METABOLISM OF 5-HYDROXY-4-KETO-VALERIC ACID IN THE RAT

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Summary. Studies in vivo and in vitro were made of the metabolism of 5-hydroxy-4-keto-valeric acid (HKV), which is formed by decarboxylation of 2-hydroxy-3-keto-adipate, the primary condensation product of the 2-keto-glutarate:glyoxylate carboligase reaction. Evidence is presented for the formation of ^{14}C-aspartate, ^{14}C-glutamate and ^{14}C-delta-aminolevulinic acid when ^{14}C-HKV is incubated with sonicated rat liver mitochondria. Formation of these products suggests that HKV may be converted to 2-keto-glutarate and delta-aminolevulinic acid via 4,5-dioxo-valerate. In vivo oxidation of ^{14}C-HKV to $^{14}CO_2$ is also shown in the rat.

A 2-keto-glutarate:glyoxylate carboligase, which catalyzes the following reaction, has been found in the mitochondrial and cytoplasmic fractions of human liver, spleen and kidney (1):

$$HOOC\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}C^*OOH + CHO\text{-}COOH \xrightarrow{TPP,\ Mg^{+2}} HOOC\text{-}CH_2\text{-}CH_2\text{-}\overset{O}{\overset{\|}{C}}\text{-}\overset{OH}{\overset{|}{C}}H\text{-}COOH + C^*O_2$$

The activity of the cytoplasmic carboligase is reduced in humans with primary hyperoxaluria, a genetic disorder of glyoxylate metabolism that results in formation of excess oxalate (1). 2-keto-glutarate:glyoxylate carboligase activity has been found in the mitochondrial fraction of rat liver (2) and beef heart (3) and in Mycobacterium takeo (4), Mycobacterium phlei (5) and Rhodopseudomonas spheroides (6).

The initial reaction product, 2-hydroxy-3-keto-adipate, is rapidly decarboxylated non-enzymatically to form 5-hydroxy-4-keto-valeric acid (7). The metabolism of 5-hydroxy-4-keto-valerate (HKV), to our knowledge, has not been investigated. Yamasaki and Moriyama (5) recently reported that HKV inhibits porphyrin formation from delta-aminolevulinic acid (ALA) in M. phlei and suggested that HKV may have a regulatory function in porphyrin synthesis.

This paper presents evidence for the formation of ^{14}C-aspartate,

^{14}C-glutamate and ^{14}C-ALA when ^{14}C-HKV is incubated with sonicated rat liver mitochondria. In vivo oxidation of ^{14}C-HKV to $^{14}CO_2$ is also shown in the rat.

METHODS

Mitochondria. Rat liver mitochondria were isolated by the method of Hogeboom (8) with these modifications: (a) the sucrose media were adjusted to pH 7.4 with 0.5 M Na_2HPO_4; (b) resuspension of the mitochondria in 0.34 M sucrose, centrifugation at 24000 x g, and removal of the supernatant and slowly sedimenting material were repeated once more. The mitochondrial pellet was suspended in 0.05 M potassium phosphate buffer, pH 7.0. One volume of buffer was used for each g of wet liver taken for the original homogenate. The suspension was sonicated 1 minute (10 kcycle). All operations were done at 0°C.

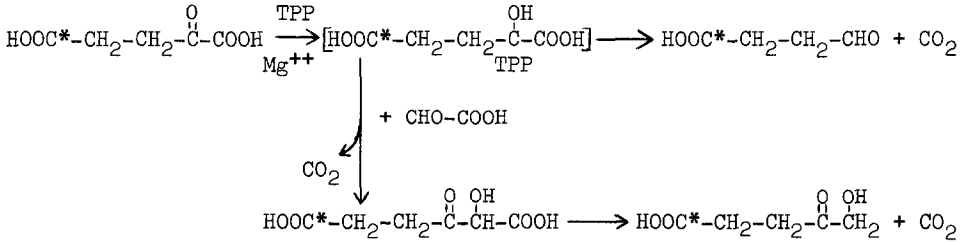
Enzymatic synthesis of ^{14}C-HKV. HKV labeled with ^{14}C at C-1, C-5 or C-1 and C-5 (radiochemicals, Amersham/Searle) was made by incubating 20 µmoles 5-^{14}C-2-keto-glutarate (10 µC) or 20 µmoles unlabeled 2-keto-glutarate with a preparation of 370-fold purified 2-keto-glutarate:glyoxylate carboligase (9) in Warburg flasks under H_2 in the presence of 10 µmoles $MgCl_2$, 1 µmole TPP and 100 µmoles phosphate buffer (pH 7.0, 10^{-3} M mercaptoethanol) with KOH in the center well. The reaction was started by adding from one sidearm either 20 µmoles unlabeled glyoxylate or 20 µmoles (20 µC) U-^{14}C-glyoxylate (final vol., 3.0 ml). After 2 hr at 37°C, the reaction was stopped by adding a mixture of 0.1 ml trichloroacetic acid (TCA), 25% and 0.1 ml 4-amino-antipyrine (0.2 M). The mixture was made to pH 7.0 with NaOH and centrifuged.

Isolation and purification of ^{14}C-HKV. The supernatant was applied to a column of Dowex 50x8 (200 mesh, 10x40 mm), H^+ form. The column was washed with water, the first 20 ml of effluent collected and applied to a Dowex 1x8 column (100-200 mesh, 11x90 mm), formate form, and eluted with a linear water: formic acid gradient (200 ml H_2O:200 ml 2 N formic acid), flow rate 4 ml/10 min, 4 ml fractions. This method was a modification of the method of Busch et al. (10). HKV was eluted in tubes 14-15, glycolate in tubes 19-20, and glyoxylate in tubes 25-27. (^{14}C-glycolate was a contaminant of U-^{14}C-glyoxylate).

These fractions were lyophilized to remove formic acid, the residue dissolved in water, pH adjusted to 7.0 with NaOH, and radioactivity determined by counting in Bray's Solution (Beckman CPM-100). HKV gave only one spot after paper chromatography in two solvent systems, (a) upper phase of n-butanol:acetic acid:H_2O (4:1:5), Rf 0.59 and (b) n-amyl alcohol:formic acid:H_2O (70:15:15), Rf 0.47. The dinitrophenylhydrazone of HKV also migrated in a single zone, Rf 0.41, in n-butanol:NH_3 (2N):ethanol (7:2:3).

The distinctness of separation of HKV and glycolate with Dowex 1x8 and the formate gradient depended upon the batch of Dowex used. When the separation was not distinct, HKV was then separated from glycolate on a Dowex 1x10 column (100-200 mesh, 0.9x30 cm), acetate form, with elution with 0.5 M acetic acid (11), flow rate 6 ml/hr, 3 ml fractions. HKV was eluted in tubes 28-30, and glycolate and glyoxylate were not eluted. The specific activity of the ^{14}C-HKV was adjusted by addition of unlabeled HKV (3).

Absence of ^{14}C-succinic semialdehyde in ^{14}C-HKV. Succinic semialdehyde might be a possible side reaction product of the carboligase reaction (2,9):



This possibility was eliminated by separation of HKV and succinic semialdehyde on a Dowex 1x10 column (100-200 mesh, 0.9x30 cm), acetate form, by elution with 0.5 M acetic acid (11), flow rate 6 ml/hr, 3 ml fractions. Standard succinic semialdehyde (detected as the dinitrophenylhydrazone) was eluted in tubes 13-14, and HKV in tubes 28-30. No detectable radioactivity appeared in tubes 13-14 when 1-^{14}C-HKV was applied to this column.

RESULTS AND DISCUSSION

The evidence of Kissel and Heilmeyer (12) that 4,5-dioxo-valeric acid could be converted to ALA suggested that HKV might also serve as a precursor

Table 1. Radioactivity from ^{14}C-HKV in the amino acid-aminoketone fraction and in the pyrrole derivatives of this fraction

Substrate	Amino acid-aminoketone cpm	Pyrrole cpm
1-^{14}C-HKV	4600	620
1-^{14}C-HKV, plus aspartate	11060	600
5-^{14}C-HKV	4690	680
5-^{14}C-HKV, plus aspartate	5780	672

The incubation mixture contained ^{14}C-HKV (3 µmoles, 4×10^4 cpm/µmole), 10 µmoles $MgCl_2$, 2 µmoles NAD, 100 µmoles phosphate buffer (pH 7.0), 15 mg mitochondrial protein (sonicated mitochondria). Aspartate, 10 µmoles, was added as indicated. The final volume was made to 3.0 ml with H_2O. The mixture was incubated aerobically at 37°C for 2 hrs. The reaction was stopped with 0.2 ml TCA (25%) and neutralized with NaOH. The mixture was centrifuged, and the supernatant applied to a Dowex 50x8 column (200-400 mesh, 11x50 mm), H^+ form (Irving and Elliott, 1969). The column was washed with 30 ml H_2O. The amino acid-aminoketone fraction was eluted with 30 ml 2M pyridine-acetate buffer, pH 6.0, collected in flasks containing 1 drop concentrated HCl, and evaporated twice to dryness under vacuum. The residue was dissolved in 1 ml H_2O. This was treated with acetylacetone for conversion of aminoketones to pyrrole derivatives, followed by extraction of pyrroles into ethyl acetate (Irving and Elliott, 1969). The ethyl acetate was washed with water, evaporated to dryness, the residue dissolved in methanol, and aliquots counted.

of ALA via oxidation to 4,5-dioxo-valeric acid. Incubation of 1-^{14}C-HKV or 5-^{14}C-HKV with sonicated mitochondria (table 1) resulted in incorporation of radioactivity into the amino acid-aminoketone fraction obtained from the deproteinized incubation mixture. With 5-^{14}C-HKV as substrate, addition of aspartate caused only a small increase in incorporation of ^{14}C into this fraction, but with 1-^{14}C-HKV, addition of aspartate increased the incorporation by 100%. Glutamate produced a 60% increase in incorporation. Asparagine, glycine, lysine, oxaloacetate or fumarate had no stimulatory effect.

The amino acid-aminoketone fraction was treated with acetylacetone for conversion of aminoketones to pyrrole derivatives, followed by extraction of pyrroles into ethyl acetate (13). The radioactivity in the pyrrole fraction was the same with both 1-^{14}C-HKV and 5-^{14}C-HKV (table 1). The yield of ^{14}C in the pyrrole fraction was very low, with only 5-15% of the radioactivity

in the amino acid-aminoketone fraction recovered in the pyrrole fraction. Addition of aspartate to the incubation mixtures did not increase the radioactivity obtained as pyrrole.

The pyrrole fractions from 1-^{14}C-HKV and 5-^{14}C-HKV were recrystallized (methanol-water) with unlabeled ALA-pyrrole (14). The specific activity was constant from the second through the fifth recrystallization, but decreased by approximately 65% in the first recrystallization, although these pyrrole fractions and unlabeled pyrrole from standard ALA gave the same Rf (Ehrlich reagent (13)) after paper chromatography in 3 solvent systems: (a) Rf 0.91 in the upper layer of n-butanol:glacial acetic acid:H_2O, 4:1:5 (13); (b) Rf 0.21 in the upper layer of n-butanol:NH_3 (1.5 M), 1:1 (13); (c) Rf 0.88 in the upper layer of n-amyl alcohol:H_2O:formic acid, 20:12:1. In the acidic solvents, all of the radioactivity applied was found in the spot with the Rf of ALA-pyrrole. In the n-butanol:NH_3 solvent, the radioactivity in the spot with Rf of ALA-pyrrole was less than the amount applied.

Substrate ^{14}C-HKV did not contaminate the amino acid-aminoketone fractions. These fractions from incubation mixtures containing 1-^{14}C-HKV or 5-^{14}C-HKV with added aspartate were chromatographed in ethanol:NH_3:H_2O (8:1:1). No radioactivity appeared at the spot corresponding to HKV (Rf 0.52). The aspartate spot (Rf 0.18) from the mixture containing 1-^{14}C-HKV showed considerable radioactivity, but no radioactivity appeared in the aspartate spot from the incubation mixture containing 5-^{14}C-HKV.

Recrystallization of the amino acid-aminoketone fraction provided evidence that approximately 40-50% of the radioactivity from 1-^{14}C-HKV was present in aspartate. When this fraction from an incubation mixture containing 1-^{14}C-HKV without added aspartate was recrystallized with unlabeled aspartate, the specific activity (739 cpm per mg initially) decreased to 400 cpm per mg after the first recrystallization from ethanol-water, a decrease of 45%. However, the specific activity then remained constant (400 cpm per mg) through the next 4 recrystallizations. Aspartate was measured by ninhydrin reaction.

The amino acid-aminoketone fraction obtained after incubation of 1-^{14}C-HKV was fractionated on a Dowex 1x10 column (100-200 mesh, 0.9x30 cm), acetate form, by elution with 0.5 M acetic acid, as described before. Aspartate was eluted in tubes 25-27 and glutamate in tubes 12-13. Other amino acids were eluted in tubes 4-5. The eluates in tubes 12-13 and 25-27 were lyophilized, the residue dissolved in water and treated with chloramine-T (15). Approximately 70% of the radioactivity found in aspartate was recovered as $^{14}CO_2$ after this treatment (table 2). In contrast, the radioactivity in the glutamate fraction was not released as $^{14}CO_2$ after treatment with chloramine-T. This observation indicates that the radioactivity found in glutamate was not located in position 1.

When the amino acid-aminoketone fraction obtained after incubation of 5-^{14}C-HKV was fractionated in the same way (table 2), radioactivity was found in the glutamate fraction but not in the aspartate fraction. The release of $^{14}CO_2$ after treatment of the glutamate fraction with chloramine-T showed that more than 50% of the radioactivity in this fraction was in position 1.

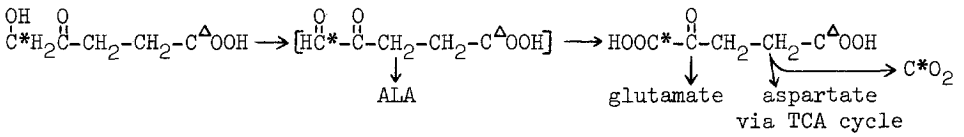
The data above show that C-5 of HKV is not incorporated into aspartate, and C-1 of HKV is not in position 1 of glutamate. These facts suggest that HKV is converted to 2-keto-glutarate via 4,5-dioxovalerate. In this process,

Table 2. Radioactivity from ^{14}C-HKV in aspartate and glutamate and $^{14}CO_2$ released after chloramine-T treatment

Substrate	Aspartate fraction		Glutamate fraction	
	total	released as CO_2	total	released as CO_2
	cpm	cpm	cpm	cpm
1-^{14}C-HKV ($5.4x10^4$ cpm, 4 µmoles) plus aspartate, 10 µmoles	1376	950	300	---
5-^{14}C-HKV ($5.4x10^4$ cpm, 4 µmoles) plus aspartate, 10 µmoles	---	---	362	192

Incubation and separation of amino acid-aminoketone fraction as in table 1.

C-1 of HKV becomes C-5 of 2-keto-glutarate, and aspartate can be formed from 2-keto-glutarate via TCA cycle intermediates, as follows:



The greater conversion of 1-^{14}C-HKV to ^{14}C-aspartate, compared to the conversion of 5-^{14}C-HKV to ^{14}C-glutamate, suggests that oxaloacetate may be formed from HKV without 2-keto-glutarate as an intermediate. However, we have been unable to show formation of a radioactive 1-C unit from 5-^{14}C-HKV.

Oxidation of ^{14}C-HKV to $^{14}CO_2$ *in vivo* was shown by injecting 6 μmoles of 1-^{14}C-HKV (1.6 μC) or 5-^{14}C-HKV (1.4 μC) intravenously (tail vein) into 150-200 g male rats (16,17). The cumulative $^{14}CO_2$ excretion (% of injected dose) was 2.5% for 1-^{14}C-HKV and 1.9% for 5-^{14}C-HKV after 2.5 hours. These values are comparable to that from the *in vivo* oxidation of (2-^{14}C-imidazole)-histidine (16) although they are much lower than that from 3-^{14}C-DL-leucine (17).

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