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Dependence Upon the Gel-Sol State of the Ion-Exchange Properties of Alginates

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The ion exchange reaction involving calcium and magnesium ions was investigated for an alginate fragment containing 90 % L-guluronic acid residues. An increase in the selectivity coefficient, k_{Mg}^{Ca}, with increasing equivalent fraction of calcium, X_{Ca}, in the fragment up to $X_{Ca}=0.4$ was observed. This increase was found to be associated with an increasing amount of the alginate fragment forming a precipitate with a much higher k_{Mg}^{Ca} than the soluble fraction. Results of experiments with crosslinked fragments, with fragments contained in an agarose gel, and with fragments crosslinked to a large excess of dextran, showed that the selectivity increased with increasing possibility of the fragment forming interchain linkages. The comparison of results of fractionation of the Ca-Mg-polyguluronates with a theoretical model for ion-binding; involving near-neighbour auto-cooperative effects, suggested that calcium ions were selectively bound in long sequences inbetween polyguluronate chains. The results of experiments with partially carboxyl reduced samples were in agreement with such a model. An observed lack of reversibility in the binding reaction could be explained by assuming that the inter-chain bridges containing calcium ions were kinetically very stable.

Previously we have examined the correlation between the uronic acid composition of alginates and their ion-exchange properties.[1] The selectivity coefficients describing the exchange-reactions between the alkaline-earth ions could generally be expressed in terms of two selectivity coefficients, one for the D-mannuronic acid residues and one for the L-guluronic acid residues. However, in exchange between calcium and magnesium ions we found, for alginate fragments of low molecular weight ($P_n=20$ and 60), a deviation from this rule when the proportion of calcium ions bound to the polymer was low. In these cases, the alginate fragments were partly soluble, and the observed selectivity coefficients were very much lower than expected. This observation suggested that the selectivity of alginates depended upon whether the alginate was in the sol or the gel state. In the present work, this phenomenon is investigated further, using a fragment of alginate rich in L-guluronic acid residues as material.

EXPERIMENTAL

Materials. A fragment of alginate rich in guluronic acid (*ca.* 90 %) was prepared as described previously.[2]

Partially carboxyl-reduced, guluronic acid rich fragments were prepared as described by Painter and Larsen for alginate.[3] Two samples, of equivalent weights 420 and 650, corresponding to 27 and 42 % reduction of the carboxyl groups, respectively, were prepared.

The fragment of alginate was cross-linked as follows: The material (3 g with $DP_n = 47$ and $M/G = 0.11$) was mixed into a paste with epichlorohydrin (10 ml) and 20 ml 40 % aqueous NaOH was slowly added with continuous mixing (pestle and mortar) over 15 min. After 1 h an additional 10 ml of epichlorohydrin and 20 ml 40 % NaOH was added as before. This addition was repeated twice at intervals of 10 h. Two portions (10 ml) of epichlorohydrin were then added after another 6 and 16 h, respectively. The product was washed with 50 % aqueous ethanol, then several times with water, then with ethanol and ether, and dried. The equivalent weight of the product was 400, suggesting that the sample contained some homopolymerised epichlorohydrin.

The fragment was crosslinked to dextran by dissolving 3 g of the material ($DP_n = 80$ and $M/G = 0.11$) together with 7 g Dextran 2000 (Pharmacia AB, Uppsala, Sweden) in water and precipitating with a large excess of ethanol. The precipitate was washed 3 times with ethanol and once with epichlorohydrin. Epichlorohydrin (30 ml) was mixed into the swollen precipitate and 40 % NaOH (5 ml) was slowly added. Addition of 5 ml of NaOH was repeated 3 times after 3, 13, and 21 h, respectively. The reaction was then allowed to proceed for another 10 h and the product was washed and dried as before. Potentiometric titration indicated an equivalent weight of 3300, corresponding to 6 % uronic acid in the material.

The agarose was supplied by Litex, Copenhagen, Denmark.

Methods. The ion-exchange experiments were carried out at 20°C by the dialysis technique described previously.[1] The dialysis bag always contained 2 ml of water and 0.1 mequiv. of polyanion. The insoluble materials were weighed directly into the bag. In the experiments in which the alginate fragments were dispersed in an agarose gel, control experiments with the same amount of pure agarose were carried out. The agarose contained a small amount of charged groups, corresponding to approx. 2.5 % of the sugar residues having a negative charge. The content of the dialysis bag was 1 % and 2 % with respect to alginate fragments and agarose, respectively. The agarose had a very low selectivity in the Ca-Mg-exchange and, in order to calculate the selectivity coefficient of the alginate fragment, the amounts of Ca and Mg found in the control experiment with pure agarose were subtracted from the total amount found in the experiment with alginate fragments present. During the experiments it was observed that, whereas the 2 % agarose yielded a clear, firm gel, the alginate fragment containing agarose had an appearance more like a turbid, swollen precipitate.

RESULTS

The selectivity coefficients (k_{Mg}^{Ca}) of the exchange reaction $Ca^{2+} - Mg^{2+}$ were determined by equilibrium dialysis as described previously. The results are given in Fig. 1 as a function of the equivalent fraction (X_{Ca}) of calcium in the polymer phase and demonstrate a marked drop in k_{Mg}^{Ca} as the value of X_{Ca} decreases, in the range of X_{Ca}-values lower than 0.4. As X_{Ca} approaches zero, k_{Mg}^{Ca} approaches a value of approximately 7.

For values of X_{Ca} below 0.4, a significant amount of the alginate fragment is soluble. The soluble and insoluble phases were separated by centrifugation and the ionic composition of each was examined separately. The selectivity coefficients of the soluble and the insoluble phases can, thus, be calculated separately, and the results of two sets of experiments are shown in Table 1.

Table 1. Fractionation of a guluronic-acid rich fragment, $\overline{DP_n} = 50$.

Salt solution M Mg^{2+}	Salt solution M Ca^{2+}	Insoluble fraction % of total	Insoluble fraction X_{Ca}	Insoluble fraction k_{Mg}^{Ca}	Soluble fraction X_{Ca}	Soluble fraction k_{Mg}^{Ca}	Total X_{Ca}	Total k_{Mg}^{Ca}	k_{Mg}^{Ca} Fig. 1
0.2	0.0005	4.6			0.017	7.0	0.019	7.6	7.6
0.2	0.001	4.6			0.033	6.9	0.036	7.4	8.5
0.2	0.002	41.5	0.34	51.2	0.067	7.2	0.18	22.6	22.0
0.2	0.003	82.7	0.41	46.6	0.065	4.7	0.35	36.0	43.0
0.2	0.004	90.7	0.45	41.4			0.42	35.5	45.0
0.2	0.01	94.5	0.65	37.1			0.62	32.0	34.0
0.2	0.001	2.8			0.036	7.5	0.045	9.4	9.0
0.2	0.0015	5.8	0.36	74.0	0.061	8.6	0.078	11.3	11.5
0.2	0.002	40.3	0.38	60.0	0.073	7.9	0.195	24.2	24.0

The selectivity coefficients of the total alginate fragment given in the table were calculated from the results obtained for the soluble and insoluble fractions, and the relative amounts of the two fractions. For comparison, the values of k_{Mg}^{Ca} taken from Fig. 1 are included in the table. In some cases the amounts of one of the fractions were too small to allow the determination of k_{Mg}^{Ca} with sufficient accuracy. The results were, however, used in the calculation of the selectivity coefficients of the total alginate fragment.

The results demonstrate that the selectivity coefficients of the soluble and the insoluble phases are remarkable different and that the variation in the selectivity coefficient of the total alginate fragment in the region of low X_{Ca} values is due to the variation in the relative amounts of the soluble and insoluble phases. The selectivity coefficient of the soluble phase is very similar to the value of k_{Mg}^{Ca} found by extrapolation to $X_{Ca} = 0$ in Fig. 1.

Experiments were carried out to show whether the soluble part of the alginate fragment behaved differently from the total alginate fragment under identical conditions. After separation by centrifugation, the soluble phase was concentrated by evaporation to the same concentration of alginate as in the first dialysis (1.0 %). The procedure was repeated and the amount of soluble and insoluble alginate determined. The results are given in Table 2, and show that the soluble phase behaves as the unfractionated sample when dialysed under the same conditions.

Table 2. Solution (1 %) of guluronic acid rich alginate fragment, $\overline{DP_n} = 50$, dialysed against (0.2 M $MgCl_2$ + 0.0015 M $CaCl_2$). Soluble phase concentrated to 1 %, dialysis repeated.

	% insoluble
1. dialysis	18.3
2. dialysis	20.4

The results given above indicate that the selectivity for calcium in the $Ca^{2+} - Mg^{2+}$ ion-exchange reaction depends upon the physical state of the alginate, and that alginate in the gel state has a much higher selectivity coefficient than soluble alginate.

In the gel state, the calcium ions are supposed to form bridges between the alginate molecules,[4] while few such bridges can exist when the alginate is in solution. It is a reasonable assumption that the difference in selectivity of the gel and the sol phase is caused by a higher selectivity when the divalent cations are bound to two different alginate chains. Changing the conditions in a way which changes the probability of calcium ions forming inter-chain linkages should, therefore, be expected to influence the selectivity coefficient of alginate.

Table 3. Dialysis experiments with varying concentration of alginate fragments ($\overline{DP_n} = 50$) against (0.2 M $MgCl_2$ + 0.002 M $CaCl_2$).

Concentration of alginate fragment, %	X_{Ca}	k_{Mg}^{Ca}	% insoluble
0.2	0.106	11.9	46
0.5	0.175	21.1	78.5
1.0	0.22	28.3	79.5
2.0	0.23	29.7	86

Table 3 gives the results of experiments with varying alginate concentrations in the dialysis bag. The proportion of the material rendered insoluble by dialysis against the salt solution increases with increasing alginate concentration, and the observed selectivity coefficient also increases.

Cross-linking the alginate molecules, *e.g.* by treatment with epichlorhydrin, prevents the alginate from passing into solution, and might, thus, be expected to lead to an increase in k_{Mg}^{Ca} for low values of X_{Ca}. Treating the alginate fragment with a cross-linking reagent in the presence of a large excess of a neutral polysaccharide like dextran, might be expected to give a cross-linked product in which the alginate molecules are prevented from getting in sufficiently close contact to allow the formation of inter-chain, calcium-ion bridges. The ion-binding properties of cross-linked samples of these two types are shown in Fig. 2. Another method of preventing the formation of inter-chain bridges is to dissolve the alginate fragment in a solution of agarose and let it gel. The ion-exchange experiments were carried out with the agarose gel containing dissolved alginate. The results are shown in Fig. 2.

The results of the experiments with the two latter types of preparation, in which the formation of inter-chain bridges was prevented, showed a lower value of k_{Mg}^{Ca} than that corresponding to Fig. 1 for a given X_{Ca}. As expected, k_{Mg}^{Ca} of these samples approached the same value as the untreated alginate fragment as X_{Ca} approached zero. The cross-linked alginate fragment, on the other hand, showed a significantly higher value at low X_{Ca} than the untreated sample. At higher values of X_{Ca}, however, also this sample showed somewhat lower values of k_{Mg}^{Ca} than the untreated fragments.

The effect of a partial reduction of the carboxyl groups to primary alcohol groups on the selectivity was also investigated. In Fig. 3 the selectivities of

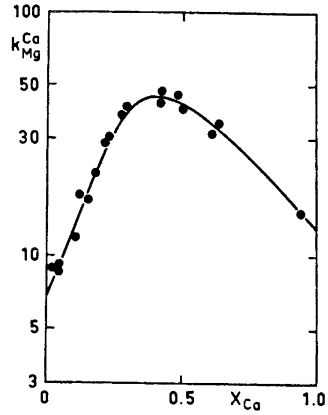

Fig. 1. Selectivity coefficients, k_{Mg}^{Ca}, for a guluronic acid rich fragment, $\overline{DP_n} = 50$, as a function of the equivalent fraction, X_{Ca}, of calcium in the fragment.

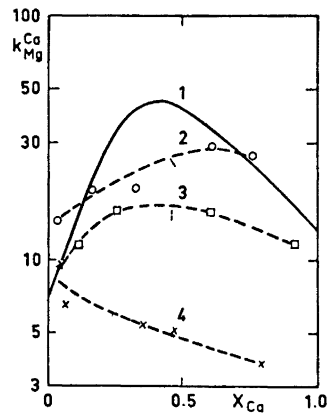

Fig. 2. Selectivity coefficients, k_{Mg}^{Ca}, as a function of the equivalent fraction, X_{Ca}, of calcium on the polyelectrolyte. Curve 1: Unmodified alginate fragment (from Fig. 1). Curve 2: Crosslinked alginate fragment. Curve 3: Alginate fragment crosslinked to dextran. Curve 4: Alginate fragment, $\overline{DP_n} = 80$, in 2 % agarose gel.

two samples, in which 27 and 42 % of carboxyl groups are reduced, are shown as a function of X_{Ca}. The results demonstrate that the reduction leads to a very marked decrease of selectivity. The value of k_{Mg}^{Ca} corresponding to $X_{Ca} = 0$ is, however, only slightly lower than for the untreated alginate fragments. The results indicate that partial reduction decreases the tendency for the formation of inter-chain calcium-ion bridges, but has only a small effect on the ion-binding properties of the isolated alginate molecules.

The transition between the sol and the gel state of a polymer is often associated with a large hysteresis, *e.g.* the melting and setting of an agar gel. If a similar lack of reversibility is the case in the transition between the sol and the gel state of calcium-magnesium alginate, it should be expected that the selectivity coefficient observed for the whole alginate will depend upon whether the alginate is in the form of an insoluble calcium alginate or a soluble sodium (or magnesium) alginate at the start of the dialysis against the salt solution. Fig. 4 gives the selectivity coefficients observed when the alginate fragment was dialysed against a 0.1 M calcium chloride solution prior to the dialysis against the calcium chloride-magnesium chloride solution. In neither case did a significant amount of alginate pass into solution. For values of X_{Ca} above 0.5, the selectivity coefficients obtained in this way are identical with those obtained by the ordinary procedure. For $X_{Ca} < 0.5$, much higher selectivity coefficients are obtained when calcium alginate gels are used as the starting material. Care was taken to obtain a practical equilibrium, and the same selectivity coefficients were obtained after 5, 10, and 15 times 24 h of dialysis against the calcium chloride-magnesium chloride solution.

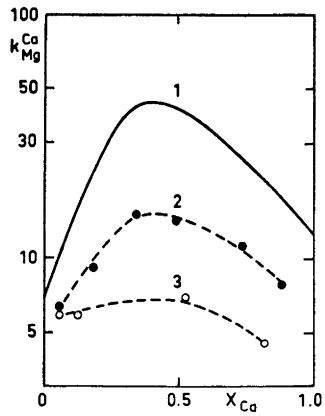

Fig. 3. Selectivity coefficient, k_{Mg}^{Ca}, as a function of the equivalent fraction, X_{Ca}, of calcium on the polyelectrolyte. Curve 1: Unmodified alginate fragment (from Fig. 1). Curve 2: Alginate fragment, $\overline{DP_n} = 80$, with 27 % of the carboxyl groups reduced. Curve 3: Same fragment with 42 % of the carboxyl groups reduced.

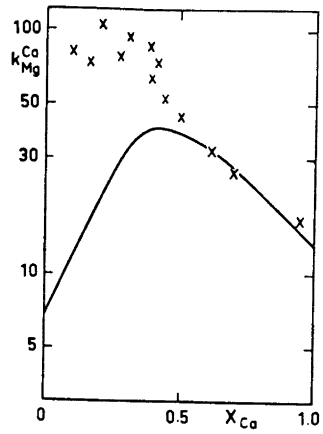

Fig. 4. Selectivity coefficient, k_{Mg}^{Ca}, as a function of the equivalent fraction, X_{Ca}, of calcium on the polyelectrolyte. Fully drawn line: Data from Fig. 1. ×: The alginate fragment converted into the calcium form before the dialysis experiment.

DISCUSSION

The main result of the experiments described above is the demonstration that alginate in solution has a significantly lower selectivity for calcium in the $Ca^{2+} - Mg^{2+}$ ion-exchange reaction than has alginate in the gel form. It is significant, however, that also in solution the guluronic-rich alginate fragment has a selectivity coefficient, k_{Mg}^{Ca}, as high as 7. We have previously [5] compared the selectivities of a number of anionic polymers and found that polyguluronic acid (including alginates containing a significant amount of guluronic acid residues) and pectate were the only polyanions investigated having a selectivity coefficient in the $Ca^{2+} - Mg^{2+}$ ion exchange reaction significantly different from unity. A remarkably high selectivity for calcium compared to magnesium is, thus, a characteristic feature of the soluble polyguluronate molecules, and not solely a property of the gel phase. It is interesting to note that the activity coefficient of calcium in dilute solutions of calcium polyguluronate is also very low [6] compared to solutions of polymannuronate, which is devoid of any selectivity in the calcium-magnesium exchange reaction.

The formation of a gel or a gel-like precipitate when calcium ions are added to a solution of sodium alginate is supposed to be due to the formation of calcium-ion bridges between the alginate chain molecules.[1] A similar gel-formation does not occur upon addition of magnesium ions. The increased selectivity for calcium ions when the alginate forms a gel may indicate that the condition in which calcium ions are bound to two different alginate molecules is energetically more favourable than that in which they are bound to isolated

alginate molecules. Magnesium ions probably have a much smaller tendency to form energetically-favourable, inter-chain linkages.

The results given above allow us to draw some conclusions about the nature of these inter-chain, calcium-ion linkages. According to Ling,[7] an increase in the selectivity coefficient, k_{Mg}^{Ca}, with increasing values of X_{Ca}, as observed here for low values of X_{Ca}, is strong indication of auto-cooperative effects being involved in the binding of calcium ions. This is strongly supported by the fractionation experiments described in Table 1. The experimental results given in this table, together with some other fractionation results were compared with results of calculations based on models with different distributions of calcium and magnesium ions along the polymer chain. The calculation is described in detail below, and the results are in agreement with an auto-cooperative type of binding, leading to a blockwise distribution of the two ionic species.

These results indicate that sequences of several calcium ions are binding the alginate chain together.

The results of the experiments with partially reduced samples are in agreement with such a model. The high selectivity for calcium, ascribed to the formation of auto-cooperative, inter-chain, calcium-ion linkages, is virtually absent in a sample in which 42 % of the carboxyl groups are reduced. If the increased selectivity could be caused by inter-chain linkages formed between isolated uronic acid residues in the alginate molecules, a sample with 58 % of the uronic acid residues intact should still exhibit a high selectivity. If several contiguous uronic acid residues are needed for the formation of a structure leading to increased calcium selectivity, a complete destruction of this selectivity might be expected by a reduction of 42 % of the residues.

The values of k_{Mg}^{Ca} obtained after extrapolation to $X_{Ca}=0$ for the partially reduced samples were only slightly lower than that of the untreated sample. By assuming a random distribution of the reduced groups, only 33.5 % of the remaining uronic acid residues would be expected to have another uronic acid residue as a nearest neighbour. Following the same reasoning as above, this indicates that the calcium selectivity of isolated alginate molecules depends only to a very small extent upon whether the uronic acid residues have a neutral sugar residue or another uronic acid residue as a nearest neighbour. In contrast to this insensitivity to chemical modification of some of the carboxyl groups, we have previously found [1] that a modification of the hydroxyl groups by acetylation completely removes the calcium selectivity of isolated alginate molecules, reducing the selectivity coefficient, k_{Mg}^{Ca}, to unity. The method we have used for cross-linking alginate molecules involves a modification of the hydroxyl groups, and this may possibly be the cause of the somewhat lower selectivity of the cross-linked sample compared to the untreated alginate fragment at intermediate values of X_{Ca}.

The discussion above leads thus to a model of calcium-magnesium alginate with two different types of binding of calcium ions, one with a selectivity coefficient, k_{Mg}^{Ca}, of approximately 7 and another, in sequences of calcium ions forming inter-chain bridges, with a much higher selectivity coefficient. In the soluble fraction, only binding with a low selectivity coefficient is possible. In the insoluble gel phase, however, both possibilities exist, and the selectivity

coefficients found for the insoluble fraction are thus a function of both selectivity coefficients.

The results in Fig. 4, where the samples were converted into calcium alginate prior to the dialysis against the calcium-magnesium salt solution, clearly illustrate the lack of practical reversibility of the ion-exchange at values of $X_{Ca} < 0.5$. This is most probably due to the auto-cooperatively formed sequences of inter-chain calcium-ion linkages, which, once formed, may be expected to be kinetically very stable. The amount of such linkages, at a given value of X_{Ca}, may therefore depend upon the previous history of the sample, leading to varying selectivity coefficients observed for the whole sample, as illustrated in Fig. 4. The low reproducibility when calcium alginate was used as the starting material (Fig. 4) is also most probably caused by variation in the relative amounts of the two types of binding sites.

The model of calcium binding of alginate emerging from this study is considerably more complicated than that reported previously,[1] where the calcium-magnesium selectivity of the guluronic acid residues was supposed to be described by one selectivity coefficient only. For values of X_{Ca} of 0.5 or higher, this gives a satisfactory description of calcium-magnesium selectivity of alginate, but for low values of X_{Ca}, it is necessary to take into account that at least two different types of binding to guluronic-acid residues are possible, and that the observed, average selectivity coefficient may depend upon the mode of preparation of the calcium-magnesium alginate.

APPENDIX

Comparison of the results of fractionation of the Ca-Mg-polyguluronates with a theoretical model for ion-binding, involving near-neighbour auto-cooperative effects.

Ling [8] has treated a simple model of a polyelectrolyte molecule, consisting of a unidimensional chain of uniformly spaced adsorption sites where each site could bind either one of two species. Cooperativeness in the binding process was introduced by considering the free energy of binding to one site to be dependent upon the type of adsorbed species on the immediately adjacent sites. In the present case, the site may be defined either as two neighbouring carboxyl groups on the polyguluronate molecule or as two carboxyl groups from two different molecules lying close to each other. The treatment to be given below contains the same basic assumptions as that of Ling, but is different from his because he related the cooperativity to the adsorption isotherms, whereas we are interested in its relationship to the heterogeneity in the ionic composition of the polymer molecules.

Of the two species, A and B, one of them, B, may occur in the middle of four triplets BBB, ABA, ABB, and BBA, and this may be exchanged for an A according to the following four reactions:

$$\text{I.}\quad \text{BBB}+\text{A} \rightleftharpoons \text{BAB}+\text{B},\ \Delta G_0^{\text{I}}$$
$$\text{II.}\quad \text{ABA}+\text{A} \rightleftharpoons \text{AAA}+\text{B},\ \Delta G_0^{\text{II}}$$
$$\text{III.}\quad \text{ABB}+\text{A} \rightleftharpoons \text{AAB}+\text{B},\ \Delta G_0^{\text{III}}$$
$$\text{IV.}\quad \text{BBA}+\text{A} \rightleftharpoons \text{BAA}+\text{B},\ \Delta G_0^{\text{IV}}$$

By assuming, as did Ling, that the energy of interaction between A and B is the same in an AB-pair as in a BA-pair, the free energies of reactions III and IV are the same, and the sum of these energies is equal to the energy of reaction II. It is therefore necessary to consider reactions I and II, only. By subtracting reaction I from II we get

$$\text{V. ABA} + \text{BAB} \rightleftharpoons \text{BBB} + \text{AAA}, \quad \Delta G_0^{\text{II}} - \Delta G_0^{\text{I}} = 4\Delta G_{0\text{P}}$$

Here no net uptake of A or B occurs, and $\Delta G_{0\text{P}}$ is the gain in free energy when one mole of AB-pairs is converted into $\frac{1}{2}(\text{AA} + \text{BB})$: it therefore characterizes the distribution of A's and B's along the molecules. We may relate $\Delta G_{0\text{P}}$ to the sequence of A's and B's by introducing the probability of occurrence (p(ABA), p(BAB) *etc.*) of the different types of sequence, and by treating reaction V as an equilibrium reaction.

$$\Delta G_{0\text{P}} = -\tfrac{1}{4} RT \ln K_{\text{P}} \tag{1}$$

$$\Delta G_{0\text{P}} = -\tfrac{1}{4} RT \ln \left[\frac{p(\text{BBB})\, p(\text{AAA})}{p(\text{ABA})\, p(\text{BAB})} \right] \tag{2}$$

By introducing the conditional probabilities, p(A|B), p(A|A) *etc.*, where p(A|B) is the probability of finding an A-species given that the preceding species is a B *etc.*, we may write

$$\Delta G_{0\text{P}} = -\tfrac{1}{4} RT \ln \left[\frac{(p(\text{B})\, p(\text{B}|\text{B})\, p(\text{B}|\text{B}))\,(p(\text{A})\, p(\text{A}|\text{A})\, p(\text{A}|\text{A}))}{(p(\text{A})\, p(\text{B}|\text{A})\, p(\text{A}|\text{B}))\,(p(\text{B})\, p(\text{A}|\text{B})\, p(\text{B}|\text{A}))} \right] \tag{3}$$

Since $p(\text{A}|\text{A}) + p(\text{B}|\text{A}) = 1$ and $p(\text{B}|\text{B}) + p(\text{A}|\text{B}) = 1$ we may simplify this equation by introducing the two ratios

$$a = \frac{p(\text{A}|\text{A})}{p(\text{B}|\text{A})} \text{ and } b = \frac{p(\text{B}|\text{B})}{p(\text{A}|\text{B})}$$

which are commonly used [9–13] to describe the sequence in binary heteropolymers of the "ultimate unit" type. Then we get

$$\Delta G_{0\text{P}} = -\tfrac{1}{2} RT \ln a/b \tag{4}$$

The sequence of the two species must now depend on the product ab. For $ab = 1$ ($\Delta G_{0\text{P}} = 0$) the distribution of A's and B's will be entirely random, (Bernoullian) for $ab > 1$ ($\Delta G_{0\text{P}} < 0$) a "block" type of sequence will be favoured (auto-cooperative binding) and for $ab < 1$ ($\Delta G_{0\text{P}} > 0$) the alternating AB-sequence will be favoured (hetero-cooperative binding). The average sequence length, $\bar{N}$, of blocks of A's or B's is simply [12] $\bar{N}^{\text{A}} = a + 1$ and $\bar{N}^{\text{B}} = b + 1$. The ratio between the equivalent fraction of the two species is [12]

$$\frac{X_{\text{A}}}{X_{\text{B}}} = \frac{p(\text{A})}{p(\text{B})} = \frac{a+1}{b+1} \tag{5}$$

Formulas have been developed [13,14] which relate a and b to the distribution of the two species among chains of given lengths. The composition-distribution may be calculated with the aid of a digital computer,[13] with results such as

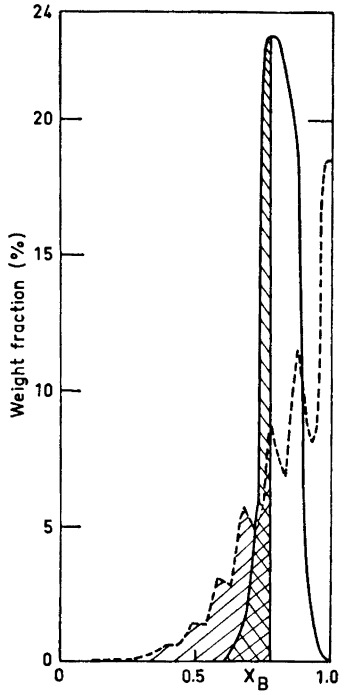

Fig. 5. Theoretical composition distribution for a random and a non-random distribution of A's and B's along the molecule. Both curves: $X_A = 0.180$; $\overline{DP}_n = 50$ with a molecular weight distribution according to the Kuhn formula [15] between DP = 1 and 100. The vertical line at $X_B = (X_B)_{crit.}$ corresponds to 58.5 % of the molecules containing more B's than $(X_B)_{crit.}$. ———: "Random", $a = 0.22$, $b = 4.55$, $ab = 1$. – – –: "Non-random", $a = 5$, $b = 26.3$, $ab = 131.5$.

those in Fig. 5, where the distribution curves for a random and a non-random case are given. One may now carry out a theoretical fractionation by assuming that all molecules containing more than a critical fraction, $(X_A)_{crit.}$, of one species is insoluble, and the rest is soluble. The value of $(X_A)_{crit.}$ is determined by the computer for a given set of a- and b-values, from knowledge of the experimental amount of soluble and insoluble material. The amount of A's in the two fractions, $(X_A)_S$ and $(X_A)_I$, may then be calculated and compared with experimental results.

The difference between $(X_A)_S$ and $(X_A)_I$ will depend not only upon the product ab (Fig. 1), but also upon the degree of polymerization, DP, of the polymer [13] and, to some extent, upon the polydispersity with respect to chain

Table 4. Theoretical fractionation of Ca-Mg-polyguluronate. Experimental values: $\overline{DP}_n = 50$, $X_{Ca} = 0.18$, $(X_{Ca})_I = 0.34$, $(X_{Ca})_S = 0.07$, and 41.5 % insoluble material. The calculation is done with $ab = 1$, and with different assumptions regarding the molecular weight distribution. The value of $(X_{Ca})_{crit.}$ was in all cases computed to be 0.22.

	DP = 1 → 100	DP = 11 → 100	DP = 50 → 50
$(X_{Ca})_I$	0.262	0.248	0.249
$(X_{Ca})_S$	0.130	0.134	0.134

length. The effect of polydispersity may be studied with the aid of programs [13] that calculate the composition-distribution for all chains between any two limiting lengths in a population of molecules obtained by random degradation.

The present polyguluronate fractions were prepared by acid-degradation of alginate and are therefore polydisperse. Knowledge of the exact distribution is lacking, but, in Table 4, three different distributions are treated with $ab = 1$ and with values of a and b obtained from eqn. (5) with $X_A = X_{Ca}$ taken from one experiment. It is assumed in these calculations that the binding site for both calcium and magnesium is two neighbouring carboxyl groups on the polyguluronate molecule.

Table 4 shows that the effect of changing the molecular weight distribution is small. The polyguluronate fragments were prepared by extensive dialysis, and the calculation with DP from 11 to 100 should therefore approximate the real situation most closely. However, since the monodisperse case yielded identical fractionation results, the rest of the calculation were, for the sake of saving computer time, performed assuming that the polyguluronates are monodisperse.

It is clear from Table 4 that a random distribution of Ca- and Mg-ions along the molecule cannot explain the large difference between $(X_{Ca})_S$ and $(X_{Ca})_I$ obtained experimentally. In Table 5 the theoretical results obtained

Table 5. Theoretical fractionation of Ca-Mg-polyguluronates. The calculation is performed in connection with the same experiment as that in Table 4.

ab	1	8.1	25.3	51.5	131.5	249	490
$\overline{N^{Ca}}$	1.22	2	3	4	6	8	11
$\overline{N^{Mg}}$	5.55	9.1	13.6	18.2	27.3	36.4	50
$(X_{Ca})_I$	0.230	0.258	0.280	0.300	0.335	0.353	0.377
$(X_{Ca})_S$	0.142	0.124	0.108	0.095	0.074	0.057	0.040

with a series of ab-values are given. In this case it is assumed that all the molecules exist as dimers and that the binding site is two carboxyl groups from the two parallel chains in the dimer.

Table 6. Comparison of experimentally and theoretically obtained fractionation results. An ab-value of 131.5 is used in all the calculations. The value of $(X_{Ca})_{crit}$ was calculated to be 0.20 ± 0.02 in all cases, based upon the observed amounts of soluble and insoluble material.

	Exp. 1		Exp. 2		Exp. 3	
	Exp. values	Calc. values	Exp. values	Calc. values	Exp. values	Calc. values
$\overline{DP_n}$	80		47		50	
% I	58.3		40.3		82.7	
X_{Ca}	0.27		0.195		0.35	
$(X_{Ca})_I$	0.40	0.372	0.38	0.364	0.412	0.416
$(X_{Ca})_S$	0.0875	0.129	0.073	0.082	0.065	0.064
$\overline{N^{Ca}}$		7.66		6.3		9.2
$\overline{N^{Mg}}$		20.7		25.8		17.1

Table 5 shows that an *ab*-value slightly higher than 131.5 is needed to obtain the experimentally observed $(X_{Ca})^I$- and $(X_{Ca})_S$-values. This suggests that the average number of neighbouring monomer units involved in the binding of calcium, $\overline{N^{Ca}}$, is higher than 6 in this experiment ($X_{Ca} = 0.180$).

In Table 6 are given results from three other fractionation experiments, together with calculated values obtained by putting $ab = 131.5$. It is seen that the experimentally obtained fractionation in each case is equal to or better than the calculated one. An *ab*-value equal to 130 corresponds to a value of ΔG_{0P} of -1.4 kcal/mol at 20°C (eqn. (4)). This means (reaction scheme V) that the exchange of magnesium for calcium is 5.6 kcal/mol more favourable when both near-neighbours are calcium ions (reaction scheme II) than when they are magnesium ions (reaction scheme I). This figure indicates that a sequence of contiguously linked calcium ions has a selectivity coefficient which is about 10^4 times higher than that for a sequence of contiguous magnesium ions.

The actual magnitude of the above figures should be treated with caution. It seems, however, quite clear that a strong auto-cooperative effect must be involved in the binding of calcium ions. For getting a better estimate of the energies involved, one has to consider at least three additional effects which may be of importance in the real situation: (1) That the fractionation process is not ideal in separating all molecules above and below a certain calcium content, (2) that all molecules most probably cannot associate into perfect dimers and that both monomers and oligomers exist in the real situation, (3) that the monomers near to the chain-ends may have properties different from the rest of the monomers.

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