# Influence of Cationic Triphenylmethane Dyes upon DNA Polymerization and Product Hydrolysis by *Escherichia coli* Polymerase I†

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ABSTRACT: The cationic triphenylmethane dyes crystal violet, methyl green, and malachite green inhibited DNA synthesis catalyzed by *Escherichia coli* B polymerase I (polymerase I). Lower concentrations of the dyes inhibited DNA replication as a direct function of the proportion of AT to GC in the DNA of *Clostridium perfringens, Escherichia coli,* and *Micrococcus lysodeikticus.* When the intercalant proflavin was employed, the GC-rich micrococcal DNA was most severely inhibited. In addition, both the triphenylmethanes and proflavin inhibited product hydrolysis catalyzed by polymerase I.

Biophysical studies have shown that the cationic triphenylmethane dyes, for example, methyl green or crystal violet, bind to DNA externally, possibly by two modes, and possess a greater affinity for AT-rich DNA than for GC-rich DNA (Kurnick and Radcliffe, 1962; Muller and Gautier, 1975; Krey and Hahn, 1975; Muller and Crothers, 1975; Muller et al. 1975). The AT affinity of these triphenylmethanes is also characteristic of a variety of other nonintercalating DNA ligands, including berenil, hydroxystilbamadine, and the *N*-methylpyrroles distamycin and netropsin, whether the affinity of the compounds is analyzed by biophysical techniques or by the inhibition of enzymes which utilize DNA templates (Festy et al., 1970; Chandra et al., 1970; Puschendorf et al., 1971; Zimmer et al., 1971a,b; Krey et al., 1973; Wartell et al., 1974; Wahnert et al., 1975). However, correlative enzymatic studies determining the influence of the cationic triphenylmethanes upon DNA synthesis appear to be lacking and the first purpose of this communication, therefore, was to test the hypothesis that cationic triphenylmethane dyes will inhibit polymerase I catalyzed DNA replication. A corollary to this hypothesis might include the supposition that the triphenylmethanes will more potently inhibit AT-rich DNA than GC-rich DNA. The results confirm the hypothesis and reveal that the lower concentrations of these dyes, in particular, inhibit polymerase I catalyzed DNA synthesis more effectively as the proportion of AT to GC increases in the tested bacterial DNAs.

*E. coli* polymerase I catalyzes five reactions (Kornberg, 1969), including the exonucleolytic cleavage of the free 3′ terminus of its reaction product (Lehman and Richardson, 1964). The influence of DNA ligands upon this reaction has not been tested and, therefore, a second objective of these investigations was to determine the influence of both the triphenylmethanes and a typical intercalant upon such hydrolysis. This second purpose has received added impetus from the following diverse considerations. (1) The property of intercalants to eliminate plasmids in bacteria (Hirota and Ijima, 1957; Mitsuhashi et al., 1961), to induce destruction of mitochondrial DNA in yeast (Mehrotra and Mahler, 1968; Goldring et al., 1970), and to stimulate the hydrolysis of DNA by a mitochondrial enzyme fraction (Paoletti et al., 1972), suggests a potential importance to the hypothesis that DNA biosynthetic or repair enzymes, as well as nucleases, might catalyze product or template hydrolysis during ligand inhibition of DNA replication in vivo. The property of intercalants, in particular, to differentially eliminate extrachromosomal elements has yet to be fully or satisfactorily explained. (2) One method of study of DNA hydrolysis in vitro has been the measurement of decolorization of the DNA–methyl green complex (Kurnick, 1950, 1962). Within unpublished limits, the binding of methyl green to DNA was reported to have no influence on the activity of pancreatic deoxyribonuclease (Kurnick, 1954), an endonuclease. However, the intercalants quinacrine and chloroquine inhibit this enzyme, but also release methyl green from its complex with DNA (Kurnick and Radcliffe, 1962; Krey and Hahn, 1975). Thus, these DNA complexers appear to influence DNA differently, although some relation also appears to exist between their attachment to or influence on a template.

These considerations in toto have led to an investigation of the influence of representatives of internal and external DNA ligands on product hydrolysis by polymerase I. The results show that the cationic triphenylmethanes, and the intercalant proflavin, inhibit this reaction, acting as typical template poisons.

## Materials and Methods

*E. coli* B DNA polymerase I and unlabeled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals, Milwaukee, Wisc., while tritiated compounds were obtained from the New England Nuclear Corp., Boston, Mass. *M. lysodeikticus* DNA was obtained from Miles Laboratories, Kankakee, Ill., while *C. perfringens* and *E. coli* DNA were purchased from ICN Pharmaceuticals, Cleveland, Ohio. The Allied Chemical Co. was the source of methyl green, proflavin, and crystal violet, while malachite green was obtained from Fisher Chemical Co., Fair Lawn, N.J.

Typical reaction systems were as follows: volume 0.3 ml; concentrations (M): KCl 0.066, $Mg(OAc)_2$ 0.0066, $KPO_4$ 0.025, pH 7.5; *E. coli* B DNA polymerase I, 0.131 enzyme unit (specific activity 0.158 unit/$\mu$g (Richardson et al., 1964)); 50 $\mu$g of DNA; dATP,[1] dGTP, or dTTP, 0.019 mmol; and [$^3$H]dCTP, 0.104 $\mu$Ci (for low specific activity systems 27

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[1] Abbreviations used are: dATP, dTTP, dGTP, and dCTP, deoxyadenosine, -thymidine, -guanosine, and -cytidine triphosphates.

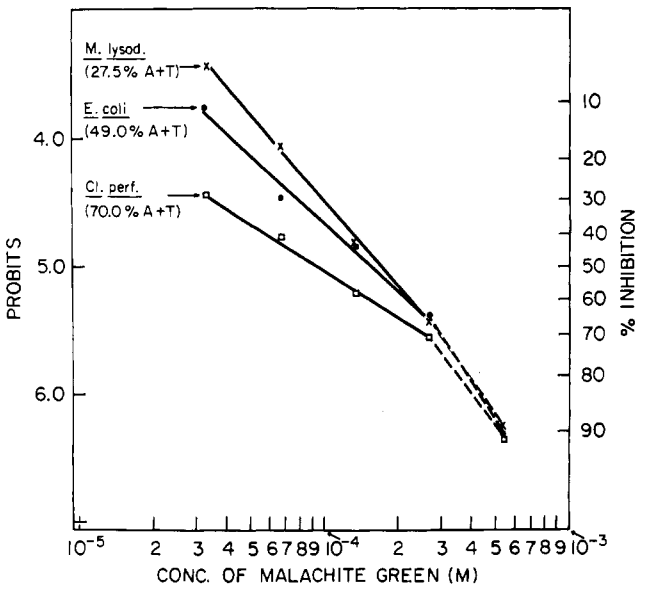


FIGURE 1: The dose-response to malachite green of *E. coli* B polymerase I catalyzed DNA synthesis utilizing bacterial DNA of various base proportions. The incorporation of uninhibited systems was as follows (in nanomoles): *C. perfringens* 4.12; *E. coli*, 4.09; *M. lysodeikticus*, 7.08. Low specific activity [$^3$H]dCTP was used.

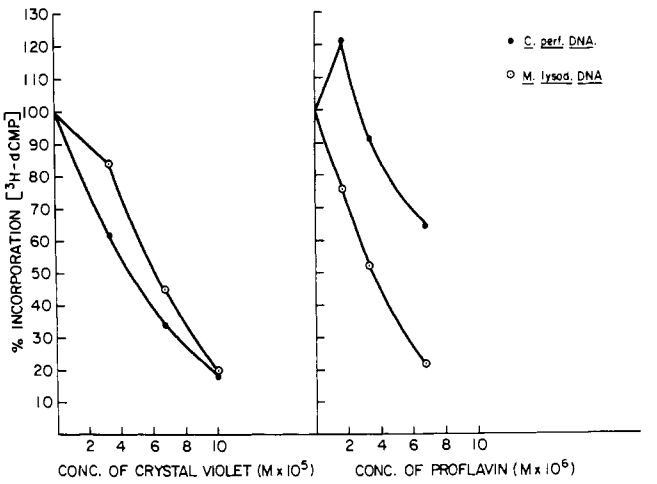


FIGURE 2: A comparison of the influence of crystal violet and proflavin upon polymerase I catalyzed DNA synthesis utilizing DNA from *C. perfringens* and *M. lysodeikticus*. The results obtained in proflavin-containing systems are similar to those obtained by Arca et al. (1970). Uninhibited system incorporation for crystal violet series (in nanomoles): *C. perfringens* 8.32, *M. lysodeikticus* 13.06; for proflavin series: *C. perfringens* 8.84, *M. lysodeikticus* 16.93. Low specific activity [$^3$H]dCTP was used.

mCi/mmol; high specific activity systems: 27 Ci/mmol). The standard incubation assays were linear for more than 10 min; hence, reaction systems were incubated at 37 °C for 10 min, 2.0 ml of cold 5% trichloroacetic acid containing 40 μg/ml of bovine serum albumin was added to stop the reaction, and these mixtures, in turn, were incubated at 4 °C for 20 min. The precipitated mixtures were filtered through Whatman glass filters, grade GF/D, the filters were then washed with 10 ml of additional cold 5% $Cl_3CCOOH$, suspended in 10 ml of hydromix scintillation cocktail (Yorktown Research Co., Hackensack, N.J.), and the scintillation vials were vigorously shaken and allowed to stand overnight. Filters were then assayed for radioactivity in a Nuclear-Chicago Mark II scintillation counter.

## Results

Figure 1 illustrates the dose–response to graded concentrations of malachite green of polymerase I catalyzed DNA polymerization utilizing bacterial DNA primer templates of markedly different base composition. The results are the averages of three or more experiments. The linearity of the inhibitions throughout a large range of dye concentrations is evidence that a single class of target sites is involved in that range, rather than, for example, both enzyme and DNA. The extent of inhibition was a function of the AT content of the tested DNAs, particularly at the lower dye concentrations, while the higher concentrations, regardless of the dye tested, tended to produce more uniform inhibition, a result which is reasonable because the triphenylmethanes also bind to GC but more weakly than to AT (Krey and Hahn, 1975). The change in slope of the curves shown in Figure 1 may indeed be taken as evidence of binding of the dye to the lower affinity sites, particularly since the final inhibition slope most closely resembles that obtained with use of the GC-rich micrococcal DNA. Qualitatively similar results were obtained with the other triphenylmethane dyes, thus confirming the inhibitory potency of this class of compounds; for *C. perfringens* DNA their 50% inhibitory concentrations were: crystal violet 4.2 × $10^{-5}$ M, methyl green 7.7 × $10^{-5}$ M, and malachite green 9.4 × $10^{-5}$ M. In the experiments of Muller and Gautier (1975), methyl green was found to have the greatest affinity for DNA, but also was the most sensitive of the tested dyes to high ionic strength buffers similar to those presently employed.

An alternate explanation for the bimodality depicted by Figure 1 possibly involves inhibition of DNA polymerization through dye binding to the enzyme itself, in the manner of the anionic triphenylmethanes, such as aurintricarboxylic acid, which inhibits a reverse transcriptase (Liao et al., 1975) and gallin, which additionally inhibits RNA polymerase (Liao et al., 1974). However, in the latter instance, the activity of the dye was found to depend upon the presence of the anionic catechol substituents (Liao et al., 1974), while these dyes more generally are considered enzyme, rather than template poisons (Liao et al., 1975; Bina-Stein and Tritton, 1976).

Figure 2 compares the influence of crystal violet with that of the intercalant proflavin upon DNA replication using two standard bacterial templates. An extensive series of investigations (Muller and Crothers, 1975; Muller et al., 1975) has revealed that intercalants, in general, possess a greater affinity for GC-rich DNA than for AT-rich DNA. It was deemed necessary to show that the present reaction systems were indeed capable of distinguishing base affinities by contrasting internal and external DNA ligands, and the results demonstrate the expected inhibitions. Thus, crystal violet was most potent with clostridial DNA, while proflavin was most potent with the GC-rich micrococcal DNA. Intercalation of the triphenylmethanes themselves was at one time considered (Lerman, 1964), but such complexes are now thought improbable (Neville and Davies, 1966; Krey and Hahn, 1975).

Table I reveals the influence of the triphenylmethanes and proflavin upon polymerase I product hydrolysis. These experiments were carried out using an excess of enzyme and a limiting quantity of high specific activity [$^3$H]dCTP to rapidly synthesize DNA, and then to render the product progressively more $Cl_3CCOOH$ soluble as a function of enzymatic hydrolysis. The results clearly show inhibition of hydrolysis by all the compounds tested, and render unlikely in vivo destruction of DNA by polymerase I type enzymes during inhibition of DNA

TABLE I: Inhibition of Polymerase I Product Hydrolysis by Triphenylmethane Dyes And Proflavin.[a]

| Inhibitor | Inc. Time (Min) | System | pmol of [$^3$H]dCMP Remaining | pmol of [$^3$H]dCMP Hydrolyzed | % Inhibition |
|---|---|---|---|---|---|
| | | Experiment I: | | | |
| | 0–3 | Uninhibited | 243 | | |
| | 3–60 | Uninhibited | 30 | 213 | 0 |
| Methyl green | 3–60 | $2.38 \times 10^{-5}$ M | 89 | 154 | 18 |
| | 3–60 | $7.86 \times 10^{-5}$ M | 207 | 36 | 84 |
| Malachite green | 3–60 | $3.33 \times 10^{-5}$ M | 119 | 124 | 42 |
| | 3–60 | $1.00 \times 10^{-4}$ M | 196 | 47 | 78 |
| | 3–60 | $1.66 \times 10^{-4}$ M | 227 | 16 | 93 |
| Crystal violet | 3–60 | $3.33 \times 10^{-5}$ M | 176 | 67 | 69 |
| | 3–60 | $1.00 \times 10^{-4}$ M | 227 | 16 | 93 |
| | | Experiment II: | | | |
| | 0–10 | Uninhibited | 233 | | |
| | 10–60 | Uninhibited | 96 | 137 | 0 |
| Methyl grreen | 0–10 | | 253 | | |
| | 10–60 | $1.66 \times 10^{-4}$ M | 263 | 0 | 100 |
| Proflavin | 0–10 | | 183 | | |
| | 10–60 | $1.00 \times 10^{-4}$ M | 193 | 0 | 100 |

[a] Methodology: time of uninhibited incubation in experiment I was 3 min, and in experiment II 5 min. At these times, 0.25-ml aliquots of such reaction systems were distributed into tubes containing either 0.05 ml of the respective inhibitors, or water, and incubations were continued for the specified periods. Initial incubation volume in experiment I was 3.38 ml, and in experiment II 4.44 ml. Standard concentrations of components were used, except that each 0.30-ml reaction mixture contained 1.05 enzyme units in experiment I and 0.53 enzyme unit in experiment II. The data in experiment II were obtained from an assay of the time course of inhibition, and the reaction mixtures actually contained the inhibitors for the period btween the transfer of aliquots at 5- and 10-min samples. Reaction mixtures containing inhibitor revealed absolute suppression of hydrolysis throughout the incubation period, in contrast to control mixtures in which hydrolysis was continuous.

synthesis by these or related drugs. Such results also suggest that it might be appropriate to reinvestigate the influence of methyl green and of triphenylmethane dyes, more generally, upon the endonucleolytic cleavage of DNA by pancreatic DNase, although it should be noted that measuremnt of DNA hydrolysis through decolorization of methyl green is carried out with the isolated complex rather than in the presence of high concentrations of the dye.

## Discussion

These experiments have shown that the cationic triphenylmethane dyes inhibit in vitro DNA replication catalyzed by polymerase I and, additionally, inhibit the hydrolysis of the polymerase I reaction product. Such inhibitions are reasonable in view of the extensive binding of these dyes to DNA. Thus, the replication of clostridial DNA, containing the highest proportion of AT to GC, was most severely inhibited by the three dyes tested, while the least inhibited template contained the lowest proportion of AT to GC. Recently, it was proposed that methyl green bound to DNA by two modes, a hypothesis based upon bimodal displacement curves of methyl green from DNA by a series of DNA organic ligands or $Mg^{2+}$ (Krey and Hahn, 1975), while Muller and Gautier (1975) inferred that crystal violet bound to DNA by two modes. It is noteworthy that the inhibition curves presented in Figure 1 are also essentially bimodal and, therefore, possibly reflect the apparent base affinities of these dyes.

Dye interference with the attachment of $Mg^{2+}$ to DNA may also play a major role in inhibition, for, although $Mg^{2+}$ could release methyl green from DNA (Krey and Hahn, 1975), this release required hours and is in contrast to the present 10-min reaction period. It is not unlikely that the dyes inhibit DNA replication by cationic attachment to the phosphoric acid residues of these polymers, in part, as a function of the proximal bases and their relative hydrophilic properties. Both enzyme progression along the template as well as enzyme binding to it may thus be blocked, although it should be noted that exonucleolysis occurred subsequent to enzyme binding, as evidenced by prior product formation.

It has been found that methyl green binds to the B form of DNA (Krey and Hahn, 1975) (a characteristic of AT regions (Arnott and Selsing, 1974)), and it was suggested that in so doing the dye spans the minor groove. However, were polymerase I to bind to nicks in the major groove, then inhibition of DNA synthesis through occupancy of the minor groove would be problematic, although it is reasonable to argue that by spanning the minor groove methyl green could prevent strand separation necessary to polymerization, and, also, thereby immobilize the free 3′ termini necessary to exonucleolytic hydrolysis. Experimental evidence indicating that methyl green prevents strand separation is found in the large increase in $\Delta T_m$ induced by this compound upon thermal denaturation of calf thymus DNA (Krey and Hahn, 1975), although the highly potent crystal violet (Krey and Hahn, 1975) and malachite green (Wolfe, unpublished observation) caused little change in the thermal denaturation profile of this DNA, thereby casting doubt upon the general applicability of this mechanism. Indeed, the inhibition of a nuclease reaction by the triphenylmethanes and intercalants suggests that their suppression of DNA synthesis may occur through obstruction of many reactions catalyzed by polymerase I, rather than by a single mechanism.

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# Osmium-Labeled Polynucleotides. The Reaction of Osmium Tetroxide with Deoxyribonucleic Acid and Synthetic Polynucleotides in the Presence of Tertiary Nitrogen Donor Ligands†

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ABSTRACT: Osmium tetroxide in the presence of pyridine or 2,2′-bipyridine has been found to react completely with the pyrimidine moieties (thymine, uracil, and cytosine) in polynucleotides. Pyrimidine osmate ester moieties, $L_2OsO_4$-pyrimidine, were formed. The $OsO_4$ has added across the 5,6 double bond and L = pyridine or ½-bipyridine. The pyridine derivatives were not stable and decomposed slowly after the $OsO_4$-pyridine reagent was removed by gel chromatography. Labeled poly(uridylic acid) lost osmium completely during gel chromatography unless the eluent contained a high concentration of pyridine. The products formed between $OsO_4$-bipyridine and polynucleotides were much more stable and the Os label was retained during and after gel chromatography. Both the $OsO_4$-pyridine and $OsO_4$-bipyridine reagents reacted more rapidly than the $OsO_4$-$CN^-$ reagent.

The biological importance and utility of polynucleotides derivatized by inertly attached heavy metals have recently been summarized (Daniel and Behrman, 1976; Marzilli, 1977; Dale and Ward, 1975). Osmium labeling of polynucleotides has proved particularly useful and has found application in both electron microscopic (Highton et al., 1968; Whiting and Ottensmeyer, 1972) and x-ray crystallographic (Rosa and Sigler, 1974; Schevitz et al., 1972; Kim et al., 1972) investigations of nucleic acids.

Osmium tetroxide selectively degrades pyrimidine bases of nucleic acids (Beer et al., 1966; Burton and Riley, 1967). In the presence of added ligands, L = $CN^-$, pyridine, ½-bipyridine, etc., stable osmate ester derivatives are formed containing the moiety $L_2OsO_4$-pyrimidine (Highton et al., 1968; Subbaraman et al., 1971; Daniel and Behrman, 1975, 1976). For L = pyridine, recent crystallographic investigations have

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