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Purification and Properties of D-4-Deoxy-5-oxoglucarate Hydro-lyase (Decarboxylating)

BY R. JEFFCOAT, H. HASSALL AND S. DAGLEY
Department of Biochemistry, University of Leeds, and Department of Biochemistry, University of Minnesota, St Paul, Minn. 55101, *U.S.A.*

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1. An enzyme extracted from *Pseudomonas acidovorans* was purified and shown to catalyse the simultaneous dehydration and decarboxylation of D-4-deoxy-5-oxoglucarate. It is proposed to name the enzyme D-4-deoxy-5-oxoglucarate hydro-lyase (decarboxylating), trivial name 'deoxyoxoglucarate dehydratase'. 2. No added cofactors were required, and the enzyme was inactivated when incubated with its substrate in the presence of sodium borohydride. Under these conditions the substrate and enzyme appeared to be bound covalently. 3. The action of the enzyme is readily explained if it is assumed that D-4-deoxy-5-oxoglucarate forms a Schiff base with a lysine residue in the enzyme.

Two successive dehydrations occur when D-glucarate is catabolized to 2,5-dioxovalerate by *Pseudomonas acidovorans*. We have now purified the enzyme that catalyses the second of these dehydrations and have confirmed the suggestion of Jeffcoat, Hassall & Dagley (1969) that carbon dioxide and water are lost simultaneously from one molecule of the substrate. The name D-4-deoxy-5-oxoglucarate hydro-lyase (decarboxylating) is proposed for this enzyme, with deoxyoxoglucarate dehydratase as a trivial name.

MATERIALS AND METHODS

Extraction and purification of deoxyoxoglucarate dehydratase. The preparation of extracts from *Pseudomonas acidovorans* grown with D-glucarate and the assay used for the substrate of the dehydratase (D-4-deoxy-5-oxoglucarate) were described by Jeffcoat *et al.* (1969). After treatment with protamine sulphate, extracts were brought to 30% saturation with $(NH_4)_2SO_4$ and the precipitated protein, being rich in D-glucarate dehydratase, was removed and used to prepare D-4-deoxy-5-oxoglucarate. The supernatant solution was brought to 50% saturation with $(NH_4)_2SO_4$ and the precipitate discarded; the protein precipitating at 75% saturation was then dissolved in 5mM-phosphate buffer, pH 7·2, and dialysed against the same buffer until free from NH_4^+ ions. The cell extract was applied to a column (10cm. × 1cm.) of DEAE-cellulose and washed with different solutions of NaCl in 5mM-phosphate buffer, pH 7·2: the enzyme was eluted completely with 0·1M-NaCl. The solution from the column was brought to 80% saturation with $(NH_4)_2SO_4$ and the precipitate was dissolved in phosphate buffer, pH 7·2, and dialysed. The extract (30mg. of protein/ml.) was then applied to a column (14cm. × 1cm.) of DEAE-cellulose that had equilibrated with 5mM-phosphate buffer, pH 7·2, and was eluted with a 0–0·15M-NaCl linear concentration gradient in the same buffer (total volume 200ml.). Protein was eluted throughout the first 50 fractions (4ml.) but the enzyme was confined to tubes 30–35 eluted at 0.08–0·10M-NaCl: these were pooled and brought to 80% saturation with $(NH_4)_2SO_4$. The precipitate was dissolved in phosphate buffer, pH 7·2, and dialysed to give a solution of enzyme purified 28-fold (Table 1). In our initial experiments, the enzyme was eluted stepwise from DEAE-cellulose to ascertain its behaviour with various solutions of NaCl. However, it was found later that batch elution with 0·1M-NaCl, namely steps 4 and 5 of Table 1, could be omitted without significant loss in the final purification achieved. These steps were therefore eliminated from subsequent procedures, and the dialysed solution of precipitate obtained at 75% saturation with $(NH_4)_2SO_4$ was applied directly to the DEAE-cellulose column and eluted with a linear concentration gradient as described. When the enzyme was examined by disc electrophoresis on gels of three pore sizes (7%, 10% and 15%; Davis, 1964) a single band was given in each system. Further details of methods for disc electrophoresis are described by Dagley, Geary & Wood (1968). The preparation also appeared to be homogeneous when examined in a Spinco model E ultracentrifuge (Fig. 1). The enzyme showed optimum activity on D-4-deoxy-5-oxoglucarate at pH 7·0–7·3 (Fig. 2). Rates of formation of 2,5-dioxovalerate were also shown to be optimum in this pH range.

Radiochemical methods. [U-^{14}C]Glucose and L-[4,5$_{(n)}$—^{3}H]lysine monohydrochloride were from The Radiochemical Centre, Amersham, Bucks. [U-^{14}C]Glucarate was prepared by the procedure used for [1-^{14}C]glucarate (Jeffcoat *et al.* 1969). Solutions containing ^{14}C from chrolmatography columns were counted in a Nuclear-Chicago gas-flow counter. ^{3}H, and ^{14}C in the presence of ^{3}H, were counted in a Beckman scintillation counter with a scintillation fluid of 5g. of 2,5-diphenyl oxazole and 100g. of naphthalene in 1l. of dioxan. C.p.m. were converted into d.p.m. by

Table 1. *Purification of deoxyoxoglucarate dehydratase*

The enzyme was assayed by two methods: from rates of disappearance of the substrate and from rates of evolution of CO_2 measured respectively as follows. (a) Solutions of 5 μmoles of D-4-deoxy-5-oxoglucarate in 2·8 ml. of 0·1 M-phosphate buffer, pH 7·2, were incubated with enzyme at 20° for 10 min., deproteinized by adding 0·2 ml. of 3 M-metaphosphoric acid and the amount of D-4-deoxy-5-oxoglucarate remaining was determined (Jeffcoat *et al.* 1969). One unit of enzyme is the amount that catalyses the removal of 1 μmole of 4-deoxy-5-oxoglucarate/hr. (b) The same incubation mixtures were used in Warburg respirometers, reactions were stopped at 10 min. by tipping 3 M-metaphosphoric acid and the CO_2 evolved was measured. On unit of enzyme is the amount that catalyses the release of 1 μmole of CO_2/hr. All values based on CO_2 evolution are given in parentheses.

Step	Method	Total protein (mg.)	Enzyme (total units)	Sp. activity (units/mg. of protein)	Yield (%)	Purification
1	Crude extract	3500	42000 (38500)	12	100	1
2	Protamine sulphate treatment	3130	45250	14	107	1·2
3	50–75%-satd.-$(NH_4)_2SO_4$ ppt., dissolved and dialysed	328	35730 (36460)	109 (111)	85 (94)	9·1 (10·1)
4	Absorbed on DEAE-cellulose, batch eluted with 0·1 M-NaCl	70	18000	256	43	21·4
5	80%-satd.-$(NH_4)_2SO_4$ ppt., dissolved and dialyzed	58	14700 (13090)	254 (225)	35 (34)	21·0 (20·4)
6	DEAE-cellulose chromatography, pooled fraction precipitated, dissolved and dialysed	13	4620 (4250)	355 (308)	11 (11)	28 (28)

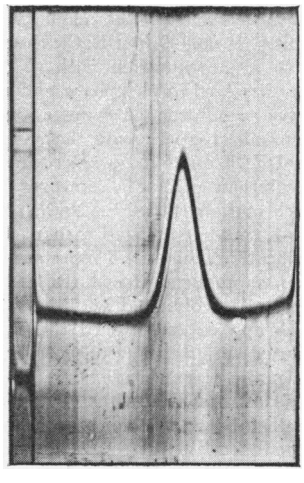


Fig. 1. Ultracentrifuge pattern for deoxyoxoglucarate dehydratase. The concentration was 12·4 mg. of protein/ml. in 0·15 M-KCl–0·05 M-KH_2PO_4, adjusted to pH 7·0 with NaOH. The photograph was taken at a phase-plate setting of 60°, 109 min. after reaching a speed of 50740 rev./min. at 20°. Sedimentation was from left to right.

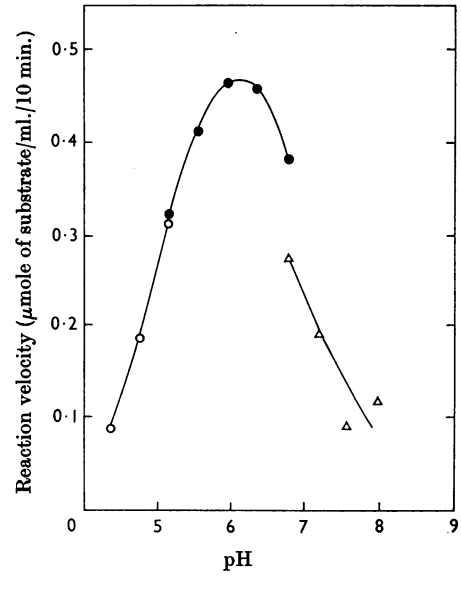


Fig. 2. Effect of pH on the activity of deoxyoxoglucarate dehydratase. Reaction mixtures (0·5 ml.) at 25° contained 0·5 μmole of D-4-deoxy-5-oxoglucarate, 2·5 μg. of enzyme protein and 30 μmoles of one of the following buffers: ○, sodium succinate; ●, KH_2PO_4–NaH_2PO_4; △, sodium borate. Reactions were stopped by adding 3 M-metaphosphoric acid after 10 min., when the amounts of D-4-deoxy-5-oxoglucarate that remained were determined. Rates measured in borate were about 30% lower than those in phosphate at the same pH.

means of internal standards, namely [^{3}H]nicotinic acid and [1-^{14}C]acetate. When only one radioisotope was present in the sample, the d.p.m. was calculated from the increase in c.p.m. after adding 1 μl. of standard. When ^{3}H and ^{14}C were both present this procedure was followed and the energy overlap of ^{14}C into the ^{3}H channel of the instrument was

also observed and allowed for. When radioactive enzyme was submitted to gel electrophoresis, ^3H and ^{14}C were located by freezing the polyacrylamide gels at $-40°$ and slicing into sections with a razor blade. Each piece was placed in a scintillation vial with 0·5ml. of 30% (w/v) H_2O_2 (Tishler & Epstein, 1968) and 0·33ml. of aq. 17M-NH_3, which was found to shorten the time required to dissolve the gel from 5hr. to about 1hr. and also served as a trap for any $^{14}CO_2$ released. The vial was sealed, kept at 50° for 60–90min. and cooled to room temperature before addition of 10ml. of scintillation fluid.

RESULTS

Enzymic decarboxylation of D-*4-deoxy-5-oxoglucarate.* Purified deoxyoxoglucarate dehydratase catalysed the formation of 1mole of 2,5-dioxovalerate from 1mole of D-4-deoxy-5-oxoglucarate with the simultaneous evolution of 1mole of carbon dioxide. 4,5-Dihydroxy-2-oxovalerate, which would arise from a direct decarboxylation of the substrate, is not an intermediate in the reaction (Jeffcoat *et al.* 1969). The evidence against a separate enzymic dehydration of deoxyoxoglucarate, which would precede the decarboxylation step, is less direct and is based on the fact that the expected product of dehydration, 2,5-dioxoadipate, possesses terminal carboxyl groups that would not be distinguished by the action of an enzyme. Accordingly the decarboxylation of such a proposed metabolite could not be reconciled with the observed preferential release of carbon dioxide from C-1 of D-glucarate (Dagley & Trudgill, 1965). However, the possibility that 2,5-dioxoadipate might be formed as a dissociable intermediate was directly eliminated when measurements of carbon dioxide evolution and deoxyoxoglucarate disappearance were used separately for assaying the enzyme during its purification. Thus the second of these procedures depends on the formation of 3-formylpyruvate when deoxyoxoglucarate is oxidized by periodate, a reaction that is not given by 2,5-dioxoadipate. Therefore if the last-named compound were formed and decarboxylated by separate enzymes the specific activities determined by the two assays might be expected to diverge during purification. It was found that specific activities were almost identical at each stage (Table 1); the two reactions were therefore apparently catalysed by a single enzyme.

Comparison of glucarate dehydratase and deoxyoxoglucarate dehydratase. Purification of deoxyoxoglucarate dehydratase failed to reveal a requirement for a coenzyme, and the enzyme was not significantly affected by 3mM-EDTA, which largely abolished the activity of glucarate dehydratase. This was shown by using a cell extract that contained both of the dehydratases: rates of reduction of NAD^+ by D-glucarate and D-4-deoxy-5-oxoglucarate were measured in the presence and absence of 3mM-EDTA (Fig. 3). Further, 90% of the activity of the purified deoxyoxoglucarate dehydratase was retained after dialysis for 24hr. against mM-EDTA in 50mM-phosphate buffer, pH7·2, followed by another dialysis for 12hr. against the same buffer containing no EDTA.

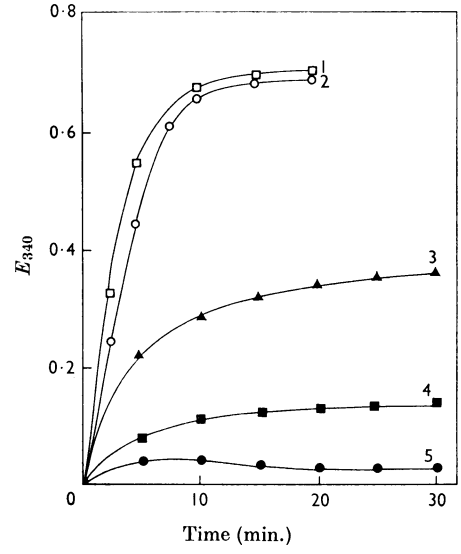


Fig. 3. Effect of EDTA on the metabolism of D-glucarate and D-4-deoxy-5-oxoglucarate. Each cuvette contained, in 3ml., 50μmoles of phosphate buffer, pH7·2, 5μmoles of NAD^+ and either 0·35μmole of D-4-deoxy-5-oxoglucarate (1 and 2) or 0·175μmole of D-glucarate (3,4 and 5). Reactions were started by adding 2·4mg. of crude-extract protein and extinctions were measured at 340nm. Other additions were: □ and ■, none; ○ and ●, 10μmoles of EDTA; ▲, 15μmoles of $MgSO_4$. The metabolism of D-4-deoxy-5-oxoglucarate (1) was unchanged by adding 15μmoles of $MgSO_4$.

Sodium (+)-tartrate inhibited glucarate dehydratase but had little effect on deoxyoxoglucarate dehydratase. Thus in a reaction mixture containing 0·8mg. of crude-extract protein, 3·6mM-magnesium sulphate and 0·09mM-glucarate the rate of reduction of 1·6mM-NAD^+ was inhibited 95% when 1·8mM-sodium (+)-tartrate was added. When glucarate was replaced by 0·05mM-deoxyoxoglucarate in a similar experiment, (+)-tartrate caused only 5% inhibition.

Treatment of deoxyoxoglucarate dehydratase with sodium borohydride. These differences in properties may be due to different types of binding of the substrates to their enzymes. Thus if D-glucarate were bound to its dehydratase at C-1 and C-2 it might be displaced by (+)-tartrate, which possesses carbon atoms in the same configuration; Mg^{2+} might serve the dual function of withdrawing

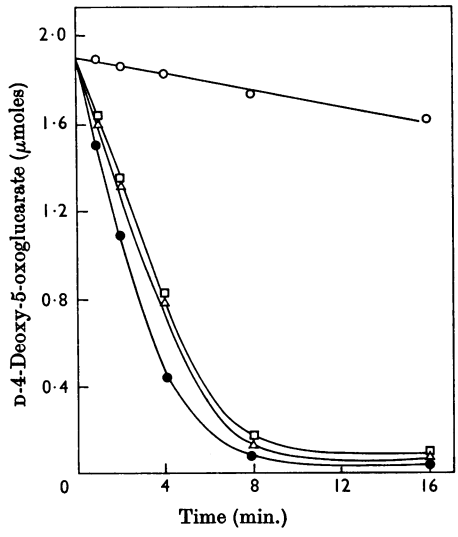
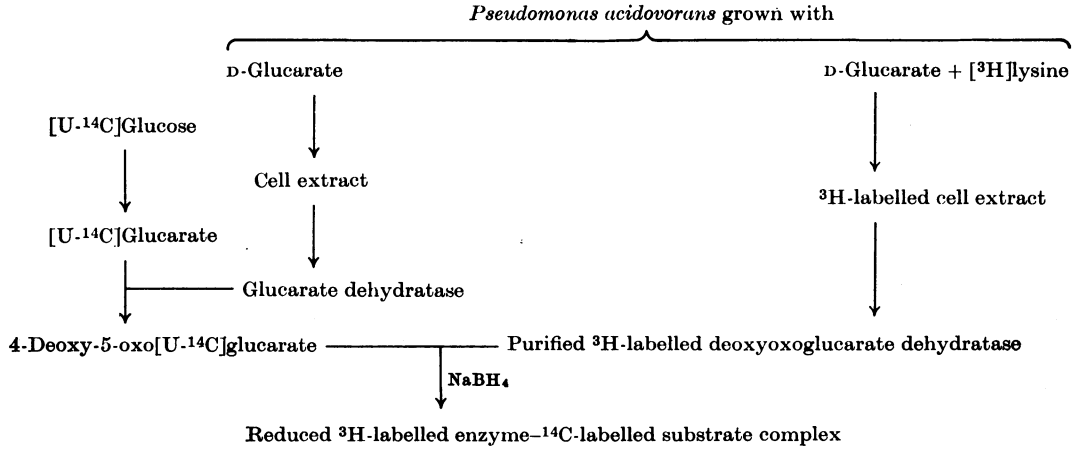


Fig. 4. Loss of enzymic activity when deoxyoxoglucarate dehydratase was incubated with sodium borohydride and D-4-deoxy-5-oxoglucarate. The enzyme (0·1 mg. of protein) in 1 ml. of phosphate buffer (50 μmoles, pH 7·2) was incubated at 25° for 30 min. with the following: □, no addition; △, 10 μmoles of $NaBH_4$; ○, 2 μmoles of D-4-deoxy-5-oxoglucarate plus 10 μmoles of $NaBH_4$. Mixtures were then dialysed against 50 mM-phosphate buffer, pH 7·2, for 18 hr., After dialysis, each solution was made up to 1·8 ml. with distilled water and 2 μmoles of D-4-deoxy-5-oxoglucarate were added. The amounts of this compound were then determined at intervals and are shown in the figure, together with measurements for a reaction mixture containing enzyme that had not been incubated and dialysed (●). The results for an experiment in which enzyme was preincubated with 2 μmoles of D-4-deoxy-5-oxoglucarate were identical with those when no addition was made.

electrons during the reaction and of helping to link C-1 to the enzyme site. On the other hand, D-4-deoxy-5-oxoglucarate possesses a carbonyl group through which it could bind to the enzyme by forming a Schiff base with a lysine residue and thereby provide a grouping to serve as an 'electron sink'. This suggestion received support from the observation that the rate of removal of D-4-deoxy-5-oxoglucarate by its dehydratase was immediately decreased from 0·68 μmole/min. to 0·05 μmole/min. when mM-sodium borohydride was added to the reaction mixture. It was then shown by the following experiment that the enzyme was inactivated by sodium borohydride only when its substrate was present. Equal quantities of purified enzyme were incubated for 30 min. with D-4-deoxy-5-oxoglucarate, with a mixture of D-4-deoxy-5-oxoglucarate and borohydride, and with borohydride alone; the respective solutions were then dialysed for 18 hr. to remove the added compounds and the kinetics of enzymic decomposition of D-4-deoxy-5-oxoglucarate were studied. It was found that the activity of the enzyme was scarcely affected by dialysis or by incubation with either borohydride or D-4-deoxy-5-oxoglucarate when these were added separately. However, the activity of the enzyme was decreased by about 95% when D-4-deoxy-5-oxoglucarate was added together with sodium borohydride (Fig. 4).

Covalent bonding of deoxyoxoglucarate dehydratase to its substrate in the presence of sodium borohydride. The object of the following experiment, summarized in Scheme 1, was to show that D-4-deoxy-5-oxoglucarate became bound covalently to the enzyme when the Schiff base was reduced. [U-^{14}C]Glucose of specific radioactivity 16·2 μc/mg. was mixed with



Scheme 1. Methods used to isolate the reduced enzyme–substrate complex formed between 4-deoxy-5-oxo-[U-^{14}C]glucarate and ^{3}H-labelled deoxyoxoglucarate dehydratase.

16·7mg. of glucose in 5ml. of water, the solution was freeze-dried and the residue oxidized to [U-^{14}C]glucarate and purified on Dowex 1 (X8; formate form) (Jeffcoat *et al.* 1969). D-Glucarate dehydratase, partially purified from an extract of glucarate-grown *Ps. acidovorans*, was used to convert [U-^{14}C]glucarate into 4-deoxy-5-oxo-(U-^{14}C]glucarate to serve as substrate for ^{3}H-labelled deoxyoxoglucarate dehydratase, which was prepared as follows. A culture (5l.) of *Ps. acidovorans*, which had received 1ml. (1mc) of L-[4,5$_{(n)}$ – ^{3}H]-lysine at the onset of the exponential phase, was harvested about 1hr. before the stationary phase was due and an extract was prepared from 5g. wet wt. of cells. Deoxyoxoglucarate dehydratase was purified from this extract as described in the Materials and Methods section except that pooled fractions from the DEAE-cellulose column were concentrated with Aquacide 1 (Calbiochem Ltd., London, W.1), dialysed against 0·01M-phosphate buffer, pH8·0, and rechromatographed on DEAE-cellulose. The radioactivity of fractions was measured and the elution pattern of radioactive material was shown to be identical with that of the enzyme. Fractions eluted at 0·07–0·10M-NaCl were pooled and concentrated to 3ml. with Aquacide 1 and a small portion (giving about 25000d.p.m.) was examined by disc electrophoresis. Radioactivity was confined essentially to one area that coincided with the single protein band.

The reduced reaction intermediate formed between enzyme and substrate was prepared by incubating 5μmoles ($6·6 \times 10^{6}$d.p.m.) of 4-deoxy-5-oxo-[U-^{14}C]glucarate and 3ml. of purified ^{3}H-labelled deoxyoxoglucarate dehydratase (10^{6}d.p.m.) with 0·4ml. of 0·1M-sodium borohydride in 4ml. of 0·1M-phosphate buffer, pH7·2, for 30min. The reaction mixture was then dialysed against 3·5l. of 0·05M-phosphate buffer, pH7·2, until excess of substrate was removed. Of the 5ml. of dialysed solution, 0·05ml. was subjected to disc electrophoresis, when radioactivity due to ^{14}C and ^{3}H in each of 30 sections of the sliced gel was counted as described in the Materials and Methods section. The bands for ^{14}C and ^{3}H were coincident (Fig. 5*a*); further, they occupied the same position as the protein of the sample as revealed by Amido Black. The remainder of the dialysed enzyme was chromatographed on a DEAE-cellulose column (10cm. × 1cm.) and eluted with a 0–0·2M-sodium chloride concentration gradient. Radioactivity was counted in 0·1ml. portions of each 2ml. fraction and ^{14}C was found to be eluted at the same concentrations of sodium chloride as was ^{3}H (Fig. 5*b*). It is evident that in the presence of sodium borohydride D-4-deoxy-5-oxoglucarate is attached to its dehydratase by a bond that survives the procedures of dialysis, electrophoresis and chromatography.

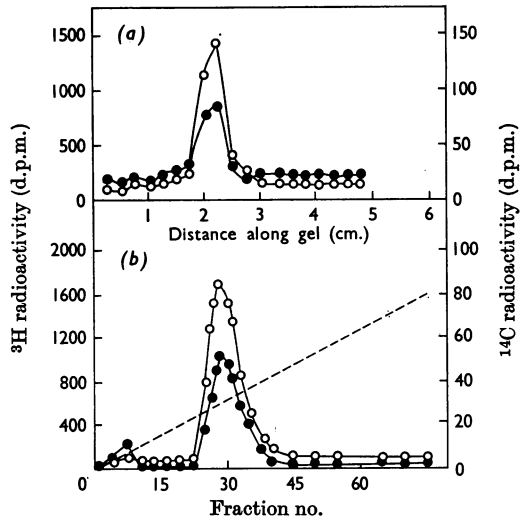
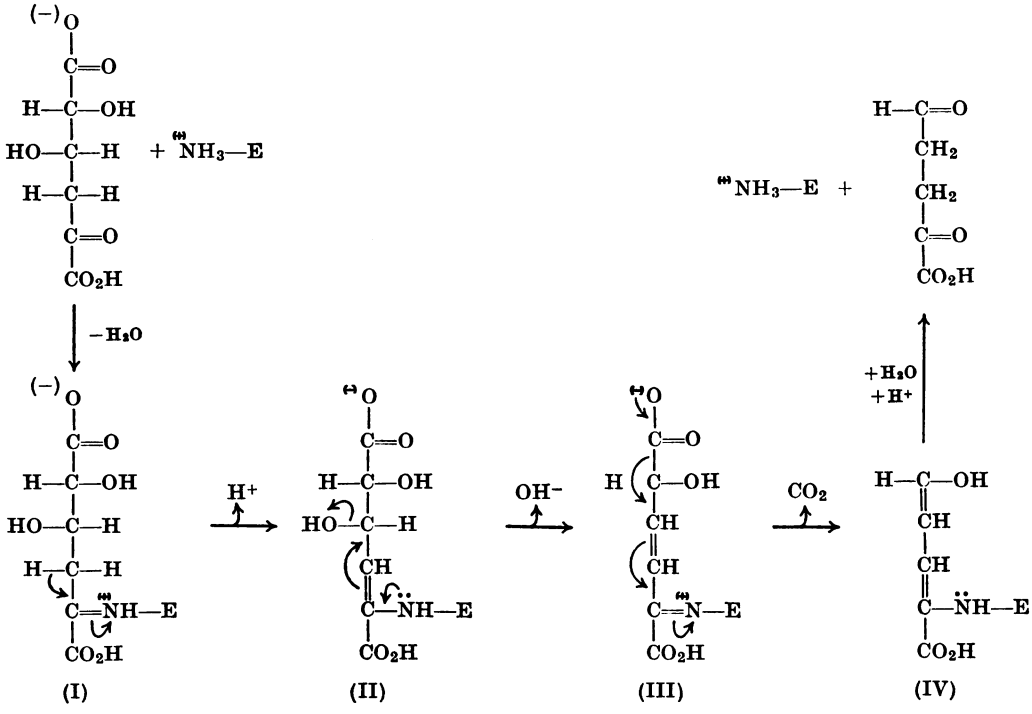


Fig. 5. Radioactivity in ^{3}H-labelled deoxyoxoglucarate dehydratase after incubation with D-4-deoxy-5-oxo-[^{14}C]-glucarate in the presence of $NaBH_4$. The enzyme was submitted to (*a*) disc electrophoresis, (*b*) chromatography on DEAE-cellulose. The broken line indicates a 0–0·2M-NaCl concentration gradient in 0·01M-phosphate buffer, pH8·0. Measurements of radioactivity: ○, ^{3}H; ●, ^{14}C.

DISCUSSION

Several decarboxylases are single proteins that exhibit a dual function: they decarboxylate a substrate and also simultaneously catalyse a second chemical transformation. Thus *threo*-D$_{s}$-isocitrate, L-malate and 6-phospho-D-gluconate are substrates that are oxidized as well as decarboxylated by their respective enzymes, namely isocitrate dehydrogenase (EC 1.1.1.41), malate dehydrogenase (decarboxylating) (EC 1.1.1.40), malate dehydrogenase (decarboxylating) (EC 1.1.1.39) and phosphogluconate dehydrogenase (decarboxylating) (EC 1.1.1.44). Kohn & Jakoby (1968) purified an enzyme that converts oxaloglycollate into D-glycerate by simultaneous reduction and decarboxylation. The present work appears to be the first concerned with a single enzyme that removes both water and carbon dioxide, and its dual action can be attributed to the shifts of electrons that are initiated when the substrate is bound to the enzyme (Scheme 2). We have demonstrated the nature of this binding by the method developed by Fischer, Horecker and co-workers (for reviews see Fisher, 1965; Horecker, 1962; Morse & Horecker, 1968), whereby the enzyme–substrate reaction intermediate, probably a Schiff base formed with a lysine residue of the enzyme, was reduced by sodium borohydride. Electrons are withdrawn from C-5 by the mechanism



Scheme 2. Suggested mechanism for D-4-deoxy-5-oxoglucarate hydrolyase (decarboxylating).

suggested in Scheme 2 so that a proton is lost from C-4 (I); elimination of water is then completed by expulsion of OH^- from C-3 (II). A similar withdrawal of electrons from C-5 of the dehydration product (III) will result in decarboxylation to give structure (IV), which would be expected to tautomerize and then release 2,5-dioxovalerate from its Schiff base, as described by Portsmouth, Stoolmiller & Abeles (1967) for the metabolism of 4,5-dihydroxy-2-oxovalerate (L-3-deoxy-2-oxoarabonate).

Several bacterial dehydratases and aldolases are similar in function and mechanism. Thus when D-glucarate is metabolized either by Pseudomonadaceae or by Enterobacteriaceae the first reaction is a dehydration; in pseudomonads a second molecule of water is then abstracted from the product, whereas enterobacteria catalyse an aldolase reaction (Trudgill & Widdus, 1966). *Pseudomonas fluorescens* likewise dehydrates 6-phosphogluconate and cleaves the reaction product to pyruvate and 3-phosphoglyceraldehyde (Entner & Doudoroff, 1952) by the action of 3-deoxy-2-oxo-6-phosphogluconate aldolase (EC 4.1.2.14) (Kovachevich & Wood, 1955). As to mechanism, aldolases and certain dehydratases are similar insofar as their reactions appear to be initiated by a shift of electrons that causes a proton to be labilized. The dehydratases for D-glucarate (Blumenthal & Fish, 1963; Dagley & Trudgill, 1965) and 6-phospho-D-gluconate (Meloche & Wood, 1964) require bivalent metal ions that probably provide electron sinks by means of which electrons can be withdrawn from C-1 to enable a proton to be withdrawn from C-2. The dehydratase of the present investigation, and that for L-3-deoxy-2-oxoarabonate (Portsmouth *et al.* 1967), depend on resonance in a Schiff base to disperse the negative charge attending the liberation of a proton from the carbon atom adjacent to the carbonyl group of the substrate. Electron sinks for aldolases are also provided either by bivalent metal ions or by Schiff base formation (Morse & Horecker, 1968); 3-deoxy-2-oxo-6-phosphogluconate aldolase is in the second of these two categories (Ingram & Wood, 1965). Deoxyoxoglucarate dehydratase removes carbon dioxide as well as water from its substrate, and it may be noted that a Schiff base is also formed during the enzymic decarboxylation of acetoacetate (Fridovich & Westheimer, 1962; Warren, Zerner & Westheimer, 1966). On the other hand some decarboxylases, such as oxaloacetate decarboxylase (EC 4.1.1.3), require metal ions. Ingram & Wood (1966) showed that the Schiff base-forming 3-deoxy-3-oxo-6-phosphogluconate aldolase also had the ability to decarboxylate oxaloacetate.

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