THE ULTRAVIOLET LIGHT INACTIVATION OF ΦX174 BACTERIOPHAGE AT DIFFERENT WAVE LENGTHS AND PH'S

RICHARD SETLOW *and* RICHARD BOYCE

From the Biophysics Department, Yale University, New Haven

ABSTRACT The bacterial virus, ΦX174, which contains a single strand of DNA has been inactivated by different wave lengths of monochromatic ultraviolet light at pH 7, 2, and 12. The action spectra for inactivation at these three pH's all showed minima at 2400 A rather than at 2300 A, which is the characteristic absorption minimum of DNA. The shapes of the action spectra have been analyzed in terms of the effects of absorbed light on the pyrimidines and purines rather than the effect on nucleoprotein. In this interpretation the pyrimidines are *at least* 2 to 3 times more sensitive than the purines. The quantum yield for inactivation of the virus at 2650 A and pH 7 is 0.006. The quantum efficiency for quanta absorbed in the pyrimidines is 0.0085 and for the purines 0.0035. It is pointed out that action spectra for single- and double-stranded polynucleotides should have minima at different wave lengths, and that this difference may be used to distinguish between these two configurations *in vivo*.

The small bacterial virus ΦX174 contains a DNA polynucleotide which is single-stranded (1). It will be useful to know the relations and differences between the physical properties of single- and double-stranded DNA. Such differences are not only of interest in their own right, but also may provide physical means for distinguishing between single- and double-stranded DNA *in vivo*. The effects of radiation on a single-stranded DNA may be simpler to analyze than those on the double-stranded configuration because of the smaller likelihood of energy migration up and down the single-stranded chain. It should be possible, by analyzing the effects of different wave lengths of ultraviolet (UV) light on ΦX174 irradiated at different pH's, to assess the relative importance, in the inactivation process, of quanta which are absorbed in the various bases of DNA. The data to be presented on the inactivation of ΦX174 may explain the action spectra obtained for more complex biological systems.

This work was assisted in part by a grant from the United States Public Health Service No. 1285-C3.

Received for publication, April 28, 1960.

Studies of the effects of UV light on biologically inactive polynucleotides have indicated that the pyrimidines are altered more rapidly than the purines (2, 3). One might expect, therefore, that for single-stranded DNA the action spectrum for the destruction of biological activity would follow the absorption spectrum of the pyrimidines rather than the absorption spectrum of the purines plus pyrimidines. This seems to be the case for ΦX174. By changing the pH of the medium it is possible to make large changes in the absorption spectra of thymidine and cytosine deoxyriboside. From the changes in the action and absorption spectra with pH it is possible to assess the relative importance of these two bases in the inactivation process. The resolution of the action spectrum for ΦX174 into the sum of component bases is similar in principle to the resolution of the action spectrum for the inactivation of enzymes into the sum of two parts, one representing an effect on the amino acid cystine and the other on the aromatic groupings in the molecules (4).

The differences between the action spectra for single-stranded DNA and double-stranded DNA may make it possible to distinguish between biological processes which take place *via* the single- *versus* the double-stranded configuration (5). Such differences in action spectra have been observed in the inactivation of complexes of bacteriophage T2 and *Escherichia coli* at various times in the latent period and have been interpreted in terms of a change in the configuration of viral DNA from the double- to single-stranded configuration (6).

EXPERIMENTAL PROCEDURE

The virus ΦX174 and the sensitive host cells (*E. coli strain* C) were obtained from I. Tessman. In the work reported here, the virus used for irradiation was obtained from a single lysate inoculated with virus from a single plaque. The general techniques of assaying and handling the virus are described by Zahler (7). Dilutions, after irradiation, were made into Difco nutrient broth. Plates were incubated at 37°C in the dark.

The virus for irradiation was diluted over 500 times from the lysate medium (tryptone, glucose, yeast extract) into the appropriate buffer, and, as a result, the absorption of the irradiated samples was less than 20 per cent. The initial virus titer for irradiations was between 10^7 and 10^8 plaque-forming units per ml. Three irradiation media were used: pH 7, obtained with 0.07 M phosphate buffer; pH 2, obtained with HCl-KCL buffer; and pH 12, obtained with 0.01 M NaOH. Control samples of the virus were not inactivated in these media for times equal to the duration of an experiment.

Monochromatic light was obtained from a large water prism monochromator (8). The intensity at each wave length was measured with a photocell which had been calibrated in absolute units. The average intensity throughout the sample, which was irradiated in a 1.0 cm light-path quartz cell kept at 15°C, was obtained from the incident intensity by using the corrections calculated by Morowitz (9). During irradiation the virus suspension was stirred with a small magnetic stirrer. Intensities ranged from 3 ergs/(mm² sec.) at 2650 A to 0.1 erg/(mm² sec.) at 2262 A. Irradiation times ranged from a total of 5 minutes at the high intensities to 40 minutes at the low intensities.

The inactivation of the virus depended only on the product of the intensity and the

time for intensities, at 2650 A, which varied over a factor of 20. There was no observed recovery of the virus on standing 18 hours at 4°C at pH 2. (Such a recovery might be expected from the fact that the UV photolysis of cytosine shows a rapid reversible effect at low pH's (10).)

RESULTS AND DISCUSSION

Action Spectrum at pH 7. Typical data for the inactivation of ΦX174 at pH7 are shown in Fig. 1. It is seen that the relative survival, over a factor of 10^4, decreases exponentially with the incident dose of radiation. There is no evidence for a slight shoulder at low doses, as is found for T2 bacteriophage, or for a decrease in sensitivity at high doses as found for T1 bacteriophage (11). This fact

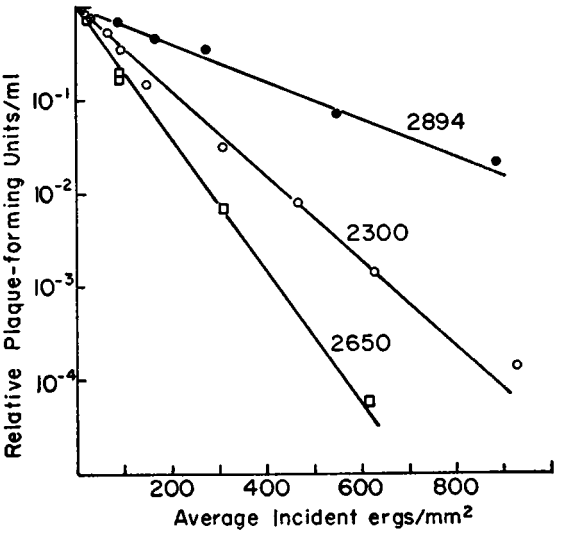

FIGURE 1 Dose-survival curves for ΦX174 at pH 7 for several different UV wave lengths.

makes it a relatively simple matter to obtain precise values for the sensitivity of the virus to UV inactivation. The data shown in Fig. 1 may be represented by the expression

$$n/n_0 = e^{-\sigma D}$$

where n/n_0 represents the relative survival of the virus, D the incident radiation in quanta/cm², and σ the sensitivity parameter which, because it has the dimensions of cm²/quantum, will be called the inactivation cross-section. The inactivation cross-section may be found from the slopes of the curves shown in Fig. 1 after the data have been converted to quanta/cm². The values of σ obtained in this way are precise. For example, four inactivation runs at 2650 A similar to that shown in Fig. 1

gave a mean value of σ for which the probable error of the mean was 0.7 per cent. The fewer repeat runs which were carried out at other wave lengths indicated probable errors of less than 5 per cent. In this experiment, because of the difficulties of determining absolute intensities over a wide wave length range, the biological error appears to be less than that associated with the physical measurements.

A graph of the inactivation cross-section *versus* wave length is the action spectrum. Fig. 2*a* shows the action spectrum for the inactivation of ΦX174 irradiated at

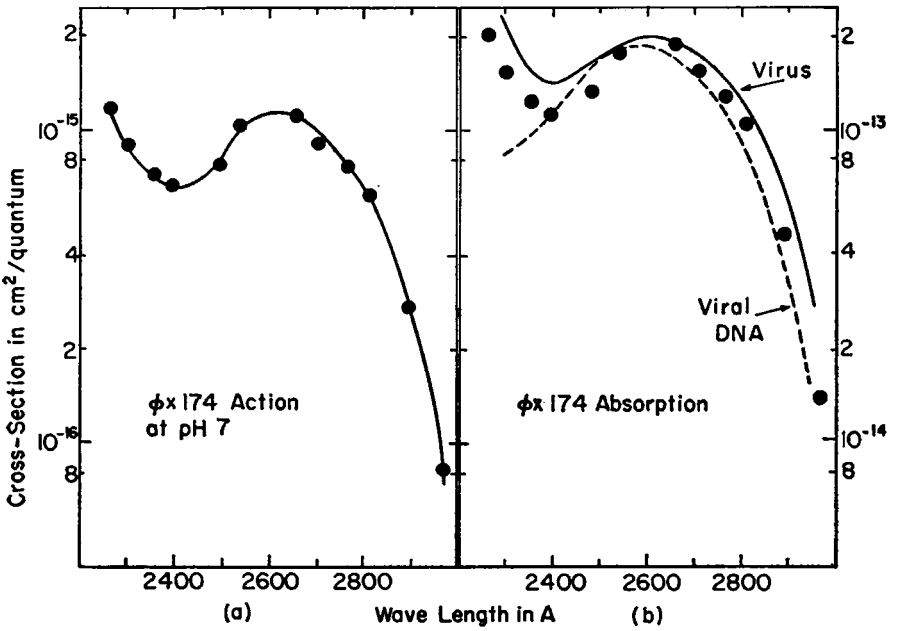

FIGURE 2*a* The action spectrum for inactivation of ΦX174 at pH 7. FIGURE 2*b* The absorption spectrum of the virus and of viral DNA from reference (1). The *relative* action spectrum, shown by the solid points, has been shifted so as to coincide with the absorption spectrum at 2650 A.

pH 7. Each point in this spectrum is obtained from an inactivation curve similar to the curves shown in Fig. 1. Fig. 2*b* shows, for comparison, the absorption spectrum of the virus, and of the viral DNA recalculated from data obtained by Sinsheimer (1). The absorption coefficient has been expressed in terms of the absorption cross-section per molecule, *s*, which is defined from the equation

$$I/I_0 = e^{-nsx}$$

where I/I_0 is the relative light transmission through a sample of thickness x with n units/cm³. In Fig. 2*b*, the action spectrum has been shifted so as to coincide with the virus absorption spectrum at 2650 A. The efficiency of *absorbed* photons in inactivating the virus may be expressed in terms of the quantum yield—the number

of virus particles inactivated per quantum absorbed. It may be found from the ratio of the inactivation cross-section σ, to the absorption cross-section (4):

$$\phi = \sigma/s. \tag{1}$$

On the logarithmic scale shown in Fig. 2 the action and absorption spectra are *not* parallel. Therefore the quantum yield at pH 7 is *not* independent of wave length but varies as shown in Fig. 3. At 2650 A its value is 0.006, which means that one out of 170 absorbed quanta inactivates the virus. This is a high efficiency compared to the efficiencies obtained for larger viruses T2 and T1 bacteriophages which have quantum yields of 3×10^{-4} and 6×10^{-4} respectively (12).

Of more interest than the absolute values of quantum yields is the fact that the action spectrum does not have the same shape as the absorption spectrum of either

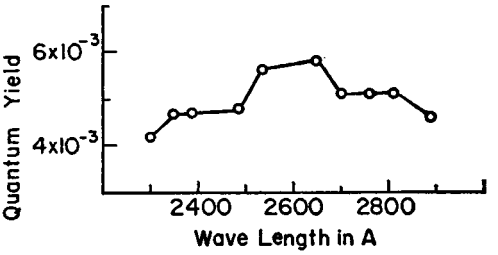

FIGURE 3 The quantum yield as a function of wave length at pH 7.

the virus or the viral DNA. The quantum yield is not constant if computed, as in Fig. 3, on basis of quanta absorbed in the virus or, as may be seen from Fig. 2, if computed on the basis of quanta absorbed in the nucleic acid portion of the virus. There are two possible extreme interpretations of the variation in ϕ with wave length. One is that both the protein and nucleic acid parts of the virus are sensitive to ultraviolet light and by choosing the sensitivity of these components appropriately we can obtain the observed action spectrum. Against this interpretation are the facts that all enzymatic proteins need much larger incident doses of radiation to affect them (13) than are used to inactivate the virus, and that there is no evidence for energy transfer from proteins to nucleic acids (14). Moreover, while the increase in virus sensitivity at wave lengths below 2400 A may be explained in terms of light absorbed in protein, the decrease at 2480 and 2400 A is inexplicable in these terms. If, therefore, we reject the possibility that the action spectrum for inactivation of ΦX174 is nucleoprotein in nature, we are left with the second possibility—a description of the action spectrum in terms of the sum of effects on the different bases which make up the DNA of ΦX174.

Analysis in Terms of the Effects on Individual Bases. It is reasonable to assume, from the results of the irradiation of free bases and polynucleotides (ably reviewed in reference 3), that the pyrimidines are the most sensitive units. Therefore, we shall attempt to describe the action spectrum in terms of a sum of light-

action on the cytosine and thymine components. There exist conceptual difficulties to this analysis. First, it is known that the effects of light on the free bases are wave length–dependent. Effects produced by longer wave lengths are at least partially reversible and those produced by wave lengths below 2300 A are irreversible (15). The relative magnitudes of these two wave length sensitivities are not known and we do not know how to evaluate the different rates of the reverse reactions in irradiated cytosine and thymine (16). Second, if we wish to analyze the spectrum in terms of the individual bases, we must assume, in the absence of more detailed information, that the bases act as independent absorbers. This is only approximately true for ΦX174-DNA because the absorption spectrum of the extracted DNA changes with the configuration of the molecule (1). The increase in extinction upon hydrolysis is, however, only 11 per cent compared to the increase of 30 to 35 per cent observed for double-stranded DNA.

We list below the explicit assumptions we shall make in order to arrive at a simple description of the effects of ultraviolet light on ΦX174. As we indicated above, these assumptions are known to be only approximately true where data relating to them are available. Therefore, it should not be suprising if the analysis yields only approximate results.

1. The bases are independent of one another and of the protein shell.

2. The quantum yield for affecting an individual base is independent of wave length.

3. The quantum yield for affecting a base at a particular wave length is independent of pH. (See reference 3 for evidence against this assumption.)

4. The ionization of the bases inside the virus is the same as if they were in the external solution.

As the pH is decreased from 12 to 2, the pyrimidine but *not* the purine nucleotides show big changes in absorption spectra (17). In going from pH 7 to 2 the absorption spectrum of cytosine changes, whereas that of thymidine does not. And the absorption spectrum of thymidine changes if the pH is raised from 7 to 12, wheras cytosine shows only a minor change. At this high pH guanosine's absorption spectrum changes slightly, but we shall ignore this change. (We note in passing that the changes in the guanosine absorption, an increase at 2700 A and a decrease between 2600 and 2300 A, do *not* agree with the changes in the action spectrum.) Fig. 4 shows the spectra of the two pyrimidines at the pH's we are interested in. The molar ratio of thymidine to cytosine in ΦX174 is 1.6 to 1 (1). Fig. 5 shows the computed absorption spectrum of a mixture of thymidine and cytosine in these ratios at the three pH's of interest. We would expect, if our limiting assumptions held, and if the pyrimidines are the most sensitive bases, that the action spectra of the virus at these three pH's would look like the absorption spectra shown in Fig. 5. At the beginning of the analysis it is worth noting that at pH 7, as a comparison of Figs. 2 and 5 shows, the action spectrum for inactivation of the virus does *not* look like the sum of thymine plus cytosine absorption. It shows a disproportionately

higher sensitivity at wave lengths below 2400 A which may be due to the increase in quantum yield of the individual bases at these wave lengths.

Action Spectra at pH 2 and pH 12. Fig. 6 shows the action spectra, determined from data similar to those shown in Fig. 1, for the inactivation of ΦX174 at pH 2, 7, and 12. The changes in the action spectra from the values at pH 7 are not drastic but, because of the high precision of the data, the differences in the spectra are significant. Thus, for example, at pH 2, cytosine's absorption coefficient at 2400 A decreases drastically and we find that the UV sensitivity has also de-

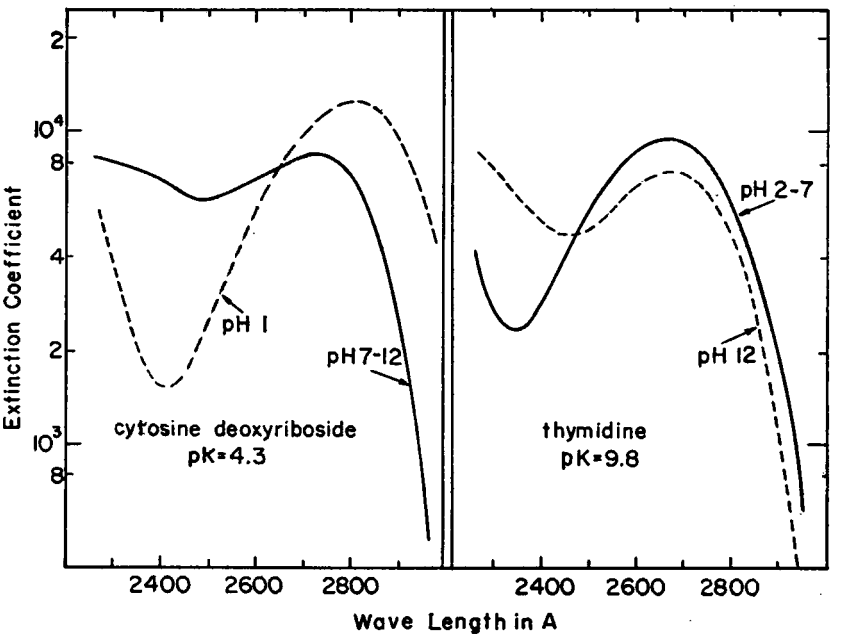

FIGURE 4 The spectra of cytosine deoxyriboside at pH 7 and 1, and the spectra of thymidine at pH 7 and 12 from reference (17). (The data at pH 1 were taken from reference 17 which reports negligible change between pH 1 and pH 2.)

creased markedly. Moreover, in agreement with the absorption spectrum at long wave lengths, the wave length of maximum sensitivity has shifted from 2600 to 2700 A. At pH 12, the UV sensitivity has increased at the lowest wave lengths, as expected qualitatively from the change in the thymidine spectrum, and is decreased significantly in the intermediate spectral range, around 2600 A. The explanation for the slightly higher sensitivity at wave lengths above 2900 A is not available. Qualitatively the changes in the action spectra parallel the changes in the absorption spectra of cytosine and thymidine. However, we would expect the changes in the action spectra to be similar to those of the absorption spectra even if the pyrimidines were not the most sensitive bases. The differences in the shapes of action and absorption spectra at pH 7 (see Fig. 2) and the *quantitative* argument given below

indicate that the pyrimidines are the important bases to consider in the absorption of light which leads to UV inactivation.

The Contributions of Pyrimidines and Purines. The relative contribution to the inactivation process of quanta absorbed in cytosine, thymine, guanine, and adenine may be found in the following way. Assume that the inactivation cross-section of the virus is the sum of the inactivation cross-sections for light absorbed in the individual bases.

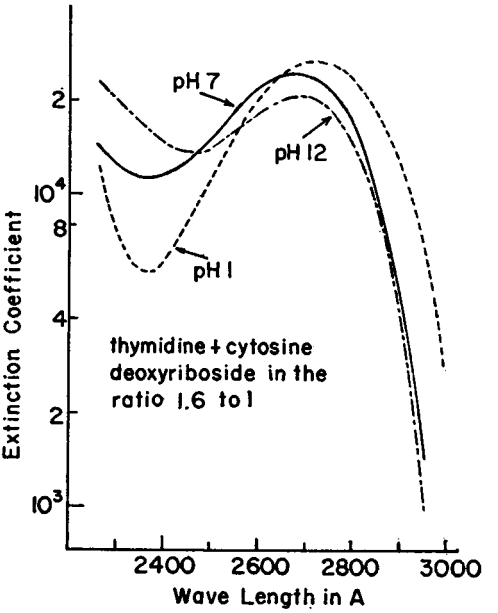

FIGURE 5 The computed absorption spectra, at pH 1, 7, and 12, of thymidine plus cytosine deoxyriboside in the molar ratio of 1.6 to 1.

$$\sigma = \sigma_C + \sigma_T + \sigma_G + \sigma_A \,. \tag{2}$$

The subscripts stands for the four bases. We use equation (1) to rewrite (2) in terms of the absorption cross-sections of the individual bases s, the number of bases n, and the quantum yield ϕ for inactivation by light absorbed in a particular type of base.

$$\sigma = \phi_C n_C s_C + \phi_T n_T s_T + \phi_G n_G s_G + \phi_A n_A s_A \,. \tag{3}$$

In so far as the absorption coefficient of the viral DNA is the sum of the coefficients of the four bases, this is a valid relation. We assume that the only parameters in equation (3) which are pH dependent are the absorption and inactivation cross-sections. If $\Delta\sigma_{7-2}$ and $\Delta\sigma_{7-12}$ represent the changes in inactivation cross-sections for the pH changes 7 to 2 and 7 to 12,

$$\Delta\sigma_{7-2} = \phi_C n_C \, \Delta s_C \,, \tag{4}$$

and

$$\Delta\sigma_{7-12} = \phi_T n_T \Delta s_T \,. \tag{5}$$

We can measure Δs_C, Δs_T, and the corresponding values of $\Delta\sigma$ and, from a knowledge of n_C and n_T we may compute ϕ_C and ϕ_T. The values of ϕ_C and ϕ_T found in this way may be substituted back into (3) so as to find $(\phi_G + \phi_A)$. Since the changes in absorption spectra of the bases inside the virus will be *less* than in solution, the values of ϕ_C and ϕ_T which we calculate will be *minimum* values.

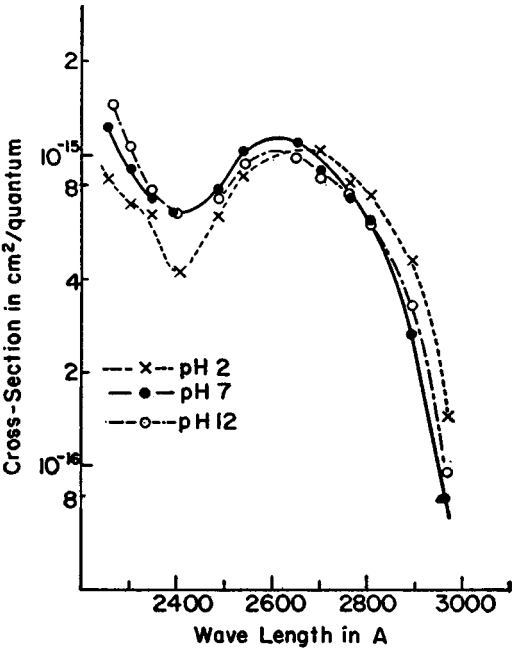

FIGURE 6 The action spectra for the inactivation of ΦX174 at pH 2, 7, and 12.

The assumption that ϕ_C *and* ϕ_T are pH-independent is verified in the following way. In changing the pH from 7 to 2 the absorption spectrum of cytosine does not change at 2650 A (see Fig. 4). If ϕ is pH-independent, this wave length should show the same inactivation cross-sections at pH 7 and 2. Actually the wave length of constant σ is 2680 A. For the change from pH 7 to 12 we would expect, from the thymidine spectrum, similar values of σ at 2470 A. The wave length of constant σ is found near 2400 A.

The numerical values that shall be substituted into equations (3, 4, 5) are obtained from Sinsheimer's (1) data on the base composition of ΦX174 and from the absorption and action spectra shown in Figs. 4 and 6. The data and derived values of ϕ_C and ϕ_T are shown in Tables I and II. The computed absorption cross-section

TABLE I

DATA USED FOR THE CALCULATION OF ΦX174 ABSORPTION AT 2650 A, pH 7

Base	No. of bases	Absorption per base	Absorption by all bases
Cytosine	970	3.2×10^{-17} cm²	3.1×10^{-14} cm²
Thymine	1550	3.7	5.7
Guanine	1250	3.8	4.8
Adenine	1230	4.6	5.6
		Sum	19.2×10^{-14}

is within 5 per cent of the observed value for viral DNA shown in Fig. 2*b*. It should be clear from an inspection of Fig. 6 that the values of $\Delta\sigma$ for thymidine, shown in Table II B, are subject to large experimental errors, perhaps as large as 50 per cent, because the changes between the action spectra at pH 7 and 12 are small. The average value for the quantum yield for the inactivation of the virus by the quanta absorbed in the two pyrimidines is 0.0085. If we assume this value is the actual value for thymine and cytosine we may use it to obtain, from equation (3), an estimate for the quantum yield for quanta absorbed in adenine and guanine. There is no practical way to determine the effects on the bases separately so we assume, in addition, that the quantum yields for inactivation by absorption in the two purines are the same and are wave length- and pH-independent. Equation (3) takes the form

$$\sigma = 0.0085(n_C s_C + n_T s_T) + \phi_{G,A}(n_G s_G + n_A s_A).$$

At pH 7 and 2650 A the value of σ, 1.1×10^{-15} cm², and the other numbers shown in Table I yield $\phi_{G,A} = 0.0034$. If similar computations, using the proper values of absorption and action cross-sections, are made at other wave lengths, we obtain at

TABLE II

DATA USED TO CALCULATE ϕ_C AND ϕ_T

A. pH 7–2

λ	Δs_C	$\Delta\sigma_{7-2}$	ϕ_C Calculated from (4)
2400 A	2×10^{-17} cm²	2.3×10^{-16} cm²	0.012
2900	3×10^{-17}	1.9×10^{-16}	0.006

B. pH 7–12

λ	Δs_T	$\Delta\sigma_{7-12}$	ϕ_T Calculated from (5)
2300 A	1.9×10^{-17} cm²	1.7×10^{-16} cm²	0.006
2650	0.8×10^{-17}	1.2×10^{-16}	0.010
		Average of ϕ_C and ϕ_T	0.0085

2480 A, $\phi_{G,A} = 0.0038$ and at 2400 A, $\phi_{G,A} = 0.0042$. The facts that the absorption spectrum of guanosine changes in the pH range 7 to 12 and that ϕ_G is not negligible do *not* affect equation (5) because the spectral changes are very small at 2300 and 2650 A. Because the value 0.0085, which represents ϕ_C and ϕ_T in the above equation, is a minimum value, the computed $\phi_{G,A}$ represents a *maximum.*

The values of $\phi_{G,A}$ are very sensitive to the value used for the quantum yield for cytosine and thymidine. If, for example, we had used a value of 0.012 for ϕ_C and ϕ_T, we would have obtained for $\phi_{G,A}$ a value of 3×10^{-4} at 2650 A. These calculations indicate that quanta absorbed in the pyrimidines have at least a 2 to 3 times greater chance of inactivating the virus than those absorbed in the purines. This result is the quantitative foundation for the statement that the action spectra for single-stranded polynucleotides should differ from double-stranded ones because in the later the non-localization of absorbed energy means that *all* absorbed quanta have equal inactivation probabilities.

Quantum yields for the spectral alteration of the free nucleotides are about the same as the numbers for affecting the bases of ΦX174. They range from 0.01 for cytosine, to about 0.0001 for the purines (3). The value for the alteration of thymidine is 0.001 but its interpretation is complicated by the fact that in solution there is a rapid reversible reaction which may not occur in the virus. A value of $\phi_{G,A}$ for the virus which is greater than values found for free bases indicates, in our method of interpretation, that the bases are really *not* completely independent and that a quantum absorbed in a purine has a non-zero probability of affecting a pyrimidine.

The alternative explanation for the shape of the action spectra, which we discarded above, namely that the spectra are the sum of action on nucleic acid and protein, does not stand up to quantitative examination. For example, the spectrum of tyrosine (18) changes drastically in going from pH 7 to 12. There is a big increase in absorption at 2900 A and a maximum absorption at 2600 A. These changes do not show up in the action spectrum of the virus. The spectral changes of tryptophan are somewhat similar to those found in the action spectra in going from pH 7 to 12, but available data on the quantum yield for an inactivation of structures by absorption of light in tryptophan (4) indicate that the quantum yield is about 0.002. This is too low an efficiency to produce the effects observed here. The spectrum of neither tyrosine nor tryptophan changes much on acidification and thus it cannot account for the observed change in the action spectrum in going from pH 7 to 2. It looks as if the only simple explanation for the action spectra for inactivation of ΦX174 is that most of the effect of the absorbed light takes place owing to quanta absorbed in the two pyrimidines and that the intrinsic sensitivity of the pyrimidines increases at short wave lengths.

If, contrary to the above analysis, the proper interpretation of the action spectra is in terms of the effect of light on nucleoprotein, one would still expect single-

stranded polynucleotides to have minima at longer wave lengths than those of double-stranded DNA. Any energy transfer or reaction between proteins, which have absorbed light between 2300 and 2400 A, and nucleic acid polymers should be more efficient with the more intimate contact between protein and single-stranded structures. This indirect mechanism may be the explanation for the shape of the action spectrum of tobacco mosaic virus (19). It is improbable that a direct effect of ultraviolet light on viral proteins would affect virus survival because, for example, in the T-even bacterial viruses the majority of the viral protein does not enter the host (20) and in the plant viruses the protein is made up of many identical subunits (21), any one of which is probably dispensable.

CONCLUSION

The action spectra for the inactivation of ΦX174 at three pH's have been analyzed in terms of contributions of the four bases—cytosine, thymine, guanine, and adenine. The action spectra all show minima at 2400 A rather than the characteristic absorption spectrum minimum of DNA at 2300 A. The action spectra, which heretofore have been interpreted in terms of the effects of light on nucleoprotein, are simpler to interpret in terms of the preferential action of light on cytosine and thymine. If this interpretation is correct, action spectra offer a convenient experimental way of distinguishing biological actions which take place *via* single- or double-stranded structures. Single-stranded structures should have action spectra minima at 2400 A; double-stranded DNA should have a minimum closer to 2300 A.

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