JOURNAL OF BACTERIOLOGY, Apr. 1970, p. 85-96
Vol. 102, No. 1

D-Glucaric Acid and Galactaric Acid Catabolism by *Agrobacterium tumefaciens*

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Received for publication 13 January 1970

Cell-free extract (crude extract) of *Agrobacterium tumefaciens* grown on D-glucuronate or D-glucarate converts D-glucarate and galactarate to a mixture of 2-keto-3-deoxy- and 4-deoxy-5-keto-D-glucarate. These compounds are then converted by partially purified crude extract to an intermediate tentatively identified as 2,5-diketoadipate. The same enzyme preparation further decarboxylates this intermediate to α-ketoglutarate semialdehyde, which is subsequently oxidized in a nicotinamide adenine dinucleotide-dependent reaction to α-ketoglutaric acid. Since *A. tumefaciens* converts D-glucuronic acid to D-glucarate, a pathway from D-glucuronate to α-ketoglutarate in *A. tumefaciens* was determined.

Hexuronic acids are metabolized by a number of bacteria (4, 22). In addition, the metabolism of certain hexaric acids, the oxidation products of hexuronic acids, has been described in bacteria (5, 6, 11, 20). These pathways, although different, all bear a degree of resemblance.

Hexuronic acid metabolism by *Agrobacterium tumefaciens* was first reported by Zajic in 1959 (49). Subsequently Chang and Feingold showed that the first step in the utilization of D-glucuronic acid (GlcUA) and D-galacturonic acid (GalUA) by the organism involves their nicotinamide adenine dinucleotide (NAD)-linked conversion to the corresponding hexaric acids (9). In this paper is described the further metabolism of D-glucaric acid (GlcA) by *A. tumefaciens*.

MATERIALS AND METHODS

Bacteria. *A. tumefaciens* strain II BN-V6 was used. Cells were maintained on stock slants of yeast extract-potato-dextrose and were cultured and harvested as described earlier (9).

Chemicals and enzymes. The following materials were obtained as gifts: 4-deoxy-5-keto-D-glucaric acid (KDGA) from H. J. Blumenthal, Department of Microbiology, the University of Michigan, Ann Arbor; α-ketoglutaric acid semialdehyde (KGSA) from E. Adams, Department of Biological Chemistry, University of Maryland, Baltimore [also prepared from methyl 2,5-dimethyl-tetrahydro-2-furoate by the method of Murakami et al. (28)]; methyl 2,5-dimethyl-tetrahydro-2-furoate from P. W. Trudgill, Division of Biochemistry, University of Illinois, Urbana; and 2-keto-4,5-dihydroxy-valeric acid from R. H. Abeles, Graduate Department of Biochemistry, Brandeis University, Waltham, Mass.

Lactate dehydrogenase (EC 1.1.1.27; L-lactate: NAD oxidoreductase), crystalline suspension in ammonium sulfate from rabbit muscle-type 1, was purchased from Sigma Chemical Co., St. Louis, Mo. Glutamate dehydrogenase [EC 1.4.1.3; L-glutamate: NAD(P) oxidoreductase (deaminating)]; ammonium sulfate suspension from bovine liver was a product from Boehringer and Soehne GmBH, Mannheim, Germany. GlcUA-*U*-^{14}C and GlcUA-*6*-^{14}C were products of New England Nuclear Corp., Boston, Mass. All other chemicals were analytical grade commercial products unless otherwise stated.

Compounds prepared. ^{14}C-labeled GlcA was synthesized from GlcUA-*6*-^{14}C or -*U*-^{14}C either by the method of DeMoss (13) or by incubation with hexuronate dehydrogenase in the presence of NAD (9). The resulting ^{14}C-labeled GlcA was purified by paper electrophoresis in 0.2 M ammonium acetate buffer, *p*H 5.8. KDGA-*6*-^{14}C and -*U*-^{14}C were prepared with partially purified hexarate dehydratase. The 2,4-dinitrophenylhydrazone (DNPH) of glyoxylate was prepared and purified by a modified method of Cavallini et al. (8). KDGA-DNPH was prepared and purified by the method of Cavallini and Frontali (7) with some modification. KGSA-bis-DNPH was prepared by the method of Dagley and Trudgill (11).

Preparation of bacterial extracts. Cell-free extracts for enzyme study were prepared from Tris buffer-washed cells of a 24-hr culture of *A. tumefaciens* as described previously (9).

Enzyme purification. All operations were performed at 0 to 4 C; in ammonium sulfate precipitations, 30 min elapsed between addition of ammonium sulfate and centrifugation. A typical purification was as follows. To 46 ml of cell-free extract prepared from GalUA-grown cells by sonic disintegration (9) was added with stirring 4.6 ml of ice-cold 1 M $MnCl_2$. The

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mixture was stirred for 1 hr, after which the precipitate was removed by centrifugation. The supernatant fluid was brought to 35% ammonium sulfate saturation with 10 g of solid enzyme-grade ammonium sulfate (Mann Research Laboratories, New York, N.Y.), and the precipitate was centrifuged and discarded. The supernatant liquid was then brought to 55% ammonium sulfate saturation by addition of 6.23 g of ammonium sulfate, and the precipitate was centrifuged down and dissolved in 10 ml of 0.02 M Tris buffer (*p*H 7.5), 0.01 M in ethylenediaminetetraacetate (EDTA). This solution was dialyzed overnight against 0.005 M Tris buffer (*p*H 7.5), 0.001 M in EDTA and 0.005 M in 2-mercaptoethanol. The dialyzed enzyme solution was adsorbed successively three times with calcium phosphate gel (gel-protein, 20:1, w/w); each time after centrifugation the supernatant solution was discarded. The combined calcium phosphate gel precipitate was then washed with 10 ml of 0.02 M Tris buffer (*p*H 7.5), and successively extracted with 10 ml each of 1 and 2% ammonium sulfate solution. Most of the enzyme activity was present in the 2% extract. When this preparation was incubated with GlcUA in the presence of NAD, only two reaction products, corresponding in mobility to GlcA and KDGA, were detected in the reaction mixture by paper electrophoresis. The purification is summarized in Table 1.

Enzyme assays. A unit of enzyme is defined as that amount of enzyme which catalyzes the transformation of 1 μmole of substrate per min under the conditions of the particular assay at 30 C.

Hexarate dehydratase (D-hexarate hydro-lyase) activity was assayed by determining the rate of keto-deoxyhexaric acid (KDHA) formation from hexaric acid. The reaction vessel contained: hexaric acid, 4 μmoles; Tris buffer (*p*H 8.0), 50 μmoles; and enzyme preparation, 25 μliters (about one unit) in a total volume of 0.8 ml. When hexuronic acid was used as substrate, 0.4 μmole of NAD was added to the reaction mixture. [This is an assay employing the coupled action of hexuronate dehydrogenase (9) and hexarate dehydratase. Since high levels of hexuronate dehydrogenase were present in all enzyme preparations, hexarate dehydratase was rate-limiting for the over-all conversion of hexuronic acid to KDHA.] The reactions, which were started by the addition of enzyme, were terminated after 10 min by the addition of 0.2 ml of 25% trichloroacetic acid. The difference between the assay value obtained with the complete system and the value for the control (in which substrate was added after the trichloroacetic acid) was used to calculate the hexarate dehydratase activity. After removal of protein by centrifugation, 0.2-ml samples were added to 0.25 ml of 0.025 N HIO_4 in 0.125 N sulfuric acid. After 20 min at room temperature, 0.5 ml of 2% sodium arsenite in 0.5 N HCl was added with shaking; 2 min later, 2 ml of 0.3% thiobarbituric acid (*p*H 2.0) was added, the mixture was mixed and heated at 100 C for 10 min and cooled to room temperature. The KDHA concentration was calculated from the absorbance at 548 nm, employing a molar absorptivity of 60,000 for KDHA (6).

KGSA dehydrogenase (2-oxoglutarate-semialdehyde:NAD oxidoreductase) activity was assayed by measuring the rate of absorbancy increase at 340 nm caused by the reduction of NAD by the enzyme in the presence of KGSA. Reaction vessels contained (in μmoles): KGSA, 2; $MgCl_2$, 4; NAD, 0.4; Tris buffer (*p*H 8.0), 50; and enzyme preparation, 0.1 ml (about 10^{-3} unit) in a total volume of 1 ml.

Chromatography. Paper chromatography was carried out on Whatman no. 1 or 3MM filter paper. For thin-layer chromatography, Eastman Chromagram sheet, type K301-R (Distillation Products Industries, Division of Eastman Kodak Co.) and Silica Gel G plates were used (37). The following solvent systems (v/v) were employed: water-saturated *n*-butyl alcohol–formic acid, 95:5 (A); *n*-butyl alcohol–*n*-propanol–water, 10:7:3 (B); 96% ethyl alcohol–25% ammonia–water, 100:16:12 (C); 2-butyl alcohol–30% ammonia, 150:60 (D); 2-butyl alcohol–formic acid–water, 150:30:20 (E); *n*-butyl alcohol–water–glacial acetic acid, 25:25:6 (chamber saturated with the lower phase of the solvent) (F); 95% methanol (G); *t*-butyl alcohol–80% formic acid–water, 4:1:1.5 (H); 80% aqueous phenol (I); *n*-propanol–0.2 N NH_4OH, 3:1 (J); *t*-butyl alcohol–formic acid–water, 70:15:15 (K); ethyl alcohol–water, 77:23 (L); phenol–water–28% ammonia, 100:20:0.03 (M); phenol–water–formic acid, 75:25:1 (N); ether–formic acid–water, 5:2:1 (O); benzene–methanol–glacial acetic acid, 90:16:8 (P); benzene–dioxane–glacial acetic acid, 90:25:4 (Q).

Paper electrophoresis. Paper electrophoresis was carried out on a high-voltage electrophoretor (model D, Gilson Medical Electronics) on Whatman no. 1 or 3MM paper in 0.2 M ammonium acetate buffer, *p*H 5.8 (17). The electrophoretic mobility of individual compounds is given relative to the mobility of D-glucuronic acid (M_{ga}).

Detection and location of sugar acids and radioactive compounds. Sugar acids on paper chromatograms, paper electrophorograms or thin-layer chromatograms generally were detected with the periodate-benzidine reagent (18) unless otherwise stated. ^{14}C-labeled radioactive compounds were located by means of autoradiography using Eastman No-Screen X-Ray Film (Eastman Kodak Co.). Radioactivity was counted in a Tri-Carb liquid scintillation spectrophotometer (Series 300; Packard Instrument Co., Inc., Downers Grove, Ill.) with a scintillation liquid containing 4 g of 2,5-phenyl-oxazole and 0.1 g of 1,4-bis-2(4-methyl-5-phenoxazolyl)-benzene per liter of toluene.

Protein determination. Protein was estimated by the ultraviolet spectrophotometric method of Waddel (42).

Concentration of solutions. All solutions were concentrated by vacuum evaporation in a rotary evaporator at a temperature not exceeding 45 C.

RESULTS

Hexarate dehydratase. Crude cell-free extracts from cells grown on GalUA, GlcUA, GalA, or

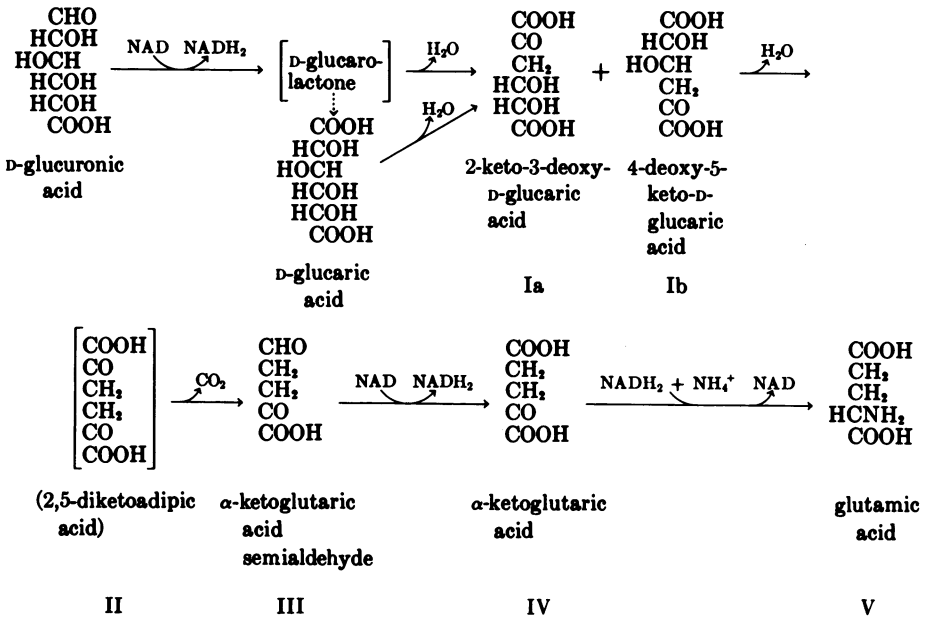

FIG. 1. *Proposed pathway of D-glucuronic acid metabolism by cell-free extract of Agrobacterium tumefaciens Compounds in parentheses are only tentatively identified; broken arrow indicates possible nonenzymatic reaction*

GlcA can convert GalA and GlcA to I (Fig. 1). (The products of metabolism of GalA and GlcA by *A. tumefaciens* will be designated by Roman numerals corresponding to the order in which they occur in the metabolic sequence.) Crude extracts could catalyze the conversion of GalUA, GlcUA, GalA, and GlcA to compound I; with the uronic acids, NAD was required. In general, higher yields of I (as judged by the relative size of spots on electrophorograms) were obtained when the uronic acids were used as substrate. Attempts to purify crude extracts invariably led to loss of their ability to catalyze the reaction of GalA or GlcA. NAD could not restore the lost activity with GalA and GlcA; however, in the presence of NAD, GalUA and GlcUA were still converted to I by the partially purified enzyme. We were unsuccessful in our attempts to stabilize the enzyme by shaking the cells with 0.3 mmole of ferrous sulfate per liter of culture for 10 min before harvesting by the method of Blumenthal and Jepson (6) for preparation of hexarate dehydratase from *Escherichia coli*. When GlcA-*U*-^{14}C alone was incubated with crude extract compound I could not be detected among the reaction products unless a large amount of nonradioactive KDGA was added to the reaction mixture as a trapping agent. When GlcA-*U*-^{14}C was incubated with partially purified enzyme preparations in the presence of nonradioactive GlcUA, no radioactive product was detected, indicating that GlcUA is not an activator for the reaction.

It was proposed by Marsh (27) that the D-glucuronolactone dehydrogenase from rat liver acts on glucofuranurono-(6 → 3)-lactone to produce the glucaro-(1 → 4)-(6 → 3)-dilactone, which subsequently decomposes spontaneously in aqueous solution to glucaro-(1 → 4)-lactone, glucaro-(6 → 3)-lactone and dicarboxylic GlcA. To test whether the stable forms of GlcA (presumably the monolactone and dicarboxylic forms) are involved in the conversion of GlcUA to compound I catalyzed by the partially purified enzyme system, an isotope dilution experiment was performed. A portion of GlcUA-*U*-^{14}C (1.7×10^{-3} μc) was mixed with 1 μmole of Tris buffer (*p*H 8.0), 0.4 μmole of $MgCl_2$, 0.4 μmole of NAD, and 5 μliters of 2% ammonium sulfate extract from the calcium phosphate gel step (Table 1; total volume, 30 μliters). The reaction mixture was incubated in the presence and absence of 0.4 μmole of nonradioactive GlcA [mostly the dicarboxylic acid form, as judged by electrophorograms (27)], in a sealed capillary tube at 25 C. The reaction was terminated at 0, 5, 10, 30, and 120 min by heating at 100 C for 3 min, and the products were examined by paper

TABLE 1. *Purification of hexarate dehydratase*

Fraction	Volume	Activity[a]	Protein	Specific activity	Yield
	ml	*units/ml*	*mg/ml*	*units/mg*	%
(I) Crude extract	46	35.4	35.7	1	100
(II) 35–55% Ammonium sulfate (dialyzed)	15	100	20	5	92
(III) 2% Ammonium sulfate extract from calcium phosphate gel	10	26.8	2.25	12	16.5

[a] Enzyme activity was assayed in the presence of 0.4 μmole NAD per assay using GalUA as substrate (cf. "Enzyme assays").

electrophoresis and autoradiography. There was no observable difference between the reaction mixtures with added GlcA and those without, indicating that the formation of I is not affected by the presence of nonradioactive GlcA. In addition, when GlcA-*U*-^{14}C (either chemically or enzymatically prepared) was incubated with the same enzyme preparation, no reaction products were detected.

Preparation of I. I could be prepared from either hexuronic acid or hexaric acid. When GalUA or GlcUA was used as substrate, increased yields of I could be obtained by coupling the activity of the 2% ammonium sulfate extract (Table 1) with lactic dehydrogenase and pyruvate in the presence of a catalytic amount of NAD (9). When GalA or GlcA was used as substrate, it was necessary to use crude cell-free extract, but no exogenous NAD was required. The reaction mixture contained 0.28 mmole of substrate, 1.15 mmoles of potassium pyruvate, 0.4 mmole of $MgCl_2$, 0.01 mmole of NAD, 3.0 mmoles of Tris buffer (*p*H 8.0), 1 ml of enzyme solution (about 30 units), and 0.1 ml of lactic dehydrogenase (about 100 units; potassium pyruvate, NAD, and lactate dehydrogenase were omitted when crude extract was used) in a total volume of 40 ml. After incubation at 25 C for 12 hr in the presence of several drops of toluene, the reaction mixture was treated with charcoal (when NAD was present) and 10 ml of 25% trichloroacetic acid and held at 0 to 4 C for 30 min. The mixture was filtered, and the filtrate was extracted with diethyl ether to remove trichloroacetic acid, concentrated to 1/10 its volume, and added to a 1.5 by 25 cm Dowex (formate form, 100 to 200 mesh) column (1 × 1). The column was eluted at the rate of 3 ml/min with a formic acid gradient obtained with a mixing vessel containing 200 ml of water and a reservoir containing 6 N formic acid. Fractions of 3 ml were collected. The order of emergence of relevant compounds from the column is: hexuronic acid, hexaric acid, pyruvic acid, I [detected by the periodate-benzidine method (17) and the periodate-thiobarbiturate test of Warren (44) combined with paper electrophoresis]. The fractions containing I were pooled and lyophilized to yield a light yellow solid. Radioactive I was prepared by incubating GlcUA-*U*-^{14}C or GlcUA-*6*-^{14}C (10 μc) with 2% ammonium sulfate extract (0.1 ml, Table 1) and NAD (1 μmole) in 1 ml of 0.1 M Tris buffer (*p*H 8.0). After 4 hr at 25 C, the mixture was held at 100 C for 5 min, protein was removed by centrifugation, the supernatant fluid was concentrated to 1/10 its volume, and radioactive I was isolated by paper electrophoresis.

Characterization of I. Reaction products formed from the enzymatic dehydration of GlcA and GalA were characterized as follows.

(i) *Reducing sugar and specific color test.* Compound I readily reduced Benedict reagent and alkaline triphenyltetrazolium chloride spray (39), suggesting the presence of an aldehyde or ketone function vicinal to an OH group. Compound I showed fluorescence under ultraviolet light after spraying with semicarbazide-acetate (41) or *o*-phenylenediamine reagent (12). Since these reagents are specific for α-keto acids (12, 41), these results suggest that the carbonyl group of I is a ketone rather than an aldehyde group. Periodate-benzidine color spray (17) gave a distinctive orange color reaction with I which showed up before the blue background developed. Authentic KDGA gave identical results with this spray. Compound I reacted weakly with silver nitrate spray reagent (39) and not at all with urea phosphate color reagent (47).

(ii) *Paper electrophoresis and paper chromatography.* I had the same electrophoretic mobility at *p*H 5.8 as authentic 4-deoxy-5-keto-D-glucaric acid (M_{gal} 1.48). I prepared from either GalA or GlcA had the same R_F (0.55 to 0.59) as that of authentic KDGA in solvent H, which separates KDGA, GlcA (R_F 0.39 to 0.42), and GlcUA (R_F 0.4 to 0.45) well.

(iii) *DNPH derivatives and periodate oxidation.* The DNPH of nonradioactive I had the same R_F as authentic KDGA-DNPH in solvent G (R_F 0.5); both compounds gave a brown color with 0.5 N alcoholic NaOH spray. Periodate cleavage of I under mild conditions (room temperature, 0.125 N H_2SO_4, 40 min; ratio of periodate to KDGA, 4), followed by conversion to the DNPH, yielded glyoxylate-DNPH (solvent G, R_F 0.65, brick-red color reaction with alkaline spray) and an unknown (presumably β-formylpyruvate-DNPH, solvent G, R_F 0.79, yellowish-gray color reaction with alkaline spray), in addition to a trace of the DNPH of unconverted compound. Identical results were obtained with KDGA. When uniformly ^{14}C-labeled I was mixed with authentic unlabeled KDGA and oxidized with periodate, the DNPH derivatives of the resulting products showed co-chromatography in solvent G.

(iv) *Periodate-thiobarbiturate · color reaction.* A positive periodate-thiobarbiturate test suggests that I possesses a deoxy moiety inside the carbon chain (43). In the case of deoxyribose or deoxyglucose, the malondialdehyde formed in this oxidation is coupled with thiobarbituric acid to give a chromogen with a λ_{max} at 532 nm. Ketodoxy sugar acids, which contain a -CHOH-CH_2-CO-COOH structure, react in the periodate-thiobarbiturate test to give a chromogen with a λ_{max} at 545 to 550 nm rather than 532 nm (45). In Fig. 2, the absorption spectrum of the periodate-thiobarbiturate reaction product of I prepared from GlcUA is shown together with the spectra of the products obtained from authentic KDGA and 2-deoxy-D-glucose. The reaction product of I prepared from GalUA, GlcA, or GalA showed an identical spectrum with an absorption maximum at 548 nm. The α-keto-β-deoxy configuration of I was further confirmed by the absorption spectrum of the product obtained by treating borohydride-reduced I with the periodate-thiobarbiturate reagent. When KDGA was reduced with an excess of aqueous sodium borohydride at *p*H 6.5 [30 min at room temperature, excess borohydride destroyed by acidification to *p*H 3 with Dowex 50-(H^+), boric acid removed by repeated addition and evaporation of dry methanol], the reduced product, 3-deoxyhexarate, showed a shift of absorption maximum from 548 to 532 nm in the periodate-thiobarbiturate color reaction, indicating the conversion of a ketodeoxy to a hydroxymethylene structure. Identical results were obtained with I (Fig. 3). Reduced I and reduced KDGA showed identical electrophoretic mobility

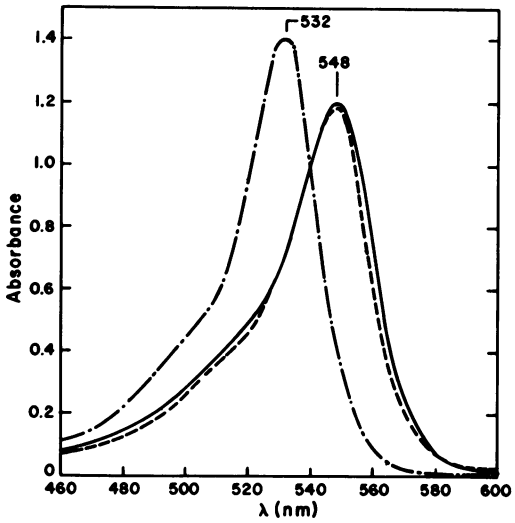

FIG. 2. *Absorption spectra of 2-deoxyglucose* (-·-), *KDGA* (— — —), *and compound I* (——) *in the periodate-thiobarbiturate color reaction. Concentrations were* 10^{-4} *to* 1.5×10^{-4} M; *compound I was prepared from GlcUA.*

(M_{ga} 1.8). Reduction also caused loss of the characteristic color reaction with periodate-benzidine.

(v) *Oxidative decarboxylation of I by hydrogen peroxide.* Decarboxylation of KDHA at the carbon atom vicinal to the keto group can be achieved specifically by treating the α-keto acid with H_2O_2 at *p*H 4 (reference 32; 1 ml of 3% H_2O_2/30 μmoles of compound, 1 hr at 25 C, add equal volume of 1 N HCl to complete CO_2 liberation, remove excess H_2O_2 by treating with catalase or by paper electrophoresis). The decarboxylation product obtained from KDGA, 2,3-dihydroxyglutaric acid, was identical in electrophoretic mobility (M_{ga} 2.16) and R_F in solvent H (0.65) to that obtained from I. After removal of excess H_2O_2, the product of the decarboxylation of I or of KDGA failed to give a color reaction with the periodate-thiobarbiturate test. When oxidized with hypoiodite solution, 2-deoxyglucose yields 2-deoxygluconic acid, which likewise does not give a characteristic color reaction with this test.

The foregoing data characterize I as α-keto-β-deoxy-D-hexaric acid, in which the configuration of the γ and δ-OH groups remains unidentified. Determination of the configuration of these groups will be described in the next section.

Site of dehydration and configuration of I. The product which results from the enzymatic dehydration of GlcA could have two different struc-

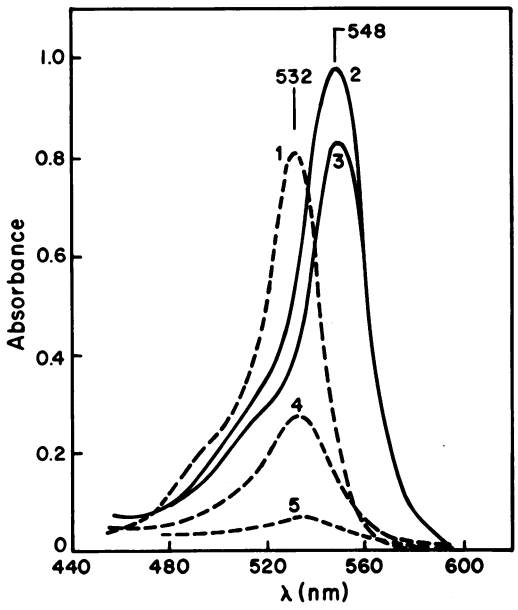

FIG. 3. *Change of absorption spectra of KDGA and compound I in periodate-thiobarbiturate color reaction by borohydride reduction. Curves: (1) 2-deoxyglucose; (2) KDGA before reduction; (3) compound I (from GlcUA) before reduction; (4) KDGA after reduction; (5) compound I after reduction.*

tures: 2-keto-3-deoxy-D-glucaric acid (Ia) or 4-deoxy-5-keto-D-glucaric acid [2-keto-3-deoxy-L-glucaric acid (Ib)], or both, depending on the site of dehydration. Determination of the site of dehydration as well as the structure of I was accomplished by using I labeled with ^{14}C in a specific atom, and treating it with H_2O_2 to release a molar equivalent of CO_2 specifically from the end of the carbon chain vicinal to the keto group.

KDHA-*U*-^{14}C and KDHA-*6*-^{14}C were prepared enzymatically from GlcUA-*U*-^{14}C and GlcUA-*6*-^{14}C, respectively. The experiment was conducted in a Conway dish as follows. A piece of Whatman no. 1 filter paper impregnated with 10% KOH was placed into the central well. The chemical reaction was carried out in the side well. The radioactive compounds were mixed with 0.2 μmole of nonradioactive KDHA and the *p*H of the mixture was adjusted to 4 with HCl; the reaction was started by the addition of 20 μliters of 3% H_2O_2. The vessel was covered tightly and incubated at room temperature for 18 hr, at which time 2 N HCl was added to stop the reaction and complete CO_2 evolution, and the vessel was covered and incubated at 37 C for another 12 hr. Analysis of the reaction mixture (neutralized with 1 N NaOH) by paper electrophoresis and radioautography showed that the decarboxylation had gone essentially to completion. The filter paper discs containing trapped $^{14}CO_2$ were counted for radioactivity.

Since H_2O_2 decarboxylates α-keto acids specifically at the α-keto group end of the carbon chain (32), $^{14}CO_2$ produced from KDHA-*U*-^{14}C should have ⅙ of the total radioactivity of KDHA-*U*-^{14}C before reaction, assuming complete decarboxylation, whereas KDHA-*6*-^{14}C should give different results depending on the relative proportion of Ia and Ib. The percentage of Ia and Ib present in the enzymatic dehydration product of GlcA thus can be determined as follows:

$$\% \text{ of Ib} = \frac{\text{radioactivity of } ^{14}CO_2}{\text{total radioactivity of KDHA-}6\text{-}^{14}C}$$

$$\% \text{ of Ia} = 100 - \% \text{ of Ib}$$

Table 2 shows that the enzymatic dehydration product of GlcA consists of 79% 2-keto-3-deoxy-D-glucaric acid and 21% 4-deoxy-5-keto-D-glucaric acid.

Formation of II from KDHA. When KDHA-*U*-^{14}C was incubated in the absence of NAD with the 35 to 55% ammonium sulfate fraction (Table 1) from GalUA-grown cells, a new compound, II, was the major enzymatic reaction product (M_{ga} 1). The same result also was obtained when GlcA-*U*-^{14}C was incubated with the crude cell-free extract from cells grown on GalUA or GalA. When the CO_2-evolution test of this enzyme reaction was performed with KDGA in a Conway vessel by using the technique previously described, less than 1% of the total radioactivity of KDGA-*U*-^{14}C was detected as radioactive CO_2, indicating that conversion of KDHA to II does not involve a decarboxylation step.

Preparation of II. II was prepared by incubating KDHA-*U*-^{14}C or GlcA-*U*-^{14}C (1 to 2 μc) with 35 to 55% ammonium sulfate fraction or crude extract (10 μliters, Table 1), respectively, in 0.2 ml of 0.1 M Tris buffer (*p*H 8.0). After incubation at room temperature for 4 hr, ^{14}C-labeled II was isolated from the reaction mixture by the same procedure employed for ^{14}C-labeled I.

Tentative identification of II. The electrophoretic mobility at *p*H 5.8 of II was identical with that of GlcUA. However, it could be separated from GlcUA by paper chromatography in solvent J (II, R_F 0.53; GlcUA, R_F 0.2). When II was subjected to hypoiodite oxidation, there was no change in its electrophoretic mobility, suggesting that it does not contain a terminal aldehyde group (21). Oxidative decarboxylation of II

TABLE 2. *Distribution of dehydration product compound Ia and Ib*

Radioactive KDGA	Experiment	Radioactivity[a]		A/B	Compound Ia	Compound Ib
		Total (A)	CO_2 (B)			
					%	%
KDGA-*U*-^{14}C[b]	1	34,894	7,360	4.75/1		
	2	34,894	7,063	5/1		
	3	5,762	914	6.3/1		
	4	6,002	1,017	6/1		
KDGA-*6*-^{14}C[c]	1	50,968	11,549		77.5	22.5
	2	50,968	11,930		76.7	23.3
	3	5,203	998		80.8	19.2
	4	5,166	985		80.9	19.1

[a] Counts per minute of ^{14}C as measured in a liquid scintillation spectrophotometer (80.05% efficiency, 1 μc, 1.77 $\times$ 10^6 counts/min).
[b] Specific activity, 4.78 mc/mmole. Average A/B, 5.5/1 theoretical A/B, 6/1.
[c] Specific activity, 1.13 mc/mmole.

with H_2O_2 at *p*H 4 resulted in release of CO_2 and 60 to 70% conversion to a product identical in electrophoretic mobility with authentic succinic acid (M_{ga} 2.1). These results indicate that II is an α-keto acid. Since II is not a decarboxylation product of KDHA, it might be a diketodideoxyhexaric acid, e.g., 2,5-diketoadipic acid derived from KDHA by another dehydration at the two remaining hydroxylated carbons; such a compound upon oxidative decarboxylation would give rise to succinic acid. Dehydration of KDHA would yield a compound which would not react in the periodate-thiobarbiturate test, due to the lack of vicinal hydroxyl groups which provide the site for periodate cleavage. This is consistent with the observation that when GlcUA or GlcA was incubated with any enzyme preparation, KDHA was the only periodate-thiobarbiturate test-positive compound detectable in the reaction mixture. The product obtained from II by oxidative decarboxylation was further characterized as succinic acid by paper chromatography and thin-layer chromatography (Table 3). These data suggest that II is 2,5-dioxoadipic acid.

Formation of III. When GlcA-*U*-^{14}C was incubated with crude cell-free extract, a minor product, III, was present in addition to II in the reaction mixture. When KDHA-*U*-^{14}C was incubated with 35 to 55% ammonium sulfate fraction (Table 1) in the absence of NAD, only very low formation of III was detected. However, in the presence of NAD, the formation of III was enhanced with concomitant release of $^{14}CO_2$, which was detected in a Conway vessel by trapping with KOH.

TABLE 3. *Paper and thin-layer chromatography of succinic acid and oxidative decarboxylation product from II*

Solvent	R_F[a]	
	Succinic acid	II
Paper chromatography		
N	0.69	0.69
O	0.78	0.77
Thin layer chromatography[b]		
P[c]	0.38	0.38
C[d]	0.43	0.43
Q[e]	0.58	0.58

[a] Succinic acid was revealed by either aniline-xylose color reagent (31) or bromocresol green spray (Sigma Chemical Co); radioactive compound was located by radioautography.
[b] Samples were dissolved in 50% aqueous methanol (v/v) for application.
[c] Carried out on Silica Gel G plate; chamber, with saturation; length of run, 18 cm; time of run, 180 min.
[d] Carried out on Eastman Chromagram sheet, type K301-R; chamber, without saturation; length of run, 16.2 cm; time of run, 170 min; showed co-chromatography.
[e] Same as *d* except: length of run, 17.5 cm; time of run, 90 min.

Preparation of III. Radioactive III was prepared in the same manner as radioactive II, except that a small amount of NAD (1 μmole) was included in the reaction mixture. (NAD caused marked stimulation of this reaction; the reason for its action is not at present apparent.) Non-

radioactive III was prepared from GlcA or GalA (40 μmoles of substrate incubated with 0.2 ml of crude extract and 500 μmoles of Tris buffer, *p*H 8.0; final volume 7 ml). It was isolated as its DNPH by incubating the deproteinized and concentrated reaction mixture with an excess of 0.1% (w/v) DNPH for 1 hr at 30 C. The orange-yellow precipitate was centrifuged, washed with 2 N HCl and cold water, and drained and dried in a vacuum.

Characterization of III. III (10^{-4} μc) was mixed with 0.2 μmole of authentic KGSA and was oxidized to α-ketoglutaric acid (KGA) by incubation with a sixfold molar excess of $KMnO_4$ (0 C, *p*H 7.5). The reaction mixture was analyzed by paper electrophoresis and autoradiography. The autoradiograms showed co-electrophoresis of the oxidized radioactive compound and the nonradioactive compound produced from KGSA [M_{ga} 2.32, alkaline silver nitrate color spray (39)]. The KGA produced (about 70% yield) was identified by its reaction with reduced NAD ($NADH_2$), NH_4^+, and L-glutamate dehydrogenase (specific activity, 3.1 IU/mg of protein) to form L-glutamic acid (31). The latter was identified by paper electrophoresis (M_{ga} 1.2) and paper chromatography in solvents A and B (R_F 0.5 and 0.32).

The absorption spectrum of the DNPH of compound III in 0.3% (w/v) sodium ethoxide resembles that of the bis-2,4-dinitrophenyl-hydrazone of authentic KGSA similarly prepared (Fig. 4); both have absorption maxima at 433 and 517 nm (8). DNPH of authentic KGA and KDGA, other keto compounds which might be present in the reaction mixture, showed λ_{max} at 390 and 444 nm, respectively, without an obvious shoulder; DNPH itself showed a maximum absorption at 397 nm with a shoulder at 520 nm.

The results support the view that KGSA is an intermediate in the pathway and that III is KGSA. However, III as isolated could not be converted to KGA enzymatically, and its electrophoretic and chromatographic behavior differed from that of chemically prepared KGSA.

KGSA dehydrogenase. KGSA dehydrogenase activity in cell-free extract of GalUA-grown cells was demonstrated as follows. When authentic KGSA was incubated with the 35 to 55% ammonium sulfate fraction (Table 1) in the presence of NAD in the standard assay system, there was an absorbance increase at 340 nm, corresponding to 2.25×10^{-3} units/ml. There was no absorbance increase in reaction mixtures lacking either NAD or KGSA (Fig. 5). Similar results were obtained when GlcA was incubated with the crude extract from cells grown either in GalUA or GalA (Table 1) in the presence of NAD (activity 1.4×10^{-2} units/ml); activity was also present with GalA or KDHA.

Quantitative study of KGA formation. To determine whether the NAD consumed in the KGSA-dehydrogenase reaction was stoichiometrically used for KGA formation, a quantitative study of these two compounds was performed. Measurement of KGA formation was done by incubating the reaction mixture from the KGSA-dehydro-

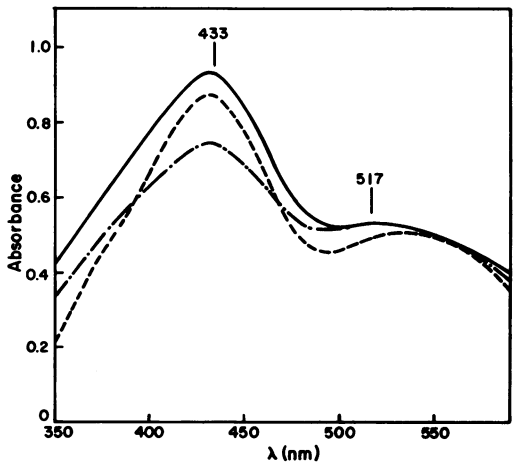

FIG. 4. *Absorption spectra of DNPH derivatives of KGSA (—) and Compound III prepared from GlcA (— — —) and GalA (-·-). Samples were dissolved in 0.3% (w/v) sodium ethoxide to a proper concentration for absorbance measurement.*

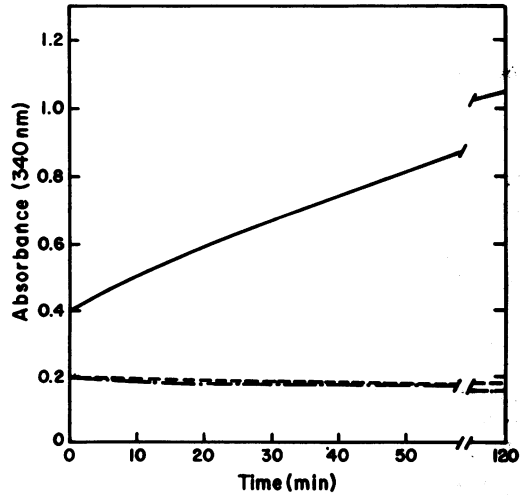

FIG. 5. *KGSA-dehydrogenase activity of 35 to 55% ammonium sulfate fraction (Table 1). Reaction conditions are described in the text; complete system contained 2 mM KGSA as substrate (—); controls lacked either KGSA (— — —) or NAD (-·-).*

genase reaction with an excess of L-glutamate dehydrogenase (2.5 μg, 3.1 IU/mg of protein) in the presence of excess $NADH_2$ (0.3 μmole) and NH_4^+ (150 μmoles; reference 38). The quantitative relationship between NAD consumption and KGA formation is shown in Table 4. The results indicate that 1 mole of NAD is required for the formation of 1 mole of KGA from KGSA or GlcA. These results show that only one NAD-linked oxidation step is involved in the course of the conversion of GlcA to KGA, and this step is the oxidation of KGSA to KGA.

Preparation of IV. IV was prepared by incubating 50 μliters of crude extract (Table 1) with (μmoles): GlcA, 4; $MgCl_2$, 4; NAD, 4; and Tris buffer (*p*H 8.0), 50; (total volume, 1 ml) at 25 C for 4 hr. The reaction mixture then was held at 100 C for 5 min; the precipitate was removed by centrifugation, the supernatant fluid was concentrated to 1/10 of its original volume, and IV was separated from the mixture by paper electrophoresis. It was located with periodate-benzidine (17) and eluted from the paper with distilled water. Radioactive IV was prepared essentially in the same manner, autoradiography was used for its location.

Characterization of IV. IV had the same electrophoretic and chromatographic behavior as authentic KGA (electrophoresis, M_{ga} 2.32; solvent A, R_F 0.57; solvent B, R_F 0.032). DNPH derivative of radioactive IV and KGA-DNPH showed co-chromatography in solvent G. Compound IV was further identified as KGA by its reaction with L-glutamate dehydrogenase to give a ninhydrin-positive compound, and also by electrophoretic and chromatographic comparison of the product of the action of glutamate dehydrogenase with authentic L-glutamic acid (electrophoresis, M_{ga} 1.2; solvent F, R_F 0.24; solvent I, R_F 0.3; solvent K, R_F 0.61; solvent L, R_F 0.49; solvent M, R_F 0.24; solvents D and E, co-chromatography with radioactive IV).

Preparation of V. The reaction product of glutamic dehydrogenase (V) was prepared, separated, and isolated by the same procedure employed for IV.

Glutamate-dehydrogenase and characterization of V. When cell-free crude extract of GalA-grown cells was incubated with KGA in the presence of $NADH_2$ and NH_4Cl, a rapid absorbance decrease at 340 nm was observed, indicating that the L-glutamate dehydrogenase is operative in this system. Upon subsequent examination of the reaction mixture by paper electrophoresis, a ninhydrin-positive compound, V, was isolated. V was identified as L-glutamic acid by paper electrophoresis and paper chromatography (solvents F, I, K, L, M, D, and E) as described above for the product of L-glutamate dehydrogenase action on KGA.

TABLE 4. *Quantitative determination of NAD consumption and KGA formation*

Substrate	NAD consumption	KGA formation
	μmole	*μmole*
KGSA[a]	0.11	0.11
	0.12	0.11
GlcA[b]	0.70	0.71
	0.76	0.83
	0.38	0.34

[a] The 35 to 55% ammonium sulfate fraction from GalUA-grown cells (Table 1) was used.
[b] Crude extract from GalA-grown cells was used.

DISCUSSION

The entire scheme, including both confirmed and tentatively identified intermediates, of D-glucuronic acid metabolism in *A. tumefaciens* is shown in Fig. 1. The first step of the metabolic pathway, the conversion of GlcUA to GlcA, has been described in detail elsewhere (9).

Since I gives a positive reaction in the periodate thiobarbiturate test, it must be a five- or six-carbon compound with at least two adjoining hydroxyl groups providing a site for periodate cleavage. I yields the five-carbon compound KGA by a series of reactions involving a decarboxylation; therefore, it must contain more than five carbon atoms. Dehydration only can occur at the 2,3 or 4,5 position of the carbon chain, because only these structures will yield β-formyl pyruvate upon periodate cleavage and give a chromogen with an absorption maximum at 548 nm (43). The α-keto structure of I is supported by the results of H_2O_2 oxidation (31), semicarbazide-acetate (41) and *o*-phenylenediamine tests (12). Finally, analysis of the DNPH derivative of the periodate oxidation products confirms the structure of I as 2-keto-3-deoxy-D-glucaric acid or KDGA, or both.

A possible explanation for failure of the stable form(s) of GlcA to serve as substrate for partially purified hexarate dehydratase, or to be involved in the transformation of GlcUA to KDHA in this system, is that the crude cell-free extract may contain two hexarate dehydratases, one responsible for the catalysis of the dehydration of the stable GlcA (mostly open chain dicarboxylic acid form) and the other responsible for the dehydration of the unstable lactone form of GlcA. The former dehydratase might be so labile that it is

destroyed during purification procedures, whereas the latter survives purification. The "true" substrate for the latter dehydratase, an unstable lactone form of hexaric acid (possibly a dilactone), may be formed instantaneously during the oxidation of hexuronic acid to hexaric acid. Since lactonization of GlcUA occurs spontaneously at physiological *p*H, according to Yamada (48), oxidation of hexuronolactone [i.e., gluconofuranurono-(6 → 3)-lactone] would yield the hexarodilactone [i.e., glucaro-(1 → 4)-(6 → 3)-dilactone], which subsequently decomposes spontaneously in aqueous solution to yield the monolactones, glucaro-(1 → 4)-lactone and glucaro-(6 → 3)-lactone as initial products (27). In this sense, hexuronate dehydrogenase would serve to produce hexaro-dilactone, which in turn would be the substrate for the stable dehydratase in the partially purified system. The instability of glucaro-(1 → 4)-(6 → 3)-dilactone, produced from gluconofuranurono-(6 → 3)-lactone by rat liver D-glucuronolactone dehydrogenase, has been discussed by Marsh (27) and Hirasaka et al. (19).

The existence of different hexarate dehydratases was not tested for in the *A. tumefaciens* system; however, in *Escherichia coli* both D-glucarate and galactarate dehydratases were detected (6). *A. tumefaciens* dehydratase attacks the two different positions of GlcA at different rates, position 2,3 is favored over position 4,5 by a ratio of approximately 4:1, to yield two different products. In *E. coli* (6) as well as in *Pseudomonas* species (20), dehydration occurs at both 2,3 and 4,5, with the 4,5 position being greatly favored. It has been suggested that only one rather unspecific dehydratase is present in *E. coli* (6). On the other hand, the work of Smiley and Ashwell (33) with *E. coli* grown in GlcUA or GalUA indicated that two different dehydratases for aldonic acids were present, each specific for either the *cis* or *trans* configuration of the hydroxyl group.

It is interesting that, in *E. coli*, hexuronic acid and hexaric acid catabolism differ greatly. GalUA and GlcUA are first isomerized to D-tagaturonic acid and D-fructuronic acid, respectively (4). The isomerization is followed by a reduction of the keturonic acids to the hexonic acids, D-tagaturonic acid to D-altronic acid, and D-fructuronic acid to D-mannonic acid. Subsequently these hexonic acids are dehydrated to the common intermediate, 2-keto-3-deoxy-D-gluconic acid, which is then phosphorylated to the 6-phosphate, and the phosphate is split by 2-keto-3-deoxy-6-phosphogluconate aldolase into equimolar amounts of pyruvate and glyceraldehyde phosphate (4, 22). A recent study of *Aeromonas* species showed a pathway of GalUA and GlcUA metabolism identical to that of *E. coli* (15). Metabolism of GlcA in *E. coli* resembles that in *A. tumefaciens* in the first step of the pathway, i.e., dehydration to KDHA. However, in *E. coli*, subsequent steps differ: KDHA is split by an aldolase to form equimolar amounts of tartronate semialdehyde and pyruvate and the semialdehyde is then reduced to tartronate in the presence of $NADH_2$ (5).

In *Pseudomonas* species, GlcA is converted to KDHA (20) which undergoes simultaneous dehydration and decarboxylation to KGSA (18); the latter is oxidized to KGA by an NAD-linked dehydrogenase (11). In *A. tumefaciens*, also, KDHA is decarboxylated to KGSA and subsequently oxidized to KGA. However, in *A. tumefaciens*, KDHA is converted to KGSA via a demonstrable intermediate and not by a simultaneous dehydration and decarboxylation. This intermediate (II), which was isolated as a labeled compound, is formed from KDHA without loss of CO_2. It was tentatively identified as 2,5-diketoadipic acid, mainly on the basis of its oxidative decarboxylation by H_2O_2 to yield succinic acid. In addition, demonstration that II is not a decarboxylation product of KDHA is supported by the following considerations. Nonoxidative decarboxylation of KDHA could give rise to (i) 2-keto-4,5-dihydroxy-valeric acid ($HOOCCOCH_2CHOHCH_2OH$) or (ii) 2-deoxypenturonic acid ($OHCCH_2CHOHCHOHCOOH$), depending on the site of decarboxylation. The first compound can be ruled out because chemically prepared 2-keto-4,5-dihydroxy-valerate is periodate-thiobarbiturate test-positive (λ_{max} 548 nm) and differs in electrophoretic mobility from II (M_{ga} 1.5). The product of oxidative decarboxylation of this compound (3,4-dihydroxybutyric acid) and of II (succinic acid) also show different electrophoretic mobility (M_{ga} 1.5 and 2.1, respectively). 2-Deoxypenturonic acid can be eliminated on the basis of susceptibility to hypoiodite oxidation, which would convert a carbonyl function to a carboxyl with a concomitant increase in electrophoretic mobility (21). That II is not the oxidative decarboxylation product of KDGA also can be ruled out, since the electrophoretic mobility of the oxidative decarboxylation product of KDHA is greater than that of II.

In 1939, Wille (46) reported the synthesis of 2,5-diketoadipic acid. The compound was metabolized by rat liver and kidney slices, pigeon breast muscle, and by crude extracts of brewers' yeast. Although no information was available concerning the further metabolism of the compound, Wille suggested that it might be involved in succinic and pyruvic acid metabolism, in other words, in the Krebs cycle. The present tentative

demonstration of 2,5-diketoadipic acid as an intermediate in a metabolic pathway is, to our knowledge, the first time the compound has been suggested to be involved in an actual metabolic sequence.

III was identified as a derivative of KGSA by its conversion to KGA and L-glutamate and chromatographic comparison of the resulting compounds. It was also identified by comparison of the absorption spectra of the DNPH derivatives of III and of KGSA. The equivocal electrophoretic and chromatographic behavior of radioactive III can be explained by the observation of Singh and Adams (32) who found that KGSA in the presence of Tris-buffer is converted nonenzymatically to pyrrole-2-carboxylate and other compounds, including a probable Δ^1-pyrroline. These compounds, although differing from KGSA in electrophoretic and chromatographic behavior, probably can be converted to KGA upon $KMnO_4$ oxidation. A recent publication by Adams and Rosso (1) notes the complete loss of enzymatically assayable KGSA after 6 hr of incubation with Tris-buffer, *p*H 8.0 (the same buffer used in the present work), at 25 C. Radioactive III, presumably a pyrrole-like compound, probably is converted to KGA by $KMnO_4$ oxidation.

The stimulation by NAD of the conversion of II to KGSA plus CO_2 is difficult to explain, since the decarboxylation is nonoxidative. NAD may function as an allosteric effector or it may play a direct role in the reaction. Such a function for NAD has been demonstrated in two nonoxidative decarboxylations: the conversion of tartrate to glycerate by an enzyme from *Pseudomonas* (10) and the conversion of uridine diphosphate-D-glucuronate to uridine diphosphate-D-xylose catalyzed by an enzyme from wheat germ (2) or *Cryptococcus laurentii* (3). In both cases, H is removed and is then reintroduced into the reactant (24; J. S. Schutzback and D. S. Feingold, J. Biol. Chem., *in press*); perhaps a similar mechanism is operative in the decarboxylation of II.

The participation of KGSA in a number of biological pathways has been reported. It has been identified as a product of D-glucarate and galactarate metabolism in a *Pseudomonas* (11, 18, 20) and as an intermediate in the conversion of 2-keto-3-deoxy-L-arabonate to KGA in *P. saccharophila* (38). It has also been reported by Singh and Adams (31, 32) as an intermediate of hydroxyproline metabolism which led to the formation of KGA in *P. putida*.

All known metabolic pathways which involve KGSA as an intermediate lead to the formation of KGA. In *A. tumefaciens*, KGA formation was demonstrated by the isolation of the compound and the subsequent identification by chromatographic comparison and conversion to L-glutamate. A study of the stoichiometry of $NADH_2$ and KGA formation from KGSA and GlcA showed a 1:1 relationship between NAD consumption and KGA formation. These results also show that the conversion of KGSA to KGA is the only NAD-linked oxidation step in the conversion of GlcA to KGA.

Both GlcUA and GalUA are converted to the corresponding aldaric acids by *A. tumefaciens* (9). Most of the studies described in this paper have been done with GlcA rather than GalA, since ^{14}C-labeled GlcA is more readily available. However, the further metabolism of GalA does not differ from that of GlcA, for both are converted to KDGA. Therefore, GalUA and GlcUA are converted to KGA via essentially identical pathways.

It is noteworthy that GlcUA and GalUA are metabolized by such a diversity of pathways by different microorganisms. These pathways, although different, all involve dehydration of a pair of vicinal OH groups adjacent to a carboxylic acid to yield the α-keto-β-deoxy acid structure. This type of reaction, first demonstrated by Entner and Doudoroff in *P. saccharophila* (14), is doubtless of general significance in biochemistry.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grants GM-08820 and 1K3-GM-28,296 from the National Institute of General Medical Sciences.

We are grateful to E. Adams for helpful criticism and suggestions.

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