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# UNIVERSITY OF CINCINNATI

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*I hereby recommend that the thesis prepared under my supervision by* Elizabeth V. Wright

*entitled* Some Biophysical Studies of Bacteriologic Material

*be accepted as fulfilling this part of the requirements for the degree of* Doctor of Philosophy

*Approved by:*

Leetooka

Harold J. Kersten

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# SOME BIOPHYSICAL STUDIES OF BACTERIOLOGIC MATERIAL

A dissertation submitted to the

Graduate School

of the University of Cincinnati

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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by

Elizabeth V. Wright

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A. M. University of Kentucky 1931

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SOME BIOPHYSICAL STUDIES OF BACTERIOLOGIC MATERIAL

Contents

## Foreword

The application of physical methods to the study of biological material has in the last two decades become a very productive and important branch of scientific research. The biological scientist for many years observed living material under various experimental conditions and described its behavior in qualitative terms. Many of his observations could not be explained, his results were only moderately consistent, and he had little knowledge of how to influence or control the behavior of his material. Because of this apparent variability of living substance the physical scientist would have little to do with it and the two great branches of study proceeded independently of each other. Certain outstanding relationships, however, could not be ignored and gradually the biologist began to seek the aid of the physicist in their interpretation. Many observers noticed that light was essential to the propogation of some forms of life and yet other forms were injured by it. From this developed extensive studies of the effects of the different components of visible light, infra-red and ultra-violet light, x-ray and radium emanations on the growth and reproduction of living cells. Electricity by various means and in many forms may profoundly influence the activity of living cells. Light and electricity are two major branches of pure physical study and in order for the biologists to be

able to use them as tools of research he must know something of their properties. The study of the effects of physical agents on the growth, reproduction and metabolism of living cells is now the field of the biophysicist.

The following biophysical studies made use of high electrical potentials for the production of x-rays and the generation of ozone. Bacteria and some of their products were subjected to the action of these agents.

# I. EFFECTS OF SOFT X-RAY RADIATIONS ON B. PRODIGIOSUS AND B. PYOCYANEUS

## A. Historical

Radiations, such as x-rays are able to produce marked effects on the cells of a wide variety of living forms. X-rays are produced by ejecting streams of electrons from a high voltage current against a metal target which, in turn, emits characteristic rays. When the rays are absorbed by a cell, high speed secondary electrons are thrown out of the atoms making up the constituents of the cell. These electrons, endowed with a large kinetic energy, plough through the cells ionizing the atoms with which they collide until their energy is exhausted and they come to rest. Ionization of the constituents of the cell produce chemical changes which in turn produce the biological effects. The biological effects produced may be morphologic, physiologic, histologic and cytologic and may or may not result in the death of the cell depending upon the dosage and length of time of exposure to x-rays. Maynard (40).

Histological changes produced in human skin subjected to x-irradiation were described by Ewing (14). There is a swelling of subcutaneous collagen. Epidermal cells swell, show abnormal mitosis and begin to necrose. These changes are followed by necrosis of subcutaneous tissue. There is an enormous swelling of epithelial nuclei following large doses of irradiation. A change in the permeability

of the cell wall is also observed.

Goodspeed and Olson (18), Komuro (30) and many others have described the cytologic changes produced in higher plants. Among these is accelerated protoplasmic streaming with short exposures. Long exposures check this acceleration without return to normal. Lowering of viscosity of protoplasm, vacuolization of nucleus and cytoplasm in the early stages, and later, contraction of the nucleus is produced. With weak doses mitosis is decreased, chromosomal rearrangement and translocation is produced. With strong doses swelling of the cell and degeneration take place. The nucleus is the first to be affected. Young cells are more susceptible to these changes than are older cells. Intermittent doses are less harmful than the same continuous dose. All metabolic activities are accelerated and if over accelerated return to normal is impossible and death results followed by a dissolution of the cell. Soft x-rays produce immediate effects while the same effects are not produced until several hours later by hard rays.

The theories of the causes of physiologic changes occurring in x-irradiated cells were discussed by Packard. (49) One theory is the point-heat hypothesis. The major part of the energy absorbed by tissues is transformed into heat by the collision of electrons with atoms of the cells. The heat generated at the point of collision coagulates the protoplasm and results in many minute dead areas of

protoplasm. The basis for this assumption is the appearance of granular material in the protoplasm of irradiated cells.

Another theory claims the precipitation of colloids of protoplasm by disturbing the potential between the particles of protoplasm and the fluid in which they are suspended. This change in electrical charge may result in altered cell viscosity, surface tension, permeability and pH concentration of the cell contents.

Observations on the effects of x-rays on bacteria have been largely on the bactericidal or lethal effects. Smith and Lisse (53) using a Coolidge type tube with molybdenum and copper targets activated by 30 K.V. and 22 ma. at 13 cm. distance killed E. coli. The rate of killing was not the same for the two targets. Molybdenum required 120 minutes for 100% killing while copper required 20 minutes for 100% killing. The bacteria were exposed on the surface of nutrient agar plates and in aqueous suspensions. Exposures of 120 to 240 minutes decreased the electrophoretic mobility of E. coli cells.

A few workers reported no difference in growth of irradiated cultures as compared with non-irradiated cultures. Others observed that the infectivity of certain pathogens such as the human tuberculosis organism, Staphylococcus aureus and B. diphtheriae was reduced when irradiated before inoculation into laboratory animals.

In most of the early work radiations were supplied by tubes producing the so-called hard or penetrating x-rays. One of the theories to explain the lethal effect of x-rays is that a cell will die when it has received a certain critical amount of radiation. Wyckoff (61) (62), in careful quantitative studies on the lethal action of x-rays for bacteria found that the absorption of one 155 K.V. electron from a Coolidge tube is sufficient to cause death of the bacterial cell. Also, the absorption of 1 quantum of energy from gas x-ray tubes with a number of different metal targets emitting soft x-rays, kills. The quantum absorbed must strike a certain vital portion of the bacterial cell. The vital portion occupies about one-twentieth of the total volume of the cell so that only about one quantum in twenty is lethal. X-rays of this type then should have a greater modifying effect on the physiology, morphology and cultural characteristics of bacteria than the so-called hard x-rays. Little significant work has been reported on the effects of sub-lethal doses of the soft x-rays.

Having available a source of soft x-rays a series of studies were made to observe some of the effects of this type of radiation in sublethal doses.

B. Experimental

1. A number of exposures to soft x-rays from a copper target gas x-ray tube operated at 30 K.V. and 10 ma.

were made in order to determine the sub-lethal dose for two species of bacteria, B. prodigiosus and B. pyocyaneus.

Exposures were made for varying periods of time at 1.5 cm. distance from the focal spot of a copper target gas x-ray tube operated at 28 peak K.V. and 10 ma. Kersten (26). An eighteen hour old agar slant culture was suspended in sterile tap water and one drop of the suspension placed on a flamed glass slide. The drop was then placed beneath the window of the x-ray tube directly in the path of the radiations and 1.5 cm. from the focal spot. After radiation, the drop of suspension was taken up with a sterile swab, brushed onto the surface of nutrient agar plates, and incubated for 48 hours at 30° C.

The following table shows the growth on plates following irradiation for the periods of time indicated.

| Irradiation Time | B. prodigiosus | | B. pyocyaneus | |
|---|---|---|---|---|
| | 24 hrs. | 48 hrs. | 24 hrs. | 48 hrs. |
| 10 min. | - | - | - | - |
| 6 min. | - | - | - | - |
| 5 min. | - | - | - | - |
| 4 min. | - | - | - | - |
| 3½ min. | - | - | - | - |
| 3 min. | ++ | +++ | - | - |
| 1 min. | +++ | ++++ | - | + |
| ½ min. | +++ | ++++ | + | + |
| ¼ min. | ++++ | ++++ | ++ | ++ |

Thus the shortest lethal time for B. prodigiosus was 3½ minutes. The sub-lethal time or length of time of irradiation after which growth from some of the organisms would occur was three minutes or less.

The sub-lethal time for B. pyocyaneus under the same conditions was 1 minute or less. Even after ¼ minute irradiation there was marked killing of this organism.

Apparently the lethal effects of this type of radiation were markedly greater for B. pyocyaneus than for B. prodigiosus. This difference may be partially accounted for in the different pigments possessed by these two species; the fluorescent pigment of B.pyocyaneus and the non-fluorescent, anthocyanin pigment of B. prodigiosus. Fluorescent substances are known to increase the degree of absorption of radiations from various sources and increased absorption enhances radiation effects. Also the lethal time at this distance was much shorter than that observed by Smith and Lisse (53).

2. A series of sub-lethal irradiations and platings was then made on strains of B. prodigiosus which produced only deep red colonies on agar plates and on another strain which produced both pigmented and non pigmented colonies. Tap water suspensions were irradiated for ½ minute then brushed on 3% glycerol agar of pH 7.2. One hundred and sixty successive irradiations and subcultures at 24 hour intervals over four months time were made on the two original strains and their progeny. At the end of the series the

original red pigmented strain still produced good red pigment and the mixed strain remained variable in its pigment producing characteristics. Irradiation for as long as ½ minute delayed pigment production for about 24 hours as compared with non-irradiated controls.

Stained preparations of cultures from irradiated suspensions were also made at frequent intervals. No marked morphologic changes were observed until near the end of the series when cells from some of the colonies appeared to possess heavy capsule like coverings. These did not appear in the non-irradiated controls. This response may be compared to the swelling of cells of the skin and of higher plants noted by other investigators.

Suspensions of B. pyocyaneus were irradiated for 5 to 15 seconds through forty series of plates. Plates of the tenth series with this organism began to show marked differences in colony forms as compared with plates made from non-irradiated suspensions. The colonies from non-irradiated suspensions at 48 hours were 1 to 3 mm. in diameter, dew-drop, butyrous, bluish by transmitted light, edges slightly frayed and centers coarsely granular. Good blue-green pigment was always produced. The new colony type was much more spreading, 5 to 6 mm. in diameter at 48 hours, margins more frayed, and so translucent as to be almost imperceptible microscopically. Two plainly visible concentric rings developed near the periphery. The colonies were markedly viscid. When

allowed to age ten days to two weeks many glistening dew-drop, pin point secondary colonies appeared over the surface of the old mother colonies.

Stained preparations from these colonies showed long, slender, slightly curved, solidly stained rods, 0.8 to 1 x 2-4 mu in size. Plates of the twelfth series developed another colony type, similar to that of the tenth series, with clear plaque-like areas. Stained slides from these plaques showed heavily encapsulated, slender, granular or beaded rods. Non-irradiated cultures did not produce these heavily encapsulated forms. The non-irradiated organisms were also much smaller, 0.5 to 1 to 2 mu in size, and uniformly stained. Cells from plaques and from viscid colonies were only occasionally motile in the hanging drop while the organisms from butyrous colonies were actively motile. These colonies persisted to the end of the series and no additional colony types were observed throughout the remainder of the series.

As was noted earlier encapsulated organisms appeared in irradiated cultures of B. prodigiosus. Apparently this was the most outstanding effect of soft x-ray radiations by this method on these two species of bacteria.

3. In the previous study it was observed that slight changes in the pH value of the agar produced marked changes in the color and amount of pigment produced by B. prodigiosus.

A series of eight plates was then prepared containing

agar varying by 0.2 pH from 6.8 to 8.2. These plates were divided into halves, one half streaked with suspensions of B. prodigiosus which was x-rayed for 30 sec. at 1 cm. distance, the other half streaked with non-irradiated B. prodigiosus. The plates were incubated for 48 hrs. at 30° C. Suspensions were made from the irradiated segments of each plate and re-irradiated for 30 sec. and streaked on plates containing agar of the same pH value as that from which the organisms were taken. The other half of each plate was again streaked with non-irradiated organisms. Eight series of such plates were made, thus subjecting the original culture to eight irradiations and subculture on agar of eight different pH values. The pigment production on agar of the same pH value remained constant through the series. The pigment production on agar of different pH values varied greatly. Pigment on pH 6.8 series was the palest pink color of the series. pH 7.8 to 8.2 produced the darkest red. pH 7.0 to 7.6 produced colonies of a bright red color. There was no marked difference in the pigment produced on irradiated and non-irradiated segments on any series of plates. All the plates of each pH value were prepared from agar from one flask so there was no variation in pH or composition from plate to plate in the series.

4. In both the preceding studies only the inoculum was irradiated. The cells in colonies produced from irradiated cells received no radiations. All changes which might have

been initiated in the cells were either lost by the time macroscopic colonies were formed or the intensity of irradiation was not great enough to initiate changes in the characteristics of the organism.

Another series of plates containing agar of pH 7.6 to 8.2 was inoculated by streaking the agar evenly over the entire surface. Half of each plate was covered with sheet lead to serve as a non-irradiated control and irradiation continued during the entire incubation period. A small incubator with a varnished paper top which was permeable to x-rays was placed 45 inches below the window of the x-ray tube. The first set of plates was irradiated continuously for 8½ hours. Pigment appeared earlier and was of a deeper color than on the non-irradiated segments. New plates inoculated with organisms from the irradiated plates were incubated and irradiated simultaneously for 5½ hours with the distance from the window of the x-ray tube reduced to 27 inches. This greatly increased the radiation intensity as the rays from the window were divergent and decreasing the distance increased the dosage. Plates containing agar of pH 8.0 and 8.2 showed definitely less pigment on the irradiated portions as compared with the non-irradiated portions. This series also showed 50% fewer colonies on irradiated halves as on non-irradiated halves.

Subcultures to a third series of plates irradiated and incubated for 22 hours produced very little pigment on rayed

portions and good red pigment on control portions. Similar results were obtained on two subsequent transfers and irradiations of 16 and 12 hours each making a total of 64 hours of continuous radiation during incubation. Transfers were discontinued at this point because of the appearance of spontaneous lysis of the colonies on the irradiated plates. The series was repeated with approximately the same periods of irradiation and similar results were obtained. The colonies lost their discrete form and became so fluid that they flowed across the surface of the agar when the plate was tilted. This phenomenon did not appear on any non-irradiated or intermittently irradiated cultures at any time.

Stained preparations of these fluid cultures showed conglomerate masses of poorly stained material and intact bacterial cells.

5. Attempts were made to determine the cause of this spontaneous autolysis. Infusion broth suspensions of the fluid material were filtered first through Berkefeld N candles then through Berkefeld W candles. Candles of these grades never yielded sterile filtrates of the lysed cultures. Growth of red pigment producing B. prodigiosus occurred when the filtrates stood at room temperature for a few days. The candles were tested for filtering efficiency with non-irradiated cultures and sterile filtrates were readily obtained. Apparently there were minute filter passing forms of

B. prodigiosus in irradiated cultures.

The filtered suspensions were added to young actively growing broth cultures of B. prodigiosus and the mixtures incubated 24 hours. Neither clearing nor inhibition of growth was ever observed, indicating the absence of a transmissible lytic agent in the irradiated cultures.

Apparently the phenomenon was a radiation necrosis of the bacterial cells similar to the necrosis produced by over irradiation of higher plant and animal cell structures.

C. Summary:

1. Radiations from a copper target gas x-ray tube are more lethal for B. pyocyaneus than for B. prodigiosus.
2. B. prodigiosus and B. pyocyaneus retained their pigment producing ability after a long series of ½ minute irradiations.
3. Irradiation for ½ minute appeared to delay pigment production about 24 hours in the two species studied.
4. Heavy capsule-like coverings appeared on cells in cultures from irradiated suspensions of B. prodigiosus. The cells possessing capsule-like coverings were non-motile.
5. Colonies from irradiated suspensions of B. pyocyaneus produced colonies showing clear plaques containing encapsulated cells and viscid colonies.

These colonies showed non-motile or only occasionally motile cells.

6. There were no apparent differences due to irradiation in cultures of B. prodigiosus grown on agar of different pH values.
7. Irradiation of B. prodigiosus during the entire incubation period produced a marked decrease in pigment production on irradiated portions of agar plates. Radiation for 64 hours produced a dissolution of the cells so that discrete colonies became fluid. A growth inhibiting or transmissible lytic agent was not responsible for this phenomenon.
8. Filterable viable forms were produced by long term irradiation.

## II. EFFECTS OF SOFT X-RAY RADIATIONS ON BACTERIOPHAGES.

### Historical

The study of the phenomenon of bacteriophage had its beginning in 1915 when Twort (56) described the appearance and properties of a white micrococcus isolated from glycerinated calf vaccinia.

> "Inoculated agar tubes, after 24 hours at 37° C. often showed watery-looking areas, and in cultures that grew micrococci, it was found that some of these colonies could not be subcultured but if kept they became glassy and transparent. On examination of these glassy areas nothing but minute granules, staining reddish with Giemsa, could be seen. Further experiments showed that if a colony of the white micrococcus that had started to become transparent was plated out instead of being subcultured as a streak then the micrococci grew, and a pure streak culture from certain of these colonies could be obtained. On the other hand if the plate cultures (made by inoculating the condensation water in a series of tubes and floating this over the surface of the medium) were left, the colonies, especially in the first dilution, soon started to turn transparent, and the micrococci were replaced by fine granules. This action, unlike an ordinary degenerative process, started from the edge of the colony, and further experiments showed that when a pure culture of the white or the yellow micrococcus, isolated from vaccinia is touched with a small portion of one of the glassy colonies the growth at the point touched soon starts to become transparent or glassy and this gradually spreads over the whole growth, sometimes killing out all the micrococci and replacing these with fine granules. Experiments showed that the action is more rapid and complete with vigorous-growing young cultures than with old ones, and there is little action on dead cultures or on young cultures that have been killed by heating at 60° C. --- The transparent material when diluted (one in a million) with water or saliva was found to pass the finest porcelain filters with ease, and one drop of the filtrate pipetted over an agar tube was sufficient to make that tube unsuitable for the growth of the micrococcus. That is, if the micrococcus was inoculated down the tube as a streak, this would start to grow, but would soon

become dotted with transparent points which would rapidly extend over the whole growth. The number of points from which this starts depends upon the dilution of the transparent material. --- This condition or disease of the micrococcus when transmitted to pure cultures of the micrococcus can be conveyed to fresh cultures for an indefinite number of generations; but the transparent material will not grow by itself on any medium. --- When placed on a fresh agar tube without micrococci it will retain its powers of activity for over six months. It also retains its activity when made into an emulsion and heated to 52° C, but when heated to 60° C for an hour it appears to be destroyed."

D'Herelle (12) in 1917 described an epizootic naturally occurring in locusts in Mexico. In mass cultures of coccobacilli isolated from intestinal contents of locusts dying of the infection, colonies which were of indented irregular contour and showing areas entirely free of growth were observed. Extensive studies were made in an attempt to explain these phenomena and an hypothesis was formulated. To d'Herelle it seemed that the coccobacillus and the agent causing these cultural peculiarities were associated but that the unknown agent was an ultramicroscopic pathogenic organism. This he was never able to demonstrate.

In 1916 d'Herelle (12) still interested in the problem arising from the locust epizootic continued to make the observations on cultures from some human diseases. Stool from a patient suffering from bacillary dysentery (Shiga) was mixed with bouillon and incubated over night. The suspension was filtered through a Chamberland candle, and

the filtrate transferred to a previously inoculated tube of Shiga bacilli. The series of tubes gave normal cultures of B. dysenteriae until the patient began to convalesce when one day an apparently sterile tube appeared. To this tube another suspension of Shiga bacilli was added and after 10 hours it was again clear. D'Herelle then decided the fecal cultures contained an agent which dissolved the Shiga bacilli. To this substance he gave the name Bacteriophage. Though d'Herelle stoutly maintained this phenomenon was not the same as that described earlier by Twort there can be no doubt at the present time that the two workers were studying the same principle. In view of this, it has become known as the Twort-d'Herelle phenomenon. However d'Herelle continued a very precise series of studies on all of the phases of the problem and all of the noteworthy early work was done and described by him.

There were three questions which d'Herelle and many others have attempted to answer: (1) What is the physical nature of the agent causing lysis of bacterial cells? (2) What is its biological nature? (3) How does it arise and from what does it come?

D'Herelle proposed many of the answers to these questions and furnished the major incentives to other workers in attempts to prove or disprove his ideas.

Physical properties:

(1) Bacteriophage is corpuscular. The particles vary

in size from 20 to 90 milli-microns in diameter. (2) A temperature of 75° C for 10 minutes completely inactivates it. (3) It is quickly destroyed by ultra violet light. The resistance is slightly greater than that of the homologous bacteria. (4) Resistance to chemicals is intermediate between that of the vegetative cells and bacterial spores. (5) It is protein in nature, colloidal, and bears a negative electric charge. (6) Its diffusibility depends upon the porosity of the membrane. (7) It is non-volatile.

Biological Nature:

(1) It is an obligate parasite on bacterial cells. (2) The clear areas produced in plate cultures are colonies of bacteriophage. (3) It may be preserved in a viable form in sealed ampoules for as long as nine years. (4) It may be increased in virulence by subculturing serially on young, actively growing, homologous bacteria. (5) It is species specific. (6) The lytic activity depends upon the vigor with which it is able to attack susceptible bacteria. That is, all phages do not show the same degree of lytic ability nor are all phages active to the same high dilution. (7) Bacteriophage when introduced parenterally stimulates the production of antibacteriophagic antibodies. It may also sensitize an animal inducing an anaphylactic condition.

Theories concerning the nature of bacteriophage.

(1) Bacteriophage may be a chemical not derived from the bacterial cell.

(2) Bacteriophage may be a chemical agent resembling an enzyme derived from the bacterial cell.

(3) Bacteriophage may be an autonomous living form foreign to the bacterial cell and parasitic upon it.

1. As to the first theory, there is no possibility that such is the case. All investigators on the phenomenon know that the titre may be increased by serial transfers which, if continued indefinitely, would remove the slightest trace of the original agent rather than increase it.

2. As to the second theory: - There are three possibilities, (a) It is an autolysin resulting from bacterial metabolism. (b) It is a fragment of the bacterial cell which retains its capacity to multiply. (c) It is a diastatic enzyme-like substance elaborated by dissolution of the bacterial cell. (3) (5) (6) (10) (11) (32) (33) (34) (36) (45) (59). (d) It is a lysin elaborated by a foreign cell.

If any one of these were tenable, then all bacterial cultures at one time or another would show the effects of bacteriophagy. This is not the case. Of these theories, however, that of a bacterial enzyme elaborated upon lysis of the bacterial cell has many supporters.

The third theory, that of a living foreign agent, to many workers is the least contradictory of the greatest number of experimental facts discovered concerning the

nature and action of the phenomenon. (2) (7) (12) (15) (17) (24) (53) (56).

The mechanism of phage lysis has been observed photomicrographically at short intervals of time.

The lysis of bacterial cells by bacteriophage appears to proceed in several stages. The phage particle is adsorbed by the bacterial cell during a short interval of time following mixture with the culture. The phage then apparently penetrates the cell and begins multiplication. This causes the cell to swell tremendously to three or four times its original size. The cell then suddenly and violently explodes and leaves a residue of amorphous material which later completely dissolves. This process requires from 2 to 4 hours for completion. Bayne-Jones and Sandholzer (1); Clifton & Morrow (17).

Fisher & McKinley (15) and McKinley and Holden (41) have shown that the lytic activity of B. coli bacteriophage is reduced or destroyed by exposure to ultra-violet light from an Alpine sun lamp and the resistance of the phage is directly proportional to the concentration of the lytic agent.

Gates (16), working with ultra-violet light, found that the energy required for destruction of the lytic activity of Staphylococcus aureus phage was slightly more than that required for destruction of the bacterial cell and for each wave length studied, readings showed progressive killings

with increased exposures.

Beckwith, Olson and Rose (3), working with six strains of B. coli and seven B. coli bacteriophages found that exposure to rays from a Roentgen tube for 30 minutes reduced but did not destroy the lytic activity of three of the possible 42 combinations.

The x-rays used in these experiments were probably the penetrating or short wave-length radiations of medical radiology so that only a small part of the radiant energy was absorbed by the bacteriophages. If it is the energy absorbed by the bacteriophages which reduces their activity, and if other conditions are the same, one would expect the degree of inactivation to be greater when the x-rays were of the less penetrating long wave-length or soft variety. Mayneord (40), Wyckoff (61).

Having available a source of soft x-rays, a series of studies were made to investigate the effects of such radiations on several bacteriophages.

Apparatus.

A gas x-ray tube having a copper target and window of aluminum foil and cellophane was operated at 30 peak Kv. and 10 ma. The most intense parts of the radiations transmitted by the window were the $K_{\alpha}$ (1.54Å) and $K_{\beta}$ (1.38A) lines of the copper x-ray spectrum (Kersten) (17). Fig. 1 shows the relative position of the target, window, and material to be irradiated.

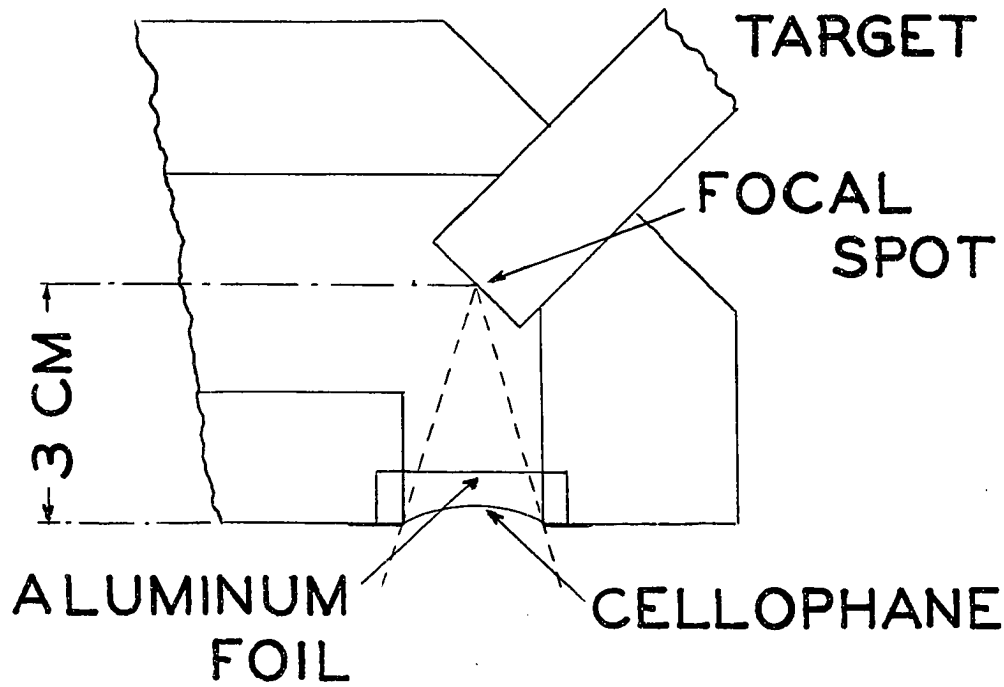



Fig. 1 X-ray tube showing target and window.

In order to measure the relative lytic activity of x-irradiated and normal bacteriophages it was necessary to have some device for measuring the changes in turbidities of suspensions of bacteria in the presence of the phages.

An inexpensive apparatus for measuring very small changes in turbidities in convenient numerical units was constructed. Fig. 2 Wright & Kersten (60).

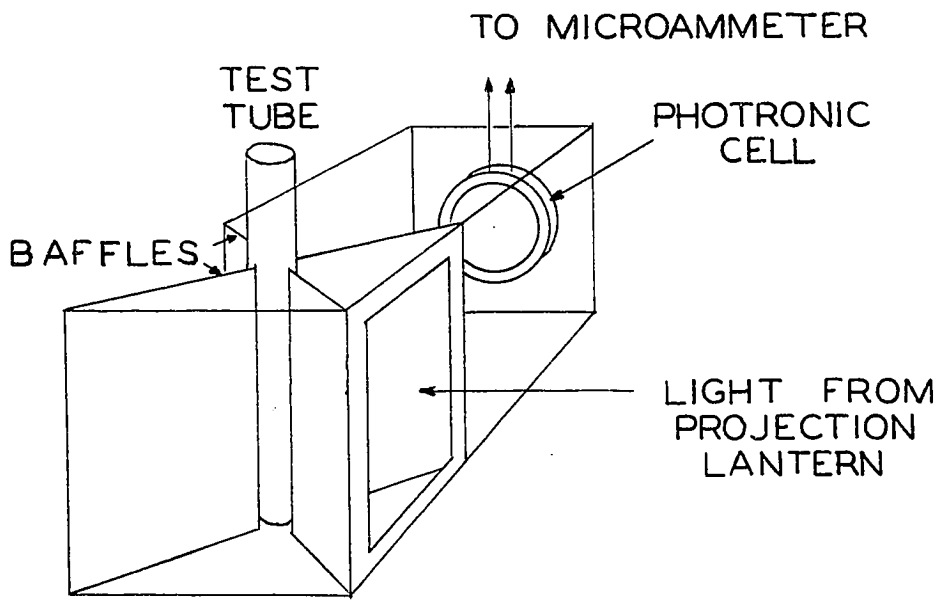



Fig. 2. Apparatus for measuring turbidities of bacterial suspensions.

It was essentially a black box 2.5 x 3.5 x 10 inches in size. In one end was mounted a Weston Photronic cell connected to a microammeter. In the other end four baffles extending from the sides towards the center pressed firmly against a test tube admitted through a hole in the top. The ends of the baffles against the test tube were covered with black plush to make a light proof seal against the glass of the tube. An intense beam of light from a projection lantern entered the box through an opening in the side between two of the baffles and illuminated the test tube. Part of the

light from the lantern was reflected at right angles by particles in suspension in the test tube and illuminated the Photronic cell producing a reading in microamperes on the ammeter. A test tube of bubble free distilled water reflected very little light so that the reading on the microammeter was practically zero. A tube containing a suspension of bacteria produced a higher reading depending upon the degree of turbidity of the suspension.

The use of the apparatus is illustrated by the curves of Fig. 3. To obtain readings on a bacterial culture the test tube containing the culture was removed from the incubator at fifteen minute intervals, shaken, and placed in the apparatus, and the microammeter reading recorded. Curve A shows how the readings for a broth culture of Staphylococcus aureus increased during incubation at 37° C. Curve B shows the results for a similar suspension to which an antiseptic was added at the time indicated by the arrow. Curve C is that of a suspension to which an homologous bacteriophage was added at the time indicated by the arrow. The curves clearly show that the readings are progressively higher when growth proceeds uninterruptedly. When growth is stopped by the antiseptic the turbidity immediately becomes constant, while after the addition of the bacteriophage the turbidity increases for an interval and then decreases during the clearing period to the original value.

One may use tubes containing standardized suspensions of Fuller's earth to calibrate this apparatus so that it will

read the turbidity in terms of numbers of bacteria rather than microamperes.

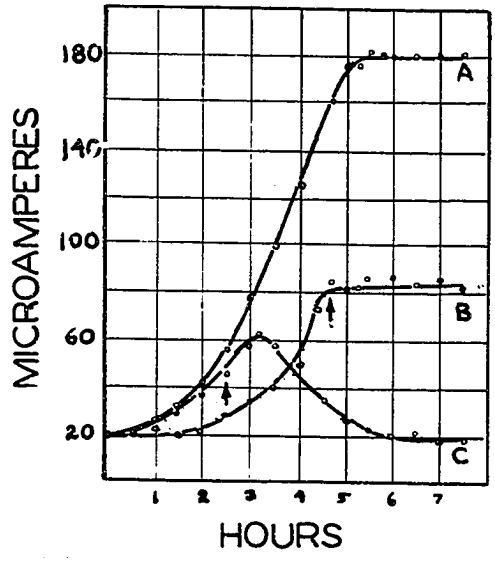



Fig. 3. Reading of the microammeter for a broth culture of Staphylococcus aureus during incubation at 37° C. Curve A, normal culture; Curve B, culture to which an antiseptic had been added at the time indicated by the arrow; Curve C, culture to which an homologous bacteriophage had been added at the time indicated by the arrow.

Experimental.

Bacteriophages for hemolytic streptococcus, Staphylococcus aureus, B. coli, and Shiga dysentery were chosen because of their high lytic activity and ready transmissibility.

The phages were prepared by inoculating test tubes containing 10 cc. of brain-heart infusion broth with ½ cc. of a 24 hour broth culture of the organism. This new

culture was incubated at 37° C. for 2 hours, then 1 cc. of phage from a previous run added and incubation continued until complete clearing was produced. One cc. of the young phage was placed in a small pyrex cup about 1.5 cm. in diameter. This quantity insured an amount of the lytic principle adequate for producing clearing in normal control tubes within 2 to 4 hours after addition to a susceptible culture. The cup, open at the top, was placed directly beneath the window of the X-ray tube so that the surface of the liquid in it was 412 cm. from the focal spot. After irradiation, the material was transferred by pipette to a test tube containing a 2 hour old broth culture of homologous bacteria. A similar tube of broth culture to which 1 cc. of non-irradiated bacteriophage was added acted as a lytic control while another culture tube containing no bacteriophage acted as a normal growth control. All tubes were incubated at 37° C. and readings of turbidity taken every 15 minutes until the maximum clouding or clearing was reached. Figures 4, 5, 6 and 7 show the results of irradiation of 4 different bacteriophages for varying periods of time. Each graph shows a normal growth curve in the presence of the irradiated and non-irradiated lytic agent. During growth the microampere readings increase with increasing clouding, while during lysis, as the culture clears, the microampere readings decrease until a low reading is obtained upon completion of lysis.

Tubes containing bacteriophages irradiated for 1 hour show only slight modification of the lytic activity while those containing bacteriophages which had been subjected to 4 to 6 hours of irradiation have growth curves approximating those of cultures in the absence of the lytic agent. Those irradiated for periods between these extremes have intermediate growth curves.

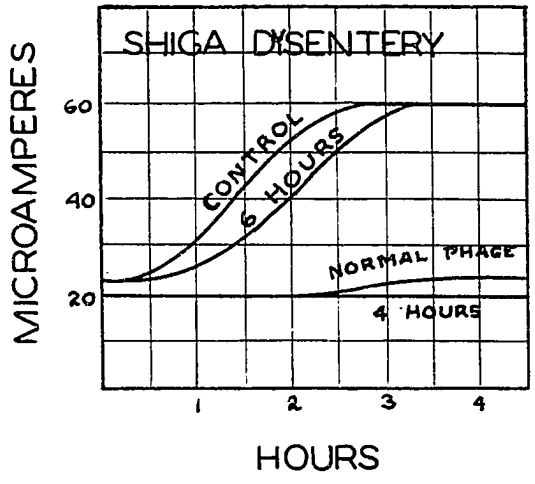



Fig. 4.

Control Experiments.

With the x-ray tube used, the sample was warmed slightly during irradiation by the heat which passed through the window with the x-rays. In order to show that the effect

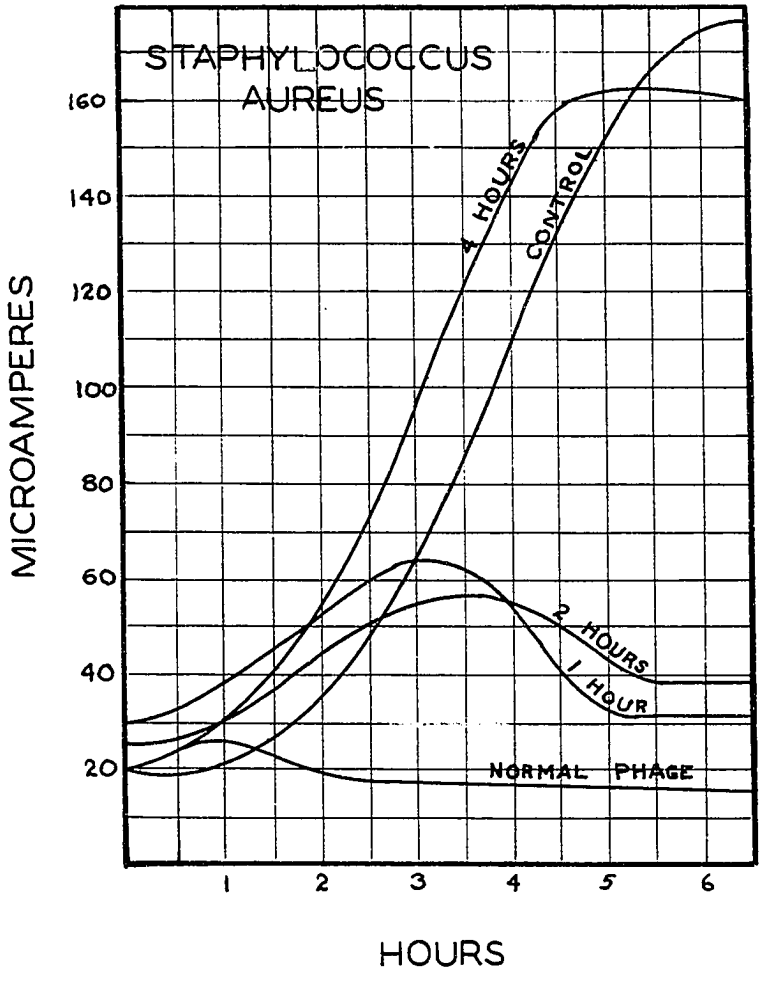



Fig. 5.

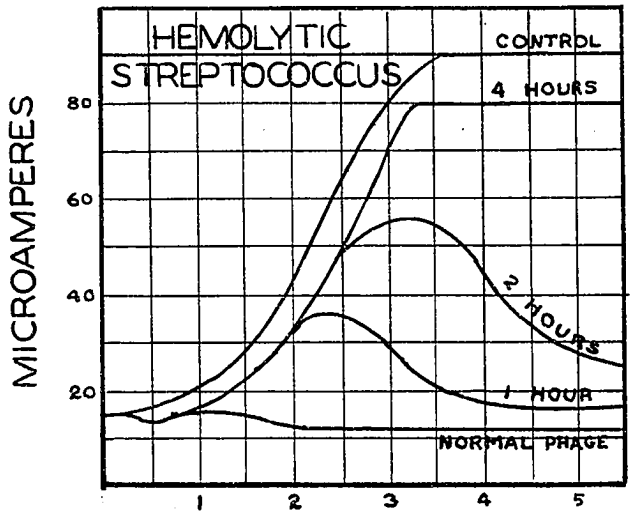



Fig. 6.

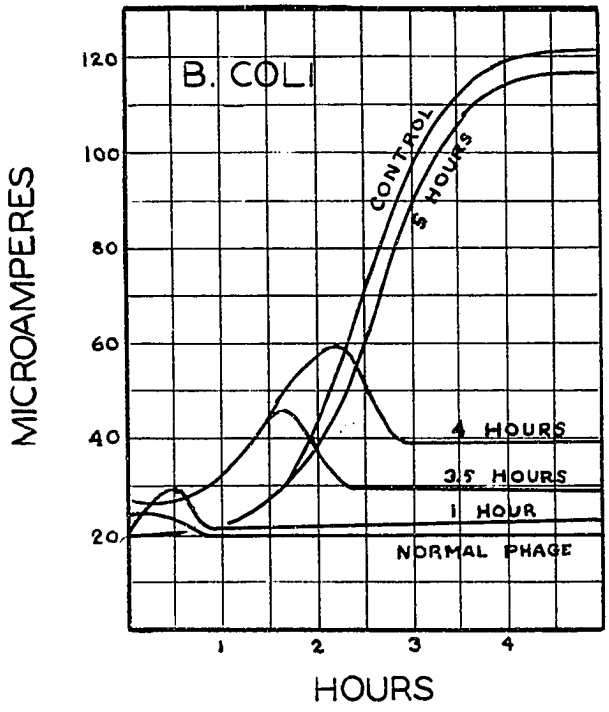



Fig. 7.

of irradiation on the lytic activity of the bacteriophages was due to the x-rays and not to the small quantity of heat accompanying them, the following additional experiments were done. The temperature just beneath the window of the x-ray tube was measured by means of a thermocouple and was found to increase not more than 3° above room temperature during a five hour period of irradiation. In another experiment the bacteriophage was held at a temperature below 14° C., by means of an ice-salt mixture, during irradiation. Reduction of the lytic activity was of the same degree as that of an uncooled bacteriophage irradiated for the same length of time. A similar bacteriophage cooled during the irradiation period in an identical manner, but protected from X-rays showed no alteration in its ability to produce lysis. Bacteriophages were also incubated for 5 hours at 45° C. and found to retain their normal lytic activity. From these experiments it may be concluded that the bacteriophages were not inactivated by heat from the X-ray tube.

There was also the possibility of an inhibiting substance being produced in the broth by the long periods of irradiation. Blank and Kersten, (4). To investigate this possibility, 1 cc. of double-strength broth was irradiated for 4 hours, 1 cc. of bacteriophage added, and the mixture held at 37° C. for 4 hours. This was then added to a susceptible young culture. No reduction of lytic activity was produced, indicating the absence of inhibiting substances in

the irradiated broth.

Finally, there was the possibility of the pH of the medium being changed by irradiation and this in turn affecting the activity of the bacteriophages. Pratt (50). Broth which had been irradiated for 4 hours showed no change in pH value as compared with non-irradiated broth from the same tube, when matched with standard brom-thymol blue indicator ampoules.

The growth curves obtained by Lin (38) using diluted bacteriophages were similar in shape to those obtained in the above experiments. Bacteriophages which have been partially inactivated by irradiation with soft x-rays correspond to the type of curve produced when the phage is diluted.

Conclusions.

From these results it appears that :

1. the lytic activity of bacteriophages for Staphylococcus aureus, Streptococcus hemolyticus, Escherichia coli and Shigella dysenteriae may be partially or completely inhibited by exposure to soft x-rays.
2. All of these bacteriophages are not equally affected by the same dose of x-rays.
3. The degree of inhibition for any one bacteriophage increased with increased doses of x-rays.

## III. LAUE PHOTOGRAPHIC METHODS APPLIED TO A STUDY OF YEAST NUCLEIC ACID AND BACTERIOPHAGE.

### A. Theoretical Discussion.

Laue, in 1912, discovered that a narrow beam of x-rays, when passed through a crystal, was split up into several beams of different intensities. A photographic plate exposed to these emergent beams gave a characteristic pattern depending upon the positions of atoms in the crystal.

Amorphous organic compounds on photographing with x-rays yield pictures showing diffraction rings from polar, rather than from atomic groups.

A study was undertaken in an attempt to detect nucleic acid from bacteriophage by x-ray diffraction methods. If nucleic acid is contained in bacteriophage some light may be thrown upon the nature of the lytic agent. All living material contains nucleic acid. The presence of nucleic acid in bacteriophage may then indicate that the lytic agent is derived from the nuclear material of the bacterial cell by an autocatalytic process or it is contained in the phage unit by virtue of its living state. In order to obtain sufficient bacteriophage for such a study it was necessary to separate the lytic agent from nucleo-proteins from autolysed bacterial cells and concentrate the active principle.

### B. Methods for Purification of Bacteriophage.

Several methods have been used for obtaining bacteriophage in a highly purified form, Wyckoff (63) by ultra-

centrifugation of staphylococcus bacteriophage obtained a lytic fraction with a molecular weight of not less than 200 million and a sedimentation constant, $S_{20} = 650 \times 10^{-13}$ cm. $dyne^{-1}$ $sec.^{-1}$ This fraction lost its lytic ability when inactivated with chymo-trypsin, alkali or heat. Chymo-trypsin inactivation did not disrupt the heavy component, alkali disrupted the heavy component, and heat produced a sharply sedimenting dilute gel.

Northrop (46), working independently or Wyckoff, obtained from lysed staphylococci cultures a nucleoprotein with transmissible lytic properties which had the same sedimentation constant ($S_{20} = 650 \times 10^{-13}$ cm. $dyne^{-1}$, $sec.^{-1}$) and a molecular weight calculated at 300 million. This substance was very unstable, was denatured at pH greater than 5, by temperatures more than $50^{\circ}$ C. for 5 min., and by chymo-trypsin but not by trypsin. Diffusion rate and sedimentation rate were not altered by denaturation. Ultra-violet of wave lengths from 2500 $A^{\circ}$ to 3000 $A^{\circ}$ inactivated agreeing, with the work of Gates (17), (16), on natural bacteriophage. Northrop suggested that in the light of this evidence bacteriophage formation may be analogous to the autocatalytic formation of pepsin and trypsin.

Northrop (45) in another piece of work isolated from lysed staphylococci a protein with a molecular weight of 500,000 which was absorbed by homologous bacteria to the same extent as bacteriophage. It was elaborated only in the presence of young actively growing bacteria. No conclusive

evidence was obtained that this substance was a phage precursor as was obtained in studies on enzyme precursors. These studies indicate that a nucleoprotein is contained in bacteriophage.

Methods for physio-chemical purification of bacteriophage are numerous. Kleiger and Olitzki (29) employed a buffered kaolin suspension for adsorption then eluted the adsorbed phage with dilute ammonium hydroxide. The eluate was dialyzed for 6 days against distilled water. By this process a phage of high titre and low nitrogen content was obtained.

Meyer, Thompson et al. (42) precipitated the protein constituents of a phage culture with colloidal iron, naphthol yellow, and barium chloride at various pH levels and obtained finally a precipitate of barium sulfate on which was adsorbed phage of good titre.

Le Mar and Myers (36) claimed to have obtained a transmissible lytic agent active to $10^{-11}$ dilution from autoclaved cultures of bacteria by oxidation with hydrogen peroxide and incubation then extraction of the oxidized material with ether. The author tried this method but was unable to obtain a lytic agent.

C. Preparation of Purified Shiga Bacteriophage.

The method of Hasoya, Nagase, and Yoshizuni (21) was used for the preparation of a purified Shiga dysentery bacteriophage.

Five litres of highly active Shiga bacteriophage was

filtered through infusorial earth then through Berkefeld N candles. To the clear filtrate was added 2% $ZnCl_2$ until no more precipitate was formed. The precipitate was removed by filtration and discarded. Ten per cent $NH_4OH$ was added until no more zinc hydroxide was precipitated. The bacteriophage was adsorbed by the gel as it precipitated. The precipitate was separated from the supernatent solution by centrifugation and washed with distilled water by suspending and recentrifugating the sediment five times. The final sediment was resuspended in 100 cc. of distilled water and 10% ammonium citrate added until it was completely dissolved. A 10% solution of ammonium sulfide was then added until no more white zinc sulfide was formed. The precipitate was removed by centrifugation and the clear solution evaporated in vacuo at 37° C. until the volume was reduced to 200 cc. This was filtered through Berkefeld candles, and the filtrate dialyzed in 25 cc. portions in collodian sacs in distilled water for 6 days. The dialysate was then evaporated to dryness in vacuo, at 37° C. A portion of the residue obtained was tested for lytic activity and was found to be highly lytic for homologous Shiga cultures.

D. Preparation of Pure Nucleic Acid.

The remainder of the residue from the above procedure and a sample of commercial yeast nucleic acid were treated simultaneously by the method of Johnson and Harkins (23) for the preparation of pure nucleic acid. 100 gms. of

commercial yeast nucleic acid was suspended in water cooled to 0° C. 100 cc. of 30% aqueous solution of sodium hydroxide cooled to 0° C. was added slowly to the cold nucleic acid suspension and the temperature kept below 5° C. for 1 hour. The solution was diluted to 500 cc. and glacial acetic acid added until the mixture was acid to litmus. The sediment formed was filtered out and discarded. Ninety-five per cent alcohol was then added to give about 4% solution and the sediment removed by filtration and discarded. Concentrated hydrochloric acid was added to just acid to congo red. An equal volume of 95% alcohol was added, the precipitate collected, and washed three times with ether. A snowy white fluffy powder of purified nucleic acid was obtained.

E. Photographs of Nucleic Acid from Yeast and Extract of Bacteriophage.

Some of the nucleic acid from the above procedure was placed in a steel cylinder, a steel plunger inserted, and the powder compressed into a disc about 0.5 mm. in thickness. The disc was placed over the pin hole in the photographic camera of a gas x-ray tube. Kersten (27). Exposure of a flat film at 5.0 cm. distance for 4 hours was made, the tube being operated continuously at 35 KV. and 20 ma.

The film on developing showed no absorption rings as are obtained with some other organic compounds.

Bacteriophage was treated by the same procedure for

extraction of nucleic acid as for yeast. A very small sample of amorphous material was obtained and photographed in the same manner as the yeast extract. The negative on development likewise showed no absorption bands.

Since ultraviolet absorption sometimes yields characteristic pictures of small quantities of substances in solution attempts were made to obtain absorption photographs with a Hilger type spectrograph. Neither yeast nucleic acid nor bacteriophage extract gave pictures with the apparatus available.

F. Summary

Attempts to obtain photographs of yeast nucleic acid and extract of bacteriophage by X-ray and ultra-violet absorption methods were not successful.

## IV. PREPARATION OF VACCINE FOR SHIGA DYSENTERY WITH OZONE.

### A. Purpose of the study.

Many methods have been employed for the preparation of bacterial vaccines. One of the most generally used is heat killing of saline suspensions of the organisms. Another common procedure is the use of chemicals such as phenol, formaldehyde, Wherry and Bowen (58), and nitrous acid. Foshay (15a). Chemicals when added in quantities sufficient to kill may over inactivate the antigenic properties of the bacteria through contact long after the bacteria are all killed and thus render the vaccine practically useless. Chemicals are also an unnecessary irritant to the tissues on injection of the vaccine and often an intense burning sensation is produced because of their presence. When used for inactivation of vaccines chemicals can not be removed so that the undesirable effects must be tolerated.

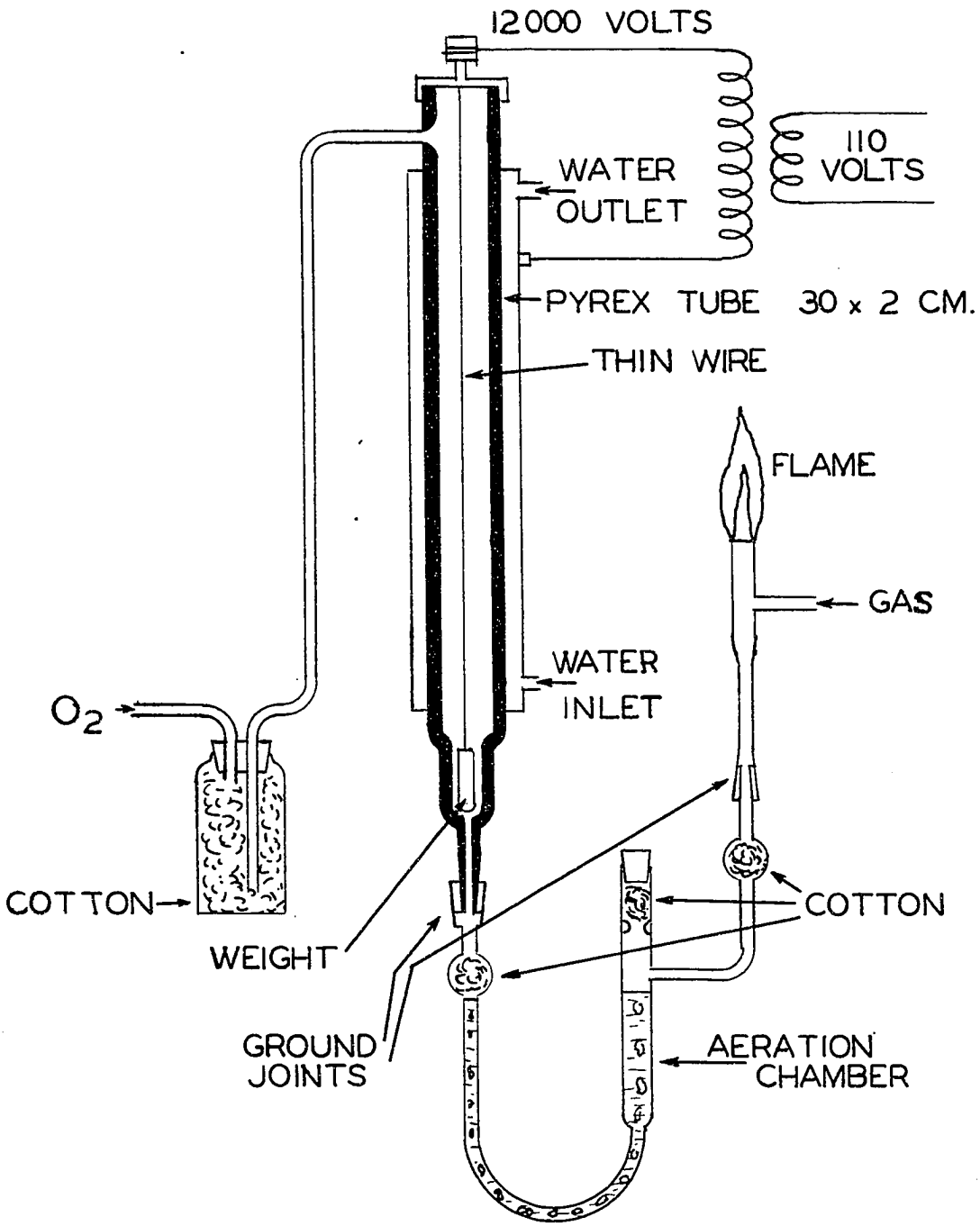
There are a few gaseous disinfectants such as chlorine, formaldehyde and sulfur dioxide which are sufficiently bactericidal to be dependable. These are all soluble in water and remain dissolved in it. There is another gaseous bactericide which may well be used for vaccine preparation which theoretically should be ideal. Ozone was shown by Heise (20) to be highly efficient as a germicide. 1-1000 volume % killed 95% of coliform organisms in one minute, 1-270,000 volume % in one hour, 1-720,000 volume % in three hours. As a highly active oxidizing agent it is capable of

modifying the cell constituents so that toxicity is reduced which in some cases is very necessary. After it has accomplished killing and modification of toxicity then its action may be immediately discontinued and no chemical residual is left in contact with the antigenic constituents of the vaccine to continue to modify their properties. The residue left after the ozone has come into contact with organic matter is oxygen which passes out of the solution.

The Cooper-Hewitt mercury vapor lamp has been used extensively for the generation of ozone for the purpose of water purification. There is no record of the application of ozone in the preparation of bacterial vaccines.

Preparation of a vaccine for Shiga dysentery by the action of ozone on a saline suspension of the organisms was undertaken in an attempt to prepare a highly antigenic, moderately toxic immunizing agent. It is generally known and may be specifically cited in the work of Moore and Kersten (43) and Wherry and Bowen (58) that heat killed Shiga suspensions are more toxic for rabbits than are live suspensions.

Shigella dysenteriae was chosen for this work because of the apparent dual nature of its toxin. Okell and Blake (47) in notable studies on the toxic properties of Shiga dysentery reported two phases in the production of the toxin. First, an endotoxic phase which corresponded with the logarithmic growth phase of the organism and lasted only 12 to 18 hours.




OZONE GENERATOR

Fig. 1.

The M.L.D. for mice during this phase was 1 cc. of a live broth culture. Second, an autolytic phase during which time the bacteria rapidly autolyzed and the toxin became exotoxic. This phase began in 24 hours and reached a maximum in 14 days at which time 0.005 cc. of the broth culture was lethal for mice. Thus the Shiga toxin according to these authors is endotoxic in the "Pfeiffer sense" but resembles the exotoxins in specificity of its toxophore and haptophore groups and in its ability to stimulate the production of a potent antitoxin when injected into susceptible animals. Olitsky and Kliger (48), however, believe there are two separate toxins.

One is a neurotoxin acting on the central nervous system of the rabbit producing paralysis and believed to be an exotoxin; the other produces severe hemorrhagic dysentery in rabbits and is endotoxic in nature.

B. Apparatus.

An apparatus for the generation of ozone in fairly high concentrations was developed for this work, Fig. 1.

A fine wire was suspended from a binding post mounted at the top of a pyrex tube 30 cm. long by 2 cm. in diameter. The lower end of the tube was fitted by ground joint into a microburner. The pyrex tube was enclosed in a reflux water jacket for cooling during operation. The wire when activated with 12,000 volts of electricity produced a glow or

corona around the wire. Pure oxygen from a cylinder when passed through the corona was partially converted to ozone. The unused oxygen and ozone was mixed with fuel gas and ignited in the microburner. The aeration chamber was easily removed for sterilization and filling. Cotton plugs sterilized in place in all openings in the gas chambers prevented contamination during the aeration process.

This device was found to produce about 5 parts of ozone per 1,000,000 parts of oxygen. The quantity of ozone produced was determined by passing the oxygen-ozone mixture through 20 cc. portions of N/24 potassium iodide in N/24 sulfuric acid solution. The following reaction takes place.

$$O_3 + 2KI + H_2 \longrightarrow I_2 + 2KOH + O_2$$

The $I_2$ liberated by the ozone was then titrated with N/24 sodium thiosulfate.

$$I_2 + 2Na_2S_2O_3 \longrightarrow 2NaI + Na_2S_4O_6$$

Thus one mole of $I_2$ liberated is equivalent to one mole of ozone produced.

Fig. 2 shows the calibration curve for the apparatus.

The following volumes of N/24 $Na_2S_2O_3$ were required to titrate the iodine liberated by ozone acting for:

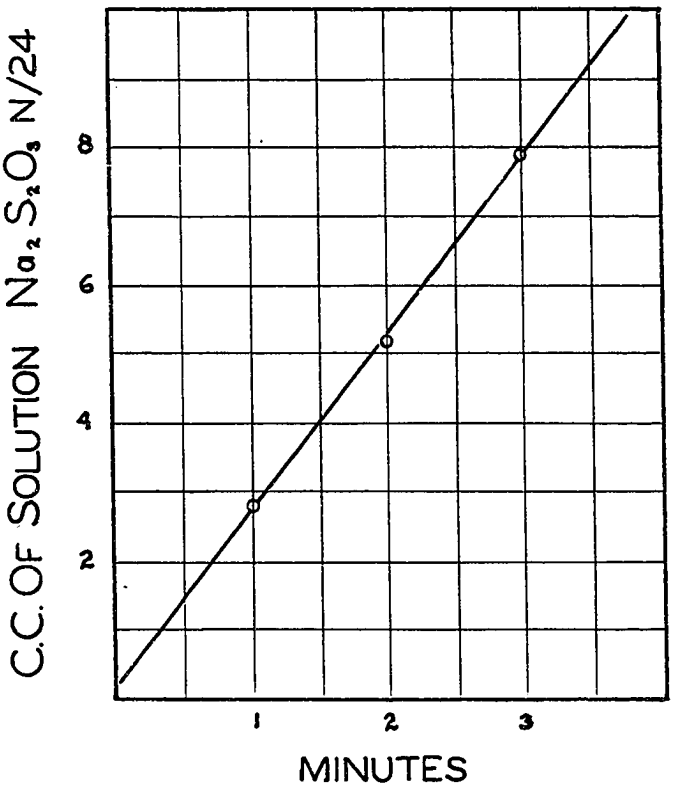
1 min. - 2.74 cc.

2. min. -5.14 cc.

3 min. - 7.89 cc.

An average of 2.63 cc. N/24 $Na_2S_2O_3$ was required to titrate the $I_2$ liberated by $O_3$ produced per minute. The

# CALIBRATION CURVE FOR OZONE GENERATOR




POTASSIUM IODIDE N/24
SODIUM THIOSULFATE N/24
OXYGEN PRESSURE - 8 CM.
OXYGEN FLOW - 3000 C.C./10 MIN.
12000 VOLTS

Fig. 2.

oxygen pressure was regulated to produce a flow of oxygen equivalent to 3000 cc. in 10 minutes. At 21° C.R.T. and 734 mm. B.P. and 18.7 mm. V.P. the volume of ozone produced was calculated as follows:

$$\frac{\text{cc.}Na_2S_2O_3 \times \text{me.eq.wt.}O_3 \times \text{min.} \times \text{Mol.vol.}O_3 \times \text{press.} \times \text{temp.}}{24 \times 48{,}000 \times (734-18.7) \times 273^\circ}$$

$$\text{mg.mol.wt.}O_3 \qquad \text{press.} \quad \text{temp.}$$

$$\frac{2.63 \times N \times .024 \times 10 \times 22{,}400 \times 760 \times 294}{24 \times 48{,}000 \times (734-18.7) \times 273^\circ}$$

.0146 cc. ozone per 3000 cc of oxygen, or approximately 5 parts ozone / 1,000,000 oxygen.

C. Procedure:

1. The growth from a 24 hour brain-veal infusion agar slant culture of Shiga dysentery was suspended in normal saline to give a density of two billion bacteria per cc. The suspensions were aerated for lengths of time varying from 15 minutes to 6 hours in the chamber of the ozone generator previously described. Aeration of cloudy suspensions for as long as thirty minutes produced complete clearing of the suspension. Aeration for less than thirty minutes produced only partial clearing. Stained slide preparations of the cleared suspensions showed a few intact cells scattered through disintegrated cellular debris which stained poorly.

After aeration 1 cc. portions of the treated suspensions were transferred to brain-veal infusion broth and incubated for sterility. Growth never occurredin the sterility

tests of ozone treated suspensions indicating complete sterilization of the vaccines with ozone.

2. Half grown rabbits were then given series of injections of the vaccines intravenously, starting with a vaccine treated with ozone for three hours or longer, then increasing the doses and decreasing the time of ozone treatment until the rabbit would tolerate large doses of short period treated vaccine. The following outline represents a typical series of injections over 30 days time.

3 injections of 3 hour treated vaccine

2 injections of 2 hour treated vaccine

2 injections of 1 hour treated vaccine

2 injections of 30 min. treated vaccine

2 injections of 15 min. treated vaccine.

The same vaccine was given until the rabbit ceased to react with loss of weight or appetite. Seven to ten days after the last injection of vaccine $\frac{1}{4}$ to $\frac{1}{2}$ cc. of a live suspension of Shiga bacilli was given. The rabbits were weighed daily and the apparent condition recorded.

D. Tables I to VII show the records of inoculation, weights and reactions of some of the rabbits.

E. Discussion

1. Ozone treated suspensions of Shigella dysenteriae were greatly reduced in toxicity for rabbits. No rabbit administered ozone treated suspensions ever showed paralysis

or hemorrhagic diarrhea. Large doses of vaccine sometimes produced loss of appetite and loss of weight but never death. Small doses produced no ill effects.

All non-vaccinated rabbits administered live suspensions of Shiga bacilli died in one to four days with the typical symptoms of Shiga intoxication. Large live doses produced hemorrhagic diarrhea and paralysis before death; small live doses produced paralysis with slight or no diarrhea.

Seven rabbits were administered ozone treated vaccine; two showed only slight signs of diarrhea following a live test dose of Shiga dysentery; three developed paralysis following the test dose and died; four of the seven rabbits lived.

Suspensions of Shiga dysentery treated with ozone retain a high degree of enterotoxic and neurotoxic antigenicity. The neurotic antigen is apparently more readily destroyed than the enterotoxic antigen since vaccinated rabbits show less protection against the neurotoxin than against the enterotoxin when tested with live doses.

Suspensions of Shigella dysenteriae become clear when treated with ozone for 20 minutes or longer. Stained preparations of ozone treated suspensions show poorly stained cells which have apparently lost most of the cellular contents and amorphous material; probably completely disintegrated cells.

TABLE I

| Rabbit Number | Date | Weight | Dose | Condition |
|---|---|---|---|---|
| 4 | 1/3/38 | 2626 gms. | 2 cc. Live Suspension | |
| | 1/4/38 | | | Dead-24 hrs.-Paralysis & Diarrhea |
| 5 | 1/5/38 | 2742 gms. | 2 cc. Suspension Heated 60°C. 30 min. | Dead after 4 hours. Complete paralysis. Viscera normal in appearance |
| | 4/27/38 | 2500 gms. | ¼ cc. HCHO treated suspension | |
| | 4/28/38 | 2476 | | Condition good |
| | 4/29/38 | 2469 | | |
| | 4/30/38 | 2300 | | |
| | 5/1/38 | 2322 | | |
| | 5/2/38 | 2342 | ½ cc. HCHO suspension | |
| | 5/3/38 | 2352 | | Condition good |
| | 5/4/38 | 2433 | | |
| | 5/5/38 | 2469 | | |
| 42 | 5/6/38 | 2503 | ¾ cc. HCHO suspension | |
| | 5/9/38 | 2620 | | Condition good |
| | 5/10/38 | 2657 | | |
| | 5/12/38 | 2730 | | |
| | 5/14/38 | 2841 | 2 cc. HCHO suspension | |
| | 5/20/38 | 2972 | | Condition good |
| | 5/30/38 | 3036 | ½ cc. Live suspension | |
| | 5/31/38 | 3062 | | Condition good |
| | 6/1/38 | 3100 | | Partially paralyzed |
| | 6/2/38 | 2972 | | Paralysis - slight diarrhea. Dead 72 hrs. after test dose |
| | 4/7/38 | 1906 | ¼ cc. Heated Filtered $O_3$ Treated 5 min. | |
| | 4/8/38 | 1947 | ½ cc. same | Good |
| | 4/9/38 | 1945 | ½ cc. same | Good |
| | 4/10/38 | 1990 | ¾ cc. same | Good |
| | 4/11/38 | 2000 | 1 cc. same | Good |
| 45 | 4/12/38 | 2020 | 1½ cc. same | Good |
| | 4/13/38 | 2060 | 2 cc. same | Good |
| | 4/16/38 | 2128 | 1 cc. Live Suspension | Good |
| | 4/17/38 | 2114 | | Good |
| | 4/18/38 | 1987 | | Good |
| | 4/19/38 | | | Bloody Diarrhea, no paralysis. Dead-72 hr. extensive hemorrhage of large intestine |

TABLE II

| Rabbit No. | Date | Weight | Dose | Condition |
|---|---|---|---|---|
| | 1/5/38 | 2627 | 2 cc. 2 hr. $O_3$ treated | Excellent |
| | 1/6/38 | 2460 | | |
| | 1/7/38 | 2430 | | |
| | 1/8/38 | 2378 | | Good-eating well |
| | 1/9/38 | 2405 | | Good |
| | 1/10/38 | 2445 | 2 cc. 2 hr. $O_3$ treated | Good |
| | 1/11/38 | 2409 | | Good |
| | 1/12/38 | 2370 | | Good |
| | 1/13/38 | 2433 | | Good |
| | 1/14/38 | 2444 | 2 cc. 2 hr. $O_3$ treated | Excellent |
| | 1/15/38 | 2495 | | Good |
| | 1/17/38 | 2547 | 2 cc. 2 hr. $O_3$ treated | Good |
| | 1/18/38 | 2745 | | Good |
| | 1/21/38 | 2700 | 1 cc. 1 hr. $O_3$ treated | Good |
| | 1/22/38 | 2632 | | Eating Poorly |
| | 1/24/38 | 2708 | | Good |
| 3 | 1/25/38 | 2761 | 1½ cc. 1 hr $O_3$ treated | Good |
| | 1/26/38 | 2700 | | |
| | 1/28/38 | 2750 | | |
| | 1/29/38 | 2777 | 1 cc. 30 minutes $O_3$ | Good |
| | 1/30/38 | 2822 | | Good |
| | 1/31/38 | 2818 | 2 cc. 20 min. $O_3$ | Good |
| | 2/1/38 | 2843 | | Good |
| | 2/2/38 | 2843 | | Good |
| | 2/3/38 | 2864 | | |
| | 2/6/38 | 2966 | | |
| | 2/10/38 | 2970 | ½ cc. 15 min. $O_3$ | Good |
| | 2/11/38 | 3017 | | Good |
| | 2/14/38 | 2980 | | |
| | 2/15/38 | 2946 | 1 cc. 15 min $O_3$ | Good |
| | 2/16/38 | 2965 | | Good |
| | 2/19/38 | 2972 | 2 cc. 15 min. $O_3$ | Good |
| | 2/20/38 | 3114 | | Good |
| | 2/25/38 | 3156 | | Good |
| | 3/1/38 | 3000 | ½ cc. Live Suspension | Good |
| | 3/2/38 | 2907 | | Good |
| | 3/3/38 | 2842 | | Weak No Diarrhea |
| | 3/4/38 | 2728 | | Paralyzed No Diarrhea Dead in 72 hrs. |

Rabbit was given each series of doses until well tolerated.

Rabbit showed apparent immunity to enterotoxic fraction - Susceptible to neurotoxic fraction.

TABLE III

| Rabbit No. | Date | Weight | Dose | Condition |
|---|---|---|---|---|
| | 12/15/37 | 1989 | 2 cc. 6 hr. $O_3$ treated | Good |
| | 12/16/37 | 1935 | | Good |
| | 12/18/37 | 1897 | | Good |
| | 12/21/37 | 2020 | 2 cc. 6 hr. $O_3$ treated | Good |
| | 12/22/37 | 2004 | | Good |
| | 12/28/37 | 2236 | 2 cc. 6 hr. $O_3$ treated | Excellent |
| | 12/31/37 | 2349 | 2 cc. 6 hr. $O_3$ treated | Excellent |
| | 1/3/38 | 2397 | 2 cc. Live Dysentery bacilli | |
| | 1/4/38 | 2335 | | Not eating, no paralysis, no diarrhea |
| 2 | | | | |
| | 1/5/38 | 2227 | | Not eating Some diarrhea. |
| | 1/6/38 | 2168 | | No paralysis. Bloody diarrhea |
| | 1/7/ 38 | 2083 | | Better, no paralysis |
| | 1/8/38 | 1972 | | Better, no paralysis |
| | 1/9/38 | 1868 | | |
| | 1/10/38 | 1724 | | |
| | 1/13/38 | 1704 | | |

Rabbit showed immunity to neurotoxin.

TABLE IV

| Rabbit No. | Date | Weight | Dose | Condition |
|---|---|---|---|---|
| | 5/5/38 | 2076 | ¼ cc 20 min. $O_3$ treated | Good |
| | 5/6/38 | 1963 | | |
| | 5/16/38 | 2100 | ¾ cc 20 min. $O_3$ treated | |
| | 5/17/38 | 2023 | | |
| | 5/24/38 | 2206 | 1 cc 20 min. $O_3$ treated | Good |
| | 5/25/38 | 2255 | | |
| | 5/31/38 | 2327 | 2 cc 20 min. $O_3$ treated | Good |
| 43 | 6/1/38 | 2305 | | |
| | 6/4/38 | 2346 | 2 cc 20 min. $O_3$ treated | Good |
| | 6/11/38 | 2483 | ¼ cc Live Suspension | |
| | 6/12/38 | 2463 | | Good |
| | 6/13/38 | 2480 | | Good |
| | 6/24/38 | 2555 | | Good-No apparent ill effects. |

Rabbit remained in good condition. No evidence of paralysis at any time. No evidence of diarrhea at any time. Immunizing and live doses all given intravenously.

| | | | | |
|---|---|---|---|---|
| | 5/5/38 | 1338 | 1 cc 20 min $O_3$ treated | Good |
| | 5/6/38 | 1347 | | |
| | 5/13/38 | 1579 | 1-1/3 cc 20 min $O_3$ treated | Good |
| | 5/14/38 | 1600 | | |
| | 5/19/38 | 1713 | 2 cc 20 min $O_3$ treated | Good |
| | 5/26/38 | 1840 | 2 cc 20 min $O_3$ treated | |
| | 5/27/38 | 1881 | | Good |
| 46 | 6/4/38 | 1979 | 2 cc 20 min $O_3$ treated | Good |
| | 6/11/38 | 2200 | ¼ cc Live Suspension intravenously | Good |
| | 6/12/38 | 2172 | | Good |
| | 6/13/38 | 2200 | | Good |
| | 6/16/38 | 2200 | | Good |
| | 6/21/38 | 2271 | | Good |

Rabbit remained in good condition. No evidence of paralysis or dysentery at any time. Immunizing injections were all made subcutaneously. Live dose given intravenously.

TABLE V

| Rabbit No. | Date | Weight | Dose | Condition |
|---|---|---|---|---|
| | 1/10/38 | 1889 | 1 cc 3 hr $O_3$ treated | Good |
| | 1/11/38 | 1865 | | |
| | 1/12/38 | 1877 | | |
| | 1/13/38 | 1904 | 1½ cc 3 hr $O_3$ treated | Good |
| | 1/14/38 | 1900 | | |
| | 1/15/38 | 1939 | | |
| | 1/17/38 | 2007 | 2 cc 3 hr $O_3$ treated | Good |
| | 1/19/38 | 2038 | | |
| | 1/20/38 | 2051 | 1½ cc 2 hr $O_3$ treated | Drowsy |
| | 1/21/38 | 1997 | | Not eating well |
| | 1/22/38 | 2016 | | Good |
| | 1/25/38 | 2123 | 1½ cc 2 hr $O_3$ treated | Good |
| | 1/26/38 | 2123 | | |
| 7 | 1/29/38 | 2092 | 1 cc 1 hr $O_3$ treated | Not eating well |
| | | | | Good |
| | 1/30/38 | 2117 | | |
| | 2/1/38 | 2194 | 1½ 1 hr $O_3$ treated | Good |
| | 2/2/38 | 2214 | | |
| | 2/5/38 | 2305 | ½ cc 30 min $O_3$ treated | Good |
| | 2/6/38 | 2303 | | |
| | 2/10/38 | 2332 | 1 cc 30 min $O_3$ treated | Good |
| | 2/11/38 | 2392 | | |
| | 2/12/38 | 2414 | | |
| | 2/15/38 | 2400 | 1cc 30 min. $O_3$ treated | Good |
| | 2/16/38 | 2414 | | |
| | 2/19/38 | 2467 | 1 cc 15 min. $O_3$ treated | Good |
| | 2/21/38 | 2504 | | |
| | 2/26/38 | 2505 | | |
| | 3/1/38 | 2540 | ½ cc. Live Suspension | |
| | 3/2/38 | 2454 | | Drowsy |
| | 3/3/38 | 2370 | | Drowsy, No Dia. |
| | 3/4/38 | 2310 | | Sl. bloody Dia. |
| | 3/6/38 | 2304 | | Dia. much less |
| | 3/7/38 | 2319 | | No Dia. Eat well |
| | 3/8/38 | 2319 | | Good - continued good. |

Rabbit showed a good degree of immunity to a large live dose of dysentery organisms.

TABLE VI

| Rabbit No. | Immunizing Agent | Imm. Dose | Imm. Time(da.) | Days Rest | Test Dose | Diarrhea | Paralysis | Lived | Died |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Live sus. | - | - | - | 1cc | + | + | - | 4da. |
| 4 | " " | - | - | - | 2 cc | + | + | - | 1da. |
| 9 | " " | - | - | - | ½cc | - | + | - | 2da. |
| 25 | " " | - | - | - | 1 cc | + | + | - | 2da. |
| 5 | Heat 60° 30 min. | | | | 2cc | - | + | - | 4 |
| 40 | Sus. 70° 30 min. Filtered | | | | 1cc | - | - | + | -- |
| | " " " " Unfiltered | | | | 1cc | | | | |
| 42 | HCHO sus. | 4cc | 14 | 18 | ½cc | + | + | - | 72hrs. |
| 45 | Heat and $O_3$ 5min. | 6½cc | 7 | 4 | 1cc | + | - | - | 72" |
| 2 | $O_3$ treated 6 hrs. | 8cc | 16 | 4 | 1cc | 2days - + | - | + | -- |
| 3 | $O_3$ 2 hrs. | 8cc | 14 | | | | | | |
| | $O_3$ 1 hr. | 2½cc | 5 | | | | | | |
| | $O_3$ 30 min. | 3cc | 4 | | | | | | |
| | $O_3$ 15 min. | 3½cc | 9 | | | | | | |
| | Total | 18cc | 18 | 10 | ½cc | - | + | - | 72hr. |
| 6 | $O_3$ 3 hrs. | 4cc | 7 | | | | | | |
| | $O_3$ 2 hrs. | 3cc | 5 | | | | | | |
| | $O_3$ 1 hr. | 2½cc | 3 | | | - | + | - | 30hr. |
| | $O_3$ 30 min. | 2½cc | 10 | | | | | | |
| | $O_3$ 15 min. | 1½cc | 4 | | | | | | |
| 8 | Total | 13½cc | 30 | 10 | ½cc | - | + | - | 36hr. |
| 7 | $O_3$ 3 hrs. | 5½cc | 10 | | | | | | |
| | $O_3$ 2 hrs. | 3cc | 8 | | | | | | |
| | $O_3$ 1 hr. | 2½ | 4 | | | - | - | + | - |
| | $O_3$ 30 min. | 3½ | 15 | | | + | | | |
| | $O_3$ 15 min. | 1 | 4 | | | | | | |
| 3 | Total | 15½cc | 41days | 10 | ½cc 2 days | | | | |
| 43 | $O_3$ treated 20 min. intravenous | 5¼cc | 30 | 7 | ¼cc | - | - | + | - |
| 46 | $O_3$ treat. 20 min. subcu. | 8-¾cc | 30 | 7 | ¼cc | - | - | + | - |

| TABLE VII | | Diar-rhea | Paral-ysis | Lived | Died |
|---|---|---|---|---|---|
| Rabbits receiving live doses | 4 | 3 | 4 | 0 | 4 |
| Rabbits receiving ozone treated vaccine | 7 | 2 (slight) | 3 | 4 | 3 |
| Rabbit receiving formaldehyde killed vaccine | 1 | 1 | 1 | 0 | 1 |
| Rabbit receiving suspension heated 70° 30 min. | 1 | 0 | 0 | 1 | 0 |
| Rabbit receiving suspension heated 60° 30 min. | 1 | 0 | 1 | 0 | 1 |
| Rabbit receiving vaccine heated 60° 30 min. + 5 min. ozone | 1 | 1 | 0 | 0 | 1 |

F. Conclusions: Nontoxic vaccines of Shigella dysenteriae with a good degree of neurotoxic and enterotoxic antigenicity can be prepared by treating saline suspensions of the organisms with ozone. Ozone is an effective bactericide for use in preparation of bacterial vaccines.

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## Autobiographical Sketch

Elizabeth Van Meter Wright, the author, was born February 18, 1906 at Shelbyville, Kentucky. She attended the Shelbyville public schools, graduated with A.B. degree from Transylvania College, Lexington, Kentucky, 1929, taught in the Lexington public schools 1930 and received M.A. degree from the University of Kentucky 1931. From 1931 to 1936 she taught at Transylvania College and in 1936 entered the University of Cincinnati to pursue work towards Ph.D.