SOME PHYSIOLOGICAL CHANGES OCCURRING DURING THE SPORULATION OF <u>CLOSTRIDIUM BOTULINUM</u> TYPE A

Thesis for the Degree of Ph. D.

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Lawrence E. Day

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This is to certify that the

thesis entitled

SOME PHYSIOLOGICAL CHANGES OCCURRING
DURING THE SPORULATION OF
CLOSTRIDIUM BOTULINUM,
TYPE A

presented by

Lawrence E. Day

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ABSTRACT

SOME PHYSIOLOGICAL CHANGES OCCURRING
DURING THE SPORULATION OF
CLOSTRIDIUM BOTULINUM,
TYPE A

by Lawrence E. Day

An investigation of the sporogenesis of Clostridium botulinum, type A, was conducted to determine some of the fundamental physiological events taking place during this process. The final step in sporulation, from the immature forespore to the mature spore, was studied by placing the immature forespores in a simplified environment. It was observed that the only exogenous nutrient requirement for this step was fulfilled by L-alanine and L-proline and that these amino acids were metabolised via the Stickland Reaction. It appeared that these amino acids were required only as an energy source. L-arginine and L-alanine, and L-isoleucine and L-proline would also serve in this capacity, but L-ornithine and L-alanine would not. Neither L-arginine alone nor L-citrulline alone would furnish the energy requirements for this final step. With the use of chloramphenicol it was found that no protein synthesis was

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occurring during this developmental stage. Radioactive phosphorus studies coupled with known inhibitors of nucleic acid synthesis, mitomycin C and 8-azaguanine, led to the conclusion that deoxyribonucleic acid (DNA) synthesis is completed almost simultaneously with the end of the log growth phase and the initiation of sporulation, while ribonucleic acid (RNA) synthesis is required through the formation of the immature forespore but not during the maturation process. A study of the synthesis of some of the end products of growth and sporulation in a medium of 4% Trypticase revealed that valeric, propionic, and acetic acids were being formed. Using acetic acid-1-C^{14}, it was found that acetic acid was being utilized during vegetative growth at a slower rate than that at which it was being formed. However, during the early phase of sporulation acetic acid was being utilized at the same rate but the production of this material had slowed, resulting in a net decrease in the level of acetic acid in the culture. Fractionation of cells indicated that the acetate was being used for the synthesis of cell wall material, although this was not verified conclusively. The synthesis of dipicolinic acid was studied, and it was found that this material was formed prior to the appearance of heat resistant spores but after the appearance

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of refractile spores. Two enzymes, acetokinase and reduced diphosphopyridine nucleotide (DPNH) oxidase, were studied to determine the time of formation of heat resistance. Acetokinase was observed to have no resistance to heat in either the vegetative cell, intact spore, or an intermediate stage, or in cell-free extracts of these forms. The DPNH oxidase was found to develop stability towards heat during sporulation, the development of this resistance being initiated at the onset of sporulation. The study did not elucidate the mechanism(s) which initiate sporulation. If the pH and concentration of end products known to be present at the time of sporulation were preset in a fresh culture, sporulation was observed to occur no sooner than in a control culture.

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DURING THE SPORULATION OF

<u>CLOSTRIDIUM</u> <u>BOTULINUM</u>,

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By

Lawrence E. Day

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INTRODUCTION

The current interest in bacterial spores can be attributed to the remarkable properties of these unique biological entities. Spores possess an extremely high level of resistance to various chemical and physical agents, and a great amount of research has been carried out to determine the nature of this resistance. Also, the events taking place during sporulation are of interest to the biologist because sporulation is fundamentally a process of cell differentiation, a problem currently under much study. Sporulation studies have been approached from many different viewpoints, and usually each investigation has been concerned with only one facet of the problem. These detailed studies are certainly desirable and give insight into some of the problems under investigation, but the results are not always related to other events occurring in the cell during the same period. This study, therefore, was undertaken to view the process of sporulation in its entirety. The ultimate goals were to relate some of the major events occurring during sporulation, and to determine the event which initiates sporulation if possible. With this in mind, many aspects of sporulation were investigated. This involved using the technique of replacement (Hardwick and Foster, 1952) in an attempt to simplify environmental conditions during sporulation. Studies were made of the nutritional requirements for sporulation, the production and utilization of metabolic end products, the synthesis of nucleic acids during sporulation, and the effects of inhibitors of these synthetic processes. An investigation concerning the heat resistance of certain enzymes of the vegetative cell, forespore, and mature spore, and the time of appearance of this resistance was also carried out.

From these data certain conclusions were made, and some of the major steps occurring during sporogenesis were clarified. Although the more subtle changes were not studied, it is hoped that this investigation might give further impetus to the elucidation of the processes taking place during the transition from the vegetative cell to the spore.

HISTORICAL REVIEW

Nutritional and Environmental Requirements for Sporulation

Ordal (1956) stated three requirements necessary for sporulation to take place; *viz*., (a) the cells must be of the sporogenous type, (b) the cells must be in the proper physiological condition, and (c) the cells must be in the proper environment. Schaeffer et al. (1959) demonstrated that the first of these requirements is under genetic control by observing that a non spore-forming strain of *Bacillus subtilis* could be transformed to the sporogenic type by the deoxyribonucleic acid (DNA) extracted from a spore-forming strain of the same organism. Not much is known of the second requirement. In a well-synchronized culture of a spore-forming bacterium, all cells will sporulate simultaneously over a relatively short period of time, but it is not known what event or mechanism triggers the initial process leading to the formation of spores. Nakata and Halvorson (1960) have shown that large amounts of acetic acid and lesser amounts of pyruvic acid were produced and accumulated in the medium in a culture of *Bacillus cereus* growing in a glucose-yeast extract-salts

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medium. After the period of logarithmic growth when sporulation was initiated, the acids rapidly disappeared from the medium, and the pH of the medium increased. It was observed that during the transition from the vegetative cell to the spore, a system which could oxidize acetate was induced; this induction being the first recognizable step leading to the formation of spores. Gollakota and Halvorson (1960) demonstrated that this induction could be inhibited by α-picolinic acid with the result that the formation of spores was also inhibited.

A great deal of investigation has been carried out concerning the proper environment and the necessary mineral and nutritional requirements for sporulation of the aerobic spore-formers, and these have been reviewed extensively by Cook (1932), Knaysi (1948), Williams (1952), Stedman (1956), and Halvorson(1957a).

Less is known of the nutritional and environmental requirements for sporulation of the anaerobes. A medium of 4% Trypticase[1] was found to support sporulation of Clostridium botulinum, type A, of about 95% of the cells if the medium was fortified with 1 μg/ml of thiamine (Day, 1960). Lund

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[1]Baltimore Biological Laboratories, Baltimore, Maryland.

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(1956) also observed the necessity of added thiamine in a Trypticase medium in order to obtain a high degree of sporulation with the Putrefactive Anaerobe, PA 3679.

Wynne (1948) demonstrated that as the concentration of salts or the ionic strength of the medium increased, the degree of sporulation decreased with a culture of C. botulinum using Na_2SO_4, NaCl, and KCl. At a concentration of 2% NaCl in brain-heart-infusion medium, sporulation was completely inhibited. The inhibitory effect of NaCl was also noted by Leifson (1931), who reported that C. botulinum would sporulate in a basal medium of 1% peptone only when NH_4^+ and $PO_4^{\equiv}$ were added. The addition of $SO_4^{=}$ stimulated sporulation somewhat.

The inhibition of sporulation by glucose has been observed many times (Leifson, 1931; Kaplan and Williams, 1941; Blair, 1950), but the reasons for this inhibition are not clear. Leifson (1931) has stated that the inhibitory effect is due to the formation of acid, but Costilow (1962) has shown that sporulation will take place after all of the glucose has been utilized. Wynne (1948) reported that 0.8% glucose in brain-heart-infusion broth did not adversely affect sporulation of C. botulinum.

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Roessler and Brewer (1956) demonstrated that the deletion of methionine and phenylalanine from this medium suppressed the sporulation of C. botulinum. Sporulation was enhanced by the addition of L-alanine, L-glutamic acid, glycine, L-ornithine, sodium butyrate, sodium valerate, L-arginine and L-proline. Perkins and Tsuji (1962) could not demonstrate spores in the synthetic medium of Williams and Blair (1950), but obtained good growth in a more complex synthetic medium. This medium would support sporulation when the level of L-arginine was raised to about 70 μmoles/ml of medium. Further study by these investigators revealed that the L-arginine was metabolized through citrulline to ornithine, and that the greater requirement for this amino acid for sporulation was not due to incorporation of the amino acid into spore material. Rather, it was thought that a mole of adenosine triphosphate (ATP) was formed for each mole of citrulline broken down to ornithine, which furnished additional energy for the syntheses leading to sporulation.

Vinter (1962) found that the cystine requirement for spores of B. cereus was about five times higher than that for the vegetative cells. This demand for cystine occurred in the period prior to the appearance of young refractile forespores. At this time the organisms showed increased resis

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resistance to X-rays. The requirement for alanine or methionine during the sporulation process did not increase (Vinter, 1960).

The pH optimum for the sporulation of C. botulinum was established by Mohrke (1926) and Collier (1956) to be 7.0-7.2. Leifson (1931) stated that the pH at the time of sporulation is of greater importance than the initial pH, but he did not state what this value should be.

The effect of oxygen tension on the sporulation of the anaerobes is not clear. It was observed that various anaerobic organisms exhibit different responses under increasing oxygen tensions (Leifson, 1931). Sommer (1930) noted that a broth culture of C. botulinum will sporulate more quickly when exposed to the air than when the culture is sealed with vaseline, and Esty and Meyer (1922) reported that small amounts of oxygen in the culture medium were beneficial to sporogenesis. However, Wynne (1948) observed that the level of sporulation in broth cultures of C. botulinum was identical in either oxygen or in a natural gas atmosphere.

Replacement and Endotrophic Sporulation

Although it had been observed for many years that the vegetative cells of the aerobic spore-forming bacteria w

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will sporulate when placed in distilled water (Buchner, 1890; Schreiber, 1896; and Knaysi, 1945), it was not until 1952 that this phenomenon was studied in detail (Hardwick and Foster, 1952). They designated the technique of transferring vegetative cells to another solution as replacement, and the sporulation of an organism in the absence of exogenous nutrients as endotrophic sporulation.

Collier (1956) was the first to observe endotrophic sporulation in an anaerobic spore-former (*Clostridium roseum*). Lund (1956) reported that the Putrefactive Anaerobe (PA-3679-h) would not sporulate in distilled water and Day (1960) reported that *C. botulinum* would not sporulate when transferred to distilled water or buffer at various intervals of growth. Neither was sporulation observed when the vegetative cells of this organism were replaced in other solutions containing various combinations of amino acids and vitamins, solutions of enzymatically hydrolyzed casein, and solutions of various anions and divalent cations known to be of importance to sporulation in other and related species. However, Day (1960) showed that 4% Trypticase broth used in replacement supported sporulation to a high degree, and when the individual amino acids known to be present in Trypticase were combined as a synthetic medium and used as a replacement sc

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solution, a significant degree of sporulation occurred.

The Role of Dipicolinic Acid in Sporulation

Powell (1953) was the first to isolate and identify dipicolinic acid (DPA), pyridine-2,6-dicarboxylic acid, from extracts of spores of *Bacillus megaterium*. Martin and Foster (1957) attempted to elucidate the biochemical pathway leading to the synthesis of DPA by testing various amino acids and carboxylic acids as precursors of this compound. They found L-glutamic acid, L-aspartic acid, L-alanine, L-serine and L-proline to be the most efficient precursors; and that CO_2 could contribute 6.5% of the carbon in DPA. Significantly, L-tryptophane did not contribute to the synthesis. These workers felt that the formation of DPA may be the result of a C_4 and a C_3 condensation, involving either L-aspartic acid and pyruvic acid or L-alanine and oxalacetic acid. Powell and Strange (1959) have postulated the formation of DPA from α, ϵ- diketopimelic acid.

Dipicolinic acid is a strong chelating agent and occurs only in bacterial endospores in high concentrations (4-15% dry weight). It is synthesized just prior to the formation of resistant spores and is completely lost during germination (Halvorson and Howitt, 1961). Its function in

sporogenesis is not known although several have been suggested. The first of these is that DPA has some relationship to the thermostability of the spore. It was noted early that the thermoresistance of the spore became apparent immediately after the synthesis of DPA (Halvorson, 1957b; Black et al., 1960). Lechowich (1958) pointed out that sporulation at elevated temperatures resulted in spores which were more thermostable and which contained a higher level of DPA. It has also been known for some time that the heat resistance of the spore is related to the calcium content of the spore. Curran et al. (1943) have noted the relationship of the presence of Ca^{++} and heat stability in several organisms. There may be an interrelationship between the role of calcium and DPA in the heat resistance of the spore, and the several mechanisms proposed for this have been discussed by Halvorson and Howitt (1961). Young and Fitz-James (1959a) found that the cortex of the spores of _B. cereus_ was formed between double layers of the forespore membrane. When the cortex was about one-third formed, the spore coat and the exosporium were laid down on the outer side of the membrane; and at this time the forespore became refractile and the uptake of calcium commenced. The synthesis of dipicolinic acid followed. In a medium which was deficient in calcium, DPA was synthesized only in amounts

proportional to the calcium present with the subsequent lessening of refractility. However, Halvorson (1957) reported that the synthesis of DPA preceded or accompanied the accumulation of Ca^{++}. Shinagawa (1960) suspended the vegetative cells of _B. cereus_ in $CaCl_2$ and observed complete sporulation after 18 hours at 37 C. In this technique, the calcium was essential and irreplaceable. It was found that the spores thus produced contained a reduced amount of DPA, were less heat resistant, and showed increased sensitivity to $HgCl_2$ and phenol. Analyses of various aerobic spore-formers for nitrogen, carbohydrate, DPA, and phosphate showed little correlation between the concentrations of these materials and the heat resistance of the spores (Walker, 1961). It was observed that as the molar concentration of Mg^{++} increased with respect to the concentrations of DPA and Ca^{++} the heat resistance decreased. Portellada (1961) has made the interesting observation that 60 μg/ml of DPA inhibited endotrophic sporulation, but in a chemically defined medium concentrations of DPA up to 80 μg/ml did not interfere with sporulation. In fact, it was reported that 20-25% of the DPA was consumed (Portellanda, 1960).

DPA has also been shown to stimulate the electron transport system in _B. cereus_ (Halvorson et al., 1958), and that this involved a soluble DPNH oxidase (Doi and

Halvorson, 1961). However, Simmons (1961) observed that the DPNH oxidase of _C. botulinum_ was no more active in the presence of DPA than in its absence.

Other roles that have been assigned to DPA include stimulation of germination (Rieman and Ordal, 1960; O'Conner and Halvorson, 1960); the masking of activity of dormant enzymes in spores (Riemann, 1961); and the rendering of stability to enzymes through calcium DPA complexes (Young, 1959).

It is apparent that the function of DPA in the spore is not yet clear and that it may have more than one role. The fact that heat resistance develops after the synthesis of DPA (Halvorson, 1957; Black et al., 1960; Hashimoto et al., 1960) indicates that DPA may play some other primary role which affects thermoresistance secondarily.

Nucleic Acids and Sporulation

Only a minimum amount of work has been reported which describes the nucleic acid metabolism of the cell undergoing sporogenesis. The investigations of Delaporte (1950), Robinow (1956), and Fitz-James and Young (1959) indicate that the nuclear material of the spore is preformed in the vegetative cell and is enclosed in the spore during sporulation. The latter workers demonstrated that during the sporulation of _B. cereus_ two nuclear bodies of DNA were present but only one of these was incorporated into the mature spore. They found some variants of the same organism which did not synthesize DNA during sporulation and some that did, however the spore DNA always arose from that of the vegetative cell and was not a _de novo_ synthesis. Fitz-James (1954) reported that ribonucleic acid (RNA) comprises 50% of the total phosphorus of the spores of _B. cereus_ and 3-4% of the dry weight. However in _B. megaterium_ the RNA accounted for only 25% of the phosphorus. The DNA of the spores of both species was about the same: 1% of the dry weight. In endotrophically produced spores of the same two organisms, Hodson and Beck (1960) found the DNA content to be less than one-half of that found in the vegetative cells, and their results indicate that one discrete nuclear body preformed in the vegetative cell is enclosed in each spore. They also found that about 99% of the nucleic acids of the spores which were formed during endotrophic sporulation were synthesized from the pre-existing nucleic acids of the cell, allowing the degree of new syntheses to be about 1%. Other results indicate, however, that synthesis of some nucleic acid is essential to sporulation. Foster and Perry (1954) have shown that the purine analogue, 2,6-diaminopurine, will inhibit endotrophic sporulation of Bacillus mycoides. Young and Fitz-James (1959b) working with B. cereus var alesti demonstrated that there was no synthesis of RNA once sporulation was initiated, although there may have been some RNA turnover. The purine analogue, 8-azaguanine, inhibited sporulation at a concentration of 100 μg/ml at any time during the vegetative growth of the organism, but if the antimetabolite was added to the culture after it had become committed to sporulation no inhibition occurred. Day (1960) noted that the addition of vancomycin to a sporulating culture of C. botulinum resulted in the inhibition of sporulation after a lag period of about 10 hours. Vancomycin at that time was believed to act specifically through the inhibition of net RNA synthesis (Jordan and Innis, 1959). Thus, it was theorized that RNA synthesis was necessary through some intermediate stage of sporulation and those cells sporulating in the presence of the RNA inhibitor had already completed this step in sporogenesis. More recent data on the mode of action of this compound casts some doubt on the validity of this conclusion. This will be discussed later.

Heat-resistant Enzymes of Spores

Most of the reports concerned with the enzymes of spores have considered only the aerobic spore-formers; few of these have reported observations on the thermostability of the individual enzymes or enzymic systems involved. Stewart and Halvorson (1953) first demonstrated the presence of alanine racemase in the spores of *Bacillus terminalis* which retained 97% of its original activity after heating for 120 min at 80 C. Lawrence and Halvorson (1954) isolated two species of catalase from the spores of the same organism, *B. terminalis* later shown to be *B. cereus*, which differed in heat resistance but could not be separated by centrifugation. Sadoff (1961) reported that the heat resistant catalase first appeared at the end of the exponential growth phase at a rate equal to the rate of synthesis of DPA; however, the maximum in catalase activity had been achieved by the time that only 2% of the DPA had been formed. Using the Ouchterlony immuno-diffusion technique, it was shown that the heat labile and the heat resistant enzymes were the same proteins immunologically. Bach and Sadoff (1962) isolated a glucose dehydrogenase from cells of *B. cereus* in the initial stages of sporogenesis, but the enzyme was not present in cells during the logarithmic phase of growth. The enzyme extracted from cells in an early stage of sporulation was identical to that extracted from mature spores in kinetic characteristics, immunologic characteristics, and in chromatographic and electrophoretic experiments. However, the enzyme from mature spores was less heat stable than that extracted from an early stage in sporulation, and the same enzyme from germinated spores was quite labile and possessed a higher pH optimum for enzymatic activity. Simmons (1961) detected a number of the enzymes of the Embden-Meyerhof-Parnas (EMP) pathway in the extracts of the spores of C. botulinum. These enzymes along with diaphorase, acetokinase, phosphotransacetylase and coenzyme A transphorase were present in the spores at lower levels of activity than that found in the vegetative cells; but DPNH oxidase in the spore extracts had higher activities and much more thermostability than a DPNH oxidase of vegetative cells.

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MATERIALS AND METHODS

Clostridium botulinum, 62 A, originally obtained from the American Type Culture Collection was used in all investigations. The culture was maintained in spore suspensions which were produced in 4% Trypticase broth (Day, 1960). When spores were present in the growth medium at levels of about 90-95% of the final population, the spores were harvested by centrifugation, washed twice in sterile distilled water, and resuspended in 0.067 M phosphate buffer, pH 7.0. The suspensions were stored at 5 C until needed. Subsequent inoculations were made from portions of the suspension which had been heated to 80 C for 10 min.

A high degree of synchrony in the culture was desired in order to study the various aspects of sporogenesis. This was achieved by first inoculating 50 ml of 4% Trypticase broth containing 1 µg/ml of thiamine hydrochloride and 1 mg/ml of sodium thioglycollate with 5 ml of a heat-shocked spore suspension. This culture was incubated at 37 C for 10 hr, after which it was added to 150 ml of the same medium without the sodium thioglycollate. Anaerobiosis was then maintained in this 200 ml culture by fitting the flask with appropriate gas inlet and exhaust tubes, and allowing natural gas to flow slowly through the culture. The entire apparatus was placed in a water bath at 37 C. Samples of the culture were then removed at various times for further study as required. Depending on the type of study being carried out, variations in the volumes of cultures used were sometimes required.

It was shown by Day (1960) that replacement of the vegetative cells of C. botulinum could be carried out only in an atmosphere devoid of oxygen. The replacement was achieved by manipulation of the cultures in a plexiglass chamber described by Day (1960) which was filled with an atmosphere of nitrogen. The cells were removed from the growth medium by centrifugation in a type G Servall Angle Centrifuge,[2] and the cells resuspended in an equal volume of the appropriate replacement medium. Anaerobiosis was maintained either by the addition of 0.1% sodium thioglycollate to the replacement solution or by the use of the Brewer anaerobic jar.

The numbers of cells and spores were counted by two methods. Refractile spores could be counted in a highly reproducible manner by use of the Petroff-Hausser Bacteria Counter.[3] When the cells present in a suspension numbered

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[2] Ivan Sorvall, Inc., Norwalk, Conn.

[3] C. A. Hausser & Son, Philadelphia, Pa.

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above 10^8/ml, a 1:10 dilution of the suspension was made and the resulting suspension utilized for enumeration of cells. Twenty squares of the chamber were counted for both total counts and for spores and the average of this count multiplied by 2×10^7 gave the number of cells per ml. The bodies counted as mature spores were those appearing to be highly refractile with a well-defined edge. Free spores were those which were free of the sporangia. Immature forespores were those bodies which showed a slight degree of refractility and a diffuse edge and were still within the sporangium.

Since the Petroff-Hausser Bacteria Counter does not provide the means of differentiating between viable and non-viable cells, the determination of heat resistant spores required the use of a method which counts viable cells. The yeast-extract-starch-bicarbonate agar (YESB agar) of Wynne et al. (1955) and oval tubes were utilized for this purpose. In order to enumerate the number of spores, samples of the suspension to be counted were first subjected to 80 C for 10 min. Appropriate ten-fold dilutions were made, and four tubes of YESB agar were poured per dilution.

The method of Kinnory et al. (1958) was used to assay C^{14} activity in various cell fractions. Spores which had been grown in 4% Trypticase broth containing acetic acid-1-C^{14} were collected by centrifugation and fractionated as follows:

Spores

- washed twice, resuspended in 0.067 M phosphate buffer, pH 7.1
- heated 80 C, 10 min, centrifuged

  - dipicolinic acid (DPA)
  - heat-shocked spores
    - broken in 5% trichloracetic acid (TCA) in Omnimixer, centrifuged
      - TCA soluble fraction, including residual DPA
      - protein, lipid, nucleic acids, mucocomplexes
        - 75% ethyl alcohol 50 C, 1 hr, centrifuged
          - lipids
          - proteins, nucleic acids, mucocomplexes
            - 5% TCA, 100 C, 30 min, centrifuged
              - nucleic acids
              - proteins, mucocomplexes
                - 8N H_2SO_4, 120 C, 5 hr, filtered on membrane filter
                  - amino acids
                  - humins

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A 1 ml sample of each of the cell fractions was placed in a 20 ml radioactivity counting bottle provided with a foil-lined cap. The fraction was evaporated at 60 C, and the residue redissolved in a 1 ml of formamide. To this was added 8.6 ml of absolute alcohol and 10.4 ml of 0.58% 2,5-diphenyloxazole in toluene. All samples were prepared in duplicate and were counted in a Tricarb Scintillation Spectrometer with Control Model 314-DC[4]. The high voltage setting was 880 volts.

The insoluble humin which was collected on a type G-5 membrane filter[5] in the last step of the cell fractionation was assayed for C^{14} activity using a Nuclear-Chicago thin-window gas-flow counter,[6] Model 3037B, and the counts registered on a Nuclear-Chicago Model 182 scaling unit. The voltage setting was 1300 volts.

When the C^{14} was present as dl-alanine-1-C^{14} another method of assay was used. The radioactive amino acid was added to a replacement solution of L-alanine and L-proline containing a sporulating culture of _C. botulinum_. Any CO_2 evolved was flushed from the culture by passing a

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[4]Packard Instrument Company, Inc., La Grange, Illinois.

[5]Gelman Instrument Company, Chelsea, Michigan.

[6]Nuclear Instrument & Chemical Corporation, Chicago, Illinois.

stream of natural gas through the culture and then through a column containing 20% KOH. The culture was acidified at the end of sporulation in order to release dissolved CO_2. To assay for C^{14} activity in CO_2, a 1 ml sample of the KOH solution was placed in a 20 ml counting bottle with 15 ml of a scintillation solution. This scintillation solution allows for the counting of alkaline aqueous samples. The composition of the solution is given in the Appendix, Table A-III. The counts were made in the Tricarb Scintillation Spectrometer as described above.

In those experiments in which radioactive phosphorus (P^{32}) was used to assess the synthesis of nucleic acids, the activity of the various fractions was measured by use of the Geiger-Mueller Counter[7] with an end-window tube, model 3, 1.6 mg/cm^2. The counts were recorded by a Berkeley Decimal Scaler,[8] Model 100, with a high voltage setting of 1250 volts. Counting was carried out for varying lengths of time depending on the level of activity of the samples. The method of Hanawalt (1959) was used in assaying the uptake of P^{32} by various cell fractions. This method allows the

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[7]Radiation Counter Laboratories, Inc., Skokie, Illinois.

[8]Berkeley Scientific Corporation, Richmond, California.

assay of small amounts of the cell fractions on membrane filters directly. A 0.2 ml sample of cell suspension was filtered by vacuum through a type G-5 filter, 1 inch in diameter, immediately after being added to 5.0 ml of ice cold 5% TCA. The residue was then rinsed with 5.0 ml of distilled water. The filter was air-dried and counted. This count represented step 1 and contained the TCA insoluble fraction. The filter was then placed in a polyethylene cup containing 75% ethyl alcohol and allowed to remain for 2 hr at room temperature. The filter was then placed on the filter apparatus and rinsed with 5.0 ml of 50% ethyl alcohol. The filter was again dried and counted. This step removed the lipid portion and was designated step 2. The filter was placed in the polyethylene cup, 3.0 ml of 2N KOH were added, and the mixture incubated at 37 C for 18 hr. This treatment dissolves the membrane filter. The mixture was chilled to 5 C in an ice bath, 3.0 ml of 10% TCA added and filtered through a new membrane filter. The residue was rinsed with 5.0 ml of 5% TCA, and the filter was dried and counted. This was designated as step 3. The radioactivity incorporated into RNA was considered to be the counts/minute of step 2 minus the counts/minute of step 3. The radioactivity due to the P^{32} in DNA was the counts/minute of step 3 minus 2% of the counts/minute of step 1 (Hanawalt, 1959).

The metabolic activity of cells in the various stages of growth and sporulation was determined using the Warburg respirometer and manometric techniques as outlined by Umbreit, Burris and Stauffer (1959).

The organic acids resulting from the metabolism of C. botulinum in 4% Trypticase broth were isolated on a column of Celite[9] containing an internal indicator of 3-(4-anilino,1-naphthylazo) 2,7-naphthylene disulfonic acid monoammonium salt. The column was prepared as described by Wiseman and Irvin (1957), the only modification being the substitution of the above mentioned indicator for alphamine red-R indicator due to the lack of availability of the latter. The developing solvents consisted of various percentages by volume of acetone in Skellysolve B.[10] These were also prepared as described by Wiseman and Irvin (1957). The lower concentrations of acetone elute the higher molecular weight organic acids. The resulting eluates were titrated with 0.005N $Ba(OH)_2$. Standards of known amounts of acetic and butyric acids placed on the column and eluted resulted in 95-105% recovery.

The method for the assay of the DPA of bacterial

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[9] Johns-Manville Products, Detroit, Michigan.

[10] Skelly Oil Company, St. Louis, Missouri.

cells and spores was that of Janssen, Lund and Anderson (1958). The cells harvested from 200 ml of medium were resuspended in 5 ml of distilled water and autoclaved for 15 min at 15 psi. This suspension was acidified with 0.1 ml of 1.0 N acetic acid and allowed to stand at room temperature for 1 hr. The suspension was clarified by centrifugation. One ml of the color reagent was added to 4 ml of the supernatant. This consisted of a 1% solution of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1% ascorbic acid in 0.5 M acetate buffer, pH 5.5. The optical density was read at 440 mμ. A standard curve was prepared using known amounts of DPA.

Poly-β-hydroxybutyric acid was assayed for by the method of Law and Slepecky (1961). The cells were harvested by centrifugation in polypropylene tubes which had been washed in ethanol and hot chloroform. The cells were then placed in a volume of sodium hypochlorite equal to the volume of the original growth medium and allowed to stand for 1 hr at 37 C. The suspension was again centrifuged, washed with water and with acetone and alcohol. Boiling chloroform was then added to extract the polymer. This step was repeated three times. The chloroform was then evaporated and 10 ml of concentrated H_2SO_4 added. The tube was stoppered and heated for 10 min at 100 C in a water bath. After cooling, the sample was placed in a silica cuvette and the absorbancy at 235 mμ measured in a Beckman, model DU,[11] spectrophotometer.

Acetyl posphate was assayed by the method described by Lipmann and Tuttle (1945) and is based on the reaction of acyl phosphates with hydroxylamine to form hydroxamic acids. These complex in the presence of ferric chloride to form violet compounds. The absorbancy was measured at 540 mμ in a colorimeter, and succinic anhydride was used as a standard.

Paper chromatography was used for the isolation and identification of several volatile organic acids and several amino acids. The method of Kennedy and Barker (1951) was used for the identification of unknown volatile acids. Due to the volatility of these acids, they were first converted to the non-volatile ammonium salts. After the acids had been eluted from the Celite column, 1.0 ml of concentrated NH_4OH was added to each sample. The solvent was evaporated on a steam bath and the aqueous portion used to spot the chromatogram. A spot of 0.01 ml was applied at the origin on a 45 x 25 cm piece of Whatman No. 1, chromatographic grade filter paper. The paper was fixed into a cylinder, stapled and placed in a battery jar containing

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[11]Beckman Instruments, Inc., Fullerton, California.

200 ml of a solution of ethanol and concentrated NH_4OH in the ratio of 100:1. This was allowed to develop at room temperature for 6-8 hr. The paper was then dried at 100 C for 5 min, and sprayed with a solution of 50 mg of bromphenol blue and 211 mg of citric acid in 100 ml of distilled water. Samples of known acids were run in the same manner.

Two-dimensional ascending paper chromatography was also used in the identification of amino acids, the solvent system being a solution of isopropanol-acetic acid-water in a ratio of 3:1:1 by volume. The papers in this case were dried in the hood at room temperature for 3 hr. The ninhydrin spray was prepared by dissolving 0.2 g of ninhydrin in 100 ml of water which was saturated with n-butanol. After spraying the chromatograms, the papers were placed in an oven at 100 C for 5 min. Standards of known amino acids were also prepared in the same manner.

Cells and spores were broken by use of either the VirTis "45" Homogenizer[12] or the Servall Omni-Mixer[13] fitted with a Micro-Attachment. With the former, about 3 g of size no. 100 Superbrite glass beads[14] and 3 ml of cell

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[12] The VirTis Company, Inc., Gardiner, New York.

[13] Ivan Sorvall, Inc., Norwalk, Connecticut.

[14] Minnesota Mining and Manufacturing Company, St. Paul, Minnesota.

suspension were placed in the glass cup. The cup was placed in the plexiglas cylinder of the homogenizer, and ethanol from a cold bath at -20 F was circulated around the cup. The homogenizer was run at a setting of 100 for about 3 min for cells and for about 10 min for spores. With the Omni-Mixer, the ratio of beads to cells by weight was about 6:1. In this case, the cup was chilled in an ice bath prior to and during breakage of the cells.

Cell-free extracts were prepared by breakage of the cells in 0.05 tris buffer, pH 7.1. After breakage the suspension was clarified by centrifugation at 30,000 rcf for 1 hr, and the supernatant dialyzed against distilled water at 5 C for 15-18 hr.

DPNH oxidase activity was assayed by measuring the decrease in optical density at 340 mμ with DPNH as substrate. To determine the thermostability of the enzyme, it was assayed immediately after breakage of the cells. The extract was then heated at 80 C for 1 min and again assayed. The activities were compared.

The assay for acetokinase activity was that given by Rose et al. (1954) and is based on the principal described previously for the assay of acetyl phosphate (Lipmann and Tuttle, 1945). The thermostability of acetokinase was determined as for DPNH oxidase.

Protein concentrations were measured by the method of Stadtman et al. (1951) and involves the precipitation of protein by trichloroacetic acid with the resulting turbidity being measured in the colorimeter at 540 mμ. A standard curve was prepared using known quantities of lysozyme.

RESULTS

Sporulation of *C. botulinum* is first indicated morphologically by the swelling of the vegetative cell at one extremity. This occurs at the conclusion of the logarithmic growth phase when the maximum number of cells are present in a culture of 4% Trypticase. In a well synchronized culture about 90% of the cells initiate the sporulation sequence at the same time. Two to three hours after swelling has begun, the larger end of the cell begins to show differences in refractility. This particular stage of development has been termed the immature forespore (Vinter, 1962a), and is characterized by a refractile area with a diffuse edge within the swollen end of the cell. This refractility continues to increase, and the edge becomes clearly delineated until the highly refractile spore is observed within the sporangium. During the next 12-36 hr the spore is freed from the sporangium by autolysis. This is the sequence of sporulation from a morphological viewpoint and is illustrated in Fig. 1. These are the phases occurring during the transition from the vegetative cell to the free spore that were considered in this investigation.

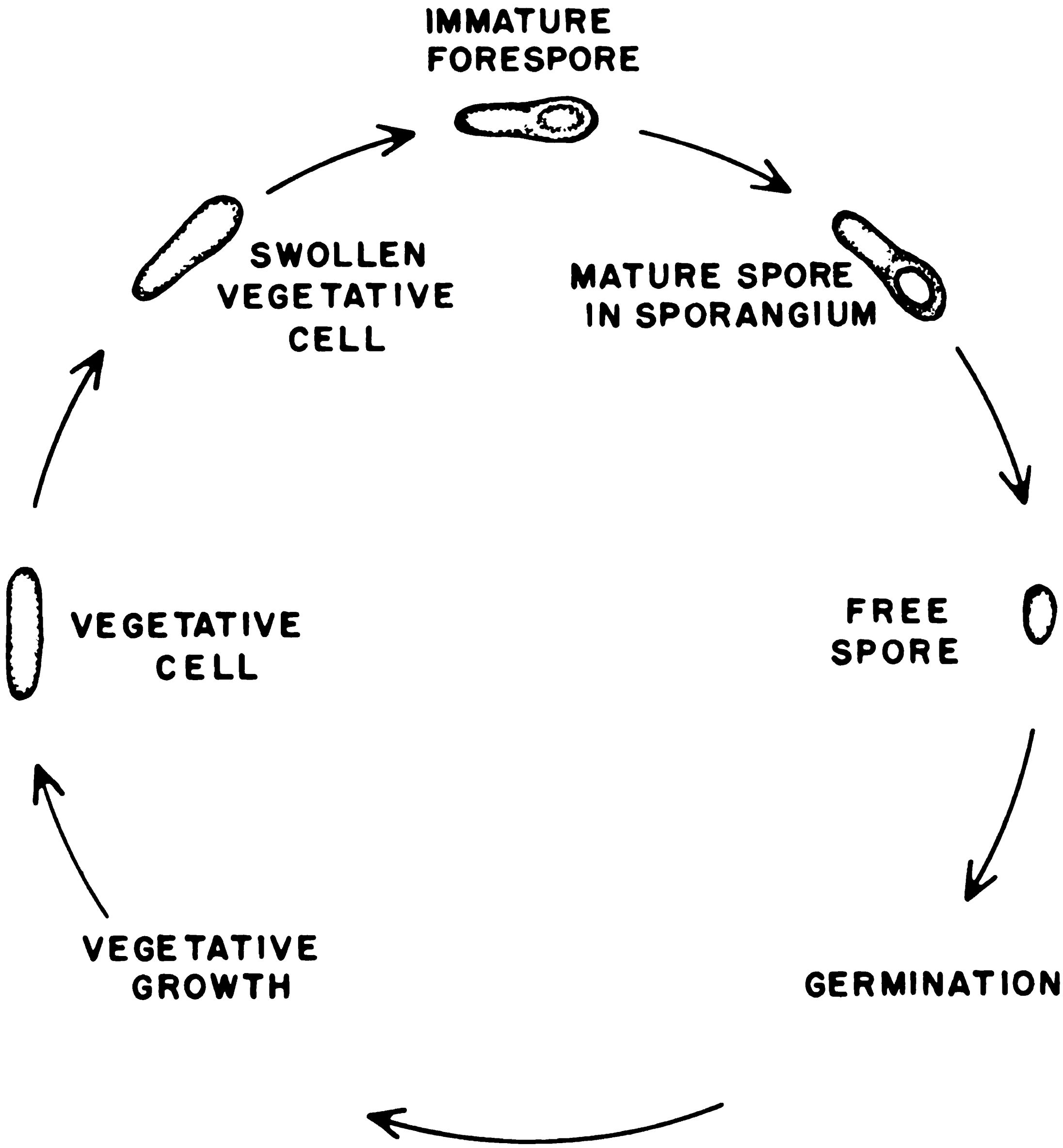


Figure 1. Sporulation cycle of C. botulinum.

Sporulation in Replacement Media

Previous work (Day, 1960) demonstrated that when cells of *C. botulinum* were replaced in fresh 4% Trypticase broth, sporulation occurred at the same time as in an unreplaced control culture. The amino acids known to be present in Trypticase could be combined into a synthetic medium which would support some sporulation in replacement. It was noted during the course of the present investigation that it was the immature forespore that could complete the final stages of sporulation to the mature spore in this amino acid replacement medium. Those cells which had not yet reached the forespore stage could progress no further in the sporulation cycle in this particular replacement medium. The first phase of this investigation was to determine the minimum nutrient requirements for this spore maturation process.

Acetate as a replacement nutrient: Previous work indicated that acetate might play some important role in sporulation. In order to establish if acetate would support sporulation in replacement, a 15 hr culture in which about 4% of the cells had completed sporulation was harvested and resuspended in a solution of potassium acetate containing 6 µc of acetic acid-1-C^{14}. The system was agitated with natural gas which was passed through 20% KOH in order to trap any evolved CO_2. A similar experiment was run utilizing sodium acetate in phosphate buffer, and in this experiment the acetic acid was isolated by column chromatography (Wiseman and Irvin, 1957) and titrated to determine the extent of utilization. The replacement cultures were acidified at the end of the incubation period to release any dissolved CO_2. The results of these two experiments are given in Table 1. There were no spores formed in either replacement solution and no utilization of acetate.

It was known from data obtained from the Baltimore Biological Laboratories that Trypticase contains a high level of acetate, and analysis of various samples of this product revealed that acetate was present in amounts from 3.5-5.4% by weight. Since the acetate was not observed to support sporulation when used alone in a replacement system, it was added to a synthetic medium containing the amino acids of Trypticase, and the cells replaced in this medium. The replacement medium of amino acids and acetate resulted in no significantly higher level of sporulation than did the control medium containing the amino acids alone (Table 2).

<u>Chemical groups of amino acids as replacement nutrients</u>: In an attempt to determine which amino acids contribute to the excellence of Trypticase as a sporulation medium, the

Table 1. Tests for sporulation and acetic acid utilization following replacement of C. botulinum cells in potassium acetate and sodium acetate solutions.

	Total Population*	Spores*	Cts/min acetate	Cts/min CO_2	mg/ml acetate
Experiment 1**					
Initial (15 hr)	1.4×10^9	5.0×10^7	5.4×10^6	185	---
Final (39 hr)	1.0×10^9	$<1.0 \times 10^7$	5.5×10^6	247	---
Experiment 2***					
Initial (15 hr)	7.2×10^8	4.3×10^7	4.2×10^5	101	2.898
Final (37 hr)	1.3×10^8	$<1.0 \times 10^7$	4.2×10^5	229	2.876

*Counts were made with the Petroff-Hausser Bacteria Counter.

**The replacement solution contained 2.0 mg/ml potassium acetate and 6.0 µc acetic acid-1-C^{14} in aqueous solutions at pH 7.3, cultures were incubated at 37 C in a water bath.

***The replacement solution contained 3.0 mg/ml sodium acetate, 5.0 µc acetic acid-1-C^{14} in 0.067 M phosphate buffer, pH 7.4; incubated at 37 C in water bath.

Table 2. Effect of 0.5% sodium acetate on sporulation of *C. botulinum* following replacement in a synthetic medium containing the amino acids of Trypticase.

	Amino acids* and acetate	Amino acids* alone
Culture age at replacement	24 hr	24 hr
Time of incubation	23 hr	23 hr
Initial population**	3.0×10^8	4.2×10^8
Final population**	8.0×10^7	9.8×10^7
Initial spores**	1.0×10^6	2.0×10^6
Final spores**	1.1×10^7	1.3×10^7

*Composition of the amino acid medium is given in the Appendix, Table I-A. Media were in screw-cap vials with 0.1% sodium thioglycollate and 0.067 M phosphate buffer, pH 7.2, final volume 10 ml. Incubated at 37 C.

**Counts were made with the Petroff-Hausser Bacteria Counter.

amino acids known to be present in Trypticase were grouped according to their distinctive chemical characteristics, i.e., the monoaminomonocarboxylic amino acids, monoaminodicarboxylic amino acids, sulfur-containing amino acids, basic amino acids, aromatic amino acids, and the heterocyclic amino acids. The acids in each group were combined in the same concentrations as found in Trypticase to prepare media to be used in replacement. Table 3 gives the results of replacement in these groups of amino acids. None of these would support further sporulation. The control in this instance was the increase in the number of spores observed in a medium of 4% Trypticase.

<u>L-alanine and L-proline as replacement nutrients</u>: The Stickland Reaction is one of the principal means by which <u>C</u>. <u>botulinum</u> can obtain energy when growing on an amino acid medium as the sole source of carbon and nitrogen (Clifton, 1940). This is a coupled reaction between two amino acids, one acting as a hydrogen acceptor and the other as a hydrogen donor. One of the most common pairs of amino acids used by the clostridia is L-alanine and L-proline (Stickland, 1935a,b), and it is known that <u>C</u>. <u>botulinum</u> rapidly ferments this pair of amino acids (Costilow, 1962). Thus, if an energy source was the only limitation, these two amino acids would be expected to support sporulation to the same extent

Table 3. Tests for sporulation of *C. botulinum* following replacement in solutions of various groups of amino acids.

Medium*	Culture age at Replacement	Time of Incubation	Initial Population**	Final Population**	Initial Spores**	Final Spores**
Monoamino-monocarboxylic acids	19 hr	24 hr	4.0×10^{8}	8.0×10^{7}	5.0×10^{6}	1.0×10^{6}
Monoamino-dicarboxylic acids	19 hr	24 hr	4.1×10^{8}	1.4×10^{7}	4.0×10^{6}	1.0×10^{6}
Sulfur containing amini acids	19 hr	24 hr	4.9×10^{8}	1.2×10^{8}	4.0×10^{6}	3.0×10^{6}
Basic amino acids	18 hr	21 hr	2.8×10^{8}	2.2×10^{8}	2.5×10^{7}	2.0×10^{7}
Aromatic amino acids	18 hr	21 hr	2.9×10^{8}	1.0×10^{8}	2.0×10^{7}	2.0×10^{7}
Heterocyclic amino acids	18 hr	21 hr	4.0×10^{8}	9.0×10^{7}	4.6×10^{7}	4.5×10^{7}
Control	18 hr***	22 hr	2.9×10^{8}	3.6×10^{8}	1.5×10^{7}	3.1×10^{8}

*The composition of each replacement medium is given in the Appendix, Table II-A. Each medium was in a screw-cap vial and contained 0.1% sodium thioglycollate; pH 7.0; final volume 10.0 ml; incubation temperature 37 C.

**Counts were made with the Petroff-Hausser Bacteria Counter.

***Control not replaced; time of initial count.

as did the synthetic amino acid medium previously described.

Fig. 2 shows the results of replacement of the cells of a 12-hr culture of C. botulinum in a solution of L-alanine and L-proline. At the time of replacement about 40% of the cells were in the immature forespore stage and about 1% of the total population had completed sporulation. During the next 25 hr most of those cells which had reached the forespore stage developed to maturity in the L-alanine, L-proline solution, while those which had not yet reached the forespore stage showed no further development. The level of sporulation in the control reached about 93% of the final population.

If a culture was allowed to proceed in development until practically all of the cells had reached the fore-spore phase before replacement in an L-alanine and L-proline replacement solution, sporulation in replacement proceeded as in the control culture (Fig. 3). At the time of replacement there were about 10% of the final mature spore population already present, and practically all cells had advanced beyond the swollen state. In this particular experiment, cells were also replaced in a sodium acetate solution and in an L-proline solution. There was no increase in spore numbers in either of these replacement solutions.

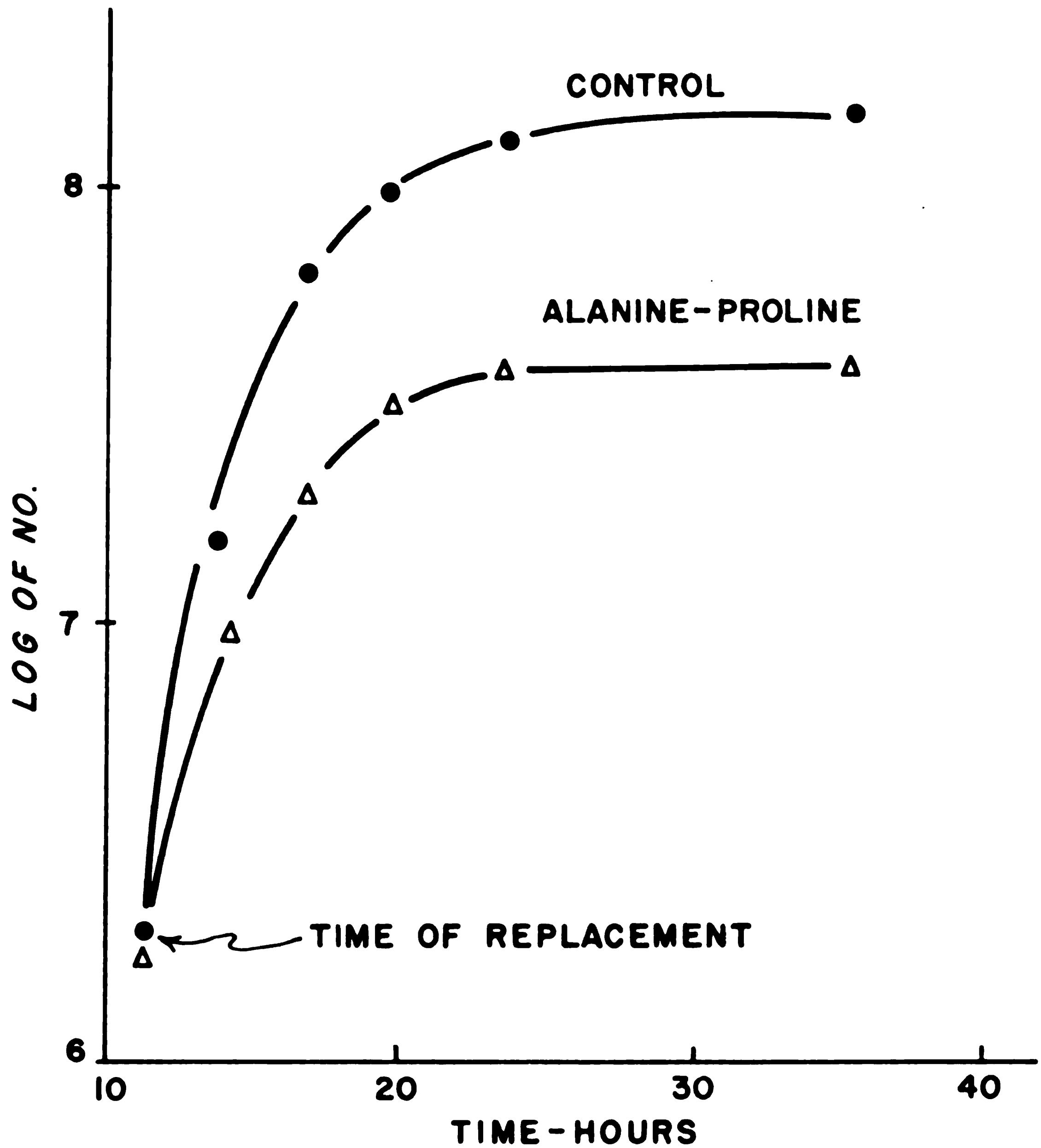


Figure 2. The formation of refractile spores of C. botulinum in a replacement solution containing L-alanine and L-proline. The concentration of amino acids was 2 mg/ml in 0.067 M phosphate buffer, pH 7.2. The final volume of 10 ml was in screw-cap vials with 0.1% sodium thioglycollate. Incubation temperature was 37 C. Replacement was at 11 hr. The control was replaced in the same sporulation medium.

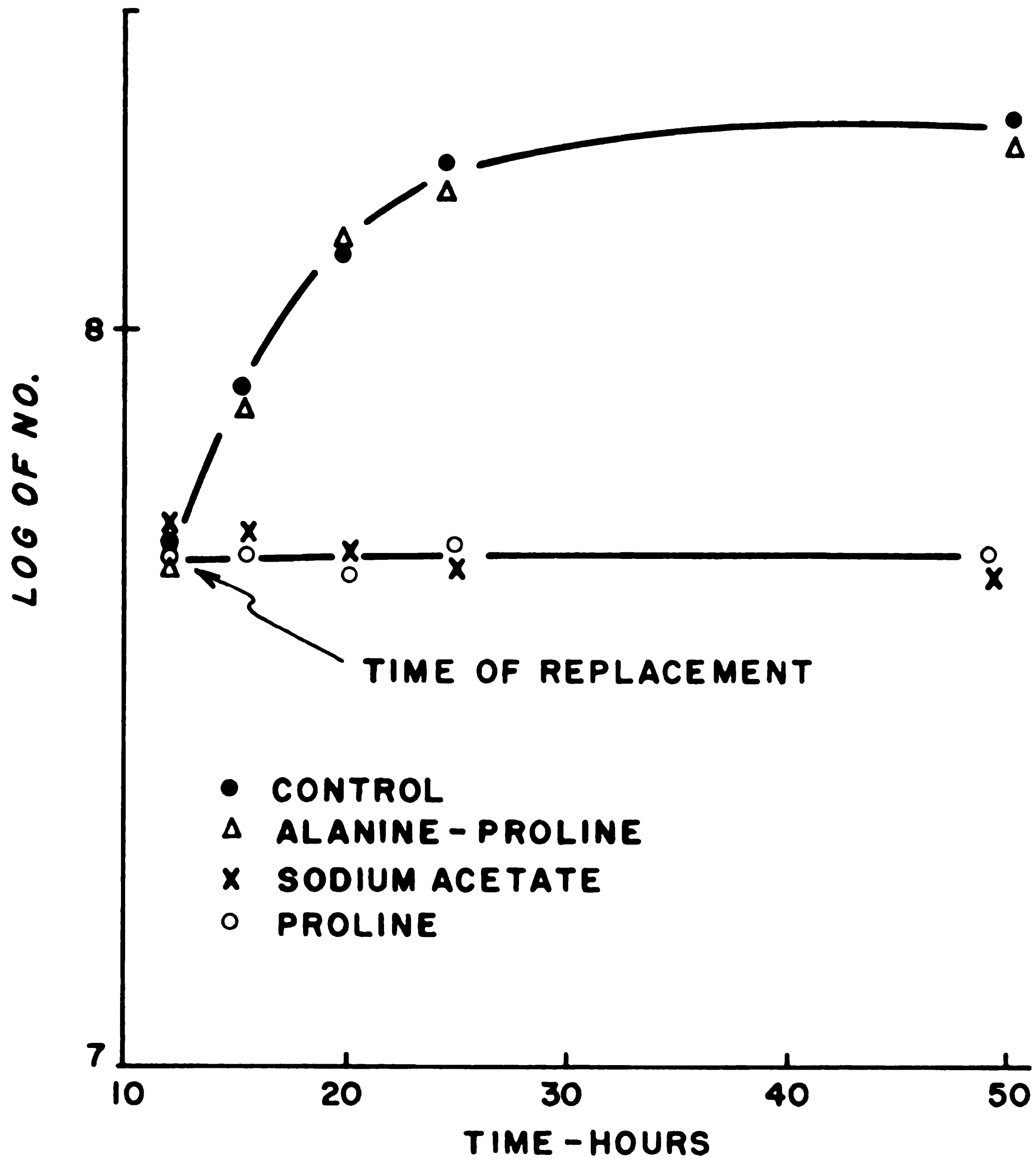


Figure 3. The formation of refractile spores of C. botulinum in a replacement solution containing L-alanine and L-proline, and in L-proline solution and in sodium acetate solution. The concentration in all solutions was 2 mg/ml in 0.067 M phosphate buffer, pH 7.2. The final volume of 10 ml was in screw-cap vials with 0.1% sodium thioglycollate. Incubation temperature was 37 C. Replacement was at 13 hr. The control was replaced in the same sporulation medium.

The spores that were observed in these experiments were spores that were counted in the Petroff-Hausser counting chamber; and, thus, there was no assurance that they were heat resistant spores. In order to determine if these were fully mature, heat resistant spores and not just refractile spore forms, cells were again replaced in a solution of L-alanine and L-proline, and the numbers of heat resistant spores determined. This was achieved by heat-shocking samples of the replacement culture at 80 C for 10 min and plating appropriate dilutions of the samples in oval tubes with the YESB agar of Wynne (1948). Fig. 4 illustrates that the spores thus formed in a replacement medium of L-alanine and L-proline were heat resistant. The numbers of spores formed in the replacement environment were not as great as those formed in the Trypticase control due to the fact that a large fraction of the cells had not yet attained the proper stage of development.

The results to this point indicated that the energy-yielding Stickland Reaction involving L-alanine as reductant and L-proline as oxidant could support that step in the sporulation cycle of *C. botulinum* from the immature forespore to the mature spore. The overall reaction is illustrated as follows:

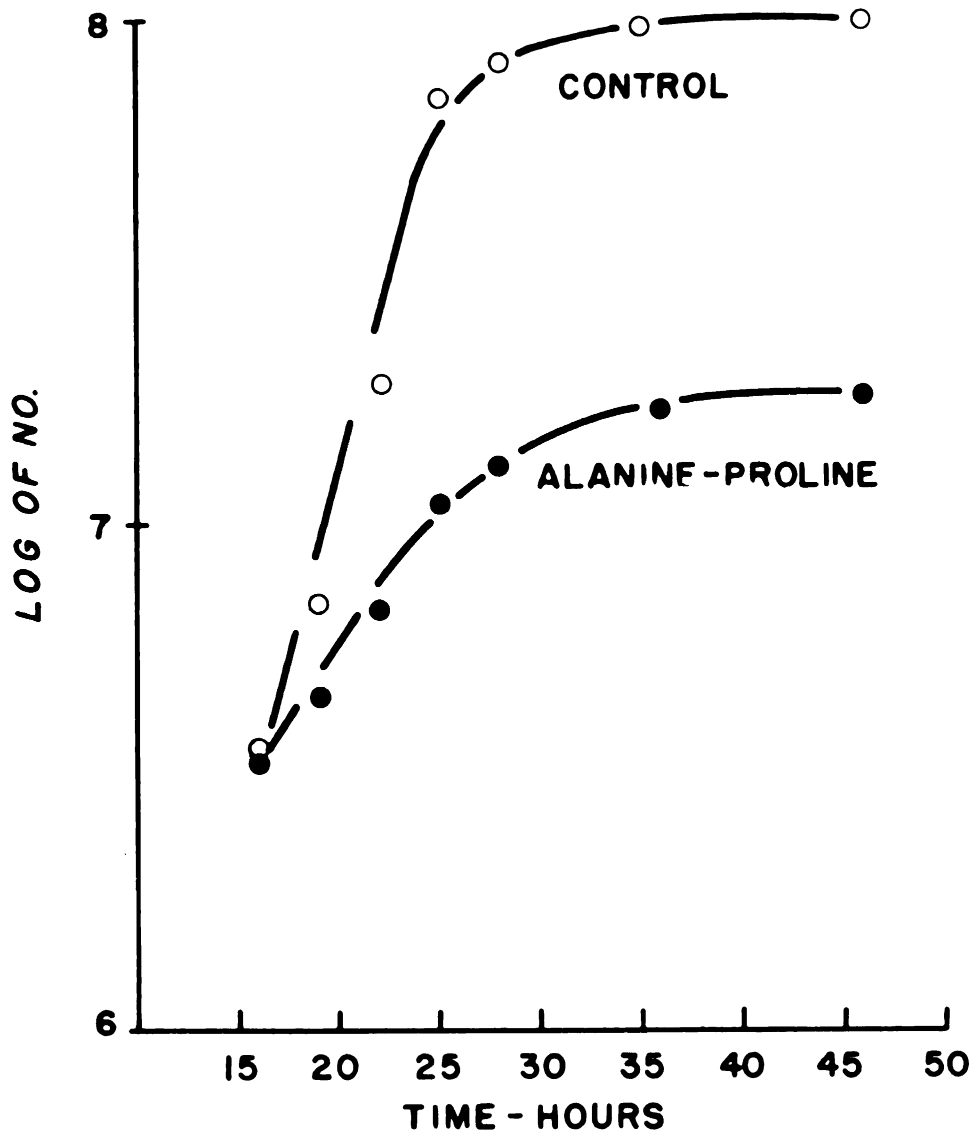
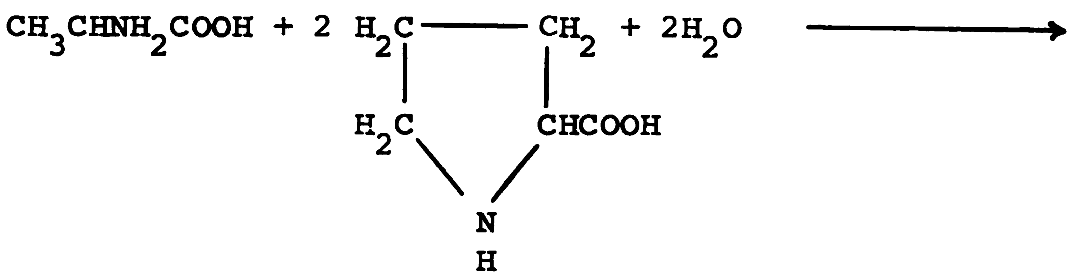


Figure 4. The formation of heat resistant spores of C. botulinum in a replacement solution containing L-alanine and L-proline. The concentration of amino acids was 2 mg/ml in .067 M phosphate buffer, pH 7.2. The final volume was 50 ml in a flask stirred with natural gas. Incubation was at 37 C in a water bath. Replacement was at 16 hr.



L-alanine L-proline

$CH_3COOH + 2NH_2(CH_2)_4COOH + NH_3 + CO_2$

acetic acid δ-aminovaleric acid

This did not, however, preclude the possibility that some synthetic reactions requiring either the amino acids or the products of fermentation were taking place at this time. In order to determine if proteins were being synthesized during the developmental step in sporulation which could be supported solely by L-alanine and L-proline, chloramphenicol was added to a culture replaced in L-alanine and L-proline and to a culture replaced in the same sporulation medium. In this experiment, one culture was replaced early in the sporulation cycle when about 50% of the cells were in the forespore stage and another culture when almost the entire population was at this stage of development. Figure 5a represents sporulation in replacement of those cultures which were replaced before all cells had reached the forespore stage. The presence of chloramphenicol had no

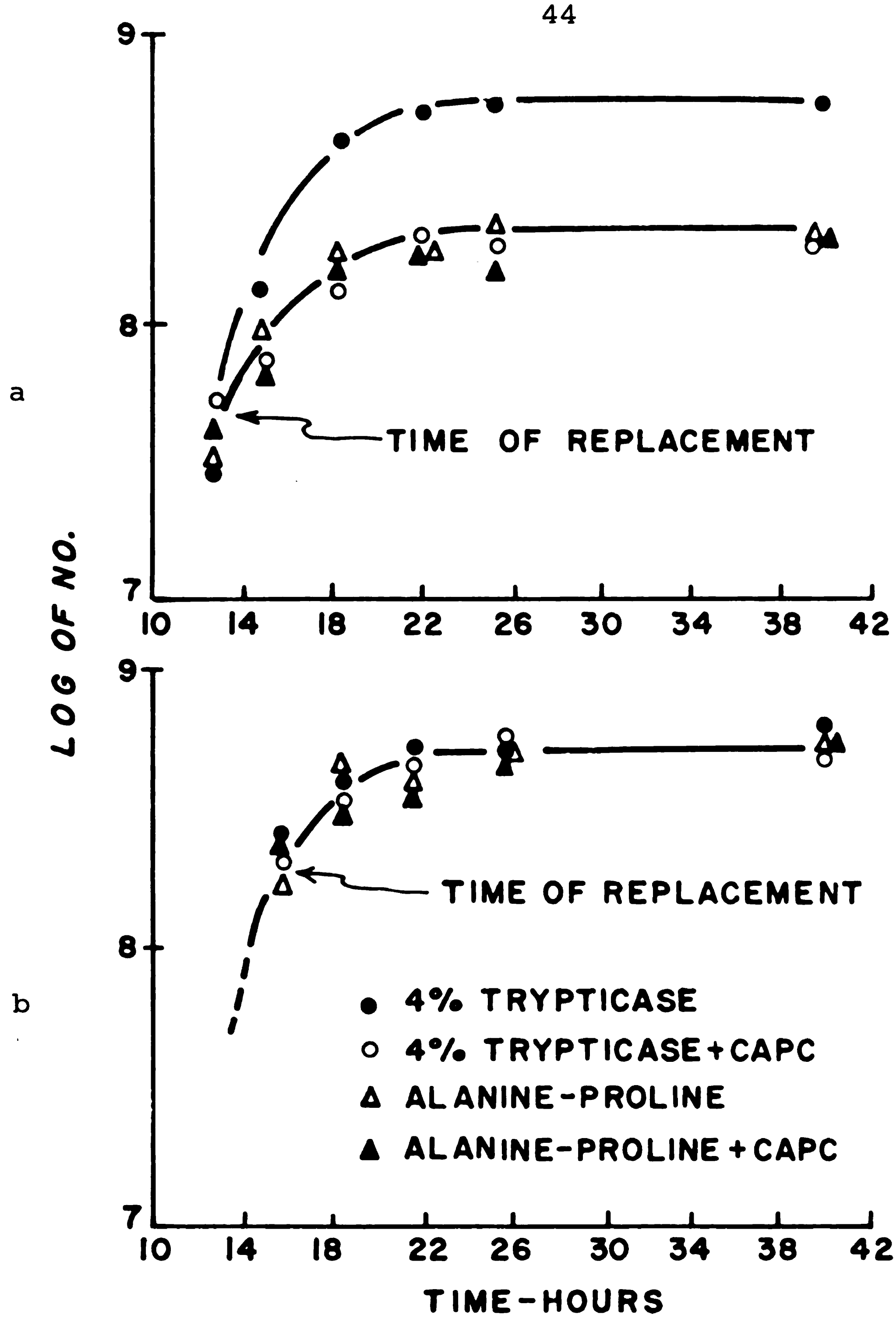


Figure 5. The effect of chloramphenicol on sporulation of C. botulinum, following replacement in a solution containing L-alanine and L-proline. The amino acid concentration was 2 mg/ml in 0.067 M phosphate buffer, pH 7.3. The final volume of 10 ml was in screw-cap vials with 0.1% sodium thioglycollate. The concentration of chloramphenicol (CAPC) was 100 μg/ml. Replacement was 13.5 hr (a) and 16 hr (b). The incubation temperature was 37 C. Cell counts were made with the Petroff-Hausser Bacteria Counter.

effect on the maturation of spores in solutions containing L-alanine and L-proline. However, the culture in 4% Trypticase with added chloramphenicol reached only the level of sporulation observed in the cultures in L-alanine and L-proline, indicating that protein synthesis was necessary for that step in sporulation from the swollen vegetative cell to the immature forespore. When almost all cells were in the forespore stage at the time of replacement, sporulation reached the same final level in all cultures even in the presence of chloramphenicol (Fig. 5b). These data indicate that no protein synthesis occurs during sporulation in a solution of L-alanine and L-proline.

Assays were run for Stickland reaction products of L-alanine, L-proline fermentation to insure that the reaction was in operation during the spore maturation process. Samples were removed from a culture replaced in a solution of L-alanine and L-proline at various times, the cells removed by centrifugation, and the supernatant fractionated on a Celite column (Wiseman and Irvin, 1957). Only one band appeared on the column during elution with solvents containing concentrations of acetone at 5% or higher. This band was identified as acetic acid by the method of Kennedy and Barker (1951). The accumulation of this acid during sporulation is evident in Fig. 6.

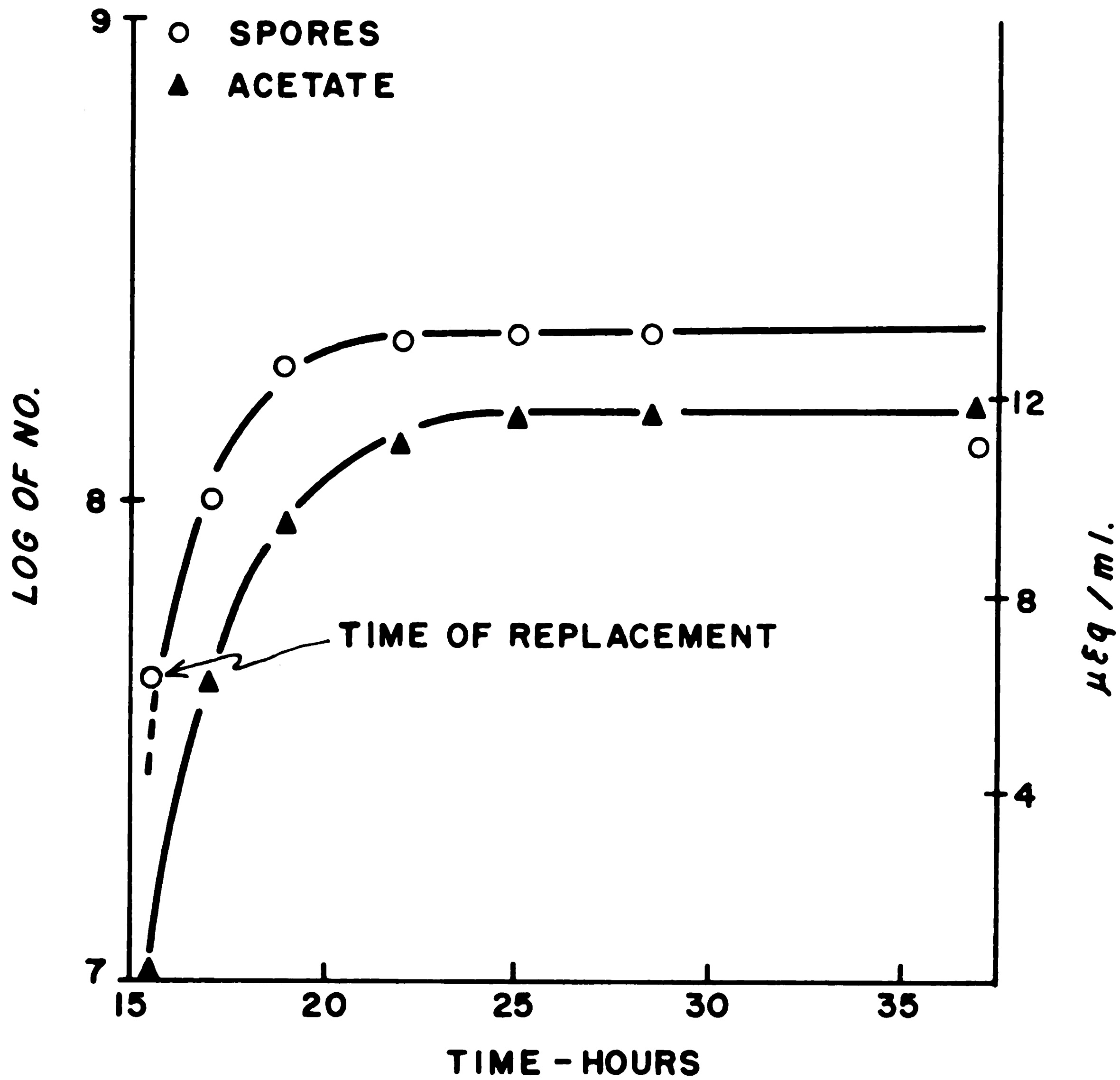


Figure 6. The formation of refractile spores of C. botulinum and the production of acetic acid in a replacement solution containing L-alanine and L-proline. Amino acid concentration was 2 mg/ml in 0.067 M phosphate buffer, pH 7.3. Final volume was 10 ml in screw-cap vials. Incubation was at 37 C in Brewer jar containing nitrogen.

Utilizing known samples of L-alanine, L-proline, and δ-aminovaleric acid, it was shown that these acids could not be eluted from the column with the solvents used. In order to show the production of δ-aminovaleric acid as another reaction product of the Stickland Reaction, samples of the replacement medium before the cells were replaced and after maximum sporulation had been attained were chromatographed. Table 4 summarizes the results of these chromatographs. Only L-alanine and L-proline were present before sporulation, while δ-aminovaleric acid was also present in the reaction mixture after sporulation.

Table 4. Chromatography* of L-alanine, L-proline replacement medium before and after sporulation of C. botulinum.

Sample	No of spots	Color	Rf
Medium before	1	purple	.34
sporulation	1	yellow	.67
Medium after	1	purple	.34
sporulation	1	yellow	.67
	1	purple	.44
L-alanine	1	purple	.34
L-proline	1	yellow	.68
δ-aminovaleric acid	1	purple	.43

*Solvent system used was isopropanol-acetic acid-water (3:1:1 v/v) and the chromatograph was allowed to develop for 6 hr at room temp. Ninhydrin, 1 g/500 ml n-butanol, was used as developer.

Samples removed from a replacement culture were also analyzed for acetyl phosphate since this is an intermediate in the Stickland Reaction (Nisman, 1954); however, the assay used did not reveal the presence of this compound.

Even though Fig. 6 indicates the production of acetic acid during sporulation in alanine and proline, this does not preclude the possibility that acetate was being utilized at a rate less than the rate of production. Therefore, cells were replaced in L-alanine and L-proline containing acetic acid-1-C^{14}, and the medium assayed at various times for the radioactive acetate. That acetate was not used in sporogenesis in the replacement medium is evident in Fig. 7. An increase in the numbers of spores was observed, but the activity of the C^{14} acetate remained constant. This was determined by isolating the acetic acid by the method of Wiseman and Irvin (1957) and counting the radioactivity by the method of Kinnory et al. (1958).

The next step was to determine if the L-alanine in the medium was being utilized directly by the cells in some synthetic process in addition to its utilization in the Stickland Reaction. Cells were replaced in an L-alanine and L-proline solution containing 5 μc of dl-alanine-1-C^{14}. Since the carboxy carbon of L-alanine is released as CO_2 in the Stickland Reaction, it would be expected that all of

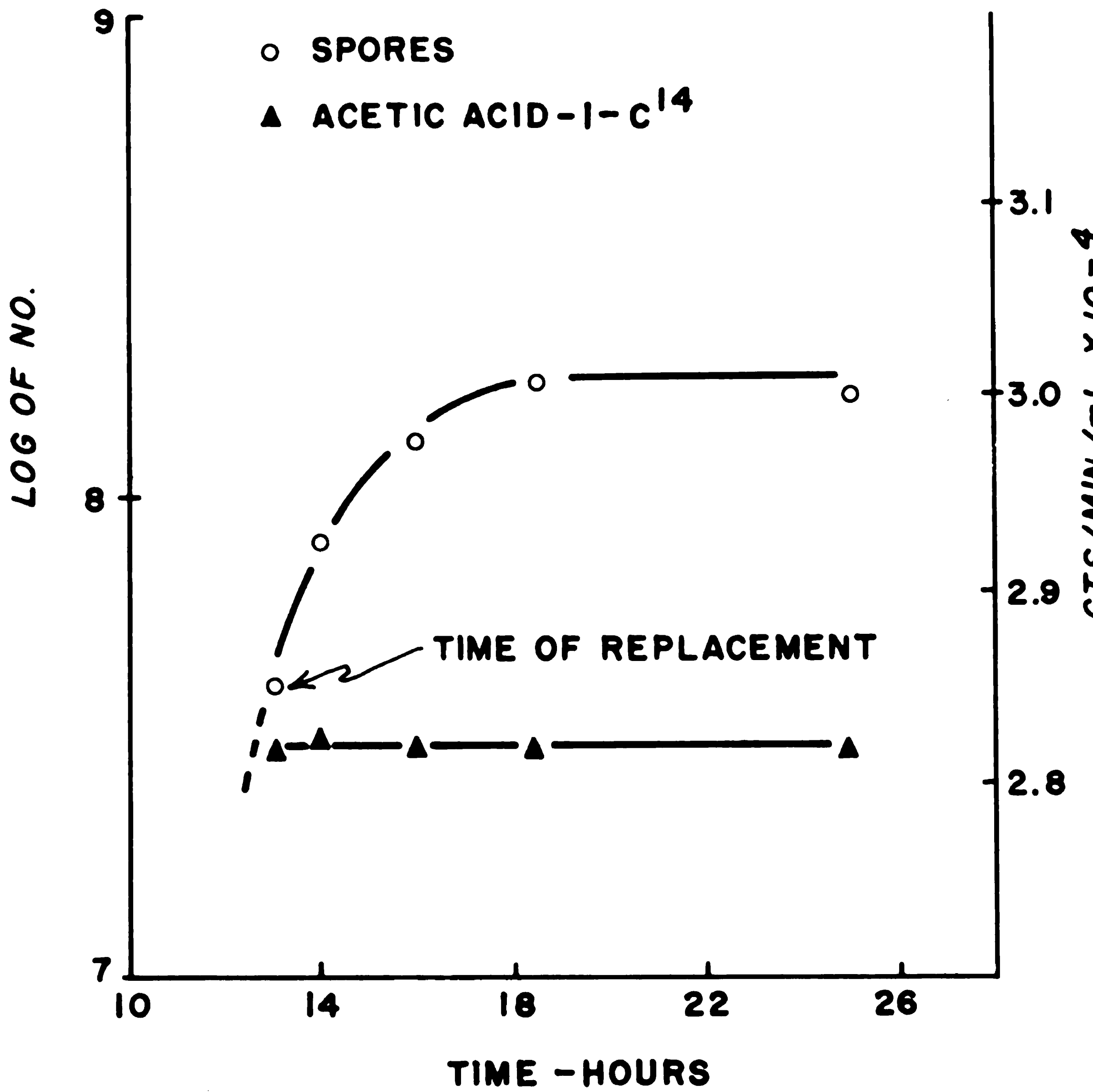


Figure 7. The formation of refractile spores of C. botulinum in a replacement solution containing L-alanine, L-proline and acetic acid-1-C^{14}. The amino acid concentration 2 mg/ml in 0.067 M phosphate buffer, pH 7.3. The solution contained 5 µc acetic acid-1-C^{14}. The final volume was 50 ml in a flask stirred with natural gas. Incubation was at 37 C in a water bath.

the C^{14} label utilized would be recovered as $C^{14}O_2$ if no alanine per se were being used for synthesis. The replacement culture was agitated with natural gas to remove CO_2 and to provide anaerobiosis. The effluent gases were passed through a column containing 20% KOH to trap the CO_2 released. After sporulation had reached the maximum, the culture was acidified to release all CO_2. This experiment indicated that all of the label utilized was found in CO_2 (Table 5), adding to the evidence that L-alanine per se was not used for synthesis.

The above data did not exclude the possibility that the acetyl-coenzyme A (acetyl-Co A) or acetyl phosphate produced as intermediates in the oxidation of L-alanine were used in synthetic processes. While exogenous acetate was not used, these high energy intermediates formed intracellularly might be utilized preferentially over exogenous acetate in synthetic processes. If this were true, another amino acid which can act as the reductant but not yield acetic acid as a product would not be able to support the sporulation when used in replacement with L-proline. *C. botulinum* is known to be active in the presence of L-isoleucine and L-proline (Costilow, 1962), and the products are valeric and δ-aminovaleric acids respectively (Nisman, 1954). Therefore, these two amino acids were used in

Table 5. The distribution of C^{14} in a replacement culture of C. botulinum in L-alanine, L-proline, and dl-alanine-1-C^{14}.

Total population, initial*	1.1×10^9
Total population, final*	4.4×10^8
Spores, initial*	7.4×10^7
Spores, final*	2.7×10^8
Cts/min, alanine, initial	3.57×10^6
Cts/min, CO_2, initial	2520
Cts/min, alanine, final	2.40×10^6
Cts/min, CO_2, final	1.11×10^6
Total cts/min, initial	3.57×10^6
Total cts/min, final	3.51×10^6
Percent C^{14} recovery	98.6%

The replacement medium contained 2 mg/ml of L-alanine, 2 mg/ml of L-proline, 5 μc of dl-alanine-1-C^{14} in 0.01 M phosphate buffer, pH 7.4. The culture was incubated at 37 C for 13 hours.

*Counts were made by the Petroff-Hausser Bacteria Counter.

replacement solutions and the resulting level of sporulation was compared to the level of sporulation observed in a solution of L-alanine and L-proline. The results show that sporulation proceeded to the same level in both cultures (Table 6). This indicates that an active form of acetate is not essential to this step of sporulation.

<u>The L-arginine to L-ornithine cycle and spore maturation</u>: The report of Perkins and Tsuji (1962) that the L-arginine to L-ornithine cycle is an important energy-yielding reaction in the sporulation of <u>C. botulinum</u> prompted the study of this cycle as a means of supporting sporulation in replacement. Cells undergoing sporulation were replaced in a solution containing L-arginine and also in a solution of L-citrulline. Since the energy-yielding step is from L-citrulline to L-ornithine, L-ornithine was not used in replacement. The results of this study (Table 7) indicate that neither of these amino acids alone can support the process of spore maturation.

Both L-arginine and L-ornithine can serve as acceptor amino acids in the Stickland Reaction, although it is believed that L-arginine is degraded to L-ornithine which acts as the final acceptor. When these two were used in conjunction with L-alanine in replacement systems, it was found that the L-arginine, L-alanine solution supported sporulation but the

Table 6. Replacement of _C_. _botulinum_ in solutions containing L-alanine and L-proline, and in L-isoleucine and L-proline.

Replacement medium	Time of replacement	Time of incubation	Initial population*	Final population*	Initial spores*	Final spores*
L-alanine, L-proline**	10 hr	15 hr	$7.7X10^{8}$	$5.2X10^{8}$	$1.0X10^{7}$	$1.5X10^{8}$
L-isoleucine, L-proline**	10 hr	15 hr	$7.5X10^{8}$	$4.7X10^{8}$	$2.0X10^{7}$	$1.6X10^{8}$
Control	10 hr***	15 hr	$8.6X10^{8}$	$5.1X10^{8}$	$2.0X10^{7}$	$4.7X10^{8}$

*Counts were made by the Petroff-Hausser Bacteria Counter.

**Each replacement solution contained 2 mg/ml of each amino acid indicated in 10 ml of 0.067 M phosphate buffer, pH 7.4. Solutions were in screw-cap vials and incubated under nitrogen in a Brewer jar at 37 C.

***The control was not replaced; time of initial count.

Table 7. Replacement of C. botulinum in L-arginine and in L-citrulline solutions.

Replacement medium	Time of replacement	Time of incubation	Initial population*	Final population*	Initial spores*	Final spores*
L-arginine**	13 hr	12 hr	1.2×10^{9}	7.0×10^{8}	1.0×10^{7}	2.0×10^{7}
L-citrulline**	13 hr	12 hr	9.6×10^{8}	5.0×10^{8}	1.0×10^{7}	1.0×10^{7}
Control	13 hr***	12 hr	1.4×10^{9}	5.1×10^{8}	1.0×10^{7}	4.8×10^{8}

*Counts were made by the Petroff-Hausser Bacteria Counter.

**Each replacement solution contained 5 mg/ml of the amino acid indicated in 10 ml of 0.067 M phosphate buffer, pH 7.4, and 0.1% sodium thioglycollate. Solutions were in screw-cap vials and incubated at 37 C.

***The control was not replaced; time of initial count.

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L-ornithine, L-alanine solution did not (Table 8).

The Role of End Products of Cell Metabolism in Sporulation

The possibility exists that the qualities inherent in a medium that render it suitable for sporulation may not be due to the presence of any nutrient added initially, but rather to the products of catabolism by the organism. Therefore, the metabolic picture of a culture undergoing sporulation was investigated to determine what role, if any, the products of metabolism of a culture in 4% Trypticase broth play in the sporulation of C. botulinum.

The metabolic activity of cells during vegetative growth and sporulation was determined by manometric techniques. Cells removed from culture at various stages of growth were placed in Warburg vessels containing an L-alanine, L-proline solution and the evolution of CO_2 and H_2 determined. The $Q^{He}_{CO_2}$ (μl of CO_2 evolved in an atmosphere of helium per mg dry wt of cells per hr) and the $Q^{He}_{H_2}$ (μl H_2 evolved in an atmosphere of helium per mg dry of cells per hr) were calculated and plotted against the time of culture age as were the numbers of total cells and the numbers of spores (Fig. 8). The cells show maximum metabolic activity after 16 hr incubation, and at this time 90% of the cells were in the

Table 8. Replacement of C. botulinum in a solution containing L-arginine and L-alanine, and in a solution containing L-ornithine and L-alanine.

Replacement medium	Time of replacement	Time of incubation	Initial population*	Final population*	Initial spores*	Final spores*
L-alanine, L-arginine**	11 hr	12 hr	8.1×10^{8}	5.9×10^{8}	2.0×10^{7}	1.6×10^{8}
L-alanine, L-ornithine**	11 hr	12 hr	8.2×10^{8}	6.0×10^{8}	3.0×10^{7}	5.0×10^{7}
Control	11 hr***	12 hr	8.0×10^{8}	3.7×10^{8}	2.0×10^{7}	3.3×10^{8}

*Counts were made by the Petroff-Hausser Bacteria Counter.

**Each replacement solution contained 1 mg/ml of the amino acids, 15 ml 0.067 M phosphate buffer, pH 7.4, and 0.1% sodium thioglycollate. Solutions were in screw-cap vials and incubated at 37 C.

***The control was not replaced; time of initial count.

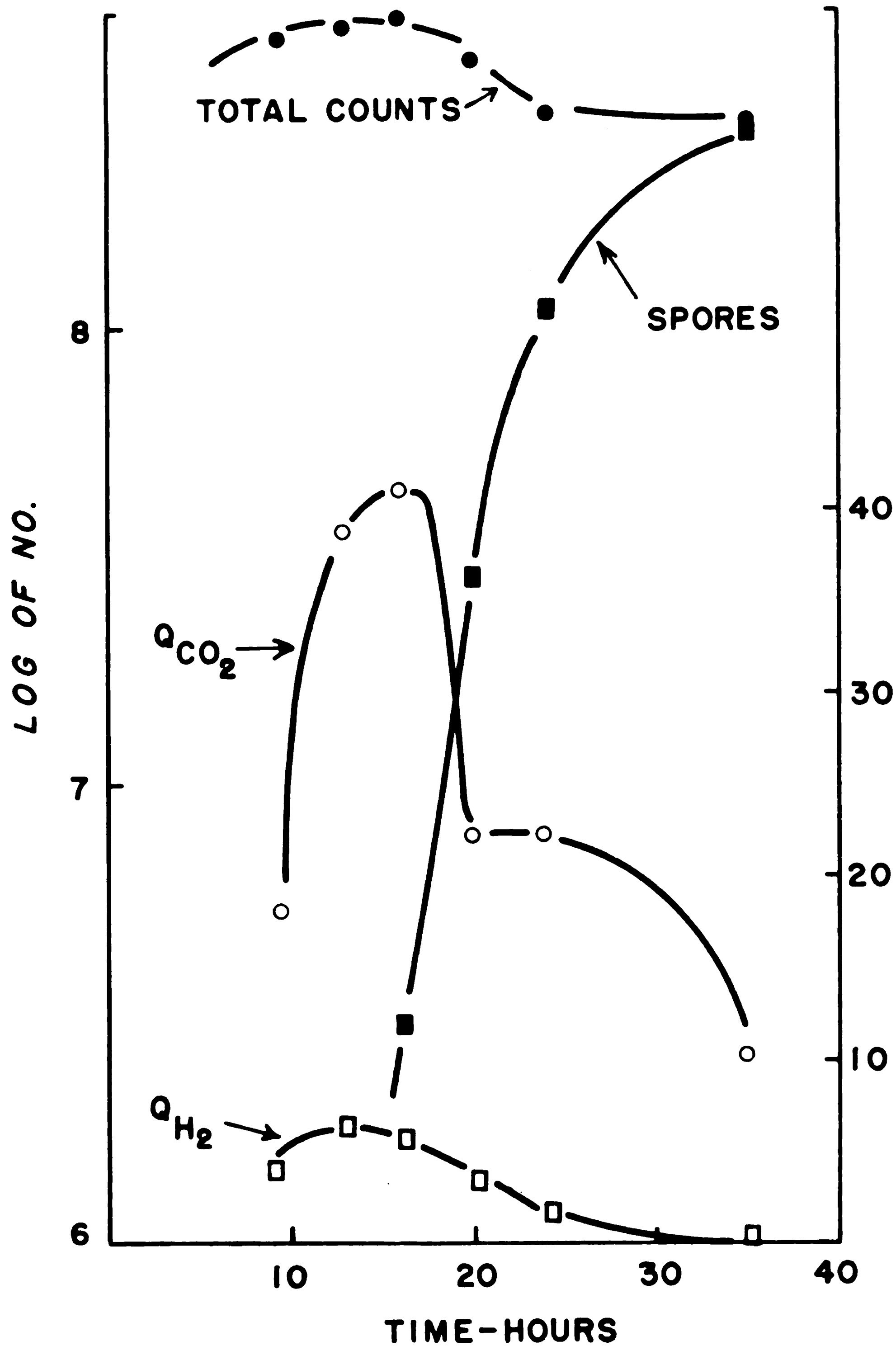


Figure 8. The metabolic activity of cells of C. botulinum at various stages of growth and sporulation. The CO_2 and H_2 evolution rates were determined manometrically, the reaction mixtures containing 100 μmoles of L-alanine, 200 μmoles L-proline, and 0.067 M phosphate buffer, pH 7.2. The total volume was 3 ml and the incubation temperature was 37 C. The atmosphere was helium. Cell counts were made with the Petroff-Hausser Bacteria Counter.

swollen state. As the cells progress in development toward the mature spore, their metabolic capacity decreases rapidly to a constant level.

Fig. 9 illustrates the relationship between the pH of the culture medium, the production of ammonia, and the formation of spores. The production of ammonia was observed to increase throughout growth and sporulation. Since one of the primary energy sources in this type of medium is from the Stickland Reaction, a great deal of ammonia production would be expected. The pH of the culture also increased during this time. Samples from the same and similar cultures were assayed for various acids. When the samples were placed on a Celite column (Wiseman and Irvin, 1957), three bands were observed on elution with varying concentrations of acetone. Using the chromatographic method of Kennedy and Barker (1951), the acids were identified as valeric, propionic and acetic acids. No butyric acid was observed (Table 9).

Samples taken from a culture at various times during growth and sporulation were then assayed quantitatively by fractionating the samples into the component acids and titrating the resultant fractions. There was a steady increase in acetic and valeric acids until the time sporulation began as indicated by the appearance of refractile spores (Fig. 10). At this time there was a decrease in the rate of production

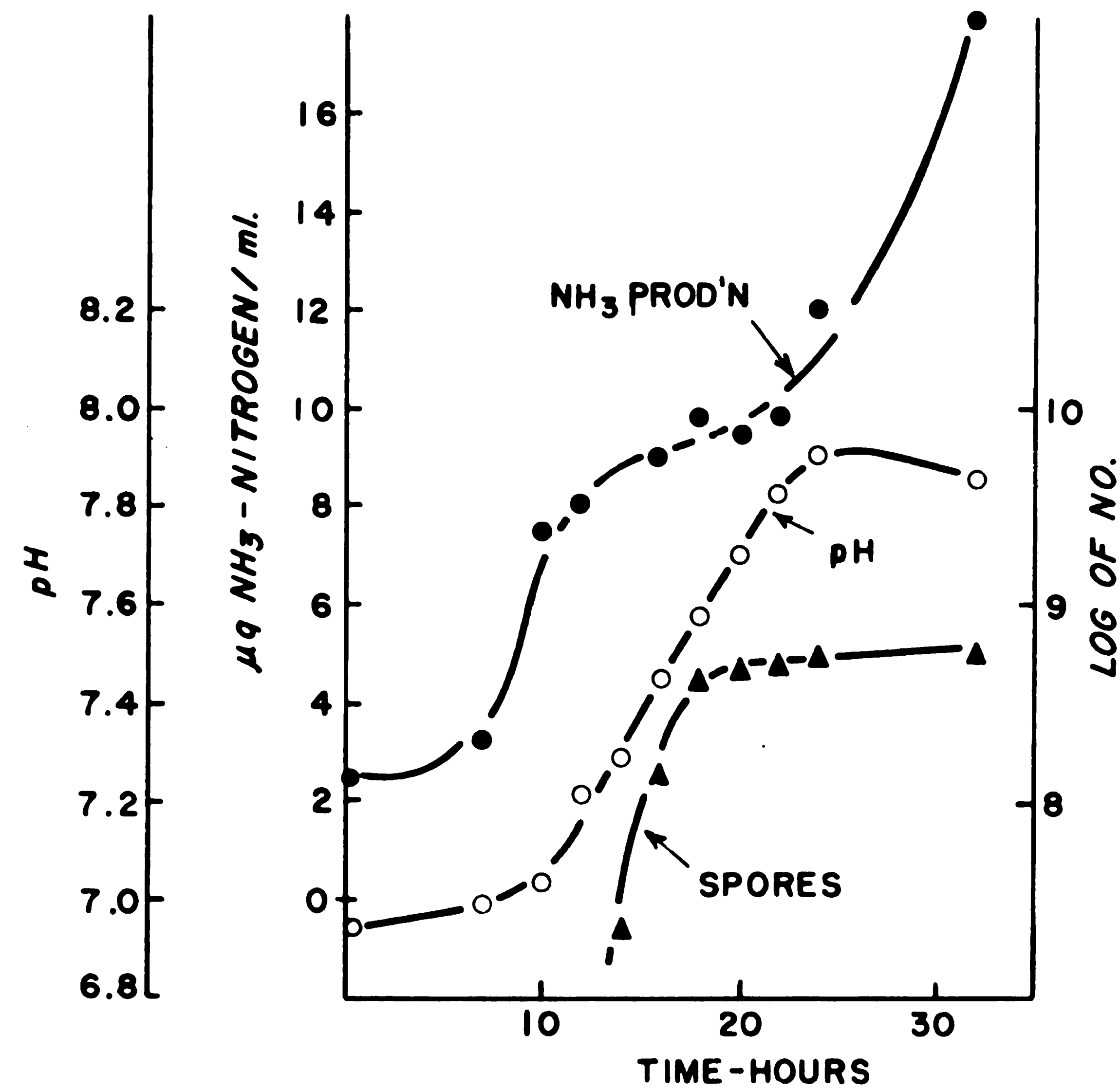


Figure 9. The relationship of culture pH and ammonia production during the sporulation of C. botulinum in 4% Trypticase broth. Incubation was at 37 C in a water bath. Cell counts were made with the Petroff-Hausser Bacteria Counter. NH_3 was analyzed by Nessler's reagent.

Table 9. The identification of acids isolated from a sporulation medium following sporulation of *C. botulinum*.

Standard acids	Rf	Corresponding unknown acids	Rf
Acetic acid	.32	Fraction 3	.32
Propionic acid	.43	Fraction 2	.43
Butyric acid	.50		
Valeric acid	.55	Fraction 1	.56

2 ml of concd NH_4OH were added to each fraction of solvent (acetone and Skellysolve) from a Celite column and the sample evaporated on a steam bath. .01 ml of each fraction was then chromatographed on Whatman #1 chromatographic grade filter paper. Solvent was NH_4OH-ethyl alcohol (1:100); and the chromatograph was allowed to develop for 3.5 hr at room temp. It was then dried at 100 C for 5 min and sprayed with bromphenol blue, 50 mg/100 ml H_2O, and 200 mg citric acid.

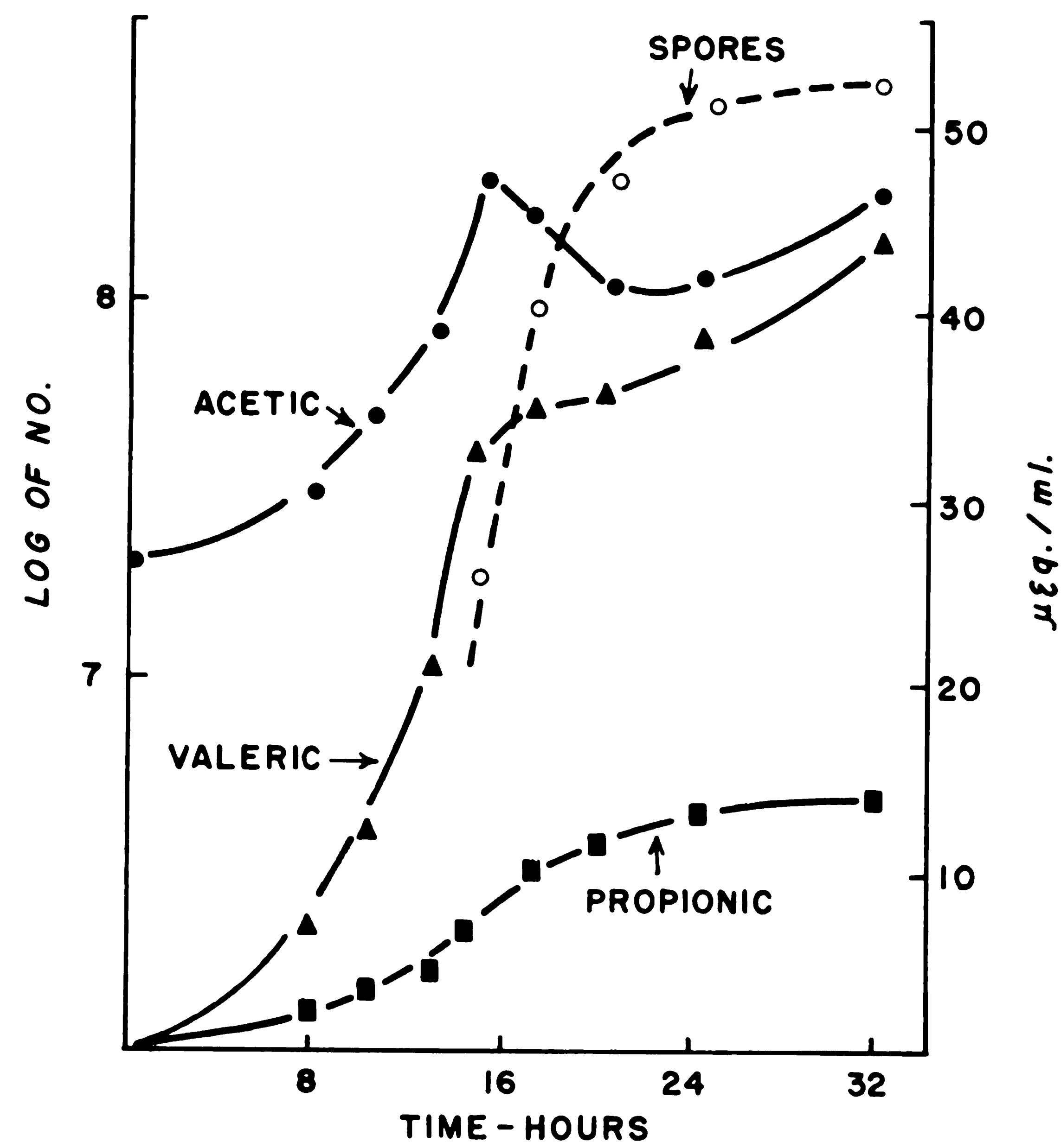


Figure 10. Acid production and sporulation of C. botulinum. Incubation was at 37 C in a water bath. Cell counts were made with the Petroff-Hausser Bacteria Counter. Organic acids were isolated by column chromatography.

of valeric acid, while there appeared to be a slight but detectable utilization of the acetate present in the culture. Propionic acid was produced at a slow rate during growth and sporulation.

Even though Fig. 10 shows the production of excess acetate during the growth of the vegetative phase of the culture, this does not preclude the utilization of acetate at a slower rate. In order to determine if acetate were being used for either growth or sporulation, acetic acid-1-C^{14} was added to a culture and samples collected and assayed as described above. The C^{14} activity of the acetate was also determined. Fig. 11 shows that acetate was utilized during vegetative growth. This utilization continued until about the time sporulation was initiated, when there appeared to be a cessation in the demand for acetate. At this time, the maximum level of acetate in the medium had been reached. As sporulation continued, more acetate was utilized.

Spores which were produced in 4% Trypticase containing acetic acid-1-C^{14} were collected and fractionated in order to determine into which fraction or fractions the radioactive carbon had been incorporated. Initial results indicated that only insignificant amounts of the activity could be recovered in any of the spore fractions; i.e., dipicolininc acid, protein, nucleic acid, or lipid. The only fraction

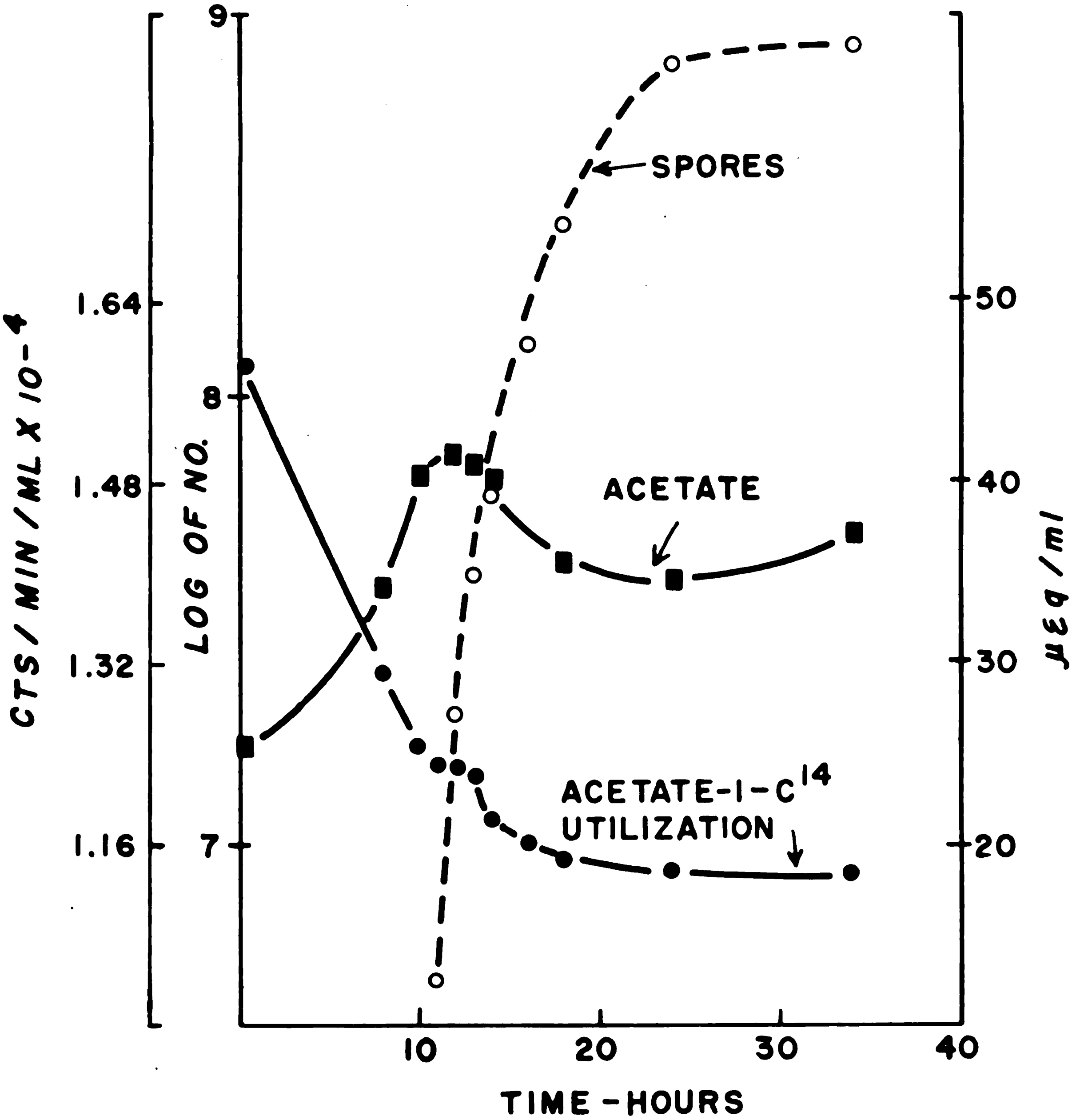


Figure 11. Acetic acid concentration and acetic acid-1-C^{14} utilization during growth and sporulation of C. botulinum. The 200 ml culture contained 10 μc acetic acid-1-C^{14}; with natural gas passed through the culture; incubation temperature was 37 C. Cell counts made with the Petroff-Hausser Bacteria Counter. Acetic acid was isolated by column chromatography.

not assayed was the insoluble portion which remained after acid hydrolysis of the spore protein. This portion was in very fine suspension, and could not be removed by centrifugation at 30,000 rcf, but it could be collected on a Millepore filter by vacuum filtration.

Another experiment was run using 10 µc acetic acid-l-C^{14} in 180 ml of sporulation medium. The activated culture technique was used, with the evolved gases being collected in 20% KOH. After sporulation was complete, the spores were collected by centrifugation, and fractionated. The various fractions were assayed for C^{14} activity by the method of Kinnory et al. (1958). The results of this experiment are given in Table 10. The only fraction containing any significant activity compared to the amount of activity which was utilized appeared to be the insoluble fraction which remained after hydrolysis of the protein.

The activity assayed in the insoluble fraction was low due to greater inefficiency of the gas-flow counter as compared to the liquid spectrometer scintillation counter, and due to self-absorption by the solid sample. A standard C^{14} source was counted on both the liquid scintillation spectrometer and the gas-flow counter, and it was observed that the gas flow-counter had about one-half of the efficiency of the scintillation counter. The standard,

Table 10. Distribution of C^{14} in fractions from spores of *C. botulinum* produced in sporulation medium containing 10 μc of acetic acid-1-C^{14}.

Fraction	Activity, cts/min
Acetate, initial in medium	8.078×10^6
Acetate, final in medium	7.267×10^6
CO_2, initial	$.001 \times 10^6$
CO_2, final	$.001 \times 10^6$
1st spore wash	$.013 \times 10^6$
2nd spore wash	$.004 \times 10^6$
Supernatant from spores heated at 80 C for 10 min	$.009 \times 10^6$
TCA soluble (Supernatant from breakage in 5% TCA)	$.008 \times 10^6$
Lipids (70% ethyl alcohol extraction)	$.021 \times 10^6$
Nucleic acids (5% TCA for 30 min at 100 C)	$.009 \times 10^6$
Protein (8N H_2SO_4 for 5 hr at 120 C)	$.007 \times 10^6$
Insoluble residue	$.112 \times 10^6$
Activity of C^{14} utilized	$.811 \times 10^6$
Activity of C^{14} recovered	$.185 \times 10^6$
Percent of C^{14} recovered	22.7%

benzoic-C^{14}-acid, was dissolved in toluene, and 10 µl counted with each counter. With the gas flow-counter, the sample was evaporated on a membrane filter; and with the liquid scintillation counter, the sample was placed in a 20 ml counting bottle with 8.6 ml of absolute alcohol and 10.4 ml of 0.58% 2,5-diphenyloxazole in toluene. The standard was calculated to emit 2,000 cts/min. The liquid scintillation counter registered 1,082 cts/min for an efficiency of 54%, while the gas-flow counter registered 487 counts/min, giving an efficiency of 24%. It should be noted also, that the evaporation of the standard on the membrane filter resulted in a very thin sample, allowing for a minimum of self-absorption. With the residue from the cell fractionation, a much heavier layer was collected on the filter, resulting in even greater loss in efficiency with the gas-flow counter. Thus, it is likely that practically all of the C^{14} from the acetate utilized was in the insoluble fraction remaining after protein hydrolysis.

The chemical nature of the residue left after hydrolysis of protein with strong acid is not known with certainty, but it is probably a protein-polysaccharide complex. The material after hydrolysis is black to dark brown in color, and further studies revealed that this residue contained from 10-15% of the original dry weight.

It is believed that the material consisted of mucopeptide complexes which are common to bacterial cell walls, and that it was synthesized during vegetative growth of the cells. If this were the case, the mucopeptide complex could be an artifact of the spores.

Upon examination of the previous results, it was realized that the spores were collected before the sporangia had been allowed to lyse. Therefore, the spores would be highly contaminated with the cell walls of the vegetative phase of growth. In view of this, another study was made in which the culture was allowed to proceed until the culture contained 95% free spores. When this culture was centrifuged at high speeds, the spores were layered in the lower level of the pellet while a black layer was observed on the upper strata. These two fractions were partially separated by washing off the black layer with water. This procedure was repeated twice with the spore layer, after which the pellet appeared fairly homogeneous. Microscopic examination of the spore fraction revealed that about 90% of the population was free spores, the rest being comprised of spores in sporangia or vegetative cells. The fraction which had been removed by washing showed mostly debris of no structural organization and barely visible cells or ghost cells. This fraction was probably the remains of cells and sporangia after lysis. These two fractions were dried and weighed, and then placed in 10 ml of 8N H_2SO_4 and autoclaved for 5 hr. The residues after hydrolysis were collected on Millepore filters and counted in the gas-flow counter. The results of this study are given in Table 11.

Table 11. Distribution of C^{14} in free spores and in debris of a culture of C. botulinum grown in sporulation medium containing 4 μc of acetic acid-1-C^{14}.

Fraction	Description	Weight	Activity
Spore	90% free spores, 10% vegetative cells and spores in sporangia	6.05 mg	2.09×10^4
Debris	Ghost cells, structures of little or no organization	5.95 mg	3.10×10^4
Activity of C^{14} utilized			31.21×10^4
Activity of C^{14} recovered			5.19×10^4
Percent of C^{14} recovered			16%

The data of Table 11 are not conclusive in determining whether the acetate utilized was used in synthesis of spore material, although it does indicate that most of it was probably used in the synthesis of cell wall material, since 60% of the recovered C^{14} was found in the debris fraction and the spore fraction was not free of debris.

Figure 11 indicated that acetate was being used to some degree during sporogenesis. This led to an experiment in which the labeled acetate was added to the culture at the conclusion of vegetative growth, at a time when the cells were undergoing the initial steps of sporulation. At the time of addition of acetate, there was about 1% spores in the population, and the rest of the cells were swollen. The spores and debris were partially separated and the fractions digested as described in the above experiment. The results of this investigation are given in Table 12.

Table 12. Utilization and distribution of C^{14} in spores and in the debris of a culture of C. botulinum grown in a sporulation medium with 10 μc of acetic acid-1-C^{14} added at the onset of sporulation.

Fraction	Activity
Spore	$.838 \times 10^4$
Debris	1.699×10^4
Percent C^{14} utilized during sporulation	4.8%
Activity of C^{14} utilized	1.580×10^5
Activity of C^{14} recovered	$.254 \times 10^5$
Percent C^{14} recovered	17%

About 5% of the available acetate was used by the sporulating culture; however, the activity found in the hydrolysate residue of the lysed cells and sporangia was about twice that observed in the acid insoluble portion of the hydrolysate of free spores. The activity of the latter portion may represent contamination by vegetative cells and debris since these could not be completely separated from the free spores by centrifugation. At the time the culture was harvested there were about 10% vegetative cells present.

It appeared possible that the mechanism initiating sporulation might be a function of the pH and the concentration of end products in the environment. To test this hypothesis a sporulation medium was prepared with the pH and the concentrations of acetic, propionic, and valeric acids adjusted to that observed in a culture at the time of sporulation. If these conditions are instrumental in initiating sporulation, a medium thus prepared should support sporulation at a significantly earlier time than a control culture. However, sporulation occurred no sooner in the adjusted medium than in the control culture (Table 13).

Nucleic Acid Snythesis and Sporulation

The synthesis of both RNA and DNA by *C. botulinum* prior to and during sporulation was followed by the method

Table 13. Sporulation of C. botulinum in a sporulation medium containing valeric, propionic, and acetic acids.

Time of Observation	Adjusted Medium*		Control Medium	
	Total Population**	Spores**	Total Population**	Spores**
8 hr	$6.4X10^{8}$	$1.0X10^{7}$	$9.4X10^{8}$	$2.0X10^{7}$
10 hr	$8.3X10^{8}$	$3.0X10^{7}$	$1.0X10^{9}$	$3.0X10^{7}$
12 hr	$8.8X10^{8}$	$6.0X10^{7}$	$6.4X10^{8}$	$7.0X10^{7}$
15 hr	$4.3X10^{8}$	$1.1X10^{8}$	$5.7X10^{8}$	$1.4X10^{8}$
20 hr	$5.5X10^{8}$	$3.2X10^{8}$	$4.5X10^{8}$	$3.2X10^{8}$
32 hr	$4.2X10^{8}$	$4.0X10^{8}$	$3.6X10^{8}$	$3.5X10^{8}$

*The adjusted medium contained 5.0 meq of valeric acid, 2.5 meq of propionic acid, 6.4 meq of acetic acid, and 1 µg/ml of thiamine, and the pH was 7.5. The control medium was 4% Trypticase containing 1 µg/ml of thiamine with a pH of 7.1.

**All counts were made by the Petroff-Hausser Bacteria Counter.

of Hanawalt (1959). To a 250 ml activated culture of this organism, 140 μc of P^{32} was added at 8 hr of growth. Fig. 12 illustrates the results of this study. RNA was synthesized at a more rapid rate than DNA, and at 12 hr of incubation, when the first swollen cells were observed, both nucleic acids were being formed at an undiminished rate. DNA synthesis was complete by the time the first mature spores were observed. RNA synthesis continued at a decreasing rate for the next 5 hr but was complete well before the maximum number of spores were observed.

Earlier work carried out by the author (Day, 1960) had indicated that RNA synthesis was essential during the final sporulation process, but this was based on the assumption that vancomycin was a specific inhibitor of RNA synthesis. However, Jordan (1961) reported that the primary effect of this antibiotic is the inhibition of cell wall mucopeptide, and that the inhibition of RNA synthesis is secondary. Experiments in this study revealed that vancomycin inhibited vegetative growth of C. botulinum and concomitantly the synthesis of RNA, but no study was made concerning the effect on cell wall. Vancomycin added to cultures during sporogenesis only partially inhibited sporulation and RNA synthesis. Since it could not be positively stated that the primary point of action of

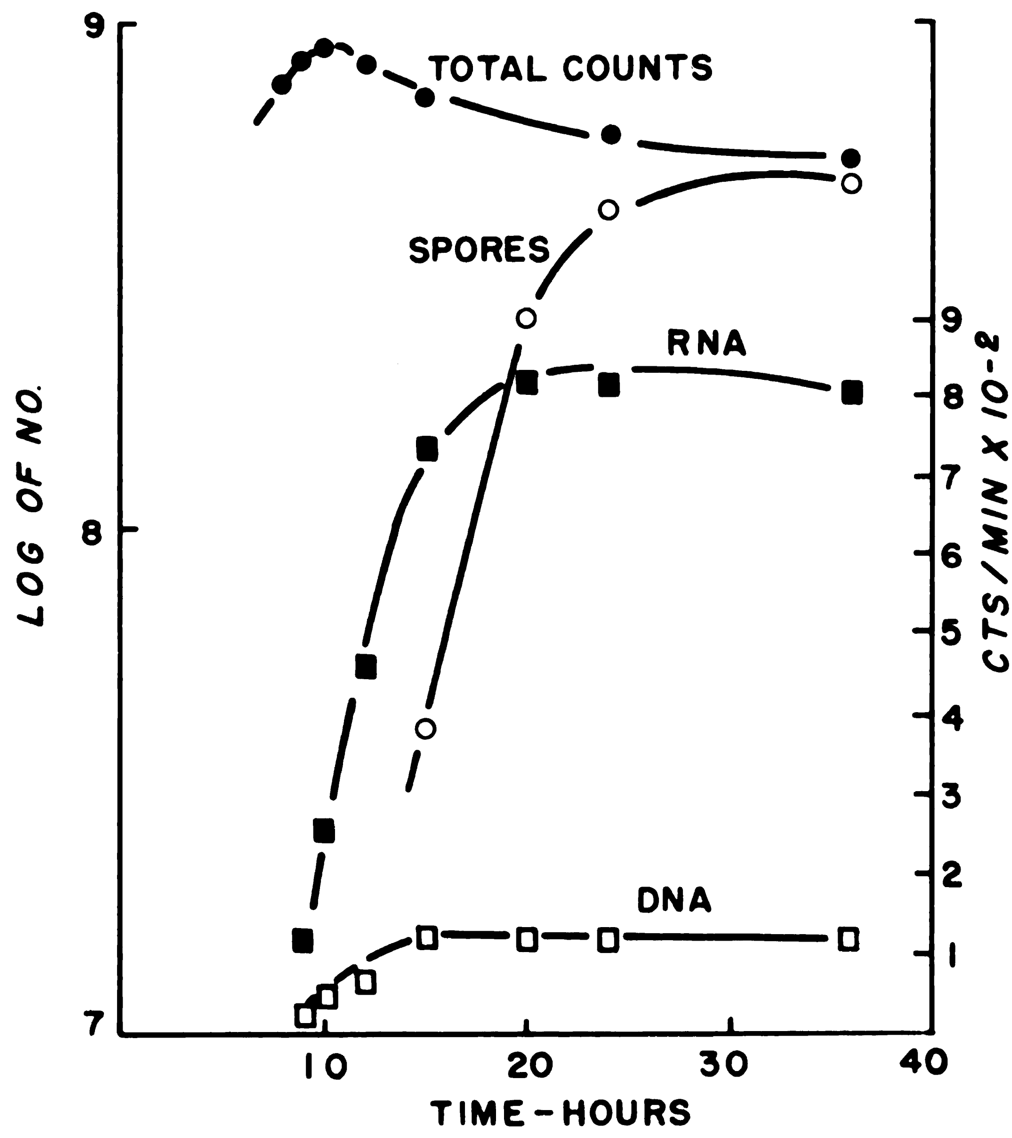


Figure 12. Nucleic acid synthesis during the sporulation of C. botulinum. Medium utilized was 4% Trypticase broth containing 1 μg/ml of thiamine; incubation temperature was 37 C. Cell counts were made by the Petroff-Hausser Bacteria Counter.

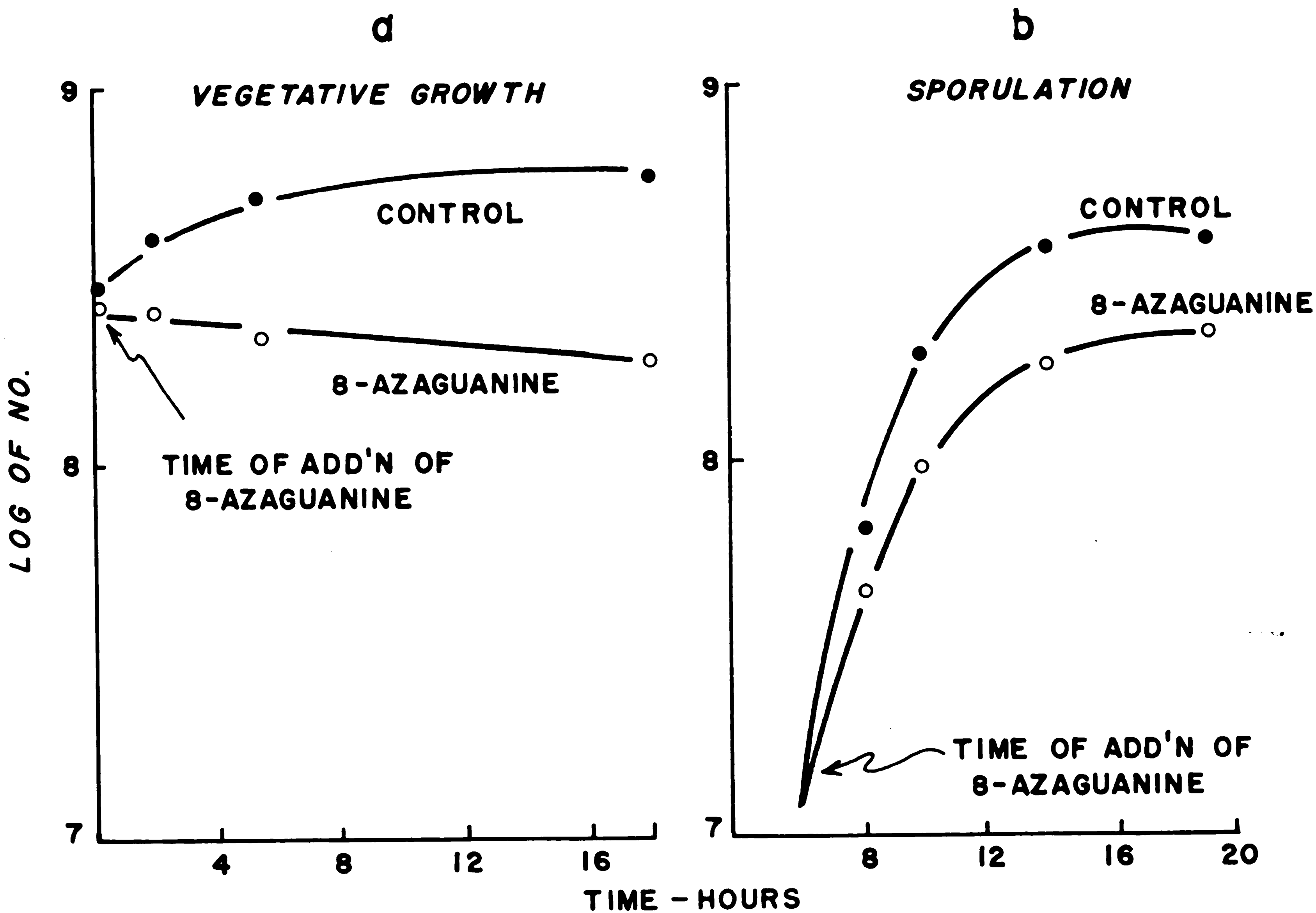
vancomycin in the metabolism of *C. botulinum* was in the inhibition of RNA synthesis, this line of investigation was not pursued further.

Since no specific inhibitor of RNA synthesis could be found, 8-azaguanine, a purine analogue was utilized as a nucleic acid antimetabolite. Figure 13a shows that this compound did inhibit vegetative growth of *C. botulinum* immediately upon addition to the culture. However, when the purine analogue was added to the culture at the time sporulation was just beginning, it had only a partial effect on further sporulation (Fig. 13b). This would indicate that some nucleic acid synthesis is essential during the early phase of sporogenesis.

According to Shiba et al. (1959) mitomycin C will inhibit the formation of DNA with no effect on RNA synthesis. This antibiotic also inhibited the vegetative growth of *C. botulinum* (Fig. 14a). However, when a culture undergoing sporulation was similarly treated, no effect was observed (Fig. 14b). The culture with added mitomycin C showed the same level of spores as did a control culture.

Finally, the synthesis of both RNA and DNA during sporulation was followed in a culture which had been replaced in an L-alanine, L-proline solution when the predominance of the cells were in the forespore phase of

Figure 13. The effect of 8-azaguanine on the growth and on the sporulation of C. botulinum. Concentration of 8-azaguanine was 100 µg/ml. Medium was 4% Trypticase with 1 µg/ml thiamine; incubation temperature was 37 C. Cell counts were made with the Petroff-Hausser Bacteria Counter.


a
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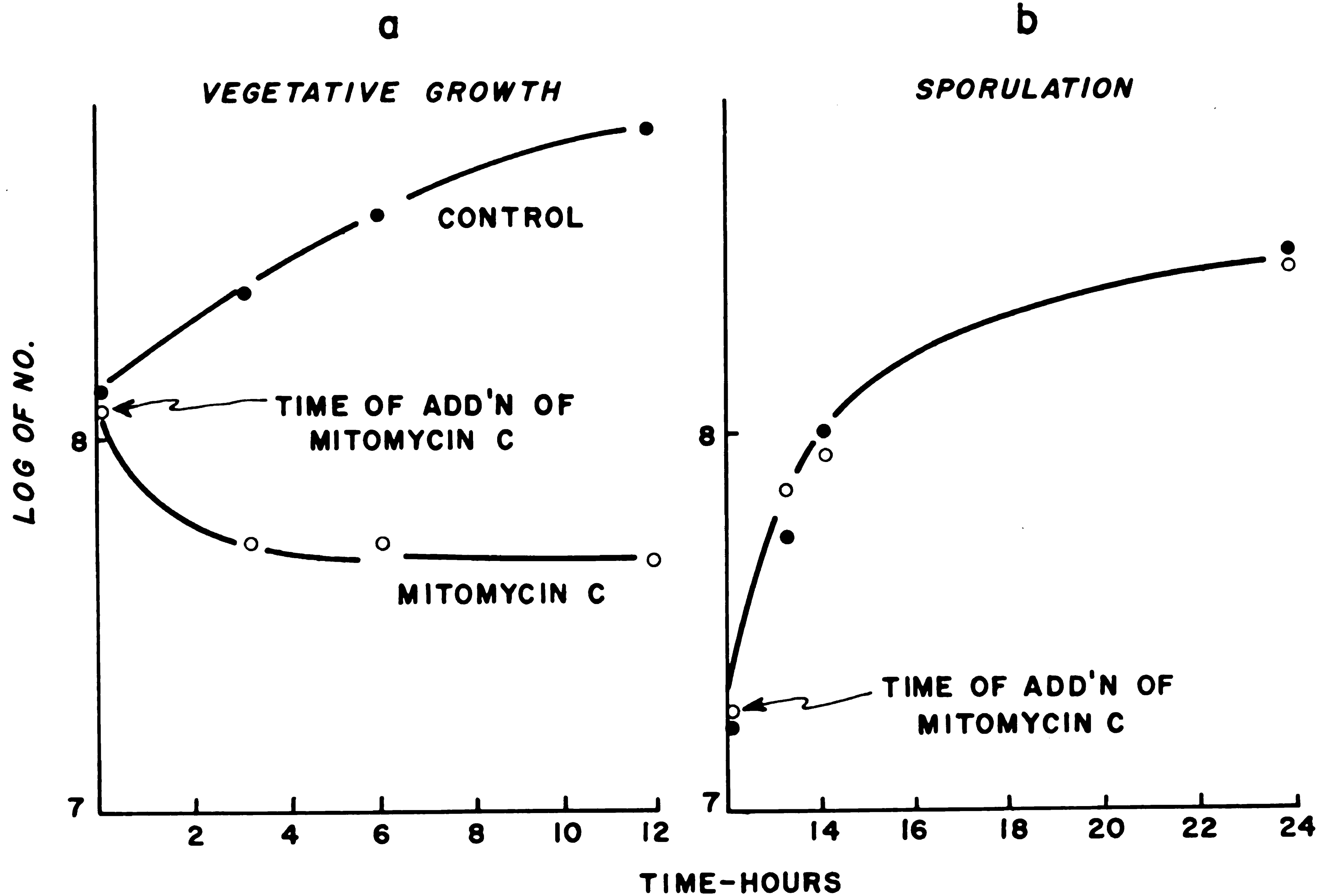
16
20
TIME - HOURS

Figure 14. The effect of mitomycin C on the growth and on the sporulation of _C_. _botulinum_. Concentration of mitomycin C was 40 μg/ml. Medium was 4% Trypticase with 1 μg/ml of thiamine. Incubation temperature was 37 C. Cell counts were made with the Petroff-Hausser Bacteria Counter.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development by use of radioactive phosphorus by the method of Hanawalt (1959). The control was a culture removed from the growth medium by centrifugation and resuspended in the original medium with 36.1 μc P^{32} added. In neither culture could any synthesis of DNA be observed, and only in the control was RNA synthesis found (Fig. 15). However, sporulation was observed in the L-alanine, L-proline replacement solution in the absence of nucleic acid synthesis, but not to the level of sporulation as seen in the control.

The Synthesis of DPA and Poly-β-hydroxybutyric Acid

The synthesis of DPA was also investigated to determine the relationship of its time of formation to the appearance of refractile and heat resistant spores. Previous results had indicated that DPA was formed simultaneously with the refractile spores (Day, 1960) but Fig. 16 demonstrates that these data were in error. This evidence indicates that refractile spores were present before the synthesis of DPA was initiated, and that the heat resistant entities were not formed until after the synthesis of DPA had begun. The level of DPA reached about 28 μg/ml of culture which is in very close agreement with the level attained in cultures of other spore-formers (Halvorson, 1957).

Law and Slepecky reported (1961) that the accumulation

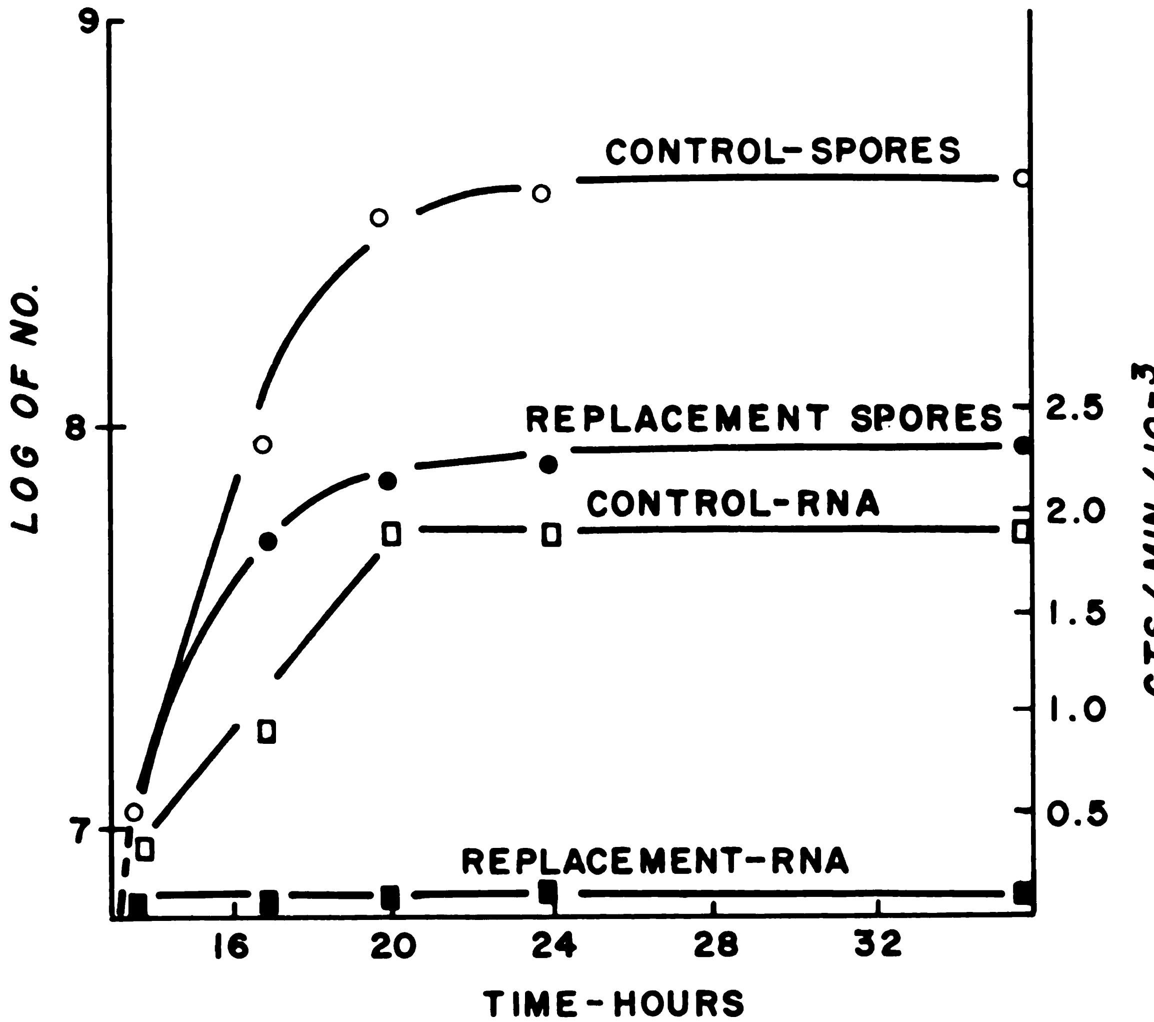

Figure 15. The synthesis of nucleic acids by C. botulinum during sporulation in a replacement solution containing L-alanine, L-proline and P^{32}. Solution contained 36.1 μc P^{32} and 2 mg/ml of amino acids in 0.067 M phosphate buffer, pH 7.4, final volume 20 ml. Solutions in screw-cap vials. Incubated at 37 C. Cell counts were made with the Petroff-Hausser Bacteria Counter.

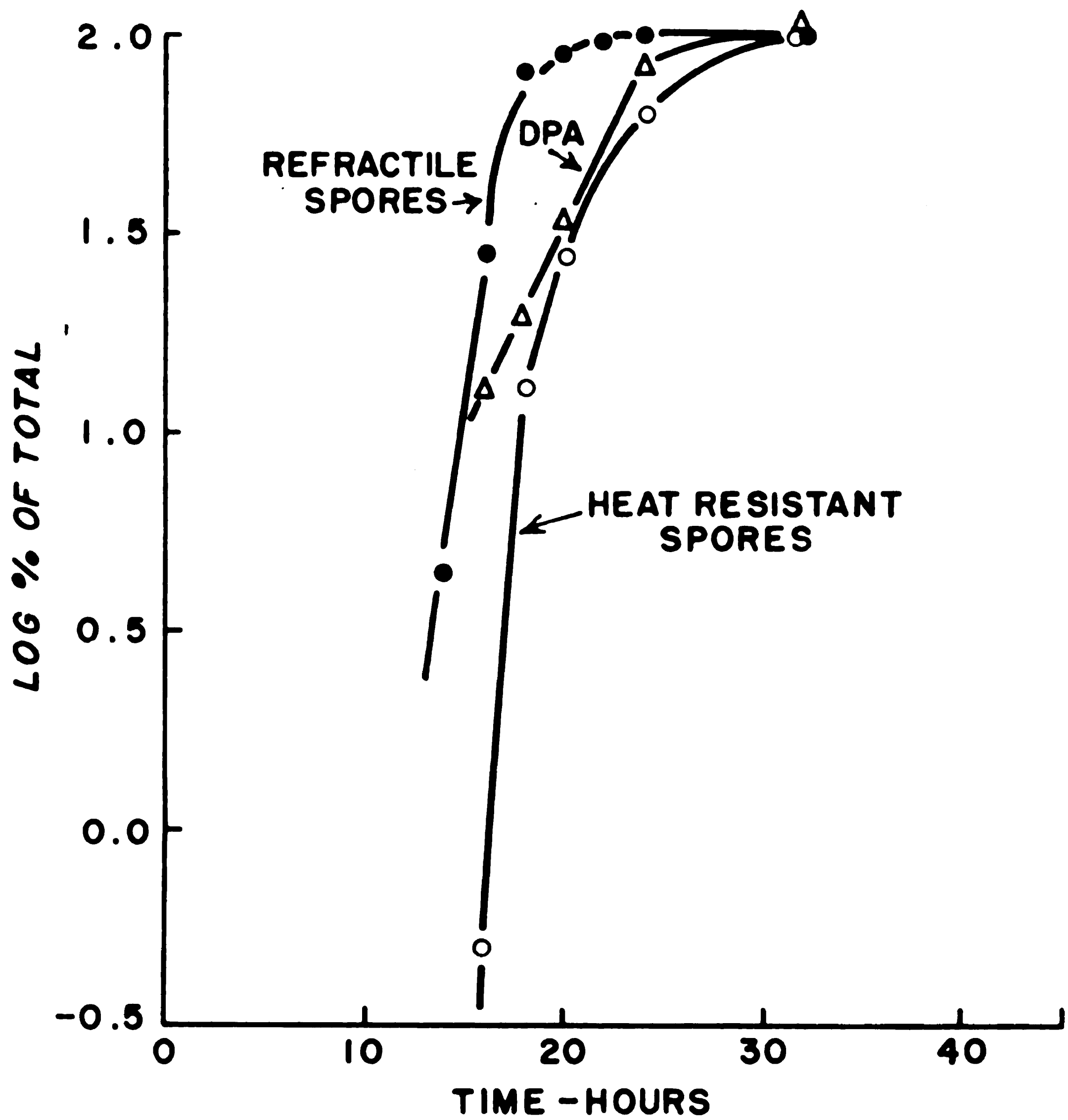

Figure 16. The time of appearance of DPA, refractile spores, and heat resistant spores of C. botulinum in a sporulation medium. Refractile counts were made by the Petroff-Hausser Bacteria Counter; heat resistant counts were heat shocked samples plated with YESB agar in oval tubes. The medium was 4% Trypticase with 1 μg/ml of thiamine, incubated at 37 C.

of β-hydroxybutyric acid in polymerized form in the cells of the aerobic bacilli just prior to sporulation. It was suggested that these granules serve as a reserve energy source, and an assay for the quantitative determination of this compound was described. When this assay was applied to the cells of C. botulinum which were approaching sporulation, the resulting spectrum was identical to that described by Law and Slepecky (1961) for spores containing no poly-β-hydroxybutryic acid.

The Formation of Heat Resistant Enzymes During Sporulation

The report of Simmons (1961) that a heat stable DPNH oxidase could be demonstrated in the spores of C. botulinum prompted an investigation to determine when this enzyme was being stabilized. Another enzyme, acetokinase, reported by Simmons to possess no stability towards heat when heated in spore extracts, was chosen for assay in order to compare a labile and a stable system.

Activated cultures in a 4% Trypticase medium were prepared and allowed to progress until the desired stage of cell development had been reached. The cells were then harvested by centrifugation, washed twice in sterile distilled water, and divided into two equal portions. One portion was placed in an 80 C water bath for 10 min, and the other portion was left unheated. The cells were then washed once more, broken in the micro-cup of the Omni-mixer, the extract centrifuged, and dialyzed for 15-18 hr against distilled water at 5 C. The extracts thus prepared were assayed for protein, acetokinase activity, and DPNH oxidase activity. This procedure was carried out for vegetative cells (8 hr culture), swollen vegetative cells (cells showing swelling but little or no refractility), and mature spores (spores allowed to stand until free of the sporangia). Table 14 summarizes the results of these experiments, and the activities therein represent the average of the values obtained for two different extracts of each of the three cultural stages.

Several aspects of the results given above are at variance with the results of Simmons (1961). The DPNH oxidase studied in these experiments was found to have a higher activity in vegetative cells than in spores. Also, the actokinase in the spore appears to be completely heat labile, although Simmons (1961) reported this enzyme to be active in extracts of heat shocked spores. It is noted in Table 14 that acetokinase was inactivated in the spore by heating for 10 min at 80 C, while the DPNH oxidase was not completely inactivated by this heat treatment either in the vegetative or the spore state. However, the DPNH

Table 14. Specific activities of the DPNH oxidase and acetokinase extracted from vegetative cells, swollen vegetative cells, and mature spores of C. botulinum.

Cell Stage	Heated at 80 C for 10 min before breakage		Unheated before breakage	
	Acetokinase*	DPNH oxidase**	Acetokinase	DPNH oxidase
Vegetative	0.0	.011	9.68	.121
Swollen vegetative cells	0.0	.014	6.10	.105
Mature spores	0.0	.022	4.67	.074

*Acetokinase activity; μmoles of acetyl phosphate formed per min per mg protein. Reaction mixtures contained: 800 μmoles of potassium acetate, .05 ml of 1.0 M tris buffer, .25 ml extract, 10 μmoles of $MgCl_2$, 10 μmoles of ATP, 700 μmoles of hydroxylamine, and 720 μmoles of KOH. Incubation temperature was 30 C; reactions were stopped with 1 ml of 10% TCA.

**DPNH oxidase activity: Change in O.D. per min per mg protein. Reaction mixtures contained: 2.3 ml of 0.067 M phosphate buffer, pH 7.4; 0.5 μmoles of DPNH; and 0.2 ml of extract. Change in O.D. at 340 mμ was followed; reaction temperature was 25 C.

oxidase in spores did have greater resistance to heat than did that in the vegetative cells or in the swollen vegetative cells. The latter appeared to be somewhat more stable to heat than the enzyme in vegetative cells.

The enzymes in extracts of the three cell forms were then assayed further for thermostability by heating the cell-free extracts for 80 C for 1 min. The acetokinase activity in extracts of spores unheated before breakage showed no heat stability as expected. Thus only the data for DPNH oxidase is presented (Table 15). The values given in the table are the averages obtained with two different extracts for each of the cell forms.

Table 15. DPNH oxidase activity of extracts of vegetative cells, swollen vegetative cells, and mature spores of _C. botulinum_ before and after heating at 80 C for 1 min.

Cell Stage	Heated at 80 C for 10 min before breakage		Unheated before breakage	
	Unheated	80 C for 1 min	Unheated	80 C for 1 min
Vegetative	.011	.009	.121	.025
Swollen vegetative	.015	.009	.105	.035
Mature spore	.022	.010	.074	.041

DPNH oxidase activity: Change in O.D. per min per mg protein. Protein determinations were not made after heating the extracts at 80 C for 1 min. Reaction mixtures contained: 2.3 ml of 0.067 M phosphate buffer, pH 7.4; 0.5 μmoles of DPNH; and 0.2 ml of extract. Change in O.D. at 340 mμ was followed; reaction temperature was 25 C.

It appears that low levels of heat resistant DPNH oxidase occurs both in cells and spores. It is obviously more stable in spores, and the enzyme extracted from spores appeared more stable to heat than that from cells. However, the high stability of this enzyme from spores observed by Simmons (1961) was not reflected in these data.

DISCUSSION

The purpose of this investigation was to define some of the major physiological events occurring during the sporulation of C. botulinum. Sporulation, like other developmental biological processes is gradual and cannot be observed to proceed in discrete, well defined steps. Many different systems are functioning during the course of sporulation, and it is probably only at the molecular level where the individual steps can be observed. The morphological stages of spore development present problems in determining the stage of cell development. The swollen vegetative cell, the first observable stage in sporulation, varies in its appearance from the slightly swollen cell with no refractility to the "club-shaped" body containing a poorly defined area with some refractility. The assessment of the number of immature forespores in a suspension is very difficult. The definition calls this stage the swollen cell containing an area of refractility with a diffuse edge. Many cells with varying degrees of refractility are observed in this stage, and undoubtedly each form represents a different stage in the development of the mature spore. Even the mature spore cannot always be recognized by visual observation. Heat resistance, one of the requirements of spore maturity, cannot be determined by visual observation since refractility precedes heat resistance.

In addition to the difficulty in determining the stage of spore development, another problem is encountered in the synchrony of the culture. The ideal situation is to have all cells in exactly the same stage of development at the same time. This was not possible to achieve in this investigation. About the best that could be obtained was to have 50-60% of the cells in approximately the same stage of development at the same time. The rest of the culture would then be slightly ahead or behind of this portion of the population in spore development. A small portion of the population would never develop beyond the vegetative stage. Therefore, it can be seen that these problems present difficulties in defining discrete steps that take place during the sporulation of _C. botulinum_. At best the results indicate what is occurring in the majority of the cells in a specific stage of spore development.

The initial event which takes place in sporogenesis was not determined. The data first indicated that acetate might play a role in initiating sporulation as was observed by Nakata and Halvorson (1960) in _B. cereus_. In a culture of _B. cereus_, maximum growth and maximum acetate concentration is reached at the same time, after which an inducible enzyme system is activated and sporulation takes place. The enzyme system is responsible for the oxidation of acetate. With C. botulinum, a maximum of acetate concentration was reached concomitantly with maximum population and the initiation of sporulation, and the level of acetate in the culture decreased thereafter. However, the use of acetic acid-1-C^{14} indicated that acetate was utilized throughout the growth period and through the initial stages of sporulation; and the C^{14} activity could only be recovered in the residues remaining after protein hydrolysis. On the basis of this evidence, it appears that acetate does not play the same or a similar role in the sporulation of C. botulinum as it does in the sporulation of B. cereus.

Srinivasan and Halvorson (1962) isolated a factor from the cells of B. cereus in the initial stages of sporulation which, when added to cells not yet committed to sporulation in a non-growth medium, caused these cells to sporulate. This material is a low molecular weight compound, but it has not yet been identified. This indicates that a compound was formed within the cell which could trigger the sporulation mechanism in other cells which would not normally sporulate at that time. Since a non-growth medium was required, evidently this compound could

not exert its control over the sporulation mechanism during growth. This may indicate that only after conditions become unsuitable for growth, either by the exhaustion of nutrients or through the accumulation of end products, that the control mechanism for sporulation can function. With _C. botulinum_ the concentration of end products and the culture pH alone would not initiate sporulation. When these conditions were preset in a culture, sporulation occurred no sooner than in a control culture. Previous work (Day, 1960) demonstrated that when the cells of _C. botulinum_ were removed from 4% Trypticase broth and replaced in a fresh 4% Trypticase medium, sporulation proceeded as in the control culture. This indicates that neither the accumulation of end products nor the exhaustion of nutrients is a necessary factor in the sporulation of _C. botulinum_. It would appear that the initial mechanism involves the interplay of several events, and that more detailed studies are required for the elucidation of this process.

The next stage of development of the spore, from the swollen vegetative cell to the immature forespore, requires no exogenous nutrients in _Bacillus_ species (Hardwick and Foster, 1952), This was shown by replacing these cells in distilled water and observing complete sporulation. It was the theory of Hardwick and Foster that the proteins

of the cells were degraded and remobilized for spore protein. However, C. botulinum will not sporulate in such a simple system (Day, 1960). The present data indicate that both protein and RNA synthesis occur during this period. An energy source alone would not support the development of cells from the swollen vegetative cell to the immature forespore, chloramphenicol inhibited this stage of development, and a purine analogue (8-azaguanine) also inhibited this development. Thus these data agree with present concepts that RNA must accompany protein synthesis. Although these results do not preclude the possibility that some protein of the vegetative cells is being degraded and reformed into spore protein, an exogenous amino acid source is required. The synthesis of DNA does not appear to be essential to this stage of development of the spore, and this data is in agreement with the theory of Delaporte (1950), Robinow (1956), and Fitz-James and Young (1959) that the DNA of the spore is preformed in the vegetative cell and is enclosed in the spore during sporogenesis. The metabolic activity of the cells was at a maximum value during this phase of sporulation, so abundant energy was available for synthetic processes.

After the formation of the immature forespore, the requirements for sporulation were less complex. While no spore maturation occurred in buffer or water, the addition of an energy source (L-alanine and L-proline, L-isoleucine and L-proline, or L-arginine and L-alanine) did result in the formation of mature spores. This energy could not be stored since it had to be furnished to the cells exogenously. This may be another fundamental difference between the aerobic spore-forming organisms and the anaerobic spore-formers. Evidence indicates that the former group can sporulate in the absence of all nutrients, indicating an ability to store energy. This may be furnished by β-hydroxybutyrate as suggested by Slepecky and Law (1961), although the presence of this material is not a prerequisite for sporulation and energy can be stored in other materials. No β-hydroxybutyrate was found in C. botulinum cells. Anaerobes, of course, require much more substrate to obtain the same amount of energy than is required by aerobes. It is difficult to speculate as to the reason L-arginine alone would not serve as an energy source for this stage of spore maturation. Other workers (Perkins and Tsuji, 1962) have observed that C. botulinum, type A, actively utilize this amino acid, and it was observed during this investigation that the cells of C. botulinum are motile in a replacement solution of L-arginine. Although the energy requirements for motility are undoubtedly small, this observation does indicate that the cells at this stage of sporulation of

C. botulinum are able to use L-arginine as an energy source.

The step in sporulation from the immature forespore to the mature spore was marked by the lack of net protein and RNA synthesis, and a decreased metabolic activity. However, during this stage of development the refractility of the spore became intensified, and the spore became well delineated within the sporangium. DPA was being formed during this stage, and it was evidently being synthesized entirely from the pre-existing materials of the cell. The cell became resistant to heat shortly after the formation of DPA, and this would indicate a role for DPA in heat resistance as postulated by Halvorson (1957) and Black et al. (1960); however, the development of the heat resistance of individual enzymes, as discussed later, complicates this picture. It is believed that the exogenous energy source was required to support these processes of final spore maturation, which appears to be the development of heat resistance and the organization of previously synthesized materials into the dense, highly refractile spore body.

One other aspect of sporulation in replacement culture must be considered. It is felt by some investigators (Powell and Hunter, 1953; Black et al., 1960) that the replacement solution is actually a dilute nutrient medium due to the lysis of vegetative cells after the transfer to the replacement solution. Lysis of C. botulinum did occur in replacement cultures; and, in the presence of an energy source, such products may be utilized by sporulating cells. However, previous work (Day, 1960), showed that the products of cell lysis alone would not support replacement sporulation. They must not be used in protein or nucleic acid synthesis, since these processes were not active at this stage. If they serve as an energy source, they are much too dilute for this anaerobe. The possibility does exist that DPA precursors are made available through lysis of cells which do not sporulate, and that these precursors are utilized by immature forespores in the synthesis of DPA. The concentration of such compounds in a replacement medium would be extremely low since the maximum lysis observed amounted to about 5 X 10^8 cells/ml. This would result in a great variety of compounds, the total dry weight of which could not exceed about 0.5 mg/ml. When an L-alanine and L-proline replacement medium was chromatographed after sporulation, no other simple amino compounds could be detected other than these two amino acids and δ-aminovaleric acid.

The thermostability of the enzymes studied revealed some interesting aspects. The acetokinase, although present in the mature spore at fairly high levels of activity, had no appreciable resistance to thermal inactivation even when the heat was applied to the intact spore. Thus, this enzyme apparently has no role in the germination of the spore. These data indicate that the enzyme was either present in the spore in an unstabilized form or was only associated with the sporangial and cellular debris contaminating the spore suspensions. The DPNH oxidase of the spore develops a resistance to thermal inactivation during the entire sporulation process, and this resistance appears to be at least partially inherent in the enzyme itself. This enzyme may be one truly significant enzyme of the spore. A definite role in spore germination has been demonstrated for a similar enzyme found in aerobic spore-formers (Doi and Halvorson, 1961). These investigators showed that the DPNH oxidase of the vegetative cells of _B. cereus_ was different from that of the spores in that the vegetative enzyme was particulate while the spore DPNH oxidase was soluble. Thus, it appears that there is a transition in enzymes during sporulation. The DPNH oxidase of _C. botulinum_ may exist in different forms in the vegetative cell and in the spore, and the development of heat resistance of the enzyme may be due to the synthesis of a new enzyme rather than the stabilization of the vegetative enzyme. The present data indicates that heat resistance of DPNH oxidase

is initiated at the time sporulation first begins. This is well before the synthesis of DPA has begun, and during a period of protein synthesis. It would appear that heat resistance involves more than the presence of DPA.

Simmons (1961) demonstrated the presence of both acetokinase and DPNH oxidase in the extracts of heat shocked spores of *C. botulinum*, and observed no inactivation of the DPNH oxidase from heat shocked spores on heating of the extracts at 80 C for 1 min. The data presented here are in obvious disagreement with these findings. Two possible explanations of these variations are (a) the method of extraction, and (b) the culture used. The extraction procedures used were similar but differed in volumes. Simmons (1961) used the 50 ml cup with the Omnimixer, while results presented here were obtained with extracts prepared in the 5 ml Micro-attachment of the Omnimixer. The culture of *C. botulinum* used for both investigations was from the same original source, but the culture used in this study had been cultivated in the laboratory for an additional year. These variables need more investigation.

This is by no means a complete picture of the processes taking place during sporogenesis, and there are undoubtedly many subtle control mechanisms in operation which will remain obscure until more refined experimental techniques are available. It is believed that this work does give a more general view of the over-all sporulation process in an anaerobe, and the manner in which some of the events involved are related. A great amount of experimental work could have been carried out in many directions to further clarify the physiological events occurring in the sporulating bacterial cell, but the solution to one problem invariably leads to another problem. It is hoped that this work will lead to more detailed and more fruitful studies in this fundamental question of cell differentiation.

SUMMARY

This investigation was carried out in order to gain insight into the over-all processes taking place during sporulation of C. botulinum, type A. The processes were viewed from several different aspects including studies of sporulation in replacement cultures; the role of end products in sporulation; the syntheses of proteins, RNA and DNA during sporulation; and the development of heat resistance of certain enzymes during the transition from the vegetative cell to the mature spore.

The replacement studies revealed that an exogenous source of energy as provided by L-alanine and L-proline, or L-isoleucine and L-proline in coupled deamination reactions (Stickland Reaction) supported the development of spores from the immature forespore stage to the final free spore stage. Using C^{14} labeled L-alanine, it was shown that no L-alanine per se was being utilized for synthetic processes during this time since all the label was recovered in CO_2. Acetic acid-1-C^{14} studies showed that this compound was not used further by the cells in a replacement culture of L-alanine and L-proline. Acetate was not utilized in replacement, but was used by cells during growth and the initial stage

of sporulation in 4% Trypticase broth plus 1 μg/ml of thiamine, and the fractionation techniques used indicated that acetate was involved in mucocomplex synthesis. It was observed that L-alanine and L-arginine would support forespore maturation also, but that L-alanine and L-ornithine would not, nor would L-arginine or L-citrulline alone.

Studies with chloramphenicol indicated that protein synthesis was not required during the final step of sporulation but that protein was being formed up to the time of the appearance of immature forespores. Dipicolininc acid was synthesized after the appearance of refractile spores but before the resistance to heat of the spores was developed.

The synthesis of RNA was required through the time of formation of the immature forespore, this conclusion being arrived at by the use of radioactive phosphorus and certain inhibitors of nucleic acid synthesis. Mitomycin C, a specific DNA inhibitor (Shiba, 1959) would inhibit vegetative growth, but had no effect on sporulation. Using P^{32} and the assay of Hanawalt (1959), it was concluded that DNA synthesis was completed during the initial sporulation stage and was not required for the formation of the immature forespores. Since the purine analogue, 8-azaguanine, inhibited sporulation to a certain level at this time, it was concluded that RNA synthesis was essential to sporulation during the transition from the swollen vegetative cell to the immature forespore.

Two enzymes, acetokinase and DPNH oxidase, were studied in the vegetative cell, spore, and the transition state between these two forms. Acetokinase was found to be readily inactivated by heat either in the cell or in cell-free extracts of any of the stages of development. DPNH oxidase had a higher specific activity in the vegetative cells than in the spore, but became more stable to heat during sporogenesis. The resistance to heat developed during the initial stages of sporulation and reached a maximum in the free spores. This resistance appeared to develop before the formation of DPA by the spores.

BIBLIOGRAPHY

Bach, J. A., and H. L. Sadoff. 1962. Aerobic sporulating bacteria. I. Glucose dehydrogenase of Bacillus cereus. J. Bacteriol. 83:699-707.

Black, S. H., T. Hashimoto, and P. Gerhardt. 1960. Calcium reversal of the heat susceptibility and dipicolinate deficiency of spores formed "endotrophically" in water. Can. J. Microbiol. 6:213-224.

Blair, E. B. 1950. Observations on bacterial spore formation in synthetic media. M.A. thesis, University of Texas. (Abstr.) Texas Repts. Biol. Med. 8:361.

Buchner, H. 1890. Ueber die Ursache der Sporenbildung beim Milzbrandbacillus. Zentr. Bakt. Parasitenk., Orig. 8:1-6., cited by Knaysi, 1948.

Clifton, C. E. 1940. The utilization of amino acids and of glucose by Clostridium botulinum. J. Bacteriol. 39:485-497.

Collier, R. C. 1956. An approach to synchronous growth for spore production in Clostridium roseum. In H. O. Halvorson [ed.], Spores. American Institute of Biological Sciences, Washington, D. C.

Cook, R. P. 1932. Bacterial spores. Biol. Revs. Cambridge Phil. Soc. 7:1-23.

Costilow, R. N. 1962. Fermentative activities of control and radiation-"killed" spores of Clostridium botulinum. J. Bacteriol. 84(6):In press.

Curran, H. R., B. C. Brunstetter and A. T. Myers. 1943. Spectrochemical analysis of vegetative cells and spores of bacteria. J. Bacteriol. 45:484-494.

Day, L. E. 1960. Studies on the sporulation of Clostridium botulinum, type A. M.S. Thesis, Michigan State University.

Delaporte, B. 1950. Observations on the cytology of bacteria. Adv. Genet. 3:1-32.

Doi, R. H. and H. Halvorson. 1961. Comparison of electron transport systems in vegetative cells and spores of Bacillus cereus. J. Bacteriol. 81:51-58.

Esty, J. and K. F. Meyer. 1922. The heat resistance of B. botulinum and allied anaerobes. J. Infect. Dis. 31:650-663.

Fitz-James, P. C. 1954. The phosphorus fractions of Bacillus cereus and Bacillus megaterium. Can. J. Microbiol. 1:502-519.

Fitz-James, P. C. and E. I. Young. 1959. Origin of initial spore wall and the enclosed DNA in sporulating Bacillus cereus. Bacteriol. Proc. 1959:38.

Foster, J. W. and J. J. Perry. 1954. The non-involvement of lysis during sporulation of Bacillus mycoides in distilled water. J. Bacteriol. 37:401-409.

Gollakota, K. G. and H. O. Halvorson. 1960. Inhibition of sporulation of Bacillus cereus. J. Bacteriol. 79:1-8.

Halvorson, H. O. 1957a. [ed.], Spores. American Institute of Biological Sciences, Washington, D. C.

Halvorson, H. O. 1957b. Rapid and simultaneous sporulation. J. Appl. Bacteriol. 20:305-314.

Halvorson, H., R. Doi and B. D. Church. 1958. Dormancy of bacterial endospores: regulation of electron transport by DPA. Proc. Natl. Acad. Sci. 44:1171-1180.

Halvorson, H. and C. Howitt. 1961. Role of DPA in bacterial spores. In H. O. Halvorson, [ed.], Spores II. Burgess Publishing Company. Minneapolis 15, Minnesota.

Hanawalt, P. 1959. P^{32} assay for nucleic acids in bacterial cells. Science 130:386-387.

Hardwick, W. A. and J. W. Foster. 1952. On the nature of sporogenesis in some aerobic bacteria. J. Gen. Phys. 35:907-927.

Hashimoto, T., S. H. Black and P. Gerhardt. 1960. Development of fine structure, thermostability and dipicolinate during sporogenesis of a Bacillus. Can. J. Microbiol. 6:203-212.

Hodson, P. and J. Beck. 1960. Origin of DNA of bacterial endospores. J. Bacteriol. 79:661-665.

Janssen, F. W., A. J. Lund and L. E. Anderson. 1958. Colorimetric assay for dipicolinic acid in bacterial spores. Science 127:26-27.

Jordan, D. C. 1961. Effect of vancomycin on the synthesis of cell wall mucopeptide of Staphylococcus aureus. Biochem. Biophys. Res. Commun. 6:167-170.

Jordan, D. C. and W. E. Innis. 1959. Selective inhibition of ribonucleic acid synthesis in Staphylococcus aureus by vancomycin. Nature 184:1894-1895.

Kaplan, I. and J. W. Williams. 1941. Spore formation among the anaerobic bacteria. I. The formation of spores by Clostridium sporogenes in nutrient agar. J. Bacteriol. 42:265-282.

Kennedy, E. P. and H. A. Barker. 1951. Paper chromatography of volatile acids. Anal. Chem. 23:1033-1034.

Kinnory, D. S., E. L. Kanabrocki, J. Greco, R. L. Veatch, E. Kaplan, and Y. T. Oester. 1958. A liquid scintillation method for measurement of radioactivity in animal tissue and tissue fractions. In C. G. Bell and F. N. Hayes, [ed.], Liquid scintillation counting. Pergamon Press, New York.

Knaysi, G. 1945. A study of some environmental factors which control endospore formation by a strain of Bacillus mycoides. J. Bacteriol. 49:473-493.

Knaysi, G. 1948. The endospore of bacteria. Bacteriol. Rev. 12:19-77.

Law, J. H. and R. A. Slepecky. 1961. Assay of poly-β-hydroxybutyric acid. J. Bacteriol. 82:33-36.

Lawrence, N. L. and H. O. Halvorson. 1954. Studies on the spores of aerobic bacteria. IV. A heat resistant catalase from spores of Bacillus terminalis. J. Bacteriol. 68:334-337.

Lechowich, R. L. 1958. Studies on thermally induced changes in the bacterial endospore and on the relationship of its chemical composition to thermal resistance. Ph.D. Thesis, University of Illinois.

Leifson, E. 1931. Bacterial spores. J. Bacteriol. 21:331-356.

Lipmann, F. and L. C. Tuttle. 1945. A specific micromethod for the determination of acyl phosphates. J. Biol. Chem. 159:21.

Lund, A. J. 1956. Discussion In H. O. Halvorson, [ed], Spores. American Institute of Biological Sciences, Washington, D. C.

Martin, H. H. and J. W. Foster. 1957. Biosynthesis of dipicolinic acid in Bacillus megaterium. J. Bacteriol. 76:167-178.

Mohrke, W. 1926. Ein neues Verfahren sur Einsporenkultur anaerober Bakterien, nebst Bemerkungen uber das Versporungsoptimun der Anaerobier. Zent. Bakt. Parasitenk., Abt. I, Orig., 98:533-547., cited by Knaysi, 1948.

Nakata, H. M. and H. O. Halvorson. 1960. Biochemical changes occurring during growth and sporulation of Bacillus cereus. J. Bacteriol. 80:801-810.

Nisman, B. 1954. The Stickland Reaction. Bacteriol. Rev. 18:16-42.

O'Conner, R. J. and H. Halvorson. 1960. L-alanine dehydrogenase: the possible binding site for L-alanine in aerobic spores. Bacteriol. Proc. 1960:66.

Ordal, Z. J. 1956. The effect of nutritional and environmental conditions on sporulation. In H. O. Halvorson, [ed.], Spores. American Institute of Biological Sciences, Washington, D. C.

Perkins, W. E. and K. Tsuji. 1962. Sporulation of Clostridium botulinum. II. Effect of arginine and its degradation products on sporulation in a synthetic medium. J. Bacteriol. 84:75-83.

Portellada, P. C. L. 1960. Studies on sporogenesis. V. Consumption of DPA. An. Microbiol. 8:75-83.

Portellada, P. C. L. 1961. Studies on sporogenesis. IV. Inhibition of endotrophic sporulation. An. Microbiol. 8:65-73.

Powell, J. F. 1953. Isolation of dipicolinic acid (pyridine-2,6-dicaroxylic acid) from spores of Bacillus megaterium. Biochem. J. 54:210-211.

Powell, J. F. and J. R. Hunter. 1953. Sporulation in distilled water. J. Gen. Phys., 36:601-606.

Powell, J. F. and R. E. Strange. 1959. Synthesis of dipicolinic acid from α,ϵ-diketopimelic acid. Nature 184:878-880.

Riemann, H. 1961. Germination of bacteria by chelating agents. In H. O. Halvorson, [ed.], Spores II. Burgess Publishing Company, Minneapolis 15, Minnesota.

Riemann, H. and Z. J. Ordal. 1960. Studies on the germination of clostridial spores. Bacteriol. Proc. 1960:44.

Robinow, C. R. 1956. The chromatin bodies of bacteria. Symp. Soc. Gen. Microbiol. 6:181-214.

Roessler, W. G. and C. R. Brewer. 1946. Nutritional studies with Clostridium botulinum, toxin types A and B. J. Bacteriol. 51:386-391.

Rose, I. A., M. Grunberg-Manago, S. R. Korey, and S. Ochoa. 1954. Enzymatic phosphorylation of acetate. J. Biol. Chem. 211:737-756.

Sadoff, H. L. 1961. Some properties of the spore catalase and some heat resistant enzymes in bacterial glucose dehydrogenase. In H. O. Halvorson, [ed.], Spores II. Burgess Publishing Company, Minneapolis 15, Minnesota.

Schaeffer, P., H. Ionesco and F. Jacob. 1959. The genetic determination of bacterial spore formation. Compt. Rend. Acad. Sci. 249:577-578.

Schreiber, O. 1896. Ueber die physiologischen Bedingungen der endogenen Sporenbildung bei Bacillus anthracis, subtilis und tumescens. Zentr. Bakt. Parasitenk., Abt. II, Orig., 20:353-374., cited by Knaysi, 1948.

Shiba, S., A. Terawaki, T. Taguchi, and J. Kawamata. 1959. Selective inhibition of formation of deoxyribonucleic acid in Escherichia coli by mitomycin C. Nature 183:1056-1057.

Shinagawa, T. 1960. Studies on the sporulation of Bacillus cereus var mycoides. Jour. Osaka City Med. Center 9:4047-4054.

Simmons, R. J. 1961. Glucose metabolism of cells, spores and germinated spores of Clostridium botulinum. Ph.D. Thesis, Michigan State University.

Slepecky, R. A. and J. H. Law. 1961. Synthesis and degradation of poly-β-hydroxybutyric acid in connection with sporulation of Bacillus megaterium. J. Bacteriol. 82:37-42.

Sommer, E. W. 1930. Heat resistance of the spores of Clostridium botulinum. J. Infect. Dis. 46:85-114.

Srinivason, V. R., and H. O. Halvorson. 1962. Personal communication.

Stadtman, E. R., G. D. Novelli and F. Lipmann. 1951. Coenzyme A function in and acetyl transfer by the phosphotransacetylase system. J. Biol. Chem. 191:365-376.

Stedman, R. L. 1956. Biochemical aspects of endospore formation and germination. Am. J. Pharm. 128:84-97.

Stewart, B. T. and H. O. Halvorson. 1953. Studies on the spores of aerobic bacteria. I. The occurrence of alanine racemase. J. Bacteriol. 65:160-166.

Stickland, L. H. 1934. The chemical reactions by which Cl. sporogenes obtains its energy. Biochem. J. 28:1746-1759.

Stickland, L. H. 1935a. The reduction of proline by Cl. sporogenes. Biochem. J. 29:288-290.

Stickland, L. H. 1935b. The oxidation of alanine by Cl. sporogenes. Biochem. J. 29:896-898.

Umbreit, W. W., R. H. Burris and J. F. Stauffer. 1959. Manometric Techniques. Burgess Publishing Company, Minneapolis 15, Minnesota.

Vinter, V. 1960. Spores of microorganisms. VIII. The synthesis of specific calcium-and cystine-containing structures in sporulating cells of bacilli. Folia Microbiol. 5:217-230.

Vinter, V. 1962. Spores of microorganisms. IX. Gradual development of the resistant structure of bacterial endospores. Folia Microbiol. 7:115-120.

Walker, H. W., J. R. Matches and J. C. Ayres. 1961. Chemical composition and heat resistance of some aerobic bacterial spores. J. Bacteriol. 82:960-966.

Williams, O. B. 1952. Symposium on the biology of bacterial spores. Backteriol. Rev. 16:89-143.

Williams, O. B. and E. Blair. 1950. Spore formation in synthetic media by Clostridium botulinum. Bacteriol. Proc. 1950:62-63.

Wiseman, H. G. and H. M. Irvin. 1957. Determination of organic acids in silage. Agricul. Food Chem. 5:213-215.

Wynne, E. S. 1948. Physiological studies on spore formation in Clostridium botulinum. J. Infect. Dis. 83:243-249.

Wynne, E. S., W. R. Schmieding, and G. T. Daye, Jr. 1955. A simplified method for counting Clostridium spores. Food Res. 20:9-12.

Young, E. I. 1959. A relationship between the free amino acid pool, dipicolinic acid, and calcium from resting spores of _Bacillus megaterium_. Can. J. Microbiol. _5_:197-202.

Young, E. I. and P. C. Fitz-James. 1959a. Chemical and morphological studies of bacterial spore formation. I. The formation of spores in _Bacillus cereus_. J. Biophys. Biochem. Cytol. _6_:467-482.

Young, E. I. and P. C. Fitz-James. 1959b. Formation of parasporal protein crystals in _Bacillus cereus_ var _alesti_. Bacteriol. Proc. _1959_:38.

APPENDIX

TABLE I-A

The Amino Acid Composition of Trypticase*

Amino Acids	Percent
Arginine	2.6
Aspartic acid	5.1
Cystine	0.3
Glycine	1.8
Glutamic acid	17.0
Histidine	2.4
Isoleucine	5.0
Leucine	7.1
Methionine	2.4
Phenylalanine	3.8
Proline	11.5
Threonine	3.5
Tryptophan	0.9
Tyrosine	2.3
Valine	5.6

*Reproduced from the table "Approximate Composition of B-B-L Peptones" furnished by the Baltimore Biological Laboratory, Inc., of Baltimore, Maryland.

TABLE II-A

The Composition of Amino Acid Replacement Solutions.

Monoaminomonocarboxylic amino acids:

glycine	.007 g
L-alanine	.010 g
L-valine	.014 g
L-leucine	.028 g
L-isoleucine	.020 g
L-serine	.010 g
L-threonine	.014 g
H_2O	10 ml

Monoaminodicarboxylic amino acids:

L-aspartic acid	.020 g
L-glutamic acid	.068 g
H_2O	10 ml

Sulfur-containing amino acids

L-cystine	.001 g
L-methionine	.010 g
H_2O	10 ml

Basic amino acids

L-lysine	.021 g
L-arginine	.010 g
L-histidine	.010 g
H_2O	10 ml

Aromatic amino acids

L-phenylalanine	.015 g
L-tyrosine	.010 g
H_2O	10 ml

Heterocyclic amino acids

L-tryptophan	.004 g
L-proline	.046 g
H_2O	10 ml

TABLE III-A

The Composition of Scintillation Liquid Used for C^{14} Assay.

xylene	385 ml
dioxane	385 ml
ethyl alcohol, absolute	230 ml
2,5-diphenyloxazole	5 g
ANPO	50 mg
naphthalene	80 g
thixotropic gel powder	40 g

All materials were placed in a Waring Blendor and mixed for five minutes. The material was then stored in glass bottles at 5 C until used.