BINDING OF CADMIUM AND COPPER(II) IONS TO OLIGOGALACTURONIC ACIDS*

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The binding of Cu^{2+} and Cd^{2+} ions to potassium oligogalacturonates of polymerization degree *DP* 1 to 9 was investigated by ion-specific electrodes and by the metallochromic indicator method. The interaction was evaluated on the basis of degree of association β of cations (M^{2+}) with carboxyl groups of the compounds examined and the signle-ion activity coefficients of counterions $\gamma_{M^{2+}}$. The results were compared with values corresponding to an electrostatic bond of Ca^{2+} ions to these oligomers. The Cd^{2+}, and especially Cu^{2+} ions were bound in a considerable measure already to mono- and oligogalacturonates of a low polymerization degree very probably by a chelate bond. Precipitation of Cu- and Cd-oligogalacturonates takes place at a polymerization degree *DP* 5 and 8, respectively. Interpretation of results by the multiple equilibria theory evidenced that the Cu^{2+} ions were stoichiometrically bound to mono- and oligogalacturonates (one cation to two carboxyl groups) similarly, as with polymeric D-galacturonan. The results revise the early conception concerning the formation of a positively charged complex of Cu^{2+} ions (similarly also of Cd^{2+} and Pb^{2+} ions) with monogalacturonic acid of $(MA)^+$ type, where A^- is the anion of uronic acid. The results are a contribution to the elucidation of the elimination mechanism of these toxic cations from human body involving the oligomeric fragments of pectin as products of its enzymic degradation in the digestive tract.

Our preceding papers[1,2] investigated the binding of Pb^{2+} and Cd^{2+} ions to pectin and of Pb^{2+} to plant tissues of some nutritionally important vegetables and fruits[3] aiming to throw more light to the function of pectin as a prophylactic and remedy against poisoning caused by those toxic cations. Elucidation of the complex mechanism of lead elimination from the body of an experimental animal, or human at a peroral application of pectin (*e.g.* ref.[4-6]), which can proceed also outside the digestive tract, required more investigation of the binding of lead cations to oligomeric precisely defined pectin fragments[7].

Makridou and coworkers[8] evidenced that Pb^{2+}, Cd^{2+} and Cu^{2+} (M^{2+}) ions are relatively firmly bound even to monomeric uronic acids (D-galacturonic and D-glucuronic acids); they assume a formation of a positively charged complex of $(MA)^+$ type, where A^- is the anion of uronic acid. Aruga[9] has proved on the basis of calorimetric investigations that in addition to the carboxyl group also the oxygen atom

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of the pyrane ring participates in the formation of the complex through an outer-sphere electrostatic bond. As we have already found[7] the increasing polymerization degree of oligogalacturonic acids strongly decreases the solubility of their lead salts in water; the strength of the Pb^{2+} bond to carboxyl groups of oligomers, on the other hand, increases. The Pb^{2+} ions were bound to the pentamer quite firmly, virtually in the same way as to the polymeric chain of D-galacturonan (pectate), where the stoichiometric bond of Pb^{2+}, Cd^{2+}, Ca^{2+} (ref.[1,2,10]) and further bivalent cations[11] was evidenced. Keeping these facts in mind, the binding mode of Pb^{2+}, Cd^{2+} and Cu^{2+} ions to D-galacturonic acid and to low-molecular oligogalacturonic acids remains open; it is to be decided whether stoichiometric bond of these bivalent cations (one M^{2+} cation per 2 carboxyl groups), or a complex of $(MA)^+$ type are involved.

In continuation to the afore-mentioned papers, this contribution deals with the examination of binding of Cd^{2+} and Cu^{2+} ions to a broader series of oligogalacturonic acids of a polymerization degree *DP* 1 to 9. Interaction of these cations with carboxyl groups of oligogalacturonates was considered according to the activities of the respective counterions determined by ion-specific electrodes, or by the metallochromic indicator method.

EXPERIMENTAL

The purified commercially available pectin (Genu Pectin, type A, Medium Rapid Set, Københavns Pektinfabrik, Denmark) was the starting material for preparation of oligogalacturonic acids. Sodium salts of these acids of polymerization degree *DP* 2 to 9 were prepared and characterized by methods already reported[12], by a partial acid hydrolysis of pectic acid, by rechromatography and desalting on Sephadex G-25 (Fine) and Sephadex G-10 columns. Chromatographic separation of higher oligomers (*DP* 7 to 9) was 3 to 4 times repeated with a narrow selection region of suitable fractions. Sodium oligogalacturonates were chemically pure; purity of preparations was considered according to the ratio of terminal reducting and carboxyl groups with lower oligomers, and according to a linear course of the function $\log (1/R_F - 1) = f(DP)$ at chromatography on Whatman No 1 paper and silica gel sheets (Silufol, Kavalier, Czechoslovakia) with the higher ones.

D-Galacturonic acid (monohydrate), *puriss.*, was a Fluka (Swiss) preparation, tetramethylmurexide was prepared from caffeine *via* tetramethylalloxanthine[13,14]. Chemicals were of *p.a.* grade, 0·05M-KOH carbonate free and redistilled water carbon dioxide free. Compensation spectrophotometer UVISPEC (Hilger), digital pH-meter Radiometer (Copenhagen), PHM 64, glass electrode Radiometer, type 222 B, and reference saturated calomel electrode, type K 401 were used. The ion-specific electrodes Crytur (Monokrystaly, Turnov, Czechoslovakia), type 48—17 (Cd), type 29—17 (Cu) and a reference saturated calomel electrode with a double salt bridge Radiometer, type K 711, were employed for determination of activity of Cd^{2+} and Cu^{2+} ions. The outer cell of the reference electrode was filled with a 10% KNO_3 solution.

Determination of Cd^{2+} and Cu^{2+} Ion Activities in Cadmium and Copper Oligogalacturonate Solutions by Ion-specific Electrodes

Cadmium and copper oligogalacturonate solutions were prepared as already reported[7]. Sodium oligogalacturonate solution was transformed into potassium salt by percolation through a Dowex

50W $(H)^+$ column followed by neutralization with 0·05M-KOH. An equivalent amount of 0·01M-$Cd(NO_3)_2$ or 0·01M-$Cu(NO_3)_2$ was added to the potassium oligogalacturonate solution. The ionic strength of the solution was adjusted by addition of 0·1M-KNO_3. The Cd^{2+} and Cu^{2+} ion activities were determined in 3 mmol $(COOM_{0.5})\,l^{-1}$ (M = Cd, Cu) oligogalacturonate solutions at a starting ionic strength $I_0 = 0{\cdot}01$ mol l^{-1}. If a partial precipitation of copper oligogalacturonates ($DP \geqq 5$) or cadmium oligogalacturonates ($DP \geqq 8$) during preparation of solutions took place, the suspension of oligogalacturonates was centrifuged at 20 000*g* for 15 min. The $a_{Cu^{2+}}$, and $a_{Cd^{2+}}$ activities were determined in supernatants in which simultaneously the total concentration of copper or cadmium was estimated.

The $a_{Cd^{2+}}$ and $a_{Cu^{2+}}$ activities were determined by ion-specific electrodes at 25·0 ± 0·1°C the solutions being stirred at a constant rate. The equilibrium potential of the indication electrode was read after 10 and 15 min. The calibration curves mV = f(pCd, pCu) were separatedly determined for each series using fresh solutions. (The calibration solutions contained $Cu(NO_3)_2$, KNO_3, $I_0 = 0{\cdot}01$, or 0·02 mol l^{-1}; $Cd(NO_3)_2$, KNO_3, $I_0 = 0{\cdot}01$ mol l^{-1}). Tabulated values of the single-ion activity coefficients $\gamma_{Cd^{2+}}$ and $\gamma_{Cu^{2+}}$ calculated by Kielland according to the theory of strong electrolyte solutions[15] were employed.

The total concentration of copper and cadmium in the 0·01M-$Cu(NO_3)_2$, 0·01M-$Cd(NO_3)_2$ stock solutions and in supernatants of the corresponding oligogalacturonates after centrifugation of the suspension were determined chelatometrically by a spectrophotometric indication of the point of equivalence; employed were 0·01M, or 0·002M-Complexon IV solutions, an interference filter 1F 600 nm (Zeiss, Jena), and murexide as an indicator. Concentration of cadmium was corrected against the blank. The total copper content was determined in solutions of 0·1 to 1·2 mmol (Cu) l^{-1} with an error not exceeding ±0·8%, that in cadmium solutions of 0·1 mmol (Cd) l^{-1} concentration ±2·0%, and at higher concentration less than ±1%.

Concentration of free Cd^{2+} *and* Cu^{2+} *ions* in oligogalacturonate solutions was calculated from the measured activities $a_{Cd^{2+}}$ and $a_{Cu^{2+}}$ using single-ion activity coefficients $\gamma_{Cd^{2+}}$ and $\gamma_{Cu^{2+}}$ corresponding to the ionic strength of the equilibrium solution. The ionic strength of the starting solution $I_0 = 0{\cdot}01$ mol·l^{-1} undergoes a little change due to the binding of counterions to carboxyl groups, this being reflected in some decrease of its value. The corrected ionic strength I corresponding to the equilibrium solution was calculated by the iterative procedure described in the preceding paper[7].

The degree of association β of bivalent cations (M^{2+}) with carboxyl groups is given by expression

$$\beta = (c_{M^{2+}} - [M^{2+}])/c_{M^{2+}}, \qquad (1)$$

where $c_{M^{2+}}$ is the starting concentration of bivalent cations of oligogalacturonates under investigation ($c_{M^{2+}} = 1{\cdot}500$ mmol l^{-1}) and $[M^{2+}]$ the concentration of free cations in the equilibrium solution, or in the supernatant.

The number of binding sites n of Cu^{2+} cations linked to one uronic acid unit in the oligogalacturonate molecule was determined using the multiple equilibria theory[16], equation (*2*):

$$r^{-1} = (nK[Cu^{2+}])^{-1} + n^{-1}, \qquad (2)$$

where r stands for the number of Cu^{2+} ions bound to one uronic acid unit in the oligomer molecule, or to another suitably chosen iterative segment of the molecule with n binding sites, K is the stability constant of the respective copper oligogalacturonate and $[Cu^{2+}]$ the concentration of free copper ions in the equilibrium solution. The ordinata section was determined and the

n value calculated by extrapolation of the linear function (*2*), $r^{-1} = f([Cu^{2+}])^{-1}$, to value $[Cu^{2+}]^{-1}$ limiting to zero.

Determination of Free Cd^{2+} Ion Concentration in Solutions of Cadmium Oligogalacturonates by the Metallochromic Indicator Method

A similar technique was employed for measurement as reported[17,18] for Ca^{2+} and Sr^{2+} activity determinations using tetramethylmurexide (TMM) as the metallochromic indicator in cadmium oligogalacturonate solutions of 0·2 mmol $(COOCd_{0.5})\,l^{-1}$ concentration, containing 0·01 mmol TMM in 1 l. Absorbances (A) of the solutions investigated and calibration solutions were determined at wavelengths $\lambda = 484$ and 520 nm corresponding to the absorbance maximum of the tetramethylmurexide–cadmium complex and tetramethylmurexide itself, respectively, with a $\pm 0{\cdot}001$ error; the starting ionic strength of solutions $I_0 = 0{\cdot}5$ mmol l^{-1} (KNO_3 the auxiliary electrolyte), cell-width 4 cm. Concentration of free cadmium ions $[Cd^{2+}]$ in an equilibrium solution was estimated using the calibration curve $\varphi = f([Cd^{2+}])$; $\varphi = A_{484}/A_{520}$ (ref.[19]). The total starting concentration of cadmium ions $c_{Cd^{2+}}$ for calculation of the degree of association (β) of Cd^{2+} ions with carboxyl groups of the oligomer (equation (*1*)) was corrected with respect to the amount of cadmium bound to the metallochromic indicator.

Degree of association β' of Cd^{2+} ions with the metallochromic indicator (TMM) was calculated according to equation:

$$\beta' = \frac{A - A_{(TMM)^-}}{A_{(TMMCd)^+} - A_{(TMM)^-}} \qquad (3)$$

A stands for absorbance of the solution of cadmium salt investigated, $A_{(TMM)^-}$ and $A_{(TMMCd)^+}$ for absorbances of the solution of metallochromic indicator itself and its complex with cadmium ions at $\lambda_{max} = 484$ nm. The $A_{(TMMCd)^+}$ value was determined in a solution containing $1 \cdot 10^{-5}$ mol TMM l^{-1} and 0·25 mol $Cd(NO_3)_2\,l^{-1}$.

RESULTS AND DISCUSSION

The binding of Cd^{2+} ions to carboxyl groups of oligogalacturonates was first investigated by the metallochromic indicator method (tetramethylmurexide), which was found to be well suitable for determination of Ca^{2+} and Sr^{2+} ion activities in solutions of the respective oligo- and polyuronates[12,20]. The murexide complex (ammonium 5,5-nitrilodibarbiturate) with cadmium ions[21], and also its tetramethyl derivative reveal an absorption spectrum very close to that of the calcium complex, the absorbance maximum being at 484 nm. The molar absorption coefficient for the complex $(TMMCd)^+$ was estimated $\varepsilon_{(TMMCd)^+} = 19\,980$ ($\varepsilon_{(TMMCa)^+} = 20\,000$, ref.[17]). The advantage of tetramethylmurexide over murexide is that the colour of the solution of this metallochromic indicator[22] in reaction with the above-mentioned cations is pH independent in the pH 4·7–8·1 range.

The stability constant K of the murexide complex with cadmium ions is relatively high ($\log K = 4{\cdot}15-4{\cdot}20$, pH 4–6, $I = 0{\cdot}1$ mol l^{-1}; KNO_3, ref.[21,23]); this shifts necessarily the concentration range of cadmium salt solution suitable for activity

$a_{Cd^{2+}}$ measurements towards very low values. To limit this region, the dependence of the degree of association β' of cadmium ions with metallochromic indicator on the concentration of Cd^{2+} in solution (Fig. 1) was estimated. The possibly lowest concentration of the metallochromic indicator in solution ($1 . 10^{-5}$ mol TMM l^{-1}) and a 4 cm-cell were used. The course of the curve in Fig. 1 shows that analyses can be sufficiently exact in solutions containing 0·01 to 0·10 mmol of free Cd^{2+} ions in 1 l. The stability constant of the complex tetramethylmurexide–cadmium, $\log K = 4{\cdot}54 \pm 0{\cdot}02$ ($I = 0{\cdot}1-0{\cdot}3$ mmol l^{-1}) was calculated from β' values (interval $\beta' = 0{\cdot}38-0{\cdot}74$). This, a little higher value of the stability constant K, when compared with that determined for the complex murexide–cadmium is first of all associated with considerably different values of ionic strengths in the equilibrium solutions examined. The error of one determination of the concentration of free Cd^{2+} ions in solution at a 0·05 – 0·10 mmol $(Cd^{2+})\, l^{-1}$ was $\pm 1{\cdot}5$ to $\pm 2{\cdot}0\%$.

Concentration of free Cd^{2+} ions in solutions of cadmium oligogalacturonates was determined in very dilute solutions of 0·2 mmol $(COOCd_{0.5})\, l^{-1}$ at an ionic strength $I_0 = 0{\cdot}5$ mmol l^{-1}; analyses were 6 to 8 times repeated. The total starting concentration of cadmium in solution ($c_0 = 0{\cdot}1$ mmol $(Cd)\, l^{-1}$) was corrected by the amount of Cd^{2+} ions bound to the metallochromic indicator, calculated using the function $\beta' = f([Cd^{2+}])$ described in Fig. 1. The corrected ionic strength I, corresponding to equilibrium solutions and varying within 0·47 to 0·37 mmol l^{-1} served for calculation of $[Cd^{2+}]$ and β values; results are summarized in Table I; c_{Cd} is the corrected value of the total concentration of cadmium in the equilibrium solution, $[Cd^{2+}]$ the concentration of free cadmium ions and β the degree of association of cadmium ions with carboxyl groups of oligogalacturonates. Dependence of β of these oligomers on the polymerization degree DP is presented by curve 2 in Fig. 2. For comparison purposes this dependence is shown for calcium ions, as well (curve 1); employed were values of calcium ion activities in calcium oligogalacturonates already determined[12,24]. Since the activities $a_{Ca^{2+}}$ were determined at a substantially higher concentration of calcium oligogalacturonate solutions ($c = 3$ mmol $(COOCa_{0.5})\, l^{-1}$), values corresponding to 0·2 mmol $(COOCa_{0.5})\, l^{-1}$) concentration were obtained employing the multiple equilibria theory providing a stoichiometric binding of Ca^{2+} ions to carboxyl groups of oligogalacturonates.

As it follows from comparison of the course of both curves, the Cd^{2+} ions are bound in a far greater measure to carboxyl groups of oligogalacturonates than the Ca^{2+} ions. It could be assumed that here a chelate bond of cadmium was involved, whereas calcium ions were bound to these oligomers by a pure electrostatic bond[24,25]. The degree of association of calcium ions continuously increases with the polymerization degree. Curve 2 describing the binding of cadmium ions shows a flexure towards higher β values in the range of $DP \geqq 4$, this being an evidence for a stronger bond of Cd^{2+} ions to these oligomers. So far, we are unable to explain unequivocally this phenomenon.

TABLE I

Binding of Cd^{2+} ions to oligogalacturonates (metallochromic indicator method) $c = 0{\cdot}200$ mmol $(COOCd_{0.5})\,l^{-1}$, $I_0 = 0{\cdot}5$ mmol l^{-1}

DP	c_{Cd} mmol l^{-1}	$[Cd^{2+}]$ mmol l^{-1}	β
1	0·0927	0·0894 ± 0·0008	0·036
2	0·0928	0·0858 ± 0·0012	0·075
3	0·0928	0·0829 ± 0·0009	0·107
4	0·0930	0·0752 ± 0·0009	0·191
5	0·0932	0·0665 ± 0·0010	0·286
7	0·0935	0·0548 ± 0·0007	0·414

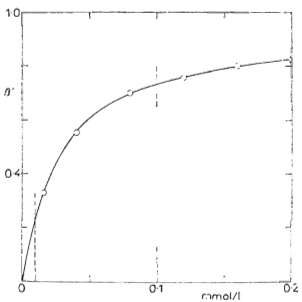

FIG. 1

Binding of Cd^{2+} to tetramethylmurexide (TMM) ($c_{TMM} = 1 \cdot 10^{-5}$ mol l^{-1}). Degree of association β' of Cd^{2+} ions with tetramethylmurexide; $[Cd^{2+}]$ concentration of free Cd^{2+} ions in solution

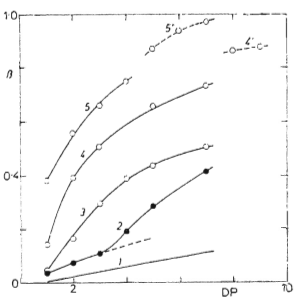

FIG. 2

Binding of Ca^{2+}, Cd^{2+} and Cu^{2+} ions to potassium oligogalacturonates. Degree of association β of M^{2+} ions with carboxyl groups of oligogalacturonates; *DP* polymerization degree of oligomers; *1*, *2* $c = 0{\cdot}200$ mmol $(COOM_{0.5})\,l^{-1}$. $I_0 = 0{\cdot}5$ mmol l^{-1}; *3*, *4*, *5* $c = 3{\cdot}00$ mmol $(COOM_{0.5})\,l^{-1}$, $I_0 = 0{\cdot}01$ mol l^{-1}; *1*, *3* Ca^{2+}; *2*, *4* Cd^{2+}; *5* Cu^{2+}; *4'*, *5'* formation of Cd- or Cu-oligogalacturonate precipitate

Binding of Cd^{2+} ions to potassium oligogalacturonates was further studied by an ion-specific electrode (Crytur). This method enabled to determine the activity of cadmium counterions at a substantially higher concentration of solutions (c_0 = 3 mmol $(COOCd_{0.5})\,l^{-1}$, I_0 = 0·01 mol l^{-1}, t = 25°C), *i.e.* at a concentration used in our preceding studies. The pH of calibration and studied solutions varied within 6·0–6·5. The function EMF = f(pCd) was linear in the investigated range of activities ($a_{Cd^{2+}} = 1 \,.\, 10^{-3} - 1 \,.\, 10^{-4}$); the line slope $\Delta mV/\Delta pCd$ was a little higher (−31 mV to −35 mV) than that corresponding to the Nernst factor 29·6 mV at 25°C. The stabilization of the electrode potential was not quite satisfactory – the error of one activity determination $a_{Cd^{2+}}$ was ±8%. The measurements were several times repeated and results listed in Table II. Values I refer to the corrected ionic strength corresponding to the equilibrium solutions. The concentration of free Cd^{2+} ions in solution and degree of association β were calculated using I values. The counterion activity coefficients $\gamma_{Cd^{2+}}$ could be mutually compared at the same concentration of solutions of cadmium oligogalacturonates only, since the value $\gamma_{Cd^{2+}}$ is a function of the concentration of the cadmium salt of oligomers in contrast to polymers. This is the reason why Table II lists the $\gamma_{Cd^{2+}}$ values exclusively for solutions of oligomers of polymerization degree DP 1–7.

The relationship of β and $\gamma_{Cd^{2+}}$ upon polymerization degree of oligogalacturonates DP is plotted in Fig. 2 (curves *4* and *4'*) and in Fig. 3 (curve 2); curve *4'* corresponds to solutions where a partial coagulation of cadmium oligogalacturonates took place. The values β and $\gamma_{Ca^{2+}}$ are presented for comparison purposes (Fig. 2, curve 3, Fig. 3, curve *1*); they correspond to binding of calcium ions to these oligogalacturo-

TABLE II

Binding of $Cd^{2}{}_{+}$ ions to oligogalacturonates (ion-specific electrode) c_0 = 3·00 mmol $(COOCd_{0.5})$ l^{-1}, I_0 = 0·01 mol l^{-1}

DP	c_{Cd} mmol l^{-1}	$a_{Cd^{2+}} \,.\, 10^{-3}$	$\gamma_{Cd^{2+}}$	I mol l^{-1}	β
1	1·500	0·868 ± 0·033	0·579	0·0094	0·15
2	1·500	0·628 ± 0·051	0·419	0·0083	0·39
3	1·500	0·516 ± 0·015	0·344	0·0077	0·51
5	1·500	0·364 ± 0·035	0·243	0·0071	0·66
6	1·500	0·337 ± 0·000	0·225	0·0069	0·68
7	1·500	0·284 ± 0·007	0·189	0·0067	0·74
8[a]	0·762 ± 0·015	0·151 ± 0·008	—	0·0061	0·86
9[a]	0·794 ± 0·027	0·137 ± 0·011	—	0·0061	0·87

[a] Precipitation of Cd-oligogalacturonate.

nates, as calculated from data determined by the metallochromic indicator method[12,24]. (Application of metallochromic indicator TMM and ionspecific electrodes (Orion) for activity determination of Ca^{2+} ions in solutions of calcium oligogalacturonates[25,26] and acid polysaccharides[27] offers identical results). Results summarized in Fig. 2 and 3 document that already oligomers with a low polymerization degree bind selectively Cd^{2+} ions at a $Cd^{2+} \rightarrow Ca^{2+}$ cation exchange similarly, as found with polymeric D-galacturonan, or pectin[2]. The Cd^{2+} ions are bound in a lesser extent even to monomeric D-galacturonic acid in accordance with results by Makridou and coworkers[8]. The ion-specific electrode does not offer a sufficiently precise measurement of $a_{Cd^{2+}}$ activity and therefore, it was impossible to apply this method to a more detailed study of the mechanism of Cd^{2+} bond to oligogalacturonates.

Binding of Cu^{2+} Ions to Potassium Oligogalacturonates

The activity of Cu^{2+} ions in copper oligogalacturonates was determined by ion-specific electrodes at the same experimental conditions as with binding of Cd^{2+} to these substances. The pH values of equilibrium solutions varied within 5·25–5·71 due to hydrolysis of copper salts. The indication electrode gave extraordinarily stable and reproducible EMF values ($\Delta EMF \leqq \pm 0{\cdot}2$ mV). The function $EMF = f(pCu)$ has a linear course in the activity range under investigation ($a_{Cu^{2+}} = 1 \,.\, 10^{-3}$ to $2 \,.\, 10^{-5}$) with the line slope $\Delta mV/\Delta pCu$ in the $-29{\cdot}6$ to $-30{\cdot}7$ mV range. The error of one activity determination $a_{Cu^{2+}}$ at $I_0 = 0{\cdot}01$ mol l^{-1} was less than $\pm 2\%$ and $\pm 1\%$ at $I_0 = 0{\cdot}02$ mol l^{-1}.

The results are summarized in Table III; the particular symbols have an analogous meaning as given for determination of cadmium ion activities. Greater deviations of $a_{Cu^{2+}}$ values in solutions of higher oligomers (DP 5–7) are due to a greater variation of $c_{Cu^{2+}}$ concentration in supernatants. The dependence of degree of association β of Cu^{2+} ions with carboxyl groups of oligomers and of activity coefficient of counterions $\gamma_{Cu^{2+}}$ upon polymerization degree of oligogalacturonates is plotted in Fig. 2 (curves *5* and *5'*) and Fig. 3 (curve *3*). A relatively large amount of Cu^{2+} ions is bound already to D-galacturonic acid, $\beta = 0{\cdot}38$. Starting with the pentamer ($DP \geqq 5$), the solubility of Cu-oligogalacturonates in water drops strongly, similarly as does the activity of couterions Cu^{2+} in equilibrium solutions. Only 9% of the starting concentration of Cu-oligogalacturonate remains dissolved with hepta-D-galacturonic acid; 97% of the Cu^{2+} ions added are bound to carboxyl groups of the oligomer in the precipitate and solution ($\beta = 0{\cdot}97$). Values determined in our preceding paper[7] for the binding of Pb^{2+} ions to oligogalacturonates and polymeric D-galacturonan (pectate) are presented just for comparison: DP 1, $\beta = 0{\cdot}43$; $DP = 5$, $\beta = 0{\cdot}96$; pectate, $\beta = 0{\cdot}96-0{\cdot}97$. Considered by analogy, the Cu^{2+} ions are bound to the heptamer by virtually the same way as to polymeric D-galacturonan chain.

Comparison of curves $\beta = f(DP)$, $\gamma_{M^{2+}} = f(DP)$ corresponding to binding

of ions Cu^{2+}, Cd^{2+}, and Ca^{2+} to oligogalacturonates also shows the order of binding selectivity of these cations to carboxyl groups of oligogalacturonates ($Cu^{2+} > Cd^{2+} > Ca^{2+}$), in agreement with findings by Makridou and coworkers[8], who found the same order of selectivity of binding of copper and cadmium cations to monomeric D-galacturonic and D-glucuronic acids. (Binding of calcium ions ($\beta = 0{\cdot}04$) and strontium ions ($\beta = 0{\cdot}01$) to D-galacturonic acid[20] is very small; the values measured are close to the experimental error.)

TABLE III

Binding of Cu^{2+} ions to oligogalacturonates (ion-specific electrode), c_0 3·00 mmol ($COOCu_{0.5}$) . l^{-1}; $I_0 = 0{\cdot}01$ mol l^{-1}

DP	c_{Cu} mmol l^{-1}	$a_{Cu^{2+}} . 10^3$	$\gamma_{Cu^{2+}}$	*I* mol l^{-1}	β
1	1·500	0·648 ± 0·011	0·432	0·0083	0·38
2	1·500	0·468 ± 0·004	0·312	0·0075	0·56
3	1·500	0·369 ± 0·002	0·246	0·0071	0·66
4	1·500	0·268 ± 0·006	0·179	0·0066	0·75
5[a]	0·473 ± 0·018	0·139 ± 0·001	—	0·0061	0·87
6[a]	0·173 ± 0·006	0·071 ± 0·004	—	0·0058	0·94
7[a]	0·132 ± 0·014	0·038 ± 0·005	—	0·0057	0·97

[a] Precipitation of Cu-oligogalacturonate.

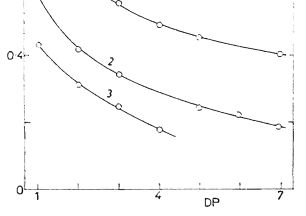

FIG. 3

Relationship of single-ion activity coefficient of counterions $\gamma_{M^{2+}}$ upon polymerization degree *DP* of cadmium and copper oligogalacturonates. ($c = 3{\cdot}00$ mmol ($COOM_{0.5}$) l^{-1}; $I_0 = 0{\cdot}01$ mol l^{-1}); *1* Ca^{2+}, *2* Cd^{2+}, *3* Cu^{2+}

Mechanism of the Interaction of Cu^{2+} Ions with Carboxyl Groups of Oligo galacturonates

The strong bond of Cu^{2+} ions, as found with oligomeric fragments of pectin, is typical also of other macromolecular carboxyl possessing polysaccharides[28], or synthetic polyelectrolytes (*e.g.*[29]). Stoichiometric bond of Cu^{2+} ions to free (unesterified) carboxyl groups of pectates and pectinates constitutes the basis for an analytical determination of esterification degree (E) of pectin carboxyl groups with methanol by the method of precipitation of insoluble copper pectates and pectinates[30,31].

Gelation of both alginate and pectin with Cu^{2+} is accompanied by spectral changes that are opposite in sign to those observed with the other bivalent cations[32]. This is an argument for a distinct and less specific mechanism of binding in accordance with the low selectivity of Cu^{2+} ions towards polyuronates of different primary structure[28]. It could be assumed that most probably the intramolecular chelate binding is involved, because the Cu^{2+} ions are bound even to monomeric D-galacturonic acid to a considerable extent and also to more or less "isolated" free carboxyl groups in the pectin molecule of a high esterification degree ($E = 90\%$), in contrast to Ca^{2+}, or Sr^{2+} ions (ref.[20]). Recently published results of calorimetric measurements of Cu^{2+} ion binding to monomeric uronic acids and acid polysaccharides are in favour of this conclusion. According to Aruga[9], a bidental action of two different ligands is involved in formation of D-galacturonate–Cu^{2+} ion complexes: carboxylate group and oxygen atom of the pyrane ring. Results of Paoletti and coworkers[33] lead to a conclusion that a loss of considerable amount of water of the hydrate shells of both kinds of ions occurs at a strong binding of Cu^{2+} to COO^- groups of microbial "alginates". A fully hydrated gel of Cu-D-galacturonan displays a formation of a carboxylate inner-sphere complex[34] as evidenced by ESR and IR spectra.

A strong intramolecular binding of Cu^{2+} ions to pectin carboxyl groups is backed by the effect of ammonia on the Cu-pectate, or pectinate gels (results so far unpublished). An easy transition of a gel to a sol is taking place when these copper gels are treated with ammonia in a very dilute solution (0·1%). On the other hand, copper ions bound to an insoluble pectate (epichlorohydrine cross-linked) cannot be extruded by washing with dilute ammonia; the Cu^{2+} ions remained strongly bound to pectate carboxyl groups. This provides evidence on a following mechanism of Cu-pectate, or pectinate gel formation: first of all, a strong intramolecular binding of Cu^{2+} ions to pectin carboxyl groups takes place followed by an aggregation of macromolecules by a much weaker intermolecular bonds.

So far, a formation of a positively charged complex according to equation (A) is considered for binding of Pb^{2+}, Cu^{2+}, and Cd^{2+} ions to monouronic acids (D-galacturonic and D-glucuronic acids)

$$[M^{2+}] + [A^-] \rightleftharpoons [(MA)^+], \qquad (A)$$

where M^{2+} is a bivalent cation and A^- the uronic acid anion[8]. On the other hand, we have proved that Ca^{2+}, Pb^{2+}, Cd^{2+}, and Cu^{2+} ions (ref.[10,1,2,30,31]) are bound to polymeric D-galacturonan (pectate) and pectinates of a high esterification degree

of carboxyl groups exactly stoichiometrically according to equation (*B*)

$$[M^{2+}] + [L^{2-}] \rightleftharpoons [ML] \qquad (B)$$

where L^{2-} is the iterative segment of the macromolecule chain, which comprises just 2 free carboxyl groups. It could be presumed from the determined β values that Pb^{2+} and Cu^{2+} ions were stoichiometrically bound already to penta-, or hepta-galacturonate, where practically an equally strong bond of these cations to the oligomer, as well as to polymeric D-galacturonans is involved.

The high exactness of determination of $a_{Cu^{2+}}$ activities in solutions of copper salts by the ion-specific electrode (Crytur) enabled us to apply this experimental technique to clear the afore-mentioned problem. The 0·01M-$Cu(NO_3)_2$ was added in a series of analyses in an amount equivalent to 30 to 130% per carboxyl groups to the starting solution of potassium mono-, di- and tetragalacturonate (3 mmol (COOK) l^{-1}, I_0, 0·02 mol l^{-1}). Concentration of carboxyl groups in the equilibrium solution varied within 2·88 to 2·53 mmol l^{-1} due to a low dilution caused by addition of the copper salt solution. The ionic strength of equilibrium solutions I remained virtually constant for each series of analyses, since the decrease of ionic strength resulting from dilution and binding of Cu^{2+} ions to oligomer was roughly compensated by addition of the copper salt (*DP* 1, 2 and 4. I = 0·0201, 0·0197 and 0·0191 mol l^{-1}; ΔI = = ±0·0002 mol l^{-1}).

The activity of ions $a_{Cu^{2+}}$ was determined, concentration of free Cu^{2+} ions was calculated in the equilibrium solution and results were evaluated by the multiple equilibria theory (equation (*2*)); Fig. 4. First of all, the reaction mechanism (*A*)

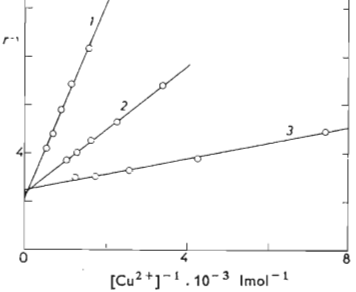

FIG. 4

Binding of Cu^{2+} ions to potassium oligogalacturonates (multiple equilibria theory)

$$[Cu^{2+}] + [A^-] \rightleftharpoons [(CuA)^+]$$

r number of Cu^{2+} ions bound to 1 uronic acid unit; $[Cu^{2+}]$ concentration of free Cu^{2+} ions in equilibrium solution; *1*, *2*, *3* mono-, di- and tetragalacturonate, respectively

was considered leading to a positively charged complex $(CuA)^+$; one uronic acid unit in monomer or oligomer was put for the ligand unit. The linear course of function $r^{-1} = f([Cu^{2+}]^{-1})$ with minimum scattering of the measured values evidences that the interaction of Cu^{2+} ions with carboxyl groups of mono- and oligomers proceeds in full agreement with the multiple equilibria theory. Therefore, the procedure described in the experimental section enables to determine the number of binding sites n of Cu^{2+} ions at the ligand unit chosen. The section at ordinata ($r^{-1} = n^{-1}$) corresponds to $r^{-1} = 2{\cdot}20$, $2{\cdot}36$ and $2{\cdot}47$ for copper mono-, di- and tetragalacturonate, respectively. The number of binding sites is then $n = 0{\cdot}450$, $0{\cdot}424$ and $0{\cdot}405$. The theoretical value for reaction mechanism (A) $n_0 = 1{\cdot}00$; for the mechanism (B), representing a stoichiometric bond of one bivalent cation to two carboxyl groups, at one uronic acid unit as the ligand unit, the value $n_0 = 0{\cdot}50$. The experimentally determined values $n = 0{\cdot}405$ to $0{\cdot}450$, close to $n_0 = 0{\cdot}50$ indicate that the Cu^{2+} ions are stoichiometrically bound even to mono- and oligogalacturonates of a low polymerization degree.

A little lower values n determined, in comparison with the theoretical value $n_0 = 0{\cdot}50$, are due to a lower pH of equilibrium solutions (pH $5{\cdot}25-5{\cdot}71$) resulting from the hydrolysis of copper salts. Dissociation of carboxyl groups of oligogalacturonic acids, and consequently the total concentration of dissociable carboxyl groups of uronates is a little lower (81 to 90% of the starting value) at lower pH. Considering this correction when calculating r, a full agreement between the measured values n and theoretical ones n_0 ($n = 1{\cdot}00$, $n_0 = 1{\cdot}00$, Fig. 5) was achieved for reaction mechanism described by equation (B) (the ligand unit is the molecule segment with two carboxyl groups, L^{2-}). The validity of the multiple equilibria theory was purposedly examined with oligomers with an even number of carboxyl groups (DP 2 and 4),

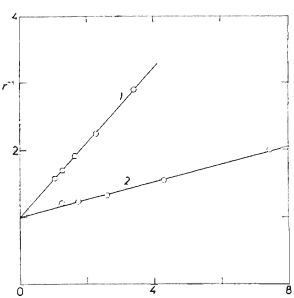

FIG. 5

Binding of Cu^{2+} to potassium oligogalacturonates (multiple equilibria theory)

$$[Cu^{2+}] + [L^{2-}] \rightleftarrows [CuL]$$

r number of Cu^{2+} ions bound to one oligomer segment, including 2 free carboxyl groups (L^{2-}); $[Cu^{2+}]$ *cf.* Fig. 4; *1*, *2* di- and tetragalacturonate, respectively

where the calculation of ligand concentration (L^{2-}) is unambiguously defined. The stability constants K for the complex (CuL), calculated from line slopes in Fig. 5 are:

$$\log K_{DP2} = 3{\cdot}26\,, \quad \text{and} \quad \log K_{DP4} = 3{\cdot}88\,(I = 0{\cdot}02 \text{ mol } l^{-1})\,.$$

Binding of Cu^{2+} ions to monomeric D-galacturonate proceeds stoichiometrically:

$$[Cu^{2+}] + 2[A^-] \rightleftharpoons [CuA_2]\,, \qquad (C)$$

with a stability constant $\log K_{DP1} = 5{\cdot}17 \pm 0{\cdot}02\,(I = 0{\cdot}02 \text{ mol } l^{-1})$. (The numerical values $\log K'_{DP1}$ and $K'_{DP2,4}$ cannot be mutually compared, as various binding mechanisms are involved.)

Bearing these facts in mind, one is entitled to presume that even Pb^{2+} and Cd^{2+} ions are bound stoichiometrically to mono- and oligogalacturonates in accordance with their stoichiometric bond to polymeric D-galacturonan chain[1,2]. Providing the stoichiometric bond of cation, the stability constant K was also calculated for the complex of cadmium cation bound to potassium digalacturonate from data obtained either by the metallochromic indicator method in very dilute solutions ($\log K_{DP2} = 2{\cdot}91$, $c = 0{\cdot}2$ mmol $(COOCd_{0.5})\,l^{-1}$, $I_0 = 0{\cdot}5$ mmol l^{-1}), or by ion-specific electrode in more concentrated solutions ($\log K_{DP2} = 2{\cdot}85$, $c = 3$ mmol $(COOCd_{0.5})\,.\,l^{-1}$, $I_0 = 0{\cdot}01$ mol l^{-1}). Both methods gave comparable values.

The presented results demonstrate a relatively strong binding of Cd^{2+} and particularly of Cu^{2+} ions to low-molecular fragments of pectin (mono- and oligogalacturonic acids). These are formed in human body by an enzymic degradation of pectin present in the food, mainly by the large intestine microflora. The results together with our preceding investigation dealing with binding of Pb^{2+} ions to oligogalacturonates are a contribution to the knowledge on the complex elimination mechanism of toxic cations from the human body through the action of pectin. The relatively strong binding of Pb^{2+}, Cu^{2+}, as well as Cd^{2+} ions to mono- or oligogalacturonic acids of the lowest polymerization degree, which are partly resorbable from the intestine, favours the conception that these substances can take part in elimination of toxic cations in another way than through the intestinal tract, *i.e.* by urine.

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