CROSS-LINKED PECTIC ACID. THE EFFECT OF CROSS-LINKING ON CATION EXCHANGE, BINDING OF ENDOPOLYGALACTURONASE AND BIODEGRADABILITY

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Pectic acid was cross-linked to different degrees by reaction with epichlorohydrin. The series of samples thus obtained was characterized by the swelling volumes, cross-linking densities, numbers of side chains and ion-exchange capacities. We have investigated the ion-exchange $Ca^{2+} \rightarrow K^+$ in differently cross-linked preparations and the exchange of K^+ for Mg^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Pb^{2+} and Cu^{2+} in a highly cross-linked preparation. The ion-exchange equilibrium was characterized by the selectivity coefficient K_K^{Me}, calculated from equivalent fractions of the cations in the cation exchanger and in the solution. The $Ca^{2+} \rightarrow K^+$ exchange was further characterized by the corrected coefficient of selectivity K_K^{Me} (AC), calculated from mole fractions of the cations in the exchanger and from the single-ion activities in an equilibrious solution. Selectivity of the $Ca^{2+} \rightarrow K^+$ exchange increased with the increasing cross-linking density of the exchanger. Cross-linked pectic acid proved to be a highly selective exchanger of bivalent cations, except the pair Ca^{2+}—Sr^{2+}. Selectivity of the $Me^{2+} \rightarrow K^+$ exchange increased with decreasing portion of Me^{2+} in the exchanger. Affinity chromatography of endopolygalacturonase proceeded best on the preparation of a medium cross-linking degree, in which enough enzyme was bound and no enzymic degradation of the matrix to oligomeric fragments yet occurred. This result accords with the findings on the size of the active centre of endopolygalacturonase.

Polyuronic acids and their salts (alginates and pectates) function as highly selective cation exchangers. This property makes them very useful in medicine and in analytical chemistry. The use of alginic acid and alginates for chromatographic separation of cations and some organic bases have been described in many papers, whereas to chromatography on pectic acid has been given little attention. The binding and exchange of cations on pectin was studied mainly in pectinate solutions (*e.g.*[1-3]).

Pectic (polyuronic) acid to be used as an ion exchanger in analytical chemistry or to remove traces of metallic cations from waste waters[4] has to be applied in an insoluble form, such as pectic acid itself (acid medium), its salts with some bivalent and polyvalent cations, pectates in suitable polar organic solvents and cross-linked preparations of pectic acid.

Films of partially neutralized pectic acid were employed to study the ion-exchange capacity for cations of the alkaline earths[5]. The selectivity coefficients of the exchange for these cations were investigated in gels of the corresponding pectates[6,7].

With suitable organic solvents a column packed with polygalacturonic acid resolves racemic bases[8,9].

A special form of the insoluble polyuronide is protopectin, *i.e.* pectin bound in tissues of plants. Extracted sugar beet slices, rich in pectin, can therefore be used to decontaminate radioactive waste waters[10]. Their capacity and ion-exchange selectivity can substantially be increased by alkaline deesterification[11].

The insoluble ion exchangers were prepared from pectic acid by cross-linking with formaldehyde[12,13]. On these preparations was studied the effect of esterification of carboxyl groups[14] and the effect of some anions[15] on the exchange selectivity of univalent and bivalent cations. Tibenský investigated the effects of the esterification degree of protopectin in beet slices[16], of pectin cross-linked with formaldehyde and of the carboxylic cation exchanger Zerolit 226 on $Ca^{2+} \rightarrow K^{+}$ exchange selectivity[17]. In contrast to the findings reported by Deuel and coworkers[14], Tibenský has proved that the affinity of any of these ion exchangers to Ca^{2+} decreases with decreasing charge density of cross-linked macromolecules. These results fully agree with our data on ion exchange in pectinate solutions[1,2]. Hydroxamic acids prepared from cross-linked pectic acid[18] are ion exchangers selective for Fe^{3+}.

Tibenský and Kuniak[19] have worked out a procedure for the preparation of cross-linked pectic acid by the action of epichlorohydrin in the gaseous phase; a very even cross-linking was attained. Epichlorohydrin was also used to prepare a copolymeric cation exchanger (polymethacrylate–pectin)[20]. The exchange capacity of pectic acid, whether free or cross-linked, can be increased by oxidation of its secondary alcoholic groups to carboxyl groups[21–23]. The effect of the cross-linking degree of pectic acid and alginic acid on their ion-exchange equilibria has not yet been studied.

Rexová, Tibenský and Markovič[24,25] have further shown that cross-linked pectic acid lends itself very well to affinity chromatography of pectic enzymes, of both microbial and plant origins, for a selective purification of endopolygalacturonase.

Since cross-linked pectic acid finds use as an ion exchanger (in analytical chemistry, food-stuff chemistry, *etc.*) and as an insoluble substrate in affinity chromatography of pectic enzymes it has turned out necessary to characterize the exchange selectivity for various cations and to elucidate the effect of the cross-linking degree of pectic acid on cation exchange, on the binding of endopolygalacturonase and on biodegradability of this substance.

EXPERIMENTAL

Chemicals and Apparatus

Potassium pectate and cross-linked pectic acid were prepared from a commercial citrus pectin (Genu Pectin-Type B, Rapid set, Københavns Pektinfabrik, Denmark).

The preparation of pectic enzymes was a partially purified mixture of enzymes isolated from microorganisms *Aspergillus niger*. It contained hydrolases, exo- and endopolygalacturonase and pectinesterase. Isolation and characterization of the preparation were decribed earlier[26].

Complexon IV (diaminocyclohexanetetraacetic acid) was an imported chemical (Lachema). Solutions of KOH (0·2M and 0·1M) were carbonate-free. Solutions of $MgCl_2$ were prepared by dissolution of MgO (A.G.) in hydrochloric acid. The excessive (unreacted) MgO was filtered off and the clear solution was brought to pH 7·0—7·1 with hydrochloric acid. The other chemicals were commercial A.G. products. Distilled water had a conductivity of $2 . 10^{-4}$ S/m.

The apparatus employed included a potentiometer Radelkis OP-205 with an antimony and a glass electrode (Radiometer), a compensation spectrophotometer UVISPEC-Hilger and a special photometer for chelatometric titration, constructed in Development Workshops of the Institutes of Chemistry, Slovak Academy of Sciences. The apparatus was equipped with a set of interference filters (Zeiss, Jena) and a galvanometer Multiflex DG 20 (Metra, Blansko), sensitivity $9 . 10^{-9}$ A/mm.

Analytical Methods and Procedures

Pectic substances were analysed (polyuronide content, esterification degree E and average molecular weight of pectin) by methods described previously[27].

Concentrations of Mg^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Pb^{2+} and Cu^{2+} in dilute equilibrium solutions were determined chelatometrically by photometric titration with 0·01M Complexon IV. To determine concentrations of Ca^{2+}, Co^{2+} and Cu^{2+} an interference filter IF 600 nm and murexide as indicator were used. With ions Mg^{2+}, Sr^{2+} and Pb^{2+} we used eriochrome black T and the interference filter IF 650 nm. Unless otherwise stated the analytical procedure was as previously described[28]. Sr^{2+} ions were determined with the addition of a magnesium salt. In determining a concentration of Co^{2+} ions we alternated small additions of 0·1M-NH_4OH and the Complexon solution. In a Pb^{2+} determination the solution was first mixed with an excess of Complexon IV, followed by the addition of an ammonium buffer solution (pH 9·5); the excess of Complexon IV was then determined by titration with a solution of $MgCl_2$.

Photometric indication of the titration end point made it possible to determine concentrations of the given cations with a high accuracy, depending almost exclusively on the error in reading-off the volume of the titrant.

Preparation of Cross-linked Pectic Acid

Potassium pectate (87 to 90% of polygalacturonate in the dry substance) was obtained by alkaline deesterification of citrus pectin in a heterogeneous phase (60% ethanol)[29]; the remaining degree of esterification was 1 to 4%, the average molecular weight $\overline{M}\eta$, determined viscosimetrically, was 17000—23000.

The potassium pectate, finely pulverized, was then cross-linked to different degrees by exposure to different amounts of gaseous epichlorohydrin in an alkaline medium[19]. The preparations were washed with distilled water until the washings were neutral, then with acetone, followed by drying at 60°C. They were yellowish and had a powder consistence similar to that of the starting potassium pectate. All the cross-linked preparations were insoluble in dilute acids or bases.

Characterization of Preparations of Cross-linked Pectic Acid

The swelling volumes of the cross-linked preparations in water (ml of swollen gel per gram of the dry substance in K^+ form) were determined as previously described[30].

The numbers of cross-links were calculated from the total amount of the reacted epichlorohydrin and from the portion of epichlorohydrin bound in side reaction as monoglycerol ethers. The numbers of the monoether groups were determined after periodate oxidation of the cross-linked preparations by quantitation of formaldehyde as the oxidation product[30].

Ion-exchange capacity of cross-linked pectic acid (H^+ form) was determined alkalimetrically in 0·5M-KNO_3 by potentiometric titration of the hydroxide excess with hydrochloric acid. In the course of the titration the alkaline suspension was protected from atmospheric carbon dioxide by a layer of toluene.

Ion-exchange capacities of the cross-linked preparations were determined specially for each Me^{2+} cation. In the exchange of K^+ for Co^{2+}, Pb^{2+} and Cu^{2+} resp., the capacity and the exchange equilibrium were investigated at lower pH (pH 6·0 with Co^{2+} and pH 5·0 with Pb^{2+} and Cu^{2+}). The cross-linked preparations were first brought to the desired pH by washing with a 0·1M acetate buffer (K^+ salt) and CO_2-free distilled water. The solutions employed were 0·15M-$Co(NO_3)_2$, 0·15M-$Pb(NO_3)_2$ and 0·02M-$Cu(NO_3)_2$, the pH values being adjusted to 6·0, 5·0 and 5·0 respectively.

Determination of the Selectivity Coefficients of Cation Exchange

The cation exchange selectivity coefficients of cross-linked pectic acid were determined by the batch procedure. The preparations were first brought to K^+ and H^+ forms by percolation with 0·1M-KOH and 0·1M-HCl. Swollen samples in the K^+ form were employed. The ion-exchange equilibria were investigated in suspensions; 100- ml samples of these contained: a cross-linked preparation in the quantity corresponding to an exchange capacity of 1 mequiv. [Me^{2+}], increasing amounts of the Me-salt and such an excess of KCl (or KNO_3) that the ionic strength of the solution, I, was 0·15. The salts employed were $MgCl_2$, $CaCl_2$, $SrCl_2$, KCl; $Co(NO_3)_2$, $Pb(NO_3)_2$, $Cu(NO_3)_2$ and KNO_3. The ion-exchange equilibria were measured after 24 hours' stirring of the suspensions at 22–25°C.

The coefficients of selectivity of the exchange $Me^{2+} \rightarrow K^+$ were calculated from the equation

$$K_K^{Me} = \frac{\bar{X}_{Me}(X_K)^2}{(\bar{X}_K)^2 X_{Me}}, \qquad (1)$$

where $\bar{X}$ and X are equivalent fractions of the corresponding cation in the ion exchanger and in the equilibrium solution, respectively.

In the exchange $Ca^{2+} \rightarrow K^+$ we calculated not only the selectivity coefficient defined by equation (*1*), but also the molar corrected coefficient of selectivity, $K_K^{Ca}(AC)$ (see[31,32]).

$$K_K^{Ca}(AC) = \frac{\bar{X}_{Ca(m)}(a_{K^+})^2}{(\bar{X}_{K(m)})^2 a_{Ca^+}}, \qquad (2)$$

where $\bar{X}_{(m)}$ designates the mole fractions of the corresponding cations in the ion exchanger, and a_{K^+} and $a_{Ca^{2+}}$ the single-ion activity of K^+ and Ca^{2+}, respectively, in the equilibrium solution.

Ionic strength, I, was calculated for each equilibrium solution. The single-ion activity coefficients γ_{K^+} and $\gamma_{Ca^{2+}}$ for a ionic strength I were calculated from tabulated values of the mean activity coefficients[33], $\gamma_{\pm KCl}$ and $\gamma_{\pm CaCl_2}$, determined experimentally at 25°C, according to the equations

$$\gamma_{Ca^{2+}} = (\gamma_{\pm CaCl_2})^3/(\gamma_{\pm KCl})^2 \qquad (3)$$

$$\gamma_{K^+} = \gamma_{Cl^-} = \gamma_{\pm KCl} \quad \text{(Mac Innes convention)} . \tag{4}$$

Activities a_K and $a_{Ca^{2+}}$ were calculated from molar concentrations of the ions K^+ and Ca^{2+} in equilibrium solutions. (Theoretical ground of the method will be given further).

The selectivity coefficients K_K^{Me} and $K_K^{Ca}(AC)$ given in this paper are averages from two parallel determinations, the analytical error being $\pm 5\%$.

The corrected selectivity coefficient of ion exchange can also be expressed by means of the selectivity coefficient (K'), which can be calculated from the molar cation concentrations (mol/l) used instead of the mole fractions of the cations in the ion exchanger ($\overline{X}_{(m)}$). The two selectivity coefficients are correlated by the equation

$$K' = \frac{1}{m} K_K^{Ca}(AC) = \frac{\overline{X}_{Ca(m)}(a_{K^+})^2}{m(\overline{X}_{K(m)})^2 a_{Ca^{2+}}} , \tag{5}$$

where m is the total molar concentration of cations in the ion exchanger.

Determination of Biodegradability of Cross-linked Pectic Acid

The effect of pectic enzymes on a suspension of cross-linked pectic acid was investigated at room temperature in a 0·1M acetate buffer, pH 4·2, the concentration of carboxyl groups being 0·025M. A suitable quantity of the enzyme preparation was determined beforehand by its action on a solution of sodium pectate of the same concentration. At time intervals aliquot portions of the substrate were withdrawn. The enzyme was inactivated by boiling; after centrifugation the supernatant was analysed for concentration of reducing terminal groups (—CHO) and tested for the presence of oligomeric fragments of polygalacturonic acid.

The reducing terminal groups were determined by Somogyi–Nelson's method[34]. Oligomeric fragments of polygalacturonic acid were detected chromatographically[35] on a thin layer of silica gel (Silufol plates) in a system n-butyl alcohol–formic acid–water (2 : 3 : 1); the spots were developed by means of an aniline phthalate reagent. The polymerization degrees of the oligomeric fragments were determined by R_F values in comparison with those of standards of oligogalacturonic acids (DP 1—7); for details see[36].

Binding of Endopolygalacturonase to Cross-linked Pectic Acid

Cross-linked pectic acid in a column equilibrated with 0·1M acetate buffer, pH 4·2, was percolated with a solution containing a mixture of pectic enzymes. Endopolygalacturonase, which got selectively adsorbed, was further eluted from the column with an acetate buffer, pH 6·0. The amount of the adsorbed endopolygalacturonase was estimated from the activity of this enzyme in the eluate brought to pH 4·2; the activity was determined using a 0·5% solution of sodium pectate as substrate. The procedure was described in a previous paper[37]. The specific activity of the enzyme is given in micromols of reducing groups liberated by 1 mg of the protein in 1 min.

RESULTS AND DISCUSSION

Characterization of Samples of Cross-linked Pectic Acid

We have prepared a series of samples of pectic acid cross-linked to different degrees by the action of epichlorohydrin in gaseous phase. The samples of the ion exchanger

in K^+ form were characterized *1*) by their swelling volumes, *V* (ml/g); *2*) by their cross-linking degree, expressed as the mean number of cross-links —CH_2—CH(OH).CH_2—R per unit of D-galacturonic acid ($\overline{DS}$(C.L.)); *3*) the extent of the side reaction was expressed as the number of monoglycerol ether groups —CH_2.CH(OH).CH_2OH bound to a D-galacturonic acid unit ($\overline{DS}$ (S.C.)); *4*) by ion-exchange capacity in mequiv. H^+ per gram of the dry substance (H^+ form).

The results of the analyses are summarized in Table I. The cross-linking degrees of a sample and the extent of the side reaction were calculated from the total amount of bound epichlorohydrin and from the number of side chains; the calculations are based on the assumption that both the cross-links and the side chains include only the three-carbon skeleton of epichlorohydrin molecule. In our view this assumption is more or less justified, since the reaction of gaseous epichlorohydrin proceeded slowly and produced preparations homogeneously cross-linked. The swelling volume, *V*, is related to the cross-linking degree (Fig. 1), so that it can be used as a measure of cross-linking.

The ion-exchange capacities ranged between 3·83 and 4·08 mequiv. H^+ per gram of a dry sample (H^+ form). The individual samples had the same capacity for H^+ and Ca^{2+} ions, the differences being less than 1%. With sample 2, having a high degree of cross-linking, the capacities were determined for more cationic species, *viz.* H^+, Mg^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Pb^{2+} and Cu^{2+}. The exchange capacities for these ions were also practically equal, the differences not exceeding $\pm 1\%$. These results prove that the carboxyl groups of cross-linked pectic acid participating in ion exchange are accessible to all the above mentioned cations, irrespective of their size and hydration.

Solutions of $Pb(NO_3)_2$ and $Cu(NO_3)_2$, used to determine the exchange capacities for Pb^{2+} and Cu^{2+}, were acid (pH 5·0), as a result of hydrolysis. Since cross-linked

TABLE I

Characterization of Samples of Cross-linked Pectic Acid

Sample	Swelling volume, *V* ml/g dry sample	$\overline{DS}$ (C.L.)	$\overline{DS}$ (S.C.)	Exchange capacity (H^+-form) mequiv./g
1	3·5	0·488	0·022	4·08
2	4·6	0·392	0·027	4·05
3	5·6	0·230	0·029	4·05
4	8·0	0·152	0·037	3·83
5	11·4	0·095	0·026	3·84

pectic acid represents a weak acidic (carboxylic) cation exchanger it would be expected with the acid solutions of lead(II) and copper(II) nitrates that the ion-exchange capacities will be lower, which is not the case. In determining the capacity an excess of the corresponding solution is passed through the column. In addition, as will be shown further, the ions Pb^{2+} and Cu^{2+} are bound very firmly to the carboxyl groups of the cross-linked preparation. As a result, the equilibrium is shifted and the Pb^{2+} and Cu^{2+} ions get bound to all carboxyl groups, despite the lower pH of the solutions.

Effect of Cross-linking of Pectic Acid on Selectivity of the Exchange $Ca^{2+} \rightarrow K^+$

Samples of cross-linked pectic acid, described in Table I, were investigated for the exchange $Ca^{2+} \rightarrow K^+$. The ionic strength, I, of the equilibrium solutions ranged between 0·150 and 0·146, depending on the exchange degree of the cations.

The exchange $Ca^{2+} \rightarrow K^+$ was characterized by the selectivity coefficient K_K^{Ca} according to formula (*1*). In this definition it is also called the rational coefficient of equilibrium (ref.[31], p. 149). The $Ca^{2+} \rightarrow K^+$ exchange was further characterized by the molar corrected selectivity coefficient $K_K^{Ca}(AC)$, according to equation (*2*).

We have previously shown[27,38] that the single-ion activity coefficients $\gamma_{Ca^{2+}}$ determined with the aid of a metallochrome indicator in mixed solutions ($CaCl_2$ + + KCl) of ionic strengths $I \leq 0{\cdot}15$ are practically identical with those calculated

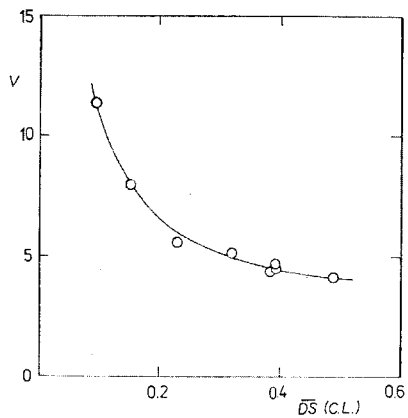

FIG. 1

Swelling Volume, V, of Cross-linked Pectic Acid (K^+ form) in Relation to Cross-linking Degree

V ml of a swollen exchanger per g of the dry exchanger. $\overline{DS}$ (C.L.) number of cross-links per unit of D-galacturonic acid.

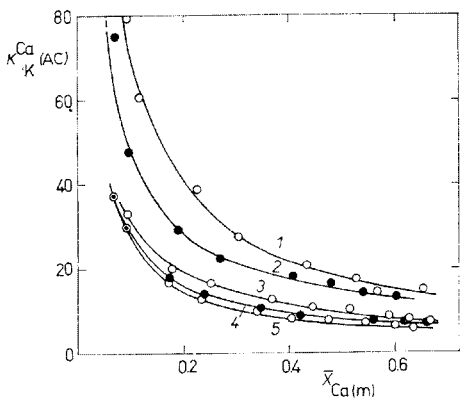

FIG. 2

Corrected Selectivity Coefficients, K_K^{Ca} (AC) of Differently Cross-linked Pectic Acid Plotted *vs* Mole Fraction of Ca^{2+} in the Exchanger, $\bar{X}_{Ca(m)}$

The samples (1—5) are specified in Table I.

from the mean activity coefficients, $\gamma_{\pm CaCl_2}$ and $\gamma_{\pm KCl}$, or from the Debye–Hückel theory of strong electrolytes. These facts show that the corrected selectivity coefficient $K_K^{Ca}(AC)$ can be calculated from the single-ion activity coefficients of Ca^{2+} and K^+ in equilibrious solutions. The activities were calculated from the molar concentrations of the cations in the solution; in these dilute solutions the molal and the molar activity coefficients are practically equal[27]. In the exchange of bivalent and univalent cations it is not yet clear[32] whether it is better to express the quantity of the cations in the ion exchanger by means of equivalent fractions, $\bar{X}$, or mole fractions, $\bar{X}_{(m)}$. Since the activities $a_{Ca^{2+}}$ and a_{K^+} were calculated from the molar concentrations we decided to calculate the corrected selectivity coefficient $K_K^{Ca}(AC)$ from the mole fractions $\bar{X}_{(m)}$.

The results of the measurements are given in Table II and Fig. 2. The values for a sample on a line correspond to the same ion-exchange equilibrium. The corrected selectivity coefficients $K_K^{Ca}(AC)$ are several times lower than the selectivity coefficients K_K^{Ca} calculated from the equivalent fractions of the cations in the ion exchanger and in the solution. However, the two cation-exchange selectivity coefficients depend in similar fashions on both the cross-linking degree of pectic acid and on the mole fraction of Ca^{2+} ions bound to the exchanger. The higher the cross-linking degree the higher the selectivity for Ca^{2+} ions.

The dependence of $K_K^{Ca}(AC)$ on the cross-linking degree of pectic acid is better illustrated in Fig. 3, where the values of $K_K^{Ca}(AC)$ correspond to half-saturation

TABLE II
Selectivity Coefficients Exchange of $Ca^{2+} \rightarrow K^+$ for Cross-linked Pectic Acid

Sample	1				2			
$\overline{DS}$ (C.L.)	0·488				0·392			
	$\bar{X}_{Ca}$	$\bar{X}_{Ca(m)}$	K_K^{Ca}	$K_K^{Ca}(AC)$	$\bar{X}_{Ca}$	$\bar{X}_{Ca(m)}$	K_K^{Ca}	$K_K^{Ca}(AC)$
	0·171	0·093	382	79·7	0·136	0·073	353	75·3
	0·210	0·117	299	60·7	0·174	0·095	231	47·8
	0·363	0·222	210	38·7	0·318	0·189	154	29·3
	0·467	0·304	161	27·6	0·424	0·269	127	22·4
	0·604	0·433	135	20·8	0·577	0·406	116	18·3
	0·690	0·526	125	17·8	0·647	0·478	114	16·9
	0·723	0·566	104	14·2	0·700	0·539	102	14·4
	0·789	0·652	116	14·9	0·751	0·602	100	13·2

of the ion exchanger with Ca^{2+} ions ($\bar{X}_{Ca(m)} = 0{\cdot}5$). These results are in good accordance with those obtained with various synthetic ion exchangers (ref.[31], p. 156). Thus Reddy and Marinsky[39] observed a similar dependence of the corrected selectivity coefficients for the exchanges $Ca^{2+} \rightarrow H^+$ and $Sr^{2+} \rightarrow H^+$ on the cross-linking degree of a polysulphostyrene cation exchanger, whereas the ion exchange of H^+ for Co^{2+}, Ni^{2+} and Zn^{2+} was practically independent of cross-linking. This difference can be explained by a change in activity coefficients of the cations in the exchanger, produced by interaction of hydrated cations with the polyanion.

The selectivity coefficients K_K^{Ca} and $K_K^{Ca}(AC)$ depended much more on the portion of Ca^{2+} ions bound to the cation exchanger. With decreasing $\bar{X}_{Ca}$ or $\bar{X}_{Ca(m)}$ the affinity of cross-linked pectic acid to Ca^{2+} ions considerably increased (Table II, Fig. 2); for details see further.

Exchange Selectivity of Some Bivalent Cations Me^{2+} *Replacing* K^+ *Ions in Cross-linked Pectic Acid*

Exchange of K^+ ions in cross-linked pectic acid in K^+ form for ions Mg^{2+}, Ca^{2+}, Sr^{2+}, Co^{2+}, Pb^{2+} and Cu^{2+} was investigated. These experiments were carried out with the highly cross-linked sample 2, $\overline{DS}$ (C.L.) = 0·392, at an ionic strength $I = 0{\cdot}15$.

Solutions of potassium pectate, salt of a weak polyacid and a strong base, have pH ≈ 7·5. A similar pH is probable in the phase of the cross-linked pectate. Pre-

TABLE II
(*Continued*)

3				4				5			
0·230				0·152				0·095			
$\bar{X}_{Ca}$	$\bar{X}_{Ca(m)}$	K_K^{Ca}	K_K^{Ca} (AC)	$\bar{X}_{Ca}$	$\bar{X}_{Ca(m)}$	K_K^{Ca}	K_K^{Ca} (AC)	$\bar{X}_{Ca}$	$\bar{X}_{Ca(m)}$	K_K^{Ca}	K_K^{Ca} (AC)
0·170	0·093	160	33·2	0·129	0·069	175	37·2	0·132	0·070	174	37·0
0·300	0·177	104	19·9	0·166	0·091	144	29·6	0·168	0·092	145	30·2
0·402	0·251	93	16·7	0·293	0·172	92	17·8	0·293	0·171	88	16·8
0·539	0·368	80	12·9	0·383	0·237	76	13·7	0·376	0·232	69	12·5
0·613	0·442	70	10·6	0·514	0·346	66	10·8	0·505	0·338	61	10·0
0·680	0·515	74	10·4	0·592	0·421	59	9·1	0·576	0·405	52	8·0
0·732	0·587	67	8·9	0·716	0·558	59	7·9	0·642	0·473	54	7·8
0·770	0·625	63	8·0	0·764	0·619	60	7·6	0·703	0·543	52	7·0
0·797	0·663	59	7·1	0·794	0·658	58	7·1	0·750	0·601	52	6·7
								0·776	0·632	45	5·6

liminary experiments revealed that at low concentrations of Pb^{2+} and Cu^{2+} ions in equilibrious solutions basic salts of these cations precipitated in the ion exchanger phase. In view of this fact we investigated the exchange equilibria $Pb^{2+} \rightarrow K^+$ and $Cu^{2+} \rightarrow K^+$ in a cross-linked preparation whose pH was first brought to 5·0; for the same reason the exchange Co^{2+}—K^+ was studied at pH 6·0.

For most of the enumerated cations we had not enough experimental data to justify use of the single-ion activity coefficients, $\gamma_{Me^{2+}}$, by proofs given earlier[27,38] for ions Ca^{2+} and Sr^{2+}. For this reason the cation-exchange selectivity coefficients were expressed by equivalent fractions of the cations (X) according to equation (*1*). Fig. 4 gives the selectivity coefficients K_K^{Me} for exchange equilibria $Mg^{2+} \rightarrow K^+$, $Ca^{2+} \rightarrow K^+$, $Sr^{2+} \rightarrow K^+$ and $Co^{2+} \rightarrow K^+$.

Like with the exchange equilibria in Fig. 2, the selectivity coefficient K_K^{Me} increases with decreasing equivalent fraction of Me^{2+} ions in the exchanger, particularly in the region of low values of $\bar{X}_{Me}$. A similar dependence of the selectivity coefficient was observed with cations of alkaline earths exchanged for Mg^{2+} or K^+ on non-cross-linked gels of alginates[40,41] and pectates[6,41]. Unlike these systems in solutions of Ca-pectinates (Ca-salt of partially esterified pectic acid), which do not gellify, the interaction of Ca^{2+} ions with carboxyl groups of the pectinate strictly obeys the law of multiple equilibria. The stability constant of Ca-pectinate as well as the

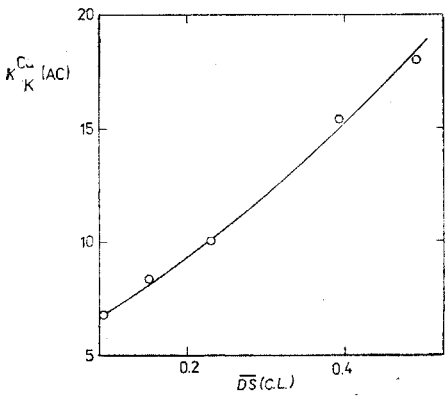

FIG. 3

Corrected Selectivity Coefficient, K_K^{Ca} (AC), Plotted *vs* Cross-linking Degree of Pectic Acid

$\bar{X}_{Ca(m)} = 0{\cdot}5$, $\overline{DS}$ (C.L.) is the number of cross-links per unit of D-galacturonic acid.

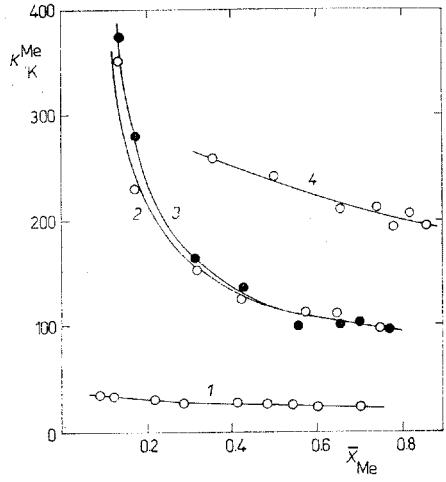

FIG. 4

Selectivity Coefficients, K_K^{Me}, of Exchange of $Me^{2+} \rightarrow K^+$ for Cross-linked Pectic Acid Plotted *vs* Equivalent Fractions of Me^{2+} Ions in the Exchanger, $\bar{X}_{Me}$

1 Mg^{2+}, *2* Ca^{2+}, *3* Sr^{2+}, *4* Co^{2+}.

selectivity coeficient K_K^{Ca} are, in this case, independent of the portion of Ca^{2+} ions bound in the macromolecule of the pectinate (*cf.*[6]). Hence we believe that the dependence of the selectivity coefficients K_K^{Me} and $K_K^{Ca}(AC)$ on the portion of cations Me^{2+} bound in an exchanger is given by the heterogeneous spatial distribution of $—COO^-$ groups in the cross-linked preparation. This heterogeneity gives rise to differences in charge density in the exchanger, forming loci with different possibilities of intermolecular binding of bivalent cations to carboxyl groups of two different chains. The mechanism of the binding of cations to polyuronides has been given more attention elsewhere[3,42,43].

The folloving Table III the selectivity coefficients K_K^{Me} for the exchange reactions of K^+ ions for a number of bivalent cations Me^{2+} in cross-linked pectic acid (sample 2) at $I = 0{\cdot}15$:

Me^{2+}:	Mg	Ca	Sr	Co	Pb	Cu
$K_K^{Me}(\bar{X}_{Me} = 0{\cdot}5)$:	26	121	120	241	2 580	3 300

The binding of ions Cu^{2+} and Pb^{2+} to cross-linked pectic acid is extraordinarily firm, which is important from the practical point of view. Firm binding of Cu^{2+} ions is also typical of other synthetic polyanions containing carboxyl groups. By contrast, polyanions containing groups $—SO_3H$ exhibit little selectivity[7] for Cu^{2+} ions. The selectivity coefficient K_K^{Cu} determined with the cation exchanger Dowex 50W X 2 (functional groups $—SO_3H$) under the same conditions had a very low value, $K_K^{Cu} = 12{\cdot}3$, compared to that found with cross-linked pectic acid, $K_K^{Cu} = 3300$. We believe that the very strong linkage of Cu^{2+} and Pb^{2+} ions to the carboxyl groups of pectin is essentially of a coordinative nature.

The foregoing results reveal that except the pair Ca^{2+}—Sr^{2+} cross-linked pectic acid is highly selective for bivalent metallic cations, which is in agreement with earlier papers[1,6,7], concerning the ion exchange of non-cross-linked pectin preparations.

Effect of Cross-linking of Pectic Acid on the Binding of Endopolygalacturonase and on Biodegradability of the Preparation

Cross-linked pectic acid has proved to be very useful for affinity chromatography of endopolygalacturonase to isolate this enzyme from a mixture of pectic enzymes, whether of a microbial or a plant origin[24,25]. Cross-linked pectic acid (insoluble substrate) binds endopolygalacturonase at the pH of its maximum activity. After washing the column endopolygalacturonase is quantitatively eluted with a buffer of pH 6·0. A prerequisite for the binding of the enzyme to a cross-linked matrix is a minimum segment length of D-galacturonic acid units, carrying no cross-links or side chains. This minimum length of a segment corresponds to the size of the active centre of the enzyme, involving its binding and its catalytic centres. What is also

important with cross-linked preparations is the mutual arrangement of macromolecular chains of pectic acid, which has to offer enough space for the enzyme to bind to the insoluble matrix. It can, therefore, be expected that with the increasing density of the cross-links the binding capacity for the enzyme will decrease, and *vice versa*.

The effect of cross-linking degree of pectic acid on selective adsorption of endopolygalacturonase was investigated with a mixture of pectic enzymes produced by microorganisms *Aspergillus niger*. The amounts of the bound endopolygalacturonase were assessed from the activity of this enzyme eluted from a column of the cross-linked preparations, as described in Experimental. The results are given in Fig. 5. As expected, the amount of the bound enzyme increased with decreasing cross-linking $\overline{DS}$ (C.L.); (samples 2–4). With sample 5, having the lowest cross-linking degree, the pectic enzymes considerably degraded the matrix, so that the binding of endopolygalacturonase was much lower than the expected level (– – –).

What is especially important to take into account in considering the application of cross-linked pectic acid in food-stuff industry and affinity chromatography of endopolygalacturonase is the biodegradability of the material. In our experiments suspensions of cross-linked samples were exposed to a mixture of pectic enzymes *A. niger*, containing both endo- and exoenzymes, at pH 4·2 The chosen pH is optimum for activity of either kind of these enzymes. Increase in the content of oligomeric fragments of pectic acid in the solution was investigated in relation to time by determining the reducing terminal groups of the oligomers (—CHO) and by characterization of the fragments by thin-layer chromatography on silica gel.

The results of the measurements are given in Fig. 6. For the sake of comparison the enzyme action was also measured on a solution of non-cross-linked sodium pectate (PA). The thin-layer chromatography showed that at high cross-linking degrees (samples 2 and 3) only monomeric D-galacturonic acid split off from the non-cross-linked ends of the macromolecule by the action of the exoenzyme, the extent of the splitting being inversely proportional to the degree of cross-linking (sample 3). At relatively low cross-linking degrees (samples 4 and 5), in which case longer segments of uronic acid units are present in the preparations, both endopolygalacturonase and the exoenzyme were active. The endopolygalacturonase split off oligomeric fragments the more the lesser the cross-linking degree of pectic acid. With these samples the thin-layer chromatography detected fragments of polymerization degrees DP 1 to DP 7. At the lowest cross-linking degree (sample 5) a partial desintegration of the matrix was observed.

Our findings concerning the binding of endopolygalacturonase to cross-linked preparations of pectic acid and the data on biodegradability of the samples are in full accordance with the views on the size of the active centre of this enzyme. Rexová[36] has demonstrated that the smallest oligomer of D-galacturonic acid to which the enzyme still binds and which it splits is a tetramer. Consequently, the enzyme

will bind to the cross-linked preparations provided their segments contain sequences of at least four units of non-substituted D-galacturonic acid. From the degrees of cross-linking we calculated the average numbers ($\bar{n}$) of non-substituted units of the uronic acid between two cross-links in samples 2–5; these were 1·6; 3·4; 5·6 and 9·5 respectively (Fig. 5).

If the distribution of cross-links in macromolecules of cross-linked pectic acid were absolutely even, sample 2 should not bind the enzyme yet and in sample 3 the quantity of the bound enzyme should correspond to about 40% of its adsorption capacity. As, however, the distribution is random, even the most cross-linked sample had a few segments that were long enough for the binding of the enzyme. Samples 2 and 3 bound 17% and 75%, respectively, of the maximum quantity of the enzyme bound to sample 4. However, in these two samples the binding segments were still so short that no oligomeric fragments appeared in the solution. In view of the random distribution of the cross-links in the samples our results seem to us to be in good

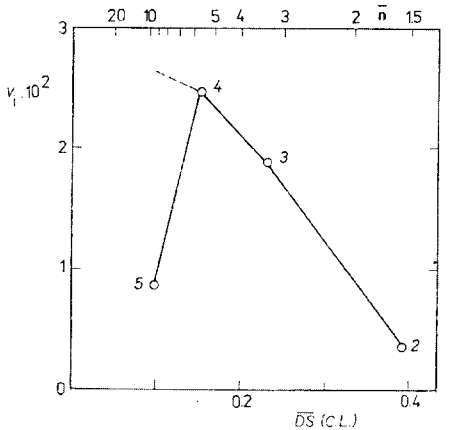

FIG. 5

Effect of Cross-linking Degree of Pectic Acid on the Binding of Endopolygalacturonase

v_i Activity of bound endopolygalacturonase after its elution from the column (μmols of reducing groups liberated by 1 mg of the protein in 1 min), $\overline{DS}$ (C.L.) number of cross-links per unit of D-galacturonic acid, $\bar{n}$ the average number of non-substituted units of the uronic acid between two cross-links, 2–5 samples of differently cross-linked pectic acid (Table I).

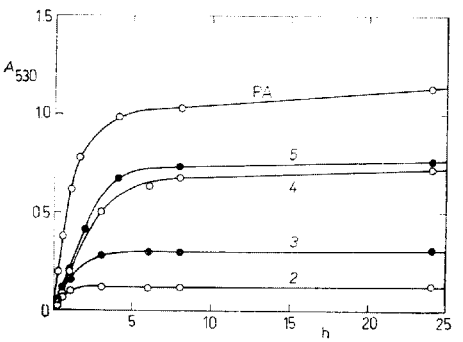

FIG. 6

Degradability of Cross-linked Pectic Acid by Pectic Enzymes *Aspergillus niger* at pH 4·2

A_{530} Absorbance at 530 nm, proportional to concentration of the liberated reducing terminal groups, 2–5 samples of differently cross-linked pectic acid (Table I), PA a solution of non-cross-linked sodium pectate.

agreement with the present-day knowledge of the size of the active centre of endopolygalacturonase.

Data on the effect of cross-linking of pectic acid upon cation exchange, binding of endopolygalacturonase and upon biodegradability of the matrix are of considerable importance for practical use of these materials. If they are to be used as selective cation exchangers, highly cross-linked products ($\overline{DS}$ (C.L.) = 0·4 to 0·5) of low swelling volumes (V = 3·5 to 4·6 ml/g) are useful. Affinity chromatography of pectic enzymes proceeds best at a medium cross-linking ($\overline{DS}$ (C.L.) = 0·20 to 0·25; $V \approx$ $\approx$ 5·5 ml/g), at which enough endopolygalacturonase gets bound, while no oligomeric fragments are yet liberated. The presence of monomeric D-galacturonic acid, split off by the exoenzymes from non-cross-linked termini of the macromolecules, does not interfere with the isolation of endopolygalacturonase. Besides, the monomeric acid appears only in the first use of the adsorbent.

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