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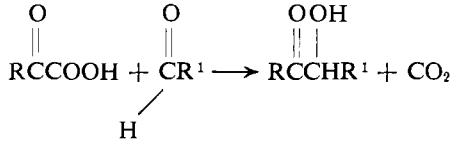
A Study of the Enzymatic Reactions Involved in the Formation of 5-Hydroxy-4-ketohexanoic Acid and Its Isomer, 5-Keto-4-hydroxyhexanoic Acid*

Leslie M. J. Shaw and W. W. Westerfeld

ABSTRACT: The enzymatic formation of 5-keto-4-hydroxyhexanoic acid (KHH) has been shown to occur by the condensation of succinic semialdehyde (SSA) with pyruvic acid (Pyr). This reaction was catalyzed by a beef heart particulate preparation and was markedly dependent upon the presence of thiamine pyrophosphate (TPP). Mg^{2+} was found to increase the formation of KHH by an additional 18%. In addition to kinetic data for the KHH reaction, kinetic studies of the analogous condensation of α-ketoglutaric acid (KG) with acetaldehyde (AcH) to form 5-hydroxy-4-ketohexanoic acid (HKH) are presented. The latter reaction was shown to occur also in a nonenzymatic model system with an absolute requirement for thiamine. Evidence is presented which indicates that it is likely that KHH formation is catalyzed by pyruvate decarboxylase, the same enzyme that serves as a catalyst in the formation of acetoin from Pyr and AcH. The inhibition of the KHH, HKH, and acetoin reactions by various reagents is described. The possible physiological significance of the KHH condensation as well as of KHH itself is not known at present.

The enzymatic condensation of α-keto acids with various aldehydes by the particulate fraction of mammalian tissues follows the general reaction



The reaction between pyruvate and acetaldehyde to form acetoin is well known, and the reaction between pyruvate and succinic semialdehyde to form 5-keto-4-hydroxyhexanoic acid is described in this paper. The analogous reaction between α-ketoglutarate and aldehydes was recognized recently when Bloom and Westerfeld (1966) identified 5-hydroxy-4-ketohexanoic acid as the product of the KG plus AcH[1] reaction; Koch and Stokstad (1966), Stewart and Quayle (1967),

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[1] Abbreviations used in this paper that are not defined in *Biochemistry 5*, 1445 (1966), are: HKH, 5-hydroxy-4-ketohexanoic acid; KHH, 5-keto-4-hydroxyhexanoic acid; Pyr, pyruvic acid; AcH, acetaldehyde; KG, α-ketoglutaric acid; SSA, succinic semialdehyde.

and Schlossberg *et al.* (1968) have presented evidence showing that 2-hydroxy-3-ketoadipate is the product of the condensation of KG with glyoxylate. Kinetic data for the KG plus AcH reaction, as well as a non-enzymatic model system for this reaction, are also reported in this paper.

Methods

Purification of KHH and HKH was achieved by column chromatography on Dowex 1-X10, Dowex AG 1-X4, and Celite according to the procedures described by Bloom and Westerfeld (1966). The Voges–Proskauer colorimetric procedure employed by the latter authors was also used for the quantitative determination of acetoin, HKH, and KHH. KHH and HKH gave identical quantities of color per micromole and authentic HKH was used as a standard in the quantitative colorimetric analysis of KHH.

SSA was prepared by a modification of the procedure of Witt and Holzer (1963). Final purification was achieved by chromatography on Celite. Most of the SSA was eluted in the forerun fraction (tubes 3–8) peaking at 5; a small quantity of the compound was also eluted in tubes 16–22 peaking at 19. SSA in these two fractions was quantitated by titration with dilute NaOH and also by means of the spectrophotofluorometric method of Salvador and Albers (1959). The forerun fraction was found to titrate at a much slower rate than the second fraction; this is most likely due to the formation of a ringed compound. Preparation of the 2,4-dinitrophenylhydrazone of SSA from both peaks resulted in the formation of a derivative whose lemon yellow color and melting point (205°) were identical with that of an authentic derivative. The melting point of a mixture of authentic derivative with each of the two did not result in a lowering of the melting point.

Preparation of Beef Heart Particulate Fraction. Trimmed beef heart was minced in the cold with a meat grinder, mixed with 0.1 M potassium phosphate buffer (pH 7.1) in a 1:5 dilution, and homogenized with the Teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 105,400*g* in the Spinco ultracentrifuge for 2 hr; the resulting precipitate was rehomogenized in phosphate buffer (1–5 dilution) and centrifuged for an additional 2 hr.

Crude Mitochondrial Preparation. Mitichondria were prepared by a modification of the method of Cleland and Slater (1953). Minced beef heart (70 g) in a 1:5 dilution with 0.1 M potassium phosphate buffer (pH 7.1) was homogenized with the all-glass Potter-Elvehjem apparatus. The homogenate was spun at 12,000*g* for 30 min in the Servall ultracentrifuge and the resulting precipitate was resuspended in the same buffer and centrifuged at 755*g* for 10 min. Supernatants from this and the subsequent resuspension and centrifugation of the precipitate at 755*g* for 10 min were combined and centrifuged at 12,000*g* for 30 min. The resulting pellets were resuspended and recentrifuged at 12,000*g* for 30 min.

pH 4.9 Mitochondrial Precipitate. Three mitochondrial pellets were homogenized with 35 ml of 0.5 M potassium phosphate buffer (pH 7.1). This suspension was brought to a temperature of −2° in a sodium chloride–ice bath and 10% acetic acid was carefully added with stirring to a final pH of 4.9. After spinning the mixture for 10 min at 1000*g* the resulting precipitate was homogenized in 0.5 M potassium phosphate buffer while the supernatant was neutralized with 4.5 N KOH. This procedure was carried out in a cold room at a temperature of 3–4°.

Protein Determination. Protein nitrogen was determined by the Kjeldahl procedure. The protein weight was estimated by multiplying the nitrogen weight by 6.25.

Results

Large-Scale Synthesis of KHH. Eight particulate pellets from 18 g of beef heart were homogenized with 80 ml of 0.01 M potassium phosphate buffer (pH 7.1) and placed in a 1-l. ehrlenmeyer flask. SSA (1.73 mmoles) from tubes 16–22 of the Celite column were added to this in addition to 3 mmoles of pyruvate-2-^{14}C, 65 μmoles of TPP, and 70 ml of the same buffer. Two hours later, 3 mmoles of pyruvate-2-^{14}C was added to the reaction mixture. At the end of 1.5 hr more, 3 mmoles of pyruvate-2-^{14}C was added. The specific activity of the labeled Pyr was 132 cpm/μmole. Total incubation time was 7 hr. The yield of chromogenic material in the 1 N formic acid eluate of a Dowex 1-X10 column was 240 mg or 1.64 mmoles (based on mol wt 146 and 0.185 μg of HKH/Klett unit). The material (172 mg) contained in the 1 N formic acid eluate was flash evaporated to dryness at 40°, diluted with distilled water, neutralized, and chromatographed on a 17 × 320 mm Dowex AG 1-X4 column. Colorimetric and radioactivity measurements indicated a major peak of KHH (152 mg) at tube 27 and two unidentified minor components in tubes 9–15 (9 mg) and 35–41 (8 mg) as estimated colorimetrically. The fraction collected in tubes 24–34 was flash evaporated and rechromatogrammed on a Celite column. Two different peaks of KHH were obtained and quantitated by the colorimetric procedure, titration, and radioactivity assay. The results of one such Celite chromatogram are as follows (20 × 700 cm column): fraction 1 contained in tubes 3–8, peak at 5: 80 mg of KHH; fraction 2 in tubes 17–26, peak at 22: 13 mg of KHH. The radioactivity in each tube of both fractions was determined by counting with the gas-flow Geiger-Mueller counter neutralized aliquots from the aqueous phase dried on aluminum planchets. In each fraction, the specific activity of KHH was 131 cpm/μmole. The material in fraction 1 titrated very slowly and was believed to be KHH in the lactone form. Preparation of the 2,4-dinitrophenylosazone of KHH from each peak yielded the same derivative obtained from HKH (mp 276°) and gave the same mixture melting point. The phenylosazone of KHH was also the same derivative obtained from HKH (mp and mmp 188°).

Periodate Oxidation of KHH. Periodate oxidation of KHH from each Celite peak gave similar results.

TABLE I: Incubations with Particulate Fraction of Beef Heart.[a]

α-Keto Acid (μmoles)	Aldehyde (μmoles)	0.22 μmole of TPP	Acetoin (μmoles/hr)	HKH or KHH (μmoles/hr)
KG (20)	AcH (107)	+	1.50	9.72
KG (20)	AcH (107)	−	0.33	7.29
KG (20)		+	0.01	0.04
Pyr (40)	AcH (107)	+	4.65	0.13
Pyr (40)	AcH (107)	−	2.87	0.00
Pyr (40)		+	0.49	0.09
Pyr (40)		−	0.19	0.00
	AcH (107)	+	1.27	0.00
	AcH (107)	−	0.25	0.00
KG (20) + Pyr (40)		+	0.39	0.59
KG (20) + Pyr (40)		−	0.04	0.19
	SSA (20) + AcH (107)	+	1.07	0.40
	SSA (40) + AcH (107)	+	0.27	1.03
Pyr (40)	SSA (20)	+	0.33	1.76
Pyr (40)	SSA (40)	+	0.01	3.89
Pyr (40)	SSA (40)	−	0.05	0.23
	SSA (40)	+	0.00	0.02
KG (20)	SSA (40)	+	0.00	0.05
Pyr (40)	SSA (20) (no enzyme)	+	0.00	0.00
		+	0.00	0.00

[a] Total volume in each capped, 20-ml beaker was 3.0 ml. Particulate protein (15.3 mg) and potassium phosphate buffer (150 μmoles) (pH 7.1) were present in each beaker; temperature 37.5°. The reaction was stopped at the end of 1 hr by the addition of 0.5 ml of 10% sodium tungstate and 1.0 ml of 0.66 N sulfuric acid. A 0.5-ml aliquot from each reaction mixture was neutralized with 0.1 N potassium hydroxide, diluted to a total volume of 5.0 ml, and put on an 8 × 45 mm Dowex column in order to separate acetoin from HKH or KHH. Each reaction was run in duplicate.

Oxidation of the 0.19-mmole peak 2 KHH (131 cpm/μmole) in 6 ml of aqueous solution was carried out by reaction with 0.39 mmole of $NaIO_4$ contained in a volume of 4 ml. After neutralizing this mixture with dilute base, it was allowed to react at room temperature for 20 min, at which time 0.205 mmole of ethylene glycol was added to reduce the excess $NaIO_4$. Celite chromatography of the reaction mixture resulted in two titratable peaks; tubes 3–10 contained 0.15 mmole of SSA as determined by the spectrophotofluorometric method and was free of radioactivity. The 2,4-dinitrophenylhydrazone prepared from this fraction was identical in color, melting point, and mixture melting point with the authentic 2,4-dinitrophenylhydrazone of SSA. The second peak (tubes 12–20) contained 0.16 mmole of acetic acid (by titration) which formed a *p*-phenylphenacyl ester with a specific activity of 155 cpm/μmole and a melting point of 111° (no depression on mixing with the acetic acid derivative). No succinic acid was detected in the reaction products.

Pyruvate Plus SSA Reaction. The reaction between SSA and Pyr was markedly dependent upon the presence of TPP when the beef heart particulate fraction was used as enzyme source (Table I) and, subsequent to the experiment reported in Table I, the yield of KHH was shown to be increased by an additional 18% when 5 μmoles of $MgSO_4$ was added to the incubation mixture. Since it is known that KG alone, incubated with the pig heart particulate preparation of Green *et al.* (1942) is converted into SSA, the condensation of SSA with AcH was also tested. If non-TPP-bound SSA were an intermediate in the formation of HKH from KG and AcH, then a substantial yield of HKH should be obtained from the condensation of SSA with AcH. As can be seen from the data in Table I, the yield of HKH was substantially less than that obtained from KG plus AcH and free SSA is not a likely intermediate in the HKH-forming reaction.

In an attempt to determine whether or not the Pyr plus SSA condensation reaction is catalyzed by pyruvate decarboxylase, the enzyme which is known to catalyze the formation of acetoin from Pyr plus AcH, AcH was incubated in the presence of Pyr plus SSA (Table II). A 7.5-fold inhibition of KHH formation was observed in the presence of 71 μmoles of AcH while only a slight inhibition of acetoin formation occurred in the presence of 80 μmoles of SSA. Figure 1 shows some kinetics of the reaction as well as the Lineweaver–Burke plot of 1/KHH *vs.* 1/SSA in the absence and in the presence of 5.35 and 10.7 μmoles of AcH; the competition between AcH and SSA for Pyr is of the "mixed" type (Webb, 1963). Thus, AcH is a potent inhibitor of the Pyr plus SSA condensation

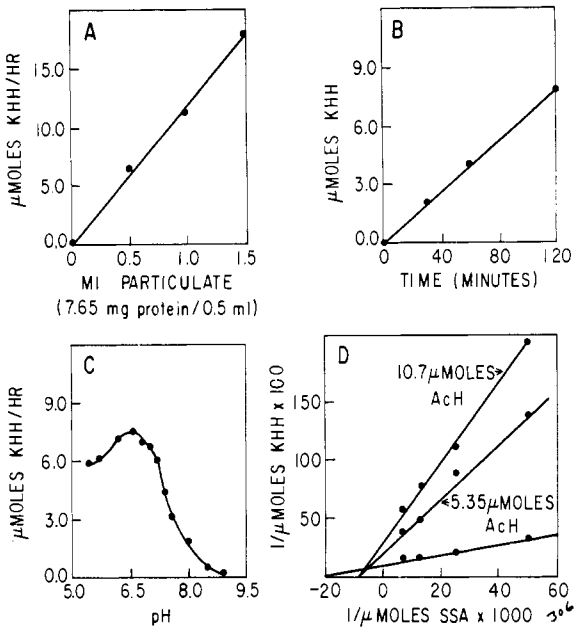

FIGURE 1: Kinetic data for the KHH reaction. Contents in a total volume of 3.0 ml/flask: 7.65 mg of beef heart particulate protein except where enzyme concentration was varied, 80 μmoles of SSA, 60 μmoles of Pyr, 0.22 μmole of TPP, and 10 μmoles of $MgSO_4$ except where SSA concentration was varied. In C 300 μmoles of potassium phosphate buffer at each pH value tested for was used. Borate buffer (pH 8.9) and acetate buffer (pH 5.4) were used at those values. In all other flasks 85 μmoles of potassium phosphate buffer (pH 7.1) was used. All other conditions were exactly the same as those described in Table I.

while SSA has only a small inhibitory effect on the Pyr plus AcH condensation. It is likely but not certain from these data that the condensation of Pyr with both AcH and SSA is catalyzed by the same enzyme, and that hydroxyethyl-TPP (Krampitz *et al.*, 1962; Holzer *et al.*, 1962) is the enzymatic intermediate in the Pyr plus SSA reaction as well as the Pyr plus AcH reaction.

TABLE II: The Effect of AcH on the Pyr Plus SSA Condensation.[a]

Flask Contents	Acetoin (μmoles/hr)	KHH (μmoles/hr)
Pyr + SSA	0.00	5.18
Pyr + AcH	3.35	0.00
Pyr + SSA + AcH	3.15	0.69

[a] Substrate concentrations: 60 μmoles of Pyr, 80 μmoles of SSA, and 71 μmoles of AcH except for 3.0–120 μmoles of Pyr was used. Each flask contained 0.22 μmole of TPP, 10 μmoles of $MgSO_4$, 85 μmoles of potassium phosphate buffer (pH 7.1), and 7.65 mg of particulate protein. Subsequent analysis of KHH and acetoin was done according to the method indicated in Table I.

TABLE III: The Effect of Added TPP upon the Condensation of α-KG with AcH by pH 4.9 Precipitated Mitochondria.[a]

Flask Contents	Acetoin (μmoles/hr)	HKH (μmoles/hr)
Crude Mitochondria		
α-KG + AcH	0.01	2.70
α-KG + AcH + TPP + Mg^{2+}	0.56	3.52
pH 4.9 Precipitate		
α-KG + AcH	0.08	0.29
α-KG + AcH + TPP + Mg^{2+}	0.61	1.30
Supernatant		
α-KG ± AcH	0.07	0.05
α-KG + AcH + TPP + Mg^{2+}	0.11	0.08

[a] Incubation procedure: 8 μmoles of KG, 107 μmoles of AcH, 500 μmoles of potassium phosphate buffer (pH 7.1), ±2.2 μmoles of TPP and 4 μmoles of $MgSO_4$, and either crude mitochondria, pH 4.9 precipitated mitochondria, or supernatant. The conditions of incubation and subsequent analysis are the same as described in Table I.

KG Plus AcH Reaction. Either crude beef heart mitochondria (A and B) or the pH 4.9 precipitated mitochondria (C) were used in obtaining the kinetic data for the condensation of KG with AcH (Figure 2). Bloom *et al.* (1966) reported that the rate of acetoin formation was markedly depressed in various tissues of thiamine-deficient rats and that this activity could largely be restored by adding TPP to the incubation mixture. On the other hand, there was no major depression in HKH formation in the thiamine-deficient rat. The addition of TPP to the HKH-forming system of mitochondria resulted in only an approximately 30% increase in activity (Table III). However, when the mitochondria were subjected to a pH of 4.9 in the cold and then neutralized rapidly, the catalytic effect of TPP on the KG plus AcH reaction was readily demonstrated; approximately 90% of the original activity was lost by the acid treatment and approximately 40% of the original activity was restored by the TPP (Table III). The amount of TPP required to reactivate the acid-treated mitochondria is shown in Figure 2C, and a comparison of these data with that obtained for the acetoin reaction (Figure 3D) illustrates the marked difference in the binding of TPP to the enzymes catalyzing these two reactions; 0.022 μmole of TPP was optimal for the acetoin reaction in the particulate fraction whereas at least 2.2 μmoles of TPP was needed for optimal HKH formation in the acid-treated mitochondria.

In addition to HKH, acetoin was also produced

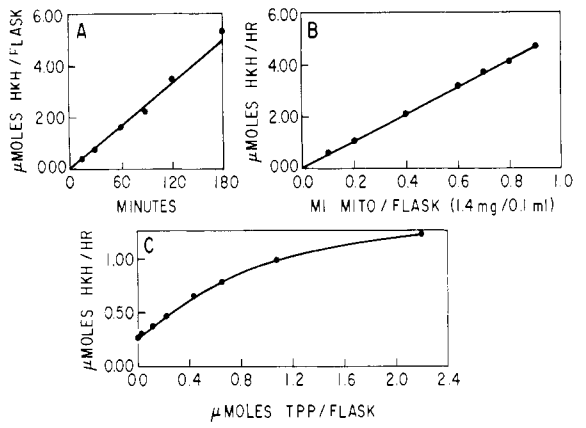

FIGURE 2: Kinetic data for the HKH reaction. Each flask in the *V vs.* time experiment (A) contained 5.6 mg of crude mitochondrial protein. In A and B each flask contained 8 μmoles of KG, 71 μmoles of AcH, and 150 μmoles of potassium phosphate buffer (pH 7.1), whereas 8 μmoles of KG, 107 μmoles of AcH, 500 μmoles of potassium phosphate buffer, 4 μmoles of $MgSO_4$, and pH 4.9 precipitated mitochondria were contained in the flasks used in C. All other reaction and analytical procedures used were identical with those described in Table I.

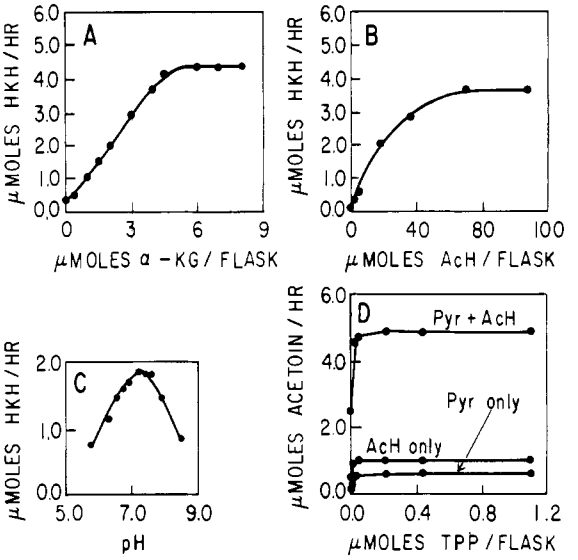

FIGURE 3: Kinetic data for the HKH and acetoin reactions. In A the KG concentration was varied while the amount of AcH was held constant at 71 μmoles in the presence of 4.6 mg of crude mitochondrial protein. All other conditions were the same as described in Figure 2. In B the AcH concentration was varied at a constant KG concentration of 8 μmoles and under the same conditions as in A. The optimal substrate concentrations of 8 μmoles of KG and 71 μmoles of AcH were used in C in the presence of 240 μmoles of phosphate buffer at each pH value indicated. The mitochondrial preparation used had a lower activity than that in A and B. Pyr (40 μmoles), AcH (71 μmoles), and particulate protein (15 mg) were used in D with all other conditions the same as in A.

when KG plus AcH was incubated with the particulate fraction. In one experiment the quantity of acetoin produced from KG plus AcH was almost four times that from AcH alone. Some of the KG was presumably converted into Pyr which then condensed with AcH to produce a substantial quantity of acetoin. In this same experiment, the Kreb's cycle intermediates succinate, malate, and oxaloacetate gave rise to acetoin when incubation in the presence of AcH. Thus, the cofactors required for the enzymatic conversion of KG into Pyr, namely NAD and coenzyme A, were still present in sufficient quantity in the particulate preparation for the enzymatic reactions involved in the conversion of KG into Pyr to occur.

Inhibitors. Several compounds were tested for their ability to inhibit the KG plus AcH condensation reaction. Glutamic and glutaric acids had no effect upon the reaction. *p*-Mercuribenzoate inhibited the reaction by 80% at a concentration of 1×10^{-4} M. Lineweaver–Burke plots (reciprocal of HKH formation *vs.* the reciprocal of AcH concentration) showed that 20 μmoles of glyoxylate acted as a competitive inhibitor.

Some of these inhibitors were also tested in the Pyr plus AcH condensation. *p*-Mercuribenzoate (1×10^{-4} M) produced a 92% inhibition. Glyoxylate (20 μmoles) gave rise to mixed inhibition when incubated in the presence of a constant Pyr concentration and varying AcH concentration. Glyoxylate also inhibited the Pyr plus SSA reaction. When incubated in the presence of constant Pyr and varying SSA concentration glyoxylate gave rise to mixed inhibition.

Nonenzymatic Formation of HKH. The incubation of 10 mmoles of KG, 10 mmoles of AcH, 1 mmole of thiamine, and 10 mmoles of borate buffer (pH 8.9), in a total volume of 136 ml for 10 hr at 40° resulted in the formation of 0.24 mmole of HKH which was purified on the three columns as described in the Methods section. The melting point and mixture melting point of the 2,4-dinitrophenylhydrazone derivative were identical with that of authentic HKH. Periodate oxidation of 54 μmoles of this HKH as previously

TABLE IV: The Nonenzymatic Formation of HKH and Acetoin.[a]

	Acetoin (μmoles)	HKH (μmoles)
α-KG + AcH + thiamine	0.12	1.16
Pyr + AcH + thiamine	2.47	0.00
Oxalacetate + AcH + thiamine	0.50	0.08
α-KG + AcH + no thiamine	0.00	0.01

[a] Reaction conditions: 200 μmoles of neutralized KG, 200 μmoles of AcH, 20 μmoles of neutralized thiamine chloride hydrochloride, and 200 μmoles of borate buffer (pH 8.9) were added to a 20-ml beaker in a total volume of 3.9 ml. The reaction vessels were then capped and evacuated. Incubation was carried out at 50° for 3 hr. The quantitation of HKH and acetoin was done in the same manner as described in Table I.

described (Bloom and Westerfeld, 1966) gave 50 μmoles of AcH as determined by the method of Stotz (1943); melting point of the 2,4-dinitrophenylhydrazone, 164–165° (no melting point depression on mixing with AcH derivative). Chromatography of the reaction mixture on Celite gave 102 μequiv (51 μmoles) of a titratable product in tubes 25–38, peak at 30. The *p*-phenylphenacyl ester prepared from this fraction was identical with the same derivative of succinic acid in melting point and mixture melting point (214°). Hence, HKH was produced by the nonenzymatic condensation of KG and AcH in the presence of thiamine. This nonenzymatic system was similar to the one used by Yatco-Manzo *et al.* (1959) to study the acetoin reaction. As shown in Table IV, thiamine is an absolute requirement for the nonenzymatic formation of HKH.

Discussion

At the moment, there is no known physiological role of the Pyr plus SSA condensation. Salvador and Albers (1959) determined the distribution of glutamic–γ-aminobutyric transminase activity in the nervous tissue of the rhesus monkey. The latter enzyme catalyzes the reaction 1. SSA is subsequently oxidized to

$$\alpha\text{-aminobutyric acid} + \text{KG} \longrightarrow \text{glutamic acid} + \text{SSA} \quad (1)$$

succinic acid. This sequence was shown to occur in substantial quantities in the grey matter of the central nervous system, but not in any other tissue. Thus, the Pyr plus SSA condensation provides a new pathway by which SSA can be metabolically utilized in the central nervous system. The pharmacological and physiological effects of the product of the condensation, KHH, if any, are unknown.

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