A STUDY OF THE BACTERICIDAL ACTION OF ULTRA VIOLET LIGHT

III. The Absorption of Ultra Violet Light by Bacteria

By FREDERICK L. GATES

(*From the Laboratories of The Rockefeller Institute for Medical Research, New York, and the Department of General Physiology, Harvard University, Cambridge*)

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In this study of the bactericidal action of ultra violet light the first paper (1) described the reaction of an 18 hour culture of *Staphylococcus aureus* to monochromatic radiations. It was shown that the course of the reaction among large numbers of organisms was approximately the same at each wave length studied but that widely different incident energies were required at different wave lengths to produce these similar effects.

The second paper (2) discussed the limits of the bactericidal zone, showed that the reaction had a low temperature coefficient, (approximately 1.1), gave evidence that within the variations of the methods used no significant errors were introduced by differences in the measured intensity of the source or in the hydrogen ion concentration of the medium, and indicated that plane polarization of the incident light had no effect upon the reaction.

The present paper deals with the absorption of ultra violet light by intact bacteria. A final paper in this series will discuss structural and chemical units of bacterial protoplasm that may prove to be involved in the reaction which results in the organism's death.

Incident Energy Relationships at Various Wave Lengths

Text-fig. 1, reproduced from the first paper of this series, shows that although the course of the bactericidal reaction was approximately the same at each wave length studied, these similar curves were found at very different incident energy levels at different points in the ultra violet spectrum.

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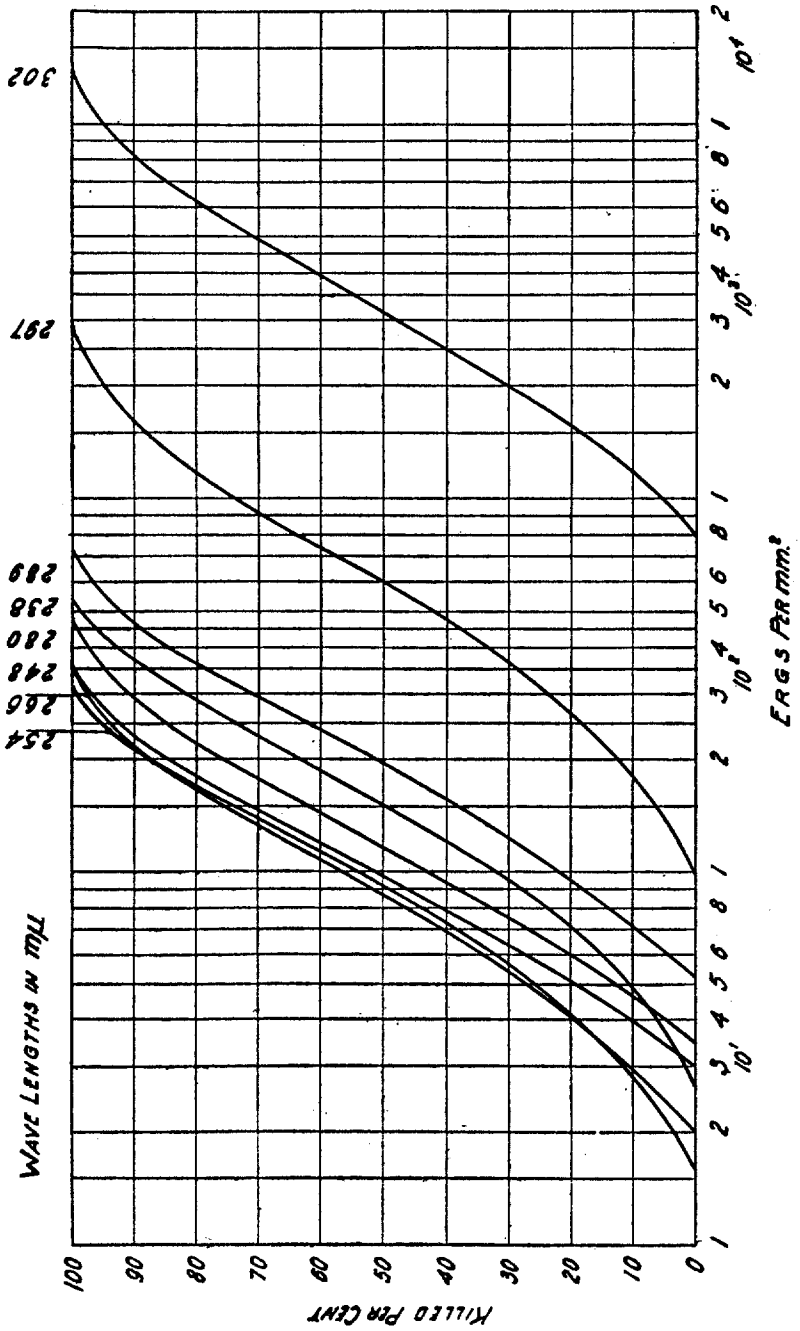

Text-Fig. 1. Incident energies required for bactericidal action at various wave lengths in the ultra violet spectrum

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For example, if the incident energy required to kill half the exposed staphylococci be taken as an index, this energy requirement ranged from 3,150 ergs per mm.^{2} at 302 m.μ, a wave length near the limit of bactericidal action, to 88 ergs at 266 m.μ. At other wave lengths, either above or below 266 m.μ, more incident energy was required. The destruction of 50 per cent of the microorganisms is chosen as the index because it is in the most accurately determined part of the curves where the mortality rate is least affected by variations in individual resistance.

If the incident energies involved in 50 per cent destruction be plotted and joined by a continuous line the resulting curve appears as in Text-fig. 2A. Parallel experiments on an 18 hour culture of *Bacillus coli* gave bactericidal energy curves similar in trend to those for *S. aureus*, and although complete statistics were not obtained on this bacterium the middle or exponential portion of the lethal reaction curves was determined by repeated observations at each wave length studied. Text-fig. 3A shows the incident energies involved in the destruction of 50 per cent of the exposed *coli* organisms. Its essential similarity to the corresponding *aureus* curve is apparent. Both curves would probably be somewhat modified in detail if more wave lengths were available for study.

These characteristic curves (Text-figs. 2A and 3A) show clearly that less incident energy is required between 260 and 270 m.μ than in any other region of the bactericidal zone examined, and point toward a second minimum below 230 m.μ. The presence of a sharp peak in the energy requirement near 240 m.μ appears to be equally significant. Due apparently to the use of but a few wave lengths in the bactericidal zone, or to failure to measure spectral intensities, or to crude methods of estimating bacterial destruction, the occurrence of a minimum at about 266 m.μ, and of the peak in the curve near 240 m.μ has been overlooked by most investigators. Usually they have been content with the conclusion that the shorter the wave length the more marked the bactericidal action (3).

Bang (4) was apparently the first to observe regions of special bactericidal "effectiveness" or of corresponding bacterial susceptibility. Using a 30 ampere carbon arc through a spectrograph at 20 m.μ intervals, and estimating bactericidal efficiency mainly by relative exposure, he found two regions of maximal action, an

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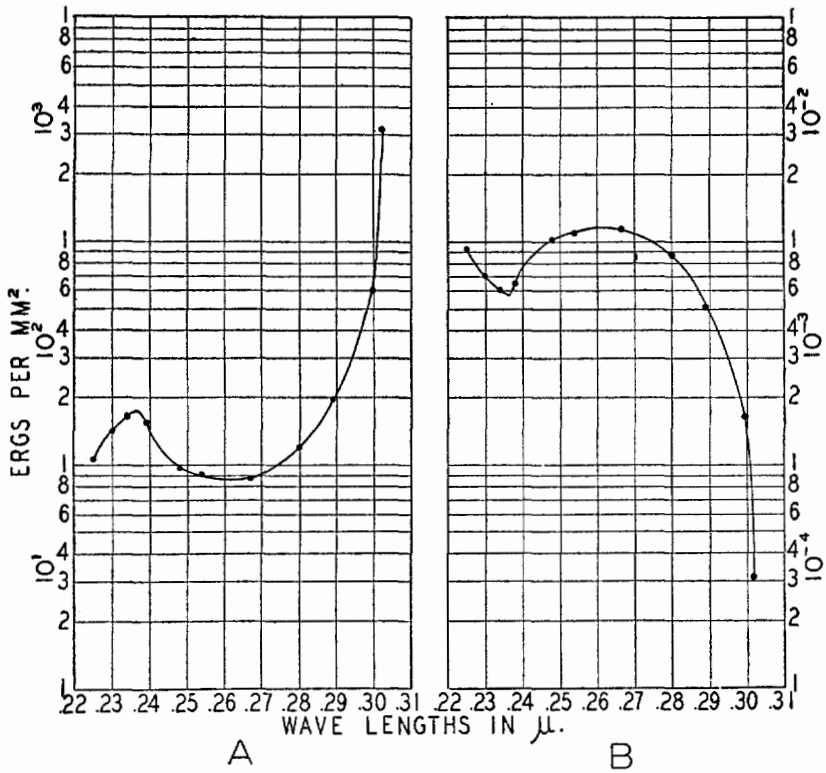
"inner maximum" between 340 and 360 m.μ, and an "outer maximum" between 240 and 260 m.μ. The lethal exposure varied from 1920 seconds at 330 to 300 m.μ through 120 seconds at 300 to 280 m.μ to 4 seconds at 280 to 260 m.μ and 2 seconds at 260 to 240 m.μ. Then longer exposures were required. The zone from 240 to 220 m.μ needed 20 seconds, that between 220 and 210 m.μ, 30 seconds, and that between 210 and 200 m.μ required 120 seconds exposure to kill the organisms.



TEXT-FIG. 2. A. Curve of incident energies involved in the destruction of 50 per cent of *S. aureus*.

B. Curve of the reciprocals of 2A.

These longer exposures at short wave lengths were evidently due to the rapid decrease in intensity of the source employed. Mme. Henri (3) thought that both of Bang's maxima should be ascribed to variations in intensity of his carbon arc.

Newcomer (5) exposed *B. typhosus* in quartz capillary tubes to narrow bands of the iron arc spectrum and counted surviving bacteria plated in agar, after exposures of 5 or 10 minutes. His figures, like Bang's, indicate a region of maximum effect

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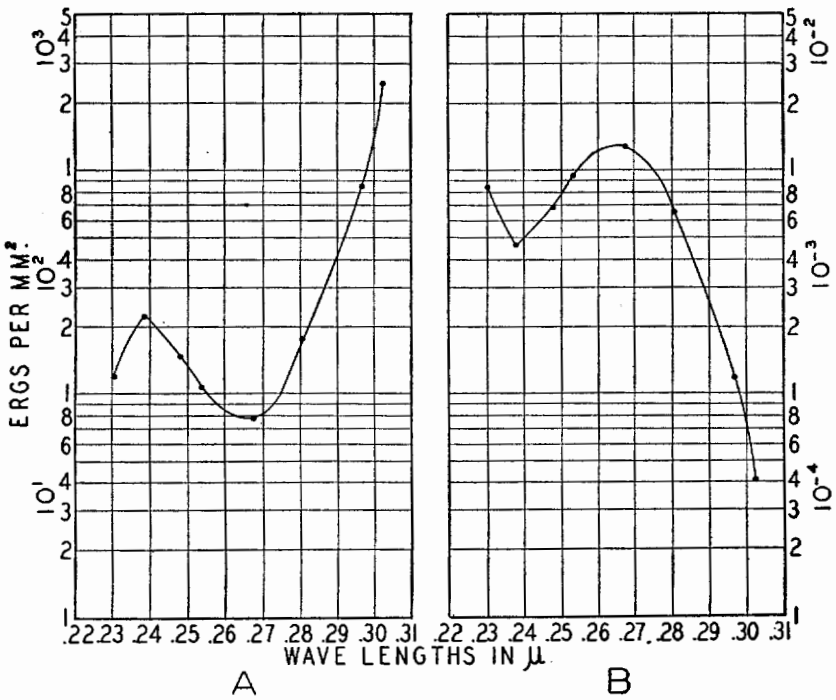
between 254 and 268 m.μ, but his iron arc varied so much in spectral intensity that he did not find any significance in this peak of effectiveness. He concluded that "equal intensities produce equal effects in the regions 2100–2800. If there is a maximum in this region it is at most only slight and would be in the neighborhood of 2600."

When Mashimo (6) varied the exposure of bacteria in a spectrograph from 15 seconds to 80 minutes he found the first evidence of bactericidal action at 275 m.μ.



TEXT-FIG. 3. A. Curve of incident energies involved in the destruction of 50 per cent of *B. coli*.

B. Curve of the reciprocals of 3A.

With somewhat longer exposures the zone widened rapidly so that with a 3 minute exposure it extended from below 210 to above 280 m.μ. The marked action at 275 m.μ was evidently due to a relatively high intensity of his source at this wave length, as an examination of his published photograph shows.

Thus variations in spectral intensity, with no adequate methods of measurement or control, made it difficult to interpret the maxima found by Bang, Newcomer, and Mashimo, and these authors laid no stress upon them.

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In the present study the measurement of the monochromatic radiant energy in absolute units focuses attention upon the marked differences in the incident energies required at different wave lengths to kill *S. aureus* and *B. coli*. The shape of the energy curves for 50 per cent destruction immediately suggests that it is the specific absorption of the ultra violet radiations that gives the curves significance. This relation of incident energy to its specific absorption is made the more striking by plotting the reciprocals of these energies (Text-figs. 2B and 3B), by which graphs not unlike absorption curves, with maxima at 266 and beyond 230 m.μ are produced.

As a first step in the further analysis of the bactericidal reaction it is obviously necessary to compare these curves with those for the absorption of ultra violet energy by the corresponding bacteria.

The Absorption of Ultra Violet Light by Bacteria

Apparently studies of the absorption spectra of bacteria have been confined hitherto to bacteria in suspension in a fluid medium (7, 8). Suspensions in liquids are unsuitable for such examinations. Reflection and refraction from the bacterial bodies, with consequent scattering of light so that bacterial suspensions are opaque even in the visible region of the spectrum, and the difficulty of estimating the number of organisms traversed, frustrate any attempt to obtain results of quantitative significance.

But a loopful of bacteria may be taken *en masse* from the surface of an agar slant and pressed between quartz plates into a layer so thin that is is all but colorless in visible light, and so transparent that objects may be seen through it clearly and without distortion. The bacteria are in optical contact and form a homogeneous medium for the transmission of visible or ultra violet light. Such a film is composed almost entirely of bacterial cells and the immediate products of their metabolism. Tests show that films of like thickness of the agar medium from which the bacteria were removed absorb no significant amounts of ultra violet light.

Such a film of bacteria may be set up in one optical path of a quartz photometer, with similar plates of quartz and a drop of glycerol in the other path as a control, and the absorption coefficient of the organisms readily obtained in the manner commonly employed for chemical solutions (9).

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Two beams of light from the same source, which have passed through the specimen under examination or its control in the photometer, are spread by a quartz spectrograph into parallel spectra in the plane of a photographic plate. The energy that traverses the control path is subject to quantitative variation by means of a sector shutter, and with the shutter set at predetermined openings a series of photographs is made. Then the point, or points, of equal blackening in each pair of spectral photographs indicate the wave lengths at which the test specimen and the sector shutter in the control path have reduced the original intensity of the light to the same degree.*

In such experiments it is necessary to know accurately the depth of the medium traversed in order to calculate the coefficients of absorption for a layer of unit thickness. The standard or unit of thickness in these observations was chosen as 0.8 μ, the average diameter of *S. aureus* (10), so that the coefficients of absorption were obtained for a single layer of bacteria. The shape of *B. coli* and its wide variations in size precluded even so crude an estimate of a "single layer," so the coefficients for *B. coli* were figured arbitrarily for a layer of the same thickness (0.8 μ) to permit a comparison with the results for *S. aureus*.

The thickness of these films of bacteria between quartz plates was found to lie between 5 and 15 μ. Since the method of measurement employed (11) is easy, and is accurate (in microns) to the second or third decimal place it may be described in brief. The method is based on interferometry, namely, the measurement of the interference bands formed by the coincident spectra of white light reflected into a wavelength spectroscope from the two quartz surfaces enclosing the bacterial film.

A point source of white light (carbon arc, concentrated tungsten filament, or even a flashlight bulb with the filament vertical), is set up at a measured angle of incidence (60°) to the film specimen, so that its light is reflected from the back surface of the first quartz plate and from the front surface of the second (the surfaces enclosing the film), into a wavelength spectroscope. The reflecting surfaces must be chosen near the bacterial film, but not to include it, since adequate reflection occurs only when air is the medium between the two plates. With a proper set-up, and films of suitable thickness a series of vertical interference bands will cut across the spectrum.

* Measurements of absorption by such films made in 1923 with thermocouple and galvanometer (*Proc. Soc. Exp. Biol. and Med.*, 1923, **21**, 61), were evidently not as accurate as those obtained by the present method and have been discarded.

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Then the distance (t) between the two plates (the thickness of the included film) is found by noting the wave lengths (λ_1 and λ_2) between which any convenient number (n) of interference bands is counted for substitution in the following equation:

$$t = \frac{n \lambda_1 \lambda_2}{2 \mu \cos r (\lambda_1 - \lambda_2)}$$

μ is the refractive index of the medium, and r is the angle of incidence and reflection. Since air is the medium $\mu = 1$, and with r at 60° $\cos r = 0.5$ so that the equation becomes

$$t = \frac{n \lambda_1 \lambda_2}{(\lambda_1 - \lambda_2)}$$

For example, with the apparatus set up as described 5 interference bands are counted between $\lambda_1 = 7241$ Å u. and $\lambda_2 = 4638$ Å u. It is only necessary to multiply these waves lengths together, divide by their difference, and multiply the result by 5 to determine that the distance between the plates *in the area examined* is 6.45 μ or 0.00645 mm.

In these experiments the films of bacteria, pressed out by hand, were not strictly plane parallel, but often thicker at one edge than at the opposite one, so four readings were taken at 90° intervals around the rim of each film and combined for an average thickness. The differences in thickness in the small central area exposed in the photometer were not so great as to make it desirable to obtain a mean exponentially according to Lambert's law. For example:

Film 1. *B. coli.* 90° readings at edge of film: 5.58, 6.17, 5.63, and 5.66 μ. Average 5.76 μ.

Film 2. *S. aureus.* 90° readings at edge of film: 9.21, 11.66, 11.24, and 10.53 μ. Average 10.66 μ.

Given the average thickness of each film, the number of layers (n) of bacteria it contained was then available for the determination of the coefficients of absorption for a layer 0.8 μ thick. For this purpose the familiar equation of Lambert's law was used, to which Wood (11) says that no exception has ever been found that was not attributable to experimental error. Thus if a is the fraction of the original intensity (I_o) transmitted by each unit layer, the intensity (I_t) observed after passage through n layers is given by the equation:

$$I_t = I_o a^n$$

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The relation of I_t to I_o is chosen at appropriate intervals on the sector shutter of the quartz photometer, as described above, and this equation solved for a gives the coefficient of transmission at the wave length where the two spectra match. Then 1-a is the corresponding coefficient of absorption for a single layer of bacteria 0.8 μ thick.

The mean absorption curve from five series of determinations with *S. aureus* is given in Text-fig. 4, and from five series with *B. coli* in Text-fig. 5. The curves are characteristic, and similar to those found for various biological tissues and fluids containing proteins or protein derivatives (12, 13, 14) on which their main features evidently depend. Differences in the curves for the two organisms are apparent, but in view of the experimental errors involved in such determinations they probably should not be stressed.

The general similarity of these absorption curves to the reciprocals of the curves for bactericidal incident energy is obvious. All four curves rise rapidly from low levels beyond 300 m.μ to a maximum between 260 and 270 m.μ, then drop to a minimum near 240 m.μ and rise again toward a limit beyond the range of experimental observation. One is tempted to correct the incident energies involved in the bactericidal reaction by these absorption coefficients for the entire organisms in order to obtain an approximation of the total energies absorbed. Yet the two sets of curves show important points of difference also, especially in the location of the dip near 240 m.μ, and a closer consideration of their relationship indicates that such a quantitative correction would be futile. The sum of the absorption coefficients of all the chemical entities in the bacterial cell cannot be expected exactly to correct the wide differences found in the bactericidal incident energies at different wave lengths unless every chemical group is involved in the bactericidal reaction in exact proportion to its contribution to total absorption. Such a hypothesis is hardly tenable. It seems more probable that it is the effect of ultra violet energy on a single vital and sensitive structural or chemical unit that results in subsequent failure in cell multiplication. Rahn (15) has recently figured from the curves of abiotic reactions among large numbers of single cell organisms that death of the cell probably involves only a single chemical entity. As Coblentz and Fulton (16) have suggested, it is to be

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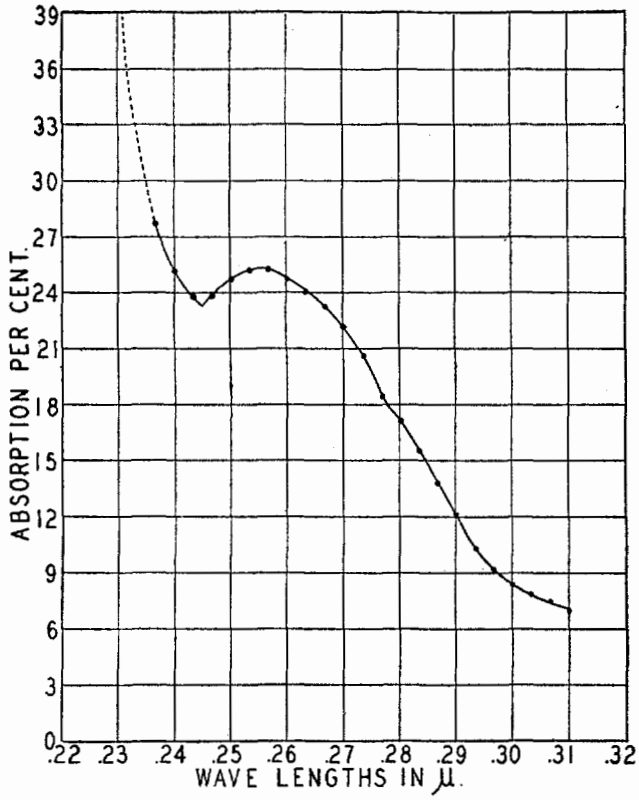
presumed that only a small fraction of the total absorbed energy first affects such an essential structure, and so leads to the death of the cell. Neither the reciprocals of the energy curves, which are undoubtedly modified by the absorption of light by elements not involved in the



TEXT-FIG. 4. The coefficients of absorption of ultra violet light by a layer of *S. aureus* 0.8 μ thick.

bactericidal reaction, nor the curves for total absorption give an accurate picture of the absorption curve of this vital substance, whatever it may be. Yet their similarities and their differences are alike useful in the further search for such an essential and sensitive element in the cell's structure and economy.

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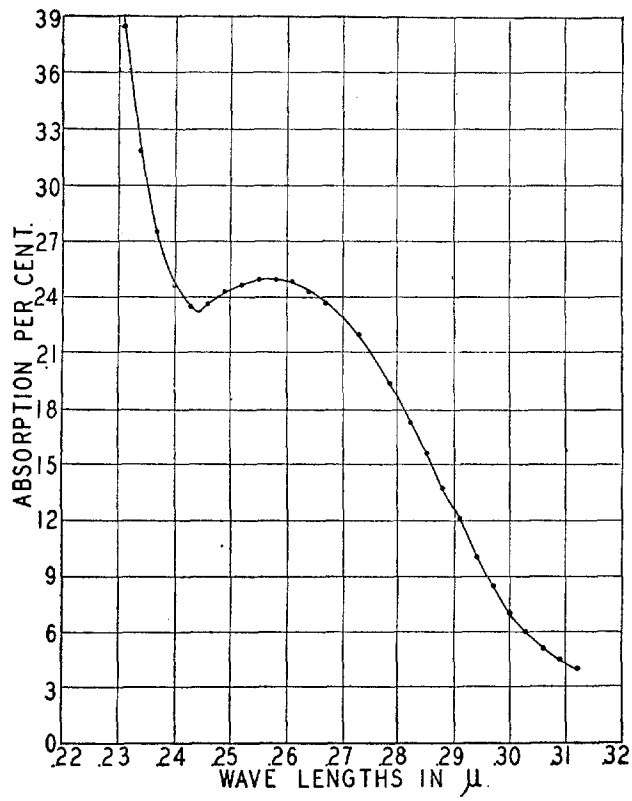
Thus it may be predicted that there are a number of chemical entities or aggregates in the living cell which have rather similar coefficients of ultra violet absorption, and that the sum of these similar absorption curves largely determines the shape of the curve for the entire cell.



TEXT-FIG. 5. The coefficients of absorption of ultra violet light by a layer of *B. coli* 0.8 μ thick.

Among these substances will be found the one essential element first affected by ultra violet light in the bactericidal reaction, and its absorption curve will be similar to, though not identical with, the reciprocal of the lethal energy curve. Finally it seems improbable that this sensitive substance is uniformly distributed throughout the cell's pro-

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toplasm. An examination of the evidence for its concentration in the cell nucleus, and the further search for evidence of its chemical character are reserved for the final paper of this series.

SUMMARY

The simple conclusion of former investigators that the shorter the wave length of ultra violet light the greater the bactericidal action is in error. A study with measured monochromatic energy reveals a characteristic curve of bactericidal effectiveness with a striking maximum between 260 and 270 m.μ. The reciprocal of this abiotic energy curve suggests its close relation to specific light absorption by some single essential substance in the cell.

Methods are described for determining the absorption curve, or absorption coefficients, of intact bacteria. These curves for *S. aureus* and *B. coli* have important points of similarity and of difference with the reciprocals of the curves of bactericidal incident energy, and point the way in a further search for the specific substance, or substances, involved in the lethal reaction.

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