BACTERICIDAL FLUORESCENCE EXCITED BY X-RAYS.

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INTRODUCTION.

The purpose of this article is to report an experiment which, coupled with underlying theoretical considerations, points to a mechanism by which the x-rays may be made to have a strong bactericidal effect.

It was suggested to me over a year ago that the fluorescence of dyes be studied from the point of view of determining whether there might be a relationship between this property and bactericidal action. The photodynamic effect of various dyes had previously been investigated, especially by Jodlbauer and von Tappeiner.[1] Dilute solutions of the dyes containing suspensions of various organisms were subjected to strong diffuse daylight and the results compared with controls kept in the dark. In general these experiments have given contradictory and unsatisfactory results and the influence of fluorescence as a factor was open to doubt.

Various forms of radiation have been and are being studied from the point of view of abiotic effect. Considering the spectrum as a whole from the very short or x-rays up through the ultra-violet and visible to the long infra-red and heat waves there are only two regions in which the rays have any considerable abiotic effect; namely, in the ultra-violet and infra-red. Most work has shown that the other spectral regions have very little bactericidal effect. Of these two regions the infra-red is probably not of practical value for our purposes, heat being harmful to all protoplasm. Of all radiation the ultra-violet is by far the most bactericidal and the least penetrative. Unfortunately, to be of practical value abiotic radiation must be

[1] Jodlbauer, A., and von Tappeiner, H., *Münch. med. Woch.*, 1904, li, 1096. Mettler, E., *Arch. Hyg.*, 1905, liii, 79.

capable of penetrating tissue. To alter the penetrative qualities of radiation is hopeless on the face of it.

From the point of view of utilization of radiant energy as a therapeutic bactericidal agent the problem therefore reduces itself to seeking to secure a transformation of penetrative energy into some form of energy having abiotic properties. Heretofore the attack has been along chemical lines. Transformation of energy is essentially a physical phenomenon and there is already at hand a very definite mechanism by which a transformation of this character may be produced; namely fluorescence. In order that this mechanism may be here applied, obviously two conditions must hold. In the first place, the fluorescent light, the light emitted by the substance when excited, should of itself have abiotic properties. In the second place, the exciting light, the light causing the fluorescence, must have considerable penetrative property. If these conditions hold, then abiotic light can be produced at any point to which the substance can be brought and the imposition of further physiological conditions would give a practical solution of the problem.

HISTORICAL.

The abiotic properties of radiant energy are best shown by ultra-violet light. The known ultra-violet extends from about 600 to 4000 Ångström units (0.06μ to 0.4μ). Its abiotic properties have been given considerable attention, perhaps most precisely by Victor Henri and his associates.[2] He investigated the effect on microorganisms from about 3600 down to about 2100 Ångström units, using a few filter screens to obtain an idea as to the relative effect of different portions of the region. He concluded that the abiotic effect first became appreciable somewhere between 2800 and 3000 Ångström units and increased progressively with decrease in wave-length. Lyman[3] has shown that the effect increases markedly below 2000, being almost instantaneous somewhere below 1750.

We have repeated the experiments of Henri using a more precise method and have come to the same general conclusions, placing the upper limit at about 2800 Ångström units and finding an approxi-

[2] Cernovodeanu, P., and Henri, V., *Compt. rend. Acad.*, 1910, cl, 52, 549. Henri and Henri, *ibid.*, 1912, clv, 315.

[3] Lyman, T., Spectroscopy of the extreme ultraviolet, London and New York, 1914, 103.

mately constant effect from there to 2100. In this region the typhoid bacillus is about one two-hundredth as sensitive as the photographic plate. Below 2000 Ångström units all materials become rapidly increasingly opaque. 2 cm. of water transmits at 2000, 86 per cent; at 1930, 75 per cent; at 1860, 30 per cent; and at 1729 Ångström units, 0.5 mm. of water is opaque.[4] It is therefore probably safe to set the figures 1800 to 2800 as defining the useful limits of the abiotic spectrum.

While the employment of red light as a penetrating radiation offers certain ulterior advantages, physical considerations discourage the prospect of producing ultra-violet fluorescence by this means. Fluorescence is almost invariably of longer wave-length than its excitant. A good example of this is the dye fluorescein. Dilute solutions of this fluoresce a bright green in daylight but by lamplight lose the property, lamplight containing plenty of green rays but no shorter blue waves. This same general law of fluorescence renders it probable that the x-ray should produce ultra-violet fluorescence, and in fact oftener than it does visible fluorescence. The latter type of x-ray fluorescence has long been known; the discovery of x-rays was due to this property.

The fluorescence problem thus resolves itself into the discovery or manufacture of substances which shall have desirable biological properties and at the same time emit light of wave-length less than 2800 Ångström units when subjected to x-radiation. The problem is therefore one which is rational and which at the same time offers far reaching possibilities.

It is well at this point to give an outline of the work as to the effect of x-rays alone on microorganisms. The literature on this subject seems to have stopped in 1906 with the publication by Russ[5] of a long article which also summarizes the results of previous workers. They all, with one exception, had obtained negative results, even with very long exposures to the x-rays. A great many pathogenic organisms were tried and in the presence of various culture media. Rieder[6] in 1898, using an apparatus incompletely described, was able to almost sterilize agar and gelatin plates of cholera, diphtheria, typhoid, and colon organ-

[4] Lyman,[3] p. 60.

[5] Russ, V. K., *Arch. Hyg.*, 1906, lvi, 341.

[6] Rieder, H., *Münch. med. Woch.*, 1898, xlv, 101, 773.

isms, with exposure for about 1 hour. Bouillon cultures were not so clearly affected. These results are in disagreement with those of everyone else including ourselves. A study of Rieder's article, however, does not reveal any probable cause for the discrepancy. Russ performed four types of experiments. He exposed the media to determine whether exposure rendered them unfit for culture purposes. He exposed organisms under the objective of the microscope in order to observe the effect upon their motility and clumping. He exposed the organisms in the various culture media and he inoculated exposed organisms into animals. All his experiments were entirely negative except for increased motility under the microscope in some cases. His exposures were from ½ to 2 hours, using a fairly powerful apparatus and soft, medium, and hard tubes. Our experiments incidentally check the work of Russ.

It was shown in 1905 by Schuhknecht[7] that the fluorescence in fluorite excited by x-rays extends from 2310 to 3900 Ångström units with a maximum at 2840. This mineral, therefore, should be suitable to use in experiments designed to demonstrate the essential correctness of the above considerations.

We have therefore undertaken the experiments reported in the following pages. The results embody an answer to the following questions. (1) Have the x-rays any bactericidal value and if so under what conditions is it demonstrable? (2) Can the x-rays be shown to have an increased bactericidal value when the bacteria are exposed to them in contact with a substance (fluorite) of such chemical and physical constitution that the x-rays excite fluorescence in it? (3) The second question being answered in the affirmative, is the increased activity due to chemical products contributed to the medium in which the bacteria are suspended, or is it due to physical agencies, presumably rays of light in the ultra-violet region of the spectrum?

The general conditions governing the experiments were as follows. The x-ray apparatus[8] consists of a coarse focus Coolidge tube operated by a Snook interrupterless transformer. The tube was operated on a 4 milliampere current backed up by a voltage equivalent to a 9 inch spark-gap. A 1 mm. aluminum filter plus black paper was used and the objects were placed at a distance of 7 inches from the anticathode. The doses were in multiples of 5 minutes, a 5 minute pause occurring between each 5 minute exposure. Fresh 18 hour agar

[7] Schuhknecht, P., *Ann. Physik.*, 1905, xvii, 717.

[8] The x-ray apparatus was kindly placed at my disposal by Dr. David R. Bowen of the Pennsylvania Hospital.

slant cultures of *Bacillus typhosus* were used, a suspension of the organisms in some medium, usually distilled water, being made. One of these suspensions stays nearly constant in count throughout the day. Each figure in the tables gives the average bacterial count per square centimeter for an agar plate made from the suspension in question.

In earlier experiments with small capillary tubes of glass as containers, certain specimens of glass gave much lower bacterial counts than the average. As glass of certain kinds is known to fluoresce under the x-rays, in order to avoid this effect paraffin was chosen as a sufficiently inert substance from which to construct containers. In order to demonstrate its inertness holes of two sizes were made, one $\frac{1}{2}$ inch in diameter and the other $\frac{1}{10}$ inch in diameter. These two have a considerably different ratio of wall to volume and figures obtained with them should therefore serve to demonstrate any influence of the wall. Experiments showed no difference for exposures in the two sizes of holes (Tables I and II). This at the same time serves to eliminate the size of the hole as a factor in determining the outcome of the experiment. In order to prevent evaporation from the holes they were sealed with thin sheets of paraffin.

Action of X-Rays Alone.

Table I gives the results of the exposure of water and normal salt solution suspensions of the bacteria to the x-rays.

TABLE I.

25 Minute Exposures of Salt Solution and Water Suspensions of Bacillus typhosus.

Medium.	Exposed.	Controls.	
		Before.	After (2 hrs.).
Salt solution, small hole.....	40, 43	180, 200	
Water, large hole.....	17, 12, 17, 17	55, 54, 60	60, 70, 70
" small "	15, 18, 17	61, 70	74
" " "	39, 38, 41	140, 135	130
" " "	44, 43	140, 145	

The figures are bacterial counts of a suspension of *B. typhosus* in water and normal salt solution exposed to the x-rays, the containers being $\frac{1}{2}$ and $\frac{1}{10}$ inch holes in paraffin blocks.

In my hands there has been uniformly a considerable reduction in the bacterial count of these suspensions. As the typhoid organism was supposed to be unaffected by the x-rays, experiments were made to determine the cause of this discrepancy. Water had never been used before as a medium. Usually the observations were made by exposing agar or gelatin plates. Even Rieder did not demonstrate an effect with bouillon as a medium. An agar plate does not make a good experiment. Presumably each individual organism after fixation in the media has already started to multiply before the necessary procedures incident to exposure are completed. As a result an agent which will kill 30 per cent of the organisms will presumably still leave in each potential colony at least one live organism and the full number of colonies will be produced. At any rate the plate experiments were tried using 25 minute exposures and protecting half the plate with $\frac{1}{2}$ inch of lead. The experiment was entirely negative whether the plate was incubated 1 hour before radiating or exposed immediately; that is, in about 15 minutes.

Table II shows that bouillon may have a protecting effect.

TABLE II.

25 Minute Exposures in Water and Bouillon of Bacillus typhosus.

Medium.	Exposed.	Controls.	
		Before.	After (2 hrs.).
Bouillon culture 4½ hrs. old.....	900, 1,150, 1,300	750, 700	1,500, 1,700, 2,000
" suspension, large hole.....	50, 45, 45, 47	80, 76, 80	85, 87, 92
" " small "	36, 25, 36	75, 60	70, 82
Water, large hole.....	17, 12, 17, 17	55, 54, 60	60, 70, 70
" small "	15, 18, 17	61, 70	74

The figures are bacterial counts of typhoid bacilli grown in bouillon or suspended in bouillon and in water, exposure being made to the x-rays in containers consisting of $\frac{1}{2}$ and $\frac{1}{10}$ inch holes in paraffin blocks.

A 4 hour bouillon culture exposed 25 minutes merely had its growth during the time of exposure partially inhibited. On the contrary, a bouillon suspension made just before exposure was reduced almost one-half in count. Water suspensions under similar conditions had their bacterial counts decreased to from one-third to one-fourth.

While these experiments are not conclusive as fixing the exact action of the x-rays alone, they nevertheless demonstrate the relative constancy of an effect much less potent than that presently to be described.

It should be noted in experiments of this sort that if the number of organisms dying in successive short intervals of time during which a destructive agency is acting be plotted, the points constitute a locus which tends to have the form of a probability curve, a curve having somewhat the shape of the profile of a wide flanged bell. This is the ordinary probability relationship familiar to everyone. The organisms die rapidly during part of the time causing a heaping up of the number of deaths in a short interval. In this interval of high mortality the total number killed increases very rapidly. This period is preceded and followed by intervals in which the additions to the figures for the total dead are less rapid. If the figures giving the total dead up to any time be plotted they form a locus having a different form from the above locus for rate of death. This new locus approximates a curve whose slope (tangent of the gradient angle) at any point is given by the height of a corresponding point on the probability curve, in other words by the death rate.[9] It is a curve, therefore, which starts with a low slope, gradually increasing to a maximum of steepness at the point of greatest mortality, then rising less and less rapidly to approach asymptotically a horizontal repre-

[9] The probability curve has a form given by the equation $y=e^{-x^2}$. For a mathematical discussion of the subject see the article on "Probability" in the Encyclopædia Britannica, 9th edition. In this article Fig. 1 outlines the shape of the curve. The area of each quadrilateral would represent the number of deaths occurring in an interval of time represented by the length of its short side. The total number of deaths caused by a dose acting up to any instant of time would be represented by the sum of all the preceding quadrilaterals; that is, by the area under the curve up to that point. The values of the second half of this integral from the mid-point of the curve on, are given in the table under paragraph 9. Plotted, they give the second half of the total mortality curve, the first half of the curve being the symmetrical extension backwards of this locus. It is on a curve of this form that the figures in my tables tend to lie if they are plotted against the length of dose. The Encyclopædia Britannica, 11th edition, under "Probability" gives in Fig. 10 the form of the probability curve and in paragraph 99 the table of values of its integral.

senting the total number of organisms subject to death. The total number dead increases slowly at first becoming rapidly larger as the point of 50 per cent sterilization is approached. From here on the number of dead increases less and less rapidly until finally all are dead.

Slight variations in the conditions of the experiment may shift this period of rapid sterilization a little one way or the other, thus producing excessive variations in the results of experiments ending within the period. Conversely large doses will require considerable increments to produce additional deaths. The increase in energy required to kill the last 10 per cent is greater than that required to kill the middle 50 per cent.

Influence of Fluorite.

The first experiments with fluorite were made with the native powder. They proved unsatisfactory on account of the controls, the powder alone killing a large percentage of the organisms. This was probably an agglutinating effect produced by shaking with the very fine microscopic crystals. Larger chipped crystals were tried but floating crystals here disturbed the uniformity of size of the drop on the plating loop. A large greenish crystal of fluorite weighing 122 gm. was then obtained from the collection of the Drexel Institute and into this a hole 1 cm. deep was drilled with a No. 42 drill (about $\frac{1}{10}$ inch). This hole when thoroughly washed out has smooth glass-like walls and organisms suspended in water placed in it for 2 hours were uninfluenced as shown by Table III.

TABLE III.

Influence of Fluorite Container Alone.

Control before	340, 320, 330
Fluorite hole, 2 hrs.	360, 340, 320
Paraffin " 2 "	320, 330, 360
Control after	360, 310, 340

Water suspensions of *B. typhosus* placed in $\frac{1}{10}$ inch holes in fluorite and paraffin and left there for 2 hours. The suspensions were then syringed out and plated.

Some difficulty was experienced in obtaining uniform loops of material for plating under the varying conditions. When plating from ordinary test-tubes the surface of the liquid is sufficiently large so that its surface tension does not influence irregularly that of the loop. With small holes or small drops the varying surface tension influences considerably the size of the drop on the loop. By placing equal quantities of material on cover-slips a fairly uniform loopful could be obtained. Suspensions placed in small holes in fluorite or paraffin were syringed out well and placed on cover-slips. The angle and velocity of the loop in leaving the drop on the cover-slip were kept about the same and the size of the drop was observed as satisfactory before plating. Control experiments were made with No. 42 holes in paraffin blocks. Table IV gives the results of exposures with the Drexel crystal.

TABLE IV.

Bactericidal Effect of Fluorite Fluorescence.

Exposure.	Fluorite.	Paraffin.	Control, not exposed.
min.			
15	2, 1, 2	80, 75, 75, 75	105, 110, 115, 111, 117
20	2, 3, 4,	70, 100, 100	160, 170
20	8, 7	140, 140	170, 170, 190

Exposures to the x-rays of water suspensions of *B. typhosus* in $\frac{1}{10}$ inch holes in fluorite and in paraffin.

One of the 20 minute exposures giving a 95 per cent mortality (7, 8 colonies per sq. cm.) is relatively the weakest bactericidal effect obtained with this crystal in any of the experiments. The corresponding controls in paraffin showed a mortality of from 22 to 45 per cent, depending upon the length of exposure and upon ordinary experimental variations. As indicated above the 95 to $98\frac{1}{2}$ per cent mortality found here means relatively more than the 50 per cent mortality of the x-rayed controls.

Nature of the Effect.

In order to limit the possibility of a chemical effect induced by the x-rays the procedure was adopted of placing the organisms in small

quartz capillaries and sealing with a tiny bit of paraffin at each end. This operation is entirely without effect of itself. The quartz is transparent to ultra-violet light and the arrangement limits the possible interpretations of the experiment.

The results of these exposures are given in Table V. They paralleled those made without the quartz protection. The difference between the quartz and paraffin exposures may be accidental or it may be due to some effect of the quartz itself. It is not impossible that the quartz may be slightly fluorescent under the x-rays.

TABLE V.

The Bactericidal Effect of Fluorite Fluorescence Is Not Chemical.

Exposures.				Controls.		
Exposure.	Fluorite and quartz.	Quartz.	Paraffin.	Stock.	Fluorite 2 hrs.	Quartz 2 hrs.
min.						
15	15, 14	60, 45, 40	54, 52, 48	140, 145, 130		130, 140, 130, 160
25	0, 0	40, 34	40, 43	180, 200	170, 180	
25	1, 1, 1	15, 19, 16, 17, 21, 19, 16, 18	39, 38, 41, 61	140, 135	120, 240	120, 160

Exposures to x-rays of water suspensions of *B. typhosus* enclosed in quartz tubes and placed in a hole in the fluorite crystal.

Table VI gives the results of 15 minute exposures of other crystals, with protection.

These crystals are all average specimens as seen in mineralogy collections, not being very perfect in form. They included clear white, green, and purple specimens. All were about equally active except a rather deeply colored light green crystal from Deming, New Mexico. Their visible fluorescence under the x-rays varied, being green, blue, and violet. Their weights ranged from 14 to 122 gm.

Table VII gives the results of an exposure of certain of these crystals, the suspensions being placed in the holes without quartz protection.

TABLE VI.

Variations in Crystals, Quartz Protection.

Origin.	Exposures.								Controls.	
	Fluorite crystals.						Quartz.	Paraffin.	Stock.	Quartz.
	Drexel.	Wear, England.	Deming, N. M.	Freiberg, Saxony.	Freiberg, Saxony.	Macomb, N. Y.				
Weight in gm.	122	97	37	14	52	46				
Color.	Green.	Bluish.	Green.	White.	Purple.	Slightly green.				
Fluorescence.	Green.	Blue.	Green.	Violet.	Violet.	Green.				
Bacterial count.	15, 14	16, 15	28, 35	4, 5	8, 7	8	60, 45, 40	54, 52, 48	140 145 130	130, 140, 130, 160

Exposures to x-rays of water suspensions of *B. typhosus* in $\frac{1}{10}$ inch holes in various crystals, the suspensions being protected by quartz.

TABLE VII.

Variations in Crystals, without Quartz Protection.

Exposure.	Crystals.					Controls.
	Drexel.	Freiberg (white).	Macomb, N. Y.	Deming, N. M.	Paraffin.	Stock.
15 min.....	2, 1, 2	1, 1, 1	2, 2, 2	10, 10, 8	80, 85, 75, 75	105, 110, 115, 111, 117
None.....	180, 180	140, 180	150, 160, 150	140, 160		180, 160

Exposures to the x-rays of water suspensions of *B. typhosus* in holes in the various crystals, together with 2 hour controls, without exposure.

The rather high mortality in fluorite shown here is probably not significant of anything but ordinary experimental variations.

There seems to be no insurmountable obstacle to the development of substances having this property of fluorite and at the same time being biologically more appropriate. It is hoped that a new and productive field of experimental therapy has been opened up.

CONCLUSIONS.

X-ray fluorescence has been pointed out as a mechanism offering exceptional possibilities in the development of physicochemical therapy.

Experiments are given which demonstrate that under these conditions the x-rays may have a strong bactericidal effect.

The x-rays alone have a partial bactericidal action on water suspensions of typhoid bacilli.

I take pleasure in expressing my indebtedness to Dr. Paul A. Lewis for his advice in the development of these conceptions and the carrying out of the experiments.