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Catabolism of D-Glucaric Acid to α-Ketoglutarate in *Bacillus megaterium*

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Crude cell-free extracts of D-glucarate-grown cells of *Bacillus megaterium* converted D-glucarate to α-keto-β-deoxy-D-glucarate (KDG). Charcoal-treated cell-free extracts or partially purified enzyme preparations converted KDG to an intermediate which was isolated and identified as 2,5-diketoadipate (DKA). This compound was synthesized, and the cell-free extracts of D-glucarate grown cells were found to catalyze the reduction of nicotinamide adenine dinucleotide (NAD) in its presence. In the absence of NAD, the same enzyme preparation catalyzed the decarboxylation of the DKA to α-ketoglutarate semialdehyde (KGS), whereas in the presence of NAD the KGS was subsequently oxidized to α-ketoglutarate by α-ketoglutarate semialdehyde dehydrogenase. Since galactarate-grown *B. megaterium* contains a galactarate dehydrase forming KDG, the complete pathway for the metabolism of D-glucarate or galactarate to α-ketoglutarate and CO_2 is now known in a gram-positive bacterium.

D-Glucaric acid, a naturally occurring dicarboxylic acid, serves as a sole source of carbon and energy for the growth of many microorganisms (10). Induced pathways of glucaric acid (GlcA) utilization have been studied and identified in many enterobacteria by Fish and Blumenthal (3, D. C. Fish, Ph.D. thesis, University of Michigan, Ann Arbor 1964), and by Trudgill and Widdus (17); in *Pseudomonas acidovorans* by Dagley and Trudgill (9); and in *Agrobacterium tumefaciens* by Chang and Feingold (Y. F. Chang, Ph.D. thesis, Univ. of Pittsburgh, 1966: reference 8). The pathway of D-glucaric acid degradation via α-keto-β-deoxy-D-glucarate (KDG) and tartronic acid semialdehyde (TAS), called the glycerate pathway (17), has been found in many members of *Enterobacteriaceae* (D. C. Fish, Ph.D. thesis, Univ. of Michigan, 1964; reference 3). A modified pathway of D-glucaric acid and galactaric acid utilization was originally identified in single strain of *P. acidovorans* (9) and subsequently in a wide variety of pseudomonads (17); this was termed the α-ketoglutarate pathway (17). The pathways for glucarate degradation through various intermediates have been reported only in gram-negative bacteria. A preliminary report of the conversion of GlcA and galactaric acids by *Bacillus megaterium* to the end product α-ketoglutarate (KG), with KDG as the first intermediate, was made by Blumenthal and Jepson (H. J. Blumenthal and T. Jepson, Bacteriol. Proc., p. 82, 1965).

The present study (portion of a thesis presented by B.S.S. in partial fulfillment of the requirements for the M. S. degree, Loyola University of Chicago, Ill., 1970) was initiated to identify all of the intermediate steps of D-glucaric acid catabolism in *B. megaterium* and to compare the GlcA metabolism in gram-negative and gram-positive organisms.

MATERIALS AND METHODS

Organism. The KM strain of *B. megaterium* was obtained from P. Gerhardt. This strain uses glucaric acid as a sole source of carbon and energy, and the ability to utilize glucaric acid is inducible. The organism was maintained on Trypticase soy agar (BBL) slants, and the culture was transferred every 15 days.

Chemicals. The monopotassium salt of glucaric (saccharic) acid, oxidized nicotinamide adenine dinucleotide (NAD), oxidized NAD phosphate (NADP), reduced NAD, tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, and bovine liver glutamate dehydrogenase (type I) were obtained from Sigma Chemical Co. (St. Louis, Mo.). Methyl-2,5-dimethoxy-tetrahydro-2-furoate was gift from P. W. Trudgill, and 2-keto-3-deoxy-DL-arabonic acid was a gift from R. Abeles. Anion exchange resin (Dowex-1-

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Cl^-; X8-200-400 mesh) was obtained from Baker Chemical Co., Phillipsburg, N.J. Enzyme-grade ammonium sulfate was obtained from Mann Lab., New York, N.Y. Enzymatically prepared KDG (5) was used after the calcium salt was converted into the sodium salt with cation exchange resin. Disodium D-glucarate, prepared from dicyclohexylammonium D-glucarate as described by Fish and Blumenthal (11), was used in enzyme experiments since commercial potassium acid D-glucarate is often impure. The ethyl succinate and ethyl oxalate used in the synthesis of diketoadipic acid were products of Eastman Chemical, Rochester, N.Y. Nuchar 190-N charcoal was obtained from Fisher Scientific Co., Pittsburgh, Pa.

Growth medium. The growth medium was (grams per liter): monopotassium glucarate, 8.0; Na_2HPO_4, 16.5; KH_2PO_4, 1.5; $(NH_4)_2SO_4$, 2.0; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.01; and $FeSO_4$, 0.0001. All the minerals were dissolved in 250 ml of deionized distilled water, and then the monopotassium glucarate was added. The pH of the medium was then adjusted to 6.9 with NaOH and made up to 1 liter. Erlenmeyer flasks (2 liter) containing 900 ml of the medium were inoculated with 100 ml of a 24-h culture and incubated on a rotary shaker at 37 C for 16 h. The cells were harvested by centrifugation, washed twice with 0.05 M KCl, and either stored at −20 C or used immediately.

Preparation of cell-free extracts. Each gram of cells was suspended in 10 ml of 0.05 M Tris-hydrochloride buffer, pH 7.5; for some studies, 0.05 M potassium phosphate buffer of the same pH was used. The cell suspension was disrupted for 10 min in a Branson S-75 sonic oscillator equipped with a cooling chamber to maintain the suspensions at 0 C. Cell debris and unbroken cells were removed by centrifugation at 20,000 × *g* for 10 min. The supernatant fluid was either used directly as the crude extract or treated with charcoal in a ratio of 1 mg of charcoal per mg of protein, in experiments in which the accumulation of intermediates was desired.

Ammonium sulfate fractionation. All fractionations were carried out at 0 to 4 C with saturated ammonium sulfate that had been neutralized with ammonia. A period of 15 min elapsed between the addition of ammonium sulfate and centrifugations. Successive 0 to 25%, 25 to 55%, and 55 to 75% saturation fractionations were performed. In each instance, the precipitate was dissolved in 0.05 M Tris-hydrochloride buffer, pH 7.5. In some instances, the 55 to 75% fraction was dissolved in potassium phosphate buffer. All ammonium sulfate fractions were used shortly after their preparation.

Chromatography. Whatman no. 1 chromatographic paper was used in the following solvent systems (vol/vol): 1-butanol-88% formic acid-water, 4:1:1.5 (A); 1-propanol-0.2 N NH_4OH, 3:1 (B); 1-butanol-ethanol-water, 52:32:16 (C); water-saturated *n*-butanol-88% formic acid, 95:5 (D); and 1-butanol-glacial acetic acid-water, 12:3:5 (E). Gelman chromatographic sheets (I.T.L.C. type SG) were used for thin-layer chromatography. Compounds possessing adjacent hydroxyl group were detected by the periodate-benzidine spray (19). Keto acids were detected with semicarbazide (16), and organic acids were detected with bromcresol green (D. C. Fish, Ph.D. thesis, University of Michigan, 1964). Free α-ketoglutarate semialdehyde (KGS) was detected under ultraviolet (UV) light by its white fluorescence.

Ion exchange chromatography. Dowex-1-Cl^- (X8; 200 to 400 mesh) anion exchange resin was converted to the formate form and used for the separation of various intermediates and end products in the incubation reaction mixtures. A column (2.5 by 25 cm) was packed with the resin, and the deproteinized incubation-reaction mixture was placed onto the column and allowed to flow into the resin. The sample was washed into the column with 10 ml of deionized, distilled water. When the last of the washed liquid just entered the resin, 10 ml of water was carefully layered on the top of the column and the gradient was started. The 1-liter reservoir containing 6 N formic acid was connected to the mixing vessel containing 175 ml of deionized, distilled water. A magnetic stirring bar within the mixing vessel provided adequate mixing. Fractions of 7 ml were collected in tubes placed within a refrigerated fraction collector with the column cooled by circulating fluid at 4 C.

Preparation of KGS. KGS was prepared by hydrolysis of methyl-2,5-dimethoxy-tetrahydro-2-furoate. The hydrolysis was performed by refluxing 1.0 g of the compound with 14 ml of 0.1 N H_2SO_4 for 15 min. The flask contents were immediately cooled and then neutralized with 5 N NaOH. Methanol and unreacted methyl-2,5-dimethoxy-tetrahydro-2-furoate were removed by being shaken with diethyl ether after which the ether phase was removed in a separatory funnel. The aqueous solution was then concentrated under vacuum at 30 C until a semisolid crystalline mass was obtained. This was stored at −20 C.

Preparation of DKA. 2,5-Diketoadipic acid (DKA) was prepared by the method of Haworth et al. (13). The low yield of DKA that had crystallized from the ether-insoluble fraction was dissolved in water and neutralized with NaOH before use as a substrate. The uncorrected melting point of the crystalline preparation of DKA was 219 C (reported melting point of the acid is 225–227 C), as determined on a Fisher-Johns melting point block. The difference in melting point probably is due to the impurities. The semicarbazone of this compound had an absorption maximum at 258 nm, indicating the presence of an α-keto acid.

Preparation of 2,4-dinitrophenylhydrazones. The KGS bis-2,4-dinitrophenylhydrazone (KGS-DNPH) was prepared by the method of Dagley and Trudgill (9), and the KG-2,4-dinitrophenylhydrazone (KG-DNPH) was prepared by a modification of the Cavallini and Frontali method (7) used by Fish (D. C. Fish, Ph.D. thesis, University of Michigan, 1964) for the preparation of pyruvate-DNPH. DKA bis-2,4-dinitrophenylhydrazone (DKA-DNPH) was prepared by incubating the solution with 0.1% DNP at 37 C for 1 h. The precipitate that formed was removed by centrifugation and washed twice with 2 N HCl and once with distilled water.

Analytical methods. The protein content of cell-free preparations was measured by the spectrophoto-

metric method of Waddell (18). Formation of $NADH_2$ from NAD at 30 C was measured spectrophotometrically in semimicro cuvettes of 1.5 ml capacity after the change of absorbance at 340 nm in a Gilford recording spectrophotometer (model 2000) with a temperature-controlled cuvette compartment. The semicarbazide test of MacGee and Doudoroff (16) was employed for the detection of α-keto acids, and a modified (2) thiobarbituric acid (TBA) test of Weissbach and Hurwitz (19) was used for the estimation of keto-deoxy sugar acids. Absorption spectra of the DNPH of KG, KDG, and other compounds were measured with a recording Beckman spectrophotometer (model DB) after dissolving the DNPH in 95% ethanol.

Formation and estimation of KDG. The conversion of GlcA to KDG was performed with the D-glucarate dehydrase in cell-free extracts of GlcA-grown cells. The reaction mixture contained (micromoles): D-glucarate, 2.5; Tris-hydrochloride buffer (pH 8.0), 40; $MgCl_2$, 2; and charcoal-treated, cell-free extract containing 1 mg of protein (about 0.01 U of D-glucarate dehydrase). After 10 min the reaction was terminated by the addition of 10% trichloroacetic acid. The protein precipitate was removed by centrifugation, and 0.2 ml of the supernatant fluid was analyzed by the TBA test. The KDG concentration was calculated by using the extinction coefficient of 60,000 for KDG at 551 nm (2).

Enzymatic conversion of KDG or KGS to KG. GlcA or KGS was incubated with cell-free extract or with partially purified preparations of enzyme in the case of KGS. The incubation mixture contained (micromoles) in a final volume of 2 ml: GlcA or KGS, 10; Tris-hydrochloride (pH 7.5), 160; $MgCl_2$, 12; and NAD, 10. The reaction was started by the addition of enzyme preparation containing 2 mg of protein to the incubation mixture equilibrated at 30 C. Samples of 0.3 ml were removed at 0-, 10-, 15-, 20-, 40-, and 80-min intervals. Each sample was placed in boiling water for 2 min, and the precipitate was removed by centrifugation. The KG formed in each sample was estimated enzymatically.

Estimation of KG. The formation of KG was measured by its conversion to L-glutamate with L-glutamic dehydrogenase in the presence of $NADH_2$. Since for each mole of KG 1 mol of $NADH_2$ is oxidized, the amount of $NADH_2$ consumed would be equal to the amount of KG. Reaction vessels contained (micromoles): $NADH_2$, 2.5; Tris-hydrochloride buffer (pH 7.5), 80; $(NH_4)_2SO_4$, 1; solution containing KG (50 μliters) and L-glutamic dehydrogenase (5 μliters of undiluted enzyme preparation) in a total volume of 1.5 ml.

KGS-dehydrogenase. KGS-dehydrogenase activity in cell-free extracts or in partially purified preparation of GlcA-grown cells (B.S. Sharma, M.S. thesis, Loyola University of Chicago, 1970) was demonstrated by measuring the rate of reduction of NAD at 340 nm. Each 1.2 ml of assay mixture contained: Tris-hydrochloride buffer (pH 8.5), 40 μmol; NAD, 2.5 μmol; and enzyme preparation. The reaction was started by adding 5 μmol of KGS. The rate of change in absorbance at 340 nm was measured for several minutes. The reaction rate was linear with time and enzyme concentration, using either crude extract or partially purified preparation. One unit of enzyme was defined as the quantity that catalyzed the reduction of 1 μmol of NAD per min. The extinction coefficient of NAD was taken as 6.2×10^3 in a 1-cm cuvette at 340 nm.

RESULTS

Compound I. Compound I is the product of reaction 1 (Fig. 1). Crude cell-free or charcoal-treated cell-free extracts of cells grown on GlcA were found to convert GlcA to compound I; extracts from glucose-grown cells had no activity. Compound I accumulated in an incubation reaction mixture containing, in a final volume of 12 ml: monopotassium glucarate, 40 μmol; Tris-hydrochloride (pH 7.5), 1.6 mmol; $MgCl_2$, 50 μmol, and 2.5 ml of charcoal-treated cell-free extract, containing about 20 mg of protein. After incubation for 30 min at 30 C, the reaction was terminated by placing the incubation vessel in a boiling-water bath for 2 min. The deproteinized solution was placed on a Dowex-1-formate column, and the intermediates were eluted with a 0 to 2 N formic acid gradient and then with a 2 to 6 N formic acid gradient. Samples of all the tubes were assayed for α-keto acids by the semicarbazide procedure, and the appropriate tubes were pooled and lyophilized. Usually compound I (P-3, Fig. 2) was eluted after compound II (P-2).

Keto-deoxy sugar acids react in the TBA test to give a chromogen with an absorption maximum at 551 nm (2, 19). The demonstration of a TBA-positive material after periodate oxidation of compound I with an absorption maximum at

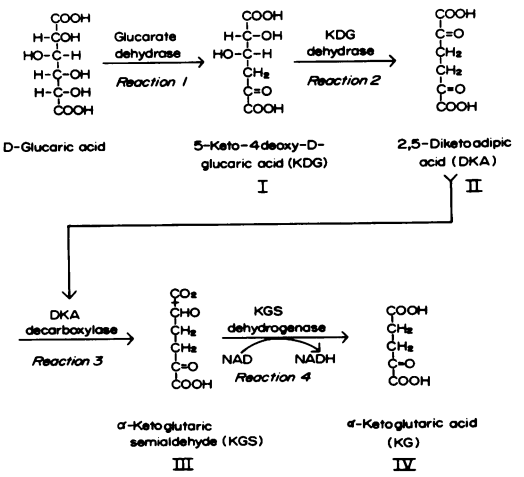

FIG. 1. *Proposed pathway for the catabolism of D-glucaric acid in Bacillus megaterium.*

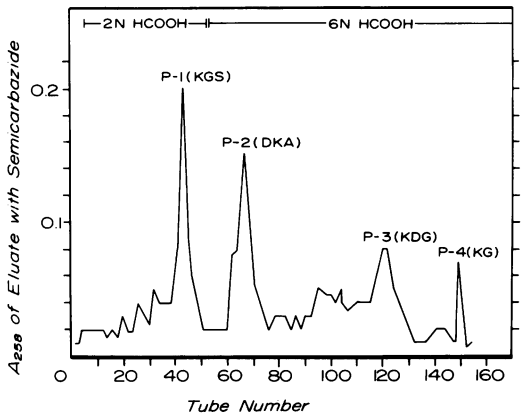

FIG. 2. *Isolation of intermediates in the reaction mixture by column chromatography on Dowex-1-formate. The enzymatically formed compounds from glucaric acid or keto-deoxy-D-glucaric acid were separated by Dowex-1-formate column using 0 to 2 and 2 to 6 N double formic acid gradients. Average fraction volume was 7.0 ml/tube. Samples of all tubes were assayed for α-keto acids by the semicarbazide procedure, and the appropriate tubes were pooled and lyophilized.*

551 nm (Fig. 3) implicated the existence of an α-keto-β-deoxy-sugar acid, with two adjacent hydroxyl groups. Since compound I is enzymatically converted to α-ketoglutaric acid, through a series of reactions involving a decarboxylation, it must contain at least six carbon atoms. The dehydration of GlcA can occur at the hydroxyl groups attached to carbon atoms 2,3 and/or 4,5 because only these structures will yield β-formyl pyruvate upon periodate cleavage and give a chromogen with an absorption maximum at 551 nm (2). That compound I is an α-keto acid is also supported by the fact that it gave a positive reaction in the semicarbazide test (16). Enzymes in the cell-free extract were able to form and subsequently utilize the KDG formed from GlcA (Fig. 4). The utilization of authentic KDG added to the incubation mixture also demonstrated the presence of an enzymatic system capable of metabolizing KDG (Fig. 4). The R_f values of authentic KDG and the TBA-positive compound prepared enzymatically from GlcA were identical in solvents A (0.44) and B (0.13) after paper chromatography and, in solvent A, upon thin-layer chromatography (0.82). The 2,4-dinitrophenylhydrazone prepared from authentic KDG or from the compound I had identical properties. On paper chromatography these prepared hydrazones migrated with the same R_f values (0.28 and 0.12), in solvents A and C. The 55 to 75% ammonium sulfate fraction of the cell-free extract catalyzed the reduction of

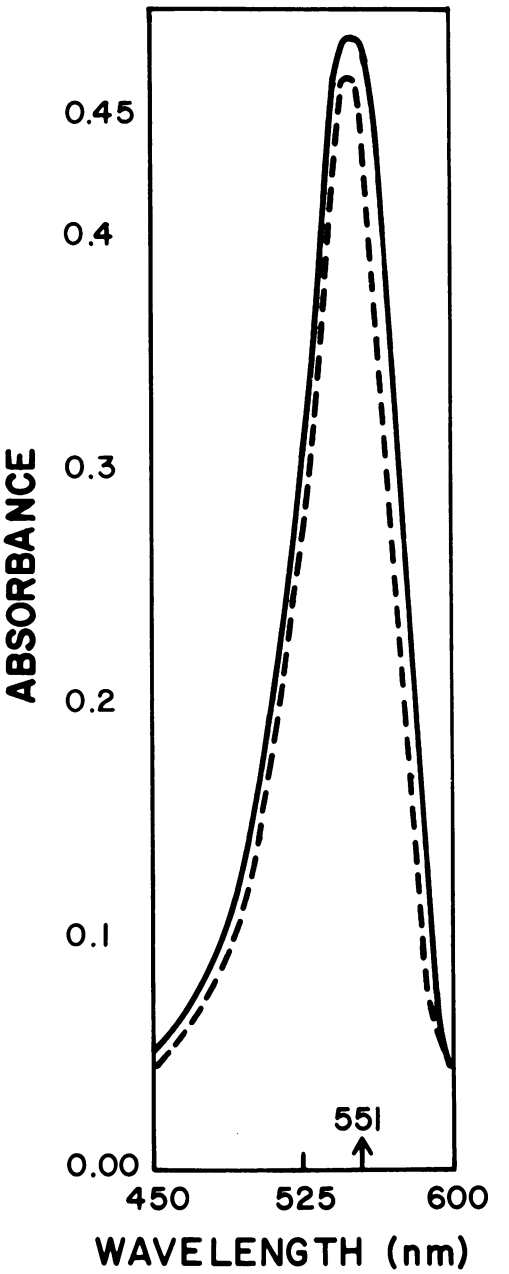

FIG. 3. *Absorption spectra of enzymatically prepared compound I (-----), and authentic KDG (——) in the periodate-thiobarbiturate test. Compound was prepared from GlcA. The reaction mixture contained (in micromoles): D-glucarate, 2; Tris-hydrochloride buffer (pH 8.0), 40; $MgCl_2$, 2; and charcoal-treated, cell-free extract containing 1 mg of protein.*

NAD to $NADH_2$ in the presence of KDG, providing further evidence that the KDG is an intermediate. Finally, authentic KDG was

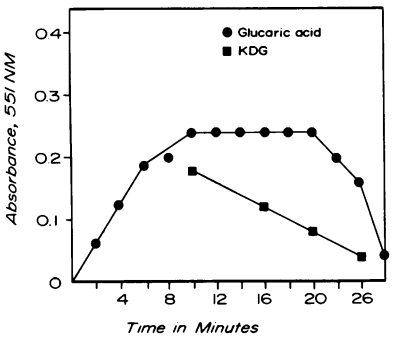

FIG. 4. *Formation and utilization of KDG with time. Curves:* (●), *KDG formation from GlcA. GlcA was incubated with a crude cell-free extract, as described in Materials and Methods, and KDG was determined in samples removed at various intervals.* (■), *KDG utilization. Authentic KDG was incubated with charcoal-treated cell-free extract, and its disappearance was measured at various time intervals by the TBA procedure. The incubation mixture contained (in micromoles): KDG, 2; Tris-HCl, 40; $MgCl_2$, 2; and charcoal-treated cell-free extract.*

shown to be converted by cell-free extracts to DKA and KGS, both intermediates in the pathway, and to the end product KG.

Although the *B. megaterium* glucarate dehydrase was not purified further, the formation of KDG and its subsequent conversion to KG suggest that glucarate dehydrase is involved in the metabolism of glucaric acid. In the many organisms previously examined, including *B. megaterium* (2, 3, 5), noninduced (glucose grown) cells possessed neither glucarate nor galactarate dehydrases. Both D-glucarate dehydrase and galactarate dehydrase are induced by growth of *B. megaterium* in either D-glucarate or galactarate (2, 6).

Compound II. Compound II was formed when KDG was incubated in the absence of NAD with charcoal-treated, crude, cell-free extract from the GlcA-grown cells. During paper chromatography of the enzymatically prepared compound in solvent B, there was always a spot present that possessed an R_f value different from KGS or KG and migrated with nearly the same position as KDG (II, R_f 0.1 to 0.15 and 0.5; KDG, R_f 0.13). However, it gave a negative reaction with the periodate-benzidine spray (KDG was positive with this spray, suggesting the conversion of KDG to compound II).

Preparation of compound II. Compound II was prepared by incubating KDG with charcoal-treated, crude, cell-free extract for 30 min at 30 C. The incubation mixture consisted of: KDG, 40 μmol; Tris-hydrochloride (pH 7.5), 3 mmol; charcoal-treated, crude, cell-free extract; 75 mg of protein. Since KGS dehydrogenase requires NAD for activity, its absence allowed accumulation of larger amounts of earlier intermediates. At the end of the incubation period, compound II was isolated from the reaction mixture by the same anion exchange chromatographic procedure employed for compound I.

Identification of compound II. Compound II gave a positive semicarbazide test, indicating the presence of an α-keto acid. It did not give a positive reaction with periodate-benzidine spray, indicating the lack of adjacent hydroxyl groups in the molecule.

If compound II is a direct decarboxylation product of KDG, KDA would be expected; if it is a dehydration product of KDG, it should be 2,5-diketoadipic acid. Compound II does not appear to be a decarboxylation product of KDG since the GlcA grown-cell did not have an enzyme system capable of converting authentic KDA to either KGS or KG. To show that compound II is a dehydration product of KDG, DKA was synthesized by the method of Haworth et al. (13). Cell-free crude extracts prepared from GlcA-grown cells were able to catalyze the reduction of NAD to $NADH_2$ in the presence of synthetic DKA (see Fig. 6). Compound II was identified as an α-keto acid which forms a DNPH derivative with an absorption spectrum different from the DNPH derivatives of KDG, KGS, or KG, but similar to that of DKA-DNPH prepared from synthetic DKA (Fig. 5). The keto acid accumulated during incubation of KDG with either charcoal-treated crude extract or a partially purified enzyme preparation in the absence of NAD. It was noted, however, that compound II-DNPH did not give a sharp spectrum. It seems likely that the isolated compound II was either unstable, as previously suggested by Chang (Y. F. Chang, Ph.D. thesis, University of Pittsburgh, 1966), or that some of the DKA was converted to its enol form during paper chromatography. After chromatography of either isolated compound II or the α-keto acid accumulated at various time intervals during incubations, two spots were obtained, one of which had an R_f value similar to that reported by Chang and Feingold (8) for their tentatively identified DKA. Chang and Feingold did not prepare the DNPH derivatives of DKA but identified succinic acid after the oxidative decarboxylation of DKA by hydrogen peroxide (8). In the presence of chemically prepared DKA, the NAD was slowly reduced to $NADH_2$ by the cell-free extract of D-glucarate-grown cells (Fig. 6). These data collectively suggest that DKA is the dehydration product of KDG.

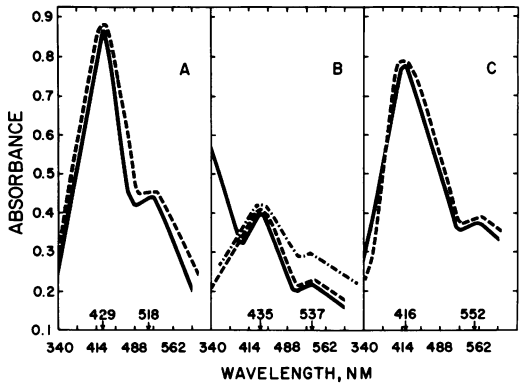

FIG. 5. *Absorption spectra of DNPH derivatives of authentic keto acids, keto acids isolated by column chromatography, and/or keto acids isolated and purified as DNPH derivatives directly from enzyme reaction mixtures without prior purification of the keto acids. Curves: (A) -----, authentic KGS-DNPH; ——, column isolated compound III-DNPH. (B) -----, authentic DKA-DNPH; -·-·-, column isolated compound II-DNPH; ——, compound II-DNPH formed from KDG without prior purification of compound II. (C) -----, compound IV-DNPH prepared from KGS without prior purification of compound IV; ——, authentic KG-DNPH. The incubation conditions for generating the keto acids are described in Materials and Methods, except that the reactions were allowed to proceed to completion. The DNPH samples were dissolved in 0.3% (wt/vol) sodium ethoxide or alkaline 95% ethanol to a proper concentration for absorbance measurement.*

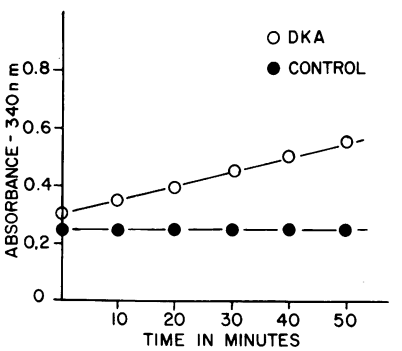

FIG. 6. *Reduction of NAD to $NADH_2$ by the cell-free extract of GlcA grown cells in the presence of chemically prepared 2,5-diketoadipic acid (DKA). Reaction mixture (1.2 ml) contained (in micromoles): DKA, 5; Tris-hydrochloride buffer (pH 7.5), 40; NAD, 2.5; $MgCl_2$, 4; and cell-free extract (7 mg of protein) (O——O); control lacking DKA (●——●).*

Formation of compound III. When KDG was incubated with charcoal-treated, cell-free extract in absence of NAD, an α-keto acid always accumulated in the incubation mixture which was eluted first from the Dowex-1-formate column. The enzymatic formation of compound III involves a decarboxylation step, and the site of decarboxylation seems to be carbon 1 since [*1-*^{14}C] glucaric acid yielded $^{14}CO_2$ when incubated with cell-free extract (Blumenthal and Jepson, unpublished results).

Preparation of compound III. Compound III was prepared by incubating KDG with charcoal-treated, cell-free extract for 30 min at 30 C. The incubation reaction mixture contained: KDG, 40 μmol; Tris-hydrochloride (pH 7.5), 1.6 mmol; and the enzyme preparation containing 80 mg of protein. The reaction was terminated by placing the reaction vessel in boiling water for about 2 min. After removal of the protein by centrifugation, α-keto acids in the supernatant were separated by column chromatography as described earlier. An elution pattern is shown in Fig. 2; peak 1 was identified as KGS.

Identification of compound III. The reaction produce of KDG formed from the enzymatic dehydration and decarboxylation of KDG was identified and characterized as follows: (i) conversion of compound III and authentic KGS to KG. Both synthetic KGS and compound III were converted to KG in the presence of NAD by a partially purified KGS-dehydrogenase preparation. After 1 h of incubation, 70 to 75% of the KGS was oxidized to KG measured by the glutamate dehydrogenase procedure described in Materials and Methods. (ii) The absorption spectrum of the DNPH of compound III in 0.3% (wt/vol) sodium ethoxide was identical to that of the bis-2,4-dinitrophenylhydrazone of authentic KGS (Fig. 5); both have absorption maxima at 429 and 518 nm. (iii) Compound III and authentic KGS migrated the same distance in solvents A and B on paper chromatography (III, R_f 0.31, 0.79, and 0.59; KGS, R_f 0.33, 0.8, 0.15, and 0.59) and in solvent A with thin-layer chromatography (III, R_f 0.65, KGS, R_f 0.65). (iv) Both the cell-free extract and a partially purified KGS-dehydrogenase were capable of reducing NAD to $NADH_2$ or NADP to $NADPH_2$ in the presence of chemically prepared KGS, indicating that KGS was oxidized to KG. These data are consistent with the identification of compound III as KGS.

Compound IV. Compound IV was demonstrated to be the oxidative product of KGS. The NAD- or NADP-dependent oxidation of KGS is catalyzed by the enzyme KGS-dehydrogenase.

Preparation of compound IV. Compound IV was prepared by incubating 40 μmol of sodium or potassium glucarate, 800 μmol of Tris-hydrochloride (pH 7.5), 50 μmol of $MgCl_2$, 12 μmol of sodium arsenite, and 15 μmol of NAD with crude, cell-free extract of GlcA-grown-cells. KG

TABLE 1. *Identification of α-ketoglutaric acid by paper and thin-layer chromatography*

Compound	Solvent[a]	R_f
Descending paper chromatography		
Authentic KG	A	0.65
Isolated compound (peak 4)	A	0.66
Reaction mixture[b]	A	0.66
Authentic KG	B	0.26
Reaction mixture[b]	B	0.20
Authentic KG	D	0.75
Isolated compound (peak 4)	D	0.75
Ascending paper chromatography		
Authentic KG	B	0.15
Reaction mixture[b]	B	0.10
Thin-layer chromatography		
Authentic KG	A	0.87
Reaction mixture[b]	A	0.865
The paper chromatography of peak 4 after conversion with L-glutamate dehydrogenase.		
Isolated peak 4 after conversion to L-glutamate	E and D	0.04 and 0.17, respectively
Authentic L-glutamate	E	0.04
Authentic L-glutamate	D	0.17
Co-chromatography	E	0.03
Co-chromatography	D	0.16

[a] See Materials and Methods.

[b] Samples were taken at different time intervals from the reaction mixture and contained (in micromoles): GlcA or KGS, 10; Tris-hydrochloride (pH 7.5), 160; $MgCl_2$, 12; sodium arsenite, 2.5; and cell-free extract containing 2 mg of protein.

was separated by anion exchange chromatography as already described.

Identification and characterization of compound IV. Compound IV had the same chromatographic behavior as authentic KG in three solvent systems (Table 1). The DNPH derivative of compound IV and of authentic KG-DNPH also showed similar behavior on ascending and descending paper chromatography. These derivatives had the same absorption spectra, with a maximum at 412 to 416 nm and a shoulder at 552 nm, in alkaline ethanol (Fig. 5).

Compound IV was enzymatically converted to glutamate by glutamate dehydrogenase and the glutamate formed was identified by chromatography and co-chromatography (Table 1).

Quantitative study of KG formation. When GlcA or KGS was incubated with cell-free extract and NAD, 70 to 80% of the GlcA was converted into KG in 80 min, as measured by the procedure described in the text. Partially purified KDG dehydrogenase was also found to catalyze the oxidation of KGS with about 70% of the synthetic KGS converted to KG in the presence of NAD in 1 h. The reaction, however, had not completely ceased at that time. The quantitative relationship between the NAD utilization and the formation of KG suggested that only one NAD-linked step is involved in the course of the conversion of GlcA to KG and that this step is the oxidation of KGS to KG.

DISCUSSION

The proposed pathway of D-glucaric acid (GlcA) utilization in *B. megaterium*, based on the data presented, is shown in Fig. 1. The first intermediate in GlcA utilization in *B. megaterium* is identified as KDG, a product of D-glucarate dehydrase. Although the *B. megaterium* D-glucarate dehydrase was not purified further, the formation of KDG and its subsequent conversion to KG strongly suggested that D-glucarate dehydrase is involved in the metabolism of D-glucaric acid. The presence of both GlcA and galactarate dehydrases was reported to exist in a variety of bacteria, including *B. megaterium* and *B. subtilis* (2, 5, 6). The GlcA dehydrase reaction appears to be the first step in the metabolism of GlcA by all bacteria studied, as well as at least one fungus, *Aspergillus niger* (B. Zakes, M.S. thesis, Loyola University of Chicago, 1969), regardless of whether the microbes subsequently use the glycerate pathway, as in enteric bacteria (2, 6, 17), or the α-ketoglutarate pathway, as in pseudomonads, *Agrobacterium* or *Bacillus* species (6, 8, 17).

In *Escherichia coli*, the KDG product of the glucarate dehydrase reaction is an approximately 85:15 mixture of 5-keto-4-deoxy- and 2-keto-3-deoxy-D-glucarate (2, 3), indicating that the preferred site for the dehydration is on the hydroxyl groups attached to carbon atoms 4 and 5 of GlcA. Similar results were obtained with *Pseudomonas acidovorans* (14). However, in *Agrobacterium tumefaciens* the preferred site (about 80%) for dehydration was at the hydroxyl groups attached to carbon atoms 2 and 3 (8). Although this problem was not specifically studied in the present report, the fact that very little activity appeared in KG after incubation

of [1-^{14}C]GlcA with *B. megaterium* extracts (Jepson and Blumenthal, unpublished results) suggests that *B. megaterium* resembles *E. coli* and *P. acidovorans*, yielding mainly 5-keto-4-deoxy-D-glucarate as the product of the glucarate dehydrase reaction. In *E. coli*, the galactarate dehydrase yields only 5-keto-4-deoxy-D-glucarate as the product, proving the asymmetry of galactarate (6).

The catabolism of GlcA in *B. megaterium* resembles that in *E. coli* in the first reaction. However, in *E. coli* the KDG is cleaved by KDG aldolase (3, 12) to give equimolar amounts of TAS and pyruvate, and the TAS is then subsequently reduced by TAS dehydrogenase to glycerate in the presence of $NADH_2$ (3). Jeffcoat et al. (14, 15) reported that KDG was converted to KGS by a single, purified enzyme preparation from *P. acidovorans* that simultaneously dehydrated and decarboxylated KDG yielding KGS. However, in *B. megaterium* as in *A. tumefaciens* (8), there is strong evidence for the existence of an additional intermediate, DKA before the formation of KGS from KDG. We have consistently observed that DKA was one of the four major α-keto acid peaks obtained while separating the intermediate compounds accumulating during GlcA catabolism in cell-free extracts by column chromatography (Fig. 2). Further support for the role of DKA as an intermediate was obtained by the reduction of NAD to $NADH_2$ in the presence of synthetic DKA (Fig. 6).

Dagley and Trudgill (9) reported that cell-free extracts of *P. acidovorans* converted D-glucarate into KGS only in the absence of NAD and that no KGS was detected in the presence of NAD. We also found that KGS accumulated only when NAD was absent and when the charcoal-treated, cell-free extracts or the partially purified enzyme preparations were used. On the other hand, in *A. tumefaciens* the conversion of the tentatively identified DKA to KGS and CO_2 was stimulated by NAD (8; Y. F. Chang, Ph.D. thesis, University of Pittsburgh, 1966).

The enzymatic formation of KGS in *B. megaterium* involves a decarboxylation step. As discussed earlier, the site of decarboxylation is likely to be carbon 1 of the original hexarate molecule. In *P. acidovorans* (14, 15) three quarters of CO_2 was found to arise from carbon 1, whereas in *A. tumefaciens* the site of the decarboxylation was not definitely established (Y. F. Chang, Ph.D. thesis, University of Pittsburgh, 1966).

The enzyme which catalyzes the oxidation of KGS to KG was identified both in *Pseudomonas* sp. and *A. tumefaciens*. The KGS dehydrogenase was also isolated, partially purified, and characterized from glucarate-grown cells of *B. megaterium* (B. S. Sharma, M.S. thesis, Loyola University of Chicago, 1970). Its properties closely resembled those of the KGS dehydrogenase described by Adams and Rosso (1) from either hydroxyproline- or glucarate-grown cells of *P. striata*.

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