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Competition for Polymeric Binding Sites between Acridine and Triphenylmethane Dyes

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SUMMARY

Acridine and triphenylmethane dyes are known to display therapeutic interference in combination, but the basis for this interaction is not understood. When added to DNA, representatives of each group of dyes, which alone are optically inactive, displayed strong signals as monitored by optical rotatory dispersion. However, when trypaflavin and crystal violet were both added to the polymer, only the signal due to bound trypaflavin was observed. This competition for the polymer was explored on the one hand by alterations in the concentrations and structures of the dyes, and on the other by the use of different polymers: poly-α-L-glutamic acid, apurinic acid, and polylysine. It was found that the two amino groups attached to positions 3 and 6 of the acridine ring greatly influenced the competition. For example, 10-methylacridine orange did not abolish the signal of crystal violet in the presence of DNA. The polymer was also influential, so that the competition appeared reversed on poly-α-L-glutamic acid. Supplementary data obtained from spectroscopy and fluorescence quenching are also presented. These observations are discussed in terms of the biological phenomenon of therapeutic interference.

INTRODUCTION

The acridine and triphenylmethane dyes are both known to bind to DNA. If these dyes produce their toxic actions by this mechanism, their combined effects might be expected to reinforce each other. However, these two groups of chemicals show antagonistic action. This was demonstrated by Browning and Gulbransen (1), utilizing a strain of *Trypanosoma brucei* which was resistant to parafuchsin. In this case infected mice were cured by trypaflavin alone, but died when fed on parafuchsin plus trypaflavin. Other investigators extended these observations to non-parafuchsin-resistant strains *in vivo*, and to studies of oxygen and glucose uptake *in vitro* (2, 3).

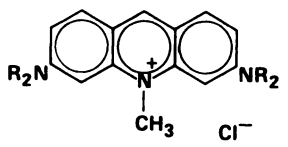
Albert (4) has discussed possible molecular bases for this interaction, including common or dual drug receptor systems, and drug-drug interactions which cause a decrease in the concentration of the monomolecular form of the active drug. In a previous paper, evidence was presented for the interaction of

these dyes *in vitro* as detected by physicochemical methods, and the possibility of complex formation was discussed (5).

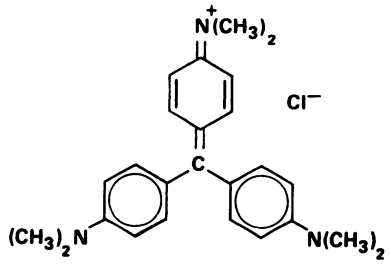
In the present study the competitive interactions of these groups of dyes with certain biopolymers were analyzed by optical spectroscopy and spectropolarimetry. The latter method has been shown by Stryer and Blout (6) and Yamaoka and Resnik (7) to give a sensitive measure of dye binding to optically active biopolymers. Neither dye alone in solution displays optical activity. However, when combined with the polymers there appears a marked degree of optical rotation about the visible absorption maximum of the dyes. Since these optical activity changes are undoubtedly related to the process of the binding of dyes (although the direct and quantitative relationship may not be established by optical methods alone and this paper is not intended for reporting such results), these studies were undertaken to help define further the possible mechanisms of therapeutic interference. Supplementary data from absorption spectroscopy and the quenching of fluorescence are also presented.

MATERIALS AND METHODS

The dyes and polymers used here have been previously described (5, 7, 8). 10-Methylacridine orange was prepared from acridine orange with dimethyl sulfate in boiling toluene (9). The structures of the dyes are shown in Fig. 1. Apurinic acid was prepared as described by Tamm *et al.* and by Petersen and Burton (10). The phosphate concentration was determined by the method of Chen *et al.* (11). A highly polymerized calf thymus DNA preparation was purchased from Worthington Biochemical Corporation. Samples of poly-α-L-glutamic acid (sodium salt) and poly-L-lysine (hydrobromide salt) were brought from Pilot Chemicals, Inc., Watertown, Mass. The concentration of DNA was determined photometrically with a molar extinction coefficient of 6400 at 259 nm and is thus expressed in terms of phosphate residues. The molar concentrations of the polypeptides were determined by weighing dry samples. The mean residue weights were 169 for poly-α-L-glutamic acid and 209.1 for poly-L-lysine.



TRYPAFLAVIN: R = H
10-METHYL ACRIDINE ORANGE: R = CH_3



CRYSTAL VIOLET

FIG. 1. *Structures of trypaflavin, 10-methylacridine orange, and crystal violet*

The ratio of polymeric site to dye, P/D, was defined as the molar concentration of the ionizable group of the polymer (phosphates for DNA and apurinic acid, carboxyls for poly-α-L-glutamic acid, and amines for poly-L-lysine) relative to the total concentration of dye in the solution. Other details are given in reference 7.

Optical rotatory dispersion (ORD) and circular dichroism (CD) were measured at 25 ∽ 27° with a Cary model 60 spectropolarimeter, while a Cary 14 recording spectrophotometer was utilized for absorption spectroscopy. Fluorescence spectra, with the molecule excited at the most efficient wavelength (obtained by studying excitation spectra, and usually about 460 nm) and corrected for "inner filter" effects, were obtained at room temperature on an Aminco-Bowman spectrofluorometer, using 1.00-cm cuvettes.

Low salt concentrations, which favor dye binding, were used. The details are given with each study. Generally, experiments were conducted in acid or neutral solution, so that the dyes were in the monocationic form. In all cases the dyes were mixed before addition of the polymer, so that both dyes were given an equal opportunity to "find binding sites." Approximately ½ hr elapsed between

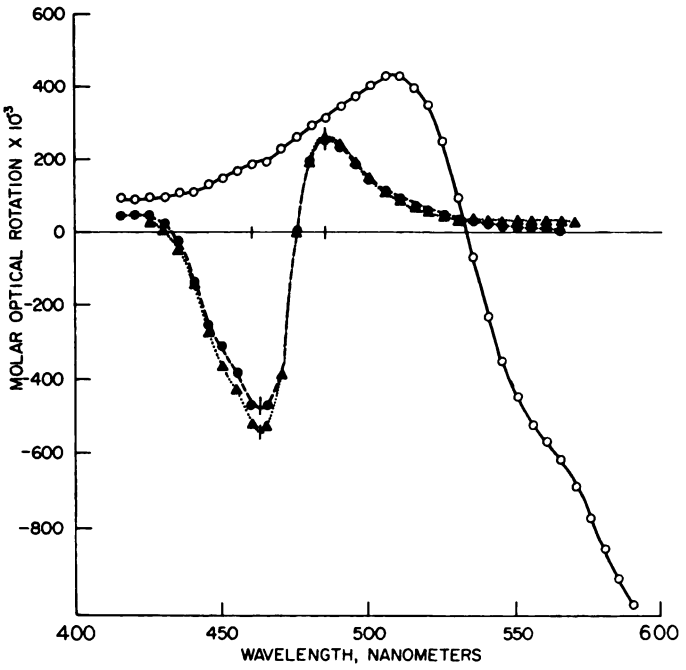


FIG. 2. *Optical rotatory dispersion patterns caused by dyes in the presence of calf thymus DNA* [DNA], 50 μM; [NaCl], 0.4 mM; pH 6.55. No buffer was present. ○, 22.6 μM crystal violet, P/D = 2.21; ▲, 10.5 μM trypaflavin, P/D = 4.76; ●, 10.5 μM trypaflavin and 22.6 μM crystal violet.

the time of mixing of dyes with polymer and the measurements. Neither the order of mixing nor time of equilibration seemed critical. Dyes and mixtures were kept in dim light except when measurements were made. Because of its stronger ORD signal, crystal violet rather than parafuchsin was more often used as the representative triphenylmethane dye.

RESULTS

Competitive binding of trypaflavin and crystal violet for calf thymus DNA. On mixing crystal violet with native calf thymus DNA, an ORD signal was observed at 533 nm; it increased with dye concentrations in the range of 10–100 μM, at a DNA to dye ratio (P/D) between 20 and 2. These results are shown in Figs. 2 and 3. However, in the presence of trypaflavin the signal due to crystal violet was abolished. In the experiment represented there were in solution 4.5 molecules of crystal violet and 2.1 molecules of trypaflavin per 10 phosphate groups at a DNA phosphate concentration of 50 μM. The signal of trypaflvin seemed only slightly affected, but no rotation due to crystal violet was detectable.

The nature of this competition can be seen in Fig. 3. Here the molar rotation[1] due to crystal violet alone at 600 nm is plotted against the crystal violet concentration. With addition of a constant amount of trypaflavin there was a marked suppression of the signal of crystal violet, far beyond that expected from a 1:1 competition for binding sites. With 2 crystal violet molecules for every trypaflavin in solution there was 90% suppression, and at a 1:1 ratio there was nearly complete abolition of the ORD signal. The blockade was not lifted even when there were 5 crystal violet molecules for every trypaflavin molecule. Since optimal dye binding occurred at P/D values between 3 and 5 (7) and in Fig. 2 both dyes together brought the ratio only up to 1:4, it was

[1] The definition of the molar rotation of the dye in the presence of the polymer is given in reference 7.

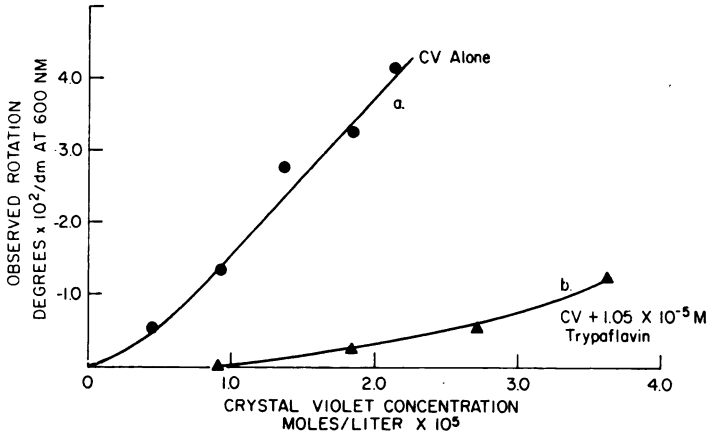


FIG. 3. *Effect of trypaflavin on ORD signal of crystal violet (CV) as a function of dye concentration in the presence of calf thymus DNA*

[DNA], 50 μM; [NaCl], 0.4 mM; pH 6.55. No buffer was present. ● (*a*), no trypaflavin; ▲ (*b*), 10.5 μM trypaflavin.

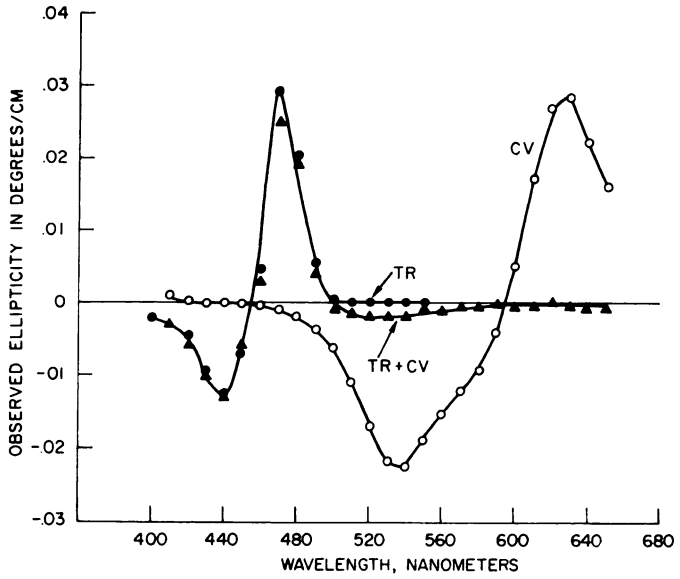


FIG. 4. *Circular dichroism studies of trypaflavin (TR) and crystal violet (CV) in the presence of calf thymus DNA*

[DNA], 61.4 μM; [NaCl], 0.4 mM; pH 6.55. No buffer was present. ●, 28.7 μM trypaflavin, P/D = 2.14; ○, 16.1 μM crystal violet, P/D = 3.81; ▲, 28.7 μM trypaflavin and 16.1 μM crystal violet.

possible that all the available sites were occupied. However, in the experiment in Fig. 3 and in other studies that follow, in which there were many sites still available, trypaflavin made these sites inaccessible to crystal violet in some manner.

Circular dichroism studies also showed the same phenomenon. Figures 4 and 5 summarize similar competitive studies in which circular dichroism was used to monitor the crystal violet interaction with DNA. Crystal violet concentrations of 4–16.1 μM showed binding (P/D between 15.4 and 3.81), and again equal concentration of trypaflavin markedly repressed the crystal violet signal. There was little reciprocal effect of the crystal violet on the trypaflavin signal.

The spectral data reveal a complicated

pattern of changes (Fig. 6). At low ratios of trypaflavin to crystal violet there was a small drop in intensity (compare curves *a* and *d* in Fig. 6A), which was indicative of the occurrence of competitive binding to DNA between these two dyes, but as the concentration of trypaflavin was increased the curve approached that of crystal violet in solution (Fig. 6C). The shoulder at 550 nm reappeared first, while the maximum at 590 nm appeared last.

Effect of alteration of chemical structure of dye on competition. When the free amino groups of trypaflavin were converted to the dimethyl-substituted form, 10-methylacridine orange (chemical structure No. 2), the Cotton effect was still manifest. Both crystal violet and 10-methylacridine orange at equimolar concentrations displayed their respective ORD signals in the three-component system (Fig. 7). However, the signal of crystal violet was somewhat decreased. Parafuchsin, as well, gave an ORD signal in the presence of 10-methylacridine orange.

Effects of other biopolymers. Other biopolymers were utilized in competition studies to determine whether the structure of the polymer also influenced the nature of the competiton. DNA was converted to apurinic acid. This polymer is essentially a denatured, single-stranded DNA free of purine bases (9). In this case (Fig. 8) the Cotton effect was observed for both dyes in a three-component system: apurinic acid, trypaflavin, and crystal violet.

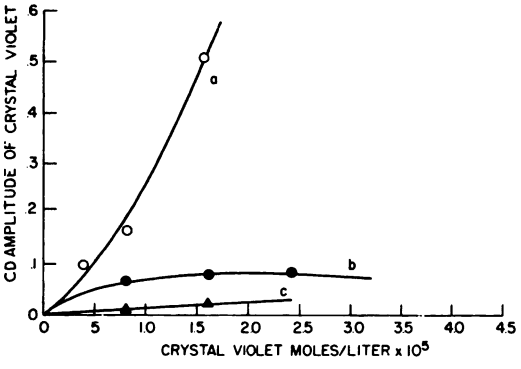


FIG. 5. *Effect of trypaflavin on circular dichroism amplitude of crystal violet in the presence of calf thymus DNA*

The amplitude is the difference in the readings of ellipticity at 630 and 520 nm. [DNA], 61.4 μM; [NaCl], 0.4 mM; pH 6.55. No buffer was present. ○ (*a*), no trypaflavin; ● (*b*), 14.4 μM trypaflavin; ▲ (*c*), 28.7 μM trypaflavin.

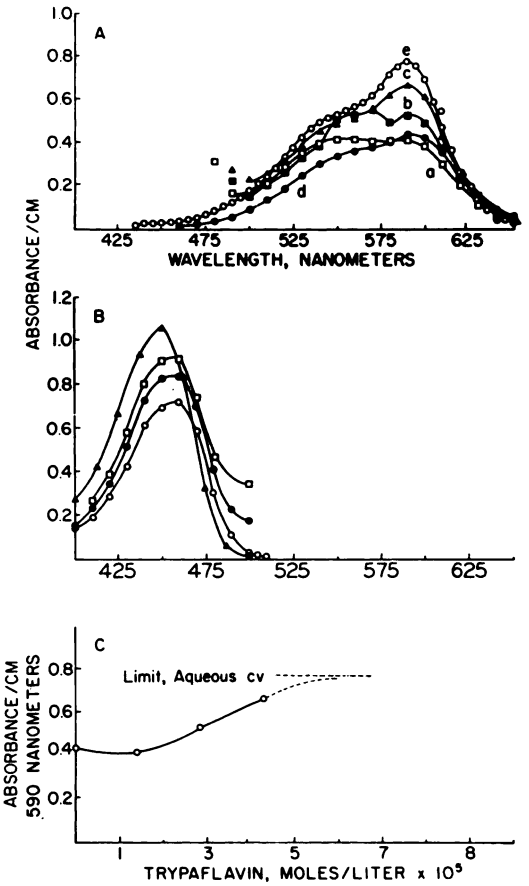


FIG. 6. *Effect of trypaflavin on absorption spectra of crystal violet in the presence of calf thymus DNA*

A. Absorption spectra of crystal violet at various levels of trypaflavin concentration. [Crystal violet], 8.1 μM; [NaCl], 0.4 mM; [DNA], 61.4 μM (except for spectrum *e*). □ (*a*), 14.4 μM trypaflavin; ■ (*b*), 28.7 μM; △ (*c*), 43.1 μM; ● (*d*), no trypaflavin; ○ (*e*), aqueous crystal violet in the absence of DNA and trypaflavin.

B. Absorption spectra of trypaflavin at various levels of crystal violet concentration. [Trypaflavin], 28.7 μM; [NaCl], 0.4 mM; [DNA], 61.4 μM (except for ▲). ●, 8.1 μM crystal violet; □, 16.1 μM; ○, no crystal violet; ▲, aqueous trypaflavin in the absence of DNA and crystal violet.

C. Influence of trypaflavin concentration on the intensity of the absorption maximum of crystal violet (cv) at 590 nm. [Crystal violet], 8.1 μM; [DNA], 61.4 μM; [NaCl], 0.4 mM. Note that the curve approaches the value of aqueous crystal violet as a limit.

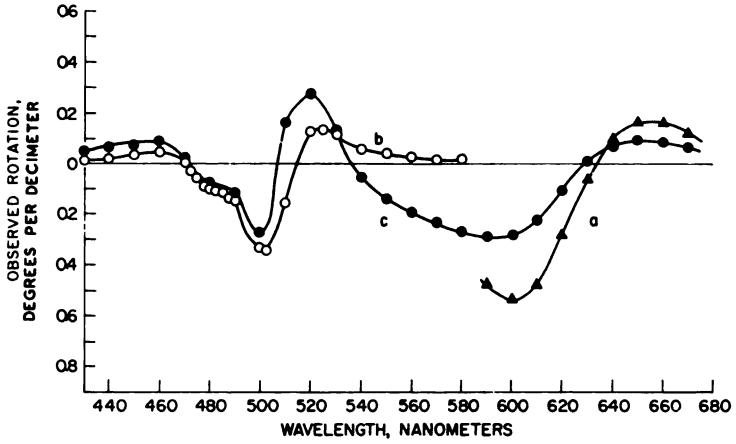


FIG. 7. *Optical rotatory dispersion measurements of crystal violet and 10-methylacridine orange in the presence of calf thymus DNA, showing role of dye structure and competitive binding*

[DNA], 90.7 μM; [NaCl], 0.8 mM; pH 6.5. No buffer was present. ▲ (*a*), 22.6 μM crystal violet; ○ (*b*), 22.6 μM 10-methylacridine orange; ● (*c*), both crystal violet and 10-methylacridine orange at the same concentrations.

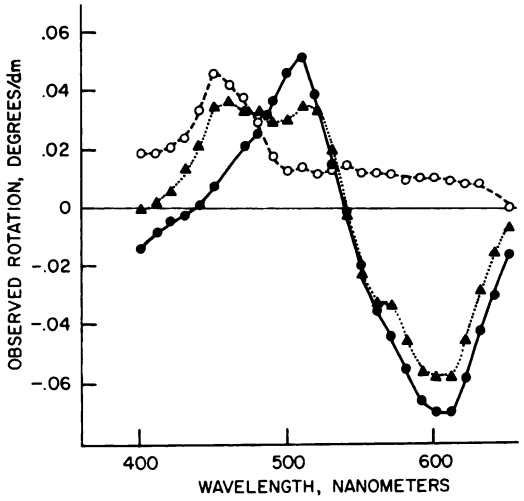


FIG. 8. *Optical rotatory dispersion curves of apurinic acid in the presence of dyes*

[Apurinic acid], 0.5 mM in all samples. The solutions were buffered at pH 8.0 in 0.005 M sodium phosphate buffer. ●, 33.6 μM crystal violet, P/D = 14.9; ○, 61.6 μM trypaflavin, P/D = 8.12; ▲, crystal violet and trypaflavin at the same concentrations.

Of special interest was a marked quenching of the fluorescence of trypaflavin when combined with crystal violet in the presence of apurinic acid. (Crystal violet itself is nonfluorescent.) In Fig. 9 it can be seen that quenching by crystal violet alone or by apurinic acid alone is considerable (curve *a* compared with curves *b* and *c*), but this is further enhanced by presence of both substances (curve *d*). We have discussed elsewhere (5) the nature of the quenching in the absence of polymers. Figure 10 illustrates the less dramatic spectral alterations that occurred when crystal violet and trypaflavin were both bound to apurinic acid. There was a marked hypochromicity in both cases, and

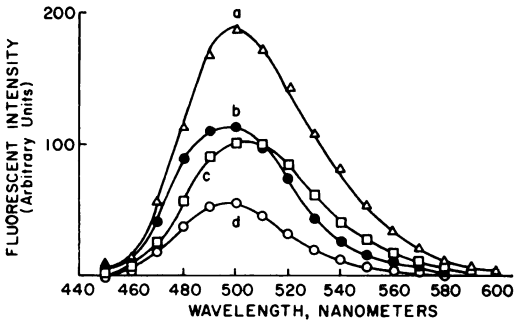


FIG. 9. *Fluorescence spectra of trypaflavin in the presence and absence of apurinic acid and crystal violet*

△ (*a*), 30.8 μM trypaflavin only; ● (*b*), 30.8 μM trypaflavin and 16.8 μM crystal violet; □ (*c*), 30.8 μM trypaflavin and 0.5 mM apurinic acid, solution buffered as in Fig. 8; ○ (*d*), 30.8 μM trypaflavin, 16.8 μM crystal violet, and 0.5 mM apurinic acid, solution buffered as in Fig. 8. The excitation wavelength was 460 nm.

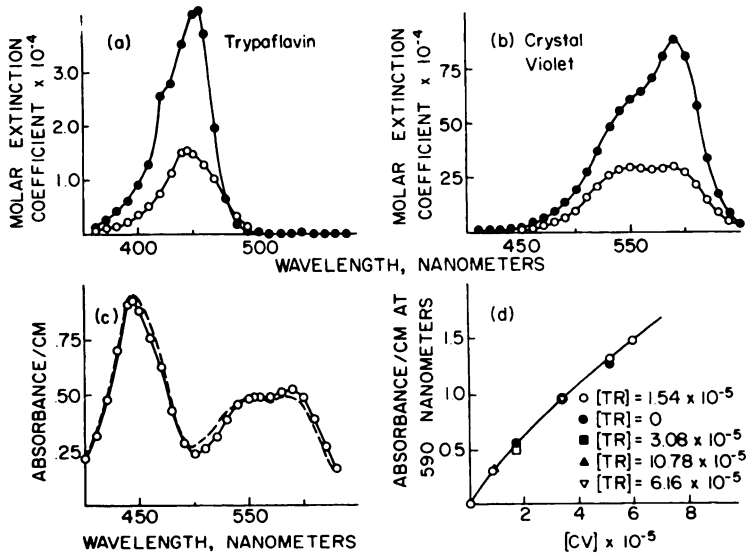


FIG. 10. *Absorption of dyes in the presence and absence of apurinic acid, showing effect of binding on spectra*

[Apurinic acid], 0.5 mM; [NaCl], 1 mM; pH 8.1. a. ●, 30.8 μM trypaflavin only; ○, 30.8 μM trypaflavin and apurinic acid. b. ●, 16.8 μM crystal violet only; ○, 16.8 μM crystal violet and apurinic acid. c. ○——○, 61.6 μM trypaflavin, 16.8 μM crystal violet, and apurinic acid; - - -, simple summation of open circles in parts a and b. d. Absorption of crystal violet (CV) at 590 nm in the presence of apurinic acid, showing the effect of various concentrations of trypaflavin (TR).

changes were observed in the positions of the absorption maxima. The crystal violet maximum and satellite shoulder developed a bimodal appearance. In Fig. 10c it can be seen that slight changes did occur from the expected simple summation in the three-component system, but these were not prominent within the concentration range of this study. Figure 10d emphasizes the failure of trypaflavin to influence the absorption intensity of crystal violet at 590 nm.

Poly-α-L-glutamic acid, with ionizable carboxyl side chain groups, has a random coil conformation in neutral or alkaline solution but exists as an α-helix at pH 5 or lower. With this molecule competition between crystal violet and trypaflavin was greatly reduced, and showed a reverse trend (Fig. 11). Whereas only 0.5 molar equivalent of trypaflavin was sufficient to reduce the signal of the crystal violet–DNA system by 90%, 4.5 eq of trypaflavin were necessary to reduce the signal of crystal violet–poly-α-L-glutamic acid by only 50%. Conversely, crystal violet suppressed the signal of trypaflavin greatly when poly-α-L-glutamic acid was present, just as trypaflavin did for crystal violet in the presence of DNA.

The spectroscopic data regarding the interaction of poly-α-L-glutamic acid and the dyes revealed certain quantitative and qualitative differences. The spectrum of trypaflavin bound to poly-α-L-glutamic acid appeared to resemble the hypochromicity observed with DNA, but the spectrum of crystal violet was quite different (Fig. 12). The peak at 590 nm essentially disappeared, and the former shoulder at 550 nm became the dominant peak. A new maximum, not previously seen, appeared at 505 nm. Studies will be published later concerning these spectral changes, but at the present time they are best explained by formation of a crystal violet aggregate, which takes place on the polymer chain at concentrations lower than that of the aqueous solution. The fluorescence of trypaflavin was enhanced by the presence of the polymer (Fig. 13). However, in the presence of crystal violet and the polymer there was quenching.

As one might expect, poly-L-lysine, a positively charged polymer, gave no ORD signal

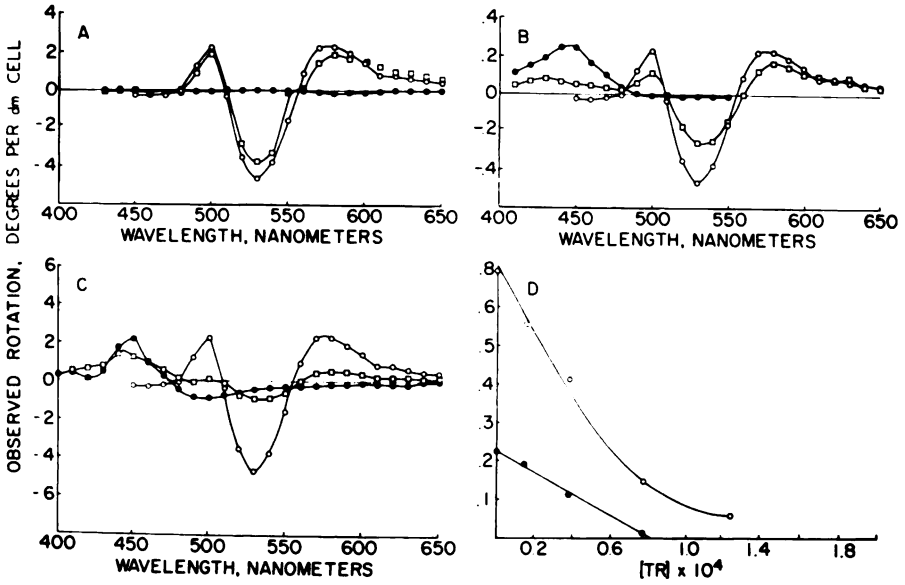


FIG. 11. *Optical rotatory dispersion measurements of crystal violet and trypaflavin in the presence of 5.9 mM poly-α-L-glutamic acid, pH 6.4, showing effect of competitive binding*

A. ○, 8.4 μM crystal violet only; ●, 38.0 μM trypaflavin only; □, 8.4 μM crystal violet and 15.4 μM trypaflavin.

B. ○, 8.4 μM crystal violet only; ●, 38.0 μM trypaflavin only; □, 8.4 μM crystal violet and 38.0 μM trypaflavin.

C. ○, 8.4 μM crystal violet only; ●, 77.0 μM trypaflavin only; □, 8.4 μM crystal violet and 77.0 μM trypaflavin.

D. Effect of trypaflavin (TR) on the Cotton effect of crystal violet. [Crystal violet], 8.4 μM. ○, observed rotation at 575 nm minus observed rotation at 530 nm; ●, observed rotation at 500 nm.

with either dye singly or in combination under the present experimental conditions.

DISCUSSION

The data presented here stress how marked the influences of dye and polymeric structure are on the competitive binding of two groups of dyes. These effects may not be seen clearly, if at all, when studies are done with one dye alone. For example, Löber (12) has found that 10-methylacridine orange has a 5-fold higher binding constant with DNA than does trypaflavin at concentrations near those used in this study. However, 10-methylacridine orange fails to show strong competition for binding sites when used together with crystal violet. It is possible to suggest a basis for this distinction when one considers how 10-methylacridine orange differs from trypaflavin: the reactive free amino groups are no longer available, and the bulk and cross-sectional projection of 10-methylacridine orange is greater. It has, in fact, been suggested (12) that acridine orange is externally bound while proflavin is intercalated in the DNA helix. Whether the free amino groups of proflavin assist in intercalation by some energetic mechanism, as suggested by Gilbert and Claverie (13), or whether the proflavin reaction is due only to the removal of the steric hindrance of the four additional methyl groups is difficult to say.

If bulk or cross-sectional projection are important, there must be a variation in the availability of dye-binding sites. As Yamaoka and Resnik (7) have pointed out, binding does not have to be random. For an explanation of our results, binding may not only be nonrandom but may involve selective availability of sites. Trypaflavin, for example, has only to bind a few of the available sites to make the rest inaccessible to crystal violet, since in the absence of trypa-

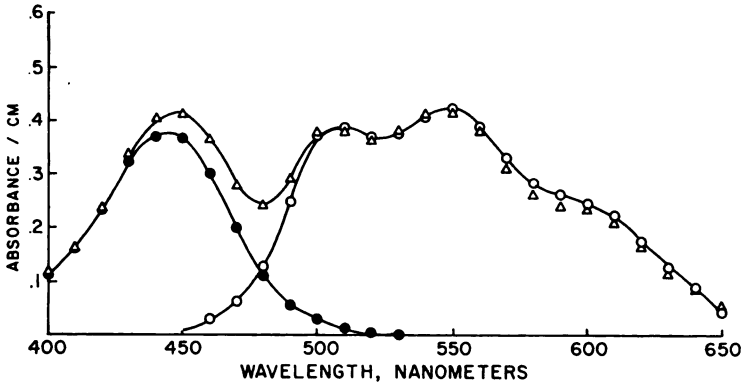


FIG. 12. *Absorption spectra of trypaflavin and crystal violet in the presence of 5.9 mM poly-α-L-glutamic acid, pH 6.4*

●, 30.7 μM trypaflavin only; ○, 16.8 μM crystal violet only; △, both dyes combined at the same concentrations given above.

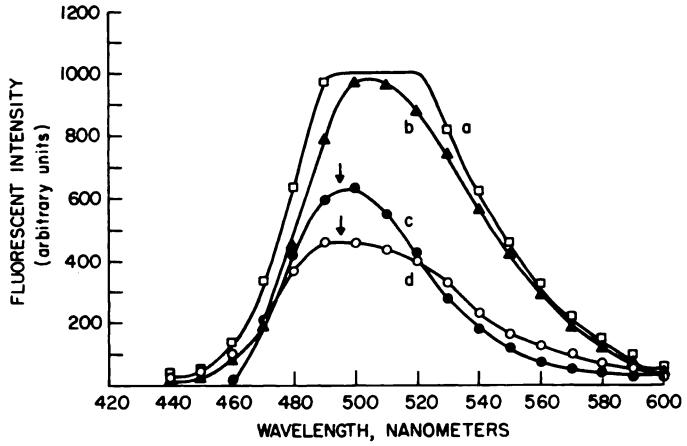


FIG. 13. *Fluorescence spectra of trypaflavin and crystal violet in the presence and absence of poly-α-L-glutamic acid at an excitation wavelength of 460 nm*

□ (*a*), 30.7 μM trypaflavin, 5.9 mM poly-α-L-glutamic acid, no crystal violet present; ▲, (*b*), 30.7 μM trypaflavin, neither crystal violet nor poly-α-L-glutamic acid present; ●, (*c*), 30.7 μM trypaflavin, 16.8 μM crystal violet, no poly-α-L-glutamic acid present; ○ (*d*), 30.7 μM trypaflavin, 16.8 μM crystal violet, and 5.9 mM poly-α-L-glutamic acid.

flavin crystal violet can bind DNA at *P/D* values lower than the ratio of trypaflavin required for blockade.

Presumably, steric effects of the DNA helix would be involved in the concealment of sites. This is made clear by the studies of apurinic acid and poly-α-L-glutamic acid. Here the "opening" of the molecular conformation of apurinic acid reduces the aggressive competition of trypaflavin seen with native DNA. With poly-α-L-glutamic acid, there is even a reversal of the effect. Here it may be that, with accessibility ensured, strong ionic and hydrogen bonds can be formed between crystal violet and the biopolymer. The sum of these bonds may be greater with crystal violet, with its greater cross-sectional projection, than with trypaflavin (2.5×10^2 A^2 compared to 2.0×10^2 A^2). On the open coil of apurinic acid it is also possible to see interaction between trypaflavin and crystal violet in a somewhat clearer fashion than previously observed in aqueous systems (5). The mutual competi-

tion in altering ORD signal and fluorescence quenching may be taken as evidence for such interaction.

In our attempts to find models *in vitro* for chemotherapeutic interferences, admittedly we can at present only speculate on the identity of the biopolymers involved. However, we can conclude from the evidence presented here with four kinds of polymers that no binding occurs with poly-L-lysine, and no interference in binding of the dyes with apurinic acid, but considerable interference takes place with native DNA and with poly-α-L-glutamic acid. With a plausible assumption that the loss of optical activity is associated with the release of dye from the polymer surface, native DNA, having ionizable phosphate groups as well as bases, binds to trypaflavin preferentially, whereas poly-α-L-glutamic acid, having only carboxylic groups as probable binding sites, binds to crystal violet in just the reverse manner. If chemotherapeutic interference should occur by simple exclusion of trypaflavin by crystal violet, poly-α-L-glutamic acid is a possible candidate and carboxyls are the site of the action. If the interference is caused by some interaction between bound trypaflavin and unbound crystal violet, DNA is a possible model polymer for the situation *in vivo*.

Reference to the biological system, which first stimulated this study, also indicates the profound influence of the nature of the polymer. In hemoflagellates, where therapeutic interference is manifest, there are two prominent DNA sites, the nucleus and the kinetoplast. The latter, an organelle involved in mitochondrial replication, constitutes a relatively large mass of extranuclear DNA. A characteristic pattern of acridine action at low concentrations on the hemoflagellates is kinetoplast loss without cell death. Tubbs *et al.* (14) investigated the relationship between the base content of DNA and the binding of acridines. They concluded that an AT:AT site (a pair) would have a relatively stronger affinity for acridines than a GC:GC site or a mixed locus (however, grossly, and at higher dye to DNA ratios, the percentage of GC in a DNA molecule determines the amount of acridine bound). Noting this study, Guttman and Eisenman (15) pointed out that a correlation exists between the AT content of the kinetoplast and its percentage loss in various strains of crithidia (forms related to the pathogenic hemoflagellates).

Finally, it should be noted that ORD and CD methods are greatly superior to conventional absorption spectroscopy for studies of interaction between therapeutically potent but optically inactive dyes and optically active biopolymers, as is clearly demonstrated in the present work.

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