*Biochem. J.* (1969) **115**, 969
*Printed in Great Britain*



# The Metabolism of D-Glucarate by *Pseudomonas acidovorans*

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(*Received* 17 *June* 1969)

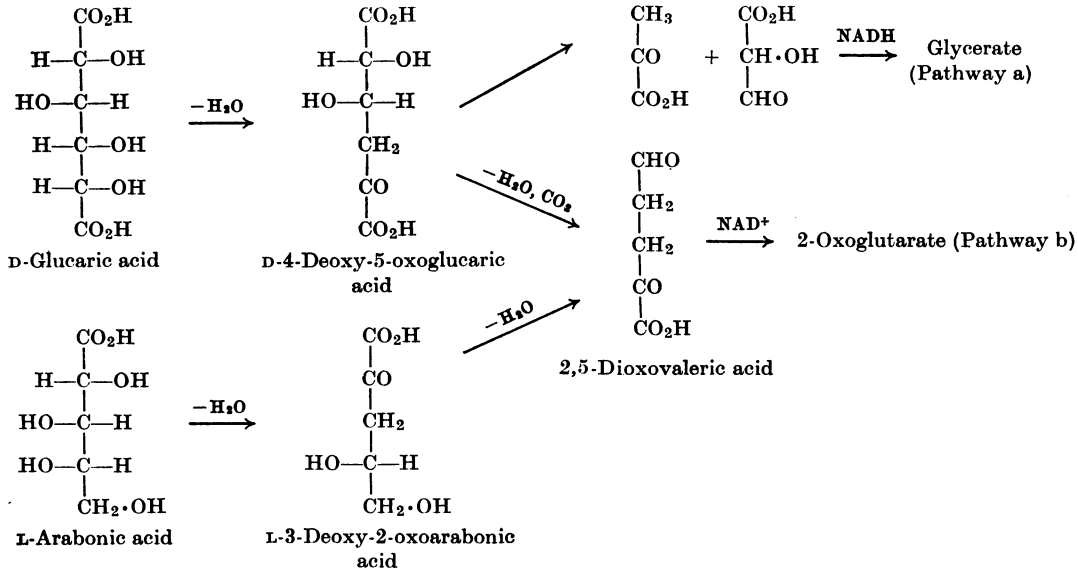
1. Dehydratases that converted D-glucarate into 4-deoxy-5-oxoglucarate were partially purified from *Klebsiella aerogenes* and *Pseudomonas acidovorans*. 2. When D-glucarate was metabolized to 2,5-dioxovalerate it appeared that water and carbon dioxide were removed from 4-deoxy-5-oxoglucarate in one enzymic step: 4,5-dihydroxy-2-oxovalerate was not an intermediate in this reaction. 3. A method for the enzymic determination of D-glucarate is described.

D-Glucaric acid can serve as a carbon source for growth of certain species of Enterobacteriaceae and Pseudomonadaceae, but the two families appear to use different pathways of metabolism. Thus Blumenthal & Fish (1963) reported that nine strains of *Escherichia coli* and six other species of enterobacteria converted D-glucarate into pyruvate and glycerate (Scheme 1, pathway a), whereas Trudgill & Widdus (1966) found that all 14 strains of the various species of *Pseudomonas* they investigated metabolized D-glucarate to 2-oxoglutarate (Scheme 1, pathway b). When Dagley & Trudgill (1965) proposed the reactions of pathway b their main evidence consisted in the observations that cell extracts, when supplemented with $NAD^+$, converted D-glucarate into 2-oxoglutarate, whereas 2,5-dioxovalerate (2-oxoglutarate semialdehyde) was formed when $NAD^+$ was omitted from reaction mixtures. Blumenthal & Fish (1961) partially purified a dehydratase from *E. coli* that converted D-glucarate mainly into 4-deoxy-5-oxoglucarate; but Dagley & Trudgill (1965) reported no direct evidence for the participation of this compound in the reaction sequence catalysed by *Pseudomonas* (A), although their demonstration that D-glucarate did not undergo direct decarboxylation indicated that the metabolic pathway was initiated by a dehydratase. We have now established that 4-deoxy-5-oxoglucarate is formed and have studied its metabolism to give the loss of one molecule each of carbon dioxide and water. If carbon dioxide were lost first the expected intermediate would be D-3-deoxy-2-oxoarabonic acid, the L-isomer of which has been shown by Stoolmiller & Abeles (1966) to be formed in *Pseudomonas saccharophilla* from L-arabonate and then dehydrated to give 2,5-dioxovalerate (Scheme 1). However, we find that D-3-deoxy-2-oxoarabonate is not metabolized by extracts of *Pseudomonas acidovorans* grown with D-glucarate and it appears that water and carbon dioxide are lost from 4-deoxy-5-oxoglucarate in one enzymic step.

## MATERIALS AND METHODS

*Maintenance and growth of organisms. Pseudomonas* (A) (Bachrach, 1957; Dagley & Trudgill, 1963, 1965) has been identified by Stanier, Palleroni & Doudoroff (1966) as *Pseudomonas acidovorans* and is listed in type culture collections as N.C.I.B. 10 013 and A.T.C.C. 17 455. The maintenance and growth of the organism with D-glucarate and the preparation of cell extracts have been described by Dagley & Trudgill (1965). Similar conditions of growth were used for *Klebsiella aerogenes* N.C.I.B. 418 and extracts were prepared from cells crushed in a bacterial press (Hughes, 1951) by centrifuging at 30000$g_{av.}$ for 1·5 hr. after incubating for 30 min. with ribonuclease and deoxyribonuclease (Koch-Light Laboratories Ltd., Colnbrook, Bucks.). The enzymes for D-glucarate catabolism remained fully active when the clear yellow solution was stored for several months at −20°. Except where stated otherwise, the phosphate buffer used throughout this work contained 0·1 M-$KH_2PO_4$ brought to pH 7·2 by addition of NaOH. The methods of Sols (1947) and of Lowry, Roseborough, Farr & Randall (1951) respectively were used to determine protein in crude extracts and during enzyme purification. Changes in the protein concentrations of successive fractions from chromatography columns were followed by measuring extinctions at 280 nm.

*Chromatography and electrophoresis.* DNP-hydrazones of oxo acids were examined by paper chromatography, with solvents A and B of Dagley, Geary & Wood (1968), or by t.l.c. on Kieselgel G with solvents A, B and C of Bayly, Dagley & Gibson (1966). Glutamic acid was identified by one-dimensional chromatography on Whatman no. 1 paper with five ascending systems, namely: propionic acid–1M-acetic acid (3:1, w/v) (Feinberg & Greenberg, 1959); phenol–aq. 0·4 M-$NH_3$ soln. (4:1, w/v); solvents C, D and E of Chamberlain & Dagley (1968). For paper chromatography of deoxyoxo-sugar acids, two solvents of Fish (1964) were used: 2-methylpropan-2-ol–formic acid–water (8:2:3, by vol.) and ethyl acetate–acetic acid–water (3:1:3, by vol.). These chromatograms were sprayed with the reagents of




Scheme 1. Bacterial metabolism of D-glucarate and L-arabonate.

Warren (1960). The identities of various compounds were confirmed by high-voltage paper electrophoresis with a Pherograph-Original-Frankfurt type 1963–64 instrument (Northern Media Supplies Ltd., Blanket Row, Hull). The buffer used was water–pyridine–acetic acid (90:5:0·2, by vol.) and an e.m.f. of 55 v/cm. was applied for 30–120 min.

When 4-deoxy-5-oxo[$^{14}$C]glucarate was oxidized by periodate to give [$^{14}$C]glyoxylate and [$^{14}$C]3-formylpyruvate, solvent B of Dagley *et al.* (1968) was used to give a good separation of the DNP-hydrazones. In this solvent the glyoxylate derivative gives two spots of $R_F$ 0·33 and 0·52, but only the slower-moving spot gives a red colour with alkali. This behaviour was further investigated since we needed to determine specific radioactivities for [$^{14}$C]-glyoxylate DNP-hydrazone, and this involved making measurements of the amounts that could be separated by paper chromatography. In experiments with authentic DNP-hydrazone of glyoxylate it was found that, when the two yellow spots were eluted and pooled, there was complete recovery of the DNP-hydrazone as determined from the extinction at 365 nm. ($\epsilon$ 16000) in neutral solution. This procedure was therefore adopted. If the eluted solutions were made alkaline and extinctions were measured at 450 nm., recoveries of 55–60% were obtained. From this observation it was concluded that in the presence of this solvent a proportion of the DNP-hydrazone of glyoxylate is converted into material that does not turn red with alkali. This conversion did not occur when the solvent used was 95% (w/v) methanol, and in this case complete recovery from eluted spots was indicated by determinations at 450 nm. in alkali.

*Chemical analyses.* Of three different assays reported for D-glucaric acid or the lactone that is formed when its acidic solutions are boiled (Marsh, 1963; Ishidate, Matsui & Okada, 1965; Blumenthal & Jepson, 1964), none seemed free from possible interference by products of glucarate catabolism. For our determinations we used crude extracts of glucarate-grown *Ps. acidovorans* to convert D-glucarate (1 mole) into 2-oxoglutarate with the simultaneous reduction of NAD$^+$ (1 mole), and we confirmed the observation of Dagley & Trudgill (1965) that the correction for oxidation of NADH in the absence of substrate is small for such extracts. Increases in extinction at 340 nm., which were measured in cuvettes in 3 ml. of phosphate buffer, were due to the metabolism of D-glucarate, and of 4-deoxy-5-oxoglucarate and 2,5-dioxovalerate if these were present. Measurements were then repeated for the same reaction mixtures with the addition of mM-EDTA. This reagent inhibited D-glucarate dehydratase completely and we were therefore able to determine $\Delta E_{340}$ due to the other metabolites and so obtain by difference the change due solely to D-glucarate.

4-Deoxy-5-oxoglucarate was determined by the method of Weissbach & Hurwitz (1959) for sugar acids containing the 3-deoxy-2-oxo grouping. The solution (0·25 ml.) was treated with 0·25 ml. of 25 mM-periodic acid in 62·5 mM-$H_2SO_4$ for 20 min. at 20°. Excess of periodic acid was removed by adding 0·5 ml. of 2% (w/v) sodium arsenite; 2 ml. of 0·3% (w/v) thiobarbituric acid was added and a pink colour was developed by heating at 100° for 10 min. Extinctions were measured at 546 nm. and concentrations of 4-deoxy-5-oxoglucarate were calculated. A range of molar extinction coefficients has been reported for the chromophores resulting from various deoxyoxo-sugar acids when submitted to this procedure. The value we used ($\epsilon$ 60000) agrees with that of Fish (1964). It was obtained by correlating decreases in extinction at 546 nm., when 4-deoxy-5-oxoglucarate was oxidized to 2-oxoglucarate by cell extracts, with the increases in extinction at 340 nm. as NAD$^+$ was simultaneously reduced to NADH.

Table 1. *Purification of* D-*glucarate dehydratase from* K. aerogenes

One unit of enzyme is the amount that catalyses the production of 1 $\mu$mole of 4-deoxy-5-oxoglucarate/min. at 20° and pH 7·2 from D-glucarate. Assay mixtures (3 ml.) contained 10 $\mu$moles of potassium hydrogen D-glucarate, 5 $\mu$moles of magnesium sulphate and 50 $\mu$moles of phosphate buffer, pH 7·2.

| Step | Method | Total protein (mg.) | Enzyme (total units) | Sp. activity (units/mg. of protein) | Yield (%) | Purification |
|---|---|---|---|---|---|---|
| 1 | Crude extract | 3100 | 434 | 0·14 | 100 | 1·0 |
| 2 | Protamine sulphate treatment | 2800 | 450 | 0·16 | 104 | 1·2 |
| 3 | Heat treatment | 851 | 458 | 0·54 | 106 | 3·9 |
| 4 | 0–50%-satd.-$(NH_4)_2SO_4$ ppt., dissolved and dialysed | 312 | 430 | 1·38 | 99 | 9·8 |
| 5 | DEAE-cellulose chromatography, pooled fractions precipitated, dissolved and dialysed | 90 | 258 | 2·87 | 59 | 20·5 |

2,5-Dioxovalerate (2-oxoglucarate semialdehyde) was determined from the extinction at 430 nm. of alkaline solutions of its bis-DNP-hydrazone in ethanol (Dagley & Trudgill, 1965).

*Purification of cell extracts.* For our initial investigations of the metabolism of 4-deoxy-5-oxoglucarate by *Ps. acidovorans* we prepared this substrate from D-glucarate by the action of a dehydratase purified from *K. aerogenes*. A crude extract of *K. aerogenes* grown with D-glucarate was treated with protamine sulphate to remove nucleic acids. To each 10 ml. of extract at 4° was added, with stirring, 2 ml. of 2% (w/v) protamine sulphate and the precipitate was removed by centrifuging at 26000$g_{av.}$ for 10 min. at 2°. The clear supernatant solution was then heated at 55° for 10 min. and the precipitate centrifuged and discarded. The extract was brought to 50% saturation with $(NH_4)_2SO_4$ and the precipitate was dissolved in 5 mM-phosphate buffer, pH 7·2, and dialysed against the same buffer until $NH_4^+$ ions were no longer detected. The solution (300 mg. of protein in 12 ml. of buffer) was applied to a column (22 cm. × 2 cm.) of DEAE-cellulose, washed with 84 ml. of 5 mM-phosphate buffer, pH 7·2, followed by 120 ml. of 0·1 M-NaCl in the same buffer and then eluted with a 0·1–0·3 M-NaCl linear concentration gradient in this buffer. Fractions containing D-glucarate dehydratase activity were brought to 50% saturation with $(NH_4)_2SO_4$, and the precipitate was dissolved in 0·1 M-phosphate buffer, pH 7·2, and dialysed against the same buffer. The purified enzyme, which required $Mg^{2+}$ ions, was free from 4-deoxy-5-oxoglucarate aldolase (Table 1). A preparation of hydratase sufficiently pure to give 4-deoxy-5-oxoglucarate in 80% yield from D-glucarate was obtained by treating a crude extract of glucarate-grown *Ps. acidovorans* with protamine sulphate; the supernatant solution was then brought to 30% saturation with $(NH_4)_2SO_4$ and the precipitate dissolved in phosphate buffer, pH 7·2. This enzyme was also $Mg^{2+}$-dependent.

*Materials.* D-[$^{14}$C]Glucarate was prepared as described previously (Dagley & Trudgill, 1965) and was chromatographed on a column (10 cm. × 1 cm.) of Dowex 1 (X8; formate form) and eluted with a 0–2 M-formic acid linear concentration gradient (total vol. 300 ml.). Most of the radioactive material was [$^{14}$C]glucarate, eluted at 1·3 M-formic acid; smaller amounts of glucose and also materials thought to be gluconate and glucuronate were eluted with the solvent front and at 0·5 M-formic acid and 0·75 M-formic acid respectively. Fractions containing glucarate were pooled, evaporated to dryness on a rotary evaporator, dissolved in water and delactonized by bringing to pH 9 for at least 5 min. before adjustment to pH 7·2. They were assayed by means of a crude extract of *Ps. acidovorans*.

4-Deoxy-5-oxoglucarate was obtained from D-glucarate, either by the action of D-glucarate dehydratase purified from *K. aerogenes*, or more often by using a partially purified extract of *Ps. acidovorans*. Protein was removed from incubation mixtures by means of metaphosphoric acid, the solution was adjusted to pH 9 with 2 M-NaOH for 5 min., returned to pH 6 with 2 M-$H_2SO_4$ and then applied to a column (10 cm. × 1 cm.) of Dowex 1 (X8; formate form). When chromatographed with a 0–6 M-formic acid linear concentration gradient (total vol. 600 ml.), deoxyoxoglucarate was eluted by 3·5 M-formic acid and was well separated from unchanged glucarate. Fractions were pooled and freeze-dried, and their purity was checked by paper chromatography. Since deoxyoxoglucarate readily lactonizes, solutions at pH 7 were held at 100° for 8 min. before they were used in experiments. 4-Deoxy-5-oxo-[$^{14}$C]-glucarate was prepared from [$^{14}$C]glucarate by this method.

4,5-Dihydroxy-2-oxovaleric acid (3-deoxy-2-oxoarabonic acid) was obtained by condensing glycollic aldehyde (from Koch-Light Laboratories Ltd., Colnbrook, Bucks.) with oxaloacetic acid as described by Stoolmiller & Abeles (1965). The bis-DNP-hydrazone of 2,5-dioxovalerate (2-oxoglutarate semialdehyde) was synthesized from methyl tetrahydro-2,5-dimethoxy-2-furoate (Dagley & Trudgill, 1965). Potassium hydrogen D-glucarate, DEAE-cellulose and Dowex 1 were from Sigma (London) Chemical Co. Ltd., London, S.W.6.

## RESULTS AND DISCUSSION

*Metabolism of* D-*glucarate by* Klebsiella aerogenes. Since all the strains of *K. aerogenes* examined by Blumenthal & Fish (1963) and Trudgill & Widdus (1966) metabolized glucarate by pathway a (Scheme 1) it was expected that these reactions would be utilized by *K. aerogenes* N.C.I.B. 418. This was confirmed by isolating tartronate semialdehyde

Table 2. *Oxidation of NADH by tartronate semialdehyde in the presence of extracts of* K. aerogenes *and* Ps. acidovorans

The cell extracts used were: A, crude extract of *K. aerogenes* grown with D-glucarate; B, heat-treated extract of glycine-grown *Ps. acidovorans* that converted glyoxylate into tartronate semialdehyde; C, crude extract of *Ps. acidovorans* grown with D-glucarate. All reaction mixtures contained 15 $\mu$moles of potassium phosphate buffer, pH 7·2, and 0·5 $\mu$mole of NADH in a total volume of 2·5 ml. Approximately linear decreases in extinction at 340 nm. were observed over 14 min.

| Cell extract ... | Extract added (mg. of protein) | | | | |
|---|---|---|---|---|---|
| | ... A | B | C | Glyoxylate | |
| Expt. no. | | | | ($\mu$moles) | $-\Delta E_{340}$/min. |
| 1 | 1·6 | 0 | 0 | 0 | 0·031 |
| 2 | 0 | 1·4 | 0 | 4 | 0·031 |
| 3 | 1·6 | 1·4 | 0 | 4 | 0·335 |
| 4 | 0 | 1·4 | 2·9 | 0 | 0·032 |
| 5 | 0 | 1·4 | 2·9 | 4 | 0·060 |

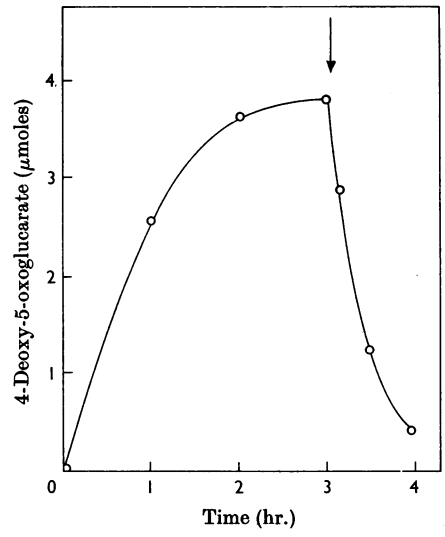



Fig. 1. Ability of *Ps. acidovorans* to metabolize 4-deoxy-5-oxoglucarate produced by *K. aerogenes*. Reactions were started by adding D-glucarate dehydratase (0·5 mg. of protein) from *K. aerogenes* to 4 $\mu$moles of D-glucarate in phosphate buffer, pH 7·2. At the time indicated by the arrow, crude extract of *Ps. acidovorans* (3 mg. of protein) was added.

as its bis–DNP-hydrazone (Dagley, Trudgill & Calley, 1961) from incubation mixtures of D-glucarate with extracts of cells grown with this substrate. 2,5-Dioxovalerate was not produced, nor was the compound metabolized by extracts even when $NAD^+$ was added. Further, these extracts contained tartronate semialdehyde reductase, whereas the enzyme was effectively absent from glucarate-grown *Ps. acidovorans* as shown by the following experiments. Rates of oxidation of NADH by extracts of *K. aerogenes* and *Ps. acidovorans* were measured in the presence and absence of tartronate semialdehyde, prepared *in situ* from glyoxylate by the action of a heat-treated extract of glycine-grown *Ps. acidovorans* as described by Dagley & Trudgill (1963). When the rate of oxidation observed in Expt. 3 (Table 2) was corrected by subtracting the sum of the rates for Expts. 1 and 2, the specific activity of tartronate semialdehyde reductase was calculated to be 3·9 $\mu$moles of NADH/hr./mg. of protein for *K. aerogenes*. The rate for Expt. 5, when that for Expt. 4 was subtracted, gave a specific activity of 0·2 $\mu$mole of NADH/hr./mg. of protein for glucarate-grown *Ps. acidovorans*.

*Evidence that 5-deoxy-4-oxoglucarate is a metabolite of both pathways of D-glucarate catabolism.* The following experiments were performed to show that the deoxyoxoglucarate formed from D-glucarate by *K. aerogenes* was also metabolized by *Ps. acidovorans* and conversely that the compound formed by *Ps. acidovorans* was a metabolite for *K. aerogenes*. It was then demonstrated that the precise chemical structure of the compound was that of 4-deoxy-5-oxoglucarate.

Glucarate dehydratase was prepared from *K. aerogenes* by the purification procedure described in the Materials and Methods section, except that the heat-treatment step was omitted. Seven identical mixtures containing 50 $\mu$moles of phosphate buffer, pH 7·2, 40 $\mu$moles of magnesium sulphate and 4 $\mu$moles of potassium hydrogen D-glucarate in 3 ml. were incubated at 25°. Of these reaction mixtures, four were used to show that deoxyoxoglucarate accumulated by the action of glucarate dehydratase; the disappearance of this compound, when an extract of *Ps. acidovorans* was added, was followed in the other three. The reactions were started by adding glucarate dehydratase (0·5 mg. of protein) to each mixture. At 0, 60, 120 and 180 min. after starting the reactions in the first four mixtures, 0·2 ml. of 50% (w/v) trichloroacetic acid was added, protein was removed by centrifuging and deoxyoxoglutarate was then assayed in 0·05 ml. of each supernatant solution. At 3 hr. from the start of the experiment, crude extract (3 mg. of protein) of glucarate-grown *Ps. acidovorans* was added to each of the three remaining mixtures and they were incubated for a further 10, 30 and 60 min. respectively before the reactions were stopped. The deoxyoxoglucarate formed by the partially purified extract of *K. aerogenes* after 3 hr. was almost completely metabolized in 1 hr. by the

extract of *Ps. acidovorans* (Fig. 1). A second experiment was performed in a similar manner, except that D-glucarate was first converted into deoxyoxoglucarate by the dehydratase from *Ps. acidovorans* and a crude extract of glucarate-grown *K. aerogenes* was then added. As in the previous experiment, deoxyoxoglucarate was rapidly metabolized at first; but in this case the rate of disappearance diminished greatly after 1hr., so that 27% of the metabolite still remained after further incubation for 2hr. This failure of extracts of *K. aerogenes* to metabolize deoxyoxoglucarate completely is apparently due to the fact that the aldolase reaction attains an equilibrium. Thus, when D-glucarate was incubated with these extracts without prior addition of the dehydratase, the reaction proceeded to the same position of equilibrium since 24% of the substrate could still be accounted for as accumulated deoxyoxoglucarate even when incubation was prolonged. However, when NADH was added to remove one of the equilibrium products, namely tartronate semialdehyde, the amount of NADH oxidized was that expected for complete decomposition of the D-glucarate.

Additional experiments provided further evidence that D-glucarate is dehydrated by the two organisms to give the same deoxyoxoglucarate. Two solutions containing 20$\mu$moles of D-glucarate in 2·5ml. of phosphate buffer, pH7·2, were incubated at 30° with crude extracts of glucarate-grown *K. aerogenes* and *Ps. acidovorans* (8·8 and 12·5mg. of protein respectively) and reactions were stopped after 5min. by adding 0·1ml. of 3M-metaphosphoric acid. Samples (50$\mu$l.) were examined by high-voltage paper electrophoresis and by paper chromatography in the two solvents of Fish (1964) (see the Materials and Methods section); deoxyoxo-sugar acids were then revealed by the method of Warren (1960). A single spot was given for each reaction mixture in every case, and in each of the three systems the material produced by the extract of *K. aerogenes* had the same electrophoretic mobility or $R_F$ as that from *Ps. acidovorans*.

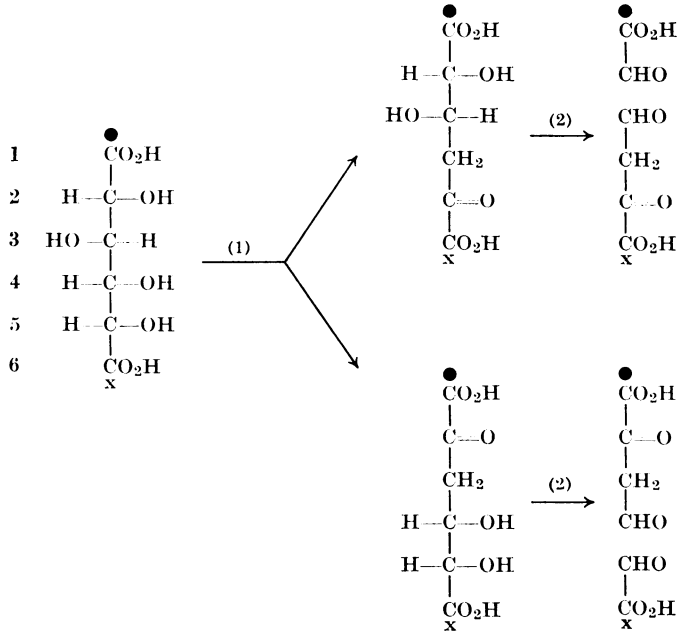
*Identification of 4-deoxy-5-oxoglucarate.* Although *K. aerogenes* and *Ps. acidovorans* form the same compound when they dehydrate D-glucarate, the evidence that the elements of water are removed from C-4 and C-5 is based on the brief report of Blumenthal & Fish (1961) that the product formed by *E. coli* is 4-deoxy-5-oxoglucarate; further, the assumption is made that *K. aerogenes* and *E. coli* metabolize D-glucarate by the same reactions. Accordingly, we sought more direct evidence relating to the chemical structure of deoxyoxoglucarate formed by *Ps. acidovorans*. Oxidation of deoxyoxoglucarate by periodate yields a mixture of glyoxylate and 3-formylpyruvate, which can be separated as DNP-hydrazones by paper chromatography in solvent B of Dagley *et al.* (1968). In particular, the DNP-hydrazone of [$^{14}$C]glyoxylate is to be expected when the product formed by dehydrating [1-$^{14}$C]glucarate at C-4 and C-5 is treated in this manner, whereas glyoxylate arising from [6-$^{14}$C]glucarate will not be labelled. Conversely, Scheme 2 predicts no labelling of glyoxylate when dehydration of [1-$^{14}$C]glucarate occurs at C-2 and C-3. Samples of [1-$^{14}$C]glucarate and [6-$^{14}$C]-glucarate were prepared from 22·5mg. (20$\mu$c) of [1-$^{14}$C]glucose and [6-$^{14}$C]glucose respectively, as described in the Materials and Methods section. A sample (5$\mu$moles) of each glucarate was metabolized by an extract of *Ps. acidovorans* and the products were then oxidized to glyoxylate and formylpyruvate by means of periodate. The average specific radioactivity of spots due to the DNP-hydrazone of glyoxylate formed from [1-$^{14}$C]glucarate (Table 3) was 17478c.p.m./$\mu$mole whereas the corresponding value for the derivative formed

Table 3. *Incorporation of radioactivity from* D-[$^{14}$C]*glucarate into the DNP-hydrazone of glyoxylate*

Samples of [1-$^{14}$C]glucarate and [6-$^{14}$C]glucarate were metabolized by an extract of *Ps. acidovorans* and the resulting 4-deoxy-5-oxo[$^{14}$C]glucarate was then oxidized with periodic acid. DNP-hydrazones of products were chromatographed on paper and each spot of glyoxylate DNP-hydrazone was eluted with water and made up to 0·5ml. A portion of each solution (50$\mu$l.) was plated out and its radioactivity measured, and the remainder was used to determine the amount of the DNP-hydrazone from its extinction at 360nm.

| | | Glyoxylate DNP-hydrazone eluted | | | |
|---|---|---|---|---|---|
| Substrate | $R_F$ | $^{14}$C incorporated (c.p.m./ml.) | Concentration ($\mu$mole/ml.) | Sp. activity (c.p.m./$\mu$mole) | Average sp. radioactivity (c.p.m./$\mu$mole) |
| [1-$^{14}$C]Glucarate | 0·33 | 3646* | 0·203 | 17962 | 17478 |
| [1-$^{14}$C]Glucarate | 0·52 | 8462* | 0·498 | 16993 | |
| [6-$^{14}$C]Glucarate | 0·33 | 157 | 0·118 | 1330 | 1570 |
| [6-$^{14}$C]Glucarate | 0·52 | 214 | 0·118 | 1810 | |

* Mean of duplicate determinations.




Scheme 2. Incorporation of $^{14}C$ into glyoxylate formed from [$^{14}C$]glucarate by (1), enzymic dehydration, followed by (2), oxidation of each of the two isomers of deoxyoxoglucarate by means of periodate.

from [6-$^{14}C$]glucarate was 1570c.p.m./$\mu$mole. In a second experiment these values were 13553 and 1113c.p.m./$\mu$mole respectively. It is therefore concluded that 4-deoxy-5-oxoglucarate constitutes more than 90% of the mixture of isomers that may arise from the enzymic dehydration of D-glucarate.

*Enzymic conversion of 4-deoxy-5-oxoglucarate into 2,5-dioxovalerate and 2-oxoglucarate,* Crude extracts of *Ps. acidovorans* convert D-glucarate into 2,5-dioxovalerate, which, when $NAD^+$ is added, is further metabolized to 2-oxoglutarate (Dagley & Trudgill, 1965). In the following experiments 4-deoxy-5-oxoglucarate was shown to give rise to these same products. Cell extract (150mg. of protein) was incubated at 30° with 0·1m-mole of 4-deoxy-5-oxoglucarate and 0·03m-mole of magnesium sulphate in 25ml. of 1·5m-moles of phosphate buffer, pH7·2. After 30min. the reaction was stopped by adding 5ml. of 3M-metaphosphoric acid, the precipitate was removed by centrifuging and the supernatant solution was incubated for 60min. at 30° with 40ml. of 0·1% (w/v) 2,4-DNP-hydrazine in 2M-hydrochloric acid. The precipitate was washed successively at 0° with 2M-hydrochloric acid, water and methanol, crystallized from ethyl acetate and dried at 50° under vacuum. DNP-hydrazones were similarly prepared from synthetic 2,5-dioxovalerate and from the reaction mixture obtained when D-glucarate was incubated with crude extract of *Ps. acidovorans*. The i.r.-absorption spectra showed that all absorption peaks were common to the three DNP-hydrazones, which also showed identical absorption in 0·3% (w/v) sodium ethoxide at 350–650nm. Details of these spectra are given by Dagley & Trudgill (1965).

A reaction mixture, similar to that of the last experiment but with smaller quantities of materials, was used to show that 2-oxoglutarate was formed from 10$\mu$moles of 4-deoxy-5-oxoglucarate when 15$\mu$moles of $NAD^+$ were present during the incubation. The DNP-hydrazone was not precipitated in this experiment, but was extracted into ethyl acetate, then into 10% (w/v) sodium carbonate and finally, after acidification, into a small volume of ethyl acetate. Some of this solution on examination by paper chromatography (two solvents) and t.l.c. (three solvents) was shown to contain a single DNP-hydrazone, that of 2-oxoglucarate; the remainder, on reduction with platinum oxide and hydrogen (Chamberlain & Dagley, 1968), gave glutamic acid, which was identified by paper chromatography in five solvents. High-voltage electrophoresis was used to confirm the identity of the DNP-hydrazone of 2-oxoglutarate and to show that glutamic acid was the sole ninhydrin-positive product of its reduction.

*Metabolism of D-glucarate and 4-deoxy-5-oxoglucarate.* If D-glucarate is first converted into 4-deoxy-

5-oxoglucarate by *Ps. acidovorans* the metabolism of these two compounds will be expected to follow similar courses. This was investigated for reaction mixtures (3ml.) in ten Warburg vessels, each of which contained 10mg. of crude extract protein, 10$\mu$moles of magnesium sulphate and 0·1m-mole of phosphate buffer, pH 7·2. In five flasks reactions were started by adding 10$\mu$moles of D-glucarate from side arms, and in the other flasks 10$\mu$moles of 4-deoxy-5-oxoglucarate were added. Reactions were stopped at intervals by tipping 0·2ml. of 3M-metaphosphoric acid from the centre wells of flasks. Released carbon dioxide was measured and amounts of 2,5-dioxovalerate formed were also determined after removing protein. These measurements were corrected for the small amounts of metabolites present in control flasks to which extract, but no substrate, was added; the largest correction was for carbon dioxide, of which 1·2$\mu$moles were released in 30min. When the substrate metabolized was 4-deoxy-5-oxoglucarate the amount of this compound was also determined. It was found that the production of either metabolite followed essentially the same course whichever substrate was decomposed (Fig. 2), thus confirming the status of 4-deoxy-5-oxoglucarate as an intermediate in the catabolism of D-glucarate by *Ps. acidovorans*.

*Enzymic decarboxylation of 4-deoxy-2-oxoglucarate.* The conversion of 1mole of 4-deoxy-2-oxoglucarate into 2,5-dioxovalerate occurs with the release of 1mole of carbon dioxide (Fig. 2). A direct decarboxylation would yield 4,5-dihydroxy-2-oxovalerate (3-deoxy-2-oxoarabonate), the L-isomer of which has been shown to be the precursor of 2,5-dioxovalerate when L-arabinose and L-arabonate are degraded by *Ps. saccharophila* (Stoolmiller & Abeles, 1966; Portsmouth, Stoolmiller & Abeles, 1967). This possibility was studied by means of a crude extract of *Ps. acidovorans* that converted D-glucarate into 2-oxoglutarate when $NAD^+$ was added (Fig. 3). In the presence of synthetic 4,5-dihydroxy-2-oxovalerate alone there was no significant reduction of $NAD^+$ by the extract; the further possibility that one isomer of the mixture might abolish the metabolism of the other was eliminated by showing that the D-glucarate-dependent reduction of $NAD^+$ was not affected by synthetic 4,5-dihydroxy-2-oxovalerate.

Although carbon dioxide is released from 4-deoxy-5-oxoglucarate when 2,5-dioxovalerate is formed, these experiments eliminate the possibility that the substrate undergoes a prior decarboxylation to give 4,5-dihydroxy-2-oxovalerate as an intermediate. Further, it is evident from the overall equation for the conversion that, in addition to carbon dioxide, water must also be lost from 4-deoxy-5-oxoglucarate. However, prior dehydration to give 2,5-dioxoadipate is also most unlikely; this is a symmetrical molecule,

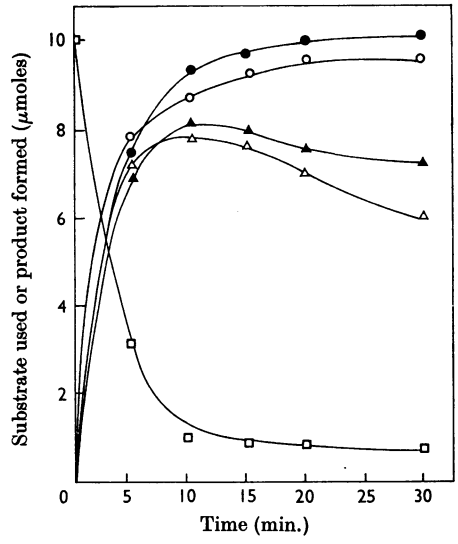



Fig. 2. Comparison of the course of formation of metabolites from D-glucarate and 4-deoxy-5-oxoglucarate. Each Warburg vessel contained 10$\mu$moles of the substrate and 10mg. of extract protein from *Ps. acidovorans*. Carbon dioxide evolved from: ○, D-glucarate; ●, 4-deoxy-5-oxoglucarate. 2,5-Dioxovalerate formed from: △, D-glucarate; ▲, 4-deoxy-5-oxoglucarate. □, Disappearance of 4-deoxy-5-oxoglucarate.

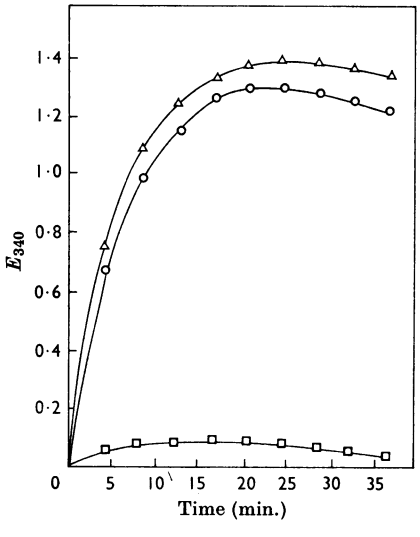



Fig. 3. Metabolism of D-glucarate and 4,5-dihydroxy-2-oxovalerate by a crude extract of *Ps. acidovorans*. Each cuvette contained, in 3ml., 50$\mu$moles of phosphate buffer, pH 7·2, 10$\mu$moles of $MgSO_4$ and 5$\mu$moles of $NAD^+$ with the following additions: ○, 0·5$\mu$mole of D-glucarate; □, 1$\mu$mole of 4,5-dihydroxy-2-oxovalerate; △, 0·5$\mu$mole of D-glucarate plus 1·0$\mu$mole of 4,5-dihydroxy-2-oxovalerate.

and Dagley & Trudgill (1965) showed that 80% of the carbon dioxide is released from C-1 during the catabolism of D-glucarate. The possibility remains that 4-deoxy-5-oxoglucarate is attacked by an enzyme that removes water and carbon dioxide in one concerted reaction.

We are most grateful to Dr D. C. Fish, who sent us a copy of his Ph.D. Thesis. We also thank the following for financial support: the Science Research Council (R. J.) and the U.S. Public Health Service (S. D.).

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