THE PATHWAY FOR L-GULONATE CATABOLISM IN *ESCHERICHIA COLI* K-12 AND *SALMONELLA TYPHIMURIUM* LT-2

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1. Introduction

The pathway for the catabolism of glucuronate in bacteria is distinct from that in animals. In bacteria glucuronate is converted into glyceraldehyde 3-phosphate and pyruvate by the sequential formation of fructuronate, mannonate, 2-oxo-3-deoxygluconate and 2-oxo-3-deoxygluconate 6-phosphate [1]. In animals glucuronate is catabolised to xylulose 5-phosphate by the sequential formation of L*-gulonate, 3-oxo-L-gulonate, L-xylulose, xylitol and xylulose [2].

In *Escherichia coli* K-12 L-galactonate is catabolised to give glyceraldehyde 3-phosphate and pyruvate [3]. L-Gulonate is the 3-epimer of L-galactonate and if its catabolism were analogous to that of L-galactonate it would be converted into fructuronate and thus enter the route for bacterial glucuronate degradation thereby yielding glyceraldehyde 3-phosphate and pyruvate (fig.1). On the other hand the only reported route for L-gulonate catabolism is as part of the glucuronate degradation sequence of animals which leads to xylulose 5-phosphate formation.

We have observed that various enteric bacteria can grow on L-gulonate and this paper shows that in *E. coli* K12 and *Salmonella typhimurium* LT-2 L-gulonate is catabolised to give glyceraldehyde 3-phosphate and pyruvate rather than xylulose 5-phosphate.

2. Materials and methods

The bacteria used were: *E. coli* strains, NCTC 9001 (neo-type), B, C, Crookes, W and the K-12 derivatives K10, X289 [4], PB1 [5], CM8 [6], JB1 [7]; *Salmonella typhimurium* LT-2.

The growth of bacteria and the preparation of cell-free extracts was as in [3]. Phage P1 *vir*-mediated transduction of *E. coli* K-12 was carried out by standard procedures [8]. Phage P22-mediated transduction of *S. typhimurium* LT-2 was carried out as in [9]. Mutants defective in 2-oxo-3-deoxygluconate 6-phosphate aldolase were isolated and characterised as in [10].

The assay for L-gulonate oxidoreductase (EC 1.1.1.?) contained in 1 ml: sodium–potassium phosphate buffer (pH 8.0) (80 μmol), NAD (3 μmol), L-gulonate (25 μmol) and bacterial extract (~100 μg protein). The increase in A_{340} concomitant with the formation of $NADH_2$ was monitored in a recording spectrophotometer. L-Galactonate oxidoreductase (EC 1.1.1.?) was measured as in [3] but using phosphate buffer pH 7.0 instead of pH 6.0. Fructuronate reductase (EC 1.1.1.57) was measured as for tagaturonate reductase [3] but with 2 μmol fructuronate instead of tagaturonate. Uronic acid isomerase (EC 5.3.1.12) was assayed as in [3] but with glucuronate as substrate. All the enzyme assays were carried out at 30°C and 1 unit enzyme activity is defined as that amount of enzyme that transforms 1 μmol substrate/min.

L-Gulonate was prepared by the reduction of glucuronate with an excess of $NaBH_4$. For this 11.7 g sodium glucuronate in 100 ml distilled water was cooled to 2°C and 1.9 g $NaBH_4$ was added with mixing. The reaction mixture was left at 2°C for 1 h then kept at room temperature (21°C) for a further 40 h. After this time all gas evolution had ceased and no reducing sugar was present in the reaction mixture. The pH of the mixture was adjusted to pH 7.4 by addition of 4 M HCl and L-gulonic acid was obtained

* Where no stereo-isomeric form is indicated the sugars are of the D-series

after passage of the mixture down a Dowex-50 cation-exchange column in the H^+ form. Fructuronate was prepared as in [11]. L-Galactonolactone was purchased from Sigma (London) and converted to sodium L-galactonate by titration with NaOH. Other biochemicals were purchased from Boehringer (London).

3. Results and discussion

When tested on solid media at 37°C *E. coli*, strain W and *S. typhimurium* LT-2 grew overnight on L-gulonate as sole source of carbon and energy. Other *E. coli* strains such as K-12, B, C, Crookes and the neotype NCTC 9001 failed to grow. However, after incubating the plates for a further 3–4 days certain of these strains, including strain K-12 showed individual colonies that had gained the ability to grow on L-gulonate.

In liquid medium at 37°C the doubling time for various K-12 L-gulonate positive mutants such as C0509 and C0519 was 180–200 min. Under the same conditions *S. typhimurium* LT-2 had a doubling time of 160 min.

To distinguish between the various possible routes for L-gulonate degradation mutants that were unable to grow on gluconate due to a defect in 2-oxo-3-deoxygluconate 6-phosphate aldolase were isolated from strain C0509. Such mutants, as expected, were unable to grow on glucuronate, galacturonate and L-galactonate, compounds whose catabolism produces 2-oxo-3-deoxygluconate 6-phosphate (fig.1). They also failed to grow on L-gulonate, although growth on xylose was normal. When glucuronate-positive transductants of one such aldolase mutant, strain C0510, were selected, they had simultaneously regained the ability to grow on L-gulonate, L-galactonate, galacturonate and gluconate. When L-gulonate positive transductants were selected all could also grow on the other 4 compounds.

Similarly when a 2-oxo-3-deoxygluconate 6-phosphate aldolase negative mutant of *S. typhimurium* LT-2 was selected and tested it was unable to grow on L-gulonate or glucuronate but grew normally on xylose (wild-type *S. typhimurium* LT-2 cannot grow on galacturonate [12] or L-galactonate [3]). Again glucuronate-positive transductants had simultaneously regained their ability to grow on L-gulonate and gluconate. These results suggest that L-gulonate catabolism involves the cleavage of 2-oxo-3-deoxygluconate 6-phosphate as an essential step.

Further support for the idea that L-gulonate is catabolised via 2-oxo-3-deoxygluconate 6-phosphate was obtained by study of extracts prepared from L-gulonate-grown cells. Such extracts from *E. coli* strain C0509 and *S. typhimurium* LT-2 showed activities for uronic acid isomerase and fructuronate reductase 10–50-fold greater than those in extracts from the corresponding glycerol-grown cells and equivalent in activity to extracts from glucuronate-grown cells, (table 1). These results are consistent with L-gulonate catabolism via the glucuronate catabolic pathway.

Consideration of the structure of L-gulonate (fig.2) shows that it may be converted by 1-step reactions to any of 3 compounds, glucuronate, fruc-

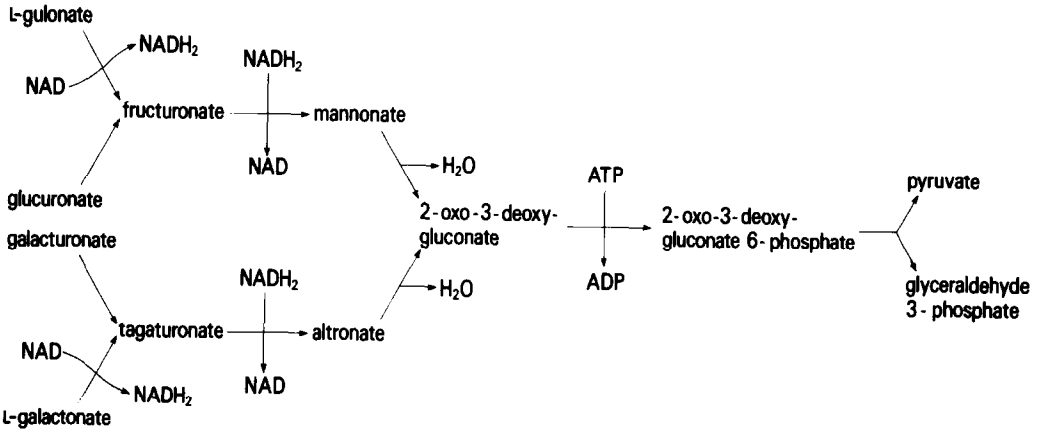

Fig.1. Schematic representation of the routes for the catabolism of L-gulonate, glucuronate, L-galactonate and galacturonate.

Table 1
Induction of uronic acid isomerase and fructuronate reductase in *E. coli* K-12 strain C0509 and in *S. typhimurium* LT-2

Growth substrate	C0509		LT-2	
	Uronic acid isomerase[a]	Fructuronate reductase[a]	Uronic acid isomerase[a]	Fructuronate reductase[a]
Glycerol	0.04	0.23	0.07	0.18
Glucuronate	0.52	6.9	2.5	6.5
L-Gulonate	0.97	12.9	3.6	12.0

[a] Specific activity in μmol . min^{-1} . mg $protein^{-1}$

turonate or mannonate, whose catabolism is already well understood (fig.1) [1]. To identify the point of entry of L-gulonate into the glucuronate catabolic sequence *E. coli* mutants with known defects in particular enzymes of the glucuronate pathway were used. Since the parental strains of these mutants would be unable to grow on L-gulonate without mutation all such strains were initially L-gulonate negative. Therefore to see whether the defect in glucuronate catabolism prevented growth on L-gulonate the mutants were treated in two ways.

(1) They were spread onto L-gulonate media to see whether they could mutate spontaneously to L-gulonate positivity.
(2) They were infected with phage P1 *vir* grown on strain C0509 and L-gulonate positive transductants were sought.

Only the uronic acid isomerase mutant, strain PB1, was able to mutate to give L-gulonate positive clones or be transduced to L-gulonate positivity. Neither strain CM8, a fructuronate reductase mutant nor strain JB1, a mannonate dehydratase mutant was able to mutate, or be transduced, to L-gulonate positivity. These results suggested that L-gulonate entered the glucuronate catabolic pathway after the isomerase acted and before the fructuronate reductase step, that is at fructuronate.

To confirm that it was the absence of fructuronate reductase or mannonate dehydratase that prevented growth of strains CM8 and JB1 on L-gulonate, both strains were transduced to glucuronate-positivity using phage P1 *vir* grown on the L-gulonate positive strain C0509. Of 87 glucuronate-positive transductants tested in either case, none grew on L-gulonate. Thus the ability to grow on L-gulonate is not readily co-transducible with *uxu* A or *uxu* B, genes specifying mannonate dehydratase and fructuronate reductase, respectively [6,7]. When the *uxu* A^+ and *uxu* B^+ transductants were spread onto L-gulonate plates, there was no growth initially but in both cases after several days, individual colonies appeared that could now grow on L-gulonate. This supports the view that it was defects in fructuronate reductase and mannonate dehydratase that prevented further mutation of strains CM8 and JB1 to L-gulonate positivity.

Conversion of L-gulonate to fructuronate would involve the operation of an oxidoreductase similar to L-galactonate oxidoreductase and crude extracts prepared from L-galactonate-grown wild-type cells have activity against both L-galactonate and L-gulonate. However, various pieces of evidence suggest that the L-gulonate oxidoreductase activity in this situation is a property of the L-galactonate oxidoreductase. The pH-activity response for the L-gulonate oxidoreductase (fig.3) is identical to that for the

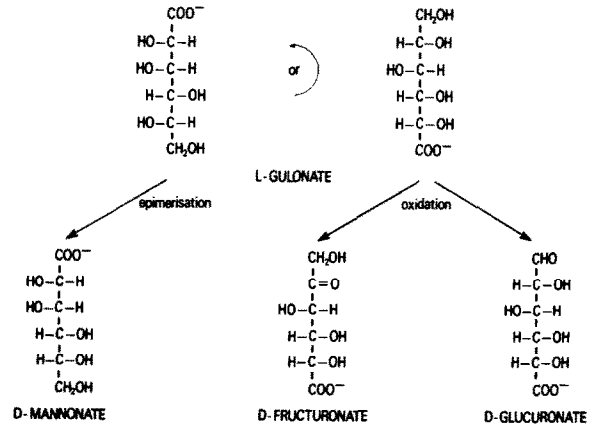

Fig.2. Structural relationships between L-gulonate, D-mannonate, D-fructuronate and D-glucuronate.

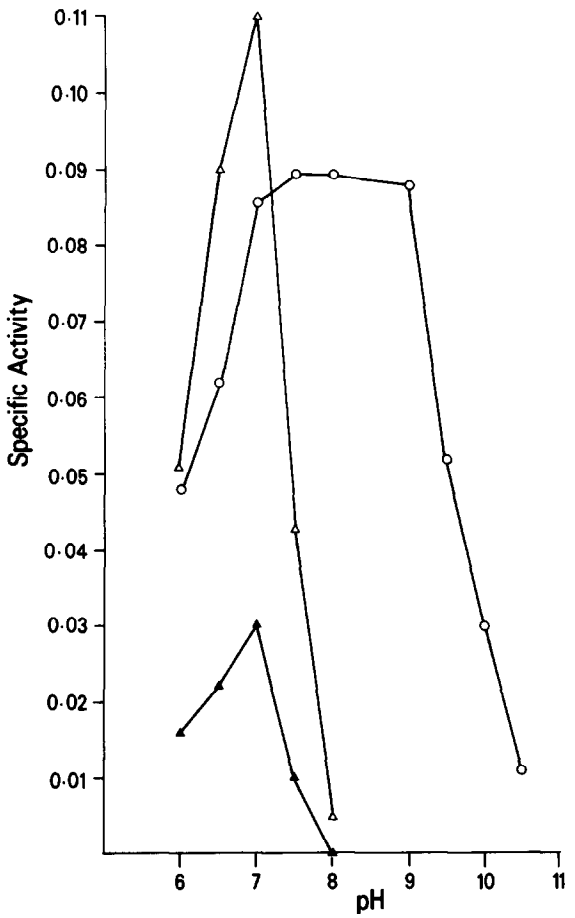

Fig.3. Effect of pH on the activity of L-galactonate oxidoreductase (△——△) and L-gulonate oxidoreductase (▲——▲) of L-galactonate-grown cells and of L-gulonate oxidoreductase of L-gulonate-grown cells (○——○). Phosphate buffers (0.05 M) were used for pH 6–8 and 0.05 M glycine–NaOH buffers for pH 9–10.5.

L-galactonate oxidoreductase with optimal activity at pH 7.0 and only slight activity at pH 8.0. Both activities elute in identical positions when crude extracts prepared from L-galactonate-grown cells are fractionated on Sephadex G-100, G-150 and G-200 columns. When wild-type *E. coli* K12, strain K10, is grown on glycerol plus L-galactonate, cell extracts show both L-galactonate oxidoreductase and L-gulonate oxidoreductase activity. However, when the L-galactonate oxidoreductase-negative mutant of strain K10 (strain C0131) is so grown the crude extracts have neither L-gulonate oxidoreductase nor L-galactonate oxidoreductase activity. Further, a hybrid ColE1 plasmid from the Carbon-Clark collection [13] that complements the L-galactonate oxidoreductase mutant strain C0518 causes 10–15-fold higher L-galactonate oxidoreductase activity and an identical increase in L-gulonate oxidoreductase activity, after growth of the complemented strain on L-galactonate.

However, a different NAD-dependent L-gulonate oxidoreductase activity was seen in extracts prepared from L-gulonate positive strains after growth on L-gulonate. Unlike the L-galactonate oxidoreductase this enzyme was highly active at pH 8.0 (fig.3) and was present at a specific activity of 0.1 μmol . min^{-1} . mg protein^{-1}. The activity separates into 2 peaks during gel filtration on Sephadex G-150 columns but neither peak corresponded to the position for L-galactonate oxidoreductase.

The L-gulonate positive strains showed no L-gulonate oxidoreductase activity (at pH 7.0 or 8.0) after growth on other compounds such as glucose, glycerol or lactate, suggesting that the enzyme was inducible.

The evidence presented here suggests that the catabolism of L-gulonate in *E. coli* K-12 and *S. typhimurium* LT-2 leads to the eventual formation of glyceraldehyde 3-phosphate and pyruvate. The pathway proposed involves the operation of only one novel enzyme, an L-gulonate oxidoreductase, to produce fructuronate, since the subsequent reactions are those of glucuronate catabolism [1]. The results from the various mutants argue strongly against the animal route for L-gulonate catabolism occurring in *E. coli* K-12.

This finding complements our report of the pathway for L-galactonate catabolism in *E. coli* K-12 [3] and allows a symmetrical addition to the known routes for both galacturonate and glucuronate catabolism in *E. coli* K-12 (see fig.1).

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References

[1] Ashwell, G., Wahba, A. J. and Hickman, J. (1958) Biochim. Biophys. Acta 30, 186–187.

[2] Hollman, S. and Touster, O. (1964) in: Non-glycolytic pathways of metabolism of glucose, pp. 95–101, Academic Press, New York.
[3] Cooper, R. A. (1979) FEBS Lett. 103, 216–220.
[4] Essenberg, M. K. and Cooper, R. A. (1975) Eur. J. Biochem. 55, 323–332.
[5] Portalier, R. C., Robert-Baudouy, J. M. and Nemoz, G. M. (1974) Mol. Gen. Genet. 128, 301–319.
[6] Robert-Baudouy, J. M. and Portalier, R. C. (1974) Mol. Gen. Genet. 131, 31–46.
[7] Robert-Baudouy, J. M., Portalier, R. C. and Stoeber, F. R. (1972) Mol. Gen. Genet. 118, 351–362.
[8] Miller, J. H. (1972) in: Experiments in molecular genetics, Cold Spring Harbor Lab., NY.
[9] Hartman, P. E., Loper, J. C. and Serman, D. (1960) J. Gen. Microbiol. 22, 323–353.
[10] Faik, P., Kornberg, H. L. and McEvoy-Bowe, E. (1971) FEBS Lett. 19, 225–228.
[11] Ashwell, G., Wahba, A. J. and Hickman, J. (1960) J. Biol. Chem. 235, 1559–1565.
[12] Gutnick, D., Calvo, J. M., Klopotowski, T. and Ames, B. N. (1969) J. Bacteriol. 100, 215–219.
[13] Clarke, L. and Carbon, J. (1976) Cell 9, 91–99.