Isolation and Identification of 5-Hydroxy-4-ketovaleric Acid as a Product of α-Ketoglutarate:Glyoxylate Carboligase*

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ABSTRACT: Incubation of glyoxylate-1,2-^{14}C and α-ketoglutarate for 2.5 hr at pH 7.1 and 37° with a beef heart particulate preparation gave $^{14}CO_2$ and two labeled intermediates. One of these has been isolated by column chromatography and identified as 5-hydroxy-4-ketovaleric acid (I). Periodate oxidation of I yielded formaldehyde and succinic acid in equimolar quantities; all of the ^{14}C was found in the formaldehyde (C-5 of I). Nonlabeled I was assayed quantitatively by the periodate–chromotropic acid procedure. Large quantities were prepared by incubating glyoxylate and α-ketoglutarate with a washed beef heart homogenate. Compound I was isolated by chromatography on three columns as white crystals, soluble in water, alcohol, and acetone, insoluble in Et_2O (mp 104–105°, pK = 4.7), 2,4-dinitrophenylosazone (mp 264–265°), and *p*-nitrophenylosazone (mp 265–266°). Elemental analysis and nuclear magnetic resonance spectrum were consistent with the assigned structure. Compound I was synthesized chemically by deamination of δ-aminolevulinic acid with nitrous acid and isolated by chromatography on Dowex and Celite columns. This synthetic I gave the same 2,4-dinitrophenylosazone as enzymatically prepared I.

The importance of glyoxylate as a metabolic intermediate was demonstrated by Ratner *et al.* (1944), who showed that glycine was oxidatively deaminated to glyoxylate. The finding that glyoxylate and glycine were interconvertible by transamination (Cammarata and Cohen, 1950; Nakada and Weinhouse, 1953) further implicated glyoxylate in glycine metabolism. In other studies (Weinhouse and Friedmann, 1951; Nakada and Weinhouse, 1953) it was shown that glyoxylate is metabolized in rat liver by one of two pathways: (a) oxidation to oxalic acid and (b) oxidation to formate and carbon dioxide. The former pathway is of little physiological significance, while the latter represents the major catabolic route of glyoxylate.

Nakada (1953) has demonstrated that the oxidation of glyoxylate in rat liver was markedly increased by the addition of L-glutamic acid. Nakada and Sund (1958) later postulated that glyoxylate catabolism proceeded *via* a decarboxylation of the carboxyl carbon concomitant with a condensation of the α-carbon with glutamic acid to yield *N*-formyl-L-glutamic acid. The latter product is then converted to glutamic acid and formate. However, Crawhall and Watts (1962) observed that when α-ketoglutarate and glyoxylate were incubated with rat liver mitochondria, the decarboxylation of both compounds was more rapid than when they were incubated separately. They also noted that α-ketoglutarate was more effective than L-glutamic acid in stimulating decarboxylation of glyoxylate.

Earlier, Franke and Jilge (1961) tentatively identified 2-keto-3-hydroxyadipic acid as an intermediate in the condensation between glyoxylate and α-ketoglutarate in *Aspergillus niger*. Using similar techniques, Okuyama *et al.* (1965) demonstrated an analogous reaction with extracts of *Rhodopseudomonas spheroides* and further postulated a cyclic mechanism for the oxidation of glyoxylate involving 2-keto-3-hydroxyadipic acid and α-hydroxyglutarate. A similar system was found in rat liver mitochondria (Kawasaki *et al.*, 1966). Recently, Koch and Stockstad (1965) reported partial purification from rat liver mitochondria of an enzyme, glyoxylate carboligase, which catalyzed the synergistic decarboxylation of glyoxylate and α-ketoglutarate. However, they suggested that the product in this reaction was 2-hydroxy-3-ketoadipic acid.

In this laboratory, it was recently found with preparations from beef kidney and other tissues that acetaldehyde and α-ketoglutarate condense to form 5-hydroxy-4-ketohexanoic acid with concomitant decarboxylation of α-ketoglutarate (Bloom and Westerfeld, 1966). Noting the structural similarity between glyoxylate and acetaldehyde, it was thought that an analogous reaction between glyoxylate and α-ketoglutarate to yield 2-hydroxy-3-ketoadipic acid could occur. A further decarboxylation of the β-keto acid would then yield 5-hydroxy-4-ketovaleric acid (HKV).[1] This paper reports our findings as related to: (1) the isolation of large quantities of HKV, and (2) the chemical identification of HKV.

* From the Department of Biochemistry, State University of New York, Upstate Medical Center, Syracuse, New York. *Received August 28, 1967.* This study was supported by U. S. Public Health Service Grants PHS-CA-01852 from the National Cancer Institute and PHS-M-1947 from the National Institute of Mental Health.

[1] Abbreviations used: HKV, 5-hydroxy-4-ketovaleric acid.

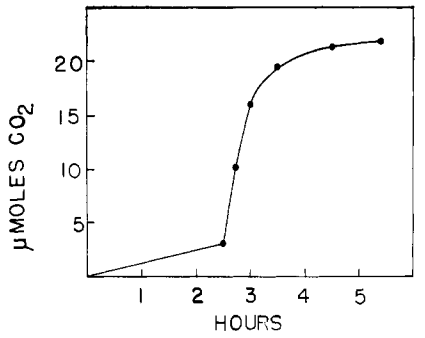

FIGURE 1: Time course of $^{14}CO_2$ production upon acidification. Procedure as described in the text. Incubations were for 2.5 hr under air in a Dubnoff metabolic shaker at 37°. The reaction was stopped by adding 0.3 ml of 50% perchloric acid. $^{14}CO_2$ was measured as $Ba^{14}CO_3$ at the times indicated. Each point represents the average of two determinations.

Methods

Glyoxylic acid was determined by the method of Dekker and Maitra (1962). The periodate–chromotropic acid procedure of Frisell and MacKenzie (1958) was used to assay samples containing between 100 and 1000 μg of HKV. Melting points were determined on a Fisher-Johns melting point apparatus.

Experimental Section

Preparation of a Particulate Fraction from Beef Heart. For small-scale incubations, a particulate fraction was prepared in the following manner (all operations were performed at 4°). A 1:5 beef heart homogenate (w/v) in 0.1 M potassium phosphate buffer (pH 7.1) was prepared with a Teflon pestle tissue grinder and centrifuged at 100,000*g* for 2 hr in a Spinco preparative ultracentrifuge. The supernatant solution was discarded, the tightly packed pellet was rehomogenized in phosphate buffer and recentrifuged, and the resulting pellet was used for incubation. The pellets were stored at 4° and retained activity for about 5 days.

Incubation Medium and $^{14}CO_2$ Measurement. The incubation medium was prepared by mixing the following: 20 μmoles of glyoxylate-1,2-^{14}C (48,000 cpm), obtained from Calbiochem; 20 μmoles of α-ketoglutarate; 1 ml of 0.10 M potassium phosphate buffer (pH 7.1); 1 ml of particulate suspension (equivalent to 200 mg of fresh heart); and enough water to make a final volume of 3 ml. Incubations were carried out in a Dubnoff metabolic shaker at 37° under air, using special 20-ml beakers without lips and covered with tight rubber caps. The center well contained a strip of filter impregnated with 0.2 ml of 8 N potassium hydroxide. The incubation mixture was shaken for 2.5 hr and the reaction was stopped by injecting 0.3 ml of 50% perchloric acid into the main compartment. The vessels were shaken for an additional 2 hr to ensure complete decarboxylation of any susceptible enzymatically formed intermediate products. The contents of the center well were collected quantitatively, mixed with 1 ml of 0.25 M Na_2CO_3, and precipitated with 3 ml of 5% $BaCl_2$. The resulting barium carbonate was collected on glass fiber filter paper disks, washed with hot water, dried with acetone, assayed in a flow counter, corrected to infinite thinness (Yankvich *et al.*, 1947), and expressed as micromoles of $^{14}CO_2$.

Results

When glyoxylate-1,2-^{14}C (ten vessels, 48,000 cpm/vessel) was incubated, a total of 266,000 cpm was found in $^{14}CO_2$ and 235,000 cpm was found in the perchloric acid filtrates. It had been previously demonstrated in *R. spheroides* (Okuyama *et al.*, 1965), rat liver mitochondria (Kawasaki *et al.*, 1966), and pig liver mitochondria (Stewart and Quayle, 1967) that glyoxylate-1-^{14}C but not glyoxylate-2-^{14}C yielded $^{14}CO_2$ when incubated with α-ketoglutarate. Since our system produced approximately equal molar amounts of radioactivity in $^{14}CO_2$ and in the perchloric acid filtrate from glyoxylate-1,2-^{14}C, it was assumed that the carbon dioxide was derived almost entirely from the carboxyl carbon of glyoxylate while the α-carbon was incorporated into some water-soluble intermediate(s).

Time Course of $^{14}CO_2$ Production in the Presence of Perchloric Acid. Twelve reaction mixtures were incubated for 2.5 hr and then acidified with perchloric acid. The center well contents were counted in duplicate at 0, 15, 30, 60, 120, and 180 min after acidification. Figure 1 shows that approximately 15% of the carboxyl carbon was converted to CO_2 after a 2.5-hr incubation without acidification. Acidification caused an increase in carbon dioxide production until a maximum level was reached in about 2 hr. Most of the $^{14}CO_2$ released by acid apparently came from a decarboxylation rather than from bicarbonate. In subsequent experiments with rat heart preparations incubated with unlabeled substrates, acidification increased the amount of HKV by as much as sixfold. Koch and Stokstad (1965) also found a relatively slow release of $^{14}CO_2$ with acid alone, but in the presence of 4-aminoantipyrine, the release was completed within 10 min.

Chromatography. The perchloric acid filtrates from the main compartments of the incubation flasks were pooled. The protein precipitate was removed by centrifugation and washed with 5% perchloric acid. The supernatant solutions and washings were combined, neutralized with 4.5 M potassium hydroxide, chilled overnight, and filtered. The filtrate was then chromatogrammed sequentially on Dowex 1-X10, Dowex 1-X4 AG, and Celite columns as previously described by Bloom and Westerfeld (1966).

Perchlorate filtrate (100 ml) at pH 7 was passed through a Dowex 1-X10 column (Busch *et al.*, 1952) at 4 ml/min followed by 50 ml of water and 100 ml each of 1, 2, 4, and 6 M formic acid. The effluent was collected in 10-ml fractions and 0.5-ml aliquots were plated on aluminum pans, dried under a lamp, and counted in a Nuclear-Chicago D-47 gas-flow counter. Approximately 90%

of the radioactivity was found in two poorly resolved peaks at tubes 19 and 22. The entire fraction from 19 to 30 was collected and evaporated to dryness in a flash evaporator and chromatogrammed on a Dowex 1-X4 AG column (Berl *et al.*, 1962).

A 20-ml sample at pH 7 was passed through the column and eluted with 35 ml of 0.05 N, 80 ml of 0.10 N, and 60 ml of 0.30 N acetic acid followed by 120 ml of 0.10 N HCl. Fractions of 5 ml were collected and 0.5-ml aliquots were plated and counted. The chromatogram showed the existence of two peaks of about equal size. The first peak (designated A) was found in tubes 32–43 (peak at 37) while the second peak (designated B) was found in tubes 49–60 (peak at 49), although this peak showed considerable variation as to its exact location. Fractions A and B gave negative tests for glyoxylate.[2] The periodate–chromotropic acid test for HKV was positive for fraction A and negative for B. Fraction A was subsequently identified as HKV while the chemical nature of B is still under investigation.

Fractions A and B were individually concentrated to 1 ml and chromatographed separately on Celite as described by Ueno *et al.* (1960). When 5-ml samples were collected, fraction A (HKV) peaked in tube 32 and was collected in tubes 28–34; fraction B peaked at 81 and was collected in tubes 75–94. These fractions were then evaporated to dryness in a flash evaporator, dissolved in water, transferred to a beaker, and dried by a stream of warm air.

Products of Periodate Oxidation of HKV. Approximately 13 mg of unlabeled HKV (prepared from nonradioactive glyoxylate and α-ketoglutarate and purified by the three chromatographic columns) was dissolved in 3 ml of water and mixed with 6 ml of 1% periodic acid. The mixture was allowed to stand for 5 min at room temperature, neutralized with potassium hydroxide, and diluted to 10 ml with distilled water. The formaldehyde formed by this reaction was precipitated as the dimedon derivative (Frisell and MacKenzie, 1958). The weight of the derivative was equivalent to 106.4 μmoles of formaldehyde and gave the theoretical melting point of 189–190°. There was no depression of the melting point upon mixing the derivative with a dimedon derivative prepared from commercial formaldehyde.

The filtrate from the dimedon-precipitated formaldehyde was neutralized, evaporated to a volume of 1 ml, and chromatogrammed on a Celite column. The acid in the eluate was determined by titrating each tube with 0.005 N KOH. The only detectable acid appeared in tubes 26–37 (peak at 31) which by comparison with known acids was suggestive of succinic acid. The amount was equivalent to 96.1 μmoles of succinate. Ueno *et al.* (1960) showed that the recovery of succinic acid on Celite is 92.1% (89% in our experiment) and thus the corrected value of 105–108 μmoles of succinic acid agrees with that obtained for formaldehyde. When the labeled HKV-5-^{14}C was oxidized with periodate, 99+% of the radioactivity was found in formaldehyde.

Positive identification of the acid as succinic acid was accomplished by preparing the *p*-phenylphenacyl ester from fractions 26–37. The silvery flakelike crystals obtained melted at 211–212° which was identical with the melting point of the ester prepared from authentic succinic acid. There was no depression on mixing of the two derivatives.

Large-Scale Preparation of Unlabeled HKV for the Preparation of Derivatives. Well-trimmed beef heart (500 g) was ground in a meat grinder, placed in a double thickness of 60-gauge cheesecloth, and dialyzed against cold (4°) running tap water for 6 hr and 10 l. of distilled water for 18 hr. The washed particulate fraction prepared in this way was homogenized with 1500 ml of distilled water in a Waring Blendor and incubated in an 8-l. round-bottom flask at 37° in air in the following proportions: 2 l. of beef heart preparation, 200 ml of 1.0 M potassium phosphate buffer (pH 7.2), 10 g of α-ketoglutaric acid neutralized with KOH and diluted in 250 ml of water, and 5 g of glyoxylic acid monohydrate dissolved in 250 ml of water. Aliquots of 50, 50, 90, and 60 ml of each substrate were added at 0, 90, 180, and 270 min after the start of the incubation. One hour after the last addition, enough 70% perchloric acid was added to give a final concentration of 5%. The mixture was allowed to stand for an additional 8 hr with occasional stirring to ensure complete decarboxylation of any enzymatically formed intermediates. The mixture was then filtered, neutralized with solid potassium hydroxide, refrigerated for 12 hr, and filtered; yield of HKV is 3.8 g (53% of theory) as determined by periodate–chromotropic acid. The yellow filtrate was diluted to 4 l. and chromatogrammed on Dowex 1-X10, Dowex 1-X4 AG, and Celite columns which were scaled up proportionately. This material was then used for the preparation of derivatives and crystallization of the free acid.

HKV (270.5 mg) was recrystallized several times from acetone and dried for 48 hr over phosphorus pentoxide under reduced pressure: yield 100 mg, mp 104–105°, neut equiv 135 (calcd 132), pK = 4.7. The free acid ($HOCH_2COCH_2CH_2COOH$) was found to be very soluble in water, soluble in ethanol and hot acetone, and insoluble in diethyl either. *Anal:* Calcd for $C_5H_8O_4$: C, 45.46; H, 6.10. Found: C, 45.54; H, 6.18.

The nuclear magnetic resonance spectrum in D_2O with 5% K_2CO_3 showed a singlet at τ = 5.64 ppm for the C-5 methylene protons and an eight-line pattern centered at τ = 7.5 ppm for the C-2 and C-3 methylene protons. The respective integral ratios were 2:4.

Derivatives

2,4-Dinitrophenylosazone Derivative. HKV (46 mg) was refluxed with 400 mg of 2,4-dinitrophenylhydrazine

[2] In early experiments, glyoxylate was not detected colorimetrically. It was later discovered that the unreacted glyoxylate was lost during flash evaporation, and/or from the lamp under which the planchets were dried. When the enzymatic reaction did not proceed to completion and care was taken to avoid volatilization, the excess radioactive glyoxylate peaked at tube 22 from the Dowex 1-X10, tube 63 from Dowex 1-X4 AG, and tube 32 from Celite.

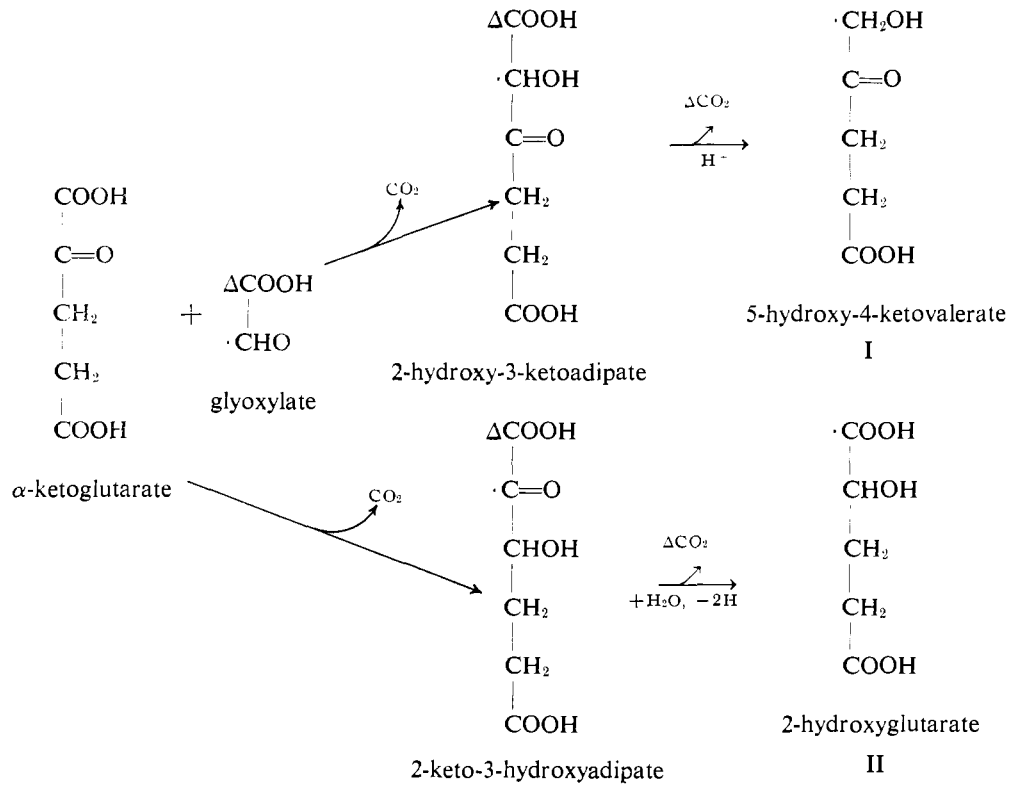

FIGURE 2: Postulated products of glyoxylate–α-ketoglutarate condensation. Mechanism I occurs in beef heart; mechanism II has been postulated by other investigators.

dissolved in 100 ml of 2 N HCl for 1 hr; yield, 82% of theory. Recrystallization from boiling nitrobenzene gave short yellow rods, mp 264–265°. *Anal.* Calcd for $C_{17}H_{14}N_8O_{10}$: C, 41.64; H, 2.88; N, 22.85. Found: C, 41.92; H, 2.95; N, 23.33.

p-Nitrophenylosazone Derivative. *p*-Nitrophenylhydrazine (400 mg) in 5 ml of absolute alcohol was added to 142 mg of HKV in 5 ml of absolute ethanol. The resulting solution was refluxed for 1 hr, cooled, and filtered. Water (20 ml) was added to the filtrate which was then stored for 1 week at 0°. The orange-red precipitate was filtered and yielded 53%. Recrystallization from nitrobenzene gave a brown precipitate, mp 265–266°. *Anal.* Calcd for $C_{17}H_{16}N_6O_6$: C, 51.00; H, 4.03; N, 20.99. Found: C, 50.73; H, 4.09, N, 20.52.

Preparation of HKV from δ-Aminolevulinic Acid. Deamination of δ-aminolevulinic acid by nitrous acid with the formation of 5-hydroxy-4-ketovaleric acid was accomplished with about a 20% yield by the following procedure. $NaNO_2$ (50%) (1 ml) was added dropwise (10 min) to 100 mg of δ-aminolevulinic acid (128 mg of the hydrochloride) dissolved in 5 ml of 1 N H_2SO_4. The solution was allowed to stand at room temperature for 25 min and in boiling water for 5 min. Excess nitrous acid was removed by transferring the solution to a round-bottom flask, adding 20 ml of 0.25 N H_2SO_4, and flash evaporating at 40° to 12 ml.

This yielded 20–25 mg of HKV which was purified by the previously described chromatography on Dowex and Celite columns. Recrystallization from acetone gave mp 102.5–104.5; 2,4-dinitrophenylosazone, mp 264.5–265°.

Discussion

The results reported in this paper have shown the presence of an enzyme in the particulate fraction of beef heart that catalyzes a condensation between glyoxylate and α-ketoglutarate. Several investigators (Koch and Stockstad, 1965; Stewart and Quayle, 1967) have demonstrated the occurrence of a similar system in both rat liver and pig liver mitochondria. Koch and Stockstad (1965) suggested that glyoxylate and α-ketoglutarate undergo an initial condensation to yield 2-hydroxy-3-ketoadipic acid which then decarboxylates to HKV in the presence of acid. Kawasaki *et al.* (1966) have suggested that in rat liver mitochondria the initial condensation product is 2-keto-3-hydroxyadipic acid which could be converted enzymatically to α-hydroxyglutaric acid. These alternate possibilities are shown in Figure 2.

Since the 2-hydroxy-3-ketoadipic acid was readily decarboxylated in the presence of acid it was necessary to isolate the decarboxylated condensation product and locate the positions of the hydroxyl and keto functions. The identification of the hydroxyl group at C-5 was established by the fact that formaldehyde is obtained by periodate oxidation and the nuclear magnetic resonance

spectrum contains a singlet at τ = 5.64 ppm for the C-5 methylene group. Furthermore, the unknown compound was identical with HKV formed by the deamination of δ-aminolevulinic acid with nitrous acid. Rappe (1959) has synthesized HKV. The melting point and nuclear magnetic resonance data are consistent with that obtained for our compound. All of this provides evidence that the primary condensation product from glyoxylate and α-ketoglutarate is 2-hydroxy-3-keto-adipic acid and the acid decarboxylation product is HKV.

Paper chromatography of the 2,4-dinitrophenylosazones of the reaction products from human kidney by Koch *et al.* (1967) suggested the presence of two compounds. On the basis of labeling patterns and optical properties, they were tentatively identified as the osazones of HKV and 2-hydroxy-3-ketoadipic acid. Stewart and Quayle (1967) chromatographed the reaction products and observed two spots, neither of which incorporated radioactivity from glyoxylate-1-^{14}C. In this paper, we have also demonstrated the existence of at least one other product in addition to HKV. The chemical nature of this compound is presently under investigation.

Moriyama and Yui (1966) have isolated and identified HKV as a product of glyoxylate metabolism in *Mycobacterium takeo*. Our data confirm their findings with regard to the structure of HKV. They have suggested that HKV is formed *via* a direct one-step condensation of glyoxylate and α-ketoglutarate. In our system, it appears that most of the HKV is formed by a chemical decarboxylation of 2-hydroxy-3-ketoadipic acid.

Acknowledgments

We thank Mr. A. L. Vulcano of Bristol Laboratories Research for the nuclear magnetic resonance spectrum and its interpretation, and for the elemental analyses.

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