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Pulse-Radiolysis and Spectral Studies of the Interaction of Cetylpyridinium Chloride and Methylene Blue with Connective-Tissue Glycosaminoglycans and Related Compounds

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1. Hydrated electrons produced by pulse radiolysis were used to study the interaction of polyanionic glycosaminoglycans and related compounds with the counterions Methylene Blue and cetylpyridinium chloride. 2. The effect of added salt (potassium chloride) on the interaction indicates that the relative binding affinities, with respect to the types of anionic site present, increases for both counterions in the order $CO_2^- < CO_2^- + O \cdot SO_3^- < CO_2^- + O \cdot SO_3^- + N \cdot SO_3^- < O \cdot SO_3^-$. The interactions of the polyanions with cetylpyridinium chloride are considerably stronger than those with Methylene Blue. 3. The effects of added salt on the metachromasia resulting from polyanion–Methylene Blue interaction were examined spectrophotometrically. 4. The collective results demonstrate a direct relationship between anionic site–dye binding and metachromasia, although a residual dye binding can be detected by pulse radiolysis when metachromasia is completely removed.

The ability of cationic compounds such as MB^+* or CPC to interact with polyanionic compounds has been of considerable value in the study of acidic glycosaminoglycans of connective tissues. CPC is used as a glycosaminoglycan precipitant, and the technique can be refined to allow separation of individual glycosaminoglycans by taking advantage of the fact that precipitation is markedly affected by the presence of inorganic salts such as potassium chloride or magnesium chloride (Scott, 1965). The stronger the interaction between the cation and the polyanion, the greater the concentration of salt required to dissociate the complex.

In an analogous manner, the interaction of dyes with glycosaminoglycans has formed the basis of a number of histological staining techniques. Refinement of these techniques by successive elution of stained tissues with different concentrations of inorganic salt solutions allows some degree of differentiation between individual glycosaminoglycans (Scott & Dorling, 1965; Scott & Willett, 1966; Scott, 1968). Many of the cationic dyes that have been used for the histological staining of these polymers exhibit the phenomenon of metachromasia. Interaction of dye and polyanion results in the formation of a new dye absorption band. This new band has been accounted for by considering the interaction between negative sites on the polyanion with the cationic dye molecule (Young, Phillips & Balazs, 1967) and also has been discussed in terms of interaction between dye molecules at adjacent sites of the polyanion. The latter has been termed the aggregation or 'stacking' theory. This theory originates from observations that spectral shifts are observed in concentrated solutions of dyes, due to the formation of dimers or higher aggregates. Michaelis (1947, 1950), by analogy, suggested that similar aggregates could be formed when two or more dye molecules bind to neighbouring anionic sites of the polyanion. This simple model has subsequently been defined and evaluated in more quantitative terms (e.g. Stone & Bradley, 1961, 1967; Curran, 1964; Scheibe, 1938; Bradley, 1961; Pal & Schubert, 1962; Van Duuren, 1966).

Information about dye–polyanion interaction may also be obtained by use of the technique of pulse radiolysis (Balazs, Davies, Phillips & Scheufele, 1968*a*,*b*). The technique can also provide information about interactions of polyanions with counterions that do not exhibit the phenomenon of metachromasia. The nucleophilic hydrated electrons (e_{aq}^-) produced during pulse radiolysis of aqueous solutions react extremely rapidly with

* Abbreviations: MB^+, Methylene Blue; CPC, cetylpyridinium chloride.

appropriate cationic compounds such as MB^+ or CPC, but much more slowly when a polyanion is also present, despite the fact that there is no change in the concentration of the cation. The marked decrease in rate induced by the presence of a polyanion indicates that a partial or complete charge neutralization of the cation has occurred and forms a basis for studying the interaction. The phenomenon is entirely dependent on the presence of the polyanion, since no such decrease in rate is found when the appropriate monomer, at a concentration equivalent to that present in the polyanion, is added to the solution of the cation (Balazs *et al.* 1968*a*,*b*).

In the present work pulse radiolysis was used to examine the interactions of certain acidic glycosaminoglycans and other polyanions with CPC, and the concentrations of potassium chloride required to reverse the interaction were determined. Analogous interactions with MB^+ were followed by both pulse-radiolysis and spectrophotometric techniques. In these cases it was possible to correlate the reversal of interaction that occurs in the presence of potassium chloride with changes in metachromasia. A preliminary account of this work has already been presented (Moore, Phillips, Dodgson & Davies, 1967).

MATERIALS AND METHODS

Chondroitin 4-sulphate and chondroitin 6-sulphate were separated from bovine tracheal cartilage by a method based essentially on that of Dodgson, Lloyd & Spencer (1957). De-*N*-sulphated heparin was prepared from commercial heparin (Boots Pure Drug Co. Ltd., Nottingham) by the procedure of Danishefsky, Eiber & Carr (1960). Sulphated hyaluronic acid (SO_4^{2-}, 9·4%) was prepared by sulphation of umbilical-cord hyaluronic acid (Seravac Laboratories, Maidenhead, Berks.) according to the directions provided by Professor A. G. Lloyd (personal communication). All the above compounds were isolated as potassium salts. Sodium alginate (Mannucol SS/LH; Alginate Industries, Girvan, Ayrshire) was dialysed, reprecipitated with acetone and dried *in vacuo* before use. Sodium dextran sulphate (mol.wt. 2×10^6; SO_4^{2-}, 51%) was supplied by Pharmacia Fine Chemicals (Uppsala, Sweden) and MB^+ was purified as described by Balazs *et al.* (1968*a*).

For both pulse-radiolysis and spectroscopic studies the complexes were prepared by mixing aqueous solutions of counterion and the polyanions so that the final concentrations were 0·01 mM and 0·1 or 1·0 'milliequivalents/anionic site'/l. respectively. The term 'equivalents/anionic site' refers to the molecular weight of the disaccharide repeating unit of the polyanion divided by the number of anionic sites present in the unit. The ratio of anionic sites to cationic dye sites (S/D ratio) under these circumstances is 10:1 or 100:1 and precipitation of the complex is not apparent.

Pulse radiolysis was carried out at the Paterson Laboratories, Christie and Holt Radium Institute, Manchester, and full details have been given by Keene (1964). An appropriate computer programme was designed for the processing of experimental results. Each experimental point quoted is the mean of three determinations. The decay of $e^-_{aq.}$ is first recorded as an oscilloscope trace. From the semilogarithmic plot of E_{700} against time after the pulse (see Fig. 1 for example) the half-life, $t_{\frac{1}{2}}$, and the first-order rate constant for the disappearance of $e^-_{aq.}$ may be calculated (see also Balazs *et al.* 1968*a*,*b*).

RESULTS

Pulse-radiolysis studies. (*a*) MB^+ complexes. The half-life, $t_{\frac{1}{2}}$, of $e^-_{aq.}$ in aqueous solutions of MB^+ (0·01 mM) after pulse irradiation with 4 Mev electrons (approx. 250 rads in 2 μsec.), calculated from the pseudo-first-order plot for the disappearance of the electron absorption, is 2·8 μsec. Addition of the polyanions alginate, chondroitin 4-sulphate, chondroitin 6-sulphate, de-*N*-sulphated heparin, heparin, hyaluronic acid sulphate and dextran sulphate to MB^+ (S/D ratios 10:1 and 100:1) is accompanied by a marked increase in the half-life of $e^-_{aq.}$ (Table 1). Addition of potassium chloride in increasing concentrations to the polyanion–dye complex results in an increasing rate of disappearance of $e^-_{aq.}$, as indicated by a decrease in $t_{\frac{1}{2}}$, until a concentration of potassium chloride is reached at which the value of $t_{\frac{1}{2}}$ reverts to that exhibited by MB^+ alone. This phenomenon is illustrated graphically in Fig. 1 for the chondroitin 4-sulphate–MB^+ complex at S/D ratio 100:1. Fig. 2 shows the changes in $t_{\frac{1}{2}}$ for all the polyanion–dye complexes at S/D ratios 10:1 and 100:1 respectively plotted as a function of potassium chloride concentration. As a specific example, $t_{\frac{1}{2}}$ for the chondroitin 4-sulphate–MB^+ complex (S/D ratio 100:1) is 7·2 μsec. and the

Table 1. *Effect of various polyanions on the reactivity of hydrated electrons with MB^+ and CPC*

Experimental details are given in the text. Half-lives, $t_{\frac{1}{2}}$, of $e^-_{aq.}$ for 0·01 mM-MB^+ alone and for 0·01 mM-CPC alone are 2·8 and 2·0 μsec. respectively. Abbreviations: ALG, sodium alginate; CSA, chondroitin 4-sulphate; CSC, chondroitin 6-sulphate; DSH, de-*N*-sulphated heparin; SHA, sulphated hyaluronic acid; Hep, commercial heparin; DS, sodium dextran sulphate.

	$t_{\frac{1}{2}}$ for $e^-_{aq.}$ (μsec.)			
	MB^+		CPC	
Polyanion	S/D 10:1	S/D 100:1	S/D 10:1	S/D 100:1
ALG	11·7	9·1	18·8	11·6
CSA	11·2	7·2	19·6	20·2
CSC	10·4	7·2	19·6	20·3
DSH	6·6	—	—	14·9
SHA	18·4	—	19·8	—
Hep	23·0	14·0	24·5	15·9
DS	16·6	25·1	26·4	26·2

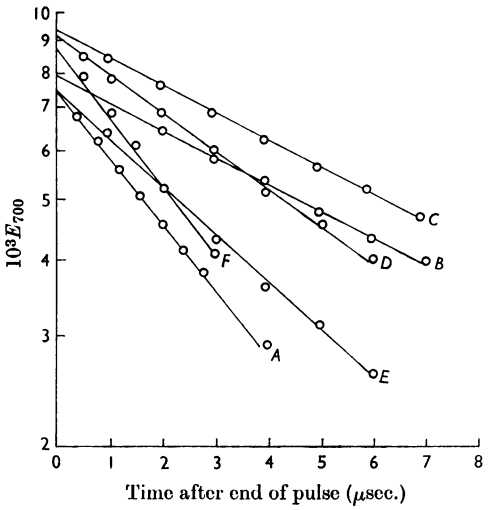

Fig. 1. First-order plots of the disappearance of hydrated electrons ($e^-_{aq.}$) in the presence of MB^+ and chondroitin 4-sulphate–MB^+ complex at increasing concentrations of KCl. Curve *A*, MB^+ (0·01 mM); curve *B*, chondroitin 4-sulphate–MB^+ complex (S/D ratio 100:1); curve *C*, chondroitin 4-sulphate–MB^+ complex plus 0·1 mM-KCl; curve *D*, chondroitin 4-sulphate–MB^+ complex plus 1·0 mM-KCl; curve *E*, chondroitin 4-sulphate–MB^+ complex plus 10 mM-KCl; curve *F*, chondroitin 4-sulphate–MB^+ complex plus 100 mM-KCl.

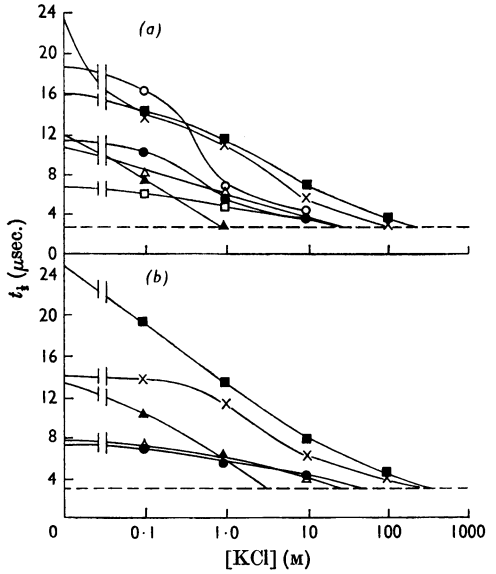

Fig. 2. Effect of KCl on the half-life, $t_{\frac{1}{2}}$, for the disappearance of hydrated electrons ($e^-_{aq.}$) in polyanion–MB^+ solutions at S/D ratios 10:1 (*a*) and 100:1 (*b*). ○, Sulphated hyaluronic acid; ●, chondroitin 4-sulphate; △, chondroitin 6-sulphate; ▲, alginate; □, de-*N*-sulphated heparin; ■, dextran sulphate; ×, heparin. The broken line indicates the value of $t_{\frac{1}{2}}$ for disappearance of $e^-_{aq.}$ in the presence of MB^+ alone and the curves have been extrapolated to this line.

concentration of potassium chloride for this value to revert to that exhibited by MB^+ alone is 40 mM, a concentration that may be regarded as that required to release all bound MB^+ completely from the complex. For the polyanions studied the concentration of potassium chloride required to achieve this complete release of MB^+ varies with the nature of the binding sites available for interaction and to a smaller degree with the S/D ratios (Table 2). The salt concentration necessary to remove all the bound counterion from the complex is here referred to as the limiting salt concentration, which, although analogous, is not identical with the critical electrolyte concentration (see Scott, 1965), which is based on the solubilization of complexes where the S/D ratio is 1:1.

(*b*) CPC complexes. The half-life for the disappearance of $e^-_{aq.}$ in aqueous solutions of CPC (0·01 mM) is 2·0 μsec., which corresponds to $k_2 = 3{\cdot}5 \times 10^{10}$ mole^{-1} sec.$^{-1}$ for the reaction of $e^-_{aq.}$ + CPC. The effect of polyanion on this reaction is shown in Table 1 for S/D ratios 10:1 and 100:1. Addition of potassium chloride releases CPC from the polyanion complex with consequent increase in the rate of reaction of $e^-_{aq.}$ (see Fig. 3 for the results at S/D ratio 10:1). As with the polyanion–MB^+ complexes the limiting salt concentration may be obtained and the relative binding affinities of the individual polyanions towards CPC may be calculated (Table 2).

Spectroscopic studies. The visible-absorption spectra of the chondroitin 4-sulphate–MB^+ complex (S/D ratio 10:1) in the presence of increasing concentrations of potassium chloride are recorded in Fig. 4. At this S/D ratio for all the other polyanion–MB^+ complexes the families of curves obtained were similar and the wavelengths of the particular isosbestic point were noted (Table 3). Similar results were obtained at S/D ratios 100:1, but the isosbestic points are not so well-defined in these cases. In Fig. 5 the progressive reversion of the metachromatic absorption to that of the free dye is expressed as a function of potassium chloride concentration for complexes where the S/D ratio is 10:1. Thus the limiting salt concentrations may be obtained for each polyanion–MB^+ complex, from the complete release of MB^+ as indicated by the E_{665} value. The effects of potassium chloride concentrations on metachromasia and on the release of MB^+ from the polyanion–dye complexes as determined by pulse radiolysis may now be compared and typical examples of such correlations are shown in Figs. 6 and 7.

Table 2. *Limiting salt concentrations and relative binding affinities for polyanion–MB⁺ complexes measured by pulse-radiolysis and spectrophotometric methods, and for polyanion–CPC complexes measured by pulse radiolysis*

Abbreviations: L.S.C., limiting salt concentration; R.B.A., relative binding affinity; others as defined in Table 1.

	Pulse radiolysis				Spectrophotometry			
	S/D 10:1		S/D 100:1		S/D 10:1		S/D 100:1	
Polyanion	L.S.C. (mM-KCl)	R.B.A.	L.S.C. (mM-KCl)	R.B.A.	L.S.C. (mM-KCl)	R.B.A.	L.S.C. (mM-KCl)	R.B.A.
MB⁺ complexes								
ALG	1·0	1	3·0	1	1·2	1·0	3·0	1·0
CSA	30	30	40	13	2·0	2·0	8·0	2·0
CSC	30	30	20	7	2·0	2·0	4·5	1·5
DSH	30	30	—	—	2·0	2·0	4·0	1·3
SHA	60	60	—	—	2·5	2·5	7·0	2·3
Hep	100	100	200	70	10	8·0	10	3·0
DS	200	200	250	85	15	12·0	45	15·0
CPC complexes								
ALG	340	1·0	350	1				
CSA	750	2·0	720	2				
CSC	750	2·0	650	2				
DSH	—	—	770	2				
SHA	1000	3·0	—	—				
Hep	860	2·5	1400	4				
DS	> 1000	>3·0	2600	7				

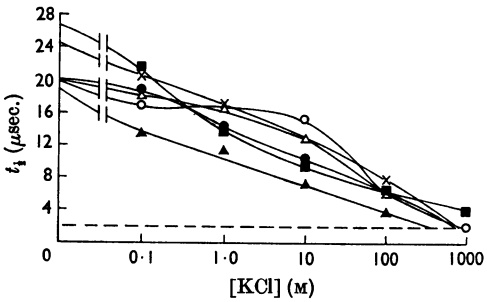

Fig. 3. Effect of KCl on the half-life, $t_{\frac{1}{2}}$, for the disappearance of hydrated electrons ($e^-_{aq.}$) in polyanion–CPC solutions at S/D ratio 10:1. ○, Sulphated hyaluronic acid; ●, chondroitin 4-sulphate; △, chondroitin 6-sulphate; ▲, alginate; ■, dextran sulphate; ×, heparin. The broken line indicates the value of $t_{\frac{1}{2}}$ for disappearance of $e^-_{aq.}$ in the presence of CPC alone and some of the curves have been extrapolated to this line. Some experimental points have been omitted for the sake of clarity.

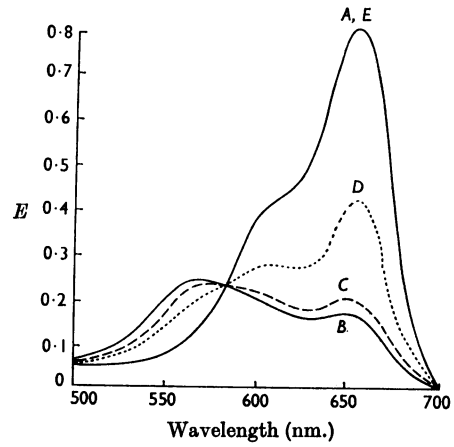

Fig. 4. Effect of increasing concentrations of KCl on the metachromatic behaviour of the chondroitin 4-sulphate–MB⁺ complex at S/D ratio 10:1. Curve *A*, absorption spectrum of MB⁺ alone; curve *B*, complex without added KCl; curve *C*, complex plus 0·1 mM-KCl; curve *D*, complex plus 1·0 mM-KCl; curve *E*, complex plus 10 mM-KCl.

DISCUSSION

Interaction between MB⁺ and all the polyanions studied is evident from the results shown in Table 1. The reaction of $e^-_{aq.}$ with MB⁺ may be represented as:

$$e^-_{aq.} + MB^+ \rightarrow MB^\cdot$$

where MB· represents the half-reduced semiquinone form of the dye (Balazs *et al.* 1968*a*). At 0·01 mM-MB⁺ the first-order kinetics are to be expected, since the concentration of MB⁺ is large

Table 3. *Effects of various polyanions on the metachromatic behaviour of MB⁺ at S/D ratio* 10:1 *and* 100:1

Abbreviations are defined in Table 1.

Polyanion	S/D 10:1 Wavelength of metachromasia max. (nm.)	S/D 10:1 Isosbestic point (nm.)	S/D 100:1 Wavelength of metachromasia max. (nm.)
ALG	580	600	580
CSA	570	588	600
CSC	570	587	600
DSH	572	586	600
SHA	557	582	585
Hep	561	584	583
DS	547	578	585

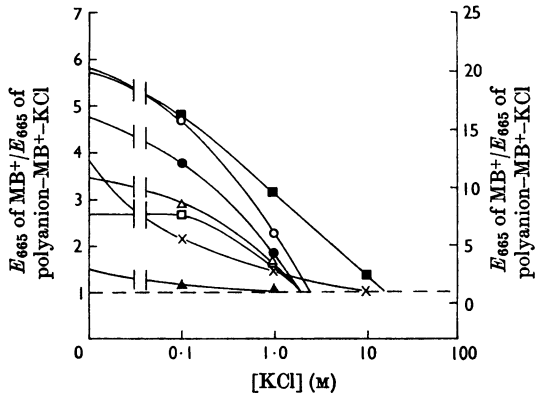

Fig. 5. Effect of increasing concentrations of KCl on the E_{665} value of polyanion–MB⁺ solutions at S/D ratio 10:1. ○, Sulphated hyaluronic acid; ●, chondroitin 4-sulphate; △, chondroitin 6-sulphate; ▲, alginate; □, de-*N*-sulphated heparin; ■, dextran sulphate; ×, heparin. Values for sulphated hyaluronic acid and heparin should be read from the right-hand ordinate. The broken line represents the situation when the extinction of the polyanion–MB⁺ complex has reverted to that of MB⁺ alone and the curves have been extrapolated to this line.

compared with the initial concentration of radicals produced in the pulse. The charge-neutralization process occurs with $t_{\frac{1}{2}}$ 2·8 μsec., which corresponds to a second-order rate constant of $2{\cdot}5 \times 10^{10}$ mole^{-1} sec.$^{-1}$ for the bimolecular reaction and is therefore indicative of a process that approaches the diffusion-controlled limit. On addition of the polyanion the rate of disappearance of $e^-_{aq.}$ is markedly decreased, despite the fact that the MB⁺ concentration is unchanged in the solution. The interaction with the polyanion thus appears to result in a change in electronic configuration over

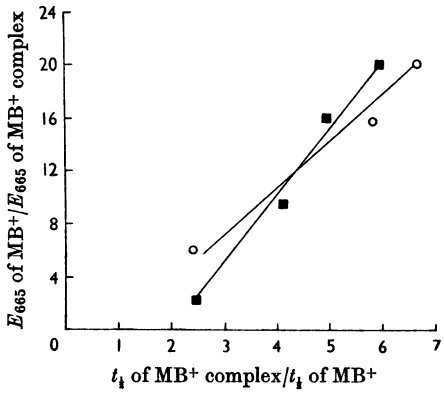

Fig. 6. Correlation between the changes in the E_{665} value and the changes in the half-life, $t_{\frac{1}{2}}$, of the hydrated electron ($e^-_{aq.}$) that occur when KCl is added to polyanion–MB⁺ complexes at S/D ratio 10:1. ○, Sulphated hyaluronic acid; ■, dextran sulphate. For the sulphated hyaluronic acid curve the experimental points, reading from left to right, refer to KCl concentrations of 1·0 mM, 0·1 mM and zero respectively. For the dextran sulphate curve the experimental points, reading from left to right, refer to KCl concentrations of 10 mM, 1·0 mM, 0·1 mM and zero respectively.

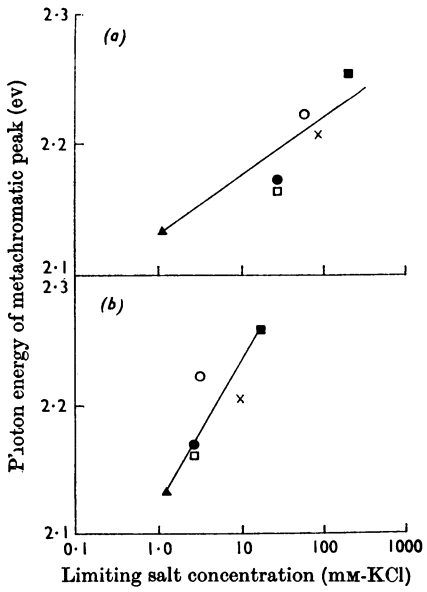

Fig. 7. Relationship between the wavelength of metachromasia (expressed as photon energy of the metachromatic peak) of various polyanion–MB⁺ complexes at S/D ratio 10:1 and limiting salt concentrations for these complexes obtained by (*a*) pulse-radiolysis and (*b*) spectrophotometric measurements. ○, Sulphated hyaluronic acid; ●, chondroitin 4-sulphate; ▲, alginate; □, de-*N*-sulphated heparin; ■, dextran sulphate; ×, heparin.

the entire chromophoric system of MB^+ with partial or complete neutralization of the charge. It has been suggested that ion-pair formation, where the ions are in physical contact, could adequately account for this behaviour (Balazs *et al.* 1968*a*,*b*).

The results reported here show that individual polyanions influence $t_{\frac{1}{2}}$ for $e^-_{aq.}$ disappearance to differing degrees. Qualitatively the polyanions known to bind organic cations most strongly (Scott & Willett, 1966) give rise to the most pronounced changes in $t_{\frac{1}{2}}$. For example, at S/D ratio 10:1 heparin changes $t_{\frac{1}{2}}$ to 23 μsec. compared with $t_{\frac{1}{2}}$ 11·7 μsec. for the alginate complex having the same S/D ratio. For the reaction $e^-_{aq.}$ + polyanion, k_2 is approx. 3×10^7 mole^{-1} sec.$^{-1}$, which demonstrates that this reaction does not contribute significantly to the removal of $e^-_{aq.}$ in the polyanion–dye complexes in solution at the concentrations used. The residual reaction with $e^-_{aq.}$ is therefore due to free dye associated with the ion-binding equilibrium, which may schematically be represented:

$$[MB^+] + [\text{polyanion}] \rightleftharpoons [\text{MB–polyanion}]$$

This cannot be considered as a simple equilibrium obeying the Mass Action Law (see, e.g., Dey & Palit, 1968), since a multiple-equilibria situation exists. The stability of the intermediate complexes will determine the position of equilibrium at any particular polyanion concentration. For the systems we have examined here, there is generally less free dye present when the S/D ratio is 10:1 than when it is 100:1. However, as the results show (Table 1), this pattern is not rigorously obeyed. Some indication of the relative binding affinities of the polyanions can be obtained from a consideration of this equilibrium as indicated by the relative values of $t_{\frac{1}{2}}$ (Balazs *et al.* 1968*a*). However, since more than one type of anionic site exists in the range of polyanions used, more reliable information can be obtained by an examination of the effect of added salt on the equilibrium. There is progressive dissociation of the complex on increasing potassium chloride concentration (Fig. 2). Certain advantages over the methods (Scott, 1965) based on solubilization or direct observation of complexes in which the S/D ratio is 1:1 are inherent in the method described. First, complexes with a range of S/D values may be studied and the ion-binding process directly observed at any particular salt concentration. Also, when more than one anionic group is present in the polyanion, some indication of multiple binding is given by the departure from linearity of the dissociation curves at potassium chloride concentrations between 0·1 mM and the limiting salt concentration (see Fig. 2). In addition, the salt concentration required to fully release all bound dye (limiting salt concentration) can be accurately determined. Such values give directly the relative binding affinities of the various polyanions and are shown in Table 2. At S/D ratios 10:1 and 100:1 the order of binding affinity of MB^+ with respect to the types of anionic sites present increases in the order $CO_2^- < CO_2^- + O \cdot SO_3^- < CO_2^- + O \cdot SO_3^- + N \cdot SO_3^- < O \cdot SO_3^-$, which is consistent with the observations by Scott (1965) and Scott & Willett (1966) for the order of binding affinities based on the critical electrolyte concentration for 1:1 complexes. A significant feature is the similar limiting salt concentrations for complexes with S/D ratios 10:1 and 100:1. The strength of interaction is thus more closely associated with the anionic-site interaction than on the degree of stacking of the counterion.

Similar dissociation curves may also be obtained for CPC complexes (Fig. 3). The order of increasing binding affinity for the polyanions is the same for CPC as for MB^+ (Table 2). However, the binding affinities of the various polyanions, relative to that of alginate, are always greater for the MB^+ complexes. As with the MB^+ complexes, the limiting salt concentrations for the CPC complexes do not alter significantly when the S/D ratio is changed from 10:1 to 100:1, but the binding of CPC to any particular polyanion is consistently and markedly stronger than the binding of MB^+ with the same polyanion (Table 2). For the binding of CPC, the limiting salt concentrations for the various polyanions are again broadly in agreement with the critical electrolyte concentrations found by Scott (1965).

The present results demonstrate that there is a direct relationship between site–dye binding and metachromasia. Reversal of the site–dye interactions on addition of salt, as indicated by the changes in $e^-_{aq.}$ rates, is accompanied by the reversal of metachromasia and the two types of behaviour can be linearly related. Although the limiting salt concentrations, as given by full reversion of the absorption spectrum to that of free MB^+, are in the same relative order $CO_2^- < CO_2^- + O \cdot SO_3 < CO_2^- + O \cdot SO_3^- + N \cdot SO_3^- < O \cdot SO_3^-$, the absolute values are consistently lower than the limiting salt concentrations determined by pulse radiolysis. Moreover, the S/D ratio influences the strength of interaction and the absorption maximum at which metachromasia occurs (Table 3). Two expressions of the strength of polyanion–dye interaction are therefore given by the energy of the metachromatic absorption maximum and the limiting salt concentration (given by the pulse-radiolysis method) and these we have sought to correlate in Fig. 7. Previously there has been no demonstration that the strength of the site–dye interaction is directly related to the metachromatic shift as shown in this correlation. The limiting salt concentration represents the amount of salt required to remove

completely the site–dye interaction and, as shown in Fig. 7, increases, as does the energy of the metachromatic shift. The photon energy (in ev) of the metachromatic peak has been adopted here since it gives a more accurate measurement of the interaction energy in metachromasia than the simple wavelength shift. It should be pointed out that some dye binding can be still detected by the pulse-radiolysis method when metachromasia has been fully removed. The facts therefore point to ion binding as the prerequisite in any aggregation of the dye that may occur on the polyanion. The residual ion binding still observable by pulse radiolysis when metachromasia is completely removed could, on this view, be associated with individual MB^+ molecules along the polyanion chain. Such monomeric bound MB^+ molecules would give rise to diminished reactivity towards $e^-_{aq.}$. However, this situation would not necessarily give rise to a spectral shift, since the probability of interaction with a nearest-neighbouring dye molecule is considerably decreased. On the exciton model (Stone & Bradley, 1961; Bradley, 1961) such interaction is a necessary condition for the degeneracy, which ultimately is responsible for the spectral shift.

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