THE ACTIVITY OF CALCIUM IONS IN AQUEOUS SOLUTIONS OF THE LOWER CALCIUM OLIGOGALACTURONATES

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ABSTRACT

The activity of calcium ions in aqueous solutions of calcium mono-, di-, tri-, and tetra-galacturonates was determined by a spectrophotometric method in which tetramethylmurexide was used as an auxiliary ligand. The concentration of the solutions was 3.00 mequiv./l with respect to [$-COOCa_{0.5}$]. In this system, the activity of calcium ions in the solution of calcium galacturonate was very close to that of calcium chloride, while the activity of the calcium ions decreased in a continuous manner with increasing degree of polymerisation of the oligogalacturonate anion. The results are interpreted as evidence that, in calcium pectate, an *intra*molecular chelate bond of calcium involving two consecutive galacturonic acid units is not very probable.

INTRODUCTION

There are two points of view concerning the mechanism whereby calcium ions are bound to the polygalacturonate (pectate) anion. The observation that partial acetylation of the hydroxyl groups of the polyanion diminished its capacity to form a gel with calcium ions led Schweiger[1–3] to propose that the binding mechanism was primarily one of chelation, in which both the vicinal hydroxyl groups of a galacturonic acid unit were directly involved (see also Ref. 4).

On the other hand, Kohn and Furda[5] have argued that the interatomic distances involved are too large to be compatible with chelation in the ordinary sense. Quantitative measurements of the stability constants of partially acetylated calcium pectate and pectinate showed that, although acetylation does indeed diminish the affinity of the polyanions for calcium, a rather high affinity nevertheless remains, even when the degree of acetylation approaches the theoretical maximum of 2 (Ref. 5). It was suggested that the binding mechanism is primarily one of electrostatic attraction, and that the effect of acetylation can best be explained on the assumption that the bulky substituents sterically hinder the bond between the carboxyl groups and calcium ions[5].

In the present work, an attempt has been made to adduce further evidence on this point, by measuring the activity of calcium ions in aqueous solutions of monomeric calcium galacturonate and of some of the lower, α-(1→4)-linked calcium oligogalacturonates.

EXPERIMENTAL

Materials. — Tetramethylmurexide was prepared from caffeine, via tetramethylalloxantin, and characterised as described earlier[6]. D-Galacturonic acid was purchased from Nutritional Biochemicals Corporation, Cleveland, U. S. A. The 0.1M sodium hydroxide was carbonate-free. The aqueous calcium hydroxide was a clear, saturated solution, having a concentration of *ca.* 21.5mM. The other reagents were all of analytical grade. The water was redistilled. Pectic acid, prepared[5] from commercial apple-pectin by repeated alkaline de-esterification, contained 90% of polygalacturonic acid and had a viscosity-average molecular weight of 16,000.

Analytical methods. — The polygalacturonic acid content of the pectic acid was determined by potentiometric titration with 0.1M sodium hydroxide, and the average molecular weight was determined by viscometry[7].

Fractions obtained in gel-permeation chromatography were analysed* with 3,5-dinitrosalicylic acid[8]. They were examined qualitatively by chromatography on Whatman No. 1 paper with ethyl acetate–acetic acid–water (18:7:8, v/v) and detection with aniline hydrogen phthalate. Desalting of the fractions by gel-permeation chromatography was monitored conductimetrically. The degree of polymerisation (d.p.) of the isolated oligogalacturonates was deduced from the ratio of their carboxyl and aldehyde groups and from their chromatographic mobilities (R_F) with the help of the relationship[9–11] $\log [(1/R_F)-1] = k$ (d.p.). The content of carboxyl groups in the oligouronides was determined by potentiometric titration, as with pectic acid, after conversion into the free acids with Dowex-50W (H^+) resin.

The aldehyde groups were estimated by a modified, semimicro, hypoiodite method[12,20], using a phosphate buffer (pH 11.4). Into a 25–ml, glass-stoppered Erlenmeyer flask were pipetted 1 ml of the phosphate buffer, 1 ml of the solution containing about 1–1.5 μmoles (–CHO) of oligogalacturonic acid, and then 0.4 ml of 25mM iodine. After 3 h at 25°, the reaction mixture was acidified with 0.4 ml of M sulphuric acid, and the liberated iodine was titrated with 25mM sodium thiosulphate with starch as indicator. The consumption of iodine for a solution of D-galacturonic acid was 100.8% of the theoretical value.

Total hydrolysis of the oligogalacturonic acids was effected by heating aqueous solutions in sealed tubes with Zerolit 225(H^+) resin at 105° for 36 h.

The spectrophotometer was a Uvispec–Hilger instrument.

Preparation of di-, tri-, and tetra-galacturonic acids. — Pectic acid was converted into its sodium salt by neutralisation with sodium hydroxide, and a 1% w/v solution of the product was brought to pH *ca.* 3.3 by the cautious addition of dilute sulphuric acid. This pH was sufficiently high not to cause precipitation of pectic acid in its insoluble, gel form. The resulting solution was heated for 17 h at 100°, the pH being

*Although this reagent is considered to be specific for reducing end-groups, the alkaline peeling suffered by the lower oligogalacturonides under the conditions of the assay was so extreme that the reagent reacted sensitively with them, and provided a convenient means of following their elution from the column.

kept at 3.3 by the periodic addition of small amounts of sulphuric acid. The cooled solution was filtered and then neutralised with the minimum of barium carbonate. The filtered solution was passed down a column of Zerolit 225(H^+) resin, and the acidic effluent and washings were concentrated *in vacuo* to one third of the starting volume. Four volumes of ethanol were then added, and the higher oligomeric material that was precipitated was removed by filtration. Ethanol was removed from the filtrate by repeated distillation *in vacuo*, with periodic addition of water.

The resulting solution of lower oligosaccharides was passed down a column of Dowex-1 (acetate) resin, which was then washed with water to remove neutral sugars. Elution with 6M acetic acid then gave the oligouronides. The effluent was concentrated to dryness *in vacuo* below 35°, and residual acetic acid was removed by repeated distillation of added water. The residue was dissolved in water, the solution was neutralised to pH 7.3 with aqueous sodium hydroxide, and the sodium oligogalacturonates were isolated by freeze-drying.

Fractionation of this product was effected[13] on columns of Sephadex G-25 (fine) with a 50mM sodium phosphate buffer (pH 7.0). Appropriate fractions were combined and desalted on columns of Sephadex G-10. The individual components were further purified by a second elution from Sephadex G-25, followed by a twice-repeated desalting on Sephadex G-10. This procedure yielded the pure, sodium salts of the di-,tri-, and tetra-galacturonic acids, which were isolated as white powders by freeze-drying.

Determination of calcium-ion activity. — The sodium salt of the oligogalacturonic acid was converted into the acidic form by passage of an aqueous solution through a column of Dowex-50W(H^+) resin. The effluent was then neutralised with 21.5mM calcium hydroxide by potentiometric titration to the point of inflexion, thereby yielding a solution of the calcium salt of known concentration.

The activities of the calcium ions in the solutions of calcium oligogalacturonates were determined by a method using tetramethylmurexide as an auxiliary ligand[6]. The measurements were performed in solutions containing 3.00 mequiv./l of [–$COOCa_{0.5}$], corresponding to 1.50mM calcium. A spectrophotometric (metal–indicator) method[14,15], elaborated for the determination of the concentration of ionised calcium in biological liquids, was used. This method has been successfully applied to a study of the interaction of calcium ions with acid mucopolysaccharides[16] and with pectinic acids[17]. It has been shown[6] that this procedure can be applied directly to the determination of calcium-ion activities in solutions of calcium pectinates, and that the absorbance of a solution containing a coloured complex of tetramethylmurexide with calcium is a function of the activity of calcium ions in the solution. The calibration curves for the determination of activities of calcium ions were constructed using solutions containing calcium and potassium chlorides. Single ion activity coefficients, $\gamma_{Ca^{2+}}$, were calculated with the help of the Debye and Hückel theory of strong electrolytes, using data for mean activity coefficients, $\gamma_{\pm CaCl_2}$ and $\gamma_{\pm KCl}$, determined by electrochemical measurements. The absorbances were measured at 490 and 530 nm (see Ref. 6).

It has been pointed out[21] that the distribution of indicator anion is unlikely to be the same in the presence of highly charged polyanion as in its absence, due to the negative Donnan effect, with the result that the calculated activities may differ from the true ones. We are of the opinion that the Ca^{2+} activities, determined by means of the metal–indicator method, have the same physico-chemical meaning as activities of counterions in pure polyelectrolyte solutions without added salt, *e.g.* as determined by means of an ion-exchange membrane electrode[22–26]. They are therefore a quantitative measure of the interaction of counterions with a polyanion, and a quantitative measure of the free Ca^{2+} ions in solutions of calcium oligo- and poly-uronates.

RESULTS AND DISCUSSION

The isolated oligouronides were obtained in a chromatographically pure form, completely free from adventitious salt. Upon hydrolysis, they yielded galacturonic acid as the only monomeric product detectable by chromatography. Their membership of a polymer-homologous series was shown by their chromatographic mobilities (R_F), which, when plotted as R_M values [$\log(1/R_F - 1)$], gave an exact straight-line relationship (Fig. 1). For the ratio [–COOH]/[–CHO] of the di-, tri-, and tetra-mers, values of 2.00, 2.90, and 4.03, respectively, were found.

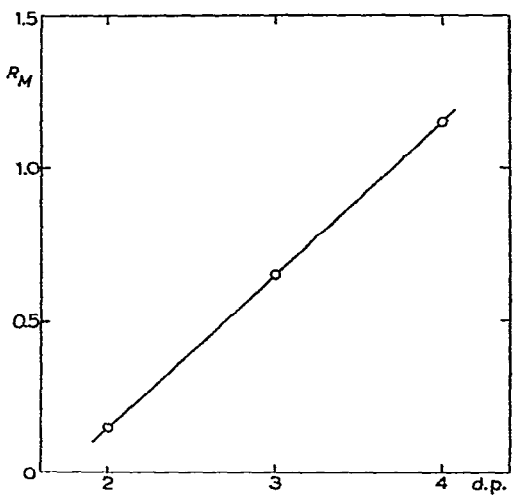

Fig. 1. Relationship between the degree of polymerisation (d.p.) of oligogalacturonic acids and the value R_M obtained by paper chromatography $R_M = \log(1/R_F - 1)$; R_F refers to D-galacturonic acid.

Table I shows the calcium-ion activities of aqueous solutions of the calcium salts of the three oligouronides, and of calcium galacturonate, all of which are 1.50mM with respect to calcium ions. The corresponding activity for 1.50mM calcium chloride, $a_{Ca^{2+}}$, was 1.12 mmoles/l, and the activity coefficient $\gamma_{Ca^{2+}}$ was 0.748. In Fig. 2, the same data are plotted graphically, and show that, whereas the calcium-ion activity of calcium galacturonate is very close to that of calcium chloride, it decreases in a smooth manner as the degree of polymerisation of the anion increases.

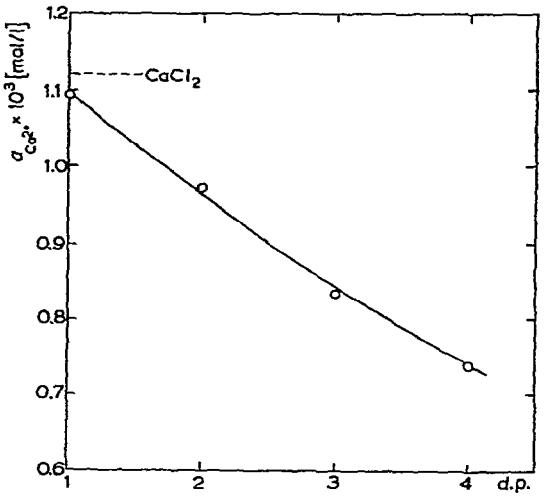

Fig. 2. Calcium-ion activity in solutions of calcium oligogalacturonates as a function of the degree of polymerisation (d.p.) of the anion; [-$COOCa_{0.5}$] = 3.00 mequiv./l; [Ca] = 1.50 mmoles/l; --- = $a_{Ca^{2+}}$ in a solution containing 1.50 mmoles of calcium chloride per litre.

TABLE I

CALCIUM-ION ACTIVITY ($a_{Ca^{2+}}$) AND ACTIVITY COEFFICIENTS ($\gamma_{Ca^{2+}}$) IN SOLUTIONS OF CALCIUM MONO-, DI-, TRI-, AND TETRA-GALACTURONATES CONTAINING 1.50MMOLES OF CALCIUM PER LITRE

Calcium oligogalacturonate (d.p.)	$a_{Ca^{2+}} \times 10^3$ *(mol./l)*	$\gamma_{Ca^{2+}}$	*Percentage*
1	1.095±0.010	0.730	100.0
2	0.972±0.008	0.648	88.8
3	0.834 ±0.005	0.556	76.2
4	0.737 ±0.005	0.491	67.3

Using a special treatment[18], it was possible to prepare a dilute solution of calcium pectate having a high degree-of-polymerisation (d.p. 91). The calcium pectate contained 90% of D-galacturonic acid residues in the macromolecule; the concentration of the solution corresponded to ~0.7mmole of calcium/l. The activity coefficient ($\gamma_{Ca^{2+}}$ 0.063) of calcium ions in these solutions of calcium pectate was very low in comparison with values obtained for solutions of the monomer, calcium galacturonate (0.730), and of calcium chloride (0.748).

The activities $a_{Ca^{2+}}$ and the activity coefficients $\gamma_{Ca^{2+}}$ in corresponding solutions of calcium galacturonate and calcium chloride are very close. It is evident that if calcium ions form a chelate complex with monomeric galacturonic acid, it is exceedingly unstable. This result is in good agreement with data of Buddecke and Drzeniek[16], who found very low stability constants for calcium galacturonate and calcium glucuronate when measured in *ca.* molar solutions. Gould and Rankin[19] recently determined the stability constants of the same calcium monouronates. The values obtained were also small, but varied with the structure of the uronic acid.

Similarly, any chelate involving the functional groups on two consecutive galacturonic acid units, according to the conception of Schweiger[1-3], must also be unstable, since the activity of the calcium ions in the calcium digalacturonate is still close to that of calcium monogalacturonate and approximately ten times higher than the activity of calcium ions in solutions of calcium pectate[18].

On the other hand, the steady decrease in the activity of the calcium ions with the increasing charge of the anion, caused by the cumulation of carboxyl groups along the chain, is fully consistent with the known behaviour of polyelectrolytes. The results are interpreted as evidence that, in calcium pectate, an *intra*molecular chelate bond of calcium involving two consecutive galacturonic acid units is unlikely.

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