Catabolism of Galacturonic and Glucuronic Acids by Erwinia carotovora*

WENDELL W. KILGORE† AND MORTIMER P. STARR‡
From the Department of Bacteriology, University of California, Davis, California

(Received for publication, March 9, 1959)

Our interest in the microbial breakdown of uronic acids stems from this group's earlier studies (1, 2) on the catabolism of pectin by soft-rot bacteria of the genus Erwinia. In those investigations it had been shown that Erwinia carotovora, when previously grown on pectin, possesses enzymes which can convert pectin to galacturonic acid, and that the galacturonate is further metabolized by galacturonate-grown cells to a mixture of end products similar to that formed by Aerobacter.

Early experiments suggested that the first detectable step in the catabolism of n-galacturonate and n-glucurionate by cell-free extracts of E. carotovora and Aerobacter cloacae was a reduction of the aldehydic group with either DPNH or TPNH to the corresponding L-hexonic acid (3). We later found (4, 5) that extracts of Erwinia contain an enzyme which isomerizes galacturonic acid to 5-keto-L-galactonic acid, and glucuronic acid to 5-keto-L-gulonic acid. As indicated in a preliminary report, the isomerization is followed by the reduction of the keturonic acids to n-mannonic acid and n-altronic acid, respectively (6). The keturonic acid reductase, which catalyzes the reductive step, acts either with DPNH or TPNH. The a-hexonates are further metabolized to triosephosphate and pyruvic acid. The present paper summarizes our evidence for the identification of the intermediate steps involved in the metabolic sequence.

It has recently been found, independently of our own investigations, that extracts of uronic acid-adapted cells of Escherichia coli also catalyze the breakdown of uronic acids by this pathway (7, 8).

EXPERIMENTAL

Cultural Procedures—The strain of E. carotovora (EC 153) used in this study was isolated from soft-rot spoilage of green peppers and is the same strain used by Kraght and Starr (1, 2) in their studies on pectic enzymes and galacturonate fermentation. Aside from its inability to "produce gas" in the usual fermentation tests, this strain is typical of soft-rot Erwinia, and actively grows on pectin, possesses enzymes which can convert pectin to galacturonic acid, and that the galacturonate is further metabolized by galacturonate-grown cells to a mixture of end products similar to that formed by Aerobacter.

Early experiments suggested that the first detectable step in the catabolism of n-galacturonate and n-glucuronate by cell-free extracts of E. carotovora and Aerobacter cloacae was a reduction of the aldehydic group with either DPNH or TPNH to the corresponding L-hexonic acid (3). We later found (4, 5) that extracts of Erwinia contain an enzyme which isomerizes galacturonic acid to 5-keto-L-galactonic acid, and glucuronic acid to 5-keto-L-gulonic acid. As indicated in a preliminary report, the isomerization is followed by the reduction of the keturonic acids to n-mannonic acid and n-altronic acid, respectively (6). The keturonic acid reductase, which catalyzes the reductive step, acts either with DPNH or TPNH. The a-hexonates are further metabolized to triosephosphate and pyruvic acid. The present paper summarizes our evidence for the identification of the intermediate steps involved in the metabolic sequence.

It has recently been found, independently of our own investigations, that extracts of uronic acid-adapted cells of Escherichia coli also catalyze the breakdown of uronic acids by this pathway (7, 8).

EXPERIMENTAL

Cultural Procedures—The strain of E. carotovora (EC 153) used in this study was isolated from soft-rot spoilage of green peppers and is the same strain used by Kraght and Starr (1, 2) in their studies on pectic enzymes and galacturonate fermentation. Aside from its inability to "produce gas" in the usual fermentation tests, this strain is typical of soft-rot Erwinia, and actively grows on pectin, possesses enzymes which can convert pectin to galacturonic acid, and that the galacturonate is further metabolized by galacturonate-grown cells to a mixture of end products similar to that formed by Aerobacter.

Early experiments suggested that the first detectable step in the catabolism of n-galacturonate and n-glucuronate by cell-free extracts of E. carotovora and Aerobacter cloacae was a reduction of the aldehydic group with either DPNH or TPNH to the corresponding L-hexonic acid (3). We later found (4, 5) that extracts of Erwinia contain an enzyme which isomerizes galacturonic acid to 5-keto-L-galactonic acid, and glucuronic acid to 5-keto-L-gulonic acid. As indicated in a preliminary report, the isomerization is followed by the reduction of the keturonic acids to n-mannonic acid and n-altronic acid, respectively (6). The keturonic acid reductase, which catalyzes the reductive step, acts either with DPNH or TPNH. The a-hexonates are further metabolized to triosephosphate and pyruvic acid. The present paper summarizes our evidence for the identification of the intermediate steps involved in the metabolic sequence.

It has recently been found, independently of our own investigations, that extracts of uronic acid-adapted cells of Escherichia coli also catalyze the breakdown of uronic acids by this pathway (7, 8).

EXPERIMENTAL

Cultural Procedures—The strain of E. carotovora (EC 153) used in this study was isolated from soft-rot spoilage of green peppers and is the same strain used by Kraght and Starr (1, 2) in their studies on pectic enzymes and galacturonate fermentation. Aside from its inability to "produce gas" in the usual fermentation tests, this strain is typical of soft-rot Erwinia, and actively grows on pectin, possesses enzymes which can convert pectin to galacturonic acid, and that the galacturonate is further metabolized by galacturonate-grown cells to a mixture of end products similar to that formed by Aerobacter.

Early experiments suggested that the first detectable step in the catabolism of n-galacturonate and n-glucuronate by cell-free extracts of E. carotovora and Aerobacter cloacae was a reduction of the aldehydic group with either DPNH or TPNH to the corresponding L-hexonic acid (3). We later found (4, 5) that extracts of Erwinia contain an enzyme which isomerizes galacturonic acid to 5-keto-L-galactonic acid, and glucuronic acid to 5-keto-L-gulonic acid. As indicated in a preliminary report, the isomerization is followed by the reduction of the keturonic acids to n-mannonic acid and n-altronic acid, respectively (6). The keturonic acid reductase, which catalyzes the reductive step, acts either with DPNH or TPNH. The a-hexonates are further metabolized to triosephosphate and pyruvic acid. The present paper summarizes our evidence for the identification of the intermediate steps involved in the metabolic sequence.

It has recently been found, independently of our own investigations, that extracts of uronic acid-adapted cells of Escherichia coli also catalyze the breakdown of uronic acids by this pathway (7, 8).
L-galactonic and n-galacturonic acids. The absorption spectrum of these compounds after reaction with orcinol was measured with the enzyme.

Under the conditions of this procedure, pentoses and keturonates do not produce an extractable color.

Lactones were determined by the procedure described by Hestrin (11).

5-Keto-L-galactonic acid was determined by its reaction with carbazole as described by Suda and Watanabe (12). Although it was not so described by the authors, this test seems to be specific for 5-ketohexonic acids; uronic acids or 2-ketohexonic acids do not react detectably with carbazole under the conditions of this procedure.

Pyruvate was determined with crystalline lactic dehydrogenase.

The orcinol reaction (13, 14) was used to measure both 5-keto-L-galactonic and n-galacturonic acids. The absorption spectrum of these compounds after reaction with orcinol was measured with a Beckman model DK2 recording spectrophotometer.

Protein was determined by the method of Folin and Ciocalteau (15). All samples were compared with standard solutions of crystalline egg albumin.

Chromatography—Radial and descending chromatographs on acid-washed Whatman No. 1 or No. 20 filter paper were used for the identification and purification of various compounds. Before application on paper, the solutions were usually treated with Dowex 50 (H+). The following solvents were used routinely: Solvent 1, ethyl acetate-acetic acid-water (2:1:2, upper phase); Solvent 2, pyridine-ethyl acetate-acetic acid-water (5:3:1:3); Solvent 3, butanol-acetic acid-water (4:1:1); Solvent 4, butanol-ethanol-water (5:1:4, upper phase); Solvent 5, ethyl acetate-pyridine water (2:1:2, upper phase); Solvent 6, acetone pyridine-water (2:1:1).

Alkaline silver nitrate spray (16) was used to detect substances capable of reducing silver. Reducing sugars were revealed by spraying the chromatograms with p-aminophenyluronic acid (17). Lactones were detected by spraying with hydroxylamine-ferric chloride reagent (18).

Sources of Biochemicals—The basic calcium salt of 5-keto-α-galacturonic acid was prepared according to the method of Ehrlich and Guttmann (19), and converted to the soluble neutral calcium salt.

Pure α-galacturonic acid was obtained from the commercial product by several recrystallizations from acetic acid. α-Galacturono-γ-lactone and α-gulono-γ-lactone were the generous gifts of Dr. E. W. Putnam kindly supplied the α-altroono-γ-lactone, and Dr. M. Doudoroff very generously provided the 2-keto-β-deoxy-β-glucuronate and the 2-keto-β-deoxy-β-phospho-β-glucuronate. All other compounds used in this study were obtained as commercial products.

Enzymatic Analyses—Reductase or dehydrogenase activity with DPN or TPN was determined spectrophotometrically at 340 nm in a Beckman DU spectrophotometer, or in a Bausch and Lomb Spectronic 20 adapted for automatic recording by using the voltage across the meter to drive a Varian Recorder (model G-11).

Kinase activities were measured manometrically by following the production of CO₂ from a bicarbonate buffer (20).

RESULTS
Early in this investigation, several possible pathways were investigated by appropriate experimental means, with the conclusion that none of the following reactions are evident when E. carotovora acts upon α-galacturonate: decarboxylation to α-arabinose, phosphorylation with ATP, oxidation to muco acid, reduction of the carboxyl group to yield α-galactose. On the other hand, reduction of the aldehydic group to an hexonic acid seemed to be the most likely first step in the metabolism of α-arabinose. Cell-free extracts prepared from E. carotovora were able to catalyze the oxidation of DPNH and TPNH in the presence of either α-galacturonic or α-glucuronate but did not act upon α-mannuronate or L-iduronate; these oxidations were carried out by substrate-induced enzymes. When a preparation from α-galacturonate-grown cells was used, DPNH reacted faster with α-galacturonic than it did with α-glucuronate; when TPNH was the reductant, the reaction with α-glucuronate was faster than it was with α-galacturonic. This difference was less distinct with enzyme preparations from cells grown with α-glucuronate. The enzyme(s) which catalyzed the reduction
Uronic Acid Isomerase Activity

Reduction Lag Period—The experimental findings revealed that E. carotovora possesses an enzyme which can isomerize D-galacturonic acid to 5-keto-D-galactonate and D-glucuronate to 5-keto-D-gulonate in the absence of any cofactor. The presence of an isomerase was suggested by the appearance of a lag period when the alduronic acids were acted upon by "uranic acid reductase" and reduced pyridine nucleotides. This lag could be eliminated by preincubating the substrate with "uranic acid reductase" before the addition of either of the reduced pyridine nucleotides (Fig. 1). No lag occurred, however, when 5-keto-L-galactonate was the substrate. Unfortunately, the keturonic analogue of D-glucuronate, 5-keto-L-gulonate, was not available at that time for a similar trial.

Identification of Isomerase Product—The actual isomerization

of the uronates with DPNH or TPNH was tentatively named "uranic acid reductase."

Uronic Acid Isomerase

Reduction Lag Period—The experimental findings revealed that E. carotovora possesses an enzyme which can isomerize D-galacturonic acid to 5-keto-D-galactonate, and D-glucuronate to 5-keto-D-gulonate in the absence of any cofactor. The presence of an isomerase was suggested by the appearance of a lag period when the alduronic acids were acted upon by "uranic acid reductase" and reduced pyridine nucleotides. This lag could be eliminated by preincubating the substrate with "uranic acid reductase" before the addition of either of the reduced pyridine nucleotides (Fig. 1). No lag occurred, however, when 5-keto-L-galactonate was the substrate. Unfortunately, the keturonic analogue of D-glucuronate, 5-keto-L-gulonate, was not available at that time for a similar trial.

Identification of Isomerase Product—The actual isomerization

of D-galacturonate to its keturonic analogue was first revealed during examination of the activity of cell-free extracts with 5-keto-D-galactonate. With the use of the carboxylic acid reaction, it could be shown that most of the keturonic acid disappeared when it was incubated with the enzyme (Fig. 2).

The orcinol reaction was also used to follow the disappearance of the keturonic acid and the appearance of D-galacturonic acid. By incubating the enzyme with 5-keto-D-galactonate, it could be shown that the keturonic acid disappeared as another compound, presumably D-galacturonic acid, appeared. From Fig. 3, it will be noted that the peak at 542 mμ, typically given by authentic 5-keto-D-galactonate, disappeared completely whereas the peak at 670 mμ, characteristic of alduronic acids, increased. Since the end product of 5-keto-D-galactonate isomerization did not lactonize by boiling for 5 minutes in N HCl, it was tentatively concluded that the product formed was D-galacturonic acid.

Chromatographic evidence served to substantiate the conclusion that D-galacturonic acid is formed from 5-keto-D-galactonate. By spotting samples of a reaction mixture on Whatman No. 20 filter paper and developing the chromatograms with a number of solvents, it was shown that the keto acid disappeared during
TABLE I

<table>
<thead>
<tr>
<th>Rα values of alduronic and keturonic acids and experimental compounds</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>------------------</td>
</tr>
<tr>
<td>D-Glucose</td>
</tr>
<tr>
<td>D-Galacturonic acid</td>
</tr>
<tr>
<td>D-Glucuronic acid</td>
</tr>
<tr>
<td>5-Keto-L-galactonic acid</td>
</tr>
<tr>
<td>5-Keto-L-galactonic acid</td>
</tr>
<tr>
<td>5-Keto-L-gulonic acid</td>
</tr>
</tbody>
</table>

α Rα is relative to D-glucose = 1.

* See “Experimental” section of text for solvent mixtures.

† Isomerase product formed from 5-keto-L-galactonate.

‡ Isomerase product formed from D-galacturonic acid.

§ Isomerase product formed from D-glucuronic acid; no reference sample of authentic 5-keto-L-gulonic acid was available.

The course of incubation and another compound appeared having the same migration rate as D-galacturonic acid (Table I).

That the compound which is formed from the keto acid is indeed D-galacturonic acid has been shown by its ready convertibility to mucic acid, the dicarboxylic acid corresponding to galacturonic acid. Since the equilibrium of the reaction (D-galacturonic acid ⇌ 5-keto-L-galactonate) favors the formation of D-galacturonic acid, it was possible to prepare a quantity of this compound by action of the isomerase upon 5-keto-L-galactonate. This was accomplished by incubating 200 mg. of sodium 5-keto-L-galactonate, 800 μmoles of Tris-HCl buffer, pH 7.2, and 5.0 ml. purified enzyme (0.60 to 0.65 (NH₄)₂SO₄ fraction) in a final volume of 30.0 ml. When 60 per cent of the keturonic acid had disappeared as determined by the carbazole reaction, 5.0 ml. of 50 per cent trichloroacetic acid were added to stop the reaction and to precipitate the protein which was removed by centrifugation. Upon passage through a small column of Dowex 50 (H+), the supernatant liquid was concentrated under reduced pressure to 2.0 ml. Upon addition of an equal volume of concentrated nitric acid and after heating in a boiling water bath for 1 hour, the product was converted to mucic acid. Crystallization occurred at 4°C. In the controls (without enzyme) only the sample containing galacturonic acid formed a crystalline precipitate when treated as described; no precipitate formed in the control tube containing 5-keto-L-galactonic acid. The crystals in the experimental mixture were collected and washed several times with ethanol; yield 68 mg.; m.p., 213°; mixed m.p. with authentic mucic acid, 213–214°.

Reversibility of Reactions and Stoichiometry—In order to demonstrate the formation of 5-keto-L-galactonate from D-galacturonic acid it is necessary to incubate the enzyme with a high concentration of D-galacturonic acid. A compound then appears which reacts with carbazole in a manner indistinguishable from authentic 5-keto-L-galactonate (Fig. 2). Several hundred μg. of the keto acid were prepared enzymatically from D-galacturonate with the use of the procedure described in the protocol accompanying Fig. 2; however, this experiment was on a much larger scale and purified enzyme (0.60 to 0.65 (NH₄)₂SO₄ fraction) was used. The keturonic acid prepared in this manner was purified by paper chromatography (Solvent 1). The band formed by the acid was located and visualized by spraying with alkaline silver nitrate a thin strip cut from the edge of each chromatogram. The corresponding materials on the unsprayed sheets were eluted with water, pooled, and condensed under reduced pressure. Table I shows that the migration rates of the unknown keto acid and 5-keto-L-galactonic acid, with Solvents 1, 2, and 3, were identical.

The a-naphthoresorcinol reagent, which reacts with alduronic acids but not with keturonic acids, was used to demonstrate the appearance of D-galacturonate from 5-keto-L-galactonate. The disappearance of the keto acid was followed by its reaction with carbazole. Under conditions similar to those described in preceding sections, 34 μg. of galacturonate were formed in 5 minutes at 23°C when 32 μg. of keto acid had disappeared. After 10 minutes of incubation, when 50 μg. of 5-keto-L-galactonate had disappeared, 50 μg. of D-galacturonate were found.

* Purification and Properties of Uronic Acid Isomerase—Further purification of the “d-s” enzyme by (NH₄)₂SO₄ fractionation, showed that the precipitate formed at 0.60 to 0.65 saturation contained the highest isomerase activity, as determined by following, with the carbazole reagent, the rate of formation of galactu-
This enzyme, together with either DPNH or TPNH, does not and a slow reduction with TPNH (Fig. 5). When 5-keto-n-galactonate, provisionally named keturonic acid reductase, which and glucuronate-grown cells of Erwinia, there is also an enzyme carbon 5 of 5-keto-L-galactonate, either r.-galactonate or D-gulonate, 5-keto-n-gluconate, 2-keto-n-gluconate, and 2-keto-n-gulonate. The rate of migration of this lactone in Solvent 3 was indistinguishable from that of either d-altrono-γ-lactone or L-galactono-γ-lactone. However, in Solvents 2 and 4 both the unknown lactone and the authentic n-altrono-γ-lactone moved at the same rate, slightly in advance of L-galactono-γ-lactone. This indicated that the unknown lactone formed from n-glucuronate is indeed 5-keto-n-gulonate. The rate of migration of this lactone in Solvent 3 was indistinguishable from that of either d-altrono-γ-lactone or L-galactono-γ-lactone. However, in Solvents 2 and 4 both the unknown lactone and the authentic n-altrono-γ-lactone moved at the same rate, slightly in advance of L-galactono-γ-lactone. This indicated that the unknown lactone formed from n-glucuronate is indeed 5-keto-n-gulonate.

**Keturonic Acid Reductase**

In addition to the uronic acid isomerase found in galacturonate- and glucuronate-grown cells of *Erwinia*, there is also an enzyme present, provisionally named keturonic acid reductase, which catalyzes the rapid reduction of 5-keto-L-galactonate with DPNH and a slow reduction with TPNH (Fig. 5). When 5-keto-L-galactonate is the substrate, TPNH reacts faster than DPNH. This enzyme, together with either DPNH or TPNH, does not act upon n-galacturonate, n-glucuronate, nor upon the other available ketohexonates, 5-keto-n-glucuronate, 2-keto n-glucuronate, and 2-keto-L-galactonate.

**Identification of Hexonic Acid**—Because of the symmetry of carbon 5 of 5-keto-L-galactonate, either L-galactonate or D-altronate, or both compounds, could arise from the enzymatic reduction of the keto acid. The hexonic acid resulting from reduction of 5-keto-L-galactonate was tentatively identified as D-altrono after converting it to the corresponding lactone. Ten pmol of 5-keto-L-galactonate, 12 to 15 pmol of DPNH, 0.5 ml. of enzyme, 200 pmol of Tris-HCl buffer, pH 7.2, in a final volume of 2.0 ml. were incubated at room temperature for 1 hour. After the incubation, the mixture was treated with Dowex 50 (H⁺) and centrifuged. The supernatant solution was acidified to pH 1 by the addition of HCl and then placed in a boiling water bath for 5 minutes. This treatment is sufficient to convert the free hexonic acid to the lactone. The lactone was purified by chromatography in Solvent 4 and then rechromatographed in several solvents to aid in establishing its identity.

The rate of migration of this lactone in Solvent 3 was indistinguishable from that of either D-altrono-γ-lactone or L-galactono-γ-lactone. However, in Solvents 2 and 4 both the unknown lactone and the authentic D-altrono-γ-lactone moved at the same rate, slightly in advance of L-galactono-γ-lactone. This indicated that the unknown lactone was not L-galactono-γ-lactone and increased the likelihood that it was D-altrono-γ-lactone. The identity of D-altrono was further substantiated by identifying the pentose which was formed after the decarboxylation (Ruff degradation) of the hexonate.

The Ruff degradation (21) of hexonates consists of an iron-
catalyzed decarboxylation of the calcium salts; i.e., the decarboxylation of calcium n-altronate would give rise to D-ribose and calcium L-galactonate would yield L-lyxose. Several mg.
of the hexonate acid were prepared from 5-keto-L-galactonate by coupling the reaction with the glucose 6-phosphate dehydrogenase system also present in cell free extracts. 5-Keto-L-galactonate, 1.0 mmole, 1.2 mmoles of glucose 6-phosphate, 10 mg. of TPN, 75 μmoles of phosphate buffer, pH 7.8, 5.0 ml. of enzyme (0 to 0.50 (NH₄)₂SO₄ fraction of "d-s" enzyme) in a total volume of 30 ml. were incubated at room temperature until the reaction had stopped. Protein was removed by the addition of trichloroacetic acid and the resulting supernatant solution condensed under reduced pressure to a syrup. After the hexonone acid was lactonized by several additions of butanol, followed by evaporation, the syrup was diluted with water and the resulting solution passed through columns of Dowex 50 (II⁺) and Dowex 2 (formate) respectively. The hexono-lactone was further purified by chromatography in Solvent 4. Crystalline calcium hexonate was prepared from the lactone which was eluted from the chromatograms. The calcium salt was used for the Ruff degradation. The degradation product was reducing, did not lactonize, and gave a positive orcinol test for aldopentose. When the product was chromatographed in Solvent 4, the migration rate (Rf = 0.27) was identical with that of D-ribose (the pentose expected after decarboxylation of n-altronate) and not like that of L-lyxose (Rf = 0.24).

Stoichiometry—The stoichiometry of the reaction, 5-keto-L-galactonate + DPNH + H⁺ → D-altro-n-lactone + DPN⁺, was easily established by determining the disappearance of the keturonic acid with carbazole and the appearance of the hexonate as the lactone. In a reaction mixture containing keturonic acid, DPNH, buffer, and reductase, it was found that after 60 minutes of incubation, 1.1 μmoles of 5-keto-L-galactonate disappeared and 1.2 μmoles of hexonate (calculated as D-altro-n-lactone) appeared.

Reductase Activity with Enzymatically Prepared 5-Keto-L-gulanonate and Identification of Product—The partially purified fraction which catalyzes the reduction of 5-keto-L-galactonate with DPNH or TPNH can also act upon 5-keto-L-gulonate. When 5-keto-L-gulonate is the substrate, TPNH reacts faster than DPNH.

Several mg of the hexonate formed after 5-keto-L-gulonate reduction, were prepared for identification purposes. The isolation methods and incubation mixture were essentially the same as described for the formation of D-altro-n-lactone except that 5-keto-L-gulonate was prepared enzymatically from glucuronate with the purified uronic acid isomerase.

Either L-gulonate or D-mannono-n-lactone could arise from the enzymatic reduction of 5-keto-L-gulonate with DPNH or TPNH. However, the compound formed is actually D-mannono-n-lactone (identified as the lactone) and not L-gulonate, as previously suggested; this has been shown by paper chromatography in three different solvent systems (Table II).

Reversibility of Reductions and pH Optimum—that the compounds formed upon the enzymatic reduction of the keturornates are actually the n-hexonates and not the L-hexonates is substantiated by the fact that reverse reactions have been demonstrated with the n-hexonates but not with the L-hexonates. When purified keturonic acid reductase is incubated with high concentrations of either of the n-hexonates and DPNH or TPNH, a reduction of the pyridine nucleotides occurs (Fig. 6); no such reduction is evident with the L-hexonates.

The pH optimum of the reductase was found to be between 7.2 and 7.8 when determined with 5-keto-L-galactonate and DPNH. The activity drops sharply on either side of this peak.

Purification of Keturonic Acid Reductase and Separation from Uronic Acid Isomerase—The keturonic acid reductase has been purified 10-fold by ammonium sulfate precipitation and DEAE-cellulose column chromatography. Ammonium sulfate fractions were prepared and the activity per mg. of protein of each of these fractions with reduced pyridine nucleotides and 5-keto-L-galactonate was determined by measuring the decrease in absorption at 340 μ. The 0.50 to 0.55 (NH₄)₂SO₄ fraction showed the greatest reductase activity with both DPNH and TPNH; however, some activity was also present in the 0.55 to 0.60 and 0.60 to 0.65 fractions. This clearly indicated that the 0.60 to

### Table II

<table>
<thead>
<tr>
<th>Solvents</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-Gulono-</td>
<td>0.61</td>
<td>0.20</td>
<td>0.22</td>
</tr>
<tr>
<td>D-Mannono-</td>
<td>0.63</td>
<td>0.24</td>
<td>0.26</td>
</tr>
<tr>
<td>Experimental lactone</td>
<td>0.63</td>
<td>0.24</td>
<td>0.26</td>
</tr>
<tr>
<td>L-Galactono-</td>
<td>0.68</td>
<td>0.28</td>
<td></td>
</tr>
<tr>
<td>D-Altronon-</td>
<td>0.71</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>D-Glucuronon-</td>
<td>0.72</td>
<td>0.30</td>
<td>0.34</td>
</tr>
</tbody>
</table>

* See "Experimental" section of text for solvent mixtures. Whatman No. 1 filter paper was used with all three solvents.

---

Fig. 6. Oxidation of hexonates by keturonic acid reductase from E. carotovora with di- and triphosphopyridine nucleotides. Cuvette contents: 20 μmoles of substrate (no substrate in endogenous); 8 μmoles of DPN or TPN; 0.2 mg. of "d-s" enzyme, 0.50 to 0.55 (NH₄)₂SO₄ fraction; 180 μmoles of Tris-HCl buffer, pH 8.0; final volume, 3.0 ml.
pyruvate was formed. Evidence was obtained that the other hyde a-phosphate and pyruvate. In a mixture which contained poses 2-keto-3-deoxy-n-gluconate to a mixture of n-glyceraldehyde via 5-keto-n-gulonate-l-phosphate. Notwithstanding these discrepancies, the latest note from Ashwell's group (7), and these two enzymes could well account for the "uronic acid reductase" first revealed in the preliminary phase of this investigation.

In order to establish that the activity of the 0.60 to 0.65 (NH₄)₂SO₄ fraction with the alduronates and reduced pyridine nucleotides was an isomerization followed by a reduction, and not due to a single enzyme catalyzing the direct reduction of the alduronates, it was necessary to remove the contaminating keturonic reductase from the isomerase in this fraction. Several attempts with starch electrophoresis at various pH values failed to show any separation of these two enzymes.

The contaminating keturonic reductase was eventually separated from the isomerase by absorbing both enzymes on a column of DEAE-cellulose followed by elution with 1.0 to 2.0 per cent NaCl. One ml. of the 0.60 to 0.65 (NH₄)₂SO₄ fraction was absorbed on the column and elution was begun by washing the column with 0.05 M Tris-HCl buffer, pH 7.2, containing 1.0 per cent NaCl. Ten 2.0-ml. fractions were collected. This was followed by washing the column with 0.05 M Tris-HCl buffer, pH 7.2, containing 2.0 per cent NaCl and collecting ten more 2.0-ml. fractions. All the isomerase activity was found to be in Tubes 3, 4, and 5 and the keturonic reductase activity in Tubes 12, 13, and 14. No fraction contained any activity with the alduronates and reduced pyridine nucleotides. Therefore, it is clear from these data that the "uronic acid reductase" is in fact two enzymes, a uronic acid isomerase and a keturonic acid reductase.

**Further Catabolism of β-Hexonates**

The subsequent steps in the catabolism of β-altronate and β-mannionate by *Erwinia* are apparently identical to those described by Ashwell's group (7, 22) for *E. coli* acting on these hexonates, and by Entner and Doudoroff (23) and MacGee and Doudoroff (24) for *Pseudomonas* acting on n-glucuronate. The non-dialyzed Spinco supernatant fluid of *Erwinia* extracts ("s" enzyme) can act upon β-altronate and β-mannionate forming a compound which seems to be identical to 2-keto-3-deoxy-β-glucuronate. A typical reaction mixture contained: 40 μmoles of substrate (β-altronate or β-mannionate); 20 μmoles of cysteine; 20 μmoles of MgCl₂; 100 μmoles of Tris-HCl buffer, pH 7.2; 1.0 ml. of "s" enzyme, in a total volume of 4.0 ml. The reaction was incubated for 60 minutes at 25°C.

The "s" enzyme in the presence of excess ATP rapidly decomposes 2-keto-3-deoxy-n-glucuronate to a mixture of n-glyceraldehyde 3-phosphate and pyruvate. In a mixture which contained initially 0.4 μmole of 2-keto-3-deoxy-n-glucuronate, 0.34 μmole of pyruvate was formed. Evidence was obtained that the other product of cleavage was n-glyceraldehyde 3-phosphate, since DPN was reduced by the enzyme preparations in the presence of ascorbate and cysteine (26), but not in the absence of these additional substances. 2-Keto-3-deoxy-n-glucuronate was not cleaved in the absence of added ATP, suggesting that a prior phosphorylation of this compound is necessary.

Further evidence was obtained to support the conclusion that 2-keto-3-deoxy-6-phosphogluconate is the immediate precursor of pyruvate and triose phosphate. "s" enzyme acts upon 2-keto-3-deoxy-6-phosphogluconate to yield equimolar quantities of the triose phosphate (0.53 μmole) and pyruvate (0.59 μmole); the triose phosphate was measured as alkali labile phosphorus (27) and the pyruvate with crystalline lacto dehydrogenase.

**DISCUSSION**

It is apparent from this study that the initial steps in the catabolism of either n-galacturonate or n-glucuronate by *E. carotovora* are isomerization to the keturonic analogue followed by reduction to the corresponding n-hexonic acid. This conclusion stems from a thorough examination of the "uronic acid reductase" first demonstrated in cell-free extracts of *Erwinia* and *Aerobacter* during the preliminary phase (3) of this investigation. A direct reduction of the aldehydic group of either n-galacturonate or n-glucuronate would, of course, yield l-galactonate and l-gulonate, respectively. However, as a result of purification of the extracts and a more positive identification of the products, it is now shown that "uronic acid reductase" is actually two enzymes, a uronic acid isomerase and a keturonic acid reductase, and that the products of isomerization and reduction of galacturonate and glucuronate are α-altronate and α-mannonate, respectively.

That the initial step in the catabolism of uronates by *Enterobacteriaceae* is indeed an isomerization is now clear from the independent work of several groups (8, 28, 29, 30) which shows that *E. coli*, *Serratia marcescens*, *Shigella flexneri*, and *Aerobacter aerogenes* also possess uronic isomerase. McRorie et al. (29, 30) state that the product resulting from enzymatic reduction of 5-keto-l-gulonate is l-gulonate; however, they may have made the same error we made earlier (3) in identifying the hexonate as l-gulonate rather than d-mannionate. McRorie and Novelli (31) have indicated that *Aerobacter aerogenes* may metabolize glucuronate to dihydroxyacetone phosphate and tartronic semialdehyde via 5-keto-l-gulonate-l-phosphate. Notwithstanding these discrepancies, the latest note from Ashwell's group (7), which appeared as this report was readied for press, shows that *E. coli* metabolizes hexuronates to β-hexonates; this is in complete agreement with the results reported here for *Erwinia*. In addition, they found that *E. coli* can convert the β-hexonates to 2-keto-3-deoxy-glucuronate and that this compound is converted to pyruvate and triose phosphate in the presence of ATP. A similar system exists in *Erwinia*, and the role of the ATP has been clarified as necessary for the phosphorylation of 2-keto-3-deoxy-glucuronate; hence, in *Erwinia*, as in *E. coli* (22), 2-keto-3-deoxy-6-phosphogluconate is the immediate precursor of pyruvate and triose phosphate (Fig. 7).

Apparently, the *Erwinia-Escherichia* uronate-metabolizing system differs from those described in plants and animals. In plants, methyl n-galacturonate is converted to l-galactono-γ-lactone (32); and in animal tissues the uronic acids are the precursors of L-xylulose (33, 34). The L-xylulose-L-xylitol-L-xylulose interconversion which is also involved in the catabolism of
Catabolism of Galacturonic and Glucuronic Acids

The initial step in the catabolism of both d-galacturonate and d-glucuronate by Erwinia carotovora is an isomerization to the keturonic analogue. The enzyme(s) responsible for this step, provisionally named uranic acid isomerase, has been purified.

It can act upon either alduronate to form the corresponding keturonic acid; i.e. d-galacturonate is isomerized to 5-keto-L-galactonate, and d-glucuronate to 5-keto-L-gulonate.

The equilibrium constant for the isomerization of galacturonate was found to be 0.28. There is no sharp pH optimum for this enzyme.

The keturonic acids formed by the isomerase from the alduronic acids are reduced with either reduced diphosphopyridine or triphosphopyridine nucleotide by keturonic acid reductase. D-Altronate has been identified as the product formed after the enzymatic reduction of 5-keto-L-galactonate with either reduced diphosphopyridine or triphosphopyridine nucleotide and D-mannonate after the reduction of 5-keto-L-gulonate. The pH optimum for this enzyme was found to be between 7.2 and 7.8 when determined with 5-keto-L-galactonate and reduced diphosphopyridine nucleotide.

Both enzymes, uranic acid isomerase and keturonic acid reductase, are present in both d-galacturonate- and D-glucuronate-grown Erwinia. The quantity of these enzymes is, however, much less in cells grown with glucose.

D-Altronate and D-mannonate are dehydrated to form the same compound, 2-keto-3-deoxy-6-phospho-D-gluconate. This intermediate is then phosphorylated with adenosine triphosphate to form 2-keto-3-deoxy-6-phosphogluconate which is cleaved to yield pyruvate and triose phosphate, thus linking the metabolism of galacturonate and glucuronate to the glycolytic cycle at the triose level.

REFERENCES

Catabolism of Galacturonic and Glucuronic Acids by *Erwinia carotovora*
Wendell W. Kilgore and Mortimer P. Starr


Access the most updated version of this article at http://www.jbc.org/content/234/9/2227.citation

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/234/9/2227.citation.full.html#ref-list-1