STUDIES ON BACTERIAL UTILIZATION OF URONIC ACIDS

I. *Serratia marcescens*

WILLIAM J. PAYNE

Department of Bacteriology, University of Georgia, Athens, Georgia

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The hypothesis of Salkowski and Neuberg (1902) that glucuronic acid may be decarboxylated by bacteria was not substantiated by the work of Cohen (1949), who found that *Escherichia coli* strain K-12 was not simultaneously adapted to uronic acids and homologous pentoses. Heald (1952*a*) reported that fermentation of glucuronic acid by coliform bacteria is not comparable to that of homologous hexose or pentose. Moreover, Heald (1952*b*) found that cells grown on glucose and adapted to glucuronic acid in cups of the Warburg apparatus were not adapted to xylose which was tipped in from a second side arm. The latter paper indicated that washed suspensions of *Aerobacter aerogenes* grown on xylose could not be induced to ferment glucuronic acid. De Ley (1953), however, demonstrated that *Aerobacter cloacae* grown on glucose could be adapted to uronic acids after short lag periods.

In studies with *Erwinia carotovora*, Kraght and Starr (1952) noted considerable qualitative and quantitative difference in the products of fermentation of galacturonic acid and glucose. Potter and McCoy (1955) detected decreases in the galacturonic acid content of pectic acid and pectin cultures of *Bacillus polymyxa*.

We have found that *Serratia marcescens* will grow on a mineral medium containing glucose, glucuronate, or galacturonate as the sole source of carbon. This paper outlines some metabolic relationships that exist among cells cultured on these media, and describes a compound which may be an intermediate metabolite isolated from a reaction mixture of dried glucuronate grown cells and glucuronate.

MATERIALS AND METHODS

Bacteria and media. A strain of *S. marcescens* used in a previous study (Payne *et al.*, 1953) was maintained on trypticase soy agar slants. A basal medium containing $(NH_4)_2SO_4$, 0.05 per cent; NH_4Cl, 0.05 per cent; $MgSO_4 \cdot 7H_2O$, 0.05 per cent and KH_2PO_4, 0.05 per cent was prepared in quantity, neutralized with NaOH, and autoclaved. Solutions of glucose, sodium glucuronate (Argo), or galacturonic acid (Eastman) neutralized with NaOH were sterilized by filtration and added to a final concentration of 0.25 per cent.

Respirometry. Standard manometric procedures were employed. Suspensions of bacteria were prepared from a glucose medium, which had been inoculated with bacteria from agar slopes, or from glucuronate or galacturonate media after no fewer than 10 subcultures in homologous media. The cultures to be assayed were incubated at room temp for 18 hr with agitation. The cells were centrifuged, washed twice with water, and taken up in 0.067 M phosphate buffer at pH 7.0 to give a suspension with 10 per cent light transmittance (9–11 mg dry wt). Each manometer cup was charged with 1 ml of bacterial suspension, an additional ml of buffer, 1 ml of 0.02 M substrate in the side arm, and 0.2 ml of 15 per cent KOH in the center well. The cups were equilibrated for a period of 30 min at 30 C, and the endogenous respiration was determined for an additional 30 min before the substrates were tipped in.

In a concluding experiment inocula of glucuronate grown cells, after a minimum of 10 transfers, were cultured once on glucose and assayed for ability to oxidize glucose, uronic acids, and xylose.

Assay for possible intermediates. Carboys containing 8 L of the glucuronate medium were inoculated with bacteria from agar slants and incubated at room temp with aeration from a stream of sterile air bubbling through the cultures. After 72 hr the cells were harvested with a Sharples super-centrifuge. Thin films of bacterial paste were spread on large watch glasses which were placed in vacuum desiccators over P_2O_5. The cells were dried rapidly and used immediately.

Reaction mixtures were prepared containing 1 ml of 0.5 M sodium glucuronate, 1 ml 0.067 M

phosphate buffer at pH 7.0, and 1 ml of bacterial suspension containing 50 mg dried cells. Controls lacking either cells or substrate were incubated with the complete systems at 30 C. In preliminary experiments samples of 0.5 ml were taken at 10-min intervals and mixed with equal volumes of acetone to stop the reaction. The samples were centrifuged and the supernatant from each applied evenly as a band 4 in long on a reference line on Whatman no. 1 paper. The ethanol-acetic acid solvent of McNair Scott and Cohen (1950) was permitted to irrigate the chromatograms. The chromatogram of each sample was cut into strips 1 in wide to be sprayed with different reagents.

Reagents for the detection of reducing sugars, keto sugars, sugar phosphates and gluconic acid derivatives and compounds sensitive to ammoniacal silver nitrate were applied to strips from each chromatogram. After the establishment of nearly optimum time of incubation at 45 min, reaction mixture was prepared of 20 ml with substrate in buffer and 0.5 to 1 g of dried cells. The reactions were stopped as before and a number of chromatograms prepared on thick Whatman no. 3 paper with a butanol-acetic acid solvent descending. The band on which the product was deposited was cut out and eluted with water in the manner described by Smith and Pollard (1952).

Analyses of the product. The eluate containing the unknown material was subjected to a variety of qualitative colorimetric and chromatographic analyses (table 1) along with the eluates of barren areas of the chromatograms as controls in some cases.

Sensitivity of the material to glucosidases was determined by incubating aliquots of the eluate with α- and β-glucosidases and β-glucuronidase. The reaction mixtures containing 1 ml of eluate,

TABLE 1

Response of unknown compound to colorimetric and chromatographic analyses

Test	Response	Reference for Procedure
1. Anthrone reaction	+	Morris (1948)
2. Barfoed's copper acetate	As oligosaccharide	Harrow *et al.* (1950)
3. Specific groupings		
a. Reducing sugar		
1. Saturated picric acid	Reduced	Harrow *et al.* (1950)
2. Aniline biphthalate	+	Partridge (1949)
b. Keto sugars	0	Johanson (1953)
c. Sugar phosphates	0	DeLey (1954)
d. 1,6- or 1,4-linkage	As 1,6- linked oligosaccharide	Buchanan and Savage (1952), Schwimmer and Benevenue (1945)
4. Assay for hydrolytic products of:		
a. α-Glucosidase*	0	
b. β-Glucosidase	0	
c. β-Glucuronidase	Glucuronate (Rf value and naphthoresorcinol test)	Kapp (1940)
d. Acid	Glucuronolactone, pentose	
e. Alkali	Glucuronate	
5. No. of "monosaccharide" units after hydrolysis†	2	Blass *et al.* (1950)

* Enzymes purchased from Nutritional Biochemicals Corporation. Glucosidases were tested for activity with sensitive disacchrides.

† A quantitative application of the aniline biphthalate reagent was necessary since the usual reducing sugar tests require boiling in alkali. It was found that the concentration of reducing sugar doubled after hydrolysis with β-glucuronidase.

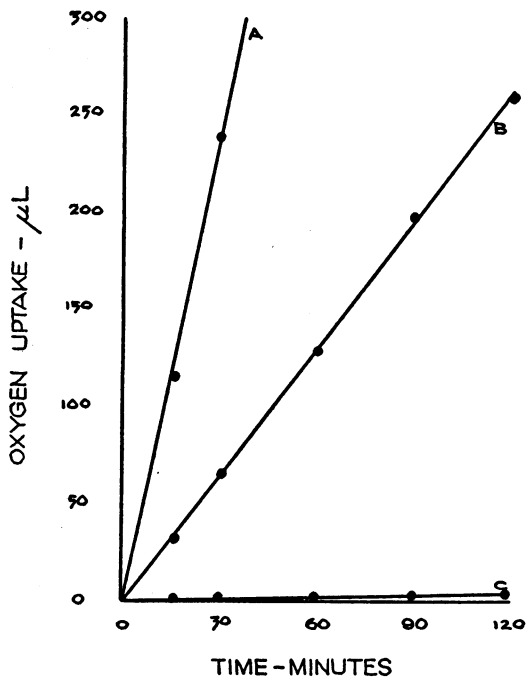

Figure 1. Oxidation of substrates by glucose-grown cells of *Serratia marcescens*. *A*, Glucose. *B*, Xylose or L-arabinose. *C*, Uronic acids. Endogenous respiration subtracted.

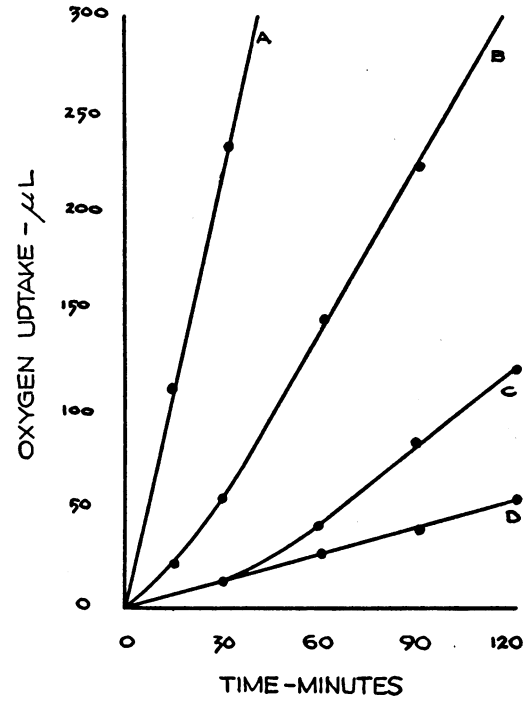

Figure 2. Oxidation of substrates by galacturonate- or glucuronate-grown cells of *Serratia marcescens*. *A*, Glucose. *B*, Glucuronic acid. *C*, Galacturonic acid. *D*, Homologous pentose. Endogenous respiration subtracted.

1 mg of enzyme, and 1 ml of 0.001 M phosphate buffer at or near the optimum pH for each enzyme were incubated at 30 C for 30 min. Controls without enzymes or without substrates were treated identically. Acetone was added to stop the reactions, the sediment centrifuged and the supernatant chromatographed.

The effects of boiling the unknown substance in dilute H_2SO_4 and NaOH were ascertained by making a few ml of the eluate 0.5 N in each and boiling for 30 min. The resulting hydrolyzates were neutralized and chromatographed.

RESULTS

Effect of cultural conditions on oxidative metabolism. Washed cells of *S. marcescens* grown on glucose were not induced to oxidize the uronic acids in the absence of a source of nitrogen (figure 1). Nor did extension of the period of incubation to 4 hr result in adaptation. These data apply also to cells cultured on glucose after prolonged exposure to glucuronate. However, the pentoses were oxidized quite rapidly. Utilization of either uronic acid as the sole source of carbon through at least 10 cultural cycles served to diminish the ability of the cells to oxidize the homologous pentoses (figure 2). The ratio of the rates of oxidation of glucose to pentoses by cells grown on glucose was 4–5:1 routinely. With uronate grown cells the ratio increased to 15–16:1 with little or no change in the rate of oxidation of glucose as the ability to oxidize the pentoses became greatly diminished. The Q_{O_2} for glucuronate was increased from virtually zero to 13–14. Adaptation to either uronic acid yielded cells which oxidized glucuronate rapidly and galacturonate slowly with the rate increasing with time.

Isolation of a possible intermediate of uronic acid metabolism. Preliminary experiments revealed no detectable change in the composition of reaction mixtures for 30 min. At 40 and 50 min a spot other than sodium glucuronate appeared on the chromatograms. The compound reacted with alkaline silver nitrate and with sprays sensitive to reducing sugars. At 60 min the spot was less intense. Chromatograms made from controls

were barren and boiled cells were unable to produce a change in the composition of the reaction mixture. Cells grown on trypticase soy broth did not synthesize the product when incubated with glucuronate.

Results in table 1 suggest that the compound isolated from the dried-cell reaction mixtures may be a dihexuronic acid with a 1,6- "ester" glucosiduronic acid linkage. The compound disappeared rapidly when it was eluted from chromatograms and incubated with dried adapted cells in buffer. No additional reducing sugars, no sugar phosphate, gluconic acid derivatives, or compounds sensitive to ammoniacal silver nitrate were recovered during this time.

DISCUSSION

The diminished rate at which the bacteria oxidized pentoses after an extended period of culture on uronic acids permits the conclusion that *S. marcescens* does not decarboxylate the acids. That conclusion was reached previously (Cohen, Heald), concerning coliform bacteria. Moreover, Hough and Pridham (1955) using plum mesocarp tissue found no evidence for conversion of glucuronate to xylose by decarboxylation. On the other hand, Rabinowitz (1955) noted that enzymes from rat tissues release $C^{14}O_2$ from C_6 labeled glucuronate with six times the radioactivity of that from the uniformly labeled compound. He concluded that the acid was decarboxylated. There was, however no attempt to characterize the remaining fragments, and it may well be that the reaction is not so simple as it would appear on the surface.

The behavior of the galacturonate grown cells may sustain the hypothesis that this sugar acid is isomerized to glucuronate before being metabolized by *S. marcescens*. Cohen (1949) established with a high degree of probability that an intermediate is formed prior to the oxidation of glucuronic acid. Heald (1952*b*) obviated the possibility that α- or β-glucuronic acid-l-phosphate may be the intermediate but suggests that glucuronic acid may be oxidized differently by various of the Enterobacteriaceae. The compound isolated in this study may be the intermediate for *Serratia* species since it was synthesized and apparently metabolized by glucuronate grown cells.

The 1,6- "ester" linkage, which seems probable in view of the positive anthrone test, the response to the reagent of Buchanan and Savage, and the sensitivity to alkali, could conceivably lead to the production of glucose. Reductior at C_6 and hydrolysis of the glucosiduronic acid bond would yield glucose and glucuronic acid. The former could then be rapidly oxidized by the cells and the latter reconjugated to form new molecules of aldobiouronic acid.

Experiments are in progress which are designed to establish the identity of the isolated dihexuronic acid by methylation, to assay for possible degradation products of the acid, and to investigate the possibility of a galacturonic acid isomerase occurring in galacturonate grown cells.

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SUMMARY

Washed suspensions of *Serratia marcescens* cultured on glucose minimal medium were not induced to oxidize glucuronic or galacturonic acids, but were found to oxidize pentoses quite rapidly. Prolonged culture on either uronic acid yielded cells capable of oxidizing glucuronic acid rapidly and galacturonic acid slowly; the ability to oxidize pentoses was greatly diminished. Glucose was oxidized equally well by cells from any of the substrates. Growth of cells on glucose for a single cultural cycle after repeated transfer on glucuronic acid resulted in the immediate loss of their ability to oxidize the uronic acids.

Incubation of adapted dried cells with buffered glucuronate yielded a compound whose properties suggest a 1,6- "ester" linked dihexuronic acid. This compound disappeared rapidly when isolated and incubated anew with dried cells. The possibility is suggested that the compound may be an intermediate in the oxidative metabolism of glucuronic acid by *S. marcescens*.

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