Comparison of the *in vitro* Action of Ethidium Chloride on Animal Viruses with that of other Photodyes

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**SUMMARY**

Four viruses were irreversibly photosensitized by $4 \times 10^{-2}$M-ethidium chloride but not by more dilute solutions. Photosensitization was greatest with vaccinia, pseudorabies, herpes tamarinus and reoviruses, intermediate with western equine encephalitis virus, and least with fowl plague and Newcastle disease viruses. Pseudorabies virus was also inactivated by ethidium chloride at $37^\circ$ in the dark. The order of susceptibility of the viruses to other dyes varied according to the concentration of dye. Native thymus DNA, but not heated DNA, inhibited the action of ethidium chloride on viruses. Toluidine blue and neutral red were more strongly inhibited by heated DNA than by native DNA, and both DNAs inhibited the action of proflavine to the same extent.

Toluidine blue and proflavine catalysed the degradation of guanine by light in both native and heated thymus DNA. The action of neutral red differed in two respects: at a low ratio of dye to nucleotide molecules, the cytosine of native DNA was also degraded; at a higher ratio, only the guanine of heated DNA was destroyed. There was no effect of ethidium chloride on any of the four bases of DNA.

**INTRODUCTION**

Dyes specifically combining with nucleic acids may display one or both of the following biological activities: (1) they catalyse the photoinactivation of viruses, (2) disturb the metabolism of cells in the dark. Altered cellular metabolism may or may not result in the inhibition of virus growth, depending on which virus is being synthesized. This paper compares the *in vitro* action of a phenanthridine (ethidium chloride) with that of an acridine (proflavine), a thiazine (toluidine blue) and a phenazine (neutral red), and attempts to correlate virus susceptibility with the known mode of interaction of dyes with isolated nucleic acids. Peacocke & Skerrett (1956) showed that proflavine-DNA complexes are bound by a strong interaction process at low concentrations of proflavine; when $r$ (i.e. the ratio of dye molecules bound per atom of DNA phosphorus) is greater than 0·2 a weaker binding process occurs which probably depends on proflavine-proflavine interactions. Optical studies of the complexes at low values of $r$ indicated that the acridine molecules are intercalated between base pairs, with their rings parallel to those of the bases (Lerman, 1963). Using refined viscosimetric method, the intercalation process was correlated with an increased stiffness of the DNA molecules, while no increased viscosity occurred for $r > 0·2$ (Drummond *et al.* 1966). In spectrophotometric assays denatured DNA bound
more proflavine molecules than native DNA, although the strength of binding was similar with both DNAs (Drummond, Simpson-Gildemeister & Peacocke, 1965). The mode of interaction of ethidium bromide with nucleic acids was found by Waring (1965) to be similar to that of proflavine; in addition, this author showed that, for $r < 0.2$, DNA bound more ethidium than RNA, while the reverse situation occurred for $r > 0.2$; native and denatured DNA displayed similar binding capacities for ethidium, but synthetic nucleic acid homopolymers required a hydrogen-bonded secondary structure in order to combine with the drug (Le Pecq & Paoletti, 1965; Waring, 1966a, b). The mode of reaction of neutral red and toluidine blue with nucleic acids is not well understood.

When the above dyes are combined with biologically active nucleic acids and then excited with light waves, inactivation occurs. The susceptibilities of different viral nucleic acids to photoinactivation vary over a wide range and seem to depend on extrinsic and intrinsic factors.

Complete virus particles treated with very high concentrations of toluidine blue (6 μg./ml.) fall grossly into two groups, according to their susceptibility to light (Hiatt, 1960); enteroviruses mostly formed the resistant group, a fact which was ascribed to impermeability of the protein coat to the dye, since the isolated RNA of poliovirus was photosensitized by toluidine blue and the same virus became photosusceptible after growth in the presence of proflavine (Schaffer, 1962). Photosensitization of poliovirus RNA in vitro is not, however, necessarily identical to that achieved during intracellular growth in vivo, since neutral red photosensitized the viral RNA in the latter and not in the former situation (Wilson & Cooper, 1965). Another extrinsic factor was found to influence the photosensitization by dyes: Wallis & Melnick (1963) showed that complete poliovirus particles could be rendered photosusceptible, provided that no organic substance was present in the dye + virus mixture; Sprecher-Goldberger (1965) first described the photosensitization of some viruses by ethidium chloride and noticed that the action of the dye on Newcastle disease virus was decreased by the presence of serum, while no such influence occurred with arboviruses.

On the other hand, two intrinsic factors may influence the susceptibility of viral nucleic acids to photodyes. First, the above mentioned physicochemical studies on the nucleic acid + dye complexes indicated that secondary structure might play a part at least in the amount of drug bound, although there is no indication that inactivation by light is solely dependent on the quantity of combined dye. Secondly, a specific damage of guanine was observed after the photodynamic action of methylene blue on bacterial DNA (Simon & Van Vunakis, 1962) and of acridine orange on TMV RNA (Sastry & Gordon, 1966). Singer & Fraenkel-Conrat (1967) found a similar action of proflavine on TMV RNA.

METHODS

Viruses. Fowl plague and pseudorabies viruses were received from Dr H. G. Pereira and herpes tamarinus virus from Dr F. Deinhardt. Type 2 reovirus, strain D-5 (Jones) was obtained from Dr E. H. Lennette. The other viruses were those previously used in this laboratory.

Preparation of virus stock. All viruses, except reovirus, were grown on monolayers of chick embryo fibroblasts in Eagle's medium without serum or phenol red. When cytopathic effect occurred, the cultures were frozen and thawed three times; cellular debris was discarded by low speed centrifugation. Vaccinia virus was partially purified
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by centrifugation through a 36% solution of sucrose for 80 min. at 23,000 g; the pellet was resuspended in phosphate buffered saline at pH 7.3 (PBS). Type 2 reovirus was adapted to the human amnion cell line FL; after three passages in these cells, virus stocks were prepared as described for the other viruses.

Virus assay. All the viruses except reovirus were titrated by the plaque method in monolayers of chick embryo fibroblasts overlaid with Eagle's medium + dialysed calf serum. Neutral red was added after 2 days for western equine encephalitis virus, 3 days for fowl plague virus, 4 days for Newcastle disease virus, vaccinia and pseudorabies viruses and 5 days for herpes tamarinus.

Owing to the difficulty of titrating reoviruses by the plaque method, assays of type 2 reovirus were made by an indirect immunofluorescence method derived from those of Spendlove et al. (1963) and of Brown et al. (1964). In short, the virus was assayed on confluent monolayers of FL cells grown on cover slips in Leighton tubes. After 20 hr incubation at 37° the cover slips were washed in PBS, fixed in acetone and dried in air. Each cover slip was then completely covered with two drops of rabbit antiserum against type 2 reovirus diluted 1/10 and incubated for 1 hr at 37° in humidified Petri dishes placed in a humidified incubator. The cover slips were then washed in PBS and covered with three drops of a 1/30 to 1/40 dilution of a fluorescein-labelled duck antiserum against rabbit y-globulin (kind gift from Dr A. Betz). They were again incubated for 1 hr at 37° in the same humid conditions and then washed in PBS and mounted in a 10% solution of glycerol in PBS. All preparations were examined under a binocular Zeiss fluorescence microscope with a Neoflu 40 x objective. For each preparation the number of fluorescent cells in a minimum of ten perfectly confluent microscopical fields was counted and the titre was expressed in arbitrary units: average number of infected units per microscopical field multiplied by the dilution factor. Each result shown in the text is the mean of the figures obtained with four different dilutions. As the results were only utilized for calculation of k (see below), the utilization of arbitrary units for titre expression was justified.

Chemicals. Ethidium chloride was a kind gift of Dr Gurd, of Boots Pure Drug Company, Nottingham, England. Other chemicals were of commercial origin and were used without further purification.

Photosensitization of viruses. Fresh solutions of dyes were prepared in 0.1 N-phosphate buffer at pH 8 and added to virus preparations diluted in the same buffer. One ml. samples of the mixtures were introduced into cotton-stoppered narrow tubes; they were preincubated in the dark at 37° (or at other temperature if specified) for various periods and then exposed to light at a distance of 7 cm. from two 150 w daylight lamps. The tubes were placed in a glass vessel and cooled with running water. The virus survivors were then titrated in dim light. The constant of the rate of inactivation was calculated by the formula: k = \[\log_{10} (v_0/v)\]/t where v_0 is the virus titre before irradiation and v is the titre of survivors after irradiation for time t min.

Quantitative analysis of DNA bases after the photodynamic action of dyes. Thymus DNA was dissolved in 0.05 M-borate buffer at pH 9, at a concentration of 500 µg./ml. DNA was denatured by heating at 100° for 5 min. followed by cooling in an ice-bath. Various concentrations of dye were added to parallel samples of native or denatured DNA; the mixtures, in total volumes of 1 ml., were placed in narrow tubes and illuminated for 8 hr with a 150 w lamp while being cooled by running tap water. The DNA was then precipitated by two volumes of ice-cold ethanol, redissolved in
citrate saline (0.15M-NaCl + 0.015M-sodium citrate solution) and precipitated a second time to wash the dye out. The DNA was hydrolysed in 88% formic acid at 175° for 30 min.; the four bases were isolated by paper chromatography (Smith & Wyatt, 1951) and estimated quantitatively by optical density at their maximal absorption wavelength.

RESULTS

Photosensitization of various viruses by ethidium chloride

To estimate the photosusceptibility of the viral nucleic acids to dyes, the speed of penetration of the dye through the virus capsid must be taken into account. For this reason the virus preparations were pretreated in the dark with 15 μg./ml. of ethidium chloride, at 37°, for periods of time varying from 1 to 150 min.; they were titrated and then exposed to light; the residual infectivity was determined after 5, 10 and 15 min. illumination and logarithms of survivors were plotted against time. For each virus preparation a series of inactivation curves was thus obtained, corresponding to various times of pretreatment with the dye. In the present series of experiments these

![Fig. 1. Comparison of the photosusceptibility of six different viruses after pretreatment with ethidium chloride for different periods. The pairs of points relate to two separate experiments. The curves obtained with Newcastle disease and fowl plague viruses were superposable. • Vaccinia virus; ○, reovirus type 2; ■, pseudorabies virus; □ western equine encephalitis virus; △, Newcastle disease and fowl plague viruses.

Fig. 2. Further comparison of the rate of photosensitization of western equine encephalitis and vaccinia viruses by ethidium chloride. A: concentration of ethidium chloride reduced from 15 μg./ml. to 10 μg./ml. B: the temperature of incubation during treatment with ethidium chloride was decreased from 37° to 30°. •, vaccinia virus; ○, western equine encephalitis virus.](image)
curves were straight lines. The constants of the rate of inactivation \((k)\) first increased according to the length of pretreatment with dye, and then reached, or tended to reach, a plateau (Fig. 1). The slope of the rising part of the curves is taken as indicating the rate of access of the dye through the virus envelope, and the level of the plateau as indicating the intrinsic susceptibility of the virus nucleic acid to the photodynamic action of the dye. If such premises are true, the envelopes of Newcastle disease and fowl plague viruses are a more efficient barrier to the dye than the envelopes of western equine encephalitis, pseudorabies and vaccinia viruses. The curve obtained with herpes tamarinus was similar to that of pseudorabies virus. On the other hand, if one considers the constants of inactivation obtained at the moment the graphs curve towards a plateau, the viral nucleic acids can be arranged according to the following order of increasing susceptibility to photosensitization by ethidium chloride: Newcastle disease and fowl plague viruses, western equine encephalitis virus, reovirus, pseudorabies, herpes tamarinus and vaccinia virus. With reovirus, owing to the difficulties of titration by the immunofluorescence technique, the constants of inactivation were only determined after one period of pretreatment with the dye (10 min. at 37°C).

It is noteworthy that the above arrangement of viruses corresponds to an arrangement according to the amount of secondary structure of the viral nucleic acids: double stranded DNA and RNA viruses (vaccinia, pseudorabies, herpes tamarinus and reovirus) are the most susceptible to photosensitization by ethidium chloride, while single-stranded Newcastle disease and fowl plague viruses are the least susceptible. The intermediate susceptibility of western equine encephalitis virus is in agreement with several points of evidence indicating that the RNA of arboviruses is specially rich in secondary structure (Sprecher-Goldberger, 1965; 1967) and in particular with the fact that Sindbis RNA shows a greater hyperchromic effect upon heating than Newcastle disease virus does (Sokol, Skacianska & Pivec, 1966; Sprecher-Goldberger, 1967). From Fig. 1, it seems that western equine encephalitis and vaccinia viruses differ by their constant of photoinactivation, for each point of the abscissa, i.e. for each period of pretreatment with the dye, but that the slope of the curves, i.e. the rate of access to the photosusceptible target, is the same with both viruses. This was confirmed by experiments comparing the constants of photoinactivation of the two viruses, when they were either pretreated with 15 \(\mu g./ml\) ethidium chloride at 30° instead of 37° (Fig. 2B), or with 10 \(\mu g./ml\) ethidium chloride instead of 15 (Fig. 2A). The constants were lower with western equine encephalitis than with vaccinia virus, but the slopes of the curves according to pretreatment time were identical for both viruses.

For most of the viruses shown in Fig. 1, treatment of the viruses by ethidium chloride at 37° in the dark did not significantly inactivate infectivity, except for pseudorabies and herpes tamarinus virus, which were very susceptible to the action of the dye at 37°, although its toxic effect was reduced in the presence of 10 % calf serum and even more when treatment was at 0° (Fig. 3). The fact that ethidium chloride only slightly inactivated pseudorabies virus at 0° could mean either that, at 0°, little or no fixation of dye occurred on the susceptible target or that ethidium chloride actually sensitized the virus to heat. The former hypothesis was supported by an experiment also shown in Fig. 3: pseudorabies virus was treated with ethidium chloride for 30 min. at 0° and then diluted 1/10 and placed in a water bath at 37°: after a lag, slight inactivation occurred, indicating that it took some time for the low concen-
tration of residual ethidium chloride (1.5 μg./ml.) to exert some effect on the virus. At high concentration, therefore, ethidium chloride did not adsorb on the virus at 0°, but it is not known whether the lethal effect observed at 37° is due only to a much higher efficiency of adsorption, or to sensitization to heat.

![Figure 3. The action of ethidium chloride on pseudorabies virus in the dark.](image)

Irreversibility of photosensitization by ethidium chloride

Wallis & Melnick (1964) studied the binding of neutral red, proflavine and toluidine blue with various viruses and found that the dye could be removed from some of the virus + dye mixtures by cationic resins. After this treatment, the mixtures which consisted of reversible complexes lost their photosusceptibility while irreversible complexes were still inactivated by light. The property of reversibility depended on both the virus and the dye. Using the same experimental procedure, Sprecher-Goldberger (1965) showed that ethidium chloride formed irreversible complexes with Sindbis virus, while toluidine blue was eluted from the same virus by cationic resins.

We have experimented with some combinations of virus and dye not studied by the above-mentioned authors. The virus preparations were treated for 30 min. at 37° in the dark with dyes at various concentrations (Fig. 4). Half of each virus + dye mixture served as a control and was left in the dark at 0°, while the other half was gently agitated at pH 9 in the presence of prewashed Dowex resin 50 W-×4 (H+), 50 to 100 mesh (Wallis & Melnick, 1964). The supernatant fluid, containing the virus
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without free dye, was assayed for photosusceptibility and compared with the control preparations. Newcastle disease, western equine encephalitis and vaccinia viruses treated with ethidium chloride showed identical inactivation curves, whether they had been in contact with Dowex resin or not. In contrast, western equine encephalitis virus photosensitized by proflavine or neutral red became completely resistant to light after removal of the dyes by resin. Finally, the action of proflavine, neutral red or toluidine blue on Newcastle disease virus was partly reversed by resin treatment. From these results, and those obtained by Sprecher-Goldberger (1965), it cannot be said that ethidium chloride necessarily forms irreversible complexes with all viruses, while

Fig. 4. Assay of the reversibility of dye action. A, Newcastle disease virus; B, western equine encephalitis virus; C, vaccinia virus; continuous line, control mixtures; broken line, mixtures treated with Dowex resin. ○, ethidium chloride, 15 μg.; ●, ethidium chloride, 13 μg.; ★, ethidium chloride, 10 μg.; □, proflavine, 0.2 μg.; ■, proflavine, 0.01 μg.; △, neutral red, 0.5 μg.; ▲, neutral red, 0.001 μg.; ▼, toluidine blue, 0.1 μg.

Fig. 5. The influence of dye concentration on the photosensitization of viruses. ———, vaccinia virus; ———, pseudorabies virus. ○, proflavine, 10⁻¹M; ●, proflavine, 10⁻³M; □, neutral red, 10⁻⁸M; ■, neutral red, 10⁻⁶M.

The order of susceptibility of various viruses to photosensitization by toluidine blue or proflavine: variations of this order according to the dye and to its concentration

Attempts to compare the action of ethidium chloride with that of other dyes met with difficulties, since strictly comparable conditions could not be applied. Photosensitization was obtained after pretreatment of the viruses for 10 to 15 min. at 37°C in the dark with 4 × 10⁻⁸M-ethidium chloride (15 μg./ml.). Since the other dyes are much more potent photosensitizers, their action under similar conditions resulted in photoinactivation curves which were very steep and did not allow the comparison between different viruses. In order to obtain convenient photoinactivation, smaller doses of dyes were
used, but it was then found that, for a given dye, the order of susceptibility of the various viruses varied with the concentration of the dye (Fig. 5). Pseudorabies virus was more susceptible than vaccinia virus to $10^{-7} M$ solutions of the dyes, while the order of susceptibilities was reversed with $10^{-8} M$ solutions. Experiments were repeated with pseudorabies, vaccinia, Newcastle disease and western equine encephalitis viruses under conditions slightly different from those reported in Fig. 5. With $10^{-8} M$ solutions of the dyes, the viruses were pretreated for 30 min. at $0^\circ$, since at these high doses toluidine blue inactivated western equine encephalitis and pseudorabies viruses at $37^\circ$ in the dark. However, dye treatment at $0^\circ$ precluded a comparison with Newcastle disease virus, since at this temperature, penetration of the dyes through the capsid of

![Diagram](image-url)

**Fig. 6.** The influence of dye concentration on the photosensitization of vaccinia, pseudorabies and western equine encephalitis viruses. With $10^{-8} M$ solutions of the dyes, the viruses were pretreated for 30 min. at $0^\circ$ instead of 10 min. at $37^\circ$ for low doses of the dyes. In the case of Newcastle disease virus the results presented here concern only the lower concentration of the three dyes (see text). To illustrate the results, the $k$ obtained at two concentrations were bound by straight line. □—□, western equine encephalitis virus; ○—○, pseudorabies virus; ●—●, vaccinia virus; ■, Newcastle disease virus; A: Toluidine blue; B: proflavine; C: neutral red.

Newcastle disease virus is poor and a maximum of photosensitization was not unambiguously reached. With low concentrations of dyes (toluidine blue $6 \times 10^{-8} M$, neutral red $2 \times 10^{-7} M$ and proflavine $10^{-7} M$), the viruses were left in contact with the dyes for 10 min. at $37^\circ$ before illumination; this period was sufficient for maximum photosensitization. The treated viruses were then illuminated. The photoinactivation curves obtained were, in a few instances, multisloped; the constants of the rate of photoinactivation were calculated for the initial slope of the curves. These constants
were plotted against the logarithm of the concentration of dye used during pretreatment of the viruses (Fig. 6). The following facts are noted: (a) at the lower concentration of neutral red and of proflavine used (2 \times 10^{-7}M and 10^{-7}M, respectively), saturation of pseudorabies virus with dye has already been reached, since doses of dye 50 to 100 times higher do not result in an increased photosensitization of this virus. With toluidine blue, there is a slight increase of photosensitization when the dye concentration is raised from 6 \times 10^{-8}M to 10^{-7}M; (b) in contrast, photosensitization of western equine encephalitis virus increases steeply according to the concentration of neutral red or toluidine blue whereas saturation is nearly reached at the low concentration of proflavine; (c) vaccinia virus behaves almost identically towards the three dyes, and the variation of photosusceptibility according to dye concentration is intermediate between that of pseudorabies and western equine encephalitis viruses.

Table 1. Inhibition of the action of dyes by native or heated DNA

<table>
<thead>
<tr>
<th>Pretreatment of western equine encephalitis virus with</th>
<th>Illumination (min.)</th>
<th>Virus survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium chloride 4 \times 10^{-4}M; 15 min.; 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Citrate saline</td>
<td>10</td>
<td>0.003</td>
</tr>
<tr>
<td>+ DNA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ heated DNA</td>
<td></td>
<td>0.001</td>
</tr>
<tr>
<td>Proflavine 2 \times 10^{-7}M; 2 min.; 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Citrate saline</td>
<td>15</td>
<td>0.01</td>
</tr>
<tr>
<td>+ DNA</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>+ heated DNA</td>
<td></td>
<td>0.7</td>
</tr>
<tr>
<td>Toluidine blue 2 \times 10^{-7}M; 2 min.; 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Citrate saline</td>
<td>5</td>
<td>0.01</td>
</tr>
<tr>
<td>+ DNA</td>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>+ heated DNA</td>
<td></td>
<td>0.3</td>
</tr>
<tr>
<td>Neutral red 2 \times 10^{-7}M; 2 min.; 37°C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ Citrate saline</td>
<td>30</td>
<td>0.22</td>
</tr>
<tr>
<td>+ DNA</td>
<td></td>
<td>0.38</td>
</tr>
<tr>
<td>+ heated DNA</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

The influence of different dye concentrations explains the permutations in virus photosusceptibilities obtained when the concentrations of proflavine and neutral red were modified within the range of 10^{-7} to 10^{-6}M. Within this range, the order of virus photosusceptibilities to toluidine blue was not modified; however, from Fig. 6, it may be expected that at concentrations of toluidine blue lower than 6 \times 10^{-8}M, photosensitization of western equine encephalitis virus would be less than that of pseudorabies virus.

The action of dyes probably depends on more factors than one; among possible factors are the amount of secondary structure and the base composition of the viral nucleic acids. Attempts were thus made to correlate the above results with the action of dyes on a commercial preparation of DNA.

Photosensitization of western equine encephalitis virus in the presence of added DNA

A solution containing calf thymus DNA 0.16 mg./ml. was prepared in citrate saline. Half of the solution was heated for 15 min. at 100°C and quickly cooled. Both preparations of DNA were preincubated with dyes and then incubated with western equine encephalitis virus 2 \times 10^6 p.f.u./ml. The preparations were illuminated and surviving...
fractions determined (Table 1). DNA competed with the virus for the dyes; however, the comparison of native with heated DNA shows varied results according to the dye. Ethidium chloride alone was inhibited only by native DNA. Such competition experiments grossly evaluate the binding capacity of the dyes for single-stranded or double-stranded structures; they demonstrate a specific binding capacity of ethidium chloride for hydrogen-bonded nucleic acid chains and a preferential binding capacity of toluidine blue and neutral red for single-stranded structures; in this test, proflavine did not differentiate between the two structures. The binding capacity of dyes for nucleic acids depends on both the amount of binding sites on the nucleic acid and the dissociation constant of the reaction, i.e. on its reversibility. The following experiment showed that the low fixation of ethidium chloride on single-stranded DNA is mostly due to the greater reversibility of this reaction, as compared to the binding with native DNA. A $3.8 \times 10^{-4}$M solution of native or heated DNA in citrate saline was mixed with $6.9 \times 10^{-5}$M ethidium chloride. The amount of bound ethidium chloride was estimated by measuring the absorbency at 510 nm, the wavelength at which maximum absorption occurs with ethidium chloride+nucleic acid complexes; the figures were roughly the same ($E = 0.18$) with the two solutions of DNA. The two mixtures were then dialysed for 4 days against citrate saline. The results were: $E = 0.04$ for double-stranded DNA and $E = 0.02$ for single-stranded DNA; i.e. double-stranded DNA retained twice as much ethidium chloride as single-stranded DNA.

**Modification of DNA bases after the photodynamic action of dyes**

Native or heated thymus DNA was mixed with dyes and illuminated for 8 hr; then base residues were isolated and quantitatively analysed. The proportions of guanine, cytosine and thymine versus adenine were compared in the DNA+dye complexes after illumination or after the same period in the dark.

<table>
<thead>
<tr>
<th>Molar ratio, dye: nucleotide</th>
<th>Native DNA</th>
<th>Heated DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>G/A C/A T/A</td>
<td>G/A C/A T/A</td>
</tr>
<tr>
<td>Proflavine $2 \times 10^{-4}$M</td>
<td>1:6</td>
<td>0.65 0.81 0.94</td>
</tr>
<tr>
<td>Toluidine blue $2 \times 10^{-4}$M</td>
<td>1:4</td>
<td>0.64 0.82 1.11</td>
</tr>
<tr>
<td>Neutral red $2 \times 10^{-5}$M</td>
<td>1:18</td>
<td>0.64 0.67 1.16</td>
</tr>
<tr>
<td>Neutral red $5 \times 10^{-6}$M</td>
<td>1:7.5</td>
<td>0.85 0.81 1.15</td>
</tr>
</tbody>
</table>

*No dye, light only; or dye in dark.*

The molar ratio of thymine to adenine was in every case equal to $1 \pm 0.15$. The ratio of guanine to adenine residues was reduced to about 0.65 in DNA ($500 \mu g./mL.$) illuminated in the presence of $2 \times 10^{-4}$M-proflavine or toluidine blue whether the DNA had been heated or not (Table 2), but only for molar ratios of dye:nucleotide of 1:6 and 1:4. Neutral red gave different results: at a molar ratio of 1:7.5, this dye catalysed the photodegradation of about 20% of the guanine residues in denatured but not in native DNA. With lower molar ratios, however (1:18 and 1:15) photodegradation
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of guanine (about 12%) occurred in both DNAs and was, moreover, accompanied by a similar degradation of cytosine in native DNA.

Molar ratios of ethidium chloride to nucleotides ranging from 1:8 to 1:0.6 were assayed with DNA concentrations from 130 to 500 µg/ml: no modification of normal base ratios was found.

DISCUSSION

The action of various concentrations of dyes on a known number of p.f.u. was studied; since the number of physical particles of viruses was not counted, the results will not be discussed in terms of ratios of dye molecules to nucleotides.

Some comparison between the intrinsic susceptibilities of various viruses to a given photodynamic can be made by estimating the photosusceptibilities obtained at saturation, i.e. when raising the dye concentration does not cause any further increase in virus photosensitization. The present data indicate that the amount of dye necessary to achieve this saturation is not related to the molecular weight of the viral nucleic acid. Photosensitization of pseudorabies virus (mol. wt of nucleic acid = 7 × 10^7 D) reached a maximum at concentrations of neutral red and proflavine ≤ 2 × 10^{-7} or 10^{-5} M. In contrast, photosensitization of western equine encephalitis virus (mol. wt of nucleic acid = 2 × 10^6 D) was still increased above these concentrations; a neutral red or toluidine blue concentration of 2 × 10^{-8} M increasing to 10^{-4} M increased greatly the constants, k, of inactivation of this virus while, within these ranges of concentrations, the photosensitizing action of proflavine was only slightly modified.

Extrapolation of the results in Fig. 6 to lower concentrations of dyes predicts that at the lowest doses capable of inducing photosensitization (k > 0), the viruses would be arranged in the following order of increasing susceptibility to neutral red, proflavine and toluidine blue: vaccinia virus (35% guanine + cytosine), western equine encephalitis virus (26% G; 25% C) and pseudorabies virus (74% G+C). The correlation between virus susceptibilities to low concentrations of dyes and their guanine contents may not be fortuitous, since other results obtained here indicate that, in the presence of light, toluidine blue and proflavine specifically degrade the guanine of DNA and that this action disappears when the dye concentration is increased. The target for neutral red action was G+C or G alone, depending on the experimental conditions. On the other hand, using several concentrations of ethidium chloride no photodegradation of any purine or pyrimidine was obtained and the photosusceptibilities of the viruses were not arranged according to the base composition of their nucleic acids.

At high dye concentrations of 10^{-4} M, western equine encephalitis is the virus most susceptible to neutral red, proflavine and toluidine blue. Extrapolation towards higher concentrations indicates that vaccinia virus tends to become as susceptible to proflavine as western equine encephalitis virus. Possibly, therefore, when the dye concentration becomes too high to achieve degradation of guanine the secondary structure of viral nucleic acids may become prominent in determining photosensitization. This hypothesis agrees with the three following findings: (1) toluidine blue and neutral red showed greater binding capacity for denatured than for native DNA; therefore, they might have been expected to react more with single-stranded RNA of western equine encephalitis virus than with double-stranded DNA of vaccinia and pseudorabies viruses; (2) proflavine had similar binding capacity for both denatured and native
DNA; this may explain why the differences between the susceptibilities to this dye of vaccinia and western equine encephalitis viruses tend to disappear at the high dye concentrations; (3) ethidium chloride bound more firmly to native than to denatured DNA; since this action was not complicated by a selective effect on one purine or pyrimidine, the influence of hydrogen-bonded structures on virus susceptibilities is more conspicuous; it accounts for the high susceptibilities of double-stranded DNA viruses (vaccinia, pseudorabies, herpes tamarinus) and double-stranded RNA viruses (type 2 reovirus), as compared to that of viruses with single-stranded RNA (western equine encephalitis and Newcastle disease).

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