

A COMPARATIVE EVALUATION OF THE ANTIMICROBIAL
EFFICACY OF SOME SULPHUR DIOXIDE-GENERATING
SALTS IN LIQUID MEDIA

BY

AYODELE ABIODUN AKINSANYA
BSC BIOCHEMISTRY, A.B.U. ZARIA.

A Thesis Submitted in Partial Fulfilment
of the Requirements for the Degree of:

MASTER OF SCIENCE
(BIOCHEMISTRY)

DEPARTMENT OF BIOCHEMISTRY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY
ZARIA - NIGERIA-

MAY, 1983

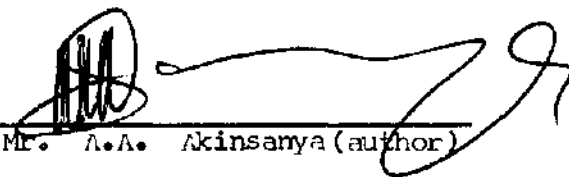
Deservedly dedicated to my mother - FLORENCE. E. AKINSANYA-

a mother to the letter.

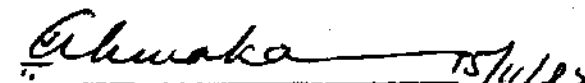
DECLARATION

I declare that this work is wholly the author's and has not been part of any presentation for any other qualification.

Date: 15/11/83

Signed by:- 
Mr. A.A. Akinsanya (author)


Dr. J.A. Abalaka (Supervisor) 15/11/83


Dr. J.A. Abalaka
Head of Department. 15/11/83

ACKNOWLEDGEMENT

I wish to thank Dr. Joseph A. Abalaka, who supervised this work, for his constructive criticism and useful suggestions at every stage of the work.

I wish to express gratitude to the Department of Biochemistry for providing the laboratory facilities. My sincere thanks to my friends and colleagues, too numerous to mention, for increasing my 'entropy'!

ABSTRACT

The lethal efficacy of some sulphur dioxide-generating antimicrobial preservatives in liquid media has been evaluated. These preservatives were sodium sulphite, potassium sulphite, sodium metabisulphite, potassium metabisulphite, and sodium bisulphite. Four bacterial species (Bacillus subtilis, Bacillus cereus, Escherichia coli and Staphylococcus aureus) were used as test organisms.

Results have shown that these antimicrobials may be rated in order of increasing potency as sulphites, bisulphite and metabisulphites. At low pH values, all the preservatives showed higher lethal efficacy thus hydrogen ion concentration has a significant effect on the antimicrobial efficacy of these preservatives. Similarly, factors such as the inoculum size of test organisms, media composition, and time influenced the potency of the sulphur dioxide-generating salts.

TABLE OF CONTENTS

<u>CONTENTS</u>	<u>PAGE</u>
ABSTRACT.....	v
<u>Chapter 1, INTRODUCTION & LITERATURE REVIEW.....</u>	<u>1</u>
Test organisms.....	6
<u>Bacillus subtilis & Bacillus cereus.....</u>	<u>6</u>
<u>Staphylococcus aureus.....</u>	<u>10</u>
<u>Escherichia coli.....</u>	<u>13</u>
Microbiology of milk.....	17
Antimicrobial agents.....	24
<u>Chapter 2, MATERIALS & METHODS.....</u>	<u>29</u>
Microorganisms.....	29
Antimicrobial agents.....	29
Liquid media.....	29
Cultivation & Stock-culture preparation of microorganisms.....	29
Preparation of stock solution of the antimicrobial preservatives.....	33
Preparation of the Nido powdered milk solution.....	33
Inoculation of microorganisms and addition of antimicrobial agents.....	34
Measurement of bacterial growth and counts.....	34
<u>Chapter 3, RESULTS.....</u>	<u>35</u>
Effect of some antimicrobial preservatives on the viability and growth of bacteria in liquid media ...	35

Table of Contents contd.

	<u>PAGE</u>
Effect of varying pH on the antimicrobial efficacy of the preservatives.....	40
Effect of varying inocula on the lethal efficacy of the antimicrobial preservatives.....	44
Viability of the test organisms as a function of time.....	47
Antimicrobial efficacy of the sulphur dioxide-generating preservatives in full cream powdered milk solution	49
Simulation of repeated contamination in 10% milk solution.....	53
Relative growth of the four bacterial species in preservative-treated milk solution.....	57
<u>Chapter 4, DISCUSSION</u>	61
Antimicrobial efficacy of the sulphur dioxide-generating salts in liquid media.....	61
Minimal lethal concentration of the preservatives as a function of pH.....	64
Minimal lethal concentration of the preservatives as a function of inocula of test organisms.....	66
<u>Chapter 5 CONCLUSION</u>	70
SUGGESTIONS FOR FURTHER STUDIES.....	71
REFERENCES.....	72

List of Tables

<u>TABLE</u>	<u>PAGE</u>
1. Effect of the antimicrobial agents on the viability and growth of <u>Escherichia coli</u> in lactose broth.....	36
2. Effect of the antimicrobial agents on the viability and growth of <u>Bacillus subtilis</u> in nutrient broth.....	38
3. Effect of the antimicrobial agents on the viability and growth of <u>Bacillus cereus</u> in nutrient broth.....	39
4. Effect of the antimicrobial agents on the viability and growth of <u>Staphylococcus aureus</u> in BHI.....	41
5. Effect of pH changes on the antimicrobial efficacy of the preservatives.....	42
6. Effect of varying inocula on the lethal potency of the preservatives.....	45
7. Effect of exposure time on the lethal efficacy of the preservatives.....	48
8. Effect of the sulphur dioxide-generating antimicrobial agents on the viability of four bacterial species in 10% solution of powdered milk.....	52
9. Effect of repeated bacterial contamination on the antimicrobial efficacy of the preservatives in 10% solution of powdered (Nido) milk.....	54-55
10. Relative growth of four bacterial species in the 10% milk solution.....	59

C H A P T E R

O N E

INTRODUCTION AND LITERATURE REVIEW

The need to improve the lot of the ever-increasing world population of mankind by way of increased food supply cannot be overstressed especially when the resultant population explosion is paralleled by increased pressure on food supplies. Indeed, to effect a meaningful step-up in the present food supply, it is necessary to gear up food production and combat, with unabated vigour, the shortcoming posed by food spoilage agents. This desired goal also demands that a concerted effort be made to increase and improve yields per acre of agricultural lands as well as improve the methods of preservation of foods and food products. Unfortunately the countries most affected by food shortages and low food quality belong to the developing countries which explain why hunger and starvation are most rampant in such countries. The predicament of the third world countries is aggravated by inadequate technology of preservation. Thus the use of chemical preservatives in such countries would prove highly invaluable.

Because mankind has been constantly under pressure from microbial contaminants of food, it is hardly surprising that preservation of foodstuffs has been practised since the start of recorded history. However, it is only recently that the problem of food contamination, which had been and is still responsible for gross food wastage, has been subjected to rigid scientific scrutiny. Food, with a few exceptions, would remain edible indefinitely if kept free from micro-organisms or if the organisms were prevented from multiplying. Unfortunately, this is scarcely practical and

has made the search for adequate methods for preventing spoilage of food the desired goal of both scientific workers and the food industry. Nevertheless, mankind must concede the useful role of some microorganisms in the production of foods and drinks such as bread, vinegar, wine and beer.

Preservation of food is likely to be associated with refrigeration, deep freezing, and canning processes all of which are nineteenth and twentieth century developments whereas the problem of preserving foods has lent itself to fascination as well as difficulty since ancient times when salting, smoking, and drying were widely employed for this purpose. In practical life, all kinds of microorganisms are potential contaminants considering the variety of food substances and the methods by which each is handled during processing. The method by which a food substance is processed and preserved, which in turn depends largely on the type of food substance, may favour contamination by certain groups of microorganisms. In this regard, the sporeforming microorganisms are of special interest and because their spores are highly resistant, their control is of great economic concern in the food processing industry and in the preparation of all sterile products. However, there is no correlation between bacterial count and sanitary quality of a food item as the kind rather than the number of contaminating microorganism may be more important (Bryan, et al, 1962).

Changes produced in food quality as a result of microbial spoilage are not limited to the results of degradation, as they

may also be effected by microbial synthesis with resultant liberation of foul-smelling products. The degradation processes that accompany such changes may be summarized as follows:

Protein foods + Proteolytic microorganisms \longrightarrow Amino acids + Amines + Ammonia + H_2S (Hydrogen sulphide).

Carbohydrate foods + Carbohydrate - fermenting microorganisms \longrightarrow Acids + Alcohols + Gases.

Fatty foods + Lipolytic microorganisms \longrightarrow Fatty acids + Glycerol (Pelczar, et al, 1977)

Consumption of microbe-infested foods has been identified as the cause of several serious health problems. Food poisoning refers to the condition caused by the ingestion of food contaminated with harmful or pathogenic bacteria or their exotoxins. It embraces toxemia from ingestion of foods containing poisons produced by Clostridium botulinum and certain micrococci, and from food infections or, more accurately, food borne infections. The latter are of two general types:

(1) Salmonella infections, in which the symptoms are typical of food poisoning, and (2) those diseases, such as typhoid fever, cholera, and dysentery, which are caused by agents transmitted by many vectors of which food is but one, and whose symptoms are not those usually associated with food poisoning. Microbial contaminants are either saprophytic and render foods organoleptically unfit for human consumption or they may be pathogenic and may therefore cause

serious infections or poisoning when the food is eaten (Bryan, et al, 1962). Contamination by microorganisms and their by-products alone has been responsible for about 90% of food borne diseases (Bergdoll, et al, 1974).

The dreadful disease, botulism, having a mortality of 60-85% is caused by the exotoxin of Clostridium botulinum. As the toxin is destroyed by heat freshly cooked foods do not cause the disease. This most potent toxin is the only such toxin known that is not destroyed by gastrointestinal secretions. In the United States and Canada between the period 1899 - 1943, 404 outbreaks of botulism involving 1125 cases and 732 deaths were recorded. Commercially packed foods were involved in 50 of these outbreaks, with olives and spinach or chard the worst offenders. Home-canned foods were responsible for 315 outbreaks, with stringbeans and corn the most frequent culprits (Bryan, et al, 1962).

Certain micrococci, generally haemolytic strains of Staphylococcus aureus, cause food poisoning with characteristic gastrointestinal disturbances. The symptoms are often associated with consumption of contaminated foods containing starch thickening, such as eclairs, cream puffs, cake fillings, ham and ham products, salad dressings, cedar cheese, chicken products, potted meat pies, milk, gravy, and even ice cream. The organisms produce soluble toxic substances or enterotoxins and may contaminate foods via boils or abscesses on the skin, nose, or via the mouth of the food handlers. Elaboration and toxin formation is favoured by inadequate refrigeration and subsequent inadequate heating may kill

the organisms but not the toxin, the latter being highly heat-resistant (Frazier and Westhoff, 1978).

Enteric diseases, such as typhoid, paratyphoid, and dysentery are often spread by contaminated oysters and other shellfish and vegetables like celery and watercress, grown in association with contaminated water. Tuberculosis may also be spread to human beings by the ingestion of meat from infected animals. From the foregoing, it is imperative to conduct a microbiological examination of food before and after processing in order to assess its keeping quality with respect to duration of storage. This may provide information concerning the quality of the food, the sanitary condition under which the food was processed and the effectiveness of the method of preservation. Such information would ipso facto lend credence to ways of improving sanitation and preservation methods so that food loss through product deterioration would be kept at a minimum.

Modern methods of preserving foods employ elaborate and stringent refinements of primitive processes reinforced with newer techniques.

The various methods used for food preservation include:

1. asepsis
2. high temperatures (which embrace boiling, pasteurization, and use of steam under pressure)
3. low temperatures (i.e. refrigeration, chilling, cold storage or freezing)
4. radiation and 5) chemical application (Frazier and Westhoff, 1978).

TEST ORGANISMS

The choice of the above organisms is justified because of their undesirable activities and proven nuisance as regards food spoilage and food poisoning. Generally speaking, food hazards caused by these microorganisms are either by microbial food intoxication or by bacterial enterotoxigenic food infection.

Bacillus subtilis and Bacillus cereus

The gram positive rod-shaped bacteria that aerobically form refractile endospores are assigned to the genus Bacillus. The endospores of the bacilli, like those of the clostridia and a few other taxa (Cross, 1970), are more resistant than their vegetative cells to heat, drying, disinfectants, and other destructive agents, and thus may remain viable for many years without detectable metabolism (Thompson and Thames, 1967). The genus Bacillus encompasses a great diversity of strains which, with a few exceptions, form catalase. The exceptions, that is, those strains that produce no catalase or only trace amounts, are strains of B. larvae, B. lentimorbus, B. popilliae, and some strains of B. stearothermophilus. Some species are strictly aerobic while others are facultatively anaerobic. It was thought that B. anthracis, which played a memorable role in the history of microbiology, is the only species of the genus that is virulent but evidence of infections due to strains of species hitherto considered as non-pathogenic is fast accumulating. At least four species of the genus viz. B. thuringiensis, B. larvae, B. popilliae, and B. lentimorbus, are pathogenic for insects. Several countries now produce B. thuringiensis insecticides on commercial scale as it is effective against

many pests of agricultural crops, forests, and stored food (Norris, 1969).

Bacillus subtilis and Bacillus cereus can survive pasteurization and thus are capable of causing spoilage in refrigerated pasteurized products (Jay, 1978). Bacillus subtilis is widely employed for testing the efficiency of disinfecting apparatus (Davis, et al, 1948). Moreover, the subtilial spores are capable of withstanding the baking temperature of bread. Consequently, B. subtilis causes ropiness in bread via the production of a levan (a polymer of fructose), and food poisoning especially after consumption of heavily contaminated bread (Frazier and Westhoff, 1978). Bryan (1969) recovered viable spores of B. subtilis from an 18-pound turkey cooked at 176.7°C for 8 hours.

Milk secreted into the udders of healthy cows is sterile, but by the time it finds its way into the milking pail, it has acquired a sizeable bacterial population which includes B. subtilis (Bryan, et al, 1962). Bacillus cereus is also a common contaminant of milk. Upon invasion of milk it causes "bitty cream" or "sweet curdling" (Davis and Wilkinson, 1973) and has been implicated as food poisoning agent in meat, liver sausage, vegetable soups, and fried rice by some workers (Goepfert, 1974; Eade, 1974; Gilbert and Taylor, 1976). Most strains of B. subtilis are non-pathogenic but may give rise to conjunctivitis, iridochoroiditis and panophthalmitis in man. Bacillus subtilis occasionally invades the bloodstream in cachectic diseases. Symptoms of food poisoning by B. subtilis include diarrhoea, abdominal cramps, nausea, prostration and vomiting without fever (Tong, et al, 1962). Textile microbiology

has also identified B. subtilis and other common aerobic sporeformers with huge losses of textile goods in process or in the finished state following textile spoilage (Bryan, et al, 1962).

Bacillus cereus ranks as the third most common causative agent of food poisoning in Hungary (Ormay and Novotny, 1968). In 88 incidents the organism was responsible for 3,560 cases which represent 4.4% of the total incidents recorded around this period in Hungary. Moreover, 4 outbreaks of B. cereus gastroenteritis involving 600 persons have been recorded in Norway (Hauge, 1955).

Complex argument still surrounds the type of food poisoning resulting from the ingestion of foods containing large numbers of viable cells of B. cereus. While some scientists viewed it as food infection (Frazier and Westhoff, 1978) others suggested food intoxication (Nygren, 1962; Hauge, 1955; Goepfert, et al, 1972) considering the fact that a significant level of growth is accompanied by synthesis of adequate amount of extra-cellular toxin (Bonventre and Johnson, 1970). In her views, Hobbs (1969) has suggested that B. cereus food poisoning involves both intoxication and infection following the observation that seitz filtrate from culture of B. cereus failed to produce either food poisoning symptoms or appreciable toxicity in mice and guinea pigs but when the author (Hobbs) consumed vanilla sauce containing 92×10^6 cells of B. cereus per ml, symptoms similar to those described for B. cereus food poisoning developed after 13 hours.

A commonplace food poisoning syndrome of B. cereus develops within 8-16 hours and lasts for 6-12 hours (Hauge, 1955). This rather mild syndrome bears a spectacular similarity to that of

Clostridium perfringens food poisoning (Gilbert and Taylor, 1976).

The symptoms are nausea, cramp-like abdominal pain, tenesmus, and watery stool but fever is absent. Another syndrome of B. cereus food poisoning develops within 1-6 hours. It is more acute and severe than the common one and similar to staphylococcal food poisoning syndrome. Strains that are the etiologic agents of this ailment may be mutants of the less virulent organisms.

The control of spore-forming bacteria has lent itself to difficulty as well as fascination particularly in the food and health sectors. The ability of bacterial spores to withstand near extremes of adversities is quite remarkable. Bond and Favero (1975) found that a temperature of 150°C (dry heat) destroyed 90% of 5×10^2 spores of a Bacillus species ATCC 27380 only after an extended period of 2½ hours. A comparison of the heat resistance of spores with vegetative cells has revealed that spores of some species may be 10^5 -fold more resistant than their vegetative cells (Gould, 1977). The higher resistance of some spores to ultraviolet and ionizing radiations (Roberts and Hitchins, 1969) as well as high hydrostatic pressures (Sale, et al, 1970) than their vegetative cells has also been documented. Borick and Fogarty (1967) observed that using 10^8 spores per ml of spore suspension of B. subtilis E152, B. globigii E154, and B. cereus E190, 90% of the spores were destroyed by 0.146 Mrad, 0.165 Mrad, and 0.107 Mrad doses of gamma irradiation respectively. Spores have also demonstrated higher resistance to a wide range of chemical agents; for example, chlorine water, ethanol, and ethylene oxide to mention but a few. The basis of the spore's resistance and longevity,

its formation, morphology, composition, and stages of germination continue to attract many investigations (Gould and Hurst, 1969; Halvorson, et al, 1971).

Staphylococcus aureus

The gram positive cocci growing in grape-like clusters, or pairs of opaque, white or coloured bacterial colonies with smooth surface, and which may be facultative in their oxygen demands in a complex glucose medium, belong to the genus Staphylococcus and the family Micrococcaceae. The staphylococci are ubiquitous, pathogenic microorganisms and are the commonest cause of localized suppurative infections (Cohen, 1972; Jeljaszewicz, 1976; Symposium, 1965). They do not form spores and are invariably non-motile. Certain strains of this genus that are saprophytic have been associated with useful agricultural processes owing to their fermenting activity. Some strains have become adapted to parasitic mode of living and have been identified as commensals or pathogens in humans and animals alike. The heat resistance of the vegetative cells of the staphylococci is higher than that of the vegetative cells of most other pathogenic bacteria. They are also resistant to drying, may remain infectious for extended periods, and are able to grow even in the presence of high sodium chloride concentration (up to 15% sodium chloride). This means that salting as a method of food preservation may not preclude staphylococcal food poisoning - a property already taken advantage of in the preparation of selective media for isolation of staphylococci (Buchanan and Gibbons, 1974).

The staphylococci are classified primarily on the basis of coagulase production and heat stable DNase. This coagulase is an enzyme that possesses thromobokinase-like activity. Most of the coagulase positive strains also produce α , β , γ and δ - haemolysins (Arbuthnott, 1970; Kreger, et al, 1971; Wiseman, 1970; Wiseman, 1975; Wiseman and Caird, 1968) and are known as Staphylococcus aureus. A few strains of S. aureus are known to form capsules, present as morphological entities or detectable by immunological means, though the vast majority are non-capsulated (Freeman, 1979) and non-sporeforming. The coagulase negative strains, 95% of which form ϵ - haemolysin, are known as Staphylococcus epidermidis. These are frequently the cause of stitch abscesses and are thought to be a less virulent variety of S. albus. Staphylococcus epidermidis is a normal skin inhabitant and only occasionally is it found in infectious processes (Baird-Parker, 1972).

Staphylococcal food poisoning is probably the most common food poisoning ailment (Nickerson and Sinskey, 1972; Frazier and Westhoff, 1978). Staphylococcus aureus, the most pathogenic of the genus, is responsible for food intoxication the causative agent being the preformed enterotoxin which is but one of the many biologically active substances produced by this organism. About 40% of normal adult humans harbour staphylococcal organisms in the nose and throat which could explain why the fingertips of humans are so easily contaminated with the organism (Williams, 1963). A major feature of staphylococcal food contamination is the absence of appreciable organoleptic changes in the

infested food items. The corollary is that food containing millions of the organism appear, smell, and taste normal compared to food free of the organism (Nickerson and Sinskey, 1972). Symptoms of staphylococcal food poisoning include gastroenteritis (inflammation of the lining of the GIT), nausea, violent diarrhoea and vomiting, prostration, and abdominal cramp (Cecil and Loeb, 1959). The true incidence of staphylococcal poisoning is not accurately known; since the post-ingestion incubation period of the toxin is usually 2-6 hours and recovery occurs in one or two days except in debilitated persons. Consequently, some cases of the illness never received the attention of a physician and therefore escape unrecorded. However, Morbidity and Mortality Weekly Reports (1972) has documented that 45% of the total outbreaks of food borne diseases reported to the Centre for Disease Control (CDC) Atlanta, U.S.A. in 1971 were due to staphylococcal food poisoning.

A large number of enterotoxigenic strains of S. aureus that produce unidentifiable enterotoxins have been isolated from culture collections. The initial recognition of the fact that staphylococci produce more than one enterotoxin was preceded by the successful purification of the protein responsible for emesis in monkey (Bergdoll, et al, 1974); although it is not clear whether a single toxin has more than one action. Moreover, the actual number of enterotoxins produced by S. aureus is not known, but six serologically distinct toxins that differ in toxicity have been identified (Frazier and Westhoff, 1978). These are enterotoxin A, B, C, C₂, D and E differentiated on the basis of their reaction with specific antibodies.

The enterotoxins are simple proteins of low molecular weight, high heat-resistance, readily soluble in water and salt solutions and are resistant to proteolytic enzymes such as trypsin, chymotrypsin, renin, and papain. Their activity is destroyed by pepsin at pH 2.0 (Bergdoll, 1970)

The presence of S. aureus in foods is largely dependent on human factor which reflects unhygienic handling of the foods. It was noted that a large number of S. aureus must be present to cause poisoning and staphylococcal count in the range of 50×10^6 to 200×10^6 cells per g of food has been documented (Casman and Bennett, 1965). Enterotoxin A and D are responsible for most food poisoning (Frazier and Westhoff, 1978) and less than one microgram (1 ug) of the former is sufficient to cause sickness in humans (Bergdoll, 1968).

Escherichia coli

The family Enterobacteriaceae (Enteric bacteria), comprising of about twelve genera, has stimulated more research in determinative methods than any other group of bacteria because of the biological and morphological similarity which renders their identification and differentiation often very difficult. The significance of physiological differentiation of these coliform bacilli in both medical and sanitary microbiology has led to the development of a tremendous battery of biochemical tests. A primary differentiation of practical but not absolute value is made on the basis of lactose fermentation such that production of gas and acid within 24 hours usually indicates nonpathogenic

species while those which do not ferment lactose are essentially pathogens (Edwards and Ewing, 1972; Ewing, 1973). Most species also ferment glucose, reduce nitrate to nitrite, and lack a cytochrome C oxidase.

The coliform bacilli are widely distributed, even ubiquitous, in the sense that many exist universally in the intestinal tract of man and animals (Roggendorf and Muller, 1976). Some exist as saprophytes in nature while others are parasitic for plants, causing blights and soft rots. The enteric bacteria are aerobic, facultatively anaerobic, gram negative, non-sporulating bacilli. They encompass motile organisms with peritrichous flagella as well as non-motile varieties.

Only one species of *Escherichia*, *E. coli* (Cooke, 1974), is recognized in the Bergey (1974) classification and it conforms to the general morphological description for the coliform bacilli. It is the only important species of the genus and has been found to be a common inhabitant of the lower bowel of man and animals. *Escherichia coli* is often present in man in concentrations of 10^7 or more viable organisms per g of faecal material and serves as a good index of faecal pollution of water supplies and food when found in either of these consumer goods (Pelczar, et al, 1977). Clinical isolates of *E. coli* may be conveniently grouped into three categories: opportunistic, enteropathogenic, and enterotoxin-producing. The latter are unable to invade the intestinal mucosa, but they release an enterotoxin that adsorbs to epithelial-cell membranes and stimulates adenyl cyclase activity.

Serological studies on E. coli have elucidated the antigenic structure as composed of three different classes of antigens (Edwards and Ewing, 1972). Serotypes may be identified with a high degree of precision (Ewing and Davis, 1961) based on the distribution of the three different antigens viz. O, K, and H antigens. The O antigens are somatic antigens; stable to heat at 100°C or 121°C ——— serological O groups rather than types are designated since several O groups contain related, but immunologically specific, antigens called O factors. The K antigens are also somatic antigens but are somewhat more complex. They occur as sheaths, envelopes or capsules and may act as inhibitors of O agglutination. Not all *Escherichia* contain K antigens and whenever they are present only one type is found in a given strain. Such strains appear, in general, to be more toxic, as well as more resistant to phagocytosis (Rottini, et al, 1975). The H antigens are flagellar, sometimes poorly developed upon primary isolation and are heat labile at 100°C. Their use completes the serotyping of this group.

The presence of enteropathogenic E. coli (EEC) in any food item is considered highly hazardous especially to young children. Diarrhoeal disease which occur in infants may be associated with infection of the gastrointestinal tract (GIT) by EEC (Hanson, 1976; Sack, 1975). This disease may assume epidemic proportions especially in institutional outbreaks as it frequently does, for example, in nurseries. A particular serotype of E. coli was first noted by Bray (1945) to be associated with infantile diarrhoea and using commercial typing sera it has been possible to identify ten

different serotypes some of which are also found in diarrhoea of adult. Their effect can be so severe sometimes that it is misconstrued to mean cholera.

The enteric bacteria produce bacteriocins, metabolites that are lethal for other strains of the same or closely related species. The bacteriocins are named after the strains that produce them, for example, E. coli produces colicins which presumably play a role in the maintenance of normal flora by conferring selective advantage particularly in the competitive environment of the intestinal tract (Hardy, 1975). However, in vivo activity of the bacteriocins is yet to be thoroughly investigated and there appears to be no significant relation between colicinogen and pathogenicity in E. coli.

Escherichia produce several soluble toxins apart from the classical endotoxin common to most gram negative bacteria. Most of the enteropathic strains produce an enterotoxin (Dorner, 1975) which mediates the movement of water and ions from the tissues into the bowel lumen to give a net secretion manifested as diarrhoea. The similarity of this process to that which occurs with cholera toxin has been documented (Skerr, et al, 1973; Moon, et al, 1971). Two of such toxins are produced by enteropathic strains, one heat-stable (Smith and Halls, 1967), and the other heat labile (Gyles and Barnum, 1969). Some strains of E. coli are haemolytic-producing two kinds of haemolysins (McGeachie, 1966; Smith, 1963). These are designated α - and β -haemolysins. The α -haemolysin, a soluble haemolysin found in cell-free culture

supernatants, occurs as two molecular species which differ in size. It is sensitive to trypsin and requires calcium ions for activation (Short and Kurtz, 1971) while the β -haemolysin is cell-bound. These haemolysins do not appear to be associated with pathogenesis of infection with haemolytic strains (Snyder and Koch, 1966).

Escherichia coli is the etiologic agent of a variety of human and animal infections (Hanson, 1976; Meyers and Guinee, 1976; Moon, et al, 1966; Moon, et al, 1968). It is the most common causative agent of cystitis and other infections of the urinary tract and also one of the most commonly recovered organisms from peritonitis consequent upon rupture of the appendix or other bowel perforations. Escherichia coli generally lends itself to laboratory manipulation and has served as a "model" organism for many types of studies in bacterial physiology, cytology by electron microscopy, genetics and chromosome mapping etcetera.

MICROBIOLOGY OF MILK

It has been observed that, other than the ramifications of a common water supply, the most frequent vehicles for the dissemination of microbial diseases are the impressively wide variety of foods consumed by man. Bearing in mind this observation it has been deemed necessary to review the microbiology of milk, which was one of the liquid media used for this study.

Milk has been regarded as the "most nearly perfect" food for humans due to its exceptional nutritive value. The chief constituents of milk are proteins, carbohydrates, fats, minerals,

vitamins, and water which buttress its suitability as an excellent medium for the growth of many bacteria. It differs from water and other foods in that it is the only major food of animal origin that is consumed largely in the 'raw' state (Foster, et al, 1957). Since large quantities of milk are consumed - about 16% of the average diet in the United States consists of milk and milk products - the importance of this food item as a vehicle of infectious disease is quite evident.

Unlike water, milk has no native bacterial flora and there is no bacterial flora that is characteristic of milk. It has previously been stated that milk as secreted into the udders of healthy cows is sterile. This implies that the presence of microorganisms in milk is always a consequence of contamination and the types of bacteria present would depend upon the source of contamination. Some of the organisms come from the cow's udder or skin, via the milk ducts of the teats, others from sources external to the animal, for example, dust, fodder, milk utensils, hands and clothing, etc of the person that performed the milking process. These bacteria are normally nonpathogenic and many of them produce chemical changes that are desirable in the manufacture of dairy products, for example, butter, cultured milks, cheese etc (Frazier and Westhoff, 1978).

Considering the various sources of contamination of milk, it is apparent that the health of dairy cattle is of primary importance. Milk drawn aseptically from a healthy cow contains few bacteria which are saprophytic whereas milk from a cow with an infected udder usually contains large numbers of pathogenic

bacteria. Since the saprophytes are of little significance so long as their multiplication is controlled, the presence of pathogenic microorganisms in milk calls for a periodic inspection of the herds to ascertain the state of health of each animal (Hammer and Babel, 1957). Of these microorganisms perhaps the most important is the tubercle bacillus and this organism may gain access into the milk either as a consequence of tuberculosis of the udder or by ingestion of infectious sputum discharged with the cow faeces which contaminate cow manure. In either case, the milk is infective for humans and gives rise to bone and joint tuberculosis. Therefore, in view of the above fact, bovine tuberculosis and its subsequent transmission to man may be controlled at its source by the elimination of infected cattle from dairy herds as commonly practised in the United States.

Brucella abortus, the most frequent agent of bovine brucellosis which causes a contagious abortion in cattle, is shed into the milk of infected cattle during the course of the disease. This organism causes undulant or Malta fever in man. More important are the streptococcal infections of the udder known as garget or mastitis. Streptococcus agalactiae, the streptococci of bovine mastitis, are rarely the causative agent of human disease but milkborne epidemics of streptococcal infection may be associated with acute udder inflammation in the dairy herd. If milk is obtained under aseptic conditions it contains only a few hundred bacteria per ml whereas with careless manipulation, even freshly drawn milk may be highly contaminated. The temperature response

of bacterial contaminants in milk is noteworthy. If kept at 0°C, milk shows a decrease in bacterial content during the first few hours, but at elevated temperatures enormous numbers of bacteria result due to an enhancement in the rate of multiplication, especially of thermophilic bacteria. The heterogeneous bacterial flora that may be detected in milk, consequent upon the multiplicity of the sources of contamination, lend credence to the sanitary conditions associated with a batch of milk. In addition to the microorganisms of soil and water, the bacteria carried by humans have relatively ready access to milk. Contamination with the bacteria of human disease is a constant possibility. The bacterial contaminants do not only survive, as in water, but actively multiply into huge numbers in pooled milk of which but a single part was originally contaminated (Smith, 1957). It is apparent that the kind and degree of initial contamination, and also, the temperature at which milk is kept are the most important factors governing the number of bacteria that may be present in the milk. Therefore the production of hygienically satisfactory milk involves cleanliness, in the first instance, and immediate cooling and storage at a low temperature in the second.

The nonpathogenic bacteria usually found in milk may be conveniently differentiated on physiological basis as follows:

1. Acid - forming bacteria
2. Alkali - forming bacteria
3. Proteolytic bacteria
4. Inert bacteria

The first group, which includes fermentative bacteria, most commonly

effect lactic acid fermentation - the process by which milk usually sours under natural conditions. In this regard, certain streptococci and gram negative bacilli have been implicated. Most abundant in naturally soured milk and particularly when the acidity is high is Streptococcus lactis while the responsible gram negative bacilli are frequently the Aerobacter aerogenes now placed in the genus Klebsiella. Other bacteria occasionally associated with soured milk include S. aureus, E. coli, and Streptococcus pyogenes (Pelczar, et al, 1977).

The alkali-forming bacteria do not ferment lactose but presumably act on the nitrogenous substances present in milk with the liberation of ammonia. Certain other bacteria, such as some of the aerobic spore formers, also produce lipase and thus decompose the fats present thereby converting the milk to a yellow transparent fluid.

Hydrolysis of milk protein (peptonization) is accomplished by proteolytic bacteria most of which also produce alkaline reaction. Two groups of enzymes are involved: a renin-like enzyme which precipitates the protein with the formation of a soft curd, and casease, which hydrolyzes the protein thereby transforming the milk to a clear fluid. Peptonization, however, does not follow precipitation when the microorganism is devoid of casease. The bacteria producing these changes include Bacillus subtilis, certain strains of staphylococci, and Proteus vulgaris (Frazier and Westhoff, 1978).

The inert bacteria produce no visible change in milk and include certain non-pigment forming bacteria from water and other

sources, and, in addition, most of the pathogenic bacteria found in milk. Presence of such dangerous contaminants is inapparent without bacteriological examination of milk.

Various chromogenic bacteria may be present in milk and confer a series of unusual or abnormal changes collectively termed "diseases" of milk. These bacteria form "blue milk", "brown milk", "red milk", and "yellow milk" due to the production of a number of different pigments many of which are phenazines (DeLey, 1964). The pigments are commonly produced by bacteria assigned to the genus Pseudomonas and relevant examples are P. syncyanea, P. putrefaciens, and P. synxantha which are responsible for "blue", "yellow", and "brown" milk respectively. The products of certain other microorganisms are termed "bitter milk" which is characterized by a bitterness that sometimes develops after a short interval. Milk sometimes suffers from a ropy or slimy fermentation mainly due to the capsule-forming activities of Alcaligenes viscolactis and often regarded as undesirable although such a fermentation is intentionally produced in the manufacture of Edam cheese in Holland (Dairy Products Laboratory, 1969).

It has been established that though freshly drawn milk is both bactericidal and bacteriostatic owing to the presence in milk of various antibodies these activities are thermolabile and disappear a few hours after the milk has been drawn.

The quality of milk is determined by several methods but the best index of quality is the number of bacteria it contains.

Plate count technique is one of the methods employed for this purpose and has proved highly invaluable in the bacteriological grading of milk. Detailed description of this method may be found in the standard procedure developed under the auspices of the American Public Health Association (A.P.H.A., 1972). Other methods used for the determination of milk quality are microscopic counts, coliform counts (usually under special circumstances), cell count and sedimentation test all of which are well described in the A.P.H.A. standard methods. It is sometimes desirable to isolate pathogenic bacteria when an outbreak of disease is suspected to be milkborne. Finally, the reduction of methylene blue by raw milk is also used for the assessment of milk quality based on the fact that there exists a direct relationship between reduction time and number of bacteria present. This method, however, cannot be used for grading milk with any degree of precision but it is generally accepted that a milk sample decolourizing in less than two hours is poor in quality while that which does not decolourize in eight hours is excellent (Nilsson, 1959).

By far the most satisfactory method of controlling milkborne infection is the process of destroying pathogenic bacteria. The use of a temperature high enough to kill most microorganisms but not sufficient to cause undesirable alterations in the substance heated was first applied for preservation of wines by Pasteur. A grateful scientific world has long termed this process as pasteurization. The process is now chiefly used for the treatment of milk with a view to improving the shelf life.

Many preservative methods cannot be used for milk without causing undesirable changes or forming different food products because milk is a delicately flavoured and easily changed food. In fact, most of the various milk products evolved in order to improve the keeping quality of milk. Despite the efficiency, versatility, and cheapness of sulphur dioxide as a preservative it is yet to be used for preservation of milk hence the inclusion of milk as one of the liquid media in this study.

ANTIMICROBIAL AGENTS

"A food additive is a substance or mixture of substances, other than the basic food stuff, which is present in food as a result of any aspect of production, processing, storage or packaging. The term does not include chance contamination" (WHO, 1965). Some food additives are specifically added to prevent the deterioration or decomposition of foods. These are referred to as chemical preservatives and are capable of inhibiting the growth and activity of microorganisms (Frazier and Westhoff, 1978).

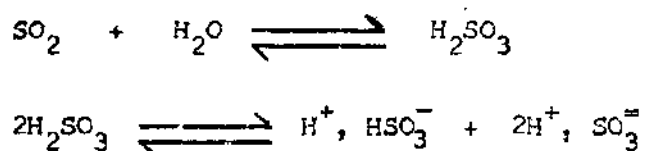
Sulphur dioxide has long been used as an antimicrobial preservative and as an antioxidant in foods and beverages to prevent both enzymatic (Mapson, 1962), and non-enzymatic (Burton, et al, 1963; McWeeny and Burton, 1963; Reynolds, et al, 1962; Fox, 1972) discolouration as well as spoilage. The major sources of sulphur dioxide as an antimicrobial agent in foods are often the sulphite, bisulphite, and metabisulphite ions of sodium and potassium and, less frequently, of calcium (Allen and Brook, 1970; Fennema, 1976).

In practice the addition of 1,000 - 2,000 ppm sulphur dioxide to fruit pulps and juices, 500 - 1,000 ppm to dried fruits and vegetables and, 100 - 500 ppm to concentrated fruit juices, wines and beers has proved to be of adequate antimicrobial efficacy (Joint FAO/WHO, 1971). It has been observed that palm wine could be preserved for six months by the addition of 0.05% (w/v) of sodium metabisulphite (Okafor, 1977). The antibotulinal efficacy of sulphur dioxide in meat has also been evaluated (Tompkin, et al, 1980). The latter study has revealed that the use of about 100ug of sulphur dioxide per g of meat is necessary to obtain significant inhibition of spore germination when a target level of 100 botulinal spores per g was exposed to sulphur dioxide. In gelatin, 800 ppm of metabisulphite rendered the product salmonella-free and 2,000 ppm was required to kill off E. coli while Pseudomonas aeruginosa was destroyed by 1,000 ppm of metabisulphite (Abalaka and Heibel, 1980). The same authors have shown that the three bacterial species were susceptible to the same level (4,000 ppm), of metabisulphite, in bone meal.

Differences in opinion have emerged as to what component, sulphur dioxide versus sulphurous acid, is responsible for lethality. It is assumed that the free gas yields sulphurous acid, having dissolved in the water present in food, which is the active antimicrobial agent (Allen and Brook, 1970; Jay, 1978). Other schools of ^{thought} / .. expressed disagreement with this assumption and have argued that the free sulphur dioxide released by sulphites, bisulphites, and metabisulphites is the antimicrobial agent (Hammond and Carr, 1976). However, this study does not intend

to verify or propose an active component.

The reactions of sulphur dioxide in solution are thought to conform to the following equations:



It was therefore inferred that the relative proportion of each species depends on the pH of the medium such that at pH 4.5 or lower the HSO_3^- ion and the undissociated sulphurous acid are the predominant species (Joslyn and Braverman, 1954). Because sulphur dioxide is most effective as an antimicrobial agent in acid medium it has been suggested that the higher effectiveness is sulphurous acid-mediated (Brown, 1972). This is because the undissociated sulphurous acid is the predominant species at pH 3 or below and readily penetrates the cell wall of microorganisms. At higher pH values the HSO_3^- ion is effective against bacteria but not yeast (Dyett and Shelly, 1966) and permits the growth of certain selected type of wild yeasts at carefully monitored concentrations in wine (Nickerson and Sinskey, 1972).

The addition of sulphur dioxide to flour results in reversible cleavage of the protein disulphide bond in the flour (Robert and McWeeny, 1972) which is thought to have desirable effects on bread dough. Wade (1972) has traced the effect of metabisulphite on the properties of hard biscuit dough to the rupture of intermolecular disulphide linkages in the gluten network of the dough. It was earlier demonstrated that the

disulphide is usually split into two parts, one containing the thiol group, and the other an S-Sulphonic acid group (Swan, 1957). Sulphur dioxide reacts generally with disulphide groups in protein but forms addition complexes with carbonyl group as found in aldehydes, ketones, and sugars. Sulphur dioxide is endowed with a self-regulatory attribute which has placed it at an advantage over any other food additive. This is reflected in the fact that the addition of sulphur dioxide beyond a certain level to food results in an offensive odour. Limits of sulphites, bisulphites, and metabisulphites in foods and drinks are prescribed in terms of sulphur dioxide. The use of sulphur dioxide in foods, beverages, and pharmaceuticals has been reviewed extensively by Schroeter; (1966) and more recently by Hammond and Carr (1976).

The level of sulphur dioxide presently in use has been accepted as safe (Joint FAO/WHO, 1965) and this chemical preservative has won a place amongst the least harmful antimicrobials employed in food preservation (Report, HMSO, 1924). The acute and chronic toxicity of sulphur dioxide (Allen and Brook, 1970), metabolism and elimination of sulphites by mammals (Gibson and Strong, 1973a) and the safety of sulphited foods (Gibson and Strong, 1973b; Wilkins, et al, 1968) have been evaluated. These studies have uncovered the fact that humans can tolerate higher levels of sulphur dioxide than the unconditionally acceptable daily intake of 0.35 mg per kg body weight (Joint FAO/WHO, 1967). Moreover, bisulphite is easily converted to sulphate by the enzyme sulphite oxidase in the liver (Macleod, et al, 1961;

Schroeter, 1966). The sulphate is often voided in urine.

The role of sulphur dioxide in carcinogenic or mutagenic hazard is yet to be deciphered. The possible participation of this preservative in any of these problems has long been the subject of extensive investigation. With this imposing labyrinth, the exact role of sulphur dioxide will have to await the availability of reliable and retrievable experimental findings especially at the level of sulphur dioxide consumed through foods and beverages. However, sulphur dioxide has been implicated in genetic mutation by a few science authorities (Hayatsu and Miura, 1970; Hayatsu, et al, 1970; Mukai, et al, 1970; Shapiro, et al, 1970a; Shapiro, et al, 1970b).

There has been a good deal of speculations concerning the mechanism by which sulphite, bisulphite, and metabisulphite exert lethal effect on microorganisms. Some of the postulated mechanisms include :- leakage of ATP (Luker, et al, 1975), formation of bisulphite addition compounds which may interfere with respiratory reactions that are NAD-linked (Chichester and Tanner, 1972), derangement of cell wall septation apparatus (McCready, et al, 1976) and multiple factors (Hammond and Carr, 1976). Akinsanya (1979) investigated the possibility of ATP leakage from metabisulphite-treated cultures of B. subtilis. The investigation suggested that ATP leakage contributed, at least in part, to the death of bacterial cells.

C H A P T E R

T W O

MATERIALS AND METHODS

Microorganisms. Bacillus subtilis NCTC 7476 and Bacillus cereus W18 were obtained from the laboratory of Dr Abalaka in this department. Escherichia coli NCTC 10418 and Staphylococcus aureus NCTC 6571 were obtained from stock cultures maintained in Microbiology department, Ahmadu Bello University, Zaria.

Antimicrobial agents. Anhydrous sodium metabisulphite, Anhydrous sodium sulphite, Sodium hydrogen sulphite or Sodium bisulphite (40% w/w solution), Potassium sulphite, and Potassium metabisulphite were all of reagent grade (BDH Chemicals Ltd. Poole England).

Liquid media. Nutrient broth (Difco), Lactose broth (Oxoid), and Brain heart infusion (BHI; Oxoid). These media were prepared according to manufacturers' directives. Nido full cream powdered milk (Nestle) was purchased from one of the supermarkets in Samaru Village, Zaria and its solutions were used for further studies as described under Results.

Cultivation and Stock Culture Preparation of Microorganisms

Bacillus subtilis

Lyophilized culture (1g) was first suspended in 1ml of 0.1% peptone water (Oxoid) and sub-cultured overnight in nutrient broth at 37°C in a Gallenkamp orbital incubator maintained at 100 rpm. The cells were then plated on nutrient agar (Oxoid) and isolated colonies were picked and sub-cultured in nutrient broth.

Confirmation and re-isolation of *B. subtilis*: The following tests were further carried out to confirm the identity of *B. subtilis*.

Microscopic examination: Samples were prepared for Gram stain reactions and from the examination under Zeiss Standard 14 Compound microscope it was observed that the cell were gram positive rods.

Catalase reaction: A sample of the sub-culture was dispensed into a test tube and to this was added 1ml of 3% (w/v) hydrogen peroxide solution in accordance with the procedure of Deibel and Evans (1960). A positive catalase reaction was indicated by the effervescence of gas bubbles ($\text{H}_2\text{O}_2 \xrightarrow{\text{catalase}} \text{H}_2\text{O} + \frac{1}{2}\text{O}_2$).

Oxidase reaction: This followed the procedure of Kovacs (1956), with a positive/^{result}indicating that the organism in question possesses the terminal cytochrome oxidase in the electron transport system. *Bacillus subtilis* was oxidase positive hence aerobic.

Following the confirmation of the microorganism as *B. subtilis*, streaks of the sub-culture were made on nutrient agar and incubated at 37°C for 24 hours. Isolated colonies were then picked and re-subcultured in nutrient broth and samples from the latter were used as working stock cultures.

Bacillus cereus

Other than the fact that it was necessary to differentiate between *B. subtilis* and *B. cereus*, the preparation of working stock cultures of the latter followed the same procedure as previously described for *B. subtilis*. However, *B. cereus* cells when lightly stained were filled with unstained globules whereas

the cells of B. subtilis were not. Moreover, the individual cells of B. cereus were usually fatter than those of B. subtilis under the microscope. The actual differentiation was carried out using egg-yolk and polymyxin medium (Kim and Goepfert, 1971) on which B. cereus colonies were surrounded by clear halos, indicating ability to hydrolyse lecithin in the medium while B. subtilis colonies showed opaque surrounding. Thus B. cereus was positive for lecithinase production while B. subtilis was negative.

Escherichia coli

From the original stock on agar slant, a resuspension of the micro-organism was made in 0.1% peptone water and grown overnight in lactose broth at 37°C. The microorganism was then streaked on to eosin methylene blue (EMB; Difco) agar and incubated at 37°C for 24 hours. Formation of colonies with metallic sheen on EMB agar plates was taken to represent E. coli. Further confirmation of E. coli was undertaken using the modified Eijkman procedure, a 24 - hour culture of E. coli was inoculated into fresh lactose broth maintained at $45.5^{\circ}\text{C} \pm 0.2^{\circ}\text{C}$ in a pre-warmed water bath. Production of gas and acid within 24 hours of incubation was taken as a positive result. Representative colonies on EMB agar plate were picked and subcultured in lactose broth and samples from this were used as working stock cultures.

Staphylococcus aureus

A resuspension of the microorganism in 0.1% peptone water was also made from original stock on agar slant and cultivated in brain heart infusion (BHI) broth at 37°C. The bacterial cells were observed to be gram positive cocci. Further identification of the microorganism was done by conducting the coagulase test. This followed the procedure of Jawetz, et al (1972). In this procedure, dilute human plasma (1:5) was mixed with an equal volume of BHI culture of the microorganism and incubated at 37°C along with a second test tube containing dilute plasma plus equal volume of sterile BHI which served as a control. After about 2 hours, the plasma in the tube containing BHI culture of the microorganism clotted thus confirming the microorganism as Staphylococcus aureus. A further confirmation employed the heat-stable DNase test which was performed as follows. Into two clean test tubes were added 5ml of 2% solution of DNA. One millilitre of S. aureus culture was added to one of the test tubes and the mixture was boiled for 10 minutes while 1ml of sterile distilled water was added to the other. Both tubes were then incubated at 37°C for 1 hour, following which 1ml of 1N hydrochloric acid (HCL) was added to each tube. The tube to/^{which}S. aureus culture was added remained very clear indicating that the DNA had been digested by the heat-stable DNase whereas the control tube (that to which water was added) showed heavy turbidity, an indication that the DNA reacted with the HCL thereby forming some precipitate.

Streaks of the culture were made on Baird-Parker agar base (Difco) to which had been added egg-yolk tellurite enrichment (50%, Difco) as directed by the manufacturer. The agar plates were then incubated at 37°C for 24 hours and isolated colonies showing clear halos were picked and subcultured in BHI for use as working stock culture.

To ensure that the bacterial cells were in exponential growth phase, cultures of all organisms used in this investigation were transferred into fresh media at every 24 to 30 hours interval.

Preparation of stock solution of the antimicrobial preservatives

Ten thousand ppm (1% W/V) of each of the preservatives was prepared and used as stock solution. From this stock, volumes that would give the desired antimicrobial concentrations were added to the appropriate sterile liquid media. In cases requiring higher concentrations of the preservatives (1,000 ppm and above) the appropriate quantities were weighed separately and added to the sterile media.

Preparation of the Nido powdered milk solution

The milk solution was prepared in two different solvents; sterile distilled water and sterile peptone water (0.1% W/V). In each case 10% milk solution was prepared by dissolving the appropriate amount of the powdered milk in warm sterile solvent.

Inoculation of microorganisms and addition of antimicrobial agents.

The appropriate inoculum size, often determined by one tenth serial dilution procedure, was introduced into the growth media after the desired concentration of antimicrobial agent had been aseptically added to the media.

Measurement of bacterial growth and counts.

Growth of the microorganisms was followed by measuring turbidity spectrophotometrically at 650nm on SP6 - 400 spectrophotometer (Pye-Unican) and using a calibration curve which relates A_{650} to bacterial growth and number. This was further checked by withdrawing known volumes of the culture at specific time intervals and conducting viability count using the standard plate count (SPC) agar (Oxoid). As there were no wide variations between counts derived from the spectrophotometric procedure and the viable plate count method, values of counts recorded under results represented the mean of the two procedures.

C H A P T E R

T H R E E

TABLE 1: Effect of the antimicrobial agents on the viability and growth of *Escherichia coli* in lactose broth

Antimicrobial agents	Concentration (ppm)	Bacterial population (Counts/ml) at				
		12	24	36	48	72
None	0	9.2×10^7	1.1×10^8	1.4×10^8	1.3×10^8	1.2×10^9
	200	7.8×10^7	9.3×10^7	1.0×10^8	1.2×10^8	1.2×10^8
Sodium sulphite	5000	2.4×10^7	4.8×10^7	6.7×10^7	8.1×10^7	1.0×10^7
	10000	1.3×10^7	3.2×10^7	5.0×10^7	7.3×10^7	9.6×10^7
Potassium sulphite	200	8.4×10^7	1.1×10^8	1.3×10^8	1.4×10^8	1.3×10^8
	5000	4.6×10^7	6.4×10^7	8.6×10^7	9.7×10^7	1.0×10^8
Sodium metabisulphite	10000	3.8×10^7	5.9×10^7	6.5×10^7	8.1×10^7	1.0×10^8
	200	2.6×10^7	3.0×10^7	3.5×10^7	6.4×10^7	9.5×10^7
Potassium metabisulphite	400	1.9×10^6	4.0×10^6	4.0×10^6	2.9×10^6	2.0×10^6
	500	0	0	0	0	0
Sodium metabisulphite	600	0	0	0	0	0
	200	3.2×10^7	4.1×10^7	6.3×10^7	8.0×10^7	1.0×10^8
Potassium metabisulphite	400	2.8×10^6	5.2×10^6	5.5×10^6	5.3×10^6	4.2×10^6
	500	0	0	0	0	0
Sodium bisulphite	600	0	0	0	0	0
	200	3.6×10^7	5.1×10^7	6.8×10^7	9.0×10^7	1.2×10^8
Potassium bisulphite	800	8.3×10^5	9.7×10^5	1.1×10^6	8.0×10^6	1.2×10^7
	1000	0	0	0	0	0
Sodium bisulphite	2000	0	0	0	0	0

with 2×10^8 cells of the appropriate bacterial species derived from an exponentially growing stock culture. One flask of culture containing no antimicrobial preservative served as a control. The flasks were incubated at 37°C for up to 72 hours in an orbital rotary incubator (Gallenkamp) maintained at 100 rpm. Five ml samples were taken from each flask at 12, 24, 36, 48 and 72 hours after incubation. Growth of test organism in each sample was estimated spectrophotometrically, as described under Materials and Methods, against the appropriate sterile liquid medium to which no antimicrobial preservative had been added. The effect of the preservatives in liquid media against E. coli, B. subtilis, B. cereus and S. aureus has been recorded in Tables 1, 2, 3 and 4 respectively.

Results indicated that sodium metabisulphite and potassium metabisulphite were the most efficient of all the antimicrobial agents used for this study. Escherichia coli was not susceptible to 400 ppm of sodium metabisulphite and potassium metabisulphite while the growth and viability of B. subtilis and B. cereus were effectively arrested at this level of the metabisulphites.

Staphylococcus aureus was not susceptible to 2,000 ppm of either of the metabisulphites and was therefore found to be the most resistant of the test organisms employed. It was observed that after three days of exposure of the microorganisms at 37°C , 10,000 ppm of either sodium sulphite or potassium sulphite failed to exert

TABLE 2: Effect of the antimicrobial agents on the viability and growth of *Bacillus subtilis* in nutrient broth

Antimicrobial agents	Concentration (ppm)	Bacterial population (Counts/ml) at time intervals (hours)				
		12	24	36	48	72
Iodine	0	9.5×10^7	1.1×10^8	1.2×10^8	1.3×10^8	1.3×10^8
Sodium sulphite	200	8.0×10^7	1.0×10^8	1.1×10^8	1.2×10^8	1.1×10^8
	5000	3.8×10^7	5.0×10^7	6.6×10^7	8.8×10^7	9.4×10^7
	10,000	2.4×10^6	1.1×10^7	2.5×10^7	5.4×10^7	7.2×10^7
Potassium sulphite	200	8.6×10^7	1.04×10^8	1.2×10^8	1.4×10^8	1.3×10^8
	5000	4.6×10^7	6.8×10^7	9.8×10^7	1.3×10^8	1.2×10^8
	10,000	4.0×10^6	1.7×10^7	3.5×10^7	6.8×10^7	8.2×10^7
Sodium metabisulphite	200	2.5×10^7	3.4×10^7	6.2×10^7	9.3×10^7	1.0×10^8
	300	5.4×10^6	8.0×10^6	1.1×10^7	3.0×10^7	4.8×10^7
	400	0	0	0	0	0
	500	0	0	0	0	0
Potassium metabisulphite	200	3.1×10^7	4.2×10^7	7.1×10^7	9.6×10^7	1.1×10^8
	300	6.2×10^6	8.0×10^6	1.2×10^7	3.5×10^7	6.7×10^7
	400	0	0	0	0	0
	500	0	0	0	0	0
Sodium bisulphite	200	2.8×10^7	4.0×10^7	6.9×10^7	9.7×10^7	1.2×10^8
	600	7.5×10^5	9.3×10^5	3.2×10^6	6.1×10^6	2.2×10^7
	800	0	0	0	0	0
	1000	0	0	0	0	0

TABLE 3: Effect of the antimicrobial agents on the viability and growth of *Bacillus cereus* in nutrient broth.

Antimicrobial agents	Concentration (ppm)	Bacterial population (counts/ml) at time intervals (hours)				
		12	24	36	48	72
None	0	1.0×10^8	1.2×10^8	1.3×10^8	1.4×10^8	1.4×10^8
Sodium sulphite	200	8.8×10^7	1.0×10^8	1.2×10^8	1.1×10^8	1.1×10^8
	5,000	4.2×10^7	6.1×10^7	7.3×10^7	8.6×10^7	1.1×10^8
	10,000	3.1×10^6	1.8×10^7	3.0×10^7	5.2×10^7	8.0×10^7
Potassium sulphite	200	9.0×10^7	1.1×10^8	1.3×10^8	1.3×10^8	1.2×10^8
	5,000	5.1×10^7	9.2×10^7	1.2×10^8	1.1×10^8	1.1×10^8
	10,000	1.3×10^7	3.0×10^7	6.5×10^7	9.0×10^7	1.1×10^8
Sodium metabisulphite	200	3.0×10^7	4.8×10^7	7.6×10^7	9.5×10^7	1.0×10^8
	300	6.0×10^6	8.1×10^6	1.0×10^7	2.5×10^7	5.4×10^7
	400	0	0	0	0	0
	500	0	0	0	0	0
Potassium metabisulphite	200	4.1×10^7	5.3×10^7	8.1×10^7	1.0×10^8	1.1×10^8
	300	7.5×10^6	9.8×10^6	1.1×10^7	3.7×10^7	7.2×10^7
	400	0	0	0	0	0
	500	0	0	0	0	0
Sodium bisulphite	200	3.8×10^7	5.6×10^7	7.0×10^7	9.3×10^7	1.0×10^8
	600	8.0×10^5	2.3×10^6	6.6×10^6	2.4×10^7	5.0×10^7
	800	0	0	0	0	0
	1,000	0	0	0	0	0

lethal effect on any of the four bacterial species. Experiment using sodium bisulphite as the antimicrobial agent showed that 800 ppm of the bisulphite was sufficient for killing B. subtilis and B. cereus. Escherichia coli and Staphylococcus aureus were killed by 1,000 and 3,000 ppm of the bisulphite respectively.

Effect of varying pH on the antimicrobial efficacy of the

preservatives. Because the antimicrobial activity of sulphur dioxide is pH sensitive and the pH of each of the liquid growth media employed in this study is near neutrality, the effect of pH changes on the lethal efficacy of the sulphur dioxide-generating preservatives was determined. Five hundred millilitres of liquid growth medium was dispensed into one litre pyrex beaker and dilute hydrochloric acid (about 1.2N) was added dropwise with thorough mixing to bring the growth medium to the desired acidity level. The pH was read on Corning pH meter model 7 (Evans Electroelenium Ltd). The growth medium was then portioned into 100ml in 250ml Erlenmeyer flasks and sterilized. The antimicrobial efficacy of the preservatives at the adjusted pH was evaluated as earlier described. Each bacterial species was subjected to the antimicrobial agents at pH 4, 5 and 6. An inoculum of 2×10^8 cells/100ml was used for this determination.

Results indicated that the effect of low pH (pH 4.0) alone was capable of destroying all the test organisms (Table 5). Sodium sulphite and potassium sulphite demonstrated a clearly destructive

TABLE 4: Effect of the antimicrobial agents on the viability and growth of Staphylococcus aureus in BHI

Antimicrobial agents	Concentration (ppm)	Bacterial population (counts/ml) at time intervals (hours)				
		12	24	36	48	72
None	0	1.1×10^8	1.2×10^8	1.3×10^8	1.35×10^8	1.5×10^8
Sodium sulphite	8,000	4.5×10^7	8.0×10^7	9.2×10^7	1.0×10^8	1.3×10^8
	10,000	3.3×10^7	7.1×10^7	8.7×10^7	1.0×10^8	1.4×10^8
Potassium sulphite	8,000	5.0×10^7	8.5×10^7	1.0×10^8	1.2×10^8	1.42×10^8
	10,000	4.4×10^7	7.7×10^7	9.8×10^7	1.12×10^8	1.4×10^8
Sodium metabisulphite	1,000	6.2×10^7	8.0×10^7	1.0×10^8	1.1×10^8	1.2×10^8
	2,000	1.8×10^7	2.2×10^7	2.4×10^7	3.0×10^7	3.8×10^7
	2,500	0	0	0	0	0
	3,000	0	0	0	0	0
Potassium metabisulphite	1,000	7.0×10^7	9.0×10^7	1.0×10^8	1.2×10^8	1.2×10^8
	2,000	4.0×10^7	4.8×10^7	6.2×10^7	5.4×10^7	4.6×10^7
	2,500	0	0	0	0	0
	3,000	0	0	0	0	0
Sodium bisulphite	2,000	5.8×10^7	8.0×10^7	9.2×10^7	7.0×10^7	6.8×10^7
	2,500	1.0×10^7	4.2×10^7	5.4×10^7	4.6×10^7	4.0×10^7
	3,000	0	0	0	0	0
	4,000	0	0	0	0	0

TABLE 5: Effect of pH changes on the antimicrobial efficacy of the preservatives.

Antimicrobial agents	pH of growth medium and minimal lethal concentrations (ppm)											
	<i>B. subtilis</i>			<i>B. cereus</i>			<i>E. coli</i>			<i>S. aureus</i>		
	pH4	pH5	pH6	pH4	pH5	pH6	pH4	pH5	pH6	pH4	pH5	pH6
Sodium sulphite	- ^a	400	800	-	400	800	-	500	>1000	-	800	5000
Potassium sulphite	-	400	800	-	400	800	-	500	>1000	-	800	>5000
Sodium metabisulphite	-	100	400	-	100	400	-	150	500	-	300	>2000
Potassium metabisulphite	-	100	400	-	100	400	-	150	500	-	300	>2000
Sodium bisulphite	-	150	800	-	150	800	-	200	1000	-	500	3000

^aNo viable cell even at 0 ppm of the preservatives.

action on the test organisms under such low pH values. It was observed that 400, 500 and 800 ppm of either sulphite destroyed the bacilli, E. coli and S. aureus respectively at pH 5. Bacillus subtilis and B. cereus were killed by 800 ppm of either sulphite at pH 6 while E. coli was not susceptible to 1,000 ppm of sulphite at the same pH value. Staphylococcus aureus evidenced resistance to 5,000 ppm of potassium sulphite while this same amount of sodium sulphite destroyed staphylococcal at pH 6. The metabisulphites of sodium and potassium also showed increase in antimicrobial efficacy at low pH values. At pH 5, 100, 150 and 300 ppm of either metabisulphite destroyed the bacilli, E. coli and S. aureus respectively. It was also observed that 400 and 500 ppm of either metabisulphite destroyed the bacilli and E. coli respectively while S. aureus was not susceptible to 2,000 ppm at pH 6. The bacilli were susceptible to 150 ppm of sodium bisulphite while E. coli required 200 ppm of the bisulphite at pH 5. Staphylococcus aureus was completely destroyed by 500 ppm of bisulphite at pH 5. At pH 6, the bacilli, E. coli and S. aureus were susceptible to bisulphite concentrations of 800, 1,000 and 3,000 ppm, respectively.

inoculum

Effect of varying inoculum sizes on lethal efficacy of the antimicrobial preservatives. Since the efficacy of lethal agents is often dependent on the inoculum level, the effect of inocula on the lethal potency of the sulphur dioxide-generating salts in liquid growth media was investigated. The concentration of the antimicrobial preservatives was steadily increased until where possible minimal lethal concentrations were established using the test organisms at inocula of approximately 10^2 to 2×10^8 cells per 100ml of growth media. Varying amounts of the antimicrobial preservatives were added to 100ml portions of growth media in 250ml Erlenmeyer flasks and the organisms were inoculated into the flasks. The flasks and their contents were then incubated at 37°C for three days. Control flasks differing in inoculum size but containing no preservative were similarly set up. Growth and viability of bacterial cells were assayed as described under Materials and Methods.

The experiment was conducted in triplicate and identical results were obtained. Growth of the test organism was observed in all control samples. Generally, higher concentrations of the antimicrobial preservatives were required to kill the test organisms as the size of the inoculum increased (Table 6). It was observed that the concentrations of the preservatives that killed 10^2 and 10^3 cells of test organisms/100ml of growth media

TABLE 6: Effect of varying inocula on the lethal potency of the preservatives.

Antimicrobial agents	Test organisms	Inocula of test organism and minimal lethal concentration (ppm)				
		10 ²	10 ³	10 ⁴	10 ⁵	2x10 ⁸
Sodium sulphite	<u>E. coli</u>	6,500	6,500	>10,000	>10,000	>10,000
	<u>B. subtilis</u>	6,000	6,000	>10,000	>10,000	>10,000
	<u>S. aureus</u>	6,000	6,000	>10,000	>10,000	>10,000
Potassium sulphite	<u>E. coli</u>	6,500	6,500	>10,000	>10,000	>10,000
	<u>B. subtilis</u>	6,000	6,000	>10,000	>10,000	>10,000
	<u>S. aureus</u>	6,000	6,000	>10,000	>10,000	>10,000
Sodium metabisulphite	<u>E. coli</u>	400	400	500	500	500
	<u>B. subtilis</u>	300	300	400	400	400
	<u>S. aureus</u>	300	300	400	400	400
Potassium metabisulphite	<u>E. coli</u>	400	400	500	500	500
	<u>B. subtilis</u>	300	300	400	400	400
	<u>S. aureus</u>	300	300	400	400	400
Sodium bisulphite	<u>E. coli</u>	800	800	1,000	1,000	1,000
	<u>B. subtilis</u>	600	600	800	800	800
	<u>S. aureus</u>	600	600	800	800	800

were not different. The bacilli were susceptible to 6000 ppm of either sulphite while E. coli was killed when exposed to 6500 ppm of the sulphite at 10^2 cells/100ml. Moreover, 10^2 staphylococcal cells/100ml of BHI showed resistance to the sulphites of sodium and potassium even at 10,000 ppm of either sulphite. Bacillus subtilis, B. cereus and E. coli were not susceptible to 10,000 ppm of either sulphite only at inocula of 10^4 to 2×10^8 cells/100ml. It was noted that 300 ppm of either metabisulphite destroyed the bacilli and 400 ppm destroyed E. coli while S. aureus required 2000 ppm at inocula of 10^2 and 10^3 cells/100ml. At inocula between 10^4 and 2×10^8 cells/100ml the bacilli, E. coli and S. aureus were susceptible to metabisulphite concentrations of 400, 500 and 2,500 ppm respectively.

Investigation into the lethal efficacy of sodium bisulphite (Table 6) as a function of inoculum size indicated that the bacilli, E. coli and S. aureus were susceptible to bisulphite concentrations of 500, 800 and 2200 ppm respectively at inocula of 10^2 and 10^3 cells/100ml. At inocula between 10^4 and 2×10^8 cells/100ml, the bacilli, E. coli and S. aureus were susceptible to bisulphite concentrations of 800, 1000 and 3000 ppm respectively. It was observed that the killing concentrations of the preservatives remained constant from 10^4 to 2×10^8 cells/100ml of growth media.

Viability of the test organisms as a function of time.

All the previous experiments were performed by incubating the preservative-treated liquid growth media at 37°C for three days after inoculation with the appropriate inocula of test organisms. Since this length of time was a random choice it was deemed necessary to determine experimentally how rapidly the test organisms died following initial contact with the sulphur dioxide-generating salts.

The inoculum level used in this phase of the investigation was 2×10^8 cells/100ml of growth medium since this size of inoculum was used in previous experiments. Into 100ml portions of sterile growth media in 250ml Erlenmeyer flasks was added different amounts of the antimicrobial preservatives followed by inoculation with the appropriate bacterial species. Prior to incubation at 37°C, a zero hour control sample was removed from each flask immediately following thorough mixing of the liquid growth medium, the preservative and the inoculated cells. Viability assay was carried out at 0-96 hours after incubation.

In the liquid growth media (Table 7), all control samples showed viability. At 10,000 ppm of sodium sulphite or potassium sulphite, none of the four bacterial species was destroyed within 96 hours. Bacillus subtilis and B. cereus were killed after 24 hours of exposure to 400 ppm of either of the metabisulphites

TABLE 7: Effect of exposure time on the lethal efficacy of the preservatives.

Antimicrobial agents	concentration (ppm)	Viability of bacteria in liquid media after exposure time(hours)																					
		<i>B. cereus</i>			<i>B. subtilis</i>			<i>E. coli</i>			<i>S. aureus</i>												
None	None	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96	0	24	48	72	96		
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		^a																					
	Sodium sulphite	10,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Potassium sulphite	10,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
	Sodium metabisulphite	400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potassium metabisulphite	400	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sodium bisulphite	800	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sodium bisulphite	1,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sodium bisulphite	2,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sodium bisulphite	2,500	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
Sodium bisulphite	3,000	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	
		+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	

^a Presence of viable bacteria.^b Viability was not determined.^c Absence of viable bacteria.

while E. coli was not destroyed within 96 hours of exposure to this metabisulphite level. However, 500 ppm of either metabisulphite destroyed E. coli after 24 hours of exposure. Similarly, S. aureus was not susceptible to 2,000 ppm of the metabisulphites after 96 hours of exposure while 2,500 ppm of metabisulphite rendered BHI Staphylococcus - free after 24 hours of exposure. Generally, all the bacterial species tolerated higher concentrations of sodium bisulphite than the metabisulphites. The bacilli were not susceptible to 600 ppm of the bisulphite within 96 hours while E. coli was not susceptible to 800 ppm of this salt within 96 hours of exposure. Staphylococcus aureus was not destroyed within 96 hours when exposed, to 2,500 ppm of the bisulphite. However, the bacilli, E. coli and S. aureus were destroyed after 24 hours of exposure to bisulphite concentrations of 800, 1,000 and 3,000 ppm respectively. The results indicated that the incubation period of three days was sufficient.

Antimicrobial efficacy of the sulphur dioxide-generating preservatives in full cream powdered milk solution (Nido; Nestle make) Ten per cent (%) milk solution was prepared as follows:

The lid top of the Nido tin was disinfected by wiping with ethanol-treated cotton wool. The lid was then removed and

the seal underneath was similarly also disinfected after which a pair of sterile forceps was used to puncture the seal and tear off a small section of the aluminium foil cover. Using sterile spatula the appropriate amount of the powdered milk was often weighed and dissolved in warm sterile solvent as described under Materials and Methods. Prior to replacement of the lid, the inner surface of the lid was also disinfected with absolute ethanol to further preclude chance contamination especially now that the seal has been broken.

In a preliminary experiment, 1ml of each of the 10% milk solutions was separately plated in quadruplicate on plate count agar (oxoid) by the spread plate method using sterile hockey glass rods. The plates were incubated overnight at 37°C to determine the general 'load' of the milk solutions, that is, number of microbial contaminants. In all cases it was observed that only 3 to 5 microbial colonies were present on plates. However, no attempt was made to identify these few colonies of contaminants. Each of the milk solutions was then portioned into 100ml fractions in sterile 250ml Erlenmeyer flasks and subjected to 'flash sterilization' (15lb per sq. in at 121°C for 2 minutes) (Abalaka, 1977). This period was found to be sufficient for destruction of the microbial contaminants in the milk solutions as determined by the viable plate count method but was short enough to prevent coagulation of the milk.

pH of the milk solutions: The pH values of the milk solutions were found to be identical. These were determined using 10ml sample of each solution and measuring the pH on Corning pH meter model 7 (Evans Electro-selenium Ltd) and were found to be 6.7 ± 0.1 . This pH range was close to the pH values of most of the commercially prepared liquid growth media used for the previous experiments.

Varying concentrations of the sulphur dioxide-generating antimicrobial agents were then incorporated into the flasks containing 100ml of sterile 10% milk solutions after which the desired test organism was inoculated to give 10^4 cells/100ml of the milk solution. A control flask (containing inoculated cells but without antimicrobial agent) was set up. All the flasks were then incubated at 37°C for up to 96 hours after which viability assay was performed on 1ml milk sample from each flask.

Results indicated that higher concentrations of the antimicrobials were required to destroy identical inocula of test organisms in milk than in the liquid growth media used for previous experiments (Table 8). It was also observed that each of the test organisms was destroyed by the same concentration of antimicrobial agent in either of the milk solutions, that is, irrespective of the solvent used for dissolving the powdered milk. Further studies was, however, performed using 10% milk solution prepared by dissolving milk powder in sterile 0.1% peptone water (oxid). Sodium sulphite and potassium sulphite failed to kill

TABLE 8: Effect of the sulphur dioxide-generating antimicrobial agents on the viability of four bacterial species in 10% solution of powdered milk.

<u>Antimicrobial agents</u>	<u>Test organisms^a and minimal lethal concentrations (ppm)</u>			
	<u>B. cereus</u>	<u>B. subtilis</u>	<u>E. coli</u>	<u>S. aureus</u>
Sodium sulphite	> 10,000	> 10,000	> 10,000	> 10,000
Potassium sulphite	> 10,000	> 10,000	> 10,000	> 10,000
Sodium metabisulphite	500	500	600	3,000
Potassium metabisulphite	500	500	600	3,000
Sodium bisulphite	1,000	1,000	1,500	4,000

^aInoculum size of 10^4 cells/100ml was used.

any of the bacterial species in milk even at 10,000 ppm use level. Similar results were previously obtained when commercially prepared liquid growth media were used. Bacillus subtilis and B. cereus were susceptible to 500 ppm of sodium metabisulphite while E. coli and S. aureus were susceptible to 600 and 3,000 ppm of sodium metabisulphite respectively. With potassium metabisulphite results paralleled those observed when sodium metabisulphite was used. In sodium bisulphite-treated milk samples, B. subtilis and B. cereus were killed by 1,000 ppm of the bisulphite while E. coli and S. aureus were killed by bisulphite concentrations of 1,500 and 4,000 ppm respectively.

Simulation of repeated contamination in 10% milk solution.

Because there exists the possibility of repeated exposure of food products to contamination during storage the stability of antimicrobial potency of the sulphur dioxide-generating preservatives was evaluated. The minimal lethal concentrations (MLC) of each of the antimicrobial preservatives, previously determined, and higher concentrations were incorporated into 100 ml portions of the milk solution. Sodium and potassium sulphites were excluded from this phase of the comparative evaluation since none of the test organisms was susceptible to as much as 10,000 ppm of either of these salts. Inocula of 10^4 and 2×10^4 cells/100 ml of milk solution were used in each case. Immediately after

the addition of antimicrobial preservatives, the desired test organism was added and the cultures were incubated at 37°C for 4 days after which viability assay was performed. Contamination was repeated, using the same level of inoculum, immediately after the viability assay and the cultures were further incubated for 4 days and then assayed for viability of the test organisms. A third simulated contamination was effected immediately after the second viability assay, that is, on the eighth day from the initial contamination, and samples were assayed for viability of test organisms at the end of the twelfth day.

Viable cells of B. cereus and B. subtilis were observed after the third simulated contamination at 500 ppm of sodium metabisulphite (Table 9). However, 600 ppm of metabisulphite destroyed the bacilli even after the third simulated contamination in milk solution. Escherichia coli and S. aureus were viable in metabisulphite concentrations of 600 and 3,000 ppm respectively, after the third contamination. It was observed that E. coli and S. aureus were not viable in metabisulphite concentrations of 800 and 4,000 ppm respectively, even after the third simulated contamination. Identical results were obtained with potassium metabisulphite when used in place of sodium metabisulphite. The bacilli also showed viability in 1,000 ppm of sodium bisulphite while E. coli showed viability in 1,500 ppm bisulphite, after the third contamination. Staphylococcus aureus was viable in 4,000 ppm of bisul-

phite but 5,000 ppm of the bisulphite destroyed the staphylococcal cells, after the third simulated contamination in milk solution.

With each of the test organisms, viability was observed after the third contamination at preservative concentrations previously established as MLCs but not at higher concentrations. Therefore, viability observed at these MLC levels might be due to loss of available sulphur dioxide with extended time of incubation.

Relative growth of the four bacterial species in preservative-treated milk solution. An investigation into the relative growth and/or percentage inhibition of Escherichia coli, Bacillus subtilis, Bacillus cereus and Staphylococcus aureus in preservative-treated 10% milk solution was undertaken. The levels of the sulphur dioxide-generating salts that were found to be sublethal or lethal against the test organisms at 10^4 cells/100ml of commercially prepared liquid growth media (Table 6) were used in this phase of the comparative study. In this investigation therefore, 400 ppm of each of the metabisulphites and 800 ppm of sodium bisulphite were used against B. subtilis and B. cereus while E. coli was tested against 500 ppm of metabisulphite and 1,000 ppm of bisulphite. Staphylococcus aureus was exposed to 2,500 ppm of each of the metabisulphites and 3,000 ppm of sodium bisulphite. Although none of the sulphites was lethal against the test organisms, it was decided to evaluate the degree of

inhibition obtainable when the test organisms were exposed to 10,000 ppm of each of the sulphites.

Four sets of six-250ml Erlenmeyer flasks containing 100ml of sterilized 10% milk solution were set up (one set for each test organism). Into five flasks were added the appropriate concentrations of the antimicrobial preservatives such that only one flask in the set of six was set up for each of the preservatives. The sixth flask to which no preservative was added served as a control. All the flasks were then inoculated with the appropriate test organism to give 10^4 cells/100ml of milk and incubated at 37°C for three days. The number of viable cells of the test organism in 1ml milk sample from each flask was determined by the viable count method and the relative percentage growth of the test organism was determined as follows:-

Number of viable cells in preservative-treated sample x 100

Number of viable cells in control sample. The value obtained for each antimicrobial preservative was then subtracted from 100% which was considered as an ideal inhibition to obtain the approximate percentage inhibition of the test organisms. Thus, in the above procedure the percentage growth of test organism in the control sample was arbitrarily set at 100.

Observation on the percentage inhibition of the four bacterial species has been recorded in Table 10. Two trials were conducted, and since there were no wide variations in the results obtained,

the values recorded represented the mean of the two determinations. Results indicated that the relative bacteriostatic or bacteriocidal efficacy of the sulphur dioxide-generating salts in increasing order could be rated as potassium sulphite, sodium sulphite, sodium bisulphite, potassium metabisulphite, and sodium metabisulphite.

C H A P T E R

F O U R

DISCUSSION

The data indicated that none of the test organisms subjected to the antimicrobial action of the sulphur dioxide-generating salts was completely refractory to the killing effect of the salts. Generally, in performing the viability assay for each bacterial species, the presence of one colony of test organism on agar plates was considered a positive result. This is because such a colony has a chance to proliferate to a significant ^{numerical} level under favourable conditions. However, some aspects of this investigation considered the reduction in microbial population as a useful parameter in assessing the antimicrobial efficacy of the sulphur dioxide-generating salts. Results suggested that there exists a definite relationship between the lethal efficacy of the salts and the inoculum size of the contaminating microorganism, between the lethal efficacy and pH of culture media, between the lethal efficacy and exposure time and between the lethal efficacy and media composition. Thus, any discussion of death inflicted on the test organisms by these salts must take into account the relationship amongst these four factors.

Antimicrobial efficacy of the sulphur dioxide-generating salts in liquid media.

Under practical conditions, the antimicrobial efficacy of food additives is determined by the intrinsic antimicrobial

spectrum of the additive which is mostly described in terms of minimal inhibitory concentration (at given pH, water activity, nutrient concentration, etc.), against a broad taxonomic group of food spoilage organisms (Joint FAO/WHO, 1971). However, this investigation described the antimicrobial efficacy of the sulphur dioxide-generating salts in terms of minimal lethal concentration for two reasons. In the first instance, four bacterial species (B. subtilis, B. cereus, S. aureus and E. coli) were used as test organisms and these cannot be considered as representing a broad taxonomic group of food spoilage organisms. Secondly, a preservative effectively suppressing one type of microorganism may have no inhibitory effect at all on a different type: no preservative effect can be expected from a bacteriostatic agent, if the spoilage association of the food in which the preservative is to be used is almost purely fungal. The lethal efficacy of the salts was tested at the optimum growth conditions for the test organisms (neutral pH, 37°C) and using a large inoculum size of the test organisms (2×10^8 cells/100ml of growth media).

Results have shown that in commercially prepared growth media (Tables 1 to 4) and in 10% solution of Nido powdered milk (Table 8), S. aureus was the most resistant of the test organisms. This investigation did not attempt to confirm the relative degree of resistance of S. aureus in the other commercially prepared growth media used since the 10% milk solution afforded a common

growth medium for the four test organisms. The difference in degree of susceptibility of the four bacterial species to the antimicrobial action of the sulphur dioxide-generating salts may be attributed to variations in morphological and physiological characteristics. Moreover, resistance is an inherent genetic characteristic that varies from species to species.

The finding that all the preservatives were less potent in milk solution by a factor of 1.2 to 1.5 than in commercially prepared growth media lends credence to the fact that there exists a definite relationship between susceptibility and media composition. Abalaka (1977) has reported that the antimicrobial effect of sulphur dioxide is dependent on the composition of the substrate in which it is incorporated and this, in turn, depends on the formulation. Particularly significant is the level of carbonyl group and the sulphur-containing amino acid composition of the substrates. Sulphur dioxide participates in addition reactions leading to the formation of aldehyde-metabisulphite addition complex and to the oxidation of the disulphide of the amino acids. Such reactions reduce the availability of sulphur dioxide, and therefore, its lethality on the microorganisms (Burton, et al., 1963). On the basis of composition, it would appear that the Nido milk solution is richer in carbonyl and disulphide groups than the other growth media thereby providing greater protection to the test organisms. Moreover, Nido powdered

milk, as inscribed on the tin container, contains 28% fat which may partly extract some preservatives from the aqueous phase of the food and hence reduce their antimicrobial activity since the microorganisms are in the aqueous phase (Joint FAO/WHO, 1971).

Minimal lethal concentration of the preservatives as a function of pH.

The most important parameter of a food determining the activity of a given preservative is its pH value. Although there are many potential antimicrobial food additives, most of the preservatives used are acids. The activity of such preservatives is virtually nil in the neutral pH area and increases with decreasing pH. Similarly, the antimicrobial effect of sulphur dioxide (the antimicrobial component of metabisulphite, bisulphite and sulphite) is pH dependent, being more potent at lower pH values than higher ones (Allen and Brook, 1970).

Preliminary experiments revealed that the pH of the growth media without any modification was near neutrality. None of the four bacterial species thrived at pH 4.0 even in the absence of any preservative (Table 5). Thus the acidity level at this pH was sufficiently high to prevent viability of the bacterial species by itself alone. At pH values between 5.0 and 6.0, the killing concentration of the sulphur dioxide-generating salts increased with increase in pH. The lethal effect of the metabisulphites on

the test organisms except S. aureus was increased 3.3 - 4 folds more at pH 5.0 than at pH 6.0. Sodium sulphite and potassium sulphite were about 50% as effective against B. subtilis, B. cereus and, E. coli at pH 6.0 as at pH 5.0 and about 16% as effective against S. aureus at pH 6.0 as at pH 5.0. Sodium bisulphite was 18.75% as effective against the bacilli at pH 6.0 as at pH 5.0 and about 16.7% as effective against S. aureus at pH 6.0 as at pH 5.0. The bisulphite was 20% as effective against E. coli at pH 6.0 as at pH 5.0. These findings are encouraging since they clearly depict the relationship of acidity to antimicrobial potency. Particularly noteworthy is the greatly enhanced potency of the sulphites at low pH values compared to near neutral pH at which the sulphites failed to exert lethality on any of the test organisms even at 10,000 ppm. It could be speculated that the solubility of the sulphites is greatly enhanced in acidic medium, being less soluble in water (pH 7.0) at 20°C. At the pH of water, anhydrous sodium sulphite has a solubility of 25g/100ml while potassium sulphite has a solubility of 28.5g/100ml (Joint FAO/WHO, 1971). This solubility effect might account for the increased availability of the undissociated acid (H_2SO_3) rather than HSO_3^- ion, the former being the active antimicrobial species in acidic medium.

Minimal lethal concentration of the preservatives as a function of inocula of test organisms.

As in the physical preservation of foods, the efficiency of chemical preservation depends largely on the initial numbers of potential spoilage agents in the food: the higher the initial load of potential spoilage, the higher the concentration of an antimicrobial preservative required to attain a desired keeping quality. However, it is desirable to limit the amount of preservatives to the lowest possible level for toxicological as well as for microbiological reasons.

Table 6 shows that higher concentrations of the sulphur dioxide-generating salts were required to kill the test organisms as the inocula were increased. In all cases, the concentrations of the preservatives required to kill 10^2 and 10^3 cells/100ml of culture were identical. Results have shown that much higher concentrations of the preservatives were required to kill even 10^2 staphylococcal cells/100ml compared to the same inoculum level of the other three test organisms. This further confirms the outstanding resistance of S. aureus. It was observed that between the inocula of 10^4 and 2×10^8 cells/100ml, the test organisms showed higher resistance to the antimicrobials than at 10^2 or 10^3 cells/100ml. Moreover, the killing concentrations of the antimicrobials remained constant over this inocula range, that is, 10^4 to 2×10^8 cells/100ml. Generally, about 25% to

36.7% more of the antimicrobial preservatives (except the sulphites) were required to kill the test organisms at 10^4 than at 10^3 cells/100ml.

Abalaka (1977) had reported that at the marginally lethal concentrations of metabisulphite, the lethal efficacy or potency of the metabisulphite was a function of the size ^{of} inoculum until a critical concentration was obtained at which the inoculum had no direct effect on the lethal potency of metabisulphite. This was explained as due to the fact that at such a critical concentration metabisulphite action was all or none since the concentration that caused death of 1 cell/g of sample also caused the death of 10^8 cells/g. In this investigation (Table 6), results tend to support these observations. The non-inoculum dependent concentration of the metabisulphite for the bacilli, E. coli and S. aureus was 400, 500 and 2,500 ppm respectively. Similarly, the non-inoculum dependent concentration of bisulphite for the bacilli, E. coli and S. aureus was 800, 1,000 and 3,000 ppm respectively. It was not possible to establish such concentration with respect to the sulphites for any of the test organisms. Moreover, these concentrations would also depend on pH of the growth media employed, media composition and the available sulphur dioxide.

The available sulphur dioxide is a very important factor in discussing the lethal potency of metabisulphites, bisulphites and sulphites. At a first glance the use of up to 10,000 ppm of the

sulphites in this investigation might appear excessive. This was due to the fact that the sulphites contain the lowest amount of sulphur dioxide and are most unstable compared to the bisulphite and metabisulphite. Sodium sulphite contains about 48% sulphur dioxide (Joint FAO/WHO, 1971). It has been documented that only 33.7% of a given amount of metabisulphite is the theoretically available sulphur dioxide (Abalaka, 1977). In practice some of the sulphur dioxide is wasted when it combines with certain organic components of foods and feeds (Burton, et al., 1963; Reynolds, et al., 1962). This implies that the free sulphur dioxide is a fraction of the theoretically available sulphur dioxide. However, mention must be made of the fact that the amount of bound and free sulphur dioxide in the liquid media used for this comparative study was not determined, but this notwithstanding, the free sulphur dioxide should be considerably lower than the total sulphur dioxide.

Mammalian tissues are known to be endowed with very efficient mechanisms for metabolizing sulphur dioxide and sulphur dioxide-containing foods especially to sulphate (Schroeter, 1966). Data from monkeys, rats, mice and dogs (Gibson and Strong, 1973a) clearly showed that mammals have a large capacity to metabolize sulphites, bisulphites and metabisulphites. Wilkins, et al (1968) have demonstrated that in dogs, the liver alone was capable of metabolizing at least 50mg SO₂/kg/hr, and Macleod, et al (1961) have

argued earlier that in rats, the liver is not the only tissue capable of metabolizing appreciable quantities of sulphite. Moreover, in fruit dip (a method of preserving fruit against microbial spoilage) about 2,500 ppm of sulphur dioxide (equivalent to about 8,000 ppm of metabisulphite) is used routinely (Potter, 1968). Therefore, the minimal lethal concentrations established in this investigation appear quite safe for animals and human beings.

C H A P T E R

F I V E

CONCLUSION

All the preservatives employed in this comparative study are generally regarded as safe (GRAS) for addition to foods and are non-toxic at the levels permitted in foods. The levels of the preservatives used in this investigation are within those permitted in foods (Joint FAO/WHO, 1971). In concluding this study, it is only proper that an attempt is made to identify which of the sulphur dioxide-generating salts is closest to an ideal preservative based on the results of this comparative evaluation. Needless to say, of all the recognised food additives, the ideal chemical preservative has not been found. Moreover, the number of new, simple and 'ideal' preservatives being suggested are few, and many of those suggested, for one reason or another, do not develop into commercially acceptable additives (Frazier and Westhoff, 1978). Consequently, many of the compounds still widely used are also the oldest preservatives.

The data obtained from this investigation have shown that the metabisulphites of sodium and potassium are the most effective sulphur dioxide-generating antimicrobials. The data also suggested that the sulphites of sodium and potassium are equally potent antimicrobials at low pH values.

SUGGESTIONS FOR FURTHER STUDIES

Despite the fact that the metabisulphites of sodium and potassium have clearly demonstrated a promising antimicrobial potency, the need to carry out further investigations along the following suggestions cannot be over-emphasized.

1. The effect of media composition on metabisulphite lethality against a broad taxonomic group of food spoilage organisms.
2. Possible synergistic effects of metabisulphite in combination with other classes of antimicrobials on the destruction of food spoilage organisms.
3. The mechanism of lethal action of metabisulphite on microorganisms.
4. Feed-trial experiments to establish the definite role of metabisulphite in carcinogenic and mutagenic hazards.
5. The effect of metabisulphite on the organoleptic
of
quality of a number/locally produce food items.

REFERENCES

1. Abalaka, J.A. 1977. Combined effect of moisture and sodium metabisulphite on the destruction of Salmonella and other bacteria in bone meal and gelatin. Ph.D dissertation, University of Wisconsin, Madison, U.S.A.
2. Abalaka, J.A. and Deibel, R.H. 1980. A comparative study of the lethal effect of metabisulphite on the viability of three bacterial species in bone meal and gelatin. *Microbios.* 27, 79-88.
3. Akinsanya, A.A. 1979. Investigation into the mechanism of killing of Bacillus subtilis by sodium metabisulphite. B.Sc dissertation, Ahmadu Bello University, Zaria, Nigeria.
4. Allen, R.J.L. and Brook, M. 1970. Effects of sulphur dioxide in animals and man, pp. 799-805. In Proc. SOS/70, 3rd Internl. Cong. Food Sci. Technol., Inst. Food Technol., Chicago, Illinois, U.S.A.
5. American Public Health Association, 1972. Standard Methods for the Examination of Dairy Products. 13th ed. American Public Health Association, New York.
6. Arbuthnott, J.P. 1970. Staphylococcal β -toxin. pp.189-236. In Montie, T.C., Kadis, S., and Ajl, S.J. (Eds): Microbial toxins. Vol. III, Bacterial Protein Toxins. Academic Press, New York.
7. Baird-Parker, A.C. 1972. Classification and identification of staphylococci and their resistance to physical agents. pp. 1-10. In Cohen, J.O. (Ed.): The Staphylococci. Wiley-Interscience, New York.
8. Bergdoll, M.S. 1968. Bacterial toxins in foods presented at the I.F.T. Annual Meetings in Philadelphia, U.S.A.

9. Bergdoll, M.S. 1970. In Montie, T.C., Kadis, S., and Ajl, S.J. (Eds.): Microbial Toxins. Vol III. Bacterial Protein Toxins. Academic Press, New York.
10. Bergdoll, M.S., Huang, I.Y., and Schantz, E.J. 1974. Chemistry of the staphylococcal enterotoxins. J. Agri. Fd. Chem. 22(1), 9 - 12.
11. Bond, W.W. and Favero, M.S. 1975. Thermal Profile of a Bacillus species ATCC 27380 extremely resistant to dry heat. Appl. Microbiol. 29, 859 - 860.
12. Bonventre, P.F. and Johnson, C.E. 1970. Bacillus cereus. In Montie, T.C., Kadis, S., and Ajl, S.J. (Eds.): Microbial Toxin. Vol III. Bacterial Protein Toxins. Academic Press, New York.
13. Borick, P.M., and Fogarty, M.G. 1967. Effects of continuous and interrupted radiation on microorganisms. Appl. Microbiol. 15, 785 - 789.
14. Bray, J. 1945. Isolation of Antigenically Homogeneous strains of Bact. coli neapolitanum from Summer Diarrhoea of Infants. J. Pathol. Bacteriol. 57, 239.
15. Brown, B.I. 1972. In Fennema, O.R. 1976. Principles of Food Science. pp.490. Marcel Dekker Inc., New York.
16. Bryan, F.L. 1969. Bacillus subtilis. pp.244-245. In Food Borne Infections and Intoxications. Rieman, H. (Ed.). Academic Press, Inc., New York.
17. Bryan, H.A., Bryan, A.C., and Bryan, G.C. 1962. Bacteriology. Principles and Practice (Sixth edn.). Barnes and Noble, Inc., New York.

18. Buchanan, R.E. and Gibbons, N.E. (Eds.). 1974. *Bergey's Manual of Determinative Bacteriology*. 8th ed. William and Wilkins Co., Baltimore.
19. Burton, H.S., McWeeny, D.J., and Biltcliffe, D.O. 1963. Non-enzymic browning; the role of unsaturated carbonyl compounds as intermediates and of SO₂ as an inhibitor of browning. *J. Sci. Food Agric.* 14, 911 - 920.
20. Casman, E.P. and Bennett, R.W. 1965. Detection of staphylococcal enterotoxin in food. *Appl. Microbiol.* 13, 181-189.
21. Cecil, R.L. and Loeb, R.F. 1959. *Textbook on Medicine*. 10th ed. Saunders, Philadelphia.
22. Chichester, D.F. and Tanner, F.W. Jr. 1972. In *Handbook of Food Additives*. Furia, T.E. (ed.). 2nd edition. CRC Press, Cleveland, pp. 115.
23. Cohen, J.O. (Ed.). 1972. *The staphylococci*. Wiley-Interscience, New York.
24. Cooke, E.M. 1974. Escherichia coli and Man. Churchill Livingstone, Edinburgh.
25. Cross, T. 1970. *J. Appl. Bacteriol.* 33, 95. In *Handbook of Microbiology*. Vol. I. Organismic Microbiology. Laskin, A.I. and Lechevalier, H.A. (eds.). CRC Press, Cleveland, Ohio.
26. Dairy Products Laboratory. 1969. *Cheese Varieties and Descriptions*. In Carr, J.G., Cutting, C.V., and Whiting, G.C. 1975. *Lactic acid bacteria in beverages and food*. Academic, New York.

27. Davis, F.L., Wyss, O., and Williams, O.B. 1948. In Microbial Ecology. Alexander, M. (ed.). John Wiley and Sons Inc., New York, London, Sydney, Toronto. pp. 194.
28. Davis, F.L. and Wilkinson, G. 1973. Bacillus cereus in milk and dairy products. pp. 57-67. In Microbiological Safety of Foods. Hobbs, B.C. and Christian, J.H.C. (eds.). Academic Press, London.
29. Deibel, R.H. and Evans, J.B. 1960. Modified benzidine test for the detection of cytochrome-containing respiratory systems in microorganisms. J. Bacteriol. 79, 356-358.
30. DeLey, J. 1964. Annu. Rev. Microbiol. 18, pp. 17. In Handbook of Microbiology. Vol. I. Organismic Microbiology. Laskin, A.I. and Lechevalier, H.A. (eds.).
31. Dorner, F. 1975. Escherichia coli enterotoxin: purification and partial characterization. pp. 242-251. In Schlessinger, D. (ed.). Microbiology - 1975. American Society for Microbiology. Washington, D.C.
32. Dyett, E.J. and Shelly, D.D.M. 1966. J. Bacteriol. 29 (3), 439. In Fennema, O.R. 1976. Principles of Food Science. pp. 490. Marcel Dekker Inc., New York.
33. Eade, S.A. 1974. Bacillus cereus food poisoning. British Food J. 76, 860 - 880.
34. Edwards, P.R. and Ewing, W.H. 1972. Identification of Enterobacteriaceae. 3rd ed. Burgess, Minneapolis, Minn.
35. Ewing, W.H. 1973. Differentiation of Enterobacteriaceae by biochemical reactions. Revised, DHEW Pub. No. (CDC) 74-8270. U.S. Public Health Service, Centre for Disease Control, Atlanta, Ga.

36. Ewing, W.H. and Davis, B.R. 1961. The O antigen groups of Escherichia coli cultures from various sources. U.S. Public Health Service, Centre for Disease Control, Atlanta, Ga.
37. Fennema, O.R. 1976. Principles of Food Science. Part 1. Food Chemistry. Chp. XIV, Antimicrobial agents. Marcel Dekker Inc., New York.
38. Foster, E.M., et al. 1957. Dairy Microbiology. In Burrows Textbook of Microbiology. 21st ed. Freeman, B.A. 1979. W.B. Saunders Company. Philadelphia. London. Toronto.
39. Fox, B.A. and Camerton, A.G. 1972. Food Science - A Chemical Approach. 2nd ed. Unibooks, University of London Press Ltd. London.
40. Frazier, W.C. and Westhoff, D.C. 1978. Food Microbiology. 3rd ed. Tata McGraw-Hill Pub. Company Ltd. New Delhi.
41. Freeman, B.A. 1979. Burrows Textbook of Microbiology. 21st ed. W.B. Saunders Company. Philadelphia. London. Toronto.
42. Gibson, W.B. and Strong, F.M. 1973a. Metabolism and elimination of sulphite by rats, mice and monkeys. Food Cosmet. Toxicol. 11, 185 - 198.
43. Gibson, W.B. and Strong, F.M. 1973b. Safety of sulphited foods. pp.256-258. In Food Res. Inst. Annual Rept. 1973, issued June 1974 (Univ. Wisconsin, 1925 Willow Drive, Madison, WI 53705).
44. Gilbert, R.J. and Taylor, A.J. 1976. Bacillus cereus food poisoning. pp. 197-213. In Microbiology in Agriculture, Fisheries and Food. Skinner, F.A. and Carr, J.G. (eds.). Academic Press, New York.

45. Goepfert, J.M. 1974. Monkey feeding trials in investigation of the nature of Bacillus cereus poisoning. IV. Intern. Cong. Food Sci. Technol. 4C, 1-3. In Food Sci. Technol. Abstr. 7(4), 1975. Abstr. No. 4C 132.
46. Goepfert, J.M., Spira, W.M., and Kim, H.U. 1972. Bacillus cereus food poisoning organism. A review. J. Milk Fd. Technol. 35, 213-227.
47. Gould, G.W. 1977. Recent advances in the understanding of resistance and dormancy in bacterial spores. J. Appl. Bacteriol. 42, 297-309.
48. Gould, G.W. and Hurst, A. 1969 (Eds.). The Bacterial Spore. Academic Press, London. England. New York.
49. Gyles, C.L. and Barnum, D.A. 1969. A heat labile enterotoxin from strains of Escherichia coli enteropathogenic for pigs. J. Infect. Dis. 120, 419 - 426.
50. Halvorson, H.O., Hanson, R., and Campbell, L.L. 1972. (Eds.). Fifth International Spore Conference. Fontana, Wisconsin. 1971. American Society for Microbiology, Washington, D.C.
51. Hammer, B.W. and Babel, F.J. 1957. Dairy Bacteriology. 4th ed. John Wiley and Sons, New York.
52. Hammond, S.M. and Carr, J.G. 1976. The antimicrobial activity of SO₂ with particular reference to fermented and non-fermented fruit juices. pp.89-110. In Inhibition and inactivation of vegetative microbes. Skinner, F.A. and Hugo, W.B. (Eds.). Academic Press Inc., New York.
53. Hanson, L. . 1976. Escherichia coli infections in childhood. Significance of bacterial virulence and immune defence. Arch. Dis. Childh. 51, 737 - 743.

54. Hardy, K.G. 1975. Colicinogeny and related phenomena. *Bacteriol. Rev.* 39, 464 - 515.
55. Hauge, S. 1955. Food poisoning caused by aerobic spore-forming bacilli. *J. Appl. Bacteriol.* 18, 591 - 595.
56. Hayatsu, H. and Miura, A. 1970. The mutagenic action of sodium bisulphite. *Biochem. Biophys. Res. Commun.* 39, 156 - 160.
57. Hayatsu, H., Wataya, Y., Kai, K., and Iida, S. 1970. Reaction of sodium bisulphite with Uracil, Cytosine and their derivatives. *Biochemistry N.Y.* 9, 2858 - 2865.
58. Hobbs, B.C. 1969. Bacillus cereus and B. mesentericus. pp. 154-173. *In* Food Borne Infections and Intoxications. Rieman, H. (ed.). Academic Press Inc., New York.
59. Jawetz, E., Melnick, J.L. and Adelberg, E.A. 1972. Coagulase test for staphylococci species. A review of Medical Microbiology. 10th ed. Blackwell Sc. Pub. Oxford, London.
60. Jay, J.M. 1978. Modern Food Microbiology. 2nd ed. (Chp. 5 The incidence and types of microorganisms in food). D. van Nostrand Co., New York. pp. 77-104.
61. Jeljaszewicz, J. (ed.). 1976. Staphylococci and staphylococcal diseases. Proceeding of III International Symposium on Staphylococci and Staphylococcal infections. *Zentbl. Bakt.* 1., Suppl. 5, pp.1-1137.
62. Joint FAO/WHO Expert Committee on Food Additives; 8th Rept. 1965. Specifications of the identity and purity of food additives, and their toxicological evaluation. *FAO Nutr. Mgt. Rept. Series No. 38; Tech. Serv. WHO* 309, 25 - 41.

63. Joint FAO/WHO Expert Committee on Food Additives; 9th Rept. 1967. Toxicological evaluation of some antimicrobials, antioxidants, emulsifiers, stabilizers, flour-treatment agents, acids and bases. FAO Nutr. Mtg. Rept. Series No. 40: WHO Tech. Rep. Ser., 1966, 339.
64. Joint FAO/WHO Expert Committee on Food Additives; 14th Rept. 1971. A Review of the Technological Efficacy of some Antimicrobial Agents. FAO Nutr. Mtg. Rept. Series No. 48: WHO Tech. Rep. Ser., 1970, No. 462.
65. Joslyn, M.A. and Braverman, J.B.S. 1954. In Advances in Food Research. Mraz, E.M. and Stewart, G.F. (eds.). Academic Press, New York. pp.97. (Cited by Fennema, O.R. 1976. pp. 489).
66. Kim, H.U. and Goepfert, J.M. 1971. Appl. Microbiol. 22, 581. In Handbook of Microbiology. Vol. 1. Organismic Microbiology. Laskin, A.I. and Lechevalier, H.A. (eds.). CRC Press, Cleveland, Ohio. pp. 87.
67. Kovacs, N. 1956. Identification of Pseudomonas pyocyanea by the oxidase reaction. Nature. 178, 703.
68. Kreger, A.S., et al. 1971. Purification and properties of staphylococcal delta haemolysin. Infect. Immun. 3, 449-465. (Cited by Freeman, B.A. 1979. pp. 444.).
69. Luker, C., Mosser, J., and Hanson, R.S. 1975. The effect of sulfite on microorganisms. pp. 378-379. In Food Res. Annual Rept. 1975. Issued June 1976 (Univ. Wisconsin, 1925 Willow Drive, Madison, WI 53706).

70. Macleod, R.M., Farkas, W., and Handler, P. 1961. Purification and properties of hepatic sulphite oxidase. *J. Biol. Chem.* 236, 1841 - 1846.
71. Mapson, L.W. 1962. Enzymic browning of potato tissue: physical, chemical and biological methods of control. *Proc. 1st Intern. Cong. Food Sci. Technol.* 2, 17 - 26.
72. McCready, R.G.L., Costerton, J.W., and Laishley, E.J. 1976. Morphological modification of cells of Clostridium pasteurianum caused by growth on sulphite. *Can. J. Microbiol.* 22, 269 - 275.
73. McGeachie, J. 1966. Hemolysis by urinary Escherichia coli. *Amer. J. Clin. Pathol.* 45, 222 - 224.
74. McWeeny, D.J. and Burton, H.S. 1963. Some possible glucose/glycine browning intermediates and their reactions with sulphites. *J. Sci. Food Agric.* 14, 291 - 302.
75. Meyers, L.L. and Guinée, P.A.M. 1976. Occurrence and characteristics of enterotoxigenic Escherichia coli isolated from calves with diarrhoea. *Infect. Immun.* 13, 1117 - 1119.
76. Moon, H.W., Sorensen, D.K., and Sautter, J.H. 1966. Escherichia coli infection of the ligated intestinal loop of the newborn pig. *Amer. J. Vet. Res.* 27, 1317 - 1325.
77. Moon, H.W., Sorensen, D.K., and Sautter, J.H. 1968. Experimental enteric colibacillosis in piglets. *Can. J. Comp. Med.* 32, 493 - 497.
78. Moon, H.W., Whipp, S.C., and Baetz, A.L. 1971. Comparative effects of enterotoxins from Escherichia coli and Vibrio cholerae on rabbit and swine small intestine. *Lab. Invest.* 25, 133 - 140.

79. Morbidity and Mortality Weekly Reports. 1972. 21, 422.
(Cited by Bergdoll, Huang, and Schantz. 1974. pp. 9).
80. Mukai, F., Hawryluck, I., and Shapiro, R. 1970. The mutagenic specificity of sodium bisulphite. *Biochem. Biophys. Res. Commun.* 39, 983 - 988.
81. Nickerson, J.T.R. and Sinskey, A.J. 1972. *Microbiology of foods and food processing*. American Elsevier Pub. Company, New York.
82. Nilsson, G. 1959. Reducing properties of normal and abnormal milk and their importance in bacteriological grading of milk. *Bacteriol. Rev.* 23, 41 - 47.
83. Norris, J.R. 1969. In the Bacterial spore. Gould, G.W. and Hurst, A. (eds.). Academic Press, London, England and New York. pp. 485.
84. Nygren, B. 1962. Phospholipase C-producing bacteria and food poisoning. *Acta Pathol. Microbiol. Scand. Suppl.* 160, 1 - 89.
85. Okafor, N. 1977. The microbiological basis of a method for palm wine preservation. *J. Appl. Bacteriol.* 43, 159 - 161.
86. Ormay, L. and Novotny, T. 1968. The significance of B. cereus food poisoning in Hungary. *Proc. VI. Intern. Symp. Fd. Microbiol.* pp. 279 - 285.
87. Pelczar, J.M., Reid, D.R., and Chan, S.C.E. 1977. *Microbiology*. Fourth ed. Tata McGraw-Hill Pub. Company Ltd. New Delhi.

88. Potter, N.N. 1968. Sulphur dioxide dip. pp. 487 - 8. In Food Science. 1st ed. The AVI Pub. Co., Westport, Connecticut, U.S.A.
89. Report of the Departmental Committee on Preservatives and colouring matters in foods. London, HMSO. 1924.
90. Reynolds, T.M., Anet, E.F.L., and Ingles, D.L. 1962. Mechanism of non-enzymic browning reactions and their inhibition by sulphur dioxide. In Proc. 1st Intern. Cong. Food Sci. Technol. 1, 209 - 216.
91. Roberts, T.A. and Hitchins, A.D. 1969. Resistance of spores. In The Bacterial Spore. Gould, G.W. and Hurst, A. (eds.). Academic Press, London. New York. pp. 611 - 670.
92. Roberts, A.C. and McWeeny, D.J. 1972. J. Food Technol. 7(3), 221 (Cited by Fennema, O.R. 1976. pp. 490).
93. Roggendorf, M. and Miller, H.E. 1976. Enterobakterien bei Reptilien. Zentbl. Bakt. 1. Orig. A. 236, 22-35. In Burrows Textbook of Microbiology. 21st ed. Freeman, B.A. 1979. W.B. Saunders Company. Philadelphia. London. Toronto.
94. Rottini, G., et al. 1975. Correlation between phagocytic activity and metabolic response of polymorphonuclear leucocytes toward different strains of Escherichia coli. (Cited by Freeman, B.A. 1979. pp. 516).
95. Sack, R.B. 1975. Human diarrhoeal disease caused by enterotoxigenic Escherichia coli. Annu. Rev. Microbiol. 29, 333 - 353.

96. Sale, A.J.H., Gould, G.W., and Hamilton, W.A. 1970. Inactivation of spores by hydrostatic pressure, *J. Gen. Microbiol.* 60, 323 - 334.
97. Schroeter, L.C. 1966. Sulphur dioxide: application in foods, beverages, and pharmaceuticals. 1st ed. Pergamon Press, New York, U.S.A.
98. Shapiro, R., Cohen, B.I., and Servis, R.E. 1970a. Specific deamination of RNA by sodium bisulphite. *Nature.* 227, 1047 - 1048.
99. Shapiro, R., Servis, R.E., and Welcher, M. 1970b. Reactions of uracil and cytosine derivatives with sodium bisulphite. A specific deamination method. *J. Amer. Chem. Soc.* 92, 422 - 424.
100. Sherr, H.P., et al. 1973. Pathophysiological response of rabbit jejunum to Escherichia coli enterotoxin. *Gastroenterology*, 65, 895 - 902. (Cited by Freeman, B.A. 1979. pp. 516).
101. Short, E.C. Jr. and Kurtz, H.J. 1971. Properties of the haemolytic activities of Escherichia coli. *Infect. Immun.* 3, 678 - 687.
102. Smith, J. 1957. Milk-borne disease. *J. Dairy Res.* 24, 121 - 133.
103. Smith, H.W. 1963. The haemolysins of Escherichia coli. *J. Pathol. Bacteriol.* 85, 197 - 211.
104. Smith, H.W. and Halls, S. 1967. Studies on Escherichia coli enterotoxin. *J. Pathol. Bacteriol.* 93, 531 - 543.
105. Snyder, I.S. and Koch, N.A. 1966. Production and characteristics of haemolysins of Escherichia coli. *J. Bacteriol.* 91, 763 - 767.

106. Swan, J.M. 1957. Thiols, disulphides, and thiosulphates: some new reactions and possibilities in peptide and protein chemistry. *Nature*. 180, 643 - 645.
107. Symposium, 1965. The staphylococci: ecological perspectives. *Ann. N.Y. Acad. Sci.* 128, 1 - 456.
108. Thompson, F.J. and Thames, O.A. 1967. Sporulation of B. stearothermophilus. *Appl. Microbiol.* 15, 975 - 979.
109. Tompkin, R.B., Christiansen, L.N., and Shaparis, A.B. 1980. Antibotulinal efficacy of sulphur dioxide in meat. *Appl. Env. Microbiol.* 39, 1096 - 1099.
110. Tong, et, al. 1962. *American J. Public Health.* 52, 976. (Cited by Bryan, F.L. 1969. pp. 245).
111. Wade, P. 1972. Action of sodium metabisulphite on the properties of hard sweet biscuit dough. *J. Sci. Fd. Agric.* 23, 333 - 336.
112. Wilkins, J.W., Green, J.A., and Weller, J.W. 1968. Toxicity of intraperitoneal bisulphite. *Clin. Pharmacol. Ther.* 9, 328 - 335.
113. Williams, R.E.O. 1963. Healthy carriage of Staphylococcus aureus: its prevalence and importance. *Bacteriol. Rev.* 27, 56 - 71.
114. Wiseman, G.M. 1970. The beta- and delta-toxins of Staph. aureus. pp. 237 - 263. In *Microbial Toxins Vol. III. Bacterial Protein Toxins*. Montie, T.C., Kadis, S., and AJL, S.J. (eds.). Academic Press, New York.
115. Wiseman, G.M. 1975. The haemolysins of Staph. aureus. *Bacteriol. Rev.* 39, 317 - 344.
116. Wiseman, G.M. and Caird, J.D. 1968. Phospholipase activity of the delta haemolysin of Staph. aureus. In *Proc. Soc. Exptl. Biol. Med.* 128, 428 - 430.