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SPORULATION AND GERMINATION OF SPORES OF *CLOSTRIDIUM PERFRINGENS*

by

Maqsud Ahmed

A Dissertation Submitted to the
Graduate Faculty in Partial Fulfillment of
The Requirements for the Degree of
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TABLE OF CONTENTS

I. INTRODUCTION

Clostridium perfringens is one of the principle causes of food-borne illness in instances when refrigeration is lacking or refrigeration is improperly applied to foods. No definite food poisoning statistics are available for most countries. During the last few years, considerable attention has been given to the isolation and quantitation of this organism in foods as well as characterization of food poisoning strains.

C. perfringens is probably the most widely spread pathogen in existence. It is present in soil, water, dust, sewage, and the intestinal tracts of animals and humans. This organism has also been isolated from foods such as spices, condiments, cooked and raw meats, fish and poultry, as well as some prepared dishes. Food poisoning outbreaks due to C. perfringens have been traced to the consumption of different kinds of meats and dishes prepared from them. Usually such foods have been precooked and allowed to cool slowly overnight.

The spore forming characteristics of C. perfringens are important in biological studies as well as for understanding food-borne illness caused by the ingestion of large numbers of these bacteria. The heat resistance of the spores of a strain involved in any one outbreak may provide information in deciding whether or not the contamination of food occurred before or after cooking. If food was contaminated before cooking, heat resistant spores are always the causative agents, whereas, if it was contaminated after cooking, either vegetative cells or spores or both can cause food poisoning outbreaks. Although spores of C. perfringens play such an important role in causing food poisoning outbreaks, production of larger numbers

of spores in laboratory media is difficult and unreliable. The first phase of the present study was to develop a suitable sporulation medium which would stimulate maximal sporulation.

C. perfringens in the spore stage apparently does not produce any harmful conditions in foods. It is only during or after germination and outgrowth that toxic substances are released in the foods. Within the past few years a number of ways have been found to induce germination of one or another species of bacterial spores. Very little information is available on the specific stimulants of germination of spores of C. perfringens. Such information as is available appears to be restricted only to the use of glucose-phosphate medium and chelating agents (calcium-dipicolinate) for germination, but rapid germination in a medium of well defined nutrients has not been reported. It was decided to observe the effects of various nitrogen sources on spore germination and to describe the effects of several common parameters giving maximal germination.

Realization that inhibition of germination is the most direct approach for preventing spoilage caused by bacterial spores has led to search for a metabolic inhibitor of germination.

The investigation reported here has three phases:

(1) the development of a medium which yields large numbers of spores of C. perfringens,

(2) the determination of the optimal conditions and minimal nutritional requirements for rapid germination of spores of C. perfringens,

(3) to study the effects of inhibitors on germination of spores of C. perfringens.

II. REVIEW OF LITERATURE

A. Sporulation

Production of spores by Clostridium perfringens in laboratory media has long been considered difficult and unreliable. Welch and Nutall (1892) who described this organism, did not record sporulation as a characteristic. Dunham (1897) was the first to succeed in obtaining spores by using coagulated blood serum as the culture medium. Working with several strains of C. perfringens, Herter (1906) found that sporulation took place with difficulty, and that the sporulation that occurred was chiefly on special media containing blood serum. Fitzgerald (1910) reported that enriched media containing 1.0% of arabinose, raffinose, inulin, mannite, dulcite, isodulcite or amygdalin induced sporulation of C. perfringens; mannite and amygdalin appeared to give best results. D-glucose, saccharose, maltose and lactose were found to be inhibitory, probably because of the acid produced by dissimilation of these sugars. A cooked meat medium was introduced by Robertson (1916) after he discovered that C. perfringens sporulated only when the medium was rich in protein and low in fermentable carbohydrate. Headlee (1931) found that an alkaline egg medium was the only medium which appeared to be satisfactory for spore production by C. perfringens.

In 1956, Ellner described a medium in which sporulation appeared to take place rapidly and quantitatively. This medium has been used widely for producing spores for laboratory studies. Collee et al. (1961) agreed that Ellner's medium encouraged sporulation but found yields to be variable. Angelotti et al. (1962) proposed SEC broth as a sporulating medium.

Hall et al. (1963) tested 83 strains of C. perfringens for sporulation in five media previously used for this purpose and only SEC broth consistently yielded spores capable of surviving 100°C for prolonged intervals. They described spore production in Ellner's medium as being atypical and unsatisfactory for heat resistance studies; Ellner's medium however, was superior to SEC broth in numbers of spores produced. Groom and Strong (1966) arrived at the same conclusion after comparing the sporulation of seven strains of C. perfringens in four laboratory media; they agreed with Perkins (1965) that no entirely suitable medium was available for production of abundant heat resistant spores of this organism. Riemann (1963a) enhanced the production of spores by C. perfringens with the addition of trypticase to spent medium in which PA 3679 had grown. Roberts (1967), however, presented evidence that the spent medium technique offered a real advantage in an only limited number of cases. The possibility of dialysis sac technique has been suggested by Schneider et al. (1963). Good spore crops of C. perfringens in a dialysis bag were obtained when the initial inoculum of cells in physiological saline solution was introduced into the dialysis bag and the dialysis bag in turn was immersed into a modified Wagenaar and Dack medium in a large pyrex tube. Collier (1957) and Halvorson (1957) utilized a procedure in which pseudo-synchronous growth was obtained to improve sporulation. Perkins (1965) reviewed the various media and methods employed in the production of clostridial spores and suggested that a combination of the relatively simple ingredients employed by Ellner (1956), Hall et al. (1963) and Riemann (1963) might be profitable in formulating a medium which would support the formation of larger numbers of heat resistant spores of C. perfringens.

Roberts (1967) prepared a cooked meat medium (CMM) consisting of Hartley's digest broth plus twice the normal quantity of meat for the sporulation of different mesophilic clostria and found that different strains of C. perfringens produced 1 to 40% spores in this medium.

Kim et al. (1967) reported the development of a modified sporulation medium in which a larger number of spores were obtained than in SEC broth and in which spores were produced with greater heat resistance than those produced in Ellner's medium. Duncan and Strong (1968) developed an improved sporulation medium in which five strains of C. perfringens exhibited significant increases in numbers of spores when compared with spore yields in SEC medium and the medium of Kim et al. (1967) under comparable conditions. In addition, three of the five strains produced increases over the numbers observed in Ellner's medium; the remaining two strains yielded approximately the same numbers of spores as in Ellner's medium. Addition of activated carbon to the medium of Duncan and Strong resulted in production of larger numbers of spores in some cases and the spores had slightly greater heat resistance than spores produced in this medium without added carbon or in SEC or Ellner's medium.

Recently, Nishida et al. (1969) used enriched cooked-meat medium for preculture and 2.0% Poly Peptone for subculture which yielded better sporulation in C. perfringens than was produced in the media of Ellner (1956), of Angelotti et al. (1962),and of Duncan and Strong (1968). Mead (1969) undertook a study to determine the effect of both temperature and pH on growth and on sporulation of C. perfringens. Sporulation of several strains of C. perfringens was generally 10-100 times greater in raw minced leg muscle (pH 6.8) than in breast muscle (pH 5.8) of the chicken. Differences in

sporulation were attributed to differences in pH and type of meat.

Quantitative data on the resistance of spores of C. perfringens to heat are lacking. Roberts (1968) pointed out that many of the published data on the resistance of spores of C. perfringens have been obtained during isolation from food stuffs implicated in food poisoning outbreaks or from fecal material, and these are not sufficiently quantitative to enable real comparisons of resistance. He further said that the common statement that "spores survived 1 1/2 hr but not 3 hr" is of little use without the indication of numbers initially present. In addition the possible protective effect of food or feces on the resistance of spores to heat has not been evaluated.

Hobbs (1965) was of the opinion that only heat resistant strains produced food poisoning, but heat sensitive as well as heat resistant strains may be implicated in outbreaks of food poisoning (McKillop, 1959; Hall et al., 1963; Yamagishi et al., 1964; and Nishida et al., 1969). Although quantitative data are lacking, it now seems more probable that resistance to heat of strains associated with food poisoning varies widely.

Roberts (1968) found that spores of a 'classical' strain of C. perfringens were more sensitive to heat (D_{90° = 3-5 min) than spores of 'food poisoning' strains (D_{90° = 15-145 min) when produced in the same sporulation medium. Similar results were reported by Duncan and Strong (1968). They further suggested that the major difference in the expressed heat resistance of different strains is genetically controlled. Collee et al. (1961) had also postulated the genetic determination of heat resistance of spores of C. perfringens.

Other reports indicate that heat resistance of spores of C. perfringens

can be influenced by the medium in which the spores were produced. Hall et al. (1963) and Kim et al. (1967) indicated that more heat resistant spores were produced in SEC broth than in Ellner's medium. Similarly Roberts (1968) obtained more heat resistant spores of C. perfringens in TPAY-GT medium than those obtained from SEC broth.

Weiss and Strong (1967) and Kim et al. (1967) reported that medium in which spores are heat treated can influence the heat resistance of spores of C. perfringens. For example spores exhibited more heat resistance when heated in SEC broth than in Ellner's medium.

1. Factors affecting sporulation

a. Effect of nutrients Clostridia are, in general, more fastidious in their growth and sporulation requirements than aerobic bacilli. Many investigators have emphasized the stimulating effects of various types of substances in the medium on the sporulation of anaerobes. These substances fall into various categories. A definite amino acid requirement has been established for the growth of several strains of C. perfringens and other clostridia (Perkins, 1965). Data presented by Perkins (1965) revealed that there are qualitative differences in minimal amino acid requirements among species as well as among strains of the same species. The author suggested that any medium in which clostridial spores are to be produced should contain a complex mixture of amino acids common to most proteins.

Kaplan and Williams (1941) pointed out the accelerating effect of utilizable sources of nitrogen (e.g. albumin, serum, etc.) in the medium for the sporulation of clostridia. Perkins (1965) compared the relative sporulation of a single strain of C. parabotulinum 62A in a beef heart infusion

and in 5% polypeptone broth and found the latter to be definitely superior. Similar results were obtained with other species such as C. perfringens and C. pasteurianum. This suggested a possibility that a suitable combination of peptones might induce sporulation in clostridia. Riemann (1963a) suggested that sporulation of C. perfringens might be significantly enhanced by increasing the peptone concentration above that used in Ellner's medium.

Glucose depresses sporulation (Gibbs and Hirsch, 1956). Depression of sporulation by the carbohydrate is probably caused by the low pH resulting from rapid degradation of the carbohydrate.

Some fermentable polysaccharides, arabinose, raffinose, inulin, mannite, dulcite, isodulcite and amygdalin, have been found to stimulate the formation of spores by C. perfringens (Fitzgerald, 1910).

No detailed study has been made on the mineral requirements for sporulation of the clostridia, but Perkins (1965) reported that except in the case of peptone concentration below about 2%, no added minerals were necessary in complex media. Certain inorganic salts have been reported to be essential or stimulatory to clostridial sporulation in dilute media (1-1.5% of peptone). These include inorganic phosphate (Leifson, 1931), $MgSo_4$ (Ellner, 1956), NaCl (Hitzman et al., 1957), and ammonium salts (Leifson, 1931; Brown et al., 1957). Leifson in his study of the effect of inorganic salts on sporulation found growth of C. botulinum occurred almost invariably at a higher concentration of inorganic salts than did sporulation.

b. Effect of moisture Little information is available on moisture requirements for sporulation and survival of bacterial spores. Williams and Purnell (1953) observed that growth and spore formation by sporulating bacteria did not

necessarily parallel each other. At a moisture concentration of 45%, sporulation of C. botulinum did not occur but growth took place. In their study on the growth, sporulation, and germination of C. perfringens, Kang et al. (1969) found that higher levels of water activity (A_w) were required for the formation of spores than for vegetative growth.

c. Effect of pH Virtually all investigators have employed an initial pH of 7.0-7.8 for the production of spores of C. perfringens and other clostridia. Fitzgerald (1910) recommended the use of an alkaline, sugar-free broth for maximum sporulation, since development of acidity prevented spore formation. Simonds (1915) stated that spore production was inconstant, occurred only in alkaline conditions, and never occurred in pure cultures containing a fermentable sugar or a free acid. Torry et al. (1930) used a sugar-free, well-buffered medium and found a pH optimum of 7.4-7.8 for sporulation of C. perfringens; no sporulation occurred below pH 6.6. Similar alkaline pH values have been found to be a controlling factor in the sporulation of other anaerobes (Möhrke, 1926; Kaplan and Williams, 1941).

Leifson (1931), however, found a neutral or slightly acidic medium more favorable for sporulation with a variety of strains. It was also suggested that pH at the time of sporulation rather than initial or final pH value was of the greatest importance.

The discrepancy in pH optima mentioned above may be due partially to the use of different strains with different backgrounds, and partially to the high nutrient concentration of Leifson's medium (2.0% sugar, 1.0% peptone, and 0.5% meat extract). Generally, media rich in albumin or other nitrogenous materials accelerate the rate of sporulation among the

anaerobes, and in some instances may even mask the inhibitory effect of an acid pH (Möhrke, 1926).

Mead (1969) reported that sporulation of C. perfringens is related to both the pH value and the type of meat concerned because at pH 5.2 there was a significant difference in the numbers of spores found in beef and turkey, while in all instances sporulation was increased by adjusting the pH value to 7.2.

d. Effect of oxygen tension The relationship of oxygen tension to the sporulation of anaerobes has not been comprehensively studied. Some evidence has been presented that the infiltration of oxygen into an "anaerobic medium" does not necessarily inhibit sporulation. Matzuschita (1902) found that exposure of actively growing cultures to oxygen resulted in an acceleration of the sporulation process. Zinsser (1906) mentioned that spores were produced only in those cultures of C. tetani and C. chauveii which contained traces of oxygen. DeSmith (1924) found that cultures of C. sporogenes which were aerated at intervals of 24 hr up to a period of 5 to 6 days sporulated better than in unaerated cultures. Leifson (1931) found that during the late stages of sporulation of anaerobes, the process becomes less sensitive to the presence of oxygen. Similar observations were reported by Collier (1957). Zoha and Sadoff (1958) felt that aeration may in fact accelerate the sporulation process since it has been observed to facilitate the lysis of sporangia and liberation of spores.

e. Effect of antisporulation factors in growth media Several investigators presented evidence that certain antisporulation factors present in growth media are removed or rendered inert by various treatments including

activated charcoal adsorption, addition of starch, semisolid or excess agar, blood, body fluids, and albumin. In 1942 Roberts and Baldwin reported that treatment of peptone with activated carbon caused a marked increase in percentage of sporulation of B. subtilis. Foster et al. (1950) and Hardwick et al. (1951) concluded that activated carbon removes factors directly inhibitory to the spore forming process. Olsen and Scott (1946) reported that soluble starch could also absorb antisporulation factors from the medium.

Foster (1956) identified fatty acids as antisporulation factors and those possessing the greatest antisporulation activity as the saturated acids containing through 15 carbon atoms. Other antisporulation factors certainly exist because media extracted with fat solvents could further be improved in sporogenic capacity by treatment with activated charcoal (Foster, 1956).

Further complicating the sporulation picture was the revelation that various species of the genus Bacillus generate a significant amount of lipoidal antisporulation activity during their growth in complex media. Foster et al. (1950) suggested that antisporulation lipid may be synthesized during growth of the culture. Davis and Dubos (1947) had pointed out that bovine serum albumin (BSA) neutralizes oleic acid by binding it. Duncan and Strong (1968) reported that activated carbon neutralized the inhibitory effect of fatty acids contained in enzymatic digest of casein and that spores produced in such a medium were of slightly increased heat resistance.

B. Germination

1. Definitions

DeBary, (1887) stated that germination of bacterial spores "consists chiefly in the development of the spores into a cell which assumes all the characteristics of the parent cell as regards conformation and vegetation." Knaysi, (1951) defined germination of endospores as "a process involving growth and [which] takes place under conditions that favor vegetative growth." Fitz-James (1954) defined germination as the period "extending from the resting stage of the 'mononucleate' spore to the vegetative or 'binucleate' cell." Fitz-James (1956) divided the germination process into two parts: "the initial activation of the resting spores into a respiring cell and the period of growth leading up to the formation of a young vegetative bacillus."

Using stainability and increase in respiratory activity as criteria of germination, Levinson and Sevag (1953) divided germination into two phases: (a) "pregermination" or the process occurring when the spore "becomes stainable and has started to consume oxygen, but before it has elongated and become typically bacillary in shape," and (b) germination which they defined as the emergence of the bacillus from the spore case. Demonstration by Mandels et al. (1956) of the rapid increase in respiratory activity coincident with stainability led Levinson and Hyatt (1956) to accept the validity of the latter as a criterion of germination. Most recent studies are based upon the definition provided by Campbell (1957). "Spore germination in bacteria may be regarded as the change from a heat-resistant spore to a heat-labile entity which may not necessarily be a true vegetative cell." Phenomena such as nuclear division, cell wall synthesis, cellular

elongation, and cellular division were considered by Campbell (1957) to be secondary and irrelevant to the germination process, and he included all these secondary changes in the generic term "outgrowth."

2. Criteria for measuring spore germination

Most investigators have recently defined germination in terms of some convenient and easily demonstrable stage. Various criteria and methods associated with the germination of bacterial spores are reviewed here.

a. Visible turbidity Most early studies on spore germination employed the appearance of visible turbidity as a criterion of germination (Williams, 1952). Such an approach obviously yields no quantitative information but can be of value in establishing that some germination has or has not occurred.

b. Loss of resistance to heat Development of heat lability by germinated spores has been applied by several workers. This criterion was first applied by Fischroeder (1909). Similar methods of germination were employed by Evans and Curran (1943) in their classical work showing the importance of preheating spores before incubation for germination, and by Hills (1949b).

Percent germination rather than number of ungerminated spores was used by Wynne and Foster (1948a) and Wynne (1952) as a basis for quantitative measurement of germination since with this standard it was felt that there was less likelihood of attaching a false significance to small deviations. Wynne (1952) has pointed out that certain precautions must be taken when loss of heat resistance is used as a criterion of germination. Heat resistance of the spores examined by this technique should be relatively great and the well known phenomenon of dormancy resulting in delayed

germination of some spores, particularly after heat treatment, must not occur in sufficient degree to be a practical obstacle. Furthermore, in testing substances for possible inhibition of germination, it must be shown that amounts of such materials remaining after dilutions do not adversely affect development of colonies in plating media. Likewise, possible sporocidal effects must be ruled out. Finally, the incubation period must be such that resporulation is not appreciable.

Since this method depends upon subsequent growth of vegetative cells, Campbell (1957) cautioned that dormancy can be broken under conditions in which the germinated form cannot survive or in which vegetative growth is inhibited.

Fernelius (1960) reported that the kinetics of germination measured by loss in resistance can be misleading since they depend to a large extent on the conditions employed for inactivation of the germinated form. He observed that the time necessary for germination of spores of B. anthracis by alanine, tyrosine, and adenosine depends upon the method used for determining germination. When loss in heat resistance was followed in thick spore suspensions, germination was complete within two minutes. However, when spores were heated in dilute solutions or inactivated by exposure to phenol, the loss of heat resistance was complete at 4 minutes. The faster germination in thick suspensions was attributed to the period required to raise the incubation temperature to that of heat shock for a thick cell suspension. This period was eliminated when spores were diluted into one percent medium at higher temperature or when they were diluted into one percent phenol. If the measurement of germination in dilute suspension reflects the true kinetics of inactivation, the inactivation both by phenol

and by heat occurred simultaneously.

Sussman and Halvorson (1966) pointed out that although the thermal death of microorganisms usually obeys first order kinetics, numerous non-logarithmic thermal death curves for some bacterial spores have been reported. Spore populations either show a lag followed by a logarithmic decline in the number of viable spores or else behave as a bimodal population regarding heat resistance. In this case the survival curve appears as the sum of two exponential functions.

c. Reduction in the optical density of spores Optical density (OD) measurements are convenient for studying the process of spore germination. The transition from dormant spore to germinated form can be observed by change in OD measured with a colorimeter or a nephelometer.

Powell (1950) observed that turbidity of spore suspensions markedly decreased on incubation with L-alanine. This phenomenon was attributed to the decrease in refractive index of the spore during germination.

Changes in optical density have been employed to quantitatively measure the kinetics of germination by the following equation developed by Hachisuka et al. (1955).

$$G_t = \frac{Ao - At}{0.7(Ao-a)} \times 100$$

where G_t = rate of germination in a given medium after t hours of incubation,

a = optical density of the medium,

Ao = optical density of the medium inoculated with spores (before incubation),

At = optical density of the medium inoculated with the spores

(after incubation for t hours).

Woese and Morowitz (1958) found that the change in the optical density could be related to the number of germinated spores by determining the ratio,

$$\frac{OD - OD_f}{OD_i - OD_f}$$

for each time interval where OD is the optical density at time t, and OD_i and OD_f are the initial and final limiting optical densities, respectively. This relation does not account for the latter stages of germination. Vary and McCormick (1965) pointed out that if the data are normalized to represent the total decrease in optical density as unity, then any part of the reaction may be expressed as some fraction (Y) of the total reaction. Thus,

$$Y = \frac{OD_i - OD_t}{OD_i - OD_f}$$

where OD_i is the initial optical density, OD_f is the limiting optical density as germination approaches completion, and OD_t is the optical density at time t.

In an attempt to find correspondence between heat sensitivity and decrease in OD on germination, Powell (1957) concluded that the loss of heat resistance occurs, on the average, in the earlier stage of change in optical properties.

d. Loss of refractility and darkening of the spores when examined by phase-contrast microscopy Extent of germination can be determined microscopically by phase-contrast microscopy, a method developed by Pulvertaft and Haynes (1951). Spores appear refractile while germinated

forms show various degrees of darkening. Spores become heat labile before they become dark under phase-contrast microscope (Powell, 1957; Riemann, (1963a).

e. Increased stainability by basic dyes Germination can also be followed by measuring the ability of the germinated spore to stain deeply while the ungerminated forms will be unstained (Powell, 1950; Levinson and Sevag, 1953).

Hills (1949a) reported that the percentage of germination determined by staining agreed with the proportion of spores which had lost their heat resistance as determined by the viable count before and after heating at 60°C for 15 mins. Sussman and Halvorson (1966) pointed out that it was difficult to measure the rate of germination by this procedure unless samples are removed frequently from the suspension and percent of unstained cells is determined. Occasionally some spores are observed in which staining is intermediate between that of dormant and germinated spores.

f. The release of dipicolinic acid (DPA) into the suspending medium Upon germination dormant spores frequently release cellular components into the suspending medium. Powell and Strange (1953) reported that DPA (or closely related compounds) is the main, or perhaps only, substance with measurable ultraviolet absorption, released upon germination. The release of DPA was related by Woese and Morowitz (1958) to decrease in OD in spores of B. subtilis. DPA was measured in the supernatant fluid by measurement of OD at 2700 Å, which is the maximum for DPA absorption. They showed that the time-course of release of DPA and decrease in OD of the spore suspension agreed very closely.

g. Enhancement of respiratory activity Glucose oxidation is one of the most frequently used systems to study the extent of germination. Dormant spores often have little or no metabolic activity against glucose (Hachisuka and Sugai, 1959). Mandels et al. (1956) observed that an increase in respiration activity on glucose peptone-yeast extract medium followed an increasing parabolic curve during germination of spores of B. megaterium and of fungus myrothecium. This increase in respiration coincided with decrease in OD and with increase in stainability. Similar results were obtained by Hachisuka and Sugai (1959).

3. Factors affecting germination

a. Heat-activation Incomplete or delayed germination has frequently been observed if spores have not been preheated or heat-activated. Evans and Curran (1943) reviewed the early literature on the accelerating effect of sublethal heat on breaking dormancy of spores and demonstrated that temperatures in the 65-95°C range with an optimum of 85°C for 10 min are effective accelerators of germination of aerobic spores. Similar results were obtained by Gibbs (1964) with spores of C. bifermentans.

Curran and Evans (1945) reported sublethal heating of spores of many thermotolerant and thermophilic aerobes had a determining influence upon the number of spores which will germinate subsequently and observed that preheating of the spores decreased the time required for germination and also the minimal temperature at which germination would take place. Powell and Hunter (1955) found that the rate of germination of preheated spores in the temperature range of 37-60°C in adenosine was roughly proportional to the temperature of preheating. Heating below 40°C had relatively little

stimulating effect. Further work on heat activation of aerobic spores has been reported (Desrosier and Heiligman, 1956; Halvorson, 1958; Murrell, 1961; O'Connor and Halvorson, 1961; Keynan et al., 1961, 1964; and Levinson and Hyatt, 1969).

Although dormancy in aerobic spores is well known, the situation with clostridial spores has not been well documented. Barnes et al. (1963) reported a 25-fold activation of a food poisoning strain of C. perfringens in blocks of meat. Roberts (1968) found activation of the order of about 20-fold with spores of food poisoning strain of C. perfringens after heat-shock at 75-80°C for 3-5 min. No heat activation was detected with a 'classical' strain of C. perfringens. On the other hand, Duncan and Strong (1968) reported a heat-shock requirement for optimal germination and out-growth in food poisoning as well as in 'classical' strains of C. perfringens; the optimum temperature for heat-shock was 80°C (10-20 min) for food poisoning strains and 70°C (10 min) for the 'classical' strain.

Halvorson (1958) has assumed that heat-shock increases the permeability of spores so that germinants can exert their effect. However, Riemann and Ordal (1961) observed that equivalent amounts of calcium and dipicolinic acid are effective germinating agents even without prior heat-activation.

b. Factors affecting heat-activation The effect of heat-activation on germination varies with the organism and is influenced by a variety of factors. The medium in which spores are heat-shocked affects the response (Curran and Evans, 1945). The heating media arranged in the order of enhancing effect upon heat-activation were: glucose or lactose, peptone, skim milk, glucose nutrient agar, beef extract, glucose nutrient broth, distilled water, NaCl. Busta and Ordal (1964a), on the other hand, observed

no effect on heat-activation of B. subtilis strain 5230 at 75°C in suspending media containing glucose, xylose, ribose, or sodium phosphate, nor did a change in pH from 5 to 8 have any effect. Treadwell et al. (1958) found that cystein increased the effect of heat-shock (at 75°C) in 5.0% yeast extract. Uehara and Frank (1965) reported that spores of PA 3679h heat-shocked in a germination medium at 80°C germinated more readily, and to a greater extent, than spores heated prior to introduction into the germination medium.

Some components of the medium may interfere with heat-activation. Splittstoesser and Steinkraus (1962) observed that potassium ions decreased heat-activation of spores of B. popilliae. Similarly Finley and Fields (1962) found that phosphate buffer at both maximal and submaximal activation temperature had a definite inhibitory effect on germination and outgrowth.

Curran and Evans (1945) observed that the medium employed for sporulation influences the heat-activation of spores which probably explains the findings that different spore preparations of the same strain differ in their temperature response. Church (1959) reported that with a higher concentration of yeast extract in G medium the level of DPA in the spore was increased from 7.0 to about 15.0% of the dry weight. Keynan et al. (1961) reported that the heat-activation requirements increased with increasing content of DPA, suggesting that DPA may be associated with the system responsible for the dormant state of the spore. Riemann (1963a) proposed that heat-shocking releases Ca-DPA which in turn stimulates germination.

Keynan et al. (1964) observed that reducing agents such as mercaptoethanol or thioglycolic acid or higher concentrations of H^+ (pH value below

5.0) accelerated activation in spores of B. cereus strain T. Both of these treatments would be expected to favor the rupture of protein-disulfide bonds (Keynan et al., 1964). Gould and Hitchins (1963) found that exposure to reducing agents or hydrogen peroxide at pH 10 in the presence of metal ions sensitized the spores to the action of lysozyme. These findings further support the suggestion that disulfide bonds may be involved in activation of spores.

c. De-activation on storage The change from a dormant to an activated state is a reversible process in most bacterial spores. Curran and Evans (1945) were the first to recognize this phenomenon and found that de-activation of the spores which occurred during storage can be reversed by a second heat treatment. Church and Halvorson (1957) made similar observations and suggested that solutes and endogenous reserves were lost during storage. The number of cycles during which spores will continue to reactivate is limited. Keynan et al. (1964) reported that reversal of heat-activation was temperature dependent; a 40% decrease in germination rate was observed at 4°C after 72 hr but at 28°C the rate of germination decreased to nearly that of dormant spores over the same period. Spores stored at 28° or 4°C could be reactivated on exposure to second heat treatment. Activation of spores by reducing agents such as mercaptoethanol was also reversible. Spores activated by this technique lost their ability to germinate after 48 hr storage in distilled water. Full reactivation of the population was achieved by reexposure to reducing agents or heat treatment.

The activation response observed by Busta and Ordal (1964b) with B. subtilis strain 5230 spores was stable during post heating storage at

5°C for 7 days in phosphate buffer, glucose solution, NaCl solution, and water. In addition, extended storage (215 days) after heating in water resulted in no loss of heat-activation response.

The reversibility of activation of metabolic activity has been questioned by Busta and Ordal (1964a). These workers suggested that de-activation may be merely an inactivation process occurring during storage; reactivation may be the initial activation of a fraction of the spores which escaped the initial activating process. On the other hand, Church and Halvorson (1957) obtained similar results on glucose oxidation and on germination rates by activated and reactivated spores, implying that the reversibility of activation applies to the entire population.

d. Effect of nutrients Within the past few years a number of nutrients have been found to induce germination of bacterial spores of one or another species.

1) Bacillus spores Schreiber (1896) reported that the elements carbon, hydrogen, oxygen, potassium, magnesium, phosphorous, and sulfur were necessary for germination of spores of 3 Bacillus species. A few years later Fischroeder (1909) found that 90% of the spores of B. anthracis lost their heat resistance after one hour incubation in nutrient broth. According to Curran (1931) the minimal concentration of peptone permitting germination of B. mycoides spores was between 0.02 and .025%. On the other hand, evidence has been presented by Knaysi (1945) that some normal mature spores of B. mycoides were able to germinate when supplied solely with a utilizable source of energy such as glucose.

The first study of the very rapid germination of spores induced by simple nutrients was performed by Hills (1949a) who isolated adenosine as

the active principle in yeast extract that induced germination in spores of B. anthracis. He found that 2 micromoles (um) of adenosine in his basal medium (a gelatine hydrolyzate with added tyrosine) gave 90% germination after 30 min at 35°C.

In his studies on species of the genus Bacillus, Hills (1949b, 1950) found that of the three compounds, alanine, tyrosine, and adenosine, L-alanine was present in all cases where highly significant stimulation of germination of spores was observed; the need for the other two compounds ranged from ability to dispense completely with both of them to a distinct need for the two together to produce maximum effect. Stewart and Halvorson (1953) obtained complete germination of spores of B. cereus in five minutes in a medium containing L-alanine and adenosine.

Subsequently, several studies cited by Riemann (1963a) revealed that many of the aerobes would germinate in the presence of L-alanine with and without adenosine and with and without glucose but many other bacillus spores did not respond to the inducing effect of L-alanine. These studies indicated that the nutrients which can induce germination may differ widely among bacillus spores.

2) Clostridial spores Comparatively little information is available on the nutritional requirements for the germination of the spores of the anaerobic genus Clostridium. Generally clostridial spores germinate more slowly than bacillus spores in simple or complex nutrient media. Progress of germination in clostridial spores therefore, has been easier to follow. Wynne and Foster (1948b) and Mehl and Wynne (1951) reported that germination apparently followed the kinetics of a first order chemical reaction.

C. roseum seems to be an exception among the anaerobes with regard to germination rate in complex media. Hitzman et al. (1957) obtained almost 100% germination in fifteen min in 5.0% trypticase. Germination of C. botulinum was very slow in casein digest medium. Yeast extract seemed to be essential for germination in this medium and attempts to replace yeast extract with compounds known to be present in this substance were not successful (Treadwell et al., 1958).

Germination of clostridial spores has not been reported with L-alanine alone. The only simple nutrient system which has been reported to give fast germination is an amino acid - thioglycolate medium formulated by Hitzman et al. (1957). This medium contains L-alanine, L-arginine, L-phenylalanine and sodium thioglycolate and gave 100% germination of spores of C. roseum and C. botulinum at pH 7-8. Treadwell et al. (1958) obtained only 12% germination in two hr in amino acid-thioglycolate medium of Hitzman et al. (1957). Kan et al. (1958) reported 99% germination of PA 3679 spores in 2 min in a solution containing L-arginine, sodium thioglycolate, and glucose. Wynne (1957) presented evidence that spores of five clostridial species including C. perfringens germinated in glucose autoclaved at alkaline or at most slightly acid pH values.

Gibbs (1964) studied the germination of spores of C. bifermantans and found that in the presence of yeast extract, L-phenylalanine, L-leucine and cystine markedly increased the rate of germination. The compounds found specifically responsible for stimulating the germination of spores of C. bifermentans included L-α-alanine and L-phenylalanine. The authors also observed that in the presence of commercial casein hydrolyzate, lactate markedly increased germination. The stimulatory effect of lactate on the

germination of clostridia was not reported previously although some dicarboxylic acids have been reported to stimulate the germination of C. botulinum (Wynne and Foster, 1948c).

Gibbs (1967) found that germination of heat-activated spores of C. bifermentans occurred only when lactate, L-phenylalanine and L-α-alanine in phosphate buffer were present simultaneously. When spores were activated at pH 10.5 and 37°C for 2 hr or longer, rapid and complete germination of washed spores could be induced by L-alanine plus either lactate or phenylalanine. All three compounds were required however, for the germination of spores activated at pH 3.0 and 37°C for 2 hr. Uehara and Frank (1965) reported that spores of PA 3679h showed 75% germination within 10 hr and maximum germination in 30 hr in a solution containing L-alanine and pyrophosphate.

Bicarbonate buffered sodium thioglycolate solution containing adenosine plus phenylalanine, tyrosine or valine induced 50-75% germination of spores of PA 3679 strain h (Lund, 1956); however, Brown (1956) could not obtain germination in other suspensions of PA 3679 strain h spores after 30 min incubation at 37°C with a number of organic compounds including amino acids, sugars, vitamins, antibiotics, purines, and pyrimidines.

The reason for the discrepancy might be that bicarbonate was used as a buffer in the experiments by Lund who also reported that bicarbonate seems to be essential for germination because there was very little germination at the same pH (pH 8.0) with other buffer systems under similar conditions. Wynne (1948a,c) found that carbon dioxide was absolutely necessary for germination of C. botulinum. Yeast extract replaced this requirement in a synthetic medium, whereas oxaloacetate replaced the carbon dioxide only in

brain heart infusion. Studies conducted by Treadwell et al. (1958) further confirmed the necessity for sodium bicarbonate for germination of the spores of C. botulinum. Holland et al. (1969) observed the distinct stimulation of germination due to carbon dioxide with PA 3679 S.2, PA 3679 h, and C. bifermentans in complex media (brain heart infusion and yeast extract) as well as in an amino acid medium (L-alanine, sodium lactate and sodium bicarbonate).

The function of bicarbonate or carbon dioxide during germination is still unknown and possibly carbonate ion is not required by all clostridial spores because rapid germination was obtained where carbonate ion was not added (Hitzman et al., 1957). Similarly PA 3679h and C. bifermentans had shown no specific carbon dioxide requirements (Gibbs, 1964; Uehara and Frank, 1965).

e. Effect of chemicals Germination induced by chemicals has been termed as chemical germination in contrast to physiological germination induced by nutrients. Rode and Foster (1962a) prepared a list of chemical compounds which have been used for one or another species. These include ethylenediaminetetraacetic acid (EDTA), ionic surfactants, especially long chain primary amines, calcium dipicolinate (Ca:DPA), hydrogen peroxide, pyridine carboxylic acid, salts of fatty acids, hydrazine, hydroxylamine and ammonium chloride.

Brown (1956) reported that spores of PA 3679, and the mutant strain (h) could be germinated with the chelating agent EDTA. Riemann and Ordal (1961) reported that Ca:DPA, a 1:1 chelate which forms in solutions containing calcium chloride and sodium dipicolinate (Na_2:DPA), can induce germination in aerobic and anaerobic bacteria over a wide pH range. Riemann (1961)

found that rate of germination of spores of PA 3679h and C. perfringens with Ca:DPA showed a temperature dependence which was similar to that found for enzymatic reactions. In addition, the degree of germination increased as the concentration of Ca:DPA increased above a certain value which was higher for the aerobes than the anaerobes. Not all spore suspensions used by the author responded to the same extent when incubated with Ca:DPA. Some strains of PA 3679 germinated in Na_2:DPA alone. Furthermore, small concentrations of calcium chloride depressed Na_2:DPA induced germination, but this inhibition was overcome when increased concentrations of calcium chloride were added.

Recently, Fields and Frank (1969) observed that Na_2:DPA, but not Ca:DPA, induced changes indicating germination in spores of B. stearothermophilus. Jaye and Ordal (1965) demonstrated that two other metal-dipicolinates, strontium dipicolinate and magnesium-dipicolinate, induce germination of spores of B. megaterium KM (ATCC13622). Other investigators (Riemann and Ordal, 1961; Lee and Ordal, 1963) have also observed the Ca:DPA induced germination of a number of mesophilic species. Hyatt and Levinson (1961) induced germination of B. megaterium spores with either KNO_3 or KNO_2, but only if the spores were first subjected to a sublethal heat treatment of 10 min at 60°C. Likewise, Black (1964) observed germination of B. cereus strain terminalis spores in 0.5 to 2.0M $NaNO_2$ or KNO_2.

Rode and Foster (1962a) examined the effects of various salts on the germination of B. megaterium QM B1551 ("glucose type"). Salts alone were found to be sufficient germinators, but not all salts were active. Cl^-, Br^-, and NO_3^- were effective germinators without glucose. Glucose was not maximally active without suitable salts. Ions apparently played a primary

role in germination and glucose a secondary role under these conditions.

In a subsequent paper, Rode and Foster (1962b) examined the germination requirements of spores of B. megaterium, Texas strain, an "L-alanine-inosine type". They found that L-alanine and inosine were devoid of germination powers in deionized water except in the presence of a large variety of monovalent and divalent alkaline metal chlorides. The potassium halides were germinative, potassium fluoride was the best. Also L-alanine was found to be replaceable by a variety of amino acids, provided suitable ions were present. In the presence of $MgCl_2$, Na_2:DPA could substitute for either inosine or L-alanine, but not both. A primary role of ions in germination is proposed by these authors and a secondary augmentative action was attributed to L-alanine and inosine.

Duncan and Foster (1968b) showed that sodium nitrite alone stimulated germination of PA 3679h spores. The process was found to be accelerated by using increased concentrations of sodium nitrite, a low pH and a high temperature of incubation. Hydroxylamine was completely inhibitory to nitrite-induced germination. Sodium nitrite, in turn, inhibited germination by L-alanine, the degree of inhibition being influenced by nitrite concentration and pH.

f. Effect of aging The effect of aging on the germination of anaerobic spores is unknown. Aerobic spores have been reported to undergo germinative changes during storage. The extent of these changes is dependent on storage temperature and on the amount of moisture present. The lower the temperature, the lower will the changes be, and the alterations are slower in a dry state than in the presence of free water (Halvorson, 1958). Church and Halvorson (1957) found that the level of glucose

oxidation by a spore preparation of B. cereus was also influenced by the age of spores. Freshly harvested, heat activated spores had very little glucose oxidative activity but this activity was increased tremendously in the same preparation after storage for 4 months at -20°C.

During storage, a change in germination requirements may also take place. The requirements, in general, become less exacting. Spores of B. cereus which required both L-alanine and adenosine for germination, germinated with less of these ingredients or with any one of these ingredients after they have been aged. In addition, aged spores germinated with substances which were not effective with fresh spores (Halvorson, 1958). L-alanine was demonstrated in the washings from such aged spores but not from fresh spores. Similarly, Schroeder (1957) found that DPA was released during storage of B. coagulans and this paralleled an increase in the ability to germinate.

Aged spores of B. cereus in contrast to fresh spores germinated without prior heat treatment (Halvorson, 1958). Such changes in the germination requirements were attributed to changes in the permeability of spores or the liberation from spores of germination stimulants through some kind of autolysis. Riemann (1963a) suggested that these germination stimulants could be calcium and DPA.

g. Effect of pH Bacterial spores normally germinate best in neutral solutions and refuse to do so in an acid environment. Ingram and Robinson (1951) found that vegetative cells and spores did not grow below pH 5.0. Wynne et al. (1954) reported that at pH 4.0 or below no discernable germination of spores of clostridia occurred in buffered glucose medium. Treadwell et al. (1958) obtained complete germination of C. botulinum in heart

infusion-yeast extract-starch medium between pH 6.3 and 7.9.

The optimum pH for germination varies not only with strain but also with the germinating agent. For example,Wolf and Thorley (1957) reported that in B. subtilis the optimum pH for germination initiated by glucose was 5.5; whereas, for L-alanine it was 8.5. Uehara and Frank (1965) with spores of PA 3679h, and O'Connor and Halvorson (1961) with spores of B. cereus strain T, found that germination in the L-alanine system was greatest at pH 8.5. Riemann (1963a) has further reviewed some examples of alkaline pH optima for L-alanine induced germination in aerobes.

h. Effect of temperature Temperature of incubation has great effect on germination processes in bacteria. The optimal temperature varies with the species involved. A limited number of studies have been made with clostridial spores. Mundt et al. (1954) observed 99% germination of spores of C. sporogenes after 2 weeks at 4°C whereas vegetative growth was completely inhibited at the same temperature. The temperature range for vegetative growth of C. botulinum has been reported to be broader than that for germination (Ohye and Scott, 1953, 1957). Wynne (1957) reported that spores of this organism germinated at 75°C whereas vegetative growth was stopped at 45°C. Such thermophilic germination was not observed in other clostridia. For example, Gibbs (1964) observed that optimal temperature for germination of spores of C. bifermentans in CMB-yeast extract medium was 37°C. The optimal temperature for alanine induced germination of spores of PA 3679h was apparently 45°C (Uehara and Frank, 1965). Although this temperature was somewhat higher than the 30°C optimum reported by O'Connor and Halvorson (1961) for alanine-induced germination of B. cereus strain T spores, it did correspond with a 40° to 45°C optimum reported for germination of PA 3679 in

a complex medium (Mehl and Wynne, 1951).

Vas and Proszt (1957) found temperature of incubation influenced both extent and rate of germination of spores of B. cereus in a complex medium. The differential effect was pH dependent; at pH 7.0, the rate of germination was strongly influenced by temperature whereas the yield was relatively unaffected. At pH 5.1, the converse was observed.

i. Effect of oxygen Germination is markedly influenced by the presence and absence of oxygen depending upon the type and the species of organisms involved. This is illustrated in the work on anaerobic bacteria. Wynne et al. (1954) reported that germination of C. botulinum in glucose-1-PO_4 occurred only under anaerobic conditions and could be stopped by the admission of oxygen. Thioglycolate did not reverse inhibition by oxygen. Similar results were obtained by Hitzman et al. (1957) in spores of C. roseum and C. botulinum when germination was induced by L-alanine, arginine and L-phenylalanine. On the other hand, C. perfringens, C. chauveii, and PA 3679 germinated slowly in either glucose phosphate (Wynne et al., 1954) or a complex medium (Wynne and Harrell, 1951) under aerobic conditions. Gibbs (1964) found that anaerobic conditions were not essential for the germination of C. bifermentans in complex or amino acid media. Similar results were obtained by Uehara and Frank (1965) with spores of PA 3679h when germination was induced by alanine-pyrophosphate. Holland et al. (1969) found that germination of PA 3679S_2 in complex media was markedly stimulated by oxygen whereas germination of PA 3679h was depressed by oxygen in complex as well as in amino acid media. The germination of C. bifermentans was stimulated by oxygen only in complex media.

j. Effect of moisture A higher relative humidity has been found to be required for the germination of bacterial spores than is required for the growth of vegetative cells (Wolf and Mahmoud, 1957). The vapor pressure of the water in equilibrium with the product in question (the water activity of the product) rather than the total moisture content of the product influences the germination. Thus water activity in any medium is affected by all the constituents which have an affinity for water. Some of these constituents can be metabolized by the organisms (Kang et al. (1969).

The following values for the lowest water activity which permitted germination of spores have been reported as: B. cereus, 0.97; B. subtilis, 0.97; B. megaterium, 0.96; and C. botulinum, 0.98 (Williams and Purnell, 1953; Halvorson, 1958). Kang et al. (1969) found that the lowest water activity supporting growth and germination of C. perfringens was between 0.97 and 0.95 in Fluid Thioglycolate Medium with added sucrose or NaCl, and 0.93 or below in the same basal medium but adjusted to required water activity with glycerol.

C. Inhibition of Germination

Inhibition of spore germination by a variety of metabolic inhibitors has been reported (Wynne et al., 1954; Schmidt, 1957; Treadwell et al., 1958; Halvorson, 1959; Curran and Knaysi, 1961; Slepecky, 1963; Parker and Bradley, 1968; and Sierra, 1968). Studies have been cited by Riemann (1963a) which indicate that bacterial spores generally germinate in the presence of antibiotics but the germinated spores are killed before they can initiate vegetative multiplication.

Anderson and Michener (1950) reported that subtilin combined with a mild heat treatment protected packs of vegetables inoculated with spores of B. stearothermophilus, C. botulinum, and B. subtilis from spoilage when stored for extended periods of time. These bacteria were inhibited from germination by concentrations of subtilin ranging from .004 umg/ml to 5 ugm/ml. Later Hawley (1962) and Gould (1964) indicated that subtilin allowed germination to proceed but prevented lysis or rupture of the spore wall. Subtilin absorbs to heat treated spores and destroys the cells at outgrowth (Hawley, 1962). Gibbs and Hurst (1964) found that nisin controlled thermophilic spoilage but it did not control mesophilic spoilage. They suggested that nisin should be used in canned vegetables and canned meats and fish. Gould (1964) reported that nisin arrested growth from sensitive spores at the same stage as subtilin. He further suggested that tylosin which is more stable than nisin should also be used for the same purposes as nisin.

Gould also determined the effects of some other common food preservatives such as diethyl pyrocarbonate, nitrite, benzoate, sorbate, metabisulfite, polyphosphate, and NaCl on growth of spores of six Bacillus species, but did not include any clostridia. He found that low concentrations of all the preservatives which were just sufficient to stop development of spores into multiplying vegetative cells did not prevent germination. Parker (1969) found that preservatives such as chlorocresol and mixed esters of p-hydroxybenzoic acid, inhibited germination of B. subtilis spores. The inhibition could be reversed by washing the spores. Other preservatives such as cetrimide, aminacrine and phenylmercuric nitrate inhibited the post germinative development and the inhibition was not

reversed by washing the spores. Probably the preservatives in the second category were bound firmly to the surface.

Gough and Alford (1965) observed that C. perfringens survives and grows in concentrations of curing salts considerably higher than those used in normal curing operations. Duncan and Foster (1968a) found that germination of PA 3679h was not inhibited even by as much as 4% of nitrite. NaCl concentrations above 6% prevented complete germination. Sodium nitrate at concentrations up to 2.0% had no apparent effect on germination and outgrowth.

III. MATERIALS AND METHODS

A. Sporulation of C. Perfringens

1. Organisms

Five strains of C. perfringens were selected for developing a sporulation medium. Four of these strains were received from Dr. K. F. Weiss (University of Wisconsin) and included: S-45, isolated from dried beef; 65, isolated from raw carrots; 214d, isolated from fecal samples of victims of food poisoning outbreaks in the United States; and a type A strain ATCC 3624 strain 26. The fifth strain, Hobbs serotype 2 (HR_2), was supplied by Dr. H. E. Hall (Robert A. Taft Sanitary Engineering Center, Cincinnati, Ohio).

All stock cultures were maintained on Cooked Meat Medium.

2. Preparation of vegetative inocula

The modified synchronous growth technique used by Groom and Strong (1966) was adapted for the preparation of vegetative inocula. Fifteen ml of Fluid Thioglycolate Medium (Difco) was inoculated with 1 ml of stock culture and incubated at 37°C for 20 hr. A transfer was then made to fresh tubes of Fluid Thioglycolate Medium and incubated for 4 hr at 37°C. At the end of this period, the cells were actively dividing, and 1 ml quantities were transferred to 15 ml portions of broth containing 1.5% yeast extract, 1.0% tryptone (Difco), 0.5% soluble starch and 1.74% K_2HPO_4. Following 16 hr incubation at 37°C, the cultures contained vegetative cells and endospores; these cultures were aerated by shaking for 4 hr at room temperature to enhance maturation of the spores (Leifson, 1931; Zoha and Sadoff, 1958). The cultures were pasteurized at 75°C for 20 min to destroy vegetative forms

(Gibbs and Hirsch, 1956); the resultant suspension was cooled immediately and transferred to 80 ml of Fluid Thioglycolate Medium and incubated for 8 hr at 37°C. Two additional serial subcultures were prepared by transferring 20 ml of previous subcultures to 80 ml of fresh Thioglycolate Medium and incubated for 8 hr at 37°C. The final subcultures contained only vegetative cells and were used as inocula for each of the test media described subsequently.

3. Sporulation medium

The medium developed for sporulation of C. perfringens and referred to as 'AW' medium contained yeast extract, 1.5%: Bacto-tryptone, 1.0%; soluble starch, 0.5%; K_2HPO_4, 1.74%; urea, 0.5%; barbituric acid, 0.1%; bovine serum albumin (crystallized and lyophilized, Sigma Chemical Company), 0.4%; and mineral mixture, 1.0% (by volume). The mineral mixture contained 2.0 g $MgSO_4.7H_2O$; 0.46 g $MnSO_4.H_2O$; 1.17 g NaCl; 6.00 g $CaCl_2.2H_2O$; 1.34 g $ZnSO_4.7H_2O$ and 1.11 g $FeSO_4.7H_2O$. Each salt was separately dissolved in 0.1 N HCl and combined to a final volume of 1 liter. BSA was filter sterilized using a Seitz filter and added along with the mineral mixture at the time of inoculation. A 10% (by volume) inoculum of vegetative cells was used in all instances.

4. Sampling and bacterial counts

To determine the degree of sporulation, a 3 ml sample of each sporulation culture was removed after 16 hr incubation at 37°C and pasteurized at 75°C for 20 min to destroy vegetative cells (Gibbs and Hirsch, 1956) and then cooled in ice water. Appropriate serial decimal dilutions were made in 1% peptone water and plated in duplicate on SPS agar without antibiotics

(Angelotti et al., 1962) in film pouches (Bladel and Greenberg, 1965), for enumeration of spores. As suggested by Duncan and Strong (1968) the results on bacterial counts presented here are reported as the total numbers of viable spores produced rather than as the percentage of total cells.

a. Pouch method Initially a laminated film designated as "Neelam" 60601 (Milprint Co. Inc., Milwaukee, Wisconsin) consisting of Mylar (polyester), 0.5 mil on the outer layer, Saran (polyvinylidine chloride), 0.1 mil as the middle layer, and an inside layer of polyethylene, 2 mil was used for the preparation of pouches. This film had an oxygen permeability of 0.4 cc per 100 in^2 per 24 hr at one atmosphere differential at 23°C. Later on another kind of film, 75 Maraflex 55 (30), manufactured by American Can Company, Neenah, Wisconsin, consisting of similar outer, middle, and inside layers as Neelam was used. This film had an oxygen transmission rate of 50 cc per $meter^2$ per 24 hr at 38°C and 90% relative humidity.

Pouches were made by placing two pieces of film with the polyethylene sides face to face and pressing firmly for a few seconds with an iron heated previously on a hot plate. The iron was in shape of a petri dish. The plastic film was free of contamination and did not need sterilization with proper handling.

One ml or 0.1 ml of the sample was placed in the pouch and 21 ml agar medium at 50°C was then added. The sample in the pouch was mixed with agar by pressing the pouch randomly; the pouch was placed in a holder with spaces set 0.5 cm apart. This technique removed entrapped air bubbles. It was not necessary to seal the pouches as the film of agar, formed in the neck of pouch after solidification of agar, produced an excellent oxygen barrier. The pouches were then removed from the holder and placed in an incubator at 37°C for 18-24 hr.

5. Heat resistance studies

For measurement of heat resistance of spores of C. perfringens, various sporulating strains of this organism were pasteurized as before. Immediately following pasteurization, 1 ml samples of the cultures were placed in 9 ml of either 0.067 M phosphate buffer, pH 6.8 or distilled water preheated to 90°C in screw-cap tubes. Samples were drawn at 1 min intervals. Duplicate counts were made as previously described.

D values on the spores were determined by plotting the percentage of the survivors against time on a semilog paper. The percentage of surviving spores was calculated on the basis of initial number of spores obtained after heating the cultures at 75°C for 20 min.

B. Germination of Spores of C. Perfringens

1. Organisms

Strain S-45 of C. perfringens was used for germination studies.

2. Production of spores

Spores of this strain were produced as previously described under 'Sporulation'.

3. Cleaning of spores

The sporulation medium containing phase-bright spores was allowed to stand overnight at 5°C. Spores were harvested by centrifugation at 7,000 RPM for 10 min in a Sorvall, Model RC2-B refrigerated centrifuge. The pellet, which contained spores, vegetative cells, and debris, was washed with cold sterile, 0.067 M, pH 6.0 phosphate buffer. During the washing procedure the upper white layer of the deposit containing largely cellular

debris and vegetative cells, was suspended in buffer solution and discarded (Long and Williams, 1958). After washing five times with buffer, the suspension was aerated for 2 hr by vigorous shaking in a reciprocal shaker at room temperature and stored overnight at 5°C to allow any remaining vegetative forms to lyse. The spores were then washed five more times with phosphate buffer. When viewed under phase-contrast microscope, the final preparation showed nearly 100% refractile spores. Although some loss of spores resulted, the method is simple. Each batch of the clean spores was consumed within a week because the spore suspensions in distilled water undergo germination at refrigerated temperatures. Or the spores were kept frozen in the pellet form and when needed, they were thawed and resuspended in distilled water. The volume of the suspension was adjusted so that when 2 ml of the suspension was added to 13 ml of distilled water in 150 x 18 mm screw-cap tubes, it gave an absorbance value of 0.20 at 610 mu (approximately 1.5×10^8 spores per ml).

4. Heat-activation

Spores, suspended in distilled water in screw-cap tubes, were heated for 20 min at 75°C for possible heat-activation (Evans and Curran, 1943) and stabilization of suspensions by destruction of partially germinated or heat-labile spores (Stumbo et al. 1950).

5. Media

Media are shown under Results and Discussion. Amino acids used were purchased from Mann Research Laboratories except cystine and cystein which were purchased from Sigma Chemical Company.

6. Aerobic and anaerobic conditions

Unless mentioned otherwise, all germination studies were undertaken under partially anaerobic conditions created by steaming the media before inoculation. All media were steamed just before inoculation to drive off dissolved oxygen and cooled immediately for inoculation of spores. Screw-cap tubes were filled with media and tightly sealed to prevent entrance of oxygen.

7. Criteria of germination

Studies on the extent of germination were conducted using decrease in OD (Powell, 1950, 1951) standardized against loss of heat stability of spores (Wynne and Foster, 1948a; Hills, 1949a) as the ultimate criterion of germination. The optical measurements provided more simple, more convenient, and more accurate measurement for studying the process of spore germination than did the use of loss of heat stability as a criterion of germination. The latter method is cumbersome and time consuming.

Decrease in OD of spores was measured at 610 mu with a Bausch & Lomb Spectronic-20 Colorimeter. The rate of germination was calculated from the equation developed by Hachisuka et al. (1955) for quantitative measurements of germination of spores. This equation is given previously in the Review of Literature.

To correlate the loss of heat stability with decrease in OD on germination, the decrease in OD was continuously followed at 37°C. During this period 1 ml samples were withdrawn at 0 hr and at intervals of 20 min and added to 9 ml of distilled water contained in screw-cap tubes, preheated to 60°C and heated for additional 15 min. Heat shock was given to the samples

in preheated distilled water because the germination rate is expected to accelerate during the time necessary to raise the temperature from that for incubation to one for heat shock. This treatment eliminated the temperature differential and also diluted the medium ten-fold. It was also expected that this technique would stop any further germination and kill the heat-labile cells.

Tubes were immediately chilled in cold water after the heat shock. Appropriate dilutions were then made and plated as previously described under 'Sporulation'. The difference between the spore levels at 0 hr and levels obtained after incubation in germination medium at different intervals of time, was assumed to represent germinated spores for the purpose of calculating percent germination.

C. Inhibition of Germination of Spores of C. Perfringens

Inhibition studies were carried out following the techniques mentioned under germination studies. Inhibitors used will be reported under Results and Discussion.

IV. RESULTS AND DISCUSSION

A. Sporulation of C. Perfringens

Initially, a basal medium containing 1% yeast extract, 1% tryptone, 0.5% soluble starch, 0.5% sodium glycerophosphate, 0.5% urea, 0.1% barbituric acid, and a separately sterilized mineral mixture (1% by volume) was used for production of spores in different strains of C. perfringens. Sporulation, as determined by phase-contrast microscopy, was poor in this medium. The pH of the media in which the different strains of C. perfringens had grown for 20 hr was checked and was found to have decreased from the initial pH of 7.4 to pH 5.5-5.7. The lower pH values persisted even after prolonged incubation periods.

Similar lowering and persistance of pH at lower levels was observed by Nakata and Halvorson (1960) and by Stahly et al. (1966) who inhibited sporulation of B. cereus by adding α-picolinic acid and 8-azaguanine (Aza G) respectively. The lowering of pH was due to the dissimilation of glucose in the medium to acetate which accumulated in the medium (Nakata and Halvorson, 1960). Gollakota and Halvorson (1960) suggested that α-picolinic acid exerts its effect by chelating with some metals essential for the synthesis of enzyme or enzyme system responsible for acetate utilization. Consequently they found that zinc, cobalt, and nickel reversed the inhibition of α-picolinic acid. The inhibition caused by α-picolinic acid was also reversed by phosphate and by increasing the concentration of yeast extract in the media. Stahly et al. (1966) also found that phosphate relieved the inhibition of sporulation by Aza G.

Persistence of low pH in our cultures was suspected to be due to the

presence of some kind of inhibitor having the characteristics of α-picolinic acid or Aza G. Thus sodium glycerophosphate in the basal medium was substituted by 0.1 M, pH 7.4 phosphate buffer. A tremendous increase in sporulation resulted in strains S-45, 214d, and ATCC 3624; sporulation in other two strains, namely HR_2 and 65, was not improved. These latter two strains were considered as representatives of strains of C. perfringens which sporulate poorly.

When yeast extract was increased to 1.5%, sporulation was further improved in the first three strains. Increasing the tryptone level to 1% resulted in an increase in sporulation in all the five strains but sporulation in strains HR_2 and 65 did not reach the levels of the other three strains. Duncan and Strong (1968) also observed that yeast extract produced increased sporulation in three of the five strains of C. perfringens. Gollakota and Halvorson (1960) suggested that yeast extract provides some organic intermediates on the metabolic pathway of acetate beyond the metabolic block caused by α-picolinic acid and thereby permitted the interrupted sequence of biochemical changes to proceed to completion. If it is assumed that the absence of sporulation in C. perfringens was due to the presence of a natural inhibitor similar to α-picolinic acid, then yeast extract seems to play similar role in C. perfringens as suggested by Gollakota and Halvorson (1960). Yeast extract also is a utilizable source of nitrogen for C. perfringens.

Peptone concentration appears to be important in spore production because on increasing the level of peptone, increased sporulation was obtained. Similar findings were reported by Riemann (1963a) and by Duncan and Strong (1968).

When seven levels of soluble starch ranging from 0.1% to 0.7% were compared, 0.5% was the optimal level. Starch was used as a carbohydrate source and as an agent to neutralize the antisporulating factors from the media. Olsen and Scott (1946) have reported that starch absorbs small amounts of antisporulating substances present in peptone media. These antisporulating substances may be generated during growth of bacteria (Foster et al., 1950). Foster (1956) identified fatty acids as an important class of antisporulating factors.

Attempts were made to further neutralize any remaining antisporulating substances from the media by use of BSA because Davis and Dubos (1947) had pointed out that BSA neutralized oleic acid by binding it. In addition, Kaplan and Williams (1941) reported that utilizable sources of nitrogen (e.g. albumin, serum, etc.) in media have an accelerating effect for sporulation of clostridia.

When added to the modified basal medium, BSA in 0.4% concentration further improved sporulation in all five strains although the numbers obtained were still less in strains HR_2 and 65. BSA also facilitated the release of spores from sporangia.

Aeration of cells of C. perfringens after 10-12 hr of growth at 37°C in the above medium was found to hasten the release of spores from sporangia. Similar observations were made by Zoha and Sadoff (1958) who reported that aeration accelerates the sporulation process since it facilitates the lysis of sporangia and liberation of spores.

Once the complete sporulation medium was developed, spores were counted by plating technique on SPS agar. A level of 10^9-10^{10} spores per ml of strains S-45, ATCC 3624, and 214d were produced on 'AW' medium and 10^8 per

ml of strains HR_2 and 65.

'AW' medium was compared for spore yields with Ellner's (1956) medium, medium of Kim et al. (1967) and medium of Duncan and Strong (1968). The results are shown in Table 1. With the five strains tested, the 'AW' medium produced approximately 300 to 30,000-fold increase over numbers of spores produced in Ellner's medium; a 300 to 16,000-fold increase over numbers of spores produced in medium of Kim et al. and 10 to 3,000-fold increase in numbers of spores produced over that of the medium of Duncan and Strong.

Table 1. Sporulation of five strains of Clostridium perfringens after 16 hrs of growth in four different media (No. of spores/ml x 10^8)

	Media			
Strains	Ellner's medium (1956)	Medium of Kim et al. (1967)	Duncan and Strong's medium (1968)	'AW' medium
ATCC	0.1	0.016	0.27	160
NCTC 8238	0.00015	0.018	0.30	5
S-45	0.002	0.012	18	190
65	0.00016	0.009	0.01	5
214d	0.1	0.03	0.01	30

The various ingredients used in 'AW' medium have resulted in the production of larger numbers of spores of different strains of C. perfringens than in other media reported in the literature. The stimulatory effect of phosphate in spore production calls for some further comment. Inorganic phosphate was reported by Leifson (1931) to be essential or stimulatory to clostridial sporulation. The role played by phosphate in enhancing sporulation is not known. Gollakota and Halvorson (1960) suggested that the

ability of phosphate to reverse the inhibition by α-picolinic acid was due to the competition between the two for the metal ions essential for sporulation. Stahly et al. (1966) found that phosphate causes a decrease in the Aza G intracellular pool size in cultures where sporulation was inhibited by addition of Aza G. It was suggested that an increase in the ratio of natural purines to Aza G occurred at the time of transition from vegetative growth to presporulation and the natural purine derivatives then are incorporated into RNA preferential to Aza G which in the absence of phosphate is incorporated solely in RNA and sporulation is inhibited. It is not clear whether the absence of sporulation in C. perfringens initially observed was due to the presence of some inhibitors having the characteristics of α-picolinic acid or Aza G and phosphate acts by relieving the inhibition caused by such hypothetical inhibitors. In addition, our results indicate that 0.1 M phosphate also avoided drastic lowering of pH resulting from the degradation of starch to acid intermediates. This lowering of pH in the absence of phosphate was found to be inhibitory to sporulation of C. perfringens. Sporulation in the presence of phosphate was a maximum when the initial pH of the media was adjusted to a range pH 7.0 to 8.5. No sporulation occurred when the initial pH of the medium was 6.0. A few spores were formed at initial pH of 6.5. These results are in agreement with Simonds (1915) who stated that spore production of C. perfringens occurred only in alkaline conditions. Torry et al. (1930) and Kaplan and Williams (1941) came to similar conclusions when they studied other clostridia.

Apart from the inhibition of sporulation at low pH, the abundant gas production, which always accompanies sporulation, did not occur. This might

give some idea as to what intermediates are accumulated when sporulation is supressed at low pH. The only known step in carbohydrate metabolism of C. perfringens which yields gases (carbon dioxide and hydrogen) is the conversion of pyruvate to acetyl phosphate (Groves and Gronlund, 1969):

$$\text{Pyruvate} + \text{phosphate} \xrightarrow[\text{enzymes}]{\text{clastic}} \text{acetyl phosphate} + CO_2 + H_2$$

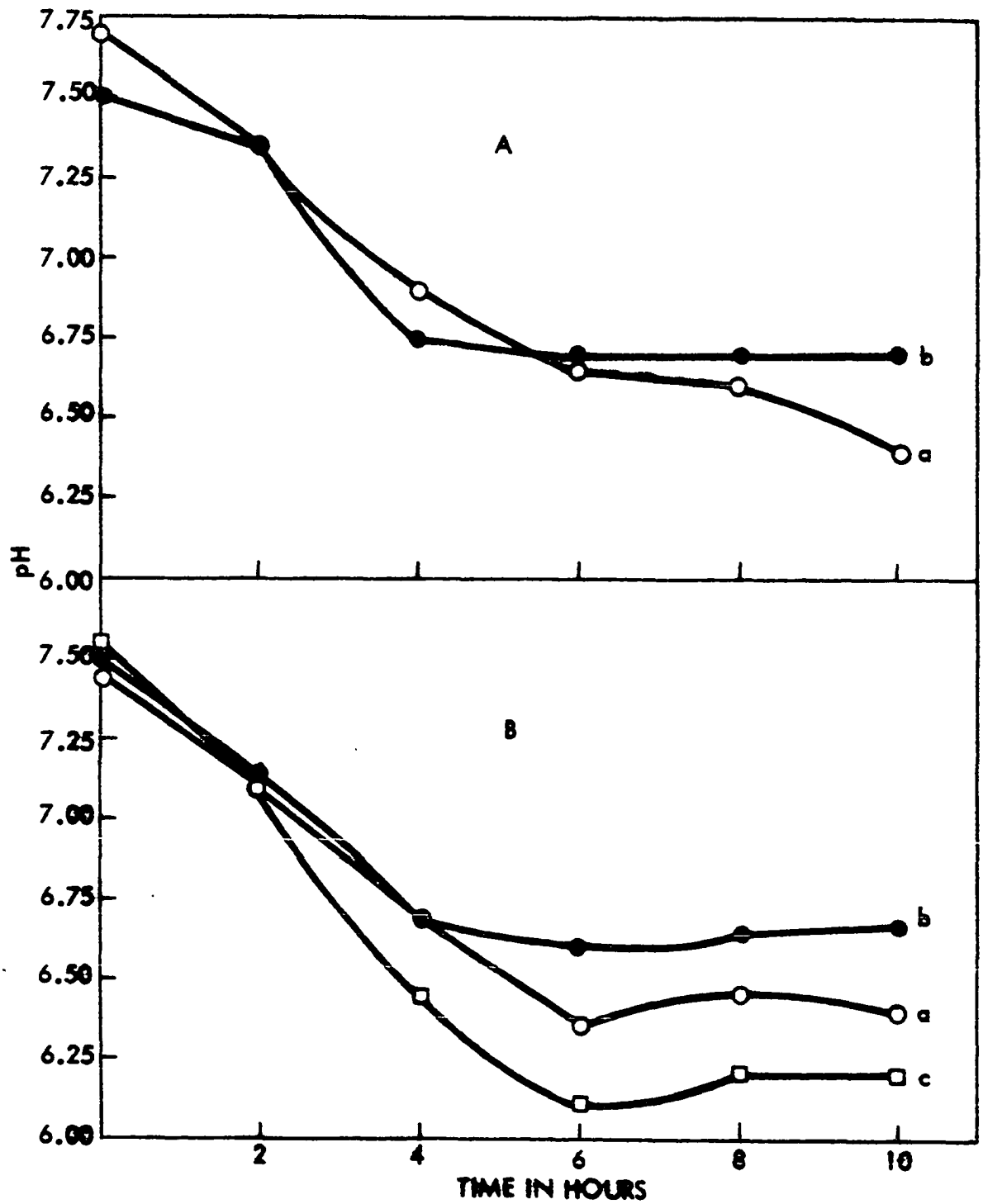
At low pH the enzyme aldolase of C. perfringens is not active; the maximal activity for this enzyme has been indicated by Bard and Gunsalus (1950) to be at about pH 7.5. The metabolism of carbohydrate at pH 6.0 or 6.5 either stops at fructose 1,6-diphosphate or at the most goes from pyruvate to lactate. All the enzymes of the Embden-Meyerhof pathway including lactic acid dehydrogenase and pyruvic clastic system have been shown to be present in cell-free extracts of C. perfringens (Groves and Gronlund, 1969). Thus, at low pH, the pyruvate phosphoclastic enzyme system is probably inactive. Pyruvate serves as source of energy for C. perfringens (Groves and Gronlund, 1969). This compound also seems to be the substrate for sporogenesis. Pappenheimer and Shaskan (1944) reported that C. perfringens grown in ferrous deficient complex media containing carbohydrates yield no gases, and such cells failed to accumulate acetate. This again indicates that the pyruvate clastic enzyme system is not active in cells which do not produce gases. The increase in sporulation in our complex medium might have been due to the increased activity of this enzyme system as Groves and Gronlund (1969) reported that this system was four times more active in cells grown in complex medium than in a semidefined medium. Whether sporulation and the activity of pyruvate clastic enzyme are related remains to be seen.

1. Effect of glucose on sporulation

It is commonly stated that glucose suppresses sporulation of clostridia. Gibbs and Hirsch (1956) suggested that the effect of glucose is indirect and largely concerned with the formation of low pH unfavorable to spore production. We endeavored to test this hypothesis by growing C. perfringens on 'AW' medium containing starch (pH 7.75) and on 'AW' medium in which starch was substituted with 0.5% glucose (pH 7.5). The pH of both culture media was recorded at two hr intervals. The results are shown in Figure 1, Part A. It can be seen that during first four hr of growth the pH dropped rapidly in both media. The growing cells were also observed under the microscope at these intervals. Cells grown on starch containing medium were predominantly ellipsoid in shape and developed refractile bodies after 6 hr, and the sporulation was complete after 10 hr; whereas, the cells grown in medium containing glucose showed no such changes in morphology and sporulation did not occur even after 48 hr. Figure 1 Part A also indicates that at the time of the induction of sporulation, i.e., after 4 hr the medium containing starch had a higher pH (pH 6.9) than in the medium containing glucose (pH 6.75). This difference in pH might have been the cause of lack of sporulation in this medium. Furthermore Leifson (1931) has reported that pH values at the time of sporulation were more important than initial or final pH values. Attempts were made to minimize changes in pH in the two media as much as possible during growth. For this reason the pH of glucose and starch containing media were adjusted to pH 7.45 and pH 7.5 respectively. The media were inoculated as before and pH recorded. It was observed that when sporulation in starch grown cultures was induced, pH values in both media were close enough to make no difference in

Figure 1. Change in pH occurring during growth of C. perfringens strain S-45, in 'AW' medium containing starch or glucose or both

a) 'AW' medium + 0.5% starch
b) 'AW' medium + 0.5% glucose
c) 'AW' medium + 0.5% starch + 0.5% glucose


7.75
7.50
7.25
7.00
6.75
6.50
6.25
6.00
pH
A
b
a
7.50
7.25
7.00
6.75
6.50
6.25
6.00
B
b
a
c
2
4
6
8
10
TIME IN HOURS

sporulation (Figure 1, Part B) yet no spores were formed in cultures grown in the presence of glucose. It was concluded that glucose does not exert its inhibitory effect on sporulation of C. perfringens by formation of low pH only. Either catabolite repression (Magasanik, 1963; Schaeffer et al., 1965) or the possible presence of antisporulating factors might have inhibited sporulation because soluble starch which has been reported to be an antisporulating factor by Olsen and Scott (1946) was absent. To observe this, a mixture of soluble starch and glucose were added to 'AW' medium and pH was recorded at intervals as before (Figure 1B, curve c). Spores were formed in the presence of the mixture of carbohydrates although the pH values had dropped to lower levels than in the presence of either of the carbohydrate alone. This again indicates that glucose inhibited sporulation not by the formation of low pH but on the contrary, it could have been due to catabolite repression or the presence of antisporulation factors in the medium. Spore formation in the presence of the mixture of the carbohydrates indicates the latter possibility to be more probable. If sporulation was inhibited due to repression, then the cells would be repressed even in the presence of a mixture of carbohydrate because glucose was expected to be utilized first. Apparently cells were not repressed by glucose and starch absorbed any antisporulating factors from the medium, thereby allowing the cells to sporulate even in the presence of glucose.

2. Heat resistance studies

Although 'AW' medium supported the production of larger numbers of spores in different strains of C. perfringens, none of the strains had an appreciable degree of heat resistance at 90°C. The D values obtained on

strains ATCC 3624, HR_2, S-45, 65, and 214d were 0.9 min, 1.27 min, 2.85 min, 1.7 min, and 1.05 min respectively. Nishida et al. (1969) also did not find any heat resistant spores in cultures isolated from unheated samples. Spores grown on 'AW' medium were consistently slightly more heat resistant than those produced on Duncan and Strong's medium. This difference was not considered particularly significant. Using strain ATCC 3624, heat resistance of spores after 16 hr in two media was compared and D values were found to be 0.9 min on 'AW' medium and 0.5 min on Duncan and Strong's medium. This slight increase in heat resistance may have resulted from the presence of BSA or differences between mineral mixture in "AW" medium and in the other media.

Duncan and Strong (1968) have observed that the inclusion of activated carbon in their proposed sporulation medium resulted in spores with slightly increased heat resistance over that of the proposed medium without added carbon. The function of the activated carbon, as reported in the literature, is to remove antisporulation factors from the media; BSA was used for the same reason. Thus, it might be possible that certain factors in the medium are responsible for low heat resistance in C. perfringens that are removed by BSA thereby producing spores of increased heat resistance.

B. Germination of C. Perfringens

1. Preliminary studies

Initially Fluid Thioglycolate Medium (Difco) was used to observe germination of spores of C. perfringens. The rate of germination in that medium, as measured by decrease in OD, was very rapid. When an attempt was made to determine percent germination after killing the germinated spores

by heat treatment, it was found that a high proportion of the spores germinated during the time required to take samples and to make inoculations for counting ungerminated spores after the heat treatment.

The proportion of the spores which became sensitive to heat and showed a decrease in OD in Fluid Thioglycolate Medium during a convenient experimental period was controlled, however, by diluting the medium. The use of this medium was later abandoned because of the content of agar and indicator present in this medium. The color of the medium turned pink on oxidation and interfered with OD measurements.

N.I.H. Thioglycolate Broth which does not contain agar and indicator, was substituted. This medium provided a gradual decrease in OD for about one hour, the OD remained constant for some time and then increased. Probably the germinated spores were growing as vegetative cells and any further decrease in OD was being nullified by the increase in OD caused by the increase in cell numbers.

Later on several investigations were carried out to formulate a medium which would allow complete germination of the spores at a moderate rate and with a minimum of outgrowth. The outcome of these investigations was a medium, designated medium 1, which contained yeast extract, 0.5%; casitone, 0.75%; glucose, 0.5% sodium chloride, 0.25%; sodium thioglycolate, 0.05%; and cystine, 0.5%. Cystine was dissolved first in water by adjusting the pH to 11.5 by the addition of 0.1N NaOH. The final pH of the medium was adjusted to pH 7.0.

The rate of germination in this medium was such that it allowed enough time to take samples at intervals for plating to count the ungerminated spores after heat shock. The germination, as determined by the decrease in

OD, approached 100% within reasonable time and outgrowth did not seem to occur. This medium was diluted 1:2 to further reduce the rate of germination and was designated as medium 2.

2. Correlation between decrease in OD and loss of heat resistance

Medium 1 (pH 7.5) was used to correlate the heat-lability and the decrease in OD observed upon germination of the spores. Percent germination by both methods was calculated by the procedures mentioned under materials and methods. It was observed repeatedly that in the early and middle phases of germination, the percent germination, as calculated from the residual spores after heat shock, was always greater than the rate of germination calculated from decrease in OD. To explain this discrepancy, it was thought that a portion of the spores may not show a decrease in OD by the loss of spore-coat materials but they may be on the verge of germination and have become sensitive to heat treatment of 75°C for 20 min and were killed by this treatment. As a result, lesser numbers of ungerminated spores were recorded in pouches. The percent germination calculated from these data will naturally be greater than that calculated from OD measurements. Certain workers (Powell, 1957; Riemann, 1963a) have reported that heat resistance of the germinated spores is lost in advance of OD decrease.

An attempt was then made to correlate heat-lability and decrease in OD of the germinated spores by using lower temperatures of heat shock. Percentages of germination calculated by OD measurements and by the plating technique after heat treatment of 60°C for 15 min were consistent with each other. The results are shown in Table 2.

After establishing the correlation between decrease in OD and the

loss in heat resistance upon germination of the spores, observations were made on the effects of certain factors on germination of C. perfringens.

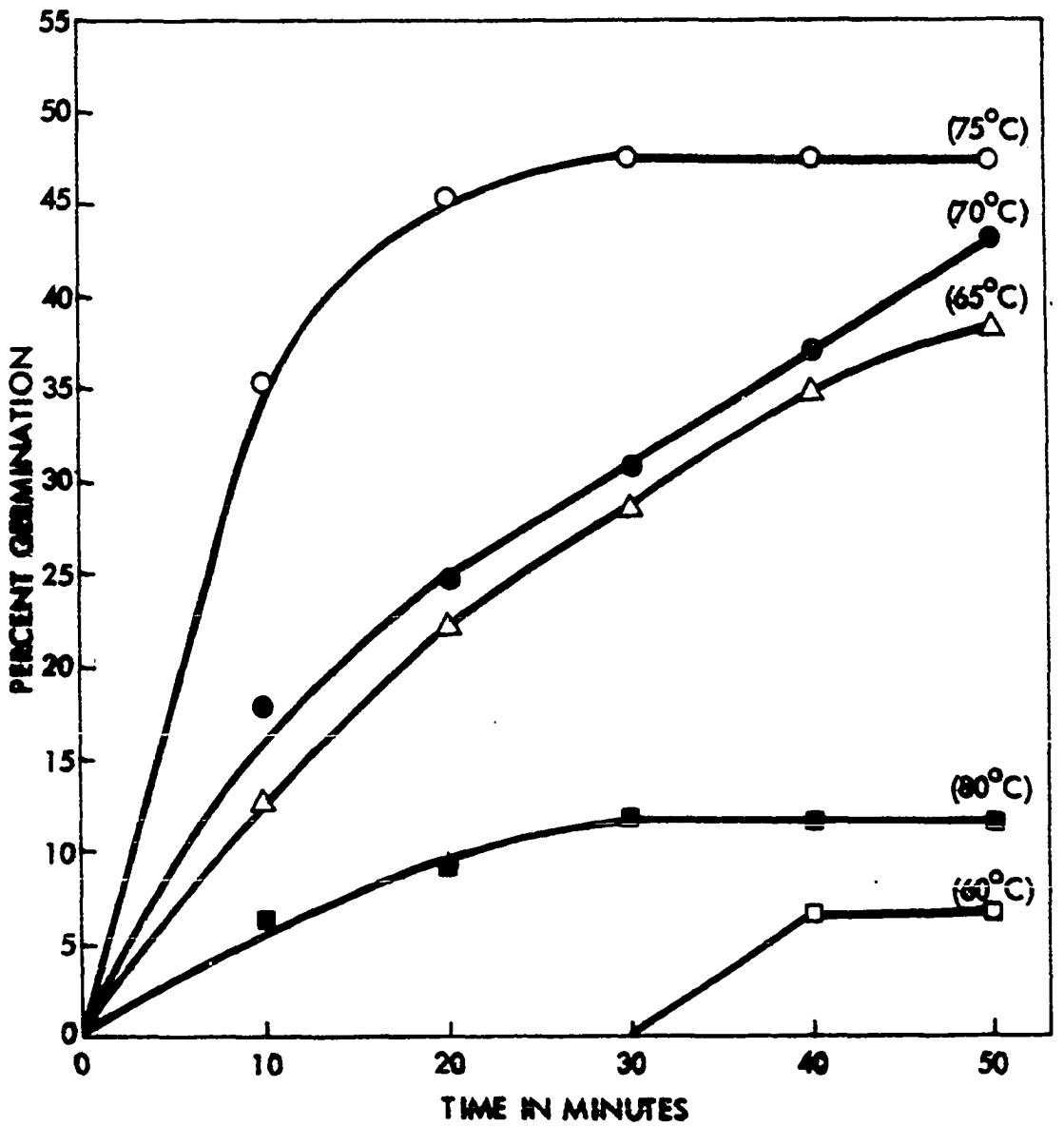
Table 2. Correlation between the rate of germination of spores of C. perfringens, calculated from the equation of Hachisuka et al. (1955) and the percent germination as determined after killing the germinated spores by heat treatment of 60°C for 15 min. Germination was carried out in medium 1, pH 7.5

Incubation time (min)	Percent germination from equation of Hachisuka et al.	Percent germination after heat killing the germinated spores
5	44	46
10	55	59
15	67	71

3. Heat-activation

Different species vary in their temperature requirements for activation. This variation exists also among different strains and even among different batches of the same strain. Curran and Evans (1945) observed that some strains of B. megaterium require only a few minutes heat treatment at 60°C for optimal germination rates, whereas B. stearothermophilus and other thermophilic and thermoduric bacteria require from 105 to 115°C for optimal activation. It was of some interest, therefore, to determine the exact time and temperature of activation of spores of C. perfringens. For this purpose, temperatures in the range 60° to 80°C for 20 min were employed. The results are summarized in Figure 2. An increasing degree of activation, as measured by the extent of germination in medium 2 of spore suspensions, was observed with an increase in temperature from 60°C to 75°C. Increasing

Figure 2. Effect of temperature of heat-activation for 20 min on the extent of germination of spores of C. perfringens in medium 2, pH 7.5 at 37°C. The spores were incubated for indicated intervals of time. Percent germination was calculated from the equation of Hachisuka et al. (1955)


55
50
45
40
35
30
25
20
15
10
5
0
PERCENT GERMINATION
0
10
20
30
40
50
TIME IN MINUTES
(75°C)
(70°C)
(65°C)
(80°C)
(60°C)

the temperature to 80°C depressed the germination. Thus a temperature of 75°C for 20 min was found to be optimal for the activation of spores of C. perfringens.

In all subsequent experiments, a 20 min heat shock at 75°C in distilled water was employed for heat-activation of the spores.

4. Effect of pH

The optimal pH value for spore germination varies widely and depends very much on the particular organism also. Therefore, the effect of pH on germination of C. perfringens in medium 2 was observed in the range pH 5.5 to 9.5. Figure 3 shows that germination took place in media with a pH range of 5.5 to 9.5 with large differences in rates, especially during the middle phases of germination. The rate of germination increased almost linearly with the decrease in pH until it dropped at pH 5.5. There appears to be an optimal pH value of 6.0 in this medium.

Duncan and Foster (1968b) also found pH 6.0 as the optimum for the rapid germination of spores of PA 3679h in a medium containing sodium nitrite. Similar pH optima were observed for the germination of B. stearothermophilus by Fields and Frank (1969) and by Ashton et al. (1968).

5. Effect of temperature

Germination of spores of different organisms can occur over a wide range of temperatures. As shown in Figure 4, germination of spores of C. perfringens can occur within a temperature range of 7° to 46°C. There was an increasing extent of germination with increasing temperature during the first 10 min; this was followed by a broad temperature optimum between 27° and 46°C. The maximum attained rate and extent of germination was lower

Figure 3. Effect of pH on the extent of germination of spores of C. perfringens in medium 2 incubated at 37°C. Percent germination was calculated as in Figure 2

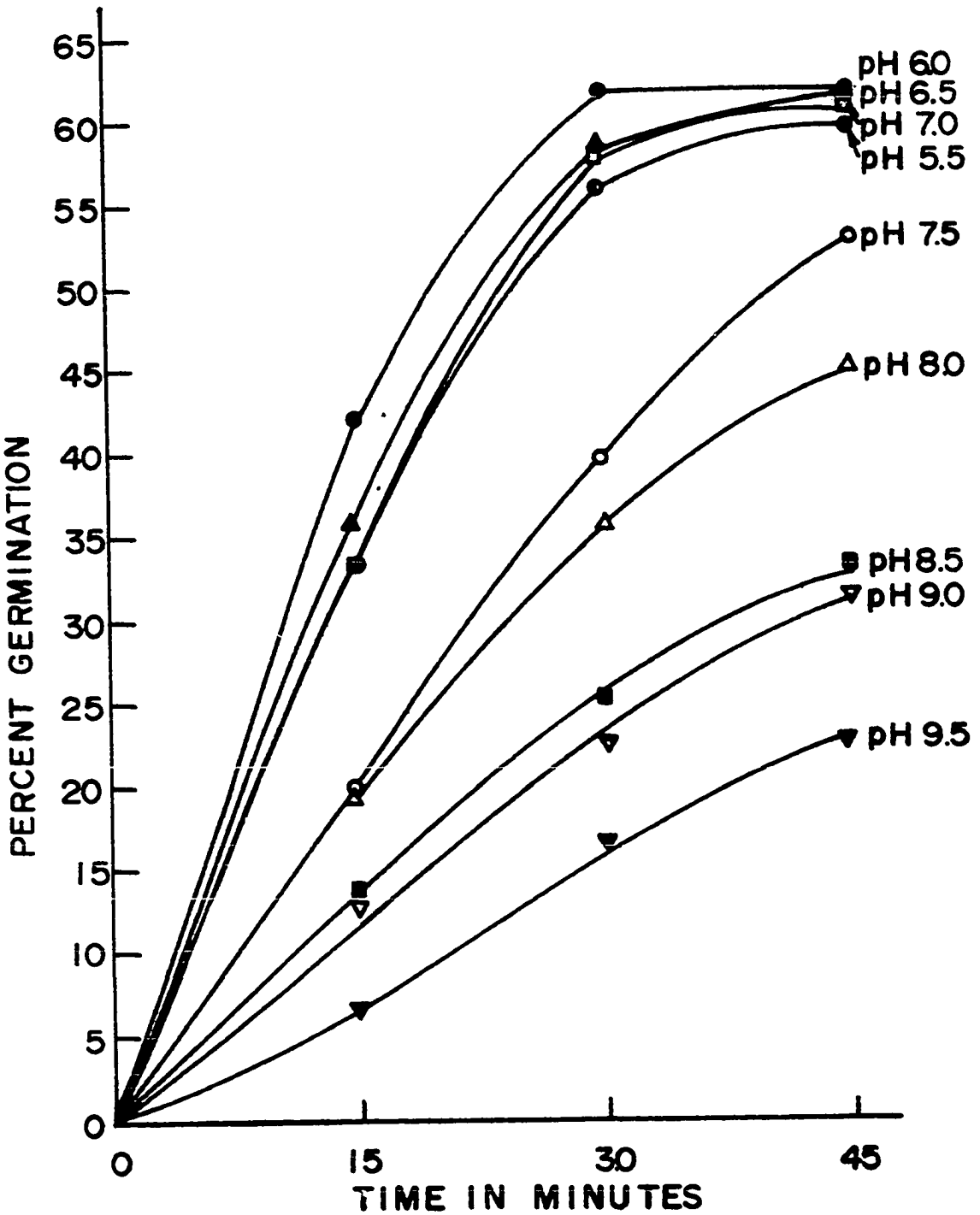
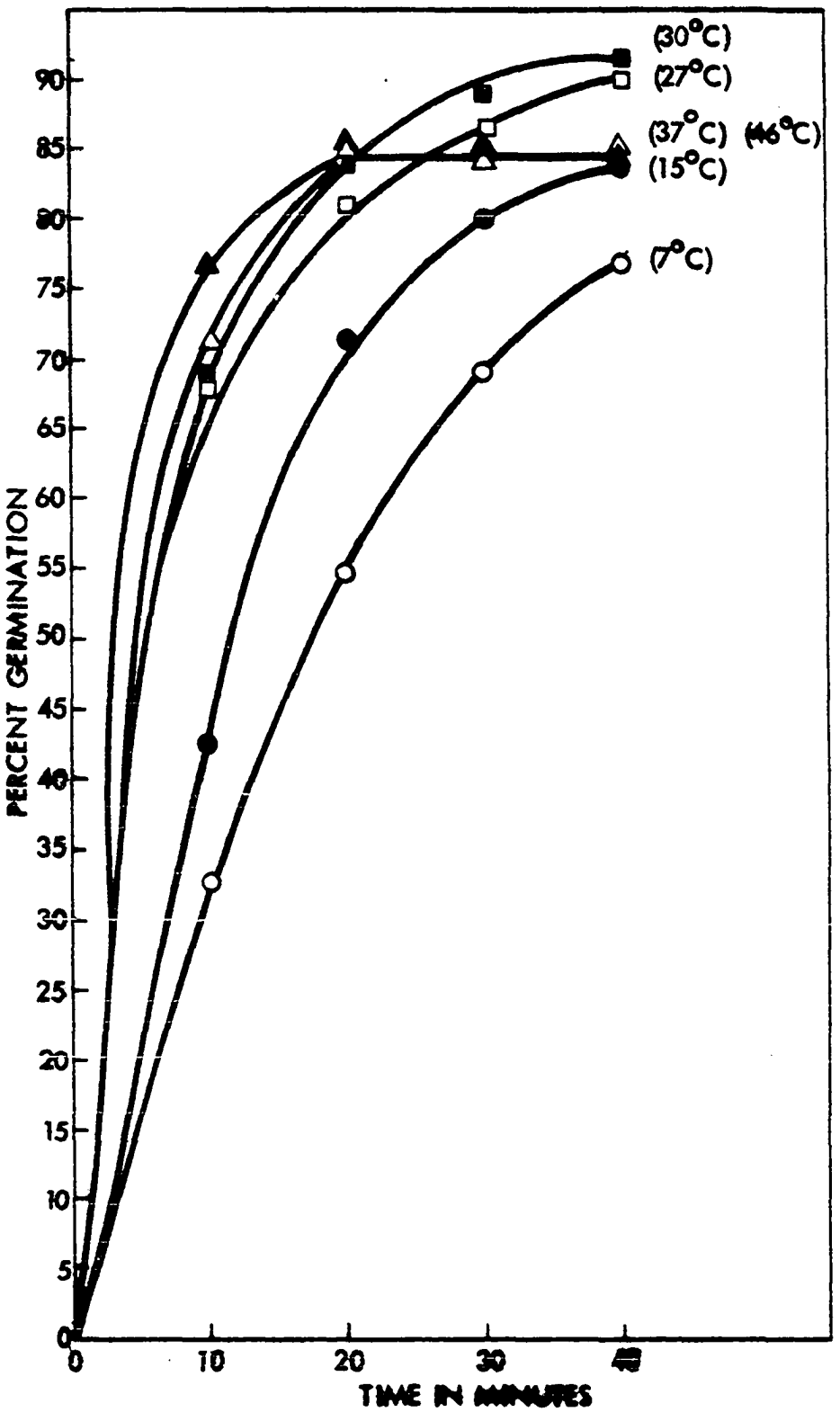

PERCENT GERMINATION
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
15
30
45
TIME IN MINUTES
pH 6.0
pH 6.5
pH 7.0
pH 5.5
pH 7.5
pH 8.0
pH 8.5
pH 9.0
pH 9.5

Figure 4. Effect of temperature on the extent of germination of *C. perfringens* spores in medium 1, pH 6.0. Percent germination was calculated as in Figure 2


(30°C)
(27°C)
(37°C)
(46°C)
(15°C)
(7°C)
PERCENT GERMINATION
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
0
10
20
30
TIME IN MINUTES

at lower incubation temperatures. The highest extent of germination was at 30°C. O'Connor and Halvorson (1961) and Knaysi (1964) also found 30°C as the optimum temperature for germination of B. cereus.

6. Effect of oxygen

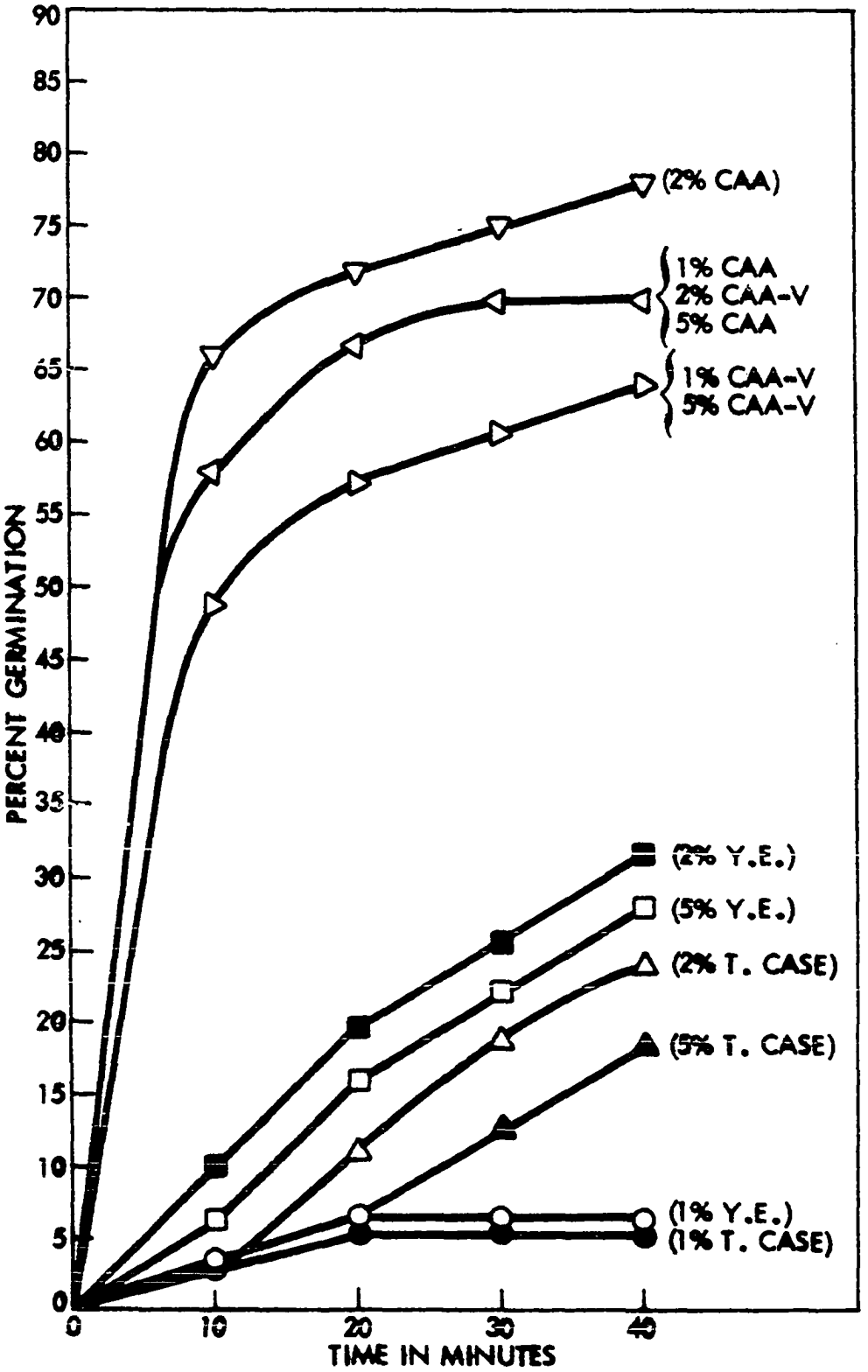
Several investigators have reported that clostridial spores germinated under aerobic conditions (Wynne and Harrell, 1951; Wynne et al., 1954; Gibbs, 1964; Uehara and Frank, 1965). Germination in medium 1 under anaerobic (nitrogen gas atmosphere), partial anaerobic (produced by heating the medium to remove oxygen before inoculation), and aerobic conditions was compared. Germination of C. perfringens spores occurred rapidly under anaerobic and partial anaerobic conditions whereas the rate and extent of germination was absent or minimal under the atmosphere of air. These results are in agreement with those obtained by Wynne and Harrell (1951) and Wynne et al. (1954) on C. perfringens.

Subsequent studies on germination were carried out at pH 6.0. Media were evacuated by steaming to drive off air and were inoculated with heat-activated spores. They were incubated at 30°C and percent germination was calculated from the equation of Hachisuka et al. (1955).

7. Investigation of the compounds specifically responsible for the germination of spores of C. perfringens

a. Effect of complex nitrogen substances Yeast extract (Difco), trypticase (BBL), vitamin-free casamino acids (acid hydrolysate of casein, Difco) and casamino acids (acid hydrolysate of casein, Difco) were tested for their ability to cause rapid germination of spores of C. perfringens. As is shown in Figure 5, vitamin-free casamino acids (CAA) produced the best

Figure 5. Effect of varying concentrations of yeast extract (Y.E.), trypticase (T. case), vitamin-free casamino acids (CAA), and casamino acids (CAA-V) on the germination of spores of C. perfringens


PERCENT GERMINATION
TIME IN MINUTES
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
0
10
20
30
40
(2% CAA)
1% CAA
2% CAA-V
5% CAA
1% CAA-V
5% CAA-V
(2% Y.E.)
(5% Y.E.)
(2% T. CASE)
(5% T. CASE)
(1% Y.E.)
(1% T. CASE)

results. Casamino acids with vitamins (CAA-V) were the second best. Yeast extract (Y.E.) and trypticase (T. case) were the inferior agents for inducing germination of spores of C. perfringens.

Vitamin-free casamino acids contain 38% sodium chloride and casamino acids with vitamins contain only 14% sodium chloride in their composition; moreover, the latter also contain 2% phosphate. These differences in sodium chloride concentration and the presence of phosphate in the latter compound may explain the superiority of vitamin-free casamino acids in supporting better germination. Also 2% vitamin-free casamino acids supported better germination. This might have been due to the optimal concentration of amino acids and salt at this concentration. Yeast extract and trypticase contain lower concentrations of amino acids and sodium chloride than casamino acids and also have several other minerals, which might be inhibitory to germination of C. perfringens. The inferiority of yeast extract and trypticase for induction of germination of C. perfringens spores might be due to the differences in chemical composition of these materials. Vitamin-free casamino acids also stimulated germination of C. bifermentans (Gibbs, 1964) but Treadwell et al. (1958) observed very slow germination of C. botulinum spores in the presence of this hydrolysate.

Results obtained here with yeast extract and trypticase are in direct conflict with the results obtained by other workers. For instance, Treadwell et al. obtained rapid germination of spores of C. botulinum in 5% yeast extract and Hitzman et al. (1957) obtained 90% germination in 15 min in 5% trypticase. These differences might be attributed to the different organisms used and the concentrations of the ingredients in these compounds.

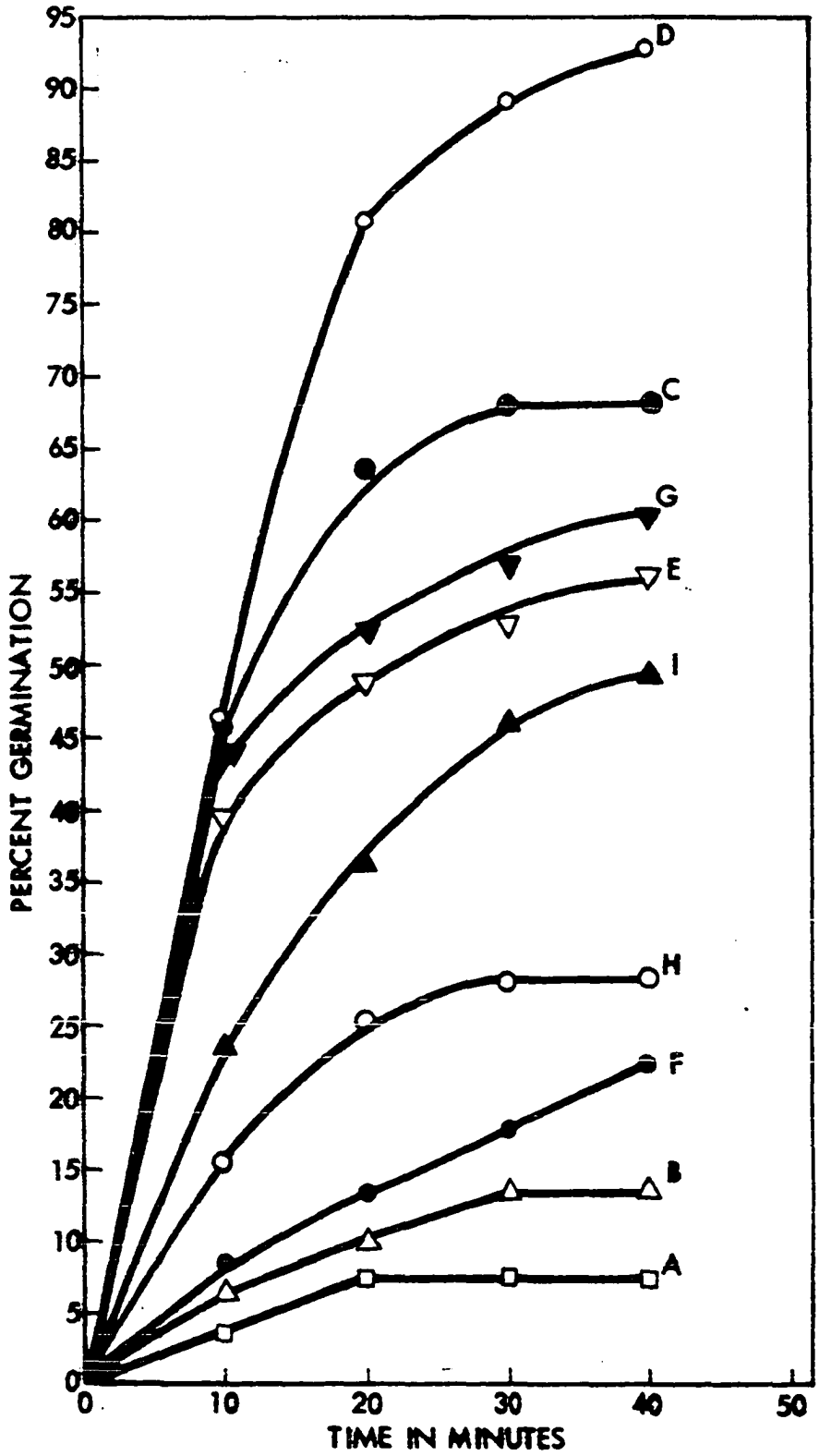
b. Amino acid requirements for germination of C. perfringens in the presence of yeast extract plus glucose As reported previously rapid germination was obtained in Fluid Thioglycolate Medium. An attempt was made to identify the specific ingredients of this medium which might cause complete germination; and germination of spores of C. perfringens approached 100% in the presence of only three ingredients of the medium namely glucose, yeast extract, and cystine. Removal of any one ingredient reduced germination considerably. Germination was very poor in the presence of yeast extract alone which was slightly improved by the addition of glucose and there was dramatic increase in the extent and rate of germination by the addition of cystine. It was concluded that cystine was specifically required in the germination of C. perfringens and its effect was augmented in the presence of glucose and yeast extract.

Twenty three L-amino acids were then tested for their ability to enhance germination of C. perfringens in the presence of glucose plus yeast extract. Of these L-cytein, L-tryptophane, and L-tyrosine enhanced germination but not to the level of L-cystine reported above. Other amino acids were without effect. Results are shown in Figure 6. Stimulation of germination of bacillus and clostridial spores by L-cystine, L-cysteine, and L-tyrosine has also been shown by other workers (Gibbs, 1964; Woese et al., 1958; Krask, 1961; O'Connor and Halvorson, 1961; Levinson and Hyatt, 1962; Hills, 1949b, 1950; Lund, 1956). Stimulation of germination of spores of C. perfringens by tryptophane is unique because such stimulation has never been reported for other bacteria.

None of the amino acids tested in the presence of yeast extract plus glucose for their effect on germination of C. perfringens produced

Figure 6. Effect of amino acids on the germination of suspensions of spores of C. perfringens in the presence of yeast extract and yeast extract + glucose

A: 1% yeast extract or 1% yeast extract + cystein
B: 1% yeast extract + 0.5% glucose
C: 1% yeast extract + 0.5 mg/ml cystine
D: 1% yeast extract + 0.5 mg/ml cystine + 0.5% glucose
E: 1% yeast extract + 1 mg/ml cystein + 0.5% glucose
F: 1% yeast extract + 1 mg/ml tryptophane
G: 1% yeast extract + 1 mg/ml tryptophane + 0.5% glucose
H: 1% yeast extract + 0.4 mg/ml tyrosine
I: 1% yeast extract + 0.4 mg/ml tyrosine + 0.5% glucose


PERCENT GERMINATION
TIME IN MINUTES
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
90
95
0
10
20
30
40
50
D
C
G
E
I
H
F
B
A

stimulatory effects in the presence of vitamin-free casamino acids. Non-stimulatory effects of amino acids in the presence of casamino acids might have been due to the self sufficiency of the latter for the amino acids. Yeast extract and casamino acids contain the same amino acids but amino acids found in the composition of vitamin-free casamino acids are in several-fold higher concentration than in yeast extract.

The mechanism whereby these amino acids enhance germination is obscure (Gould, 1969), however, largely due to our lack of knowledge at present time of the biochemical activities of spores. This probably involves deamination. Church and Halvorson (1957) emphasized that for an amino acid to be a germinant, it must be deaminated. If this is true, then apparently an alternative mechanism is available for amino acid metabolism because several amino acids can induce or enhance germination. A cystein disulfhydrase yielding pyruvate, H_2S and NH_3 is known in spores of B. cereus (Krask, 1961) and may be operative in germination by L-cystein.

Hachisuka et al. (1955) on the other hand suggested that amino acids are required as "sparking factors" for changing the resting spores to active spores. They presented evidence that amino acids effective in germination were used in other ways than materials for the synthesis of cell substrates. The suggestion made by Hachisuka et al. was supported by Riemann (1961) who proposed a spore structure-Ca-DPA or Enzyme-Ca-DPA complex to be associated with dormancy in spores. He suggested that this complex could be disrupted by chelation of calcium with agents such as ethylenediamine tetraacetic acid (EDTA), DPA, and amino acids, thus leading to germination.

Hyatt and Levinson (1961) postulated that either the activation of spores brings about the production internally of substance (s) capable of

reacting together with added compounds to initiate germination, or heating alters the permeability of spores to make them more susceptible to the triggering action of a variety of compounds.

There appears to be no outstanding structural relationship among the compounds supporting germination, nor does there seem to be structural resemblance among those compounds which do not support germination.

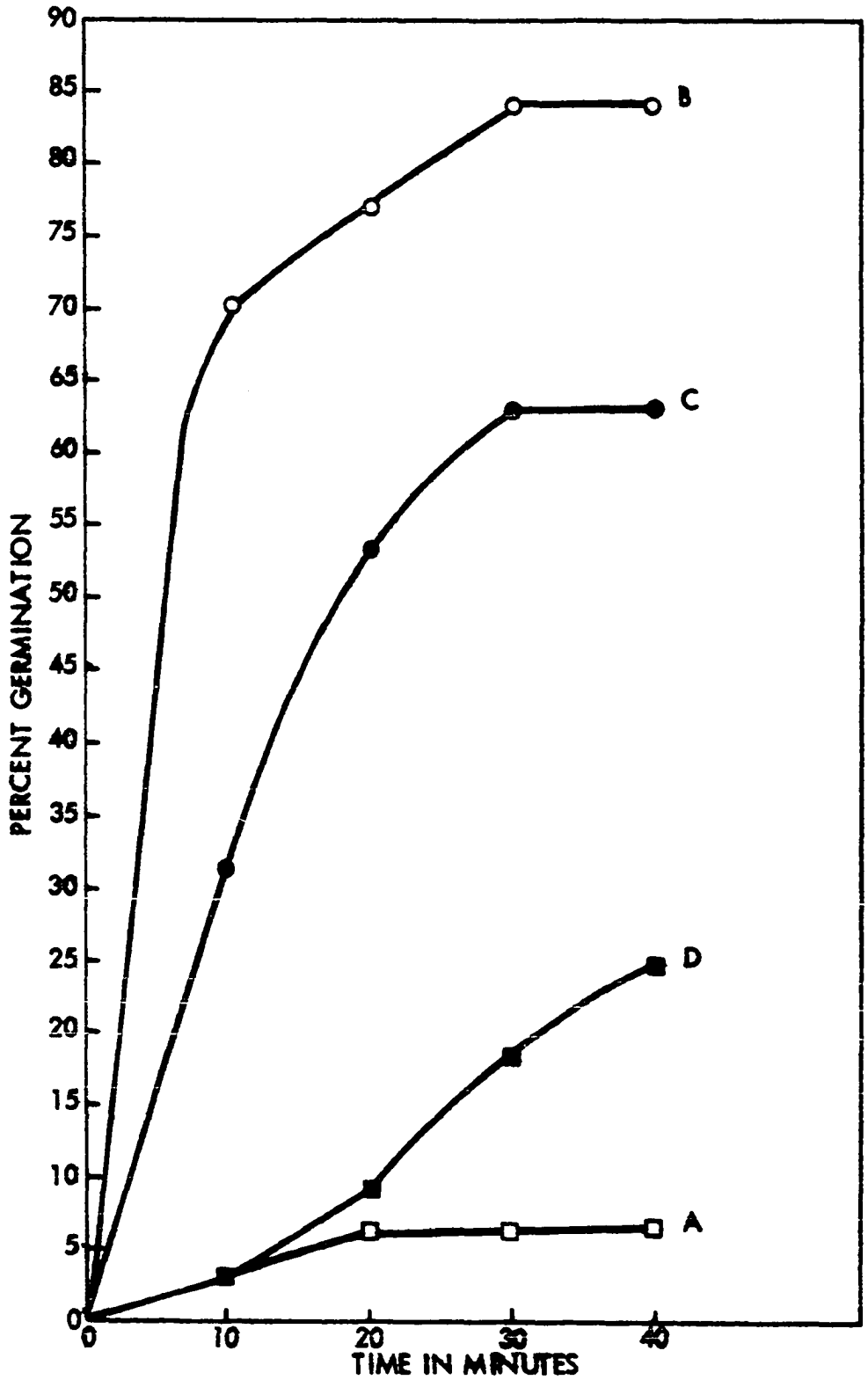
c. The effect of carbonate and carbon dioxide on germination of C. perfringens in the presence of yeast extract Bicarbonate and carbon dioxide (Wynne and Foster, 1948a,b; Treadwell et al., 1958; Roberts and Hobbs, 1968), lactate (Gibbs, 1964), and thioglycolate (Riemann, 1963a; Holland et al., 1969) have been found to increase germination of various clostridial spores including PA 3679 and C. botulinum strains.

The ability of these compounds to induce germination of C. perfringens spores when added individually to yeast extract medium was tested. The results of this experiment are shown in Figure 7. A marked increase in germination took place when 0.3% sodium bicarbonate was added to 1.0% yeast extract. A distinct stimulation of germination was also apparent when carbon dioxide was bubbled in the yeast extract medium at a vigorous rate. The stimulation of germination due to carbon dioxide was less marked than that by sodium bicarbonate. This might have been caused by some loss of carbon dioxide and the passage of oxygen into the medium during the time of inoculation.

The pH value fell considerably below pH 6.0 by passing carbon dioxide into the medium. This was avoided by adjusting the initial pH of the medium to 6.5 instead of pH 6.0 and subsequent passing of carbon dioxide for four to five min brought the pH of the medium to pH 6.0 ($\pm$ 0.1).

Figure 7. Effect of sodium bicarbonate, carbon dioxide, and sodium lactate on the germination of suspensions of spores of C. perfringens in the presence of yeast extract

A: 1.0% yeast extract
B: 1.0% yeast extract + 0.3% sodium bicarbonate
C: 1.0% yeast extract + carbon dioxide
D: 1.0% yeast extract + 0.1% sodium lactate


90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
PERCENT GERMINATION
0
10
20
30
40
TIME IN MINUTES
B
C
D
A

Sodium lactate also produced significant stimulation of germination in the presence of yeast extract but the stimulation was not as marked as in the case of carbonate and carbon dioxide. Increasing concentrations of lactate did not produce any additional stimulation.

Thioglycolate was without effect in the presence of yeast extract. No further stimulation of germination took place by glucose.

d. Effect of carbonate, carbon dioxide, lactate, and thioglycolate in the presence of vitamin-free casamino acids on germination of spores C. perfringens The ability of sodium bicarbonate, carbon dioxide, sodium lactate, and sodium thioglycolate to enhance germination of C. perfringens, when added separately to 2% vitamin-free casamino acids, was also tested. The results reported in Figure 8 indicate that all of the compounds listed above exhibited a marked increase in both the rate and extent of germination. The enhancing effect produced by these compounds on germination in the presence of casamino acids was of the same degree, whereas different degrees of stimulation were shown by these compounds in the presence of yeast extract. Moreover, sodium thioglycolate was without effect in the presence of yeast extract. The reason for this effect is not known. The addition of glucose made no difference.

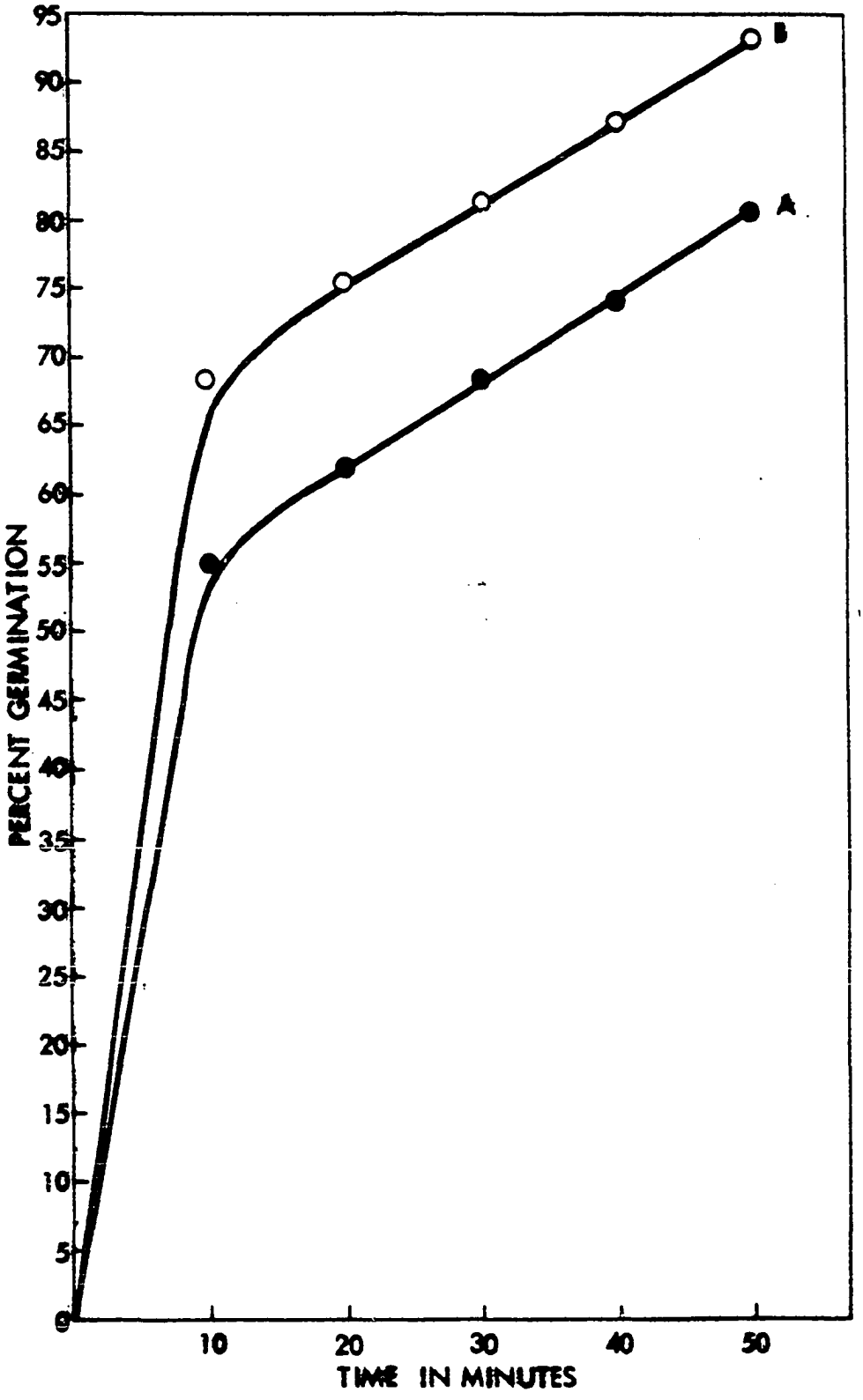
Carbonate, carbon dioxide, lactate, and thioglycolate caused little or no germination in the absence of yeast extract or casamino acids. This suggests that an unknown factor present in yeast extract and casamino acids is required to augment the germination of spores of C. perfringens by these compounds.

The role played by carbonate, carbon dioxide, and lactate during germination is obscure. Similarly the mechanism by which thioglycolate induced enhanced germination in the presence of casamino acids is unknown.

Figure 8. Effect of sodium bicarbonate, carbon dioxide, sodium lactate, and sodium thioglycolate on the germination of suspensions of spores of C. perfringens in the presence of vitamin-free casamino acids

A: 2.0% vitamin-free casamino acids
B: 2.0% vitamin-free casamino acids plus selected individual supplements:

a) 0.1% sodium bicarbonate
b) carbon dioxide which was passed in the medium as mentioned before
c) 0.1% sodium lactate
d) 0.05% sodium thioglycolate


95
90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
PERCENT GERMINATION
B
A
10
20
30
40
50
TIME IN MINUTES

However, thioglycolate is a reducing agent and it is also a fairly good chelating agent (Chaberek and Martell, 1959) and it might be by virtue of these properties that it induced germination.

e. Effect of ions plus yeast extract on germination of spores of C. perfringens Considerable data are available on the necessity and usual nonspecificity of ions in different germinative systems (Hyatt and Levinson, 1961; Rode and Foster, 1962a; Duncan and Foster, 1968b; Labbe and Duncan, 1970). Thus, in addition to the effect of "physiological germinants" such as amino acids, and glucose reported previously, ions such as sodium nitrite, sodium chloride, ammonium chloride, and sodium nitrate were also used in the presence of yeast extract to study their effects on the germination of C. perfringens. The results are given in Figure 9.

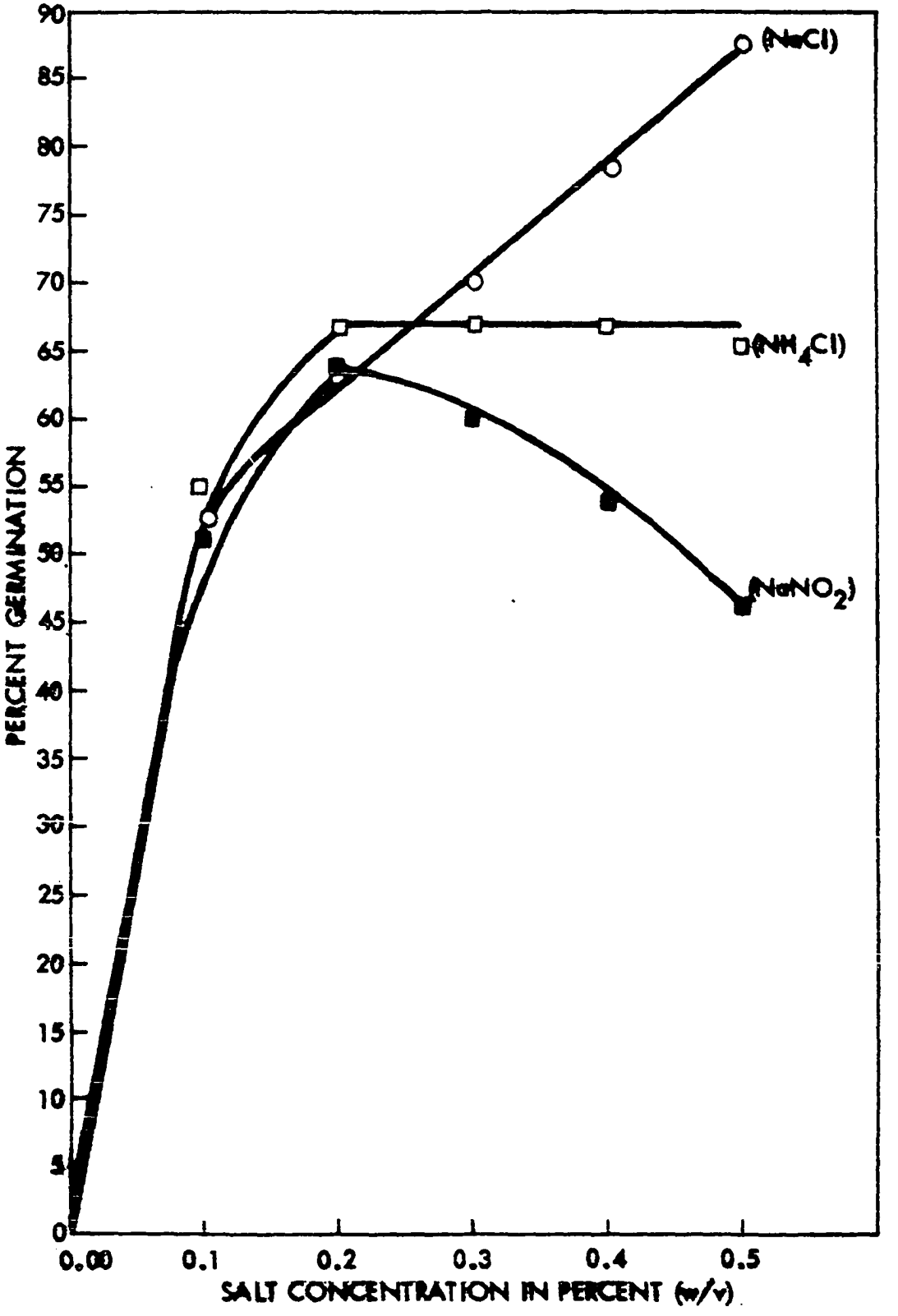
Sodium nitrite was used in concentrations from 0.01 to 1.0%. It was found that increasing concentrations from 0.1% to 0.2% accelerated germination. At higher concentrations, germination decreased.

Nitrite induced germination was also observed with C. butyricum and C. tyrobutyricum (Bester et al., 1968), PA 3679h (Duncan and Foster, 1968b), and C. perfringens (Labbe and Duncan, 1970). Our findings confirm its occurrence with another strain of C. perfringens.

Sodium chloride was tested in concentrations from 0.01 to 5%. The rate and extent of germination was increased with increasing concentrations of the salt from 0.1 to 0.5% (Figure 9), and then no further stimulation of germination by the higher concentrations from 0.6 to 3.0% of the salt. Beyond this point the stimulating effect of the salt was reduced with increase in concentration until the effect was absent at 8.0% sodium chloride.

Ammonium chloride also enhanced germination of spores of C. perfringens

Figure 9. Effect of varying concentrations of sodium chloride, ammonium chloride, and sodium nitrite on germination of spores of C. perfringens when added to 1% yeast extract medium. (Measurements taken over a period of 40 min) Salts were added to give the final concentrations shown. Germination induced by yeast extract alone was about 7%


90
85
80
75
70
65
60
55
50
45
40
35
30
25
20
15
10
5
0
PERCENT GERMINATION
(NaCl)
□(NH4Cl)
(NaNO2)
0.00
0.1
0.2
0.3
0.4
0.5
SALT CONCENTRATION IN PERCENT (w/v)

in the presence of yeast extract (Figure 9). Optimal concentration for the maximum effect was found to be 0.2%. No further enhancement of germination was observed with increasing concentrations of the salt from 0.3 to 0.5%.

Sodium nitrate, when added to yeast extract in various concentrations, exhibited no stimulatory effect towards germination of C. perfringens spores. Similar results were obtained by Duncan and Foster (1968b) with PA 3679h.

Optimum concentrations of sodium chloride (0.5%) and sodium nitrite (0.2%) when added to yeast extract produced no additive enhancing effects for germination. Salts in themselves alone or in combinations, caused little or no germination. It was concluded that some unknown factor present in yeast extract and ions combine to enhance germination by C. perfringens spores.

Various hypotheses have been put forth to explain the mechanism by which ions stimulate germination of spores. Foerster and Foster (1966) concluded that the major event in germination was ionic. Rode and Foster (1962a) suggested that ions could affect some structure protective to the core which depended on associated ions for its stability, the most likely candidate for this role being the electronegative cortex murein. Rode and Foster (1965) further suggested that a number of germinants, viz., inorganic and organic ions, chelating agents, surfactants, and hydrogen ions, induce germination by altering the conformational structures of proteins and perhaps macromolecules responsible for the dormant spore state.

As reported before, Riemann (1961) had proposed a Spore structure-Ca-DPA or Enzyme-Ca-DPA complex to be associated with dormancy in spores. Riemann's suggestion on the mechanism of germination by disruption of this

complex by various compounds was extended by Fleming and Ordal (1964) who showed that increased ionic strength of mono and multivalent ions could favor dissociation of the chelated structure.

Germination by nitrite is probably effected by reduction with nitrite serving an electron donor. Duncan and Foster (1968b) suggested that possibly nitrite causes alteration of the tertiary structure of a spore protein, which, in turn, may be involved in the Ca-DPA complex. The increased germination rate with increasing temperature and increasing nitrite concentration observed by Duncan and Foster may be a reflection of such structural alteration.

Alternatively, germination may involve the breaking of a permeability barrier to ions, or to involve action of ion-dependent enzymes. For instance, Gould et al. (1966) reported that the germinative action of B. cereus spore lytic enzyme required about 100 mM sodium chloride and relatively nonspecific ion requirements for activity of diverse enzymes are not uncommon. Hermier and Rousseau (1967) pointed out that certain amino acids caused only partial phase darkening of B. subtilis SJ2 spores unless potassium phosphate was present. They suggested that action of amino acids dehydrogenases was necessary only during the lag phase, and that the ions were necessary for the subsequent refractility change characteristic of germination.

On the other hand, Duncan and Foster (1968b) suggested that in view of the variety of ions reported to be effective in germination, any role as an enzyme activator would seem to be non-specific in nature. Moreover, the concentration of ions involved in germination is normally higher than that usually considered necessary for enzyme activation.

f. Specific stimulants of germination of spores of C. perfringens

Since amino acids cystine, cystein, tryptophane, and tyrosine had shown increased germination of spores of C. perfringens when added individually to yeast extract-glucose medium, and certain inorganic ions had also caused enhanced germination both in the presence of yeast extract or vitamin-free casamino acids, attempts were made to determine specific amino acid and/or ionic requirements for induction of complete germination of spores of C. perfringens.

All four amino acids mentioned above were tested individually and in different combinations in the presence or absence of glucose. None of the amino acids individually or in any of the different combinations tested induced germination in the absence of glucose. Whereas, different degrees of stimulation of germination were observed when these amino acids were tested individually or in combination in the presence of glucose. The results are shown in Table 3. Cystine alone in the presence of glucose induced germination which was of a higher degree than was induced by any other amino acids individually or in combination in the presence of glucose. Tyrosine was found to inhibit the germination by cystine-glucose mixture or by cystine-cystein-glucose mixture. These results emphasize the specific need of cystine-glucose mixture for the stimulation of germination of spores of C. perfringens.

Further improvement in the capacity of cystine-glucose to induce complete germination was sought by the addition of sodium bicarbonate, sodium nitrite and sodium chloride individually to the above medium. The results which are also shown in Table 3 indicate that 0.25% carbonate enhanced germination in the presence of glucose-cystine and carbonate was

Table 3. Specific stimulants of germination of spores of C. perfringens. Germination was carried out under the previously specified conditions

Media[a]	Percent germination
L-cystine + glucose	53
L-tryptophane + glucose	25
L-cystein + glucose	17
L-tyrosine + glucose	0
L-cystine + L-tryptophane + L-cystein + L-tyrosine + glucose	49
L-cystine + L-tryptophane + L-cystein + glucose	50
L-cystine + L-cystein + L-tyrosine + glucose	0
L-cystine + L-tyrosine + glucose	0
L-cystine + glucose + sodium nitrite	51
L-cystine + sodium bicarbonate[b]	45
L-cystine + sodium bicarbonate[b] + glucose	52
L-cystine + 0.25% sodium bicarbonate[c]	60
L-cystine + 0.25% sodium bicarbonate[c] + glucose	65
L-cystine + sodium chloride[d] + 0.5% glucose	82
L-cystine + sodium chloride[d]	82

[a]0.5 mg/ml cystine, 1.0 mg/ml tryptophane, 1.0 mg/ml cystein, 0.4 mg/ml tyrosine, and 0.5% glucose.

[b]0.1% sodium bicarbonate.

[c]0.25% sodium bicarbonate.

[d]0.5% sodium chloride.

also found to replace the requirements of glucose to a large extent. Nitrite did not enhance germination when added to glucose-cystine mixture. Sodium chloride enhanced the capacity of glucose-cystine mixture or cystine considerably. Germination in the presence of glucose-cystine-sodium chloride or cystine-sodium chloride was of the same level as was obtained by 2.0% vitamin-free casamino acids or by broth medium 1. Thus it was concluded that cystine and sodium chloride constitute the specific requirements for the germination of spores of C. perfringens.

C. Inhibition of Germination of Spores of C. Perfringens

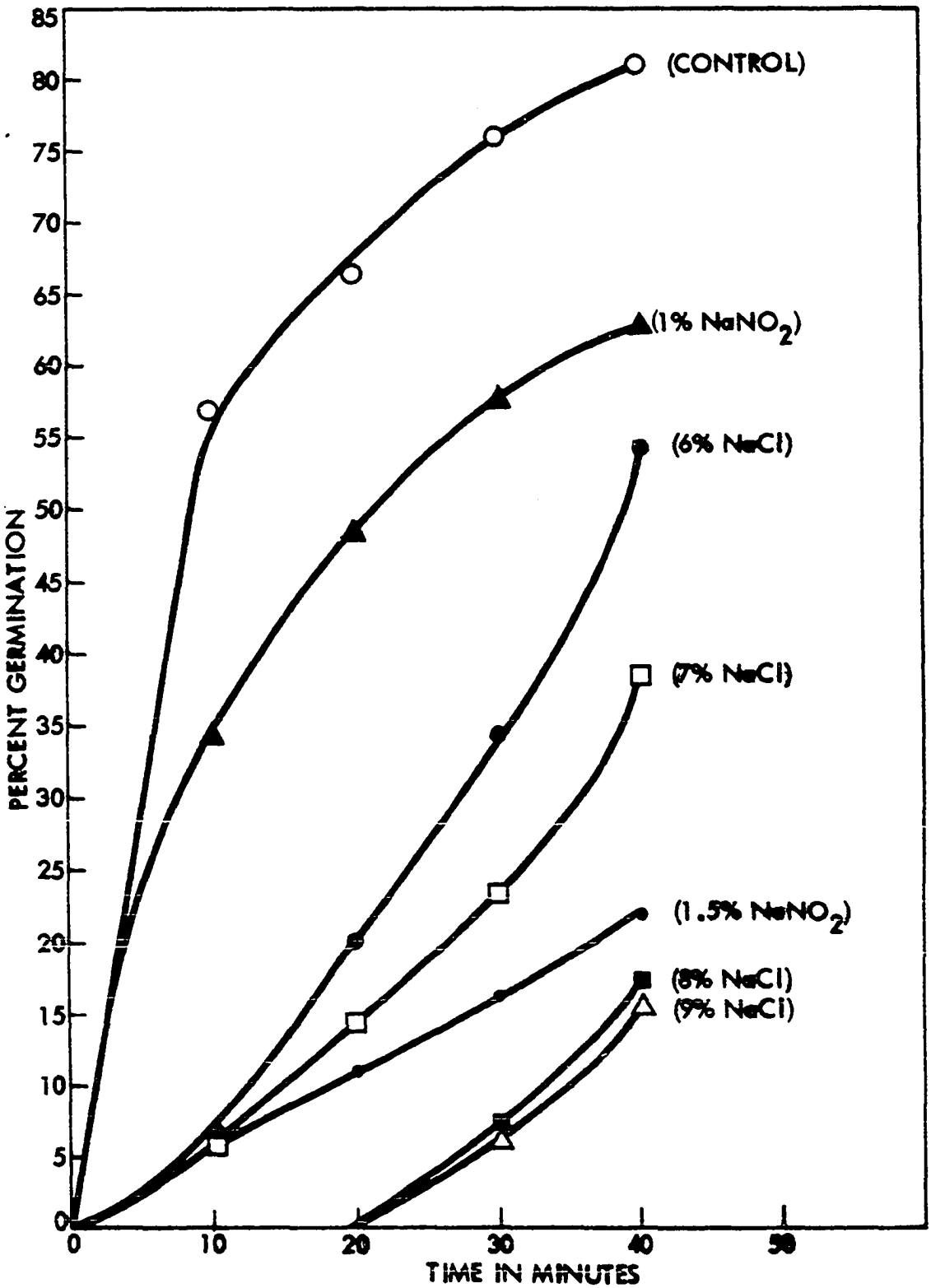
1. Inhibition of germination by sodium nitrite and sodium chloride

Sodium nitrite and sodium chloride in higher concentrations than used before were individually added to medium 1 to observe the possible inhibition of germination of spores of C. perfringens by these salts. This medium, as reported previously, allowed rapid germination of the spores of C. perfringens.

Sodium nitrite was used in concentrations from 1.0 to 5.0%. It was found (Figure 10) that germination decreased with increase in concentration from 1.0 to 1.5% and was completely inhibited at 2.0% sodium nitrite.

Gould noted that germination of several Bacillus species in yeast extract-glucose agar containing 0.075 to 0.25% nitrite was prevented altogether. Duncan and Foster, on the other hand, failed to observe inhibition of germination of PA 3679h in the presence of as much as 4.0% nitrite and Labbe and Duncan observed no inhibition of C. perfringens strain

Figure 10. Inhibition of germination of spores of C. perfringens by varying concentrations of sodium nitrite and sodium chloride when added individually to medium 1 (control). The salts were added to the medium to give final concentrations shown


PERCENT GERMINATION
TIME IN MINUTES
(CONTROL)
(1% NaNO2)
(6% NaCl)
(7% NaCl)
(1.5% NaNO2)
(8% NaCl)
(9% NaCl)

FDl when 2.0% nitrite was added to complex medium. Our results are in conflict with those of Gould, Duncan and Foster, and Labbe and Duncan.

Sodium chloride was used in concentrations ranging from 4.0 to 10%. As is shown in Figure 10, germination in medium 1 in the presence of salt concentrations from 4.0 to 9.0 was progressively reduced and was finally prevented at 10.0% salt concentration. Gould reported that up to 8.0% sodium chloride did not prevent germination of spores of any of the organisms studied. Concentrations of 10.0 to 15.0% salt progressively reduced and finally prevented germination. Comparable results were obtained by Duncan and Foster.

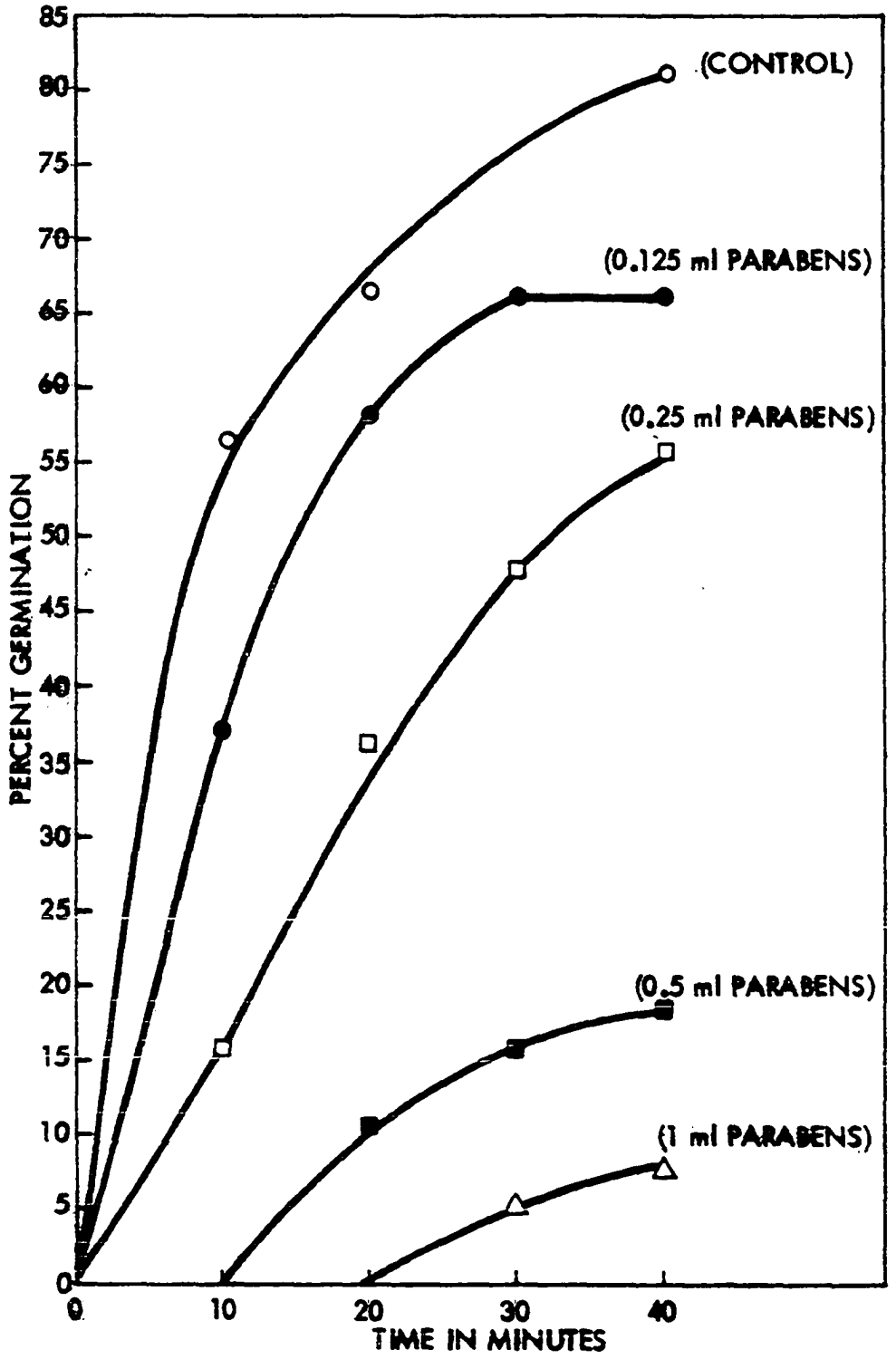
By contrast, sodium chloride levels as high as 10.0% inhibited germination of spores of C. perfringens in this study.

The various effects of sodium nitrite and sodium chloride on germination are probably due primarily to species difference and the different experimental conditions employed by these workers.

2. Inhibition of germination by parabens

Parabens, the 2:1 mixture (v/v) of the methyl and propyl esters of p-hydroxybenzoic acid containing 1.0% (w/v) of the methyl ester and 0.5% (w/v) of propyl ester were prepared. Various volumes of the mixture ranging from 0.125 ml to 2.0 ml were added to germination medium 1 to study the inhibition of germination of spores of C. perfringens. Germination was progressively decreased with increasing concentrations of parabens (Figure 11) till it was prevented by 1.0 ml of the mixture containing 0.066% of the methyl ester and 0.033% of the propyl ester in final concentration. Inhibition of germination by parabens was also observed by Parker (1969) who

Figure 11. Inhibition of germination of spores of C. perfringens by varying concentrations of parabens when added to medium 1 (control). 1.0 ml of the mixture of parabens contains in final concentration 0.066 of the methyl ester and 0.33% of the propyl ester


(CONTROL)
(0.125 ml PARABENS)
(0.25 ml PARABENS)
(0.5 ml PARABENS)
(1 ml PARABENS)
PERCENT GERMINATION
TIME IN MINUTES
0
5
10
15
20
25
30
35
40
45
50
55
60
65
70
75
80
85
0
10
20
30
40

reported that the inhibitory effect was readily reversed by aqueous washing. In an attempt to explain the role played by parabens in inhibition, Parker suggested that preservatives may antagonize lytic enzymes (Sierra, 1964, 1967; Gould and Hitchins, 1965) which are thought to be involved in germination. Slepecky (1963) studied the inhibition of sporulation and germination of B. megaterium by phenylethyl alcohol and suggested that the formation of new RNA during early stages of germination was prevented.

3. Inhibition of germination by antibiotics

Antibiotics nisin, subtilin, and tylosin were used to observe their effects on the process of germination of spores of C. perfringens. Concentrations as high as 130 ug/ml of nisin, 150 ug/ml of subtilin, and 400 ug/ml of tylosin failed to inhibit germination. On the basis of observations of others, as well as of his own, Gould (1964) concluded that these antibiotics were active at the outgrowth stage of development and did not prevent germination at lower concentrations.

4. Inhibition of outgrowth

The effect of sodium nitrite, sodium chloride, parabens, and antibiotics was tested on the outgrowth of C. perfringens. Sodium nitrite concentrations at levels of 0.03% inhibited outgrowth. No development of vegetative cells took place. Similar results were obtained by Gould (1964) who reported that spores of several Bacillus species germinated in less than 0.03% nitrite at pH 6.0 but development was arrested immediately after germination.

On the contrary, nitrite concentrations up to 0.06% at pH 6.0 allowed

emergence and elongation of vegetative cells from spores of PA 3679h but cell division was blocked (Duncan and Foster, 1968a). Labbe and Duncan (1970) reported that outgrowth of heat injured spores of C. perfringens strain FD1 was prevented by 0.02% of sodium nitrite whereas the outgrowth of similarly treated strain NCTC 8798 was prevented by 0.01% sodium nitrite.

Sodium chloride concentration as high as 5.0% allowed germination but outgrowth was completely inhibited at this concentration. Duncan and Foster reported that most of the spores of PA 3679h germinated in the presence of 3 to 6% sodium chloride and produced vegetative cells but cell division was blocked. Similar observations were made by Gould (1964).

Parabens, containing 0.016% (w/v) of methyl ester and 0.008% (w/v) of propyl ester of p-hydroxybenzoic acid slightly inhibited germination of spores of C. perfringens but the germinated spores were inhibited from further growth. On the other hand, Parker (1969) has observed that spores of B. subtilis emerged as vegetative cells in the presence of 0.04% of methyl ester and 0.02% of propyl ester of p-hydroxybenzoic acid.

Antibiotic concentrations of 1.6 ug/ml of nisin, 0.15 ug/ml of subtilin and 2.0 ug/ml of tylosin inhibited outgrowth of spores of C. perfringens. Gould also studied the effect of lower concentrations of these antibiotics on the outgrowth of spores of several Bacillus species. He found that concentrations of 0.25 to 2.5 ug/ml of nisin, 5 to 20 ug/ml of subtilin and 0.5 to 1 ug/ml of tylosin inhibited the growth of sensitive and resistant spores respectively.

As is seen above, lower concentrations of inhibitors are required to inhibit the outgrowth than the germination step. Parker pointed out that in the transformation from the spore to the vegetative cell there is a

progressive unmasking of sites vulnerable to preservative activity. The early stages involve the rapid degradative changes of germination which are not as susceptible to chemical inhibition as is the outgrowth which being dependent of biosynthesis of new cellular components, represent more metabolically sensitive sites.

Our findings can be used in relation to the control of C. perfringens in foods. Foods involved in food poisoning outbreaks by C. perfringens include various kinds of meats and dishes prepared from them. These foods may not support sporulation of C. perfringens, as Kim et al. (1967) obtained no sporulation of C. perfringens in different foods including meats.

The results obtained on the germination of C. perfringens indicate that nitrogen sources such as casamino acids containing amino acids and minerals supported rapid germination. It was also found that germination was stimulated by sodium nitrite and sodium chloride when added individually or in combination to yeast extract. It is assumed that if spores of C. perfringens are introduced into foods containing germination stimulants such as sodium nitrite and/or sodium chloride, they may undergo germination and subsequent mild heat treatment will kill them. For example, the canned luncheon meats are remarkably stable. They contain the usual curing ingredients (sodium nitrite, sodium nitrate, and sodium chloride) and they are heat processed after canning. The heat treatment is, however, far below that required to effect sterilization; and canning ingredients by themselves are not sufficient to prevent spoilage of the meats (Gough and Alford, 1965; Gross et al., 1946; Tanner and Evans, 1943).

Canned cured meats products usually contain 4.0 to 6.0% sodium chloride and 0.2% sodium nitrite along with other ingredients. Our

findings indicate that sodium nitrite and sodium chloride at greater than commercially acceptable concentrations do not prevent initiation of germination of spores of C. perfringens. Relatively lower levels of these salts can stimulate germination in a poor germination medium such as yeast extract and far lower concentrations of sodium nitrite (0.3%) and relatively high concentrations of sodium chloride (5.0%) blocked outgrowth of the germinated spores in a complex germination medium. These germinated spores will be susceptible to thermal inactivation. Spores that have not germinated and survived the heat treatment may germinate and their outgrowth may be inhibited by the residual salts.

Our results may help to explain the importance of sodium nitrite and sodium chloride in the preservation of canned meat products, and they also indicate that salt concentrations used in practice may contribute significantly to the control of C. perfringens in canned cured products.

Riemann (1963b) pointed out that the stability and safety of canned cured meats depend upon the number of cells in meats. In the present studies, concentrations of 1.5×10^8 spores per ml were used; this is much greater than the actual spore load in meats. Therefore, concentrations of sodium nitrite and sodium chloride lower than those used here may have been equally effective against germination and outgrowth had we employed a smaller number of spores.

Methyl ester and propyl ester of p-hydroxbenzoic acid are allowed in foods up to 0.1% concentration. Mixed esters in 2:1 ratio containing 0.066% (w/v) of the methyl ester and 0.033% (w/v) of propyl ester of p-hydroxybenzoic acid inhibited germination and outgrowth was inhibited by 1/4th concentration. Thus, these preservatives can also control the germination and outgrowth when added to foods in legal limits.

V. SUMMARY

This research was designed to develop a suitable medium for producing larger numbers of spores of C. perfringens with enhanced heat resistance, to study the optimal conditions and the minimal nutritional requirements for their rapid germination, and to study inhibition of germination.

A medium, designated as 'AW' medium was developed for sporulation of five strains of C. perfringens. These included strains S-45, 214d, ATCC 3624, HR_2 and 65. Actively growing vegetative inocula were prepared in Fluid Thioglycolate Medium using a modified technique adapted from Groom and Strong (1966). 'AW' medium was inoculated with 10% (by volume) of the vegetative cells and incubated at 37°C for 16 hr. Spore suspensions were heat treated for 20 min at 75°C to kill the vegetative cells. The spores were enumerated on SPS agar in film pouches.

A level of 10^9 to 10^{10} spores per ml of medium in strains S-45, 214d, and ATCC 3624 and 10^8 per ml in strains HR_2 and 65 was produced. The latter two strains were considered as representative of C. perfringens strains which sporulated poorly.

'AW' medium was compared for spore yields with Ellner's medium, medium of Kim et al. and Duncan and Strong medium. The results indicated that all five strains of C. perfringens tested produced larger numbers of spores in 'AW' medium than were produced in any of the other three media under comparable conditions.

Although 'AW' medium supported consistent production of large numbers of spores of C. perfringens, none of the strains tested at 90°C had an appreciable degree of heat resistance. The D values at this temperature

ranged from 0.9 to 2.85 min.

Spores of strain S-45 of C. perfringens were used to determine the optimal environmental factors and minimal nutritional requirements for their rapid germination. Germination was measured employing decrease in OD, standardized against loss of heat stability of spores upon germination, as the ultimate criterion of germination. The factors for optimal germination were found to be (1) heat-activation at 75°C for 20 min, (2) pH 6.0, (3) temperature of incubation at 30°C and (4) anaerobic or partial anaerobic atmosphere.

Out of the various complex nitrogen substances tested for their ability to induce rapid germination, only casamino acids were found to be good germinants. Vitamin-free casamino acids were better source than casamino acids. Eighty-four percent germination took place in 2.0% vitamin-free casamino acids; germination was increased when sodium bicarbonate, carbon dioxide, sodium lactate, and sodium thioglycolate were individually added to 2.0% vitamin-free casamino acids.

Only 7% germination occurred in the presence of yeast extract which was increased to about 14% when glucose was added to yeast extract. Out of the 24 amino acids individually tested for their ability to enhance germination in the presence of yeast extract and glucose, only four amino acids, namely cystine, cystein, tyrosine, and tryptophane enhanced germination. The extent of germination in the presence of yeast extract, glucose and any one of the four amino acids ranged from 48 to 94%, with yeast extract, glucose, and cystine giving germination of the highest level. Germination was reduced considerably in the absence of glucose.

Different degrees of stimulation of germination were also observed when

sodium bicarbonate, carbon dioxide, sodium lactate, ammonium chloride, sodium nitrite, and sodium chloride were individually added to yeast extract. The extent of germination under the above conditions ranged from 25 to 88% with sodium bicarbonate and sodium chloride stimulating the germination to the highest level. Glucose did not stimulate germination here.

Further studies indicated that a mixture of cystine and sodium chloride caused 82% germination. These two simple ingredients seem to constitute the specific requirements for the germination of spores of C. perfringens.

A complex broth medium which produced 82% germination and also supported growth of C. perfringens, was used to study inhibition of germination by different compounds. Two percent sodium nitrite, 10.0% sodium chloride, parabens at a final concentration of 0.066% (w/v) of methyl ester and 0.033% (w/v) of propyl ester of p-hydroxybenzoic acid inhibited the germination, whereas, 130 ug, 150 ug, and 400 ug per ml of antibiotics nisin, subtilin, and tylosin respectively, did not inhibit germination, higher concentrations than mentioned above were not used because of their impracticality.

Outgrowth of the germinated spores was blocked by 0.03% of sodium nitrite, 5.0% sodium chloride, 0.25 ml parabens, 1.6 ug per ml of nisin, 0.15 ug per ml of subtilin, and 2 ug per ml of tylosin in complex broth medium. The implications of these findings in the control of C. perfringens in foods have been discussed.

VI. CONCLUSIONS

The following conclusions can be made with regard to sporulation, germination, and inhibition of germination of spores of C. perfringens:

1. Sporulation of C. perfringens is enhanced by:

   a. controlling the pH of 'AW' medium within a range of an initial pH of 7.8 to a minimum pH of 6.0 with 0.1M K_2HPO_4;

   b. addition of yeast extract and tryptone;

   c. probable removal or adsorbance of antisporulation factors by addition of soluble starch and bovine serum albumin to the medium; and

   d. aeration of cultures after 12 hr of growth in 'AW' medium.

2. Sporulation is completely inhibited when soluble starch is replaced with 0.5% glucose in 'AW' medium.

3. The degree of sporulation varies with different strains of C. perfringens.

4. Spores of C. perfringens exhibited maximal germination:

   a. after heat-activation at 75°C for 20 min;

   b. at pH 6.0;

   c. at a temperature of incubation of 30°C; and

   d. under anaerobic or partial anaerobic conditions.

5. More extensive germination of spores of C. perfringens occurs in the presence of 2.0% vitamin-free casamino acids than in the presence of yeast extract, trypticase or casamino acids with vitamins.

6. Germination approaches completion when sodium lactate, sodium thioglycolate, sodium bicarbonate are individually added to 2.0% vitamin-free casamino acids or carbon dioxide is bubbled into this medium for 4 min.

7. Out of 24 amino acids tested, only L-tryptophane, L-tyrosine, L-cystein, and L-cystine increases germination when added individually to yeast extract-glucose mixture.

8. Germination increases upon addition of sodium lactate, sodium bicarbonate, or carbon dioxide (bubbled for 4 min) to 1.0% yeast extract. No stimulation of germination takes place when sodium thioglycolate is added to 1.0% yeast extract.

9. Germination in 1.0% yeast extract is increased when sodium nitrite, ammonium chloride, and sodium chloride are added to it.

10. Cystine plus sodium chloride induced germination equal to that obtained with 2.0% vitamin-free casamino acids.

11. Two percent sodium nitrite, 10.0% sodium chloride, and parabens at a level of 0.066% (w/v) of methyl ester and 0.033% (w/v) of propyl ester of p-hydroxybenzoic acid completely inhibit germination of spores of C. perfringens; whereas, the antibiotics, nisin, subtilin, and tylosin at levels of 130, 150, and 400 µg per ml respectively do not block initiation of germination.

12. Outgrowth of germinated spores is inhibited by 0.03% sodium nitrite, 5.0% sodium chloride, and parabens at level of 0.016% (w/v) of methyl ester and 0.008% (w/v) of propyl ester of p-hydroxybenzoic acid.

13. The antibiotics, nisin, subtilin, and tylosin inhibit outgrowth at a level of 1.6, 0.15, and 2.0 µg per ml, respectively.

VII. LITERATURE CITED

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