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Formation of an Adduct with Tris(hydroxymethyl)aminomethane during the Photooxidation of Deoxyribonucleic Acid and Guanine Derivatives*

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ABSTRACT: Rabbits immunized with deoxyribonucleic acid (DNA) photooxidized in Tris buffer in the presence of methylene blue and complexed with methylated bovine serum albumin produced antibodies specific for a photoproduct. The lack of serologic activity of DNA photoxidized in bicarbonate buffer suggested that Tris was participating in the formation of the photoproduct. Using the specific antibody in conjunction with suitable radioisotopic and electrophoretic techniques, it was shown that (1) Tris is incorporated into DNA during photooxidation, and (2) guanosine 5′-phosphate photooxidized in Tris gives three electrophoretically separable products. Two of these photoproducts have incorporated Tris into their structures, but only one of these Tris adducts is capable of inhibiting the immune reaction. The ability of photooxidized guanine residues to condense with molecules such as Tris may permit the controlled modification of DNA *in vitro* but will make photodynamic studies carried out *in vivo* difficult to interpret without knowledge of the intracellular macromolecular and ionic environment surrounding the photooxidized residues.

The chemical structure of the photoproducts which are produced in nucleic acids irradiated with visible light in the presence of methylene blue and O_2 has yet to be described, although evidence implicating destruction of the guanine residue exists (Simon and Van Vunakis, 1962; Wacker *et al.*, 1963; Simon, 1963). When rabbits were immunized with complexes formed between methylated bovine serum albumin and deoxyribonucleic acid (DNA) photooxidized in the presence of methylene blue and 0.1 M Tris buffer, pH 8.5, antibodies specific for an altered guanine photoproduct were produced (Seaman *et al.*, 1965, 1966). At appropriate antiserum dilutions, there is no serologic reaction between anti-$DNA_{(PO\text{-}Tris)}$[1] and nonirradiated DNA, and the serologic activities of both native and denatured DNA increase with time of irradiation. During the course of these studies, it became apparent that

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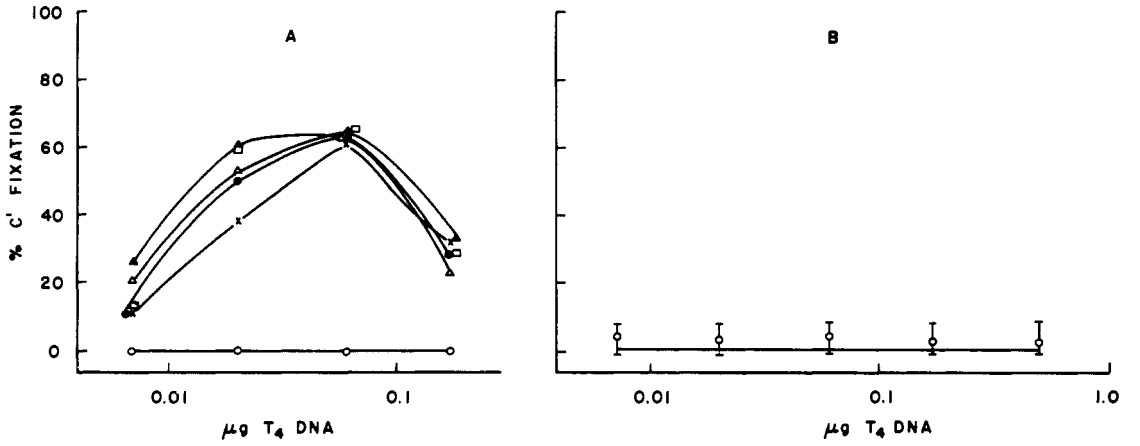

FIGURE 1: Fixation of C′ with antisalmon sperm $DNA_{(PO\text{-}Tris)}$ diluted 1:800 and increments of denatured T_4 DNA. (A) Photooxidized in Tris buffer for 0 (○), 1 (×), 2 (●), 3 (Δ), 4 (□), and 5 (▲) hr. (B) Photooxidized in bicarbonate for 0, 1, 2, 3, 4, and 5 hr.

the formation of a photoproduct which could react with this specific antibody was influenced by the buffer in which the photooxidation reaction was carried out. Evidence of some of these buffer effects and the implications of them are presented in this paper. It should be stressed that the differences found in photoproducts appear to be due solely to the different pathways of the guanine photooxidation reaction and are not attributable to the reaction of the other bases found in nucleic acids.

Materials and Methods

DNA. Calf thymus DNA was purchased from Calbiochem and used without further purification. T_4 DNA was isolated by rupturing the phage with cold phenol buffered with 0.05 M phosphate, pH 7.4 (Gierer and Schramm, 1956). Following three phenol extractions, the DNA was extracted twice with chloroform–isoamyl alcohol and dialyzed against 0.15 M NaCl.

Radioactive Compounds. [^{14}C]Guanosine 5′-monophosphate uniformly labeled, Tris([^{14}C]hydroxymethyl)aminomethane, and $NaH^{14}CO_3$ were purchased from New England Nuclear Corp. All counting was done in a Nuclear Chicago Unilux scintillation counter using Bray's fluid.

Alkaline Phosphatase. Bacterial alkaline phosphatase was purchased from Worthington Biochemical Corp. Digestions were performed for 4 hr at 37° in 0.1 M Tris, pH 8.5. The enzyme concentration was 10% (w/w) of substrate concentration (Torriani, 1960).

Photooxidation. Native or heat-denatured DNA was photooxidized at a concentration of 100–500 μg/ml in the presence of 20 μg/ml of methylene blue (zinc free, Matheson Coleman and Bell) as outlined by Simon and Van Vunakis (1962). All buffers used were 0.05 M, pH 8.5 ± 0.2. Deoxyribonucleotides were photooxidized at a concentration of 1–10 mg/ml in the presence of 20 μg/ml of methylene blue. The incubation tubes were illuminated with 6000 foot-candles using a GE or Sylvania 150-w flood lamp as the light source. The temperature was maintained below 15° 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 $DNA_{(PO\text{-}Tris)}$ in which all of the guanine residues had been destroyed by photooxidation in 0.1 M Tris, pH 8.5, in the presence of methylene blue. 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). For serologic analyses, the micro C′ fixation method of Wasserman and Levine (1960) was used.

Electrophoresis. Electrophoresis was performed according to the method of Markham and Smith (1952), Whatman No. 1 or 3MM paper was used. The paper strips were subjected to 2.5-hr electrophoresis with a voltage gradient of 20 v/cm. The buffer used was 0.05 M formate, pH 3.5. The electrophoresis strips were scanned in a Tracerlab 4π scanner. Full-scale deflection corresponds to 1000 cpm with an efficiency of counting of 10%.

Results

As previously reported (Seaman *et al.*, 1966), the ability of $DNA_{(PO\text{-}Tris)}$ to react with anti-$DNA_{(PO\text{-}Tris)}$

[1] Abbreviations used: ammediol, 2-amino-2-methyl-1,3-propanediol; PO, photooxidized; PO-Tris, photooxidized in Tris buffer; GR, guanosine or guanine ribonucleoside; GdR, guanosine deoxyribonucleoside; GMP, guanosine 5′-phosphate; dGMP, deoxyguanosine 5′-phosphate; GDP, guanosine 5′-diphosphate; GTP, guanosine 5′-triphosphate; XR, xanthosine; XMP, xanthosine 5′-phosphate; TMP, thymidine 5′-phosphate; AMP, adenosine 5′-phosphate.

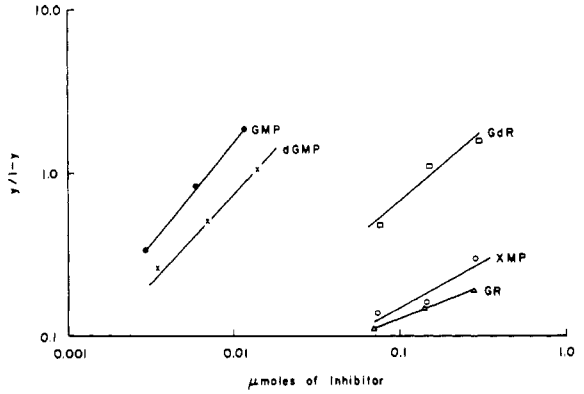

FIGURE 2: C′ fixation inhibition of the reaction between 0.03 μg of denatured T_4 $DNA_{(PO\text{-}Tris)}$ for 3 hr and anti-salmon sperm $DNA_{(PO\text{-}Tris)}$ diluted 1:650 by GMP, dGMP, GdR, GR, and XMP photooxidized in Tris in the presence of methylene blue. All samples were photooxidized until the loss of absorption at λ_{max} equalled 65% ± 3%. $Y/(1 - y)$ is plotted *vs.* hapten concentration (y is the % inhibition of C′ fixation divided by 100).

TABLE I: The Ability of Nucleosides and Nucleotides Photooxidized in Tris Buffer to Inhibit the $DNA_{(PO\text{-}Tris)}$–Anti-$DNA_{(PO\text{-}Tris)}$ Immune System.

Derivative	% Loss of Absorbance at λ_{max}[a]	mμmoles of Reaction Mixture Required for 50% Inhibn
GMP	68	8.3
GR	66	>280[b]
GR (dephosphorylated, photooxidized GMP)	—	>280[b]
GDP	59	3.6
GTP	66	5.7
dGMP	67	14
GdR	67	150
GdR (dephosphorylated, photooxidized dGMP)	—	230
XR	62	>350[c]
XMP	63	>280[d]
dAMP	0	*e*
TMP	37	*f*

[a] % OD lost = $(OD_{initial} - OD_{(terminal)})/OD_{initial}$. [b] Required for 20% inhibition. [c] Required for 9% inhibition. [d] Required for 30% inhibition. [e] No inhibition by 210 mμmoles. [f] No inhibition by 200 mμmoles.

is dependent upon the degree of photooxidation (Figure 1A) and has been shown to be related to the amount of guanine destroyed. If DNA is photooxidized in the presence of bicarbonate, however, there is no appearance of serologic activity when measured with anti-$DNA_{(PO\text{-}Tris)}$ (Figure 1B). Base analysis even on the terminal sample revealed that only guanine was destroyed.

The deoxynucleotide, dGMP, photooxidized in Tris buffer, inhibits the $DNA_{(PO\text{-}Tris)}$–anti-$DNA_{(PO\text{-}Tris)}$ immune system (Seaman *et al.*, 1966) and thus represents part, if not all, of the antigenic determinant of $DNA_{(PO\text{-}Tris)}$. To test for structural similarities among photoproducts, various guanine derivatives, including GR, GdR, GMP, dGMP, GDP, and GTP, as well as dAMP, TMP, and XMP, were photooxidized in Tris buffer at pH 8.5, and their effectiveness in inhibiting the $DNA_{(PO\text{-}Tris)}$–anti-$DNA_{(PO\text{-}Tris)}$ immune system was determined (Figure 2 and Table I). It can be seen that photooxidized GDP, GTP, GMP, and dGMP are the most effective inhibitors; 3.6–14 mμmoles (based on the concentration of the starting product) are required to inhibit 50% of the immune reaction. The guanine nucleosides are not as reactive (150 mμmoles of GdR is needed for 50% inhibition, while 280 mμmoles of GR gives only 20% inhibition). If photooxidized GMP and dGMP are treated with alkaline phosphatase, then the amount of dephosphorylated photoproduct required to give 50% inhibition rises to a level comparable to that of GdR or GR which had been photooxidized directly. The phosphate group, therefore, appears to influence the activity of the antigenic determinant either by stabilizing its structure or by actual inclusion in it.

Photooxidized TMP was not able to inhibit the reaction. Photoxidized XMP is able to inhibit the immune system only slightly, 280 mμmoles being necessary for 30% inhibition. Thus, it is at least 50-fold less effective than GMP. Only 9% inhibition was observed with 350 mμmoles of photooxidized XR. No inhibition was observed with dAMP, but none was expected since there is no evidence (*i.e.*, loss of spectrum or uptake of O_2) that this molecule can be photooxidized in the presence of methylene blue.

The inhibitory effectiveness of photooxidized GMP or dGMP depends upon the buffer in which the reaction is carried out (Table II) even though destruction of these derivatives occurs in all buffers. While GMP and dGMP photooxidized in Tris could inhibit the $DNA_{(PO\text{-}Tris)}$–anti-$DNA_{(PO\text{-}Tris)}$ to the greatest extent (9 and 11 mμmoles required, respectively, for 50% inhibition), approximately 105 mμmoles of the GMP reaction mixture and 135 mμmoles of the dGMP reaction mixture were required when the photooxidation was carried out in ammediol (an analog of Tris in which one of the three hydroxymethyl groups is replaced by a methyl group). There was only a slight inhibitory reaction when dGMP and GMP were photooxidized in borate, and GMP in phosphate. There was no detectable inhibition by GMP or dGMP photooxidized in bicarbonate, or dGMP photooxidized in phosphate. These results

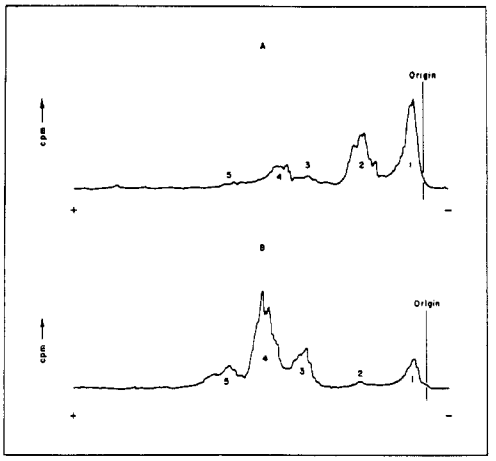

FIGURE 3: Radioactivity strip scan of an electrophoresis pattern of [^{14}C]GMP photooxidized in the presence of methylene blue in: (A) 0.05 M Tris buffer and (B) 0.05 M bicarbonate buffer. The specific activity of GMP was 1.5 μc/μmole. Electrophoresis was performed as described in Materials and Methods.

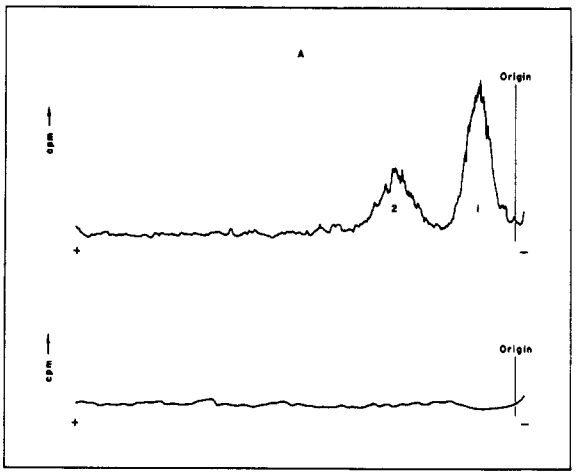

FIGURE 4: A radioactivity strip scan of an electrophoresis pattern of GMP photooxidized in the presence of methylene blue in: (A) 0.0125 M Tris buffer (sp act. 0.16 μc/μmole) and (B) 0.0111 M bicarbonate buffer (sp act. 0.18 μc/μmole).

TABLE II: The Ability of GMP and dGMP Photooxidized in Various Buffers to Inhibit the $DNA_{(PO\text{-}Tris)}$–Anti-$DNA_{(PO\text{-}Tris)}$ Immune System.

	GMP		dGMP	
Buffer	% Loss of Absorbance at 250 mμ	mμmoles Required for 50% Inhibn	% Loss of Absorbance at 250 mμ	mμmoles Required for 50% Inhibn
Tris	66	8.7	57	11
Ammediol	62	105.0	59	135
Borate	71	*a*	64	*a*
Phosphate	51	*b*	61	*b*
Bicarbonate	59	*c*	61	*c*

[a] 11% inhibition by 63 mμmoles of GMP and 12% inhibition by 125 mμmoles of dGMP in borate. [b] 7% inhibition by 250 mμmoles of GMP and no inhibition with 250 mμmoles of dGMP in phosphate. [c] No inhibition observed with either 125 mμmoles of GMP or dGMP in bicarbonate.

suggested that Tris did not act solely as a buffer during the photooxidation reaction, but may have influenced the nature of the photoproduct.

When [^{14}C]GMP was photooxidized in the presence of Tris or bicarbonate and the photoproducts were separated electrophoretically at pH 3.5 (Figure 3A,B), the number and distribution of the peaks varied depending upon the buffer used during photooxidation. If the individual peaks of the Tris run were eluted and assayed serologically, only peak 2 could inhibit the $DNA_{(PO\text{-}Tris)}$–anti-$DNA_{(PO\text{-}Tris)}$ reaction. The absence of this peak and the distribution of other photoproducts when GMP was photooxidized in bicarbonate are also shown in Figure 3B. The peaks of the bicarbonate run were not eluted individually to test for inhibition since the complete reaction mixture itself was not inhibitory.

The possibility that Tris is condensing with an intermediate formed during the photooxidation of guanine residues was approached directly by photooxidizing DNA in the presence of radioactive Tris buffer. The results in Table III show that [^{14}C]Tris has been incorporated into the photooxidized DNA but was not incorporated into the dark control containing methylene blue nor in the light control lacking the dye.

GMP was also irradiated in the presence of [^{14}C]-Tris (Figure 4A) and the photoproducts were separated by electrophoresis at pH 3.5. Two peaks were obtained. Both of these peaks were eluted and tested for their ability to inhibit the $DNA_{(PO\text{-}Tris)}$–anti-$DNA_{(PO\text{-}Tris)}$ system. Only peak 2 (corresponding in position to peak 2 in Figure 3A) was inhibitory. When the photooxidation of GMP was carried out in [^{14}C]bicarbonate buffer, no evidence of incorporation of label into the photoproducts was obtained (Figure 4B). The same results were obtained when dGMP was photooxidized in [^{14}C]Tris or [^{14}C]bicarbonate. Again, only one of the Tris–dGMP peaks was able to inhibit the immune system. Photooxidation of TMP or dAMP in [^{14}C]-Tris buffer followed by electrophoresis at pH 3.5 revealed no adduct formation.

By following the loss of spectra and/or oxygen uptake, the effect of environmental factors on the rate of photooxidation of guanine derivatives was studied (Simon and Van Vunakis, 1964; Simon, 1963). However,

TABLE III: Incorporation of [^{14}C]Tris into DNA during Photooxidation.[d]

Time of Incubn (hr)	Cpm/ml Incorporated		
	Exptl[a]	Dark Control[b]	Light Control[c]
0	5040	3760	4560
22	81,400	4880	5580

[a] The experimental tube containing the dye was illuminated with 6000 foot-candles. [b] The dark control tube was wrapped in several thicknesses of aluminum foil. [c] The light control was illuminated with 6000 foot-candles in the absence of dye. [d] Denatured *Pseudomonas aeruginosa* DNA at a concentration of 500 μg/ml was photooxidized in the presence of 20 μg/ml of methylene blue in Tris buffer (10^{-2} M, pH 8.5, sp act. 41 μc/μmole). Water-saturated oxygen was passed through all incubation tubes. Aliquots were taken at the indicated times and dialyzed exhaustively in the dark against unlabeled Tris (10^{-2} M, pH 8.5). The dialyzed samples were counted in a scintillation counter.

the extent to which the hypothetical intermediate forms, as well as its ability to condense with Tris (or other molecules), may not be reflected in the over-all reaction rate. The effect of environmental factors on the formation of the adduct is currently being investigated.

Discussion

The availability of antibody specific for a photoproduct produced when DNA is irradiated with visible light in the presence of Tris buffer (pH 8.5), methylene blue, and O_2 permitted us to explore some of the properties of the reaction (Seaman *et al.*, 1965, 1966). When DNA was photooxidized in carbonate buffer, no serologic activity was obtained with the anti-$DNA_{(PO\text{-}Tris)}$ although base analysis revealed that guanine had been preferentially destroyed.

Since photooxidation of dGMP in Tris buffer led to the production of an inhibitory photoproduct (Seaman *et al.*, 1966), it was possible to study some of the properties of the reaction on the nucleoside and nucleotide level. The photooxidized nucleoside mono-, di-, and triphosphates of guanine were inhibitory at low concentrations. The nucleosides were inhibitory at higher concentrations, suggesting that the phosphate group is important in the antigenic site. Enzymatic removal of the phosphate from photooxidized dGMP or GMP gave an inhibitory product equal in effectiveness to dG or GR photooxidized directly. In addition to showing the importance of the phosphate group in the antigenic center (assuming no unknown side reactions of the enzyme), this experiment suggests that the photoproduct is also formed at the nucleoside level but is a less potent immunological inhibitor. Previously it has been shown that the photooxidation of guanosine led to a multiplicity of photoproducts; at least three were detected chromatographically (Simon and Van Vunakis, 1962).

Photooxidation of XMP in Tris buffer resulted in the formation of a slightly inhibitory photoproduct. When this nucleotide was photooxidized in the presence of radioactive Tris, no adduct formation in the photoproduct could be observed by electrophoretic analyses at pH 3.5. The serologic assay of the XMP photoproduct indicates formation of no more than 2% of the inhibitory activity observed in photooxidized GMP. A comparison of the results obtained with GMP and XMP indicates that the difference between these molecules, *i.e.*, the presence of an amino rather than a keto group at C-2 of the purine ring, is important in determining the formation of the intermediate which eventually condenses with Tris to give the adduct. The photoproducts formed during the photooxidation of dGMP and GMP in ammediol were inhibitory to a lesser extent than those formed in Tris. Electrophoresis of the photoproducts formed upon photoxidation of [^{14}C]GMP in the presence of ammediol gives a distribution pattern similar to [^{14}C]GMP photooxidized in Tris. Thus, both GMP and dGMP probably form adducts when photooxidized in ammediol. The structural differences of the ammediol and Tris adducts may account for the difference in serologic activity. It is also possible that peak 2 (Figures 3A and 4A) consists of more than one photoproduct. The buffer may greatly affect the relative amount of a minor inhibitory component without changing the observed distribution of radioactivity.

Some of the photooxidation products of purine and guanine derivatives that have been identified in our laboratory include 1,3-dimethylallantoin from theophylline (Simon and Van Vunakis, 1964), allantoin from uric acid, and 9-β-D-ribofuranosylpurine-6-sulfinate from 6-mercaptopurine nucleoside. Photooxidation of guanine derivatives with other dyes has yielded guanidine, CO_2, and parabanic acid among the products (Sussenbach and Berends, 1964).

The multiplicity and diversity of the photoproducts formed when dGMP or GMP is photooxidized in different buffers presents several problems which are open to attack by the judicious use of chemical, immunological, and radiochemical techniques. An antibody directed toward a photoproduct (or other lesion in DNA) provides a specific and sensitive tool for monitoring the reaction in the *intact* DNA molecule and can guide the chemical studies toward the isolation of photoproduct(s) from DNA in as "native" a form as possible. For example, the stability of the inhibitory photoproduct (peak 2) can be determined both by immunological and electrophoretic methods, and this information can be used to isolate and characterize the products in photooxidized DNA. In addition, we are not certain that all the photoproducts produced upon photooxidation of the nucleotide are present in DNA. An assay is provided for at least one class of

photoproducts in DNA by the use of radioactive Tris. A comparison of the amount of [^{14}C]Tris incorporated into the DNA and the amount of guanine destroyed will indicate the extent to which this condensation reaction occurs in the macromolecule and will indicate if a significant amount of guanine is photooxidized to give other product(s). At present, we do not know whether the lack of inhibition by the second Tris adduct (peak 1 in Figure 4A) is due to the fact that its formation is specifically precluded in the DNA by steric or other factors, or whether it has escaped detection by serologic techniques because it is not immunogenic.

In view of the evidence presented herein, it is obvious that the experimental conditions under which photooxidation is carried out may determine the character of the photoproduct(s) formed both on the monomeric and macromolecular level. Thus, there exists a possibility that once the chemistry of the condensation reaction is understood, specific chemical structures could be introduced into nucleic acids *in vitro* by presenting the photointermediate with a suitable condensing molecule. Such specifically modified nucleic acids should be valuable for various biological studies.

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