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2-Keto-4-hydroxybutyrate. Synthesis, Chemical Properties, and as a Substrate for Lactate Dehydrogenase of Rabbit Muscle*

Roger S. Lane and Eugene E. Dekker

ABSTRACT: 2-Keto-4-hydroxybutyric acid, obtained by reaction of DL-homoserine with pyridoxal in the presence of Cu^{2+} ions and isolated by ion-exchange column chromatography, has been crystallized as the γ-lactone and also as its 2,4-dinitrophenylhydrazone derivative. The physicochemical properties of these crystalline compounds have been examined. Crystalline lactate dehydrogenase from rabbit muscle catalyzes the reduction of 2-keto-4-hydroxybutyrate in the presence of reduced diphosphopyridine nucleotide. Maximal rates of reduction relative to pyruvate are 61, 43, and 25% for glyoxylate, 2-ketobutyrate, and 2-keto-4-hydroxybutyrate as substrates, respectively. The apparent K_m for 2-keto-4-hydroxybutyrate is 4.6×10^{-3} M in 33 mM potassium phosphate buffer at pH 7.4 and 25° compared with values of 3.3×10^{-4} M for pyruvate, 1.8×10^{-2} M for glyoxylate, and 4.8×10^{-3} M for 2-ketobutyrate. Oxamate competitively inhibits 2-keto-4-hydroxybutyrate reduction; in contrast, oxalate is a noncompetitive inhibitor. The dissociation constants for the enzyme–inhibitor complexes (K_i values), measured in 33 mM Tris-Cl buffer at pH 7.4 and 25°, are 8.0×10^{-5} and 8.1×10^{-4} M for oxamate and oxalate, respectively. Competitive inhibition by oxamate also occurs with pyruvate, glyoxylate, and 2-ketobutyrate (K_i values: 8.3×10^{-5}, 7.6×10^{-5}, and 7.4×10^{-5} M, respectively) whereas oxalate is noncompetitive (K_i values: 8.0×10^{-4}, 8.4×10^{-4}, and 8.0×10^{-4} M, respectively). The equilibrium for the reaction of 2-keto-4-hydroxybutyrate with reduced diphosphopyridine nucleotide in the presence of lactate dehydrogenase favors reduction of the ketohydroxy acid; the K_{equil} is 1.2×10^{-10} M determined in 0.30 M Tris-Cl buffer at 25°. Reduced diphosphopyridine nucleotide oxidation is also favored with pyruvate, glyoxylate, and 2-ketobutyrate as substrates (K_{equil} values: 4.8×10^{-12}, 1.0×10^{-9}, and 3.0×10^{-11} M, respectively). One mole of reduced diphosphopyridine nucleotide is oxidized for every mole of 2-keto-4-hydroxybutyrate reduced; 2,4-dihydroxybutyric acid has been identified as the product of the reaction. The metabolic significance of this enzymic reduction of 2-keto-4-hydroxybutyrate is discussed.

2-Keto-4-hydroxybutyric acid has been identified as a normal constituent in extracts of certain plants (Virtanen and Alfthan, 1955); its role in plant biochemistry, however, is unknown. This same compound has been suggested as a possible intermediate in the metabolism of homoserine by mammals (Meister, 1957).

In earlier studies, Hift and Mahler (1952) detected an enzyme in beef liver extracts that catalyzed the condensation of pyruvate with formaldehyde yielding 2-keto-4-hydroxybutyrate. Meister (1956) noted, in a review article, that 2-keto-4-hydroxybutyrate participates in the glutamine transaminase system; data supporting this statement have not been published. In 1966, Finkelstein *et al.* reported that crude extracts of human liver catalyze the formation of 2-keto-4-hydroxybutyrate from L-homoserine. Liver extracts of a patient with cystathionuria were found by these authors to be abnormally deficient in homoserine dehydratase activity but catalyzed the formation of 2-keto-4-hydroxybutyrate at a rate comparable with that observed with control extracts.

Studies carried out recently in our laboratories have pro-

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vided direct evidence that 2-keto-4-hydroxybutyrate is readily and efficiently metabolized in mammals by processes of transamination and aldolytic cleavage (Lane and Dekker, 1968a).[1] No information is presently available, however, concerning methods for preparing this compound nor regarding other enzymic reactions utilizing 2-keto-4-hydroxybutyrate. We report here a procedure for the facile chemical synthesis of 2-keto-4-hydroxybutyrate by reaction of DL-homoserine with pyridoxal in the presence of cupric ion; some chemical properties of the isolated compound are described. In addition, this paper describes the properties of the pyridine nucleotide-linked reduction of 2-keto-4-hydroxybutyrate in the presence of lactate dehydrogenase and compares the effectiveness of this keto acid as a substrate with other compounds (pyruvate, 2-ketobutyrate, and glyoxylate) previously shown to be especially effective substrates for the dehydrogenase (Friedmann *et al.*, 1951; Meister, 1952; Banner and Rosalki, 1967). A preliminary report of some of these findings has appeared (Lane and Dekker, 1968b).

Materials

Lactate dehydrogenase from rabbit muscle was obtained from Calbiochem as a crystalline suspension in 2.2 M $(NH_4)_2SO_4$; the specific activity was listed as 550 EU/mg of protein. Before use, the enzyme was either dialyzed or diluted with an appropriate buffer. DPNH (disodium salt) and DPN^+ were purchased from P-L Biochemicals; bovine serum albumin, sodium pyruvate, sodium phenylpyruvate, sodium glyoxylate, sodium 2-ketobutyrate, DL-homoserine, pyridoxal hydrochloride, and oxamic acid were products of Nutritional Biochemical Corp.; sodium DL-2-hydroxybutyric acid was procured from Calbiochem. All other materials not listed were commercial products of the highest analytical quality available.

The γ-lactone of 2-keto-3-phenyl-4-hydroxybutyric acid was synthesized by condensing formaldehyde with sodium phenylpyruvate according to the method of Hemmerle (1917). The product was recrystallized three times from ethanol. *Anal.* Calcd for $C_{10}H_8O_3$: C, 68.18; H, 4.58. Found: C, 68.49; H, 4.66; mp 204°.

2,4-Dihydroxybutyric acid was prepared by nitrous acid deamination of DL-homoserine, following the procedure of Akobe (1936). The product was crystallized as its amide with concentrated aqueous ammonia (Nagai and Flavin, 1967); 2,4-dihydroxybutyramide, twice recrystallized from anhydrous methanol, gave the following analytical values. *Anal.* Calcd for $C_4H_9NO_3$: C, 40.33; H, 7.61; N, 11.76. Found: C, 40.27; H, 7.53; N, 11.87; mp 121–122°; the infrared spectrum showed strong absorption bands at the following wavelengths: 2.98, 3.25, 5.98 (primary amide), 6.31, 7.55, 8.98, 9.29, 9.44, 10.08, and 11.10 μ.

Methods

General Methods. Protein was measured by the method of Lowry *et al.* (1951) with crystalline bovine serum albumin as a standard. Carbonyl compounds were quantitatively determined according to the procedure of Friedemann and Haugen (1943); sodium 2-ketobutyrate served as reference compound to estimate the concentration of 2-keto-4-hydroxybutyrate in solution. α-Keto acids were visualized on paper chromatograms by spraying with a 0.4% solution of 2,4-dinitrophenylhydrazine in 2 N HCl followed by 10% NaOH solution. 2,4-Dinitrophenylhydrazone derivatives were prepared and extracted with ethyl acetate in the usual manner (Block *et al.*, 1958). The concentration of 2,4-dihydroxybutyrate in solution was measured by a modification of the method of Barker and Summerson (1941), using sodium DL-2-hydroxybutyric acid as a standard. A molar extinction coefficient of 6.22×10^3 (Horecker and Kornberg, 1948) at 340 mμ was used to calculate the concentration of DPNH solutions. Absorbancy measurements at a single wavelength were made with a Gilford spectrophotometer (1-cm light path) equipped with a thermostated cuvet holder and a digital absorbance meter; a Cary model 15 recording spectrophotometer was used to obtain continuous ultraviolet absorption spectra. Infrared absorption spectra were recorded in Nujol using a Perkin-Elmer Model 237 double-beam spectrometer. A Fisher-Johns hot plate apparatus was used to measure melting points. Spang Microanalytical Laboratory, Ann Arbor, Mich., carried out the elemental analyses.

Enzyme Assay. The rate of DPNH oxidation at 340 mμ was followed to measure initial reaction velocities. Unless otherwise indicated, the reaction mixtures (3.0 ml) contained 33 mM potassium phosphate buffer (pH 7.4) and 0.067 mM DPNH, together with substrate and dehydrogenase at varying concentrations. Addition of lactate dehydrogenase usually initiated the reaction; continuous readings were taken for 3–5 min thereafter. One unit of enzyme activity is defined as the amount of protein causing the oxidation of 1 μmole of DPNH/min at 25°.

Synthesis of 2-Keto-4-hydroxybutyric Acid. 2-Keto-4-hydroxybutyric acid was prepared in solution as follows. DL-Homoserine (5 mmoles), pyridoxal hydrochloride (5 mmoles), and $CuCl_2 \cdot 2H_2O$ (2.5 mmoles) were dissolved in 25 ml of water and the pH of the mixture was immediately adjusted to 5.0 with KOH solution. Acetate buffer (10 mmoles, pH 5.0) was added and the solution was heated for 20 min at 100°. The resulting suspension was chilled in ice and the pH was raised to 6.8 by addition of alkali. The neutralized reaction mixture was then applied to a column (2.0 × 66 cm) of Dowex 50 (H^+) resin. 2-Keto-4-hydroxybutyrate was washed from the resin column with water; 8.5-ml fractions were collected. A typical elution profile is shown in Figure 1. The contents of those tubes containing keto acid were pooled, the pH of the solution was raised to 6.9 by adding alkali, and the product was concentrated to a small volume (about 15 ml) by lyophilization. A 30–35% yield of 2-keto-4-hydroxybutyrate is obtained; the compound is stable in solution for at least 2 months when frozen at neutral pH.[2]

Solutions of 2-keto-4-hydroxybutyrate were tested by the following procedures. (a) Large aliquots showed a negative reaction in the quantitative ninhydrin determination (Rosen,

[1] R. S. Lane, A. Shapley, and E. E. Dekker, to be published.

[2] We have also prepared 2-keto-4-hydroxybutyrate, in yields comparable with those obtained by chemical synthesis, by reaction of L-homoserine with snake venom L-amino acid oxidase. Finkelstein *et al.* (1966) previously reported the enzymic preparation of 2-keto-4-hydroxybutyrate; no details of their procedure, however, have appeared in the literature.

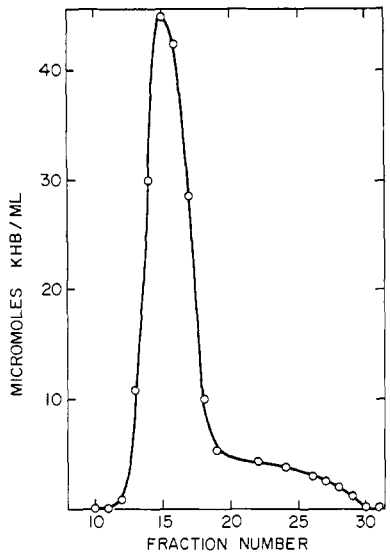

FIGURE 1: Elution pattern of 2-keto-4-hydroxybutyrate (KHB) from a column of Dowex 50 (H^+) ion-exchange resin. The components in the mixture and the conditions of the reaction are outlined in the text.

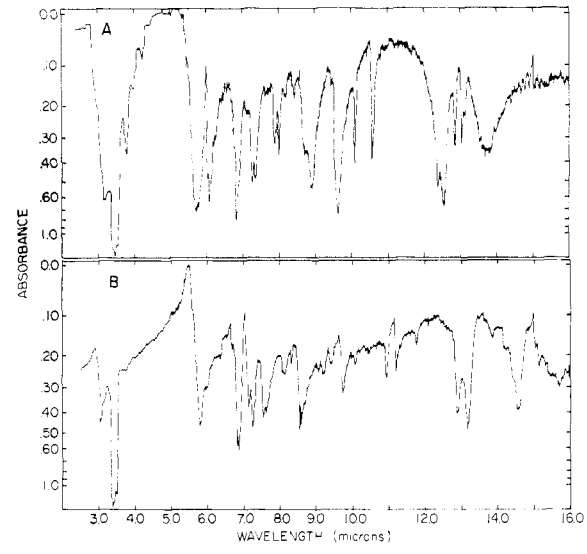

FIGURE 3: Infrared absorption spectra of (A) γ-lactone of 2-keto-4-hydroxybutyrate and (B) γ-lactone of 2-keto-3-phenyl-4-hydroxybutyrate.

1957). (b) Samples of increasing size gave a linear response in the Friedemann–Haugen (1943) test for carbonyl compounds. (c) The compound was lactonized by heating in 0.1 N HCl for 10 min at 100°, giving a quantitative lactone test as determined by the hydroxamate reaction (Cori and Lipmann, 1952). (d) One homogeneous spot, responding positively as a carbonyl compound, was detected by paper chromatographic techniques in the following solvent systems (Whatman No. 1 filter paper, ascending solvent flow): 1-butanol–acetic acid–water (12:3:5, v/v), R_F 0.36; water-saturated 1-butanol–formic acid (95:5, v/v), R_F 0.03; and 1-butanol–pyridine–water (6:4:3, v/v), R_F 0.12. The paper chromatographic properties of 2-keto-4-hydroxybutyrate were compared with and determined to be different from those of pyruvate, oxaloacetate, 2-ketobutyrate, α-ketoglutarate, and 2-keto-4-hydroxyglutarate. (e) The ultraviolet absorption spectra of 2-keto-4-hydroxybutyrate recorded at acidic and alkaline pH values were very similar to those obtained for 2-ketobutyrate under the same conditions (Figure 2). (f) L-Homoserine was formed when 2-keto-4-hydroxybutyrate and L-glutamate were incubated in the presence of either glutamate–oxaloacetate aminotransferase (EC 2.6.1.1) or glutamate–pyruvate aminotransferase (EC 2.6.1.2) (both highly purified from pig heart).[1]

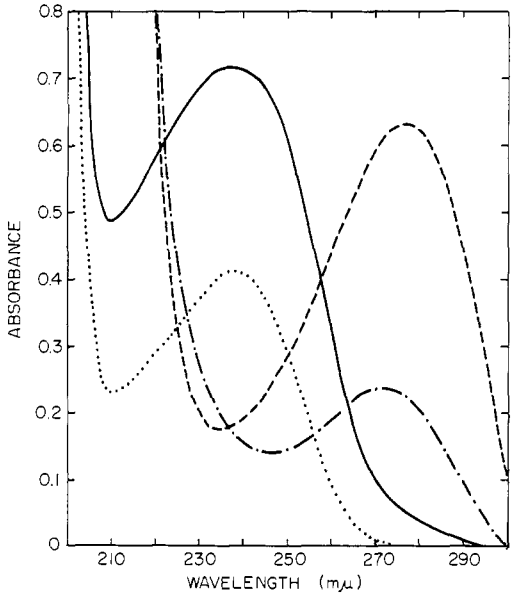

FIGURE 2: Ultraviolet absorption spectra of 2-ketobutyrate (6.0×10^{-4} M) and chemically synthesized 2-keto-4-hydroxybutyrate (9.6×10^{-4} M), (———) 2-keto-4-hydroxybutyrate, pH 1.2; (– – – – –) 2-keto-4-hydroxybutyrate, pH 12.9; (· · · · ·) 2-ketobutyrate, pH 1.1; and (–·–·–) 2-ketobutyrate, pH 12.8.

Crystallization of 2-Keto-4-hydroxybutyrate as 2-Keto-4-oxobutyrolactone. 2-Keto-4-hydroxybutyrate was crystallized from solution as its γ-lactone by the following procedure. Pooled fractions from the column of Dowex 50 (H^+) resin (1150 μmoles of 2-keto-4-hydroxybutyrate, pH 1.5) were lyophilized under acidic conditions to a small volume (about 20 ml); the yellow solution obtained was extracted four times with 100-ml portions of anhydrous ether. The combined ethereal extracts were dried with solid anhydrous Na_2SO_4, filtered, and allowed to evaporate at room temperature. White crystals began to separate from the concentrated solution; crystallization was allowed to continue at 4° an additional 4 days. The crystals were removed by filtration and recrystallized twice in the same manner from ethereal solution. The final product, obtained as white needles, was dried at 4° *in vacuo* over $CaCl_2$: yield 69 mg, mp 106–107°. *Anal.* Calcd for $C_4H_4O_3$: C, 48.01; H, 4.03. Found: C, 47.68; H, 4.06. The infrared absorption spectrum is shown in Figure 3; the spectrum of 2-keto-3-phenyl-4-oxobutyrolactone was taken for comparative purposes. The ultraviolet absorption spectrum showed λ_{max} at 277 mμ (pH 1.4) and at 317 mμ (pH 12.4). The compound reacted quantitatively in the hydroxamate test for lactones (Cori and Lipmann, 1952); 2-keto-4-hydroxybutyrate was identified by paper chromatography after

TABLE I: Paper Chromatographic Properties of the 2,4-Dinitrophenylhydrazone Derivatives of 2-Keto-4-hydroxybutyrate and Other Keto Acids.[a]

Solvent	Ratio	R_F Values: Pyruvate–DNP	2-Keto-4-hydroxybutyrate–DNP	2-Ketobutyrate–DNP
1-Butanol–acetic acid–H_2O	15:3:7	0.88	0.77	0.90
t-Amyl alcohol–absolute ethanol–H_2O	5:1:4	0.65	0.73	0.87
Ethanol–H_2O	7:3	0.65	0.72	0.77
1-Butanol–pyridine–H_2O	2:1:1	0.71	0.76	
1-Butanol–absolute ethanol–0.5 N NH_4OH	7:1:2	0.55	0.62	

[a] Aliquots of concentrated ethanolic solutions were applied to Whatman No. 1 filter paper; chromatograms were developed for 18 hr, ascending solvent flow. Compounds were detected by visible yellow color or by absorption of ultraviolet light.

brief treatment of the lactone with 3 N NaOH at 100° followed by prompt neutralization.

Preparation of the 2,4-Dinitrophenylhydrazone of 2-Keto-4-hydroxybutyrate. 2-Keto-4-hydroxybutyrate 2,4-dinitrophenylhydrazone was prepared by incubating the keto acid (350 μmoles, pH 6.8) with 100 ml of 0.1% 2,4-dinitrophenylhydrazine solution in 2 N HCl for 45 min at room temperature. The precipitate was removed by filtration and recrystallized twice from ethanol–water (1:5, v/v); the long yellow needles so obtained were dried *in vacuo* over $CaCl_2$ at 25°: yield 60 mg, mp 238–239°. *Anal.* Calcd for $C_{10}H_{10}N_4O_7$: C, 40.27; H, 3.38; N, 18.79. Found: C, 40.24; H, 3.28; N, 18.61. This derivative showed one homogeneous spot on paper chromatograms (Table I).

When the crystalline 2,4-dinitrophenylhydrazone of 2-keto-4-hydroxybutyrate (6 mg dissolved in 50 ml of ethyl acetate) was reduced at 50 psi of H_2 gas for 6 hr at 25° with PtO_2 as catalyst, DL-homoserine, the expected product, could be identified by paper chromatographic techniques in these solvent systems: (Whatman No. 1 filter paper, ascending solvent flow) ethanol–water (7:3, v/v), R_F 0.46; pyridine–acetic acid–water (10:7:3, v/v), R_F 0.39; 1-butanol–acetic acid–water (4:1:1, v/v), R_F 0.16 on Whatman No. 3 filter paper, descending solvent flow.

Results

Reaction of 2-Keto-4-hydroxybutyrate with Lactate Dehydrogenase. A significant reduction of 2-keto-4-hydroxybutyrate occurs in the presence of lactate dehydrogenase and DPNH. The rate of reduction of 2-keto-4-hydroxybutyrate, as measured by decreasing absorbancy at 340 mμ, is linear with time and protein concentration (Figure 4). No reaction occurs when either 2-keto-4-hydroxybutyrate, DPNH, or the dehydrogenase is omitted nor when heat-denatured enzyme is added. TPNH is about 1% as effective as is DPNH in this system. No increase in absorbancy at 340 mμ is observed when 2-keto-4-hydroxybutyrate is incubated with DPN^+ in the presence of lactate dehydrogenase; a lactate dehydrogenase

TABLE II: Relative Rates of Reduction, Apparent Michaelis Constants, and Equilibrium Constants for Lactate Dehydrogenase Substrates.[a]

Substrate	Apparent K_m[b] (M)	Relative V_{max}[b]	K_{equil}[c] (M)
Pyruvate	3.3×10^{-4}	1.00	4.8×10^{-12}
Glyoxylate	1.8×10^{-2}	0.61	1.0×10^{-9}
2-Ketobutyrate	4.8×10^{-3}	0.43	3.0×10^{-11}
2-Keto-4-hydroxybutyrate	4.6×10^{-3}	0.25	1.2×10^{-10}

[a] K_m and V_{max} values were calculated from Lineweaver–Burk double-reciprocal plots as shown in Figure 5. [b] Measured in 33 mM potassium phosphate buffer (pH 7.4) at 25 ± 0.2°. [c] Measured in 0.3 M Tris-Cl buffer (pH range 7.4–9.2) at 25–26°.

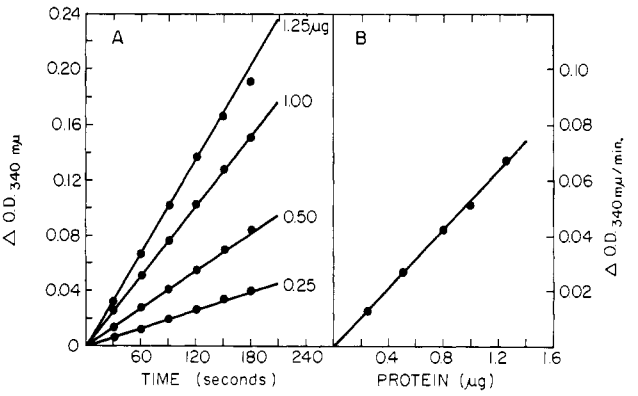

FIGURE 4: Enzymic reduction of 2-keto-4-hydroxybutyrate by DPNH in the presence of lactate dehydrogenase as a function of (A) time and (B) protein concentration. The reaction mixtures (3.0 ml) contained 3.3×10^{-2} M potassium phosphate buffer (pH 7.4), 6.7×10^{-5} M DPNH, 2.8×10^{-3} M 2-keto-4-hydroxybutyrate, and lactate dehydrogenase at the levels indicated. Addition of enzyme initiated the reaction.

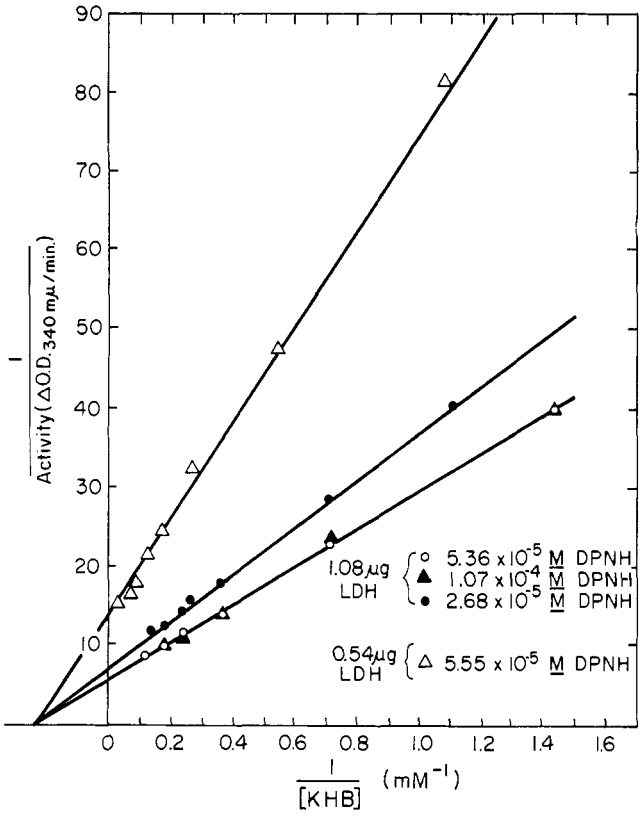

FIGURE 5: Double-reciprocal plot of lactate dehydrogenase (LDH) activity as a function of 2-keto-4-hydroxybutyrate concentration. The reaction mixtures (3.0 ml) contained 33 mM potassium phosphate buffer (pH 7.4) and the amounts of DPNH and lactate dehydrogenase listed. 2-Keto-4-hydroxybutyrate (KHB) concentration varied as shown; reaction was initiated by adding the dehydrogenase.

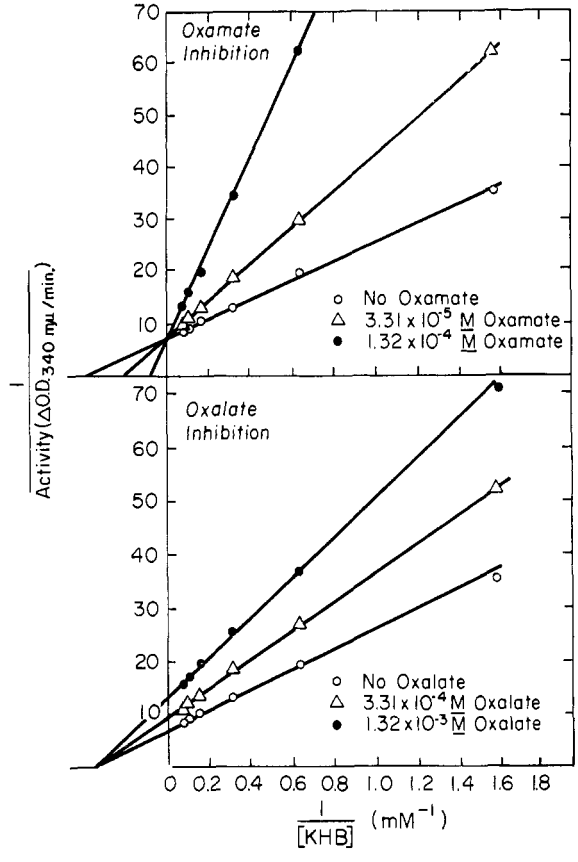

FIGURE 6: Inhibition of lactate dehydrogenase activity by oxamate and oxalate as a function of 2-keto-4-hydroxybutyrate (KHB) concentration. The reaction mixtures (3.0 ml) contained 3.3×10^{-2} M Tris-Cl buffer (pH 7.4), 5.70×10^{-5} M DPNH, 1.08 μg of lactate dehydrogenase, and 2-keto-4-hydroxybutyrate, oxamate, and oxalate concentrations as listed. Addition of enzyme, which initiated the reaction, was just preceded by addition of inhibitor.

catalyzed oxidation of the primary alcohol group of 2-keto-4-hydroxybutyrate, therefore, does not seem to occur.

Kinetic Studies. The effect of increasing concentrations of 2-keto-4-hydroxybutyrate on the rate of the dehydrogenase-catalyzed reaction is shown in Figure 5 as the conventional double-reciprocal plot (Lineweaver and Burk, 1934). The common intercept on the abscissa demonstrates that the apparent Michaelis constant for 2-keto-4-hydroxybutyrate remains unchanged when substrate saturation is determined at different concentrations of either DPNH or lactate dehydrogenase.

Table II compares the kinetic parameters of the enzymic reduction of 2-keto-4-hydroxybutyrate with those determined for other pyruvate analogs previously shown to be effective substrates for lactate dehydrogenase. K_m and V_{max} values were calculated from Lineweaver–Burk (1934) double-reciprocal plots like those shown in Figure 5. Pyruvate is by far the best substrate having the lowest K_m and the greatest V_{max}. The affinity of the dehydrogenase for 2-keto-4-hydroxybutyrate is the same as that for 2-ketobutyrate although the V_{max} for 2-keto-4-hydroxybutyrate is less. The apparent K_m value for glyoxylate is high, yet maximal glyoxylate reduction occurs more rapidly than with either 2-keto-4-hydroxybutyrate or 2-ketobutyrate. However, when initial reaction rates for these substrates are compared at a substrate concentration which is limiting, glyoxylate is utilized less effectively than either 2-keto-4-hydroxybutyrate or 2-ketobutyrate. The following rates of reduction relative to pyruvate were obtained at the substrate concentrations indicated: 1.67×10^{-3} M; 13, 7, and 5%; 3.33×10^{-3} M; 19, 11, and 9% for 2-ketobutyrate, 2-keto-4-hydroxybutyrate, and glyoxylate, respectively, in each case. These results reflect the low affinity of lactate dehydrogenase for glyoxylate. The last column of Table II lists the average values of the equilibrium constants obtained with these four compounds. In each case, several determinations were made at different pH values and substrate concentrations; pyruvate reduction was measured only at quite alkaline pH values and glyoxylate reduction at only neutral pH. The values shown were calculated as defined by Kubowitz and Ott (1943) and were measured in the direction of reduction only. The magnitude of these constants was not changed by a tenfold increase in lactate dehydrogenase concentration. DPNH oxidation is favored with all substrates; pyruvate reduction is optimal in all respects. Of the substrates listed, the extent of reduction is poorest for glyoxylate. The equilibrium of the reaction with glyoxylate as substrate, however, is more complex than with the other compounds and must be received with caution (see the section entitled Discussion).

Inhibition Studies. Oxamate and oxalate are known inhib-

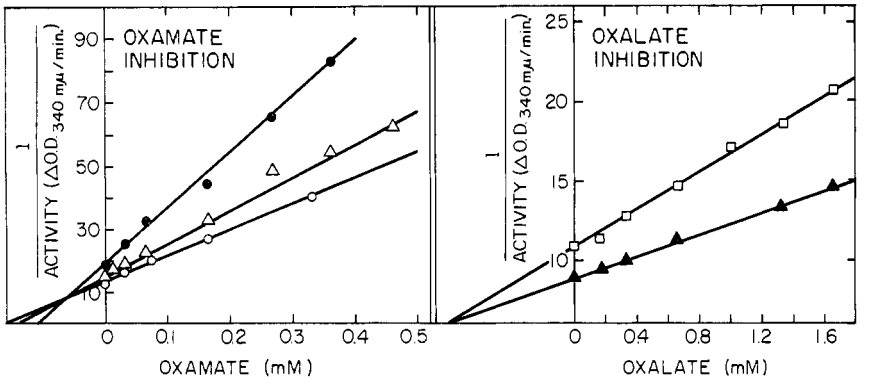

FIGURE 7: Inhibition of lactate dehydrogenase activity by oxamate and oxalate as a function of inhibitor concentration. Conditions were the same as in Figure 6, except enzyme was preincubated with inhibitor for 3 min prior to the addition of 2-keto-4-hydroxybutyrate which initiated the reaction. For a given series of points, 2-keto-4-hydroxybutyrate was constant at these concentrations: (●) 2.32×10^{-3} M, (△) 4.64×10^{-3} M, (○) 6.95×10^{-3} M, (▲) 8.94×10^{-3} M, and (□) 4.47×10^{-3} M. Inhibitor was present at the levels indicated.

itors of lactate dehydrogenase activity (Busch and Nair, 1957; Novoa *et al.*, 1959; Plummer and Wilkinson, 1963). Figure 6 shows the inhibition of the dehydrogenase-catalyzed reduction by fixed levels of oxamate or oxalate and varying concentrations of 2-keto-4-hydroxybutyrate. Inhibition by oxamate is competitive with 2-keto-4-hydroxybutyrate whereas oxalate inhibition is noncompetitive. The extent of inhibition as a function of inhibitor concentration at a constant level of 2-keto-4-hydroxybutyrate was studied according to the procedure of Dixon (1953) (Figure 7). Again, oxamate is a competitive inhibitor but oxalate exerts its inhibitory influence in a noncompetitive manner.

A summary of the dissociation constants obtained for the enzyme–inhibitor complexes is presented in Table III. The constants listed were calculated from Dixon (1953) plots, as presented in Figure 7; values determined from double-reciprocal plots (*i.e.*, Figure 6, using the appropriate equations for competitive and noncompetitive inhibition; Dixon and Webb, 1964) were essentially the same. As shown in Table III, oxamate is a more potent inhibitor of lactate dehydrogenase activity than is oxalate. As can also be seen, the K_i values for a given inhibitor are virtually identical for all substrates tested; this indicates catalytic reduction by a single enzyme. In all cases studied, oxamate was competitive and oxalate noncompetitive with the same substrate.

Identification of 2,4-Dihydroxybutyric Acid as a Product of the Reaction. In order to identify the product resulting from the reduction of 2-keto-4-hydroxybutyrate, an incubation mixture (1.0 ml) was prepared containing the following components (in micromoles): 2-keto-4-hydroxybutyrate, 9.6; DPNH, 10; and Tris-Cl buffer (pH 7.4), 200. Addition of 0.27 mg of lactate dehydrogenase initiated the reaction which was allowed to proceed for 2 hr at 37°. An additional 0.27 mg of dehydrogenase was then added and the incubation was continued for another 2 hr. The reaction, estimated to be 96% complete by the change in absorbancy at 340 mμ, was terminated by heating for 5 min at 100°. After the denatured protein was removed by centrifugation, the supernatant fluid was applied to a column (0.5 × 8 cm) of Dowex 50 (H^+) resin and the resin column was subsequently washed with water. Those fractions (0.5-ml volume) reacting positively to Congo red paper were pooled and treated with acid-washed charcoal (Darco G-60) until the absorbancy at 260 mμ was negligible. The charcoal was removed by filtration and the filtrate was reduced to a small volume *in vacuo* at 35°. The concentrated solution was examined by paper chromatographic techniques. The data in Table IV show that the product of the enzymic reaction migrates identically with authentic 2,4-dihydroxybutyrate.

Stoichiometry of 2-Keto-4-hydroxybutyrate Reduction. The stoichiometry of 2-keto-4-hydroxybutyrate reduction by DPNH in the presence of lactate dehydrogenase is shown in Table V. For expt 1, the reaction mixture (3.0 ml) contained the amounts of 2-keto-4-hydroxybutyrate and DPNH indicated plus 1.08 μg of lactate dehydrogenase in 33 mM Tris-Cl buffer (pH 7.4). The solution was incubated at 25° for 90 min, at which time the rate of reaction was minimal as judged by the decreasing absorbancy at 340 mμ. An aliquot (1 ml) was then quickly removed, diluted into 2 ml of 12% trichloroacetic acid solution, and analyzed for unreacted 2-keto-4-dihydroxybutyrate. A second aliquot (1 ml) was layered onto 6 ml of cold, concentrated H_2SO_4; the solution was then carefully mixed and the content of 2,4-dihydroxybutyrate was determined. Quantitative analyses for 2-keto-4-hydroxybutyrate and 2,4-dihydroxybutyrate were performed as described in the section entitled Methods.

Other than for the initial levels of 2-keto-4-hydroxybutyrate and DPNH used, the reaction mixture (3.0 ml) in expt 2 of

TABLE III: Dissociation Constants of the Enzyme–Inhibitor Complexes for Lactate Dehydrogenase Substrates.[a]

Substrate	K_i Value: Oxamate (M)	K_i Value: Oxalate (M)
Pyruvate	8.3×10^{-5}	8.0×10^{-4}
Glyoxylate	7.6×10^{-5}	8.4×10^{-4}
2-Ketobutyrate	7.4×10^{-5}	8.0×10^{-4}
2-Keto-4-hydroxybutyrate	8.0×10^{-5}	8.1×10^{-4}

[a] Measured in 33 mM Tris-Cl buffer (pH 7.4) at 25 ± 0.2°.

TABLE IV: Paper Chromatographic Identification of Enzymically Formed 2,4-Dihydroxybutyric Acid.[a]

Solvent	Ratio	R_F Values: Enzymic Product	R_F Values: Reference 2,4-Dihydroxybutyrate
1-Butanol–acetic acid–H_2O	12:3:5	0.55	0.53
Isoamyl alcohol saturated with 1 N formic acid		0.21	0.21
1-Butanol–propionic acid–H_2O	10:5:7	0.37	0.38
1-Butanol–pyridine–H_2O	6:4:3	0.43	0.43
1-Butanol–formic acid (95:5) saturated with H_2O		0.35	0.33

[a] Whatman No. 3MM filter paper was used with descending solvent flow. 2,4-Dihydroxybutyric acid was detected by spraying with 0.1 N $AgNO_3$–5 N NH_4OH (1:1, v/v) solution, followed by heating at 95° for 5 min.

TABLE V: Stoichiometry of the Lactate Dehydrogenase Catalyzed Reduction of 2-Keto-4-hydroxybutyrate.[a]

Compound	Initial Concn (μmoles)	Final Concn (μmoles)	Δ (μmoles)
Expt 1			
2-Keto-4-hydroxybutyrate	1.01	0.38	−0.63
DPNH	0.86	0.36	−0.50
2,4-Dihydroxybutyrate		0.54	+0.54
Expt 2			
2-Keto-4-hydroxybutyrate	0.96	0.35	−0.61
DPNH	1.00	0.37	−0.63
2,4-Dihydroxybutyrate		0.60	+0.60

[a] For experimental conditions, see Results section.

Table V differed in that it contained 1.62 μg of lactate dehydrogenase in 33 mM potassium phosphate buffer (pH 7.0) and was incubated at 25° for 53 min. Thereafter, 2-keto-4-hydroxybutyrate and 2,4-dihydroxybutyrate were determined as before. It can be seen (Table V) that in the lactate dehydrogenase mediated reaction, 1 mole of DPNH is oxidized for every mole of 2-keto-4-hydroxybutyrate reduced; concomitantly, 1 mole of 2,4-dihydroxybutyrate is formed.

Discussion

Studies regarding the substrate specificity of lactate dehydrogenase have shown that although a large number of compounds undergo a measureable degree of reaction only a few are strikingly effective as substrates. For example, the dehydrogenase from bovine heart muscle is catalytically active with many α-keto and α,γ-diketo acids (Meister, 1950, 1952); only pyruvate, hydroxypyruvate, thiopyruvate, 2-ketobutyrate, and glyoxylate, however, are utilized at a rapid and significant rate. In contrast, the rates of reaction with phenylpyruvate, *p*-hydroxyphenylpyruvate, oxaloacetate, and α-ketoglutarate are extremely slow or negligible. On the basis of the results outlined in this paper, 2-keto-4-hydroxybutyrate should also be regarded as one of those few substrates which is very efficiently reduced by DPNH in the presence of lactate dehydrogenase; the new product of this reaction has been identified as 2,4-dihydroxybutyric acid. Since crystalline lactate dehydrogenases from heart (Neilands, 1952), rat liver (Gibson *et al.*, 1953), and rat muscle (Kubowitz and Ott, 1943) are specific for L-(+)-lactate, the 2,4-dihydroxybutyrate formed by reduction of 2-keto-4-hydroxybutyrate is presumed to be the L isomer. For every μmole of 2-keto-4-hydroxybutyrate reduced, 1 μmole of DPNH is oxidized and 1 μmole of 2,4-dihydroxybutyrate is formed. Meister showed earlier that only 1 mole of DPNH reacts with 1 mole of an α,γ-diketo acid (Meister, 1950); since γ-ketovaleric acid is not a substrate for crystalline beef heart lactate dehydrogenase whereas α-ketovalerate undergoes reaction to a small but measurable extent, apparently only α-keto groups are reduced.

The equilibrium for the lactate dehydrogenase catalyzed reaction with 2-keto-4-hydroxybutyrate favors DPNH oxidation, but to a somewhat lesser extent than for 2-ketobutyrate (Table II). Carbonyl reduction is also favored with pyruvate and glyoxylate. The K_{equil} value we determined for pyruvate is in good agreement with that reported by Racker (1950). With glyoxylate as substrate, the equilibrium of the reaction is quite complex and the K_{equil} value listed in Table II should be viewed with caution. The complication is that lactate dehydrogenase also catalyzes the oxidation of glyoxylate in the presence of DPN^+ yielding oxalic acid as a terminal product (Sawaki *et al.*, 1967). Glyoxylate, therefore, may be oxidized once the level of DPN^+, formed by the initial reduction of glyoxylate by DPNH, becomes substantial; in addition, the DPNH regenerated as a result of glyoxylate oxidation may subsequently be used to further reduce unreacted glyoxylate. To calculate the K_{equil} value listed in Table II for glyoxylate, we assumed the only species in solution were reduced and oxidized pyridine nucleotides, glyoxylate, and glycolic acid.

Although the apparent K_m values for 2-keto-4-hydroxybutyrate and 2-ketobutyrate are virtually the same (Table II), 2-ketobutyrate is reduced more effectively than is 2-keto-4-hydroxybutyrate as indicated by a greater initial rate of reaction under conditions of limiting substrate concentration, a greater V_{max} and a lower K_{equil} value (Table II). These results suggest that the primary alcohol group in the γ position (C-4) of 2-keto-4-hydroxybutyrate (or 2,4-dihydroxybutyrate) may have an inhibitory effect on the dehydrogenase. Meister (1952) observed that hydroxypyruvate is also reduced to a somewhat lesser extent than is pyruvate. He further reported (Meister, 1950) that the time–activity curves for reduction of α,γ-diketo acids deviate from linearity much sooner than those for α-keto acid reduction under the same conditions. Although this observation was not investigated fully, the suggestion was made that the product of the reaction, a γ-keto-α-hydroxy

acid in this instance, may inhibit lactate dehydrogenase activity.

The relative rate of reduction of 2-ketobutyrate, when compared with pyruvate as substrate, is more rapid with crystalline lactate dehydrogenase from pig heart (isozyme 1) than with the enzyme from rabbit muscle (isozyme 5) (Rosalki and Wilkinson, 1960; Plummer *et al.*, 1963). This observation has been used as a powerful diagnostic tool in cardiac disorders (Rosalki, 1963; Elliott and Wilkinson, 1961). Although Banner and Rosalki (1967) could also demonstrate a somewhat better level of reactivity of glyoxylate with the LD-1 isozyme than with LD-5, the difference they observed was minor when compared with that obtained using 2-ketobutyrate. Differential utilization of glyoxylate as a substrate for lactate dehydrogenase isozymes would appear, therefore, to have limited diagnostic applications. However, since 2-keto-4-hydroxybutyrate and 2-ketobutyrate are structurally very similar, a preferred utilization of 2-keto-4-hydroxybutyrate by one of the two isozymes of lactate dehydrogenase may be sufficiently great to be of clinical value. Such tests remain to be done.

Oxamate, as an inhibitor, has been studied extensively with lactate dehydrogenase from bovine heart (Hakala *et al.*, 1953; Novoa *et al.*, 1959), rabbit erythrocytes (Ottolenghi and Denstedt, 1958), ox heart (Plummer and Wilkinson, 1963), and rabbit muscle (Papaconstantinou and Colowick, 1961). In every case, oxamate inhibited pyruvate reduction competitively and lactate oxidation noncompetitively. Quastel and Wooldridge (1928), using crude extracts of *E. coli*, first reported the inhibition of lactate dehydrogenase by oxalic acid. Subsequent studies with lactate dehydrogenase from ox heart (Neilands, 1954), beef heart (Novoa *et al.*, 1959), rat liver (Baptist and Vestling, 1957), rabbit erythrocytes (Ottolenghi and Denstedt, 1958), and rabbit muscle (Busch and Nair, 1957; Plummer and Wilkinson, 1963) showed that oxalate functions competitively with lactate and noncompetitively with pyruvate as substrate. With 2-keto-4-hydroxybutyrate as substrate for rabbit muscle lactate dehydrogenase, we also observe inhibition of a competitive type with oxamate whereas inhibition by oxalate is noncompetitive. Oxamate and oxalate inhibition of lactate dehydrogenase activity with 2-ketobutyrate and glyoxylate as substrates confirm the findings of Plummer and Wilkinson (1963) and Banner and Rosalki (1967), respectively. Although oxamate is uniformly a more potent inhibitor of lactate dehydrogenase activity than is oxalate, the degree of inhibition caused by either compound is the same with all four substrates tested (Table III).

The reduction of 2-keto-4-hydroxybutyrate by DPNH in the presence of lactate dehydrogenase, yielding 2,4-dihydroxybutyrate, raises the question as to what the metabolic role of this reaction may be. As indicated earlier in this paper, we have found that highly purified glutamate–oxaloacetate and glutamate–pyruvate aminotransferases from pig heart readily catalyze the reversible formation of 2-keto-4-hydroxybutyrate from L-homoserine. Either α-ketoglutarate, oxaloacetate, or pyruvate function as amino group acceptors in this process; a detailed report of these findings is being prepared. Furthermore, Nagai and Flavin (1967) recently reported that when grown in the presence of [^{14}C]homoserine, *Neurospora* accumulates labeled *O*-acetylhomoserine and 4-*O*-acetyl-2,4-dihydroxybutyrate. These two compounds are believed to be formed by O acetylation of homoserine, followed by a deamination of *O*-acetylhomoserine to the α-keto analog and a reduction of the latter compound by lactate dehydrogenase. Similarly, transamination of free homoserine liberates 2-keto-4-hydroxybutyrate which can then be reduced by lactate dehydrogenase to yield 2,4-dihydroxybutyrate as a product of homoserine metabolism in mammals.

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Utilization of Volatile Fatty Acids in Ruminants. III. Comparison of Mitochondrial Acyl Coenzyme A Synthetase Activity and Substrate Specificity in Different Tissues*

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ABSTRACT: Studies were undertaken to establish the important rate-limiting steps controlling acetate, propionate, and butyrate metabolism in ruminants. As an initial approach to this problem, the activity of acetyl, of propionyl, and of butyryl coenzyme A synthetase in mitochondria from various ruminant tissues was studied. Mitochondria were frozen and thawed three times to solubilize the enzymes. The acyl coenzyme A synthetase preparation was partially purified and its substrate specificity was studied. The results show that the activity of the enzyme active on acetate is high in highly aerobic tissues such as heart and mammary gland and low in liver, in brain, in aorta, in skeletal muscle, and in rumen muscle. In heart, in mammary gland, in kidney, and in testes, the enzyme preparations were all about equally active on acetate and on propionate. In liver the enzyme preparation was equally active on propionate and on butyrate and least active on acetate. The enzyme from rumen epithelium was most active on butyrate, less active on propionate, and least active on acetate. Lung differs from all the other tissues in that the enzyme is very active on propionate and shows equal but lower activity for acetate and for butyrate. Marginal enzyme activity was found in skeletal muscle, in rumen muscle, in brain, and in aorta. The results offer an explanation for the preferential utilization of acetate by extrahepatic tissues, propionate by the liver, and butyrate by rumen epithelium. Butyrate, if present in peripheral blood, has a pharmacological effect on the pancreas. The ruminant has a unique mechanism for preventing butyrate appearance in peripheral blood, *i.e.*, a high butyryl coenzyme synthetase activity in rumen epithelium ensures rapid activation of butyrate for subsequent oxidation to β-hydroxybutyrate by the epithelium. If some butyrate escapes metabolism by the rumen epithelium, it is readily removed from portal blood by the liver where a high butyryl coenzyme A synthetase activity ensures rapid activation and subsequent metabolism to ketone bodies. Substrate specificity studies show that in addition to the three well-known acyl coenzyme A synthetases active on straight-chain fatty acids (acid:ligase (adenosine monophosphate), EC 6.2.1.1, 6.2.1.2, and 6.2.1.3) there are at least three other acyl coenzyme A synthetases with distinct substrate specificities. The acyl coenzyme A synthetase from sheep liver is specific for C_3 to C_7 straight-chain fatty acids. The acyl coenzyme A synthetase from sheep kidney is specific for C_2 to C_7 straight-chain fatty acids. Sheep lung has an acyl coenzyme A synthetase specific for propionate. This work along with earlier studies by others would suggest that there are at least six acyl coenzyme A synthetases in animal tissues with varying substrate specificities.

This laboratory is engaged in a study of control of acetate, of propionate, and of butyrate utilization in ruminants. These short-chain acids are produced in the rumen by microbial fermentation of carbohydrates and serve as primary metabolic substrates in ruminants. It has been shown that under normal conditions the ruminant liver is the major body organ metabolizing propionate. Most of the acetate present in portal blood is not removed by the liver and is available to extrahepatic tissues (Cook and Miller, 1965; Black *et al.*, 1961). Butyrate is not present in significant amounts in rumen vein or in portal blood (Cook and Miller, 1965; Annison *et al.*, 1957) and is believed to be oxidized to β-hydroxybutyrate on absorption from the rumen (Ramsey and Davis, 1965).

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