Carboligase Activity of α-Ketoglutarate Dehydrogenase*

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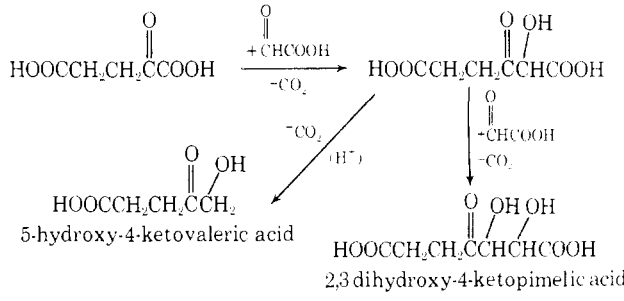
ABSTRACT: The enzyme which catalyzes a decarboxylative condensation of α-ketoglutarate with glyoxylate has been purified about 100-fold from beef heart. It also catalyzes the simple decarboxylation of α-ketoglutarate as well as the formation of 5-hydroxy-4-ketohexanoic acid from acetaldehyde plus α-ketoglutarate; all three enzymatic activities were parallel during the purification procedure. In addition to 5-hydroxy-4-ketovaleric acid (which is formed by a secondary decarboxylation of 2-hydroxy-3-ketoadipic acid) another product formed in the reaction between α-ketoglutarate and glyoxylate has now been identified as 2,3-dihydroxy-4-ketopimelic acid.

A condensation reaction between α-ketoglutarate[1] and acetaldehyde to form 5-hydroxy-4-ketohexanoic acid was originally detected in studies of alcohol metabolism *in vitro* (Westerfeld and Bloom, 1966; Bloom and Westerfeld, 1966; Bloom *et al.*, 1966). This reaction is analogous to the formation of acetoin from pyruvate plus acetaldehyde, or the formation of other acyloins from α-keto acids plus aldehydes (Berg and Westerfeld, 1944); the reaction between pyruvate plus succinic semialdehyde to form 5-keto-4-hydroxyhexanoic acid has also been demonstrated (Shaw and Westerfeld, 1968). KG also reacts with some aldehydes other than acetaldehyde, and 5-hydroxy-4-ketovaleric acid (HKV) has been identified (Schlossberg *et al.*, 1968) as an acid–decarboxylation product of one of the major metabolites (2-hydroxy-3-ketoadipic acid) formed in this type of condensation reaction between KG and glyoxylate. The synergistic decarboxylation of glyoxylate and KG was first demonstrated by Crawhall and Watts (1962), and the enzyme which catalyzes this reaction has been purified about 100-fold by Koch and Stokstad (1965) and by Stewart and Quayle (1967) from rat and pig liver mitochondria, respectively. Schweet *et al.* (1951) showed that the formation of acetoin was catalyzed by the pyruvate dehydrogenase complex, and an analogous relationship seemed likely for the KG condensation reactions.

The purpose of this communication is to report that: (1) the same enzyme complex, ketoglutarate dehydrogenase (2-oxoglutarate:lipoate oxidoreductase (acceptor acylating), EC 1.2.4.2), is responsible for the condensation reaction between KG and either acetaldehyde or glyoxylate; (2) the purified enzyme forms two products in the reaction with glyoxylate, and (3) the second product, in addition to HKV has now been identified as 2,3-dihydroxy-4-ketopimelic acid (DHKP).

The reaction between KG and glyoxylate can, therefore, be given as:



Methods

Glyoxylic acid was determined by the method of Dekker and Maitra (1962). HKV was assayed by the periodate–chromotropic acid procedure of Frisell and MacKenzie (1958). HKH and acetoin were separated and determined as described by Bloom *et al.* (1966). Protein was determined by the method of Lowry *et al.* (1951) using crystalline bovine serum albumin as a standard.

Enzyme Assays

KG Dehydrogenase. The incubation medium was prepared by mixing: 100 μmoles of potassium phosphate buffer, pH 6.5; 50 μmoles of neutralized 1-[^{14}C]KG (222,000 dpm) obtained from Calbiochem; 0.4 μmole of TPP; 20 μmoles of $MgCl_2$; 30 mg of bovine serum albumin (fraction V from Armour & Co.); tissue sample and water to a final volume of 2.9 ml. Incubations were carried out with shaking in a closed system at 37.5° and with Hyamine hydroxide (0.2 ml of a 1 M solution in methanol) and a filter paper in the center well. The reaction was started after temperature equilibration by injecting 0.1 ml of 0.5 M potassium ferricyanide into the main compartment, and was stopped after 15 min with 0.3 ml of 50% trichloroacetic acid. The beakers were shaken for an additional 30 min to permit the complete trapping of the [^{14}C]CO_2 by the Hyamine. The center well contents were mixed with 11 ml of Bray's solution as the scintillant and counted in a Beckman LS-150 liquid scintillation system with externa

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[1] Abbreviations used are: HKH, 2-hydroxy-3-ketohexanoic acid; HKV, 5-hydroxy-4-ketovaleric acid; TPP, thiamine pyrophosphate; KG, α-ketoglutarate; HKA, 2-hydroxy-3-ketoadipic acid; DHKP, 2,3-dihydroxy-4-ketopimelic acid.

standardization. One unit was defined as that amount of enzyme which would produce 1 μmole of [^{14}C]CO_2/hr at pH 6.5 and 37.5°.

KG–Glyoxylate Carboligase. The incubation medium contained 20 μmoles of neutralized 1-[^{14}C]glyoxylate (44,400 dpm) obtained from Nuclear-Chicago Corp.; 20 μmoles of neutralized KG; 100 μmoles of potassium phosphate buffer, pH 7.1; 0.4 μmole of TPP; 10 μmoles of $MgCl_2$; tissue and distilled water to a final volume of 3 ml. After incubation for 15 min at 37.5°, the reaction was stopped by injecting 0.15 ml of 25% trichloroacetic acid plus 0.15 ml of 0.2 M 4-aminoantipyrine. The [^{14}C]CO_2 was collected and assayed as described for the ketoglutarate dehydrogenase assay. One unit was defined as that amount of enzyme which would produce 1 μmole of [^{14}C]CO_2/hr at pH 7.1 at 37.5°.

KG–Acetaldehyde Carboligase. The incubation medium contained: 20 μmoles of neutralized KG; 214 μmoles of acetaldehyde; 100 μmoles of potassium phosphate buffer, pH 7.1; 0.4 μmole of TPP; 10 μmoles of $MgCl_2$; 20 mg of albumin; tissue and water to 3 ml. The reaction was initiated by the addition of the acetaldehyde. After incubation for 1 hr at 37.5°, the proteins were precipitated by injecting 0.3 ml of 70% perchloric acid and were removed by centrifugation. The supernatant was neutralized with potassium hydroxide (4.5 M) to phenol red, refrigerated overnight, and centrifuged to remove potassium perchlorate. The separation and analysis of both HKH and acetoin were performed as described by Bloom *et al.* (1966). One unit of HKH activity was defined as that amount of enzyme which would produce 1 μmole of HKH/hr at pH 7.1 and 37.5°.

Pyruvate–Acetaldehyde Carboligase. The pyruvate enzyme was measured by the HKH procedure except that 40 μmoles of sodium pyruvate plus 107 μmoles of acetaldehyde replaced the KG and acetaldehyde listed above. One unit was defined as that amount of enzyme which would produce 1 μmole of acetoin/hr at pH 7.1 and 37.5°.

Purification of KG–Glyoxylate Carboligase.[2] Several hundred grams of trimmed beef heart was homogenized in a Waring Blendor for 5 min at high speed and then 10 min at low speed with about two volumes of a medium consisting of 0.005 M potassium phosphate buffer, pH 7.2, made 0.2 M with respect to KCl. The temperature was maintained at 4° by the addition of crushed ice, and enough homogenizing medium was added to give a final 1:3.5 homogenate. After centrifuging at 14,600*g* for 60 min in a Servall refrigerated centrifuge using 250-ml polycarbonate bottles with a GSA head, the precipitate was discarded.

The thick, dark red extract was brought to 50% saturation with solid ammonium sulfate, and cold 1 M KOH was added in order to maintain the pH at 7. After stirring for an additional 15 min the mixture was centrifuged at 14,600*g* for 30 min. The resulting precipitate was suspended in enough 0.03 M phosphate buffer, pH 7.2, to give a final ammonium sulfate concentration of 5–10%. The suspension was either frozen overnight and thawed the next day or allowed to stand for 0.5 hr. It was then centrifuged at 14,600*g* for 90 min. The yellow supernatant was brought to 40% saturation with ammonium sulfate, centrifuged for 30 min at 14,600*g*, and the resulting precipitate was homogenized in 200–300 ml of 0.03 M phosphate buffer, pH 7.2, made 0.001 M with respect to iodoacetate. It was then dialyzed overnight against 15 l. of the same buffer.

After dialysis, the gelatinous mixture was frozen for several days at −20°. On thawing, the bulky precipitate was centrifuged at 24,000*g* for 20 min using an SS-34 head. The supernatant was adjusted to a protein concentration of 10–15 mg/ml with 0.03 M phosphate buffer, pH 7.2. One volume of calcium phosphate gel[3] was then added and the mixture was stirred for 15 min and then centrifuged. Impurities were eluted from the gel by stirring for 15 min with an amount of 0.1 M phosphate buffer, pH 7.0, equal to the volume of enzyme solution originally adsorbed. After centrifugation this was repeated and both supernatants were discarded. The enzyme was then eluted with the same volume of 0.2 M phosphate buffer, pH 8.0, and precipitated with 40% ammonium sulfate. After centrifuging at 14,600*g* for 20 min the precipitate was dissolved in 20–30 ml of 0.03 M phosphate buffer, pH 7.2, made 0.001 M with respect to dithiothreitol.

The yellow enzyme solution was centrifuged at 105,000*g* for 6 hr in a No. 40 head in a Spinco Model L ultracentrifuge. The resulting bright yellow precipitate was frozen overnight in the centrifuge tubes. After thawing, the precipitate was homogenized in 4 or 5 ml of 0.03 M phosphate buffer, pH 7.2, containing 0.001 M dithiothreitol. The mixture was frozen again overnight in the homogenizing vessel, then thawed, rehomogenized, and centrifuged for 20 min at 24,000*g* to obtain the enzyme in solution.

Chromatography on DEAE-Cellulose. DEAE-Cellulose was prepared by leaching it with 1 N NaOH, and washing with alkali, 1 N HCl, and H_2O. After further washing with alkali and H_2O, the cake was suspended in 100 ml of 0.02 M phosphate buffer, pH 7.0, and 0.2 M KH_4PO_4 was added until the pH was 7.0 ± 0.1. The cellulose was then washed with about three volumes of 0.02 M phosphate buffer, pH 7.0, suspended in 500–600 ml of the buffer, poured into a 1.5-cm wide column, and washed with an additional 300 ml of buffer.

The bright yellow enzyme solution obtained from the freeze-thawing procedure was applied to such a DEAE-cellulose column, and was followed by 120 ml of 0.02 M phosphate buffer, pH 7.0, and then by 200 ml of 0.2 M phosphate buffer, pH 6.5; both buffers contained 0.001 M dithiothreitol. The active enzyme was eluted by the 0.2 M phosphate buffer.

Results

Table I shows that the relative distribution of the KG–glyoxylate carboligase activity in different rat tissues is the same as that previously reported for the KG–acetaldehyde reaction (Bloom *et al.*, 1966). The results of a typical fractionation of the beef heart enzyme are given in Table II. The overall purification from the initial extract was usually between 80- and 110-fold, and the overall yield between 1 and 7%. This is comparable with the 85–100-fold purification of the carboligase activity obtained by Stewart and Quayle (1967) from pig liver and by Koch and Stokstad (1965) from rat liver mitochondria. It also appears to be comparable with the 73–

[2] Some of the steps used in this purification are similar to those used by Kaufman *et al.* in the purification of KG dehydrogenase.

[3] The gel was suspended in enough 0.03 M phosphate buffer, pH 7.0, to give a final concentration of 19–20 mg/ml.

TABLE I: Distribution of KG–Glyoxylate Carboligase Activity in Different Rat Tissues.[a]

Tissue	Percent Incorporation of 1-[^{14}C]Glyoxylate into [^{14}C]CO_2	
	Homogenate	Particulate
Heart	62.6	56.4
Kidney	57.3	54.3
Liver	21.0	20.5
Skeletal muscle	13.3	6.4
Brain	8.5	6.4

[a] Each flask contained 20 μmoles of 1-[^{14}C]glyoxylate (44,400 dpm), 20 μmoles of KG, 80 μmoles of potassium phosphate buffer, pH 7.1, and 1 ml of tissue suspension equivalent to 100 mg of tissue wet wt. Incubation was for 1 hr at 37.5°. Carbon dioxide was trapped as described previously. [^{14}C]CO_2 (1 mole) is released in the formation of either HKV or DHKP. The particulate fraction was the pellet obtained by high-speed centrifugation (105,000*g* for 2 hr) of the whole homogenate.

140-fold purification achieved with KG dehydrogenase (Kaufman *et al.*, 1953; Hiroshima *et al.*, 1967). Electrophoresis of the DEAE-cellulose eluate (after dialysis of 2 ml of a 7% enzyme solution *vs.* barbiturate buffer, pH 8.6; 225 V; 3 mA) showed, after 1 hr, a trailing shoulder (on an otherwise single symmetrical peak) which represented about 5% of the total protein.

A comparison of the four different enzyme activities at various stages of the purification procedure showed that the pyruvate dehydrogenase activity was lost, but all three KG activities were concentrated together in a relatively constant ratio. While two-thirds of the KG–carboligase activity was solubilized by the KCl–phosphate, one-fourth or less of the pyruvate dehydrogenase was extracted, and essentially all of the latter was eliminated by the time the KG enzyme was eluted from the calcium phosphate gel. The KG decarboxylase and the KG–acetaldehyde carboligase activities paralleled the KG–glyoxylate carboligase activity during purification; a comparison of these activities in the original homogenate and the final DEAE-cellulose eluate is shown in Table III.

Attempts to remove the last small amount of impurity from 0.2 ml of the enzyme by layering it on 4.5 ml of 5–25% sucrose gradient containing 0.1 M phosphate buffer, pH 7.2, and centrifuging for 5 hr (25,000 rpm; SW-50 head; Spinco L) gave only a single broad peak of protein and enzyme activity in tubes 8–22, with peak at tube 14; fractions were collected by puncturing the bottom of the tube and collecting 15

TABLE II: Purification of α-KG–Glyoxylate Carboligase.

	Units (μmoles of CO_2/hr)	Protein (g)	Sp Act. (units/mg of Protein)
Homogenate	33,330	66.0	0.5
KCl extract	22,680	21.2	1.17
50% $(NH_4)_2SO_4$ precipitate	22,368	13.8	1.6
40% $(NH_4)_2SO_4$ precipitate	17,600	9.8	1.8
FT[a] supernatant	14,364	5.2	2.8
$CaPO_4$ eluate	3,021	0.212	14.2
FT[a] supernatant	1,744	0.051	34.1
DEAE eluate	1,044	0.022	47.8

[a] Freeze-thaw.

TABLE III: Specific Activities and Ratios of the Three KG Enzyme Activities in the Original Homogenate and the DEAE Eluate.

	Specific Activities		
	KG–Glyoxylate Carboligase	KG–Acetaldehyde Carboligase	KG Decarboxylase
Homogenate	0.674	0.360	0.176
DEAE eluate	54.800	25.450	11.000
	Activity Ratios		
	KG–Glyoxylate/ Decarboxylase	KG–Acetaldehyde/ Decarboxylase	KG–Glyoxylate/ KG–Acetaldehyde
Homogenate	3.83	2.05	1.87
DEAE eluate	4.98	2.31	2.15

TABLE IV: Specific Activities and Ratios of the Three KG Enzyme Activities in Fractions from the Sucrose Density Gradient Centrifugation.

	Specific Activities		
Fraction	KG–Glyoxylate Carboligase	KG–Acetaldehyde Carboligase	KG Decarboxylase
Tubes 1–12	91.379	59.561	18.182
Tubes 13–21	45.106	22.500	8.511
Pellet	34.839	19.484	7.741
	Activity Ratios		
	KG–Glyoxylate/ Decarboxylase	KG–Acetaldehyde/ Decarboxylase	KG–Glyoxylate/ KG–Acetaldehyde
Tubes 1–12	5.03	3.28	1.53
Tubes 13–21	5.30	2.64	2.00
Pellet	4.50	2.54	1.79

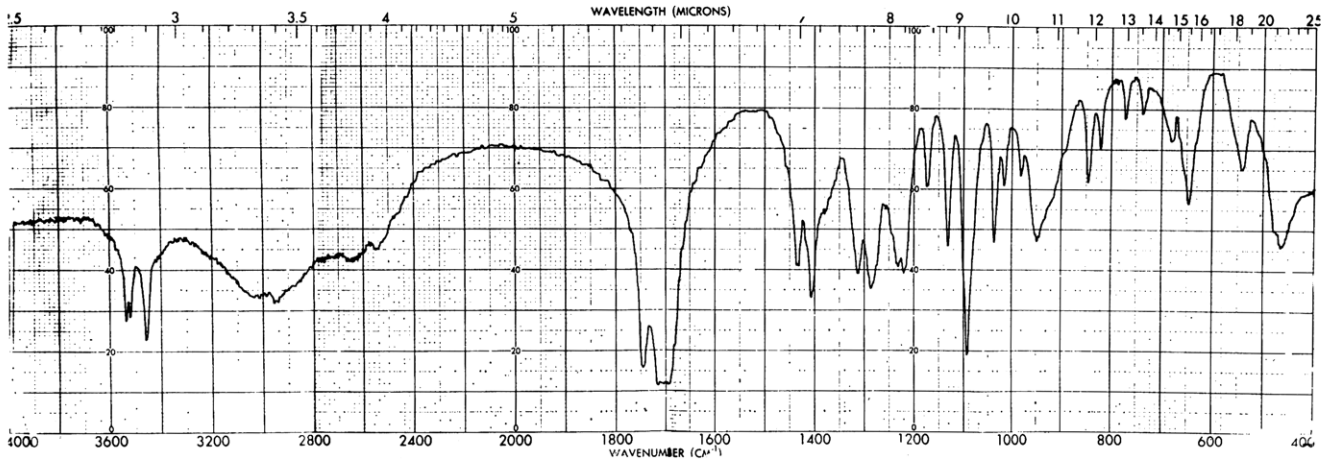


FIGURE 1: The infrared absorption spectra for compound A in KBr pellets.

drops/tube. However, there appeared to be slightly more protein in relation to enzyme activity on the trailing side of the peak. Centrifugation for 10 hr gave a bright yellow pellet (0.62 mg of protein) and two overlapping peaks in tubes 1–12 (1.28 mg of protein) and 13–21 (0.94 mg of protein). While the specific activity of the enzyme in tubes 1–12 was twice that of the pellet or the slower moving peak, the ratios of the three enzyme activities remained relatively constant in all three fractions (Table IV). Aggregation of the enzyme appeared to influence its activity with respect to all three reactions in the same way.

Isolation and Identification of DHKP

Both HKV and DHKP were isolated from incubations of KG plus glyoxylate with washed rat heart and beef heart particulate fractions by the following procedure. Rat heart (60 g) was homogenized with 360 ml of 0.06 M sodium phosphate buffer, pH 7.1, and centrifuged for 2.5 hr in a Spinco preparative ultracentrifuge at 21,000 rpm. The pellet was rehomogenized with buffer and centrifuged as before, and was finally resuspended by homogenizing with 300 ml of buffer. This rat heart particulate homogenate was incubated with 100 ml each of 0.2 M KG (pH 7.1) and 0.2 M glyoxylate (pH 7.1) at 37° for 2.5 hr with shaking. One preparation was made from 5-[^{14}C]KG; others were made from glyoxylate labeled in either C-1 or C-2 or both. Perchloric acid (40 ml, 70%) was then added and the mixture was shaken for an additional 2 hr; the solution was flushed frequently with N_2 to remove the CO_2 which otherwise interfered with subsequent chromatography. The protein precipitate was removed by centrifugation and washed with 5% perchloric acid. The supernatant and wash were combined, neutralized with solid potassium hydroxide to a pH of approximately 7, chilled overnight, and the precipitate of $KClO_4$ was removed by filtration.

The $KClO_4$ filtrate was chromatographed on Dowex 1-X10 (formate form) and Celite as previously described (Bloom and Westerfeld, 1966), and was followed by radioactivity. The elution from Dowex gave the following peaks and ranges of tubes over which each substance was eluted: HKV = 22 (20–25); unchanged glyoxylate = 25 (23–28); DHKP = 30 (27–33); unchanged KG = 50 (45–55); oxalate = 74 (72–78). The elution pattern for glyoxylate was determined colorimetrically (Dekker and Maitra, 1962) since it largely evaporated while drying the samples for counting in a Nuclear-Chicago D-47 gas-flow counter. The DHKP eluate (tubes 25–35) was evaporated to remove formic acid and all traces of glyoxylate, and the residue was rechromatographed on Dowex 1-X10 (formate form) and developed only with 0.5 M formic acid. Under these conditions the DHKP fraction separated into two peaks of about equal size: compound A peaked at tube 45 (range 40–55) and compound B at 65 (60–75). Each of these two fractions was chromatographed separately on Celite to provide further purification; compound A was eluted with a peak at tube 95 (range 80–100); compound B = 85 (75–98).

Compounds A and B were recrystallized from acetone with the addition of chloroform. Both were colorless, soluble in water, ethanol, and acetone, and insoluble in chloroform: A, mp 156–157°; B, mp 148–149°. The infrared spectra[4] of both A and B (Figures 1 and 2) have the same general features of strong acid, ketone, and alcohol absorptions, but they also show some differences. The nuclear magnetic resonance spectrum in D_2O of compound A gave a ten-line symmetrical pattern centered at δ 2.79 ppm (measured from an external tetramethylsilane reference) which is consistent for RCH_2-CH_2R' and a doublet, J = 3 Hz, at δ 4.55 ppm for a O—C—H adjacent to another O—C—H. The other CH to which it is coupled is obscured by the strong DOH resonance at δ 4.74 ppm. The nuclear magnetic resonance of compound B run under the same conditions showed a nearly identical ten-line pattern at δ 2.90 ppm for the RCH_2CH_2R' and an almost completely obscured pattern under the DOH resonance at δ 4.76 ppm which could be the RC(O)HC(O)HR'.

Except for the differences in melting points, chromatographic behavior, and small differences in spectra, compounds A and B were found to be identical, and are considered to be stereoisomers of DHKP. Neither rotated polarized light. *Anal.* Theory ($C_7H_{10}O_7$): C, 40.77; H, 4.85; compound A: C, 40.60; H, 5.06; compound B: C, 41.07; H, 5.16.

[4] We are indebted to Mr. A. L. Vulcano of the Research Division, Bristol Laboratories, Syracuse, New York, for the infrared and nuclear magnetic resonance spectra and their interpretation.

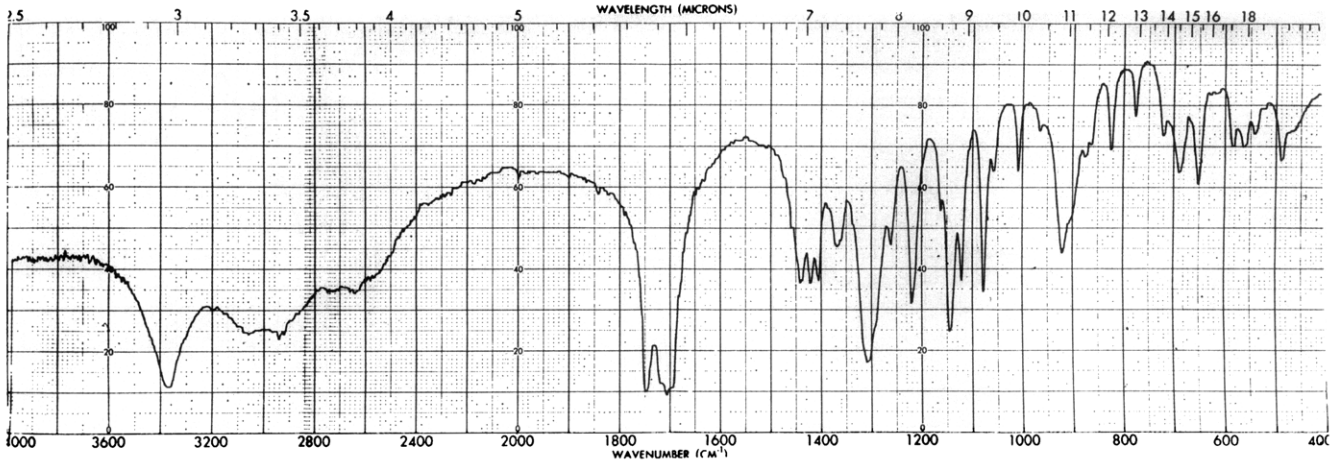


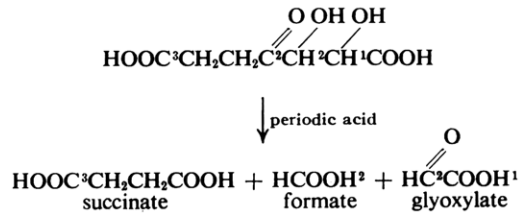
FIGURE 2: The infrared absorption spectra for compound B in KBr pellets.

Both A and B had the same titration curve, and each required 2 equiv of base for complete neutralization at pH 7–8. The orange-red 2,4-dinitrophenylosazones were identical and gave no depression in melting point on mixing; mp 280–283°. *Anal.* Found for compound B: N, 19.8. Theory ($C_{19}H_{16}N_8O_{13}$): N, 19.9. Each gave the same derivative with phenylhydrazine (mp 225–226°), but the exact composition of this derivative was not determined; it was atypically orange-red and probably a cyclized osazone (Bloom and Westerfeld, 1966).

Periodate Oxidation. Approximately 30 mg of compound A or B in 19 ml of water was treated with 1 ml of 0.55 M periodic acid at room temperature for 15 min. An aliquot was then removed to determine the periodate utilized (Jackson, 1944), while the remainder was neutralized to pH 7.2 with saturated $Ba(OH)_2$, chilled, and centrifuged to remove the barium periodate–barium iodate precipitate. Glyoxylate was determined colorimetrically (Dekker and Maitra, 1962) on an aliquot of the filtrate, and the remainder was chromatographed on Celite; each tube of eluate was titrated, counted for radioactivity, and analyzed colorimetrically for glyoxylate and formate (Snell and Snell, 1953). Periodate (2 moles) was used for each mole of DHKP added, and the three products of the reaction were identified as 1 mole each of glyoxylate, formate, and succinate (average of three experiments 0.93, 0.95, and 0.91, respectively).

Formate was eluted from the Celite at peak 18 (range 16–21) and was further identified by two colorimetric procedures (Snell and Snell, 1953) and by reduction with Mg + HCl to formaldehyde; the dimedone derivative of the formaldehyde (Frisell and MacKenzie, 1958) melted at 194° and showed no depression on mixing with an authentic sample. The second peak from the Celite at tube 32 (range 28–36) contained 1 mole of glyoxylate by colorimetric analysis, and 1 mole of succinic acid on the basis of 3 equiv of alkali required for neutralization. The glyoxylate was removed from this fraction by reacting it with an equivalent amount of 2,4-dinitrophenylhydrazine in HCl, extracting the hydrazone with ethyl acetate, and crystallizing the derivative from ethanol–H_2O; mp and mmp 193–194°. The succinic acid remaining in the aqueous phase was recovered by chromatography on Dowex 1-X10; its phenylphenacyl ester (Shriner *et al.*, 1964) had mp and mmp 214–215°.

When the DHKP was prepared from 5-[^{14}C]KG and unlabeled glyoxylate, only the succinate obtained by periodate oxidation was radioactive. When prepared from 1-[^{14}C]glyoxylate and unlabeled KG, only the glyoxylate fragment was labeled. When prepared from 2-[^{14}C]glyoxylate, both the glyoxylate and formate fragments were radioactive while the succinate was unlabeled. The following diagram[5] illustrates the periodate splitting and the origin of the labeled carbons in DHKP.



Reduction with P. Further proof of the structure of DHKP was obtained by reduction with phosphorous and identification of the major reaction product as 4-ketopimelic acid. DHKP (120 mg) (A or B) was refluxed with 6 ml of glacial acetic acid, 11.5 mg of iodine, and 35 mg of red phosphorus for approximately 8 hr. After filtering off the phosphorus and evaporating to dryness, the material was chromatographed on Dowex 1-X10. The radioactivity (71%) was eluted as a peak between fractions 26 and 35; rechromatography on Celite gave a peak between 15 and 29; recrystallization from acetone with the aid of chloroform gave colorless crystals (mp 143.5°). There was no depression of mp on mixing with 4-ketopimelic acid prepared from 2-furanacrylic acid (Marckwold, 1887; Komppa, 1938). Both methyl esters melted at 52°, and the semicarbazones at 185–187° without depression on mixing.

Formation of DHKP by Purified Enzyme. Incubation of the purified enzyme with KG plus glyoxylate formed DHKP as well as HKV. The system contained: 400 μmoles of 1,2-[^{14}C]-

[5] $^1C_1$ of glyoxylate; $^2C_2$ of glyoxylate; $^3C_5$ of KG.

glyoxylate; 400 μmoles of KG; 8 μmoles of TPP; 200 μmoles of MgCl ; 2000 μmoles of potassium phosphate buffer, pH 7.2; 400 mg of bovine serum albumin, and 660 units of KG–glyoxylate carboligase, purified 102-fold, in a total volume of 30 ml. After 2 hr at 37°, 3 ml of 70% perchloric acid was added and shaking was continued for 2 hr to collect a total of 302 μmoles of $[^{14}C]CO_2$ in the Hyamine trap. Chromatography on Dowex as previously described gave 152 μmoles of HKV, 14 μmoles of unchanged glyoxylate, and two-thirds as much radioactivity in the DHKP fraction (34 μmoles) as in HKV. The DHKP fraction was further resolved into compounds A and B by rechromatographing on Dowex and elution with 0.5 M formic acid. Hence the purified enzyme gave the same reaction products as the crude particulate fraction, and the amount of $[^{14}C]CO_2$ released in the reaction was approximately twice the amount of HKV formed.

Discussion

The results show that a highly purified KG dehydrogenase complex is also responsible for catalyzing the condensation reactions with acetaldehyde and glyoxylate. The ratios of these three activities remained relatively constant throughout a 100-fold purification of the enzyme. Koch and Stockstad (1965) suggested that KG and glyoxylate condensed to form 2-hydroxy-3-ketoadipic acid (HKA) which was then decarboxylated to HKV by acid. The establishment of DHKP as another product of the KG plus glyoxylate condensation shows that the initial product is undoubtedly 2-hydroxy-3-ketoadipic acid rather than 2,3-dihydroxyadipic acid (Kawasaki *et al.*, 1966). This intermediate can then be decarboxylated to HKV or it can react with another molecule of glyoxylate to form DHKP. It seems probable that the two products tentatively identified by Koch *et al.* (1967) as the osazones of HKV and HKA were the derivatives of HKV and DHKP, since the HKA would be unstable in acid.

The condensation of 2-hydroxy-3-ketoadipic acid (HKA) with a second molecule of glyoxylate to form DHKP is an interesting reaction from the standpoint of substrate specificity in TPP-catalyzed decarboxylations. The length of the carbon chain is important but not critical for both the pyruvate and KG dehydrogenases, and the latter can decarboxylate α-ketoadipate at a slower rate than KG (Kanzaki *et al.*, 1969). A shift of the β-keto group in HKA to the α position during the reaction seems improbable since the expected product would then be 2,4-dihydroxy-3-ketopimelic acid. It is possible, but unlikely, that the HKV formed by nonenzymatic decarboxylation of HKA, could react with glyoxylate to form DHKP. Hence, the α,β-ketol group in HKA seems to meet the enzyme requirements for an appropriate substrate, and the 5-carbon decarboxylated fragment must be bound to the TPP and/or enzyme since it is available in an "active form" for the further reaction with glyoxylate. The lack of optical activity in DHKP suggests a meso compound or a more random configuration of the hydroxyl groups than is usually associated with enzyme reactions.

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