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EXCLUSION CHROMATOGRAPHY OF ANIONIC DYES

ANOMALOUS ELUTION PEAKS DUE TO REVERSIBLE AGGREGATION

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SUMMARY

Several anionic dyes, especially the barbiturate and pyrazolone oxonol dyes, were shown by exclusion chromatography to form large, monodisperse, polymeric aggregates in dimethylformamide. Their size was approximately that of polystyrene of molecular weight 10^6 daltons. They were in labile equilibrium with monomer and dissociated in salt solution. The anomalous elution profile with a threshold characteristic of the dye and a peak increasingly retarded with increasing dye quantity was explained in terms of continuing concentration-dependent monomer–aggregate equilibrium during chromatography. This aggregation was contrasted with the association in water which accompanies a change in optical absorption spectrum and gives smaller particles. A vesicular structure of membrane made of associated dye molecules, with an electrical double layer was suggested on the basis of the monodisperse large size of the aggregates, their instability in salt solution and the ionic nature of the dyes. Exclusion chromatography allows the study of aggregation phenomena which cannot be followed by measurements of colligative properties or absorption spectra.

INTRODUCTION

Oxonol dyes[1] can serve in biological studies as indicators of membrane potential[2] and in photographic chemistry as antihalation or filter dyes and as spectral sensitisers[3]. A consideration of the state of aggregation of these dyes is relevant to these applications. While the aggregation of cationic dyes, particularly the cyanines, has been studied extensively[4], the aggregation of oxonol dyes is not so well known.

The difficulty of separating dyes by chromatography[5] is increased by their tendency to form pure aggregates as well as mixed complexes and aggregates. The source of anomalous smears and elution volumes in the exclusion (gel permeation) chromatography of oxonol dyes was the reversible formation of large aggregates. Studies of the aggregation of these anionic dyes or the use of exclusion chromatography to study dye aggregation have not previously been reported.

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EXPERIMENTAL

Chromatography of the dyes with dimethylformamide (DMF) solutions using Styragel (polystyrene) stationary phase was performed with a Waters high-pressure liquid chromatograph using three 4-ft. columns with given nominal exclusion limits of $1 \cdot 10^4$, $3 \cdot 10^4$ and $1 \cdot 10^5$ Å chain length polystyrene and a refractive index detector. The system was calibrated with polystyrene of known molecular weight (Fig. 1). The sample was injected as a 2-ml loop of varied concentration.

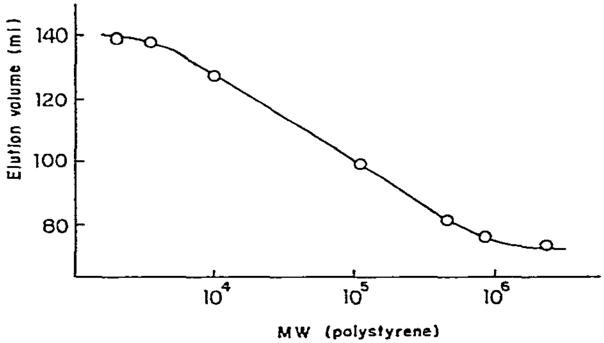


Fig. 1. Calibration of the Styragel columns with standard polystyrene of known molecular weight (MW).

Chromatography of the dyes with aqueous solutions using Sephadex and with dimethyl sulphoxide solutions using Sephadex was performed with vertical columns calibrated with Gaspar yellow 1350 (polymer) and copper sulphate (monomer). Where necessary the aqueous solutions were prepared by dissolving the solid in a little methanol or DMF and diluting with water. All measurements were made at room temperature.

Molecular weights according to the vapour pressure method were determined in the concentration range 0.1–1.0% using a Perkin-Elmer instrument.

RESULTS

The oxonol dyes in DMF, when added at a low concentration ($<10^{-4}$%), chromatograph as large monodisperse aggregates with a size equivalent to that of polystyrene with molecular weight *ca.* 10^6 daltons. As the injected concentration is increased, the elution peak is retarded to a larger elution volume but the threshold of the leading edge is independent of the amount injected and is characteristic of the dye. Above 10^{-3}% initial concentration, the different amounts share a common leading edge while the trailing edge is further retarded with increasing amount injected. This behaviour is illustrated in Fig. 2 with a typical example, a barbiturate oxonol dye (No. 1, Table I). In Table I are listed the threshold values, characteristic of the dyes, for a number of anionic dyes. The molecular weight of the equivalent size polystyrene can be read from Fig. 1.

The aggregate formation was completed immediately on formation of the solution and there was no slow change on standing as evidenced by the chromatograms

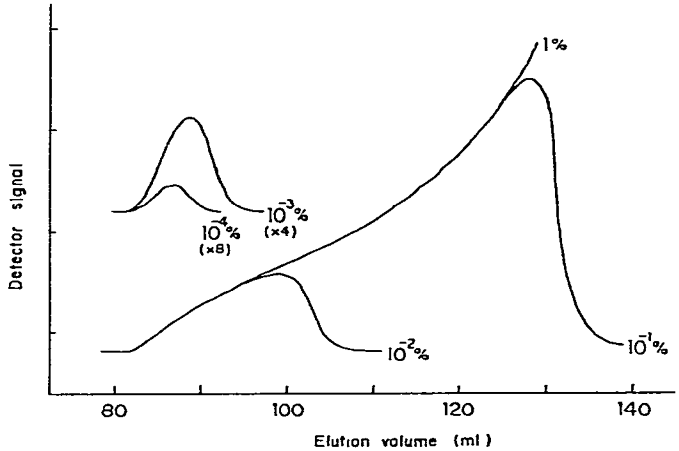
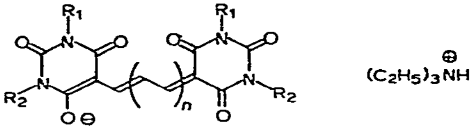


Fig. 2. The elution peak as a function of amount injected for a typical oxonol dye. The solvent is DMF with Styragel stationary phase. The percentage concentration of the dye injected is indicated. In parenthesis is the attenuation of the trace. The dye is No. 1 (Table I), structure I ($n = 2$, $R_1 = R_2 = -CH_3$), injected at concentrations of 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1} and 1 %, in 2-ml volume. For clarity the baseline is displaced for the two smallest amounts. The anomalous peak shape is attributed to a concentration-dependent reversible aggregation (see Fig. 5).

I



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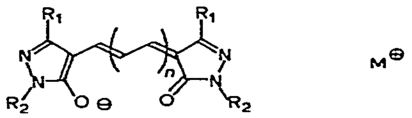

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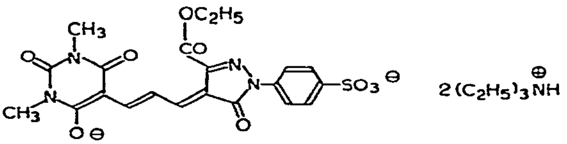

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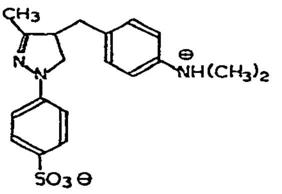

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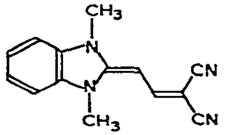
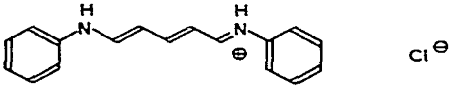


VI



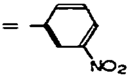
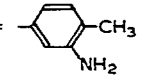
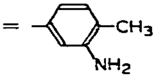
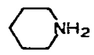
(dye No. 6). The addition of salts to the solution inhibited aggregate formation. In the presence of 1 % lithium bromide, the dye No. 1 eluted completely as monomer at $1.3 \cdot 10^{-1}$ % and $1.3 \cdot 10^{-3}$ % injected concentrations, with an elution volume of 140 ml. The presence of 10 % dimethylbarbituric acid (VII), which is not itself aggregated, in the injected sample of dye No. 1, a blue pentamethine barbiturate oxonol at 10^{-2} %, increased the elution volume from 82.5 to 90 ml. The elution volume of dye No. 6 was increased from 80 to 115 ml by 6 % VII and to 135 ml by 18.5 % VII when VII was added with the dye. Since VII was not added to the pumped solvent in these experiments, the disaggregation is not quantitatively determined, but is significant. The oxonol anions are very weak bases. Spectroscopy showed that dye No. 1 was not protonated by 1 % oxalic acid in DMF.

While the barbiturate oxonols (I) showed essentially similar behaviour, the pyrazolone oxonols (II) appeared to form larger aggregates. For comparison, while IV showed aggregation, V and VI, which was deprotonated to the neutral molecule in the DMF, chromatographed as monomers. Gaspar yellow 1350, a covalent polymer, Gaspar cyan 802B and Pontamine blue gave elution volumes of 70 ml, 71–5 ml and 72–5 ml respectively. Covalent polymeric dyes with structure I ($n = 2$, $R_1 = H$, $R_2 = (CH_2)_n$) gave elution volumes of *ca.* 70 ml, and similar results were obtained with dye No. 3 copolymerised with succinyl chloride or hexamethylene diisocyanate.

In water the tendency to aggregate was less apparent, the molecular sizes being less than those of globular proteins of molecular weight 5000 daltons, with some exceptions such as dye No. 7 which gave large aggregates. In DMF, the absorption spectrum did not appear to change on aggregation. The specific optical absorption of dye No. 1 at 595 nm in DMF decreased in intensity only marginally between $4 \cdot 10^{-5}$ % and $4 \cdot 10^{-2}$ % concentration and no new peak appeared. In water on the other hand there was a dramatic and complex change in optical absorption with increasing concentration (Fig. 3). Above 10^{-4} % concentration a new peak appeared at lower wavelength, 520 nm, to reach a maximum absorption by 10^{-2} %. The 585 nm absorption decreased in intensity above 10^{-3} % concentration. These deviations from Beer's law are plotted for dye No. 1 in Fig. 3. The absorption spectra for dyes No. 1 and No. 4 at an intermediate concentration where both peaks are visible are shown in Fig. 4. The spectral change in water is not always as clearcut as with the barbiturate oxonol dyes. Dye No. 7 at $4 \cdot 10^{-2}$ % and $4 \cdot 10^{-4}$ % concentrations had the same absorption peak at 615 nm, although the higher concentration had a larger shoulder

TABLE I

ELUTION VOLUMES OF SOME ANIONIC DYES

Structure		*DMF (Styragel) (ml)**	*Water (Sephadex) (ml)***
No. 1	I, $n = 2$ $R_1 = R_2 = CH_3$	82.5	79
No. 2	I, $n = 2$ $R_1 = CH_3$ $R_2 =$ 	87.0	–
No. 3	I, $n = 2$ $R_1 = CH_3$ $R_2 =$ 	80.0	70
No. 4	I, $n = 2$ $R_1 = R_2 = C_2H_5$	86.0	73
No. 5	I, $n = 2$ $R_1 = C_2H_5$ $R_2 =$ 	85.0	90
No. 6	I, $n = 1$ $R_1 = R_2 = CH_3$	80.0	–
No. 7	II, $n = 2$ $R_1 = CH_3$ $R_2 = p\text{-}C_6H_4SO_3^-K^+$ $M^+ = K^+$	71.0	50
No. 8	II, $n = 2$ $R_1 = NH_2$ $R_2 = C_6H_5$ $M^+ = (C_2H_5)_3N^+H$	75.0	135 (G-75)
No. 9	II, $n = 1$ $R_1 = CH_3$ $R_2 = C_6H_5$ $M =$ 	80.0	90
No. 10	III	82.5	–
No. 11	IV	81.5	–

* Threshold value, see text. See Fig. 1 for calibration.

** Sephadex G-25 except where otherwise stated. With G-25 monomer ($CuSO_4$) eluted at 90 ml and high polymer (Gaspar yellow 1350) at 49 ml. With G-75 monomer eluted at 135 ml and high polymer at 33 ml.

on the low wavelength side. Aggregation of these dyes is also seen when the solvent is dimethyl sulphoxide.

The apparent molecular weight of ionic dyes calculated from colligative properties increases only up to a point on their aggregation. Assuming separation of the counter-ion from the aggregated dye, the maximum value for dye No. 1, an ion pair,

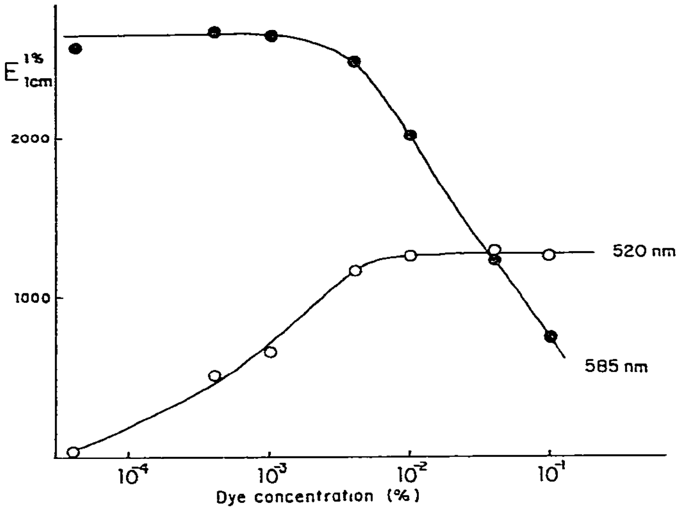


Fig. 3. The optical absorption of dye No. 1 (Table I) at the two absorption maxima, $\lambda_{max.}$ = 585 nm and $\lambda_{max.}$ = 520 nm in water as a function of concentration. These absorption changes are attributed to reversible aggregation of the dye.

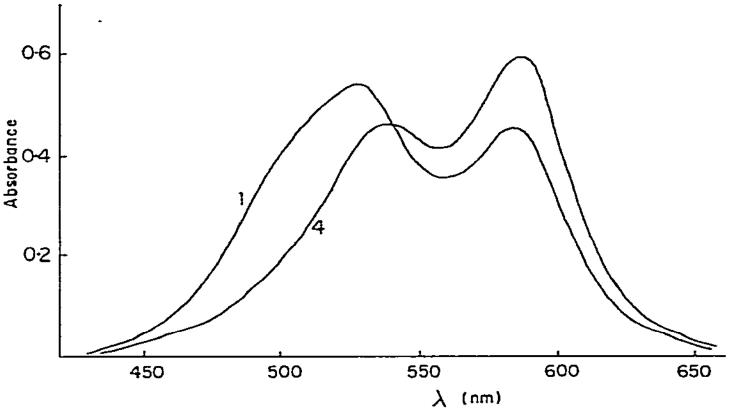


Fig. 4. The optical absorption of dyes No. 1 and No. 4 (Table I) in water at intermediate concentrations where both peaks are visible. The concentration is 0.04%, pH 6, 0.01-cm path length cuvette.

is 475 and for dye No. 7, an ion quadruplet, 228, although incorporation of counterions in the aggregate would give higher results. Molecular weights determined by the vapour pressure method for dye No. 7 were 180 in water and 273 in DMF. Dye No. 1 gave 327 in DMF.

DISCUSSION

That a number of dyes, particularly the pyrazolone and barbiturate oxonol dyes, form monodisperse polymeric aggregates in solution in DMF as well as dimethyl sulphoxide and to a lesser extent water has been shown by exclusion (gel permeation) chromatography. The rapid formation of the aggregates, their instability in salt solutions even at fairly low concentrations of salt and the exclusion chromatography elution volumes and peak shapes support the formation of large, labile, monodisperse aggregates, unstable in salt solution and in equilibrium with monomer. This model explains the anomalous skewed elution peaks. Since the dyes are themselves salts, there is a dye concentration above which the aggregates, stable in DMF, are dissociated to monomer. A low injected concentration (*ca.* 10^{-4}%) is completely aggregated and chromatographs as a monodisperse polymer. Above 10^{-3}% concentration there is appreciable dissociation to monomer, particularly at the center of the peak where the concentration is highest. Aggregate concentration is optimal at the front and rear of the peak and since the aggregate travels faster than the monomer its transport counteracts backward diffusion at the rear of the peak and extends the front of the peak towards a lower elution volume. This behaviour is illustrated schematically in Fig. 5. This model skews the peak in the opposite sense to a reversible aggregation independent of ionic strength.

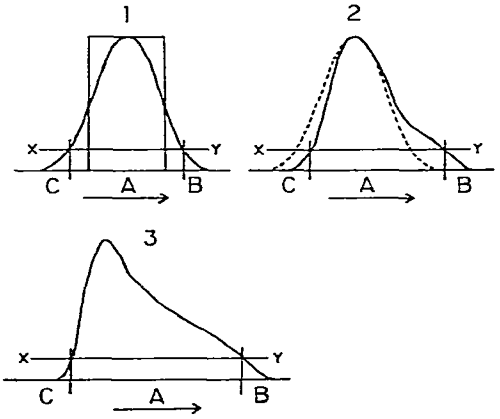


Fig. 5. The proposed origin of the anomalous exclusion chromatography elution peaks for ionic dyes which aggregate at low salt concentration only. The ordinate represents the dye concentration and the abscissa the distance travelled through the column. Direction of motion is indicated by the arrows. The lines XY represent the concentration above which dissociation of the aggregates occurs. In 1 the plug has begun to spread by diffusion in the solvent. Regions B and C of low concentration are aggregate while A is largely monomer. In 2, after some transport, the aggregate travels faster than monomer, B draws ahead of A but C catches up A and disaggregates. C is replenished by back diffusion and B is increased by diffusion. Continuation of this process gives rise to the shape in 3, and the experimental curves in Fig. 2. The final shape depends on the amount injected and the distance travelled.

Unlike the association of these dyes in water, which gives smaller particles and is accompanied by changes in the optical absorption peaks, this high aggregation in DMF occurs without significant change in absorption spectrum. It cannot be followed by colligative properties.

The dyes which aggregated were ionic. The largest aggregates were formed by the triionic species VII, and the neutral species V and VI were not aggregated in these conditions. The results suggest that the associated ions form aggregates and that dissociation of ion pairs with increasing salt (dye) concentration is accompanied by disaggregation. The monodisperse and large size of the aggregates, equivalent to that of polystyrene of molecular weight 10^6 daltons, suggests the formation of vesicles (microsacs) by the packing of parallel dye chains perpendicular to the surface in a membrane the surface of which is an electrical double layer. This structure would be analogous to that well documented for lipids in water[6], although in this case there is a dye monolayer rather than a lipid bilayer. This aggregate structure may be different from those formed in other solvents at different concentrations.

Effects of the association of oxonol dyes have been prevented by the addition of zwitterionic detergents[7], and this improved their performance when used as photographic filter dyes. In chromatography the smear due to aggregation of these dyes is completely removed by the presence of 1 % lithium bromide in which the dyes elute as monomers rather than polymeric aggregates. Thus other species, such as the dye as part of or attached to a covalent polymer, may be clearly resolved.

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