A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

I. The Reaction to Monochromatic Radiations

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During the past fifty years, many investigators have studied the bactericidal activity of ultra violet light, but a review of their reports shows that little has been learned about many essential factors in the reaction. There have been few contributions to a knowledge of the quantitative relations involved. Little precise information is available on differences in resistance of individual bacteria to monochromatic light and the consequent reaction curve of large numbers of organisms; on the effective range of ultra violet frequencies; on the relation between incident and absorbed energies at various wave lengths; on relations between time and intensity; on the temperature coefficient; on the action of polarized light; or indeed on any of the factors essential to the reaction on which an examination of its biophysical significance must be based.

But the ultimate object of the present study has not been simply to determine a set of coefficients for the bactericidal action of ultra violet light. This particular reaction was chosen for study because less individual variation is to be expected among bacteria than among higher forms of life. They are easily handled in quantity, and the death or survival of microorganisms provides a clearcut endpoint which may be observed and statistically recorded with unusual accuracy for biological material. They seemed to afford the best available opportunity for a quantitative study of an effect of certain frequencies in the ultra violet on protoplasm: with the prospect that the reaction of the bacterial cells might open leads for further studies on higher organisms.

It is obvious that in such work a considerable number of factors

must be controlled, and all but a selected one kept constant under standard conditions, while that one is varied experimentally. In the present study the measured variables were especially wave length, intensity, time, and lethal action, and, except when subject to special experiment, the factors kept as constant as possible were the character and age of the bacterial cultures employed and the composition, temperature, and hydrogen ion concentration of the medium on which the bacteria were exposed and subsequently allowed to multiply.

In quantitative studies energy of a single frequency, or a very narrow range of frequencies is essential: the selective absorption of radiant energy is characteristic of biological materials and a fundamental factor in their reaction to it. Since Grotthus it has been axiomatic that only energy that is absorbed can do work. The exposure of test objects to a whole gamut of radiation frequencies, even when the range is restricted by selective filters, introduces so many unknowns in partial absorption as to void any measurements of the effective energies. The importance of separating the radiations monochromatically has been recognized by Ward (1), Barnard and Morgan (2), Browning and Russ (3), Mashimo (4), and more recently by Bayne-Jones and von der Lingen (5), who exposed bacteria on a nutrient substrate in a quartz spectrograph, and so obtained spectral images of the result. But the equal importance of determining the incident energy required at each frequency to produce comparable effects has apparently been emphasized only by Hertel (6), who reported, however, on only six lines between 210 and 440 mμ. Bang (7) measured the periods of exposure (in seconds) necessary to kill at 20 mμ intervals (with the carbon arc), and tried to correlate the bactericidal effects with bolometric observations on his light source, and Coblentz and Fulton (8) have recently made careful radiometric observations on a few wide regions in the ultra violet defined by filters. But intensity measurements of monochromatic radiations in absolute units, and the correction of incident energies by the absorption coefficient of the objects under test apparently have not been undertaken. Yet it is obvious that physical and chemical analyses of the reaction, and quantitative comparisons with other biological ultra violet reactions can only be made on the basis of such information.

Methods

Light Source. Monochromatic ultra violet radiations were obtained from a vertical, quartz, air-cooled, high intensity mercury vapor arc, with tungsten anode, operating at 67 v., 5.5 amp. direct current. The mercury arc was chosen rather than a spark or a metallic open arc source, because of its steadiness and the intensity and separation of its principal spectral lines. While single frequencies or groups of frequencies within narrow limits are thus easily available, the ultra violet region between wave lengths 2253 and 3126 Å. u. is fairly well-covered by

the radiations at 2302, 2345, 2379, 2482, 2536, 2675, 2804, 2894, 2967, and 3022 Å. u.—all lines, or groups of lines, of sufficient intensity for practical use.

Quartz Monochromator. These specific ultra violet energies were separated and focused by means of a large quartz monochromator of special design.

The vertical quartz mercury lamp is placed on an optical bench, directly behind an adjustable entrance slit, curved to correct the spherical aberration of the quartz optical train. Radiations through the slit are rendered parallel by a crystal quartz planoconvex lens of 75 mm. aperture, and then pass through a Cornu quartz prism of 66 × 41 mm. face, and a large photographic shutter. A second 75 mm. crystal quartz lens then focuses the spectrum on a straight, vertical, adjustable exit slit, by which the desired frequency is isolated and passed to the surface of exposure. The bench supporting the shutter, collimating lens, and exit slit remain stationary, and different wave lengths are thrown upon the slit opening by rotation of the lamp housing, entrance slit, and collecting lens around the axis of the Cornu prism, maintained at minimum deviation by rotation through half the angle. Focus is obtained automatically by a synchronous adjustment of the lamp and entrance slit during their angular travel.

Energy Measurement. The intensity of the monochromatic radiations passed by the exit slit is measured, in the plane of the receiving surface, by thermopiles of special design, patterned after those described by Pfund (9), who kindly gave personal instruction in making them. These compensated, linear thermopiles are of 1.5 × 12 mm. surface, of about 5 seconds period, practically without creep at ordinary room temperatures, and, connected through a ballast resistance with a Leeds and Northrup high sensitivity galvanometer, give readings of about 1 cm. deflection at 2 meters scale distance for a flux of 1 erg mm.^{2} second (10^{-7} watt), a sensitivity ample for these energy measurements, as the protocols will show. At frequent intervals the thermopile in use was calibrated in absolute units against a Bureau of Standards carbon filament, incandescent lamp, (No. S 26), or a similar secondary standard.*

Test Objects. Although some measurements were made on a laboratory strain of *B. coli communis*, most of the observations here reported were made on a strain of *S. aureus*, originally obtained from Dr. C. G. Bull, and maintained on standard laboratory media for a period of years before these experiments were undertaken. This strain of cocci was chosen because of long adaptation to its environment, and because of the spherical shape of the single organisms, since orientation could not be controlled. During the experiments the strain was transplanted daily on beef infusion peptone 2 per cent agar, buffered at pH 7.4. This same medium was used as the substrate and covering layer in the tests.

For the quantitative determination of a bactericidal effect, it was essential, of

* When energy measurements are given, it should be noted that the energy reported is the total per square millimeter of surface illuminated, and not the energy that falls upon each bacterium.

course, that no absorbing medium should intervene between the measured incident energy and the exposed bacteria. This, and the necessity that all organisms lie approximately in a plane, both during exposure and for subsequent colony counts, precluded the use of a fluid medium, and the following technique was finally adopted.

Experimental Procedure

Small Petri plates, 5.5 cm. in diameter, were attached with wax to 5.1 × 7.6 cm. glass microscope slides and partly filled with a layer of nutrient 2 per cent agar. A carefully made suspension of an 18 hour culture of *S. aureus*, with a transparency limit, or depth-of-disappearance of 30 cm. (10) was washed over the agar surface, the excess drained off, and the plate was allowed to stand in a vertical position until the excess fluid on the surface had evaporated. This method was found to give the most uniform distribution of organisms, so separated that subsequent colony counts could be readily made. The Petri plate was then covered with a crystal quartz plate (to compensate for the quartz window of the thermopile) and the glass slide was set vertically in a mechanical stage, adapted for the purpose, which was rigged on the monochromator so that the agar surface and the thermo-elements could be brought into position alternately, in the same plane, behind the exit slit. The five areas, 4 × 24 mm., to be exposed on each plate, separated by 4 mm. control areas, were then successively located and centered by readings on the mechanical stage. Timed exposures, with monochromatic radiation, and at measured intensities, were made at room temperature, between 20° and 22°C. Tests showed no appreciable variations within this temperature range.

After exposure, the bacteria-strewn surface of the Petri plates was covered with a second layer of nutrient agar at 39°C. In the earlier experiments the use of this covering layer of agar had not been developed, and it was necessary to incubate the plates at 37.5°C. under frequent inspection and to count the colonies in the exposed and control areas as soon as they became clearly visible and before confluent growth occurred. Then it was found that a second layer of agar, flowed on after exposure to cover the bacteria, dislodged an inconsiderable number of them (tens, out of the many thousands on the plate) and that no significant error was introduced by its use. On the contrary, the fixation of the bacteria between two layers of agar practically prevented confluence, and made possible an overnight incubation and a more accurate colony count.

For counting colonies, the exposed and control areas were located under the microscope by corresponding readings on a mechanical stage similar to that on the monochromator. A small central section of each area, measured between parallel lines and between stops on the mechanical stage, and corresponding approximately to the area of the thermopile junctions, was covered in each count.

The experimental errors which this method of estimating bactericidal action involves are obvious. Variations in the house current affect the light source.

Incident energy may not be uniform over the entire surface exposed. Bacteria may occasionally overlie and partly protect one another, and uneven distribution makes appreciable variations in the counts used as controls. This last source of error is the most conspicuous and is largely responsible for the common variations in the results of single exposures in parallel experiments, as shown in Chart 2. If single experiments were used at each wave length, irregular bactericidal curves would result. But each of the curves reported is the average of smoothed curves from a number of parallel experiments, and since errors due to irregular distribution fall indifferently above or below the line, the averages of these smoothed curves approximate closely the true course of the reaction. On the other hand, the use of such smoothed curves (Chart 1) to obtain the average of a number of experiments at each wave length precludes the inclusion of points on the final curves, lest they be interpreted as points of observation, rather than of statistical summary, and thus give a false impression of experimental accuracy. Therefore it will be noted that a number of such curves in this series are reported without points. The alternate method of presentation, when many observations in parallel experiments are scattered irregularly along a common energy gradient, is to collect the points into groups and average them at successive energy levels. For comparison this has been done in Table II, and the results plotted in Chart 2, together with all the single observations and with the curve obtained as the average of smoothed curves from each experiment. The close coincidence of the results of the two methods is apparent and would appear to justify the use of smoothed curves as the more acceptable representation of a continuously progressing reaction.

Before proceeding to an analysis of the experimental results, it may be stated that the effects reported are due to direct action of ultra violet light on the exposed bacteria. Browning and Russ (3), and Coblentz and Fulton (8) reported, and experiments in this series have confirmed the observation that exposure of an uninoculated agar surface to bactericidal wave lengths and intensities of ultra violet light has no measurable effect on the growth of microorganisms subsequently spread on the exposed areas, as compared with adjacent, unexposed controls.

In reports on the bactericidal action of ultra violet light, it is usual to consider first the range of frequencies which are effective. But, as will be shown later, this element in the problem is so intimately related to the specific absorption of energy at different wave lengths that it seems best to defer a consideration of the energies involved at different wave lengths until the typical reaction of bacteria at a single wave length has been examined.

The Reaction to Monochromatic Ultra Violet Light

The first experiments, then, deal with the typical reaction to monochromatic ultra violet light of an 18 hour culture of *S. aureus*, spread on nutrient agar plates, at a temperature of 20°C. and a hydrogen ion concentration of 7.4. The protocol of a single experiment at wave length 266 mμ is given in Table I and the smoothed curve of the findings in Chart 1.

TABLE I

A Single Experiment to Illustrate the Bactericidal Action of Monochromatic Ultra Violet Energy

S. aureus λ266 mμ 7/12/23

Galv. defl. 9.3 cm. Factor: 1 cm. defl. = 1.18 ergs per mm.^{2} sec.

Incident energy = 11 ergs per mm.^{2} sec.

Exp. sec.	2	4	6	8	10	12	16	20	24	30	Controls*	
Energy ergs.	22	44	66	88	110	132	176	220	264	330	Plate	
Plate No.	1					2					1	2
Colonies	52	42	38	28	23	20	13	10	2	0	54–67	54–58
Per cent killed	15	31	38	54	62	64	77	82	96	100+	61	56
Plate No.	3					4					3	4
Colonies	70	64	56	43	39	32	19	11	7	0	87–80	78–82
Per cent killed	17	24	32	49	54	60	76	86	91	100+	84	80
Average killed per cent	16	28	35	52	58	62	77	84	94	100+		

* In later experiments control counts were made between each two adjacent exposed areas and averaged in pairs across the plate.

The ends of the curve would have to be obtained by extrapolation, for the least energy used killed some bacteria, and the greatest may have been more than sufficient to kill them all. The general trend of the reaction is to be seen, however, and the curve suggests that for the most part the relation of the incident ultra violet energy to its bactericidal action is logarithmic.

A more complete and accurate curve is obtained by averaging smoothed curves from a number of experiments at the same wave

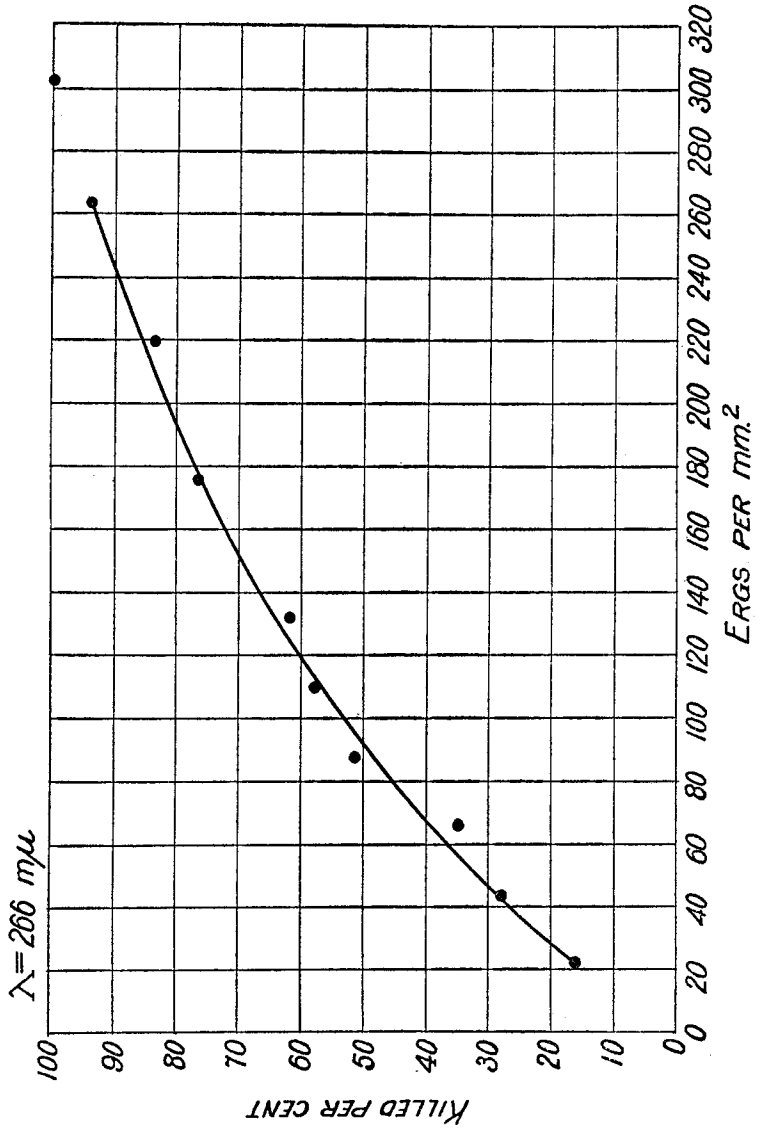

CHART 1. Course of the bactericidal action of monochromatic ultra violet energy in a single experiment.

length, which has been done at each wave length studied. Then the initial and the total bactericidal energies are much more closely approximated, and a curve may be drawn to illustrate the lethal effect from 0 to 100 per cent. Such a curve, the average of smoothed curves from 17 series of observations at λ254 mμ is shown in Chart 2. In this chart all the experimental observations are recorded, and points have been included which were obtained by an alternate method of summarizing these observations, as is shown in Table II. Although approximately the same range of monochromatic energy was covered in each experiment, the separate exposures were timed differently on different days. Hence all the single points in these 17 experiments had to be collected into groups, and averaged at the mid-point of each successive range of incident energies, as indicated.

TABLE II

Summary of Observations on the Bactericidal Action of Ultra Violet Energy at λ254 mμ

Energy levels (ergs per mm.²)	20 - 40	40 - 60	60 - 80	80 - 100	100 - 120	120 - 140	140 - 180	180 - 220	220 - 260	260 - 300	300 - 340
Point of average (ergs)	30	50	70	90	110	130	160	200	240	280	320
No. of observations...	22	18	17	11	13	10	17	12	10	4	6
Average per cent killed	13	25	36	50	59	66	76	88	94	97	99

In Chart 3 this curve at λ254 mμ has been included with similar curves obtained at each wave length examined, all drawn with the incident energy recorded on a logarithmic scale. The significance of the fact that very different energies are involved at different wave lengths is reserved for later consideration. But regardless of the absolute energies involved, the curves are so similar as to indicate that the reaction at any one wave length is typical of them all. Each curve shows four successive periods of reaction, clearly seen also in Chart 4 in which all the curves of Chart 3 have been made comparable, and averaged, by expressing the energies, as well as the bacteria killed, in terms of 100 per cent.

1. In an initial period of exposure no bacteria succumb. The energy incident before any bactericidal effect is observed is between 6 and 7 per cent of that required to kill all the organisms.

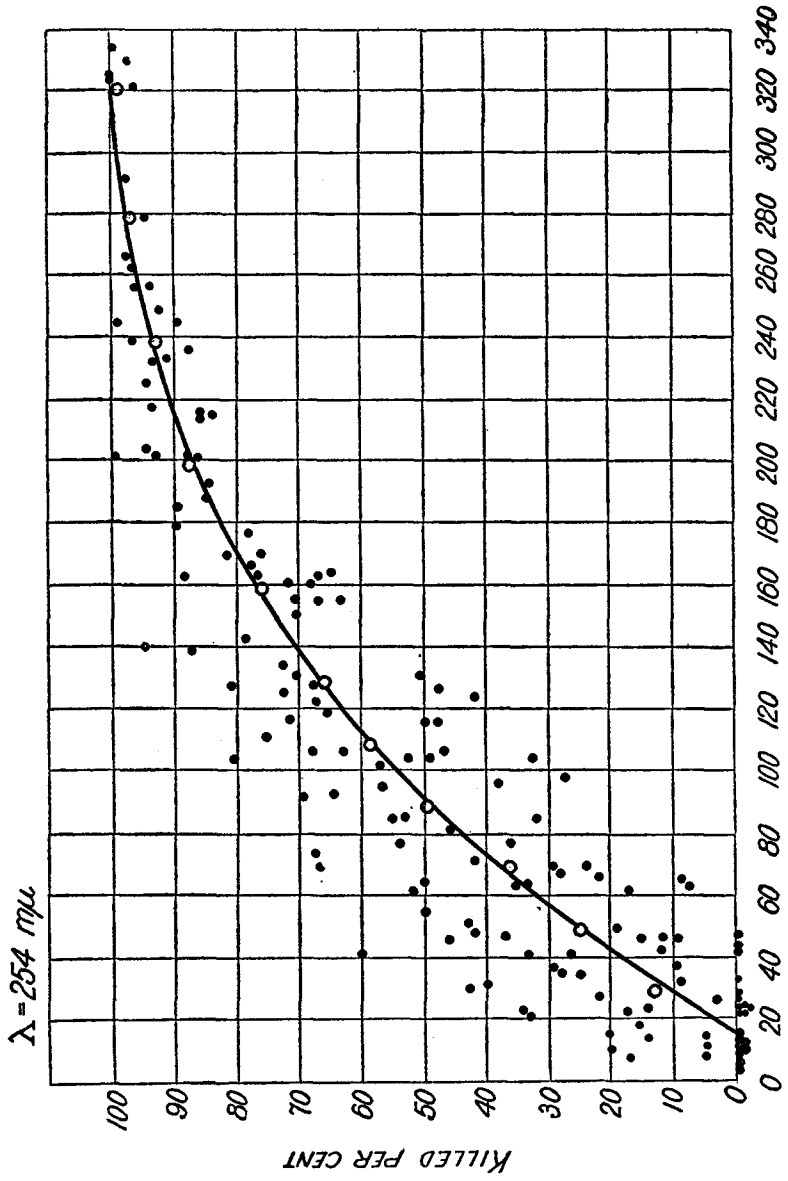

CHART 2. Smoothed curve of bactericidal action from a series of experiments. Points indicate single observations. Circles from Table 2.

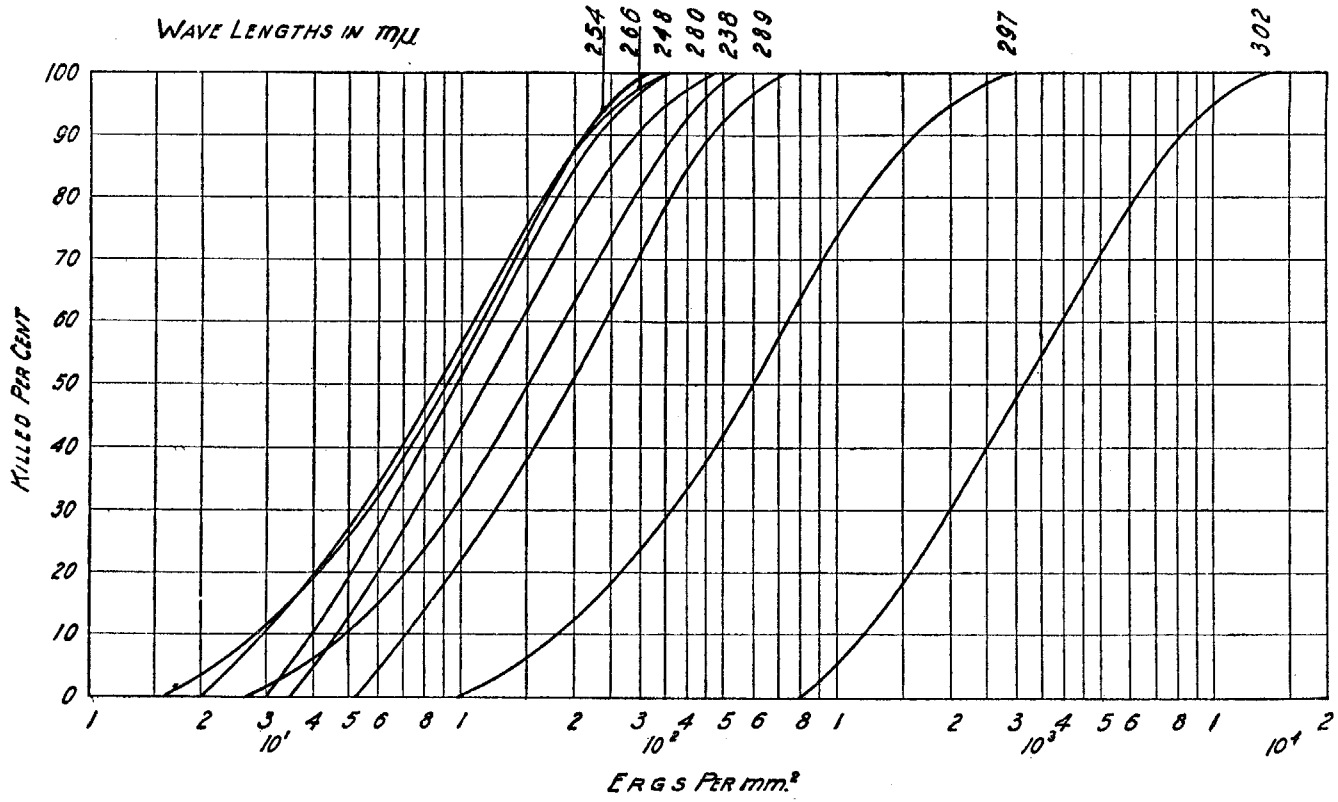

CHART 3. Incident energies required for bactericidal action at various wave lengths in the ultra violet.

2. After this initial exposure a considerable number of bacteria, between 20 and 30 per cent, are destroyed by less ultra violet energy than would be predicted from the rate of destruction for the remainder of the group. They seem to be less resistant than the rest.

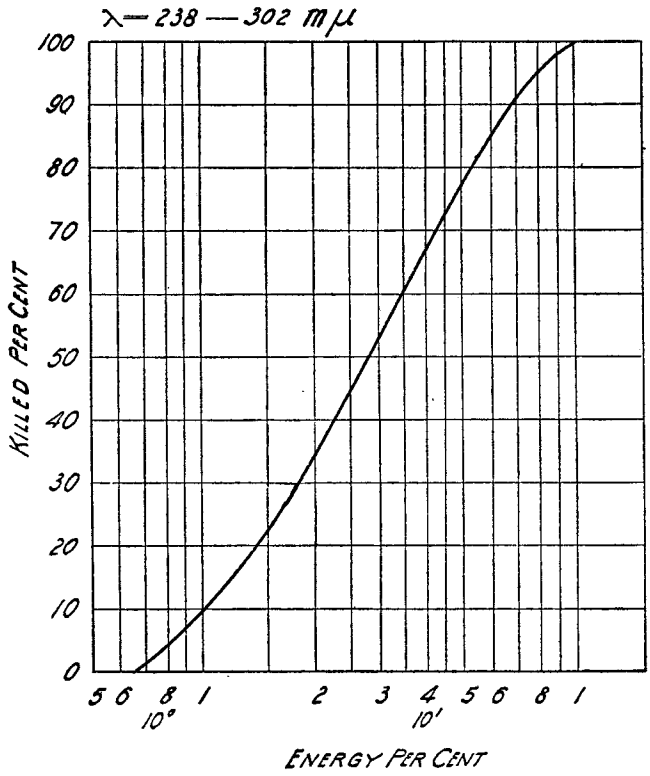

CHART 4. Smooth curve for all wave lengths shown in Chart 3, averaged by figuring the different incident energies from 0 to 100 per cent.

3. Then a considerable number of the remaining bacteria, to about 70 or 80 per cent of the total number, succumb along an energy gradient that appears to bear an exponential relationship to its lethal effect. In Charts 3 and 4, this section of the curves is a straight line.

4. In the final period a number of organisms remain which require an excess of energy to kill them.

Before the significance of the curve, as a whole, can be discussed,

consideration must be given to a number of factors that obviously affect it.

The presence of an initial period of exposure from the effects of which no bacteria succumb was attested by every experiment in which small energies were used. The error introduced by variations in distribution hindered the attempt to determine the initial energies involved in single experiments, so the effect of small energies was subjected to special investigation.

In an experiment at λ254 mμ, 23 plates of *S. aureus* were exposed to small energies, from 7.6 to 32.8 ergs per sq. mm., and it was found that the counts approached the control figures within the common limit of error, and no progressive action was evident in this experiment, until the 32.8 ergs exposure was reached. Then 18.2 per cent of the exposed bacteria failed to multiply.

The fact that bacteria must be exposed to an appreciable ultra violet energy before any of them are killed is evidence that a summation of reactions is involved which finally results in the death of the organisms. But since all the bacteria are exposed to an equal energy, this initial summation effect does not explain why certain organisms are the first to succumb, or why the organisms are not all killed by the same total energy.

The observation that the first 20 to 30 per cent of the cocci succumbed to unduly small energies, but at an increasing rate, until an exponential energy relationship was established, seems to indicate a special susceptibility. And the apparently increased resistance of the last 10 per cent to be killed also suggests that the age and relative resistance of individual bacteria in an 18 hour culture must be taken into consideration. An agar slant culture of *S. aureus*, incubated at 37.5°C., for 18 hours, has not yet reached the limit of multiplication, and contains many organisms but recently divided and in an active metabolic state. It is known that young bacteria are less resistant to ultra violet light than are older, resting organisms (11), and the following experiment shows the greater susceptibility of young individuals in the strain of *S. aureus* under test.

Plates were seeded with distilled water suspensions of broth cultures of *S. aureus*, grown at 37.5°C. for 4, 28, and 52 hours respectively, and were exposed to λ254 mμ. No differences could be detected in the

morphology of colonies which subsequently grew out in the various exposed and control areas, but Chart 5 (from smoothed curves) shows that the recently divided and genetically and metabolically active

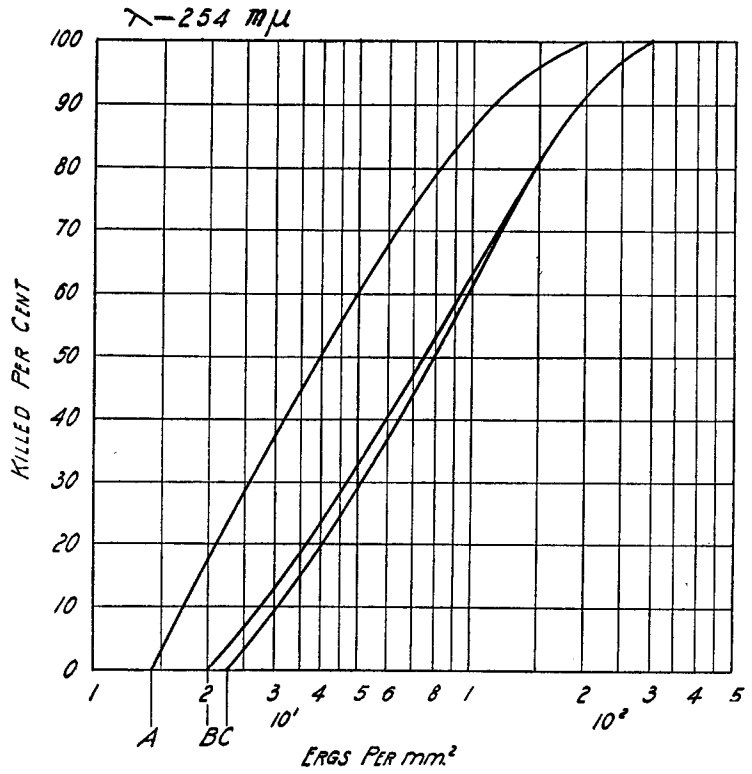

CHART 5. Differences in the course of the bactericidal reaction due to differences in age of the cultures employed.

A = 4 hrs. old.
B = 28 " "
C = 52 " "

bacteria in the 4 hour cultures were appreciably less resistant to the ultra violet energy. This relation between genetic activity and susceptibility to ultra violet radiation will be discussed in the final paper of this study.

It is interesting to note that the incident energy involved in the destruction of the 4 hour culture, in which most of the organisms would

fall in the same genetically active age group, apparently increases logarithmically from the beginning. This is additional evidence that the initial deaths in older cultures are of young and especially susceptible organisms. The cause of the terminal decrease in the rate of destruction cannot be stated with equal assurance. Unduly long exposures were required to kill the last survivors from each culture. But it is not certain whether, due to age or metabolic condition, these bacteria were individually more resistant, or whether they were partially protected in some way from the light. It is possible that small clumps of organisms may persist in the suspensions used, so that some bacteria are overlaid and partially protected. But microscopic slide preparations of many of the suspensions have failed to disclose clumps large enough to afford such protection, and, on the other hand, cocci from colonies of the last surviving organisms have proved to be inherently no more than normally resistant to ultra violet light.

Here may be mentioned an observation which has sometimes led investigators astray. If plates are thickly stewn with bacteria, the resulting colonies are smaller than when nutrient substances are not so limited per colony, and waste products do not accumulate so fast. When few bacteria survive in the middle of an exposed area, or when relatively few are left at the edges, where the intensity falls away, the colonies they produce are always much larger than those in the more crowded control areas of the plate. This has been interpreted to indicate a stimulating action of the light on exposed organisms that were not actually killed (3). But it is only necessary to dilute the original suspension and plate it out on the same medium, so that unexposed organisms are spaced as widely as the survivors in exposed areas are, to determine the source of the more active multiplication and consequent large colonies. Coblentz and Fulton (8) have noted the large colonies from surviving bacteria and have given this explanation of their development.

These experiments, therefore, give no evidence of a stimulating effect of ultra violet light in sublethal doses on the subsequent multiplication of bacteria. They show that there must be a summation of reactions due to radiant energy before any organisms succumb, and that the bacteria of an 18 hour culture show individual variations in their resistance to monochromatic ultra violet light.

DISCUSSION

The characteristic shape of the empirical curve for the bactericidal action of ultra violet light on *S. aureus* (Chart 4) invites an inquiry as to its significance. For it is another example to add to a growing list of experimental biological reactions in which, during most of its course, the reaction rate seems to depend upon the number of reacting units present at the given time.

It is typical of these reactions, such as the killing of bacteria by disinfectants (12, 13, 14), the limitation of the duration of life of fruit flies by untoward conditions of environment (15), or the hemolysis of erythrocytes by specific antibodies, or by ultra violet light (16) that the middle portion of the experimental curve may be reproduced mathematically by the equation that also describes the course of monomolecular chemical reactions. The formula tempts one to speculate, by analogy, on the nature of the fundamental biological reactions involved. But it is equally typical, and important, that toward the ends of the reaction curve the monomolecular reaction formula does not hold good. For example, in the reaction under discussion, there is an initial period of exposure, and consequent summation of its first effects, before any bactericidal action becomes apparent, and after the beginning of the reaction the reaction rate lags for a time before its maximum velocity is attained, although at first the maximum number of cells is exposed to the ultra violet energy. As the surviving units become relatively few, the velocity of the reaction again drops below prediction, and an excess of energy is required before the final bacteria succumb. The characteristic shape of these experimental curves for various biological reactions, with its similarity to the monomolecular reaction curve, and its equally essential differences, has given rise to extended discussions of its significance. It is generally recognized that differences in resistance must and do occur in heterogenous groups of biological units, such as fruit flies or bacteria, or erythrocytes, *in vitro*. If these differences in resistance are essential factors in the reaction of the individual, they must essentially modify the course of the reaction of the group. Under such circumstances, the course of the reaction depends on the distribution among the units of the factors causing resistance, and so the rate is

determined by probability rather than by the fundamental character of the reaction, and the appropriate curve with which to compare the rate is the "mortality curve" of insurance statistics (Chart 6). Then

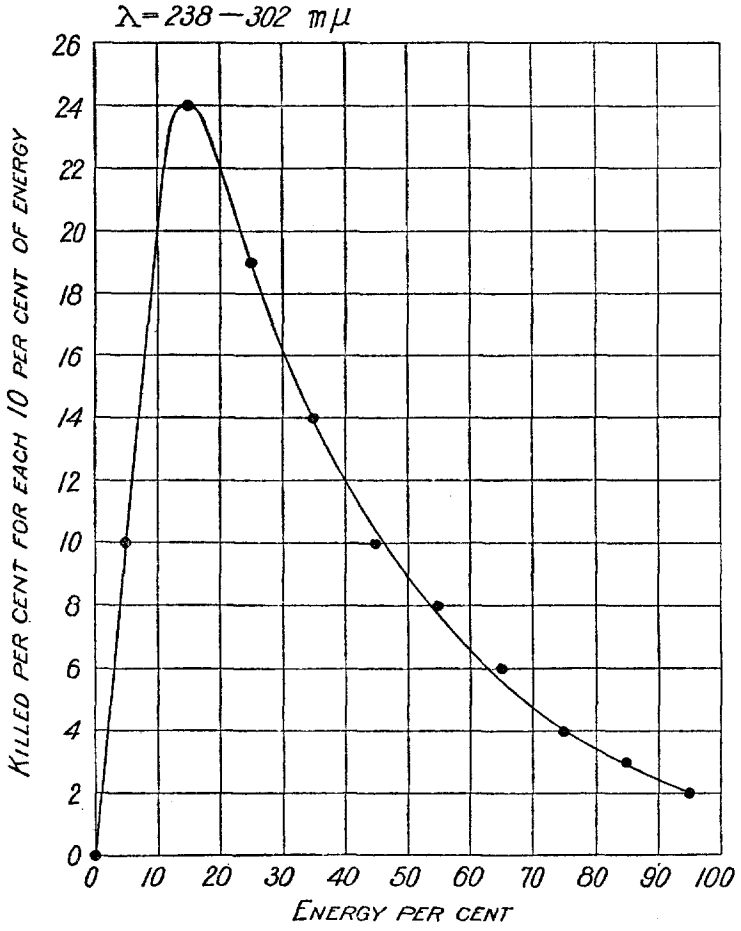

CHART 6. The data of Chart 4 refigured as a curve of probability.

the similarity of its middle portion to the course of a monomolecular reaction is to be ascribed to coincidence within the limits of experimental error—a distribution of resistance factors that simulates a logarithmic curve (15). On the other hand, proponents of the mono-

molecular reaction hypothesis insist that individual resistance varies only within relatively narrow limits, and when large numbers of units are under observation the differences in resistance may be disregarded, while the similarity to a monomolecular reaction persists.

These two opposed points of view are fully reviewed and defended by Brooks (16) and by Cohen (14). At best, the two interpretations affect only the theory of the course of the reactions and throw no light upon their fundamental nature in the various objects under test, as both writers are careful to insist.

The curves for the bactericidal effect of ultra violet light on *S. aureus* are evidently open to either interpretation. Experimental evidence of variations in individual resistance according to age stresses the importance of this factor in determining the course of the reaction. Partial elimination of the age factor by the use of young bacteria produces a reaction curve more nearly logarithmic at the start. But the most that can be said is that the rate of the fundamental reaction, whatever it may be, is undoubtedly modified by variations in individual resistance, and by variations in experimental conditions that mask its true course and make futile any attempt at exact interpretation. And the particular biophysical reactions in each bacterium that result in its death cannot be further analyzed merely from a series of observations on the incident energies that are involved at single wave lengths. A second essential factor in such an analysis—the relation of the incident energy at each wave length to that absorbed by the exposed bacteria—will be considered in a later paper of the series.

SUMMARY

In this first paper of a series on the bactericidal action of ultra violet light the methods of isolating and measuring monochromatic radiations, of preparing and exposing the bacteria, and of estimating the effects of exposure, are given in detail.

At all the different wave lengths studied the reactions of *S. aureus* followed similar curves, but occurred, at each wave length, at a different energy level. The general similarity of these curves to those for monomolecular reactions provokes a discussion of their signifiance, and emphasis is laid upon variations in susceptibility of individ-

ual organisms, due especially to age and metabolic activity, so that the typical curve seems to be best interpreted as one of probability.

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