BIOSYNTHESIS OF ALGINATE

PART II. POLYMANNURONIC ACID C-5-EPIMERASE FROM *Azotobacter vinelandii* (LIPMAN)

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ABSTRACT

An enzyme preparation was isolated from liquid cultures of *Azotobacter vinelandii* by precipitation with ammonium sulphate after removal of cells by centrifugation. When incubated in the presence of calcium ions with alginate prepared from brown algae, the enzyme epimerizes D-mannuronic acid residues to L-guluronic acid residues in the polymer chain. An assay is described, based on the difference in colour intensity of the two uronic acids in the Dische carbazole reaction. The influence of calcium, strontium, magnesium, and sodium ions, and of pH and temperature on the activity was determined. The energy required for the transformation of D-mannuronic to L-guluronic acid residues is thought to be supplied by the stronger binding of calcium ions to the latter type of monomer. By using polymannuronic acid as a substrate, attempts were made to determine the end-point of the reaction and to follow the formation of the two different types of L-guluronic acid-containing blocks during the epimerization reaction. The results demonstrate that both homopolymeric blocks of L-guluronic acid and blocks having an alternating sequence are formed. No transformation of the latter to the former type could be demonstrated, and both block types thus appear to be end-products of the enzymic reaction.

INTRODUCTION

In the previous paper[1], we reported the presence of an enzyme in the culture medium of *Azotobacter vinelandii*, capable of epimerizing the D-mannuronic acid residues in alginate to L-guluronic acid residues. The present work is a further study of this enzyme. A preliminary report of some of these results has been published[2].

MATERIALS AND METHODS

Alginate preparations. — In most experiments, an alginate prepared from the intercellular substance of the medulla of *Ascophyllum nodosum* receptacles[3] was used (Sample *A*); this contained[4] 93% of D-mannuronic and 7% of L-guluronic acid, and the weight-average molecular weight[5] was approximately 10^6. In some experiments, three other alginate samples were used: *B*, from *Laminaria digitata*, 62% of D-mann-

uronic acid; *C*, from *Ascophyllum nodosum*, the part of the thallus between the holdfast and the lowest vesicle, 61% of D-mannuronic acid; and *D*, from *Laminaria hyperborea* stipes, 27.5% of D-mannuronic acid. The weight-average molecular weight for these samples was between 5×10^5 and 8×10^5.

Enzyme preparation. — *Azotobacter vinelandii*, strain E, was grown on medium *B* (containing 0.1mM $CaCl_2$)[1] in liquid culture at 30° with vigorous shaking for 30 h. The culture was cooled in an ice bath, cells were removed by centrifugation, ammonium sulphate was added (to 30% saturation at 0°), and the resulting precipitate was discarded. The ammonium sulphate concentration was increased to 50% of saturation, and the precipitate was collected by centrifugation, washed with ammonium sulphate solution (50% saturation), and dissolved in water. This solution was dialysed against a solution containing the same amounts of inorganic constituents as the nutrient, but with calcium chloride omitted. The volume after dialysis was approximately 1.5% of the total volume of the original culture. All operations were carried out at temperatures below 4°. The enzyme preparations were stored at −20° and were stable for at least three months. The amounts of carbohydrate and protein in the precipitates were determined by the phenol–sulphuric acid method[6] and the Folin–Ciocalteu method[7], respectively, using glucose and bovine serum albumin as standards.

Enzyme assay. — Alginate solution (0.25%, 0.5 ml, Sample *A*) was mixed with collidine buffer (2 ml, pH 6.8, 50mM), 34mM calcium chloride (0.35 ml), enzyme preparation (0.1–0.4 ml), and water (to 3.5 ml). The composition of this standard medium was varied as described in the text with respect to pH, calcium concentration, or presence of other ions. The standard incubation time was 3 h at 20°. The solution was analysed by the carbazole method at 55° without addition of borate[8], and the ratio of the reading at the termination of the incubation time and at the start of the reaction (P) is used as a measure of the activity. The activity is given as 100 $(P-1)$/h/0.1 ml of enzyme solution.

End-point determinations. — Alginate solution (0.25%, 1.5 ml) was mixed with collidine buffer at pH 6.8 (6 ml), aqueous calcium chloride (1.05 ml) of the desired strength, enzyme preparation (0.4 ml), and water (to 10 ml). Enzyme preparation (0.2 ml) was added after 6, 12, and 25 h. The incubation temperature was 20°. Samples (2.0 ml) were removed at the start of the reaction, and after 24 and 48 h. The samples were analyzed by the carbazole reaction at 55°, with and without borate[8]. The ratio of these readings can be used for determining the ratio of D-mannuronic to L-guluronic acid in the preparation[9]. Three alginate samples having known compositions of uronic acid, determined by separation on ion-exchange columns[4], were used as standards.

Partial hydrolysis. — The enzymic reaction was carried out as described above. Samples (2.5 ml, containing 0.9 mg of alginate) were removed and mixed with ethanol (2.5 ml) and M potassium chloride (0.4 ml). At the same time, samples were removed for determination of the uronic acids, as described above. The precipitated alginate was collected by centrifugation, suspended in 0.3M hydrochloric acid (1 ml), and heated in a boiling-water bath for 35 min. After centrifugation, the centrifugate

was decanted through a porous glass filter, leaving as much as possible of the precipitate in the centrifuge tube. The precipitate was washed with 0.3M hydrochloric acid (1–1.5 ml), suspended in water, and dissolved by addition of dilute alkali to neutrality. The precipitate on the filter was removed by pouring a small amount of dilute alkali through the filter, and the amounts of polysaccharide in the soluble and insoluble fraction were determined, after partial hydrolysis, by the phenol–sulphuric acid reaction[6].

Enzymic modification of alginate on a larger scale. — Two experiments were carried out with 50–100 mg of alginate. The experimental procedure used with Sample *A* is described in detail in our preliminary report[2]. Sample *C* (50 mg in 20 ml of water) was mixed with collidine buffer at pH 6.6 (40 ml), 60mM calcium chloride (1.5 ml), and 2 ml of an enzyme preparation (activity 10). After 20 h at 20°, more of the enzyme preparation (2 ml) was added, and the mixture was left for 20 h. The enzyme-modified alginate was prepared by precipitation with ethanol, washed with ethanol and ether, and dried. The yield was 52 mg.

RESULTS

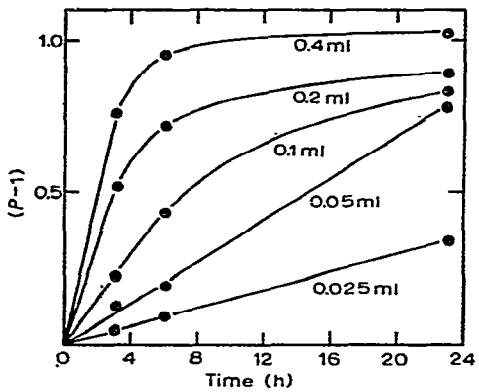
Enzyme assay. — The well-known carbazole reaction[10] for uronic acids gives a very different colour intensity for the different uronic acids, and mannuronic acid gives a particularly low intensity of colour. The increase of colour intensity may therefore be used as a measure of the degree of conversion of mannuronic into guluronic acid units. If E_G and E_M are the extinction coefficients of L-guluronic and D-mannuronic acid residues, respectively, a is the amount of polymannuronic acid at the start of the reaction, and b is the amount of L-guluronic acid residues after the time t, the proportion (P) between the colorimetric readings at time t and at the start of the reaction is

$$P = \frac{(a-b)E_M + bE_G}{aE_M} = 1 + \frac{b}{a}\left(\frac{E_G}{E_M} - 1\right).$$

$(P-1)$ is thus proportional to the degree of conversion, and we have chosen this value as a measure of the enzyme activity, using Sample *A* as the standard substrate.

Fig. 1 shows $(P-1)$ as a function of time for five different enzyme concentrations. In Fig. 2, the results are plotted as a function of enzyme concentration. Below a value of 0.5, $(P-1)$ shows a satisfactory linearity both with time and with enzyme concentration. We therefore describe the activity of our enzyme preparation as $A = 100\ (P-1)$/h per 0.1 ml of enzyme preparation, measured under conditions giving values of $(P-1) < 0.5$ in 3 h.

Enzyme preparation. — The supernatant, after removal of bacterial cells by centrifugation, was used for all enzyme preparations. The activities of precipitates obtained by the addition of different concentrations of ammonium sulphate were determined as follows. The supernatant was divided into six parts, and each was mixed with the appropriate amount of ammonium sulphate. The precipitates were



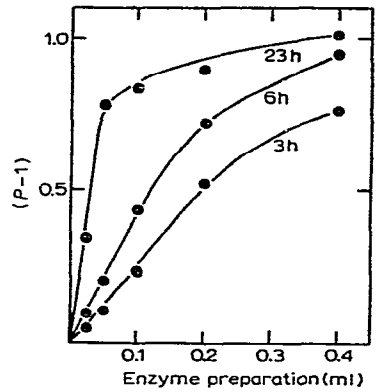


Fig. 1. Degree of conversion as a function of time for different concentrations of enzyme.

Fig. 2. Degree of conversion as a function of enzyme concentration.

collected by centrifugation, dissolved in water, and dialysed as in the standard procedure. The volume was adjusted to 5% of that of the supernatant used for the precipitation, and the activities and the amounts of protein and carbohydrate were determined (Table I).

TABLE I

ACTIVITIES AND PROTEIN AND CARBOHYDRATE CONCENTRATIONS IN AMMONIUM SULPHATE PRECIPITATES

Saturation (%)	A	*Protein* (*mg/ml*)	*Carbohydrate* (*mg/ml*)	A *per* *mg of protein*
20	0.1	0.12	0.12	8.3
30	0.1	0.17	0.13	6.0
40	2.1	0.43	0.38	49.0
50	2.6	0.48	0.39	54.2
65	1.4	0.29	0.31	48.3
80	1.7	0.33	0.39	51.5

No significant activity was found in the precipitates with 20 and 30% ammonium sulphate saturation. Above 40% saturation, the activity per mg of protein was virtually constant. The decrease in total activity and protein in the precipitates at 65 and 80% ammonium sulphate saturation is due to losses during centrifugation caused by part of the protein floating in the ammonium sulphate solution.

Based on these results, our standard procedure for enzyme preparation (Materials and Methods) was developed. No further purification has been carried out in this work. The activity was found to vary considerably from one preparation to another; values of A fell typically between 5 and 15, measured at 20°, in the presence of 3.4mM calcium chloride.

Activity determinations. — The correlation between enzyme activity and calcium concentration is shown in Fig. 3. A marked correlation was observed over a wide

range of calcium concentrations, with a particularly pronounced increase between 0.5 and 1.0mM (1–2 mequiv./l). The alginate concentration in the test solution corresponds to 1.8 mequiv./l.

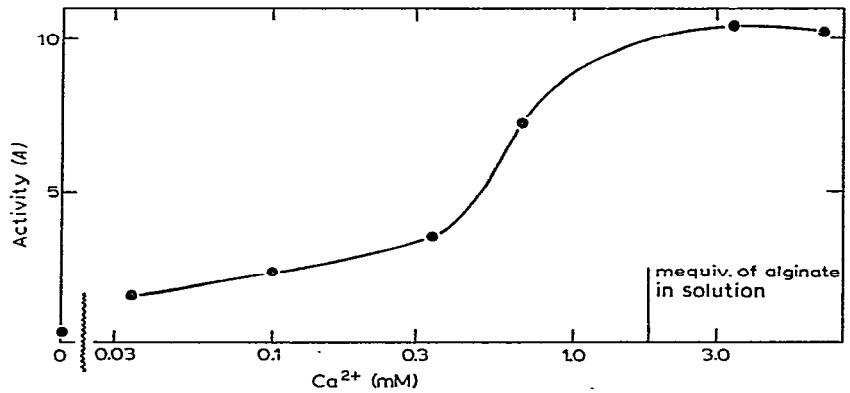


Fig. 3. Activity as a function of calcium concentration.

In the absence of calcium ions, magnesium and sodium ions gave only 10–20% of the activity in the presence of 3.4mM calcium chloride. The concentration range tested for magnesium ions was from 2 to 100mM, and for sodium ions 100–1000mM. A significant activity was, however, observed in the presence of strontium ions (Fig. 4). Concentrations higher than 3mM could not be tested, as these caused a partial precipitation of the alginate. In the presence of 3.4mM calcium chloride, the addition of magnesium or sodium ions led to a decrease in activity (Figs. 5 and 6). At calcium ion concentrations that were too low to give maximal activity, small amounts of magnesium and sodium ions led to an increase in activity, whereas a decrease was observed at higher magnesium or sodium concentrations (Figs. 5 and 6).

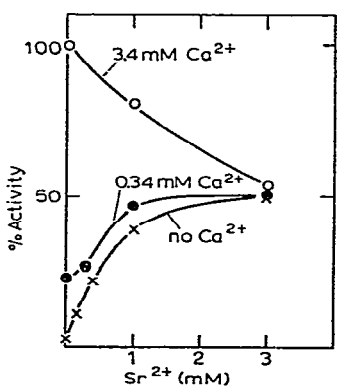


Fig. 4. The effect of Sr^{2+} on activity, given as percentage of activity in the presence of 3.4mM $CaCl_2$.

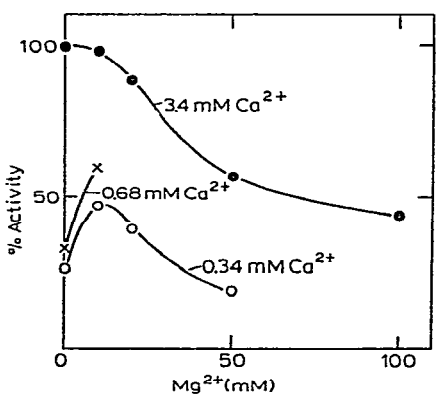
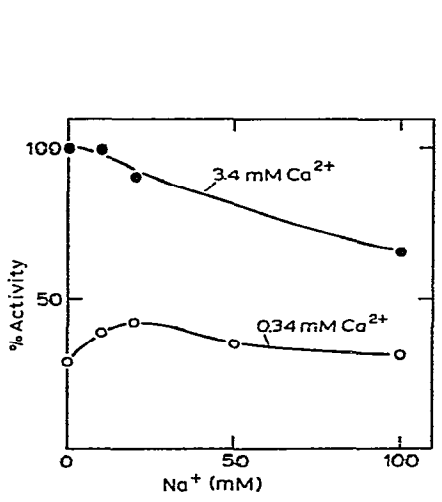


Fig. 5. Activity in the presence of calcium and magnesium ions, as percentage of activity in the presence of 3.4mM $CaCl_2$.

Addition of strontium ions also led to a decrease of activity at 3.4mM Ca^{++}, whereas at lower calcium concentrations, the activity was primarily determined by the strontium ions (Fig. 4).



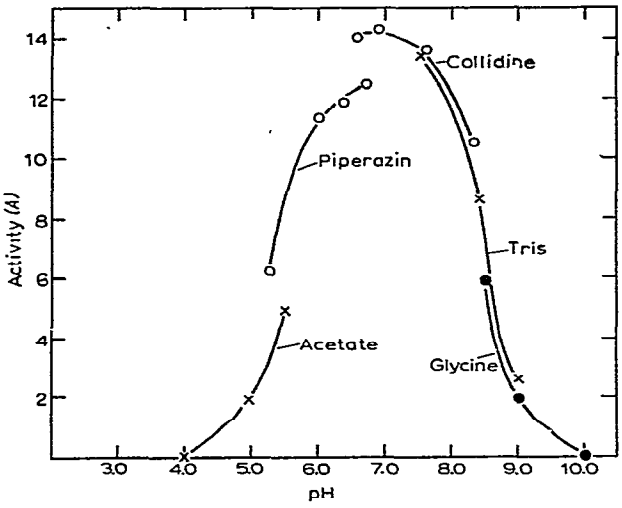


Fig. 6. Activity in presence of calcium and sodium ions, as percentage of activity in the presence of 3.4mM $CaCl_2$.

Fig. 7. Activity as a function of pH.

The pH dependence was determined in the presence of 3.4mM calcium chloride. The enzyme activity has a maximum around pH 7 (Fig. 7), and significant differences between the different type of buffers were observed.

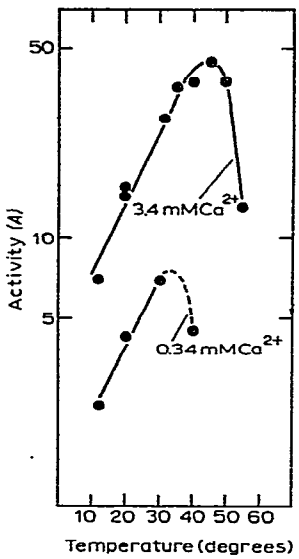
The temperature dependence was determined for two different calcium concentrations (Fig. 8). The temperature optimum was significantly higher in the presence of 3.4mM calcium chloride than in 0.34mM calcium chloride, indicating an effect of calcium ions on the stability of the enzyme. This was further investigated as follows. Samples containing buffer, enzyme, and the desired amount of calcium ions were kept for 2 h at 40°. After cooling to 20°, alginate was added, the calcium chloride concentration was adjusted to 3.4mM, and the activity was determined by our standard procedure. The results, given in Table II, demonstrate a decrease of stability at the lowest concentration of calcium.

Determination of the end-point of the reaction. — Attempts were made to force the reaction to the end-point by prolonged incubation and repeated additions of

TABLE II

STABILITY OF ENZYME FOR 2 H AT 40° AT DIFFERENT CONCENTRATIONS OF CALCIUM CHLORIDE

Pretreatment with Ca^{2+}	None	3.4mM	0.34mM	0.07mM
Activity (*A*)	11.4	11.4	11.7	6.3



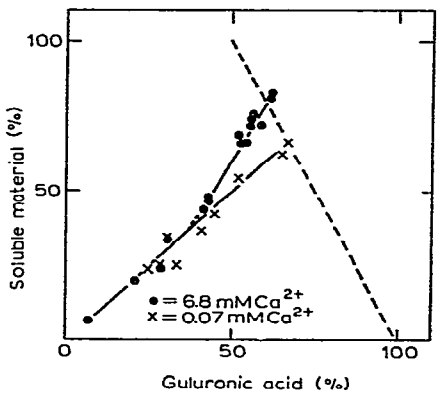


Fig. 8. Activity as a function of temperature at two different calcium concentrations.

Fig. 9. Correlation between the amount of material soluble by partial, acid hydrolysis and the conversion of D-mannuronic into L-guluronic acid residues.

enzyme preparation. The results (Table III) all correspond to incubation times of 40 h, and in no case was a significant change in composition observed between 20 and 40 h. It may, therefore, be assumed that the results correspond closely to the end-point of the reactions under the conditions used.

TABLE III

END-POINT OF THE ENZYMIC REACTION

Alginate sample	Ca^{2+} (mM)	*Mannuronic acid* (%)	
		Before treatment	*After treatment*
A	0	92	89
	0.07	92	28
	0.34	92	32
	3.4	92	39
B	0	62	59
	0.07	62	29
	3.4	62	39
C	0.07	61	43
	0.14	61	37
D	0	27.5	27.5
	0.07	27.5	27.0
	3.4	27.5	26.5

Whereas the activity of the enzyme increases with increasing calcium concentration (up to 3.4mM), Table III shows that the end-point of the reaction lies at a higher content of guluronic acid at low concentrations of calcium ion. Of the samples having a higher content of guluronic acid than sample *A*, a significant transformation of mannuronic to guluronic acid residues was observed for Samples *B* and *C*, where the original content of guluronic acid was approximately 40%, whereas no significant change in composition was observed for Sample *D*, which had an original content of guluronic acid residues of 72.5%. In no case was a change in the direction of increased content of mannuronic acid observed when samples having a significant content of guluronic acid were incubated with enzyme in the absence of calcium ions.

Sequence of residues in enzyme-modified alginate. — As discussed previously[1], the sequence of monomers in alginates from *Azotobacter vinelandii* resembles closely that of marine alginates, *i.e.*, it too consists of homopolymeric blocks of both D-mannuronic and L-guluronic acid residues, and also contains parts having a predominantly alternating structure[11,12]. Experiments were carried out to establish whether the same type of block structure is formed by the action of the epimerase.

Enzyme-modified alginate was subjected to partial hydrolysis with acid and fractionation as described previously[11]. The experiment carried out with Sample *A* has been described in detail[2], and a similar experiment was carried out with Sample *C*, in an attempt to let the reaction proceed to the end-point. This sample was particularly rich in blocks having an alternating sequence. The purpose of this experiment was primarily to investigate whether a decrease in the amount of this type of sequence, and an increase in the amount of homopolymeric blocks of L-guluronic acid, could be detected.

The results of both experiments are given in Table IV. For both samples, the enzyme treatment led to a change in the fractionation pattern after partial hydrolysis, indicating an increase in the amount of blocks having an alternating structure and in blocks of L-guluronic acid residues, and a decrease in blocks of D-mannuronic acid residues. No indication of transformation of the blocks having an alternating sequence to L-guluronic acid blocks was observed.

TABLE IV

PARTIAL HYDROLYSIS OF ENZYME-TREATED ALGINATES

	Mannuronic acid (%)	*Soluble 0.3M HCl, 2 h 100°*	*Insoluble*	
			Soluble pH 2.85	*Insoluble pH 2.85*
Sample *A*				
Before treatment	92	9.1	80.5	10.4
After treatment	49	31.0	17.2	51.8
Sample *C*				
Before treatment	60	59.0	27.0	14.0
After treatment	45	65.0	12.0	23.0

The fractionation pattern indicates that the sequence of the enzyme-modified alginates is of the same general type as previously described for marine alginates and for *Azotobacter* alginates. The free-boundary electrophoresis patterns[2] of the insoluble part of the enzyme-modified Sample *A* after partial hydrolysis agree with this conclusion. The soluble part after partial hydrolysis was investigated as follows. Sample *A*, before and after enzyme modification, was hydrolysed for 20 min in M oxalic acid at 100°, and the centrifuged solution was kept for 1 h at 100°. The solution was then neutralized with calcium carbonate and concentrated by evaporation. Paper electrophoresis in borate buffer containing calcium chloride[13] demonstrated the presence of two new components in the enzyme-modified alginate, which had the same mobility as L-guluronic acid[13] (M_M: 0.80*) and a diuronide containing both monomers[11] (M_M 1.43), respectively, in addition to the two components present in the untreated sample *A*, mannuronic acid and a dimannuronic acid (M_M 1.22).

The formation of blocks was further investigated by following the increase, during enzyme treatment, in the amount of material rendered soluble by partial hydrolysis with acid. Table V shows the results obtained by use of this method on three different alginate samples, each before and after enzyme treatment. Assuming that the soluble material corresponds to the amount of blocks having an alternating sequence, the total amount of homopolymeric blocks ("G-blocks" and "M-blocks" in Table V) may be calculated. Even if this assumption is an over-simplification, the results indicate the changes in the relative amounts of the three types of blocks. The action of the enzyme on Sample *A* was investigated in more detail, and the results are given in Fig. 9, showing the amount of material made soluble by the partial, acid hydrolysis, as a function of the L-guluronic acid content of the enzyme-modified samples. The experiments were carried out at two different concentrations (0.07 and 6.8mM) of calcium chloride. With the same assumption as above, the dotted line gives the theoretical end-point of the reaction, corresponding to conversion of all blocks of mannuronic acid residues into one of the two types of block containing guluronic acid. At the lower concentration of calcium, the linear plot shown in Fig. 9 clearly indicates that the relative rates of formation of the two types of block containing guluronic acid are constant throughout the epimerization reaction. The slope of the curve corresponds to the same rate of formation of guluronic units in the two types of block. At the higher calcium concentration, the results indicate that the relative rate of formation of the two types of block changes in the course of the epimerization reaction. In the last part of the reaction, the slope of the curve indicates that no homopolymeric blocks containing guluronic acid are formed; the epimerization reaction leads only to formation of blocks having an alternating sequence.

DISCUSSION

In the enzymic reaction studied in this work, the substrate is the D-mannuronic acid residues in the alginate chain, and the product is L-guluronic acid residues in the

*M_M = mobility with respect to that of mannuronic acid.

TABLE V

EXTENT OF SOLUBILISATION BY PARTIAL HYDROLYSIS

	Mannuronic acid (%)	*Soluble material* (%)[a]	*Calculated*	
			"M-blocks"	*"G-blocks"*
Sample *A*				
before	92	6	89	5
after	29	40	9	51
Sample *B*				
before	62	22	49	29
after	27	47	3.5	49.5
Sample *C*				
before	61	58	32	10
after	44	70	9	21

[a]After treatment for 35 min with 0.3M hydrochloric acid at 100°.

same chain. No method is available by which the product can be analysed without interference from the substrate. The assay we have used is based on the different extinctions of substrate and product in the Dische carbazole reaction. This reaction has recently been carefully studied by Knutson and Jeanes[8,9], and we have used their experimental procedure, with alginate samples of known uronic acid composition[4] for standardization. Figs. 1 and 2 demonstrate that the degree of transformation is a linear function both of enzyme concentration and time for values of $(P-1) < 0.5$, corresponding to degrees of transformation below 25%. All activity measurements were carried out at enzyme concentrations giving less than 25% transformation in 3 h. In order to obtain a satisfactory accuracy, conditions have been chosen, when possible, to give a degree of transformation of no less than 10%.

No attempts have been made to rigorously purify the enzyme. As mentioned previously[2], the alginate produced by *A. vinelandii* was usually of relatively low viscosity, and the thiobarbituric acid reaction indicated a certain activity of alginases of the β-eliminase type. The degradation taking place[2] during the enzymic epimerization reaction was small, but significant. We have found a variable amount of alginase activity in our enzyme preparations. However, in all cases, the alginase activity is small when compared to the epimerase activity, and, at the maximum, corresponds to 0.02 elimination for each monomer unit epimerized. Care was taken to select preparations having low alginase activity for end-point determinations and for examination of block structure, and comparisons between preparations having different alginase activity showed that no influence of the elimination reaction on the results could be detected.

The effect of calcium ions on the activity of the epimerase is remarkable, and no significant activity was observed in the absence of calcium ions. The transformation of D-mannuronic acid residues into L-guluronic acid residues, assuming the former

to be in the *C1* (D) conformation[14], leads to an increase in the number of axial groups carrying heavy substituents, and would, therefore, be expected to be thermodynamically unfavourable. No energy-rich compounds are involved in the epimerization reaction, however, and it seems reasonable to assume that the higher affinity of calcium ions for guluronic acid residues than for mannuronic acid residues[15,16] might provide the energy required for the reaction. A similar argument, has recently been brought forward as additional support of experimental results[17] that indicate a higher affinity of calcium ions for monomeric L-guluronic acid than for D-mannuronic acid. However, as the epimerization takes place on the polymer level, and the difference in ion-binding properties of the two types of uronic acid residues, when present in a polymer chain, is well established[15], we do not agree that the reaction of the epimerase described here can be used as an argument for the calcium-binding properties of the monomers.

Selectivity studies have shown that the difference in affinity of the two types of uronic acid residue is even more pronounced for strontium than for calcium ions[15]. Strontium ions can replace calcium ions in the epimerization reaction, but are markedly less effective. This may indicate that calcium ions also function in a way other than by providing energy for the reaction. This is supported by the effect of magnesium and sodium ions on the activity in the presence of calcium ions. Because of the ion-exchange reactions taking place, the addition of sodium or magnesium ions would decrease the amount of calcium ions bound to the carboxyl groups of the polymer, and thereby increase the calcium activity in the solution. If the sole effect of calcium ions was to provide energy by the binding to L-guluronic acid residues, addition of magnesium or sodium ions would be expected to lead to a decrease of enzyme activity. This was the case at high concentrations (3.4mM) of calcium ion. At low concentrations (0.34mM) of calcium ion, however, addition of sodium or magnesium ions leads to an increase in activity followed by a decrease at higher concentrations. This increase may indicate that calcium ions are also involved in the enzymic process in a way other than by making the reaction thermodynamically favourable.

The lower temperature optimum at low (0.34mM) than at high (3.4mM) concentrations of calcium ion indicates that calcium ions are important for the stability of the enzyme (Fig. 8). This is supported by the results in Table II. No decrease in stability was observed at 0.34mM calcium in the absence of substrate. Only when the calcium concentration was still lower (0.01mM) was the stability of the enzyme diminished. The calcium-binding properties of the substrate should, however, be taken into account, making comparison of results at the same calcium concentration with and without substrate misleading.

The effect of calcium demonstrated above requires that comparisons be carried out at the same calcium activity in solution. A detailed study of the kinetics of the reaction is thus very difficult, since the calcium-binding properties of the substrate results in an increase in affinity to Ca^{2+} as the conversion into L-guluronic acid residues proceeds. This applies also to the effect of substrate alone, since a variation of the substrate concentration will influence the calcium activity in solution. We have,

therefore, not attempted to determine the Michaelis–Menten parameters of the reaction. Another complication is the precipitation or gel formation with calcium ions, which depends upon the uronic acid composition of the alginate[18]. At higher concentrations of substrate, we have observed the transformation of the whole solution into a gel during the enzyme reaction, as the increasing content of L-guluronic acid leads to increased tendency for gel formation. At the alginate concentrations used in this work, however, no visible precipitation was observed for calcium concentrations below 7mM.

In agreement with this, the results in Table V and Fig. 4 seem clearly to indicate that the end-point of the reaction, at the conditions used in this work, corresponds closely to the composition obtained when no more mannuronic acid-rich blocks are present. No tendency of transformation of blocks having alternating structure into guluronic acid-rich blocks has been observed, and the end-point of the reaction will, therefore, depend upon the relative rate of formation of the two block types. Increasing the concentration of calcium ion from 0.07 to 6.8mM leads to a significant change in the relative rates of formation of the two types of block in the last part of the epimerization reaction (Fig. 9) and, consequently, in the end-point of the reaction. This is in qualitative agreement with the results in Table V. The possibility of changing the relative rates of formation of the two block-types by varying the conditions in the epimerization reaction has not yet been further explored, but the composition of alginates produced by *Azotobacter vinelandii*[1] indicates that samples may be formed that are richer in blocks of L-guluronic acid residues than those corresponding to the results given in Fig. 9.

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