The Formation and Determination of 5-Hydroxy-4-ketohexanoic Acid*

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ABSTRACT: The Voges–Proskauer color reaction was utilized for the colorimetric determination of 5-hydroxy-4-ketohexanoic acid (HKH), and was applied to a study of the formation of this metabolite from α-ketoglutarate (KG) plus acetaldehyde (AcH) by various rat tissue homogenates. The simultaneous formation of acetoin from pyruvate (Pyr) plus AcH was also determined. Heart, kidney, and skeletal muscle formed significant amounts of HKH when KG alone was added to the homogenate. All tissues formed relatively large amounts of HKH when both KG and AcH were supplied as substrates; the relative activities were in the order: heart > kidney > liver or skeletal muscle > brain > lung or spleen. Except for brain, these tissues had the same relative activities in forming acetoin from Pyr plus AcH; brain was relatively more active in forming acetoin than HKH. The system responsible for the condensation of KG plus AcH to form HKH was located primarily, if not exclusively, in the particulate fraction of all tissues. Substitution of glucose or fructose for KG in the presence of AcH gave no more HKH than AcH alone; however, glutamate increased the yield of HKH in liver, kidney, and skeletal muscle. The addition of KG plus alcohol (as a potential source of AcH) gave the expected large amounts of HKH with liver or kidney, but also gave a small increase in the amount of HKH formed by lung and spleen (as compared with KG alone). None of the tissue homogenates destroyed any appreciable quantity of added HKH or acetoin, and there was no interconversion between them. Thiamine deficiency markedly decreased the formation of acetoin from Pyr plus AcH by all tissue homogenates, and this defect was largely reversed by the addition of thiamine pyrophosphate (TPP) *in vitro*. The formation of HKH from KG plus AcH was not affected seriously by thiamine deficiency in most tissues; the activities in liver and heart were depressed, but neither was restored by TPP *in vitro*. Pyruvate alone gave rise to HKH in heart homogenate, and good yields were obtained from Pyr plus KG. The formation of HKH from Pyr plus KG could not be effected by the particulate fraction alone, but this activity was restored to the particulate fraction by the addition of TPP or a control supernate (but not by a thiamine-deficient supernate). Essentially all of the activity in the supernate could be attributed to the amount of TPP normally present. Hence, the particulate fraction of rat heart homogenate was able to convert Pyr to AcH, when TPP was added, as well as condense the latter with KG.

A new metabolite from ethanol was recently isolated and identified (Bloom and Westerfeld, 1966) as 5-hydroxy-4-ketohexanoic acid.[1] It appears to be formed by a decarboxylation–condensation reaction between acetaldehyde and α-ketoglutarate through a mechanism which is analogous to the formation of acetoin from pyruvate plus AcH (Berg and Westerfeld, 1944). Nothing is yet known about the role of this substance in ethanol metabolism *in vivo*, and the biological properties of this metabolite have yet to be evaluated. The present report describes: (1) a colorimetric procedure for the quantitative determination of HKH; (2) the formation of HKH by homogenates of various rat tissues; and (3) the effect of thiamine deficiency on this reaction.

Determination of 5-Hydroxy-4-ketohexanoic Acid

The ketol grouping in HKH reacts with creatine and α-naphthol to give a positive Voges–Proskauer reaction, and the colorimetric procedure previously developed for the determination of blood acetoin (Westerfeld, 1945) has been applied to the determination of HKH. A separation of acetoin and HKH prior to the development of the color was accomplished with a Dowex 1-X10 column in the formate form. Acetoin ran through such a column freely; HKH was adsorbed by the column because of the presence of a carboxyl group, and was subsequently eluted with formic acid. The procedure developed for HKH will, therefore, determine acidic substances which also possess a chromogenic ketol or diketone structure. While 5-hydroxy-4-ketohexanoic acid is the only known metabolite which meets these specifications at the present time, others are theoretically possible.

Colorimetric Procedure. Creatine (1 ml of 5%)

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[1] Abbreviations used: HKH, 5-hydroxy-4-ketohexanoic acid; KG, α-ketoglutarate; AcH, acetaldehyde; Pyr, pyruvate; TPP, thiamine pyrophosphate.

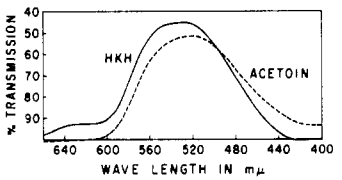

FIGURE 1: The absorption spectra of the colors produced by reacting acetoin or HKH with creatine and α-naphthol in alkali (Voges–Proskauer reaction). The solution contained 10 μg of acetoin or 36.84 μg of HKH in 5 ml of water. After adding 1 ml of creatine plus 1 ml of α-naphthol, and waiting 60 min for acetoin and 90 min for HKH, the colors were read in a 1-cm cuvet in a recording Beckman spectrophotometer. The optical density per milligram of compound at 520 mμ was 28.4 for acetoin and 9.42 for HKH. At 620 mμ the optical density per milligram of HKH was 0.87.

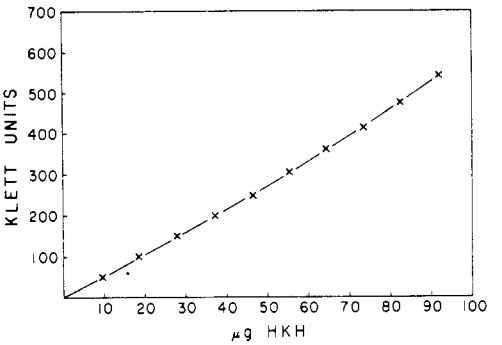

FIGURE 2: Standard curve for 5-hydroxy-4-ketohexanoic acid. The indicated amount of HKH was dissolved in 5 ml of water; 1 ml of 5% creatine and 1 ml of 5% α-naphthol in 3.5 N NaOH were added. After standing for 90 min at room temperature with occasional shaking, the colors were read on a Klett–Summerson colorimeter with filter no. 54 against a reagent blank.

was completely mixed with 5 ml of the test solution which contained between 1 and 100 μg of HKH. α-Naphthol (1 ml of 5%)[2] was added, and the color was allowed to develop at room temperature for 90 min with occasional shaking. It was then read against a reagent blank with filter no. 54 on a Klett–Summerson colorimeter.

Color development appeared to involve an air oxidation since the color was darker at the surface when high concentrations of HKH were used, and the maximum color was reached faster with small amounts of HKH. The color faded slowly on standing, but not enough to prevent a reasonably good linear relationship in the standard curve when all concentrations were run at 25° and read at 90 min. The rate of color development and fading were hastened by higher temperatures, and the time for development of the maximum color with HKH could be shortened to 60 min at 30°.

Figure 1 shows the spectra of the colors produced by the reaction of HKH or acetoin with creatine and α-naphthol in alkali. Both colors had a broad absorption peak at 520–540 mμ, but the HKH color also had a small shoulder at 610–630 mμ; this additional absorption gave a purplish-red color to the HKH product instead of the cherry red color obtained with acetoin. A spectrophotometric reading at 520 and 620 mμ could be used to detect HKH in the presence of acetoin (without separating them), but such a procedure would be relatively insensitive to HKH.

Standard Curve. Figure 2 shows that the standard curve obtained with HKH was essentially linear with less than 70 μg of HKH, but concentrations above 70 μg deviated slightly. Using Klett filter no. 54 acetoin produced 2.84 times as much color in 60 min as HKH did in 90 min on a weight basis, and 1.7 times as much color on a molar basis. With a Beckman DU spectrophotometer at 520 mμ, the relative chromogenic value was 3.0 on a weight basis and 1.82 on a molar basis.

Since the isolated HKH was not crystalline, its purity and therefore its value as a primary standard were initially in doubt. When the crystalline dehydroabietylamine salt was used as the primary standard, it gave only 83% as much color as an equivalent amount of the HKH from which it was formed. When dehydroabietylamine was added in stoichiometric amounts to aqueous solutions of HKH, the color reaction was inhibited 16%. Hence, the results were consistent when the isolated HKH was accepted as a pure standard, or when the amount of color obtained with the crystalline amine salt was corrected for the small inhibition produced by the amine itself.

Preparation of Sample. Tungstic and perchloric acid filtrates gave quantitative recoveries of HKH in the protein-free filtrates of tissue homogenates. An aliquot of the tungstic acid filtrate (usually 5 ml containing no more than 1 mg of HKH) was neutralized with 0.1 N KOH to pH 7 with phenol red indicator, and run through an 8 × 45 mm column of well-washed Dowex 1-X10 in the formate form (Busch *et al.*, 1952). This was followed by 5 ml of water to wash through all the acetoin, and an aliquot of this combined effluent was utilized for the colorimetric determination of acetoin (Westerfeld, 1945). The HKH was then eluted quantitatively from the column with 9 ml of 1 M formic acid. NaOH (1 ml, 4.5 N) was added to the eluate to partially neutralize the formic acid, and an aliquot of this solution was used for the colorimetric determination of HKH. The presence of formate in the solution had no effect on the color reaction, and the phenol red previously added to the filtrate was retained by the Dowex column. Appropriate studies showed

[2] Powdered, colorless α-naphthol (1 g, recrystallized from chloroform) was dissolved in 20 ml of 3.5 N NaOH. This reagent was not prepared until after the creatine had been added to the test solution, and was used as soon as the α-naphthol dissolved.

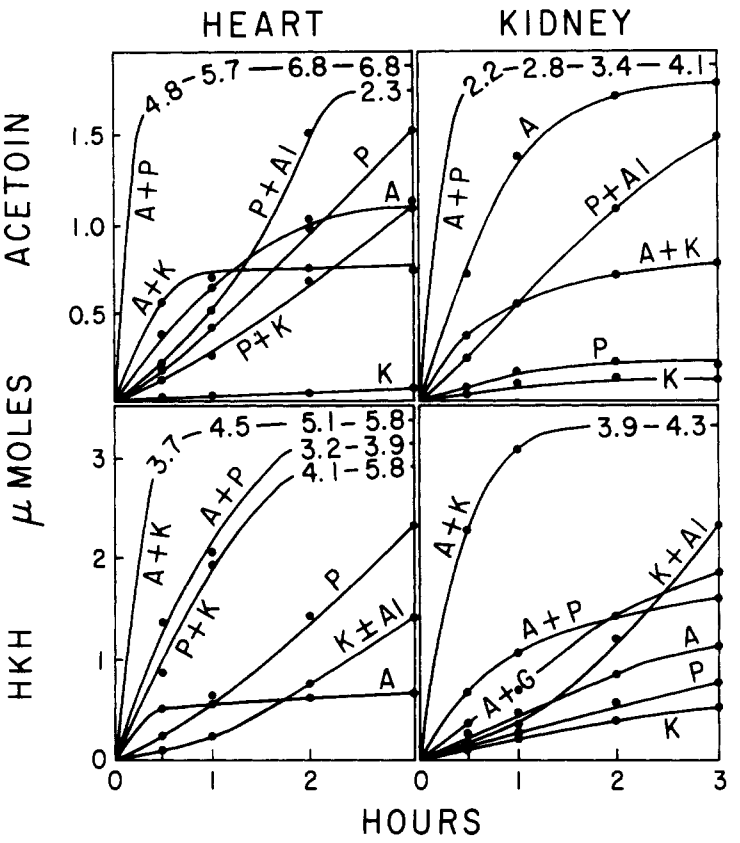

FIGURE 3: The simultaneous formation of acetoin and HKH by heart and kidney rat tissue homogenates in the presence of various combinations of the following substrates: 68 μmoles of acetaldehyde (A); 33 μmoles of α-ketoglutarate (K) or pyruvate (P); 6 μmoles of glutamate (G); 2 mg of methylene blue (MB); 200 mg of alcohol (Al)/100 ml final concentration. Values exceeding the scale on the ordinate have been written in along a broken line; 250 mg of tissue. phosphate buffer, pH 7.1, total volume 3.2 ml, 38°.

that both acetoin and HKH were recovered quantitatively by this procedure. The maximum loading of the column was not determined, but recoveries up to 1 mg of HKH were complete.

Formation of 5-Hydroxy-4-ketohexanoic Acid by Tissue Homogenates

The following studies demonstrated the relative activities of various rat tissue homogenates in the formation of HKH from α-ketoglutarate plus acetaldehyde. The formation of acetoin from pyruvate plus AcH was determined simultaneously for comparative purposes. Several other substrates were also tested with added AcH, and in some experiments ethanol was used as a potential precursor of AcH.

Procedure. The following solutions were placed in a 20-ml beaker: (1) 2.5 ml of a 1:10 tissue homogenate, prepared in 0.05 M potassium phosphate buffer, pH 7.1; (2) 0.3 ml of a solution containing 33 μmoles of sodium pyruvate or potassium α-ketoglutarate (adjusted to pH 7); (3) 0.3 ml of a solution containing 7 μmoles of acetaldehyde, or sufficient ethanol to provide a final concentration of 200 mg/100 ml. Sufficient water was added to bring the final volume to 3.2 ml.

The beaker was closed with a rubber cap and incubated at 38° for 0.5, 1, 2, or 3 hr with shaking. The proteins were then precipitated by the addition of 0.8 ml of 10% sodium tungstate plus 1 ml of 0.66 N H_2SO_4, and were removed by centrifugation. A 3-ml aliquot of the supernate was analyzed for acetoin and HKH as previously described.

Results

In the absence of any added substrates, the various tissue homogenates formed little or no HKH. Heart muscle formed about 17 μg in 3 hr; skeletal muscle, liver, and kidney formed about one-half as much; lung, spleen, small intestine, brain, and adipose tissue formed none. The simultaneous formation of acetoin under these conditions was: liver 18, kidney 10, heart 10, skeletal muscle 10, brain 7, and others 2 (μg/250 mg of tissue in 3-hr incubation).

The results obtained (Figures 3 and 4) when either AcH or KG alone was added to the tissue homogenate showed that the formation of AcH was usually the

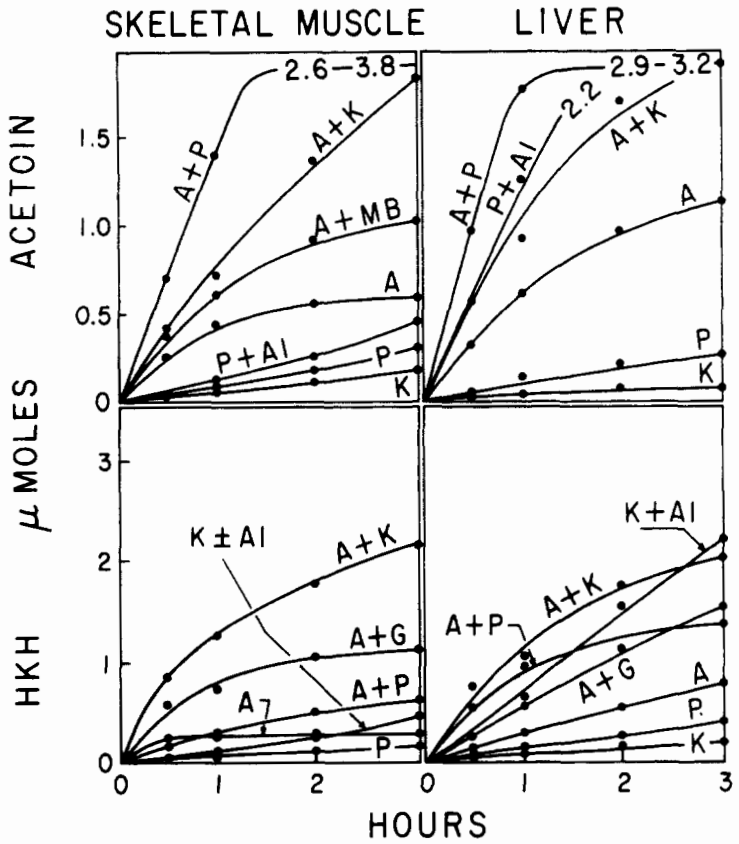

FIGURE 4: The simultaneous formation of acetoin and HKH by skeletal muscle and liver rat tissue homogenates in the presence of various combinations of substrates. All conditions are the same as in Figure 3.

limiting factor in the amount of HKH produced. Kidney, skeletal muscle, and especially heart formed significant amounts of HKH from KG alone, presumably through a conversion of some of the KG to AcH prior to the condensation reaction. When AcH alone was added to the homogenates, all the tissues supplied enough KG and Pyr from endogenous substrates to form at least small amounts of HKH and acetoin.

When both AcH and KG were supplied as substrates, relatively large amounts of HKH were formed by all tissues (Figures 3–5). Kidney and especially heart were rich in the enzyme system responsible for this condensation reaction; both formed more than 400 μg (2.74 μmoles) of HKH in 1 hr. Liver and skeletal muscle formed about 1.2, while brain, lung, and spleen formed about 0.75 μmole in 1 hr. Some of the tissue homogenates also converted some of the added KG to Pyr because more acetoin was formed by liver, skeletal muscle, and brain when KG was added along with the AcH; however with kidney and heart, the acetoin formation in the presence of AcH plus KG was less than with AcH alone, presumably because the AcH was converted to HKH before the additional Pyr became available.

A particulate fraction of the various homogenates was prepared by centrifuging a 1:5 homogenate in the phosphate buffer at 40,000 rpm in a Spinco preparative ultracentrifuge with head no. 40 (105,000*g*) for 2 hr; the supernate was discarded, and the pellet was rehomogenized and incubated with KG plus AcH. With all five tissues (heart, liver, kidney, brain, and skeletal muscle), the formation of HKH by the particulate fraction was very similar to that shown in Figures 3–5 for the whole homogenates (not plotted separately). Substances in the soluble supernate contributed little or nothing to the condensation reaction between KG and AcH. The simultaneous formation of acetoin from added KG and AcH showed that the particulate fraction from heart, kidney, or skeletal muscle did not convert KG to Pyr readily, but the particulate fraction from liver and brain did convert some KG to Pyr.

Pyruvate alone gave little HKH with brain or skeletal muscle, but unexpectedly gave rise to quite large amounts of HKH with heart. In fact, Pyr alone was a better precursor of HKH with heart homogenate than was KG alone, even though the Pyr had to be converted to both AcH and KG prior to the formation of HKH. Heart was also unusually good in forming acetoin from Pyr alone.

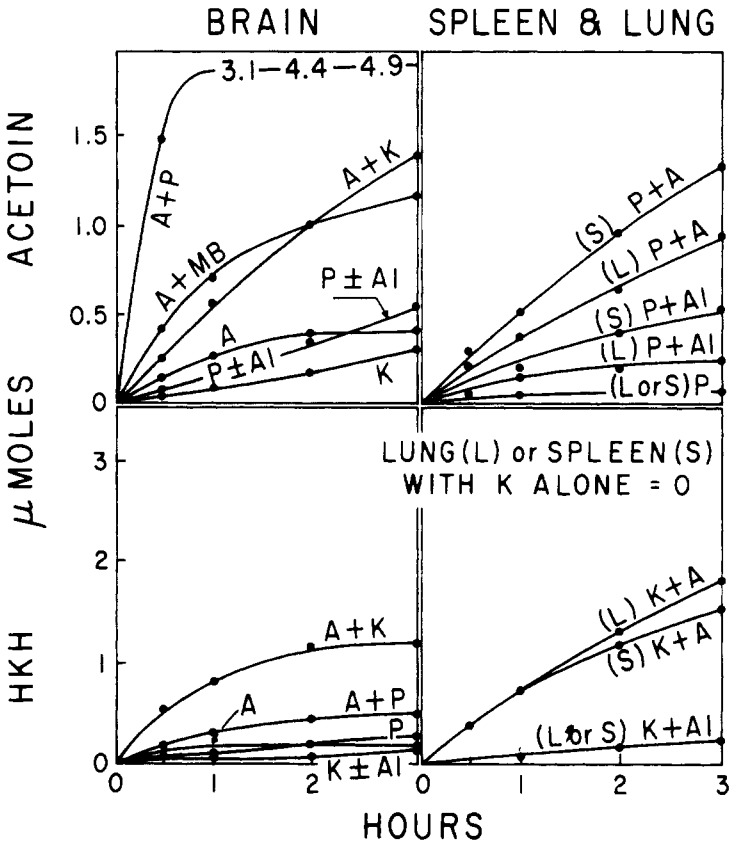

FIGURE 5: The simultaneous formation of acetoin and HKH by brain and spleen plus lung rat tissue homogenates in the presence of various combinations of substrates. All conditions are the same as in Figure 3.

In the presence of Pyr plus AcH all of the tissue homogenates formed large amounts of acetoin. Except for brain, their relative activities in forming acetoin from AcH plus Pyr were approximately the same as their relative activities in forming HKH from AcH plus KG. Heart was about four times and kidney about three times as active as liver or skeletal muscle with respect to both reactions; brain was one-half as active in forming HKH but two times as active as liver or skeletal muscle in forming acetoin. The particulate fraction from each of these homogenates formed about two-thirds as much acetoin from Pyr plus AcH as did the whole homogenates (not shown separately in Figures 3–5); the simultaneous formation of HKH by the particulate fraction from Pyr plus AcH was roughly equivalent to the curves shown for AcH alone in the whole homogenate.

When Pyr and KG were added together as the substrates in the absence of any added AcH, all of the tissues except heart formed about as much HKH as they did with KG alone, and they formed about as much acetoin as they did with Pyr alone (not shown separately). These tissues did not form large amounts of AcH from Pyr. While whole heart homogenate (Figure 3) did form a large amount of HKH from Pyr plus KG, the particulate fraction from heart was unable to carry out this reaction unless the supernatant fraction or thiamine pyrophosphate was added.

Other Substrates. When substrates other than Pyr or KG were added to the homogenate along with AcH, the formation of increased amounts of HKH and acetoin presumably reflected an increased supply of KG or Pyr for the condensation reaction. The addition of AcH plus 6 μmoles of glucose or fructose, or 2 mg of methylene blue, produced no more HKH than was obtained with AcH alone in any of the tissues. The addition of 6 μmoles of sodium glutamate also had little effect on the yield of HKH in brain or heart, as compared with AcH alone, but gave substantial increases with liver, kidney, and skeletal muscle (Figures 3 and 4).

The amount of acetoin formed by all the tissues in the presence of AcH was not increased by more than 30% when glucose, fructose, or glutamate was added simultaneously. Hence the endogenous supply of Pyr in these tissues was not increased greatly by such additions. The addition of methylene blue along with the AcH had no effect on the yield of acetoin in heart or liver, but gave sizeable increases with skeletal muscle and brain (Figures 4 and 5). Supplying an

autoxidizable dye to these two homogenates increased the Pyr formation from endogenous substrates.

Ethanol Substrate. Rat liver and kidney contain ethanol dehydrogenase and are responsible for the oxidation of most of the ethanol to acetaldehyde *in vivo*. The addition of ethanol plus Pyr or KG to these homogenates reflected this oxidation of ethanol to AcH by increased yields of acetoin and HKH. Both liver and kidney homogenates gave large increases in acetoin when Pyr plus ethanol was compared with Pyr alone; larger amounts of HKH were also formed from KG plus ethanol as compared with KG alone (Figures 3 and 4). Similar studies with lung and spleen (Figure 5) showed a small but definite effect of ethanol on the yield of acetoin and HKH from Pyr and KG, respectively; these tissues apparently have a weak ethanol-oxidizing capacity. Heart homogenate formed increased amounts of acetoin when ethanol was added with Pyr, but did not form more HKH when ethanol was added with KG. It is not known whether the ethanol was oxidized to AcH by heart homogenate or whether the ethanol affected the endogenous formation of AcH from Pyr. Any effect of ethanol on the formation of acetoin or HKH by skeletal muscle was small or negligible, and similar studies with brain homogenate were completely negative.

Stability of HKH in Tissue Homogenates. None of the rat tissue homogenates was capable of destroying any appreciable quantity of added HKH. When 200 μg (1.37 μmoles) of HKH was added to the various tissue homogenates in place of other substrates, there was no change whatsoever in this HKH concentration during 0.5-, 1-, 2-, or 3-hr incubation with brain, lung, spleen, or small intestine; liver and skeletal muscle destroyed less than 20% in 3 hr, while kidney and heart actually increased the HKH concentration by 20%. No acetoin appeared in the lung, spleen, or small intestinal homogenate during the 3-hr incubation with HKH, and only 5–10 μg of acetoin was produced by brain, skeletal muscle, heart, kidney, or liver. These latter tissues also formed an additional 5–15 μg of acetoin from endogenous substrates when 70 μg (0.80 μmole) of added acetoin was incubated with the homogenates for 3 hr. No significant formation of HKH (8–17 μg) occurred during the 3-hr incubation of acetoin with any tissue homogenate except heart (the latter formed 48 μg). The results showed a very poor utilization of either HKH or acetoin by these tissue homogenates and no interconversion between them.

Thiamine Deficiency

Weanling male rats (Holtzman) were fed either a purified control diet (Westerfeld and Richert, 1954) or the same diet without thiamine. Deficient rats were sacrificed by decapitation when growth ceased after 2–3 weeks on the deficient diet, and a corresponding control group was sacrificed simultaneously. The tissues from several rats were pooled, and a 1:5 homogenate was prepared with the 0.05 M potassium phosphate buffer, pH 7.1. The incubation flask contained: 1 ml of tissue homogenate (200 mg of fresh tissue), 6 μmoles of α-ketoglutarate (or pyruvate) plus 223 μmoles of acetaldehyde (0.6 ml), and additional phosphate buffer to a total volume of 3.2 ml. Thiamine pyrophosphate (1 mg) was added to duplicate flasks in order to determine the *in vitro* TPP effect. With heart homogenate, an additional substrate combination composed of 6 μmoles of Pyr plus 6 μmoles of KG (no AcH) was also tested. All flasks were capped and incubated with shaking for 1 hr at 38°. Tungstic acid filtrates were prepared and analyzed for acetoin and HKH as previously described. The activity of liver and kidney in forming HKH from KG plus AcH was the same when rats were fed either the control purified diet or chow.

The average results obtained in three to five thiamine-deficient experiments are given in Table I. The marked effect of thiamine deficiency on the formation of acetoin from Pyr plus AcH was evident in all tissues. The

TABLE I: The Formation of Acetoin and 5-Hydroxy-4-ketohexanoic Acid (HKH) by Tissue Homogenates Obtained from Rats Fed a Purified Control or Thiamine-Deficient Diet.[a]

			Control Diet		Thiamine Deficient	
Tissue	Substrate	TPP	Acetoin	HKH	Acetoin	HKH
Heart	KG + AcH	−	2.84	6.03	0.34	5.55
		+	4.15	6.23	2.90	5.24
	Pyr + KG	−	0.34	0.48	0.06	0.02
		+	0.45	0.68	0.34	0.31
	Pyr + AcH	−	6.14	1.16	1.53	0.79
		+	6.65	1.16	5.85	0.96
Liver	KG + AcH	−	0.85	4.18	0.17	1.64
		+	1.02	4.28	0.91	2.05
	Pyr + AcH	−	1.93	0.89	0.45	0.96
		+	2.10	0.96	1.48	1.06
Kidney	KG + AcH	−	0.97	4.86	0.11	3.49
		+	1.65	4.76	1.25	4.18
	Pyr + AcH	−	3.47	0.68	0.45	0.38
		+	4.32	0.79	2.39	0.48
Brain	KG + AcH	−	0.91	2.43	0.57	1.95
		+	1.19	2.43	0.91	2.23
	Pyr + AcH	−	2.67	0.58	1.53	0.38
		+	2.84	0.58	2.56	0.48

[a] The incubation system consisted of: 200 mg of tissue, 6 μmoles of α-ketoglutarate or pyruvate, 233 μmoles of acetaldehyde, and 1 mg of thiamine pyrophosphate (TPP); 1 hr at 38°; phosphate buffer, pH 7.1; total volume 3.2 ml. Values for acetoin and HKH are given in μmoles/200 mg of tissue/hr. The standard error of the mean for these determinations was about 11% of the mean for the acetoin values and 12% of the mean for HKH.

TABLE II: The Formation of Acetoin and 5-Hydroxy-4-ketohexanoic Acid from α-Ketoglutarate (Plus Acetaldehyde) by Control and Thiamine-Deficient Rat Heart Homogenates and Fractions Thereof.[a]

	Control				Thiamine Deficient			
	HKH[b]		Acetoin		HKH		Acetoin	
	−	+TPP	−	+TPP	−	+TPP	−	+TPP
Whole homogenate	33.7	33.6	3.18	6.25	8.8	11.6	0.23	4.15
Particulate fraction[c]	24.2	24.2	0.45	4.09	6.3	6.6	0.06	2.78
Supernate	5.9	6.0	0.06	0.17	0.8	0.9	0.06	0.11
Particulate plus supernate	30.3	30.3	1.93	5.11	7.2	8.5	0.17	3.47

[a] Incubation process: 200 mg of rat heart + 33 μmoles of KG + 223 μmoles of AcH ± 200 μg of thiamine pyrophosphate (TPP) in 3.2 ml at 38° for 1 hr. [b] HKH and acetoin: μmoles/200 mg of tissue/hr. [c] The particulate fraction was the pellet obtained when a 1:5 homogenate in 0.05 M phosphate buffer, pH 7.1, was centrifuged twice for 2 hr each time at 40,000 rpm in a Spinco preparative ultracentrifuge.

thiamine-deficient heart homogenate formed only 25% (1.53/6.14) as much acetoin as its control, and the corresponding values for other tissues were: liver 23%, kidney 13%, and brain 58%. The *in vitro* addition of TPP had little effect on this reaction when added to tissue homogenates from control rats, but TPP restored the activity of the deficient homogenates to 70–95% of their control values.

The formation of HKH from KG plus AcH was affected relatively little by thiamine deficiency in kidney or brain (70–80% of control values), but was clearly inhibited (40% of control) in the liver homogenate. The addition of TPP *in vitro* had no effect on this reaction in the control tissue homogenates, and it had relatively little effect in restoring the activity of the deficient liver. Either the apoenzyme of this system in liver was lost along with the cofactor, or the cofactor was not TPP alone. Clearly the acetoin- and HKH-forming systems were different, and only the former was severely depleted by thiamine deficiency.

The formation of HKH from Pyr plus KG by heart homogenate was practically eliminated by thiamine deficiency (Table I) and this activity was partially restored by the *in vitro* addition of TPP. The decreased activity of the Pyr plus KG reaction was due to a block in the conversion of Pyr to AcH, since the condensation of the latter with KG was still vigorous in the thiamine-deficient heart.

A defect in the formation of HKH from AcH plus KG by a thiamine-deficient heart was demonstrated when the amount of KG added to the incubation system was increased from the previous 6 to 33 μmoles (Table II). At these higher substrate concentrations (33 μmoles of KG plus 223 μmoles of AcH), a control heart homogenate formed 33.6 μmoles of HKH in 1 hr; the heart homogenate from thiamine-deficient rats formed only 8.9 in 1 hr, and this was increased to 11.6 μmoles by the *in vitro* addition of 200 μg of TPP. As with liver the defect produced in this system in heart by thiamine deficiency was not reversed in any major way by the addition of TPP *in vitro*.

Particulate Fraction. The system responsible for the condensation of KG plus AcH to form HKH was located primarily in the particulate fraction of the heart homogenate obtained from both control and thiamine-deficient rats. Rat heart was homogenized with 4 volumes of 0.05 M potassium phosphate buffer, pH 7.1, and the 1:5 homogenate was centrifuged for 2 hr at 40,000 rpm in a Spinco preparative ultracentrifuge with head no. 40. The supernate was decanted, and the pellet was rehomogenized with an original volume of buffer and centrifuged again for 2 hr at 40,000 rpm. The wash supernate from this centrifugation was discarded. The pellet was resuspended in buffer and diluted to its original volume for testing of the particulate fraction.

The results obtained from the incubation of these fractions with 33 μmoles of KG plus 223 μmoles of AcH are given in Table II. In a normal rat heart most, but not all, of the HKH-forming activity was sedimented by the two centrifugations. One-half of the activity remaining in the supernate could be sedimented by an additional 2-hr centrifugation, and all of it was destroyed by heating for 2 min in a boiling water bath. Similar studies with the heart homogenate obtained from thiamine-deficient rats gave much lower yields of HKH, but the activity was again localized in the particulate fraction. Added TPP had relatively little effect on the HKH-forming activity of any fraction.

The simultaneous formation of acetoin in these experiments (Table II) depended upon endogenous Pyr or the formation of Pyr from KG, as well as a condensation of the Pyr with the added AcH. In the control homogenate considerable acetoin was formed, and this was increased appreciably by added TPP. However, the particulate fraction formed little acetoin

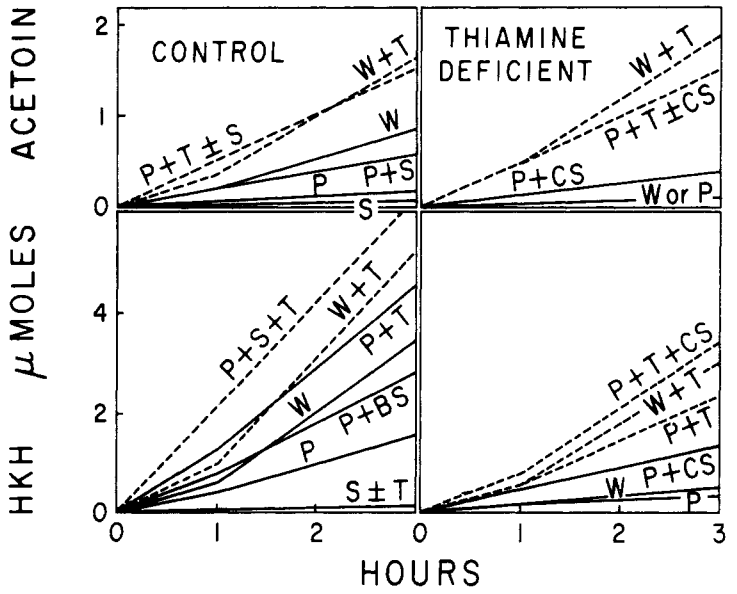

FIGURE 6: The formation of acetoin and HKH from pyruvate plus α-ketoglutarate by control or thiamine-deficient rat heart homogenates and fractions thereof. Incubation process: 200 mg of tissue, 33 μmoles of pyruvate plus 33 μmoles of α-ketoglutarate, phosphate buffer, pH 7.1, ±200 μg of thiamine pyrophosphate, in a total volume of 3.2 ml at 38°. W = whole homogenate; P = particulate fraction or the washed pellet obtained from centrifuging the homogenate for 2 hr at 40,000 rpm; S = supernate of original homogenate; T = thiamine pyrophosphate; CS = control supernate; BS = boiled supernate.

from KG plus AcH unless TPP was added. None of the thiamine-deficient fractions formed much acetoin unless TPP was added.

Pyruvate Plus KG Reaction. The formation of HKH from Pyr plus KG by heart homogenate was found in the previous studies to be dependent upon some factor in the supernate in addition to the condensing system in the particulate fraction. This factor in the supernate was possibly an enzyme which converted Pyr to AcH, but the following additional studies showed that TPP alone was responsible for this effect. In the presence of added TPP, the particulate fraction converted Pyr to AcH as well as condensed the latter with KG to form HKH.

A 1:5 rat heart homogenate in 0.05 M potassium phosphate buffer, pH 7.1, was centrifuged twice as previously described to separate the particulate and supernate fractions. Aliquots equivalent to 200 mg of original tissue were incubated at 38° with 33 μmoles of Pyr plus 33 μmoles of KG (both neutralized to pH 7.1) ± 200 μg of TPP in a total volume of 3.2 ml. After 1–3-hr incubation, tungstic acid filtrates were prepared and analyzed for acetoin and HKH.

The amount of HKH formed from Pyr plus KG by the whole homogenate of normal rat heart was increased by the *in vitro* addition of TPP (Figure 6). The activity of the particulate fraction was much less than that of the whole homogenate, but it was restored almost completely by the *in vitro* addition of TPP. The supernate had little or no activity by itself with or without added TPP. Hence the particulate fraction alone was able to convert Pyr to AcH when TPP was added, as well as condense the latter with KG. In the whole homogenate the supernate provided the TPP for this reaction, and when the supernate was recombined with the particulate fraction, the HKH formation was approximately the same as that obtained by adding TPP to the particulate fraction (not shown separately). A boiled supernate was less effective, and the supernate from a thiamine-deficient heart homogenate had relatively little effect when added to the control particulate fraction. The small additional effect of the supernate, beyond that which was due to the TPP, can be attributed to the incomplete sedimentation of the AcH–KG condensing system.

The formation of acetoin under these circumstances (Figure 6) apparently involved a conversion of some of the Pyr to AcH prior to a condensation of the latter with more Pyr since the results were similar to those obtained with HKH formation. The activity of the whole control homogenate was increased by added TPP; the particulate fraction alone was relatively inactive but was largely or completely restored by TPP or a fresh or boiled supernate (but not by a thiamine-deficient supernate). In the presence of added TPP, none of the supernates had any additional effect. These results clearly established TPP as the only factor in the supernate which contributed to the formation of acetoin from Pyr by the particulate fraction. Additional TPP was not required by the particulate

fraction to form acetoin from Pyr plus AcH, although the yield with the particulate fraction alone was only two-thirds that obtained with a whole homogenate.

A heart homogenate from a thiamine-deficient rat formed little HKH or acetoin from Pyr plus KG, but both activities were increased markedly by TPP added *in vitro* (Figure 6). The addition of control supernate plus TPP to the deficient particulate fraction had more effect than TPP alone on the formation of HKH but not acetoin, and this was consistent with the presence of a small amount of the AcH–KG but not the AcH–Pyr condensing system in the supernate; this additional activity in the supernate for the formation of HKH was lost on boiling.

The amount of TPP added in the previous experiments was in large excess and the following additional experiments were conducted to see if the amount of thiamine present in normal rat heart could account for the activity of the supernate. A control heart particulate fraction which formed 1.67 μmoles of HKH in 3 hr from Pyr plus KG formed 3.64, 4.51, 4.84, and 5.48 μmoles upon the addition of 1, 5, 10, or 50 μg of TPP. At the same time, the addition of 0.5-, 1.0-, or 1.5-ml aliquots of a control supernate (which had been centrifuged twice at 40,000 rpm for 2 hr each) to the particulate fraction gave HKH values of 3.34, 4.86, and 5.91; boiled supernate gave 3.25, 4.45, and 5.27 μmoles, respectively. From these results, 0.6–0.8 μg of TPP would completely account for the activity of 0.5 ml of the fresh or boiled supernate, and this concentration is within the normal range of 5–10 μg of thiamine/g of heart (Olson *et al.*, 1948; Schultz *et al.*, 1939). A small amount of the AcH–KG condensing activity was also present in the supernate since the total amount of HKH formed with 1.5 ml of supernate was more than that formed with pure TPP, and this extra activity was abolished by boiling. The simultaneous formation of acetoin in these studies indicated that about 0.5 μg of TPP would account for the activity of 0.5 ml of fresh or boiled supernate. Glutathione had no effect on the formation of HKH or acetoin by the particulate fraction in the presence or absence of added TPP. Thiamine chloride had no activity when used in place of TPP.

Discussion

Two different systems are involved in the formation of acetoin from Pyr plus AcH as compared with the formation of HKH from KG plus AcH, although both are located in the particulate fraction of the cell. The thiamine cofactor was readily removed from the acetoin-forming system during thiamine deficiency, and it appeared to be washed away to some extent in the preparation of the particulate fraction; it was readily replaced by the addition of TPP *in vitro*. Thiamine deficiency had relatively little effect on HKH formation, and this is consistent with a tenacious retention of the cofactor by the ketoglutarate oxidase system (Gubler, 1961). It is possible that the formation of acetoin and HKH are effected by the pyruvate oxidase and ketoglutarate oxidase systems, respectively, when AcH is added along with their usual substrates, but no evidence has yet been obtained on the identity of the HKH-forming system. It seems probable that pyruvate oxidase is also responsible for the formation of acetaldehyde from pyruvate when TPP is added to the particulate fraction, but the possibility of a separate pyruvate decarboxylase in the particulate fraction has not been ruled out. The endogenous ethanol produced by rat heart (McManus *et al.*, 1966) undoubtedly arises from this reaction.

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