Effect of Sorbitol on Interaction of Phenolic Preservatives with Polysorbate 80

JAMES BLANCHARD ˣ, WILLIAM T. FINK, and JAMES P. DUFFY

Abstract □ The effect of sorbitol on the binding of several commonly used phenolic preservatives (*i.e.*, *p*-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben) with the nonionic surfactant polysorbate 80 was investigated using an equilibrium dialysis technique. The binding data were expressed in the form of Scatchard plots utilizing a modified form of the Scatchard equation. The data analysis indicated that all four phenolic preservatives were bound to two distinct loci within the polysorbate micelle; one exhibited a high affinity and a low capacity for the preservative molecules, while the other appeared to have a near-zero affinity but an almost infinite binding capacity. The high affinity site was assumed to be located near the junction of the hydrocarbon core with the polyoxyethylene region of the micelle. The interaction of the preservatives with the second class of sites apparently involved a nonspecific and nonsaturable partitioning of the preservative molecules into the polyoxyethylene region of the micelle. Sorbitol was ineffective in displacing significant amounts of bound preservative from either binding site, presumably because it was too polar to partition into the micelle sufficiently to displace bound preservative.

Keyphrases □ Sorbitol—effect on binding of various phenolic preservatives to polysorbate 80 □ Binding—various phenolic preservatives to polysorbate 80, effect of sorbitol □ Polyols—sorbitol, effect on binding of various phenolic preservatives to polysorbate 80 □ Polysorbate 80—binding to various phenolic preservatives, effect of sorbitol □ Preservatives, phenolic—*p*-hydroxybenzoic acid, methylparaben, ethylparaben, and propylparaben, binding to polysorbate 80, effect of sorbitol □ Surfactants, nonionic—polysorbate 80, binding to various phenolic preservatives, effect of sorbitol

The interference of nonionic surfactants with the preservative activity of parabens was first reported in 1950 by Bolle and Mirimanoff (1). Since that time, several reports confirmed their observations (2–4). It is now generally acknowledged that many preservatives commonly used in pharmaceutical formulations undergo similar complexation reactions and resultant inactivation.

This complexation may be inhibited by certain biologically inactive molecules such as the polyols (5–8). Some polyols, such as sorbitol, are not only compatible with many ingredients common to pharmaceutical formulations but are widely used for their humectant, solubilizing, stabilizing, and anti-cap-lock properties. Thus, a systematic study of the ability of such agents to inhibit the complexation of preservatives by nonionic surfactants seems highly desirable.

In this study, a model system consisting of polysorbate 80, sorbitol, and several phenolic preservatives was utilized to determine the ability of sorbitol to activate complexed paraben molecules.

EXPERIMENTAL

Reagents—The following reagents were used: methylparaben[1] (I), mp 130°; ethylparaben[2] (II), mp 117°; propylparaben[3] (III), mp 96–97°; *p*-hydroxybenzoic acid[4] (IV), mp 214–215°; polysorbate 80[5]; and crystalline sorbitol[6]. Compounds I–IV were recrystallized from distilled water (9, 10) and dried in a desiccator for at least 48 hr prior to use. Polysorbate 80 and sorbitol were used without further purification. All solutions were prepared using double-distilled water from an all-glass apparatus.

Procedures—The dialysis procedures and equipment were similar to those of Patel and Foss (11). Commercially available[7] acrylic plastic dialysis cells were employed. Nylon[8] membranes, previously washed for 1 hr in distilled water heated to 80° to remove any contaminants, proved to be satisfactory. These membranes previously were shown to be impermeable to the polysorbate yet permeable to the parabens, thereby allowing equilibration of the latter (2, 12).

After the cells were assembled, 5 ml of distilled water was added to one cell compartment (the aqueous compartment) and 5 ml of a mixture of varying volume ratios of preservative in 10% (w/v) polysorbate 80 and 10% (w/v) polysorbate 80 was added to the other cell compartment (the surfactant compartment). This procedure was employed to vary the amount of preservative present in the surfactant compartment while maintaining the polysorbate concentration at 10% (w/v). The cells were then placed on a water bath shaker[9] and agitated at 120 oscillations/min for 20 hr at 30° to achieve equilibrium. Preservative samples were removed from both sides of the membrane and assayed spectrophotometrically at 255 nm (2, 11, 13).

Sorbitol was added as a possible competitor to the preservative in 10% (w/v) polysorbate 80 solution so that the molar ratio of sorbitol to paraben was constant in any aliquot. The assay procedure was identical to those in the experiments involving no competitor, since neither polysorbate 80 (2) nor sorbitol interfered with the assay of the preservatives.

RESULTS AND DISCUSSION

Binding phenomena in undersaturated systems, such as those utilized here, can be considered to obey the law of mass action (14). For a macromolecule with n independent and equivalent binding sites, each having an association constant K, the following expression may be written:

$$r = \frac{[DM]}{[M_t]} = \frac{nK[D]}{1 + K[D]} \qquad \text{(Eq. 1)}$$

where r is the number of moles of preservative bound per mole of surfactant; $[DM]$ and $[D]$ are the concentrations of bound and free (unbound) paraben, respectively; and $[M_t]$ is the concentration of macromolecule (surfactant). Theoretically, $[M_t]$ should be the concentration of micelles, n the number of binding sites per micelle, and K the association constant for the reaction with the micelles, since the binding interaction reportedly occurs between the preservative and the surfactant micelles rather than the monomer surfactant molecules (12, 15). From a practical standpoint, however, it is more convenient to express K, n, and M_t in terms of the surfactant concentration, since the micellar molecular weight is not precisely known. Since the critical micelle concentration of polysorbate 80 is very low (15, 16), the monomer concentration may be neglected (12). In analyzing the data, the molecular weight of polysorbate 80 was assumed to be 1470[10].

If there are m classes of independent sites with each class i having n_i sites with an intrinsic association (binding) constant K_i, then Eq. 1 can be generalized to:

$$r = \sum_{i=1}^{m} \frac{n_i K_i [D]}{1 + K_i [D]} \qquad \text{(Eq. 2)}$$

[1] Lot L49260, Ruger Chemical Co., Irvington, NJ 10533.
[2] Eastman Organic Chemicals, Rochester, NY 14650.
[3] Lot C708922, Ruger Chemical Co., Irvington, NJ 10533.
[4] Lot 750543, Calbiochem, La Jolla, CA 92037.
[5] Lot 1546, ICI America Inc., Atlas Chemicals Division, Wilmington, DE 19899.
[6] Lot 7702D, ICI America Inc., Atlas Chemicals Division, Wilmington, DE 19899.
[7] Model 289, Bel-Art Products, Pequannock, NJ 07440.
[8] Capran 77C, 0.6-mil, Allied Chemical Corp., Morristown, NJ 07960.
[9] Model 50, GCA/Precision Scientific, Chicago, IL 60647.
[10] Dr. Paul Becher, ICI America Inc., Wilmington, DE 19897, June 1976, personal communication.

Table I—Binding Parameters for the Interaction of Various Preservatives with Polysorbate 80 at 30° in the Presence of Varying Concentrations of Sorbitol

Preservative	Competitor[a]	n_1, Sites/Molecule	K_1, liters/mole × 10^{-3}	C, liters/mole	$P_{octanol-water}$
p-Hydroxybenzoic acid	None	0.074 ± 0.036[b]	3.01 ± 0.271	69.8 ± 6.3	38.02
	Sorbitol (1:1)	0.078 ± 0.031	3.06 ± 0.264	74.4 ± 5.2	
Methylparaben	None	0.098 ± 0.050	2.64 ± 0.326	84.0 ± 15.1	91.2
	Sorbitol (1:1)	0.123 ± 0.040	2.69 ± 0.205	72.9 ± 8.3	
Ethylparaben	None	0.044 ± 0.011	45.39 ± 1.10	204.2 ± 38.1	295.1
	Sorbitol (50:1)	0.044 ± 0.021	44.30 ± 2.49	179.3 ± 52.8	
Propylparaben	None	0.087 ± 0.016	123.5 ± 11.5	445.6 ± 39.7	1096.5
	Sorbitol (5:1)	0.107 ± 0.015	97.01 ± 5.25	468.0 ± 38.8	

[a] Molar ratio of competitor to preservative shown in parentheses. [b] All parameter uncertainties are expressed as standard deviations.

A Scatchard (17) plot results when $r/[D]$ *versus* r is plotted. By rearranging Eq. 1, it can be seen that such a plot should be linear with a slope of $-K$ and ordinate and abscissa intercepts of nK and n, respectively, when only one class of binding sites is present.

This simplest of binding models is referred to as the "two-parameter model" (18) since only two parameters, n and K, need to be determined to describe the binding process adequately. However, as noted previously (9, 12, 19), Scatchard plots frequently exhibit curvature indicative of the presence of more than one class of binding sites. To describe these more complex binding processes, Eq. 2 may be rewritten as:

$$r = \sum_{i=1}^{m-1} \frac{n_i K_i [D]}{1 + K_i [D]} + n_m K_m [D] \qquad \text{(Eq. 3)}$$

when one association constant becomes very small. The second term on the right-hand side of Eq. 3 is, therefore, indicative of a class of binding sites that possesses an "infinite" (nonsaturable) binding capacity but "zero" affinity, thereby producing a horizontal asymptote on the Scatchard plot.

In analyzing these more complex interactions, the binding data are fitted to a three-, four-, or five-parameter model (18, 20–22). The four-parameter model merely corresponds to a value of $m = 2$ in Eq. 2, whereas the three- and five-parameter models correspond to values of $m = 2$ and 3, respectively, in Eq. 3. In a five-parameter model, the parameters are n_1, n_2, K_1, K_2, and C, where $C = n_m K_m$.

The four-parameter model has been most commonly used to describe binding data in which a nonlinear (curved) Scatchard plot was observed. However, in many cases it appears to have been used inappropriately. Scatchard plots exhibiting a horizontal asymptote (*i.e.*, constant values of $r/[D]$ at high values of r) can best be fitted to a three- or five-parameter model, since, without the inclusion of the $n_m K_m [D]$ term of Eq. 3 to describe the horizontal asymptote (*i.e.*, the nonsaturable binding site), many parameter fitting programs frequently fail to converge as $K_2 \rightarrow 0$ and $n_2 \rightarrow \infty$ (18).

The selection of the most appropriate model to describe the binding data is critical if meaningful estimates of the binding parameters are to be obtained and erroneous inferences avoided (23). An attempt was made to fit the data in this study to three-, four-, and five-parameter models; the data were best described by the three-parameter model, which can be written as:

$$\frac{r}{[D]} = \frac{n_1 K_1}{1 + K_1 [D]} + C \qquad \text{(Eq. 4)}$$

where $C = n_2 K_2$. The best-fit values of the parameters (n_1, K_1, and C) were calculated by means of a nonlinear least-squares procedure (24) executed on a digital computer[11]. These values were then used to construct Scatchard plots for the various binding interactions.

The Scatchard plots depicted in Figs. 1–4 illustrate the interaction between 10% (w/v) polysorbate 80 and IV, I, II, and III, respectively, in the presence and absence of sorbitol. The binding parameters for the interaction of the various preservatives with polysorbate 80 are shown in Table I.

The use of Eq. 4 to fit the binding data seems reasonable in view of the NMR data of Corby and Elworthy (13), who postulated that parabens were solubilized in both the oxyethylene region and the micellar core of the related nonionic surfactant cetomacrogol, which is believed to exhibit binding properties similar to those of polysorbate 80 (25). Several other investigators observed that the interaction of phenolic preservatives with surfactants involves two distinct classes of binding sites (9, 12, 19), but they fitted their data to a four-parameter model in contrast to the three-parameter model used here.

A micelle of polysorbate 80 may be visualized as consisting of a densely

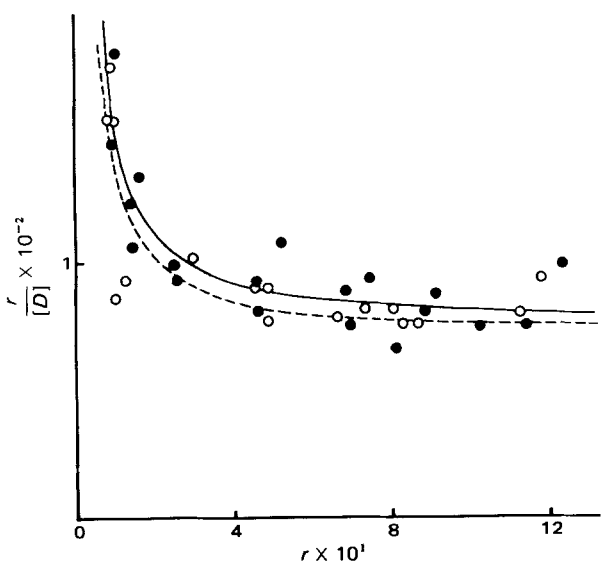

Figure 1—*Influence of sorbitol on the binding of* p-*hydroxybenzoic acid to 10% (w/v) polysorbate 80 at 30°. Key:* ○, p-*hydroxybenzoic acid alone; and* ●, p-*hydroxybenzoic acid plus sorbitol (1:1).*

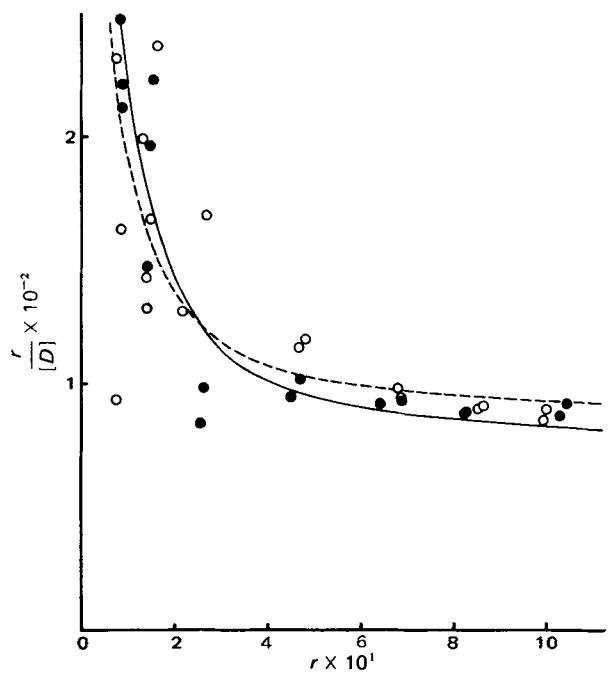

Figure 2—*Influence of sorbitol on the binding of methylparaben to 10% (w/v) polysorbate 80 at 30°. Key:* ○, *methylparaben alone; and* ●, *methylparaben plus sorbitol (1:1).*

[11] Control Data Corp. 6400.

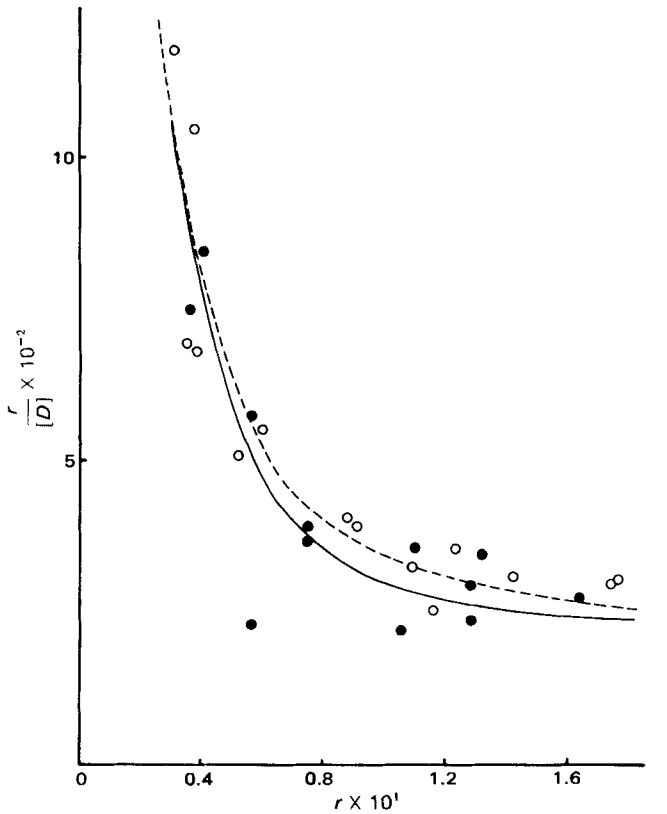

Figure 3—*Influence of sorbitol on the binding of ethylparaben to 10% (w/v) polysorbate 80 at 30°. Key: ○, ethylparaben alone; and ●, ethylparaben plus sorbitol (50:1).*

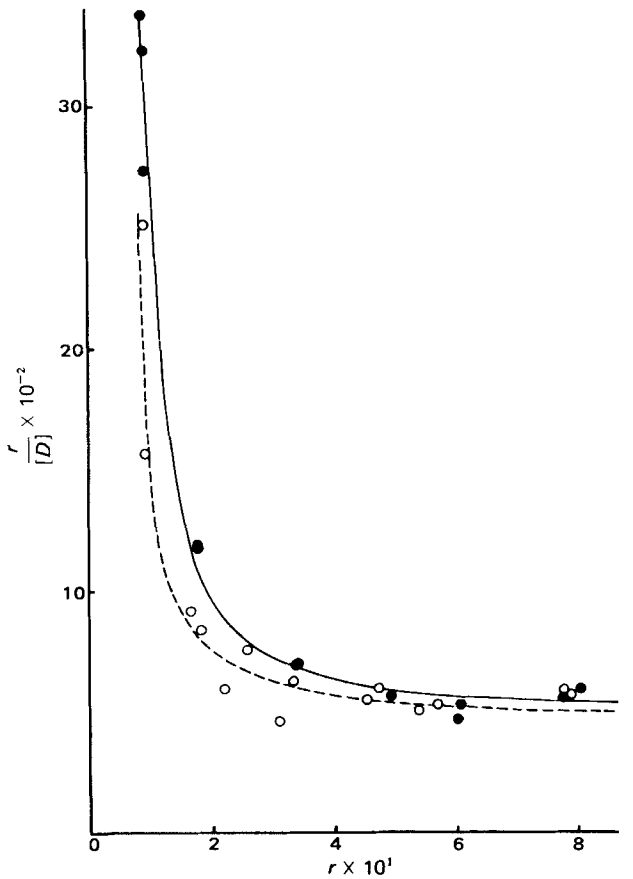

Figure 4—*Influence of sorbitol on the binding of propylparaben to 10% (w/v) polysorbate 80 at 30°. Key: ○, propylparaben alone; and ●, propylparaben plus sorbitol (5:1).*

packed central lipid core of hydrophobically bonded oleate chains surrounded at the periphery by a layer of sorbitan moieties from which emanate the polar polyoxyethylene chains in the form of apically truncated spiral cones (13, 26). Shimamoto and Ogawa (15), utilizing NMR studies, indicated that preservative molecules may reside in one or more of three different loci of a surfactant micelle: (*a*) within the lipid hydrocarbon core, (*b*) within the polyoxyethylene network, and (*c*) at the junction of these two regions.

The horizontal asymptote (C) observed in the Scatchard plots (Figs. 1–4) at high values of r may be interpreted as being indicative of a weak nonspecific (nonsaturable) binding process, analogous to a partitioning of the preservative between the micellar and aqueous phases (18, 22). Therefore, the value of C may be considered to represent a type of partition coefficient describing the distribution of the preservative molecule between the micellar and aqueous phases. This partitioning phenomenon may be envisioned as a process whereby preservative molecules, to lower their free energy, migrate from a thermodynamically less stable aqueous environment in the bulk phase to a more stable (less polar) micellar phase, with the degree of penetration and affinity being proportional to their lipophilicity.

At low r values, the preservative molecules preferentially orient themselves in the vicinity of the high affinity–low capacity sites, which are believed to be located near the junction. When these sites become saturated, the preservative molecules then partition into the somewhat more polar but still relatively stable (in relation to the aqueous phase) class of high capacity–lower affinity sites, which are believed to be located in the polyoxyethylene region of the micelle. A possible explanation for the higher affinity of the junction binding sites relative to those in the polyoxyethylene region may be that it is possible for a molecule situated at the junction to form bonds with several different structures, *i.e.*, apical ethylene oxide units, sorbitan moieties, and the outer surface of the lipid core. The capacity of this class of sites is necessarily low because of the limited number of these structures.

The data in Table I indicate that the C values observed for the various compounds are in good rank order agreement with their reported (27) octanol–water partition coefficients. This finding further substantiates that the binding process described by the C term is a type of partitioning phenomenon. The rank-order agreement between the C value and the degree of lipophilicity of the preservative molecules also substantiates that the binding sites are less polar than the surrounding aqueous environment. Furthermore, the binding parameters shown in Table I are in good inverse rank-order agreement with the relative order in which the preservatives are inactivated by the polysorbate, as measured by their inability to inhibit microbial growth (28).

Crooks and Brown (19) studied the interaction between the polar chloro and dichloro derivatives of xylenol and methylparaben in a cetomacrogol system. The more polar xylenol derivatives were able to displace a portion of the methylparaben from the polyoxyethylene region yet were unable to displace it from the postulated more specific, high affinity site believed to be located near the junction. These findings seem reasonable in view of the relative intramicellar polarity and the polarity of the competitors involved. In light of these and other findings (5–8), certain polar polyols such as sorbitol might be expected to act as competitors for bound preservative in a manner similar to chloroxylenol and dichloroxylenol.

The binding data shown in Figs. 1–4 and Table I illustrate that sorbitol has little influence on the binding of the phenolic preservatives with either class of sites; the plots are virtually superimposable, and the binding parameters in the presence and absence of sorbitol are in close agreement. The sorbitol is probably unable to displace bound phenolic preservatives because it is too polar to partition deeply enough into the micellar phase to displace significant amounts of the bound preservative. The use of other, somewhat less polar molecules that are compatible with various pharmaceutical formulations seems to be a worthwhile area for future research.

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Binding of *N*-Substituted Anthracenecarboxamides to Double-Stranded DNA: An Electronic Spectral Study

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Abstract □ The electronic absorption and fluorescence spectra of two isomeric anthracenecarboxamides substituted with quaternary alkylammonium groups were studied as a function of solvent polarity, solvent rigidity, and state of protonation. These relatively simple environmental perturbations of the electronic spectra were employed to interpret spectral shifts caused by complexation with the bases or base pairs of DNA. The exocyclic side chains of the compounds studied have some freedom of movement when the spectroscopic probes are bound to double-stranded DNA.

Keyphrases □ Anthracenecarboxamides, *N*-substituted—binding to DNA, electronic absorption and fluorescence spectral study □ Binding—*N*-substituted anthracenecarboxamides to DNA, electronic absorption and fluorescence spectral study □ DNA—binding to *N*-substituted anthracenecarboxamides, electronic absorption and fluorescence spectral study □ Electronic absorption spectrometry—binding of *N*-substituted anthracenecarboxamides to DNA □ Fluorescence spectrometry—binding of *N*-substituted anthracenecarboxamides to DNA

Many drugs active against microorganisms and neoplasms are polycyclic aromatic or heteroaromatic cations, which bind reversibly to the polyanionic nucleic acids *in vitro* and *in vivo* (1). Their modes of action are generally believed to be related, directly or indirectly, to their ability to be bound to DNA, resulting in errors of transcription or replication in the affected organism (1). However, the process of binding to DNA often entails interactions different from, and in addition to, the electrostatic interaction between ions of opposite charge.

Various techniques such as low-shear viscometry, ultracentrifugation, and NMR spectroscopy have yielded information indicating that the aromatic portions of the drugs can become inserted (intercalated) between base pairs of the double helix of DNA. This insertion results in a partial unwinding and linear extension of the double helix to accommodate the intruding drug molecule (2–5).

Several studies of binding believed to result in intercalation of drug or dye molecules with DNA have been carried out, with the binding parameters often estimated absorptiometrically or fluorometrically. However, little has been established concerning the nature of the changes in the electronic absorption or fluorescence spectra of the drug or dye molecules upon such binding. The failure of electronic spectroscopy to deliver useful information about nucleic acid binding has been due in part to the complex structures of the drug and dye molecules involved; the environmental perturbations of these spectra are difficult to interpret.

In the present study, the changes in the electronic absorption and fluorescence spectra of two cationic, *N*-substituted anthracenecarboxamides occurring upon binding to calf thymus DNA were examined. The observed changes were compared to the changes occurring in the spectra of these compounds upon protonation and as a function of solvent rigidity and polarity.

The compounds studied, *N*-(2-anthroyl)-*N'*,*N'*-dimethyl-*N'*-[(3-trimethylammonium)propyl]ethylenediamine dibromide (I) and *N*-(9-anthroyl)-*N'*,*N'*-di-