ULTRAVIOLET SENSITIVITY OF THE BIOLOGICAL ACTIVITY OF ΦX174 VIRUS, SINGLE-STRANDED DNA, AND RF DNA

MICHAEL YARUS *and* ROBERT L. SINSHEIMER

From the Division of Biology, California Institute of Technology, Pasadena, California. Dr. Yarus's present address is Department of Biochemistry, Stanford School of Medicine, Palo Alto, California.

ABSTRACT Action spectra for inactivation of ϕX virus, free ϕX single-stranded DNA, and double-stranded ϕX DNA (RF) have been measured using light of wavelength 225–302 mμ. The sensitivity of RF has been determined using bacterial hosts both capable and incapable of reactivation of UV damage. The inactivation of ϕX virus is due, at all wavelengths, to damage to its DNA; it appears that, below 240 mμ, energy absorbed by viral structural protein may inactivate the viral DNA. The variation of the probability of inactivation by an absorbed quantum (quantum yield) with wavelength, in the case of free-single-stranded ϕX DNA, suggests that energy absorbed by pyrimidine residues is more likely to yield inactivation than absorption by purines. This implies that energy transfer is not so extensive as to make all absorbed energy available to pyrimidines.

INTRODUCTION

ϕX174 is a minute bacteriophage which attacks *Escherichia coli*. The virus particle contains one molecule of single-stranded DNA (Sinsheimer, 1959 *a*, *b*) which, when extracted from the virus and purified, is infective to bacteria previously converted to spheroplasts by means of lysozyme and versene (Guthrie and Sinsheimer, 1960; Guthrie and Sinsheimer, 1963; Hofschneider, 1960; Sekiguchi, Taketo, and Takagi, 1960; Wahl, Huppert, and Emerique-Blum, 1960); the DNA is therefore the complete phage genome. This infective viral DNA is a ring (Fiers and Sinsheimer, 1962; Freifelder, Kleinschmidt, and Sinsheimer, 1964) containing about 5500 nucleotide residues (Sinsheimer, 1959 *b*). On entering its host, the viral single strand is transformed into a double-stranded structure, called RF, which is also infective to bacterial spheroplasts (Sinsheimer, Starman, Nagler, and Guthrie, 1962) and is, like the parental single strand, a ring (Chandler, Hayashi, Hayashi, and Spiegelman, 1964; Kleinschmidt, Burton and Sinsheimer, 1963), apparently formed by addition of a closed, complementary strand to the viral DNA ring.

The availability of a relatively small, homogeneous, infective, single-stranded, well-characterized viral DNA and the same DNA in a double-stranded form makes this system an attractive one for the study of the effects of ultraviolet (UV) radiation on nucleic acid. Presented here are the results of measurements of the sensitivity of the infectivity of the ϕX virus, of the single-stranded DNA (SS), and of the RF to inactivation by monochromatic ultraviolet light at several wavelengths. We know of no other such data on a homogeneous DNA free in solution.

MATERIALS AND METHODS

Irradiation

Irradiations were performed in a 1 × 1 × 4 cm quartz cell in the Young-Thollen double prism monochromator described in Winkler, Johns, and Kellenberger (1962). Techniques were generally as described by these authors except that the slit widths used in this work were characteristically smaller and thereby the band of wavelengths passed narrower. The experiments were done in a room whose temperature was controlled at 22°C and DNA samples were allowed to come to this temperature before irradiation. Since the DNA solutions were vigorously stirred by a quartz propellor during an experiment, suitable control experiments were performed which showed that the processes of handling and stirring the samples did not cause significant inactivation.

The solvent for most inactivation studies was 0.05 M Tris buffer, pH 7.5 or 8.1. Some experiments were performed with single-stranded ϕX DNA in high salt concentration; for these experiments the medium was 0.05 M Tris plus the desired molarity of NaCl. The presence of versene at 10^{-3} M in either type of solvent did not change the sensitivity of SS.

The energy reaching the solution in the cell was measured with a photocell and photometer previously calibrated by comparison with a thermopile and also by comparison to the rate of bleaching of malachite green leucocyanide (Calver and Rechen, 1952) exposed to the UV beam. Readings were taken with the photocell placed behind the sample position before and after each irradiation and the two measurements averaged to get the true dose rate. Corrections were commonly applied for the absorption of light by the sample (Morowitz, 1950) and for volumes withdrawn in sampling.

Values for the ultraviolet sensitivity (cross-section) are derived from a least squares line fitted to the logarithm of the observed fractional, surviving infectivities with the aid of an IBM 7094 computer. The points on the action spectra (Fig. 1) represent two to six full inactivation curves at each wavelength, except for the several points obtained by assay of irradiated RF on *E. coli* K12 AB1886, each of which is derived from one inactivation curve.

Virus and DNA

The ϕX virus used was isolated and assayed as by Sinsheimer (1959 *a*).

Single-stranded DNA was obtained from preparations of ϕX virus by the Ca^{++} or phenol procedures (Guthrie and Sinsheimer, 1960). RF was typically obtained from drop collection of an equilibrium CsCl density gradient into which had been put a phenol-treated lysate of infected cells. Usually these lysates were of cells in which mature virus synthesis had been inhibited with chloramphenicol. RF preparations isolated without phenol treatment and preparations purified from lysates by selective precipitation and column chromatography (Burton and Sinsheimer, 1965) have also been used and found to have the same cross-sections.

In most determinations reported here, no attempt was made to separate the RF from host

DNA, so the major DNA species in solution was *E. coli* DNA. No role in the inactivation or assay is attributed to this DNA because experiments at different DNA concentrations gave consistent cross-sections.

To isolate DNA from irradiated phage, extractions were performed by the phenol procedure detailed by Guthrie and Sinsheimer (1963), with the following changes. The phage samples contained 10^{10}–10^{11} PFU/ml in 0.05 M Tris, pH 8.1. After irradiation, BSA (Armour Pharmaceutical Co., Kankakee, Ill., fraction V) and denatured calf thymus DNA (Sigma Chemical Co., St. Louis, Mo.) carrier (dissolved in the same solvent and heated to 100° for 10 min) were added to final concentrations of 6 and 1 mg/ml respectively. Sometimes a mutant of ϕX which could be plated selectively was added to each tube at this point to serve as a control on recovery; the recovery of infectivity from this virus was the same in all samples. One extraction with phenol was performed and the separated phenol phase was washed with saturated sodium borate and this was added to the first aqueous phase, before extraction with ether.

Infectivity Assay

Assays of DNA were performed by the spheroplast procedures described by Guthrie and Sinsheimer (1960, 1963); the later procedure was employed particularly in the determination of the action spectra for single-stranded DNA. Release of phage from infected spheroplasts is characteristically slow and incomplete (Guthrie and Sinsheimer, 1960), so osmotic shock or freezing and thawing or both were used to free the phage still bound in spheroplasts at the end of the assay. In all experiments, a series of assays at several dilutions was made with the unirradiated DNA sample being used and in all cases the assay was linear, i.e., the phage output was proportional to the input concentration of DNA. The concentrations of infective single-stranded and RF DNA used in the irradiation cell and in the assays were in the range 10^6–10^9 particles/ml as judged by comparison to a reference preparation of viral SS DNA. The phage yield/input DNA particle varied from 0.1 to 1.0 in different experiments. The cells converted to spheroplasts when reactivation of UV damage was desired (HCR^+ cells) were *E. coli* K12W6 obtained from Dr. Jean Weigle, or when it was desired to minimize reactivation (HCR^- cells), *E. coli* K12 AB1886 obtained from Dr. Paul Howard-Flanders.

It was established that the infectivity of irradiated DNA samples of both kinds (SS and RF) decreased linearly upon dilution exactly as did an unirradiated standard, i.e., irradiated samples behaved simply as populations of molecules which had reduced numbers of infective particles, without interference from damaged molecules. Therefore, the assay used in routine inactivation experiments was not a complete dilution curve, but an assay in duplicate or triplicate of the sample from the irradiation cuvette. Assays were carried out in dim light to minimize possible photoreactivation.

In the spheroplast assay as usually performed the quantity determined and used as a measure of the input DNA is the phage yield obtained after incubation of spheroplasts with the DNA. It seemed possible that the inactivation of a DNA molecule by ultraviolet radiation might not be an all-or-none effect, reflected simply in the phage yield, but that there might be sublethal damage which decreased the burst size but did not abolish phage production entirely. Therefore, comparisons were made, using irradiated DNA, of the reduction of the ability to initiate an infective center (spheroplast) and of the reduction of final phage production. Dilution and soft-agar assays of infected spheroplasts were perfromed by methods described previously (Guthrie and Sinsheimer, 1963). These experiments showed that, using either SS or RF, these two functions have the same sensitivity to UV.

These results, taken together with the single-hit kinetics of inactivation, mean that a com-

plete loss of the ability to complete the infective process and release phage occurs as a result of a single photochemical alteration.

A comparison of the growth curves of irradiated and unirradiated ϕX virus in cultures synchronized by starvation (Denhardt and Sinsheimer, 1965) has also been made: the number of infective centers obtained upon infection at low multiplicity is that expected from survival as measured in the plaque assay, and the time at which there is one phage per cell, the time of lysis, the rate of phage synthesis, and the burst size are all, within experimental variation, the same for the survivors of heavy irradiation (eight to nine hits at 260 mμ) as for an unirradiated population. Apparently, as for free DNA, few, if any, sublethal damages result from UV irradiation of ϕX phage. This is in contrast to the situation in T phages, where the survivors of UV irradiation are observed to have extended latent periods (Luria, 1944; Setlow, Robbins, and Pollard, 1955).

Absorption Spectra

Absorption spectra presented here as "corrected for scattering" were obtained with the Beckman DU spectrophotometer and corrected by extrapolation from wavelengths where there was no absorption, using the procedure suggested by Dulbecco (1950). An exponential variation of absorbance, due to scattering, with wavelength above 310 mμ was observed in all cases, with an exponent of approximately (minus) 4.0. Spectra of the DNA were taken on the Beckman DK-2 recording spectrophotometer; no scattering correction was necessary.

The spectrum for ϕX protein presented in Fig. 3 was obtained as follows: the 70S-light component (Carusi and Sinsheimer, 1963) from several preparative RbCl equilibrium density gradients was centrifuged to equilibrium (ρ = 1.31) twice in the RbCl (Sinsheimer, 1959 *a*) density gradient in the presence of saturated sodium tetraborate. After the first centrifugation only the material lighter than "70S-heavy" component (Carusi and Sinsheimer, 1963) was selected for recentrifugation by entering through the side of the gradient tube with a syringe and needle. After the second centrifugation the sharp band called "70S light" was collected in the same way. The 70S-light fractions were pooled and dialyzed into 1/20 saturated sodium tetraborate. The volume of these fractions was reduced to 0.2 ml by evaporation under vacuum and then the concentrated sample was layered over a 5–20% sucrose gradient containing saturated borate plus 10^{-3} M EDTA. After centrifugation for 110 min at 38,000 RPM and 5°C in the SW39 head of the model L ultracentrifuge, drops were collected and a single symmetrical peak at a calculated S = 66 was found. Those fractions containing material of $s_{20,w}$ = 50–75 Svedbergs were pooled and dialyzed exhaustively against deionized distilled water. A sample was then diluted volumetrically into 0.05 M Tris, pH 8.1 for the spectrum. Absolute absorption cross-sections per particle are based on 4.5×10^6 avograms of coat protein/virus and a specific absorptivity of 1.3/mg/ml protein at 275 mμ. This absorptivity is that of the 5S coat subunit of Carusi and Sinsheimer (1963).

RESULTS

Data consistent with simple (Sinsheimer, Starman, Nagler, and Guthrie, 1962) exponential or "single-hit" inactivation was always obtained for virus, viral DNA, and RF in these experiments. The back extrapolates of the least squares lines fitted to the data did not vary systematically from a fractional survival of 1.0. Some typical data are presented in Fig. 4.

The action spectra for ϕX virus, for single-stranded ϕX DNA in a solvent of low ionic strength, and for RF in two hosts are presented in Fig. 1. The inactivation

cross-section of SS irradiated in a solvent of low ionic strength is 16.6 times that of RF at 235 mμ and about seven times that of RF in the 280–290 mμ region. The SS spectrum is quite similar to that of the whole virus above 240 mμ, suggesting that the viral DNA absorbs most of the energy effective in inactivation. This last point is considered further in Fig. 4, which shows that the DNA extracted from virus after irradiation at several wavelengths declines in infectivity at the same rate as does the viral particle itself.

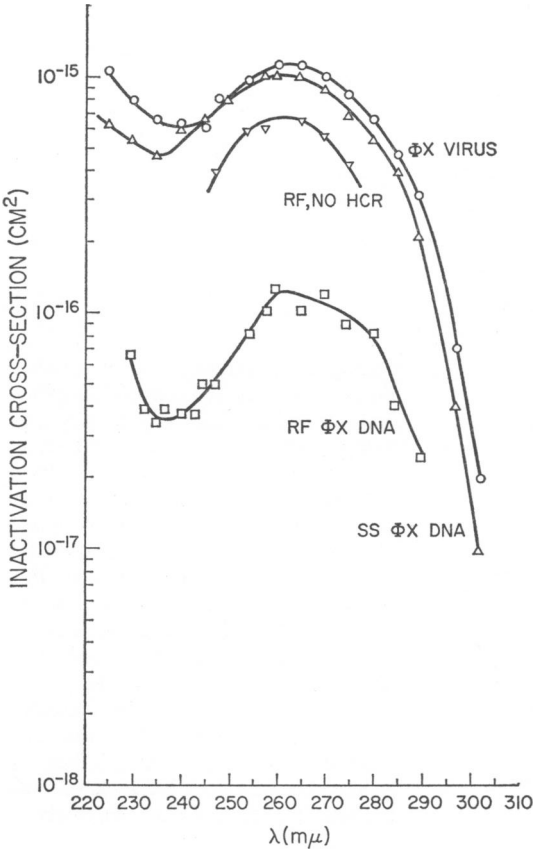

FIGURE 1 Action spectra for inactivation of φX virus, free SS DNA, and RF in Tris, 0.05 M, pH 8.1. Assays performed using *E. coli* C., a host-cell-reactivating host: ○, φX virus. Assays of free DNA performed using *E. coli* K12W6, a host cell-reactivating host: △, single-stranded DNA; □, RF. Assays performed on *E. coli* K12 AB1886, a host lacking reactivation capacity: ▽, RF.

The single-stranded DNA is flexible and responds to changes in ionic strength by expansion and contraction (Sinsheimer, 1959 *b*). Fig. 2 contains quantum yield data for the SS DNA in 0.05 M Tris, pH 8.1, and also in that buffer plus 0.5 M NaCl. Only minor changes in quantum yield are observed, though the polynucleotide chain should be in the one case extended, and in the other folded into a much more compact form. Fig. 2 also presents the quantum yields for the virus as a function of wavelength. The pyrimidines are the most UV sensitive of the constituents of DNA (for a review, see Shugar, 1960; Wacker, 1963); therefore, we have also plotted the

fraction of total energy absorbed by pyrimidines, calculated on the basis of the spectra of nucleotides and the composition (Sinsheimer, 1959 *b*) of ϕX SS DNA.

In Fig. 3 are absorption spectra for ϕX virus and its component DNA and coat protein. Also presented is the summed absorption of the coat protein and SS DNA. The measured spectrum agrees well with the sum of the spectra of purified DNA and coat protein; the DNA is, therefore, probably packaged into the virus particle

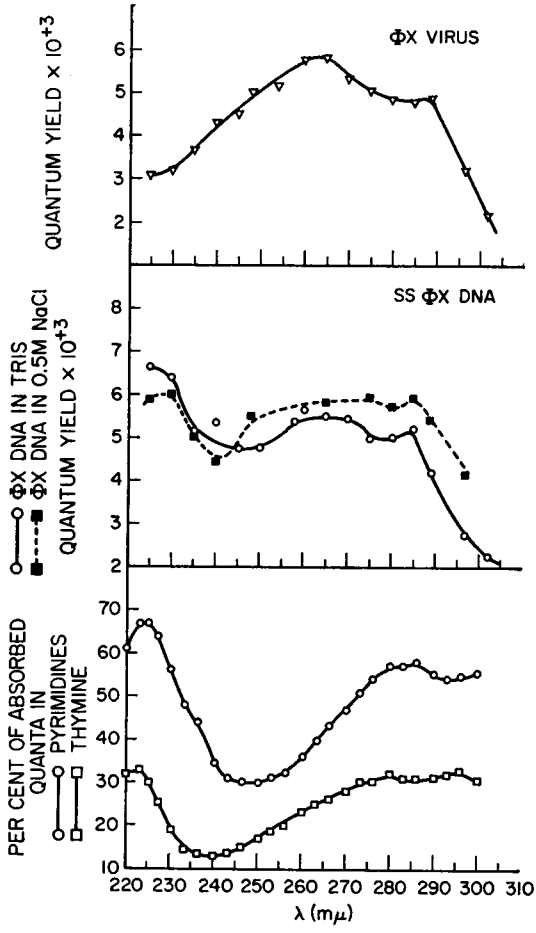

FIGURE 2 Quantum yields for inactivation of ϕX virus and free ϕX SS DNA, and calculated fraction of total absorbed light absorbed by pyrimidines. The fractional absorption was calculated from the relation: (fractional absorption in pyrimidines = $(0.328\ E_\lambda^T + 0.185\ E_\lambda^C)/(0.246\ E_\lambda^A + 0.328\ E_\lambda^T + 0.241\ E_\lambda^G + 0.185\ E_\lambda^C)$ in which E_λ^C, E_λ^A, E_λ^T, and E_λ^G are the extinction coefficients of the deoxyribonucleotides of cytosine, adenine, thymine, and guanine respectively. Spectra for the nucleotides are from data partially presented by Sinsheimer (1954). The quantum yield, or probability of inactivation by an absorbed quantum, is calculated from the relation: yield = inactivation cross-section/absorption cross-section, using data in Figs. 1 and 3.

without gross alterations of the DNA structure from that in solution. [Tobacco mosaic virus is a contrary example: the spectra of its separated protein and RNA sum to a lower absorption coefficient than that of the intact virus. This result presumably reflects the ordered configuration of the intraviral RNA and its intimate association with the structural protein (Bonhoeffer and Schachmann, 1960; Ginoza, 1958)].

DISCUSSION

φX Virus

In the range of wavelengths 240–302 mμ, the evident similarity of the action spectra of φX virus and that of SS (Fig. 1) [and, in a general way, of both to the absorption spectra of φX SS DNA (Fig. 3)] indicates that the quanta absorbed by the viral

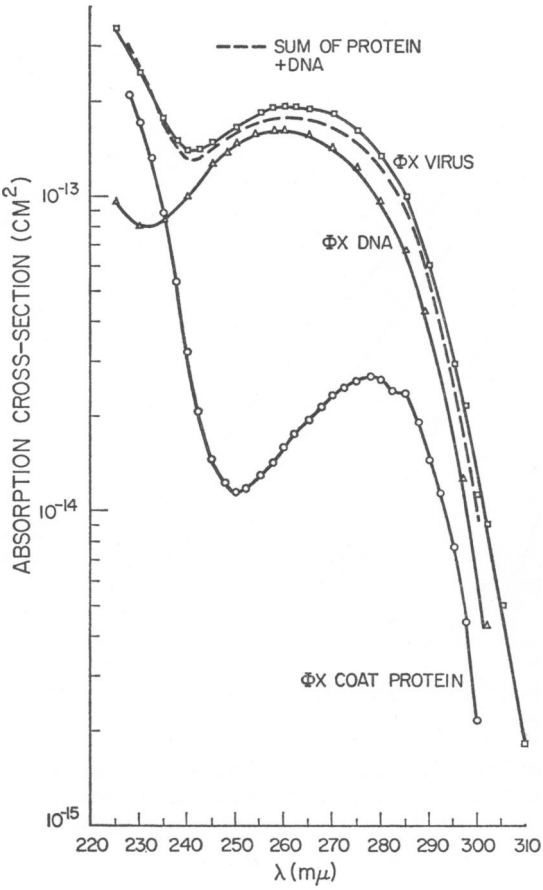

FIGURE 3 Absorption spectra for φX virus and its components, corrected for scattered light. The SS spectrum was measured in Tris + 0.5 M NaCl at 20°C. The solvent for the protein was also 0.05 M Tris, pH 8.1. The absolute value of the absorption cross-section of the DNA was calculated by equating its ϵ (*P*) in 0.2 M NaCl + 0.05 M Tris, pH 8.1, 37°C, to the value previously found (Sinsheimer, 1959 *b*) for mM phosphate buffer + 0.2 M NaCl at 37°C and pH 7.5: □, φX virus; △, φX DNA; ○, φX coat protein; ---, calculated sum of protein and DNA.

DNA are those which lead to the observed inactivation of the virus particle. Experimentally, small but significant disparities between the inactivation cross-sections of SS and φX virus do occur in the spectral region 260–302 mμ in which the virus protein has an absorption maximum (Fig. 3); however, at these wavelengths small increases in the inactivation cross-section of the DNA itself are observed when the DNA in solution assumes a more compact configuration, as it must in the virus

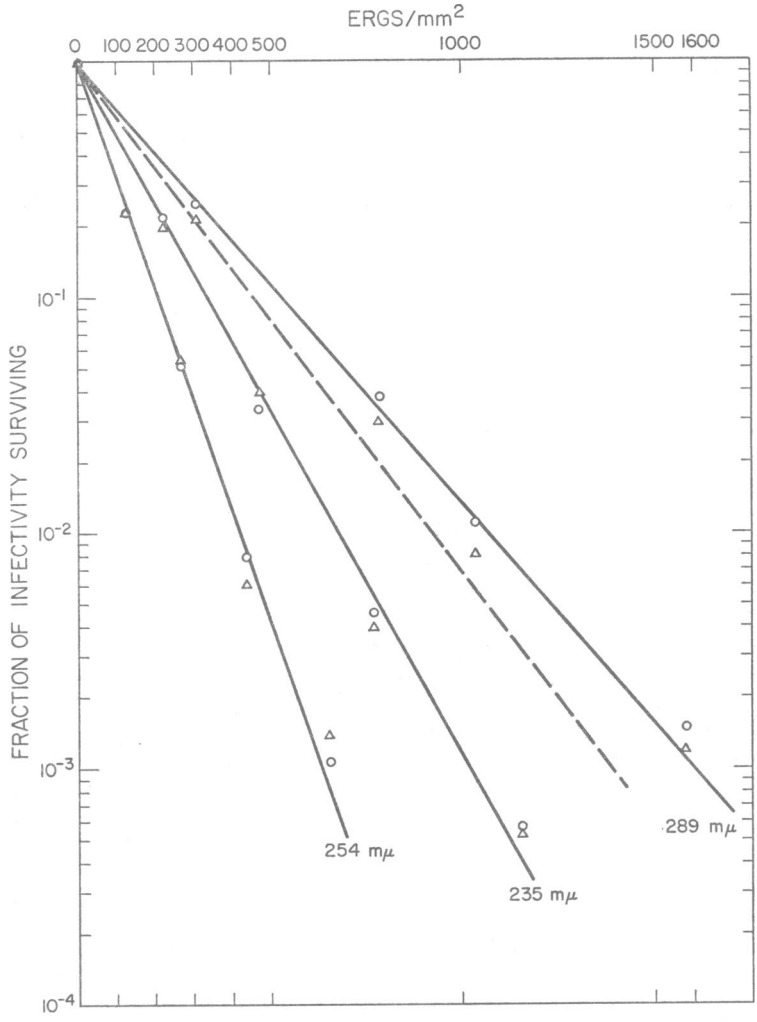

FIGURE 4 Comparison of survival after irradiation at three wavelengths of irradiated phage and of their DNA, extracted after irradiation. ○, survival of virus; △, survival of DNA extracted from irradiated virus; ---, survival of free DNA irradiated in 0.5 M NaCl, with 235 mμ radiation.

particle.[1] Therefore, we conclude that on irradiation with UV light of wavelength 240–302 mμ, the lethal quanta are those which cause primary excitations in viral DNA.

In Fig. 4, φX inactivation by 254 and 289 mμ light is shown to be accompanied

[1] The effects of the addition of NaCl to 0.5 M are to increase the quantum yield (Fig. 2) and to decrease the absorption cross-section (Sinsheimer, 1959 *b*). The net effect is to slightly enlarge the inactivation cross-section at long wavelengths. For example, the cross-sections of SS DNA are increased from those of Fig. 1 by about 5% at 275 mμ and nearly 30% at 297 mμ.

quantitatively by inactivation of the intraviral DNA. Thus, the action spectra indicate that quanta absorbed in the DNA are the cause of lethal damage and the data of Fig. 4 show that the site of the lethal damage initiated by the absorption of light is also in the DNA itself.

The action spectra of the virus particle and its DNA diverge in the range 225 to 240 mμ, the virus being more sensitive to UV than is free DNA (Fig. 1). Small changes observed in the sensitivity of SS on contraction in solution (Figs. 2 and 3) cannot account for this difference between virus and DNA. Extraction of DNA from virus irradiated at 235 mμ (Fig. 4) yields DNA with the same residual infectivity as had the whole, irradiated virus from which it was derived. Thus, the inactivation of the virus by irradiation at 235 mμ is due, as at longer wavelengths, entirely to damage in the DNA; the incorporation of SS into virus must sensitize it to 235 mμ light (Fig. 4). It seems likely therefore that radiant energy at 235 mμ absorbed by another chromophore is transferred to and inactivates the DNA within the virus particle. From the absorption spectra in Fig. 3 one may identify the steep rise of absorption in the coat protein as the cause of the higher absorption of the virus at low wavelengths; around 235 mμ, about half the light absorption by φX virus is attributable to the protein. The quantum efficiency of inactivation by light absorbed by the the virus definitely *decreases* in the 240–225 mμ spectrum interval even though the quantum yield for free viral DNA *increases* (Fig. 2). From these facts, it seems likely that the quanta absorbed by the virus structural protein can produce lethal alterations in the intraviral DNA, but are more easily dissipated without inactivation of the particle than are quanta absorbed by the DNA.

These results suggest that the primary event in *virus* inactivation by short UV wavelengths is, in a large fraction of inactivations, the absorption of a quantum by the protein. The resulting inactivation could be a result of a cross-link to the DNA formed by the UV-excited protein, or conceivably, could represent true energy transfer from protein to DNA. Damage mediated by protein when the irradiation was performed with light of short UV wavelength has also been detected in phage T4 as a morphological alteration (Winkler, Johns, and Kellenberger, 1962).

Several other action spectra of φX virus have previously been published (Denhardt and Sinsheimer, 1965; Rauth, 1965; Setlow and Boyce, 1960). The cross-sections and quantum yields presented here are in good agreement with these other determinations. The wide agreement on the absolute sensitivity of φX virus suggests its use as a biological dosimeter.

Single-Stranded DNA

The small ultraviolet hyperchromicity on DNase digestion or heating of φX SS (Sinsheimer, 1959 *b*) implies that the interactions between nucleotides are weaker than the interactions between base pairs in the more ordered DNA's. If the spectra of the 5′-mononucleotides are summed in the proportions in which they are found

in ϕX DNA, the calculated spectrum has the same maximum, minimum, and general shape as the observed spectrum of ϕX DNA. In Fig. 2 the fraction of absorbed energy absorbed by pyrimidines, based on the summed spectra of mononucleotides, is plotted and compared with the variation of quantum yield with changes in wavelength. It can be seen that for SS the two quantities are related. The quantum yield and fractional absorption in pyrimidines rise in concert at low wavelength, and both have minima at 240–250 mμ and maxima at longer wavelength. However, the quantum efficiency of inactivation declines markedly at wavelengths above 285 mμ, while the fractional light absorption in pyrimidines (or in thymine) does not. The fall in the quantum yield above 285 mμ, in the absence of a parallel decrease in the fraction of absorbed energy in pyrimidines, could be a result of a fall in the intrinsic quantum yield for photochemical change in pyrimidines at longer wavelengths. Deering and Setlow (1963) found that the quantum yield for thymine dimerization (Beukers and Berends, 1961) in dithymidylic acid and polythymidylate decreases at long wavelengths.

A relation of high pyrimidine absorption to high quantum yield might be expected, since pyrimidines are the most UV-sensitive components of DNA (Shugar, 1960; Wacker, 1963). The persistence of this property of pyrimidines in a highly polymerized DNA may be taken as evidence that quanta absorbed by purines are less effective for inactivation than those causing excitation of pyrimidines, and thus that the absorbed energy of excitation is not equally available to all bases through energy transfer. This apparent limitation on energy transfer is not very rigorous, however, for energy migration among purines or among pyrimidines would not be detected here at all.

The quantum yield for SS is affected by ionic strength; the more compact molecule is inactivated slightly more efficiently than the extended SS by absorbed energy of wavelengths above 250 mμ (Fig. 2). One possible explanation is that the collapse of the SS into a more compact ordered structure would enhance the likelihood of damage requiring interaction of different parts of the molecule, such as formation of photodimers between two pyrimidine residues (Beukers and Berends, 1961).

ϕX SS DNA (Yarus and Sinsheimer, 1964) and ϕX virus (Sauerbier, 1964) are not reactivated by the host cell reactivation system(s). Therefore, even though the amounts and types of UV-induced alteration of ϕX DNA may be similar to those in other organisms, a greater fraction of the primary photochemical effect will be lethal to ϕX. To this extent, these inactivation cross-sections are representative of the *total* rate of potentially lethal damage.

RF

As expected, the action spectrum of RF in Fig. 1 has the maximum, minimum, and general shape of absorption spectra of DNA.

The infectivity of RF is much more resistant to ultraviolet inactivation than SS

at all wavelengths examined; this has been shown by Yarus and Sinsheimer (1964) and Jansz et al. (1963) to be a result of the presence in bacterial cells of a repair process (HCR) which can act only on RF, neutralizing most of the potential lethal damages in it. In Fig. 1 the sensitivity of RF assayed on hosts possessing and lacking HCR may be compared.

Since the K12W6 spheroplasts usually used to assay the irradiated RF possessed the ability to reactivate ultraviolet damages (Sauerbier, 1962), the cross-sections comprising the lower, more complete RF spectrum in Fig. 1 represent the rate at which nonreactivatable damages are done.

Although RF when assayed using a bacterial host incapable of reactivation is about 5% as sensitive as SS, RF is expected to absorb about twofold more light than SS. Therefore, the difference in the quantum efficiency of inactivation for the two types of DNA, even when both are assayed on HCR^- hosts, is still quite significant. This contrasts with the reported similarity of the quantum yield for formation of thymine dimers in single- and double-stranded DNA (David, 1964).

This difference between the quantum yields for inactivation of RF and SS (assayed in a HCR^- strain) cannot be attributed in any simple way to the redundancy of information in the two strands of RF. For if the duplication of information provided a means of relief from photochemical damage, we would expect the inactivation curves to be multihit rather than exponential. It is possible that the quantum yields for the formation of lethal lesions (other than thymine dimers) are significantly different in the two DNA's.

Other qualitative differences in the action spectra of RF and SS are unexplained; the 260 $m\mu$/235 $m\mu$ ratio of cross-sections is larger for RF than for SS, the 260 $m\mu$ band seems broader, as if it were composed of more than one overlapping band, and the limb of the RF spectrum below 235 $m\mu$ seems to rise more rapidly. Detailed photochemical comparisons could be performed to clarify these points.

This work is drawn from a thesis by M. Yarus submitted in partial fulfillment of the requirements for the Ph. D. degree.

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