EXPERIMENTS ON LIGHT-REACTIVATION OF ULTRA-VIOLET INACTIVATED BACTERIA

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Many types of microorganisms are killed by ultra-violet light and the number of survivors falls off with increasing dose. A. Kelner[1] reported recently on his discovery that, if exposure to ultra-violet light is followed by exposure to visible light, the number of survivors is very much larger. A similar discovery was reported by R. Dulbecco[2] for bacteriophage.

We investigated this phenomenon of light-reactivation of ultra-violet inactivated bacteria and found some very simple regularities.

In our experiments we used a strain of coli, B/r, originally isolated by E. M. Witkin.[3] Cultures were grown in a lactate-ammonium phosphate medium under aeration to between 5×10^7 and 10^8 bacteria per cc., then transferred into saline, and incubated under aeration at 37°C. from 14 to 18 hours. The cultures were then placed in the icebox, kept at about 6°C. and used for experiments over a period of about one week.

Figure 1 shows in semilogarithmic plot the result of one series of experiments. Eight other such series were conducted with similar results.

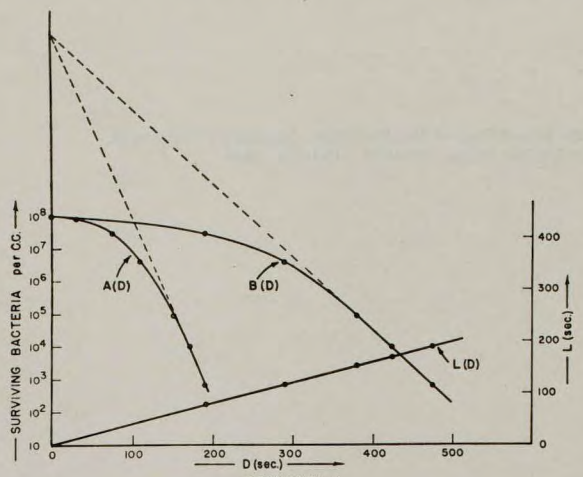

FIGURE 1

Experiment of May 11, 1949. $A(D)$, left scale, gives in semi-log plot the number of survivors as function of ultra-violet dose, D, in seconds of exposure to 15-watt germicidal lamp at 50 cm. distance (no light reactivation). $B(D)$, left scale, gives in semi-log plot the number of survivors as function of ultra-violet dose, D, with light reactivation by 1-hour exposure to light of 1000-watt projection lamp at 8 inches distance. $L(D)$, right scale, gives in linear co-ordinates L as function of D satisfying equation $B(D) = A(L)$.

In these series of experiments the survivor curves, $A(D)$, were obtained in the following manner: The bacteria were exposed in saline to different ultra-violet doses, D, characterized by the number of seconds of exposure, then plated on nutrient broth agar, incubated, and the survivors determined by colony count. A 15-watt germicidal lamp was used as the source of ultra-violet radiation with the mercury line at 2536 Å. predominating in the spectrum. A 15-cm. Petri dish containing 20 cc. of the bacterial suspension

was exposed at a distance of 50 cm. from the lamp and was agitated during exposure.

The survivor curves, $B(D)$, were obtained in a similar manner, except that immediately following exposure to the ultraviolet lamp the bacterial suspensions were exposed for one or two hours, at 8-inch distance, to the light of a 1000-watt projection lamp, and then plated on nutrient broth agar. The color temperature of the lamp was close to 3350°K. The bacterial suspensions were kept at 37°C. during exposure to the light of the projection lamp. In most of the series a 1-inch layer of a 0.05 molar solution of $CuCl_2$ was interposed between the lamp and the bacterial suspension.

As can be seen in figure 1, in semilogarithmic plot, both survivor curves A and B drop with increasing ultra-violet dose, D, slowly at first, then faster, and finally go over into a straight line.

As figure 1 shows, light-reactivation is a striking phenomenon. For an ultra-violet dose of $D = 190$ sec., for instance, the number of survivors falls in the absence of light-reactivation (curve A) from 10^8 to 700. With light reactivation, however, (curve B) the number of survivors only falls from 10^8 to 3×10^7; i.e., due to light-reactivation the titre of viable bacteria increases by a factor of about 40,000. In order to obtain the same drop in the survivors (from 10^8 to 3×10^7), in the absence of light-reactivation, a *lower* ultra-violet dose of $D = 75$ sec. would have to be employed. For every ultra-violet dose, D, which is followed by light-reactivation and thus leads to a certain number of survivors, $B(D)$, there can be found a lower ultra-violet dose, L, which in the absence of light-reactivation would lead to the same number of survivors; i.e., for which we have $B(D) = A(L)$.

Plotting L as a function of D in figure 1 we obtain for $L(D)$ a straight line going through the origin, for which we may write $L(D) = qD$, where q is a dose-independent constant smaller than 1. This means that there is a simple relationship between the survivor curves A and B which we may express by writing

$$B(D) = A(qD). \qquad (1)$$

This relationship is confirmed by the nine series of experiments that were carried out. In any one of them the bacteria were light-reactivated by an exposure to the light of a projection lamp, at 8-inch distance, either of one hour or of two hours. After exposure of two hours the number of survivors obtained is, within the limits of experimental error, indistinguishable from the maximum reactivation that can be obtained with longer exposures to light. After exposure of one hour the number of survivors is lower, but the difference is not great. The q values obtained in these experiments vary slightly from series to series within the range of $0.32 < q < 0.42$. In the experiments shown in figure 1 we have $q = 0.4$.

If the light intensity used for light-reactivation is doubled by placing

the bacterial suspension closer to the projection lamp, the maximum number of survivors obtainable remains the same, only it is obtained with a shorter exposure to the light.

From the relationship (1) it follows that if we extrapolate in the semi-logarithmic plot the straight line portions of the survivor curves A and B to zero ultra-violet dose, we must be led back to the *same point.* This is shown by the dotted straight lines in figure 1.

In order to interpret our results we assume for the *sake of argument* that when bacteria are irradiated with ultra-violet light a "poisonous" chemical compound, P, is produced in an amount which is *proportionate* to the ultra-violet dose, D. We assume that this "poisonous" compound is produced in two forms: a form P_x, which is not sensitive to light and which is produced in the amount x_0, and a form P_y, which can be destroyed by light and which is produced in the amount y_0, so that $D = x_0 + y_0$. We further assume that the ratio x_0/y_0 is independent of the ultra-violet dose D. And finally we assume that the number of survivors after exposure to ultra-violet irradiation, with or without subsequent light-reactivation, is determined by the amount of "poison" that is present in the bacteria *at the time* they are incubated with nutrient medium and permitted to multiply.

On the basis of these assumptions, we may now account for the relationship (1) between the survivor curves A and B by saying that if we follow up the ultra-violet irradiation of a bacterial suspension by exposure to strong light for one or two hours we destroy all the poison present in the form P_y, and leave only the poison P_x, and by further saying that the amount of the poison present in the form P_x is given by $x_0 = qD$, where q is a dose-independent constant. The amount of "poison" left in the bacteria after light-reactivation is then given by qD, and therefore the number of survivors after light-reactivation, $B(D)$, can be taken from the survivor curve obtained in the absence of light-reactivation, $A(D)$, by writing $B(D) = A(qD)$.

If we now further assume that the light-sensitive variety of the poison, P_y, which is present in an amount y, is destroyed by light at a rate which is proportionate to its amount, y, we may write $\frac{dy}{dt} = -\alpha y$, where α is a function of the light intensity and independent of the dose, D. From this assumption it follows that if a bacterial suspension is exposed to an ultra-violet dose, D, and immediately afterward is exposed to strong light (say, to the light of a 1000-watt projection lamp at 8-inch distance) for a period of time, t, the total amount of poison, $L = x + y$, present in the bacteria is at any time, t, given by

$$L = x_0 + (D - x_0)e^{-\alpha t}. \qquad (2)$$

For an infinitely long exposure to light, we have $L_\infty = x_0$. We may then

write

$$\ln(L-L_\infty) = \ln(D-L_\infty)-\alpha t. \qquad (3)$$

The result of an experiment undertaken to check this relationship (3) is shown by curve I in figure 2. In this experiment we first exposed the bacterial suspension to the ultra-violet lamp for $D = 250$ sec. and then to the light of a 1000-watt projection lamp at 8-inch distance. We determined the number of survivors as a function of t, the time of exposure to the projection lamp, by plating on broth agar from aliquots taken from the bacterial suspension at different times, t, up to a time of 2 hours. From the number of survivors, we computed by means of the survivor curve, $A(L)$, the corresponding L values. As L_∞ we took the L value corresponding to the number of survivors obtained after 2 hours of exposure to the projection lamp.

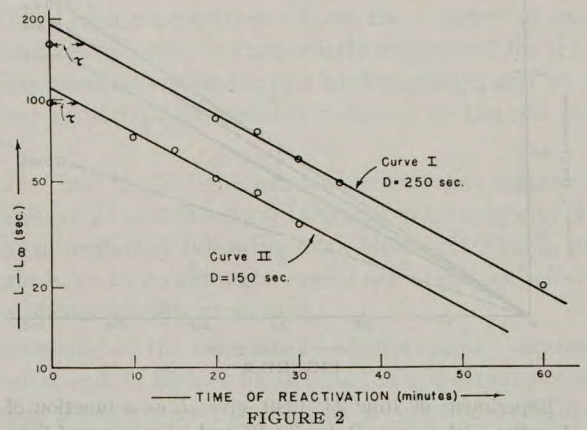

FIGURE 2

Experiment of June 23, 1949. Curve I gives in semi-log plot $L - L_\infty$ as a function of time of reactivation at 8 inches from 1000-watt projection lamp for bacteria that had received an ultra-violet dose, D, of 250 sec. Curve II is the same for bacteria that had received an ultra-violet dose of 150 sec. The interval τ represents the latent period of the process of reactivation.

As can be seen from curve I in figure 2 the points obtained fall on a straight line in accordance with equation (3). This type of relationship was confirmed in five series of experiments, giving values of α ranging from 2.19 to 2.38 per hour.

It should be noted however that the straight line I in figure 2 does not extrapolate back to the experimental point for zero exposure to light and the discrepancy corresponds to a latent period, τ, of about 3 minutes.

Line II in figure 2 differs from line I only in so far as it was obtained with an exposure to the ultra-violet lamp of $D = 150$ seconds. The fact that these two lines are parallel is in accord with the thesis that α is independent of the ultra-violet dose, D. The latent period τ manifested by line II is again about 3 minutes. Two series of experiments were performed to demonstrate in this manner the independence of α of the ultra-violet dose D.

Figure 3 shows the result of three experiments in which light-reactivation was carried out by exposing the ultra-violet irradiated bacterial suspension to the light of the projection lamp for $t = 20$, 25 and 30 min., respectively. From the number of survivors observed we computed, by means of the survivor curve, $A(D)$, the corresponding L values, and in each case we obtained for $L(D)$, as can be seen from figure 3, a straight line passing through the origin.

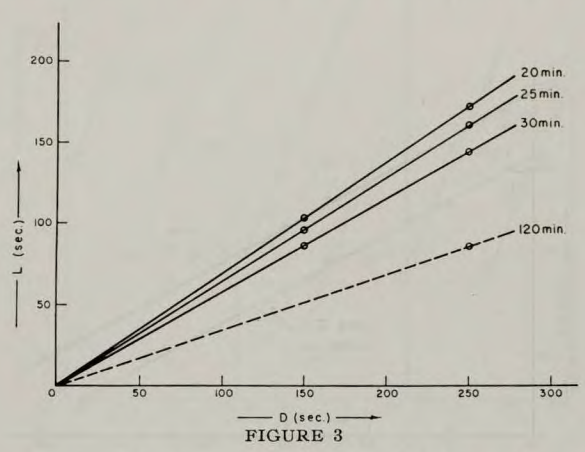

FIGURE 3

Experiment of June 23, 1949, gives L as a function of the ultra-violet dose, D, for 20, 25 and 30 minutes of light reactivation at 8 inches from 1000-watt projection lamp.

This result is to be expected provided that both α and τ are independent of the ultra-violet dose, D. Two such series of experiments were performed with identical results.

We were also interested to see in what manner the value of α depends on the intensity of the light which is used for reactivation. We varied this intensity by placing the bacterial suspension at different distances from the projection lamp. In such experiments we found that the value of α decreased with decreasing light intensity, but the decrease was less than strict proportionality with intensity would lead one to expect. The latent period τ increased in these experiments with decreasing light intensity.

We did not, however, in any of these experiments lower the light intensity very much below the value that obtains at 8inches from the pro-

jection lamp. We kept the intensity comparatively high in order to be able to neglect a reaction which takes place in the dark and therefore, of necessity, must also manifest itself if light-reactivation is carried out by means of very weak light. This dark reaction is easily observed if the bacterial suspension is irradiated with ultra-violet and then incubated in the dark at 37°C., for instance for 3 hours, prior to being reactivated at 8-inch distance from the projection lamp. The number of survivors after light-reactivation is then found to be much lower than is obtained when no dark incubation is interposed between ultra-violet inactivation and light-reactivation.

This concludes the report on our findings concerning the light-reactivation of ultra-violet inactivated bacteria in so far as these findings relate to the effect of light-reactivation on the number of survivors.

It is known that ultra-violet radiation not only kills bacteria but also produces mutants among the progeny of the survivors. We were interested to find out how light-reactivation affects the number of mutants which appear among these progeny. Particularly convenient for this purpose are mutants which manifest resistance to a bacteriophage, and we chose for the purpose of our investigation mutants resistant to the coli phages *T*4, *T*6 or *T*1.

M. Demerec[4] had studied the ultra-violet-induced mutations in coli to resistance to phage *T*1 and discovered that the great majority of the mutants do not appear immediately following ultra-violet irradiation, but that most of the bacteria have to go through several cell divisions before their mutation becomes phenotypically expressed.

A small minority of the mutants is phenotypically expressed prior to any cell division and A. Kelner finds that the appearance of such mutants is suppressed if the ultraviolet irradiation is followed by light-reactivation (see A. Kelner, abstracts of papers presented at the 49th General Meeting of the Society of American Bacteriologists, page 14, May 1948).

In our experiments we allowed, following irradiation, the survivors to go *through ten generations in liquid culture* and determined among the progeny the number of phage-resistant mutants per 10^8 bacteria.

If we are right in assuming that a poison is produced by the ultra-violet rays employed in our experiments, that the amount of this poison is reduced by light-reactivation, and that the amount of poison which is present in the bacteria when they are allowed to multiply determines the number of survivors, it then seems possible that this same "poison" might also determine the number of mutants—resistant to one of the phages—that appears among the progeny of the survivors. In this case we should expect that the number of mutants appearing among the progeny of the bacteria which were given a certain ultra-violet dose, D, and which were subse-

quently light-reactivated will be the same as the number of mutants appearing among the progeny of the bacteria which were exposed to a *lower* ultra-violet dose, qD, but which were *not* light-reactivated.

In our experiments the bacteria were exposed in saline suspension to different ultra-violet doses, D. Aliquots were then taken which were either incubated with lactate-ammonium phosphate medium at 37°C. under aeration or they were first exposed to the light of the projection lamp at 8-inch distance for one hour and were subsequently thus incubated. In either case the bacteria were allowed to go through about *ten generations* in liquid culture and were then assayed for mutants resistant to one of the coli phages $T4$, $T6$ or $T1$.

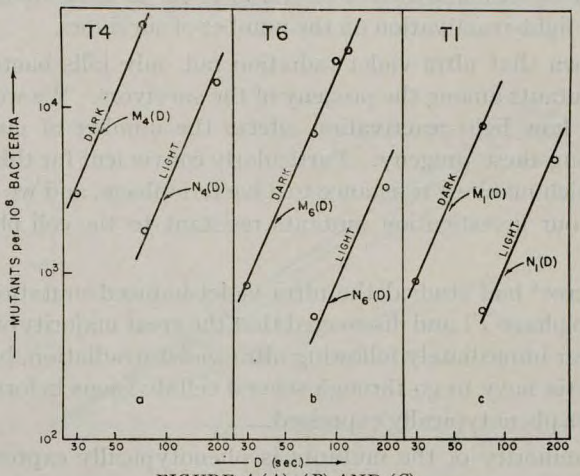

FIGURE 4 (*A*), (*B*) AND (*C*)

Experiment of July 2, 1949, gives in log-log plot the number of phage-resistant mutants per 10^8 bacteria as a function of the ultra-violet dose D both without (*DARK*) and with (*LIGHT*) light reactivation for phages $T4$, $T6$ and $T1$ in figures *a*, *b*, and *c*, respectively. The bacteria went through 10 generations in liquid culture prior to assaying for the mutants. The values shown in the graph are corrected for the spontaneous mutants which amounted to 800, 40 and 170 per 10^8 bacteria for $T4$, $T6$ and $T1$, respectively.

Figure 4 shows the result of one series of experiments in log log plot. The symbols $M_4(D)$, $M_6(D)$ and $M_1(D)$ relate to bacteria which were exposed to ultra-violet, were *not* light-reactivated, and went through ten generations in liquid culture (corresponding to an increase in bacterial titre by a factor of about 1000). Similarly $N_4(D)$, $N_6(D)$ and $N_1(D)$ relate to bacteria which were exposed to ultra-violet, were light-reactivated, and went through ten genera-

tions in liquid culture. These symbols designate the number of mutants resistant to phages $T4$, $T6$ and $T1$, respectively, which were found per 10^8 bacteria in the cultures—corrected for the spontaneous mutants that were obtained for zero ultra-violet dose—and plotted as a function of the ultra-violet dose, D.

Within the range of this experiment and within the limits of its accuracy, the lines $M(D)$ and $N(D)$ are represented in the log log plot by straight lines which are *all parallel* to each other. For each phage and for any given ultra-violet dose, D, the value of M is higher than the value of N. For every ultra-violet dose, D, which leads, when followed by light-reactivation, to a certain number of mutants, $N(D)$, there can be found a *lower* ultra-violet dose, L, which in the absence of light-reactivation leads to the same number of mutants; i.e., for which we have $N(D) = M(L)$.

Because the lines $M(D)$ and $N(D)$ are represented by *parallel* straight lines in the log log plot we may write:

$$N_4(D) = M_4(m_4D); \qquad N_6(D) = M_6(m_6D); \qquad N_1(D) = M_1(m_1D) \quad (4)$$

where m_4, m_6 and m_1 are dose-independent constants. The values of m_4, m_6 and m_1 taken from the experimental series plotted in figure 4 are 0.3, 0.3 and 0.32, respectively. For the same series of experiments the value of q was also determined and was found to be 0.35. Within the limits of our experimental accuracy we have thus found: $m_4 = m_6 = m_1 = q$. Two other series of experiments which were undertaken also gave, within the limits of experimental error, identical values for m and q.

It should be noted, however, that our experiments establish the validity of relationship (4) with much less accuracy than they establish the validity of relationship (1). In general, in our experiments the determination of the number of the mutants in any one single measurement was affected by an error of $\pm 50\%$, whereas the determination of the number of survivors in any one single measurement was affected only by an error of $\pm 20\%$. And more important, due to the shape of the survivor curve $A(D)$, a large percentage error in the number of survivors leads only to a small percentage error in the corresponding L values taken from the survivor curve A, whereas an error of 50% in the number of mutants, according to the curves M and N plotted in figure 4, leads to an error in the corresponding ultra-violet dose, D, of about 25%.

We have not investigated the ultra-violet-induced mutations to phage resistance with the same thoroughness as we have the effect of ultra-violet exposure on the number of survivors. Therefore at this time we can only say that our results to date are entirely consistent with the view that the effect of light-reactivation on the appearance of mutants among the progeny of the ultra-violet-irradiated bacteria is the same as is its effect on the number of survivors and that this effect consists in the reduction of the

effectiveness of the ultra-violet dose by the dose-independent factor q. This makes it possible to surmise that in our experiments the killing of the bacteria and the production of the mutants might be due to the *same* chemical effect produced by the ultra-violet irradiation.

[1] Kelner, A., PROC. NATL. ACAD. SCI., **35**, 73 (1949).
[2] Dulbecco, R., *Nature*, **163**, 949 (1949).
[3] Witkin, E. M., *Genetics*, **32**, 221 (1947).
[4] Demerec, M., PROC. NATL. ACAD. SCI., **32**, 36 (1946); Demerec, M., and Latarjet R., *Cold Spring Harbor Symposia*, **11**, 38 (1946).