THE KILLING OF COLON BACILLI BY ULTRAVIOLET LIGHT

By RALPH W. G. WYCKOFF

(From the Laboratories of The Rockefeller Institute for Medical Research)

(Accepted for publication, November 6, 1931)

The experiments described in this paper are measurements of the survival ratios of *B. coli* irradiated for different lengths of time by monochromatic ultraviolet light. As such they are an extension to the ultraviolet region of observations previously made with cathode rays[1] and with X-rays.[2]

There are several qualitative investigations of the destructive action of ultraviolet radiation upon bacterial cells. A quantitative study[3] also has been published of the killing of *S. aureus* by a number of ultraviolet wave lengths. When plotted on a proper scale the resultant death curves are of a multiple-hit-to-kill type. These curves give incorrect data unless each cell is sufficiently separated from its neighbors so that it can produce an independent colony. The well known tendency of staphylococci to cling together in chains makes such a spread almost impossible to achieve. Suitable irradiations of the motile *B. coli* accordingly may be expected to yield a better index of the course of bacterial death.

Technique

The culture was obtained from the American Type Culture Collection. Its cultivation and the method of spreading dilute suspensions of the organisms on agar surfaces are those used in the earlier experiments.[4] Because the areas that could be irradiated were smaller, however, the concentration of the seeded cells has been somewhat increased.

[1] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 921.

[2] Wyckoff, R. W. G., *J. Exp. Med.*, 1930, **52**, 435, 769.

[3] Gates, F. L., *J. Gen. Physiol.*, 1929–30, **13**, 231, 249; 1930–31, **14**, 31.

[4] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 921.

Intense ultraviolet light of a single wave length was obtained from a large quartz mercury vapor lamp and a special monochromator. For measurements of survival ratios it is obviously of the greatest importance that the radiation be of constant intensity throughout an experiment. The current for the lamp therefore was drawn from a large 220 V storage battery. In addition the arc was operated for at least an hour before use to insure that it was running steadily. The total output of light was controlled during every experiment by adjusting, when necessary, the current through the lamp so as to give constant readings from a conveniently placed quartz sodium photoelectric cell.

Statistically significant data will be obtained only when the irradiations are short compared with the organism's reproductive cycle. To obtain such an intense monochromatic ultraviolet beam of sufficient cross section a monochromator of great light-gathering power is required. The one used in the present experiments had quartz lenses 6 inches in diameter and prisms of corresponding size. With it an area, 3 × 30 mm., could be flooded with a single spectral line by the proper adjustment of its position and that of the slit. As in earlier experiments, this area was defined and marked on the irradiated surface by cutting edges attached to the spectrometer and set in a screen capable of shielding the rest of the agar plate. Several exposures were made on a single seeded plate. Counts of survivors on these areas compared with the number of colonies growing out on a similarly stamped but not irradiated control area gave survival ratios. Failure to have a beam of equal intensity over the entire exposed field represents one of the greatest sources of error in such experiments. In the present instance this was checked by a fluorescent screen and more sensitively by the requirement that different parts of the same field should give like amounts of destruction.

For each experiment the energy flux in the beam striking the irradiated organisms was found by a thermopile which replaced the cutting edge and lay in the plane of the agar surface. This thermocouple was calibrated with a carbon lamp of known energy output obtained from the U. S. Bureau of Standards.

Experimental Results

Survival ratios measured for each of the wave lengths 3132 A, 2900 A, 2803 A, 2699 A, 2652 A and 2536 A are recorded in Table I and plotted in Figs. 1–5. Every ratio is the average of counts on at least 10,000 organisms. It is evident that, with the possible exception of the data from 3132 A, all these results can be closely represented by straight lines plotted on semilogarithmic paper.

In Table II the effective radiant energies are stated as the amounts needed to kill half the bacteria upon which they fall. They are to be taken as approximate only. Precise estimates require the exercise of considerable care—more than is warranted by the present accuracy of

the biological results. The previously recorded[5] lethal doses for *B. coli* are of the same order of magnitude but for most wave lengths they are less than those of Table II. Bacteria of different strains were used and the irradiations were carried out under such unlike experimental conditions that it is not evident wherein lies the cause of this discrepancy.

TABLE I

Survival Ratios of Bacteria Irradiated with Ultraviolet Light

Time	Wave length				
	3132 A	2900 A	2803 A	2699 A	2536 A
	B. coli				
sec.					
5	0.977	0.926	0.900	0.882	0.884
10	0.945	0.808	0.795	0.756	0.793
20	0.907	0.632	0.621	0.536	0.596
40	0.794	0.396	0.422	0.251	0.305
60	0.697	0.236	0.266	0.132	0.191
80	0.601	0.162	0.187	0.082	0.111
100	0.459	0.124	—	—	—
Energy incident per mm.^{2} per sec.....	47.8 ergs	11.3 ergs	7.5 ergs	7.8 ergs	8.0 ergs
	B. aertrycke				
10	0.949	—	0.818	—	—
40	0.799		0.326		
80	0.644		0.102		
Energy incident per mm.^{2} per sec.....	46.3 ergs		8.2 ergs		

A few experiments have been made with *B. aertrycke* to compare their killing with that of *B. coli*. The data of Table II and of Figs. 1–5 indicate that these two organisms cannot thus be distinguished. Their equal sensitivity, already found with X-rays, is more striking with ultraviolet light because it must mean practical identity in the specific absorption of their protoplasms for the wave lengths used.

[5] Gates, F. L., *J. Gen. Physiol.*, 1929–30, **13**, 231, 249; 1930–31, **14**, 31.

Analysis

The foregoing experiments indicate that colon bacilli under ultraviolet irradiation, just as through the action of X-rays and electrons, die at a rate which is semilogarithmically linear. For cathode rays such a result was interpreted in terms of probability theory[6] to show that practically every high speed electron absorbed by a bacterium was lethal. The same kind of interpretation applied to the data from soft X-rays led to the plausible explanation that though cell death

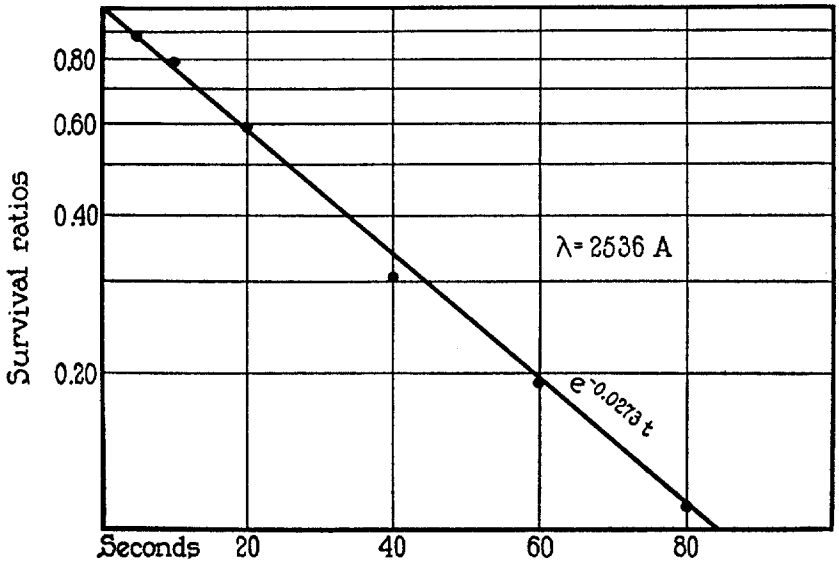

FIG. 1. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2536 A.

takes place through the absorption of a single quantum of energy, this absorption to be deadly must occur within an especially sensitive volume which cannot be greater than 1 per cent of that of a colon bacillus.

[6] Crowther, J. A., *Proc. Roy. Soc. London, Series B*, 1926, **100**, 390; Condon, E. U., and Terrill, H. M., *J. Cancer Research*, 1927, **11**, 324; Holweck, F., *Compt. rend. Acad.*, 1929, **188**, 197; Rahn, O., *J. Gen. Physiol.*, 1929–30, **13**, 179, 395; Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 921.

It is instructive to carry through a similar analysis based on the results of ultraviolet killing. This will be done using the data from $\lambda = 2699$ A. The survival ratios A/A_o of Table I and Fig. 2 follow the equation $A/A_o = e^{-0.0325t}$. In the language of a quantum interpretation[7] the average number of effective absorptions per bacterium per second therefore is 0.0325. The thermocouple measurements have shown that in this experiment the energy incident per second is

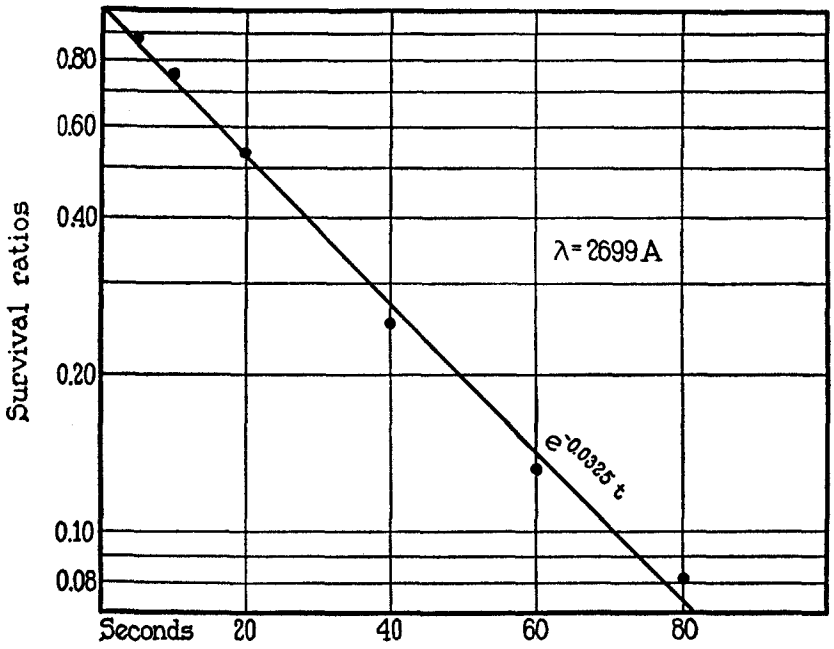

FIG. 2. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2699 A.

7.79 ergs per sq. mm. Taking the colon bacillus to be a rod 0.5μ in diameter and 2μ long the energy striking a single bacterium will then be 7.79×10^{-6} ergs/sec. The absorption of a thin film of *B. coli* has been measured[8] for several wave lengths. As in the X-ray experiments it is sufficiently accurate to consider the absorption of a

[7] Wyckoff, R. W. G., and Rivers, T. M., *J. Exp. Med.*, 1930, **51**, 921.
[8] Gates, F. L., *J. Gen. Physiol.*, 1929-30, **13**, 231, 249; 1930-31, **14**, 31.

single organism as equivalent to that of a block of protoplasm 0.5 × 2μ in cross section and 0.42μ thick. Since the absorption of wave length 2699 A in such a layer is 0.127, it follows that the radiant energy absorbed per bacterium per second is 0.99×10^{-6} ergs. The voltage equivalent of the 2699 A line is 4.55_7 volts. Converting to appropriate units, the $h\nu$ value found from the usual quantum relation $Ee = h\nu$ is directly calculated to be $7.25_5 \times 10^{-12}$ ergs. Hence the average number of quantum absorptions per bacterium per

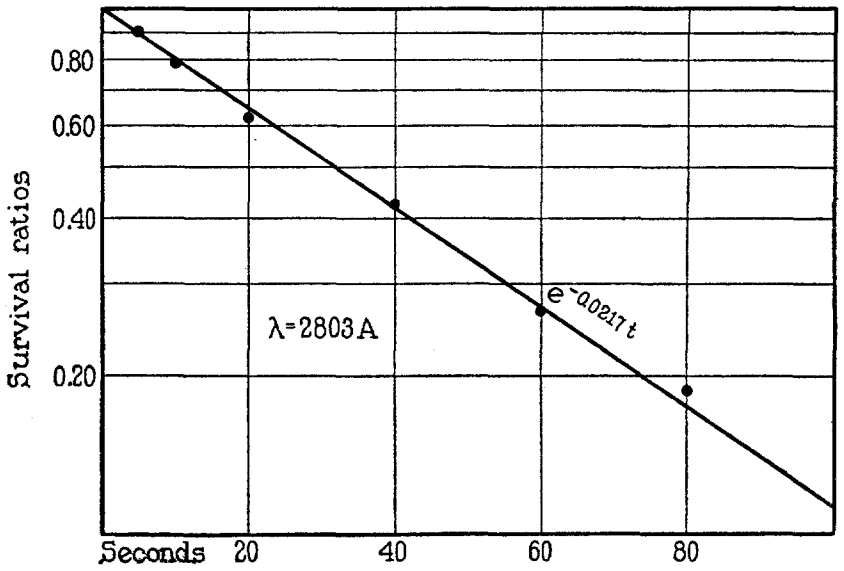

FIG. 3. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2803 A.

second is $\frac{0.99 \times 10^{-6}}{7.25 \times 10^{-12}} = 136{,}300$. Because the average number of absorptions that are effective is 0.0325 per sec., only $\frac{0.0325}{136{,}300}$, or one in 4.19×10^6, of the absorbed quanta is capable of causing cell death.

This interpretation may be completed by comparing the "sensitive volume" provided by these calculations with the size of possible structures within an irradiated bacillus. In doing this it must always be borne in mind that what is really being measured is a purely physical

quantity—the sphere of influence within which the quantum acts. The result may be biologically significant in that it sets a definite upper limit to the size of those elements whose injury or destruction results in death. For the present experiments the "sensitive volume" is $\frac{0.0325}{136,300} \times 3.93 \times 10^{-13}$ cc. (the volume of one bacterium), or 9.38×10^{-20} cc.

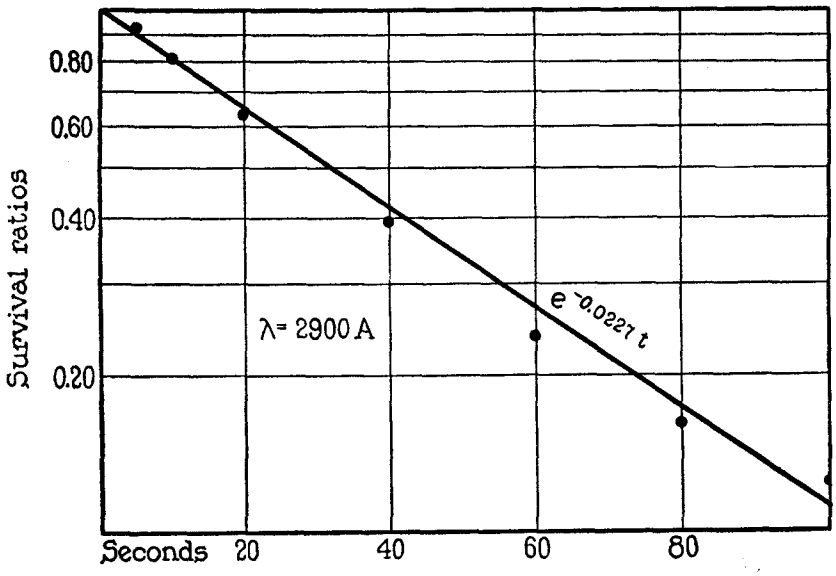

FIG. 4. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 2900 A.

A single protein molecule has approximately this size. Neglecting hydrogen atoms to permit calculation, the average atomic weight of a typical protein is not far from 15. One with a molecular weight of 120,000 will thus contain about 8,000 atoms. Assuming the molecule to be cubic it would have 20 atoms on a side. The interatomic distances in organic crystals vary between *ca* 1 A and *ca* 3 A. Taking the intermediate value of 2 A, the protein molecule 40 A on a cubic side would have the volume 6.4×10^{-20} cc. For longer wave lengths than 2699 A the calculated "sensitive volume" becomes even less than such molecular volumes. For 3132 A it is only 1.5×10^{-20} cc.

It should therefore be concluded that if the lethal action of ultraviolet light upon *B. coli* is to be explained in terms of a quantum-hits-to-kill mechanism, the sensitive unit whose well-being is essential to the cell's continued growth and multiplication cannot be larger and may be much smaller than an aggregate of two or three protein molecules of moderate size.

Further data bearing upon the destructive action of ultraviolet radiation can be obtained through comparisons of the amounts of absorbed energy required to kill with ultraviolet light of different wave lengths and with X-rays. Table II records the ultraviolet energies absorbed when 50 per cent of the irradiated organisms are destroyed. These quantities would be expected to be equal only in case killing were directly proportional to the amount of absorbed

TABLE II

Approximate Energies Necessary to Kill 50 Per Cent of Irradiated Colon Bacilli

Wave length	Incident energy	Energy absorbed/bacterium
2536 A	200 ergs/mm.^{2}	2.75×10^{-6} ergs
2652	110	1.50 "
2699	160	2.10 "
2803	240	2.50 "
2900	340	2.30 "
3132	5200	10.9 "

energy or in the highly improbable event that the "vital material" of the cell had exactly the same ultraviolet absorption curve as the entire organism. Nevertheless they are of the same order of magnitude. In one of the previous experiments[9] with copper K series X-rays, 0.22_8 quanta were absorbed per bacterium per second. Since the $h\nu$ equivalent of these X-rays ($\lambda = 1.537$ A) is $1.27_8 \times 10^{-8}$ ergs, the average energy absorbed per cell per second is directly $0.22_8 \times 1.27_8 \times 10^{-8} = 2.91 \times 10^{-9}$ ergs. Under these circumstances 50 per cent killing is achieved in 48 seconds. The energy to produce this killing, $2.91 \times 10^{-9} \times 48$ ergs $= 1.40 \times 10^{-7}$ ergs absorbed per bacterium, is little more than one per cent of that required to produce the same effect in the ultraviolet region.

[9] Wyckoff, R. W. G., *J. Exp. Med.*, 1930, **52**, 435.

DISCUSSION

The foregoing analysis might be thought to show that colon bacilli are killed through the absorption of one quantum of ultraviolet energy within a volume not greater than a single large protein molecule.[10] The most important reason for believing such an explanation improbable lies in the fact that no injurious effects have been observed in the growth of the surviving organisms which, nevertheless, must have absorbed millions of quanta apiece. These ultraviolet radiations are known to coagulate protoplasm and if one properly placed absorption

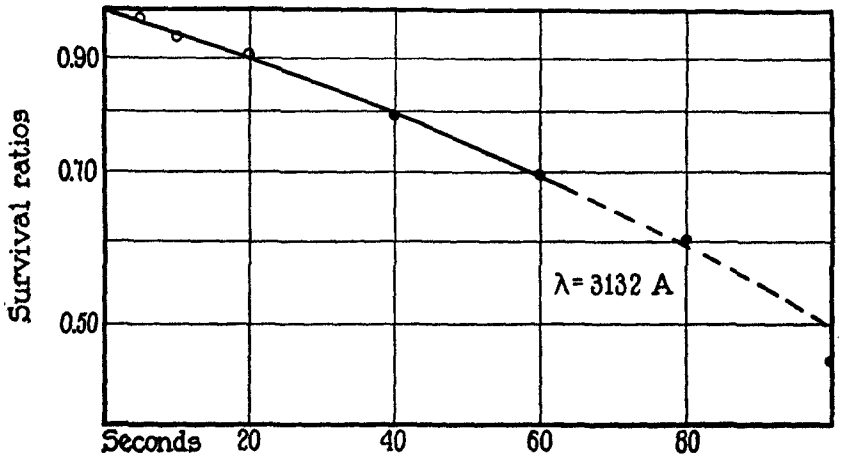

FIG. 5. Survival ratios of *B. coli* irradiated with ultraviolet light of wave length 3132 A.

can kill, several million similar ones ought to reveal themselves by changes in the rate of growth, in colony shape or in some of the other so-called "dissociative changes" often observed among colon bacilli. No abnormalities have been seen during the present experiments but it might be profitable to carry out additional irradiations under conditions especially favorable to the perpetuation of such variants.

If the foregoing one-hit-to-kill explanation is not the correct one for these ultraviolet data, some other interpretation must be found for the semilogarithmically linear destruction of *B. coli*. Two possibilities

[10] Rahn, O., *J. Gen. Physiol.*, 1929–30, **13**, 179, 395.

suggest themselves. According to one, of a type often encountered in biological discussions, death is due to a process which follows the course of a monomolecular chemical reaction. It is very possible that the ultraviolet coagulation of protoplasm proceeds exponentially. Because this rate is independent of the distribution of the protoplasm it has sometimes been urged that irradiated cells should die according to the demands of a semilogarithmically linear relation. Unless death is held to be the consequence of the destruction of a single one of a cell's molecules, this conclusion is clearly fallacious. Provided such pictures based on analogies with simple chemical reaction rates are devoid of significance, as may well be the case, it would appear that the primary factor influencing the rate of bacterial death by ultraviolet light is to be found in the relative sensitivities of the bacteria themselves. Experiments have not yet been made which show whether or not this is true, but varying resistance clearly determines the rate of destruction of bacteria by heat and by certain chemical agents.[11]

The killing experiments with bacteria thus furnish a striking example of some of the pitfalls which attend efforts to give detailed interpretations of biological reactions even when quantitative data are at hand. The rate of death is the same whether cathode rays, X-rays or ultraviolet light be used. With cathode rays the simple statistical explanation of this relation based on probability theory must quite certainly present a correct picture of the phenomena since at a sufficiently high voltage every absorbed quantum will be lethal. With X-rays, current physical ideas of the processes consequent on absorption make this type of interpretation a highly probable one, especially with the harder rays. In the ultraviolet region, however, it is likely that exactly the same quantitative biological results have a totally different explanation and one which resides not so much in the mode of action of the killing agent as in biological characteristics inherent in the organisms themselves. So little is now known of the internal constitution of bacteria that discussions of whether or not death results from the decomposition of one molecule or from some other cause involve speculations which are at the moment incapable of verification.

[11] Reichenbach, H., *Z. Hyg.*, 1911, **69**, 171.

The writer is indebted to James R. Lucas and to C. G. Porskieves for help in carrying out these experiments.

SUMMARY

The survival ratios of colon bacilli subjected to several monochromatic ultraviolet radiations follow semilogarithmic straight lines. For each wave length approximate observations have been made of the energy involved in cell destruction. This energy varies somewhat with frequency in the ultraviolet region; it is furthermore nearly one hundred times as great as the amount of X-ray energy required to bring about the same killing.

Preliminary experiments show no measurable difference either in rate of killing or in lethal energy between *B. coli* and *B. aertrycke*. Parallel results have already been obtained with X-rays and electrons.

The data from colon bacilli are interpreted in terms of the assumptions employed for X-rays. They indicate that though bacterial death should result from a single quantum absorption, millions more such absorptions seemingly are without injurious effect on cell growth and multiplication. The "sensitive volume" within which, according to this picture, the lethal quantum must be stopped proves to be about the same as that of a single protein molecule. If this is the correct description of the phenomena of ultraviolet killing, it seems strange that the millions of non-deadly quanta absorbed per bacillus should not show themselves by altered growth rates or in other ways. That they apparently do not suggests the inapplicability of the statistical picture. The death rate under this kind of radiation then would be primarily an expression of the relative sensitivities of the bacterial population. Additional experiments are required to determine this question.