

ASYMMETRIC DEHYDRATION OF GALACTARATE  
BY BACTERIAL GALACTARATE DEHYDRATASE\*

Harold J. Blumenthal and Theresa Jepson  
Department of Microbiology, The University of Michigan  
Ann Arbor, Michigan

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It has been known for some years that galactaric (mucic) acid, the naturally occurring dicarboxylic acid analogue of D-galactose (cf Anet and Reynolds, 1954), can support the growth of various microorganisms (den Dooren de Jong, 1926), particularly enteric bacteria (Koser, 1923; Sternfeld and Saunders, 1937; Clapper and Poe, 1947). The point was further clarified in our laboratory, by means of experiments involving resting cell suspensions or cell-free extracts of Escherichia coli grown in the presence of galactarate (Blumenthal and Campbell, 1958). It was demonstrated that under those conditions each mole of galactarate or D-glucarate was converted to one mole of pyruvate and unidentified products. The sequence of enzymes responsible for the conversion of galactarate to pyruvate was rapidly inactivated, although those enzymes in the extract that were responsible for the conversion of D-glucarate to pyruvate were stable.

Subsequent studies (Blumenthal, 1960) showed that the initial step in the utilization of D-glucarate was catalyzed by the relatively stable D-glucarate dehydratase yielding ketodeoxyglucarate (KDG). Although this enzyme was induced by either D-glucarate or galactarate, galactarate could not serve as a substrate for the purified D-glucarate dehydratase. The enzymes KDG aldolase and tartronate semialdehyde reductase were also shown to be involved in the metabolism of D-glucarate to pyruvate and glycerate (Blumenthal and Fish, 1963).

Further evidence has now been gained concerning the nature of galactarate dehydratase, the labile enzyme that catalyzes the formation of KDG from galactarate in E. coli and a number of other bacteria. This report

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describes (1) the isolation and certain properties of galactarate dehydratase, and (2) results showing that the galactarate molecule is dehydrated asymmetrically by this enzyme. The interrelationship of galactarate and glucarate metabolism is shown in Fig. 1.

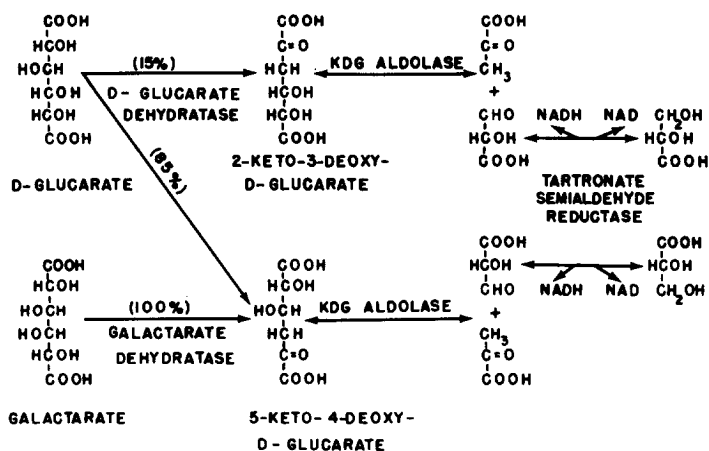


Fig. 1. Pathways for the catabolism of galactarate and D-glucarate by *E. coli*.

*E. coli* CR63MA was grown aerobically for 16 hr. on a rotary shaker at 37° C. with 0.8% galactaric acid as sole carbon source in a salts medium (Cohen, 1949) adjusted to pH 6.8 with NaOH. Five min. before harvesting, 300  $\mu$ moles of ferrous sulfate were added to each liter of culture medium and shaking was resumed. The cells were then harvested by centrifugation and washed twice with 0.02 M Na<sub>2</sub> galactarate. To each g. of cells, 9 ml. of 0.02 M Na<sub>2</sub> galactarate were added and the suspension was disrupted for 10 min. in a 10 KC sonic oscillator. The enzyme was purified by addition of sodium acetate (0.4M) and protamine sulfate (60 mg.) and then precipitated with 5.3 M potassium acetate, pH 6.3. After a partial desalting by addition of Ca<sup>++</sup>, the enzyme was treated batchwise successively with calcium phosphate gel and XE-64 anion exchange resin equilibrated with 0.05 M potassium acetate, pH 5.7. More enzyme was eluted from the resin by washing with 0.02 M Tris-acetate, pH 6.0. Although the galactarate dehydratase was completely separated from the glucarate dehydratase by this procedure, these fractions were very labile and had to be used immediately. Unlike the labile pseudomonad L-tartrate dehydratase (Hurlbert and Jacoby, 1964), galactarate dehydratase could not be reactivated by Fe<sup>++</sup> or protected with reducing agents. The results of a fractionation are presented in Table 1.

TABLE 1  
PURIFICATION OF *E. COLI* GALACTARATE DEHYDRATASE

Fraction	Galactarate dehydratase			D-Glucarate dehydratase Units/ml.	Galactarate dehydratase Glucarate dehydratase
	Units/ml.	Spec. act.	Yield, %		
1. Crude extract.	3.19	0.38	100	9.32	0.33
2. K acetate ppt.	1.58	3.0	49.0	0.53	3.00
3. Ca <sub>3</sub> (PO <sub>4</sub> ) <sub>2</sub> supernatant	1.28	6.0	40.1	0.19	6.75
4. XE-64 fractions					
a) Supernate	0.64	17.0	20.0	0.0	∞
b) 0.02 M Tris-ac pH 6.0	0.26	22.6	8.1	0.0	∞
c) 0.02 M Tris-ac pH 7.5	0.11	9.6	3.4	trace	>36.0
d) 0.05 M Tris-ac pH 7.5	0.05	4.0	1.5	0.004	12.5

Galactarate dehydratase assay: Na<sub>2</sub> galactarate, 2.0 μmoles; Tris-acetate buffer, pH 8.0, 40 μmoles; Na<sub>2</sub>EDTA, 1.0 μmoles; enzyme; final vol. 0.4 ml. D-glucarate dehydratase assay; Na<sub>2</sub>D-glucarate; 2.0 μmoles; Tris-acetate buffer, pH 8.0, 40 μmoles; MgSO<sub>4</sub>, 4 μmoles; enzyme; final vol. 0.4 ml. The reactions, which were started by addition of enzyme, were terminated by addition of 0.1 ml. of 10% TCA after an incubation period of 10 min. at 30° C. The difference between the glucarate dehydratase assay and the control lacking substrate (galactarate was added after the TCA) was used to determine the true glucaric dehydratase values. For analysis of the KDG-formed, 0.1 ml. samples were used for the periodate-thiobarbituric test of Weissbach and Hurwitz (1959) employing an extinction coefficient of 60,000 for KDG (Fish, 1964). One unit of either enzyme was defined as that amount of enzyme which would catalyze the formation of 1 μmole of KDG/min. under these conditions. Proteins were determined nephelometrically with sulfo-salicylic acid (Fish, 1964).

A convenient and important difference between the two hexarate dehydratases is their response to inhibitors (Table 2) reflecting a cation requirement of D-glucarate dehydratase and the absence of such a requirement for galactarate dehydratase. The galactarate dehydratase activity was not stimulated when any of 17 cations, including Fe<sup>++</sup>, were added in the absence of EDTA.

When galactarate is examined by the criteria of Hirschmann (1960), it does have reflective, but not rotational, symmetry. Evidence that this is indeed the case was obtained by isolating C<sup>14</sup>-labeled glycerate and

TABLE 2  
EFFECT OF INHIBITORS ON HEXARATE DEHYDRATASES

Inhibitor	Inhibitor concentration, M	Galactarate dehydratase, relative activity	D-Glucarate dehydratase, relative activity
None		100	100
Na <sub>2</sub> EDTA	0.005	100	0
CaCl <sub>2</sub>	0.0012	100	0
KCN	0.005	0	80
NaF	0.005	100	50
Na pyrophosphate	0.005	15	0

XE-64 fraction b) was used for the source of galactarate dehydratase and a fraction eluted with 0.2 M Tris-acetate, pH 7.5, for D-glucarate dehydratase. The latter fraction possessed properties similar to those of a 40-fold purified D-glucarate dehydratase prepared by other procedures (Blumenthal, 1960). Assays were performed as indicated in Table 1 with the exception that EDTA and Mg<sup>++</sup> were not included in the galactarate and glucarate assay mixtures, respectively. D-glucarate dehydratase activity was ca. 30% of that with Mg<sup>++</sup> added.

unlabeled pyruvate following the metabolism of galactarate-1-C<sup>14</sup> by crude extracts of galactarate-grown *E. coli* (Fig. 2). A similar experiment employing galactarate-6-C<sup>14</sup> of lower specific activity, made by oxidation of biosynthetically prepared D-galacturonate-6-C<sup>14</sup> (kindly provided by Dr. F. Loewus) with HNO<sub>3</sub>, yielded C<sup>14</sup>-labeled pyruvate. The results provide evidence that galactarate is an asymmetric molecule that is dehydrated by the enzyme yielding only 5-keto-4-deoxy-D-glucarate (Fig. 1). This contrasts with the D-glucarate dehydratase which dehydrates D-glucarate at either of 2 sites, although 5-keto-4-deoxy-D-glucarate is also the major product (Blumenthal and Fish, 1963; Fish, 1964).

When glucarate-grown cells are sonicated in 0.05 M KCl, or a variety of other salts, the extracts are rich in glucarate dehydratase but no galactarate dehydratase can be demonstrated. However, when the glucarate grown cells are treated with ferrous ions five minutes prior to harvest, and sonicated in the presence of galactarate, the galactarate dehydratase is stabilized and can be demonstrated. Thus, glucaric and galactaric acids can each induce the formation of both dehydratases even though each serves as a substrate for only one dehydratase. Employing the same techniques used with *E. coli*, both D-glucarate and galactarate dehydratases have now been demonstrated in cell-free extracts of either glucarate- or galactarate-

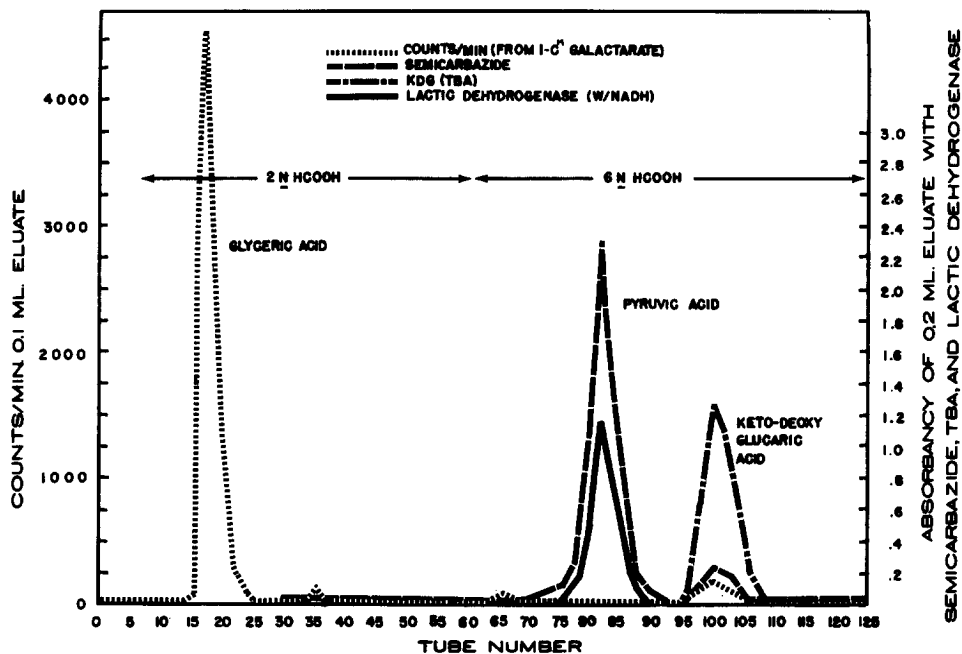


Fig. 2. Anion exchange chromatography of the products of galactarate- $1\text{-C}^{14}$  metabolism. The main compartment of the incubation flask contained, in a volume of 22.0 ml. galactarate- $1\text{-C}^{14}$ , (prepared by  $\text{HNO}_3$  oxidation of D-galactose- $1\text{-C}^{14}$ ) 180  $\mu\text{moles}$ , containing 9,521 counts/min. 7  $\mu\text{moles}$ ;  $\text{CoCl}_2$ , 35  $\mu\text{moles}$ ; Tris-HCl, pH 7.5, 4.0 mmoles; sodium arsenite, 60  $\mu\text{moles}$ ; NADH, 25 mg. The side arm of the flask contained in a volume of 5 ml.:  $\text{Na}_2\text{EDTA}$ , 50  $\mu\text{moles}$ ; Tris-HCl, pH 6.5, 4.0 mmoles; NADH, 25 mg. The flask was evacuated for 15 min. to remove dissolved oxygen. The source of enzymes was a freshly-prepared extract of galactarate-grown *E. coli*. The freshly harvested cells were washed with, and sonicated in, 0.05 M KCl, centrifuged 10 min. at 20,000  $\times$  g., and 10 ml. of cell-free enzyme extract immediately added to the main compartment. The flask was again evacuated for 15 min. and then further incubated at room temperature in the dark for 90 min. The contents of the side-arm were then tipped into the main compartment, providing conditions more favorable for the reduction of tartronate semialdehyde, and the incubation was continued for an additional 15 min. After terminating the reaction in a boiling water bath the deproteinized fluid was placed on a 2  $\times$  21 cm. column of Dowex-1-formate. The organic acids were eluted using a 2-stage gradient of formic acid and 7 ml. fractions were collected (Blumenthal and Fish, 1963). Samples (0.2 ml) were assayed for  $\alpha$ -keto acids using semicarbazide (MacGee and Doudoroff, 1954); KDG was determined by the periodate-thiobarbituric acid assay of Weissbach and Hurwitz (1959); neutralized samples were assayed for pyruvate using lactic dehydrogenase and NADH; and 0.1 ml. samples were counted with a liquid scintillation spectrometer using DAM 611 (Davidson and Feigelson, 1957). The tubes in the region where glycerate is eluted were combined, lyophilized and the solids dissolved and treated with charcoal (Nuchar) to remove NAD and NADH. The solution was then rechromatographed on another Dowex-1-formate column and the main fraction, which contained 84% of the  $\text{C}^{14}$  applied to this column, was again lyophilized. A solution of this material was analyzed for glycerate content (Bartlett, 1959) and identified as glycerate by paper chromatography (Blumenthal and Fish, 1963).

grown suspensions of Erwinia caratovora, Aerobacter aerogenes, Escherichia freundii, Paracolobactrum sp., Klebsiella pneumoniae, Bacillus subtilis and B. megaterium.

Pseudomonads possess a modified pathway of hexarate metabolism (Kilgore and Beckman, 1962; Dagley and Trudgill, 1964). However, in Pseudomonas syringae extracts KDG is utilized (Kilgore, personal communication) and the properties of extracts from another pseudomonad system are consistent with the operation of a labile galactarate dehydratase and a stable glucarate dehydratase (Dagley, personal communication). Following the initial dehydratase reactions, the pseudomonads apparently do not use the KDG aldolase and tartronate semialdehyde reductase sequence found in many other bacteria (Blumenthal and Fish, 1963).

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