STUDIES ON THE SPORULATION AND GERMINATION OF PUTREFACTIVE ANAEROBE 3679 (PA 3679)

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Syed Mohammad Shamsuz-Zoha

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STUDIES ON THE SPORULATION AND GERMINATION OF PUTREFACTIVE ANAEROBE 3679 (PA 3679)

by

Syed Mohammad Shamsuz-Zoha

An Abstract of

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Submitted to the School of Advanced Graduate Studies of
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ABSTRACT

A study of the sporulation and germination of spores of a Putrefactive Anaerobe has been initiated. This strain is designated as PA 3679 by the National Canners Association and is used as a test organism in safe processing of medium acid canned foods.

PA 3679 does not sporulate rapidly in usual laboratory media. In addition these infusion media contain particles of proteinaceous material which are difficult to separate from spores.

An aparticulate medium and a technique has been developed for the rapid production of clean spores of PA 3679. The sporulation medium contained trypticase, 1.5%; peptone, 1%; glucose, 0.2%; sodium chloride, 0.5% and dipotassium phosphate, 0.25%. Synchronization and stirring of the cultures had a pronounced effect on the sporulation and complete sporulation was obtained in 44 hours. After sporulation, the cultures were aerated briefly which resulted in the freeing of the spores from the sporangia and caused lysis of the few remaining vegetative cells. The spores were harvested and washed by centrifugation and could be stored at 4°C. in water or phosphate buffer without germinating.

The thermal properties of the spores obtained by the above procedure were compared to those prepared from brain heart infusion. It was observed that PA 3679 spores which had been freed of growth medium and vegetative cells have Z values of 17.1-17.9 and D values of 0.83-0.95, regardless of the medium in which they have been produced.

Spores of PA 3679 germinate in 10-15 minutes in 4% yeast extract solution. Yeast extract could be partially replaced by alanine, adenosine, glucose and phosphate solution, in which complete germination was observed in 45-55 minutes.

Attempts were made to characterize the germination accelerating factor present in yeast extract. The component is dialysable, heat stable and stable toward mild hydrolysis with N/10 acid or base, but loses its activity when hydrolyzed with 4N H_2SO_4.

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INTRODUCTION

A bacterial endospore is considered as "a structure that is a veritable fortress against most of the detrimental effects of the environment."[1] These endospores are resting bodies that are formed within the bacterial cell. Their reproductive role is limited since each sporulating bacterial cell is capable of forming only a single spore, and this in turn gives rise to a single vegetative cell. The spore's biological role is not clearly understood and at the present stage of inconclusive studies, it appears to serve a protective function. However the spores' peculiar nature and particularly their great tolerance to extremely unfavorable conditions have afforded grounds for speculation and experimentation as to their mode of formation and their germination processes. Spores have drawn the attention of microbiologists for more than seventy years, and have been of interest to investigators in all phases of bacteriology. As Lamana points out, "the endospore has had a major role in historical development of bacteriology, for only after their discovery and recognition as exceptionally resistant bodies did it become possible to devise crucial experiments that decisively disposed of theories of spontaneous generation."[2]

Structurally endospores appear as spheroidal, dense, refractile bodies and they contain nuclear material, carbohydrate, fat,

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[1] Oginsky, <u>Bacterial Physiology</u> (San Francisco: W.H. Freeman & Co., 1954), p. 365.

[2] Lamana and Mallette, <u>Basic Bacteriology</u> (Baltimore: Williams and Wilkins & Co., 1953), p. 169.

protein, and relatively large amounts of calcium and 2,6-dicarboxy pyridine.

With the exception of some species of Vibrio, Spirillium, and Sarcina, only two bacterial genera are known to form spores. These are Bacillus and Clostridium. Some of the spore forming bacteria are of much importance in pathogenic bacteriology because they produce diseases in humans and animals while others are of main concern in industrial processes. The spores' extreme resistance to all factors known to be lethal to vegetative cells of bacteria, e.g., heat, dessication and toxic chemicals, have posed a great problem in sterilization processes used in various food industries.

During the past decade a number of investigators have initiated basic studies into various phases of physiology of bacterial spores. Preceding this work most of the investigations of spores had been confined to an evaluation of their heat tolerance to supply data needed in the canning industry. These data were of empirical nature since the very nature of the heat resistance phenomenon is not clearly understood. This has led to the general realization that more basic information is needed regarding the nature of sporogenesis and the germination process of bacterial spores.

Many food microbiologists feel that the degree of heat resistance of spores is a function of the medium in which they are produced. There are early reports that infusion media produce spores of higher heat resistance than "clear" broths. These observations may have been due to the lack of correct methodology needed for the study of resistant properties of the spore.

Extraneous proteinaceous material present in spore suspensions that are used for heat tolerance studies may have an affect on the apparent heat resistance of spores. It is well known that the materials which lower the oxidation-reduction potential of a medium, enhance the recovery of heat or radiation damaged cells. Furthermore, in the biochemical studies of spores it is desirable to have them uncontaminated with non-spore material. With this view in sight, studies were undertaken to develop a process for the production of "clean spores" in a clear liquid medium which would facilitate the study of the germination process.

As mentioned before, limited studies appear in the literature with regard to the physiology of sporulation and germination of bacterial spores. Although there is general agreement with Knaysi's view that sporulation is a normal process in organisms which characteristically form spores, opinions seem to differ with regard to the fundamental nature of the process. Knaysi (1948) assumes that sporulation occurs in the members of family Bacillaceae when healthy cells face starvation. Foster and Heiligman (1949) have proposed that sporulation is a sequence of integrated biochemical reactions which are independent of vegetative growth. Schmidt (1950) believes that sporulation is a function both of environment and of "cellular factors" determining reaction to the environment. Wynne (1948), on the other hand, has drawn attention to an essentially correct concept, the "Behring hypothesis," which suggests that spore formation can be considered as an intermediate stage in normal cellular development. Spore development may be partially or completely inhibited by any physiological damage short of total prevention of growth.

The transition from the vegetative cell to the spore probably requires more stringent environmental conditions than comparable growth requirements for a given species. Many reagents which have no effect on growth, inhibit or stimulate sporulation of bacterial cells. Variations in pH, which may not have appreciable effects on vegetative multiplication, may stop sporulation. While the range of temperature for vegetative growth may vary from 20-40°C., temperature ranges for sporulation are generally much narrower. In addition, the sporulation temperature is usually lower than that for optimum growth.

During the process of germination, which may be described as the transition from heat stable spore to a heat labile form, there is an initiation of measurable metabolic activity in the spores. At no time during the germination is this activity comparable to that of a vegetative cell. From this it may be inferred that there is a fundamental difference between the process of spore germination and vegetative growth. In fact the findings described in the succeeding pages lead to the need for a visualization of some process by which the protein and enzymatic structure of the spore is transformed from the state of inactive and heat resisting protoplasm, to the enzymatically active protein of the vegetative cell. The purpose of the present studies was to develop a process for the production of clean spores which could be used in the study of physiology of germination. In addition a comparison was made between the thermal properties of the spores obtained from clear medium and those from infusion media.

LITERATURE REVIEW

General. Although spores may represent a stage in the normal development of certain bacteria, the production of a spore is controlled by the environment in which the cell is grown. Under certain conditions, it is possible to produce only vegetative cells, from members of those genera which will normally form spores. Collier (1956) has shown that by reducing the concentration of trypticase and phosphate in a complete medium, cultures of *Clostridium roseum* can be maintained in a vegetative stage. Similarly Greenberg (1954) observed that the omission of manganese from a semi-synthetic medium resulted in complete inhibition of sporulation of *Bacillus cereus* (*terminalis*). There are also reports in literature of the development of permanently sporeless strains by a process of mutation (Knaysi, 1938).

Despite the above examples, there is general agreement among the workers in this field that sporulation is a normal physiological process of cells of those organisms which characteristically form spores.

Sporulation. Studies in the last seventy years seem to indicate that the conditions required for sporulation may often be quite different from those considered optimal for growth, and the metabolic activity of vegetative cells may not be at their maximum at the time spores are produced.

The effect of physical and chemical factors on sporulation is difficult to interpret since the classical method of single fac-

tor determinants is inadequate. Too little is known of the effect of any factor on the sporulation process or of the interaction of two or more factors.

The methodology for the quantitative study of sporulation employs either microscopic counts of total cells and spores or viable counts of the number of heat stable spores. The first method is limited in its precision by sampling errors and by cell autolysis. Therefore, viable counts are more desirable.

The effect of temperature on sporulation. Cook (1932) Knaysi (1948) and Schmidt (1955), who have reviewed the earlier works of German authors, have reported that Cohn (1876) and Koch (1888) studied the effect of temperature on the sporulation of *Bacillus subtilis* and *Bacillus anthracis* respectively. Cohn (1876) reported the growth and sporulation of *B. subtilis* at 47-50°C. Koch (1888) showed that cultures of *B. anthracis* develop rapidly at 35°C. and that sporulation starts after less than 20 hours of growth. Schriber (1896) concluded from his studies on *B. anthracis*, *B. subtilis*, and *Bacillus tumesans* that the effect of temperature on sporulation is slight and is due to the extension of vegetative growth. Similarly in Migula's (1897) studies with *B. subtilis,* the most rapid sporulation occurred at temperatures of most rapid vegetative growth. On the other hand, spores are not formed at all temperatures that allow growth. More intensive and apparently exact studies on the effect of temperature on vegetative growth and spore formation were made by Holzmeuller (1909). He showed that germination took place within narrower intervals of temperature than did germination. From these studies as well as those of Knaysi (1945, 1946, 1948) it can be con-

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cluded that the optimum temperature for sporulation is close to that of vegetative cell growth, but the range is generally narrower. Similar results are reported by Esty and Meyer (1922) and Curran and Evans (1937, 1945). Later workers observed that a temperature slightly lower than optimum for vegetative growth was most favorable for the sporulation of anaerobes (Hitzman, 1954; Zoha, 1954; Collier, 1955; and Sugiyama, 1951).

The effect of pH on sporulation. The optimum pH for sporulation in aerobes has been observed to be in the range of pH 7.0-7.5 (Knaysi, 1945; Foster et al., 1948; and Curran, 1934). Spore formation is very sensitive to the development of acidity in the media. (Fitzjames, 1955) In case of the anaerobic spore formers, the optimum pH for sporulation was reported to be in the range of 6.9-7.9 (Wynne and Foster, 1948; Esty and Meyer, 1922). Schmidt (1952) observed that the pH for sporulation of Clostridium sporogenes is 7.7-7.9. A pH of 7.0-7.2 was observed optimum for sporulation in Cl. roseum, Clostridium botulinum, Clostridium tetani and Clostridium putrificum (Hitzman, 1954; Collier, 1955; Zoha, 1955; Mohrke, 1926). The optimum pH for sporulation of Putrefactive Anaerobe 3679 (PA 3679) is reported to be 7.5-7.8 (Brown, 1956). The sporulation of anaerobic bacteria has not been reported below pH of 6.1.

The effect of surface tension on sporulation. The effect of surface tension on the sporulation of Cl. botulinum was studied by Wynne (1948). He showed that the lowering of surface tension by means of lauryl sulfate causes no significant depression of sporulation at tensions above 35 dynes/cm. At values lower than 35 dynes/cm, a logarithmic decrease in sporulation occurred. The nature of depres-

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sant however seemed more important than the actual surface tension. This is in contradiction to the observation made by Larson (1919) who showed marked depression of sporulation in *B. subtilis* at surface tension values of less than 45 dynes/cm. Wynne (1948) stated that Larson's results may have been due to a diminution of oxygen supply.

Effect of available moisture on sporulation. No direct relationship has been established between the availability of water and the process of sporulation since the effect of added salts varies. Migula (1897) has stated that moisture is necessary for sporulation. On the other hand, Holzmeuller (1909) observed that sporulation was hastened when material containing aerobic spore forming bacteria was allowed to dry on a cover glass. Other authors (Leifson, 1931; Williams and Purnell, 1953) have demonstrated that spore formation is more sensitive to conditions of low water availability than is vegetative growth.

Effect of visible light on sporulation. Holzmeuller (1909) found that diffuse light had no effect on sporulation of *Bacillus mycoides*, and similar results were reported by Schreiber for certain other bacilli. Schreiber (1896) claimed that sunlight exerted a deleterious effect on sporulation of *Bacillus* spp. Wynne (1948) did not find any significant difference in the extent of sporulation of *Cl. botulinum* in presence or absence of light.

Effect of oxygen availability on sporulation. Aerobic spore formers require oxygen for sporulation. However Knaysi (1948) believes that the absolute necessity of molecular oxygen for sporulation of *Bacillus* spp. has not been conclusively proven, even though earlier workers (Migula, 1897; Holzmeuller, 1909; Bayne-Jones, 1933) showed definite increases in sporulation in Bacillus spp. on aeration. The need for oxygen was also confirmed by the studies of Hardwick and Foster (1952) with washed cells of B. cereus. Roth et al. (1955) working with B. anthracis and Bacillus globigii, showed that 0.7-1.0 mMol. of O_2/L/min was required to complete the sporulation of 24 hour old culture, which had been initiated with a heat shocked spore inoculum. When cultures were inoculated with vegetative cells at their maximum growth levels (just prior to sporulation), only 0.1-0.2 mMol of O_2/L/min was required for complete sporulation.

Leifson (1931) showed that anaerobes exhibit considerable difference in their ability to sporulate under increased oxygen tension. The sporulation of Cl. tetani and Clostridium novii was inhibited at a partial pressure of oxygen equivalent to 1 cm of Hg, while Cl. sporogenes and Clostridium chauveri were inhibited at slightly higher levels. Somer (1930) has reported that broth cultures of Cl. botulinum, when exposed to air, sporulate more rapidly than control cultures. Traces of oxygen in the medium have been reported to be beneficial to spore formation in other anaerobes (Esty and Meyer, 1926). Wynne (1948) however observed no difference in the spore yields of Cl. botulinum in an atmosphere of air or natural gas. Collier (1956) has shown that oxygen will inhibit sporulation in Cl. roseum.

Effect of medium composition on sporulation. Since sporulation is considered as a normal phenomenon in the life processes of spore forming bacteria, the nutritional requirements for the growth and multiplication of vegetative forms must play a role in their metamorphosis to spore forms. Conflicting reports exist in the literature dealing with the effect of nutrient concentration of the growth medium on the rate and relative amount of sporulation that will occur in a given culture. Recent studies with organisms of the genus Bacillus show that the spore yield is increased when nitrogen and carbon sources are not in extreme excess (Knaysi, 1945). This has also been found true in case of Cl. botulinum (Wynne, 1948). Kaplan and Williams (1941) who studied Cl. sporogenes showed that an increase in the concentration of peptone beyond certain concentration levels inhibited sporulation.

Williams (1931) was unable to get satisfactory yields of spores with B. subtilis in several synthetic media. Recently some chemically defined media containing growth factors and amino acids have been described by various authors (Williams and Harper, 1949; Frank and Campbell, 1953). These media gave satisfactory yields of spores of B. cereus and other aerobes, as well as some anaerobes. In general, the extent of sporulation in dilute media is proportional to the concentration of the nutrients while in 'concentrated' media the accumulation of inhibitors like fatty acids reduce the number of spores (Hardwick and Foster, 1949).

Very few studies have been carried out regarding chemically identified nutritional factors required for sporulation. Foster and Heiligman (1949) have stated that nutritional factors favoring sporulation do not necessarily "stimulate" the onset of sporulation per se, but instead provide a metabolic shift leading to the transformation of vegetative cells to spores. Hardwick and Foster (1952) in studies of the sporulation process in B. cereus showed that sporogenesis could take place in the absence of exogenous nutrients.

They labeled the process as "endotrophic sporulation" and recognized that the exogenous nutrition of the sporulating cells had been completed prior to actual sporulation. Cells are literally "committed" to sporulation!

Stimulatory and inhibitory effects of some factors on sporulation are reported in literature. The recent work of Grelet (1950, 1952) suggests that a complex balance of suitable ions and energy source is required for sporulation; e.g., sporulation of B. megatherium occurs when concentrations of nitrate, sulphate and iron are at low levels, while calcium, sodium and chlorine have no effect. With low levels of glucose, potassium, magnesium, and manganese are required for sporulation. Leifson (1931) reported that phosphate and ammonium ions, and to some extent sulphate ions, increased spore yields in cultures of Cl. botulinum. Univalent cations such as sodium, potassium and lithium stimulate sporulation in Bacillus spp. (Fabian and Bryan, 1933). Foster and Heiligman (1949) found that the addition of potassium to an enzymatically hydrolyzed casein medium resulted in an increase of more than ten-fold in the spore yield of B. cereus, while the effect on vegetative growth was slight. In the case of Cl. roseum, ferrous iron stimulates spore formation (Hitzman, 1955), and ammonium ions and sulphur in various forms are necessary for spore formation in the National Canners' Association (NCA) strain of PA 3679 (Brown, 1956).

Few investigations of amino acid requirement for sporulation have been made. DL-alanine inhibits spore formation in a strain of B. cereus as reported by Foster and Heiligman (1949). These authors found that only leucine and isoleucine, of nineteen amino acids tested, were active in the reversal of the inhibitory effect of DL-alanine. The beneficial effect of leucine in the sporulation of B. cereus has been confirmed by Williams and Harper (1951). Blair (1950) reported that the omission of methionine from a synthetic medium suppressed spore formation in Cl. botulinum, but it should be noted that vegetative development was also lessened. Lysine was found to replace arginine for growth of Cl. botulinum, but no spores were noted. A somewhat similar relationship was found when an increased tyrosine concentration was used to fulfill a phenylalanine requirement.

Glucose, in low concentrations, is known to stimulate the sporulation of aerobic spore formers (Foster et al., 1949; Grelet, 1952). Oxalate seems to be required specifically for the formation of heat stable spores of B. megatharium (Powell, 1951).

Virtually no confirmed knowledge exists concerning the role of growth factors in sporulation. Hayward (1943) noted a slight beneficial effect of inositol for a strain of B. subtilis, and Williams and Harper (1951) have noted a similar effect with p-aminobenzoic acid with two strains of B. cereus. Mellon (1926) reported that sporulation in a species of B. cereus occurred earlier and more abundantly when a filtrate of a culture of symbiotic strain was added. Increased sporulation has been reported by other authors in mixed cultures (Powell et al., 1955). Schmidt (1952) suggested that there are factors present in medium in which cells have been grown which have an important effect on spore formation. Lund (1954) has used a "spent media" for the sporulation of a mutant strain of PA 3679.

Effect of antisporulation factors on sporulation. Knaysi (1948) believes that bacterial spores are formed when nutritive substances are depleted from growth medium. This hypothesis has been used to explain poor sporulation results in concentrated media.

However, recent investigations have indicated that the presence of antisporulation factors may account for poor spore crops. Baldwin and Roberts (1942) reported that peptone concentrations allowing growth but not sporulation gave good yields of spores following adsorption of the medium with charcoal. Similarly Foster *et al.* (1950) found that a treatment of complex media with charcoal or soluble starch gave substantial increases in growth and percentages of spores in *Bacillus larvae*. Later Hardwick *et al.* (1951) showed that the non-volatile saturated fatty acids (C_{10}-C_{14}) and the unsaturated fatty acids (oleic, linoleic, and linolenic) were strongly inhibitory to sporulation at concentrations of 50 mg/ml, while concentrations three times this value were inhibitory for vegetative multiplication. Alanine and β-alanine in concentrations of 1-2 mg/ml, prevent the stimulation of sporulation of *B. cereus* by glucose (Foster and Heiligman, 1949). Much earlier, Tarr (1932) found that addition of asparagine to casein hydrolysate prevents spore formation of several *Bacillus* species. Krask (1953) showed that methionine sulfoxide, which is a specific antagonist of the conversion of glutamic acid to glutamine, inhibited spore formation in *B. subtilis* in simple glucose-salts-glutamate medium. The effect of antibiotics on sporulation has not been studied extensively; however, Collier (1956) reported that penicillin will inhibit sporulation of anaerobic bacteria.

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Often the presence in the growth medium of nutritional factors which are known to stimulate germination, may inhibit sporulation by a process analagous to the competitive inhibition by methionine sulfoxide. Powell (1950) has reported high yields of spore-like heat labile cells when B. megatherium was grown in a medium containing glucose, glycerophosphate, pyruvate and lactate. These compounds are also germination initiators for B. megatherium spores.

Germination of bacterial spores. When spores are stored under suitable conditions, they remain viable for many years without appreciable loss of heat stability. But if they are transferred to favorable environment, the spores will become permeable to stain and lose their heat stability. This change in the spore's property, followed by its transformation into a vegetative form, is usually referred to as germination. With most organisms, at least an hour elapses before the first vegetative forms appear. The germination process is slow compared to vegetative growth, but faster than spore formation. The gradual process of change of a dormant, inactive, heat stable spore to an actively respiring heat labile cell has been visualized to involve intermediate stages of transition. These intermediate stages show varying biochemical properties. However, when spores become permeable to basic stains, they also lose heat stability, and quantitative measurements of germination by either process are quite well related.

Quantitative methods of study of germination. In earlier studies, the outgrowth of cells following the incubation of spores in a nutrient medium was used to indicate germination. However this method is not quantitative and therefore not suitable for the present study. In older literature, (Knaysi, 1933) the period of germination was defined as the time which elapses between the beginning of incubation of the spores in growth medium and first division of the germ cells. However, modern studies utilize the spore's loss of heat stability, the permeability to basic stains, and change in optical properties as criteria for germination.

Powell (1950), Levinson (1955), Stewart and Halvorson (1954) have measured the rate of germination of spores by staining procedures. After treatment with nutrient solutions, the germinated spores were able to take up simple dyes like methylene blue while the ungerminated spores did not.

The most accurate method of determining the germination rate is to follow the loss of heat stability of the spores being tested. Yet this method depends considerably on the various conditions under which the spores are heated and also on the choice of recovery media.

Germinating spores undergo changes in refractive index and therefore their germination can be followed by phase contrast microscopy (Pulvertaft and Haynes, 1951; Brown, 1956). Powell (1950) observed that the turbidity of spore suspensions decreased markedly on incubation with germinating nutrients. These measurements in the change in light transmission can be observed in any photoelectric colorimeter over most of the visible spectrum. This procedure has been found to be very effective in measuring the rate of germination at short intervals. The method correlates with either staining or heat lability techniques as described above.

Resting spores do not respire at a rate detectable by commonly used manometric techniques. However, germinated spores show res-

Sugiyama (1951) also found lower temperatures of incubation to be more favorable for the growth of Cl. botulinum. Exposure to sublethal temperatures often stimulates both the rate of germination and number of spores that germinate (Evans and Curran, 1934; Curran and Evans, 1937, 1945; Murrell, Olsen and Scott, 1950). Mefferd and Campbell (1951) found that furfural at a concentration of 1 p.p.m. increased the percentage of germination and replaced the effect of preheating or heat shocking in thermophilic bacilli. The exposure of spores to low or high pH is reported to have effect similar to heat shocking. (Murrell, 1954; Fitzjames, 1954; Zoha, 1955) Heat activation of spores of PA 3679 has been reported by Reynolds (1941) and Stumbo et al. (1950). Such heat treatment has been reported to reduce the resistance of spores to chemical agents like phenol and formaldehyde (Reddish, 1950).

Role of metals in germination. The effect of heavy metals on the growth, morphology, and metabolism of the vegetative form of micro-organisms has been studied in some detail, but not much work has been done on the influence of heavy metals on the germination of bacterial endospores. Keilin and Hartree (1947) reported that 20 mM 8-hydroxyquinoline (oxine) inhibited the germination of B. subtilis spores in glucose-yeast extract media. The inhibition could be reversed by washing and resuspending spores in fresh medium. Powell (1950) studied the effect of oxine on the germination of B. subtilis spores in a synthetic media consisting of L-alanine, glucose and phosphate and found complete inhibitions in presence of 10 mM oxine, British anti-lewisite, 2-3 dimercaptopropanol, (B.A.L.) inhibits germination partially at 4 mM and completely at 10 mM/ml. A partial piratory activity which can be easily detected. This initiation of measurable respiration can also be utilized as a measurement for the rate of germination.

Requirements for spore germination. Little work is reported in earlier literature on the nutrition of spore germination. A comprehensive list of various compounds that initiate germination is presented by Stedman (1956) and Schmidt (1955) has also made a survey of the subject.

The physical environment which is necessary for germination is usually very similar to that for vegetative growth (Holzmeuller, 1909; Cook, 1932; Knaysi, 1948; Wynne, 1952).

Effect of temperature on germination. Temperature changes affect the rate of spore germination and vegetative growth to different degrees. Mehl and Wynne (1951) reported the rate of germination of spores of PA 3679 to be a function of temperature over the range of 20-45°C., when thermal lability was used as a criterion for germination. Using the Arrhenius equation, they calculated a value of 10,300 calories for the activation energy of that reaction which was rate limiting in the germination of spores of PA 3679. Similar experiments performed in our laboratory by Sadoff (1957) showed a value of 15,900 calories as an activation energy for the germination of spores of PA 3679 (NCA strain). It should be pointed out that in the latter case the germination was followed turbidimetrically and obviously a different reaction was rate limiting. Reed (1942) found that incubation temperatures lower than 37°C. were more favorable for the maximum recovery of spores of *Cl. botulinum* when colony counts were used to determine survivors from heat processing.

reversal of inhibition was observed by the addition of soluble salts of zinc, magnesium, copper and iron. The effect of mercuric chloride was also studied. It was found to inhibit germination and this inhibition could be overcome by washing and inclusion of thiolactate or B.A.L. in the germination medium. Powell (1951) found that 10 mM/ml also completely inhibited the germination of B. megatherium spores. Levinson and Sevag (1953) found that iron and copper inhibited germination and respiration in spores of B. megatherium. They also noted a stimulation of germination and respiration by manganese, cobalt, and zinc. Although manganese stimulated germination of B. megatherium spores, it had no effect on spores of B. subtilis or B. cereus.

The effect on spore germination of several inorganic ions inhibitory to glycolysis or respiration has also received some attention. Powell (1951) incubated spores of B. megatherium in buffer with test substances for 30 minutes before adding glucose and followed the germination by staining. Cyanide, fluoride, iodoacetate and azide in concentrations of 1.0-10 mM had no effect.

Levinson and Sevag (1953) noticed an inhibition of germination and respiration of B. megatherium spores by a high phosphate concentration (0.05 mM) which could be overcome by chloride or other univalent ions.

Hachisuka et al. (1955) observed during their studies on B. subtilis that M/10 iodoacetate, arsenite, fluoride and cyanide did not inhibit germination but did inhibit vegetative growth of the germinated spore. Harrell and Halvorson (1955) found that arsenate and azide at levels which completely inhibit vegetative cells (10^{-2}M) have no effect on the germination of spores of *B. cereus* (*terminalis*).

Krishnamurty (1957) had difficulty in germinating spores of *B. cereus* (*terminalis*) which had been grown in semi-production equipment. These spores would germinate in L-alanine and adenosine after repeated washing with pH 7 phosphate buffer or dialysis in versene (ethylene diamine tetracetic acid). Manganese and magnesium ions stimulated the rate of germination in versene-washed spores. Copper, chromium, iron and mercury inhibited germination of versene or phosphate treated spores. Using arsenate to overcome the inhibition of germination by the metals, he observed that iron would also inhibit germination. Similarly Brown (1956) observed that spores of PA 3679 germinated rapidly in versene and that beryllium was found to be inhibitory.

Organic substances stimulating germination. Some 95 to 100 organic compounds including amino acids, carbohydrates, purines, pyrimidines, nucleic acids, nucleotides, nucleosides, and di- and tri-carboxylic acids have been used as stimulatory and inhibitory agents in the study of germination of bacterial spores (Keilin and Hartree, 1947; Hills, 1949, 1950; Powell, 1950; Powell and Hunter, 1953; Stewart and Halvorson, 1953; Pulvertaft and Haynes, 1951; Levinson and Sevag, 1953; Wynne and Mehl, 1956; Hachisuka, 1955; Hitzman and Halvorson, 1955; Lawrence, 1955; Church and Halvorson, 1956). Most of these studies were concerned with the germination of spores of aerobic bacteria and relatively few studies appear on the role of nutrients in the germination of spores of anaerobic bacteria.

Wynne and Foster (1948) using heat sensitivity as a criterion of germination, studied the factors affecting germination of spores of Cl. botulinum and other clostridia. Germination of spores of Cl. botulinum in brain heart infusion medium was markedly delayed when CO_2 was not present. Carbon dioxide could be replaced by oxaloacetate in the germination of Cl. botulinum spores. Although yeast extract could also replace carbon dioxide in synthetic media, Anderson (1951) reported that the addition of $NaHCO_3$ (0.10 to 0.15%) to medium containing spores of Cl. botulinum was essential for the prompt development of colonies. Reynolds (1952) obtained higher spore counts of PA 3679 when $NaHCO_3$ was added to tryptone-yeast-extract-thioglycollate medium. Similar effects of the addition of $NaHCO_3$ to Yessir pork infusion media is reported by Wynne et al. (1955) for Cl. botulinum types A and B.

Long chain fatty acids (oleic, linoleic, linolenic) were found by Foster and Wynne (1948) to inhibit germination of spores of Cl. botulinum but not spores of aerobic bacilli. This inhibition could be relieved by the addition of starch in the germination medium. Roth and Halvorson (1952) found that unsaturated fatty acids do not inhibit spore germination unless they are rancid. Benzoyl peroxide produced similar inhibition of germination but its effect could be partially overcome by catalase. The formation of colonies on solid media was used as the index of germination and organisms tested in the study were PA 3679, Cl. botulinum, B. subtilis, and B. stearothermophilus. Wynne et al. (1953) have reported the germination of spores of several clostridial species in buffered glucose pH 7.0 under anaerobic conditions in 48 hours. This germination was inhibited by 1 mg/ml oleate of pH 4.8.

Mundt et al. (1954) have studied germination of spores of a strain of Cl. sporogenes and observed rapid germination in 60% glucose and 8% sodium chloride, conditions which do not allow the germinated spores to mature into vegetative cells.

Wynne and Gaylen (1955) have reported rapid germination of spores of several clostridial species in phosphate buffered glucose at 75°C. This effect was more pronounced when glucose was autoclaved with buffer.

Hitzman, Zoha and Halvorson (1955) reported that spores of Cl. roseum and Cl. botulinum types A and B will germinate rapidly in a 5-10 minute period, when incubated with L-alanine, L-arginine and L-phenylalanine at room temperature. Alanine could be replaced by pyruvate with a decrease in the rate of germination. Arginine was essential to the germination process (Zoha, 1955).

Effect of antibiotics on germination. A number of studies have been conducted in recent years concerning the effect of antibiotics on the heat resistance of bacterial spores (Curran and Evans, 1940, 1946; Anderson et al., 1950; Adams, Hyeres and Tischer, 1951; Cameron and Bohun, 1951; Williams and Campbell, 1951; Kaufman, Ordal and El Bisi, 1954; Wynne et al., 1953). The results have been somewhat variable but in general antibiotics are neither sporostatic nor sporocidal.

EXPERIMENTAL

Studies on the Sporulation of Putrefactive Anaerobe 3679

Materials and Methods:

1. Organism. A National Canners Association strain of Putrefactive Anaerobe 3679, obtained from our laboratory culture collection, was used during this investigation. The requirement for serine for growth in a defined synthetic media (Frank and Campbell, 1955) and blackening of meat particles was used as a criterion for the authenticity of the strain.

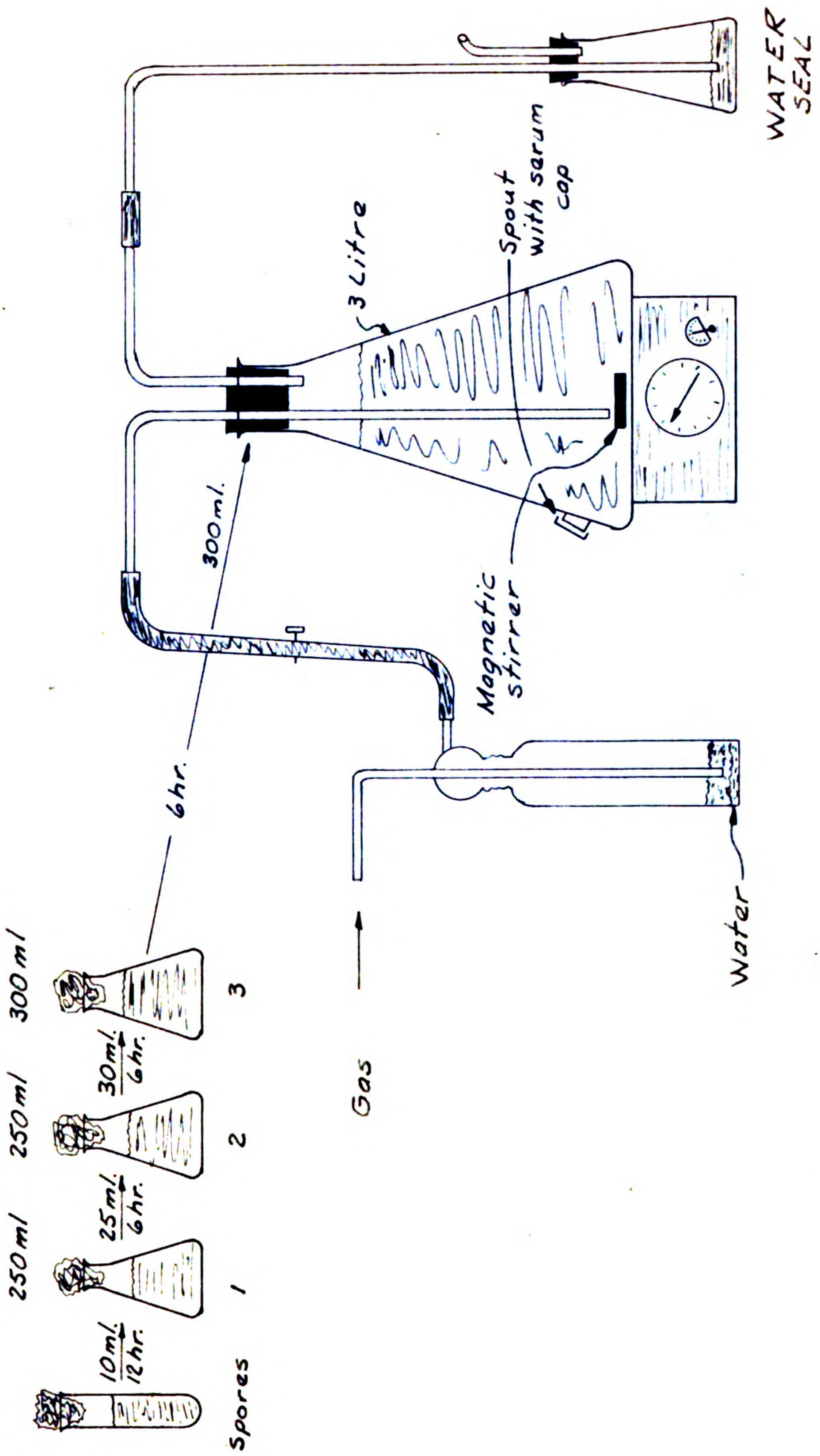
2. Media. Considerable difficulty is encountered in obtaining good yields of spores of some spore forming anaerobes in common laboratory media. Putrefactive Anaerobe 3679 (PA 3679) is known to form spores only after a considerable period of time of incubation. In the general practice of cultivating this organism, media containing various amounts of solids and tissues are employed. These media include thioglycollate broth (B.B.L.), brain-heart infusion (Difco), veal infusion, peptone-liver infusion with added particles of liver, and pork-pea infusion with added starch. All these media increase the difficulty of obtaining clean spore suspensions since they contain particles of solids, tissues or agar which are difficult to separate from spores. The clear liquid medium described by Hitzman, Zoha, and Halvorson (1955) for the sporulation of Cl. botulinum, and other anaerobes, hereto referred to as 'TSZ' medium, was used in these studies. It contained trypticase 1.5%, sodium chloride 0.5%, dipotassium phosphate 0.25% and dextrose 0.2%. Several modifications of TSZ medium, with decreases and increases in each component and the

addition of peptone were also used. The TSZ medium containing peptone will be referred to as TSP. In the inoculation of the culture medium, a heavy cell suspension amounting to 10% of the total culture volume was introduced into a freshly prepared flask of the medium. In TSP medium, 0.1% sodium thioglycollate was added if no anaerobic precautions were used during the cultivation of PA 3679.

Synchronization technique. Since a heavy inoculum is used in cultivation of this anaerobe, the culture resulting is not of a uniform physiological age, and cells will sporulate at various times during the course of the growth of the culture. A portion of the spores formed earlier during the period of incubation will germinate and probably start another life cycle. This naturally would result in a poor spore crop. To overcome this difficulty, a procedure described by Collier (1956) for the synchronization of sporogenesis was used. This technique was used for *Cl. roseum* and consisted of 4-6 transfers of an actively growing culture at 4 hour intervals. The procedure used in this study was slightly modified and carried out as follows: A spore inoculum of PA 3679 was introduced into a test tube of TSP medium. At the end of twelve hours, 10 ml of the culture was transferred into a 250 ml Erlenmyer flask containing 200 ml of fresh TSP medium. Transfers were made at 6 hour intervals and the entire contents of the third flask were introduced into the sporulation flask (Fig. I). This vessel contained three litres of TSP medium and was agitated by means of a magnetic stirrer. Anaerobic conditions were initiated in stirred cultures by bubbling city gas through the medium for 30-60 minutes after inoculation.

Growth flasks which were used for inoculum were maintained at

FIG. I SYNCHRONIZATION AND STIRRING TECHNIQUE



37°C. while sporulation was always carried out at approximately 33-35°C. The initial pH of the medium was 7.2-7.3 and this was maintained all through the period of sporulation.

Population studies. These studies were directed toward the determination of the growth rate of vegetative cells and the rate of spore formation in stirred and unstirred cultures of PA 3679. A synchronized inoculum was used for both the stirred and unstirred culture. Counts were taken at two hour intervals over 24 hours of incubation and then at every 4 hours until the fiftieth hour. A final count was taken at the end of one week period.

Total counts and spore counts were made in Pricket tubes which were prepared as follows: The counting medium, which consisted of TSP and 0.2% starch with 2% agar, was heated and poured into Pricket tubes. Methylene blue was also added as an oxygen indicator in the tubes. These tubes were autoclaved at 121°C. for 30 minutes. Samples were withdrawn from stirred culture flask by means of a sterile syringe, and dilutions were made in distilled water blanks. One-half ml of the dilutions was poured into melted TSP agar in Pricket tubes and shaken thoroughly to insure proper mixing. After the tubes cooled and the agar solidified, 3 ml of plain agar containing 1% sodium thioglycollate were poured over the TSP agar in the tube to insure an anaerobic seal. This "capping agar" removed traces of oxygen in the tube and kept it anaerobic. The tubes were then incubated for 48 hours at 37°C. and colonies counted.

For spore counts, samples withdrawn from the culture were heated in sterile tubes for 15-20 minutes at 90°C. This treatment was sufficient to destroy the vegetative cells. Microscopic examinations were also made to detect the onset of sporulation in the cultures.

Cleaning and harvesting of spores. Small quantities of the medium could be directly centrifuged in a Serval Super Centrifuge and the few vegetative cells and cell debris (which appear black or gray) could be mechanically separated from the white compact layer of the spores. This method was too cumbersome for harvesting spores from 6-12 litres of culture. Besides, even though 95-99% sporulation was obtained from the TSP medium, it was desirable to destroy the remaining vegetative cells. Lysozyme was effective against these cells but, unfortunately, the spores germinated at the conditions necessary for enzyme activity. A 15-20 minute aeration of the culture at the end of 44 hours incubation period was found to be a more effective treatment since it resulted in the lysis of nearly all vegetative cells. The spores were harvested by centrifugation in an electrically driven Sharples Super Centrifuge which was placed in a hood. This precaution was necessary to maintain the habitability of the laboratory and to prevent contamination of the laboratory with the spore-laden aerosol. The spores were washed 8 to 10 times with distilled water and stored at 4°C.

Spores obtained from brain heart infusion, after two weeks' incubation at 37°C., were filtered through cheese cloth padded with glass wool and then washed several times in the water. Microscopic examination showed that the spore suspension contained a considerable amount of extraneous material. These spores were further purified by gradient centrifugation at 4°C. This technique consists of centrifuging spores in a density and viscosity gradient established by layering various concentrations of sucrose in the centrifuge tube. The clear band of clean spores which was formed was withdrawn from the sucrose solution by means of a hypodermic needle and finally washed free of sugar.

Heat resistance studies. The heat resistance of the cleaned spores originally grown in infusion medium was compared to that of spores produced by the synchronization culture technique. The following procedure based on that of Bigelow and Esty's method was used for thermal death time determination. Aliquots of the stock spore suspension were diluted in sterile medium and M/250 phosphate buffer separately to give suspensions containing 10,000 spores/ml. Two ml of suspension was put in each thermal death time tube (TDT) by means of a 5 ml sterile syringe. The TDT tubes consisted of 9 ml pyrex glass tubes, 15 cm in length. Before use they were cleaned in dichromate cleaning solution, thoroughly rinsed in water, and sterilized. The tubes containing spore suspension were sealed and sets of 5 tubes were placed in metal holders. The tubes were heated by total immersion in an electrically heated and constantly stirred constant temperature oil bath ($\pm 0.15°C.$). The sets were heated for various times, at each of 4 temperatures in the range of 230 F.-250 F. A time correction of 2.5 minutes, which had been previously determined, was allowed for the time lag involved in heating the contents of the tube to bath temperature. At the end of the specified heating time, the holder and tubes were removed from the oil bath and rapidly immersed in a cold water bath. The cold tubes were wiped clean, flamed, and tubes containing buffer suspension were opened and the contents transferred to freshly prepared and exhausted B.B.L. fluid thioglycollate tubes. The tubes containing the medium suspension were kept sealed. Growth was observed after 36 hours of incubation.

Treatment of Data. A logarithmic order of death was assumed and the data obtained were treated according to the conventional method of Townsend, Esty and Baselt (1938) to construct a thermal death time curve. The following formula as suggested by Stumbo (1948) was also used to calculate D values, which is applied in the construction of thermal resistance curve.

$$D = \frac{U}{\log a - \log b} \qquad (1)$$

D = time in minutes to accomplish 90% reduction in the number of spores.

U = time of heating in minutes

a = number of spores subjected to one time-temperature relationship (the number of spores present per sample multiplied by the number of replicate samples).

b = number of spores surviving at the end of heating time U.

The value of b was derived by applying the equation of Halvorson and Zeigler (1932) to data obtained for samples subjected to time-temperature conditions that destroyed only a portion of the total number of organisms in the sample.

$$X = 2.3 \log \frac{n}{q} \qquad (2)$$

where X = most probable number (MPN) of spores surviving per replicate sample.

n = total number of replicate samples.

q = number of sterile samples as evidenced by lack of growth in subculture tubes.

then b = X multiplied by the number of replicates.

D values calculated from the data obtained corresponding to each time temperature relationship were used to construct a thermal resistance curve.

Germination studies - (methods). Germination of a spore suspension was followed in a Cenco-Sheard photometer by the change in optical density which occurred when they germinated. The procedure was correlated with the uptake of dye by the germinated spores. Aliquots measuring 0.5 ml of 10^{12}/ml spore suspension were pipetted into 13x100 pyrex test tubes and heated to 80°C. for 10 minutes. The tubes were removed from the water bath and allowed to cool down to room temperature. The germinating solution was then boiled with 0.01% sodium-thioglycollate and 4.5 ml of this suspension was added to the heat shocked spores while the solution was still warm. A spore control was similarly made with water and 0.01% sodium thioglycollate. Readings were taken at 5-minute intervals. Slides were made 30 minutes after the additiion of the nutrients using crystal violet stain. This procedure was followed all through the germination studies.

RESULTS

Studies on Sporulation of PA 3679

Effect of various media. Of those media which are reputed to induce sporulation of anaerobes, only TSZ medium and Brewers thioglycollate medium showed a reasonable response (Table 1). Furthermore, the addition of peptone markedly increased the percentage of sporulation in cultures of PA 3679 grown in TSZ medium. In Table 2 it is seen that the use of TSZ plus 1% peptone (TSP) yielded 90-99% sporulation. This medium, (TSP), was therefore used during further studies on growth or sporulation of PA 3679. Cultures of the test organism in this medium sporulated in 72 hours.

Sporulation in stirred and unstirred culture. Since spores germinate rapidly in TSP medium there was always a problem of harvesting the spores from still cultures without germination. This difficulty could be overcome by chilling the sporulated culture for 24-48 hours and harvesting the spores at 4°C. However it was observed that, if the medium was constantly stirred under anaerobic conditions, the percentage of sporulation was increased and the spores produced could be harvested at room temperature.

The total viable counts and spore counts in stirred cultures remained constant while the unstirred culture showed a rise in total count and decrease in spore count after one week of incubation (Fig. II). This undoubtedly was due to the germination of a considerable number of the spores.

Synchronization Technique. Spores of PA 3679 can be obtained

TABLE 1

GROWTH AND SPORULATION OF PA 3679 IN VARIOUS MEDIA

Media	Time of incubation									
	12 Hours		24 Hours		48 Hours		72 Hours		1 Week	
	*Growth	Spores	Growth	Spores	Growth	Spores	Growth	Spores	Growth	Spores
Brain heart infusion dehydrated(Difco)	+	-	++	-	+++	-	+++	1-2%	+++	15-20%
**TSZ medium	++	-	++	-	+++	-	++++	5-10%	++++	20-30%
TSZ medium containing half of original phosphate concentration	+	-	++	-	++	-	++	1-2%	++	5%
TSZ medium with manganese, magnesium and sodium phosphate	+	-	+	-	++	-	+++	5-10%	+++	5-10%
Brewers thioglycollate (Difco)	+++	-	++++	-	++++	-	++++	20-30%	++++	50%
TSZ + 1% lactose	+	-	+	-	++	-	++	2-5%	++	2-5%
TSZ + 1% soluble starch	+	-	+	-	++	-	++	10%	+++	10-15%
TSZ + 1% peptone	++++	-	++++	5%	++++	40-50%	+++	90-95%	+++	99%
TSZ + 5% peptone	++++	-	++++	1-2%	++++	10-15%	++++	50%	+++	90-99%
Peptone 5% + 1% lactose	+	-	+	-	++	-	++	2-5%	++	2-5%
Peptone 5% + trypticase 10%	++++	-	++++	1-2%	++++	5-10%	++++	20%	+++	40-50%

* + = poor growth; ++ = growth; +++ = good growth; ++++ = very good growth; - = no spores

**Trypticase 1.5%, NaCl 0.5%, Glucose 0.2%, K_2HPO_4 0.25%

TABLE 2

EFFECT OF PEPTONE CONCENTRATION ON SPORULATION

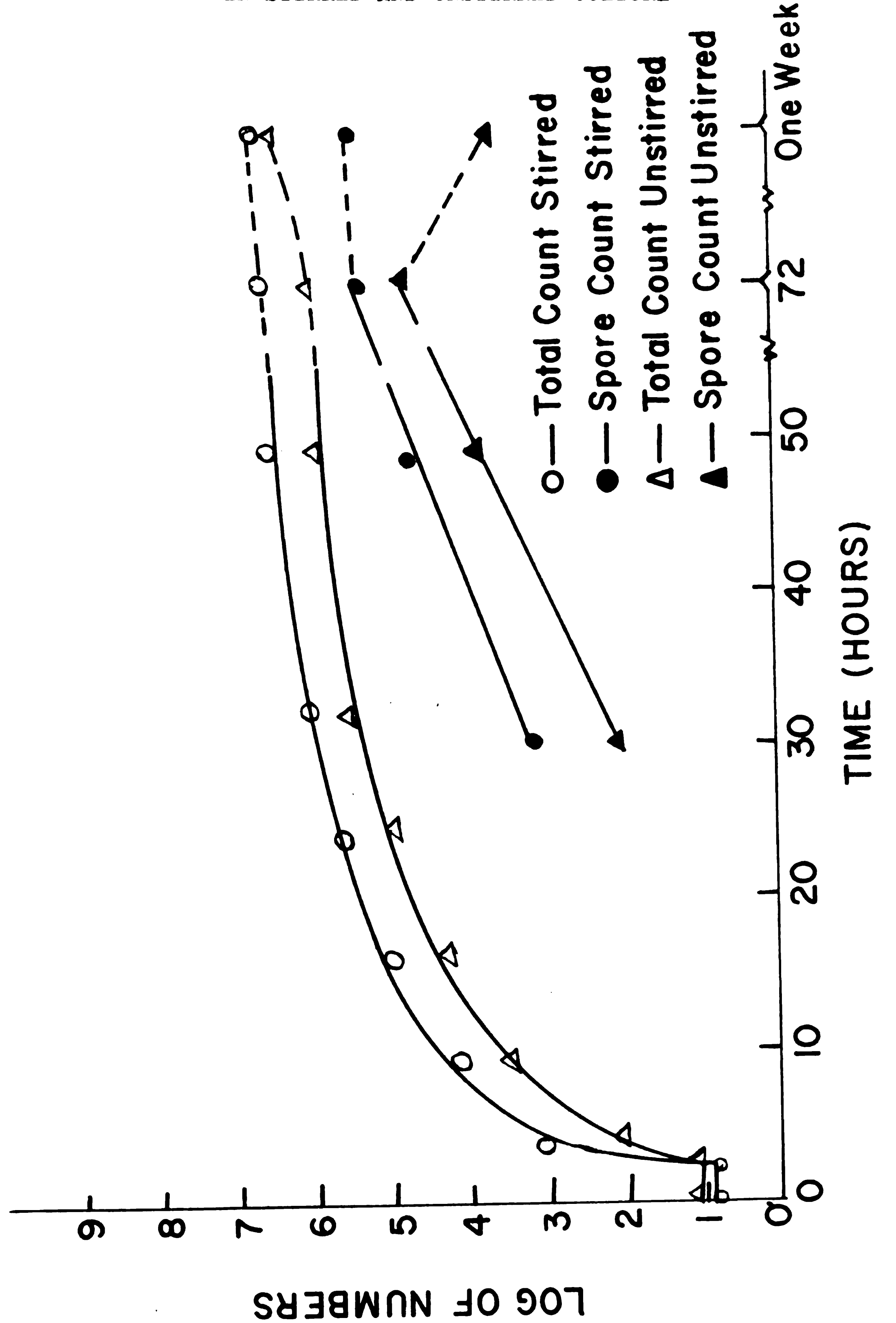
Media	Time of incubation									
	12 hours		24 hours		48 hours		72 hours		1 week	
	*Growth	Spores	Growth	Spores	Growth	Spores	Growth	Spores	Growth	Spores
TSZ	++	-	+++	-	+++	-	+++	5-10%	+++	20-30%
TSZ + 0.5% peptone	++++	-	++++	-	++++	5-10%	++++	60-70%	++++	60-70%
TSZ + 2% peptone	++++	-	++++	-	++++	40-50%	++++	90-95%	++++	99%
TSZ + 5% peptone	+++	-	++++	-	++++	20-30%	++++	70-80%	++++	80%
TSZ with 10% trypticase and 1% peptone	++	-	+++	-	++++	-	++++	-	++++	5-10%
**Peptone medium	++	-	+++	-	+++	-	+++	-	+++	5-10%
Peptone medium with 10% peptone	++	-	+++	-	+++	-	+++	-	+++	5-10%

* + = poor growth
++ = growth
+++ = good growth
++++ = very good growth
\- = no spores

** Peptone medium:

Peptone	5%
NaCl	0.5%
K_2HPO_4	0.25%
Glucose	0.20%

FIG. II TOTAL VIABLE COUNT AND SPORE COUNT
IN STIRRED AND UNSTIRRED CULTURE



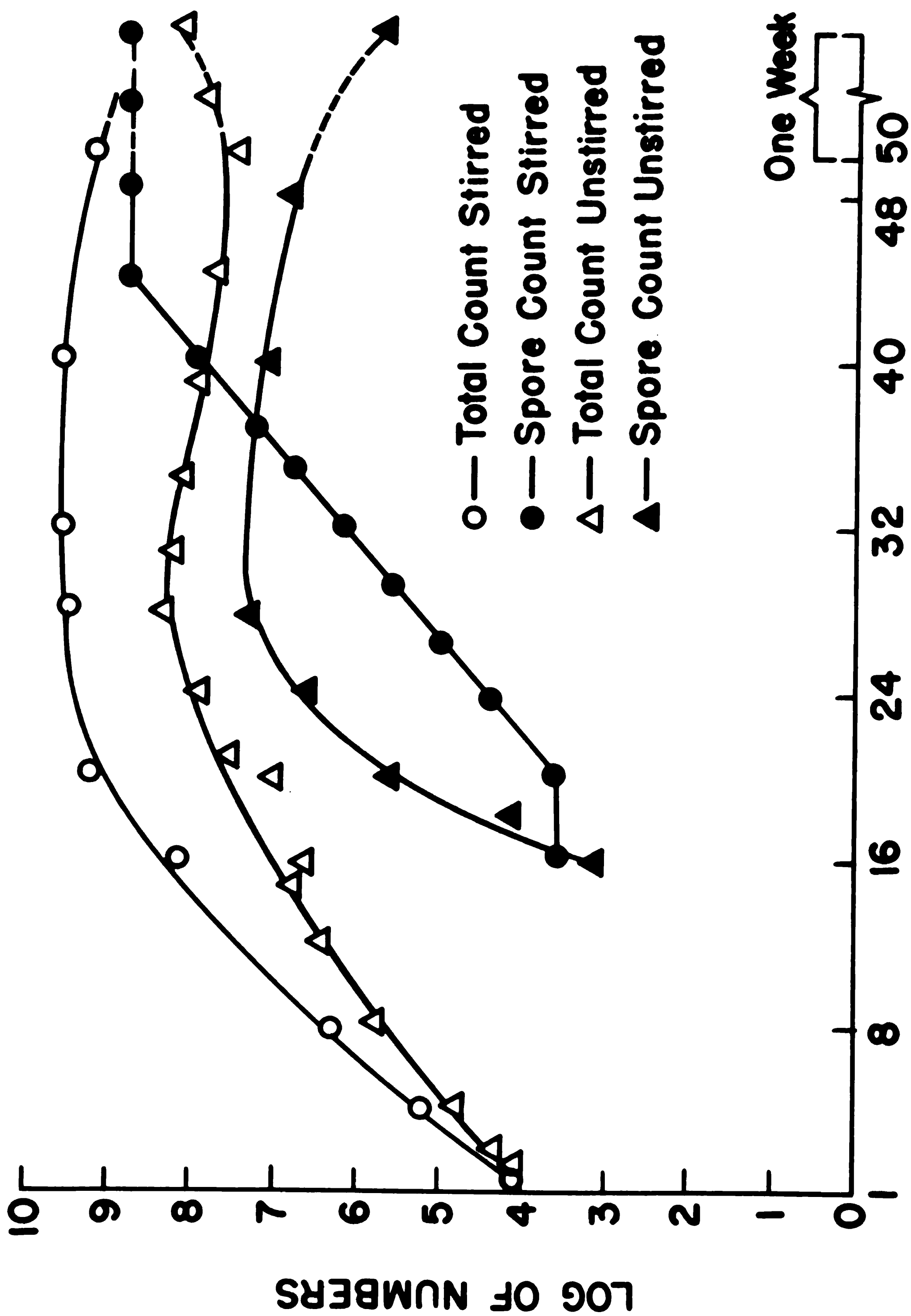
from stirred TSP medium in 72 hours while cultures of Cl. roseum and Cl. botulinum will sporulate completely in 8-12 hours. The spores of the latter organisms were obtained by synchronization technique. Application of the same procedure resulted in the sporulation of PA 3679 in 44 hours. It was necessary to modify Collier's (1956) procedure for use with the PA 3679 because more than 4 transfers at intervals of less than 6 hours resulted in lysis of the culture. Gassing was observed in the sporulation flask in 1 hour, after the transfer of the inoculum, and sporulation began at the 16th hour of incubation. It was interesting to note that the exponential growth of vegetative cells had not quite ceased at this time. Sporulation occurred exponentially in the stirred culture and was completed after 44 hours of incubation. The total viable counts and spore counts of the stirred, synchronized cultures were approximately 10 times those of the unstirred cultures (Fig. III). These results are in accord with those obtained from unsynchronized cultures.

The synchronized culture technique for the production of spores of PA 3679 has been successful with final culture volumes of 10-12 litres.

In TSP or TSZ medium, various levels of glucose, lactose and starch were tested for their effect on sporulation of PA 3679. Of the three carbohydrates, only glucose affected sporulation. High concentrations of glucose, which have been found inhibitory for the sporulation of Bacillus spp., also inhibited sporulation in PA 3679. A glucose concentration of 0.2% was critical because lower levels resulted in poor cell growth and higher levels in poor sporulation.

Similarly, the phosphate and sodium chloride concentration

FIG. III TOTAL VIABLE COUNT AND SPORE COUNT IN STIRRED AND UNSTIRRED CULTURE INITIATED WITH SYNCHRONIZED INOCULUM



described for TSP medium was found to be optimal for sporulation of the test organism. An increase in trypticase concentration had no effect on the degree of sporulation while a decrease resulted in poor growth and poor sporulation. Peptone could not replace trypticase nor could the enhancement of sporulation due to peptone be obtained with yeast extract in the medium. A 1% concentration of peptone in TSP was found to be optimal for spore production.

Population studies. The generation time of PA 3679 was approximately 1 hour in the stirred synchronized culture. The generation time in synchronized unstirred culture was 65 minutes, and the doubling rate in unsynchronized culture showed a similar difference between stirred and unstirred culture. The standing, unsynchronized culture showed a doubling rate for PA 3679 cells to be 78 minutes and the stirred one of 72 minutes. However, a lag of approximately 2½ hours was noted when cultures were initiated by spore suspensions although germination occurred in an hour or less in TSP medium.

In synchronized, stirred cultures, spore counts up to 10^9/ml were obtained and in the exponential rate of spore formation, there was a doubling in number every 84 minutes.

Heat-resistance studies. Thermal death time curves were plotted according to the method of Townsend, Esty and Baselt (1938). These showed that clean spores of the test organism had a Z value of 18.1-18.5 and F_{250} of 4.8-5.1. A Z value of 18 means that for each 18°F. rise in temperature, the death rate of the spores will be increased ten-fold. The F_{250} value is the time in minutes to sterilize the standard spore suspension (10,000 spores/ml).

FIG. IV THERMAL RESISTANCE CURVE OF SPORES OF PA 3679 OBTAINED FROM TSP AND BRAIN HEART INFUSION (BHI) WHEN HEATED IN BUFFER

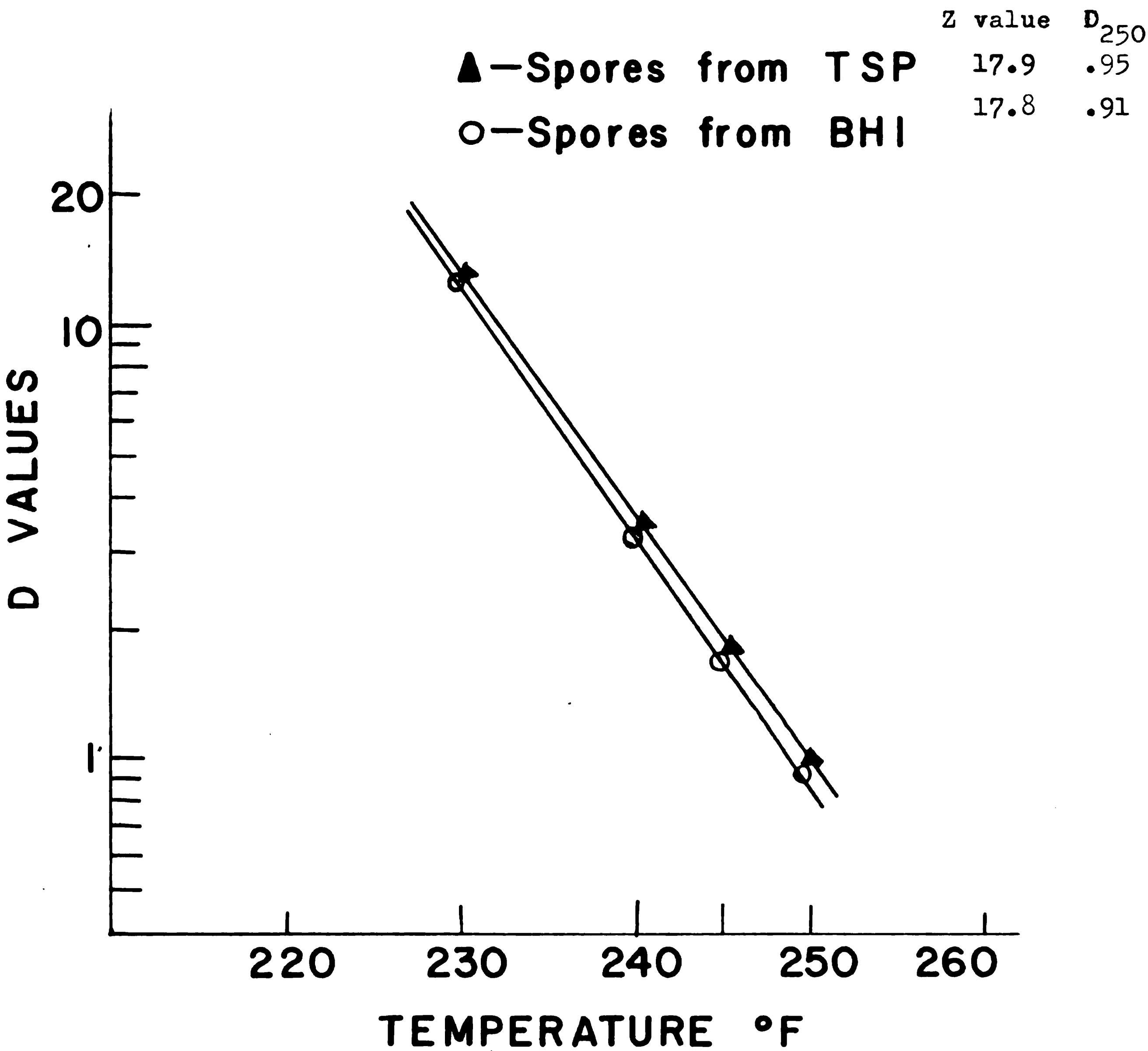
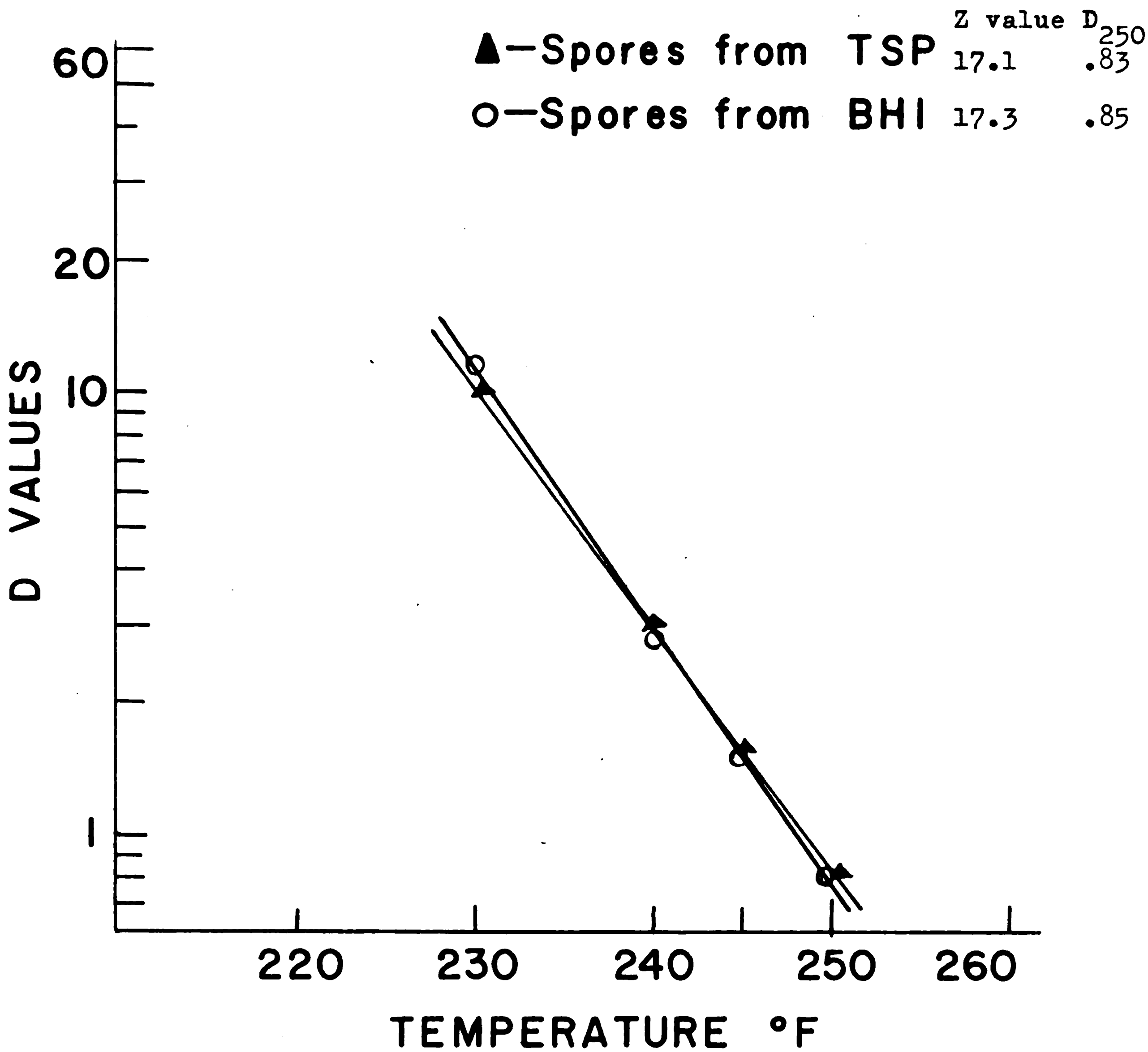


FIG. V THERMAL RESISTANCE CURVE OF SPORES OF PA 3679 OBTAINED FROM TSP AND BRAIN HEART INFUSION (BHI) WHEN HEATED IN TSP



Thermal resistance curves are more accurate representations of the thermal death rate of the spores than thermal death time curves. The curves (Figs. IV and V) which were drawn on the basis of calculated D values showed Z values of 17.5 and 17.9 and D_{250} in the range of 0.83-0.95. These data, which are given in Table 3, showed that spores obtained from brain heart infusion and TSP medium have D_{250} of 0.85 and 0.83 respectively, when heated in TSP medium, and D_{250} of 0.91 and 0.95 respectively, when heated in buffer. The difference observed within a set is not significant and differences between sets (D_{250} of 0.83-0.95) may only be due to experimental error.

<u>Germination studies</u>. Preliminary studies of germination were performed on spores harvested from a one litre culture of TSP medium. The results are summarized in Tables 4 and 5. These spores had been harvested, washed and heated to 80°C. for 15 minutes in an effort to accelerate their release from the sporangium. These spores failed to show rapid germination in either complex or defined nutrients. Repeated washings with distilled water or versene (EDTA) or longer heat shock had no effect on the germination of these spores. However, they showed complete germination in 5 hours in complex media like thiotone or peptone and yeast extract. During germination, these spores were stained gradually, while with normally germinating spores, staining is an all-or-none proposition. The slowly germinating spores took deep stains only in the periphery in the first hour followed by light staining in the spore center during the second and third hours. At the end of the fifth hour, the spores were completely stained and they also showed a change in their refractive index as evidenced by increased light transmission.

TABLE 3

D VALUES OF SPORES OF PA 3679 IN TSP MEDIUM AND BRAIN HEART INFUSION

Temperature in degrees F.	Average D values			
	Spores from brain heart infusion heated in		Spores from TSP medium heated in	
	TSP	Buffer	TSP	Buffer
230	11.5	12.1	10	13
240	2.8	3.1	3	3.41
245	1.5	1.6	1.6	1.75
250	0.85	0.91	0.83	0.95

TABLE 4

GERMINATION OF SPORES OF PA 3679, OBTAINED WITHOUT LYSIS OF SPORANGIA*

As Observed by Light Transmission

Media	Per cent Transmittance										
	Time in Minutes										
	0	5	10	15	20	25	30	60	120	12 hr	24 hr
1. Thiotone 5%	14	14	14	14	14	14	14	14	14	<u>26</u>	<u>30</u>
2. Peptone 5%	15	15	15	15	15	15	15	16	<u>18</u>	<u>29</u>	<u>35</u>
3. Yeast extract 5%	18	18	18	18	18	18	18	18	20	<u>24</u>	<u>30</u>
4. TSZ	21	21	21	21	21	21	21	22	26	growth	growth
5. TSP	20	20	21	21	21	21	21	21	<u>25</u>	growth	growth
6. Yeast extract 5%	18	18	18	18	18	18	18	18	19	<u>26</u>	<u>39</u>
**CONTROL	25	25	25	25	25	25	25	25	25	25	25

* Spores heat shocked for 10 minutes at 80°C.
0.01% sodium thioglycollate added to each germinating mixture.

Temperature 25-26°C.

pH of suspension 6.9-7.1

**Control - spore suspension in distilled water with 0.01% sodium thioglycollate.

TABLE 5

GERMINATION OF SPORES OF PA 3679 OBTAINED WITHOUT LYSIS OF SPORANGIA*

As Observed by Staining Reaction

	Per cent Spores Stained			
Media**	Time - 2 hr	4 hr	12 hr	24 hr
Control	3-4%	3-4%	3-4%	3-4%
1.	3-4%	5-10%	10-20%	20-30%
2.	3-4%	5-10%	10-20%	25-35%
3.	3-4%	3-4%	10-20%	20%
4.	3-4%	20%	70-80%	Growth
5.	3-4%	25%	Growth	Growth
6.	3-4%	5-10%	10%	30-40%

*.01% Sodium thioglycollate added to all mixtures including control.

Spores were heat shocked for 10 minutes at 80°C.

Temperature 25-26°C.

pH of suspension 6.9-7.1

**See Table 4.

When spores of the test organism were obtained after complete lysis of the sporangium and vegetative cells, they germinated rapidly in casamino acid, tryptone, thiotone, peptone, beef extract, and yeast extract solutions. The results,which are summarized in Table 6,indicate that spores of PA 3679 germinate in 60 minutes at room temperature in a complex nutrient solution. Yeast extract solution initiated more rapid and complete germination than any other complex medium. Since casamino acid in combination with 2% yeast extract was as good a germinating agent as 5% yeast extract, further experiments were performed with yeast extract and casamino acid combinations. The results of these studies are summarized in Table 7 and show that something more than amino acids(Casamino acids) are required for the rapid germination of PA 3679 spores.

Replacement of yeast extract by adenosine. Hills (1949) had shown that yeast extract could be replaced by adenosine in the germination of spores of aerobic bacilli, and a similar situation could be envisaged in spores of anaerobic bacteria. Experiments were then planned with purines, pyrimidines, and nucleosides, in combination with casamino acids. The following compounds were used in M/250 concentration with 5% casamino acids: adenosine, thymidine, inosine, adenine, guanadine, creatinine, xanthine, uracil, and guanine. Only adenosine produced complete germination after 24 hours and partial germination in 30 minutes. Partial germination was also observed in other mixtures, after 24 hours, but this effect was probably due to the casamino acid itself. However, further experiments with defined mixtures of amino acids confirmed that adenosine could partially replace yeast extract (Table 8) as a germinating agent.

TABLE 6

GERMINATION OF SPORES OF PA 3679 OBTAINED AFTER AERATION TREATMENT OF CULTURE AND CELL LYSIS*
As Observed by Light Transmission and Staining

Germinating Solution	Per cent Transmittance Time in Minutes 0	5	10	15	20	25	30	60	120	12 hrs	24 hrs	Staining after 30 min.
1. Beef extract 5%	11	12	14	14	<u>16</u>	<u>16</u>	<u>18</u>	<u>25</u>	<u>30</u>	31	Growth	5-6%
2. Peptone 5%	14	14	16	16	18	<u>20</u>	<u>20</u>	<u>24</u>	<u>30</u>	<u>35</u>	Growth	10-15%
3. Thiotone 5%	16	17	17	17	17	18	18	20	<u>24</u>	<u>40</u>	Growth	5-10%
4. Yeast extract 5%	20	28	<u>31</u>	<u>40</u>	<u>44</u>	<u>46</u>	<u>47</u>	<u>48</u>	<u>48</u>		Growth	90-95%
5. Casamino acid 5% + Yeast extract 2%	21	24	25	<u>30</u>	<u>31</u>	<u>35</u>	<u>42</u>	<u>44</u>	<u>45</u>		Growth	85-90%
CONTROL**	25	25	25	25	25	25	25	25	25	25	26	1-2%

* 0.01% Sodium thioglycollate added to each germinating solution.

Temperature 25-27°C. Spores heat shocked for 10 minutes at 80°C.

pH 6.9-7.1

** See Table 4.

TABLE 7

GERMINATION OF SPORES OF PA 3679 IN CASAMINO ACID AND YEAST EXTRACT SOLUTION*
As Observed by Light Transmission and Staining

	Per cent Transmittance Time in Minutes										Staining after 30 minutes
Germinating Mixture	0	5	10	15	20	25	30	60	120	240	Per cent stained spores
1. 5% Casamino acid thioglycollate	21	23	21	24	24	24	25	26	29	30	30-40%
2. 5% Casamino acid yeast extract	22	24	28	36	45	52	54	55	55	56	80-90%
3. Yeast extract 0.5% thioglycollate	26	26	27	27	28	28	28	30	32	46	10-15%
4. Yeast extract 2% 0.01% thioglycollate	27	29	29	30	32	33	35	36	37	42	10-15%
5. Yeast extract 1% 0.01% thioglycollate	29	34	36	36	38	40	41	49	50	50	40-50%
6. Yeast extract 0.01% thioglycollate	29	39	45	51	56	59	59	60	62	62	95-99%
7. Yeast extract 0.01% thioglycollate	28	37	49	55	58	61	61	61	61	62	95-99%
8. Yeast extract 0.01% thioglycollate	29	39	41	49	52	58	58	58	61	61	95-99%
CONTROL - water 0.01% thioglycollate	16	16	16	16	16	16	17	15	15	15	1-2%

*0.01% Sodium thioglycollate added to each germinating solution.
Temperature 25-27°C. Spores heat shocked for 10 minutes at 80°C. pH 6.9-7.1

TABLE 8

REPLACEMENT OF YEAST EXTRACT BY PURINES AND PYRIMIDINES NUCLEOSIDES IN GERMINATION OF SPORES OF PA 3679*
As Observed by Light Transmission and Staining

	Germinating Mixture	Per cent Transmittance Time in Minutes 0	5	10	15	20	25	30	60	120	24 hrs	Per Cent Stained Spores Staining after 30 minutes	Staining after 24 hours
1.	C.A. + Yeast extract	30	36	40	46	46	50	56	58	58		70-80%	Growth
2.	C.A. + Adenosine	30	32	32	34	38	38	38	41	44	68	50-60%	95-99%
3.	C.A. + Thymidine	30	30	31	31	31	32	32	33	33	40	2-3%	60-70%
4.	C.A. + Inosine	31	31	31	31	31	31	31	32	32	38	2-3%	50-70%
5.	C.A. + Adenine	31	31	31	31	31	31	33	32	33	40	2-3%	60-70%
6.	C.A. + Guanadine	31	31	31	31	31	31	31	31	31	39	2-3%	60-70%
7.	C.A. + Creatine	30	30	30	30	31	31	32	32	32	39	2-3%	60-70%
8.	C.A. + Xanthine	30	31	32	32	32	33	34	35	35	41	2-3%	50-60%
9.	C.A. + Uracil	28	28	28	28	28	30	32	34	34	42	2-3%	60-70%
10.	C.A. + Guanine	30	30	30	30	31	31	31	31	31	36	2-3%	50-60%
11.	C.A. + Asparagine	29	29	29	29	30	30	30	32	34	41	2-3%	60-70%
12.	Casamino Acid 5%	30	32	32	34	34	34	34	36	38	49	2-3%	60-70%
	CONTROL**	28	28	28	27	27	28	27	28	28	30	1-2%	2-3%

* 0.01% Sodium thioglycollate added to each germinating solution.
Temperature 25-27°C. Spores heat shocked for 10 minutes at 80°C. pH 6.9-7.1

C.A. = Casamino acid.

<u>The effect of amino acids on germination</u>. In an effort to characterize the amino acids or acid that were necessary for the germination of spores of PA 3679, experiments were performed with sixteen amino acids which are known to be present in yeast extract, as well as casamino acids. Mixtures of amino acid solutions were prepared so that concentrations were in the same proportions as are found in yeast extract. Each mixture lacked one amino acid normally present in the yeast extract. The following key is used for the accompanying Table 9.

	Amino Acids	Concentration
1.	Alanine	0.50 %
2.	Arginine	0.78 %
3.	Phenylalanine	2.2 %
4.	Aspartic Acid	5.1 %
5.	Glutamic Acid	6.5 %
6.	Histidine	0.94 %
7.	Isoleucine	2.90 %
8.	Leucine	3.60 %
9.	Lysine	4.00 %
10.	Methionine	0.79 %
11.	Tryptophane	0.88 %
12.	Threonine	3.40 %
13.	Tyrosine	0.60 %
14.	Valine	3.4 %
15.	Glycine	4.4 %

TABLE 9

GERMINATION OF SPORES OF PA 3679 IN AMINO ACID SOLUTIONS AS OBSERVED BY LIGHT TRANSMISSION*

Amino Acid Omitted	Per cent transmittance									
	Time in Minutes									
	0	5	10	15	20	25	30	60	120	24 hours
1. None	25	25	25	25	25	26	28	28	28	40
2. None + 1% Yeast Extract	24	26	28	34	36	37	38	42	45	59
3. None + 2% Yeast Extract	23	25	27	33	40	46	55	56	57	
4. None + Adenosine	26	26	26	26	26	27	29	36	39	48
5. Alanine Omitted	29	29	29	29	29	29	29	29	29	31
6. Other amino acid omitted**	28	28	28	28	28	29	31	33	36	41
7. Yeast Extract 2%	24	24	24	24	25	26	30	33	36	46
Control*** (Aqueous spore suspension)	30	30	30	30	30	30	30	30	30	32

*Spores heat shocked for 10 min. at 80°C. Sodium thioglycollate 0.01% added to each germinating solution. pH of the germinating solution 6.9. Temperature incubation 25-27°C.

**Omission of any of the amino acids e.g., arginine, aspartic acid, glutamic acid, glycine, histidine, iso-leucine, leucine, lysine, methionine, threonine, tryptophane, tyrosine and valine, showed the same rate of germination.

***Control - see Table 4.

The pH of the mixtures was brought to neutrality. Table 9 indicates that the amino acid mixture which did not contain alanine did not germinate the spores.

One per cent yeast extract solution would not replace alanine in the germination of PA 3679 spores. Two per cent yeast extract solution in the absence of alanine germinated spores at a lower rate than 4% yeast extract. However 2% yeast extract in a complete mixture of amino acids showed the same rate of germination as a 4% yeast extract solution. These results indicate that the spore required alanine for germination.

Experiments were then planned with L-alanine, L-arginine and L-phenylalanine mixtures which had been shown to bring about germination of spores of Cl. botulinum types A & B. The data given in Table 10 show that only L-alanine with the addition of 1% yeast extract produced some germination. The yeast extract in these experiments could be replaced with adenosine. Further experiments with alanine, adenosine mixture with addition of 1 and 2% yeast extract resulted in germination responses comparable to 4% yeast extract as is shown in Table 11.

Effect of carbohydrates and other carbon sources on germination of PA 3679. Various carbohydrates have been known to initiate and stimulate the germination of spores of aerobic bacteria. Some of the best known are glucose, maltose, sucrose, mannose, ribose, and various caramelized preparations. Similarly acetate, succinate, fumarate, malate, and pyruvate are known to stimulate the germination of spores of B. subtilis. The effect of these compounds was observed in the presence or absence of alanine and adenosine. Table

TABLE 10

GERMINATION OF SPORES OF PA 3679 IN ALANINE ARGININE AND PHENYLALANINE IN PRESENCE OF 1 AND 2% YEAST EXTRACT OR ADENOSINE*

Germinating Solution	Per cent Transmittance										
	Time in Minutes										
	0	5	10	15	20	25	30	40	60	120	24 hrs
Control**	24	24	24	24	24	24	24	24	24	24	26
L-Alanine, L-Arginine, L-Phenylalanine	24	24	24	25	25	25	26	26	28	28	40
L-Alanine, L-Arginine and yeast extract 1%	24	26	28	28	30	30	36	40	42	44	58
L-Alanine, L-Arginine, L-Phenylalanine and yeast extract 2%	25	22	29	32	34	40	41	44	46	57	Growth
L-Alanine, L-Arginine, L-Phenylalanine and Adenosine	25	25	25	26	26	26	28	28	34	40	61
L-Alanine and yeast extract 1%	22	23	26	26	27	28	32	41	44	49	69
L-Alanine and yeast extract 2%	21	26	28	39	40	42	46	48	51	60	Growth
L-Alanine and Adenosine	24	24	24	26	27	29	34	35	41	44	46
3% Yeast extract	20	26	30	38	46	54	66	66	67	67	Growth

*Spores heat shocked for 10 minutes at 80°C.

0.01% Sodium thioglycollate added to the mixture.

pH 7.2
Temperature of incubation 25-27°C.

** See Table 4

TABLE 11

GERMINATION OF SPORES OF PA 3679 IN ALANINE, ADENOSINE, GLUCOSE AND PHOSPHATE (AAGP) WITH ADDITION OF YEAST EXTRACT*

Germinating Solution	Per cent Transmittance								
	Time in Minutes								
	0	5	10	15	20	25	30	60	24 hrs.
AAGP + 1% yeast extract	18	24	30	38	39	40	41	42	+***
AAGP + 2% yeast extract	16	20	27	29	32	34	39	40	+
AAGP	19	21	24	26	28	34	35	41	44
Yeast extract 4%	12	20	31	35	37	38	38	38	+
Control**(water)	20	20	20	20	20	20	21	22	24

* Spores heat shocked for 10 minutes at 80°C.
0.01% Sodium thioglycollate added to the mixture.
pH 7.2
Temperature 25-26°C.

**See Table 4

*** + = growth of cells into vegetative cells

12 shows the effect of some of the above mentioned compounds on the germination of PA 3679 spores. Glucose or caramelized glucose stimulated the germination of spores of the test organism. This confirms a previous observation by Wynne (1954) who reported the germination of PA 3679 spores in buffered glucose in 48 hours. Germination in the above case was noted in buffered glucose in 48 hours.

Solutions containing alanine, adenosine, glucose and phosphate effected complete germination in 30-45 minutes. Various levels of each component were tried to accomplish a rate comparable to 4% yeast extract. The optimal level of the nutrients induced complete germination in 20-45 minutes and initiated measurable germination as early as 5 minutes after the addition of the reagents to the spore. The concentrations were as follows:

L-Alanine	0.001%
Adenosine	0.00025%
Glucose	0.05%
K_2HPO_4	0.005%

If 1% yeast extract was added to the above mixtures, the rate of germination was comparable to 4% yeast extract. The process was complete in 20 to 30 minutes (Fig. VI).

<u>Characterization of germination accelerating factor present in yeast extract</u>. When 1% yeast extract was added to the germinating solution of alanine, adenosine, glucose and phosphate (AAGP), the rate of germination was comparable to that obtained in 4% yeast extract solution. The characterization of the accelerating component was attempted in the following manner.

Yeast ribonucleic acid (RNA) is known to stimulate the germination of spores of some aerobic bacilli (Hills, 1949) after mild

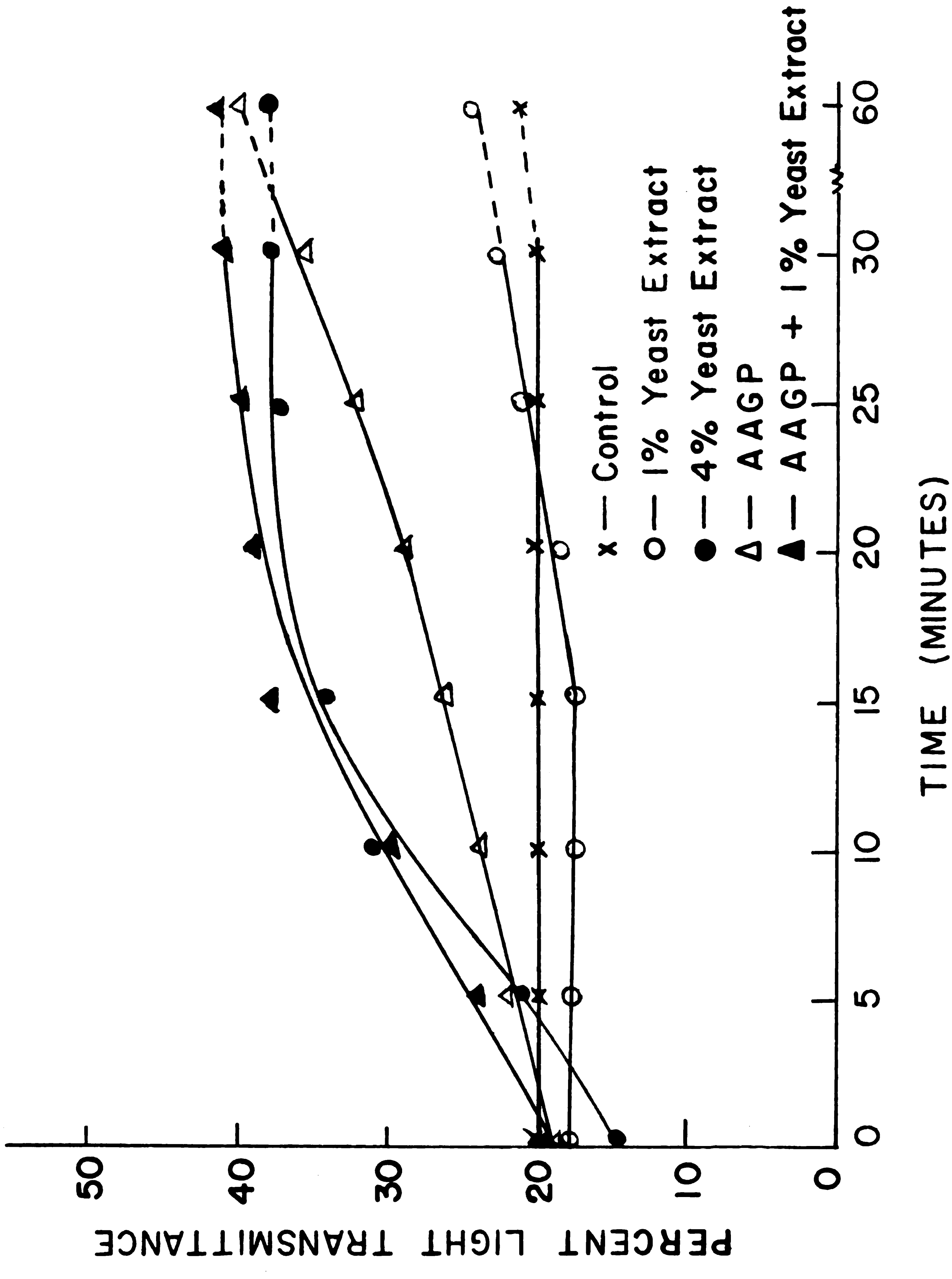
TABLE 12

EFFECT OF CARBOHYDRATE AND OTHER CARBON SOURCES ON GERMINATION OF PA 3679 SPORES AS OBSERVED BY LIGHT TRANSMISSION*

Germinating Solution	Per Cent Transmittance — Time in Minutes								24 hours	Spores stained after 30 minutes
	0	5	10	15	20	25	30	60		
Yeast extract 3% + 0.05% Glucose	9	16	22	25	29	31	32	34	Growth ++	90-99%
Yeast extract 3% + 0.05% Lactose	10	12	14	17	19	21	22	30	Growth +	60-70%
Yeast extract 3% + 0.05% Starch	8	10	11	16	19	24	27	31	Growth +	60-70%
Yeast extract 3%	9	13	18	21	22	24	28	30	Growth +	80-90%
Alanine, Adenosine, Glucose 0.05% + Phosphate	15	17	18	20	24	24	26	36	42	90-95%
A.A.P.** + 0.05% lactose	14	16	16	17	17	19	22	25	40	15-20%
A.A.P. + 0.05% Starch	14	14	14	14	15	15	16	22	40	15-20%
A.A.P. + 0.2% Acetate	15	15	15	15	15	15	17	20	32	5-10%
A.A.P. + 0.05% Ribose	15	15	15	15	16	18	18	20	40	5-10%
CONTROL***	16	16	16	16	16	16	17	17	18	1-2%

*Spores heat shocked for 10 minutes at 80°C.
0.01% Sodium thioglycollate added to each germinating mixture and pH was 7.2.
Temperature of incubation - 25-27°C.
**Alanine, adenosine and phosphate.
***See Table 4.

FIGURE VI RATE OF GERMINATION OF PA 3679 SPORES



hydrolysis with N/10 NaOH. Therefore, hydrolysis by both 1N KOH and 1N H_2SO_4 was performed and the hydrolysate neutralized and tested for its ability to germinate the spores of PA 3679. These hydrolysates did not compare with 4% yeast extract or AAGP solution in their germinating ability. Furthermore, the addition of these hydrolysates to AAGP solution did not enhance the rate of germination of PA 3679 spores.

It was assumed that yeast extract could act as either chelating agent and thus suppress the inhibitory role of the metal on germination or act as metal donor to enhance the rate of germination. Four grams of yeast extract were dissolved in 100 ml of 5% versene solution and tested for germination. This solution did not show any increase or decrease in its germinating capacity. This indicated that if the yeast extract was supplying a metal it was not possible to remove it by chelation. The reverse control did not increase the ability of AAGP to germinate spores and from this it was concluded that the yeast extract was not acting as a simple chelating agent. The fact that the yeast extract was not a metal donor was confirmed by the addition of ash of yeast extract to AAGP solution which had no effect on the rate of germination by that solution.

Yeast extract was first boiled and then autoclaved for one hour at 121°C. and the suspensions prior to and after autoclaving were tested. Neither prolonged heating nor autoclaving of yeast extract affected the rate of germination of PA 3679 spores in yeast extract solution.

One hundred ml of 4% yeast extract solution (1) freshly prepared and (2) autoclaved were each treated with animal charcoal and filtered. This was repeated three times. The filtrate after three treatments with animal charcoal retained the original color of yeast extract and no change in its germinating capacity was noted.

<u>Dialysis of yeast extract</u>. Fifty ml of 4% yeast extract were dialysed against 500 ml of distilled water. The dialysate was concentrated to 50 ml and tested for germination along with the material in the dialysis tube. Both fractions showed activity approximately comparable to 2% yeast extract. Further dialysis of the fraction that was retained in the tube resulted in a solution of little activity and germination comparable to 3-4% yeast extract was observed in the mixture of first and second dialysate upon concentration to the original volume.

<u>Hydrolysis of yeast extract</u>. Hydrolysis of yeast extract with either N/10 base or acid at 121°C. had no effect on its ability to germinate spores. When yeast extract was treated with 6N H_2SO_4 and autoclaved for 1 hour, a black, sirupy liquid was obtained. This, on precipitation with $Ba(OH)$ for the removal of sulfate, gave deep red, clear liquid which was neutralized with NH_4OH. The liquor was then treated with animal charcoal, resulting in a straw-colored suspension. The suspension was adjusted to original volume and tested for germination. The suspension, either alone or in combination with the components of AAGP mixture, failed to stimulate germination.

DISCUSSION

Putrefactive anaerobe 3679 has been widely used as a test organism in thermal resistance studies. Various workers have used a wide variety of media for spore production, spore count determinations, and thermal resistance studies. However, very few authors have reported the use of a liquid medium without particles for the growth and sporulation of PA 3679. Brown (1956) and Lund (1954) attempted to use a "spent media" or simple trypticase media, but failed to obtain good sporulation. The TSP medium described in this thesis was effective in supporting good growth as well as a high percentage of sporulation of the test organism. No attempts were made to characterize the nutritional factors stimulating sporulation. The effect of peptone however was pronounced and although peptone could not replace trypticase in the medium, trypticase alone failed to yield good sporulation. Further studies on the effect of peptone fractions on stimulation of sporulation are needed.

The higher yield of spores in stirred cultures may be due to more complete utilization of the substrate under these conditions than in standing culture. PA 3679 is a strict anaerobe and grows at the bottom of the culture flask where utmost anaerobic conditions prevail. Although gassing in the initial stages will bring about circulation of the nutrients through the culture medium, cells tend to settle down as soon as the gassing stops (4-6 hours in unstirred cultures). Diffusion of the nutrients to the cells at the bottom of the flask may become the growth limiting factor. Since one cell forms one spore, limiting growth will also limit the spore crop. Moreover a sporulating organism may produce some activators for sporulation, since "spent media" has been known to increase sporulation in anaerobes.

However stirring alone does not yield as high a population of spores as does synchronization plus stirring. The process of sporulation in synchronized culture is completed in 44 hours as compared to 6 days in unsynchronized cultures.

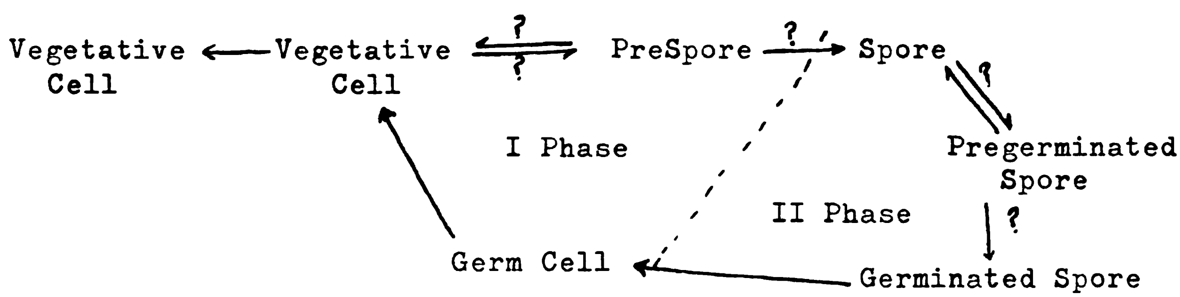
The exponential rate of sporulation which was observed in the stirred cultures lends an interesting insight into the problem of the formation of spores. Spores are formed after the exponential growth of the organism has ceased. We have thought in the past that the metabolic activity of the organism in this period was at a low ebb and yet it is capable of the protoplasmic rearrangement that leads to a spore.

Sporulation appears to be autocatalytic. It must be necessary to build up levels of key intermediates in the cells or in the culture medium prior to sporulation. This is borne out by the observation that the cells which have "settled out," as in unstirred cultures, sporulate at a greater initial rate than those of stirred cultures. The cells which have "settled out" are at higher unit concentration than corresponding stirred cultures.

The exponential rate of spore formation argues against depletion of nutrients as a cause of sporulation. During logarithmic growth, the substances are disappearing from the medium at an exponential rate. On the other hand, spores are only produced in the stationary phase at which time the metabolic activity of a constant population of cells is assumed to be constant. These cells would produce a linear decrease in nutrient concentration which is difficult to reconcile with exponential spore production.

The study of the mechanism of sporulation in the absence of growth media, "endotrophic sporulation," has not been possible with the anaerobes. Handling of the vegetative cells exposes them to oxygen and they lyse. Moreover, although the sporulation of Bacillus mycoides in absence of exogenous nutrients is well established, such phenomenon may not be general. In absence of any study of the process and nature of sporogenesis in anaerobes, one may conclude in words of Cook (1932) that "sporulating bacteria form spores because they form spores."

Oginsky (1955) has described sporogenesis as "defensive mechanism." However such "defense mechanisms" are not to be compared with the cyst formation in protozoa, where an organism develops a resistant coat over its body to protect itself from adverse conditions. Since spores are so radically different from the vegetative cells in their biochemical activity, they must be separate and distinguishable entities that could be pictured as an alternating generation in the life cycle of bacteria. The following diagram illustrates the above statement.



Spores grown in TSP medium were found to have the same heat resistance properties as of those obtained from infusion media provided the latter were freed from vegetative cells and debris. This however is in apparent contradiction to previous observations made by several authors that the heat resistance properties of the spores are dependent on their cultural history. Most of the workers in food microbiology feel that the spores produced in liquid medium free from particles have a lower heat resistance than those prepared in particulate media. But the one prime factor which has been neglected by almost all workers in their heat resistance studies is use of "clean spore" suspensions.

It is important that the spores which are used in heat resistance studies be free of debris which might aid in their recovery after heat damage. Heat resistance studies with highly purified spores of PA 3679 indicate that heat resistance is an inherent property of the spore despite its cultural history. Substances inhibitory or stimulatory to germination present in a recovery media will affect the results in heat resistance studies.

The nature of the heat resistance of spores is unknown, and has been attributed to the impermeability of the spore coat, the lipoidal components of the spore, the state of the enzyme and protein components in the spore and possibly the spore's bound water content. None of the above theories are adequate and fundamental study of the mechanism of heat resistance must be initiated.

The cleaning procedure as noted before involves aeration of the culture which results in the lysis of vegetative cells. The effect of oxygen may be two-fold. The anaerobes,which possess flavoprotein system of hydrogen transport, will form H_2O_2 (hydrogen peroxide) in presence of oxygen. Because of the absence of catalase activity in these organisms, peroxide might accumulate, and may cause lysis. In addition, the presence of peroxide may "Lwoff" the organism and stimulate the release of bacteriophage. Since most clostridia are lysogenic for phage, this would result in mass lysis.

It was also noted that rapid transfers of an actively growing culture for more than four times resulted in mass lysis. This could be due to carryover of enough dissolved oxygen during the transfer, since no strict anaerobic precautions were mentioned. Further studies are necessary to prove the exact role of oxygen in lysis of cultures of PA 3679.

Preliminary experiments to show rapid germination of spores of PA 3679 in either complex or defined nutrients were unsuccessful. These spores as noted before had been harvested and heated to 80°C. for 15 minutes in an effort to accelerate their release from sporangia. This may have resulted in the shrinkage of the vegetative cell around the sporangium rather than its rupture with the freeing of spores. There appeared to be a physical barrier to the uptake of germinating nutrients by the spores. This was indicated when the germination of the spores in nutritive media was studied by staining.

In later experiments when "clean spores" were obtained after the aeration treatment, and heat shocked for 10 minutes at 80°C., they germinated rapidly at room temperature in solution of peptone, thiotone, beef extract and yeast extract. At pH 7.2 rapid germination of PA 3679 spores in yeast extract solution could not be observed at temperatures above 45°C., although Wynne (1956, 1957) has reported germination of PA 3679 and other mesophillic clostridia at 75°C. Wynne has based his report of germination on his inability to recover spores incubated at 75°C., a temperature which is lethal to germinated spores after 10-15 minutes exposure. Buffered glucose solution without the addition of yeast extract did not bring about germination, even at the end of 24 hours incubation at room temperature.

A solution containing alanine, adenosine, glucose, and phosphate has been reported to germinate spores of the thermophillic bacteria B. coagilii, B. stearothermophillus and B. thermoacidurans in 5 to 24 hours. The same compounds caused the germination of spores of PA 3679 in 20-45 minutes. However yeast extract (4%) will germinate these spores in 10-15 minutes. This action of yeast extract at 4% concentration, which resulted in complete germination in 10-15 minutes, could not be achieved even after changing the levels of concentration of alanine, adenosine, glucose and phosphate (AAGP) mixture. Although no exhaustive attempts to characterize the accelerating factor in yeast extract have been made, the preliminary studies indicate that the compound may be a small peptide. It is destroyed by hydrolysis with 4 normal acid and not by autoclaving at 121°C. for 15 minutes in the presence of N/10 acid or base. It is dialysable and soluble in 70% alcohol, but insoluble in other organic solvents. The compound must be needed in a very small quantity, since the germination rate of AAGP mixture plus 1% yeast extract is equivalent to that of 4% yeast extract solution. One per cent yeast extract does not induce germination in less than 24 hours. The possibility that metallic ions were stimulatory or inhibitory to germination could not be demonstrated. Spores washed with versene or the inclusion of versene in yeast extract solution had no effect on the rate or percentage of germination. Moreover, the addition of yeast extract ash to AAGP mixture had no effect on rate of germination. Oginsky (1955) has described germination as a reversal of sporulation, but has put forward no evidence for her statement. The only work describing intracellular changes during the process of spore germination is reported by Fitzjames (1955). He observed changes in the distribution of phosphorus content of the spores during germination. The initial stages of spore germination or "awakening" proceed with a sudden rise in detectable respiratory activity, a loss in heat resistance, and permeability to stains. Fitzjames noted that there was an increase in cold-acid soluble phosphate fraction and a decrease in hot trichloroacetic acid soluble phosphate fraction. This effect was found to be independent of the phosphate supplied in the germinating solution. This indicates that during this initial phase of germination, protein bound phosphorus of the dormant spore becomes acid soluble. The other changes that have been noted during the process of spore germination are loss in weight, release of solids (dipicolinic acids), uptake of water, and alteration in response to acid hydrolysis. (Powell, 1953; Robinnow, 1953; Foster, 1956) The exact mechanism by which the dormant spores change into the actively metabolising form is still not understood.

The role of the simple organic compounds which initiate germination is unknown. These compounds perhaps supply energy to bring about unlocking of the occlusion structure of the spore coat and thus expose an active enzyme site to start the sequence of reactions (?) leading to the germinated spore. Although no conclusive studies are

available on the enzyme nature of the germination process in spores of bacteria, such an assumption could be accepted on the basis of the initiation of metabolic processes observed during the germination. The cleavage of adenosine by germinating spore (Lawrence, 1955), the presence of ribosidase enzyme in the spore (Powell, 1956), the oxidation of glucose by heat shocked spores (Church, 1955) and alanine deaminating activity of the spores (Levinson, 1956) are proof that the "awakening" process is accompanied by enzyme catalysed reactions. The "awakening" or pre-germination is reversible, and can be achieved by heat shocking or exposing the spore to sub-optimal concentration of the germinating solutions.

The philosophical question that then presents itself is, whether the spores' "awakening" is the first step in the germination process or whether germination is the sum total of the enzymic reactions leading to a germinated spore. The question will only be resolved when considerably more critical studies have been made on spore germination.

SUMMARY

The study of the physiology of spore formation and germination in a spore forming anaerobic bacterium has been initiated. A medium, TSP, and a technique was devised by which spores of Putrefactive Anaerobe 3679 (PA 3679) could be obtained as 'clean' preparations in short time. This medium contained trypticase but the addition of peptone had a pronounced effect on rate and extent of spore formation.

PA 3679 sporulated in TSP medium in 44 hours, as compared to the conventional two week period of incubation in particulate medium. Spores were obtained in concentrations of 10^9/ml of the medium. Stirring and synchronization of the cultures were effective in increasing the spore yield.

A 15-20 minute aeration of the sporulated cultures resulted in the lysis of sporangia and vegetative cells, facilitating the harvesting of 'clean' spores. Further cleaning was performed by repeated washings with distilled water.

Spores obtained from TSP medium were compared for their heat-resistance properties to those obtained from particulate medium. 'Clean' spores from either source were found to have the same heat resistance. Z values were of the order of 18.1-18.5, D_{250} values were 0.85-0.95, and F_{250} values were 4.9-5.1.

No previous studies have appeared on the rapid germination of the spores of PA 3679. Brown (1956) showed germination of PA 3679 spores in versene after the spores had been stored for nine months.

Brown's freshly prepared spores, however, did not germinate rapidly. On the other hand, PA 3679 spores obtained from TSP medium germinated rapidly in 4% yeast extract, and germination was completed in 10-15 minutes. A mixture of alanine, adenosine, glucose and phosphate solution germinated the spores of PA 3679 completely in 40-50 minutes.

The rate of germination in alanine, adenosine, glucose and phosphate mixture was comparable to those of 4% yeast extract when 1.0% yeast extract was added to the mixture. Attempts were made to characterize the accelerating factor present in yeast extract. It was dialysable, heat stable, and stable toward hydrolysis with N/10 acid or base, but labile to strong hydrolysis with 4N H_2SO_4. Metallic ions did not seem to effect spore germination.

The studies described in the thesis have afforded an insight into the process of sporulation and have demonstrated that sporulation is an autocatalytic process.

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