can be evaluated only on the basis of limited data. The known bacterial *trans*-eliminases have all been shown to attack polygalacturonic acid. *Clostridium multifermentans* evidently does not produce a hydrolytic enzyme. Other bacterial species may produce polygalacturonase but a positive identification of this hydrolytic enzyme in bacteria has not yet been made. Prior to the discovery of *trans*-eliminases it was generally observed that crude bacterial pectic enzymes attack polygalacturonic acid at *p*H values above neutrality and that they are stimulated by the addition of calcium. Since these observations were on the basis of either reducing-group production or decreases in viscosity, conceivably either polygalacturonase or *trans*-eliminase could be involved. Proven fungal polygalacturonases, however, have low *p*H optima and no calcium dependence. Thus bacterial polygalacturonases, if they exist, either differ considerably from the fungal type or are not widespread. All of the known bacterial *trans*-eliminases agree with the earlier observations by having both high *p*H optima and calcium dependence. Therefore it is suspected that polygalacturonic acid-*trans*-eliminase, in combination with pectinesterase, constitutes the major enzymic system for pectinolysis by bacteria.

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# The Pattern of Action of an Exopolygalacturonic Acid-*trans*-eliminase from *Clostridium multifermentans**


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A bacterial polygalacturonic acid-*trans*-eliminase (PATE) of *Clostridium multifermentans* has been shown to remove units of *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid (or α,β-unsaturated digalacturonic acid) from the reducing ends of polygalacturonate chains. The reaction goes to completion. This terminally splitting enzyme has been named "exopolygalacturonic acid-*trans*-eliminase" (exo-PATE) in contrast to the already known randomly splitting endo-PATE produced by other bacteria. Tetra- and trigalacturonic acids are degraded at the same initial rate as polygalacturonate. Each produces 1 mole of unsaturated digalacturonic acid. The balance of the reactions consists of 1 mole of normal digalacturonic acid in case of the tetramer and 1 mole of D-galacturonic acid in case of the trimer. Digalacturonic acid is not attacked by the enzyme. Viscosity decrease of polygalacturonate in relation to per cent cleavage is typical of an end-group attack. Fully esterified pectin is not attacked by exo-PATE unless a source of pectinesterase is also added. By determining the extent of cleavage of partially esterified pectin N.F. some information has been obtained on the distribution of the free carboxyl groups in this substrate.


Within the last decade it has become increasingly apparent that the glycosidic bonds of pectic substances are subject to degradation by a variety of enzymes which differ markedly in both substrate specificity and mode of attack. In 1957, prior to the discovery of enzymes which split pectic substances by a *trans*-elimination mechanism, Demain and Phaff (1957) proposed a classification of the pectic glycosidases based on whether the substrate molecule was split in a terminal or a random manner. Random degradation, the most common type of breakdown encountered, is typified by the endopolygalacturonase produced by *Saccharomyces fragilis*. Demain and Phaff (1954b) found that this enzyme hydrolyzed polygalacturonic acid initially to higher oligouronides, which ultimately became degraded to a mixture of D-galacturonic and digalacturonic acids. Saito (1955) showed that *Asper-*

* These results were presented at the Symposium on Recent Developments in Pectin Chemistry and Pectin Degradation at the 114th American Chemical Society meeting in Los Angeles, California, March 31 to April 5, 1963.

*gillus niger* produced both an endopolygalacturonase and an exopolygalacturonase. The endopolygalacturonase was similar to that produced by *Saccharomyces fragilis*. The exopolygalacturonase, however, produced galacturonic acid initially before higher oligouronides were detected and the latter were ultimately hydrolyzed completely to galacturonic acid.

In addition to analysis for the sequence in which the products of degradation occur a second approach employed for determining the mode of attack is based on viscosity measurements. When the substrate has undergone a small percentage of cleavage, randomly splitting enzymes cause a large decrease in viscosity, whereas terminally splitting enzymes cause a relatively small drop in the viscosity of the substrate.

The enzymes which degrade pectic substances by *trans*-elimination have so far been found to act randomly upon the substrate molecules. Thus, pectin-*trans*-eliminase from *Aspergillus fonsecaeus* (Edstrom, 1962) and polygalacturonic acid-*trans*-eliminase from *Bacillus polymyxa* (Nagel and Vaughn, 1961a,b) caused 50% reduction in relative viscosity of their respective substrates when only 2–3% of the bonds were broken. The random-splitting activity of the above enzymes and that of *Erwinia carotovora* (Moran, 1963) was also shown by paper-chromatographic examination of the products produced during reactions with their respective substrates.

The present publication deals with the pattern of action of polygalacturonic acid-*trans*-eliminase from *Clostridium multifermentans* on pectic acid and on pectins with different degrees of esterification. It will be shown that the enzyme removes unsaturated digalacturonic acid units from the reducing end of polygalacturonic acid and of certain oligogalacturonides and thus may be classified as an exopolygalacturonic acid-*trans*-eliminase (exo-PATE).[1]

## Materials and Methods

The materials and methods employed for the production, assay, and purification of PATE and general procedures for paper chromatography are described in the accompanying publication (Macmillan and Vaughn, 1964). Spots were detected by spraying with silver nitrate (Trevelyan *et al.*, 1950).

Polygalacturonic acid with a low DP was prepared by heating pectin in dilute sulfuric acid according to the procedure of McCready and Seegmiller (1954). Its average DP was 11.94, based on the ratio of carboxyl groups to aldehyde groups. Its moisture content was 9.4%.

Tetragalacturonic acid was prepared by treating commercial polygalacturonic acid with purified yeast endopolygalacturonase according to the procedure of Demain and Phaff (1954a). Digalacturonic and trigalacturonic acids were prepared by the procedure of Phaff and Luh (1952).

A sample of *O*-(4-deoxy-β-L-*threo*-hexopyranos-4-enyluronic acid)-(1 → 4)-D-galacturonic acid was kindly supplied by Dr. C. W. Nagel. The structure of this compound was elucidated by Hasegawa and Nagel (1962). It will be referred to as unsaturated digalacturonic acid in the experimental section.

A polygalacturonic acid (no. 3491) of high purity was purchased from the Sunkist Growers, Inc., Corona, Calif. It was used without further treatment. Pectin N.F. was purchsed from Exchange Lemon Products Co., Corona, Calif. Polymethylpolygalacturonate methyl glycoside (Link pectin) was prepared by the method of Morell and Link (1932) and Morell *et al.* (1934).

A solution of crude alfalfa pectinesterase was prepared by macerating leaves and stems in a blender with 0.1 M potassium phosphate buffer at *p*H 7.0 to form a thick slurry which was then filtered. The filtrate was frozen and refiltered prior to use. A solution of crude tomato pectinesterase was prepared by macerating 200 g fresh tomato tissue in a blender with 2 g of NaCl. The homogenate was filtered and the clear yellow serum was used as the enzyme source without further treatment.

## Results

*Viscosity Decrease versus per cent Cleavage.*—First a comparison was made of the decrease in viscosity and the per cent degradation of pectic acid as a function of time. Thirty ml of reaction mixture was prepared as described in Figure 1. Immediately after addition of enzyme, 10 ml of the reaction mixture was placed in an Ostwald-Cannon-Fenske capillary viscosimeter previously standardized by determining the flow times for water and for a reaction mixture containing boiled instead of active enzyme. Flow times for the active preparation were determined periodically. Other samples were removed from the remaining 20 ml of reaction mixture and diluted 50-fold with 0.1 M sodium acetate buffer at *p*H 3.7 to inactivate the enzyme. The absorbancy at 235 mμ of these diluted samples was used for determination of the per cent degradation of polygalacturonic acid by the method described in the accompanying publication. A comparison of the two sets of data (Fig. 1) shows that 22.5% degradation had occurred at 50% reduction in viscosity. Complete conversion to unsaturated dimer was taken as 100% degradation.

The high percentage of degradation at 50% reduction of the viscosity gives a strong indication for terminal action of the enzyme, since various figures in the literature indicate approximately 3% cleavage for the same viscosity change in the case of random cleavage of pectins.

*Paper Chromatography of Pectic Acid Treated with PATE.*—When pectic acid (0.5%) was treated with PATE in Tris buffer (0.033 M) at *p*H 8.0 and samples of the reaction mixture were analyzed by paper chromatography, unsaturated digalacturonic acid was the only product which could be detected very early during the reaction. Its concentration increased to high levels as the reaction proceeded. This observation confirms a terminal mechanism of cleavage, since randomly splitting pectic enzymes produce a number of higher oligogalacturonides during the early stages of a reaction.

If the polygalacturonate chain is attacked from the nonreducing end by a *trans*-elimination mechanism, the first oligouronide produced should be a saturated product, the balance of the reaction being a chain with an α,β-unsaturated unit at the nonreducing end. From there on, all subsequent dimers removed should constitute unsaturated digalacturonic acid. If, on the other hand, the polymer is degraded from the reducing end of the chain, the first product and subsequent ones are unsaturated digalacturonic acid units. When the end of the chain is reached a saturated monomer or oligogalacturonide fragment results, its size depending on the smallest oligomer which the enzyme can cleave and on the fact whether the chain contains an odd or even number of monomer units. Since the DP of polygalacturonate ranges between

[1] Abbreviations used in this work: PATE, polygalacturonic acid-*trans*-eliminase; DP, degree(s) of polymerization.

TABLE I

PAPER CHROMATOGRAPHIC ANALYSIS OF THE PRODUCTS FROM POLYGALACTURONIC ACID DEGRADED BY PATE AT 30°[a]

| Time | Products | | | | | Per cent Degradation |
|---|---|---|---|---|---|---|
| | Unsaturated Digalacturonic Acid[b] | D-Galacturonic Acid[b] | Di-galacturonic Acid[b] | Tri-galacturonic Acid[b] | X[c] | |
| 5 min | (+) | | | | | 2.7 |
| 15 | + | (+) | (+) | | | 6.5 |
| 30 | ++ | (+) | (+) | | | 10.2 |
| 45 | ++ | (+) | (+) | | | 13.8 |
| 1 hr | +++ | (+) | (+) | (+) | (+) | 17.7 |
| 1.5 | +++ | + | + | (+) | (+) | 25.1 |
| 2 | +++ | + | + | (+) | (+) | 31.7 |
| 4 | ++++ | ++ | + | (+) | (+) | 60.2 |
| 6 | ++++ | ++ | + | (+) | (+) | 72.0 |
| 14 | ++++ | ++ | ++ | (+) | (+) | 84.3[d] |

[a] The concentration of PATE was 0.031 unit per ml of 0.46% substrate in 0.033 M Tris buffer at *p*H 8.0. [b] (+), Represents very weak spots; +, weak spots; ++++, very intense spots. [c] Presumably unsaturated trigalacturonic acid by relative position on the paper. [d] This figure does not represent maximal degradation—upon longer incubation values up to 98% were obtained.

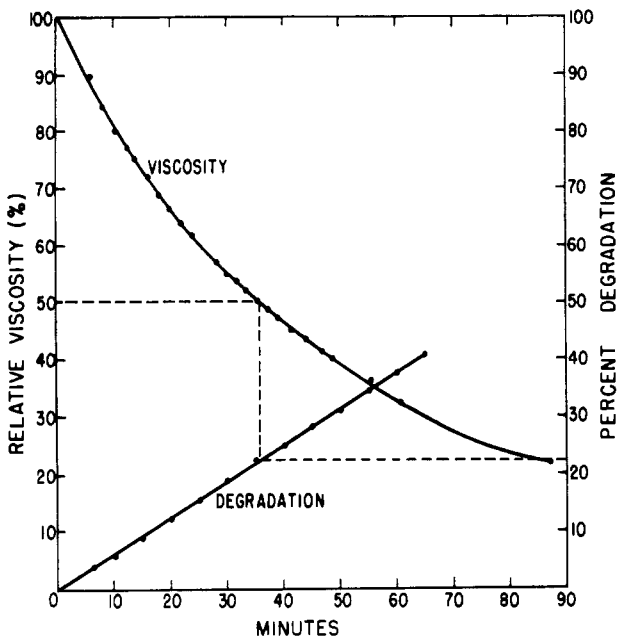


FIG. 1.—Viscosity changes and per cent degradation of polygalacturonate by PATE as a function of time. The reaction mixture contained 0.46% polygalacturonic acid in 0.0005 M $CaCl_2$ and 0.033 M Tris buffer at *p*H 8.0. The enzyme concentration was 0.075 unit per ml and the temperature was 30°. The initial flow time through the viscosimeter was 82.3 seconds (100%). Water had a flow time of 54.9 seconds (0%).

60 and 100, the saturated products may be expected to occur in a relatively low concentration. Since normal applications of aliquots of reaction mixtures on the chromatograms gave no clear-cut evidence for saturated products the experiment was repeated and larger samples of 2 ml were taken. After dilution with 2 ml of 0.2 M acetate buffer at *p*H 3.7 to stop the reaction, cations were removed with Dowex 50 ($H^+$ form) and the samples were evaporated to dryness over $CaCl_2$ in a vacuum desiccator. The residues were taken up in 0.2 ml of distilled water and 10 μl of each sample was chromatographed on Whatman No. 4 paper with ethyl acetate–pyridine–water–acetic acid (5:5:3:1). Toward the end of the reaction, which was followed by measurement of the increase in absorbancy at 235 mμ, small amounts of compounds with mobilities identical to D-galacturonic acid and digalacturonic acid were shown to be present (Table I). In addition, minute amounts of two other compounds were detected. One of these corresponded to a control spot for trigalacturonic acid and the other one was presumably unsaturated trigalacturonic acid, i.e., trigalacturonic acid with an α,β unsaturated monomeric unit at the nonreducing end.

If the above experiments are done with 0.5% polygalacturonate of considerably shorter chain length, the relative concentrations of products other than unsaturated digalacturonic acid should increase. When polygalacturonate with an average DP of 12 was used as the substrate under otherwise identical conditions, the relative concentrations of galacturonic and digalacturonic acids were considerably higher than before. The other two faint spots referred to above did not significantly increase in intensity.

*Action of PATE on Oligogalacturonides.*—Initial experiments were performed by measuring absorbancy changes at 235 mμ with di-, tri-, tetra-, and polygalacturonic acid (DP-12) at approximately equimolar concentrations. The reaction mixtures were dissolved in 0.033 M Tris buffer at *p*H 8.0 and contained 0.0005 M $CaCl_2$. It was found that digalacturonic acid produced no change in absorbancy at 235 mμ, but the other three substrates underwent *trans*-elimination at the same initial rate and the reaction continued linearly until nearly completed. Thus trigalacturonic acid appears to be the smallest oligouronide which can undergo *trans*-eliminative cleavage by the PATE of *C. multifermentans*.

Next the products of the reaction were investigated by paper chromatography as described for the polymer. The reaction mixtures contained 0.75% trigalacturonic acid or 1% tetragalacturonic acid in 0.0005 M $CaCl_2$ and 0.033 M Tris buffer at *p*H 8.0. The enzyme concentration was 0.06 unit of PATE per ml and the temperature was 30°. After 17 hours tetragalacturonic acid was nearly quantitatively converted into normal and unsaturated digalacturonic acids, whereas trigalacturonic acid yielded D-galacturonic and unsaturated digalacturonic acids as the only detectable products. The accumulation of normal digalacturonic acid from the tetramer further confirmed the lack of activity of clostridial PATE on digalacturonic acid.

*Establishment of PATE Action on the Reducing End of Polygalacturonate Chains.*—Two pieces of evidence were obtained in the preceding results which indicated terminal action at the reducing end of polygalacturonate: (1) Action of PATE on trigalacturonic acid produced D-galacturonic acid and unsaturated dimer.

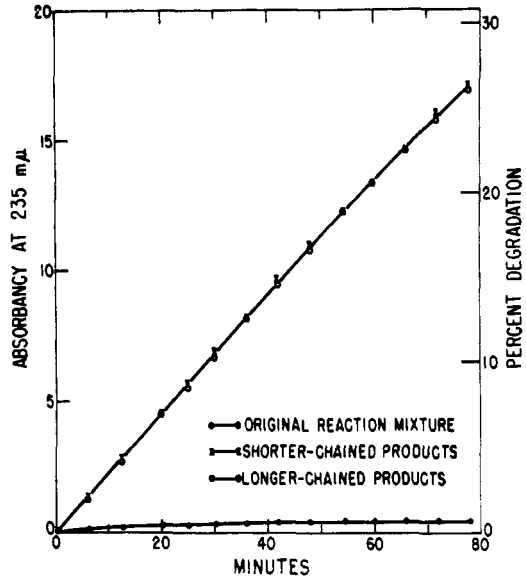


FIG. 2.—Comparison of absorbancy changes at 235 mμ in a reaction mixture of PATE with polygalacturonic acid (DP-12) and absorbancy changes in fractions representing the longer-chained galacturonides and the shorter-chained galacturonides isolated from the same reaction mixture. The reaction mixture contained 0.5% substrate, 0.033 M Tris buffer at *p*H 8.0, 0.0005 M $CaCl_2$, and 0.06 unit of PATE per ml. Temperature, 30°.

Action from the nonreducing end would have given digalacturonic acid plus unsaturated monomer which would have rearranged itself to deoxyketouronic acid (Preiss and Ashwell, 1963) with resulting loss in absorbancy at 235 mμ; and (2) the occurrence of both D-galacturonic acid and digalacturonic acid during the degradation of polyuronide chains can be explained most logically by assuming that they represent terminal fragments from the nonreducing ends of odd- or even-numbered polymers, respectively.

The following experiment was designed to obtain more direct evidence. Attack at the reducing end of the substrate molecule should result in the production of unsaturated digalacturonic acid and absence of a double bond in the chain after unsaturated dimer units were removed from the molecule. The reverse would be the case if the chains were attacked from the nonreducing ends. The measurement of absorbancy in the longer-chained residual galacturonides, isolated in purified form from samples obtained at intervals from a reaction mixture, could give confirmatory evidence for one of the two possibilities. In preparation for this experiment, a method was developed for precipitating polygalacturonic acid, DP-12, from a solution containing purified unsaturated digalacturonic acid. In addition to these two components the solution also contained 0.0005 M $CaCl_2$, Tris buffer, and 0.1 M acetate buffer, *p*H 3.7. Upon addition of an equal volume of 95% ethanol to this solution polygalacturonic acid, DP-12, precipitated as a dense white voluminous material. Unsaturated digalacturonic acid was not precipitated under these conditions as all of the absorbancy at 235 mμ, present prior to the addition of alcohol, could be accounted for in the supernatant liquid after centrifugation. No further precipitation was observed by the addition of more ethanol. After the precipitate was washed twice with 95% ethanol it was dissolved in distilled water. The absorbancy of this solution at 235 mμ was not significantly higher than that of a control sample of polygalacturonic acid, DP-12. Thus,

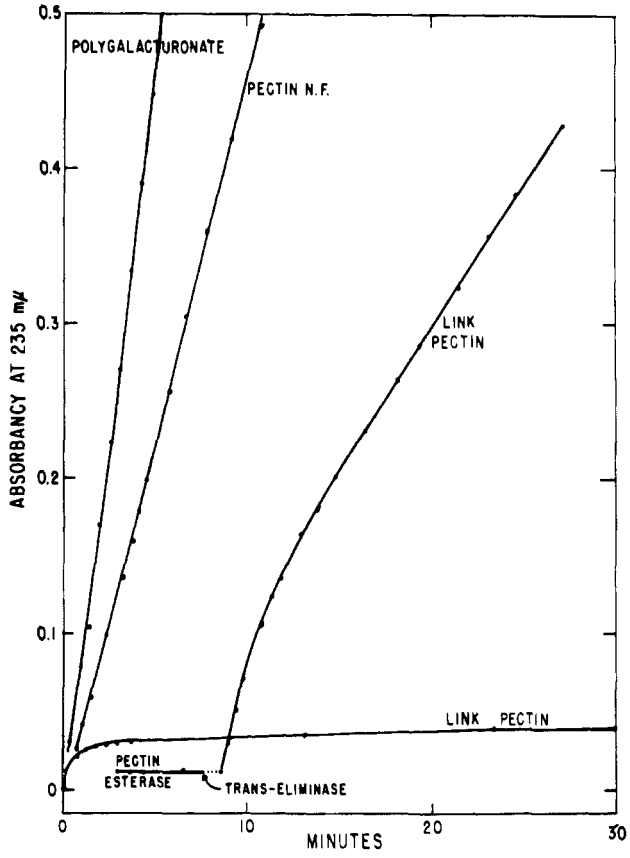


FIG. 3.—The action of PATE on Link pectin with and without added alfalfa pectinesterase compared to the action of PATE on polygalacturonic acid and pectin N.F. All reaction mixtures contained approximately 0.5% substrate in 0.001 M $CaCl_2$, 0.033 M Tris buffer at *p*H 7.0, and 0.05 unit of PATE per ml. Temperature was 30°.

this procedure effectively removed the added unsaturated digalacturonic acid.

This method of separation was then applied to a reaction mixture containing 0.5% polygalacturonic acid (DP-12) and PATE. Aliquots were removed from the reaction mixture at various times and treated with 95% ethanol as described above. Absorbancy at 235 mμ of both the redissolved precipitates (longer-chained products) and the supernatant fluids (shorter-chained products) was measured and compared with the absorbancy found for unfractionated samples removed concomitantly from the reaction mixture. The results are shown in Figure 2. The absorbancy values of the shorter-chained products (unsaturated digalacturonic acid) are in excellent agreement with those of the unfractionated reaction mixture. The small amount of absorbancy in the precipitated longer-chained products is probably due to incomplete washing out of unsaturated dimer. The data clearly show that the longer-chained products resulting from the action of PATE do not contain double bonds in significant concentration, and polygalacturonate, therefore, must be attacked by clostridial PATE from the reducing end of the molecule.

*Percentage Conversion of Substrates of High and of Low DP to Unsaturated Digalacturonate.*—Since it has been established that the enzyme starts its action at the reducing end of the chains by successively splitting off unsaturated digalacturonic acid units, it can be concluded that D-galacturonic acid is formed as the final unit from trigalacturonic acid in odd-num-

TABLE II

RATE AND EXTENT OF DEGRADATION OF PECTIC SUBSTANCES OF VARYING DEGREES OF ESTERIFICATION WHEN ACTED UPON BY PATE AT 30°[a]

| Substrate | Enzyme Concentration (PATE units/ml) | Substrate Concentration (%) | $\frac{\Delta OD_{235}}{Minute}$ | Relative Rate | Final Degradation (%) |
|---|---|---|---|---|---|
| Polygalacturonic acid | 0.02 | 0.46 | 0.045 | 100 | 99[b] |
| Pectin N.F. (68% esterified) | 0.02 | 0.43 | 0.019 | 42 | 8[b] |
| Polymethylpolygalacturonate methyl glycoside (Link pectin, 96% esterified) | 0.04 | ~0.5 | trace | ~0 | <0.1[c] |

[a] Reaction mixtures contained 0.0005 M $CaCl_2$ and 0.033 M Tris buffer at *p*H 7.0 in addition to the substrate and enzyme concentration shown. [b] Value at 50 hours; only slight changes in absorbancy between 30 and 50 hours were detected. [c] Value at 30 minutes; no change in absorbancy occurred between 30 minutes and 5 hours.

bered chains and digalacturonic acid from the degradation of tetragalacturonic acid in even-numbered chains. At the same time the final concentration of these saturated products must increase as the DP of the substrate is lowered. This reasoning was subjected to experimental analysis. As explained in the accompanying paper (Macmillan and Vaughn, 1964), the percentage conversion of polygalacturonate to unsaturated dimer can be calculated on the basis of a molar extinction coefficient of 4800 for the latter compound. With polygalacturonate the maximum conversion to unsaturated digalacturonic acid was 98%. In a comparable experiment with polygalacturonate of DP-12, a value of 87% was obtained. Since the percentage degradation was based on absorbancy measurements, the differences between these percentages and 100% was interpreted as the percentage of substrate which was not converted into unsaturated digalacturonic acid. Thus 13% of the substrate with DP-12 went to D-galacturonic and digalacturonic acids, which were identified chromatographically. Since the DP value is an average figure one might assume that an equal number of odd- and even-unit chains are present (say, 50% DP-12, 25% DP-11, 25% DP-13), which would result in equimolar concentrations of galacturonic and digalacturonic acids. Thus, on the average, 1.5 monomer units from polygalacturonic acid with an average DP of 12 would theoretically result in saturated products. This value represents 12.5% of the substrate and is in good agreement with the value of 13%, which was found experimentally.

Paper chromatographic analysis of the breakdown of tri- and tetragalacturonic acids suggests that 66.7% of the former and 50% of the latter substrate are converted to unsaturated digalacturonic acid.

*Extent of Cleavage of Substrates of Different Degrees of Esterification.*—In the accompanying paper it was shown that purified PATE had essentially no activity on fully esterified pectin (Link pectin). Since pectin N.F. contained approximately 32% unesterified anhydrogalacturonic acid units, it was of interest to compare the rate and extent of degradation of this substrate with those found for polygalacturonic acid and Link pectin (Table II).

Although 32% of pectin N.F. is unesterified, PATE can degrade only 8% of the chains to unsaturated digalacturonic acid (100% being taken as complete degradation to unsaturated dimer). Since PATE action starts at the reducing end of the chains, an average of 8% of the anhydrogalacturonic acid units at the reducing ends must be blocks of unesterified units, unless the enzyme is able to bypass single esterified units. However, paper chromatographic analysis of the reaction products (after precipitation of residual pectin with 60% ethanol) in ethyl acetate–acetic acid–water, 2:1:2 (Demain and Phaff, 1954a) revealed no products other than unsaturated digalacturonic acid.

*Action of Mixtures of Pectinesterase and PATE on Link Pectin.*—Whereas purified PATE has virtually no activity with Link pectin as the substrate, crude clostridial PATE, which contains pectinesterase, can degrade fully esterified pectin. In the following experiment the effect of an extraneous source of pectinesterase (alfalfa) was tested in a reaction mixture containing purified PATE and Link pectin. Link pectin was incubated with alfalfa pectinesterase for approximately 7 minutes prior to the addition of PATE. During this preliminary incubation no increase in absorbancy at 235 mμ was detected, indicating that the crude pectinesterase contained no *trans*-eliminase activity (Fig. 3). Upon addition of PATE there was a rapid increase in absorbancy showing that during the first 7 minutes sufficient deesterification of the Link pectin had occurred to allow PATE to operate at approximately the same rate as when pectin N.F. was the substrate. This rate, however, soon decreased to a lower value which was most likely a function of the rate of deesterification by the esterase.

This point was confirmed in another experiment in which varying amounts of tomato pectinesterase were added to reaction mixtures of Link pectin which all contained the same amount of purified PATE. Increases in absorbancy at 235 mμ were followed for 10 minutes, during which period the optical density increased as a linear function of time. The rate of *trans*-elimination as a function of the concentration of pectinesterase is shown in Figure 4. These results demonstrate that pectinesterase is the rate-limiting factor in the cleavage of the glycosidic bonds of Link pectin by clostridial PATE.

## DISCUSSION

The results presented above show that the PATE from *Clostridium multifermentans* splits unsaturated digalacturonic acid units from the reducing end of polygalacturonate chains. It may therefore be classified as an exo-PATE in contrast to the randomly splitting PATE (endo-PATE) from *Bacillus polymyxa* (Nagel and Vaughn, 1961a) and *Erwinia carotovora* (Moran, 1963). Exo-PATE attacks tri-, tetra-, and polygalacturonic acid (DP-12) at approximately the same initial rate. This unusual behavior is in sharp contrast to the randomly splitting *trans*-eliminases which degrade oligogalacturonides at rapidly declining rates as the DP of the substrate decreases. Of particular interest is the fact that trigalacturonic acid is the smallest oligouronide which can be split by exo-PATE and the reaction products are D-galacturonic acid

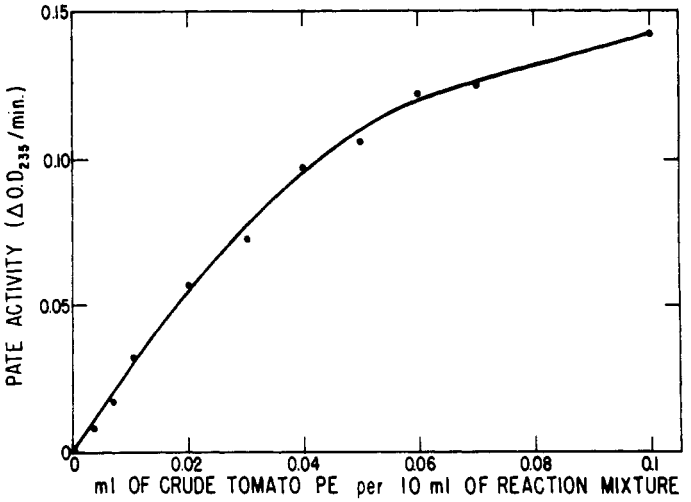


FIG. 4.—Effect of various concentrations of crude tomato pectinesterase on the activity of PATE. The substrate was 0.5% Link pectin dissolved in 0.0005 M $CaCl_2$ and 0.033 M Tris buffer at *p*H 7.0. Each reaction mixture contained 0.08 unit of PATE per ml. Temperature, 30°.

(a substrate which cannot be utilized by the living clostridial cells) and unsaturated digalacturonic acid. The latter product is presumably metabolized by the growing organism as it does not accumulate *in vivo*. Moran (1963) discovered an intracellular enzyme in *Erwinia carotovora* which specifically splits unsaturated digalacturonic acid to 2 moles of 4-deoxy-L-*threo*-5-hexosuloseuronic acid.

Besides the difference in action pattern with polygalacturonate, exo-PATE and endo-PATE also differ in their behavior toward tetragalacturonate. With this substrate endo-PATE produces D-galacturonate, unsaturated di- and tri-galacturonate, and a trace of normal digalacturonate (Nagel and Vaughn, 1961b). In contrast, exo-PATE can split only the central bond of tetragalacturonate producing equimolar quantities of normal and unsaturated digalacturonate. Thus endo-PATE can cleave either the second or third bond from the reducing end of the molecule, while exo-PATE action is limited to bond 2.

Since the clostridial exo-PATE produces exclusively unsaturated digalacturonic acid units from the ends of polygalacturonate chains and the reaction rapidly goes to completion with the formation of only very small quantities of saturated by-products, this system is ideal for the production of unsaturated dimer in quantity. The product can simply be precipitated from reaction mixtures as the strontium salt with 73% ethanol, since there are no significant quantities of other oligouronides which complicate isolation (Nagel and Vaughn, 1961b). After washing with 96% ethanol and ether it was dried and appeared to be nearly pure when tested paper chromatographically. After removal of $Sr^{2+}$ with Dowex 50 ($H^+$ form) it can be reprecipitated in the same manner for further purification.

The action of exo-PATE on pectin N.F. revealed some features about the distribution of the methoxyl groups in this substrate. In pectin N.F., which is 68% esterified, about one out of three carboxyl groups is free. If these free carboxyl groups were distributed evenly along the pectin chain, exo-PATE would not be expected to show any action on this substrate, since the enzyme would require at least two free anhydrogalacturonic acid residues at the reducing end of the chains. Experimental evidence obtained has shown that 8%, rather than a theoretical maximum of 32%, of pectin N.F. can be degraded to unsaturated digalacturonate. This suggests that one quarter of the available free carboxyl groups are located as a block at the reducing end of the molecule. Since no products other than unsaturated digalacturonic acid could be detected on paper chromatograms, it appears that exo-PATE cannot bypass units in the chain of which the carboxyl groups are esterified by methanol. Solms (1954) and Solms and Deuel (1954) have shown that plant pectinesterases hydrolyze methyl ester groups successively along a pectin chain, starting at a position where there is initially a free carboxyl group. This results in a product containing blocks of free carboxyls and esterified portions. It may be that during some stage prior to or during the manufacture of pectin N.F., citrus pectinesterase exerts a limited action on the pectin of the albedo, resulting in the formation of the above-mentioned blocks. Our work indicates that about one-fourth of the free carboxyl groups in pectin N.F. are located at the reducing end of the molecule.

When tomato pectinesterase was added to a reaction mixture of fully esterified pectin and purified PATE, activity of the latter enzyme was initiated in proportion to the amount of pectinesterase added (Fig. 4). Since this procedure is relatively simple, it may prove useful, upon standardization with known amounts of pectinesterase, as a method for determining unknown concentrations of this enzyme spectrophotometrically.

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# The Oxidation of Glucose and Related Compounds by Glucose Oxidase from *Aspergillus niger**

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Glucose oxidase from *Aspergillus niger* was purified by ammonium sulfate fractionation and chromatography on DEAE-cellulose. The purified enzyme was homogeneous on ultracentrifugation, density-gradient centrifugation, and starch-gel and paper electrophoresis. The enzyme was capable of oxidizing D-aldohexoses, monodeoxy-D-glucoses and *O*-methyl-D-glucoses at varying rates. Differences in the rates of oxidation of these compounds have been interpreted to indicate that the structural features of the substrate of particular importance in the enzymatic reaction are a pyranose ring in the chair or Cl conformation, an equatorially orientated hydroxyl group at position 1, and an equatorially orientated hydroxyl group at position 3. These structural features are probably involved in the formation of the enzyme-substrate complex.

Since the detection of glucose oxidase in extracts from *Aspergillus niger* (Müller, 1928), significant progress has been made on methods for the purification of this enzyme from various fungal sources (Franke, 1944; Keilin and Hartree, 1952; Underkofler, 1958; Kusai *et al.*, 1960) and on the elucidation of the mode of action of the enzyme (Keilin and Hartree, 1952; Bentley, 1955; Gibson *et al.*, 1963). In our laboratory a rapid and effective method based on ammonium sulfate fractionation and chromatography on DEAE-cellulose (Pazur and Ando, 1959) has been utilized for the preparation of glucose oxidase from *Aspergillus niger* in a highly purified form. The chromatography step was found to be particularly effective for separating the glucose oxidase from catalase, a separation difficult to achieve by other techniques (Underkofler, 1958). The purified glucose oxidase was homogeneous on ultracentrifugation, density-gradient centrifugation, and starch-gel and paper electrophoresis. Like glucose oxidase from other organisms (Keilin and Hartree, 1952; Kusai *et al.*, 1960), the enzyme from *Aspergillus niger* contains two flavin adenine dinucleotide (FAD) moieties per molecule and has a molecular weight of approximately 150,000. The *p*H optimum for the enzyme is 5.5 and the isoelectric point is 4.2.

Conflicting reports have appeared in the literature on the ability of glucose oxidase to oxidize hexoses other than D-glucose (Adams *et al.*, 1960; Hlaing, *et al.*, 1961). Consequently the action of the purified enzyme has been examined on some fifteen glucose isomers and derivatives with the view of determining which compounds are oxidized by the enzyme and which structural features of the substrate are of importance in the enzyme reaction. Included in the list of compounds were the monodeoxy derivatives, the epimers, a sulfur-containing derivative, and several *O*-substituted derivatives of D-glucose. A new procedure has been used for the preparation of 4-deoxy-D-glucose and 6-deoxy-D-glucose via mesyl- and iodotetraacetyl-D-glucose. Since the replacement of a secondary hydroxyl group with an iodo group is difficult to achieve, the reaction conditions are described in detail. Most of the compounds tested in this study were found to be slowly oxidized by pure glucose oxidase. Differences in the rates of oxidation of the compounds have been interpreted to indicate that certain structural features of the hexose molecule are of particular importance in the enzyme reaction. These structural features are a pyranose ring most probably in the chair or Cl conformation (Reeves, 1951), an equatorially orientated hydroxyl group at position 1, and an equatorially orientated hydroxyl group at position 3. Alteration of the hexose structure in any of these aspects has a greater effect on the rate of enzyme action than an alteration at other parts of the D-glucose molecule.

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## MATERIALS

*Enzyme Source.*—A preparation of glucose oxidase (Dee-O) was provided by the Miles Chemical Co., Elkhart, Ind. This enzyme preparation was isolated from a strain of *Aspergillus niger* by extraction of the enzyme with water and precipitation with a nonaqueous solvent. Examination of the preparation by density-gradient centrifugation showed that it contained several ultraviolet-absorbing components of different molecular size (Pazur *et al.*, 1962). Assays for enzyme activities showed also that the preparation contained catalase and hydrolytic carbohydrases as well as glucose oxidase. The starting material for the purification work contained approximately 100 units of glucose oxidase activity per mg of nitrogen. A unit of glucose oxidase activity is defined as that quantity of enzyme which will cause the uptake of 11.2 $\mu$l of oxygen at STP per minute in a Warburg manometer at 30° and 760 mm Hg in the presence of excess catalase and oxygen with a 3% solution of D-glucose in 0.1 M potassium phosphate buffer, *p*H 5.9. This defini-