THE MECHANISM OF *BACILLUS MEGATERIUM* M1 PHAGE PROTECTION PROVIDED BY SOME GAS ATMOSPHERES DURING GAMMA IRRADIATION

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN RADIATION BIOLOGY IN THE GRADUATE SCHOOL OF THE TEXAS WOMAN'S UNIVERSITY

COLLEGE OF

ARTS AND SCIENCES

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DENTON, TEXAS

MAY, 1971

Texas Woman's University

Denton, Texas

May, 19 71

We hereby recommend that the dissertation prepared under our supervision by Rosemary Callan Mainland entitled "The Mechanism of Bacillus megaterium M1 Phage Protection Provided by Some Gas Atmospheres During Gamma Irradiation"

be accepted as fulfilling this part of the requirements for the Degree of Doctor of Philosophy.

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ACKNOWLEDGMENTS

The author wishes to acknowledge her appreciation to Dr. Robert Fuerst, under whose direction this research was performed. She also wishes to thank Dr. Kenneth Fry, Dr. Alan Cockerline, Dr. Mohamed Aboul-Ela, and Dr. Ray Burkett for their helpful suggestions and constructive criticisms in regard to this manuscript.

Appreciation is also due to Mr. Larry Wiggin for his assistance in drafting the graphs used in this dissertation.

This research was supported in part by a National Defense Education Act Fellowship, The Robert A. Welch Foundation Research Grant, M-190, and by institutional grants awarded by the Texas Woman's University.

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INTRODUCTION

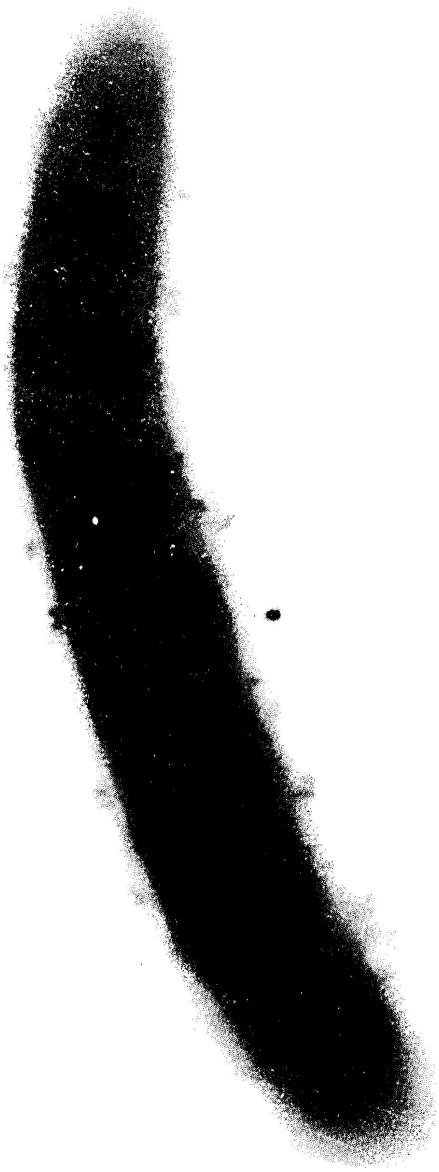
The purpose of this research was to help elucidate the mechanisms of protection against gamma irradiation effects in M1 phage of Bacillus megaterium by some low molecular weight gaseous oxidizing agents. M1 phage of Bacillus megaterium was selected for this study because its bacterial host is easily grown and maintained on regular bacterial media with no additional growth factors required. Large plaques are formed on the bacterial lawn by the agar layering technique, and these clear areas are easily enumerated. This phage has a stability range from pH 5 to 10 which practically eliminates pH as a factor to consider in evaluating the number of plaque-forming units. The burst size of M1 phage is usually about 105 particles and the latent period is 93 min. The size of a single M1 phage particle is 620 A by 2,900 (Friedman and Cowles, 1953).

Many studies have been made on the morphology and chemical composition of the cell wall of Gram-positive organisms. This knowledge makes it easier to study the mechanisms of phage attachment to the bacterial cell wall.

Some of the simple, low molecular weight gases were selected to test their effect on the ability of the phage to be adsorbed to the bacteria, enter the bacterial cell and reproduce therein.

Radical formation of these gases has been analyzed in a pure chemical system and in conjunction with gamma radiation exposure (Lind, 1961). This information might aid in the explanation of some of the biochemical interactions between a simple chemical system and a simple biological organism. A complete study of this nature has not been made on Ml phage.

Figure 1--An electron micrograph of phage sensitive strain of Bacillus megaterium S with T2 phage of Escherichia coli adsorbed. X 10,000.



REVIEW OF LITERATURE

Phage morphology and the infective process.

Bacteriophages were discovered independently by Twort in England and d'Herelle in France. The lysis of specific sensitive bacteria is the process by which the lytic agent, now called phage, which has grown within the bacterium is liberated from the cell and dispersed in the solution (Adams, 1959). Much of the research with bacterial-phage systems particularly with Escherichia coli has been cited in reviews by Stent (1963) and Hayes (1968).

Gratia (1936) developed an agar layer method by which each phage particle which entered a sensitive bacterium could be enumerated. When the phage multiplied in the bacterial cell the bacterium was lysed and a clear area was formed in the bacterial lawn of the petri plate. These clear areas, plaques, can be counted and each represents an original phage particle. This method provides an accurate indicator system by which to access quantitative experimental results on phage. The mature phage consists of a DNA core surrounded by a protein capsid. When examined under the electron microscope, tail fibers are seen wrapped around the base of the phage tail (Kozloff, Lute and Henderson, 1957).

When the phage particles interact with cell wall material the fibers unwind. There is evidence that zinc complexes in the cell wall may be the triggering mechanism for this unwinding (Kozloff and Lute, 1957; Kozloff et al., 1969).

A pH dependent process has been reported by Cummings and Kozloff (1960) in their studies with T2 phage of E. coli. These investigators determined by differential centrifugation and subsequent electron micrographs of T2 phage that at pH values below 5.8 the phage particles had only short heads, while at pH values greater than 5.8 the phage heads were much longer. Cummings (1963) believed that this transformation of the phage head was a result of changes in the configuration in the protein part of the head and not in the DNA since phages with either long or short heads were both capable of infecting sensitive bacteria. The contraction of the phage head precedes the injection of DNA and is an essential condition for phage infection of the bacterial cell. It was also determined by Cummings (1963) that the monomeric form of the head protein is a particle having a molecular weight of 42,000. Change from the monomer to the dimer form of the molecule could be made by adding Zn^{++}. It was thought that these ions were responsible for this polymerization.

After the fibers unwind an enzyme similar to lysozyme is exposed and this enzyme is believed to catalize the reaction permitting entry of the phage DNA through the cell wall.

The capsid remains attached to the bacterial cell wall. In the T-even phages and in lambda phage, Fisher (1959) was able to liberate this enzyme by freezing and thawing the phage. The site of action of the enzyme is the deeper lipopolysaccharide layer of the cell wall releasing large amounts of alanine, glutamic acid, and diaminopimelic acid which constitute the chemical links holding together the network of large molecules to which E. coli T3, T4, and T7 phages attach (Barrington and Kozloff, 1954).

Lwoff and Gutman (1950) analyzed phage induction in a lysogenic strain of Bacillus megaterium. The exposure of the lysogenic cultures to ultraviolet irradiation was followed after a latent period by complete liberation of 70 to 150 phage particles per bacterium (Lwoff, Siminovitch, and Kjelgaard, 1950).

Most phages contain double-stranded DNA which in many instances forms a circular chromosome (Hayes, 1968). Two of the known reactions of radiation on genetic material are the formation of pyrimidine dimers induced by UV light, and the structural change brought about as a result of the decay of incorporated radiophosphorus. This latter effect may be the result of atomic disintegration in which sulfur is substituted for a phosphorus atom (Stent and Fuerst, 1955).

Radical formation in organic chemical systems.

In organic systems the characteristic feature of all free radical processes is that the reactive species has an

odd electron. Radical-forming sources most commonly are peroxides, azonitriles, and light. Initiation or acceleration of a reaction by a radical source is characteristic of a free radical process (Sosnovsky, 1964). Radical formation caused by bond dissociation depends mainly on temperature. Some molecules with covalent bonds dissociate at room temperature, for example, peroxides, organic azo compounds, and some organometallic compounds, and disulfides.

As a rule radical reactions are not sensitive to polar solvents and acids and bases generally have no catalytic effect. Many peroxides can be used to initiate radical reactions in the laboratory. Those most frequently used are dialkyl peroxides, diacyl peroxides, hydrogen peroxides, and peresters. Hydrogen peroxide produces radicals at 50 to 115 C. The diacyl peroxides most frequently used are acetyl peroxide and benzoyl peroxide.

Sosnovsky (1964) said that when light is absorbed many molecules produce radicals at temperatures below those at which thermal decomposition occurs. The energy of a quantum of light is sufficient for the disassociation of most covalent bonds. Therefore the absorption of a light quantum of appropriate wavelength by a molecule with a covalent bond results in cleavage of the bond to form two radicals. Free radical reactions have been studied with sulfur compounds. Under free radical conditions the reaction of hydrogen sulfide with oelfins initially yields mercaptans (Vaughn and Rust, 1942).

Irradiation of bacterial medium before inoculation has resulted in decreased survival of the organisms. This may be partially the result of a pH change in the medium, but organic peroxides and hydrogen peroxides have also been detected in the medium (Wagner et al., 1950).

Measurement of radiation reaction in phage indicator systems.

When radiation effects are measured in terms of biological inactivation the yield is determined by plotting the logarithm of the surviving fraction of phage against radiation dose. In the dry state or in suspensions in which the phage is protected from indirect effects either by high concentration or by added protective materials so that the damage is mostly from the direct effect, it was found that the survival curves for phage S13 were exponential (Alper, 1955). Lea and Salmon (1964) determined that inactivation of a single phage particle may occur as a result of ionization taking place anywhere within the particle. In aqueous suspensions at high dilution, inactivation occurs mainly by an indirect effect.

Alper (1954) studied the inactivation of phage by hydrogen peroxide. In general the effect of radiation produced peroxide was greater than that of an equal concentration of commercial hydrogen peroxide, possibly because of the addition of different stabilizers in the commercial product. These stabilizers are not easily removed and they

alter the biological effects of hydrogen peroxide considerably. From evidence Alper (1954) has obtained there is indication that hydrogen peroxide attacks phage by the reducing action of the free radicals into which it decomposes.

Shapes of survival curves from indirect effects depend on the conditions of the experiment. If oxygen is present during irradiation the shape of the survival curve will be dependent on the dose rate. During long-term irradiation the hydrogen peroxide formed will inactivate part-inactivated phage, thus increasing the overall effect. With X-rays at 130 rad/min the rate of inactivation of S13 phage increased with increasing radiation dose (Alper, 1952). Watson (1952) also concurred with these findings. Watson used T2 phage irradiated with X-rays at 5.6 rad/min. The survival curves were not exponential. But when catalase was present during irradiation an exponential curve was obtained. At 3,400 rad/min all survival curves obtained were exponential.

Since the presence of oxygen is necessary for the formation of hydrogen peroxide, its purpose may be that of preventing the loss of radicals. Overall radiation effects which are measured by allowing a time lapse after irradiation, may be enhanced by the presence of oxygen, even when changes other than oxidations occur in the test system.

Effect of gases on biological systems.

Powers (1963) showed a relationship between gaseous atmosphere and survival of spores of Bacillus megaterium

exposed to gamma radiation. He found that spores in an atmosphere of 20% oxygen and 80% nitrogen are more sensitive to gamma radiation than spores irradiated in an atmosphere of 2% nitric acid. If spores were irradiated in a nitrogen atmosphere and nitric oxide was added to the atmosphere prior to the oxygen it was found that the spores also had a low sensitivity to radiation. Powers (1963) therefore thought that radiation induced the formation of something which was removed by the addition of nitric oxide after irradiation and which became permanently fixed in a toxic state if oxygen was introduced into the system.

In free radical reactions it has been found that oxygen can play a dual role. Oxygen is a diradical and as such it can react in many free radical processes. A small amount of oxygen may initiate a free radical reaction while a large amount of oxygen may retard or completely inhibit the reaction and induce oxidation processes instead (Sosnovsky, 1964).

Energy absorbed in or very near the nucleic acids of phage is more effective than energy adsorbed in the cytoplasm. In several different phages, the relationship between phage survival surves in atmospheres of different gas and gas mixtures have indicated that in many systems the phage particles are inactivated by reducing radicals but are not affected by the presence of hydroxyl or hydroperoxyl radicals (Alper, 1956).

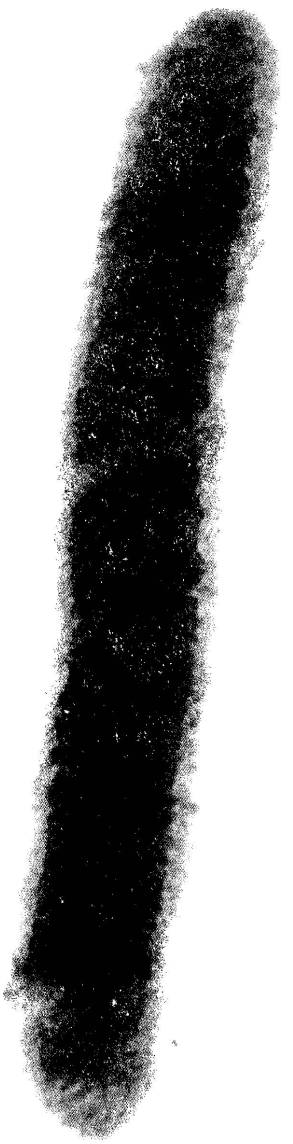
Since the phage particle is composed mostly of protein and DNA with relatively small amounts of RNA and lipids and is synthesized in the bacterial cell, it would seem that the reaction of phage might be similar to that observed in the degradation of animal DNA. However, Daniels, Scholes, and Weiss (1953) used X-rays to determine the post irradiation degradation of animal DNA, and it was found that degradation was more rapid when irradiation was carried out in the presence of oxygen. Ephrussi-Taylor and Latarjet (1955) noted that X-irradiation damage in the transforming virus of Dipolococcus pneumoniae was not enhanced by the presence of oxygen. Kimball (1955) irradiated paramecia containing kappa particles which are composed of DNA and protein. His results indicated that the particles were protected from radiation damage produced by radicals if oxygen were present and provided the irradiation occurred while the particles were inside the Paramecium. If the kappa particles were removed from the host cell and irradiated the oxygen protection against the radicals was not as effective.

In spite of the evidence to the contrary it is sometimes reported as general knowledge that HO_2 radicals are damaging to DNA. Primary involvement of HO_2 radicals is not indicated if a delayed action of radiation is enhanced by oxygen. In phage it is thought that immediate inactivation by radiation is due to reducing radicals (Alper, 1956). There is good evidence that hydrogen peroxide acts as a reducing agent on

phage, and that it does so on decomposition. With X and gamma rays, the incorporation of hydrogen into peroxide required the presence of oxygen during irradiation so that oxygen is in effect an agent for saving radicals from recombination into water.

Howard-Flanders and Alper (1957), using techniques of Ebert and Alper (1954) with E. coli, Shigella flexneri, and a haploid yeast, determined the variation in radiosensitivity by a series of mixtures in which the concentration of oxygen was different. This oxygen effect enhanced the effect of radiation in many organisms by reacting with ionized target molecules which in the absence of oxygen might be restored to normal function. Under anoxic conditions a reaction leading to effective damage might take place between the ionized target molecule and another ionized molecule nearby so that the enhancing effect of oxygen would be less with densely ionizing radiations. Ebert and Alper (1954) feel that even though hydrogen peroxide formation by irradiation is maximum in aqueous solutions with oxygen saturation, many phages are not inactivated. On the other hand, inactivation of many phages such as S13 and T3 is maximum in oxygen-free solutions saturated with hydrogen. The mechanism of inactivation is through the reduction action of free radicals. Bachofer and Pottinger (1956) also indicated that E. coli phage T1 is sensitive to reduction.

Figure 2--An electron micrograph showing the lysis of Bacillus megaterium S after 50 min incubation with M1 phage. X 10,000.



Landry and Fuerst (1968), in a study of the effect of over twenty gases on several genera of bacteria, found that perfluoropropane had an inhibitive effect on the survival of E. coli to UV irradiation. Nitrous oxide, carbon tetrafluoride, and perfluoropropane when used without radiation produced almost no change in the rate of survival or in colony morphology in some strains of E. coli.

The effect of several hydrocarbon gases on Neurospora crassa were analyzed by Fuerst and Stephens (1970). They found that carbon dioxide inhibited ascospore germination and perithecia production while oxygen enhanced the gamma irradiation damage in this organism.

Effect of radiation on organic compounds.

Latarjet and Ephrati (1948) tested the protective action of some compounds in aqueous solutions on T2 phage irradiated with X-rays. They assumed that these protective substances acted against the indirect effect of the X-rays. In order of most protective to least protective the compounds were thioglycollic acid, tryptophan, glutathione, cystine, and cysteine. Ascorbic acid and glucose did not seem to have any protective effect on the phage. The presence of a saturated oxygen solution or its absence did not affect the protective action of the compounds tested.

The action of radicals produced from ethanol, formate, and methanol by OH radical attack is to destroy the thymine

chromophore. If small quantities of cysteine are added the thymine is almost completely protected. Loman, Voogal, and Blok (1970) believed that the explanation for this might be that a hydrogen atom is transferred from the sulfhydryl compound to the organic radical thus restoring the parent organic molecule and preventing the reaction of the organic radical with thymine. This type of indirect protection of DNA may be important in its radioprotection of living cells. The experimental results of Rafi, Weiss, and Wheeler (1968) indicate that radiation sensitivity decreases with a decrease in the A-T content of the native DNA. Seven DNA's of different origin were used in these experiments with gamma irradiation.

In a mass spectrometric analysis of the products yielded by the X-irradiation of methane in an excess of argon, Meisels et al. (1956) identified the following radicals: H_2, C_2H_6, C_3H_8, and C_4H_{10}. Iodine was used as a scavenger to capture and identify the free radicals formed in the radiolysis of methane.

Stevens, Tolbert, and Bergstrom (1970) used gamma radiation of egg-white lysozyme to analyze its inactivation in the presence of oxygen in vacuo. Oxygen enhanced the radiosensitivity of the lysozyme molecule to gamma radiation.

Alper and Hodgkins (1969) found a protective action of acriflavine on E. coli B when the bacteria were exposed to

the dye before UV irradiation. There seemed to be evidence that the protection was a result of insertion of the dye molecules between bases which are consequently inhibited from photochemical interaction between neighbor molecules.

Since much work has been reported on the radiolysis of pure gas, it was thought that perhaps this information could help explain the reaction of M1 phage when gamma irradiated in the presence of different gaseous atmospheres.

MATERIALS AND METHODS

Determination of the experimental design.

Some preliminary studies were conducted to determine the exact experimental design that would be used in this research project. It was necessary to decide several factors concerning the phage: the concentration of the phage, whether the organisms should be irradiated in a lyophilized condition or whether they should be irradiated in water or in peptone broth. It was decided to use a concentration of approximately 7×10^7 phage particles per ml suspended in a 2% peptone broth. Ten ml of phage suspended in 2% peptone broth were selected to be irradiated because the dose had been calibrated for this volume of fluid in the Turner bulb which was the chamber used for maintaining the gas atmospheres (Bowen, 1968). This volume was also sufficiently large so that the gases could be introduced directly into the liquid medium as they entered the gassing chamber. In addition, experiments on other phage systems had been done under similar conditions (Latarjet and Ephrati, 1948; Watson, 1952), and therefore some comparisons could be made.

Strains employed.

Lyophilized cultures of Bacillus megaterium ATCC #11478 (Hershey's Bacillus megaterium S), which is the host strain

for the bacteriophage Bacillus megaterium ATCC #11478-b (M1 phage of P. B. Cowles derived from culture filtrates of B. megaterium strain 899, the lysogenic strain of den Dooren der Jong) were obtained from the American Type Culture Collection, Rockville, Maryland. These strains were used in all experiments.

Media used.

All B. megaterium stocks were cultured and maintained on 2% Difco peptone agar slants in 18 x 150 mm screw top test tubes. This medium was also used in experiments where the bacteria were grown in 150 mm petri dishes. Twenty ml of the 2% peptone were poured into each of the petri dishes. The aluminum tops of the petri dishes were lined with absorbent cardboard discs as described by Brewer (1950).

Nutrient broth was prepared by adding 20 g of Difco bacto-peptone per liter of distilled water. All media were sterilized in the autoclave at 124 C and 18 lb pressure for 15 min. All dilutions of the bacterial stocks were made using this 2% peptone broth.

Concentration of bacterial cultures.

The bacterial cultures grown on 2% peptone agar slants for 18 hr were suspended in 3 ml of peptone broth. The concentrations of the bacterial cultures were determined on the Bausch and Lomb Spectronic 20 Spectrophotometer at a

wavelength of 525 mμ. The concentration of bacterial cells used in all experiments was approximately 1 X 10^8 cells/ml.

Preparation of high titer phage stocks.

Phage stocks were grown by the agar layer method (Gratia, 1936) on B. megaterium M1 host strain. After 18 hr incubation, 5 ml of 2% peptone broth was added to each petri plate containing phage plaques and allowed to stand for 30 minutes. Then the soft agar and broth mixture was placed in 50 ml tubes and centrifuged for 20 min at 4500 RPM/min. At the end of this time the supernatant containing the phage was decanted and filtered through a Falcon plastic disposable membrane filter with a pore size of 0.22μ. This process removed all the bacteria and yielded a phage stock with a titer of approximately 7 X 10^{10} phage particles/ml. For experimental purposes this stock was diluted with 2% peptone broth to a titer of approximately 7.0 X 10^7 phage particles/ml.

The plaque counting method.

Viable phage particles were counted by the agar layer method of plaque counting (Gratia, 1936). Two hundred fifty ml of sterile nutrient agar were cooled to 47 C in a water bath. Twenty ml aliquots were then poured into sterile petri plates and allowed to solidify. Test tubes containing 4.0 ml of melted 0.7% agar were cooled to 47 C in a water bath and viable phage particles were counted by mixing 1 ml

of treated phage in a suitable dilution with 0.5 ml of a concentrated broth suspension (approximately 1 X 10^8 bacterial cells per ml) of the host organism, B. megaterium. Since an excess of bacteria was present in this mixture each of the phage particles infected a single bacterium. The mixture was then poured over the surface of a 2% peptone agar plate to form a thin layer on the surface of the petri plate which became a semi-solid so that the bacteria were immobilized. The petri plate was incubated right side up for 24 hr at 37 C. Uninfected bacteria multiplied to form a solid film of growth over the surface of the plate. Each infected bacterium will burst after a period of time and liberate phage particles. As a result of this bacterial lysis, circumscribed areas of clearing called plaques occur in the confluent bacterial growth.

Gases utilized in this study.

Initial experiments indicated that molecular oxygen, carbon dioxide and methane were protective to M1 phage during gamma irradiation. Therefore, it was decided to select other gases to be tested according to the following criteria: (1) Some gases would be used which were oxides. (2) Some of the gases should have a low molecular weight. (3) At least one gas should be a noble gas. (4) One gas should be a reducing gas. (5) Some simple fluoronated gases should be used. (6) Ethane was used because in a

pure chemical system methane when gamma irradiated forms some ethane (Meisels, Hamill and Williams, 1956). The gases selected for this study are listed in Table 1.

The gases used in this research were obtained from the Matheson Company, Inc., East Rutherford, New Jersey, and La Porte, Texas, in lecture bottles. The purity of the gases was at least 97.0% or greater (The Matheson Company, 1969).

Method of exposing phage to gas.

The method of gas exposure was based on previous experiments with other microorganisms performed in this laboratory (Fuerst and Landry, 1967; Fuerst and Stephens, 1970). The 100 ml Turner bulb which was used as a chamber for the gas experiments permitted the gas to enter directly into the 10 ml of fluid medium containing the phage, and the gas was exhausted from the flask above the fluid. The rate of gas flow was set so that there would be complete displacement of air in the chamber by the gas which was introduced. By letting the gas flow directly into the fluid it was considered that the medium was saturated with the gas. At the end of 10 min the ground glass top on the Turner bulb was sealed and the gas atmosphere was maintained for 3 hr before opening the bulb and testing the medium. The gas flow was monitored by a Hastings mass Flowmeter and was introduced at a rate of 30 ml/min into

Table 1--Gases tested for biological effects on strains of Bacillus megaterium M1 phage.

Name of gas*	Formula	Molecular weight
Argon	Ar	39.94
Nitrogen	N_2	28.01
Oxygen	O_2	32.00
Methane	CH_4	16.04
Carbon dioxide	CO_2	44.01
Nitrous oxide	N_2O	44.02
Tetrafluoromethane; Freon-14	CF_4	88.01
Perfluorobutene-2; perfluoro-2-butene	C_4F_8	200.04
Ethane	C_2H_6	30.00
Compressed air		

*Descriptive data published by Matheson Gas Products (1969), East Rutherford, New Jersey.

Turner bulbs containing 10 ml aliquots of phage stock. After 10 ml of gas flow the bulbs were sealed and the gas was maintained at 27 C in the bulbs for 3 hr in some experiments and for 8 hr in other experiments.

Source of gamma irradiation.

The source of Co-60 irradiation was from a U. S. Nuclear Corporation Model GR-9 gamma irradiator. Source dosimetry (Bowen, 1968) indicates an exposure of 943 R/min. The dosage was calibrated for 10 ml of fluid in each Turner bulb.

Technique of phage irradiation.

Because of the very large numbers of organisms being irradiated and the method of assaying them, it was desirable to have a higher irradiation kill than an LD/50. In addition, other investigators using similar systems with microorganisms had used doses of 100 kR to 400 kR gamma irradiation (Howard-Flanders and Alper, 1957; Powers, 1963). A 10 ml aliquot of phage stock was pipetted into the Turner bulb. The top was sealed and the bulb was placed in the Co-60 gamma irradiator. In some experiments the phage was gassed and then irradiated. Immediately after the gas treatment the sealed Turner bulb containing phage was placed in the Co-60 irradiation chamber. The total dose of gamma irradiation was 170 kR or 450 kR.

At the end of the treatment period the Turner bulb was opened, the phage stock was diluted immediately, and plated

by the agar layer method (Gratia, 1936). For each treatment 5 to 15 petri dishes were prepared. The phage was diluted sufficiently so that there were between 30 to 300 plaques per plate. At least five of the plates were assayed for plaques for each sample.

Enzyme treatment of phage.

The enzymes used in these experiments were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. The enzymes tested in some experiments were deoxyribonuclease (activity 250-300 Kunitz units/mg); ribonuclease (activity 50-100 Kunitz units/mg); and lysozyme (activity 17,000-30,000 units/mg). One mg of each enzyme was added to a 10 ml aliquot of phage stock. This mixture was incubated in Turner bulbs at 37 C for 3 hr. One ml of the treated phage stock was then diluted and plated by the agar layer method (Gratia, 1936) and incubated 18 hr before counting the plaques.

The phage stock containing the enzyme was exposed to different gases by the same techniques as have been previously described for the gas exposure of the phage stock. The phage stock containing the enzyme was then incubated at 37 C for 3 hr in the gas atmosphere before plating the sample.

Preparation of electron micrographs.

An 18 hr culture of B. megaterium S grown on a 2% peptone agar slant was washed with 1 ml of physiological

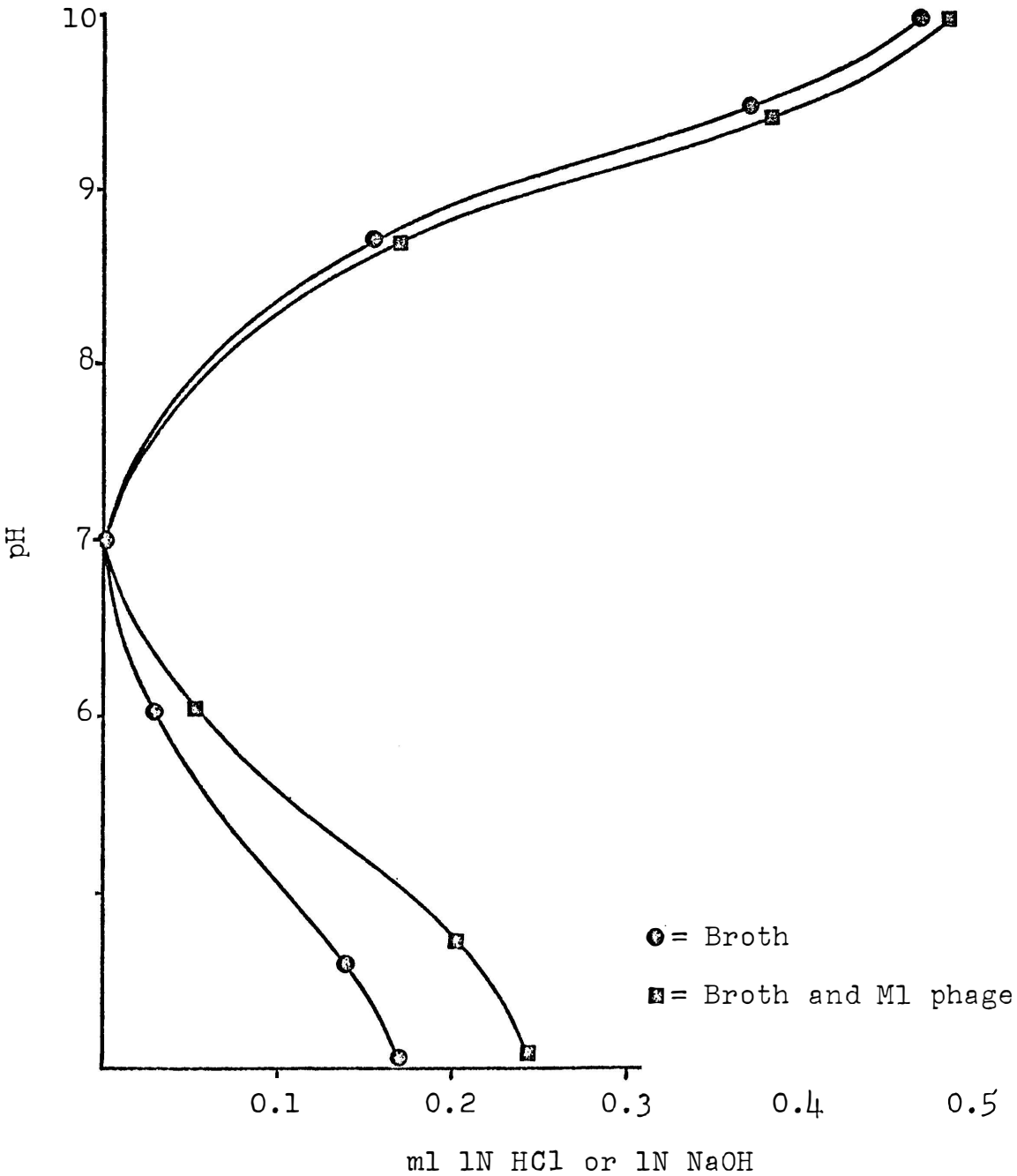
saline. To this suspension 0.2 ml of phage stock was added and allowed to adsorb for 50 min. One drop of the adsorbed mixture was placed on a formvar coated grid which was then dried under vacuum and shadowed with chromium and observed under the electron microscope.

EXPERIMENTAL RESULTS

One condition which is thought to have a measurable effect on living systems or chemical compounds when irradiated in aqueous solutions is a change in the pH of the solution. Therefore, the following determinations were made on the nutrient medium in which M1 phage was suspended. The peptone broth was buffered in air at a pH of 7. In order to determine the amount of acid or the amount of base that would have to be formed in the medium before the pH of the broth could be changed from pH 7 to 5, or from pH 7 to 10, (this range is the viable pH range for M1 phage according to Friedman and Cowles, 1953) 1N solutions of HCl or NaOH were used in titrating the broth. Figure 3 shows the results which were obtained. It took 0.47 ml of 1N NaOH to change the pH of 10 ml of broth without phage and 0.48 ml of 1N NaOH to change the pH of 10 ml of phage suspended in broth from pH 7 to 10. Titration with 1N HCl required 0.11 ml of acid to change the pH of 10 ml of broth from 7 to 5 and 0.18 ml 1N HCl to change 10 ml of phage suspended in broth from pH 7 to 10.

Determinations of pH were made on the broth and phage in broth under each different experimental condition. The

Figure 3--The quantity of 1N HCl necessary to change the ph of 10 ml of broth or 10 ml of M1 phage in broth from ph 7 to 4, and the amount of 1N NaOH necessary to change the ph of 10 ml of broth or 10 ml of M1 phage in broth from pH 7 to 10.


10
9
8
7
6
pH
0.1
0.2
0.3
0.4
0.5
= Broth
= Broth and M1 phage
ml 1N HCl or 1N NaOH

pH determinations were made immediately following the end of the gas or irradiation treatment.

Ten ml aliquots of 2% peptone broth were used in all pH tests in order to correspond with the volume of phage in broth used in all experiments. In addition all pH tests were carried out in Turner bulbs and the total dose of 170 kR gamma irradiation was used to standardize these conditions in all phases of experimentation. Gas treatments for pH effects were carried out in Turner bulbs and the gas exposure time was 3 hr. Table 2 gives the results of tests on the pH of the medium under different experimental conditions. According to these data the peptone broth maintained a pH of 7 under atmospheric conditions whether it received 170 kR gamma irradiation or not. The pH of the broth without phage in other gas atmospheres was not tested. Five of the gas atmospheres did not change the pH of the broth with phage but maintained the pH at 8.5. These gases were tetrafluoromethane, perfluorobutene-2, methane, compressed air, and air at atmospheric pressure. Under an oxygen atmosphere the broth containing the phages changed from pH 8.5 to pH 8. With an argon atmosphere the pH change was from 8.5 to 7.0. In an atmosphere of nitrous oxide the pH change was from 8.5 to 6.5. Two of the gases, carbon dioxide and nitrogen, caused the greatest change in pH from 8.5 to 6.0.

Table 2--Effects on pH of broth* with M1 phage after 3 hr exposure to various gases only, 170 kR Co-60 irradiation, and Co-60 administered in a gas atmosphere.

Gas used for treatment	Un-irradiated broth	Irradiated broth	Bacillus megaterium M1 phage**		
			Phage in broth	With gas only	Gas and Co-60
	pH	pH	pH	pH	pH
Argon			8.5	7.0	7.0
Tetrafluoromethane			8.5	8.5	8.5
Perfluorobutene-2			8.5	8.5	6.0
Oxygen			8.5	8.0	7.0
Methane			8.5	8.5	8.5
Carbon dioxide			8.5	6.0	6.0
Nitrous oxide			8.5	6.5	6.5
Nitrogen			8.5	6.0	
Compressed air			8.5	8.5	7.0
Atmospheric air	7.0	7.0	8.5	8.5	7.0

*2% peptone broth.
**M1 phage can tolerate a pH change of 5 to 10 (Friedman, 1953).

In comparing the effects of the pH change in the different gas atmospheres and in the gas atmosphere with gamma irradiation these data show that tetrafluoromethane and methane did not produce any pH change in the medium. The pH of the medium saturated with argon was 7 and gamma irradiation did not change this condition. Carbon dioxide and nitrous oxide in the medium maintained the same pH conditions with a gas or with gas and gamma irradiation. Gamma irradiation lowered the pH of the medium when oxygen, compressed air, and air under atmospheric conditions were present.

According to these data tetrafluoromethane and methane do not produce any change in the pH of the medium under any experimental conditions used.

The plaque-forming units of gamma irradiated M1 phage were determined by the agar layer method of Gratia (1936) as described in the materials and methods section of this dissertation. Five plates were assayed for plaque formation from each sample taken at intervals during the gamma irradiation. There were no surviving phage after 890 kR gamma irradiation.

The surviving numbers of M1 phage capable of lysis of Bacillus megaterium strain S after being exposed to Co-60 gamma irradiation at 943 R/min are shown in Figure 4.

Figure 5 is a diagram showing the general structure of an M1 phage particle. There are three distinct types

Figure 4--The surviving number of plaque-forming units of M1 phage of Bacillus megaterium after exposure to Co-60 gamma radiation at 943 R/min in atmospheric air.

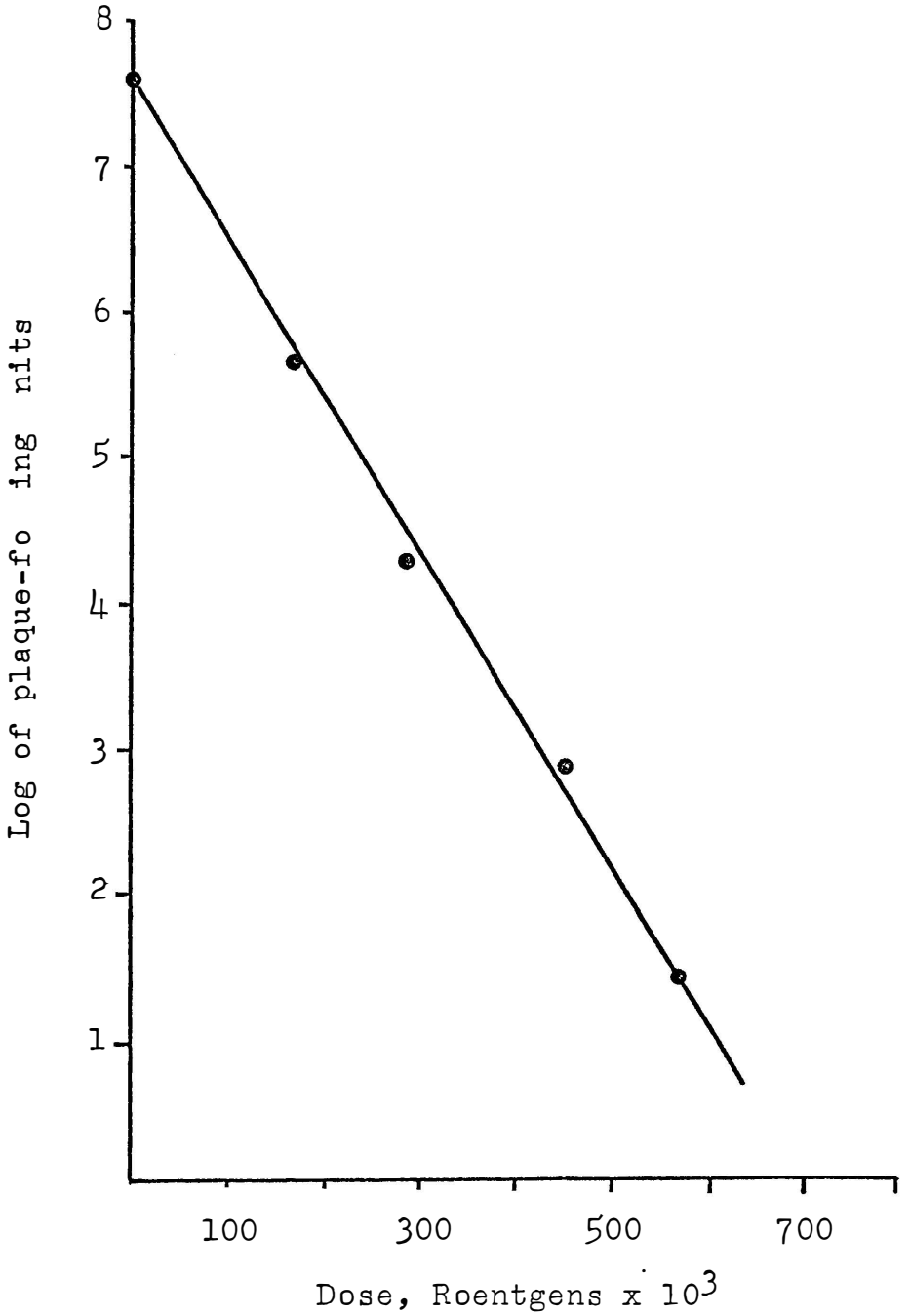
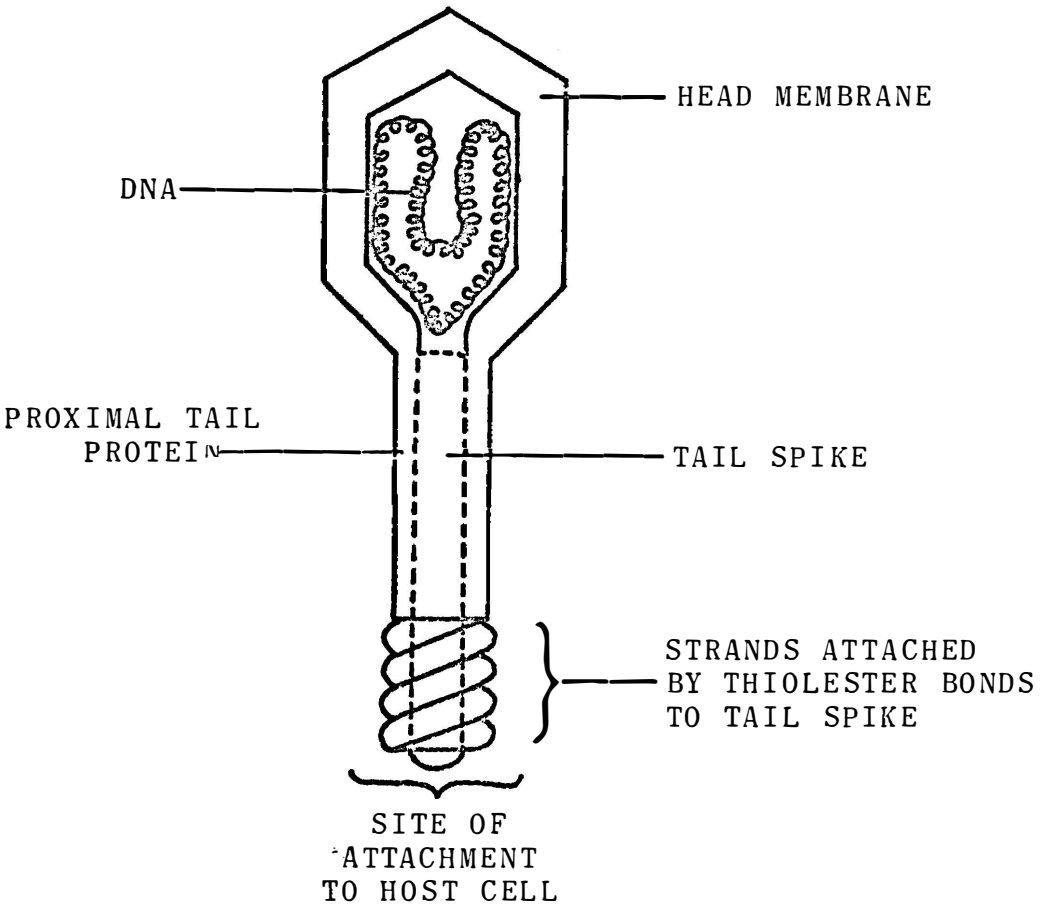

8
7
6
5
4
3
2
1
Log of plaque-fo ing nits
100
300
500
700
Dose, Roentgens x 10^3

Figure 5--Diagrammatic representation of the structure of M1 phage (Kozloff, Lute, and Henderson, 1957).


HEAD MEMBRANE
DNA
PROXIMAL TAIL
PROTEIN
TAIL SPIKE
STRANDS ATTACHED
BY THIOLESTER BONDS
TO TAIL SPIKE
SITE OF
ATTACHMENT
TO HOST CELL

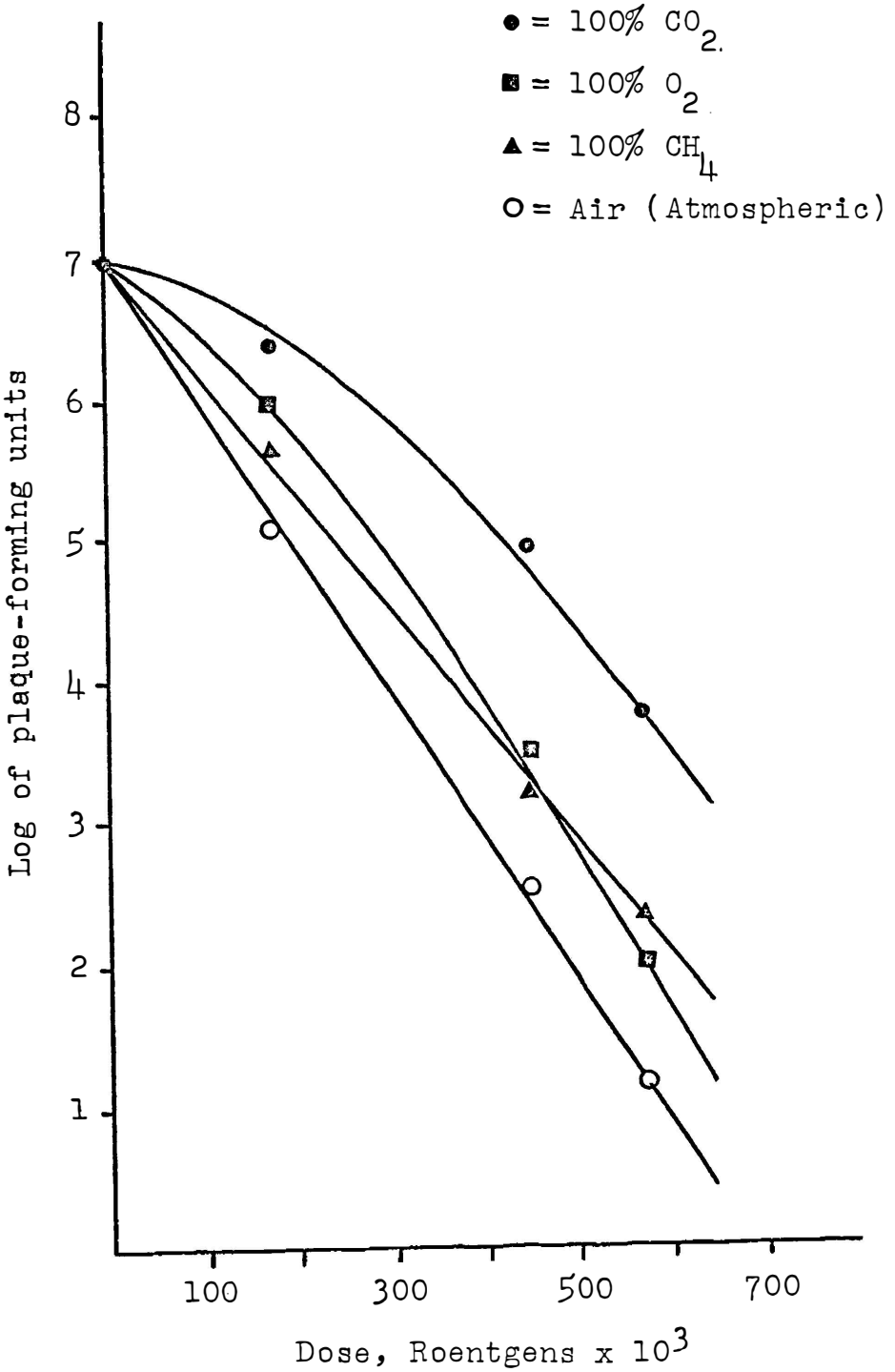
of protein in the capsid surrounding the phage particle. The DNA is located in the head of the phage. There are thiol ester bonds in the distal tail portion of the phage. It is thought that the tail spike of the phage is a hollow tube-like structure.

An experiment was performed with three of the protective gases in order to obtain surviving fractions of M1 phage after gamma irradiation in the protective gas atmospheres. The gases used were carbon dioxide, oxygen, and methane. The number of phage particles surviving after gamma irradiation in atmospheric air was used as a control. These data are plotted on the graph in Figure 6.

The shape of the survival curve of the phage in the carbon dioxide atmosphere initially does not seem to be exponential. However, after approximately 170 kR gamma irradiation the survival curve for M1 phage in carbon dioxide becomes exponential.

The survival curve for the phage in an atmosphere of oxygen is also initially not exponential. This again may indicate some radical formation which is protective to the phage against gamma irradiation. After approximately 170 kR of gamma irradiation the survival curve again becomes exponential and seems to drop off very rapidly. This may indicate that with gamma irradiation doses greater than 225 kR the protective value of oxygen to the phage may decrease.

Figure 6--The surviving fraction of M1 phage of Bacillus megaterium after Co-60 gamma radiation in different gas atmospheres.


● = 100% CO_2
■ = 100% O_2
▲ = 100% CH_4
○ = Air (Atmospheric)
Log of plaque-forming units
8
7
6
5
4
3
2
1
100
300
500
700
Dose, Roentgens x 10^3

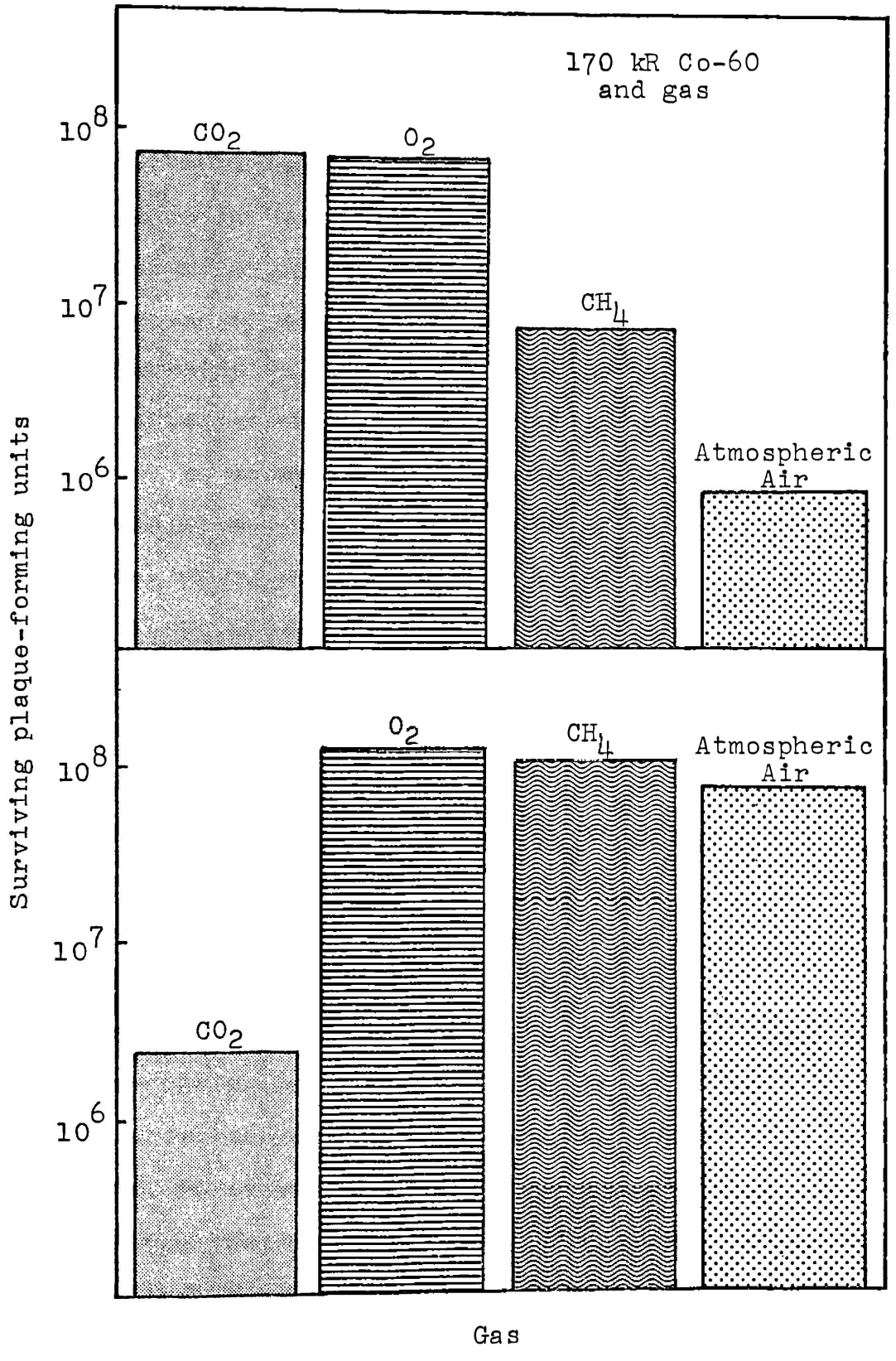
The survival curve for the M1 phage in an atmosphere of methane was exponential.

In making a comparison between the protective effect of a gas atmosphere to M1 phage when exposed to 170 kR gamma irradiation, the surviving numbers of phage per ml of medium were compared to the surviving numbers of phage per ml in air at atmospheric pressure. A t-test was made on these data to determine whether the gas atmospheres were harmful, protective, or did not seem to effect the survival of phage when exposed to 170 kR gamma irradiation or when exposed to the gas atmospheres for 3 hr. The phage in air at atmospheric pressure was used as an estimate of the population (u) when compared to the effect of phage exposure in different gas atmospheres. M1 phage in air at atmospheric pressure which was gamma irradiated at a dose of 170 kR was used as the radiation control.

No statistical analysis was made to compare the differences between the protective gases or the differences between the harmful gases.

A comparison was made on the effect of some gas atmospheres which are protective to M1 phage when exposed to a total dose of 170 kR gamma irradiation, and the effect of these same gas atmospheres on the survival of M1 phage when not irradiated. Figure 7 shows the results of these comparisons.

Figure 7--A comparison of 170 kR gamma irradiated and non-irradiated gas atmospheres on the survival of M1 phage.


170 kR Co-60
and gas
10^8
10^7
10^6
CO_2
O_2
CH_4
Atmospheric Air
10^8
10^7
10^6
O_2
CH_4
Atmospheric Air
CO_2
Surviving plaque-forming units
Gas

It is interesting to observe that while carbon dioxide was very protective to M1 phage during 170 kR gamma irradiation, when the phage was exposed to carbon dioxide gas without being irradiated there was a significant decrease in numbers of phage particles surviving as compared to the air control.

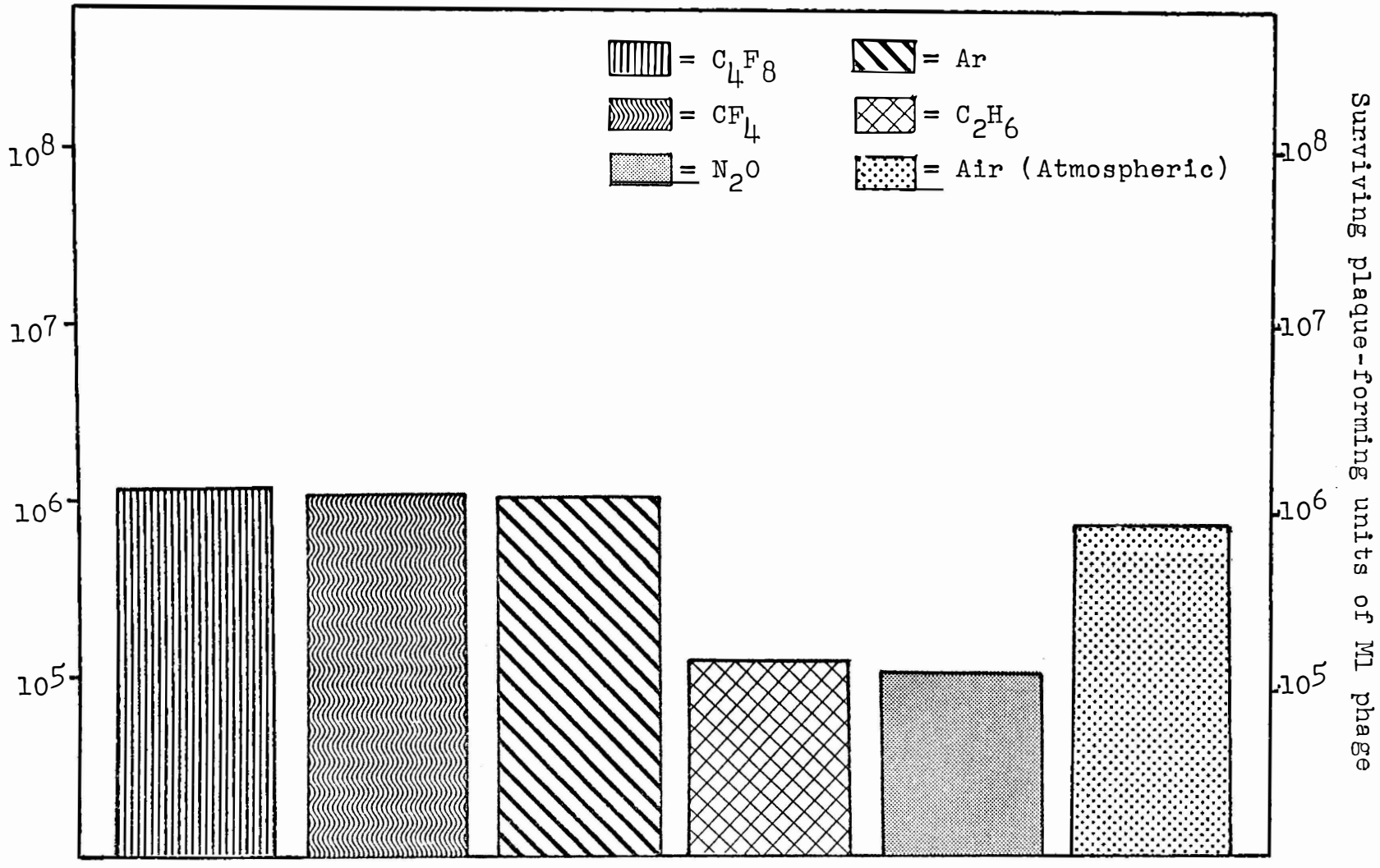
An atmosphere of methane while protective to M1 phage during exposure to gamma irradiation was neither protective nor harmful to the phage in a methane atmosphere without the gamma irradiation.

Oxygen was protective to phage during gamma irradiation and when M1 phage was exposed to the gas without any gamma irradiation there was a significant increase in phage numbers over the unirradiated control.

Figure 8 gives the results of M1 phage exposure to five additional gas atmospheres during 170 kR of gamma irradiation. Perfluorobutene-2, tetrafluoromethane, and argon did not change the survival rate of M1 phage.

Ethane and nitrous oxide decrease the surviving numbers of M1 phage. M1 phage exposed to an atmosphere of nitrogen gas for 3 hr but not gamma irradiated gave a mean survival of 20 organisms per ml. This gas was not tested by this investigator for its effects on gamma irradiated M1 phage. However, nitrogen has been reported by Alper (1955) as being very harmful to S13 phage when present in the atmosphere during gamma irradiation.

Figure 8--Surviving plaque-forming units of M1 phage after exposure to 170 kR gamma irradiation in different gas atmospheres.


Gas and 170 kR gamma irradiation
= C_4F_8
= Ar
= CF_4
= C_2H_6
= N_2O
= Air (Atmospheric)
10^8
10^7
10^6
10^5
Surviving plaque-forming units of M1 phage

Three enzymes, lysozyme, deoxyribonuclease, and ribonuclease, were tested for their effect on the pH of the M1 phage broth medium after 3 hr exposure of the enzyme-phage mixture to two different gas atmospheres. Table 4 gives the results of these tests. Carbon dioxide caused a change in pH from 7 to 6 in the medium. However, the presence of methane in the atmosphere did not seem to change the pH of the medium from 7.

Table 3 summarizes the effects of all treatments on the survival of plaque-forming units of M1 phage.

Table 3--Effects on the survival of M1 phage of *Bacillus megaterium* plaque-forming units after 3 hr gas treatment, 170 kR Co-60 irradiation in gas atmosphere, and enzyme treatment in gas atmosphere.

Treatment with gas	Gas only	Gas and Co-60	Enzyme* only	Gas and enzyme
Ar	no** effect	no effect		
CF_4	no effect	no effect		
C_4F_8	no effect	no effect		
O_2	enhanced	protective		
CH_4	no effect	protective		no effect
CO_2	harmful	protective		harmful
N_2O	harmful	harmful		
N_2	harmful			
Air (compressed)	no effect	harmful		
Air (atmospheric)	control	control	no effect	

*One mg of the enzyme was incubated with 10 ml aliquots of phage for 3 hr at 37 C. Identical results were obtained whether the enzyme preparation was lysozyme, ribonuclease, deoxyribonuclease.

**No change in the number of phage surviving when compared to the number surviving in atmospheric air.

Table 4--Effects on pH of broth with M1 phage of *Bacillus megaterium* after 3 hr incubation at 37 C with enzyme in gas atmosphere.

Enzyme*	Gas**	Phage***	pH****
None		M1	7.0
Lysozyme	CO_2	M1	6.0
Deoxyribonuclease	CO_2	M1	6.0
Ribonuclease	CO_2	M1	6.0
Lysozyme	CH_4	M1	7.0
Deoxyribonuclease	CH_4	M1	7.0
Ribonuclease	CH_4	M1	7.0

*One mg of the enzyme was incubated with 10 ml of phage. Lysozyme (17,000-30,000 units/mg), DNase (250-300 Kunitz units/mg), or RNase (50-100 Kunitz units/mg).

**The gas was introduced into 100 ml Turner bulbs at a rate of 30 ml/min for 10 minutes. At the end of 10 min the bulbs were sealed for 3 hr.

***Ten ml of phage stock containing 7.0×10^7 particles/ml.

****pH at the end of 3 hr incubation period.

DISCUSSION

There are several factors which should be taken into consideration when analyzing data presented in this dissertation. One of the first considerations was to determine if there was any relationship between the low molecular weights of a gas and its ability to protect M1 phage from the effects of gamma irradiation. The most protective gases had molecular weights ranging from 16 for methane, 32 for oxygen, to 44 for carbon dioxide.

The gases which increased the detrimental effects of gamma irradiation on M1 phage also had a comparable range of molecular weights to the gases which were protective to the phage survival. For example, nitrogen with a molecular weight of 28, ethane with a molecular weight of 30, and nitrous oxide with a molecular weight of 44.

The two gases with the highest molecular weights were tetrafluoromethane, 88 MW, and perfluorobutene-2, 200 MW; they did not seem to have any effect on M1 phage survival. Argon with a much lower molecular weight of 40 did not have any effect on the surviving plaque-forming units of M1 phage either.

From these data it would appear that the molecular weight of the gas was not a factor in the protection of M1 phage to gamma irradiation.

Since molecular oxygen was found to be protective to phage during gamma irradiation and to significantly increase the number of phage particles capable of forming plaques when present without gamma irradiation, the question arose whether other oxygen containing gases would also be protective to M1 phage against gamma irradiation. Carbon dioxide was found to be protective to the phage during gamma irradiation. However, it was determined that nitrous oxide significantly reduced the surviving phage numbers both when gamma irradiated in a gas atmosphere or when treated with the gas and not irradiated. The mere presence of an oxygen containing gas according to these data apparently is not the determining factor in the activity of the protective mechanism during phage irradiation.

A second factor to consider is whether a change in pH within the limits tolerated by M1 phage has any effect on the protective action of the gas atmosphere on M1 phage against gamma irradiation. The tolerance range of M1 phage as determined by Friedman and Cowles (1953) is pH 5 to 10. Data reported in this dissertation indicate that while three of the gases lowered the pH of the medium in which the phage was suspended one of these gases, carbon dioxide, was protective to the phage against gamma irradiation; another of

these gases, nitrous oxide, was harmful to M1 phage; and the third gas, perfluorobutene-2, did not seem to be either protective or harmful to M1 phage when present during gamma irradiation of the phage. In addition, the lowest pH reading of any gas atmosphere during gamma irradiation was a pH of 6. This figure was well within the known range of pH tolerance for this phage system. As is indicated by data given in Figure 1, the medium in which the phage is suspended is quite stable, therefore, it seems that in the M1 phage system used for these gas studies pH was probably not a determining factor in the interaction of gas atmospheres with gamma irradiation in the protection of the M1 phage.

Carbon dioxide in the atmosphere was protective to M1 phage during gamma irradiation but it decreased the ability of the phage to form plaques when there was no gamma irradiation present. Methane was also protective to the phage during gamma irradiation, and it did not seem to affect the plaque-forming ability of the phage particles when there was no irradiation present.

A comparison was made of methane and carbon dioxide during gamma irradiation by Anderson and Dominey (1968), and they have found that the amount of carbon dioxide was very constant during gamma irradiation. Their studies showed that carbon monoxide and oxygen are formed from carbon dioxide during gamma irradiation. However, rapid reoxidation of CO stabilized the amount of CO_2. It was also determined

that methane when exposed to gamma irradiation at temperatures ranging from 17 C to 26 C in the presence of oxygen containing gases formed carbon monoxide and oxygen was given off.

The fact that one of the products of radiolysis of these gases is molecular oxygen whether produced directly as by carbon dioxide or indirectly by the interaction of carbon dioxide with methane, may explain why these two gases give protection to M1 phage during gamma irradiation.

Additional studies (Lind, 1961) reveal that ethane is also among the products of the radiolysis of methane. The radiolysis of ethane produces hydrogen gas in considerable quantities. The action of nitrous oxide when gamma irradiated yields molecular nitrogen, nitrous oxide, and a small amount of molecular oxygen.

Compressed air without gamma irradiation seemed to have no effect on the production of M1 phage plaque-forming units, but when compressed air was exposed to gamma irradiation the M1 phage plaque-forming ability was significantly reduced. This harmful reaction could be the result of interaction between the nitrogen in the compressed air and the phage particles.

Nitrogen was so harmful to M1 phage even without gamma irradiation exposure that at the end of 3 hr in the gas atmosphere an average of only 20 plaque-forming units per ml of M1 phage had survived. Since the effect of this gas

has been reported by Howard-Flanders and Alper (1957) to be harmful to other phage systems while being gamma irradiated, this gas was not tested on M1 phage during gamma irradiation. In addition to testing nitrogen Alper also tested the effect of other reducing gas atmospheres on the survival of S13 phage plaque-forming units. He found that these gases decreased the survival of this phage.

In many biological systems the harmful effect of gas atmospheres can be attributed to a change in pH of the system being tested. This research had indicated that in the M1 phage system the change in pH does not seem to be a prime factor which was causing the harmful effect on this phage. As to why nitrogen in the atmosphere has such a detrimental effect on the survival of the phage can perhaps be attributed to the fact that it is a reducing agent and as such may interfere with some vital process in phage plaque formation.

The three enzymes, DNase, RNase, and lysozyme, which were added to M1 phage and incubated for 3 hr in a gas atmosphere of CO_2 or CH_4 did not change the pH of the medium containing M1 phage. A pH of 6 for CO_2 and 7 for CH_4 was the same for the enzyme phage mixture as it was for the phage alone in the two gas atmospheres (Table 3).

In most microorganisms with more complex metabolism than in phage the mechanism of the oxygen effect occurred whereby the radiosensitivity of the organism was increased when free oxygen was present during gamma irradiation. It

was thought that this enhancement by oxygen of the radiosensitivity of living cells could be attributed to the action of free HO_2 radicals formed in water. Alper (1956) suggested that under the conditions of free oxygen during gamma irradiation, the cell death may be attributed to a chemical change in a vital target molecule as put forth by the classical "target theory." He believed that after the ejection of an electron the target molecule would be very excited and if an oxygen molecule were close to the ionized target the two would react to produce an organic peroxide which could not be restored to its original state. According to Howard-Flanders and Alper (1957) yeast is more sensitive to gamma irradiation if oxygen is present in the atmosphere during radiation exposure. He believes that approximately two-thirds of the target molecules ionized by X or gamma rays would be in anoxic condition and would be able to eventually recover and function normally provided oxygen was not present during irradiation.

Another hypothesis put forth by Howard-Flanders and Alper (1957) is the "direct effect" hypothesis in which they think that when two molecules which are close together become ionized a reaction may take place between these molecules. This reaction may be similar to the molecular reactions which occur in water and lead to the production of hydrogen peroxide in the denser parts of ionization tracts. Therefore an ionized molecule in the vicinity of

an ionized target molecule may react with it and make any subsequent restoration impossible. If the ionizations take place singly in target molecules sufficiently far away from other ionized molecules then their fate is influenced by the presence or absence of oxygen.

Some conclusions concerning the chemical structure of compounds which are able to capture free radicals were summarized by Alexander et al. (1955). They believed that in general a free amine group in the molecule, particularly beta substituted ethylamines such as are found in cysteamine and beta-phenylethylamine, is especially effective in capturing free radicals. In general the compounds which have the best radical capturing ability are those containing divalent sulfur such as thiols. Aliphatic mercaptans seem to be equally effective in capturing radicals and also thiols in which the ionized form is part of a resonance system wuch as thiouracil and thiosemicarbazide (Errera and Forssberg, 1960).

The removal of oxygen has been suggested by Lengerova and Zeleny (1958) to be the cause of the protection afforded by thiols in vitro, since these compounds are known to be rapidly oxidized to the corresponding disulfides. This removal of oxygen by thiols does not, however, seem to be the only protective mechanism in vivo since according to Errera and Forssberg (1960) this reaction has not been experimentally confirmed.

Evidence exists that in simple living systems other mechanisms of protection besides the radical scavenger may be operating. Cysteamine and other protective compounds may be reacting with yeast proteins, bacterial or bacteriophage proteins to form complexes which may enable the organism to become resistant to the action of radicals (Hollaender and Stapleton, 1956).

Rafi, Weiss and Wheeler (1968) studied aqueous solutions of DNA from calf thymus, Staphylococcus epidermidis, Enterobacter aerogenes, Pseudomonas fluorescens, Micrococcus radiodurans, and Micrococcus lysodeikticus and tested the effect of gamma irradiation on those solutions of DNA with different nitrogen base ratios. It was found that the radiation sensitivity of DNA decreased as the A-T content of the DNA decreased. Therefore, the sensitivity to gamma irradiation was determined by the ratio of adenine to thymine present in the DNA.

Electron micrographs have been prepared for this dissertation which indicate that E. coli T2 phage will adsorb to Bacillus megaterium S the host strain for the M1 phage. Friedman and Cowles (1953) obtained electron micrographs of M1 phage of B. megaterium which reveal that this phage is similar in appearance to T2 phage of E. coli. It has been suggested by Brown (1968) that the M1 phage is dependent upon Zn^{++} complexes for attachment to the bacterial cell

wall. Therefore, some analogies may be drawn between these two phage systems.

Kozloff and Lute (1957) studied the effects of several chemical compounds on T2 phage by observing the phage with the electron microscope. Treatment of T2 phage with cadmium cyanide complexes caused contraction of the tail protein. Compounds having a free amine group such as glucosamine, lysine hydrochloride, and ornithine hydrochloride caused the phage to release as much as 90% of its DNA. These authors also tested the effect of ions such as Na^{+}, Ca^{++}, and Mg^{++} on the phage after contraction of the tail protein to determine whether these ions could cause the release of the DNA from the phage. They did not find that these ions had any effect on the release of DNA.

In T2 and T4 phages the inactivation by cyanide complexes is under genetic control according to Kozloff et al. (1957), and it was determined by this group of investigators that the thiol ester bonds were broken rather than the disulfide bonds. The binding site of Zn^{++} in the cell wall of the phage host bacteria is usually considered to be the sulfur in the phage tail fibers. It has been determined that the high concentration of cysteine sulfur is located in the phage tail (Hayes, 1968).

In discussing metal ion control of chemical reactions Busch (1971) believes that there are three possible types of interaction between a metallic atom and a reactant.

First, a metal ion may alter the character of the medium by changing its ionic structure. Second, the metal ion may become part of the reactant molecule which produces a reaction upon encountering the substrate. The third type of reaction of a metal ion is its function as an enzyme activator.

Zinc probably does not function as a coenzyme for the viral tail enzyme because when the phage was altered so that the lysozyme-like enzyme was exposed, the enzyme easily digested the zinc deficient cell walls in the absence of added metal ions (Kozloff and Lute, 1957). Therefore, zinc may be one of the reactants forming part of the ligand molecule. However, zinc has been identified as an activator of a large number of dehydrogenases. In bacteria it has been determined that the cytochrome system and some dehydrogenases are located in the bacterial cell membrance (Prasad, 1966). Kozloff and Lute (1957) found some indication that there is a zinc dependent dehydrogenase in the cell wall of E. coli T2 phage host which has thiol esterase activity.

Other zinc sensitive enzymes are a hexokinase which has been reported in Neurospora by Medina (1957). Bertrand and de Wolf (1959) observed the effect of zinc on an aldolase in Aspergillus niger. Alkaline phosphatase of Escherichia coli is also a zinc metaloenzyme (Plocke, Levinthal and Vallee, 1962).

The property of the caudal sheath of bacteriophage which is most characteristic of this protein is its ability to contract. Since phages are probably non-metabolizing organisms except when within a living cell it is thought that phage proteins are almost inert (Prasad, 1966). In T2 bacteriophage there is some structural similarity between the motor reactions of the tail sheath, the muscles of higher animals, and the motor organelles of individual cells (Poglazov, 1966).

The first indication of the presence of a similar function of these proteins of different origin was determined by Dukes and Kozloff (1959) when they found that there was adenosine triphosphatase (ATPase) activity in highly purified preparations of phages T2, T4 and T5 of E. coli. The properties of this enzyme system were studied not only in the T phage group but also in a number of other phage groups which had different organization of the caudal processes.

Two phage systems of Bacillus mycoides and the SD phage of E. coli strain SK were also investigated. ATPase was pH dependent and its activity was very low at pH 6 or below. Maximum activity of the enzyme seemed to be reached at pH 8. This enzyme's activity was dependent on the concentration of Ca^{++} and Mg^{++}.

Discovery of a relationship between ATPase activity and the tail structure was made by Poglazov (1966). He ascertained that the enzyme was concentrated in the caudal

portion of the phage protein by using the alkaline method of partition of phage T2 particles into separate elements.

The presence of this myosin-like protein with ATPase properties in the sheath of the caudal process of T2 phage was thought to assure the powerful contractile movements which lead to puncture of the bacterial cell membrane. The sheath protein of phage T2 is apparently a very simple form of contractile protein which shows a resemblance to myosin and actin (Poglazov, 1966).

Since the effects of some simple gas atmospheres on perhaps the simplest form of living organisms have been discussed in this dissertation, it would be interesting to speculate on the evolutionary role played by these gases when present in primitive earth's atmosphere. Could bacteriophages be the first living matter found on primitive earth or could they represent a primitive type of sexual reproduction which has evolved from a more complex living system. Some possibilities will be discussed.

According to Dauvillier (1965) it is not correct to think that the primitive atmosphere which existed after the condensation of the oceans is the same as our present-day atmosphere and to believe that oxygen is only a residue which did not react during the earth's formation. The galactic nebulae reveal the presence of oxygen and nitrogen as combustion residues from the stars. Oxygen can only exist in the free state in the stars and nebulae and it

is impossible to extract any chemical energy from it in this state. Lightning unites oxygen and nitrogen in the form of NO, which falls to earth with the rain. Arrhenius believed that the primitive free oxygen was formed by the result of the combustion of a primitive cyanogen in this oxygen. The products of this reaction, CO_2 and N_2 are the two gases existing on Venus and Mars. Other theories attribute the free oxygen to the disassociation of water vapor produced in the upper atmosphere by ultraviolet solar radiations shorter than 1850 A.

It is known that high temperature lavas release combustible reducing gases such as hydrogen, methane, and hydrogen sulfide. The earth's crust contains sulfides such as HgS, Cu_2S, Fe_2S_3, PbS, and ZnS (Dauvillier, 1947).

Although oxygen is the gas characteristic of life from the cosmic point of view, it does not appear on any planet in which the atmospheres contain more CO_2 than the earth's. Argon-40 makes up 1% of present day earth's atmosphere and this gas is not of primitive origin but has been produced gradually by the disintegration of potassium-40 of the lithosphere.

Among the gases found on primitive earth were H_2, CH_4, NH_3, and water vapor (Miller and Urey, 1959). With energy supplied by lightning and ultraviolet light these gases could have eventually formed polymers such as Oparin's coacervates (1957) and Fox's microspheres (1960).

Bernal (1965) does not believe that life had its origin in an aqueous medium but instead he thinks that the polymers were first formed by adsorption on clay. He also postulates that protoenzymes containing iron, copper, cobalt, and manganese evolved in this manner, and these could have polymerized into the basic metabolic enzymes such as the oxidases and cytochromes. Later these compounds may have become oxygen carriers such as hemoglobins or photon traps such as chlorophyll.

According to Fox (1960) the first living organism was probably an anaerobe which could metabolize by a simple membrane bound fermentative phosphorylation. This process was thought to be the predecessor of oxidation chain phosphorylation which developed with aerobic respiration. It is thought that ferredoxin was a primitive catalyst for these reactions.

The microspheres as postulated by Fox and Yuyama (1963) formed from proteinoids which were condensations of primitive molecules made by cooling the proteinoid solution from 25 C to 0 C. This change in temperature caused budding chains of molecules and eventually gave rise to structures which resemble electronmicrographs of thin sections of Bacillus subtilis.

The first primitive self-reproducing systems may have had something like RNA for the self replicating molecule and in addition to this self replicating molecule another

necessary molecule would have been a peptide synthetase or a t-RNA (Mora, 1963).

One of the best schemes of microbial evolution has been proposed by Kluyver and Van Niel (1965). Their hypothesis is based on the assumption that Micrococcaceae are the most primitive forms of bacteria. The Pseudomonadaceae evolved as one side branch, the Bacillaceae as an equivalent side branch, and the Mycobacteriaceae in direct line of evolution with the Micrococcaceae.

Since it has been reported in this dissertation that M1 phage is protected against gamma irradiation by several simple gases this relationship might have enabled the phage to survive the environment of primitive earth. Bacteriophage in general are rather simple living organisms containing several very primitive types of compounds. For example, the capsid of phage is made up of a very primitive type of contractile protein. This protein has been reported to polymerize when in the presence of Zn^{++} and Ca^{++} (Poglazov, 1966). This organism also reacts to a cyanogen in the medium by releasing its DNA externally (Kozloff and Lute, 1957). Cyanogens have been reported as being precusors of organic molecules on primitive earth by Oparin (1957). M1 phage might be considered a primitive type of organism also because it cannot carry on its metabolism outside a living cell.

There are some molecules in this phage, however, which are not believed to be among the first formed organic compounds. For example, the double strand DNA molecule which M1 phage contains is thought to have evolved at a later time than the RNA molecule which is believed by many to be the perpetuating mechanism in the first living organism.

Since phage does contain a double stranded DNA molecule and since it does have a very specialized mechanism of entering its host bacteria it seems more likely that bacteriophages were not the first living organisms on earth but have perhaps evolved as an off shoot of the main evolutionary pathway.

SUMMARY

1. The purpose of the research reported in this dissertation was to study the effects of some gases with and without Co-60 gamma irradiation on Bacillus megaterium M1 phage. The gases tested were O_2, CH_4, CO_2, N_2, C_2H_6, CF_4, C_4F_8, Ar, N_2O and compressed air. Effects of these gases on M1 phage were all compared with the effect of atmospheric air which was used as the control. The bacterial strain used in all experiments was Bacillus megaterium strain S (ATCC# 11478), which is the host for the bacteriophage Bacillus megaterium (ATCC# 11478-b) M1.
2. One method used in this study was the agar layer method of counting phage plaques. The phage was treated with the gas at a rate of 30 ml/min in a 100 ml Turner bulb for 10 min. The Co-60 gamma irradiator was used to give a total dose of 170 kR or 450 kR. Immediately after the treatment period the phage suspension was diluted usually to 10^{-5} and five petri plates were prepared by the agar layer method (Gratia, 1936). After 18 hr growth the plaques formed in the bacterial lawn of the petri plates were counted.
3. Because of the design of the experiment several factors were eliminated which would normally be expected to

have some bearing on the results of gamma irradiation experiments. For example, the phage particles were irradiated and diluted in a stable 2% peptone broth which maintained the pH of the medium within the normal tolerance range of M1 phage. The combination of a high gamma irradiation dose rate of 943 R/min and a high concentration of phage particles, 7×10^7 particles per ml, in the medium were considered to practically eliminate the possibility of indirect radiation as the predominate reaction. The radiation reactions were considered to be a direct target molecule reaction. Shapes of the survival curves confirmed this assumption. Oxygen tension in this simple non-metabolizing organism was not believed to be as critical as it might have been in a more complex organism. Experimental results indicated that the molecular weight of the gas was not a determining factor in its radioprotection to M1 phage.

4. Molecular oxygen in the medium during gamma irradiation seemed to be the mechanism protective to M1 phage against the effect of gamma irradiation. All the protective gases such as O_2, CH_4, and CO_2 which were tested in these experiments either released molecular oxygen upon exposure to gamma irradiation or these gases interacted with some oxide present at the target site to release molecular oxygen.

5. The gases which were harmful to M1 phage during gamma irradiation were either strong reducing agents or upon radiolysis significant amounts of reducing agents were evolved. The harmful gases were N_2O and Ethane.

6. Because of some known similarities between T2 phage of E. coli and M1 phage of B. megaterium some comparisons were made between these two phage systems. It is known that thiol ester bonds have a high affinity for oxygen and that these bonds bind the tail fibers of T2 bacteriophage together. It has also been determined that these tail fibers must unwind in order to provide the attachment site of the phage to the cell wall of the host bacterium. Therefore, it is proposed that the increased amount of oxygen during gamma irradiation initiates an essential oxidation reaction by which the S-H bonds in the tail protein of M1 phage are broken thus permitting attachment of the phage tail to the bacterial cell wall. Even though the tail fibers are released before the phage is in contact with the host bacterial cells perhaps because of the buffering ability of the peptone broth medium in which irradiation occurred there did not seem to be any loss of DNA from the phage. Subsequently at the end of the irradiation period the altered phage were still able to infect the host bacterial cells.

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