ION BINDING ON POLYURONATES—ALGINATE AND PECTIN

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ABSTRACT

The influence of both the structure and the conformation of polyuronates and the linear charge density of these macromolecules upon the interaction of some divalent cations with their carboxyl groups is discussed. Several presumed mechanisms of cation binding to polyuronates have been mentioned and evaluated from our cited experimental data: determination of activity coefficients of Ca^{2+} and Sr^{2+} counterions in solutions of oligo- and polyuronates, examination of circular dichroism of these substances and ultracentrifugation. In this way it is shown that, in addition to the linear charge density, the conformation of macromolecules and the *inter*molecular binding of divalent cations determine the bond strength of cations to polyuronates and the selectivity in ion exchange reactions on these natural ion exchangers. In addition, the ion exchange properties of crosslinked pectate, the affinity chromatography of pectolytic enzymes on this insoluble support and its biodegradability are presented.

INTRODUCTION

It is generally known that polyuronides (alginates and pectic substances) are natural ion exchangers of outstanding properties. Recently, many experimental data concerning this subject have been collected (for survey of papers see, e.g., references 1–4). These substances play an important role in plant physiology and phytopathology.

The polyuronides and their low molecular fragments are of special interest in human medicine as prophylactic substances and drugs against intoxication by radioactive strontium and heavy metals. As early as 1825 Braconnot suggested that pectic substances might be good antidotes for heavy metal poisoning because of the insolubility of the compounds formed. In connection with this problem a further note can be found in the monograph *The Pectic Substances*, by Z. I. Kertesz[5]: 'Since pectic constituents are a normal part of our diet, one may well wonder to what extent we are all indebted to these compounds for health or at least for being alive'. Many scientific institutes, among which at least those in Canada. the Soviet Union, Great Britain, the USA and Yugoslavia should be mentioned, successfully investigated in detail the clinical application of alginates and pectic substances.

Some several hundreds of papers refer to all those problems. My contribution does not aim to survey these investigations or point out results of

clinical examination but primarily to throw more light on the mechanism of binding of cations to polyuronides. This mechanism, as will be shown later, is different from that of ion binding to classical polyelectrolytes.

STRUCTURE OF POLYURONIDES AND ION BINDING

Alginates are composed of D-mannuronic acid and L-guluronic acid units, which are present in the linear macromolecule in homopolymeric blocks of each monomer, together with blocks of the alternating sequence[6–8].

For many mono- and divalent cations the selectivity of ion exchange is closely related to the uronic acid composition of alginates. In most cases the higher the content of L-guluronic acid units in the alginate, the higher also the selectivity in ion exchange reactions[1, 9–12], as shown, e.g., in *Figure 1*, for the exchange of Ca^{2+} and K^+ ions[11].

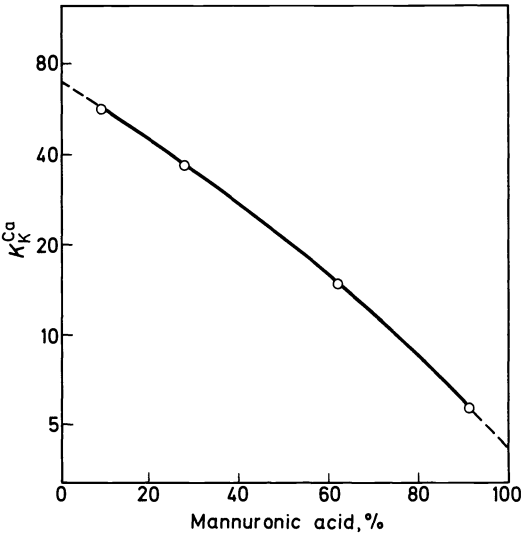


Figure 1. Selectivity coefficients K_K^{Ca} of alginates with varying content of mannuronic and guluronic acids[11].

The basic skeleton of the pectin molecule is the polygalacturonic acid, the carboxyl groups being partially esterified with methanol. In addition, a small amount of neutral sugar units, mainly L-rhamnose and D-galactose, is bound in the molecule. Since the degree of esterification (E) of pectin determines the linear charge density of the macromolecule, it is the most important factor influencing the ion binding properties of pectin.

Figure 2 shows the stability constant K of calcium pectinates in dependence on the degree of esterification E (reference 13). The concentration of free Ca^{2+} was determined by means of Raaflaub's metal-indicator method, using tetramethylmurexide as an auxiliary ligand[14, 15]. With decreasing degree of esterification, i.e. with increasing linear charge density of the pectin molecule, the stability constant K strongly increases in a function close to a

logarithmic relationship. This function is of general validity regardless of the origin of pectin (apple, wild apple, citrus, sunflower, sugar beet), the polyuronide content in the sample, its molecular weight (29000–109000) and the character of neutral saccharide units bound in the pectin molecule.

Similar conclusions hold for the dependence of the selectivity coefficient K_K^{Ca} on the degree of esterification of pectin (*Figure 3*). The cation concentration in the polyelectrolyte phase (p) and in the solution (s) was expressed uniformly by means of equivalent fractions X. The fully de-esterified pectin ('polygalacturonate') is highly selective for Ca^{2+}, while pectin with a high degree of esterification is no more selective in the Ca^{2+}–K^+ exchange reaction[13].

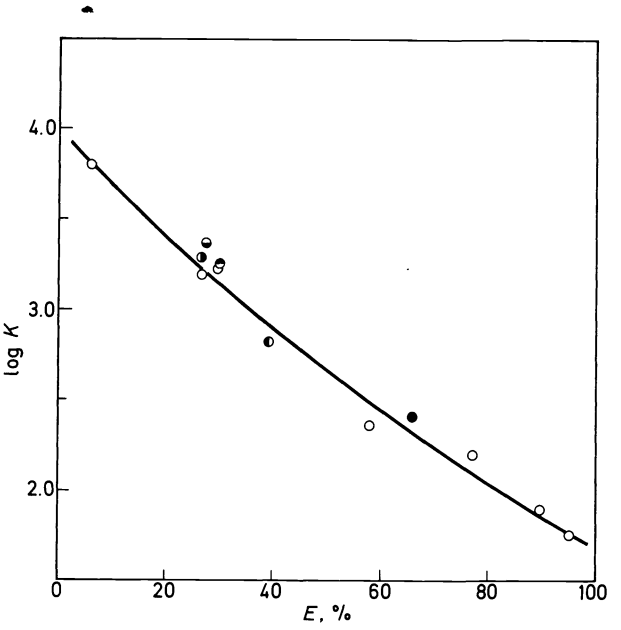


Figure 2. Dependence of stability constant K of calcium pectinate on the degree of esterification E of pectin[13].
Origin of pectin: ○, apple; ◐ wild apple; ◓, ●, citrus; ◑, sunflower; ◒, sugar beet.
[—COOH] = 4.0 mequiv./l; ionic strength I = 0.02 (KCl).

Multiple equilibria law. The interaction of Ca^{2+} with free carboxyl groups of pectin in solutions of pectinates can well be expressed by the multiple equilibria law. According to it the following equation can be applied[16]:

$$1/r = 1/\{nK[\mathrm{Me}]\} + 1/n \qquad (1)$$

where r stands for mol bound cation/total repeating segments, n is the number of binding sites per repeating segment, [Me] is the concentration of free counterions in the solution and K is the intrinsic stability constant. This function is represented by a straight line. If $1/[\mathrm{Me}] = 0$, then $1/r = 1/n$.

It was convenient for the calculation of the stability constant (K) to choose

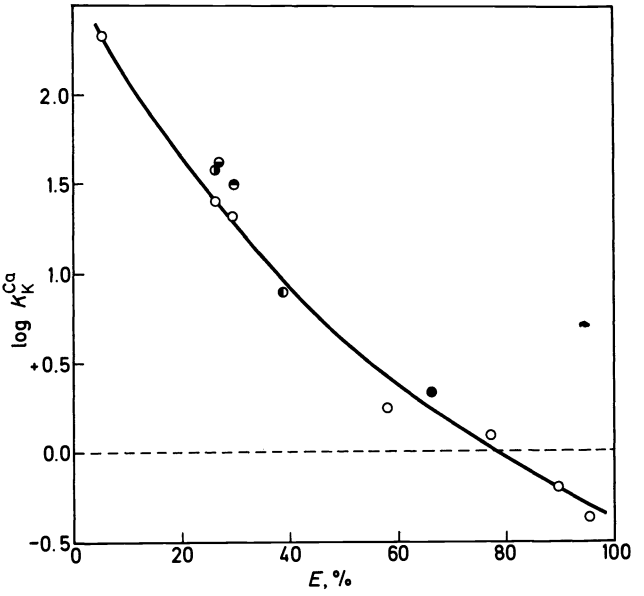


Figure 3. Dependence of selectivity coefficient K_K^{Ca} of Ca^{2+}–K^+ exchange reaction in pectin on its degree of esterification E (reference 13). Origin of pectin: see *Figure 2*;

[—COOH] = 4.0 mequiv./l; ionic strength I = 0.02 (KCl)

$$K_K^{Ca} = X_{Ca}^{p}(X_K^{s})^2/X_{Ca}^{s}(X_K^{p})^2$$

as ligand unit a segment of the macromolecule which contained two free carboxyl groups binding just one divalent cation (complex 1:1); then $n = 1$. The course of the function $1/r = f(1/[Ca^{2+}])$ for calcium pectinates with various degrees of esterification[13, 17] is shown in *Figure 4*. Both the linear course of the function and the experimentally confirmed value of $n = 1$ indicate that in solutions of calcium pectinates the interaction of Ca^{2+} with free carboxyl groups coincides exactly with the multiple equilibria law. If, however, the interaction of Ca^{2+} with carboxyl groups of pectin results in a formation of gel (e.g. calcium pectate), then a deviation from the multiple equilibria law takes place and the stability 'constant' K is no longer constant[3] but undergoes alteration with the molar fraction of Ca^{2+} bound in the pectin molecule.

Some selectivity coefficients K_K^{Ca} for typical representatives of the polyuronates including polymannuronate, polyguluronate and polygalacturonate were estimated[11] with the following results:

	K_K^{Ca}
Polymannuronate	4.2
Polyguluronate	70.8
Polygalacturonate	67.8

A remarkable difference in affinity of the polymannuronate for calcium ions on the one hand and polyguluronate and polygalacturonate on the

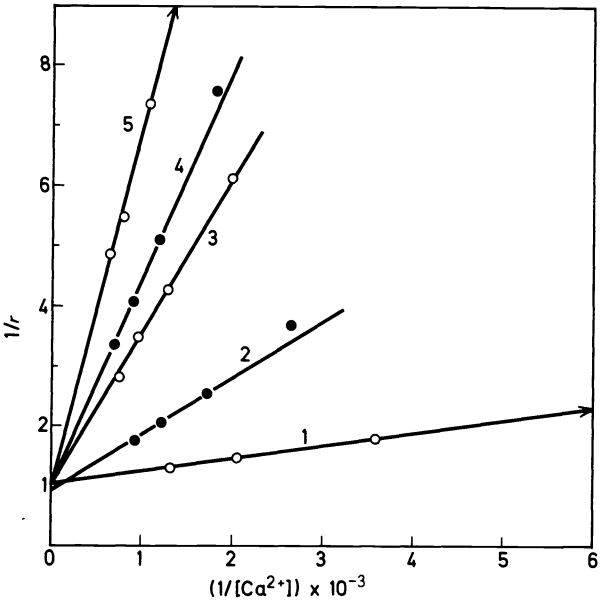


Figure 4. Interaction of Ca^{2+} with free carboxyl groups of pectin in 0.5 M solution of sucrose[17]. (multiple equilibria law). *r*, mol bound cation/total repeating segments; $[Ca^{2+}]$, concentration of free Ca^{2+}-counterions. Degree of esterification *E* per cent: 1, 10.3; 2, 30.8; 3, 45.5; 4, 60.5; 5, 75.0. [—COOH] = 3.0 mequiv./l; ionic strength $I = 0.02$ (KCl).

other was observed. (The alginate fragments with alternating sequence of mannuronic and guluronic acid units exert only a low selectivity in ion exchange reactions[46]. This problem will not be further discussed.) The affinity of the monomers (D-mannuronate, L-guluronate and D-galacturonate) to calcium ions was found to be virtually the same. Single-ion activity coefficients $\gamma_{Ca^{2+}}$ in solutions of these calcium uronates are very close to that of a calcium chloride solution of the same concentration[11]. This result is in good agreement with data of Buddecke and Drzeniek[18], who found very low stability constants for calcium galacturonate and calcium glucuronate when measured in *ca.* 1 M solutions. Gould and Rankin[19] determined the stability constant of the same calcium uronates. The values obtained were also small, but nevertheless varied with the structure of the uronic acid.

Table 1. Activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ in dilute solutions of calcium and strontium salts of D-galacturonic acid and its derivatives (3.0 mequiv. $[-COOMe_{0.5}]$/l) (references 23, 24)

Sample	$\gamma_{Ca^{2+}}$	$\gamma_{Sr^{2+}}$
Calcium (strontium) D-galacturonate	0.730	0.746
Calcium (methyl α-D-galactopyranosid)uronate	0.758	—
Calcium (methyl 4-deoxy-β-L-threo-hex-4-enopyranosid)uronate	0.748	—
Theoretical values according to Debye and Hückel	0.759	0.754

Triffitt[20] found that the monomeric L-guluronic acid had no detectable binding capacity for calcium and strontium.

The activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ were estimated by means of the metal-indicator method[21,22] in dilute solutions of the respective calcium and strontium D-galacturonate and its α-methyl glycoside and 4,5-unsaturated derivative[23,24], and are listed in *Table 1*. The last of them serves as a model for non-reducing terminal units formed by a β-eliminative alkali cleavage of pectin. The $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ values determined experimentally are very close to those calculated according to Debye and Hückel[25] for solutions of strong electrolytes of equal ionic strength. All these results indicate that the ion-binding properties of polyuronates are due to their polymeric nature and that the difference between them must be in some way caused by differences in the steric arrangement of the active groups in the polymer chain.

MECHANISM OF ION BINDING INCLUDING VICINAL HYDROXYL GROUPS OF URONIC ACIDS

The first hypothesis concerning the mechanism according to which divalent cations are bound to polyuronates was suggested by Schweiger[26–28]. The observation that partial acetylation of the hydroxyl groups of a polyanion diminished its capacity to form a gel with calcium ions led Schweiger to propose that the binding mechanism was primarily one of chelation, in which both the vicinal hydroxyl groups of the uronic acid unit were directly involved (*Figure 5*); see also reference 29. The approach of carboxyl groups

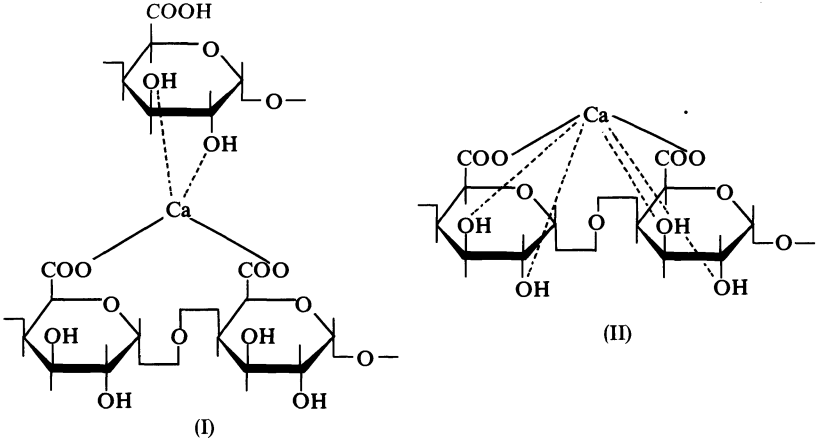


Figure 5. Chelate bond of calcium in calcium pectate[27]. (I), intermolecular bond; (II), intramolecular bond.

to the distance necessary for the formation of such a chelate bond of calcium requires free rotation of the D-galacturonic acid units around the glycosidic bond. Owing to the *trans* diaxial α(1 → 4) glycosidic bonds, the pectin molecule can be considered as a relatively rigid linear chain with restricted flexibility. We have shown on a model of the pectin molecule[30] that the shortest possible distance between dissociated carboxyl groups is about

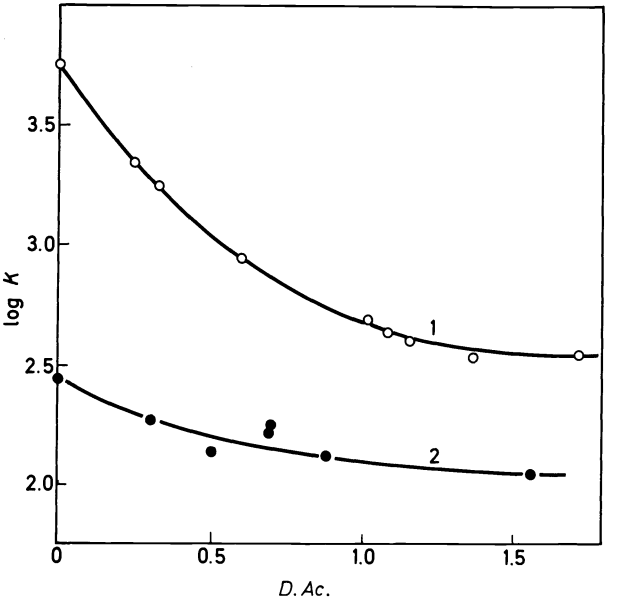


Figure 6. Dependence of the stability constant K of calcium pectate and pectinate on the degree of their acetylation[30]: 1, acetyl derivatives of calcium pectate (E 2%); 2, acetyl derivatives of calcium pectinate (E 58%).

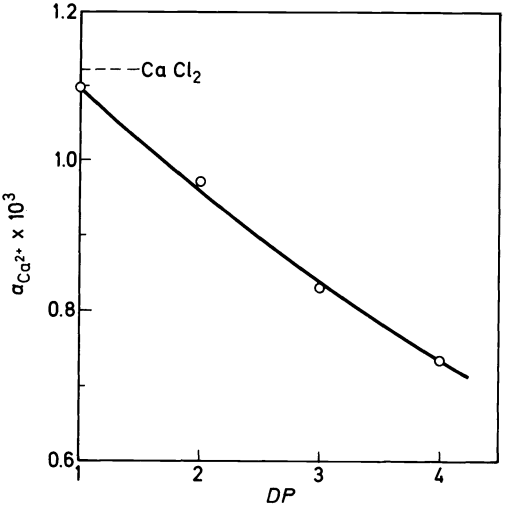


Figure 7. Calcium ion activity in solutions of calcium oligogalacturonates as a function of the degree of polymerization (DP) of the anion[31]. [—$COOCa_{0.5}$] = 3.0 mequiv./l; [Ca] = 1.5 mmol/l; dashed line, $a_{Ca^{2+}}$ in a solution containing 1.5 mmol $CaCl_2$ per litre.

5.5–5.8 Å. This distance is too large for a chelate bond of calcium, considering the binding mechanism of Schweiger.

Our determination of the stability constant K of partially acetylated calcium pectate and pectinate[30] showed that, although acetylation does indeed diminish the affinity of the polyanion for calcium, a rather high affinity remains, even when the degree of acetylation approaches the theoretical maximum of two (*Figure 6*; curve 1). With calcium pectinate ($E = 58$ per cent), where approximately each second unit of uronic acid bears a free carboxyl group, the effect of acetyl groups on the stability of the calcium binding is substantially lower. The stability constant K decreases only slowly with increasing degree of acetylation (curve 2).

We attempted to add further evidence on the mechanism of ion binding by measuring the activity of calcium ions in aqueous solutions of lower calcium oligogalacturonates[31] (*Figure 7*). These experiments were intended to show whether calcium forms a firm complex with still lower oligosaccharides, as is the case with, e.g., calcium citrate[32]. Any chelate involving the functional groups of two consecutive galacturonic acid units must be unstable, since the activity of calcium ions in the calcium digalacturonate is still close to that of calcium monogalacturonate. On the other hand, the steady decrease in the activity of calcium ions with the increasing charge of the anion, due to the cumulation of carboxyl groups along the chain, is fully consistent with the known behaviour of polyelectrolytes. The results were interpreted as evidence that calcium ions in these lower calcium oligogalacturonates are bound first of all by electrostatic attractive forces and that, in calcium pectate, an *intra*molecular chelate bond of calcium involving two consecutive galacturonic acid units is unlikely. The possibility of forming *inter*molecular bonds between calcium ions and uronic acid units belonging to two linear polyuronate macromolecules will be discussed where appropriate.

THE EFFECT OF DEGREE OF POLYMERIZATION OF POLYURONATES ON THE INTERACTION OF Ca^{2+} WITH THEIR CARBOXYL GROUPS

All polyuronates are to some extent similar in their primary structure; in spite of this, great differences in ion exchange properties have been observed. To be able to understand the ion exchange mechanism, further data were needed, especially those referring to the interaction of cations with carboxyl groups of the three characteristic polyuronates (polymannuronate, polyguluronate and polygalacturonate), depending upon the degree of polymerization, from the monomeric uronic acid up to the polyuronate. The isolation of pure oligo- and polyuronate fractions with defined molecular weights was laborious and rather time-consuming. The oligo- and polymannuronates and -guluronates were prepared and characterized by Dr Larsen of the Norwegian Institute of Seaweed Research, NTH, Trondheim; the oligo- and polygalacturonates by my co-worker, Dr Luknár[31, 33, 34].

Alginates with a high content of L-guluronic acid and D-mannuronic acid, respectively, were prepared from the seaweed *Laminaria hyperborea* (stipes) and *Fucus vesiculosus* (receptacles). Since the content of L-guluronic or

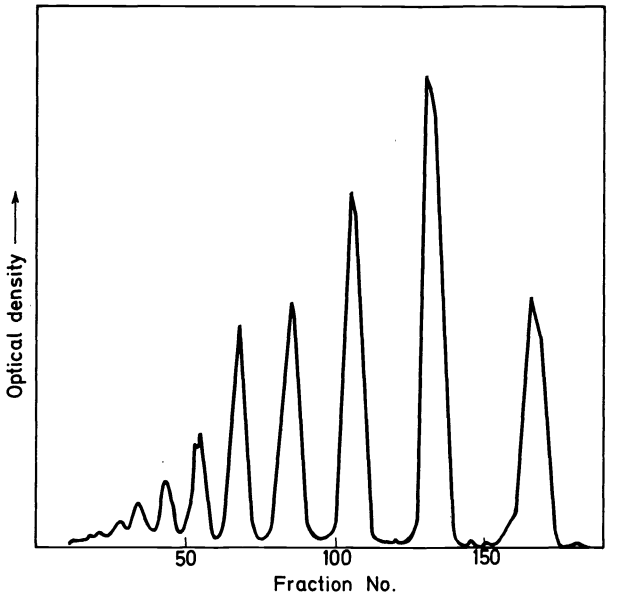


Figure 8. Separation of oligoguluronates on a column of Bio-Gel P4 (reference 33).

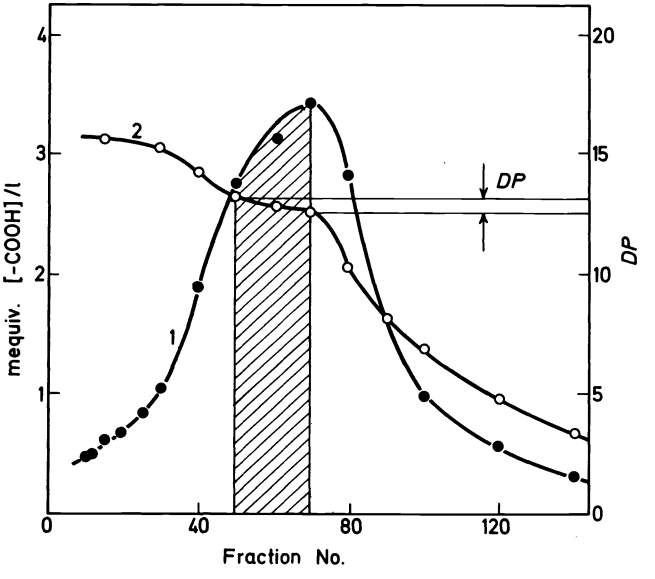


Figure 9. Rechromatography of the polygalacturonate fraction (*DP* 13) on a column of Sephadex G-50 (reference 34): 1, concentration of [—COOH]; 2, degree of polymerization (*DP*).

D-mannuronic acid in these alginate samples was at least 90 per cent the terms 'polyguluronate' and 'polymannuronate' are used below. The pectate ('polygalacturonate') prepared by alkaline de-esterification of apple pectin contained about 91 per cent of D-galacturonic acid.

The oligouronates and low molecular weight polyuronates were prepared by a partial acid hydrolysis of the appropriate polyuronate samples and gel-permeation chromatography on columns of Bio-Gel and Sephadex[33,34]. The oligomers were monodisperse, as is evident from the separation of oligoguluronates[33] on a column of Bio-Gel P4, shown in *Figure 8*. The polymeric fractions were prepared as cut-outs from a molecular distribution obtained by gel-permeation chromatography. The rechromatography of the polygalacturonate fraction ($DP = 13$) illustrated in *Figure 9* demonstrates that in addition the fractions with a higher degree of polymerization are polydisperse only to a very low extent[34].

The most direct way to investigate the interaction between polyanion and counterions in polyelectrolyte solution is to study the activity of counterions. Calcium ion activities and activity coefficients $\gamma_{Ca^{2+}}$ were determined by the spectrophotometric metal-indicator method, mentioned earlier. We have shown[21,22] that this procedure can be applied directly to the determination of single-ion activities of Ca^{2+} and Sr^{2+} in solutions of corresponding salts. Single-ion activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ determined in mixed solutions of $CaCl_2$–KCl and $SrCl_2$–KCl, respectively, were in excellent agreement with the theoretical $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ values calculated according to Debye and Hückel.

Solutions of polyuronic acids of a low concentration prepared by the ion exchange technique were carefully neutralized with a clear solution, of

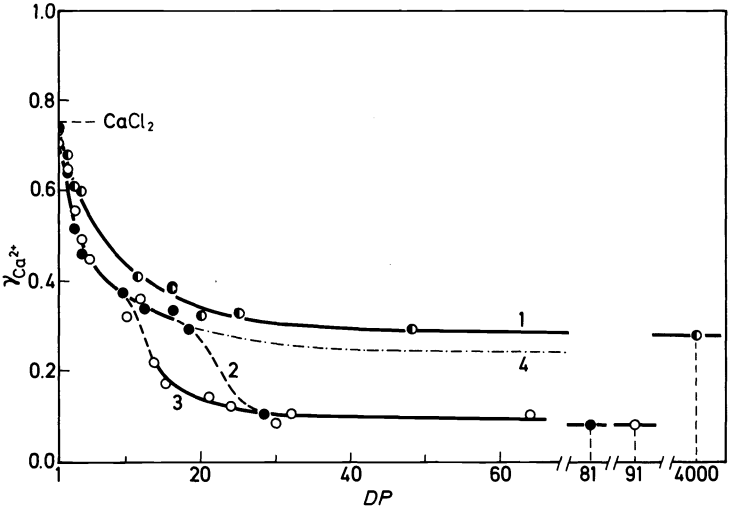


Figure 10. Activity coefficient $\gamma_{Ca^{2+}}$ in solutions of calcium oligo- and polyuronates as a function of the degree of polymerization (DP)[33,34]: 1, mannuronate; 2, guluronate; 3, galacturonate; 4, theoretical $\gamma_{Ca^{2+}}$ values in solutions of calcium polyguluronate and calcium polygalacturonate.

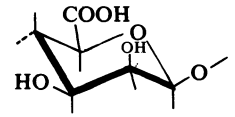
calcium hydroxide. Within the concentration range tested, oligo- and polymannuronates gave soluble calcium salts. The polyguluronates and polygalacturonates, however, gave a partial coagulation during the conversion process. The insoluble part was removed by centrifugation at 13000 *g*. The clear supernatant fluid containing calcium polyuronates in a soluble form was then used for Ca^{2+} activity measurements. The degree of polymerization of polyuronates in the insoluble part and in the solution was found to be the same.

The results of activity [$a_{Ca^{2+}}$] measurements are seen in *Figure 10* (see references 33, 34). The activity coefficient $\gamma_{Ca^{2+}}$ of the monomeric calcium uronates is close to the $\gamma_{Ca^{2+}}$ value in a solution of calcium chloride of the same concentration. For calcium mannuronates (curve 1) there is a steady decrease in the activity coefficient $\gamma_{Ca^{2+}}$ until it becomes practically independent of the chain length at a $DP > 30$. Calcium guluronates, on the other hand, show a much more complicated dependence on the molecular weight (curve 2) by a pronounced drop in the activity coefficient $\gamma_{Ca^{2+}}$ in the *DP* region between 18 and 28. In solutions of calcium oligo- and polygalacturonates the dependence of activity coefficient $\gamma_{Ca^{2+}}$ on the degree of polymerization *DP* similar to that of calcium polyguluronates was found[34] (curve 3). The pronounced drop of the activity coefficient $\gamma_{Ca^{2+}}$ occurs in the region of rather lower *DP* values. These phenomena need to be explained.

THE EFFECT OF LINEAR CHARGE DENSITY OF THE POLYURONIDE MACROMOLECULE ON THE BINDING OF Ca^{2+} AND Sr^{2+}

The interaction between molecules of polyelectrolytes and counterions has been studied and reported in many monographs and review articles[35–39]. This interaction is influenced by the linear charge density of the macromolecule, expressed by the distance between adjacent charged groups in a perpendicular projection on the main axis of the macromolecule. The higher the linear charge density the stronger the interaction of counterions with ionic groups and the lower the activity coefficient of counterions. It seems, therefore, useful to compare the activity coefficients $\gamma_{Ca^{2+}}$ with linear charge densities of the corresponding polyuronates.

Figure 11 shows the structural characterization of typical polyuronides. The polymannuronic acid (III) contains D-mannuronic acid units in the conformation *C*1 linked by diequatorial *trans*-glycosidic bonds $\beta(1 \rightarrow 4)$. The polyguluronic acid (IV) contains L-guluronic acid units in the conformation 1*C* with diaxial *trans*-glycosidic bonds $\alpha(1 \rightarrow 4)$. The D-galacturonic acid units in the molecule of pectic acid (V) have conformation *C*1; they are linked by diaxial *trans*-glycosidic bonds $\alpha(1 \rightarrow 4)$. In oriented gels of calcium polymannuronate a threefold screw symmetry, and in gels of calcium polyguluronate and pectate a twofold screw symmetry, were found. The repeating distance (*b*) referring to one sugar unit of these calcium polyuronates is 5.0, 4.36 and 4.35 Å, respectively. It is evident that calcium polyguluronate and calcium pectate have the same conformation of the macromolecule. This structural characterization of polyuronates is based on x-ray diffraction data[40–44] and p.m.r. measurements[45] (see also reference 46).



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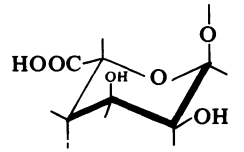

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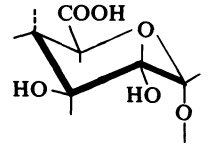


(V)

Figure 11. Structural characterization of typical polyuronides: (III), polymannuronic acid, D-β-(1e → 4e), *C1*; (IV), polyguluronic acid, L-α-(1a → 4a), *1C*; (V), pectic (polygalacturonic) acid, D-α-(1a → 4a), *C1*.

The activity coefficients $\gamma_{Ca^{2+}}$ in solutions of calcium salts of polyacids under investigation are collected in *Table 2* (reference 33). In the solution of calcium polymannuronate a relatively high activity coefficient ($\gamma_{Ca^{2+}} = 0.281$) was found, indicating a weaker interaction of Ca^{2+} with carboxyl

Table 2. Activity coefficient $\gamma_{Ca^{2+}}$ in solutions of calcium polyuronates and calcium polymethacrylates[33]

Polyacid	$\overline{DP}$	[Ca], mmol/l	$\gamma_{Ca^{2+}}$	*b*, Å
Polymannuronic	4000	1.50	0.281	5.0
Polyguluronic	81	0.68–0.73	0.083	4.36
Pectic	91	0.60–0.76	0.063	4.35
Polymethacrylic	390	1.50	0.075	2.5
Polymethacrylic	3200	1.50	0.066	2.5

groups. The activity coefficients $\gamma_{Ca^{2+}}$ in solutions of calcium polyguluronate and calcium pectate on the contrary are several times lower ($\gamma_{Ca^{2+}} = 0.083$ and 0.063, respectively). These findings are in agreement with the ion exchange properties of these polyuronates. The polyguluronate and pectate exert a similar high selectivity for Ca^{2+} in ion exchange reactions of calcium and potassium, while the polymannuronate has only a low selectivity for this cation.

Let us now compare the activity coefficients $\gamma_{Ca^{2+}}$ of calcium polyuronates and calcium polymethacrylate with the linear charge densities of the corresponding macromolecules expressed as a repeating distance of the adjacent carboxyl groups (*b*). At first sight it is evident that the activity coefficients $\gamma_{Ca^{2+}}$ of calcium polyguluronate and calcium pectate are clearly lower than predicted on the basis of linear charge densities compared especially with

calcium polymethacrylate. This demonstrates that the linear charge density is not the only factor controlling the interaction between the polyanion and counterions.

Calcium and strontium pectinates. The charge density of the pectin molecule changes to a large extent with its degree of esterification (E). The dependence of the activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ in solutions of calcium and strontium pectinates on the degree of esterification is shown in *Figure 12* (reference 23). At a high degree of esterification ($E > 90$ per cent) the free carboxyl groups are very distant from each other. Such a so-called '*isolated*' carboxyl group has a similar affinity to divalent cations as has the monomeric galacturonate. With the decreasing degree of esterification, i.e. with the increasing charge density, the activity coefficient decreases smoothly. In the range of degree of esterifIcation $E = 42$–35 per cent a deflection of the curve from the expected course appears; a sudden drop of activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ was found. In the range of degree of esterification E below 20 per cent the activity coefficients $\gamma_{Ca^{2+}}$ and $\gamma_{Sr^{2+}}$ change but little.

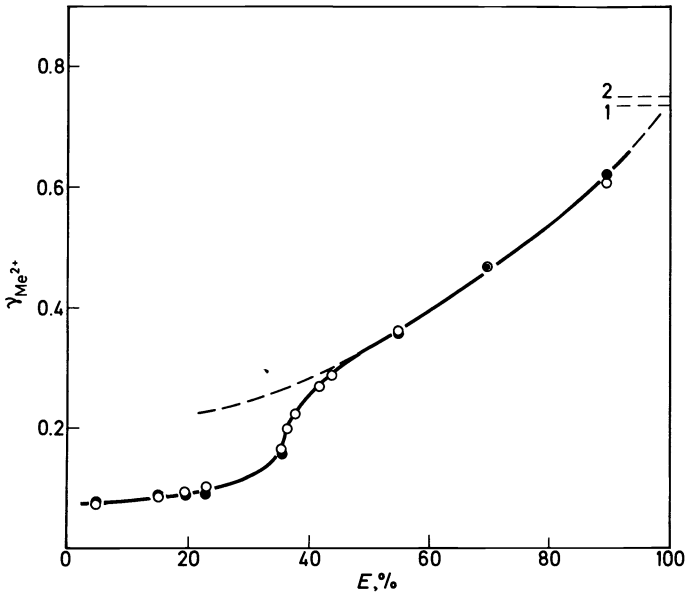


Figure 12. Activity coefficient of calcium and strontium ions ($\gamma_{Me^{2+}}$) in solutions of corresponding pectinates as a function of their degree of esterification E (reference 23): ○, Ca^{2+}; ●, Sr^{2+}; 1, Ca-galacturonate (monomer); 2, Sr-galacturonate (monomer).

With respect to the interpretation of results, it is of great importance that the unexpected drop of activity coefficients $\gamma_{Me^{2+}}$ starts as soon as a partial coagulation of calcium or strontium pectinates occurs during the neutralization process of pectinic acids with the corresponding hydroxides. The degree of esterification E in the insoluble fraction and in the solution of pectinate was found to be the same. The anomalous drop of the activity

coefficients $\gamma_{Me^{2+}}$ cannot therefore be caused by fractionation of pectinates with respect to their degree of esterification, which could take place during the coagulation process. The results summarized in *Figure 12* demonstrate further that the affinity of pectinates to calcium and strontium ions is roughly the same[23]. This finding agrees well with our previous results concerning the stability constants and selectivity coefficients determined in gels of calcium and strontium pectates[3,12].

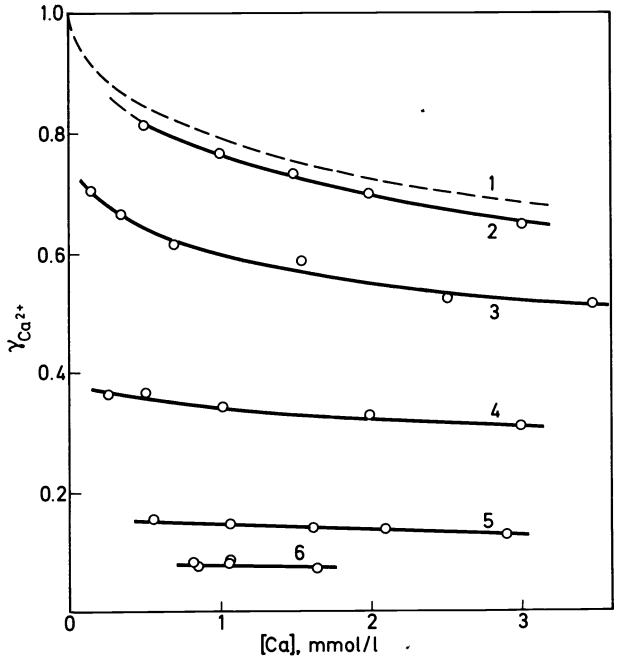


Figure 13. Activity coefficient $\gamma_{Ca^{2+}}$ as a function of the concentration of calcium pectinate solutions with different degrees of esterification E (reference 23): [Ca], total concentration of calcium in the solution of calcium pectinates; 1, theoretical $\gamma_{Ca^{2+}}$ values calculated according to Debye and Hückel for a strong electrolyte; 2, calcium D-galacturonate (monomer); 3.4.5.6, calcium pectinate of esterification degree E 89.7, 54.8, 35.4 and 4.6 per cent respectively.

Figure 13 shows the relationship between the activity coefficient $\gamma_{Ca^{2+}}$ and concentration of calcium pectinate solutions ([Ca]) of various degrees of esterification[23]. Curve 1 relates to the theoretical $\gamma_{Ca^{2+}}$ values as calculated according to Debye and Hückel[21,25] for a simple strong electrolyte. The activity coefficient $\gamma_{Ca^{2+}}$ estimated in the solution of calcium D-galacturonate (curve 2) is concentration-dependent in the same way as is $\gamma_{Ca^{2+}}$ of a simple calcium salt. Also similar is the case with highly esterified pectin (E 89.7 per cent, curve 3), which contains more distant carboxyl groups. The lower the degree of esterification of pectinates, in other words the higher the linear charge density of the macromolecule (curves 4–6), the less $\gamma_{Ca^{2+}}$ changes

with the concentration of the solution, which is characteristic for polyelectrolyte solutions[47–49].

The course of curves $\gamma_{Ca^{2+}} = f([Ca])$ plotted in *Figure 13.* together with earlier results (*Figures 2, 3, 12*), shows that the ability of pectinates to bind cations varies over a broad range depending on the linear charge density of the macromolecules. Pectinates of a high degree of esterification behave as do simple electrolytes, whereas pectinates of a low degree of esterification reveal the typical properties of a polyelectrolyte.

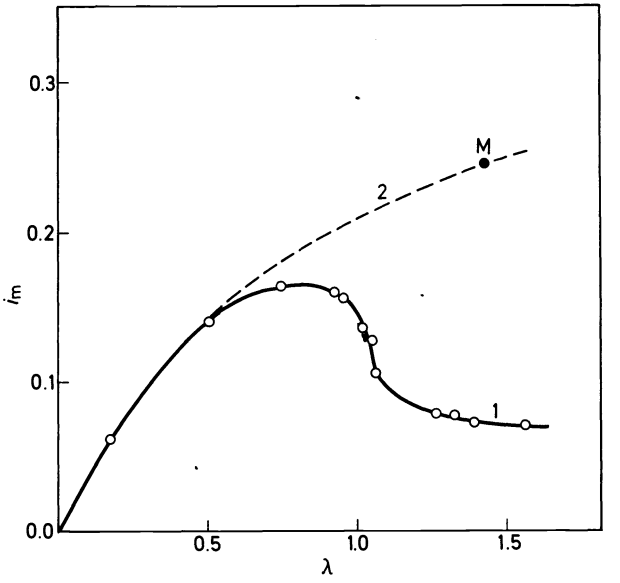


Figure 14. The effective degree of ionization of a monomeric unit (i_m) of calcium pectinate as a function of the charging parameter (λ)[23]: 1, calcium pectinates; 2, theoretical curve; M, calcium polymannuronate

The anomalous drop of the activity coefficients $\gamma_{Me^{2+}}$ becomes even more evident if the activity measurements are evaluated applying the theory of polyelectrolyte solutions. The dependence of the effective degree of ionization of the monomeric unit (i_m) of calcium pectinate on the charging parameter (λ) is shown in *Figure 14* (curve 1)[23].

$$i_m = \alpha \gamma_{Me} \overline{DS} \tag{2}$$

The ionization degree α refers to the ratio of the number of ionized groups to the number of total ionizable groups of the polyelectrolyte. (For calcium pectinate solution $\alpha = 1$, since a salt of polyacid is involved.) The γ_{Me} is the single-ion activity coefficient of counterions and $\overline{DS}$ is the mean substitution degree of monomeric units of the macromolecule by an ionizable group.

The charging parameter λ is an important structural characteristic of a polyelectrolyte solution, as it follows from the quantitative evaluation of the interaction of counterions with the polyelectrolyte molecule according to the rodlike model of Lifson and Katchalsky[36, 50].

$$\lambda = e_0^2/\varepsilon k T b \tag{3}$$

where e_0 is the electronic charge, ε the dielectric constant of solvent, k the Boltzmann constant,

T the absolute temperature and b the repeating distance between neighbouring ionized groups along the main axis of the linear molecule of the polyelectrolyte.

The dashed line (*Figure 14*, curve 2) expresses theoretical values; point M corresponds to the i_m value determined in the solution of calcium polymannuronate. This theoretical curve is similar to that found by Rinaudo and co-workers[51] in a solution of calcium carboxymethyl cellulose. The interaction of Ca^{2+} with both these polyanions, the polymannuronate and the carboxymethyl cellulose, is supposed to be of a pure electrostatic nature[51]. The anomalous course of function $i_m = f(\lambda)$ found in calcium pectinate solutions provides further evidence that the linear charge density is not the only factor controlling the interaction of Ca^{2+} with carboxyl groups of this polyuronide.

THE EFFECT OF THE CONFORMATION OF POLYURONATES ON THE BINDING OF CATIONS

Atkins and co-workers[40,41,52,53] have shown that, in molecules of polyuronic acids, there exist some possibilities of *intra* residue hydrogen bonds. The molecular chain of polymannuronic acid is a flat ribbon-like molecule whose conformation appears to be stabilized by the formation of an intramolecular hydrogen bond between the —O(3)H of one unit and the ring oxygen atom O(5)′ of the next sugar unit in the chain. In the *zig-zag*-shaped macromolecule of polyguluronic acid there exist also several possibilities for formation of *intra* residue hydrogen bonds between the equatorial hydroxyl group of the C(2) atom and either oxygen atom in the carboxyl group of the adjacent sugar unit in the chain.

These findings were of great importance for further studies. Calculation of the conformational energy maps for linear polyuronate macromolecules showed that these macromolecules are very rigid and extended[54,55]. Smidsrød, Haug and Whittington[46] and Rees with co-workers[56,57] have attempted further to interpret the ion binding on polyuronates by means of calculations of the conformation of these macromolecules. *Figure 15* (reference 46) illustrates a projection of the L-guluronic acid dimer in an *1C* conformation (VI) as obtained from the computer when the torsion angles of the two single bonds of the glycosidic linkage satisfy the condition that no overlap of van der Waals radii occurs. According to the above-mentioned authors[46], there is a 'cavity' between the sugar rings, including the carboxyl

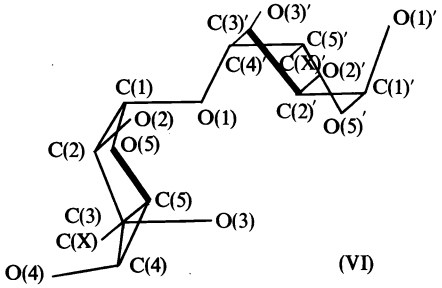


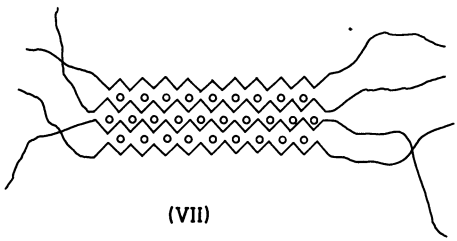
Figure 15. Projection of the L-guluronic acid dimer in *1C*-conformation[46].

group in the reducing end marked C(X)′, the ring oxygen O(5)′, the bridge oxygen O(1) and two hydroxyl groups in the non-reducing end, —O(2)H and —O(3)H.

The authors[46] suggest that the binding energy of calcium ions is lowered as a result of the interaction with one or more oxygen atoms in the cavity. The calculations have shown that a calcium ion may simultaneously contact the carboxyl group in one sugar unit and the two oxygens of the hydroxyl groups in the preceding sugar unit. Neutralization of the 'other' charge of the calcium ion from direct contact with a neighbouring carboxyl group is impossible, because this carboxyl group is situated on the other side of the sugar ring. A non-specific neutralization of the surrounding carboxyl groups was, therefore, suggested. Smidsrød, Haug and Whittington[46] assume that the high selectivity of polyguluronate for Ca^{2+} in the exchange reaction of Ca^{2+} and Mg^{2+} is due to the calcium binding to isolated chain molecules, even though they admit the existence of an intermolecular binding of divalent cations. On the other hand, the same authors[58] have demonstrated that the selectivity coefficient K^{Ca}_{Mg} determined in a gel of polyguluronate is very high in comparison with that found in a polyguluronate solution containing isolated molecules. An autocooperative interchain contact is supposed to be most probably the cause of the strong Ca binding.

Katchalsky and co-workers[59] found that the osmotically active fraction ϕ_p of calcium ions in oriented calcium alginate gels reached only one per cent of the total calcium concentration in the gel. The authors supposed that calcium ions in an alginate gel form bridges between the alginate chain molecules (intermolecular binding of Ca^{2+}).

Rees and co-workers[56, 57] have found on the basis of calculation of the conformations of polyuronate macromolecules similar 'cavities' or 'nests', as mentioned earlier. Based upon these findings and on circular dichroism studies of the sol → gel transition for calcium polyuronates[57, 60], the authors explain the selectivity of polyguluronate and pectate in ion exchange reactions by a cooperative mechanism of binding, involving two or more chains in terms of the 'egg-box' model (VII), as shown schematically in *Figure 16*. The



(VII)

Figure 16. 'Egg-box' model of the intermolecular binding of Ca^{2+} on polyguluronates[57]: ○, Ca^{2+}; ⌐⌐⌐⌐⌐, polyguluronate chain.

selectivity of cooperative binding is determined by the comfort with which cations (the 'eggs') of the particular size may pack into the 'box'. It was shown that the conformation of the polyguluronate chain offered four-oxygen coordination. With polygalacturonate there exist fewer coordination possibilities, most of which involve only three oxygens. The coordinating

oxygens are more widely spaced for polyguluronates. According to the above-mentioned authors[57], these stereochemical concepts explain why polyguluronates form stronger complexes with metal cations than polygalacturonates and why polyguluronate binds preferentially the larger Sr^{2+} ions in ion exchanging of Ca^{2+} and Sr^{2+}, whereas polygalacturonate does not reveal exchange selectivity for those cations[3, 12, 23].

In contrast to the polyguluronate, the polymannuronate molecule, owing to diequatorial glycosidic bonds, is a flat ribbonlike chain with more shallow 'nests' for the cations to occupy. This would explain both the inability of such chains to complex except at higher ion concentrations and the low selectivity in cation exchange reactions.

When studying the crystalline structure of polyguluronic acid, Atkins and co-workers[41, 53] showed that the *inter*molecular hydrogen bond could only arise by inclusion of water molecules between the chains of macromolecules. According to these authors, it seems very probable that metal cations might occupy the same sites in polyguluronate salts like the water molecules in the polyacid. This idea is in accordance with the 'egg-box' model.

INTERMOLECULAR BOND OF Ca^{2+} IN CALCIUM POLYGULURONATE AND POLYGALACTURONATE

The extremely low activity coefficients $\gamma_{Ca^{2+}}$ found in solutions of calcium polyguluronate and calcium pectate may be due to three factors having an additive character: (1) the higher linear charge density of macromolecules of these polyuronates in comparison with the calcium polymannuronate, (2) the *inter*molecular binding of Ca^{2+} with carboxyl groups of different chains in small *soluble* aggregates and (3) the distance between the carboxyl and hydroxyl group or groups participating in the binding process[33].

Let us now discuss these hypotheses of ion binding mechanism in the light of our results (*Figure 10*).

The chain-dotted line (curve 4) represents the theoretical $\gamma_{Ca^{2+}}$ values in solutions of calcium polyguluronate and calcium pectate, calculated for pure electrostatic interaction of Ca^{2+} with carboxyl groups, by analogy with other published data[35, 51] (reference 35; p 396). In the range of lower degree of polymerization (polyguluronate $DP \leqslant 18$; polygalacturonate $DP \leqslant 11$) the $\gamma_{Ca^{2+}}$ values lie on the theoretical curve. At a higher degree of polymerization a sharp unexpected drop of the activity coefficient $\gamma_{Ca^{2+}}$ occurs; the activity coefficients $\gamma_{Ca^{2+}}$ are several times lower than the theoretical values. If the binding mechanism, involving only *intra* residue interaction of two consecutive uronic acid units of an isolated chain molecule[46], is taken into consideration, the decrease of $\gamma_{Ca^{2+}}$ with increasing degree of polymerization should be smooth, without any sudden drop in the activity coefficient $\gamma_{Ca^{2+}}$.

As mentioned earlier, this drop in the activity coefficient $\gamma_{Ca^{2+}}$ in solutions of polyuronates (*Figure 10*) as well as in solutions of pectinates (*Figure 12*) is closely related with the appearance of a partial coagulation of calcium polyguluronate, polygalacturonate and pectinate, respectively, during the neutralization process of these polyacids with calcium hydroxide. Moreover, if an excess of calcium chloride is added to a dilute solution of sodium

polyguluronate and sodium pectate. an irreversible quantitative coagulation occurs. The excess of calcium chloride also causes a precipitation of calcium polymannuronate. In contrast to the irreversible coagulation. this process is most probably a 'salting-out' process, since the precipitate of calcium polymannuronate is easily dissolved in distilled water. All these findings demonstrate that the conformation of macromolecules of polyguluronate and polygalacturonate are more suitable for clustering chains together in a network with local limited crystallization.

In our opinion, the extremely low $\gamma_{Ca^{2+}}$ found in solutions of calcium polyguluronate and calcium polygalacturonate can be explained by an *inter*molecular binding of calcium ions with carboxyl groups of different chains in small soluble aggregates, including 'blocks' of oriented uronate units.

All activity measurements were carried out in perfectly *clear* solutions of calcium or strontium polyuronates obtained by centrifugation at 13000 *g*. These solutions passed easily through a dense filter paper without change in concentration, or with a slight change close to that of experimental error. It remains an open question whether clear calcium polyuronate solutions with anomalously low activity coefficients $\gamma_{Ca^{2+}}$ are molecular-disperse, or contain small aggregates of macromolecules.

Circular dichroism spectra. Morris, Grant, Rees and co-workers[57, 60] have

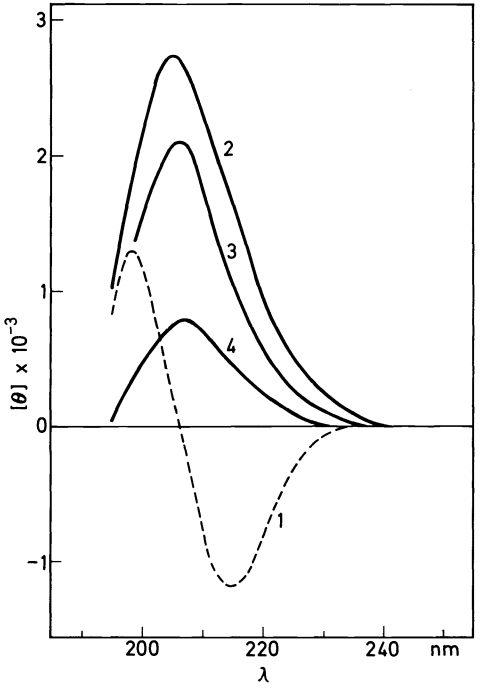


Figure 17. Dichroic spectra of dilute solutions of polymannuronates and pectates[61]: $[\theta]$, molecular ellipticity (degree cm²/decimole); 1, K-, Mg-, Ca-polymannuronate; 2, K-pectate; 3, Mg-pectate; 4. Ca-pectate; $[-COO^-] = 2.0$ mequiv./l.

demonstrated that the transition of sodium alginate and pectate sols to a gel of calcium polyuronates is accompanied by a great change in the spectra of circular dichroism in the $n \rightarrow \pi^*$ band of the carboxylate group. The authors suggest that the n-orbitals in gels of polyguluronates and polygalacturonates with oriented macromolecules are perturbed by the proximity of specifically bound Ca^{2+}. Polymannuronate sequences in alginate rich in mannuronic acid units can also be perturbed spectroscopically by Ca^{2+} binding at higher Ca^{2+} concentrations.

We therefore examined the spectra of circular dichroism in *clear* dilute solutions of pectates and polymannuronates in their K^+, Mg^{2+} and Ca^{2+} forms. Dichroic spectra were taken also of solutions of K, Mg and Ca D-galacturonate and its α-methyl glycoside[61]. Salts of the monomeric D-galacturonic acid and its α-methyl glycoside gave spectra independent of the cation involved (K, Mg, Ca). Dichroic spectra of pectates and polymannuronates are seen in *Figure 17*. The K, Mg and Ca polymannuronate solutions displayed nearly identical spectra (curves 1). The dichroic spectra of K, Mg and Ca pectate (curves 2, 3, 4) differ markedly, however. An extremely large decrease in intensity of the $n \rightarrow \pi^*$ band occurs with the calcium pectate solution. This spectral difference is due to a very intense interaction of Ca^{2+} with carboxyl groups.

The difference in dichroic spectra of polymannuronates and pectates entitles us, with respect to the results of Rees and co-workers[57, 60], to conclude that solutions of calcium polymannuronates are very probably of molecular-disperse character and those of calcium pectates occur in a micro-gel state.

Ultracentrifugation. Our last experiments have provided direct evidence that all solutions which exhibited the extremely low activity coefficients $\gamma_{Ca^{2+}}$ contained small aggregates of macromolecules[34]. The clear solutions of calcium polyuronates (supernatants; 13000 *g*) were treated by further centrifugation up to 190000 *g*, using a preparative ultracentrifuge (centrifugation time 30 min). The concentration of polyuronates in supernatants was determined. The results are shown in *Figure 18*. In solutions of all potassium polyuronates, as well as of potassium carboxymethyl cellulose, no concentration change after centrifugation up to 190000 *g* was observed. The same result was found with the solution of calcium polymannuronate and calcium carboxymethyl cellulose (curve 1). This indicates that solution of calcium polymannuronate and calcium carboxymethyl cellulose may be considered as molecular-disperse. On the other hand, the concentration of solutions of calcium pectate (curve 2) as well as calcium polyguluronate (curve 3) decreased markedly with the increasing centrifugal acceleration; increasing amounts of a transparent gel were separated. These results demonstrate that the anomalously low activity coefficients $\gamma_{Ca^{2+}}$, reflecting a strong binding of calcium ions on these polyuronates, are closely related with the existence of calcium pectate and calcium polyguluronate in small aggregates, in a *micro-gel* state[34].

Having thrown further light on this problem, we can also rationalize in terms of these findings the effect of acetylation of pectin on the binding of calcium on its carboxyl groups (*Figure 6*, curve 1). The increasing degree of acetylation of calcium pectate resulting in a significant decrease of the stability

constant K is due to the fact that acetyl groups provide steric hindrance and avoid the formation of aggregates of macromolecules. In contrast to this, in the solution of calcium pectinate (E 58 per cent, curve 2), which could be considered molecular-disperse, no intermolecular bond of calcium can form

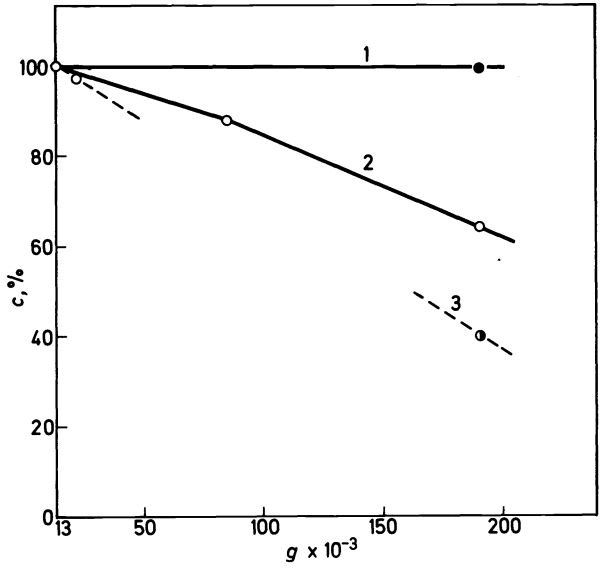


Figure 18. Concentration change (c%) in solutions of polyuronates after ultracentrifugation[34]. 1, Ca-polymannuronate; Ca-carboxymethyl cellulose; K-polyuronates: 2, Ca-pectate; 3, Ca-polyguluronate.

and therefore the steric effect of acetyl groups could be manifested only slightly (the greater distance of free carboxyl groups in the molecule of pectinate could also be taken into account).

The results obtained entitle us to conclude that the binding of cations to *isolated* chains is above all controlled by electrostatic attractive forces, as evidenced by the activity coefficients $\gamma_{Ca^{2+}}$ in solutions of low molecular polyuronates and in solutions of pectinates with a higher degree of esterification.

The firm binding of some divalent cations on polyguluronate and pectate, and the high selectivity of these polyuronates in ion exchange reactions, are mainly due to the *intermolecular binding* of cations, in accordance with the 'egg-box' model of Rees and co-workers[57]. This intermolecular bond involves the interaction of Me^{2+} with two carboxyl groups, belonging to two different chains, and also offers the best possibility of bringing Me^{2+} into contact with oxygen atoms of uronic acid units in 'cavities' or 'boxes' in the sense of the above-mentioned mechanisms. Schweiger[27] presumed, in contrast to this concept, an interaction of Me^{2+} with two neighbouring carboxyl groups of one chain and two vicinal hydroxyl groups of another chain in *inter*molecular chelation. Results shown in *Figures 10* and *12* led us

to conclude that in the *inter*molecular bond of Me^{2+} aggregates of oriented segments of macromolecules containing 'blocks' of free carboxyl groups are formed.

The knowledge of laws controlling the interaction of Ca^{2+} with free carboxyl groups of pectin is notable also from another viewpoint. The value of the stability constant K was used as a characteristic of the distribution pattern of free and esterified carboxyl groups in the molecule of pectin. By means of this method it has been shown that the enzyme pectinesterase reacts by a one-chain mechanism, forming long segments with a blockwise arrangement of free carboxyl groups[62]. Similarly, it has been shown that in the esterification process of both pectic and pectinic acids with methanolic sulphuric acid the accessibility of free carboxyl groups in starting preparations is the main factor influencing the distribution pattern of free and esterified carboxyl groups[63]. Details of these investigations would exceed the scope of this contribution.

CROSSLINKED PECTATE

A crosslinked insoluble ion exchanger, exhibiting a high selectivity in ion exchange reactions, could be prepared from sodium or potassium pectate[64] (see also reference 69). In addition, Drs Rexová and Tibenský[65, 66] of our Institute have adduced evidence that this preparation is an excellent tool for the selective purification of enzyme endopolygalacturonase (affinity chromatography). From this point of view it was interesting to examine the selectivity of exchange of some divalent cations, the binding of endopolygalacturonase and also the biodegradability of this crosslinked pectate.

Ion exchangers based on pectic acid, differing to a large extent in the number of crosslinks, were prepared and characterized by Dr Kuniak[67]. Sodium pectate containing more than 90 per cent of polygalacturonate in dry substance was crosslinked, applying epichlorohydrin in the vapour phase[64]. The resulting preparations were crosslinked quite homogeneously. The samples were characterized (*Table 3*) by the swelling volume V (ml/g), the

Table 3. Characterization of crosslinked pectates[67]

Sample No.	Swelling volume V, ml/g	UAU/CL	UAU/SC
1	4.6	5.1	37
2	5.6	8.7	35
3	8.0	13.2	27
4	11.4	21.1	38

UAU/CL, number of uronic acid units per one crosslink; UAU/SC, number of uronic acid units per one glycerol monoether side chain.

number of uronic acid units belonging to one crosslink and the degree of side reactions, i.e. the number of uronic acid units belonging to one glycerol monoether side chain. Calculations were made provided that the side chains, due to this crosslinking technique, were not of polymeric character.

The selectivity of the exchange of Ca^{2+} and K^+ as functions of both the degree of crosslinking of pectate and the equivalent fraction of Ca^{2+} bound in the resin phase ($\bar{X}_{Ca}$) is shown in *Figure 19* (reference 67). With increasing degree of crosslinking the selectivity of the ion exchanger to Ca^{2+} increases.

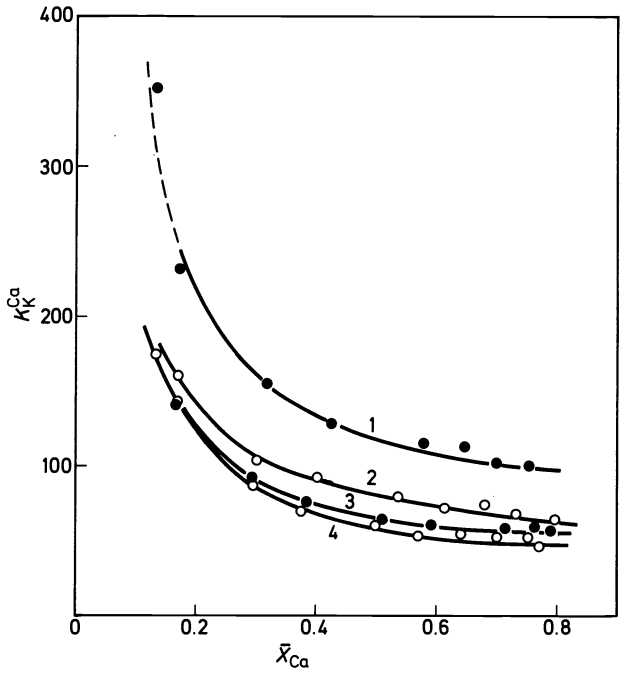


Figure 19. Selectivity of the exchange of Ca^{2+} and K^+ as a function of the degree of crosslinking of pectate and on the equivalent fraction of Ca^{2+} bound in the resin phase ($\bar{X}_{Ca}$)[67]: 1,2,3,4, samples of crosslinked pectate (see *Table 3*).

Similar results were found by Reddy and Marinsky[68] in the exchange of calcium and strontium with hydrogen ion in variously crosslinked polystyrene sulphonate cation exchangers. With the decreasing amount of calcium ions bound in the resin phase, the selectivity to Ca^{2+}, as well as to Mg^{2+} and Sr^{2+} strongly increases. This phenomenon, which was also observed in gels of calcium and strontium pectates[3], can be caused by an inhomogeneous distribution of carboxyl groups in the ion exchanger, forming loci with different charge densities and different possibilities of *inter*molecular binding of divalent cations. In contrast to these insoluble ion exchangers, in solutions of pectinates the interaction of calcium ions with free carboxyl groups obeys the multiple equilibria law exactly, as was shown earlier.

Selectivity coefficients K_K^{Me} of the ion exchange reaction of several divalent cations and potassium ions on crosslinked pectate (sample No. 1) are listed in *Table 4* (reference 67). The selectivity coefficients relate to the equivalent

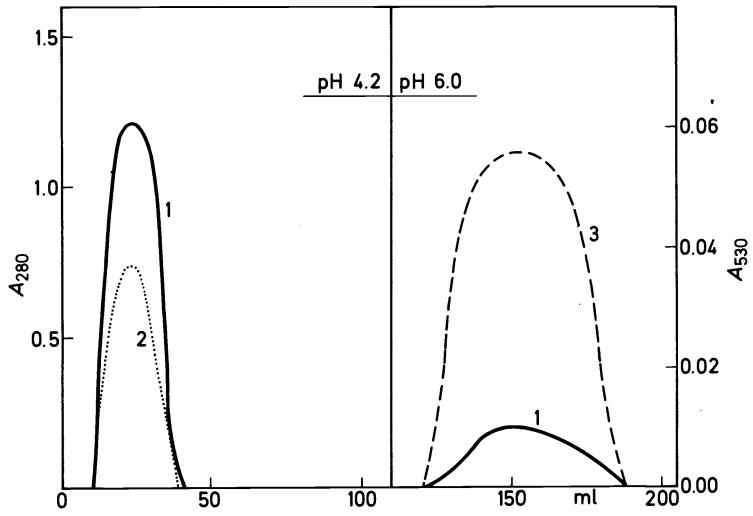


Figure 20. Affinity chromatography of pectolytic enzymes on column of crosslinked pectate[65]: 1, absorbance at 280 nm (proteins and impurities); 2, exopolygalacturonase (A_{530}); 3, endopolygalacturonase (A_{530}).

fraction of divalent metal cations bound in the resin phase $\overline{X}_{Me} = 0.5$; ionic strength $I = 0.15$. As evidenced by these results, the crosslinked pectate is highly selective for cations of divalent metals with the exception of the pair calcium–strontium, this being in accordance with previous findings[3, 12, 23].

Figure 20 shows the affinity chromatography of pectolytic enzymes from *Aspergillus niger* over crosslinked pectate[65]. At pH 4.2, which is the pH optimum of activity of endopolygalacturonase, this enzyme is quantitatively captured in the column, whereas other pectolytic enzymes (exopolygalacturonase and pectinesterase) pass out in the effluent. The specific endopolygalacturonase is extruded from the column at pH 6.0 (curve 3); curves 1 show the absorbance at 280 nm. The absorbance A_{530} is proportional to the activity of enzymes, i.e. to the reducing end groups formed during the hydrolysis process (method of Somogyi–Nelson).

Dr Rexová tested the biodegradability of crosslinked pectate preparations by pectolytic enzymes of *Aspergillus niger*[67]. Results are seen in *Figure 21* and *Table 5*. Samples of crosslinked pectate (Nos. 1–4) are the same as those

Table 4. Selectivity coefficients K_K^{Me} on crosslinked pectate (sample No. 1)[67]

Me^{2+}	K_K^{Me} ($\overline{X}_{Me} = 0.5$)	Me^{2+}	K_K^{Me} ($\overline{X}_{Me} = 0.5$)
Mg	26	Co	241
Ca	121	Pb	2580
Sr	120	Cu	3300

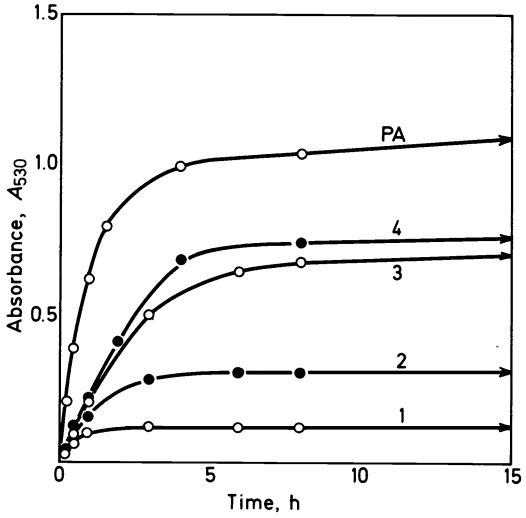


Figure 21. Degradation of crosslinked pectates by pectolytic enzymes of *Aspergillus niger* (pH 4.2)[67]: 1,2,3,4, samples of crosslinked pectate (see *Table 3*); PA, solution of sodium pectate.

used in previous experiments; PA corresponds to a solution of sodium pectate. The absorbance A_{530} is proportional to the reducing end groups of mono- and oligomers formed by the action of enzymes. The *DP* in *Table 5* is the polymerization degree of oligogalacturonates as degradation products. Pectates with a low degree of crosslinking (curves 3, 4) are degraded by both

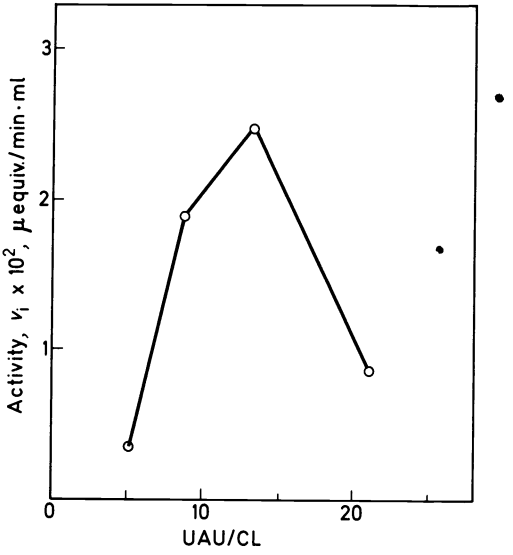


Figure 22. Adsorption of endopolygalacturonase of *Aspergillus niger* on crosslinked pectate (pH 4.2)[67]: UAU/CL, the number of uronic acid units per one crosslink.

endopolygalacturonase and exopolygalacturonase, while pectates with a high content of crosslinks (curve 1) are attacked to a small extent only by exopolygalacturonase (see *Table 5*).

The effect of crosslinking on the binding of endopolygalacturonase[67] is

Table 5. Degradation of crosslinked pectates by pectolytic enzymes of *Aspergillus niger* (pH 4.2)[67] (oligomeric products of degradation)

Sample No.	Oligogalacturonates, *DP*						
	1	2	3	4	5	6	7
1	+						
2	+++						
3	+++	+	+	+	+	+	+
4	+++	++	++	++	++	++	++
PA	+++	++	++	++	++	++	++

shown in *Figure 22*. The degree of crosslinking (UAU/CL) is given again by the number of uronic acid units belonging to one crosslink and the amount of adsorbed enzyme by its activity (v_i) after elution. The affinity of the enzyme to the insoluble support decreases with the increasing degree of crosslinking (in the direction from right to left). The anomalously low amount of enzyme adsorbed on sample No. 4, having the lowest degree of crosslinking (UAU/CL 21), is due to the high degradation of this ion exchanger, resulting in loss of the insoluble material.

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