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The Metabolism of D-Glucaric Acid in Bacillus Megaterium

Brahma Shanker Sharma
Loyola University Chicago

THE METABOLISM OF D-GLUCARIC ACID IN *BACILLUS MEGATERIUM* KM

BY

Brahma Shanker Sharma

Department of Microbiology

Stritch School of Medicine

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LIST OF ABBREVIATIONS

DNP	2,4-Dinitrophenylhydrazine
DNPH	2,4-Dinitrophenylhydrazone
EDTA	Ethylenediamine tetra-acetate
DKA	2,5-Diketoadipic acid
DTT	Dithiothreitol
GA	D-Glucaric acid
KDA	2-Keto-3-deoxyarabonic acid
KDG	α-Keto-beta-deoxy-D-glucaric acid
KG	α-Ketoglutaric acid
KGS	α-Ketoglutarate semialdehyde
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
TAS	Tartronic acid semialdehyde
TBA	2-Thiobarbituric acid
Tris	Tris-(hydroxymethyl)aminomethane

1.0 INTRODUCTION

1.01 Occurrence of D-glucaric Acid

The first isolation from nature of D-glucaric acid (saccharic acid), the dicarboxylic acid analogue of D-glucose, was from rubber plants as its magnesium salt (22). In 1926 Grüss (23) found that several nectar yeasts were able to oxidize glucose to glucaric acid. In 1927 Challenger *et al.* (12) reported the isolation of glucaric acid from the culture media of glucose-grown *Aspergillus niger*. However, their identification was not complete by modern standards. More recently, D-glucaric acid has been isolated from plants (29) and from urine of humans, rat and other mammals (34). Using enzymatic methods for its identification, DiFilippo (15) has also reported the presence and estimation of the D-glucarate content of human blood.

1.02 Biosynthesis of D-glucaric Acid

By 1911, (38) it was suggested that glucarate arose from the oxidation of glucuronic acid in animals and the oxidation of glucose to glucarate was later demonstrated in plants (20). In 1927, Challenger *et al.* (12) reported the isolation of glucarate from a glucose-grown culture filtrate of *A. niger*. In 1959, Zajic (54) presented some indirect evidence that NAD-linked hexuronic dehydrogenase of *Agrobacterium tumefaciens* oxidized glucuronic acid, galacturonic and mannuronic acids, to the corresponding hexaric acids. Kilgore and Starr (30) isolated a NAD-requiring uronic acid dehydrogenase from cell

free extracts of glucuronate or galacturonate grown cells of *Pseudomonas* sp. which oxidized galacturonic acid to galactaric acid. In 1966, Chang (10) purified the enzyme from *A. tumefaciens* and identified the product. Thus, the biosynthesis of D-glucarate appeared to proceed via the oxidation of D-glucuronic acid.

1.03 Metabolism of Glucaric Acid

Glucaric acid served as sole source of carbon and energy for the growth of a variety of bacteria (14). Although the fermentation of glucarate by *Escherichia coli* (40) and *Clostridium* (28) was known to form the same fermentation products as those formed from glucose, the intermediates were unknown. In 1958, Blumenthal and Campbell (4) noted that the disappearance of one micromole of glucarate was accompanied by the concomitant production of one micromole of pyruvate in *E. coli*. The first step in glucarate metabolism was later shown to be the dehydration of adjacent hydroxyl groups on carbons 2, 3, and/or 4, 5 by means of a specific D-glucarate dehydrase to yield α-keto-β-deoxyglucarate (KDG) (6).

The further metabolism of KDG by *E. coli* involved cleavage by a specific KDG aldolase (19) yielding equimolar quantities of pyruvic acid and tartronic acid semialdehyde (TAS) (5,18). TAS in turn was reduced to glyceric acid by tartronate semialdehyde reductase in presence of NADH. Presumably, then, the glycerate was phosphorylated and the glycerate-3-phosphate metabolized by known pathways.

This pathway of glucarate metabolism, hereafter called the glycerate pathway (Fig. 1), was found to be common in the enteric bacteria (5,18). It has been reported to be present in many strains of *E. coli* and in *E. freundii, Erwinia carotovora, Aerobacter aerogenes, Paracolobactrum arizonae, Klebsiella pneumoniae* and *Salmonella typhosa*. The presence of the glycerate pathway has also been confirmed by Trudgill and Widdus (48) in three different strains of *A. aerogenes*.

In addition to the glycerate pathway, Dagley and Trudgill (13) found that there was a modified pathway for glucarate metabolism, the α keto-glutarate (KG) pathway, in a single strain of a *Pseudomonas* species (Fig. 2). In this KG pathway, D-glucarate was not cleaved to form two C_3 units. Instead, the KDG was dehydrated and decarboxylated to form α-ketoglutarate semialdehyde (KGS), which was then oxidized by a NAD-dependent dehydrogenase to yield KG. Recently, Jeffcoat *et al.* (27) reported that the dehydration and decarboxylation of 4-deoxy-5-ketoglucarate to KGS in *Pseudomonas* was catalyzed by a single enzyme. Trudgill and Widdus (48) subsequently found the KG pathway to be present in a wide variety of *Pseudomonas* species.

Thus far, only gram-negative organisms have been shown to utilize glucaric acid as sole source of carbon and energy. Fish (18) attempted to induce the formation of D-glucarate dehydrase and KDG alsolase in *Staphylococcus aureus* and *Staphylococcus epidermidis* using manometric techniques to detect substrate utilization. He found that washed bacterial cell suspensions previously incubated in buffered glucarate for 16 hr oxidized glucarate slowly.

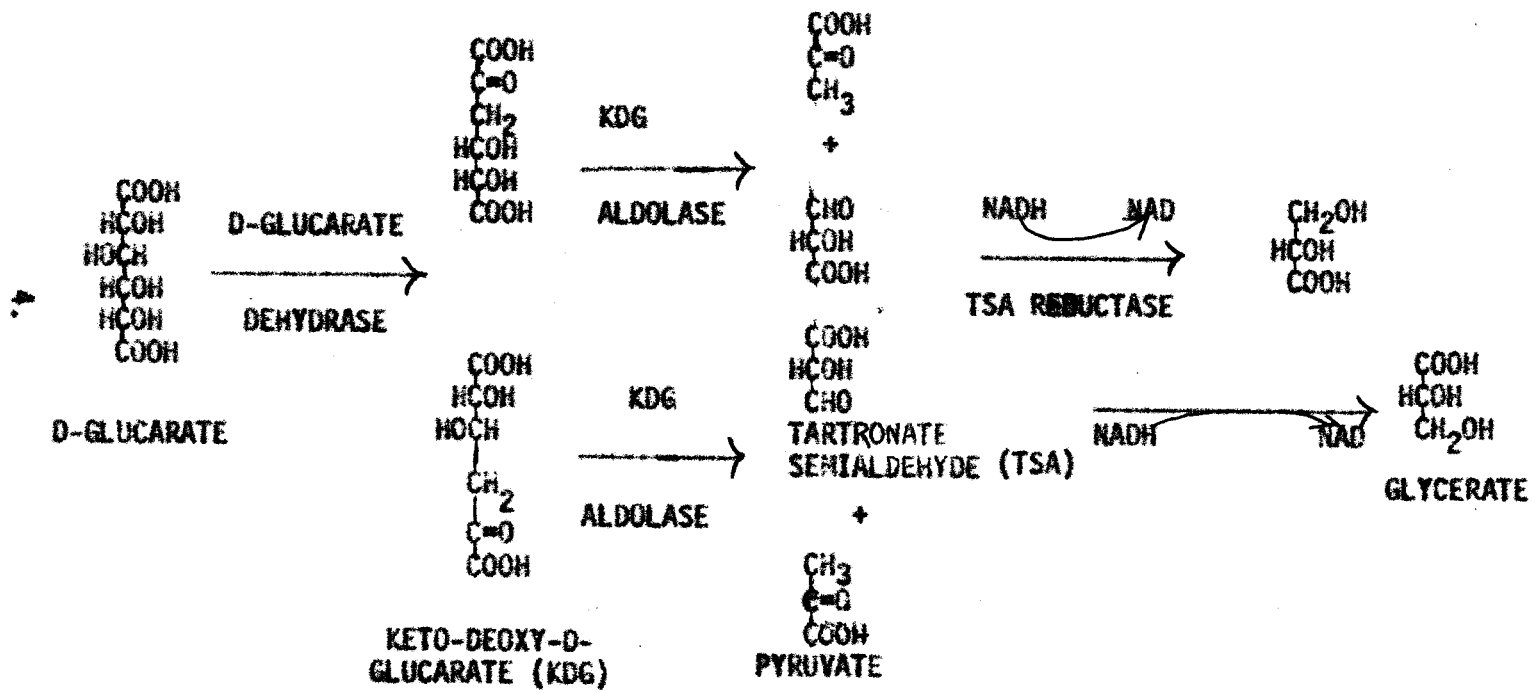


FIGURE 1. METABOLISM OF D-GLUCARIC ACID IN *ESCHERICHIA COLI* BY THE GLYCERATE PATHWAY.

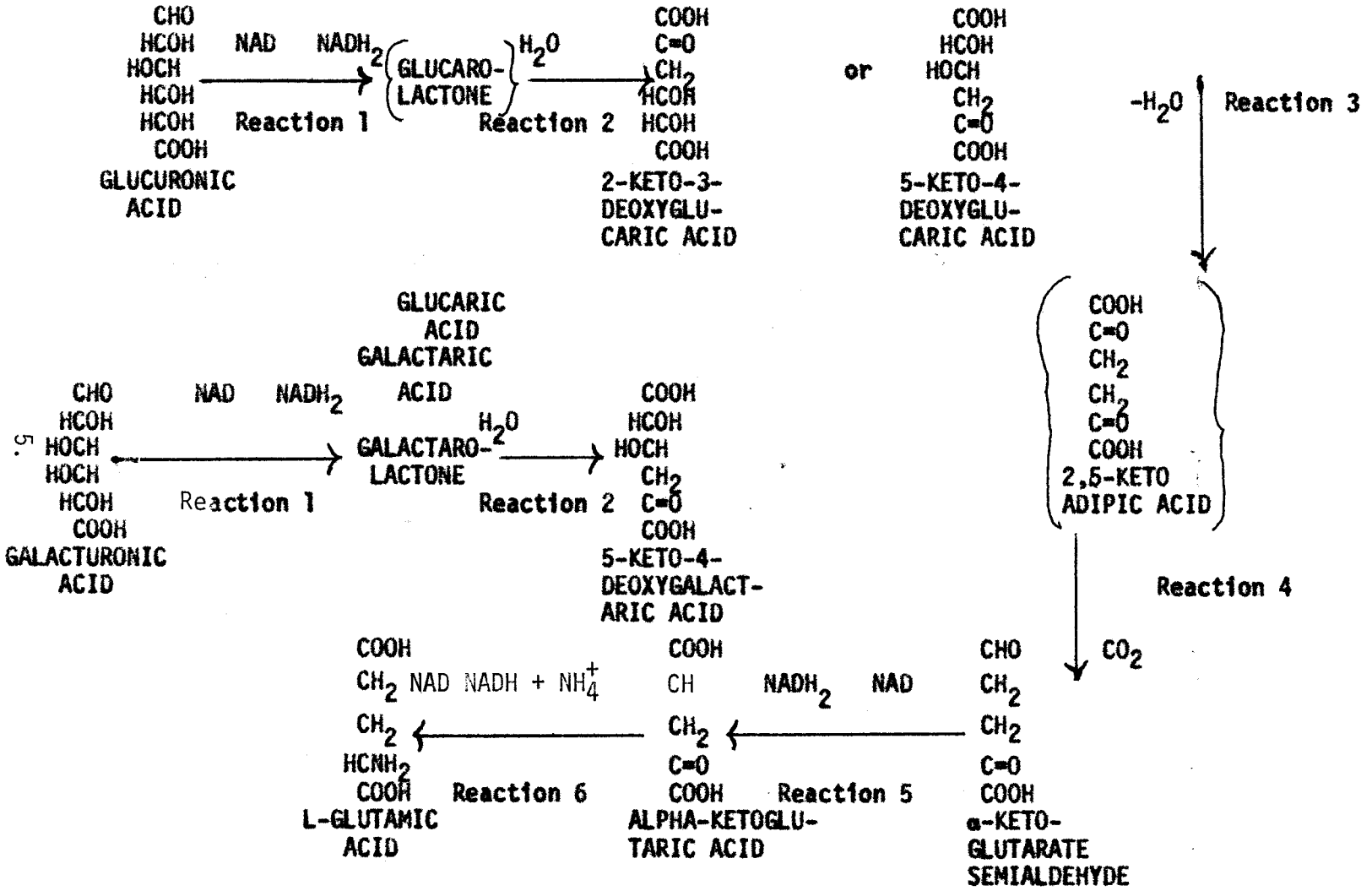


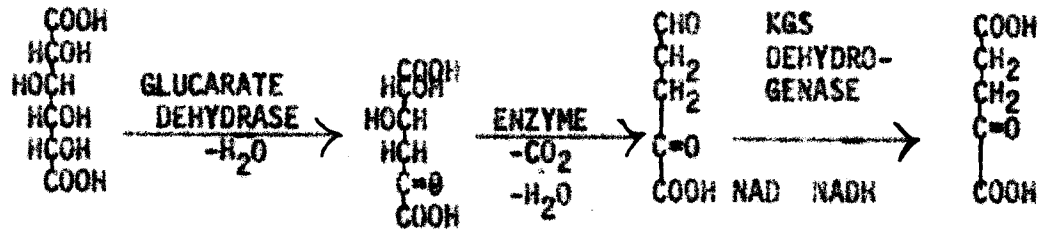
FIGURE 3. PROPOSED PATHWAY OF GLUCURONIC ACID AND GALACTURONIC ACID METABOLISM BY *AGROBACTERIUM TUMEFACIENS* (COMPOUNDS IN BRACKETS ARE ONLY TENTATIVELY IDENTIFIED)

Blumenthal and Jepson (8) reported that *Bacillus megaterium* strain KM also utilized glucaric acid through KDG yielding KG. A number of other intermediates were isolated by anion exchange column chromatography but were not identified.

In 1966, Chang and Feingold (11) proposed a pathway for glucuronic acid and galacturonic acid metabolism in *A. tumefaciens* (Fig. 3) in which they suggested that 2,5-diketoadipic acid (DKA) was an intermediate between KDG and KGS. This conclusion was based upon limited studies involving electrophoresis, paper chromatography and chemical degradation of the electrophoretically isolated compound to yield succinic acid. This intermediate was reported to be very labile.

KGS has been identified as a metabolic intermediate in many induced catabolic pathways, such as a product of hydroxyproline metabolism (41-43) and as an intermediate in the metabolism of L-arabonate (47) and of D-glucarate and galactarate (48) by *Pseudomonas* strains. Recently, it has also been identified as an intermediate in the catabolic pathway of hexuronic acids (via the hexaric acids) in *A. tumefaciens* (10,11). We have also identified KGS in the present study as an intermediate in the metabolism of D-glucaric acid in *Bacillus megaterium*.

Singh and Adams (1,2) reported the NADP-dependent enzymatic oxidation of KGS to yield KG and have partially purified KGS-dehydrogenase from the extracts of hydroxyproline grown cells of *Pseudomonas striata*. Dagley and Trudgill (13) noted a similar reaction in crude extracts of *Pseudomonas*



D-GLUCARATE

4-DEOXY-5-KETO-D-GLUCARATE (KDG)

α KETOGLUTARATE SEMIALDEHYDE (KGS)

α KETOGLUTARATE (KG)

FIGURE 2: METABOLISM OF D-GLUCARIC ACID IN *PSEUDOMONAS* SP. BY THE α-KETOGLUTARATE PATHWAY

strains grown in glucarate or galactarate. Stoolmiller and Abeles (47) reported the oxidation of KGS by the enzyme aldehyde dehydrogenase partially purified from *Pseudomonas oleovorans*. Extracts of hexuronate grown cells of *A. tumefaciens* also catalyzed the oxidation of KGS to KG (10). Recently Adams and Rosso (1) have reported the properties of the purified induced and constitutive KGS dehydrogenases.

The purpose of the present study was to determine the intermediates in D-glucarate metabolism between KDG and KG in *Bacillus megaterium*. During this study, some evidence for the participation of DKA was obtained and KGS was established as an intermediate. KGS-dehydrogenase, which catalyzed the NAD or NADP-dependent oxidation of KGS to KG, was isolated, partially purified and characterized. The proposed pathway for the metabolism of D-glucarate is shown in Fig. 4.

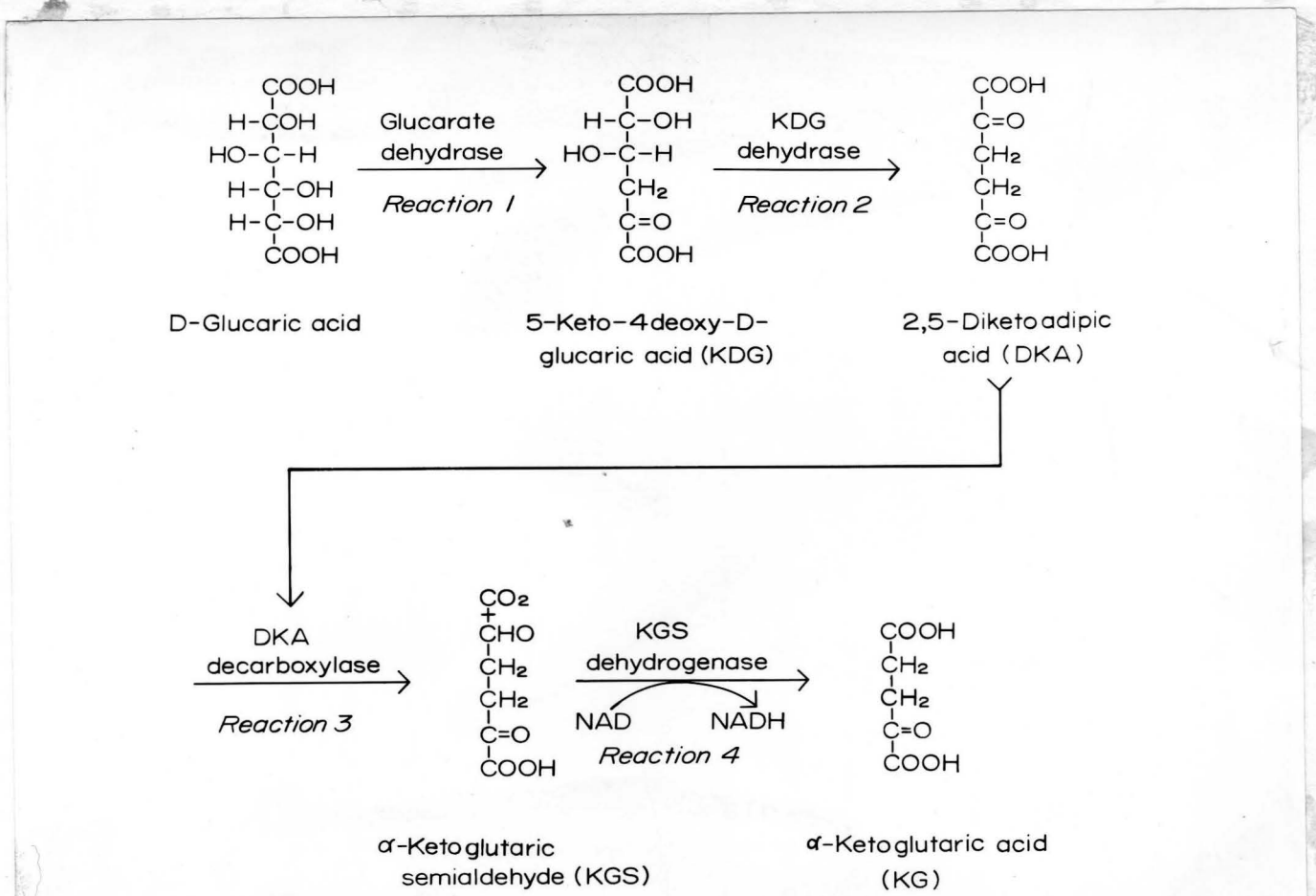


FIGURE 4: Proposed pathway for the metabolism of D-glucarate in *Bacillus megaterium* KM.

2.0 MATERIALS AND METHODS

2.01 Chemicals

KGS was prepared from methyl-2,5-dimethoxy-tetrahydro-2-furoate (gift from Dr. P. W. Trudgill, Division of Biochemistry, University of Illinois, Urbana) by hydrolysis with dilute sulfuric acid, as described later (35). Enzymatically prepared 5-keto-4-deoxy-D-glucarate (6) was used after converting the calcium into the sodium salt with cation exchange resin. 2,5-Diketoadipic acid was synthesized by two different methods, one of which will be described later (24). Ethyl succinate and ethyl oxalate were products of Eastman Chemicals, Rochester, N.Y. 2-Keto-3-deoxy-D,L-arabonic acid was a gift from Dr. R. Abeles, Graduate Department of Biochemistry, Brandeis University, Waltham, Massachusetts. Monopotassium saccharate (glucarate), oxidized nicotinamide adenine dinucleotide (NAD), oxidized nicotinamide adenine dinucleotide phosphage (NADP), reduced NAD, tris(hydroxymethyl)aminomethane and glutamate dehydrogenase were obtained from Sigma Chemicals (St. Louis, Mo.). Whatman microgranular DEAE-cellulose DE 52 (preswollen), was obtained from Reeve Angel, Clifton, N.J. Anion exchange resin (Dowex-1-Cl^-; X8; 200-400 mesh) was obtained from Baker Chemical Co., Phillipsburg, N.J. Nuchar-C190-N charcoal was obtained from the Fisher Scientific Co., Pittsburg, Pa., and dithiothreitol (DTT) from Calbiochem, Los Angeles, Cal. Enzyme grade ammonium sulfate was obtained from Mann Laboratories, New York, N.Y. All other chemicals were commercial reagent grade chemicals and were used without further purification.

2.02 Organism

The metabolism of glucaric acid (GA) was studied in the KM strain of *Bacillus megaterium* originally obtained from Dr. P. Gerhardt. The ability to utilize GA in this organism is inducible. This strain uses GA as sole source of carbon and energy.

B. megaterium KM was maintained on trypticase soy (BBL) agar slants. The culture was transferred every fifteen days and stored at 4 C. Before using, the purity of the organism was checked on solid media by gram staining and by the typical morphology of the bacillus.

2.03 Medium

A synthetic medium containing 0.8% GA was used to grow the cells for metabolic studies. This medium contained the following ingredients per 2 L: 16.0 g GA, 33.0 g Na_2HPO_4, 3.0 g KH_2PO_4, 4.0 g $(NH_4)_2SO_4$, 0.4 g $MgSO_4 \cdot 7\ H_2O$, 0.02 g $CaCl_2 \cdot 2\ H_2O$, 0.2 mg $FeSO_4 \cdot 7\ H_2O$. The Na_2HPO_4, KH_2PO_4 and $(NH_4)_2SO_4$ were first dissolved in 250 ml of deionized water in a 2 L Erlenmeyer flask and then GA was added. To this solution, 2.0 ml of 10 mg/ml $CaCl_2 \cdot 2\ H_2O$, 2.0 ml of 200 mg/ml $MgSO_4 \cdot 7\ H_2O$ and 2.0 ml of 25 mg/500 ml of $FeSO_4 \cdot 7\ H_2O$ solutions were added. The pH of this medium was then adjusted to 7.0 with dilute NaOH, as measured with a pH meter, and then, the volume was made up to 2 L with deionized distilled water. It was distributed in 900 ml amounts in each 2 L flask and 100 ml amounts in each 250 ml flask. The flasks containing the medium were then autoclaved at 121 C for 15 min.

2.04 Preparation of cells

The starter flask, 100 ml of medium in a 250 ml flask, was inoculated with a loopful of cells from a 24 hr old slant of *B. megaterium*. The flask was incubated for 24 hr on a rotary shaker at 37 C. The entire flask contents were then used as the inoculum for 900 ml of the same medium in a 2 L Erlenmeyer flask, which was then incubated for 16 hr under the same conditions. After the growth for 16 hr, the cells were harvested by centrifugation at 10,000 x g for 10 min at 4 C and then washed twice by suspending in cold 0.05 M KCl and recentrifugation. The washed cells were either stored at -20 C or used immediately.

2.05 Preparation of Cell-Free Extracts

Either frozen or fresh washed cells were suspended (1: 10 w/v) in a centrifuge tube in 0.05 M Tris-HCl buffer, pH 7.5; for some studies 0.05 M potassium phosphate buffer of the same pH was used in place of Tris-HCl buffer. The cell suspension was disrupted for 10 min in a Branson S-75 sonic oscillator using the macro tip. The tube was cooled by fluid circulating around it at -4 to 0 C. Cell debris was removed by centrifugation at 20,000 x 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 charcoal/mg protein for some experiments involving the accumulation of intermediates.

2.06 Ammonium Sulfate Fractionation

All fractionations were carried out at 0-4 C. Neutral ammonium sulfate was prepared by adjusting the pH of a saturated ammonium sulfate solution

to ca. 7 with ammonium hydroxide. The pH was determined on a 1:100 dilution with a pH meter. To prepare the 0-25% saturated ammonium sulfate fraction, neutral saturated ammonium sulfate was added to cell free extracts while slowly stirring until the concentration of ammonium sulfate reached 25% saturation. The contents of the tube were occasionally stirred for 15 min and then the precipitate was removed by centrifugation at 20,000 x g for 10 min. The prepare the 25-55% saturated ammonium sulfate fraction, neutral ammonium sulfate was added to the supernatant fluid of the 0-25% fraction until the concentration reached 55%. It was stirred for 15 min and the precipitate was collected by centrifugation. The precipitate was dissolved either in 0.05 potassium phosphate or Tris-HCl buffer, pH 7.5. This fraction was used for the accumulation of DKA in some experiments.

2.07 Paper Chromatography

Whatman number 1 chromatographic paper was used for both descending and ascending paper chromatography. For ascending chromatography the chamber was saturated with solvent prior to the addition of paper while for descending chromatography the paper was equilibrated with solvent in the tank prior to the development. The composition of solvent systems used are shown in Table 1.

2.08 Thin Layer Chromatography

Gelman chromatographic sheets I.T.L.C. type SG, were used for thin layer chromatography. An Eastman chromatogram chamber set was employed for developing the thin layer chromatograms.

2.09 DEAE-Cellulose Column Chromatography

DEAE-cellulose was used for the purification of the enzyme KGS-dehydrogenase. The desired amount of DEAE-cellulose was first equilibrated with either 0.05 M phosphate or Tris-HCl, pH 7.5, buffer in a batch process. The DEAE-cellulose was suspended in the buffer and then allowed to settle for 20-30 min. The slightly turbid supernatant fluid was decanted and the cellulose was resuspended in more buffer. This procedure was repeated 4-5 times to remove the fine particles of cellulose. The slurry was then packed into a column and equilibrated with buffer at 5 C until the pH of the washings coming through the column remained unchanged.

2.10 Ion Exchange Chromatography

Dowex-1-Cl^- X8 (200-400 mesh) anion exchange resin was converted to the OH^- form by batch treatment with sodium hydroxide at 70 C. The OH^- form was then converted to the formate form by passing 4 N sodium formate through a column of OH^- form resin until the eluate was neutral. After washing the resin with water to remove excess sodium formate, it was stored in water at room temperature.

A column of the desired size (usually 2.5 x 25 cm) was packed with resin by gravity and washed with 3 or more column volumes of water. Such a column was then used for the separation of the intermediates of the glucarate catabolic pathway. The deproteinized incubation reaction mixture was placed onto the column and allowed to flow into the resin. The sample was then washed into the column with 10 ml of water. When the last of the

Table 1. Solvents used for paper chromatography

Solvent system	Composition
A	t-Butanol : 88% formic acid : water (4:1:1:5 by volume)
B	n-Propanol : 0.2 N NH_4OH (3:1, by volume)
C	n-Butanol : ethanol : water (52:32:16, by volume)
D	n-Butanol : formic acid : water (95 : 5, saturated with water)
E	n-Butanol : glacial acetic acid : water (12 : 3 : 5, by volume).

liquid had just entered the resin, 10 ml of water was carefully placed on the top of the column and the gradient was started. The 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. Every six min fractions of 7 ml were collected in tubes placed within a refrigerated fraction collector at 4 C.

2.11 Detection of Compounds on Chromatograms

A. Periodate-benzidine spray: Compounds possessing adjacent hydroxyl groups were detected by the periodate-benzidine spray (52). Such compounds showed pale yellow to orange spots on a blue-violet background; KDG gave an orange-yellow spot.

B. Semicarbazide spray (50): After spraying with 0.1% semicarbazide-HCl the chromatograms were dried and observed under ultraviolet light. Keto acids which formed semicarbazones with the spray reagent appeared as dark quenching spots against the lightly fluorescing background on the paper.

C. Brom cresol green spray: Organic acids were detected by spraying with an 0.04% ethanolic solution of brom cresol green. The acids appeared as yellow spots against blue background.

D. Visualization by ultraviolet light: Free KGS was visible under ultraviolet light due to its white fluorescence; it was only visible when present in large amounts.

2.12 Materials Prepared

2.121. Preparation of α-ketoglutarate semialdehyde. KGS was prepared by hydrolysis of methyl-2,5-dimethoxy-tetrahydro-2-furoate. The hydrolysis was performed by gently refluxing 1 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 extracted by shaking with diethyl ether and removing the ether phase in a separatory funnel. The aqueous solution was then concentrated under vacuum at 30 C until a semi-crystalline mass was obtained. This semi-crystalline KGS was stored at -20 C.

2.122. Preparation of 2,5-diketoadipic acid (24). 2,5-Diketoadipic acid was prepared by condensing 80 ml ethyl oxalate and 43 ml ethyl succinate in 400 ml etheral solution of sodium ethoxide. After 48 hr the reaction mixture was poured into water and acidified with HCl and left for some time. The oil which separated was then extracted with ether. The syrup obtained after evaporation of the ether was dissolved in cold, concentrated HCl and left for 24 hr. It was then diluted with an equal part of water and heated at 90 C until the evolution of CO_2 had ceased. HCl was removed by distillation with frequent addition of water. After the concentrated solution was cooled, the oxalic acid and succinic acid which deposited were removed by filtration. Upon further concentration, yellow crystals were deposited which were triturated with ether. By continual extraction with ether, the yellow material was separated in an ether soluble and an ether insoluble fraction; the latter fraction contained the DKA. The ether insoluble frac-

tion was further concentrated and the DKA crystallized on cooling. DKA was dissolved in water and neutralized with NaOH before using as a substrate of the enzyme system present in cell free extract. The melting point of the crystalline preparation of DKA was 219 C, as determined on a Fisher-Johns melting point block. The semicarbazone of this compound had an absorption maximum at 258 nm, indicating the presence of α-keto acid.

2.123. Preparation of KGS bis-2,4-dinitrophenylhydrazone (KGS-DNPH). KGS-DNPH was prepared by adding 5% excess 0.1% 2,4-dinitrophenylhydrazine in 2 N HCl to the chemically prepared KGS. After incubation for 1 hr at 37 C the precipitated KGS-DNPH was collected by centrifugation and washed twice with 2 N HCl and twice with water.

The enzymatically prepared KGS was also used to prepare KGS-DNPH in a similar manner. A 5% excess of DNP was added to either a deproteinized incubation reaction mixture or peak-1 isolated by column, and incubated for 1 hr at 37 C. The precipitated KGS-DNPH was removed by centrifugation and washed with 2 N HCl and then with water.

2.124. Preparation of KG-2,4-dinitrophenylhydrazone (KG-DNPH). KG-DNPH was prepared by the method used by Fish (18) for the preparation of pyruvate-DNPH. KG was incubated with a 5% excess of 0.1% DNP in 2 N HCl for 1 hr. Following the extraction of the KG-DNPH with diethyl ether, the ether was allowed to evaporate. The residue was then dissolved in chloroform and extracted with 1 N NH_4OH. The combined NH_4OH extracts were cooled and the pH was reduced below 2 with cold 6 N H_2SO_4. The KG-DNPH was finally re-extracted with ether and the ether was then allowed to evaporate.

2.125. Preparation of KDG 2,4-dinitrophenylhydrazone (KDG-DNPH). KDG-DNPH was prepared by the method described by Fish (18).

2.13 Analytical Methods

2.131. Determination of protein. The protein content of cell-free preparations and enzyme fractions was measured by the spectrophotometric method of Waddell (51) based on the ultraviolet absorption at 215 and 225 nm in a Gilford 2000 spectrophotometer.

2.132. Formation and utilization of NADH. NADH formation and utilization was measured spectrophotometrically following the change of absorbance at 340 nm in a Gilford spectrophotometer. A molar absorptivity of 6,220 for NADH was used (26).

2.133. Detection of keto acids. The semicarbazide test of MacGee and Doudoroff (31) was employed for the detection of α-keto acids. A 0.2 ml sample was added to a tube containing 1.3 ml of deionized distilled water and this was followed by 1.5 ml of 1% semicarbazide hydrochloride in 1.5% sodium acetate·3 H_2O. After mixing, the tube was incubated for 30 min at room temperature and then the absorbancy was measured at 258 nm.

2.134. Periodate-thiobarbituric acid (TBA) test. The TBA test for keto-deoxy sugar acids was performed by the method of Weissbach and Hurwitz (53) as modified by Fish and Blumenthal (6). To the sample in 0.2 ml solution, 0.25 ml of 0.025 N HIO_4 in 0.125 N H_2SO_4 was added. After careful mixing the tube was allowed to remain for 20 min at room temperature. Following this 0.5 ml of sodium arsenite in 0.5 N HCl was added while shaking

and the solution was allowed to stand for 2 min. Then 2 ml of 0.3% thiobarbituric acid was added and the tubes were placed in a boiling waterbath for 10 min. After cooling the absorbancy was measured at 551 nm and the KDG concentration was calculated employing the extinction coefficient of 60,000 for KDG.

2.135. Absorption spectra. Absorption spectra of the DNPH derivatives of KG, KDG, KGS and other compounds were measured with a recording Beckman spectrophotometer, model DB, after dissolving the derivatives in 95% ethanol. The spectra were also measured after making the DNPH solutions alkaline. In the case of KGS-DNPH, it was dissolved in 0.3% sodium ethoxide.

2.14 Enzymatic Procedures

2.141. D-Glucarate dehydrase assay. D-Glucarate dehydrase enzyme activity was measured according to the method of Blumenthal (7). The enzyme, catalyzing the dehydration of glucaric acid to give KDG, was detected quantitatively by the TBA test. KDG is cleaved by periodate to yield formylpyruvic and glyoxylic acids. The chromogen formed by the reaction of thiobarbituric acid and formylpyruvate was measured at 551 nm.

The incubation mixture in a 10 x 75 mm test tube contained 0.1 ml sodium or potassium D-glucarate (0.02 M), 0.05 ml Tris-HCl buffer (0.8 M, pH 8.0) and 0.05 ml $MgSO_4$ (0.08 M). About 0.01 unit of dehydrase was added to start the reaction. After 10 min the reaction was terminated by the addition of 10% TCA. The protein precipitate was removed by centrifugation, and 0.2 ml of this incubation mixture was used for the TBA test. KDG

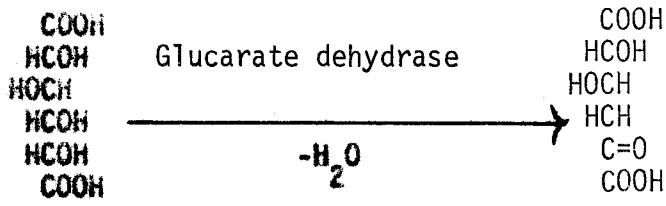
concentration was calculated by using an extinction coefficient of 60,000 for KDG at 551 nm. The difference between the glucarate dehydrase assayaand a control lacking substrate was used to determine the true glucarate dehydrase activity.

2.142. KGS dehydrogenase assay. KGS dehydrogenase activity was assayed measuring the rate of reduction of NAD at 340 nm in a Gilford model 2000 recording spectrophotometer with a temperature controlled cuvette compartment. Each 1.2 ml of assay mixture contained; Tris-HCl buffer, pH8.5, 40 micromoles; NAD, 0.2 ml(10 mg/ml); amd enzyme preparation. The reaction was started by adding 5 micromoles of KGS. The rate of change in absorbancy at 340 nm was measured for several min at 25-28 C. The reaction rate was linear with time and enzyme concentration using either crude extract or partially purified enzyme preparation. One unit of enzyme was defined as the quantity that catalyzes the reduction of 1 micromole of NAD per min. The extinction coefficient of NADH was taken as 6.2×10^3 in 1 cm cuvette at 340 nm.

3.0 Results

3.1 Metabolic Pathway. The proposed pathway of D-glucaric acid utilization by Bacillus megaterium is shown in Fig. 4. The experimental evidence for the operation of each of these reactions will now be presented.

3.11. Reaction 1. The first reaction in the metabolism of D-glucaric acid has been shown to be a dehydration yielding KDG, (8). The reaction is catalyzed by the enzyme glucarate dehydrase.



D-Glucaric acid KDG

The product of this reaction, KDG, has been isolated by anion exchange chromatography and identified. In order to isolate KDG, D-glucarate was incubated without NAD using as the enzyme source either crude, cell-free extract, charcoal-treated crude extract or a 0-40% ammonium sulfate fraction. A typical incubation contained, in a final volume of 12 ml: monopotassium glucarate, 40 micromoles; Tris-HCl, pH 7.5, 1.6 millimoles; $MgCl_2$, 50 micromoles and 2.5 ml of charcoal-treated crude extract, containing about 20 mg protein. Following incubation for 30 min at 30 C with occasional stirring, the reaction was terminated by placing the incubation vessel in a boiling water bath for 2 min. AFter removal of protein precipitate by centrifugation, the deproteinized solution was placed on a Dowex-1-formate column and the organic acids were eluted with a 0-2 N and then with a 2-6N formic acid double gradient. Samples of all tubes were assayed for -keto acids by the semicarbazide procedure and the appropriate tubes were pooled and lyophilized. Usually KDG (P-III) was eluted after DKA (P-II); typical elution pattern is shown in Fig. 5.

Keto-deoxy sugar acids with a $-CHOH-CN_2-CO-COOH$ moiety react in the TBA test to give a chromogen with an absorption maximum at 551 nm. Authentic KDG, the compound in the reaction mixture and the compound

isolated by the column chromatography from the incubation reaction mixture all formed chromogens in the TBA test which absorbed maximally at 551 nm (Fig. 6). The formation and subsequent utilization of KDG by other enzymes in the crude extract is shown in Fig. 7. In one experiment authentic KDG was added in place of GA and its disappearance was measured (Fig. 7).

In paper and thin layer chromatography the Rf-values of authentic KDG and the TBA positive compound isolated from various reaction mixtures were the same. 2,4-Dinitrophenylhydrazones prepared from authentic KDG, from the reaction mixture, or from the compound isolated from P-III on the column all had identical properties. On paper chromatography all of these prepared hydrazones migrated with the same Rf in 2 different solvent systems (Table 2). When KDG was tested as a substrate for the enzyme system, there was a gradual disappearance of KDG using either the crude extract or the charcoal treated crude extract of glucarate grown cells (Figure 7). This indicated that KDG is an intermediate in the pathway of glucaric acid utilization. Also, a 55-75% ammonium sulfate fraction of the cell free extract catalyzed the reduction of NAD to NADH in the presence of KDG, providing further evidence that the KDG is an intermediate. Finally authentic KDG was shown to be converted to DKA and KGS, both intermediates in the pathway and to the end product, KG.

Although the *B. megaterium* glucarate dehydrase was not purified or studied further, the formation of KDG and its subsequent conversion to KG served as proof that glucarate dehydrase is involved in the metabolism of glucarate first to KDG, and subsequently to KG.

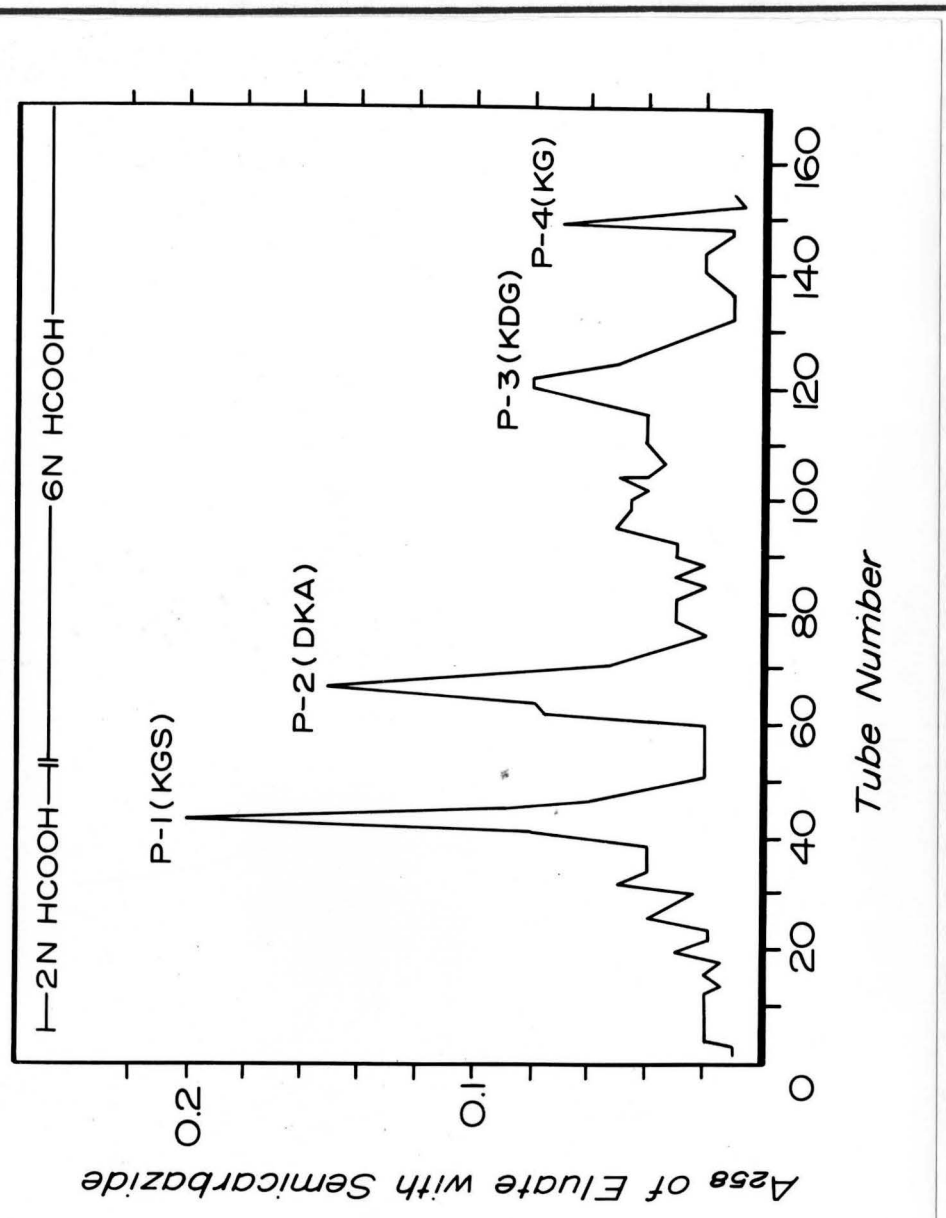


FIGURE 5: Isolation of Intermediates in the Reaction Mixture By Column Chromatography on Dowex-1-Formate. The enzymatically formed intermediates were separated by column chromatography using 0-2 and 2-6 double formic acid gradients. Average fraction volume was 7.0 ml per tube.

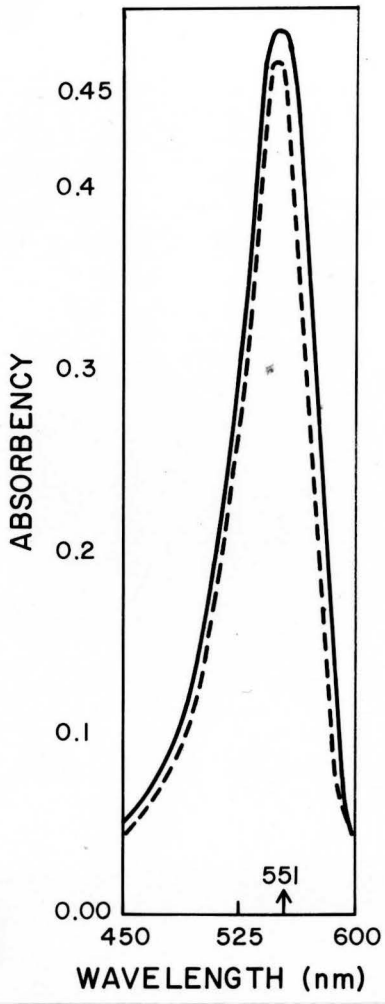


FIGURE 6: The absorption spectra of either enzymatically formed KDG in the incubation reaction mixture and authentic KDG after the periodate-thiobarbituric acid color reaction. Authentic KDG(___), compound in reaction mixture (----).

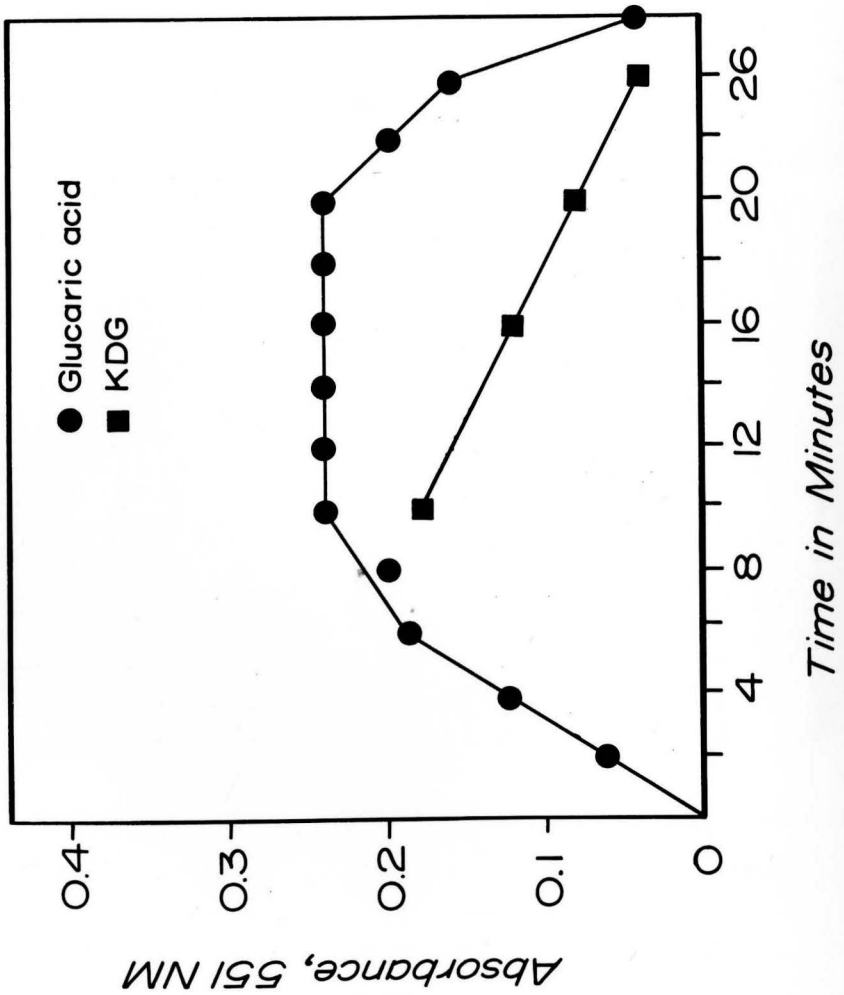


FIGURE 7: Formation and utilization of KDG with time. --O--O-- GA was incubated with a crude cell-free extract as described in the test and samples were removed at time intervals and KDG measured by the TBA procedure; --□--□-- authentic KDG was incubated with charcoal-treated crude extract and KDG utilization was measured by the TBA procedure.

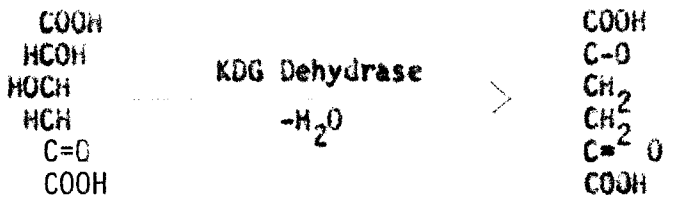
Table 2

Identification of KDG in reaction mixture by paper and thin layer chromatography

Compound	Rf-value	Solvent
Authentic KDG	0.44	A
Reaction mixture	0.45	A
Authentic KDG	0.13	B
Authentic KDG-DNPH	0.12	C
Reaction mixture DNPH	0.11	C
P-III DNPH	0.28	A
Authentic KDG-DNPH	0.28	A
Reaction mixture-DNPH	0.28	A
Ascending paper chromatography		
Authentic KDG	0.05	B
Reaction mixture	0.055	B
Thin layer chromatography		
Authentic KDG	0.82	A
Reaction mixture	0.82	A

* DNPH of keto acid isolated from reaction mixture by column chromatography

3.12 Reaction 2. The second reaction in the pathway of D-glucaric acid utilization appeared to be another dehydration. In this reaction KDG is dehydrated to yield DKA.



KDG

2,5-Diketoadipic acid (DKA)

Compound 2(P-2) was prepared from KDG and isolated by anion exchange chromatography. KDG was incubated with charcoal-treated crude extract in the absence of NAD for 30 min. The incubation mixture consisted of: KDG, 40 micromoles; Tris-HCl, pH 7.5, 3 mmoles; charcoal-treated crude extract, 75 mg protein, and 18 ml deionized distilled water. The crude enzyme fraction was treated with charcoal to remove as much NAD as possible from the enzyme. Since KGS dehydrogenase requires NAD for activity, its absence allowed accumulation of larger amounts of earlier intermediates. At the end of the incubation period the enzyme system was inactivated in a boiling water bath and the denatured protein formed was removed by centrifugation. The supernatant solution was placed onto a column of Dowex-1-formate and the organic acids were eluted with a formic acid gradient as described in

Methods. Fractions of 7 ml were collected and the tubes with α-keto acids, as revealed by the semicarbazide test, were pooled and lyophilized. The DKA (P2) appeared after KGS has been eluted from the column (Fig.5).

The presumed DKA-DNPH was prepared by incubating a solution of P-2 with 0.1% DNP at 37 C for 1 hr. The precipitate that formed was removed by centrifugation, and then washed twice with 2 N HCl and then with distilled water. The dry DNPH derivative was then dissolved in alkaline ethyl alcohol and its spectrum was measured in a recording spectrophotometer. It showed a maximum at 437 nm and a shoulder at 536 nm (Fig. 8).

The presumed DKA-DNPH was also obtained by direct treatment of an incubation reaction mixture with the same manner KDG was incubated without NAD using as an enzyme source either a ammonium sulfate fraction or a charcoal-treated crude extract. The DNPH absorbed with a maximum at 435 nm and a shoulder between 537-540 nm (Fig. 9, 10).

During paper chromatography of samples of the reaction mixture in a n-propanol, NH_3 solvent, there was always a spot present that possessed a different Rf value from KGS, KG and migrated with nearly the same position as KDG. However it gave a negative reaction with the periodate-benzidine spray whereas KDG was positive with this spray. When isolated P-2 was chromatographed it gave two spots, one spot with a Rf value the same as above, while the other spot had approximately the same Rf value as reported by Chang (10) for DKA. These results are shown in Table 3. Synthetic DKA was able to serve as a substrate for the enzyme system present in cell-free crude extracts. Cell-free crude extracts prepared from

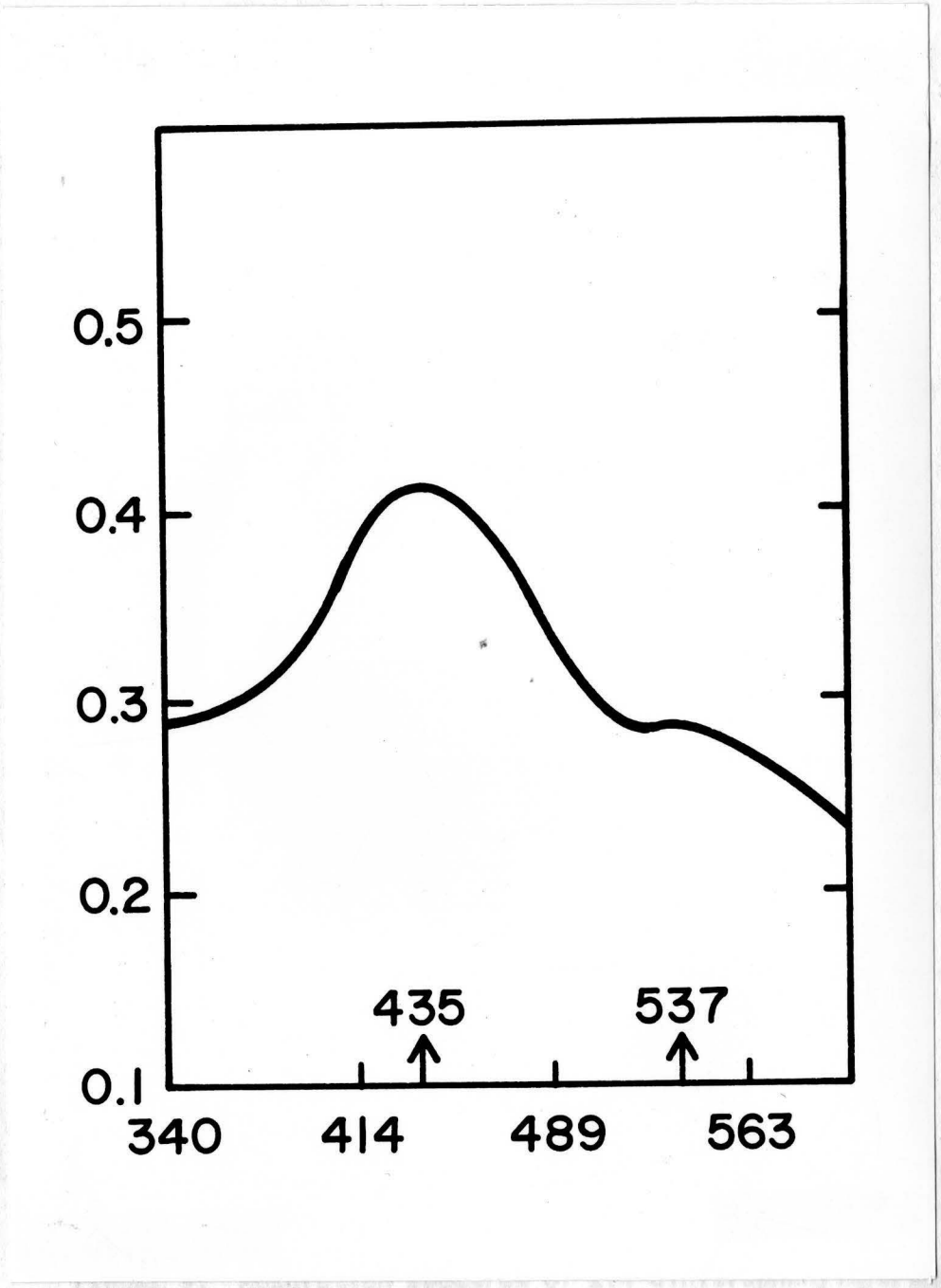


FIGURE 8: Absorption spectrum of the 2,4-dinitrophenylhydrazone of a compound (Peak-2) isolated from the reaction mixture in alkaline ethyl alcohol.

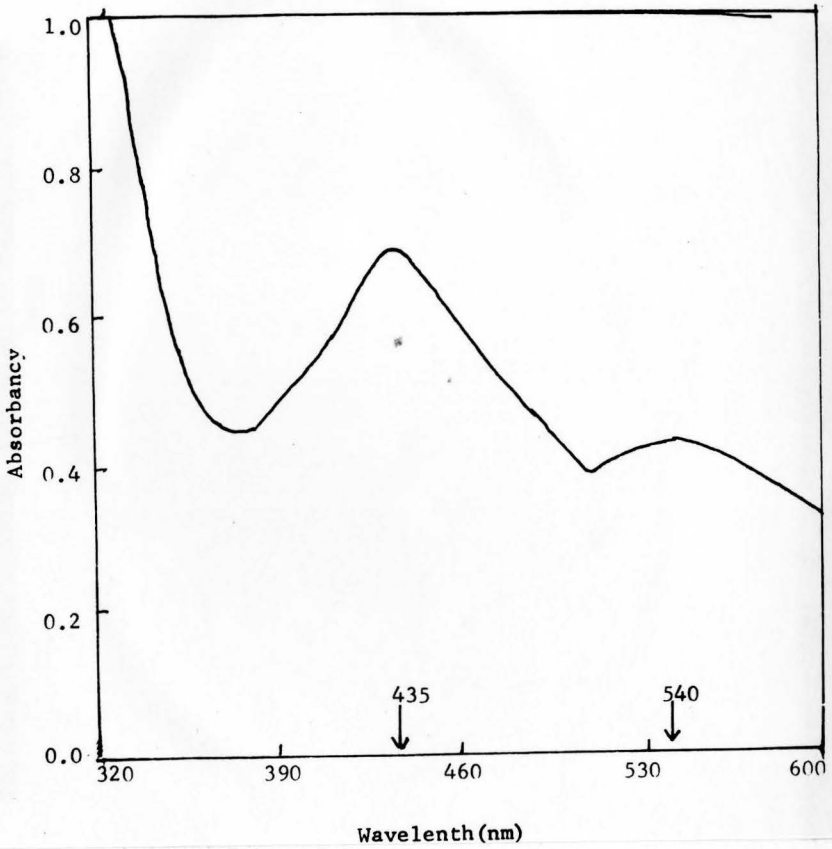


FIGURE 9: Absorption spectrum of 2.4-dinitrophenylhydrazone of a compound formed enzymatically in the reaction mixture. Sample was dissolved in 95% ethyl alcohol and made alkaline by a drop of 50% sodium hydroxide solution for absorbancy measurement.

Table 3

Chromatographic behavior of P-2 and a keto acid accumulated in the reaction mixture.

Descending paper chromatography

Compound	Rf-valve	Experiment	Solvent
P-2	0.56 and 0.35	2	A
P-2	0.69 and 0.25	2	A
Reaction mixture	0.52	1	A
	0.09	4	B
Ascending paper chromatography			
P-2	0.09-0.16 and 0.5	2	B
Reaction mixture	0.06-0.1	8	B

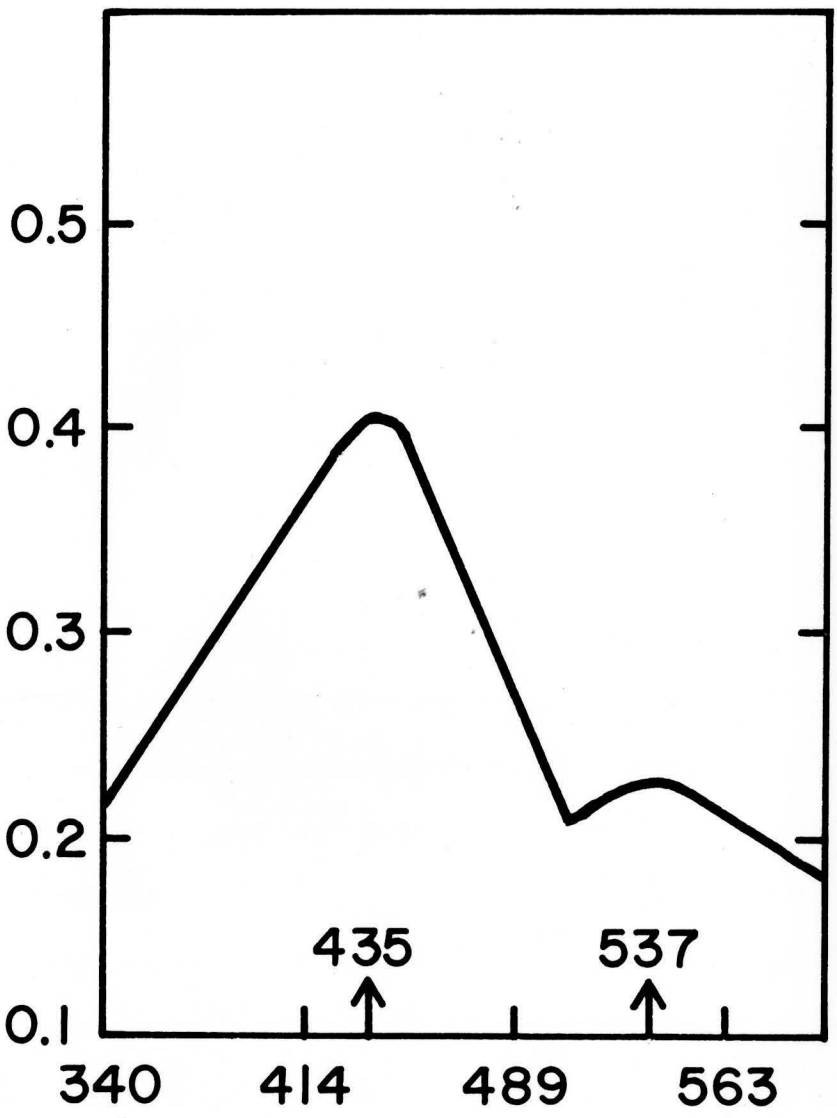
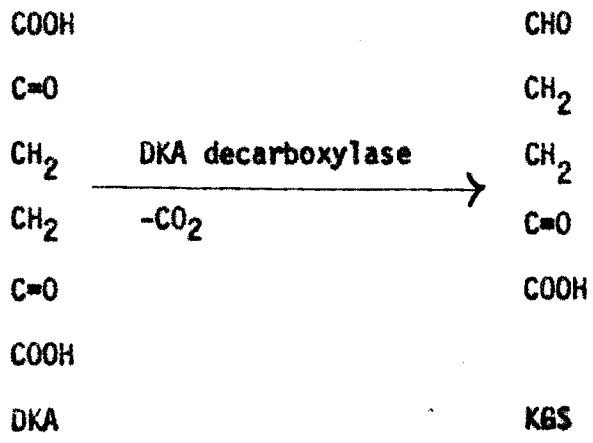


FIGURE 10: Absorption spectrum of the 2,4-dinitrophenylhydrazone of synthetic 2,5-diketoadipic acid in alkaline 95% ethyl alcohol.

glucarate grown cells catalyzed the reduction of NAD to NADH in the presence of synthetic DKA indicating that DKA is an intermediate in the pathway of glucarate utilization.

3.13. Reaction 3. The third reaction in the pathway of glucarate utilization was the decarboxylation of DKA to KGS.



The product of this reaction was isolated by Dowex-1-formate column chromatography and identified as α-ketoglutarate semialdehyde. KDG was incubated with charcoal-treated cell free extract for 30 min. The incubation reaction mixture contained: KDG, 40 micromoles; Tris-HCl, pH 7.5, 1.6 mmoles; and deionized distilled water 9 ml. The reaction was initiated by the addition of enzyme preparation and it was terminated by placing the reaction vessel in boiling water for about 2 min. After removal of the protein by centrifugation, the supernatant fluid was placed on the column. A double gradient of formic acid was started and fractions of about 7 ml were collected. Those tubes containing semicarbazide-positive α-keto acids were pooled and lyophilized. An elution pattern is shown in Fig. 5; peak-1 was identified as α-ketoglutarate semialdehyde.

The DNP-hydrazone of the reaction mixture containing P-1 was prepared by incubating the deproteinized reaction mixture with an excess amount of 0.1% DNP for 1 hr at 37 C. The reaction mixture, in the absence of NAD, contained either GA or KDG as substrate and used either crude extract or charcoal-treated drude extract as the enzyme source. An orange, yellow precipitate formed which was separated by centrifugation and washed twice with 2 N HCl and twice with water. The DNP-hydrazones of isolated P-1 and authentic KGS were also prepared in the same way.

The DNP-hydrazone derivatives were dissolved in 0.3% sodium ethoxide and their spectra were measured. It was observed that the spectra of the derivatives prepared from P-1, deprotenized incubation mixture and from authentic KGS were almost identical with maxima at 429 or 430 and at 517-519 nm (Fig. 11-13).

Upon paper chromatography of P-1, authentic KGS, and the compound in the reaction mixture, all migrated at same Rf in two solvent systems (Table 4)- This indicated that both P-1, and the compound accumulated in the reaction mixture, were KGS.

Further evidence for the identity of KGS was obtained by its conversion to KG. Both synthetic KGS and P-1 were converted to KG in the presence of NAD, by a partially purified KGS-dehydrogenase preparation. KGS was incubated with 55-75% ammonium sulfate fraction in the presence of NAD for 1 hr and70-75% of the KGS was oxidized to KG, as measured by the glutamate dehydrogenase assay, Table 6. Also, both the cell free extract and a partially

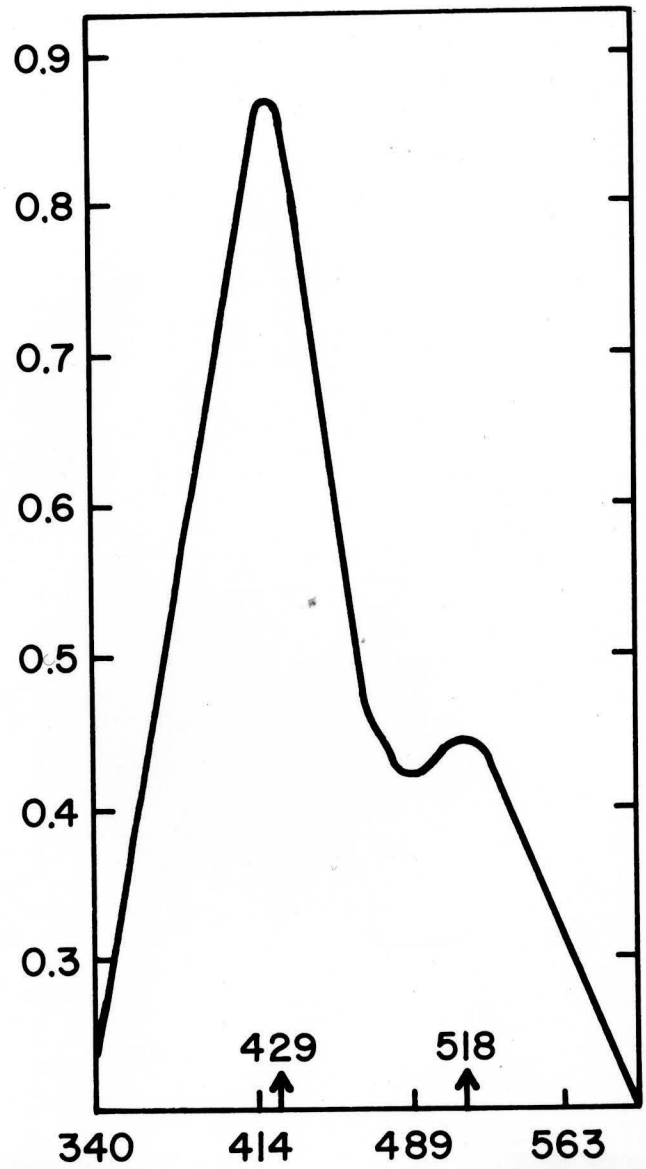


FIGURE 11: Absorption spectrum of 2,4-dinitrophenylhydrazone of isolated Peak 1 from the reaction mixture in 0.3 sodium ethoxide.

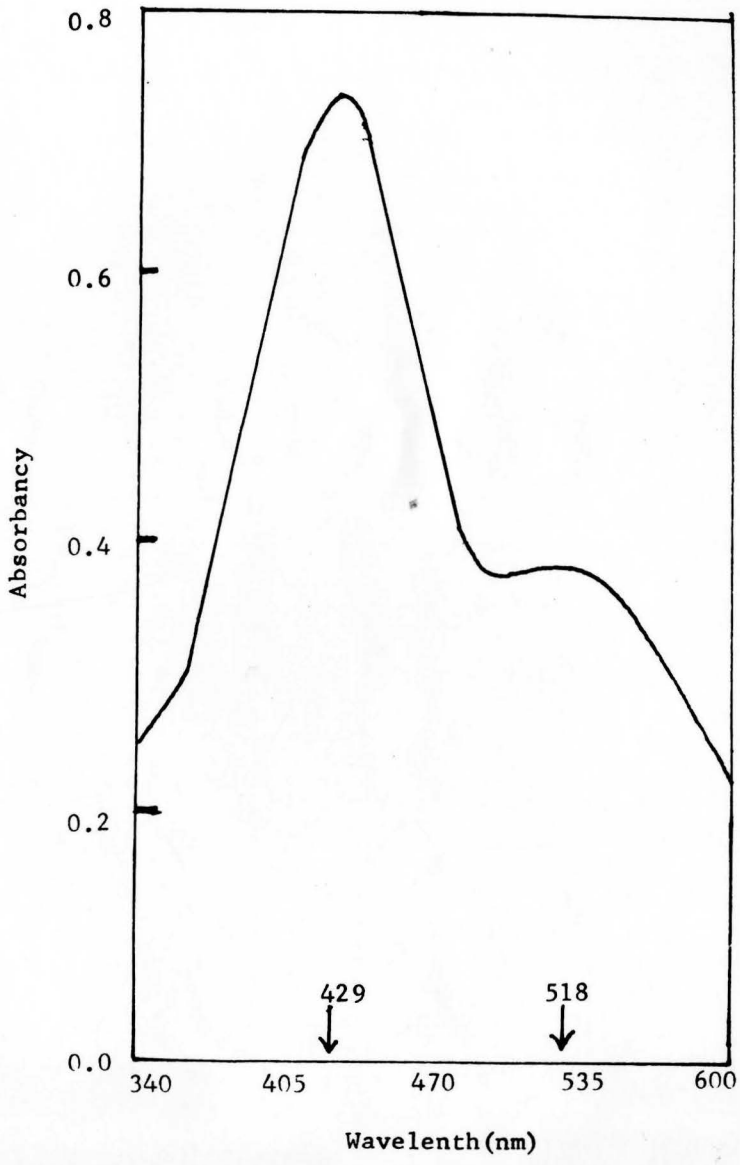


FIGURE 12: Absorption spectrum of the 2,4-dinitrophenylhydrazone derivative of the compound formed enzymatically in the reaction mixture.The sample was dissolved in 0.3%(w/v) sodium ethoxide.

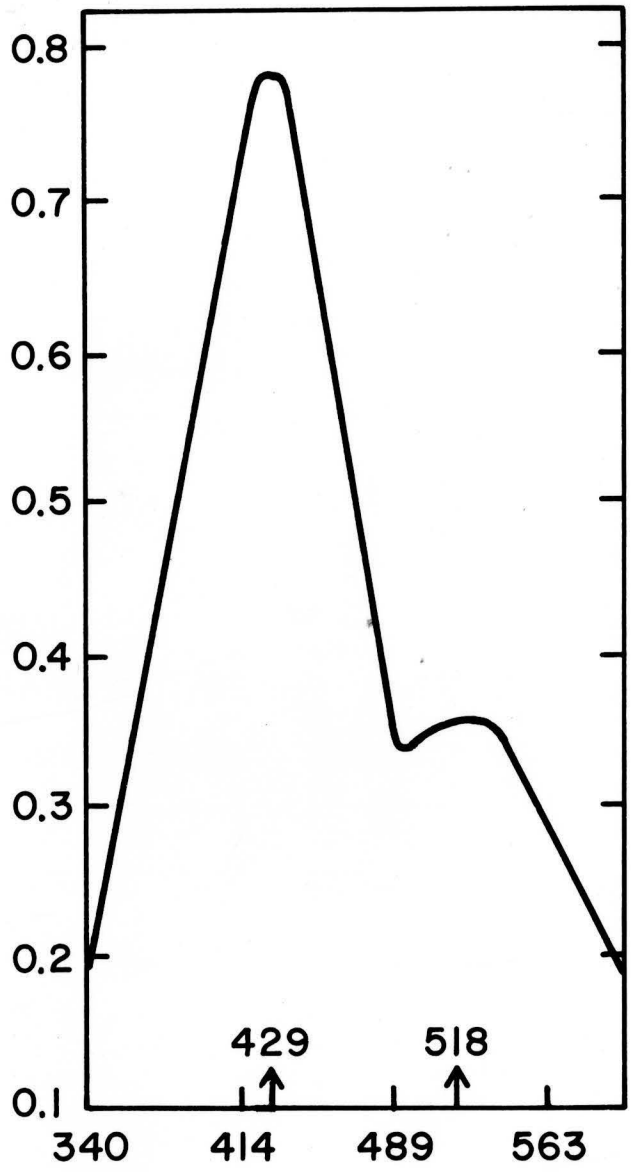


FIGURE 13: Absorption spectrum of 2,4-dinitrophenylhydrazone of synthetic α-ketoglutarate semialdehyde in 0.3%(w/v) sodium ethoxide.

Table 4

Paper chromatography and thin layer chromatography of authentic KGS, isolated compound (peak 1) and reaction mixture

Compound	Solvent	Rf
Descending paper chromatography.		
Authentic KGS	A	0.33 and 0.8
P-1	A	0.31 and 0.79
* Reaction mixture	A	0.78
Authentic KGS	B	0.15 and 0.59
Reaction mixture	B	0.59
Ascending paper chromatography		
KGS	B	0.39 and 0.72
Reaction mixture	B	0.72
Thin layer chromatography		
KGS	A	0.65
Reaction mixture	A	0.65

* KDG was incubated with charcoal-treated crude extract in absence of NAD for 30 min.

Table 5

Formation of alpha-ketoglutarate from synthetic alpha-ketoglutarate semialdehyde by partially purified KGS-dehydrogenase.

Initial A_{340}	Final A_{340}	Difference A_{340}	KG µmoles 1.5 ml
1.41	0.63	0.78	0.186

10 micromoles of KGS were converted to 7.44 micromoles of KG i.e. 74.4% of KGS was converted to KG in 1 hr. The alpha-ketoglutarate formed was measured by glutamate dehydrogenase assay.

purified KGS-dehydrogenase were capable of reducing NAD to NADH or NADP to NADPH in the presence of chemically prepared KGS, indicating that KGS was oxidized to KG. All these data were consistent with the identification of the product of reaction 3 as KGS.

3.131 α-Ketoglutarate semialdehyde dehydrogenase of *Bacillus megaterium*. α-Ketoglutarate semialdehyde dehydrogenase is an enzyme which catalyzed the NAD- or NADP-dependent oxidation of KGS to α-ketoglutarate. The KGS-dehydrogenase was prepared and partially purified by the following procedure.

Step 1. Preparation of cell-free extract. Frozen or fresh cells were suspended in 0.05 M potassium phosphate buffer, pH 7.5, containing 0.008 EDTA and 1.6 mM DTT. This suspension was sonicated as described in Methods and the sonicated suspension was centrifuged at 20,000 x g at 4 C for 10 min. The supernatant fluid (S_1) was further centrifuged at 1000,000 x g for 1 hr at 4 C. The resulting supernatant fluid (S_2) was then used for further purification.

Step 2. Neutral ammonium sulfate fractionation. All procedures were carried out at 4 C. Cold saturated ammonium sulfate, adjusted to pH 8 with NH_4OH, was slowly added to S_2 while stirring until the concentration reached 45% saturation. After 15 min in an ice bath the precipitated protein was removed by centrifugation for 10 min at 20,000 x g. To the supernatant fluid further neutral saturated ammonium sulfate was added to bring the concentration to 75% saturation. After 15 min stirring the precipitate thus formed was collected by centrifugation. The resulting precipitate was dis-

solved in an appropriate volume of either pH 7.5, Tris-Hcl or potassium phosphate yielding fraction S_3 at a concentration of about 6 mg protein/ml. This S_3 fraction was further purified by adsorbtion on an aged calcium phosphate gel in a ratio of 2.5 mg S_3 protein per 24 mg (16 mg/ml) gel. The S_3 was added to the calcium phosphate gel and stirred for 10 min and then centrifuged for a few min to remove the gel. The enzyme adsorbed onto the gel was eluted by increasing concentrations of pH 7.5 potassium phosphate buffer. Most of the enzyme activity was eluted from the gel when it was washed three times, each time with 5-10 ml of 0.25 M buffer, and twice with the same volume of 0.3 M buffer. By this method the enzyme was purified 6- to 7-fold.

Step 4. Column chromatography on DEAE-cellulose. Preswollen DEAE-cellulose was equilibrated with 0.02 M Tris-HCl buffer, pH 8.4, containing 0.005 M EDTA and 1.6 mM DTT. The 2.4 x 25 cm column was packed by gravity and equilibrated with buffer at 4 C. The temperature was maintained at 4 C throughout the procedure. A sample of 16 ml of S_4 was placed on the column and washed into the column with 10 ml of 0.02 M buffer. The column was then washed with the same buffer, collecting 10 ml fractions, until tube 12 at which time the linear gradient was started. The reservoir contained 0.4 M KCl in the washing buffer and the mixing chamber contained 170 ml of the washing buffer without KCL. Each tube was frozen and protein analyses were performed at a later time after thawing. All tubes containing protein were assayed for enzyme activity. It was noted that the enzyme activity was eluted in two distinct peaks, in tubes 5-9 and 32-37. However, there was no significant purification as judged by the specific activity of the enzyme, as a result of the DEAE-cellulose column chromatography.

Purification of

α-ketoglutarate semialdehyde dehydrogenase

	Fraction	Volume, ml	Protein, mg	Enzyme units	Specific activity	Enzyme recovery %	Purification, -fold
S 1	Cell-free extract	39	203	132	0.45	100	-
S 2	100,000 x g supernate	38	192	156	0.62	-	1.2
S 3	45-75% Ammonium sulfate	8	50	95	1.90	71.0	4.2
S 4	Calcium phosphate gel eluate	20	22.9	80	3.30	60.0	7.3
S 5	DEAE-cellulose peak fractions	28	14.0	44	3.7	33.0	8.2

3.1311 Properties of KGS-dehydrogenase

The properties of the KGS-dehydrogenase enzyme were determined using an enzyme preparation purified through the ammonium sulfate step (S_3), unless otherwise noted.

The activity of the enzyme was assayed using three different concentrations of S_3 (7-fold purified preparation). As one would expect with a valid enzymatic assay, there was a proportionality between the enzyme concentrations and the initial reaction rates (Fig. 16.). Similar results were obtained with assays of crude enzyme or enzyme purified at each subsequent step. There was a linear relationship between the amount of enzyme and the increase in absorbancy at 340 nm for a period of 3 min(Fig. 17.), after which there was a slight reduction in the reaction rate. The initial reaction rate (first min) was always used to assay the enzyme concentration.

Effect of pH. KGS-dehydrogenase was found to be most stable after storage at -10 C for 30 min at Tris-HCl at pH 8.5, and its stability decreased rapidly at more acidic or alkaline pH values (Fig.18.). The enzymatic activity was also maximal at this pH value in the same buffer (Fig.19.) Potassium phosphate buffer seemed to inhibit the enzyme activity; at pH 7.5 the activity in phosphate buffer was less than half of that of Tris-HCl buffer at the same pH. In Tris-maleate buffer there was increased enzyme activity as the pH pf buffer increased (Fig. 19.).

Effect of cations. The enzyme does not seem to have any requirement for cations such as Ca^{++}, Mg^{++}, and Fe^{+++}. Also, since the chelating agent

FIGURE 16: Reaction rate as a function of enzyme concentration. The reaction mixture contained samples of diluted S-3 enzyme.

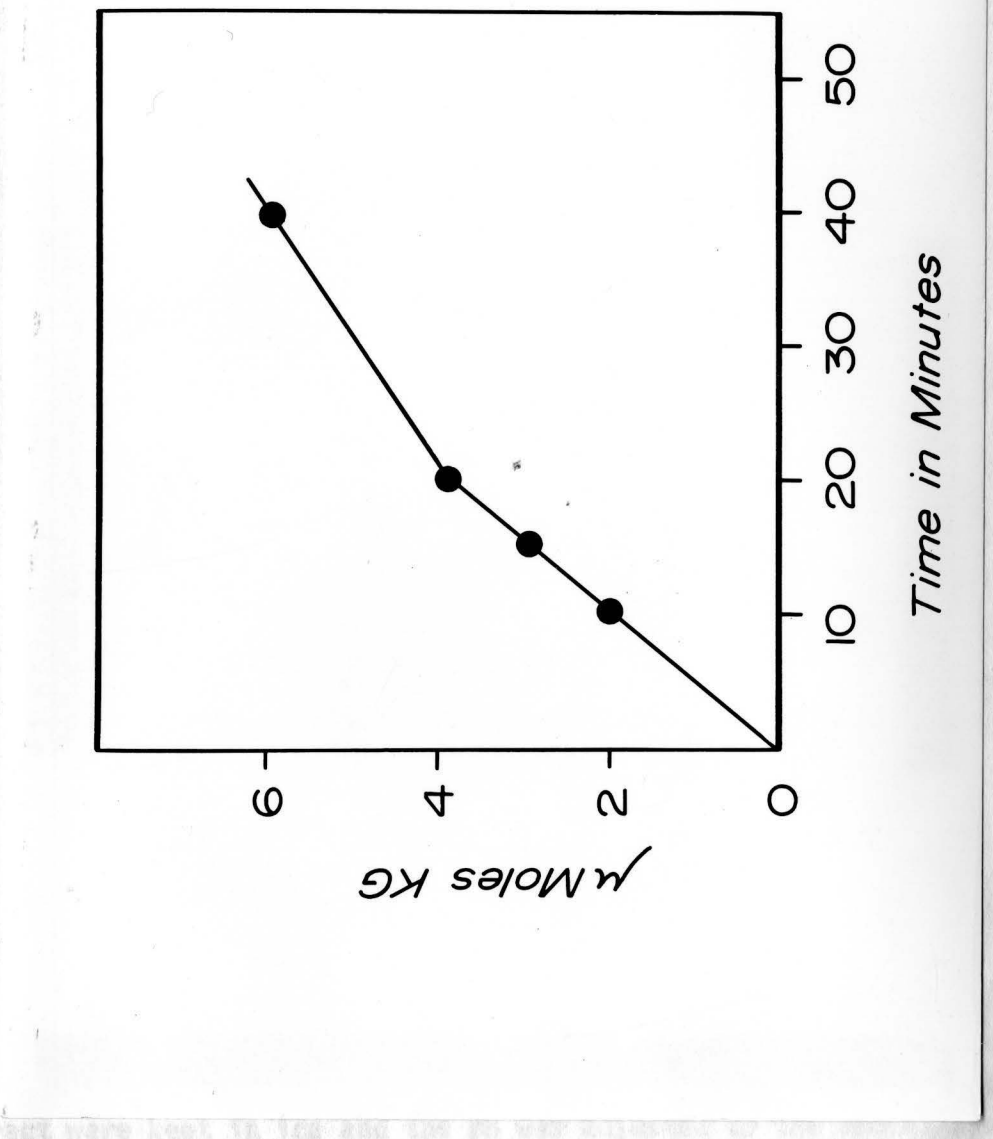


FIGURE 17: Reaction rate as a function of time. A quantity of 40 ug of S-3 enzyme was used in the standard assay.

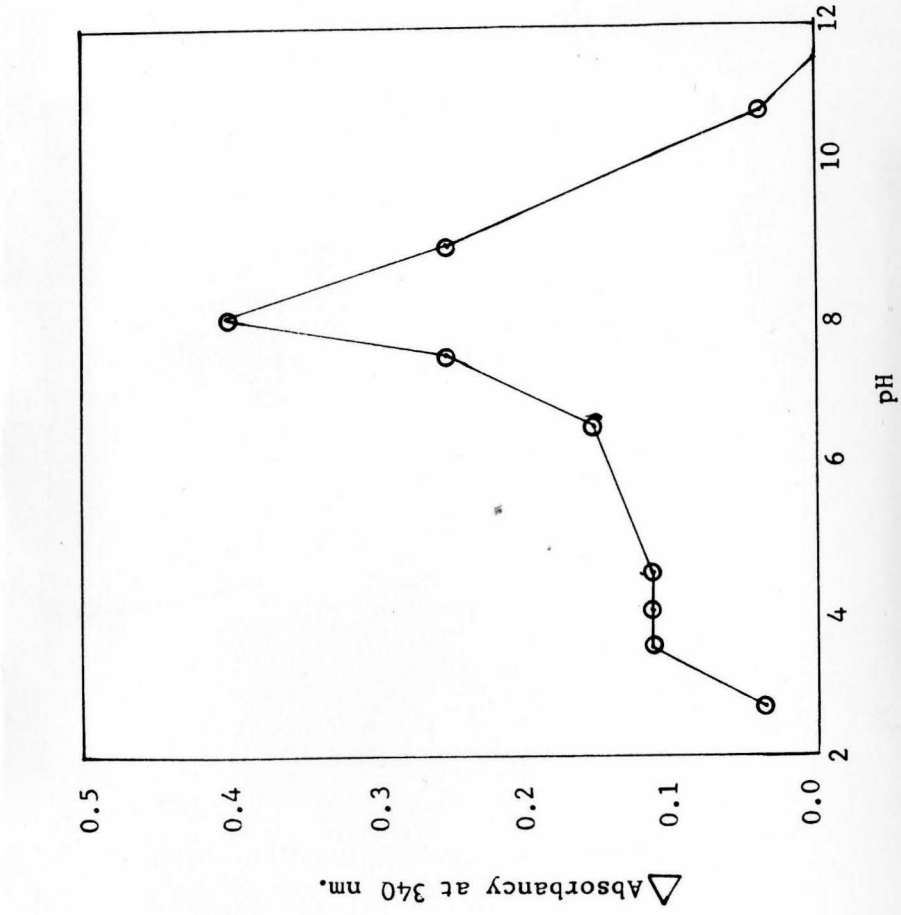


FIGURE 18: pH stability curve. Tubes containing the cell-free extract were kept in ice and the pH was adjusted to the above values. After 30 min., the pH was adjusted to 7.5 by cold NaOH and HCl solutions. The specific activities of all the samples were measured in order to find out at which pH the enzyme remained most stable.

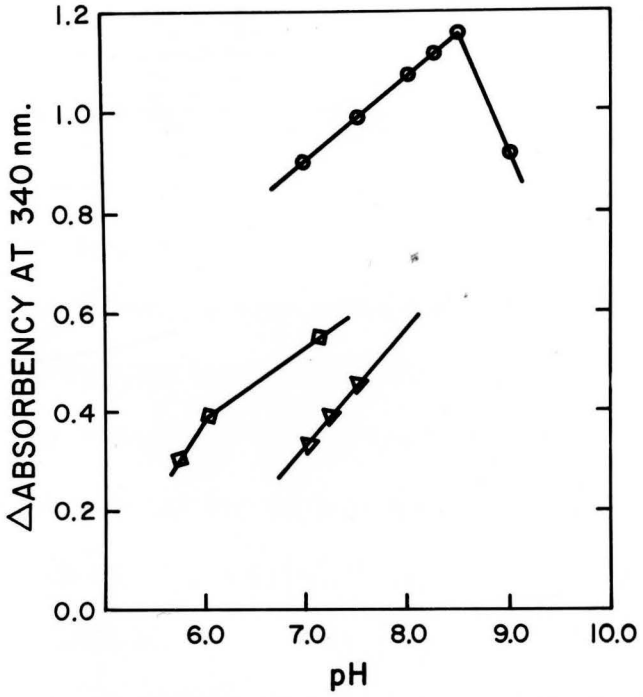


FIGURE 19. Effect of pH on KGS-dehydrogenase activity. S-3 enzyme preparation was used. Buffers tested were Tris-HCl (o), potassium phosphate (Δ), and Tris-maleate (◇).

EDTA does not inhibit the enzyme activity at a concentration of 8.0×10^{-3}M, it appears unlikely that cations are still present in the S_3 enzyme preparation.

Effect of various compounds and ions on enzyme stability and activity. The activity of KGS dehydrogenase was reduced after storage at 4 C and -20 C (Table 7). However, EDTA, DTT and NAD in combination not only stabilized the enzyme, but also stimulated its activity. There was a 10-fold increase in activity when the enzyme was stored for 64 hr at 4 C with these 3 compounds present compared to the activity of the enzyme kept for the same length of time in their absence. The enzyme activity was more than twice as high after 64 hr.at 4 C with these three stabilizers present than at it was with them after only 16 hr.

Freezing and thawing also affected the activity of enzyme. However, with KGS-dehydrogenase there was little decrease in activity when the S_3 preparation was frozen and thawed twice after storage at -20 C (Table 6.). EDTA and GSH stabilized the enzyme somewhat while NAD and NADP also protected the enzyme, both working more effectively at 4 C than at -20 C. KG, the product of the enzyme, together with NADP, did not stabilize the enzyme. The enzyme seemed to be stable in HEP buffer although EDTA and DTT together stabilized the enzyme but did not stimulate it as well as when NAD was also included.

Effect of substrate concentration. The effect of KGS concentration on the activity of KGS-dehydrogenase was studied with an enzyme preparation purified through step S_3. The data, plotted by the method of Lineweaver and Burke, are presented in Fig. 20. The K_m value was 1.15×10^{-4}M and there was an inhibitory effect with high concentrations of substrate.

Table 7

Effect of various compounds and ions on the stability and enhancement of enzyme activity after storage of the partially purified enzyme at 4 C and -20 C.

Additions to enzyme	Specific activity + units/mg, after storage for					
	16 hr		64 hr		112 hr	
	-20 C	4 C	-20 C	4 C	-20 C	4 C
None	0.19	0.19	0.18	0.09	0.08	0.08
EDTA(8mM)+GSH(5mM)	0.29	0.30	0.73	0.37	0.40	0.19
EDTA(8mM)+ DTT(1.6mM)						
DTT+Ca^{2+} $+Mg^{2+}$ $+Fe^{3+}$	0.08	0.40	0.13	0.37	0.08	0.19
NADP(1.6 mM)	0.40	0.70	0.30	0.40	0.30	0.50
NAD (1.6 mM)	0.40	0.50	0.40	0.52	0.30	0.40
EDTA (8 mM) +DTT(1.6 mM)						
+NAD(0.16 mM)	0.6	0.40	0.85	0.97	0.60	0.70
*HEP +Ca+Mg	0.29	0.29	0.40	0.42	-	-
KG + NADP	0.19	0.20	0.25	0.18	-	-
HEP(0.02 M) $+Ca^{2+}$ $+Fe^{3+}$	0.18	0.18	0.43	0.18	-	-

*HEP = N-2 hydroxyethyl piperazine, N-2 ethanesulphonic acid
DTT = Dithiothreitol

†The initial specific acticity was 0.215.

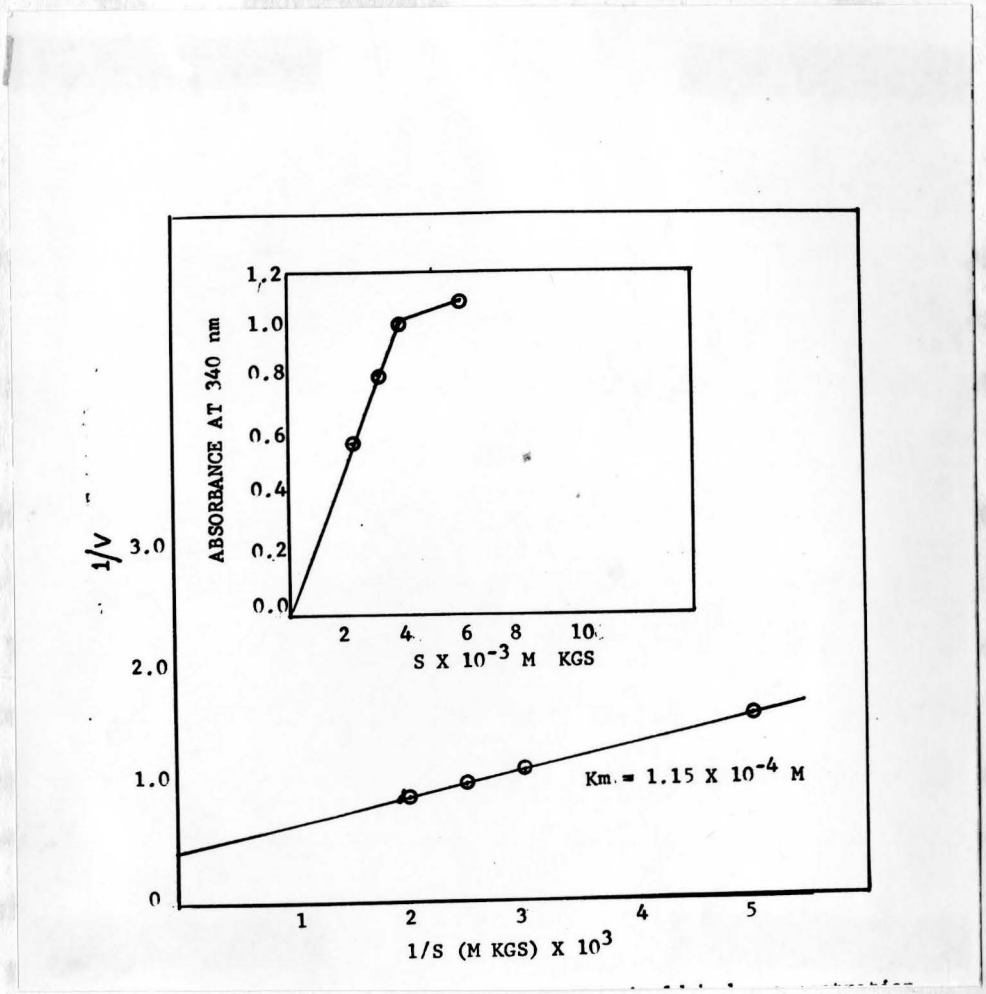
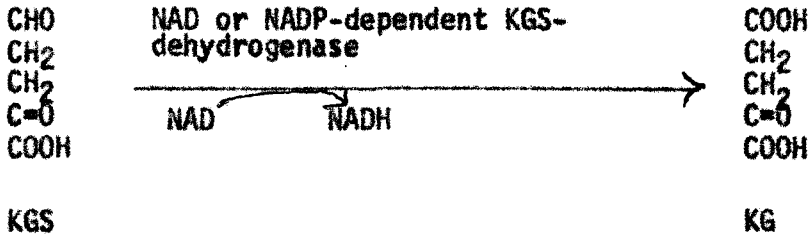


FIGURE 20: Effect of alpha-ketoglutarate semi-aldehyde concentration on KGS-dehydrogenase. Reaction conditions are those described in the standard assay. The enzyme used was purified through Step S_3.

3.14. Reaction 4. The fourth reaction in glucarate metabolism is the oxidation of KGS to KG in the presence of NAD or NADP. This NAD or NADP-dependent oxidation of KGS is catalyzed by the enzyme KGS-dehydrogenase.



The product of this reaction was isolated and identified as α-ketoglutarate. Glucaric acid or KDG was incubated with cell-free extract or with partially purified preparations of the enzyme system in the case of KDG or KGS. The incubation mixture contained in a final volume of 12 ml: sodium or potassium glucarate, 40 micromoles; Tris-HCl buffer, pH 7.5, 800 micromoles; $MgCl_2$ 50 micromoles; sodium arsenite, 12 micromoles; NAD, 1.2 ml of a 10 mg/ml solution. The reaction was started by the addition of enzyme and terminated by placing the reaction vessel in boiling waterbath for 2 min. KG was separated and isolated by anion exchange chromatography as already described. The tubes containing semicarbazide positive material were lyophilized.

KG was identified as the product of reaction 4 by chromatography of the keto acid accumulated; the reaction was incubated for one hour in the presence of NAD. The compound had the same Rf value as authentic KG in three solvent system while the compound isolated by column chromatography also had the same Rf (Table 8.).

Table 8

Paper and thin layer chromatography of authentic KG, a compound in reaction mixture and a alpha-keto acid isolated from the reaction mixture by column chromatography

Compound	Solvent	Rf
Authentic KG	A	0.65
Isolated compound (Peak 4)	A	0.66
Authentic KG	B	0.26
Reaction mixture	B	0.20
Reaction mixture	A	0.66
Authentic KG	D	0.75
Isolated compound (Peak 4)	D	0.75
Ascending paper chromatography		
Authentic KG	B	0.15
Reaction mixture	B	0.10
Thin layer chromatography		
Authentic KG	A	0.87
Reaction mixture	A	0.865
The paper chromatography of Peak IV after conversion with glutamate dehydrogenase.		
Isolated Peak 4 after conversion to glutamate	E and D	0.04 and 0.17 respectively
Authentic KG	E	0.04
Authentic KG	D	0.17
Co-chromatography	E	0.03
Co-chromatography	D	0.16

The 2,4-dinitrophenylhydrazones of authentic KG, of the keto acid accumulated in the reaction mixture, and of the isolated compound, were prepared by incubating with 0.1% DNP for 30 min at room temperature. The precipitate formed was treated in the manner described in methods (2.124) upon ascending paper chromatography in solvent C the Rf values of the DNPH derivatives of authentic KG of isolated keto acid and the compound in the reaction mixture were 0.43, 0.44, 0.45, respectively. Upon descending chromatography in solvent A the Rf values were 0.74, 0.72 and 0.75 respectively. All three derivatives had the same absorption spectra, with maxima at 372 nm in 95% ethanol and 412-416 nm, with a shoulder at 552 nm, in alkaline ethanol (Fig. 14, 15).

The isolated compound was cozymatically converted to glutamate by glutamate dehydrogenase and the glutamate was identified by chromatography and co-chromatography.

3.141. Quantitative study of KG formation. When GA was incubated with cell-free extract and NAD, 75-80% of the GA was converted into KG, as measured by the glutamate dehydrogenase assay (Table 9.). Also, partially purified KGS-dehydrogenase catalyzed the oxidation of KGS with about 70% of the synthetic KGS converted to KG in the presence of NAD (Table 5.).

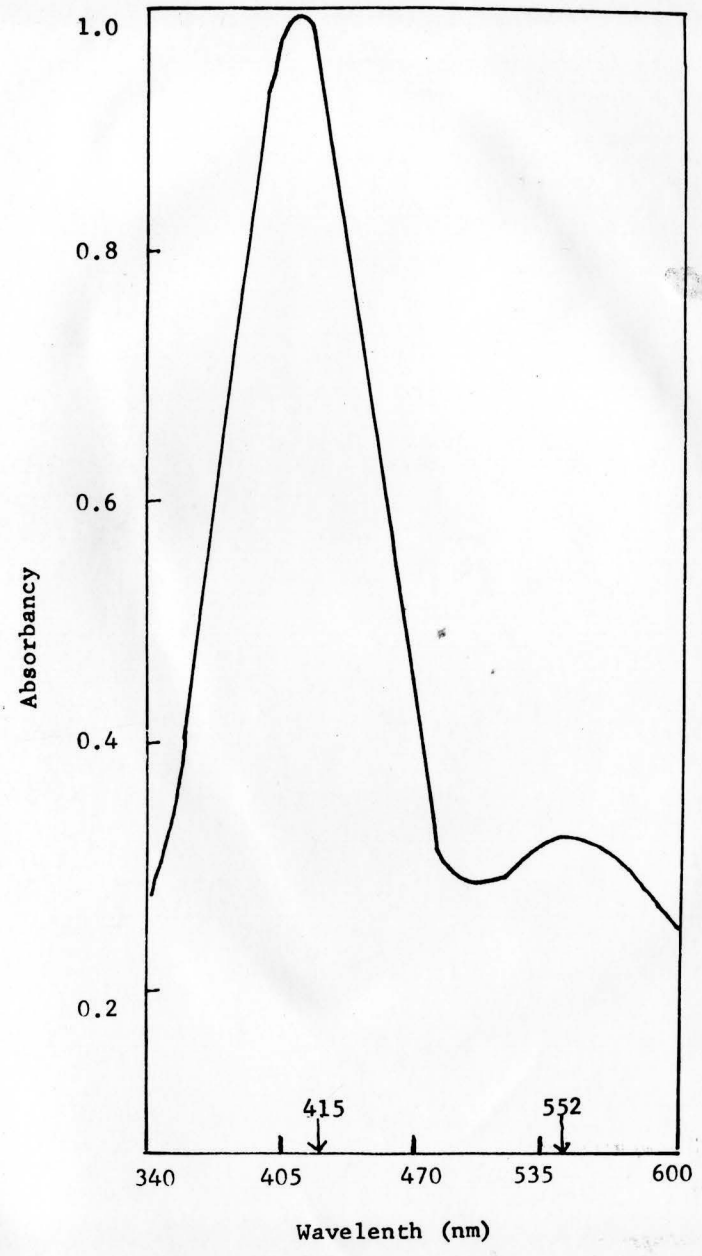


FIGURE 14: Absorption spectrum of the 2,4-dinitrophenylhydrazone of enzymatically formed peak 4 in alkaline ethyl alcohol.

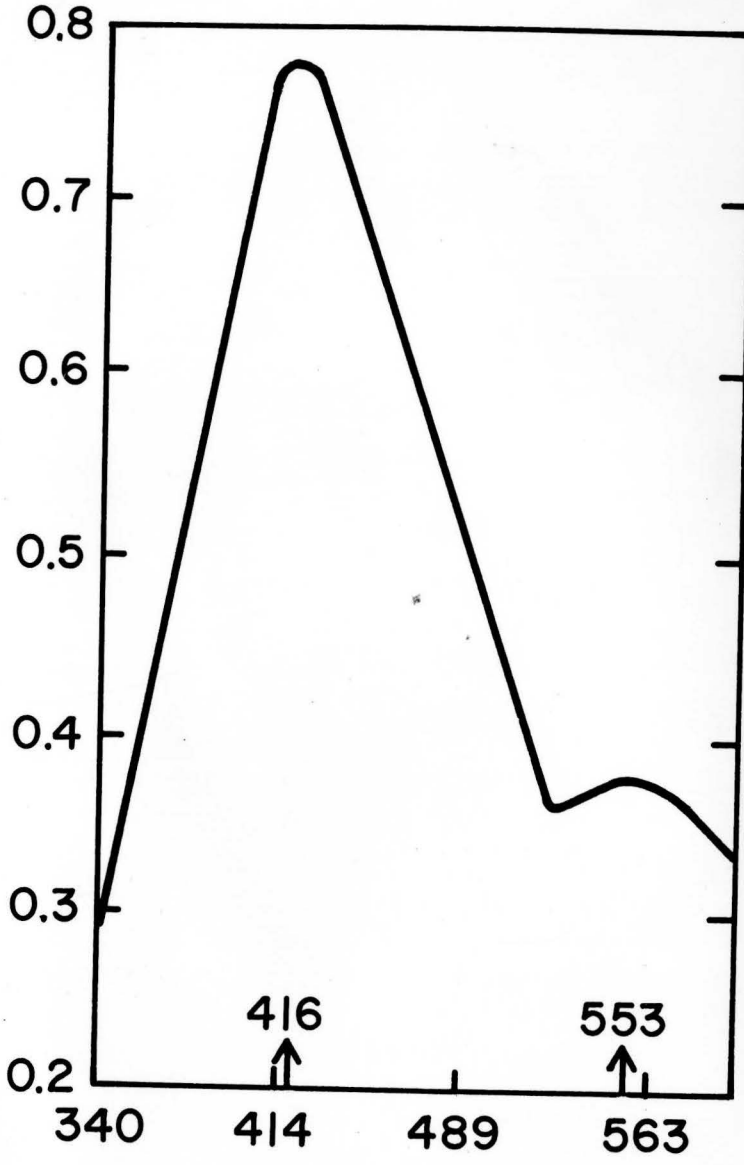


FIGURE 15: Absorption spectrum of the 2,4-dinitrophenylhydrazone of authentic alpha-ketoglutaric acid. Sample was made alkalin by 10% NaOH to a proper concentration for absorbancy measurement.

Table 9

Time course of alpha-ketoglutaric acid formation by a cell-free extract of *B. megaterium* in the presence of NAD.

Time, min.	Initial A_{340}	Final A_{340}	Difference in A_{340}	KG*
0.0	0.9	0.874	0.026	0.004
10	0.95	0.74	0.21	0.05
15	0.99	0.69	0.30	0.075
20	0.99	0.60	0.39	0.094
40	1.0	0.51	0.58	0.14
80	1.1	0.40	0.70	0.17

*KG in micromoles/1.5 ml. 10 micromoles of glucaric acid were converted to 7.0 micromoles of KG, i.e. 70% conversion, in the glutamate dehydrogenase assay.

4.0 DISCUSSION

The proposed pathway of D-glucaric acid utilization in *Bacillus megaterium* strain KM is based on the experimental evidence presented in this thesis and on the evidence of Blumenthal and Jepson (Fig. 4).

During studies on the time course of D-glucaric acid catabolism by cell-free extracts of glucarate grown *Bacillus megaterium*, there was an initial appearance and then disappearance of a TBA-positive compound with a maximum at 551 nm indicating that an intermediate with an α-keto-β-deoxy sugar acid was involved in glucarate catabolism.

Ketodeoxy sugar acids are now known to be intermediates in the catabolism of glucose (16), glucarate and galactarate (18), polygalacturonate (36), D-altronic and mannonic acids (44), hyaluronic acid (32), chondroitin sulfate (25), pectin (3,46) and alginic acid (49,37). Also, ketodeoxy sugar acids are known to be intermediates in the biosynthesis of 2-keto-3-deoxyoctonic acid-8-phosphate (31), 2-keto-3-deoxy-D-araboheptonic acid-7-phosphate (45) and N-acetylneuraminic acid-9-phosphate (28). The 2-keto-3-deoxy sugar acids which contain 5 and 6 carbon atoms are formed by dehydration of the corresponding aldonic or aldaric acids and are primary intermediates in the degradation of the parent compounds while the 2-keto-3-deoxy sugar acids containing 7-9 carbon atoms are primarily formed by aldol condensations.

According to Weissbach and Hurwitz (53), a TBA-positive material should have a structure which on cleavage with periodate forms β-formylpyruvate, yielding a pink chromogen with thiobarbituric acid with an absorption maximum near 550 nm. Compound 1, the production of reaction 1 in glucarate catabolism in *B. megaterium*, has an α-keto-β deoxy moiety in its structure since it yielded a chromogen with an absorption maximum at 551 nm (Fig.6). Compound 1 also formed a semicarbazone which absorbed at 258 nm indicating that it was an α-keto acid. Since compound 1 was formed after enzymatic dehydration of glucarate, it must contain at least two adjoining hydroxyl groups providing a site for periodate cleavage. Also, since the TBA-positive material, as well as authentic KDG or glucaric acid, formed KG on incubation with cell-free extracts by a series of reactions involving decarboxylation at 1-C^{14}, compound 1 must contain six carbon atoms.

On separating the intermediates in the incubation reaction mixture by column chromatography, four major peaks were eluted from the column (Fig.5). Peak 3, probably contains 4,5-KDG although in *E. coli* both 2-keto-3-deoxy- and 5-keto-4-deoxy-D-glucarate are formed, with the latter predominating. This reasoning is based on the findings of Blumenthal and Jepson (6) that *B. megaterium* KM yielded $^{14}CO_2$ and unlabeled KG from $^{1-14}$C-glucarate. The TBA-positive compound from P-3 and authentic KDG both formed DNP-hydrazones which had the same Rf values during paper and thin layer chromatography (Table 2).

Finally, there was a gradual disappearance of authentic KDG when it was incubated with either a crude extract or a charcoal-treated crude extract of glucarate grown cells (Fig.7) and 55-75% ammonium sulfate fraction catalyzed the reduction of NAD to NADH in the presence of KDG. These data prove that compound 1 is KDG. Although the glucarate dehydrase of *B. megaterium* was not purified, the formation of KDG and its ultimate conversion to KG, which was identified by paper chromatography and by its conversion to glutamate by glutamate dehydrogenase, served as a proof that glucarate dehydrase was involved in the metabolism of D-glucarate. Thus, in all microorganisms studied thus far, including the present gram positive bacterium and fungi (55), the first product of D-glucarate catabolism is KDG, even though the subsequent metabolic routes may vary somewhat.

In *E. coli*, KDG is cleaved by KDG aldolase to form molecules of tartronate semialdehyde and pyruvate. The TAS is then reduced to glycerate in the presence of NADH(Fig.1). In *B. megaterium* the second step seemed to be another dehydration, this time of KDG, and the product of this dehydration, the product of reaction 2, was tentatively identified as 2,5-diketoadipic acid. Chang (10) and Chang and Feingold (11) have presented some preliminary evidence that such a reaction occured during the metabolism of glucaric acid by *Agrobacterium tumefaciens*.

During time sequence incubation studies it was found that a keto acid was accumulated when either D-glucaric acid or KDG was incubated with charcoal-treated crude extract or with a partially purified enzyme preparation

without NAD. In examining the molecular structure of KDG there seemed to be two possible ways for the further catabolism of KDG: (1) decarboxylation, or (2) dehydration. In the first instance, the product of decarboxylation would be 2-keto-3-deoxy-D-arabonate (KDA), while in the second case the dehydration should yield 2,5-diketoadipic acid (DKA).

Both of the possibilities of KDG degradation were examined. When samples were taken during incubation at various time intervals and then chromatographed, no benzidine-periodate positive spot other than KDG was detected. Since KDA yields a positive spot in the benzidine-periodate test, while DKA does not, this finding favored DKA as an intermediate. Moreover, if we assumed that KDA was one of the intermediates, the glucarate grown cells should have had an enzyme system capable of converting the synthesized KDA to KGS or KG. However, NAD was neither reduced to NADH in the presence of authentic D,L-KDA by enzyme preparations, nor was it capable of converting KDA to KG. It seemed, therefore, that KDA was not an intermediate in the pathway of glucarate utilization in *B. megaterium*.

Jeffcoat et al (27) recently presented a preliminary report suggesting that KDG is converted to KGS by a single purified enzyme preparation that both dehydrated and decarboxylated KDG without any intermediate between KDG and KGS. They reported that this purified enzyme behaved like a single enzyme in many tests. However, in *B. megaterium* there is good evidence that there is an additional step, a dehydration of KDG(Reaction2) yielding DKA. This is based on the observation of four major peaks of keto acids on separating the intermediates accumulated in the reaction mixture and on the paper chromatography of the accumulated keto acids (Table 3).

On further studies of the compounds in these four peaks, after lyophilization, it was found that peak 2 was a keto acid that formed a DNP-hydrazone with an absorption spectrum different from the DNPH derivatives of KG, KGS or KDG. The keto acid also accumulated during incubation of KDG, in the absence of NAD, with either charcoal-treated crude extract or with partially purified enzyme preparations. It was noted that the isolated keto acid yielded small quantities of the DNPH and hence no sharp spectra were obtained. It seemed that eitherthe isolated compound was unstable, as previously reported by Chang(10), or that a major portion of this keto compound was converted to its enol form, which does not form a DNPH. However, the crude synthetic DKA also formed a DNPH derivative with a spectrum identical to that of the DNPH derivative prepared directly from the keto acid accumulated by incubation. During paper chromatography of the peak 2 keto acid, both the isolated compound and the accumulated keto acid accumulated in the incubation medium yielded two spots, one of which had a Rf value similar to that reported by Chang(10) for DKA.

Finally, in the presence of chemically prepared DKA and a cell-free extract of glucarate grown cells, NAD was slowly reduced to NADH. Thus, the accumulated experimental evidence indicated that DKA was one of the intermediates in glucarate metabolism by *B. megaterium*.

The third reaction in glucarate catabolism was the decarboxylation of DKA yielding KGS. The KGS was identified as follows: 1a. The isolated compound 3(peak 1, Fig.5) formed a DNPH derivative which had an absorption

spectrum identical to that of authentic KGS-DNPH(Fig. 11); b. Upon incubation of KDG or glucarate with crude extract in the absence of NAD, a keto acid accumulated which yielded a DNPH derivative with an absorption spectrum similar to the DNPH of compound 3(Fig.12); (2) a. Authentic KGS was converted to KG with partially purified KGS-dehydrogenase in the presence of NAD. (Table 5).b. NAD was reduced to NADH in the presence of authentic KGS and either a crude extract or a partially purified enzyme preparation of glucarate grown cells (Fig.16,17); (3) KGS-dehydrogenase was isolated, partially purified and characterized.

The enzymatic formation of KGS involves a decarboxylation step (reaction 3). The site of the decarboxylation seems to be carbon 1 since 1-C^{14} glucaric acid yielded $^{14}CO_2$ when incubated with cell-free extract (6). In *Pseudomonas* species three quarters of the CO_2 arose from 1-C of either glucaric or galactaric acids in the enzymatic decarboxylation. However, in *A. tumefaciens* the site of decarboxylation has not been established.

The product of reaction 4, the end product of D-glucarate metabolism in *B .megaterium*, was identified as KG. KG has been reported to be an end product formed through KGS oxidation in a number of metabolic pathways(10, 12, 37-38,43). KG formation in *B. megaterium* was demonstrated following its isolation from reaction mixtures. It was identified by paper chromatography of the free acid in 4 solvent systems, and by chromatography of its DNPH derivative in three solvent systems. Also, the isolated compound and the keto acid accumulated in the reaction were converted to glutamic acid with glutamate dehydrogenase, NH_4^+, and NADH. The glutamic acid formed was then identified by paper chromatography in 3 solvent systems. Quantitative

studies of the formation of KG from glucaric acid and KGS showed that 70-80% of these compounds were converted to KG(Table 5,9).

Thus, glucarate metabolism in *B. megaterium* closely resembles the hexarate metabolism in various *Pseudomonas* species and in *A. tumefaciens* (Fig.2,3). In all three pathways the glucarate is converted to KGS which is in turn oxidized to KG through NAD or NADP-dependent dehydrogenase.However, glucarate metabolism in *B. megaterium* differs from that in *Pseudomonas* sp. by an additional step between KDG and KGS. In the *pseudomonads*, glucarate is dehydrated to KDG which in turn is converted to KGS, whereas in *B. megaterium* KDG is further dehydrated to DKA and then is decarboxylated to KGS. *A. tumefaciens* apparently has the same pathway as that present in *B. megaterium*.

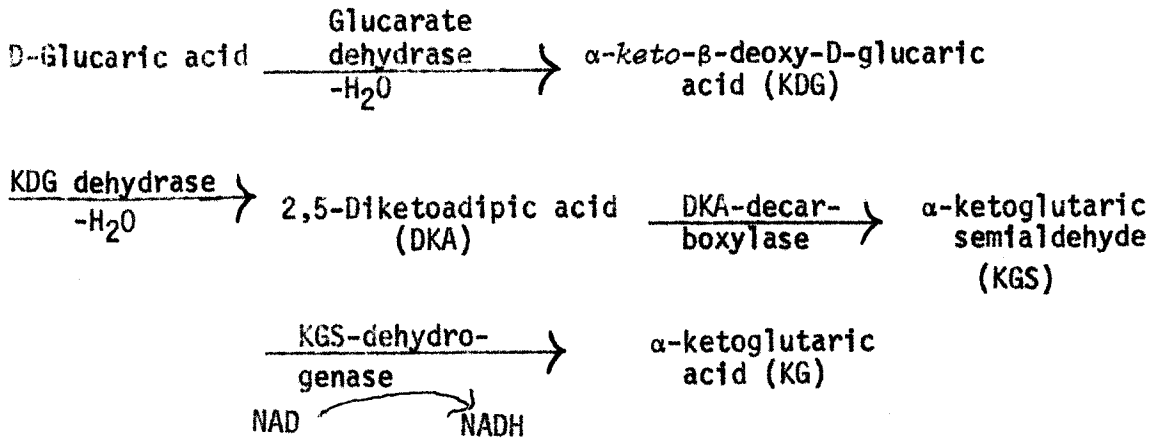
The enzyme which catalyzed the oxidation of KGS to KG was identified both in a *Pseudomonas sp.* and in *A. tumefaciens*. However, the purification and characterization of this enzyme has not been described from either of these sources. The KGS-dehydrogenase was isolated, partially purified and characterized in the present study, from glucarate grown cells of *B. megaterium*. It resembled the KGS-dehydrogenase described by Adams and Rosso prepared from hydroxyproline or glucarate grown cells of *Pseudomonas striata* (1,2). The observation that the KGS-dehydrogenase of *B. megaterium* was eluted in two separate peaks during DEAE-cellulose column chromatography was similar to the findings of Adams and Rosso(2). They reported that the *P. striata* KGS-dehydrogenase existed in two forms, one constitutive and the other induced mainly by the growth of cells on hydroxyproline or glucarate. They further suggested that the constitutive isoenzyme, which

differed from the induced enzyme in physico-chemical properties, was related to lysine metabolism. The constitutive enzyme was not examined in the present study.

Due to its instability, the induced *B. megaterium* KGS-dehydrogenase was not highly purified. However this preparation resembled the purified KGS-dehydrogenase of *P. striata* in many properties. Not enough characteristics of the enzyme have been observed to compare it with the KGS-dehydrogenase of hydroxyproline grown cells of *P. striata*.

5.0 SUMMARY

The inducible metabolism of D-glucaric acid in *Bacillus megaterium* strain KM was found to follow the pathway:



The presence of glucarate dehydrase, which catalyzed the dehydration of glucaric acid to KDG, was demonstrated in the cell-free extracts of glucaric acid grown cells. KDG was identified chromatographically, chemically and enzymatically.

KDG was dehydrated to the metabolic intermediate DKA, which was isolated by anion exchange chromatography. It was tentatively identified by paper chromatography following its conversion to the 2,4-dinitrophenylhydrazone (DNPH) derivative. The derivative had an absorption spectrum that was different from that of the KGS-DNPH and KG-DNPH, but was similar to the spectrum of the DNPH derivative of synthetic DKA.

KGS was shown to be involved in the glucarate metabolic pathway by the demonstration of its enzymatic formation. The KGS was isolated and converted to its DNPH derivative, which had the same absorption spectrum as the DNPH derivative of synthetic KGS.

The relatively labile induced KGS-dehydrogenase was isolated, partially purified and some of its properties were determined. This enzyme catalyzed the NAD or NADP-dependent oxidation of KGS to KG. KG was identified, following its isolation, by the chromatographical chemical properties of the free acid and its DNPH derivative. The isolated KG was also converted by glutamic dehydrogenase to glutamic acid, which was identified by paper chromatography and co-chromatography.

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APPROVAL SHEET

The thesis submitted by Brahma Sharma has been read and approved by three members of the faculty of the Stritch School of Medicine, Loyola University.

The final copies have been examined by the director of the thesis and the signature which appears below verifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

1/16/20
Date

Signature of Adviser