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TECHNICAL REPORT  
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**MODE OF RADIATION RESISTANCE  
OF  
CLOSTRIDIUM BOTULINUM SPORES**

by

Brooks D. Church, Gregory R. Germaine  
and

Durwood B. Rowley

North Star Research

and

Development Institute  
Minneapolis, Minnesota

Contract No. DAAG 17-68-C-0094

August 1970

UNITED STATES ARMY  
NATICK LABORATORIES  
Natick, Massachusetts 01760



FOOD LABORATORY  
FL-112

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Food Laboratory  
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## FOREWORD

In developing a new food preservation technology such as "Radiation Sterilization", the process must impart microbiological safety and stability to the product. The prevailing "Safety Concept" requires a "Minimal Radiation Dose" (MRD) equivalent to a computed 12-log cycle reduction (12-D) in a Clostridium botulinum spore population. Since Cl. botulinum spores, types A and B are the most radiation resistant among the bacteria of public health concern they have been selected as the microbial index for determining the MRD requirement for radiation sterilized meats. The MRD presently required may stress the organoleptic qualities of certain foods such as beef. In order to alleviate or minimize this disadvantage, one has two potential approaches: (a) attempt to establish radiation conditions under which the radiation effect becomes relatively selective towards spore destruction; and (b) attempt to establish means of reducing the spore's resistance to radiation. In either case the spore's reaction to or interaction with its irradiation environment is a key factor. Thus the objective of this study was to attempt to elucidate the mechanism(s) of Cl. botulinum spore resistance to radiation energy.

The experimental effort described herein was performed at the North Star Research and Development Institute, Minneapolis, Minnesota 55406, under Contract Number DAAG 17-68-C-0094. This research was done under Project Number LJ062110A033, Radiation Preservation of Food. The Project Officer and Alternate Project Officer for the U. S. Army Natick Laboratory were Dr. Durwood B. Rowley and Mr. Abe Anellis, respectively.

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## ABSTRACT

The purpose of this project was to investigate possible mechanism(s) of the radiation resistance of Clostridium botulinum spores. A better understanding of the radioresistance of this important food pathogen would enhance ones ability to reduce the minimal radiation dose (MRD) presently required for the radiation sterilization of meats. The ultimate reduction of the MRD would result in an improvement in the organoleptic quality of certain established prototypes, particularly those sensitive to the prevailing dose levels.

During germination the loss of heat resistance preceded the loss of radiation resistance. Changes in radioresistance during germination were not due to loss of calcium, dipicolinic acid (DPA) or disulfide-rich protein. Studies with DPA-less spores of Bacillus cereus showed that they were just as resistant to radiation as the wild type (DPA<sup>+</sup>) spores. Radiosensitization during germination occurred prior to synthesis of ribonucleic acid, proteins and deoxyribonucleic acid (DNA) and therefore was concluded to be independent of macromolecular synthesis.

Spores whose disulfide sulfur was reduced to sulfhydryl sulfur remained radioresistant. The form of sulfur in spores may play a small role in radioresistance but was not responsible for the spores high resistance relative to the vegetative cell.

Conditions (spores produced in the presence of bromodeoxyuridine) known to specifically sensitize vegetative cell DNA to irradiation similarly sensitized spores. It follows that DNA is indeed a target in spores of Cl. botulinum. Changes in the hydration state of the spore and the physical state (dehydrated) of the target molecule (DNA) are discussed as possible mechanisms of the radiation resistance of Cl. botulinum spores. However, further research is required to actually delineate the mechanism of Cl. botulinum spore radioresistance.

## INTRODUCTION

The purpose of this study was to determine the reason(s) for the greater resistance of Clostridium botulinum spores to ionizing radiation compared to the vegetative form of the same organism. It was hoped that information of this kind would serve as a guide to the development of suitable food radiation sterilization procedures. Presently, the total dosages required for food sterilization are set at that dose which is sufficient to yield less than  $1/10^{12}$  of the original population as survivors(1). In general, the doses required for this level of killing (depending on the food material to be sterilized) are in the megarad (Mrad) range. Many food materials are appreciably changed by such radiation doses (i.e., taste, smell)(1). By understanding the mechanism of spore radioresistance, it seemed reasonable that environmental manipulation of foods to bring about increased spore radiosensitivity might proceed on a less empirical basis than in the past.

This report covers Phases I and II of a study of the "Mode of Radiation Resistance of Clostridium botulinum Spores" under sponsorship of U. S. Army Natick Laboratories. It covers the period 14 March 1968 to 15 April 1970.

## MATERIALS AND METHODS

### Organisms

The organism used in most of this study was Clostridium botulinum 62A. The culture was obtained from Dr. Durwood Rowley of the Natick Laboratories. One experiment is reported in which Bacillus cereus T was used. Two strains, a wild type and a mutant (HW 3) that produced heat sensitive, dipicolinic acid-less spores<sup>(2)</sup>, were obtained from Dr. H. Orin Halvorson of the University of Minnesota.

### Media

Compositions of the media used in these studies are given in the following tables:

Table 1  
Sporulation Medium (SM)

Component	g/liter
Trypticase (BBL)*	40
Bacto-Peptone (Difco)	5.0
KH <sub>2</sub> PO <sub>4</sub>	1.25
Yeast extract (BBL)	1.0

\* Baltimore Biological Laboratory

Table 2  
Thiotone-TES-Thioglycollate Medium (TTT)

Component	g/liter
Thiotone (BBL)	30
Sodium thioglycollate (BBL)	0.5
N-tris (hydroxymethyl) methyl-2-amino-ethane sulfonic acid (TES)	23
Sodium bicarbonate	10

Table 3  
Trypticase-Yeast Extract-Phosphate Medium (TYP)

Component	g/liter
Trypticase (BBL)	50
Yeast extract (BBL)	5.0
Bacto-Peptone	5.0
Soluble starch	1.0
Glucose	0.10
K <sub>2</sub> HPO <sub>4</sub>	1.25
Sodium thioglycollate (BBL)	0.50
Bacto-Agar	10.0

Table 4  
Cysteine-TES-Thioglycollate Germination Medium

Component	Concentration (mM)
L-cysteine	8.0
Sodium thioglycollate	4.4
TES (pH 7.0)	100
Sodium bicarbonate	11.9

Table 5  
Gelatin-Phosphate (G·P) Diluent

Component	g/liter
Gelatin	2
Na <sub>2</sub> HPO <sub>4</sub>	4

SM broth was used for spore production. The pH was adjusted to 7.5 with N NaOH prior to sterilization by autoclaving. The pH was not changed by autoclaving.

TTT broth was used for routine growth of the organism and spore germination. The final pH of this medium was 7-7.5. All components except the bicarbonate were mixed and filter sterilized (autoclaving reduced both the growth rate and extent and rate of germination of spores). The bicarbonate-lacking broth was, in many cases, steamed for 20 minutes (to remove dissolved oxygen) and rapidly cooled to 37°C prior to addition of a filter sterilized bicarbonate solution. The steaming procedure reduced the time for emergence of germinated spores from 5 to 3 hours. Finally, TTT broth was used as a semisolid medium (0.8 percent Bacto-Agar) for titering the organism with the tube procedure (see next section) in the early phase of this work.

TYP agar<sup>(1)</sup> was used for plating exclusively in the latter phases of the study. This medium was superior to TTT since colonies could be counted after 24 hours incubation. TTT medium, on the other hand, required 36-48 hours for full colonial development. For enumeration of spores and germinated spores, sodium bicarbonate (0.5 mg/ml) was included in the medium. As in TTT, a solution of bicarbonate was added to the autoclaved and cooled (48°C) agar medium. The final pH of this medium was 7-7.5.

The cysteine germination broth was developed and communicated to us by Dr. Durwood Rowley (Natick Laboratories). A solution containing L-cysteine, thioglycollate, and TES was filter sterilized, steamed (20 minutes), and cooled to 37°C. Then, bicarbonate solution was added.

In all cases, the air head space of broth cultures was flushed with pure N<sub>2</sub> gas through a sterilized cotton filter prior to inoculation.

The enumeration of B. cereus was done on Bacto-Nutrient Broth solidified with Bacto-Agar (2 percent).

G·P was used in all cases for dilution. The final pH (after autoclaving) was 7-7.5.

#### Viability Determinations

Three methods of titering were used during the course of this study. The first method (tube) was used prior to the purchase of a Thelco anaerobic incubator. In this method, an aliquot of diluted organisms (1 ml) was added to a 12 x 250 mm tube. Then TTT medium (containing 0.8 percent agar) at 48°C was added (20 ml) in such a manner that thorough mixing of the media and organisms occurred. Finally, an agar plug (1-2 ml) was added to the top of the solidified media column. The tubes were incubated at 37°C for 36-48 hours. With this method, it was difficult to accurately count more than 50 colonies per tube, thus we turned to methods allowing the use of petri dishes.

In a few cases, spread plates were used. Aliquots of diluted organisms (0.10 ml) were spread with sterile glass spreaders onto the surface of 1.5 or 2.0 percent agar plates. The plates were incubated in a nitrogen atmosphere at 37°C. To replace the air atmosphere with nitrogen gas, the incubator was evacuated to the vapor pressure of water, and the chamber was filled with nitrogen gas. This process was repeated 6-7 times, resulting in less than 0.001 percent air in the incubator (calculated as a dilution problem based on information supplied with the incubator). The evacuating process pulled the agar in the plates into the cover of the dish if they were incubated upside down, thus, the plates were not inverted. Occasionally, due to the high relative humidity in the incubator, the surface colonies spread over the surface of the plates in a thin film of water. The spreading problem plus the time required to spread the dilutions on the plates discouraged this method.

We finally settled on using the pour plate method with 1 percent agar medium. Aliquots of the dilutions (1 ml) were pipetted into the petri dish, then about 20 ml of molten TYP media (48°C) poured into the dish. The dishes were swirled to facilitate even mixing and then the agar allowed to solidify. The plates were incubated (upright) in the anaerobic incubator as described above. The disadvantage of this method lies in the tendency of the organism to spread and grow between the agar and plate. Gas bubbles occasionally formed but were not troublesome. The bulk of the viability studies were done using the pour plate method.

#### Production of Spores

Sporulated cells grown in SM broth were harvested by centrifugation (10,000 x g, 4°C, 10 min), washed twice with sterile, deionized H<sub>2</sub>O and suspended in one-tenth the original culture volume. Lysozyme was added to a concentration of 0.5 mg/ml. After 60 minutes at 37°C, pronase was added to a final concentration of 1 mg/ml. Incubation continued for 37°C for 5-10 minutes. If the spores were exposed to pronase for more than 15-20 minutes, a slow phase-darkening occurred. Next, sodium lauryl sulfate was added to a concentration of one percent, and the detergent-treated spore suspension was heated at 60°C for 15 minutes. The spores were collected by centrifugation and washed with H<sub>2</sub>O until no evidence of detergent residues was seen as determined by excess foaming upon vigorous mixing. The spore pellet was resuspended in one-half the above volume of water and washed twice more by low-speed centrifugation (5,000 x g, 4°C, 5 min). Finally, the spores were dialyzed against distilled water overnight and the spore dialysate was collected and stored at 4°C for experimental use. Stock spore suspensions (~2 x 10<sup>9</sup>/ml) contained 1-2 percent germinated forms.

Alternatively, if most (>90 percent) of the spores were released from the sporangium, the pronase procedure could be omitted, and water washes together with differential centrifugations could be employed directly. No

differences in radiation sensitivity were noticed after either of these methods was used for obtaining clean spores. The studies involving sulfur analysis and germination-induced radioresistance changes were done on enzyme- and detergent-treated spores.

### Germination of Spores

Clostridium spores were germinated in TTT broth at the desired temperature. Unless otherwise stated, the germination was done in sterile Klett tubes (10 ml size) containing either 5 or 10 ml of broth plugged with rubber serum caps. Germination was measured by following the decrease in optical density (Klett-Summerson Colorimeter, #66 filter) of the spore suspension. As stated earlier, in some cases the TTT broth was steamed before adding spores, and in others it was not. The effect of steaming on heat activated (80°C/10 minutes) spore germination at 37°C is shown in Figure 1. The initial rates of germination are similar (up to 30 minutes), thereafter the process is faster in the steamed broth. Outgrowth occurs 3 hours sooner in steamed broth than in the unsteamed system. The extent of germination (maximum percent drop in optical density) is similar in both cases.

The requirement for bicarbonate and a reduced oxygen concentration ( $N_2$  flush) for extensive spore germination is shown in Figure 2. Spores were inoculated into unsteamed TTT broth at 25°C that (a) received no additions ( $\Delta$ ), (b) received only  $N_2$  ( $\blacktriangle$ ), (c) received only bicarbonate ( $HCO_3^-$ ) and (d) received both bicarbonate and  $N_2$  ( $N_2 + HCO_3^-$ ). Germination did not occur in the absence of bicarbonate ( $N_2$  or control) and to only a limited extent in the presence of bicarbonate but absence of a  $N_2$  flush ( $HCO_3^-$ ). Germination proceeded at a faster rate and to a greater degree in the complete system ( $N_2 + HCO_3^-$ ) than in the  $HCO_3^-$  system.

Effect of heat activation time at 80°C on spore germination at 37°C in Rowley's cysteine system is shown in Figure 3. In this system, heat activation at 80°C for 10 minutes was insufficient for good germination. At the suggestion of Dr. Rowley, we heat activated the spores for 60 minutes at 80°C and obtained germination typical of his results (Rowley, personal communication). Microscopic examination of 60 minute heat-activated spores germinated in this system for 285 minutes revealed the spores were phase dark, but not swollen.

### Chemical Analyses of Spores

Dipicolinic acid was determined in spores using a modification<sup>(3)</sup> of the original method devised by Janssen, Lund and Anderson<sup>(4)</sup>. Protein content of spores was determined following extraction of spores with cold ten percent trichloroacetic acid (TCA) followed by extraction at 90°C for 30 minutes with ten percent TCA. The final pellet (containing the protein)

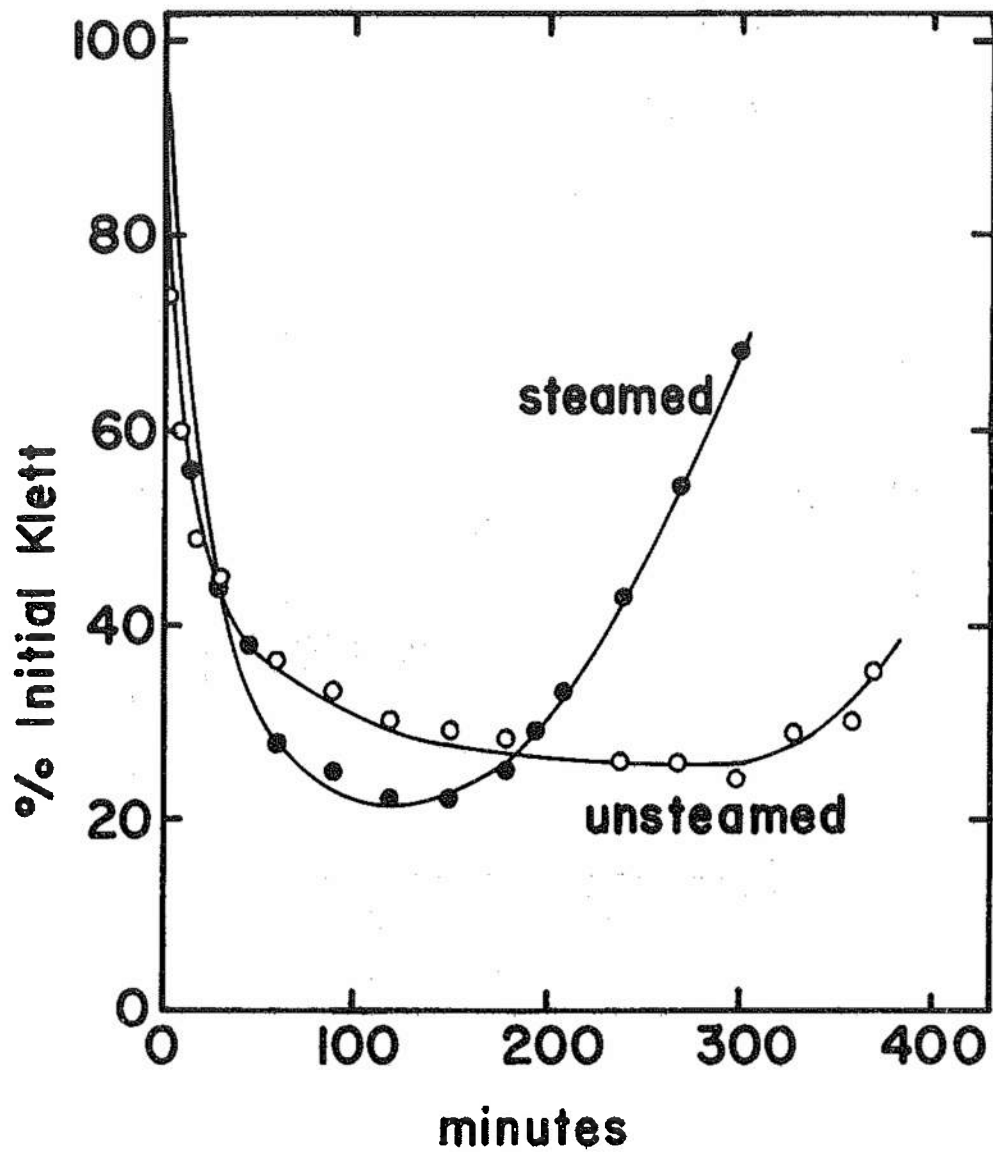


Figure 1. Germination of *Cl. botulinum* spores in deaerated (steamed) and non-deaerated (unsteamed) TTT broth.

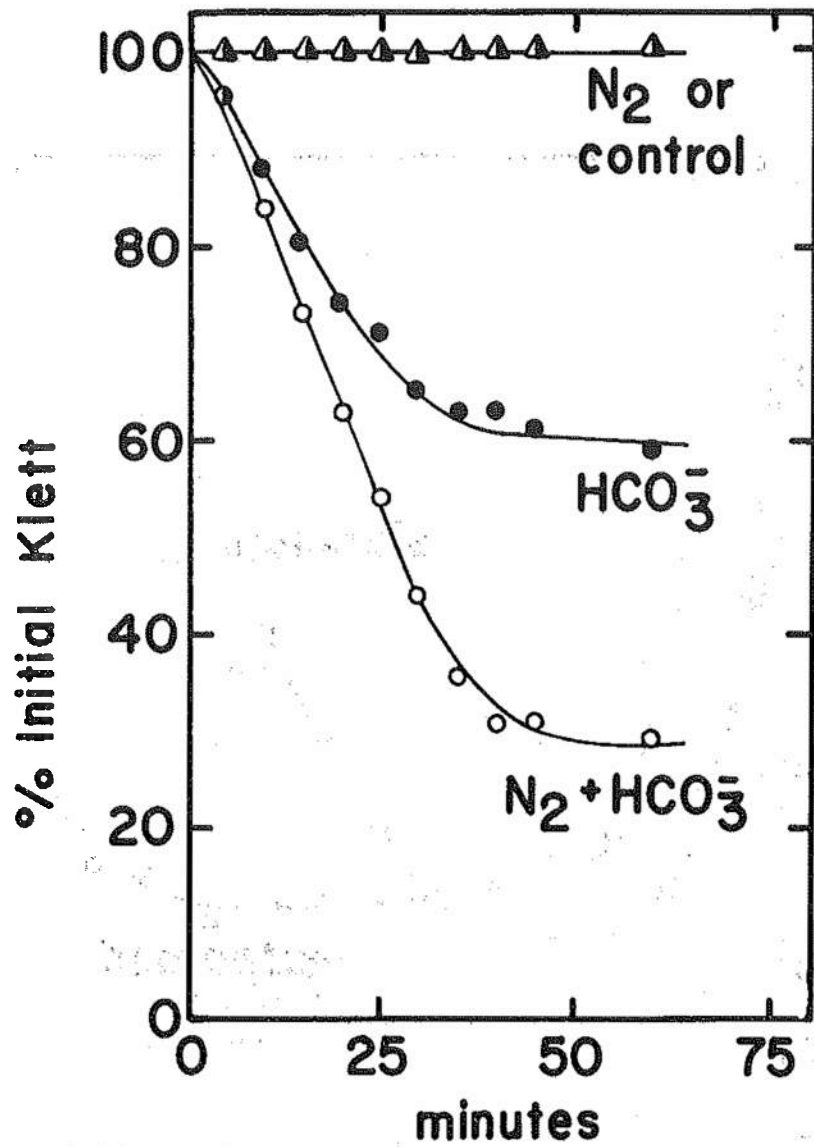


Figure 2. Effect of N<sub>2</sub> and bicarbonate on germination of *Cl. botulinum* spores in TTT broth.

- a) TTT broth without HCO<sub>3</sub><sup>-</sup> or N<sub>2</sub> flush (Δ)
- b) TTT broth without HCO<sub>3</sub><sup>-</sup>, with N<sub>2</sub> flush (▲)
- c) TTT broth with HCO<sub>3</sub><sup>-</sup>, without N<sub>2</sub> flush (●)
- d) TTT broth with HCO<sub>3</sub><sup>-</sup> and N<sub>2</sub> flush (○).

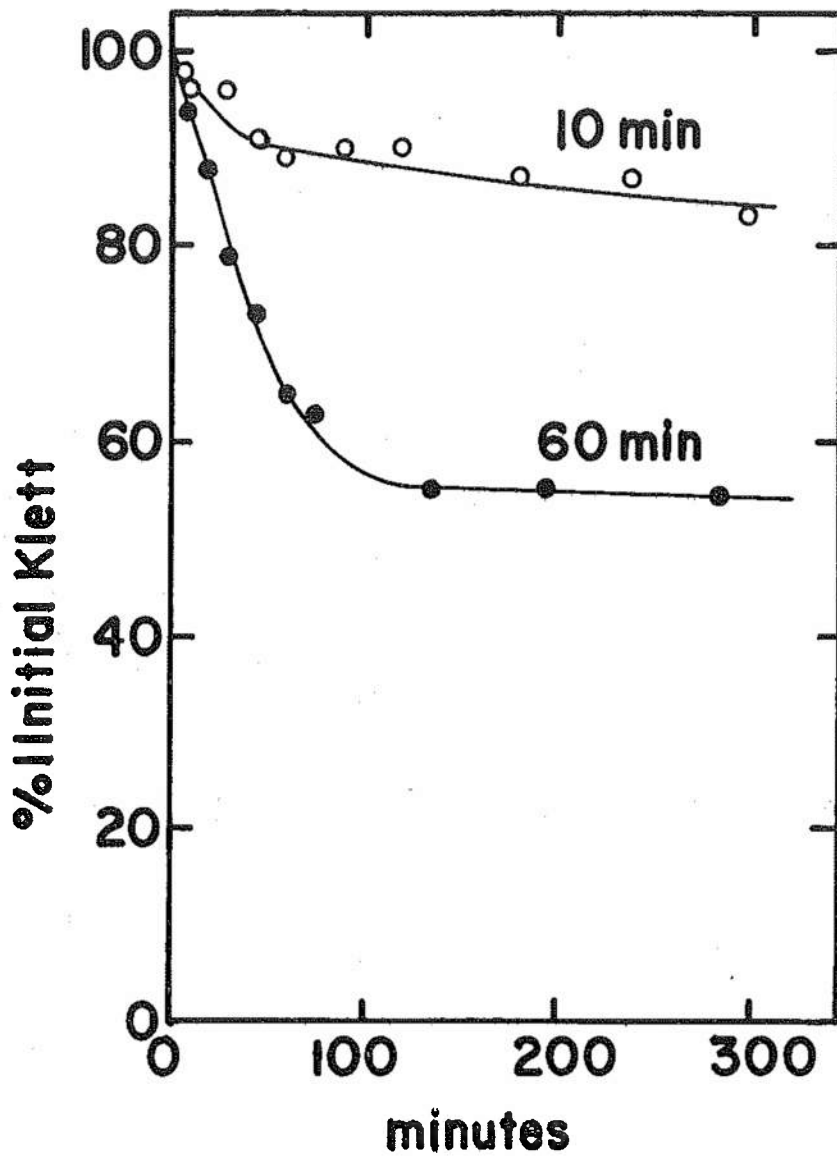


Figure 3. Effect of length of heat activation time at 80°C on germination of *Cl. botulinum* spores in synthetic media.

was dissolved in 0.1 N sodium hydroxide. The protein content of the pellet was estimated by the method of Lowry et al. (5) using human serum albumin as the standard. Alternatively, a micro-Kjeldahl was used. Results were slightly higher than obtained by the Lowry method.

Spore sulfhydryl (SH) groups were measured by the method of Ellman (6,7). One volume of an aqueous spore suspension (~3 mg/ml), three volumes of water, two volumes of phosphate buffer (0.1 M, pH 8.0) and 1/20 volume of 0.01 M 5, 5'-dithiobis (2-nitrobenzoic acid) in 0.1 M phosphate buffer (pH 7.0) were incubated for 30 minutes at room temperature. The system was centrifuged and the optical density of the yellow supernatant solution determined at 410 m $\mu$ . Calculation of SH concentration was made by comparison to a standard curve prepared with cysteine hydrochloride as the primary standard.

Reduction of spore disulfide bonds (S-S) for determination as SH was done using dithiothreitol (8). One volume of an aqueous spore suspension (~3 mg/ml) was added to three volumes of phosphate buffer (0.1 M, pH 8.0) and one-tenth volume of 0.1 M dithiothreitol (DTT). The mixture was incubated at 37°C for one hour with occasional shaking. After incubation, the spores were washed by centrifugation five times with water and finally suspended in one volume of water. All detectable DTT was removed by the third wash as determined by sulfhydryl analysis of the washes. Disulfide bonds were determined by subtracting the SH of nonreduced spores from the SH of DTT-reduced spores. It is important to note that all SH determinations were made on intact spores and thus, may not represent the total SH content of the spores, however, from certain considerations discussed later, we feel most of the SH was detected.

#### Measurement of DNA and RNA Synthesis in Germinating Spores

RNA synthesis of spores germinating in either TTT broth or the cysteine system was followed using ( $C^{14}$ )-uracil incorporation into cold ten percent trichloroacetic acid (TCA) insoluble material. Typically, 1  $\mu$ C of uracil-2- $^{14}C$  (47 mC/mM) was added per ml of broth. At appropriate intervals, duplicate 0.20 ml samples were removed and added to 10 ml ice cold ten percent TCA. Following at least 30 minutes incubation at ice bath temperatures, the precipitated spores were collected on millipore membrane filters, washed with 10 ml ice cold TCA and the filters dried for liquid scintillation estimation of radioactivity. Production of radioactively labeled spores was achieved through sporulation in SM broth containing ( $H^3$ )-thymidine (10.5 C/mM) at 1  $\mu$ C/ml.

### Zonal Centrifugation

Zonal centrifugation (through preformed linear density gradients of Renografin (E. R. Squibb & Sons, Inc.) was performed on germinating spore populations. Gradients were built at 25°C using a Universal density gradient former (Buchler Instruments). Samples were layered (0.2 ml sample/4.5 ml gradient or 1 ml sample/25 ml gradient) onto the gradients and the tubes centrifuged 30 minutes (20,000 rpm, 20°C) in either the SW65 swinging bucket rotor or the #30 fixed angle rotor in a L265B Beckman preparative ultracentrifuge. Following centrifugation, gradients were collected with the aid of a Universal piercing unit (Buchler Instruments). Two methods of analyzing the collected fractions were employed. The first method was to determine the optical density (540 m $\mu$ ) of the fractions. In the second method, radioactive spores (labeled with (H<sup>3</sup>)-thymidine) were centrifuged. The collected fractions were millipore filtered and the filtered material washed with water on the filter. Then the filters were dried and assayed for radioactivity. The filtering and washing procedure was developed to remove Renografin since the compound quenched more than 90 percent of the radioactive count rate.

### Radioactive Counting Procedure

Samples containing radioactive material (contained on millipore filters or filter paper) were submerged in scintillation cocktail (2,5-diphenyl-oxazole and 1,4-bis-[2-(5-phenyloxazolyl)] benzene at 4 gm and 100 mg per liter of toluene, respectively). On occasion, aqueous samples were assayed in which the organic solvent was toluene and ethanol at 80 and 20 percent (v/v) respectively. Samples were assayed by liquid scintillation spectrometry in a Packard Tri-Carb instrument.

### Gamma Irradiation Procedure

Vegetative cell or spore material to be irradiated was suspended in the desired material (TTT broth, G·P or water) and irradiated with a Cs<sup>137</sup> source on the University of Minnesota main campus. Temperatures during irradiation were ambient (about 25°C). The suspensions were in contact with air. The configurations we employed gave dose rates of 0.125, 0.4 and 1 Mrad per hour. Following irradiation, viability determinations were begun within two hours.

## EXPERIMENTAL

### The Development of Resistance to Ionizing Radiation During Sporulation

The marked radiation resistance of the spore was shown to develop after the appearance of cystine-rich structures (CRS)<sup>(9)</sup>, but prior to the acquisition of heat resistance<sup>(9,10)</sup> in sporulating Bacilli. We found a similar situation existed with Clostridium botulinum. An overnight SM broth culture was inoculated (1/500) into fresh SM in a nephoflask, the atmosphere was replaced with N<sub>2</sub>, and the culture was incubated at 37°C. At appropriate times samples were removed with a syringe and assayed for total viable (T), sucrose resistant\* (S<sup>R</sup>), heat resistant (80°C/10 minutes) (H<sup>R</sup>), and gamma radiation resistant (0.08 Mrad) (γ<sup>R</sup>) colony forming units. In addition, sulfur in the disulfide and sulfhydryl form was also measured. Forms resistant to sucrose and radiation began to increase just after stationary phase was reached in the culture (Figure 4a). The development of H<sup>R</sup> forms lagged behind that of γ<sup>R</sup> forms, at 28 hours less than 10<sup>5</sup> H<sup>R</sup> forms per ml were present. The increase in SH content of the culture closely resembled the growth rate (Figure 4b) and leveled off when the culture reached stationary phase. Sulfur in disulfide bonds was detected shortly after the optical density of the culture leveled off and continued to increase throughout the duration of the experiment (29 hours). During exponential growth, the cells were slender and motile. At 17 hours (post-exponential phase) the rods appeared shorter and plumper. Swelling was first detected at 22 hours, and spore coats were visible by 25 hours. Developing refractility was detected at 28 hours. Since the full development of γ<sup>R</sup>, H<sup>R</sup>, and S-S in the culture was not achieved in this experiment, it is difficult to correlate such changes with the morphological changes. However, it is clear that radioresistance develops prior to complete spore maturation in agreement with the work cited earlier<sup>(9,10)</sup>. Thus, one characteristic of spores -- disulfide content -- may be related to their radioresistance based on the temporal relationship seen in Figure 4a, b and the work of Vinter<sup>(9)</sup>. Based on a similar argument, radioresistance and heat resistance appear not to be related in Clostridium or Bacillus<sup>(9)</sup> spores.

During the course of this work, we attempted to dehydrate cells in aqueous suspension with high concentrations of sucrose. This led to the observation that resistance to sucrose developed very early in sporulating Clostridium cultures. The basis for sucrose resistance is not known (for example, inability to remove water and/or resistance to rupture from osmotic shock upon dilution out of the sucrose suspension) at this time. It seems possible that development of sucrose resistance coincides with spore coat completion. Evidence for this notion will be presented in a later section.

\* for determination of S<sup>R</sup>, a culture sample was diluted 1/10 into a 50 percent (w/v) sucrose solution, allowed to sit 20 minutes at room temperature and then diluted further in G·P diluent and plated in TYP.

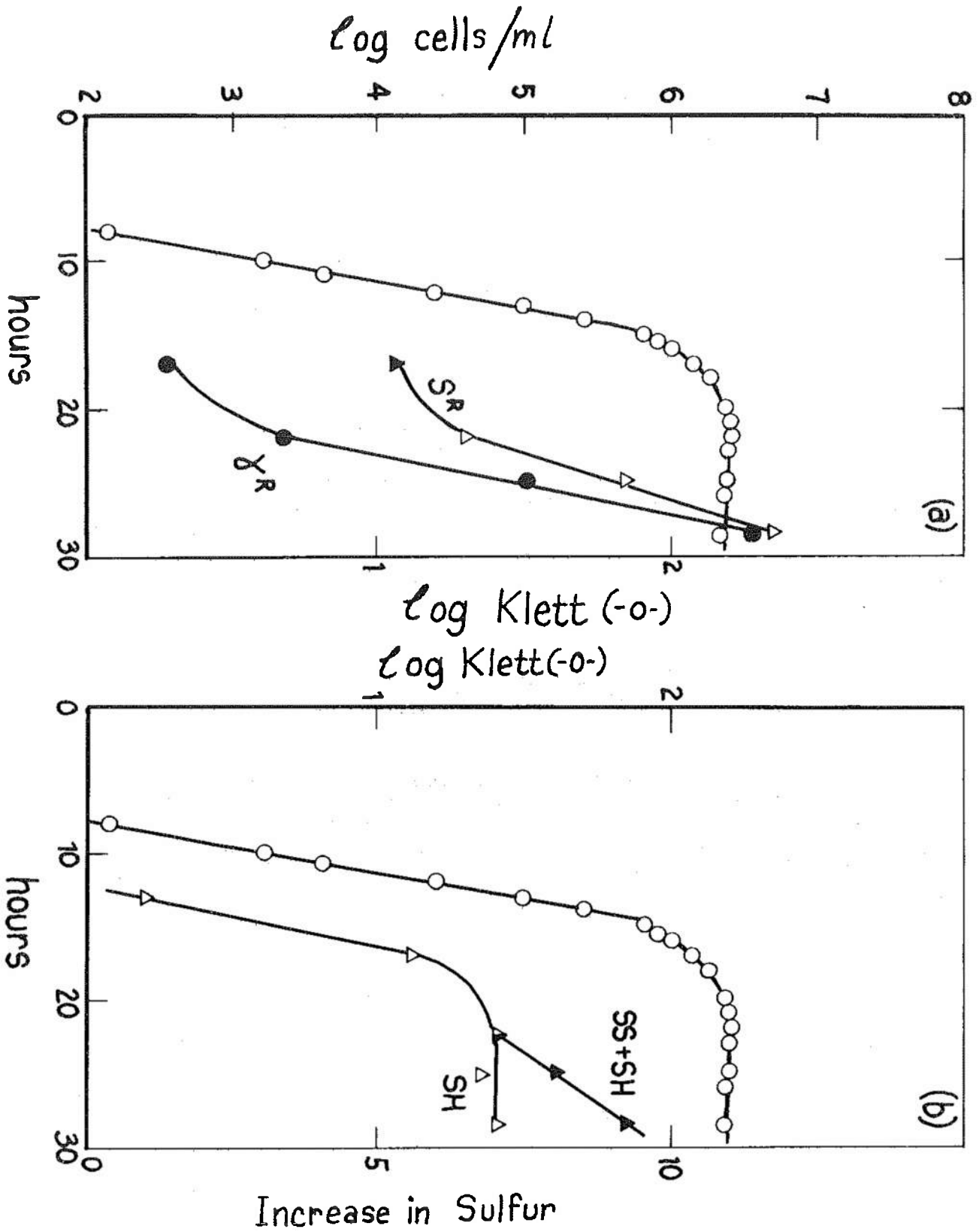


Figure 4. Development of  $s^R$ ,  $\gamma^R$  and disulfide-containing structures in sporulating *Cl. botulinum*. Open circles indicate growth as measured by optical density (Klett).

Radiation Resistance of Heat Sensitive  
Dipicolinic-Acid-less Spores of *Bacillus cereus* T

A correlation between heat resistance and the amount of dipicolinic acid (DPA) in spores of *Bacillus cereus* was noted by Church and Halvorson<sup>(11)</sup> and Black, Hashimoto and Gerhardt<sup>(12)</sup>. The latter authors reported endotrophically produced spores that were deficient (but not totally lacking) in DPA were more resistant to  $CO^{60}$  irradiation than normal spores. Rowley and Levinson<sup>(13)</sup> reported DPA containing,  $Ca^{++}$  deficient spores remained radioresistant. Recently, Wise, Swanson and Halvorson<sup>(2)</sup> reported the isolation of mutants of *B. cereus* T that were unable to synthesize DPA and produced heat sensitive, DPA-less spores. The availability of such spores provided an opportunity to re-examine the role of the presence of dipicolinic acid in spore radioresistance. Spores of wild type ( $DPA^+$ ) and mutant HW-3 ( $DPA^-$ ) were obtained from Dr. H. Orin Halvorson. The spores were suspended in water and irradiated at a dose rate of 0.4 Mrad/hour. Survivors were enumerated on nutrient agar plates (Figure 5). It is clearly shown that  $DPA^+$  and  $DPA^-$  spores are identical in their radiation resistance. This result is consistent with those obtained in sporulating cultures of *Bacilli*<sup>(9)</sup> and *Clostridia*. Dipicolinic acid, therefore, is not a factor in spore radioresistance.

The Effect of Reduction of Spore Disulfide  
on Radiation Resistance in *Clostridium botulinum*

Since the discovery<sup>(14)</sup> that spores of *B. megaterium* and *B. cereus* contain disulfide (cystine) in high amounts, speculations as to its function(s) have been abundant. Stabilization of spore structures<sup>(14,15,16,17)</sup> and function as a free-radical sink<sup>(9,18,19,20)</sup> have been postulated. As previously discussed, formation of cystine-rich structures (CRS) is an early event in spore formation and precedes the development of resistance to ionizing radiation. The possibilities exist that (a) disulfide forms of sulfur are radioprotective and, thus, responsible for spore radioresistance, or (b) CRS involvement in spore radioresistance is absent or only minor. Reduction of disulfides in CRS to sulfhydryl groups would result in radiosensitization of spores if (a) were true. Suggestions that reduction of spore disulfide does not alter spore radioresistance<sup>(20)</sup> and that radioresistance does not correlate with spore disulfide content<sup>(19)</sup> have appeared. However, lack of convincing evidence of actual disulfide reduction<sup>(20)</sup> and generally inconsistent results<sup>(19)</sup> leave the role of disulfide bonds in spore radioresistance in an uncertain state. We felt, therefore, a re-examination of the effect of spore disulfide reduction on radioresistance was in order.

Cleaned spores of *Clostridium botulinum* were treated with dithiothreitol (see Materials and Methods) to effect disulfide reduction. It is important to note the reduction was done under the slightly alkaline conditions

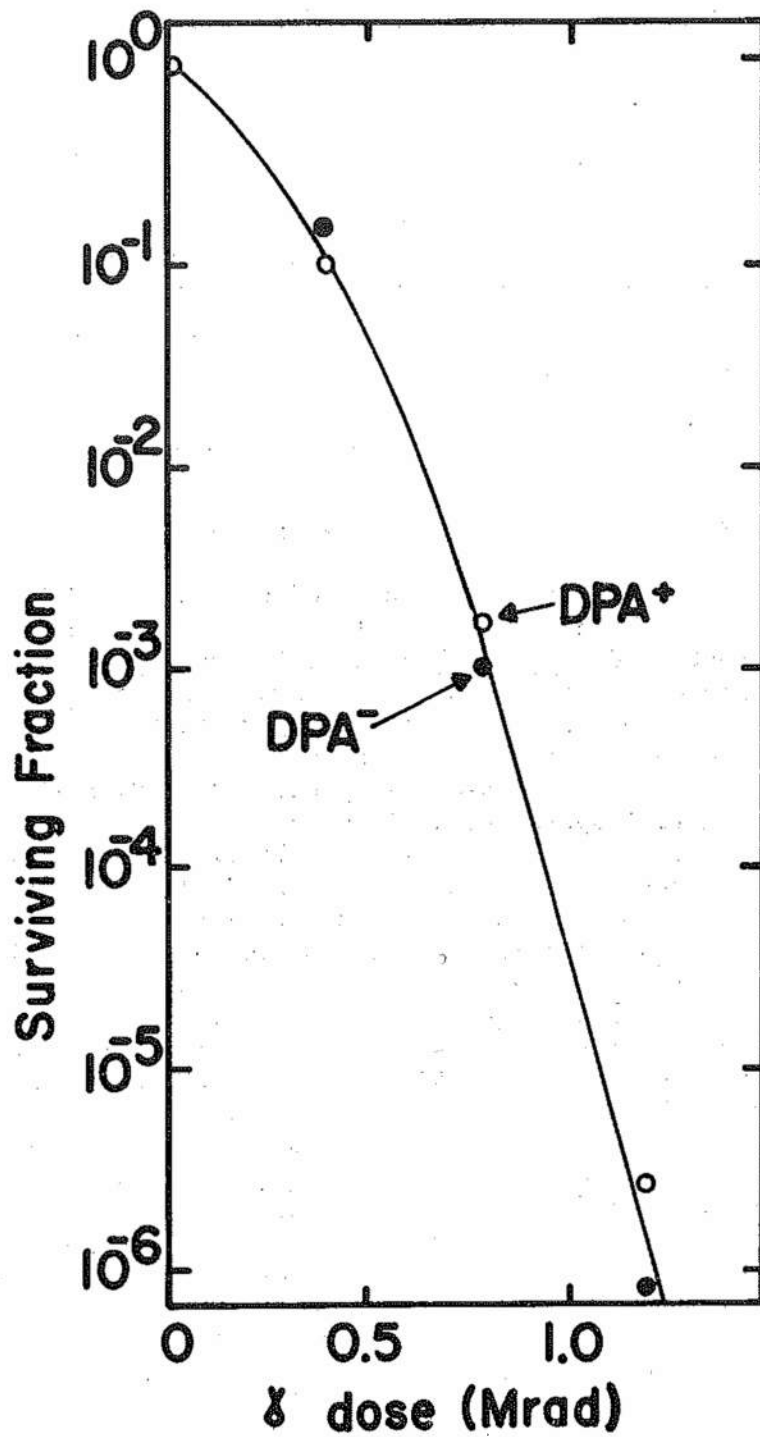


Figure 5. Radioresistance of wild type ( $DPA^+$ ) and mutant ( $DPA^-$ ) spores of *B. cereus* T.

that favor reduction<sup>(21)</sup> as opposed to the acidic conditions used by Hitchins *et al.*<sup>(20)</sup>. Evidence that actual reduction of spore disulfide occurred is shown in Table 6.

Table 6  
Reduction of Spore Disulfide by Dithiothreitol

Spores	S <sub>heq.</sub> (μmole/mg spores)	
	as SH	as SS/2 <sup>†</sup>
Control (non-reduced)	3.7	47.4
Reduced <sup>††</sup>	47.2	0

<sup>†</sup> half disulfide

<sup>††</sup> reduction did not affect heat resistance

In control (nonreduced) spores, 93 percent of the sulfur, detectable after reduction as SH, was present in the disulfide form. Reduced spores, on the other hand, contained only sulfhydryl sulfur and no disulfide bridges. Since intact spores were used for sulfur assays, the possibility exists that all of the sulfur was not detected. Comparison of our results with those of Setlow and Kornberg<sup>(22)</sup> for *B. megaterium* indicates we detected 72 percent of the SS + SH (expressed as μmoles SH in SS+SH/mg spores) they reported for intact spores using fluorescein mercuric acetate<sup>(23)</sup>. It is important to note that only sulfhydryl levels in spores were increased by assay of ruptured spores<sup>(22)</sup>, disulfide levels were identical in intact or ruptured spores. Bott and Lundgren<sup>(19)</sup> reported nearly four times the amount of disulfide in *B. cereus* spores as found in *B. megaterium*<sup>(22)</sup>. However, a value nearly 10 times smaller than that found by Bott and Lundgren<sup>(19)</sup> was reported for *B. cereus* by Hitchins *et al.*<sup>(20)</sup>. Blankenship and Pallansch<sup>(23)</sup> reported values of disulfide for *B. megaterium* which were similar to those of Setlow and Kornberg<sup>(22)</sup>. Data reported by Vinter<sup>(9)</sup> are difficult to compare with those of others due to the units employed by him (μg S/mg N of hydrolyzed TCA precipitate as opposed to μg or μmoles SH/mg spores). However, his data for a number of *Bacilli* indicate that spores contain about five<sup>(9,14,18)</sup> times more cystine and cysteine than the corresponding vegetative cells. Others<sup>(19)</sup> have estimated spores contain from ten to twenty times more disulfide than vegetative cells. It is evident that work on spore disulfide is confused. Differences may be related to the different organisms or assay methods used by the various authors and ourselves. Thus, since our values are not unreasonable compared to those reported by others for *Bacillus* spores and also due to our inability to observe higher levels of sulfhydryl and disulfides in spores made more permeable through germination<sup>(22)</sup>, we argue that most (>90 percent) of the disulfide present in *Cl. botulinum* spores was detected.

Control and reduced C1. botulinum spores were suspended in water and irradiated at 1.0 Mrad/hour. The results (Figure 6) indicated reduction had little, if any, effect on the shoulder region of the survival curve. Reduced spores did exhibit increased sensitivity on the exponential portion of the curve (reduced spore rates is 1.6 times the control rate). Reduced spores were, however, still markedly radioresistant compared to cells taken from an exponentially growing culture (Figure 6). The dosages required to reduce populations of control and reduced spores and vegetative cells to 10 percent survival level ( $D_{10}$ ) and for reduction by 90 percent on the exponential portion of the curves [ $D_{10}(\text{exp})$ ] are given in Table 7. To reduce the viable population by 90 percent, normal and reduced spores require 22 and 17 times, respectively, the dosage required by a vegetative population. The dosage required to inactivate one log cycle of organisms on the exponential portion of the curves is 14 (normal spores) and 9.5 (reduced spores) times that for vegetative cells. By subtracting  $D_{10}(\text{esp})$  from  $D_{10}$  for normal and reduced spores, the shoulder dosage is seen to be identical (0.165 Mrad).

Table 7  
Comparison of Radioresistance Among  
Normal and Reduced Spores and Vegetative Cells

Organism	Dose (Mrad)	
	$D_{10}$	$D_{10}(\text{exp})$
Normal spores	0.462	0.297
Reduced spores	0.363	0.198
Vegetative cells	0.021	0.021

The contribution of disulfide to total spore radioresistance is at most 35 percent  $\left[ \left( 1.00 - \frac{0.198}{0.297} \right) \times (100) \right]$  considering only the slope of the exponential portion of the survival curve and 25 percent when the first log cycle of kill is only considered. Generally, spore radioresistance is not due to the presence of high disulfide concentrations.

It has been suggested by many authors (see, for example, Ref. 9 and 24-29) that sulfhydryl and disulfide sulfur were radioprotective via trapping free radicals. In order to determine if ionizing radiation caused changes in the state of spore sulfur, we determined the amounts of sulfhydryl and disulfide after 0, 0.25, 0.50 and 1.0 Mrad exposure of normal and reduced spores (Figure 7). Control spores did exhibit a splitting of disulfide with the production of sulfhydryl. At a total dose of 1.0 Mrad, 9  $\mu\text{moles}$  of half-disulfide were lost, accompanied by the new appearance of 5  $\mu\text{moles}$  of sulfhydryl. This suggested one sulfur of the disulfide bond was reduced and the other oxidized, perhaps to

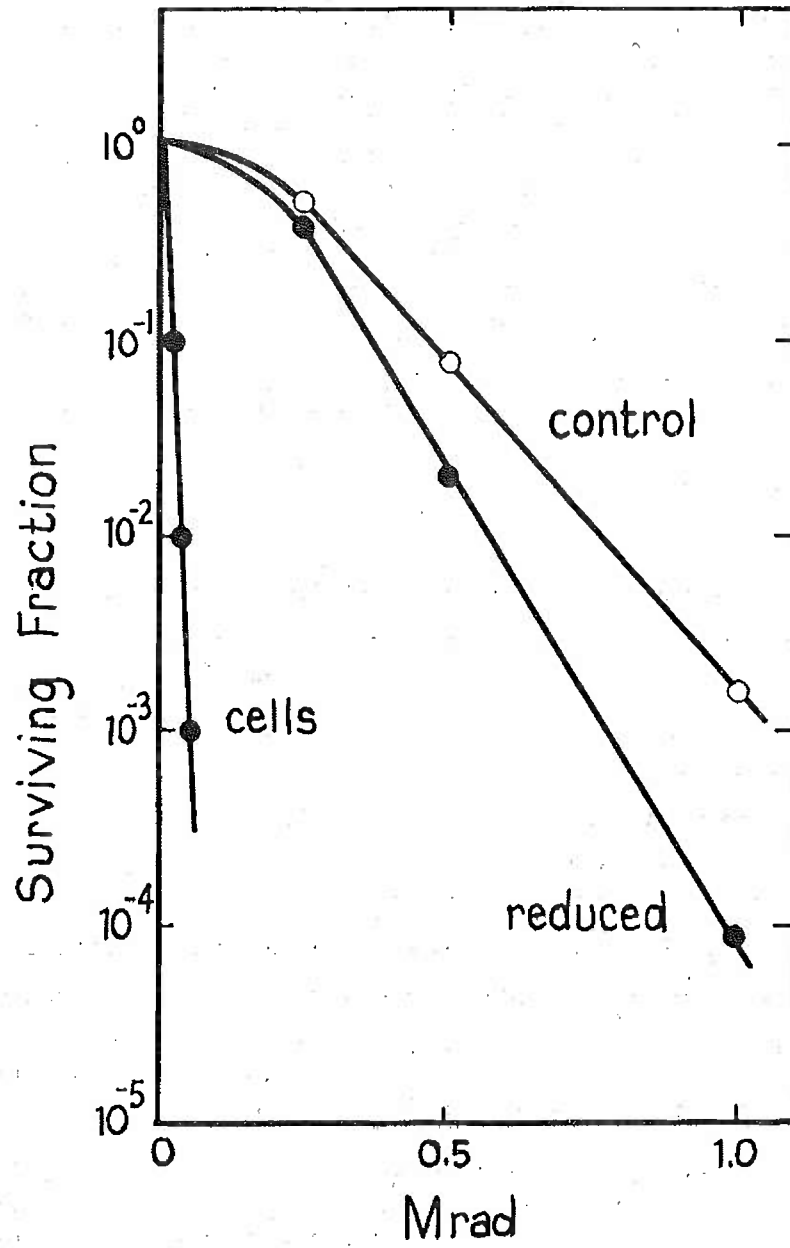


Figure 6. Radioresistance of non-reduced spores (control) and vegetative cells and reduced spores of *Cl. botulinum*.

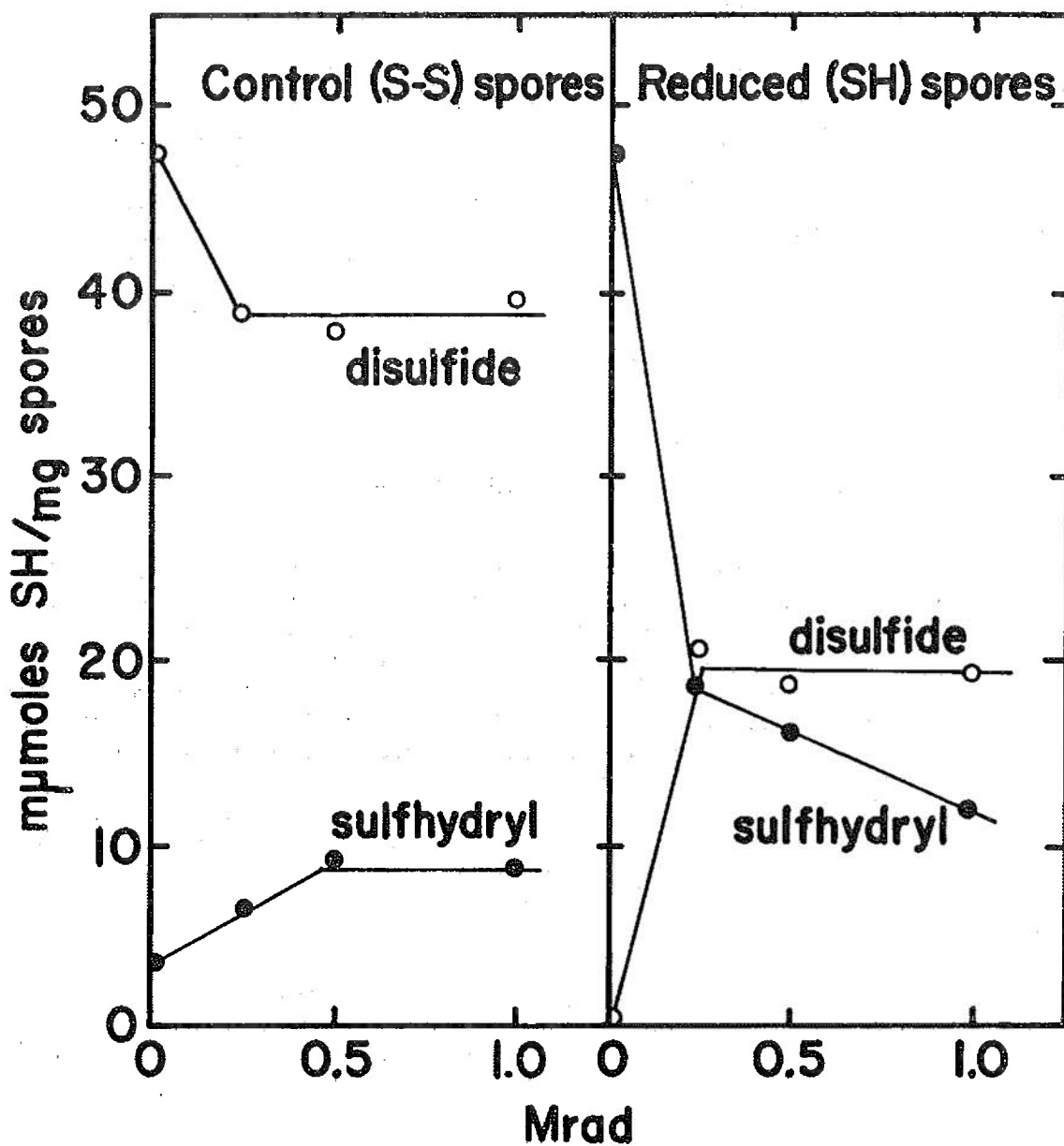
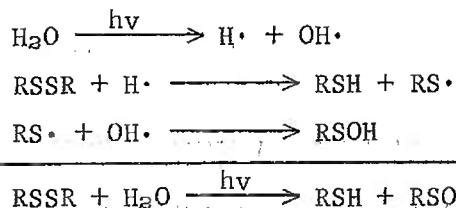


Figure 7. Effect of irradiation on sulfhydryl and disulfide contents of control and reduced spores.



cysteine sulfenic acid<sup>(30)</sup>. Reduced spores showed a marked loss of sulfhydryl with the concomitant appearance of disulfide upon irradiation. The curve (Figure 7) describing sulfhydryl loss shows two distinct rates of loss and thus may represent two distinct environments in which spore sulfhydryl groups are found. Total loss of sulfur recoverable as half-disulfide and sulfhydryl was 35 percent at 1.0 Mrad. Comparison of control and reduced spores indicate sulfhydryls are more radiolabile than disulfide bridges. The expectation<sup>(9)</sup> that disulfide-sulfhydryl interchanges occur in normal spores was thus borne out.

#### Changes in Radiation Resistance of Germinating Spores of *Clostridium botulinum*

Stuy<sup>(31)</sup> reported that spores of *B. cereus* became sensitive to X-irradiation upon exposure to germination conditions. Sensitization was characterized by a continuous increase in the slope of the dose-survival curves as the time of germination increased. Changes in slope were detected in one minute of germination (at which time about 90 percent were heat sensitive). Woese<sup>(32)</sup> similarly observed radiosensitization of *B. mesentericus*, *B. brevis*, *B. megaterium*, and *B. anthracis* spores following germination. He observed that spores whose inactivation kinetics exhibited a shoulder gave rise to germinated spores (at the stage of elongation) that also exhibited a shoulder on the survival curve. These observations were interpreted as reflecting the number of targets per spore. The targets' inherent sensitivity to inactivation was postulated to increase during germination.

As shown in Figure 6, spores of *Clostridium botulinum* exhibit a shoulder on the dose-survival curve. Vegetative cells from an actively growing culture, on the other hand, exhibited strictly exponential inactivation. Careful analysis of radiosensitization during germination may provide valuable information on the mechanism of spore radioresistance. Unfortunately, the former authors<sup>(31,32)</sup> did not adequately describe the courses of spore germination for their radiation studies. Thus, we sought to describe in detail the changes in radioresistance of germinating populations of *Cl. botulinum* spores.

Spores of *Cl. botulinum* were heat activated (80°C/10 minutes) and inoculated into TTT broth. Germination was carried out at 26°C to decrease the rate of germination. At appropriate intervals, samples were removed by syringe and diluted 1/100 into cold gelatin-phosphate diluent. The

percentage of heat resistance (80°C/10 minutes) and  $\gamma$  resistant (80 Krad) forms were determined as previously described. Germination of the population was measured by the decrease in optical density. The percentage of spores germinated was estimated by taking the ratio  $(1.00 - \frac{OD_t}{OD_{max}}) \times 100$  where  $OD_t$  and  $OD_{max}$  are the decrease in optical density at any particular time and the maximum optical density decrease obtained (65 percent), respectively. At 5 minutes of germination, 98 percent of the spores were heat sensitive. The rate of appearance of  $\gamma^S$  spores (Figure 8) was slower than the germination rate. Extrapolation of the  $\gamma^S$  curve indicated about 80 minutes would be required to obtain 99 percent of  $\gamma^S$  forms. These data further support the notion that heat resistance and  $\gamma$  resistance are independent of each other (see also Figures 4 and 5).

Although the data in Figure 8 clearly indicate spores become  $\gamma^S$  upon germination, the shape of the curve is meaningless unless the dose used was on a similar portion of a dose-survival curve for each germinated spore sample. Additionally, the mode or pattern of radiosensitization of germinating spores is not revealed by data of the type in Figure 8. Thus, in order to examine these parameters, complete dose-survival relationships were done on spores from a germinating population.

Heat-activated spores were inoculated into prewarmed TTT broth at 37°C. All procedures for removing samples, stopping germination, and irradiation (0.125 Mrad/hr) were as previously described in Materials and Methods. The results of several experiments are summarized in Figure 9. The course of germination and postgerminative development is given at the top of the figure. The germination and growth profile has been divided into four sections: early (E) and late (L) germination phase, emergence (Em) phase in which emergence is detectable microscopically and also by a slight increase in turbidity, and growth (G) phase in which vegetative cells are predominant.

Ungerminated (heat activated) spores exhibit the typical shoulder and relatively low inactivation rate. Germinating spores in the E phase of germination are composed of two populations. The majority of spores exhibit a low inactivation rate, but the remaining population was inactivated at a considerably higher rate. Notice, however, that both populations had lost the large spore shoulder. Spores in L phase of germination are a relatively homogeneous (>95 percent) population which is inactivated at a high rate. A small shoulder (about 20 percent of the spore shoulder) is still evident. Spores in the emergence phase have a slightly greater rate of inactivation and smaller shoulder than L phase spores. Finally, cells removed from the system (G) after the minimal turbidity (100-150 minutes) had doubled (~270 minutes), exhibit full vegetative cell sensitivity as shown by comparison to a sample from exponentially growing cells (solid circles on the G line).

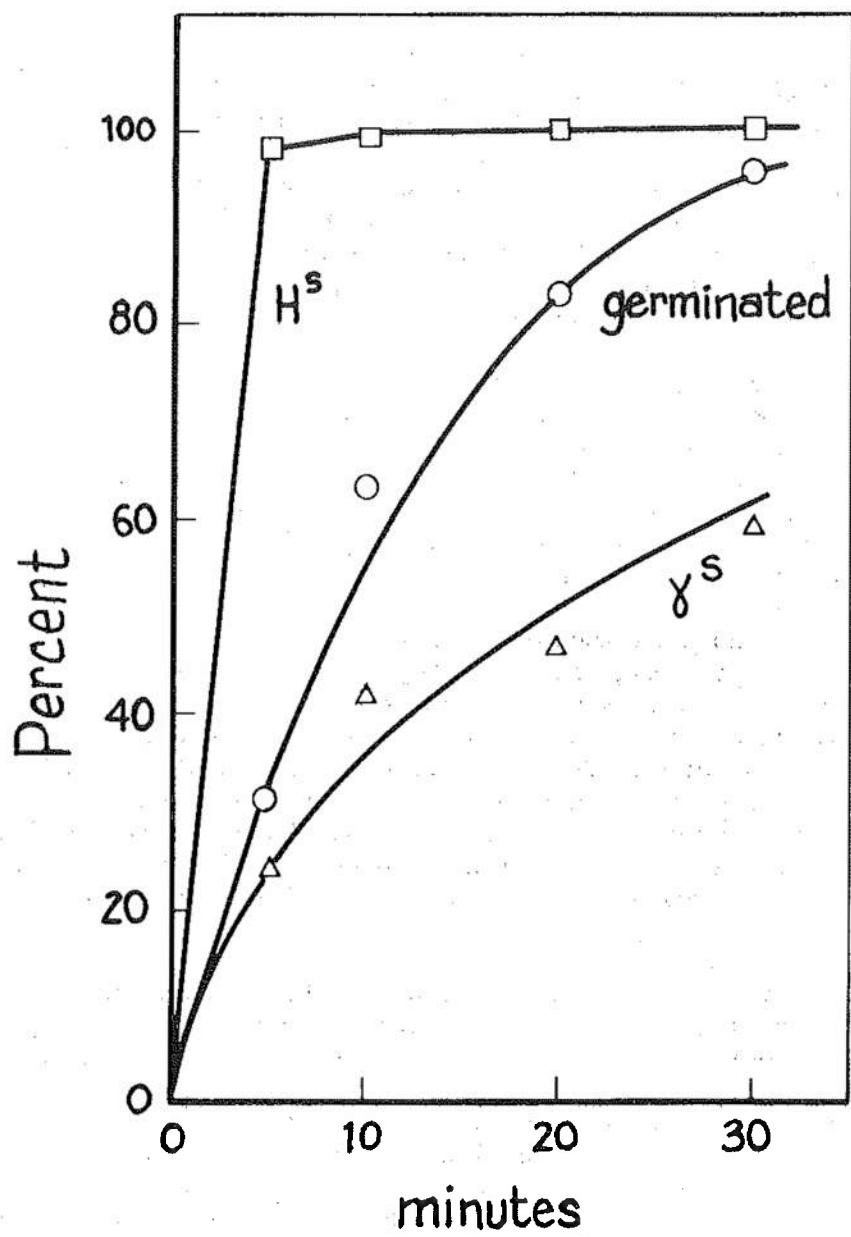


Figure 8. Acquisition of heat and radiation sensitivity of germinating *Cl. botulinum* spores.

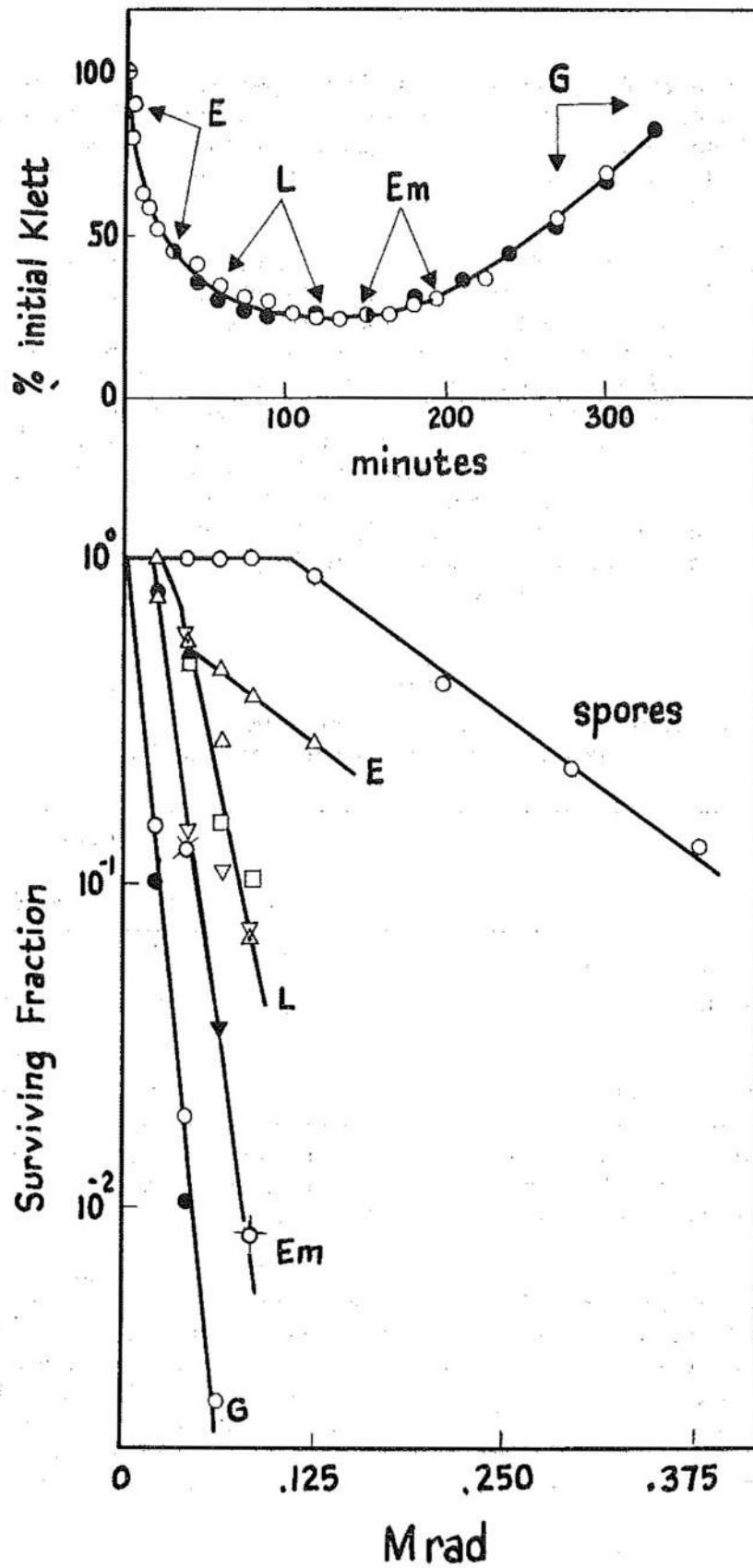


Figure 9. Dose-survival relationships of spores undergoing germination and postgerminative development in TTT broth.

Upper: germination of spores and subsequent outgrowth.

Lower: radioresistance of resting spores, germinated spores and vegetative cells.

See text for explanation of symbols.

Three phenomena were distinguished in this experiment, (1) loss of the typical spore type shoulder early in germination but with the conservation of the spore-type inactivation rate, (2) a marked increase in the inactivation rate, and (3) eventual loss of the small shoulder upon the appearance of vegetative cells.

The quantitative relationships among the various radioresistance classes are obtainable from the data in Table 8. The shoulder dose (Sh), dose to inactivate the first log cycle ( $D_{10}$ ) and the dose required to inactivate one log cycle on the exponential ( $D_{10}(\text{exp})$ ) portion of the dose-survival curves are given. The shoulder dose of vegetative cells, Em and L phase spores was 0, 11 and 20 percent of the spore value, respectively. The  $D_{10}(\text{exp})$  of cells, Em and L phase spores was 7.5, 15 and 17 percent of the spore value, respectively. The  $D_{10}(\text{exp})$  of the major population (low inactivation rate) of E phase spores (Figure 9) was 97 percent (271 Krad) of the spore value (data not in Table 8). The results reported by Woese<sup>(32)</sup> and our results are consistent; ungerminated spores and spores at the stage of emergence both exhibited shoulders (refer to beginning of this section).

Table 8  
Comparisons of Radioresistance Among Spores,  
Germinated Spores and Vegetative Cells

	Dosage (Krad)		
	Sh <sup>†</sup>	$D_{10}$	$D_{10}(\text{exp})$
Spores (ungerminated)	115	395	281
Spores (L phase)	31	78	47
Spores (Em phase)	16	57	42
Vegetative cells	0	21	21

<sup>†</sup> extent of shoulder on dose-survival curve determined by intersect of 100 percent survival line and extrapolated exponential portion of survival curve.

The change in slope of the survival curves has been interpreted to indicate a change in the state<sup>(32)</sup> (i.e., inherent sensitivity) of the lethal target. In addition, the values obtained by extrapolation of the exponential portion of the curves to zero dose (i.e., the target number) actually increases during germination (ungerminated spores = 2.3, L and Em phase spores  $\approx$  9). This casts doubt on attaching significance to the values as indicating the number of identical targets<sup>(32)</sup>, since they at first increase (2.3 to 9) and then decrease to 1.0 (9 to 1) for vegetative cells.

The spore shoulder may reflect a repair system(33-38), but it is difficult to visualize a repair system that is lost upon germination. Eighty percent of the spore shoulder was lost after one minute of exposure to germination conditions (data not shown), indicating the loss of the shoulder was unrelated to a decrease in optical density (less than 5 percent decrease in one minute germination) and that the shoulder was probably not due to enzymatic repair of DNA lesions. The characteristics of the shoulder loss are more consistent with a change in target state or a decreased protection against indirect radiodamage due to a change in spore structure.

Finally, analyses of spore SH and S-S through the emergence phase of germination revealed that disulfide was neither reduced nor released from spores. Similar results were reported for B. megaterium by Setlow and Kornberg(22). Thus, since neither a change in state nor amount of spore disulfide occurred under conditions (germination) that resulted in marked changes in radioresistance, we conclude that neither the disulfide content nor disulfide form of sulfur plays a major role in overall spore radioresistance. At most, spore disulfide may contribute to the difference in radioresistance of emerging spores and vegetative cells. This difference accounts for a total contribution of 7 percent to the  $D_{10}(\text{exp})$  and 11 percent of the total shoulder dose (Sh) of ungerminated spores. Comparison of these values with the estimated 30 percent contribution (see sulfur section) of disulfide vs. sulfhydryl to spore radioresistance may indicate (1) reduction caused other changes in spores that affected radioresistance in addition to simply effecting disulfide reduction or (2) germination caused a decrease in any radioprotective effect of the disulfide content of spores. A choice between these alternatives cannot be made at this time. In summary, the quantitative contribution of disulfide bridges to total spore radioresistance lies between 7 and 30 percent.

A chemically defined germination medium for Cl. botulinum was recently developed by Dr. D. Rowley at the Natick Laboratories, Department of the Army. Germination in this medium has already been given (Figure 3). The pattern of radiosensitization in this medium during germination (Figure 10) was found to be different from that found for the complex TTT broth (Figure 9). Germination (Figure 10, top) was characterized by a 60 percent drop in turbidity and complete lack of emergence. Spores germinated 30 minutes  $37^{\circ}$  were composed of two populations, (a) a very sensitive and (b) a more resistant population. The  $D_{10}(\text{exp})$  of the population with the lower rate of death was 0.103 Mrad. This figure is nearly a three-fold higher rate of inactivation than the  $D_{10}(\text{exp})$  of ungerminated spores (Table 8). Notice the total absence of the small shoulder seen in the TTT system (Figure 9). Analysis of spores germinated 180 minutes again revealed two populations composed of (a) germinated spores whose estimated sensitivity ( $D_{10}(\text{exp}) = 0.021$  Mrad) was identical to vegetative cells ( $D_{10}(\text{exp}) 0.021$  Mrad) and (b) ungerminated spores. Thus, in the chemically defined medium the small shoulder (0.031 to 0.016 Mrad) was not observed. This finding was unexpected and is difficult to interpret. Apparently the germination process in TTT vs. the chemically

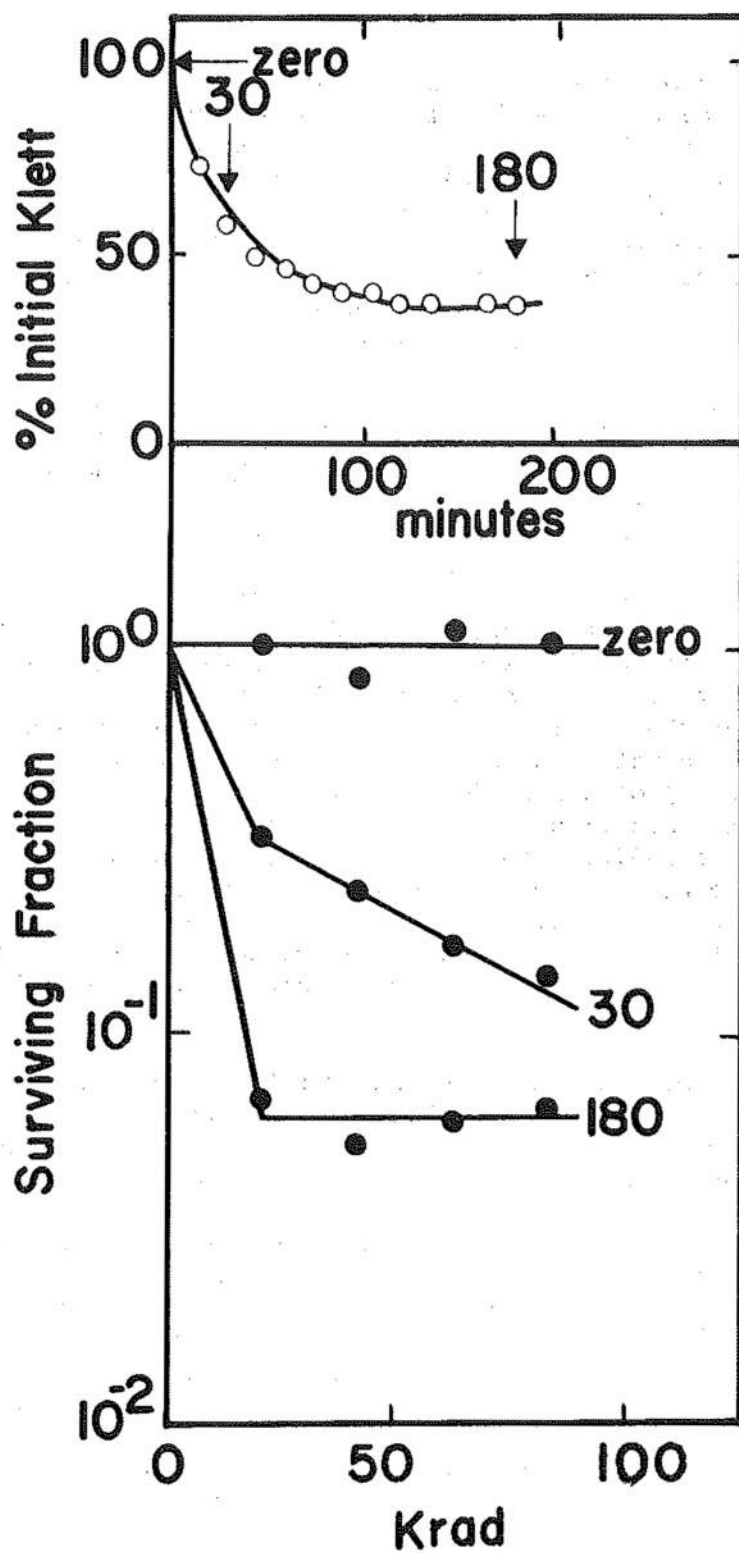


Figure 10. Dose-survival relationships of spores germinating in synthetic media.

Upper: germination of spores

Lower: radioresistance of germinating spores

defined system is sufficiently different to yield the different results. One point which may be important is that spores germinated in Rowley's system require 60 minutes of heat activation (Figure 3). Perhaps the small shoulder is due to an enzymatic repair or a physical quenching of radicals by some spore structure. Prolonged heat activation could inactivate a repair system or alter a spore structure such that the small shoulder would be lost.

As stated earlier (see discussion of Figure 9), the large shoulder was lost within one minute of germination at 37°C. The processes of spore activation, germination, and outgrowth are temperature dependent<sup>(39,40)</sup>. Activation energies for spore germination are of the order of those found for enzymatic systems (see Ref. 43). Doi<sup>(41)</sup> discussed the evidence for enzymatic reactions in spore germination and the probable effects of heat activation on initiating the germination process. We decided to examine two aspects of the major shoulder loss, (1) the effect of germination at 10°C and (2) the  $N_2$  and  $HCO_3^-$  requirements.

Spores were heat activated and introduced into steamed TTT broth that had been rapidly cooled and equilibrated at 10°C. The course of germination is given at the top of Figure 11. Germination was markedly slowed. Heat sensitive spores similarly appeared very slowly (compare >98 and 10 percent HS after 10 minutes of germination at 37°C (Figure 8) and 10°C, respectively). Samples were removed after 10, 20 and 40 minutes of germination and irradiated as before. The results (Figure 11, bottom) indicate a few spores (about 20 percent) lost the major shoulder by 10 minutes. This fraction of the population did not increase up to 40 minutes of germination. The remaining population gave evidence of a larger shoulder since the survival curve leveled off. Thus, the loss of the major shoulder was temperature dependent, similar to the whole germination process.

#### The Relationship of RNA Synthesis to Changes in Radioresistance of Germinating Spores

Germinating spores initiate macromolecular synthesis in the order: RNA, protein and DNA<sup>(39-41)</sup>. Spore DNA is in a different physical state than vegetative cell DNA as evidenced by the nature of the UV photoproducts of thymine obtained from spores as compared to cells<sup>(45-47)</sup>. The commencement of RNA synthesis during spore germination most likely follows the transition of the spore DNA to cell-type DNA. We sought to determine the temporal relationship between spore radiosensitization and the commencement of RNA synthesis.

Heat activated spores (10 and 60 minutes at 80°C for TTT and Rowley's system, respectively) were germinated as previously described. RNA synthesis was estimated by the incorporation of ( $C^{14}$ )-uracil into acid insoluble material (see Materials and Methods). RNA synthesis (Figure 12, right) was first detected after 60 minutes of germination in TTT broth

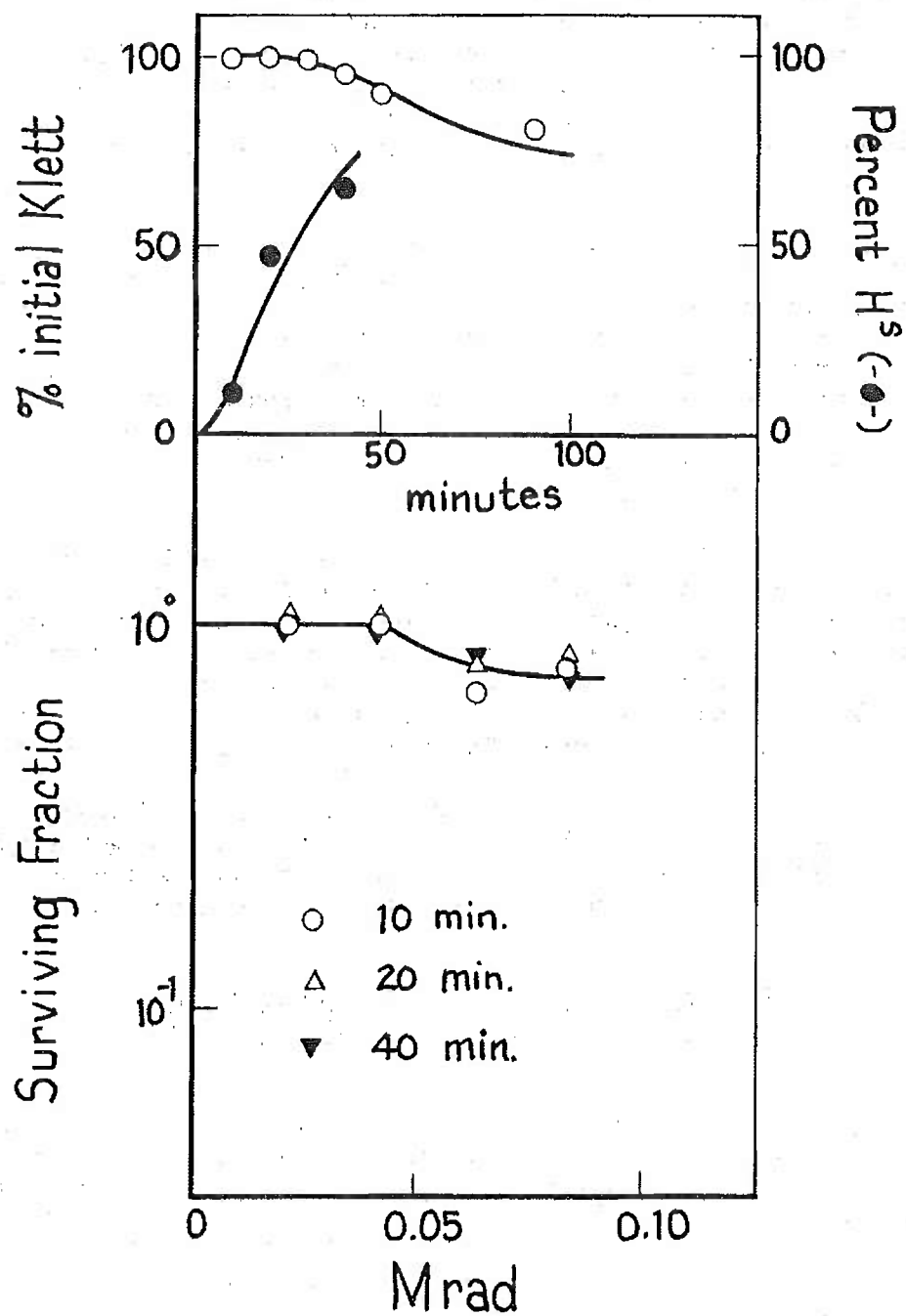


Figure 11. Radioreistance of spores germinated 10, 20 and 40 minutes in TTT broth at 10°C.

Upper: germination and appearance of heat sensitivity  
 Lower: radioreistance of germinating spores

and then continued linearly for the duration of the experiment. In the chemically defined germination medium, spores germinated (Figure 12, left) but failed to synthesize RNA (Figure 12, right). Germination-dependent radiosensitization of spores, thus, occurred prior to the commencement of RNA synthesis (compare Figures 9 and 12) in TTT and in the total absence of RNA synthesis in the synthetic medium (compare Figures 10 and 12). Thus, macromolecular biosynthesis appears not to be involved in the radiosensitization of germinating spores. Further support for this notion is derived from the following experiment. C. botulinum spores were germinated in TTT containing 50 µg/ml Actinomycin D. This concentration of Act. D inhibits over 90 percent of the RNA synthesis and completely blocks emergence and outgrowth (data not shown). The results clearly indicate, (a) germination was unaffected by the presence of the antibiotic (Figure 13, left) in agreement with others<sup>(48)</sup>, and (b) that very similar survival responses (Figure 13, right) were obtained in control and Act. D spores germinated 120 minutes (arrow, Figure 13, left). Thus, in complex broth, inhibition of RNA synthesis did not alter the development of radio-sensitive spores during germination.

#### Physical and Structural Changes Associated with Spore Germination

Generally, spore germination is characterized by release of  $\text{Ca}^{++}$ , DPA<sup>(49, 50, 51)</sup>, small molecules<sup>(52)</sup>, and DPA-containing peptides in addition to loss of refractility and acquisition of stainability<sup>(49, 50)</sup>. In addition, germinated<sup>(2)</sup>,  $\text{Ca}^{++}$ -deficient<sup>(53)</sup> and DPA-less<sup>(2)</sup> spores are less dense than ungerminated, normal spores. In order to determine whether discrete density changes of germinating spores correlated with changes in radiosensitivity, spore density changes were analyzed by zonal centrifugation through preformed linear Renografin density gradients<sup>(2, 53)</sup>.

Spores labeled with ( $\text{H}^3$ )-thymidine were used so that label would not be released during germination and confuse the density profile. Heat activated spores were inoculated into TTT broth and germination followed at 25°C (in order to slow down germination). Samples were removed at 8 and 30 minutes. A control sample that was heat activated but not introduced into TTT was also taken. Each sample was immediately diluted into cold water, and the spores were collected by centrifugation. The pellets were taken up in 0.2 ml water and layered onto gradients covering the density range of 1.12 to 1.36 g/cc. Centrifugation, fraction collection, and assay of the gradients was as already described (Materials and Methods). Control spores (Figure 14) banded at a density position of about 1.30 g/cc. Spores germinated 8 minutes had redistributed to a position in the gradient corresponding to a density of 1.25 g/cc. A further small density shift (to about 1.22 g/cc) was evident in 30 minute spores. The first density change observed in 8 minute spores was detected in 1 minute spores in another experiment (data not shown). Further changes in density between 60 and 240 minutes (prior to elongation) were not seen. The first density change (1-8 minutes) is temporally related to the loss of the major shoulder

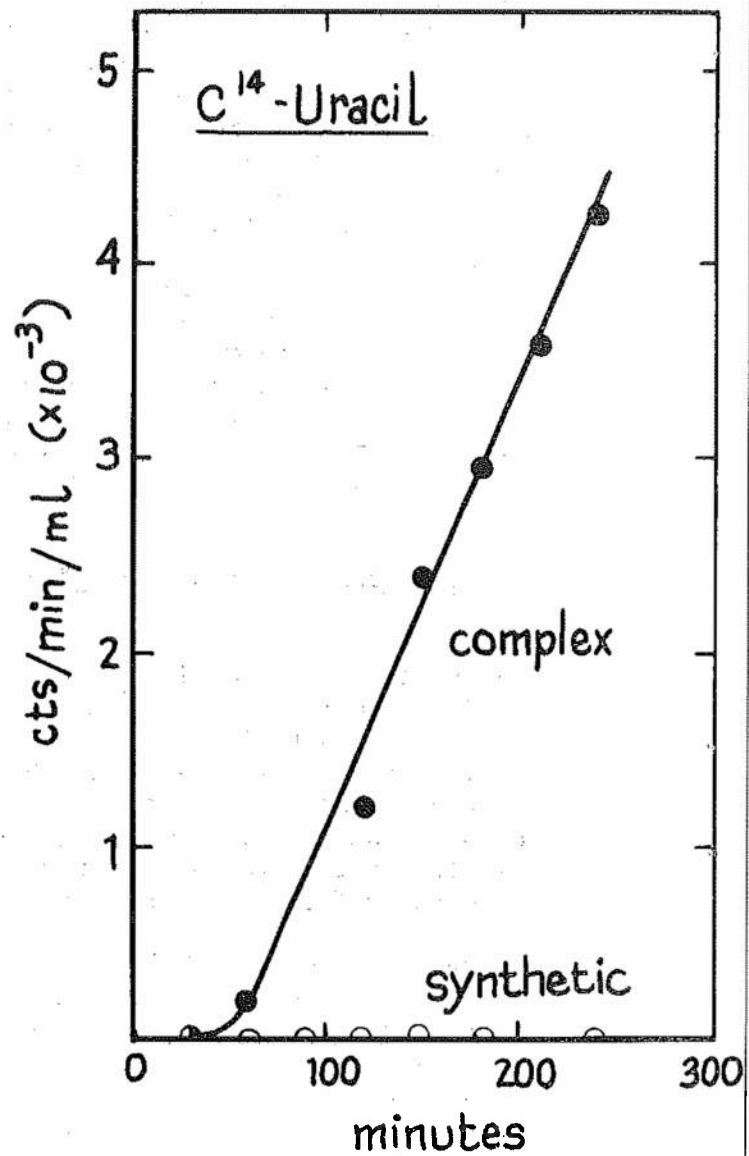
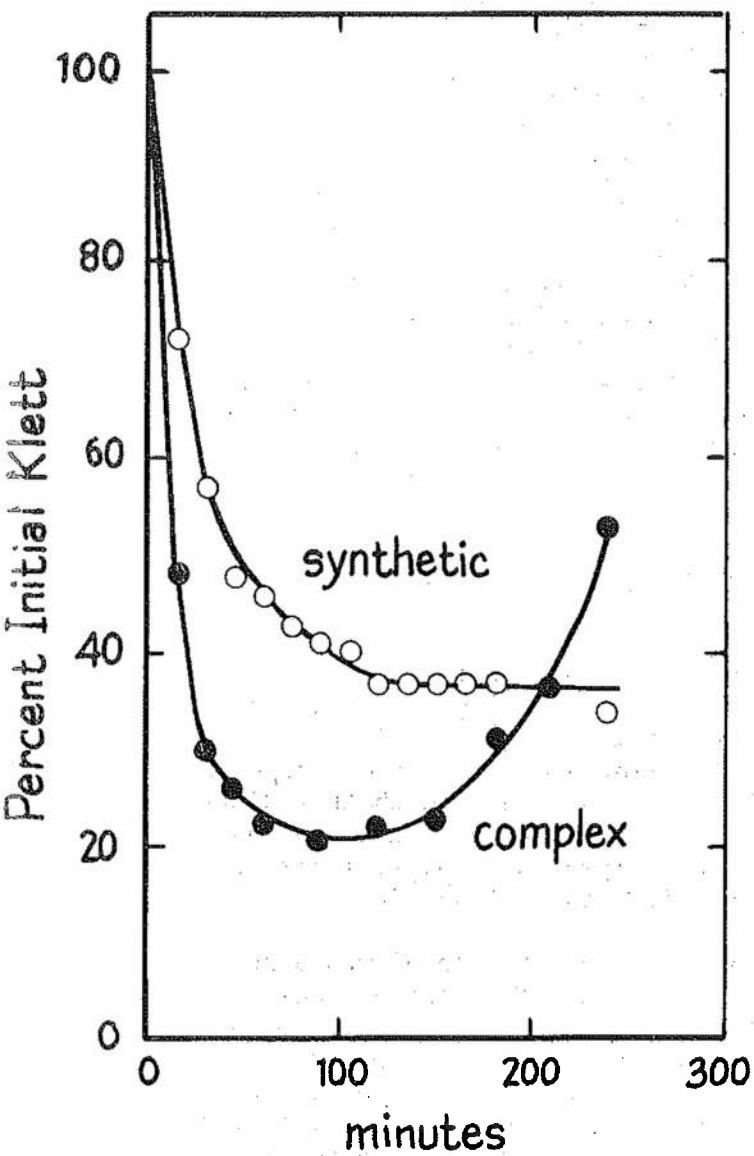


Figure 12. Onset of RNA synthesis in populations of spores germinating in TTT (complex) and synthetic media.

Left: germination of spores  
 Right: RNA synthesis

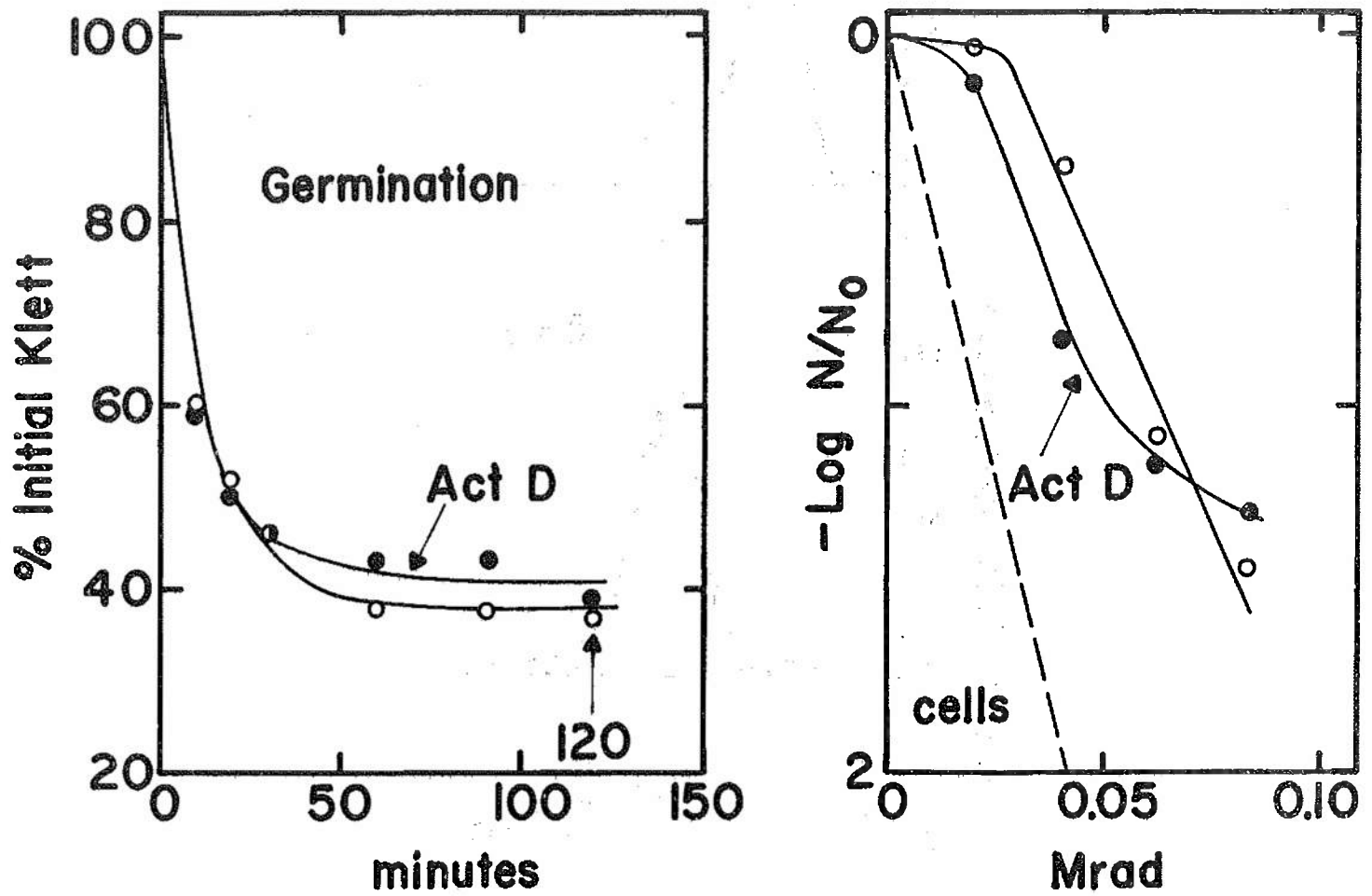


Figure 13. Effect of Actinomycin D on spore germination and (right) radiosensitivity of spores exposed to germinating medium for 120 minutes. The radiosensitivity of vegetative cells is indicated for reference (dashed line).

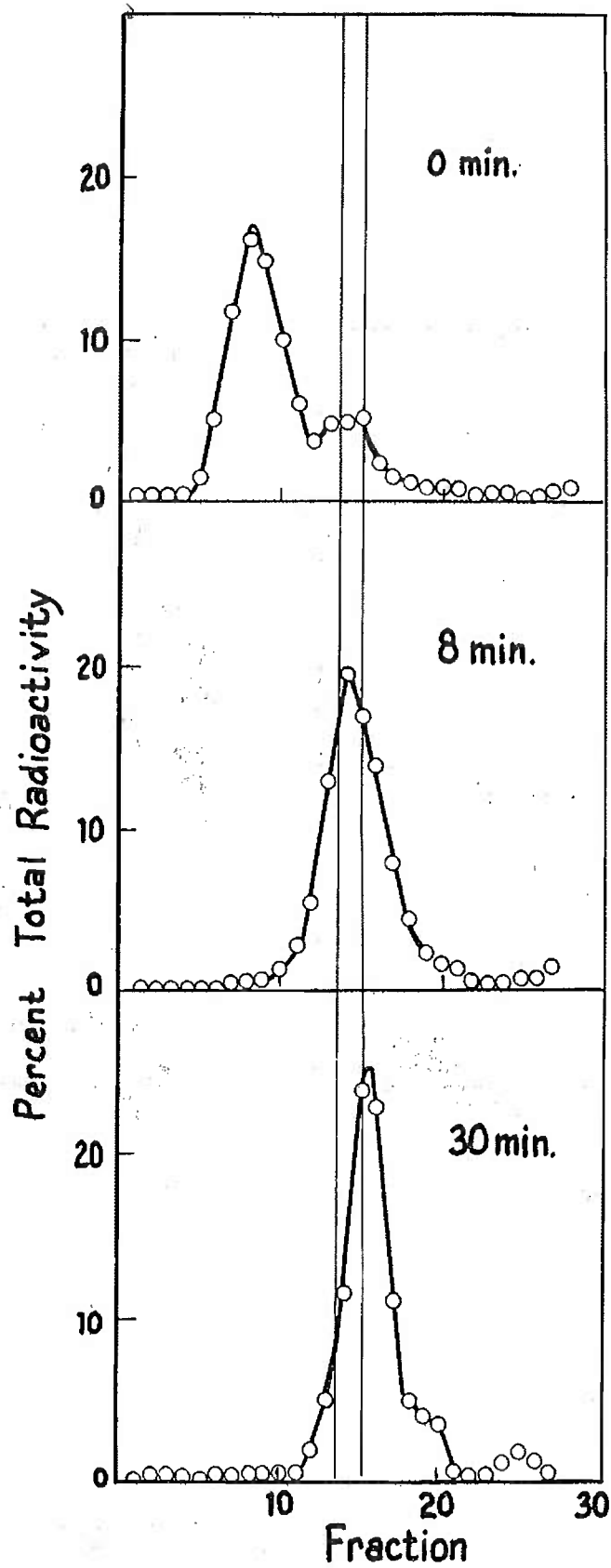


Figure 14. Density changes occurring in germinating *Cl. botulinum* spores.

on the spore survival curve. However, the most probable reason for a density decrease ( $\text{Ca}^{++}$ -DPA release)<sup>(2,53)</sup> early in germination seems (see discussion of DPA-less spores) not to be responsible for the shoulder loss. In addition, spores whose  $\text{Ca}^{++}$  but not DPA is lost are normal in radioresistance<sup>(13)</sup>. Dramatic changes in radioresistance of spores germinated more than 30 minutes appear not to correlate with density changes. Spore hydration and loss of other spore components may also contribute to the decrease of spore density, since DPA<sup>-</sup> spores of B. cereus are intermediate in density between DPA<sup>+</sup> ungerminated spores and germinated spores<sup>(2)</sup> (data not shown).

Many attempts were made to physically separate various classes of differentiating Clostridium botulinum 62A in sporulating cultures by zonal centrifugation. Although distinct bands were obtained, Renografin proved bacteriocidal to these organisms. This is in contrast to Clostridium spores which are not killed. Tamir and Gilvarg<sup>(53)</sup> reported B. megaterium cells and spores were not killed by Renografin. The reason(s) for the sensitivity of Cl. botulinum cells to this compound is unknown.

As shown in Figure 4, sporulating Cl. botulinum became resistant to 45 percent (w/v) sucrose just prior to acquiring radiation resistance. Originally, this result was taken to indicate that point at which the sporulating cell contained no free water in the protoplast. We have also determined the time at which germination spores (in nonsteamed TTT) become sucrose sensitive. The results (Figure 15) indicate sucrose resistance is lost much later in germination after the spores are phase dark and just before emergence in the population is detectable. This result contradicts our previous notion that the dry state of the spore protoplasm and sucrose resistance are related. More likely, sucrose resistance may depend upon a resistance to osmotic rupture following dilution out of the sucrose solution. The development of the spore coat may account for the appearance of sucrose resistance in sporulating cultures. In germinating spores, nicking of the spore coat prior to emergence may account for loss of sucrose resistance. Thus, sucrose resistance is temporally (but probably not causally) related to appearance of radiation resistance in sporulating populations, and not causally related to loss of spore radiation resistance during germination.

#### Sensitization of Spores to Radiation by Bromodeoxyuridine (BUdR)

One of the classical arguments in favor of DNA being a target of ionizing and ultraviolet irradiation in cells is the enhancement of sensitivity of cells which have incorporated halogenated uracil derivatives into their DNA in place of thymine<sup>(54,55,56)</sup>. Recently, direct evidence of ionizing radiation-induced lesions (single strand breaks) in DNA in spores of B. subtilis<sup>(57)</sup> was published.

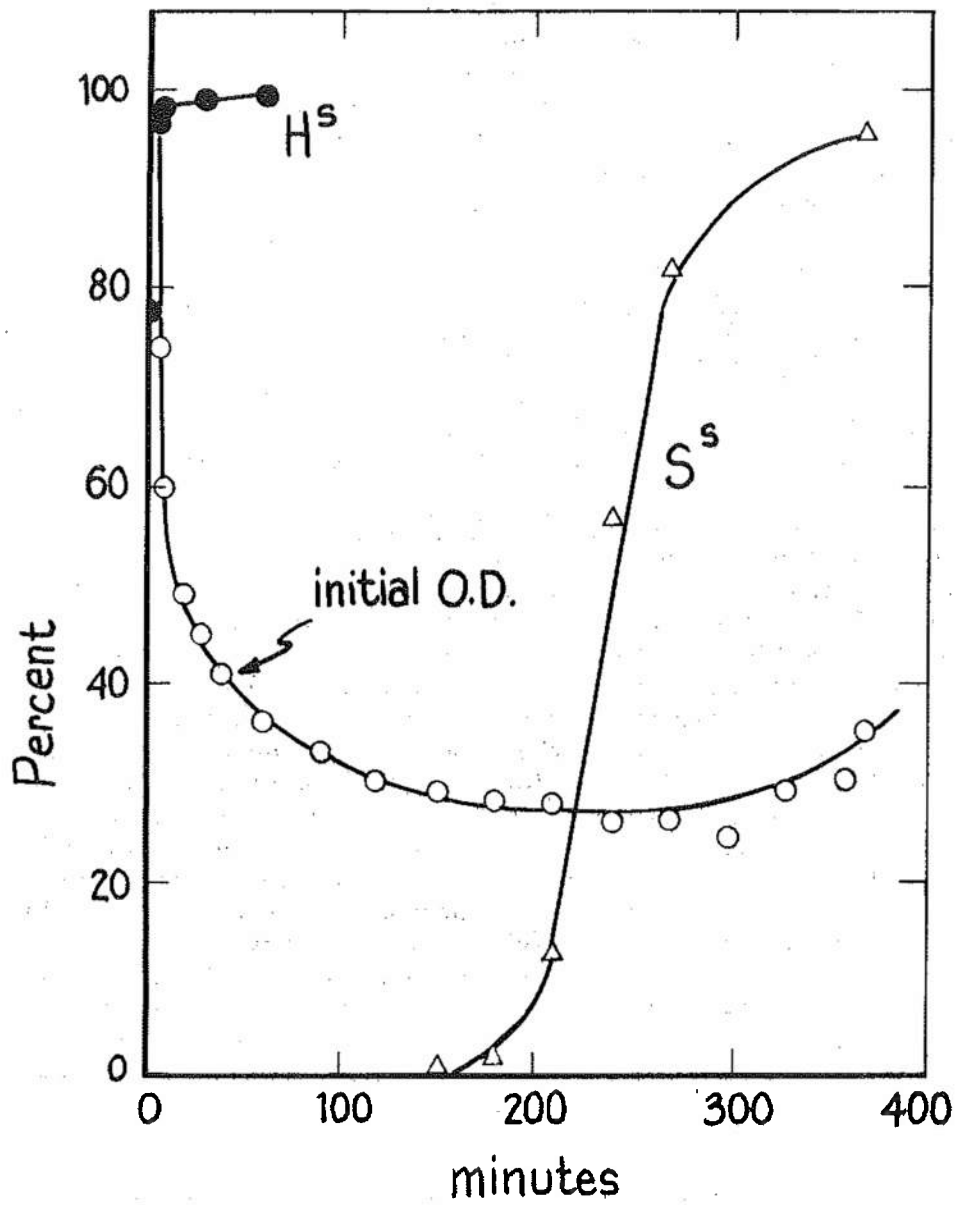


Figure 15. Appearance of sucrose-sensitivity in germinating *Cl. botulinum* spores.

We sought to find the effect of sensitizing spore DNA by BUdR replacement of thymine on spore radioresistance. An overnight culture of Cl. botulinum was inoculated into five tubes of SM broth containing 1, 20, 40, 80 and 120  $\mu\text{g/ml}$  BUdR (filter sterilized). After 46 hours of growth, the percent sporulation was determined by microscopic count (Figure 16, left). Sporulation was 83 percent in the control. The percent sporulation in the BUdR containing cultures decreased as the concentration of BUdR increased. Spores were harvested from each culture and irradiated to 0.375 Mrad (a dose corresponding to the exponential portion of the dose survival curve of normal spores). The results (Figure 16, right) clearly indicate spores produced in BUdR containing broth were more radiosensitive (7-fold at 80 and 120  $\mu\text{g/ml}$  BUdR) than control spores. Thus, conditions known to specifically sensitize DNA to irradiation in vegetative cells similarly sensitized spores. It follows that DNA is indeed a target in spores of Cl. botulinum.

#### Attempts to Isolate Radiation Sensitive, Mature Spore Mutants

One of the original goals of this project was to isolate radiation sensitive spores of Cl. botulinum. A number of methods were utilized in this mutant search - all without success.

Vegetative cultures exposed to mutagenic treatment (Mitomycin C (MC) at 20  $\mu\text{g/ml}$  for 60 minutes, ultraviolet irradiation or gamma irradiation) were screened for sensitivity to gamma radiation by three methods.

- (1) Overnight broth cultures of mutagenized strains were spotted (0.03 ml) on SM agar plates divided into quadrants. After the spots had dried, the plates were irradiated for 10 seconds with UV and incubated under an anaerobic atmosphere. Growth was scored 48 hours later. It was previously determined that 10 seconds of UV exposure with the geometry of our irradiation source gave only a slight decrease in growth of control cultures. Those strains which exhibited only a limited growth (<10 colonies/spot) were recovered from the original nonirradiated overnight broth cultures and then examined for their  $\gamma$  sensitivity. Fresh broth cultures were titered and irradiated with .041 Mrad. A post-radiation titer was done to assess their  $\gamma$  sensitivity. Alternately, single colonies were picked from spread plates of each strain, suspended in gelatin-phosphate, titered, irradiated, and titered again.
- (2) Colonies derived from mutagenized cultures were suspended in either (a) gelatin-phosphate (GP) diluent or (b) SM broth. The GP suspensions were swabbed onto SM agar slants. Cell suspensions in SM broth

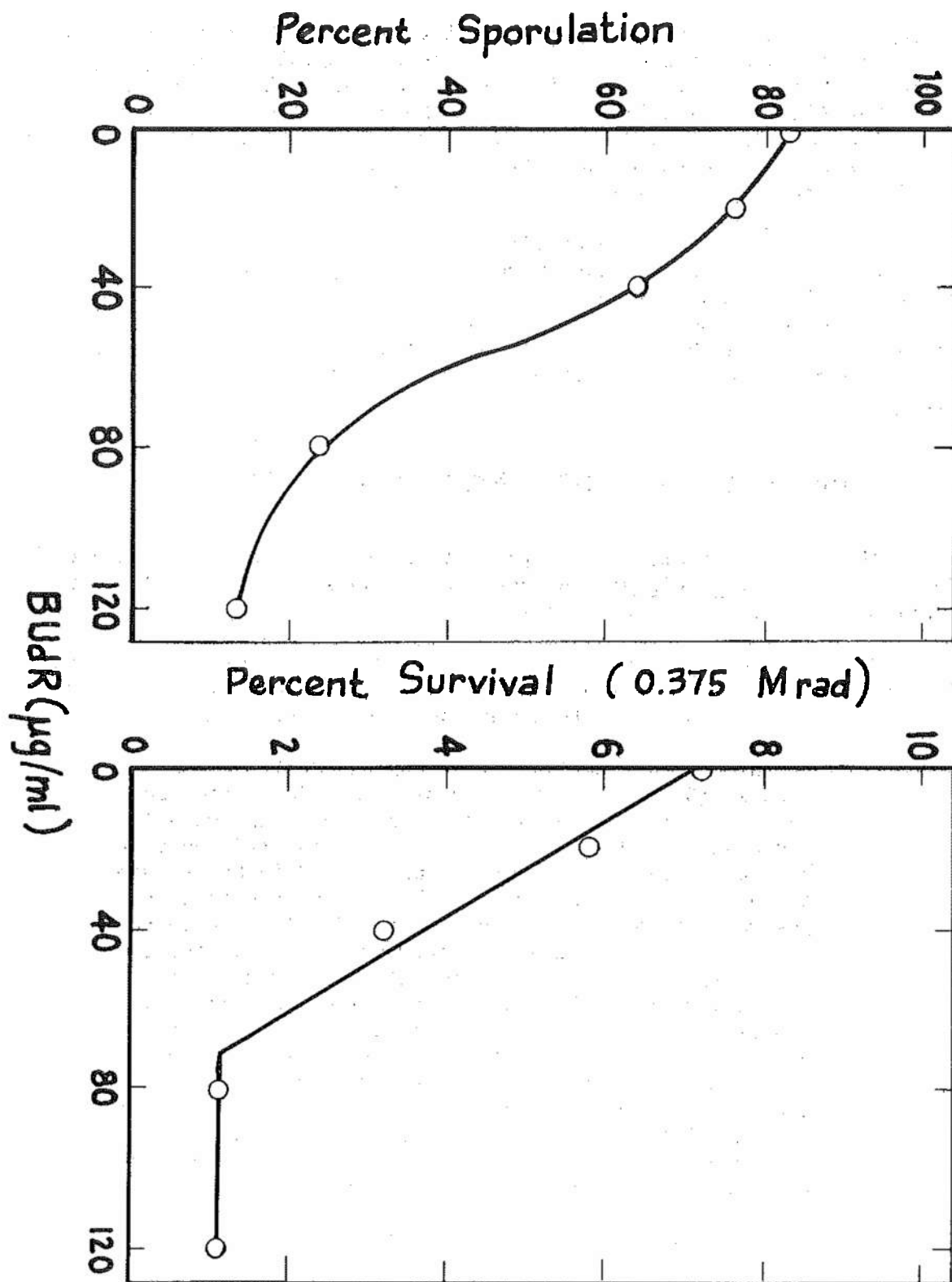


Figure 16. Effect of Bromodeoxyuridine (BUdR) on sporulation and (right) radioresistance of spores produced in its presence.

were either swabbed directly onto SM agar slants or swabbed onto slants after overnight growth. The inoculated slants were then irradiated in a special holder with  $Cs^{137}$ . Previously we had determined the extent of growth as a function of exposure time for control (nonmutagenized) cells. In order to recognize mutant strains which were markedly more sensitive to gamma radiation than the control strain, we elected to use an exposure time which produced a barely detectable effect on controls. The nonmutagenized controls gave >200 colonies to confluent growth. Thus, we could expect to detect differences in sensitivity at least as small as two logs.

- (3) We employed replica plating to facilitate our search for mutants. Briefly, mutagenized (UV) vegetative cultures were diluted and plated. The plates were incubated for approximately five days to allow all capable colonies to sporulate. Typically, about 10 percent of the colonies remained transparent. The plates were then replica plated to fresh cold plates. The "copies" containing transferred spores were irradiated in a special holder to a dose which did not visibly affect growth on the plate. The "copies" were incubated 30-48 hours at 37°C. After incubation "master" and "copy" plates were compared and those colonies which failed to develop on the copy plates were subcultured from the master plate. These colonies were tentatively considered to be radiation sensitive. Broth cultures were grown from these master plate colonies and new plates inoculated with sufficient cells to provide confluent growth. Incubation was allowed to proceed for 5 days. The organisms (cells, sporulated cells, free spores) were harvested from the plate by adding 5 ml of water and scraping with a glass spreader. The suspension was heated (80°C for 10 min) and irradiated for assessment of its radioresistance.

Mutagenized clones treated according to the procedures of (1) and (2) yielded strains that gave evidence of UV sensitivity, but they were similar or more resistant to gamma radiation than untreated controls (Table 9).

Table 9  
Survival of Strains to  $\gamma$  Radiation

UV mutagenesis		MC mutagenesis	
Strain	N/No <sup>†</sup> ( $\times 10^3$ )	Strain	N/No* ( $\times 10^3$ )
Control	2.6	Control	0.02
UV-57	6.3	MC-5	0.50
UV-60	>2.5	MC-7	0.24
UV-64	>2.9	MC-8	0.08
UV-65	3.3	MC-9	2.0
UV-66	>3.0	MC-10	0.32
UV-71	>3.5	MC-12	0.47
UV-74	>3.5	MC-15	0.19
UV-77	2.9	MC-31	0.85
UV-78	1.3	MC-33	0.08
UV-79	2.1	MC-34	0.10
UV-80	>1.7		
UV-83	>2.2		

<sup>†</sup> irradiated (0.041 Mrad) in broth

\* irradiated (0.041 Mrad) in gelatin-phosphate

Survival in gelatin-phosphate was about 100-fold less than in broth. Striking differences in survival were not seen in the UV strains. The MC strains, however, all appeared more resistant to  $\gamma$  radiation than the control. Some strains (MC-9 and MC-31) were 50 to 100 times more resistant. Spores of MC-9 and MC-31, however, were only slightly more resistant than control spores.

Results obtained by procedure (3) are outlined in Table 10.

Table 10  
Radiosensitivity of Spores  
from Apparent  $\gamma^S$  Colonies

Strain	Percent Survival*
R-1	30.3
R-2	52.3
R-3	56.2
R-4	40.3
R-5	48.3
R-6	75.0
R-7	52.5
R-8	31.2
R-9	34.0
R-10	44.5

\* 0.375 Mrad

To date, we have examined approximately 3000 colonies and obtained 10 which failed to grow on the "copy" plate. All of these colonies were transparent and gave evidence of very poor sporulation on the master plate. Spores were obtained, however, on subculture and examined for radioresistance. It is evident that gamma sensitive ( $\gamma^S$ ) spores were not obtained. Wild type spores under these conditions gave between 15 and 30 percent survival.

Radiation sensitive ( $\gamma^S$ ) organisms might be expected to recover from radiation exposure and commence growth at a slower rate than wild type organisms. Thus, a selection mechanism designed to enrich for slow recovery cells was devised. Generally, the procedure was to irradiate a cell suspension and then allow recovery and growth to occur in the presence of penicillin. These organisms which initiate growth earliest would be killed by the penicillin, whereas, the gamma sensitive ( $\gamma^S$ ) members of the population should be more refractory to the presence of penicillin. After a period of penicillin exposure, the antibiotic was removed and growth allowed to proceed normally. The population should be enriched for  $\gamma^S$  types. To further enrich for  $\gamma^S$  types the treatment was repeated. Finally, single colonies were isolated and analyzed for  $\gamma$  sensitivity.

An overnight SM broth culture was harvested and suspended in sterile  $H_2O$ . Following UV irradiation (to be a survival level of about 5 percent) the irradiated cells were diluted 1/50 into SM broth containing 5 U/ml Penicillin G. After overnight incubation little growth occurred. The cells were harvested by filtration and resuspended in 40 ml SM broth. Again, little growth occurred after overnight incubation. The culture was centrifuged and resuspended in sterile water, UV irradiated as previously and the cell suspension diluted 1/20 into SM broth containing 5 U/ml Penicillin G. Following overnight incubation, the culture was filtered, washed with 40 ml of SM broth and the cells suspended in 40 ml of SM broth. Following 3 days' incubation the culture consisted of mainly vegetative cells and a few (<1 percent) sporulated cells. After 10 days' incubation a sample was removed, heat shocked ( $80^\circ C/10$  min), and streak plates were made. No growth appeared on the plates. Another sample was pour plated. Colonies developed and were inoculated into SM broth and incubated anaerobically. The culture was transferred to fresh SM broth and, following sporulation, streak plates were prepared. Next, 83 single colonies were inoculated into 1 ml portions of SM broth and incubated anaerobically overnight. The broth cultures were inoculated onto SM agar slants with a cotton swab. The slants were irradiated as in method (2) above with the  $C^{137}$  source. Following irradiation, the slants were incubated anaerobically for 48 hours. Three strains (P-18, P-68 and P-75) gave 10-30 colonies, whereas the others gave >200 to confluent growth. Two of the strains, P-18 and P-75, produced spores whose radioresistance was identical to control spores. The third strain, P-68, failed to sporulate.

Thus, our attempts at obtaining gamma sensitive spores failed. Spores markedly sensitive to UV have recently been isolated<sup>(58)</sup> from UV sensitive cell lines of B. subtilis. The UV sensitive spores, however, were only slightly more sensitive to Cs<sup>137</sup> irradiation than wild type spores. The implications of these results and our failure to obtain  $\gamma^S$  spore mutants will be covered in the Discussion.

## GENERAL DISCUSSION

The results of this study indicated radioresistance and heat resistance of spores were unrelated. Evidence for this includes, (1) the temporal relationship of the acquisition of  $\gamma^R$  and  $H^R$  during sporulation, (2) sequence of loss of  $H^R$  and  $\gamma^R$  in germinating spores and, (3) studies with the DPA-less B. cereus spores. Our findings were in complete agreement with the studies of Vinter<sup>(9)</sup> and Romig and Wyss<sup>(10)</sup> who showed radiation resistance was acquired prior to heat resistance in sporulating B. cereus cultures. In addition, spores of B. megaterium, rendered heat sensitive by leaching cations from the spores at low pH, exhibited normal resistance to  $^{60}\text{Co}$  gamma irradiation<sup>(13)</sup>. Our results with heat sensitive, DPA-less spores of B. cereus indicated normal radiation resistance was independent of heat resistance. One may conclude neither calcium<sup>(13)</sup> nor DPA play a role in spore radioresistance.

The role of spore disulfide content was shown to contribute (at most) 30 percent to spore radioresistance. More likely, since the content of disulfide does not change during germination (through emergence), only 7 percent of spore radioresistance is contributable to the presence of disulfide bridges. Vinter<sup>(9)</sup> and Hitchins, et al.<sup>(20)</sup> reached similar qualitative conclusions.

The most dramatic changes in spore radioresistance during germination, namely loss of 80 percent of the large shoulder and the major increase in slope of the exponential inactivation, occurred prior to the onset of RNA synthesis. Since RNA synthesis precedes other macromolecular syntheses<sup>(39-41)</sup> in germinating spores, this is taken as evidence that radiosensitization occurs prior to the commencement of RNA, protein, or DNA synthesis. Further support for this notion is found in the germination studies in the synthetic medium of Rowley. Although RNA synthesis, emergence, and elongation did not occur during germination, radiosensitization most certainly occurred. We attempted to determine the time of initiation of DNA synthesis, however, our attempts failed due to lack of uptake of ( $\text{H}^3$ )-thymidine; even in elongating spores. Addition of deoxyadenosine<sup>(59)</sup> did not change the situation.

The loss of 80 percent of the large shoulder occurred very rapidly upon exposure of spores to germination conditions. In TTT broth, the phenomenon coincided with a 30-40 percent decrease in the difference in density between spores and vegetative cells. In addition, the shoulder loss was shown to be temperature dependent. Since the phenomenon (shoulder reduction) also occurred in the synthetic medium, it is possible the event is dependent only upon the activation of some germination enzyme system.

The only statement we can make about the rate ( $D_{10}(\text{exp})$ ) change is that it occurred in TTT prior to the onset of RNA synthesis and in Rowley's

medium in the total absence of RNA synthesis. A strong possibility is that the slope change is indicative of a change in the sensitivity of a lethal target (i.e., DNA) as a result of a change in the physical properties of the spore protoplast.

Spores of Cl. sporogenes<sup>(60)</sup> contain a featureless core that changes during germination to one in which ribosomes are detectable. Maeda et al.<sup>(61)</sup> concluded from dielectric and NMR measurements that water in spores of B. megaterium was less mobile (bound) than water in the corresponding vegetative cells. Evidence<sup>(45-47)</sup> that the DNA in spores is in a physical structure different from that found in vegetative cells has been cited earlier. In spores, a UV photoproduct containing thymine is found that is not found in the photoproducts formed in vegetative cells<sup>(45)</sup>. The spore photoproduct is, however, obtained in DNA irradiated as a dry film<sup>(46)</sup>, DNA films equilibrated to 65 percent and less relative humidity<sup>(62)</sup>, and in aqueous DNA irradiated at very low (-100 to -196°C) temperatures<sup>(63,64)</sup>. Rahn and Hosszu<sup>(62)</sup> postulated the transition in DNA structure involved a change from the B form<sup>(65)</sup> of DNA, not to the A<sup>(65)</sup> form as has been suggested<sup>(66)</sup>, but to a form in which the DNA is disorganized and mostly dehydrated. DNA in dried films was shown to be denatured, but due to lack of water, the strands remain in register. The DNA in spores was suggested to be in a similar state<sup>(62)</sup>. The general concept, then, is that spore DNA (and perhaps the protoplast) is in a dehydrated state.

Ionizing radiation resistance of bacterial spores is dependent upon temperature and the nature of the gases present during and after irradiation<sup>(67)</sup> of dry spores. Additionally, the water vapor pressure during spore irradiation in oxygen or nitrogen followed by post-irradiation oxygen treatment markedly affects spore survival<sup>(68)</sup>. Conditions that allow only direct damage to essential molecules (Class I damage)<sup>(67)</sup> reveal this type of damage is independent of water vapor pressure<sup>(68)</sup>. This conclusion, however, does not contradict a hypothesis in which the "dry state of spore DNA" is invoked to account for spore radioresistance. The spore protoplast does not equilibrate with external water conditions, since aqueous spore suspensions (liquid state) yield dry DNA-type UV photoproducts<sup>(45)</sup>.

That DNA is indeed a lethal target in spores is supported by the sensitization<sup>(69)</sup> of Cl. botulinum spores by BUdR (which replaces thymine in DNA) described earlier. Terano et al.<sup>(57)</sup> showed spores of B. subtilis acquired single-strand breaks in their DNA by exposure to ionizing radiation. The breaks were repaired during germination. Finally, DNA extracted from irradiated (6 MeV electron beam) spores was indeed damaged (as assayed by transformation), but more resistant than DNA from irradiated vegetative cells<sup>(70)</sup>. Thus, evidence indicating spore DNA is a target (Class I type)<sup>(67)</sup> of ionizing as well as UV irradiation is not lacking.

Many authors have suggested germinating spores take up water. Stafford and Donnellan<sup>(66)</sup> have shown with B. megaterium the transition from spore-type DNA to cell-type DNA began with the initiation of germination and reached completion within 4 (based on loss of spore photoproduct) to

greater than 15 minutes (based on maximal yield of cell-type photoproduct). These results<sup>(66)</sup> constitute fairly convincing evidence that water-associated events take place in spore protoplasts during germination.

Generally, two types of lesions in DNA result from exposure of an organism to ionizing radiation: (a) modification of a nucleotide base (chiefly pyrimidines), and (b) rupture of the phosphodiester backbone<sup>(71)</sup>. The predominant effect appears to be a rupturing of the backbone (single strand break) as determined in bacteriophage and isolated DNA (see Ref. 54). Single strand breaks are not lethal events<sup>(54)</sup>; on the other hand, double strand breaks, which occur as a result of the juxtaposition of single strand breaks on opposite chains, correlate well with lethality<sup>(54)</sup>. In a similar manner, nucleotide modifications are not thought to be lethal since the information lost in the modified region of one strand remains intact on the opposite strand. The minor or least frequent lesion (nucleotide modification) produced by ionizing radiation may be dealt with by the organism in a manner identical to that used for the repair of UV lesions<sup>(71)</sup> (which are of the nucleotide modification type)<sup>(55)</sup>.

Dark repair of UV lesions (for example, of cyclobutane-type thymine dimers) proceeds in four distinct steps<sup>(56)</sup>. First, the lesion is recognized and a single strand incision is made by an endonuclease. Second, the dimer and adjacent nucleotides are excised by an exonuclease. Third, the resulting gap is filled by DNA polymerase to give an exact replica of the opposite strand. The last bond, however, remains open since DNA polymerase is unable to complete it. Finally, the remaining strand break may be filled by DNA ligase<sup>(56)</sup>. Once a nucleotide modification has been recognized and removed, repair may proceed by a common mechanism, regardless of the type of nucleotide modification. Thus, modified pyrimidines produced by ionizing irradiation (for example, hydroperoxides) might be expected to be repaired by the so-called UV repair system. Single strand breaks would require only the action of DNA ligase. Double strand breaks are not repairable by these mechanisms.

Thus, if a common mechanism is employed to repair UV and ionizing radiation base modifications (see Ref. 71), a strain of bacteria sensitive to UV should also exhibit sensitivity to ionizing radiation. This is the case with many strains (see Ref. 71 for discussion); however, the magnitude of increased sensitivity to UV is usually several times greater than that for ionizing radiation. This, it seems, further indicates that base modifications are only a minor fraction of the total damage produced by ionizing radiation. Thus, on the basis of mutation studies with cells, chemical studies on both the free bases in solution and free DNA in solution, genetic studies with free DNA in solution, and physical studies on the DNA of virus particles, the evidence heavily supports the idea that the single strand break is the primary effect of ionizing radiation on DNA (for a discussion of this evidence see Ref. 54).

It is currently thought that DNA ligase is necessary for the normal replication of the bacterial chromosome<sup>(72,73)</sup>. Thus, any mutation in this enzyme which seriously affects DNA replication would be lethal.

Therefore, assuming (a) single strand breaks are the predominant lesions in DNA following gamma irradiation of cells and spores, (b) DNA ligase is utilized to repair single strand breaks, and (c) DNA ligase is necessary for normal DNA replication and hence viability, it follows that a mutation which renders a strain markedly gamma radiation sensitive would also markedly affect its viability. Thus, from the standpoint of repairing ionizing radiation damage to DNA, mutants with orders of magnitude increased radiosensitivity are not expected to be found.

Indeed, we have screened a number of clones of Clostridium botulinum 62A which gave evidence of UV sensitivity; however, differences with respect to gamma irradiation were either minimal or lacking.

It should be noted that DNA ligase requires adjacent 5'-phosphoryl and 3'-hydroxyl termini in order to close a gap via formation of 3'5'-phosphodiester linkage. Recent evidence<sup>(74)</sup> suggests that single strand breaks in DNA produced by ionizing radiation in vitro may not have the required chemical structure for direct action by DNA ligase. They postulate a nuclease action is required to "clean up" the ends prior to DNA ligase action. This possibility may explain why UV sensitive strains exhibit increased ionizing radiation sensitivity; in addition to repair of modified bases caused by ionizing radiation, a nuclease employed in the UV repair system may serve the "cleaning up" role postulated above<sup>(74)</sup>.

Our general conclusion is that the spore-specific state of the protoplasm is largely responsible for spore radioresistance. The next step, therefore, in continuation of this work would be an examination of the transition from spore-type DNA to cell-type DNA during Cl. botulinum spore germination. The UV thymine photoproduct spectrum of samples removed during germination would be an excellent indicator of such a transition<sup>(66)</sup>. The transition, if responsible for loss of spore radioresistance, should correspond with the onset and time course of radiosensitization of germinating spores.

## SUMMARY

Work has been conducted to delineate the mechanisms responsible for the extraordinary resistance of Clostridium botulinum spores to ionizing radiation as compared with vegetative cells. The findings indicate that the progression from the high resistance of spores to the sensitivity of vegetative cells proceeds in at least three discrete steps. Several biochemical events have been shown not to play a key role in the resistance and the state of hydration and configuration of DNA has emerged as a prime candidate to explain the changes in resistance.

Sporulating cultures of Cl. botulinum were found to become resistant to gamma irradiation ( $Cs^{137}$ ) prior to development of heat resistance. In addition, loss of radiation resistance during spore germination was preceded by a rapid loss of heat resistance. These observations suggested radiation and heat resistance were dependent on different mechanisms. Further support for this notion emerged when it was found that heat sensitive, DPA-less spores of B. cereus were identical to wild type, DPA<sup>+</sup> spores in radiation resistance.

The effect of spore disulfide reduction upon spore radioresistance was examined in Cl. botulinum. Plots of the log of spore survival vs. irradiation dose, yield a curve in which survival remains at 100 percent up to a dose of 120-170 Krad (shoulder) followed by a constant decrease in survivors (slope) as the dosage is further increased. Reduced and nonreduced spores exhibited identical shoulders, but reduced spores had a slope 1.6 times greater than control spores. These data indicate that reduction of disulfide linkages decreased (assuming this was the only result of reduction) radioresistance by 25-30 percent. Data obtained during spore germination (in which 93 percent of the difference between vegetative cell and spore radiation resistance was lost) showed disulfides to remain unchanged during germination (neither reduced nor released). These data cast doubt on the disulfide contribution to spore radioresistance (~1/3) estimated earlier. Disulfide contribution to spore radioresistance is estimated at about 10 percent, based on germination data.

Patterns of radioresistance of germinating populations of Cl. botulinum spores revealed three general phenomena occurred sequentially (1) reduction of the ungerminated spore shoulder seen on dose-survival plots by 80 percent within minutes after commencement of germination; this phenomenon was temperature dependent. (2) Change in slope of the inactivation curve to a value six times greater than ungerminated spores and, (3) complete loss of the remaining shoulder and further increase in slope of inactivation curve (to 12-13 times the ungerminated spore slope). Changes (1) and (2) occurred prior to commencement of RNA synthesis but concomitant with spore density changes. During germination in a synthetic medium, only changes (1) and (3) were seen. RNA synthesis did not occur in spores germinated in synthetic medium. Actinomycin D

did not affect spore germination and subsequent radiosensitization in the complex broth system. Thus, all three phenomena appear to be independent of RNA (macromolecular) synthesis. Spores produced in the presence of BUdR were up to seven times more sensitive (at a total dose of 0.375 Mrad) than control spores. Thus, spore DNA was shown to be a lethal target. Attempts to isolate radiation-sensitive spores of C1. botulinum were unsuccessful. The changes in sensitivity upon germination are discussed in terms of changes in the physical state of the spore protoplast.

These findings have served to eliminate several possible mechanisms of radioresistance and to incriminate others. Conceivably, the state of hydration and configuration of DNA may play a significant role. The finding that changes in spore radioresistance occur in discrete steps provide a powerful tool for further delineation of the mechanisms of radiation resistance.

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13. ABSTRACT <p>The purpose of this project was to investigate possible mechanism(s) of the radiation resistance of <u>Clostridium botulinum</u> spores. A better understanding of the radioresistance of this important food pathogen would enhance ones ability to reduce the minimal radiation dose (MRD) presently required for the radiation sterilization of meats. The ultimate reduction of the MRD would result in an improvement in the organoleptic quality of certain established prototypes, particularly those sensitive to the prevailing dose levels.</p> <p>During germination the loss of heat resistance preceded the loss of radiation resistance. Changes in radioresistance during germination were not due to loss of calcium, dipicolinic acid (DPA) or disulfide-rich protein. Studies with DPA-less spores of <u>Bacillus cereus</u> showed that they were just as resistant to radiation as the wild type (DPA<sup>+</sup>) spores. Radiosensitization during germination occurred prior to synthesis of ribonucleic acid, proteins and deoxyribonucleic acid (DNA) and therefore was concluded to be independent of macromolecular synthesis.</p> <p>Spores whose disulfide sulfur was reduced to sulfhydryl sulfur remained radioresistant. The form of sulfur in spores may play a small role in radioresistance but was not responsible for the spores high resistance relative to the vegetative cell.</p>			

(See continuation sheet)

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	ROLE	WT	ROLE	WT	ROLE	WT
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Radiation Effects	8					
Clostridium Botulinum	9,7					
Spores	9,7					
Irradiation	6					
Food Irradiation	4					

### 13. Abstract (continued)

Conditions (spores produced in the presence of bromodeoxyuridine) known to specifically sensitize vegetative cell DNA to irradiation similarly sensitized spores. It follows that DNA is indeed a target in spores of C1. botulinum. Changes in the hydration state of the spore and the physical state (dehydrated) of the target molecule (DNA) are discussed as possible mechanisms of the radiation resistance of C1. botulinum spores. However, further research is required to actually delineate the mechanism of C1. botulinum spore radioresistance.

