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EFFECT OF CHEMICAL ENVIRONMENT ON THE LETHALITY OF GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

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INTRODUCTION

As with all scientific research, many factors were responsible for the initiation of this investigation. For example, early in this decade, waste fission products became available in sufficient quantity to make their peacetime use by various industries feasible. Because of this, the problems connected with such uses of ionizing radiations needed evaluation. One phase of this study involved an evaluation of the factors influencing the survival of microorganisms subjected to ionizing radiations. The food industry was particularly concerned with the fate of anaerobic bacteria present in foods that might be prepared for public consumption by radiation sterilization. It was as part of this latter program that Kempe was studying the spores of Clostridium botulinum in canned meat subjected to combined heat and radiation sterilization. During the course of this research, he observed that the number of Clostridium botulinum spores surviving irradiation with cobalt-60 was greater in a gelatin solution than in a solution of phosphate buffer (Kempe, 1955). This same effect had been reported previously by Proctor and Goldblith (1951) with cells of Staphylococcus aureus. The observation that a protective effect could be elicited with anaerobic bacterial spores as well as with aerobic, vegetative cells stimulated an interest in alteration of the lethal effects of ionizing radiations on bacteria by chemical agents. It was considered desirable to determine if this phenomenon was widespread in its occurrence and, if so, the reason for it.

At the time this research was begun, a survey of the existing literature showed that only a few studies even mentioned the chemical factors influencing survival of microorganisms during exposure to ionizing radiations. The most extensive study dealing with bacteria was reported by Hollaender and his co-workers (Hollaender et al., 1951; Hollaender and Stapleton, 1953). These investigators, working with the aerobic, vegetative cells of Escherichia coli, strains B and B/r, and using X-radiation in dosages between 5,000 and 90,000 roentgens, reported protection of their organisms by certain chemicals.

Subsequent to the work of these investigators and at the same time the present research was being conducted, a few reports were published that considered the effect of the composition of the medium on the survival of bacteria exposed to ionizing radiations (Proctor and Goldblith, 1954, 1954a) and the protection phenomenon noted when substances of a protein nature were incorporated into the irradiation medium (Edwards et al., 1954). These reports, however, dealt only with aerobic organisms and contained no detailed studies.

Since it is not advisable to draw analogies between different species of bacteria with regard to the factors affecting their radiation sensitivity, it was considered desirable to do a study using spores of Clostridium species as the test organisms. These organisms had not been studied by previous investigators. It was planned to determine whether alteration of the radiation sensitivity of these spores could be obtained by altering the chemical

composition of the medium in which they were suspended during irradiation and, if so, if this alteration followed the same pattern reported by other investigators using vegetative cells of aerobic bacteria.

Radiation dosages of between 300,000 rep and 1,440,000 rep were used. These were much higher dosages than had been used by previous workers studying chemical protection of bacteria. Furthermore, gamma radiation from cobalt-60 was the type of ionizing radiation employed whereas previous investigators had used cathode rays or X-radiation. Cobalt-60 offered the advantage that it emits gamma radiation of only two energies, 1.17 and 1.33 Mev (mega-electron-volts)(Nehemias et al., 1954). The beta rays which are also emitted by cobalt-60 were absorbed, in this case, by the aluminum jacketing used to house the rods of radioactive material. Therefore, only the gamma radiation from cobalt-60 acted upon the bacterial spores.

The objectives of this research were to collect qualitative data on a variety of chemical compounds to determine whether alteration of the radiation sensitivity of anaerobic bacterial spores by chemicals was possible. If protection effects were found, the chemicals which provided this protection were to be classified into groups and, if possible, quantitative data collected. It was considered likely that from generalizations based upon such data, the limitations inherent in the use of ionizing radiations for sterilization of biological substances could perhaps be more clearly understood and, as a consequence, rationally controlled.

HISTORICAL

I. Discovery of Radioactivity

In the last decade of the nineteenth century, two discoveries in the field of physics opened an entirely new area for research. The first of these discoveries was made in 1895 by Wilhelm Konrad Röntgen while studying cathode rays in a Hittorf-Crookes vacuum tube. Röntgen observed that a distant fluorescent screen was caused to glow as a result of the operation of the vacuum tube, despite the fact that the tube was at that time surrounded by heavy paper that was opaque to ordinary light rays. He concluded that these rays, of great penetrating power, were due to some unknown form of radiation to which he gave the name X-rays (Röntgen, 1895). Röntgen's discovery, reported in December of 1895, was the instigating factor for the investigations of Antoine Henri Becquerel. Early in 1896, while studying the connection between the phosphorescence of certain minerals after illumination and their ability to darken a photographic plate through a light-absorbing substance, Becquerel discovered that certain phosphorescent uranium salts were effective in this regard. Further investigation revealed that this property of emitting rays detectable by a photographic plate did not depend on exposure of these salts to light but was an inherent characteristic of the element uranium itself (Becquerel, 1896, 1896a, 1896b). This was an entirely new property of matter and for its discovery Becquerel is given the title of "the father of radioactivity".

II. Early Studies of the Effect of Radiation on Bacteria

The relationship of radioactivity to other scientific fields was quickly investigated. The earliest report of the effect of radiation

from radioactive substances on bacteria came in 1899 when Pacinotti and Porcelli exposed various aerobic, vegetative bacterial cells to preparations of powdered uranium and found that the organisms were killed (Pacinotti and Porcelli, 1899). Through the research of Rutherford on alpha and beta rays (from Glasser et al., 1952) and the work of Villard on gamma rays (from Glasser et al., 1952), the presence of these three types of rays in radioactive material was known at the time Pacinotti and Porcelli conducted their experiments. These latter investigators made no attempt, however, to separate the effects of the three in their work with bacteria.

Following the research of Pacinotti and Porcelli, numerous studies were conducted during the early years of the twentieth century on the effect of a newly discovered radioactive element, radium (Curie, P. and M.S., 1898), on bacteria. The interpretation of the results obtained in these early studies varied, however. Iredell and Minett (1909) stated that staining, growth, and reproduction of Bacillus pyocyaneus were completely unaffected by radium emanations. Aschkinass and Caspari (1901), working only with Micrococcus prodigiosus, and later Dixon and Wigham (1904), using several different organisms, reported that while no death resulted, a temporary inhibition of bacterial growth was produced by the beta rays emitted by radium. Jansen (1910), Chambers and Russ (1912), Lequeux and Chome (1919) and Bruynogne and Mund (1925), in contradiction to the conclusions of the previous workers, reported that radium emanations were lethal for bacteria. Bruynogne and Mund even went so far as to state that this lethal effect was the result of the inability of the cells to divide subsequent to irradiation. In no case were the gamma rays

of radium considered of importance, however, and Bruynogne and Mund (1925) actually stated that these rays were without action on bacteria. This statement was in agreement with the conclusions of all the earlier workers, who had ascribed all effects noted to either the alpha or beta particles emitted by radium.

Between 1925 and 1940 a few investigators reported studies of the effect of radium on bacteria. It was during this period, however, that X-rays almost completely superseded naturally occurring radioactive elements as the source of ionizing radiations used for studies on microorganisms. The prohibitive cost of radium made large sources of this element unavailable; on the other hand, advances in the field of physics between 1900 and 1925 resulted in considerable improvement of X-ray producing machines. Undoubtedly, these factors contributed to this changing research trend.

Spencer (1934, 1935) studied broth cultures of vegetative cells of the aerobes Salmonella typhosus, Streptococcus scarlatinae and Proteus vulgaris OX-19 irradiated with radium. He stated that rapidly multiplying cultures of these bacteria underwent a temporary retardation of growth (first 6 hours) if subjected to irradiation but that after 24 hours this difference in growth was no longer detectable when these cultures were compared with the unirradiated controls. On the contrary, bacteria held at 0 C during irradiation to prevent multiplication were gradually killed while the controls showed neither reduction or multiplication of numbers. Spencer concluded from these results that resting cells of bacteria were more susceptible to irradiation with radium than actively growing cultures. As had the previous workers, he attributed the observed effects

to the action of the beta rays emitted by radium rather than to the gamma rays. It was not until the study of Dozois et al. (1936) that the gamma rays of radium were finally implicated as a causative agent for the death of bacteria.

III. Major Theories of the Mechanism of Action of Ionizing Radiations

During the 1930's an increasing number of investigators studied the effects of ionizing radiation on microorganisms. From the results obtained in these studies, an hypothesis was formulated to explain the mechanism of action by which ionizing radiations produced the lethal effects which were noted. Wyckoff (1930, 1930a) first proposed this hypothesis to explain the results he obtained while studying the effect of X-rays on cells of Bacillus aerotryke and Escherichia coli. He found that death of these cells occurred in an exponential fashion and, in terms of the known absorption of X-rays in quanta, interpreted this to mean that one absorption of any of these radiations was sufficient to kill. According to his calculations, the sensitive area of the cell was approximately 0.01 per cent of the total cell volume. This theory became known as the "one-quantum-hit-to-kill" or "target" theory and, during the decade from 1930 to 1940, gained prominence as the preferred explanation of the mode of action of ionizing radiations on living cells. Ionizing radiations are any electromagnetic or particulate radiations capable of producing ions in their passage through matter. The target theory assumes the direct dissipation of energy within a molecule subsequent to its collision with such an ionizing radiation. The molecule in which the ionization is produced is the "target" and the production of ionization is termed a "hit".

Such investigators as Pugsley et al. (1935) and Lea et al. (1936, 1937), in studying bacteria exposed to X-radiation, interpreted their results in terms of this theory as did investigators who worked with other test systems. Lea became one of the leading proponents of the target theory and later published a book summarizing much of the data reported in the literature which substantiated this interpretation of the results obtained with single celled organisms (Lea, 1947).

In the decade from 1940 to 1950 a second theory began to receive attention as a possible explanation for some of the results obtained in studies of biological materials. This theory was first suggested as an "activated solvent" hypothesis by Risse (1929, 1930). He postulated that the primary action of ionizing radiations was on the suspending medium rather than directly on the solute; that irradiation of water, for example, produced atomic hydrogen and OH radicals which, in turn, acted upon the materials present in the solution. In 1931, Stenstrom and Lohmann reported experiments in which a protective effect was noted in solutions of inorganic chemicals exposed to X-radiation (Stenstrom and Lohmann, 1931). Later, Fricke and Brownscombe (1933), in studying the reduction of chromate solutions by X-rays, concluded that this reduction was due to a primary production of an activated form of hydrogen peroxide from the water. In 1934, Fricke gave official recognition to his adoption of Risse's theory by stating (Fricke, 1934):

> An important role is played by water. While water is not changed chemically as the result of irradiation with X-rays, nevertheless the water molecules are activated by the substances present in solution, resulting in their chemical transformation. Since so large a part of the cell is water, reactions of this type probably play an important role in the biological effects of the rays.

During the 1930's, however, this theory was not employed to explain the results obtained in studies on living cells. It was not until the work of Dale and his co-workers that the findings of the earlier investigators on solutions of inorganic chemicals were applied to biologically active materials (Dale, 1940, 1942, 1943, 1943a; Dale et al., 1943, 1949a). Until Dale's work, it was believed that X- and gamma rays influenced enzymes only when the irradiation dose was enormous. The conclusion was drawn that enzymes played little or no part in the inhibition of cell division by irradiation (Scott, 1937). Dale's work contradicted the earlier reports. Working with low concentrations of highly purified enzymes, he found that the concentration of enzyme and its state during irradiation were both factors in X-ray sensitivity (Dale, 1940). He observed a "concentration effect"; a definite amount of radiation energy absorbed corresponded to a constant amount of enzyme inactivated. He also noted a "protective effect" with a great number of compounds, including the protection of the prosthetic group of d-amino-acid oxidase by the specific protein of the enzyme (Dale, 1942). From these and later investigations, Dale concluded that the effect of radiation on substances dissolved in water must be an indirect one; that the results, especially protection, were very difficult to explain on the basis of direct action (Dale, 1943a; Dale et al., 1943, 1949a). Dale extended his work on enzymes to a non-protein, non-enzymatic substance, acetylcholine, and obtained similar results (Dale, 1943). He did not, however, attempt to specify the nature of the intermediate product formed in the water or its mode of action. This was done by Weiss.

Weiss (1944, 1946) advanced the theory that the primary action of ionizing radiations resulted in the splitting of water molecules to form the free radicals

$$H_2O \rightarrow \ldots\ldots\ldots \rightarrow OH + H,$$

presumably through the ions H_2O^+ and H_2O^-. If this mechanism were correct, then the ionic yield (number of molecules liberated for every 32.5 electron-volts of X-ray absorbed) should not exceed unity if one OH radical were involved in, for example, deamination of a mono-amino-mono-carboxylic acid molecule. Stein and Weiss (1948) reported such a yield for the deamination of certain simple amino acids. This picture of the mechanism of action of radiation on living cells was found inadequate, however, to explain the results reported later by Dale and his co-workers (Dale and Davies, 1949; Dale et al., 1949). They obtained an ionic yield of 3 for the deamination of glycine and so concluded that, in irradiated solutions of glycine, either: (1) one ion pair gave rise to more than one OH radical; or (2) that some of the radiation energy could form radicals by excitation rather than by ionization.

Subsequent to World War II, research into all aspects of radiation received a noticeable impetus. In line with this, the radiation chemistry of aqueous solutions was thoroughly studied by a number of investigators and the identity of the intermediate products formed in water by ionizing radiations was fairly well established (Allen, 1948; Allen et al., 1952; Hart, 1954). As a result of such investigations, the following scheme has now become fairly well accepted as representative of the reactions that occur when water is subjected to ionizing radiations:

$$2\ H_2O \rightarrow H_2 + H_2O_2$$

$$H_2O \rightarrow H + OH$$

$$2\ H_2O \rightarrow H + H + H_2O_2.$$

If oxygen is also present in the water, the possible radical reactions that may then follow are:

$$H_2O \rightarrow H + OH$$

$$H + O_2 \rightarrow HO_2$$

$$H + HO_2 \rightarrow H_2O_2$$

$$HO_2 + HO_2 \rightarrow H_2O_2 + O_2$$

$$OH + HO_2 \rightarrow H_2O + O_2$$
$$OH + H_2O_2 \rightarrow H_2O + HO_2.$$

This explanation of the radiation chemistry of water supplemented Weiss's original hypothesis.

IV. Effects of Varying Chemical and Physical Conditions On Survival of Bacteria During Irradiation

As well as the studies on the radiation chemistry of aqueous solutions, a voluminous quantity of literature appeared within this decade on the effects of radiation on living cells. The discussion here will be limited to those studies of the effects of radiations on bacteria that have direct applicability to the present study of alteration of the radiation sensitivity of anaerobic spores by chemicals.

A study of the effect of chemicals on bacteria was done by a group of workers at the Oak Ridge National Laboratory. Burnett et al.

(1951), using a Maxitron 250 kV X-ray machine as the source of radiation and Escherichia coli B/r as the test organism, reported that the chemicals 2,3-dimercaptopropanol (BAL), sodium hydrosulfite and ethanol all protected Escherichia coli B/r. The log of the survival of this organism was a linear function of the X-ray dose over the range of 15,000 to 90,000 roentgens whether the suspensions were chemically treated or untreated. The compound 2,3-dimercaptopropanol provided the greatest protection and the authors postulated that this might be due to its two sulfhydryl groups. The protection afforded by sodium hydrosulfite they attributed to its ability to remove oxygen from the suspension. No really satisfactory explanation was given for the protection observed with ethanol, a protection which was of lesser magnitude than that obtained with the other two compounds. Later in 1951, Hollaender reported that further study of chemicals giving protection illustrated that they could be categorized on the basis of chemical structure as follows (Hollaender et al., 1951):

1. compounds containing sulfhydryl groups
2. alcohols and glycols
3. sodium hydrosulfite
4. metabolic intermediates and products.

Both alcohols and glycols and the metabolic intermediates and products gave markedly better protection if they were incubated with the bacterial cells prior to irradiation. Hollaender, therefore, postulated that the protective action of these compounds was dependent upon their entering the metabolic cycle of the cell and thereby either replacing basic cell constituents destroyed by irradiation or depleting the oxygen supply (Hollaender et al., 1951). Later work seemed to favor the

postulation of enzymatic oxygen removal by these compounds (Stapleton et al., 1952; Hollaender and Stapleton, 1953). Invariably, if the compounds could be oxidized by the organism, they would protect; where oxidation did not occur, there was no protection. This same effect of protection by a metabolic intermediate was also reported by Thompson et al. (1951) using pyruvate with cells of Bacillus anthracis, Escherichia coli and Micrococcus pyogenes var. aureus.

Hollaender found that, in contrast to the alcohols and glycols or metabolic intermediates, neither sodium hydrosulfite nor sulfhydryl-containing compounds required previous incubation with the bacterial cells in order to give maximum protection (Hollaender et al., 1951). Burnett conducted further investigations using sodium hydrosulfite and, from the results obtained in this later work, stated that removal of oxygen from the medium was the sole protective mechanism of this compound (Burnett et al., 1952).

Still another effect was noted by this same group (Hollaender et al., 1951a). If cells of E. coli were grown anaerobically and then irradiated under anaerobic conditions, the shape of the survival curve was sigmoid rather than linear. They further studied this effect, subjecting E. coli, strains B and B/r, to X-radiation under conditions of lowered oxygen tension by replacing the air atmosphere with nitrogen, helium, hydrogen, or carbon dioxide gas (Hollaender et al., 1951a). Lowering the oxygen tension resulted in increased radiation resistance of their organisms. The results reported by these workers also seemed to indicate that the actual mechanism of action of the radiation was different under anaerobic and aerobic conditions. In this same study they also

observed increased sensitivity to X-rays with decreasing concentration of the bacteria in the irradiated suspensions.

During the 1950's, studies were also done on the effects of different physical factors on survival of both spores and vegetative cells of bacteria exposed to ionizing radiations. Lawrence et al. (1953) studied aerobic, spore-forming bacteria. They found that organisms which formed spores were more difficult to kill than vegetative cells. Morgan and Reed (1954) later studied the resistance of both aerobic and anaerobic spores to gamma radiation. This was the first report in the literature in which anaerobic bacterial spores were used as test organisms in evaluating the effects of ionizing radiations on bacteria. Morgan and Reed worked with a putrefactive anaerobe (P.A. 3679), Clostridium botulinum Types A and B, a thermophilic anaerobe (T.A. 3814) and a thermophilic aerobe (F.S. No. 1518). The gamma radiation used by these workers was delivered either by cobalt-60 or tantalum-182 in doses ranging in intensity from 27,000r per hour to 367,000r per hour. Two effects were noted: (1) that the intensity with which the radiation was delivered had no effect on the lethality of a given dosage for spores; and (2) if the concentration of the spores was varied initially, the percentage of the original population killed by a given dosage of radiation remained constant. Morgan and Reed also studied the effect of varying concentrations of oxygen in contact with their spores during irradiation. They found that lowering the oxygen tension by varying the gaseous atmosphere above the liquid surface resulted in greater protection for the anaerobic than for the aerobic spores. If, on the contrary, the oxygen tension was lowered by suspending the spores in a 0.5 per cent aqueous solution of

ascorbic acid-thiourea, a chemical combination which lowered the oxidation-reduction potential of the medium, aerobic spores were protected to a greater degree than the anaerobic spores. Morgan and Reed interpreted these results in terms of oxygen toxicity and the relative efficiencies of the two methods in decreasing the oxygen concentration around or in the spore itself.

When radioactive disintegration products became available in sufficiently large quantities to permit serious consideration of their use for sterilization of foods, pharmaceuticals and other biological products, studies of the effects of the constituents of the biological materials on the lethality of gamma radiation for microorganisms were necessary. In line with this, Proctor and Goldblith investigated the influence of (1) different conditions of atmosphere and medium, and (2) the physical state in which samples were irradiated on the radiation sensitivity of bacteria. Their results depended upon the organism studied. Escherichia coli was susceptible to both changing conditions of medium and atmosphere and to changing physical state, being most resistant to radiation when frozen (Goldblith et al., 1955). Spores of Bacillus thermoacidurans, on the contrary, were affected little if any by changing environmental conditions (Proctor and Goldblith, 1954). Opposed to the aerobic spores of B. thermoacidurans, the anaerobic spores of Clostridium sporogenes were markedly affected by different atmospheres (air, oxygen, vacuum)(Proctor and Goldblith, 1954a). The results obtained by Proctor and Goldblith with anaerobic spores were contradictory to the results reported by Morgan and Reed (1954) who found greater protection of anaerobic than aerobic spores when the gaseous atmospheres were changed. Proctor and Goldblith (1954a)

also observed a sigmoid type of death curve when working with C. sporogenes spores, the same type of curve that was noted by Hollaender et al. (1951) when they grew and irradiated E. coli under anaerobic conditions. In studying the effect of suspending organisms in different media during irradiation, Proctor and Goldblith found that the radiosensitivity of E. coli to cobalt-60 gamma radiation was different in three media in an air atmosphere. These media, in order of decreasing protective capacity for E. coli, were: pea puree, nutrient broth, and saline (Proctor and Goldblith, 1956). Bacillus subtilis spores, on the contrary, decreased in radiosensitivity in the same order in these media.

Moos (1952) conducted a similar study on the effect of drying and freezing on the survival of Pseudomonas aeruginosa and E. coli cells subsequent to irradiation. He found that either drying or freezing resulted in an increase in survivor numbers. Moos attributed this, in part at least, to the reduction of "indirect action" effects from water in the dried or frozen state.

As part of a program to determine the effect of the constituents of foods on the lethality of gamma radiation for microorganisms which might be present in these foods, Kempe and his co-workers studied the effects of combined heat and cobalt-60 gamma radiation on C. botulinum spores inoculated into cans of cooked ground beef (Kempe, 1955; Kempe et al., 1954, 1957). In the course of this investigation, these workers noted a greater survival of these spores when gelatin rather than phosphate buffer was used as the suspending medium during irradiation. The present study was initiated as a result of this observation that C. botulinum spores exhibited varying susceptibility to gamma radiation if suspended in chemically different media.

At the time this study was undertaken, a relatively small amount of work was reported in the literature on the chemical protection of bacteria; no studies were available on chemical protection of anaerobic spores. Furthermore, the results reported from studies of the effect of physical factors on radiation sensitivity of microorganisms seemed to indicate that this sensitivity was dependent upon the oxygen requirements (aerobic or anaerobic) of the organisms and whether the test system was vegetative cells or spores. These factors were indicative of the need for more studies on the alteration of radiation sensitivity of bacteria. The lack of literature with regard to anaerobic spores indicated that the need was especially great in this area. As one phase of this type of investigation, the present study was undertaken to attempt to elucidate the effects of chemical environment on the lethality of cobalt-60 gamma radiation for anaerobic bacterial spores. Two strains of C. botulinum, an organism which is the source of one type of food poisoning, were included among the test organisms used so that the results obtained in this research would have practical as well as theoretical applicability.

METHODS AND MATERIALS

I. Preparation of Spores

A. Organisms Used

Spores of three strains of anaerobic bacteria were used in this study. Their sources and types were as follows:

1. *Clostridium botulinum* 62A; and
2. *Clostridium botulinum* 213B; both originally obtained from the Hooper Foundation for Medical Research at the University of California, San Francisco, California.
3. *Clostridium* species, putrefactive anaerobe, PA 3679; originally obtained from the National Canners Association, Washington, D. C.

The species and type identity of these organisms were checked periodically throughout this study. Small quantities of the same media used for large scale production of spores were aseptically inoculated with pure cultures of the organisms. These cultures were grown under anaerobic conditions and examined under the oil immersion lens of a binocular light microscope. Developing spores were found to be subterminal to terminal in position and swelled the sporangium before their release. The vegetative cells were Gram positive rods varying in size between 0.5 to 0.8 by 3.0 to 5.0 microns.

Supernates of the two *C. botulinum* strains were also injected intraperitoneally into mice to test for toxicity. The type of toxin produced was verified by neutralization tests in mice. The type specific antitoxin used was supplied by the New York State Department of Health.

B. Growth Medium for Spores

1) Putrefactive anaerobe 3679

This organism was grown in pork infusion broth prepared as follows. Fresh pork ham was trimmed of fat, ground and mixed with water in the ratio of 1 pound of pork per liter of water. This mixture was boiled for 1 hour and then filtered through an 8 layer cheesecloth filter to remove the meat particles, which were dried and saved. The pork extract was cooled overnight in a refrigerator to allow any fat still present to solidify as a layer on top of the medium. The following day this fat was removed by skimming and the medium again made up to original volume by the addition of distilled water. The following ingredients were then added:

Bacto-peptone (Difco).....	5	g/l
Bacto-tryptone (Difco).....	1.5	g/l
Glucose.....	1	g/l
K_2HPO_4.....	1.25	g/l
Soluble starch.....	1	g/l
Sodium thioglycollate.....	1	g/l.

These were dissolved by heating and the pH of the medium adjusted to 7.4 by the addition of an appropriate volume of a 10 per cent sodium hydroxide solution. The medium was then dispensed in 5 l quantities into large Erlenmeyer flasks and autoclaved for 30 minutes at 15 psig steam pressure. The organisms were then cultured in this medium using the following schedule.

a. Two ml of a culture of Putrefactive anaerobe 3679 were transferred into each of 3 tubes, each containing 10 ml of

medium (medium sterilized with a small amount of dried pork in the bottom of the tube). This was stratified with pork agar (pork medium to which 1.5 per cent agar had been added) and incubated at 37 C.

When the tubes showed good growth as indicated by gas production, (usually in about 1 day), 2 ml were transferred into each of six 10 ml tubes of medium that contained the pork medium, dried pork and an iron wire* freshly cleaned with hydrochloric acid. Stratification of these tubes was unnecessary. Incubation was at 37 C for 1 day.

c. The contents of 3 of the tubes of the second step were transferred to a 50 ml volume of medium (medium, dried pork and 5 iron wires). Two such cultures were made. Again stratification was not necessary. The cultures were incubated at 37 C for 2 days.

d. The contents of the 2 cultures of the third step were transferred to a flask containing 5 l of medium (medium plus dried pork plus 10 to 15 iron wires). This was incubated at 37 C for 1 week followed by incubation at 30 C for 2 additional weeks.

e. After the period of incubation, the pork was removed by filtering the culture through a sterile, thin cheesecloth-cotton pad filter. The liquid was centrifuged in order to collect the spores in a small volume.

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* Iron Wire, Analytical Reagent (Mallinckrodt)

2) Clostridium botulinum 62A and 213B

These strains were grown in a medium modified from Reed, Bohrer and Cameron (1951). It consisted of:

Bacto-Casitone	100	g/l
Liebig's beef extract (Difco)	5	g/l
Na_2HPO_4	5	g/l
Distilled water	to volume.	

The medium was prepared in 5 l quantities, dispensed into Erlenmeyer flasks and autoclaved for 45 minutes at 15 psig steam pressure.

Cultures of C. botulinum 62A and 213B were first grown in 10 ml of medium at 30 C and the larger flasks seeded from these cultures. Flasks were incubated for 3 weeks: C. botulinum 62A at 37 C; C. botulinum 213B at 30 C.

C. Preparation of Stock Spore Suspensions

Spore crops were harvested from the growth medium when 80-90 per cent of the vegetative cells had sporulated as determined by a microscopic examination of the culture. The spores were collected from the growth medium by centrifuging the liquid for 30 minutes at 1300 G. After siphoning off the mother liquor, the spores were washed by resuspending the pellet in 200 ml of cold, sterile distilled water followed by centrifugation in a refrigerated centrifuge. This procedure was repeated a minimum of 20 times. After 20 washings, the resultant spore crop was examined under a binocular light microscope. If no more than 1 vegetative cell per 100 spores was visible per microscopic field, the spores were not subjected to further washing. If the number of vegetative cells

present exceeded this figure, however, the spore suspension underwent further washings until such time as only 1 vegetative cell per 100 spores remained. The spores were then stained with 1 per cent aqueous methylene blue and, in the absence of dye uptake, used in the radiation experiments.

For storage, the spores were suspended in a small volume of sterile distilled water and kept at 4 C in 250 ml Erlenmeyer flasks containing sterile glass beads. The number of viable spores present in these stock suspensions varied between 1×10^7 and 5×10^8 spores per ml depending both upon the strain of organism and the particular crop harvested. This viability count was obtained using the dilution count method in anaerobic media. Similar counts were obtained both with and without heating of the spore suspension at 85 C for 15 minutes prior to titration.

Changes in radiation sensitivity and viability of these stock suspensions were checked periodically. It was found that the spores could be stored for as long as 2 years without a significant change in either of these two factors.

II. Experimental Arrangements

A. Treatment of Spores for Experimental Use

When spores were needed for an experiment, the flask containing the stock spore suspension was placed on a rotary shaker* having a 1 inch radial stroke. This flask, containing glass beads, was shaken for 5 minutes at 250 rpm to disperse any spore clumps that

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* New Brunswick Scientific Co.

may have formed. An appropriate quantity of stock solution was then aseptically pipetted into a sterile 15 ml test tube and placed in an 85 C water bath for 15 minutes. This treatment activated the spores and, at the same time, killed any vegetative cells that might have been present. The desired experimental dilution of the spores was then prepared by aseptically pipetting from this heated stock suspension to the solutions being tested.

B. Control Solution

Unless otherwise noted, a phosphate buffer solution served as the control in all experiments. The phosphate buffer was prepared as needed from these two stock solutions.

1. 11.87 g of $Na_2HPO_4 \cdot 2\ H_2O$ or 9.47 g of Na_2HPO_4 anhydrous (Merck reagent) were dissolved in distilled water and made up to 1 liter in a volumetric flask.
2. 9.08 g of KH_2PO_4 (Merck reagent) were dissolved in distilled water and made up to 1 liter in a volumetric flask.

These stock solutions could be stored in stoppered bottles for prolonged periods of time.

Immediately prior to use in an experiment, 63.9 ml of Na_2HPO_4 solution were added to 36.1 ml of KH_2PO_4 to give a 0.15M buffer solution. The pH was determined using the Beckman pH meter, Model G, and always approximated ($\pm$ 0.03 pH units) the theoretical pH value of 7.05 for this combination. This solution was sterilized by autoclaving at 15 psig steam pressure for 15 minutes.

C. Preparation of Chemicals

Structural formulae of all chemicals used in this research are listed in Appendix A. All the chemicals were purchased specifically for this project so that only fresh, unopened materials were used. These were of reagent grade. Chemicals were dried before being weighed; weight measurements were made on either a Torsion or analytical balance so were accurate to $\pm$ 0.001 g.

Phosphate buffer was the suspending medium used for all chemicals. Sterilization was accomplished by various means, depending upon the physical properties of the specific chemical employed. In most cases Seitz filtration was found to be satisfactory. A sterility check was always run on these solutions subsequent to their sterilization and just prior to inoculation with anaerobic spores. This check was done by pipetting a small quantity of the solution into a tube of anaerobic medium and incubating for seven days at 30 C.

D. pH Determinations

For the experiments which were done on the effect of pH on spore survival during exposure to cobalt-60 gamma radiation, use was made of two different buffer solutions. Phosphate buffer was prepared as described in the section on Control Solution except that the proportions of KH_2PO_4 and Na_2HPO_4 were varied as necessary to obtain the different pH values desired. The pH ranged from 5.0 to 8.4 in phosphate buffer.

Acetate buffer was prepared as follows:

1. Six g of acetic acid (CH_3COOH) were dissolved in distilled water and made up to 1 liter volume in a volumetric flask.
2. 8.2 g of sodium acetate (CH_3COONa) were dissolved and made up to 1 liter volume by the same procedure.

These stock solutions were stored until needed for use in an experiment when they were mixed in various proportions, depending upon the pH desired. The pH values in acetate buffer ranged from 3.4 to 6.0.

E. Preparation of p-Chloromercuribenzoate-Treated Spore Suspensions

For those experiments in which spores were treated with p-chloromercuribenzoic acid prior to irradiation, the following procedure was followed. An appropriate quantity of the powdered form of the chemical was weighed out on an analytical balance. The powder was then dissolved in a small volume of a 1M sodium hydroxide solution. The spores were added to sterile phosphate buffer and a sufficient quantity of the sodium hydroxide solution of p-chloromercuribenzoate added to give the desired molar concentration of the chemical. This solution was allowed to stand at room temperature for 30 minutes, then placed in a refrigerator overnight.

The following day the mercury-soaked spores were centrifuged and washed. All centrifugation was performed at 4 C in the International refrigerated centrifuge at 1700 G using cold, sterile phosphate buffer (pH 7.02) for washing. The spores were spun down from the mercury solution for 1 hour, then resuspended three successive times in 25 ml of phosphate buffer and centrifuged for 1 hour after each resuspension. After the final washing, they were resuspended

again in 25 ml of cold phosphate buffer and this suspension dispensed in five ml glass vials for irradiation. The pH of the final suspension was determined and found to be 7.02.

Subsequent to irradiation, mercury treated spores were first diluted in water blanks and then grown in anaerobic medium, both containing sodium thioglycollate. The addition of sodium thioglycollate was to permit reaction between any mercury remaining bound to the spores and the sodium thioglycollate. Sites on the spore bound by mercaptide formation would be freed by this second reaction.

F. Preparation of Samples for Irradiation

All the solutions being tested in a particular experiment were initially prepared in sterile 25 ml Erlemeyer flasks. The desired concentration of anaerobic spores was added before dispensing individual samples; this insured a uniform distribution of organisms.

Five ml glass vials were used for actual irradiation of samples. These were constructed with a long, narrow neck through which the test solution could be introduced by means of a capillary pipette. Prior to use, the vials and capillary pipettes were sterilized by heating for ten minutes at 210 C in a dry heat oven. Four ml aliquots of a solution were dispensed into each vial using aseptic technique. When all the samples for an experiment were thus apportioned, the vials were sealed by fusing the narrow neck in an oxygen flame. The speed with which such a flame was capable of melting the glass prevented heating of the test solution itself. After sealing, all vials were tested for leakage; if no leaks were found, the vials were either

stored at 4 C until time for irradiation or were immersed in an ice water bath and irradiated immediately.

As is indicated by the method of preparation, all samples contained oxygen both in the solution itself and in the air space above the liquid in the vials. The air equilibrium atmosphere of test suspensions was altered in those experiments where the effect of different gaseous atmospheres on spore survival during irradiation was studied.

III. Gamma Irradiation

A. Source

The large cobalt-60 gamma radiation source at the University of Michigan was used for irradiation of samples in this investigation. Brownell et al., (1953) and Nehemias et al., (1954) have described the design, installation and operation of this source. Unless otherwise specified, samples were irradiated in the center well of the source where 1.55×10^5 rep (roentgens-equivalent-physical) were developed per hour. A rep is that amount of radiation which will result in the absorption of 93 ergs per gram of tissue.

B. Dosimetry

The radiation dose being delivered at different distances from the cobalt-60 source was determined by a variation of the ferrous sulfate dosimetry method as described by Weiss (1952). The basis of this method is the fact that ferrous ions are oxidized to ferric ions by ionizing radiations. This has been shown by other investigators (Fricke and Morse, 1927; Lea, 1947).

As a basis for this method, it was necessary to construct a graph showing optical density vs. micromoles of ferric iron per liter. The ferric ion concentration of the standard solutions used was determined by quantitative titration. The Beckman spectrophotometer, with a wave length setting of 304 mμ and using quartz cells and a hydrogen lamp, was employed in determining the optical density of these solutions. Once the accuracy of this curve was established, it became the reference standard by which radiation delivered from the ionizing radiation source was measured. For this purpose the abscissa of the graph was converted from micromoles of ferric iron per liter to radiation dosage using a standard conversion factor of 15.4 micromoles/liter/1,000 rep.

C. Dosimetry Reagents

The stock solution, which was stable for a few days, was made up using a Torsion balance, a 100 ml graduate and a pipette. The chemical constituents were:

$FeSO_4(NH_4)_2SO_4 \cdot 6\ H_2O$ (Merck reagent).....3.9 g

NaCl (B & A reagent).....0.12 g

Concentrated sulfuric acid (reagent).....2.2 g

Triply-distilled water.....100 ml

The sodium chloride was used to prevent trouble from dust, etc., in the triply distilled water and the solution was made up in sulfuric acid to overcome sensitivity of the ferrous ammonium sulfate to pH. The exact concentration of ferrous ions did not need to be known since only the ferric ion concentration was determined subsequent to radiation. The unirradiated blank reading was used as a zero reference.

The dosimetry solution, which was stable for only 1 or 2 days, was always freshly prepared. It consisted of:

Aerated triply distilled water.....	200	ml
Concentrated sulfuric acid (reagent).....	4.4	ml
Stock solution (from above).....	1	ml

The distilled water was acidified before aeration. To aerate the solution, compressed air was used with the insertion of a glass wool plug between the air source and the solution in order to remove oil which might be introduced from the compressed air line. Saturation of the solution with oxygen is necessary since the quantity of iron oxidized depends upon the oxygen concentration.

Total dosage of radiation received when measuring radiation by means of the ferrous dosimeter should not exceed 50,000 rep; this dosage utilizes all the oxygen in the solution and oxidation yield drops by a factor of two thereafter.

D. Sampling Arrangement

Since equivalent radiation dosage of samples was impossible to obtain unless all samples were placed equidistant from the cobalt-60 source, a metal container was devised by J. T. Graikoski to fit the center aperture of the source. This container was a hollow metal cylinder. It was insulated by an asbestos wrapping around the outside and held a two-tiered circular metal rack. Each tier of the rack was capable of holding 14 of the 5 ml glass vials used in this research. When fitted inside the outer cylinder, the circular metal rack left a center opening approximately 4 inches in diameter into which ice

could be placed for cooling water. Upon removal of sample vials from the cylinder, the vacant slot created was filled with a similarly prepared glass vial containing 4 ml of distilled water. This was done in order to maintain geometric similarity and, therefore, constant dosimetry.

Samples were irradiated for various lengths of time as the experiment demanded. Radiation times usually varied between a minimum of 3 and a maximum of 8 hours.

E. Radiation Temperature

Unless otherwise specified in a particular experiment, samples were immersed in a 4 C ice water bath at all times during the irradiation process. This insured the maintenance of a uniformly low temperature during the entire handling process of the samples from the time of preparation until titration.

IV. Spore Titration

A. Medium

All irradiated spores were grown in pork extract medium. This was prepared by adding 1.5 per cent agar to the pork infusion broth described for growth of Putrefactive anaerobe 3679 in the section on Growth Medium for Spores. The medium was sterilized after addition of the agar by autoclaving for 30 minutes at 15 psig steam pressure.

After sterilization the flask was placed in a 55 C incubator in a tilted position for several hours during which time a flocculent sediment formed at the lowest point in the flask. The clear

supernatant liquid was decanted from the sediment through a filter of cotton and cheesecloth. It was tubed in 25 ml quantities in a modified form of agar slant tubes*, sterilized at 15 psig steam pressure for 30 minutes and stored at 4 C until needed.

B. Dilution Method

Samples were prepared for titration by aseptic transfer of the solution (irradiated or control) to a sterile test tube from the glass vial used during the actual irradiation process. The medium used for growth of the spores was melted in boiling water, then cooled and kept at 55 C in a water bath. Appropriate 100-fold serial dilutions of spore samples were then made using sterile 99 ml distilled water blanks. From these, the correct aliquot (either 1 ml or 0.1 ml) was aseptically pipetted to the titration medium. This medium was then agitated by vigorous rolling of the test tube between the palms of the hands. This insured even dispersion of the spores throughout the liquid. The tube of medium was immediately placed in an ice water bath to insure rapid solidification. This prevented the spores from settling to the bottom of the tube.

Upon solidification of the nutrient medium, each tube was sealed with a plug composed of a sterile 2 per cent agar solution containing 0.1 per cent sodium thioglycollate and 10 ml per 1 of a 1:500 dilution of methylene blue. The latter acted as an oxidation-reduction indicator. If samples were to be stored for longer than a week, this agar plug

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* Fisher Scientific Co.

was then overlaid with a mixture made of one part paraffin and one part petrolatum* to prevent dehydration of the medium. A cotton plug was then placed in the top of the tube and the tubes were incubated for seven days at 30 C.

C. Counting Method

The original irradiated samples (4.0 ml each) were titrated as described in the previous section on Dilution Method. Duplicate tubes were made for each of the three dilutions prepared from each sample, a 10-fold difference existing between the dilutions. Dilutions of the spore suspensions were used which would give countable numbers of colonies in two of the three dilutions. For purposes of this research, colony counts between 10 and 200 per tube were considered usable. If a tube of medium contained more than 200 colonies, the counts were considered unreliable. Wherever possible, the counts of the two tubes of the dilution in which the number of colonies present was between 100 and 200 were averaged and used in plotting the data. The counts obtained were considered suitable for plotting if they met the following two requirements: (1) the two tubes of the dilution used gave the same count of number of organisms surviving the irradiation treatment (i.e., within 2 standard deviations of the mean) [See Appendix D], and (2) the same survival ratios were obtained upon running a duplicate experiment. It was found that data were reproducible even when spore survival numbers were low. This condition was frequently met in control solutions after eight hours irradiation

* Stanolind petrolatum, snow white U.S.P. alba (The Standard Oil Co.)

or in chemical solutions where little protection was afforded the spores. All experiments were performed at least twice.

When the colonies were ready for counting, as evidenced by visible growth after seven days incubation, the tubes were removed from the incubator and counted under a $1\frac{1}{2}$ x magnifying glass on a dark-field Quebec colony counter. In several experiments, cultures were reincubated for as long as 30 days after counting to check for any change in numbers of colonies. No changes were ever observed.

EXPERIMENTAL RESULTS

I. A Study of the Effect of Two Experimental Variables on Spore Survival During Irradiation

At the beginning of this study, it was considered advisable to determine the effect on spore survival of two factors which could not be held constant throughout the research. If the effects of chemical environment were to be studied, other factors which might vary during the course of the investigation would either need to be evaluated or eliminated as being significant. It was possible to control certain of the physical factors which might have influenced results. The temperature at which samples were prepared and irradiated was maintained at 4 C by the use of an ice water bath. The oxygen content of the samples for irradiation was regulated in all experiments by a uniform method of sample preparation. Immediately prior to use in an experiment, the phosphate buffer used as the solvent was freshly exhausted of oxygen by autoclaving. All samples were then dispensed into 5 ml glass vials to insure a fairly equivalent air space above the aqueous phase of the suspending medium. This factor changed only in the experiments where gaseous atmospheres were being specifically studied or where the chemical under investigation was capable of altering the oxygen content of the medium.

Photoreactivation, as observed with ultraviolet irradiation, has not been reported to occur with bacteria subjected to gamma radiation from cobalt-60. However, any possibility that this might have occurred was eliminated by storage of irradiated samples in the dark until they were titrated.

At the inception of this study, it was planned to investigate the mechanisms by which gelatin afforded protection to anaerobic spores against the lethal effects of gamma radiations. Gelatin is a protein substance which can be prepared as a semi-solid gel by the addition of water. It was felt that the distribution of free radicals developed by the ionizing radiation as well as the rate of their subsequent destruction might differ in such a medium from that seen in a liquid. Therefore, possible effects due to the semi-solid nature of the medium required study. Furthermore, it was desirable to know how the lethality of gamma radiation for anaerobic bacterial spores varied with change in the hydrogen ion concentration of the suspending solution. Thus a study of these two factors was undertaken.

A. Effect of a Semi-Solid Medium on Spore Survival

Experiments were performed using two different substances, guar gum and gelatin, to determine whether such semi-solid media afforded greater protection to spores than an otherwise similar liquid medium. Guar gum was tested first. This substance is a polysaccharide composed of 35 per cent galactose and 63 per cent mannose. It was prepared in a 1 per cent solution by weighing out 2 grams ($\pm$ 0.001 g) of the powder and dissolving this in 198 ml of phosphate buffer. The pH of the solution was determined prior to sterilization and was found to be 7.10. Sterilization was accomplished by autoclaving the guar gum solution for 15 minutes at 15 psig steam pressure. The buffer solution used as a control was sterilized similarly. Three 50 ml quantities of the sterile guar gum solution were then placed in large

test tubes and appropriate quantities of a spore suspension were added to each tube to produce a final dilution of 1 x 10^6 spores per ml. Three different organisms were used: C. botulinum 62A, C. botulinum 213B, and Putrefactive anaerobe 3679.

Irradiation of these samples was performed on the outside of the radiation source. This was accomplished by attaching the tubes, by means of rubber bands, to the wire cage which surrounds this source. Here radiation was delivered at an intensity of 169,000 rep per hour. Samples were irradiated for from 0 to 6 hours, portions being removed at appropriate intervals by pipetting 4 ml quantities from the large tube into sterile 10 ml test tubes. These were refrigerated until ready for titration. The results of the experiment using C. botulinum 62A are shown in Figure 1; the similar results obtained when C. botulinum 213B and Putrefactive anaerobe 3679 were used are given in Table I. A comparison of number of survivors in the presence of guar gum to the number in the control solution shows that no great difference existed between the two. Therefore, trapping the spores in a semi-solid medium did not appear to materially enhance the number of spores surviving irradiation over the number obtained using a liquid suspending medium.

A similar series of experiments was performed using gelatin. This material is chemically different from guar gum, being protein rather than carbohydrate in nature, but solutions of the two compounds can be prepared which have the same semi-solid consistency. The gelatin was prepared in a 10 per cent suspension. Twenty g of Bacto-gelatin were dissolved in 180 ml of distilled water and the pH adjusted to 7.06 by the addition of 20 ml of 0.15M K_2HPO_4. The gelatin solution

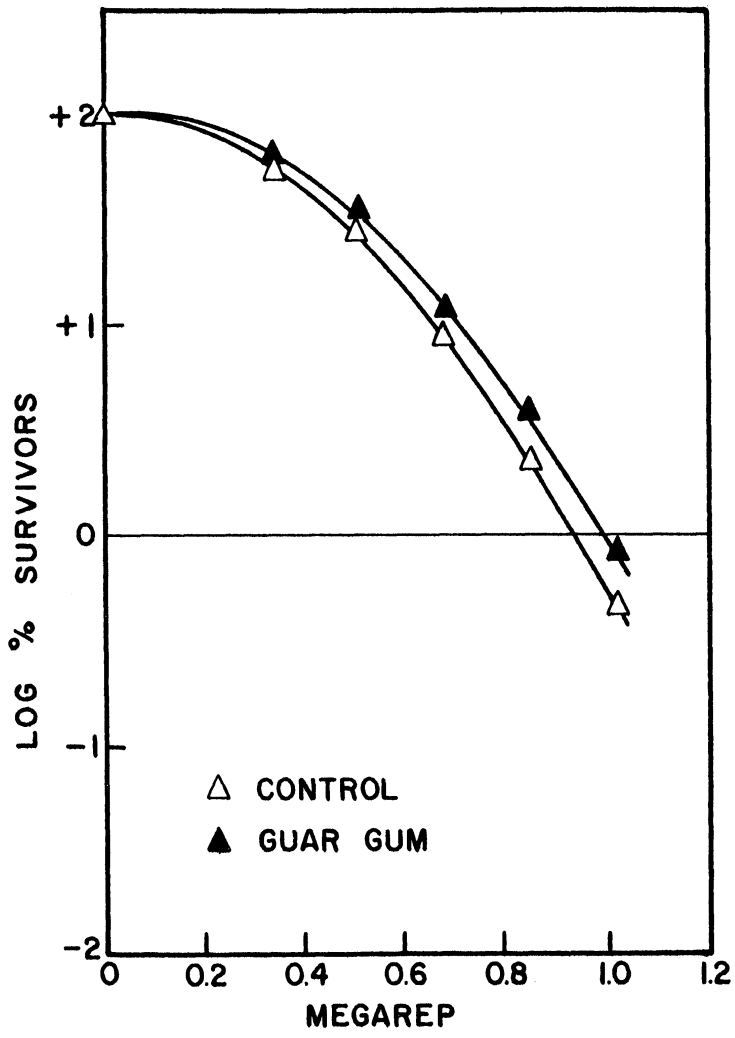


Figure 1. The effect of the presence of guar gum in the suspending solution on the lethality of cobalt-60 gamma radiation for spores of C. botulinum 62A.

TABLE I

THE EFFECT OF A SEMI-SOLID MEDIUM ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

Organism	Hours Radiation	REP	No. of Organisms Surviving per ml	Log Percent Survivors
C. botulinum 62A				
Control	0	0	640,000	2.000
	2	338,000	350,000	1.738
	3	507,000	190,000	1.473
	4	676,000	56,500	0.946
	5	845,000	14,800	0.364
	6	1,014,000	2,900	$\bar{0}$.344
Guar Gum	0	0	740,000	2.000
	2	338,000	445,000	1.779
	3	507,000	272,000	1.565
	4	676,000	87,000	1.070
	5	845,000	30,000	0.607
	6	1,014,000	6,050	$\bar{0}$.087
C. botulinum 213B				
Control	0	0	3,600,000	2.000
	2	338,000	2,090,000	1.764
	3	507,000	1,190,000	1.519
	4	676,000	260,000	0.859
	5	845,000	70,000	0.289
	6	1,014,000	11,500	$\bar{0}$.496
Guar Gum	0	0	4,900,000	2.000
	2	338,000	3,520,000	1.856
	3	507,000	1,965,000	1.603
	4	676,000	595,000	1.084
	5	845,000	170,000	0.540
	6	1,014,000	21,700	$\bar{0}$.354
Putrefactive anaerobe 3679				
Control	0	0	925,000	2.000
	2	338,000	575,000	1.794
	3	507,000	285,000	1.489
	4	676,000	74,000	0.903
	5	845,000	23,000	0.396
	6	1,014,000	3,700	$\bar{0}$.398
Guar Gum	0	0	960,000	2.000
	2	338,000	625,000	1.814
	3	507,000	370,000	1.586
	4	676,000	125,000	1.115
	5	845,000	35,500	0.568
	6	1,014,000	7,100	$\bar{0}$.131

was then sterilized by autoclaving. The gelatin and a control solution were each dispensed, in 25 ml quantities, into large test tubes. Spores of C. botulinum 62A were then added to each tube. The tubes were irradiated on the outside of the radiation source in the same manner as those in the guar gum experiments with radiation times running from 0 to 6 hours. As shown in Figure 2, gelatin protected the spores whereas guar gum did not. This was interpreted to mean that some chemical constituent of the gelatin rather than its semi-solid nature gave protection since, if the consistency of the medium had been the key to the protection, the same protection should have been obtained with guar gum. Gelatin, however, is a complex substance containing 18 amino acids plus nitrogen (18 per cent) and sulfur (0.5 per cent) which are not part of the amino acid molecules (Block and Bolling, 1950). It was impossible to determine if only one or a combination of these factors was responsible for this protection so research in this area was directed toward the study of structurally simpler compounds.

B. Effect of pH on Spore Survival

Since the problem under consideration involved the use of different chemicals, it was considered probable that solutions having different pH values would be used. It was, therefore, necessary to determine what effects, if any, the hydrogen ion concentration itself would have on the lethality of cobalt-60 gamma radiation for the three strains of anaerobic spores being tested. To obtain the range of pH over which it was desired to test, two different buffers were used. These were phosphate and acetate. At pH 5.0 and pH 6.1 both acetate and phosphate buffer solutions were tested. This procedure allowed

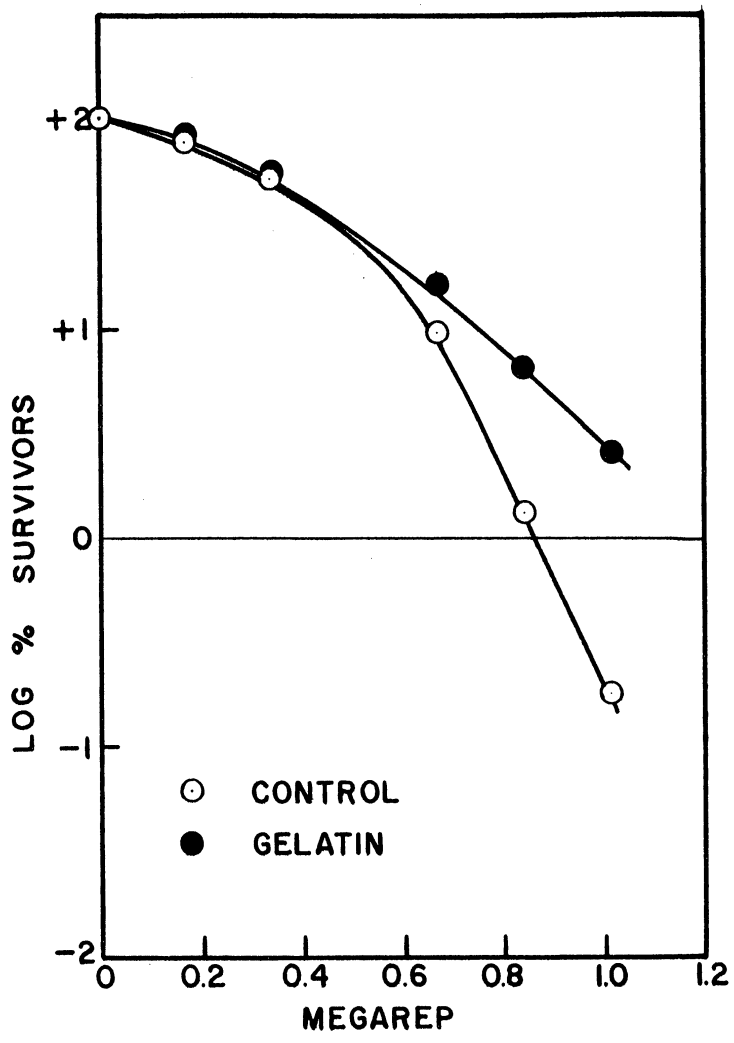


Figure 2. The effect of gelatin in the suspending solution on the lethality of cobalt-60 gamma radiation for spores of C. botulinum 62A.

comparison of effects due to the buffer solutions themselves so that this factor could be taken into consideration in the evaluation of the results. However, no difference in susceptibility to radiation in the two buffers were found. As can be seen in Figure 3, which shows the results obtained with C. botulinum 62A, pH values ranging from 3.23 to 8.40 had no effect on number of spores surviving the gamma irradiation treatment. Table II, in which the results for all three organisms used are shown, indicates, however, that the lower pH values were themselves toxic for spores of C. botulinum 62A and C. botulinum 213B. The total number of spores surviving in the solution steadily decreased as the pH dropped as evidenced by the reduced count in the unirradiated controls as well as in those subjected to treatment with cobalt-60 gamma radiation. At pH 3.23 only 26 per cent of the original number of viable C. botulinum 213B spores with which the solution was inoculated survived the time interval (24 hours) necessary to complete the experiment. With C. botulinum 62A the effect was not so great, 72 per cent of the original inoculum of spores remaining viable. With Putrefactive anaerobe 3679 no apparent decrease occurred due to the changes in pH. This would lead to the conclusion that the three strains of spores vary in their susceptibility to acid pH values, Putrefactive anaerobe 3679 being quite resistant to the lethal effects of increased hydrogen ion concentration, C. botulinum somewhat resistant and C. botulinum 213B the most susceptible of these three strains. Those spores which remained viable at lowered pH values did not have an altered radiation sensitivity. Their death curve was the same as that seen with organisms in media with higher pH values where no death due to the medium itself occurred.

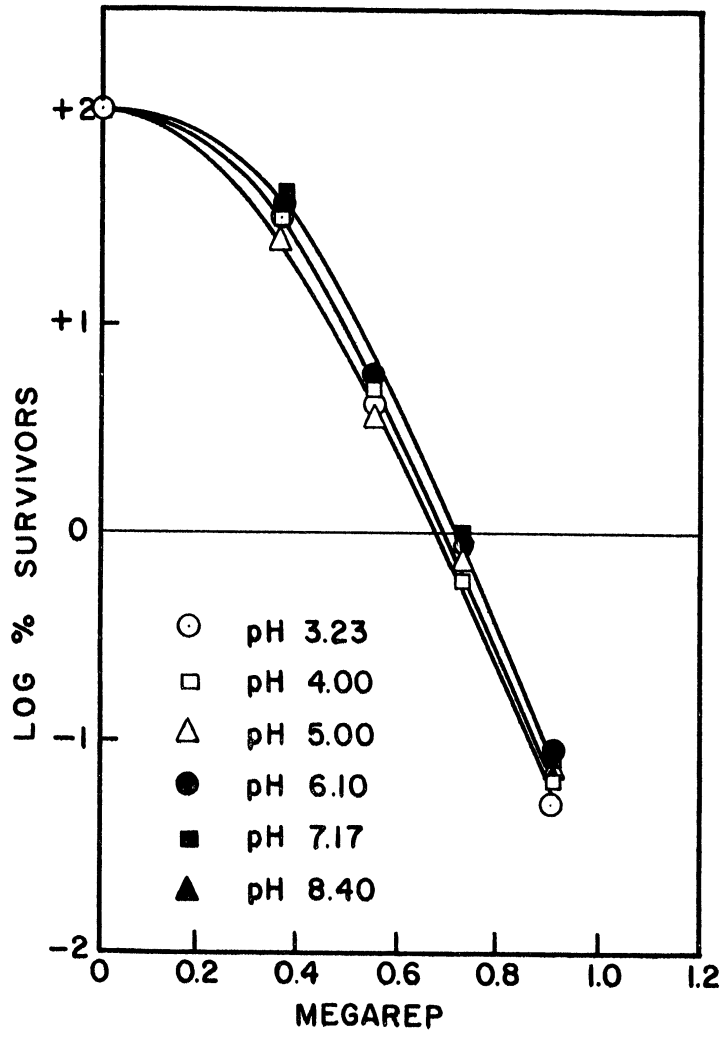


Figure 3. Survival of spores of C. botulinum 62A irradiated with gamma rays from cobalt-60 in solutions of varying pH.

TABLE II

THE EFFECT OF pH ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

REP Rec'd	pH 3.23			pH 4.00		
	62A	213B	P.A. 3679	62A	213B	P.A. 3679
0	800,000	1,553,000	--	880,000	3,900,000	3,250,000
360,000	250,000	420,000	--	280,000	845,000	1,340,000
540,000	34,500	75,000	--	43,000	139,500	--
720,000	6,800	5,500	--	5,200	8,650	40,000
900,000	410	850	--	630	1,760	2,500
1,080,000	--	--	--	--	--	140

REP Rec'd	pH 5.00			pH 6.10		
	62A	213B	P.A. 3679	62A	213B	P.A. 3679
0	950,000	4,730,000	3,100,000	1,050,000	4,870,000	3,670,000
360,000	250,000	1,180,000	1,235,000	375,000	1,910,000	1,300,000
540,000	36,500	174,000	--	56,000	255,000	--
720,000	7,050	20,400	67,000	8,700	56,000	46,500
900,000	725	1,625	4,950	1,000	3,740	4,600
1,080,000	--	--	745	--	--	700

REP Rec'd	pH 7.17			pH 8.40		
	62A	213B	P.A. 3679	62A	213B	P.A. 3679
0	1,110,000	5,800,000	3,300,000	1,113,000	6,000,000	3,870,000
360,000	425,000	2,155,000	1,330,000	416,000	2,230,000	1,735,000
540,000	57,500	240,000	--	62,000	330,000	--
720,000	11,000	69,500	42,500	9,400	42,000	49,500
900,000	910	5,650	2,850	835	3,100	4,450
1,080,000	--	--	150	--	--	480

II. Effect of Reducing Compounds on the Lethality of Cobalt-60 Gamma Radiation for Anaerobic Spores

The ionization of water into active radicals was postulated as early as 1944 (Weiss, 1944) and, more recently, the theory has been substantiated by the work of other investigators (Allen, 1954; Dewhurst et al., 1954). The nature of these free radicals is presented in the "History" section of this dissertation. Other investigators have shown that alteration of chemical compounds occurs in aqueous solutions subjected to ionizing radiations (Kinsey, 1935; Stein and Weiss, 1948). These systems were shown to undergo change by reaction with the high-energy particles from the radiation. The work of still other investigators has shown that oxidation-reduction systems are among the chemical compounds which are altered by different types of ionizing radiations (Fricke and Morse, 1927; Phillips, 1954; Maxwell et al., 1955) and much work has been done on such systems.

Many chemical compounds that can be classified as reducing agents are either added to foods as preservatives or are present as actual constituents of the food itself. In this investigation, some of these reducing compounds were chosen for study on the hypothesis that if the ionizing radiation was absorbed by these products, present in the medium, protection of the bacterial cell itself might result.

A. Effect of Food Preservatives

The first substances tested were four chemicals used at present in the preservation of different foods:

1. sodium nitrite - used to reduce "off-flavor" development during irradiation of meats (Proctor and Goldblith, 1952; Brownell et al., 1953).

2. propyl gallate - a reducing agent used to prevent rancidity of fats (Higgins and Black, 1944; Lundberg and Halvorson, 1945).

3. sodium ascorbate - a reducing agent in general use (Gray and Stone, 1939; Olcott, 1941; Bickoff and Williams, 1946).

4. nordihydroguaiaretic acid (NDGA) - a reducing agent used in preservation of meats, milk and milk products (Smith et al., 1945; Stull et al., 1948, 1951).

Sodium nitrite was tested first. One g of reagent grade sodium nitrite was dissolved in 20 ml of phosphate buffer to give a 5 per cent solution with a pH of 6.5. The solution was sterilized by Seitz filtration and a 12 ml aliquot removed. To this was added 3 ml of a stock spore suspension of C. botulinum 62A so that the final sodium nitrite concentration was 4 per cent. The final concentration of spores in the test solution was approximately 1 x 10^6 spores per ml. Three ml samples of the suspension were placed in sterile 5 ml screw cap vials and irradiated in the cobalt-60 source at 10 C for 0,1,2,3, and 4 hours. A control solution was irradiated simultaneously. The results are shown in Table III. As can be seen from a study of this table, the number of spores surviving after 4 hours irradiation (720,000 rep) was four times greater in the presence of the sodium nitrite than in the control solution. This seemed to be due to the initial delay in death of the spores as evidenced during the first hour of irradiation in the presence of the chemical; after that the death curve duplicated almost exactly that seen for the control solution.

TABLE III

SURVIVAL OF C. botulinum 62A SPORES IN MEDIUM CONTAINING SODIUM NITRITE AS COMPARED TO A PHOSPHATE BUFFER CONTROL MEDIUM

Medium Used	No. of Hrs. Radiation	REP	No. of Organisms Surviving per ml	Percent Survivors	Log Percent Survivors
Control	0	0	850,000	100%	2.000
	1	180,000	495,000	58.24	1.765
	2	360,000	190,000	22.35	1.349
	3	540,000	64,000	7.53	0.877
	4	720,000	10,000	1.19	0.076
Sodium Nitrite	0	0	885,000	100%	2.000
	1	180,000	910,000	100	2.000
	2	360,000	520,000	58.76	1.769
	3	540,000	235,000	26.55	1.424
	4	720,000	40,000	4.52	0.655

Having shown some degree of protection with sodium nitrate, sodium ascorbate and NDGA (nordihydroguaiaretic acid) were tested to see if similar or greater protection would result in their presence. Again the test organism was C. botulinum 62A. The sodium ascorbate was prepared in a 0.05 per cent solution by dissolving 25 mg of the chemical in 50 ml of sterile phosphate buffer. NDGA was prepared in a 0.075 per cent solution using 37.5 mg of the dried powder in 50 ml of buffer. The usual phosphate buffer control was run. The spores were added in a concentration of 6×10^5 spores per ml and samples were irradiated for 0,1,2,3,4,5 and 6 hours at 10 C. The results are shown in Table IV. It can be seen that these chemicals were completely ineffectual in preventing death of the spores.

An experiment using propyl gallate as the protective chemical was then performed. Propyl gallate was prepared in a 0.015M solution by dissolving 159 mg of the powder in 50 ml of phosphate buffer. The solution was sterilized by boiling for 10 minutes in a boiling water bath. Thirty ml of the cooled solution were removed and spores added to this to give a spore concentration of approximately 1×10^6 spores per ml. Samples were dispensed into glass vials as described in the "Methods and Materials" section and irradiated for 0,1,2,3,4,5 and 6 hours at 180,000 rep per hour. The temperature during irradiation was 10 C. As illustrated in Figure 4, propyl gallate was also unable to give protection to the Clostridium spores during irradiation. The number of spores surviving the irradiation treatment was actually slightly less in the propyl gallate solution than in the control.

TABLE IV

THE EFFECT OF TWO ANTI-OXIDANTS, PRESENT IN THE SUSPENDING SOLUTION DURING IRRADIATION, ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR SPORES OF C. botulinum 62A

	Hours Radiation	REP	No. of Organisms Surviving per ml	Log Percent Survivors
Control	0	0	660,000	2.000
	1	180,000	520,000	1.896
	2	360,000	260,000	1.595
	3	540,000	58,000	0.944
	4	720,000	5,500	$\bar{0}$.079
	5	900,000	213	$\bar{1}$.491
	6	1,080,000	3	$\bar{3}$.421
Sodium Ascorbate	0	0	540,000	2.000
	1	180,000	470,000	1.940
	2	360,000	170,000	1.498
	3	540,000	27,000	0.699
	4	720,000	3,200	$\bar{0}$.227
	5	900,000	183	$\bar{1}$.470
	6	1,080,000	5	$\bar{3}$.033
Nordihydro-guaiaretic Acid	0	0	700,000	2.000
	1	180,000	550,000	1.895
	2	360,000	245,000	1.544
	3	540,000	35,500	0.704
	4	720,000	3,250	$\bar{0}$.334
	5	900,000	65	$\bar{2}$.032
	6	1,080,000	3	$\bar{3}$.447

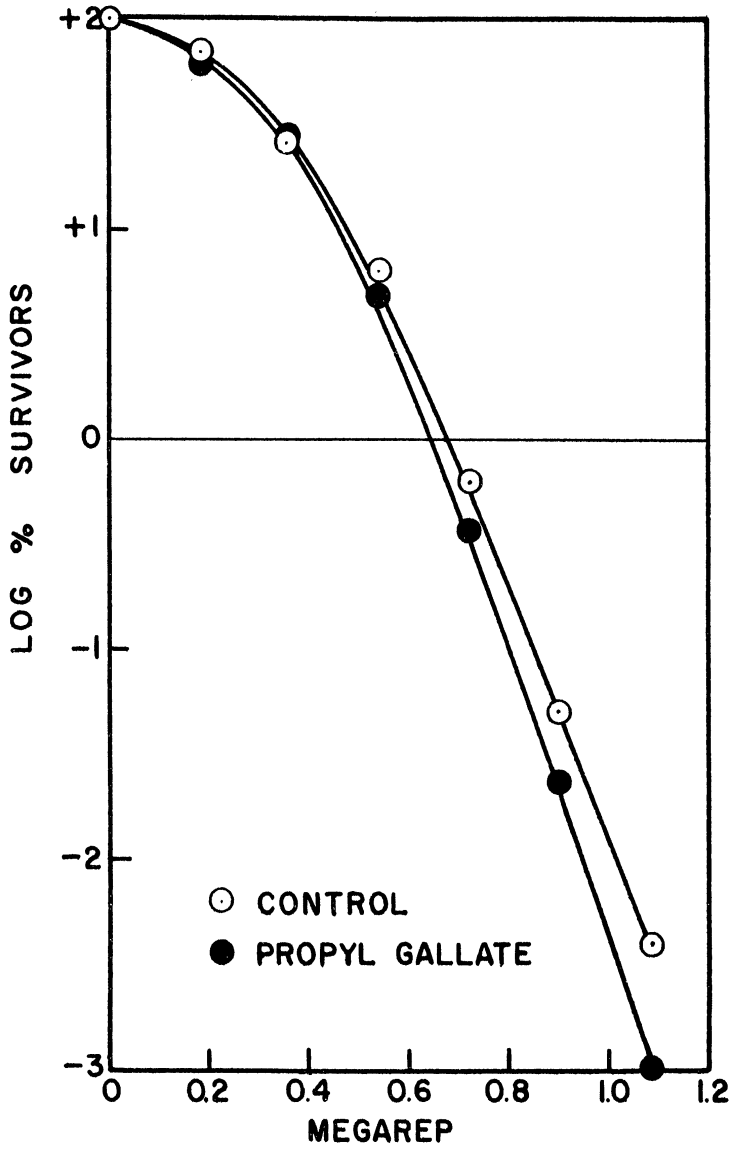


Figure 4. The effect of a propyl gallate solution on the lethality of cobalt-60 gamma radiation for spores of C. botulinum 62A.

B. Effect of Certain Amino Acids on Spore Survival

Since the reducing compounds tested had not given an unusual degree of protection to the spores, attention was again focused along the line of research suggested by the results with gelatin. The latter was too complex a substance to be used for determination of the specific site(s) responsible for its protective effect. However, from its protein nature and the fact that it had given a greater protection than any other substance tested, it was hypothesized that perhaps some protective factor lay in proteins themselves. Since gelatin contains amino acids, it was planned to test these simpler molecules. The following three were chosen: lysine, methionine and cysteine.

Lysine and methionine were tested simultaneously using 1×10^6 spores per ml of C. botulinum 62A as the test organism. Lysine was prepared in a 0.34M solution in 25 ml of phosphate buffer; methionine was prepared in a 0.0067M concentration in the same quantity of solvent. These solutions and a control were each placed in a large test tube and irradiated on the outside of the radiation source for 0,2,4,5 and 6 hours. The results are shown in Figure 5. Lysine exhibited about the same degree of protection as had been noted with the use of sodium nitrite but not as much as had been obtained with gelatin irradiated for the same length of time. Methionine, however, showed a degree of protection never before obtained. After six hours irradiation (1,014,000 rep), 39,500 spores per ml from the original population of 610,000 spores per ml were still viable in the methionine solution while only 1,075 spores per ml survived in the control. This represented a 37-fold increase in survivor numbers.

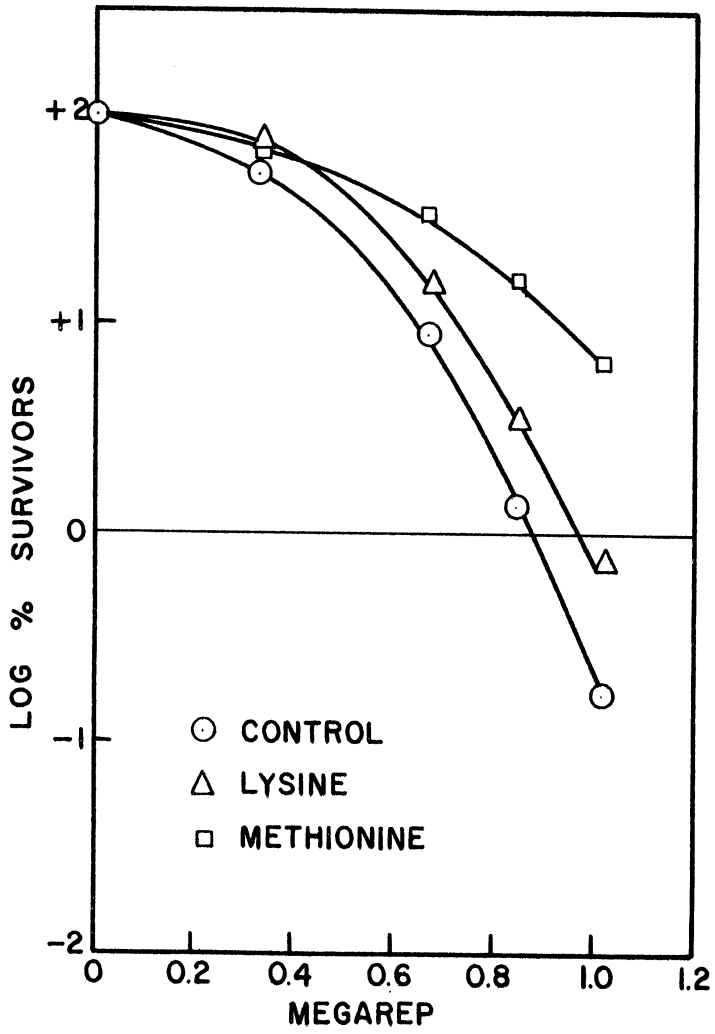


Figure 5. The effect of lysine or methionine in the suspending solution on the lethality of cobalt-60 gamma radiation for spores of C. botulinum 62A.

Following these experiments, cysteine was tested. Reagent grade cysteine hydrochloride was prepared by dissolving 1 g of the dried powder in 20 ml of phosphate buffer. However, when the pH of the resultant solution was determined, it was noted that cysteine hydrochloride in phosphate buffer gave a solution with a pH of 1.5. The pH was again brought to 7.0 by the addition of 5 ml of 1M sodium hydroxide. The final concentration of cysteine in the solution was 4 per cent. This solution was sterilized by Seitz filtration and 1×10^6 spores per ml of C. botulinum 62A added. Three ml samples of the suspension were placed in sterile 5 ml screw cap vials and irradiated in the center well of the cobalt-60 source for 0,1,2,3 and 4 hours at 4 C. The results are shown in Figure 6. Again the protection noted was comparable to that seen with both sodium nitrite and lysine. It was felt, however, that the results obtained did not give a true picture of the protective potentialities of cysteine. The physical properties of cysteine are such that this chemical was not ideal for use in this work. It is rapidly autooxidizable to cystine above a pH of 4.0. The latter compound is extremely insoluble at neutral pH values; it can only be studied in strongly acid or alkaline solution. However, it was not feasible to alter the pH of the solutions used during irradiation to either strongly alkaline or acid pH values since these solutions were toxic for the spores. This meant that with the cysteine-cystine system reversible oxidation and reduction could not take place in the pH range in which it was necessary to work since cystine immediately precipitated out as

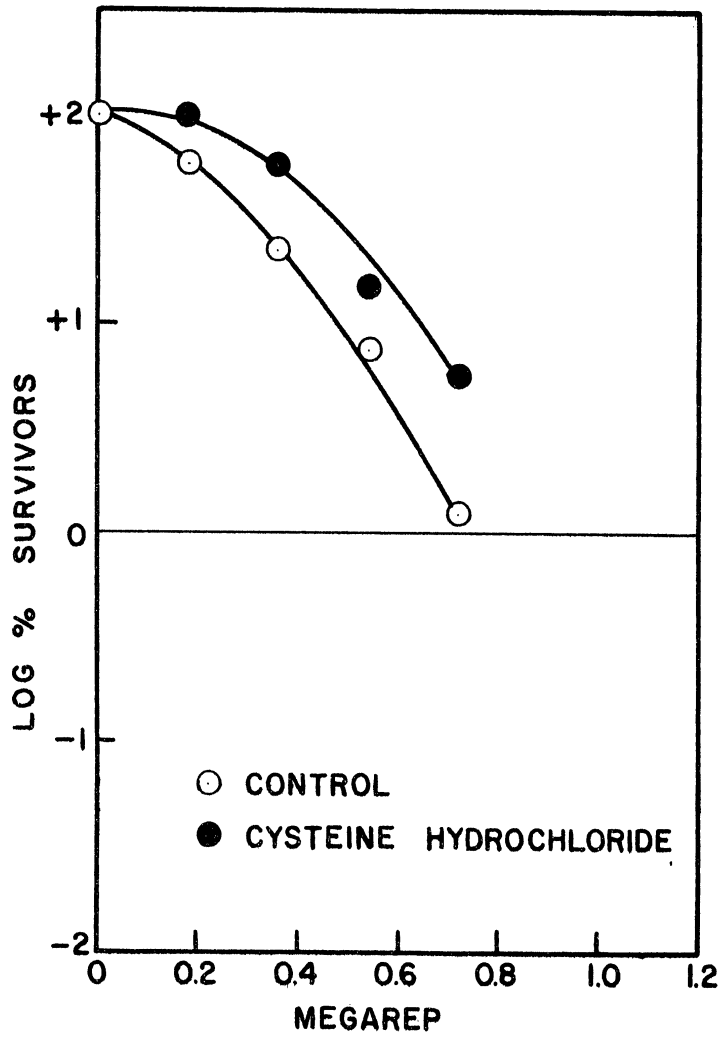


Figure 6. Survival of C. botulinum 62A spores in a cysteine hydrochloride solution as compared to a phosphate buffer control.

an insoluble component. In the experiment described above, the precipitation of cystine was noted to some extent as soon as the cysteine hydrochloride solution was prepared for use by readjusting the pH to 7.0; the cystine precipitated out as silver-white crystals. It was felt the degree of protection afforded the spores during irradiation might have been greater had the cysteine all remained in the reduced form. Since, at pH 7.0, this was difficult to achieve, a system with which it would be easier to work was sought.

C. Observations on the Effect of Glutathione on the Radiation Sensitivity of Anaerobic Spores

Barron has used glutathione to obtain protection of enzymes during their irradiation (Barron and Dickman, 1949; Barron et al., 1949). This compound is a tripeptide containing cysteine as one of its three constituents (Hopkins, 1929) and is more stable at neutral pH values than cysteine. It is readily available commercially and so was chosen for continuation of this work. The initial experiment was performed using C. botulinum 62A spores as the test organism. The stock solution was diluted to approximately 1 x 10^6 spores per ml for use in the experiment. Glutathione was prepared in a 0.02M concentration by dissolving 0.154 g of the dried powder in 25 ml of sterile phosphate buffer, pH 7.05. This solution was sterilized by filtration. The glutathione and control solutions were dispensed into glass vials and irradiated for 0,1,2,4,5 and 6 hours.

Upon titration, a striking increase in the number of spores surviving the irradiation treatment was noted in the presence of glutathione. The proportionate number of spores surviving increased with

increasing dosage of radiation until, at the end of 6 hours (1,080,000 rep), over a 900-fold increase in survivors was seen.

This protection was much greater than anything previously observed and was checked by a duplicate experiment. The results of this were the same as those obtained in the first experiment.

Next it was considered advisable to determine whether this effect could be repeated using other strains of organisms. Both C. botulinum 213B and Putrefactive anaerobe 3679 were used. They were tested using the same molar concentration of glutathione and the same radiation procedure employed for C. botulinum 62A. The initial concentration of spores was increased, however, so that irradiation of samples could be performed for a longer period of time; i.e., up to 8 hours. C. botulinum 213B and Putrefactive anaerobe 3679 showed protection of the same magnitude as was obtained using C. botulinum 62A spores. The results are given in Tables V and Va and are shown in Figure 7.

As can be seen from Figure 7, a certain percentage of the spores were killed even in the presence of the glutathione. Scott and Livermore (1954), using paper chromatography, have shown that cysteine breaks down when subjected to gamma irradiation to give rise to a variety of products: cystine, cysteic acid, alanine, serine, and possibly pyruvic acid. Since cysteine is one of the components of the glutathione molecule, it was postulated that a similar breakdown might occur in the glutathione molecule during irradiation. Therefore, an experiment was performed to determine whether death of the spores

TABLE V

THE EFFECT OF A SOLUTION OF GLUTATHIONE ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

Spores Used and Suspending Media	Hours Radiation	REP	No. of Organisms Surviving per ml	Log Percent Survivors
C. botulinum 62A				
Control	0	0	1,030,000	2.000
	1	180,000	765,000	1.871
	2	360,000	318,000	1.490
	4	720,000	6,490	$\bar{0}$.201
	5	900,000	218	$\bar{1}$.324
	6	1,080,000	50	$\bar{2}$.318
Glutathione	0	0	980,000	2.000
	1	180,000	965,000	1.993
	2	360,000	775,000	1.898
	4	720,000	264,500	1.431
	5	900,000	117,000	1.077
	6	1,080,000	45,500	0.667
C. botulinum 213B				
Control	0	0	7,700,000	2.000
	2	360,000	3,600,000	1.670
	3	540,000	645,000	0.923
	4	720,000	57,000	$\bar{0}$.131
	5	900,000	5,400	$\bar{1}$.154
	6	1,080,000	50	$\bar{3}$.192
	7	1,260,000	6	$\bar{4}$.108
	8	1,440,000	--	--

TABLE V-A

THE EFFECT OF A SOLUTION OF GLUTATHIONE ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

Spores Used and Suspending Media	Hours Radiation	REP	No. of Organisms Surviving per ml	Log Percent Survivors
C. botulinum 213B				
Gluthathione	0	0	7,500,000	2.000
	2	360,000	5,200,000	1.841
	3	540,000	3,700,000	1.693
	4	720,000	2,370,000	1.500
	5	900,000	1,100,000	1.166
	6	1,080,000	410,000	0.738
	7	1,260,000	112,500	0.176
	8	1,440,000	49,500	$\bar{0}$.180
Putrefactive Anaerobe 3679				
Control	0	0	3,130,000	2.000
	2	360,000	1,120,000	1.554
	4	720,000	16,200	$\bar{0}$.286
	5	900,000	1,210	$\bar{1}$.412
	6	1,080,000	130	$\bar{2}$.383
	7	1,260,000	2	$\bar{4}$.320
	8	1,440,000	1	$\bar{4}$.796
Glutathione	0	0	3,600,000	2.000
	2	360,000	1,925,000	1.728
	4	720,000	720,000	1.301
	5	900,000	260,000	0.859
	6	1,080,000	128,500	0.553
	7	1,260,000	36,500	0.006
	8	1,440,000	6,250	$\bar{0}$.760

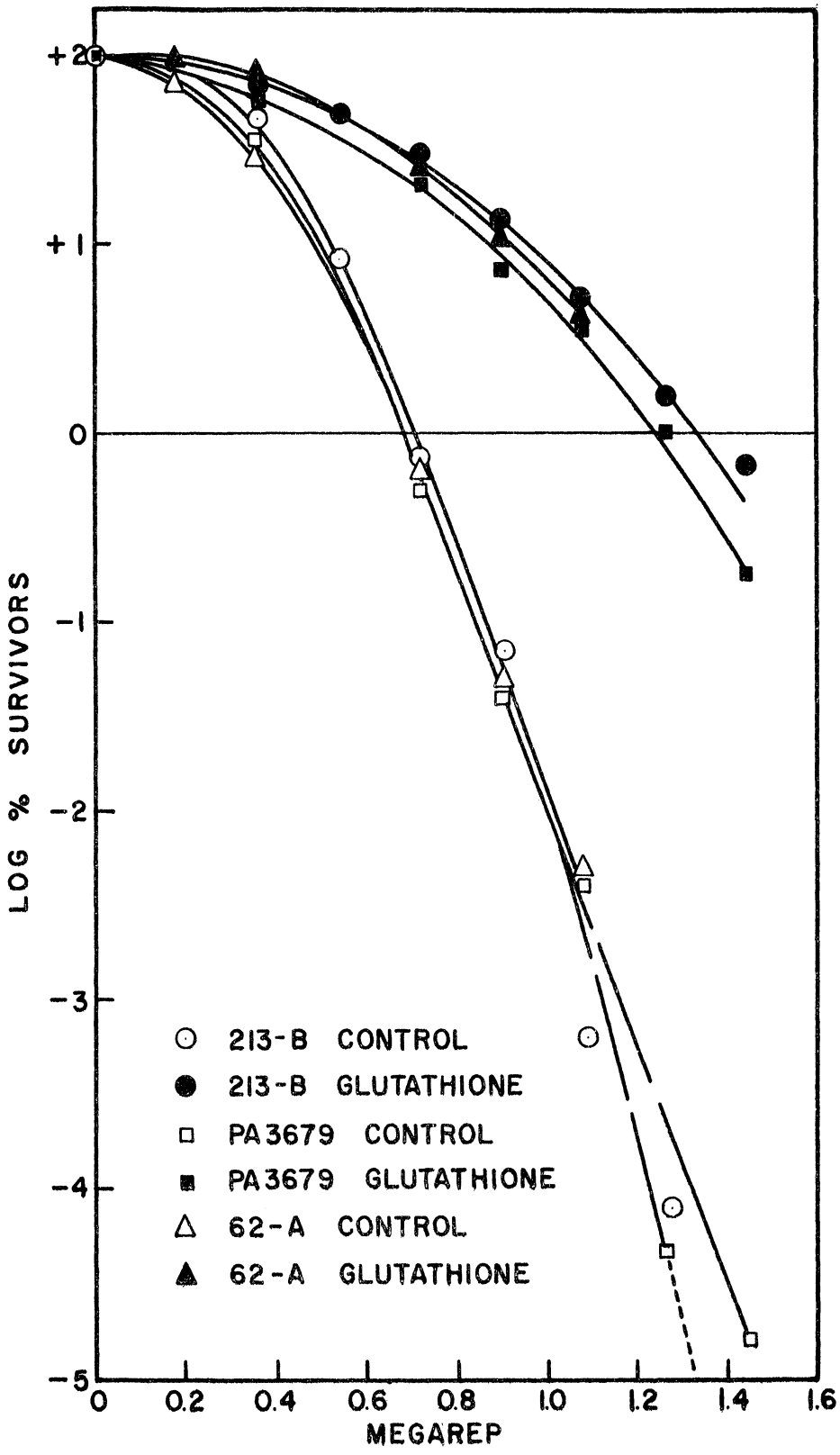


Figure 7. Survival of anaerobic bacterial spores after cobalt-60 gamma irradiation in a glutathione solution.

present in a glutathione solution during irradiation was partially the result of toxicity of the irradiated chemical itself for the spore or if death could be ascribed entirely to radiation damage. A buffered solution of glutathione was prepared in a 0.02M concentration and 4 ml quantities subjected to gamma irradiation for 4,5 and 6 hours. Immediately upon removal from the radiation source, the vial of glutathione was broken open and 3 ml of the irradiated chemical added to a test tube containing 0.03 ml of C. botulinum 213B spores. The chemical and the spores were thoroughly mixed and allowed to stand at 4 C for 72 hours with periodic shaking to insure suspension of the spores. At the end of 72 hours, all samples were titrated. A suspension of spores to which 3 ml of the original non-irradiated glutathione solution were added served as a control and to determine the original number of viable spores present. The experiment was run in duplicate. Results of these experiments are shown in Table VI. As can be seen there, no statistically significant decrease in the number of viable spores present was obtained even when the chemical had been subjected to as much as 930,000 rep. Therefore, it was concluded that glutathione did not form toxic products during exposure to cobalt-60 gamma radiation and any death of the spores observed in a glutathione suspending medium was solely due to the action of the radiation.

Barron, working with sulfhydryl enzymes in vitro has reported that a certain percentage of reactivation of enzyme activity was obtained subsequent to irradiation by the addition of sulfhydryl-containing compounds such as glutathione to the irradiated enzyme

TABLE VI

THE EFFECT OF IRRADIATION OF A GLUTATHIONE SOLUTION ON THE TOXICITY OF THE SOLUTION FOR SPORES OF *C. botulinum* 213B

No. of Hours Chemical Irradiated	Rep Received	No. of Organisms Surviving per ml.		Percent Survivors	
		RUN 1	RUN 2	RUN 1	RUN 2
0	0	5,000,000	4,600,000	100 %	100 %
4	620,000	4,850,000	4,300,000	97.00	93.48
5	775,000	4,450,000	4,400,000	89.00	95.65
6	930,000	4,300,000	4,150,000	86.00	90.22

preparation (Barron and Dickman, 1949; Barron et al., 1949). An experiment was run to determine if it was possible to increase the number of spores surviving irradiation by the addition of glutathione subsequent to irradiation of these spores. The test organism used was C. botulinum 213B with an initial concentration of 3×10^6 spores per ml. The spores, suspended in sterile phosphate buffer of pH 7.02, were irradiated for 0,3,5,6 and 7 hours. Immediately upon removal of a sample from the radiation source, the vial was opened and 3.4 ml of the spore suspension transferred to a sterile test tube. To this was added 0.2 ml of a sterile glutathione solution. This solution contained 0.1106 g of glutathione per ml so that the final concentration of the chemical when diluted by the spore suspension was 0.02M. The tubes were shaken and allowed to stand at 4 C for 72 hours. At the end of this time, a titration was run. The results are shown in Figure 8. Glutathione was completely ineffective in increasing the number of spores surviving the irradiation treatment. This experiment confirmed the necessity of the actual presence of glutathione during irradiation in order for protection to result.

Having confirmed the protective effect of glutathione for the three strains of organisms being used and having shown that in order to afford protection, glutathione must be present at the actual time of irradiation of the spores, the mechanism responsible for this phenomenon was sought. This phase of the problem was approached by the use of other chemicals in order to study the different reactive groups of glutathione. Glutathione has one sulfhydryl group per

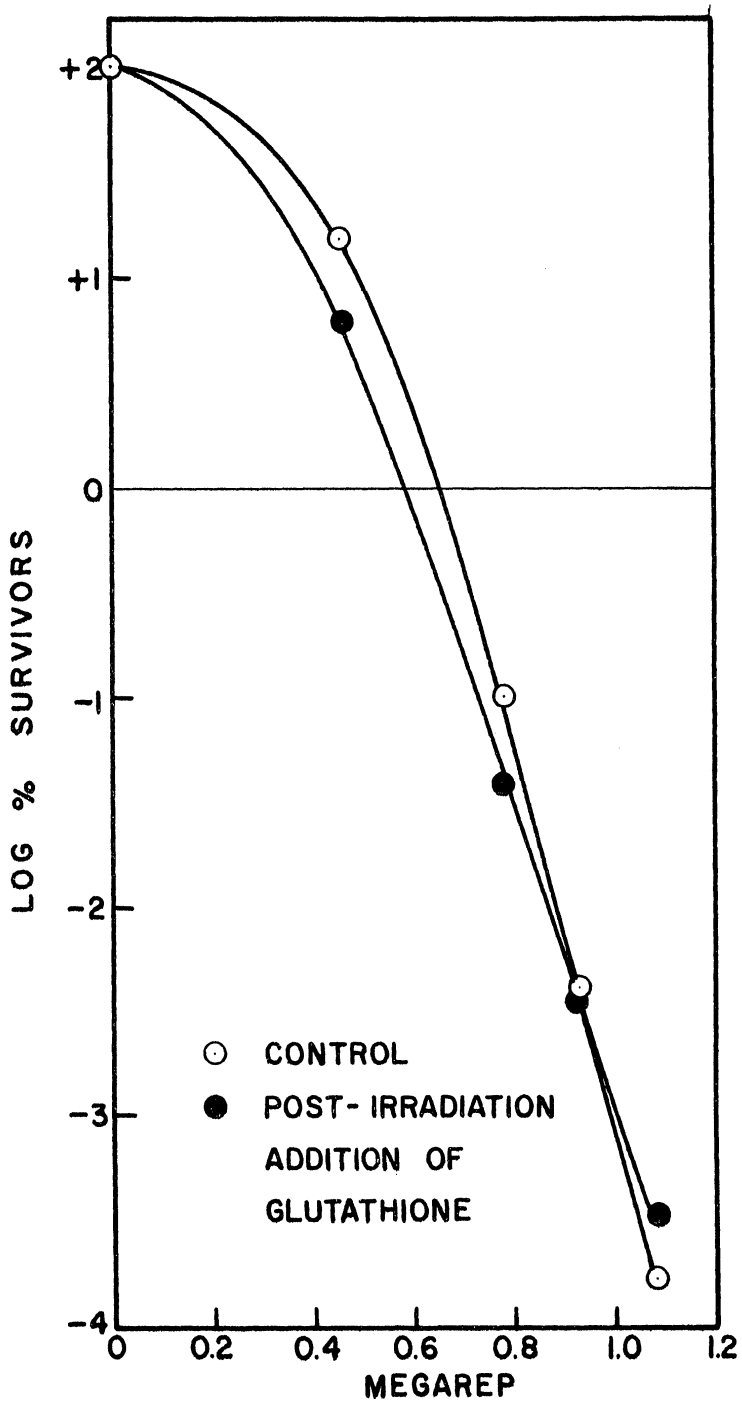


Figure 8. The effect of post-irradiation addition of a glutathione solution to spores of C. botulinum 213B exposed to cobalt-60 gamma radiation.

molecule which can be oxidized to the disulfide. Therefore, glutathione solutions were first treated with different gaseous atmospheres prior to irradiation to determine whether protection was a function of an oxidation reaction. Three atmospheres were used:

1. oxygen
2. air
3. nitrogen.

A glutathione solution was prepared in 0.02M concentration in sterile phosphate buffer and C. botulinum 213B spores in an original concentration of 6×10^6 spores per ml were added. The solution was then dispensed in 4 ml quantities into 15 of the sterile 5 ml glass vials routinely employed for irradiation. However, prior to sealing, 5 vials were exposed to each of the three gases. The vials being run in air required no further treatment; these were immediately sealed in an oxygen flame and stored at 4 C. Those vials in which the air was to be replaced by oxygen or nitrogen atmospheres were connected to appropriate tanks of these gases and the gas allowed to bubble through each vial for half an hour. Immediately upon removal from the tank of gas, the vial was closed with a clay plug to prevent gas exchange with the atmosphere and then sealed in an oxygen flame. Irradiation of all samples was performed for 0,3,5,6 and 7 hours. The results of duplicate experiments are shown in Figure 9. Here it can be seen that treatment of the glutathione solution with nitrogen did not enhance the degree of protection obtained previously in an air atmosphere. This is illustrated by a comparison of the survival curves in a nitrogen atmosphere with the survival curves shown

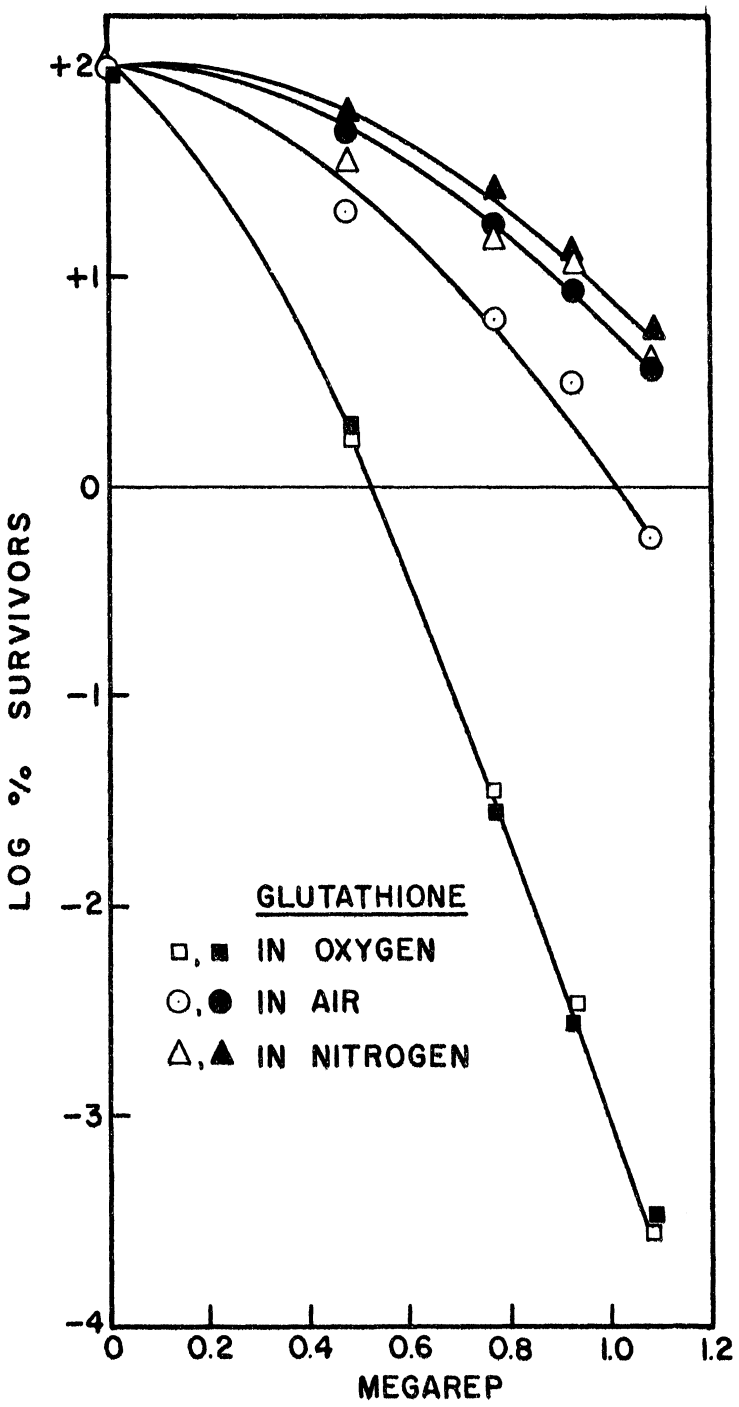
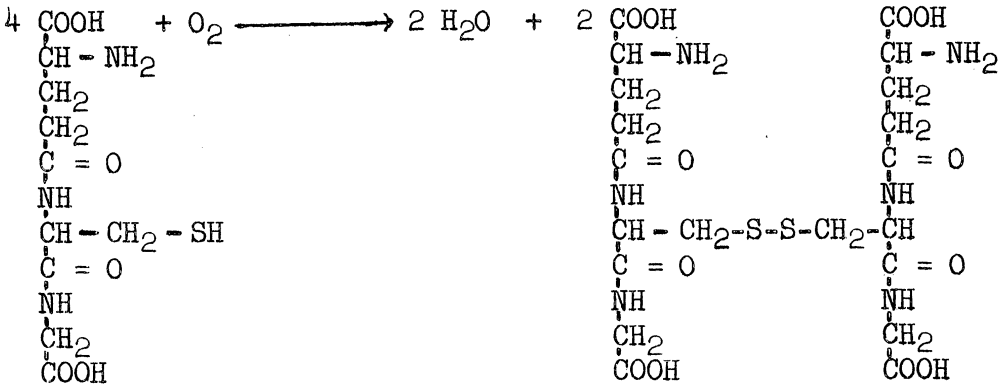


Figure 9. The effect of different gaseous atmospheres on the protection afforded spores of C. botulinum 213B by a glutathione solution during cobalt-60 gamma irradiation.

in Figure 7 as well as by the similarity of the air atmosphere and nitrogen atmosphere curves in this experiment. When the duplicate experiment was run, the vials that were irradiated in an air atmosphere showed a somewhat lower survival ratio than the corresponding set of vials in the first experiment. It was hypothesized that this was the result of an accidental partial oxidation of the glutathione during its manipulation prior to irradiation. This low a percentage of survivors was noted nowhere else during the experimentation with this chemical. Treatment of a glutathione solution with oxygen gas resulted in a complete loss of its protective capacities; this lent plausability to the assumption of partial oxidation of glutathione as an explanation for the low values obtained in air in the one experiment. The possible mechanism by which oxygen removed the protective capacity of glutathione was felt to be the following reaction:



To determine whether or not the sulfhydryl group was the specific site of the protective capacity of glutathione was the next logical step in this investigation.

It was hypothesized that cobalt-60 gamma radiation might owe its lethal effects on anaerobic spores, in part at least, to the

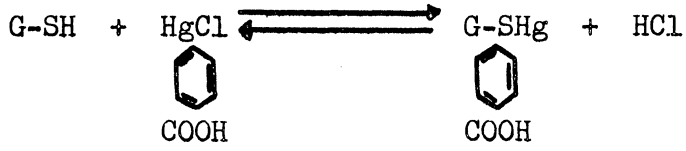
ability of gamma rays to oxidize sulfhydryl groups. The exact location of these groups in or on the cell was unknown; they might be part of essential enzyme systems of the cell or part of the actual genetic material of the cell itself. That such groups must be present somewhere within the cell was assumed from the fact that no living cell is known to exist which does not require the presence of such groups for maintenance of its life processes (Barron, 1951). If sulfhydryl groups of the cell itself were being affected by radiation, it was logical to postulate that glutathione protected the cells by preventing the gamma rays from reaching these groups. That is, when glutathione was present in the medium during irradiation, the total number of sulfhydryl groups available for potential oxidation was greatly increased. By absorption of the gamma rays passing through the medium, the glutathione was oxidized as follows:

$$2\ R\text{-}SH \xrightarrow{\text{oxidation}} R\text{-}S\text{-}S\text{-}R + 2\ H^{+}$$

and the bacterial cell itself was saved from such an oxidation.

Hellerman (Hellerman, 1937, 1939; Hellerman et al., 1943) and later Boyer (1954) have both shown that the organic mercurial, p-chloromercuribenzoic acid, combines specifically with sulfhydryl groups. This reaction was used in the present research to determine whether the protective ability of glutathione lay in the oxidation of its sulfhydryl group. Two solutions of 0.02M glutathione were prepared in sterile pH 7.02 phosphate buffer. A solution of p-chloromercuribenzoate was prepared by dissolving 0.356 ($\pm$ 0.001) g of the chemical in 3 ml of 1M sodium hydroxide and

adding 1.5 ml of this solution to one of the glutathione solutions to give an equimolar concentration of both glutathione and p-chloromercuribenzoate. This mixture was allowed to stand 24 hours at room temperature while the following reaction occurred (Hellerman, 1933):



The following day, 1.5 x 10^6 spores per ml of C. botulinum 213B were added to this medium, to the untreated glutathione solution and to a phosphate buffer control and irradiation was performed. The results of two such tests are shown in Figure 10. As can be seen there, the protective ability of the glutathione was completely removed when its sulfhydryl groups were chemically bound with mercury; evidence that these sulfhydryl groups must be free before glutathione is capable of preventing radiation damage to anaerobic bacterial spores.

To test this hypothesis by a second approach, oxidized glutathione was used. This chemical, commercially available in reagent grade, differs from reduced glutathions as follows:

Reduced Glutathione	Oxidized Glutathione
G-SH	G-S-S-G

As is the case after mercaptide formation, the sulfur is not available in the reduced -SH form. The test organism was again C. botulinum 213B with an initial concentration of 1 x 10^7 spores per ml. The oxidized glutathione was prepared in a 0.02M concentration. Samples

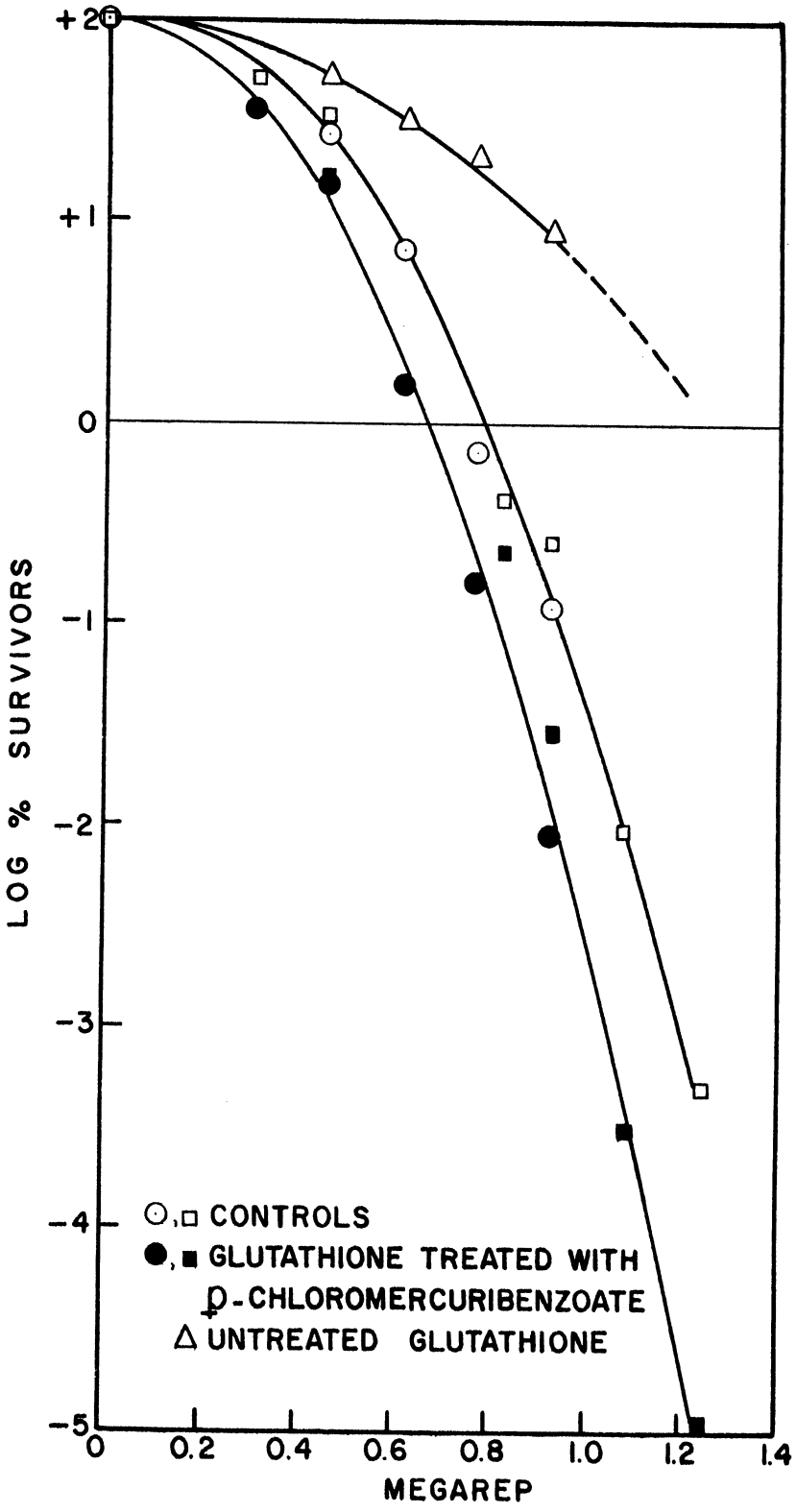


Figure 10. The effect of the pre-irradiation addition of p-chloromercuribenzoate to a glutathione solution on its protective capacity for spores of C. botulinum 213B during cobalt-60 gamma irradiation.

were irradiated for 0,3,5,6 and 7 hours. The results of this experiment are shown in Figure 11. A duplicate experiment was run to confirm these results as well as an experiment using Putrefactive anaerobe 3679 as the test organism. The results of these are given in Table VII. These results confirmed the original observation that death in the presence of oxidized glutathione was comparable to death in a control medium. No protection resulted without available sulfhydryl groups.

The effect of pH on the protective capacity of glutathione was tested by preparing buffered glutathione solutions at intervals of 0.5 pH unit over a range from pH 3.4 to 8.5. C. botulinum 213B was used as the test organism. The protection afforded by glutathione was the same in solutions ranging in pH from 8.5 to 6.0 but decreased somewhat as the pH of the solution dropped after a hydrogen ion concentration of 6.0 was reached. The decrease in survival numbers was not sufficient to warrant further investigation. Only a 3-fold difference in total number of survivors was seen between the solution of lowest pH (3.4) and that of optimum pH after 6 hours irradiation (1,080,000 rep). Since the pH of glutathione solutions used in the course of the investigation could be controlled at a value of pH 7.0 ($\pm$ 0.02 pH units) and the highest protection by the chemical was obtained in this pH range, further study of pH with respect to glutathione was not pursued.

D. Effect of p-Chloromercuribenzoate on the Radiation Sensitivity of Anaerobic Spores

Having removed the protective effect of glutathione by binding the sulfhydryl group with p-chloromercuribenzoate, the effect

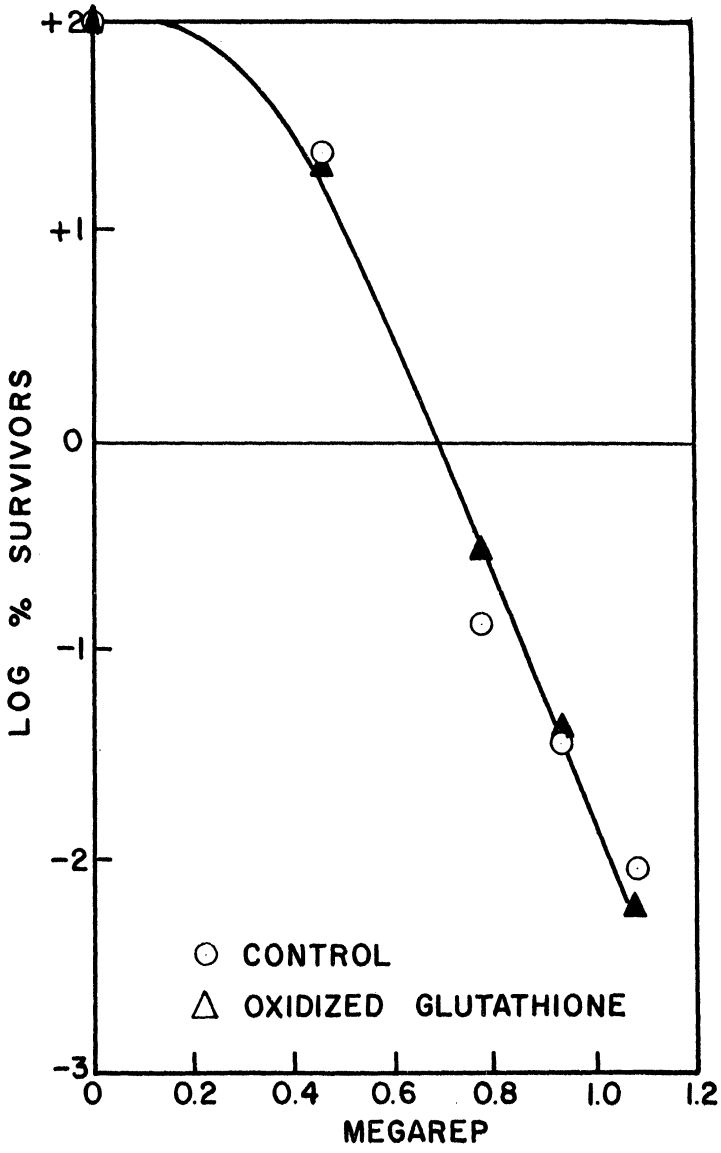


Figure 11. Effectiveness of oxidized glutathione as a protective agent for spores of C. botulinum 213B during exposure to cobalt-60 gamma radiation.

TABLE VII

THE EFFECT OF A SOLUTION OF OXIDIZED GLUTATHIONE ON THE LETHALITY OF COBALT-60 GAMMA RADIATION FOR ANAEROBIC BACTERIAL SPORES

Media Used	Hours Radiation	Rep	C. botulinum 213B		Putrefactive Anaerobe 3679	
			No. of Organisms Surviving per ml.	Log Percent Survivors	No. of Organisms Surviving per ml.	Log Percent Survivors
Control	0	0	9,530,000	2.000	3,300,000	2.000
	3	465,000	2,350,000	1.392	690,000	1.320
	5	775,000	27,500	$\bar{0}$.540	5,550	$\bar{0}$.774
	6	930,000	3,800	$\bar{1}$.399	2,000	$\bar{1}$.217
	7	1,085,000	685	$\bar{2}$.143	192	$\bar{2}$.235
Oxidized	0	0	10,500,000	2.000	2,570,000	2.000
Glutathione	3	465,000	2,410,000	1.361	515,000	1.302
	5	775,000	30,500	$\bar{0}$.537	4,100	$\bar{0}$.797
	6	930,000	3,700	$\bar{1}$.453	1,460	$\bar{1}$.246
	7	1,085,000	690	$\bar{2}$.182	134	$\bar{2}$.283

on spore survival of the use of p-chloromercuribenzoate alone was investigated. It was hypothesized that if essential sulfhydryl groups of the cell were being affected by radiation, binding these groups by mercaptide formation with the organic mercurial compound might prevent their oxidation and, therefore, increase the spore survival ratio.

A solution of p-chloromercuribenzoate was prepared in 1M sodium hydroxide and an aliquot sufficient to yield a 0.02M concentration of the mercury compound was added to 0.15M phosphate buffer containing 3×10^8 C. botulinum 213B spores per ml. The control (C. botulinum 213B spores in phosphate buffer only) was also set up and both solutions stored overnight at 4 C. The fact that a chemical reaction took place between the spores and the p-chloromercuribenzoic acid was evidenced by a change in the hydrogen ion concentration of the solution containing spores to a more acid reaction. This did not occur when the mercury compound, dissolved in a sodium hydroxide solution, was added to phosphate buffer alone. The following day the p-chloromercuribenzoate was washed from the spores as described in the "Methods and Materials" section and the spores resuspended in phosphate buffer. Samples were dispensed into glass vials and irradiated for 0,3,5,6,7 and 8 hours in the cobalt-60 source. When the samples were ready for titration, 1 ml of a 1M solution of sodium thioglycollate was added to each water blank used in the dilution procedure as well as to every tube of medium used for subsequent growth of the spores. Controls as well as mercury-treated spores were similarly treated by addition of

sodium thioglycollate to eliminate this treatment as a possible variable influencing results. The results of this experiment are seen in Figure 12. It will be noted that an increase in survivor numbers was obtained after treatment of the spores with p-chloromercuribenzoate. The increase was comparable to that seen in a sodium nitrite solution; it was checked by running two more experiments with the mercury compound under the same experimental conditions. In all cases the results were the same.

Different molar concentrations of p-chloromercuribenzoate were then tested to determine whether or not increased protection would result with increases in concentration. Four concentrations of p-chloromercuribenzoate were prepared: 0.02M; 0.05M; 0.08M and 0.1M. C. botulinum 213B was the test organism with an original concentration of 1×10^7 spores per ml. The spores were incubated with the mercury solution for 72 hours at 4 C and then washed prior to irradiation as before. The results are shown in Figure 13. An increase in number of survivors resulted with increased molar concentrations, the 0.1M concentration showing a 16-fold increase in number of survivors over the 0.02M concentration solution. Higher concentrations of the mercury compound could not be tested due to its limited solubility. This limited solubility necessitated the presence of large volumes of sodium hydroxide in the test suspension. These were toxic for the spores.

Next, rather than cleaning the spores of residual p-chloromercuribenzoate prior to irradiation, further enhancement of the protection effect was sought by irradiation of the spores in the presence

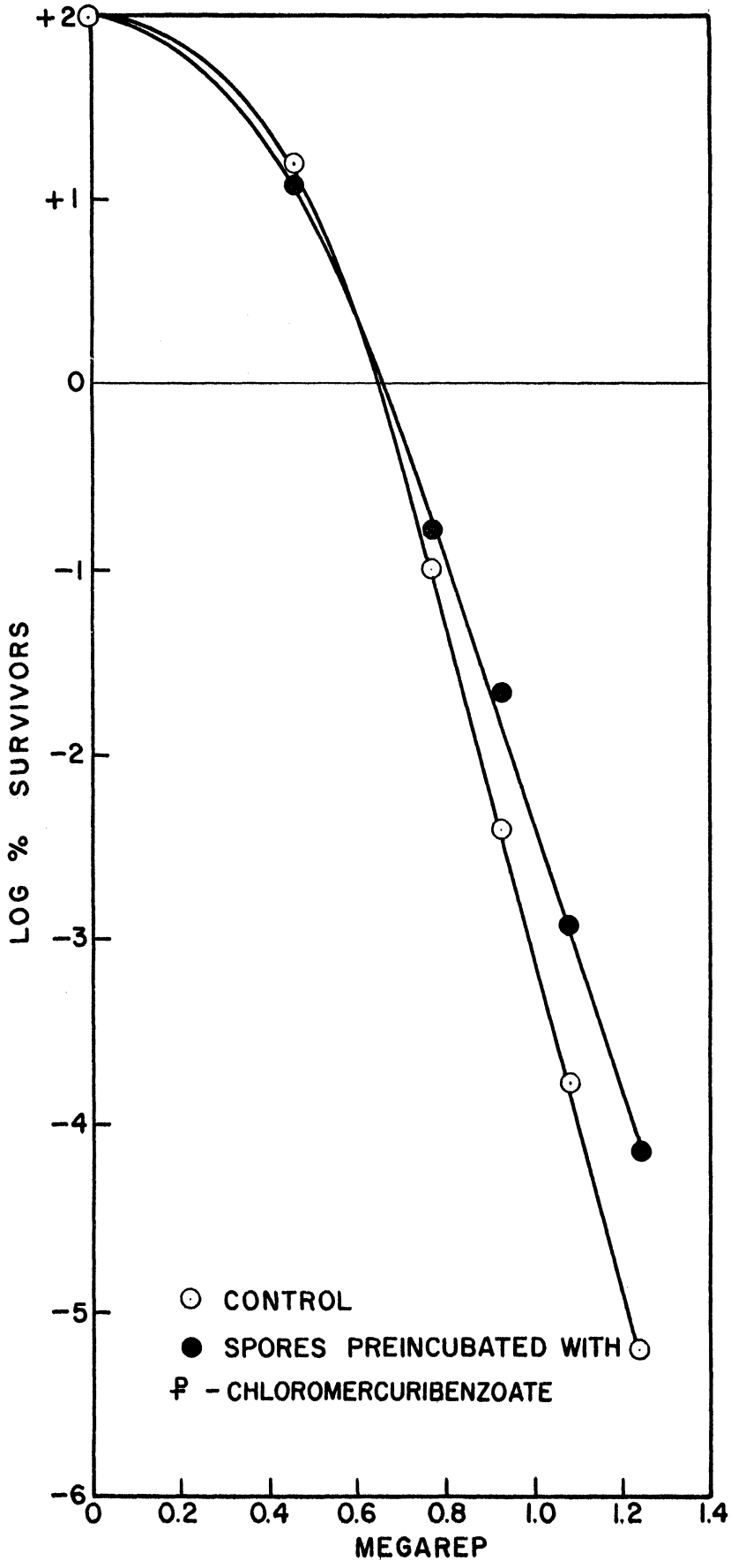


Figure 12. The effect of pre-irradiation incubation of C. botulinum 213B spores with a p-chloromercuribenzoate solution on the lethality of cobalt-60 gamma radiation for these spores.

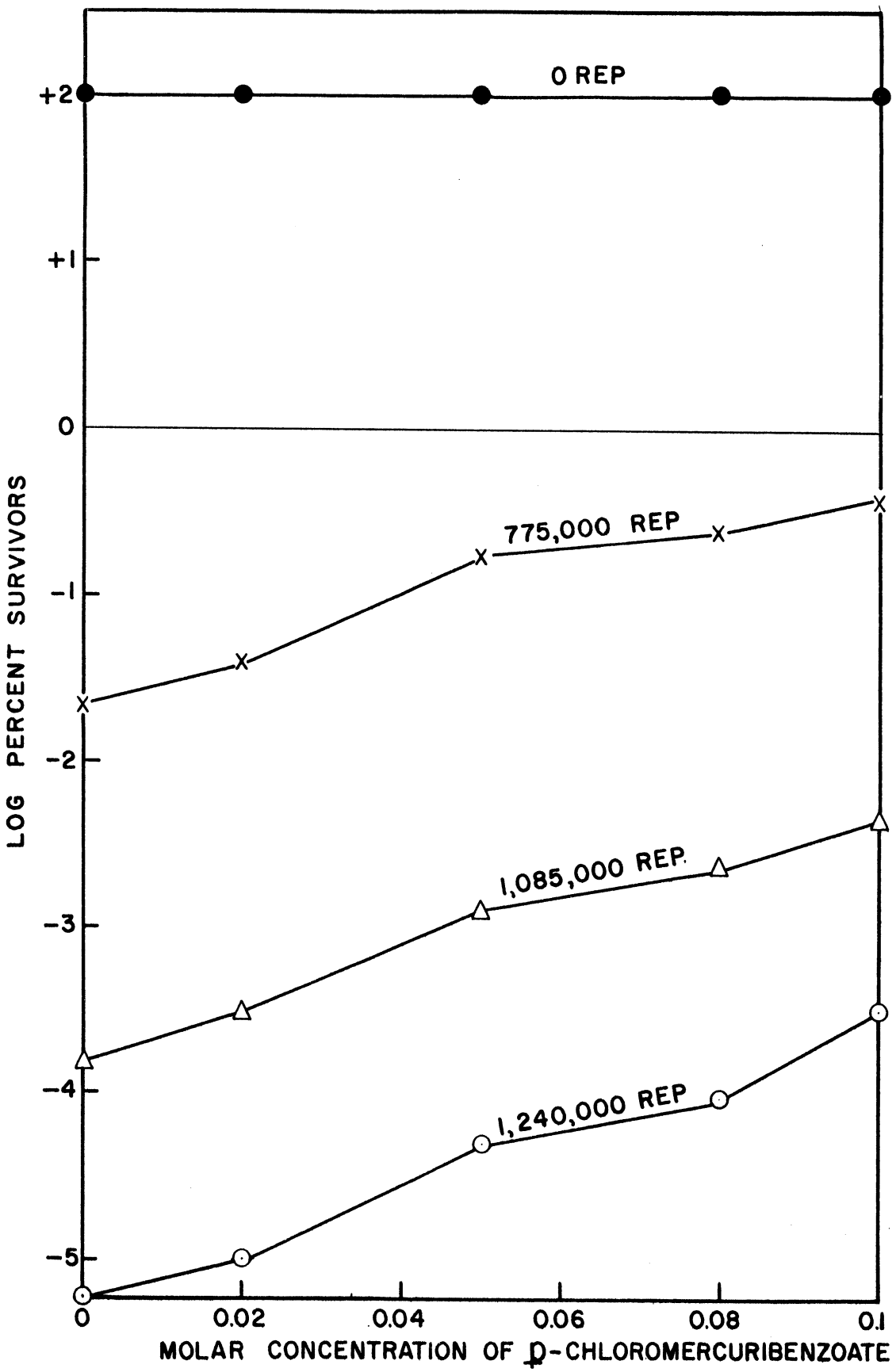


Figure 13. The effect of concentration of p-chloromercuribenzoate solutions on the lethality of cobalt-60 gamma radiation for the spores of C. botulinum 213B.

of the chemical. 1 x 10^7 spores per ml of C. botulinum 213B spores were used and the p-chloromercuribenzoate was prepared in 0.1M concentration. The samples were irradiated for 0,4,5 and 6 hours. The results are shown in Figure 14. Again there was protection of the spores in the presence of the organic mercurial but no measurable increase in protection resulted over that obtained with washed spores.

E. Observations on the Effect of Sodium Hydrosulfite on the Radiation Sensitivity of Anaerobic Spores

In reading the literature, it was noted that investigators who had worked with sodium hydrosulfite, using Escherichia coli as the test organism, had observed a protective effect of the chemical for their cells (Burnett et al., 1951; 1952; Hollaender et al., 1951). Therefore, it was decided to irradiate anaerobic spores in the presence of this chemical to determine whether protection would result. Sodium hydrosulfite was prepared in a 0.02M solution; this was sterilized by Seitz filtration. C. botulinum 213B spores were added to give a final concentration of 8 x 10^6 spores per ml and samples dispensed as described in the "Methods and Materials" section. Irradiation of the samples was performed for 0,1,2,3,4,5,6,7 and 8 hours. Upon titration it was found that a striking protection occurred in the presence of this chemical. The degree of protection even exceeded that seen with glutathione. Of an original population of 7,600,000 spores per ml only 9 survived in the control solution after 7 hours irradiation compared to 640,300 in the sodium hydrosulfite solution, an increase of over 70,000-fold. The protection was confirmed using both Putrefactive anaerobe 3679 and C. botulinum 62A spores. The results are given in Table VIII and Figure 15.

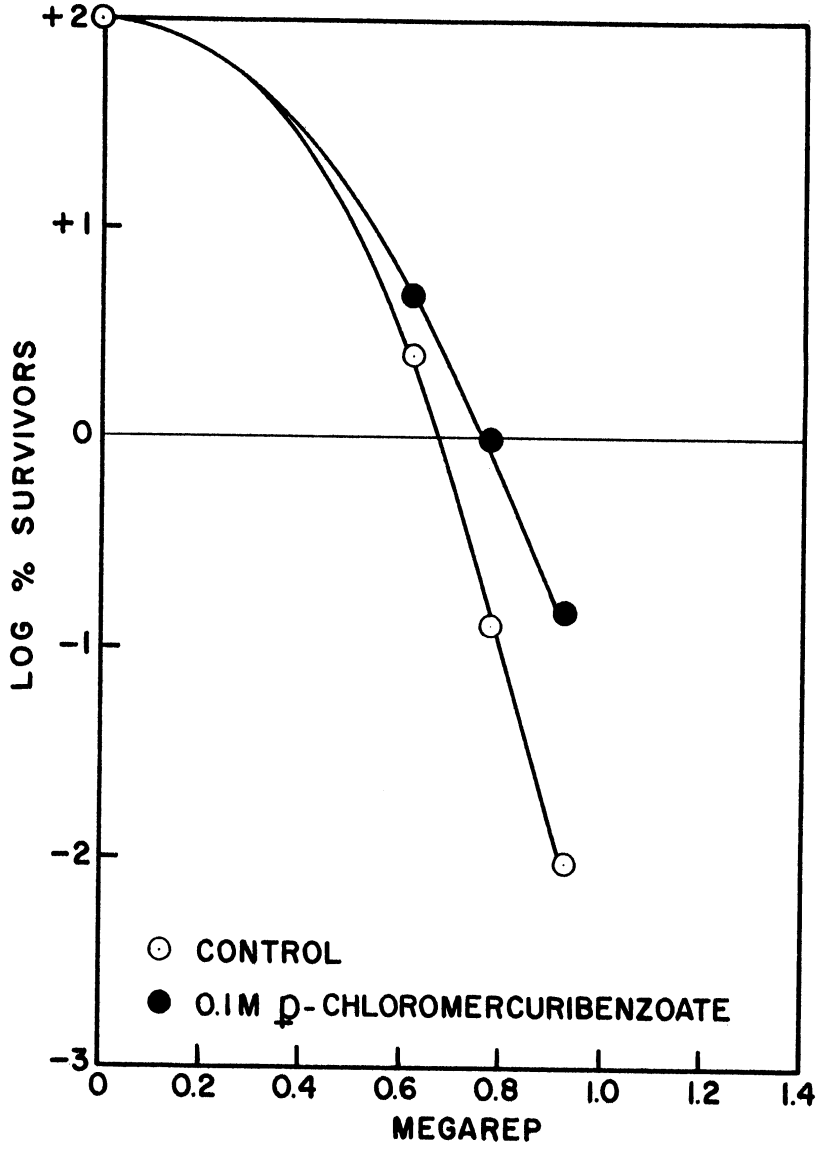


Figure 14. The effect of cobalt-60 gamma radiation on spores of C. botulinum 213B suspended in a solution of 0.1M p-chloromercuribenzoate.

TABLE VIII

THE EFFECT OF SOLUTIONS OF SODIUM HYDROSULFITE ON THE LETHALITY OF GAMMA RADIATION FROM COBALT-60 FOR ANAEROBIC BACTERIAL SPORES

Suspending Medium	Hours of Radiation	Rep	No. of Organisms Surviving per ml.	Log Percent Survivors	No. of Organisms Surviving per ml.	Log Percent Survivors	No. of Organisms Surviving per ml.	Log Percent Survivors
			C. botulinum 62A		C. botulinum 213B		Putrefactive Anaerobe 3679	
Control	0	0	10,700,000	2.000	7,600,000	2.000	3,400,000	2.000
	1	180,000	7,800,000	1.863	-	-	-	-
	2	360,000	3,450,000	1.508	3,200,000	1.624	1,240,000	1.562
	3	540,000	-	-	1,120,000	1.168	185,000	0.736
	4	720,000	71,000	$\bar{0}$.178	117,000	0.187	19,000	$\bar{0}$.253
	5	900,000	2,550	$\bar{1}$.623	16,300	$\bar{0}$.669	1,450	$\bar{1}$.370
	6	1,080,000	300	$\bar{2}$.552	164	$\bar{2}$.665	160	$\bar{2}$.327
	7	1,260,000	14	$\bar{3}$.883	9	$\bar{3}$.927	12	$\bar{3}$.452
	8	1,440,000	2	$\bar{4}$.728	-	-	1	$\bar{4}$.531
$Na_2S_2O_4$	0	0	10,900,000	2.000	7,300,000	2.000	3,570,000	2.000
	1	180,000	11,000,000	2.000	-	-	-	-
	2	360,000	10,850,000	1.998	7,100,000	1.988	2,700,000	1.879
	3	540,000	-	-	5,700,000	1.893	1,900,000	1.726
	4	720,000	5,900,000	1.733	3,875,000	1.722	1,700,000	1.678
	5	900,000	3,500,000	1.507	2,480,000	1.531	750,000	1.322
	6	1,080,000	1,630,000	1.175	1,195,000	1.214	440,000	1.091
	7	1,260,000	820,000	0.876	615,000	0.926	195,000	0.737
	8	1,440,000	325,000	0.474	241,000	0.519	88,000	0.392

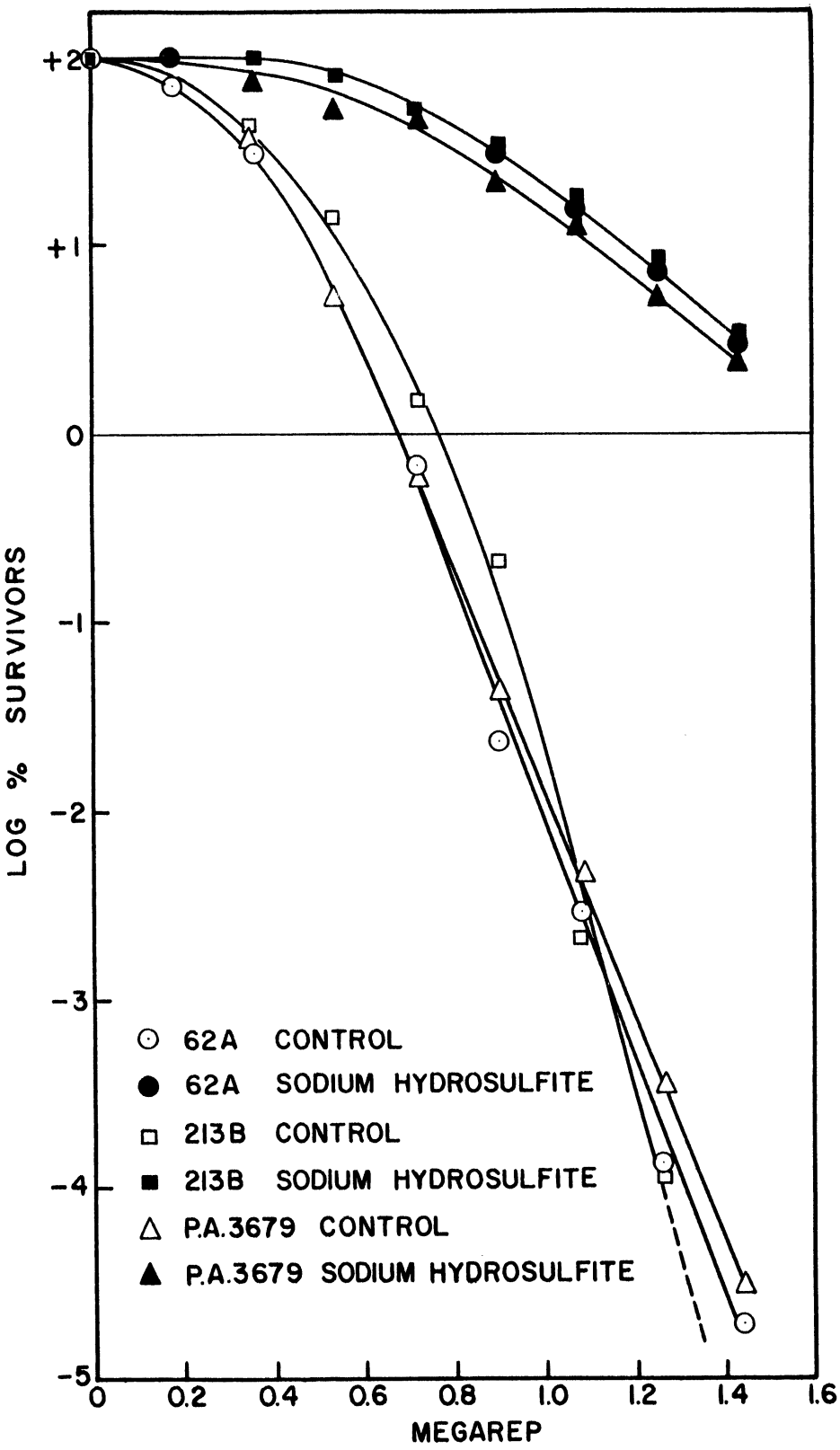


Figure 15. The effect of a sodium hydrosulfite solution on the lethality of cobalt-60 gamma radiation for anaerobic bacterial spores.

Having established its protective capacity if present during irradiation of the spores, the effect of post-irradiation addition of sodium hydrosulfite on spore survival was determined. The test organism, C. botulinum 213B, was used in an experimental concentration of 6×10^6 spores per ml. The spores were suspended in sterile phosphate buffer, pH 7.02, and irradiated for 0,3,5,6 and 7 hours. As soon as irradiation of a sample was completed, the vial was opened and 3.5 ml of the spore suspension transferred to a sterile test tube. To this was added a sufficient quantity of a freshly prepared sodium hydrosulfite solution to give a 0.05M concentration of the chemical. The tubes were shaken and kept at 4 C for 72 hours. Shaking of the samples was performed three times daily during this incubation period. The results of the subsequent titration are illustrated by Figure 16. Sodium hydrosulfite was unable to revive spores killed by gamma radiation when added to these spores subsequent to their irradiation.

An experiment was performed in which sodium hydrosulfite was irradiated alone to determine if it developed toxic products during exposure to cobalt-60 gamma radiation. Duplicate experiments were run using 0.02M sodium hydrosulfite. The solutions were irradiated at 4 C for 0,4,5 and 6 hours. Immediately upon removal from the radiation source, the vials were opened and 3 ml of the irradiated sodium hydrosulfite added to 0.03 ml of C. botulinum 213B spores. The final spore concentration was 4×10^6 spores per ml. The samples were shaken periodically while undergoing a subsequent 72 hour incubation at 4 C. The results of the titrations are shown in Table IX where

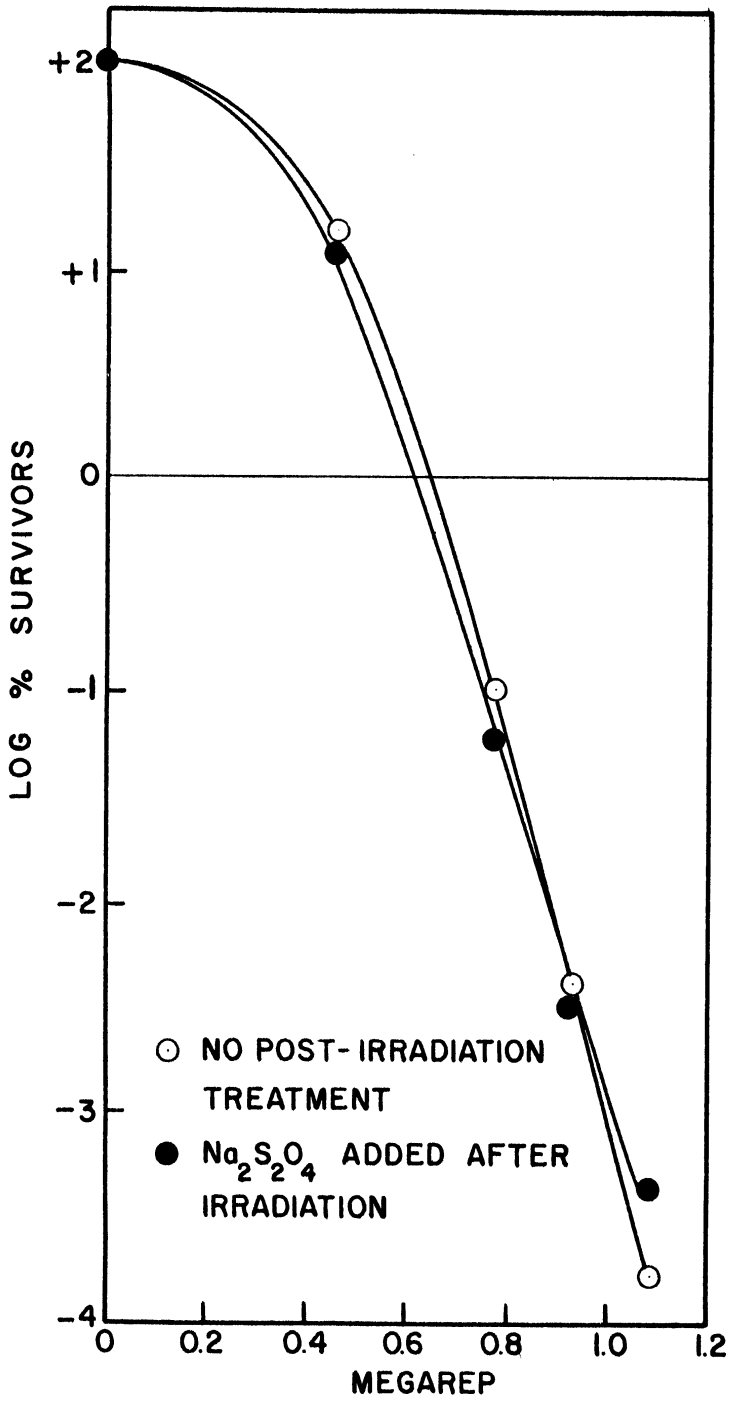


Figure 16. The effect of post-irradiation addition of a sodium hydrosulfite solution on the survival of C. botulinum 213B spores previously exposed to cobalt-60 gamma radiation.

TABLE IX

THE EFFECT OF IRRADIATION OF SODIUM HYDROSULFITE ON THE TOXICITY OF THAT CHEMICAL FOR SPORES OF *C. botulinum* 213B

No. of Hours Chemical Irradiated	Rep Received	No. of Organisms Surviving per ml.		Percent Survivors	
		RUN 1	RUN 2	RUN 1	RUN 2
0	0	3,500,000	4,500,000	100 %	100 %
4	620,000	3,100,000	4,350,000	88.57	96.67
5	775,000	3,550,000	4,350,000	100	96.67
6	930,000	3,200,000	3,700,000	91.43	82.22

it can be seen that no statistically significant decrease in spore numbers resulted in the presence of irradiated sodium hydrosulfite when compared with survival numbers in the unirradiated controls. As with glutathione, the results of this experiment served to eliminate the possibility that death of spores in the presence of sodium hydrosulfite was due to toxic products in the medium rather than to the radiation itself.

Sodium hydrosulfite, like glutathione, is a reducing agent. The two compounds do not accomplish their action by the same mechanism, however. Glutathione acts by giving up a hydrogen ion from its sulfhydryl group in the course of an oxidation-reduction reaction and is thereby transformed into oxidized glutathione. Sodium hydrosulfite, on the other hand, absorbs an oxygen ion when in solution and becomes sodium metabisulfite according to the following reaction (Martin, 1946):

$$2\ Na_2S_2O_4 + O_2 \longrightarrow 2\ Na_2S_2O_5 \quad .$$

Once formed, this latter product can also react with oxygen but at a much slower rate than does sodium hydrosulfite.

Since sodium hydrosulfite acts by absorbing oxygen, an experiment was run using the chemical in three different gaseous atmospheres:

1. oxygen
2. air
3. nitrogen.

It was considered likely that oxygen might reduce and nitrogen enhance the protective effect of sodium hydrosulfite. A 0.02M solution of this chemical was prepared and aseptically inoculated

with 6 x 10^6 spores per ml of C. botulinum 213B spores. Samples were dispensed into glass vials as described in the "Methods and Materials" section and those vials being irradiated in an air atmosphere were immediately sealed and stored at 4 C. Five vials each were exposed to either oxygen or nitrogen gas for a half hour. Immediately upon removal from the tank of gas, the vials were closed with a clay plug to prevent gas exchange with the atmosphere and sealed in an oxygen flame. All samples were irradiated for 0,3,5,6 and 7 hours. The results are shown in Figure 17. Treatment of the sodium hydrosulfite solution with oxygen prior to irradiation did decrease its protective effect somewhat but not to the extent anticipated. Irradiation in a nitrogen atmosphere gave no enhancement of the protection obtained when performing the experiments in air. These results were checked by a duplicate experiment.

Burnett et al., (1952) in experiments using Escherichia coli and X-rays have reported that sodium hydrosulfite present in the irradiation medium in concentrations below 0.0001M gave no protection from X-radiation to the organisms, in concentrations between 0.0001M and 0.0004M the protection increased logarithmically, and above a molar concentration of 0.0004M no further increase in protection of the organisms from radiation effects resulted. Since this upper concentration is very close to the theoretical amount of sodium hydrosulfite (0.0005M) necessary to remove all the dissolved oxygen from a phosphate buffer solution saturated with air at 25 C, Burnett concluded that the protective mechanism was entirely one of removal of oxygen from the medium.

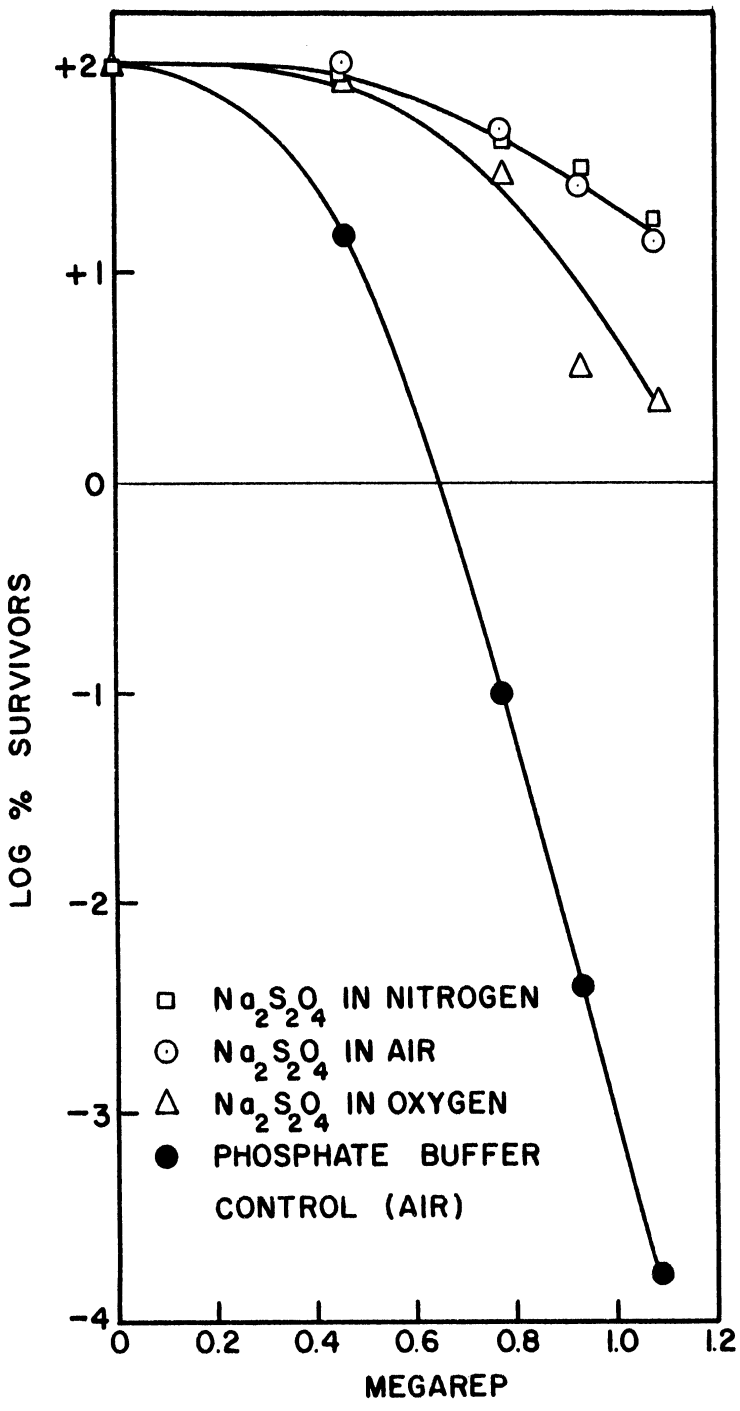


Figure 17. The effect of different gaseous atmospheres on the protection afforded C. botulinum 213B spores by a sodium hydrosulfite solution during cobalt-60 gamma irradiation.

The results obtained with spores of C. botulinum 213B in an oxygen atmosphere did not seem to corroborate Burnett's conclusion so an experiment was set up along the same lines he had used in an attempt to duplicate his work.

Sodium hydrosulfite was prepared in concentrations ranging from 0.00005M to 1M by adding appropriate amounts of the chemical (weighed to the nearest milligram) to measured volumes of 0.15M phosphate buffer (pH 7.0). Twenty ml quantities of each molar concentration were placed in sterile Erlenmeyer flasks and 0.2 ml of C. botulinum 213B spores were added to each flask to give a final spore concentration of 1×10^6 spores per ml. The solutions were then dispensed into vials and irradiated for 0,2,4,5 and 6 hours in the cobalt-60 source. The results of the subsequent titration are shown in Figure 18. A definite concentration effect was observed. Its range did not, however, agree with that reported by Burnett using Escherichia coli as the test organism. Although no difference was noted in the degree of protection afforded by sodium hydrosulfite between concentrations of 0.00005M and 0.0001M, the protective effect which began after a concentration of 0.0001M was reached continued above a concentration of 0.0005M. An increase in protection was noted with concentrations of sodium hydrosulfite as high as 0.01M. The greatest single increase in spore survivors resulted when the concentration of sodium hydrosulfite in the solution was increased from 0.001M to 0.005M. The maximum protection possible seemed to have been achieved using sodium hydrosulfite in a concentration of 0.01M. Molar concentrations above this failed to further increase the degree of protection obtained with this concentration.

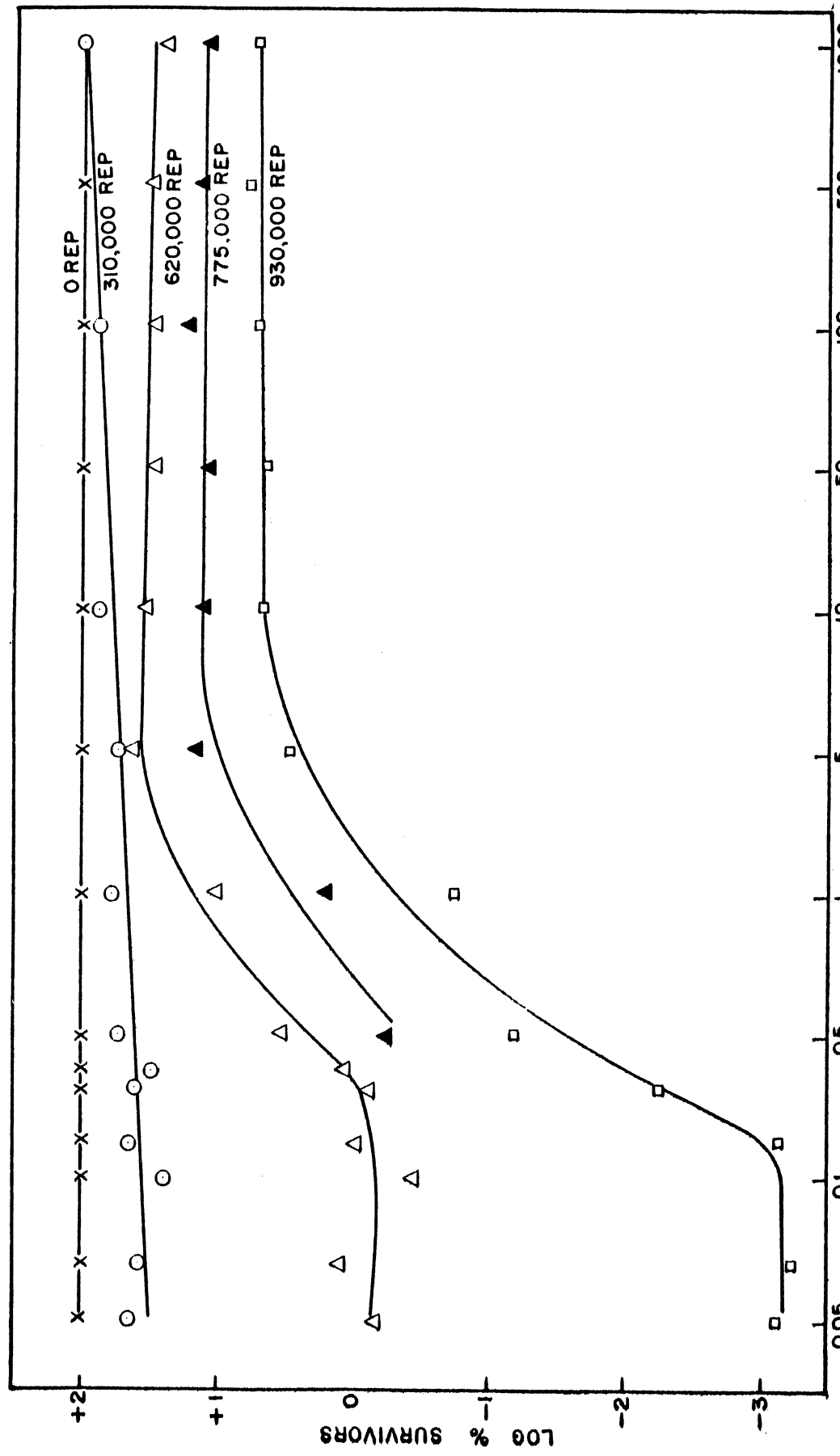


Figure 18. Effect of the molar concentration of sodium hydrosulfite in the suspending solution on the lethality of gamma radiation from cobalt-60 for anaerobic bacterial spores.

It was felt that the relation of oxygen removal to protection could be studied by yet another approach. If sodium hydrosulfite acted solely by the mechanism of oxygen removal from the medium, it was hypothesized that protection comparable to that seen in the presence of sodium hydrosulfite could be obtained by removing the oxygen from a phosphate buffer suspension of spores. An experiment was set up using 6×10^6 spores per ml of C. botulinum 213B. Sample vials containing 4 ml of the test suspension were prepared and exposed to either an oxygen, air, or nitrogen atmosphere using the same techniques employed in the experiments in which glutathione and sodium hydrosulfite were prepared in different gaseous atmospheres. Samples were then irradiated for 0,3,5,6 and 7 hours. The results, as illustrated in Figure 19, indicated that replacement of air by a nitrogen atmosphere increased the survival ratio of anaerobic spores. An increased oxygen content of the atmosphere, on the contrary, had a deleterious effect. The protection afforded by nitrogen in phosphate buffer was not the same as that attained with sodium hydrosulfite, however. Even when irradiation was performed on a sodium hydrosulfite solution which was exposed to oxygen, the resultant protection was greater than that attained with phosphate buffer in a nitrogen atmosphere.

Glutathione and sodium hydrosulfite were combined in solution to see if the presence of both chemicals would enhance the spore survival obtained when they were used separately. Sufficient quantities of glutathione and sodium hydrosulfite were weighed out

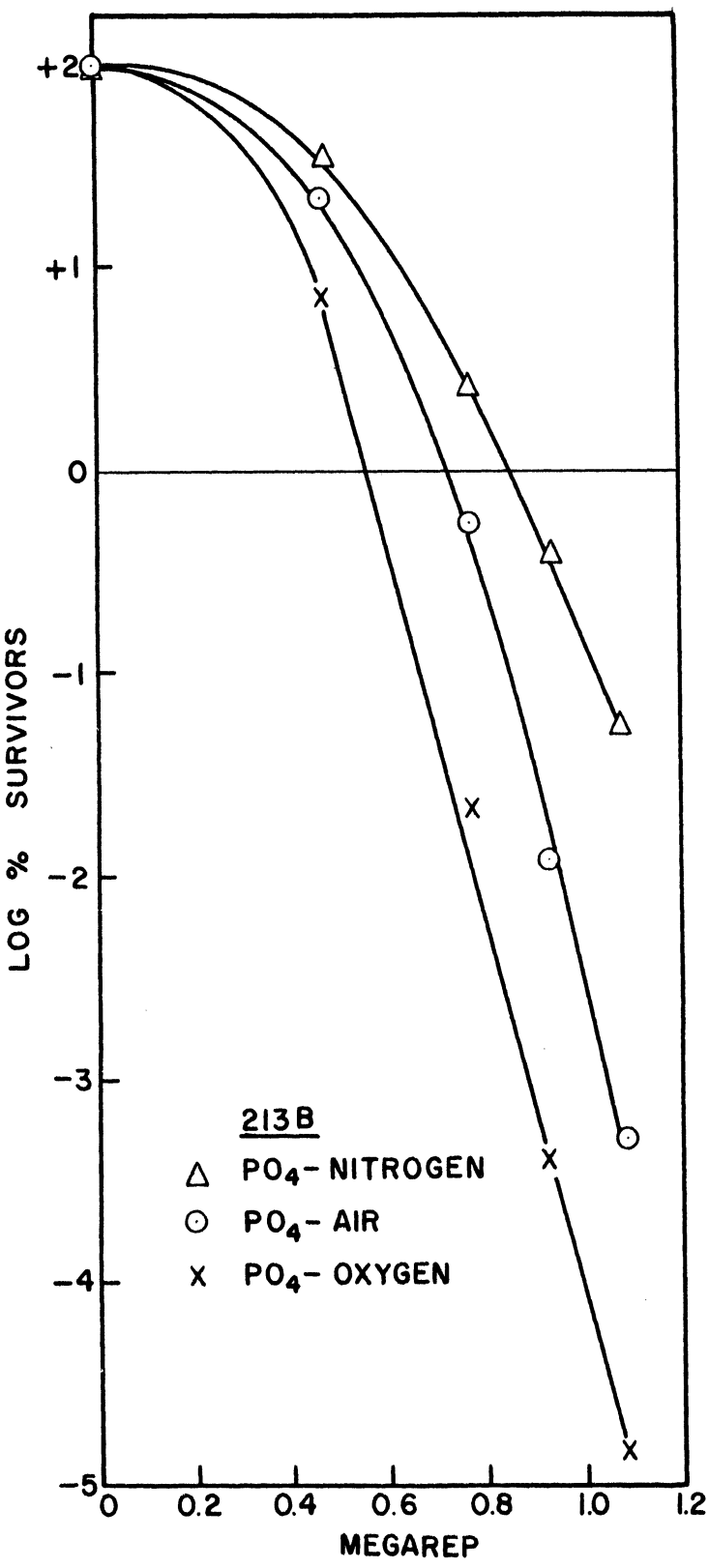


Figure 19. Influence of the gas present in and above phosphate buffer on the lethality of gamma radiation from cobalt-60 for C. botulinum 213B spores suspended in the solution.

(±0.001 g) to give a 0.02M concentration of each in a solution. These powders were then added to 0.15M phosphate buffer (pH 7.02), dissolved, and the resulting solution inoculated with 6 x 10^6 spores per ml of C. botulinum 213B. Irradiation was performed for 0,3,5,6, 7 and 8 hours. The combination of the two chemicals gave no greater protection than that obtained with sodium hydrosulfite alone. This was a greater protection than was obtained with glutathione when it alone was present during irradiation but represented no enhancement of the protective ability of sodium hydrosulfite. The experiment was repeated using Putrefactive anaerobe 3679 in a concentration of 1 x 10^6 spores per ml. The results of experiments with both organisms are shown in Table X.

III. Study of Oxidizing Free Radicals Responsible for the Lethality of Cobalt-60 Gamma Radiation for Anaerobic Bacterial Spores: Hydrogen Peroxide

All the results obtained pointed to an oxidation of the bacterial cell by the free radicals produced in the suspending solution during irradiation. This oxidation could be prevented to varying degrees depending upon the chemical present in the medium at the time the irradiation was being performed. Workers who have studied the radiation chemistry of aqueous solutions state that the four products formed upon irradiation of water are the two radicals H and OH and the molecular water decomposition products H_2 and H_2O_2. In the presence of oxygen, the powerful oxidizing agent, O_2H, is also formed, (Allen, 1954; Dewhurst et al., 1954). It was hypothesized that one or two of these oxidizing agents was responsible for the majority of the lethal effects observed with bacterial spores, rather

TABLE X

THE EFFECT OF A SOLUTION CONTAINING BOTH GLUTATHIONE AND SODIUM HYDROSULFITE ON THEIR PROTECTIVE CAPACITY FOR ANAEROBIC BACTERIAL SPORES DURING EXPOSURE TO COBALT-60 GAMMA RADIATION

Spores	Hours of Radiation	Rep	No. of Organisms Surviving per ml.	Log Percent Survivors
C. botulinum 213B				
Control	0	0	7,430,000	2.000
	3	465,000	1,390,000	1.272
	5	775,000	10,500	$\bar{0}$.850
	6	930,000	54	$\bar{3}$.139
	7	1,085,000	2	$\bar{1}$.570
	8	1,240,000	-	-
$Na_2S_2O_4$ +	0	0	6,700,000	2.000
Glutathione	3	465,000	6,350,000	1.977
	5	775,000	2,660,000	1.599
	6	930,000	1,825,000	1.435
	7	1,085,000	1,275,000	1.279
	8	1,240,000	455,000	0.832
Putrefactive Anaerobe 3679				
Control	0	0	2,900,000	2.000
	3	465,000	580,000	1.301
	5	775,000	12,100	$\bar{0}$.380
	6	930,000	730	$\bar{1}$.599
	7	1,085,000	36	$\bar{2}$.906
	8	1,240,000	2	$\bar{1}$.161
$Na_2S_2O_4$ +	0	0	3,200,000	2.000
Glutathione	3	465,000	2,300,000	1.857
	5	775,000	1,120,000	1.544
	6	930,000	700,000	1.340
	7	1,085,000	425,000	1.123
	8	1,240,000	184,000	0.760

than all products being equally responsible for death. Hydrogen peroxide was chosen for investigation since it was postulated that its formation might be controlled by the presence of catalase in the suspending solution during irradiation. Catalase is an enzyme which acts exclusively on a hydrogen peroxide substrate. The use of catalase to protect another enzyme, phosphoglyceraldehyde dehydrogenase, from in vitro inactivation by very low radiation dosages of alpha rays (maximum radiation delivered was 1,080 roentgens) has been reported by Barron and Dickman (1949). Also, catalase has been reported to be relatively resistant to inactivation by ionizing radiations (Tytell and Kersten, 1941; Barron, 1954). Therefore, it was considered possible that this enzyme might be a good tool for determining what percentage of the death of anaerobic bacterial spores from high dosages of gamma radiation (maximum dosage of 1,240,000 rep) was due to hydrogen peroxide.

Phosphate buffer (pH 7.02) was sterilized by autoclaving and 100 mg of purified crystalline catalase was dissoved in 24.15 ml of the cooled buffer solution. To this was added 0.85 ml of a stock suspension of C. botulinum 213B spores to give an initial spore concentration of 1×10^7 spores per ml. Four ml quantities of this preparation and its control were then dispensed for irradiation and irradiated for 0,3,5,6,7 and 8 hours in the center well of the large cobalt-60 source. The results obtained were striking. The degree of protection seen in the presence of the catalase was almost the same as that seen when glutathione was present in the suspending solution. The experiment was run again, this time allowing a delay

between the time of initial preparation of the samples and their actual irradiation. They were allowed to stand at 4 C for 48 hours so that any inactivation of the catalase which might have developed due to factors in the medium or the spore could occur. The results of this experiment duplicated those of the previous one.

A second organism, Putrefactive anaerobe 3679, was then tested. The same procedure was followed as had been used with C. botulinum 213B except that the stock spore suspension of Putrefactive anaerobe 3679 was concentrated 10-fold for use in the experiment. This concentration of the spores insured a high spore count in the control solution at the end of 7 hours irradiation. Sixty mg of catalase and 0.5 ml of spores were added to 14.5 ml of buffer, samples were dispensed and irradiation was performed for 0,5,6 and 7 hours. The results obtained with both organisms have been reported in the literature (Williams and Kempe, 1957) and are shown in Figure 20 and Table XI.

Further experiments with catalase were not performed beyond the initial discovery that the phenomenon of protection of living cells from the lethal effects of ionizing radiations could be elicited by its use. Experiments based upon this discovery would appear to be a good starting point for future studies in this phase of radiation research. The possibility is certainly worthy of serious consideration.

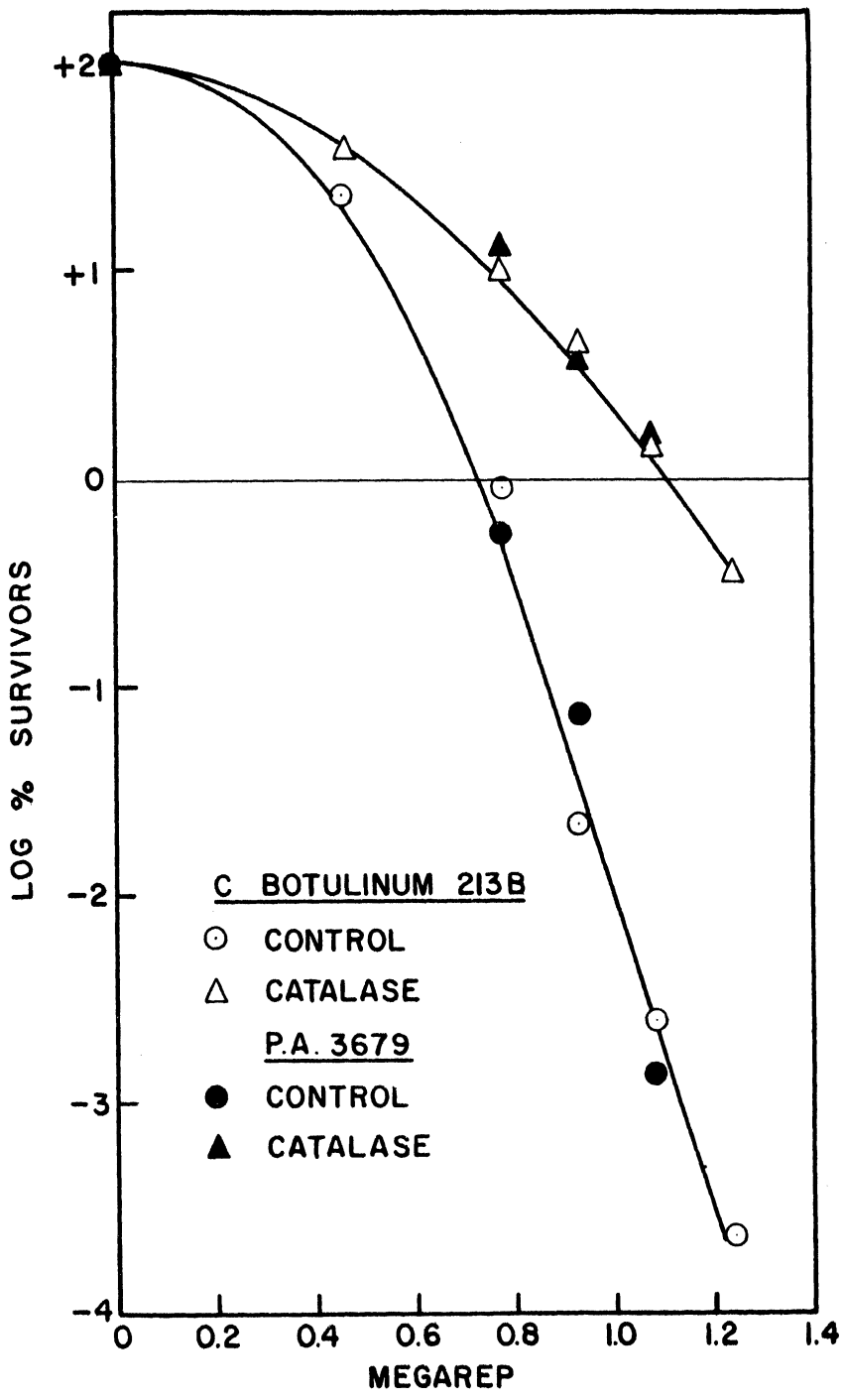


Figure 20. The effect of catalase on the lethality of gamma radiation from cobalt-60 for anaerobic bacterial spores.

TABLE XI

THE EFFECT OF CATALASE ON THE LETHALITY OF GAMMA RADIATION FROM COBALT-60 FOR ANAEROBIC BACTERIAL SPORES

Spores	Hours Radiation	REP	No. of Organisms Surviving per ml	Log Percent Survivors
C. botulinum 213B				
Control	0	0	15,000,000	2.000
	3	465,000	3,500,000	1.368
	5	775,000	136,000	$\bar{0}$.043
	6	930,000	3,330	$\bar{1}$.654
	7	1,085,000	380	$\bar{2}$.596
	8	1,240,000	35	$\bar{3}$.632
Catalase	0	0	16,900,000	2.000
	3	465,000	6,800,000	1.605
	5	775,000	1,690,000	1.000
	6	930,000	710,000	0.623
	7	1,085,000	233,000	0.139
	8	1,240,000	59,000	$\bar{0}$.456
Putrefactive Anaerobe 3679				
Control	0	0	10,700,000	2.000
	5	775,000	61,500	$\bar{0}$.241
	6	930,000	8,100	$\bar{1}$.121
	7	1,085,000	145	$\bar{2}$.868
Catalase	0	0	11,600,000	2.000
	5	775,000	1,460,000	1.100
	6	930,000	410,000	0.548
	7	1,085,000	179,500	0.189

DISCUSSION

This research was an outgrowth of investigations into the effects of combined heat and radiation sterilization on spores of C. botulinum strains and Putrefactive anaerobe 3679 in cooked, canned meat (Kempe et al., 1954, 1958; Kempe, 1955). The observation that increased survival of spores resulted when they were irradiated using gelatin rather than phosphate buffer as the suspending medium stimulated an interest in alteration of the lethality of ionizing radiations for anaerobic bacteria. At the time this work was begun, studies on actual chemical protection of bacteria during exposure to ionizing radiations were limited in number. Moreover, reports in the literature of studies using spores as the test organism were almost totally lacking. From the industrial aspect, such studies were definitely needed. Any alteration of the lethality of ionizing radiation for microorganisms is of paramount interest to the food industry since such alteration can affect the dose of radiation necessary to sterilize food products. Such studies have similar applicability to the fields of pharmaceuticals and biological materials sterilzied by radiation.

The same organisms used by Kempe and his co-workers were considered the most logical choice for study of another phase of the same project. Therefore, the same three strains of anaerobic bacterial spores, C. botulinum 62A, C. botulinum 213B, and Putrefactive anaerobe 3679, were used in this study. It was considered possible that phenomena might be observed using such spores which were not seen with the aerobic, vegetative cells of bacteria, with

viruses, and with the more complex organisms which had been studied by previous investigators.

Two studies were done to determine the effects of (1) semi-solid nature of the medium; and (2) variations in pH of the suspending solution on the survival of spores during irradiation. No enhancement of survivor numbers was obtained by the use of a semi-solid medium. The protection noted with gelatin was ascribed to its chemical nature rather than its physical state since guar gum, a carbohydrate medium used in these experiments as a semi-solid gel, gave no protection. In later experiments it was shown that methionine, one of the amino acid constituents of gelatin, had protective properties when irradiated with the spores used in this study. This lent experimental support to the a priori reasoning that some chemical constituent of the gelatin gave the protection which was noted. Gelatin also contains sulfur (0.5%) which may be a factor in its protective effect. Dale has shown that elemental sulfur will afford protection to certain enzymes if added to these enzymes prior to irradiation (Dale et al., 1949a). Similarly, inorganic, sulfur-containing compounds have been shown by other investigators (Burnett et al., 1952) to protect vegetative cells of bacteria from ionizing radiation.

The radicals OH and O_2H are among the oxidizing agents formed during irradiation as decomposition products of water. Although increased hydrogen ion concentration theoretically increases the oxidizing power of such radicals (Barron et al., 1949), this increase

was apparently insignificant in comparison to the total oxidizing capacity of these radicals formed by high energy ionizing radiations acting on phosphate buffer. In studying the effect of variation of pH on spore survival, it was found that decreasing the pH of the suspending medium had no effect on the damage to the spores caused by cobalt-60 gamma radiation. Within the pH range studied, pH values between 3.4 and 8.5, no change in spore survivors was seen as a result of the alterations in hydrogen ion concentration.

Protection of anaerobic bacterial spores was observed with nine of the reducing agents tested. The degree of protection obtained was variable, apparently being dependent upon the relative efficiencies of the different chemicals for removing oxidizing free radicals from the medium. Isoefficiency dose ratios as low as 1.10 for lysine and as high as 2.40 for sodium hydrosulfite were obtained (See Appendix B). Isoefficiency dose ratio is a mathematical expression developed by the group at Oak Ridge, Tennessee. For purposes of this discussion it is defined as the ratio of the gamma radiation dose required to kill a given number of cells in the presence of a compound to that required in its absence. Of those chemicals that protected, glutathione was studied most extensively and the results obtained with this chemical will be considered first.

Although early reports stated that the glutathione molecule is extremely resistent to alteration by gamma irradiation (Woodward, 1933), more recent work has shown that it is chemically

changed upon exposure to ionizing radiation (Barron and Flood, 1950; Dale and Davies, 1951). With the high dosages of gamma radiation delivered during the course of this work (155,000 to 1,440,000 rep) it is probable that breakdown of the glutathione into a variety of simpler chemical compounds resulted. It was shown experimentally, however, that none of these products were toxic for the bacterial spores being studied. Survival numbers were identical for spores incubated in irradiated and unirradiated glutathione solutions.

The effect of solutions of varying hydrogen ion concentration on the protective properties of glutathione was studied. The results obtained indicated that pH values near neutrality were optimum both for viability of the spores used in this study and for the protective capacity of reduced glutathione. The decrease in effectiveness of glutathione in solutions having a pH below 6.0 might have been the result of partial oxidation of the compound. It has been shown (Kailan, 1921; Loiseleus, 1942) that the concentration of hydrogen peroxide, an oxidizing agent capable of oxidizing glutathione (Pirie, 1931), increases with decreasing pH.

Glutathione is a tripeptide, γ-glutamyl-cysteinyl-glycine. From a survey of the work of Barron and his group (Barron et al., 1949; Barron and Dickman, 1949) it was postulated that the protective capacity of glutathione might be a function of its sulfhyryl group. Barron and his co-workers had not worked with living organisms. They limited themselves to studies of the effects of alpha, beta, gamma and X-rays on enzyme solutions and found the mechanism of action of all these radiations to be the same; i.e., an initial oxidation of the sulfhydryl

group of the enzyme by the products of water irradiation followed by more destructive changes which they considered to be protein denaturation. These workers hypothesized from these results that sulfhydryl groups were of paramount importance in effects of ionizing radiations on living cells since these sulfhydryl groups were the most sensitive to radiation damage. Barron's primary use of glutathione and other sulfhydryl compounds was to reactivate irradiated sulfhydryl enzymes subsequent to the exposure to these enzymes to ionizing radiations. He also tested the protective capacity of glutathione if irradiated in the same medium with certain enzymes. When the radiation emitted was either alpha or X-rays, he obtained protection of enzymes when glutathione was present in the suspending medium. Glutathione was also capable of reactivating the enzymes urease and phosphoglyceraldehyde dehydrogenase, up to a certain limiting dosage, after their exposure to alpha or X-rays. On the contrary, Barron could not protect the enzyme urease by the presence of glutathione during irradiation with gamma rays (Barron and Dickman, 1949). Similarly, glutathione was unable to reactivate either urease or phosphoglyceraldehyde dehydrogenase when added after exposure of these enzymes to either beta or gamma rays. The inability of glutathione to reactivate enzymes above a particular radiation dosage, independent of the type of radiation, was explained as the result of oxidation of the sulfhydryl bonds of the enzyme by the radiation to steps beyond disulfide formation. Barron felt that the oxidative step from sulfhydryl to disulfide was reversible. Beyond this, the oxidation was too severe to be reversed by addition of glutathione.

If an analogy is to be drawn between the conclusions of Barron and his group and the results obtained in this investigation, it must be said that: (1) the sulfhydryl groups of the anaerobic spores which are affected by gamma irradiation are unavailable to glutathione added to the medium and are, therefore, not reactivated; or that (2) oxidation of the sulfhydryl groups of the anaerobic spores by gamma radiation is carried beyond the disulfide formation step. Otherwise, post-irradiation addition of glutathione should reactivate at least a certain percentage of the spores. Such a result was not obtained. When glutathione was added to spores which were subjected to irradiation in phosphate buffer, the survival ratio was identical with that seen in the control. Reactivation was actually not expected. It was felt that even if sulfhydryl groups of the cell were available to the glutathione, oxidation of these groups by the products of gamma radiation had been carried beyond the disulfide formation step. Barron and his co-workers were working with radiation dosages of between 1 and 1,000 roentgens and got irreversible inactivation of their enzymes in the upper portion (500 to 1,000r) of this range. Much higher radiation dosages were employed in the present study so oxidation of sulfhydryl linkages, if this was a factor in the death of the cell, undoubtedly had progressed to stages beyond disulfide formation.

Contrary to the results of Barron and Dickman (1949) with enzymes, glutathione present in the suspending medium <u>during</u> irradiation resulted in a marked increase in anaerobic spore survival in the present study. In fact, glutathione was the second most effective chemical

studied in its ability to protect the anaerobic spores from the lethal effects of cobalt-60 gamma radiation. It had an isoefficiency dose ratio of 1.89; i.e., approximately 90 per cent more radiation had to be delivered in the presence of glutathione to effect the same percentage kill of organisms obtained in a control solution. Although glutathione is a sluggish reducing agent (Barron, 1951), it was nevertheless hypothesized that the protection which this compound afforded bacterial spores was due to the absorption of oxidizing free radicals developed in the medium by the ionizing radiation. At least two such oxidizing agents are believed to be developed in water during irradiation, OH and H_2O_2, as well as the reducing agent, H. In the absence of a solute; i.e., in pure aqueous solutions, these products will react with each other to again form water according to the following back reactions (Hart, 1954):

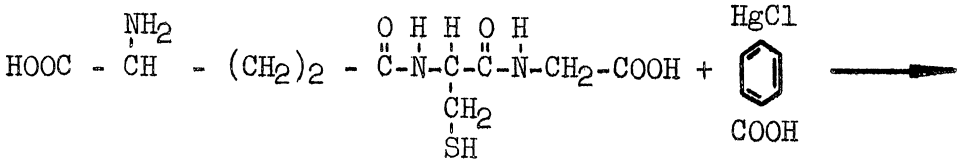
$$OH + H_2 \longrightarrow H_2O + H$$

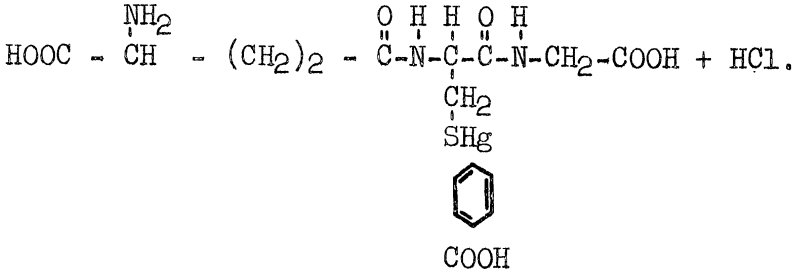
$$H + H_2O_2 \longrightarrow H_2O + OH$$

$$H + OH \longrightarrow H_2O$$

However, if other substances are present in the aqueous solution, some of the oxidizing radicals, though short-lived, may react with this material in preference to recombination. Should a bacterial cell be present in the aqueous medium, irreversible changes may be produced which result in death of the cell subsequent to irradiation. When a reducing agent such as glutathione is present in the suspending medium during irradiation, however, the opportunity for oxidation of cell components is decreased; i.e., if a glutathione molecule is in the vicinity of an oxidizing radical formed by the passage of

an ionizing radiation through the medium, the glutathione molecule may absorb the radical and undergo oxidation, thus saving the cell from such a change. It was felt that this oxidation reaction was at the site of the sulfhydryl group rather than at other possible reactive sites, such as amino groups or carboxyl groups, on the glutathione molecule. Hollaender et al., (1951) have shown that compounds possessing a sulfhydryl group have protective properties for Escherichia coli but the specific site of this protective effect was not studied by these workers. It was felt that experimental substantiation of the theory that the sulfhydryl end group was responsible for the protection of anaerobic bacterial spores seen with glutathione could be obtained by removing the reactivity of this end group. This was done with p-chloromercuribenzoic acid, a chemical compound which reacts specifically with sulfhydryl groups. It reacts with glutathione as follows (Hellerman et al., 1943):





In the present research, the fact that a reaction occurred upon mixing of the organic mercurial and glutathione was evidenced by a drop in the pH of the solution as hydrochloric acid was liberated. The addition of p-chloromercuribenzoate to glutathione before use in an

irradiation experiment led to complete loss of the protective capacity of the glutathione for spores.

Confirmation of the necessity of glutathione being in the reduced state to afford protection was obtained by the use of the oxidized form of this chemical. In oxidized glutathione the sulfhydryl linkage has been converted to a disulfide; two reduced glutathione molecules being linked together through this bond. Barron and Flood (1950) have shown that, although reduced glutathione is oxidized by ionizing radiations, oxidized glutathione is not reduced, in spite of the fact that ionizing radiations produce both oxidizing and reducing radicals. The results obtained by the use of oxidized glutathione in the present study not only indicated that the sulfhydryl group was essential for glutathione to protect the anaerobic spores but also led to the conclusion that further oxidation of the disulfide linkage had no protective effect on the organisms present at the time this occurred. Death rates were the same in oxidized glutathione as in a control solution for all strains of anaerobic spores tested.

This same negation of protection could be achieved by still another method. Oxidation of a solution of reduced glutathione was accomplished by bubbling oxygen gas through an aqueous solution of the chemical prior to irradiation. After such treatment, the glutathione was incorporated in the solution used for suspension of the spores during irradiation. The protective properties of the chemical were completely absent under these circumstances.

In addition to treatment with an oxygen atmosphere, nitrogen gas was also bubbled through glutathione solutions. This was an attempt to increase the protection obtained in air atmosphere solutions of glutathione. The use of a nitrogen atmosphere failed to enhance the protection seen with glutathione prepared in air. Since precautions were taken to maintain a uniformly low oxygen content of the suspending phosphate buffer in experiments conducted in an air atmosphere, this result was anticipated. Unless the sulfhydryl group of the reduced glutathione was partially oxidized in its solvent, no enhancement of its protective properties would be expected in a nitrogen atmosphere. That such oxidation might occur with improper handling of samples was evidenced by a drop in the protection afforded the spores by glutathione in one experiment. The fact that no enhancement of the protection was obtained in a nitrogen atmosphere over that seen in other experiments performed in an air atmosphere was evidence that the glutathione was primarily in its reduced state in solutions in equilibrium with air.

The evidence accumulated through the study of the chemical glutathione lent support to the conclusion, reached during the progress of the experimentation, that the results obtained using cysteine were not indicative of its full protective capacity. Cysteine is an amino acid which contains a sulfhydryl group. Its chemical formula is given in Appendix A. It is a relatively simple molecule whose end groups are the same as those found on glutathione. Therefore, it could be predicted that it would afford the same degree of protection to anaerobic bacterial spores as that obtained with glutathione.

Furthermore, cysteine should accomplish this result through the same mechanism seen in glutathione; i.e., oxidation of its sulfhydryl group. The fact that a greater degree of protection was not obtained using cysteine (isoefficiency dose ratio of 1.29 compared to one of 1.89 for glutathione) was apparently due to spontaneous autooxidation of the molecule from cysteine to cystine in an air atmosphere. Further evidence that cysteine possesses protective properties comparable to those of glutathione is the successful use of both chemicals by other investigators to protect animals from the biochemical lesion caused by irradiation (Patt et al., 1949; Chapman et al., 1949). In this investigation, the autooxidation of cysteine might have been lowered somewhat by special handling of the compound. Glutathione, however, is a more stable compound with which to work; it is autooxidizable only in the presence of added catalysts such as iron or copper (Harrison, 1924; Lyman & Barron, 1937). Since results were more reproducible in its presence, it was chosen for the more intensive study.

The statement has been made that sulfhydryl groups essential to life processes are present in all living cells (Barron, 1951). Having used the organic mercurial, p-chloromercuribenzoate, to successfully bind such a group on the glutathione molecule, it was felt anaerobic bacterial spores should be irradiated after treatment with p-chloromercuribenzoic acid. It was hypothesized that any protection of spores which could be obtained during irradiation after such treatment of the spores would indicate that sulfhydryl groups of the cell itself were involved in the lethal effects produced by

radiation. Although mercury compounds are themselves known to be toxic for microorganisms, it was considered probable that the mercaptide formation induced by suspending spores in a solution containing p-chloromercuribenzoate could be reversed subsequent to irradiation of the spores. The reversibility of the reaction between p-chloromercuribenzoate and sulfhydryl groups upon addition to a compound having free sulfhydryl groups has been demonstrated by other investigators (Hellerman et al., 1943; Sibly & Wood, 1951). There are reports in the literature that sodium thioglycollate is the most efficient of the compounds capable of reversing this reaction (Mirsky & Anson, 1935). For this reason, sodium thioglycollate was chosen for post-irradiation addition to the spores which had been prepared with p-chloromercuribenzoate.

The results of the irradiation of spores pre-treated with this mercury compound indicated that it provided some protection (isoefficiency dose ratio of 1.21). This relatively low value may be a reflection of the limited role of sulfhydryl groups of the cell in the lethal action of ionizing radiations but it is more likely that it is a reflection of the limited accessibility of such groups to the mercurial compound.

The degree of protection afforded by 0.02M p-chloromercuribenzoate could be increased by raising the molar concentration of the compound. Such experiments were performed in an attempt to reach more sulfhydryl groups on the cell by higher concentrations of the chemical. This approach to the problem had practical limitations

and it was not possible to determine the upper limit of this concentration effect. Because of the limited solubility of p-chloromercuribenzoate, even in such strong alkali as 1M sodium hydroxide, it was not feasible to test concentrations of the chemical higher than 0.1M. To do so would have necessitated the addition of such large volumes of concentrated sodium hydroxide to the test suspension that death of the spores would have resulted. Also, the mercurial was in itself somewhat toxic for the spores and very high concentrations would have reduced the initial numbers of viable cells to a level which would not have permitted prolonged irradiation.

A further increase in spore survival numbers was sought over that obtained by incubation of the spores with p-chloromercuribenzoate followed by washing of the spores prior to their irradiation. Spores were irradiated in the presence of the p-chloromercuribenzoic acid but this failed to enhance the protection of the spores previously obtained. This was interpreted to mean that p-chloromercuribenzoate must be in chemical combination with the spore to afford protection. Such a combination was assumed to occur during initial contact between the compound and the spores based upon the change in pH of the solution which was noted when the two were mixed. Apparently the presence of any residual p-chloromercuribenzoate was valueless in decreasing the lethal effects of the cobalt-60 gamma irradiation. Continued presence of the organic mercurial during irradiation offered no advantage over pre-irradiation incubation of the spores with the compound followed by successive washings prior to irradiation.

Other investigators (Hollaender et al., 1951) have stated that sulfhydryl-containing compounds and sodium hydrosulfite are representatives of two separate classes of chemicals capable of protecting Escherichia coli from X-radiation. It was, therefore, considered advisable to study the effect that sodium hydrosulfite would have on anaerobic spores. The initial experiments performed with sodium hydrosulfite in the irradiation medium showed it to be the most protective of the compounds tested. The isoefficiency dose ratio (2.4) was over 20 per cent greater than that of any other compound studied. This high degree of protection was confirmed with all three strains of the spores used in this work. Protection occurred, however, only if the sodium hydrosulfite was present during the actual irradiation of the cells.

Those radicals which were responsible for the fraction of the lethal effects upon the cell which could be reversed by sodium hydrosulfite were apparently not present in the medium subsequent to its irradiation. If such were the case, it seems probable that sodium hydrosulfite would have had some protective action upon addition to irradiated cells. However, such results were not obtained. Sodium hydrosulfite, if added to irradiated cells, was completely ineffectual in reducing the lethal effects produced by the radiation.

Similarly, it was shown that none of the lethal effects seen after irradiation of spores in a solution containing sodium hydrosulfite were due to the toxic decomposition products of the chemical itself. An experiment in which a sodium hydrosulfite solution was subjected to prolonged irradiation showed that no toxicity of the chemical for spores developed as a result of this treatment.

An extensive study of the protective effect of sodium hydrosulfite for bacteria during exposure to ionizing radiation has been done by Burnett et al., (1951, 1952). They obtained approximately the same degree of protection by bubbling nitrogen through a suspension of Escherichia coli prior to irradiation as by addition of sodium hydrosulfite to the irradiation medium (isoefficiency dose ratio of nitrogen = 3.2; isoefficiency dose ratio of sodium hydrosulfite = 3.7). It is known that sodium hydrosulfite absorbs dissolved oxygen from water (Martin, 1946). Knowing this, and having obtained such similar isoefficiency dose ratios for a nitrogen atmosphere and a sodium hydrosulfite solution, Burnett interpreted the results of his preliminary experiments as indicating that the protective action of sodium hydrosulfite was entirely due to its ability to remove dissolved oxygen from the solution. He explained the discrepancies in isoefficiency dose ratios of a nitrogen atmosphere and a sodium hydrosulfite solution as the result of their different efficiencies in absorbing this dissolved oxygen. To confirm this hypothesis, he did a study in which various molar concentrations of sodium hydrosulfite were employed.

Sodium hydrosulfite reacts with oxygen in a moleculr ratio of 2:1 according to the following equation (Martin, 1946):

$$2\ Na_2S_2O_4 + O_2 \longrightarrow 2\ Na_2S_2O_5\ .$$

Therefore, 0.0005M is the theoretical quantity of sodium hydrosulfite required to remove 0.00025M of oxygen from a phosphate buffer medium. This is the amount of oxygen dissolved in phosphate buffer saturated with air at 25 C and 740 mm pressure (Burnett et al., 1952). In

Burnett's study, the dissolved oxygen content of phosphate buffer solutions containing different molar concentrations of sodium hydrosulfite was assayed chemically. A direct correlation was established between the protective capacity of sodium hydrosulfite solutions of varying molar concentration for cells of Escherichia coli during irradiation and the ability of these solutions to remove oxygen from the medium. Molar concentrations below 0.0001M removed almost no oxygen from the medium and, concomitantly, gave no protection. The protective capacity of the chemical was a linear function of concentration between values of 0.0001M and 0.0004M. This upper concentration of sodium hydrosulfite was that amount which reduced the oxygen content of the medium almost to zero (0.5 mg per l). In all sodium hydrosulfite solutions higher than 0.0004M in concentration, no oxygen could be detected in the medium; at the same time, no further increase in protection of Escherichia coli was obtained although concentrations as high as 0.4M were tested. Burnett attributed the fact that 0.0004M was the upper limiting concentration of sodium hydrosulfite in his experiments, instead of the 0.0005M expected stochiometrically, to the entrance of a second compound, the newly formed metabisulfite ($Na_2S_2O_5$), into the reaction between sodium hydrosulfite and oxygen.

Based upon this background, experiments were planned for the present study to determine if removal of oxygen from the medium was the sole mechanism by which sodium hydrosulfite protected anaerobic bacterial spores. In the experiment in which various molar concentrations of sodium hydrosulfite were tested, protection was obtained with

sodium hydrosulfite solutions having concentrations higher than 0.0005M. Increases in the number of spores surviving the irradiation treatment were noted until a molar concentration of 0.01M sodium hydrosulfite was reached. Above this concentration, no further increase in protection could be obtained although concentrations as high as 1M were studied. These results were not in agreement with Burnett's work. The question might logically be asked: If 0.0005M sodium hydrosulfite removes all the oxygen from a phosphate buffer solution saturated with air at room temperature, why did the degree of protection obtained increase with concentrations of the chemical up through 0.01M in the present study? A logical answer might be found in a residual effect due to some other chemical reaction of sodium hydrosulfite.

As a second approach to the problem, sodium hydrosulfite was subjected to three different gaseous atmospheres. If the compound was protecting solely by absorption of the dissolved oxygen in the medium, it was postulated that its protective capacity could be negated by bubbling oxygen through the solution. This treatment would oxidize the sodium hydrosulfite before its use in a radiation experiment. At the same time that some sodium hydrosulfite solutions were subjected to oxygen gassing, sodium hydrosulfite solutions in buffer equilibrated with air and in buffer in which the air was replaced by nitrogen were also prepared. The air atmosphere was used as a control to determine the ratio of survivors under these conditions compared to survivors in a sodium hydrosulfite solution in which the air was replaced by oxygen. A nitrogen atmosphere was used to study

potential increases in the survival numbers over air equilibrium solutions. The results obtained indicated that although exposure to gaseous oxygen reduced the protective capacity of sodium hydrosulfite solutions, the lethal effect of the radiation was still not as great in oxygen treated sodium hydrosulfite solutions as in phosphate buffer solutions. Thus, as illustrated in Figure 17, after 1,085,000 rep, 7.8×10^5 organisms per ml survived in a sodium hydrosulfite solution with an air atmosphere. Compared to this, 1.27×10^5 organisms per ml from the same original population survived in a sodium hydrosulfite solution in which the air was replaced by oxygen. In contrast, only 9 organisms per ml were viable after similar treatment in a phosphate buffer solution equilibrated to air. These data were in accord with the earlier results on the effect of molar concentrations of sodium hydrosulfite on anaerobic bacterial spores; they suggested that oxygen removal was not the only mechanism by which sodium hydrosulfite protected these organisms.

In another experiment, the oxygen was removed from a phosphate buffer solution by bubbling nitrogen through it in an attempt to increase protection in phosphate buffer to levels similar to those obtained in sodium hydrosulfite solutions. It was not possible to do so. Sodium hydrosulfite in an air atmosphere had an isoefficiency dose ratio seven times that obtainable with phosphate buffer solutions,

even when the latter were prepared in a nitrogen atmosphere. Even if sodium hydrosulfite was oxidized by exposing a solution of the chemical to oxygen gas, the protective capacity remained above that obtained with a deaerated phosphate buffer solution. This again was not in agreement with the work of Burnett and his co-workers who had obtained almost the same protection when using deaerated phosphate buffer solutions as when using sodium hydrosulfite solutions of optimum molar concentration.

In this present investigation, all the evidence obtained pointed to more than removal of oxygen from the medium as the mechanism by which sodium hydrosulfite afforded protection to the anaerobic bacterial spores during gamma irradiation. Removal of oxygen played some role in its protective capacity as evidenced by the drop in protection seen when sodium hydrosulfite was exposed to oxygen prior to irradiation, thus converting it to metabisulfite. The remainder of its protective capacity appeared to be the result of other reactions.

The exact nature of the residual effect was not determined in this study. Therefore, to say with certainty what reactions of the sodium hydrosulfite contribute to its protective capacity aside from absorption of oxygen is impossible in the light of the experimental evidence. But a study of the results obtained with the food preservative, sodium nitrite, and the amino acid, methionine, gives evidence upon which to base a tentative hypothesis.

Sodium nitrite is an extremely simple molecule that can be oxidized to sodium nitrate by the reaction:

$$2\ NaNO_2 + O_2 \rightleftharpoons 2\ NaNO_3 \ .$$

It was felt that the protective property of this chemical for bacterial spores was due solely to its ability to absorb oxygen from the medium by the above reaction. The end group -ONa was considered to be ineffectual in affording protection since a similar group is possessed by sodium ascorbate, a chemical which gave no protection to spores irradiated in its presence. The fact that an isoefficiency dose ratio (1.22) was obtained for sodium nitrite which was almost identical to that of a phosphate buffer solution in which the air atmosphere was replaced by nitrogen (1.20) lent support to the hypothesis that oxygen absorption was the sole protective mechanism of the sodium nitrite.

Turning to the work on methionine, its protective properties seemed to derive from the presence of its sulfur atom. All the end groups of the methionine molecule are found in other chemical compounds studied where no protection was obtained. The methyl group (CH_3), for example, is present on propyl gallate which had no protective property when tested with the anaerobic bacterial spores in this study. Similarly, the $-CH_2-CH_2-\underset{NH_2}{CH}-COOH$ portion of the methionine molecule is duplicated in the glutathione molecule. Yet it was illustrated with the latter compound that this portion of the molecule played no role in protection of the bacterial spore from radiation damage. Also, it is only in the possession of a sulfur atom that methionine differs markedly from lysine, yet the latter was the least protective of any of the compounds which could be said to increase survival (isoefficiency dose ratio of 1.10) while methionine was one of the most efficient chemical protectors studied (isoefficiency dose ratio of 1.43). If conclusions are to be drawn from these observations, it seems reasonable to assume that the

protective capacity of methionine is a function of its sulfur atom even though this sulfur is an integral part of the molecule rather than a free end group.

Using the observations noted in studying methionine and sodium nitrite to develop a theory of the means by which sodium hydrosulfite protects the anaerobic bacterial spore, one might consider the protection as occurring in two ways. Sodium hydrosulfite owes its protection, in part, to removal of oxygen from the medium. The remainder of its protective capacity is due to some unknown reaction. The possibility presents itself that this reaction may involve the two sulfur atoms of its molecule. Assume that the ionizing radiations cleave the sodium hydrosulfite molecule at the sulfur bond; i.e.,

$$Na_2S_2O_4 \xrightarrow[\text{radiation}]{\text{gamma}} 2\ NaSO_2.$$

If this were the case, then the oxidizing free radicals produced by ionizing radiations might be absorbed according to any of the following reactions:

$$NaSO_2 + O_2H \longrightarrow NaHSO_4$$

$$NaSO_2 + OH \longrightarrow NaHSO_3$$

$$2\ NaSO_2 + H_2O_2 \longrightarrow 2\ NaHSO_3.$$

This, however, is merely presented as a tentative hypothesis and much experimental data would be necessary to confirm it.

The experimentation with catalase (Williams and Kempe, 1957) was designed to determine the relative importance of the oxidizing radical, H_2O_2, in the total lethal effect produced on the bacterial spore by the cobalt-60 gamma radiation. The formation of hydrogen peroxide as a product of irradiation of water with X-rays has been

demonstrated by Frilley (1947). The fact that the protective action of catalase in an irradiated solution is due to destruction of this hydrogen peroxide has been shown by Barron and Dickman (1949) with the enzyme, phosphoglyceraldehyde dehydrogenase. Catalase, present in the suspending solution during irradiation of phosphoglyceraldehyde dehydrogenase with alpha rays, reduced the inactivation of the enzyme preparation by 45-55 per cent while addition of another protein, egg albumin, to a similar enzyme solution during irradiation did not reduce inactivation of the enzyme. From this, Barron and Dickman concluded that the action of catalase was not a function of its protein nature but rather was due to its reaction with its substrate, hydrogen peroxide. In the present investigation, catalase, present in the suspending medium during irradiation, gave a high degree of protection (isoefficiency dose ratio of 1.53), third in effectiveness only to glutathione and sodium hydrosulfite. Such protection illustrated the significant role played by the oxidizing agent, hydrogen peroxide, in the death of the anaerobic spore due to ionizing radiations from cobalt-60 gamma radiation.

In studying the effect of chemicals on spores during exposure to cobalt-60 gamma irradiation, 7 chemicals were tested which either gave no protection or were actually slightly deleterious in their effect. These compounds are listed in Appendix C with notations as to the reason for choosing them for study and their resultant effects. Since this research developed as a study of chemicals which gave protection to anaerobic bacterial spores and the reason for this

effect, compounds which were found to give no increase in spore survival numbers were not tested further.

While an evaluation of the relative merits of the two major theories of action of ionizing radiations on living cells was not the objective of this investigation, some remarks on this subject may be in order. The fact that a protective effect was observed with chemicals would be difficult to explain if one attributed death of all the bacteria to direct dissipation of the energy from the ionizing radiation within a molecule of these cells. In the light of the evidence accumulated, it seems more likely that the strong oxidizing agents, which are produced in media by the passage of ionizing radiations through the solutions, react with at least a portion of the spores to bring about one or more unknown intermediate steps responsible for death. Chemicals present in the medium during irradiation then protect by reacting with these oxidizing agents. It is possible that a part of the lethal effect which was observed even in the presence of chemicals is due to direct damage produced by the gamma rays on essential cell components. Whether these essential components are genetic in nature is not definitely known at present. There is experimental evidence reported in the literature which favors a genetic alteration of cells (Lea, 1955). There is also evidence of interference with non-genetic components, e.g., the enzyme systems, by ionizing radiations (Billen and Lichstein, 1952; DuBois et al., 1951). Ionizing radiations produce oxidizing radicals in the aqueous medium surrounding the cell; it is also possible that similar radicals are produced in aqueous phases within the cell. If so, these oxidizing radicals could then produce lethal effects within the cell. Since there is no evidence

either for or against permeability of the anaerobic spores for the chemicals investigated, some of the lethal effects noted in this study may have been the result of such reactions.

If investigation of these problems were to be pursued, organisms other than anaerobic spores would be a wiser choice as the test vehicle. These spores are not ideal for investigations into the mechanisms of action of ionizing radiation since at present their growth phase cannot be so uniformly controlled as is possible with vegetative cells and the pathways of their metabolism have not, as yet, been clearly enough elucidated to permit advanced studies. It does, however, present a field for future investigations.

SUMMARY

This investigation was designed to determine the effect of the chemical nature of the environment on survival of anaerobic bacterial spores during irradiation with gamma rays from cobalt-60. C. botulinum 62A, C. botulinum 213B and Clostridium sp., P.A. 3679 were the test organisms used throughout the study. The technique employed for determining the number of viable spores present in solutions both before and after irradiation was the conventional 10-fold serial dilution method followed by plate counts.

Experimental results obtained early in the study indicated that changing the irradiation medium from a liquid to a gel state had no effect on the lethality of gamma radiations for the organisms tested. Similarly, varying the hydrogen ion concentration of the solutions in which the spores were irradiated did not affect their radiation sensitivity although buffer solutions having pH values ranging from 3.4 to 8.5 were used.

Reducing agents were included among the chemicals studied for two reasons: (1) reducing agents are widely used throughout the food industry as preservatives, and (2) other investigators have stated that the oxidizing free radicals are more active than the reducing radicals produced by the passage of ionizing radiations through solutions. A total of 21 chemicals were tested; nine of these had a protective effect (See Appendix B). All nine of the protective compounds can be classified as reducing agents. The degree of protection afforded spores by these compounds appeared to be a function of their relative efficiencies in removing oxidizing free radicals from the medium.

The two most protective compounds found, in order of their effectiveness, were the reducing agents sodium hydrosulfite and glutathione. Experiments were conducted in which both chemicals were present in the suspending medium during irradiation of spores. The results obtained indicated that this procedure did not enhance the protective effect noted in the presence of sodium hydrosulfite alone. It was also experimentally shown that in order to afford protection, sodium hydrosulfite or glutathione must be present in the medium at the actual time of irradiation of spores; addition of either of these compounds to phosphate buffer suspensions of irradiated spores subsequent to irradiation gave no increase in survival numbers over that obtained in a control solution. The lethal effects of gamma radiation for bacterial spores observed even when glutathione or sodium hydrosulfite were present in the suspending medium were due solely to the ionizing radiations. Any breakdown products which may have formed as a result of the irradiation of the chemicals were not toxic for the bacterial spores as shown by comparable survival of organisms in both irradiated and unirradiated solutions of the chemicals.

Reasons for the observed protective effects were sought. Although other investigators have used sulfhydryl-containing compounds to protect many different types of organisms from radiation effects, the specific site responsible for the protective capacity of these compounds has not been critically studied. Experiments were conducted in this present study to determine the importance of the sulfhydryl group in the protection afforded anaerobic bacterial spores by glutathione. It was found that: (1) the sulfhydryl group must be present in a reduced state to afford protection, and (2) this end group of the glutathione molecule

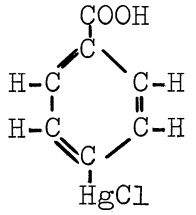
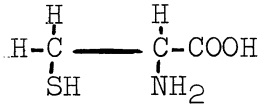
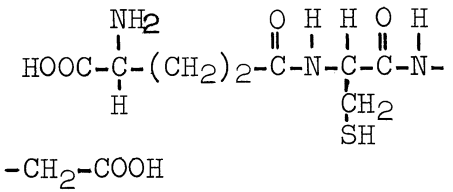
alone is responsible for the protective capacity of the chemical. The results of three types of experiments were presented to substantiate these conclusions: (1) the use of oxidized glutathione as a protective agent; (2) the addition of a mercaptide-forming agent, p-chloromercuribenzoate, to glutathione prior to irradiation; and (3) bubbling oxygen gas through a solution of reduced glutathione before use of the solution in an irradiation experiment. All three treatments are capable of changing the sulfhydryl group of glutathione to an oxidized state and all three treatments completely removed the protective effect for bacterial spores seen in solutions of reduced glutathione. If p-chloromercuribenzoate was added directly to bacterial spores, the survival of these organisms during irradiation was actually increased. Since p-chloromercuribenzoate is a specific sulfhydryl group reagent, these results were interpreted as presumptive evidence that sulfhydryl groups are present in or on the bacterial spore and that these groups play some role in the lethal effects of radiation on spores.

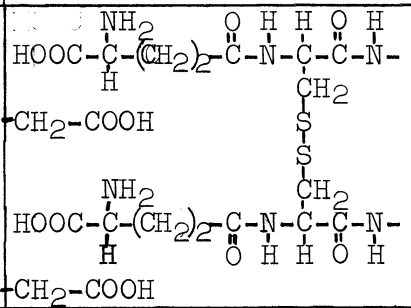
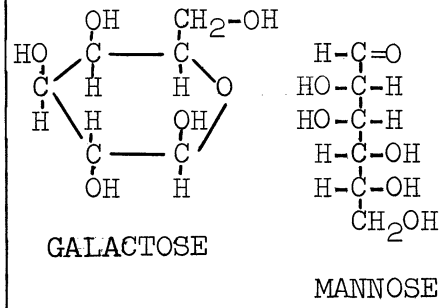
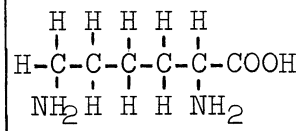
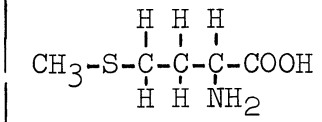
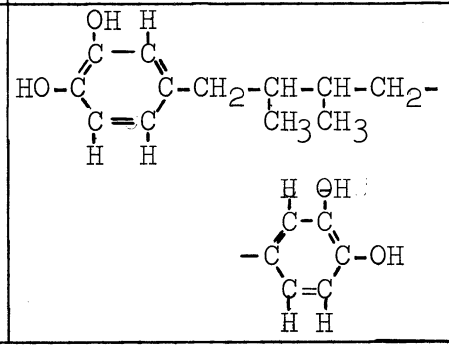
The exact mechanisms responsible for the protection afforded anaerobic bacterial spores by sodium hydrosulfite were not established. The experiments that were conducted seemed to indicate, however, that this protection was not due solely to removal of oxygen from the suspending solution by sodium hydrosulfite. Theoretically, 0.0005M sodium hydrosulfite is the maximum concentration necessary to remove all oxygen from a phosphate buffer solution saturated with air. Therefore, if oxygen removal were the sole protective mechanism of sodium hydrosulfite, no increase in the protection afforded bacterial spores should be obtained by increasing the molar concentration of the chemical in solution above

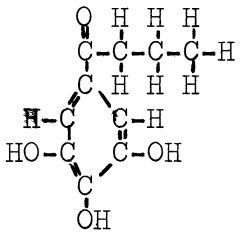
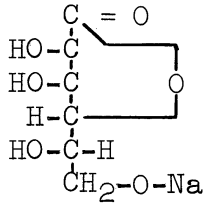
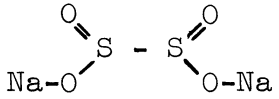
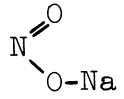
0.0005M. In the experiments in which sodium hydrosulfite solutions of varying molar concentration were studied, however, increasing protection of spores with increasing molar concentration was noted until a molar concentration of 0.01M was reached. A theoretical mechanism was postulated to explain the residual effect that was observed.

As far as is known, the experiments with catalase represent the first use of this chemical as a protective agent for living cells during their exposure to ionizing radiations. Catalase was found to be a very efficient protective agent when used in this capacity. These results would seem to indicate the significant role of the oxidizing agent, H_2O_2, in the lethal effects produced by cobalt-60 gamma radiation on the anaerobic spores tested.

GENERAL INFORMATION ON THE CHEMICAL COMPOUNDS USED THROUGHOUT THIS STUDY

Common Name of the Compound	Chemical Name	Source of Supply	Structural Formula
Catalase	A heme-protein	General Biochemicals, Inc. Chagrin Falls, Ohio	Exact structure unknown
p-chloromercuribenzoic acid	p-chloromercuribenzoic acid	Delta Chemical Company St. Louis, Missouri	
Cysteine	α-amino-β-thiopropionic acid	Eastman Organic Chemicals Rochester, New York	
Gelatin	Gelatin	Difco Laboratories, Inc. Detroit, Michigan	Exact structure unknown Contains the following: Nitrogen 18% Sulfur 0.5% Amino Acids 81.5%
Glutathione	γ-glutamyl-cysteinyl-glycine	General Biochemicals, Inc. Chagrin Falls, Ohio	

Common Name of the Compound	Chemical Name	Source of Supply	Structural Formula
Oxidized Glutathione	γ-glutamyl-cysteinyl-glycine	General Biochemicals, Inc. Chagrin Falls, Ohio	
Guar Gum	35% β-D-galactose 63% D-mannose	Stein Hall New York 17, New York	 GALACTOSE MANNOSE
Lysine	α-ε-diamino-n-caproic acid	Nutritional Biochemicals Corporation Cleveland, Ohio	
Methionine	α-amino-γ-methyl-thio-n-butyric acid	Nutritional Biochemicals Corporation Cleveland, Ohio	
Nordihydroguaiaretic acid	4,4'-(2,3-dimethyl-tetramethylene)-dipyrocatechol	William Stange, Co. Chicago, Illinois	

Common Name of the Compound	Chemical Name	Source of Supply	Structural Formula
Propyl gallate	3,4,5-trihydroxy-benzoate	Heyden Chemical Corporation New York, New York	
Sodium Ascorbate	2,3-enediol-L-gulono-1,4-lactone	Merck & Co. Rahway, New Jersey	
Sodium Hydrosulfite	Sodium Hydrosulfite	Eastman Kodak Corporation Rochester, New York	
Sodium Nitrite	Sodium Nitrite	Mallinckrodt Chemical Works St. Louis, Missouri	

APPENDIX B

ISOEFFICIENCY DOSE RATIOS* OF THE CHEMICAL COMPOUNDS THAT PROTECTED ANAEROBIC BACTERIAL SPORES FROM COBALT-60 GAMMA RADIATION

CHEMICAL COMPOUND	Isoefficiency Dose Ratio
Sodium hydrosulfite	2.40
Glutathione	1.89
Catalase	1.53
Methionine	1.43
Gelatin	1.30
Cysteine	1.29
Sodium nitrite	1.22
p-Chloromercuribenzoate	1.21
Nitrogen (PO_4 buffer)	1.20
Lysine	1.10

* Isoefficiency dose ratio is the ratio of the gamma radiation dose required to kill a given number of cells in the presence of the compound to that required in its absence.

A LIST OF THE CHEMICALS STUDIED THAT GAVE NEGLIGIBLE PROTECTION TO ANAEROBIC BACTERIAL SPORES DURING EXPOSURE TO COBALT-60 GAMMA IRRADIATION

Name of Chemical	REP Delivered	Reason for Testing Compound	Degree of Protection Obtained
Vitamin A	600,000	Used in foods to protect the fatty acids during irradiation	No protection
Vitamin K	600,000	Used in foods to protect the fatty acids during irradiation	No protection
Biotin	600,000	To compare the effect of water soluble vitamins with that of fat soluble vitamins	Two-fold increase in survivors
Riboflavin	600,000	To compare the effect of water soluble vitamins with that of fat soluble vitamins	Four-fold increase in survivors
Nicotinic acid	600,000	To compare the effect of water soluble vitamins with that of fat soluble vitamins	Two-fold increase in survivors
Plumbous chloride	600,000	A reducing agent	Deleterious effect
Lecithin	600,000	Used in foods to protect the fatty acids during irradiation	Three-fold increase in survivors

APPENDIX D

A STATISTICAL DETERMINATION OF THE COUNTING ERROR INHERENT IN VIABLE PLATE COUNTS AS A FUNCTION OF NUMBER OF COLONIES PRESENT

Strain of *C. botulinum* Sampled	Number of Tubes Counted (N)	Mean Number of Colonies Per Tube ($\overline{Y}$)	Variance (θ^2)	Standard Deviation (θ)	Counting Error (%)
62A	26	53	67.8	8.2	15%
213B	27	105	120.3	10.9	10%
213B	15	192	372.1	19.3	10%

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