MONOCHROMATIC ULTRAVIOLET ACTION SPECTRA AND QUANTUM YIELDS FOR INACTIVATION OF T1 AND T2 ESCHERICHIA COLI BACTERIOPHAGES

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Much of the interest in the biological effects of ultraviolet radiation arises from the fact that different wavelengths are absorbed selectively by different chemical groups. The possibility exists, therefore, of gaining some clues as to the cellular constituents that absorb the ultraviolet radiation leading to a particular effect by comparing the efficiency of different wavelengths in producing the effect—the action spectrum—with the absorption spectra of different cellular constituents. Thus, on the basis of the relative bactericidal efficiency and absorption of different regions of the ultraviolet spectrum, Henri as early as 1914 was led to the hypothesis that the seat of the bactericidal effect was in the proteins of the nucleus. The further possibility of inducing heritable modifications by sublethal exposures to ultraviolet radiation was evident to her and was realized, apparently, in experiments with *Bacillus anthracis* designed expressly to test this hypothesis.

Gates (1928) was the first to publish a biological action spectrum determined by the more refined technique of employing monochromatic beams of ultraviolet light. His results on the relative bactericidal effectiveness of different wavelengths focused attention on the nucleic acids. Since that time, the action spectrum technique has been applied to a number of the biological effects of ultraviolet radiation. Giese (1945), Loofbourow (1948), and Blum (1950) reviewed the different types of action spectra observed.

Action spectra for the inactivation of bacteriophages have been published by a number of authors (Gates, 1934; Fluke and Pollard, 1949; Franklin *et al.*, 1953). In general, a maximum of efficiency at about 2600 A is observed, suggesting nucleic acids as the absorbing chromophore. These results are similar to those obtained with other viruses (Hollaender and Oliphant, 1944) and for the bactericidal effects of radiation by numerous workers (see Zelle and Hollaender, 1954, for review).

Although Brackett and Hollaender (1939) discussed earlier certain aspects of the question, Loofbourow (1948) presents the most complete discussion of the various assumptions underlying the action spectrum technique which must be fulfilled if the method is to give accurate clues to the biologically important molecules absorbing the ultraviolet. One of these assumptions is that the quantum efficiency or quantum yield is independent of the wavelength for the spectral region studied. No information on the question of quantum yields for the inactivation of bacteriophages at different wavelengths is available in the literature so far as the authors are aware. The present paper presents some data bearing on this question, and, in addition, action spectra are presented that were obtained under conditions permitting accurate comparisons between two bacteriophages of the well known T series attacking *Escherichia coli*.

MATERIALS AND METHODS

The T1 and T2 bacteriophages and their host, *E. coli*, strain B, were employed in this study. The phages were irradiated in M/15 phosphate buffer, pH 6.8, which is transparent to the wavelengths used in these studies. Assays of viable bacteriophages were made by the agar layer method and in such a manner that multiplicity reactivation (Luria, 1947) would not affect the results until very small survival ratios were observed. Adequate precautions were taken to prevent photoreactivation.

The source of radiation was a homemade, water-cooled, high-intensity mercury arc of the

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Daniels-Heidt type, which was focused upon the entrance slit of a large crystal-quartz monochromator (Hollaender, 1938). The bacteriophages were exposed to the emergent beam of monochromatic ultraviolet radiation which was directed downward by means of a right angle quartz prism. The incident energy was measured by a thermopile and sensitive galvanometer after calibration against a standard lamp.

The experiments were performed in two different laboratories: the National Institutes of Health and the Oak Ridge National Laboratory. Three different thermopiles were employed, and there were other less important differences in physical setup. Although there are some discrepancies, in general, the data obtained in the two laboratories after an interval of more than two years agree remarkably well.

EXPERIMENTAL RESULTS

Action spectra for inactivation of T1 and T2. In the original work done at the National Institutes of Health, nine wavelengths between 2220 and 3022 A were used in determining the action spectrum. To insure more accurate comparison, the two phages were mixed in approximately equal numbers and irradiated together. Assays for surviving phages of the two strains were made by plating upon appropriate resistant-mutant indicator strains. Thus, although inaccuracy in the measurement of the incident energy affects comparisons between wavelengths and between replications, it does not influence the comparison of sensitivity of the two phages at a particular wavelength. Hence, any differences in action spectra observed could not be due to errors in energy measurements. Similarly, nonspecific absorption also is eliminated as a contributing factor to any possible difference in action spectra since both phages were exposed to the identical incident energy. The absorption of ultraviolet radiation by the two bacteriophages will be discussed later. Ten milliliters of the mixture of phages were irradiated in a 50 ml quartz flask rotating slowly in the monochromatic beam. The energy incident on the phage particles was corrected for the geometry.

No significant deviation from exponential inactivation was observed in the present data at any of the wavelengths until very low survival ratios were obtained. At such large doses, there was a slight "tailing off" or apparent decrease in efficiency of killing due, in all probability, to mutual reactivation (Luria, 1947) of some of the inactivated particles. Values of the inactivation dose ($E_{.37}$) or the dose for $e^{-1} = 0.37$ survival were estimated by fitting the least-squares line to the logarithm of the survival ratio and the ultraviolet dose for the apparently exponential portion of the inactivation curves. At least three replicated inactivation curves at each wavelength were obtained.

Sigmoidal survival curves, extrapolating to a value of about 1.6 for the survival ratio at zero dose, have been reported for T2 after careful studies at 2537 A (Luria, 1953, p. 153). Such sigmoidal survival curves are commonly interpreted as "multihit" curves, indicating that inactivation is not a one quantum effect. If this is the correct interpretation, the whole concept of quantum yield is invalidated.

Atwood and Norman (1949) have interpreted sigmoidal survival curves on a multiunit, single hit per unit hypothesis. In this view, the value of the extrapolate at zero dose is an estimate of the average number of units which must be inactivated to cause inactivation of the cell, and the slope of the exponential portion of the survival curve is an estimate of the rate of exponential inactivation of the individual units. This ultimate slope permits calculation of the quantum yield for inactivation of the individual units, not of the cell as a whole.

These implications of the sigmoidal survival curves reported for T2 should be remembered when considering the data presented below.

The action spectra for the National Institutes of Health data are presented in figure 1. To facilitate comparison, the efficiency of inactivation of the different wavelengths relative to the efficiency of 2650 A is plotted rather than the absolute value. The absolute values of $E_{.37}$ can be computed from the plotted relative efficiency and the values of $E_{.37}$ for 2650 A given in the legend of figure 1.

Basically, the action spectra for both T1 and T2 are similar with a broad maximum of efficiency at 2650 A and an increasing efficiency at wavelengths shorter than 2378 A. Slight differences appear between the T1 and T2 action spectra at 2804 A, 2537 A, and especially at the shortest wavelengths employed. Similar differences were observed in a completely independent

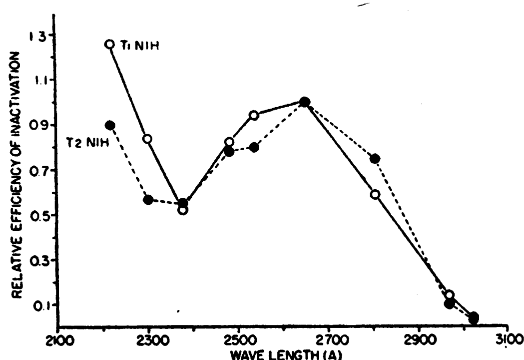

Figure 1. Ultraviolet action spectra for T1 and T2 bacteriophages. Relative efficiency $= \frac{E_{.37}2650\ A}{E_{.37}\ \lambda}$ where $E_{.37}$ is the 37 per cent survival incident dose in quanta per square centimeter. $E_{.37}2650$ A for T1 is 1.05×10^{15}; $E_{.37}2650$ A for T2 is 3.55×10^{14}.

determination of the action spectrum at six wavelengths at the Oak Ridge National Laboratory. In this replication, bacteriophage suspensions purified by differential centrifugation were irradiated, after dilution in buffer by a factor of 10^{-4}, to further reduce nonspecific absorption. The magnitude of the difference between T1 and T2 was smaller at 2537 A and especially at 2804 A, but the difference at the shortest wavelengths was even more pronounced in the Oak Ridge National Laboratory data. It is impossible to know if these differences are biologically significant, but it is of interest that similar differences are evident in the absorption spectra of the two bacteriophages.

In contrast to the T1 action spectrum published by Fluke and Pollard (1949), no decrease in efficiency was observed for wavelengths below 2300 A. Nonspecific absorption in the broth in which Fluke and Pollard dried the samples for irradiation may perhaps account for their result, for such nonspecific absorption would increase relatively rapidly at the shorter wavelengths. Since the present work did not employ any wavelengths between 2650 and 2804 A, a possible second maximum at about 2804 A suggested by Fluke and Pollard's data for T1 and by Franklin *et al.* (1953) for a *Bacillus megaterium* phage could not be detected. Comparisons of absolute sensitivity between the present data and Fluke and Pollard's results are of dubious value since their irradiated material was dried in dilute broth resulting in some nonspecific absorption.

Estimation of quantum yield. For the present study, the quantum yield (QY) is defined as the number of phage particles inactivated per quantum absorbed in viable phage particles. For any given dose, the number of particles inactivated is determined simply from the survival ratio, the initial concentration per cm^3, and the quantity of material irradiated. Computation of the number of quanta absorbed in viable phage particles is somewhat more difficult and involves the incident energy in quanta per cm^2, the absorption coefficient or absorbancy (A_p) of the phage in square centimeters per particle, the total number of particles exposed, and a correction factor to compensate for absorption in already inactivated particles. Fortunately, for a survival ratio of 0.37 ($N/N_0 = e^{-1}$), the expression simplifies to

$$QY = \frac{1}{(E_{.37})(A_p)}$$

where $E_{.37}$ is the inactivation dose (0.37 survival ratio) in quanta incident per cm^2, and A_p is the absorbancy in units of cm^2 per particle. Hence, accurate estimates of quantum yields can be made if accurate values of these two factors can be determined.

Although the values of $E_{.37}$ of the present data may not be extremely accurate, it is possible to determine such values with reasonable accuracy if one is willing to invest the work required to accumulate sufficient data to reduce the statistical errors involved.

Obtaining accurate estimates of the absorbancy, A_p for the particular wavelength, however, is not such a simple matter. An accurate value of A_p must be based on measurements of absorption made on a purified sample of the bacteriophage; that is, a sample in which there is no nonspecific absorption. Nonspecific absorption can arise from two sources: from impurities still present in the sample despite the purification procedures employed and from inviable phage particles that still absorb ultraviolet but that are unable to cause plaque formation. Such nonspecific absorption results in too large values of A_p and, hence, in too low values for the quantum yield.

A second major difficulty encountered in determining A_p is the fact that photoelectric

spectrophotometers measure not just absorption but absorption plus scattering. Since few laboratories have available the apparatus required for measuring light scattering in the ultraviolet, the usual practice, if scattering is not ignored, is to correct the absorption spectrum as measured in the spectrophotometer for scattering (see, for example, Luria *et al.*, 1951). This correction is made by extrapolating a line fitted to the logarithm of the optical density versus the logarithm of the wavelength for a spectral region where only scattering and no appreciable absorption occur, generally from about 3200 to 4000 A, to the ultraviolet region. From the extrapolated line, which theoretically should have a slope of -4, the value of the logarithm of the density due to scattering is determined for each wavelength, and the density corresponding is subtracted from the observed optical density.

Several workers have published ultraviolet absorption spectra for various T phages. The most critical approach, however, has been that of Luria *et al.* (1951) who have applied to some of the T phages a technique (Backus and Williams, 1950) for counting with the electron microscope the total number of virus particles in a purified suspension. Thus, chemical composition and light absorption can be related to the actual number of particles rather than to the number of particles capable of plaque formation. They found the ratio of infectious units to total particles to vary from about 0.4 to unity with the most frequently observed values lying near 0.5 to 0.6.

Luria *et al.* (1951) published several estimates of A_p for T2 at 2600 A, the values being corrected for scattering and related to the actual number of particles. Dr. Luria has kindly made available an absorption spectrum of a T2 preparation for which the total number of particles was known. Unfortunately, when the extrapolation for correction for scattering is made with these data, the corrected absorption for wavelengths in the 2300 A region seems improbably small, casting doubt on the validity of the extrapolation in this particular case.

So far as the authors are aware, no really good estimates of A_p for T1 and T2 at the different ultraviolet wavelengths are available in the literature. Accordingly, the values of A_p obtained from purified preparations of T1 and T2 made simultaneously by the present writers have been employed for the calculation of the quantum yields. The preparations are the best the authors have made, and their infectivity ($10^{-16.28}$ and $10^{-15.85}$ g N per infectious unit for T1 and T2, respectively) compares reasonably well with values reported in the literature (Hook *et al.*, 1946; Kozloff and Putnam, 1949; Kerby *et al.*, 1949; Putnam *et al.*, 1952). The values of A_p used are shown in table 1. Those for T2 are slightly higher than similar values estimated from Dulbecco's absorption spectrum (1950) or from Luria's preparation.

TABLE 1

Absorbancy (A_p) corrected for scattering, and quantum yield (QY) for T1 and T2 bacteriophages at different wavelengths

WAVELENGTH A	T1			T2		
	A_p (cm²/particle × 10^{-12})	QY (× 10^{-4}) NIH*	QY (× 10^{-4}) ORNL†	A_p (cm²/particle × 10^{-12})	QY (× 10^{-4}) NIH*	QY (× 10^{-4}) ORNL†
2220	2.41	4.95		9.51	2.65	
2250	2.12		4.97	8.22		1.90
2300	1.58	5.08		6.00	2.69	
2378	0.917	5.47	4.53	4.10	3.75	2.73
2483	1.13	6.88		6.34	3.49	
2537	1.39	6.41	6.24	7.52	2.99	3.29
2650	1.48	6.42	6.74	8.62	3.26	3.64
2804	0.988	5.70	6.32	5.93	3.55	3.52
2967	0.167	8.05	5.95	1.17	2.43	2.50
3022	0.0536	7.26		0.464	1.76	

* The National Institutes of Health Laboratory.

† The Oak Ridge National Laboratory.

Although Luria *et al.* (1951) mention that the correction for scattering at 2600 A is generally 10 to 20 per cent, it is often somewhat larger in the writers' experience. Furthermore, the relative size of the correction increases for both the shorter and longer wavelengths and is smallest for the wavelengths in the 2600 A region. Consequently, the extrapolation over so long an interval is a rather unsatisfactory procedure and may lead to serious errors. However, it is even less accurate to ignore the correction for scattering. Hence, the values of A_p employed in the calculations were corrected for scattering.

There is no way to adjust these values of A_p for inviable particles since the total particle count is not known. It seems doubtful, however, that the values would be incorrect for this reason by a factor much larger than two.

This rather extended discussion of the difficulty involved in obtaining accurate values of A_p has been made to focus attention on the problem and to caution against too trusting acceptance of scattering corrections based on the extrapolation procedure.

Table 1 presents the values of A_p employed in the computations and of the quantum yields obtained for the T1 and T2 phages at the National Institutes of Health and later in the partial replication at Oak Ridge National Laboratory. The quantum yield values are plotted in figure 2. In general, the estimates obtained in the two laboratories agree remarkably well. Although there are fluctuations in the values observed at the different wavelengths, there seems to be little indication of a systematic change in quantum yield over the wavelength range studied. Systematic increases in quantum yields with decreasing wavelengths have been reported for inactivation of some enzymes (see McLaren, 1949, for discussion). With both T1 and T2 bacteriophages, however, the quantal efficiency for inactivation seems constant even though the quantal energies differ by about 30 per cent for the extreme wavelengths.

Despite the fluctuation apparent at different wavelengths, it appears that the quantum yields for T1 are about twice those for T2. However, until values of A_p based on the total number of particles are available for both phages, this conclusion must be regarded as tentative since the difference in quantum yields would disappear if the number of particles per plaque forming unit were twice as high in the T2 preparation as in the T1 preparation. This does not seem highly probable. Certainly, however, the 2.5 to 3.0 times greater incident energy required for inactivation of T1 than for T2 does not appear to be a good index of the true relative resistance of these phages to ultraviolet, for, on the basis of energy absorbed, T1 actually may be the more sensitive.

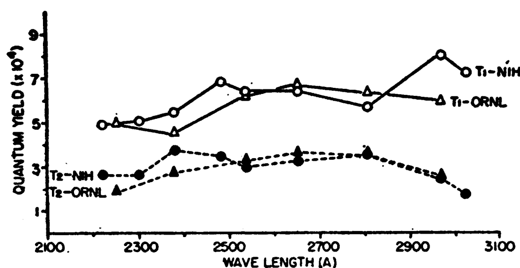

Figure 2. Quantum yields for T1 and T2 bacteriophages.

DISCUSSION

The significance of quantum yields is discussed by McLaren (1950) who demonstrates an approximate inverse relation between quantum yield and molecular weight first suggested by Uber (1941). Quantum efficiency estimates for inactivation of tobacco mosaic virus, T2 bacteriophage, and *E. coli* roughly agree with the relation that is based largely on inactivation of various enzymes. McLaren suggests that the relation could be understood if each molecule had a single sensitive volume and that the larger the molecule, the lower is the probability of absorption of a quantum in the sensitive volume. In the case of the T1 and T2 phages and bacteria with a complex morphology rather than a simple macromolecular structure, absorption in nonvital molecules could reduce the quantum yield still further. Hence, it appears that quantum yields are likely to be related inversely to chemical and biological complexity. Bearing in mind the reservations imposed by the absorption coefficients discussed earlier, it is interesting, in this respect, that T1 apparently exhibits a higher quantum yield than T2 which on the basis of multiplicity reactivation, genetic recombination, and internal morphology appears to be a more complex and differentiated entity than T1.

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SUMMARY

Action spectra of the nucleic acid type were obtained for the inactivation of coliphages T1 and T2 by monochromatic ultraviolet light of wavelengths between 2220 and 3022 A.

Data regarding the quantum efficiency of the inactivation of the two bacteriophages are presented. The data show that the quantum yield is essentially constant over the range of wavelengths studied. Higher quantum yields are obtained with T1.

The possible significance of the observations is discussed briefly. Certain difficulties encoun-

tered in the accurate determination of quantum yield are pointed out.

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