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Quantitation of Pyrimidine Dimer Contents of Nonradioactive Deoxyribonucleic Acid by Electrophoresis in Alkaline Agarose Gels†

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ABSTRACT: We have developed a method of quantitating the pyrimidine dimer content of nonradioactive DNAs. DNA samples are treated with the UV-endonuclease from *Micrococcus luteus* and then separated according to molecular weight by electrophoresis on alkaline agarose gels. From their migration relative to known molecular weight standards, their median molecular weights and thus the number of dimers per DNA molecule in each sample can be calculated. Results of action spectra for dimer formation in T7 bacteriophage measured by this method agree well with action spectra for T7 killing. In addition, the method gives dimer yields in good agreement with those obtained by others using alkaline sucrose gradient sedimentation.

Cis-syn cyclobutylpyrimidine dimers are major lesions induced in DNA by ultraviolet light (UV; 220-320 nm). Classical methods of pyrimidine dimer measurement require radioactive labeling for location and quantitation after subsequent chromatographic or sedimentation analysis. It is important to be able to measure UV-induced pyrimidine dimer levels in DNAs of cells or tissues not amenable to radioactive labeling.

Achey et al. (1979) developed a method for detecting pyrimidine dimers in nonradioactive DNA of fish cells: extracted DNAs were treated with the *Micrococcus luteus* UV-endonuclease [which makes a single-strand nick adjacent to each dimer (Ahmed & Setlow, 1977)] and electrophoresed in alkaline agarose (McDonell et al., 1977). Dimer formation was deduced from the decrease in molecular weight in endonuclease-treated UV-irradiated samples. They also used radioactively labeled *Escherichia coli* DNA to analyze dimer contents by using three methods: (a) alkaline sucrose gradient sedimentation; (b) acid hydrolysis and chromatography; (c) gel electrophoresis. Although they found that the DNA profiles on gels reflected the dimer contents measured by other methods, they could not obtain absolute molecular weights from their gel samples. Sutherland et al. (1980) obtained estimates of dimer contents from gel electrophoresis of nonradioactive human skin DNA by comparison of the mid positions of the DNA profiles with the migration distances of molecular weight standards.

To examine this method further, we have chosen "ideal" conditions for experimental and theoretical analysis: a homogeneous population of small DNA molecules, well-defined dosimetry, and irradiation with monochromatic light. We also used DNA concentrations and agarose gel concentrations to allow optimal separation and display of intact and cleaved molecules. We show that pyrimidine dimer determination by our analysis method gives accurate measures of dimer yields (i.e., comparable to those obtained by others by alkaline sucrose gradient sedimentation). The technique also gives results of sufficient precision for action spectroscopy; action spectra obtained by this method on T7 bacteriophage and its DNA implicate a nucleic acid as chromophore and agree well with those for phage killing.

Materials and Methods

Bacteriophage. T7 bacteriophage were obtained from F. W. Studier, Brookhaven National Laboratory, and grown on *Escherichia coli* AB2500. Viable virus titer was determined by plating a dilution of phage and 0.2 mL of *E. coli* in 2 mL of soft agar (10 g of Bactotryptone, 5 g of NaCl, and 7 g of Bacto Agar per L) over a hard agar base (10 g of tryptone, 5 g of NaCl, and 10 g of Bacto Agar per L). Plates were incubated overnight at room temperature.

Irradiation. Purified phage were diluted to 3.2×10^{10}/mL in 50 mM tris(hydroxymethyl)aminomethane (Tris), pH 8, and 200 mM NaCl. Two-milliliter samples were exposed with constant stirring to monochromatic radiation from a Johns' monochromator (Johns & Rauth, 1965); incident light was monitored with a silicon photodiode. Incident intensities (in photons per second) for the various wavelengths (in nanometers) were the following: 248, 1.29×10^{14}; 265, 1.89×10^{14}; 280, 1.84×10^{14}; 289, 4.48×10^{14}; 297, 1.76×10^{15}; 313, 2.44×10^{16}. Irradiations and intensity measurements at 313 nm were of light filtered through a thin Mylar filter to exclude shorter wavelengths.

DNA Analysis. After each sample was irradiated, a small amount was removed for determining phage survival (see above). The remaining phage were concentrated by centrifugation for 1 h in a Brinkmann microcentrifuge. The phage

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pellet was resuspended in 10 μL of 30 mM Tris, pH 8, 40 mM NaCl, and 10 mM ethylenediaminetetraacetic acid (EDTA) and heated to 65 °C for 20 min, with cooling every 5 min on ice, to release the phage DNA. After being cooled to 37 °C, 20 μg of a preparation of *Micrococcus luteus* UV-endonuclease (Carrier & Setlow, 1970) was added to each sample. This preparation quantitatively produces single-strand nicks at the site of each pyrimidine dimer (Ahmed & Setlow, 1977) and does not nick unirradiated DNA (Sutherland et al., 1980). After a 20-min incubation at 37 °C, the reaction was stopped by the addition of 2 μL of a mixture containing 2.4 mM NaOH, 0.24% Bromocresol green, and 40% glycerol. Molecular weight standards included intact T7 DNA (M_r 13.2 × 10^6, single strand) and the three *BglI* cleavage fragments of T7 DNA, with single-strand molecular weights of 1.32 × 10^6, 4.48 × 10^6, and 7.42 × 10^6, respectively. The samples were applied to the wells of 13 × 14 cm horizontal agarose gels containing 0.29 g of agarose, in 70 mL of 60 mM NaOH, and 6 mM EDTA. The electrode buffer was 4.6 mM NaOH and 0.46 mM Na_3EDTA. Electrophoresis was 22 h at 16 V. The gels were neutralized by soaking in 500 mL of 100 mM Tris, pH 8, for 30 min, and then stained in 100 mM Tris, pH 8, containing 0.5 μg/mL ethidium bromide. Excess ethidium bromide was removed by soaking the gel in H_2O for 2 h at room temperature. The gel was placed on an Ultraviolet Products C63B transilluminator, and fluorescence from DNA-bound ethidium was photographed through a red filter by using Polaroid type 55 positive–negative film. Negatives were scanned with a Joyce-Lobel densitometer.

Results

The irradiated phage were analyzed by liberation of the DNA, treatment with UV-endonuclease, and electrophoresis in alkaline agarose gels. Figure 1 shows densitometer tracings for data from a typical gel. Trace a shows unirradiated DNA (extracted and treated identically with all irradiated samples except for UV exposure); traces b–f show DNAs exposed to 2.69 × 10^{18}, 5.38 × 10^{18}, 10.8 × 10^{18}, 21.5 × 10^{18}, or 42.9 × 10^{18} photons/m^2 of 265-nm radiation, respectively. The positions of the molecular weight markers are indicated by the arrows at the top of the figure.

The molecular weights of these DNA populations, and thus the number of dimers per molecule, were calculated as follows: The peak positions of the molecular weight standards (DNAs of homogeneous size and known molecular weights) were plotted vs. the logarithms of their molecular weights. The molecular weights of the control and irradiated DNA populations were then determined by (a) tracing the lanes on the film negative with a Joyce-Lobel densitometer to obtain a profile of each DNA, (b) cutting out the resulting profile and determining gravimetrically[1] the midpoint of the mass of the DNA, (c) determining the migration distance of each DNA population from the midpoint of the DNA mass, by using the densitometric tracing, with the position of half the height of the inner edge of the well as zero migration distance, (d)

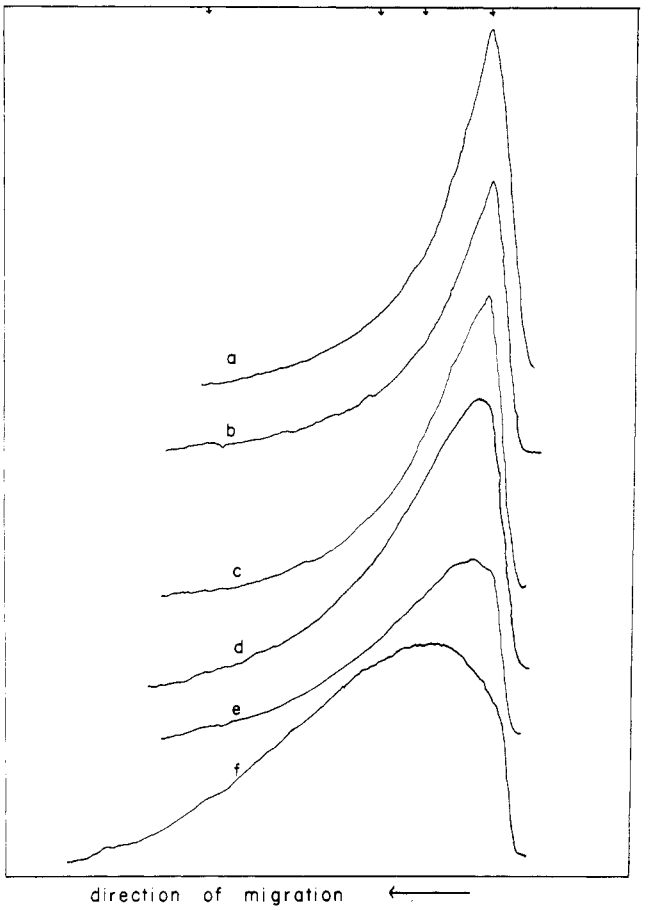

FIGURE 1: Profiles of T7 DNA from bacteriophage exposed to 265-nm radiation, released by heating, treated with UV-endonuclease (20 μg), and electrophoresed in alkaline agarose. UV exposures were as follows: (a) 0, (b) 2.69 × 10^{18}, (c) 5.38 × 10^{18}, (d) 10.8 × 10^{18}, (e) 21.5 × 10^{18}, and (f) 42.9 × 10^{18} photons of 265-nm radiation. Molecular weight markers are indicated by arrows at the top of the figure, and the corresponding molecular weights (from right to left) are the following: intact T7 DNA, 13.2 × 10^6; three *BglI* cleavage products of T7 DNA, 7.42 × 10^6, 4.48 × 10^6, and 1.32 × 10^6, respectively.

determining the median molecular weight ($M_{1/2}$)—the molecular weight which is exceeded by half of the mass of molecules in the distribution—of the DNA by comparing the migration distance determined in (c) with the migration distances of the DNA standards, and (e) determining the net number of breaks (b) per molecule by the equation of Veatch & Okada (1969)

$$b = \frac{M_r}{0.6}\left[\frac{1}{M_{1/2(\text{irradiated})}} - \frac{1}{M_{1/2(\text{unirradiated})}}\right] \quad (1)$$

where M_r is the molecular weight of the intact DNA. Since the UV-endonuclease nicks quantitatively at dimers, the number of breaks equals the number of dimers.

This analysis assumes that the weight of the densitometric profile is proportional to the mass of the DNA in that profile. We tested this assumption by electrophoresing known quantities of T7 DNA, photographing the gels, tracing the negatives, and weighing the resulting profiles. Figure 2 shows that the weights obtained by this method are proportional to the amount of DNA on the gel.

The dose–response curves for dimer production in T7 DNA by the various wavelengths are shown in Figure 3. These data show the high efficiency of 265 nm in dimer production compared to other wavelengths. Since these curves are similar in shape, we can compute an action spectrum for pyrimidine

[1] The midpoint of mass of the DNA was determined as follows: each densitometer tracing was copied by Xerox; the resulting profiles were cut out by using fine scissors. The profiles were handled with forceps during all manipulations. The weight of the entire profile was determined on a Mettler HL52 analytical balance. The midpoint of the mass of a profile was then estimated and the profile sliced vertically at this position. One "half-profile" was weighed; if the resulting weight was more than half of the total weight of the total, successive vertical slices were removed and the resulting half-profiles reweighed until the profile was exactly half of the total weight. The inner edge of the half-profile was the midpoint of the DNA mass.

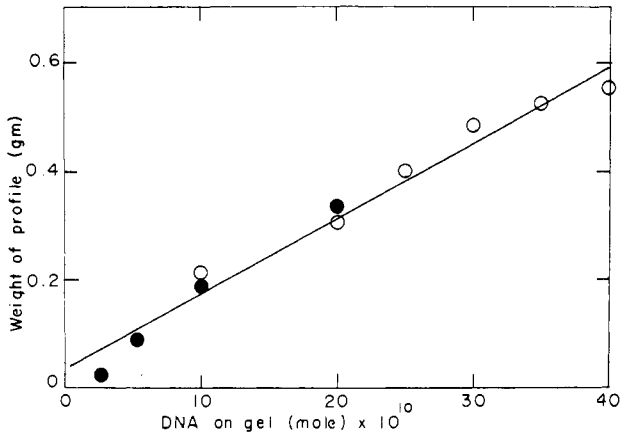

FIGURE 2: Weight of densitometric profiles of DNA samples as a function of the quantity of DNA in the sample. Purified T7 DNA was electrophoresed, the gel photographed, and the resulting negative traced. Each profile was cut out and its weight determined. Data from two independent experiments are shown; the line was determined from linear regression analysis of the data.

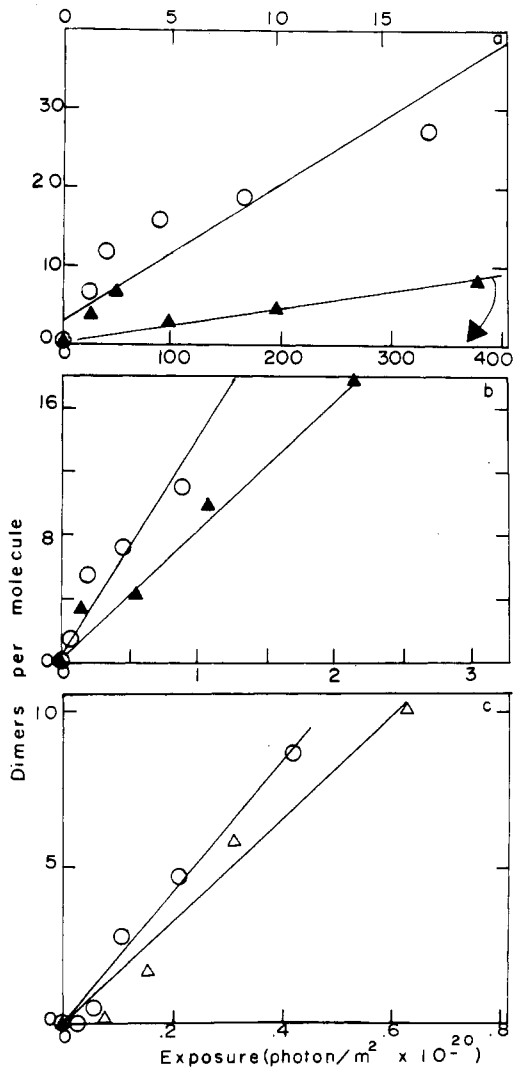

FIGURE 3: Pyrimidine dimer yields as a function of UV exposure. Panel a: 313 [(▲) lower scale] and 297 nm [(○) upper scale]. Panel b: 280 (○) and 289 (▲) nm. Panel c: 265 (○) and 248 (△) nm.

dimer formation in T7 DNA (Jagger, 1967). Action spectra obtained from two independent experiments are shown as closed symbols in Figure 4. This action spectrum shows a maximum at 265 nm, with decreasing efficiency of dimer formation at shorter and at longer wavelengths.

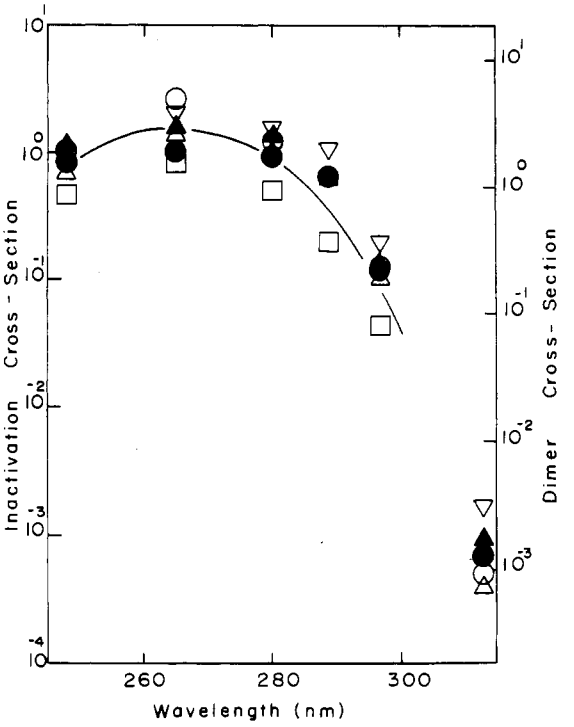

FIGURE 4: Action spectra for pyrimidine dimer formation in T7 phage (closed symbols, two independent experiments; units are m^2/photon $\times 10^{-14}$ for production of four dimers per T7 molecule) and for T7 phage killing (open symbols, three independent experiments; units are m^2/photon $\times 10^{-15}$ for 50% inactivation). The curved line is the absorption spectrum of purified T7 DNA, measured in our laboratory.

The phage were also analyzed for survival after the various UV exposures. Figure 5 shows the dose–response curves for killing of T7 phage by wavelengths in the wavelength range 248–313 nm. The 265-nm wavelength is clearly the most efficient in viral inactivation, followed by 280 and 248 nm (see panel c); panel b shows that 289 nm (upper dose scale) is more effective than 297 nm (lower dose scale), and panel a shows that 313 nm is the least effective of the wavelengths tested. The similarity of shapes of the dose–response curves allows the determination of an action spectrum for viral killing; an action spectrum obtained from data of three independent experiments is shown as the open symbols of Figure 4. These data clearly indicate a maximum at 265 nm, with decreasing efficiency at shorter or longer wavelengths.

Discussion

Electrophoresis in agarose gels offers the advantages of separation according to size and visualization by fluorescent staining of nonradioactive DNAs. In addition, the molecular weights of DNAs can be determined by comparison of the relative migration of unknowns with those of molecular weight standards. Achey et al. (1979) and Sutherland et al. (1980) used the agarose gel technique to detect pyrimidine dimer formation in nonradioactive DNAs; we have examined the gel system to determine if it could be used to quantitate small differences in dimer content as is demanded, for example, by action spectroscopy. This presented two major problems: first, the development of a valid measure of mobility of a population of heterogeneous molecules (produced by dimer formation and endonuclease cleavage of DNA), and second, use of these mobility determinations to determine dimer content.

We chose T7 bacteriophage and its DNA as an ideal simple system for examining the method: the DNAs in the virus capsids are a homogeneous population of molecular weight 26.4×10^6. We first developed a method for obtaining a

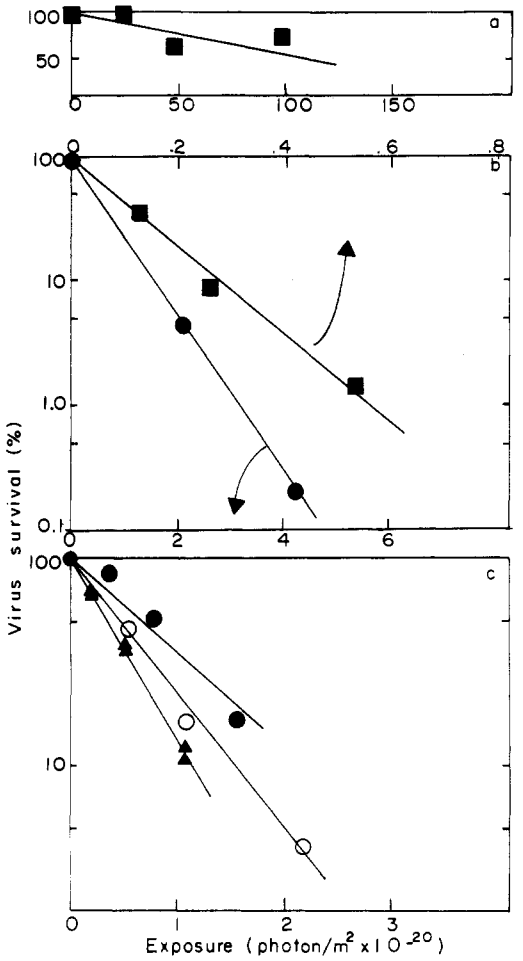

FIGURE 5: T7 phage survival as a function of UV dose for the wavelengths 313 nm (panel a); 297 [(●) lower scale] and 289 nm [(■) upper scale] (panel b); and 248 (●), 280 (○), and 265 (▲) nm (panel c).

reproducible measure of the midpoint of the heterogeneous fragments produced by dimer formation and UV-endonuclease cleavage. We obtained absorbance profiles of each DNA by tracing with a Joyce-Lobel densitometer. We found it important to use consistent background levels, using a trace taken adjacent to a particular lane if necessary to check for possible gel absorbance heterogeneity. We found that use of a longer wavelength (~300 nm) transilluminator (Ultraviolet Products C63B), as well as soaking the stained gel in H_2O, was of great value in reducing the background fluorescence of the gel and facilitating choice of the base line.

We determined gravimetrically the midpoint of the net absorbance profile of the DNA sample. We ascertained that the total weight obtained for each DNA trace was proportional to the amount of DNA on the gel. Using as zero migration distance the position of half the height of the inner edge of the well, we calculated the midpoing migration distance of each DNA sample. Singer et al. (1979) used the midpoints of migration of restriction fragments to obtain molecular weight estimates from gels. Since migration conditions and times might vary slightly from one gel to another, we included molecular weight standards on each gel. In addition, since the reciprocal of the molecular weight of the unirradiated DNA is subtracted from the corresponding value for each irradiated DNA, we found it helpful to include on each gel two or three samples of unirradiated phage, heated and treated with UV-endonuclease, to obtain a better estimate of that molecular weight. Finally, we noted that the two outside lanes sometimes gave anomalous migration and have avoided using them whenever possible.

We next analyzed these midpoint values to obtain the dimer content of each DNA sample. McDonell et al. (1977) showed that migration of DNAs on agarose gels was linearly proportional to the logarithm of the molecular weight of the DNA over a limited range, with curvature at higher and lower molecular weight regions. It is thus imperative to include molecular weight markers which span the range of all the migration distances of the midpoints of the DNA profiles in a given experiment. We used intact T7 DNA as the largest DNA molecule expected and its 1.32×10^6 *BglI* fragment as the lower limit of population molecular weights to be examined. Comparison of the position of the midpoint of each UV-irradiated or unirradiated DNA with the plot of migration distance vs. molecular weight of each molecular weight standard yielded a median molecular weight, $M_{1/2}$.

The number of breaks (b) in the DNA is related to the number-average molecular weight (M_n) as follows:

$$b = M_r\left[\frac{1}{M_{n(after)}} - \frac{1}{M_{n(before)}}\right] \quad (2)$$

where M_r is the molecular weight of the phage DNA and $M_{n(after)}$ and $M_{n(before)}$ are the number-average molecular weights of the irradiated and unirradiated DNAs, respectively [see Veatch & Okada (1969)]. M_n can be obtained from M_w (the weight-average molecular weight) or from $M_{1/2}$ (the median molecular weight); Ehmann & Lett (1973) have shown that after the first two or three breaks per molecule, both these measures give good estimates of the true molecular weight as determined by calculation from a theoretical profile with a known number of breaks or by direct calculations from an experimental profile with controlled strand breakage. According to Veatch & Okada (1969), M_n is related to $M_{1/2}$ as follows: $M_n = 0.6M_{1/2}$. The number of dimers ($d \equiv$ number of breaks) per molecule is thus calculated from the $M_{1/2}$ according to

$$d = \frac{M_r}{0.6}\left[\frac{1}{M_{1/2(irradiated)}} - \frac{1}{M_{1/2(unirradiated)}}\right] \quad (3)$$

where M_r is the molecular weight of the intact T7 DNA molecule. Our combination of heat treatment to release the DNA from the viral particle, endonuclease treatment, alkali treatment for strand separation, and layering onto the gel generates one to two breaks per T7 molecule. At values of $d \leq 1$, it is difficult to obtain $M_{1/2}$ values which provide good estimates of d. We have thus determined our action spectrum at the level of four dimers per T7 molecule. Comparison of this action spectrum, shown as closed symbols in Figure 4, with that for UV-induced killing of the phage indicates that both have maxima at 265 nm and similar shapes.

We have used three criteria to assess the gel method of dimer quantitation: First, does it give results which are consistent among the various wavelengths? Second, are the results consistent with the phage survival data? Third, do the dimer yields obtained from this method agree with those obtained by others using different methods of dimer measurement? Examination of the action spectrum for dimer formation indicates that the spectrum is consistent with one in which nucleic acid is the major absorbing chromophore. Furthermore, comparison of the dimer action spectrum with that for phage killing indicates that the gel data are internally consistent with the biological results obtained from the same T7 samples. Ahmed & Setlow (1979) measured the pyrimidine dimer yield

at 254 nm by alkaline sucrose sedimentation of V79 hamster cell DNA. They obtained values of about 2.5 dimers per J/m^2 per 10^8 daltons. Determination of dimer yields at 254 nm by the gel method gave a value of 2.3 dimers per J/m^2 per 10^8 daltons, in good agreement with those obtained by Ahmed and Setlow.

We thus conclude that for simple systems (homogeneous populations of small DNAs), this agarose gel technique and analysis procedure can give an accurate measure of pyrimidine dimer formation in nonradioactive DNAs. However, many DNAs of biological interest (e.g., eucaryotic cells or tissues) pose additional problems: (1) they are higher in molecular weight and would not enter easily the 0.4% gels used for T7 DNA; (2) because of their high molecular weight, they are easily sheared during extraction and thus are heterogeneous in size even without irradiation or endonuclease treatment. Of course, agarose gels as low as 0.1% gels are useful for DNAs of higher molecular weight, and it seems likely that a theoretical treatment similar to that used in analyzing the T7 DNA would also be applicable (we note that even unirradiated T7 DNAs suffered ~1–2 nicks per molecule during the heat liberation from the viral capsid, so indeed the unirradiated DNA was heterogeneous). In addition, molecular weight markers spanning the range of molecular sizes of the untreated and cleared molecules would be required. Of course, some tissues may also contain nondividing or necrotic cells with degraded DNA which would interfere with dimer determination.

The simple system we have used does not address these problems of highly heterogeneous, high molecular weight DNA populations. It does show that the gel technique and our analysis procedure give good measures of pyrimidine dimer content under carefully chosen experimental conditions. It seems likely that with appropriate modification of experimental conditions for other DNA populations, the same methodology and analysis can provide the foundation for a wide range of DNAs not amenable to radioactive labeling.

Acknowledgments

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Registry No. *Micrococcus luteus* UV-endonuclease, 9073-83-0.

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Isolation and Characterization of a Hemorrhagic Proteinase from Timber Rattlesnake Venom†

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ABSTRACT: A protein isolated from timber rattlesnake (*Crotalus horridus horridus*) venom by ion-exchange and high-pressure liquid chromatography is hemorrhage inducing and lethal to mice (LD_{50} of 10 μg/g of body weight). It is a Ca^{2+}- and Zn^{2+}-containing proteinase and has the ability to hydrolyze hide powder azure. Atomic absorption spectroscopy shows 2.5 Ca^{2+} and 1 Zn^{2+} per protein monomer. The proteinase activity is destroyed by incubation with disulfide-reducing agents and by dialysis against ethylenediaminetetraacetate. Coincident with the loss of proteinase activity is a corresponding loss of lethal and hemorrhagic activities, suggesting that all three are related. Attempts to replace the metals and restore activity have been unsuccessful. Amino acid analysis and isoelectric focusing reveal that this component is an acidic protein (p*I* = 5.1) containing about 20 disulfide bonds and 507 residues. Reduction of one disulfide bond per molecule decreases proteinase activity by 50% while reduction of eight disulfide bonds decreases activity by 80%. Loss of hemorrhagic activity parallels the decrease in proteinase activity.

Rattlesnake bites in the United States result in considerable damage to the tissues adjacent to the site of envenomation. Hemorrhage is one of the most common manifestations of snakebite and is of clinical concern since antivenom treatments may not prevent hemorrhage (Minton, 1954). A number of hemorrhage-inducing components have been isolated from North American rattlesnake venoms. An even larger number of proteases have been isolated from these venoms, but very few of the hemorrhagins have been shown to contain proteolytic activity.

Fabiano & Tu (1981) have reported the isolation of a tissue-damaging toxin from prairie rattlesnake (*Crotalus viridis viridis*) venom which was lethal and hemorrhagic in mice and catalyzes limited hydrolysis of dimethylcasein. Five hemor-

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