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SPECTROSCOPIC PROBE OF THE DISTRIBUTION OF CATIONIC DRUG OR DYE BETWEEN ANIONIC POLYMERS

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ABSTRACT

Knowledge of the distribution of a drug (or dye) between different biopolymers may be useful in understanding the mechanism of drug action. We have studied the distribution of drugs or dyes between different anionic polymers as model systems. Spectrophotometric and circular dichroism have been used as techniques to monitor the distribution of intercalating, partially intercalating, and nonintercalating cationic dyes or drugs between DNA and other anionic polymers. Both spectrophotometric and circular dichroic evidence indicates that the partially intercalating dye dimethylmethylene blue distributes itself between DNA and heparin with a preference for DNA. In the case of the intercalating drug actinomycin-D, DNA uses its intercalating ability to win heparin in competitive binding of the drug. The picture is somewhat different when pinacyanol (PCYN) chloride, a nonintercalating dye, is used as the ligand in the competitive binding of DNA and poly(styrene sulfonate) (PSS); DNA loses to PSS in competitive binding of this ligand. The equilibrium constant of the distribution of PCYN between DNA and PSS has been determined from a method developed here.

INTRODUCTION

There are various mechanisms by which cells are inactivated or killed by antibiotics or drugs in general, e.g., by inhibiting the replication of DNA or the synthesis of proteins, RNA, or cell wall materials, or by damaging membranes, or by affecting the enzyme systems. If the drug action by any of these mechanisms is preceded by binding of the drug to a selected biomolecule of the cell organells, the question of the distribution of a drug for its preferential binding to some particular biomolecule arises. Besides such a distribution among the biomolecules of the target cell, the question of the competitive binding of the drug itself to biomolecules in the environment of the target cell, for example, biochemicals present in the plasma, also arises. In the case of the killing of cells by photodynamic treatment using different photosensitizers [1], precise knowledge of the distribution of a photosensitizer among different biomolecules of the cells may be useful in understanding the mechanism of photodynamic treatment.

Some reports on earlier work on the competitive binding of two ligands to one biopolymer can be cited. In 1967 Lepecq et al. [2] studied the competition between ethidium bromide and the acridine dye quinacrine for DNA to determine the relation between the binding sites of the two ligands with DNA. In 1978 Reinhardt et al. [3] examined the binding interaction of ethidium bromide with DNA in the presence of actinomycin or actinomine to get information about the specific binding sites of the drugs to DNA from fluorescence spectroscopy. Wartell et al. [4] studied the simultaneous binding of netropsin and actinomycin to four natural DNAs to determine the influence of one ligand on the binding of the other. Thus, from a competition study of two ligands for DNA, the influence of one ligand binding on the other can be ascertained. Further, if the specific binding sites of one ligand is known, the other's binding sites can be speculated about from such a competitive binding study. It would also be interesting to study the reverse system; namely, the distribution of one particular ligand between two biopolymers. In this paper we report on our results of spectroscopic probes of the distribution of a drug or a dye between two different biopolymers as a model study. In other reports [5, 6] we showed that the intercalation ability of DNA has a dominating role in the competitive binding of a ligand. In this report we show that the intercalating ability of DNA is almost the sole factor in deciding the results of the competitive binding of the drug actinomycin-D between the two anionic biopolymers DNA and heparin. However, with a nonintercalating ligand, the reverse is the case, as shown by the results obtained with pinacyanol as the ligand and DNA and poly(styrene sulfonate) as the competing polymers.

EXPERIMENTAL

1,9-Dimethylmethylene blue [3,7-bis(dimethylamino)-1,9-dimethylphenothiazinylium chloride] (Serva Feinbiochemica), pinacyanol chloride (PCYN, 1-ethyl-2[(1-ethyl-2(1*H*)-quinolylidene) propenyl)quinolinium chloride] (Sigma), and actinomycin-D from streptomyces species, also a product of Sigma Chemical Co., were used as such. Calf thymus DNA and heparin (sodium salt), both from Sigma Chemical Co., and sodium salt of poly(styrene sulfonate), Serva Feinbiochemica,

were also used without any purification. Stock solutions of DNA, heparin, actinomycin-D, and poly(styrene sulfonate) were prepared in distilled water and were stored in a freezer until use. Stock solutions of dyes ($\approx 10^{-3}$ M) were made in water and stored in the dark at room temperature when not in use. The pHs of the experimental solutions were maintained around 7.2 by adding dilute NaOH solution, without using buffer, but in equilibrium studies a Tris-HCl buffer of pH 7.0 was used. P/D means the ratio of the concentration of polymer to dye. In the situation where the polymer molecular weight is replaced by the equivalent weight, this is the average weight containing one anionic site. The molar ellipticity $[\theta]$ was calculated with respect to the dye or drug concentration by using the equation

$$[\theta] = \frac{\text{displacement (mm)} \times \text{amplification (m°/cm)}}{\text{path length (cm)} \times \text{molar concentration} \times 100} \text{degree} \cdot \text{cm}^2 \cdot \text{dmol}^{-1}$$

Absorbances of the experimental solutions were read with respect to water or buffer as reference.

A Shimadzu Spectrophotometer UV-150-02 and a Jasco Spectropolarimeter-500-C were used for recording the absorption and circular dichroism (CD) spectra, respectively. All spectra were recorded at the laboratory temperature (25°C).

RESULTS AND DISCUSSION

It has been shown [5] that when DNA is pipetted into a heparin-acridine orange mixture, the absorption and the CD spectra change to those absolutely characteristic of the DNA-acridine orange system. All dyes bound to heparin are completely transferred to DNA. However, our results with the poorly intercalating dye, 1,9-dimethylmethylene blue (DMMB) (Figs. 1 and 2) show that the situation is somewhat different; DNA is not completely successful in snatching any DMMB bound to heparin.

In Fig. 1, Spectra A, B, and C are, respectively, of 1.20×10^{-5} M DMMB in water, in the presence of 6.00×10^{-5} M DNA, and 6.00×10^{-5} M heparin. DMMB is a strongly aggregating dye due to its extra methyl groups on the aromatic rings, and it exhibits its dimeric band at 595 nm even at this low concentration with the monomeric band at 650 nm. Upon adding DNA, Spectrum A of the dye changes to B with a prominent peak at the position of the dimer band of the dye, and the monomer band is red-shifted to 660 nm with reduced absorbance. The red shift presumably arises from the fraction of the dye intercalated, and the prominent peak at 593 nm is possibly due to dimers formed between outer bound dyes as well as between outer bound and intercalated dye monomers [7]. Upon adding heparin to DMMB, the spectrum changes to C with its very sharp metachromatic peak at 533 nm and there is a drastic reduction in absorbance in the normal absorption region of the dye. Comparison of Spectra B and C shows that heparin is a much stronger polyelectrolyte in inducing metachromasia in DMMB compared to that by DNA. However, when DNA is added to the solution responsible for Spectrum C, the spectrum drastically changes to D with prominent peaks at 600 and 660 nm, and the sharp peak at 533 nm in Spectrum C is reduced to a shoulder. Thus, it seems that DNA takes away a major portion of the dye bound to heparin and forms its own complex. In the reverse system when heparin is pipetted to a DNA-DMMB mixture,

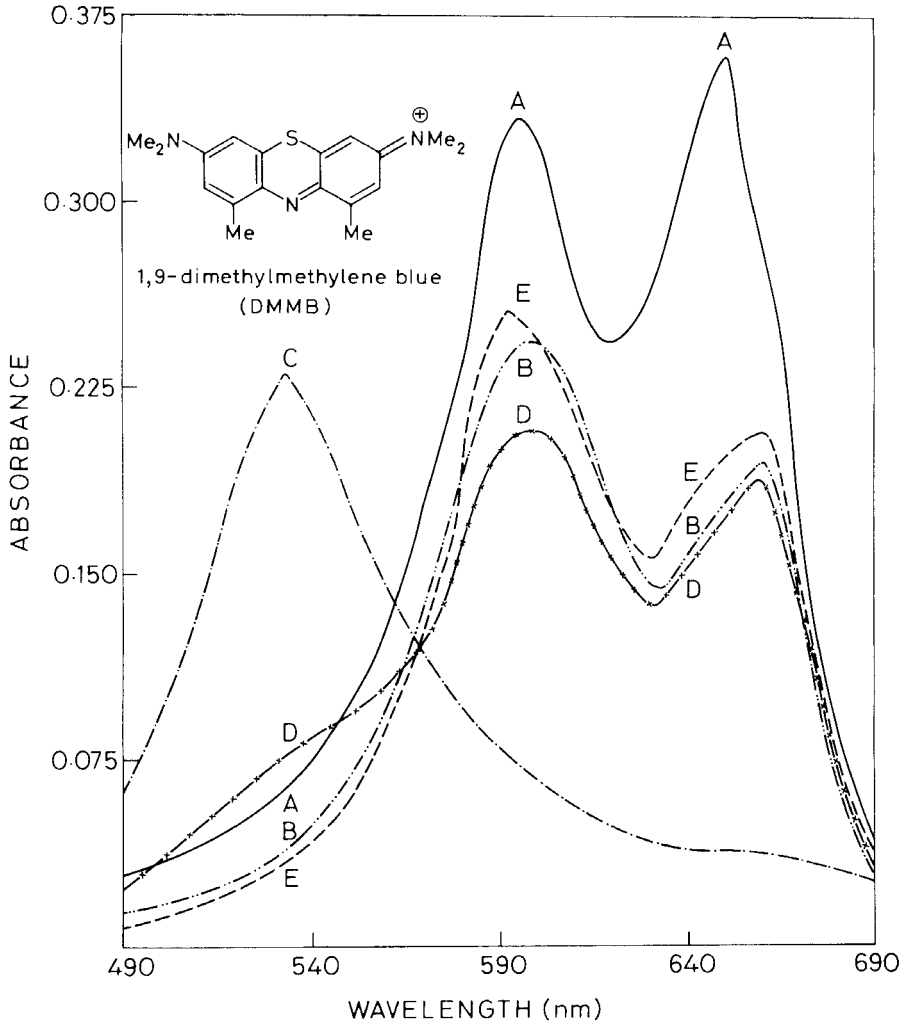

FIG. 1. Absorption spectra of 1.20×10^{-5} *M* DMMB in water (A), in the presence of DNA at a polymer/dye (P/D) mole ratio of 5.0 (B), in the presence of heparin at a P/D of 5.0 (C), in the presence of DNA pipetted to Hep-DMMB (D), and Hep pipetted to DNA-DMMB (E). Concentration of Hep as well as DNA in individual or mixed solutions is 6.0×10^{-5} *M*. Distilled water was taken as the reference.

the resulting solution gives Spectrum E which is very similar to Spectrum B of DNA-DMMB and not identical with Spectrum D. The difference between Spectra D and E is not an artifact and is very much reproducible. The dependence of the shapes of Spectra D and E on the order of pipetting indicates the partly irreversible nature of binding of the dye in spite of an apparent isosbastic point at 567 nm.

The CD spectra summarized in Fig. 2 elucidate the same picture. The CD Spectra B and C of DNA-DMMB and Hep-DMMB, respectively, are characteristically different due to the induction of chirality in the achiral dye DMMB by the two

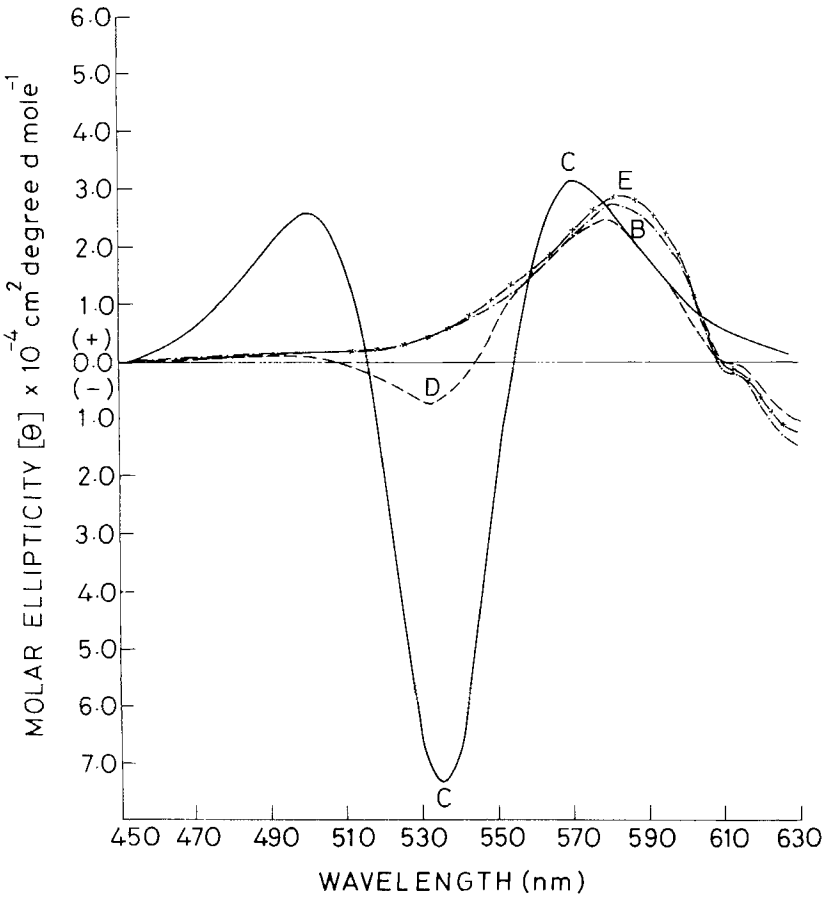

FIG. 2. The CD spectra of Solutions A–E of Fig. 1. Molar ellipticity values (θ) were calculated with respect to DMMB concentration.

chiral polymers with different conformations [8–12]. On pipetting DNA into Hep-DMMB, the CD Spectrum C changes to D, which is qualitatively similar but not exactly the same as the CD Spectrum B of DNA-DMMB. Spectrum D maintains a weak trough at 535 nm, characteristic of Hep-DMMB, with the peak of D positioned intermediate between those in Spectra B and C. Thus, in support to the conclusion derived from absorption spectra, the CD spectra also show that when DNA is pipetted into Hep-DMMB, not all the dye molecules migrate to DNA. However, when heparin is pipetted into DNA-DMMB, it fails to dislodge any dye bound to DNA, as shown by the identical shapes of Spectra D and E. While for the strongly intercalating dye acridine orange there is almost complete migration of dye from the heparin-dye complex to DNA [5], with DMMB as the ligand the dye seems to be distributed between the two biopolymers, with a preference to DNA.

With the completely nonintercalating ligand pinacyanol (PCYN), the picture is reversed; DNA loses completely to the synthetic polyelectrolyte poly(styrene sulfonate) (PSS) in the competitive binding of the dye. This is proved beyond doubt

from the sets of absorption and CD spectra summarized in Figs. 3 and 4. Pinacyanol is also a strongly aggregating dye. It shows its prominent dimer band at 550 nm in 1.20×10^{-5} M aqueous solution and has its monomer band at 600 nm (Fig. 3A). On adding DNA to the dye at a P/D ratio of 4.0, the spectrum changes to B with two peaks at 488 and 560 nm and a shoulder around 635 nm with a drastic reduction of the absorbance at the monomer band of the dye. This is the characteristic metachromatic spectrum of DNA-PCYN [13]. Spectrum C is the metachromatic spectrum of PCYN induced by poly(styrene sulfonate) (PSS) at P/D = 4.0. This spec-

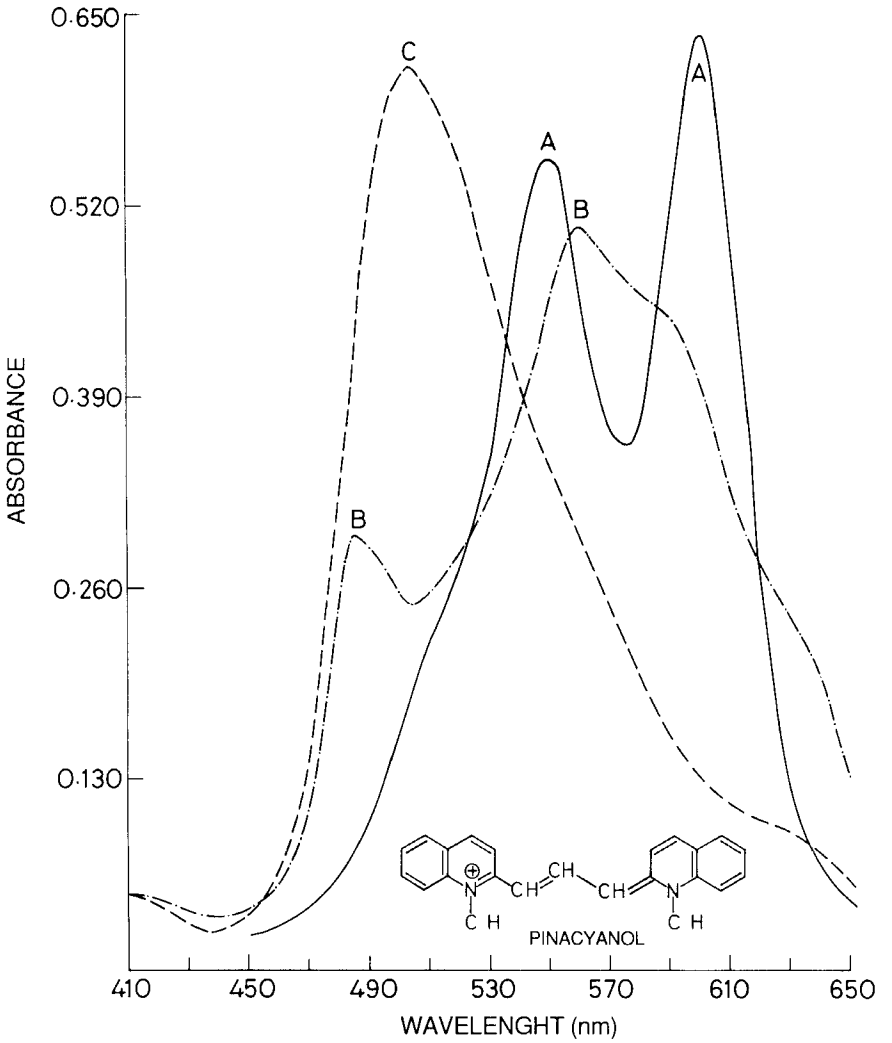

FIG. 3. Absorption spectra of PCYN (1.20×10^{-5} M) in water (A), in the presence of DNA (B), and in the presence of PSS (C). The spectrum of DNA pipetted to PSS-PCYN or PSS pipetted to DNA-PCYN is identical to Curve C. The concentration of PSS and of DNA is 4.8×10^{-5} M. Distilled water was taken as the reference.

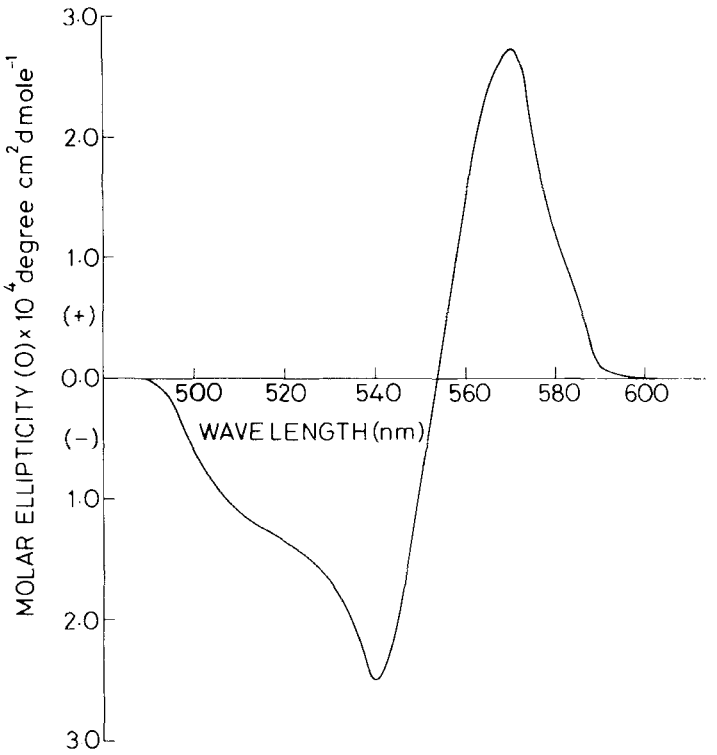

FIG. 4. CD spectra of 1.20×10^{-5} *M* PCYN induced by 4.80×10^{-5} *M* DNA. PSS-PCYN, DNA pipetted to PSS-PCYN, or PSS pipetted to DNA-PCYN does not exhibit any dichroism. The concentration of PCYN was used to calculate the molar ellipticity.

trum is distinctly different from that of DNA-PCYN. Note that PCYN discriminates between polyanions regarding the shapes of metachromatic spectra induced by them, i.e., those polyanions which form complexes with the dye with a 2:1 P/D stoichiometry induce sharp and single-banded matachromasia similar to Spectrum C [14]. Other polyanions, including heparin, which bind the dye at all anonic sites, induce broad and multiple-banded metachromasia similar to Spectrum B. Since the metachromatic spectra of PCYN induced by DNA and heparin respectively are similar, we selected PSS in place of heparin to study the competitive binding of this nonintercalating dye between DNA and another polyanion. When DNA is added to PCYN-PSS, the spectrum is identical to C, which shows that DNA fails to transfer any dye bound to PSS. Thus, DNA loses in the competition when the ligand is nonintercalating. This is confirmed from the absorption spectrum obtained by pipetting PSS to the PCYN-DNA system; the spectrum becomes identical to C.

Thus, PSS apparently removes all PCYN bound to DNA to form its own complex. This conclusion is confirmed from Fig. 4. PSS is achiral in all respects, and hence it is not expected to induce chirality in the achiral dye PCYN. However, double helical DNA induces a biphasic CD in the dye (Fig. 4) as was reported earlier [15]. On adding PSS to PCYN-DNA, the CD spectrum is reduced to the baseline,

presumably indicating that PSS deprives DNA of its bound PCYN. When DNA is pipetted into PCYN-PSS, the system does not show any dichroism. This confirms our earlier conclusion [5] that DNA is a weaker polyelectrolyte and uses its intercalating ability to win in the competitive binding of a ligand if the ligand is capable of intercalating between base pairs of a DNA helix. Pal and Ghosh [13] reported that the shape of the metachromatic spectrum of PCYN-DNA drastically changes at a high P/D of around 30, 0, and the system depicts a new and very sharp metachromatic peak at 557 nm. When PSS is added to DNA-PCYN (P/D 30, 0), the spectrum surprisingly changes to one identical with the spectrum of PSS-PCYN, similar to Fig. 3(C) (figures not shown), showing the PSS snatches dyes from DNA even when the latter is present in a large excess. Our dichroic probe also shows that even a large excess of DNA cannot inhibit the migration of all the PCYN to PSS. Thus DNA cuts a sorry picture in competition when the ligand is nonintercalating. For an intercalating ligand the reverse is the case, and we present in this report another good instance with the intercalating drug actinomycin-D (ACM) which is used in the treatment of tumors [16]. Figure 5 shows the absorption spectra of 1.00×10^{-5} *M* ACM in water (A) and in the presence of DNA (B) and heparin (C) at P/D = 5 in both individual cases. It is apparent that the spectrum of ACM is not changed appreciably in the presence of the polyanions. The observed higher absorbance in the UV range in the presence of DNA is obviously due to the absorbance of DNA itself and does not indicate any perturbation of the spectrum of the drug; the difference spectrum (B − A) reflects the absorption spectrum of DNA. Heparin seems to slightly increase the absorbances around the absorption peaks of the drug, and DNA slightly red-shifts the UV peak. The shapes of the absorption spectra of the solutions obtained by pipetting DNA to drug-Hep or adding Hep to drug-DNA are identical (D). Thus, absorption spectroscopy is not a useful tool to study the distribution of ACM between DNA and Hep.

The CD spectra of the systems (Fig. 6), however, show DNA is the winner in the competition. The drug itself is optically active and depicts its characteristic CD Spectrum A. The CD spectrum of ACM-Hep is exactly identical to A, thus the binding of the drug to Hep leaves the chirality of the drug unchanged. However, when DNA is added to the drug, the CD Spectrum B is characteristic of ACM-DNA. The change in the CD spectrum of the drug induced by DNA cannot be solely assigned to the contribution of DNA itself; if the dichroism of DNA is subtracted from the dichroism of ACM-DNA, the difference Spectrum C is obtained, which is notably different from that of the drug alone. When DNA is pipetted into ACM-Hep or Hep is pipetted to ACM-DNA, the CD spectra of the resulting solutions are identical to Spectrum B, characteristic of ACM-DNA. Thus, it seems that in competitive binding, the drug ACM migrates to DNA irrespective of the order of pipetting. ACM is an intercalating drug [17], and DNA uses its intercalating ability to win the competition.

Finally, we also made an attempt to estimate quantitatively the equilibrium constant (K) of the distribution of PCYN between DNA and PSS.

$$\text{DNA-PCYN} + \text{PSS} = \text{DNA} + \text{PSS-PCYN}$$
$$K = \frac{[\text{DNA}]_f[\text{PSS-PCYN}]}{[\text{PSS}]_f[\text{DNA-PCYN}]} \tag{1}$$

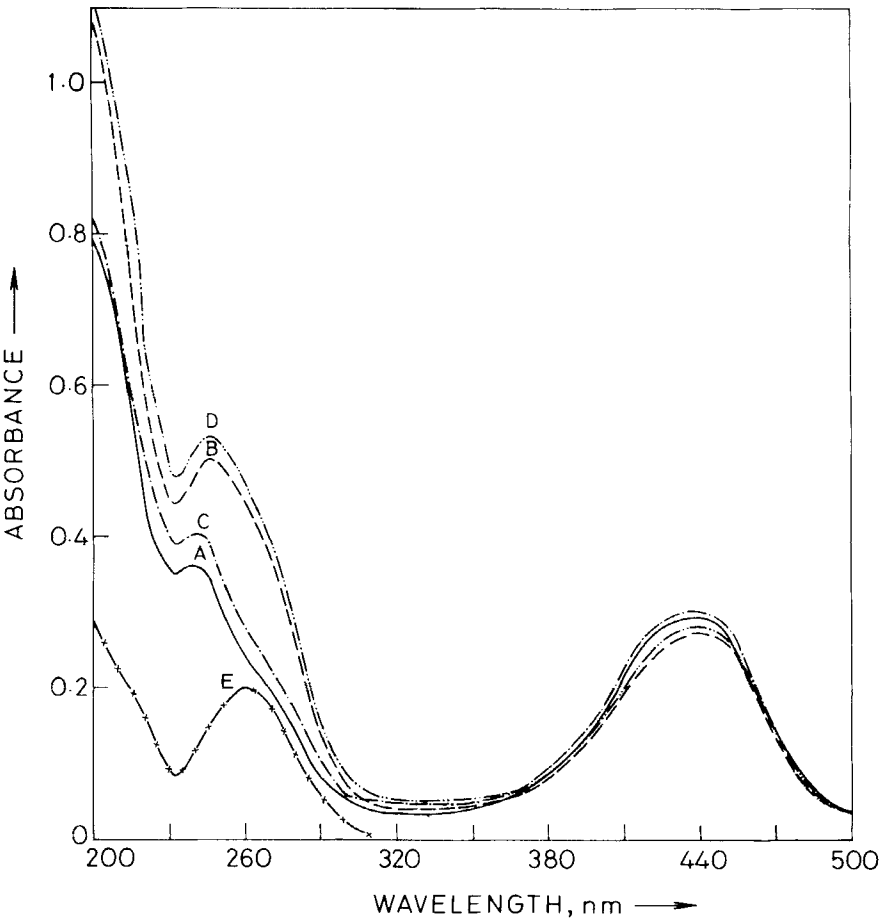

FIG. 5. Absorption spectra of 1.00×10^{-5} M actinomycin-D in water (A), in the presence of DNA (B), in the presence of Hep (C), in the presence of DNA pipetted to ACM-Hep (D), and the difference spectrum (B − A) (E). Concentration of DNA and Hep is 5.0×10^{-5} M. Distilled water was used as the reference.

For this quantitative treatment, we buffered the systems with 1.00 mM Tris buffer of pH 7.00. Our observation is that use of the buffer does not have any appreciable effect on the shapes of the metachromatic spectra compared to the unbuffered systems maintained at pH 7.0–7.2. Figure 7 shows the changes in absorption spectra of DNA–PCYN with progressive addition of PSS. The spectra pass through a single isosbestic point at 540 nm, which is identical with the crossing of Spectra B and C of Fig. 1. The suffix f in Eq. (1) indicates the concentration of the free polymers not binding any dye. To calculate K, we made the following rational assumptions: 1) There is no free dye present in the system, 2) both DNA–PCYN and PSS–PCYN complexes in the concentration range used obey Beer's law [18]. If x is the mole fraction of dye bound to PSS, $(1 - x)$ will be the mole fraction bound to DNA, so [PSS–PCYN] and [DNA–PCYN] will be given by xD_T and $(1 - x)D_T$, where D_T is

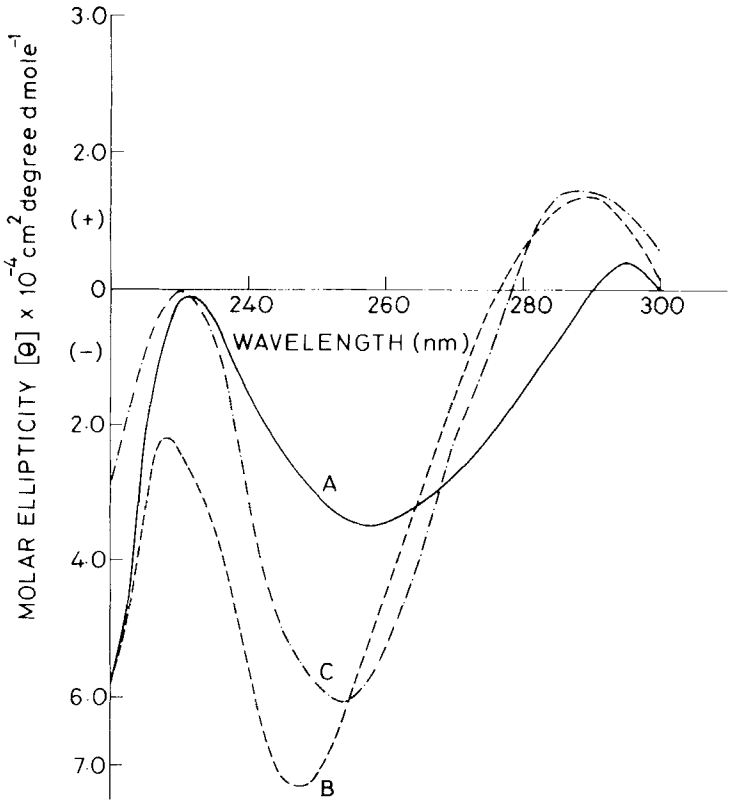

FIG. 6. CD spectra of Solutions A and B of Fig. 5 and the difference CD spectrum (ACM-DNA) − DNA (C). The molar ellipticity of ACM-DNA values were calculated with respect to ACM concentration.

the total concentration of the dye. $(DNA)_f$ will be equal to $[DNA_T - (1 - x)D_T]$; similarly $(PSS)_f = (PSS_T - xD_T)$. The suffix T indicates the total concentrations. Therefore

$$K = \frac{[DNA_T - (1 - x)D_T][xD_T]}{[PSS_T - xD_T][(1 - x)D_T]} \tag{2}$$

or

$$\frac{[DNA_T - D_T] + xD_T}{PSS_T - xD_T} = K\left(\frac{1 - x}{x}\right) \tag{3}$$

With knowledge of DNA_T, PSS_T, and D_T, a plot of the left-hand side of Eq. (3) vs $(1 - x)/x$ should give a straight line passing through the origin and with a slope of K. The values of x for the sets of Fig. 7 may be calculated from the absorbances at either of the two peaks, 500 and 560 nm, from knowledge of the absorbances at the same peaks for the individual PSS-PCYN (0.605 at 500 nm and 0.277 at 560 nm)

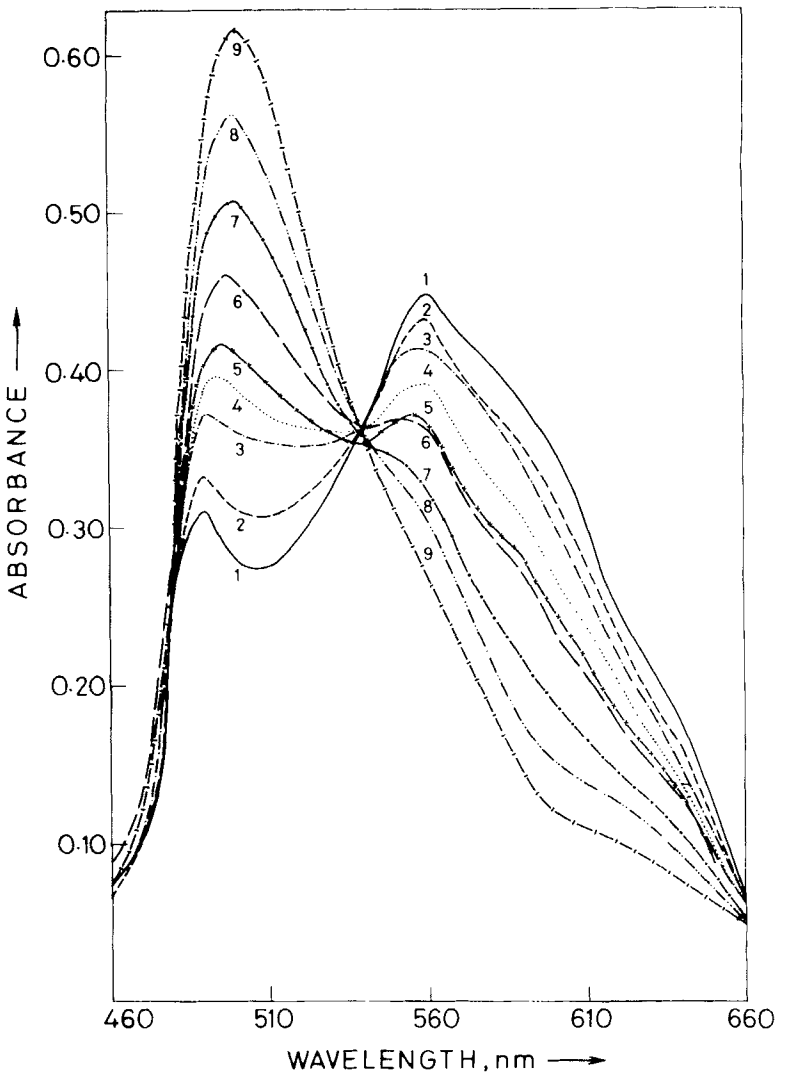

FIG. 7. Absorption spectra of PCYN (1.20×10^{-5} *M*) in the presence of 4.8×10^{-5} *M* DNA after addition of 0.2×10^{-5} *M* PSS (1); 0.40×10^{-5} *M* PSS (2); 0.60×10^{-5} *M* PSS (3); 0.80×10^{-5} *M* PSS (4); 1.00×10^{-5} *M* PSS (5); 1.20×10^{-5} *M* PSS (6); 1.60×10^{-5} *M* PSS (7); 2.00×10^{-5} *M* PSS (8); and 2.60×10^{-5} *M* PSS (9). Tris buffer of pH 7.00 taken as the reference.

and DNA-PCYN (0.273 at 500 nm and 0.479 at 560 nm), using the following relations of the absorbances at the same two wavelengths for each of the sets of Fig. 7.

$$OD_{500} = x \times 0.605 + (1 - x)0.273$$
$$OD_{560} = x \times 0.277 + (1 - x)0.479$$

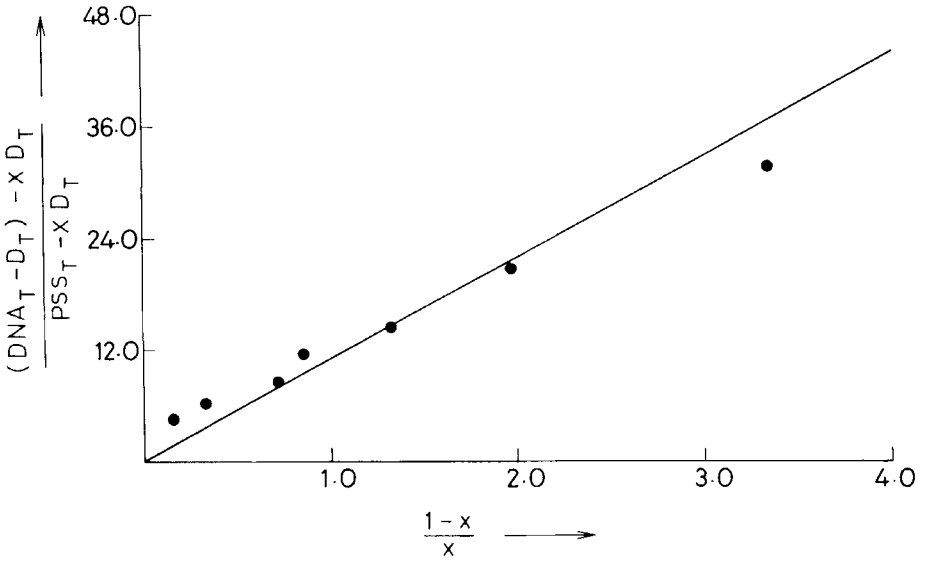

FIG. 8. Plot of $\frac{(DNA_T - D_T) + xD_T}{PSS_T - xD_T}$ vs $\frac{1-x}{x}$, where $DNA_T = 4.80 \times 10^{-5}$ M; $D_T = 1.20 \times 10^{-5}$ M, PSS_T = total concentration of PSS added, and x = mole fraction of the dye bound to PSS, calculated from the absorbances at 560 nm of the spectra in Fig. 7.

Figure 8 shows the plot of

$$\frac{[DNA_T - D_T] + xD_T}{PSS_T - xD_T} \text{ vs } \left(\frac{1-x}{x}\right)$$

where x is computed from absorbances at a wavelength of 560 nm, which gives a moderately good straight line passing through the origin at a value of $K = 11.0$, in spite of the assumptions made for the derivation of the equations. It is apparent that deviations from linearity are more prominent when either of the two polymers is in relatively large excess.

Although the absorption spectra of Fig. 3 and the CD spectra of Figs. 4 and 6 apparently give the qualitative impression of complete migration of the ligand from one polymer to another, a quantitative study of the distribution of PCYN between DNA and heparin gives a K value of 11.0, much below expectation. The linear relation shown in Fig. 8 is of some importance in view of the overlapping spectra of DNA-PCYN and PSS-PCYN and of the assumptions in deriving Eq. (3).

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