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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

GROWTH AND SPORULATION REQUIREMENTS OF *CLOSTRIDIUM ROSEUM*

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

BENNIE CECIL WOOLEY

Norman, Oklahoma

1966

GROWTH AND SPORULATION REQUIREMENTS OF

CLOSTRIDIUM ROSEUM

APPROVED BY

DISSERTATION COMMITTEE

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GROWTH AND SPORULATION REQUIREMENTS OF *CLOSTRIDIUM ROSEUM*

CHAPTER I

INTRODUCTION

Possibly the most challenging and dynamic field of biology today is the study of cellular differentiation. One of the most common forms of microbial intracellular differentiation involves the formation of bacterial endospores by the Gram-positive spore-forming rods of the genera *Bacillus* and *Clostridium* and by the coccus, *Sporosarcina*. During growth each cell possesses the genetic capacity to sporulate but, the sporogenic genome remains unexpressed until it receives the required stimuli. Upon induction or derepression this genome directs the qualitative orderly changes in phenotype which result in the formation of a semi-dormant resistant spore. In each case biochemical and morphological changes occur in response to appropriate environmental stimuli. For recent reviews on the formation of endospores see

Murrell (1961) and Halvorson (1962).

Since the environmental conditions, for example the nutritional requirements, play such a key role in the morphogenic process of sporulation, they have been the subject of a great deal of investigation. The literature concerning the factors influencing the rate of sporulation and the factors influencing the quality of spores has been reviewed recently by Halvorson (1962). However, most of the research has been limited to the nutritional requirements of the genus Bacillus. For example, only 15 of 224 references cited in the above review were concerned with the genus Clostridium.

According to Bergey's Manual of Determinative Bacteriology (Breed, Murray, and Smith, 1957) the genus Clostridium contains all but one of the human pathogens in the family Bacillaceae and the majority of species of great inportance to the food and fermentation industries. A survey of the literature concerning the production of clostridial spores shows that it has been traditional to provide amino acids in the form of proteins or protein fragments. This practice may have originated as an attempt to simulate the wound environment from which the first pathogenic clostridium was isolated (Pasteur and Joubert, 1877). Another and more probable reason is that all clostridia sporulate to some

degree in complex infusions (Perkins, 1965). However, these complex infusions prevent the examination of the chemical composition or the fine structure of the spore crop since tissue fragments present prevent the complete separation of the free spores from the medium.

In the past few years investigators have found that various peptones are as active in stimulating sporulation as the complex infusions (Sugiyama, 1951; Bowen and Smith, 1955; Brown, Ordal and Halvorson, 1957; Collier, 1957; Lund, Janssen, and Anderson, 1957; Zoha and Sadoff, 1958; Costilow, 1962; Grecz and Anellis, 1963; Hall, Angelotti, Lewis, and Fotor, 1963). These peptones offer the advantages of being completely water soluble and having relatively constant amino acid compositions.

Although the specific amino acids essential for growth have been elucidated for several species of clostridia (Burrows, 1933; Elberg and Meyer, 1939; Roessler and Brewer, 1946; Skull, Thoma, and Peterson, 1949; Williams and Blair, 1950; Mager, Kindler, and Grosswicz, 1954; Cambell and Frank, 1956; Kindler and Mager, 1956; Fuchs and Bonde, 1957; Perkins and Tsuji, 1962) sporulation is poor or non-existent in the absence of proteinaceous material. It appears that a broad spectrum of proteinaceous material may initiate and support

sporulation of a particular species of clostridia and that the main difference lies in the amount of sporulation in each. This indicates that each of the active proteinaceous substances contains a specific molecular structure in common which is required for sporulation. The difference in the amount of sporulation may then be a reflection of the number of times the required structure is repeated in a particular protein molecule.

Perkins (1965) has recently stated that "Possibly the most significant contribution to the study of anaerobic spore production in recent years has been the revelation that 'semi-defined' peptone media will support the sporulation of most species. These simple media will facilitate biochemical studies of clostridial spore production that were heretofore impossible and can be expected to lead to the development of synthetic sporulation media for many species." The development of synthetic sporulation media is an absolute requirement in the understanding of the changes in cellular metabolism which occur in the transition from vegetative cell to spore.

This investigation was designed to determine the nutritional requirements for growth and sporulation of Clostridium roseum. This organism is a predominantly saccharolytic

species which is capable of complete, rapid and synchronous sporulation in glucose, salts, and Trypticase (Collier, 1957; Wooley and Collier, 1965). Trypticase is a pancreatic digest of the milk protein, casein. Although the amino acid, vitamin, and elemental content of this protein is believed to be known (Block and Weiss, 1956; Basch and Gordon, 1961; BBL Technical Publication, 1963; Perkins, 1965) and relatively constant, the specific requirement and effect of each component cannot be known until each can be tested as a pure chemical entity. This investigation is concerned with the construction of a chemically defined medium which duplicates the growth and sporogenic activity of Trypticase.

CHAPTER II

MATERIALS AND METHODS

Culture: Clostridium roseum was used throughout this investigation. Stocks of this organism were maintained by lyophilization and spores were obtained using the "active-culture" technique and the Tris Test Medium of Collier (1957). Clean free spores are produced in this medium in 12 hr. The spore crops were harvested by centrifugation at 4 C in a Servall Refrigerated Centrifuge; washed ten times with deionized water; and then suspended at a concentration of approximately 1 X 10^8 spores per ml in deionized water.

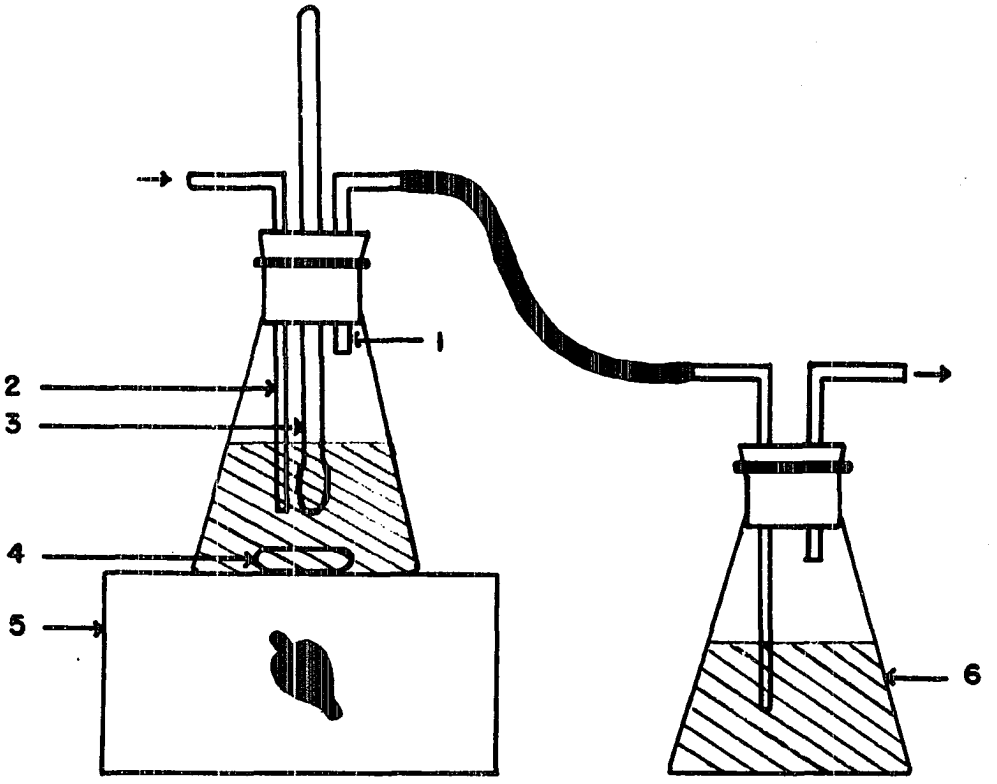
Construction of synthetic medium: Since Trypticase supports the germination, growth, and sporulation of C. roseum the ideal approach was to duplicate the reported amino acid and vitamin content of Trypticase and use the carbohydrate, salts, and buffers of the Tris Test Medium. The Trypticase used throughout this investigation was obtained from the Baltimore Biological Laboratories, Lot No. 312647.

The synthetic medium was fabricated from chromatographically pure components available from Nutritional Biochemicals Corporation, CALBIOCHEM, and Fisher Scientific Co. All glassware was washed in a commercial dishwasher and rinsed with tap water and with distilled water. The amino acids and buffer system were made up in deionized water, dispensed in screw-capped Rx bottles, and autoclaved. The glucose, salts, and vitamins were made up separately in deionized water, filter sterilized (Millipore Filter Corp., Type HA 0.45u), and stored at 4 C. The vitamins and salts were stored in screw-capped flasks covered with a double layer of aluminum foil to prevent photodecomposition and oxidation. The glucose was not autoclaved in the medium since this practice has been found to produce both growth inhibiting and growth promoting substances (Field and Lichstein, 1958). The amino acids and buffers were autoclaved and then placed in an 80 C water bath. The glucose, salts, and vitamins were added aseptically. One hundred ml of the synthetic medium was inoculated with 5 ml of the washed spore suspension and after 15 min of heat-shock the screw-caps were tightened, the bottles placed in a 37 C water bath, and incubated for 24 hr. Germination was determined by examination of a crystal violet stained smear. The amount of growth was measured

by determining the optical density at 500 mu using a B & L Spectronic 20.

Production of active culture: To accomplish this an aliquot of the 24 hr cells was inoculated into the synthetic medium in a 125 ml anaerobic culture flask (See Figure 1). Growth in the flask was determined by measuring the optical density of an aseptically removed aliquot. A sample of the uninoculated medium was used as a control. As the cells approached the end of the logarithmic growth phase, 10 ml was transferred to fresh synthetic medium. In the final transfer the temperature was dropped from 37 C to 20 C when the cells were approximately half-way through the logarithmic growth phase. This temperature was maintained for 30 min; the temperature raised to 37 C for an additional 30 min; and then lowered to 20 C. After 30 min, 10 ml of the synchronously dividing cells were inoculated into fresh synthetic medium at 37 C which was to be tested for sporogenic activity. Aliquots were removed at each hour following inoculation. The growth was determined by measuring the change in optical density. Two smears were made from each aliquot and stained with crystal violet. The per cent sporulation was determined by counting the number of non-staining spores and the number of stained vegetative cells in 10 random fields on each of

Figure 1. Anaerobic spin-flask culture apparatus



1. GAS OUTLET
2. GAS INLET
3. THERMOMETER
4. MAGNETIC STIRRING BAR
5. MAG-MIX
6. WATER TRAP

the two slides.

Testing other compounds for sporogenic activity: Since the basic synthetic medium failed to initiate and support sporulation, other available compounds were tested for sporogenic activity. The same synthetic medium and procedures were used as given above with the exception that the final medium contained the compound to be tested. This compound was either autoclaved in the medium or filter sterilized (Millipore Filter Corp., Type HA 0.45u), depending upon its heat lability, and added aseptically. The growth and sporulation was measured as previously described.

Sporogenic activity of casein: Agar plates consisting of the synthetic medium, 5% purified casein (Nutritional Biochemicals Corp.), and 2% agar were streaked with a sample of actively growing cells from the synthetic medium. To reduce spreading the plates had been incubated in an inverted position at 37 C for 24 hr prior to inoculation. After inoculation the plates were placed in an inverted position in a vacuum jar. Anaerobic conditions were created by alternately pulling a vacuum and replacing with natural gas. The final addition of natural gas did not completely displace the vacuum in order to allow expansion of the natural gas at the incubation temperature without destroying the air tight

seal. The plates were incubated at 37 C for 24 hr. Sporulation was determined in stained smears as previously described. The proteolytic activity of the cells was determined by flooding the plates with a saturated solution of picric acid.

Fractionation of Trypticase: In the following text each time a fraction was tested for sporogenic activity, the "active culture" technique was used as previously described. To insure that the active component was organic in nature, a 1 g sample of Trypticase was ashed. After the carbon was removed, the residue was tested for sporogenic activity. The solubility of the active component in acetone, ethanol, ether, benzene, dioxane, and methanol was tested by Soxhlet liquid-solid extraction. After 24 hr of extraction the solvent was removed from the extracted soluble portion and the insoluble residue by drying at 40 C in a vacuum oven and each tested for sporogenic activity. An attempt to partially purify the active fraction was made by dialyzing against deionized water. The dialysate and residue were concentrated to dryness at ambient temperature in a Nester/Faust flash evaporator and tested for sporogenic activity. The susceptibility of the sporogenic activity to acid was tested by subjecting a sample of Trypticase to refluxing for 24 hr in 6N

hydrochloric acid; dried in a vacuum desiccator over sodium hydroxide; and then tested for sporogenic activity. Another sample was refluxed for 24 hr in 6N sodium hydroxide; dried in a vacuum desiccator with sulfuric acid; and then tested for sporogenic activity.

Since casein is a phosphoprotein, it was thought that the active fraction may contain either phosphoserine or phosphothreonine. The procedures of Levine and Hill (1933) were carried out on Trypticase and the white powdery phosphopeptides obtained were tested for sporogenic activity.

Column fractionation of Trypticase: Two hundred grams of Sephadex G-25 (Lot No. TO 5340; Particle size 20-80u) were stirred with 2 liters of 0.5M phosphate buffer at pH 7.0 for 24 hr. A water tight polyethylene collar was fitted to the top of a 4.5cm X 60cm glass chromatographic column and the lower end fitted with a porous plate. The collar acted as a reservoir to permit the addition of the entire column bed at once. The slurry was poured into the column and reservoir after the delivery tip had been closed. After approximately 10 cm of the column bed had been packed, the delivery tube was opened and the remainder of the bed allowed to settle and pack. The resulting bed had the dimensions of 4.5cm X 45cm and a bed volume of approximately 1

liter. The phosphate buffer was displaced from the column with aq 0.2M ammonia solution.

The sample (10 ml of 30% Trypticase in 0.2M ammonia solution) was applied to the top surface of the column, allowed to absorb, and then washed through with the ammonia solution. For a preliminary test 100 ml fractions were collected from 0-1600 ml of effluent; dried at 50 C in a vacuum oven; placed in aq solution; and tested for sporogenic activity. The column was flushed by passage of 3 liters of the solvent. Another 10 ml sample of 30% Trypticase was passed through the column and 25 ml fractions were collected from 300-700 ml of effluent. Each fraction was dried as above; placed in aq solution; and tested for sporogenic activity. After determining the elution range of the sporogenic components, 25 ml fractions were collected and dried as above. Each was placed in aq solution and an equal aliquot of each tested for ninhydrin reactivity using the procedure of Moore and Stein (1954). The optical density of the test solutions was measured at 570 mu using a Gilford Recording Spectrophotometer.

A glass tube (1.5cm X 110cm) was drawn to a delivery tip at one end and this end was packed with a layer of glass wool. The column was packed using a slurry of Sephadex G-25

as previously described. The final bed had the dimensions of 1.5cm X 100cm. A 10 ml sample of 30% Trypticase was passed through the first column (4.5cm X 45cm) and the effluent from 350-650 ml was collected, dried as previously described and placed in solution with 10 ml of aq 0.2M ammonia solution. One ml of this solution was placed on the top surface of the 1.5cm X 100cm column and developed with the ammonia solution. Ten ml fractions were collected; treated as previously described; and tested for sporogenic activity. Another 1 ml sample was treated as above and an equal aliquot of each fraction tested for ninhydrin reactivity as previously described.

The purity of each fraction was determined by high voltage paper electrophoresis. Horizontal paper electrophoresis was run on Whatman 3 MM filter paper, 4cm X 60cm, using an E-C Apparatus Co. electrode box and power supply with 0.05M acetate buffer at pH 5.0 as the buffer system. Each of the fractions from the 1.5cm X 100cm column was dried and resuspended in 0.05M acetate buffer. A portion of each fraction was applied across the center of separate filter paper strips and the separation allowed to proceed at 200 V for 2-6 hr. After the run the strips were removed and then dried in a chromatographic oven at 50 C. Each strip

was dipped in a 0.5% solution of ninhydrin in acetone and developed by drying at 110 C for 15 min.

Since the resolving power of the second Sephadex G-25 column was not great enough to yield separate peptide entities, a glass tube (1.5cm X 110cm) was prepared as previously described and packed with a slurry of Sephadex G-10 (Lot No. TO 7610; Particle size 40-120u) to give a final bed of 1.5cm X 85cm. A sample of the effluent from 100-190 ml from the 1.5cm X 100cm Sephadex G-25 column was dried, suspended in aq 0.2M ammonia solution, and placed on the surface of the Sephadex G-10 column. The column was developed with the ammonia solution and 10 ml fractions were collected. The fractions were treated as previously described and tested for ninhydrin reactivity, sporogenic activity, and purity.

Preparative electrophoresis: Horizontal preparative electrophoresis was run on Whatman 3 MM filter paper (20cm X 60cm) using the procedures previously described. Up to 20 strips were run at one time. The sample used was the sporogenic effluents from the Sephadex G-25 (1.5cm X 100cm) column which had been dried and suspended in 0.05M acetate buffer at pH 5.0. Separation was allowed to proceed at 200 V for 4 hr. After the run the strips were dried as previously described. A 1 cm strip was cut off the entire length of each

filter paper strip and developed with ninhydrin. This strip permitted the location of each peptide band on the electrophoretic strips. Each separate band was marked, cut from the electrophoretic strips, and eluted as described below. The corresponding ninhydrin positive bands from each electrophoretic strip were pooled, cut into small fragments (approx 1mm X 5mm), and macerated in a Waring Blender for 15 min with deionized water as the solvent. The resulting suspension was first filtered through W. H. Curtin & Co. filter paper (No. 7760) and then through a Millipore Filter Corp. filter (Type HA 0.45u) to remove the macerated filter paper. The water solution of each peptide was concentrated to dryness at 50 C in a vacuum oven, resuspended in deionized water, and tested for sporogenic activity. The purity of the two sporogenic peptides (sporogens) was checked by two-dimensional descending paper chromatography. The chromatography was carried out on 18 inch X 18 inch sheets of Whatman 3 MM filter paper. The first dimension was developed with secondary-butanol, formic acid, and water (70:10:20) and the second dimension with phenol, water, and ammonium hydroxide (80:19.7:.3). After development in both directions and drying at 50 C, the sporogens were detected by dipping in 0.5% ninhydrin in acetone and drying at 110 C for 15 min. The

purity of each sporogen was also checked by horizontal paper electrophoresis in the following buffer systems: (1) Hydrochloric acid-Potassium chloride pH 2.0; (2) Sodium acetate-Acetic acid pH 5.0; (3) Monobasic sodium phosphate-Dibasic sodium phosphate pH 7.0; (4) Monobasic sodium phosphate-Dibasic sodium phosphate pH 8.0; (5) Carbonate-Bicarbonate pH 10.0. The buffers were prepared as described by Gomori (1955).

Chemical and physical methods: Elemental analyses were performed using the sodium fusion method as described by Shriner, Fuson and Curtin (1964). The presence of carbohydrate was checked by the phenol-sulfuric acid colorimetric method as described by Hodge and Hofreiter (1962) and the presence of hexosamines by the Elson-Morgan reaction as described by Dische (1962). The ultraviolet absorption spectrum of each sporogen was determined in 0.1M phosphate buffer pH 7.0 using a Gilford Recording Spectrophotometer. The buffer alone was used as the control.

For identification of the constituent components of each sporogen, each was subjected to acid hydrolysis (6N hydrochloric acid) using the procedure described by Moore and Stein (1963). After hydrolysis the excess acid was removed by repeated evaporation after the addition of deionized

water. The ultraviolet absorption spectrum of each hydrolyzed sporogen was determined as described for the unhydrolyzed samples. The hydrolyzed sporogens were tested for sporogenic activity and subjected to two dimensional descending paper chromatography. The chromatography was performed on Whatman 3 MM filter paper with secondary-butanol, formic acid, and water (70:10:20) as the first solvent and phenol, water, and ammonium hydroxide (80:19.7:.3) for the second dimension. The constituent amino acids of the sporogens were identified by simultaneous chromatography of known amino acids and related compounds and later by co-chromatographic techniques. Amino acids were detected by dipping in 0.5% ninhydrin in acetone and drying at 110 C for 15 min. Sulfur amino acids were detected by the iodine-azide reagent of Chargaff, Levine, and Green (1948). Carbohydrates were developed with the aniline hydrogen phthalate reagent of Partridge (1949). Amino sugars were detected with the Morgan-Elson reagent as modified by Partridge (1948). Sugar-phosphates and other organo-phosphate esters were detected by dipping in the molybdate reagent of Burrows, Grylls, and Harrison (1952). Each chromatogram was examined for ultraviolet absorbing or florescent spots with a Chromato-Vue at wavelengths of 3600A and 2537A before staining.

Phosphopeptide identification and isolation: A sample of each purified unhydrolyzed sporogen was spotted on Whatman 3 MM filter paper and tested with the Burrows, Grylls, and Harrison (1952) molybdate reagent for organo-phosphate esters. Since the test was positive for both sporogens, the original procedure of Levine and Hill (1933) was modified as follows. This procedure calls for the addition of phenolphthalein (pH 8.2-10; colorless-red) to the reaction mixture to detect the endpoint of each fractionation procedure. However, due to the inherent dark-brown color of a concentrated solution of Trypticase, this indicator did not produce clear reproducible endpoints. Therefore, the following procedure was used. Seventy g of Trypticase in 400 ml of deionized water was raised to pH 10.3 with a 10% solution of barium hydroxide. The precipitated barium phosphate was centrifuged out and discarded. The pH of the clear solution was adjusted to pH 8.2 with a 10% solution of neutral lead acetate. The lead salts were removed by centrifugation and the mother liquor discarded. The solid was stirred with sufficient 10% sodium carbonate to raise the pH to 10.6. The insoluble lead carbonate was centrifuged out and discarded. The clear solution containing the sodium salt of the phosphopeptides was then stirred with 3 volumes of ethanol and the precipitate

so obtained allowed to settle. The supernatant was decanted and the precipitated peptides solidified by the addition of acetone and tested for sporogenic activity.

Alterations in the synthetic medium:

Vitamins: For this determination, actively growing vegetative cells were harvested by centrifugation at 4 C; washed eight times in sterile synthetic medium minus all vitamins; and suspended at a concentration of approximately 1 X 10^8 cells per ml in synthetic medium devoid of vitamins. Five ml of the suspension was inoculated into the synthetic medium which had been prepared and cooled to 37 C as previously described. Each Rx bottle contained all but one of the vitamins to be tested. Two bottles containing all of the vitamins were used as controls. The vitamins had been prepared individually and filter sterilized (Millipore Filter Corp., Type HA 0.45u). The cultures in Rx bottles were incubated at 37 C for 24 hr and the amount of growth determined by measuring the optical density at 500 mu using a B & L Spectronic 20.

Structural requirements of vitamins: For this assay 100 ml samples of the synthetic medium containing only the amino acids and buffers at pH 7.3 were autoclaved in screw-capped Rx bottles and then placed in an 80 C water bath.

Filter sterilized glucose and salts were added aseptically. Filter sterilized test vitamins were then added with the concentration being the same as that in the normal synthetic medium. The test vitamins were divided into three groups: (1) Pantothenic acid group; (2) Niacin group; and (3) Pyridoxal group. When testing a particular group, the active member of the other two groups was present. For example, when testing the pantothenic acid group, niacin and pyridoxal were always present. All of the vitamins tested were purchased from Nutritional Biochemicals Corp. with the exception of Pantoyl-alpha-alanine which was synthesized from L pantoyl lactone and alpha-alanine using the procedure of Williams, Mitchell, Weinstock, and Snell (1940). After the proper additions, each bottle was inoculated with 5 ml of washed spores; the spores heat shocked at 80 C for 15 min; the screw-caps tightened; and the bottles placed in a 37 C water bath. After 24 hr the amount of growth was determined by measuring the optical density as previously described. Germination and the morphological characteristics of the cells were determined by the examination of crystal violet stained smears.

Amino acids required for germination and growth: To determine the amino acids required for germination and growth 100 ml samples of the synthetic medium at 80 C in Rx bottles

were inoculated with 5 ml of a washed spore suspension. Each bottle contained all but one amino acid; the normal salts, glucose and buffers; and the required vitamins. Two bottles containing all of the amino acids were used as controls. The initial pH of each bottle was adjusted to pH 7.3. After 15 min of heat shock the lids were tightened; the bottles placed in a 37 C water bath; and incubated for 24 hr. The germination and growth was measured as previously described.

Glucose requirement for growth: For this determination cells were grown in the synthetic medium until they had reached the end of their logarithmic growth phase. Ten ml of this culture was then transferred to 100 ml of the synthetic medium in the anaerobic culture flasks whose glucose concentration was varied from 0%-1%. Incubation occurred at 37 C with constant magnetic stirring. At hourly intervals aliquots were aseptically removed and the amount of growth determined by measuring the optical density change using a B & L Spectronic 20.

Alteration of buffer capacity: For this test the phosphate was reduced to 1 g per liter of monobasic potassium phosphate and 1 g per liter of dibasic potassium phosphate. The tris (2 amino-2-(hydroxymethyl)-1,3-propanediol) concentration was not altered. Ten ml of an active culture from

the synthetic medium was inoculated into the medium to be tested in the anaerobic culture flasks. Incubation proceeded at 37 C and aliquots were aseptically removed at hourly intervals and the growth and sporulation measured as previously described. The pH of each aliquot was measured using a Beckman Zeromatic pH Meter.

Effect of time of addition of Trypticase and sporogens on sporulation: A series of anaerobic culture flasks containing the synthetic medium at 37 C was prepared as previously described. Each was inoculated with 10 ml from an actively growing culture of vegetative cells. At hourly intervals beginning with the time of inoculation sufficient Trypticase was added to a flask to give a final concentration of 0.1%. At hourly intervals an aliquot of each flask was aseptically removed and checked for the amount of growth and sporulation as previously described. The above procedures were repeated using a mixture of the two pure sporogens rather than Trypticase.

CHAPTER III

RESULTS AND DISCUSSION

Construction of synthetic medium: The initial synthetic medium consisted of the amino acids and vitamins reported by Baltimore Biological Laboratories to be present in their Trypticase and the carbohydrate, salts, and buffers of the Tris Test Medium (Collier, 1957; Wooley and Collier, 1965). This medium failed to support either the germination of washed spores or the growth of washed vegetative cells. Reexamination of the BBL publication showed that there were several possible explanations for this failure. This publication does not list alanine or serine yet both are present in the parent molecule, casein (Block and Weiss, 1956; Hipp, Basch, and Gordon, 1961). Also the cysteine-cystine content is listed only as cystine which may or may not be the only form of this amino acid present in Trypticase. This publication shows that the amino-nitrogen does not account for all the nitrogen in Trypticase which indicates that some

nitrogen may be present in another form such as an amide in asparagine and glutamine. With these points in mind the medium was reconstructed as shown in Table I.

This medium supported the germination and growth processes of washed spores of Clostridium roseum. However, neither process was as rapid as that obtained in the usual germination medium consisting of 1.5% Trypticase and 0.5% yeast extract. Figure 2 shows the rate of germination and growth in the synthetic medium as compared with Trypticase-yeast extract. The initial decrease in the optical density represents the loss in refractility of the spores which occurs during germination. Since the spores had been previously cultured in Trypticase, they were capable of rapid germination and growth in the Trypticase-yeast extract medium, but both germination and growth were retarded in the synthetic medium. The germination rate was not affected to the same extent as the processes involved in post-germinative development and the subsequent growth. However, the examination of crystal violet stained smears showed that germination in the synthetic medium was far from synchronous. After 2-4 hr the culture consisted of spores, germinated spores, and vegetative cells. Although continued incubation in the synthetic medium resulted in complete germination, the culture

TABLE I

The initial synthetic medium

A. Amino acids	g/liter
* 1. Alanine	0.525
2. Arginine	0.390
3. Aspartic Acid	0.765
4. Cystine	0.060
* 5. Cysteine	0.090
6. Glycine	0.270
7. Glutamic Acid	2.550
8. Histidine	0.360
9. Isoleucine	0.750
10. Leucine	1.065
11. Lysine	0.795
12. Methionine	0.360
13. Phenylalanine	0.570
14. Proline	1.725
*15. Serine	0.780
16. Threonine	0.525
17. Tryptophan	0.135
18. Tyrosine	0.345
19. Valine	0.840
*20. Asparagine	0.610
*21. Glutamine	0.270

B. $CaNO_3$

After autoclaving add 10 ml/liter of 2% $CaNO_3$

C. Vitamins	mg/liter
1. Biotin	0.021
2. Cyanobobalamine	0.006
3. Folic Acid	0.110
4. Niacin	2.120
5. Pantothenic Acid	0.533
6. Pyridoxal	0.150
7. Riboflavin	0.337
8. Thiamine	0.500
9 PABA	0.123

D. Salts

Dissolve in 250 ml water
Use 10 ml/liter

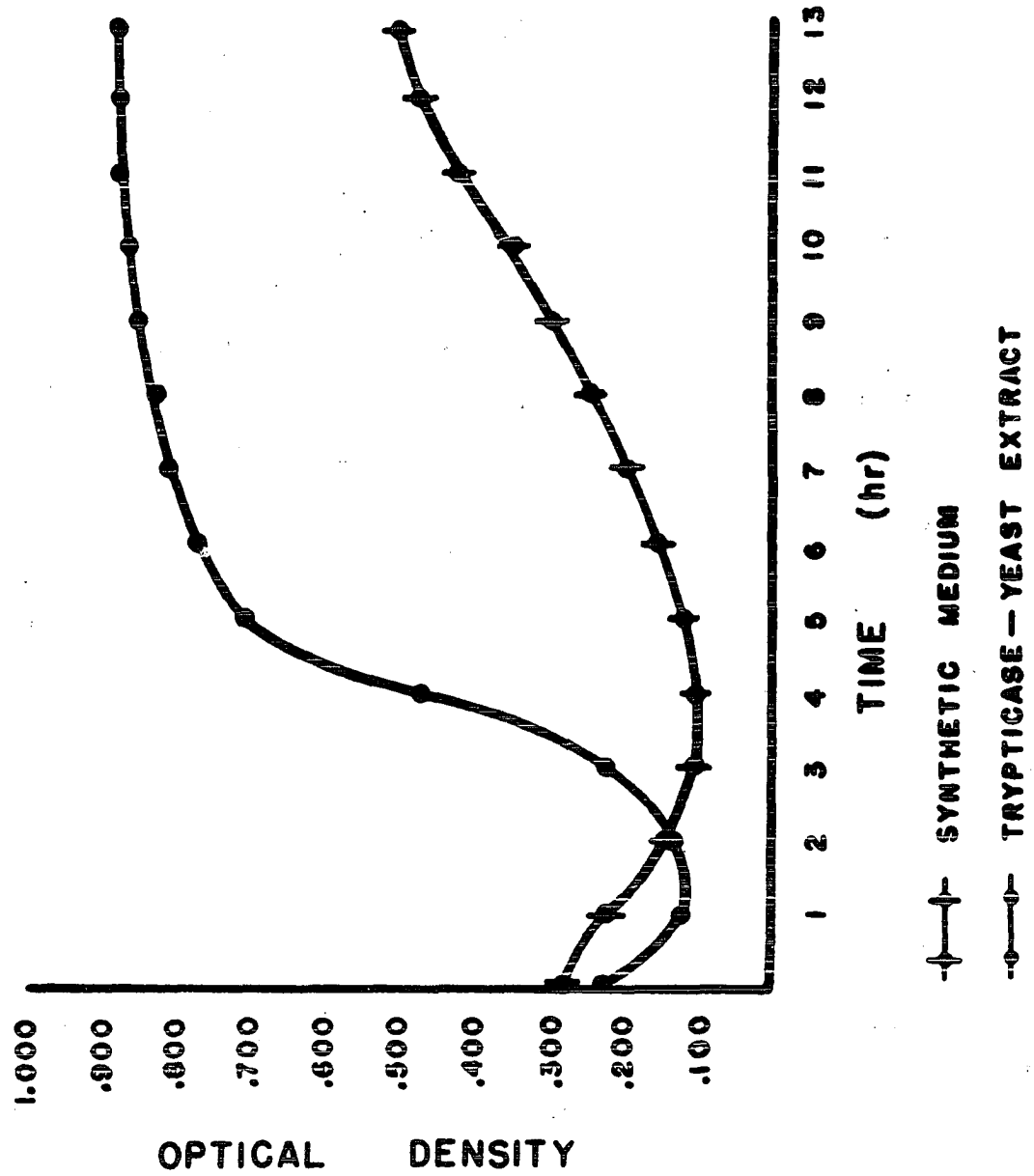
1. $FeSO_4 \cdot 7H_2O$	12.5 mg
2. $CuSO_4 \cdot 5H_2O$	125.0 mg
3. $ZnSO_4 \cdot 7H_2O$	125.0 mg
4. $MnSO_4 \cdot 4H_2O$	1.25 g
5. $MgSO_2$ anhyd	5.00 g

E. Additional	g/liter
1. K_2HPO_4	5.500
2. Tris buffer	1.500
3. Glucose	2.000
4. NaCl	5.000

* Not listed on BBL publication, but occur in casein.

Figure 2. Spore germination and subsequent growth in the synthetic medium and in 1.5% Trypticase-0.5% yeast extract.

Water washed spores were heat-shocked for 15 min at 80 C in the germination medium; incubated at 37 C; and the germination and growth determined by measuring the optical density at 500 mu.

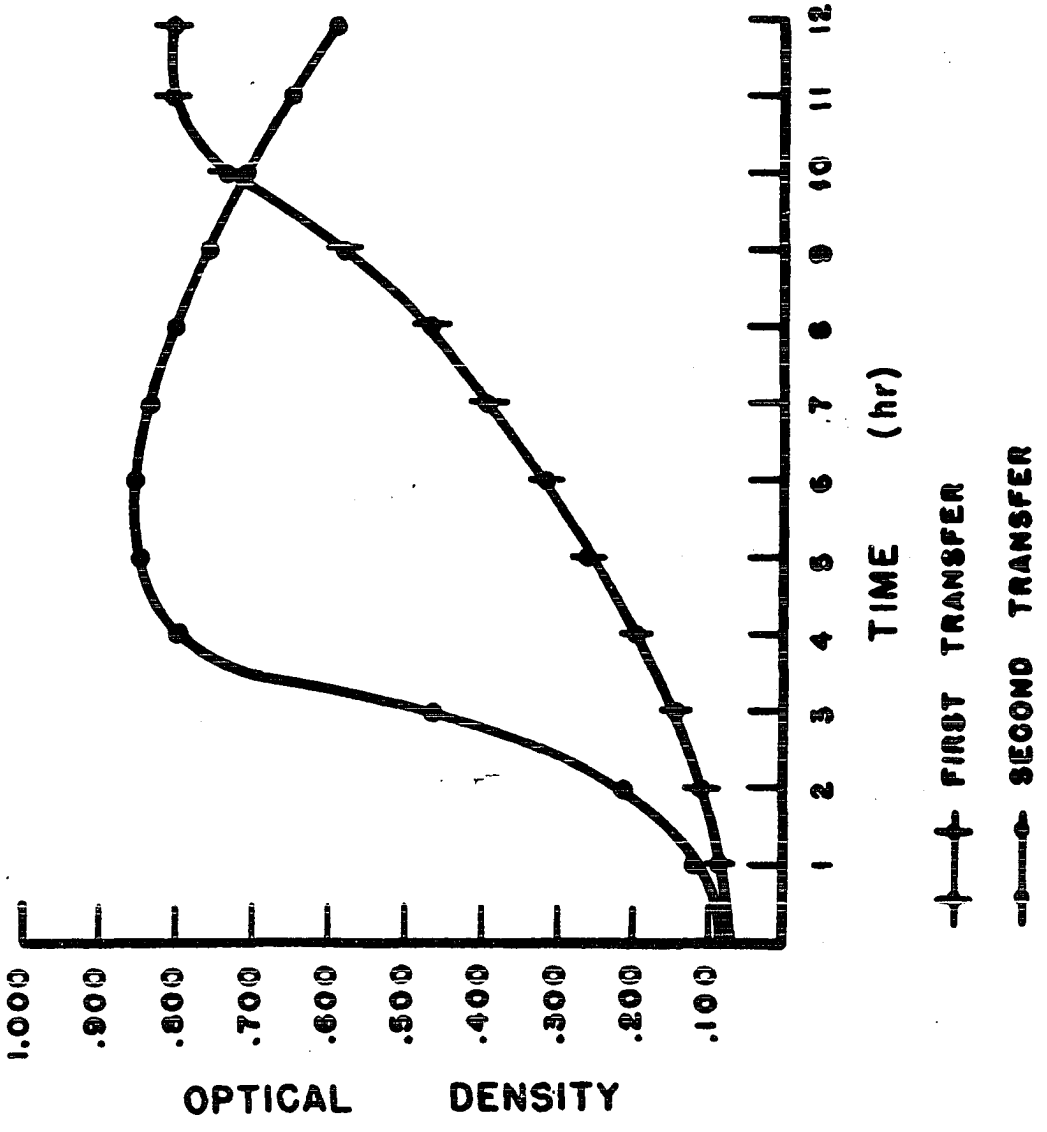

1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1 2 3 4 5 6 7 8 9 10 11 12 13
TIME (hr)
SYNTHETIC MEDIUM
TRYPTICASE—YEAST EXTRACT

consisted of a mixture of vegetative cells at different stages in the growth cycle. The clostridia are notorious for the difficulty involved in the production of vegetative cell-free spore crops. This is due primarily to the phenomenon of recycling in which a spore is formed and then immediately germinates and begins to grow and divide. This phenomenon is primarily the result of a culture of vegetative cells at different stages of development.

To obtain a culture of vegetative cells that would initiate the sporulation process synchronously it was necessary to produce an "active culture." To accomplish this the vegetative cells were transferred repeatedly in the synthetic medium before they entered the stationary phase of their growth cycle. Figure 3 shows the growth obtained in the first and second transfers after germination and growth. Growth in the first transfer was only slightly more rapid than that immediately following germination. However, if these cells were transferred to fresh synthetic media before they entered their stationary growth phase, almost no lag period was observed and the maximum population was obtained in 4-5 hr. However, as the maximum population was reached, the cells immediately exhibited lysis. It appeared that the rate of lysis was directly related to the rate of growth.

Figure 3. Adaptation to the synthetic medium.

After germination and growth the cells were transferred to fresh synthetic medium. At the beginning of the stationary phase of this first transfer, the cells were transferred again to fresh synthetic medium. Growth in this transfer is listed on the graph as second transfer. The amount of growth in each transfer was determined by measuring the optical density at 500 mu.


1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1 2 3 4 5 6 7 8 9 10 11 12
TIME (hr)
FIRST TRANSFER
SECOND TRANSFER

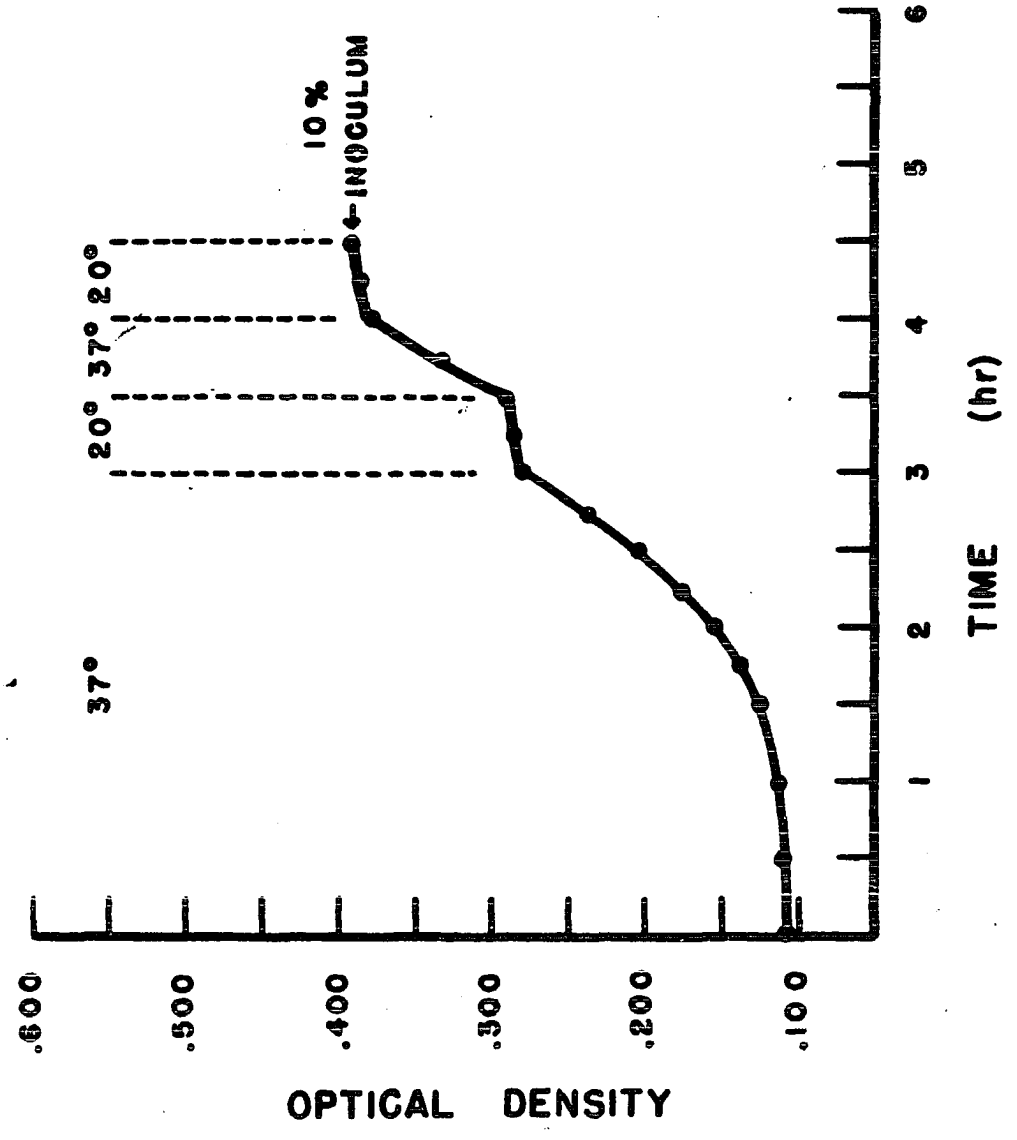
That is, a culture which required 10-12 hr to reach the stationary phase lysed at a much slower rate than one that required only 4-5 hr. However, continued incubation of all liquid cultures resulted in almost cell-free suspensions.

To obtain a culture of rapidly dividing vegetative cells that would reach the sporulation stage synchronously, cells in their logarithmic growth phase in the second transfer were transferred to fresh synthetic media and incubated at 37 C. Figure 4 shows the growth of these cells. As the culture approached the middle of its logarithmic growth phase it was subjected to temperature shifts to induce synchronous cell division. After the final 30 min incubation period at 20 C, an aliquot of the culture was transferred to the sporulation test medium at 37 C. In theory each cell transferred would exhibit an immediate cell division in the test medium and each cell would be in the same stage of development throughout the growth phase and initiate the sporulation processes synchronously.

The first synthetic medium tested for sporulation is shown in Table I. However, sporulation did not occur in this medium. When the cells entered the stationary growth phase, immediate and rapid lysis was exhibited rather than sporulation. The morphological development of the cells in the

Figure 4. Production of a culture of synchronously dividing cells.

Cells from the second transfer which were in their logarithmic growth phase were inoculated into fresh synthetic medium at 37 C. As the cells approached the middle of their logarithmic growth phase, temperature shifts were initiated to induce synchronous cell division. After the final cooling period, 10 ml of this culture was used to inoculate the sporulation test medium.


10%
INOCULUM
20° 37° 20°
37°
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1
2
3
4
5
6
TIME (hr)

synthetic medium appeared identical to that in the Tris Test Medium preceding the initiation of sporulation. In the Tris Test Medium the cells exist in chains of large deeply staining vegetative cells during the logarithmic growth phase. The chains break up into individual cells as the culture approaches the stationary phase and line up longitudinally in doublets, triplets, and sometimes tetrads. At this point the non-staining refractive spores begin to appear. In the synthetic medium this last step is replaced by roughening of the cell wall, irregular staining of the cytoplasm, and lysis. Continued incubation resulted in an almost cell-free suspension consisting of cell fragments. Two logical explanations for the failure of the synthetic medium to support sporulation were that either the medium was inhibitory or some required substrate was not present. Since the addition of small quantities of Trypticase to the synthetic medium always resulted in sporulation, it was assumed that it actually supplied some molecule required for sporulation. A similar conclusion was made by Lund (1957) in his work on the sporulation of the putrefactive anaerobe PA 3679.

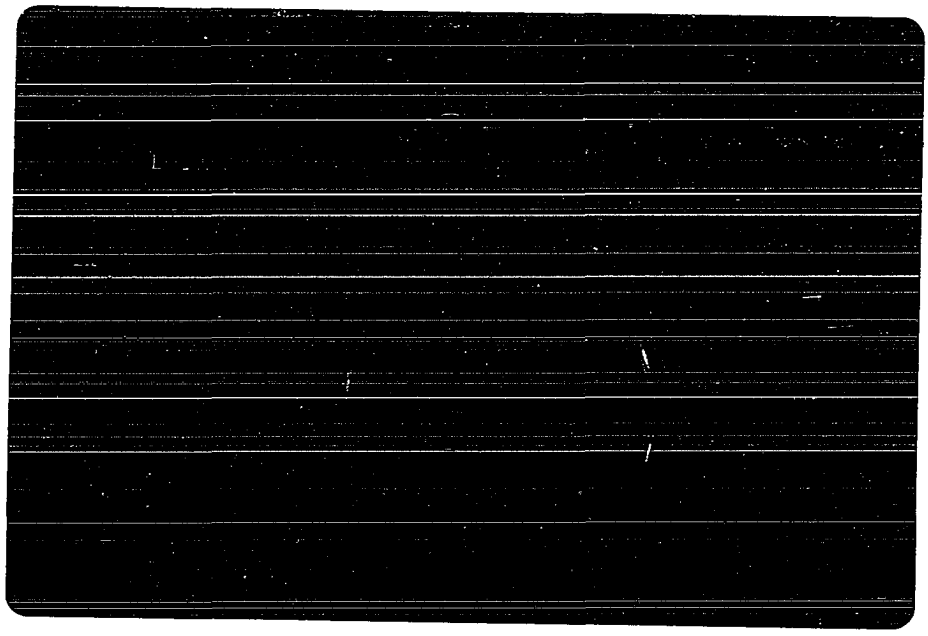
Testing other compounds for sporogenic activity: To duplicate the sporogenic activity of Trypticase numerous organic compounds of biological origin were tested for

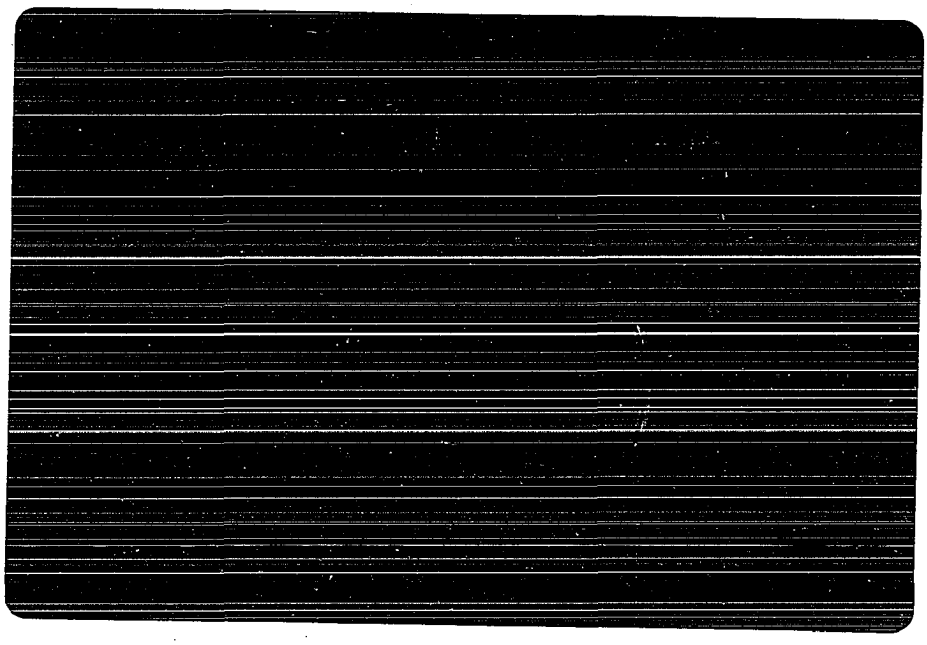
sporogenic activity. The list of compounds tested includes all of the known and commercially available vitamins, amino acids, purine and pyrimidine bases, Embden-Meyerhoff and Kreb cycle intermediates, carbohydrates, organic acids, and fatty acids. All of the synthetic peptides available from Nutritional Biochemicals Corp. were tested. All compounds were tested in the synthetic medium using the active culture technique and all were found to be non-sporogenic.

Sporogenic activity of casein: Since screening of available organic compounds did not yield the required sporogen, it was necessary to isolate and characterize the component of Trypticase which initiated and supported sporulation. To insure that the source of this component was the parent molecule, casein, and not some extraneous compound added during pancreatic digestion, the sporogenic activity of casein was determined. The examination of stained smears showed that the purified casein will initiate and support the sporulation of C. roseum. The proteolytic activity of the cells is shown on Plate 1. The clear zones show the areas in which protein was hydrolyzed whereas the opaque zones are due to undigested protein which was precipitated by picric acid. This plate shows the extracellular protease of C. roseum can diffuse and exert its activity well away from the cells.

Plate 1. Proteolytic activity of C. roseum.

C. roseum was allowed to grow and sporulate on agar plates consisting of casein and the synthetic medium. After incubation the plates were flooded with picric acid. The clear zones show the areas in which casein was hydrolyzed whereas the opaque zones are due to precipitated unhydrolyzed protein.





This proteolytic activity appears to be characteristic of the cells in the late logarithmic growth phase (pre-sporulation) since actively growing vegetative cells inoculated into the synthetic medium containing casein as the only amino acid source failed to exhibit proteolytic activity or growth. However, in the presence of free amino acids, growth was normal and the casein was solubilized during the late log phase. A similar finding was made by Bernlohr (1964) in Bacillus licheniformis in which he detected an active post-logarithmic extracellular protease which was not detectable in young vegetative cells. Extracellular proteases are common in the proteolytic clostridia (Mandl, Ferguson, and Zaffuto, 1957) and also appear to be produced by the predominantly saccharolytic anaerobe, C. roseum, as shown in this investigation.

Fractionation of Trypticase: Since the cells sporulated using the parent molecule, casein, the fractionation of Trypticase was initiated. Ashed Trypticase failed to initiate sporulation and showed that the required sporogen was organic. The sporogens were insoluble in all organic solvents tested and were not denatured by the extraction procedures. Dialysis against deionized water resulted in activity in both the dialysate and residue. The possibility existed that the sporogen was of rather large molecular weight

or that at least two sporogens were present; one which was small and dializable and another whose molecular weight prevented passage through the dialysis membrane.

The sporogenic activity was found to be completely destroyed by acid and basic hydrolysis. Commercial preparations of acid hydrolyzed casein also failed to support sporulation. The phosphopeptides isolated by the procedures of Levine and Hill (1933) also failed to initiate and support sporulation.

Column fractionation of Trypticase: Since Trypticase is composed of a mixture of amino acids and peptides of different molecular weights and soluble only in water, it was decided to try fractionation by means of filtration through cross-linked dextran gels. Filtration through these gels was introduced by Porath and Flodin (1957) and applications of this procedure to various biochemical problems have been reported (Bjorck and Porath, 1959; Gelotte and Krantz, 1959; Kisliuk, 1960; Bennich, 1961; and Phillips and Gibbs, 1961). These investigations indicate that separation of molecules in water solutions is due to differences in molecular weight and structure.

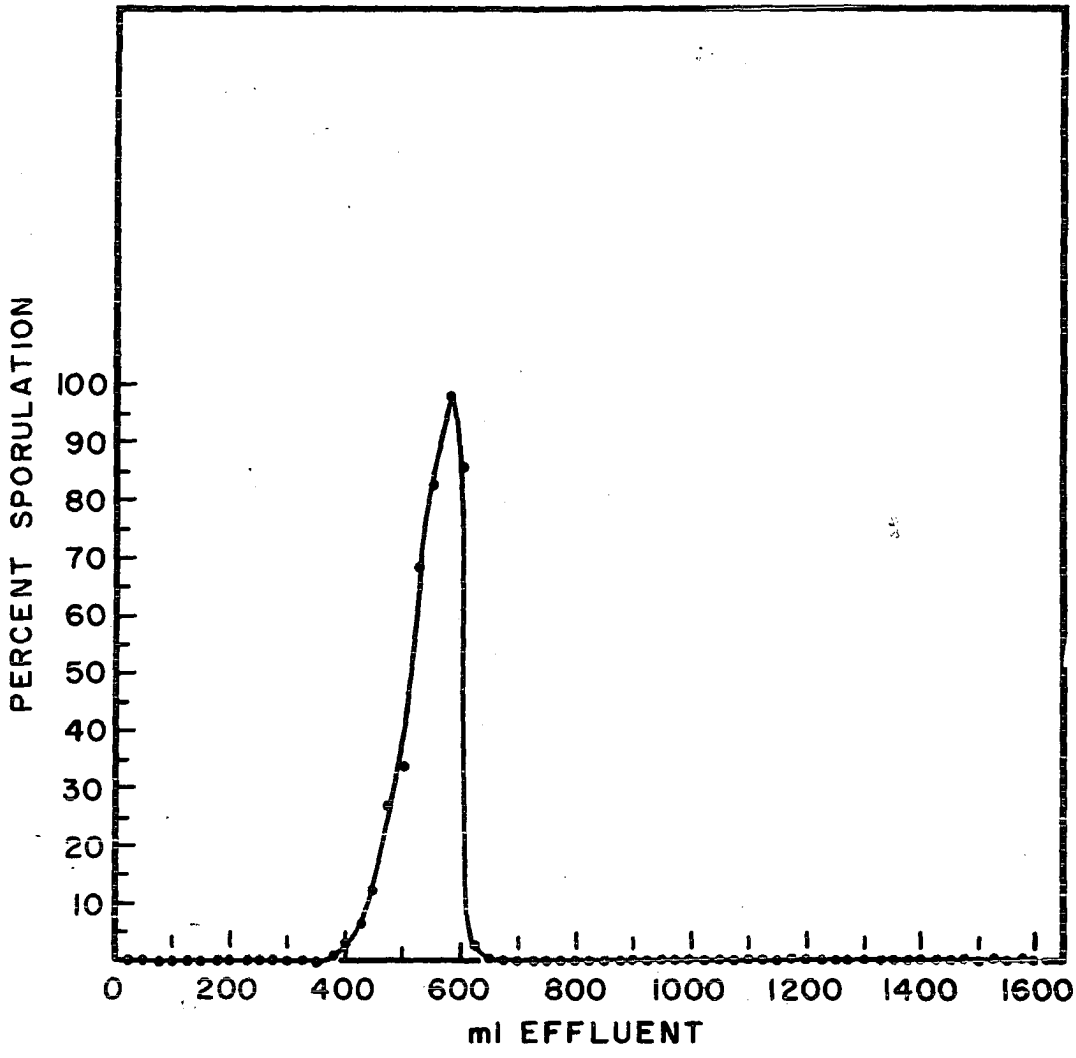
Sephadex G-25 was chosen for this separation since its molecular exclusion limit (5000) allows the passage of

undigested protein and large polypeptides and retain and separate amino acids and small peptides. Figure 5 shows the sporogenic activity of fractions collected after passage through a 4.5cm X 45cm column. This graph shows that the activity is discharged between 350 and 700 ml effluent and that the maximum activity is eluted between 550 and 600 ml. Figure 6 shows the ninhydrin reactivity of the effluent between 300 and 700 ml. It can be seen that the maximum ninhydrin reactivity also occurs between 575 and 600 ml.

Since the sporogenic activity was spread over a rather large effluent range, it was desirable to try further column separation of the sporogenic fractions to increase the resolution. Figure 7 shows the sporogenic activity of fractions after passage through a 1.5cm X 100 cm column of Sephadex G-25. The activity began to elute at 100-110 ml of effluent and increased slowly with the maximum activity being eluted between 150 and 180 ml. The leading edge of the activity from both columns appeared ill-defined and spread out whereas the trailing edge appeared sharp and well-defined since the activity dropped sharply after the maximum activity was eluted. Figure 8 shows the ninhydrin reactivity of the column effluent. The maximum reactivity again was eluted at the same point as the maximum sporogenic activity. The small

Figure 5. Sporogenic activity of Trypticase fractions after Sephadex filtration.

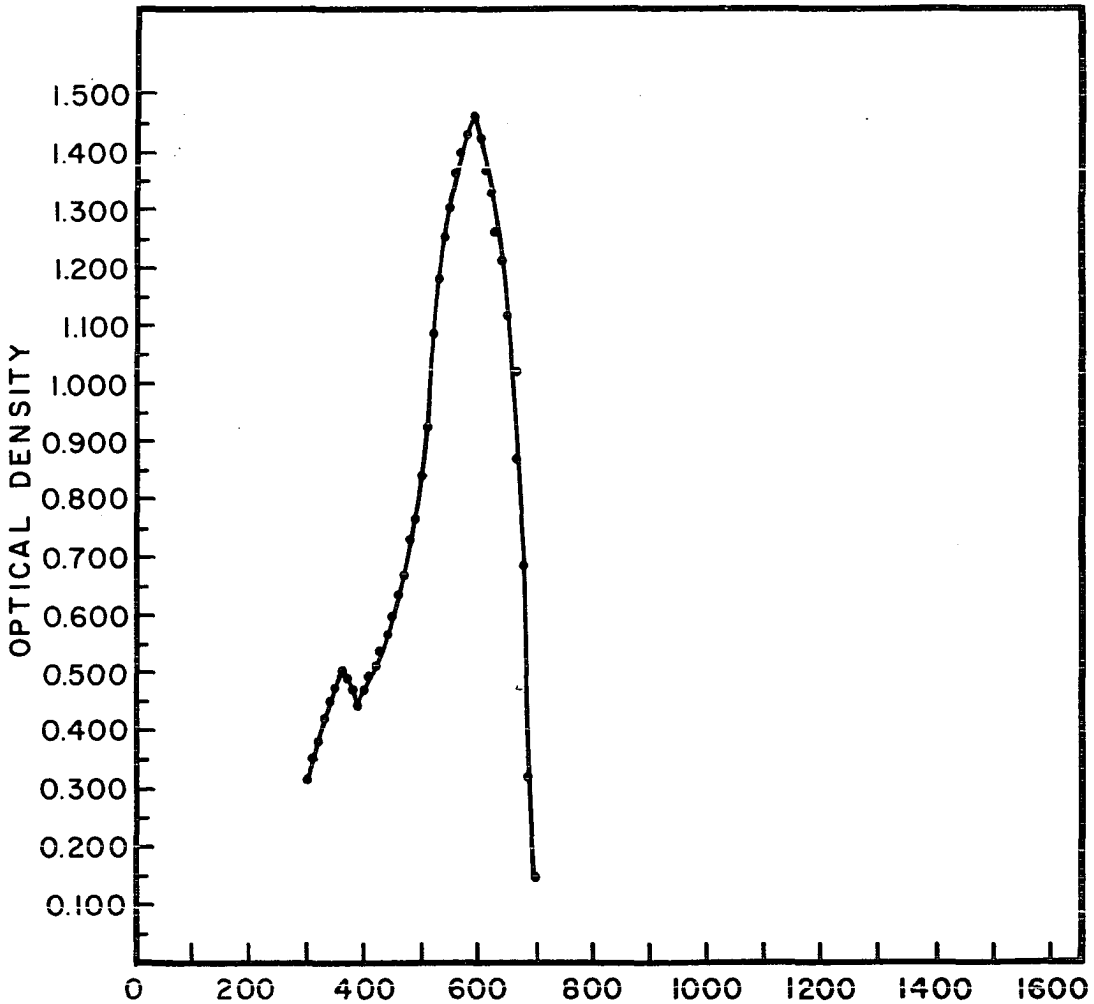
Trypticase was fractionated on a 4.5cm X 45cm column of Sephadex G-25. Twenty-five ml fractions were collected, treated as described in the text, and tested for sporogenic activity. The per cent sporulation was determined by examination of crystal violet stained smears.



SPOROGENIC ACTIVITY OF TRYPTICASE FRACTIONS AFTER SEPHADEX FILTRATION ($4\frac{1}{2}$ x 45 cm. COLUMN)

Figure 6. Ninhydrin reactivity of sporogenic fractions.

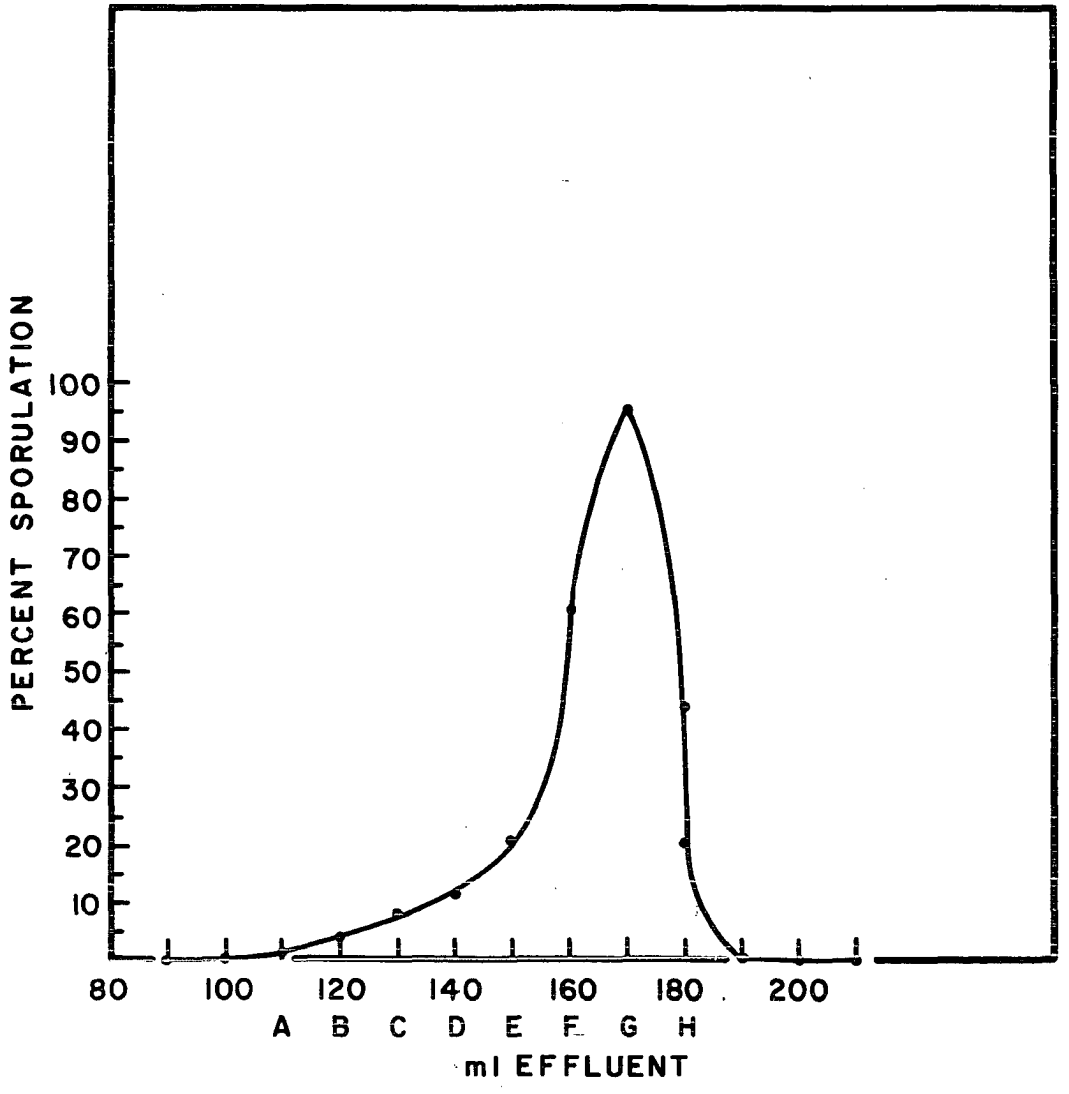
An equal aliquot of each sporogenic fraction obtained by Sephadex G-25 gel filtration (4.5cm X 45cm column) was tested for ninhydrin reactivity using the procedure of Moore and Stein (1954). The optical density was measured at 570 mu using a Gilford Recording Spectrophotometer.



NINHYDRIN REACTIVITY OF SPOROGENIC FRACTION AFTER SEPHADEX FILTRATION ($4\frac{1}{2}$ x 45 cm. COLUMN)

Figure 7. Sporogenic activity of Trypticase fractions after Sephadex filtration.

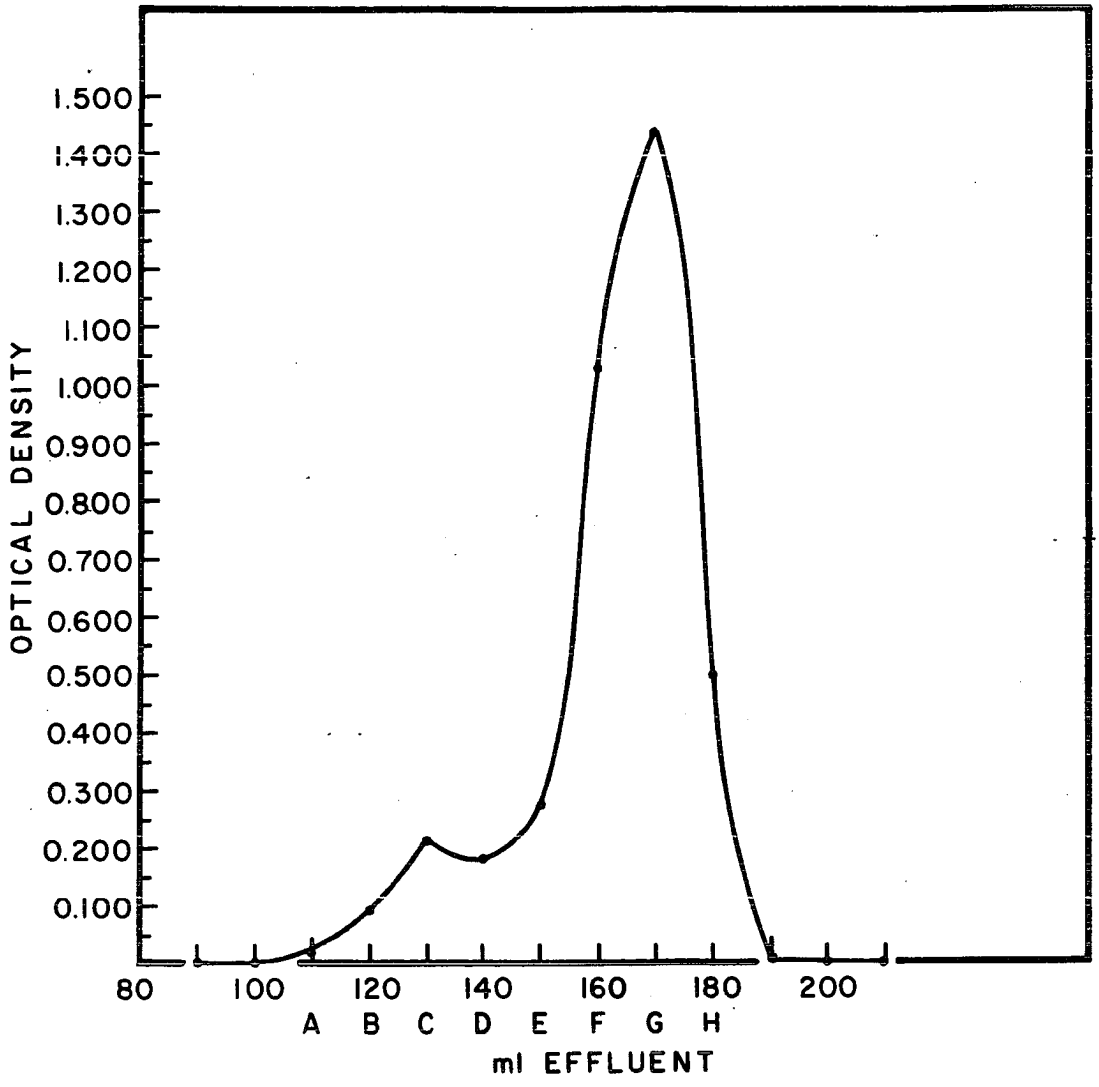
The pooled sporogenic fractions from the 4.5cm X 45cm column were subjected to further filtration on Sephadex G-25 using a 1.5cm X 100cm column. Ten ml fractions were collected, treated as described in the text, and tested for sporogenic activity. The per cent sporulation was determined by examination of crystal violet stained smears.



SPOROGENIC ACTIVITY OF TRYPTICASE FRACTION AFTER SEPHADEX FILTRATION (1.5 x 100 cm. COLUMN)

Figure 8. Ninhydrin reactivity of sporogenic fractions.

An equal aliquot of each sporogenic fraction obtained by Sephadex G-25 gel filtration (1.5cm X 100cm column) was tested for ninhydrin reactivity using the procedure of Moore and Stein (1954). The optical density was measured at 570 mu using a Gilford Recording Spectrophotometer.



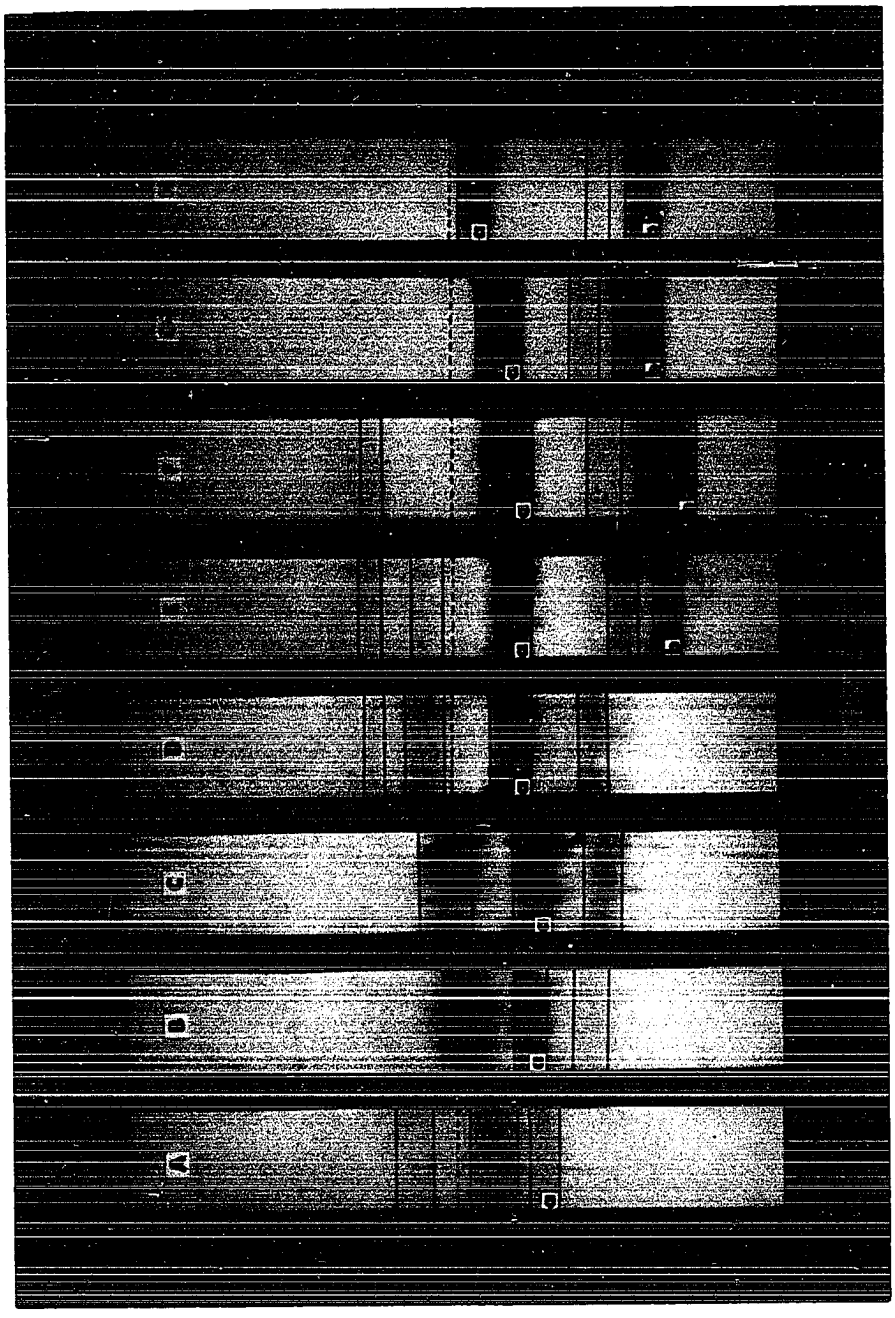
NINHYDRIN REACTIVITY OF SPOROGENIC FRACTION AFTER SEPHADEX FILTRATION (1.5 x 100 cm. COLUMN)

peak which occurred at 120-130 ml could have been the result of the elution of some non-sporogenic component since a similar peak was not recorded for sporogenic activity. Plate 2 shows the electrophoretically separated components of each of the sporogenic fractions from this column. Each sporogenic fraction on Figure 7 and 8 is labeled correspondingly. For example electrophoretic strip A shows the separated components of the fraction from 100-110 ml. These strips show that none of the fractions was composed of pure peptide entities. Only one peptide, labeled "a", was present in all of the fractions. Since all fractions exhibited sporogenic activity, it was assumed that peptide "a" was a sporogen. This peptide increased in concentration from fraction A to G and then decreased. This corresponds to the increase in sporogenic activity and ninhydrin reactivity shown on Figures 7 and 8. Another peptide, labeled "b", first appears in fraction E, increased in concentration in fractions F and G, and decreased in H. This peptide also proved to be sporogenic. Therefore, the distribution of the two peptides explains the slow increase in sporogenic activity and abrupt decrease shown on Figure 7.

Since the resolving power of the second Sephadex G-25 column was insufficient to yield separate peptide entities,

Plate 2. Electrophoretically separated components of each sporogenic fraction from the Sephadex G-25 (1.5cm X 100cm) column.

The strips were developed with 0.5% ninhydrin in acetone.



it was decided to try Sephadex G-10. This dextran gel has a molecular exclusion limit of 700 and promised to be capable of greater resolution of small molecular weight compounds. A sample of the sporogenic fractions from the second Sephadex G-25 column was developed on this column. Figure 9 shows the distribution of the activity after passage through this column and Figure 10 shows the ninhydrin reactivity. The maximum sporogenic activity and ninhydrin reactivity was eluted between 140 and 160 ml. The material passed through this column in a well-defined band since the leading and trailing edge of the effluent was symmetrical and well-defined, but electrophoresis showed that the overall resolution was not significantly greater than that obtained on the second Sephadex G-25 column.

Preparative electrophoresis: Since the columns did not yield pure peptide entities, it was necessary to resort to preparative electrophoresis to obtain sufficient quantities of each pure peptide for testing. Only the two peptides shown on Plate 2 as "a" and "b" exhibited any sporogenic activity. Both peptides in aqueous solution exhibited a bright yellow color, strong blue-white florescence at 3660 A, and formed intensely yellow, hydroscopic, amorphous solids upon drying. The purity of each sporogen was confirmed by

Figure 9. Sporogenic activity of Trypticase fractions after Sephadex filtration.

The pooled sporogenic fractions from the 1.5cm X 100cm column were subjected to further filtration on Sephadex G-10 using a 1.5cm X 85cm column. Ten ml fractions were collected, treated as described in the text, and tested for sporogenic activity. The per cent sporulation was determined by examination of crystal violet stained smears.

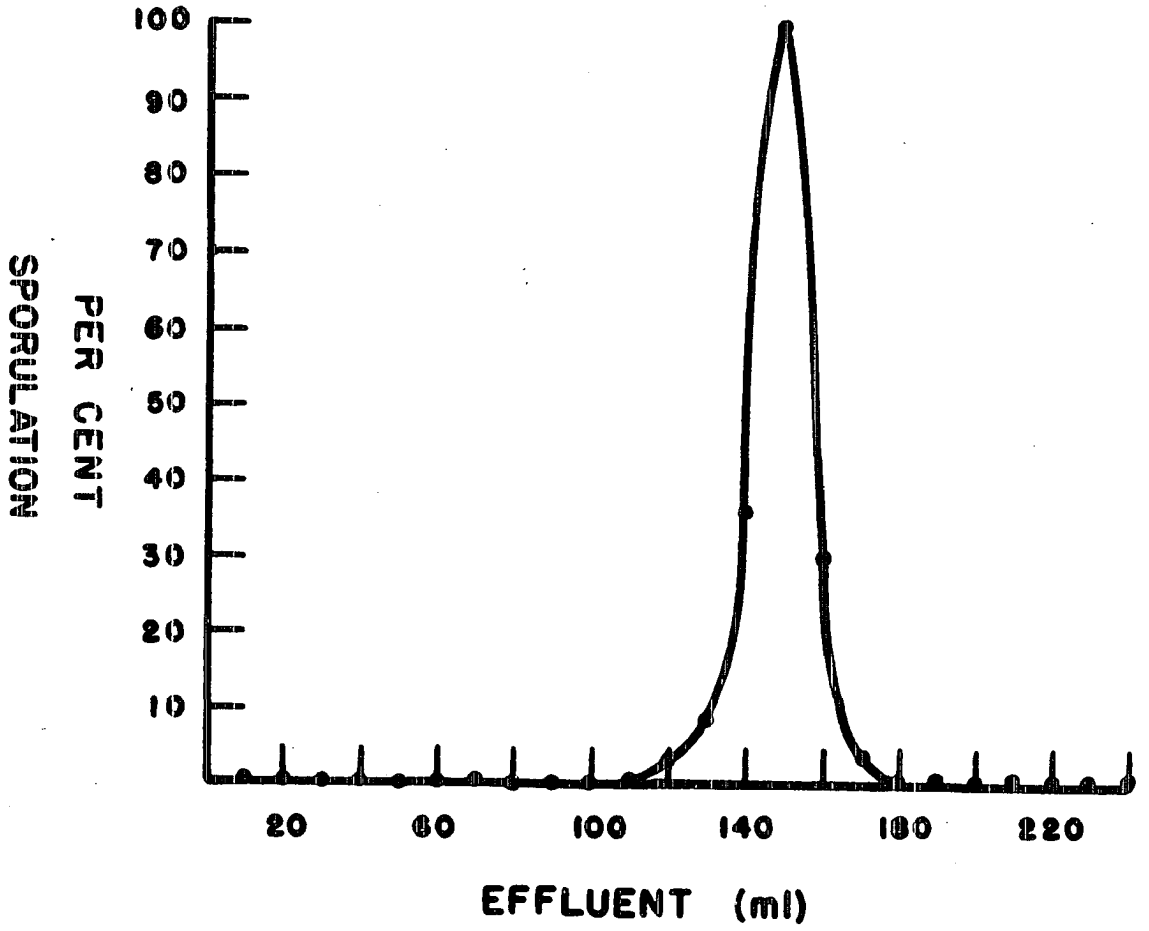
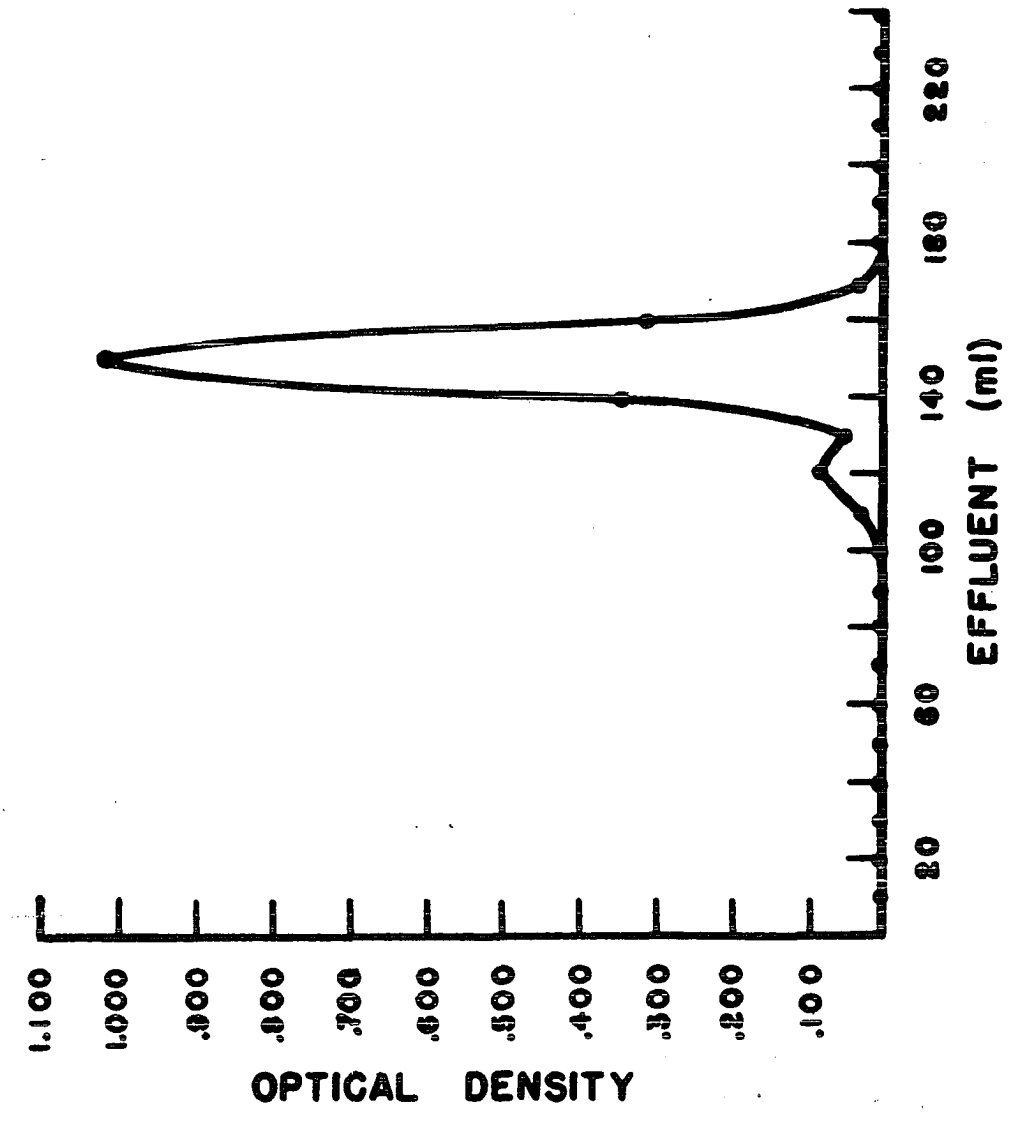

PER CENT
SPORULATION
100
90
80
70
60
50
40
30
20
10
20
60
100
140
180
220
EFFLUENT (ml)

Figure 10. Ninhydrin reactivity of sporogenic fractions.

An equal aliquot of each sporogenic fraction obtained by Sephadex G-10 gel filtration (1.5cm X 85cm column) was tested for ninhydrin reactivity using the procedure of Moore and Stein (1954). The optical density was measured at 570 mu using a Gilford Recording Spectrophotometer.


1.100
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
20
60
100
140
180
220
EFFLUENT (ml)

electrophoresis in different buffer systems and by two dimensional paper chromatography.

Chemical and physical methods: Each of the separated sporogens was subjected to various chemical and physical tests for characterization. The sodium fusion method of elemental analyses revealed only nitrogen. Preliminary isolates gave positive tests for carbohydrates, but this was later shown to be due to the presence of small fibers of filter paper from the electrophoretic strips. Filtration through the Millipore filters (Type HA 0.45u) removed these fibers and ended the positive carbohydrate tests. Although casein is known to contain hexoses, hexosamines, sialic acids, and other carbohydrate material (Johansson and Svennerholm, 1956), neither sporogen contained any detectable carbohydrate-type material.

Figure 11 shows the ultraviolet absorption spectrum of the two sporogens. Alpha and beta on this graph are used to designate peptides "a" and "b" respectively shown on Plate 2. The alpha sporogen showed the only significant absorption and this occurred at about 275 mu. Figure 12 shows the ultraviolet absorption spectrum of the two sporogens after acid hydrolysis. Again the only significant absorption was exhibited by the alpha sporogen at 270 mu. It appears that

Figure 11. Ultraviolet absorption spectrum of sporogens.

The ultraviolet absorption spectrum of each sporogen in 0.1M phosphate buffer at pH 7.0 was determined using a Gilford Recording Spectrophotometer. Alpha is used to designate the sporogen labeled "a" on Plate 2. Beta is used to designate the sporogen labeled "b" on Plate 2.

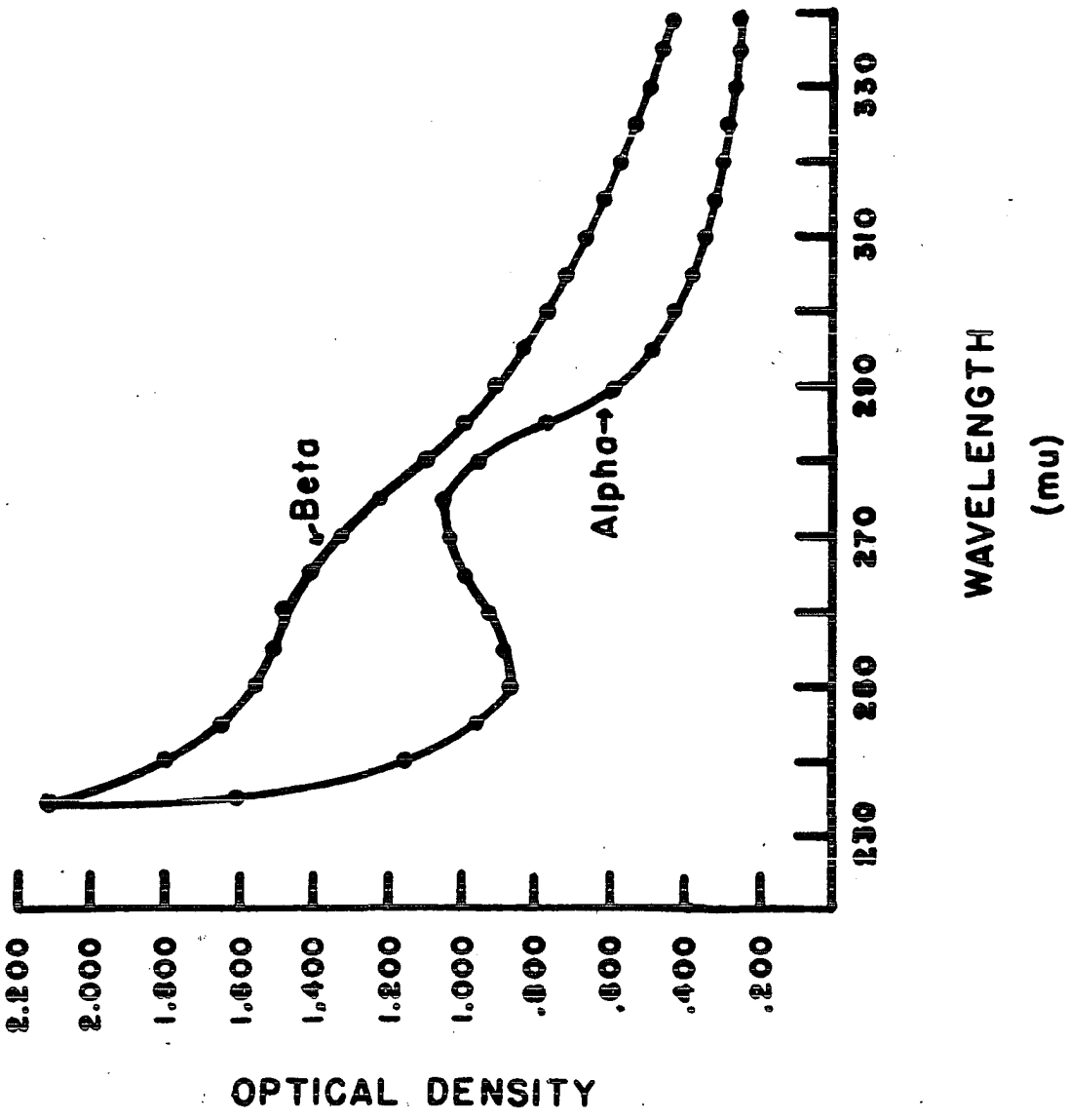
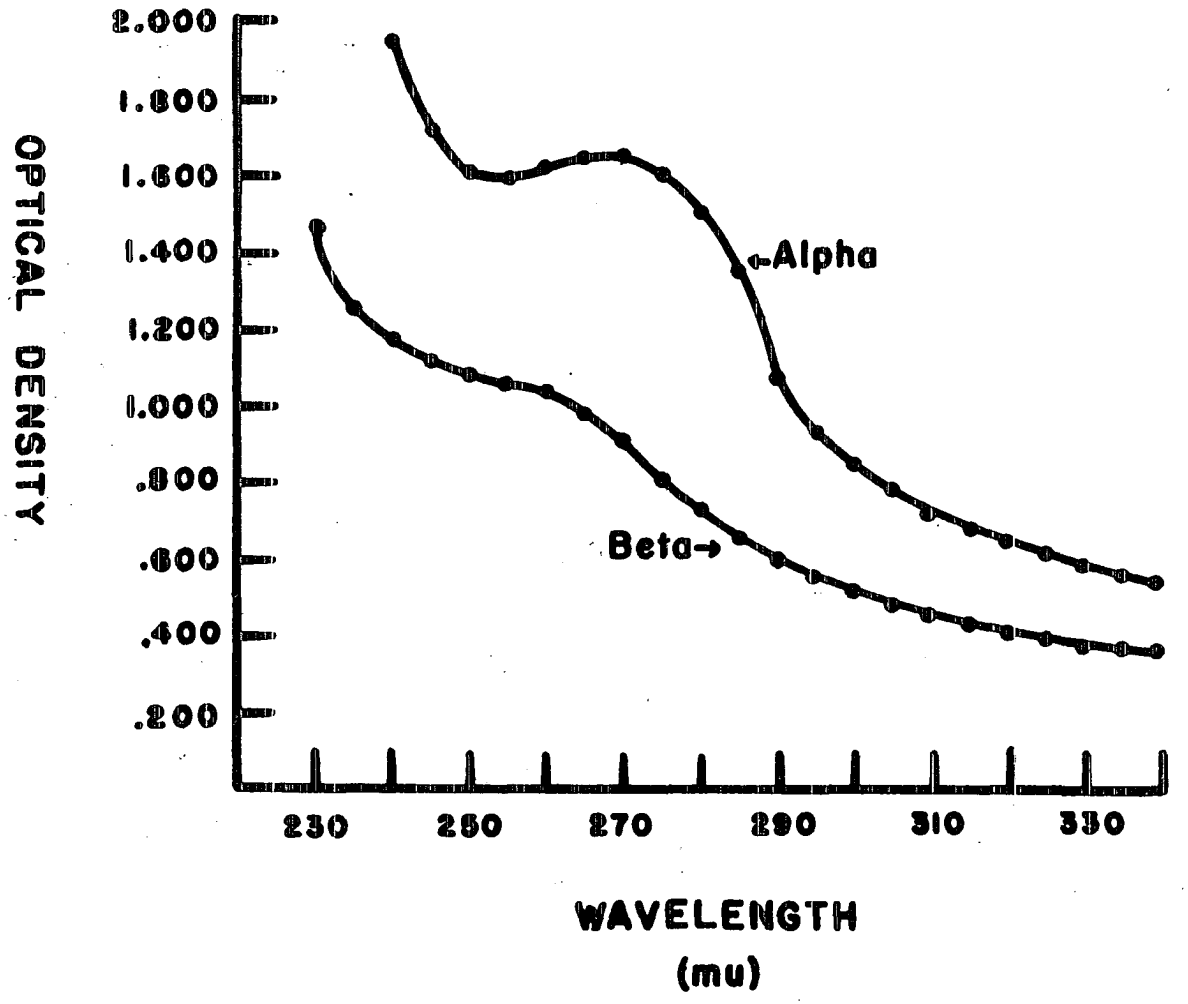

Beta
Alpha
2.200
2.000
1.800
1.600
1.400
1.200
1.000
.800
.600
.400
.200
OPTICAL DENSITY
230
250
270
290
310
330
WAVELENGTH
(mu)

Figure 12. Ultraviolet absorption spectrum of acid hydrolyzed sporogens.

The ultraviolet absorption spectrum of each sporogen was determined after hydrolysis with 6N hydrochloric acid. Measurements were made using a Gilford Recording Spectrophotometer with each hydrolysate suspended in 0.1M phosphate buffer at pH 7.0. Alpha is used to designate the sporogen labeled "a" on Plate 2. Beta is used to designate the sporogen labeled "b" on Plate 2.


OPTICAL DENSITY
2.000
1.800
1.600
1.400
1.200
1.000
.800
.600
.400
.200
←Alpha
Beta→
230
250
270
290
310
330
WAVELENGTH
(mu)

acid hydrolysis did not significantly alter the absorption spectrum of either sporogen.

Paper chromatography of the acid hydrolysate of the sporogens revealed the presence of eight amino acid residues in the alpha sporogen and five in the beta sporogen. Simultaneous chromatographic and co-chromatographic analyses of the hydrolysates showed that both sporogens were composed of alanine, aspartic acid, glutamic acid, lysine, and serine. In addition the alpha sporogen contained glycine, tyrosine, and proline. These three additional residues increase the molecular weight of alpha and explain why this sporogen began its elution from the Sephadex columns before beta. The aromatic residue of tyrosine explains the relatively large elution volume required to completely elute alpha since it is known that aromatic residues cause retardation in the column (Phillips and Gibbs, 1961). Therefore the larger molecular weight of alpha and the retardation effect of tyrosine result in the poor separation of the two sporogens in the column. The tyrosine residue also explains the ultraviolet absorption of the alpha sporogen.

Sulfur amino acids, carbohydrates, amino sugars, and organo-phosphate esters were not detected in the acid hydrolysates of either sporogen. However, the hydrolysate

of each sporogen contained a compound which after chromatography was located between glutamic acid and serine. The ninhydrin stain was either completely rejected by this compound or stained it a light cream color. All efforts to identify this spot failed. Acid hydrolyzed pantothenic acid yielded a similar spot after chromatography and staining. It has been reported that pantothenic acid occurs in nature bound to protein (Williams, 1943; Wright, 1943; King, Fels, and Cheldelin, 1949; Neilands, Higgins, King, Handschumacher, and Strong, 1950) and it always exists as pantoyl-beta-alanine. Exhaustive paper chromatography in different solvent systems has shown that the alanine in both sporogens is alpha rather than beta. Therefore, if the unidentified compound is the pantoyl moiety of pantothenic acid, it exists in this case as pantoyl-alpha-alanine rather than pantoyl-beta-alanine. However, it should be pointed out that this spot may be only an artifact which is a product of the acid hydrolysis procedure and may not represent a constituent of the sporogens.

Phosphopeptide identification and isolation: Although both hydrolyzed sporogens consistently yielded negative tests for organo-phosphate esters, the omnipresence of serine in both was noted with interest since it has been reported that serine is the main site of phosphate attachment to the casein

molecule (Rimington and Kay, 1926; Levine and Hill, 1933; Damodaran and Ramachandran, 1941; Lowndes, Macara, and Plimmer, 1941; Rimington, 1941; McMeekin and Polis, 1949; Bennich, Johansson, and Osterberg, 1959; and Osterberg, 1959) and that this ester linkage is susceptible to acid hydrolysis (Rimington and Kay, 1926). Therefore it was possible that the serine in both sporogens actually existed in the parent casein molecule and subsequently in Trypticase as phosphoserine. The molybdate reagent revealed that both sporogens did contain an organo-phosphate ester. This finding stimulated the modification of the procedure originally used for the isolation of phosphopeptides. The white, powdery phosphopeptides of Trypticase were isolated using this modified procedure and again failed to exhibit any sporogenic activity. It was at this point that it was noted that the white, powdery phosphopeptide fraction was totally different from the yellow amorphous sporogens. However, it was noted that the ethanolic supernatant was cloudy and retained the original yellow-brown color of Trypticase. A sample of this supernatant was concentrated under vacuum and the resulting yellow aqueous solution was found to initiate and support sporulation. Electrophoretic analysis showed that both alpha and beta sporogens were present as well as other phosphorous containing peptides and/or

amino acids. Since the sporogens were not cleanly precipitated by ethanol and acetone, column passage was necessary for removal of residual lead, barium, and other elements used in the isolation procedure. Although this procedure confirmed the finding that the sporogens were phosphopeptides, it did not offer any outstanding advantages over the original Sephadex-electrophoresis procedure for their isolation.

Alterations in the synthetic medium: At this point in the investigation, it was desirable to determine the minimum nutritional requirements of C. roseum to learn more about the specific requirements for growth and sporulation.

Vitamins: Table II shows that only three vitamins were required for growth. Growth was completely arrested by the absence of pantothenic acid and pyridoxal and was depressed in the absence of niacin. Apparently, the cells are capable of niacin synthesis, but the rate of synthesis inhibited the growth rate of the cells. Studies on the growth of clostridia in synthetic media have shown a wide range of growth factor requirements (Mager, Kindler, and Grossowicz, 1954; Kindler and Mager, 1956; Fuchs and Bonde, 1957; Perkins and Tsuji, 1962). These studies have shown that most clostridia require biotin and PABA and that other growth factors which are stimulatory or essential include thiamin, pyridoxine,

pantothenic acid, riboflavin, niacin, and folic acid.

TABLE II

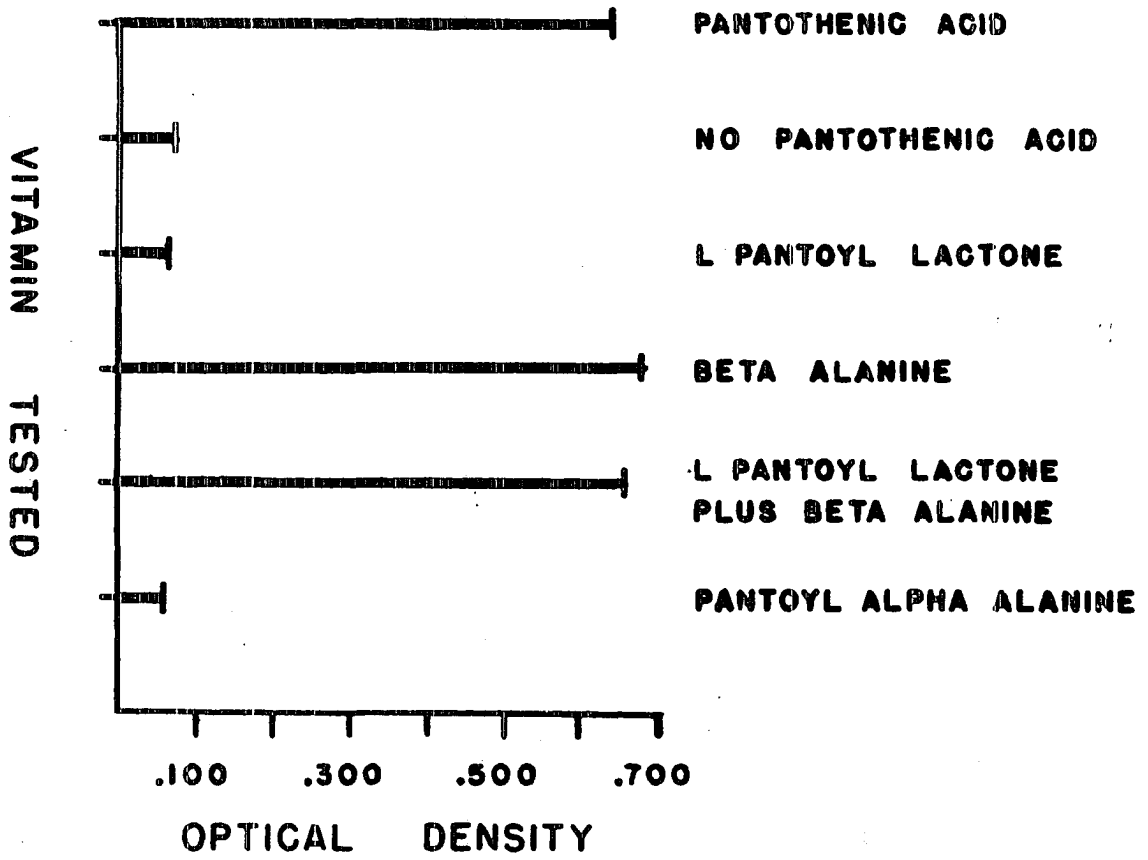
Vitamins required for growth

	Vitamin	Optical Density
1.	Control (all vitamins)	0.632
2.	Control (all vitamins)	0.595
3.	Biotin	0.590
4.	Choline	0.610
5.	Cyanocobalamine	0.623
6.	Folic acid	0.587
7.	Niacin	0.110
8.	Pantothenic acid	0.032
9.	Pyridoxal	0.041
10.	Riboflavin	0.618
11.	Thiamine	0.592
12.	PABA	0.605
13.	Inositol	0.620

Structural requirements of vitamins: Since pantothenic acid, pyridoxal, and niacin exist in several molecular forms, it was desirable to determine which form of each vitamin was most active for germination and growth of C. roseum. Figure 13 shows the relative ability of several molecular forms of pantothenic acid to satisfy the pantothenic acid requirement. These results show that only the beta-alanine moiety of pantothenic acid is required to satisfy the requirement. Evidently the cells are capable of synthesizing the pantoyl moiety, but cannot synthesize beta-alanine. Apparently

Figure 13. The ability of various molecular forms of pantothenic acid to satisfy the pantothenic acid requirement for growth.

Each vitamin form tested was placed in the synthetic medium in place of pantothenic acid. Pantothenic acid was used as the control. The amount of growth was determined by measuring the optical density at 500 mu after 24 hr of incubation at 37 C.

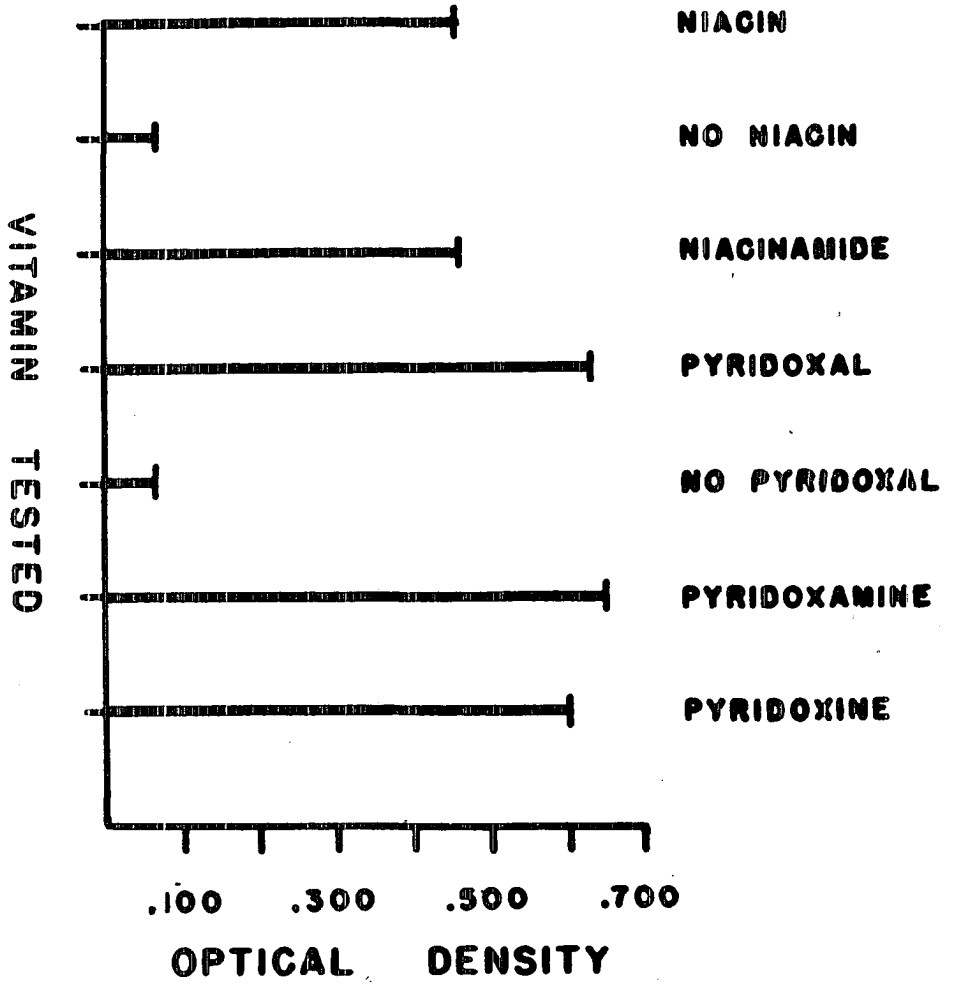

PANTOTHENIC ACID
NO PANTOTHENIC ACID
L PANTOYL LACTONE
BETA ALANINE
L PANTOYL LACTONE
PLUS BETA ALANINE
PANTOYL ALPHA ALANINE
VITAMIN TESTED
.100
.300
.500
.700
OPTICAL DENSITY

exogenous beta-alanine enters the cells and is condensed with the endogenous pantoyl moiety to supply pantothenic acid. Beta-alanine has been shown to satisfy the pantothenate requirement of some yeasts (Williams and Rohrmann, 1936; Sarett and Cheldelin, 1945) while pantoic acid can replace this requirement in Acetobacter suboxydans (Underkofler, Bantz, and Peterson, 1943; Sarett and Cheldelin, 1955) and Streptococcus hemolyticus H69G (Woolley, 1939).

Figure 14 shows the ability of various molecular forms of niacin and pyridoxal to satisfy the parent vitamin requirement. It appears that niacinamide is as active as the parent niacin for supporting growth. Most niacin-requiring microorganisms can utilize the acid and amide interchangeably; however, some organisms specifically require either the amide or acid (Guirard and Snell, 1962). All three members of the B_6 group (PAL, PAM, and PINE) are approximately equal in their ability to support the growth of C. roseum. These three forms are also equally active for animals and for most yeasts and molds (Snell and Rannefeld, 1945), but most other microorganisms have very specific requirements for members of the B_6 group (Guirard and Snell, 1962). The metabolic basis for these differences is largely unknown. However, all three forms apparently can enter the cells of C. roseum and are

Figure 14. The ability of various molecular forms of niacin and pyridoxal to satisfy the parent vitamin requirement for growth.

Each vitamin form tested was placed in the synthetic medium in place of the parent vitamin. The parent molecules were used as controls. The amount of growth was determined by measuring the optical density at 500 mu after 24 hr of incubation at 37 C.


NIACIN
NO NIACIN
NIACINAMIDE
PYRIDOXAL
NO PYRIDOXAL
PYRIDOXAMINE
PYRIDOXINE
VITAMIN TESTED
.100
.300
.500
.700
OPTICAL DENSITY

phosphorylated to the functional coenzyme form.

Microscopic examination of crystal violet stained smears showed that vitamins were not required for germination or the initial processes of post-germinative development.

Amino acids required for germination and growth: Hitzman, Halvorson, and Ukita (1957) have reported that only alanine, phenylalanine, and arginine are required for the germination of C. roseum. However, our strain of this organism would not germinate using these amino acids and it was desirable to determine the required amino acids for germination and growth. Table III shows the effect of the removal of individual amino acids on germination and growth. Arginine, alanine, cysteine, phenylalanine, proline, and valine are required for maximum germination. However, the deletion of alanine, phenylalanine, or arginine had the most pronounced effect on these processes. The effect of the deletion of specific amino acids did not have as profound an effect on growth as did the deletion of the required vitamins. Apparently C. roseum is capable of synthesizing several of the amino acids, but the rate of synthesis is limiting to growth. The only amino acids which did not have a profound effect on the growth rate were cystine, glutamine, and asparagine.

TABLE III

Amino acid requirement for germination and growth

Amino acid	Optical Density	Morphological appearance
1. Arginine	0.090	very poor germination small fat cells
2. Alanine	0.095	no germination
3. Aspartic acid	0.083	only germinated spores no sign of growth
4. Cysteine	0.135	poor germination cells normal
5. Glycine	0.099	only germinated spores no sign of growth
6. Glutamic acid	0.192	good germination cells small, but fat
7. Histidine	0.141	good germination cells small and thin
8. Isoleucine	0.132	good germination long thin cells
9. Leucine	0.111	good germination cells normal
10. Lysine	0.310	good germination cells normal
11. Methionine	0.105	good germination long chains, no sign of cell division
12. Phenylalanine	0.245	poor germination cells normal

TABLE III--Continued

Amino acid	Optical density	Morphological appearance
13. Proline	0.215	poor germination cells normal
14. Threonine	0.170	good germination long, non-segmented chains
15. Tryptophan	0.120	good germination cells normal
16. Serine	0.210	good germination cells normal
17. Tyrosine	0.110	good germination cells appear normal
18. Valine	0.190	few ungerminated spores long thin cells
19. Cystine	0.670	good germination normal cells
20. Glutamine	0.640	good germination normal cells
21. Asparagine	0.650	good germination normal cells
22. Control (All amino acids)	0.672	good germination normal cells
23. Control (All amino acids)	0.665	good germination normal cells

With the preceding results in mind the synthetic medium was reconstructed and utilized for the remainder of the investigation. Table IV shows this qualitatively minimum synthetic medium. It should be noted that thiamine is included in this medium. It was found that the synthetic medium would not induce sporogenesis in the presence of the sporogens or small quantities of Trypticase when only niacin, pantothenic acid, and pyridoxal were present. Screening of the vitamins showed that the sporulation process specifically requires thiamine. A thiamine deficiency in the growth medium has also been found to depress the sporulation of PA 3679 (Lund, Janssen, and Anderson, 1957).

Glucose requirement for growth: Since C. roseum is reported to be a predominantly saccharolytic species but exhibits proteolytic activity, it was desirable to determine the actual glucose requirements for growth. Figure 15 shows the effect of various concentrations of glucose on growth. It is apparent that glucose is required. All of the small additions (0.0001%-0.01%) had approximately the same stimulatory effect. However, larger additions (0.1%-1%) greatly increased both the rate and the total amount of growth. Since high concentrations of glucose are an almost universal sporulation inhibitor, a concentration of 2 g/liter was

TABLE IV

Qualitatively minimum synthetic medium

A. Amino acids	g/liter
1. Alanine	0.525
2. Arginine	1.500
3. Aspartic acid	0.765
4. Cysteine	0.090
5. Glycine	0.270
6. Glutamic acid	0.755
7. Histidine	0.360
8. Isoleucine	0.200
9. Leucine	0.630
10. Lysine	0.795
11. Methionine	0.360
12. Phenylalanine	0.400
13. Proline	0.415
14. Serine	0.780
15. Threonine	0.525
16. Tryptophan	0.135
17. Tyrosine	0.345
18. Valine	1.080

B. Vitamins	mg/liter
1. Niacin	2.120
2. Pantothenic acid	0.533
3. Pyridoxal	0.150
4. Thiamine	0.500

C. Salts

Dissolve in 500 ml water
use 10 ml/liter

1. NaCl	3.75 g
2. $CaNO_3$	2.60 g
3. $FeSO_4 \cdot 7H_2O$	0.10 g
4. $MnSO_4 \cdot 4H_2O$	0.50 g
5. $MgSO_4$ anhyd	2.00 g

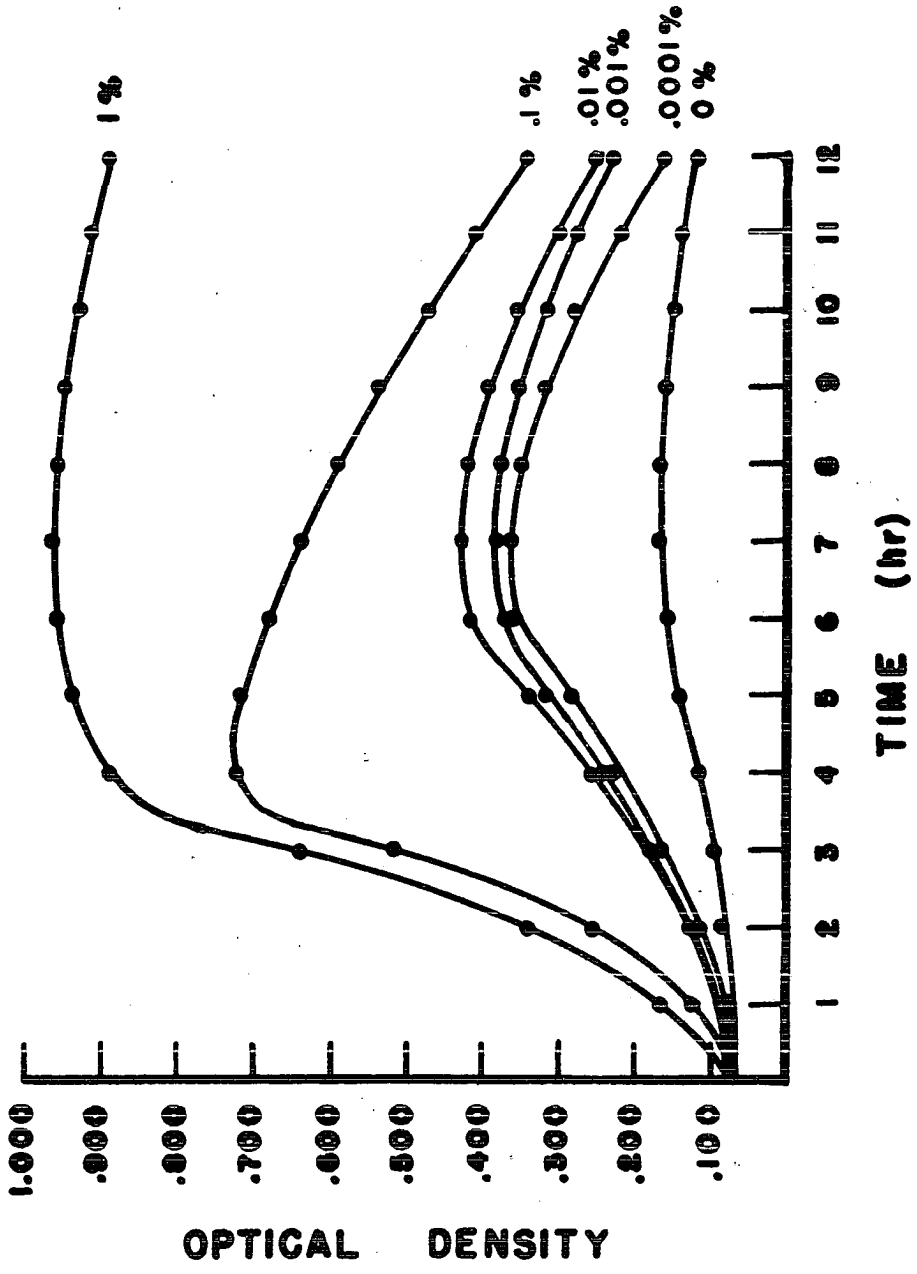
D. Additional	g/liter
1. Glucose	2.00
2. K_2HPO_4	7.00
3. Tris buffer	1.50

chosen for the synthetic medium since this concentration supports good vegetative cell growth and does not inhibit sporulation in the Tris Test Medium.

Alteration of buffer capacity: Since glucose is believed to exert its inhibitory effect on sporulation of other clostridia by lowering the pH (Gibbs and Hirsch, 1956;

Figure 15. The glucose requirement for growth.

Cells at the end of their logarithmic growth phase were inoculated into anaerobic culture flasks which contained the synthetic medium with a glucose concentration of 0% to 1%. Aliquots were removed at hourly intervals and the amount of growth determined by measuring the optical density at 500 mu.


1%
.1%
.01%
.001%
.0001%
0%
1 2 3 4 5 6 7 8 9 10 11 12
TIME (hr)
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY

Ohye and Scott, 1957), the buffer capacity of the synthetic medium was lowered to measure the pH change and effect on sporulation. Figure 16 shows the growth, pH change, and sporulation in the Tris Test Medium. In this medium the pH drops to 6.7-6.8 and sporulation occurs as the cells approach the end of the logarithmic growth phase. Figure 17 shows the growth, pH change, and sporulation in the synthetic medium with lowered buffer capacity. It is apparent that sporulation did not occur and that the culture exhibited a definite pH break. A similar break was observed in Bacillus cereus (Nakata and Halvorson, 1960). During vegetative growth glucose was converted to organic acids and led to a drop in pH. During the initial processes of sporulation the pH rose due to the utilization of the acids. In the case of C. roseum even though sporulation did not occur in the synthetic medium, the pH rose indicating that the acids produced during vegetative growth were utilized. This figure shows again that the cells experience lysis rather than sporogenesis at the end of the logarithmic growth phase. Figure 18 shows the effect of addition of Trypticase to the synthetic medium. With the addition of Trypticase, the pH break still occurs, but the cells do not lyse. Although lysis did not occur, sporogenesis was poor. Since the sporulation processes are

known to be pH sensitive, the low pH during the late log period could account for the poor sporulation. Apparently the sporogenesis process had progressed sufficiently to prevent lysis of the vegetative cells. Figure 19 shows that essentially the same effects are produced by the addition of the sporogens as by the addition of Trypticase. Halvorson (1962) has stated that sporulation can be viewed as a process in which a large number of spore specific components are synthesized sequentially. However, the results of this investigation indicate that the process may not be totally sequential in C. roseum. When the cells are grown in the synthetic medium, the sporulation process is initiated as evidenced by the characteristic pH break which occurs as vegetative cell metabolism is completed. Also the lytic system characteristic of late sporogenesis is generated and destroys the vegetative cells. This indicates that the genetic element responsible for the lysis of the sporangium is phenotypically expressed even though some of the preceding processes have not been expressed.

Effect of time of addition of Trypticase and sporogen on sporulation: To determine the time of the sporulation process which requires the sporogens, additions were made at hourly intervals during vegetative growth. Figure 20 shows

Growth, sporulation, and pH changes occurring in test media.

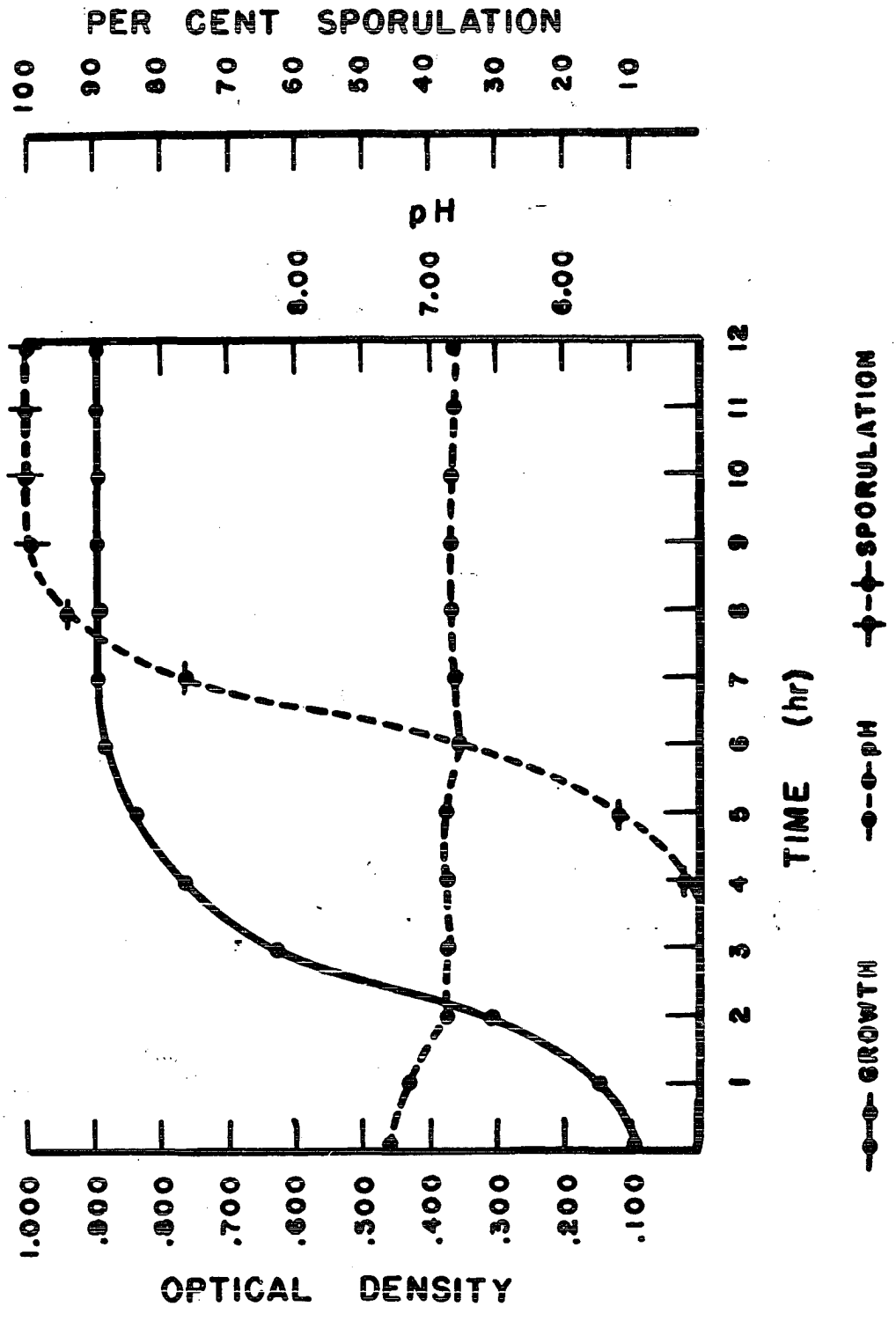
Figure 16. Tris Test Medium

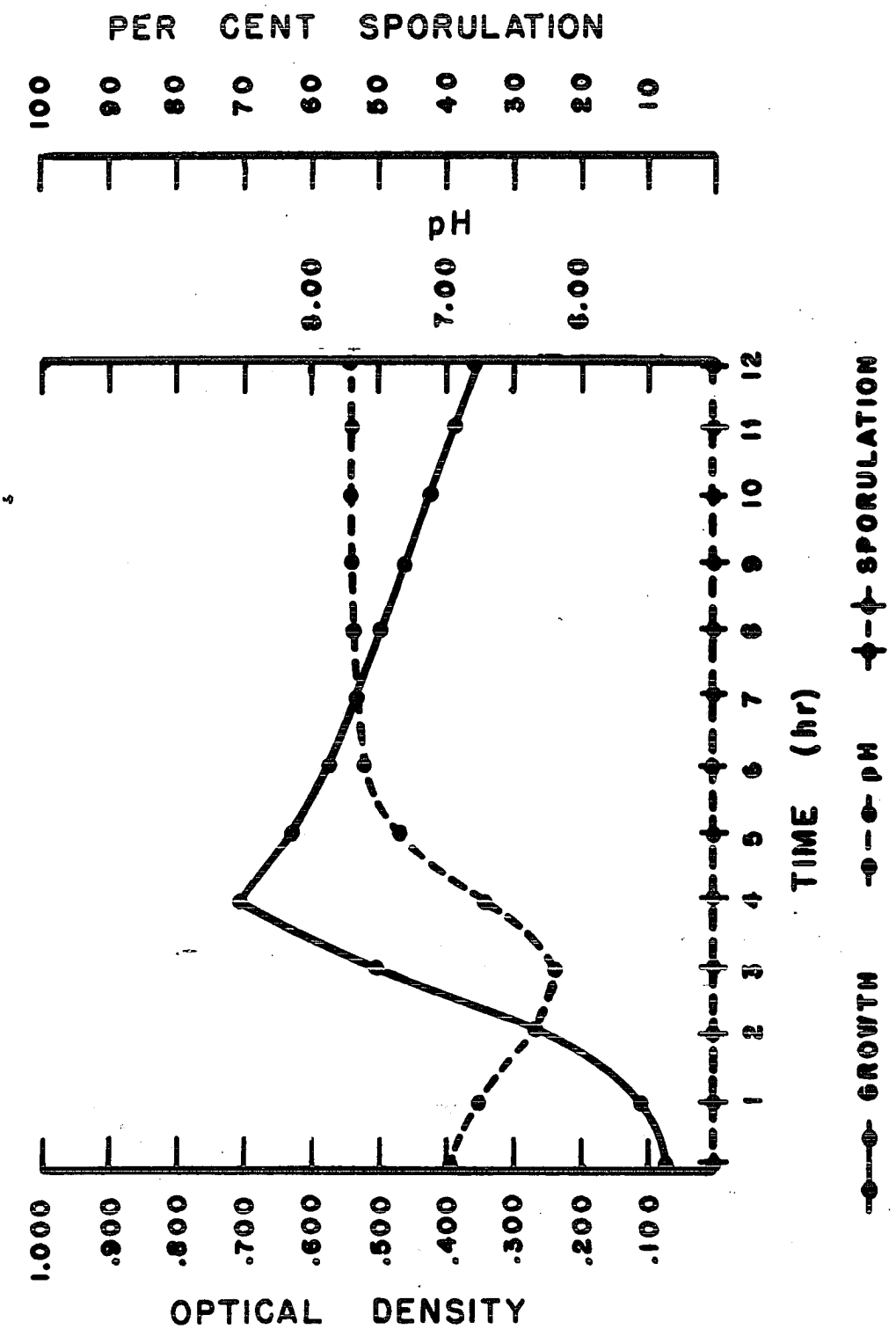
Figure 17. Synthetic Medium alone

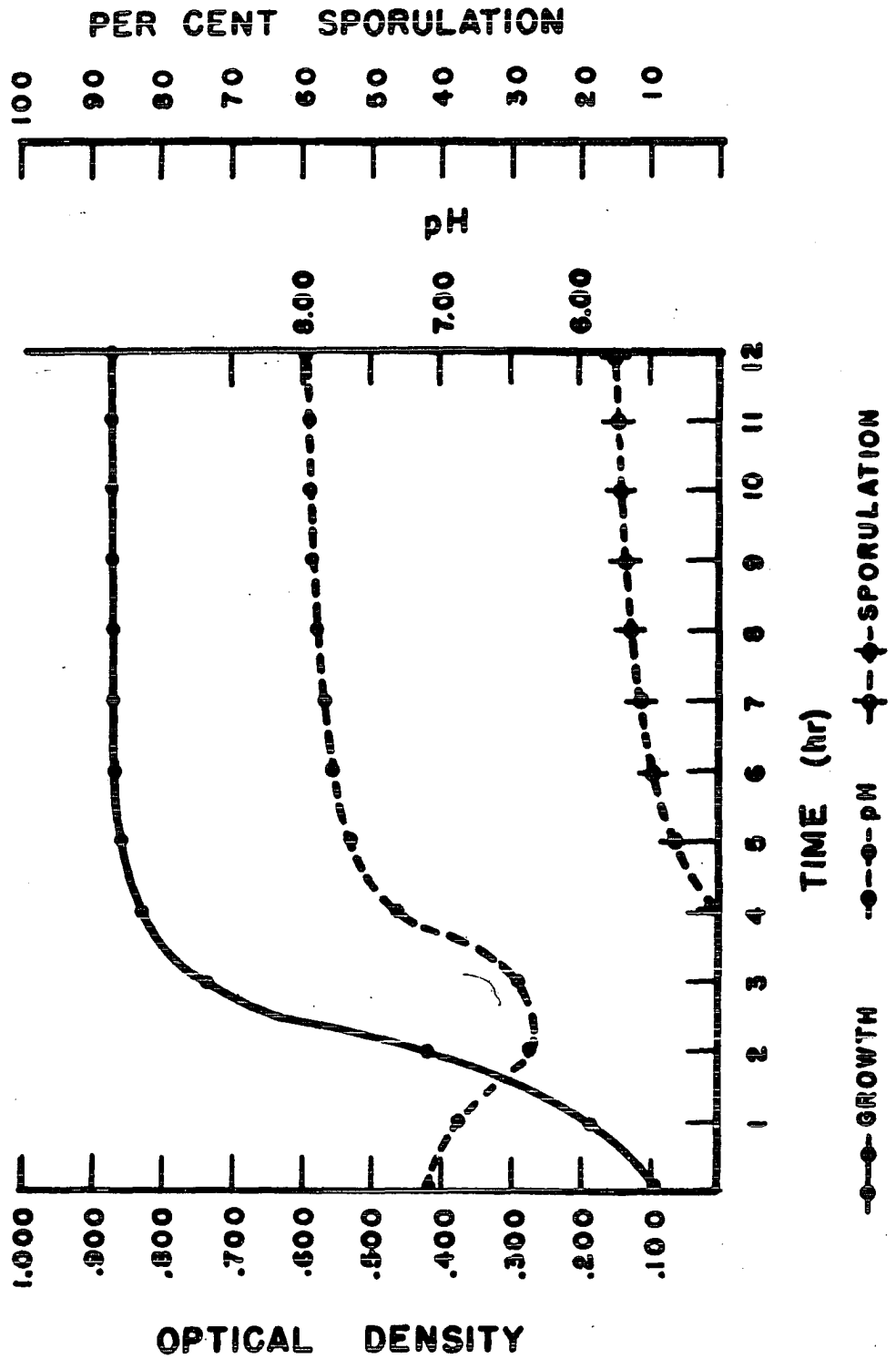
Figure 18. Synthetic Medium plus 0.1% Trypticase

Figure 19. Synthetic Medium plus sporogens

Synchronously dividing cells in their logarithmic growth phase were inoculated into the above media. Aliquots were removed at hourly intervals and the following determinations made: (1) The amount of growth by measuring the optical density at 500 mu; (2) The amount of sporulation by examining a crystal violet stained smear; and (3) The change in pH using a Beckman Zeromatic pH meter.


PER CENT SPORULATION
100
90
80
70
60
50
40
30
20
10
pH
8.00
7.00
6.00
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1
2
3
4
5
6
7
8
9
10
11
12
TIME (hr)
GROWTH
pH
SPORULATION


PER CENT SPORULATION
100
90
80
70
60
50
40
30
20
10
pH
8.00
7.00
6.00
TIME (hr)
1
2
3
4
5
6
7
8
9
10
11
12
GROWTH
pH
SPORULATION
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY


PER CENT SPORULATION
100
90
80
70
60
50
40
30
20
10
pH
8.00
7.00
6.00
1
2
3
4
5
6
7
8
9
10
11
12
TIME (hr)
GROWTH
pH
SPORULATION
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY

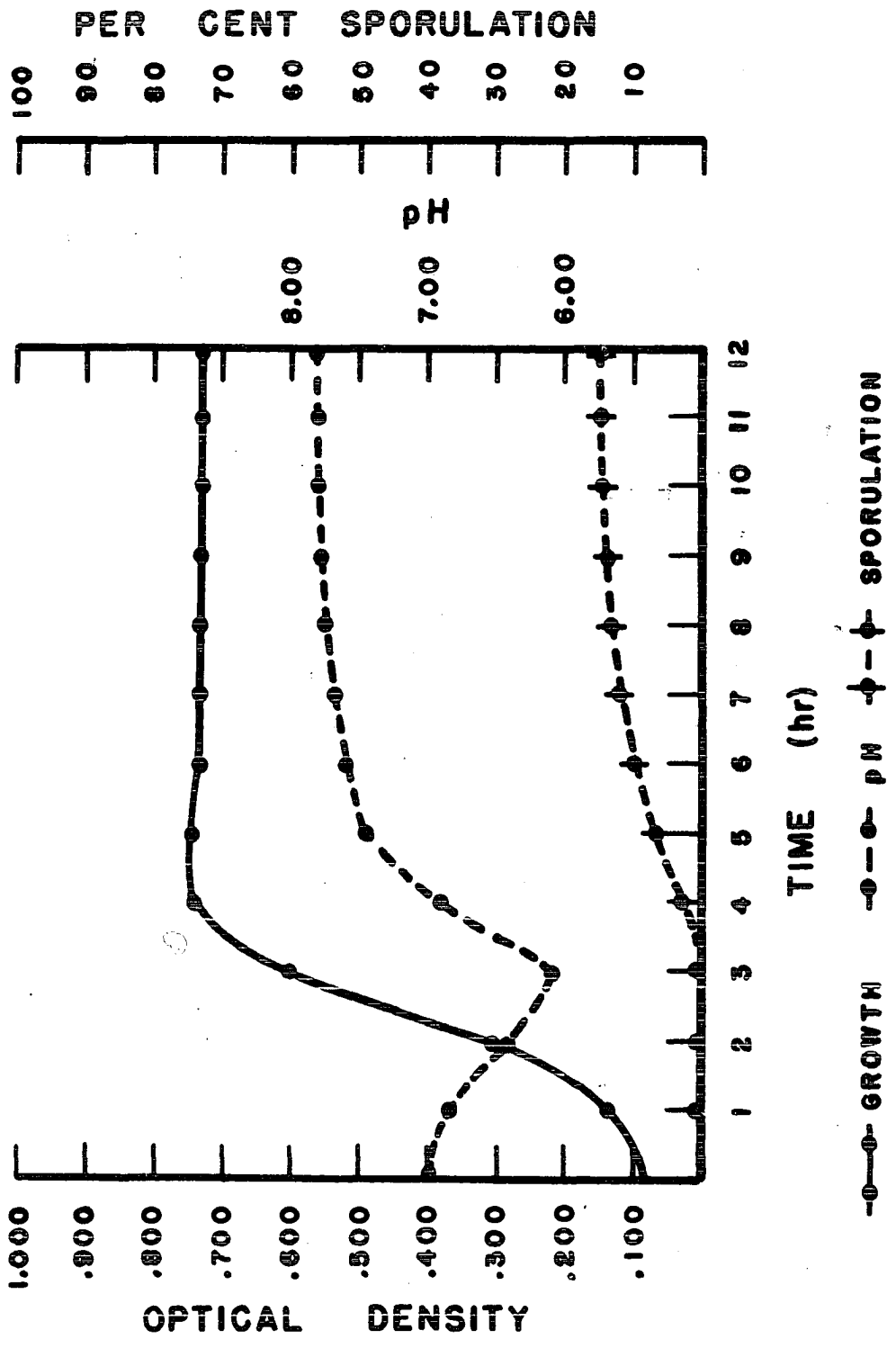
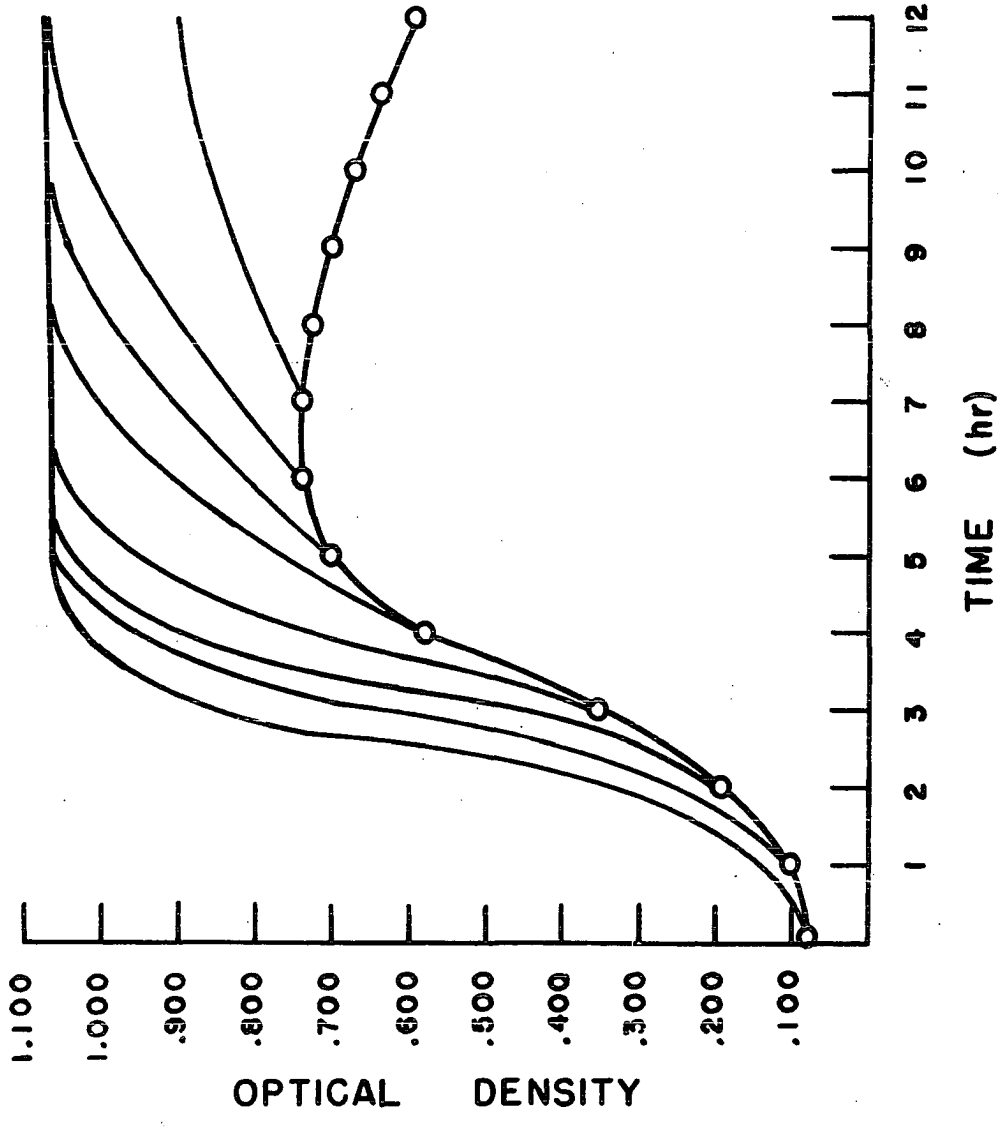

PER CENT SPORULATION
100
90
80
70
60
50
40
30
20
10
pH
8.00
7.00
6.00
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1 2 3 4 5 6 7 8 9 10 11 12
TIME (hr)
GROWTH
pH
SPORULATION

Figure 20. The effect of adding Trypticase to the synthetic medium at various times during growth.

A series of anaerobic culture flasks containing the synthetic medium were inoculated with synchronously dividing cells. At hourly intervals Trypticase was added to a flask. The amount of growth in each flask was determined by measuring the optical density at 500 mu at hourly intervals. The main line of this figure plotted with open circles represents the growth in synthetic medium without the addition of Trypticase. The remainder of the lines represent the growth obtained after the addition of Trypticase at that time interval. These lines actually represent the trend of what occurs after the addition of Trypticase. The points were purposely omitted since a point to point plot of each line would result in a maze of lines which would be almost impossible to resolve.


1.100
1.000
.900
.800
.700
.600
.500
.400
.300
.200
.100
OPTICAL DENSITY
1 2 3 4 5 6 7 8 9 10 11 12
TIME (hr)

the effect on growth of the addition of Trypticase. Additions during the early log phase result in an increase in both the rate and total amount of growth. Additions at the end of the log phase resulted in additional growth due to the availability of additional nutrients.

Figure 21 shows the importance of the time of addition of Trypticase. If the addition is made during the log phase normal and complete sporulation is initiated. However, additions after the log phase result in progressively less sporulation. Actually only the cells which were formed after the addition of Trypticase as shown by Figure 20 may have sporulated.

Figure 22 shows the importance of the time of addition of the sporogens. The additions affected neither the rate nor the total amount of growth. This experiment showed that the sporogens were required during the log phase. Additions during the late log phase resulted in poorer sporogenesis than additions during the early and middle log phase. These results lend support to the earlier conclusion that the sporogens are active only when added to dividing cells. If the cells have passed a particular stage in the sporulation process, the sporogens are no longer active.

Figure 21. The influence on sporulation of adding Trypticase to the synthetic medium at various times during growth.

The per cent sporulation is plotted against the time of addition of Trypticase. For example the column at 3 hr represents the amount of sporulation which occurred when Trypticase was added after 3 hr of incubation in the synthetic medium. The per cent sporulation was determined by examination of crystal violet stained smears.

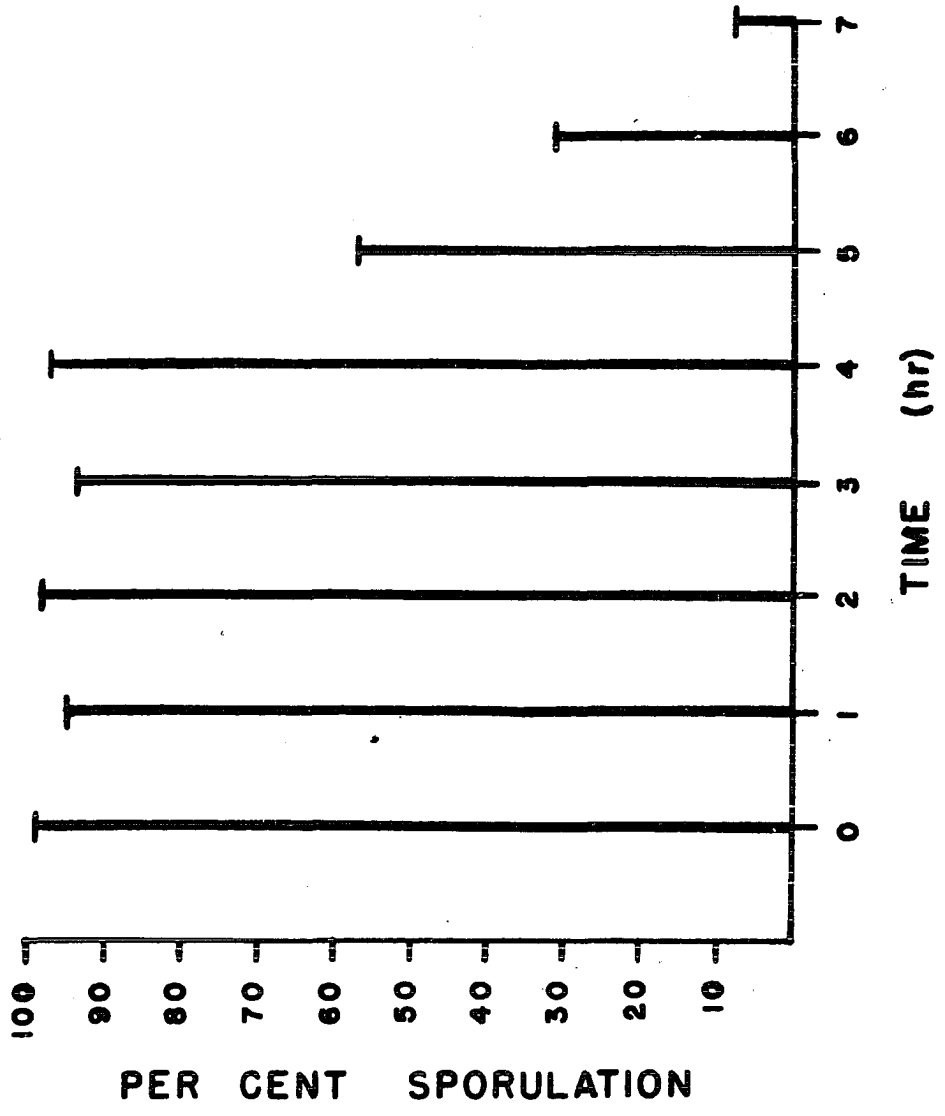
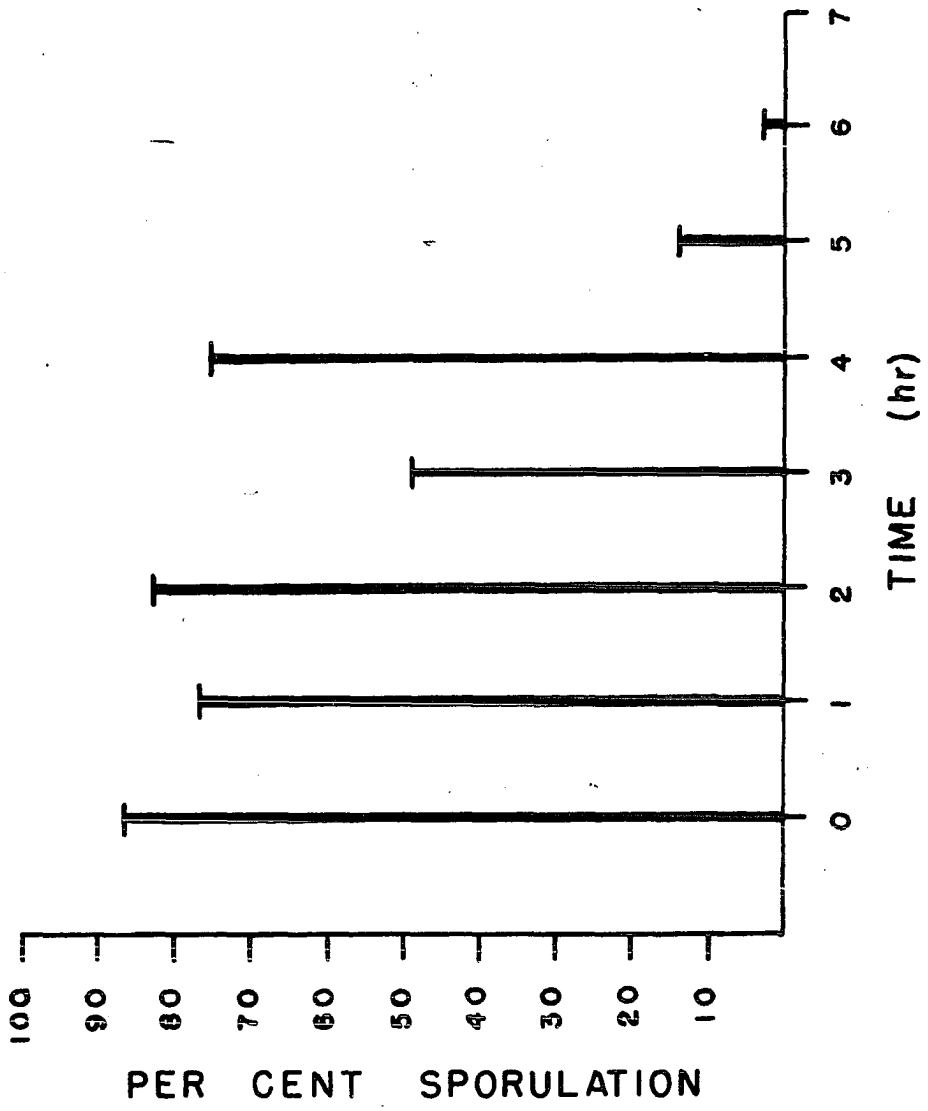

0
1
2
3
4
5
6
7
TIME (hr)
100
90
80
70
60
50
40
30
20
10
PER CENT SPORULATION

Figure 22. The influence on sporulation of adding the sporogens to the synthetic medium at various times during growth.

The per cent sporulation is plotted against the time of addition of the sporogens. For example the column at 2 hr represents the amount of sporulation which occurred when the sporogens were added after 2 hr of incubation in the synthetic medium. The per cent sporulation was determined by examination of crystal violet stained smears.


0
1
2
3
4
5
6
7
TIME (hr)
100
90
80
70
60
50
40
30
20
10
PER CENT SPORULATION

CHAPTER IV

SUMMARY AND CONCLUSIONS

This investigation has shown that spores of Clostridium roseum are capable of germination, post-germinative development, and growth in a synthetic medium consisting of eighteen amino acids, three vitamins, glucose, salts, and a buffer system. However, after the logarithmic growth phase, rapid and eventually complete lysis results rather than the formation of normal resistant spores. As in the case of other clostridia, proteinaceous material in the form of Trypticase or casein and thiamine were required for sporulation. Fractionation of Trypticase using gel filtration and paper electrophoresis resulted in the isolation of two peptides which exhibited sporogenic activity. Characterization of the two sporogens revealed that each was a phosphopeptide consisting of alanine, aspartic acid, glutamic acid, lysine, and serine. One also contained tyrosine, glycine, and proline.

The exact role of the sporogens in sporogenesis was not determined. However, it was shown that their activity is expressed only when added to a dividing culture. This finding and the fact that refractile pre-spores were never observed in the absence of the sporogens indicates that they are involved in some early sporogenic process. Initially it was thought that the sporogens may actually induce sporulation since they were only required in small amounts and since peptides have been isolated that induce morphogenesis (Pramer and Kuyama, 1963). However, the inducement or derepression of the sporogenic genome may occur in the absence of the sporogens since the cells in the synthetic medium exhibited a powerful lytic system and a definite pH break occurred, both being characteristic of sporulating cells (Greenberg and Halvorson, 1955; Powell and Strange, 1956; Nakata and Halvorson, 1960). The sporogens may then be required for the phenotypic expressing of this genome.

It has been estimated that proteins of spores are almost entirely derived de novo (Halvorson, 1965). This means that a tremendous amount of protein synthesis must occur during the initial stages of sporulation. Evidence is accumulating for the importance of peptides in protein synthesis (Fruton, 1963). The occurrence and possible role of

phosphopeptides in protein synthesis have been reported (Thoai and Pin, 1954, 1959; Kumar, Sundararajan, and Sarma, 1957; Pin, Kim Bai, and Thoai, 1960a, b). Other required and stimulatory peptides from casein have been reported (Mueller and Miller, 1955; Fox, 1961; Phillips and Gibbs, 1961). The phosphopeptide sporogens may then act as preformed, required peptide units that the sporulating cells are unable to synthesize.

The importance and role of the organic phosphate of the sporogens is unknown. However, it was determined that phosphoserine alone does not exhibit any detectable sporogenic activity. It appears that if the phosphate ester is a functional moiety of the sporogens, it is inactive unless the serine is attached to the peptide. It is known that phosphopeptides have very specific metal binding properties (Osterberg, 1957; Osterberg, 1958) and these complexes are of great physiological interest since they are believed to act <u>in vivo</u> as vehicles for metals (Mellander, 1955). Mellander found that these complexes promoted the intestinal resorption of calcium and iron and that calcium in this form was well utilized in bone calcification and rachitic children. Osterberg (1958) has found that the phosphopeptides and the metals Fe(III), Cu(II), Mn(II), Mg(II), and Ca(II) have

strong complex-forming ability. It is interesting to note that these are the main metals which are required for normal resistant spores (Halvorson, 1962).

One of the most controversial questions in the spore field today is the manner in which Ca(II) and dipicolinic acid are complexed with cell substance to produce heat resistance. It is interesting to speculate on the possibility of spore protein stabilization by the complexing of DPA, Ca(II), and the phosphate ester of phosphopeptides.

In conclusion, the inherent difficulties of studying the role of the sporogens should be pointed out. The extracellular protease of C. roseum is capable of leaching the required molecular structure from the intact casein molecule. Therefore, it is likely that enzymes alter the structure of the isolated sporogens before they enter the cell. This is supported by the finding that two peptides are active that are similar, but not identical. Therefore a precisely characterized sporogen added to the medium may be greatly altered before it enters the cell and exerts its function.

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