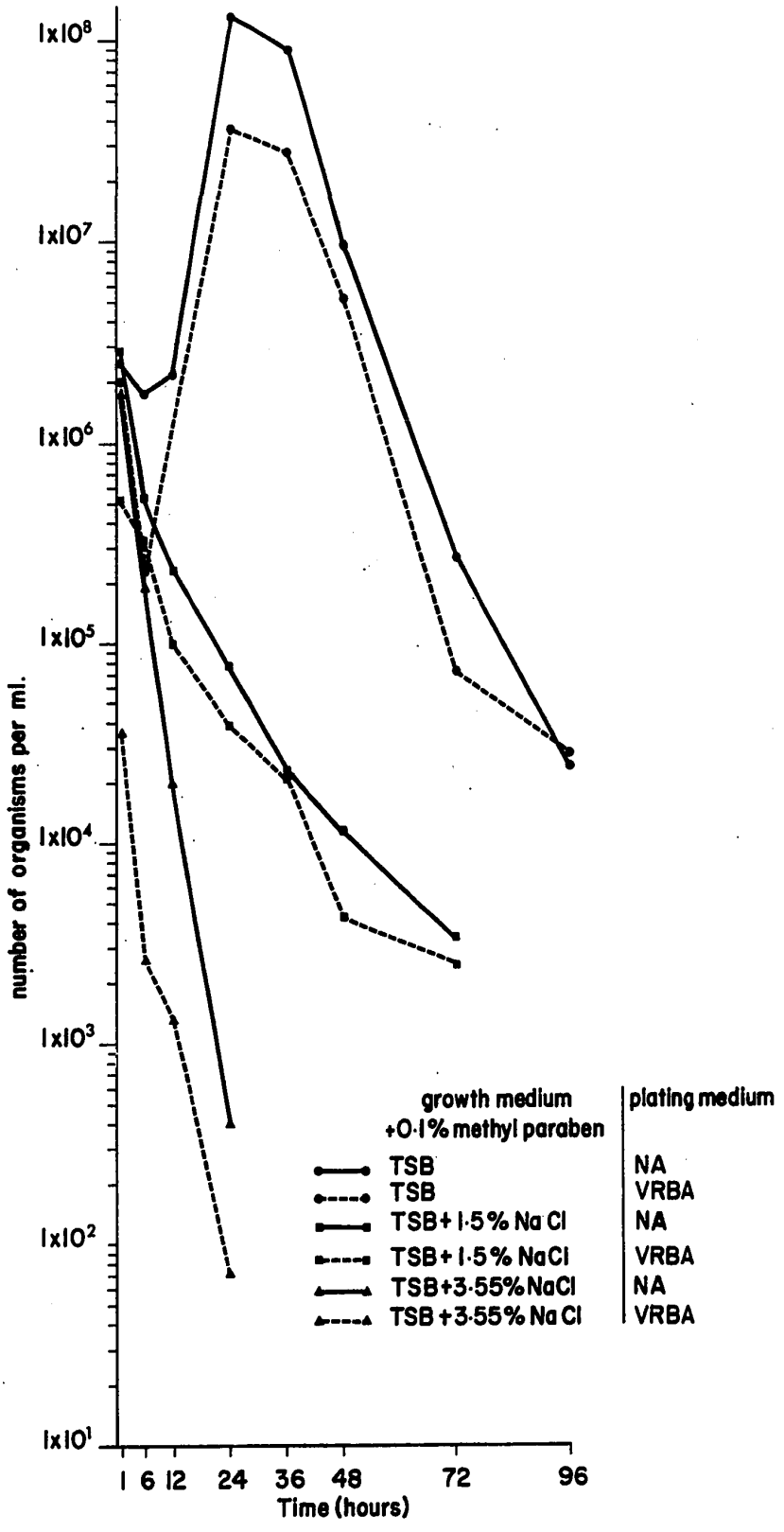
1x10⁸
1x10⁷
1x10⁶
1x10⁵
1x10⁴
1x10³
1x10²
1x10¹
number of organisms per ml.
1 6 12 24 36 48 72 96
Time (hours)
growth medium +0·1% methyl paraben
plating medium
TSB NA
TSB VRBA
TSB+1·5% Na Cl NA
TSB+1·5% NaCl VRBA
TSB+3·55% NaCl NA
TSB+3·55% Na Cl VRBA

Fig. 12 Growth curves for *E. coli* SA603 grown in TSB containing 1.5 and 3.55% NaCl and 0.1% methyl paraben, at 37°C and plated on NA and VRBA. (Based on results from duplicate trials)

difference between the counts after 24 hr incubation. Faster death rates were noted in the presence of the higher salt concentration. The effect of 7.02% added NaCl was referred to in the introduction to this experiment.

The effect of salt in the growth medium in the presence of 0.1% methyl paraben is shown in Fig. 12. In the presence of 1.5 and 3.55% added salt, the culture failed to grow, and increasing salt concentration resulted in a more rapid death rate. However, the counts on NA and VRBA, for the control and in the presence of 1.5% NaCl, were comparable, indicating that injury for growth on VRBA did not occur. At 3.55% NaCl, differences in the count on NA and VRBA, up to 2 log cycles, occurred early in the death phase.

These observations were confirmed by statistical analysis. Results of the Duncan's multiple range test are shown in Table 8. Because growth did not occur at 1.5 and 3.55% salt, in the presence of 0.1% methyl paraben, only the death phase data could be submitted to statistical analysis. From the counts (intercept data) no statistically significant difference was observed between the control and K sorbate data. The death rates indicated some statistically significant results between NA and VRBA, notably with methyl paraben, and in the presence of 1.5 and 3.55% added NaCl. In general, plating medium, added preservative and growth medium significantly influenced the

Table 8. Results of the Duncan's multiple range test for differences between plating media, preservatives and their interaction effects for *E. coli* SA603 grown in 1.5 and 3.55% NaCl and NaCl with preservatives

	Death phase (intercepts)			Death phase (slopes)		
Media	V	N		N	V	
Preservatives	P_4	P_3	C	P_4	C	P_3
Growth media	3.55%	1.5% TSB		3.55%	TSB	1.5%
Media-Preservatives interaction effect:-						
control (TSB)	V	N		V	N	
K sorbate	V	N		V	N	
methyl paraben	V	N		N	V	
Media-growth media interaction effect:-						
control (TSB)	V	N		N	V	
TSB + 1.5% NaCl	V	N		V	N	
TSB + 3.55% NaCl	V	N		N	V	
Preservatives-Growth media interaction effect:-						
control (TSB)	P_4	P_3	C	P_4	C	P_3
TSB + 1.5% NaCl	P_4	P_3	C	C	P_4	P_3
TSB + 3.55% NaCl	P_4	C	P_3	P_4	C	P_3

N - nutrient agar
V - violet red bile agar
C - control, TSB
P_3 - K sorbate
P_4 - Methyl paraben
1.5% - TSB + 1.5% NaCl
3.55% - TSB + 3.55% NaCl

growth of the *E. coli* strain SA603.

2. *E. coli* strain SA211

The results for salt containing growth media with strain 211 differed considerably from results obtained for strain 603. Furthermore, marked, between replicate differences were observed with strain 211. These differences occurred despite the changes in culture propagating procedures, and the concurrent study of experimental replicates. Counts of the cells plated on NA and VRBA from TSB and TSB with 1.5% and 3.55% NaCl, in the death phase, indicated
levels of injury ranging from 1½ log cycles (as shown in Figs. 4 and 5) for growth in TSB, up to 4 log cycles difference in TSB + 3.55% NaCl. Similar between replicate differences to those shown in Figs. 4 and 5 were also observed.

In the K sorbate containing growth media, between replicate differences were observed, but levels of injury were generally greater ranging between 2 and 3 log cycles.

The effect of 0.1% methyl paraben in the TSB growth medium was shown in Fig. 6 (p. 39). With 1.5% NaCl added to the methyl paraben containing growth medium, death was more rapid than that shown in Fig. 6. In the presence of 3.55% NaCl, the death rate was too rapid to be measured with this sampling procedure. The 1 hr sample could only be counted on NA (6.8×10^3), and on VRBA the level of

injury was sufficient that there was no growth at 10^{-1} dilution.

E. Implications

The reliability of the selective medium for the enumeration of *E. coli* was shown to be influenced by the growth phase, growth medium, and in some instances the presence of preservatives in the growth medium. In addition, between strain differences were observed for the test strains used in this study.

The selective medium generally gave lower counts than the nonselective medium, even for untreated cells. This was in agreement with the findings of Maxcy (1969, 1970); Scheusner, Busta and Speck (1971a); Roth, Stiles and Clegg (1973); and Stiles *et al.* (1973). However, Roth and Keenan (1971) noted that counts were similar when untreated cells were plated on selective and nonselective media.

This study indicated that the reliability of the selective medium was less during the death phase than during other phases of the growth cycle. Cells were not only dying, but were also undergoing a form of injury, which limited their ability to grow on the selective medium. Contrary to observations on *Staphylococcus aureus* (Stiles and Witter, 1965) the decreased count on the selective medium did not significantly influence the apparent death rate of the cultures.

The studies indicated little or no influence of Na

benzoate or K sorbate on the reliability of the counts on the selective medium. This could have been due to the pH of the growth medium. The pH of the TSB growth media was around 7.0, and both Na benzoate and K sorbate are most active at lower pH values (Bandelin, 1958; Moustafa and Collins, 1969). Roth and Keenan (1971) indicated that pH had a marked effect on the reliability of the counts on VRBA, hence the pH effect was not included in this study.

For cells grown in the presence of methyl paraben, a markedly different growth curve and a faster death rate was observed, however the reliability of the counts on VRBA appeared to be greater than that observed for the control. The effect of methyl paraben on the growth curves and death rate could be explained by the fact that the parabens are active over a wider pH range (Bandelin, 1958). However, the observation of less injury for growth on VRBA could not readily be explained. This could be due to injury of the cells for growth on NA. An indication that heat injury occurs for growth on NA was shown by Stiles *et al.* (1973). A similar phenomenon might occur in the presence of methyl paraben, bringing the counts on the selective and nonselective media closer than in the controls.

There were a few exceptions where the counts on VRBA exceeded the counts on NA. This was observed during the death phase of strain 211 grown in TSB with 0.1% methyl

paraben, and in the prolonged stationary phase of strain 211 in the skimmilk growth media with 0.1% K sorbate. Again, the best explanation of this phenomenon appears to be the injury of the cells for growth on the nonselective medium (NA).

The growth medium had a marked effect on the reliability of the counts on VRBA, and influenced the growth curves of the cultures. This observation indicated that the reliability of the count could vary with the food in which coliforms are tested. In the skimmilk studies large, between strain differences were noted in the growth curves and the reliability of the VRBA counts. In the skimmilk growth media, water activity would be reduced, so that the effective concentration of the preservatives would be increased. This might account for the results of K sorbate and methyl paraben being similar, whereas K sorbate results for other growth media were similar to the control medium. However, there were no marked differences between the results for the 10 and 20% skimmilk solids in the growth medium and pH values were 6.8 and 6.6, respectively.

The agar containing growth media were incorporated to give another measure of the water activity effect. The concentrations used in these studies, gave no significant differences between the control and up to 3% agar for strain 603. Either these concentrations of agar were

insufficient to affect the concentration of the preservatives, or conversely, there might have been an interaction effect between the agar and the preservatives, which made the preservatives ineffective in the growth medium. This was suggested by the observation that methyl paraben was effective in causing differences in TSB, but it had no effect in the presence of 1, 2 or 3% agar.

In the presence of salt, there was a marked decrease in the reliability of the VRBA counts. In addition, the growth curves were markedly affected for both strains of *E. coli*. In the presence of salt (2.0 and 4.05%), 0.1% methyl paraben was strongly toxic for the two test strains of *E. coli*. These observations indicated that in cured food, the reliability of colony counts on VRBA could be altered. In the presence of methyl paraben and 2.0% salt, the counts on VRBA remained similar to those on NA; however the VRBA count at 4.05% salt was less reliable, indicating a possible influence of salt and preservative on the reliability of the count.

The study incorporating both TSA and NA as non-selective plating media, and DA and VRBA as selective plating media indicated that for these media, either could be selected as the selective and nonselective medium, respectively. This might not apply to the other selective media for coliforms, but this result suggests

that other selective media using bile salts or sodium desoxycholate as the selective agents, might give similar results.

Both K sorbate and methyl paraben are intended primarily as preservatives for yeasts and molds (Chichester and Tanner, 1968), and parabens in particular, were noted as being "less effective against bacteria, especially gram-negative bacteria". Despite this it was shown in these studies that methyl paraben markedly affected the growth characteristics of these strains of *E. coli*, and influenced the reliability of the VRBA counts. This was generally a favorable influence, however, the effect on the NA count would need confirmation before the effect could be reliably interpreted.

In general, these results indicate a need for caution in interpreting coliform counts. It is considered that selective media for coliforms require modification equivalent to those achieved in the selective media for staphylococci.

CONCLUSIONS

The object of these studies was to determine the influence of microbial preservatives on the reliability of coliform counts on selective media. In addition, different growth media were used, and their influence on the reliability of the counts was also recorded. This study indicated that caution should be exercised in the tests for coliforms in food containing preservatives, and emphasizes another aspect of injury that might occur to the cells and cause unreliable counts to be obtained.

Na benzoate and K sorbate appeared to have a negligible effect on the reliability of the coliform counts, yet in the study of sorbate in the presence of milk solids, an effect was noted. Methyl paraben, on the other hand, was shown to have a marked effect on the growth and counts of *E. coli*, and this should be taken into consideration in testing foods for coliforms or *E. coli*.

The study has shown growth medium, preservative and growth medium x preservative interaction effects on the reliability of counts on selective media intended for the enumeration of coliform bacteria. The influence of factors not intended to affect the coliform counts emphasizes the need for improved selective media for coliforms.

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Appendix A

Table 1

Stationary phase (mean of counts)

	N	V	T	D	$\bar{x}_p$
C	21.145	19.831	21.340	19.804	20.530
P_2	20.951	19.958	21.057	19.879	20.371
P_3	21.011	19.460	21.124	19.727	20.331
P_4	18.983	18.568	19.283	18.211	18.761
$\bar{x}_m$	20.523	19.364	20.701	19.405	

C - control (TSB)
P_2 - TSB + 0.1% Na benzoate
P_3 - TSB + 0.1% K sorbate
P_4 - TSB + 0.1% methyl paraben

N - nutrient agar
T - tryptic soy agar
V - violet red bile agar
D - desoxycholate agar

$\bar{x}_m$ - means of counts for each plating medium

$\bar{x}_p$ - means of counts for each growth medium

Appendix A

Table 2[1]

Death phase 1 (intercept data)

	N	V	T	D	$\bar{x}_p$
C	20.749	19.680	21.150	19.648	20.307
P_2	20.517	19.520	20.838	19.680	20.139
P_3	20.611	19.098	20.954	19.654	20.080
P_4	18.619	18.465	19.990	17.976	18.513
$\bar{x}_m$	20.124	19.191	20.483	19.240	

[1]See Table 1, p. 72 for key to the abbreviations

Appendix A

Table 3[1]

Death phase 2 (intercept data)

	N	V	T	D	$\bar{x}_p$
C	18.317	15.399	18.467	16.044	17.057
P_2	18.814	15.556	19.515	16.092	17.494
P_3	19.410	17.051	19.965	15.702	18.032
P_4	17.925	17.334	17.993	17.356	17.624
$\bar{x}_m$	18.617	16.335	18.958	16.298	

[1]See Table 1, p. 72 for key to the abbreviations

Appendix A

Table 4[1]

Death phase 1 (slope data)

	N	V	T	D	$\bar{x}_p$
C	-0.037	-0.077	-0.038	-0.058	-0.052
P_2	-0.040	-0.081	-0.034	-0.067	-0.055
P_3	-0.040	-0.056	-0.038	-0.057	-0.048
P_4	-0.053	-0.054	-0.056	-0.042	-0.052
$\bar{x}_m$	-0.042	-0.067	-0.042	-0.056	

[1]See Table 1, p. 72 for key to the abbreviations

Appendix A

Table 5[1]

Death phase 2 (slope data)

	N	V	T	D	$\bar{x}_p$
C	-0.251	-0.206	-0.244	-0.210	-0.228
P_2	-0.141	-0.157	-0.155	-0.167	-0.155
P_3	-0.198	-0.246	-0.200	-0.164	-0.202
P_4	-0.225	-0.194	-0.189	-0.189	-0.199
$\bar{x}_m$	-0.203	-0.201	-0.197	-0.182	

[1]See Table 1, p. 72 for key to the abbreviations