

INTERMOLECULAR CALCIUM ION BINDING ON POLYURONATES-POLYGALACTURONATE AND POLYGULURONATE^a

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Received September 30th, 1975

Homopolymeric oligo- and polygalacturonic acids of polymerization degree DP 1–64 and a narrow dispersion of molecular weight in the ± 3 to $\pm 5\%$ range were prepared. The relationship between the activity coefficient $\gamma_{Ca^{2+}}$ and DP of uronates was established in dilute solutions of calcium oligo- and polygalacturonates. The activity of calcium ions was determined spectrophotometrically tetramethylmurexide being the metallochromic indicator. The anomalous course of the function $\gamma_{Ca^{2+}} = f(DP)$ was rationalized by the intermolecular Ca^{2+} ion binding on carboxyl groups of two or more polygalacturonate macromolecules of $DP > 20$. As evidenced by a preparative ultracentrifugation, the clear calcium polygalacturonate and polyguluronate solutions showing anomalously low $\gamma_{Ca^{2+}}$ values contained small aggregates of macromolecules. The aggregation of macromolecules in solutions of calcium polymannuronate and calcium carboxymethylcellulose, where an intramolecular binding of cations of pure electrostatic nature is supposed, was not proved. The results are in agreement with the conception of an intermolecular chelate binding of Ca^{2+} ions in terms of the "egg-box" model.

Pectates (poly(α -D-galacturonate)) and alginates rich in α -L-guluronic acid units behave as highly selective ion exchangers in exchange reactions of numerous mono- and bivalent cations^{1–5}. On the other hand alginates, in which poly(β -D-mannuronic acid) is the main component, are less selective in exchange of cations. Several hypotheses concerning the binding mechanism of bivalent cations on polyuronates and the selectivity of cation exchange have been presented^{6–10}. In addition to a pure electrostatic binding of cations to carboxyl groups as is the case in polymannuronate solutions, an interaction of these cations with oxygen atoms of hydroxyl groups in "cavities" or "nests" formed by two neighbouring uronic acid units in the chain of macromolecules were supposed with polygalacturonate and polyguluronate. An intramolecular⁹ and intermolecular coordination binding of this type¹⁰ was suggested (see¹¹).

Our previous papers^{12,13} dealing with the interaction of Ca^{2+} or Sr^{2+} ions with carboxyl groups of alginates and pectinates were based upon activities of counterions

^a The results were presented at the VII International Symposium on Carbohydrate Chemistry (IUPAC), Bratislava, August 1974.

in dilute solutions of corresponding calcium or strontium polyuronates. In the solution of calcium polyguluronate of polymerization degree $DP \geq 28$ and in the solution of calcium (strontium) pectinate of a low esterification degree ($E < 30$) anomalously low values of the single-ion activity coefficient $\gamma_{Ca^{2+}}$ or $\gamma_{Sr^{2+}}$ were found. As deduced from these results, the linear charge density of macromolecules of the above-mentioned polyuronates was not the only factor governing the interaction between the polyanion and counterions. The anomalously low $\gamma_{Ca^{2+}}$ ($\gamma_{Sr^{2+}}$) values were most probably due to the intermolecular Ca^{2+} (Sr^{2+}) ion binding to carboxyl groups of various chains in small soluble aggregates of macromolecules.

To throw more light on the mechanism of cation binding to polyuronates the effect of polymerization degree of polygalacturonate, which is one of the typical representatives of polyuronates, on the activity of Ca^{2+} counterions in dilute solutions of calcium polygalacturonates was investigated. The high purity of preparations (homopolymeric fragments) with the lowest possible dispersion of molecular weights was stressed.

EXPERIMENTAL

Chemicals

Citrus Pectin, Type B, Rapid Set (Københavns Pektinfabrik) was the starting preparation. Sodium polyguluronate and polymannuronate were prepared by Haug and Smidsrød¹⁴. Polyguluronic acid contained 91% of L-guluronic acid units (*G*) and 9% of D-mannuronic acid units (*M*); its average polymerization degree \overline{DP} was 47. Polymannuronic acid was composed of *M* (94%) and *G* (6%); $\overline{DP} > 3000$. Carboxymethylcellulose (Na^+) of an average substitution degree \overline{DS} 1.0 was prepared by Rinaudo and Milas¹⁵; this sample had \overline{DP} 200. Penta(α -D-galacturonic acid) was obtained by the same procedure as oligomers in¹⁶. Tetramethylurexide was synthesized and characterized as reported earlier¹⁷. The 0.1M-NaOH and 0.05M-KOH solutions were carbonate free. The concentration of the clear saturated solution of $Ca(OH)_2$ was approximately 0.021M. Other chemicals were of analytical grade. The specific conductivity of the redistilled water was $2 \cdot 10^{-4} \text{ S} \cdot \text{m}^{-1}$.

Preparation of Polygalacturonic Acid of Various Polymerization Degree

Pectic acid obtained from the citrus pectin by an alkaline deesterification in 60% ethanol¹⁸ was purified as described earlier¹⁷. The solution of sodium pectate (0.5%) was acidified with 0.3M- H_2SO_4 to pH approximately 3 up to the point of coagulation of pectic acid. The mixture was heated on a steam bath. The suitable time of hydrolysis was deduced from the preliminarily estimated relationship between the \overline{DP} of fragments and the time of hydrolysis. The \overline{DP} after 4h of hydrolysis was 17. The hydrolysate neutralized with sodium hydroxide to pH 7 at room temperature was preliminarily fractionized by addition of 96% ethanol in a 1 : 0.55 ratio. The coagulate thus formed was centrifuged at 13000 *g*. The coagulate, containing fragments of \overline{DP} 23, represented an amount of material corresponding to 66% of the starting pectic acid. The coagulate was dissolved in a greater amount of water and ethanol was removed by evaporation of the solution to dryness in a rotary vacuum evaporator at 35°C. The residue was used for a further fraction-

nation by means of gel-permeation chromatography. The supernatant corresponding to 34% of the starting material contained fragments of \overline{DP} 9; this fraction was not worked up.

The solution (12 ml) containing roughly 500 mg of sodium pectate fragments was soaked into Sephadex G-50 (two columns 120 . 3.5 cm connected in series) and eluted with 0.1M- Na_2SO_4 at a 15 ml . h⁻¹ flow-rate. The content of polygalacturonic acid and its \overline{DP} were determined in eluates, which were afterwards combined into three fractions (I, II, III). The \overline{DP} of the first fraction was 10–22, of the second and third ones 23–38 and 39–58, respectively. The solutions were concentrated by evaporation, substantially desalted by dialysis and rechromatographed under constant conditions. The first two fractions were separated over Sephadex G-50, the third one over Sephadex G-75. The first and second fractionation was repeatedly carried out under constant conditions in order to obtain a sufficient amount of individual fractions of exactly defined \overline{DP} .

The single final fractions partially desalted by dialysis were concentrated and desalted by a 2–3-fold gel filtration through a Sephadex G-10 column (220 . 2.5 cm). This procedure was checked on the basis of electric conductivity of the solutions. The desalted solutions were freeze-dried; sodium polygalacturonate preparations were stored at +5°C.

Analytical Methods

The methods employed for the analysis of pectin and pectic acid (the esterification degree E , the content of polyuronides, the viscometric determination of molecular weight, the content of neutral saccharides in pectic acid and in fragments of polygalacturonic acid) were already published¹⁷. The polymerization degree DP of polygalacturonic acid fragments was estimated on the basis of the concentration ratio of carboxyl and reducing terminal —CHO groups. The carboxyl group content was determined by the modified carbazole method¹⁹ with a $\pm 1\%$ relative error, sodium tetraborate being the colouration stabilizer. The reducing terminal groups were determined by the Nelson and Somogyi method²⁰ with a $\pm 0.5\%$ error. The concentration of carboxyl groups in solution of polyuronic acids and carboxymethylcellulose (H^+) was determined by alkalimetric potentiometric titration using a glass electrode (Radiometer Denmark). The concentration of potassium polyuronates before and after centrifugation of their solutions was estimated by the carbazole method¹⁹, the concentration of calcium salts of these acids by chelatometric titration of calcium with 0.01M Complexon IV solution and photometric indication of the point of equivalence. An interference filter Zeiss Jena IF 600 nm and murexide as metallochromic indicator were used; the relative error of analysis was $\pm 0.3\%$. Solutions were centrifuged up to 190 000 g with a VAC-601 Janetzki (GDR) preparative ultracentrifuge.

Determination of Ca^{2+} Ion Activities in Calcium Oligo- and Polygalacturonates

Solutions of sodium oligo- and polygalacturonates (concentration approximately 5 mequiv. $[-\text{COONa}] \cdot \text{l}^{-1}$) were percolated through a column of Dowex 50W $\times 2$ (H^+) and thoroughly neutralized with calcium hydroxide to the point of equivalence ($\text{pH} \sim 7.2$). A partial coagulation of the corresponding Ca-polygalacturonates was observed during the neutralization when preparing calcium polygalacturonate solutions of $DP \geq 10$. Suspensions were centrifuged at 13000 g . Supernatants served for estimation of the Ca^{2+} ion activities.

The concentration of $[-\text{COOCa}_{0.5}]$ groups in Ca-oligogalacturonate solutions was calculated from the consumption of $\text{Ca}(\text{OH})_2$ for the neutralization of the appropriate uronic acids. The concentration of $[-\text{COOCa}_{0.5}]$ in supernatants of Ca-polygalacturonates after centrifugation was determined — considering the very low consumption of the sample for analysis — by the carbazole method¹⁹. This method afforded the same results as the chelatometric determination

of Ca in the same solutions. The Ca^{2+} ion activity in calcium oligo- and polygalacturonates was determined spectrophotometrically with tetramethylmurexide as the metallochromic indicator^{17,21}. Pure calcium oligo- and polygalacturonates were employed as reference solutions. Absorbances at 490 and 530 nm were measured with a Hilger UVISPEC compensation spectrophotometer at 23–25°C; the CaCl_2 solutions for calibration were used without addition of other electrolyte. The single-ion activity coefficients $\gamma_{\text{Ca}^{2+}}$, calculated according to a modified Debye and Hückel equation²², were used for calculation of Ca^{2+} ion activities in calibration solutions; the effective diameter of the hydrated calcium ion $a' = 0.60$ nm. A computer GIER (Algol 4) calculated the $\gamma_{\text{Ca}^{2+}}$ values; a program to calculate the activity coefficients γ_i in aqueous solutions at a given temperature and ionic strength I was elaborated²³.

Estimation of Molecular Weight on Thin Layer of Sephadex

Samples of sodium polygalacturonate of various DP were dissolved in a 0.7% NaCl solution and spotted on a Sephadex G-50 (Superfine) layer (0.6 mm thick). The separation of fragments proceeded 3.5 h at a slope $\alpha = 15^\circ$ with a 0.7% NaCl solution. (Performed with a TLG Pharmacia, Uppsala Sweden, apparatus.) A print was taken from the thin layer by means of a Whatman 3 M paper. This was divided into two parts: on the first part the fragments of $DP \geq 10$ were visualized with ruthenium red²⁴, on the second one the oligomeric fragments of $DP < 10$ were detected with aniline phthalate reagent. The dependence $d = f(\log DP)$ was expressed by means of the distance of the individual spots from base line (d) and the known DP values.

RESULTS

Pectic acid obtained by an alkaline deesterification of purified citrus pectin was the material employed for preparation of pure homopolymeric polygalacturonic acids of various polymerization degree and the narrowest possible dispersion of molecular weights. Pectic acid contained 90% of polygalacturonic acid and 10% of neutral saccharides: D-galactose, L-arabinose, D-glucose, D-xylose, L-rhamnose in a molecular ratio 9.1 : 2.9 : 1.7 : 1.0 : 0.4. Its esterification degree E was 4.2%, the average molecular weight \bar{M}_n 16 500 and the sulphate ash content 0.11%. Pectic acid was partially hydrolyzed under acid conditions and its fragments fractionated by a column gel chromatography on Sephadex. Rechromatography afforded samples of Na-polygalacturonates with a narrow dispersion of molecular weights as seen in Fig. 1 exemplifying the rechromatography of a fraction with a lower polymerization degree. Shaded area shows the combined fractions. The samples did not contain any trace of inorganic salts, which might negatively influence the Ca^{2+} ion activity determination. Desalting by gel filtration was checked conductometrically. Sodium polygalacturonates are well electrically conductive and therefore, the criterion for a perfect desalting was the shape of the curve showing the electric conductivity of the individual fractions: it had to parallel that expressing the concentration of polygalacturonate in the very same fractions (Fig. 2, the shaded area shows the combined fractions).

A series of Na-polygalacturonate samples of various polymerization degree DP was obtained in this way. Four samples of a higher DP were analyzed for a possible

presence of neutral saccharides. Only D-galactose was found in the samples in minute amounts. The characterization of sodium polygalacturonate samples and their yield (mg) are listed in Table I. These results demonstrate the virtually homopolymeric chains of polygalacturonic acids containing more than 99% of polygalacturonate in the dry substance with a dispersion of molecular weights in the ± 3 to $\pm 5\%$ range.

We had fragments of polygalacturonic acid of a very low dispersion of molecular weights as reference substances on hand and therefore, we examined the possibility to apply thin layer gel chromatography for determination of molecular weight of

FIG. 1
Rechromatography of Fragments of Polygalacturonic Acid on a Sephadex G-50 Column

1 Concentration of polygalacturonate ($-\text{COOH}$ groups), 2 polymerization degree DP .

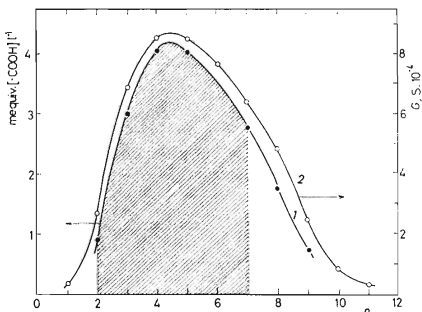
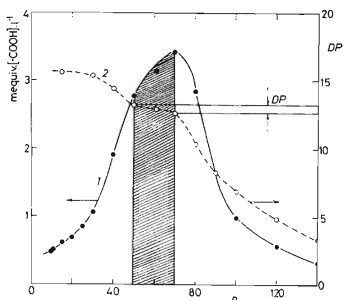


FIG. 2

Desalting of Na-Polygalacturonate (DP 15) on a Sephadex G-10 Column

1 Concentration of polygalacturonate ($-\text{COOH}$ groups), 2 electric conductivity (G).

polygalacturonic acid on Sephadex G-50 (Superfine). The development of the same samples was three times repeated; each of the analyses (I, II and III) evidenced the linear dependence of the function $d = f(\log DP)$, where d is the distance of the spot from the base line. The obtained results are plotted in Fig. 3; the results of analysis III are knowingly omitted as they are superimposable with those of analysis II. Since almost homodisperse samples are involved $M_n = M_w$. Errors of the single determinations M_w are listed in Table II.

As evidenced by results, thin layer chromatography is an useful tool for molecular weight determination of polygalacturonic acids with an error ranging from ± 3 to

TABLE I
Characteristic Data of Samples of Na-Polygalacturonates

Sample	DP	Yield, mg	D-Galactose %
1	10.0 \pm 0.5	30	—
2	11.8 \pm 0.5	35	—
3	13.9 \pm 0.6	20	—
4	15.3 \pm 0.6	20	0.39
5	21.0 \pm 0.7	25	—
6	24 \pm 1.0	26	0.64
7	30 \pm 1.0	31	—
8	32 \pm 1.0	29	0.84
9	47 \pm 1.5	6	—
10	64 \pm 1.5	34	0.90

TABLE II
Determination of Molecular Weight (M_w) of Polygalacturonic Acid Fragments by Chromatography on Thin Layer of Sephadex G-50 (Superfine)

Sample	DP	M_w	$\Delta M_w, \%$		
			I	II	III
1	10.0	1 780	—	0	+3
4	15.3	2 710	-2	+4	-5
6	24	4 240	+3	-8	-6
8	32	5 650	-1	+7	+8
9	47	8 290	-3	-8	-6

$\pm 8\%$; mean error of a single analysis $\pm 5.5\%$. Lower oligomeric fragments of polygalacturonic acid were also tried to separate on thin layers of Sephadex G-50. We found that the separation capability of Sephadex G-50 was not satisfactory for lower oligomers and the separation was not enough effective. We were unable to prepare a thin layer sheet sufficiently firm from Sephadex G-25 (Superfine).

Dependence of the Activity of Calcium Ions in Solutions of Calcium Oligo- and Polygalacturonates on Their Polymerization Degree

Pentagalacturonic and polygalacturonic acids characterized in Table I were used for Ca^{2+} ion activity determination. A partial coagulation of Ca-polygalacturonate occurred when preparing solutions of calcium polygalacturonates of $DP \geq 10$. The Ca^{2+} ion activity was estimated in supernatants containing the soluble calcium polygalacturonate as obtained by centrifugation at 13 000 *g*. The activity coefficient $\gamma_{\text{Ca}^{2+}}$ was calculated from the determined activity $a_{\text{Ca}^{2+}}$ and the concentration of calcium in the supernatant. The results are given in Table III. Due to a partial coagulation of calcium polygalacturonates the concentration of equilibrated solutions

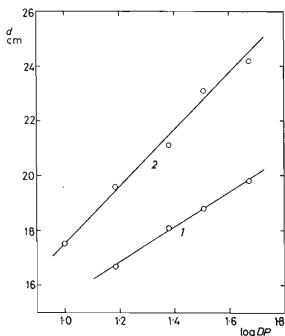


FIG. 3

Chromatography of Fragments of Polygalacturonic Acid (Na-Salt) on a Thin Layer of Sephadex G-50 (Superfine)

d Distance of spots from the base line, *DP* polymerization degree, 1, 2 analysis of I and II.

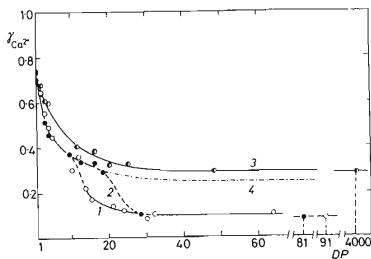


FIG. 4

Relationship between the Activity Coefficient $\gamma_{\text{Ca}^{2+}}$ in Solutions of Calcium Oligo- and Polygalacturonates and Their Polymerization Degree

1 Galacturonate, 2 guluronate, 3 mannuronate, 4 theoretical $\gamma_{\text{Ca}^{2+}}$ values in calcium polygalacturonate and polyguluronate solutions.

varied in a 0.4 to 1.2 mmol [Ca]. l⁻¹ range depending mainly on the polymerization degree of polygalacturonic acid. Different concentrations of solutions used for the $a_{\text{Ca}^{2+}}$ activity determination did not influence the $\gamma_{\text{Ca}^{2+}}$ value. As already shown¹³ the activity coefficient $\gamma_{\text{Ca}^{2+}}$ in dilute calcium pectinate solutions of a low esterification degree, *i.e.* in solutions of a polyelectrolyte with a high linear charge density of macromolecules did not depend on the concentration of solutions. The interaction of Ca²⁺ ions with the carboxyl groups of oligo- and polygalacturonates is being judged according to the single-ion activity coefficient of counterions $\gamma_{\text{Ca}^{2+}}$.

TABLE III
Activity Coefficient $\gamma_{\text{Ca}^{2+}}$ in Solutions of Calcium Oligo- and Polygalacturonates

Sample	DP	c mmol [Ca]. l ⁻¹	$a_{\text{Ca}^{2+}} \cdot 10^3$	$\gamma_{\text{Ca}^{2+}}$
1 ^a	1	1.500	1.095	0.730 ± 0.007
2 ^a	2	1.500	0.972	0.648 ± 0.005
3 ^a	3	1.500	0.834	0.556 ± 0.003
4 ^a	4	1.500	0.737	0.491 ± 0.003
5	5	1.500	0.685	0.456 ± 0.002
6	10.0	0.472	0.144	0.305
7	11.8	0.421	0.157	0.362 ± 0.011
		0.407	0.143	
8	13.9	0.530	0.106	
		0.515	0.115	0.223 ± 0.016
		0.515	0.127	
9	15.3	0.558	0.096	0.173 ± 0.002
		0.567	0.099	
10	21	1.062	0.140	0.142 ± 0.010
		0.864	0.131	
11	24	1.242	0.143	0.121 ± 0.007
		1.284	0.165	
12	30	1.399	0.128	0.086 ± 0.006
		1.405	0.113	
13	32	1.678	0.162	0.106 ± 0.009
		1.372	0.158	
14	64	1.005	0.090	
		0.855	0.102	0.106 ± 0.010
		0.969	0.105	

^a Data measured earlier¹⁶.

The relationship between $\gamma_{\text{Ca}^{2+}}$ and the polymerization degree DP of calcium oligo- and polygalacturonates is shown in Fig. 4 (curve 1). Considering a further interpretation of results the data in Fig. 4 were supplemented by the same relationship of calcium guluronates (curve 2) and manuronates (curve 3) determined earlier¹². Curve 4 displays the theoretical course of the function calculated for calcium galacturonates and guluronates providing that a pure electrostatic interaction of Ca^{2+} with carboxyl groups was involved. The theoretical values were calculated on the basis of analogy with other reported values of activity coefficients γ_M in solutions of poly-anions with different linear charge densities in which an interaction of mere electrostatic nature was supposed^{25,26}. Particularly the $\gamma_{\text{Ca}^{2+}}$ values determined in solutions of calcium salts of carboxymethylcellulose were concerned²⁶. The $\gamma_{\text{Ca}^{2+}}$ values determined in calcium polymannuronate solutions¹² and the distances b of the neighbouring carboxyl groups in the molecule of calcium polymannuronate²⁷ ($b = 0.50$ nm), polygalacturonate²⁸ and polyguluronate²⁹ ($b = 0.435$ nm) in perpendicular projection on the main axis of linear macromolecules were taken when calculating these values.

Curves 1 and 2 show that the experimentally obtained values of $\gamma_{\text{Ca}^{2+}}$ lay in the range of shorter macromolecules of polyuronates (galacturonates $DP \leq 11$; guluronates $DP \leq 18$) on the theoretical curve. At a higher polymerization degree of calcium polygalacturonates (curve 1) an unexpected drop of the activity coefficient $\gamma_{\text{Ca}^{2+}}$ took place similarly as in solutions of calcium polyguluronates¹² (curve 2). The activity coefficients $\gamma_{\text{Ca}^{2+}}$ are in the region of higher DP values of these polyuronates by several times lower than the theoretical values.

The sudden decrease of the activity coefficient $\gamma_{\text{Ca}^{2+}}$ in the solution of calcium polygalacturonate was accompanied by a partial coagulation of the polyuronate during the neutralization of polygalacturonic acid with calcium hydroxide. A similar phenomenon was encountered with calcium or strontium pectinate having a low esterification degree. The anomalous course of the curve $\gamma_{\text{Ca}^{2+}} = f(DP)$ could be caused, due to the partial coagulation of calcium polygalacturonate, by a fractional coagulation according to a different polymerization degree of the individual molecules in samples. We have already shown that all samples of polygalacturonates are polydisperse to a low extent only. Moreover, we have determined the polymerization degree both in the clear supernatant and also in the coagulate of polygalacturonate in all samples. The polymerization degree was found to be in both cases virtually the same with deviations within the analytical error.

We suppose that clear solutions of the respective polyuronates (the supernatants) are not molecularly disperse but contain soluble aggregates of macromolecules, since the anomalously low $\gamma_{\text{Ca}^{2+}}$ values in solutions of calcium polygalacturonates and also in solutions of calcium pectinates are always accompanied by a partial coagulation of polyuronates during preparation of their solutions. The anomalously low activity

coefficients $\gamma_{Ca^{2+}}$ in solutions under study can be associated with an intermolecular binding of Ca^{2+} ions in aggregates of macromolecules.

The Proof of Existence of Macromolecule Aggregates in Solutions of Calcium Pectate and Calcium Polygluronate

Activities $a_{Ca^{2+}}$ were measured in supernatants obtained by centrifugation of gel-like suspensions of Ca-polygalacturonate at 13000 *g*. These supernatants containing the soluble Ca-polygalacturonate were clear and easily passed the most dense analytical filter papers (Schleicher & Schüll, No 589³; Filtrak No 390, GDR) almost without any change in concentration. The solutions had a very low concentration (1–2.5 mequiv. $[-COOCa_{0.5}] \cdot l^{-1}$) and therefore, an analytical ultracentrifuge could not be used in evidencing the formation of aggregates; a preparative ultracentrifuge was found to be appropriate. Supernatants of various polyuronic acids, carboxymethylcellulose (H^+) and their potassium and calcium salts obtained by centrifugation at 13000 *g* were further centrifuged in a preparative ultracentrifuge up to 190000 *g* (30 min). The concentration of polyuronates determined in supernatants was compared with that of the starting solutions obtained by centrifugation at 13000 *g*. The measurements

TABLE IV
Concentration Changes of Polyacids and Their Salts at Various Centrifugal Accelerations

Sample	Concentration $[-COOM]^a$ after centrifugation		
	13000 <i>g</i> mequiv. l^{-1}	190000 <i>g</i> mequiv. l^{-1}	%
H-Carboxymethylcellulose	2.12	2.20	104
Polymannuronic acid	1.45	1.45	100
Pectic acid	4.69	4.67	99.5
Polyguluronic acid	2.41	2.05	85
K-Polymannuronate	2.50	2.48	99
K-Pectate	2.26	2.28	101
Ca-Carboxymethylcellulose	2.13	2.15	101
Ca-Polymannuronate	2.48	2.46	99
Ca-Pectate	2.40	1.63	68
	2.22	1.33	60
Ca-Polyguluronate	1.16	0.56	48
	1.22	0.40	33

^a M = H, K, Ca_{0.5}.

were mostly done with approximately 1.2 to 2.5 mequiv. $[-COOM] \cdot 1^{-1}$ solutions, where $M = H, K$ or $Ca_{0.5}$, *i.e.* at a like concentration employed in determination of the activity $a_{Ca^{2+}}$. The obtained data are listed in Table IV and seen in Fig. 5.

Solutions of carboxymethylcellulose (H^+), polymannuronic and pectic acids can be most probably considered molecular disperse systems, since no change of concentration in the solution occurred at a high centrifugal acceleration excepting polyguluronic acid which tends to form aggregates particularly at a lower polymerization degree¹².

Potassium salts of all polyacids are well soluble and do not form aggregates of macromolecules. The concentration of Ca-carboxymethylcellulose and Ca-poly-mannuronate solutions does not change upon centrifugation; this fact indicates that even in this case molecular disperse systems are probably involved. These polyelectrolytes are supposed to have Ca^{2+} cations bound to isolated macromolecules merely by electrostatic attractive forces. On the other hand, the concentration of Ca-pectate and Ca-polyguluronate solutions was substantially lowered with the increase of the centrifugation intensity and concurrently the amount of a transparent gel sedimenting in the centrifuge tube also increased. This phenomenon indicates that in solutions of calcium polygalacturonate and polyguluronate a formation of macromolecule aggregates took place. The formation of macromolecule aggregates evidences the possibility of an intermolecular Ca^{2+} binding to carboxyl groups of two or more polyuronate chains.

DISCUSSION

The interaction of cations with anionic groups of classical synthetic polyelectrolytes, similarly as in solutions of polymannuronate and carboxymethylcellulose, is considered to be of pure electrostatic nature^{25,26,30}. In polygalacturonate and polyguluronate

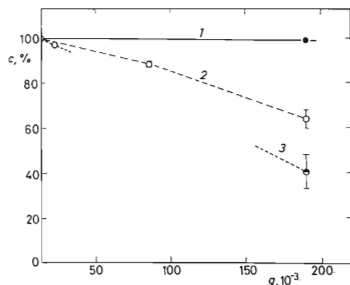


FIG. 5

Concentration Change of Ca-Polyuronate and Ca-Carboxymethylcellulose Solutions at a Various Centrifugal Acceleration

1 Ca-Poly-mannuronate, Ca-carboxymethylcellulose, 2 Ca-pectate, 3 Ca-polyguluronate.

solutions a coordination binding of Ca^{2+} ions and some other bivalent cations is involved; this includes, in addition to carboxyl groups, hydroxyl groups or oxygen atoms in "cavities" between two neighbouring uronic acid units in the macromolecular chain⁶⁻¹⁰. These "cavities" or "nests" are formed as a result of the zig-zag conformation of the linear polygalacturonate and polyguluronate macromolecules given by their diaxial *trans*-glycosidic bonds $\alpha(1 \rightarrow 4)$ (ref.^{9,10}). Various conceptions concerning the binding mechanism of bivalent cations to polyuronates were described in more detail in our previous paper¹¹.

The anomalously low activity coefficients $\gamma_{\text{Ca}^{2+}}$ found in solutions of calcium polygalacturonate ($DP > 20$) and polyguluronate ($DP > 28$) can be caused by 3 factors of additive character¹²: 1. A higher linear charge density of these polyuronates when compared with calcium polymannuronate, 2. Intermolecular binding of Ca^{2+} to carboxyl groups of various macromolecules of polyuronate to form small soluble aggregates, 3. Conformation of macromolecules. This geometry of the ligand determines both the distance between carboxyl and hydroxyl groups or between oxygen atoms involved in the process of binding, and the ability to cluster the macromolecules in aggregates with oriented chains.

Experimental results of this project offer the answer whether in solutions of calcium polygalacturonate and polyguluronate a coordination binding of Ca^{2+} ions to isolated macromolecules according to conception of Smidsrød and coworkers⁹ or a cooperative mechanism of Ca^{2+} ion binding to two or more oriented polyuronate chains as described by Rees and coworkers¹⁰ (the "egg-box" model) are involved.

Basing upon both stability constant values of acetyl derivatives of calcium pectate and pectinate³¹ and the activities $a_{\text{Ca}^{2+}}$ determined in solutions of lower calcium oligogalacturonates¹⁶ we have shown that the intramolecular chelate binding of Ca^{2+} ions according to conception of Schweiger⁶⁻⁸ is unlike due to the steric hindrance. Results summarized in Fig. 4 proved that in the region of low-molecular fragments of polyuronates (galacturonates $DP \leq 11$, guluronates $DP \leq 18$) the interaction of Ca^{2+} ions with their carboxyl groups is of electrostatic nature. The difference in $\gamma_{\text{Ca}^{2+}}$ values determined in solutions of low molecular fragments of polymannuronate on one hand and polygalacturonate and polyguluronate on the other, corresponded to the difference in the linear charge density of the polyanions under investigation. Should the Ca^{2+} ions be bound by an intramolecular chelate binding to the isolated chains of macromolecules according to the conception of Smidsrød and coworkers⁹ then the function $\gamma_{\text{Ca}^{2+}} = f(DP)$ determined in solutions of calcium galacturonates and guluronates had to have a smooth course without any sudden drop similarly, as in solutions of mannuronates.

We brought (by means of a preparative ultracentrifuge) the evidence that clear solutions of calcium polygalacturonate and polyguluronate revealing the anomalously low $\gamma_{\text{Ca}^{2+}}$ values are not molecular disperse, but contain small soluble aggregates

of macromolecules. The presence of aggregates of macromolecules has not been found either in the solution of calcium polymannuronate or Ca-carboxymethylcellulose, where the binding of Ca^{2+} ions of merely electrostatic nature has been presumed. The existence of macromolecule aggregates in solutions of calcium polygalacturonate and polyguluronate evidenced the intermolecular binding of Ca^{2+} ions to carboxyl groups of two or more chains of macromolecules in accordance with the conception of the binding mechanism as described by Rees and coworkers¹⁰ (the "egg-box" model). According to these authors the high selectivity of polyguluronate and pectate³¹ in exchange reactions of cations is given by the mutually suitable sizes of a particular cation and the cavity between the oriented chains of macromolecules.

The intermolecular binding of Ca^{2+} ions to the above-mentioned polyuronates offers further the possibility to contact directly Ca^{2+} ions with oxygen atoms in cavities between two oriented chains of macromolecules according to the conception of Rees and coworkers¹⁰. The activity coefficient $\gamma_{\text{Ca}^{2+}}$ determined in solutions of calcium polygalacturonate and polyguluronate are by several times lower than the theoretical $\gamma_{\text{Ca}^{2+}}$ values corresponding to an exclusively electrostatic binding. The obtained results prove therefore the conception of an intermolecular chelate binding. It was further shown that the Ca^{2+} binding to isolated polyuronate macromolecules involves only a pure electrostatic binding of cations, irrespective of the structure of polyuronates. The same character of cation binding is taken into consideration with classical polyelectrolytes.

Our thanks are due to Mr M. Bystran for experimental assistance.

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Translated by Z. Votický.