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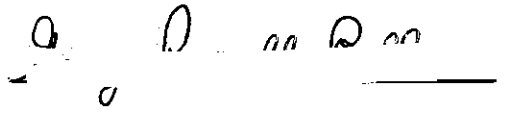

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DIFFERENCES IN CYST(E)INE CONTENT BETWEEN VEGETATIVE CELLS AND SPORES OF *CLOSTRIDIUM BOTULINUM*

A THESIS

Presented to

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DIFFERENCES IN CYST(E)INE CONTENT BETWEEN VEGETATIVE CELLS AND SPORES OF *CLOSTRIDIUM BOTULINUM*

Approved:

Chairman

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I dedicate this thesis to my extraordinary parents whose unselfishness and dedication to my educational progress have made whatever I have done possible.

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SUMMARY

Several investigators have demonstrated that spores of various species of Bacillus contain about five times more cyst(e)ine than the vegetative cells. This supports the general hypothesis that the protein structure of bacterial spores is substantially different from that of the vegetative cells and that it may contribute to the increased resistance of spores to many chemical and physical factors.

The objective of this research was to determine if such cyst(e)ine differences also exist between the spores and vegetative cells of Clostridium botulinum, an anaerobic sporeformer.

Cyst(e)ine was determined using the Kuratomi method which involves reductive decomposition of proteins with hydrazine. The hydrogen sulfide produced in this reaction was liberated by acidification and reacted with zinc acetate to yield zinc sulfide. The zinc sulfide was measured colorimetrically by Caro's method. The protein nitrogen content was determined by the Lowry method.

Based on the research reported in this text, it was concluded that the spores of Clostridium botulinum contain approximately ten times more cyst(e)ine than the vegetative cells. In addition, no correlation was found between the total amount of cyst(e)ine (cysteine plus cystine) and the resistance of the spore types to gamma irradiation. Also, no difference was found in the spore/cell cyst(e)ine content ratios between Langeland (Type F), OGB-69 (Type F), and Beluga (Type E).

CHAPTER I

INTRODUCTION

Several investigators have reported that spores of various species of Bacillus contain significantly more cyst(e)ine* than the vegetative cells. However, only a very limited amount of research of this kind has been reported for the clostridia. The purpose of this thesis research is to measure the cyst(e)ine content of spores and vegetative cells of Clostridium botulinum, type E, Beluga strain, and type F, strain Langeland, and its variant OGB-69. The results of these analyses are considered in relation to the greater resistance of spores to various chemical and physical factors.

The Bacterial Spore

The bacterial spore represents a unique form of life capable of adapting to unfavorable environmental changes and of restoring normal metabolic activity under suitable conditions. Initiation of sporogenesis normally occurs at the end of the logarithmic growth phase and seems to be activated by two interdependent external factors: reduction of the concentration of one or more growth-supporting substrates which repress the spore genome and accumulation of catabolites (Murrell, 1967).

The mature spore consists of a core, containing soluble proteins and nuclear material, surrounded by layers of a mucopeptide cortex,

*Total content of cysteine plus cystine.

proteinaceous outer coats, and an exosporium in some species. Electron micrographs show that that the spore coats are multilayered structures which appear late in sporogenesis (Kondo and Foster, 1967; Rode and Williams, 1966). The coats occupy about 50 percent of the spore volume and 40-60 percent of the dry weight, and they are composed largely, if not exclusively, of protein (Warth, Ohye and Murrell, 1963). Kadota (1965) showed that the nitrogen content of spore coat samples increases proportionally with the purity of the sample; for example, whole spores contain about 10 percent N, crude spore coats contain 11.2 percent N, and enzymatically-purified spore coats contain 14.5 percent N, a value comparable to that of pure protein. In addition, Kadota (1965) discovered unique crystalline structures in spore coats of B. subtilis, and, using crystallographical and chemical analyses, demonstrated the similarity of the structures to α and β-keratin.

Cystine-Rich Coat Protein

There are at least two types of coat proteins, each associated with a distinct structural layer (Spudich and Kornberg, 1968; Ohye and Murrell, 1962). The laminated, thioglycollate-soluble, inner coat layer of B. cereus represents 80 percent of total coat protein and consists mainly of polypeptides cross-linked by disulfide bonds (Aronson and Fitz-James, 1968). The dense, thioglycollate-insoluble, outer coat layer is synthesized during a definite time period associated with a rapid uptake of cystine from the medium and with the increased cystine content of the coat layers (Vinter, 1959a, 1959b, and 1960). Pulse-chase experiments with several labelled amino acids indicated that the kinetics of this cystine

incorporation in synchronously sporulating B. megaterium and B. cereus differed markedly from that of the other amino acids in that there was a four to five-fold increase of cystine-incorporation during the forespore stage, several hours before the appearance of the coat layers (Vinter, 1961, 1962). Because this cystine incorporation could be inhibited by reagents which compete for sulfhydryl groups, Aronson and Fitz-James (1968) concluded that cystine exchanges with available sulfhydryl groups of coat precursor proteins rather than being involved in polypeptide synthesis. These disulfide exchange reactions have been described by Eagle, Oyama, and Piez (1960), and by Smithies (1965). Aronson and Fitz-James (1968) proposed that the outer coat layers consist of cystine-rich polypeptides held together by hydrophobic bonds and with the cystine residues buried within the folded protein structure.

The coat precursor proteins are probably synthesized by messenger RNA and polysomes bound to the cytoplasmic membrane (Aronson, 1965a and 1965b).

Results of experiments by Vinter (1959a, 1960a, 1960b) indicated that spores of several species and strains of Bacillus contain four to five times more cyst(e)ine than the vegetative forms. Vegetative cells of B. subtilis, for example, have been found to contain 26 μg cyst(e)ine sulfur per mg protein nitrogen; spores contained 108 μgS/mgN; enzymatically-purified spore coats contained 292 μgS/mgN (Kadota, 1965). Tsuji and Perkins (1962) reported five times more cyst(e)ine in spores (48.3 μgS/mgN) of Clostridium botulinum, type 62A, than in vegetative cells (9.6 μgS/mgN).

Cystine-Rich Structure and Resistance

The great resistance of bacterial spores to many chemical and physical factors is the result of a complex system of protection involving both the structural and biochemical properties of the spores. However, it has been difficult to determine the contribution of the individual components of the protective system because the integration of all the components must be taken into account. Therefore, any discussion of the cystine-rich structure in the resistance of spores can only be speculative for the present.

Cystine, with its disulfide bonds, is an integral part of the tertiary structure of many proteins. Vinter (1959a, 1960b, 1969) suggests that the stabilization of the protein structure of spores by disulfide bonds may contribute to the overall resistance of the spore in much the same way in which disulfide bonds stabilize highly inert proteins, such as keratins. There is an apparent relation between the disulfide content of spore coats and regulation of the permeability of resting spores, because treatment of spores with agents which rupture disulfide bonds renders them permeable to dyes and lysozyme (Gould and Hitchins, 1963). Spores are normally 10-20 times more resistant to irradiation than parent vegetative cells (Rowley and Newcomb, 1964; Stuy, 1956; Grecz, 1965; Vinter, 1969). In addition, Vinter (1961, 1962) demonstrated that the radioresistance of sporulating bacilli appears simultaneously with the formation of the cystine-rich structure. Barron (1955) has reviewed the effects of ionizing radiation on sulfhydryl and disulfide groups, and the radioprotective effect of sulfhydryl compounds has been demonstrated in

spores (Powers and Kaleta, 1960) and other biological materials (Eldjarn and Pihl, 1957; Ormerod and Alexander, 1962). The effectiveness of some of these compounds is related to their ability to form disulfides (Eldjarn and Pihl, 1958). However, few or no sulfhydryl groups have been detected in spores of several species of Bacillus (Vinter, 1961, 1962; Mortenson and Beinert, 1953; Bott and Lundgren, 1964).

A possible role of the disulfide bond in radioresistance is suggested by a number of paramagnetic resonance studies of X-irradiated proteins which indicated that the disulfide bond can serve as an electron donor for replacing electrons knocked out of the molecule (Gordy, Ard, and Shields, 1955; Gordy and Shields, 1958). Gordy and Miyagawa (1960) proposed that the electron vacancy is led along the polypeptide chain to sulfur.

In addition, breakage of disulfide linkages might serve as a non-destructive, non-lethal energy dissipator, since the rupture of disulfide bonds by radiation has been demonstrated in simple disulfides (Cavallini et al. 1960), proteins (Ray, Hutchinson and Morowitz, 1960) and spores (Gould and Ordal, 1968).

However, cystine-rich proteins seem to be possible radioprotectors only during the early stages of sporogenesis before they are incorporated into the spore coats. Mature spores of B. cereus are as resistant to gamma irradiation after treatment with agents which rupture 20-30 percent of the disulfide bonds or when they are irradiated in the presence of reagents which block the formation of sulfhydryl groups as are untreated spores (Hitchins, King, and Gould, 1966).

Chemical Methods

The purpose of this thesis research was to measure the amounts of total protein and cysteine plus cystine in the spores and vegetative cells. The methods used were those most suitable for the accurate and reproducible estimation of these parameters from the small amount of test material available, the amount being restricted because, for instance, the process of spore collection required some two weeks and normally produced only about 2×10^{10} spores from nine liters of culture.

Kirk (1947) has reviewed most of the methods for protein determination in a perceptive and critical manner. The method of Lowry *et al.* (1951), which is based on the color produced by the biuret and Folin reactions together, was selected because it can be scaled down to estimate protein in the 5-100 μg range. In this method, treatment of protein with an alkaline copper solution and the Folin-Ciocalteau reagent (phosphomolybdic acid-phosphotungstic acid) results in the development of a color which is 100 times more intense than that produced in the biuret method and depends much less upon the protein composition (i.e. tyrosine and tryptophan residues) than does the color developed by the Folin reaction alone. Further, this method is 10-20 times more sensitive than spectrophotometric assay of protein at 280 nm, and less subject to individual variation. The digestion of protein and estimation of the released ammonia by Nesslerization can be as sensitive as the Lowry method but it is considerably more time consuming. The only limitation of the Lowry method is that the color is not directly proportional to all protein concentrations; that is, calibration curves commonly show two linear portions. This problem is overcome simply by working at protein

concentrations where the slope is linear (Lowry et al., 1951).

For the estimation of cyst(e)ine a specific method developed by Akabori et al. (1952) was used involving reductive decomposition of proteins by hydrazine at 120 C. Kuratomi et al. (1957) have shown that this method gives rise to H_2S specifically and quantitatively from cysteine and cystine and have confirmed that no H_2S is produced from taurine or methionine under the same conditions. They offer the following possible reaction:

$$\text{Cystine} + NH_2 - NH_2 \rightarrow 2\ H_2S + NH_3 + N_2 + \text{unknown substance}$$

In addition, they showed that the H_2S is retained in the hydrazine hydrate solution even at 120 C for eight hours. The H_2S is liberated by acidification of the hydrazine hydrate solution with sulfuric acid and quantitatively absorbed in zinc acetate solution. The zinc sulfide thus produced is reacted with a N, N, dimethyl-p-phenylenediamine solution resulting in the formation of methylene blue (St. Lorant, 1929). This is generally known as Caro's method (Almy, 1925). The cyst(e)ine content in a trichloroacetic acid precipitate is usually expressed as µg of sulfur per mg protein nitrogen.

CHAPTER II

MATERIALS AND METHODS

Organisms Used

Two immunological types of Clostridium botulinum were used as the test organisms in this study. The type E, Beluga strain was obtained from Dr. M. W. Eklund, Bureau of Commercial Fisheries, Seattle, Washington. The type F, Langeland strain was acquired from Dr. C. E. Dolman of the University of British Columbia, Canada; and the isolate OGB-69, a colonial variant of the Langeland strain, was obtained from Dr. Lillian Holdeman, Communicable Disease Center, Atlanta, Georgia. Stock cultures have been maintained as 2.5 ml samples of five-day cooked meat cultures which were quick-frozen at -80 C in a 2-ethoxyethanol-dry ice bath. The stock cultures were stored at -10 C in the laboratory of Dr. N. W. Walls, Engineering Experiment Station, Georgia Institute of Technology, Atlanta, Georgia.

The vegetative cells were gram positive rods varying in size from 0.5 to 0.9 micrometers in diameter and 2.0 to 5.0 micrometers in length. Spores were subterminal in position and swelled the sporangium before release.

Preparation of Spore Crops

For production of a spore crop, a tube of frozen stock culture of the particular strain was thawed and the contents of the tube were transferred to 30 ml of sterile cooked meat medium (see Appendix A). This was

incubated for four days after which 15 ml of this culture were transferred to 300 ml of cooked meat medium. After 72 hours incubation 150 ml of the culture supernate were used to inoculate a 3000 ml quantity of Type C Toxin Medium (see Appendix A) which was contained in a four liter Erlenmeyer flask. Type F cultures were incubated at 30 C and type E cultures at 25 C.

Collection of Spores

Sporulation in the growth medium was followed daily by microscopic examination of culture smears. The culture was harvested when there was no further increase in percentage of spores to vegetative cells. When the spore crop was ready for harvesting, 200 ml quantities of the culture were transferred to each of six 250 ml centrifuge bottles. These were placed in sealed centrifuge cups and spun in a refrigerated centrifuge* at 3000 x g for 30 minutes at 5 C. The supernatant was decanted and the spore sediment was washed out with a small volume of cold, sterile, deionized water into a sterile 250 ml Erlenmeyer flask held in an ice-water bath. This procedure was repeated until all the culture had been centrifuged and the sediment retrieved. The pooled sediment was washed four times with 200 ml quantities of cold, sterile, deionized water, centrifuging after each washing. The final wash water was carefully siphoned off and the spore pellet resuspended in a small volume of cold, sterile, deionized water. This was aseptically transferred to a sterile, 50 ml screw-capped Erlenmeyer flask containing glass beads. The crude spore suspension was stored at 4 C.

*Model PR-2, International Equipment Company, Needham Heights, Mass.

Cleaning of Spores

Vegetative cells and cell debris were removed from the crude spore suspensions using an adaptation of the method of Grecz et al. (1962) involving use of lytic enzymes and ultrasonic oscillation. Millipore filter-sterilized solutions of lysozyme* (10 mg per ml) and trypsin (5 mg per ml) were prepared. One ml of each enzyme solution was added to the crude spore suspension which was then diluted to 50 ml with sterile deionized water. This gave a final concentration of 0.2 mg per ml of lysozyme and 0.1 mg per ml of trypsin. The spore-enzyme mixture was incubated at 45 C. Rapid lysis of the sporangia was enhanced by ultrasonic oscillation of the mixture for five minute periods at intervals of 0, 0.5, 1 and 2 hours after initiation of incubation. The 20 khz Bronwill Biosonik III oscillator** was used at the 60 percent power setting. The cleaning procedure was completed by washing the spores five times with sterile deionized water. After each washing the spores were spun down in a refrigerated centrifuge*** at 30,000 x g and 5 C for 10 minutes. The cleaned spores were resuspended in 10-20 ml of sterile distilled water. Freedom of these spore suspensions from vegetative cells and cell debris was greater than 95 percent as estimated microscopically by the loss of crystal violet stainability.

Spore Titration

Titrations of the number of viable spores present in the clean spore suspensions were obtained with fresh pork infusion agar as the

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* See Appendix A for listing of the sources for all chemicals used in this research.

** Will Scientific, Inc., Rochester, New York.

***Model B-20, International Equipment Company, Needham, Mass.

growth medium (see Appendix A) in Fisher Scientific modified agar slant tubes. The tubes were heated in boiling water until the titration medium had melted and were then held at 45 C in a water bath. Samples (1 ml) of the clean spore suspensions were serially diluted 100-fold using sterile, 99 ml, 0.1 percent (w/v) peptone water blanks (see Appendix A) contained in milk dilution bottles. From these, three replicate volumes, either 0.1 or 1 ml, were transferred to the titration medium and each tube was vigorously rolled between the palms to distribute the spores evenly throughout the liquid. To prevent settling of the spores the tubes were placed in an ice water bath, facilitating rapid solidification of the medium, whereupon each tube was sealed with an overlay composed of a sterile two percent (w/v) Bacto-Agar (Difco) solution containing 0.1 percent (w/v) sodium thioglycollate, and the original cotton plug was then re-inserted in the top of the tube. The tubes were incubated five days at 30 C (or 25 C) after which the tubes containing approximately 10-200 colonies were counted under the 1.5 X magnifying glass of a Bactronic colony counter*. Results were accepted if the three tubes of a particular dilution gave counts which did not vary more than 10 percent.

Preparation of Vegetative Cell Crops

For growth of a vegetative cell crop, 0.2 ml of a clean spore suspension was inoculated into 20 ml of trypticase soy broth medium (see Appendix A). After 24 hours incubation at 30 C (or 25 C), 10 ml of the culture were inoculated into 100 ml trypticase soy broth (TSB) which was incubated for 24 hours. Fifty ml of this culture were inoculated into

*Model CC-110; New Brunswick Scientific Company, New Brunswick, New Jersey.

500 ml of TSB. After 18 hours at 30 C (or 25 C), this log-phase culture was spun at 30,000 x g and 5 C for 10 minutes. The cells were washed five times with sterile distilled water, resuspended in 10 ml of sterile distilled water and aseptically transferred to a sterile, 50 ml screw-capped Erlenmeyer flask.

Estimation of Total Protein

Preparation of Spore and Cell Proteins

The disruption of spores was accomplished by ultrasonic oscillation of a spore suspension for one hour at the 50 percent power setting of the 20 khz Bronwill Biosonik III oscillator*. One ml of a concentrated vegetative cell or sonicated spore suspension was added to 10 ml of 10 percent (w/v) trichloroacetic acid (TCA). After 15 minutes at room temperature, the mixture was centrifuged at 30,000 x g and 5 C for 10 minutes in a refrigerated centrifuge. The supernatant was discarded; the pellet was washed once with 10 ml of distilled water, extracted with 20 ml of ethanol-ether (3:1), and heated at 90 C for 30 minutes in 25 ml of 5 percent (w/v) TCA. This mixture was then centrifuged as above, the supernatant decanted, and the pellet dissolved in 10 ml of 0.01 N NaOH.

Reagents

A. 50 ml of 2 percent (w/v) Na_2CO_3 in 0.1 N NaOH mixed immediately before use with 0.5 ml of 1 percent (w/v) $CuSO_4$ and 0.5 ml of 2 percent (w/v) sodium potassium tartrate.

B. 5.0 ml of Folin-Ciocalteau reagent added to 6.8 ml of distilled water to give a solution N in acid.

*Will Scientific, Inc., Rochester, New York.

C. Standard solution of bovine serum albumin (11 mg protein N per ml) diluted 1:100 with distilled water.

Standard Curve for Total Protein

A modification of the method of Lowry et al. (1951) was used to determine total protein throughout this study. To prepare a standard curve for total protein, aliquots of the standard bovine serum albumin (0.05-1.0 ml) were diluted to 1.0 ml with distilled water. Five ml of reagent A were added to each sample. After 10 minutes, 0.5 ml of reagent B was rapidly added; within five seconds the mixture was vigorously agitated. A blank consisting of 1.0 ml distilled water, 5.0 ml reagent A, and 0.5 ml reagent B was prepared in the same way. After 30 minutes at room temperature the absorbance of each sample was read at 500 nm in a Bausch and Lomb Spectronic 20 colorimeter* using matched, 1.27 cm diameter Spectronic 20 tubes. The absorbances of the samples were plotted against the respective concentrations of bovine serum albumin (Figure 1). The lines fitted to these points by the method of least squares (see Appendix B) were used to estimate total protein. The standard errors of estimate ($S_{y.x}$) were 0.02 for both slopes in Figure 1.

Determination of Total Protein in Cells and Spores

Aliquots (0.1 - 1.0 ml) of a dissolved trichloroacetic acid precipitate (see Preparation of Spore and Cell Proteins) were diluted to 1.0 ml with distilled water. The same procedure was followed as described in the preparation of the standard curve for total protein.

*Bausch and Lomb, Inc., Rochester, New York.

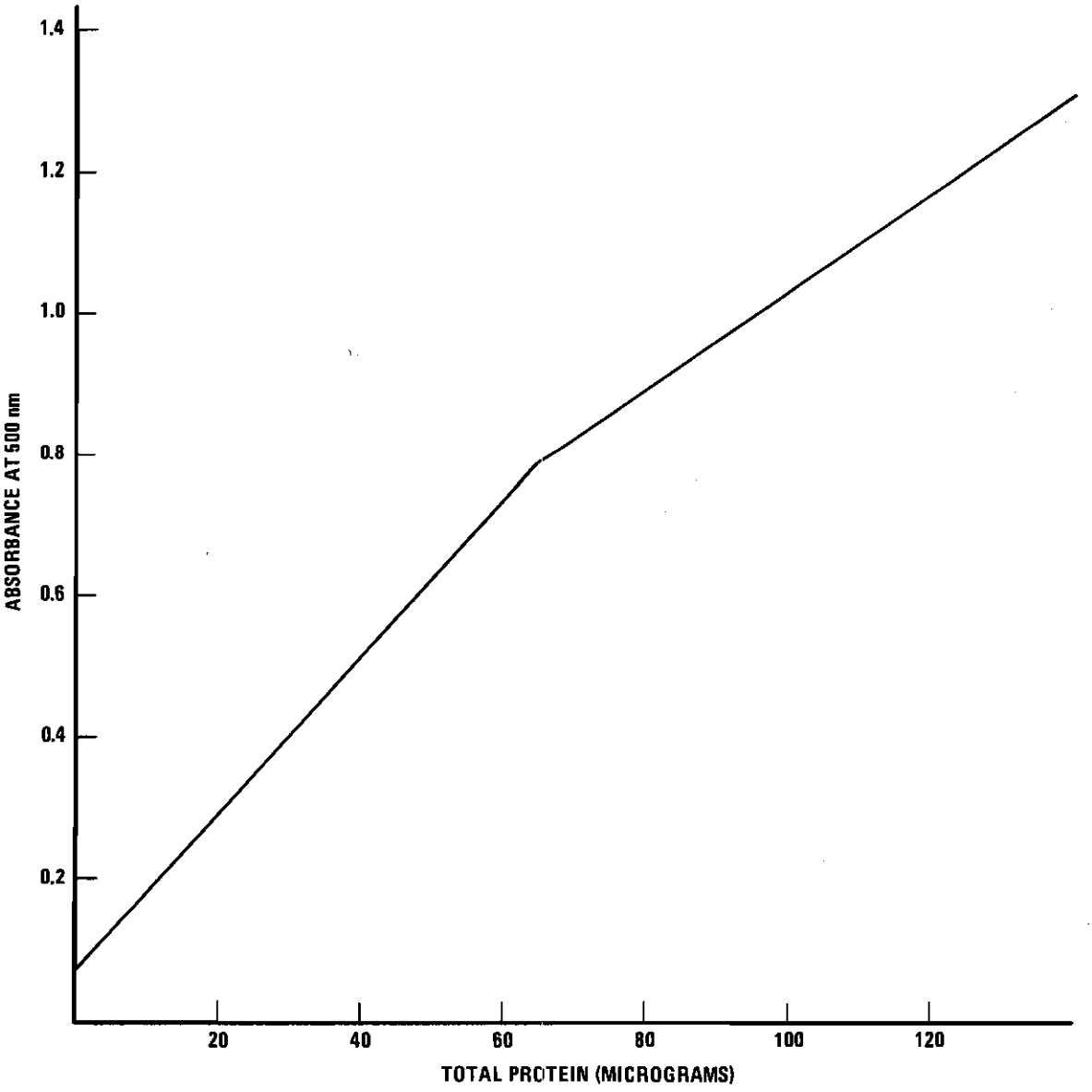

Figure 1. Standard Calibration Curve for the Determination of Total Protein (Lowry et al., 1951).
The estimating equations $Y = 0.074 + 0.011\ X$ and $Y = 0.331 + 0.007\ X$ were derived using the method of least squares (see Appendix B). The standard error of estimate ($S_{y.x}$) = 0.02 for both equations.

Determination of Cystine plus Cysteine by Hydrazinolysis

Reagents

A. Hydrazine Hydrate - 85 percent.

B. Zinc Acetate Solution - 60 g of zinc acetate, 17 g of sodium acetate and 0.05 g sodium chloride dissolved in distilled water and diluted to one liter.

C. Ferric Ammonium Sulfate Solution - 31 g of ferric ammonium sulfate dissolved in distilled water, treated with 6.3 ml of concentrated sulfuric acid and made up to 250 ml with distilled water.

D. N, N, dimethyl-p-phenylenediamine monohydrochloride solution- 0.5 g of the hydrochloride in one liter of an aqueous solution containing 200 ml of concentrated sulfuric acid.

E. 6 N Sulfuric Acid.

F. Cystine Standard - 6.00 mg (25 μmoles) l-cystine (produces 50 μmoles hydrogen sulfide)

Gas Generation and Collection Apparatus

The apparatus used for the generation and collection of hydrogen sulfide is shown in Figure 2. All joints in the assembly were standard taper 14/20 ground glass. The center joint of the three-necked, 50 ml, round bottom distilling flask (Ace #9287)* was tightly sealed with a ground glass stopper (Ace #9390). A distilling adapter with 75° angle side arm (Ace #9156) was fitted to one of the remaining joints, and a vacuum adapter (Ace #9136) was attached to the distilling adapter. A

*Ace Glass Incorporated, Vineland, New Jersey.

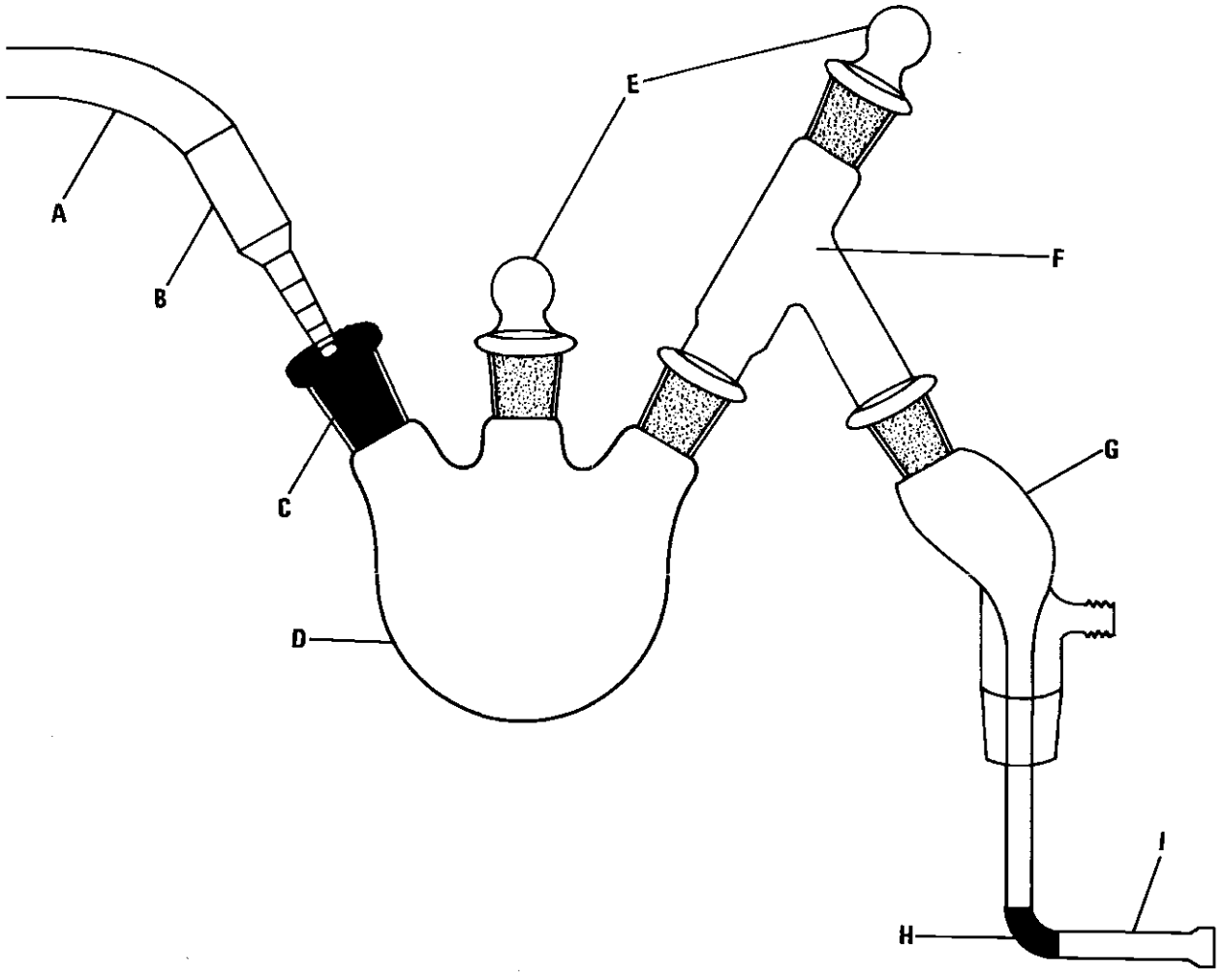

Figure 2. The Apparatus Used for the Generation and Collection of Hydrogen Sulfide. (A) Nitrogen Delivery Tube ; (B) Syringe; (C) Rubber Serum Bottle Cap; (D) Three-Necked Flask; (E) Ground Glass Stopper; (F) Distilling Adapter; (G) Vacuum Adapter; (H) Latex Tubing; (I) Gas Dispersion Tube.

glass tube 20 cm in length and 4 mm I.D. was expanded on one end and fitted with a 9 mm diameter sintered glass disc. This tube was attached to the tube of the vacuum adapter by means of a 4 cm length of latex tubing. The remaining joint was tightly stoppered with a rubber serum bottle cap. Nitrogen was introduced into the apparatus through a small syringe attached to the nitrogen delivery tube.

Calibration Curve for Hydrogen Sulfide

A cystine standard (6.00 mg) was treated with 0.5 ml of pure hydrazine hydrate and the mixture was refluxed in an oil bath at 115-120 C for 8 hours. The flask was then cooled with water, the condenser thoroughly rinsed with cold water and the flask contents and condenser washings were diluted to 50 ml with distilled water. An aliquot of this solution (0.5 - 5 ml) containing 0.5 - 5 μmoles hydrogen sulfide was transferred to the flask of the gas generation apparatus. Ten ml of 6 N H_2SO_4 was pipetted rapidly into the flask through the gas inlet port. The port was stoppered and nitrogen gas was run through the apparatus for 30 minutes at a rate of three liters per hour. Simultaneously, the flask was heated in a water bath at 50-55 C to drive out the hydrogen sulfide completely. The hydrogen sulfide was passed into a test tube (1.8 cm x 15 cm) containing 3.5 ml of the zinc acetate solution. After all the hydrogen sulfide had been driven out and absorbed in the zinc acetate solution, the gas dispersion tube was disconnected from the vacuum adapter and rinsed with 1.5 ml zinc acetate solution. This rinse was then pooled with the 3.5 ml solution containing the bulk of the absorbed hydrogen sulfide. Five ml of the N, N, dimethyl-p-phenylenediamine monohydrochloride solution and 1.0 ml of the ferric ammonium sulfate solution were added and

the mixture vigorously swirled. A blank was prepared from 5.0 ml zinc acetate solution, 5.0 ml of N, N, dimethyl-p-phenylenediamine solution, and 1.0 ml of ferric ammonium sulfate solution. Each tube was capped and read after exactly one hour at 630 nm using the Bausch and Lomb equipment previously mentioned. The absorbances of the samples were plotted against the respective hydrogen sulfide concentrations (Figure 3) and the estimating equation for hydrogen sulfide was determined by the method of least squares. The standard error of estimate ($S_{y.x}$) was 0.01.

Determination of Cystine plus Cysteine in Cells and Spores

Hot trichloroacetic acid precipitates were prepared from 2.0 ml of a concentrated cell suspension or from 1.0 ml of a clean spore crop as described previously (see Preparation of Spore and Cell Proteins). These protein precipitates were transferred to a 10 ml pear shaped distilling flask with a standard taper 14/20 joint (Ace #9293). The contents of the flask were dried at 105 C. The flask was then attached to a reflux condenser (Ace #9195), 1.0 ml pure hydrazine hydrate was added, and the flask was heated at 115-120 C for 8 hours. The flask was cooled and the condenser rinsed with cold, distilled water. The combined flask contents and condenser washings were diluted so that 5 ml of the diluted solution would contain 1-10 μmole of hydrogen sulfide. The degree of dilution used here was determined by trial and error in earlier experiments. Five ml aliquots of this diluted solution were transferred to the gas generation apparatus. The methods of evolution, absorption, and colorimetric estimation of hydrogen sulfide were conducted exactly as described in the previous sections. Conversion from μmoles hydrogen sulfide to μg S was

accomplished as follows:

(μmole H_2S) (.942 μmole S/μmole H_2S)* (32 μg S/μmole S) = μg S

*The factor 0.942 was obtained from the fact that sulfur (MW = 32) comprises 32/34 = 0.942 of H_2S (MW = 34).

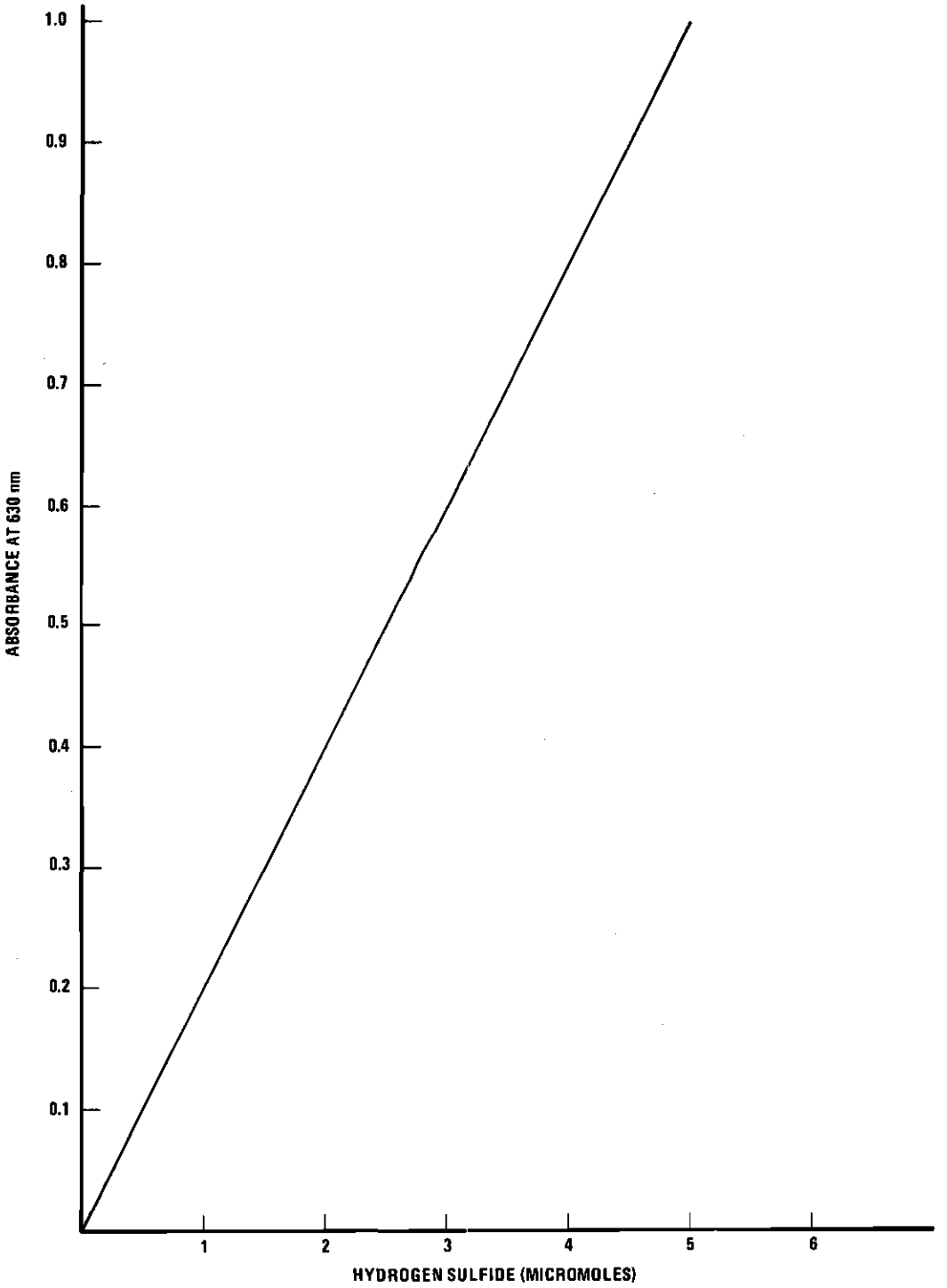

Figure 3. Standard Calibration Curve for the Determination of Hydrogen Sulfide (Kuratomi et al., 1957).
The estimating equation $Y = 0.20\ X$ was derived using the method of least squares (see Appendix B). The standard error of estimate $(S_{y.x}) = 0.01$

CHAPTER III

RESULTS AND DISCUSSION

The results of five or six chemical analyses of the spores and cells of each of the three test types of Clostridium botulinum are given in Table 1. The ratio μg S/mg N is a true expression of the cyst(e)ine content of the spore and cell proteins because (1) the amount of sulfur is a function of hydrogen sulfide liberated only from cysteine and cystine by reductive decomposition with hydrazine; (2) the amount of nitrogen represents only protein nitrogen.

The ratios of the cyst(e)ine sulfur content of protein between spores and cells of each of the three test organisms are summarized in Table 2. The errors expressed for these ratios are a function largely of the considerable scatter in the values for the sulfur content of spores and cells (Table 1). The coefficients of variation for these values are 17 percent (Langeland), 26 percent (OGB-69), and 26 percent (Beluga).

During the preparation of the hydrogen sulfide calibration curve (Figure 3) it was found that adding the reagents (see MATERIALS AND METHODS) to a standard sulfide solution (5 μg zinc sulfide/ml zinc acetate) produced a color intensity that always slightly exceeded that produced by driving over an equivalent amount of hydrogen sulfide with nitrogen. That is, there was a slight loss in the transfer process. However, this was always strictly reproducible so no error was introduced in reading from a calibration curve when the same conditions of gas

Table 1. Results of Chemical Analyses of Spores and Cells of *Clostridium botulinum*

Test Material	Analysis	Cyst(e)ine Sulfur (micrograms)	Protein Nitrogen (milligrams)	µg S/mg N
Langeland Spores (Type F)	1	122	1.35	90.4
	2	117	1.35	86.7
	3	116	1.35	85.9
	4	145	1.34	107
	5	179	1.35	133
	6	168	1.35	124
Langeland Cells (Type F)	1	34	3.4	10
	2	39	3.0	13
	3	33	3.0	11
	4	30	3.5	8.6
	5	32	4.2	7.4
OGB-69 Spores (Type F)	1	157	2.10	74.8
	2	147	2.10	70.0
	3	184	2.10	87.6
	4	256	2.10	122
	5	298	2.10	142
	6	206	2.10	98
OGB-69 Cells (Type F)	1	39	4.0	9.8
	2	60	4.2	14
	3	52	4.7	11
	4	46	4.0	12
	5	34	4.0	8.0
Beluga Spores (Type E)	1	38	0.50	76
	2	79	0.50	160
	3	61	0.50	120
	4	50	0.50	100
	5	46	0.50	92

Table 1. (Continued)

Test Material	Analysis	Cyst(e)ine Sulfur (micrograms)	Protein Nitrogen (milligrams)	µg S/mg N
Beluga Cells (Type E)	1	18	1.6	11
	2	15	1.8	8.3
	3	14	1.6	8.8
	4	11	1.5	7.3
	5	26	2.0	13

Table 2. Summary of the Ratios of the Cyst(e)ine Sulfur Content of Protein Between Spores and Cells of Clostridium botulinum

Organism	Cyst(e)ine Sulfur Content* (μg S/mg N)		
	Spores**	Cells	Spore/Cell Ratio***
Langeland (Type F)	105	10	10.5 ± 3.8
OGB-69 (Type F)	99.1	11	9.0 ± 3.9
Beluga (Type E)	110	9.7	11.3 ± 5.1

* Average values from five or six determinations (see Appendix B).
** Coefficients of variation: Langeland (17%), OGB-69 (26%), Beluga (26%).
***Determined as the average of five or six individual ratios.

transfer were used both in preparation of the calibration curve and each experimental analysis. In addition, the standard error of estimate of the hydrogen sulfide calibration curve was quite small ($S_{y.x} = 0.01$). Therefore, the scatter of sulfur content values is probably due to random errors in (1) the completeness of the hydrazinolysis reaction; (2) loss of hydrogen sulfide during the hydrazinolysis reaction or the dilution and transfer of the decomposed material to the apparatus for the generation of hydrogen sulfide.

Despite the size of the errors, it is evident from the data in Table 1 and Table 2 that the spores of the test organisms contain about ten times more cyst(e)ine than the corresponding vegetative cells. In addition, there seems to be no difference between the spore/cell ratio of the three test organisms.

Furthermore, there is no perceptible difference between the cyst(e)ine sulfur contents of the two type F spores and the type E spore (see Table 2). These findings were considered in relation to the radiation resistances of the three types of Clostridium botulinum spores used in this thesis research. The data shown in Table 3 were supplied by Dr. N. W. Walls from experiments in which the spores were exposed to cesium-137 gamma irradiation while suspended in pH 7 phosphate buffer. The D_{10} values shown in the second column of Table 3 were calculated from data from the logarithmic death cycles of these spores, where inactivation occurs with exponential kinetics relative to dose. The observation that they do not differ significantly is consistent with the notion that a common mechanism of death is involved. However, the radiation doses

Table 3. Radiation Resistance of Spores of Clostridium botulinum

Organism*	D_{10} values** (in Mrad) for Logarithmic Death	D_{10} values*** (in Mrad) for First Log Cycle
Langeland (Type F)	.150	.56
OGB-69 (Type F)	.150	.48
Beluga (Type E)	.145	.31

* Cesium-137 gamma irradiation while suspended in pH 7 phosphate buffer (Walls, personal communication, unpublished data).

** The amount of radiation required for 90 percent inactivation of the microorganisms of a test population during the exponential portion of the survival curve.

***The amount of radiation required for the first log cycle reduction. The first log cycle includes the "shoulder" region where some type of cumulative action is indicated.

necessary to effect the first log cycle reduction are significantly different, and it appears that the spores of the two proteolytic type F organisms have some radioprotective mechanism which is present to a much lesser extent in the spores of the non-proteolytic type E test organism. Because there is no perceptible difference between the total cyst(e)ine sulfur contents of the two type F spores and the type E spore, it can be concluded that the total amount of cysteine and cystine is not involved in the radioprotective mechanism of these mature spores. It should be noted, however, that the errors in the measurement of total cyst(e)ine and radiation resistance were perhaps too large to permit detection of small correlations. In addition, it is possible that the content of either cysteine or cystine alone could be correlated to the radioprotective mechanism.

CHAPTER IV

CONCLUSIONS

Based on the research reported in the preceding text, the following conclusions are made:

Spores of the types of Clostridium botulinum analyzed in this research contain approximately ten times more cyst(e)ine than do the corresponding vegetative cells.

The total amount of cyst(e)ine in Clostridium botulinum spores is not correlated with the resistance of the spores to gamma irradiation.

No difference was found in the spore/cell cyst(e)ine content ratios between Langeland (Type F), OGB-69 (Type F), and Beluga (Type E).

CHAPTER V

RECOMMENDATIONS

Based on the results of this investigation, the following extensions of this research can be recommended:

The development of efficient methods for synchronization of Clostridium botulinum growth and spore formation would allow studies of the sequential expression of biochemical events and of the development of resistance. Specifically, such synchronization methods would permit experiments which would determine if the commencement of the increase in radioresistance coincides with the phase of enrichment of cellular proteins with cystine and the development of specific morphological structures of the spore. However, when conventional cultivation techniques are used the vegetative cell population consists of cells of different ages and physiological states, and chemical analysis of such cell mixtures must be somewhat inaccurate.

Chemical analysis of coat fractions isolated after disintegration of spores of Clostridium botulinum would help ascertain if cystine-rich proteins are located in these layers.

Hydrolysis of spore and cell proteins under conditions which would not destroy cysteine or cystine would furnish data which could be compared with results of hydrazinolysis experiments. In addition, such hydrolysis would permit separate analysis of cysteine and cystine. This type of data could demonstrate a correlation between one of these amino

acids and radioresistance which can not be detected when a total of the two is measured.

Determination of the resistances of the vegetative cells of the three test organisms to cesium-137 gamma irradiation would allow the calculation of an accurate ratio of the radiation resistances between spores and cells. This type of experiment would be technically difficult, however, because it would require the production of a spore-free vegetative cell crop and the irradiation and growth of these oxygen-sensitive organisms under strictly anaerobic conditions.

APPENDICES

APPENDIX A

Formulae of Media Used

1. Type C Toxin Medium

Proteose Peptone (Difco)*.....	120 grams
N-Z-amine type B (Sheffield Farms)**....	60 "
Yeast Extract (B-B-L)***.....	60 "
Dextrose (Mallinckrodt).....	30 "
Deionized Water.....	2850 ml

All the ingredients except the dextrose were added to the deionized water and the mixture was heated with constant stirring until the solid materials had dissolved. The pH of the three liters of medium was adjusted to 7.2 with 10 N NaOH and the medium was placed in a four liter Erlenmeyer flask which was then plugged with cotton and covered with aluminum foil. The medium was autoclaved at 15 psi and 120 C for 30 minutes. When the medium had cooled somewhat 150 ml of a filter-sterile 20 percent dextrose solution were added aseptically and the flask swirled gently to insure uniform mixing.

2. Peptone Water

Bacto-Peptone (Difco).....	1 gram
Deionized water.....	1000 ml

The peptone was dissolved in the deionized water and 99 ml aliquots

* Difco Laboratories, Detroit, Michigan.
** Sheffield Chemical, Norwich, New York.
***Baltimore Biological Laboratory, Baltimore, Maryland.

were dispensed into 150 ml milk dilution bottles. These were autoclaved at 15 psi and 120 C for 20 minutes, capped with rubber stoppers, and stored at room temperature until needed.

3. Fresh Pork Infusion Medium

A whole fresh pork ham was trimmed of fat, ground using the fine attachment of an electric food chopper* and mixed with tap water in the ratio of one pound of pork per liter of water. This mixture was brought to a boil, then simmered for one hour. While still hot, the meat and broth were filtered through eight layers of cheese-cloth to remove the meat particles. The pork infusion broth was caught in a large enamel boiler, then cooled overnight in a refrigerator to allow any fat still present to solidify as a layer on top of the medium. On the next day, after the fat had been skimmed from its surface, the broth was carefully measured into a clean enamel boiler and brought up to its original volume with tap water. The following ingredients were added:

Bacto-Peptone (Difco).....	5 g/l
Bacto-Tryptone (Difco).....	1.5 "
Dextrose (Mallinckrodt).....	1 "
Soluble Starch (Merck).....	1 "
Sodium Thioglycollate (Difco).....	1 "
K_2HPO_4 (Mallinckrodt).....	1.25 "

The medium was heated with frequent stirring until the dry ingredients had dissolved, then cooled to room temperature and adjusted to pH 7.4 with 10 N NaOH. The medium was dispensed into four liter Erlenmeyer

*Model D, General Slicing Machine Company, Walden, New York.

flasks (2.5 to 3 l/flask) and to each was added 1.5 percent (w/v) Bacto-Agar (Difco). The flasks were cotton-plugged, the plugs covered with aluminum foil, and the medium autoclaved at 15 psi and 120 C for 30 minutes. After autoclaving, the flasks were placed in a 55 C water bath in a tilted position and left overnight to allow the precipitate which forms in pork infusion medium to settle to the bottom of the flasks. The following day the clear portion of the medium was carefully decanted and filtered through four layers of cheese-cloth with a 1/2 inch layer of absorbent cotton between two of the layers. Twenty five ml of the clarified medium were dispensed into each Prickett tube (Fisher Scientific modified agar slant tube). The tubes were cotton-plugged, placed in deep round wire baskets, weighted with a board and one or two bricks, and autoclaved at 15 psi and 120 C for 30 minutes.

4. Trypticase Soy Broth (B-B-L)

Fifteen grams of trypticase soy broth were added to 500 ml of deionized water in a one liter, screw-capped, Erlenmeyer flask and mixed until solution was complete. The medium was autoclaved at 15 psi and 120 C for 15 minutes.

5. Cooked Meat Medium (Difco)

Thirty milliliters of deionized water were added to 3.75 g of the dry cooked meat medium in a large screw-capped culture tube (2.5 cm x 20 cm) and the mixture allowed to stand 15 minutes to insure complete wetting of the meat particles before autoclaving at 15 psi and 120 C for 15 minutes. The sterile medium was cooled to room temperature and immediately inoculated with a thawed stock culture.

Sources of Chemicals Used

Trichloroacetic acid, sodium carbonate, sulfuric acid, anhydrous ether, hydrochloric acid and potassium phosphate dibasic were obtained from Mallinckrodt Chemical Works, New York, New York. Zinc acetate, l-cystine, ferric ammonium sulfate, sodium chloride, hydrazine hydrate and Folin-Ciocalteau reagent were obtained from Fisher Scientific Company, Atlanta, Georgia. Bovine serum albumin was supplied by Armour Pharmaceutical Company, Kankakee, Illinois; cysteine monohydrochloride by Mann Research Laboratories, Inc., New York, New York; N, N, dimethyl-p-phenylenediamine monohydrochloride by Eastman Organic Chemicals, Rochester, New York; copper sulfate and acetic acid by Merck and Company, Inc., Rahway, New Jersey; sodium potassium tartrate and sodium acetate by Baker Chemical Company, Phillipsburg, New Jersey; lysozyme by Nutritional Biochemicals Corporation, Cleveland, Ohio; trypsin and sodium thioglycollate by Difco Laboratories, Detroit, Michigan.

APPENDIX B

Table 4. Computation of Sums for Correlation of 0 to 66 Micrograms of Protein Nitrogen (X) and Absorbance (Y) at 500 nm

Case	X	Y	X^2	Y^2	XY
1	11	0.156	121	0.024336	1.716
2	11	.169	121	.028561	1.859
3	22	.323	484	.104329	7.106
4	22	.320	484	.102400	7.040
5	22	.305	484	.093025	6.710
6	33	.426	1089	.181476	14.058
7	33	.433	1089	.187489	14.289
8	44	.572	1936	.327184	25.168
9	44	.571	1936	.326041	25.124
10	44	.545	1936	.297025	23.980
11	55	.640	3025	.409600	35.200
12	55	.650	3025	.422500	35.750
13	66	.767	4356	.588289	50.622
14	66	.775	4356	.600625	51.150
15	66	.762	4356	.580644	50.292
16	66	.745	4356	.555025	49.170
TOTAL	660	8.159	33154	4.828549	399.234

Table 5. Computation of Sums for Correlation of 77-176 Micrograms of Protein Nitrogen (X) and Absorbance (Y) at 500 nm

Case	X	Y	X^2	Y^2	XY
1	77	0.853	5929	0.727609	65.681
2	77	.857	5929	.734449	65.989
3	88	.951	7744	.904401	83.688
4	88	.963	7744	.927369	84.744
5	88	.917	7744	.840889	80.696
6	88	.917	7744	.840889	80.696
7	99	1.036	9801	1.073296	102.564
8	99	1.022	9801	1.044484	101.178
9	110	1.097	12100	1.203409	120.670
10	110	1.131	12100	1.279161	124.410
11	110	1.081	12100	1.168561	118.910
12	110	1.081	12100	1.168561	118.910
13	176	1.538	30976	2.365444	270.688
14	176	1.553	30976	2.411809	273.328
TOTAL	1496	14.997	172788	16.690331	1692.152

Table 6. Computation of Sums for Correlation of Micromoles of Hydrogen Sulfide (X) and Absorbance (Y) at 630 nm

Case	X	Y	X^2	Y^2	XY
1	0.5	0.081	0.25	0.006561	0.0405
2	0.5	.093	.25	.008649	.0465
3	0.5	.104	.25	.010816	.0520
4	0.5	.101	.25	.010201	.0505
5	0.5	.080	.25	.006400	.0400
6	0.5	.110	.25	.012100	.0550
7	0.5	.098	.25	.009604	.0490
8	0.5	.089	.25	.007921	.0445
9	0.5	.109	.25	.011881	.0545
10	0.5	.110	.25	.012100	.0550
11	1.0	.200	1.0	.040000	.2000
12	1.0	.205	1.0	.042025	.2050
13	1.0	.217	1.0	.047089	.2170
14	1.0	.191	1.0	.036481	.1910
15	1.0	.210	1.0	.044100	.2100
16	1.0	.220	1.0	.048400	.2200
17	1.0	.195	1.0	.038025	.1950
18	1.0	.187	1.0	.034969	.1870
19	1.0	.207	1.0	.042849	.2070
20	1.0	.221	1.0	.048841	.2210
21	2.0	.385	4.0	.148225	.7700
22	2.0	.397	4.0	.157609	.7940
23	2.0	.407	4.0	.165649	.8140
24	2.0	.380	4.0	.144400	.7600
25	2.0	.411	4.0	.168921	.8220
26	2.0	.406	4.0	.164836	.8120
27	2.0	.391	4.0	.152881	.7820
28	2.0	.402	4.0	.161604	.8040
29	2.0	.394	4.0	.155236	.7880
30	2.0	.401	4.0	.160801	.8020
31	3.0	.611	9.0	.373321	1.8330
32	3.0	.625	9.0	.390625	1.8750
33	3.0	.609	9.0	.370881	1.8270
34	3.0	.614	9.0	.376996	1.8420
35	3.0	.595	9.0	.354025	1.7850
36	3.0	.600	9.0	.360000	1.8000
37	3.0	.612	9.0	.374544	1.8360
38	3.0	.618	9.0	.381924	1.8540
39	3.0	.589	9.0	.346921	1.7670

Table 6. (Continued)

Case	X	Y	X^2	Y^2	XY
40	3.0	.610	9.0	.372100	1.8300
41	4.0	.780	16.0	.608400	3.1200
42	4.0	.794	16.0	.630436	3.1760
43	4.0	.802	16.0	.643204	3.2080
44	4.0	.813	16.0	.660969	3.2520
45	4.0	.788	16.0	.620944	3.1520
46	4.0	.811	16.0	.657721	3.2440
47	4.0	.805	16.0	.648025	3.2200
48	4.0	.809	16.0	.654481	3.2360
49	4.0	.797	16.0	.635209	3.1880
50	4.0	.801	16.0	.641601	3.2040
51	5.0	1.003	25.0	1.006009	5.0150
52	5.0	1.011	25.0	1.022121	5.0550
53	5.0	0.984	25.0	0.968256	4.9200
54	5.0	1.002	25.0	1.004004	5.0100
55	5.0	1.014	25.0	1.028196	5.0700
56	5.0	0.995	25.0	0.990025	4.9750
57	5.0	0.990	25.0	0.980100	4.9500
58	5.0	0.992	25.0	0.984064	4.9600
59	5.0	1.016	25.0	1.032256	5.0800
60	5.0	1.009	25.0	1.018081	5.0450
TOTAL	155	31.101	552.5	22.234400	110.8175

Computation of the Estimating Equation and Standard Error ($S_{y.x}$) for the Determination of Hydrogen Sulfide

The estimating equation for hydrogen sulfide was calculated by solving the two "normal" equations:

$$\text{I.} \quad \Sigma Y = Na + b\Sigma X$$

$$\text{II.} \quad \Sigma XY = a\Sigma X + b\Sigma X^2$$

Where X = an observed value of X (μmole H_2S)

Y = an observed absorbance value

a = Y-intercept

b = slope

Using sums from Table 6

$$\text{I.} \quad 31.101 = 60a + 155b$$

$$\text{II.} \quad 110.817 = 155a + 552.5b$$

$$a = 0.00$$

$$b = 0.20$$

The estimating equation is $Y = 0.20\ X$

The standard error ($S_{y.x}$) of estimate was calculated by substituting values obtained in Table 5 into the following formula:

$$S_{y.x} = \sqrt{\frac{\Sigma Y^2 - (a\Sigma Y + b\Sigma XY)}{N}}$$

$$S_{y.x} = \sqrt{\frac{22.2344 - [(0.00)(31.101) + (0.20)(110.8175)]}{60}}$$

$$S_{y.x} = 0.01$$

Computation of the Estimating Equations and Standard Errors ($S_{y.x}$) for the Determination of Total Protein

The estimating equations and standard errors of estimate were calculated using values obtained in Table 4 and Table 5.

For 0 to 66 µg protein:

$$\text{I.} \quad 8.159 = 16a + 660b$$

$$\text{II.} \quad 399.234 = 660a + 33154b$$

$$a = 0.074$$

$$b = 0.011$$

The estimating equation is: $Y = 0.074 + 0.011X$

$$S_{y.x} = \sqrt{\frac{4.828549 - [(0.074)(8.159) + (0.011)(399.234)]}{16}}$$

$$S_{y.x} = 0.02$$

For > 66 µg protein:

$$\text{I.} \quad 14.997 = 14a + 1496b$$

$$\text{II.} \quad 1692.152 = 1496a + 172788b$$

$$a = 0.331$$

$$b = 0.007$$

The estimating equation is: $Y = 0.331 + 0.007X$

$$S_{y.x} = \sqrt{\frac{16.690331 - [(0.331)(14.997) + (0.007)(1692.152)]}{14}}$$

$$S_{y.x} = 0.02$$

Computation of the Mean ($\bar{X}$) and Standard Deviation ($\hat{\sigma}$) of Observed Data from Chemical Analyses of *Clostridium botulinum*, Type F, Langeland

Langeland Spore	
X (μg S/mg N)	X^2
90.4	8172
86.7	7517
85.9	7379
107	11449
133	17689
124	15376
627	67582

$$\bar{X} = \frac{\Sigma X}{N} = \frac{627}{6} = 104.5$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{67582}{6} - \left(\frac{627}{6}\right)^2}$$

$$\hat{\sigma} = \sqrt{343.4} = 18.5$$

Langeland Cell	
X (μg S/mg N)	X^2
10	100
13	169
11	121
8.6	74
7.4	55
50	519

$$\bar{X} = \frac{\Sigma X}{N} = \frac{50}{5} = 10$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{519}{5} - \left(\frac{50}{5}\right)^2}$$

$$\hat{\sigma} = \sqrt{3.8} = 1.9$$

Computation of the Ratio of (μg S/mg N) Between Langeland Spores and Cells

$$\text{Spore/Cell} = \frac{105 \pm 18.5}{10 \pm 1.9} = \frac{105 \pm 17\%}{10 \pm 19\%} = 10.5 \pm 36\% = 10.5 \pm 3.8$$

Computation of the Mean ($\bar{X}$) and the Standard Deviation ($\hat{\sigma}$) of Observed Data from Chemical Analyses of Clostridium botulinum, Type F, OGB-69

OGB-69 Spore	
X (µg S/mg N)	X^2
74.8	5595
70.0	4900
87.6	7674
122	14884
142	20164
98.0	9604
594.4	62821

$$\bar{X} = \frac{\Sigma X}{N} = \frac{594.4}{6} = 99.06$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{62821}{6} - \left(\frac{594.4}{6}\right)^2}$$

$$\hat{\sigma} = \sqrt{656} = 25.6$$

OGB-69 Cell	
X (µg S/mg N)	X^2
9.8	96
14	196
11	121
12	144
8.0	64
54.8	621

$$\bar{X} = \frac{\Sigma X}{N} = \frac{54.8}{5} = 10.96$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{621}{5} - \left(\frac{54.8}{5}\right)^2}$$

$$\hat{\sigma} = \sqrt{4.08} = 2.02$$

Computation of the Ratio of (µg S/mg N) Between OGB-69 Spores and Cells

$$\text{Spore/Cell} = \frac{99.1 \pm 25.6}{11 \quad \pm 2.0} = \frac{99.1 \pm 26\%}{11 \quad \pm 18\%} = 9.0 \pm 44\% = 9.0 \pm 3.9$$

Computation of the Mean ($\bar{X}$) and the Standard Deviation ($\hat{\sigma}$) of Observed Data from Chemical Analyses of *Clostridium botulinum*, Type E, Beluga

Beluga Spore	
X (µg S/mg N)	X^2
76	5776
160	25600
120	14400
100	10000
92	8464
548	64240

$$\bar{X} = \frac{\Sigma X}{N} = \frac{548}{5} = 109.6$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{64240}{5} - \left(\frac{548}{5}\right)^2}$$

$$\hat{\sigma} = \sqrt{835.8} = 28.9$$

Beluga Cell	
X(µg S/mg N)	X^2
11	121
8.3	69
8.8	77
7.3	53
13	169
48.4	489

$$\bar{X} = \frac{\Sigma X}{N} = \frac{48.4}{5} = 9.68$$

$$\hat{\sigma} = \sqrt{\frac{\Sigma X^2}{N} - \left(\frac{\Sigma X}{N}\right)^2}$$

$$\hat{\sigma} = \sqrt{\frac{489}{5} - \left(\frac{48.4}{5}\right)^2}$$

$$\hat{\sigma} = \sqrt{4.09} = 2.02$$

Computation of the Ratio of (µg S/mg N) Between Beluga Spores and Cells

$$\text{Spore/Cell} = \frac{110 \pm 28.9}{9.7 \pm 2.0} = \frac{110 \pm 26\%}{9.7 \pm 21\%} = 11.3 \pm 47\% = 11.3 \pm 5.1$$

LITERATURE CITED

AKABORI, S., OHNO, K., AND NARITA, K. 1952. Reductive decomposition of cystine by hydrazine. Bull. Chem. Soc. Japan 25: 214.

ALMY, L. H. 1925. A method for the estimation of hydrogen sulfide in proteinaceous food products. JACS 47: 1381-1390.

ARONSON, A. I. 1965a. Characterization of messenger RNA in sporulating Bacillus cereus. J. Mol. Bio. 11: 576-588.

------ 1965b. Membrane-bound messenger RNA and polysomes in sporulating bacteria. J. Mol. Biol. 13: 92-104.

ARONSON, A. I., AND FITZ-JAMES, P. C. 1968. Biosynthesis of bacterial spore coats. J. Mol. Biol. 33: 199-212.

BARRON, E. S. G. 1955. The effect of ionizing radiation on systems of biological importance. Ann. NY Acad. Sci. 49: 574-595.

BOTT, K. F., AND LUNDGREN, D. G. 1964. The relationship of sulfhydryl and disulfide constituents of Bacillus cereus to radioresistance. Radiation Research 21: 195-211.

CAVALLINI, D., MONDOVI, B., GIOVANELLA, B., AND DE MARCO, C. 1960. Disulfide interchange by ionizing radiation. Science 131: 1441.

CROXTON, F. E. 1959. Elementary statistics with applications in medicine and the biological sciences. Dover Publications, Inc., New York.

EAGLE, H. OYAMA, V. I., AND PIEZ, K. A. 1960. The reversible binding of half-cystine residues to serum protein, and its bearing on the cystine requirement of cultured mammalian cells. J. Biol. Chem. 235: 1719-1726.

ELDJARN, L., AND PIHL, A. 1957. On the mode of action of X-ray protective agents. II. Interaction between biologically important thiols and disulfides. J. Biol. Chem. 225: 499-510.

------ 1958. The cysteine-cysteamine group of protective agents: chemical structure, protective ability, and mixed disulfide formation. Radiation Research 9: 110.

GORDY, W., ARD, W. B., AND SHIELDS, H. 1955. Microwave spectroscopy of biological substances. I. Paramagnetic resonance in X-irradiated amino acids and proteins. Proc. Nat. Acad. Sci. 41: 983-1004.

GORDY, W., AND MIYAGAWA, I. 1960. Electron spin resonance studies of mechanisms for chemical protection from ionizing radiation. Radiation Research 12: 211-229.

GORDY, W., AND SHIELDS, H. 1958. Electron spin resonance studies of radiation damage to proteins. Radiation Research 9: 611-625.

GOULD, G. W., AND HITCHINS, A. D. 1963. Sensitization of bacterial spores to lysozyme and to hydrogen peroxide with agents which rupture disulfide bonds. J. Gen. Microbiol. 33: 413.

GOULD, G. W., and ORDAL, Z. J. 1968. Activation of spores of Bacillus cereus by gamma-radiation. J. Gen. Microbiol. 50: 77-84.

GRECZ, N. 1965. Biophysical aspects of clostridia. J. Appl. Bact. 28: 17-35.

GRECZ, N., ANELLIS, A., AND SCHNEIDER, M. D. 1962. Procedure for cleaning of Clostridium botulinum spores. J. Bact. 84: 552-558.

HITCHINS, A. D., KING, W. L., AND GOULD, G. W. 1966. Role of disulfide bonds in the resistance of Bacillus cereus spores to gamma-radiation and heat. J. Appl. Bact. 29: 505-511.

KADOTA, H. IIJIMI, K., AND UCHIDA, A. 1965. The presence of keratin-like substance in spore coat of Bacillus subtilis. Agr. Biol. Chem. 29: 870-875.

KIRK, P. L. 1947. The chemical determination of proteins. Advances in Protein Chem. 3: 139-167.

KONDO, M., AND FOSTER, J. W. 1967. Chemical and electron microscope studies on fractions prepared from coats of Bacillus spores. J. Gen. Microbiol. 47: 257-271.

KURATOMI, K., OHNO, K., AND AKABORI, S. 1957. A new microdetermination of the total quantity of cystine plus cysteine in protein by hydrazinolysis. J. Biochem. (Tokyo) 44: 183-190.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., AND RANDALL, R. J. 1951. J. Biol. Chem. 193: 265-275.

MORTENSON, L. E., AND BEINERT, H. 1953. Appearance of sulfhydryl groups during growth of Bacillus globigii. J. Bact. 66: 101-104.

MURRELL, W. G. 1967. The biochemistry of the bacterial endospore, p 133-251. In A. H. Rose and J. F. Wilkinson (eds.), Advances in Microbiol Physiology, Vol. 1. Academic Press, New York.

OHYE, D. F., AND MURRELL, W. G. 1962. Formation and structure of the spore of Bacillus coagulans. J. Cell. Biol. 14: 111-123.

ORMEROD, M. G., AND ALEXANDER, P. 1962. Repair of radiation damage in a nucleoprotein by cysteamine. Nature 193: 290-291.

POWERS, E. L., AND KALETA, B. F. 1960. Reduction of radiation sensitivity of dry bacterial spores with hydrogen sulfide. Science 132: 959-960.

RAY, D. K., HUTCHINSON, F., AND MOROWITZ, H. J. 1960. A connection between disulfide bond breakage and inactivation by radiation of a dry enzyme. Nature 186: 312-313.

RODE, L. J., AND WILLIAMS, M. G. 1966. Utility of sodium hypochlorite for ultrastructure study of bacterial spore integuments. J. Bact. 92: 1772-1778.

ROWLEY, D. B., AND NEWCOMB, H. R. 1964. Radiosensitivity of several dehydrogenases and transaminases during sporogenesis of Bacillus subtilis. J. Bact. 87: 701-709.

SMITHIES, O. 1965. Disulfide bond cleavage and formation in proteins. Science 150: 1595-1602.

SPUDICH, J. A., AND KORNBERG, A. 1968. Biochemical studies of bacterial sporulation and germination. J. Biol. Chem. 243: 4588-4599.

ST. LORANT, I. 1929. Uber eine neue colorimetrische micromethode zur bestimmung des schwefels in sulfiden, sulfaten. Z. Physiol. Chem. 185: 245-266.

STUY, J. H. 1956. Studies on the mechanism of radiation inactivation of microorganisms. III. Inactivation of germinating spores of Bacillus cereus. Biochimica et Biophysica Acta. 22: 241-246.

TSUJI, K., AND PERKINS, W. E. 1962. Sporulation of Clostridium botulinum. J. Bact. 84: 81-85.

VINTER, V. 1959a. Difference in cyst(e)ine content between vegetative cells and spores of Bacillus cereus and Bacillus megaterium. Nature 183: 998-999.

------ 1959b. The participation of cysteine and cystine in spore formation by Bacillus megaterium. Folia Microbiol 4: 216-221.

------ 1960a. Spores of microorganisms. VIII. Synthesis of specific calcium and cystine containing structures in sporulating cells of bacilli. Folia Microbiol. 5: 217-230.

------ 1960b. The formation of cystine-rich structure in sporulating cells and its possible role in the resistance of spores, p 127-141. In H. O. Halvorson (ed.), Spores II. Burgess Publishing Company, Minneapolis, Minnesota.

VINTER V. 1961. Changes in radioresistance of sporulating cells of Bacillus cereus. Nature 189: 589-590.

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

------ 1969. Physiology and biochemistry of sporulation, p 73-123. In G. W. Gould and A. Hurst (eds.), The Bacterial Spore. Academic Press, New York.

WALLS, N. W. Personal communication (unpublished data).

WARTH, A. D., OHYE, D. F., AND MURRELL, W. G. 1963. The composition and structure of bacterial spores. J. Cell. Biol. 16: 579-592.