Polyanions and their Complexes. Part IX.[†] Binding Affinities of Inorganic Ions to Heparin

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The effect of inorganic ions on the interaction between heparin and Methylene Blue has been studied using pulse radiolysis and visible absorption spectroscopy. The concentration of ions required fully to remove Methylene Blue from the complex, *i.e.* the limiting salt concentration, decreases in the order $Li^+ > Na^+ > Cs^+ > NH_4^+ > Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$. For inorganic ions of the same valence the strength of binding parallels the size of the hydrated ion, with the smallest ion binding most strongly. The presence of an additional charge increases the strength of binding and outweighs the size effect. Within the group of ions of identical valence the same variation in size exerts a comparable influence on binding strength.

THE interaction which occurs between certain cationic dyes and glycosaminoglycans forms the basis of many histological staining techniques. Following the early work of Landsmeer[1] on the effects of metal ions on the degree of staining of mast cells, several semi-quantitative studies were made on the influence of various cations on the quantity of dye bound to polyanionic materials,[2-4]

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with a view to differentiating between various components of connective tissue. Interaction of the dye with the polyanion leads to a change in the absorption spectrum of the dye (metachromasia).[5-11] Although Lison [12] showed, as long ago as 1935, that addition of salt suppresses this effect, no study of the relative effectiveness of a range of metal ions has been reported, apart from the work of Landsmeer.[1]

We have recently obtained further information about polyanion–counterion interactions using the technique of pulse radiolysis.[11-15] The rapid rate of reaction of the hydrated electron (e^-_{aq}), produced during pulse radiolysis, with the metachromatic dye Methylene Blue (MB^+) [16] and the detergent cetylpyridinium chloride (CPC) [14] is replaced by an extremely slow reaction when a polyanion is also present. By following changes in the rate of reaction of e^-_{aq} which occurs when KCl is added in increasing concentrations to a solution of the polyanion–counterion (MB^+ or CPC) complex the limiting salt concentration (l.s.c.),[14] *i.e.* the concentration of KCl required to destroy the complex, was obtained. Similarly the rate of release of MB^+ from its complex with increase in temperature has also been evaluated and an estimate of the thermodynamic parameters associated with complex formation has been made.[15]

In the present study, pulse radiolysis and spectral measurements have been used to examine quantitatively the influence of inorganic ions on the interaction between MB^+ and heparin. A measure of strength of binding of the inorganic ions to the polyanion can be obtained from the concentration necessary fully to replace MB^+ in the complex.

EXPERIMENTAL

Heparin (156 units mg^{-1}) was obtained from Evans Medical Ltd., and MB^+ from E. Gurr Ltd. The inorganic chlorides were of the highest grade available. Complexes were prepared by mixing aqueous solutions of MB^+ and heparin so that the final concentrations were 10 μM and 100 μ ' equivalents per anionic site ' [14] respectively. Under these conditions the ratio of anionic to cationic sites was 10 : 1 and no precipitation of the complex is apparent.

Pulse radiolysis experiments were carried out at the Paterson Laboratories, Christie Hospital and Holt Radium Institute, Manchester, using the apparatus essentially the same as that described by Keene.[17] The decay of the absorption of e^-_{aq} was recorded as an oscilloscope trace from which the semilogarithmic plot of O.D. (at 700 nm) against time after the end of the pulse could be made and the first-order rate constant, k_1, for the disappearance of e^-_{aq} could be calculated.

Visible absorption spectra of the complex with increasing concentrations of alkali halides and of MB^+ alone were recorded using a Perkin-Elmer 450 recording spectrophotometer.

RESULTS

Pulse Radiolysis Studies.—The first-order rate constant, k_1, for the reaction of e^-_{aq} with MB^+ (10 μM) following a 0·1 μs pulse of 12 MeV electrons was calculated to be 26×10^4 s^{-1}. In the presence of heparin–MB^+ complex (S/D = 10/1) the value of k_1 decreases to $3{\cdot}5 \times 10^4$ s^{-1}. Addition of increasing concentrations of the chlorides of Li^+, Na^+, Cs^+, NH_4^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} to the heparin–MB^+ complex results in a gradual increase in the value of k_1 until the value exhibited by MB^+ alone is reached; see, for example, Figure 1. Figure 2 shows the changes in k_1 for the heparin–MB^+ complex in the presence of the metal ions plotted as a function of increasing concentrations of the metal ion, and the concentration of metal

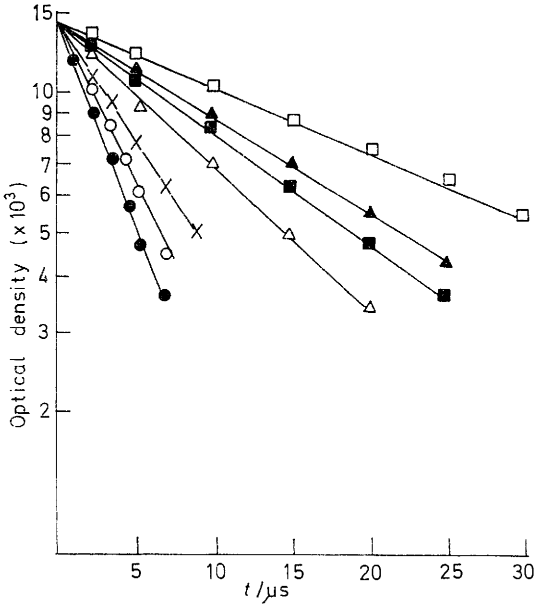

FIGURE 1 First-order plots for the disappearance of the absorption of the hydrated electron (e^-_{aq}) in the presence of MB^+ alone and MB^+–heparin complex (S/D = 10) containing increasing concentrations of CsCl: □ MB^+-heparin complex; ▲ complex + 1 × 10^{-5}M-CsCl; ■ complex + 5 × 10^{-5}M-CsCl; △ complex + 5 × 10^{-4}M-CsCl; × complex + 5 × 10^{-3}M-CsCl; ○ complex + 1 × 10^{-2}M-CsCl; ● MB^+ (1 × 10^{-5}M)

The effect of inorganic ions on heparin–Methylene Blue interaction

	Li^+	Na^+	Cs^+	NH_4^+
L.s.c. (× 10^{-3}M) from pulse radiolysis	65	55	46	27
L.s.c. (× 10^{-3}M) from spectra	26	19	16	13
	Mg^{2+}	Ca^{2+}	Sr^{2+}	Ba^{2+}
L.s.c. (× 10^{-3}M) from pulse radiolysis	18	7·5	1·2	0·9
L.s.c. (× 10^{-3}M) from spectra	3·4	1·4	0·85	0·3

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ion required to revert the original value of k_1 to that exhibited by MB^+, *i.e.* to destroy the complex and totally release the dye, can be determined (Table). This concentration has been defined as the l.s.c.[14] Addition of each fo the above ions to its l.s.c. to MB^+ does not change the rate constant for e^-_{aq} with the dye.

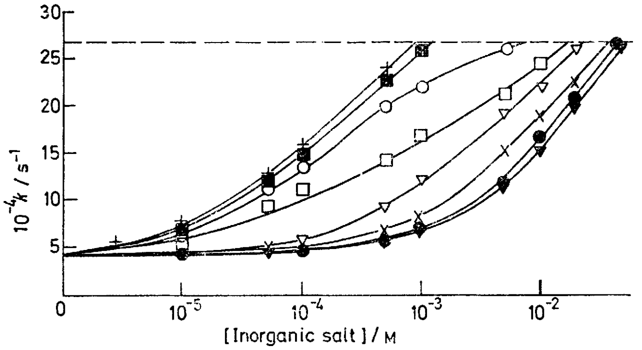

FIGURE 2 Effect of inorganic salts on the first-order rate constant for reaction of e^-_{aq} in MB^+-heparin solutions at S/D ratio 10/1. The broken line represents the value of k_1 in the presence of MB^+ alone and the curves have been extrapolated to this value: ▼ LiCl; ● NaCl; × CsCl; ▽ NH_4Cl; □ $MgCl_2$; ○ $CaCl_2$; ■ $SrCl_2$; + $BaCl_2$

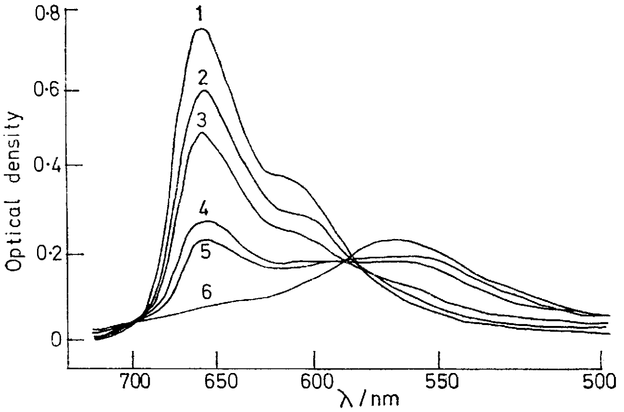

FIGURE 3 Effects of increasing concentrations of $CaCl_2$ on the metachromatic behaviour of MB^+-heparin complex at a S/D ratio of 10/1: 1 MB^+ (10^{-5}M); 2 complex + $CaCl_2$ (10^{-3}M); 3 complex + $CaCl_2$ (5×10^{-4}M); 4 complex + $CaCl_2$ (1×10^{-4}M); 5 complex + $CaCl_2$ (5×10^{-5}M); 6 complex

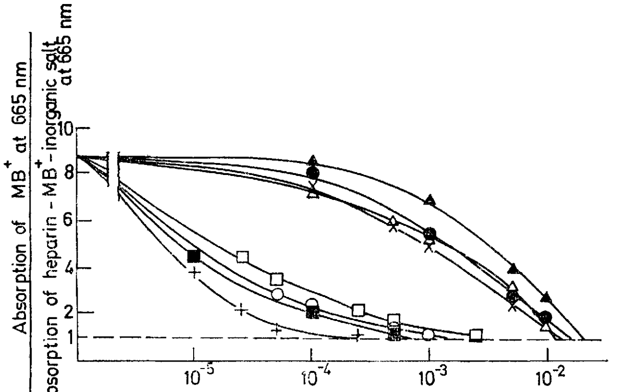

FIGURE 4 Effect of inorganic salts on the absorption at 665 nm of heparin–MB^+ solutions at S/D = 10/1. The broken line represents the situation when the absorption of the heparin–MB^+ complex has reverted to MB^+ alone, and the curves have been extrapolated to this point: ▲ LiCl; ● NaCl; × CsCl; ▽ NH_4Cl; □ $MgCl_2$; ○ $CaCl_2$; ■ $SrCl_2$; + $BaCl_2$

Spectroscopic Studies.—The visible absorption spectra of the heparin–MB^+ complex in the presence of increasing concentrations of $CaCl_2$ are shown in Figure 3. Similar curves were obtained for the other inorganic salts studied. The progressive destruction of metachromasia as indicated by the increase in the absorption at 665 nm is expressed as a function of inorganic salt concentration (Figure 4) and the l.s.c. for destruction of metachromasia are given in the Table.

In Figure 5, the changes in the absorption at 665 nm for the heparin–MB^+ complex in the presence of $CaCl_2$ and NaCl are compared with the corresponding changes in the values of k_1 for the disappearance of e^-_{aq}. A similar series of curves were obtained for the other salts.

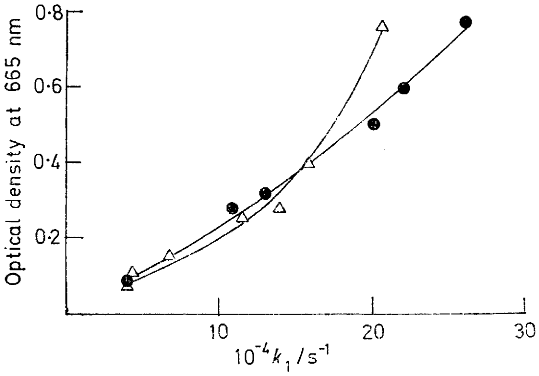

FIGURE 5 Correlation between the changes in the absorption at 665 nm and the changes in k_1 (the first-order rate constant of reaction of e^-_{aq}) that occurs when $CaCl_2$ is added in increasing concentrations to heparin–MB^+ complex (S/D = 10/1). The experimental points, reading from left to right, refer to $CaCl_2$ ● at 0, 5×10^{-5}, 1×10^{-4}, 5×10^{-4}, and 10^{-3}M and MB^+ alone (10^{-5}M), and NaCl △ at 0, 1×10^{-4}, 1×10^{-3}, 5×10^{-3}, 1×10^{-2}, and $2{\cdot}5 \times 10^{-2}$M

DISCUSSION

It is now well established that the extremely rapid reaction of e^-_{aq} with MB^+ is considerably decreased when a polyanion is also present.[11-15] This effect is again illustrated here where heparin is the added polyanion. The pseudo-first-order rate constant for reaction of e^-_{aq} with MB^+ (10 μM) decreases from 26×10^4 to $3{\cdot}5 \times 10^4$ s^{-1} when heparin (100 μequiv. l^{-1}) is added. Heparin reacts extremely slowly with e^-_{aq}[18] ($k_2 = 2{\cdot}2 \times 10^7$ l mol^{-1} s^{-1}) and the residual value of k_1 is within experimental limits identical with that obtained for the water and heparin solutions (100 μequiv. l^{-1}) used for these experiments. Addition of inorganic salts destroys the interaction between polyanions and cationic systems which may be dyes,[3,7,13-15] detergents,[19] proteins,[20-23] or polyamino-acids.[21,22] Addition of Li^+,

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Na^+, Cs^+, NH_4^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} in increasing concentrations to the heparin–MB^+ complex liberates MB^+ in differing efficiencies as indicated by increased reactivity of e^-_{aq} observed in pulse radiolysis and the increase in the visible absorption using spectral measurements. Furthermore, Pal[7] has concluded that destruction of such complexes is not only affected by variations in the ionic strength of the system. Divalent ions are more efficient than the monovalent ions and furthermore, the l.s.c. for the divalent ions decreases as the ionic radius of the aqueous ion decreases. Though this trend has been previously observed for destruction of polyanion–detergent complexes[23] no such correlation has been made for the systems examined here. Furthermore, the l.s.c. for the divalent metal ions decreases in the order $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$, which is in good agreement with the binding affinities of these ions for glycosaminoglycans.[24] This relationship indicates that replacement of MB^+ by the metal ion occurs by a non-specific electrostatic binding rather than a chelating type where the order should be $Mg^{2+} < Ca^{2+} < Sr^{2+} < Ba^{2+}$.

The l.s.c. determined by spectral measurement of the removal of metachromasia with heparin and MB^+ vary in the same order as for the removal of the interaction given by the pulse radiolysis measurements. Monovalent ions are less effective than divalent ions. However the l.s.c. for metachromasia destruction is always less than for total release of the dye as indicated by pulse radiolysis. A similar observation was made for destruction of a series of polyanion complexes by KCl.[14] Dye binding is apparent, even though metachromasia is totally destroyed, which shows that such bound dye remains in a non-aggregated monomeric form. Fluorescent measurements lead to an identical conclusion.[25] Since residual binding is present at the l.s.c. value indicated by the spectral data, the pulse radiolysis method provides a more accurate measure of the relative binding affinities of the competing inorganic ion.

Bungenberg de Jong[26] pointed out that the reversibility of the charge of sulphate and carboxy-colloids by inorganic cations varies with the nature of the cation. Increasing the valency of the cation decreases the reversal of charge concentration. The effect on charge reversal also parallels our order of complex destruction, namely $Li^+ > Na^+ > K^+$, $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$. Polarisability effects have been considered to account for the observed effects.[26] The polarizability of anionic groups decreases in the order $SO_4^{2-} < H_2O < PO_3^{2-} < CO_2^-$, and consequently, PO_3^{2-} and CO_2^- tend to displace water of hydration from the cation. Since heparin contains five SO_4^{2-} groups per tetrameric repeating unit, which do not displace water of hydration from the cation, the dominant influence appears to be ionic size, as might be anticipated if electrostatic forces controlled the interaction. Pauley[27] showed that such electrostatic forces control the order of binding to polystyrene sulphonate, with ion binding determined mainly by the size of the hydrated ions. The smallest ion is bound most strongly, and the strength of binding is thus the reverse of the size order. From the data of Stokes and Robinson[28] and Kielland[29] the size order for the hydrated ions of the alkali metals is $Li^+ > Na^+ > K^+ > (NH_4^+) > Rb^+ > Cs^+$ and that of the alkaline earths $Mg^{2+} > Ca^{2+} > Sr^{2+} > Ba^{2+}$. Apart from NH_4^+, which Kielland[29] places between K^+ and Rb^+ in size, our results in the Table show the predicted correlation between size and ability to break the complex with MB^+ (l.s.c.). This correlation between hydrated ion size and l.s.c. using the more significant pulse radiolysis data is shown in Figure 6 using the values of Kielland.[29] No value is given for NH_4^+, which is, therefore, not included. The relationship is reasonably linear since the inorganic ion replaces MB^+ in the complex, and it is the smallest ion which is bound the most strongly (lowest l.s.c.) as for polystyrene sulphonate. The hydrated magnesium ion is larger than the hydrated lithium ion, but the presence of an additional charge can more than compensate and leads to stronger binding to the polyanion. It is significant that the slopes of the hydrated ion size against l.s.c. plots for the monovalent and divalent ions are virtually identical; the slope of the monovalent ion relationship is $1{\cdot}1 \times 10^{-2}$ and $1{\cdot}2 \times 10^{-2}$ for the divalent ions in units of 1 nm mol^{-1}. Thus, although the added valency confers a greater binding affinity, within ions of the same valency, the variation in size exerts virtually an identical influence on binding strength, increasing as the ion becomes smaller.

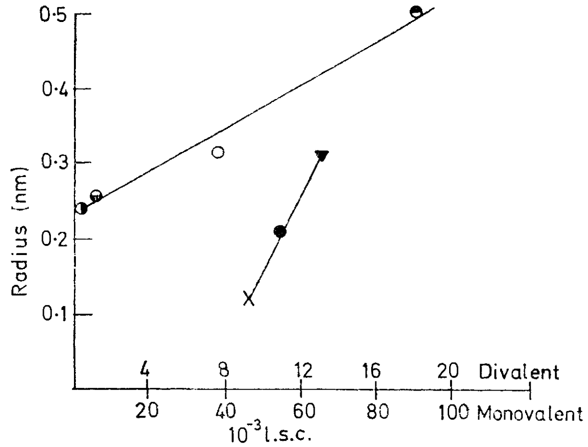

FIGURE 6 Relationship between ionic radii of metal ions and limiting salt concentration of heparin–MB^+ complex obtained by pulse radiolysis. The symbols are those given in Figure 2

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