Studies on the Methyl Green–DNA Complex and Its Dissociation by Drugs[†]

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ABSTRACT: Spectrophotometric results indicated that Methyl Green bound stably to native calf thymus DNA and to poly[d(A-T)] but to a lesser extent to ϕX 174 DNA, tRNAs, and poly(dG·dC), a copolymer that exists preferentially in the A conformation. Exposing the Methyl Green–DNA complex to graded concentrations of ethyl alcohol liberated part of the dye slowly by a zero-order reaction; higher alcohol concentrations which cause the B → A transition of DNA released the bulk of Methyl Green. The viscosity of the Methyl Green–DNA complex was significantly lower than that of the uncomplexed DNA. The dye was progressively liberated from DNA by 1.5×10^{-1} *M* NaCl and by much lower concentrations of Mg^{2+}; in its stoichiometric complex with DNA, it increased T_m by ~12°C. A series of DNA-complexing drugs displaced Methyl Green from DNA at exponential rates and to end points which were correlated. End points of displacement correlated with the abilities of drugs to unwind supercoiled DNA, to labilize ribosomes to heat, and to eliminate a kanamycin resistance determinant from an R factor carried by *Salmonella typhimurium*. Additional correlations between Methyl Green displacement and biochemical-biological activities of displacing drugs are cited. In conjunction with these findings, our results suggest that Methyl Green displacement analysis is a useful biochemical screen for the detection or development of biologically active compounds which bind to DNA.

The triphenylmethane dye, Methyl Green, MG,[1] is a histochemical stain for DNA and its complex with DNA is used as an experimental substrate for the determination of deoxyribonucleases (Kurnick, 1950, 1962). The MG-DNA complex is dissociated by drugs whose bioreceptor is DNA, and this MG displacement has been used to study the binding of synthetic drugs, antibiotics, and alkaloids to the double helix (Kurnick and Radcliffe, 1962; Rauen et al., 1965; Zeleznick and Sweeney, 1967; Zeleznick et al., 1969; Bates et al., 1969; Krey and Hahn, 1970, 1971, 1974; Zunino and di Marco, 1972).

The use of the MG-DNA complex as a DNase substrate or in displacement analysis is based upon the fact that free MG at pH values of >5 undergoes rapid spontaneous molecular rearrangement to its colorless carbinol base (Kurnick and Foster, 1950) so that the liberation of the dye from DNA by enzymatic hydrolysis or by displacement can be followed spectrophotometrically as a decrease in absorbance at 642 nm.

Information related to the structure of the MG-DNA complex is scattered and fragmentary, thus rendering it difficult to infer a mechanism of the dye's displacement from the double helix. MG binds to DNA with a stoichiometry of 1 molecule per 13 nucleotides (Kurnick and Foster, 1950), i.e., ~two-thirds of one helical turn of the double helix in its B conformation, and prevents photodimerization of pyrimidines over a critical interval of 20 base pairs (Sutherland and Sutherland, 1969). In its complex with DNA, the MG color base is stabilized and also protected from oxidation by hydrogen peroxide (Kurozumi et al., 1963). Binding is preferential for native duplex DNA: heat-denatured DNA binds little MG (Rosenkranz and Bendich, 1958; Scott and Willett, 1966; Scott, 1967). Transverse fragmentation of high-molecular DNA to shorter duplex structures with sedimentation coefficients as low as 5.5 did not diminish the binding of MG (Rosenkranz and Bendich, 1958).

Scott and Willett (1966) determined "critical concentrations" of NaCl which prevented MG complexation with anionic biopolymers, found that these molar concentrations were two orders of magnitude higher for MG-DNA complexes than for the binding of the dye to a variety of RNAs or to biological polysulfates, and speculated that MG was intercalated into DNA and electrostatically bound with two of its cationic charges "at opposite sides of the helix".

Polarization microscopy of MG-DNA fibers indicated that the light "absorbing groups" of the dye were lying parallel to the fiber axis although the orientation was imperfect (White and Elmes, 1952). Hahn and Krey (1969) observed in flow dichroism studies of the MG-DNA complex that the dichroism of the absorption band of the dye at 642 nm had the opposite sign of that of the base pairs at 259 nm. The direction of the 642-nm electronic transition was therefore arranged approximately perpendicular to the planes of the base pairs in the MG-DNA complex, i.e., parallel to the direction of flow.

Whether MG is, at least partially, intercalated into DNA or is bound peripherally to the double helix remained one central question. Citing Scott and Willett (1966), Zeleznick and Sweeney (1967) assumed that MG does intercalate into DNA and that its displacement by antibiotics was, therefore, a test for intercalation binding of these substances. Lerman (1964b) had inferred intercalation of three other triphenylmethanes, accommodated by enforced coplanarity of two of the three phenyl rings. A detailed analysis of the conformations of triphenylmethanes renders such a coplanarity unlikely because the molecules have a propeller-like conformation in which all three phenyl rings have the same sense of twist relative to a reference plane which can be placed through the three aryl carbons bonded to the central carbon atom (Gust and Mislow, 1973). An X-ray fiber

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[1] Abbreviation used is: MG, Methyl Green.

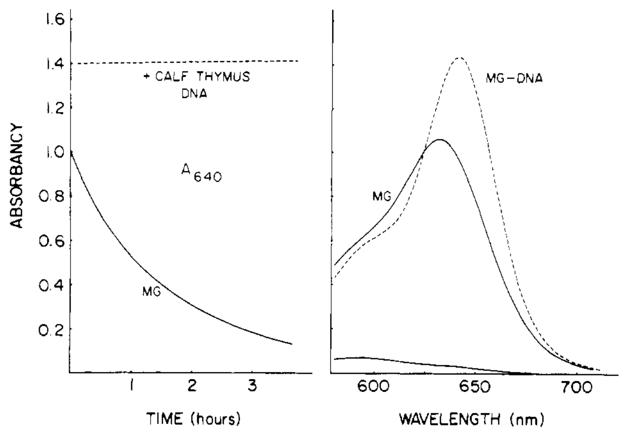

FIGURE 1: Effect of native calf thymus DNA on the absorbance of Methyl Green. At left are shown time courses of the absorbancies at 640 nm of free Methyl Green and of DNA-bound MG. At right are absorption spectra of the dye in the absence and presence of DNA; the solid line near the horizontal axis represents the spectrum of MG after the dye had undergone complete molecular conversion during 19 hr. Concentrations: 3.0×10^{-5} *M* MG, 2.8×10^{-4} *M* DNA phosphorus.

analysis of the complex of *p*-rosaniline with DNA has failed to detect indications of intercalation binding (Neville and Davies, 1966). Likewise, a recent viscometric titration with Crystal Violet of DNA fragments with a molecular weight average of ~200000 did not yield indications of intercalation binding (Müller and Gautier, 1975).

We report here results of biophysical studies on the MG-DNA complex with the major conclusions that (1) the dye is not intercalated, (2) it binds to double helices in their B conformation, and (3) the binding forces are predominantly electrostatic.

We have developed MG displacement analysis into a quantitative method which determines the kinetics and end points of this reaction; an abstract of our work has been published (Krey and Hahn, 1971). We report here the results of quantitative displacement analyses for a series of DNA-complexing drugs and correlations between these results and certain parameters of biological or biochemical actions of these drugs.

Materials and Methods

Calf thymus DNA was purchased from Worthington Biochemical Corporation, the Methyl Green-DNA complex was from the Sigma Chemical Company, tRNA was from General Biochemicals, and poly[d(A-T)], poly(dG·dC) and ϕX 174 single-stranded DNA were from Miles Laboratories, Inc.; irehdiamine was a gift from Dr. R. Goutarel, Centre National de la Recherche Scientifique, Institute des Substances Naturelles, 91-Gif-sur-Yvette, France. Nitroacridines 3582 and 2 as well as the dibenzimidazole derivative, Hoechst 33258, were generous gifts of Farbwerke Hoechst, Germany; compounds with WR code numbers were furnished by the Division of Medicinal Chemistry of our Institute. The other drugs and dyes were commercial preparations. All experiments with the exception of the viscometric studies were carried out in 5 m*M* Tris-HCl at pH 7.5. Viscosities were measured in 5 m*M* acetic acid-acetate buffer at pH 4.2.

Determinations of the displacement of Methyl Green from DNA by various chemical substances were performed in a Cary Model 14 spectrophotometer, absorption spectra were recorded in the same instrument, and thermal denaturation profiles were determined in a Gilford Model 2000 spectrophotometer which was equipped to record temperatures and optical densities automatically.

Viscosities were derived from determinations of flow times of DNA or DNA-MG test solutions in a Cannon semimicro dilution viscometer, Model K 100. An accuracy of flow times of ±0.05 sec was obtained when their determination was carried out with a Heath-Schlumberger electronic timer, Model SM-102A, coupled to the Wescan optical monitoring system of our viscometer by an electronic interface which was designed and built at the Walter Reed Army Institute of Research. The detectors of the optical system were two photosensitive resistors (Clairex CL 904L) which monitored the start and stop of the timing operation, respectively; as common light source of the system served an incandescent lamp (Chicago Miniature Lampworks 2307) which was placed relative to the detectors in such a manner that light reaching these detectors had been reflected by air or by the test solutions inside the capillary of the viscometer. A change in refractive index inside this capillary during flow of our test solutions past the detectors altered the amount of light reaching these photosensitive resistors; the resulting change in voltage drop across each photoresistor was amplified by an operational amplifier module (Teledyne Philbrick 1026), subsequently limited to ±5 V by a Zener diode and finally shaped by an integrated circuit analog voltage comparator (Signetics 526) to the appropriate trigger requirements (TTL level) of respectively the start or stop signal of the electronic timer.

Results

Effects of DNA and Other Polynucleotides on the absorption Spectrum of MG. Upon binding of MG to native calf thymus DNA, the dye's major absorption band at 642 nm underwent a bathochromic shift of 10 nm and a hyperchromic change of 40% (Figure 1). Bathochromic shifts indicate the binding of single dye molecules, rather than of dimers or aggregates (Michaelis, 1947); the observed hyperchromicity for MG is in contrast to absorbance changes usually seen for intercalated chromophores whose absorption intensity typically *de*creases when they are placed in the hydrophobic environment between DNA base pairs. The absorbance of free MG at 640 nm decreased as a first-order reaction with time while that of DNA-bound MG showed no change over an experimental period of 3.5 hr.

Reacting MG with single-stranded ϕX 174 DNA or with a population of tRNAs also produced bathochromic shifts but no drastic changes in absorption intensity of the dye's major band (Figure 2) which then decreased exponentially with a rate which for MG-ϕX 174 DNA was similar to that for free MG but was less for MG-tRNAs. While absorbance in the region between 575 and 675 nm of free MG had disappeared almost entirely after the free dye had undergone molecular rearrangement (bottom line, Figure 1, right side), the dye's spectrum was still seen after the mixtures of MG with ϕX 174 DNA or with tRNAs had been left standing in the dark for several days (Figure 2, upper right-hand corner, lower lines). Evidently, a fraction of MG became stably bound to these polymers.

When MG interacted with poly[d(A-T)] (Figure 2), the spectrographic manifestations resembled those seen with native calf thymus DNA (Figure 1). In contrast, poly(dG·dC) (Figure 2) produced a slight hypochromic change in the absorption band at 640 nm of MG; A_{640} then decreased slowly until, after several days, 33% of the original absorbances of MG-poly(dG·dC) remained (Figure 2). Besides by

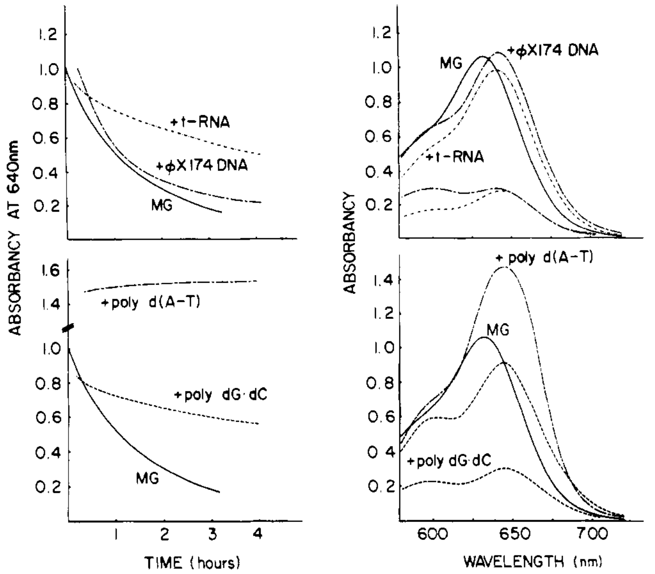

FIGURE 2: Absorbance of MG in the absence and presence of various polymers. The left-hand side of the diagram shows the time courses of the absorbances at 640 nm of MG in the presence (top) of tRNA and ϕX 174 single-stranded DNA and (bottom) of poly[d(A-T)] and poly(dG·dC). The right-hand side depicts the absorption spectra of MG bound to these same polymers; spectra were taken at zero (mixing) time and after no further absorbance change occurred. Concentrations: 3.0×10^{-5} *M* MG; 2.8×10^{-4} *M* tRNA, poly[d(A-T)], and poly(dG·dC); 3.5×10^{-4} *M* ϕX 174 DNA.

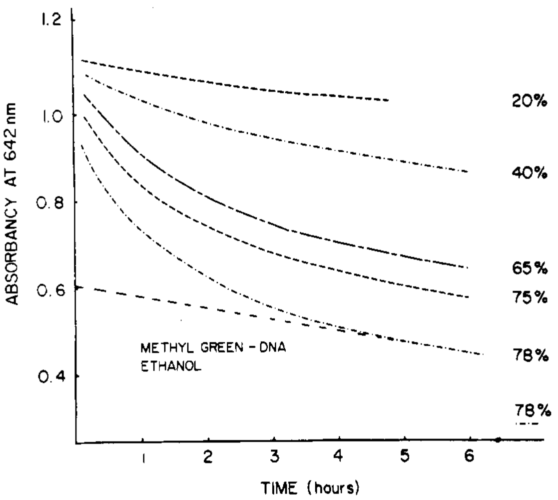

FIGURE 3: Effect of ethanol on the Methyl Green-DNA complex. Time courses for the release of Methyl Green from its complex with DNA were measured at different volume percents of ethanol; for 78% alcohol the extrapolation to zero time of the linear portion of the release kinetics is shown. Concentrations: 106 μg/ml of MG-DNA complex.

base composition and by the homopolymeric nature of its component strands, poly(dG·dC) differs from DNA and poly[d(A-T)] by its preference for the A conformation, although variable fractions coexist in the B conformation (Arnott and Selsing, 1974). The B conformation may be required for stable binding of MG to DNA or DNA-like duplex polymers. This was proved by showing that ethanol at 65-78 vol % which causes a B → A transition immediately released MG from DNA.

Effects of Ethyl Alcohol on the MG-DNA Complex. When exposed to an ethanol concentration of 20 vol % DNA shows an abrupt drop in viscosity which decreases further with increasing alcohol concentrations (Frisman et al., 1974). Up to a concentration of 40%, ethanol decreases the median thermal denaturation temperature, T_m, of DNA; beyond 40% ethanol, T_m increases again owing to a gradual transition of the B form of DNA to the A form (Usatyi and Shlyakhtenko, 1974). At 67-78% ethanol, DNA exhibits by circular dichroism the conformational change from the B to the A form (Ivanov et al., 1974). At 100% ethanol, DNA is completely denatured (Herskovits et al., 1961). Hence, within certain ethanol concentrations which are somewhat dependent upon inorganic ion concentrations, DNA undergoes gradual denaturation and a B → A transition.

Effects of ethanol, from 20 to 78%, on the MG-DNA complex were investigated with results shown in Figure 3. MG was liberated to extents which increased with increasing ethanol concentrations and also showed increasing initial curvature followed by transition to linearity beyond 4.5 hr. When the curved portions of the release kinetics were corrected by subtraction of the contribution of the linear process, extrapolated backwards, it was found that the time course of liberation of MG was a composite of a constant linear rate and of an initial burst of release of MG from DNA following addition of ethanol; the released dye then decayed as free MG according to first-order kinetics as shown in Figure 1. We assume that the progressive liberation of MG at linear rates was the result of progressive denaturation of DNA while the sudden release, upon addition of ethanol, was caused by the transition of DNA from the B to the A conformation. MG was not extracted from DNA by ethyl alcohol: placing a sample of the solid MG-DNA compound into 100% ethanol did not decolorize the sample upon prolonged exposure.

Effects of MG on the Intrinsic Viscosity of DNA. Intercalation increases the intrinsic viscosity of DNA (Lerman, 1961) because it lengthens and stiffens linear DNA. Viscosity enhancement, hence, is one of hydrodynamic criteria of intercalation binding. Lerman (1964a) tabulated one viscosity increase of chicken erythrocyte DNA (~5×10^{-5} *M* nucleotides) in the presence of 10^{-5} *M* fuchsin and referred (1964b) to this observation in speculating on the possible intercalation binding of triphenylmethane dyes. We have studied the influence of MG on the intrinsic viscosity of calf thymus DNA in a viscometer with an ultrasensitive automatic timing device (see Materials and Methods) with results shown in Figure 4. Over a DNA concentration range of from 125 to 210 μg/ml and a MG/DNA molar ratio of 1/13 nucleotides, the dye caused consistently a significant *decrease* in the intrinsic viscosity of DNA. This result argues against intercalation binding of MG.

Electrostatic Effects in the MG-DNA Interaction. When DNA was attached to cellulose, NaCl (>3 *M*) interfered with the formation of the MG-DNA complex (Scott and Willett, 1966) or dissociated it at 1-1.5 *M* (Inagaki and Kageyama, 1970). We found that the addition of 1.5×10^{-1} *M* NaCl to solutions of the MG-DNA complex liberated the dye at an exponential rate (not shown) which asymptotically approached, after 25 hr, a dissociation of 75% of the complex. Mg^{2+} at 10^{-3} *M* displaced 55% of bound MG while 5×10^{-1} *M* Mg^{2+} produced 96.5% displacement. These data are taken from Figure 5 which represents the absolute end points of MG displacement as a function of the first-order rate constants of the displacement reactions. The bimodal shape of this correlation requires comment. Ninety per cent of MG was displaced by

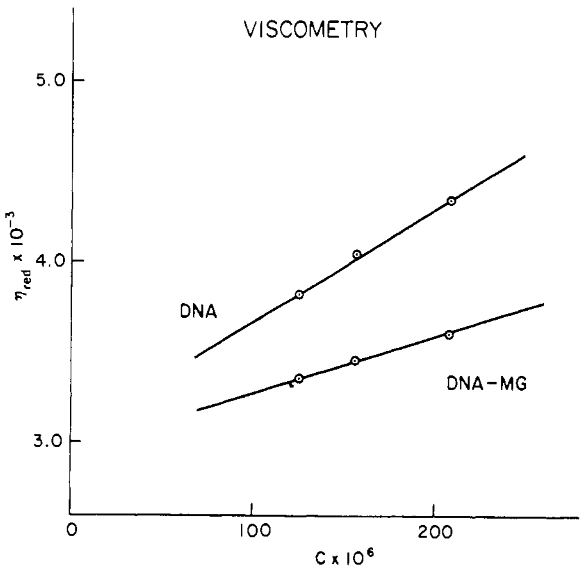

FIGURE 4: Effect of MG on the viscosity of DNA. The diagram shows reduced viscosities as a function of DNA concentrations (given in g/ml) for DNA in the absence of Methyl Green and in its presence at a constant molar ratio of DNA phosphorus to MG of 13.

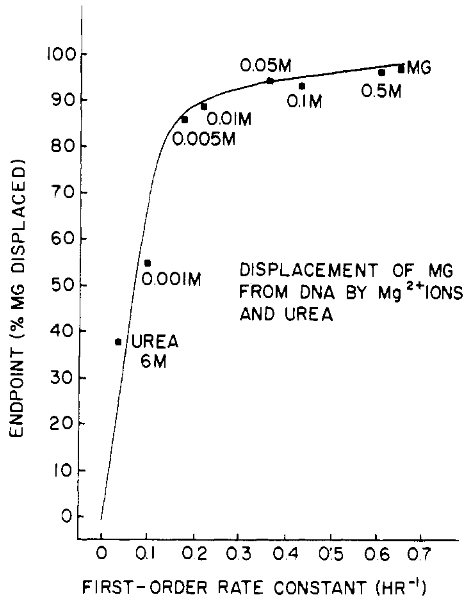

FIGURE 5: Displacement of MG from DNA by magnesium ions and urea. Mg^{2+} and urea were used at the concentrations indicated in the diagram; 1.88×10^{-5} *M* MG was considered bound to 2.45×10^{-4} *M* DNA phosphorus present.

concentrations of Mg^{2+} which caused a marked dependence of the end point on the reaction rate. The remaining 6.5% displacement was caused by higher Mg^{2+} concentrations for which the dependence of the end point upon the reaction rate was less pronounced. Differences in the kinetics of release of MG by ionic competition may point to more than one mode of attachment of MG to DNA.

We also have studied the release of MG from DNA by urea. Mirsky and Pauling (1936) have proposed that urea, by virtue of its ability to form hydrogen bonds, can break existing hydrogen bonds in biopolymers. It is perhaps more conservative to assume that urea disrupts nonpolar regions in biopolymers: the compound does not denature DNA. We found that urea, added at a concentration of 6 *M* to a solution of MG-DNA, slowly liberated the dye to an end point of 38% dissociation of the complex (Figure 5). We do not speculate on the nature of the nonionic forces which contribute to the stability of the MG-DNA complex; by comparison to ionic attraction, they are of minor importance.

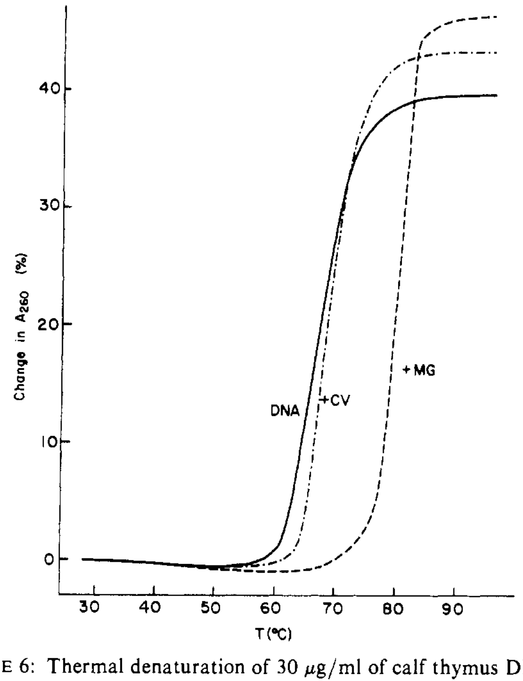

FIGURE 6: Thermal denaturation of 30 μg/ml of calf thymus DNA in the presence of Methyl Green or Crystal Violet and in their absence. Increases in absorbances at 260 nm were calculated with respect to DNAs absorbancy at room temperature. Concentrations of MG and CV, 7.5×10^{-6} *M*.

A counterionic effect of MG on the heat stability of DNA is illustrated in Figure 6 which shows the influence of the dye on the melting profile of calf thymus DNA. In its stoichiometric complex with DNA, MG shifted the thermal denaturation profile of the polymer by ~+12°C. In contrast, an equimolar concentration of Crystal Violet produced no significant shift in the melting curve of DNA although both dyes rendered the melting process marginally more cooperative. In contrast to MG, Crystal Violet does not possess a quaternary nitrogen atom.

Displacement of MG by DNA-Complexing Drugs. Our results of quantitative analyses of the displacement of MG from DNA by drugs whose bioreceptor is DNA are summarized in Figure 7. Free MG rearranged itself to its colorless carbinol base at a rate and to an end point which were greater than in any of the displacements entered in Figure 7. Hence, the decay of the liberated drug was not a rate-limiting process in displacement analysis. We reported (Krey and Hahn, 1974) that the concentrations of quinacrine (Qua) added to the MG-DNA solution (1.88×10^{-5} *M* MG) determined whether the displacement reaction was of first order (10^{-4} *M* Qua) or second order (2.5×10^{-5} *M* Qua) with time or (at 5×10^{-5} *M* Qua) underwent a change from first-order to second-order kinetics and have explained these kinetic differences as the result of the different stoichiometries by which MG and Qua are bound to DNA. All displacements, entered in Figure 7, were measured for a standard drug concentration of 5×10^{-5} *M*, and the definitive end points beyond which no further MG was displaced are plotted as percent displacement and represented as a function of the first-order rate constants of the displacement reactions.

The bimodal correlation between rate constants and end

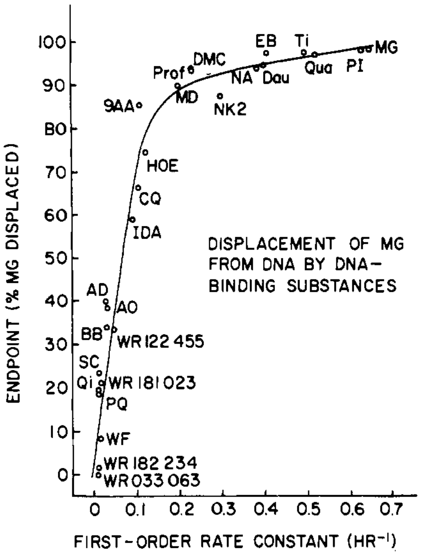

FIGURE 7: Displacement of MG from DNA by drugs and dyes. Displacing compounds: propidium iodide (PI), quinacrine (Qua), tilerone (Ti), ethidium bromide (EB), daunomycin (Dau), Nitroakridin 3582 (NA), distamycin A (DMC), proflavine (Prof), nitroakridin 2 (NK 2), Hoechst 33258 (HOE), chloroquine (CQ), irehdiamine (IDA), actinomycin D (AD), Acridine Orange (AO), berberine (BB), side chain of quinacrine and chloroquine (SC), quinine (Qi), primaquine (PQ), warfarin (WF), α-(2-piperidyl)-3,6-bis(trifluoromethyl)-9-phenanthrenemethanol hydrochloride (WR 122455), 6-methoxy-8-(4-amino-1-methylbutylamino)lepidine·4HCl·$2C_2H_5OH$·H_2O (WR 181023), 6-methoxy-8-(4-amino-1-methylbutylamino)quinaldine dihydrochloride (WR 182234), and α-(di-*n*-heptylaminomethyl)-6-bromo-9-phenanthrenemethanol hydrochloride (WR 033063); the displacing compounds were used at a concentration of 5×10^{-5} *M*; 1.88×10^{-5} *M* MG was considered bound to 2.45×10^{-4} *M* DNA phosphorus present; 1-cm light path. Miracil D (MD) and 6-chloro-2-methoxy-9-methylaminoacridine were used at a concentration of 5×10^{-6} *M*; 1.88×10^{-6} *M* MG was considered bound to 2.45×10^{-5} *M* DNA phosphorus present; 10-cm light path.

points in Figure 7 can be virtually superimposed on the same correlation, Figure 5, for the displacement of MG by graded concentrations of Mg^{2+}. As in Figure 5, there is an inflection at ~90% displacement. Up to this value, end points were strongly dependent upon the rate constants of the displacement reactions while for end points of >90% this dependence was less marked. Bimodality here suggests again the existence of discrete modes of binding of MG to DNA in addition to differences in the ability of the tested compounds to interfere with these modes.

The most important question raised by our results of displacement analyses of a number of drugs is, of course, that of the biochemical or biological significance of the numbers obtained. For several of the compounds tested, stoichiometries and apparent association constants of their binding to DNA have been determined by different methods in different laboratories. Since such results were obtained under varied experimental conditions (pH, inorganic ion concentrations, DNA, and drug concentrations) the collection of such heterogeneous data cannot be expected to show a clear-cut correlation with our parameters of the displacement reaction.

Waring (1970) determined equivalence points of binding of several intercalating drugs to supercoiled DNA, i.e., those fractions of intercalated drugs which converted supercoiled DNA to unconstrained circular DNA. Figure 8 suggests a correlation between these critical unwinding fractions and the end points of displacement of MG from our work for six compounds.

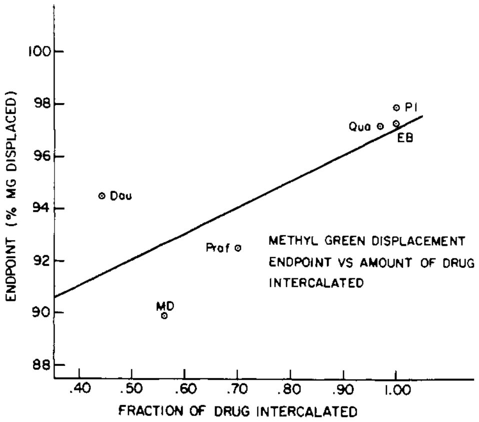

FIGURE 8: Correlation between the absolute end points of the displacement of MG produced by selected drugs (symbols as in Figure 8) and the fractional concentration of these compounds at their equivalence points of unwinding supercoiled DNA (Waring, 1970).

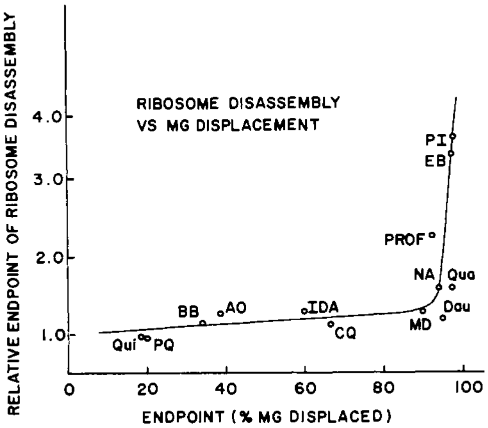

FIGURE 9: Effect of test compounds on disassembly of ribosomes (Wolfe et al., 1972) as a function of Methyl Green displacing end points.

Our laboratory reported earlier (Wolfe et al., 1972) that a series of intercalating compounds had the group property of labilizing ribosomes to thermal disassembly. The relative accelerations of this process by 13 compounds have been plotted in Figure 9 as a function of the end points of MG displacement from our present work. This correlation shows a remarkable similarity to Figures 5 and 7 in that end points of MG displacements when represented as a function of relative reaction rates, this time of ribosomal disassembly, again showed bimodality with an inflection at ~90% displacement.

Finally, our laboratory has studied the elimination of resistance determinants from R factor, R1, carried by *Salmonella typhimurium* (Hahn and Ciak, 1975), by intercalating drugs which presumably is based on selective template toxicity for plasmid DNA and its replication. The percent elimination by eight drugs of a determinant of kanamycin resistance are plotted in Figure 10 as a function of the end points of MG displacement by these compounds. One point which is somewhat distant from the linear correlation is that for chloroquine (CQ) which was proportionally less active against the kanamycin determinant than in the elimination of three other genetic markers from the R factor.

Discussion

Studies on the formation, dissociation, or properties of the MG-DNA complex have led us to conclude the fol-

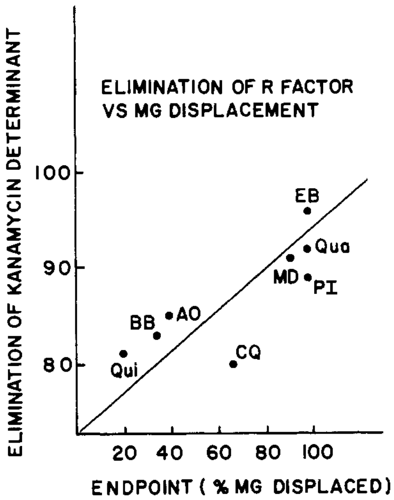

FIGURE 10: Relation between elimination by drugs of a determinant of kanamycin resistance (Hahn and Ciak, 1975) and end points of the MG displacement analysis.

lowing. (1) MG does not bind to DNA by intercalation. The molecule of the dye is not planar and coplanarity even of two of the phenyl residues is in doubt (Gust and Mislow, 1973). The *hyperchromic* change in the spectrum of MG upon its binding to DNA (Figure 1a, Scott, 1967) argues against any mode of binding, including intercalation, by which the chromophore would be placed into a hydrophobic environment. The viscosity decrease of DNA upon binding of MG (Figure 4) contradicts an assumption of intercalation binding. A viscometric titration with Crystal Violet of fragmented DNA of a molecular weight average of ~200000 revealed viscosity changes "far too small to be compatible with an intercalation process (Müller and Gautier, 1975)". (2) MG binds preferentially to double helices in the B conformation. This is suggested by preferential binding to DNA and poly[d(A-T)] in contrast to lesser binding to poly(dG-dC) which exists as a mixture of B and A conformations with preference for the latter (Arnott and Selsing, 1974). At critical ethyl alcohol concentrations which transform B-type into A-type DNA (Ivanov et al., 1974), the bulk of DNA-bound MG is released and then decays according to the kinetics of rearrangement of free MG. Netropsin, which binds preferentially to the B form of DNA, is also released at ethyl alcohol concentrations which cause the B → A transition (Luck and Zimmer, 1973). (3) MG binds to DNA prominently by ionic attraction. NaCl at 1.5×10^{-1} *M* and Mg^{2+} at lower concentrations displace MG from DNA by ionic competition. Nonionic binding, as reversed by 6 *M* urea, contributes only weakly to the coherence of the MG-DNA complex. In contrast, the intercalation complex of DNA with actinomycin D is readily dissociated by urea (Hartmann et al., 1963) but is unaffected by elevated concentrations of Mg^{2+} (Reich and Goldberg, 1964). In melting experiments, MG shifts the temperature range of the thermal denaturation process markedly to higher temperatures while Crystal Violet which does not possess a quaternary amino N fails to do so. It is assumed that the quaternary ammonium ion of MG is directly involved in binding of the dye to DNA phosphates.

MG is displaced from DNA to 90% by processes which render the end points of the reaction strongly dependent upon the rates; the remaining MG is displaced by processes which make the end points much less dependent upon the reaction rates. Since this is seen for one substance, Mg^{2+}, at different concentrations (Figure 5) as well as for a set of compounds at 5×10^{-5} *M* (Figure 7), it suggests that different displacements are results of different modes of attachment of MG to DNA such as would be caused by two cationic charges of the dye binding either to one strand of the double helix or to both strands across the minor groove. The first type of binding may be responsible for the stable binding of a fraction of the amount of MG interacting with single-stranded ϕX 174 (Figure 2). The displacement of MG from DNA by cationic drugs may, hence, be a combination effect, based in part upon counterionic competition and to another part by marked changes in the helical structure caused by intercalation binding. It should be noted that compounds which displaced MG with high rates and to end points of >90% are intercalative substances with high association constants and stoichiometries of DNA binding. One exception is the antibiotic distamycin A which does not intercalate but binds pseudoirreversibly to DNA (Krey et al., 1973).

The importance of MG displacement analysis firstly is methodological. While a preliminary and qualitative demonstration of the binding of a compound to DNA can be made by showing that DNA alters the absorption spectrum of the ligand or that such a compound sediments with DNA in analytical ultracentrifugation, MG displacement analysis yields within a few hours kinetic constants which are a measure of the affinity of the displacing compound for DNA. The end points of the displacement reactions are an expression of the stoichiometry of binding of a ligand to DNA. Such MG displacement analyses require only a simple spectrophotometer and the MG-DNA reagent. The usefulness of MG displacement in studying the interactions of DNA with drugs is documented in numerous research papers, cited in the introduction.

Secondly, the theoretical importance of results of displacement analyses with MG-DNA is that such numbers are correlated with a variety of biochemical or biological activities of DNA-complexing substances. Rauen et al. (1965) showed that MG-displacing concentrations of a series of phthalanilides were proportional to the antibacterial concentrations of these compounds. Zeleznick et al. (1969) found a correlation between the extent of MG displacement and the immunosuppressive activity of 19 substituted heterocyclic amines. Zunino and di Marco (1972) demonstrated increases in rates of MG displacements in a homologous series of distamycin congeners with from three to five *N*-methylpyrrole ring systems. Other parameters of binding to DNA (Zimmer et al., 1972) and antiviral activities of these compounds as well as their DNA template toxicity in RNA transcription (Chandra et al., 1971) increased similarly. Greenblatt et al. (1970) explained the long known interference between triphenylmethanes and aminoacridines in the experimental chemotherapy of trypanosomiasis by showing spectropolarimetrically that trypaflavine displaced Crystal Violet from DNA. Finally, we report here correlations between end points of MG displacement by DNA-binding drugs and the abilities of these compounds to unwind supercoiled DNA, to labilize ribosomes and to eliminate a resistance determinant from an R factor (Figures 8-10). We propose that MG displacement analysis may be useful as a biochemical screening method in the selection or development of biologically or therapeutically active compounds whose bioreceptor is DNA.

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