





RETURNING MATERIALS:
Place in book drop to
remove this checkout from
your record. FINES will
be charged if book is
returned after the date
stamped below.

FEB 12 1998 FEB 20 1998 MSU LIBRARIES 400 A064		
--	--	--

**STUDIES ON THE SPORULATION OF
CLOSTRIDIUM BOTULINUM, TYPE A**

**By
Lawrence E. Day**

A THESIS

**Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of**

MASTER OF SCIENCE

Department of Microbiology and Public Health

1960

ABSTRACT

STUDIES ON THE SPORULATION OF CLOSTRIDIUM BOTULINUM, TYPE A

by Lawrence E. Day

A study was made of the sporogenic process of Clostridium botulinum, ATCC 62-A, in order to determine whether or not this organism would undergo endotrophic sporulation, and also to determine the nutritive requirements for sporulation. It was demonstrated that Cl. botulinum would not sporulate after being replaced in water, phosphate buffer, several solutions of various amino acids and vitamins, a solution of acid hydrolyzed casein, and a solution of enzymatic hydrolyzed casein. A four percent solution of trypticase, a pancreatic digest of casein, was found to be highly sporogenic both as a culture medium and as a replacement medium. When the individual amino acids known to be present in trypticase were combined as a synthetic medium and used as a replacement solution, significant sporulation occurred.

A study of the effects of penicillin, chloramphenicol, and vancomycin on the sporulation process was made. Penicillin and vancomycin were found to inhibit sporulation if added to the culture before or shortly after the sporulation process had begun, however, a lag was observed before vancomycin demonstrated any effect. Chloramphenicol arrested sporulation when added to the culture at any stage of sporulation, with no lag being demonstrated.

ACKNOWLEDGEMENTS

The author wishes to extend his sincere appreciation to Dr. R. N. Costilow of the Department of Microbiology and Public Health for his kind assistance and guidance, and for his technical advice.

The many helpful suggestions of Dr. H. L. Sadoff of the Department of Microbiology and Public Health are acknowledged with much gratitude.

TABLE OF CONTENTS

	PAGE
INTRODUCTION.....	1
REVIEW OF LITERATURE.....	3
The replacement technique and endo- trophic sporulation.....	3
Nutritional and environmental require- ments for sporogenesis.....	4
The role of dipicolinic acid in sporulation.....	9
The mode of action of penicillin, chlor- amphenicol and vancomycin.....	10
EXPERIMENTAL METHODS.....	14
RESULTS.....	20
DISCUSSION.....	43
SUMMARY.....	50
BIBLIOGRAPHY.....	52

LIST OF TABLES

TABLE	PAGE
I. Summary of the results of the endotrophic sporulation of <u>Cl. botulinum</u>	29
II. Summary of the results of the sporulation of <u>Cl. botulinum</u> in chemically defined replacement media; cells cultivated in TSP medium..	32
III. Summary of the results of the sporulation of <u>Cl. botulinum</u> in chemically defined replacement media; cells cultivated in 4 percent trypticase.....	34

LIST OF FIGURES

FIGURE	PAGE
1. Comparison of growth and sporulation of <u>Cl. botulinum</u> in TSP medium and in 4 percent trypticase broth containing 1 ug per ml of thiamine.....	21
2. Comparison of growth and sporulation of <u>Cl. botulinum</u> in 4 percent trypticase and in 4 percent trypticase containing 1 ug per ml of thiamine.....	23
3. Comparison of total refractile spore counts with heat resistant spore counts and the level of dipicolinic acid present during sporulation of <u>Cl. botulinum</u>	24
4. The effect of replacement of the vegetative cells of <u>Cl. botulinum</u> in the presence of atmospheric oxygen.....	26
5. The effect of replacement of the vegetative cells of <u>Cl. botulinum</u> in the presence of nitrogen.....	28
6. The replacement of the vegetative cells of <u>Cl. botulinum</u> in a synthetic medium containing thiamine and the amino acids of trypticase.....	37
7. The effect of penicillin on the sporulation of <u>Cl. botulinum</u>	39
8. The effect of chloramphenicol on the sporulation of <u>Cl. botulinum</u>	40
9. The effect of vancomycin on the sporulation of <u>Cl. botulinum</u>	42

LIST OF APPENDICES

APPENDIX PAGE 57

INTRODUCTION

Practically all studies concerning the nature of the sporogenic process of Clostridium botulinum have been carried out in complex, artificial media, and no real distinction has been made between conditions which favor growth of the vegetative cell and those which favor sporulation. However, Blair (1950) demonstrated limited spore formation of Cl. botulinum in variations of the synthetic medium of Roessler and Brewer (1946). In order to study the processes of sporulation of the anaerobic bacteria more directly, the need exists for the development of a technique and/or a medium which allows sporulation to proceed to completion in an environment of known composition. The technique of replacement offers a method of partially segregating the growth phase from the sporogenic process. Hardwick and Foster (1952) demonstrated the practicability of the replacement procedure for aerobic spore-forming bacteria by transferring vegetative cells that were committed to sporulation to distilled water. A high percentage of sporulation was shown to occur in this non-nutritive environment. They have designated this phenomenon as endotrophic sporulation. There are no reports that this procedure can be carried out with Cl. botulinum, although Collier (1956) reported that the vegetative cells of Clostridium roseum will sporulate in an environment devoid of nutrients.

This work was undertaken to investigate endotrophic

sporulation of Cl. botulinum as a possible approach to a more detailed study of the sporogenic process of this organism; and, failing this, to determine the nutritive requirements necessary for cells to sporulate. Some of the more general aspects of sporulation of this species were also studied in order to gain more insight into the nature of sporulation of the anaerobic bacteria.

REVIEW OF LITERATURE

Replacement and endotrophic sporulation. When the vegetative cells of aerobic spore-formers are harvested and resuspended in distilled water, sporulation occurs (Buchner, 1890; Schreiber, 1896; and Knaysi, 1945). There was no experimental utilization of this observation until 1952, when Hardwick and Foster (1952) noted the value of this technique as an experimental approach to sporogenesis. Using Bacillus mycoides and Bacillus lacticola, they found that sporulation occurred over a two hour period after replacement in distilled water, and a final level of 70 - 90 percent spores was attained. The heat resistance of the spores thus produced was the same as the heat resistance of spores produced in a growth medium. However, Black et al. (1960b) observed that spores of Bacillus cereus are heat susceptible when produced endotrophically.

It is the hypothesis of Hardwick and Foster (1952) that sporulation in distilled water is accomplished by the degradation of materials already in the cell for the utilization in the sporogenic process. The absence of exogenous nutrition would support this conclusion. While it is felt by some investigators (Powell and Hunter, 1953; Black et al., 1960b) that the replacement solution is actually a dilute nutrient medium due to the lysis of vegetative cells after the transfer to water, Perry and Foster (1955) were unable

to detect lysis during endotrophic sporulation. Hardwick and Foster (1953) further observed that the enzymes of glycolysis are degraded and utilized as substrate by amino acid enzymes in the sporogenic process. When glucose is present in the environment, sporulation is inhibited, indicating that the glucose metabolism takes precedence over the metabolism essential for sporulation.

Collier (1956) removed sub-samples from a culture of Cl. roseum under anaerobic conditions when the culture was in the process of sporulation. The cells were washed, and incubated at 30°C in sterile distilled water under anaerobic conditions. The culture sporulated completely under these conditions. However, Lund (1956) observed that a mutant of the Putrefactive Anaerobe (PA 3679-h) would not sporulate in a replacement menstruum of buffered thioglycollate or in thioglycollate and glucose after being cultivated in a refortified spent medium of 2 percent trypticase broth. When the vegetative cells of this same organism were replaced in a non-sporogenic medium such as 2 percent trypticase, sporulation took place in the replacement medium before sporulation in the control medium.

Mineral, nutritional and environmental requirements for sporogenesis. Much investigation has been done in this particular area, especially with the aerobic spore-formers. Ordal (1956) has stated three requirements for sporogenesis; 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. The first of these requirements is, of course, primary. The second of the requirements, proper physiological condition, is much more difficult to assess. The right physiological condition for sporogenesis apparently requires minerals, nutrients, growth factors, and environmental conditions which differ from those required for growth. The manganese ion is of prime importance to sporulation in the genus Bacillus (Charney et al., 1951; Powell, 1956). Curran and Evans (1954) noted that the requirement for Mn^{++} could be replaced by large additions of $Fe^{++}(+)$, but Weinberg (1955) demonstrated that this quantity of $Fe^{++}(+)$ contained minute amounts of Mn^{++} as an impurity and the amount of manganese that is required for sporulation is only one part in twenty million. The function of this ion may be considered from two aspects; it may inactivate some spore inhibiting compounds, or it may be required for the action of enzymes of sporogenesis (Weinberg, 1956).

Other metallic ions have been found to be of importance in sporogenesis. In addition to manganese, Anaha et al. (1956) observed that Co^{++} and Ni^{++} enhanced sporulation in the genus Bacillus. Foster and Heiligman (1949) found that K^+ is deficient in complex organic media in the fluid state for sporulation of B. cereus; while Brewer et al. (1946) have reported that Mg^{++} would enhance sporulation of Bacillus anthracis in a synthetic medium. It was also noted

by these investigators that Ca^{++} increased sporulation 4.5 times in a synthetic medium compared to the same medium containing no Ca^{++} .

The effect of the salt concentration or the ionic strength of the medium on sporogenesis has not been studied extensively. The percent sporulation of a culture of Cl. botulinum decreased as the concentration of Na_2SO_4 , NaCl and KCl increased in the culture medium, and, when the concentration of NaCl was increased to 2 percent in brain-heart-infusion medium, sporulation was inhibited completely (Wynne, 1948). Leifson (1931) also noted the inhibitory effect of NaCl, and reported that sporulation took place in a basal medium of 1 percent peptone only when NH_4^+ and $\text{PO}_4^{=}$ were added. The addition of sulfate stimulated sporulation somewhat.

The role of glucose in sporulation is controversial. Leifson (1931) reported that a 0.2 percent concentration of this carbohydrate is inhibitory to sporulation of Cl. botulinum due to acid formation, and Kaplan and Williams (1941) noted the same effect with Clostridium sporogenes. Elair (1950) noted inhibition of sporulation of Cl. botulinum in synthetic medium, but Wynne (1948) reported that 0.8 percent glucose in brain-heart-infusion medium did not adversely affect sporulation of this organism.

The significance of growth factors in the sporogenic process has not been studied extensively. Ordal (1956) reported that the amount of folic acid required for

sporulation was greater than that required for growth of Bacillus coagulans; and Williams and Harper (1951) noted that when p-aminobenzoic acid was deleted from a medium, the sporulation of B. cereus was reduced. Lund (1956) observed that the medium in which PA 3679 had been cultivated demonstrated sporogenic activity. He believed that this could be due to exhaustion of some essential nutrient for growth of the vegetative cells; or to the utilization of some sporulation inhibitor; or to the synthesis of some factor which is required for sporulation. The medium used in this investigation was 2 percent trypticase with 0.1 percent sodium thioglycollate. When this medium was fortified with 10 µg per ml of thiamine, all cells were in the swollen state at the end of 24 hours of growth. However, the cells never completed the sporulation process, and at the end of 72 hours only vegetative cells were present in the medium. If the cells were removed from this medium after 18-20 hours of growth, and the medium refortified with the initial quantity of dry ingredients, sporulation took place to a level of about 90 percent. A control of 4 percent trypticase, which corresponded to the concentration of trypticase in the refortified spent medium, did not demonstrate as great a degree of sporulation. This seemed to eliminate the theory of the removal of a sporulation inhibitor or the exhaustion of nutrients as the factors initiating sporulation. On the basis of this observation, it was theorized that something

is synthesized and elaborated into the medium by the organism which is required for sporogenesis.

Elair (1950) working with the synthetic medium of Reessler and Brewer (1946) demonstrated that the deletion of methionine and phenylalanine from this medium suppressed the sporulation process of Cl. botulinum. The percent sporulation was increased by addition of α -alanine, glutamic acid, glycine, ornithine, sodium butyrate, sodium valerate, arginine and proline. The stimulatory effect of glucose towards sporogenesis in aerobic bacilli in a synthetic glucose-glutamate medium was reversed by the addition of DL-alanine (Foster and Heilgman, 1949).

The effect of oxygen tension on sporulation of the anaerobes is not clear. Leifsen (1931) reported that various anaerobic organisms exhibit different responses under increasing oxygen tension. Sommer (1930) working with Cl. botulinum noted that a broth culture 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, it has been observed that the level of sporulation in broth cultures is identical in either oxygen or in natural gas atmospheres (Wynne, 1948). All of these observations were made with Cl. botulinum.

The optimum pH for the sporulation of Cl. botulinum has been established to be 7.0-7.2. This was noted by Mohrke

(1926) and by Collier (1956). Leifson (1931) stated that the pH at the time of sporulation is of greater importance than the initial pH, but he did not recommend what this pH should be.

The role of dipicolinic acid in sporulation. Pyridine-2, 6-dicarboxylic acid or dipicolinic acid was first isolated from the spores of Bacillus megatherium by Powell (1953). Perry and Foster (1955) isolated DPA from endotrophically produced spores of B. cereus and Bacillus subtilis. In a rapidly sporulating culture of Cl. roseum, DPA synthesis was found to correlate directly with the sporogenic process (Collier and Krishnamurty, 1957); but in other work with this same organism, it was noted that the maximum DPA was formed an hour before heat resistant spores could be detected (Halvorson, 1957). This was also observed with sporulating B. cereus (Black et al., 1960a). Young (1959) ascribes the thermostability of spores to a complex of DPA and amino acids in the presence of calcium; however, this complex was only demonstrated on the filter paper of a chromatogram. This combination could be the means whereby dipicolinic acid renders stability to proteins and nucleic acids of the spore. The work of Black et al. (1960b) would indicate that DPA does render heat stability to the spore. These investigators demonstrated that endotrophically produced spores of B. cereus were heat susceptible and DPA deficient. If Ca^{++} were added to the replacement suspension shortly

after transfer of the vegetative cells, the DPA deficiency was relieved; however, the spores were still heat sensitive. These spores were as resistant to gamma radiation or to phenol as normal spores. Church and Halverson (1959) reported that if phenylalanine were present during sporogenesis of B. cereus, the DPA level of the spores was reduced with a concomitant lowering of the heat resistance. No other amino acids demonstrated this phenomenon. The most recent report would indicate that the DPA content of the spores of Cl. roseum is not necessarily correlated to the heat resistance. Byrne et al. (1960) reported that spores produced in the presence of L-alanine had a lower level of dipicolinic acid than spores produced in a medium containing no L-alanine; however, the spores with the lesser amount of DPA were more heat resistant. Obviously, much work needs to be accomplished on the role of DPA in the spore and on its connection with heat resistance. At one time it was surmised that DPA gave ultraviolet (U.V.) resistance to the mature spore because it absorbs strongly in the U.V. region of the spectrum, but Romig and Wyss (1957) noted that resistance to U.V. appears at the time of the formation of the forespore of B. cereus while the DPA does not appear until mature spores are present. This work was done with cultures sporulating in distilled water.

The mode of action of penicillin, chloramphenicol, and vancomycin. Most recent reports attribute the action of

penicillin to the inhibition of division of the bacterial cell by the combination with and inactivation of some essential constituent of the cell which is required for division to occur (Lederberg, 1957). It does not prevent respiration or synthesis within the cell (Pratt, 1953). The compound inactivated by penicillin which is required for cell wall synthesis is N-acetyl muramic acid (Park and Strominger, 1957). Most earlier reports, however, attributed the mechanism of action of this antibiotic to the inhibition of RNA synthesis (Mitchell and Moyle, 1951; Gale, 1949) or a disturbance in the nucleotide balance (Mitchell, 1949; Krampitz and Werlman, 1947). This disturbance of nucleic acid synthesis works by blocking assimilatory processes, thus organisms which are dependent upon assimilation rather than synthesis for growth are sensitive to this drug (Gale, 1949). Gram positive organisms lose their characteristic staining reactions. This was shown by analysis to be due to the alteration by penicillin of the ratio of ribonucleic phosphorous to deoxyribonucleic phosphorous in the gram positive sensitive cell toward the ratio in the gram negative organism (Gale, 1948).

Gale and Folkes (1953) reported that chloramphenicol acts by the inhibition of protein synthesis, but they added that the same concentration of the antibiotic stimulated the synthesis of RNA. With the use of labeled amino acids, it was found that protein synthesis was inhibited by the

prevention of amino acid utilization (Gale and Folkes, 1953; Smith, 1953). The inhibition of metabolism of fats and esters by the action of chloramphenicol has also been demonstrated to be of significant importance in the inhibition of growth of the bacterial cell (Smith, 1953).

Wissiman et al. (1952) reported that the inhibition of the assimilation of NH_3 and the concurrent oxidation of glycerol was a mechanism of this drug, while Gale and Paine (1950) found that chloramphenicol had little effect on glutamate assimilation even at high concentrations but inhibited the synthesis of protein completely. Primarily, then, the mode of action of chloramphenicol would appear to be the inhibition of the synthesis of protein materials in the cell.

A minimum amount of investigation has been reported on the mechanism of action of vancomycin. Of the information available, it is known that this antibiotic is bactericidal. This is accomplished by the inhibition of the synthesis of ribonucleic acid with the resulting inhibition of cellular division. Death is due to the unbalanced growth brought about by the inability of the organism to synthesize RNA (Inniss, 1959).

Collier (1956) removed samples of a culture of C1. roseum at various intervals of time and placed the samples in aeration tubes with penicillin at a final concentration of 100 units per ml. It was found that further sporulation was arrested by the penicillin, but no effect was observed

on the spores already in the sample at the time of transfer. Weinberg (1955) investigated the effect of nine antimicrobial drugs on the sporulation of B. subtilis cultured in a medium containing Mn^{++} . A level of penicillin which did not prevent growth had no effect on sporulation; higher levels of this antibiotic and its effect on sporulation were not discussed. Chloramphenicol inhibited sporogenesis at a lower concentration than was required to inhibit growth. This effect was not reversed by added Mn^{++} .

EXPERIMENTAL METHODS

Clostridium botulinum, 62-A, was the organism used in all aspects of this investigation. The culture was originally obtained from the American Type Culture Collection and was maintained in spore suspensions produced in either the trypticase-salts-peptone medium (TSP) of Zohn and Sadoff (1956) or in a medium of 4 percent trypticase broth (BBL). When spores were present in the culture at a level of 10^7 or 10^8 per ml, the spores were harvested from the growth medium by centrifugation, washed twice in sterile distilled water and resuspended in 0.067 M phosphate buffer. These suspensions were then stored at 5°C until needed. Subsequent inoculations were made from these suspensions.

Originally, TSP medium was used for the cultivation of vegetative cells and spores; however, subsequent work demonstrated that a medium of 4 percent trypticase containing 1 µg per ml of thiamine resulted in more rapid and complete sporulation. Yeast-extract-starch-bicarbonate agar (YESBA) of Wynne et al. (1955) was used for making colony counts with oval tubes. The composition of these media are given in the appendix, Table X.

Preliminary work indicated that an atmosphere devoid of oxygen would be necessary to carry out the transfer of the vegetative cells of Cl. botulinum from the growth medium to the replacement menstruum. This was achieved by the use of a plexiglass chamber containing an atmosphere

of nitrogen. This chamber was fitted with arm-length rubber gloves attached through openings in the front of the chamber to allow handling of equipment inside the chamber. The chamber was filled with nitrogen from a cylindrical tank of the compressed gas after passage over hot copper shavings to remove oxygen present in the nitrogen tanks. The gas entered the bottom of the chamber through gas jets fitted to the lower part of the chamber, and the atmosphere in the chamber escaped through a gas jet fitted to the top of the chamber. The chamber was equipped with an air-interlock in the rear which allowed cultures or equipment to be removed without the necessity of refilling the entire chamber with nitrogen. An electrical outlet was fitted to the inside of the chamber, and a Sorvall angle-head centrifuge was utilized for centrifuging the cells from the growth medium. The replacement technique was achieved by first cultivating the organism in TSP medium or 4 percent trypticase at 37°C for the desired length of time. Then, using the nitrogen chamber, a 10 ml sample of the culture was transferred to a sterile tube, and the cells removed from the growth medium by centrifugation for 10 minutes. The cells were washed three times in sterile distilled water, and resuspended in the appropriate replacement menstruum.

Anaerobiosis was attained by one of two methods. Sodium thioglycollate in a concentration of 0.1 percent was used in conjunction with YESBA medium used for the

enumeration of cells and spores. Sodium thioglycollate was also used in some cases with replacement solutions. This method was the most convenient to handle of those employed but was of doubtful value if the incubation times extended much beyond 72 hours. The sodium thioglycollate apparently loses its ability to maintain anaerobic conditions after this time interval. The other method of attaining anaerobiosis was by the use of the Brewer anaerobic jar. This method was utilized exclusively with replacement cultures. The cultures were placed in the jar, the jar exhausted by vacuum, and then filled with nitrogen from which the oxygen had been removed. The jar was exhausted and refilled three times to insure anaerobiosis.

Total counts and spore counts were usually made by the use of the Petroff-Hausser counting chamber. This is a highly reproducible method of enumerating cells and spores, however the method is of no value in determining viable or non-viable organisms. Also, it is of little use in differentiating heat resistant and non-heat resistant, or mature and immature spores. In those experiments concerned with the effect of antibiotics on the sporogenic process, YESEA and oval tubes were utilized to determine viable cells and viable spores. This again offered some difficulty in determining total counts since not all spores germinate when placed in germination medium without a heat shock. Samples for spore counts were heat shocked for 10 minutes

at 80°C. Four tubes were poured per dilution in running viable cell and/or spore counts.

In order to determine the DPA content of spores at various stages of development a large quantity of cells was required. Therefore, a 2L Erlenmeyer flask containing 1500 ml of 4 percent trypticase medium and fitted with appropriate inlet and exhaust tubes to allow the passage of sterile natural gas through the medium was inoculated with a 10 percent inoculum of vegetative cells of Cl. botulinum. At various time intervals 200 ml samples of the growth suspension were removed and total counts and spore counts made both by plating and by direct microscopic counts. Cells were then removed from the medium by centrifugation and resuspended in 5 ml of sterile phosphate buffer, 0.067M. The dry weight of the cells of each sample was determined by placing 1 ml in a crucible and drying at 110°C for 24 hours. The samples were then diluted to contain 4-20 mg of cells per 5 ml of sample. A dipicolinic acid assay was then run on these samples by the method of Janssen et al. (1958). The percent of DPA of the dry weight of the cells was calculated.

Penicillin at a concentration of 100 units per ml, chloramphenicol at 100 µg per ml, and vancomycin at 10 µg per ml were utilized to determine the effect of these antibiotics on sporulation. For each of these determinations four flasks containing 100 ml of 4 percent trypticase medium were used, each flask being inoculated with a 5 percent spore inoculum. To the first culture, the microbial

inhibitor being utilized was added during the logarithmic growth phase, or sometime during the interval from 10 to 16 hours of growth. During this time no sporulation was apparent and no forespores could be observed. At the end of the logarithmic growth or at about the 20th hour when almost all cells were in the swollen state but only about 1-5 percent spores could be observed, the microbial inhibitor was added to the second culture. At the point when there were from 10-50 percent spores (between the 26-30th hour), the antagonist was added to the third flask. The fourth flask served as a control. At various time intervals, samples were removed from each culture and total counts and spore counts made both by plating and by direct microscopic count.

The preparation of trypticase ash for use in replacement solution was accomplished by ashing 4 g of trypticase at 525°C in a porcelain crucible in a muffle furnace. This was then dissolved in 96 ml of water. It was found that it was necessary to lower the pH to about 3.0 in order to dissolve the ash. When the pH was again raised, precipitation of some constituent of the ash began at a pH of about 5.0. Therefore the pH was held just below this value.

A dialysate of trypticase was prepared by placing 100 ml of a 4 percent trypticase solution in dialysis tubing and suspending in a chromatography jar containing distilled water. The dialysis procedure was carried out for 72 hours at 5°C. The distilled water was stirred constantly

and changed every 24 hours. The dialyzed 4 percent trypticase solution was then sterilized for use as a replacement solution.

RESULTS

The initial phase of this investigation was concerned with the selection of a medium which would support growth and good sporulation of Clostridium botulinum. Originally, TSP medium was used for the cultivation of vegetative cells and spores of this organism because it had been demonstrated by Zoha and Sadoff (1958) that this medium would support the sporulation of a similar organism. They used Putrefactive Anaerobe 3679 (PA 3679) and achieved sporulation at a level of 10^9 per ml in 44 hours. However, these results could not be reproduced using Cl. botulinum in this work. Invariably the number of spores in the culture did not reach 80 - 90 percent of the total count until after 5 - 7 days of incubation. Therefore, a broth of 4 percent trypticase was tested as a culture medium, and it was observed to be of a much greater sporogenic nature than that of TSP medium. A comparison of the two media was made and it was found that sporulation in the TSP medium reached maximum sporulation after about 150 hours incubation, while sporulation in the 4 percent trypticase attained a level of 90 percent in about 40 hours. This comparison may be seen in Figure 1.

Trypticase is a pancreatic digest of casein marketed by the Baltimore Biological Laboratory, Inc. of Baltimore, Maryland. An approximate assay of the product shows it to be deficient in thiamine (Vera, 1960), and Lund (1956) reported that the addition of thiamine to trypticase stimulated spore

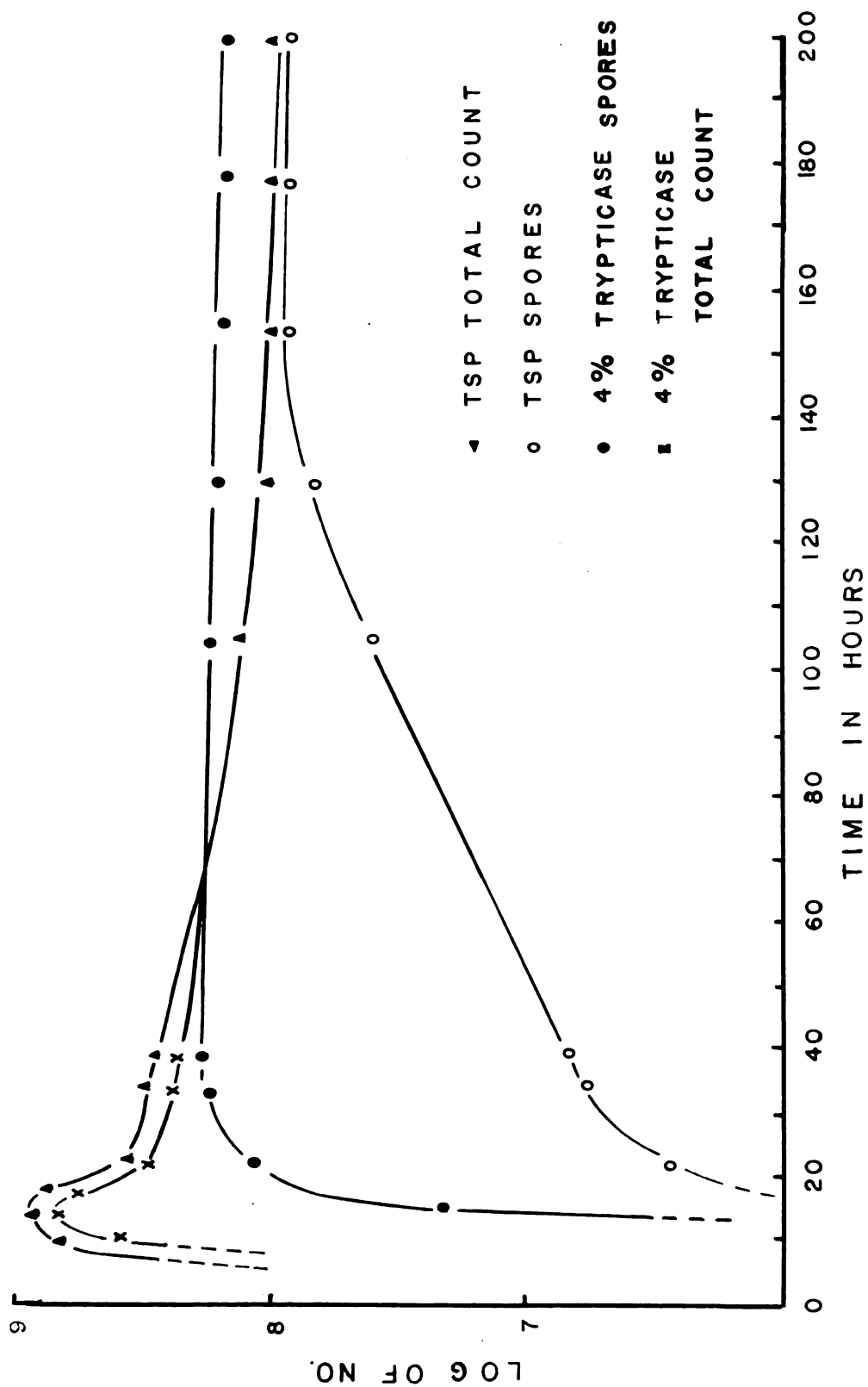


Figure 1. Comparison of growth and sporulation of *Clostridium botulinum* in TSP medium and in 4 percent trypticase broth containing 1 mg per ml of thiamine.

formation of PA 3679 and Cl. botulinum. This investigation also demonstrated that the addition of 1 µg of thiamine per ml of 4 percent trypticase medium stimulated sporulation of Cl. botulinum (Figure 2). Sporulation in the medium containing the thiamine reaches a level of 10^6 per ml about 9 hours earlier than in the medium containing no thiamine, and the final spore population was increased about ten fold by the addition of thiamine. Gas evolution was apparent in the thiamine containing medium, but none was observed in the control.

Two methods of counting spores were used in this work. The first was a direct microscopic count by use of the Petroff-Hausser counting chamber, and in this method only those spores which were highly refractile were enumerated as mature spores. This was compared with colony counts made in heat shocked samples by the oval tube method. A comparison of these two methods in conjunction with the dipicolinic acid content of the spores during various stages of the sporogenic process is presented in Figure 3. From these results it should be noted that the direct microscopic count was consistently higher, usually by about 70 percent. This difference in the number of spores can be attributed to a number of factors; viz., (a) it is highly unlikely that all spores germinate and produce colonies, (b) due to clumping, a single colony may arise from more than one spore, and (c) not all refractile bodies observed microscopically have heat resistance. A measurable concentration of DPA was

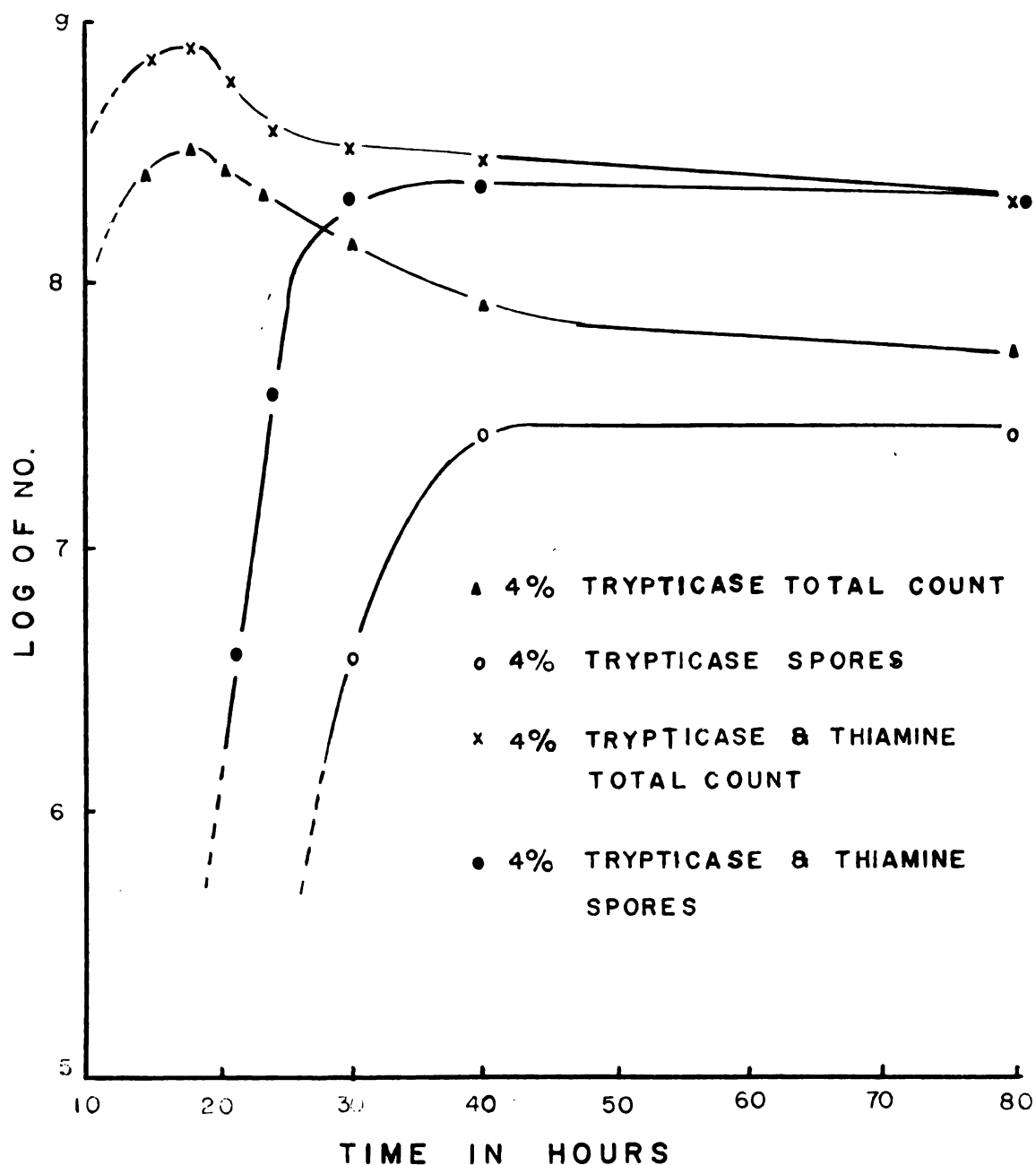


Figure 2. Comparison of growth and sporulation of *Cl. botulinum* in 4 percent trypticase and in 4 percent trypticase containing 1 µg per ml of thiamine.

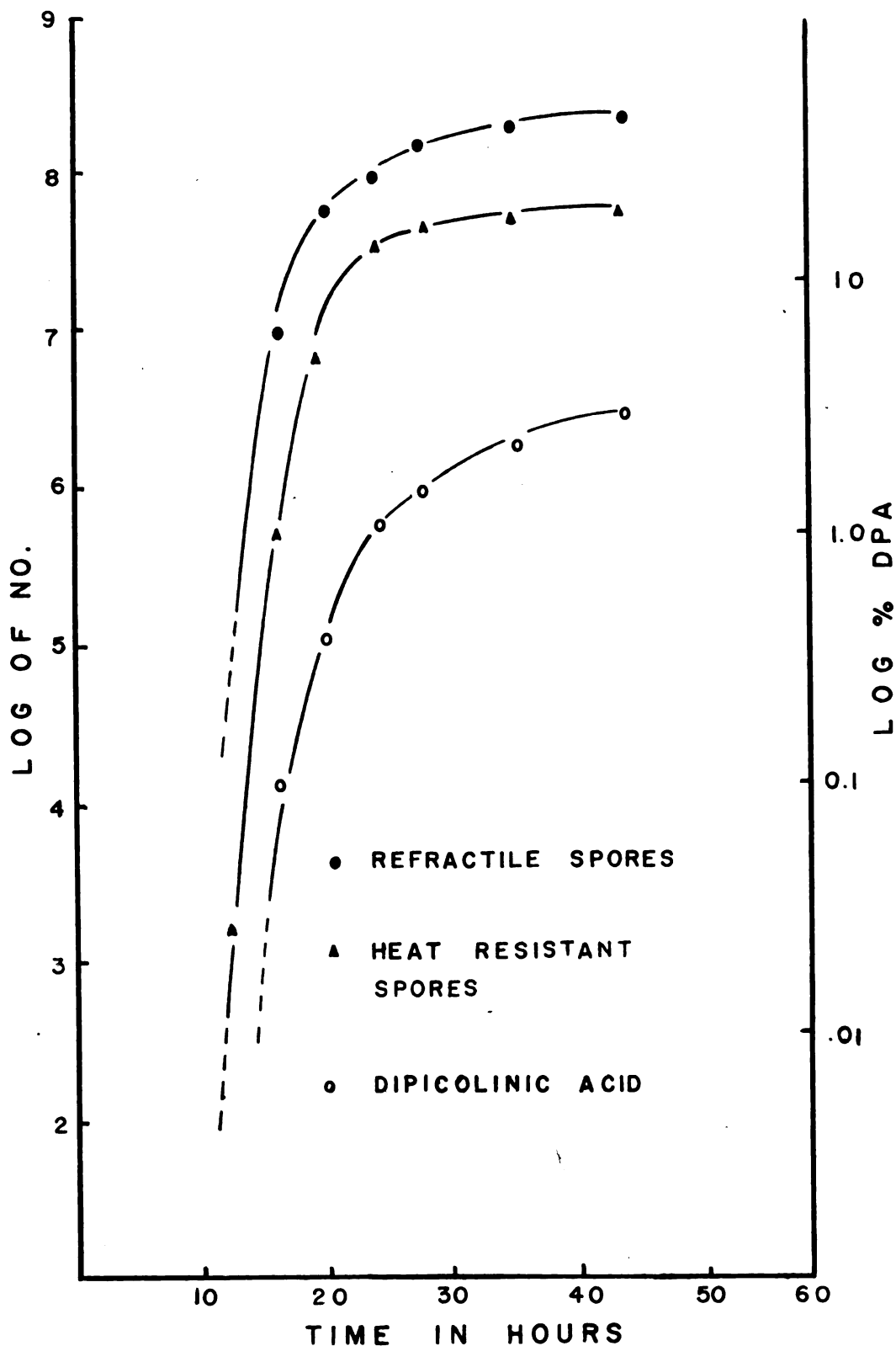


Figure 3. Comparison of total refractile spore counts with heat resistant spore counts and the level of dipicolinic acid present during sporulation of C. botulinum.

observed only after an appreciable number of spores were present. When the heat resistant spores were present in a concentration of 10^3 per ml, the amount of DPA present was not detectable by the method being utilized for the assay. At this point the spores accounted for only 0.03 percent of the total count. As sporulation proceeded, the amount of DPA present also increased. When the spores reached a level of 10^5 per ml, the DPA accounted for 0.10 percent of the total dry weight of the spores and vegetative cells, although it was assumed that the DPA was in the spore. This trend continued until spores were present at a level of 10^7 per ml and the DPA reached 3.41 percent of the dry weight.

Endotrophic sporulation. Initial experiments were designed to determine the effect of exposure of the vegetative cells of Cl. botulinum to the atmosphere during harvest and transfer (replacement) to a fresh medium. Cells were cultivated in a 4 percent trypticase medium and were removed from the growth medium at the 24th hr of growth and replaced in fresh sterile 4 percent trypticase in the presence of atmospheric oxygen. Figure 4 illustrates the results. At the time of replacement, there were 10^7 spores per ml and this level remained constant in the replacement medium while the total number of cells declined. Sporulation proceeded as expected in the control culture and reached almost 100 percent in 70 hours, with 85 percent spores at 45 hours.

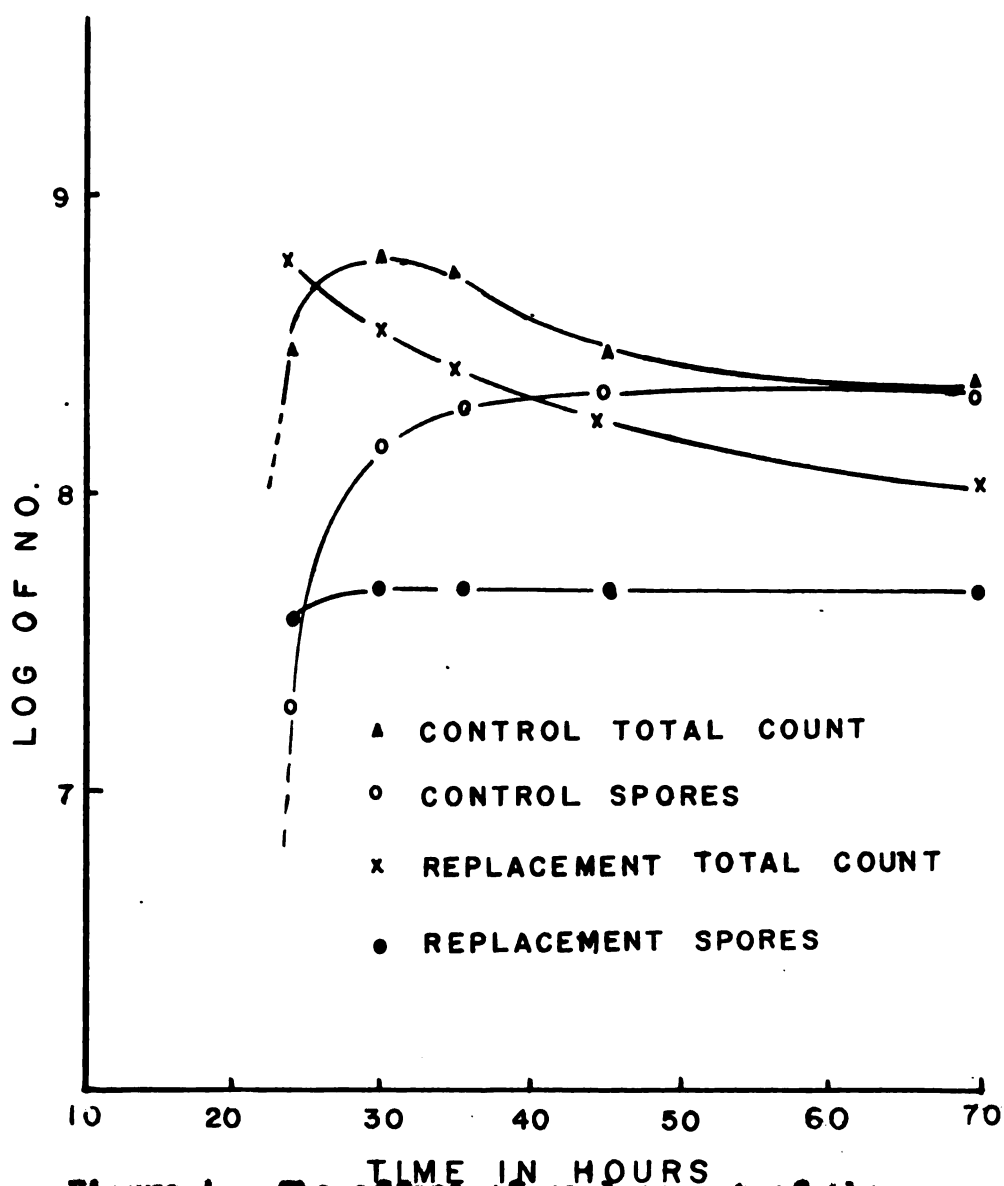


Figure 4. The effect of replacement of the vegetative cells of *Cl. botulinum* in the presence of atmospheric oxygen.

The replacement technique was then carried out using a chamber containing an atmosphere of nitrogen. This time TSP medium was used both as the cultivation medium and the replacement medium. The replacement spores in this instance reached a higher level than the control spores (Figure 5), indicating that no cell damage was incurred by the procedure of centrifugation and replacement.

The vegetative cells of Cl. botulinum were cultivated in TSP medium for use in the first attempts to sporulate this organism in an environment containing no exogenous nutrients. At various stages of growth, the cells were harvested by centrifugation under nitrogen and fractions suspended in (a) sterile distilled water, (b) .067 M phosphate buffer containing 0.1 percent sodium thioglycollate, and (c) .067 M phosphate buffer. All three suspensions were incubated at 37°C; the water suspension being incubated under nitrogen in a Brewer anaerobic jar. The results of these three experiments are given in consolidated form in Table I, and detailed results are found in the appendix, Tables I-A, II-A, and III-A. The spore counts indicate that replacement was carried out about 1 day before the onset of sporulation, again just prior to or shortly after the onset of sporulation, and lastly after sporulation had taken place in 10 percent or more of the total cells present. The total populations in both the control and replacement menstrua were found to decrease at all incubation times after the initial harvest

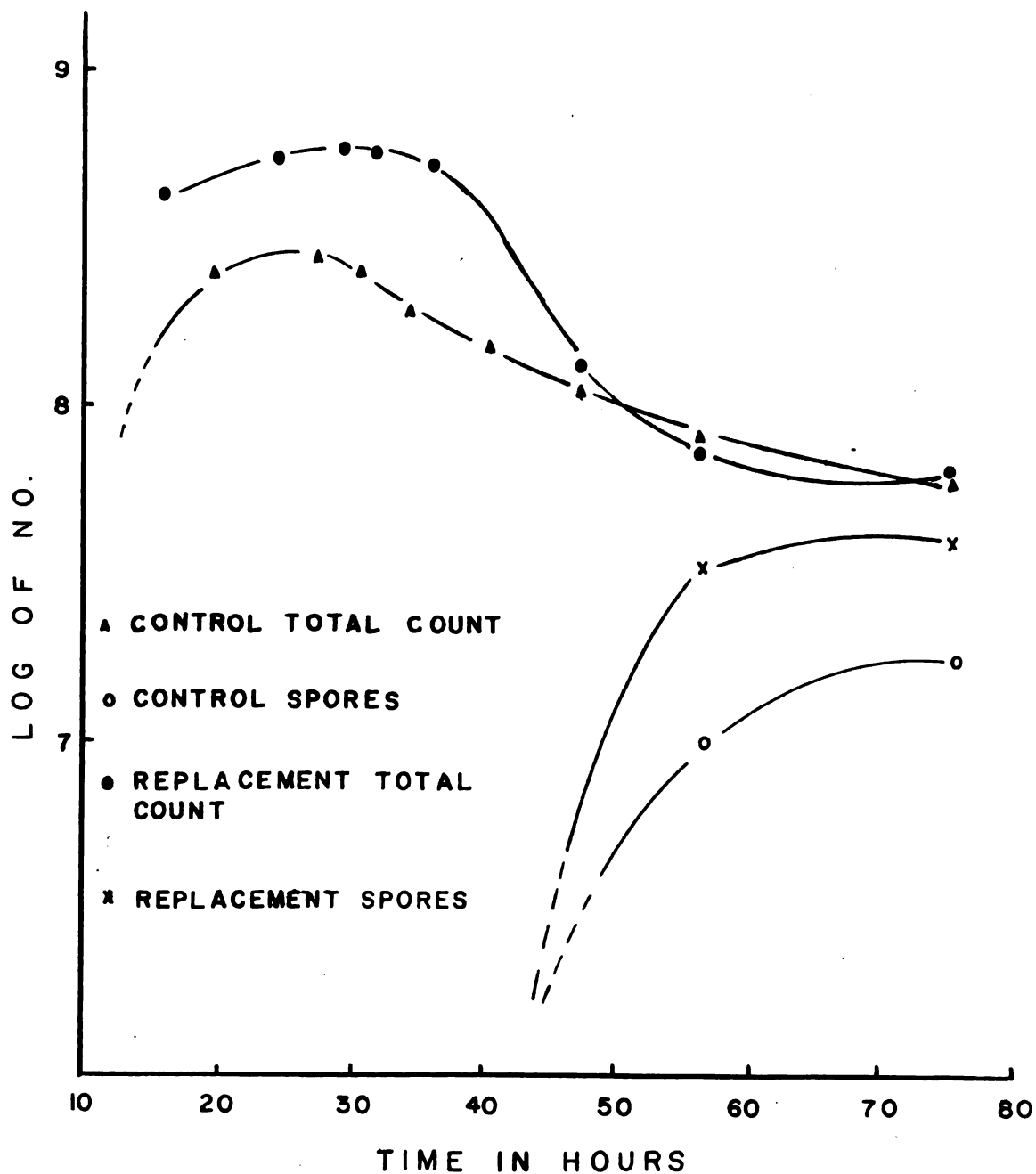


Figure 5. The effect of replacement of the vegetative cells of Cl. botulinum in the presence of nitrogen.

Table I

Summary of results of the endotrophic sporulation of Clostridium botulinum

	Replacement Menstruum					
	Distilled water incubated under N ₂	.067 M phosphate buffer pH 7.0, with 0.1% Na thioglycollate			.067 M phosphate buffer, pH 7.0	
Age of culture at replacement (hrs.)	24	48	72	50	72	120
Incubation time after replacement (hrs.)	120	96	72	70	48	50
Spore count at replacement ($\times 10^{-6}$)	<1	<1	11	<1	<1	1
Percent change in total count in: Control*	-85	-40	-25	-75	-55	-30
Replacement	-41	-69	-36	-99	-99	-95
Menstruum						
Percent change in spore count in: Control*	+90	+90	+81	+90	+90	+93
Replacement	0	0	-9	0	0	0
Menstruum						

*Control = a culture of the organism cultivated in the growth medium over the same time interval but not harvested or replaced.

time. However, while the spore counts of the control cultures increased by 80 percent or more beyond the populations at replacement time, no increase in the number of spores occurred in any of the replacement menstrua irrespective of the harvest time. Similar results were observed in five subsequent experiments using water or phosphate buffer as the replacement menstruum. Therefore, it is concluded that the culture of Cl. botulinum 62-A tested will not undergo endotrophic sporulation.

The observed decrease in total populations, which undoubtedly resulted from cell lysis, was greater in the phosphate buffer suspensions than in the water. This may have resulted from incomplete anaerobiosis in the former, since they were incubated under atmospheric conditions.

Sporulation of Clostridium botulinum in chemically defined replacement media. Since the foregoing results lead to the conclusion that endotrophic sporulation was not possible with this organism, a search was initiated for a chemically defined or simplified medium which would support final sporulation. The cells were again cultivated in TSP medium and transferred at various stages of development into media of known composition. The three replacement menstrua tested had the following composition:

No. 1	biotin	0.01 percent	
	methionine	0.03 percent	pH 7.1
	MnSO ₄	0.01 percent	

No. II	biotin	0.0001 percent	pH 7.1
	methionine	0.0600 percent	
	nicotinic acid	0.0001 percent	
	folic acid	0.0001 percent	
	pantothenic acid	0.0001 percent	
	K_2SO_4	0.1000 percent	
	p-aminobenzoic acid	0.0001 percent	
No. III	DL - α - alanine	0.2000 percent	pH 7.1
	L - glutamine	0.2000 percent	
	L - ornithine	0.2000 percent	
	glucose	0.2000 percent	
	glycine	0.0500 percent	
	biotin	0.0005 percent	
	p-aminobenzoic acid	0.0002 percent	
	thiamine	0.0002 percent	
	nicotinic acid	0.0002 percent	
	KH_2PO_4 - K_2HPO_4	0.03M	

The results of sporulation studies in these replacement menstrua are given in Table II, and the detailed results are found in the appendix in Tables IV-A, V-A, and VI-A. None of these three replacement solutions containing vitamins and amino acids would support further sporulation of the organism. A control culture exhibited normal sporulation during the same intervals of time. When spores were present at the time of transfer to menstrua II and III, a decrease was noted in the number of spores during the incubation period. This decrease was attributed to germination of the spores. The decrease in the total counts in both the controls and the replacement media undoubtedly resulted from lysis of the vegetative cells.

Since sporulation proceeded quite slowly in the TSP medium, 4 percent trypticase broth containing 1 μg per ml of thiamine was utilized in subsequent experiments as the

Table II

Summary of the results of the sporulation of Clostridium botulinum
in chemically defined replacement media

	Replacement Menstruum									
	No. I					No. II				
Age of culture at replacement (hrs.)	48	67.5	94	24	72	120	21	45	69	No. III
Incubation time after replacement (hrs.)	264	244.5	218	120	120	120	72	76	120	
Spore count at replacement ($\times 10^{-6}$)	<1	<1	3.6	<1	2.5	16	<1	8.4	39	
Percent change in total count in:										
Control*	-47	-68	-42	-82	-70	-26	-53	-58	-19	
Replacement Menstruum	-50	-57	-57	-74	-16	-7	-35	-18	-17	
Percent change in spore count in:										
Control*	+90	+90	+90	+90	+90	+50	+90	+84	+42	
Replacement Menstruum	0	0	-8	0	-36	-50	0	-32	-30	

*Control = a culture of the organism cultivated in the growth medium over the same time interval but not harvested or replaced.

initial culture medium. This medium gave highly reproducible results as to the time of the swelling of the cells and the onset of sporulation. Therefore, all succeeding replacements were carried out between the 18th and the 20th hour of incubation. At this time almost 100 percent of the vegetative cells were in the swollen state, and the number of spores in a culture accounted for about 1-10 percent of the total count. It was believed that a large percentage of the cells should be committed to sporulation at this stage of development.

A culture of Cl. botulinum cultivated for 19.5 hours in a medium of 4 percent trypticase with 1 μ g per ml of thiamine was replaced in: (a) sterile distilled water, (b) a solution of calcium acetate at a concentration of 200 ppm, and (c) a solution of thiamine at a concentration of 1 ppm. The first three columns of Table III show the results of this investigation. The total counts decreased in both the control and in the replacement media, although the per cent decrease was much less in the replacement solution of calcium acetate and the solution of thiamine than in the control. No increase in the number of spores was observed in any of the replacement menstrua, while during the same time interval the spores in the trypticase control increased over 99 percent. Detailed results of these experiments are given in the appendix, Table VII-A.

In an attempt to characterize the type of compound(s)

Table III

Summary table of the results of the sporulation of Clostridium botulinum in various chemically defined replacement menstrea.
(Cells cultivated in 4 percent trypticase)

	Replacement Menstruum						
	Dist. H ₂ O	Ca ⁺⁺ 200 ppm	thiamine 1 ppm	dialyzed 4% trypticase	4% trypticase ash	4% casamino acids acid hydrolyzed	enzymatic hydrolyzed
Age of culture at replacement (hrs.)	19.5	19.5	19.5	18	18	20	20
Incubation time after replacement (hrs.)	22.5	22.5	22.5	29	29	72	72
Spore count at replacement ($\times 10^{-6}$)	<1	<1	<1	30	40	14	14
Percent change in total count in:							
Control*	-60	-60	-60	-41	-41	-88	-88
Replacement Menstruum	-57	-19	-27	-6	-37	-86	-99
Percent change in spore count in:							
Control*	+99	+99	+99	+98	+98	+83	+83
Replacement Menstruum	0	0	0	-66	-15	-97	-99

*Control - a culture of the organism cultivated in the growth medium over the same time interval but not harvested or replaced.

vital to the final sporulation process, a dialyzed 4 percent trypticase solution and a solution containing the ash of 4 grams of trypticase per 100 ml were used as replacement media. The vegetative cells were cultured for 13 hours in a 4 percent trypticase broth with added thiamine. The results (columns 3 and 4 of Table III) show that the spores in the control increased 93 percent while in the replacement menstrua a net decrease was observed. Table VIII-A, appendix, gives the detailed results of this experiment. It must be noted that the pH of the solution of the ash of trypticase was 5.0 in order to solublize the ash. Evidence cited previously (Mohrke, 1926; Collier, 1956) indicated that the optimum pH for sporulation of this organism is about 7.0 - 7.2.

Products similar to trypticase were also used as replacement media. A 4 percent solution of a vitamin free enzymatic hydrolysate of casein (General Biochemicals, Inc.) and a 4 percent solution of Bacto-casamino acids (Difco) were both utilized in this manner. The replacement was performed after 20 hours of growth in 4 percent trypticase fortified with thiamine. At this time sporulation had taken place in about 10 percent of the cells. No further sporulation was observed beyond this level in the replacement menstrua; instead a large decrease was observed (Table III, columns 6 and 7, and appendix Tables IX-A). It was noted that after replacement in the enzymatic hydrolysate of

casein there was clumping of the cells. After the 10th hour it was impossible to enumerate the number of cells present by use of the direct microscopic count, and no spores could be observed at all. In the acid hydrolyzed casein replacement medium, there was a loss of 97 percent of the spores present at the time of transfer. No doubt, the decrease in spore counts resulted from germination.

An assay data table (Appendix, Table II) obtained from the Baltimore Biological Laboratory containing the approximate composition of B-B-L peptones was used as a reference for the preparation of a solution of amino acids of the same composition and concentration as found in a solution of 4 percent trypticase. Only the amino acids were used, along with 1 μ g per ml of thiamine. This solution was then used as the replacement solution for cells cultivated for 24 hours in 4 percent trypticase. At the time of replacement there was about 1.2 percent sporulation in the culture. Sporulation in the replacement medium increased to a level of about 83 percent over the first 16 hours after replacement (Figure 6). The spores in the control medium reached almost 99 percent of the total count in the same time interval. Although sporulation in the replacement medium did not reach the level of that in the control, it was the first time that a significant increase was noted in a replacement solution of known composition.

The effect of antibiotics on the sporulation of

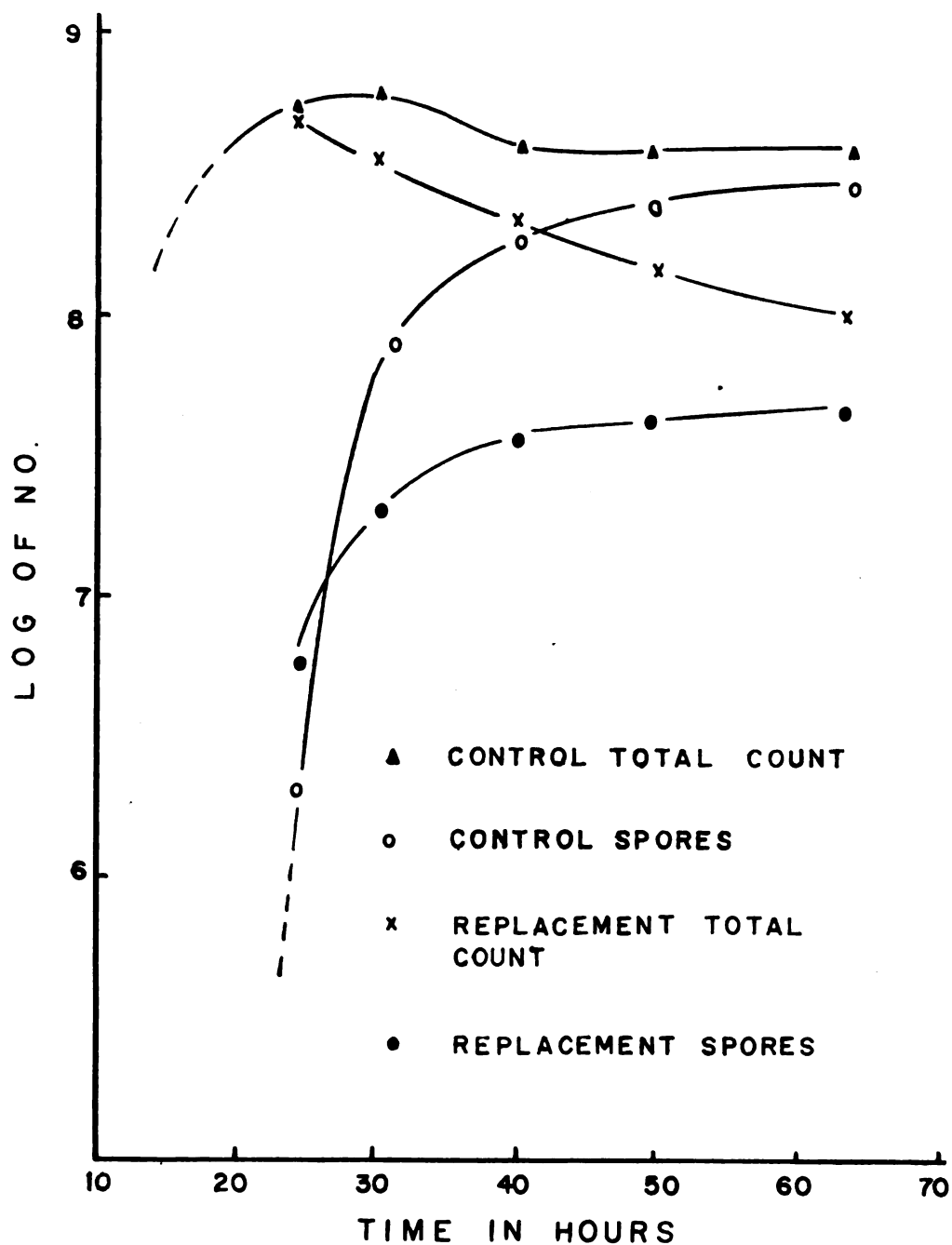


Figure 6. The replacement of the vegetative cells of *Cl. botulinum* in a synthetic medium containing thiamine and the amine acids of trypticase.

Clostridium botulinum. Three antibiotics were utilized in this phase of the investigation; viz., penicillin, chloramphenicol, and vancomycin. The first of these, penicillin, was added at various stages of growth of the culture to give a final concentration of 100 units per ml of culture medium. If penicillin was added to the culture during the log growth phase or at the 16th hour, further sporulation appeared to be inhibited, and the spores already present decreased. This population decrease might have been due to the germination of spores (Figure 7). If added at the 20th hour, when there were about 10 percent spores in culture, sporulation continued over the next four hours and then ceased. When the antibiotic was added to the culture at the 24th hour, when sporulation was about 30 percent complete, the antibiotic appeared to have no effect and sporogenesis continued. Microscopic counts of refractile spores made in conjunction with the counts of heat resistant spores showed essentially the same results.

Chloramphenicol was first used in a concentration of 10 μ g per ml, but at this level no effect was observed on sporulation. However, when the concentration was increased to 100 μ g per ml, sporulation was arrested immediately on addition. This proved to be true whether the drug was added early (10th and 20th hour) or later (30th hour) in the sporulation process of the culture (Figure 8).

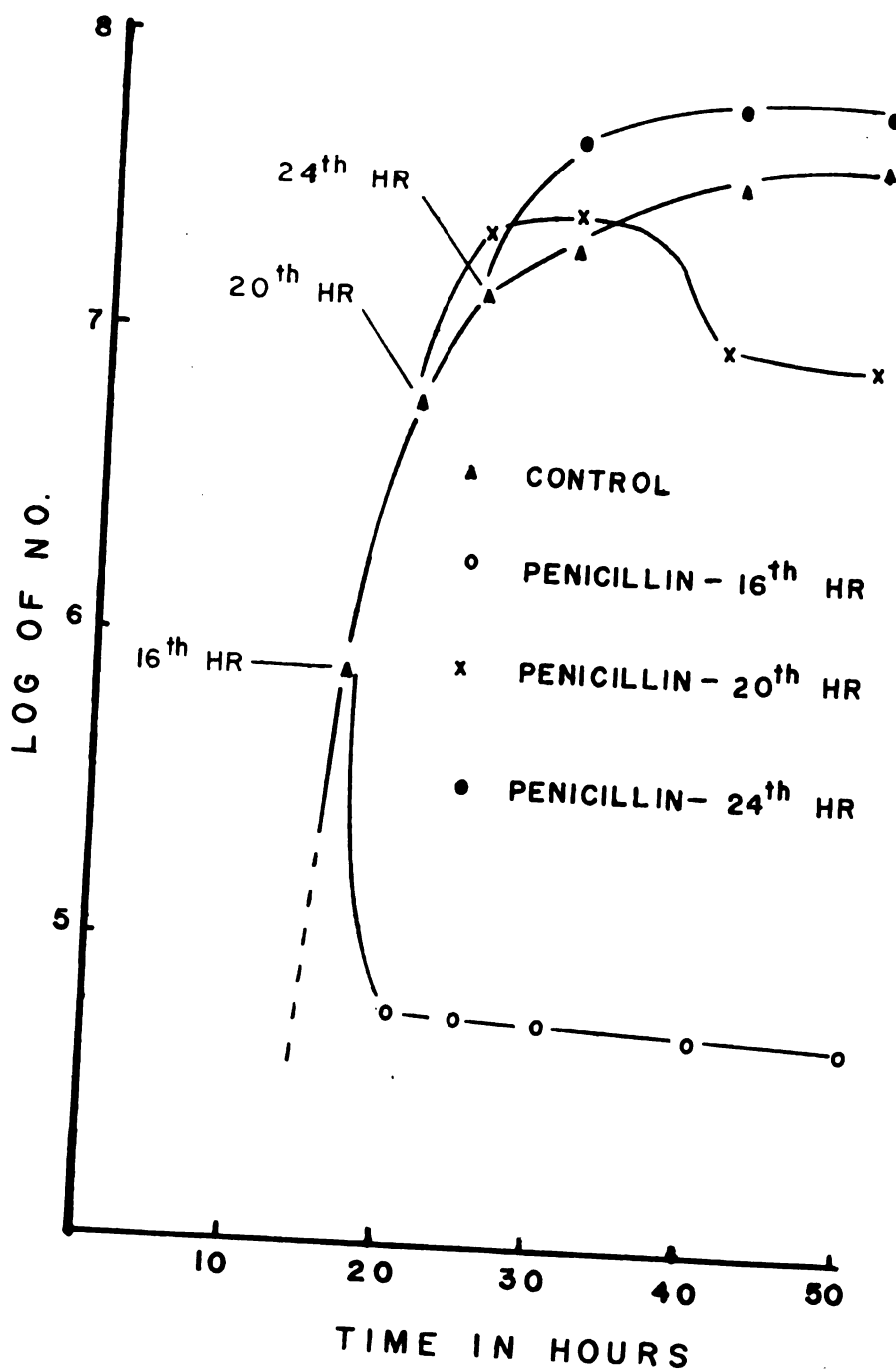


Figure 7. The effect of penicillin on the sporulation of Cl. botulinum.

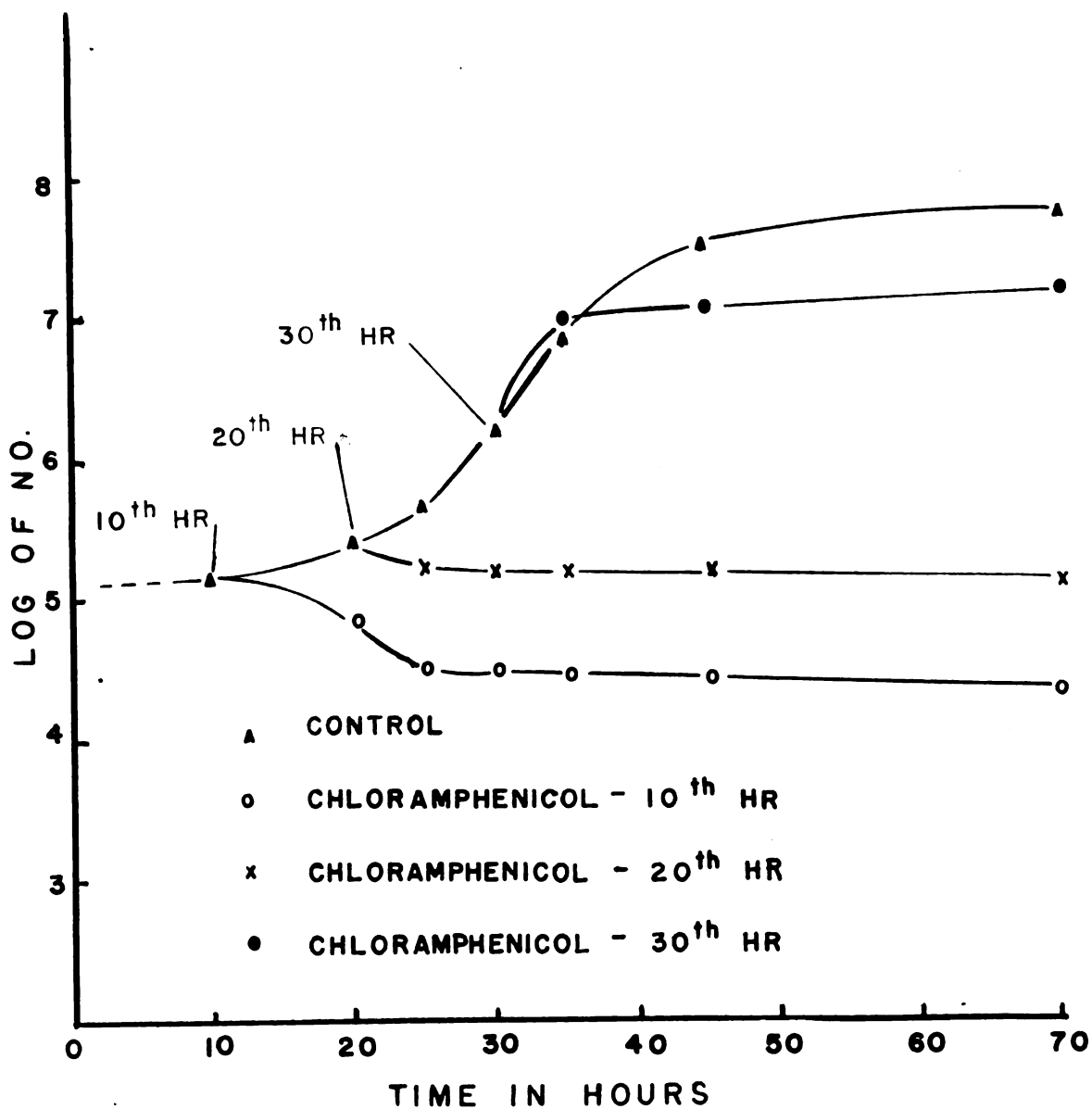


Figure 8. The effect of chloramphenicol on the sporulation of Cl. botulinum.

Vancomycin, at a concentration of 10 μg per ml, was observed to arrest sporulation. The time intervals of growth before the addition of the antibiotic were again 10, 20 and 30 hours. With the antibiotic there was an interval of about 10 hours after the addition of the antibiotic before any effect could be observed. After this time interval no further sporulation was observed (Figure 9).

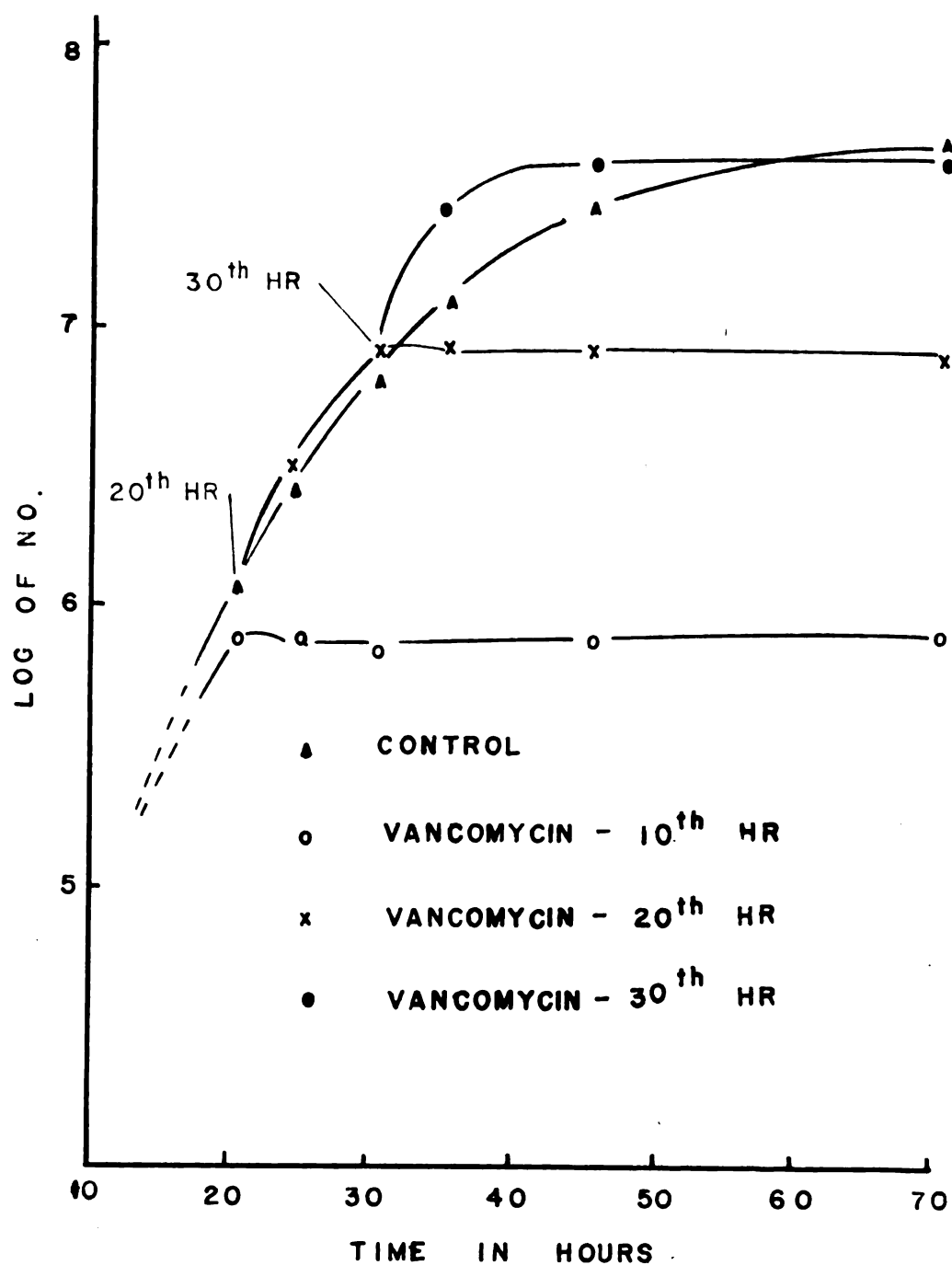


Figure 9. The effect of vancomycin on the sporulation of Cl. botulinum.

DISCUSSION

There are several points of importance in the dissimilarity of the sporogenic properties of TSP medium and of 4 percent trypticase broth. First, it should be noted that trypticase, as prepared by the Baltimore Biological Laboratory, is a highly variable product. Its exact composition is unknown, and its composition and characteristics vary from batch to batch; this depending on the casein being utilized in the preparation of the trypticase (Vera, 1960). This variability, however, appears to be overcome by the addition of thiamine to the medium in a concentration of 1 µg per ml (Lund, 1956). The concentration of trypticase in TSP is 1.5 percent, while in the trypticase medium the concentration is 4 percent. Herein may lie the difference in the sporulation supporting properties of the two media. Apparently, casein contains some factor which is necessary for the sporulation of this organism, and it would appear that concentrations of trypticase under 4 percent may not contain enough of this factor to support sporulation. Since the trypticase is a variable product as to composition, the batch of trypticase used by Zohar and Sadoff (1958) may have been particularly high in the sporogenic factor(s), and the subsequent lots used in this work were not of the same characteristics. While the original work with TSP was with PA 3679 and the present study was with Cl. botulinum, many other trials conducted in this

laboratory with PA 3679 (Costilow, 1960) resulted in failure to obtain a high percentage of sporulation. This was true even when the medium was supplemented with thiamine.

Another aspect of TSP medium to be considered is the presence of glucose in this medium at a concentration of 0.3 percent. It has been noted by several investigators (Blair, 1950) that this carbohydrate is inhibitory to sporulation of Cl. botulinum at a concentration of 0.4 percent. A 0.3 percent level may very well, then, delay and partially inhibit complete sporulation of this organism. The work of Hardwick and Foster (1952) concerning the nature of the inhibition of sporulation by the presence of glucose would also indicate this. A medium of 4 percent trypticase would not contain glucose since trypticase, as assayed by the Baltimore Biological Laboratory, is free of fermentable carbohydrates.

The acetate content of trypticase is quite high, since acetic acid is used for purposes of pH adjustment in order to avoid a high chloride content of the product (Vera, 1960). It is known from concurrent work in this laboratory (Simmons, 1960) that Cl. botulinum 62-A contains a very active acetokinase; thus the acetate content of trypticase may somehow be involved in sporogenesis as a source of energy. Future investigations will study this aspect of the sporogenic nature of trypticase.

The role of thiamine in sporulation is not well established. However, the results would indicate that it is not essential for growth and sporulation of this organism, but that it does enhance both. Again the composition of trypticase comes into play. Thiamine may be present in trypticase in minute, catalytic quantities but not in great enough concentrations to be conducive to complete sporulation. Therefore, the complete absence of this factor may result in the complete absence of sporulation. This can only be established by use of a synthetic sporulation medium.

Some things may be deduced from the results of this work about the compounds of trypticase which do lead to sporulation of Cl. botulinum. In all probability, these factors are dialyzable. Trypticase is composed of amino acids and small peptides and it would not seem unreasonable that most of the components of trypticase are dialyzable. Also, the ash of trypticase alone is not sporogenic in a replacement menstruum although it must be remembered that the pH of this solution was 5.0. Of course, the possibility exists that either the factors in ash or in the dialyzed portion of trypticase in combination with other factors or with each other might be sporogenic in the replacement medium. This possibility was not examined experimentally.

All of the various factors tested, amino acids, minerals, various inorganic ions, digests of casein other

than trypticase, and vitamins proved to be non-sporogenic when used singly or in combination in replacement solutions. On this basis, the sporogenic nature of these compounds and factors cannot be precluded. It can only be stated that they have no sporogenic value by themselves, or in combinations used in this work, as replacement menstura.

While it is obvious that this organism cannot undergo the phenomenon of endotrophic sporulation, the organism may very well become committed to sporulation, but not to the extent that it can complete sporogenesis in the absence of exogenous nutrients. While it is possible that existing proteins and nucleic acids are utilized in spore synthesis by this organism, some exogenous nutrient or factor is also required.

Perhaps the most important clue as to the requirements for sporulation of this organism is given by the work which demonstrated that a combination of the amino acids of trypticase and thiamine is at least partially sporogenic in nature. Although sporulation did not approach the level of sporulation obtained in a normal sporogenic environment, a definite increase in sporulation could be observed. In this solution of amino acids, the ionic strength would be considerably higher than in the trypticase medium, since all amino acids were added as their salts, and this condition may have a limiting effect on the

sporogenic process. Leifson (1931) and Wynne (1943) pointed out that sporulation decreased as the salt concentration increased in a culture medium. It may be that the synthetic amino acid solution is not complete enough in itself to warrant complete sporulation. If the latter is the case, the next step in the work is to add to this amino acid menstruum the various metallic ions, inorganic ions, vitamins, and other factors of known importance to the sporulation of this and similar organisms. The high level of acetate in trypticase, and the acetokinase activity of this organism indicates that a combination of the amino acids and acetate should be investigated for sporogenic properties.

The effect of penicillin on sporulation is difficult to explain in light of the hypothesis that this antibiotic prevents the synthesis of cell wall material (Pratt, 1953; Lederberg, 1957; Park and Strominger, 1957). It would be expected that if the cell were committed to sporulation, that this drug would have no effect on the process, and sporulation would proceed to completion. The possibility does exist, however, that the synthesis of the spore coat may proceed by a pathway similar to that of the cell wall, and then the action of penicillin might be explained. If this antibiotic acts by the inhibition of RNA synthesis, as reported in earlier investigations (Mitchell and Hoyle, 1951; Gale, 1949), it would affect sporulation in the same

manner as vancomycin, which is known to inhibit RNA synthesis (Inniss, 1959). However, the results do not clearly show that penicillin does act on a sporulating culture in the same manner as vancomycin. Before an explanation can be made as to how penicillin interferes with the sporulation process, the mode of action of this antibiotic must be known with certainty.

When vancomycin is added to a culture of Cl. botulinum, the inhibition of sporulation did not occur immediately. A lag of about 10 hours was observed before the sporulation ceased. If RNA is essential to protein synthesis, as is hypothesized in the template theory, then the inhibition of RNA synthesis results in the inhibition of protein synthesis, and this antibiotic ultimately acts in the same manner as chloramphenicol. The lag which is observed with the RNA inhibitor would be explained by the fact that some cells, at the time when the antibiotic is added, already have the RNA which is necessary to serve as templates for the synthesis of proteins which are essential for sporulation. These cells would then continue to synthesize spore proteins, and sporogenesis would go to completion. Presumably this would take place over the lag period, accounting for the increase in the number of spores. Those cells which had not yet formed the RNA templates required for spore protein at the time of the addition of the vancomycin would be prevented from synthesizing

these particular RNA molecules, and the sporulation would be stopped at that point.

It is felt by the author that the use of antibiotics and other antimicrobial inhibitors may offer a practical tool in the elucidation of the sporogenic process. If the mode of action of the inhibitor is known with certainty, it would be possible to determine which reactions are being inhibited and thus which pathway(s) are essential to sporogenesis. Some work has been carried out by the author with the trivalent arsenic ion and its effect on sporulation. At this time the results are not clear, but it is felt that this inhibitor of cellular metabolism may present clues as to the mechanisms of sporulation. Other compounds of this nature should be investigated as to their effect on sporulation.

A great deal of work remains to be accomplished in the area of sporulation of the anaerobic bacteria. The development of a good basal medium which can be rendered free of metallic contamination is required for the determination of the mineral requirements of sporulation. Perhaps the most valuable technique already developed for the study of sporogenesis is the technique of replacement because it offers a method of practically separating the sporulation process from growth. Future work will utilize this technique in order to further elucidate the nutritional requirements for sporulation of Cl. botulinum.

SUMMARY

Studies on the nature of sporulation of Clostridium botulinum, ATCC 62A, revealed this organism will not undergo endotrophic sporulation. Minerals, amino acids, inorganic ions, and vitamins used by themselves and in various combinations would not promote sporulation in replacement menstrua with one exception. A synthetic medium of the amino acids reported to be in trypticase, fortified with thiamine, would support sporulation to a significant extent. The nature of the sporogenic quality of this solution was not investigated further. It was also noted that trypticase with added thiamine is an excellent sporogenic medium for this organism, however trypticase which has been dialyzed does not exhibit the sporogenic property. It was concluded that spores of Cl. botulinum cannot be formed solely from the degradation of cellular material and the recombination of the products of this degradation into compounds necessary for sporulation.

Three antibiotics, penicillin, chloramphenicol, and vancomycin, were studied to ascertain their effect on the sporulation process of this organism. Penicillin inhibited sporulation if added before the onset of sporulation or during the early stages of sporulation. Added at a late stage of spore development, this antibiotic had no apparent effect on sporulation. Vancomycin affected sporulation in much the same manner, however, a lag of about

10 hours was observed after the addition of this antibiotic before sporulation was arrested. Chloramphenicol inhibited further sporulation regardless of the stage of growth when the drug was added to the culture. From these results and the hypothesized mode of action of these antibiotics, it was concluded that the synthesis of protein material is an essential mechanism in the final sporogenic process of this organism.

BIBLIOGRAPHY

- Amaha, M.Z., Ordal, J. and Touba, A. 1956 Sporulation requirements of Bacillus coagulans var thermoacidurans in complex media. J. Bacteriol., 72, 34-41.
- Black, S.H., Hashimoto, T. and Gerhardt, P. 1960a Development of fine structure, thermostability, and dipicolinate during sporogenesis in a bacillus. Can. J. Microbiol., 6 (1), 203-212.
- Black, S.H., Hashimoto, T. and Gerhardt, P. 1960b Calcium reversal of the heat susceptibility and dipicolinic deficiency of spores formed "endotrophically" in water. Can. J. Microbiol., 6 (2), 213-224.
- Blair, E.E. 1950 Observations on bacterial spore formation in synthetic media. M.A. Thesis, University of Texas. Abst. in Texas Repts. Biol. Med., 8, 361.
- Brewer, C.R., McCullough, W.G., Mills, R.C., Roessler, W.G., Herbst, E.J. and Howe, A.F. 1946 Studies on the nutritional requirements of Bacillus anthracis. Archiv. of Biochem., 10, 65-75.
- Buchner, H. 1890 Über die Ursache der Sporenbildung beim Milzbrandbacillus. Zentr. Bakteriolog. Parasitenk., Orig. 8, 1-6. (c.f. Knaysi, 1948).
- Byrne, A.F., Burton, T.H., and Koch, R.B. 1960 Relation of dipicolinic acid content of anaerobic bacterial endospores to their heat resistance. J. Bacteriol., 80, 139-140.
- Charney, J., Fisher, W.P., and Hegarty, C.P. 1951 Manganese as an essential element for sporulation in the genus Bacillus. J. Bacteriol., 62, 145-148.
- Church, B.D., and Halvorsen, H.O. 1959 Dependence of the heat resistance of bacterial endospores on their dipicolinic acid content. Nature, 183, 124.
- Costilow, R.N. 1960 Personal communication.
- Collier, R.C. 1956 An approach to synchronous growth for spore production in Clostridium roseum. In "Spores". Edited by H. O. Halvorsen. American Institute of Biological Sciences, Washington, D.C., 1957. pp 10-14.

The first part of the document discusses the importance of maintaining accurate records of all transactions. It emphasizes that every entry, no matter how small, should be recorded to ensure the integrity of the financial data. This includes not only sales and purchases but also expenses and income.

The second part of the document provides a detailed breakdown of the company's financial performance over the past year. It includes a comparison of actual results against budgeted figures, highlighting areas of both success and improvement. The analysis shows that while sales were slightly below target, operating expenses were well-controlled, leading to a positive contribution margin.

The third part of the document outlines the company's strategic goals for the upcoming year. It focuses on increasing market share through targeted marketing campaigns and improving operational efficiency by streamlining processes. The management team is committed to achieving these goals and ensuring the company's long-term growth and profitability.

The fourth part of the document discusses the company's commitment to social responsibility and environmental sustainability. It details the various initiatives implemented to reduce carbon footprint, support local communities, and ensure ethical sourcing of materials. These efforts are seen as integral to the company's overall success and reputation.

The fifth part of the document provides a summary of the key findings and recommendations. It reiterates the importance of accurate record-keeping, the need for continuous improvement in financial and operational performance, and the commitment to sustainable growth. The management team is confident that these measures will lead to a successful and profitable future for the company.

- Collier, R.E. and Krishnamurty, G.G. 1957 The correlation of DPA synthesis with sporulation of Clostridium roseum. Bacteriol. Proc., 1957, 32.
- Curran, H.R. and Evans, F.R. 1954 The influence of iron or manganese upon the formation of spores by mesophilic aerobes in fluid organic media. J. Bacteriol., 67, 489-497.
- Esty, J. and Meyer, K.F. 1922 The heat resistance of B. botulinum and allied anaerobes. J. Infect. Dis., 31, 650-663.
- Foster, J.W. and Heiligman, F. 1949 Biochemical factors influencing sporulation in a strain of Bacillus cereus. J. Bacteriol., 57, 639-646.
- Foster, J.W. and Perry, J.J. 1954 The non-involvement of lysis during sporulation of Bacillus mycoides in distilled water. J. Bacteriol., 57, 401-409.
- Gale, E.F. 1948 The nitrogen metabolism of gram-positive bacteria. Bull. Johns Hopkins Hosp., 83, 119-175.
- Gale, E.F. 1949 The action of penicillin on the assimilation and utilisation of amino acids by gram positive bacteria. Symposia of the society for Experimental Biology. III. Selective Toxicity and Antibiotics. Academic Press Inc., Publishers, New York, New York.
- Gale, E.F. and Folkes, J.P. 1953 The assimilation of amino acids by bacteria. 14. Nucleic acid and protein synthesis in Staphylococcus aureus. Biochem. J., 53, 483-492.
- Gale, E.F. and Folkes, J.P. 1953 The assimilation of amino acids by bacteria. 20. The incorporation of labelled amino acids by disrupted staphylococcal cells. Biochem. J., 59, 661-675.
- Gale, E.F. and Paine, T.F. 1950 The effect of inhibitors and antibiotics on glutamic acid accumulation and on protein synthesis in Staphylococcus aureus. Biochem. J., 47, xxvi.
- Halvorson, H.O. 1957 Rapid and simultaneous sporulation. J. Appl. Bacteriol., 20, 305-314.
- Hardwick, W.A. and Foster, J.W. 1952 On the nature of sporogenesis in some aerobic bacteria. J. Gen. Phys., 35, 907-927.

- Hardwick, W.A. and Foster, J.W. 1953 Enzymatic changes during sporogenesis in some aerobic bacteria. J. Bacteriol., 65, 355-360.
- Inniss, W.E. 1959 Studies on possible modes of action of a new antibiotic (vancomycin) on strains of Staphylococcus aureus. M.S. Thesis, University of Toronto.
- Janssen, F.W., Lund, A.J. and Anderson, L.E. 1953 Colorimetric assay for dipicolinic acid in bacterial spores. Science, 127, 26-27.
- Kaplan, I. and Williams, J.W. 1941 Spore formation among the anaerobic bacteria. I. The formation of spores by Clostridium sporogenes in nutrient agar. J. Bacteriol., 42, 265-282.
- 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.
- Krampitz, L.O. and Werkman, C.L. 1947 On the mode of action of penicillin. Arch. Biochem., 12, 57.
- Lederberg, J. 1957 Mechanism of action of penicillin. J. Bacteriol., 71, 144.
- Lefson, E. 1931 Bacterial spores. J. Bacteriol., 21, 331-356.
- Lund, A.J. 1956 Discussion in "Spores". Edited by H.O. Halvorson. American Institute of Biological Sciences, Washington, D.C. 1957. pp 26-30.
- Mitchell, P. 1949 Nature, 164, 259. (cited by: Antibiotics: a survey of their properties and uses, London: Pharmaceutical Press, 17 Bloomsbury Square, W.C. 1.)
- Mitchell, P. and Moyle, J. 1951 Relationships between cell growth, surface properties, and nucleic acid production in normal and penicillin-treated Micrococcus pyogenes. J. Gen. Microbiol., 1, 421-423.
- Mohrke, W. 1926 Ein neues Verfahren zur Einsporenkultur anaerober Bakterien, nebst Bemerkungen über das

Versporungsoptimum der anaerobier. Zent. Bakt. Parasitenk., Abt. I, Orig. 98, 533-547. (c.f. Knaysi, 1948).

- Ordal, Z.J. 1956 The effect of nutritional and environmental conditions of sporulation. In "Spores". Edited by H. O. Halvorson. American Institute of Biological Sciences, Washington, D.C. 1957. pp 18-25.
- Park, J.T. and Strominger, J.L. 1957 Mode of action of penicillin. Biochemical basis for the mechanism of action of penicillin and for its selective toxicity. Science, 125, 99-101.
- Perry, J.J. and Foster, J.W. 1955 Studies on the biosynthesis of dipicolinic acid in spores of Bacillus cereus var mycoides. J. Bacteriol., 69, 337-346.
- Powell, J.F. 1953 Isolation of dipicolinic acid (pyridine-2, 6-dicarboxylic acid) from spores of Bacillus megatherium. Biochem. J., 54, 210-211.
- Powell, J.F. 1956 Informal discussion in "Spores". Edited by H. O. Halvorson. American Institute of Biological Sciences, Washington, D.C. 1957 pp 9.
- Powell, J.F. and Hunter, J.R. 1953 Sporulation in distilled water. J. Gen. Phys., 36, 601-606.
- Pratt, R. 1953 Symposium on the mode of action of antibiotics. IV. Mechanisms of penicillin action in vitro. Bacteriol. Rev., 17, 41-45.
- Rossler, W.G. and Brewer, C.R. 1946 Nutritional studies with Clostridium botulinum, toxin types A and B. J. Bacteriol., 51, 571-572.
- Romig, W.R. and Wyss, O. 1957 Some effects of ultra-violet radiation on sporulating cultures of Bacillus cereus. J. Bacteriol., 74, 386-391.
- Schreiber, O. 1896 Über die physiologischen Bedingungen der endogenen sporen bildung die Bacillus anthracis, subtilis, and tumescens. Zentr. Bakt. Parasitenk. Abt. I., 20, 353-374. (c.f. Knaysi, 1948).
- Simmons, R.J. 1960 Personal communication.
- Smith, G.N. 1953 Symposium on the mode of action of antibiotics. I. The possible modes of action of chloromycetin. Bacteriol. Rev., 17, 19-29.

- Sommer, E.W. 1930 Heat resistance of the spores of Clostridium botulinum. J. Infect. Dis., 46, 85-114.
- Vera, H.D. 1960 Personal communication.
- Weinberg, E.D. 1955 The effect of Mn^{++} and antimicrobial drugs on sporulation of Bacillus subtilis in nutrient broth. J. Bacteriol., 70, 289-296.
- Weinberg, E.D. 1956 Discussion in "Spores". Edited by H. O. Halvorson. American Institute of Biological Sciences, Washington, D.C. 1957. pp 6-8.
- Williams, C.B. and Harper, O.F., Jr. 1951 Studies on heat resistance. IV. Sporulation of Bacillus cereus in synthetic media and the heat resistance of the spores produced. J. Bacteriol., 61, 551-556.
- Wissiman, C.L., Jr., Ley, H.L., Jr., and Hahn, F.E. 1952 The action of chloramphenicol on microorganisms. Bacteriol. Proc., 1952, 92.
- Wynne, E.S. 1948 Physiological studies on spore formation in Clostridium botulinum. J. Infect. Dis., 81, 243-249.
- Wynne, E.S., Schmieding, W.R., and Daye, G.T., Jr. 1955 A simplified method for counting Clostridium spores. Food Res., 20, 9-12.
- Young, E. 1959 A relationship between the free amino acid pool, dipicolinic acid, and calcium from resting spores of Bacillus megatherium. Can. J. Microbiol., 5, 197-202.
- Zoha, S.M.S. and Sadoff, H.L. 1958 Production of spores by a putrefactive anaerobe. J. Bacteriol., 76, 203-206.

APPENDIX

TABLE I-A.

The replacement of the vegetative cells of Clostridium botulinum in sterile, distilled water. Incubation at 37°C under nitrogen.

<u>Growth time in TSP medium</u>	<u>Time in replacement medium</u>	<u>Total count in replacement</u>	<u>Spores in replacement</u>
24 hrs.	0 hrs.	4.1×10^8	$< 10^6$
	10 hrs.	4.0×10^8	$< 10^6$
	24 hrs.	3.3×10^8	$< 10^6$
	36 hrs.	3.7×10^8	$< 10^6$
	48 hrs.	3.6×10^8	$< 10^6$
	72 hrs.	3.6×10^8	$< 10^6$
	96 hrs.	3.0×10^8	$< 10^6$
	120 hrs.	2.4×10^8	$< 10^6$
48 hrs.	0 hrs.	1.7×10^8	$< 10^6$
	12 hrs.	1.7×10^8	$< 10^6$
	24 hrs.	1.6×10^8	$< 10^6$
	48 hrs.	1.3×10^8	$< 10^6$
	72 hrs.	9.3×10^7	$< 10^6$
	96 hrs.	5.2×10^7	$< 10^6$
72 hrs.	0 hrs.	1.4×10^8	1.1×10^7
	24 hrs.	1.3×10^8	1.2×10^7
	48 hrs.	9.0×10^7	1.0×10^7
	72 hrs.	8.9×10^7	1.0×10^7

TSP CONTROL

<u>Growth time</u>	<u>Total count</u>	<u>Spores</u>
12 hrs.	7.2×10^8	$< 10^6$
24 hrs.	6.4×10^8	$< 10^6$
34 hrs.	3.0×10^8	$< 10^6$
48 hrs.	2.0×10^8	$< 10^6$
60 hrs.	1.6×10^8	4.1×10^6
72 hrs.	1.5×10^8	1.2×10^7
96 hrs.	1.5×10^8	5.5×10^7
120 hrs.	1.2×10^8	6.0×10^7
144 hrs.	1.2×10^8	6.5×10^7

TABLE II-1.

The replacement of the vegetative cells of Clostridium botulinum in 0.067 M phosphate buffer, pH 7.0 with 0.1 percent sodium thioglycollate. Incubated at 37°C.

<u>Growth time in TSP medium</u>	<u>Time in replacement menstruum</u>	<u>Total count in replacement</u>	<u>Spores in replacement</u>
50 hrs.	0 hrs.	3.1×10^8	$< 10^6$
	10 hrs.	1.1×10^8	$< 10^6$
	22 hrs.	8.9×10^7	$< 10^6$
	32 hrs.	7.5×10^7	$< 10^6$
	46 hrs.	$< 10^6$	$< 10^6$
	70 hrs.	$< 10^6$	$< 10^6$
72 hrs.	0 hrs.	1.8×10^8	$< 10^6$
	10 hrs.	9.6×10^7	$< 10^6$
	24 hrs.	$< 10^6$	$< 10^6$
	48 hrs.	$< 10^6$	$< 10^6$
120 hrs.	0 hrs.	4.3×10^7	1.0×10^6
	20 hrs.	5.6×10^6	1.0×10^6
	50 hrs.	2.0×10^6	1.0×10^6

TSP CONTROL

<u>Growth time</u>	<u>Total Count</u>	<u>Spores</u>
10 hrs.	6.1×10^8	$< 10^6$
24 hrs.	7.6×10^8	$< 10^6$
50 hrs.	3.7×10^8	$< 10^6$
60 hrs.	3.4×10^8	$< 10^6$
72 hrs.	2.0×10^8	$< 10^6$
82 hrs.	1.8×10^8	$< 10^6$
96 hrs.	1.1×10^8	1.0×10^6
120 hrs.	8.9×10^7	1.2×10^6
140 hrs.	6.3×10^7	6.1×10^6
170 hrs.	6.2×10^7	1.9×10^7

TABLE III-A.

The replacement of the vegetative cells of Clostridium botulinum in sterile 0.067M phosphate buffer, pH 7.0.
Incubated at 37°C under the normal atmosphere.

<u>Growth time in TSP medium</u>	<u>Time in replacement menstruum</u>	<u>Total count in replacement</u>	<u>Spores in replacement</u>
24 hrs.	0 hrs.	6.7×10^7	$< 10^6$
	20 hrs.	9.1×10^6	$< 10^6$
	49 hrs.	7.4×10^6	$< 10^6$
	67 hrs.	$< 10^6$	$< 10^6$
	139 hrs.	$< 10^6$	$< 10^6$
44 hrs.	0 hrs.	2.3×10^8	2.0×10^6
	29 hrs.	7.2×10^7	1.0×10^6
	47 hrs.	8.2×10^6	2.0×10^6
	119 hrs.	2.0×10^6	2.0×10^6
73 hrs.	0 hrs.	1.4×10^8	3.6×10^7
	18 hrs.	9.2×10^7	3.4×10^7
	42 hrs.	3.7×10^7	3.5×10^7
	66 hrs.	3.5×10^7	3.5×10^7
	90 hrs.	3.6×10^7	3.5×10^7

TSP CONTROL

<u>Growth time</u>	<u>Total count</u>	<u>Spores</u>
10 hrs.	8.1×10^7	$< 10^6$
24 hrs.	9.1×10^7	$< 10^6$
44 hrs.	1.2×10^8	3.0×10^6
73 hrs.	1.0×10^8	7.6×10^6
91 hrs.	9.4×10^7	8.9×10^6
115 hrs.	8.9×10^7	1.4×10^7
139 hrs.	8.6×10^7	2.8×10^7
163 hrs.	7.9×10^7	3.7×10^7

TABLE IV-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution of biotin, methionine, and KH_2PO_4 , pH 7.1 Incubated at 37°C under nitrogen.

<u>Growth time in TSP medium</u>	<u>Time in replacement menstruum</u>	<u>Total count in replacement</u>	<u>Spores in replacement</u>
43 hrs.	0 hrs.	2.6×10^7	$< 10^6$
	46 hrs.	2.0×10^7	$< 10^6$
	118 hrs.	2.0×10^7	$< 10^6$
	264 hrs.	1.3×10^7	$< 10^6$
67.5 hrs.	0 hrs.	1.4×10^8	$< 10^6$
	26.5 hrs.	1.0×10^8	$< 10^6$
	98.5 hrs.	8.3×10^7	$< 10^6$
	244.5 hrs.	6.0×10^7	$< 10^6$
94.0 hrs.	0 hrs.	8.0×10^7	3.6×10^6
	24 hrs.	7.1×10^7	3.6×10^6
	48 hrs.	6.8×10^7	3.4×10^6
	74 hrs.	5.2×10^7	3.1×10^6
	218 hrs.	3.4×10^7	3.3×10^6

TSP CONTROL

<u>Growth time</u>	<u>Total count</u>	<u>Spores</u>
24 hrs.	9.8×10^7	$< 10^6$
48 hrs.	6.2×10^7	$< 10^6$
67.5 hrs.	1.6×10^8	$< 10^6$
94 hrs.	8.8×10^7	4.1×10^6
118 hrs.	6.8×10^7	5.6×10^6
142 hrs.	5.9×10^7	2.2×10^7
168 hrs.	4.9×10^7	3.8×10^7
312 hrs.	5.1×10^7	4.1×10^7

TABLE V-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution of biotin, methionine, nicotinic acid, folic acid, pantothenic acid, thiamin, p-aminobenzoic acid and $MnSO_4$, pH 7.1. Incubated at 37°C under Nitrogen.

<u>Growth time in TSP medium</u>	<u>Time in replacement medium</u>	<u>Total cells in replacement</u>	<u>Spores in replacement</u>
24 hrs.	0 hrs.	3.8×10^8	$< 10^6$
	24 hrs.	3.6×10^8	$< 10^6$
	48 hrs.	3.6×10^8	$< 10^6$
	72 hrs.	3.4×10^8	$< 10^6$
	120 hrs.	9.6×10^7	$< 10^6$
72 hrs.	0 hrs.	1.2×10^8	2.5×10^6
	24 hrs.	1.0×10^8	1.0×10^6
	48 hrs.	1.4×10^8	3.0×10^6
	72 hrs.	1.1×10^8	2.0×10^6
	96 hrs.	1.0×10^8	2.5×10^6
	120 hrs.	1.0×10^8	1.6×10^6
120 hrs.	0 hrs.	5.5×10^7	1.6×10^7
	48 hrs.	5.5×10^7	2.0×10^7
	96 hrs.	5.0×10^7	1.0×10^7
	120 hrs.	5.1×10^7	8.0×10^6

TSP CONTROL

<u>Growth time</u>	<u>Total counts</u>	<u>Spores</u>
24 hrs.	4.2×10^8	$< 10^6$
48 hrs.	3.7×10^8	$< 10^6$
72 hrs.	2.0×10^8	3.1×10^6
96 hrs.	1.1×10^8	7.9×10^6
120 hrs.	7.6×10^7	2.2×10^7
144 hrs.	7.2×10^7	2.3×10^7
168 hrs.	6.4×10^7	2.6×10^7
192 hrs.	6.0×10^7	3.2×10^7
216 hrs.	5.7×10^7	4.0×10^7
240 hrs.	5.6×10^7	4.7×10^7

TABLE VI-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution of DL- α -alanine, L-glutamine, L-ornithine, glycine, glucose, biotin, p-aminobenzoic acid, thiamin, nicotinic acid in 0.067 M phosphate buffer, pH 7.1. Incubated at 37°C under nitrogen.

<u>Growth time in TSP medium</u>	<u>Time in replacement menstruum</u>	<u>Total count in replacement</u>	<u>Spores in replacement</u>
21 hrs.	0 hrs.	2.6×10^8	$< 10^6$
	10 hrs.	2.6×10^8	$< 10^6$
	24 hrs.	3.2×10^8	$< 10^6$
	48 hrs.	2.1×10^8	$< 10^6$
	72 hrs.	1.8×10^8	$< 10^6$
45 hrs.	0 hrs.	1.6×10^8	8.4×10^6
	24 hrs.	1.4×10^8	2.0×10^6
	48 hrs.	1.4×10^8	7.0×10^6
	76 hrs.	1.3×10^8	5.7×10^6
69 hrs.	0 hrs.	9.6×10^7	3.9×10^7
	24 hrs.	8.1×10^7	2.4×10^7
	52 hrs.	8.4×10^7	1.3×10^7
	120 hrs.	7.9×10^7	2.7×10^7

TSP CONTROL

<u>Growth time</u>	<u>Total counts</u>	<u>Spores</u>
21 hrs.	3.1×10^8	$< 10^6$
31 hrs.	3.6×10^8	$< 10^6$
45 hrs.	2.2×10^8	8.9×10^6
69 hrs.	1.6×10^8	4.6×10^7
93 hrs.	9.8×10^7	4.2×10^7
121 hrs.	9.2×10^7	5.7×10^7
189 hrs.	8.4×10^7	6.9×10^7

TABLE VII-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution of thiamin, in a solution of calcium acetate, and in sterile, distilled water. Incubated at 37°C in screw-cap vials; solutions containing 0.1 percent sodium thioglycollate.

<u>Growth time</u> <u>in 4 percent</u> <u>Trypticase</u>	<u>Replacement</u> <u>menstruum</u>	<u>Time in</u> <u>replacement</u> <u>menstruum</u>	<u>Total counts</u> <u>in replace-</u> <u>ment</u>	<u>Spores in</u> <u>replace-</u> <u>ment</u>
19.5 hrs.	sterile, distilled water	0 hrs.	4.2×10^8	$< 10^6$
		3.5 hrs.	4.1×10^8	$< 10^6$
		6.5 hrs.	3.5×10^8	$< 10^6$
		12.5 hrs.	3.0×10^8	$< 10^6$
		22.5 hrs.	1.8×10^8	$< 10^6$
19.5 hrs.	calcium acetate (200ppm)	0 hrs.	4.0×10^8	$< 10^6$
		3.5 hrs.	3.8×10^8	$< 10^6$
		6.5 hrs.	3.4×10^8	$< 10^6$
		12.5 hrs.	3.2×10^8	$< 10^6$
		22.5 hrs.	3.2×10^8	$< 10^6$
19.5 hrs.	thiamin 1 ug per ml	0 hrs.	4.3×10^8	$< 10^6$
		3.5 hrs.	4.0×10^8	$< 10^6$
		6.5 hrs.	3.4×10^8	$< 10^6$
		12.5 hrs.	3.3×10^8	$< 10^6$
		22.5 hrs.	3.0×10^8	$< 10^6$

4 PERCENT TRYPTICASE CONTROL

<u>Growth time</u>	<u>Total counts</u>	<u>Spores</u>
19.5 hrs.	5.0×10^8	$< 10^6$
23.0 hrs.	4.5×10^8	3.0×10^6
26.0 hrs.	4.4×10^8	3.2×10^7
32.0 hrs.	3.4×10^8	8.0×10^7
42.0 hrs.	2.0×10^8	1.5×10^8

TABLE VIII-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution containing the ash of 4 grams of Trypticase in 100 ml of sterile, distilled H₂O; also replacement in dialysed 4 percent Trypticase. Incubated at 37°C in screw-cap vials; solutions containing 0.1 percent sodium thioglycollate.

Growth time in 4 percent Trypticase	Replacement menstruum	Time in Replacement menstruum	Total count in replace- ment	Spore count in replace- ment
18 hrs.	dialysed 4 percent Trypticase	0 hrs.	3.2×10^8	3.0×10^7
		3.0 hrs.	3.0×10^8	3.0×10^7
		6.5 hrs.	2.2×10^8	2.0×10^7
		12.0 hrs.	2.1×10^8	1.0×10^7
		18.0 hrs.	2.0×10^8	1.0×10^7
		29.0 hrs.	2.0×10^8	1.0×10^7
18 hrs.	4 percent Trypticase ash	0 hrs.	3.2×10^8	4.0×10^7
		3.0 hrs.	3.3×10^8	4.0×10^7
		6.5 hrs.	3.2×10^8	3.5×10^7
		12.0 hrs.	3.0×10^8	3.3×10^7
		18.0 hrs.	3.1×10^8	3.4×10^7
		29.0 hrs.	3.0×10^8	3.4×10^7

4 PERCENT TRYPTICASE CONTROL

Growth time	Total count	Spores
18 hrs.	3.4×10^8	3.0×10^8
21 hrs.	3.2×10^8	9.1×10^8
24.5 hrs.	3.1×10^8	1.6×10^8
30 hrs.	2.8×10^8	1.7×10^8
36 hrs.	2.2×10^8	1.7×10^8
47 hrs.	2.0×10^8	1.8×10^8

TABLE IX-A.

The replacement of the vegetative cells of Clostridium botulinum in a solution of 4 percent casamino acids (acid hydrolysate of casein); also in 4 percent casamino acids (enzymatic hydrolysate of casein). Incubated at 37°C in screw-cap vials; solutions containing 0.1 percent sodium thioglycollate.

Growth time in 4 percent Trypticase	Replacement menstruum	Time in replacement menstruum	Total count in replace- ment	Spores in replace- ment
20 hrs.	4 percent casamino acids (acid hydrolyzed)	0 hrs.	5.4×10^8	1.4×10^7
		10 hrs.	4.0×10^8	1.0×10^7
		20 hrs.	3.8×10^8	1.1×10^7
		30 hrs.	2.2×10^8	1.0×10^7
		45 hrs.	2.0×10^7	7.0×10^6
		72 hrs.	7.2×10^7	6.0×10^6
20 hrs.	4 percent casamino acids (ensy- matic hydro- lyzed)	0 hrs.	4.8×10^8	1.0×10^7
		10 hrs.	3.0×10^8	5.0×10^6
		20 hrs.	clumping	-
		30 hrs.	clumping	-
		45 hrs.	clumping	-
		72 hrs.	clumping	-

4 PERCENT TRYPTICASE CONTROL

Growth time	Total count	Spores
20 hrs.	5.6×10^8	1.0×10^7
30 hrs.	3.2×10^8	3.1×10^7
40 hrs.	3.0×10^8	5.0×10^7
50 hrs.	2.1×10^8	5.9×10^7
65 hrs.	1.4×10^8	6.0×10^7
92 hrs.	6.2×10^7	6.1×10^7

TABLE I-A.

Formulae of media used in this investigation.

1. TSP medium, Zohar and Sadoff (1958)

trypticase	1.5 percent
peptone	1.0 percent
NaCl	0.5 percent
K ₂ HPO ₄	0.25 percent
glucose	0.30 percent
(NH ₄) ₂ SO ₄	0.10 percent
sodium thioglycollate	0.10 percent
thiamine	1 ug per ml

2. YSSB agar, Wynne et al. (1955)

yeast extract	1.0 percent
glucose	0.2 percent
methylene blue	0.0004 percent
soluble starch	0.1 percent
agar	1.5 percent

The following additive is used in 0.5 ml/tube
of YSSB agar

sodium thioglycollate	0.2 percent
K ₂ HPO ₄	0.2 percent
NaHCO ₃ (filter sterilize)	0.1 percent

TABLE XI-A.

Approximate Composition of Trypticase*

Source: Casein

Hydrolysis: Pancreatic

<u>Nitrogen</u>			
<u>Total content</u>		11.7	
Amino	3.5		
NaCl	0.5		
Ca	0.33		
Fe	0.03		
K	0.24		
Mg	0.03		
P	0.65		
S	0.73		
<u>Carbohydrates percent</u>		0.0	
<u>Amino Acids percent</u>			
Arginine	2.6	<u>Vitamins, mg/l</u>	
Aspartic Acid	5.1	Biotin	0.18
Cystine	0.3	Choline	1960.00
Glycine	1.8	Cyanocobalamin	0.50
Glutamic Acid	17.0	Folic acid	1.17
Histidine	2.4	Niacin	212.00
Isoleucine	5.0	Pantothenic acid	8.90
Leucine	7.1	Pyridoxine	3.2
Methionine	2.4	Riboflavin	19.00
Phenylalanine	3.3	Thiamine	+
Proline	11.5	PABA	0.21
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.

~~_____~~



~~AUG 11 '66~~

~~MAY 13 '67~~

~~7/67~~
~~NOV 27 '67~~

~~SEP 2 '67~~

~~MAY 25 '67~~

~~DEC 1 '67~~

~~JUL 20 '62~~

~~JUN 3 '62~~

~~SEP 27 '62~~

~~MAR 11 '63~~

~~JUN 25 '64~~

~~JUL 2 '64~~

~~_____~~

~~DEC 1 '65~~

MICHIGAN STATE UNIVERSITY LIBRARIES



3 1293 10756 8044