ALDURONIC ACID METABOLISM BY BACTERIA[1]

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In a previous study (Payne and Carlson, 1957), the metabolism of glucuronic acid and galacturonic acid by whole cells was found to proceed in a variety of enteric bacteria and plant pathogens as the result of substrate induced enzymes. Glucuronate utilization by dried cells of *Serratia marcescens* resulted in the production of an unidentified reducing acid which could be isolated by paper chromatography from incubation mixtures (Payne, 1956). This compound was utilized by cells which had been adapted to uronic acids, suggesting that it was a normal intermediate in glucuronate utilization. In a preliminary report (Payne and McRorie, 1958), it was demonstrated that similar intermediates are produced by cell-free extracts of several organisms when incubated with either glucuronate or galacturonate. The intermediates from the metabolism of these alduronic acids were identified as the corresponding keturonic acids, D-fructuronic acid (2-keto-D-glucuronic acid or 5-keto-L-gulonic acid) from glucuronate and D-tagaturonic acid (2-keto-D-galacturonic acid or 5-keto-L-galactonic acid) from galacturonate, respectively.

Extracts of adapted organisms contain enzymes, keturonic reductases, which further metabolize these intermediates in the presence of reduced pyridine nucleotides to the corresponding hexonic acids, L-gulonic acid from D-fructuronate and L-galactonic acid from D-tagaturonate, respectively.

This paper describes the occurrence of these enzymes in a number of microorganisms which are capable of adapting to the utilization of uronic acids and delineates some of the properties of the enzyme systems.

MATERIALS AND METHODS

Bacteria. Strains of *Aerobacter aerogenes*, *Erwinia carotovora*, *Escherichia coli*, and *Serratia marcescens* used in a previous study (Payne and Carlson, 1957) and a laboratory strain of *Shigella flexneri* 2 were cultured in nutrient broth supplemented with either glucuronate or galacturonate or in minimal salts media supplemented with uronic acids. An unclassified marine isolate, M11, (Payne, 1958*b*) was grown in sea water nutrient broth enriched with uronic acids. Cells were grown under vigorous aeration in 10 to 16 L volumes for 24 to 48 hr at 30 C and harvested on a Sharples supercentrifuge. Cell pastes obtained in this manner were frozen until the time of use.

Cell-free extracts. Extracts were prepared by grinding frozen cells with powdered alumina (AB levigated) and extracting with cold 0.066 M phosphate buffer at pH 7.0 in the ratio: cells, 3 g; alumina, 2 g; buffer, 6 ml. After extraction for 10 to 15 min in the cold, the extracts were clarified in a Spinco model L ultracentrifuge for 20 min at 20,000 × G. The clear supernatant solutions were stored frozen until used. Preparations obtained in this manner were stable for several weeks.

Enzyme assays. Uronic isomerase activity was readily measured by either the resorcinol (Roe *et al.*, 1949) or cysteine-carbazole (Dische and Borenfreund, 1951) methods under the same conditions used for the assay of the phosphohexoisomerases. The latter assay proved to be the more convenient, yielding a purple color reaction with an absorption maximum at 540 mμ. Appropriate adjustments were made for color response in the substrate and enzyme blanks.

Keturonic reductase was measured by the oxidation with time of reduced diphosphopyridine nucleotide (DPNH) or triphosphopyridine nucleotide (TPNH) at 340 mμ. The TPNH used in the experiments with *S. marcescens* was generated enzymatically in the reaction mixture by the action of aconitase and isocitric dehydrogenase which were present in the crude extracts.

Preparation of uronic acids. The basic calcium salt of D-tagaturonic acid was prepared from galacturonic acid and calcium oxide by the method of Ehrlich and Guttmann (1934). Treat-

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ment of the calcium salt with equivalent amounts of oxalate gave the free acid as described by these authors.

The yellow basic calcium salt of D-fructuronic acid was prepared by a similar procedure using reactants in a solution which was 10 times as concentrated to yield an amorphous, yellow powder. The free acid, obtained as a pale yellow, hygroscopic oil after treatment of the calcium salt with oxalate, did not crystallize. The magnesium, barium, strontium, and lead salts were also amorphous products.

The synthetic acids had the same migration characteristics on paper chromatograms as the enzymatically prepared compounds which were obtained from incubation mixtures by chromatography on paper and elution with methanol.

Chromatography. Paper chromatograms were made using butanol:acetic acid:water (4:1:1) and pyridine:butanol:water (3:2:1.5) as solvents. Compounds were visualized on chromatograms with the following reagents: aniline biphthalate (Partridge, 1949) for reducing sugars, naphthoresorcinol (Isherwood and Jermyn, 1951) and anthrone (Johanson, 1953) for keto sugars, and ferric-hydroxamate test (Abdel-Akher and Smith, 1951) for lactones.

RESULTS

In initial experiments, the metabolic products of glucuronate metabolism by crude cell extracts were studied by paper chromatography. Production of the first intermediate was followed by developing incubation mixtures which had been stopped by the addition of equal volumes of acetone in the pyridine-butanol solvent and spraying with the aniline biphthalate reagent. The formation of an additional product was demonstrated by stopping the reaction with HCl, boiling to lactonize the hexonic acids formed and determining the lactones with the ferric-hydroxamate reagent after chromatography in the same solvent. Typical results obtained with *A. aerogenes* extracts are shown in figure 1 where it can be seen that maximum production of L-gulonolactone occurs only in the presence of TPNH whereas reducing acid production requires only glucuronate and enzyme. The somewhat larger amounts of reducing acid produced in the presence of TPNH are possibly due to the disruption of the equilibrium which strongly favors the alduronic acids (see also figure 3).

Since the reducing acid was produced in the absence of TPNH and both reducing acid and gulonic acid were produced in the presence of TPNH, it seemed probable that the reducing acid was formed from glucuronate and subsequently reduced to gulonic acid. Confirmation of the production of an intermediate prior to reduction was obtained with extracts of *S. marcescens* by measuring the rate of DPNH (or TPNH) oxidation with time at 340 mμ as shown in figure 2. It can be seen (curve *1*) that glucuronate dependent oxidation of reduced

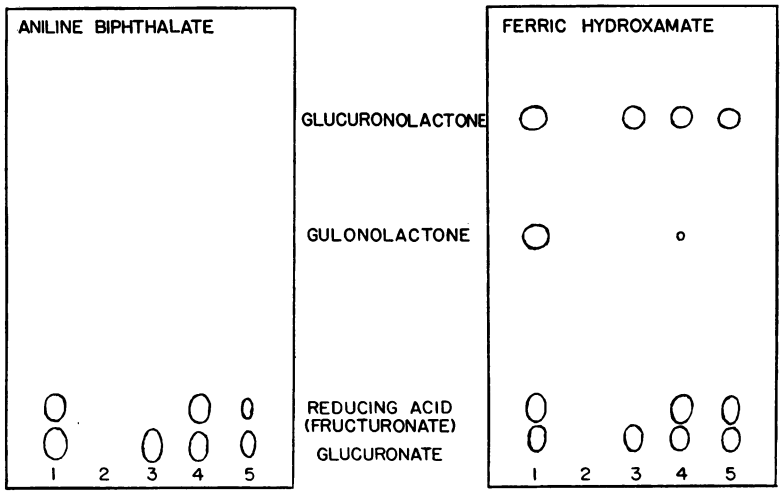

Figure 1. Production of reducing acid and lactone by extracts of *Aerobacter aerogenes*. (*1*) Complete system: 40 μmoles Na glucuronate, 40 μmoles Na citrate, 0.25 mg TPN and 0.4 ml crude enzyme in 0.066 M phosphate buffer, pH 7.0. Total volume: 1.45 ml. (*2*) No glucuronate. (*3*) No enzyme. (*4*) No citrate. (*5*) No TPN. Incubated 1 hr at 37 C.

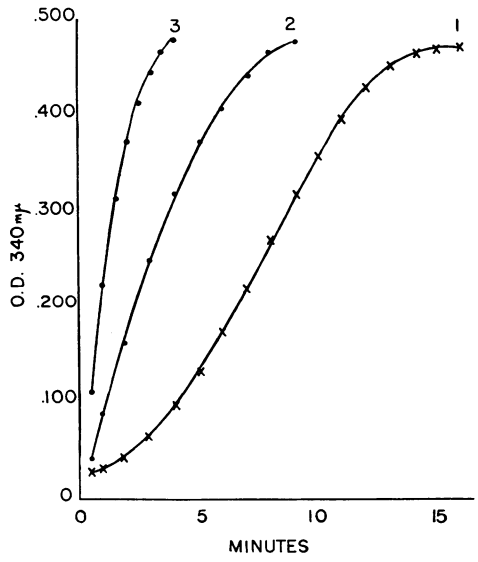

Figure 2. DPNH dependent reduction of uronic acids by extracts of *Serratia marcescens*. (*1*) 10 μmoles Na glucuronate, 0.1 μmole DPNH, 100 μmoles Tris buffer, pH 8.0, 0.1 ml crude enzyme. Total volume: 3.0 ml. (*2*) System incubated 10 min before DPNH was added. (*3*) Equimolar concentration of fructuronate replacing glucuronate.

coenzyme proceeded at a maximum rate only after a period of several minutes. This lag period was not present when glucuronate and enzyme extracts were incubated for 10 min prior to the addition of reduced coenzyme (curve *2*). Confirmation of the fact that the reducing acid previously observed was the substrate for reduction was obtained by the separation of glucuronate from the intermediate on paper chromatograms, elution with methanol, and addition to similar incubation mixtures after removal of the methanol in air. The acid was reduced in the system without a lag period as shown in curve *3*.

The intermediate reducing acid gave a weakly positive reaction with the naphthoresorcinol and anthrone keto sugar reagents on paper chromatograms and reacted with the ferric-hydroxamate reagent without treatment to produce a lactone, suggesting that the initial reaction was the result of isomerization to a keto acid. In the earliest experiments (Payne, 1956) the compound did not accumulate in the dried cell reaction mixtures in sufficient quantity to be visualized with these reagents. Confirmation of the isomerization to a keto acid was obtained with the resorcinol and cysteine-carbazole reactions under the conditions used for the assay of phosphohexoisomerase. The production of keto acid from both glucuronate and galacturonate by

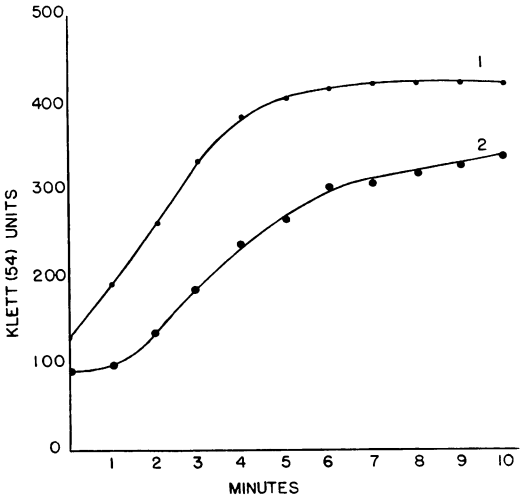

Figure 3. Uronic acid isomerase activity of extracts of *Shigella flexneri* 2 as measured by the cysteine-carbazole reaction. (*1*) 5.0 μmoles galacturonate. (*2*) 5.0 μmoles glucuronate. Total volume: 0.5 ml. Incubated 5 min at 37 C. Approximately 1.0 μmole keturonic acid produced in each system.

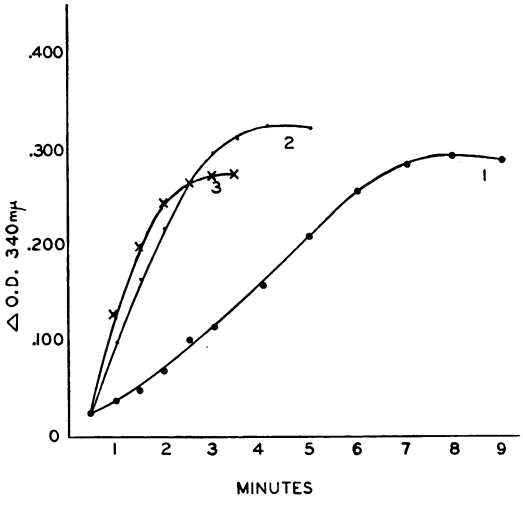

Figure 4. DPNH dependent reduction of uronic acids by extracts of *Erwinia carotovora*. (*1*) 10 μmoles galacturonate, 0.1 μmole DPHN, 100 μmoles Tris buffer, pH 8.0, 0.1 ml crude enzyme. Total volume: 3.0 ml. (*2*) System incubated for 5 min before DPNH was added. (*3*) Equimolar concentration of tagaturonate replacing galacturonate.

TABLE 1

Uronic isomerase and keturonic reductase activity of crude bacterial extracts

Organism	Substrate in Culture*	Isomerase Activity†		Reductase Activity†			
		gluc-a	gal-a	gluc-a	gal-a	fruc-a	tag-a
Aerobacter aerogenes	gluc-a	+	+	+	+	+	+
	gal-a	+	+	+	+	+	+
Erwinia carotovora	gluc-a	+	+	+	+	+	+
	gal-a	+	+	+	+	+	+
Escherichia coli	gluc-a	+	+	+	+	+	+
	gal-a	+	+	+	+	+	+
Marine isolate M11	gluc-a	+	−	−	−	−	−
	gal-a	+	+	−	−	−	−
Serratia marcescens	gluc-a	+	+	+	+	+	+
	gal-a	+	+	+	+	+	+
Shigella flexneri 2	gluc-a	+	+	+	+	+	+
	gal-a	+	+	+	+	+	+

* gluc-a = glucuronate; gal-a = galacturonate; fruc-a = fructuronate; and tag-a = tagaturonate.

† Positive (+) and negative (−) reactions under the conditions of figures 1–3.

crude extracts of *S. flexneri* 2 grown on galacturonate is shown in figure 3. It can be seen that isomerase activity for both substrates is rapid, reaching a maximum keto acid production after 5 to 7 min.

Confirmation of the production of D-fructuronate from D-glucuronate and D-tagaturonate from D-galacturonate was obtained by synthesis as described above. The synthetic compounds had the same migration on paper chromatograms as the enzymatically produced compounds and gave the same activity in the reductase system as illustrated for *E. carotovora* in figure 4 and for a variety of bacteria as summarized in table 1.

Glucuronolactone showed greatly reduced isomerase activity as compared to glucuronic acid, suggesting that the straight chain form of the acid is the true substrate for glucuronic isomerase. Mannuronolactone and mannuronic acid were not isomerized unless incubated for longer periods. Although detectable quantities of fructuronic acid are obtained after incubation for 1 hr or longer, mannuronate is apparently metabolized by a different pathway. Reduced DPN was used for the measurement of reductase activities shown in table 1 although the rate of reduction of the keto acids was 2- to 3-fold greater with TPNH using enzymes from *S. marcescens*. In the instances where DPNH was found to be inactive, TPNH also gave negative results.

DISCUSSION

From the data presented it can be seen that organisms grown on either glucuronic acid or galacturonic acid contain very active, induced enzymes which first isomerize the alduronic acids to keturonic acids. The general distribution of these enzymes in all cells studied suggest that the initial step in the utilization of alduronic acids is isomerization (reaction (1)).

D-glucuronate or D-galacturonate $\xrightleftharpoons{\text{uronic isomerase (s)}}$ D-fructuronate or D-tagaturonate (1)

Although the reactions are similar, it has not been established whether separate enzymes are present for the two substrates or whether the isomerization is the result of a single nonspecific isomerase which is induced by either substrate.

The results obtained with the marine bacterium M11 support the existence of at least one separate glucuronic isomerase. Simultaneous induction of separate enzymes may be the result of galacturonate conversion to glucuronate as previously suggested for Serratia (Payne, 1958*a*).

A subsequent reaction of the 2-keturonic acids thus formed can be mediated by another induced system, keturonic reductase, in the presence of reduced pyridine nucleotides (reaction (2)).

D-fructuronate or D-tagaturonate $\xrightarrow[\text{DPNH or TPNH}]{\text{keturonic reductase}}$ L-gulonate or L-galactonate (2)

Although the presence of reductase activity could not be demonstrated in all cells studied, the majority of these organisms seem to be able to produce L-hexonic acids.

Studies on the fractionation of these enzymes, the metabolism of the keturonic acids and the possible utilization of the L-hexonic acids are in progress.

SUMMARY

Production of the keturonic acids, fructuronic and tagaturonic, from the alduronic acids, glucuronic and galacturonic, respectively, by

cell-free extracts of various gram-negative bacteria has been demonstrated. In the presence of reduced diphosphopyridine nucleotides the extracts have been shown to reduce D-fructuronic to L-gulonic acid and D-tagaturonic to L-galactonic acid. Confirmation of these reactions has been obtained by spectrophotometric measurement of the oxidation of reduced pyridine nucleotides employing enzymatically or synthetically produced keturonic acids as substrates.

Isomerases induced by culturing the various species on either glucuronic or galacturonic acid were active on both substrates, with the exception of a specific glucuronate isomerase produced by the glucuronate-grown marine bacterium. Reductases for the keturonic acids were not produced by these cells. Extracts of the galacturonate-grown marine bacterium contained isomerizing systems for both alduronic isomers but did not contain reducing systems for either keturonic acid.

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