Antibodies to the Methylene Blue Sensitized Photooxidation Product in Deoxyribonucleic Acid*

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ABSTRACT: Irradiation of deoxyribonucleic acid (DNA) with visible light in the presence of methylene blue and oxygen produces a lesion in the molecule which can elicit the formation of specific antibodies in rabbits. At appropriate antiserum dilution, there is no serologic reaction between the antiphotooxidized DNA and nonirradiated DNA. At these antiserum dilutions, the serologic activities of native and denatured calf thymus, *Proteus vulgaris*, and *Pseudomonas aeruginosa* DNA's are observed only after irradiation and increase with time of reaction.

The serologic activities of photooxidized native DNA probably reflect exposure of single strand sequences to the antibodies. If the four nucleotides present in DNA are irradiated, only the deoxyguanylic acid (dGMP) photoproduct inhibits the serologic activity of the immune system. The inhibitory effectiveness of dGMP increases as photooxidation proceeds and is related to the loss of absorbance of the dGMP. The rate of appearance of serologic activity with DNA is dependent upon pH and the concentration of methylene blue.

Methylene blue can sensitize the specific photooxidation of guanine derivatives and the guanine residues in nucleic acids (Simon and Van Vunakis, 1962, 1964; Wacker *et al.*, 1963). With guanine derivatives, the reaction resulted in the loss of the characteristic ultraviolet absorbance spectra and the uptake of 1 mole of O_2/mole of substrate (Simon and Van Vunakis, 1962, 1964). The specific photooxidation products in the nucleic acids, however, have not been identified. At least part of the product must still be attached to the polydeoxyribophosphate backbone (Simon, 1963), otherwise specific cleavage at the point of base destruction should occur, such as that found in apurinic and apyrimidinic acids (Michelson, 1963).

The successful use of methylated bovine serum albumin–nucleic acid complexes to obtain antibodies to nucleic acids (Plescia *et al.*, 1964, 1965; Seaman *et al.*, 1965a,b) suggested that antibodies might be produced to the specific methylene blue sensitized photooxidation product, as well as to the normal constituents of deoxyribonucleic acid (DNA). Antiphotooxidized T_4-DNA did contain antibodies to glucosylated hydroxymethylcytosine, deoxyguanylic acid (dGMP), and deoxycytidylic acid (dCMP) residues and to products resulting from exposure of the DNA to visible light in the presence of methylene blue (Seaman *et al.*, 1965a).

The present report characterizes the serologic reaction between the methylene blue sensitized photooxidation product in DNA from various sources and antibodies to methylene blue photooxidized salmon sperm, *P. vulgaris* and *Ps. aeruginosa* DNA's.

Materials and Methods

DNA. Salmon sperm DNA and calf thymus DNA were purchased from Calbiochem and used without further purification. DNA from *P. vulgaris* and *Ps. aeruginosa* was isolated according to the procedure of Marmur (1961).

Photooxidation. Native or heat-denatured DNA was photooxidized at a concentration of 500 μg/ml in 0.1 M Tris buffer, pH 8.5, in the presence of 20 μg/ml of methylene blue (zinc free, Matheson Coleman and Bell) as outlined by Simon and Van Vunakis (1962). Deoxyribonucleotides were photooxidized at a concentration of 10 mg/ml in 0.1 M Tris, pH 8.5, in the presence of 20 μg/ml of methylene blue. The incubation tubes were illuminated with 6000 footcandles using a GE or Sylvania 150-w flood lamp as the light source. The temperature was maintained between 15 and 20° by immersing the incubation tubes in a regulated water bath.

Antisera. The antisera were prepared by immunizing rabbits according to the method of Plescia *et al.* (1964) with denatured salmon sperm, *P. vulgaris*, and *Ps. aeruginosa* DNA in which all of the guanine residues had been destroyed by photooxidation in the presence of methylene blue and the dye removed by Dowex 50 (Na^+) resin. Base ratio analysis showed that the other bases were not affected. The extent of guanine destruction in DNA was determined by base ratio analysis (Wyatt, 1951).

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[1] Abbreviations used: anti-PO, antiphotooxidized; MB, methylene blue.

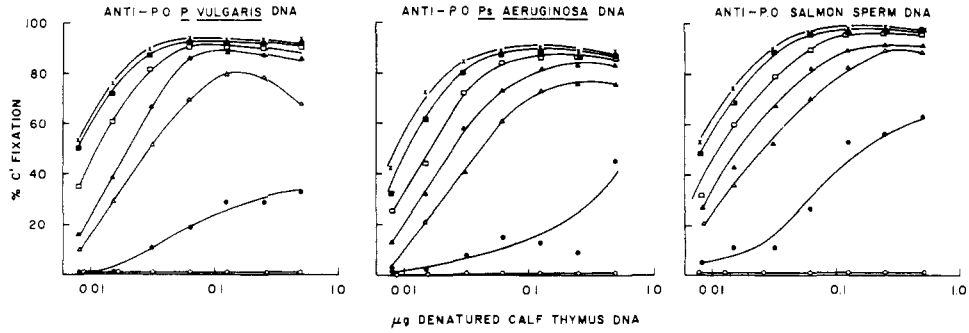

FIGURE 1: Fixation of C′ by increments of denatured calf thymus DNA photooxidized in the presence of methylene blue for 1 hr (●), 2 hr (△), 3 hr (▲), 4 hr (□), 6 hr (■), and 8 hr (×). Denatured controls, DNA alone, denatured DNA in the presence of methylene blue but kept in the dark for 8 hr, or denatured DNA in the light in the absence of methylene blue (○). Anti-PO *P. vulgaris* DNA (Ra-665B-1 diluted 1:150). Anti-PO *Ps. aeruginosa* DNA (Ra-666B-1 diluted 1:200). Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1:200). In the C′ fixation technique used throughout this paper, 1.0 ml of diluted antisera, 3.0 ml of Tris buffer, 1.0 ml of diluted C′, and 1.0 ml of diluted antigen are incubated at 2–4° overnight. In the inhibition experiments, 1.0 ml of diluted inhibitor and 2.0 ml of buffer are used instead of 3.0 ml of buffer.

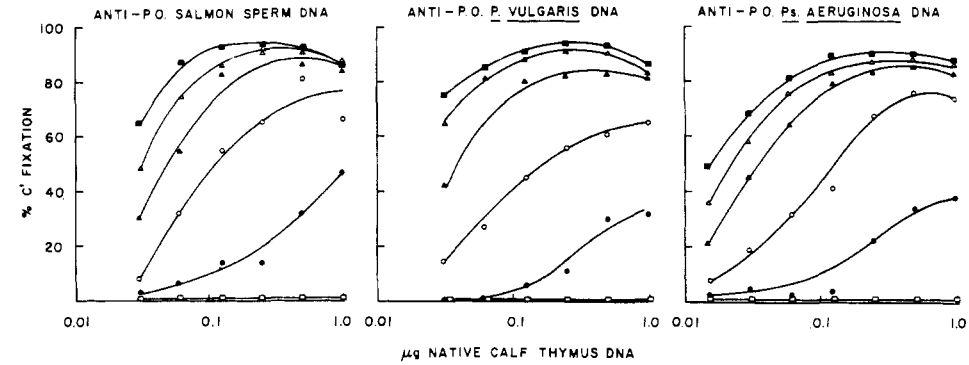

FIGURE 2: Fixation of C′ by increments of native calf thymus DNA photooxidized in the presence of methylene blue for 3 hr (●), 6 hr (○), 12 hr (▲), 18 hr (△), 25 hr (■), and control DNA's (□). Antiserum dilutions are the same as those used in Figure 1.

The micro C′ fixation method and the C′ fixation inhibition method of Wasserman and Levine (1960) were used in the serological analyses. Antiserum to guanosine was prepared by Dr. David Stollar using the procedure of Erlanger and Beiser (1964). We would like to thank Dr. Stollar for the use of the antiserum.

Results

When T_4 bacteriophage DNA which had been photooxidized in the presence of methylene blue was used as immunogen, antibodies were produced to glucosylated and normal bases and to a product resulting from the photosensitized reaction (Seaman *et al.*, 1965b). The sera of rabbits immunized with photooxidized salmon sperm, *P. vulgaris*, and *Ps. aeruginosa* DNA also contained antibodies directed toward the bases. The C′ fixing activities of the antibodies directed toward the photooxidized product are such that with an appropriate dilution of antiserum, only the events associated with the photooxidation can be studied. At the dilution used with each of the three antisera, no C′ fixation was obtained with the untreated DNA preparations used in this study.

When thermally denatured calf thymus DNA was irradiated in the presence of methylene blue for periods of 0–8 hr and assayed with high concentrations of antisera directed toward the photooxidized DNA's of salmon sperm *P. vulgaris* and *Ps. aeruginosa*, the data shown in Figure 1 were obtained. Serologic activity appears after 1 hr of irradiation, and continues to increase until maximum activity is approached at 6–8 hr. Suitable controls, *i.e.*, the complete reaction mixture kept in the dark or the reaction mixture lacking methylene blue but exposed to visible light, were negative after the terminal reaction time.

The data in Figure 2 represent the appearance of serologic activity when native calf thymus DNA was irradiated in the presence of methylene blue and assayed with the same three antisera. With native DNA

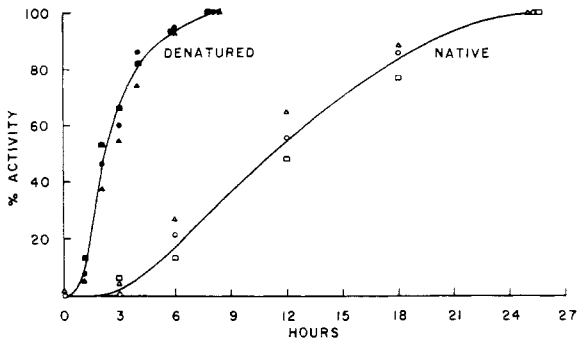

FIGURE 3: Appearance of serologic activity of denatured and native calf thymus DNA as a function of time of photooxidation. The C′ fixation values used to calculate the percentage of serologic activities (see text) were obtained with anti-PO *P. vulgaris* (●, ○), anti-PO salmon sperm (■, □), and anti-*Ps. aeruginosa* (▲, △) DNA's.

as substrate, serologic activity was observed after 3 hr of irradiation, and increased up to 25 hr of irradiation. A comparison of the appearance of serologic reactivity in native and denatured DNA photooxidized under identical conditions is shown in Figure 3. The appearance of serologic activity was calculated by using the C′ fixation results obtained with 0.01 μg of denatured DNA or with 0.05 μg of native DNA at different times of photooxidation. Since the C′ fixation approaches a constant value after 6–8 hr of irradiation for denatured DNA and 18–25 hr of irradiation for native DNA, these points were assumed to represent 100% activity. Using denatured DNA as the substrate, 50% serologic activity appeared after 2.3 hr of photooxidation. Irradiation for 12 hr was required before native DNA exhibited 50% serologic activity. Earlier studies on the relative effectiveness of methylene blue in photooxidizing denatured and native DNA also showed that denatured DNA was more susceptible to this reaction (Simon and Van Vunakis, 1962).

The antiserum dilutions used to obtain the data shown in Figures 1 and 2 were such that all of the available C′ was fixed. Thus, the regions of antigen excess inhibition were not observed. In addition, fragmentation of DNA resulting from extended periods of photooxidation of denatured DNA (Simon and Van Vunakis, 1962) was not observed. If the antisera are used at greater dilutions, however, both antigen excess inhibition and the fragmentation are observed. Data obtained with antiphotooxidized salmon sperm DNA, at $^1/_{500}$ dilution, and native and denatured calf thymus DNA photooxidized for extended periods are shown in Figure 4. The denatured DNA samples treated for 12, 18, and 25 hr are less effective serologically than the 6-hr sample. A decrease in the serologic activity of denatured DNA after extensive irradiation was also observed using anti-T_4-DNA and anti-DNA present in lupus erythematosus sera. With the latter immune systems, molecular weight measurements revealed that the DNA had fragmented (Simon and Van Vunakis, 1962).

The increasing maximum C′ fixation obtained with native DNA following varying periods of photooxidation suggests that the nonhydrogen bonded areas in the native DNA are initially small and then become larger. (See also Figures 9 and 10.) As shown by CsCl density centrifugation (Figure 5), the native DNA had not been completely denatured by this dose of irradiation.

To determine the effect of methylene blue concentration on the appearance of serologic activity in denatured DNA, a constant amount of DNA was photooxidized in the presence of varying concentrations of methylene blue. Aliquots were removed after varying times of irradiation and assayed for C′ fixation (Figure 6). The time of irradiation required to produce 50% serologic activity as a function of methylene blue con-

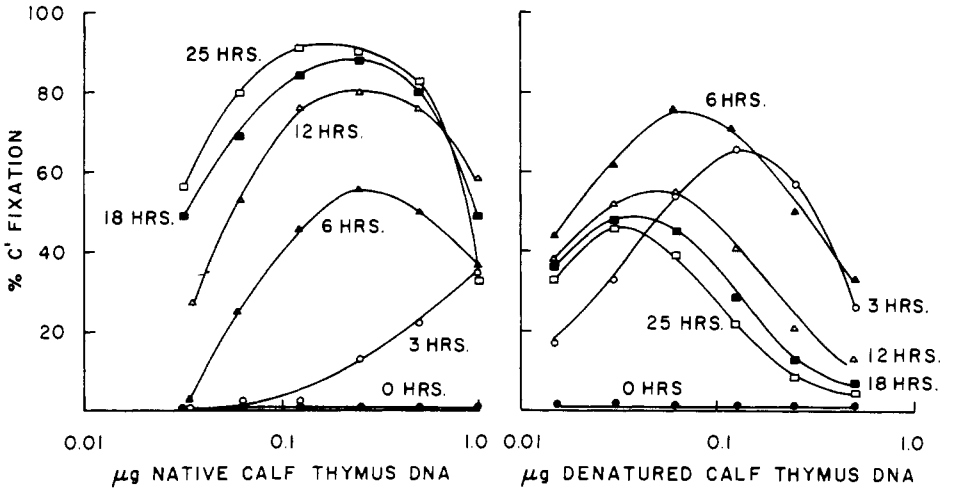

FIGURE 4: Fixation of C′ by increments of native (left) and denatured (right) calf thymus DNA photooxidized for varying periods of time. Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1:500) was used.

centration is shown in Figure 7. For calculations of the serologic activities, the C′ fixation values obtained with 0.02 μg of DNA were used. Under these experimental conditions, optimum ratios of methylene blue to DNA were found to be 10–20 μg of methylene blue/500 μg of denatured calf thymus DNA. With both of these methylene blue concentrations, 2.3 hr was required for the appearance of 50% of the serologic activity. As shown later, this time may depend on the guanine and cytosine (G + C) content of the DNA being photooxidized.

The effect of pH on photooxidation of deoxyguanosine suggested that the dissociation of a proton from the N-position influences the rate of reaction (Simon and Van Vunakis, 1964). In order to determine whether photooxidation of the guanine residue in DNA was similarly affected by pH, denatured calf thymus DNA was irradiated for 3 hr in the presence of methylene blue in buffers whose pH ranged from 6.0 to 9.5. The irradiated DNA was diluted into isotonic Tris–NaCl buffer, pH 7.4, and assayed immunologically. The C′ fixation values obtained with 0.02 μg of the irradiated DNA at various pH values are shown in Figure 8. As was found with deoxyguanosine, the extent of photooxidation as measured by serologic activity is increased from pH 7 to 9.5. The precise role of the proton dissociation could not be determined since rates of the reaction were not followed.

Irradiated native and denatured *P. vulgaris* and *Ps. aeruginosa* DNA were also tested for their activity with antiphotooxidized salmon sperm DNA. The resulting C′ fixation curves are shown in Figures 9 and 10. With *P. vulgaris* and *Ps. aeruginosa* DNA, the antiserum could be diluted $^1/_{700}$ to get comparative C′ fixation, as was obtained with photooxidized calf thymus DNA at an antiserum dilution of $^1/_{500}$. The differences in activity of the DNA's probably reflect the molecular weights of the DNA samples (Healy *et al.*, 1963). Thus, it is difficult to relate extent of reaction with the G + C content of the DNA.

The time at which 50% of serologic activity is observed, however, appears to be related to the G + C content of the DNA. The data in Figure 11 describe the appearance of serologic activities of photooxidized native and denatured *P. vulgaris* (37% G + C) and *Ps. aeruginosa* (68% G + C) DNA. Serologic activity is produced faster with the DNA with the highest G + C content; *i.e.*, *Ps. aeruginosa* DNA (50% serologic activity at 1.5 hr with denatured and 12 hr with native DNA). *P. vulgaris* DNA with the lower G + C content was the slowest to produce serologically active lesions (50% serologic activity at 3.2 hr with denatured and 14 hr with native DNA). Using native DNA's, Bellin and Grossman (1965) showed that the decrease in T_m after 1-hr illumination with methylene blue, light, and oxygen is directly proportional to the mole fraction of G + C in the nucleic acid.

If the antibody is directed toward the photooxidized lesion, then the photooxidized nucleotide should inhibit the reaction between photooxidized DNA and antiphotooxidized DNA. Therefore, the four nucleotides of DNA, thymidylic acid (TMP), dCMP, deoxy-

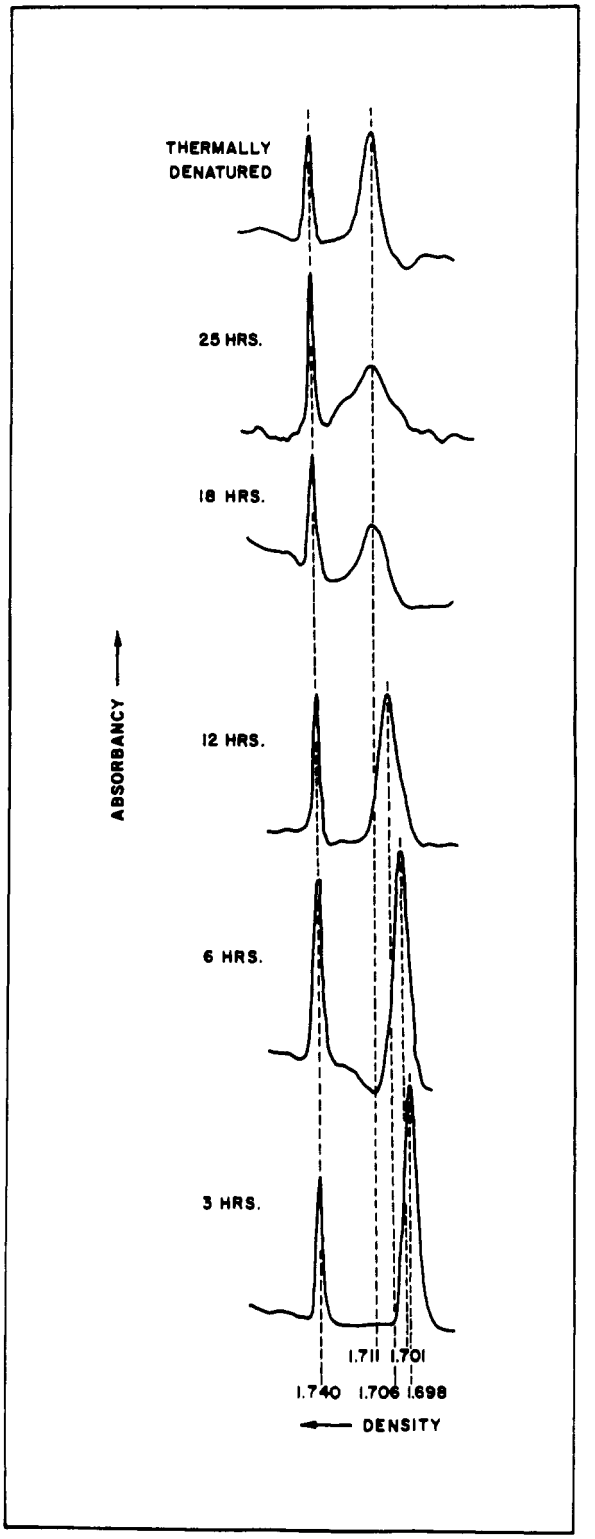

FIGURE 5: CsCl density gradient centrifugation of native *P. vulgaris* DNA photooxidized in the presence of methylene blue for varying periods of time. Reference DNA, buoyant density 1.740, is *Bacillus subtilis* SP 8 phage DNA.

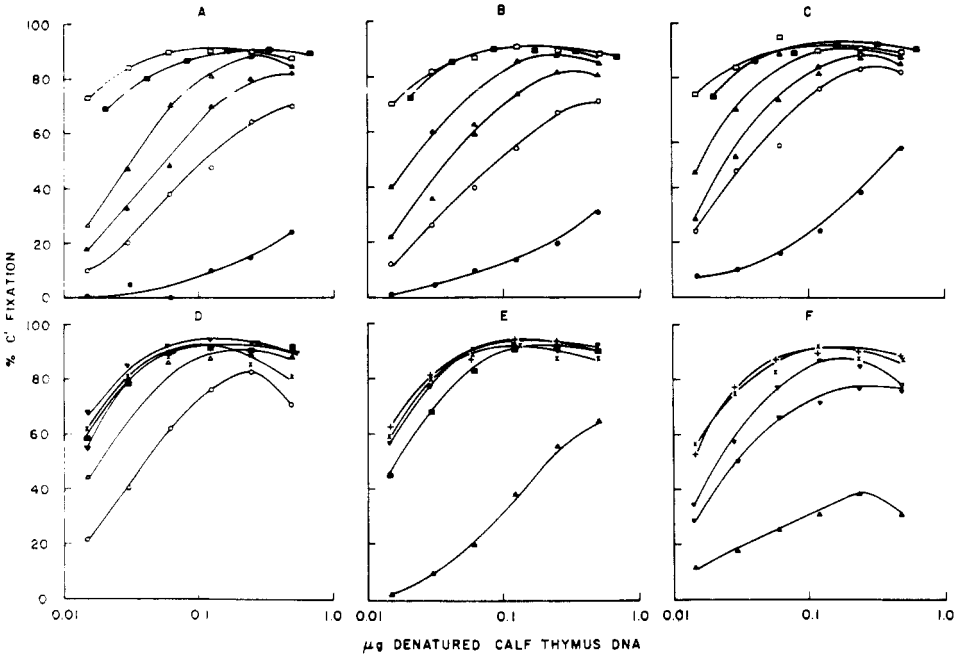

FIGURE 6: Fixation of C′ by increments of denatured calf thymus DNA photooxidized in the presence of methylene blue for 1 hr (●), 2 hr (○), 3 hr (▲), 4 hr (△), 6 hr (■), 8 hr (□), 12 hr (▽), 18 hr (×), and 21 hr (+). Control DNA's, not shown here, did not fix C′. A, 60 μg of MB/500 μg of DNA; B, 40 μg of MB/500 μg of DNA; C, 20 μg of MB/500 μg of DNA; D, 10 μg of MB/500 μg of DNA; E, 5 μg of MB/500 μg of DNA; F, 2.5 μg of MB/500 μg of DNA. Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1/200) was used for C′ fixation.

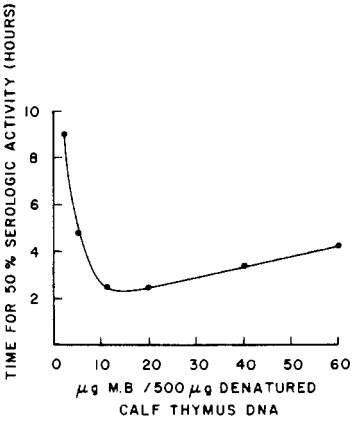

FIGURE 7: Time of appearance of 50% serologic activity (in hours) as a function of the weight ratio of methylene blue to denatured calf thymus DNA.

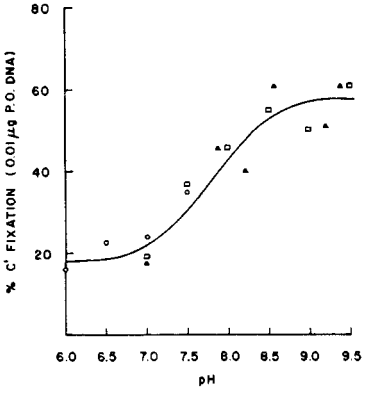

FIGURE 8: Fixation of C′ by 0.01 μg of denatured calf thymus DNA photooxidized for 3 hr in the presence of methylene blue at varying hydrogen ion concentrations. ○, 0.05 M phosphate buffer; ▲, 0.05 M barbital buffer; □, 0.05 M Tris buffer. Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1:200) was used. The values for the per cent C′ fixation obtained after irradiation in barbital buffer were lower than those found in Tris buffer and were corrected to correspond to the values obtained in Tris buffer at the same pH.

adenylic acid (dAMP), and dGMP were photooxidized. At 100-μg levels, only photooxidized dGMP was an effective inhibitor. At these levels, dAMP, dCMP, and TMP did not inhibit; dGMP kept in the dark for 6 hr did not inhibit.

The appearance of inhibitory activity of dGMP after various times of photooxidation is shown in Figure 12. As the time of photooxidation increased, the amount of dGMP required for 50% inhibition decreased. After 18 hr, there was only a slight decrease in the amount of dGMP required for 50% inhibition (3.0 μg after 8 hr, 2.5 μg after 14, 17, and 19 hr). When the same reactions were measured by loss in absorbance at 260 mμ, there

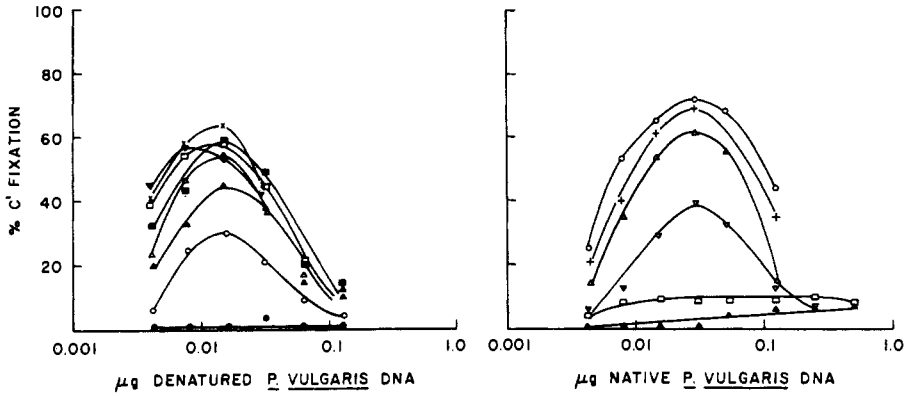

FIGURE 9: Fixation of C′ by increments of denatured and native *P. vulgaris* DNA photooxidized for varying periods of time. Left side, denatured DNA; 1 hr (●), 2 hr (○), 3 hr (▲), 4 hr (△), 5 hr (■), 6 hr (□), 7 hr (×), and 8 hr (▼). Right side, native DNA; 3 hr (▲), 6 hr (□), 12 hr (▼), 18 hr (△), 25 hr (+), and 31 hr (○). Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1:1500) was used.

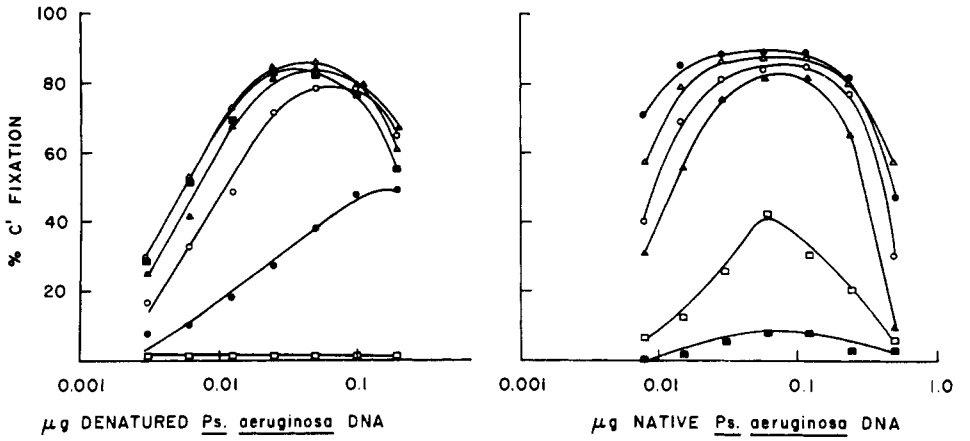

FIGURE 10: Fixation of C′ by increments of denatured and native *Ps. aeruginosa* DNA photooxidized for varying periods of time. Left side, denatured DNA; no irradiation (□), 1 hr (●), 2 hr (○), 3 hr (▲), 4 hr (△), and 5 hr (■). Right side, native DNA; 3 hr (■), 6 hr (□), 12 hr (▲), 16 hr (○), 24 hr (△), and 34 hr (●). Anti-PO salmon sperm DNA (Ra-598B-3 diluted 1:1000) was used.

was a direct logarithmic relationship between effectiveness of inhibition and loss in absorbance.

The destruction of guanine during photooxidation was shown in still another way by using an antiserum with a specificity toward this base (Erlanger and Beiser, 1964). Unlike the antiphotooxidized DNA, the serologic reaction between anti-G and denatured DNA should decrease as photooxidation proceeds and the number of guanine residues diminish. The results shown in Figure 13 confirm this prediction.

Discussion

Chemical studies had previously shown that the guanine residues in nucleic acids were preferentially attacked during the methylene blue sensitized photooxidation reaction (Simon and Van Vunakis, 1962). In the present study, the reaction between the lesion in photoirradiated DNA and the antibodies present in sera of rabbits immunized with such DNA is demonstrated. The serologic activities observed after irradiation in the presence of methylene blue increase with time of exposure, while the controls (*i.e.*, DNA and methylene blue kept in the dark or DNA exposed to light in the absence of methylene blue) exhibit no serologic activity at the dilutions of antiserum used. These results suggest that the antigenic specificity resides in altered guanine residues. Hapten inhibition experiments yield more direct evidence that the antigenic determinant is the guanine photoproduct. The four untreated nucleotides (dAMP, dGMP, TMP, and dCMP) do not inhibit the photooxidized DNA immune system. After irradiation, however, dGMP competes effectively for the combining site of the antibody. The effectiveness of inhibition reflects the extent of dGMP breakdown as measured by loss of absorbance. With an antiserum

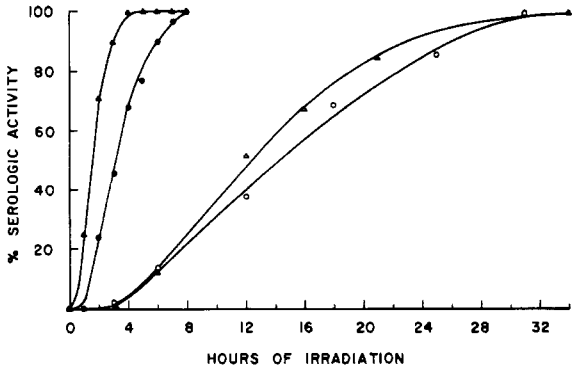

FIGURE 11: Appearance of serologic activity of denatured (▲) and native (△) *Ps. aeruginosa* DNA and denatured (●) and native (○) *P. vulgaris* DNA as a function of time of photooxidation. Values calculated from the data shown in Figures 8 and 9.

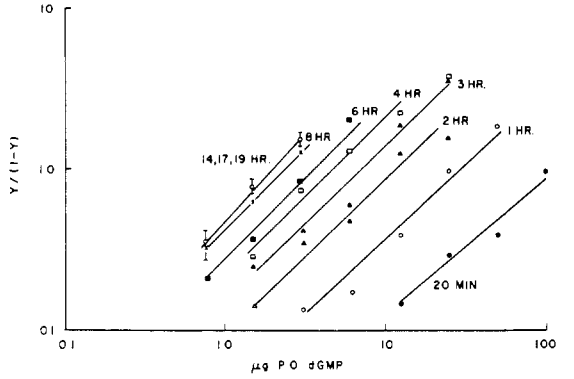

FIGURE 12: C′ fixation inhibition of the reaction between 0.1 μg of native calf thymus DNA photooxidized for 25 hr and anti-PO salmon sperm DNA (Ra-598B-3) diluted 1:500 (see Figure 4) by dGMP photooxidized in the presence of methylene blue for 20 min (●), 1 hr (○), 2 hr (△), 3 hr (▲), 4 hr (□), 6 hr (■), 8 hr (×), and 14, 17, 19 hr (⊕). dGMP alone, dGMP in the presence of methylene blue but kept in the dark for 19 hr, or dGMP exposed to the light for 19 hr but in the absence of methylene blue showed no inhibition at concentrations of 500 μg. The log of $Y/(1 - Y)$ is plotted *vs.* the log of hapten concentration (Y is the % inhibition of C′ fixation divided by 100).

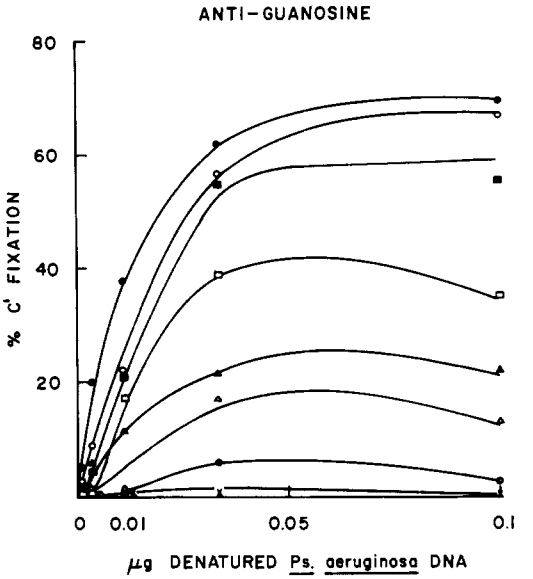

FIGURE 13: Fixation of C′ by increments of denatured *Ps. aeruginosa* DNA photooxidized in the presence of methylene blue for varying periods of time. No irradiation (●), 1 hr (○), 2 hr (■), 3 hr (□), 4 hr (▲), 5 hr (△), 6 hr (◐), and 7 hr (×). Antiguanosine (obtained from Dr. David Stollar) used at a dilution of 1:600.

specific for guanine (Erlanger and Beiser, 1964), increasing times of photooxidation of the DNA result in decreasing serologic activities, as would be predicted for a system in which guanine is the antigenic determinant.

Although the destruction of guanine in DNA can be determined directly by base analysis, the immunological technique is considerably more sensitive in detecting minute quantities of altered guanine product produced during the early irradiation times. A positive serologic response occurs after 10 min of photooxidation. This amount of lesion is not detected by base analyses since only approximately 0.5% of the guanine is affected. Attempts to correlate the destruction of guanine (*i.e.*, production of lesion) with the appearance of the serologic activities were not successful primarily because the fragmentation of DNA which occurs upon prolonged irradiation decreases the serologic activity. The dependence of the serologic reaction upon molecular weight has been discussed by Healy *et al.* (1963).

In all DNA serologic reactions, the denatured state of the DNA is more effective than native DNA (Levine *et al.*, 1960; Butler *et al.*, 1962; Stollar *et al.*, 1962; Sela *et al.*, 1964; Plescia *et al.*, 1965). Apparently, the nitrogenous bases which are oriented toward the center of the helix and stabilized by hydrophobic forces between the stacked bases and hydrogen bonds between the base pairs must be exposed to the aqueous solvent before interaction with antibody will occur. When native DNA is irradiated, the guanine residues which are destroyed can no longer participate in hydrogen bonding with the cytosine of the opposite chain or in stacking with the neighboring base on the same strand. Local sequences are then available for reaction with the antiphotooxidized antibody even though the DNA is essentially a double strand.

The size and number of the damaged sequences capable of reaction with the antibody are not known at this time. These exposed areas in native DNA are detected by the antibody before changes in buoyant density (indicative of helix–coil transition), as measured

by CsCl density centrifugation, are observed. With the use of more concentrated antiserum, single strand areas in native DNA are detected after 10 min of irradiation, whereas the first increase in buoyant density was observed only after 12 hr of irradiation. The possibility that the exposed areas are occurring at the ends of double strand DNA has not as yet been ruled out. The increasing serologic activity of native DNA with increased times of irradiation is not likely to be caused only by frayed ends as adenine–thymine rich areas would be expected to block the continued opening at the ends of the DNA. The use of an exonuclease specific for single strand DNA (Lehman, 1960) and an endonuclease specific for single strand DNA (Healy *et al.*, 1963; Ashe *et al.*, 1965), should resolve these alternatives. The latter type of enzyme, the dogfish liver endonuclease (Ashe *et al.*, 1965), has been used and found to eliminate these antigenically available areas as judged by the loss of the serologic activities of photooxidized products. Native DNA alone was not affected by the enzyme as measured with antibodies to DNA present in the serum of lupus erythematosus patients (Stollar *et al.*, 1962).

Even though the chemical mechanisms of the photodynamic reaction of methylene blue with nucleic acids still remains to be elucidated, antibodies to photooxidized DNA's are currently being used to answer such questions as (1) do all photosensitive dyes react with nucleic acid to give the same product? and (2) does photooxidation of various purine derivatives with a specific dye yield the same product?

While the antisera used in this study contain antibodies to the normal constituents of DNA, these could be diluted out and still have antibodies to the photooxidized products. Thus, with photooxidized DNA at least, the C′ fixing antibodies produced against the altered base are stronger than those produced to the natural bases. It might then be possible to produce antibodies to other chemically induced alterations in DNA. We have been able to produce antibodies in rabbits to altered bases resulting from exposure of DNA to ultraviolet light and hydroxylamine. The characterization of these antisera and their use will be presented in a future communication.

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