THE LETHAL AND MUTAGENIC EFFECTS OF ULTRAVIOLET LIGHT ON ESCHERICHIA COLI

by

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OBJECT OF THE INVESTIGATION

The lethal effects of ultraviolet light on bacteria have been studied for nearly three quarters of a century but it is only in more recent years that attention has been directed to the effects of these rays on bacterial variation. It has been shown that ultraviolet light is capable of inducing mutations in bacteria and our purpose was to determine whether any relationships exist between the lethal action and the mutagenic effect of these rays.

The bacterial mutations we have studied are a group of stable and heritable changes, but whether these changes are gene mutations is not definitely known at the present time but it seems quite probable that they are for some mutations in bacteria have been shown to be due to a gene change by recombination experiments with a sexually fertile strain of Escherichia coli. The mutations studied were quite numerous and it was not always necessary to screen large numbers of individuals and similar results could probably be obtained using higher forms of life. Generally, however, bacteria are used for radiation studies and they are particularly suitable since: (a) large populations can easily be handled; (b) there are numerous mutants which can be readily detected and counted; and (c) individual organisms result from each cell division enabling the phenotype to be determined at any time after a gene change.

INTRODUCTION AND HISTORICAL REVIEW

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1. Biological Effects of Ultraviolet Radiation on Bacteria

It would be hard to name a paper or date which might be regarded as the starting point for the study of the effects of radiation on bacteria. The history of this field has been very gradual in development and in most cases, dependent upon the advances of the other basic sciences and especially upon our increasing knowledge of the behaviour of microorganisms.

The credit for the "discovery" of the lethal action of sunlight on bacteria is generally given to Downes and Blunt (50,51) who in 1877 showed that certain organic substances in solution which would normally undergo decomposition by the action of a mixture of bacteria, would be inhibited in this decomposition by long exposures to direct sunlight. This drew the attention of biologists to the significance of spectral radiation and there immediately followed numerous qualitative investigations all serving to indicate that the lethal effects of sunlight were not limited to a few sensitive organisms. Duclaux (53,54) reported killing effects of sunlight upon bacterial spores and also noted the degrees of resistance of different species. Arloing (4) confirmed the earlier studies of degrees of resistance demonstrating the different effects of sunlight on vegetative cells and spores of the anthrax bacillus.

It was about this time that Ward (177,178) and others realized that not the whole spectrum but only part of it, the ultraviolet was the important if not the chief factor, in the bactericidal effect of sunlight

on microörganisms. The period around the turn of the century was spent mainly in determining those parts of the spectrum responsible for the observed effects. The credit for this initial work should go to Strebel (166) who isolated different parts of the spectrum and showed that strong killing action for bacteria was confined to the ultraviolet.

The ultraviolet spectrum includes all the radiations from those overlapping the x-rays (about 150 Å) to those bordering the visible (3900 - 4000 Å). The lower range of wavelengths, shorter than 2000 Å, is called the Schumann region and its biological effects have been only infrequently studied (24,25) due mainly to lack of proper equipment necessary to obtain appreciable intensity and energies for these wave lengths. The portion of the ultraviolet from 2000 - 3100 Å is often called the abiotic region because these wavelengths kill or injure cells most readily. In the range 3100 - 4000 Å the radiation is much less effective and its details have been dealt with elsewhere in a review by Loofbourow (105).

The majority of investigators have found the region of bactericidal activity to lie between the wavelengths 2100 - 2960 Å. Newcomber (130) working with the bacillus of typhoid fever gives the effective limits as 2100 - 2800 Å. Browning and Russ (30) exposed agar plates coated with bacteria and found the effective region to be between 2380 and 2940 Å. The most extensive work of this nature was carried out by Bayne-Jones and Van der Lingen (14) who worked with Staphlyococcus aureus and Escherichia coli; they defined the limits of bactericidal activity to be 2380 - 2960 Å. Up to this time the tungsten arc was generally employed but little was known regarding the distribution of intensities in the lines of this arc. With the advent of the mercury vapour lamp more accurate and detailed data could be obtained.

Coblentz and Fulton (39) made a very careful radiometric study of ultraviolet radiation on several organisms and found slight differences in the wavelengths effective for killing different organisms. For Escherichia coli the range for the lethal effects extended from 2200 to 2960 Å; for Salmonella typhosa from 2100 to 3000 Å; and for Staphylococcus aureus it extended from 2380 to 2960 Å. They also found that intermittant exposures had the same effects as a continuous exposure for the same total dose, that is, the action is cumulative. Numerous detailed reviews are available on the effective wavelengths responsible for ultraviolet killing (55,57,58,67,105) but most authors agree that the limits are from 2100 to 2960 Å with a maximum between 2510 and 2801 Å. The definition of the lower wavelength limit in most of these studies is very rough because of poor equipment and methods and most workers have only studied wavelengths greater than 2000 or 2100 Å.

The first quarter of the twentieth century has generally been marked by rather sporadic studies, after which intensive interest was revived. It is now well recognized that all types of animal cells are similarily sensitive to ultraviolet radiations, that is, any protoplasm exposed to short ultraviolet radiations is injured or destroyed. The center of interest in studies of the effects of ultraviolet radiations upon organisms has varied from time to time as specific phases were solved and investigators moved on to other problems.

Detailed Action Spectra: The wavelength which is most efficient in producing a given effect must be absorbed by some compound of the cell, since only light that is absorbed can produce activated molecules which lead to photochemical reactions. The photochemical reactions which

take place in living systems underlie a number of phenomena which may be classed together as photobiological processes. In most of these processes the intermediate steps between the primary reaction and the final response are unknown which is a disadvantage for we can only observe the end response and so to determine the absorption of quanta by the light absorber we must assume that there is some proportionality between the number of quanta absorbed in the primary reaction and the final step in the photobiological reaction.

We do not intend to go into the theory and mechanisms of photochemical reactions which in many cases are long and complicated, but we will only give the basic assumptions of photochemistry which are easily understood.

The first step in any photochemical process, be it in vitro or in vivo is known as the primary reaction, and is represented by the following equation:

$$A + h\xi = A^* \qquad (I)$$

where A is a molecule of a given substrate which will be referred to as the light absorber. A* is the same molecule activated, after it has been raised to an excited state by the capture of a quantum of radiant energy $h\xi$, where h is Planck's constant (6.6236×10^{-27} erg sec.), and ξ is the frequency of the radiation. The frequency is related to the wavelength λ as follows:

$$v = c/\lambda \qquad (II)$$

where c is the velocity of light (3×10^{10} cm. per sec.)

The primary reaction (I) is the initial event in any photochemical process, that is, a molecule must absorb a light quantum in order to reach the excited state from which it may proceed to take part

in a chemical reaction. Molecules in solution absorb only quanta of certain energy ranges, determined by their internal structure. Hence, a given light-absorbing substance can only participate in a photochemical reaction when exposed to quanta of the appropriate energy capable of bringing about excitation of its molecules, presumably due to the activation of the same type of molecule. The action spectrum for a photobiological process is an expression of the variation of the effectiveness of the exciting radiation with the wavelength.

The exact determination of action spectra requires an appropriate source of monochromatic radiation. "Line" sources, for example the mercury arc, emit only a limited number of wavelengths which appear in the spectrum as discrete lines. However, it is a relatively easy matter to isolate virtually monochromatic radiation by means of filters. The wavelengths correspond to the size of quanta emitted when electrons jump from higher to lower energy levels in the atom, and since the levels are characteristic of the emitting atom, the wavelengths of the lines emitted by a given kind of arc are always the same, although their relative intensities may vary.

None of the absorption spectra completely correspond to any of the action spectra, the fungicidal and bactericidal effects coming closest to matching. This indicates that injury results not from gross and general absorption by the cell but rather from absorption by some particular substance, the absorption spectrum of which corresponds to the action spectra. There are numerous reasons why action spectra, measured the only way in which we are able to measure them, i.e. outside of the living system, may not agree accurately with the absorption spectrum of that substance when inside the living system. There is the

possibility that part of the incident light is absorbed by non-participating substances that absorb selectively with respect to wavelength. An example of this is that of the action of ultraviolet on pollen grains where the pollen wall absorbs strongly the wavelengths that affect the living protoplasm within (175). It is still also possible for a non-participating substance inside the cell to act as an internal filter, a still more difficult effect to estimate quantitatively. Fluorescence is another possible source of difficulty, since not only does it serve to diffuse the light, but introduces chromatic impurity, as the fluoresced light has a different wavelength from that of the incident. Antagonistic effects of radiation from different parts of the spectrum have even been reported (59) and may be another source of discrepancy. Here several wavelengths may be effective and different parts of the light absorber may be involved in different reactions.

In review, there are many factors which may affect the action spectrum and may also interfere with an exact interpretation of its meaning. This makes one wonder if there is any value in determining such spectra for there are only a few cases of close agreement between action and absorption spectra. However, in some cases with carefully controlled systems, exact measurements may yield direct information but this must be tempered according to the problem on hand.

About seven different types of action spectra have been described and are discussed elsewhere (67). Those which are most commonly found suggest effects on two extremely important components of living systems, the non-conjugated proteins and the nucleic acids. The spectra for most proteins have maxima around 2800 Å, while those for nucleic acids is about 2600 Å. The effect on proteins is largely held responsible

for gross structural changes in the protoplasm. Action spectra which resemble absorption by nucleic acids are those for mutagenic effects (84,162); retardation of division (68); killing of viruses (82,83,146); the bactericidal effects (39,57,185); and the fungicidal effects (63,64, 94).

The action spectra resembling absorption by proteins and nucleic acids which underlie most of the ultraviolet studies make it difficult to escape the conclusion that these compounds, either alone or together, are involved in mediating the observed effects.

2. Suggested Modes of Bactericidal Action

Over the past quarter of a century there have been many theories advanced regarding possible modes of the bactericidal action of ultraviolet radiation on the bacterial cell. Among these, those most commonly cited are:

(a) Indirect effects through changes in the medium

(b) Decomposition of cellular material

(c) Inactivation of enzymes

(d) Lethal mutations

The order in which these are presented is not necessarily the order of their importance or of sequence of action. It is highly probable that radiations may act against the bacterial cell by more than one of these mechanisms either together or as a chain reaction. Each of the above mechanisms will be discussed briefly with regard to their place in the literature and their value in the light of recent knowledge.

(a) Indirect Effects Through Changes in the Medium:

The early explanations for the indirect lethal mechanisms of radiation assumed that the organisms were killed as a result of oxidation and the formation of hydrogen peroxide in the medium (3,16,177). The view was current that, in general, the destruction of organic material results primarily through oxidative processes and most workers believed that the presence of oxygen was essential for bactericidal action (153); this idea persisted until 1892 when it was corrected by Ward (177,178). At this time this theory was confirmed by the fact that observers noted that stirring or shaking exposed fluids enhanced killing efficiency.

Today we know that stirring or shaking cultures is essential in order that all organisms may be equally exposed to the radiation. Experimental evidence soon grew against the above view and Bie (18) showed that strong bactericidal action of ultraviolet could be demonstrated in the absence of air. Moore and Webster (122) attributed the germicidal power of ultraviolet rays to the production of formaldehyde in the medium but this theory never received any further support.

Many workers believed that irradiation of the culture medium previous to inoculation lessened its ability to support growth (39). Blank and Arnold (20) believe that radiation changes agar or agar-water gels and that there is formed a non-volatile, thermostabile material capable of diffusing through the gel. In confirming these results, Baumgartner (13) notes that irradiation of carbohydrates is accompanied by a marked production of acid, approximately half of which is formic. However, in the light of present day studies, the effects of radiation on carbohydrates can be ruled out as a source of the lethal action of radiation on bacteria for it is known that the ultraviolet energy required to appreciably affect carbohydrates far exceeds that necessary to kill a bacterial cell (20).

Bayne-Jones and Van der Lingen (14) were the first to determine the possibility of altering either the velocity of the bactericidal effect or the wavelength limits by exposure of bacteria in graded series of the pH range from pH 5.5 to 8.0. They found that there is no change in either effect within this range. Others have found that variation in the pH of the medium between 4.5 and 7.5 are without influence on the lethal action but at pH 9 and 10 there is a slight increase in bacterial sensitivity (64). Marchal (111) defines the pH limits as

between pH 6 and 8 in which there is no change in sensitivity. It is generally believed that below pH 4.5, the iso-electric point of bacteria, the time to kill drops off suddenly, so that acceleration of the killing action is marked in the more acid media. In the basic range, above pH 9 no definite conclusions can be drawn due to the many conflicting results in the literature. In the more recent work all experiments on ultraviolet radiation have been carried out in buffered solutions at or near neutrality where it is known that the media has no effect on the bactericidal action.

Such effects on the medium have little to do with the mechanism by which organisms on the surface of a plate or in a non-nutrient media are killed within a few seconds of exposure to ultraviolet rays. In this case, it is necessary to seek some other mechanism for the action of these rays on bacteria.

(b) Decomposition of Cellular Materials:

A great deal of work has been done to determine which of the many chemical constituents of bacteria absorb the light in the primary process by comparing the absorption spectra of various cellular constituents with the spectral regions of greatest lethal activity (140). These spectra show that proteins and nucleic acids absorb strongest in this region and therefore may be decomposed after being subjected to radiation. No attempt will be made to review all of the literature on the effects of ultraviolet light on proteins; this can be obtained in several comprehensive reviews (5,37,113,157), but we will outline some of the more obvious and important effects.

There have been quite a number of diverse effects ascribed

to proteins, however, in most cases little information appears as to the intensities employed. Examples of such effects are change in solubility (52), viscosity (36,40,120), absorption spectrum (113), surface tension (36,113), optical rotation and dispersion coefficients (113), electrical conductivity (113), and in molecular weight (5). Chemical changes include oxidation-reduction (157), ammonia formation (113) and liberation of sulphhydryl groups (118).

It is not known just how and where protein denaturation takes place. Neurath et al. (124) point out that the changes proteins undergo are so numerous that the term denaturation is ambiguous unless (a) the nature and magnitude of the changes are defined; (b) criteria for their recognition are established; and (c) the agents which cause these changes are known. Probably the field most extensively covered is that of the amino acids. Harris and Hoyt (79) have shown that absorption of ultraviolet light in the lethal regions by proteins is due very largely to their ring-containing amino acids -- tryptophane, phenylalanine and tyrosine; the influence of most of the remaining amino acids is evident chiefly in the absorption at wavelengths less than 2300 Å. The question arises, at what point is the protein molecule split? This is a moot question but calculations indicate that the peptide bond is the weakest and is therefore considered the one most likely to be broken (135). Most of the evidence for this theory comes from studies on analogues such as peptides of proteins on a surface.

In the past, the effect of exposure of nucleoproteins and nucleic acids to ultraviolet has not been too extensively studied or analysed and the literature in this field is very disorganized and

scanty. It is known that nucleic acids absorb strongest in the 2537 Å line where the lethal effects for vegetative cells of bacteria is most pronounced (95). Nucleic acids and nucleoproteins absorb light much more effectively than do proteins when both are compared by unit weight; the specific absorption by nucleoproteins and nucleic acids is said to be due to the chromophores in the purines and pyrimidines (26). It is thought that absorption of light by nucleic acid results in photodecomposition (81) followed by depolymerization of the acid (133) and it is possible that depolymerization of the acid may initiate all of the observed effects of the destructive action of ultraviolet on cells, but at present there is no evidence to link retardation of cell division, or the lethal and mutagenic effects with depolymerization. The possibility exists that ultraviolet light in some way interferes with the conversion of ribonucleic acid into desoxyribonucleic acid, as has been postulated for the ionizing radiations (105).

(c) Inactivation of Enzymes:

In the study of the effect of ultraviolet light on cellular enzymes, little use has been made of quantitative methods. Further, most studies have been in relation to their absorption spectra and thus isolated from the bacterial cell which in all probability does not give a true picture of the end effect achieved after irradiation of the cell as a whole. It was as early as 1879 when Downes and Blunt (51) first realized the importance of the effect of ultraviolet on enzymes. Since that time there have only been a few enzyme systems studied systematically. These are diastase (74,136), sucrase (70,71), lipase (137), pepsin (1,66), trypsin (33,87) and urease (170). In all of these cases

where the enzyme was studied isolated from the cell, there was found to be inactivation by ultraviolet in the lethal region of the spectrum. This work is further reviewed in detail in several monographs (55,58,113).

There is very little literature on the effects of radiation on respiratory enzyme systems, and what there is consists of a number of conflicting reports. This is unfortunate because probably the best approach to the study of irradiation effects on enzymes is through the respiratory enzyme systems. One drawback to good quantitative studies at this time was that methods and equipment used were not developed to the high state of refinement which they are today. However, no one in recent years has done any extensive work on this problem and it has just lay dormant. In view of the fact that inactivation of enzymes has been suggested as a possible mode of death by ultraviolet, this problem is important and it should be shown whether these enzymes are either unaffected and if they are to determine where the inactivation occurs.

(d) Lethal Mutations:

Most of the literature relating to lethal mutations as the cause of death of irradiated microörganisms deals with the effects of ionizing radiations. However, a few analogies can be made with the ultraviolet effects. With both x-rays and ultraviolet the survival curves are exponential over short ranges and the effect of a given dose is independent of the intensity (97,185). If, as with ionizing radiations, the lethal effect is caused by a single hit (95,185) which is quite unlikely, it would mean that with ultraviolet the absorption of a single quantum would be sufficient and the action would have to take place in a very small area. This area would have to have control over the rest of

the cell, or would contain the precursor for this controlling factor, and we might suppose it to be the genetical apparatus of the bacterium. If this was the case, it would be reasonable to believe that this mechanism would be concentrated in one spot and not scattered throughout the cytoplasm. Wyckoff (185) has calculated that the total number of quanta for the 2699 Å line absorbed per bacterium per second to be 136,000. He also determined what he considered to be a sensitive volume of a cell, this being of the order of 10^{-20} ml. and is approximately the volume occupied by an average size protein molecule. The interpretation that the absorption of one quantum would be lethal to the cell is unlikely for of the other thousands of quanta absorbed some at least must be effective.

Other evidence against lethal mutations as the cause of death in bacteria can be obtained from radiation resistant strains and a comparison with sensitive strains. The radiation resistant strain B/r of Escherichia coli which is capable of producing high mutations rates per cell division is not as easily killed by radiation as the sensitive parent strain B. If lethal mutations were responsible for death one would expect a lower survivor rate in the B/r strain. Also in the dose effect curve of strain B/r a plateau is formed by the percent mutations produced over a certain dose range, while at the same time there is an increase in killing. This does not rule out the possibility that lethal mutations may in part, but not wholly, be responsible for death. This latter case has been given for lethal effects in Neurospora crassa (6,7).

At present it is not universally accepted that bacteria have genes and chromosomes although recent evidence points to their existence and arrangement in bacteria similar to that of higher forms (43,98,100).

If this is true there is no reason not to believe that gene mutations may result in the death of a bacterium in the same manner as in higher organisms. Generally, a strong case for lethal gene mutations as a cause of death in irradiated bacteria cannot be given now and much of our interpretation has to rest on analogies.

3. Theories of Bacterial Variation

(a) Adaptation:

In surveying the literature in the general field of bacterial variation, one find that there has been a great deal of controversy about the theory of adaptation, the requirements for such a theory, and the role it plays in variation. Probably the greatest confusion exists in a basic disagreement characterizing the various interpretations of experimental results in variation phenomena.

That school which favours the theory of adaptation have endowed bacteria with a hypothetical inherent potential of sufficient flexibility to permit bacteria to adapt themselves to a multitude of environmental conditions (92,163,146,186). To quote Knight (92), "New enzymes are produced as a direct reaction to the chemical stimulus of the new nutrients, in the absence of the normal nutrients." Virtanen (176) points out that there are limits to the possibilities of adaptation by a given bacterial species. Adaptation widens the scope of enzyme action but only in accordance with well defined rules and a given strain of a bacterium can only be adapted to form certain enzymes. The ability to synthesize certain enzymes is thus a characteristic of a given strain and that irrevelent new enzymes can never be produced by organisms. Some workers believe enzymic changes take place without cell multiplication but by protoplasmic synthesis during the training period; others believe enzymes are only produced during propagation. It seems hard to conceive that a single cell could synthesize a new enzyme or enzyme system to cope with the varied environments it meets within its short life.

Recently greater emphasis has been put on the genetical aspect

of adaptation. The general assumption is that simple relations exist between genes and enzymes; this is at least true in the field of biochemical genetics where the one gene - one enzyme theory proposed by Beadle (15) has been largely adopted. Recent results obtained by Lederberg (99) seem to invalidate the identity hypothesis as it is understood in the case of an adaptive system. Lederberg studied the ability to ferment lactose in the sexually fertile K-12 strain of Escherichia coli which is associated with the production of an adaptive enzyme of the β-galactosidase type. The results obtained by the recombination of this and other characters indicate that the corresponding genes are held in a single linear linkage group. Monod (121) furthered this work by studying the ability to ferment glucose in the same organism. Here again, adaptation to glucose did not depend on one gene alone, however, specific genes may be implicated in determining the competence to adapt. The one gene - one enzyme theory may hold true for enzymes involved in millimicromolar reactions in the cell.

In a review of Braun (28) the list of arguments against the concept of adaptation includes: (a) the lack of direct correlation between specific environmental conditions and the type of change produced in populations submitted to them; (b) the occurrence of widely different types under identical environmental conditions (8,92,142); and (c) the fact that in dissociation, completely different environmental agents can produce identical results (46,73,76,96).

(b) Life-Cycles:

The life-cycle or 'cyclogenic' theory advanced by Enderlein (60) and extended by Mellon (114,115,116) has not been very widely accepted by bacteriologists. It states that the bacterial cell is potentially polyphasic,

and maintains that culture phases are stages in an orderly life cycle in which the expression of each stage is dependent on the environmental conditions. Thus, when the environment around a bacterium in the vegetative stage changes, particularly to a less favourable one, the first effect of the change would be to initiate a new reproductive process and a change in the nucleus to adapt itself to the new conditions of growth. The reorganization of the nucleus would then be to suppress, partially or wholly, reproduction by binary fission and to introduce a new form of sexual reproduction in order to give a new basis for variability and the new adaptation. The forms brought about by the new method of reproduction would be the filamentous, beaded, and 'involution' forms commonly seen in cultures.

With the gradual stabilization of the new form, the common vegetative form and reproduction by fission returns. Hadley (77), the greatest exponent of this theory states, "that each culture phase represents a stage in the development of the individual bacterium, whose span of life extends from the gonidium (or similiar reproductive entity) to the reproductively mature rough phase culture. On these grounds, the bacterium should not be conceived as a single cell, but as the entire range of successive culture developments from the gonidium to the mature form."

The main evidence against this theory is: (a) the frequent absence of an orderly succession of culture phases. A great deal of work has been carried out to determine whether there is a life-cycle in the pneumococci (19,42), Escherichia coli (173), Brucella abortus (80), Serratia marcescens (142), Micrococcus tetragenus (145), Haemophilus influenzae (35), and the lactobacilli (12) and in no case was any cycle

found; (b) the occurrence of independent changes of characteristics as shown in the Friedlaender and coli-aerogenes groups (134, Serratia marcescens (142), Micrococcus tetragenus (145), Corynebacterium diphtheriae (86); and (c) by more recent work which provides substantial proof for the existence of undirected, heritable changes in bacteria (45, 47,73,108,152).

(c) Mutation and Selection

The concept of mutations, i.e. permanent, spontaneous and undirected hereditary changes affecting one or more properties of a bacterial cell and its offspring, entered into the bacteriological literature almost as soon as the mutation theory was advanced for higher organisms by DeVries in 1900 (48). There is support for this theory by studies which fail to agree with either the 'adaptation' or 'cyclogenic' theories, but to which the mutation concept could be applied (143).

The first problem to arise is that of proving the spontaneous origin of mutants at more or less constant rates per cell division, for this is an important means of establishing the underlying similarity of these changes to those in higher organisms. The theory of spontaneous mutation assumes that variants arise spontaneously during growth under normal conditions, the part played by the adverse environment being purely selective. This problem was analyzed by Luria and Delbrück (108) on the basis of the frequency distribution of mutants and is based on the rate of growth of the mutants. The occurrence of rare mutants, in the order of approximately 1 in 10^{-5} per generation, obeying the laws of chance, is subject to large fluctuations, and successive samples taken from the same culture or from similar cultures give very erratic results.

These fluctuations themselves are a distinguishing feature of spontaneous mutations. If the change were introduced by the test environment in a certain proportion of the cells, this proportion should not differ from sample to sample, whether the samples come from the same culture or from a series of similar ones. If, however, the variants originate by mutation prior to the test, the chance occurrence of rare mutations will be reflected in large variations in their time of occurrence and, therefore, in the number of individuals present in each mutant clone. This, in turn, will result in large fluctuations in the proportion of mutants in different wild type cultures. The presence of such fluctuations in the number of mutants between cultures that have grown from one or two wild type cells is strong evidence of clonal grouping and hence of the mutational origin of variants. The spontaneous occurrence of mutations comparable in rates to those of higher organisms has been studied and applied by this 'fluctuation' test to the acquired resistance to bacteriophage (47,108), resistance to penicillin (44,45), sulphonamide resistance (132), histidine dependence to histidine independence (155), uracil dependence to independence (156), radiation resistance with x-rays and ultraviolet (73,168,181), growth factor requirements (151,152), and with environmental factors (159). The mutations investigated involved changes in resistance and metabolic processes rather than the smooth, rough, and mucoid changes typical of dissociation. Zelle (187), however, working with Salmonella typhimurium contributed direct observations in morphological variation of the spontaneous occurrence of R cells from an initially pure S culture by separating the cells in vitro as they divided.

Although most of the evidence is for the spontaneous occurrence of mutations rather than as an environmental response, the environment is

a very important factor in determining the course of bacterial variation. The environment may act in one of two ways: (a) bacterial mutations of an adaptive nature may require the environment to render phenotypical a change that in a different environment would have remained masked, or (b) the environment may act selectively by favouring the growth of certain phenotypes, as with bactericidal agents.

Population dynamics of how normal and mutant types compete in an environment in which they both can grow, depends on the effects of the mutation upon metabolic processes governing growth characteristics. Mutations producing apparent increases in biochemical activities (110,168), and growth rates (106) may prove unfavourable for growth under normal conditions although useful in exceptional environments. Growth rates, however, may not always be different (182) and one cannot always determine which of two phenotypes will establish itself in a mixture as the predominant one. Growth rate differences of various cell types within a colony will give rise to sectors (160), the size and shape of the sectors depending upon the growth rates of the variant cells. Luria (107) believes that further research on this type of variation will explain most cases of apparent 'directional' phase variation, in which different cultures of the same type appear to undergo a similar series of orderly changes. This may offer an alternate explanation to Hadley's work (77) and the cyclogenic theory.

It is generally believed that dissociation does not occur in the absence of propagation (27,78,134,158) which is further support for the theory of the mutational origin of variants. However, there is still some disagreement over this point (85,93,165) probably due to the lack of proper definition and a misinterpretation of terms.

There have been a number of claims in the earlier literature for the induction of bacterial variants by various environmental agents, destructive agents (72,75,76) and nutritional deficiencies (73,152,168), but in most of these cases due to their method of selection it is often very hard to determine whether the effect described is due to selective killing or really induced and most are better explained by the differential selection of spontaneous mutants. There are, however, a number of agents, x-rays, ultraviolet light and chemicals that are capable of producing increased mutation rates and new mutants, i.e. induced mutants, in higher organisms and some of these agents are also known to affect mutability in bacteria; perhaps the most useful agent of this type is radiation. The first critical work in this field to establish the true induction of mutations in bacteria is that of bacteriophage resistance after ultraviolet radiation described by Demerec (46) and since then numerous radiation induced mutants selected by destructive agents have been studied. With these mutations the spontaneous rates can be determined quite accurately and also all of the mutant individuals can be detected. After irradiation there is a substantial increase in the mutation rate, which increase becomes greater with increasing doses of radiation. Some of the induced mutations manifest themselves by the time the treated bacteria begin to divide, but the greater number are expressed after the bacteria have passed through several generations.

Bacterial genetics differs from the genetics of higher organisms chiefly because of the lack of information about the mechanism of orderly transmission of characters from generation to generation. Although direct genetic tests which might prove the occurrence of mutations in bacteria

cannot be made except in certain strains of Escherichia coli, it seems reasonable to assume from the accumulated evidence that in this group of microörganisms, changes that are comparable to gene mutations in higher organisms do occur. The case necessarily rests on analogies, and among the similarities between variations in bacteria and mutations in higher organisms which are most suggestive of a common underlying genetic basis are the following: (a) many variations in morphological and physiological characters in cultures of bacteria are transmissible, unchanged through generations, and are therefore considered to be stable and heritable (159); (b) the spontaneous origin of certain heritable and stable variations, independent of the specific treatments used to detect them, has been proved in some cases by means of special technics (44,108,156); (c) different characters within a strain may vary independently of one another (28,142); (d) physical and chemical agents known to be effective in increasing the rates of mutation in higher organisms have similar effects on bacteria (46, 73,76,168), the variants induced by these agents in bacteria, as in higher organisms, seem to be random and non-specific; (e) certain variations in bacteria leading to altered growth requirements and synthetic abilities form a series similar to those biochemical mutations in higher forms in which gene-controlled heredity has been established; (f) mutations in bacteria have a definite rate of occurrence per cell division (108,125); and (g) bacterial variations are sometimes reversible showing that the variation is not necessarily a loss, caused by unequal division or some similar process.

4. The Genetic Mechanism of Bacteria

The subject of whether the bacterial cell possesses a nucleus or nuclear material has long been of great interest to cytologists, bacteriologists and geneticists and as yet no satisfactory and accepted answer has been found. The literature on the subject is quite voluminous, contradictory and controversial and it is hard to put all of the reports and theories advanced on the same plane for study. Lewis (101) has divided the numerous theories advanced over the years into eight groups as follows:

1. Bacteria do not possess a nucleus or its equivalent (62).
2. The cell is differentiated into a chromatin-containing central body and peripheral cytoplasm (32).
3. The bacterial body is a nucleus devoid of cytoplasm; a naked nucleus or nuclear cell (154).
4. The nucleus consists of several chromatin bodies, a chromidial system scattered throughout the cytoplasm (61).
5. The form of the nucleus is not constant throughout the growth cycle; it may appear as a discrete spherical body, an elongated chromatin thread, or scattered chromidia depending on the stage of development; a polymorphic nucleus (49).
6. The nuclear substance consists of fine particles of chromatin dispersed uniformly in the cytoplasm but not distinguishable as morphological units; a diffuse nucleus (117).
7. The protoplast contains one or more true vesicular nuclei (188).
8. The nucleus is a naked invisible gene string, or a chromatin-encrusted gene string analogous to a single chromosome (104).

There is no one theory which has been accepted and all have met a great deal of criticism. Much of the early work must be regarded critically for at that time, many of our present-day cytological methods were unknown and evidence for nuclei were based mainly on staining reactions and cell inclusions, immature spores and cytoplasmic structures were often regarded as true nuclei.

There is no reason why we should not believe the bacterial cell to possess a nucleus and genetic apparatus similar to that of cells of higher organisms and now, with newer methods of cytological investigation and carefully controlled experiments, there is a great deal of data accumulating which points to the existence of a true nucleus which undergoes division and reproduction like cells of higher forms. Studies by Robinow (148,149) using rod-shaped bacteria show nucleus-like structures readily distinguishable from the cytoplasm and undergoing various stages of division during the log phase of multiplication of the bacterium. These chromatinic bodies are more or less dumbbell shaped and undergo division lengthwise in a plane parallel with the short axis of the bacterium - one dumbbell giving rise to two whole daughter dumbbells. Division of the bacterium by constriction of the cell wall may follow division of the chromatinic rod but is usually deferred until the chromatinic body has undergone one or two further divisions. There is growing evidence that this chromatinic body is of nuclear nature. Bacteria from young cultures in the logarithmic growth phase are usually free from cell inclusions, especially fat globules and volutin which take up nuclear stains; these chromatinic bodies have been seen in widely different species of bacteria; their division precedes cell division, and they take up nuclear stains. Ultraviolet microphotography

and the 'nucleal' reaction of Feuglen have demonstrated the presence of such a structure to have the morphological value of a nucleus. Boivin (23) and Tulasne and Vendrely (174) have furnished quite substantial proof of the cytoplasmic localization of ribonucleic acid and of the nuclear localization of desoxyribonucleic acid in bacteria by the digestion of one in the presence of the other followed by specific staining reactions. This is analogous to higher cells where the cytoplasm contains ribonucleic acid and desoxyribonucleic acid is present in the genes and chromosomes of the nucleus. Quite recently, DeLamater (43) has published data pointing to the occurrence of true mitosis in bacteria and the recognition of several phases of the mitotic cycle in prepared slides. This work cannot be reviewed critically nor evaluated at the present time as it is only based on initial studies and has not been substantiated by others as yet.

Recently Cavalli and Maccacaro (34) have presented quite good evidence for a true gene mutation in bacteria with chloromycetin resistance in Escherichia coli. These authors used crossing experiments and found that several loci were involved in gaining full resistance, and that the many loci had a cumulative action. Mapping loci of resistance is an indicative test for the linear arrangement of genes on the chromosome. Numerous experiments by Lederberg (98,99), Lederberg and Tatum (100), Tatum and Lederberg (169), Newcombe and Nyholm (128,129) and Rothfels (unpublished data) demonstrate the clearcut occurrence of chromosome linkage and segregation in the bacterium Escherichia coli by crossing several of the sexually fertile strains of this organism. If the same mechanism is in operation here as it is in higher, sexually fertile microorganisms and animals, the results of linkage are good evidence for chromosomes, and segregation is evidence for genes in bacteria, similar to those of higher forms.

5. Light Induced Recovery from Ultraviolet Radiation Injury

The discovery of light-induced recovery or 'photoreactivation' (88) of the lethal effects of ultraviolet radiation has become one of the most interesting problems to date in radiobiology. This phenomenon has been known for nearly a quarter of a century but it went unnoticed for a long time and its significance overlooked. It has only been in the latter part of the past decade that interest has been renewed, older data re-evaluated and the problem put on a quantitative basis. An excellent critical review of the early work in this field has been given by Prat (138).

Photoreactivation is the post-treatment with visible light and the near ultraviolet of microorganisms previously irradiated with ultraviolet of the bactericidal range. The first pertinent work was that of Whitaker (180) who found that lengthening of the lag phase in fertilized ultraviolet irradiated eggs of the alga Fucus furcatus could be partially prevented by subsequent treatment with white light. The problem was not investigated further as the effect was believed to be due to photosynthesis. Kelner's finding that conidia of Streptomyces griseus, an actinomycete, made non-viable by ultraviolet radiation,could be reactivated by while light, has stimulated much current interest, especially from the photochemical viewpoint, as it is known that here photosynthesis is not responsible for recovery (88). This has resulted in numerous studies attempting to determine how widespread the phenomenon is and the possible mechanism of action of ultraviolet and visible light. The majority of these studies have been concerned with reactiviation of ultraviolet killed organisms.

White light and near ultraviolet recovery has been shown for the apparent lethal effects and mutagenic effects to bacteriophage,

streptomycin, and other deleterious agents in Escherichia coli (89,90, 129,131,147) and other organisms and the phenomenon appears to be basically similar in all organisms for these effects. It has been demonstrated for recovery from apparent lethal effects in the yeast, Saccharomyces cerevisiae (89), in the mould Penicillium notatum (89,150) and in bacteriophage (56). There are also reports on the photoreactivation of injurious effects other than killing. Delay in cleavage of fertilized ultraviolet irradiated eggs of the sea urchins Arbacia punctulata (21,22,112), and Strongylocentrotus purpuratus (179) can be reversed by white light. Reactivation of ultraviolet induced delay in rhizoid formation and the photoreactivation of ultraviolet retarded adaptive galactozymase enzyme formation in yeast (167) are other effects.

It can be hypothesized that the photoreversable effects of these radiations such as killing, inhibition of adaptive enzyme formation, cleavage delay, are all manifestations of a basic disruption of the synthetic processes of the cell. Failure to obtain complete photoreactivation suggests a multiple effect of ultraviolet light.

Outline of Experimental Studies

The experimental investigations reported in this paper will be of a preliminary and limited nature of the lethal and mutagenic effects of monochromatic ultraviolet light upon the test organism Escherichia coli, strain B/r and of the methods and apparatus used in this study.

The report of the experimental work will be presented in four parts:

Part One: Growth characteristics, fermentative and biochemical properties of the test organism used throughout the experimental studies.

Section A: Growth curves of Escherichia coli B/r .

Section B: Fermentative and biochemical properties of Escherichia coli B/r and selected mutants.

Part Two: The lethal and mutagenic action of ultraviolet light on Escherichia coli B/r.

Section A: The lethal action of ultraviolet light.

Section B: The mutagenic action of ultraviolet light.

Section C: Photoreactivation of the ultraviolet induced mutagenic and lethal effects.

Part Three: The effect of monochromatic ultraviolet light on several respiratory enzyme systems of Escherichia coli B/r.

Part Four: Final discussion and summary of experimental results.

EXPERIMENTAL STUDIES

Part One

GROWTH CHARACTERISTICS, FERMENTATIVE AND BIOCHEMICAL PROPERTIES OF THE TEST ORGANISM USED THROUGHOUT THE EXPERIMENTAL STUDIES

The Growth Characteristics, Fermentative and Biochemical Properties of the Test Organism used Throughout the Experimental Studies

Test Organism: For our experiments we have employed Escherichia coli, strain B/r - a radiation resistant mutant strain derived from strain B by Witkin (181). This organism and strain is that most commonly used for bacteriological, biochemical and genetical studies employing radiation as a mutagenic agent. It is generally known that the coliform group of organisms are not fastidious in their growth requirements, grow rapidly on most media, give fairly constant and reliable tests of their metabolic activity and in most cases are non-pathogenic.

Escherichia coli strain B/r is known to be stable both in broth and on agar and no change in sensitivity to ultraviolet or in other characteristics has been observed. Resistance is, therefore, considered to be a stable, heritable character (182). The growth rates of the parent strain B and the resistant strain B/r show a slight difference in the length of their lag phase - that of B/r being about 25 percent shorter than the parent strain although their generation time is the same, about 20 minutes. Another difference between the sensitive and resistant strains is observed by examining cultures irradiated with low doses of ultraviolet, less than 100 ergs per mm^2, under the microscope. Gates (65) has shown that ultraviolet light exerts a specific inhibiting effect on the division of bacterial cells without affecting growth in length, resulting in the production of long filaments. Cells of the resistant strain treated in such a manner show no production of filaments and a normal lag phase and division occurs. The two strains are similar in that they have the same rate of spontaneous

mutation to antibacterial agents.

Method Employed for Growth of the Test Organism:

There is no problem in finding a culture medium in which the test organism used will grow but in finding one which does not absorb ultraviolet rays or contain large particles suspended in it which would mask the bacterial cells during the irradiation period and thus reduce the effectiveness of a given dose. Most of our common laboratory media - as peptone broths - fall in this latter class. That is, they contain protein molecules or aggregates as large as, or larger, than bacterial cells and thus may protect the bacteria from radiation.

This problem can be met in one of two ways: we may grow the bacteria in broth, wash them several times and resuspend them in saline or distilled water before irradiation; or we may grow the bacteria in a synthetic medium which does not contain particles or molecules which would offer protection to bacteria. For our purpose, we found the latter solution the best as it eliminates unnecessary handling of the culture, and is faster. The medium we have employed for growing the test organism is the M-9 synthetic medium of Anderson (2), the formula for which may be found in Appendix A of this report.

Test tubes, 30 mm. in diameter and 180 mm. in length are employed. These are fitted with a two hole stopper containing a 3 mm. aeration tube extending to the bottom of the culture tube and a short plugged air outlet tube. Fifty ml. of culture medium are used per tube; sterile, washed, compressed air is bubbled through the tube at the rate of 120 bubbles per minute. All cultures are grown in a water bath at 37°C.

Stock Culture and Inocula:

A single cell isolation from a saturated culture of Escherichia coli, strain B/r, was made and inoculated into ten ml. of peptone broth. This was the basis for the stock cultures used as the inocula throughout these experiments. The broth culture was incubated at 37°C for six hours and ten stock agar slants were prepared. These slants were the only source of inocula, thus the bacterial population should be fairly homogenous.

The test organism was twice subcultured in peptone broth for three hours each at 37°C and 0.1 ml. from the second subculture served as the inoculum for each subsequent culture prepared. The object of the subcultures was to obtain a uniform bacterial population consisting of young cells with which to inoculate the test culture. In this way the majority of the bacterial population at any given period of incubation should therefore be of the same age and consequently show the same resistance to deleterious effects.

Determination of Viable Cell Count:

For all estimations of viable cell count under any conditions, we employed the "drop plate" method using calibrated dropping pipettes as described by Reed and Reed (144). With this method a great number of counts can be made in a very short time with consistent results. Nutrient agar plates were used with this method, the formula for the agar being given in Appendix A. Plates to be used were incubated at 37°C after pouring for twenty-four hours and then let stand at room temperature for a further twenty-four hours before use. The plates at this stage were quite dry and the drops did not run over the surface but soaked in immediately. Colony counts were made after twenty-four hours incubation and the plates

were then re-incubated for another twenty-four hours to detect any change in count from late growing cells.

Section A

Growth Curves of Escherichia coli B/r

In starting this problem, one of the first things to be determined were growth curves for the test organism, Escherichia coli strain B/r. At the same time, with the methods used, it was convenient to study pH changes in the medium during growth of the culture and to determine the optimum age and size of inoculum to use.

At this time the question was raised as to whether aerating the cultures during growth increased the viable cell count or led to a more uniform cell count in similar cultures when compared with unaerated cultures. As Escherichia coli is generally considered a microaerophilic organism we did not believe that increased availability to oxygen would result in an increased cell count. However, our contentions were that bubbling air would act as a physical agitator, and would possibly remove, by oxidation, some waste or toxic products, but moreover, serve to keep the bacteria in suspension rather than let them settle out on the bottom of the vessel, thus giving them an optimum biological space relationship in turn resulting in a maximum concentration. How far our convictions proved to be correct are best seen in the composite growth curves summarized in Tables VII and XIV and Fig. 1.

Experiments to Determine the Growth Curve of the Test Organism *Escherichia coli* strain B/r and the Effect of Aeration During Growth

Experiment One

The culture medium for these experiments is the glucose-phosphate synthetic M-9 medium previously described. All cultures were 50 ml. in volume and grown in the water bath at 37°C. One ml. samples were removed aseptically at given time intervals, serial dilutions made, and viable cell counts determined by the "drop plate" method. Inoculated plates were incubated twenty-four hours at 37°C, the viable cell count determined and the plates incubated again for a further twenty-four hour period. No changes in colony counts were observed after the first incubation.

The culture for this experiment was grown *without* aeration; using a 3 mm. loopful of surface growth from a fourteen-day-old agar slope.

The results of Experiment One are given in Table I.

Table I

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	1.4×10^6	6.15
1	7.1	1.9×10^6	6.28
2	7.1	4.2×10^6	6.62
3	7.1	4.1×10^6	6.61
4	7.1	2.0×10^7	7.30
5	7.0	6.1×10^7	7.78
6	6.8	2.7×10^8	8.43
8	6.8	4.9×10^8	8.69
10	6.8	5.6×10^8	8.75
18	6.8	6.0×10^8	8.78
24	6.8	7.2×10^8	8.86

Experiment Two

This experiment is similar to Experiment One for materials, method and viable cell counts. The culture was grown without aeration, the inoculum used was 0.1 ml. of a 1:10 dilution made by emulsifying one 3 mm. loopful of surface growth from a fourteen-day-old agar slope.

The results of Experiment Two are given in Table II.

Table II

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	3.2×10^5	5.50
1	7.0	1.1×10^6	6.04
2	7.0	4.0×10^6	6.60
3	7.1	9.3×10^6	6.97
4	7.1	2.4×10^7	7.38
5	7.1	5.9×10^7	7.77
6	7.0	8.7×10^7	7.94
8	7.0	3.0×10^8	8.48
10	6.8	5.5×10^8	8.74
12	6.8	5.9×10^8	8.77
24	6.8	8.3×10^8	8.92

Experiment Three

Determination of a growth curve for Escherichia coli B/r in synthetic M-9 medium. The culture was grown without aeration with 1 ml. of a three hour broth culture as the inoculum.

The results of Experiment Three are given in Table III.

Table III

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	1.0×10^6	6.00
1	7.1	1.5×10^7	7.18
2	7.0	4.2×10^7	7.62
3	6.8	1.6×10^8	8.20
4	6.8	4.4×10^8	8.64
5	6.9	5.1×10^8	8.71
6	6.9	4.8×10^8	8.68
8	6.8	5.9×10^8	8.77
10	7.0	6.1×10^8	8.78
12	6.9	3.8×10^9	9.58
24	6.8	4.2×10^9	9.62
48	6.7	4.6×10^9	9.66

Experiment Four

This experiment is similar to Experiment Three. The culture was grown without aeration, however, the size of the inoculum differs. In this case we used 0.1 ml. of a 1:10 dilution of a three hour broth culture.

The results of Experiment Four are given in Table IV.

Table IV

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	2.6×10^5	5.42
1	7.0	1.1×10^6	6.04
2	7.0	3.2×10^6	6.50
3	7.1	8.9×10^6	6.95
4	7.1	4.2×10^7	7.62
5	7.0	5.3×10^7	7.72
6	7.0	1.9×10^8	8.28
8	6.9	3.6×10^8	8.56
10	6.9	4.1×10^8	8.61
12	6.8	8.5×10^8	8.93
24	6.8	1.5×10^9	9.18
48	6.9	3.7×10^9	9.56

Experiment Five

In this experiment and the next one, the experimental conditions are the same; both cultures being grown in synthetic M-9 medium without aeration. The volume of each inoculum is equal, they are from the same source - however, their age differs. In this case, the growth curve was determined using 0.1 ml. of a three hour broth culture as the inoculum.

The results from this experiment are presented in Table V.

Table V

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	8.8×10^5	5.94
1	7.0	3.1×10^6	6.49
2	7.1	3.9×10^6	6.59
3	7.2	8.4×10^6	6.92
4	7.0	3.3×10^7	7.52
5	6.8	5.0×10^7	7.70
6	6.8	9.2×10^7	7.96
8	6.7	2.9×10^8	8.46
10	6.6	6.1×10^8	8.78
12	6.8	8.2×10^8	8.91
24	6.8	8.6×10^8	8.93
48	6.8	7.5×10^8	8.88

Experiment Six

Determination of growth curve for Escherichia coli B/r, in synthetic M-9 medium using 0.1 ml. of a seventy-two hour broth culture as an inoculum. Culture grown at 37°C without aeration.

The results of this experiment are given in Table VI.

Table VI

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium Without Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.2	2.0×10^6	6.30
1	7.2	2.8×10^6	6.45
2	7.0	3.9×10^6	6.60
3	7.0	7.6×10^6	6.88
4	6.8	4.6×10^7	7.66
5	6.8	2.2×10^8	8.34
6	6.7	3.4×10^8	8.53
9	6.7	3.6×10^8	8.56
12	6.6	7.8×10^8	8.89
24	6.6	6.0×10^8	8.78
48	6.8	6.4×10^8	8.80

The following table is a compilation of the average viable cell counts from Experiments One to Six. In all experiments the cultures were grown in synthetic M-9 media without aeration at 37°C.

Table VII

Average Results of Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium With Aeration. The Data is Compiled from Results of Experiments One to Six

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	9.8×10^5	5.99
1	7.0	4.2×10^6	6.62
2	7.0	1.0×10^7	7.00
3	7.0	3.3×10^7	7.52
4	6.9	1.0×10^8	8.00
5	6.9	1.6×10^8	8.20
6	6.8	2.4×10^8	8.38
8	6.8	3.9×10^8	8.59
10	6.8	5.2×10^8	8.72
12	6.7	1.1×10^9	9.04
24	6.7	1.5×10^9	9.18
48	6.8	2.4×10^9	9.38

Experiment Seven

Determination of growth curve for Escherichia coli, strain B/r. Culture was grown in 50 ml. of synthetic M-9 medium at 37° C. with aeration (approximately 120 bubbles per minute). The inoculum was a 3mm. loopful of surface growth from a fourteen-day-old agar slope.

The results of this experiment are given in Table VIII.

Table VIII

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium With Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	3.2×10^6	6.50
1	7.0	5.6×10^6	6.75
2	7.0	5.7×10^6	6.76
3	7.1	6.4×10^7	7.81
4	7.0	1.5×10^8	8.19
5	6.9	3.0×10^8	8.48
6	6.9	4.7×10^9	9.67
8	6.8	4.6×10^9	9.66
10	6.8	1.6×10^{10}	10.20
12	6.8	2.3×10^{10}	10.36
16	6.8	1.1×10^{10}	10.04
26	6.9	5.4×10^9	9.73
48	7.0	5.2×10^9	9.72

Experiment Eight

This experiment is similar to Experiment Seven. The cells were in 50 ml. synthetic medium with aeration and the same age of inoculum used. It differs in the volume of inoculum in this case being 0.1 ml. of a 1:10 dilution made by emulsifying a 3 mm. loopful of surface growth from a fourteen-day-old agar slope in 10 ml. sterile distilled water.

The results of this experiment are recorded in Table IX.

Table IX

Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium with Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Counts (organisms per ml.)	Log of Count
0	7.0	5.9×10^5	5.77
1	7.0	1.1×10^6	6.04
2	7.1	4.6×10^6	6.66
3	7.2	5.7×10^6	6.76
4	7.1	3.6×10^7	7.56
5	7.0	9.2×10^7	7.96
6	7.0	4.5×10^8	8.65
8	6.8	1.0×10^9	9.00
10	7.0	6.2×10^9	9.79
12	6.8	8.4×10^9	9.92
24	6.8	1.5×10^{10}	10.18
48	7.0	3.4×10^{10}	10.53

Experiment Nine

The experimental conditions and procedure for Experiment Nine are the same as for the previous experiments, the cultures being grown in synthetic medium with aeration. Experiments Nine and Ten are similar, using the same age inoculum but with different volumes. In this case the inoculum is 1 ml. of a three hour broth culture.

The results of Experiment Nine are given in Table X.

Table X

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synethetic Medium With Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Counts (organisms per ml.)	Log of Count
0	7.2	2.8×10^6	6.45
1	7.2	2.1×10^7	7.32
2	7.2	7.8×10^7	7.89
3	7.2	1.9×10^8	8.28
4	7.0	7.6×10^8	8.88
5	7.0	1.0×10^9	9.00
6	7.0	3.5×10^9	9.54
8	6.8	2.3×10^{10}	10.36
10	6.8	2.9×10^{10}	10.46
12	6.8	2.8×10^{10}	10.45
24	6.8	3.0×10^{10}	10.48
36	6.6	4.2×10^9	9.62
48	6.6	2.8×10^9	9.45

Experiment Ten

An experiment similar to Experiment Nine but with a different volume of inoculum. Here 0.1 ml. of a 1:10 dilution from a three hour broth culture was used as the inoculum.

The experimental results are recorded in Table XI.

Table XI

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synthetic Medium with Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.1	2.1×10^5	5.32
1	7.1	1.9×10^6	6.28
2	7.2	6.5×10^6	6.81
3	7.1	2.0×10^7	7.30
4	7.0	7.4×10^7	7.87
5	7.0	3.6×10^8	8.56
6	7.0	9.1×10^8	8.96
8	6.8	4.0×10^9	9.60
10	6.9	6.1×10^9	9.78
12	6.8	7.9×10^9	9.90
24	6.8	1.0×10^{10}	10.00
36	6.8	2.4×10^{10}	10.38
48	6.6	9.7×10^9	9.98

Experiment Eleven

Growth curve determination for the test organism in synthetic medium with aeration. The inoculum used was 0.1 ml. of a three hour broth culture.

The experimental results are given in Table XII.

Table XII

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synthetic Medium With Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.2	1.1×10^7	7.04
1	7.2	4.1×10^7	7.61
2	7.2	7.9×10^7	7.90
3	7.1	3.8×10^8	8.58
4	7.1	8.2×10^8	8.91
5	7.0	1.2×10^9	9.08
6	7.1	5.2×10^9	9.72
8	7.0	5.9×10^9	9.77
10	6.8	7.1×10^9	9.85
18	6.8	8.8×10^9	9.94
24	6.8	5.1×10^9	9.71

Experiment Twelve

This experiment is similar to Experiment Eleven. The culture was grown in M-9 medium with aeration at 37°C. The inoculum is of the same volume but of a different age -- in this case we used 0.1 ml. of a forty-eight hour broth culture as the inoculum.

The results of Experiment Twelve are given in Table XIII.

Table XIII

Viable Cell Counts During Growth of *Escherichia coli* B/r in Synthetic Medium With Aeration

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.0	3.2×10^7	7.50
1	7.2	3.7×10^7	7.57
2	7.2	2.8×10^8	8.45
3	7.1	3.9×10^8	8.59
4	7.0	7.2×10^8	8.86
5	6.8	2.8×10^9	9.45
6	6.8	5.2×10^9	9.72
8	6.9	5.1×10^9	9.71
10	7.0	6.3×10^9	9.80
18	7.0	6.9×10^9	9.84
24	6.8	5.4×10^9	9.73

The following table is a compilation of the average viable cell counts from Experiments Seven to Twelve. The culture medium in all cases was synthetic M-9, the cultures being grown at 37°C with aeration.

Table XIV

Average Results of Viable Cell Counts During Growth of Escherichia coli B/r in Synthetic Medium With Aeration. The Data is Compiled From Results of Experiments Seven to Twelve

Age of Culture (hours)	pH of Medium	Viable Cell Count (organisms per ml.)	Log of Count
0	7.1	8.3×10^6	6.92
1	7.1	1.8×10^7	7.25
2	7.2	7.6×10^7	7.88
3	7.1	1.7×10^8	8.23
4	7.0	4.3×10^8	8.63
5	7.0	9.6×10^8	8.98
6	7.0	3.3×10^9	9.52
8	6.8	7.3×10^9	9.86
10	6.9	1.2×10^{10}	10.08
12	6.8	1.7×10^{10}	10.23
24	6.8	1.3×10^{10}	10.14
48	6.8	1.3×10^{10}	10.14

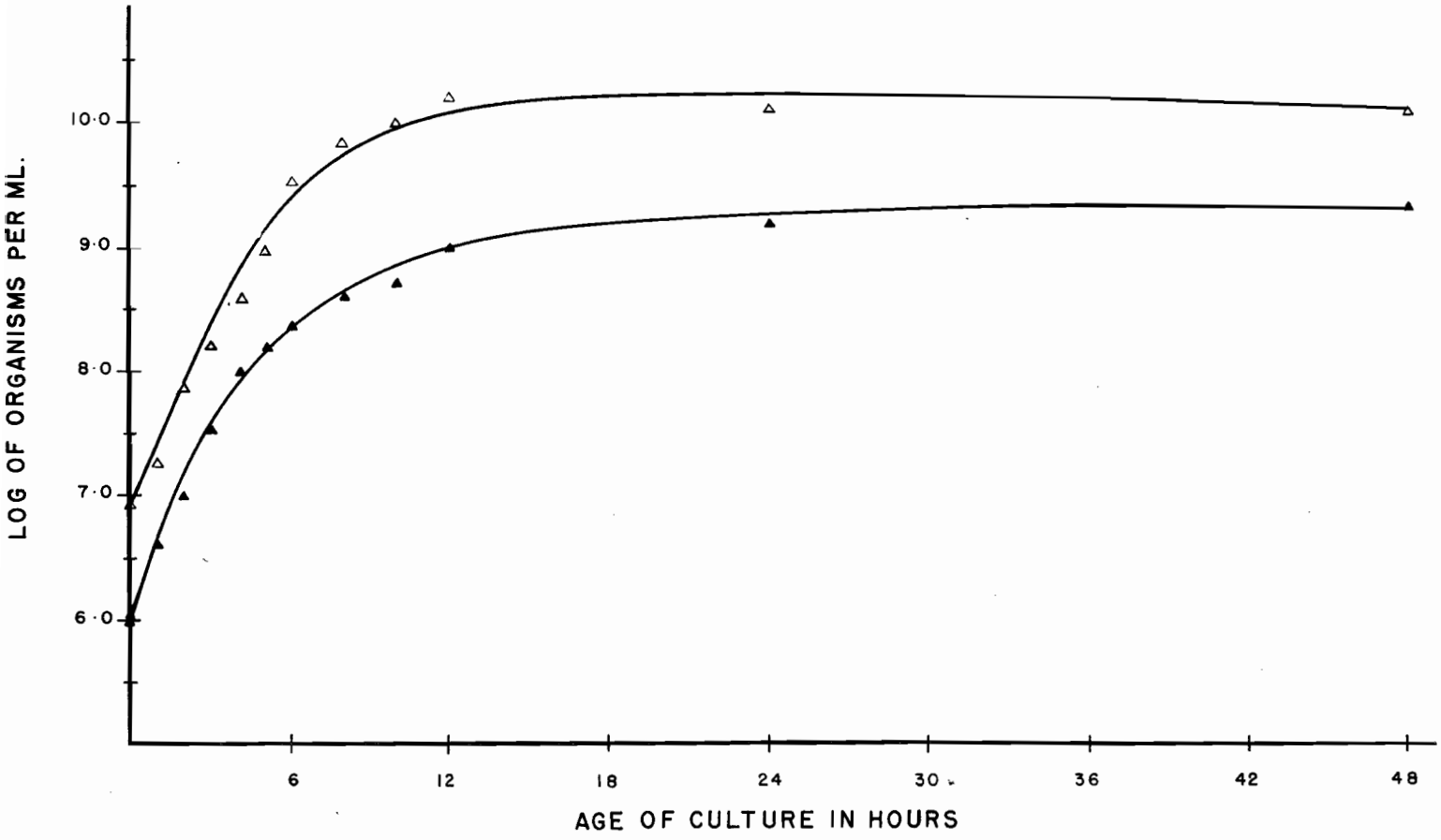


FIG. I. GROWTH CURVES OF Escherichia coli, STRAIN B/r

△ = with aeration ▲ = without aeration

Discussion of Results

The age and size of inoculum used in these experiments has very little apparent effect on the lag phase of the growth curve. It is to be noted that the lag phase is very short, usually less than an hour; this has previously been stated as a characteristic of this strain (181). The pH change of the medium during growth of the culture is very slight -- seldom varying more than a few tenths of a point in any culture even after prolonged incubation.

From Tables VII and XIV, it can be seen that aeration of the cultures does have a pronounced effect on the growth curve; it generally increases the population tenfold. Cultures grown with aeration usually have a shorter lag, a steeper and longer logarithmic phase and a higher titre in the stationary phase of growth. It is uncertain why aeration has this effect and our hypothesis is that it acts as an agitator to keep the bacteria suspended thus giving them the maximum biological space relationship.

The general procedure used in all later experiments which we have derived from these results is quite simple. It is to grow the cultures with aeration in synthetic M-9 media with aeration; the inoculum used is 0.1 ml. of a three hour broth culture previously passed through two successive three hour subcultures. This we find usually gives us constant and uniform cell counts for any culture.

Section B

The Fermentative and Biochemical Properties of *Escherichia coli* B/r and Selected Mutants

Introduction

The following experiments were designed and carried out to determine whether mutant colonies induced by radiation and selected by colour response on mannitol-tetrazolium chloride agar (formula in Appendix A) differed from the normal unirradiated colonies in their common fermentative and biochemical properties generally given for the classification of this organism. There is no reason to believe, however, that just because we select a mutation to one character, in this case colour response to mannitol tetrazolium agar, we should expect a linked change with some other character, although in some cases there are linked changes.

The majority of the colour mutants differ very slightly from the non-mutants and are detectable only where they occur as sectors in otherwise non-mutant colonies. A few of the more striking ones could be identified as whole colony mutants but to make the test as objective as possible only sectored colonies were picked.

It is unlikely that any appreciable proportion of these colour mutants are associated with change in mannitol fermentation since the more striking ones are distinguishable when streaked on eosin methylene blue-lactose agar or xylose-tetrazolium agar. Mannitol-tetrazolium agar was chosen because of the ease in scoring on this medium, and because the colour is less likely to become too deep with prolonged incubation, or to fade with storage in the cold.

Methods

Synthetic M-9 medium was inoculated with 0.1 ml. of a three hour broth culture from the stock culture of Escherichia coli B/r. This culture was grown aerated in a 37°C waterbath for twenty-four hours. Assays made at this time show a viable count of approximately 5×10^9 viable cells per ml.

Undiluted suspensions were irradiated with a 15 Watt, General Electric Germicidal Lamp, this being estimated to deliver 95 percent of the ultraviolet energy in the 2537 Å line. For irradiation 10 ml. quantities of the suspension were placed in Petri dishes of 100 mm. diameter and 20 mm. in depth and shaken mechanically in order to ensure uniform exposure of all cells. The frequency and amplitude of the shaker were such as to produce standing waves. The apparatus used is described in Appendix B of this paper.

Dosage was measured in ergs per mm^2, exposure timed by a stopwatch and previously measured by a Westinghouse photoelectric ultraviolet meter. The doses used in these experiments ranged from 500 to 3000 ergs per mm^2.

After irradiation mannitol-tetrazolium agar plates were spread in suitable dilutions and incubated for twenty-four hours. After incubation, the plates were chilled for twelve hours to allow the colour to deepen to facilitate picking the mutant colonies. From the irradiated plates ten sectored colonies were picked at random for each dose used; the sectors were picked, inoculated into 2 ml. M-9 medium, incubated for twenty-four hours, and spread in suitable dilution on mannitol-tetrazolium agar; the colonies which grow should not be sectored but should be pure

mutants. If they were not the process was repeated. To test whether these cultures were pure mutants or not, equal numbers of these cultures and non-mutant cultures were spread together on plates. These plates, following incubation, should show approximately equal numbers of mutant and non-mutant colonies.

Experiment Thirteen

Control Culture

From an unirradiated culture spread on a mannitol-tetrazolium agar plate, five non-mutant colonies were picked and incubated in 2 ml. M-9 medium for six hours. From this culture the biochemical test media and sugars given in Tables XV and XVI were inoculated and their reactions determined.

From these tests it can be seen that there are no differences between any cultures or from the characteristic reactions given in Bergey's Manual for *Escherichia coli*.

Table XV

Biochemical Reactions of Unirradiated Cultures

Culture Number	gelatin liquefaction	litmus milk	indole produced	nitrite reduction	utilize citric salts	urea decomposed	H_2S produced	methyl red	Voges-Proskauer
M-500-TTC-1	-	a	+	+	-	-	-	+	-
M-500-TTC-2	-	a	+	+	-	-	-	+	-
M-500-TTC-3	-	a	+	+	-	-	-	+	-
M-500-TTC-4	-	a	+	+	-	-	-	+	-
M-500-TTC-5	-	a	+	+	-	-	-	+	-

Table XVI

Fermentation Tests of Unirradiated Cultures

Culture Number	glucose	fructose	galactose	lactose	maltose	arabinose	xylose	rhamnose	mannitol	sucrose	raffinose	salicin	dulcitol	glycerol	inulin	dextrin	starch	inositol
O-TTC-1	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
O-TTC-2	ag	ag	ag	a	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
O-TTC-3	ag	a	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
O-TTC-4	ag	ag	ag	ag	-	ag	ag	a	a	-	-	-	-	-	-	-	-	-
O-TTC-5	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-

a = acid; g = gas; - = no reaction

Irradiated Samples

The methods described herein are for Experiments Fourteen to Seventeen in which the biochemical and fermentation tests are carried out on mutant cultures induced by irradiation and selected on mannitol-tetrazolium agar.

Ten ml. volumes of undiluted culture were irradiated with appropriate doses (500 - 3000 ergs per mm^2) of ultraviolet in the 3537 Å line. After irradiation, the cultures were suitably diluted and five plates spread for each culture using 0.1 ml. inocula. After incubation and refrigeration, ten mutant sectored colonies for each series or irradiation doses were selected at random, a small inoculum picked from the sector of each colony and seeded separately into 2 ml. volumes of broth. These cultures were passed through two subcultures as previously described and pure mutant colonies picked for the test cultures.

From the test mutant cultures the biochemical test media and sugars were inoculated. The results of these experiments are given in Tables XVII to XXIV in Experiments Fourteen to Seventeen.

Experiment Fourteen

Table XVII

Biochemical Reactions

Ultraviolet Irradiation With 500 ergs per mm^2 at 2537 Å

Culture Number	gelatin liquefaction	litmus milk	indole produced	nitrite reduction	utilize citric salts	urea decomposed	H_2S produced	methyl red	Voges-Proskauer
M-500-TTC-1	-	a	+	+	-	-	-	+	-
M-500-TTC-2	-	a	+	+	-	-	-	+	-
M-500-TTC-3	-	a	+	+	-	-	-	+	-
M-500-TTC-4	-	a	+	+	-	-	-	+	-
M-500-TTC-5	-	a	+	+	-	-	-	+	-
M-500-TTC-6	-	a	+	+	-	-	-	+	-
M-500-TTC-7	-	a	+	+	-	-	-	+	-
M-500-TTC-8	-	a	+	+	-	-	-	+	-
M-500-TTC-9	-	a	+	+	-	-	-	+	-
M-500-TTC-10	-	a	+	+	-	-	-	+	-

a = acid; - = no reaction; + = positive reaction

Table XVII

Fermentation Tests

Ultraviolet Irradiation With 3000 ergs per mm^2 at 2537 Å

Culture Number	glucose	fructose	galactose	lactose	maltose	arabinose	xylose	rhamnose	mannitol	sucrose	raffinose	salicin	dulcitol	glycerol	inulin	dextrin	starch	inositol
M-500-TTC-1	ag	ag	ag	a	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-2	ag	ag	ag	ag	-	ag	ag	ag	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-3	a	ag	ag	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-4	ag	ag	a	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-5	ag	ag	a	ag	-	a g	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-6	ag	ag	ag	a	-	a	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-7	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-8	ag	ag	ag	ag	-	a	a	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-9	ag	a	ag	a	-	a	ag	a	ag	-	-	-	-	-	-	-	-	-
M-500-TTC-10	a	ag	a g	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-

a = acid; g = gas; - = no reaction

Experiment Fifteen

Table XIX

Biochemical Reactions

Ultraviolet Irradiation With 1000 ergs per mm^2 at 2537 Å

Culture Number	gelatin liquefaction	litmus milk	indole produced	nitrite reduction	utilize citric salts	urea decomposed	H_2S produced	methyl red	Voges-Proskauer
M-1000-TTC-1	-	a	+	+	-	-	-	+	-
M-1000-TTC-2	-	a	+	+	-	-	-	+	-
M-1000-TTC-3	-	a	+	+	-	-	-	+	-
M-1000-TTC-4	-	a	+	+	-	-	-	+	-
M-1000-TTC-5	-	a	+	+	-	-	-	+	-
M-1000-TTC-6	-	a	+	+	-	-	-	+	-
M-1000-TTC-7	-	a	+	+	-	-	-	+	-
M-1000-TTC-8	-	a	+	+	-	-	-	+	-
M-1000-TTC-9	-	a	+	+	-	-	-	+	-
M-1000-TTC-10	-	a	+	+	-	-	-	+	-

a = acid; - = no reaction; + = positive reaction

Table XX

Fermentation Tests

Ultraviolet Irradiation With 1000 ergs per mm^2 at 2537 Å

Culture Number	glucose	fructose	galactose	lactose	maltose	arabinose	xylose	rhamnose	mannitol	sucrose	raffinose	salicin	dulcitol	glycerol	inulin	dextrin	starch	inositol
M-1000-TTC-1	ag	ag	ag	ag	-	ag	ag	ag	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-2	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-3	ag	ag	ag	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-4	ag	ag	a	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-5	ag	ag	ag	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-6	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-7	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-8	a	ag	a	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-9	ag	ag	ag	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-1000-TTC-10	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-

a = acid; g = gas; - = no reaction

Experiment Sixteen

Table XXI

Biochemical Reactions

Ultraviolet Irradiation With 2000 ergs per mm^2 at 2537 Å

Culture Number	gelatin liquefaction	litmus milk	indole produced	nitrite reduction	utilize citric salts	urea decomposed	H_2S produced	methyl red	Voges-Proskauer
M-2000-TTC-1	-	a	+	+	-	-	-	+	-
M-2000-TTC-2	-	a	+	+	-	-	-	+	-
M-2000-TTC-3	-	a	+	+	-	-	-	+	-
M-2000-TTC-4	-	a	+	+	-	-	-	+	-
M-2000-TTC-5	-	a	+	+	-	-	-	+	-
M-2000-TTC-6	-	a	+	+	-	-	-	+	-
M-2000-TTC-7	-	a	+	+	-	-	-	+	-
M-2000-TTC-8	-	a	+	+	-	-	-	+	-
M-2000-TTC-9	-	a	+	+	-	-	-	+	-
M-2000-TTC-10	-	a	+	+	-	-	-	+	-

a = acid; - = no reaction; + = positive reaction

Table XXII

Fermentation Tests

Ultraviolet Irradiation With 2000 ergs per mm^2 at 2537 Å

Culture Number	glucose	fructose	galactose	lactose	maltose	arabinose	xylose	rhamnose	mannitol	sucrose	raffinose	salicin	dulcitol	glycerol	inulin	dextrin	starch	inositol
M-2000-TTC-1	ag	ag	ag	a	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-2	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-3	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-4	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-5	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-6	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-7	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-8	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-9	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-2000-TTC-10	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-

a = acid; g = gas; - = no reaction

Experiment Seventeen

Table XXIII

Biochemical Reactions

Ultraviolet Irradiation With 3000 ergs per mm^2 at 2537 Å

Culture Number	gelatin liquefaction	litmus milk	indole produced	nitrite reduction	utilize citric salts	urea decomposed	H_2S produced	methyl red	Voges-Proskauer
M-3000-TTC-1	-	a	+	+	-	-	-	+	-
M-3000-TTC-2	-	a	+	+	-	-	-	+	-
M-3000-TTC-3	-	a	+	+	-	-	-	+	-
M-3000-TTC-4	-	a	+	+	-	-	-	+	-
M-3000-TTC-5	-	a	+	+	-	-	-	+	-
M-3000-TTC-6	-	a	+	+	-	-	-	+	-
M-3000-TTC-7	-	a	+	+	-	-	-	+	-
M-3000-TTC-8	-	a	+	+	-	-	-	+	-
M-3000-TTC-9	-	a	+	+	-	-	-	+	-
M-3000-TTC-10	-	a	+	+	-	-	-	+	-

a = acid; - = no reaction; + = positive reaction

Table XXIV

Fermentation Tests

Ultraviolet Irradiation With 3000 ergs per mm^2 at 2537 Å

Culture Number	glucose	fructose	galactose	lactose	maltose	arabinose	xylose	rhamnose	mannitol	sucrose	raffinose	salicin	dulcitol	glycerol	inulin	dextrin	starch	inositol
M-3000-TTC-1	ag	ag	ag	ag	-	ag	a	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-2	ag	ag	ag	a	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-3	ag	ag	a	a	-	a	ag	a	a	-	-	-	-	-	-	-	-	-
M-3000-TTC-4	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-5	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-6	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-7	ag	ag	ag	a	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-8	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-9	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-
M-3000-TTC-10	ag	ag	ag	ag	-	ag	ag	a	ag	-	-	-	-	-	-	-	-	-

a = acid; g = gas; - = no reaction

Summary

It is apparent from the results presented in the previous tables that the test organism, Escherichia coli strain B/r, and the mutants induced by radiation and selected on mannitol-tetrazolium agar behave in a similar manner in their biochemical and fermentative reactions. This is indicative that the mutants, selected at random, are not contaminants nor is the mutation to colour response linked with any of the biochemical or fermentative properties of the organism which were tested for.

Part Two

THE LETHAL AND MUTAGENIC ACTION OF ULTRAVIOLET LIGHT ON *ESCHERICHIA COLI*, STRAIN B/r

Section A

The Lethal Action of Ultraviolet Light on Escherichia coli, B/r

Introduction

The lethal effects of the far ultraviolet radiations on bacteria has been a controversial question for many years. Toxic effects of parts of this region have been reported by Ward (178), Bayne-Jones and Van der Lingen (14) and Coblentz and Fulton (39) while negative results have been reported by Gates (64), Ehrismann and Noethling (57) and by Bachem and Dushkin (11). Additional literature is reviewed in the historical introduction of this report.

Rahn and Barnes (141) in an experimental comparison of various criteria of death in yeasts, noted that by definition these are mostly negative. "We call an organism dead when it shows no more the symptoms which we consider characteristic of life." The criteria which these workers studied were: (a) loss of reproductive power as determined by plate counts; (b) loss of fermentative ability; (c) change in staining reactions; (d) loss of selective permeability. In our work we have studied the effects of radiation on the viable cell count after varied periods of exposure to the harmful influence as determined by the 'drop plate' method.

In observations on single cells of Escherichia coli in the first few hours after irradiation with ultraviolet rays (2537 Å), Gates (65) has shown that there is a wide zone of behaviour between cells which are not visibly affected and those which seem to be killed outright by the exposure. Organisms which have apparently been unaffected grow and divide

regularly on the surface of nutrient agar. Organisms which have been subjected to lethal doses show no increase in size and no cell division, soon lose their high refractility, become beaded and degenerate into ghosts or shadows which are undoubtedly dead. Those organisms in the intermediate zone between these two extremes increase in size, especially in length without apparent inhibition, but do not divide when the normal adult stage is reached; so that long filaments of clear protoplam are formed. After a few hours the cells reach a limit of individual growth and cell division or degenerative changes begin: (a) the long cells may undergo a gradual degeneration with beading and ghost formation; (b) they may divide by cross fission into a number of large or small units which degenerate without further growth; or (c) one or more normal looking daughter cells may pinch off at one end and multiply rapidly to colony formation.

Here then, is an apparent separation of two coördinate functions commonly essential to life. Cell division is regulated by a mechanism which is more sensitive to these ultraviolet wavelengths than is the concomitant function of growth. Unless the division mechanism is restored the cell soon degenerates.

These effects, however, are not observed with the resistant strain used throughout this work. Cells of the resistant strain after exposure to ultraviolet radiation are either (a) killed outright or fail to grow on agar, or (b) begin to grow immediately with a very short lag phase.

In this work it has been our practice to do viable cell counts for all plates at twenty-four, forty-eight, and seventy-two hours of incubation. In this way, any increase in cell count due to inhibition of

division which occurs as a result of the treatment with radiation is determined. However, in no case have we found an increase in cell count after the first twenty-four hour incubation.

Experimental Procedure

The experimental procedure used to determine the effect of increasing doses of ultraviolet radiation on the test organism is quite simple and straightforward. It is as follows:

1. The test organism was grown in aerated M-9 media as described in Part One of this paper.
2. Ten ml. samples of culture were irradiated with increasing doses of ultraviolet light in the 2537 Å line.
3. After irradiation the samples were shaken on an electric vibrating machine to break up any cell aggregates and ensure a uniform suspension for cell counts. This electric vibrating machine is described in Appendix C. The optimum rate of vibration for our purpose was found to be approximately 1300 vibrations per minute. At this rate maximum turbulence results. Three minutes vibration using this machine is sufficient to produce satisfactory results.
4. Estimation of the total cell count using the Thoma counting chamber, and determination of the viable cell count by the 'drop plate' method.

Rather than include numerous similar tables and figures of the lethal effect of ultraviolet on the test organism, only three average tables will be presented and one figure - the data for which is the average of the tables given. These results are now given in Tables XXV to XXVII.

Experiment Eighteen

Table XXV

Survival of Escherichia coli B/r with Increasing Doses of Radiation

Total Cell Count = 4.1×10^8 org./ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survival Ratio (Viable to Total Count)	Percent Survival
0	3.7×10^8	1 : 1.1	100
500	8.0×10^7	1 : 5.1	21.62
1000	1.8×10^5	1 : 2.3×10^3	4.8×10^{-2}
3000	1.6×10^4	1 : 2.5×10^4	4.3×10^{-3}
5000	1.9×10^2	1 : 2.1×10^6	5.1×10^{-5}

Experiment Nineteen

Table XXVI

Survival of Escherichia coli B/r with Increasing Doses of Radiation

Total Cell Count = 1.2×10^9 org./ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survival Ratio (Viable to Total Count)	Percent Survival
0	9.6×10^8	1 : 1.2	100
500	1.7×10^8	1 : 7.0	17.70
1000	5.1×10^5	1 : 2.4×10^3	5.3×10^{-2}
3000	1.3×10^4	1 : 9.2×10^4	1.3×10^{-3}
5000	5.8×10^2	1 : 2.0×10^6	6.1×10^{-5}

Experiment Twenty

Table XXVII

Survival of *Escherichia coli* B/r with Increasing Doses of Radiation

Total Cell Count = 1.7×10^9 org./ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survival Ratio (Viable to Total Count)	Percent Survival
0	1.1×10^9	1 : 1.5	100
500	1.5×10^8	1 : 10.1	13.63
1000	5.4×10^5	1 : 3.1×10^3	4.9×10^{-2}
3000	4.2×10^4	1 : 4.0×10^4	3.8×10^{-3}
5000	1.5×10^3	1 : 1.0×10^6	1.4×10^{-5}

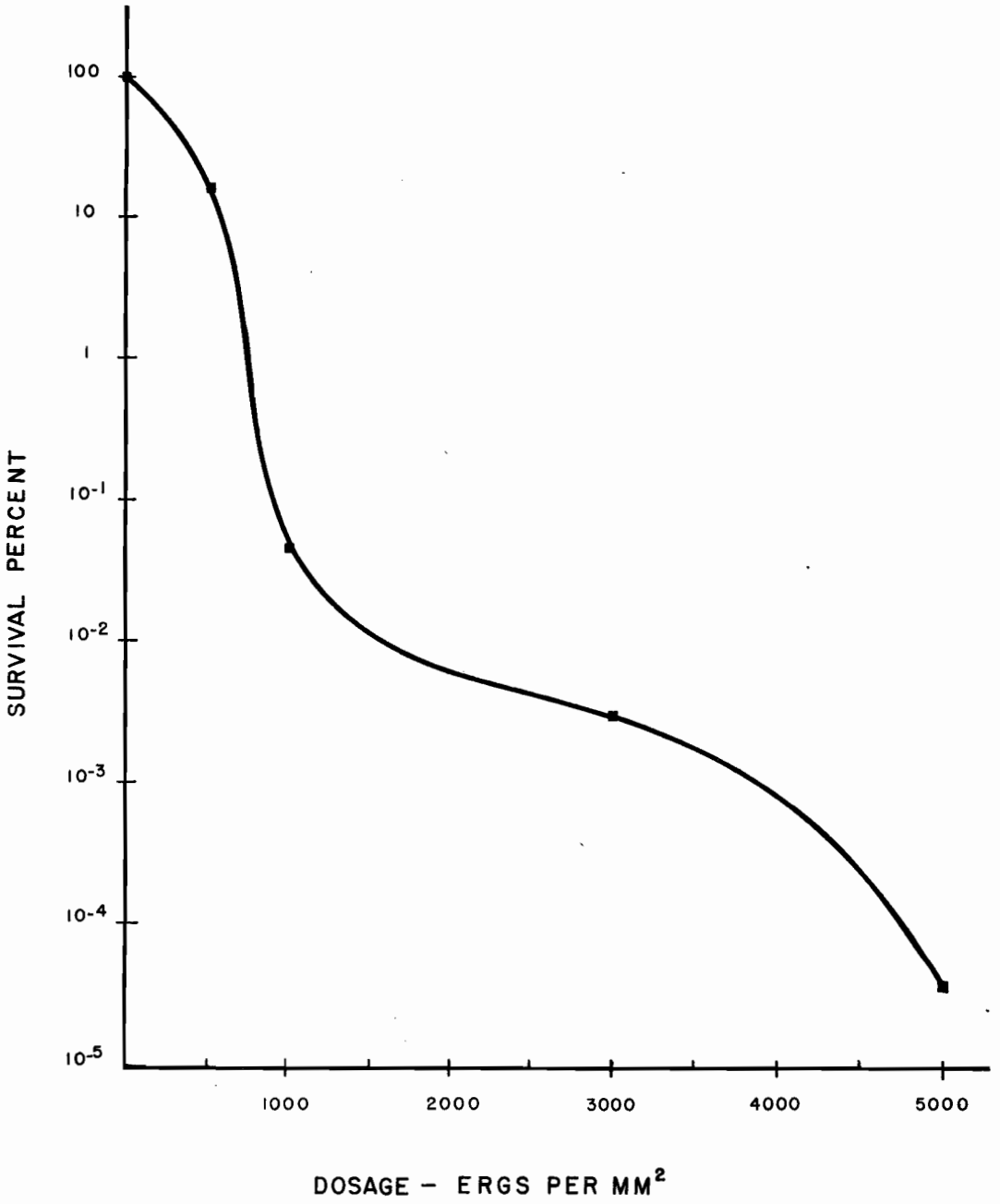


FIG. 2. ULTRAVIOLET (2537 Å) SURVIVAL CURVE OF *Escherichia coli* STRAIN B/r

Section B

The Mutagenic Action of Ultraviolet Light on *Escherichia coli* B/r

When living cells are irradiated with ultraviolet light, some are killed and some of the survivors are caused to mutate. These mutations result from an increase in mutation rate and are not due to selective action in favour of pre-existing mutants in the culture. The changes induced by irradiation cannot be distinguished from those which occur spontaneously..

The mutation studied is to a change in colour response on mannitol-tetrazolium agar. The colour mutants were obtained simply by plating suitable numbers of the treated bacteria on the mannitol-tetrazolium agar, and incubating until the colonies developed. After the incubation period it was our practice to refrigerate the plates overnight, allowing the colour to deepen and thus facilitate scoring. In scoring the number of mutations only the sectored colonies were counted; the whole colony mutants differing very slightly from the non-mutant colonies. The results of the mutagenic action of ultraviolet light on *Escherichia coli* B/r are presented in Table XXVIII and Fig. 3. The data given are the results of numerous experiments carried out over the period of one year. It can be seen that with lower doses, 0 - 1000 ergs per mm^2, the percentage of mutations rises quite rapidly until a threshold is reached, the mutagenic effect becomes stable and a plateau is formed between 1000 - 5000 ergs per mm^2.

Experiment Twenty-One

Table XXVIII

The Effect of Increasing Ultraviolet Light - 2537 Å - on the Percent of Induced Mutations of *Escherichia coli* B/r

UV Dose ergs per mm^2	Colonies Examined	Mutant Colonies	Percent Mutation
0	7832	18	0.2
500	7206	838	11.6
1000	1487	210	14.1
1500	3899	423	10.9
2000	2211	297	13.4
2500	1228	163	13.3
3000	2733	345	12.6
5000	1016	136	13.4

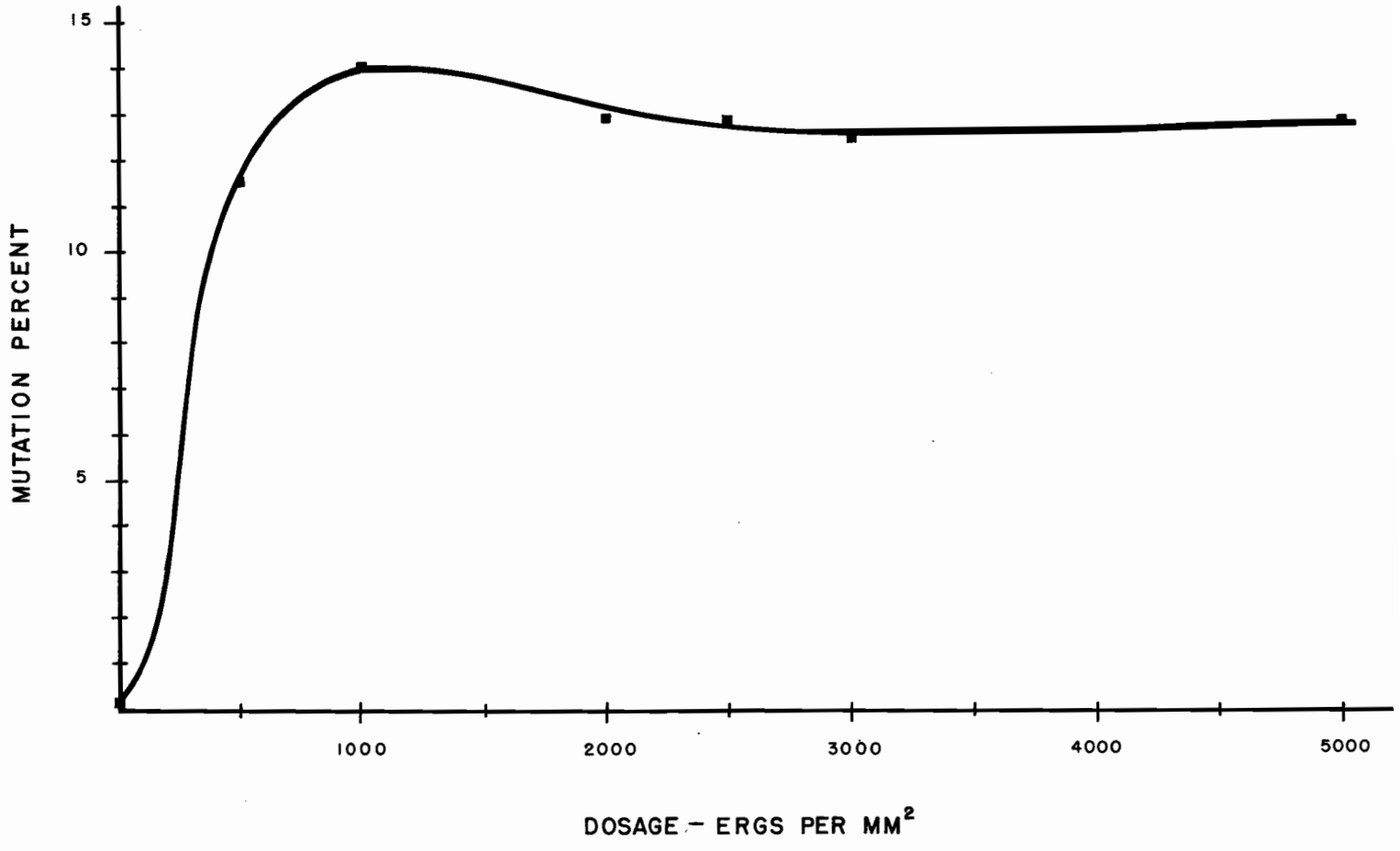


FIG. 3. MUTAGENIC EFFECT OF ULTRAVIOLET (2537 Å) ON Escherichia coli STRAIN B/r

Section C

Photoreactivation of the Ultraviolet Induced Mutagenic and Lethal Effects in Escherichia coli B/r

Introduction

In the past few years numerous workers have found that both the apparent lethal and induced mutation effects of ultraviolet light in the 2537 Å line, can be partially reversed by post-treatment with visible light and the near ultraviolet. The reversal of the suppression of growth is termed "photoreactivation" (88) and has been demonstrated to occur in a wide range of organisms. More recently a similar reversal of the mutagenic effect has been reported to occur in most organisms which show the photoreactivation phenomenon. A literature review of this is to be found in the historical review of this thesis.

We have studied the effects of post-treatment with ultraviolet light of the 3650 Å line on the lethal effects, and of induced mutations to a change in colour response by sectorial colonies on mannitol-tetrazolium agar, with the resistant strain B/r of Escherichia coli.

Experimental

In our early experiments, subsequent light treatment was by means of a General Electric B-H6 mercury vapour lamp. This lamp delivers approximately 1000 Watts and has a strong emission in the 3500 - 4500 Å region (the portion of the spectrum most active in photoreactivation). High intensities were obtained by keeping the distance between the material and the lamp at a minimum, approximately two inches. The material was

held in a waterbath at 37°C during light treatment and the light was passed through a Corning H-R Red Purple Ultra filter, which transmits mainly the 3650 and 3663 Å lines, and a sheet of Diamond window glass to remove any wavelengths below 3000 Å. In some later experiments with this lamp the filter was omitted and two sheets of Diamond window glass were used; with this latter arrangement the exposure required to produce the maximum reactivation was reduced but otherwise the effect of the treatment was unchanged.

In our later work we employed a General Electric A-H5 lamp, delivering 250 watts. This emits ultraviolet light in the same region as does the B-H6 but is of a lower intensity - the principle line of ultraviolet emission being 3650 Å. The effects produced by this lamp are identical with the B-H6 but being of a lower intensity the time required to produce the same results is longer; no filters are needed with this lamp. The material was held in a 37°C waterbath and the light passed through a sheet of window glass and one of lead glass. This apparatus is described in Appendix B.

Experiment Twenty-Two

Preliminary experiments were carried out to determine whether post-treatment with ultraviolet light in the 3650 Å line with our procedure and equipment had any lethal effects or mutagenic action on the test organism. In this experiment, 5 ml. samples of a twenty-four hour culture were subjected to light treatment for varying periods of time, viable cell counts made before and after the light treatment, and suitable dilutions plated and the mutants scored.

The results presented in Table XXIX are from experiments using the B-H6 lamp and show that the lethal effects of this wavelength are negligible. It is also shown that the percent mutations after light treatment is not significantly greater than the spontaneous ones from untreated samples. In all later experiments two minutes exposure was found sufficient to produce the maximum photoreactivation effect.

Table XXX tabulates the results from a similar experiment using the A-H5 lamp. Exposures under ten minutes fail to give the maximum reversal of the suppression of growth or photoreactivation; this is due to the lower intensity of this type of lamp. In all later experiments using this treatment we used a fifteen minute exposure period and found no lethal or mutagenic effects from this wavelength alone.

Differences between viable cell counts before and after light treatment which do appear in Table XXIX and Table XXX are within the realm of the inherent error present in all counting methods.

Table XXIX

Lethal and Mutagenic Effects of Ultraviolet Light in the 3650 Å Line

Using a G.E. B-H6 Lamp

(Colour response mutants on mannitol-tetrazolium agar)

Exposure to Light (minutes)	Viable Cell Counts (organisms per ml.) Before Exposure	After Exposure	Colonies Examined	Mutant Colonies	Percent Mutation
0	4.3×10^9	4.2×10^9	2123	2	0.09
5	4.5×10^9	4.3×10^9	1253	2	0.16
10	4.3×10^9	4.1×10^9	1675	4	0.24
15	4.4×10^9	4.6×10^9	1696	4	0.23
30	4.5×10^9	4.6×10^9	1070	2	0.18
60	4.0×10^9	4.2×10^9	968	2	0.20

Table XXX

Lethal and Mutagenic Effects of Ultraviolet Light in the 3650 Å Line

Using a G.E. A-H5 Lamp

(Colour response mutants on mannitol-tetrazolium agar)

Exposure to Light (minutes)	Viable Cell Counts (organisms per ml.) Before Exposure	After Exposure	Colonies Examined	Mutant Colonies	Percent Mutation
0	1.9×10^9	1.9×10^9	1297	2	0.15
5	2.0×10^9	2.6×10^9	1862	1	0.05
10	2.2×10^9	2.0×10^9	1251	3	0.23
15	2.0×10^9	1.9×10^9	1525	4	0.26
30	2.3×10^9	2.4×10^9	1404	3	0.21
60	2.1×10^9	2.2×10^9	1449	2	0.13

Experiment Twenty-Three

In this experiment varying time exposures of post-treatment with the near ultraviolet were used on aliquots of a sample previously irradiated with a dose of 500 ergs per mm^2 of ultraviolet in the 2537 Å line. This treatment was carried out using the B-H6 mercury vapour bulb. Viable cell counts were made before and after light treatment and the percent mutation determined.

The results of this experiment are presented in Table XXXI.

Table XXXI

Post-Treatment with Increasing Exposures of the Near Ultraviolet of a Previously Irradiated Culture

Exposure to Light (minutes)	Viable Cell Count (organisms per ml.) Before Exposure	After Exposure	Colonies Examined	Mutant Colonies	Percent Mutation
Unirrad. Control	4.5×10^9	4.5×10^9	1654	4	0.24
Irrad. only	8.7×10^8	-	914	93	10.17
2	8.7×10^8	1.3×10^9	1963	101	5.14
5	8.7×10^8	1.8×10^9	2200	108	4.90
7	8.7×10^8	1.1×10^9	2180	79	3.62
10	8.7×10^8	1.4×10^9	1971	83	4.21
13	8.7×10^8	1.2×10^9	2073	82	3.95
15	8.7×10^8	1.6×10^9	3726	187	5.01
30	8.7×10^8	1.3×10^9	1452	68	4.68
60	8.7×10^8	1.7×10^9	1656	71	4.29
120	8.7×10^8	1.5×10^9	1848	63	3.40

These results show that the minimum exposure for the maximum reactivation time to be about two minutes. This time was used in all later experiments.

Experiment Twenty-Four

Photoreversal of the Lethal Effect: As previously stated, the reversal of the suppression of growth by the near ultraviolet has been shown to occur in a wide range of organisms, including bacteria. Our interest lay in determining to what extent photoreactivation did occur in Escherichia coli B/r which had previously been irradiated with ultraviolet in the 2537 Å Line.

Three representative experiments will be given from the results of many carried out over a considerable period of time. It is felt that there is no need to give a great number of similar experiments for all data follow the same trend.

The cultures were grown as given in the general procedure in Part One. Ten ml. samples of a twenty-four hour culture were irradiated with increasing doses of ultraviolet in the 2537 Å line; the range covered was from 0 - 5000 ergs per mm^2. Five ml. portions of each sample were post-treated with the near ultraviolet light in the 3650 Å line for two minutes. Viable cell counts were made after the initial irradiation period and after post-treatment with light. The results of this experiment are given in Tables XXXII to XXXIV and a survival curve from the combined data is presented in Fig. 4.

It is to be seen that post-treatment with light has a pronounced effect on the apparent lethal effect, the increase in the survival factor becoming greater with increasing dose.

Table XXXII

Photoreversal of the Lethal Effect by the Near Ultraviolet in *Escherichia coli* B/r

UV Dose ergs per mm^2	Viable Cell Count After Irradiation	Viable Cell Count After Light Treatment	Increase Factor
0	3.7×10^8	3.7×10^8	1
500	8.0×10^7	2.8×10^8	3.5
1000	1.8×10^5	1.0×10^6	5.5
3000	1.6×10^4	9.6×10^4	6.0
5000	1.9×10^2	1.8×10^3	9.5

Table XXXIII

Photoreversal of the Lethal Effect by the Near Ultraviolet in *Escherichia coli* B/r

UV Dose ergs per mm^2	Viable Cell Count After Irradiation	Viable Cell Count After Light Treatment	Increase Factor
0	9.6×10^8	9.6×10^8	1
500	1.7×10^8	5.4×10^8	3.1
1000	5.1×10^5	2.3×10^6	4.5
3000	1.3×10^4	7.3×10^4	5.6
5000	5.8×10^2	4.2×10^3	7.2

Table XXXIV

Photoreversal of the Lethal Effect by the Near Ultraviolet in *Escherichia coli* B/r

UV Dose ergs per mm^2	Viable Cell Count After Irradiation	Viable Cell Count After Light Treatment	Increase Factor
0	1.1×10^9	1.1×10^9	1
500	1.5×10^8	5.2×10^8	3.4
1000	5.4×10^5	2.0×10^6	3.5
3000	4.2×10^4	2.2×10^5	5.2
5000	1.5×10^3	7.6×10^3	5.0

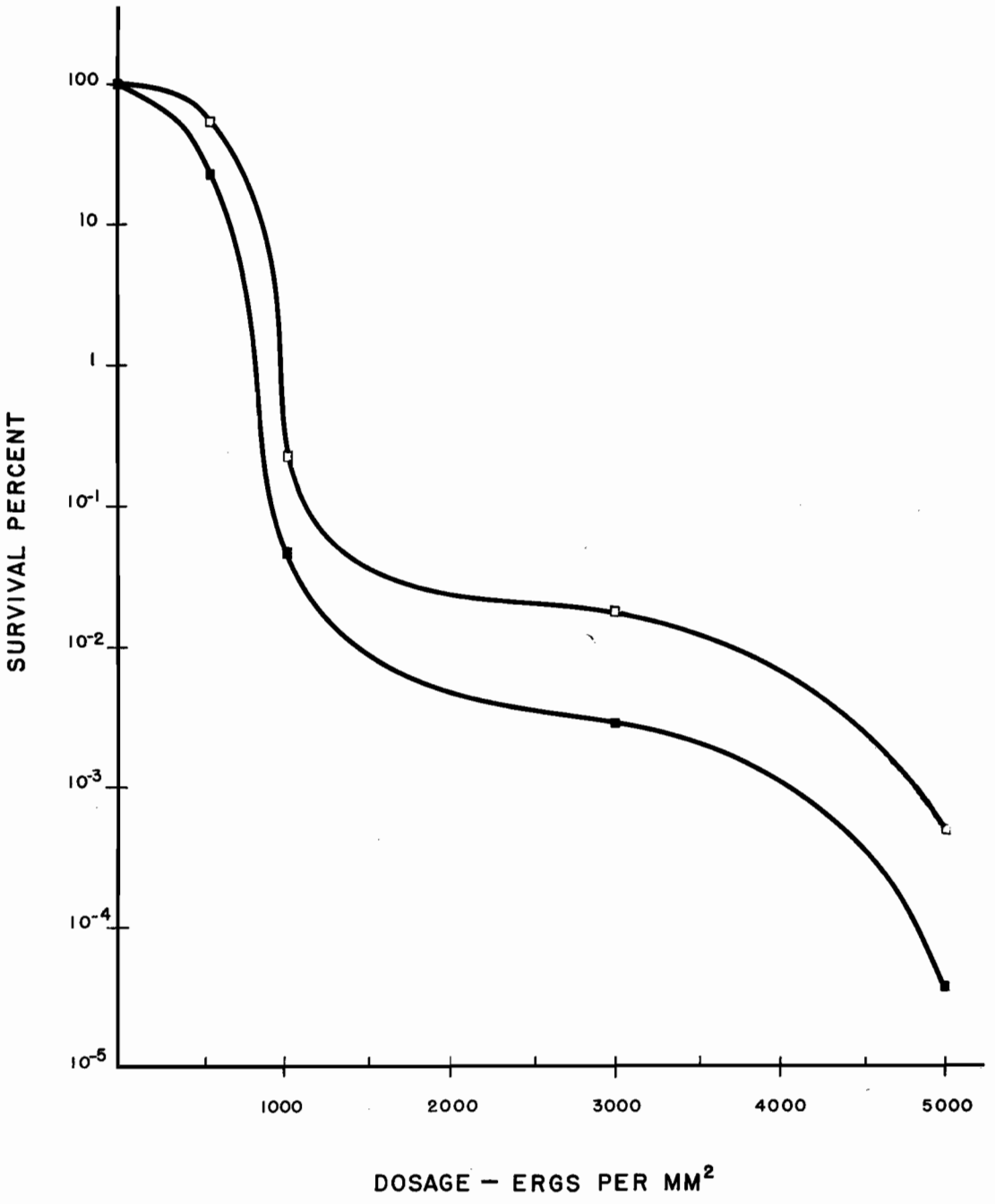


FIG.4. PHOTOREVERSAL OF THE LETHAL EFFECTS OF ULTRAVIOLET LIGHT IN *Escherichia coli* STRAIN B/r

■ = 2537 Å □ = 3650 Å

Experiment Twenty-Five

Introduction

The phenomenon of post-treatment with light of previously irradiated cultures arouses greatest interest in the effect it has on induced mutation. Here we have a case where ultraviolet rays of one wavelength produces an increased number of mutations in a strain and post-treatment with ultraviolet of another wavelength decreases this number. In this respect, ultraviolet is probably the most useful mutagenic agent to use to determine the cause and effect of induced mutations in bacteria.

Experimental

In this experiment 10 ml. samples of a culture of Escherichia coli B/r were irradiated with increasing doses of ultraviolet in the 2537 Å line. Five ml. portions of the irradiated samples were subsequently treated with the near ultraviolet in the 3650 Å line. Suitable dilutions of each sample were plated on mannitol-tetrazolium agar, incubated, and the number of mutants counted as sectored colonies, determined after irradiation and post-treatment.

With low doses of ultraviolet (less than 500 ergs per mm^2) most of the mutagenic effect could be reversed by exposure to the near ultraviolet; the proportion of mutants being reduced to less than ten percent of what it would otherwise have been. With increasing doses (from 500 to 2500 ergs per mm^2) the number of mutants which respond to post-treatment becomes less with increasing dose. With doses over 2500 ergs per mm^2 the mutagenic effect becomes stable and a plateau is

produced in the dose effect curve similar to that of the 2537 Å mutation curve (see Fig. 3) with very little alteration in the proportion of mutants.

The results of Experiment Twenty-Five are given in Table XXXV and Fig. 5.

Table XXXV

Photoreversal of the Mutagenic Effect in Escherichia coli B/r as a Function of Ultraviolet Dose

(using colour response mutants on mannitol-tetrazolium agar)

UV Dose ergs per mm^2	Not Light Treated			Light Treated			Percent Effect Remaining After Light Treatment
	Colonies Examined	Mutant Colonies	Percent Mutation	Colonies Examined	Mutant Colonies	Percent Mutation	
0	7832	18	0.2	7951	20	0.2	0
500	7206	838	11.6	24476	221	0.9	7.8
1000	1487	210	14.1	1175	54	4.6	32.6
1500	3899	423	10.9	6584	471	7.2	65.9
2000	2211	297	13.4	1994	199	10.0	74.2
2500	1228	163	13.3	783	103	13.2	99.2
3000	2733	345	12.6	4074	499	12.2	97.0
5000	1016	136	13.4	1650	218	13.2	98.7

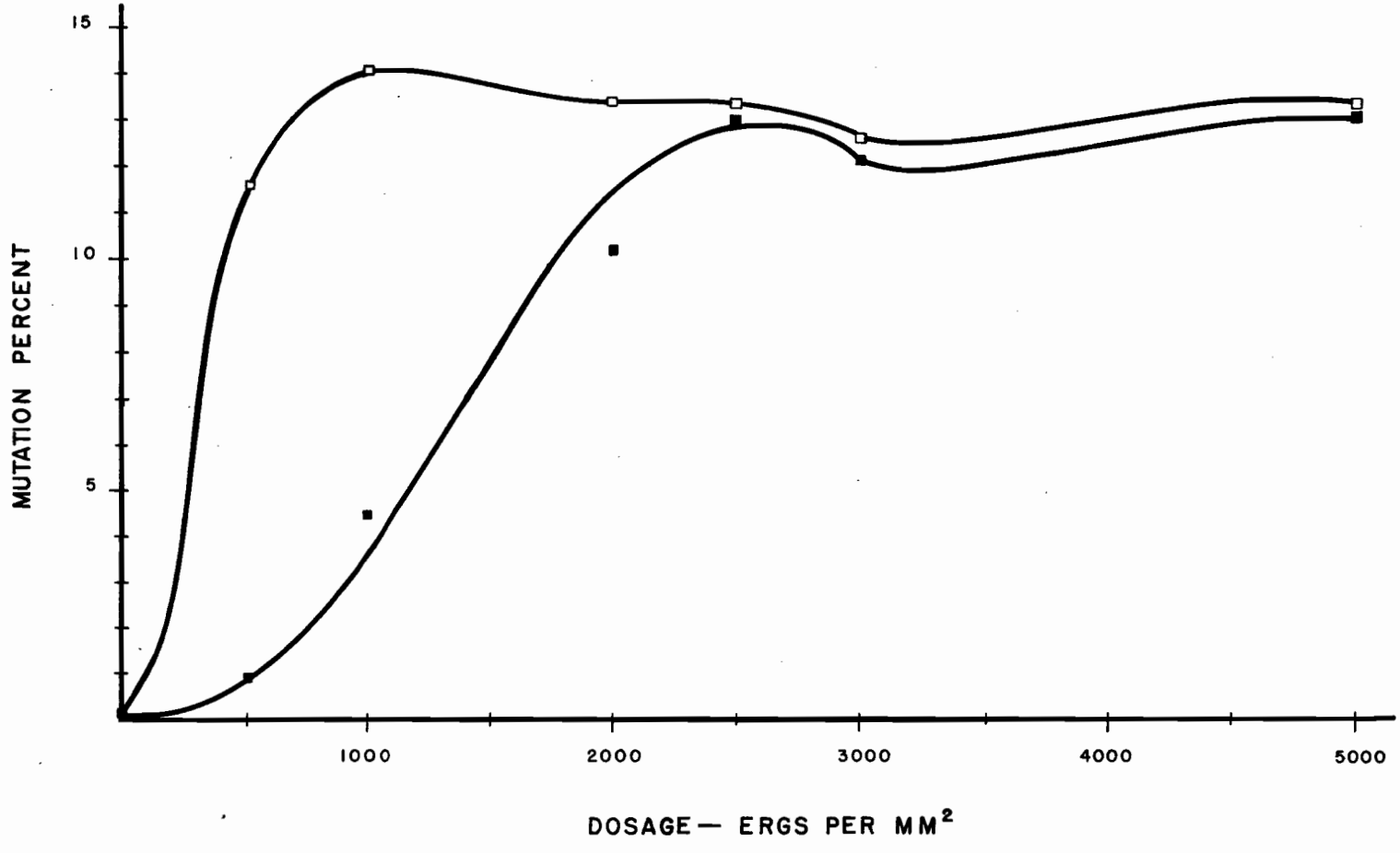


FIG. 5. PHOTOREVERSAL OF THE MUTAGENIC EFFECT OF ULTRAVIOLET LIGHT IN *Escherichia coli* STRAIN B/r

□ = 2537 Å ■ = 3650 Å

Experiment Twenty-Six

A further difference between the lethal and the mutagenic effects became apparent when the bacteria were subjected alternately to low doses (500 ergs per mm^2) of ultraviolet in the 2537 Å line and to light in the 3650 Å line. The mutagenic effect was induced and reversed in turn as expected but after the first irradiation and light treatment the bacteria became more sensitive to the killing effect of ultraviolet - killing in the vicinity of 99 percent of the population each time it was used. No explanation for this response can be offered, but it appears that killing must be a more complex phenomenon than mutation.

The results of this experiment are given in Table XXXVI.

Table XXXVI

Repeated Photoreversal of the Mutagenic Effects of Low Doses of Ultraviolet

Treatment (UV* and Light)	Colonies Examined	Mutant Colonies	Percent Mutation
500	2272	270	11.9
500 + L	3522	37	1.1
500 + L + 500	1835	202	11.0
500 + L + 500 + L	2619	39	1.5
500 + L + 500 + L + 500	2287	207	9.1

* Dose expressed in ergs per mm^2

Experiment Twenty-Seven

A final comparison was made to determine whether after high doses of 2537 Å ultraviolet, light induced restoration of viability was accompanied by a restoration of the capacity for further mutational response to low doses of ultraviolet. This, if it occurred, could mean that the plateau level of mutation might be greatly increased.

Initial doses of 1500, 2000, and 2500 ergs per mm^2 were administered and followed with light treatment at 3650 Å which increased the number of survivors by factors of approximately 1000x, 100x and 2x respectively. A second irradiation dose at 3527 Å of 500 ergs per mm^2, however, failed to raise the number of mutants to a higher level than could have been obtained with a single dose alone. The restoration of viability in these experiments was not associated with restoration of the capacity for induced mutation.

The experimental results are given in Table XXXVII.

Experiment Twenty-Seven

Table XXXVII

Failure of Light Treatment to Restore Capacity for Further Induced Mutation in Heavily Irradiated Bacteria

Treatment (UV* and Light)	Colonies Examined	Mutant Colonies	Percent Mutation
1500	1794	205	12.0
1500 + L	612	56	9.1
1500 + L + 500	988	143	14.5
2000	1848	251	13.6
2000 + L	1270	127	10.0
2000 + L + 500	1017	121	11.9
2500	1228	163	13.3
2500 + L	783	103	13.2
2500 + L + 500	980	128	13.1

* Dose expressed in ergs per mm^2

Summary and Discussion

The ultraviolet survival curves (Figs. 2 and 4) show that a sigmoidal curve is produced; it falls off very slowly with low doses (less than 500 ergs per mm^2) until an apparent threshold is reached, the curve then drops rapidly and tapers off with higher doses. The curve for light treated bacteria parallels the killing curve but the survivors are increased about tenfold.

With low doses of ultraviolet (500 ergs per mm^2), post-treatment with light reduces the final number of mutants to less than ten percent of what it would otherwise have been. This is shown in Table XXXV for colour variants on mannitol-tetrazolium agar. With increasing doses of ultraviolet, the mutagenic effect becomes more stable in the presence of light, so that with doses of 2500 ergs per mm^2 and higher, virtually all of the mutagenic effect is stable. There is no appreciable change in the proportion of mutants produced over the dosage range from 500 to 5000 ergs per mm^2: from this it follows that the maximum unstable mutagenic effect and the maximum stable mutagenic effect of ultraviolet are approximately equal.

The reversal of the mutagenic effects and of the killing effects are similar but differ in a few important respects: (a) with low doses light treatment reverses both effects; (b) with high doses light treatment reverses much of the killing but very little of the mutagenic effect; and (c) alternate irradiation and light treatment reverses the mutagenic effect but causes killing.

It seems that some common factor, responsible for both killing and mutation in the ultraviolet irradiated cells, is destroyed when the

cells are exposed to light (this has been suggested by Kelner (90), Kimball and Gaither (91) and Goodgal (69)). The supposed common factor could be termed a "photosensitive mutagen poison" and the existence of a corresponding "photostable mutagen poison" might be inferred from the effects of ultraviolet that remain after treatment with light.

The nature of the above stable and unstable mutagenic effects is not known. They could be changes in the gene molecules which under conditions favourable to mutation tend to restitute, and which when irradiated with light are partly unaltered and partly restituted by the treatment. They could represent changes in the immediate gene environment affecting the genes themselves only when they reproduce, or they could represent mutagenic substances which can diffuse through the nucleus and may cause mutations to occur only when the cell becomes metabolically active, or when the genes reproduce. Thus, the mutagenic effects of ultraviolet could be a matter of (a) gene mutations, (b) gene alterations predisposing it to mutate, and (c) alterations outside of the gene predisposing it to mutate (e.g. mutagenic chemicals).

The bulk of the killing does not seem to be due to lethal gene mutations since the lethal effects (both photostabile and photosensitive) continue to increase with increasing dose after the proportion of mutations has reached a maximum. Photoreversal would have to be explained in terms of sensitivity of a proportion of mutations to light, in terms of reverse mutation or differential sensitivity to ultraviolet, or insensitivity of a large proportion of genes to mutation. Interpretation along these lines is unlikely on a priori grounds since phenotypic expression of an induced mutation is delayed (41,102,126), and on experimental grounds

as the colour mutants tested from low doses failed to show differential sensitivity to light, or to any appreciable extent to ultraviolet.

Internal or external alterations to the gene predisposing it to mutate are indistinguishable experimentally. If the changes are internal to the gene, photoreversal would indicate that a proportion are undone by the light treatment and the plateau could be explained either as due to an equilibrium proportion of changes being produced or to all possible changes being accomplished. With external changes there would be (a) equilibrium between production and destruction; (b) a limit to the production (limited precursor); or (c) a saturation effect in the interaction of mutagen and gene such that, regardless of the quantity of mutagen present, the gene response cannot exceed a certain level. It is to be noted that the maximum effect of the photosensitive mutagen is approximately the same as that of the photostable mutagen, and this would be a somewhat surprising coincidence if the maximum level of both were determined by the rates of production and destruction for the two mutagens, particularly since the one is produced so much more abundantly than the other (in terms of effect) by low doses of radiation. It would be even more surprising if the supply of a precursor were the limiting factor for the photostable mutagen. Neither of the above suggestions can account for the loss of the unstable mutagenic effect, although (a) and (b) combined might. Repeated reactivation fails to show exhaustion of a substrate, and yet the photosensitive mutagenic effect cannot be added to the photostable effect after light treatment.

Part Three

THE EFFECT OF MONOCHROMATIC ULTRAVIOLET LIGHT ON SEVERAL RESPIRATORY ENZYME SYSTEMS OF ESCHERICHIA COLI B/r

The Effect of Monochromatic Ultraviolet Light on Several Anaerobic Respiratory Enzyme Systems of *Escherichia coli*

Introduction

A brief statement of results of some earlier experiments on the effect of ultraviolet radiation on enzyme systems of various organisms has been given in the historical introduction.

In most of the earlier work the studies were made on enzyme preparations extracted from cells rather than studying the system as it functions in the intact cell. With the belief that only the true picture can be obtained by studying the enzymes in the intact cell, we started studies to determine whether any enzyme properties were lost in cells which had been irradiated with increasing doses of ultraviolet.

The majority of the work on enzyme inactivation employs the general principle of methylene blue reduction and most experimenters have employed the Thunberg technique (171, 172) or some modification of it, in which the reduction of methylene blue in the presence of a suitable substrate, phosphorous buffer and tissue suspension is studied in a special tube from which atmospheric oxygen had been removed. Quastel and Whetham (139) used the Thunberg tube but modified the method; these workers used "resting organisms" which were defined as "organisms grown for two days in tryptic broth, separated by centrifuging, thoroughly washed with normal saline, made up to a thick emulsion with saline and finally well aerated." The reactions of these organisms under the conditions stated were considered to be the reactions of resting or non-proliferating organisms. The tests were carried out under anaerobic conditions in vacuum tubes. Braun and Wörderhoff (29) modified the Thunberg-Quastel method, assuring

the absence of oxygen from the liquids by boiling rather than by evacuation. Bach (9) developed yet another method combining the best features of the Thunberg-Quastel and the Braun and Wörderhoff techniques. This method employs non-proliferating bacterial suspensions and tubes which after evacuation at the water pump can be filled with nitrogen without their removal. After evacuation and nitrogen replacement the tubes are then removed from the vacuum ramp, sealed and placed in the incubator for the test.

In all of these methods the procedure has to be carried out in two operations: first, the removal of oxygen followed by nitrogen replacement; and second, sealing the tubes with a nitrogen atmosphere, removing them from the vacuum ramp and placing them in the test environment. The ideal method would be one in which after nitrogen replacement and without removal of the tubes, to be able to carry out the test under any experimental conditions. Such an apparatus was designed by Myers (123) and is a modification of that described by Bach. The description of this apparatus and the methods employed in its use are described in Appendix D.

With this apparatus we have studied the effects of ultraviolet radiation on several anaerobic respiratory enzymes of Escherichia coli strain B/r observing the degree of enzyme inactivation and the lethal effects. Those enzyme systems studied were:

Anaerobic dehydrogenases (Cytochrome-linked)
1. Succinic
2. Lactic

Anaerobic dehydrogenases (Pyridine-nucleotide-linked)
1. Malic
2. Glucose
3. Formic

Unclassified dehydrogenases
1. Pyruvic.

Preparation of the Cell Suspension:

The test organism, Escherichia coli B/r, was grown for six hours in synthetic M-9 medium with aeration. One ml. aliquots of this culture was used to inoculate ten nutrient agar plates. The plates are incubated for eighteen hours at 37°C, chilled, and the surface growth scraped off and suspended in distilled water. The cells were washed three times to remove any traces of nutrient material by centrifuging with the high speed centrifuge at 13,500 r.p.m. The washed cell sediment was weighed and resuspended in molar fifteen phosphate buffer at pH 7 to give a cell suspension of 10 mgm. wet weight cells per ml. This suspension was vibrated with the electric vibrating machine at approximately 1300 vibrations per minute until a homogenous suspension was obtained. The opacity of this suspension in a 1:5 dilution was checked on the Evelyn photometer using filter number 660. The undiluted suspension was then adjusted by the addition of sterile buffer so that a 1:5 dilution in distilled water gave a standard light transmission of approximately 20 percent.

After standardization of the cell suspension, both total and viable cell counts were made, the suspension then stored at 5°C for eighteen hours and viable cell counts made again before the suspension was used for an experiment. In all experiments recorded there was no change in the viable cell counts made before and after storage and the suspension was taken as a non-proliferating cell suspension.

Irradiation of Cell Suspension:

Samples of the stored cell suspension were irradiated in 10 ml. volumes in Petri dishes with monochromatic ultraviolet light in the 2537 Å line. The dose range used in these experiments was from 0 to 5000 ergs

per mm^2. After irradiation the test samples were stored in dark test tubes at 0^oC until the test was conducted. At no time were the irradiated samples stored for more than one hour.

Test for Enzyme Activity:

The degree of enzyme activity is the time taken for the treated cell sample to reduce a given concentration of methylene blue as compared with the time taken for an unirradiated sample. The time taken for the unirradiated sample is given an arbitrary value of 100 and that of the treated samples are expressed as that fraction of 100 obtained by dividing the reduction time of the unirradiated sample by that of the treated sample.

In all experiments we have used a methylene blue chloride* solution in a concentration of 1-20,000 w/v. It has been demonstrated that methylene blue in this concentration is not toxic to the enzyme systems of bacteria whereas greater concentrations generally are (10). Sterile solutions of this concentration were prepared and stored for use in amber bottles.

Substrate solutions were prepared as tenth molar solutions of the sodium salts except in the case of sodium lactate which was prepared as 0.1 percent w/v solution. One ml. volumes of the substrate solutions were used in all tests.

The reaction tube for the oxidation-reduction test consists of a heavy walled Pyrex glass test tube, 18 x 100 mm.; one small glass tube 8 x 60 mm.; one glass rod 1 x 90 mm.; and one single-hole gum rubber stopper to fit the larger tube. This stopper is fitted with a length of

*Methylene blue 1% (Oxidation-Reduction Indicator), Hartmann-Leddon Co., Philadelphia, Pa. Sold by Will Corporation, 594 Broadway, New York.

glass tubing 4 x 50 mm. in such a manner that the tubing will project into the reaction tube a distance of 10 mm.

The contents of the large tube are:

Bacterial suspension	1.0 ml.
Methylene blue, 1-20,000	1.0 ml.
Phosphate buffer, pH 7	1.0 ml.

The contents of the small tube are:

Substrate solution	1.0 ml.

With this tube successful results can only be obtained if the total volume of the reacting fluids does not exceed 4.0 ml.

Nitrogen is bubbled vigorously through the contents of the large tube for thirty seconds. The substrate solution is placed in the small tube, and the glass rod placed within it; with forceps the small tube with the glass rod is then placed in the large tube. The gum rubber stopper is fitted to the large tube and by means of the glass tubing projecting from the stopper, the tube is attached to the rubber adapter of the vacuum ramp. All rubber-glass joints are then sealed with collodion. At this stage the tubes hang freely from the vacuum ramp and are immersed in the water bath to three-quarters of their length.

In all experiments the tubes were evacuated by the water pump with a negative pressure of 18 cm. of mercury. Tubes were evacuated for a three minute period and during this period they were gently tapped, without splashing the contents, against the rubber apron extending from the vacuum ramp into the bath. Following evacuation the suction valve is closed and nitrogen allowed to flow freely into the reaction tubes until a positive pressure of 2 cm. of mercury is reached. This whole procedure is repeated three times for three minute periods each time. Following the

third evacuation the positive pressure of 2 cm. of mercury is maintained throughout the test period.

At this point the reaction tubes are inverted, thus allowing the contents of the large and small tubes to mix. The positive pressure prevents the fluid from flowing along the glass tube leading to the ramp. The glass rod resting inside the smaller tube serves to support this tube when it is inverted and also allows the substrate solution to flow freely from the tube. It has been our practice to repeat the inversion of the tubes three times in order to ensure complete washing out the substrate and adequate mixing of the substrate with the contents of the larger tube.

The time of the test period has been arbitrarily set at three hours. The time is noted when the tubes are inverted and again when reduction is complete. The time interval between these readings is given as the reduction time. With the tubes used in the experiment, one cannot estimate methylene blue reduction by photometrical methods. However, we have found that in any control experiments the reduction of methylene blue progresses until only a very thin layer, approximately 0.5 mm. deep, of unreduced methylene blue remains at the upper surface of the fluid in the tubes. In our work the end point was taken as the time required to obtain reduction of methylene blue which is complete as that obtained in the control tube with unirradiated sample (a).

The control tubes used in each experiment are: (a) unirradiated sample with all other constituents; (b) unirradiated sample with the substrate solution replaced with buffer; (c) no cell suspension, it being replaced with buffer.

Results of Experiments:

The tables which follow give the results obtained using the method described for the study of ultraviolet radiation on the viable cell count and some anaerobic respiratory enzyme systems of Escherichia coli strain B/r.

The enzyme systems studied were succinic dehydrogenase, lactic dehydrogenase, malic dehydrogenase, glucose dehydrogenase, formic dehydrogenase and pyruvic dehydrogenase.

Experiment Twenty-Eight

Table XXXVIII

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Succinic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 3.2×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survival Ratio	Reduction Time	Enzyme Activity Coefficient
0	2.7×10^9	1 : 1.2	12 min.	100
500	2.6×10^8	1 : 1.3×10^1	12 min.	100
1000	1.8×10^7	1 : 1.8×10^2	12 min.	100
2000	4.2×10^5	1 : 7.6×10^3	12 min.	100
3000	8.6×10^4	1 : 3.7×10^4	12 min.	100
4000	5.1×10^3	1 : 6.2×10^5	12 min.	100
5000	1.4×10^3	1 : 2.3×10^6	12 min.	100

Table XXXIX

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Succinic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 4.1×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.4×10^9	1 : 1.2	10 min.	100
500	2.4×10^8	$1 : 1.7 \times 10^1$	10 min.	100
1000	1.3×10^7	$1 : 3.2 \times 10^2$	10 min.	100
2000	6.3×10^5	$1 : 6.5 \times 10^3$	10 min.	100
3000	3.2×10^4	$1 : 1.3 \times 10^5$	10 min.	100
4000	1.2×10^3	$1 : 3.4 \times 10^6$	10 min.	100
5000	9.7×10^2	$1 : 4.2 \times 10^6$	10 min.	100

Experiment Twenty-Nine

Table XL

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Lactic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 3.2×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	2.7×10^9	1 : 1.2	14 min.	100
500	2.6×10^8	1 : 1.3×10^1	14 min.	100
1000	1.8×10^7	1 : 1.8×10^2	14 min.	100
2000	4.2×10^5	1 : 7.6×10^3	14 min.	100
3000	8.6×10^4	1 : 3.7×10^4	14 min.	100
4000	5.1×10^3	1 : 6.2×10^5	14 min.	100
5000	1.4×10^3	1 : 2.3×10^6	14 min.	100

Table XLI

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Lactic Dehydrogenase of Escherichia coli B/r

Total Cell Count = 4.1×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.4×10^9	1 : 1.2	11 min.	100
500	2.4×10^8	$1 : 1.7 \times 10^1$	11 min.	100
1000	1.3×10^7	$1 : 3.2 \times 10^2$	11 min.	100
2000	6.3×10^5	$1 : 6.5 \times 10^3$	12 min.	91
3000	3.2×10^4	$1 : 1.3 \times 10^5$	11 min.	100
4000	1.2×10^3	$1 : 3.4 \times 10^6$	12 min.	91
5000	9.7×10^2	$1 : 4.2 \times 10^6$	11 min.	100

Experiment Thirty

Table XLII

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Malic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 4.4×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.0×10^9	1 : 1.5	30 min.	100
500	8.2×10^8	1 : 5.3	30 min.	100
1000	2.3×10^7	$1 : 1.9 \times 10^2$	30 min.	100
2000	6.3×10^5	$1 : 6.9 \times 10^3$	30 min.	100
3000	3.0×10^4	$1 : 1.5 \times 10^5$	30 min.	100
4000	1.8×10^3	$1 : 2.4 \times 10^6$	30 min.	100
5000	9.2×10^2	$1 : 4.7 \times 10^6$	31 min.	97

Table XLIII

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Malic Dehydrogenase of Escherichia coli B/r

Total Cell Count = 2.6×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	1.9×10^9	1 : 1.4	27 min.	100
500	5.5×10^8	1 : 4.7×10^1	27 min.	100
1000	3.2×10^7	1 : 8.1×10^2	27 min.	100
2000	2.7×10^5	1 : 9.6×10^3	27 min.	100
3000	2.3×10^4	1 : 1.1×10^5	27 min.	100
4000	1.8×10^3	1 : 1.4×10^6	28 min.	96
5000	1.3×10^2	1 : 2.0×10^7	28 min.	96

Experiment Thirty-One

Table XLIV

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Glucose Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 3.2×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	2.7×10^9	1 : 1.2	10 min.	100
500	2.6×10^8	1 : 1.3×10^1	10 min.	100
1000	1.8×10^7	1 : 1.8×10^2	10 min.	100
2000	4.2×10^5	1 : 7.6×10^3	11 min.	90
3000	8.6×10^4	1 : 3.7×10^4	12 min.	83
4000	5.1×10^3	1 : 6.2×10^5	11 min.	90
5000	1.4×10^3	1 : 2.4×10^6	11 min.	90

Table XLV

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Glucose Dehydrogenase of Escherichia coli B/r

Total Cell Count = 4.1×10^9 organisms per ml.				
UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.4×10^9	1 : 1.2	7 min.	100
500	2.4×10^8	$1 : 1.7 \times 10^1$	8 min.	87
1000	1.3×10^7	$1 : 3.2 \times 10^2$	8 min.	87
2000	6.3×10^5	$1 : 6.5 \times 10^3$	7 min.	100
3000	3.2×10^4	$1 : 1.3 \times 10^5$	7 min.	100
4000	1.2×10^3	$1 : 3.4 \times 10^6$	7 min.	100
5000	9.7×10^2	$1 : 4.2 \times 10^6$	8 min.	87

Experiment Thirty-Two

Table XLVI

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Formic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 3.2×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	2.7×10^9	1 : 1.2	10 min.	100
500	2.6×10^8	1 : 1.3×10^1	10 min.	100
1000	1.8×10^7	1 : 1.8×10^2	11 min.	90
2000	4.2×10^5	1 : 7.6×10^3	10 min.	100
3000	8.6×10^4	1 : 3.7×10^4	9 min.	111
4000	5.1×10^3	1 : 6.2×10^5	9 min.	111
5000	1.4×10^3	1 : 2.4×10^6	10 min.	100

Table XLVII

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Formic Dehydrogenase of Escherichia coli B/r

Total Cell Count = 4.1×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.4×10^9	1 : 1.2	8 min.	100
500	2.4×10^8	$1 : 1.7 \times 10^1$	9 min.	88
1000	1.3×10^7	$1 : 3.2 \times 10^2$	8 min.	100
2000	6.3×10^5	$1 : 6.5 \times 10^3$	9 min.	88
3000	3.2×10^4	$1 : 1.3 \times 10^5$	9 min.	88
4000	1.2×10^3	$1 : 3.4 \times 10^6$	8 min.	100
5000	9.7×10^2	$1 : 4.2 \times 10^6$	8 min.	100

Experiment Thirty-Three

Table XLVIII

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Pyruvic Dehydrogenase of *Escherichia coli* B/r

Total Cell Count = 4.4×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	3.0×10^9	1 : 1.5	15 min.	100
500	8.2×10^8	1 : 5.3	16 min.	93
1000	2.3×10^7	1 : 1.9×10^2	15 min.	100
2000	6.3×10^5	1 : 6.9×10^3	15 min.	100
3000	3.0×10^4	1 : 1.5×10^5	15 min.	100
4000	1.8×10^3	1 : 2.4×10^6	15 min.	100
5000	9.2×10^2	1 : 4.7×10^6	15 min.	100

Table XLIX

The Effects of Ultraviolet Radiation on the Respiratory Enzyme System, Pyruvic Dehydrogenase of Escherichia coli B/r

Total Cell Count = 3.2×10^9 organisms per ml.

UV Dose ergs per mm^2	Viable Cell Count (organisms per ml.)	Survivor Ratio	Reduction Time	Enzyme Activity Coefficient
0	2.6×10^9	1 : 1.2	12 min.	100
500	4.1×10^8	1 : 7.8×10^1	12 min.	100
1000	6.1×10^7	1 : 5.2×10^2	13 min.	92
2000	7.9×10^5	1 : 4.0×10^3	13 min.	92
3000	3.1×10^4	1 : 1.0×10^5	13 min.	92
4000	3.1×10^3	1 : 1.0×10^6	12 min.	100
5000	1.3×10^2	1 : 2.5×10^7	13 min.	92

Summary of Results

The results of numerous experiments studying the effects of ultraviolet radiation on several respiratory enzymes in the intact cell of Escherichia coli strain B/r which are presented in Tables XXXVIII to XLIX indicate that the lethal action of ultraviolet is not due primarily to inactivation of enzymes. In all cases the viable cell count over the range of dosages studied is reduced by a factor of 10^6 to 10^7 and even at the highest doses the enzyme activity coefficient is the same as that for the unirradiated sample.

This work is far from complete in that only a few enzyme systems have been studied, but it does seem to indicate that we must look elsewhere at some other component or cellular system of bacteria which is responsible, at least in the primary stage, for the lethal action of ultraviolet radiation.

Part Four

FINAL DISCUSSION AND SUMMARY OF RESULTS

General Discussion and Summary

Throughout this report we have discussed the results of specific experiments at the end of each section. The discussion and summary to follow is a brief compilation of these resumes.

Generally our problem has been twofold -- to study the lethal effects and the mutagenic effects of ultraviolet light on the radiation resistant strain B/r of Escherichia coli -- and it was to our advantage and to the general understanding of the problem to study these effects simultaneously. The change studied was an induced mutation to colour response on mannitol-tetrazolium agar. This mutant is easily characterized by the formation of sectors in colonies; whole mutants are also observed but due to the difficulty in differentiation between whole colony mutants and non-mutant colonies, and to make the scoring of mutants as objective as possible, only sectored mutants were used.

The results given in Part One of this report show that the mutants selected differ from the parents only in their ability to reduce 2,3,5-triphenyl tetrazolium chloride to the dark insoluble formazan and not in their other common fermentative and biochemical properties. In working with mutations this is to be expected for there is no reason to believe that selection for one character should be linked with other unrelated characters.

In Part Two we have studied the lethal and mutagenic effects of ultraviolet radiation. The lethal effects have been determined over a wide range of doses and a sigmoidal killing curve is produced. Lethal effects have been determined in every experiment and the survival curve is reproducible, close fits being obtained each time. The mutagenic

effect of ultraviolet gives an increase in percent mutation with increasing dose until a threshold point is reached and from this point a plateau is produced in the dose effect curve with increasing doses of radiation. Post-treatment with light of irradiated cultures reduces the lethal and mutagenic effects and gives curves of similar shape. It is possible that the same common factor is responsible for both effects but their expression is influenced by some other mechanism. It does appear that killing is a more complex process than mutation but precise quantitative comparisons have yet to be made before any definite conclusions can be drawn.

The last section of the paper is a start on the attempt to determine what is the key site of the cell which is affected by radiation and which may be responsible for the many diverse effects observed in irradiated cells. At present it appears that some factor other than enzymes are responsible and it is more than probable that this substance is a precursor to many synthetic processes in the cell. If this were the case, the many effects observed might easily be accounted for.

There is a great deal of work yet to be done on this problem and so far we have only scratched the surface. Probably the most profitable avenue of approach would be more detailed studies of the phenomenon of post-treatment of irradiated cultures with light. Here we have a situation where treatment with ultraviolet of a higher wavelength undoes and alters the effects of initial treatment with ultraviolet of a lower and more bactericidal wavelength. The best approach to this problem is the study of bacterial mutability for which the field of bacterial variation offers a wide range of material.

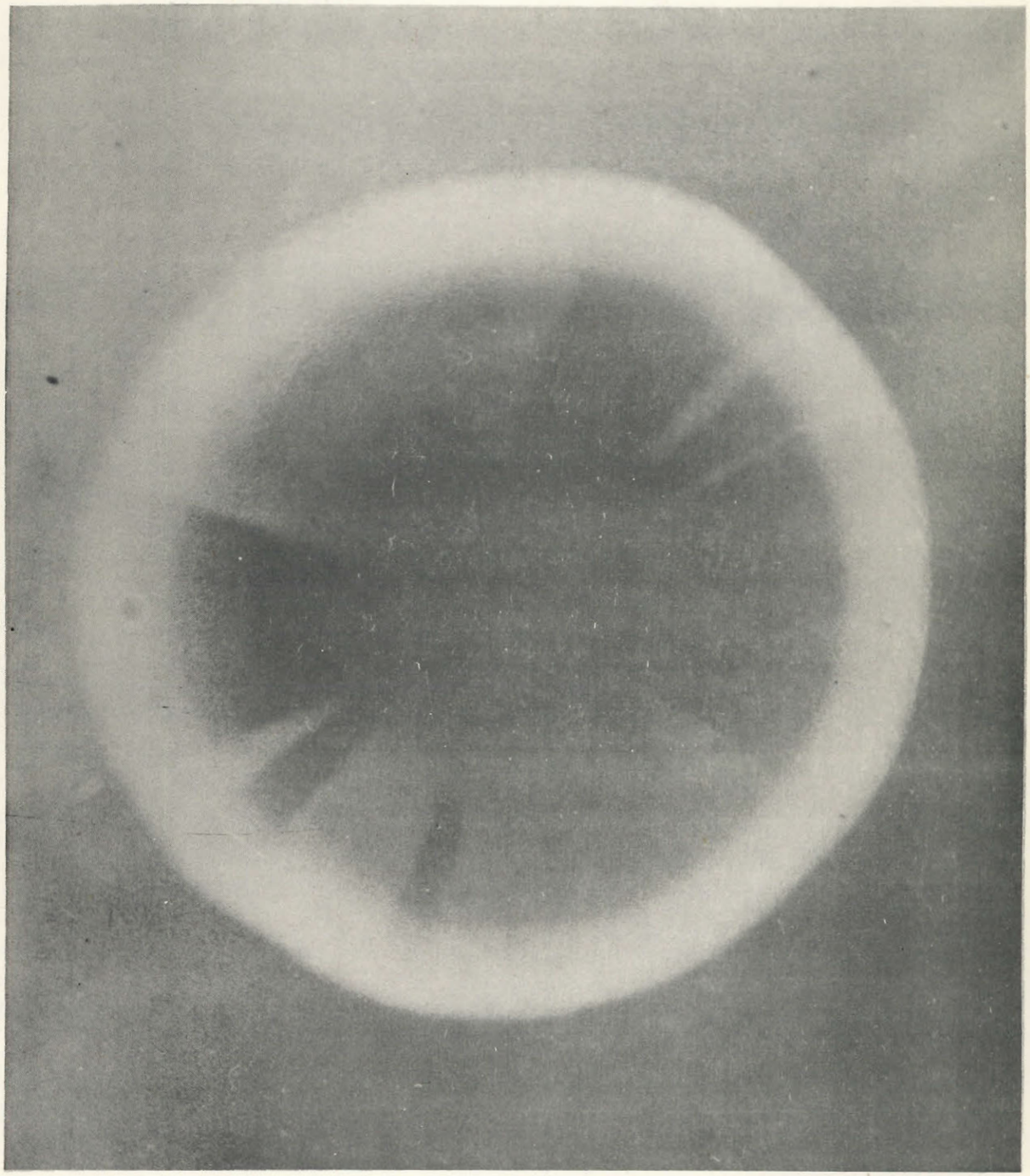


FIG.6. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

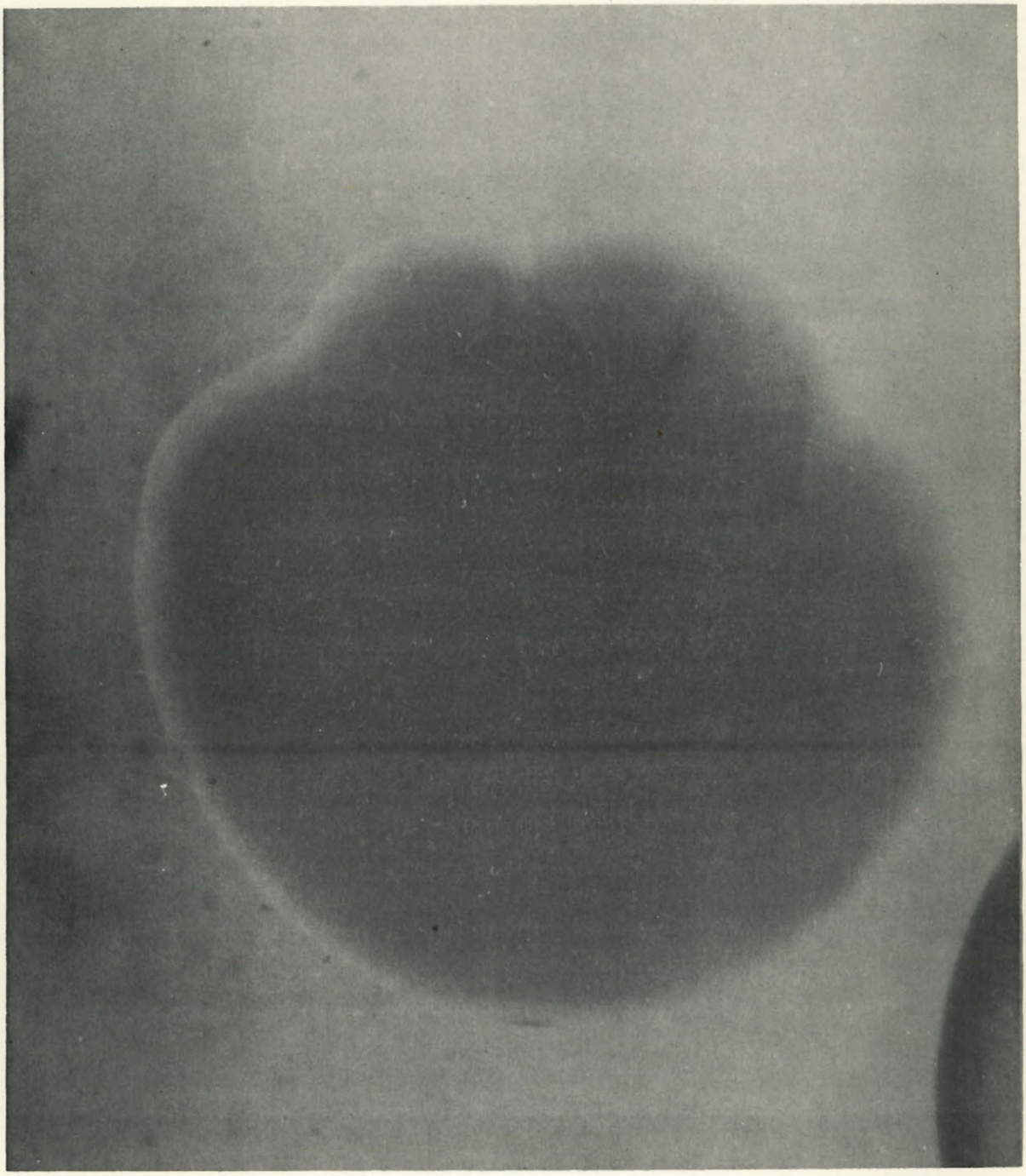


FIG.7. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

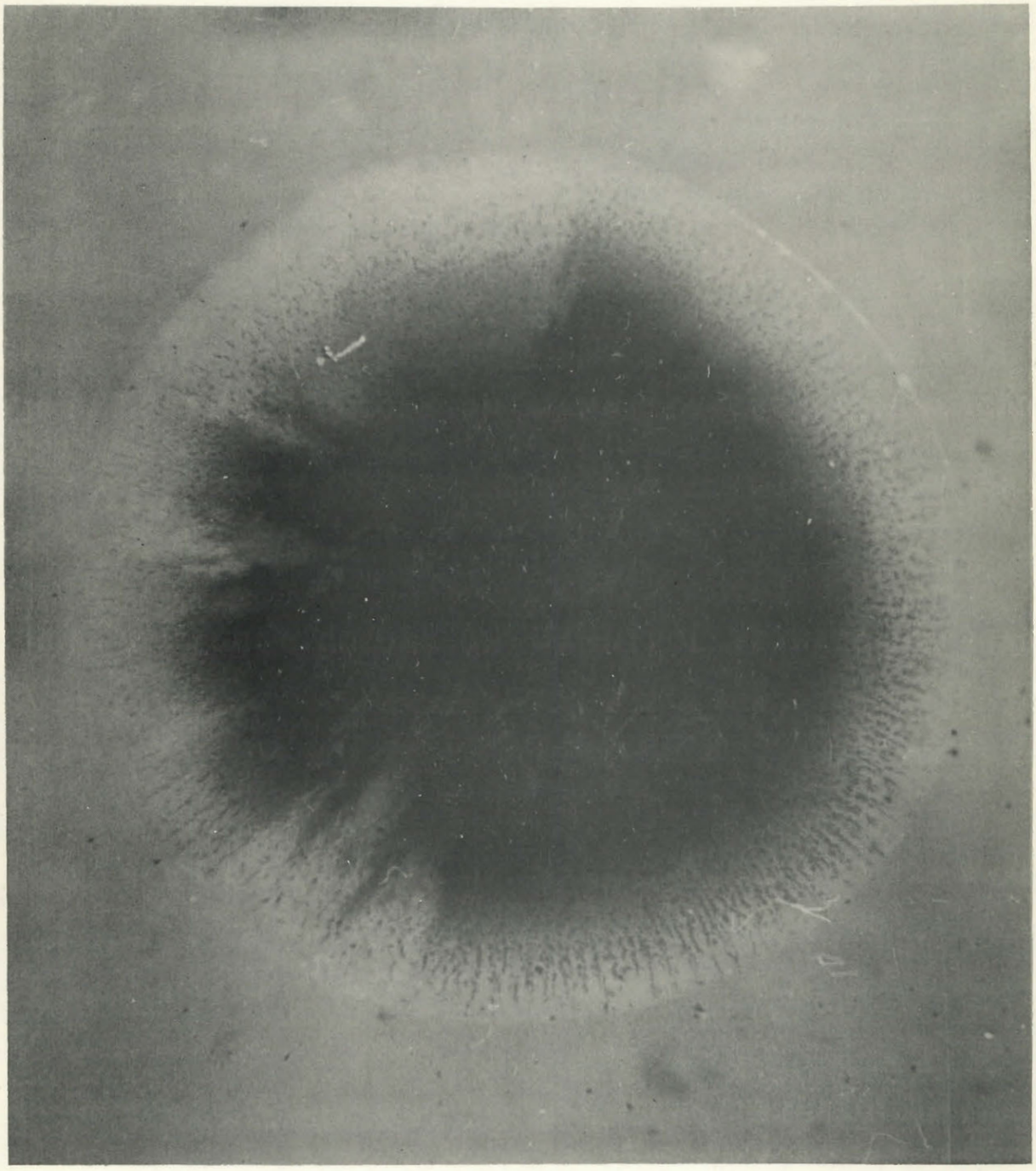


FIG. 8. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

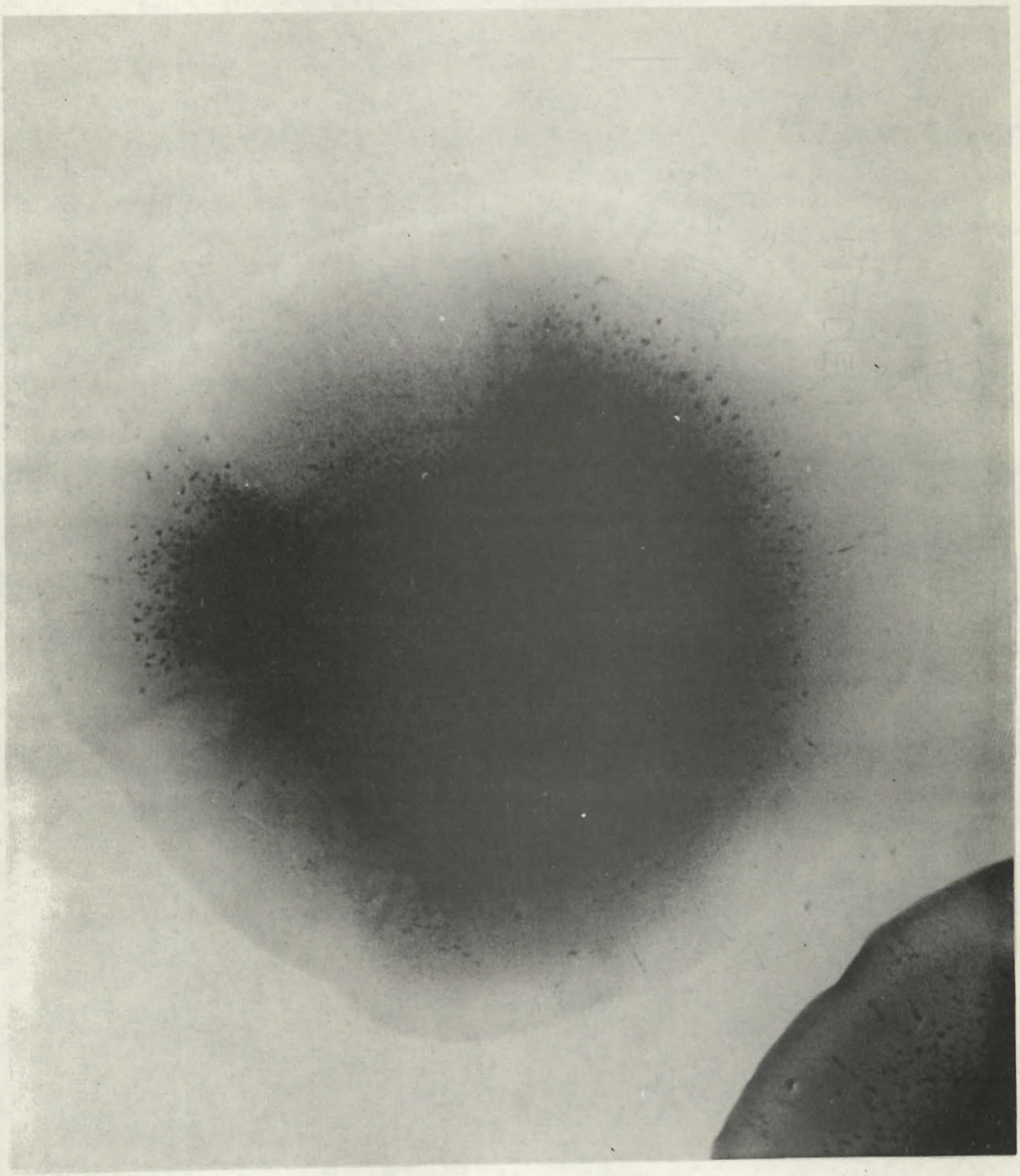


FIG. 9. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

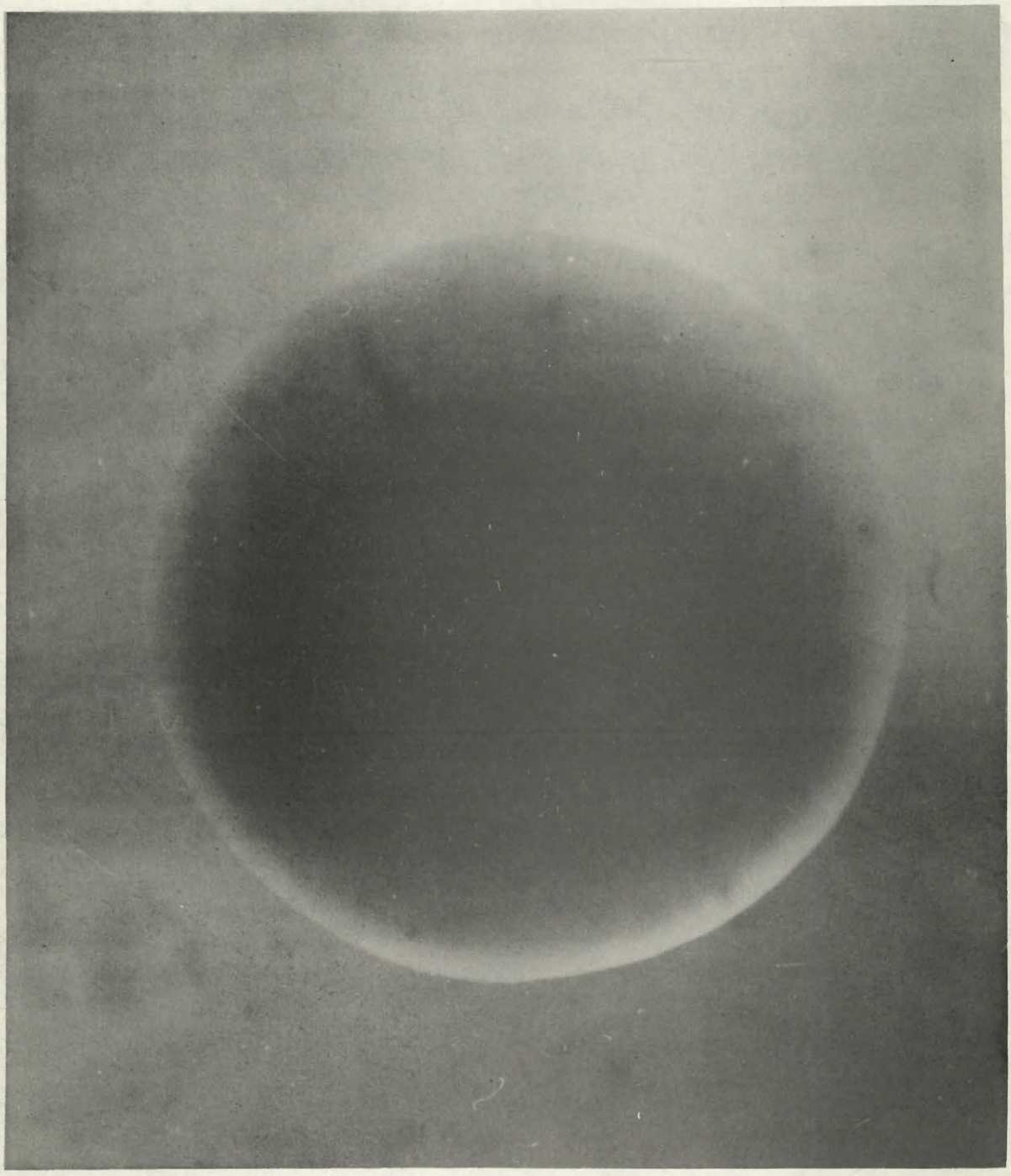


FIG. 10. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

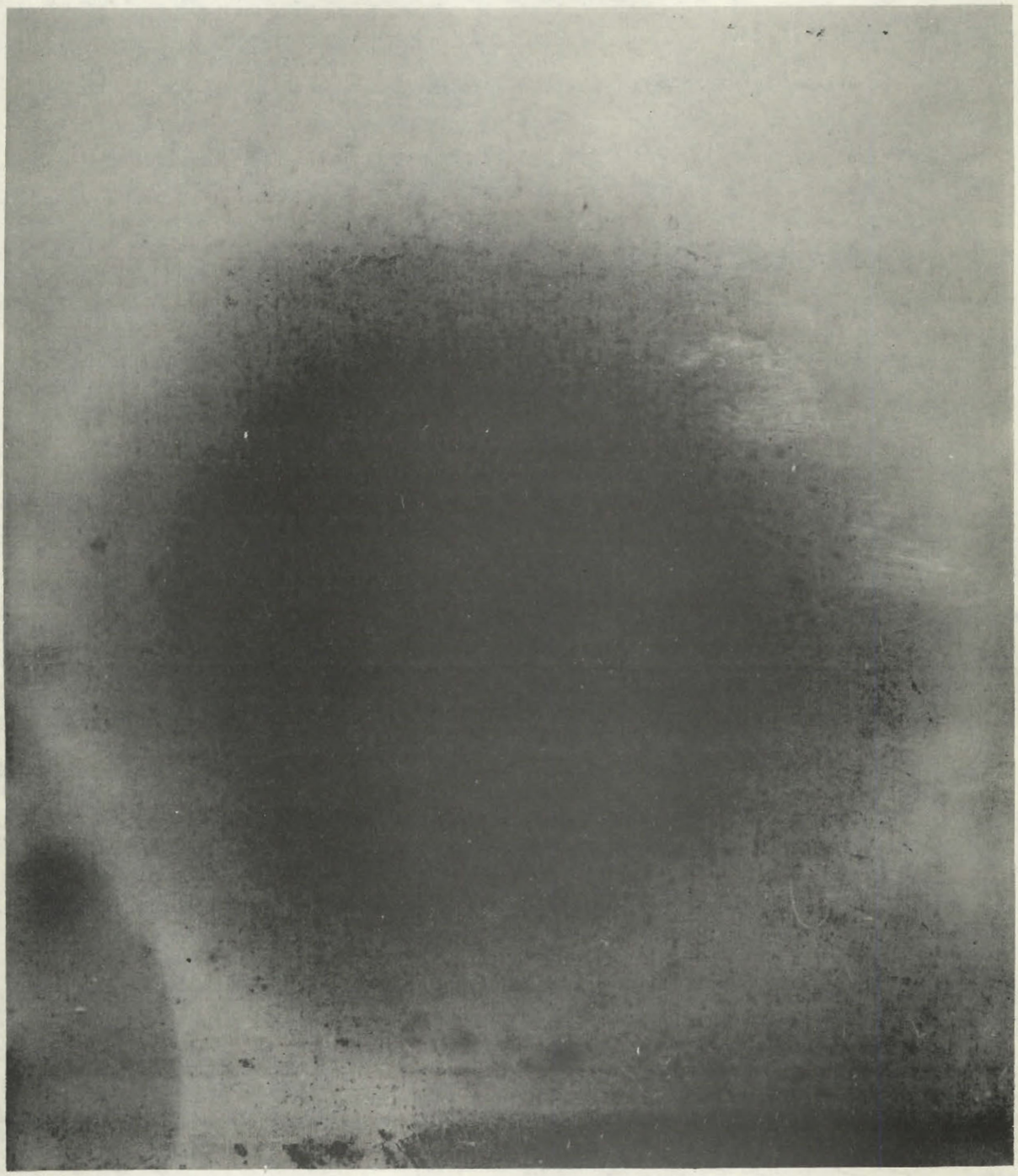


FIG. 11. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

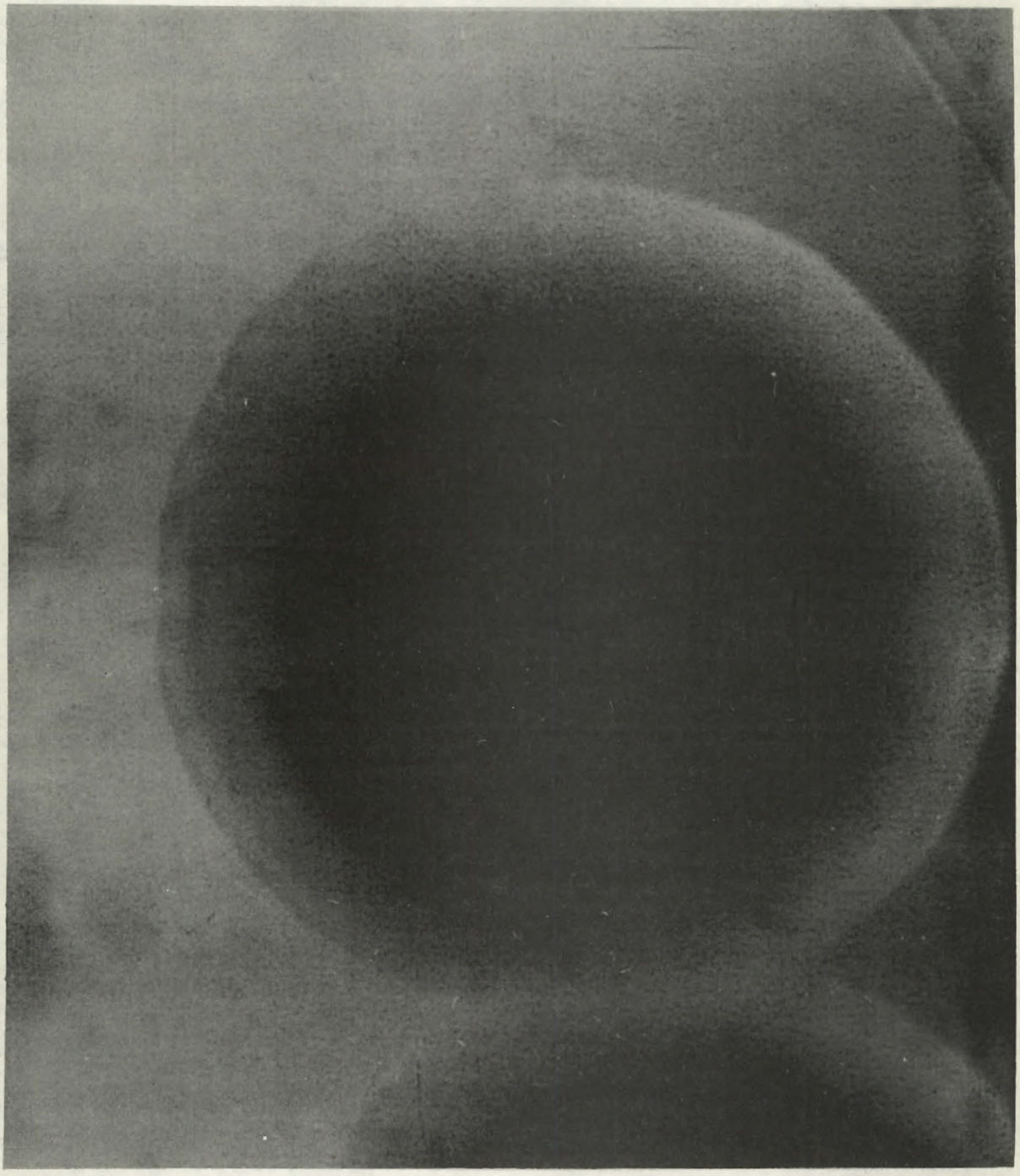


FIG.12. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

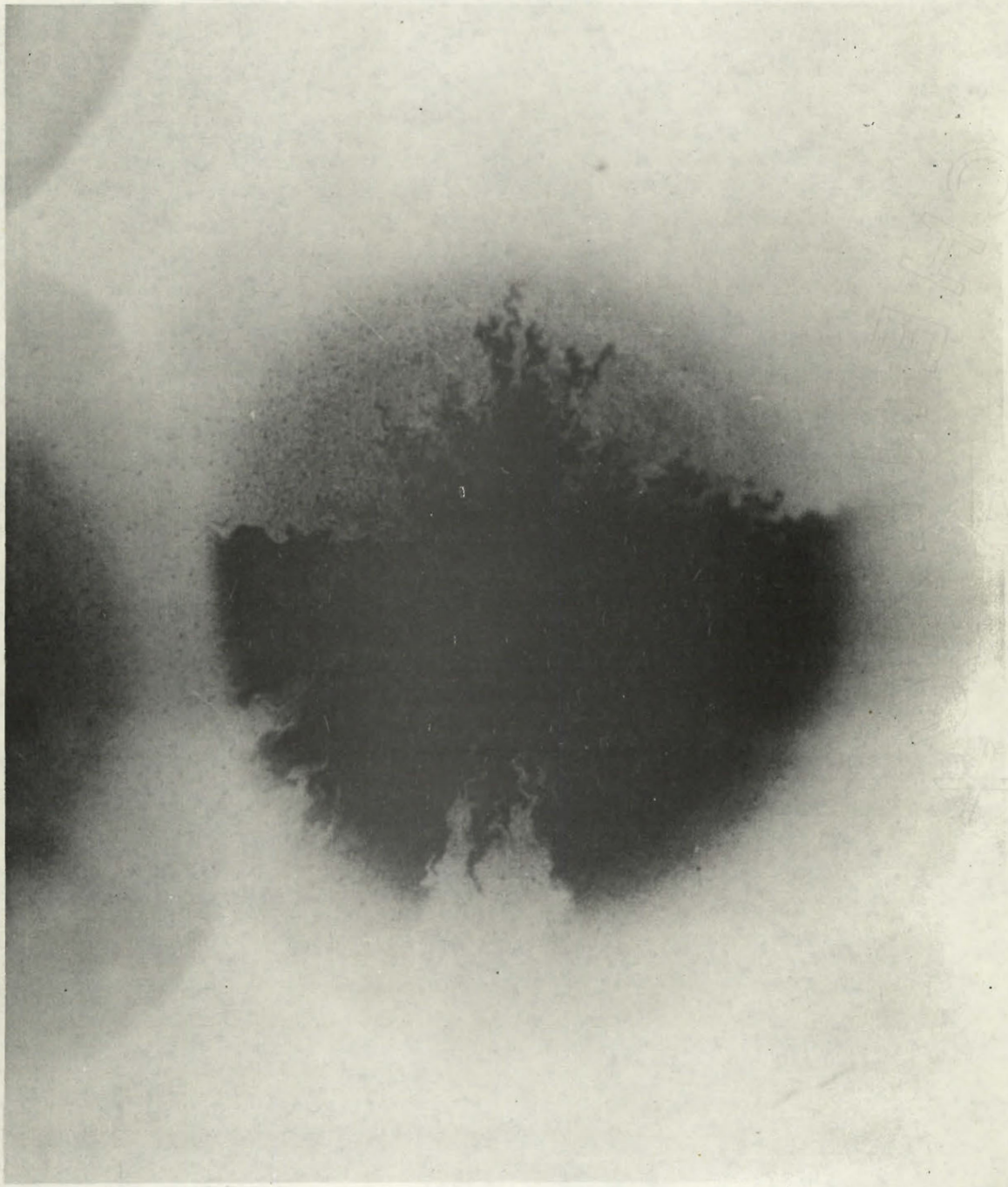


FIG. 13. SECTORED COLONY ON MANNITOL TETRAZOLIUM AGAR

APPENDICES

Appendix A

Formulae and Methods of Preparation of Media

1. M-9 Synthetic Media:

Solution I -	KH_2PO_4	3.0 gms
	$MgSO_4.7H_2O$	0.2 gms
	NaCl	1.0 gms
	$Na_2HPO_4.12H_2O$	15.0 gms
	Distilled water	900 gms
Solution II -	Dextrose	4.0 gms
	Distilled water	100 gms

No pH adjustment is necessary. Sterilize solutions separately; Solution I by autoclaving at 120°C for 20 minutes and Solution II by Seitz filtering. Store separately until used.

For use mix Solutions I and II in the ratio of 1 part Solution II to 9 parts Solution I. Add trace elements A and B, each in a concentration of 0.1 ml. per litre of media.

Trace Elements A:	$CaCl_2$	5.0 gms
	KI	0.5 gms
	Distilled water	500 gms
Trace Elements B:	H_3BO_3	5.0 gms
	$ZnSO_4$	5.0 gms
	$MnCl_2$	5.0 gms
	$FeCl_3$	5.0 gms
	$CuSO_4.5H_2O$	0.5 gms
	Distilled Water	500 gms

Use 0.1 ml. of each of Trace elements A and B per litre.

2. Nutrient Agar:

NaCl	5.0 gms
Beef Extract (Difco)	3.0 gms
Peptone (Difco)	5.0 gms
Agar	15.0 gms
Distilled water	1000 gms

Adjust pH to 6.8, sterilize in the autoclave at 120°C for 20 minutes. Agar is made up in 10 litre volumes and dispensed in 400 ml. bottles for storage.

3. Mannitol-tetrazolium chloride Agar

Nutrient agar	400 ml.
tetrazolium chloride 1% stock solution	2.0 ml.
mannitol - 20% stock solution	20.0 ml.

Tetrazolium chloride - 1% stock solution:

2,3.5-triphenyl tetrazolium chloride*	0.5 gms
35% ethyl alcohol	50.0 ml.
Hydrochloric acid (conc.)	0.05 ml.

The tetrazolium chloride stock solution is kept in a dark bottle in the cold room. It does not need to be autoclaved for use. The stock mannitol solution is sterilized by Seitz filtering. These two sterile solutions are added to the melted sterile agar and mixed just before pouring plates. The agar must be below 75°C when the tetrazolium is added.

*2,3,5-triphenyltetrazolium chloride. Lot #50727 supplied by the British Drug Houses (Canada) Ltd., Toronto, Canada.

Appendix B

Apparatus and Technique for Ultraviolet Irradiation

1. 2537 Angstroms:

Samples were irradiated with a 15-watt General Electric Germicidal Lamp which is estimated to deliver 95 percent of its energy in the 2537 Å line. Exposures were made at a distance of 74 centimeters, the intensity being measured through a photoelectric cell by a Westinghouse ultraviolet meter. The starting time to full output of this lamp is approximately five minutes. Exposures were made from 0 to 5000 ergs per mm^2 and the time period required for each dose measured. All dosages used in our experiments were taken on a time basis and are given in Table L.

Table L

Time Required for a Given Dose Using a G-E 15-Watt Germicidal Lamp

Intensity ergs per mm^2	Time	
	minutes	seconds
0	0	0
250	0	48
500	1	35
1000	3	10
1500	4	45
2000	6	20
2500	7	55
3000	9	30
4000	12	40
5000	15	50

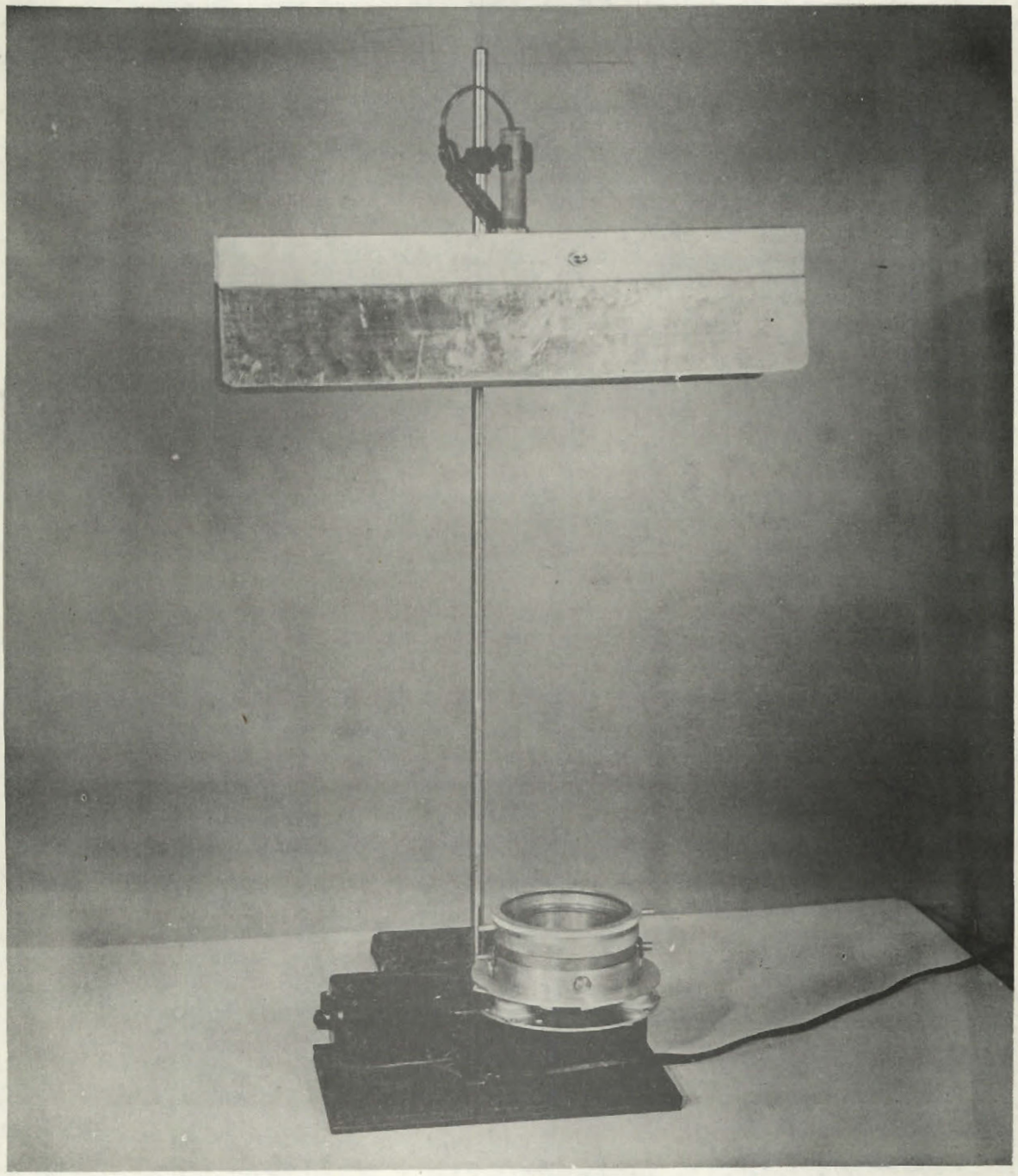
Ten ml. samples were irradiated in open Petri dishes (100 x 20 mm.) clamped in a dish holder which was vibrated in order to ensure uniform exposure of all cells. The frequency and amplitude were such as to produce standing waves. Shaking was accomplished by mounting the Petri dish holder on rubber shock absorbers fastened to a heavy brass plate. To the underside of this brass plate a spindle with a heavy eccentric weight was fastened. The spindle axle was in turn attached to a non-induction motor by means of a piece of rubber tubing. The speed of the motor is controlled by a variable rheostat which was normally adjusted so that a speed of 5,000 r.p.m. was obtained. We found this setup to be fully satisfactory for obtaining uniform standing waves.

2. 3650 Angstroms:

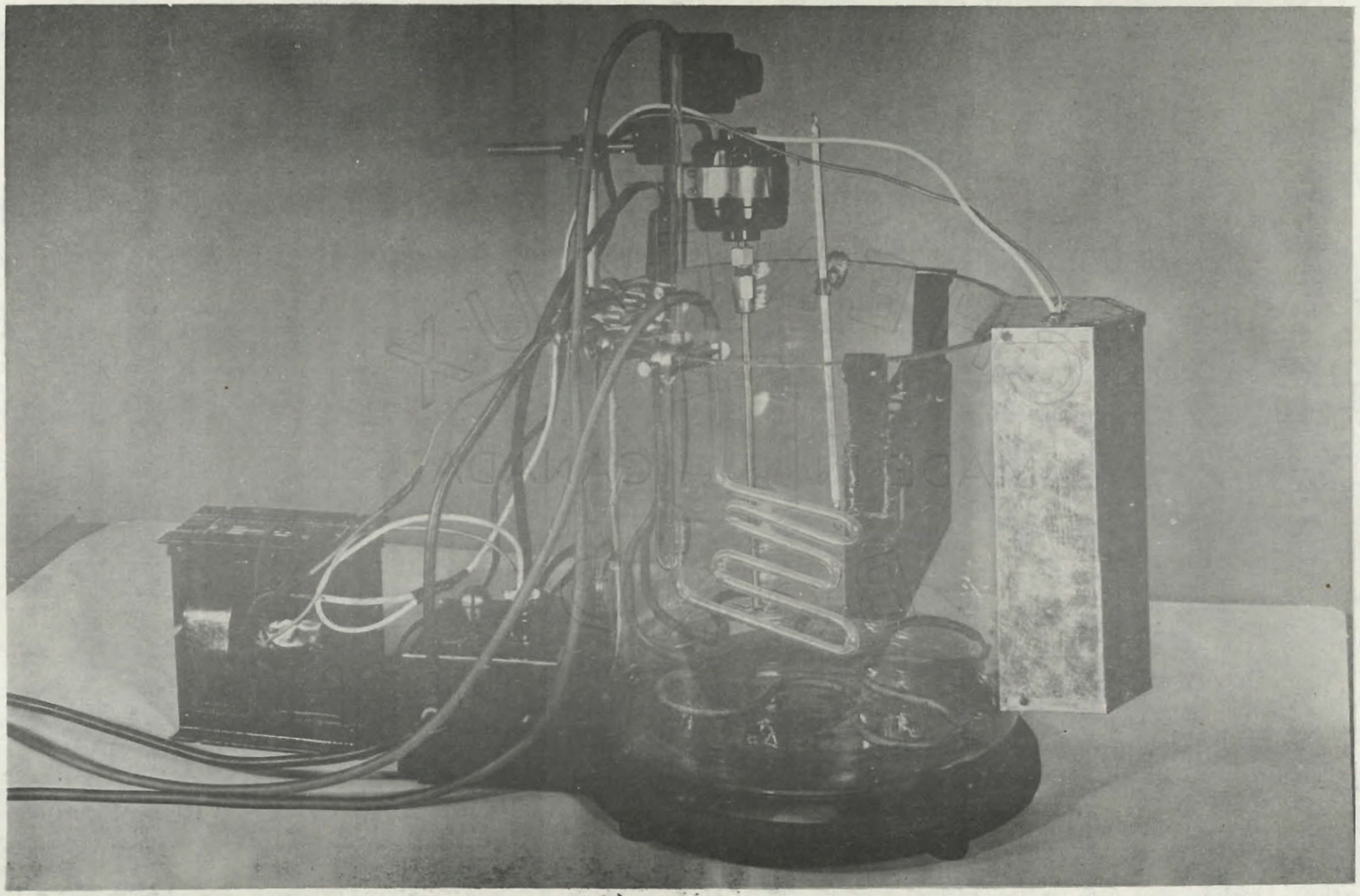
The apparatus to be described will be that using a 250-watt General Electric A-H5 mercury vapour bulb. The samples to be post-treated with light are held in a constant temperature waterbath at 37°C. Five ml. samples are treated in screw cap tubes held in a rack about four inches from the light source so as to obtain as high an intensity as possible; the duration of light treatment at this distance was 15 minutes. No filters are required with this lamp; the light is passed through a sheet of Diamond window glass and the lead glass end of the water bath to remove any wavelengths below 3000 Å.

The bulb is mounted in a polished aluminium reflector and placed against the end of the bath. The temperature of the bath is kept within 0.1°C with a mercury thermoregulator and a cooling coil. The cooling coil is necessary for after a short period of operation the heat

generated by the lamp heats the bath above the required temperature. A constant circulation of water is maintained by an electric stirrer.



2537 Å IRRADIATION APPARATUS



3650 Å IRRADIATION APPARATUS

Appendix C

An Electric Vibrating Machine

The electric vibrating apparatus to be described is that designed by Myers (123) in our laboratory. The machine consists of a Hamilton Beach Vibrator, Type C, 115 volts A.C.* The machine was formerly used for massage and for that purpose was equipped with a rubber suction cup which forms the contact between the vibrator and the skin surface being massaged. In order to adapt the apparatus for the vibration of test tubes and small bottles, the rubber suction cup was replaced by an adjustable rubber lined clamp.

The speed of the motor is controlled by means of a variable transformer which was generally adjusted to give approximately 1300 vibrations per minute.

Between the drive shaft of the motor and the test tube clamp are two working parts which are responsible for the particular type of vibration obtained; first, an eccentric ball and socket coupling, and second, a shaft which pivots universally about a ball and socket fulcrum.

In operation, the test tube clamp moves through a circle of 5 to 6 mm. diameter in both the horizontal and vertical planes. The net result of this motion is that the contents of the test tube continually swirl upward in a spiralling manner against the inner wall of the tube, while the fluid from the crest of the spiral falls downward toward the center of the tube. A turbulence results which uniformly disperses all particulate matter throughout the fluid.

* Hamilton Beach Manufacturing Co., Racine, Wisconsin, U.S.A.

Appendix D

A Modified Oxidation-Reduction Apparatus

The apparatus to be described is a modification of that designed by Bach (9). A pyrogallol-alkali reservoir has been introduced between the nitrogen source and the vacuum ramp. This permits "washing" of the nitrogen to remove any traces of oxygen before the nitrogen enters the reaction tubes. In operation, the reaction tubes are not removed from the vacuum ramp but once attached they are sealed to the rubber adapters of the ramp with collodion, and are immersed in the thermoregulated water bath at 37°C throughout the test period. A stiff rubber apron extending downward from the lower edge of the vacuum ramp makes an excellent background for observing the reduction of methylene blue when the reaction tubes are hanging in the bath.

Evacuation of Reaction Tubes:

Valve B from the nitrogen reservoir is closed and valves C and D are opened. Suction is applied by the water pump E and the extent of the vacuum obtained in the reaction tubes is measured by the mercury manometer. In all experiments 18 cm. of mercury was the negative pressure employed. Tubes are evacuated three minutes during which time they are agitated by gentle tapping against the rubber apron. Following evacuation Valve C is closed.

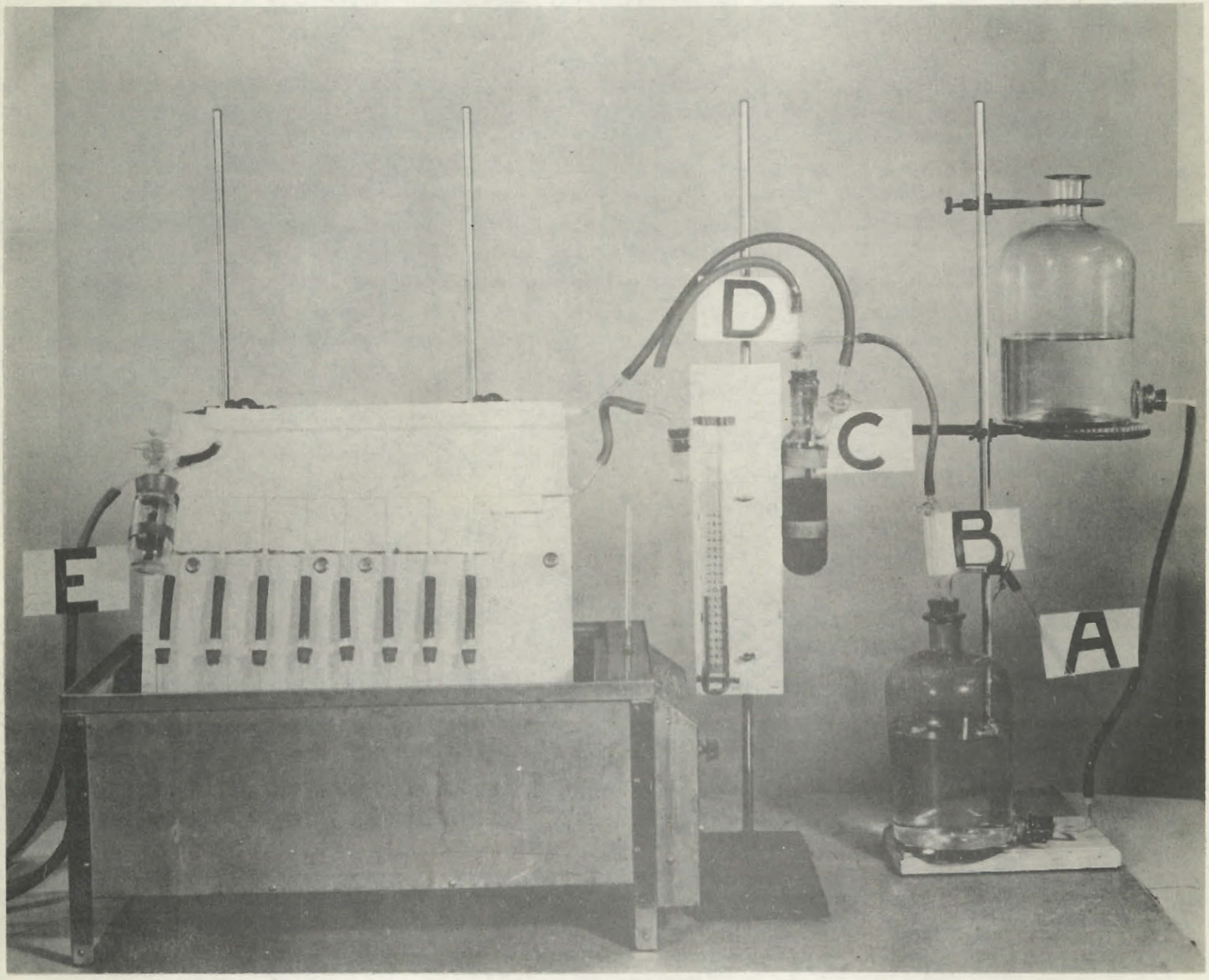
Filling Reaction Tubes with Nitrogen:

Bach has pointed out the advantage of having an intermediary nitrogen reservoir which forms a gasometer and permits filling of the

reaction tubes with a known positive pressure of nitrogen. This reservoir is represented by the two large bottles, each of approximately three litre capacity. Nitrogen is originally supplied from a tank of the compressed gas. The bottles are joined at their lower ends by rubber and glass tubing, the lower bottle is closed at its upper end with a two-holed rubber stopper. Prior to use the valve B is closed.

The lower bottle is completely filled with water by way of the tube leading from the upper bottle. Nitrogen is permitted to enter the lower bottle slowly through tube A; with valve B closed water is forced from the lower bottle to the upper bottle. When valve B is opened nitrogen flows from the lower bottle under pressure exerted by the head of water present in the upper bottle. This flow of nitrogen is controlled by adjustment of valve B. When the nitrogen reservoir is filled, there is sufficient volume present to last for several complete experiments. The nitrogen is bubbled slowly through the pyrogallol-alkali reservoir and when valve C is closed and valve D is opened nitrogen will flow into the vacuum ramp and the attached reaction tubes. The positive pressure of nitrogen in the reaction tubes is registered by the mercury manometer; in all experiments reported the positive pressure of nitrogen employed was 2 cm. of mercury. Valve B is closed following the operation of filling the reaction tubes with nitrogen.

Evacuation and refilling of reaction tubes is repeated three times as described. Following the third refilling, valve B is adjusted so that a positive pressure of nitrogen equivalent to 2 cm. of mercury is maintained throughout the entire oxidation-reduction test period.



OXIDATION - REDUCTION APPARATUS

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