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GERMINATION OF SPORES OF *Clostridium perfringens* FD1

By

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ABSTRACT

GERMINATION OF SPORES OF Clostridium perfringens FD1

By

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The purpose of this investigation was to design a chemically defined medium for the germination of spores of Clostridium perfringens FD1 and to determine the effect of several factors. Factors studied were heat shock, pH, nutrients and inhibitors.

Initial experiments demonstrated that a chemically defined medium containing 18 amino acids, glucose and some mineral salts, promoted full germination of the spores. A heat shock at 80 C for 10 minutes was necessary to activate the spores. Tests with individual amino acids indicated that alanine (30 mg/l), glutamic acid (224 mg/l) and leucine (92 mg/l) were the most effective germinants. Spores of C. perfringens FD1 also required a pH of 6.0-6.5 and the presence of sodium chloride, dibasic potassium phosphate and L-cystine for rapid germination. The addition of glucose or fructose (or a sugar containing a terminal glucose or fructose moiety) was required for germination. Sodium chloride had a stimulatory effect on germination, and its effect was only partially substituted by sodium or potassium nitrite or sodium bicarbonate.

The pH had a marked effect on germination rate and the extent of germination. Satisfactory germination occurred in chemically defined media from pH 5.5 to 7.0, or in complex media from pH 5.5 to 9.0.

Inhibitors of vegetative growth usually permitted spore germination at concentrations that were inhibitory for vegetative growth. Inhibitors of germination of aerobic spores, that act by blocking the L-alanine-induced germination system in spores of bacilli, were ineffective or only slightly effective in inhibiting germination of spores of C. perfringens FD1.

The chemically defined germination system designed for spores of C. perfringens FD1 was also adequate for spores of C. perfringens strains ATCC 3624 and NCTC 8238, but failed to support germination of spores of C. sporogenes (PA 3679) and C. perfringens strain ATCC 12195.

A mis padres

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INTRODUCTION

Studies on germination of spores of Clostridium were limited for a long time because of the need for tedious anaerobic techniques and a lack of adequate media for the production of spores of some clostridia. However, during the last fifteen years new culture media were developed thus permitting the production of considerable amounts of spores. Consequently, an increase in investigations concerning germination of spores of anaerobes has been observed in the last ten years.

The germination requirements of several species of clostridia have been investigated. Extensive studies have been conducted on germination of C. bifermentans, C. roseum, C. sporogenes and C. botulinum, but specific knowledge about the germination systems in these spores is quite limited. With regards to C. perfringens, almost nothing is known concerning its germination requirements. Germination responses of spores of C. perfringens have primarily been studied in complex media, and little information is available on germination of these spores in media of known chemical composition.

The mechanism of germination in spores of bacilli has been the subject of several investigations. However, for clostridial spores there is little information concerning the mechanism of action of germinants, the characteristics of the receptor sites and the effect of inhibitors on the germination process.

The objectives of this project were: 1) to design a chemically defined medium for the germination of spores of C. perfringens FD1; 2) to determine the minimum nutritional requirements for germination; and 3) to investigate the effects of various compounds on germination.

LITERATURE REVIEW

Clostridium perfringens

Clostridium perfringens is a nonmotile, gram-positive, anaerobic rod with blunt ends which forms spores, but many strains form few spores in normal laboratory media. Distinctive characteristics are a stormy fermentation of milk at 46 C and nonmotility (Breed *et al.*, 1957). It is more widely spread than any other anaerobic pathogenic bacterium, and its principal habitats are soil and the intestinal contents of man and animals. Since the late 1800s it has been linked with food poisoning, but it was not until 1959 that *C. perfringens* foodborne illness was oficially recognized (Smith, 1968; Duncan, 1970; Ladiges *et al.*, 1974; Matches *et al.*, 1974).

Clostridium perfringens causes a disease characterized by acute abdominal pain and diarrhea, accompanied by little or no nausea and vomiting. The incubation period is usually 9-15 hours, the illness is of short duration, and fever or other signs of infection are rarely observed (Hall *et al.*, 1963; Strong *et al.*, 1963; CDC, 1977).

Food frequently becomes contaminated from the natural sources where *C. perfringens* can be found. The main problem is the presence of spores which survive cooking,

and then germinate and grow in the contaminated food (Ladiges et al., 1974).

Because of the complex media and tedious anaerobic techniques used for the production of spores of anaerobes, studies on germination were consequently limited. In 1965 a review by Perkins (1965) indicated that 224 references cited regarding bacterial spores included only 15 dealing with clostridia. However, after 1965 improved media were designed for the production of spores of clostridia and the interest in germination of clostridial spores increased. Currently, several sporulation media are available, permitting the production of large numbers of spores of C. perfringens. Among these media are those designed by Ellner (1957), Kim et al. (1967), Duncan and Strong (1968), Ting and Fung (1972), Goodenough and Solberg (1972), and Sacks and Thompson (1975, 1977, 1978).

Transformation of Spores Into Vegetative Cells

The bacterial spore is a dormant structure that enables the cell to persist under unfavorable conditions, with little or no metabolism. A new cycle of growth and multiplication may start when the environmental conditions are adequate for these purposes (Lewis, 1969). Dormant spores germinate poorly or not at all under conditions that permit germination of aged or heat-activated spores. It has been suggested that the high concentration of cystine present in

spore coat proteins is responsible for the dormant state. Disulfide bonds present in these proteins need to be broken to promote changes in tertiary structure resulting in activation of spores and changes in permeability (Keynan and Halvorson, 1965; Keynan et al., 1965).

The release of spores from the dormant state is under precise control (Lewis, 1969). The whole process of transformation of a dormant bacterial spore into a vegetative cell can be divided into three stages: activation, germination and outgrowth. Each stage must correspond to one or more macromolecular processes which are slowly beginning to be understood (Freese and Cashel, 1965; Keynan and Halvorson, 1965; Lewis, 1969).

Activation. Spores of freshly harvested cultures usually do not germinate or do so only slowly. Often some germination can be induced by placing the spores into rich media, but a long lag period will preceed germination, which will by unsynchronized and incomplete (Keynan and Evenchik, 1969).

Activation is a reversible process based on a change in the marcomolecular structure of the spore coats. It is a nonmetabolic change and terminates the dormant state temporarily without ending cryptobiosis. When activated or aged spores are placed under germinative conditions the cryptobiotic state is lost irreversibly (Keynan and Halvorson, 1965; Keynan et al., 1965; Keynan and Evenchik, 1969).

An activation phase may not be recognizable for all spore preparations or strains, or for all germination conditions (Lewis, 1969).

Roberts (1968) demonstrated heat activation of spores of clostridia by heating at 70 C for 30 minutes. The presence of calcium dipicolinate also activates spores, presumably due to its chelating abilities (Freese and Cashel, 1965).

Methods of Activation. Three common methods used for activation of spores are: exposure to sublethal heat, pH treatments and aging. Among these, the simplest is heat activation which helps condition spores for physiological germination. Some bacterial spores are extremely dormant and require temperatures as high as 100 C or more for activation. The activation energy required is high, similar to that required for heat denaturation of macromolecules (Keynan and Halvorson, 1965; Keynan et al., 1965).

Heat activation induces three measurable changes in a spore suspension: 1) increases the germination rate, 2) activates some enzymes and 3) reduces the requirements for the induction of germination. Heat activation can be influenced by pH (the optimum for spores of bacilli is pH 2-3), adsorption of salts and media in which spores are activated (Keynan and Halvorson, 1965; Keynan et al., 1965; Keynan and Evenchik, 1969).

When changes in pH are used to promote activation, the chemical composition of the spore is changed because of the release of dipicolinic acid, which is associated with a change in permeability of the spore coat (Keynan and Evenchik, 1969). Treatments at low pH may also affect the heat resistance of the spores (Alderton and Snell, 1969).

Finally, natural activation can be achieved by aging. Upon storage spores behave as if they have been heat-activated. Aging and heat activation seem to be similar phenomena, and thermodynamically there seems to be no difference between heat activation and aging. Both end the state of dormancy and the only difference is that during heat activation dormancy is lost temporarily while during aging dormancy is lost irreversibly (Keynan and Evenchik, 1969).

Germination. Germination is a well-defined stage in the developmental cycle of sporeforming bacteria. It is essentially the conversion of a resistant and dormant spore into a sensitive and metabolically active form (Gould, 1969). Germination is a process of hydrolysis, depolymerization and exudation of low and high molecular weight substances (Lewis, 1969). The resulting cell has lost the characteristics of a bacterial spore, is metabolically active, heat-labile and non-refractile, yet readily distinct from a vegetative cell (Keynan and Halvorson, 1965). At the same time that depolymerization of the spore

coat is taking place, biosynthesis of the new cell wall is occurring (Lewis, 1969). The breakdown products can be utilized in the synthesis of the new wall in the outgrowing cell (Vinter, 1965).

Several changes are associated with germination, such as the loss of resistance to dehydration, pressure, vacuum, UV and ionizing radiation, antibiotics, chemicals and extremes of pH. There are also some cytological changes, such as the disintegration of the cortex and the appearance of some of the elements of the cytoplasm. The optical density of a spore suspension also decreases by about 60% due to the excretion of dry matter from the germinating spore. Phase darkening is the result of the loss of refractive index of spores during germination as a result of the combined effects of excretion of dry matter, swelling and redistribution of water inside the spore (Gould, 1969). These changes, together with the release of calcium, loss of heat resistance and onset of stainability, have been used as criteria to follow germination of spores (Treadwell et al., 1958).

Different conditions are required for germination and growth, indicating that there are two different processes involved. Special nutritional requirements are needed for germination and outgrowth to transform the spore into a normal vegetative cell. One important fact is that spores can germinate in the presence of inhibitors of macromolecular

synthesis, indicating that production of new compounds is not essential for germination (Keynan and Halvorson, 1965).

Outgrowth. Outgrowth is a process of synthesis of new macromolecules, particularly those not present in the resting spore. The result is the emergence of a new vegetative cell (Keynan and Halvorson, 1965; Keynan and Evenchik, 1969).

Germination Requirements of Bacterial Spores

The germination requirements for bacterial spores can be simple and specific. Amino acids, sugars, organic acids, ribosides and even mineral salts have been reported as germinants for bacterial spores. Germinants can be metabolizable and nonmetabolizable compounds. However, the specific metabolic effects of metabolizable germinants is controversial, some authors claim germination is a metabolic process while others believe germination is non-metabolic.

Germination requirements for spores of bacilli have been widely studied. Among the most common germinants, L-alanine has been the most effective. Germination by L-alanine can be competitively inhibited by its D-isomer. The mechanism of action for germination by L-alanine has been explained as a conversion of the amino acid into some compound, such as pyruvate, that can be used as an energy source (Heiligman et al., 1955; Gould, 1969). Freese and

Cashel (1965) proposed that L-alanine and its analogs act as germinants because they are deaminated by alanine dehydrogenase and, simultaneously, NAD is reduced to NADH. However, since mutants lacking the enzyme germinate in the same manner as spores of the wild type, this effect seems to be caused by some reaction other than the NAD-dependent enzymatic reaction of alanine dehydrogenase (Freese and Cashel, 1965).

Ribosides, particularly adenosine and inosine, are also excellent germinants. These compounds promote germination almost instantaneously when used at concentrations as low as ca. 10^{-5}M, with higher concentrations being inhibitory (Pulvertaft and Haynes, 1951). The main function of ribosides is probably as a source of utilizable phosphates or ribose (Gould, 1969).

Little information is available on the effect of different sugars on germination of bacterial spores. Hachisuka et al. (1955) reported that germination of spores of B. subtilis in the presence of asparagine isoleucine, serine and valine was stimulated by the presence of glucose. Heiligman et al. (1955) also reported that a mixture of glucose, L-alanine and adenosine permitted rapid germination of spores of bacilli.

Germination requirements for clostridial spores are more complex than those of Bacillus spores (Holland et al., 1969; Waites and Wyatt, 1971). L-alanine was reported to

induce 100% germination of C. bifermentans (Waites and Wyatt, 1971). In the absence of L-alanine a mixture of L-arginine, L-phenylalanine and L-lactate also induced rapid germination (Waites and Wyatt, 1971). Germination induced by L-alanine requires the presence of sodium chloride and sodium phosphate, since omission of these compounds prevented or reduced the germination rate (Waites and Wyatt, 1971). For another strain of C. bifermentans spores germinated in a mixture of L-alanine, L-phenylalanine and L-lactate (Gibbs, 1971).

Spores of C. sporogenes germinated in the presence of L-alanine and sodium ions, but germination was faster in the presence of L-phenylalanine and L-arginine. D-alanine was neither inhibitory nor induced germination alone, although it induced germination in the presence of other amino acids (Holland et al., 1969; Waites and Wyatt, 1971). Under alkaline conditions C. sporogenes strain PA 3679h was able to germinate in a medium containing L-alanine and pyrophosphate (Uehara and Frank, 1965).

For C. roseum, a mixture of alanine and arginine promoted germination. However, the same mixture was not effective for C. botulinum which required yeast extract and bicarbonate to induce rapid germination (Hitzman et al., 1957). Treadwell et al. (1958) reported that it is difficult to attain germination of C. botulinum in synthetic medium.

With respect to the effect of ribosides on germination of clostridia, apparently these spores do not require the presence of ribosides for germination. This fact confirms that the germination requirements for spores of clostridia are strikingly different when compared with spores of bacilli (Gould, 1969).

There are few publications concerning germination of C. perfringens and most of them are related to germination in complex media. Busta et al. (1973) tested several complex nitrogen sources on germination of C. perfringens, among them isolated soy protein, sodium caseinate, trypticase and casaminoacids, as well as mixtures of all amino acids. Without heat activation less than 50% of the spores germinated in 12 hours in the complex nitrogen sources, but if spores were heat activated, more than 90% of the spores germinated in one hour. A combination of 18 amino acids also permitted considerable germination (Busta et al., 1973). When soy proteins were used they had both stimulatory and inhibitory effects. Furthermore, there is an apparent relationship between treatment given to the soy proteins and their effect on germination (Busta and Schroeder, 1971; Schroeder and Busta, 1973).

Ahmed and Walker (1971) determined the optimum conditions for germination of spores of C. perfringens S45. They reported excellent germination in vitamin-free casaminoacids, trypticase or yeast extract. Germination in 1%

yeast extract was enhanced by L-cystine, L-cysteine, L-tryptophan and L-tyrosine, as well as the addition of sodium bicatbonate, glucose, lactate, thioglycollate or sodium chloride. The previous single amino acids in the presence of glucose and sodium chloride permitted good rates of germination. However, one of the best germinant solutions was a mixture of L-cystine and sodium chloride. This system promoted minimal germination without an oxygen scavenger (Ahmed and Walker, 1971).

Nonmetabolizable germinants have also been used for germination of spores of clostridia. Sodium and manganese ions apparently enhance germination by their effect on some structure protective to the core of the spore, which depends on associated ions for stability. Surfactants that alter the permeability, as well as chelates, also act as germinants (Gould, 1969). Ando (1978) reported germination of C. perfringens spores in a mixture of potassium chloride and potassium phosphate at pH 7.0.

Other Factors that Affect the Germination of Spores of Clostridia

Among other factors that can affect the rate and extent of germination, the effect of carbon dioxide in germination media has received considerable attention. Some anaerobic bacterial spores, such as those of C. botulinum, have an absolute requirement for carbon dioxide and

fail to germinate under vacuum (Treadwell et al., 1958). Spores of other clostridia such as C. chauvei, C. hystolyticum and C. perfringens do not show a requirement for carbon dioxide (Wynne and Foster, 1948; Holland et al., 1969). The requirement for carbon dioxide can be provided by sodium bicarbonate. Even though a stimulatory role for sodium ions has been postulated when sodium bicarbonate is used in germination media, the stimulatory effect of sodium bicarbonate is due to the bicarbonate ion. Recently, Enfors and Molin (1978) determined that the stimulatory effect of carbon dioxide on spore germination was more pronounced at low pH (5.2-6.0) where the primary molecular species is CO_2. Clostridium perfringens germinated in the presence of 5% carbon dioxide, and the rate of germination was faster than that observed for C. sporogenes.

Oxidation-reduction potential and water activity have been widely studied as factors which affect the growth of C. perfringens. However, little information is available concerning their effects on germination (Kliger and Guggenheim, 1938; Hanke and Katz, 1943; Reed and Orr, 1943; Mead, 1969; Ades and Pierson, 1973). For C. tetani, germination is retarded if the Eh of the medium is increased, but no conclusive remarks were made about inhibition caused by Eh or gaseous oxygen (Knight and Fidles, 1930). It seems that oxygen itself, and not Eh, is the decisive factor in the inhibition of germination of spores of C. butyricum

(Douglas et al., 1973).

Other Ways to Promote Germination

Alkali-Induced Germination. Alkaline treatment of spores removes a protein fraction from the spore coat of C. bifermentans, increasing the germination rate. Although Waites et al. (1972) reported that alkaline treatments do not affect the viability of the treated spores, Duncan et al. (1972) stated that alkaline treatments reduced the apparent viability of heat-sensitive strains of C. perfringens to about 0.0005% recovery. The alkaline treatment only affected the normal germination system and viabile spore recovery was increased to 90-95% in the presence of lysozyme (Duncan et al., 1972). Lysozyme and other lytic enzymes act on the murein of the spore coat, but the spores must be treated with reducing agents or low pH to break the disulfide bonds to facilitate the enzymatic attack. These lytic enzymes are effective in inducing germination of heat-injured, acid treated or alkali-treated spores of heat-sensitive or heat-resistant strains of C. perfringens. This enzymatic action is dependent upon incubation time, pH and temperature (Duncan et al., 1972; Adams, 1973; Ando, 1975).

Treatment with EDTA at pH 9.5 permitted lysozyme-induced germination of spores of C. perfringens by sensitizing them to the action of lysozyme. The spores are not

sensitized by breaking disulfide bonds, but rather by chelation of cations which seem to play an important role in resistance of spores to lysozyme (Adams, 1973).

Nitrites and Sodium Chloride. The effects of common food additives, such as sodium chloride and sodium nitrite, have been evaluated in the germination of spores of clostridia. Nitrite actually stimulates germination, especially under acidic conditions at elevated temperatures, but outgrowth is completely blocked (Duncan and Foster, 1968a). Germination of PA 3679 with sodium nitrite, has been achieved at temperatures as high as 90 C. Ionic germination apparently takes place by induction of a volume change permitting core hydration or by activation of some enzymes (Duncan and Foster, 1968b). The conditions needed for nitrite-induced germination are different from those promoting physiological germination or growth. This type of germination is dependent on high temperature, low pH and relatively high concentrations (2.0%) of nitrite (Labbe and Duncan, 1970; Rhia and Solberg, 1975). Concentrations of sodium chloride as high as 4.0% did not interfere with germination of spores of PA 3679 (Duncan and Foster, 1968b).

Inhibitors

Two types of inhibitors affecting the life cycle of sporeforming microorganisms are those affecting vegetative development and those inhibiting germination of spores.

The inhibitors of interest in this study are potassium sorbate and Lauricidin plus™ which inhibit vegetative growth, and methylantranilate and sodium 5-5 diethylbarbiturate which inhibit germination of aerobic spores.

Inhibitors of Vegetative Growth. Potassium sorbate has been utilized mainly for its fungistatic and bacteriostatic effects. The effective inhibitory species in the undissociated form of sorbic acid which apparently inhibits the oxidative assimilation of carbon (Frazier, 1967). A wealth of information is available on its action on vegetative growth; however little is known about the effect of potassium sorbate on germination of bacterial spores except that germination of bacterial spores takes place at concentrations which would inhibit growth (Gould, 1964).

All the published information on lauricidin concerns the effect of this compound on the vegetative growth of bacteria. The mechanism of action of lauricidin is associated with the hydrophilic and hydrophobic parts of the molecule. The hydrophobic part of the fatty acid is apparently the most important portion of the molecule and Kabara and coworkers postulate that this compound affect the fluidity of the cell membranes (Kabara, 1978; Kabara and Vrable, 1977). Lauricidin plus™, a mixture of lauricidin and sorbic acid, was recently patented as an antimicrobial agent which has activity against a wide spectrum of microorganisms.

Specific Inhibitors of Germination. Methylanthranilate inhibits germination of spores of Bacillus species. The inhibition of germination by this compound is irreversible and is not eliminated by subsequent washing of the treated spores with the inhibitor (Prasad and Srinivasan, 1969). Methylanthranilate interfers with the L-alanine-induced germination system as a competitive inhibitor. The effect can be counteracted using a combination of D-glucose, D-fructose and potassium ions, presumably to activate a separate germination system (Prasad, 1974).

Sodium 5-5 diethylbarbiturate or veronal is a well known inhibitor of germination of aerobic spores. Sierra (1968) tested the effect of veronal on the germination of spores of B. subtilis in a complex medium. Germination of heat-activated and nonheat-activated spores was prevented in the presence of 10 mM veronal. The mechanism of inhibition is reversible and seems to be associated with L-alanine induced germination. The activity of the inhibitor is increased by decreasing the pH or increasing the concentration of veronal at a constant pH. Thus the undissociated molecule is the active species. The inhibitory action of veronal is also counteracted by the combined addition of D-glucose, D-fructose and potassium ions (Sierra and Bowman, 1969).

MATERIALS AND METHODS

Organisms

Clostridium perfringens FD1 from the culture collection of the Michigan State University Food Microbiology Laboratory was used in all experiments. Also, C. perfringens strains NCTC 8238, ATCC 3624, ATCC 12195 and C. sporogenes PA 3679, obtained from the same collection, were used in some experiments.

Spore Production and Preparation of Spore Suspensions

Spores were produced by a modification of the method described by Duncan and Strong (1968). An active culture was obtained by three subsequent transfers of C. perfringens vegetative cells in Fluid Thioglycollate medium (FTG). The first culture was grown for 18 hours at 37 C, 1 ml was transferred to 9 ml of fresh FTG, incubated for 4 hours at 37 C, and finally 1 ml of this culture was inoculated into 9 ml of FTG and grown for 4 hours at 37 C. This last FTG culture (10 ml) was transferred into 100 ml of Duncan-Strong sporulation medium (DS) and incubated for 18 to 24 hours at 37 C. Spores were harvested by centrifugation in a Sorvall refrigerated centrifuge at 14,600xg for 30

minutes at 4 C. Spores were washed with chilled deionized, sterile water 5 to 7 times and stored at 4 C.

Heat Shock

Several heat shock treatments were given to the spore suspension to promote activation. Spores were heat shocked in distilled deionized water at 70 C, 75 C and 80 C in a temperature controlled (±1 C) water bath for 20, 15 and 10 minutes, respectively.

Media

Sporulation Medium. Duncan-Strong sporulation medium was prepared using the following formula: yeast extract, 0.4%; peptone, 1.5%; soluble starch, 0.4%; sodium thioglycollate, 0.1%; dibasic sodium phosphate, 1.0% (Duncan and Strong, 1968).

Plating Medium. Tryptose-Sulfite-Cycloserine basal medium (TSC); Tryptose, 1.5%; soytone, 0.5%; yeast extract, 0.5%; sodium metabisulfite, 0.1%; ferric ammonium citrate, 0.1%; agar, 2.0% (Harmon et al., 1971). No cycloserine was added.

Germination Media. Brain Heart Infusion, 2.5%; yeast extract 0.27% (BHI + YE; Duncan et al., 1972). Fluid thioglycollate (FTG) broth (Difco). FTG prepared in the laboratory (LFTG): trypticase, 2.0%; sodium chloride, 0.25%; dibasic potassium phosphate, 0.1%; sodium sulfite,

0.02%; sodium thioglycollate, 0.06%; L-cystine, 0.04%; glucose, 1.0%. Chemically defined media were prepared utilizing the inorganic ingredients of LFTG plus L-cystine. Na thioglycollate and glucose (this medium was designated FTG-base). The nitrogen source was represented by mixtures of amino acids or by a single amino acid.

Carbohydrates Used as Germinants

Ribose, xylose, fructose, lactose, sucrose, cellobiose, melibiose, melezitose, and raffinose were substituted for glucose in the germination system. Sugars were present at a final concentration of 1.0%. Non-metabolizable analogs of glucose were also used. These were represented by α- and β-methylglucopyranosides which were tested at a final concentration of 1.0%.

Nitrogen Sources Used as Germinants

Complex and simple nitrogen sources were used as germinants, trypticase and casaminoacids were used at a final concentration of 2.0%. Also individual amino acids were tested, in different combinations and concentrations. The concentrations used were based on the ones reported by Busta et al. (1973) and are listed in Table 1. D-isomers of L-alanine, L-glutamic acid and L-leucine were tested. They were added at the same concentration as the corresponding L-isomer.

Table 1. Concentrations of amino acids used as germinants in chemically defined media

Amino Acid	mg/l	Amino Acid	mg/l
Alanine	30	Serine	63
Valine	72	Threonine	49
Leucine	92	Cysteine	3
Isoleucine	61	Tyrosine	63
Proline	113	Aspartic	71
Phenylalanine	50	Glutamic	224
Tryptophan	17	Lysine	82
Methionine	28	Arginine	41
Glycine	27	Histidine	31

Mineral Salts

Several mineral salts were tested as substitutes for sodium chloride. They were tested at a concentration of 0.25% in the presence of glucose, 1.0%; glutamic acid, 0.022%; and leucine 0.018% at a final pH of 6.0. The mineral salts used were the following: potassium nitrite, potassium nitrate, sodium nitrite, sodium nitrate, potassium chloride and sodium bicarbonate.

Inhibitors

Two types of inhibitors were tested in the germination system: 1) inhibitors of vegetative growth and 2) inhibitors of germination of aerobic spores.

Inhibitors of Vegetative Growth. Potassium sorbate (KS) was tested in complex (BHI + YE and FTG) and chemically defined media, over a pH range from 5.0 to 7.0 at concentrations ranging from 0.1 to 2.0%.

Lauricidin a known inhibitor of growth of gram-positive bacteria has been combined with KS to produce an inhibitor called Lauricidin plus™. It was tested over a concentration range from 0.03 to 0.5 mg/ml in complex and chemically defined media. This compound was first dissolved in fresh media tempered to approximately 60 C and then added aseptically to fresh media tempered to the same temperature.

Inhibitors of Germination of Aerobic Spores. Sodium 5,5-diethylbarbiturate or Veronal (V) was tested at a final concentration of 10 mM (a concentration which inhibits germination of aerobic spores). It was only tested in chemically defined media. (FTG-base + L-alanine, FTG-base + L-glutamic acid, FTG-base + L-leucine and FTG-base + L-glutamic acid + L-leucine).

Methylanthranilate (MA) was tested in FTG and in FTG-base + L-glutamic acid + L-leucine at a final concentration of 0.5 mM.

pH

Adjustment of pH was carried out with 0.1N hydrochloric acid and 0.1 N sodium hydroxide. The pH range used was from 4.0 to 10.0 with intervals of 0.5. A Corning model 7 pH meter was used for measurement of pH.

Sterilization

When media with unadjusted pH were used, sterilization was performed by autoclaving at 121 C for 15 minutes. However for the chemically defined media, only some ingredients were autoclaved. Sugars and amino acids were filter sterilized and added to the media aseptically. When media with adjusted pH were used, filter sterilization with Gelman membrane filters (0.45μ) was used to avoid changes in pH which occur during autoclaving.

Inoculation

Screw-cap tubes, 13 x 10-mm, were used as germination containers. Each tube contained 5 ml of culture media and 0.5 ml of a spore suspension were added to give an initial optical density of 0.32 ± 0.04.

Incubation

All germination experiments were conducted at 45 C in a forced air incubator.

Measurement of Germination

Three criteria were used to follow germination of the spores: a) Decrease in optical density (OD) of a spore suspension at 625 nm on a Spectronic 20 (Bausch and Lomb) with a red filter. Readings were taken every 30 minutes during the first two hours and subsequently every one hour up to 6 to 10 hours. A final reading was taken after 24 hours; b) Observation under an American Optical Series 10 phase-contrast microscope for darkening of individual spores; c) Loss of heat resistance after heat shock at 80 C for 5 minutes, followed by plating on TSC. Incubation of plates was performed under anaerobic conditions at 37 C for 24 hours.

Calculation of Percent Germination and Extent of Germination (Y)

The percent change in OD was calculated as follows:

$$\% \text{ change in OD} = \frac{ODi-ODt}{ODi} \times 100 = \frac{\Delta ODt}{ODi} \times 100$$

Where:

ODi = initial OD

ODt = OD at time t

The extent of germination (Y) was calculated as follows:

$$Y = \frac{ODi-ODt}{ODi-ODf} = \frac{\Delta ODt}{ODi-ODf}$$

Where:

ODf = final OD

ΔODt = Difference of ODi-ODt at time t

The percent germination was calculated considering the relationship between decrease in OD as a percent of the initial OD, and the extent of germination measured by loss of heat resistance of the spore suspension.

A 55% decrease in the initial OD corresponded to 99.99% germination. Greater decreased in OD may occur due to loss of cell constituents and cell lysis, particularly after incubation for 24 hours.

RESULTS

Germination in Complex Media

Spores germinated relatively rapidly in BHI + YE, FTG and FTG-base + Trypticase. During the first 0.5 to 1.5 hours there was a marked drop in OD_{625} after which the spores were phase dark when viewed under phase microscopy. These complex media supported substantial outgrowth after germination.

Germination in Chemically Defined Media

Since spores of C. perfringens FD1 germinated in FTG, FTG-base plus all 18 amino acids present in trypticase was used as the basis for a chemically defined medium. As expected, this medium promoted germination of the spores and also supported some outgrowth. In comparison to FTG, there was a relatively long lag phase before the onset of germination. There was a drop in OD_{625} of 50% after 2.5 hours and by 6 hours outgrowth was also observed. However, the nutrients present in this medium were not enough to support luxuriant growth.

The 18 amino acids that supported germination were split into four groups (Table 2) based on the characteristics

Table 2. Classification of amino acids based on the characteristics of the R group

Nonpolar	Polar	Polar-charged	Basic
Alanine	Glycine	Aspartic	Lysine
Valine	Serine	Glutamic	Arginine
Leucine	Threonine		Histidine
Isoleucine	Cysteine		
Proline	Tyrosine		
Phenylalanine			
Tryptophan			
Methionine			

of their R groups (Lehninger, 1975). When media were prepared with two or more groups of amino acids, the most efficient media for germination were those having the nonpolar and polar-charged groups. The nonpolar group and the polar-charged group were able to support germination, while the polar and basic groups were unable to promote germination (Table 3). Interestingly, the nonpolar and polar-charged groups were more effective alone than in combinations containing the other two groups of amino acids. For example, the polar group completely blocked the germinative action of the nonpolar group when the two were in combination.

The effect of single amino acids in the nonpolar and polar-charged groups on germination is shown in Table 4. Only alanine, glutamic acid and leucine supported germination with a decrease in OD_{625} >50% within 24 hours at 45 C.

Effect of Alanine, Glutamic Acid and Leucine on Germination of Spores of C. perfringens FD1

During the initial stages of germination, the fastest germination rate among single amino acids added to FTG-base was obtained with leucine, followed by glutamic acid and alanine (Table 5). However, after 24 hours no marked differences were observed in the extent of germination for each of these three amino acids. When added at twice the normal concentrations, the rates of germination

Table 3. Percent change in optical density of a spore suspension of *Clostridium perfringens* FD1 in various chemically defined media after 5 and 24 hours

Media[a]	Change in OD (%)	
	Time	
	5 hours	24 hours
None	0	0
All amino acids	4	59
All except polar	6	40
All except polar-charged	0	5
All except nonpolar	3	45
All except basic	3	41
Nonpolar + polar-charged	50	53
Nonpolar + polar	0	0
Nonpolar + basic	7	63
Polar + polar-charged	3	48
Polar-charged + basic	7	48
Nonpolar	18	71
Polar-charged	13	47
Polar	0	3
Basic	0	7

[a]All media contained FTG-base plus the amino acid group(s) indicated (pH 6.8)

Table 4. Effect of the nonpolar and polar-charged groups of amino acids on germination of spores of C. perfringens FD1 after 24 hours at 45 C

Amino acid[a]	Decrease in OD (%)	Amino acid[a]	Decrease in OD (%)
Nonpolar group	70	Leucine	59
Polar-charged group	67	Isoleucine	19
Aspartic	24	Proline	13
Glutamic	71	Phenylalanine	16
Alanine	53	Tryptophan	10
Valine	20	Methionine	10

[a]All media contained FTG-base (pH 6.8) plus the amino acids indicated

Table 5. Effect of alanine, glutamic acid and leucine on the germination of spores of C. perfringens FD1 after 5, 10 and 24 hours at 45 C

Amino Acid[a]	Time in hours					
	5		10		24	
	Decrease in OD (%)	Germination (%)	Decrease in OD (%)	Germination (%)	Decrease in OD (%)	Germination (%)
Glutamic 1X	13	22	36	59	58	100
Alanine 1X	13	22	39	65	65	100
Leucine 1X	19	32	48	81	58	100
Glutamic 2X	25	42	46	77	61	100
Alanine 2X	21	35	48	81	72	100
Leucine 2X	18	29	46	77	71	100
Glu + Ala ½X	27	45	52	86	64	100
Glu + Leu ½X	43	72	57	100	57	100
Ala + Leu ½X	29	48	58	100	65	100

[a]The amino acid(s) indicated was added to FTG-base (pH 6.8)

increased for glutamic acid and alanine while the rate for leucine was essentially unchanged. When combinations of two of the three amino acids were used at half the original concentration, the mixture of glutamic acid and leucine gave the best results regarding rates of germination. The extent of germination after 24 hours was similar for single amino acids or any of the mixtures (Table 5).

D-glutamic acid and D-leucine supported rapid germination when used alone or in combination with their L-isomers. However, D-alanine caused a delay in the onset of germination, even when L-glutamic acid and/or L-leucine were used as germinants. This inhibitory action affected only the initial stages of germination (Table 6).

Effect of a Heat Shock on the Germination of Spores of C. perfringens

Initially a heat shock of 70 C for 20 minutes was given to spore suspensions. With this activation treatment spores germinated at a satisfactory rate and extent, either in FTG or in a chemically defined medium supplemented with 18 amino acids. However, germination was more rapid in FTG than in the synthetic medium. Nonheat-shocked spores germinated in an asychronous manner in FTG and did not germinate in the chemically defined medium (Fig. 1).

From the previous results it was apparent that a heat treatment at 70 C for 20 minutes modified the germination

Table 6. Effect of D-isomers of alanine, glutamic acid and leucine on the germination of spores of C. perfringens FD1 after 2, 6 and 24 hours at 45 C.

Amino acid[a]	Decrease in OD (%)		
	2 hours	6 hours	24 hours
L-ala	3	16	65
D-ala	0	10	58
LD-ala	0	7	59
L-glu	46	50	50
D-glu	46	46	50
LD-glu	46	50	54
L-leu	48	48	56
D-leu	46	50	50
LD-leu	48	48	52
L-glu + L-leu	56	56	63
D-glu + D-leu	48	52	52
LD-glu + LD-leu	44	48	48
L-glu + D-ala	3	23	60
L-leu + D-ala	3	16	61
L-glu + L-leu + D-ala	29	50	61
L-glu + L-leu + L-ala + D-ala	33	52	56

[a]The amino acid(s) indicated was added to FTG-base adjusted to pH 6.0.

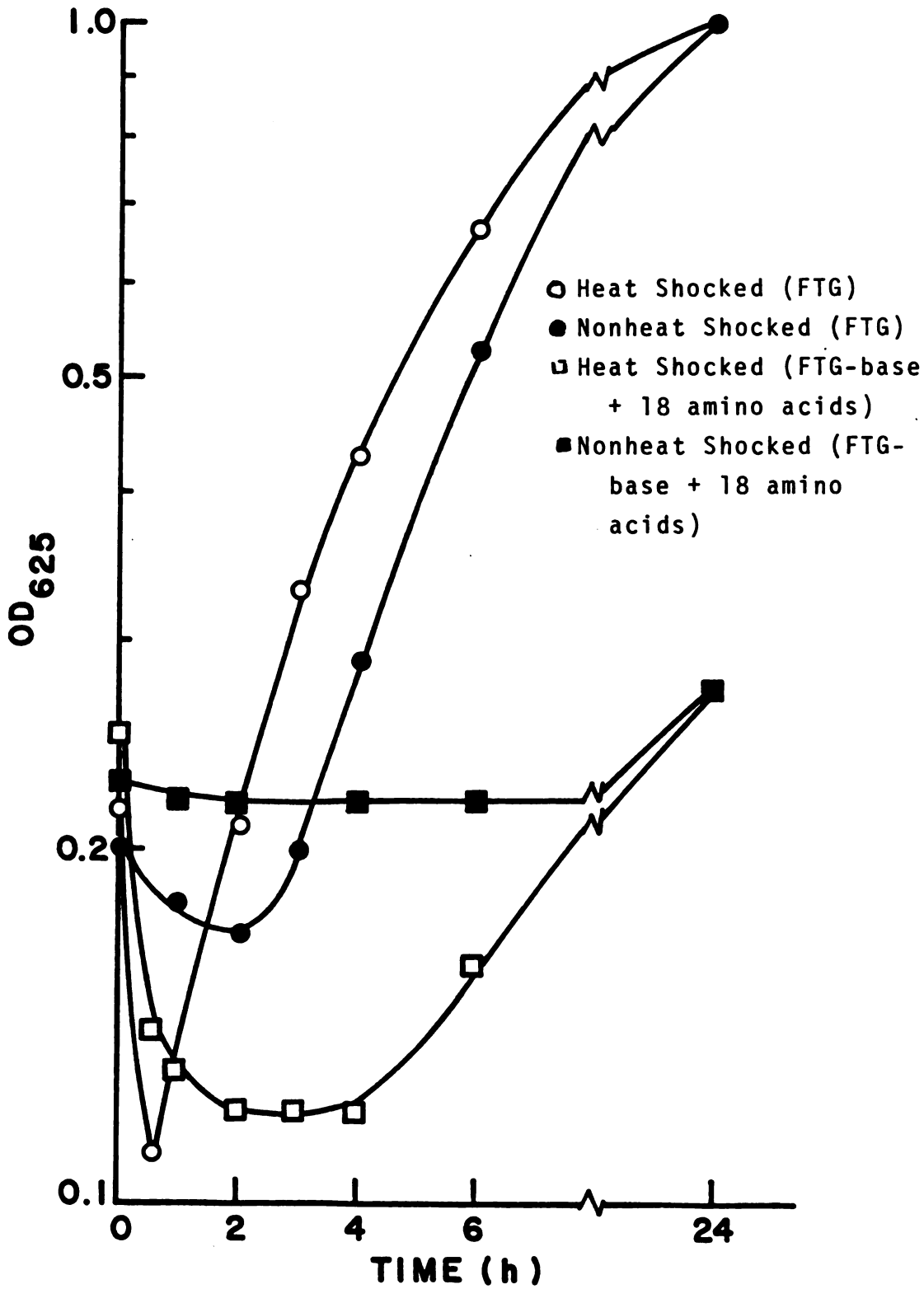


Fig. 1. Effect of a heat shock at 70 C for 20 min on germination and outgrowth of Clostridium perfringens FD1 at 45 C in FTG or FTG-base plus 18 amino acids

requirements. Using different time/temperature relationships, as the temperature used during the heat shock increased, the germination response in a chemically defined medium also increased. However, in some instances, no improvement in the ability to promote germination in media lacking a specific group of amino acids was obtained (Table 7). This was the case when the polar group was omitted, where a slight decrease in the germination extent was obtained as the heat-shock temperature was increased. Since a heat shock at 80 C for 10 minutes generally resulted in the best germination in these chemically defined media, it was selected for use in further experiments. Data presented in the following Tables and Figures was obtained using spore suspensions which had been heat shocked at 80 C for 10 minutes.

Effect of pH on Germination of C. perfringens FD1 in Complex and Chemically Defined Media

Changes in pH dramatically affected germination induced by FTG-base containing single amino acids or combinations of amino acids which promoted germination. Germination in the complex media took place over a much broader pH range. On the acid side BHI + YE lost its ability to promote rapid germination at pH 5.5, FTG at pH 5.0 and the chemically defined medium with glutamic acid and leucine at pH 4.5 (Fig. 2). On the alkaline side, the complex media supported

Table 7. Effect of various heat treatments on the germination of spores of C. perfringens FD1 in chemically defined media after 24 hours at 45 C.

Media[a]	Change in OD (%)		
	70 C/20 min	75 C/15 min	80 C/10 min
All except nonpolar	11	37	52
All except polar-charged	9	10	7
All except polar	51	42	40
All except basic	9	28	47

[a]The groups of amino acids indicated were added to FTG-base, pH 6.8.

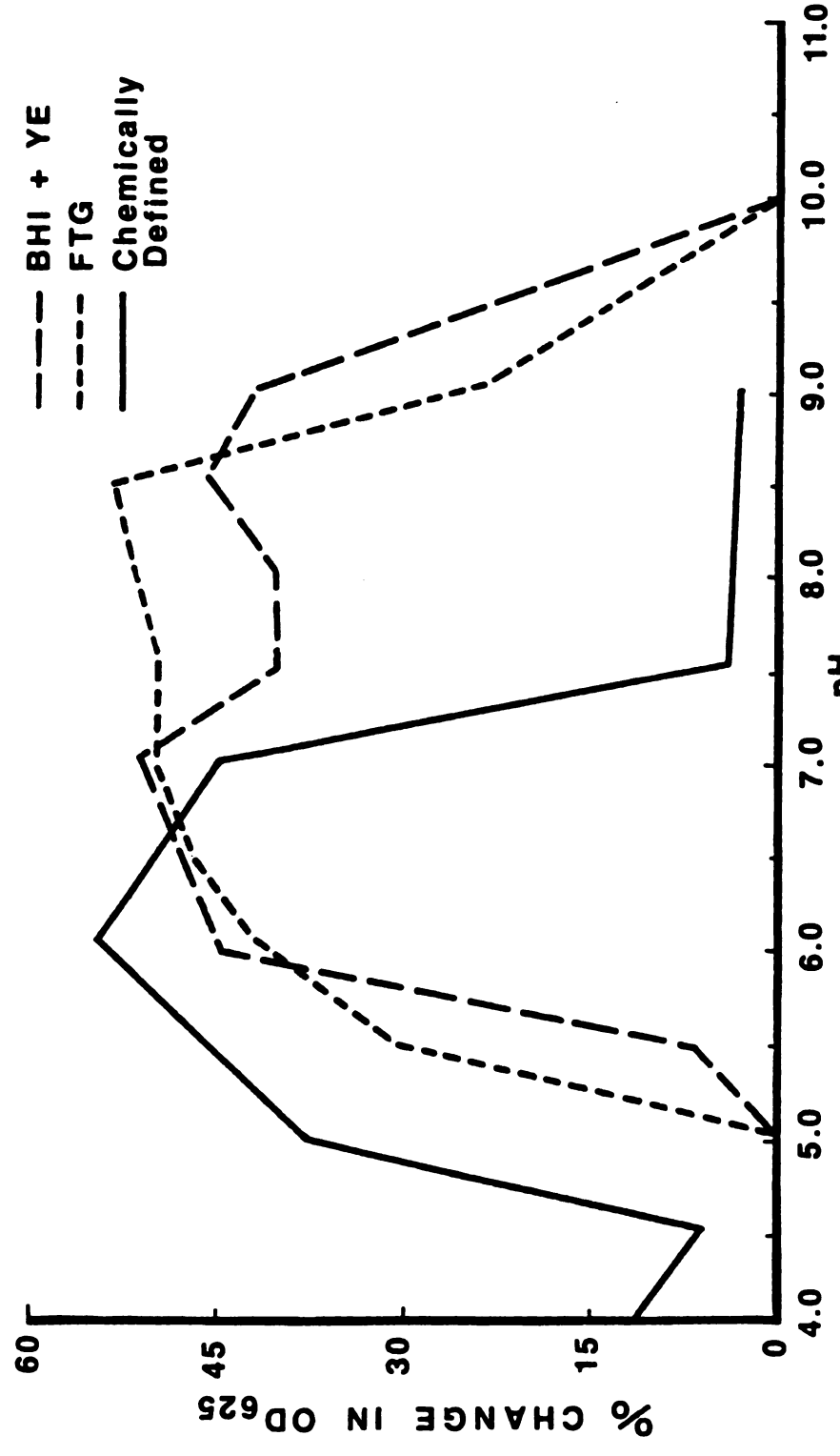


Fig. 2. Effect of pH on germination of spores of C. perfringens FD1 in complex and chemically defined media (complex media after 1 hour and chemically defined medium after 2 hours at 45 C)

relatively rapid germination at pH 9.0, but not at pH 10.0. Outgrowth was observed in the complex media even at pH 8.5. For the chemically defined media containing alanine, glutamic acid or leucine, there was a narrow optimum pH for germination around pH 5.5 to 6.5. When glutamic acid and leucine were both present a slightly wider optimum pH range was observed (Fig. 3).

Effect of Ingredients of the Basal Medium on Germination of Spores of C. perfringens FD1

Spores were washed 10 times in chilled, distilled, deionized water in an attempt to completely remove any adsorbed substances from the spores and experiments were conducted by adding ingredients to media containing glucose, glutamic acid, and leucine. When the pH was not adjusted, the extent of germination was low if only one other ingredient was added. When the pH was adjusted to 6.0, partial germination was promoted only by the addition of sodium chloride or L-cystine; after 4 hours of incubation decreases in OD_{625} of 35 and 32% were obtained with sodium chloride or L-cystine, respectively. The other ingredients did not cause any noticeable change id OD_{625} even after incubation for 24 hours at 45 C (Table 8).

When two ingredients were added to the medium containing glucose, glutamic acid and leucine, the most effective combinations contained sodium chloride. The media containing

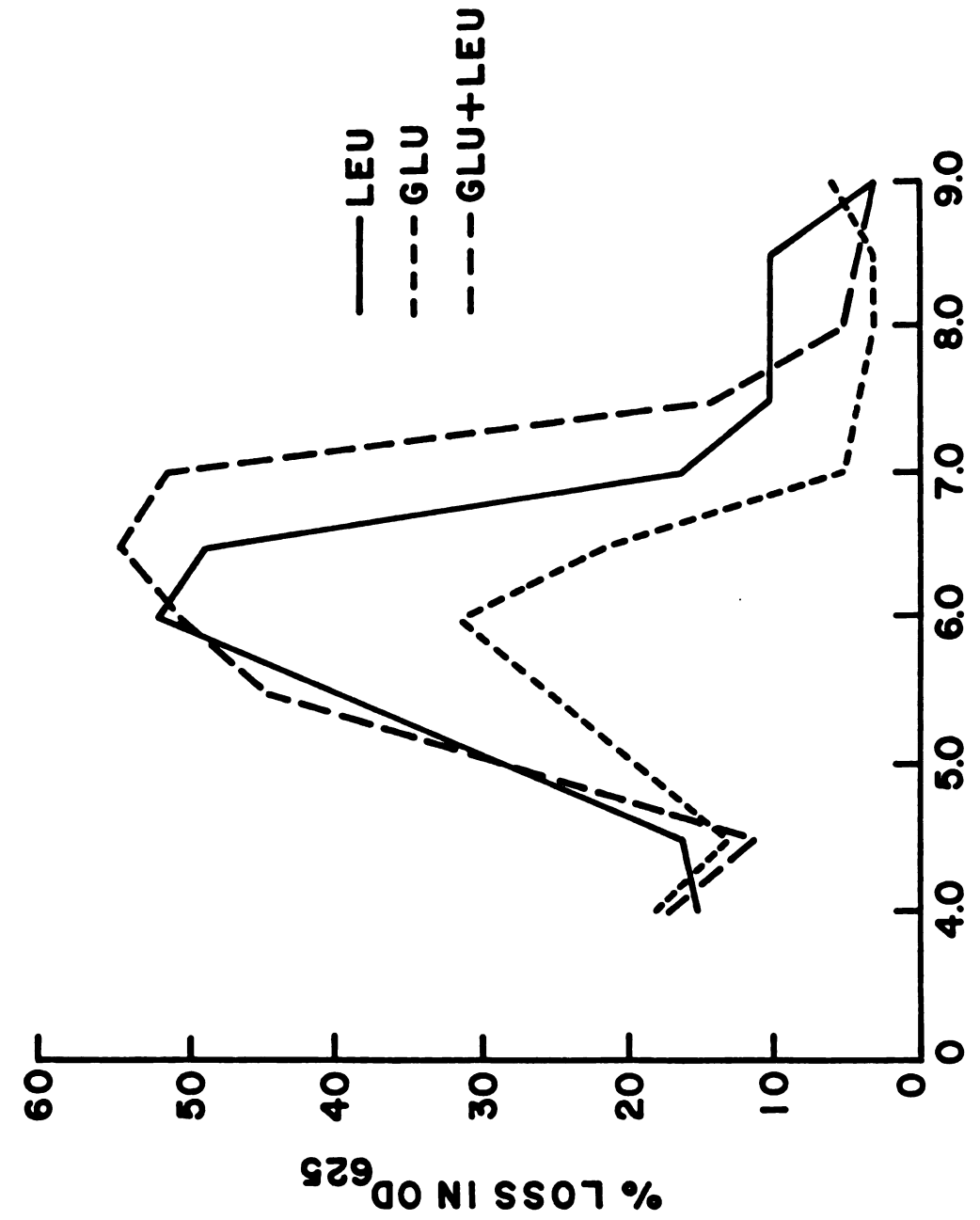


Fig. 3. Effect of pH on germination of spores of C. perfringens FD1 in chemically defined media after 5 hours at 45 C.

Table 8. Effect of single ingredients and combinations on germination of spores of C. perfringens FD1 after 1, 4 and 24 hours at 45 C

Ingredient[a]	Change in OD (%)		
	1 hour	2 hour	3 hour
NaCl (Na)	15	35	38
H_2HPO_4(K)	0	0	0
Na_2SO_2 (NaS)	0	3	6
Na-thioglycollate (NaT)	0	0	0
L-cystine (C)	27	32	32
Na + K	21	41	50
Na + NaS	3	27	41
Na + NaT	0	22	38
Na + C	35	44	47
K + NaS	0	0	6
K + NaT	0	0	6
K + C	36	42	47
NaS + NaT	0	0	3
NaS + C	18	29	30
NaT + C	17	34	46
Na + K + C	50	54	54
Na + NaS + C	13	30	35
Na + NaT + C	39	44	48
Na + K + NaS + C	46	46	50
Na + K + NaT + C	46	50	50
Na + NaS + NaT + C	32	44	44
Basal Complete	51	56	56

[a]Media contained 224 mg/l L-glutamic acid, 92 mg/l L-leucine and 10 g/l glucose adjusted to pH 6.0.

sodium chloride combined with dibasic potassium phosphate, sodium sulfite or L-cystine had decreases in OD_{625} of 50, 41 and 47%, respectively after 24 hours. Intermediate changes in OD_{625} were obtained with sodium chloride + sodium thioglycollate, and sodium sulfite + L-cystine, with 39 and 31% decrease in OD_{625}, respectively. The remaining combinations gave negligible changes after 24 hours at 45 C. The changes in OD_{625} obtained with any of the mixtures of two ingredients of the basal medium were usually less than and occurred more slowly than those obtained with the complete basal medium supplemented with glucose and amino acids. When combinations of three or more ingredients were used, combinations containing sodium chloride, L-cystine and dibasic potassium phosphate were most effective in inducing germination (Table 8).

Effect of Different Mineral Salts as Substitutes of Sodium Chloride

When a series of mineral salts were tested to determine their ability to substitute for sodium chloride in a chemically defined germination system, only sodium nitrite, sodium nitrate and potassium nitrite induced some germination in media without glutamic acid and leucine. All of the mineral salts tested induced partial germination in the presence of glutamic acid and leucine (Table 9).

Table 9. Effect of different salts on germination of spores of C. perfringens FD1 after 24 hours at 45 C in media with or without amino acids.

Salt[a]	No Amino Acids		Amino Acids[b]	
	Decrease in OD (%)	Germination (%)	Decrease in OD (%)	Germination (%)
NaCl	16	27	38	63
KNO_2	13	22	34	57
KNO_3	0	0	23	38
$NaNO_2$	17	28	34	57
$NaNO_3$	26	43	21	35
KCl	0	0	21	35
$NaHCO_3$	0	0	32	54

[a]Media contained 1% glucose adjusted to pH 6.0 plus 0.25% of the salt listed.

[b]Also contained 224 mg/l glutamic acid and 92 mg/l leucine.

Effect of Different Sugars on Germination of Spores of C. perfringens FD1

Several sugars, including nonmetabolizable glucose analogs, induced rapid germination. While the pentoses ribose and xylose, failed to support substantial germination after one hour, they supported 75 to 90% germination after 24 hours (Table 10).

Effect of Inhibitors on Germination of Spores of C. perfringens FD1

Potassium sorbate was tested to determine its effect on germination in complex and chemically defined media over a pH range from 5.0 to 7.0. BHI + YE promoted good germination from pH 5.5 to 7.0. However, the rate of germination was slow at pH 5.5 even in the control without sorbate. As the pH was increased, higher concentrations of the inhibitor were generally needed to obtain the same inhibitory effect. At pH 7.0, 1.5% potassium sorbate was needed to substantially inhibit germination during the first 4 hours of incubation (Fig. 4).

Similar results were obtained when FTG was used as the germination medium (Fig. 5). In the chemically defined medium, considerable germination was obtained for the controls in the pH range from 5.5 to 6.5. However, at pH 7.0 germination was extremely slow. When potassium sorbate was present the extent of germination decreased at the lowest

Table 10. Effect of different sugars on germination of spores of C. perfringens FD1 after 1 and 24 hours at 45 C

Sugar[a]	1 hour	24 hours
	Decrease in OD (%)	Decrease in OD (%)
None	0	6
Ribose	18	41
Xylose	6	50
Glucose	39	50
Fructose	39	46
Sucrose	46	46
Lactose	44	44
Cellobiose	45	58
Melibiose	47	52
Melezitose	48	55
Raffinose	46	54
α-Methyl glucoside	44	55
β-Methyl glucoside	47	54

[a]Media contained FTG-base, 224 mg/l glutamic acid, 92 mg/l leucine, and 1% of the sugar indicated, adjusted to pH 6.0.

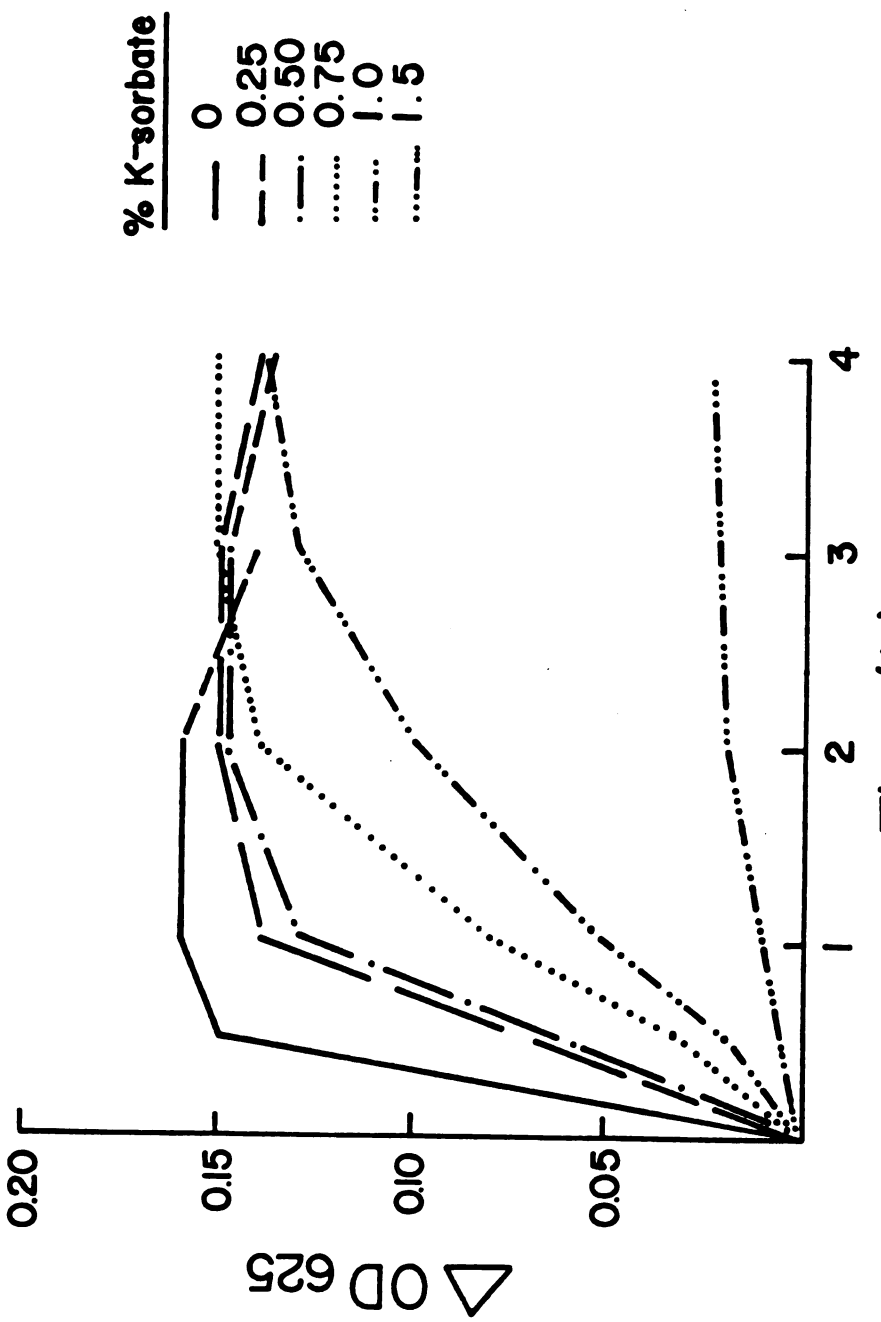


Figure 4. Germination of spores of C. perfringens FD1 in BHI + YE with different concentrations of potassium sorbate (pH 7.0).

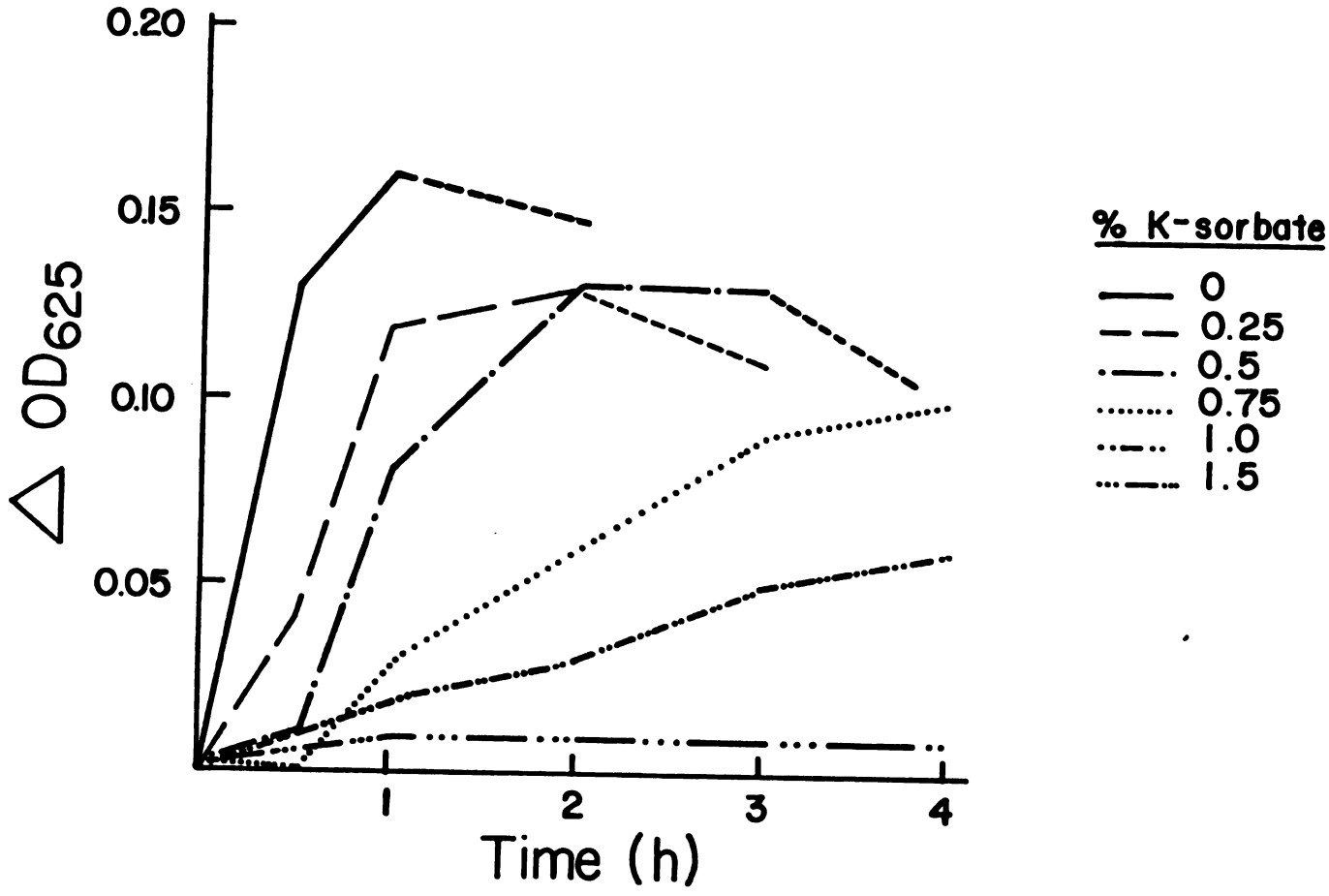


Fig. 5. Germination of spores of C. perfringens FD1 in FTG with different concentrations of potassium sorbate (pH 7.0).

concentrations tested at each pH level. At the optimum pH (6.0-6.5) germination in the chemically defined medium was only 60% in the presence of 0.25% potassium sorbate, and higher concentrations completely inhibited germination (Fig. 6).

Germination rates decreased in complex and chemically defined media as the potassium sorbate concentration increased. Table 11 shows the time needed to obtain 50% germination in media containing various concentrations of potassium sorbate. The minimum potassium sorbate concentrations needed to limit germination to 50% are presented in Table 12.

Concentrations of Lauricidin plus™ ranging from 0.03 to 0.5 mg/ml did not markedly affect the extent of germination in complex media; even at the highest concentration used more than 70% of the spores germinated within 24 hours at 45 C. However, the rate of germination decreased when media contained $\geq$ 0.12 mg/ml Lauricidin plus™. In the chemically defined medium, the extent of germination and germination rate were substantially affected by the presence of 0.5 mg/ml Lauricidin plus™ (Table 13).

Methylanthranilate did not inhibit germination in complex media. In chemically defined media the germination rate was decreased by the presence of the inhibitor and only 62% germination was observed after 24 hours at 45 C (Table 14). Sodium 5,5-diethylbarbiturate (veronal) at a

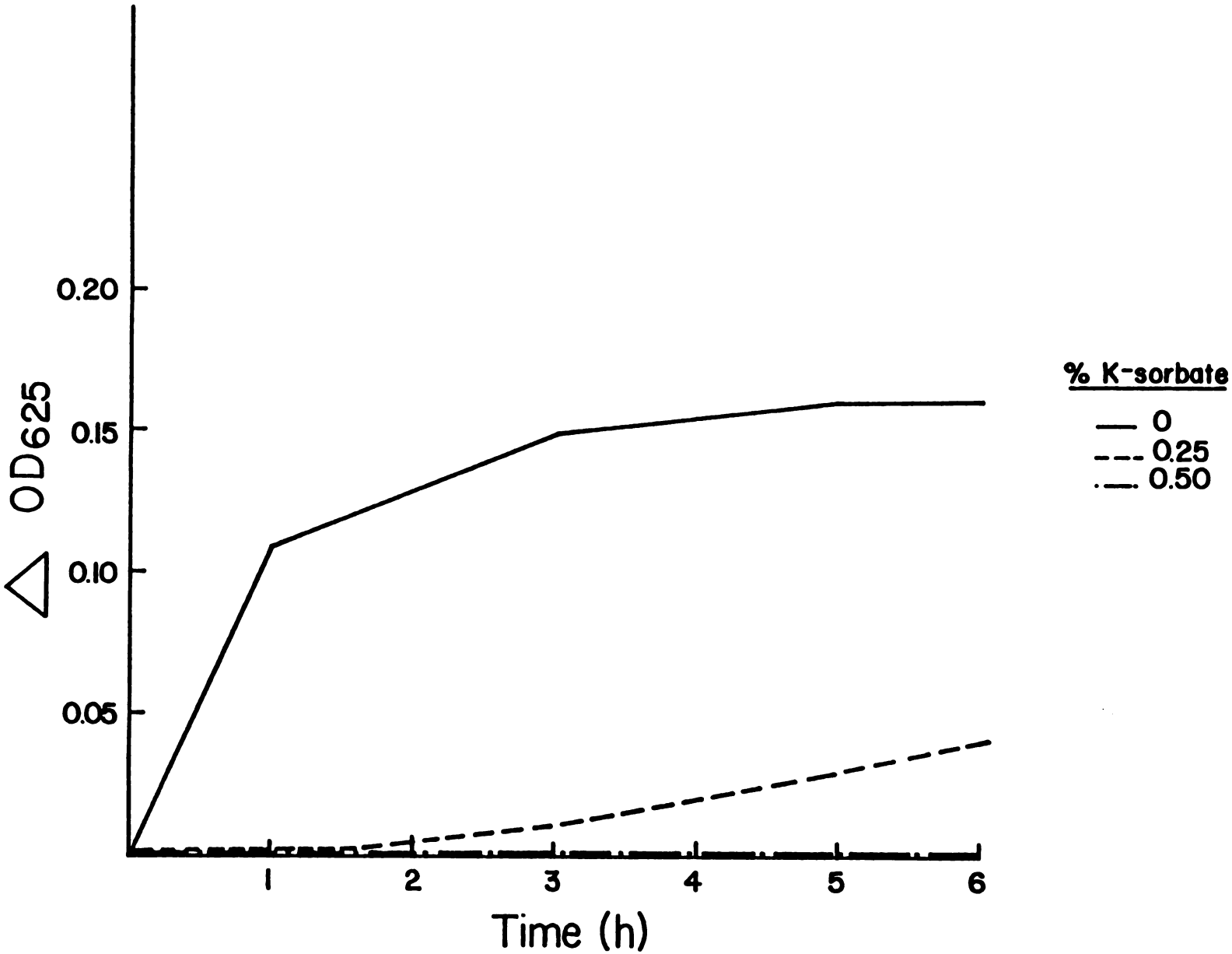


Fig. 6. Germination of spores of C. perfringens FD1 in chemically defined medium with different concentrations of potassium sorbate (pH 6.0).

Table 11. Time (in hours) needed to obtain 50% germination of C. perfringens FD1 spores in BHI + YE (pH 7.0), FTG (pH 7.0) and chemically defined medium (pH 6.0) containing various concentrations of potassium sorbate.

K-sorbate (%)	BHI + YE	FTG	Chemically Defined
0.0	<0.5[a]	<0.5	<1.0
0.25	<1.0	<1.0	24
0.50	<1.0	<2.0	>24
0.75	<1.5	3	>24
1.0	2.0	>5	>24
1.5	24	>24	>24
2.0	<24	>24	>24

[a]Readings were taken at 0.5 hours intervals during the first two hours and at one hour intervals from 2 to 10 hours. A final reading was taken after 24 hours.

Table 12. Minimum concentration of potassium sorbate (%) needed to limit germination of spores of C. perfringens FDI to ≤ 50% in three media at pH 5.0-7.0 after 24 hours at 45 C

pH	BHI + YE	FTG	Chemically Defined
5.0	-[a]	0.1[b]	0.1[b]
5.5	0.3	0.5	0.2
6.0	1.5	1.5	0.2
6.5	2.0	1.5	0.25[b]
7.0	2.0	1.5	0.25[b]

[a]Inhibition by pH

[b]Lowest concentration used

Table 13. Effect of Lauricidin plus™ on germination of spores of C. perfringens FD1 in three media after 1, 5 and 24 hours at 45 C

Media	Decrease in OD (%)		
	Time (hrs)		
	1	5	24
BHI + Ye (pH 7.5)			
Control	44	-[a]	-
0.03 mg/ml	22	24	41
0.12 mg/ml	14	16	37
0.50 mg/ml	34	37	46
FTG (pH 7.0)			
Control	40	-	-
0.03 mg/ml	29	32	44
0.12 mg/ml	22	33	39
0.50 mg/ml	22	32	43
Chemically defined (pH 6.0)			
Control	42	52	52
0.03 mg/ml	41	49	49
0.12 mg/ml	17	43	47
0.50 mg/ml	2	18	28

[a]Outgrowth occurred resulting in increases in OD.

Table 14. Effect of Veronal (V) and Methylanthranilate (MA) on germination of C. perfringens FD1 after 1, 5 and 24 hours at 45 C (pH 6.0)

Media and Inhibitors	Decrease in OD (%)		
	Time (hrs)		
	1	5	24
FTG Control	50[a]	-[b]	-
Chemically defined			
Control[a]	46	49	53
L-alanine	9	46	51
L-glutamic acid	42	50	50
L-leucine	44	52	57
L-glutamic + L-leucine (MA)	6	31	34
L-alanine (V)	11	37	39
L-glutamic (V)	44	47	37
L-leucine (V)	44	50	53
L-glutamic + L-leucine (V)	46	49	51

[a]Determined at 0.5 hours

[b]Outgrowth

final concentration of 10 mM had no effect on germination rate or extent of germination in complex or chemically defined media (Table 14).

Germination of Other C. perfringens strains and PA 3679

For three different batches of spores of C. perfringens FD1 germination varied from 87 to 91% after 24 hours at 45 C in the chemically defined medium containing glutamic acid and leucine. Clostridium perfringens strains NCTC 8238 and ATCC 3624 reached a maximum germination of about 70%. However, only 13 and 27% germination occurred for PA 3679 and C. perfringens strain ATCC 12195, respectively (Fig. 7).

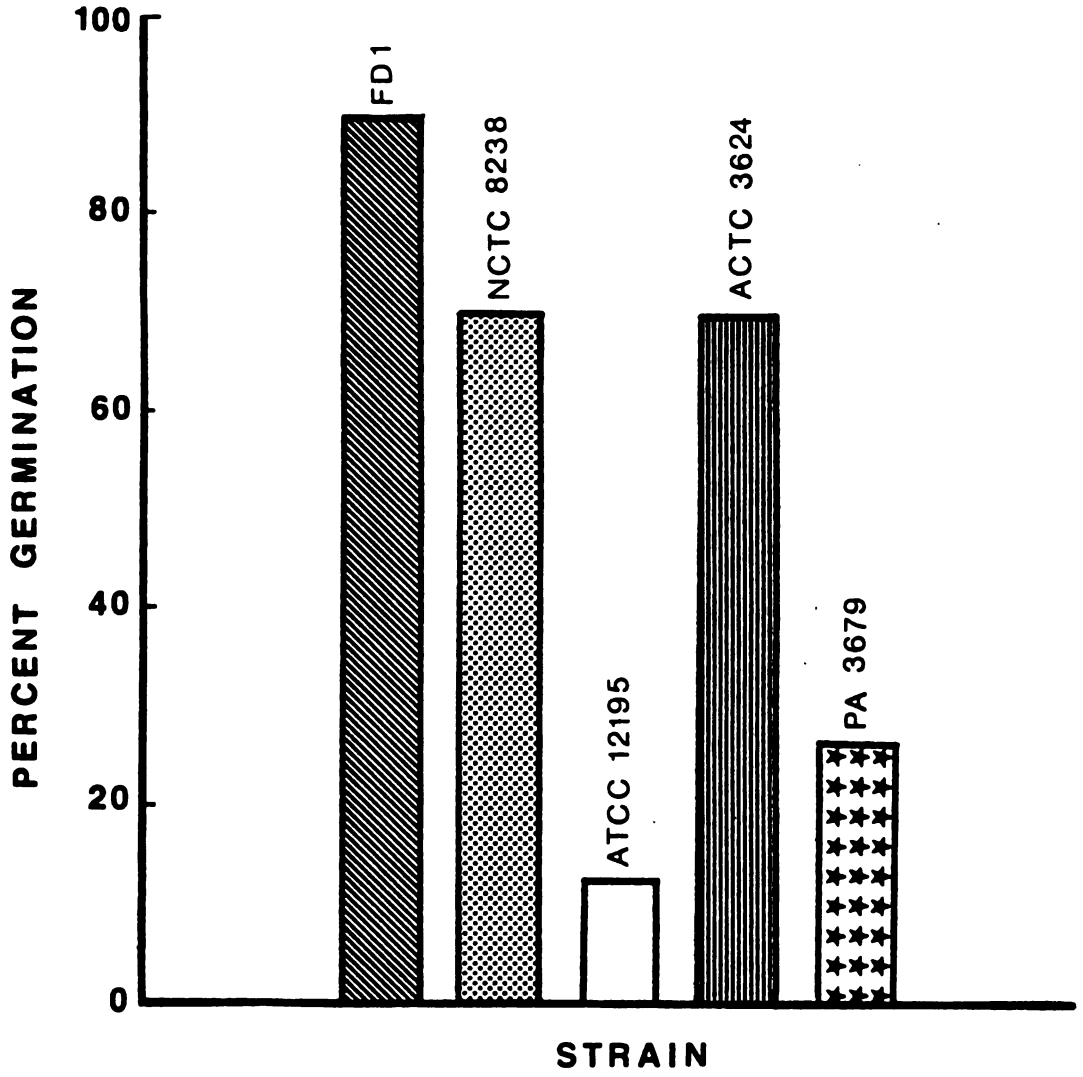


Fig. 7. Germination of different strains of C. perfringens and PA 3679 after 24 hours at 45 C in the chemically defined germination medium containing glutamic acid and leucine adjusted to pH 6.0.

DISCUSSION

There is little information available on germination of spores of C. perfringens in chemically defined media. Reports of germination of spores of clostridia in media of known chemical composition are usually related to other species of this genus.

Several amino acids have been used successfully in germination systems for spores of Clostridium species; among them L-alanine, L-phenylalanine, L-arginine and L-methionine are the most common amino acids used as germinants for C. bifermentans, C. roseum, C. sporogenes, C. septicum and C. tetani, and also for some strains of PA 3679 (Hitzman et al., 1957; Treadwell et al., 1958; Gibbs, 1964; Uehara and Frank, 1965; Holland et al., 1969 and 1970; Waites and Wyatt, 1971). However other ingredients must be present in the media to permit acceptable levels of germination. Lactate, sodium chloride and sodium phosphate were required for C. bifermentans (Holland et al., 1970). Sodium ions are necessary for germination of C. sporogenes (Waites and Wyatt, 1971). Sodium lactate and sodium bicarbonate were required for PA 3679 (Holland et al., 1969). Pyrophosphate was also required for

PA 3679 (Uehara and Frank, 1965).

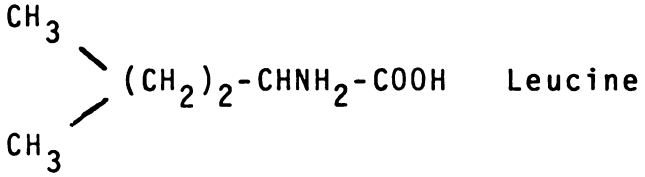
Except for ionic germination, the simplest germination system reported for C. perfringens was buffered glucose at pH 7.0 which permitted 94% germination in 46 hours at 37 C (Wynne and Foster, 1954). However, during the past 25 years there have been no further reports of germination of spores of C. perfringens under these conditions. Furthermore, C. perfringens FD1 failed to germinate under these conditions (unpublished data).

Considerations on Amino Acids and Germination

With regards to germinants found for spores of C. perfringens FD1 only L-alanine was previously reported as an effective germinant, while L-glutamic acid and L-leucine have not been reported as effective germinants even for other Clostridium species. It is important to mention that the chemical structures of these amino acids are quite different:

CH_3-$CHNH_2$-COOH Alanine

COOH-$(CH_2)_2$-$CHNH_2$-COOH Glutamic acid



Leucine

Interestingly, amino acids such as aspartic acid, that has only one methylene group less than glutamic acid, or valine and isoleucine, which have somewhat similar structures to leucine, were not effective germinants for C. perfringens FD1. This indicates that the size of the R group and/or the steric hindrance of these molecules have pronounced effects of their germinative ability. Therefore, the possibility of different receptor sites for glutamic acid, leucine and alanine should be considered. Keynan (1978) postulates that the receptor sites for the triggering of germination are located in the inner membrane surrounding the protoplast and are independent of the spore coat. Apparently, germinants bind to specific locations and promote some biophysical changes in the plasma membrane which alter the permeability of this structure (Keynan, 1978). The activity of hydrolases and proteolytic enzymes that degrade the cortex and the simultaneous activation of spore metabolism leads to the development of a new cell (Keynan, 1978).

Woese et al. (1958) evaluated the steric hindrance of molecules related to L-alanine, particularly that associated with the NH_2 and H substituents on the asymmetric carbon of the amino acid. Any steric change or change in electronegativity of the NH_2 group caused a drastic change in the ability of the amino acid to bind to the receptor site. On the other hand, the number of carbon atoms and the steric

configuration of the R group were related to the ability of the amino acids to induce germination. For spores of B. subtilis L-leucine was considered a poor germinant. Its ineffectiveness as a germinant was associated with the steric configuration of the R group. Each methyl group added to the R group of alanine resulted in a 10-fold reduction in effectiveness as a germinant (Woese et al., 1958). Thus, L-leucine had approximately 1000-times less activity as a germinant than L-alanine. However, in spores of C. perfringens FD1 L-leucine was one of the best germinants and L-glutamic acid gave comparable germination to that obtained with L-alanine. L-glutamic acid and L-leucine were needed at much lower concentrations than those reported for B. subtilis. Thus, the steric characteristics of the R group of amino acids used as germinants for spores of Bacillus do not hold true for spores of C. perfringens FD1. Furthermore, Woese et al. (1958) established that a positive or negative charge in the R group of the amino acid rendered it ineffective as a germinant. In the system designed for C. perfringens FD1 at pH 6.0, glutamic acid is negatively charged but still promoted germination.

The relationship of the four substituents of the asymmetric carbon atom of the amino acid is of great importance in the germination ability of L-alanine for spores of Bacillus species. Interchange of the NH_2 and H substituents transforms the most potent germinant (L-alanine) into a potent

inhibitor (D-alanine). However, for heat activated spores of C. perfringens FD1 this relationship does not appear to be significant because both the D-isomers and L-isomers functioned as germinants. D-alanine did not inhibit germination induced by L-alanine, L-glutamic acid or L-leucine. For spores of bacilli, D-alanine has a high affinity for the receptor sites and competitively inhibits germination. Although D-alanine apparently binds to the receptor sites in spores of C. perfringens, it promotes germination. D-leucine and D-glutamic acid also induced germination as effectively as the L-isomers. Thus, the steric disposition of the NH_2 group is not as important as structural differences in the R group of the three amino acids. Finally Woese et al. (1958) suggested that since the spores of bacilli can distinguish between D- and L-isomers, there must be at least a three point specificity for the R, NH_2 and H groups; the COOH group could also be involved as a fourth point of specificity due to its charge and not to its steric configuration. Since germination is promoted in spores of C. perfringens by D- and L-amino acids this suggests that the R group of the amino acid is an important factor in germination.

The existence of receptor sites for germinants is well recognized and some studies have been performed to determine the nature of these structures. In spores of Bacillus, Yasuda-Yasaki et al. (1978a,b) proposed the receptor sites

for L-alanine are hydrophobic; they determined this in experiments using alcohols with 8, 9 and 10 carbon atoms that inhibited germination due to their hydrophobic nature. If this assumption is correct it reinforces the fact that the R group of the amino acid may be responsible for triggering germination. In the germination of spores of C. perfringens FD1 a hydrophobic receptor site could explain the germinant characteristics of alanine and leucine. However, glutamic acid has an extra carboxylic group with hydrophilic characteristics, and thus must be attracted to receptor sites with different characteristics to permit germination. This feature again supports the existence of different types of receptor sites for various compounds which trigger germination.

Considerations on the Germination Medium and Other Compounds on the Germination of Spores of C. perfringens

The chemically defined medium designed for C. perfringens FD1 permitted full germination of heat activated spores. Even though several ingredients were present in FTG-basal medium, at pH 6.0 only sodium chloride, dibasic potassium phosphate, L-cystine, and glucose or other carbohydrates were required for germination in the presence of glutamic acid and leucine. Ahmed and Walker (1971) determined that cystine, cysteine, tryptophan and tyrosine enhanced germination of spores of C. perfringens S45 in the

presence of yeast extract. However, individually none of these amino acids were able to induce germination in the absence of glucose. Different responses were observed when the amino acids were tested in the presence of glucose, and cystine was the most effective germinant. Interestingly, tyrosine had a stimulatory effect in the presence of yeast extract but blocked germination induced by cystine + cysteine + glucose.

Regarding germination of C. perfringens FD1, it was observed that cystine in the presence of glucose, glutamic acid and leucine promoted a 50% drop in optical density, but tryptophan was unable to induce germination. Tyrosine and cysteine were included in the polar group of amino acids, and this group had poor germinative ability. Tyrosine could also have an inhibitory effect on germination of C. perfringens FD1 spores similar to the one observed by Ahmed and Walker (1971).

With respect to mineral salts, Ahmed and Walker (1971) found that sodium nitrate did not enhance germination, while sodium nitrite in the presence of yeast extract and at concentrations of 0.2% permitted almost 65% germination. For spores of C. perfringens FD1, sodium nitrite promoted almost 60% germination at concentrations of 0.25%. Glucose, glutamic acid and leucine were needed to induce germination. In the absence of amino acids only 43% germination was achieved. Potassium nitrite gave similar results as the

ones observed for sodium nitrite.

Although sodium chloride always had a stimulatory effect on germination of spores of C. perfringens FD1, usually other ingredients were required to obtain complete germination. Sodium nitrate, potassium chloride and sodium bicarbonate permitted only partial germination.

Enzymes Related to the Germination of Bacterial Spores

Several enzymes have been found in resting and germinating spores. In spores of Bacillus L-amino acids, particularly L-alanine, are involved in spore germination, and D-amino acids competitively inhibit the germinative ability of the L-isomers. This specificity indicates the involvement of binding or interactions similar to those found in some enzymatic reactions. Alanine racemase has been found in B. cereus, but its content in spores is approximately 16 times greater than in vegetative cells (Sadoff, 1969). The action of alanine racemase may hinder L-alanine-induced germination in the presence of D-alanine through the formation of L-alanine which then slowly initiates germination in the presence of D-alanine (Gould, 1969). It is unlikely that an extremely active racemase is present in clostridial spores permitting high rates of conversion of D-isomers into the L-forms, since germination rates for L- and D-isomers of glutamic acid and leucine during germination of C. perfringens FD1 were equivalent.

However, more studies must be performed at this point to confirm this assumption.

Transaminases, acting on oxoglutaric acid in the presence of L-aspartic acid, L-alanine or D-alanine are present in spores of B. subtilis, but their specific role in germination is not clear. L-amino acid dehydrogenase, catalases and nucleoside ribosidases, have also been isolated from resting or germination spores. However, their exact role in the germination process has not been elucidated (Gould, 1969; Sadoff, 1969). Therefore, although a clear pathway for the reactions that occur during germination has not been established, the germination of spores may depend on a sequence of enzyme reactions.

Specifically, the metabolism of glutamic acid in microorganisms takes place through two pathways; the methylaspartate and the hydroxyglutarate pathways. In clostridia the methylaspartate pathway is used. 3-methylaspartase is one of the enzymes involved in the metabolism of glutamic acid. Interestingly, Buckel and Barker (1974) reported that C. perfringens does not ferment glutamic acid; presumably the enzymes involved in glutamic acid metabolism would not be present in the spores. However, glutamic acid was a good germinant for spores of C. perfringens FD1. Its strong germinative ability may be due to its participation in transamination reactions.

Finally, the germinative system of spores of C. perfringens FD1 differs from the highly specific L-alanine-induced germination system (sensitive to the presence of D-alanine) reported for spores of bacilli in the following aspects: 1) the specificity of the binding sites in spores of C. perfringens FD1 is not as high as that in spores of bacilli, 2) different germinants are involved in triggering germination of spores of C. perfringens FD1 than in spores of bacilli.

Effect of pH on Germination of Spores of C. perfringens FD1

pH significantly influences the germination process of spores of C. perfringens FD1 in complex and chemically defined media. The limiting pH value in complex media on the acid side was determined to be between 4.5 and 5.0 for FTG and BHI + YE, respectively; however, satisfactory germination was observed above pH 5.5 with an increasing rate of germination up to pH 7.0. At pH 7.5 a slight decrease in the extent of germination was observed with more rapid germination at pH 8.0 to 9.0. Germination in complex media was arrested at pH 10.0. For chemically defined media the optimum pH range lies between 6.0 and 6.5 for systems containing single amino acids; when combinations of two amino acids are used (glutamic acid plus leucine) the optimum pH ranges from 5.5 to 7.0. Interestingly, at pH 7.5 germination is arrested and no response is obtained at

more alkaline levels of pH in germination media containing one or two amino acids. However, at pH 9.0 germination was obtained in media prepared with the original four groups of amino acids or with a mixture of all amino acids, although a long lag phase was observed before changes in OD_{625} were detected. The germination of spores at pH 9.0 in complex media or in chemically defined media supplemented with several amino acids indicates the presence of more than one germination system with each functioning independently over a different pH range.

The effect of pH on the germination media can be attributed to: 1) the ionic form of the germinants; 2) the electric charge of some components of the receptor sites, which can bind the germinant molecule by electrostatic forces; and 3) to the activity of enzymes which may take part in the germinative process.

Uehara and Frank (1965) reported an optimum pH of 8.5 for L-alanine-induced germination of spores of PA 3679h. However, for spores of C. perfringens FD1 the optimum pH for alanine-induced germination is 6.5 indicating the existence of a different germination system and/or a different set of enzymes involved in the germination processes for these two clostridia.

Metabolic Considerations on Germination of Spores of C. perfringens FD1

The metabolic role of germinants is controversial. Some authors suggest that germination is a metabolic process (Gould, 1969; Ando, 1978), while others state that no metabolism is required for germination to occur (Vary, 1978; Rossignol and Vary, 1978; Scott et al., 1978). Vary (1978) described the enzymes involved in glucose metabolism in spores of B. megaterium. Enzymes for glycolysis and the hexose monophosphate shunt were present plus glucose dehydrogenase. Other enzymes such as glucokinase, gluconate dehydrogenase, or the enzymes of the Entner-Duodoroff pathway were absent. He established that metabolism of glucose is not necessary for germination of spores of B. megaterium based on the following facts: 1) mutants lacking essential enzymes for glucose metabolism germinated as well as the wild type in the presence of glucose; 2) glucose analogs such as α-methylglucopyranoside and 6-deoxyglucose which are not substrates for hexokinase or glucodehydrogenase still initiated germination (Vary, 1978).

In the germination media designed for spores of C. perfringens FD1, the most readily available energy source would be glucose. In some instances amino acids can be used as energy sources; however, the concentrations at which they were present in the germination media were extremely low.

In C. perfringens FD1 germination media several sugars and α and β methylglucopyranosides permitted germination. Germination in the presence of methylglucopyranosides was equal to that obtained in the presence of metabolizable sugars. Thus, a nonmetabolic mechanism of action may be attributed to these sugars in the triggering of germination; they may act, in conjunction with other germinants, to promote configurational changes in the spore structures. This may unblock or release some enzymes for subsequent steps in germination.

The requirement for a sugar was relatively nonspecific since a variety of sugars promoted germination. However, with the exception of pentoses, the sugars had a terminal fructose or glucose residue. Further studies would have to be conducted to determine the molecular configurations which are required to promote germination in C. perfringens FD1.

Minimal Requirements for Germination

Preliminary screening of ingredients of the basal medium revealed that sodium chloride, L-cystine and dibasic potassium phosphate were required for germination while sodium sulfite and sodium thioglycollate were not required. Amino acids (glutamic acid and leucine) in water at pH 6.0 alone were unable to promote germination.

Germination of heat-resistant strains of C. perfringens Type A has been obtained in the presence of potassium chloride in phosphate buffer at pH 7.0 and in incubation temperature of 40 C (Ando, 1978). In the germination system designed for C. perfringens FD1 sodium chloride was required for satisfactory germination. The requirement for sodium chloride could not be satisfied by other potassium or sodium salts. Nitrates induced a slight drop in optical density, but only partial germination was achieved. Ando (1978) suggested the role of potassium ions in germination may be to act as a cofactor for pyruvate kinase which requires potassium ions for full activity. However, potassium chloride did not promote evident changes in optical density of spores of C. perfringens FD1. The requirement for sodium chloride during germination agrees with the results obtained by Holland et al. (1970) and Waites and Wyatt (1971) for C. sporogenes strain PA 3679 and C. bifermentans, respectively.

Anaerobic Conditions in Germination of Spores of C. perfringens FD1

During this investigation the only precautions used for establishing anaerobic conditions were the introduction of reducing agents such as L-cystine and oxygen scavengers such as sodium thioglycollate. However, germination was obtained in the presence or absence of sodium thioglycollate.

Thus, dissolved oxygen in the germination medium (sterilization was done by filtration) did not interfere with the germination process of spores of C. perfringens FD1. In addition the spores germinated without CO_2. These results agree with the findings of Wynne and Foster (1954) who reported that some clostridial spores (including a strain of C. perfringens) can germinate at a satisfactory rate in the absence of CO_2. Other investigators (Enfors and Molin, 1978) have reported that oxygen and/or the absence of CO_2 retard or inhibit germination of clostridia (including a strain of C. perfringens).

Effect of Inhibitors of Vegetative Growth on Germination

The effect of potassium sorbate on vegetative growth has been related to the presence of undissociated sorbic acid molecule which interferes with the oxidative assimilation of carbon compounds. Potassium sorbate has also been used as a selective agent for the isolation of clostridia (Emard and Vaughn, 1952; York and Vaughn, 1954).

The germination response of spores of C. perfringens FD1 in the presence of potassium sorbate differed with the type of media used. In complex media (BHI + YE and FTG) germination was obtained at pH 5.5 to 7.0, and as the pH increased the amount of potassium sorbate needed to inhibit germination generally increased. However, since the actual concentration of undissociated sorbic acid required for

inhibition decreased with increasing pH the inhibitory effect appeared to be related to the rate of germination and to the concentration of potassium sorbate and sorbic acid. In chemically defined medium at the optimum pH of 6.0 rapid and complete germination was obtained in the control without sorbate. However, concentrations of potassium sorbate as low as 0.25% affected the level and rate of germination, and 0.5% completely inhibited germination. This can be attributed to the fact that the chemically defined medium provides limiting conditions for germination and no protection by the presence of other ingredients as in the case of complex media.

The inhibitory effect of Lauricidin plus^{TM}, a mixture of monolaurin plus sorbic acid, presumably is related to changes in the fluidity of the plasma membrane of vegetative cells of gram-positive microorganisms (Kabara, 1978). However, inhibition was not observed during germination of C. perfringens FD1 at any of the concentrations tested, but the emergence of the vegetative cell or its multiplication was blocked by concentrations of the inhibitor as low as 0.03 mg/ml. The noninhibitory effect of Lauricidin plus^{TM} on the germination process may be attributed to failure of the inhibitor to reach the membrane of the spore due to steric impediment or, less likely, to a lack of penetration of the spore coats.

Effect of Specific Inhibitors of Germination

Although methylanthranilate and veronal are potent inhibitors of germination of bacilli, only methylanthranilate had an inhibitory effect on the germination process of spores of C. perfringens FD1. Germination was significantly retarded in a chemically defined medium (only 60% germination was obtained after 24 hours), but no inhibition of germination or outgrowth was observed in complex media. The inhibitory effect of methylanthranilate has been associated with the L-alanine induced germination system of spores of Bacillus, and it competitively inhibits L-alanine dehydrogenase and L-glutamate-pyruvate-transaminase (Prasad, 1974). A partial inhibition of the latter enzyme could be responsible for the slight inhibition of germination noted in the chemically defined medium. In complex media the inhibitory effect may be counteracted by interaction of methylanthranilate with compounds in the media or by utilization of other germination systems.

Responses of Other C. perfringens Strains to the Germination Conditions of C. perfringens FD1

The chemically defined medium designed for C. perfringens FD1 also promoted germination of a heat-sensitive strain (ATCC 3624) and a heat-resistant strain (NCTC 8238) of C. perfringens although the extent of germination was only 70%. Only 30% germination was obtained for PA 3679.

The lack of complete germination can be attributed to differences in heat-shock requirements or in the requirement for additional nutrients. C. perfringens strain ATCC 12195 failed to germinate in chemically defined medium and thus apparently has an absolute requirement for other germinants and/or germination conditions.

CONCLUSIONS

1. In the presence of leucine and glutamic acid, C. perfringens spores required sodium chloride, dibasic potassium phsophate, L-cystine, glucose and pH 6.0-6.5 for rapid germination.
2. Spores of C. perfringens FD1 did not germinate in chemically defined media without a heat shock. In this investigation optimal germination occurred after heat activation for 10 minutes at 80 C.
3. L- and D-isomers of glutamic acid and leucine had equivalent germinative activity for spores of C. perfringens FDI. However, D-alanine caused a delay in the onset of germination.
4. Inhibitors of vegetative growth were not effective for inhibiting germination in complex media. But potassium sorbate and Lauricidin plus™ had a slight inhibitory effect on germination in chemically defined media. Inhibitors of germination of aerobic spores permitted more than 50% germination of spores of C. perfringens FD1.
5. The germination system designed for spores of C. perfringens FD1 was also adequate for germination of spores of C. perfringens strains NCTC 8238 and ATCC 3624.

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