CESIUM CHLORIDE DENSITY GRADIENT STUDY OF SOME MODIFICATIONS INDUCED BY ULTRAVIOLET RADIATION ON THE DENATURED DNA MOLECULE OF PHAGE T_2

M. CREMONESE, C. GIAMPAOLI, M. MATZEU, *and* G. ONORI

From the Physics Laboratories, Istituto Superiore di Sanità, Rome, Italy

ABSTRACT The behaviour of the modifications due to ultraviolet (UV) radiation on denatured phagic DNA has been investigated by looking at the changes of density in CsCl gradient, after irradiation with increasing doses of monochromatic light. The spectral range studied is 2300–2900 A, with a wavelength resolution $\Delta\lambda = \pm 20$ A. The observed effect is a gradual shift toward higher densities as the UV absorbed dose increases. The experimental results show an exponential law and the analysis of the action spectra indicates that thymine is the main factor responsible for the observed effect. An evaluation of the quantum yield for the thymine-dimers formation gives a constant value of $(18 \pm 3) \times 10^{-3}$ quanta^{-1}.

INTRODUCTION

Ultraviolet (UV) radiation, when absorbed by a DNA molecule, in vivo or in vitro, native or denatured, causes some physicochemical modification. The interaction between UV radiation and DNA is essentially localized in the bases, and particularly in the pyrimidine ones (thymine and cytosine) whose cross-section for photochemical reactions is 10–100 times greater than that relative to the purine bases (adenine and guanine) (Herrera, 1952). The main photoproducts found are the thymine dimers (Beukers and Berends, 1960; Beukers and Berends, 1961; Wulff and Fraenkel, 1961; Gerdil, 1961; Setlow, 1961; Johns, Rapaport, and Delbruck, 1962; Wulff, 1963), cytosine-thymine dimers (Setlow and Carrier, 1966), partial local denaturations (Marmur and Grossman, 1961), breakages in the DNA molecule (Cremonese, 1968; Marmur et al, 1961), and cross-links between strands (Marmur and Grossman, 1961; Cremonese, 1968).

We wanted to study the behaviour of some modifications produced by UV radiation on denatured phagic DNA; this was done by following its density variations in a CsCl gradient after irradiation with increasing doses of monochromatic light of different wavelengths.

MATERIALS AND METHODS

Deoxyribonucleic Acid

DNA was extracted from phage T_2 by the method described by Mandell and Hershey (1960). Two characteristics of this DNA are high percentage of thymine (35%) (Hershey, Dixon, and Chase, 1953) and replacement of cytosine with the corresponding 5-hydroxymethyl form (Wyatt, and Cohen, 1953), which is incapable of forming the hydrates characteristic of the cytosine base (Setlow, and Carrier, 1966; Glišin, and Doty, 1967) when exposed to UV radiation.

Denaturation

The DNA (~400 μg/ml) was added to a solution of NaOH (in 0.1 M NaCl), pH = 12.3, and continuously stirred. After 3 min the pH was adjusted to 7.0 by adding HCl, pH = 2.4. This method was employed to avoid local pH's which are capable of hydrolyzing the DNA. The final solution is about 12 μg/ml ($OD_{(2600\ A)}$ = 0.31) in 0.1 M NaCl.

Ultraviolet Irradiation

Samples of denatured DNA were irradiated in a quartz cuvette having a 1 cm light path; a Hilger & Watts D 96 quartz prism monochromator (Hilger & Watts Inc., Chicago, Ill.) was used. The spectral range studied was 2300–3900 A, with $\Delta\lambda = \pm 20$ A at each wavelength. The samples were continuously mixed and cooled. Mixing was carried out by rapidly rotating the cuvette through 180° at intervals of 30″. The temperature during irradiation was about 15°C.

The absorbed dose (number of quanta per cubic millimeter of solution) was evaluated from the expression

$$D_A = D_I (1 - T_0),$$

where the incident dose D_I was measured by the thermopile nanovoltmeter method, and the transmission coefficient T_0 was determined spectrophotometrically (see footnote 1).

CsCl Density Gradient Centrifuging

The CsCl density gradient of the irradiated DNA was obtained with a Spinco model E ultracentrifuge (Spinco Div., Beckman Instruments, Inc., Palo Alto, Calif.) at 44,710 rpm for 24 hr, an UV absorption optic being used. *Bacillus megaterium* DNA was used as a reference (ρ_r = 1.695 g/cm³). The resulting photographs were surveyed with a microphotometer and its tracings where analyzed as a set of gaussian curves by a 7040 IBM computer (Cortellessa and Farchi, 1965).

The density of the banded DNA was determined from the values of the abscissas corresponding to the maxima of the gaussian curves and its homogeneity was determined from the half-width "*h*". (Frontali, 1963).

RESULTS

An example of the results obtained is displayed in Fig. 1, where we compare the CsCl buoyant density of a DNA sample irradiated at $\lambda = (2500 \pm 20)$ A and an unirradiated control.

In Fig. 2 the buoyant density is plotted as a function of the absorbed dose for two wavelengths, 2500 and 2800 A, chosen as examples. The complete DNA denaturation can be inferred by observing the perfect symmetry of the gaussian curves. The pattern of the experimental points is similar for the whole of the spectral range examined, except for $\lambda = 2300$ A, where the density increase is accompanied by a new phenomenon, i.e. an increase in the width of the gaussian curves (Fig. 3) which is highly significant for $D_A \gtrsim 10 \times 10^{14}$ quanta/mm^3.

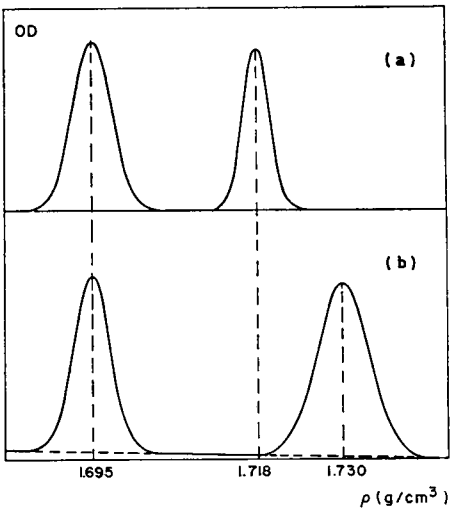

FIGURE 1 Microphotometer tracing of CsCl buoyant density of the T_2 phage denatured DNA; (*a*) unirradiated sample and (*b*) irradiated sample at 2600 A, $D_A = 11 \times 10^{14}$ quanta/mm^3. *Bacillus megaterium* DNA ($\rho = 1.695$ g/cm^3) was used as the standard.

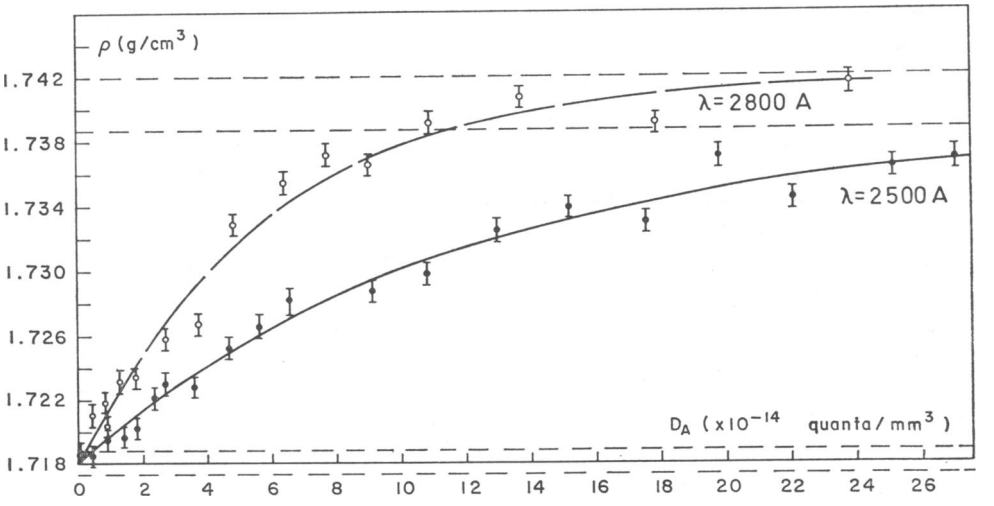

FIGURE 2 Behaviour of CsCl gradient density as a function of the absorbed dose; $\lambda = 2800$ A and $\lambda = 2500$ A. The dashed lines are the "plateau" values.

The experimental points have been interpolated with the function

$$\rho = \rho_0 + A\,(1 - e^{-\sigma D_A}), \tag{1}$$

where ρ_0 is the density corresponding to the denatured unirradiated sample and A and σ are constant for each wavelength. The computer gave us the values of A and σ, and we could therefore obtain the initial slope of the curves:

$$\left(\frac{d\rho}{dD_A}\right)_{D_A=0} = A \times \sigma. \tag{2}$$

All the curves obtained have an exponential pattern but a different initial slope and, less markedly, different values of the plateau (see Table I).

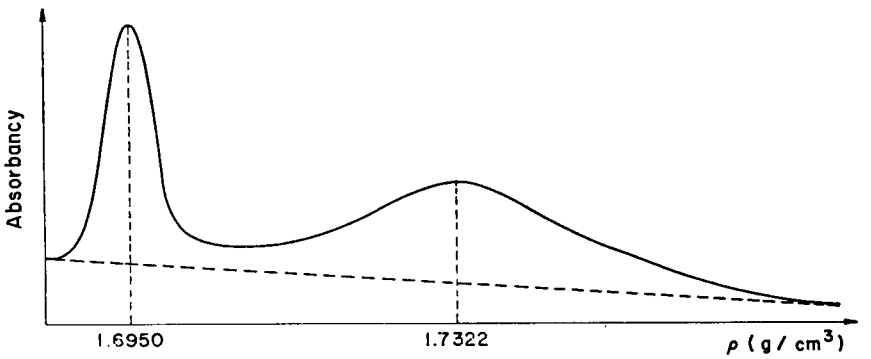

FIGURE 3 Microphotometer tracing of CsCl gradient distribution of the T_2 phage denaturated DNA irradiated at 2300 A, $D_A = 11 \times 10^{14}$ quanta/mm^2.

TABLE I

PLATEAU (A), σ, AND INITIAL SLOPE ($A \times \sigma$) VALUES AT DIFFERENT WAVELENGTHS

λ	A	σ	$A \cdot \sigma$
± 20 A	$\times 10^2$ g/cm^3	$\times 10^{15}$ mm^2/quanta	$\times 10^{17}$ g/cm^3 / quanta/mm^2
2300	—	—	1.40 ± 0.30
2400	1.93 ± 0.16	0.79 ± 0.13	1.52 ± 0.38
2500	2.10 ± 0.14	0.88 ± 0.12	1.85 ± 0.36
2600	2.26 ± 0.11	1.30 ± 0.14	2.94 ± 0.46
2700	2.33 ± 0.05	1.66 ± 0.09	3.87 ± 0.29
2800	2.39 ± 0.10	1.71 ± 0.17	4.09 ± 0.58
2900	2.18 ± 0.13	1.50 ± 0.21	3.27 ± 0.65

INTERPRETATION OF THE EXPERIMENTAL RESULTS

Identification of the Photoproducts

In interpreting the experimental results, we can first suppose that the molecular site for the photochemical process is one of the four bases in the DNA. Then it is always possible to describe the beginning of the process by

$$B + h\nu \rightarrow F, \tag{3}$$

where B is a base whose photoproduct is F. If the density ρ is a function of the number N_F of the photoproducts per mm³, we have

$$\left(\frac{d\rho}{dD_A}\right)_{D_A=0} = \left(\frac{d\rho}{dN_F}\right)_{N_F=0} \times \left(\frac{dN_F}{dD_A}\right)_{D_A=0}. \tag{4}$$

Since, for $D_A = 0$, the only contribution to the formation of F is from equation 3, we can write:

$$\left(\frac{dN_F}{dD_A}\right)_{D_A=0} = N_B \alpha_1 Q_1, \tag{5}$$

where N_B is the number of B molecules per mm³ at time $t = 0$, α_1 is the probability that a photon absorbed by the DNA is absorbed by the B molecule, and Q_1 the probability that the absorbed photon gives rise to the photoproduct F.

If we suppose that the energy transfer among bases is negligible, we have

$$\alpha_1 = \frac{\epsilon_B}{\Sigma_i \epsilon_i N_i} \propto \frac{\epsilon_B}{\mathrm{OD}}, \tag{6}$$

where ϵ_B is the molar extinction coefficient of the B nucleotide, ϵ_i is the same for the "i" nucleotide, of which we have N_i molecules per mm³ of solution, and OD is the optical density of the DNA solution. So we have

$$\left(\frac{d\rho}{dD_A}\right)_{D_A=0} \propto \frac{\epsilon_B}{\mathrm{OD}} \times Q_1 \tag{7}$$

and if Q_1 is a constant for the whole of the spectrum, the quantity

$$\left(\frac{d\rho}{dD_A}\right)_{D_A=0} \times (\mathrm{OD}) \tag{8}$$

must have a behaviour, as a function of λ, similar to that of the molar extinction coefficient ϵ_B. If, on the contrary, the energy transfers have an important role, the quantity α_1 is presumably a constant with λ and the quantity in equation 8 must behave in a similar way to the absorption spectrum of the DNA.

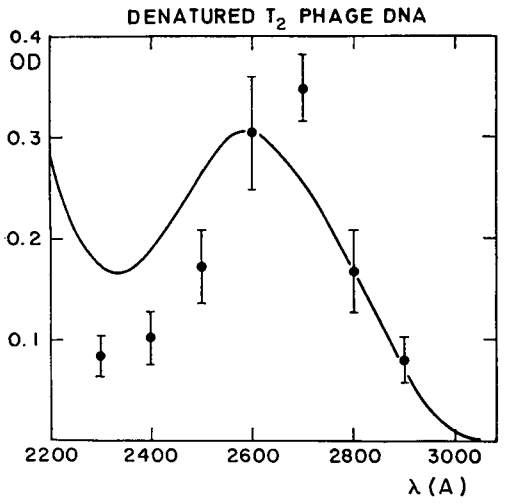

FIGURE 4 Comparison between $(dp/dD_A)_{D_A=0} \cdot (OD)$ and the absorption spectrum of the DNA.

In Figs. 4 and 5 we have plotted the experimental values obtained by 8 and have compared them with the absorption spectrum of the DNA and with the coefficient of molar extinction of the four nucleotides (Beaven, Holiday and Johnson, 1955; Wierzchowski and Shugar, 1960). We see that the agreement is good only in the case of thymidine. Such a result states that thymine is responsible for the effect observed for low doses and suggests to us that the photochemical reaction is the formation of thymine dimers. This reaction causes an equilibrium to be established between formation and breaking of thymine dimers (Setlow, 1961; Johns, Rapaport and Delbruck, 1962; Wulff, 1963); the equilibrium is a stationary state which at high doses of radiation is strongly dependent on the wavelength. The equilibrium shifts towards the dimeric form at $\lambda = 2800$ A and towards the monomeric one at $\lambda = 2400$ A.

We actually observed a higher value of plateau at $\lambda = 2800$ A than at $\lambda = 2400$ A, though the difference in the plateaus was much smaller than that observed by Wulff (1963) for dimers. We must also expect a reversibility of the effect. To check this a sample which had received a dose of 27×10^{-14} quanta/mm^3 at $\lambda = 2800$ A, was reirradiated at $\lambda = 2400$ A (Fig. 6).

Calculation of the Quantum Yield

The formation of a thymine dimer can be represented by:

$$TT + h\nu \underset{Q_2}{\overset{Q_1}{\rightleftharpoons}} \widehat{TT}, \qquad (9)$$

where Q_1 and Q_2 are the quantum yields for formation and breakage of thymine

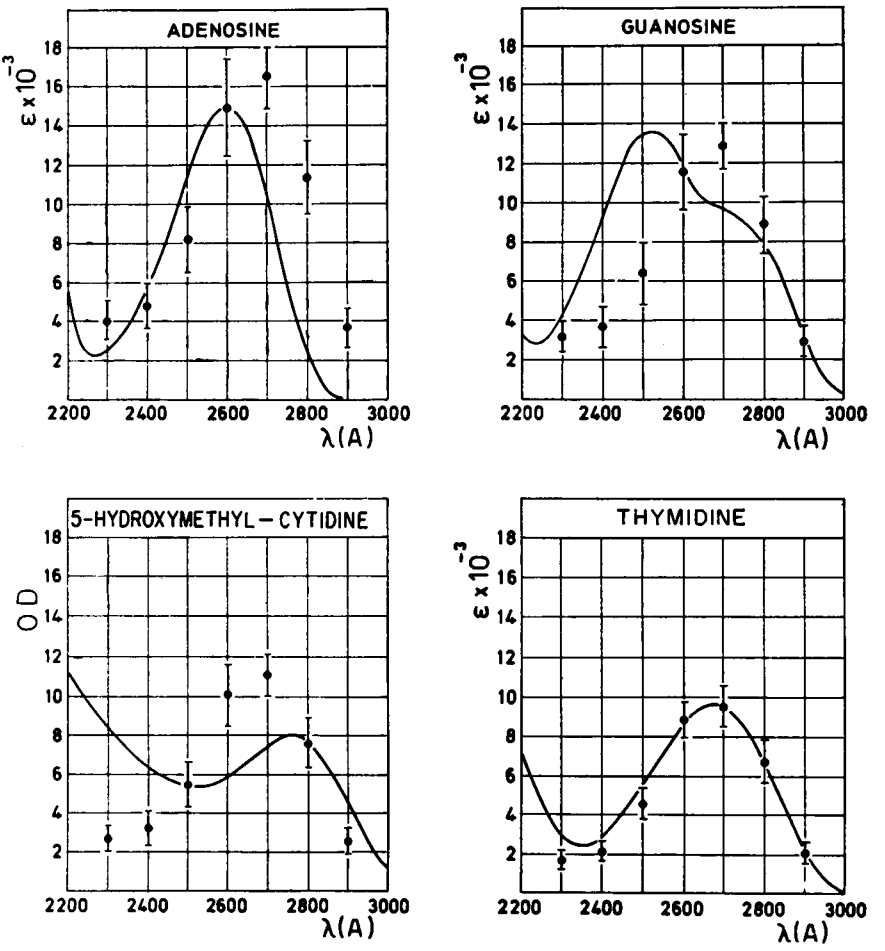

FIGURE 5 Comparison between $(d\rho/dD_A)_{D_A=0}$ (OD) and the nucleotides absorption spectra.

dimers: they are expressed as the probability of the photochemical reaction per photon absorbed by a pair (TT), or by a dimer ($\widehat{TT}$).

Our interpretation of the experimental curves assumes that the only effect of the radiation is that of dimerizing the thymine. In this way we hope to obtain information on the Q_1 and Q_2 values.

The number of thymine dimers $N_{\widehat{TT}}$ is a function of the absorbed dose; the solution of the differential equation is:

$$\frac{dN_{TT}}{dD_A} = \alpha_1 Q_1 N_{TT} - \alpha_2 Q_2 N_{\widehat{TT}} = \frac{2\epsilon_T}{\Sigma_i \epsilon_i N_i} Q_1 N_{TT} - \frac{\epsilon_{\widehat{TT}}}{\Sigma_i \epsilon_i N_i} Q_2 N_{\widehat{TT}} \quad (10)$$

These two expressions are valid: $\alpha_1 = (2\epsilon_T)/(\Sigma_i \epsilon_i N_i)$ and $\alpha_2 = \epsilon_{\widehat{TT}}/(\Sigma_i \epsilon_i N_i)$, where

the molar extinction coefficient of a thymine pair has here been assumed to be twice that corresponding to a monomer.

If the photodimerization can take place between any two thymines which are adjacent along a DNA strand, we can write:

$$\begin{cases} N_{TT} + N_{\widehat{TT}} = N_0 = \text{constant} \\ (N_{\widehat{TT}})_{D_A=0} = 0, \end{cases} \tag{11}$$

where N_0 represents the number of thymine pairs susceptible to dimerization. We can integrate equation 10[1] so that

$$\frac{N_{\widehat{TT}}}{N_0} = \frac{1}{1 + \frac{\epsilon_{\widehat{TT}}}{2\epsilon_T}\frac{Q_2}{Q_1}} \left\{ 1 - \exp\left[\left(- \frac{2\epsilon_T Q_1 + \epsilon_{\widehat{TT}} Q_2}{\Sigma_i \epsilon_i N_i} \right) D_A \right] \right\}. \tag{12}$$

If then we assume that $\rho - \rho_0 = K(N_{\widehat{TT}}/N_0)$, a linear function of $N_{\widehat{TT}}$, we find an exponential behaviour of ρ. We also have the expressions

$$\text{(a)} \qquad \left(\frac{d\rho}{dD_A}\right)_{D_A=0} = K \frac{2\epsilon_T}{\Sigma_i \epsilon_i N_i} Q_1 = K \frac{\gamma \cdot 10^6}{N} \frac{2\epsilon_T}{\text{OD}} \cdot Q_1$$

$$\text{(b)} \qquad \left(\frac{d\rho^1}{dD_A}\right)_{D_A=0} = K \frac{\epsilon_{\widehat{TT}}}{\Sigma_i \epsilon_i N_i} Q_2 = K \frac{\gamma \cdot 10^6}{N} \frac{\epsilon_{\widehat{TT}}}{\text{OD}} \cdot Q_2$$

$$\text{(c)} \qquad A = K \frac{1}{1 + \frac{\epsilon_{\widehat{TT}}}{2\epsilon_T} \cdot \frac{Q_2}{Q_1}}, \tag{13}$$

where N is Avogadro's number and γ is a constant ($\gamma = 0.72$) which takes into account the hypochromicity of the denatured DNA as compared with a mixture of free nucleotides. In equation 13 (*b*) $(d\rho^1)/(dD_A)_{D_A=0}$ is the density variation when $N_{TT} = 0$ for $D_A = 0$. This is the condition shown in the curve of Fig. 6.

From equations 13 (*a*) and 13 (*b*) we have $Q_2/Q_1 \simeq 30$; from equation 13 (*c*) we can obtain the behaviour of the A values as a function of λ by using the $\epsilon_{\widehat{TT}}/2\epsilon_T$

[1] We suppose that Q_1 and Q_2 are independent of the absorbed dose. The same is true for α_1 and α_2 because

$$D_A = D_I(1 - T); \qquad \Sigma_i \epsilon_i N_i \propto \text{OD},$$

from which we can write equation 10 as

$$\frac{dN_{\widehat{TT}}}{dD_A} = \frac{dN_{\widehat{TT}}}{(1 - T)\, dD_I} = \frac{f(N_{TT})}{\text{OD}},$$

where $f(N_{TT})$ is a function of N_{TT}. In the course of irradiation we observe that the quantity $1 - T/\text{OD}$ is practically a constant and equal to $1 - T_0/(\text{OD})_0$ measured before irradiation.

ratio found by Johns et al (1962). Our results agree well with the experimental values for $\lambda > 2600$ A, but the experimental plateaus are higher than the theoretical ones for $\lambda < 2600$ A. However, we note that for the evaluation of the quantum yield we use only the initial slopes of the curves and the value of K. This value can be evaluated at $\lambda = 2800$ A, where

$$A_{2800} = \frac{1}{1 + 8 \times 10^{-4} \times 30} K \simeq K. \qquad (14)$$

The values of Q_1 are constant for the whole spectrum. This is shown in Table II. The Q_2 value, as obtained from the curve of Fig. 6, is about 0.7. This evaluation is

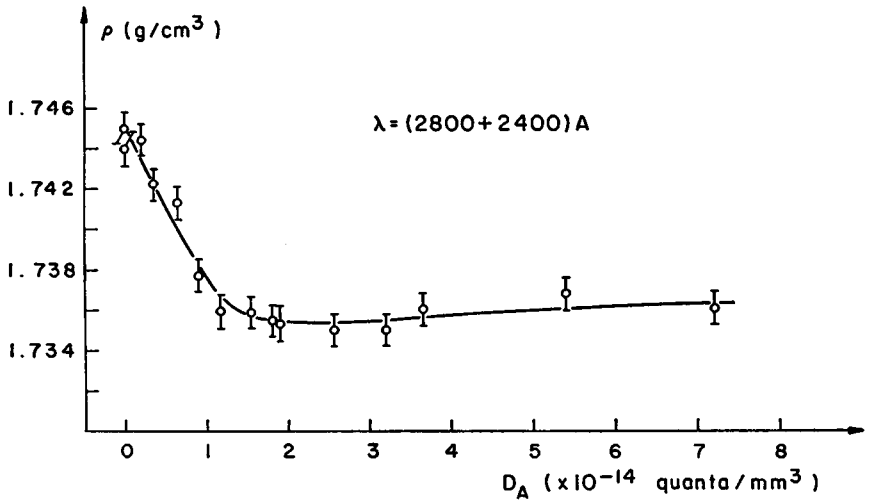

FIGURE 6 CsCl gradient density versus the absorbed dose at 2400 A of a previously irradiated sample at 2800 A, $D_A = 27 \times 10^{14}$ quanta/mm^3.

TABLE II

Q_1 AND Q_2 VALUES FOUND BY BOTH AUTHORS AND OTHERS

	T_2 denatured	*E. coli* native	$\widehat{TT} \rightarrow T + T$
Q_1	$(18 \pm 2) \times 10^{-3}$*	23×10^{-3} (2750 A)‡	
Q_2	0.7*	0.9‡	0.6§ 0.7–0.5‖

* Author's values.
‡ Wulff, 1963.
§ Setlow, 1961.
‖ Johns et al., 1962.

probably affected by a large error due to the uncertainty in estimating the initial slope of the curve. The values of quantum yields obtained by other workers on the *Escherichia coli* native DNA and on aqueous thymine dimer solutions are also shown in the same table.

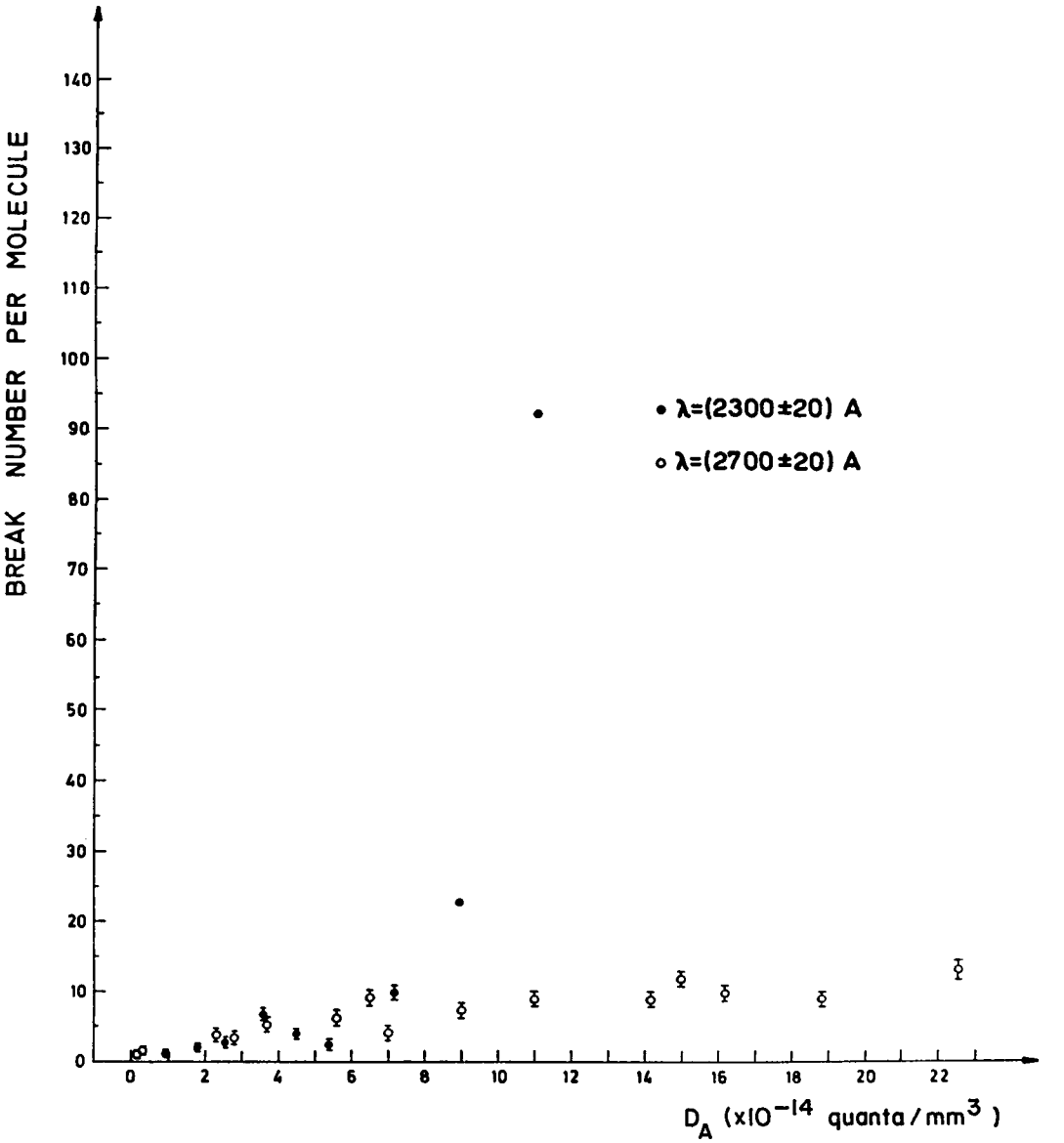

FIGURE 7 Average number of breaks per molecule versus absorbed dose at 2300 A (•) and 2700 A (○).

Broadening of the Gaussian Curves

The standard deviation "h" of the gaussian curves is related to the homogeneity of the material under study; the increase observed after the UV irradiation indicates a decrease of such homogeneity.

Because the symmetry of the curves is maintained, we can conclude that all the molecules in the solution are affected statistically by the phenomenon responsible for the increase of the "h" value. Since one of the effects of UV radiation on DNA is the creation of breaks along the sugar-phosphate backbone, the spreading of the curves experimentally observed can be considered the result of such a phenomenon.

It seems reasonable to assume that such breaks are randomly distributed throughout the irradiated material while the original sample is satisfactorily homogeneous (as revealed by boundary sedimentation: $S_{20,w} = 66$ S). Therefore the validity conditions concerning the random breaks of polynucleotides in Montroll and Simha's theory (1940) are well satisfied. An expression of the relative broadening of the gaussian curves as a function of the mean number of breaks per molecule was derived from this theory. It can be written:

$$\left(\frac{h_0}{h}\right)^2 = f(\alpha p)$$

where h_0 is the standard deviation of the gaussian curves of the unbroken molecules, while h refers to the degradated material; αp is the mean number of breaks per molecule.

"h_0" and "h" can be considered the standard deviations of the unirradiated and irradiated samples respectively. It is therefore possible on the basis of the preceding formula to calculate the mean number of breaks induced by the irradiation, at least as order of magnitude. Fig. 7 shows the behaviour of the quantity αp as a function of the dose absorbed by DNA at 2300 A and 2700 A. The behaviour at the other wavelengths is quite similar to that found at 2700 A.

DISCUSSION

Processing of the experimental data as in the previous model leads to an evaluation of the quantum yield which agrees quite well with the higher wavelength region values determined by other authors. Moreover the theoretical behaviour of the plateau values as a function of the wavelength does not coincide with our experimental values. As already noted, such a disagreement is appreciable for $\lambda < 2600$ A and particularly for $\lambda = 2400$ A, where we expect a plateau value of about one-seventh of the corresponding 2800 A value.

Two different explanations can be invoked to explain our results:
(*a*) the presence of photoproducts different from thymine-dimers;
and (*b*) a nonlinearity of CsCl density gradient variations with the number of photodimers.

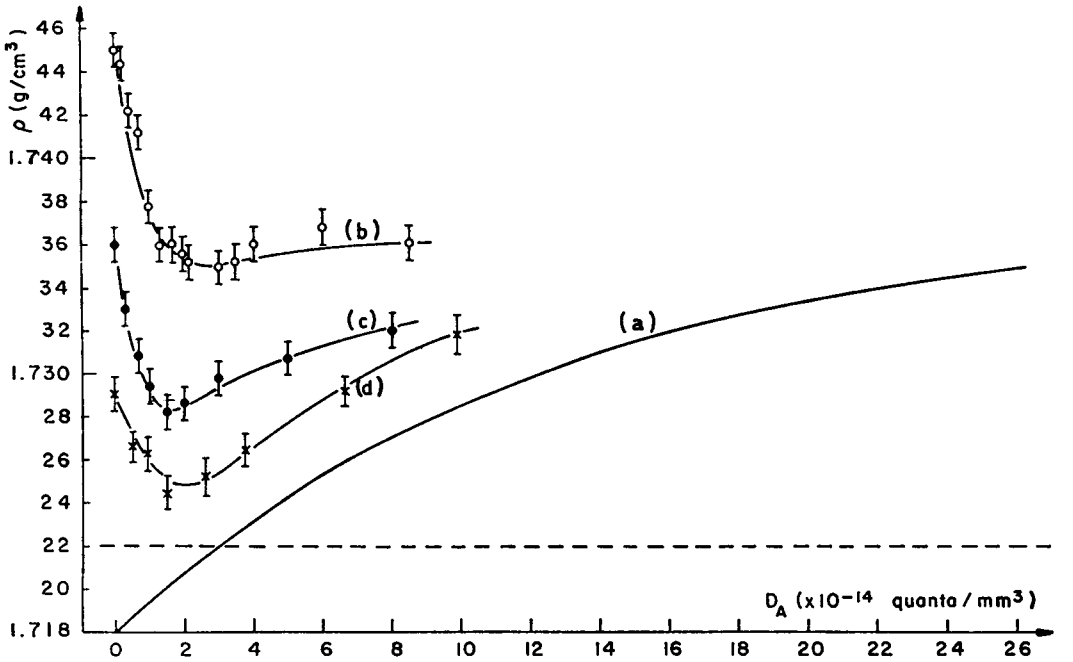

FIGURE 8 Reversion at 2400 A of radiation effect in DNA sample already irradiated with different doses at 2800 A; (*a*) direct irradiation at 2400 A; (*b*) D_A (2800 A) = 27 × 10^{14} quanta/mm²; (*c*) D_A(2800 A) = 10 × 10^{14} quanta/mm²; and (*d*) D_A(2800 A) = 3 × 10^{14} quanta/mm². The dashed line is theoretical "plateau" value for direct irradiation at 2400 A.

To discriminate between these two possibilities, samples which have already received different doses of radiation at λ = 2800 A have been reirradiated at λ = 2400 A. The results obtained are shown in Fig. 8. It is evident that in curves (*c*) and (*d*) we have a further lowering of the density with respect to the plateau value of curve (*a*).

This behaviour can be expected from the first hypothesis but not from the second one. In this second case we start with an equilibrium condition and do not expect any density change. The observed behaviour therefore seems to confirm the theory of a new photoproduct. On the other hand it is known that thymine photoproducts which are different from the dimers have been isolated. (Johns, et al, 1964; Varghese and Wang, 1967). The formation of such a photoproduct can change the plateau values, especially in the shorter wavelength region where the dimer contribution is very small. We can thus infer that the model used for the computation of the quantum yields in the shorter wavelength region probably does not work.

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