THE EFFECTS OF COMBINED PRESERVATIVE SYSTEMS ON BACTERIAL SPORES AND NON-SPOREFORMERS

by

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TO MY LOVING MOTHER MADAM WURAOLA OLOYEDE

AND MY LATE FATHER PA BUKOYE OLOYEDE

WHO SO MUCH DESIRE EDUCATION FOR THEIR

CHILDREN BECAUSE THEY NEVER

HAD THE OPPORTUNITY

PREFACE

The application of heat is one of the effective methods of food preservation. Heat involves the expenditure of much energy and in many parts of the world the cost is high. Moreover, overprocessing may severely affect the essential organoleptic and nutritive qualities of the food. In view of these problems there is increased interest in the use of reduced thermal processes combined with chemical preservation.

In the thermal processing of foods bacterial spores are generally used as the target resistant organisms. Consequently a thorough understanding of the effects of combined preservative systems involving heat and chemicals is critical to define the process parameters, to assure safety and to prevent spoilage.

The present study investigates the response of bacterial spores to mild heat treatment in the presence of potassium sorbate, salt and other chemicals. Due to their public health significance, the response of non-sporeforming organisms including Staphylococcus aureus and Escherichia coli are also considered.

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CONTENTS

ABSTRACT

The effects of the combination of heat, chemicals and pH on Staphylococcus aureus (NCIB 6571), Escherichia coli (NCIB 8114) and particularly Bacillus cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920) spores were studied in five test systems. The systems contained (a) potassium sorbate (0 - 1%) or (b) sodium chloride (0 - 10%) or (c) sodium benzoate (0 - 3%) or (d) combination of (a) and (b) or (e) combination of (a) and (c) at varying pH levels (4.2 - 7.1). The test systems were heated at varying temperatures ranging from 50° - 95°C at different times. In general inhibition of all tests strains by all systems increased with increasing levels of chemicals used and with decrease in pH. Low temperature heat treatment in the presence of sorbate at low pH caused almost total inhibition of S. aureus and E. coli but had marginal to moderate inhibition on bacterial spores. In the presence of either sodium chloride or benzoate at low pH temperatures of 85° - 95°C provided marginal to moderate inhibition of these spores. Total inhibition of the spores and synergistic inhibitory effects were obtained only with combination of sorbate and sodium chloride or combination of sorbate and benzoate at low pH with low heat treatment. The recovery of surviving spores was slightly inhibited by incubation temperatures of 37° and 60°C for B. cereus and B. stearothermophilus respectively wheras 44°C markedly inhibited both spores after exposure to selected combination treatments. The potential of the Direct Fluorescence Microscopy technique for the enumeration of spores after exposure to combination preservative systems was indicated. Evidence from Direct Fluorescence Microscopy, Scanning Electron Microscopy and spore

extraction studies revealed changes in spore wall structure. Possible mechanisms explaining the observed inhibition of spores by the combination treatments are postulated. A computer program is described for the presentation of data generated from exposure of spores to combination treatments in the form of 3D-histograms.

INTRODUCTION

Although the genera Sporolactobacillus and Desulfotomaculum have been reported as sporeformers, the two sporeforming genera of most importance to the food industry are Bacillus and Clostridium (Doores, 1983). The sporulation phenomena of species of these genera is well researched and articles by Murrell (1967, 1969), Hanson, Peterson and Youssen (1970) and Warth (1978) have reviewed the subject.

Bacterial spores are characterised by their extreme dormancy and resistance to heat and other agents (Anagnostopoulos and Sidhu, 1981). Most theories which have been proposed to explain the high resistance of spores are closely related to the spore structure (Cook and Pierson, 1983; Gombas, 1983).

1.1 STRUCTURE OF THE BACTERIAL SPORE

The bacterial spore may be spherical, ovoid or elongated (Noble, 1965) and may occupy central, subterminal or terminal positions within the cell. The endospore may be smaller or larger in diameter than the cell. A comprehensive description of the structure of the bacterial spore has been presented by Tipper and Gauthier (1972) and Warth (1978) and in a review article by Fitz-James and Young (1969). Spores of all species have the same basic structure characterised by a central core, or protoplast, surrounded in turn by a plasma membrane or inner spore membrane, germ cell-wall, cortex, coats and exosporium (Warth, 1978; Russell, 1982).

The core is regarded as a relatively normal cell in terms of its macromolecular constituents (Warth, 1978). The core contains spore

enzymes, the majority of which have similar properties to their corresponding enzymes in vegetative cells. The core is also the location of deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) (Tipper and Gauthier, 1972). The properties of the DNA from spores of B. cereus (Tsuji, Suzuki and Imahori, 1975) and B. subtilis (Sakakibara, Saito and Ikeda, 1969) were found not to differ significantly from the vegetative cell DNA. The properties of the spore and cell nucleic acids have been reviewed by Doi (1969).

The core also contains lipid; the lipid composition of spores being similar to that of vegetative cells. Setlow (1975a, b) reported the presence of a group of basic, low molecular weight proteins in the core of B. megaterium spores which are rapidly degraded on germination.

The location of some spore specific compounds, particularly the dipicolinic acid (DPA), has been a subject of much controversy amongst researchers. However, it now seems clear that the DPA and much calcium are located in the core and not the cortex. Evidence in support of this claim has been presented by Leanz and Gilvarg (1973) who studied the attenuation of β-particles emitted from tritium - labelled DPA and labelled components in the spore coats, cortex and core. A central location, in the core, was clearly indicated. Similarly ultraviolet photomicrographs of sporulating B. subtilis (Wyckoff and Ter Louw, 1931) also provide evidence for a core or core plus cortex location. Warth (1978) argued that because the cortex showed no significant capacity to bind DPA in vitro, a cortical location seemed unlikely. Furthermore Johnstone, Ellar and Appleton (1980) used the high resolution electron probe X-ray to

demonstrate that the core of B. megaterium contained most of the calcium, potassium, magnesium, manganese and phosphorus. They argued that for electrical neutrality to be achieved, a major portion of divalent metal ions must be associated with DPA, a strong chelating agent. It is believed by some workers that the DPA is responsible for the heat resistance of spores (see later).

The inner spore membrane synthesizes the next layer called the germ cell wall during sporulation. During germination, this layer develops into vegetative cell wall and has a similar lysozyme sensitivity (Tipper and Gauthier, 1972). It has therefore been inferred that it contains vegetative peptidoglycan.

The main constituent of the cortex is the peptidoglycan, often referred to as mucopeptide or murein. The peptidoglycan is a linear unbranched, β-(1-4)-linked glycan chain consisting of alternate repeating units of N-acetylglucosamine (GlcNAc), N-acetylmuramic acid (MurNAc) and muramic lactam (Mur-lactam) (Figure 1). Peptide side chains are attached to the muramic acid residues of the polysaccharide through the carboxyl group of the lactyl ether group of the muramic acid (Warth and Strominger, 1972). Although the structure is similar to that in vegetative cell walls (Ghuysen, 1968) some modifications unique to spores are present. About 45 - 60% of the muramic acid residues do not possess either a peptide or N-acetyl substituent but form an internal amide (muramic lactam). Compared with vegetative cell-wall peptidoglycans the degree of cross linking is very low (Warth, 1978). The cortex has been implicated in the heat resistance of bacterial spores (Gombas, 1983; see later).

The spore coats which are generally rigid and multilayered, consitute about 50% of the spore volume (Murrell, 1969). The

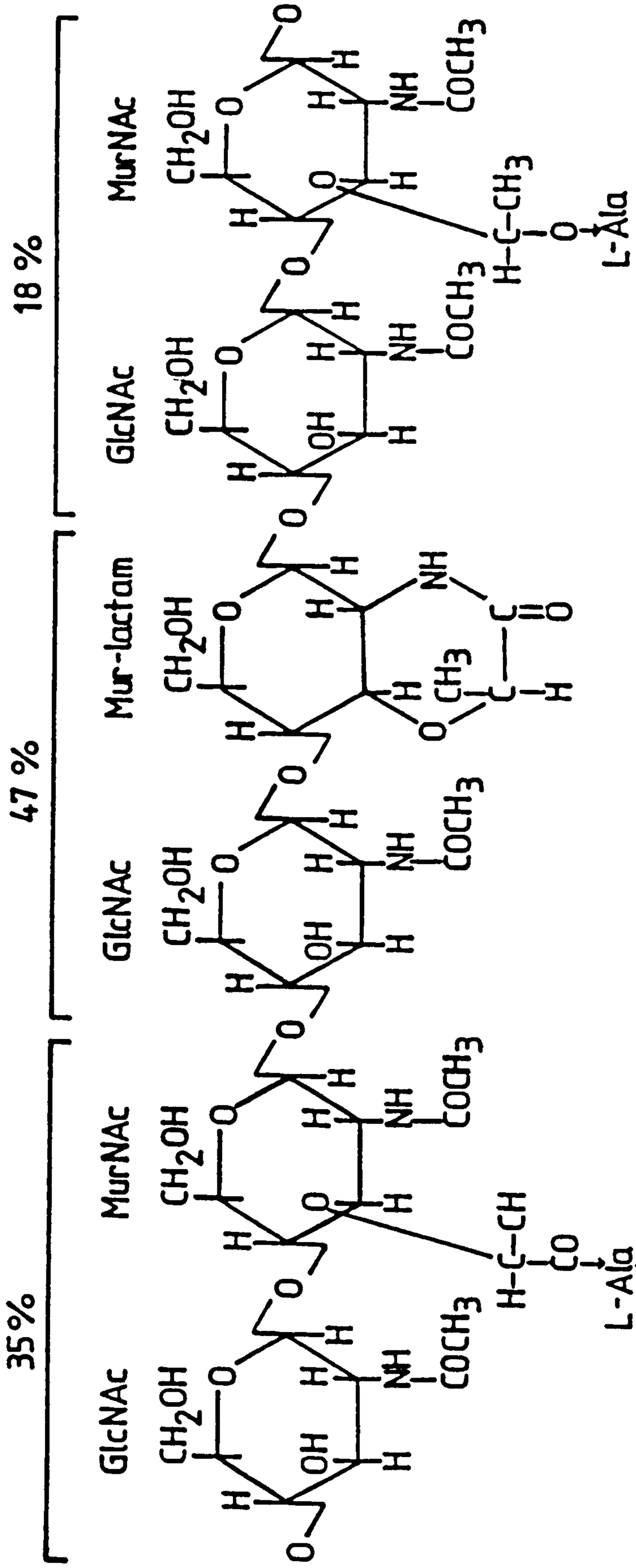

FIGURE 1. Chemical structure of cortex peptidoglycan (Russell, 1982).

morphology of the spore coat layers varies considerably between species. Three main types of layer can usually be distinguished in thin sections. The most distinctive is the middle layer which shows a very characteristic laminar pattern. A region of poorly structured material sometimes referred to as undercoat is located beneath the laminated coat layer (Aronson and Fitz-James, 1976).

The spore coats make up 30 to 60% of the spore dry weight and contain up to 80% of the spore protein (Tipper and Gauthier, 1972). They are composed of smaller amounts of complex carbohydrates, lipids and sometimes considerable amounts of phosphorus (Warth, 1978). The protein content varies from 60% for B. coagulans (Warth, Ohye and Murrell, 1963) to 80% for B. licheniformis (Bernlohr and Sievert, 1962).

The spore coats protect the cortex against physical and enzymatic attack and are themselves remarkably resistant to proteolytic enzymes (Tipper and Gauthier, 1972). However, they are not known to play a significant role in resistance of spores to heat and ultraviolet radiation (Russell, 1982) but are resistant to octanol.

The exosporium which is not present in all spores lies to the outer part of the spore coat. It is present in spores of some B. megaterium (Beaman, Pankratz and Gerhardt, 1972) and the structure has been described for pigmented Clostridium (Lund et al., 1978).

Little information is available regarding the chemical composition of the exosporium. In B. cereus T, the exosporium consists mainly of protein and significant amounts of glucose and lipid (Matz, Beaman and Gerhardt, 1970). The exosporium does not appear to influence the heat resistance of spores (Gombas, 1983).

1.2 TRANSFORMATION OF A BACTERIAL SPORE INTO A VEGETATIVE CELL

Three sequential kinds of processes are responsible for the transformation of a spore into a vegetative cell; these are termed activation, germination and outgrowth (Duncan, 1970; Cook and Pierson, 1983).

1.2.1 Activation

Activation is a process which conditions the spore to germinate under appropriate circumstances (Smoot and Pierson, 1982). This reversible process results in a spore which retains most of the properties typical of a dormant spore, viz: resistance to heat and radiation, refractility, nonstainability and retention of the DPA but it is no longer dormant (Keynan and Evenchik, 1969). Activation increases the rate and extent of germination, activates enzymes which are dormant in resting spore (Church and Halvorson, 1957), changes the chemical composition and morphology of the spore (Harrell and Mantini, 1957; Morberly, Shafa and Gerhardt, 1966) and influences the permeability of the spore (Keynan and Evenchik, 1969).

Activation of spores or the breaking of dormancy is commonly achieved by heating spores in aqueous suspension, a process first described by Curran and Evans (1945). Bacillus stearothermophilus requires a temperature from 105° to 115°C for optimal activation (Keynan and Evenchik, 1969) while Clostridium botulinum type A spores are maximally activated by heat at 80°C for 10 to 20 min (Ando, 1973).

Many workers (Keynan et al., 1965; Issahary, Evenchik and Keynan, 1970; El-Mabsout and Stevenson, 1979; Blocher and Busta, 1983) have reported that Bacillus spores are activated by low pH. Keynan et al.

(1965) found that B. cereus T was activated when heated at 65°C for 20 min in a menstruum of pH 2 to 3. Other activators that have been described include use of chemicals such as dimethyl formamide (Russell, 1982), dimethyl sulphoxide (Widdowson, 1967), chloral hydrate (Keynan and Evenchik, 1969), mercaptoethanol and thioglycollic acid (Keynan et al., 1964) and exposure to calcium dipicolinate (Ca - DPA; Lee and Ordal, 1963). Gould and Ordal (1968) also suggested the use of ionizing radiation while ageing was reported by Powell (1950) as activating agents.

In order to explain the phenomenon of activation, several hypotheses have been postulated. Lee and Ordal (1963) postulated that heat shock and other germination stimuli may directly or indirectly upset the DPA stability of the dormant spore and initiate a permeability change that would result in germination. Harrell and Mantini (1957) and Keynan, Murrell and Halvorson (1961) found a relationship between DPA content of spores and heat shock requirement for activation. Hyatt, Holmes and Levinson (1966) and Hyatt and Levinson (1968) explained the activation process by considering that water vapour enters and hydrates critical site in the spore which is essential for the functioning of the germinants. They also stated that water seems to be the common factor for activation by heat, by aqueous ethyl alcohol or by water vapour. They postulated that heat or ethyl alcohol alters the structure of liquid water giving it the ability, like the water vapour to reach and hydrate a specific critical spore site, thus resulting in activation. Maeda, Noguchi and Koga (1974) presented a similar hypotheses that water vapour might cause the dissociation of the macromolecule - Ca - DPA into Ca - DPA hydrate and a spore macromolecule.

Other workers have proposed that activation involves structural adjustment of the spore. Busta and Ordal (1964b) suggested that "heat might break dormancy by structural reorientation of components of the spore which in turn leads to the loss of the characteristic integrity of the spore". Keynan et al. (1964) also accounted for activation on the basis of reversible changes in the tertiary structure of a macromolecule. They proposed that a coat protein might be responsible for the increase in accessibility of a substrate to certain enzymatic sites necessary for germination. In support of this view, and based on electron microscopy studies, Hashimoto and Conti (1971) found striations or comblike structures in the spore coat of heat activated spores. These workers concluded that during activation certain coat components are denatured or released into the medium which might facilitate uptake of germinants. Maeda, Kaigami and Koga (1978) studied activation of spores by means of differential thermal analysis and concluded that heat activation causes denaturation or structural changes of the spore coat protein which may facilitate either the permeation of germinants or the release of germination inhibitors.

Most methods adopted for the measurements of activation follow the kinetics of germination. Germination may be followed by a decrease in optical density, loss of refractility and darkening of the spore. Another method is to measure the percentage of spores which are able to form colonies when placed on a suitable recovery medium. A number of factors may influence the extent and degree of activation including species, growth medium and age of the spore suspension.

1.2.2 Germination

Germination is an irreversible process which proceeds when properly activated spores are placed in an appropriate germination environment (Smoot and Pierson, 1982). It involves the irreversible loss of a typical pattern of spore properties (Gould, 1969; Hansen et al, 1970; Russell, 1982; Smoot and Pierson, 1982). In general, the sequence of events during germination comprises loss of heat resistance, loss of chemical resistance, loss of calcium and DPA, excretion of peptidoglycan, increase in stainability, loss of refractility and decrease in optical density (Gould, 1970). The resulting cell is still readily distinguishable from the vegetative cell.

The loss of heat resistance during germination of spores (Gould, 1970) is accompanied by changes from a phase bright (refractile) to a phase dark (non-refractile) condition (Cook and Pierson, 1983). Also there is a loss of about 30% of the spores dry weight (Powell and Strange, 1953). This loss is due to material being excreted into the medium and includes much of the typical spore components i.e. DPA, calcium, manganese and peptidoglycan. A decrease in optical density is a common feature during germination and this decrease may reach 60% (Levinson and Hyatt, 1966). Powell (1950) has attributed the decrease in optical density to the excretion and solubilization of dry matter from germinating spores. According to Vary and McCormick (1965), germination could be expressed in terms of optical density.

Germinating spores have an affinity for dilute stains (Powell, 1950) and the stainability of germinating spores has been attributed to the breaking of a permeability barrier near the outer edge of the cortex (Rode, Lewis and Foster, 1962) or to the unmasking of reactive

groups within the spore (Gould, 1969).

Germinating spores show an increase in permeability and a tendency to take up water. This permeability involves the core and depends on the accessibility of functional groups (Black and Gerhardt, 1962). According to Rode et al. (1962) there is a permeability barrier near the outer edge of the cortex in the dormant spore. Gould (1969) suggested that the dissolution of the cortex may lead to an increase in permeability.

The volume of spore has been found to increase by about 20% during the initial stages of germination, as measured by micro-haematocrit devices (Hitchins, Gould and Hurst, 1963). A Coulter counter has also been used to study the increase in volume of germinating spores (Parker and Barnes, 1967).

Phase contrast microscopy has been used for counting germinated spores, ungerminated spores appearing refractile and bright and germinated spores as dark bodies (Pulvertaft and Haynes, 1951).

Germination of spores can be initiated by various non-nutrient germinants (bicarbonate, Ca - DPA, metal ions, surfactants), enzymatic germinants (lysozyme, spore lytic enzyme, subtilin, B. cereus T initiator protein), physical germinants (abrasion and deformation, hydrostatic pressure) and nutrient germinants (Gould, 1969; 1970; Gould and Dring, 1972).

The nutrient germinants tend to be few and specific for particular strains of spores (Gould, 1970). Nutrient germinants include aminoacids particularly L-alanine, purine ribosides, bases and sugars (Gould, 1969). L-alanine initiates germination of many spores including B. stearothermophilus, B. cereus, B. subtilis and B. licheniformis. L-alanine - initiated germination is inhibited by

D-alanine. Other aminoacids acting as germinants are L-α-aminobutyric acid and L-valine (Krask, 1961; Gould, 1970). The manner in which aminoacids initiate germination is not known. Spores may also be germinated by ribosides; including inosine, adenosine, guanosine and xanthosine. Warren and Gould (1968) and Gould and Dring (1972) have suggested that the ribosides act as potentiators of amino acid induced germination. The mechanism of potentiation is not clear but it has been suggested by Smoot and Pierson (1982) that ribosides act by increasing the permeability of some site within the spore to aminoacids. Sugars such as glucose and sometimes fructose and amino sugars may be used to germinate spores (Hyatt and Levinson, 1964). Shay and Vary (1978) studied the glucose initiated germination. They suggested that the metabolism of exogenously added glucose is not the primary stimulatory effect in germination.

The mechanism of spore germination has not been established. Some workers have viewed the process as being metabolic in nature (Freese, Park and Cashel, 1964; Freese and Cashel, 1965; Rossignol and Vary, 1978) while others have viewed it as being a physicochemical event (Levinson and Hyatt, 1962; Scott et al. 1978).

1.2.3 Outgrowth

Outgrowth describes the development of a germinated spore into a vegetative cell (Duncan, 1970). Cook and Pierson (1983) summarised the three stages involved in the outgrowth process, viz: germinated spores become swollen, shed their coats as elongation occurs and elongation results in a mature vegetative cells.

Unlike germination which is mainly a degradative process, outgrowth involves the synthesis of new macromolecules and the highly

ordered sequence of biosynthetic events. This results in the differentiation of structures which are characteristic of the vegetative cells (Strange and Hunter, 1969). The macromolecular synthesis is dependent on the physicochemical conditions of the environment and proceeds in an ordered manner. Ribonucleic acid (RNA) synthesis is the first to occur after germination (Vinter, 1970; Adams, 1978), followed by protein and DNA synthesis which occur sometimes later (Russell, 1982). The synthesis of the cell wall begins after RNA and protein synthesis but before the DNA synthesis (Halvorson and Szulmajster, 1973).

Generally the requirements for outgrowth of spores are similar to those of vegetative growth and include aminoacids, ribosides and other growth factors (Strange and Hunter, 1969). An inadequate supply of nutrients may result in "microcycle sporogenesis" in which outgrowth occurs but not the normal cycle of vegetative division.

1.3 HEAT RESISTANCE OF BACTERIAL SPORES

Bacterial spores represent the most heat - resistant forms of life known (Gould, Brown and Fletcher, 1983), although different species exhibit a wide range of heat resistance. The thermal inactivation rate or D-value, which is the time required to inactivate 90% of the spore population at a particular temperature, can vary from less than 0.1 sec at 110°C for Clostridium botulinum type E spores to about 40 min for B. stearothermophilus spores (Murrell and Scott, 1966). However, the heat resistance of bacterial spores is not fixed for a given species or even within a population (Russell, 1982) but is influenced by a number of factors (Roberts and Hitchins, 1969; Molin, 1982; Russell, 1982).

1.3.1 Sporulation Effects

The environment in which bacterial spores are produced influences the resistance of the spores to heat. The studies carried out by Cook and Gilbert (1968), Abdelgadir (1974) and Warth (1978) showed that the temperature of spore formation influenced the resistance of spores to heat. Warth (1978b) found that the heat resistance of spores of Bacillus species increased with increase in the optimum growth temperature for the vegetative cells of the organism. Hence B. stearothermophilus which has a high optimum temperature for growth produces spores with the highest moist heat resistance. Bacillus stearothermophilus is a commonly used indicator for moist heat sterilisation processes (Kereluk and Gammon, 1973; Heintz et al. 1976). However, Rey, Walker and Rohrbough (1975) found that the heat resistance of Cl. perfringens was unaffected by the sporulation temperature.

The ionic composition of the sporulation medium also influences the heat resistance of spores. Amaha and Ordal (1957) reported that manganese and calcium in a sporulation medium increased the heat resistance of B. coagulans. In a similar study, Levinson and Hyatt (1964) found that calcium chloride in a sporulation medium increased the heat resistance of B. megaterium spores. However, Cook and Gilbert (1968) found that except at high concentration (0.1%) manganese sulphate in the sporulation medium had little effect on the heat resistance of B. stearothermophilus spores. In contrast, low concentration of Mn^{2+} in a sporulation medium was found to be necessary for the development of heat resistant spores of B. fastidiosus (Aoki and Slepecky, 1973) and B. megaterium (Aoki and Slepecky, 1974).

Early studies by Williams (1929) and Sommer (1930) indicated increases in the heat resistance of spores of B. subtilis and Cl. sporogenes with the incorporation of phosphate in sporulation media. On the contrary, Amaha and Ordal (1957) showed that increases in phosphate concentration in a sporulation medium reduced the heat resistance of spores of B. coagulans var. thermoacidurans. Levinson and Hyatt (1964) also reported similar finding for spores of B. megaterium.

Other constituents of the sporulation medium which have been studied in relation to the heat resistance of spores include saturated or unsaturated fatty acids (Sugiyama, 1951), protein (Put and Aalbersberg, 1957) as well as pH level (El-Bisi and Ordal, 1965; Pang, Carrorad and Wilson, 1983) and water activity (a_w) (Jakobsen and Murrell, 1977).

Thompson and Moller (1968) and Abdelgadir (1974) also found that spores produced on solid media were more resistant to heat compared to those produced in liquid media. Furthermore the age of the spores has been found to be an important factor in heat resistance. Magoon (1926) found an increase in the heat resistance of spores of B. mycoides after a storage period of 60 days. On the other hand, Abdelgadir (1974) found that storage at 4°C for 20 months actually decreased the heat resistance of B. stearothermophilus spores. The study by Navani (1970) did not indicate significant differences in the heat resistance of B. stearothermophilus spores following storage for up to two years.

1.3.2 Heating Menstruum

The heat resistance of bacterial spores is markedly influenced by the nature and composition of the suspending medium. The effects of pH, a_w and antibacterial agents on the heat resistance of bacterial spores have been studied by many workers.

There is no concensus amongst workers as to the effect of the heating menstrum pH on spore resistance. Williams (1929) reported that between pH 4.4 and 7.6 spores of B. subtilis were more heat sensitive at the lowest pH. Similarly reports by Cook and Gilbert (1968), Gibriel and Abd-El Al (1973) and Cameron, Leonard and Barrett (1980) demonstrated that increases in acidity of the heating menstruum were paralleled by decreases in heat resistance. The report by Bigelow and Esty (1920) showed that as the pH of the menstruum increased, the time for the complete destruction of a spore suspension at a given temperature also decreased. Hersom and Hulland (1980) reported no correlation over the range pH 5.2 to 6.8 and the heat resistance of Cl. botulinum spores. In addition, Nichols (1940) working within the pH range of commercial canned foods (pH 5.95 - 7.25) observed inconsistent results in relation to the heat resistance of B. subtilis spores. Levinson and Hyatt (1960) have attributed these discrepancies in results to the use of different buffer systems. An additional factor which may often be ignored is possiblity of carry-over of heating medium to the recovery medium (Cook and Gilbert, 1968).

The influence of the type of buffer system on the heat resistance of spores has also been studied. Blocher and Busta (1983) found that the D-value of spores of Cl. botulinum was influenced by the type of acidulants in the buffer. Malic acid reduced the D-value more than

citric and hydrochloric acids at given pH and concentration levels. Bacillus megaterium spores (strain QMB1551) were found to be more resistant in heated phosphate buffer than in water or cacodylate buffer at the same pH (Levinson and Hyatt, 1960). However, spores of B. megaterium strain 1A - 48 and B. polymyxa strain 1A - 39 were found to be less resistant in phosphate buffer than in citrate, phthalate or ammonium salt buffers (Walker, 1964). In addition the report by Odlaug and Pflug (1977c) indicated no significant decrease in the number of Cl. botulinum type A spores when heated in citrate - phosphate buffer and in tomato juice at the same pH. Gombas (1983) cited Sako et al.(1981) who attributed the decrease in D-value under acidic conditions to enhanced DNA damage.

Studies have also been carried out on the effect of sodium chloride on heat resistance of spores. Weiss (1921) reported a reduction in the heat resistance of Cl. botulinum spores when heated in a medium containing 3% sodium chloride. Briggs and Yazdany (1970) working with sodium chloride (2, 4 and 8%) also found that an increase in sodium chloride concentration reduced the heat resistance of B. stearothermophilus and had no effect on the heat resistance of other spores used in the study. The report by Roberts, Gilbert and Ingram (1966) indicated that the heat resistance of spores of Cl. sporogenes was the same in 3 and 6% (w/v) sodium chloride levels as in water. However, by heating the spores to 0.1% survivor level, the spores were rendered more sensitive to the sodium chloride in the recovery medium. Buhlmann, Gay and Schiller (1973) also found that suspension of B. stearothermophilus spores in physiological saline (0.9% sodium chloride) gave higher heat resistance compared with a water control.

Early studies also relate to the effects of preservatives on heat resistance of spores. Anderson, Esselen and Fellers (1949) found that 0.1% (w/v) sodium benzoate in tomato juice had no significant effect on the heat resistance of B. coagulans. The report by Michener, Thompson and Lewis (1959) showed that 0.3% (w/v) nitrite reduced the heat resistance of P.A.3679 spores heated at pH 7 in 0.025M phosphate buffer. Yokoya and York (1965) found that 0.0112% sorbic acid had no effect on the heat resistance of B. coagulans spores.

Other reports also refer to the effects of carbohydrates (Anderson et al, 1949), lipids (Molin and Snygg, 1967) and proteins (Roberts and Hitchins, 1969) in the heating medium on resistance of spores.

1.3.3 Recovery Effects

Spores which have been exposed to heat are often more demanding in their nutritional requirements than untreated spores (Adams, 1978; Russell, 1982). Thus the nature of survivor curves is greatly influenced by the composition of the recovery medium (Roberts and Ingram, 1966; Labbe, 1979).

Roberts (1970) found that supplementation of the recovery medium with sodium bicarbonate increased the colony count of heat - treated Clostridium spores. In the recovery of heat - treated B. cereus spores, Busta, Baillie and Murrell (1976; 1977) found that sucrose or Mg^{2+} was essential for their revival. Similarly Hyatt and Levinson (1957; 1959) found that sulphate and phosphate were necessary for the growth of heat - treated spores of B. megaterium. The high colony counts

obtained during the recovery of heat - treated Cl. botulinum spores in yeast extract agar, pork infusion agar and modifications of these media were attributed to the presence of sodium thioglycollate and sodium bicarbonate in the media (Odlaug and Pflug, 1977c).

On the contrary, certain constituents of the recovery media have been found to be inhibitory to the recovery of heat - treated spores. The inclusion of bromocresol purple in certain brands of dextrose tryptone broth have been found to be inhibitory to heated spores of B. stearothermophilus (Cook and Brown, 1960). Bromothymol blue was also reported to have similar effect (Bühlmann et al, 1973), although the influence is less pronounced. Ernst (1968) reported that thioglycollate broth inhibited the outgrowth of Bacillus spores after they had been exposed to steam and dry heat sterilization. Thus Davis, Carls and Gillis (1979) concluded that thioglycollate broth was unsuitable for monitoring saturated steam sterilization processes involving B. stearothermophilus as the test organism.

A study was carried out by Cook and Brown (1965) on the effect of pH on the recovery of heat - treated B. stearothermophilus spores. The results showed that recovery after severe heat treatment was greatest at pH 7.0 to 7.3 whereas untreated spores recovered better in a medium of pH 5.9.

The effect of additives in the recovery media of heat - treated spores has been reported in review articles by Roberts (1970), Adams (1978) and more recently by Foegeding and Busta (1981) and Russell (1982). A study by Duncan and Foster (1968a) showed that the presence of the curing agents NaCl, $NaNO_3$ and $NaNO_2$ in recovery medium were inhibitory to thermally injured P.A.3679h spores. A similar report

was made for heat - injured Cl. perfringens spores (Flowers and Adams, 1976; Chumney and Adams, 1980). Roberts and Ingram (1966) have also reported the inhibition of heat - treated spores of Cl. sporogenes and B. subtilis by NaCl and potassium nitrate added to their recovery media. Flowers and Adams (1976) and Chumney and Adams (1980) found that heated Cl. perfringens spores were sensitive to neomycin and polymyxin in the recovery media.

Another factor that has been found to influence the recovery of heat - treated spores is the incubation temperature (Cook and Gilbert, 1968; Prentice and Clegg, 1974). Cook and Gilbert (1968) found that heated spores of B. stearothermophilus recovered maximally at 45° - 50°C, whereas unheated at 50° - 65°C.

1.3.4 Mechanism of Heat Resistance

Although many studies have been carried out on the heat resistance of bacterial spores and the mechanisms responsible for this phenomenon are still uncertain, two main theories apply.

1.3.4(a) DPA Theory

Some workers have related DPA with the heat resistance of bacterial spores. DPA, often present at concentrations as high as 10% of the dry spore weight, forms a stable chelate with approximately equivalent amount of calcium in the spore core (Gould et al, 1983). Levinson et al. (1961) showed that as the molar ratio of Ca : DPA in B. megaterium spore decreased, the heat resistance also decreased. Thus the workers concluded that by altering the Ca and DPA content of B. megaterium, spores of differing heat susceptibility could be obtained. A study by Walker et al. (1961) showed that as the molar

concentration of magnesium was increased in relation to DPA and Ca, the heat resistance of spores of Bacillus species likewise decreased. However, DPA levels alone showed little correlation with heat resistance. Young (1959) suggested that DPA and Ca^{2+} could act together in stabilizing essential proteins and nucleic acids in spores. The study by Mishiro and Ochi (1966) confirmed this suggestion. It was found that human serum albumin solution became turbid at 60°-95°C but the occurence of turbidity was completely prevented in the presence of 0.05% solutions of DPA. It was thus inferred that DPA had a preventive effect on the heat denaturation of proteins, which in turn could be linked to the heat resistance of spores. This has yet to be proven conclusively for the bacterial spore. An earlier study by Slepecky and Foster (1959) showed that partial replacement of Ca^{2+} by other cations gave spores which were less heat resistant than those possessing normal Ca^{2+} content. The Ca^{2+} content of the sporulation medium was found to be proportional to the DPA that accumulated and this in turn was proportional to the heat resistance of spores. The isolation of a DPA - negative heat sensitive spore mutants of B. cereus T by Halvorson and Swanson (1969) confirmed the involvement of DPA in heat resistance and other workers have shown that mutants of B. megaterium (Fukuda and Gilvarg, 1968) and B. sutilis (Balassa and Yamamoto, 1970) which are unable to synthesize DPA produce heat sensitive spores. Heat resistance only becomes apparent when DPA is added to the medium.

Conversely, studies have also been performed which demonstrate that DPA may not be involved in heat resistance. Hanson et al. (1972) showed that revertants of B. cereus strains which were DPA negative and low in Ca^{2+}, had heat resistance as high as wild types. Similarly

Frank and Tonaki (1971) found that a DPA - negative mutant of B. subtilis strain 168 was as thermoresistant as the wild type strain. Wise et al (1971) and Zytkovicz and Halvorson (1972) have also decribed DPA - less mutants of B. cereus, B. subtilis and B. megaterium. However, Dring and Gould (1981) recently isolated a DPA - negative heat resistant spores of B. cereus I and they concluded that the DPA alone may not be solely responsible for the heat resistance of spores.

1.3.4(b) The Cortex Theory

Some workers have attempted to associate the heat resistance of spores with the low water content in the spores. Certain properties of the spores such as refractility (Harnulv et al. 1977; Gerhardt et al. 1982) and high specific gravity (Lewis et al. 1965; Iisa et al. 1982) are consistent with a low water content.

It has been reported by Gibson (1973) that osmotically dehydrated bacterial cells can withstand high temperatures. Some workers have suggested that the cortex is responsible for obtaining and maintaining a low water content in bacterial spores. Hence the cortex appears to be clearly associated with the heat resistance of spores. Although doubts exist as to the manner by which the cortex is able to achieve this function, the following theories have been proposed.

(i) Contractile cortex theory

Lewis, Snell and Burr (1960) first proposed the contractile cortex theory whereby the water content of the protoplast is reduced by a mechanical contraction of the cortex about the protoplast. This proposition is supported by the contractile properties of the peptidoglycan, constituent of the cortex.

Marquis (1968) and Ou and Marquis (1970) reported that peptidoglycan from isolated bacterial cell wall can be made to contract by electrostatic interactions with salt. It has been stressed by Baillie and Murrell (1974) that the conformation of peptidoglycan is dependent on the ionic strength of its environment. They found that a high ionic strength in the environment resulted in contraction of the cortex while reduction of the ionic strength led to swelling of the cortex. It is viewed that the extent of the contraction is dependent on the stability and mechanical strength of the peptidoglycan. Murrell and Warth (1965) observed that for most species with high resistance to heat a high degree of crosslinking was obtained. The degree of crosslinking was dependent on the diaminopimelic acid content as well as the diaminopimelic acid: glucosamine ratio. These workers found that the degree of crosslinking was an indicator of the mechanical strength of the cortex and therefore of the degree of shrinkage. The ultimate result of a fully contractile cortex would be dehydration of the protoplasm and a maintenance of this state; the degree of dehydration being dependent on the species of the spore.

(ii) **Expanded cortex theory**

Alderton and Snell (1963) and Gould and Dring (1975) proposed that the cortex expands by electrostatic repulsion between the free carboxyl groups of the cortex peptidoglycan, thus creating a comprehensive force which is able to expel water from the core. The greater the compression on the core the more water is expelled and the lower the water content of the core (Lewis et al. 1960). Gombas (1983) reported that the presence of a

rigid spore coat would be essential to direct the force of the expanding cortex inward. However, Gould and Dring (1975) found that mutant strains with no detectable coat retained the heat resistance of the parent strain. Therefore they concluded that the spore coat was not essential for attaining heat resistance.

(iii) Osmoregulatory expanded cortex theory

Gould and Dring (1975) considered the cortex as an osmotic pump in order to achieve and maintain the dehydration of the core. It is viewed that since the cortex contains electronegative peptidoglycan, cations will be required to act as counterions. It has been proposed by these workers that the loosely bound cations are responsible for the high osmotic potential within the cortex. The high concentration of cations in the core are assumed to be mainly in the form of low solubility salts which exert little osmotic potential of their own (Gould, 1977).

Gould considered that since the core has to be in osmotic balance with the surrounding cortex, water will be forced out of the core into the cortex until the pressures are equalized. This will create a high water content cortex surrounding a low water content core.

Other theories include the anisotrophic cortex theory and the reverse osmosis theory. Warth (1978) postulated the anisotrophic cortex theory which involves the radial expansion of the cortex brought about by the removal of peptide cross links in the peptidoglycan with the formation of muramic lactam. Also, Algie (1980) cited by Russell (1982) considered that the reverse osmosis is

achieved with the cortex. It is conceived that as the cortex grows at its inner surface it pushes upon the protoplast so that water is expelled from both the cortex and the protoplast.

Since the state of hydration of the core is a significant factor in the heat resistance of spores (Russell, 1982) and is most likely achieved by osmosis (Gould and Dring, 1975), it is considered that the difference in ionic contents between the developing spore and the surrounding mother cell is responsible for creating the osmotic potential, rather than the cortex. A study by Marquis et al. (1983) of immature B. megaterium spores i.e. in stage III, before DPA incorporation and cortex formation showed that the spores had a much lower content of osmotically active ions than the sporangium. It was therefore proposed by these workers that the relative osmotic potential of the sporangium would cause the spore cytoplasm to dehydrate.

In support of the cortex theory, Beaman et al. (1983) found that water is compartmentalized inside the spore. In addition, the cortex was found to have the highest water content out of the spore structures while the core had the least, thus agreeing with the cortex theory. In contrast, Bradbury et al. (1981) and Watt (1981) found that the core appeared to have a water content equal to or greater than that of cortex. However, the data obtained was based on isolated spore fractions and not intact spores. Moreover, there is no evidence in the literature of spore mutant devoid of cortex which has normal heat resistance.

1.4 EFFECTS OF CHEMICALS ON BACTERIAL SPORES

Many chemicals have been tested for their inhibitory effects on bacterial spores. Some of these chemicals exhibit their inhibitory effects when used in conjunction with other treatments while some are capable of inhibition on their own. The stage at which the inhibition takes place in the spore has been established for relatively few of these chemicals. Examples of these chemicals and particularly those related to foods include the following:

1.4.1 Sodium Chloride

Several workers have investigated the effects of sodium chloride on Cl. botulinum spores. Ando (1974) found that 8 - 10% sodium chloride inhibited the spore germination of Cl. botulinum type A while type E was sensitive to concentrations as low as 5%. Ohye and Christian (1967) reported that 10% sodium chloride was not enough to inhibit Cl. botulinum when the pH and temperature were optimum for germination and outgrowth. However, the report by Riemann et al. (1972) showed that with a decrease in pH from 7 to 5, decreasing concentrations of sodium chloride prevented growth and toxin production of Cl. botulinum types A, B and E spores. It has also been reported by Emodi and Lechowich (1969b) that the salt tolerance of Cl. botulinum type E is reduced by reduced temperatures. An earlier study by Duncan and Foster (1968b) showed that 6 - 10% NaCl inhibited the germination of Cl. sporogenes but allowed initial events of germination to occur, preventing complete loss of refractility.

Similarly, Gould (1964) found that germination of Bacillus species was inhibited by 10 - 15% sodium chloride while 4 - 7% was

required for the inhibition of outgrowth. The mechanism of inhibition of spore germination by sodium chloride has not been reported.

1.4.2 Nitrite

The effects of nitrite on bacterial spores have been reviewed by Sofos et al. (1979) and Benedict (1980). Labbe and Duncan (1970) reported that 0.30 - 0.40% nitrite inhibited the outgrowth of Cl. perfringens at pH 7. However, by decreasing the pH of the medium to 6, 0.02 - 0.04% nitrite was required to inhibit the outgrowth of the spores. Similarly, Duncan and Foster (1968b) showed that 0.08 - 1.0% nitrite inhibited the outgrowth of Cl. sporogenes in a medium of pH 7 and sensitivity was increased at pH 6. In general, it has been established that nitrite inhibits spore outgrowth and multiplication but does not inhibit germination (Sofos et al. 1979b). However, study by Gould (1964) demonstrated that 0.075 - 0.25% nitrite inhibited germination of Bacillus species in a medium of pH 6. Other workers like Pivnick et al. (1970) stated that nitrite appeared to act between the stages of germination and outgrowth.

Roberts et al. (1976) found that 0.03% nitrite inhibited the growth of a mixture of Cl. botulinum types A and B spores in a pasteurized pork slurry for 28 days when formulated with 1.8% NaCl. Under the same conditions only 0.004% was required for inhibition of growth when 3.5% NaCl was used. It has been proposed by Cook and Pierson (1983) that the inhibitory effect of nitrite may be due to stimulation of germination followed by death of germinated spores which fail to outgrow. Tompkin et al. (1978) also proposed that nitric oxide may react with an essential iron-containing compound within germinated spores, thus inhibiting outgrowth by interfering

with energy metabolism. This theory is supported by findings that the addition of iron to meats reduces the antibotulinal effects of nitrite (Tompkin et al., 1979).

In a more recent study, Bell and DeLacy (1984) found that 0.02% sodium nitrite enhanced the spore destruction of B. licheniformis when heated at 80°C in a medium acidified to pH 4.4. Earlier reports by Perigo et al. (1967) and Perigo and Roberts (1968) demonstrated that when nitrite was heated in laboratory media at temperatures greater than 90°C it was more inhibitory to clostridial spores than was nitrite alone. Ralph (1967) also showed that sodium nitrite and sodium nitrate reduced the heat resistance of B. stearothermophilus spores.

1.4.3 Alkyl Esters of P-hydroxybenzoic Acid (Parabens)

Dymicky and Huhtanen (1979) reported that 0.004% propyl parabens inhibited the growth of Cl. botulinum type A in a laboratory medium. Similarly Robach and Pierson (1978) also found that a lower concentration 0.002% propyl parabens was required to inhibit the growth of the same spore. It has been shown by Watanabe and Takesue (1976) that 0.007% methyl parabens inhibited germination while a higher concentration of 0.011% inhibited the outgrowth of B. megaterium spores.

1.4.4 Phenolic Antioxidants

The inhibitory effect of phenolic antioxidants on Cl. botulinum spores has been demonstrated in a laboratory medium by Reddy et al. (1982). They found that 0.02% butylated hydroxyanisole (BHA), 0.02% butylated hydroxy toluene (BHT) and 0.04% tertiary

butylated hydroxyquinoline (TBHQ) inhibited the growth of Cl. botulinum spores in tryptone glucose yeast extract broth. However, the addition of these antioxidants to comminuted pork product did not prevent the growth of the spores occurring within seven days (Pierson and Reddy, 1982).

1.4.5 Ethanol

Trujillo and Laible (1970) reported that 2% ethanol was inhibitory to the spore germination of B. subtilis. A similar effect was also reported by Yasuda-Yasaki et al. (1978) for B. pumilus which was inhibited by 9.2% ethanol. Trujillo and Laible (1970) suggested that alcohols might inhibit germination by inactivating enzymes. Boyazoglu (1969) also observed that alcohol was effective against B. coagulans spores especially at pH 5 and suggested that the effect of the alcohol might be due to the hydroxyl group, its position in the molecule and the length of the alkyl group.

Magoon (1926) found that spores of B. stearothermophilus and B. coagulans were inactivated when heated at 100° and 105°C in a buffer solution containing 8% ethanol. Coussin (1968) also recorded a decrease in D-value for B. stearothermophilus spores when treated with 10% ethanol in a medium of pH 4.6 and 5.2. The worker suggested that the effect of alcohol might be due to an attack on the lipoprotein of the spore wall.

1.4.6 Nisin

There are conflicting reports on the effects of antibiotics on bacterial spores. Most workers support the view that antibiotics

are sporostatic and not sporicidal and that outgrowth and not germination is affected by antibiotics. Hitchins et al. (1963) reported that concentrations of nisin greater than 0.00005% inhibited the spore outgrowth of B. cereus and B. subtilis in glucose yeast broth. Scott and Taylor (1981) also found that 0.02% nisin inhibited the growth of Cl. botulinum A spores in brain heart infusion broth. An earlier study by Gould (1964) showed that nisin did not inhibit spore germination but outgrowth of Bacillus species was blocked at the postgerminative stage. Recently, Bell and DeLacy (1985) found that outgrowth of B. licheniformis spores was completely inhibited by 0.000025% nisin incorporated in plate count agar. Thorpe (1960) suggested that nisin bound to the spore and caused inhibition on outgrowth. Nisin has also been shown to reduce the D-values of Cl. botulinum (O'Brien et al., 1956) and PA3679 (Michener et al., 1959) spores.

1.4.7 Organic Acids

The report by Foster and Wynne (1948) showed that several unsaturated fatty acids inhibited the germination of Cl. botulinum spores. They found that 0.001% oleic, linoleic or linolenic acid inhibited the germination of the spores. However, Bacillus species were not inhibited and Cl. perfringens, Cl. histolyticum and Cl. chauvei were only slightly inhibited. A study by Parker (1969) also showed that 0.17% chlorocresol inhibited germination of B. subtilis spores.

1.4.8 Other Chemicals

Thomas and Russell (1974) found that a mixture of formaldehyde and glutaraldehyde reduced the permeability of Cl. bifermentans spores. This was shown by the reduction in Cu^{2+} uptake and loss of DPA during subsequent heating at 121°C. In a study by Chung et al. (1979), the sporicidal activity of 5% hydrogen peroxide and iodophor was demonstrated using Cl. sporogenes spores. It was suggested by these workers that the peroxide made the spores more susceptible to the iodophor. By removing the spore coats of B. subtilis spores, McErlean et al. (1980) found that the resistance of these spores to iodophor, alkaline glutaraldehyde and a mixture of hypochlorite/methanol was reduced by twelve, eight and eight fold respectively. Thus treatments which remove coat protein render spores sensitive to chemicals which are not normally sporicidal. Hanlin et al. (1981) showed that the addition of 0.0005% ethidium bromide to B. subtilis spores heated at 85°C for 10 min reduced the survival of the spores from 85% to 50%. The same workers found that the addition of 5×10^{-7}% acriflavine and 0.001% daunomycin to the heat treated spores achieved similar results. They suggested that the action might be on the DNA. Other reports have examined the effects of UV irradiation and hydrogen peroxide on a number of Bacillus species (Bayliss and Waites, 1979) and ultrasonic radiation and hydrogen peroxide effects on B. cereus and Cl. sporogenes (Ahmed and Russell, 1975).

1.4.9 Mechanisms of Inhibition of Spore Germination

The mechanisms by which chemicals inhibit spore germination are still unknown but some theories have been proposed by Cook and

Pierson (1983).

1.4.9(a) Blockage of Germination - Promoting Sites

It is considered on activation spore coats become permeable to germinants which then act at germination promoting sites. Cook and Pierson (1983) therefore proposed that all inhibitors act in similar manner by permeating the spore coats and blocking these sites. If the germination promoting sites are located in the cortex, the effectiveness of the inhibitor will therefore depend on its ability to pass through the cortex. This in turn is a function of the nature of the cortex and the crosslinking of the peptidoglycan.

1.4.9(b) Inhibition of Metabolic Events

Since some workers have suggested that germination is a metabolic event, Cook and Pierson (1983) proposed that chemicals might act by interfering with this event and hence block germination.

1.4.9(c) Blockage of Allosteric Reaction

Certain germinants such as cations are believed to react non-specifically with sites on spore structures which are critical to germination. By these interactions, allosteric alterations could occur and cause triggering of germination or activation. It is proposed that hydrophobic compounds such as sorbic acid, alcohols and parabens may bind to hydrophobic sites on spore structures and thereby prevent germinants from acting.

1.4.9(d) Competition for Germination Sites by Germinants and Inhibitors

Compounds which promote germination may reduce the effectiveness of germination inhibitors. Smoot and Pierson (1981) have shown that germinants and inhibitors may compete for related sites which determine the ability of germination to occur.

1.5 COMBINATION OF SORBATES WITH OTHER INHIBITORY FACTORS

Many workers (Chichester et al., 1972; Sofos and Busta, 1983) have reported the use of sorbic acid and its potassium salt as fungistatic agents in the food industry. However, in recent times sorbates are also being used for the control of pathogenic and spoliage bacteria in food products. In review articles by Sofos et al. (1979b), Sofos and Busta (1981, 1982, 1983) and Busta and Foegeding (1983) many aspects of sorbic acid antibacterial effects have been highlighted.

It has been shown by Gould (1964) that 0.015 - 0.05% sodium sorbate in a medium of pH 6 allowed the germination of spores of six Bacillus species with subsequent lysing of the spore walls, emergence and elongation of vegetative cells but prevented their multiplication.

Ando (1973) reported that concentrations of potassium sorbate greater than 1% were inhibitory to the spore germinations of Cl. botulinum types A and E in a laboratory medium of pH 6.7. In a more recent study, Smoot and Pierson (1981) found that 0.52% potassium sorbate inhibited Cl. botulinum 62A spore germination when the pH of the medium was 5.7. The same workers found that 0.39% potassium sorbate was required to inhibit germination of B.

cereus under the same test condition. In addition, Seward et al. (1982) have reported that at pH 5.8 - 6.0, the germination rate of Cl. botulinum type E spores was decreased to nearly zero in the presence of 1%, 1.5% and 2% sorbate.

The antibacterial activity of sorbates has also been reported in some food products. Cunningham (1979) and To and Robach (1980) have demonstrated that a 5% potassium sorbate dip can extend the shelf life of fresh broiler chickens. The same potassium sorbate concentration was found to inhibit the growth of Salmonella and Staphylococcus aureus which were inoculated on the surface of the broiler chickens. The addition of 0.25% potassium sorbate to cooked and vacuum packed turkey breasts prevented the growth of Salmonella, E. coli and S. aureus (To and Robach, 1980). An earlier study by Raevuori (1976) showed that the addition of 0.4% potassium sorbate to a Karelian pasty rice totally inhibited growth of B. cereus while 0.26% prevented B. subtilis. Similarly Myers et al. (1983) found that the growth of Yersinia enterocolitica, a psychrotrophic and potentially pathogenic organism was inhibited when inoculated roasted pork loin were sprayed with or dipped in 5 and 10% potassium sorbate solution, vacuum-packaged and stored at 5°C.

Sorbates have been applied in combination with other chemicals. The combination of sorbate and nitrite has been found to extend the botulinal safety of cured meat products more than nitrite or sorbate alone (Sofos et al, 1979b). In a similar study, Ivey and Robach (1978) also showed that the combination of 0.1% or 0.2% sorbic acid with 0.005% nitrite greatly retarded botulinal growth and toxicity in a canned comminuted pork product. Botulinal growth and toxin

production were also inhibited in the presence of 0.2% sorbic acid. Interestingly, in the same study the addition of sodium acid pyrophosphate (SAPP) or sodium hexametaphosphate produced a synergistic effect with sorbic acid. The addition of 0.002% nitrite and 0.1 or 0.2% sorbic acid to a chicken product delayed botulinal growth more than 0.1 or 0.2% sorbic acid.

The combination of potassium sorbate and BHA has also been shown to have antibacterial effects. Morad et al. (1982) found that combination of 0.01% potassium sorbate and 0.01% BHA was more inhibitory to the growth of S. typhimurium in cooked turkey than BHA alone. A similar investigation by Parada et al. (1982) showed that a combination of 0.25% potassium sorbate and 0.01 or 0.02% BHA had no significant effect on growth of S. aureus in a model system of pH 6 and 30°C. Elliott et al. (1982) also reported that a combination of pH 5.5, 100% CO_2 and 1.5% sorbate produced a synergistic antimicrobial activity against S. aureus.

2. EXPERIMENTAL

2.1 MICROBIOLOGICAL METHODS

2.1.1 Test Cultures

The test strains listed below were obtained as freeze dried cultures from the National Collection of Industrial Bacteria (NCIB, Torry Research Station, Aberdeen).

Bacillus stearothermophilus	(NCIB 8919, 8920)
Bacillus cereus	(NCIB 6349, 7464)
Staphylococcus aureus	(NCIB 6571)
Escherichia coli	(NCIB 8114).

Each freeze dried culture ampoule was opened aseptically. Bacillus stearothermophilus and B. cereus were grown in nutrient broth CM 2 (NB, Oxoid Ltd., London) and on surfaces of predried nutrient agar (NA, Oxoid CM 3; Appendix 1) plates. Staphylococcus aureus and E. coli were grown in trypticase soy broth (TSB; BBL Microbiology Systems Becton-Dickinson Co.) and on surfaces of predried trypticase soy agar (TSA; BBL Microbiology Systems Becton-Dickinson Co.; Appendix 1). Cultures of S. aureus and E. coli were incubated overnight (18-24h) at 37°C while B. cereus and B. stearothermophilus were incubated for the same period of 30°C and 55°C respectively. Biochemical tests were performed on typical colonies picked from each plate in accordance with the procedures outlined by Harrigan and McCance (1976).

Following growth cultures of B. cereus and B. stearothermophilus were maintained on NA plates and subcultured weekly on the NA plates and incubated at 30°C and 55°C respectively. Staphylococcus aureus and E. coli were similarly maintained on TSA plates at 4°C and

subcultured weekly.

2.1.2 Cleaning and Sterlization of Material

The cleaning of glassware, sterilization of media, diluents, disposal of cultures and contaminated glassware were performed following the standard methods by Harrigan and McCance (1976).

2.1.3 Biochemical Tests

The biochemical characteristics of the test strains are presented in Table 1. These results agreed with Buchanan and Gibbons (1974) and Cowan (1974) for these strains and their typical characteristics were confirmed to be appropriate for subsequent study.

TABLE 1. BIOCHEMICAL CHARACTERISTICS OF TEST STRAINS

Tests	*B. stearothermophilus*		*B. cereus*		*S. aureus*	*E. coli*
	8919	8920	6349	7464	6571	8114
Gram reaction	+[a]	+	+	+	+	-[b]
Catalase	+	+	+	+	+	+
V-P reaction	+	+	+	+	+	-
Citrate utilization	-	-	+	+	+	-
Reduction of nitrate	+	+	+	+	+	+
Starch hydrolysis	+	+	+	+	-	-
Fermentation of sugars						
Glucose	Acid	Acid	Acid	Acid	Acid	No acid
Xylose	Acid	Acid	No acid	No acid	No acid	No acid
Mannitol	No acid	No acid	No acid	No acid	Acid	Acid

[a] positive [b] negative

2.2 SELECTION OF SPORULATION MEDIUM

The type of medium employed for production affects the properties of spores (Molin, 1982) and in turn influences their response (Waites, Stansfield and Bayliss, 1979; Bayliss, Waites and King, 1981; Molin, 1982). A number of sporulation media has been reported in the literature. For the production of B. stearothermophilus spores, dextrose tryptone agar (DTA; Navani, Scholefield and Kibby, 1970) SMS (Appendix 1 ; Mallidis, 1981) and fortified nutrient agar (FNA; Finley and Fields, 1962; Manson, 1977) have been employed.

In a study of the resistance of B. subtilis spores to a combined treatment, Briggs and Yazdany (1974) used an antibiotic assay medium (AAM) containing 0.0001% manganous sulphate as sporulation medium. Emodi and Lechowich (1969a) employed trypticase-peptone-sucrose and yeast extract medium for Cl. botulinum spore production while Roberts, Jarvis and Rhodes (1976) used trypticase-peptone-thioglycollate medium for the same organism. In these two studies the spores produced were used to evaluate responses to combined preservation systems. In the present study, selected sporulation media were compared to determine the highest percentage production of refractile spores, a widely accepted parameter of spore resistance.

2.2.1 Materials and Methods

2.2.1(a) Sporulation Medium

The sporulation medium tested include SMS, FNA, NA and plate count agar (PCA) (Appendix 1) using B. stearothermophilus (NCIB 8919) and B. cereus (NCIB 6349) as test cultures.

2.2.1(b) Production of Spore Suspensions

Plates of the different sporulation media were streaked with a heavy inoculum from overnight cultures of B. stearothermophilus and B. cereus. At intervals during incubation at 60°C for B. stearothermophilus and at 30°C for B. cereus, wet mounts of the cultures were made by emulsifying a small amount of growth in a loopful of sterile distilled water on a clean slide and gently placing a clean coverslip on top. Phase contrast microscopy examination of the wet mounts was carried out and the percentage of the refractile (phase bright) spores calculated by counting a minimum of two hundred cells.

2.2.1(c) Spore Cleaning

Spore suspensions were cleaned as described by Mallidis (1981) and shown in Figure 2. Spores were suspended in sterile distilled water in plastic conical based universal bottles (Sterilin Ltd., Middlesex, England) and centrifuged at 2500g for 15 min in a swing out centrifuge MSE model (Measuring and Scientific Equipment, Crawley, England). The supernatant which consisted mainly of vegetative cells was discarded. The pellet was resuspended in sterile distilled water and further cleaning carried out following the sequence shown in Figure 2. The process was repeated until clean spores were obtained as revealed by phase contract microscopy examination.

The spore suspensions were sonicated at 9A 20 KH_z for 30s (Soniprobe 7530A, Dawe Inst. Ltd., England) at the different steps shown in the flow chart. Between stages 2 and 3 of the centrifugation procedure, the spore suspension was also heat treated at 45°C

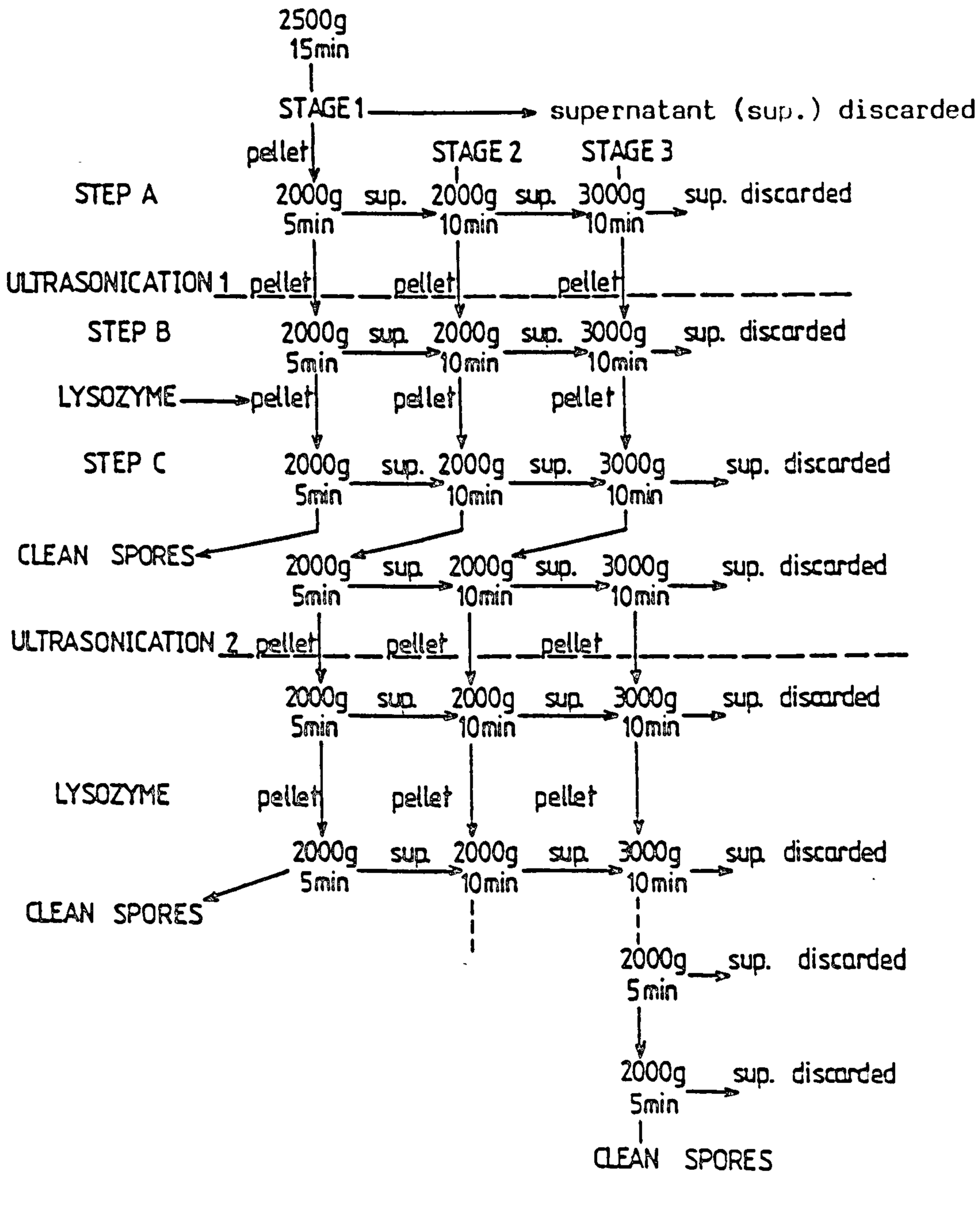

FIGURE 2. Flow sheet diagram for the cleaning of spores.

in the presence of 0.05% lysozyme for 60 min. Cleaned spore suspensions were bulked and stored at 4°C for a maximum period of two weeks before use.

2.2.1(d) Results and Discussion

The extent of the spore formation of B. stearothermophilus (NCIB 8919) and B. cereus (NCIB 6349) in test media are presented in Figures 3 and 4 respectively. PCA and NA were poor sporulation media for B. stearothermophilus but gave a relatively satisfactory result for B. cereus. This is in agreement with earlier studies by Abdelgadir (1974) and Manson (1977). In the present experiment, results showed that FNA was satisfactory as a sporulation medium for B. cereus but not for B. stearothermophilus. Finley and Fields (1962) and Manson (1977) obtained similar results in their studies. By contrast, SMS medium proved to be very satisfactory as a sporulation medium for both strains. Both Abdelgadir (1974) and Mallidis (1981) also showed SMS to be a very good medium for the sporulation of B. stearothermophilus. However, unlike Mallidis (1981), the incubation period was increased to 120h in the present study to reduce the post harvest manipulation of the spores (Waites and Bayliss, 1980). Therefore, SMS was selected as the sporulation medium for all the strains of B. stearothermophilus and B. cereus cleaned as described in Section 2.2.1(c).

2.3 "COME-UP" TIME DETERMINATION

The various techniques for measuring the moist heat resistance of bacteria have been reviewed by Stumbo (1973). Methods include the TDT-tube method, TDT-can method, tank method, flask method,

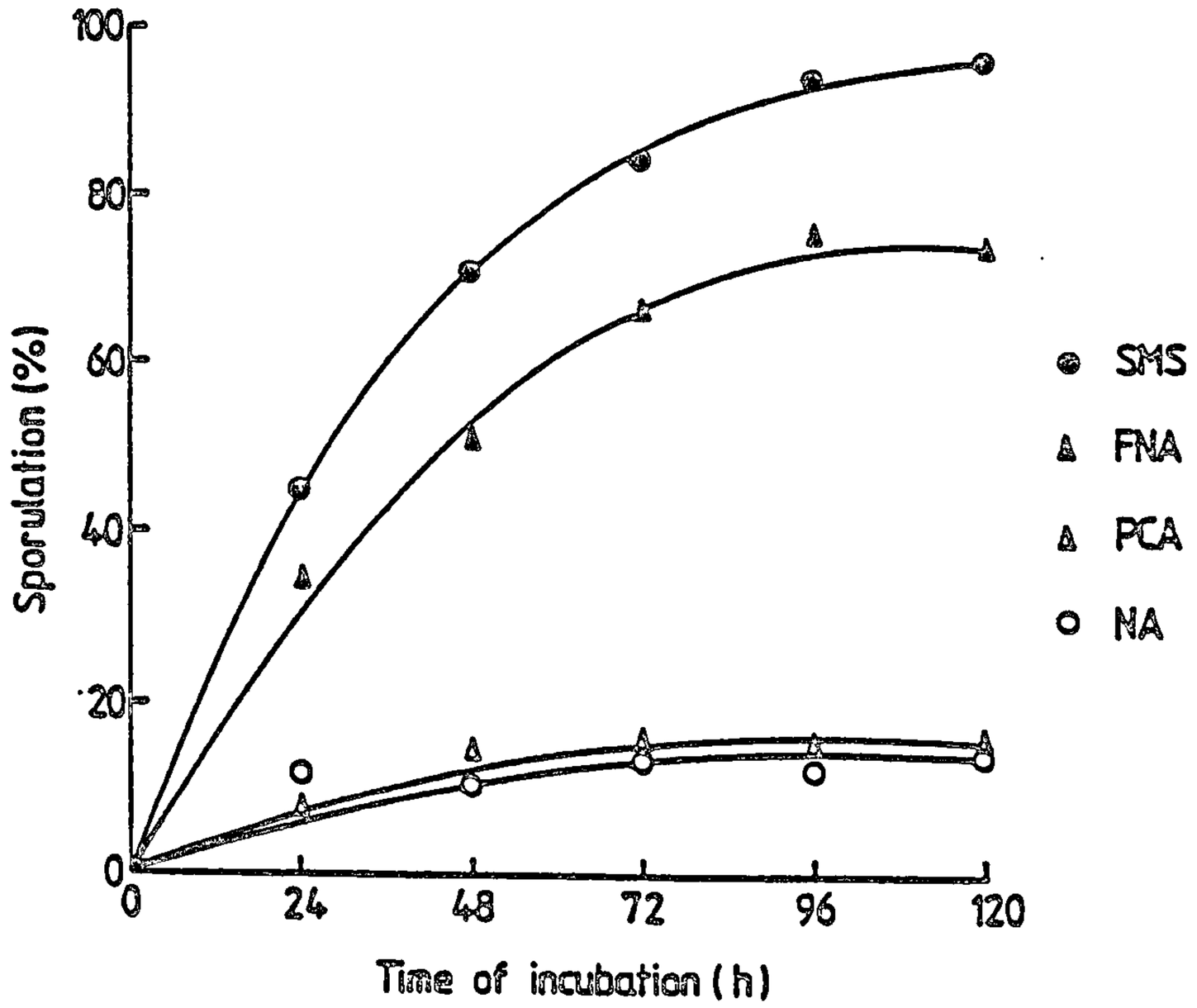

FIGURE 3. Sporulation of B. stearothermophilus (NCIB 8919) on different media.

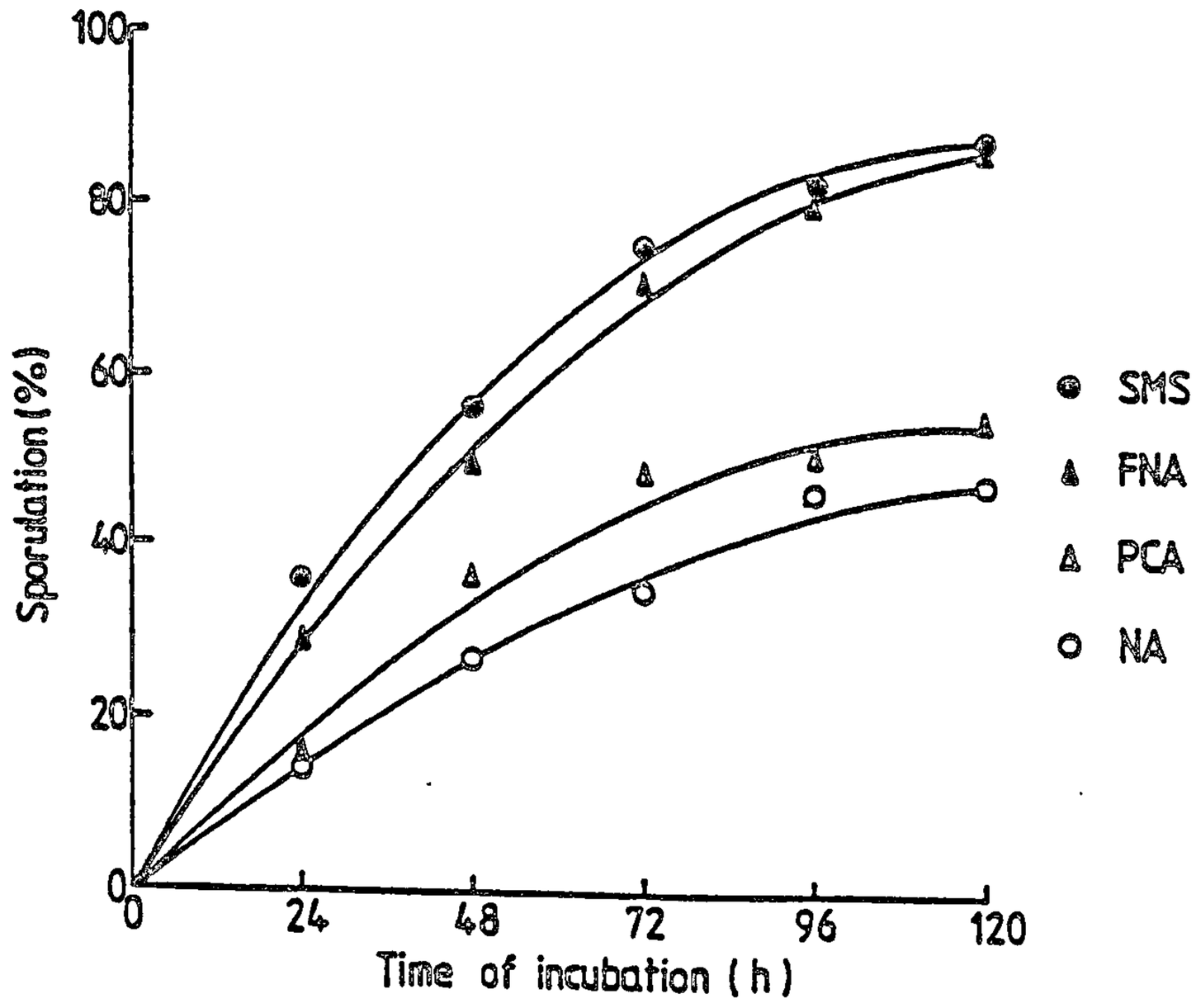

FIGURE 4. Sporulation of B. cereus (NCIB 6349) on different media.

thermoresistometer method, unsealed TDT-tube method, capillary tube method and the screw capped technique described by Kooiman and Geers (1975).

Generally, the method is named after the container used for study. The effectiveness of the container is dependent on the "come-up" time, also called 'Zero time' or 'heating lag'.

The "come-up" time respresents the time taken for a heated container (can, tube, flask etc) and its content to attain a desired test temperature. A long "come-up" time may affect the response of spores exposed to heat (Navani, 1970) and the parameters determined from the results. The objective of this preliminary experiment was to determine the "come-up" time of the tubes used in subsequent studies.

Materials and methods

Some light walled pyrex tubes (Corning Ltd., Lab. Division, Staffordshire, England) with dimensions 12 x 75 mm were selected and sterilized. These were then filled with 4.9 ml aliquots citrate - phosphate buffer (CPB) pH 7.0. To this, 0.1 ml spore suspension of B. stearothermophilus was added and gently rocked to mix. Four tubes were used for each test temperature. The tubes were placed in the holes drilled on a specially constructed aluminium plate which served as a carrier tray. The tubes were submerged in a thermostatically controlled and circulated water bath (Townson and Mercer Ltd., Croydon, England) previously heated and equilibrated to the desired temperature.

In order to determine the "come-up" time the tip of a copper-constantan thermocouple (O.F. Ecklund, Batavia, Illinois, USA) was placed at the approximate geometric centre of the spore suspension.

The thermocouple was held in place in the glass tube by clamping it to a retort stand and the time it took the tube to attain a desired temperature was recorded. The temperature was monitored using a calibrated potentiometer (Elektrolaboratoriet, Copenhagen). As soon as the required temperature was attained, the tubes were quickly removed and cooled to 10°C in an ice-water bath. The time for this to be attained was recorded. At each test temperature four determinations were made and the mean computed.

Results and Discussion

The mean "come-up" times for tubes at different temperatures are presented in Figure 5. The results indicated that the higher the temperature the longer the "come-up" time. At 95°C, a "come-up" time of 42.5 secs was obtained and at 50°C the "come-up" time was 33.5 secs.

In each subsequent study the tubes contents were initially heated for a period equivalent to the "come-up" time for the test temperature before zero time commenced for the experiment. The zero correction time was taken from the calibration graph in Figure 5. Since cooling time to reduce the temperature from 95°C to 10°C was short (3 - 5 secs), no correction factor was introduced. These tubes are referred to as "sample tubes" in subsequent studies.

2.4 EVALUATION OF PLATING TECHNIQUES

The viable plate counts is the method often used for assessing the response of bacterial cells or spores to heat, chemicals or to a combination of heat and chemicals (Stumbo, 1973; Tomlins and Ordal, 1976; Russell, 1982). In resistance studies viable counts are often achieved by the pour plate (Meynell and Meynell, 1965;

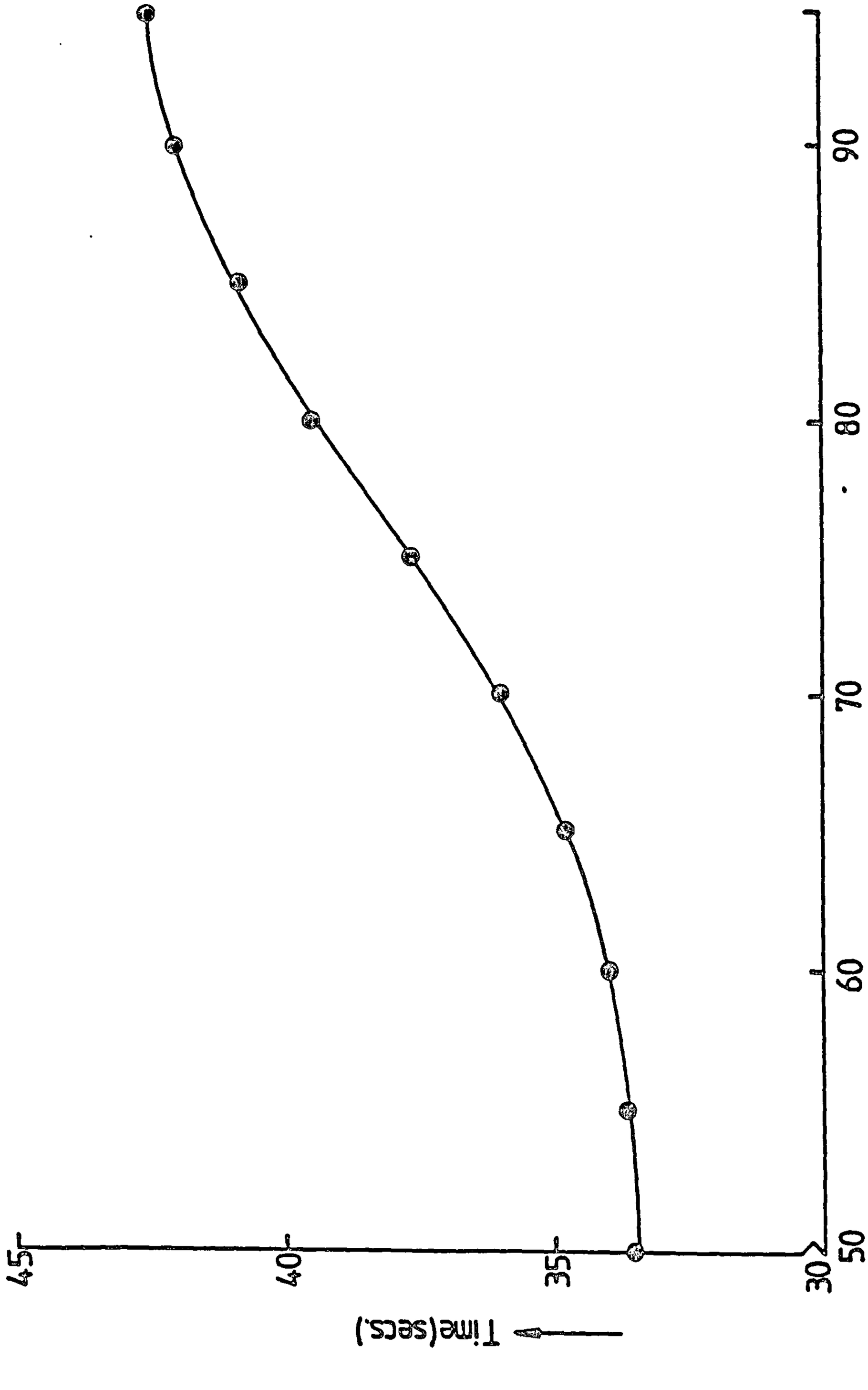

FIGURE 5. "Come-up" time for sample tubes.

Kass and Norden, 1968; Navani, 1970) and the streak plate techniques (Cook and Gilbert, 1968; Mallidis, 1981). Although these conventional techniques are statistically adequate, they are labour intensive and costly (Jarvis, Lach and Wood, 1977).

Many workers (Campbell and Gilchrist, 1973; Gilchrist et al., 1973) have shown that the spiral plate technique can be used for the quantitative estimation of viable microorganisms in foods. With this technique, the labour requirements for the enumeration of microorganisms were only 31% of the streak or pour plate techniques and there were considerable savings in materials (Jarvis et al., 1977).

In studying the response of bacterial cells or spores to combination of heat and chemicals plating techniques are often selected without much regard for their adequacy. An objective of this preliminary study was to evaluate three different plating techniques with a view of selecting the best for subsequent studies.

Materials and methods

An aliquot (0.1 ml) of B. stearothermophilus (NCIB 8919) spore suspension containing ca. 10^8 spores ml^{-1} was added to 4.9 ml citrate-phosphate buffer pH 7.0 in sample tubes. The sample tubes and contents were heated at 95°C for 45 min. The tubes were cooled in an ice bath and thoroughly mixed using the Whirlimixer (Fisons Scientific Co. Ltd., Loughborough). Serial decimal dilutions of the survivors were carried out using sterile distilled water. The survivors were recovered on YPTD agar pH 7.3 containing gl^{-1} (bacteriological peptone 5; tryptone oxoid 2.5; yeast extract powder (L-20) 1.0; Lab-lemco oxoid (L-29) 3.0; dextrose (L-71) 1.0; agar No 3 oxoid 15.0).

YPTD agar was dispensed in 15 ml aliquots in petri dishes on a carefully levelled surface and allowed to set completely before handling. Prepared plates were dried overnight at 37°C with the lids inclined. The dried plates were used for the streak and spiral plate techniques. In the case of the pour plate technique the YPTD agar was dispensed in 180 ml amounts in screw capped bottles and stored until required.

The streak plate technique involved using 0.1 ml aliquot of the heated spores, transferred into the plates using a 0.1 ml syringe (Terumo microsyringe, Terumo Corp., Japan). The inoculum was evenly distributed by the use of a bent pipette. The pour plate technique involved dispensing 1 ml aliquot of the heated spore suspension into each disposable petri dish and adding 15 ml aliquot of melted YPTD agar. The YPTD agar was tempered to 45 ± 1°C before use. An even distribution of the inoculum was achieved by gentle shaking and circular movement of the plates for 10 - 15 s. In the case of the spiral plate technique, the Spiral Plater (Don Whitney Scientific, Shipley, Yorks, UK) was used. The procedure was followed as outlined in the manual.

Plates were incubated at 55°C for 96h and the survivors counted using the digital colony counter (Gallenkamp Ltd. England).

Results and Discussion

Table 2 shows the statistical analysis of the viable counts of *B. stearothermophilus* (NCIB 8919) after subjection to heat and enumeration by three plating techniques. The results showed that the pour plate technique gave the highest mean estimate, followed by the spiral and finally the streak plate techniques. The % C.V. value for all the techniques was found to lie between 2 and 5%.

TABLE 2. Viable counts of B. stearothermophilus (NCIB 8919) using different plating techniques after exposure of spores to heat.

Summary of Statistical Analysis[a]

Plating Tech.	No of Plates	Mean Count (X̄)	Variance (S^2)	Std. Deviation (s)	Coef. Variation % (C.V.)[b]
Pour	20	6.98(9.5×10^6)[c]	0.00011	0.0104	2.38
Spiral	20	6.96(9.0×10^6)	0.00036	0.019	4.36
Streak	20	6.94(8.8×10^6)	0.00041	0.02	4.64

Analysis of Variance

Source	Sum of Squares	Degrees of Freedom	Mean Square	F-ratio
Plating tech.	0.01060	2	0.0053	17.67[d]
Residual	0.01685	57	0.0003	
Total	0.02745	59		

[a]Statistical analysis was computed using the $\log_{10}$ of estimates

[b]Coefficient variation (% C.V.) computed on the actual estimates

[c]Values in parentheses indicate the antilog values of the means

[d]Significant at the $\alpha = 0.05$

Thus it was concluded that any of the tested techniques could be used to assess the survival of the spores after exposure to heat.

The one-way analysis of variance was computed using the log values of estimate as described by Mead and Curnow (1983). The result showed that there was a significant difference ($P = 0.05$) in the plating techniques - high F ratio compared to the critical value. The studentized range test also showed that the mean values of counts obtained using the pour plate technique was significantly different ($P = 0.05$) from the mean values of counts using the remaining two techniques. A study by Soestbergen and Lee (1969) showed that pour plate technique was statistically more accurate than the streak plate. This is in agreement with the present study; the pour plate was found to have the lowest variance value, followed by the spiral and the streak plating techniques respectively. In contrast to the present finding, Gilchrist et al.(1973) and Jarvis et al.(1977) obtained no significant difference ($P = 0.05$) in variance between the pour and spiral plating techniques. A plausible reason for the difference might be in the range of microorganisms used by these workers, i.e. mainly aerobes, whereas B. stearothermophilus used in this study is facultative anaerobe (Stumbo, 1973). This may account for the mean count of survivors with the pour plate technique being slightly higher than the other two techniques.

In resistance studies a technique which gives the maximum recovery of spore or cells will give a more accurate assessment of resistance. Hence, the pour plate technique was recommended for the enumeration of B. stearothermophilus spores after exposure to heat, chemicals or combination of both. This technique was used in all the subsequent studies unless otherwise stated.

3. EFFECTS OF THE COMBINATIONS OF HEAT, POTASSIUM SORBATE AND pH ON BACTERIAL SPORES AND CELLS

The response of any given organism to heat is not only dependent on inherent genetic factors but also on a number of environmental factors including the composition of the suspension. The influence of the composition of the suspending medium on the heat response by bacterial spores have been reported by Odlaug and Pflug (1977a), Smelt et al. (1977), Gauthier et al. (1978) and Gould (1978) as well as by bacterial cells by Tomlins and Ordal (1976).

The reported studies by Townsend et al. (1938), Tsuji et al. (1960) and Alderton et al. (1976) have shown that thermal processing requirements can be reduced with the addition of chemicals. In commercial processing, organic acids are often used to adjust the pH of a heating medium and consequently reducing the thermal processing requirements (Townsend et al., 1954; Ito and Chen, 1978).

Smoot and Pierson (1981) and Seward et al. (1982) reported that Cl. botulinum spores were inhibited by sorbates. The partial replacement of nitrite in cured meats by sorbates has also been described (Sofos and Busta, 1981). In the search for chemicals that could enhance the effect of heat in reducing the number of spores or cells, it was considered that potassium sorbate should be examined. Therefore, the objective of this preliminary experiment was to determine the effects of the combinations of heat and potassium sorbate on B. stearothermophilus and B. cereus spores as well as on S. aureus and E. coli.

Materials and methods

Tests cultures: Bacillus stearothermophilus (NCIB 8919), B.

stearothermophilus (NCIB 8920), B. cereus (NCIB 6349), B. cereus (NCIB 7464), S. aureus (NCIB 6571) and E. coli (NCIB 8114). The spores were obtained using the procedure described in Section 2.2.1(c). Cells of S. aureus and E. coli were grown aerobically on TSA slants in universal bottles at 37°C for 24h in case of E. coli and for 48h in the case of S. aureus. The cells were washed from the surface of the slants with sterile distilled water, centrifuged at 2000g for 5 min for four to five times to remove debris and to obtain clean cells. The cleaned cells were bulked and diluted as appropriate to give the desired cell concentration.

Heating menstruum

The heating menstrua described below includes the various constituents examined in the main study. Potassium sorbate-KS (Koch Light, Laboratory Ltd., Colnbrook Bucks, England), sodium chloride-NaCl (BDH Chemicals Ltd., Poole, England) and sodium benzoate-SB (BDH Chemicals Ltd., Poole, England) were the chemicals employed. The pH of the menstruum was adjusted with citrate phosphate buffer-CPB; 0.1M solution of citric acid and 0.2M Na_2HPO_4) as described by McIlvaine (1921). To ensure the desired pH was attained a buffer of lower pH than the final pH was prepared so that a shift due to the addition of chemicals and heat was accommodated.

A 5 ml aliquot of the heating menstruum was prepared using 1 ml of a 5 fold of the desired final concentration of the chemical (e.g. KS) and 4 ml of the CPB. In the case of the combined chemicals, e.g. KS and NaCl, the menstruum was made up of 1 ml aliquot of a 5 fold of the desired final concentration of each chemical and 3 ml aliquot of CPB. The 5 fold concentrations of sorbate, benzoate and NaCl were prepared separately using sterile distilled water.

Potassium sorbate and sodium benzoate solutions being filter sterilized (Millipore filter; 0.45 µm) while NaCl solutions and CPB were autoclaved at 121°C for 15 min.

Heat treatment: A 0.1 ml aliquot of spore or cell suspension of approximately 10^7/ml, as determined by phase contrast microscopy (Leitz Labrolux 11), was centrifuged at 2000g for 5 min in a plastic concial based universal bottles (Sterilin Ltd., Middlesex, England) to obtain a spore or cell pellet. The spore or cell pellet was re-suspended in the 5 ml heating menstruum and thoroughly shaken using glass beads and the Whirlimixer (Fisons Scientific Apparatus Loughborough, Leiceistershire). The 5 ml aliquot of cell or spore suspension was aseptically transferred into sample tubes which were covered with aluminium foil and placed in the carrier tray. The tubes were submerged in a thermostatically controlled and circulated water bath and heated at the appropriate temperature for the required time taking cognisance of the "come-up" time. After the desired heat treatment, the sample tubes were quickly removed and cooled in an ice-water bath. Escherichia coli and S. aureus suspensions were heated at 50° and 55°C for 15 min. B. cereus spore suspension were heated at 75°, 80°, 85°C for 30 min and B. stearothermophilus spore suspension heated at 85°, 90° and 95°C for 45 min.

Estimation of survivors: Serial dilutions of the treated cells and spores were made using 0.1% (w/v) peptone water and sterile distilled water respectively. Where survivors were low in number the treated spores or cells were poured into plastic conical based universal bottles and centrifuged at 2000g for 5 min, this pro-cedure being repeated four times using sterile distilled water. The

spores or cells were resuspended in distilled water and thoroughly mixed using the Whirlimixer. Surviving spore or cell numbers were determined using YPTD agar for B. stearothermophilus and B. cereus spores while TSA was used for S. aureus and E. coli. Survivors were incubated at 30°C for B. cereus, 37°C for S. aureus and E. coli, and at 55°C for B. stearothermophilus. The survivors were incubated for a maximum period of 6 days and were counted using a digital colony counter (Gallenkamp Ltd., England).

The computed mean of triplicate plates at two dilution levels was obtained using the formula of Farmiloe et al. (1954). Counts between 30 and 300 were considered.

Treatment of results

Recovered treated cells or spores are presented as $\log_{10}$ survivors per ml. Each value representing a mean of 6 replicates. The surviving cell or spore counts are expressed on a percentage basis by considering the mean of the counts in the presence of potassium sorbate as a percentage of the counts in the absence of the chemical under the same heating conditions.

Results and Discussion

3.1 HEAT AND POTASSIUM SORBATE EFFECTS

In view of the nature of the study, temperatures below 100°C were selected to achieve mild heat treatment conditions. For the spores temperatures ranging from 70° to 95°C were selected depending on whether B. cereus or B. stearothermophilus was involved. In a study by Smoot and Pierson (1981) a test temperature of 70°C was used for B. cereus T and 80°C for Cl. botulinum 62A spores. The temperatures of 50° and 55°C were used for S. aureus and E. coli. Shibasaki and Tsuchido (1973) also employed similar temperatures in

their study which involved E. coli as the test organism.

Tables 3 and 4 show the survivor counts of S. aureus and E. coli respectively after exposure to the combination of heat and potassium sorbate. The results of this preliminary experiment showed that heating at 55°C for 15 min with 1% (w/v) potassium sorbate provided marginal inhibition (15.4%) of S. aureus but for E. coli the same treatment gave slightly higher inhibition of 23.8%. Other concentrations of potassium sorbate at the same temperature caused lesser inhibition of the two organisms. Furthermore, heating of S. aureus at 50°C for 15 min with (0 - 1%) potassium sorbate did not show an inhibition (Table 3). However, for E. coli exposure to the same heating condition in the presence of 1% sorbate caused 15.7% inhibition. (Table 4). Heat treatment in the presence of less than 1% sorbate demonstrated less than 15.7% inhibition.

The results of this preliminary experiment indicate that in general a combination of heat at 50° and 55°C with sorbate (0 - 1%) did not show marked inhibitory effect on S. aureus and E. coli.

The results of the effects of the combination of heat and potassium sorbate on bacterial spores are shown in Tables 5 - 8. It was found that heating at temperatures below 100°C in the presence of varying concentrations of sorbate showed no appreciable inhibition of the spores. A combination of heat at 95°C for 45 min with 1% sorbate caused an inhibition of 6.9% for B. stearothermophilus (NCIB 8919). Other combinations provided lower inhibition (Table 5). A similar pattern of results was obtained for strain 8920. An inhibition of 8.5% was obtained using a combination of heat at 95°C for 45 min in the presence of 1% (w/v) sorbate. Other combinations caused less inhibition (Table 6). The application of temperatures

TABLE 3. The effects of the combination of heat and potassium sorbate-KS on Staphylococcus aureus (NCIB 6571).

% (w/v) KS in heating medium	Log_{10} number of surviving cell counts recovered in TSA after heating at	
	50°C for 15 min	55°C for 15 min
0.00	6.36 (100.0)[a]	6.30 (100.0)
0.05	6.36 (100.0)	6.29 (96.0)
0.10	6.37 (102.2)	6.27 (92.5)
0.15	-[b]	6.26 (90.5)
0.20	6.37 (102.2)	6.27 (92.0)
0.25	-	6.25 (88.6)
1.00	6.36 (100.00)	6.23 (84.6)

[a]Surviving cells, calculated as a percentage of the control result at each heating temperature.

[b]Not done.

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 4. The effects of the combination of heat and potassium sorbate-KS on Escherichia coli (NCIB 8114).

% (w/v) KS in heating medium	Log_{10} number of surviving cell counts recovered in TSA after heating at	
	50°C for 15 min	55°C for 15 min
0.00	6.03 (100.0)[a]	5.57 (100.0)
0.05	6.02 (97.2)	5.54 (94.6)
0.10	6.00 (93.5)	5.53 (91.9)
0.15	6.02 (96.3)	5.49 (83.8)
0.20	5.99 (90.7)	5.48 (81.1)
0.25	6.01 (95.4)	5.47 (79.7)
1.00	5.96 (84.3)	5.45 (76.2)

[a]Surviving cells, calculated as a percentage of the control result at each heating temperature.

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 5. The effects of the combination of heat and potassium sorbate-KS on B. stearothermophilus (NCIB 8919) spores.

% (w/v) KS in heating medium	Log_{10} number of surviving spore counts recovered in YPTD after heating at		
	85°C for 45 min	90°C for 45 min	95°C for 45 min
0.00	6.06 (100.0)[a]	6.10 (100.0)	6.16 (100.0)
0.20	6.07 (101.7)	6.11 (101.6)	6.16 (99.3)
0.40	6.07 (102.6)	6.12 (104.0)	6.15 (97.2)
0.60	6.06 (100.9)	6.10 (100.0)	6.15 (96.6)
0.80	6.06 (99.1)	6.12 (104.0)	6.14 (94.5)
1.00	6.07 (102.6)	6.11 (101.6)	6.13 (93.1)

[a]Surviving spores, calculated as a percentage of the control result at each heating temperature.

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 6. The effects of the combination of heat and potassium sorbate-KS on B. stearothermophilus (NCIB 8920) spores.

% (w/v) KS in heating medium	Log_{10} number of surviving spore counts recovered in YPTD medium after heating at		
	85°C for 45 min	90°C for 45 min	95°C for 45 min
0.00	6.03 (100.0)[a]	6.05 (100.0)	6.07 (100.0)
0.20	6.05 (104.6)	6.06 (102.7)	6.05 (96.6)
0.40	6.04 (100.9)	6.06 (102.7)	6.06 (98.3)
0.60	6.03 (100.0)	6.05 (100.0)	6.05 (95.7)
0.80	6.03 (100.0)	6.05 (101.8)	6.04 (93.2)
1.00	6.04 (100.9)	6.04 (98.2)	6.03 (91.5)

[a]Surviving spores, calculated as a percentage of the control result at each heating temperature.

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 7. The effects of the combination of heat and potassium sorbate-KS on B. cereus (NCIB 6349) spores.

% (w/v) KS in heating medium	Log_{10} number of surviving spore counts recovered in YPTD medium after heating at		
	75°C for 30 min	80°C for 30 min	85°C for 30 min
0.00	6.16 (100.0)[a]	6.23 (100.0)	6.31 (100.0)
0.10	6.18 (103.4)	6.23 (100.6)	6.32 (101.5)
0.20	6.17 (102.1)	6.23 (99.4)	6.29 (96.6)
0.30	6.21 (111.0)	6.22 (98.2)	6.28 (93.6)
0.40	6.19 (107.6)	6.24 (101.8)	6.29 (94.6)
0.50	6.20 (108.3)	6.22 (97.7)	6.28 (92.7)
1.00	6.18 (103.4)	6.22 (98.2)	6.26 (89.2)

[a]Surviving spores, calculated as percentage of the control result at each heating temperature.

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 8. The effects of the combination of heat and potassium sorbate-KS on B. cereus (NCIB 7464) spores.

% (w/v) KS in heating medium	Log_{10} number of surviving spore counts recovered in YPTD medium after heating at		
	75°C for 30 min	80°C for 30 min	85°C for 30 min
0.00	6.22 (100.0)[a]	6.28 (100.0)	6.35 (100.0)
0.10	6.24 (105.4)	6.29 (102.1)	6.35 (100.9)
0.20	6.23 (103.0)	6.27 (97.4)	6.32 (93.3)
0.30	6.23 (101.8)	6.26 (95.2)	6.32 (94.6)
0.40	6.21 (98.2)	6.25 (93.7)	6.33 (95.1)
0.50	6.22 (100.6)	6.26 (96.3)	6.32 (93.7)
1.00	6.22 (100.6)	6.27 (97.4)	6.29 (87.4)

[a]Surviving spores, calculated as percentage of the control result at each heating temperature.

Each recorded log_{10} value is a mean of 6 replicates.

lower than 95°C and sorbate (0 - 1%) did not inhibit the test strains of B. stearothermophilus.

Although lower temperatures were used for B. cereus spores compared to B. stearothermophilus the combinations of heat and sorbate also showed similar trend of inhibitory effects on the former. A combination of heat at 85°C for 30 min in the presence of 1% (w/v) sorbate caused 10.8% inhibition of B. cereus (NCIB 6349) (Table 7) whereas for strain 7464 an inhibition of 12.6% was obtained (Table 8). Treatments in the presence of sorbate concentration of less than 1% showed less inhibition.

It was concluded from this preliminary experiment that combinations of mild heat and potassium sorbate caused marginal inhibition on S. aureus and E. coli and had almost no inhibition on the bacterial spores.

3.2 EFFECTS OF THE COMBINATION OF HEAT, POTASSIUM SORBATE AND pH

The effect of pH on the response of cells (Hansen and Rieman, 1963) and bacterial spores (Anderson and Friesen, 1974; Blocher and Busta, 1983; Gombas, 1983) to heat has been reported. However, there is no consensus of opinion as to the nature of this effect on bacterial spores. Some workers have found that pH had little or no influence on the heat resistance of spores (Lang, 1935; Sognefest et al., 1948; Abedelgadir, 1974). In the studies by Cook and Gilbert (1968), Anderson and Friesen (1974), El-Mabsout and Stevenson (1979) and Cameron et al. (1980) it was found that changes in pH led to significant changes in the heat response of spores.

The contribution of pH adjustment in food preservation has been reported by other workers. In a study by York et al. (1975) it was

found that storage of tomato juice containing heat - injured B. coagulans spores at pH 4.45 for 9 weeks provided a 1.5 to 3.5 log cycle reduction in spore counts. The stability of canned cured meats is considered to be partly due to the slightly acid pH of the products (Chumney and Adams, 1980). In order to reduce the thermal processing necessary to prevent spoilage canners have resorted to pH control to inhibit the growth of Cl. botulinum (Townsend et al., 1954). Similarly, Braithwaite and Perigo (1971) have shown that the thermal processing time of foods can be reduced where the pH of the food is acidic.

Furthermore, it has been demonstrated by many workers (Restaino et al., 1981; Eklund, 1983; Sofos and Busta, 1983) that the antimicrobial effectiveness of potassium sorbate is increased with reduced pH. Following the findings reported in Section 3.1 that combinations of heat and potassium sorbate provided a marginal inhibition of S. aureus and E. coli and with no apparent inhibition on the bacterial spores, it was considered that the effects of the combinations of heat, potassium sorbate and pH on these organisms should be studied.

Materials and methods

Test cultures: As described in Section 3.1.

Heat treatment: Cultures were heated at the following temperatures: S. aureus - 55°C for 15 min, E. coli - 50°C for 15 min, B. cereus spores - 85°C for 30 min and B. stearothermophilus spores - 95°C for 45 min. The pH of the menstrua were adjusted with citrate - phosphate buffer as described in Section 3.1. All other procedures were as described in Section 3.1 p. 50.

Treatment of results

The survivors of the treated S. aureus (Table 9) and E. coli (Table 10) are presented graphically in Figures 6 and 7 respectively. The survivors are compared on percentage basis. This was achieved by considering the mean of the viable counts in the presence of sorbate, sodium chloride or a combination of both as a percentage of the counts in the absence of the chemical at each pH. The survivors of the treated bacterial spores (Tables 11 - 14) are presented as 3-dimensional histograms (Figures 8 - 11; Appendix 3). Survivors of the bacterial spores exposed to other treatments are also presented as 3D-histograms.

The 3D-histograms were obtained using a developed computer program, Section 3.3 going through the following steps.

(a) Each pH was considered as a test system.

(b) The mean viable counts of bacterial spores obtained after exposure to heat with either or both potassium sorbate and sodium chloride were computed as a percentage of the viable count in the absence of chemicals at the same pH.

(c) The percentage survivors were arranged such that low values were in the "front" of the 3D-histograms and high values at the "back".

(d) These values were analysed by computer in order to generate 3D-histograms as described in Section 3.3.

Results and Discussion

The results of the effects of the combination of heat, potassium sorbate and pH on S. aureus, E. coli, B. cereus (NCIB 6349, 7464) and B. stearothermophilus spores (NCIB 8919, 8920) are shown in Figures 6 - 11.

TABLE 9. The effects of the combination of heat, potassium sorbate-KS and pH on S. aureus (NCIB 6571)

% (w/v) KS in the heating medium	Log_{10} number of surviving cell counts recovered in TSA after heating at 55°C for 15 min			
	pH 7.0	6.5	6.0	5.5
0.00	6.29 (100.0)[a]	6.25 (100.0)	6.21 (100.0)	6.13 (100.0)
0.05	6.24 (90.7)	6.17 (83.1)	6.10 (76.7)	5.83 (50.4)
0.10	6.22 (86.5)	6.12 (74.0)	5.97 (57.7)	4.32 (15.5)
0.15	6.18 (78.2)	5.99 (55.4)	5.58 (23.2)	3.17 (0.1)
0.20	6.15 (73.6)	5.83 (38.4)	4.94 (5.4)	2.39 (0.018)
0.25	6.10 (65.3)	4.34 (12.3)	3.93 (0.5)	<1.48 (0.002)

[a]Surviving cell counts calculated as percentage of the control at each pH.

Each recorded log_{10} value is a mean of 6 replicates.

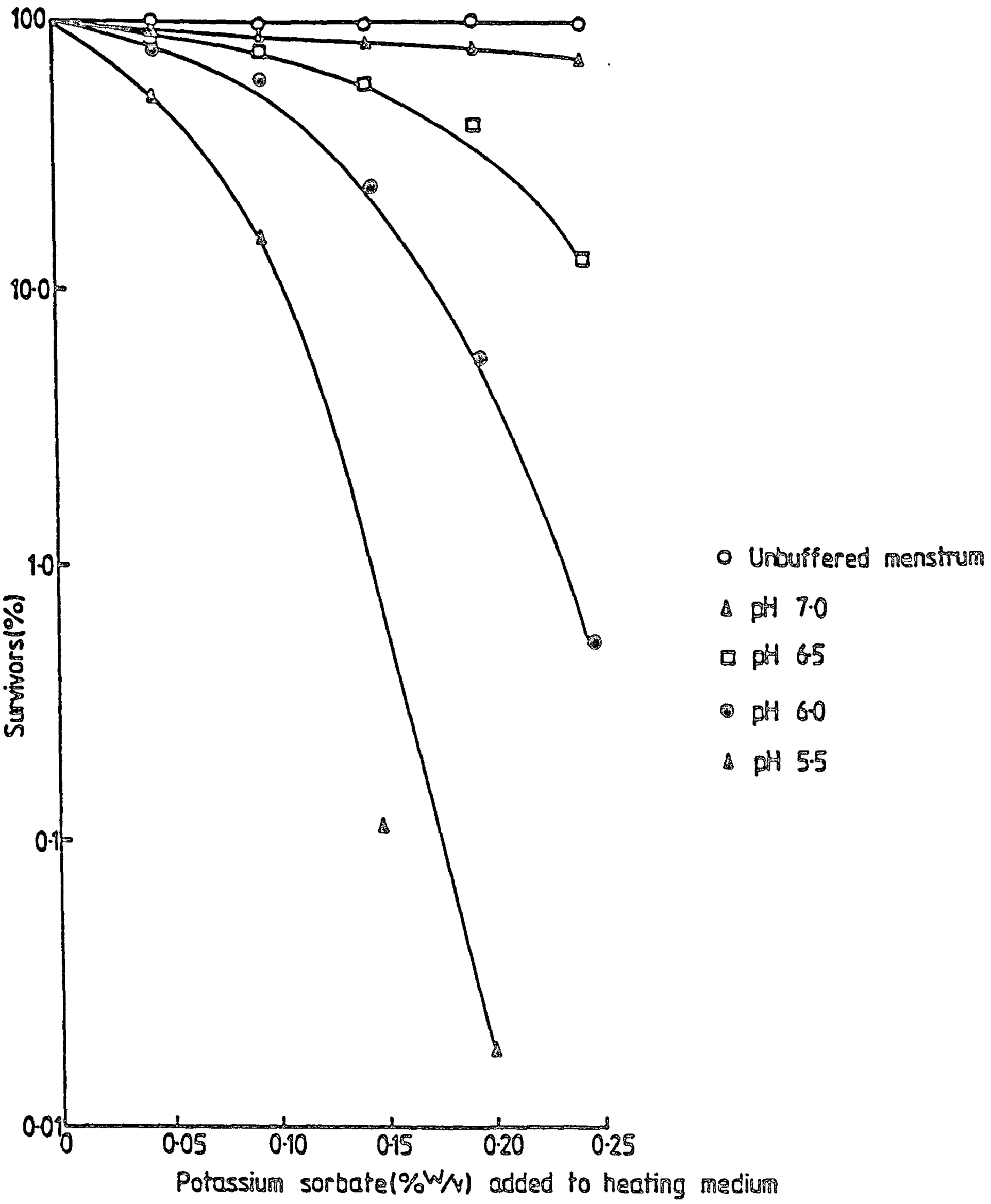

FIGURE 6. The effects of the combination of heat, potassium sorbate and pH on S. aureus (NCIB 6571). Heated at 55°C for 15 min.

TABLE 10. The effects of the combination of heat, potassium sorbate-KS and pH on E. coli (NCIB 8114)

% (w/v) KS in the heating medium	Log_{10} number of surviving cell counts recovered in TSA after heating at 50°C for 15 min			
	pH 7.0	6.5	6.0	5.5
0.00	5.83 (100.0)[a]	5.76 (100.0)	6.59 (100.0)	5.60 (100.0)
0.05	5.80 (92.7)	5.72 (90.4)	5.61 (83.7)	5.49 (77.5)
0.10	5.79 (91.2)	5.67 (82.5)	5.53 (70.3)	5.05 (28.3)
0.15	5.78 (88.2)	5.64 (77.2)	5.45 (57.1)	4.30 (5.0)
0.20	5.76 (83.8)	5.61 (71.9)	5.25 (36.1)	2.91 (0.20)
0.25	5.75 (82.4)	5.54 (61.4)	4.91 (16.5)	1.51 (0.008)

[a]Surviving cell counts calculated as percentage of the control at each pH.

Each recorded log_{10} value is a mean of 6 replicates.

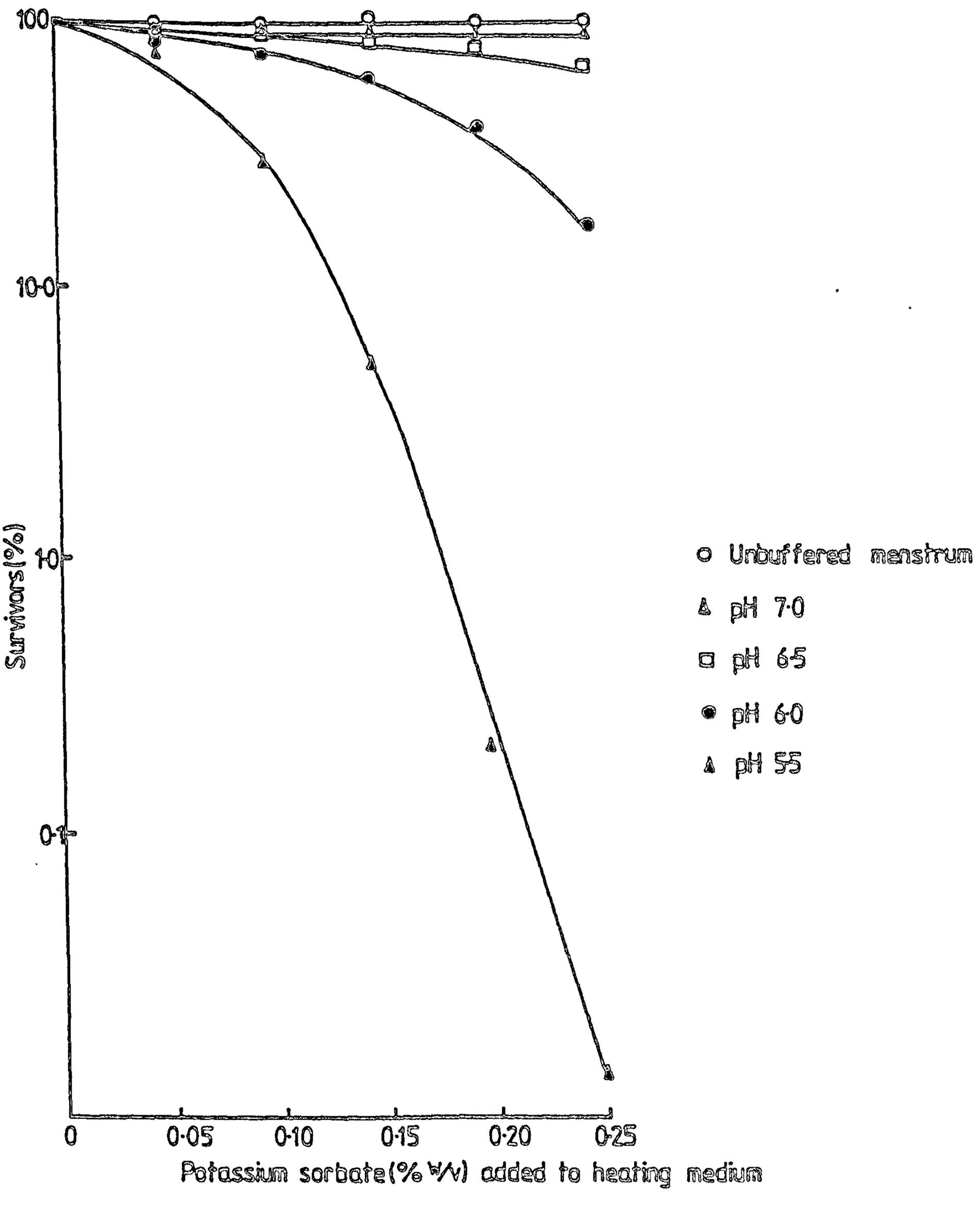

FIGURE 7. The effects of the combination of heat, potassium sorbate and pH on E. coli (NCIB 8114). Heated at 50°C for 15 min.

TABLE 11. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. cereus (NCIB 6349) spores.

pH	% (w/v) NaCl in heating medium	Log_{10} number of surviving spore counts recovered in YPID medium after heating at 85°C for 30 min					
		% w/v KS 0.0	0.10	0.20	0.30	0.40	0.50
6.5	0.0	6.26	6.27	6.24	6.24	6.23	6.22
	1.5	6.31	6.29	6.25	6.19	6.16	6.11
	3.0	6.23	6.24	6.20	6.09	6.02	5.94
	4.5	6.20	6.22	6.16	5.98	5.90	5.71
	6.0	6.17	6.19	6.11	5.89	5.74	5.62
	7.5	6.12	6.14	6.03	5.79	5.66	5.57
6.0	0.0	6.23	6.22	6.19	6.18	6.15	6.15
	1.5	6.25	6.21	6.14	6.11	6.06	6.03
	3.0	6.16	6.12	6.03	5.99	5.89	5.82
	4.5	6.13	6.08	5.94	5.83	5.70	5.54
	6.0	6.06	6.00	5.85	5.72	5.57	5.42
	7.5	6.01	5.86	5.75	5.61	5.36	5.06
5.5	0.0	6.19	6.17	6.15	6.12	6.10	5.06
	1.5	6.16	6.14	6.08	6.03	6.01	5.96
	3.0	6.08	6.00	5.92	5.83	5.74	5.69
	4.5	6.03	5.89	5.82	5.69	5.56	5.46
	6.0	5.93	5.81	5.73	5.61	5.46	5.18
	7.5	5.86	5.75	5.60	5.44	5.21	4.60
4.5	0.0	6.14	6.11	6.09	6.04	5.99	
	1.5	6.05	6.03	5.96	5.86	5.79	
	3.0	5.94	5.86	5.72	5.61	5.27	
	4.5	5.88	5.75	5.36	4.08	3.91	
	6.0	5.76	5.57	4.95	3.82	2.52	
	7.5	5.65	5.41	3.99	3.06	-[a]	

[a]No survivor

NOTE: Calculated percentage values used for plotting 3D-histograms are shown in Appendix 3
Each recorded log_{10} value is a mean of 6 replicates.

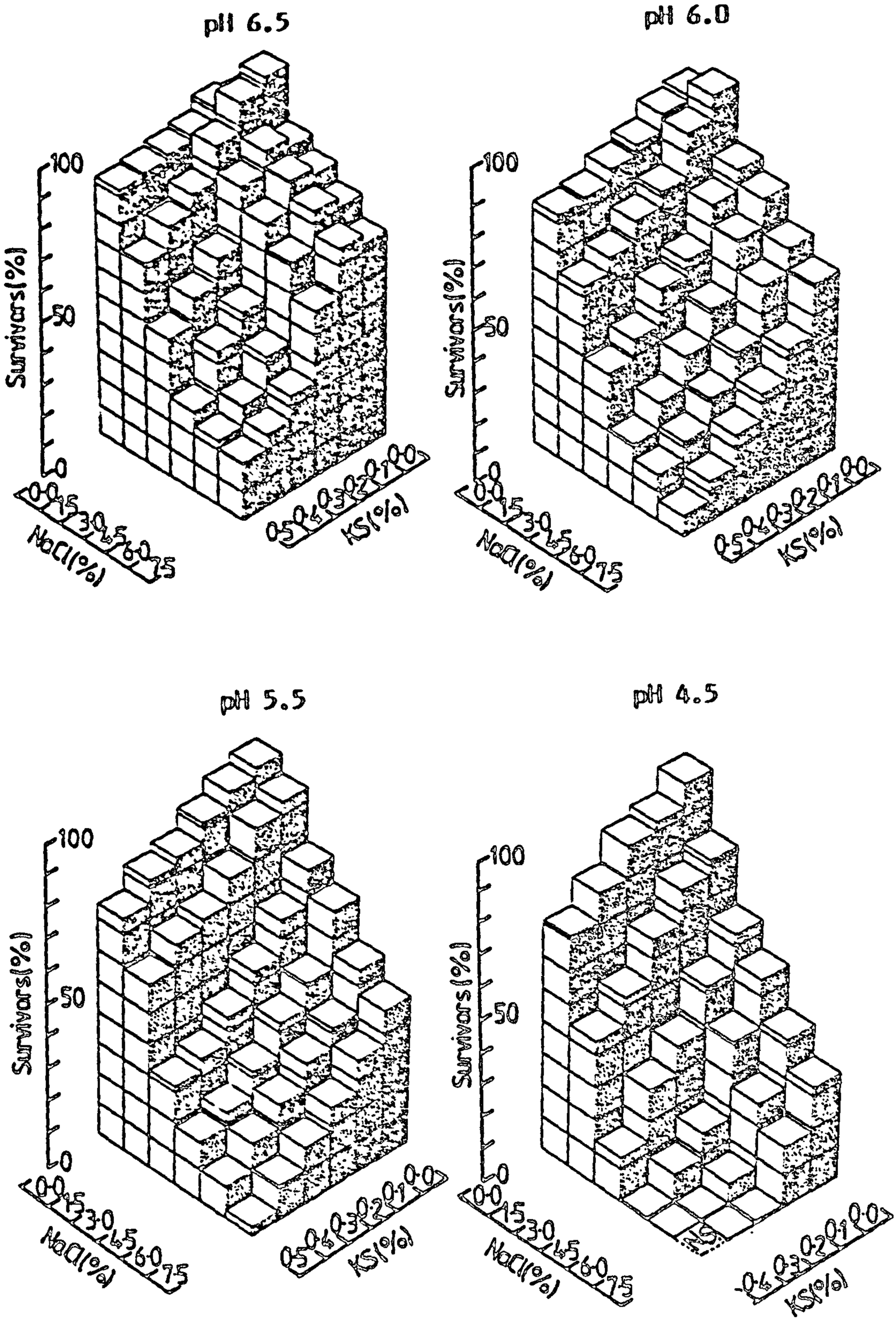

FIGURE 8. The effects of the combination of heat, potassium sorbate-KS, NaCl and pH on B. cereus (NCIB 6349) spores. Heated at 85°C for 30 min. N.S. (No survivor).

TABLE 12. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. cereus (NCIB 7464) spores.

pH	% (w/v) NaCl in heating medium	Log_{10} number of surviving spore counts recovered in YPID medium after heating at 85°C for 30 min					
		% w/v KS 0.0	0.10	0.20	0.30	0.40	0.50
6.5	0.0	6.31	6.30	6.28	6.27	6.25	6.23
	1.5	6.34	6.29	6.27	6.26	6.22	6.18
	3.0	6.29	6.27	6.23	6.15	6.11	6.07
	4.5	6.24	6.22	6.20	6.08	5.91	5.88
	6.0	6.22	6.21	6.15	5.92	5.72	5.65
	7.5	6.16	6.13	6.05	5.80	5.62	5.58
6.0	0.0	6.28	6.24	6.22	6.19	6.15	6.14
	1.5	6.27	6.25	6.20	6.18	6.14	6.13
	3.0	6.22	6.19	6.11	6.10	6.05	5.99
	4.5	6.19	6.14	6.01	5.96	5.94	5.89
	6.0	6.15	6.07	5.88	5.84	5.76	5.67
	7.5	6.11	6.02	5.73	5.61	5.57	5.49
5.5	0.0	6.25	6.20	6.16	6.13	6.09	6.07
	1.5	6.22	6.19	6.14	6.12	6.09	6.09
	3.0	6.16	6.15	6.05	5.98	5.96	5.89
	4.5	6.12	5.97	5.88	5.77	5.69	5.46
	6.0	6.09	5.91	5.67	5.53	5.46	5.00
	7.5	6.05	5.84	5.48	5.31	5.11	4.49
4.5	0.0	6.19	6.13	6.06	6.03	5.99	
	1.5	6.14	6.09	6.04	5.98	5.80	
	3.0	6.09	5.98	5.88	5.79	5.25	
	4.5	6.02	5.92	5.52	5.38	3.44	
	6.0	5.96	5.85	4.88	3.48	2.29	
	7.5	5.92	5.68	3.95	2.54	-[a]	

[a]No survivor NOTE: Calculated percentage values used for plotting 3D-histograms are shown in Appendix 3

Each recorded log_{10} value is a mean of 6 replicates.

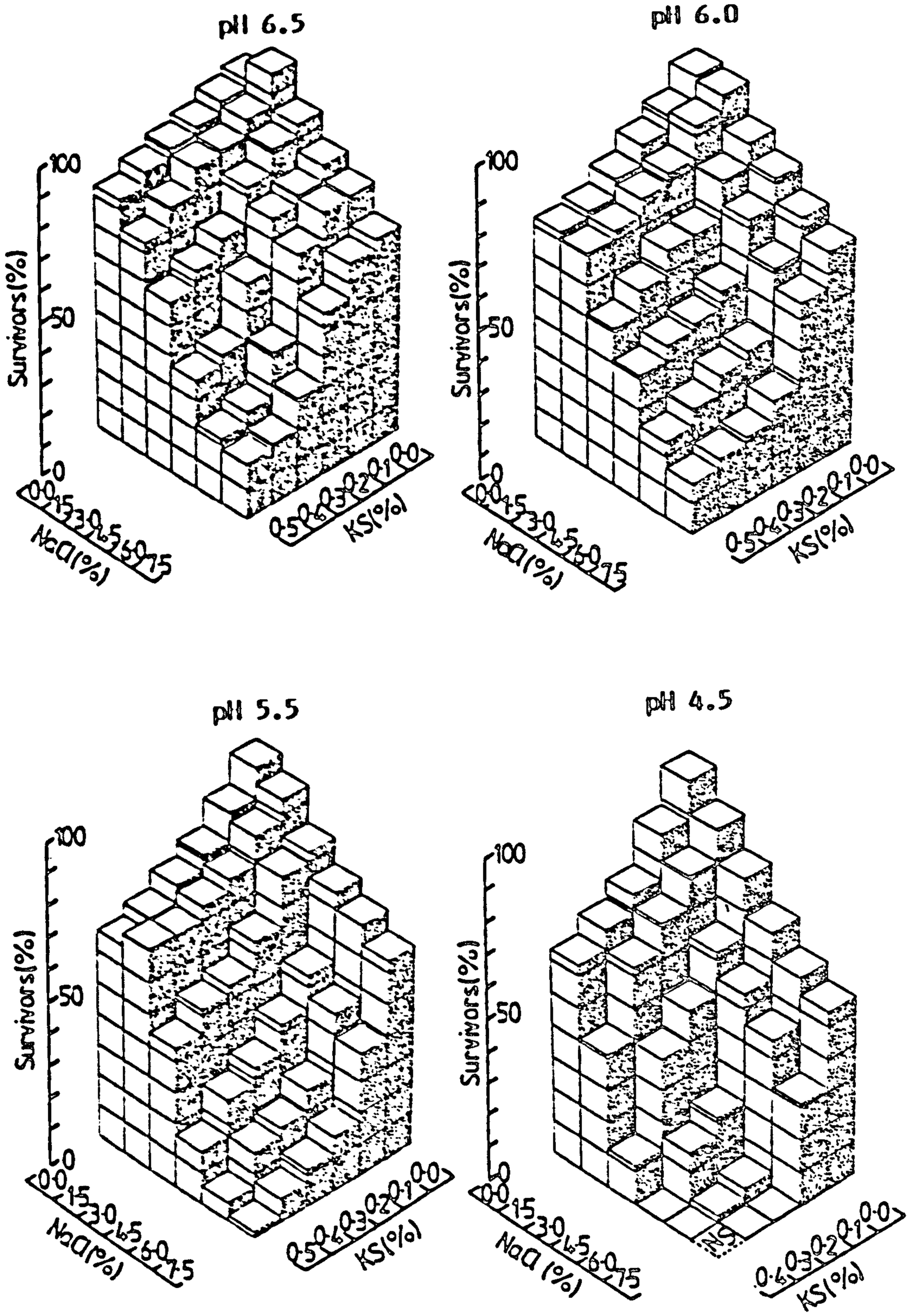

FIGURE 9. The effects of the combination of heat, potassium sorbate-KS and sodium chloride (NaCl) on B. cereus (NCIB 7464) spores. Heated at 85°C for 30 min. N.S. (No survivor).

TABLE 13. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. stearothermophilus (NCIB 8919) spores.

pH	% (w/v) NaCl in heating medium	Log_{10} number of surviving spore counts recoverd in YPID medium after heating at 95°C for 45 min					
		% (w/v) KS 0.0	0.2	0.4	0.6	0.8	1.0
6.5	0.0	6.13	6.14	6.12	6.10	6.06	6.05
	2.0	6.05	6.06	6.03	6.00	5.91	5.88
	4.0	6.02	6.02	5.97	5.91	5.85	5.85
	6.0	6.00	5.99	5.92	5.88	5.82	5.81
	8.0	5.97	5.96	5.91	5.87	5.81	5.79
	10.0	5.98	5.97	5.90	5.85	5.83	5.78
6.0	0.0	6.06	5.97	5.94	5.90	5.83	5.80
	2.0	5.93	5.82	5.73	5.62	5.52	5.43
	4.0	5.90	5.79	5.69	5.60	5.48	5.36
	6.0	5.88	5.76	5.66	5.54	5.42	5.35
	8.0	5.86	5.72	5.64	5.53	5.37	5.30
	10.0	5.87	5.71	5.62	5.51	5.38	5.28
5.2	0.0	5.91	5.77	5.71	5.65	5.53	5.45
	2.0	5.75	5.56	5.41	5.28	5.02	4.84
	4.0	5.71	5.51	5.38	5.24	4.96	4.79
	6.0	5.67	5.48	5.33	5.18	4.84	4.79
	8.0	5.68	5.46	5.30	5.12	4.80	4.76
	10.0	5.69	5.44	5.29	5.10	4.82	ND[a]
4.2	0.0	5.79	5.61	5.53	5.49	5.34	
	2.0	5.61	5.30	5.13	4.97	4.58	
	4.0	5.57	5.25	5.05	4.82	2.45	
	6.0	5.49	5.11	4.88	4.62	1.54	
	8.0	5.52	5.06	4.85	4.46	-[b]	
	10.0	5.54	5.01	4.78	ND[a]	-[b]	

[a]Not done [b]No survival NOTE: Calculated percentage values used for plotting 3D-histograms are shown in Appendix 3 Each recorded log_{10} value is a mean of 6 replicates.

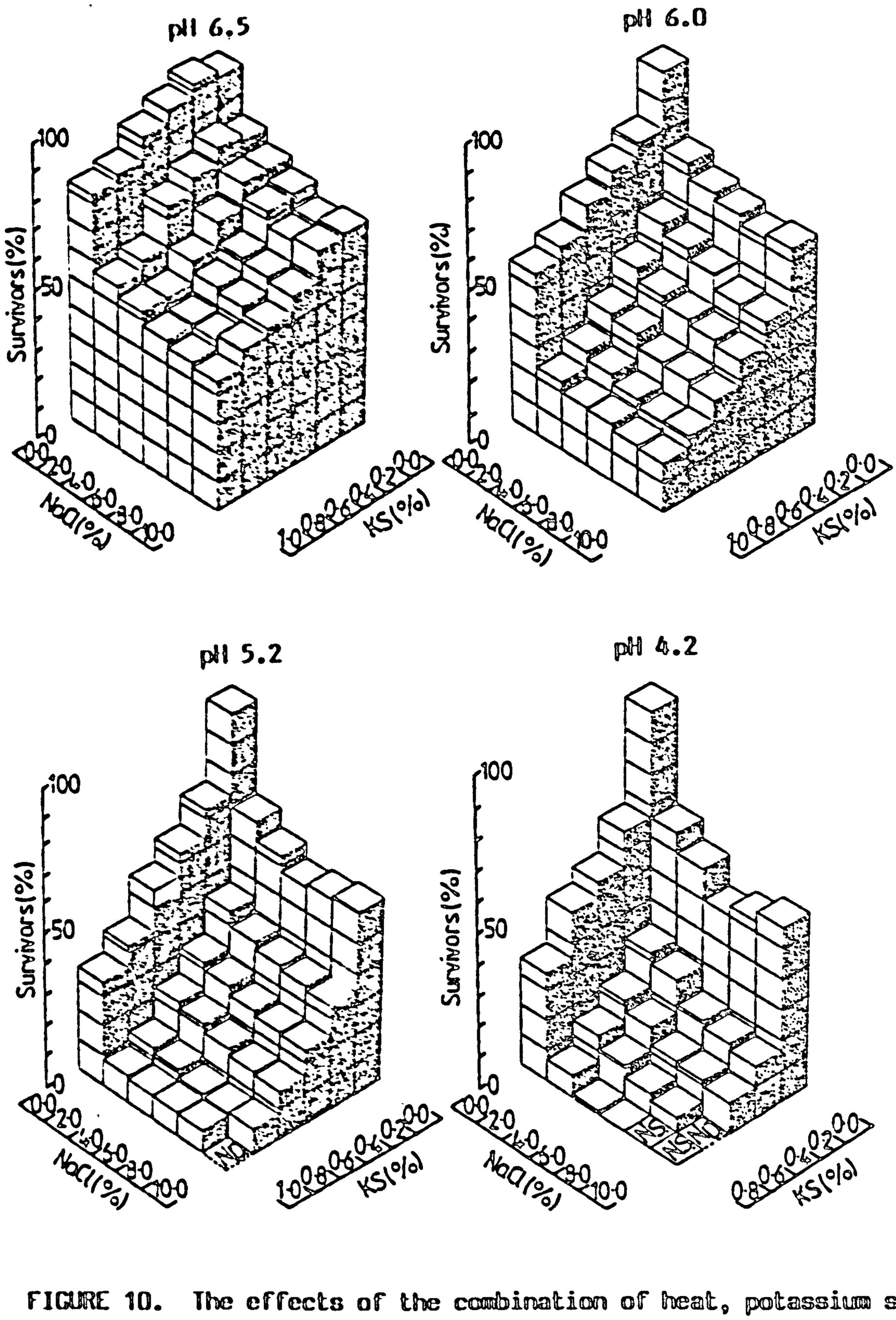

FIGURE 10. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. stearothermophilus (NCIB 8919) spores. Heated at 95°C for 45 min. N.S. (No survivor), N.D. (Not done).

TABLE 14. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. stearothermophilus (NCIB 8920) spores.

pH	% (w/v) NaCl in heating medium	Log_{10} number of surviving spore counts recoverd in YPTD medium after heating at 95°C for 45 min				
		% (w/v) KS 0.0	0.2	0.4	0.6	0.8
6.5	0.0	6.05	6.06	5.91	5.89	5.88
	2.0	6.07	5.81	5.72	5.66	5.56
	4.0	6.02	5.76	5.65	5.60	5.51
	6.0	5.85	5.69	5.59	5.49	5.41
	8.0	5.82	5.65	5.54	5.44	5.40
	10.0	5.79	5.62	5.53	5.43	5.37
6.0	0.0	6.00	5.83	5.82	5.79	5.73
	2.0	5.81	5.54	5.45	5.35	5.27
	4.0	5.78	5.47	5.36	5.23	5.11
	6.0	5.72	5.41	5.29	5.14	4.98
	8.0	5.69	5.32	5.23	5.09	4.90
	10.0	5.67	5.27	5.18	5.10	4.87
5.2	0.0	5.79	5.60	5.54	5.49	5.38
	2.0	5.59	5.14	4.89	4.76	4.48
	4.0	5.53	5.03	4.63	4.52	4.19
	6.0	5.49	4.86	4.56	4.22	3.82
	8.0	5.48	4.70	4.41	4.15	3.80
	10.0	5.46	4.52	4.36	4.14	3.79
4.2	0.0	5.72	5.51	5.45	5.37	5.12
	2.0	5.51	4.87	4.54	4.04	3.56
	4.0	5.44	4.64	4.37	3.60	1.51
	6.0	5.38	4.27	3.82	2.83	-[a]
	8.0	5.36	4.26	3.67	1.77	-
	10.0	5.35	4.25	3.63	ND[b]	-

[a]No survivor [b]Not done NOTE: Calculated percentage values used for plotting 3D-histograms are shown in Appendix 3 Each recorded log_{10} value is a mean of 6 replicates.

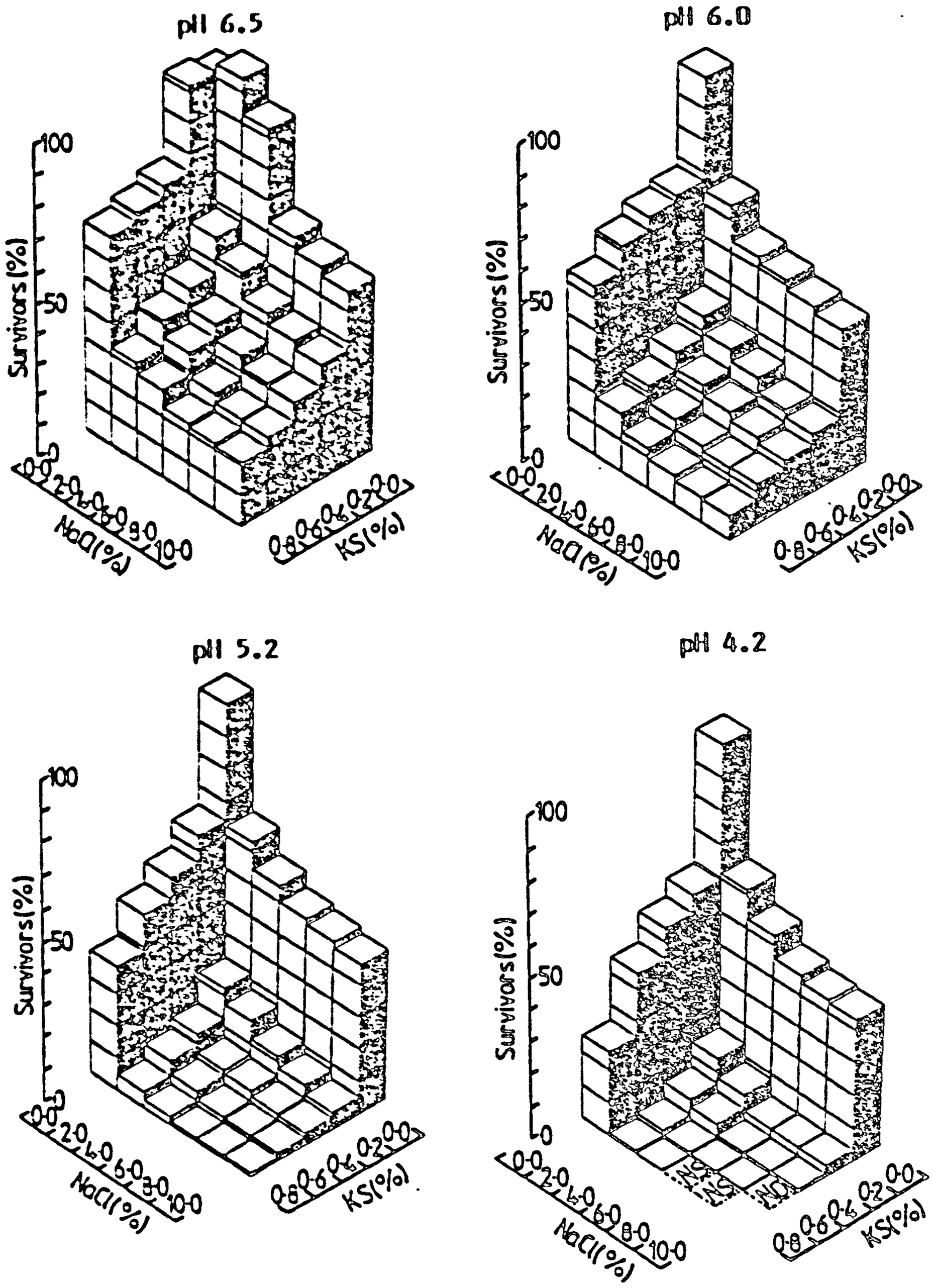

FIGURE 11. The effects of the combination of heat, potassium sorbate, sodium chloride (NaCl) and pH on B. stearothermophilus (NCIB 8920) spores. Heated at 95°C for 45 min. N.S. (No survivor), N.D. (Not done).

In this series of experiments it was found that the control sample which did not contain sorbate and NaCl provided results which showed that viable counts decreased with decrease in pH of the heating medium (Tables 9 - 14). The sensitivity of the test cultures to the combinations treatments increased with decrease in pH and increase in potassium sorbate level. The exposure of S. aureus to a combination of heat at 55°C for 15 min with 0.2% sorbate at pH 5.5 caused 99.98% inhibition whereas under the same conditions at pH 7.0 the inhibition was 26.4% (Figure 6). In studies by Elliot et al. (1982) and Eklund (1983) a combination of potassium sorbate and pH was found inhibitory to S. aureus. However, in these studies heating was not involved. The response of E. coli to the combination treatment was found to be similar to S. aureus. A decrease in pH from 7.0 to 5.5 as part of a combination treatment involving heat at 50°C for 15 min and 0.2% sorbate increased inhibition of E. coli from 16.2 to 99.8% (Figure 7). It is therefore evident from this experiment that reduction of the test system pH with citrate - phosphate buffer enhanced the antimicrobial effectiveness of the chemicals. This agrees with an earlier study by Restaino et al. (1981) who found that citrate and phosphate potentiated the antimicrobial activity of potassium sorbate. Furthermore, the results confirm the view of many workers (Huhtanen and Feinberg, 1980; Sofos et al., 1980; Eklund, 1983) that the inhibitory effect of potassium sorbate is increased at low pH.

Increase in potassium sorbate levels as part of the combination treatments of heat, potassium sorbate and pH led to increased inhibition. An increase in sorbate level from 0.05 to 0.25% as part of the combination treatment involving heat at 50°C for 15 min at

pH 5.5 increased inhibition of E. coli from 22.5% to 99.99% (Figure 7). This observation is supported by the findings of Shibasaki (1973) and Lusher et al. (1984) who indicated that increasing concentrations of potassium sorbate increased inhibition of E. coli. However, these studies did not examine pH effects.

While the present study demonstrated that other combinations also proved to be inhibitory to E. coli the most effective test systems comprised heating at 50°C for 15 min in a medium of pH 5.5 with 0.25% potassium sorbate. A similar pattern of inhibition was also found for S. aureus. A combination of heat at 55°C for 15 min in the presence of 0.05% sorbate and pH 5.5 provided inhibition of 49.6% and with increase of sorbate to 0.2% an inhibition of 99.98% was obtained (Figure 6). The latter combination treatment was found to be the most inhibitory towards S. aureus in the present study. Potassium sorbate has also been found to be inhibitory to S. aureus in other studies by Tompkin et al. (1974), Robach and Ivey (1978), Cunningham (1979), Pierson et al. (1979) and To and Robach (1980). However, the present study has for the first time assessed the effects of the combination of heat, sorbate and pH on S. aureus.

The results of the effects of the combination of heat, potassium sorbate and pH on bacterial spores are presented together with the effects of other combination treatments. These are shown in Figures 8 - 11 for B. cereus (NCIB 6349), B. cereus (NCIB 7464), B. stearothermophilus (NCIB 8919) and B. stearothermophilus (NCIB 8920) respectively.

As observed for S. aureus and E. coli the inhibitory effectiveness of the combination treatments on bacterial spores was similarly found to be dependent on pH and the concentration of potassium

sorbate. However, the inhibition was not equally pronounced for all strains of Bacillus spores. A combination of heat at 85°C for 30 min in the presence of 0.4% sorbate at pH 6.5 caused an inhibition of 6.6% of B. cereus (NCIB 6349; Figure 8) whereas with the same conditions at pH 4.5 an inhibition of 29% occurred. Similarly for B. cereus (NCIB 7464, Figure 9), the same heat treatment at pH 6.5 with 0.4% sorbate caused an inhibition of 12.2% while at pH 4.5 the inhibition increased to 37%. Increase in inhibition due to reduction in pH has also been demonstrated by Smoot and Pierson (1981) who reported that 0.39% sorbate at pH 5.7 caused 97% inhibition of B. cereus I germination whereas at higher pH levels very low inhibition was obtained. However, the extent of inhibition was found to be lower in the present study which may be due to differences in test strains. Other combinations as shown in Figures 8 and 9 also caused some inhibitory effect but none was appreciable.

Although the increase in potassium sorbate concentration as part of the combination treatment increased inhibition of B. cereus (NCIB 6349, 7464) spores the inhibition was not appreciable. A combination of heat at 85°C for 30 min with low pH 4.5 in the presence of 0.1% sorbate gave 7.2% inhibition of B. cereus while increasing the sorbate concentration to 0.4%, increased inhibition to 29% (Figures 8 and 9).

The sensitivity of B. stearothermophilus (NCIB 8919, 8920) to combined treatments was also found to be dependent on pH and the concentration of potassium sorbate (Figures 10 and 11). Lowering the pH and increasing the concentration of potassium sorbate increased the inhibitory effect of the combination treatments. An exposure of B. stearothermophilus (NCIB 8919) to a combination of

heat at 95°C for 45 min with 0.8% potassium sorbate at pH 6.5 caused an inhibition of 14.2% whereas with a decrease of the pH to 4.2, the inhibition increased to 64.7% (Figure 10). Similarly for B. stearothermophilus (NCIB 8920; Figure 11) spores the same heat treatment at pH 6.5 gave 32.1% inhibition and at pH 4.2 inhibition was 74.6%. In a related study Smoot and Pierson (1981) also showed that 0.52% sorbate at pH 5.7 caused 95% inhibition of Cl. botulinum 62A spore germination while an increase in pH reduced the inhibitory effect significantly. This was achieved after heat activation at 80°C for 60 min.

3.3 GENERATION OF THE 3D BLOCK HISTOGRAMS

The computer program required to generate a 3D block histogram is summarized in Figure 12. The program CREATE interactively requests data which is then filed. The program BLOCKS takes this data file and generates the 3D structure required by BIBLE. This program takes a 3D geometry and allows the user to produce views of the artifact from a desired eye position.

PROGRAM - CREATE

The dialogue for creating a file is as follows:

NAME OF DATA FILE? > test. dat (give a name to each test system e.g. BS 5. dat).

INPUT M AND N? > e.g. 6, 6(i.e. M represents the various concentrations of KS and N the concentrations of NaCl).

INPUT SIZE OF BLOCKS? > e.g. 10 (this represents the percentage survivor axis).

INPUT DISTANCE BETWEEN BLOCKS? > 10 (this respresents the dimensions one wants for the various concentrations of the KS and NaCl or other

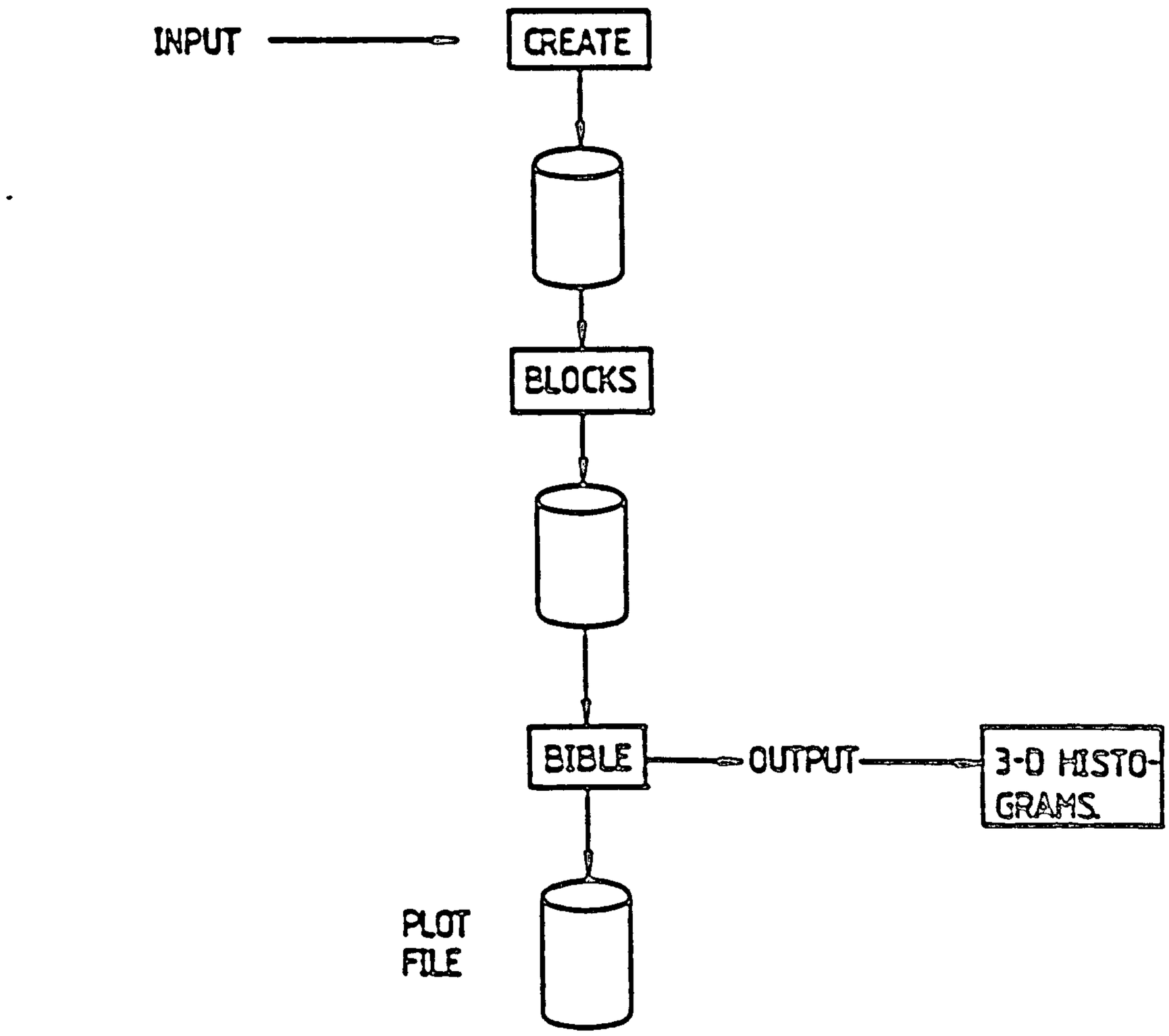

FIGURE 12. Programs for generating 3D-histograms.

chemicals).

INPUT VALUES FROM ROW 1? (the first set of percentage survivors is entered).

INPUT VALUES FROM ROW 2? (the second set of percentage survivors is entered).

INPUT VALUES FROM ROW 3? (the third set of percentage survivors is entered).

The dialogue continues until all the values have been filed. The listings of the computer program to create a data is as shown in Appendix 4.

PROGRAM BLOCKS

The program BLOCKS reads the data file and processes it into a 3D BIBLE format. The dialogue is as follows:

NAME OF INPUT DATA FILE? test. dat (i.e. the name of the file given earlier e.g. BS 5. DAT).

NAME OF BIBLE FILE? > test. bib (i.e. name of the file given earlier e.g. BS 5. BIB).

The BIBLE file is then passed into the program BIBLE to be displayed as required. The listings of the program to create BLOCKS is as shown in Appendix 4.

PROGRAM BIBLE

The listings of the program to create BIBLE data is as shown in Appendix 4.

The program was run on one of the Strathclyde University VAX B computers.

4. THE EFFECTS OF THE COMBINATION OF HEAT, SODIUM CHLORIDE AND pH ON BACTERIAL SPORES

Sodium chloride is one of the most widely used food additives and is often present in appreciable amounts in canned foods. The microbial spoilage of most canned foods is determined by the content of bacterial spores (Jakobsen and Murrell, 1977). In view of the importance of bacterial spores as potential spoilage organisms in canned foods several studies have been undertaken to examine the effect of sodium chloride on heat resistance of spores. The results of these studies have been conflicting. The studies by Viljoen (1926), Headlee (1931), Duncan and Foster (1968a), Harnulv and Snygg (1972) and Bühlmann et al. (1973) have shown that spores exhibited an increased heat resistance with increasing salt concentrations. By contrast a study by Briggs and Yazdany (1970) showed that increasing NaCl concentration (2, 4 and 8% w/v) in the heating medium decreased the heat resistance of spores of B. stearothermophilus. Similar studies by Anderson et al. (1949), Cook and Gilbert (1969), Fields (1970) and Bell and DeLacy (1984) also revealed a decrease in heat resistance with increasing sodium chloride concentrations. Other studies by Busta and Ordal (1964a) and Roberts et al. (1966) showed that spores were unaffected by the presence of sodium chloride in the heating medium.

Interestingly, some of the results of these studies were obtained using spores in phosphate buffer at pH 7.0 or were unbuffered. In practice, the pH of canned foods is usually less than 7.0 (Braithwaite and Perigo, 1971) and therefore such results may not reveal the exact response of spores to sodium chloride and heat

particularly in low-acid and acid canned foods. Because of the significance of pH in food preservation (Townsend et al. 1954; Braithwaite and Perigo, 1971; Steinbuch, 1974) it was considered that the effects of the combination of heat, sodium chloride and pH on bacterial spores should be studied.

Materials and methods

Test cultures: B. cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920).

Heat treatment: Heating of the cultures was carried out using approximately 10^7 spores/ml at the following temperatures: B. cereus spores at 85°C for 30 min and B. stearothermophilus spores at 95°C for 45 min. Other procedures are as described in Section 3, p. 50.

Treatment of results

The recoveries of the treated spores are presented as part of 3D-histograms as described in Section 3.2, p. 62.

Results

The recoveries of bacterial spores after exposure to the combination of heat, sodium chloride and pH are presented as part of 3D-histograms. These are shown in Figures 8 - 11 (p. 68, 70, 72, 74) for B. cereus (NCIB 6349), (NCIB 7464), B. stearothermophilus (NCIB 8919) and (NCIB 8920) respectively.

Results showed that the inhibitory effect of the combined treatments is dependent on the pH and NaCl levels. In general as the test pH decreased the inhibitory effect of the combined treatments increased. When B. cereus (NCIB 6349) was heated at 85°C for 30 min in the presence of 7.5% NaCl and at pH 6.5, an inhibition of 27.1% was obtained. A decrease of pH to 4.5 increased the inhibition to 67.4% (Figure 8, p. 68). A similar pattern of response was

observed for B. cereus (NCIB 7464) although less inhibition 45.4% than for strain 6349 was indicated when exposed to a combination of heat at 85°C for 30 min with 7.5% NaCl at pH 4.5 (Figure 9, p. 70). At the other pH tested and other NaCl levels, the same trend occurred but lesser inhibition for both strains (Figures 8 and 9, p. 68 and 70).

Interestingly, increasing NaCl levels at a particular pH provided an increase in the inhibitory effect of the combined treatments. A combination of heat at 85°C for 30 min in the presence of 1.5% NaCl and at pH 4.5 caused 18.1% inhibition of B. cereus 6349 spores. An increase in the NaCl level of the combination treatment to 7.5% provided 67.4% inhibition. The same trend was also demonstrated for B. cereus (NCIB 7464). On heating strain 7464 at 85°C for 30 min in the presence of 1.5% NaCl and at pH 4.5 an inhibition of 9.7% was obtained whereas increasing the NaCl level to 7.5% gave an inhibition of 45.4%. Similarly other NaCl levels as part of the combined treatment and at a particular pH also caused inhibition.

A slight protective effect by NaCl as part of the combined treatment was observed when the concentration was 1.5% and at pH 6.5. This effect was demonstrated in strains 6349 and 7464 spores.

The results of this experiment also showed that the sensitivity of B. stearothermophilus spores to the combined treatment progressively increased with decrease in pH and increase in NaCl levels. At pH 6.5 treatment at 95°C for 45 min in the presence of 8% NaCl caused 31.1% inhibition of strain 8919. Decreasing the pH to 4.2 under the same conditions increased the inhibition to 46.8% (Figure 10, p. 72). The response of strain 8920 to the combined treatments followed the same pattern as strain 8919 (Figure 11, p. 74).

It is noteworthy that with 2% NaCl and at pH 6.5 the combined

treatment conferred protection to all test strains examined except 8919 spores.

Although inhibition of all test strains by the combination of heat, NaCl and pH was observed the most effective caused a moderate inhibition. These treatments are: for B. cereus 6349, a combination of heat at 85°C for 30 min in the presence of 7.5% NaCl and at pH 4.5 caused 67.4% inhibition (Figure 8, p. 68). The same treatment provided 45.4% inhibition of strain 7464 (Figure 9, p. 70). For B. stearothermophilus 8919, a combination of heat at 95°C for 45 min with 8% NaCl and at pH 4.2 gave 46.8% inhibition (Figure 10, p. 72) whereas same treatment caused 56% inhibition of strain 8920 (Figure 11, p. 74).

Discussion

The present experiment demonstrates that the inhibitory effect of the combinations of heat, sodium chloride and pH is dependent on the pH of the medium. Earlier studies by Coussins (1968) and Braithwaite and Perigo (1971) have shown similar effect. However, the present results also showed that irrespective of the test strain inhibition by a combined treatment was most effective at acidic pH values. A related study by Braithwaite and Perigo (1971) agrees with this finding. They found that at acidic pH mild heat treatment with sodium chloride inactivated spores of Bacillus species including B. stearothermophilus and B. cereus. Similarly the studies by Cook and Gilbert (1969) and Briggs and Yazdany (1970) support the present results that increasing NaCl level increased the inhibitory effect of combined treatments, although their studies did not involve variation in pH. The protective effect of 2% or less NaCl at pH 6.5 on the heat injury of the test strains confirmed an earlier

observation by Duncan and Foster (1968a). However, in the present study this protective effect was not observed for B. stearothermophilus (NCIB 8919). It is evident from the present results that both pH and NaCl contribute to the inhibitory effect of the combined treatments. It is suggested that the mild heat sensitizes the spores to the inhibitory effects of NaCl and low pH and this in turn interferes with the germination mechanism of the spores. This would then affect the number of spores that outgrow after treatment. Such a suggested sensitization effect has also been demonstrated by Jarvis et al. (1976).

Although inhibition of B. cereus and B. stearothermophilus spores is only moderate even for the most effective combinations the results suggest that both NaCl and pH may play an important role in controlling thermophilic and mesophilic spoilage in pasteurized canned foods of low acidity. Such control is however very limited as results reveal and cannot be relied on for practical purposes.

5. THE EFFECTS OF THE COMBINATION OF HEAT, POTASSIUM SORBATE, SODIUM CHLORIDE AND pH ON BACTERIAL SPORES

Potassium sorbate - sodium nitrite combination is reportedly effective in inhibiting Cl. botulinum spore germination (Sofos et al. 1979a) and in delaying toxin formation by the same spore in canned comminuted pork (Ivey and Robach, 1978). Earlier studies by Gooding et al. (1955), Sheneman and Costilow (1955) and Costilow et al. (1956; 1957) also showed that sorbates act synergistically with salts. More recently, LaRocco and Martin (1981) found that 3% NaCl in conjunction with 0.3% potassium sorbate significantly inhibited the growth of S. typhimurium.

In view of the moderate inhibitory effect on spores of test strains by the combination treatments of heat, potassium sorbate and pH on one hand and heat, sodium chloride and pH on the other, the present experiment was designed to investigate the effects of the combination of heat, potassium sorbate, sodium chloride and pH on spores.

Materials and methods

Test cultures: B. cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920) spores.

Heat treatment: Heating of the cultures was carried out using approximately 10^7 spores/ml at the following temperatures: B. cereus at 85°C for 30 min and B. stearothermophilus spores at 95°C for 45 min. Other procedure as described in Section 3, p. 50.

Treatment of results

Survivors are presented as part of 3D-histograms as explained in Section 3.2, p. 62.

Results

The response of test strains to the combination of heat, potassium sorbate, sodium chloride and pH are shown in Figures 8 - 11 (p. 68, 70, 72, 74) for B. cereus (NCIB 6349), (NCIB 7464), B. stearothermophilus (NCIB 8919) and 8920 respectively. Results show that in general inhibition by the combined treatments is dependent on the pH, sorbate and sodium chloride levels.

As the pH of test system decreased inhibition of strains by the combined treatments increased. A combination of heat at 85°C for 30 min in the presence of 0.4% sorbate and 6% NaCl at pH 6.5 caused 69.6% inhibition of B. cereus 6349. A decrease of the pH of the test system to 4.5 increased the inhibition to 99.98% (Figure 8, p. 68). The same treatment caused the same pattern of inhibition for B. cereus (NCIB 7464) spores (Figure 9, p. 70).

The effect of a decrease in pH as part of combination treatment was also demonstrated for B. stearothermophilus (NCIB 8919) spores. A combination of heat at 95°C for 45 min in the presence of 0.8% sorbate and 8% NaCl at pH 6.5 caused 51.8% inhibition. A decrease in pH of the system to 4.2 increased the inhibition to 100% (Figure 10, p. 72). The same trend of results was obtained with B. stearothermophilus (NCIB 8920) spores. (Figure 11, p. 74).

Increasing the sorbate level as part of the combined treatments also increased inhibition. For B. cereus (NCIB 6349) a combination of heat at 85°C for 30 min in the presence of 6% NaCl and 0.1% sorbate at pH 4.5 gave 73.2% inhibition. An increase of the sorbate level to 0.4% increased the inhibition to 99.98% (Figure 8, p. 68). Similarly for B. stearothermophilus (NCIB 8919) a combination of heat at 95°C for 45 min with 8% NaCl and 0.2% sorbate at pH 4.2 provided

81.4% inhibition whereas an increase to 0.8% sorbate gave 100% inhibition (Figure 10, p. 72). Other strains also showed similar responses.

At any given pH increasing NaCl levels as component of the combined treatment increased inhibition. The results showed that with B. cereus (NCIB 6349) spores a combination of heat at 85°C for 30 min in the presence of 0.4% sorbate and 1.5% NaCl at pH 4.5 caused 55.8% inhibition. An increase in the NaCl level of the combined treatment to 6% increased the inhibition to 99.98% (Figure 8, p. 68). A similar effect with B. stearothermophilus (NCIB 8919) spores showed that a combination of heat at 95°C for 45 min with 0.8% sorbate and 2% NaCl provided 93.1% inhibition. Increase in the NaCl level of the combination system to 8% increased the inhibition to 100% (Figure 10, p. 72). Other strains also showed similar trend of response.

Significantly, the results of this study demonstrate that a combination of heat, sorbate, sodium chloride and pH caused synergistic inhibitory effects with the test strains. For B. cereus (NCIB 6349) spores a combination of heat at 85°C for 30 min in the presence of 0.4% sorbate at pH 4.5 provided 29% inhibition whereas the same combination with 6% NaCl instead of sorbate gave 58% inhibition. A combination of the treatment in the presence of the same concentrations of sorbate and NaCl caused 99.98% inhibition (Figure 8, p. 68). A similar synergistic effect was shown with B. stearothermophilus (NCIB 8919) spores. Heating at 95°C for 45 min with 0.8% sorbate at pH 4.2 provided 64.7% inhibition, the same treatment with 8% NaCl instead of sorbate gave 46.8% inhibition. A combination of the two systems increased the inhibition to 100% (Figure 10, p. 72). The

synergistic effect was also demonstrated at pH 4.5 for B. cereus (NCIB 7464) and at pH 4.2 for B. stearothermophilus (NCIB 8920) spores (Figures 9 and 11 respectively).

Furthermore, results showed that the most effective combination that caused total (100%) inhibition of the test strains were (i) for B. cereus (NCIB 6349, 7464), a combination of heat at 85°C for 30 min in the presence of 0.4% sorbate and 7.5% NaCl at pH 4.5 (ii) for B. stearothermophilus (NCIB 8919) this involved a combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2 while same treatment but 6% NaCl caused the effect with strain 8920.

Discussion

The results of this experiment clearly demonstrate that at pH 4.2 - 6.5 increasing NaCl levels potentiated the inhibitory effect of the combinations of mild heat and sorbate on all test strains (Figures 8 - 11). In a comparative study by Robach (1980b) it was shown that the inhibitory effectiveness of sorbate on outgrowth of Cl. sporogenes was similarly enhanced by increasing levels of NaCl at pH 6. In addition it was suggested by this worker that the inhibitory effect may be due to an interaction between sorbate and NaCl. This suggestion may partly account for the inhibition of the bacterial spores by the combined treatments in the present study. Moreover, the synergistic effects of the combination of sorbate,NaCl, pH and mild temperature on the spores lend support to this suggestion. Hence, to achieve inhibition of the spores by the combination treatments it is viewed that the mild heat sensitizes the bacterial spores to sorbate - sodium chloride interaction. This interferes with the germination mechanism and consequently reduces the number of spores which outgrows eventually. The extent of interference of the

germination mechanism by the sorbate - NaCl interaction is enhanced as the pH decreased.

Results obtained from the present experiment are of practical significance. The results showed that for a complete inhibition of B. stearothermophilus (NCIB 8919) spores a combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2 was required. The temperature used in the present experiment is well below the range 115° - 150°C recommended for the commercial sterilization of canned foods (Olson and Nottingham, 1980). Therefore, with the application of the combined treatments the severe heat processes commonly applied to foods destined for markets in tropical climates and subject to the hazard of thermophilic bacteria growth may be reduced. The use of the combined treatments would enable inhibition of B. stearothermophilus spores in low acid canned foods such as peas, corn and potato where they are the major spoilage organisms (Fields, 1970). However, this proposal would require further experimental study since laboratory studies do not necessarily represent a practical food processing situation.

6. THE EFFECTS OF THE COMBINATION OF HEAT, POTASSIUM SORBATE, SODIUM BENZOATE AND pH ON BACTERIAL SPORES

Most review articles on the antimicrobial activity of benzoates have shown that as with sorbates, benzoates have a primary effect on yeasts and moulds (Baird - Parker, 1980; Lueck, 1980; Chipley, 1983). However, Chipley (1983) also reported that food poisoning and spore forming bacteria are inhibited by 0.01 to 0.02% undissociated benzoic acid. An earlier study by Eklund (1980) showed that benzoates have an inhibitory effect on the growth of E. coli, B. subtilis and Pseudomonas aeruginosa. In addition a study by Gould (1964) indicated that at least 0.04% sodium benzoate at pH 6 was required for the inhibition of outgrowth of spores of certain Bacillus species.

More recently, Webster and Cooke (1984) found that a combination of sodium benzoate with sodium citrate was inhibitory to the growth of S. aureus. Synergistic effects of combinations of benzoates with SO_2, CO_2, NaCl or sucrose were reported by Chipley (1983). A study by Webster, Fowler and Cooke (1985) showed that a combination of sodium benzoate with sodium citrate enhanced the inhibitory effects of reduced water activity (a_w) and pH on a range of common food spoilage and poisoning organisms.

Furthermore, it has been shown by Beuchat (1980) that the addition of 0.03% sodium benzoate to a laboratory medium enhanced the rate of thermal inactivation of Vibrio parahaemolyticus. Lueck (1980) found that the combination of sodium benzoate with potassium sorbate was more inhibitory to acid producing bacteria than the two components individually. In a related study Beuchat (1981) showed that potassium sorbate and sodium benzoate acted synergistically with

heat to inactivate six strains of food spoilage yeasts suspended in laboratory media. However, there are no reports in the literature concerning the exposure of bacterial spores to heat in the presence of combination of potassium sorbate and sodium benzoate at different pH levels. The present study was designed to investigate specifically these aspects.

Materials and methods

Test cultures: B. stearothermophilus (NCIB 8919) and B. cereus (NCIB 6349).

Test systems: Results of the experiment Section 3.2, showed that for B. cereus (NCIB 6349) a combination of heat at 85°C for 30 min with pH (6.0, 5.5, 4.5) and sorbate (0.2, 0.4%) caused a marginal inhibition (7.7% to 29%). Also for B. stearothermophilus (NCIB 8919) a combination of heat at 95°C for 45 min with pH (6.0, 5.2, 42.) and sorbate (0.4, 0.8%) provided a marginal (24.3%) to a moderate inhibition (64.7%). These conditions were selected in this experiment in combination with varying levels of sodium benzoate as shown in Table 15. The pH was adjusted with citrate - phosphate buffer as described in Section 3 p. 50. The unbuffered system containing only sodium benzoate had pH 8.9 (Table 16). The test systems used for B. cereus are referred to as A, B, C and D and B. stearothermophilus as E, F, G and H (Table 15).

TABLE 15. Test system for studying the effects of the combination of heat, potassium sorbate-KS, sodium benzoate-SB and pH on bacterial spores.

System	pH	SB conc (%w/v)	KS conc (%w/v)	Heat treatment
A	7.1	0.0, 0.25, 0.50 0.75, 1.25, 1.75	,0,.0,2,.0.4	85°C for 30 min
B	6.0	"	"	"

continued....

System	pH	SB conc (%w/v)	KS conc (%w/v)	Heat Treatment
C	5.5	0.0, 0.25, 0.50 0.75, 1.25, 1.75	0, 0.2, 0.4	85°C for 30 min
D	4.5		"	"
E	7.1	0.0, 0.5, 1.0 1.5, 2.0, 2.5	0, 0.4, 0.8	95°C for 45 min
F	6.0	"	"	"
G	5.2	"	"	"
H	4.2	"	"	"

Heating menstrua and treatments and enumeration of survivors.

The preparation of the menstrua, heat treatments and enumeration techniques were as previously described in Section 3 p. 50. The heating was carried out using approximately 10^7 spores/ml.

Treatment of results

The recoveries of the treated B. cereus (Table 17) and B. stearothermophilus spores (Table 18) were compared by considering the mean of the viable counts in the presence of potassium sorbate, sodium benzoate or combination of both chemicals as percentage of the counts in the absence of the chemicals at each pH. The percentage survivors (Appendix 3) were then presented as 3D-histograms (see Section 3.2) as shown in Figures 13 and 14 for B. cereus and B. stearothermophilus respectively.

Results

The results of the preliminary experiment (Table 16) showed that unbuffered sodium benzoate pH 8.9 (0.25 - 1.75%) and (0.5 - 2.5%) in combination with heat had no effect on B. cereus and B. stearothermophilus spores respectively.

In the main experiment controls which did not contain sorbate and benzoate (Tables 17 and 18; p. 95 and 97) provided results which showed that viable counts decreased with the pH of the heating

TABLE 16. The effects of the combination of heat and sodium benzoate-SB on B. cereus (NCIB 6349) and B. stearothermophilus (NCIB 8919) spores.

B. cereus heated at 85°C for 30 min		B. stearothermophilus heated at 95°C for 45 min	
% (w/v) SB in heating medium	Log_{10} surviving spore counts	% (w/v) SB in heating medium	Log_{10} surviving spore counts
0.00	6.41 (100.0)[a]	0.0	6.36 (100.0)
0.25	6.40 (97.7)	0.5	6.37 (102.0)
0.50	6.43 (104.7)	1.0	6.39 (107.0)
0.75	6.42 (102.3)	1.5	6.34 (95.6)
1.25	6.45 (109.7)	2.0	6.38 (104.8)
1.75	6.43 (104.7)	2.5	6.35 (97.8)

[a]Surviving spores, calculated as a percentage of the control result

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 17. The effects of the combination of heat, potassium sorbate -KS, sodium benzoate-SB and pH on B. cereus (NCIB 6349) spores.

pH	% (w/v) SB in heating medium	Log_{10} surviving spore counts recovered in YPTD after heating at 85°C for 30 min		
		% (w/v) KS 0.00	0.20	0.40
7.1 (System A)	0.00	6.34	6.36	6.33
	0.25	6.34	6.37	6.32
	0.50	6.33	6.33	6.35
	0.75	6.33	6.35	6.31
	1.25	6.35	6.31	6.33
	1.75	6.34	6.36	6.34
6.0 (System B)	0.00	6.29	6.24	6.20
	0.25	6.27	6.26	6.23
	0.50	6.31	6.28	6.25
	0.75	6.32	6.28	6.26
	1.25	6.28	6.26	6.24
	1.75	6.26	6.25	6.23
5.5 (System C)	0.00	6.19	6.14	6.04
	0.25	6.17	6.15	5.99
	0.50	6.13	6.09	5.93
	0.75	6.11	6.05	5.90
	1.25	6.07	6.03	5.83
	1.75	6.09	5.99	5.73
4.5 (System D)	0.00	6.05	5.90	5.85
	0.25	6.00	5.80	5.69
	0.50	5.96	5.61	5.26
	0.75	5.92	5.34	3.81
	1.25	5.89	4.47	2.10
	1.75	5.88	3.97	-[a]

[a]No survivor

Each recorded log_{10} value is a mean of 6 replicates.

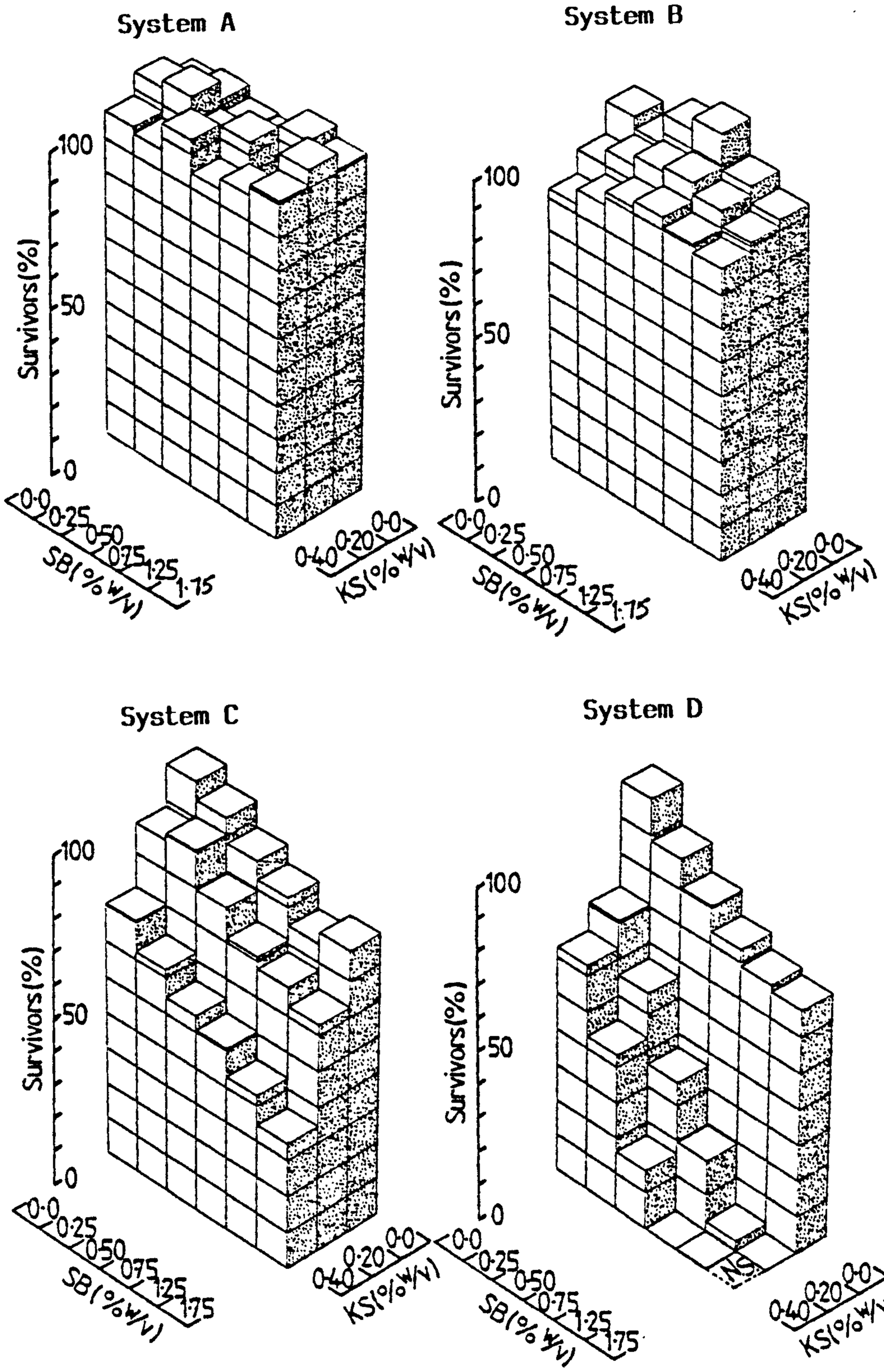

FIGURE 13. The effects of the combination of heat, sorbate, sodium benzoate and pH on B. cereus (NCIB 6349) spores. N.S. (No survivor).

TABLE 18. The effects of the combination of heat, potassium sorbate -KS, sodium benzoate-SB and pH on B. stearothermophilus (NCIB 8919) spores.

pH	% (w/v) SB in heating medium	Log_{10} surviving spore counts recovered in YPTD after heating at 95°C for 45 min		
		% (w/v) KS		
		0.0	0.40	0.80
7.1 System E	0.0	6.18	6.19	6.19
	0.5	6.18	6.20	6.19
	1.0	6.19	6.20	6.17
	1.5	6.21	6.19	6.18
	2.0	6.20	6.17	6.18
	2.5	6.22	6.20	6.17
6.0 System F	0.0	6.11	6.02	5.86
	0.5	6.12	6.04	5.91
	1.0	6.14	6.08	6.04
	1.5	6.11	6.09	6.01
	2.5	6.10	6.06	5.99
5.2 System G	0.0	6.05	5.78	5.59
	0.5	0.04	5.94	5.79
	1.0	6.02	5.91	5.72
	1.5	6.01	5.88	5.66
	2.5	5.97	5.83	5.56
4.2 System H	0.0	5.85	5.51	5.32
	0.5	5.76	5.37	4.39
	1.0	5.72	5.24	3.25
	1.5	5.66	5.04	2.93
	2.0	5.58	4.69	1.58
	2.5	5.54	4.70	-[a]
	3.0	5.47	4.32	-

[a]No survivor

Each recorded log_{10} value is a mean of 6 replicates.

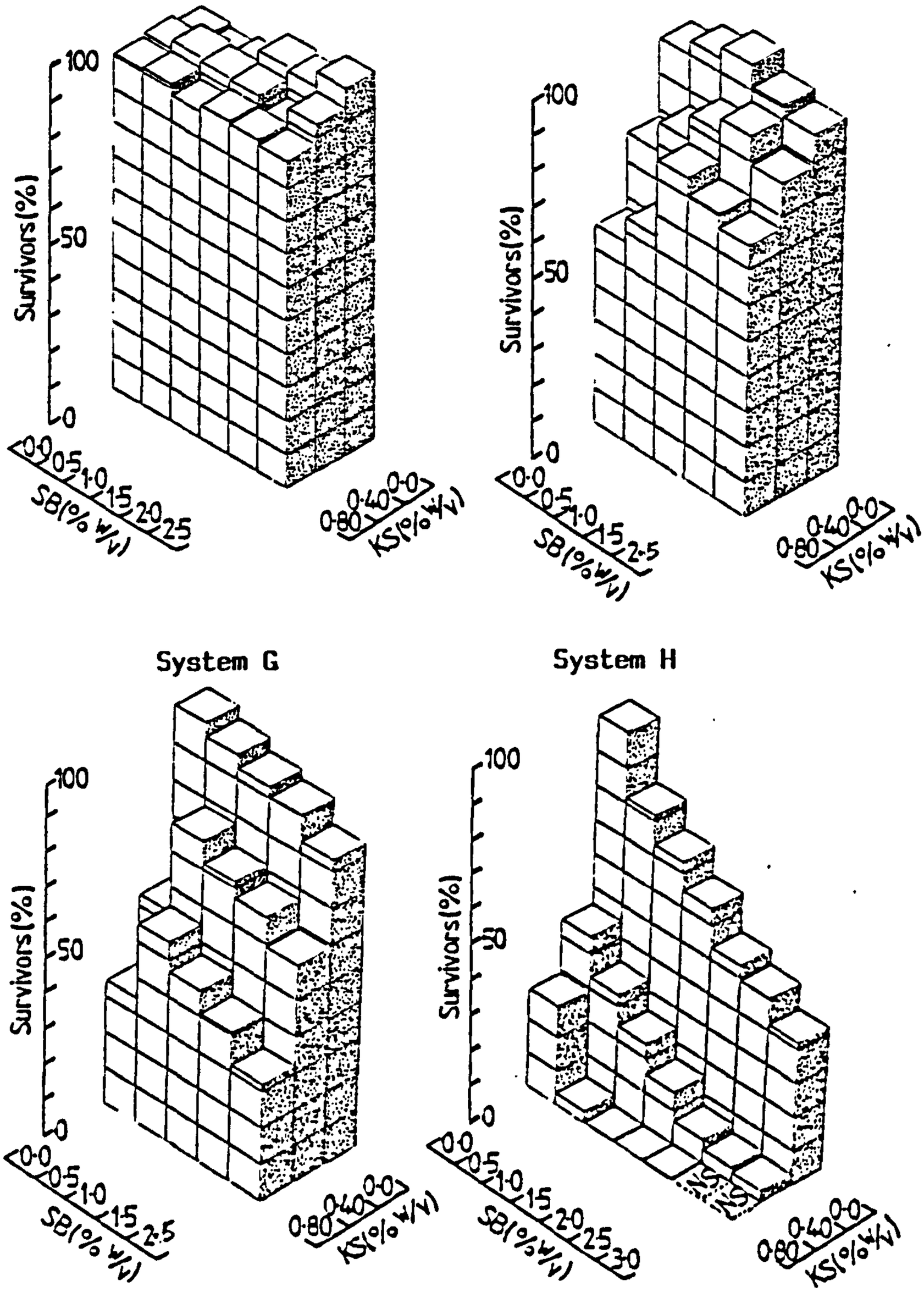

FIGURE 14. The effects of the combination of heat, sorbate, sodium benzoate and pH on B. stearothermophilus (NCIB 8919) spores. N.S. (No survivor).

medium. A similar effect was earlier shown in Section 3.2, p. 75.

With the exception of systems A (Figure 13) and E (Figure 14) the individual effects of sorbates in all systems showed that the viable counts were reduced with increasing concentration. The effect became more apparent at the lowest pH levels, 4.5 in system D for B. cereus and 4.2 in system H for B. stearothermophilus. In system D, the presence of 0.4% sorbate effected a marginal (37%) inhibition with B. cereus spores while in system H, 0.8% sorbate gave 70.8% inhibition with B. stearothermophilus. The present observation confirmed the earlier results in Section 3.2, p. 75, 68 and 72.

The individual effects of benzoate in systems C and D for B. cereus (Figure 13) and G and H for B. stearothermophilus (Figure 14) indicated that increasing benzoate concentrations led to further reductions in viable counts for these systems. In system D, 0.25% benzoate provided 11.5% inhibition while increase to 1.75% benzoate provided 32.7% inhibition. Also, for B. stearothermophilus, in system H, 0.5% benzoate gave 18.3% inhibition and increase to 2% benzoate caused 46.5% inhibition. In all the systems, the inhibition due to increasing benzoate concentrations was not well marked while in systems A and E the trend was uncertain.

Although combinations of sorbate and benzoate in systems B and C for B. cereus (Figure 13) and F and G for B. stearothermophilus (Figure 14) were found inhibitory to the spores, in most cases they were not as effective as sorbate alone. In system B (Figure 13) 0.4% sorbate alone provided 17.1% inhibition, 1.75% benzoate caused 6.7% inhibition, a combination of sorbate and benzoate gave 11.9% inhibition with B. cereus spores. Similarly system F (Figure 14) showed that 0.8% sorbate alone caused 43% inhibition, 2.5% benzoate gave 2.3% inhibition, a combination of the chemicals gave 23.4%

inhibition.

Significantly, in systems D (Figure 13) and H (Figure 14) combinations of sorbate and benzoate demonstrated synergistic effect with B. cereus and B. stearothermophilus respectively. In system D for B. cereus, 0.4% sorbate provided 37.2% inhibition, 0.75% benzoate gave 26.5% inhibition while combination of the chemicals caused 99.99% inhibition. A similar effect was shown with B. stearothermophilus. In system H (Figure 14), 0.8% sorbate gave 70.8% inhibition, 1% benzoate provided 26.8% inhibition while combination of the chemicals caused 99.75% inhibition. The combinations that were responsible for 100% inhibition of B. cereus involved heating at 85°C for 30 min with 0.4% sorbate and 1.75% benzoate in a medium of pH 4.5. For B. stearothermophilus this was found to be a combination of heat at 95°C for 45 min in the presence of 0.8% sorbate and 2.5% benzoate pH 4.2.

Discussion

In the present experiment it was found that the viable counts of the controls of the test systems decreased with decreasing pH. Similar effect was observed in Section 3.2, p. 72. Thus the present experiment confirms this earlier observation. Similarly the contribution of sorbate to the inhibition of the spores by the various test systems in this experiment was found to follow the same pattern as shown in Section 3.2, p. 72. The results demonstrated that, with the exception of system A for B. cereus and E for B. stearothermophilus, inhibition of the spores by the test systems increased with increasing sorbate levels and decreasing pH. These observations had earlier been explained in Section 3.2, p. 72. However, the lack of inhibition of the spores by heat and sorbate in

test systems A and E (pH 7.1) is in agreement with the findings of Yokoya and York (1965) who reported that 0.0112% sorbic acid at pH 7 had no effect on the heat response of B. coagulans spores. Furthermore, the pH of the heating medium 7.1 was higher than 6.5 which had been reported by Robach (1980a) and Sofos and Busta (1983) as the highest pH at which the antimicrobial effectiveness of sorbate is obtained.

The lack of inhibitory effect by the combination of heat and sodium benzoate on B. cereus and B. stearothermophilus as shown in the preliminary experiment may be associated with the pH of the unbuffered heating medium, 8.9. Related studies by Ralph (1967) and Boyazoglu (1969) similarly showed that addition of sodium benzoate had no effect on B. stearothermophilus spores. It has been reported by Ingram et al. (1956), Baird-Parker (1980), Robach (1980a) and Chipley (1983) that the antimicrobial effectivenesss of benzoates is primarily due to the undissociated acid which increases with reducing pH (Sofos and Busta, 1981). This may explain the lack of inhibition of the spores by the combination of heat and sorbates in a medium of pH 8.9. However, a reduction in pH of the test systems from pH 5.5 in system C to 4.5 in system D for B. cereus, 1.75% benzoate increased the inhibition from 21.8% to 32.7% (Figure 13). Similarly for B. stearothermophilus, 2% benzoate increased inhibition in system G, pH 5.2 from 16.8% to 46.5% in system H, pH 4.2. It is likely that the decrease in the pH increased the undissociated acid and therefore increased the inhibitory effectiveness of the combination of heat, sodium benzoate and pH. Although the inhibition of the spores by the combination of heat, sodium benzoate and pH was not appreciable, a similar effect has been demonstrated with

yeasts (Beuchat, 1981; 1982). It is proposed that the inhibition of the spores by the test systems may have been achieved as follows:- the heat sensitizes the spores which allows the undissociated benzoate to penetrate the spores which in turn affects the germination sites and consequently reduces the number of spores which outgrow after treatment. The amount of the undissociated acid molecule increases with a decrease in pH (Chipley, 1983).

It is interesting to note that synergistic effects of the combination of sorbate, sodium benzoate and low pH on heated B. cereus and B. stearothermophilus spores were demonstrated. The synergistic effects were demonstrated only at pH 4.2 and 4.5 for B. stearothermophilus and B. cereus respectively. This indicates that the undissociated acids do play an important role in the inhibition of the spores. A similar synergistic effect was demonstrated in a study involving yeasts (Beuchat, 1981). It is possible that inhibition of the spores by the combination of heat, benzoate and low pH follow the same mechanism as combination of heat, sorbate and pH. It is viewed that the heat sensitized the spores to the undissociated acids and these affected most germination sites of the spores which consequently reduced the number that outgrow. However, greater inhibition of the spores was observed with combination of heat, sorbate, benzoate and low pH compared with inhibition by a combination of heat, sorbate and pH. This may be due to the presence of the undissociated acids contributed by sorbate and benzoate in the former treatment compared with the latter which is as a result of only sorbate.

7. EFFECTS OF RECOVERY TEMPERATURES AND INCUBATION PERIODS ON SPORES EXPOSED TO COMBINED TREATMENTS

One of the factors that affects the recovery of bacterial spores after exposure to heat, chemicals or combination of both is the incubation temperature (Roberts, 1970; Prentice and Clegg, 1974; Adams, 1978; Foegeding and Busta, 1981). A study by Cook and Gilbert (1968) showed that unheated B. stearothermophilus (NCIB 8919) spores recovered at 50° to 65°C whereas spores heated at 115°C recovered maximally at 45° to 50°C. By contrast, Mallidis (1981) did not observe any significant difference in the recovery of B. stearothermophilus (NCIB 8923) at 50° and 60°C after subjection to heat at 120°C. Edwards, Busta and Speck (1965) also found that B. subtilis spores gave higher recoveries at 32°C than at 45°C after exposure to ultra-high temperatures. In a similar study by Prentice and Clegg (1974) it was found that B. subtilis (NCIB 8057) spores recovered maximally at 30°C after subjection to heat at 95°C. Poor recovery was obtained above 40°C whereas the recovery of unheated spores was similar at 16° to 48°C. Although heated B. cereus spores are often recovered at 30°C (Johnson, Nelson and Busta, 1983), 35°C has also been used (Parry and Gilbert, 1980; Smoot and Pierson, 1981).

For the recovery of B. stearothermophilus exposed to combined treatments a temperature of 56°C was used while B. megaterium, B. pantothenticus and B. subtilis were recovered at 37°C (Briggs, 1966). However, El-Mabsout and Stevenson (1979) used a temperature of 55°C for the recovery of B. stearothermophilus exposed to a combination of heat and pH treatment. This temperature was also used by Thorpe (1960) for the recovery of the spores after exposure to

a combination of heat and nisin treatments.

The following experiment was designed to investigate the influence of recovery temperature and incubation period on spores exposed to combined treatments.

Materials and methods

Test cultures: B. cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920) spores.

Test systems: In Section 5, p. 87 of this study results revealed that the following combined treatments provided an inhibition of nearly 100%.

(i) B. cereus (NCIB 6349): heating at 85°C for 30 min with 0.4% sorbate and 6% NaCl at pH 4.5 gave 99.98% inhibition.

(ii) B. cereus (NCIB 7464): heating at 85°C for 30 min with 0.4% sorbate and 6% NaCl at pH 4.5 provided 99.99% inhibition.

(iii) B. stearothermophilus (NCIB 8919): heating at 95°C for 45 min with 0.8% sorbate and 6% NaCl at pH 4.2 caused 99.85% inhibition.

(iv) B. stearothermophilus (NCIB 8920): heating at 95°C for 45 min with 0.6% sorbate and 6% NaCl at pH 4.2 gave 99.67% inhibition.

These combined treatments were selected for the present experiment. For each test the same levels of sorbate and NaCl were used while the pH was varied as shown in Table 19.

Heat treatment: The heat treatment was carried out using approximately 10^7 spores/ml in menstrua containing the varying concentrations of sorbate, NaCl and adjusted with citrate phosphate buffer to the appropriate pH level. The procedure is described in Section 3, p. 50.

Table 19. Test systems used for studying the influence of incubation temperatures and periods on spores exposed to combined treatments.

Strain	Test System	pH	% Sorbate	% NaCl	Heat Treatment
Bacillus	I	6.5	0.4	6	85°C for 30min
cereus	J	6.0	"	"	"
(NCIB 6349,	K	5.5	"	"	"
7464)	L	4.5	"	"	"
B. stearo-	M	6.5	0.8	"	95°C for 45min
thermo-	N	6.0	"	"	"
philus	O	5.2	"	"	"
(NCIB 8919)	P	4.2	"	"	"
B. stearo-	Q	6.5	0.6	"	"
thermo-	R	6.0	"	"	"
philus	S	5.2	"	"	"
(NCIB 8920)	T	4.2	"	"	"

Recovery: After exposure to the combined treatments, B. cereus and B. stearothermophilus spores were recovered using YPTD agar pour plates. Bacillus cereus was incubated at 25°, 30°, 37° and 44°C and B. stearothermophilus was incubated at 44°, 50°, 55° and 60°C. Survivors were enumerated after 2, 6, 10 and 15 days incubation.

Treatment of results

The recoveries of B. cereus (NCIB 6349; Table 20; p. 106), B. cereus (NCIB 7464; Table 21; p. 107), B. stearothermophilus (NCIB 8919; Table 22; p. 108) and B. stearothermophilus (NCIB 8920; Table 23; p. 109) after exposure to various test systems are presented as 3D-histograms. Figures 15 - 18 for B. cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920) respectively.

The 3D-histograms were obtained using the following steps:

(a) For each test system survivors obtained at day 15 at temperatures 30° and 55°C for B. cereus and B. stearothermophilus respectively were considered as the reference control (100%). Tempera-

TABLE 20. The effects of recovery temperatures and incubation periods on the counts of B. cereus (NCIB 6349) spores exposed to combined treatments.

Systems	Incubation temp °C	Log_{10} surviving spore counts recovered in YPTD at different incubation periods (days)			
		15	10	6	2
I	25	5.67	5.70	5.69	5.56
	30	5.72	5.74	5.74	5.59
	37	5.68	5.72	5.75	5.64
	44	5.32	5.34	5.38	5.36
J	25	5.51	5.54	5.52	5.48
	30	5.59	5.61	5.57	5.52
	37	5.47	5.47	5.47	5.46
	44	5.20	5.22	5.22	5.17
K	25	5.46	5.47	5.47	5.36
	30	5.49	5.47	5.46	5.32
	37	5.44	5.46	5.46	5.35
	44	4.93	4.93	4.98	4.99
L	25	2.54	2.58	2.56	2.47
	30	2.53	2.52	2.52	2.37
	37	2.16	2.19	2.19	2.21
	44	<1.48	<1.48	<1.48	<1.48

NOTE: Percentage values used for 3D-histograms in Appendix 3

Each recorded log_{10} values is a mean of 6 replicates.

TABLE 21. The effects of recovery temperatures and incubation periods on the counts of B. cereus (NCIB 7464) spores exposed to combined treatments.

Systems	Incubation temp °C	Log_{10} surviving spore counts recovered in YPTD at different periods (days)			
		15	10	6	2
I	25	5.60	5.66	5.69	5.59
	30	5.73	5.73	5.72	5.61
	37	5.65	5.69	5.72	5.66
	44	5.29	5.29	5.37	5.32
J	25	5.64	5.68	5.67	5.62
	30	5.76	5.77	5.76	5.63
	37	5.57	5.61	5.64	5.59
	44	5.36	5.44	5.44	5.32
K	25	5.46	5.48	5.51	5.39
	30	5.49	5.48	5.46	5.35
	37	5.42	5.45	5.43	5.31
	44	5.08	5.16	5.11	5.06
L	25	2.30	2.34	2.33	2.24
	30	2.31	2.32	2.29	2.16
	37	2.02	2.09	2.15	2.04
	44	<1.48	<1.48	<1.48	-[a]

[a]No growth (NG). NOTE: Percentage values used for 3D-histograms in Appendix 3

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 22. The effects of recovery temperatures and incubation periods on the counts of B. stearothermophilus (NCIB 8919) spores exposed to combined treatments.

Systems	Incubation temp °C	Log_{10} surviving spore counts recovered in YPTD at different incubation periods (days)			
		15	10	6	2
M	60	5.81	5.78	5.79	5.68
	55	5.86	5.84	5.82	5.72
	50	5.83	5.85	5.80	5.73
	44	5.52	5.57	5.58	5.53
N	60	5.19	5.21	5.25	5.26
	55	5.41	5.43	5.42	5.33
	50	5.39	5.43	5.43	5.34
	44	5.22	5.22	5.21	5.16
O	60	4.68	4.73	4.76	4.59
	55	4.87	4.86	4.84	4.70
	50	4.86	4.89	4.85	4.68
	44	4.34	4.38	4.47	4.44
P	60	2.87	2.93	-[a]	-
	55	3.04	3.01	2.96	-
	50	3.05	3.06	3.03	-
	44	-	-	-	-

[a]No growth (NG). NOTE: Percentage values used for 3D-histograms in Appendix 3

Each recorded log_{10} value is a mean of 6 replicates.

TABLE 23. The effects of recovery temperatures and incubation periods on the counts of B. stearothermophilus (NCIB 8920) spores exposed to combined treatments.

Systems	Incubation temp °C	Log_{10} surviving spore counts recovered in YPTD at different incubation times (days)			
		15	10	6	2
Q	60	5.32	5.33	5.32	5.24
	55	5.52	5.49	5.49	5.33
	50	5.46	5.54	5.48	5.36
	44	5.09	5.11	5.20	5.08
R	60	5.04	5.06	5.09	5.02
	55	5.12	5.12	5.14	5.06
	50	5.11	5.12	5.12	5.06
	44	4.63	4.76	4.84	4.81
S	60	4.03	4.05	4.06	4.00
	55	4.23	4.23	4.22	4.17
	50	4.20	4.22	4.23	4.14
	44	3.51	3.69	3.71	3.65
T	60	3.01	3.09	-[a]	-
	55	3.21	3.25	3.23	-
	50	3.25	3.25	3.26	-
	44	-	-	-	-

[a]No growth (NG). NOTE: Percentage values used for 3D-histograms in Appendix 3

Each recorded log_{10} value is a mean of 6 replicates.

tures at 30° and 55°C were the incubation temperatures used for the recovery of B. cereus and B. stearothermophilus respectively in the earlier experiment Section 3.2, p. 60, invol ving combinations of heat, sorbate, sodium chloride and pH.

(b) Survivors at other incubation temperatures at each test system was calculated as percentage of (a) using the relationship:

$$\text{Percentage spore count} = \frac{V_{(T)}}{V_{(T_R)}} \text{ at day 15}$$

where V_T = viable spore count at temperatures other than 30° and 55°C

V_{T_R} = viable spore count at 30°C and 55°C for B. cereus and B. stearothermophilus respectively.

(c) The calculated percentages were arranged such that values for day 2 were in "front" and values for day 15 at the "back" in the 3D-histograms.

(d) Values in (c) were fed into the computer to generate 3D-histograms as described in Section 3.3.

Results and Discussion

Effects on B. cereus spores: In general the recoveries of B. cereus (NCIB 6349, 7464) spores at 25° and 37°C were not markedly different from that at 30°C after exposure to systems I, J and K (Figures 15 and 16) for the 15 days incubation. However, recoveries at 44°C resulted in 41 to 73% reduction of the recoveries at 30°C. This indicates that the germination and outgrowth of treated spores were affected by elevation of the incubation temperature from 30°C. It can be inferred that a supra-optimum incubation temperature reinforces the inhibitory effectiveness of systems I, J and K. A similar effect was demonstrated with system L. Treatment of spores with system L and followed by incubation at 44°C provided 99.91%

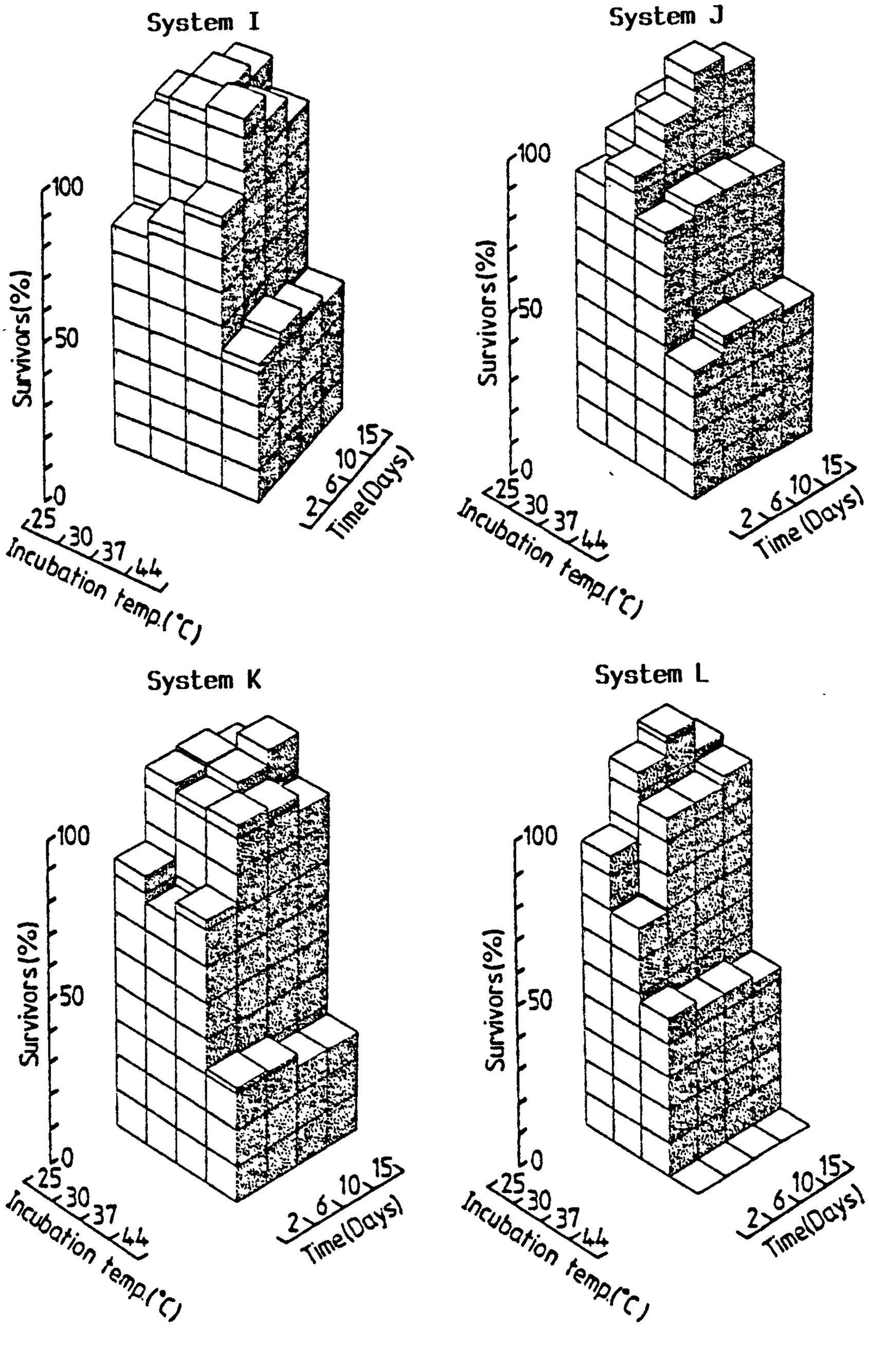

FIGURE 15. The effects of recovery temperatures and incubation periods on the survivors of B. cereus (NCIB 6349) spores exposed to combined treatments - systems I, J, K and L. (see Table 19).

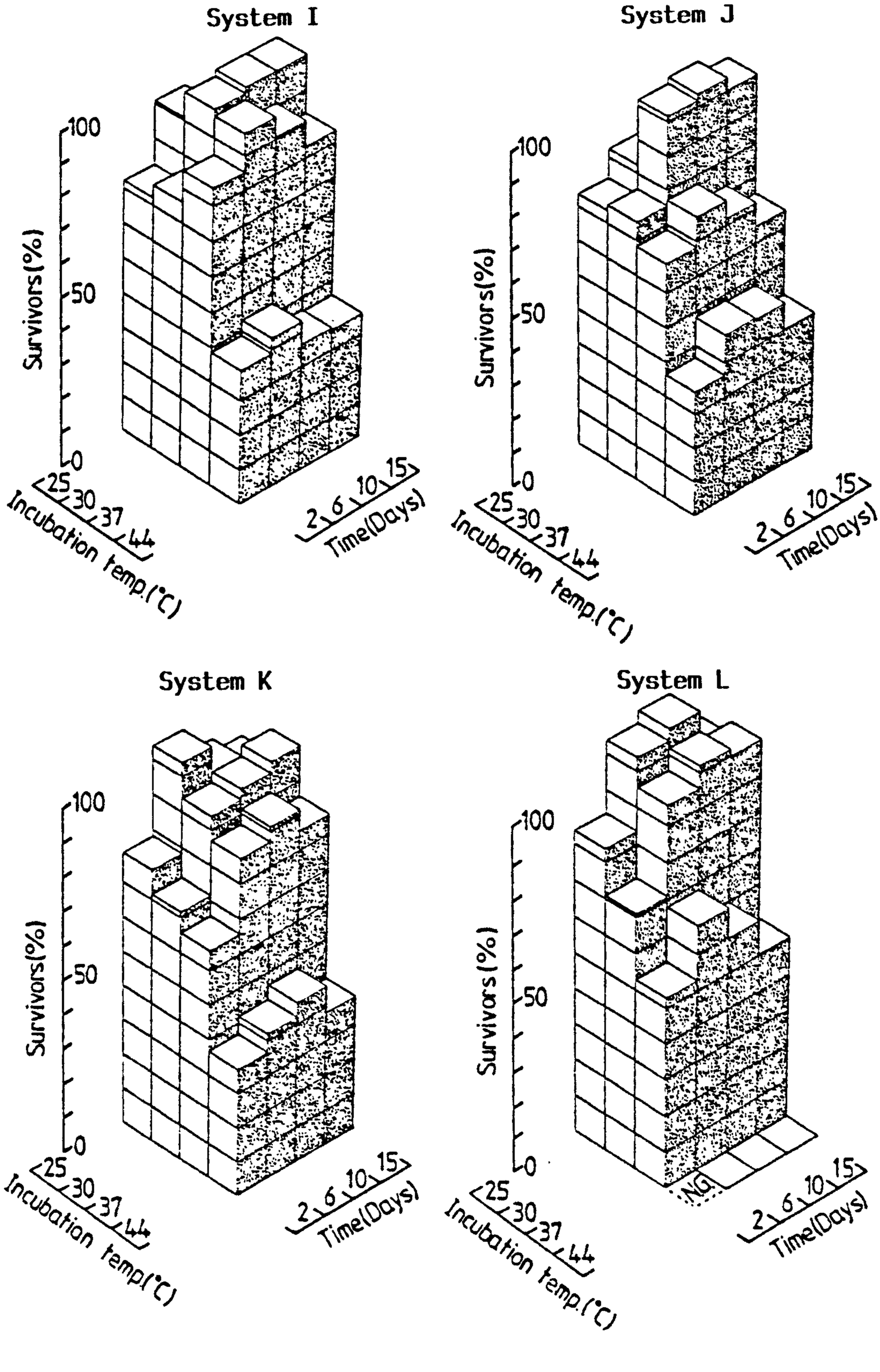

FIGURE 16. The effects of recovery temperatures and incubation periods on the counts of B. cereus (NCIB 7464) spores exposed to combined treatments - systems I, J, K and L. (see Table 19). N.G. (No growth).

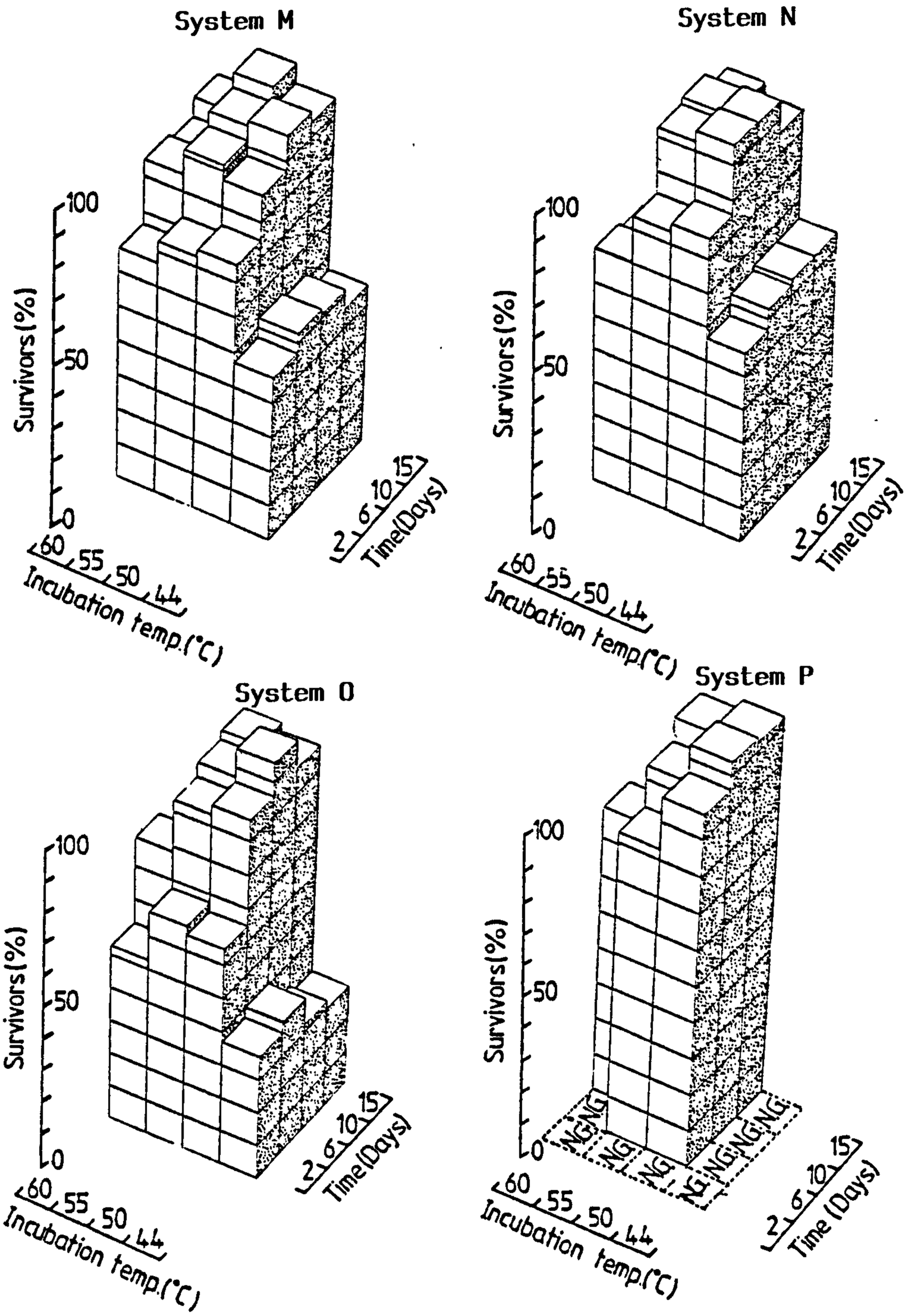

FIGURE 17. The effects of recovery temperatures and incubation periods on the counts of B. stearothermophilus (NCIB 8919) spores exposed to combined treatments - systems M, N, O and P. (see Table 19). N.G. (No growth)

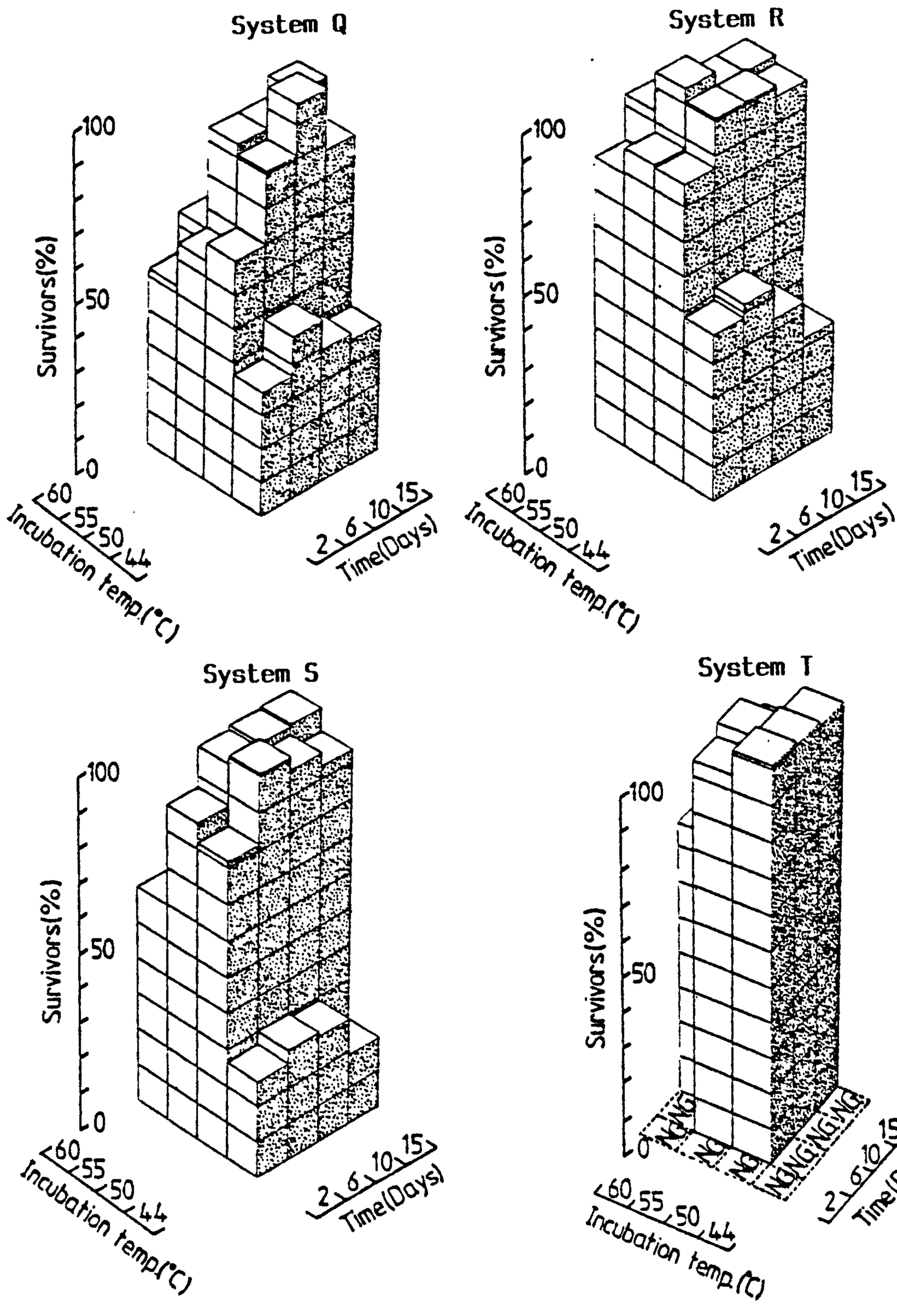

FIGURE 18. The effects of recovery temperatures and incubation periods on the counts of B. stearothermophilus (NCIB 8920) spores exposed to combined treatments - systems Q, R, S and T. (see Table 19). N.G. (No growth).

reduction in recoveries at 30°C for the 15 days incubation. In addition with B. cereus (NCIB 7464) recovery was delayed until after day 2 incubation. The marked reduction in the recoveries at 44°C may be associated with the inhibitory effectiveness of system L compared with other systems. It is viewed that the more the inhibitory effectiveness of the test systems the more selective are treated spores with respect to recovery temperatures.

Furthermore, recoveries at 25°C for B. cereus spores exposed to system L were not appreciably different from those at 30°C. Recoveries at 37°C provided 22 to 57% and 18 to 50% reductions in the recoveries at 30°C for B. cereus (NCIB 6349, Figure 15) and B. cereus (NCIB 7464, Figure 16) respectively. These results also demonstrate that recoveries at a supra-optimum temperature reinforced the inhibitory effectiveness of system L.

Effects on B. stearothermophilus: For strains (NCIB 8919 and 8920) exposed to systems 'MNO' and 'QRS' respectively (Figures 17 and 18) recoveries at 50°C were found to be similar to those at 55°C over the 15 day incubation period. This is in agreement with earlier findings by Cook and Gilbert (1968) who also employed these temperatures for the recovery of heat - treated B. stearothermophilus (NCIB 8919) spores. However, recoveries at 60°C showed reductions from 6% - 41% of those at 55°C after exposure to systems M, N and O. Similarly, for strain 8920 exposed to systems Q, R and S recoveries at 60°C showed 7% - 37% reductions.

Compared with 55°C recoveries at 44°C for strain 8919 subjected to M, N and O systems exhibited marked reductions of 27 to 70%. A similar effect was shown for strain 8920 exposed to Q, R, S systems. Recoveries at 44°C gave reductions of 28 to 81% of those at 55°C.

These effects parallel the observations for treated B. cereus spores recovered at 44°C. Thus incubation temperature 44°C reinforced the inhibitory effectiveness of systems M, N, O for strain 8919 and Q, R, S for strain 8920.

Furthermore, results showed that recoveries at 50°C were similar to those at 55°C for strains 8919 and 8920 exposed to systems P and T respectively. However, recoveries at both temperatures were delayed until after day 2 for both strains (Figures 17 and 18). Similarly recoveries at 60°C were delayed until after day 6 for both strains. The results demonstrate the need for prolonged incubation periods particularly when making quality control tests on heat processed foodstuffs. Also recoveries at 60°C were reduced compared with 55°C for strains 8919 and 8920 after exposure to systems P and T respectively. Hence the temperature 60°C potentiated the inhibitory effectiveness of systems P and T. Most significantly no recovery of either strain 8919 or 8920 was obtained at 44°C over the 15 day incubation period. The present findings demonstrate the need for careful selection of the incubation temperature for assessment of the recovery of spores exposed to combined treatments, otherwise erroneous results may be obtained.

8. DIRECT FLUORESCENCE MICROSCOPY (DFM) STUDIES OF SPORES EXPOSED TO COMBINED TREATMENTS

Most research workers including Roberts and Ingram (1966), Robach (1980b), Mallidis (1981) and Bell and DeLacy (1984) have employed the plate count technique for the enumeration of spores after exposure to heat and/or chemicals. Similarly in the present study the plate count technique was selected as the standard procedure. However, other methods including phase contrast microscopy and stainability of spores may be used for assessment. These methods have been discussed in review articles by Gould (1971) and Cook and Pierson (1983).

Parker (1969) used phase contrast microscopy for the evaluation of effects of preservatives on the development of bacterial spores. The same method was used by Seward et al. (1982) for defining the sites at which potassium sorbate and other preservatives acted to prevent the germination and outgrowth of Cl. botulinum type E.

Manson (1977) employed an acriflavine staining (ACFS) technique and found that it compared with the standard malachite green staining (MGS) procedure for the enumeration of unheated and heated bacterial spores. The present experiment was designed to evaluate the MGS and in particular the ACFS techniques for the enumeration of spores exposed to the combination of heat and chemical treatments.

Materials and methods

Test cultures: B. cereus (NCIB 6349, 7464) and B. stearothermophilus (NCIB 8919, 8920).

Test systems: Based on the results of previous experiments in this study the systems shown in Table 24 were selected. Most of

TABLE 24. Systems employed for DFM studies.

Systems	Description	Strains used	Percentage inhibition
I	Unheated	All strains	-
	Heat only		
II	85°C for 30 min	B. cereus	-
III	95°C for 45 min	B. stearothermophilus	-
	Combination of heat, sorbate, NaCl and pH		
IV	Heated (0.4% KS[a]+7.5% NaCl pH 5.5)	B. cereus 6349, 7464	89.4; 92.6
V	Heated (0.4% KS+7.5%NaCl pH 4.5)	"	100
VI	Heated (0.8% KS+6% NaCl pH 5.2)	B. stearotherm. 8920	96.2
VII	Heated (0.8% KS+6% NaCl pH 4.2)	"	100
VIII	Heated (0.8% KS+8% NaCl pH 5.2)	" 8919	92.2
IX	Heated (0.8% KS+8% NaCl pH 4.2)	" 8919	100
	Combination of heat, sorbate, benzoate and pH		
X	Heated (0.4% KS+1.75% SB[b] pH 5.5)	B. cereus 6349	65.4
XI	Heated (0.4% KS+1.75% SB pH 4.5)	"	100
XII	Heated (0.8% KS+0.5% SB pH 5.2)	B. stearotherm. 8919	54.1
XIII	Heated (0.8% KS+2.5% SB pH 4.2)	"	100

[a]Potassium sorbate.

[b]Sodium benzoate.

these systems had been shown to cause moderate, significant or complete inhibition of treated spores. Control systems were also included. The systems are grouped according to the type of combined treatments as shown in Table 24. Percentage inhibition values were extracted from the results in Sections 6 and 7. Heating of B. cereus strains was carried out at 85°C for 30 min and for B. stearothermophilus at 95°C for 45 min in menstrua containing the chemicals shown in parentheses.

Heat treatments: The menstrua used in this experiment are designated Systems I to XIII (Table 24). The preparation of the menstrua and adjustment of their pH followed the procedure described in Section 3, p. 50. For each heat treatment approximately 10^7 spores/ml was used.

Preparation of treated spores: Following exposure to the appropriate treatment, spore suspensionswere poured into disposable plastic universal bottles (Sterilin Ltd, Mid., England) and centrifuged at 2000g for 5 min. This was repeated three times using sterile distilled water. The pellet obtained was resuspended in a known volume of sterile distilled water and thoroughly mixed using a Whirlmixer (Fisons Scientific Apparatus Loughborough, Leiceistershire).

Preparation of smears: The method used was reported by Manson (1977). A 1 cm square was carefully etched on Chance glass microscope slides (76 x 25 mm) using a glass diamond marker. The slides were immersed in a 5% (v/v) aqueous solution of RBS 25 concentrate for 2 to 4 h, thoroughly washed in hot running tap water and allowed to dry. Care was taken not to allow dust to settle on the etched square on the slides.

An aliquot 10 µl of the well mixed treated spore suspension

was taken up using an Oxford Sampler Pipette (Boehringer Corp., London Ltd. Lewes, East Sussex) into a disposable plastic tip and pipetted onto the 1 cm square. The sample was spread over the whole 1 cm square using the edge of a wire loop and allowed to dry on an even surface. The dried slides were placed on a staining rack and covered with propan-2-ol to fix the treated spores. The samples were fixed for about 15 min, drained on metal racks and stored in plastic slide holders until the staining was carried out usually within 1 or 2 h.

Malachite Green Staining Procedure

The MGS procedure described by Schaeffer and Fulton (1933) was used. This involved flooding the fixed slides with a 5% (w/v) aqueous solution of MGS (British Drug Houses Ltd. Poole, England) and gently heating the underside of the slide with flaming torch until steam was seen to rise from the slide. The flaming of the slide was discontinued to allow the steam to cool before being heated again to steaming. This procedure was repeated 3 or 4 times, taking care that the stain was not allowed to dry out before washing the slide in running tap water. A 1 in 10 aqueous dilution of Ziehl Neelsen's carbol fuchsin (Appendix 2) was applied as counterstain to the sample for 30s. This was rinsed in tap water, drained and dried.

Acriflavine Staining Technique

The ACFS involved using acriflavine (British Drug Houses Ltd. Poole, England) stored at 18 - 20°C in an amber bottle as a 1% (w/v) aqueous stock solution. A 0.01% (w/v) solution of acriflavine in

0.067 M phosphate buffer pH 7.2 (Appendix 2) was prepared weekly from the stock. The propanol fixed preparations were placed on a staining rack and covered with the acriflavine stain, allowed to act for 10 min, rinsed briefly in running tap water, drained and dried.

Microscopical Procedures

The examination of the stained treated spores was carried out using Phase contrast microscope (Laborlux model 11, E. Leitz, W. Germany) fitted with an epi-fluorescence illuminator which allowed incident light examination of the preparations. Photomicrographs were taken using an Orthomat fully automatic camera (E. Leitz W. Germany) fitted to the microscope system.

Enumeration Procedures

The formula used in the enumeration of the stained spores was obtained following these steps:-

(a) Determine the number of divisions per field using a graticule = Q

(b) Determine the diameter of the field

On the graticule 100 div x 0.1 = 10 mm

$\therefore$ For Q divisions = Q/10 mm r = Q/20 mm

(c) Determine area of field = $3.14\ Q^2/400 = 7.85 \times 10^{-3} Q^2$ sq mm

(d) Since 10 µl of sample was spread over 100 sq mm

i.e. 100 sq mm $\equiv$ 10 µl

$\therefore\ 7.85 \times 10^{-3} Q^2$ sq mm $\equiv\ 7.85 \times 10^{-4} Q^2$ µl

(e) Accumulated number of spores counted in known number of fields, say C

(f) Total number of fields, say F

(g) $\therefore$ Number of spores counted/field $\equiv$ C/F

(h) $7.85 \times 10^{-4} Q^2$ µl ≡ C/F spores

$$\therefore \quad 1 \text{ ml} \equiv \frac{C}{FQ^2} \cdot 1.27 \times 10^6$$

∴ To determine no. of spores/ml, the formula

$$1.27 \times 10^6 \cdot \frac{C}{FQ^2}$$ was used.

C represents total number of spores counted

F represents no. of fields

Q represents no. of divisions equivalent to a field on the graticules

The precision of the counts was determined using the method of Cassell (1965).

Preliminary studies

In order to confirm the potential of DFM for the enumeration of spores, preliminary studies were carried out to compare counts obtained by this technique with those obtained using MGS and plate count (PC) procedures.

Bacillus stearothermophilus (NCIB 8919) spores were used. Stock spore suspension was diluted to obtain concentrations from 10^6 to 10^9 per ml estimated by phase contrast microscope. Heat treatment of each dilution was carried out as described in Section 3, p. 50. The plate counts (PC) of the heated spores were carried out on YPTD agar as described in Section 3, p. 52 while the MGS and ACFS preparations were carried out as described earlier in this experiment.

Results and Discussion

The results of the preliminary studies are presented in Table 25. The results indicate that the microscope counts were higher

TABLE 25. Total plate counts and microscope counts of B. stearothermophilus (NCIB 8919) spores.

Spore suspension[a]	Plate counts[b]	Malachite Green Stained		Acriflavine Stained	
		GS	RS	GF	OF
10^6	$4.68\pm0.13^{c}\times10^5$	$2.64\pm0.18^{d}\times10^5$	$6.83\pm0.55\times10^5$	$3.25\pm0.43\times10^5$	$5.67\pm1.03\times10^5$
10^7	$5.98\pm0.32\times10^6$	$1.93\pm0.08\times10^6$	$7.24\pm0.57\times10^6$	$2.43\pm0.56\times10^6$	$8.05\pm0.48\times10^6$
10^8	$6.14\pm0.49\times10^7$	$1.29\pm0.11\times10^7$	$8.15\pm0.93\times10^7$	$3.85\pm0.24\times10^7$	$6.17\pm0.56\times10^7$
10^9	$5.29\pm0.05\times10^8$	$2.34\pm0.36\times10^8$	$7.03\pm0.52\times10^8$	$2.71\pm0.19\times10^8$	$6.84\pm0.38\times10^8$

[a]Stock spore suspension determined by phase contrast and diluted to obtain the required estimate.

[b]Plate counts on YPTD

[c]S.D. ± spores ml^{-1}

[d]90% C.I. ± spores ml^{-1}

GS, green stained; RS, red stained; GF, green fluorescent; OF, orange fluorescent

All counts were made after heating at 95°C for 45 min.

than the corresponding plate counts. This is in agreement with the earlier observation by Scholefield, Manson and Johnston (1976) who also reported higher microscope counts than plate counts with cultures of E. coli and S. aureus. The preliminary finding can be very significant with respect to the quality control assessment of foodstuffs where the colony count technique is the standard reference for assessing bacterial numbers.

The ACFS spores were found to be green fluorescent (GF) or orange fluorescent (OF) while the MGS spores were either green stained (GS) or red stained (RS). In an earlier study involving bacterial spores Manson (1977) reported that a ratio of 0.75 - 1.25 signified correlation between two counts. In the present experiment the ratios 0.74 - 0.99, 0.69 - 0.83 and 0.90 - 1.32 were found between PC:OF, PC:RS and RS:OF counts respectively (Table 26). Thus the results showed that there was a fairly good correlation between the plate counts and the microscope counts of red stained and orange fluorescent spores. Since nonstainability is a feature of dormant spores (Gould, 1977) the counts due to the green stained (GS) and green fluorescent (GF) spores (Table 25) may be those which were not activated by heat at 95°C for 45 min. This may account for the lower PC compared with microscope counts since in most cases spores need to be activated for germination and outgrowth to occur. Thus there is an implication that a microscope count may be inherently more representative of the actual spore count than the plate count.

Main studies

In view of the observation in the preliminary studies, discrimination was made between the RS and GS for the MGS spores and with the ACFS preparations the counts for the GF were distinguished

TABLE 26. Ratios of count of B. stearothermophilus (NCIB 8919) spores using the plate count (PC), red stained (RS) and orange fluorescent (OF) counts.

Concentration of spore suspension[a]	Ratios[b]		
	PC : RS	PC : OF	RS : OF
10^6	4.68 : 6.83 = 0.69	4.68 : 5.67 = 0.83	6.83 : 5.67 = 1.20
10^7	5.98 : 7.24 = 0.83	5.98 : 8.05 = 0.74	7.24 : 8.05 = 0.90
10^8	6.14 : 8.15 = 0.75	6.14 : 6.17 = 0.99	8.15 : 6.17 = 1.32
10^9	5.29 : 7.03 = 0.75	5.29 : 6.84 = 0.77	7.03 : 6.84 = 1.03

[a]Concentration of stock spore suspension determined by phase contrast and diluted to obtain the required estimate.

[b]Values used were taken from Table 25.

from the OF spores. This was done for all the systems I to XIII (Table 24).

Microscope counts of unheated and heated spores in the presence or absence of chemicals and designated by systems I to XIII are summarized in Tables 27 - 29. The spore counts are compared on a percentage basis by considering spore counts of system I (unheated) for each type of bacterial spore and strain as 100%.

Effects of the combination of heat, sorbate, NaCl and pH on spores .

MGS spore counts: Tables 27 and 28 show that the GS spore count is highest in system I. The RS spore counts increased while the GS counts decreased with the severity of treatment. This is shown in Table 27 for B. stearothermophilus (NCIB 8919) after exposure to systems I, III, VIII and IX. This observation is supported by Plates 1, 3, 5 and 7. The highest RS spore counts represent the following percentages of GS:

System	RS (as percentage of GS)	Test Strain
V	90.8	B. cereus 6349 (Table 28)
V	95.2	" 7464 "
VII	92.3	B. stearothermophilus 8920 (Table 27)
IX	83.0	" 8919 (Table 27)

These systems represent the most effective combination for the inhibition of spores as observed in earlier experiment Section 5, p. 86.

ACFS spore counts: Results of ACFS spore counts in Table 27 and 28 follow the same trend as the MGS spore counts. The GF spore count was found to be highest in system I. The OF spore count increased and the GF spore counts decreased with the severity of treat-

TABLE 27. Microscope counts of B. stearothermophilus spores after exposure to combination of heat, potassium sorbate (KS), sodium chloride (NaCl) and pH.

Systems	B. stearothermophilus (NCIB 8919)				B. stearothermophilus (NCIB 8920)			
	No. of spores/ml (x 10^6)							
	MGS		ACFS		MGS		ACFS	
	GS	RS	GF	OF	GS	RS	GF	OF
I	7.93±0.25[a]	0	8.84±0.05	0	7.64±0.44	0	8.38±0.63	0
III	1.04±0.19	5.62±0.88	2.26±0.58	6.34±0.39	1.15±0.93	6.16±0.58	2.04±0.67	6.95±0.74
VI	-	-	-	-	1.53±0.86	6.73±0.44	1.63±0.28	7.19±0.16
VII	-	-	-	-	1.39±0.18	7.05±0.79	0.91±0.11	7.94±0.63
VIII	1.49±0.28	5.84±0.95	2.13±0.79	6.52±0.18	-	-	-	-
IX	1.03±0.55	6.58±0.38	0.64±0.17	7.34±0.86	-	-	-	-

[a]Figure denote 90% C.I.± spores ml^{-1}.

GS, green stained; RS, red stained; GF, green fluorescent; OF, orange fluorescent; MGS, Malachite green stained; ACFS, Acriflavine stained.

TABLE 28. Microscope counts of B. cereus spores after exposure to combination of heat, potassium sorbate (KS), sodium chloride (NaCl) and pH.

Systems	B. cereus (NCIB 6349)				B. cereus (NCIB 7464)			
	No. of spores/ml (x 10^6)							
	MGS		ACFS		MGS		ACFS	
	GS	RS	GF	OF	GS	RS	GF	OF
I	8.24±0.93[a]	0	8.95±0.18	0	8.38±0.69	0	8.79±0.93	0
II	2.93±0.29	6.25±0.38	1.66±0.43	7.58±0.66	2.33±0.14	6.74±0.55	2.04±0.12	6.97±0.66
IV	1.28±0.77	6.87±0.67	0.95±0.24	7.74±0.32	1.63±0.58	7.03±0.47	1.37±0.78	7.29±0.15
V	1.38±0.45	7.48±0.19	0.59±0.05	8.34±0.48	0.96±0.07	7.98±0.39	0.63±0.03	8.43±0.72

[a]Figures denote 90% C.I. ± spores ml^{-1}.

MGS, Malachite green stained; ACFS, Acriflavine stained; GS, green stained; RS, red stained; GF, green fluorescent; OF, orange fluorescent.

TABLE 29. Microscope counts of bacterial spores after exposure to combination of heat, potassium sorbate (KS), sodium benzoate (SB) and pH.

Systems	*B. stearothermophilus* (NCIB 8919)				*B. cereus* (NCIB 6349)			
	No. of spores/ml (x 10^6)							
	MGS		ACFS		MGS		ACFS	
	GS	RS	GF	OF	GS	RS	GF	OF
I	7.86±0.79[a]	0	8.46±0.11	0	7.93±0.22	0	8.65±0.59	0
II	-	-	-	-	1.96±0.03	6.77±0.51	1.34±0.72	7.82±0.42
III	1.13±0.28	5.79±0.44	2.13±0.58	6.55±0.22	-	-	-	-
X	-	-	-	-	1.46±0.08	6.94±0.66	0.86±0.03	8.05±0.17
XI	-	-	-	-	1.09±0.54	7.28±0.36	0.65±0.02	8.44±0.36
XII	1.18±0.22	6.07±0.34	1.94±0.36	6.81±0.01	-	-	-	-
XIII	0.86±0.11	6.93±0.72	0.38±0.06	8.03±0.67	-	-	-	-

[a]Figures denote 90% C.I. ± spores ml^{-1}.

MGS, Malachite green stained; ACFS, Acriflavine stained; GS, green stained; RS, red stained; GF, green fluorescent; OF, orange fluorescent.

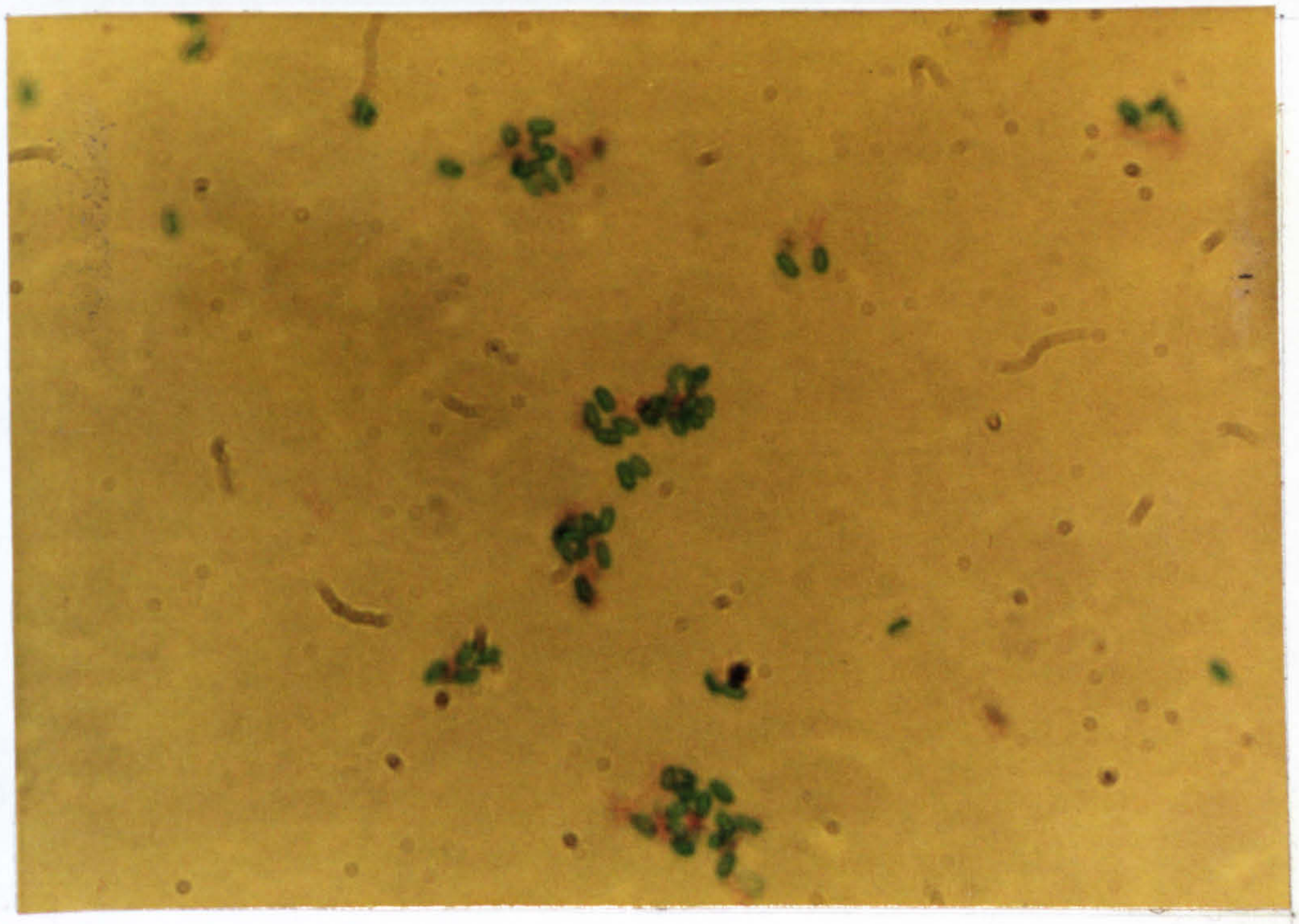

PLATE 1. Unheated spores of Bacillus stearothermophilus (NCIB 8919) stained using the malachite green stain. Magnification x 1480

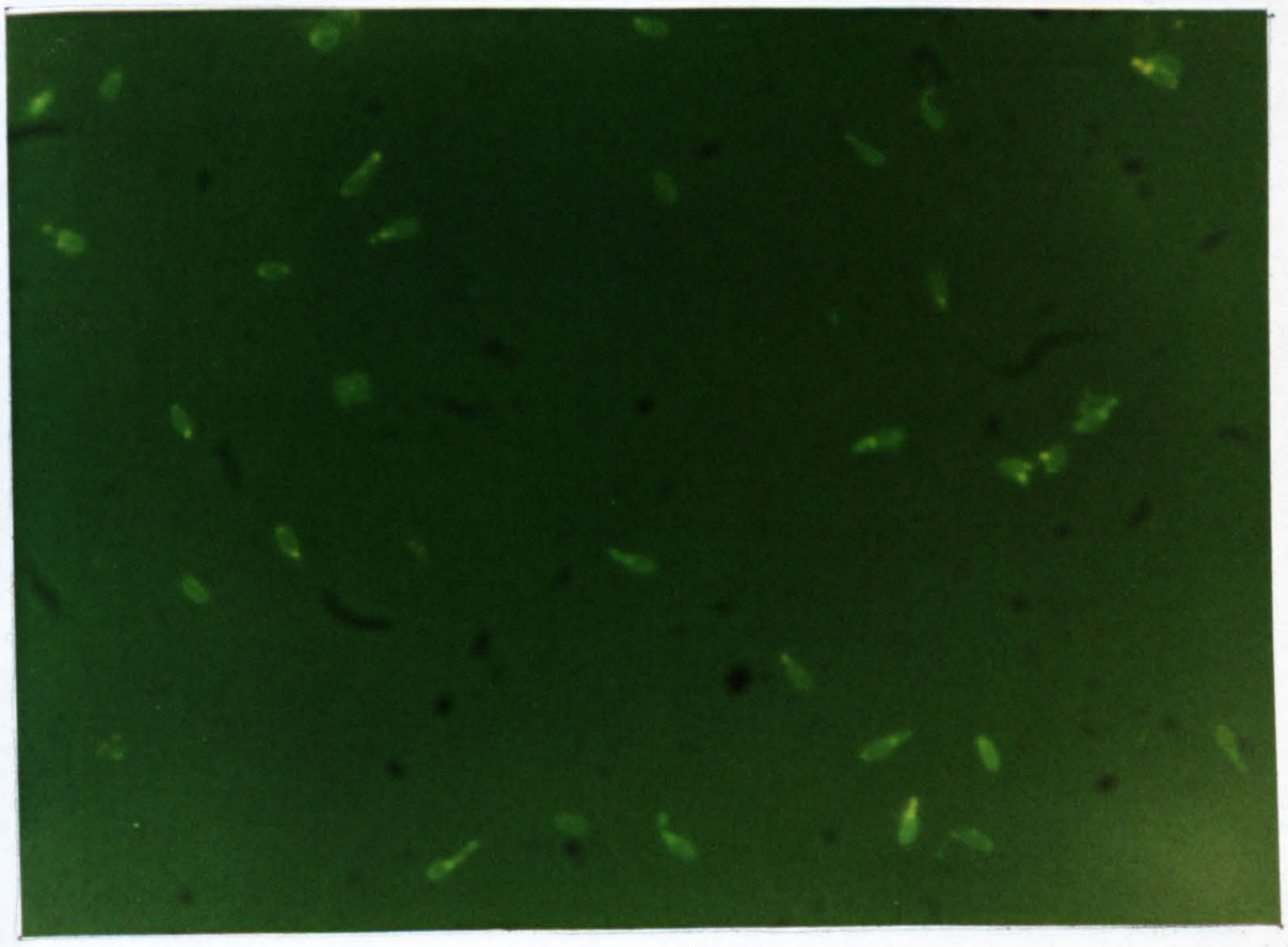

PLATE 2. Unheated spores of Bacillus stearothermophilus (NCIB 8919) stained using the acriflavine stain. Magnification x 1480

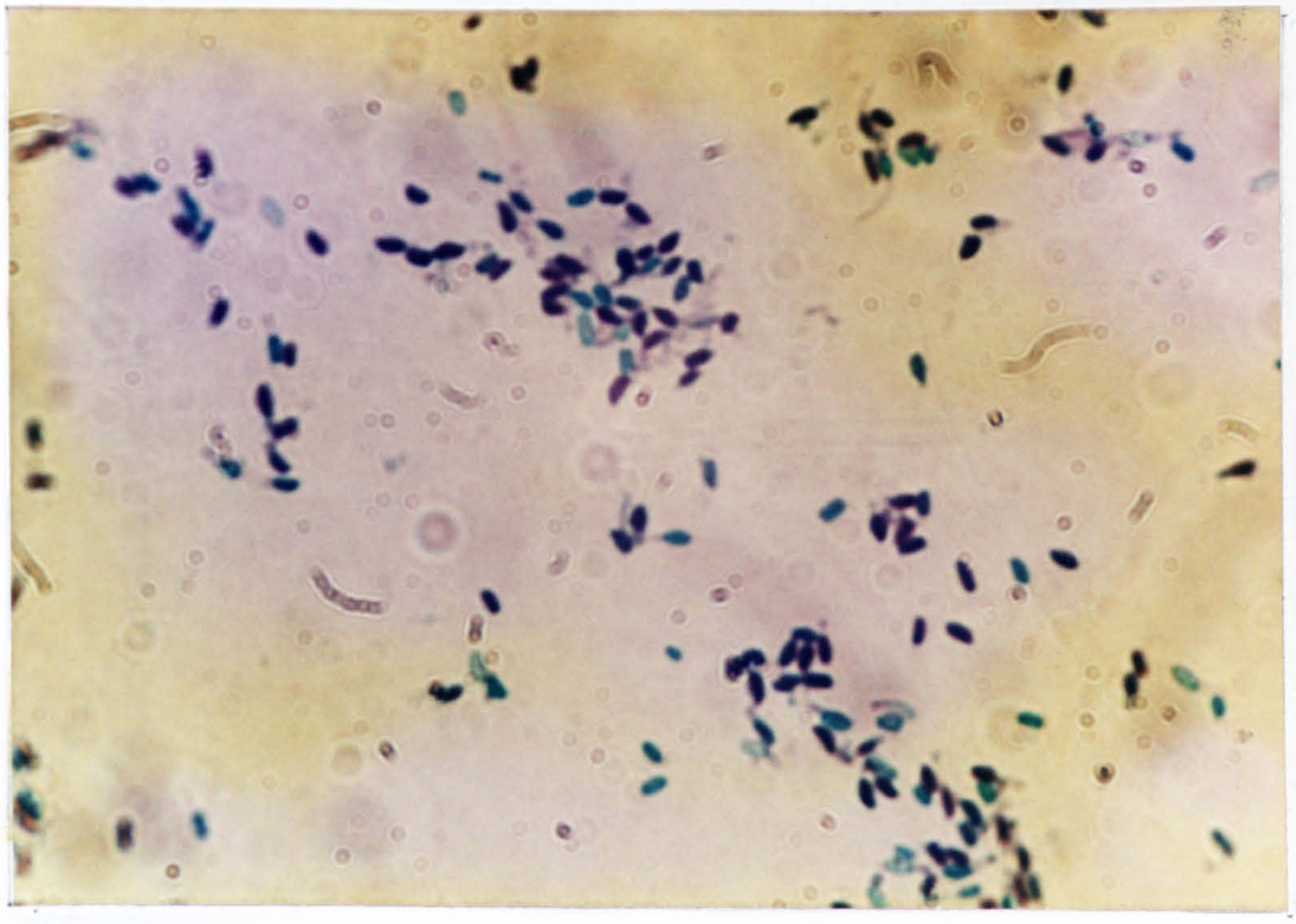

PLATE 3. Spores of B. stearothermophilus (NCIB 8919) stained using the malachite green stain after exposure to heat at 95°C for 45 min (system III). Magnification x 1480

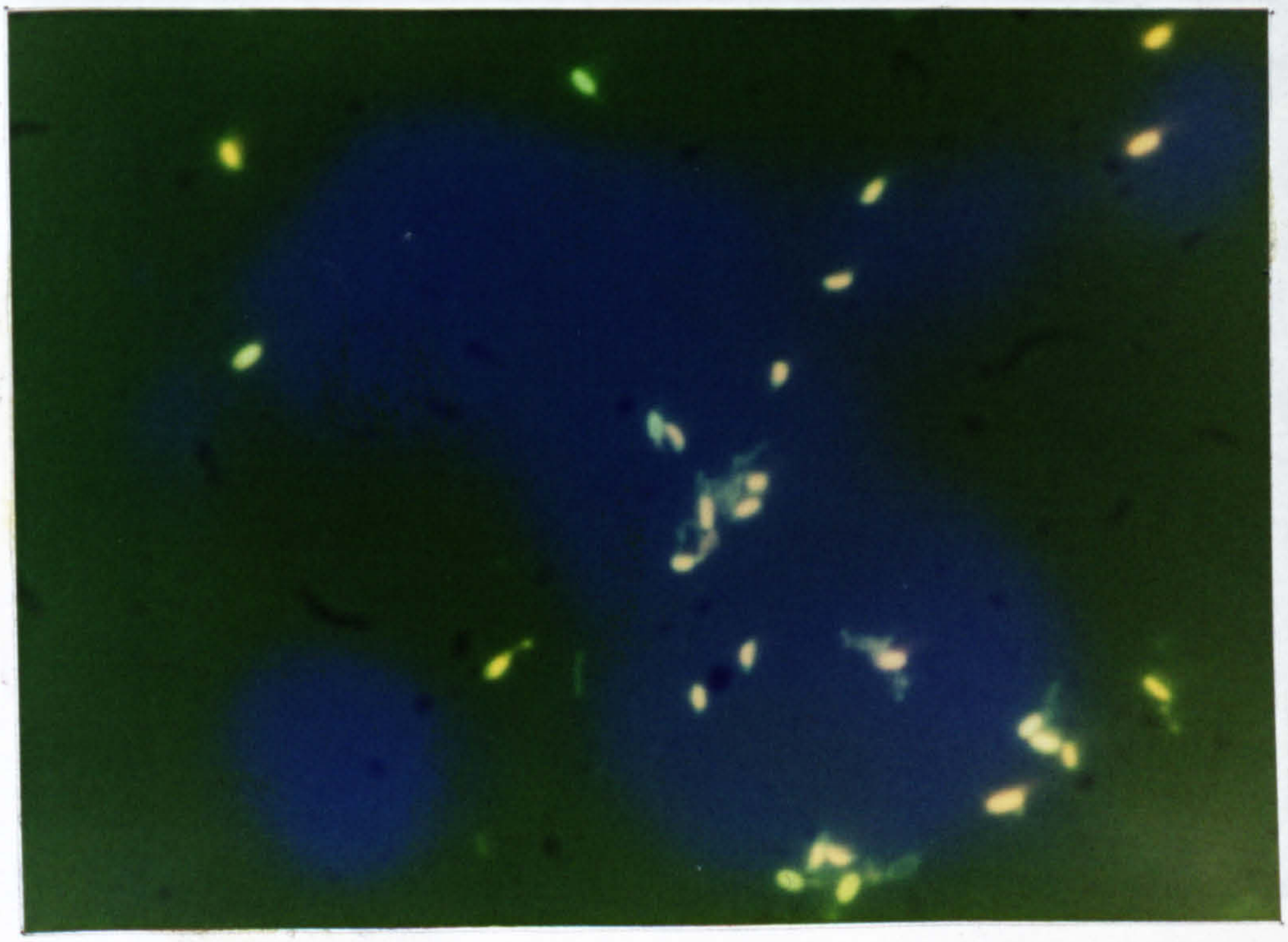

PLATE 4. Spores of B. stearothermophilus (NCIB 8919) stained using the acriflavine stain after exposure to heat at 95°C for 45 min (system III). Magnification x 1480

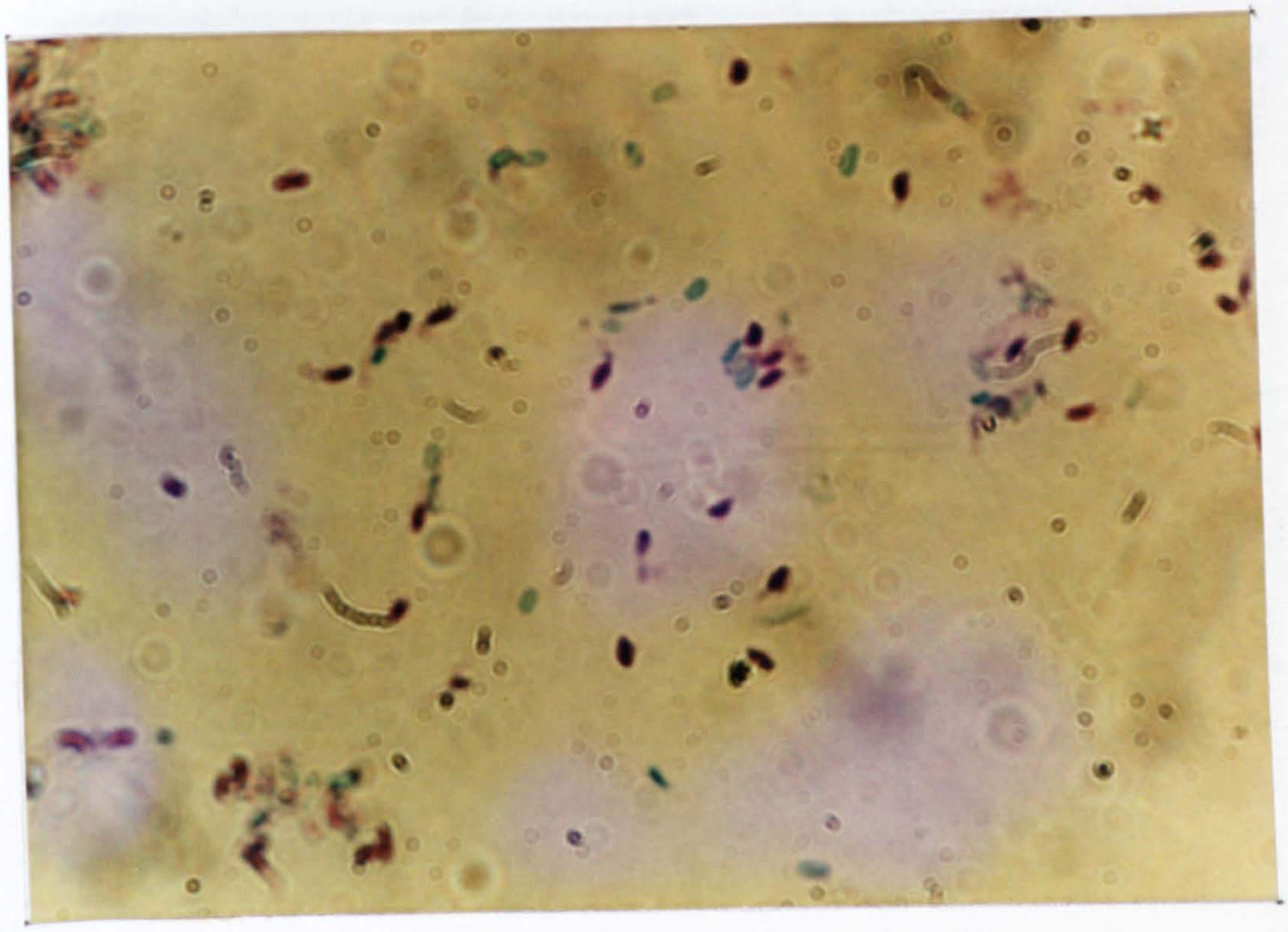

PLATE 5. Spores of B. stearothermophilus (NCIB 8919) stained using the malachite green stain after exposure to system VIII (i.e. heated 0.8% (w/v) sorbate + 8.0% (w/v) NaCl pH 5.2). Magnification x 1480

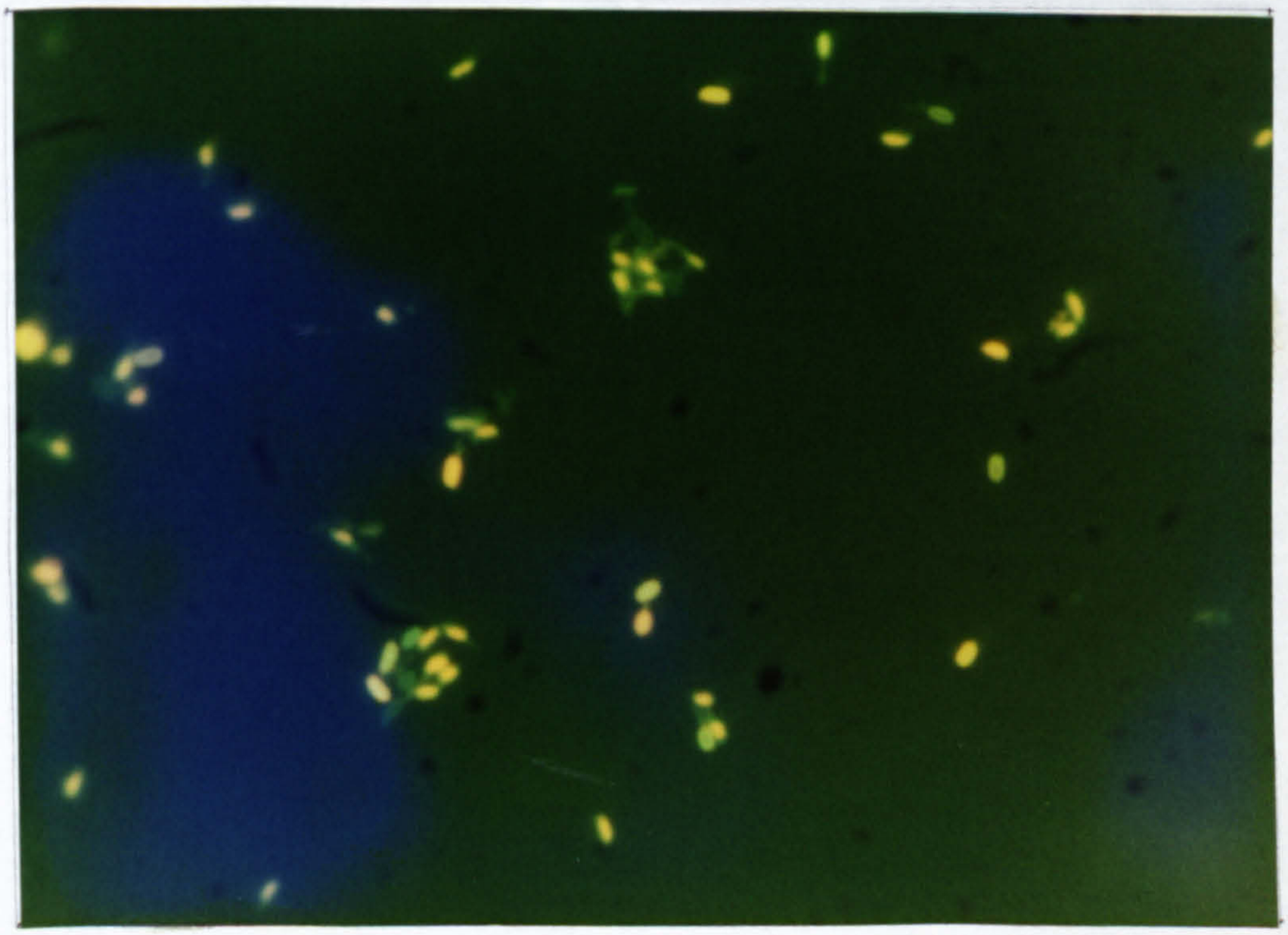

PLATE 6. Spores of B. stearothermophilus (NCIB 8919) stained using the acriflavine stain after exposure to system VIII (i.e. heated 0.8% (w/v) sorbate + 8.0% (w/v) NaCl pH 5.2). Magnification x 1480

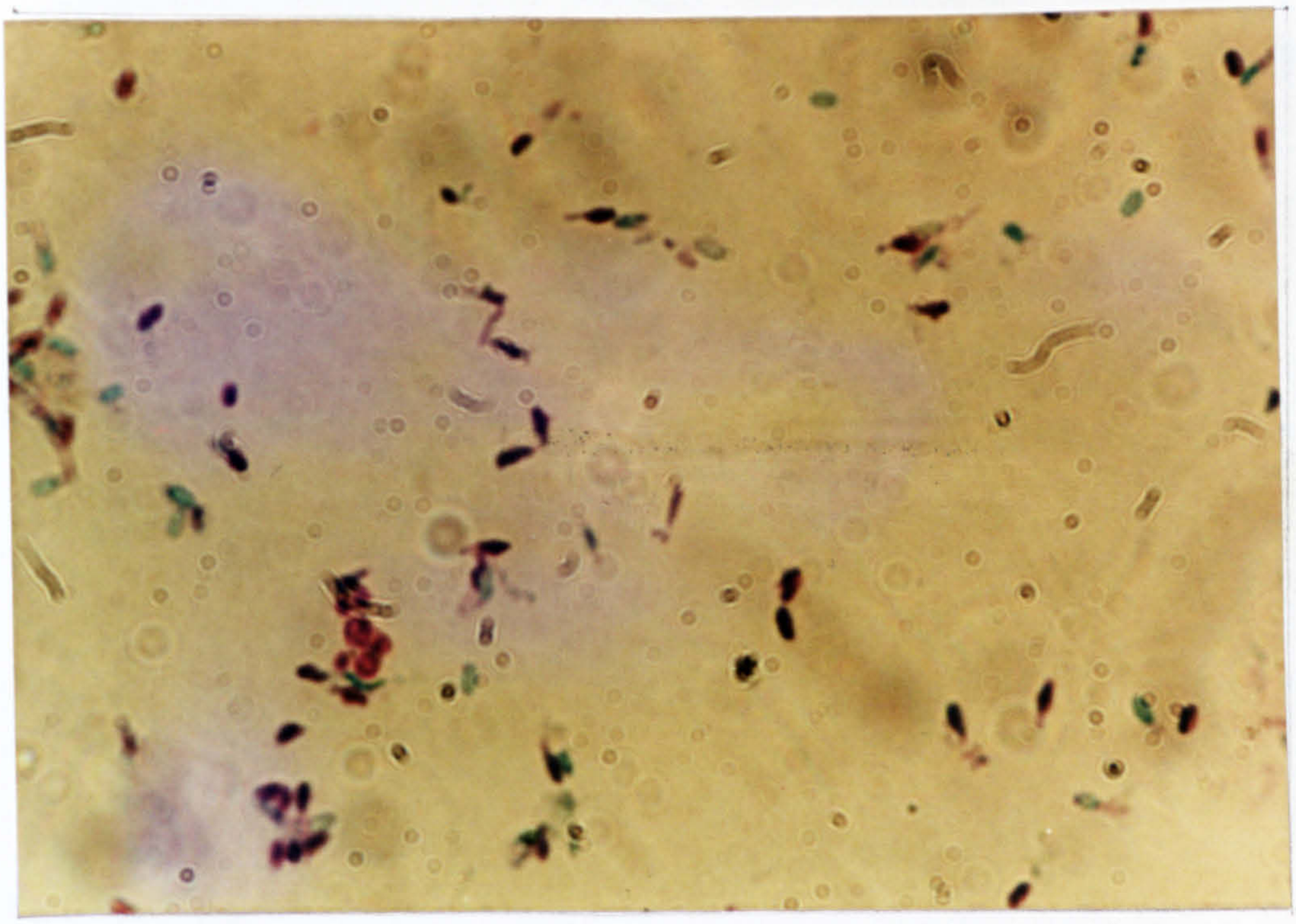

PLATE 7. Spores of B. stearothermophilus (NCIB 8919) stained using the malachite green stain after exposure to system IX (i.e. heated 0.8% (w/v) sorbate + 8.0% (w/v) NaCl pH 4.2) Magnification x 1480

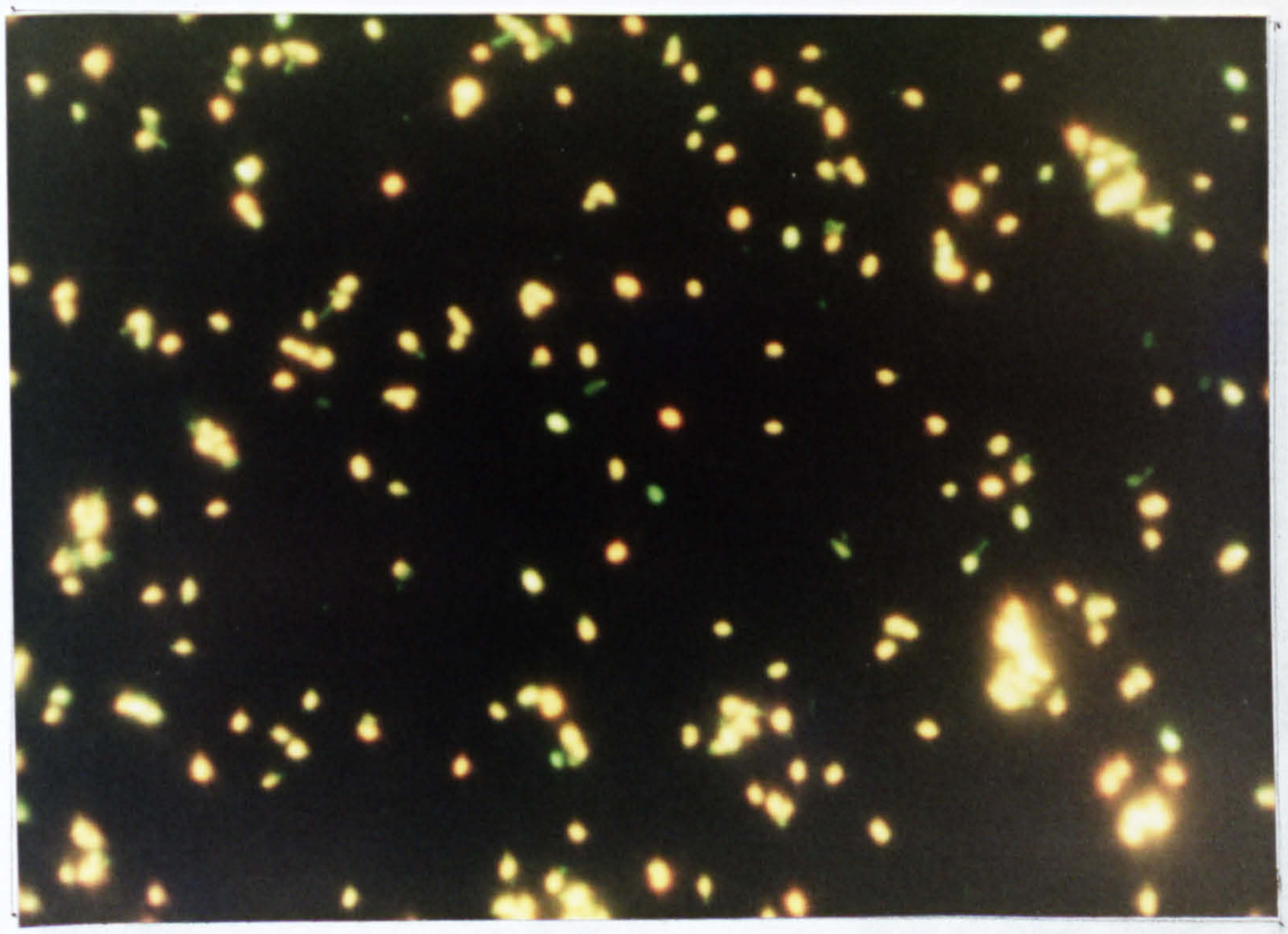

PLATE 8. Spores of B. stearothermophilus (NCIB 8919) stained using the acriflavine stain after exposure to system IX (i.e. heated 0.8% (w/v) sorbate + 8.0% (w/v) NaCl pH 4.2) Magnification x 1480

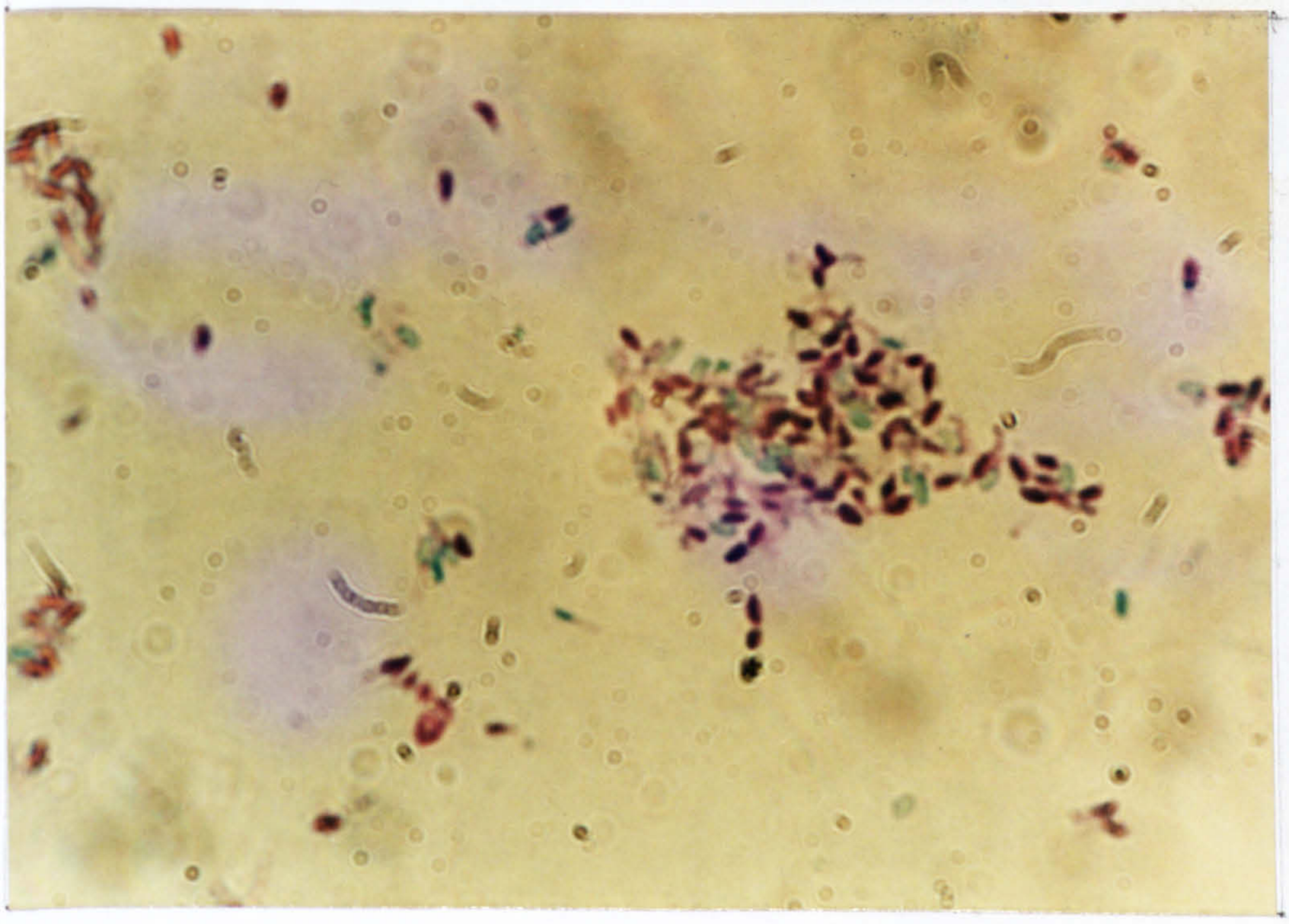

PLATE 9. Spores of B. stearothermophilus (NCIB 8919) stained using the malachite green stain after exposure to system XII (i.e. heated 0.8% (w/v) sorbate + 0.5% benzoate pH 5.2). Magnification x 1480

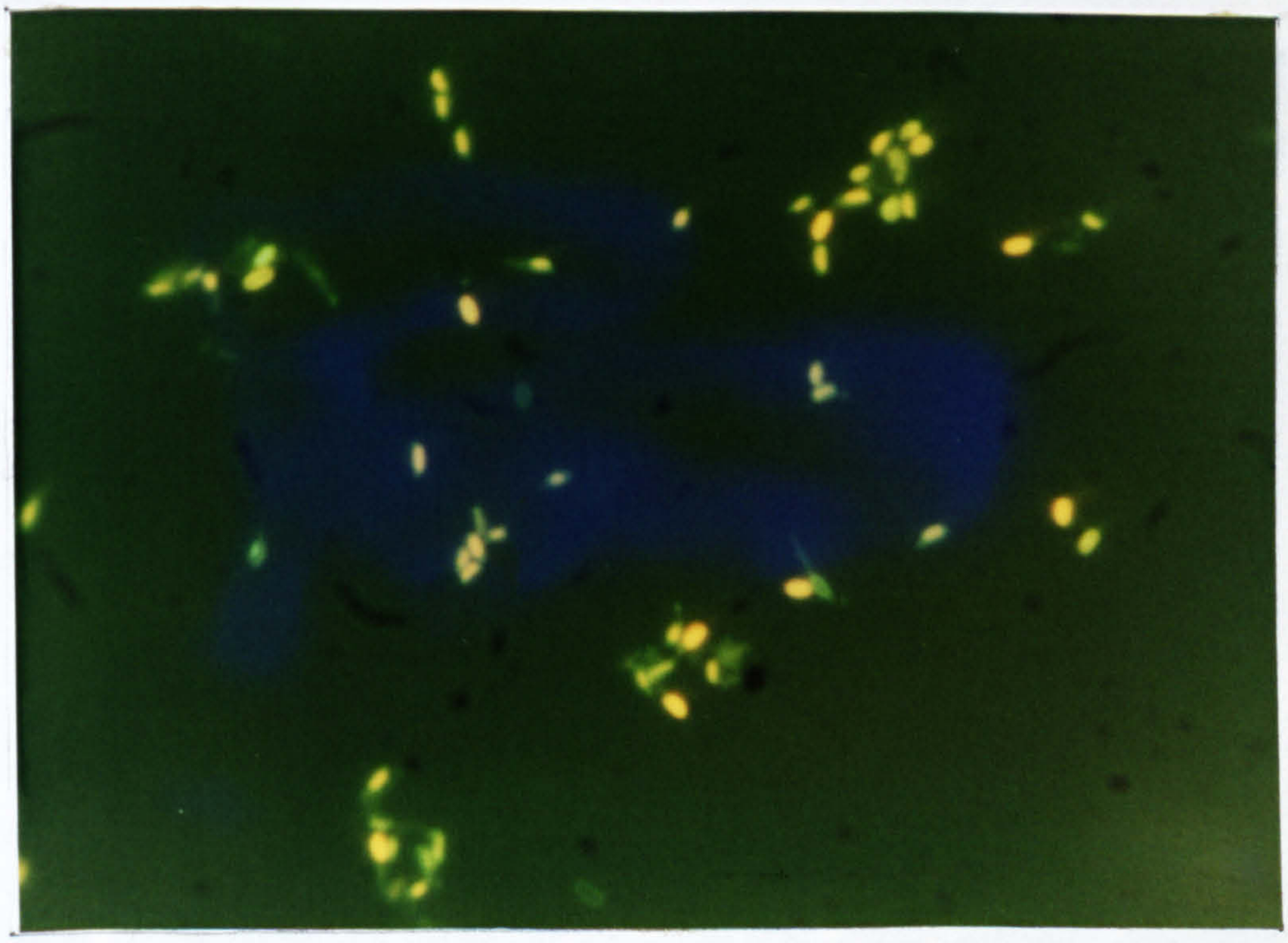

PLATE 10. Spores of B. stearothermophilus (NCIB 8919) stained using the acriflavine stain after exposure to system XII (i.e. heated 0.8% (w/v) sorbate + 0.5% benzoate pH 5.2). Magnification x 1480

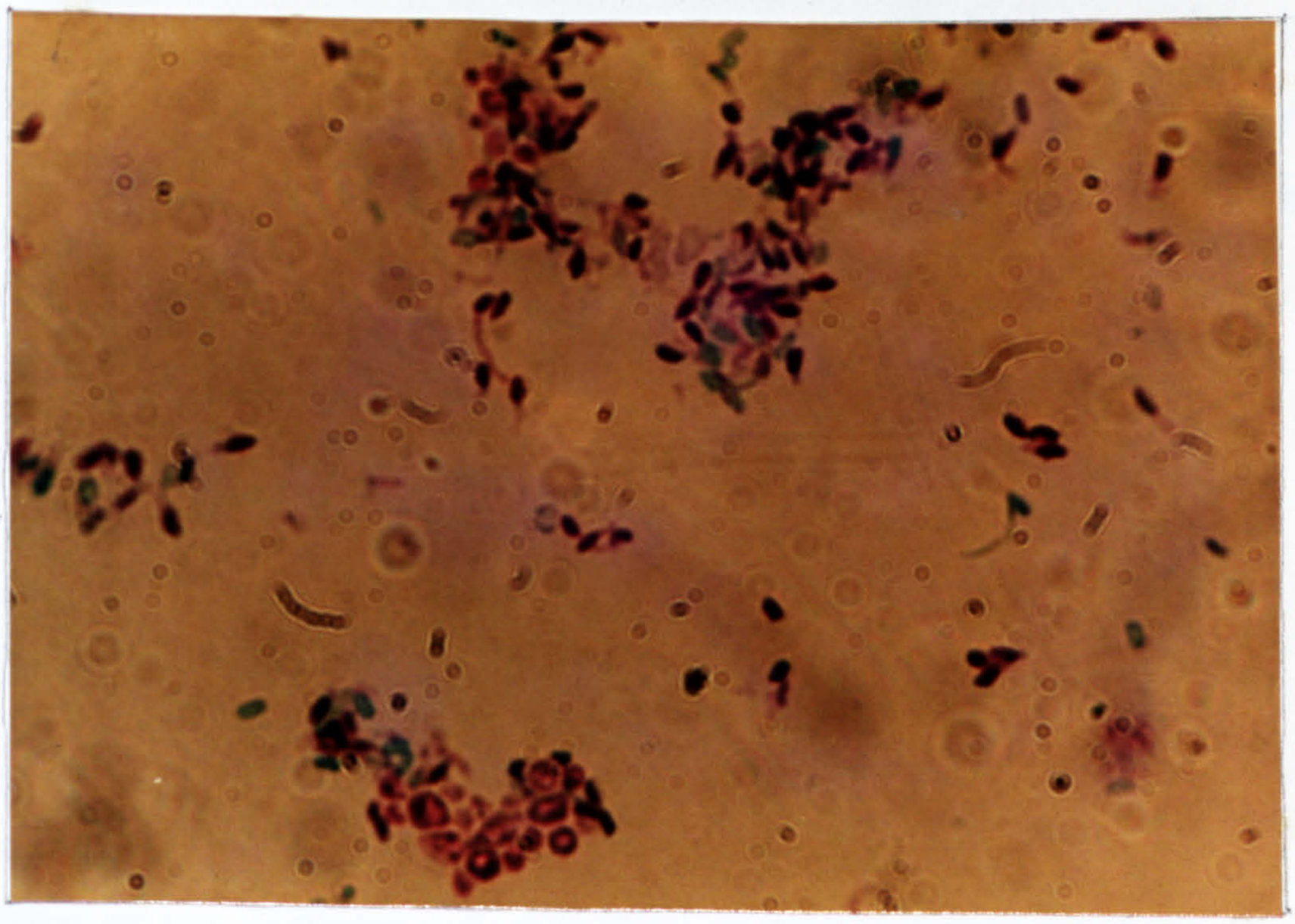

PLATE 11. Spores of B. stearothermophilus (NCIB 8919) stained using the malachite green stain after exposure to system XIII (i.e. heated 0.8% (w/v) sorbate and 2.5% (w/v) benzoate pH 4.2). Magnification x 1480

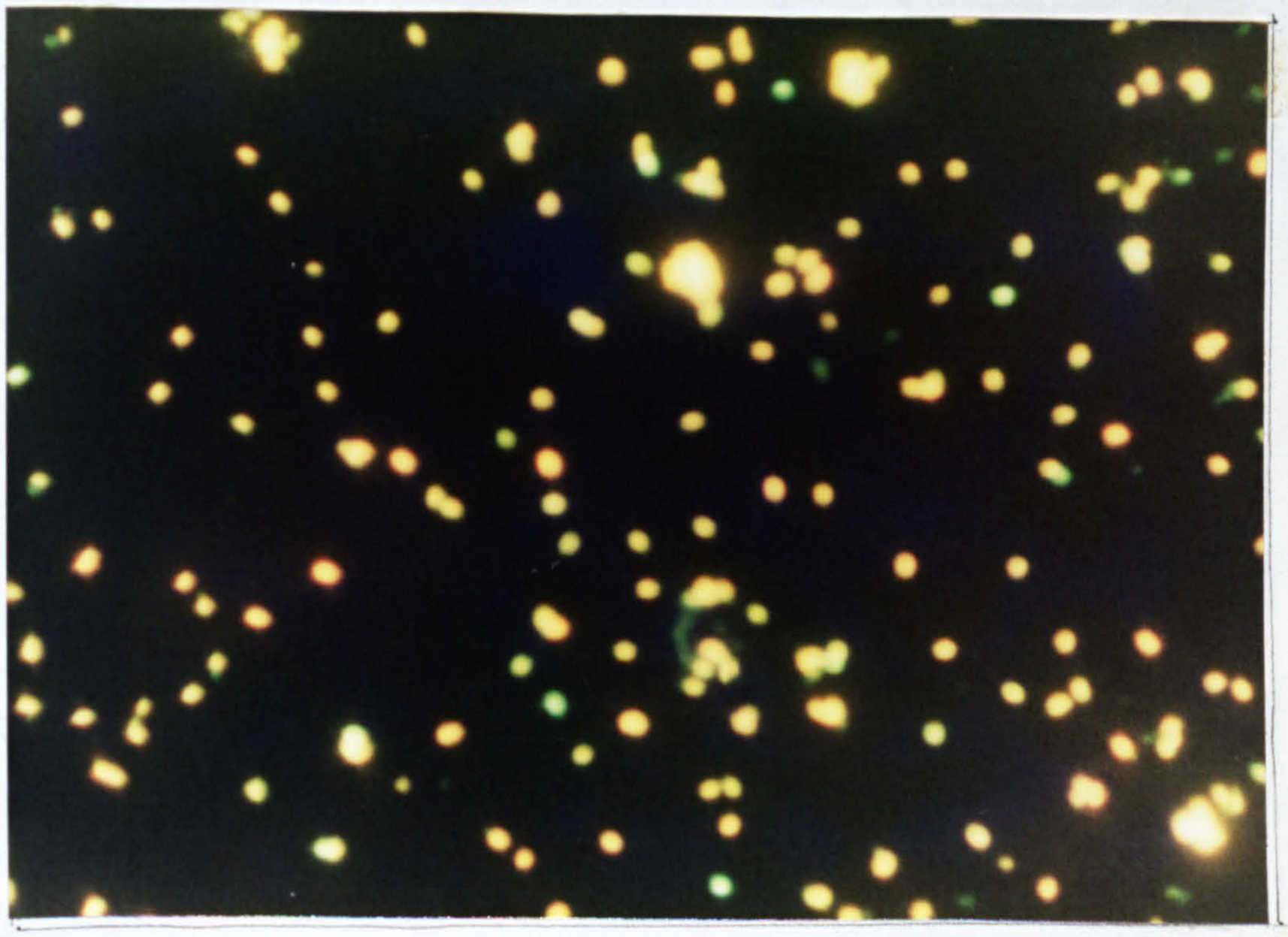

PLATE 12. Spores of B. stearothermophilus (NCIB 8919) stained using the acriflavine stain after exposure to system XIII (i.e. heated 0.8% (w/v) sorbate + 2.5% (w/v) benzoate pH 4.2). Magnification x 1480

ment of the spores. For example this is demonstrated by B. stearothermophilus (NCIB 8919) after exposure to Systems I, III, VIII and IX (Table 27). This observation is supported by Plates 2, 4, 6 and 8. The highest OF spore counts represent the following percentages of GF:

System	OF (as percentage of GF)	Test Strain
V	93.2	B. cereus 6349 (Table 28)
V	95.9	" 7464 "
VII	94.8	B. stearothermophilus 8920 (Table 27)
IX	83.0	" 8919 (Table 27)

Effects of the combination of heat, sorbate, benzoate and pH.

The microscope counts in Table 29 indicate that the GS spore count was highest in System I for both B. stearothermophilus (NCIB 8919) and B. cereus (NCIB 6349). The RS spore count increased and the GS counts decreased with the severity of treatment, e.g. compare Systems I, III, XII and XIII and also Plates 1, 3, 9 and 11. Hence the highest RS spore counts represent the following percentages of GS:

System	RS (as percentage of GS)	Test Srain
XI	91.8	B. cereus 6349 (Table 29)
XIII	88.1	B. stearothermophilus 8919 (Table 29)

The results of the ACFS spore counts also followed the same pattern as the MGS spore counts as shown in Table 29. The highest GF spore counts was found with System I. While the OF spore counts increased the GF counts decreased with severity of treatment. This is illustrated by Systems I, III, XII and XIII as well as Plates 2,

4, 10 and 12. Thus the highest OF spore counts represent the following percentages of GF:

System	OF (as percentage of GF)	Test Strain
XI	97.6	B. cereus NCIB 6349 (Table 29)
XIII	94.9	B. stearothermophilus NCIB 8919 (Table 29)

Discusson

The present experiment has confirmed the preliminary results that a correlation exists between the RS of the MGS and OF of the ACFS spore counts. For spores exposed to a combination of heat, sorbate, NaCl and pH the correlation is illustrated by the ratios 0.89 - 0.95 as below:

System	RS:OF	RATIO	Test Strain
V	7.48 : 8.34	0.90	B. cereus 6349 (Table 28)
V	7.98 : 8.43	0.95	" 7464 "
VII	7.05 : 7.94	0.89	B. stearothermophilus 8920 (Table 27)
IX	6.58 : 7.34	0.90	" 8919 (Table 27)

Similarly for spores exposed to a combination of heat, sorbate, benzoate and pH the correlation is shown by the ratio 0.86 as below:

System	RS:OF	RATIO	Test Strain
XI	7.28 : 8.44	0.86	B. cereus 6349 (Table 29)
XIII	6.93 : 8.03	0.86	B. stearothermophilus 8919 (Table 29)

Hence these results confirm the potential of the DFM for the enumeration of spores after exposure to combination preservative systems.

Interestingly, system I which represented the unheated bacterial spores of the different strains was found to contain only GS and GF

spores Plates 1 and 2 respectively. This indicates a low level non-stainability and confirm that the test cultures contained mainly dormant spores.

Stainability of the spores was demonstrated in B. stearothermophilus and B. cereus after exposure to heat alone, combination of heat, sorbate, NaCl and pH as well as combination of heat, sorbate, benzoate and pH. Stainability of spores is attributed to permeability of the dormant spores (Gould, 1969). In the present study it was found that the more inhibitory the combinations of heat and chemicals the more stainable were the spores as illustrated by B. stearothermophilus 8919 below:

System	RS (as percentage of GS)	OF (as percentage of GF)	
I	0	0	Extracted from Table 27
III	70.9	71.7	
VIII	73.6	73.8	
IX	83.0	83.0	

Hence it can be inferred that the more inhibitory the combination treatments the more permeable became the spores. It is therefore considered that the combination treatments of heat and chemicals affected the spore wall. The extent of the effect being dependent on the extent of inhibition. The systems IX and XIII which caused 100% inhibition of B. stearothermophilus (NCIB 8919) spores showed that almost all the spores were orange fluorescent (Plates 8 and 12).

9. SCANNING ELECTRON MICROSCOPY (SEM) STUDIES OF BACTERIAL SPORES EXPOSED TO COMBINED TREATMENTS

Electron Microscopy is an important tool for ascertaining the structure and composition of the bacterial spore (Aronson and Fitz-James, 1976). Evidence from the structural organization of the spore as revealed by Electron Microscopy has contributed to our present knowledge of the heat resitance of bacterial spores (Lewis, Snell and Burr, 1960; Gould, 1977; Warth, 1978). A study by Mallidis (1981) related the structure of ultrathin sections shown in electron micrographs to a proposed heat resistance mechanism.

SEM has been used by Bulla et al. (1969), Holt and Leadbetter (1969), St. Julian, Bulla and Hesseltine (1971) and Mackey and Morris (1972) to reveal the detailed surface structures of bacterial spores. Structural changes on B. subtilis spores after inactivation by microwaves have been studied using SEM (Al-Shawa, 1981).

From the results of the Fluorescence Microscopy experiments it was decided to use SEM to examine the spores before and after exposure to the combination of heat and chemicals. The investigation was undertaken to determine possible structural changes of the spores as a result of inactivation by combinations of heat and chemical treatments.

Materials and methods

Test cultures: Spore suspension of B. stearothermophilus (NCIB 8919) was employed as the test organism.

Test systems: The systems used in this study are shown in Table 30 which had been employed for the Fluorescence Microscopy studies. The percentage inhibition values were extracted from the

results in Sections 5 and 6. Heating was carried out at 95°C for 45 min in menstrua containing the chemicals shown in parentheses.

TABLE 30. Systems used for the SEM studies.

Systems	Description	Percentage inhibition
I	Unheated	-
II	Heated (95°C for 45 min)	-
III	Heated (0.8% sorbate + 8% NaCl pH 5.2)	92.2
IV	Heated (0.8% sorbate + 8% NaCl pH 4.2)	100
V	Heated (0.8% sorbate + 0.5% benzoate pH 5.2)	54.1
VI	Heated (0.8% sorbate + 2.5% benzoate pH 4.2)	100

Heat treatment and recovery procedure: The procedure employed was as described in Section 3, p. 50 whereby heating of suspersion of B. stearothermophilus containing 10^7 spores/ml was performed.

The processed spore suspensions was poured into plastic conical based universal bottles and centrifuged at 2000g for 5 min on five successive occassions using sterile distilled water to resuspend the pellet obtained. The same procedure was used for the unheated control.

Specimen preparation: The pellet which was obtained for each system was re suspended in 10 ml of 10% buffered glutaraldehyde solution (i.e. 1 ml of 2.5% (w/v) glutaraldehyde made up to 10 ml with 0.2M sodium cacodylate buffer pH 7.4) for a period of 24 h. Specimens were centrifuged on three successive occassions at 2000g for 5 min using sodium cacodylate buffer pH 7.4 to remove the buffered glutaraldehyde. The pellet was successively resuspended in increasing concentrations of 50, 70 and 100% (v/v) ethanol to

dehydrate the samples, centrifuging each time at 2000g for 5 min finally suspending in 2 ml ethanol. Exactly 0.05 ml of the spore suspension was placed on Microscope slide and allowed to dry in a desiccator.

Specimen mounting: Each Microscope slide containing the dried specimen was stuck onto the surface of an aluminium Scanning Electron Microscope stub which had been previously covered with Scotch double-sided adhesive tape.

Specimen coating: Each Microscope stub was carefully coated round the edges using evaporated carbon. The Microscope stubs were transferred to a Sputter Coater S150A apparatus (Edwards, High Vacuum Ltd. England) to be coated with vapourised pure gold. The stub specimens were placed on the stub specimen holder of the coater and coated with gold to a thickness of 100Å to 200Å. The specimens were left to cool for a few minutes before air was carefully admitted into the bell jar of the coater.

Scanning of spore preparations: The coated spore specimens were examined using the Philips PSEM - 500 Scanning Electron Microscope (Philips, Holland). The equipment was operated following the procedures as described in the manufacturers handbook. The Scanning Electron Microscope was operated at beam voltage of 12 KV for all specimens; the surface of each specimen being scanned at various magnifications and recorded photographically on an Ilford FP 4 film.

Results

The scanning electron micrographs of *B. stearothermophilus* spores (NCIB 8919) after exposure to combined treatments of heat and chemicals are shown in Plates 13 - 18.

Plates 13a and b show the general appearance of untreated

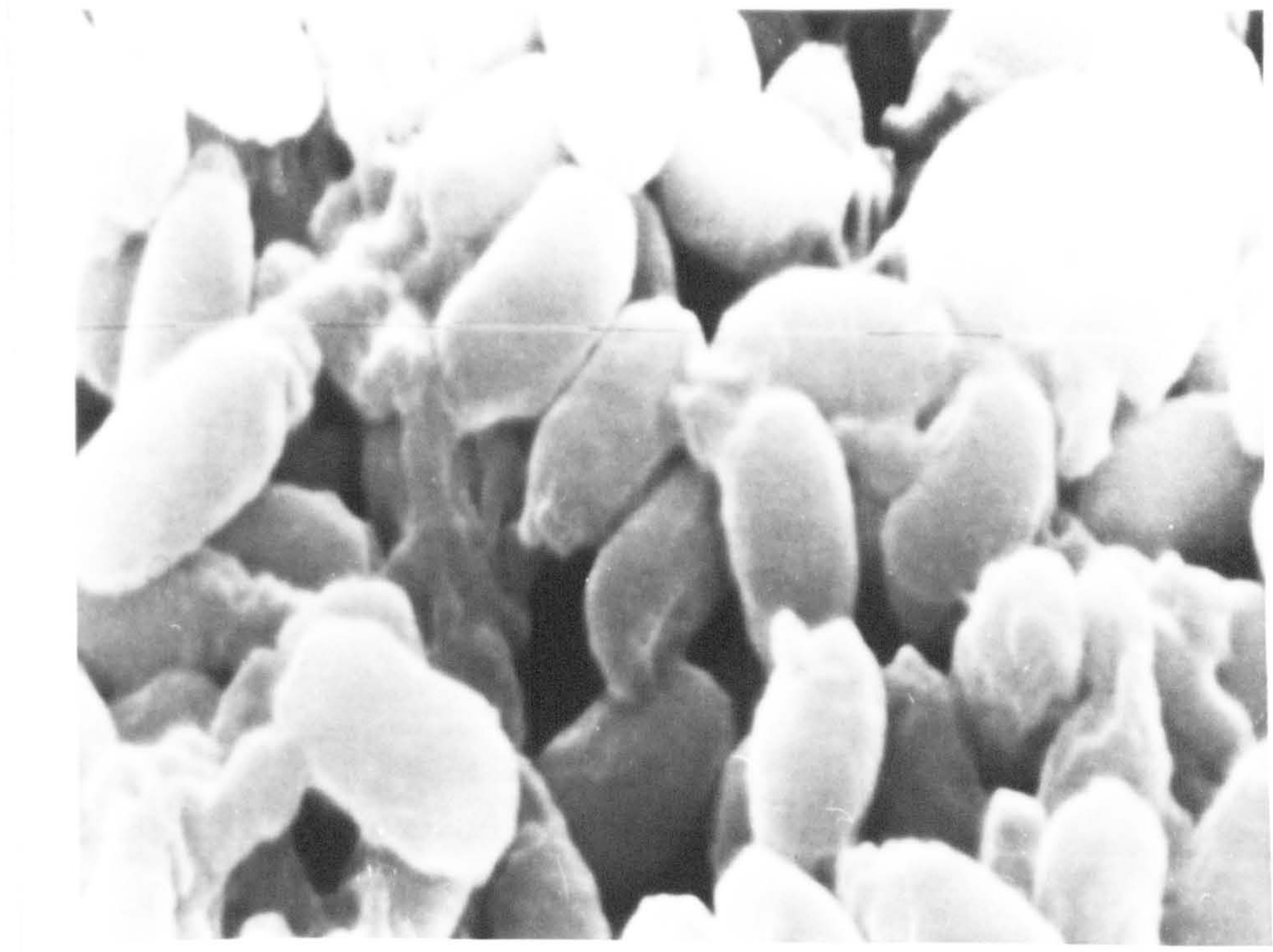

PLATE 13a. Scanning electron micrographs of untreated B. stearothermophilus (NCIB 8919) spores. Magnification x 10,000; marker represent 0.7 µm.

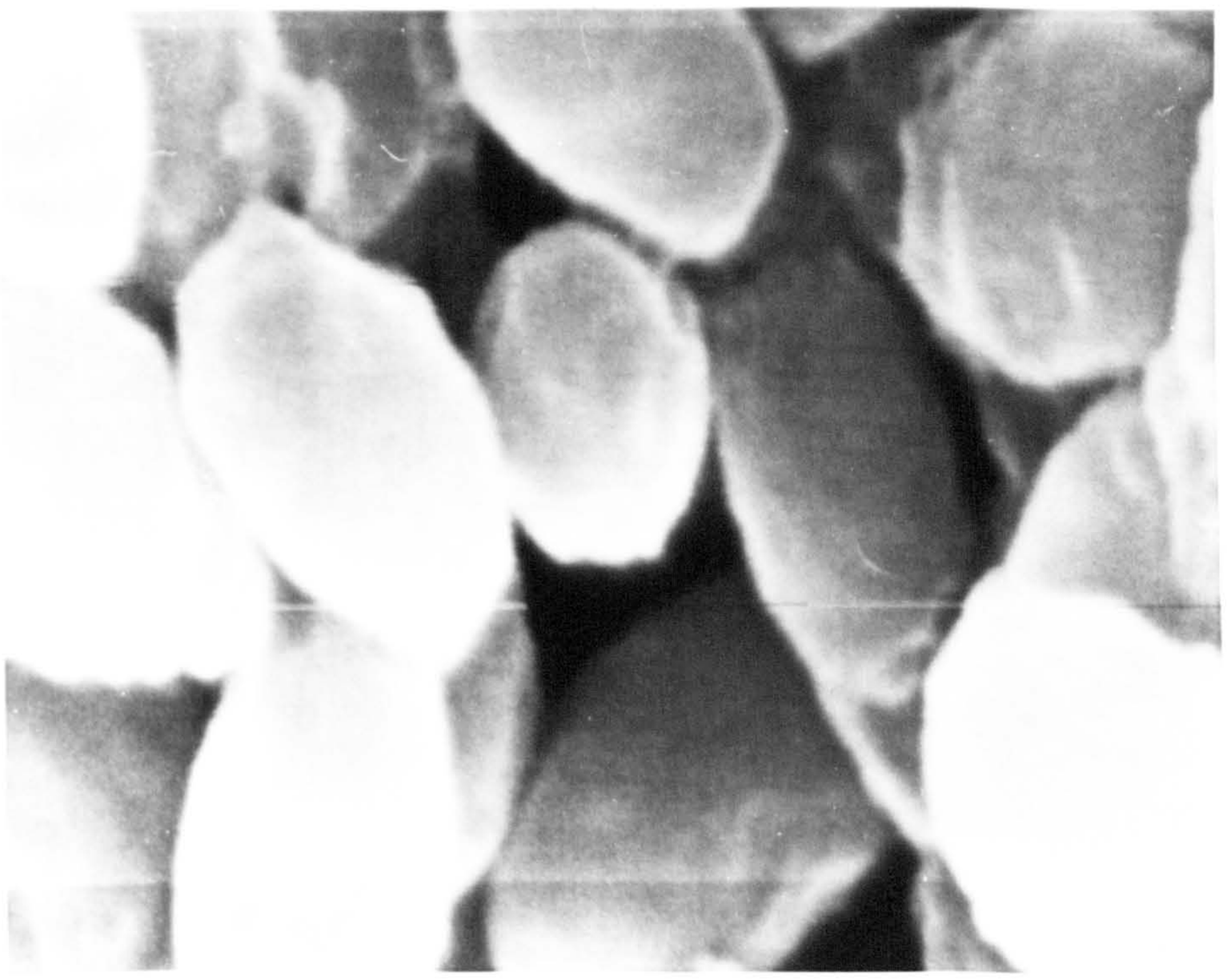

PLATE 13b. Scanning electron micrographs of untreated B. stearothermophilus (NCIB 8919) spores. Magnification x 20,000; marker represent 0.7 µm

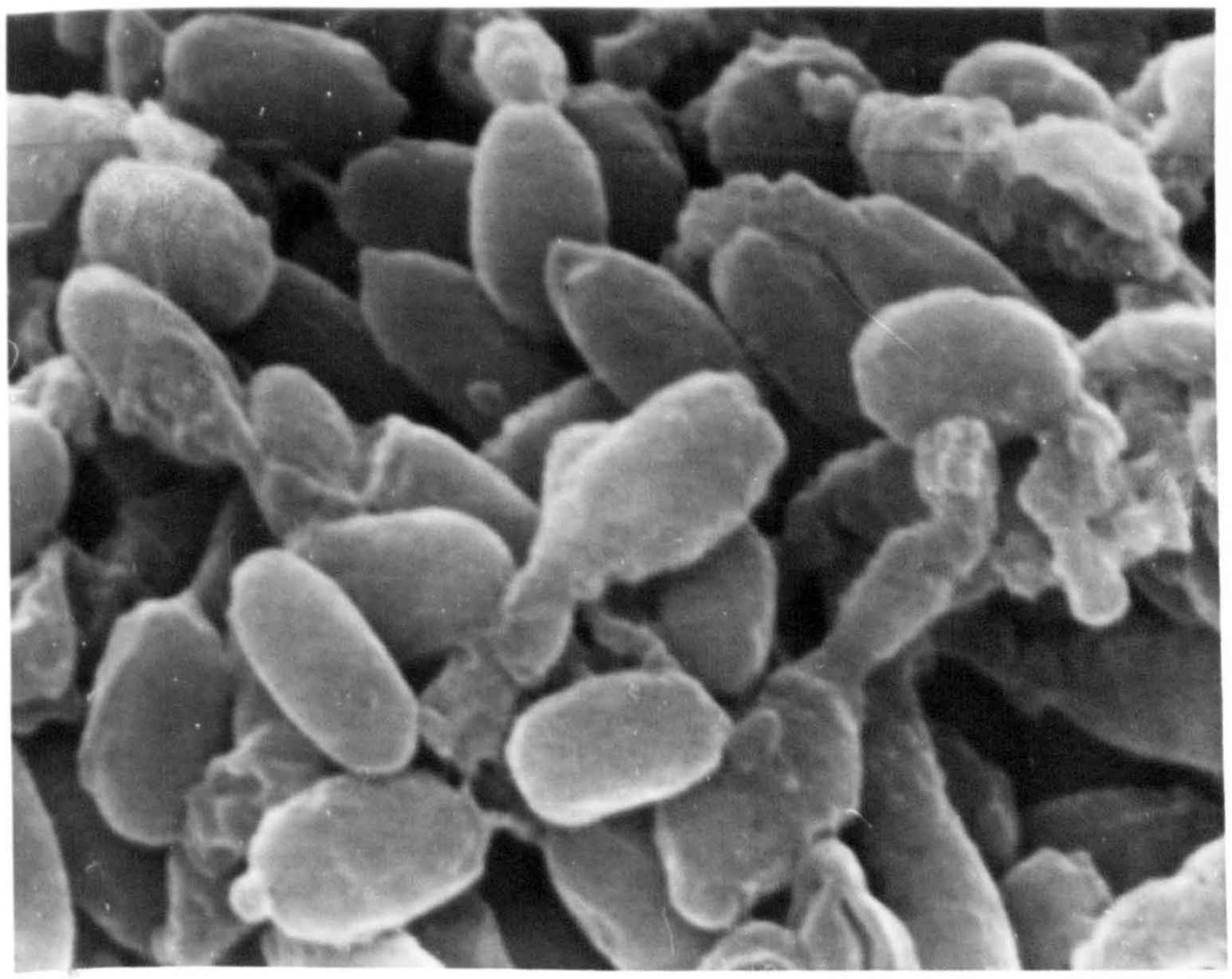

PLATE 14. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat at 95°C for 45 min (system II). Magnification x 10,000; marker represent 0.7 µm

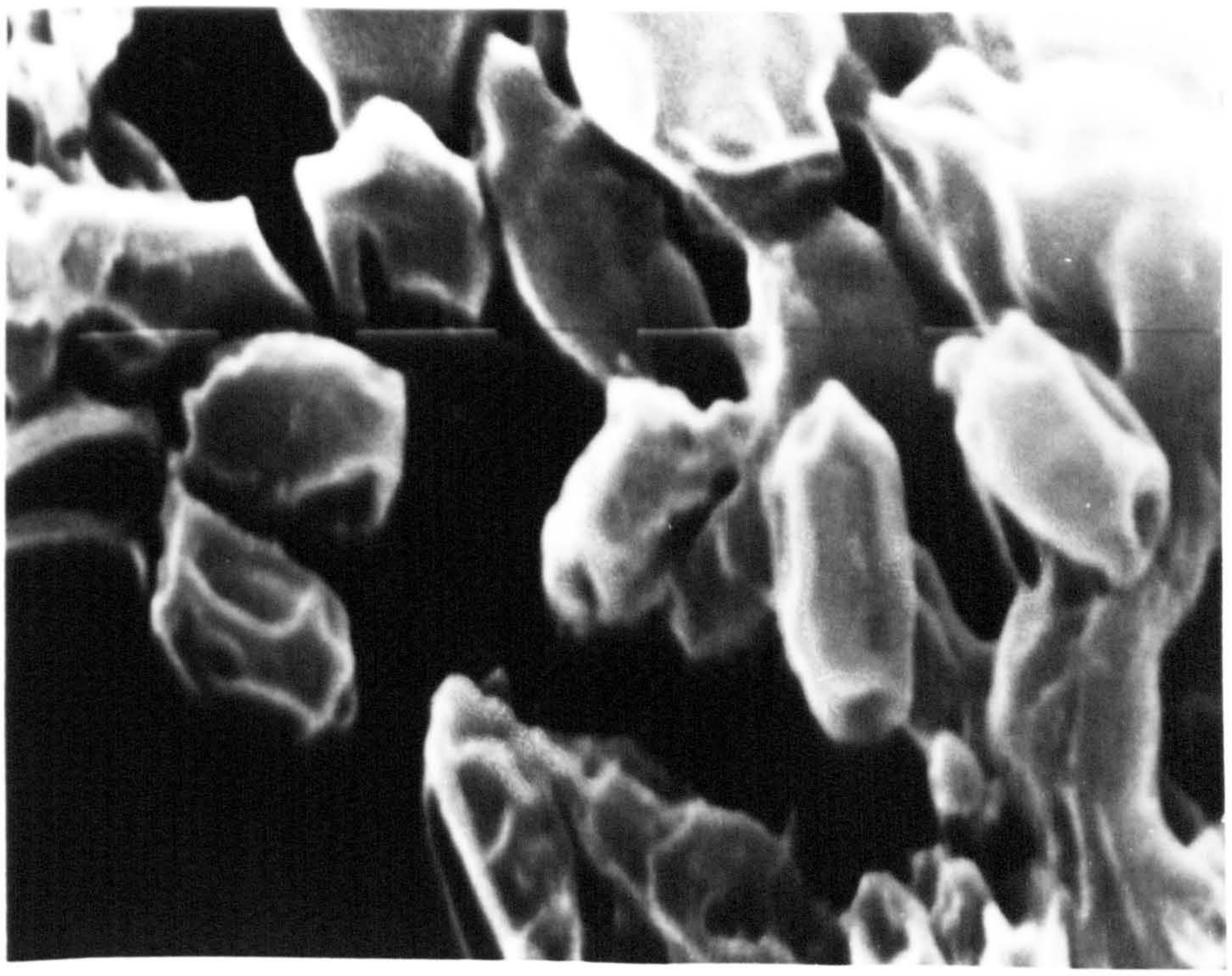

PLATE 15. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat (0.8% sorbate + 8.0% NaCl pH 5.2; system III). Magnification x 10,000; marker represent 0.7 µm

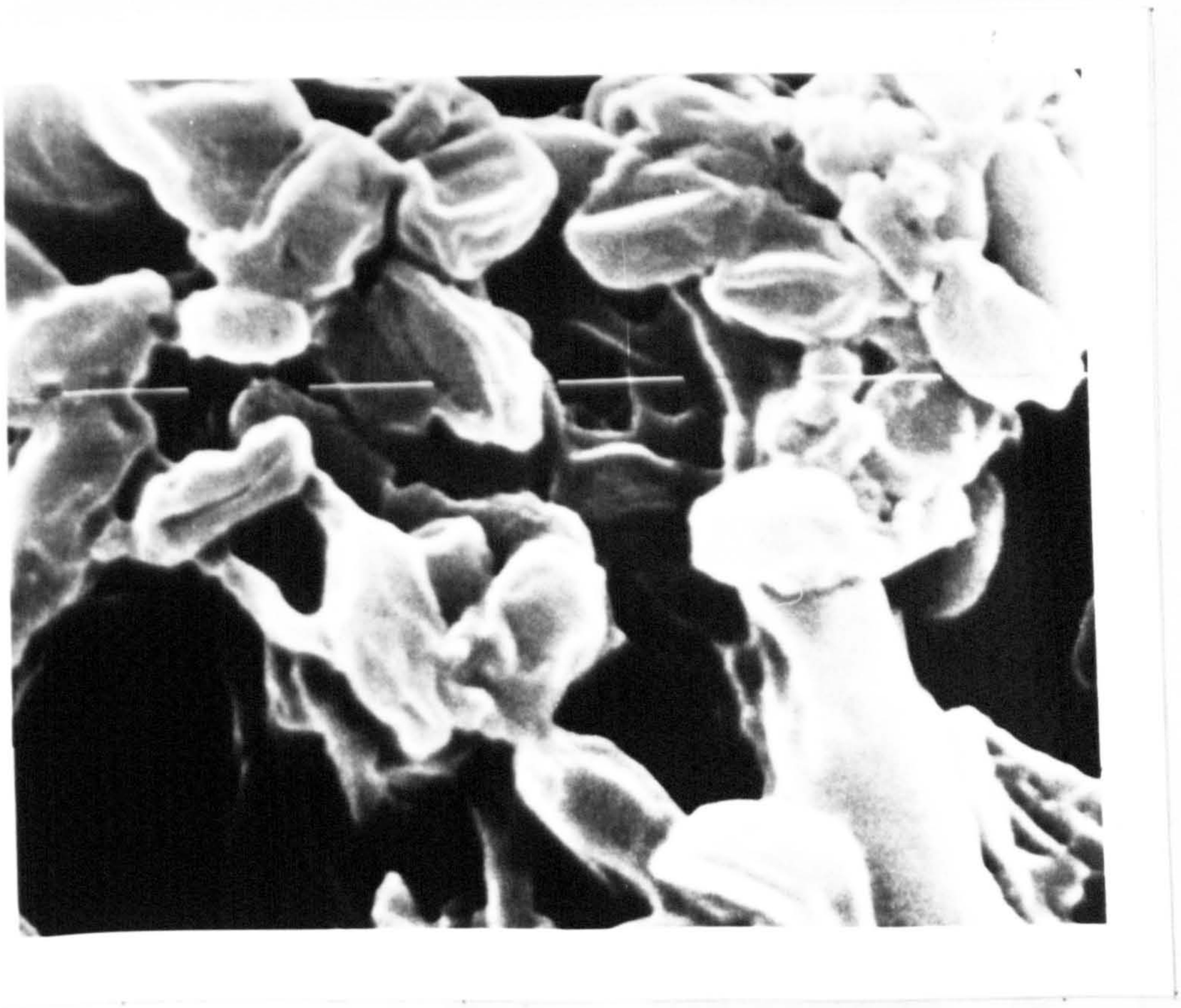

PLATE 16a. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat (0.8% sorbate + 8.0% NaCl pH 4.2; system IV). Magnification x 10,000; marker represent 0.7 µm

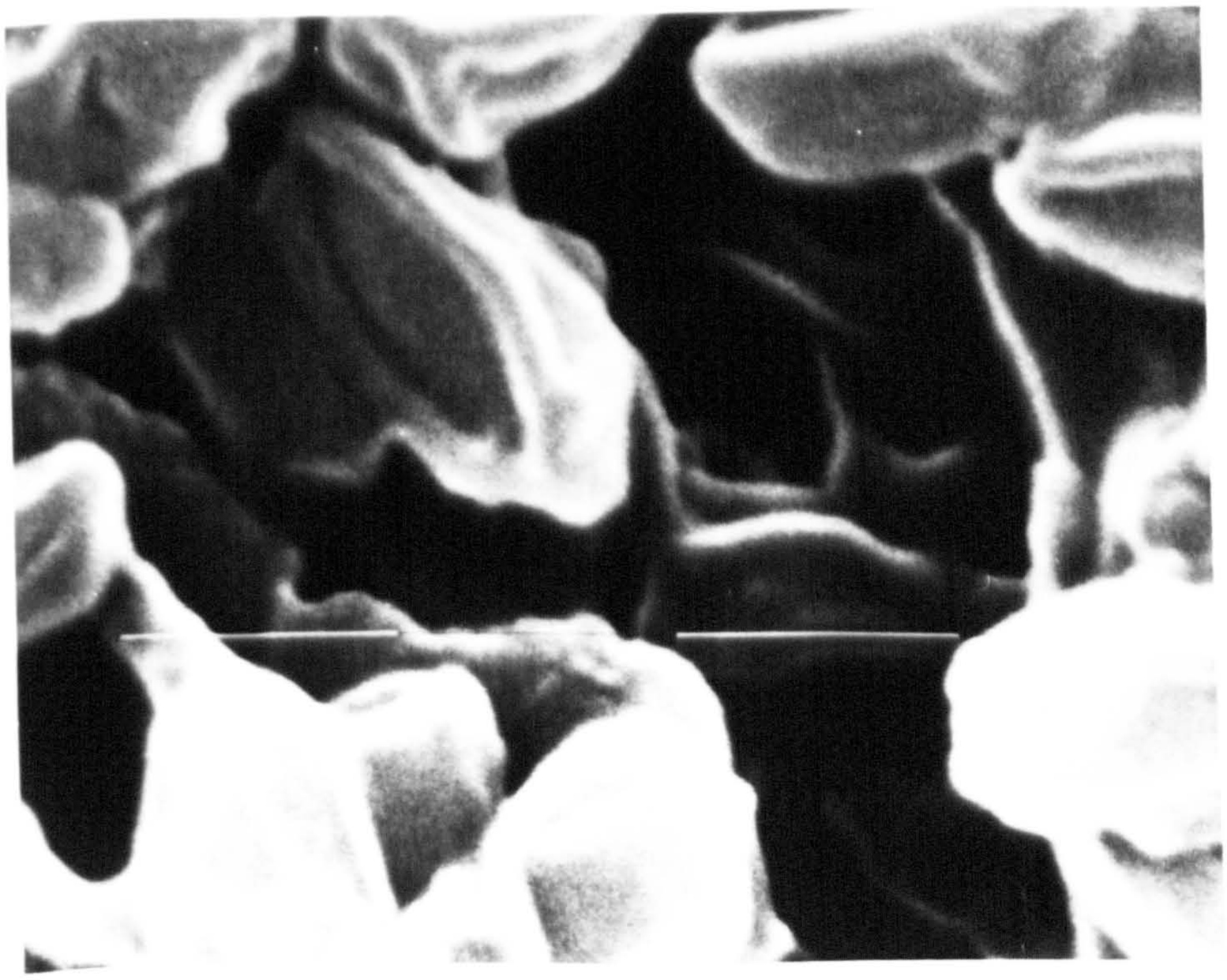

PLATE 16b. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat (0.8% sorbate + 8.0% NaCl pH 4.2; system IV). Magnification x 20,000; marker represent 0.7 µm

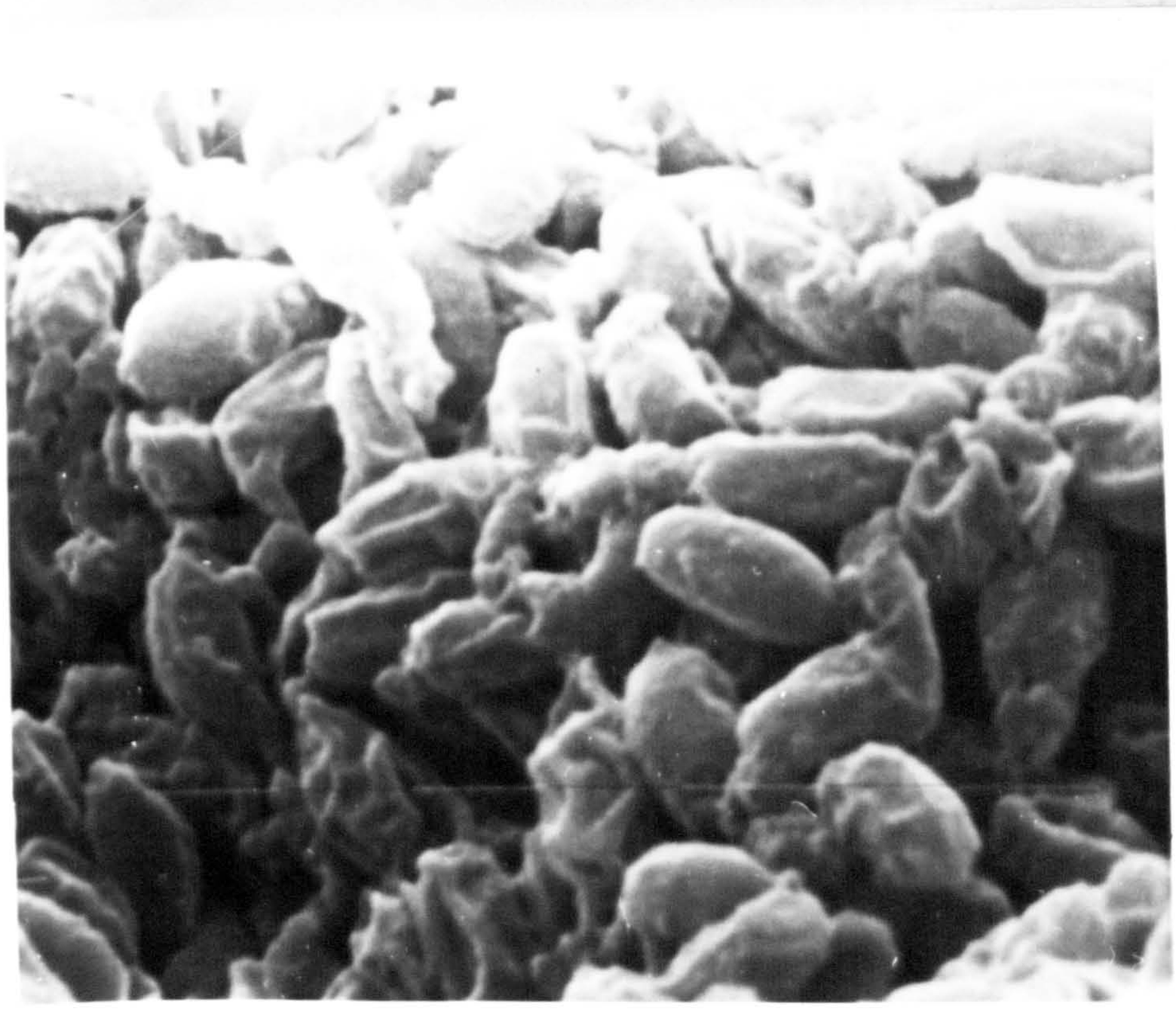

PLATE 17. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat (0.8% sorbate + 0.5% benzoate pH 5.2; system V). Magnification x 10,000; marker represent 0.7 µm

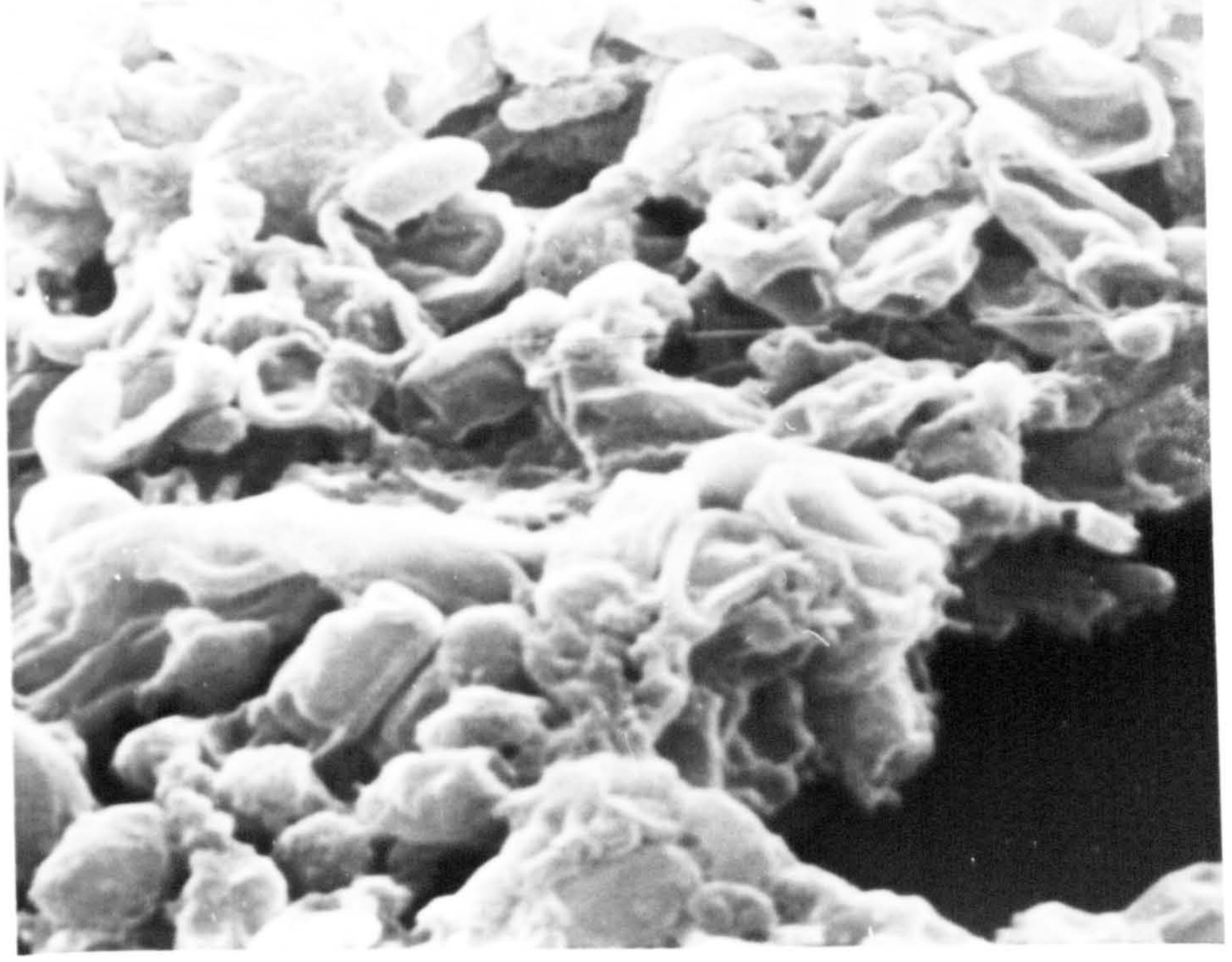

PLATE 18. Scanning electron micrographs of spores of B. stearothermophilus (NCIB 8919) after exposure to heat (0.8% sorbate + 2.5% benzoate pH 4.2; system VI). Magnification x 10,000; marker represent 0.7 µm

spores in control system I. It can be seen that the spores had an oval or rounded - corner cylindrical shape. This is better illustrated in Plate 13b, oval shapes being typical of B. stearothermophilus spores (Fields, 1970).

Spores which were exposed to system II did not demonstrate differences in the oval shape or any alteration of the surface characteristics (Plate 14) compared with the control. Plate 15 shows the appearance of the spores after subjection to system III. Some spores lost their oval shapes and a few exhibited crevices or indentations on the surface. However, the remainder maintained their integrity. By decreasing the pH of system III to 4.2 i.e. system IV, a much higher proportion of spores lost their oval shapes and were reduced in size (Plates 16a and b). Most spores exhibited a collapsed central portion and spore content were apparently lost. This is well illustrated in Plate 16b.

For spores exposed to system V a few spores showed crevices on the surfaces but most still retained their oval shapes (Plate 17). Interestingly, by decreasing the pH of system V to 4.2 and increasing the benzoate concentration to 2.5% i.e. system VI, the structure of the majority of spores collapsed and crevices on the surfaces became more pronounced. However, very few spores which still retained their original shapes were shortened in length (Plate 18). Those spores with crevices had also apparently lost their contents.

Discussion

This investigation demonstrated little alteration of the spore wall after simple heat activation at 95°C for 45 min which contrasts with a previous study by Moberley, Shafa and Gerhardt (1966) who showed that the spore coat of B. anthracis became more mottled and

the cytoplasm was less granular compared with the dormant spore. However, in the latter study only ultrathin sectioning techniques were employed and different strains were involved.

The small number of surface indentations or crevices shown by spores after exposure to system III imply that the combined treatments had affected the structure of the spore wall. A similar conclusion relates to treatment of the spores by system V. These systems did not totally inactivate the spores and SEM clearly indicated that most spores still retained their integrity. It is relevant to note that the fluorescence micrographs (Plates 6 and 10) which show that some spores are still stained green support the SEM findings. Green stained spores are indications of dormant spores which still retain their integrity.

The most effective combined treatments, system IV led to pronounced changes in the structure of the spore wall. The surface changes observed (Plates 16a and b) can be attributed to the spore wall destabilisation by the multiparametric system which lead to some spores losing their spore contents. Since exposure to system VI also led to similar effects, damage to the spore wall may account for the indentations and crevices observed and crumbling of the spore structure.

This study has clearly shown that heat activation in the presence of chemicals leads to alteration in the spore coat structure. Keynan and Evenchik (1969) reported that heat activation influences the permeability of spores. Similarly evidence from the present Fluorescence Microscopy study indicates increased permeability with increased severity of treatment. It is therefore postulated that heating of the spores in the presence of the

chemicals caused spore coat damage which increased the permeability of the spores to the chemicals. This would result in the penetration of the chemicals which may act at sensitive sites and therefore cause irreversible damage. The extent of damage may be dependent on a combination of the chemicals and the pH of the menstruum. This study has demonstrated that SEM can be useful for elucidating the mechanism of action of multiparametric systems in bacterial spores.

(b) DNA ASSAY OF SPORE SUPERNATANT AFTER EXPOSURE TO COMBINED TREATMENTS

Evidence from the Scanning Electron micrographs (Plates 16a, b and 18) clearly indicated spore wall damage with loss of spore content. This was shown when spores were exposed to combinations of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl in a menstruum of pH 4.2. A similar observation was made with spores exposed to heat at 95°C for 45 min in a menstruum of pH 4.2 containing 0.8% sorbate and 2.5% benzoate. In order to confirm this observation, the following experiment was undertaken to determine the DNA content of the menstruum after exposure of spore to the combined treatments.

Materials and methods

Test culture: B. stearothermophilus (NCIB 8919).

Test system: Systems I to VI as described in Table 30 were used.

Heat treatment: The procedure described in Section 3, p. 50 was used with citrate - phosphate buffer containing the appropriate concentrations of chemicals as the heating menstruum. An aliquot (0.1 ml) of the spore suspension containing 10^7 spores/ml was used as inoculum. After the desired heat treatment, the menstruum was poured into a conical based universal bottle (Sterilin Ltd.) and centrifuged at 2000g for 5 min. The supernatant was carefully decanted and used for the DNA estimation.

Standard curve for DNA estimation: The method of Hill and Whatley (1975) was employed. A stock solution was prepared containing 0.3 mg/ml mithramycin (Pfizer Ltd. UK) and 91.49 mg/ml $M_gCl_2.\ 6H_2O$ (BDH, Poole, UK) which promotes DNA - mithramycin

interaction. A stock solution of calf thymus DNA (Sigma type 1) 10 µg/ml was also prepared.

DNA concentrations ranging from 0 to 10 µg/ml was prepared by taking the appropriate volume from the stock solution and to 0.1 ml of the stock mithramycin, 0.1 ml of $MgCl_2 . 6H_2O$ was added. The mixture was made up to 3 ml with sterile distilled water and the fluorescence of the 3 ml aliquot was determined at 540 nm in a Perkin Elmer 3000 Fluorescence Spectrophotometer using an excitation wave length of 440 nm. Determinations were carried out in triplicate for each standard and the standard curve prepared (Figure 19).

DNA estimation in treated samples: The DNA concentration of the heated - spore supernatant was carried out using 0.6 ml aliquots. The aliquot was made up to 3 ml with sterile distilled water after the addition of 0.1 ml mithramycin and 0.1 ml $MgCl_2 . 6H_2O$. Determinations were then carried out as described for standard DNA for systems I to VI. Each determination was carried out in triplicate.

TABLE 31. Concentration of DNA from the supernatant of B. stearothermophilus after exposure to combined treatments.

System	Fluorescence Values	DNA (µg/ml)	Percentage[a]
I	108	0.54	<0.1
II	106	0.20	<0.1
III	126	3.6	12.4
IV	155	8.5	29.3
V	-[b]	-	-
VI	164	10.0	34.6

[a]Percentage obtained by calculating DNA concentration as fraction of DNA in intact spore. The DNA concentration of intact spores was calculated from the data given by Doi (1969) to be 28.98 µg/ml/10^7 spores.

[b]Not done.

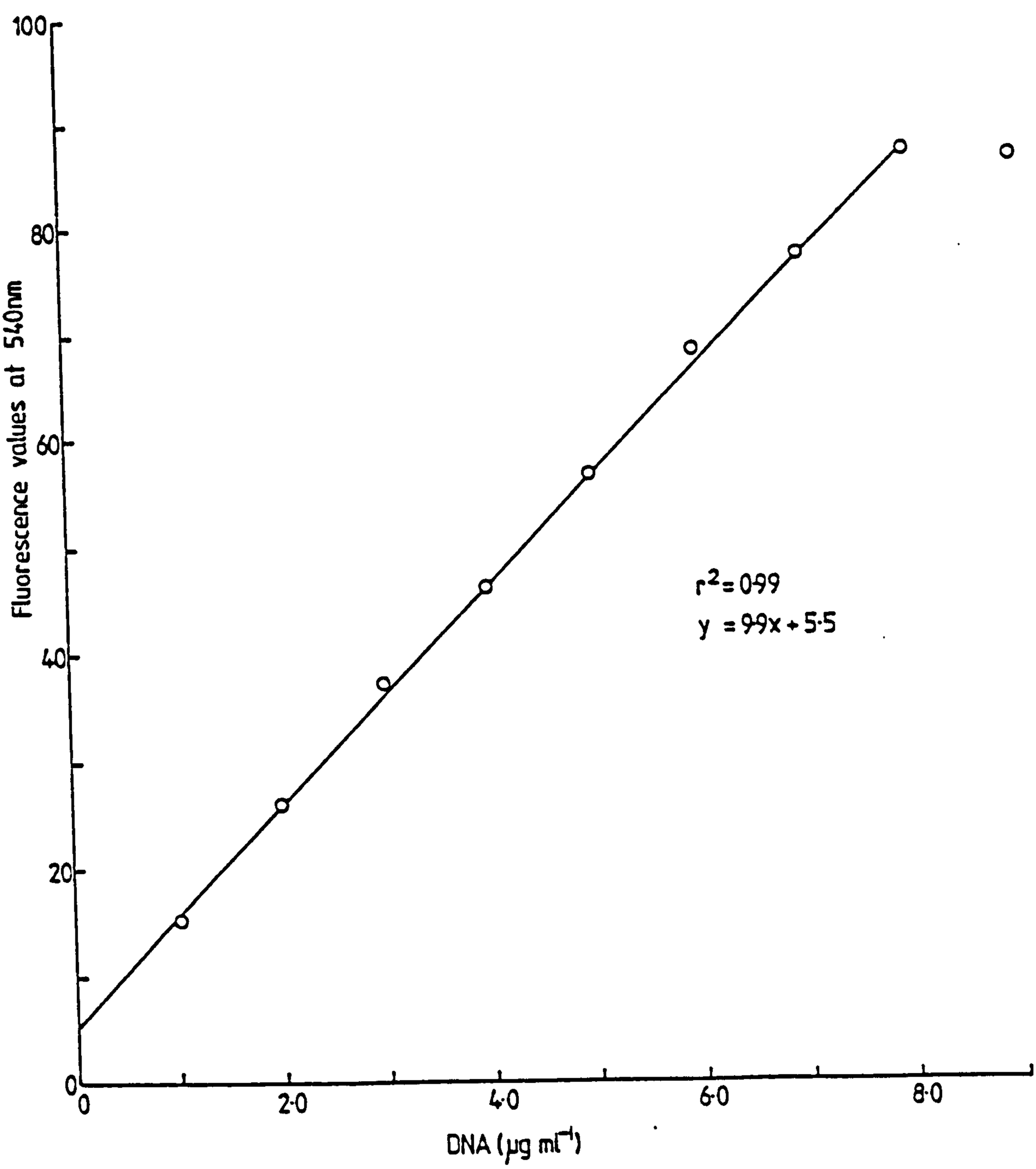

FIGURE 19. Standard curve for DNA determination.

Results and Discussion

The concentration of DNA released into the menstruum after heating under various experimental conditions is shown in Table 31.

Although the results obtained are not conclusive they show that systems IV and VI were most effective in inhibitory terms and caused the greatest release of DNA 29 and 35%, respectively of the DNA content of the intact spore. The result clearly confirms the conclusions made from the Fluorescence Microscopy and SEM experiments (Plates 16a, b and 18) that there was loss of spore content and it is now concluded that the loss was DNA or a similar material. System III which was moderately inhibitory caused the release of about 12% of the DNA content and for systems I and II little or no DNA was released. Although a small number of crevices were observed on spore surfaces (Plate 15), loss of spore content was not as apparent as demonstrated for system III treatment in the present study. However, the presence of little or no DNA in systems I and II is supported by the intact nature of the spores as shown in Plates 13a, b and 14.

The results of this experiment confirmed that the most inhibitory systems IV and VI caused the greatest release of the spore content identified in part as DNA. It can therefore be proposed that these inhibitory systems not only affected the spore wall as shown by the SEM study but also affected the spore core. DNA is located in the spore core (Russell, 1982) and while the above deduction can be made, further experimental study is necessary to confirm this novel proposition.

GENERAL DISCUSSION

In food processing heat is the most widely used agent for the destruction of food spoilage and food poisoning microorganisms including bacterial spores. However, the heat treatment required to achieve the necessary reductions in microbial load is often detrimental to the organoleptic qualities of the food. In addition the cost of energy input to achieve the desired process is high.

The combination of heat with other factors was proposed as a means to control microbial growth by Leistner and Rodel (1976) and as a practical alternative for food preservation in the 1980s (Smith and Pintauro, 1980).

The present study was conducted to investigate the effect of combinations of chemical preservatives with heat on selected microorganisms and particularly bacterial spores. The chemicals included potassium sorbate, sodium benzoate and sodium chloride.

In an initial experiment it was found that the effect of the combinations of heat at 55°C for 15 min with 1% sorbate caused a marginal inhibitory effect, 15.4% on S. aureus (NCIB 6571) and 23.8% on E. coli (NCIB 8414). Similarly the combinations of the chemical with heat at temperatures lower than 100°C had no marked effect on all strains of B. cereus and B. stearothermophilus spores probably due to the alkaline nature of the medium, pH 8.6.

Acidity is often used in food preservation in conjunction with other factors. In the present study the pH of the heating medium was adjusted with citrate - phosphate buffer, chosen because citrate is the common acidulant used in the food industry (Powers, 1975; Robach, 1980b). The results of this study showed that the sensitivity

of all test cultures, S. aureus, E. coli, B. cereus and B. stearothermophilus spores, to combinations of heat, sorbate and pH was dependent on both the pH and concentration of sorbate. The dependency of the inhibitory effect of sorbate on pH has also been reported by Robach (1978), Huhtanen and Feinberg (1980) and Sofos et al. (1980). The results showedthat reductions of pH 7.0 to 5.5 increased the inhibitory effect of combinations of heat and sorbate on E. coli and S. aureus.

A combination of heat at 50°C for 15 min and 0.2% sorbate at pH 7.0 caused 16.2% inhibition of E. coli. By decreasing the pH of the combination system to 5.5 the inhibition increased to 99.8%. The same combination treatments involving heat at 55°C for 15 min instead of 50°C for 15 min increased the inhibition of S. aureus from 26.4 to 99.98%.

Furthermore, increasing levels of sorbate as part of the combination treatments led to increased inhibition on the test strains at low pH. An increase in sorbate from 0.05 to 0.25% as part of a combination system involving heat at 50°C for 15 min and pH 5.5 increased inhibition of E. coli from 22.5% to 99.98%. Also at the same pH a combination treatment involving heat at 55°C for 15 min and increase in sorbate from 0.05 to 0.20% increased inhibition of S. aureus from 49.6 to 99.98%. In related studies by Shibasaki (1973) and Lusher et al. (1984) increasing concentrations of sorbate increased inhibition of E. coli although their studies did not involve variation in pH level. It is significant to note that while other combinations also showed to be inhibitory on E. coli and S. aureus the most inhibitory involved: a combination of heat at 50°C for 15 min and pH 5.5 with 0.25% sorbate for E. coli and combination

of heat at 55°C for 15 min and pH 5.5 with 0.2% sorbate for S. aureus. In each case an inhibition of 99.99 and 99.98% was obtained respectively. Interestingly inhibition of S. aureus by potassium sorbate has also been demonstrated in studies by Tompkin et al. (1974), Robach and Ivey (1978) and To and Robach (1980) but present study represents the first to report the effects of the combination of heat, sorbate and pH on this strain. Although the findings are preliminary they may be of importance to the food canners. Staphylococcus is a typical organism of raw beef with pH 5.4 - 5.8 (Corlett and Brown, 1982). It is suggested that the application of heat at 55°C for 15 min with 0.2% sorbate may be used to inhibit the organism if the food is to be heat processed. Earlier studies in support of this proposition include the findings by Tompkin et al. (1974) and Pierson et al. (1979) who have demonstrated that sorbate inhibited S. aureus in meat products. However, due to the limitations of laboratory media in studies of this nature, future studies are required to study this aspect.

The combinations of heat, sorbate and pH did not demonstrate a very pronounced effect on all the strains of B. cereus and B. stearothermophilus spores. However, a decrease in pH and increase in sorbate level increased the inhibitory effectiveness of the combination treatments. A decrease in pH from 6.5 to 4.5 as part of combination involving heat at 85°C for 30 min and 0.4% sorbate increased the inhibition of B. cereus 6349 from 6.6 to 29%. This was also demonstrated with B. cereus 7464 where the same combination treatments increased the inhibition from 12.2 to 37%. In a comparative study involving B. cereus T spores Smoot and Pierson (1981) found that 0.39% at pH 5.7 provided 97% inhibition of germination

while at higher pH levels very low inhibition was obtained. This was achieved after heat activation of the spores. A similar effect was also demonstrated with B. stearothermophilus. A decrease in pH from 6.5 to 4.2 as part of the combination of heat at 95°C for 45 min and 0.8% sorbate increased inhibition of strain 8919 from 14.2 to 64.7%. The same combined treatment increased the inhibition from 32.1 to 74.6% with strain 8920. In support of this finding Smoot and Pierson (1981) also found that 0.52% sorbate at pH 5.7 provided 95% inhibition of Cl. botulinum 62A spore germination while at a higher pH the inhibitory effect was decreased. The results of the present experiment may be important in the preservation of low acid and acid canned foods. Since B. coagulans and Cl. pasteurianum can cause spoilage in acid canned foods and are less heat resistant than B. stearothermophilus (Olson and Nottingham, 1980), it is suggested that the application of heat at 95°C for 45 min with 0.8% sorbate may be used to inhibit these organisms in such products. Furthermore the use of such combination treatments may be instrumental in resolving the problem of the growth of Cl. botulinum spores in tomato juice at pH 4.2 (Odlaug and Pflug, 1977). This proposition may be considered in future studies which should include the sensory evaluation of products with added sorbate.

Sodium chloride is a common component of foods and is often added to foods during processing. Reports by Viljoen (1926), Duncan and Foster (1968a), Cook and Gilbert (1969), Briggs and Yazdany (1970), Bühlmann et al. (1973) and Bell and DeLacy (1984) have considered its effects on microorganisms during heating. Conflicting results have been obtained in relation to spores. In the present study the effects of the combinations of heat, sodium chloride and pH on spores

was examined. Results have shown that the sensitivity of test strains to the combination treatments is dependent on the pH and NaCl levels. The inhibitory effectiveness of the combination treatments increased with decrease in pH. A decrease in pH from 6.5 to 4.5 as part of the combination treatments involving heat at 85°C for 30 min and 7.5% NaCl increased inhibition of B. cereus 6349 from 27.1 to 67.4%. A similar decrease in pH from 6.5 to 4.2 as part of the combination treatments involving heat at 95°C for 45 min and 8% NaCl also increased inhibition of B. stearothermophilus 8919 from 31.1 to 46.8%. Other test strains exhibited similar response patterns. The present results confirm the earlier findings by Coussins (1968) and Braithwaite and Perigo (1970) who also showed that the pH of the medium affected the response of B. stearothermophilus spores to a combination of heat and NaCl.

The effect of increasing NaCl as part of the combination treatments also provided an increased inhibition. An increase in NaCl from 1.5 to 7.5% as part of the combinations of heat at 85°C for 30 min and pH 4.5 increased inhibition of B. cereus 6349 from 18.1 to 67.4%. Other workers Cook and Gilbert (1969) and Briggs and Yazdany (1970) have also demonstrated that increasing NaCl levels increased the inhibitory effectiveness of the combinations of heat and NaCl on spores. However, their studies did not involve variation in pH. Significantly the results showed that the most effective combination of heat, NaCl and pH provided only marginal to moderate inhibition on B. cereus and B. stearothermophilus spores. It is indicated in the present study that both NaCl and pH may contribute to the control of mesophilic and thermophilic spoilage in pasteurized canned foods of low acidity.

Following the marginal to moderate inhibition of test strains by a combination of heat, NaCl and pH, subsequent experiments were designed to examine the effect of the combination of heat, sorbate, NaCl and pH on spores. Results of this experiment showed that irrespective of the test strain, sensitivity to combination treatments increased with:

(a) Decrease in pH: A decrease in pH from 6.5 to 4.5 as part of the combination treatment involving heat at 85°C for 30 min with 0.4% sorbate and 6% NaCl increased inhibition of B. cereus 6349 from 69.6% to 99.98%. Similarly a decrease in pH from 6.5 to 4.2 as part of a combination treatment involving heat at 95°C for 45 min increased inhibition of B. stearothermophilus 8919 from 51.8% to 100%. Treatments with B. cereus 7464 and B. stearothermophilus 8920 exhibited similar trend.

(b) Increase in sorbate level: An increase in sorbate form 0.1 to 0.4% as part of a combination treatment involving heat at 85°C for 30 min with 6% NaCl and at pH 4.5 increased inhibition of B. cereus 6349 from 73.2 to 99.98%. A similar increase in sorbate from 0.2 to 0.8% as part of a combination treatment involving heat at 95°C for 45 min and 8% NaCl at pH 4.2 increased inhibition of B. stearothermophilus 8919 from 81.4 to 100%.

(c) Increase in NaCl level: This followed the same trend as reported for increase in sorbate for all test strains. In a study by Robach (1980b) it was observed that the inhibition of outgrowth of Cl. sporogenes by potassium sorbate was potentiated by increasing levels of NaCl at pH 6. It is considered that the inhibitory effectiveness of the combination treatment may partly be due to interaction between sorbate and NaCl as also suggested by Robach (1980b).

Interestingly synergistic effects were demonstrated by combination of heat, sorbate, NaCl and pH with test strains. A combination of heat at 85°C for 30 min and 0.4% sorbate at pH 6.5 caused 7.3% inhibition of B. cereus 6349. The same combination treatment in the presence of 6% NaCl instead of 0.4% sorbate provided 19.6% inhibition of the same strain. However the same combination treatment in the presence of 0.4% sorbate and 6% NaCl increased the inhibition to 69.6%. The synergistic effect was also demonstrated at other pH levels and with the other test strains. For the test strains the most effective combinations in inhibitory terms were found to be:

Strain	Treatments
B. cereus 6349:	Combination of heat at 85°C for 30 min with 0.4% sorbate and 7.5% NaCl at pH 4.5
" 7464:	"
B. stearothermophilus 8919:	Combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2
" 8920:	" 6% NaCl "

Inhibition by the combination of sorbate, NaCl and storage temperature on growth of S. aureus has been reported by Robach and Stateler (1980). Roberts, Gibson and Robinson (1982) also showed that a combination of sorbate, NaCl and pH significantly reduced the growth of Cl. botulinum types A and B in pasteurized cured meats. However, the present study is the first to examine the response of B. stearothermophilus to the combination of heat, sorbate, NaCl and pH. In order to achieve a complete inhibition of these spores by the combination treatments, the temperature employed i.e. 95°C was below 115° - 150°C recommended for the sterilization of canned foods (Olson and Nottingham, 1980). This finding may offer potential for

reducing the severe heat processes used to control thermophilic bacteria in foods destined for markets in tropical climates. Since B. stearothermophilus are the major spoilage micro-organisms of canned low-acid vegetables such as peas, corn and potatoes (Fields, 1970) the inclusion of 0.8% sorbate and 8% NaCl would prevent this spoilage following heating at 95°C for 45 min. These experimental results need to be verified by considering the effect of food ingredients and additives in combination treatment processes.

In building up a picture of the effects of the combination of heat and chemicals on bacterial spores, spores were heated in the presence of sodium benzoate. The results of this experiment showed that sensitivity to the combination treatment was again dependent on pH and benzoate level. However, none of the combination treatments caused appreciable inhibition of spores. A decrease in pH from 5.2 to 4.2 as part of the combination treatment involving heat at 95°C for 45 min and 2% benzoate increased inhibition of B. stearothermophilus 8919 from 16.8 to 46.5%. This effect was also demonstrated with B. cereus 6349. It is known that a decrease in pH increases the antimicrobial effectiveness of sodium benzoate due to the increased level of undissociated acid (Chipley, 1983). This explains the increase in inhibition of the spores by the combined treatments at low pH. A similar inhibitory effect has also been demonstrated with yeasts (Beuchat, 1981; 1982). However, in the preliminary experiments no inhibition of spores was observed when the heating was carried out in an unbuffered medium containing benzoate, probably due to the alkaline pH 8.9 of the medium. Similar studies by Ralph (1967) and Boyazoglu (1969) indicated that the combination of heat and sodium benzoate had no effect on spores.

Further experiments were carried out to examine the effect of the combination of heat, sorbate, benzoate and pH on bacterial spores. The results showed that decrease in pH and increases in sorbate and benzoate levels as part of the combination treatments increased inhibition. Significantly combination treatments that caused 100% inhibition of the test strains was found to be:

Strain	Treatments
B. cereus 6349:	Combination of heat at 85°C for 30 min with 0.4% sorbate and 1.75% benzoate pH 4.5
B. stearothermophilus:	Combination of heat at 95°C for 45 min with 0.8% sorbate and 2.5% benzoate pH 4.2.

Similarly Beuchat (1981) showed that combination of heat, sorbate, benzoate and pH affected the growth of yeasts significantly.

Interestingly synergistic effect was demonstrated by a combination of heat, sorbate, benzoate and pH on test strains. The synergistic effect was shown at pH 4.5 for B. cereus and at pH 4.2 for B. stearothermophilus. It is therefore postulated that the inhibitory effect by the combination treatment is partly due to the acid condition of the heating medium and probably is due to the undissociated molecules of the acid. A combination of heat at 85°C for 30 min with 0.4% sorbate at pH 4.5 caused 37.2% inhibition of B. cereus 6349. The same combination treatment in the presence of 0.75% benzoate instead of 0.4% sorbate gave 26.5% inhibition. The synergistic effect was demonstrated by the same combination treatment in the presence of 0.4% sorbate and 0.75% benzoate by providing 99.99% inhibition of B. cereus 6349. The synergistic effect was also demonstrated with B. stearothermophilus 8919. Observations that sorbate and benzoate can combine with heat to inactivate B. stearo-

thermophilus and B. cereus spores in an acidic medium offers the potential for redefining process requirements for certain foods. The process requirement would be reduced without sacrificing sterility. However, the levels of sorbate and benzoate required to achieve the sterility may alter the sensory qualities of the foods and this aspect requires detailed study. Moreover, the response of bacterial spores in laboratory medium may differ from the response in foods under practical processing conditions.

There have been different claims by workers with regards to the most suitable recovery temperature of bacterial spores following exposure to heat or combination treatments. In the present study experiments were designed to determine the recoveries of B. cereus and B. stearothermophilus at certain temperatures following exposure to combination of heat, sorbate, NaCl and pH. Compared with recovery at 30°C, recovery of B. cereus 6349 and 7464 at 25°C and 37°C did not differ markedly after exposure to the combination treatments. However, recovery at 44°C potentiated inhibition by a combination of heat at 85°C for 30 min with 0.4% sorbate and 6% NaCl at pH 4.5. The recovery at this temperature i.e. 44°C caused reductions of 99.91% compared with that at 30°C. Furthermore, for B. cereus 7464 recovery was delayed at 44°C until after day 2.

Similarly, for B. stearothermophilus compared with 55°C recovery at 44°C enhanced the inhibition of the most effective combination treatments involving heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2. It was found that after exposure of spores to the same combination treatment no survivor was obtained at 44°C throughout the 15 days of investigation. The present finding may be significant when canned food is to be exported to tropical

climates where high ambient temperatures provide an environment conducive for the growth of thermophiles. The use of an appropriate combined treatment may adequately prevent spoilage by aerobic sporeformers. Recovery at 50°C did not differ appreciably from 55°C, the latter temperature being selected as control because it has been used in previous studies and is generally quoted as the optimum incubation temperature. Other workers Navani (1970), Abdelgadir (1974), Labbe (1979), Mallidis (1981) and Chopra and Marthur (1984) also used this temperature for recovering B. stearothermophilus.

At 60°C, 6% - 41% and 7% - 37% reductions of strain 8919 and 8920 respectively were obtained. This was achieved following exposure of strain 8919 to a combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2 while the same combination treatment with 6% instead of 8% NaCl was used for strain 8920. In addition recovery at 60°C was delayed until after day 6. These findings emphasize the need for prolonged incubation periods whenever quality control assessment of heat processed foods are being carried out. The results also demonstrate the caution required in the selection of recovery temperatures.

The experimental study of the effects of combination treatments on spores has generated much data in this study. In the present study results have been presented in terms of percentage survivors relative to a control system and have been represented in form of 3-dimensional histograms. The 3D-histogram is a novel approach for presenting spore survival data and was obtained using a developed computer program Section 3.3. Manually derived 3-dimensional block diagrams were used by Roberts et al. (1976) and Jarvis et al. (1979) in their work on the inhibition of Cl. botulinum in pasteurized cured meat by curing salts and other additives. However, this

study introduces the application of a computer program to draw 3D-histograms for the first time. This approach has the advantage that the data generated from studies can be stored, errors can be deleted and the histogram drawn out in near real time. The approach is an improvement on the cumbersome method of hand drawing block diagrams which take days to complete. The representation of data generated for combination treatments using 3D-histograms enables trends to be rapidly calculated and shown and the specific contribution by each factor can be clearly distinguished.

The application of staining techniques and particularly Direct Fluorescence Microscopy (DFM) for assessing survivors following exposure of bacterial spores to combined treatments was studied. Initial experiments indicated that correlation between the Plate Counts (PC) and the microscope counts red stained (RS) and orange fluorescent (OF) was fairly good as indicated by the ratios 0.74 - 0.99, 0.69 - 0.83 and 0.90 - 1.32 between PC:OF, PC:RS and RS:OF counts respectively. In a similar study by Manson (1977) a ratio of 0.75 - 1.25 was reported to signify reasonable correlation between two counting methods.

Correlation between the standard malachite green staining (MGS) and the DFM technique was indicated by the ratios between red stained (RS) and orange fluorescent (OF) spore counts. Ratios of 0.89 - 0.95 and 0.86 - 0.90 were obtained for B. cereus and B. stearothermophilus spores respectively. This followed exposure of test strains to a combination of heat, sorbate, NaCl and pH as well as the same combination involving benzoate instead of NaCl. The results demonstrate the potential of DFM techniques for the rapid non-cultural enumeration of spores after exposure to combined treatments. Furthermore

results of the experiment revealed that the more inhibitory the combined treatments the more permeable the spores became as inferred from the extent of stainability. Typical results indicate a range from nil orange fluorescent spores in the control system to 83% OF spores in completely inhibited B. stearothermophilus 8919 spores. It is considered that the exposure of spores to a combination of heat and chemicals affects the sporewall. Thus the DFM technique offers additional evidence to explain the mechanism of the action of combined treatments based on a consideration of the whole spore.

Although earlier study by Scholefield et al. (1976) showed that dead cells of Saccharomyces cerevisae stain dull green due to absence of binding sites, the stainability of the spores even after exposure to combined treatments may be due to the timing of the stainings. This was carried out almost immediately after treatment and with recently demised cells there are still binding sites and therefore are stainable (Scholefield, pers. commun.).

The present results indicate that with refinement the DFM technique offers an alternative to cultural counting methods for spores survival determinations. The advantage of being rapid means that automation of heat resistance determination and the on-line determination and computer analysis of data is possible. Such automated equipment is currently being developed by Scholefield (1985).

In the present study Scanning Electron Microscopy (SEM) was performed on B. stearothermophilus (NCIB 8919) spores before and after exposure to combined treatments. Results showed that exposure of spores to a combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2 caused pronounced changes in the

structure of the spore wall. These ranged from collapse of the central portion of spores to loss of spore contents (Plates 16a, b). Similar changes were observed after exposure of spores to a combination of heat at 95°C for 45 min with 0.8% sorbate and 2.5% benzoate at pH 4.2. With less effective combination treatments in inhibition terms the spore wall changes were found to be less pronounced while control samples exhibited intact spore walls. This study has shown that SEM may offer the potential for relating changes in spore structure and particularly the spore wall to combined treatments. A similar technique was used by Al-Shawa (1981) to identify structural changes in B. subtilis after inactivation by microwaves. In view of the changes observed in the present study it is considered that the observed damage to the spore wall was due to exposure of spores to the combined treatments.

Based on evidence from the DFM and SEM studies it is viewed that heating of spores in an acidic medium in the presence of chemicals caused extreme damage to the spore wall. The permeability of the spores to the chemicals is increased and with consequential action at sensitive sites causing irreversible damage and loss of viability of the spores. Thus, it has been shown that although information from SEM is contributory it supports the proposed mechanism of action of combination treatments based on information from a whole spore. Other studies have been concerned only with ultrathin sections of spores and the present study is one of the few using SEM techniques on whole spores.

The apparent loss of spore content as revealed by the SEM study necessitated confirmation. Since the DNA is known to be present in intact spores, its presence in the menstruum after exposure to

combined treatments was examined. The results of this study conclusively showed that exposure of B. stearothermophilus (NCIB 8919) to a combination of heat at 95°C for 45 min with 0.8% sorbate and 8% NaCl at pH 4.2 lead to release of up to 29% of the DNA content of the intact spore. Similarly exposure to a combination of heat at 95°C for 45 min with 0.8% sorbate and 2.5% benzoate resulted in 35% loss of the DNA content. While the levels of DNA presented were based on calculated initial levels in whole spores given by Doi (1969) the results provide strong evidence that the spore wall had been severely damaged. It seems reasonable to infer that the presence of DNA in the menstruum is an effect resulting from spore wall and spore core damage by certain combined heat and chemical treatments. The precise mechanism of action of these combined treatments requires to be investigated in future studies.

CONCLUSIONS

1. Medium SMS was found to be the best for sporulation of Bacillus cereus and B. stearothermophilus test strains.
2. The 'come-up' time of sample tubes was found to be short; 42.5s at 95°C, the highest temperature used in the study. The cooling time was very short and no correction factor was introduced.
3. The pour plating technique was found to be most statistically accurate method for the recovery of B. stearothermophilus spores after exposure to mild-heat treatments.
4. Exposure of S. aureus and E. coli to a combination of heat, potassium sorbate and pH caused almost total inhibition of these strains whereas for B. cereus and B. stearothermophilus spores inhibition was not as pronounced.
5. Exposure of Bacillus cereus and B. stearothermophilus spores to a combination of heat, sodium chloride and pH provided a marginal to moderate inhibition of the spores. Inhibition increased with increasing sodium chloride levels and decreased in pH. In the presence of 2% NaCl or less and at pH 6.5 the combination conferred a slight protective effect on spores of all test strains except B. stearothermophilus (NCIB 8919).
6. Exposure of B. cereus and B. stearothermophilus spores to a combination of heat, sodium benzoate and pH resulted in a marginal to moderate inhibition of the spores. This occurred at pH 4.5 and 5.5 for B. cereus and 4.2 and 5.2 for B. stearothermophilus. At higher pH values the effect was uncertain.
7. Exposure of B. cereus and B. stearothermophilus spores to a combination of heat, sorbate, sodium chloride and pH caused total inhibition of the spores. Inhibition increased with increasing

levels of the chemicals and decrease in pH. A maximum synergistic effect was shown by the combinations at pH 4.2 and 4.5 for B. stearothermophilus and B. cereus respectively.

8. Exposure of B. cereus and B. stearothermophilus spores to a combination of heat, sorbate, benzoate and low pH caused total inhibition of the spores. This occurred at pH 4.2 for B. stearothermophilus and 4.5 for B. cereus.

9. Recovery of B. cereus spores at supra-optimum temperatures enhanced the inhibitory effectiveness of certain combination treatments. This effect was not found at sub-optimal temperatures.

10. Recovery of B. stearothermophilus spores was markedly affected by incubation at 44°C after exposure to certain combination treatment and slight inhibition was noted at 60°C.

11. The potential of the Direct Fluorescence Microscopy (DFM) technique for spore survival determinations was indicated.

12. Evidence for spore wall interference by combination treatments was observed using the DFM technique.

13. Scanning Electron Microscopy (SEM) study showed evidence of alterations in spore wall structure after exposure to combination treatments.

14. Evidence for loss of spore content was indicated after exposure to combination treatments.

15. A computer program is described for the presentation of data generated from combination treatments in form of 3D-histograms. The presentation allows the specific contribution by each factor to be clearly distinguished.

SUGGESTIONS FOR FUTURE WORK

From the results of this study the following fields of investigation are suggested.

1. A detailed study of the effects of the combination of heat, sorbate, sodium chloride and pH on spores using model food systems as the menstrua.
2. Sensory evaluation of foods in which appropriate combination preservation treatment has been used.
3. A more comprehensive study of the Direct Fluorescence Microscopy technique for determining the survival of spores exposed to combination of heat, chemicals and pH.
4. Electron microscopy study of thin sections to follow the structural changes in spores during exposure to the combination preservation systems.
5. A detailed study of the mechanisms of inhibition of the spores by the various combination preservation systems.

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APPENDIX 1: COMPOSITION OF MEDIA

(a) NA (Nutrient agar Oxoid)

"Lab lemco" beef extract	1g
Yeast extract	2g
Peptone (Difco)	5g
Sodium chloride (BDH)	5g
Agar Oxoid No. 3	15g
Distilled water	1 litre

pH 7.4

(b) TSA (Trypticase soy agar)

Trypticase peptone (Oxoid)	15g
Phytone peptone (Oxoid)	5g
NaCl (BDH)	5g
Agar Oxoid No. 1	15g
Distilled water	1 litre

pH 7.5

(c) SMS (Sporulation medium for B. stearothermophilus)

Bacteriological peptone (L37) Oxoid	3g
Tryptone (L42) Oxoid	2.5g
Yeast extract (L20) Oxoid	4.0g
Lab-lemco (L30) Oxoid	2.5g
K_2HPO_4 (BDH)	2.0g
$MnCl_2$ (BDH)	0.01g
Agar Oxoid No. 3	15.0g
Distilled water	1 litre

pH 7.0 - 7.1

(d) FNA (Fortified Nutrient Agar)

Nutrient agar CM 3 (Oxoid)	20.0g
D - Glucose (BDH)	0.5g
$MnSO_4.4H_2O$ (BDH)	0.03g
Distilled water	1 litre

pH 7.2

(e) PCA (PLate count Agar)

Yeast extract (L21 Oxoid)	2.5g
Tryptone Oxoid	5.0g
D-Dextrose (BDH)	1.0g
Agar No. 1 (L11 Oxoid)	15.0g
Distilled water	1 litre

pH 7.0

APPENDIX 2: CONSTITUENTS OF STAINING SOLUTIONS

(a) Malachite green

Malachite green (BDH C.I. 42000)	5g
Distilled water	100ml

(b) Ziehl-Neelsen's (strong) carbol fuchsin

Basic fuchsin (BDH C.I. 42500)	1g
Phenol (crystalline) (BDH)	5g
Ethanol	10ml
Distilled water	90ml

(c) Acriflavine

Acriflavine (BDH)	0.10g
Disodium hydrogen phosphate, Na_2HPO_4 (BDH)	8.55g
Potassium dihydrogen phosphate, KH_2PO_4 (BDH)	2.54g
Distilled water	1000ml

APPENDIX 3: PERCENTAGE OF SURVIVING SPORES RECOVERED AFTER EXPOSURE TO DIFFERENT COMBINATION TREATMENTS

TABLE I . The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. cereus (NCIB 6349) spores.

pH	% (w/v) NaCl in heating medium	Percentage surviving spore counts recovered in YPTD after heating at 85°C for 30 min					
		% w/v KS 0.0	0.10	0.20	0.30	0.40	0.50
6.5	0.0	100.0	101.7	96.7	95.0	93.4	92.3
	1.5	112.7	108.3	97.8	85.6	79.0	70.7
	3.0	92.8	96.1	88.4	67.4	58.0	48.6
	4.5	87.9	90.6	80.1	53.0	44.2	28.2
	6.0	81.2	84.5	71.3	42.5	30.4	23.2
	7.5	72.9	76.2	58.6	33.7	25.4	20.4
6.0	0.0	100.0	98.2	92.3	88.8	84.0	82.8
	1.5	104.7	96.5	81.7	76.3	68.6	63.3
	3.0	84.6	77.5	63.3	58.0	45.6	39.1
	4.5	79.9	70.4	51.5	40.2	29.6	20.7
	6.0	68.1	59.8	42.0	31.4	21.9	15.6
	7.5	61.0	43.2	33.1	24.3	13.6	6.9
5.5	0.0	100.0	96.8	91.6	85.1	82.5	74.7
	1.5	94.2	90.3	78.6	70.1	66.9	59.1
	3.0	77.3	65.6	53.9	44.2	35.7	31.8
	4.5	68.8	50.7	42.9	31.8	23.4	18.8
	6.0	55.2	41.6	35.1	26.6	18.7	9.8
	7.5	47.4	36.4	26.0	17.9	10.6	2.6
4.5	0.0	100.0	92.8	89.9	79.7	71.0	
	1.5	81.9	76.8	65.9	52.9	44.2	
	3.0	63.8	52.2	38.4	29.7	13.6	
	4.5	55.1	40.6	16.7	8.7	0.6	
	6.0	42.0	26.8	6.5	0.5	0.02	
	7.5	32.6	18.8	0.7	0.08	-[a]	

[a]No survivor

TABLE II. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on *B. cereus* (NCIB 7464) spores.

pH	% (w/v) NaCl in heating medium	Percentage surviving spore counts recovered in YPTD after heating at 85°C for 30 min					
		% w/v KS 0.0	0.10	0.20	0.30	0.40	0.50
6.5	0.0	100.0	97.6	93.6	91.2	87.8	83.3
	1.5	106.9	96.1	90.7	89.2	80.9	73.3
	3.0	94.6	91.2	82.8	69.6	63.7	57.8
	4.5	85.8	80.4	77.0	59.3	40.2	36.8
	6.0	80.4	79.4	69.6	41.2	26.0	22.1
	7.5	70.1	66.7	54.9	30.9	20.6	18.6
6.0	0.0	100.0	91.6	86.8	80.5	74.7	72.6
	1.5	98.4	93.2	82.6	79.0	73.2	70.5
	3.0	87.9	80.5	67.4	66.8	58.4	51.1
	4.5	81.6	72.6	53.7	47.9	45.8	41.1
	6.0	73.7	61.6	39.5	36.3	30.5	24.7
	7.5	67.4	55.3	28.4	21.6	19.5	16.3
5.5	0.0	100.0	90.3	81.3	76.1	70.5	66.5
	1.5	94.9	87.5	78.4	74.4	69.9	70.5
	3.0	83.0	79.6	63.6	54.6	51.7	44.3
	4.5	75.0	53.4	42.6	33.5	27.8	16.4
	6.0	69.9	46.6	26.7	19.3	16.5	5.7
	7.5	63.6	39.2	17.1	11.7	7.4	1.8
4.5	0.0	100.0	88.3	74.7	68.8	63.0	
	1.5	90.3	80.5	70.8	62.3	40.8	
	3.0	79.9	62.3	49.4	39.6	11.6	
	4.5	68.2	54.6	21.5	15.5	0.18	
	6.0	59.7	45.5	4.9	1.95	0.013	
	7.5	54.6	31.2	0.6	0.2	-[a]	

[a]No survivor

TABLE III. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on *B. stearothermophilus* (NCIB 8919) spores.

pH	% (w/v) NaCl in heating medium	Percentage surviving spore counts recoverd in YPTD medium after heating at 95°C for 45 min					
		% (w/v) KS 0.0	0.2	0.4	0.6	0.8	1.0
6.5	0.0	100.0	102.2	97.8	93.3	85.2	83.0
	2.0	83.0	85.2	79.3	74.1	60.0	56.3
	4.0	77.8	77.8	68.9	60.0	52.6	52.6
	6.0	74.1	72.6	61.5	56.3	48.9	48.2
	8.0	68.9	67.4	60.0	54.8	48.2	45.9
	10.0	70.4	68.9	58.5	52.6	50.4	44.4
6.0	0.0	100.0	80.9	75.7	68.7	59.1	54.8
	2.0	73.9	57.4	47.0	36.5	28.7	23.4
	4.0	68.7	53.9	42.6	34.8	26.1	19.9
	6.0	66.1	50.4	40.0	30.4	22.9	19.4
	8.0	62.6	45.2	38.3	29.6	20.4	17.4
	10.0	64.4	44.4	36.5	27.8	20.9	16.6
5.2	0.0	100.0	72.8	63.0	55.6	42.0	34.8
	2.0	69.1	44.4	32.1	23.6	13.0	8.5
	4.0	63.0	39.5	29.6	21.5	11.2	7.7
	6.0	58.0	37.0	26.4	18.6	8.5	7.7
	8.0	59.3	35.6	24.7	16.3	7.8	7.2
	10.0	60.5	34.0	24.1	15.6	8.2	ND[a]
4.2	0.0	100.0	66.1	54.8	50.0	35.3	
	2.0	66.1	32.3	21.8	15.0	6.1	
	4.0	59.7	28.7	18.1	10.7	0.046	
	6.0	50.0	20.8	12.3	6.8	0.006	
	8.0	53.2	18.6	11.5	4.7	-[b]	
	10.0	56.5	16.5	9.7	ND	-	

[a]Not done [b]No survivor

TABLE IV. The effects of the combination of heat, potassium sorbate-KS, sodium chloride (NaCl) and pH on B. stearothermophilus (NCIB 8920) spores.

pH	% (w/v) NaCl in heating medium	Percentage number of surviving spore counts recoverd in YPTD medium after heating at 95°C for 45 min				
		% (w/v) KS 0.0	0.2	0.4	0.6	0.8
6.5	0.0	100.0	101.8	74.1	70.5	67.9
	2.0	104.5	58.0	46.4	41.1	32.1
	4.0	92.9	51.8	40.2	35.7	28.6
	6.0	63.4	43.8	34.8	27.7	23.0
	8.0	58.9	40.2	31.3	24.6	21.9
	10.0	56.3	37.5	30.4	24.0	20.9
6.0	0.0	100.0	67.3	65.4	61.4	53.5
	2.0	64.4	34.7	27.9	22.2	18.4
	4.0	53.6	29.2	22.7	16.8	12.8
	6.0	51.5	25.5	19.3	13.7	9.4
	8.0	48.5	20.7	16.8	12.2	7.8
	10.0	46.5	18.4	15.0	12.5	7.3
5.2	0.0	100.0	64.5	56.5	50.0	38.7
	2.0	62.9	22.3	12.6	9.4	4.8
	4.0	54.8	17.3	6.9	5.3	2.5
	6.0	50.0	11.6	5.8	2.7	1.1
	8.0	48.4	8.1	4.2	2.3	1.02
	10.0	46.5	5.3	3.7	2.2	1.00
4.2	0.0	100.0	59.6	54.2	45.0	25.4
	2.0	61.5	14.2	6.7	2.1	0.7
	4.0	52.9	8.5	4.5	0.8	0.006
	6.0	46.2	3.6	1.3	0.13	-[a]
	8.0	44.0	3.5	0.9	0.01	-
	10.0	43.1	3.4	0.8	ND[b]	-

[a]No survivor [b]Not done

TABLE V. The effects of the combination of heat, potassium sorbate -KS, sodium benzoate-SB and pH on B. cereus (NCIB 6349) spores.

pH	% (w/v) SB in heating medium	Percentage surviving spores recovered in YPTD after heating at 85°C for 30 min		
		% (w/v) KS 0.00	0.20	0.40
7.1	0.00	100.0	105.1	98.2
	0.25	101.8	107.4	96.3
	0.50	98.2	99.5	104.2
	0.75	97.7	103.7	94.5
	1.25	104.2	94.9	97.7
	1.75	100.9	105.5	100.9
6.0	0.00	100.0	90.2	82.9
	0.25	96.4	94.8	87.6
	0.50	105.7	97.9	91.7
	0.75	109.3	99.5	93.8
	1.25	98.5	95.9	90.7
	1.75	93.3	91.2	88.1
5.5	0.00	100.0	88.5	70.5
	0.25	94.9	90.4	62.2
	0.50	87.8	79.5	54.5
	0.75	82.7	71.2	50.6
	1.25	75.0	68.0	43.0
	1.75	78.2	62.8	34.6
4.5	0.00	100.0	70.8	62.8
	0.25	88.5	55.8	43.4
	0.50	80.5	36.3	16.1
	0.75	73.5	19.6	0.006
	1.25	69.0	2.6	0.00001
	1.75	67.3	0.01	-[a]

[a]No survivor

TABLE VI . The effects of the combination of heat, potassium sorbate -KS, sodium benzoate-SB and pH on B. stearothermophilus (NCIB 8919) spores.

pH	% (w/v) Sodium benzoate in heating medium	Percentage surviving spores recovered in YPTD medium after heating at 95°C for 45min		
		% (w/v) KS		
		0.0	0.4	0.8
7.1	0.0	100.0	102.0	100.7
	0.5	98.7	104.6	101.3
	1.0	100.7	103.3	98.0
	1.5	106.6	102.6	100.0
	2.0	105.3	97.4	99.3
	2.5	109.9	104.0	98.0
6.0	0.0	100.0	78.1	57.0
	0.5	103.9	85.9	64.1
	1.0	107.0	93.0	85.2
	1.5	100.8	96.9	80.5
	2.0	97.7	89.1	76.6
5.2	0.0	100.0	53.1	34.5
	0.5	96.5	77.9	54.9
	1.0	92.9	71.7	46.9
	1.5	90.3	67.3	40.7
	2.0	83.2	59.3	31.9
4.2	0.0	100.0	45.1	29.2
	0.5	81.7	33.2	3.4
	1.0	73.2	24.2	0.3
	1.5	64.8	15.4	0.1
	2.0	53.5	6.9	0.01
	2.5	49.3	4.2	-[a]
	3.0	41.8	2.9	-

[a]No survivor

TABLE VII.. The effects of recovery temperatures and incubation periods on the counts of B. cereus (NCIB 6349) spores exposed to combined treatments.

Systems	Incubation temp °C	Percentage spore counts recovered in YPTD at different incubation times (days)			
		15	10	6	2
I	25	88.9[a]	94.3	92.5	67.9
	30	100.0	103.8	103.8	73.6
	37	90.6	97.1	105.7	83.0
	44	39.1	41.7	45.1	43.0
J	25	82.1	89.7	84.6	76.9
	30	100.0	105.1	94.8	84.6
	37	74.9	75.9	76.2	73.1
	44	40.5	42.1	42.8	37.7
K	25	93.2	96.1	95.2	74.5
	30	100.0	95.8	92.9	67.4
	37	88.1	92.3	93.6	72.6
	44	27.4	27.4	31.0	31.6
L	25	102.9	111.8	105.9	85.9
	30	100.0	97.1	97.1	69.4
	37	42.9	45.0	45.0	47.7
	44	<0.09	<0.09	<0.09	<0.09

[a]Values used for 3D-histograms.

TABLE VIII. The effects of recovery temperatures and incubation periods on the counts of B. cereus (NCIB 7464) spores exposed to combined treatments.

Systems	Incubation temp °C	Percentage spore counts recovered in YPTD at different incubation times (days)			
		15	10	6	2
I	25	74.1[a]	85.2	90.7	72.2
	30	100.0	100.0	98.2	75.9
	37	83.3	90.7	96.3	85.2
	44	35.9	36.5	43.2	38.7
J	25	77.2	84.2	82.5	73.7
	30	100.0	103.5	101.8	75.4
	37	64.9	71.9	77.2	68.4
	44	40.2	47.7	48.3	36.5
K	25	93.2	96.8	103.9	78.4
	30	100.0	96.8	93.8	71.6
	37	85.5	91.3	87.1	65.8
	44	38.4	46.1	41.9	36.8
L	25	96.6	107.3	103.9	83.5
	30	100.0	101.9	95.6	70.9
	37	50.5	59.7	68.0	52.9
	44	<0.09	<0.09	<0.09	-[b]

[a]Values used for 3D-histograms.

[b]No growth.

TABLE IX. The effects of recovery temperatures and incubation periods on counts of B. stearothermophilus (NCIB 8919) spores exposed to combined treatments.

Systems	Incubation temp °C	Percentage spore counts recovered in YPTD at different incubation times (days)			
		15	10	6	2
M	60	88.9[a]	83.3	84.7	66.7
	55	100.0	95.8	91.7	72.9
	50	94.4	97.2	87.5	75.0
	44	45.8	51.4	52.8	47.2
N	60	59.8	63.7	68.8	70.3
	55	100.0	104.7	102.7	83.6
	50	96.1	105.5	105.9	85.9
	44	64.5	64.5	62.9	56.6
O	60	64.9	73.0	78.4	52.7
	55	100.0	97.3	93.2	67.6
	50	97.3	105.4	96.0	64.9
	44	29.9	32.3	40.3	37.0
P	60	67.3	78.2	-[b]	-
	55	100.0	93.6	82.7	-
	50	102.7	103.6	96.4	-
	44	-	-	-	-

[a]Values used for 3D-histograms. [b]No growth (NG).

TABLE X . The effect of recovery temperatures and incubation periods on the counts of B. stearothermophilus (NCIB 8920) spores exposed to combined treatments.

Systems	Incubation temp °C	Percentage spore counts recovered in YPTD at different incubation periods (days)			
		15	10	6	2
Q	60	63.3[a]	64.5	63.9	52.1
	55	100.0	93.9	93.9	64.6
	50	87.9	106.1	90.9	69.7
	44	37.6	39.4	48.2	36.7
R	60	82.6	87.9	93.2	79.6
	55	100.0	100.0	104.6	87.1
	50	97.7	100.8	100.8	86.4
	44	32.6	43.2	52.3	48.5
S	60	63.3	66.3	67.5	59.8
	55	100.0	99.4	98.2	87.0
	50	93.5	97.0	100.6	81.7
	44	18.9	29.0	30.2	26.6
T	60	63.2	76.1	-[b]	-
	55	100.0	108.0	104.3	-
	50	108.6	109.8	111.0	-
	44	-	-	-	-

[a]Values used for 3D-histograms. [b]No growth (NG).

APPENDIX 4: LISTINGS OF THE COMPUTER PROGRAM

(a) Creating data file

(b) Creating Blocks

(c) Creating BIBLE data

(a) Program for creating data file

```
      DIMENSION  VAL(25)
      CHARACTER*30  FNAME
      IUIN = 5
      IUOUT = 6
      IFIL = 10

      WRITE(IUOUT,5000)
      READ(IUIN,5100)  FNAME

      OPEN(UNIT=IFIL,FILE=FNAME,STATUS='NEW',
     &     ACCESS='SEQUENTIAL',IOSTAT=ISTAT,ERR=999)

  100 CONTINUE
      WRITE(IUOUT,5200)
      READ(IUIN,*,ERR=100)  M,N

  200 CONTINUE
      WRITE(IUOUT,5300)
      READ(IUIN,*,ERR=200)  DX

  300 CONTINUE
      WRITE(IUOUT,5400)
      READ(IUIN,*,ERR=300)  DXX

      WRITE(IFIL,6000)  M,N,DXX,DXX,DX,DX

      DO 1000 I = 1,N

 1100 CONTINUE
      WRITE(IUOUT,5500)  M,I
      READ(IUIN,*,ERR=1100)  (VAL(J),J=1,M)

      WRITE(IFIL,6100) (VAL(J),J=1,M)

 1000 CONTINUE

      STOP

  999 CONTINUE
      WRITE(IUOUT,5600)
      STOP

 5000 FORMAT(' NAME OF DATA FILE  ?  >',$)
 5100 FORMAT(A30)
 5200 FORMAT(' INPUT  M AND N  ? >',$)
 5300 FORMAT(' INPUT SIZE OF BLOCKS ? >',$)
 5400 FORMAT(' INPUT DISTANCE BETWEEN BLOCKS ? > ',$)
 5500 FORMAT(' INPUT ',I4,'  VALUES FOR ROW',I3,'  ? ',/,' >',$)
 5600 FORMAT('  FILE ERROR')
 6000 FORMAT(' ',2(I5,','),4(F8.2,','))
 6100 FORMAT(' ',8(F8.2,','))
      END
```

(b) Program for creating blocks

```
      SUBROUTINE  PRISM(XXX,YYY,ZZZ,DDX,DDY,DDZ)

      COMMON/FIL/IUOUT,IUIN,IFIL,IOFIL
      DIMENSION  X(50),Y(50),Z(50)
      DIMENSION  NVER(50),JVN(50,4)

      XX = XXX
      YY = YYY
      ZZ = ZZZ
      DX = DDX
      DY = DDY
      DZ = DDZ

      IF (DX .LE. 0.0)  GOTO  999
      IF (DY .LE. 0.0)  GOTO  999
      IF (DZ .LE. 0.0)  GOTO  999

      N = INT(DZ/10.0)+2
      IF (DZ .EQ. 10.0*INT(DZ/10.0))  N = N-1
      X(1) = XX
      Y(1) = YY
      Z(1) = 0.0
      X(2) = XX+DX
      Y(2) = YY
      Z(2) = 0.0
      X(3) = XX+DX
      Y(3) = YY+DY
      Z(3) = 0.0
      X(4) = XX
      Y(4) = YY+DY
      Z(4) = 0.0

      DO 100 I = 2,N

      DZZ = (I-1)*10.0
      IF (I .EQ. N)  DZZ = DZ

      DO 150 J = 1,4

      K = (I-1)*4 + J
      X(K) = X(J)
      Y(K) = Y(J)
      Z(K) = DZZ

  150 CONTINUE

  100 CONTINUE

      NVT = N*4
      NSUR = (N-1)*4 + 2

      NVER(1) = 4
      JVN(1,1) = 1
```

```
      JVN(1,2) = 5
      JVN(1,3) = 6
      JVN(1,4) = 2
      NVER(2) = 4
      JVN(2,1) = 2
      JVN(2,2) = 6
      JVN(2,3) = 7
      JVN(2,4) = 3
      NVER(3) = 4
      JVN(3,1) = 3
      JVN(3,2) = 7
      JVN(3,3) = 8
      JVN(3,4) = 4
      NVER(4) = 4
      JVN(4,1) = 4
      JVN(4,2) = 8
      JVN(4,3) = 5
      JVN(4,4) = 1

      DO 200 I = 2,N-1

      DO 250 J = 1,4
      K = (I-1)*4 + J

      NVER(K) = NVER(J)

      DO 275 J1 = 1,4

      JVN(K,J1) = JVN(J,J1) + (I-1)*4

  275 CONTINUE

  250 CONTINUE

  200 CONTINUE

      NVER(NSUR-1) = 4
      JVN(NSUR-1,1) = 1
      JVN(NSUR-1,2) = 2
      JVN(NSUR-1,3) = 3
      JVN(NSUR-1,4) = 4

      NVER(NSUR) = 4
      JVN(NSUR,1) = NVT
      JVN(NSUR,2) = NVT-1
      JVN(NSUR,3) = NVT-2
      JVN(NSUR,4) = NVT-3

      WRITE(IOFIL,5000)  NVT,NSUR
      DO 300 I = 1,NVT
      WRITE(IOFIL,5100)  X(I),Y(I),Z(I)
  300 CONTINUE
      DO 350 I = 1,NSUR
      WRITE(IOFIL,5200)  (NVER(I),(JVN(I,J),J=1,NVER(I)))
  350 CONTINUE

  900 CONTINUE
      RETURN

  999 CONTINUE
      STOP ' ERROR IN PRISM'
```

```
5000 FORMAT('GEN',/,' ',I3,',',I3,',')
5100 FORMAT(' ',3(F10.3,','))
5200 FORMAT(' ',I4,',',3X,4(I3,','))
5300 FORMAT('LIN',/,1X,'   5,')
5400 FORMAT('  ',3(F10.3,','))
     END
```

(c) Program for creating BIBLE data

```
      COMMON/FIL/IUIN,IUOUT,IFIL,IOFIL
      DIMENSION  VAL(10)
      CHARACTER*40  IFNM,IOFNM

      IUOUT = 6
      IUIN = 5
      IFIL = 11
      IOFIL = 12

      WRITE(IUOUT,5000)
      READ(IUIN,5100)  IFNM

      OPEN(UNIT=IFIL,FILE=IFNM,STATUS='OLD',
     &     IOSTAT=ISTAT,
     &     ACCESS='SEQUENTIAL',
     &     ERR=989)

      WRITE(IUOUT,5200)
      READ(IUIN,5100)  IOFNM

      OPEN(UNIT=IOFIL,FILE=IOFNM,STATUS='NEW',
     &     CARRIAGECONTROL='LIST',
     &     IOSTAT=ISTAT,
     &     ACCESS='SEQUENTIAL',
     &     ERR=999)


      READ(IFIL,*)  N,M,DX,DY,DXX,DYY


      DO 100 I = 1,M

      READ(IFIL,*)  (VAL(J),J=1,N)

      YY = (I-1)*DY

      DO 200 J = 1,N
      IF (VAL(J) .EQ. 0.0)  GOTO  200
      XX = (J-1)*DX

      CALL  PRISM(XX,YY,0,DXX,DYY,VAL(J))

  200 CONTINUE

  100 CONTINUE

      STOP

  989 CONTINUE

      WRITE(IUOUT,6000)  ISTAT
      STOP


  999 CONTINUE

      WRITE(IUOUT,6100)  ISTAT
      STOP


 5000 FORMAT(' NAME OF DATA FILE ? >',$)
 5100 FORMAT(A40)
 5200 FORMAT(' NAME OF OUTPUT FILE ? >',$)
 6000 FORMAT(' ERROR OPENING INPUT FILE ',I8)
 6100 FORMAT(' ERROR OPENING OUTPUT FILE ',I8)
      END
```