Cellular Localization and Characterization of Bovine Liver Branched-Chain α-Keto Acid Dehydrogenases†

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ABSTRACT: Subcellular distribution studies established that the oxidative decarboxylation activities of bovine liver with the branched-chain α-keto acids α-keto-β-methylvalerate, α-ketoisocaproate, and α-ketoisovalerate are located principally in the mitochondria. Techniques which distinguish between metabolic reductants within and outside the mitochondrial inner membrane further established that the branched-chain keto acid dehydrogenase activities are localized on the outer face of the inner membrane. Broad pH optima of these activities range from pH 6.8 to pH 7.4. The branched-chain α-keto acid dehydrogenase activities of the mitochondrial fraction were shown to depend upon added nicotinamide–adenine dinucleotide (NAD^+) and coenzyme A for maximal catalytic function. $NADP^+$ is inactive in the system. Calcium, magnesium, and inorganic phosphate appear to be required for optimal dehydrogenase activity; ATP is inhibitory. Apparent Michaelis constants for α-ketoisocaproate dehydrogenase are 3×10^{-5} M for NAD^+ and 6×10^{-5} M for coenzyme A. Some evidence is presented for the existence of a soluble branched-chain α-keto acid dehydrogenase different from the particulate activity.

The branched-chain amino acids leucine, isoleucine, and valine, which are synthesized in bacteria and plants, are essential nutrients for animals. Coon *et al.* (1955) studied the general pathways for the degradation of these three amino acids in mammalian tissues, and found that the initial steps follow a similar pattern of transamination to the respective α-keto acids, α-ketoisocaproic acid (KIC),[1] α-keto-β-methylvaleric acid (KMV), and α-ketoisovaleric acid (KIV), followed by irreversible oxidative decarboxylation of these branched-chain α-keto acids to the corresponding acyl-coenzyme A derivatives. It has been postulated that the overall oxidative decarboxylation of KIC, KMV, and KIV proceeds by a metabolic conversion analogous to that proposed by Gunsalus (1954) and Reed (1960) for pyruvate and α-ketoglutarate.

The obligatory participation of coenzyme A and NAD^+ has been observed in the enzymic oxidative decarboxylation of KMV, KIC, and KIV by extracts of *Bacillus subtilus* (Namba *et al.*, 1969) and rat liver (Wolhueter and Harper, 1970). Direct evidence for the function of lipoic acid, FAD, and thiamine pyrophosphate as prosthetic groups of the branched-chain α-keto acid dehydrogenase systems of mammalian tissues has not been available.

Liver and kidney possess the highest branched-chain α-keto acid dehydrogenase activity in the rat (Wolhueter and Harper, 1970) and in bovine tissue (Bowden, 1965); low levels were observed in heart and brain tissue, while no oxidative decarboxylation of these compounds was obtained by preparations of skeletal muscle. A knowledge of the intramitochondrial location of the enzymes responsible for the catalysis of oxidative decarboxylation of KIC, KMV, KIV, pyruvate, and α-ketoglutarate in bovine liver cells is of considerable general interest, and would contribute to an understanding of the relationships among these metabolites. Information about the spatial arrangement of these enzymes with respect to the inner membrane would provide some clarification of the question of whether the α-keto acid substrates must penetrate to the matrix prior to oxidation.

Recently, interest in the catabolism of the branched-chain amino acids and their corresponding α-keto acids has been evidenced both with respect to the role of these compounds in the metabolic anomaly branched-chain ketoaciduria and from the viewpoint of overall regulatory aspects of amino acid catabolism.

Thus, the aim of the present communication is to further characterize the nature of the oxidation of the α-keto acids KIC, KMV, and KIV in preparations of bovine liver tissue. This study attempts to elucidate the subcellular location of the branched-chain α-keto acid oxidative activities in the bovine liver cell and to identify the apparent requirements for free coenzymes and activators.

Experimental Section

Materials. The chemicals used in these studies were obtained from the following sources: β-nicotinamide–adenine dinucleotide; trilithium coenzyme A; sodium salts of α-ketoisocaproate, α-ketoisovalerate, α-keto-β-methylvalerate, α-ketoglutarate, succinate, pyruvate, and DL-α-glycerophos-

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[1] Abbreviations used are: KIC, α-ketoisocaproate; KIV, α-ketoisovalerate; KMV, α-keto-β-methylvalerate; NAD^+, nicotinamide–adenine dinucleotide (oxidized form); NADH, nicotinamide–adenine dinucleotide (reduced form); CoA, reduced coenzyme A; $NADP^+$, nicotinamide–adenine dinucleotide phosphate; α-GP, DL-α-glycerophosphate; TPP, thiamine pyrophosphate; FAD, flavin–adenine dinucleotide; FMN, flavin mononucleotide; P_i, orthophosphate.

phate; L-amino acid oxidase; D-amino acid oxidase; antimycin A; nicotinamide–adenine dinucleotide phosphate; DL-α-lipoic acid; thiamine pyrophosphate; flavin adenine dinucleotide; flavin mononucleotide (Sigma Chemical Co., St. Louis, Mo.); L-leucine-*1*-^{14}C, DL-valine-*1*-^{14}C, L-isoleucine-*U*-^{14}C (Cal-Atomic Division, Calbiochem, Los Angeles, Calif.). All other chemicals were of analytical reagent grade.

Preparation of ^{14}C-Labeled α-Keto Acids. [^{14}C]Carboxyl-labeled α-keto acids α-ketoisocaproate-*1*-^{14}C (sodium salt), α-ketoisovalerate-*1*-^{14}C (sodium salt) and α-keto-β-methylvalerate-*U*-^{14}C (sodium salt) were prepared with the following modifications of the method of Meister (1951).

Two grams of the L-amino acid with 50–100 μCi of ^{14}C-labeled amino acid added were incubated at 37° with L-amino acid oxidase for 12–24 hr. The α-keto acid separated from protein by gel filtration of the reaction mixture on a Sephadex G-25 column (3 cm × 45 cm) at a flow rate of 1.0 ml/min. Analysis of the collected fractions indicated complete separation of protein from radioactivity. Highest activity fractions (90–95% of total radioactivity) were combined and evaporated at 26-mm pressure and 35° and passed through a Dowex 50-W cation-exchange resin (H^+ form), and the α-keto acid was eluted with water at a flow rate of 0.5 ml/min. Fractions containing 90–100% of remaining radioactivity and no amino acid contaminant were combined, adjusted to pH 4.5 with 5 N NaOH, and evaporated to a volume of less than 10 ml under reduced pressure.

Radiochemical purity of the compounds was ascertained by chromatographic and spectrophotometric determination, using the 2,4-dinitrophenylhydrazone derivatives (Dancis *et al.*, 1963).

Enzyme Assay Methods. A. α-Keto acid dehydrogenase activities were estimated using modifications of the methods utilized by Wohlhueter and Harper (1970) and Snyder and Godfrey (1961), in which the amount of labeled carbon dioxide evolved from carboxyl-labeled α-keto acids is measured. The final reaction mixture contained about 2–3 mg of enzyme protein in a final volume of 1.0 ml (see Table III).

Warburg flasks, or small erlenmeyer flasks with a center well, served as reaction vessels. A small test tube (10 mm × 20 mm) containing 0.5 ml of hydroxide of Hyamine 10X was placed on the center well, enzyme solution was added by pipet to initiate the reaction, and the flask was tightly capped with a rubber serum cap. The reactions were allowed to proceed for 20 min, or other specified time interval, at 30° in a shaking water bath, after which time 0.2 ml of 2.0 N sulfuric acid was added to the reaction mixture by syringe through the rubber cap.

Distillation of CO_2-^{14}C into the Hyamine was allowed to continue, with shaking, for 1 additional hr, at which time the tube and its contents were transferred to a scintillation vial containing 10 ml of a scintillation fluid [0.4% 2,5-diphenyloxazole, 0.01% 1,4-bis[2-(5-phenyloxazolyl)]benzene, in toluene, w/v], and counted three times for 10 min in a Packard Tri-Carb liquid scintillation spectrometer (Automatic Model 500-E). The average background was 30 counts per minute (cpm). Samples were run in duplicate or in triplicate. Blank values of 10–30 cpm above background determined by carrying boiled enzyme preparations through an identical procedure were consistently observed for any one α-keto acid preparation, possibly resulting from traces of CO_2-^{14}C in the α-keto acid solutions. Typical assays measured in the range of 500–1500 cpm. The amount of CO_2 produced was calculated from the specific activity of the carboxyl-labeled substrate-*1*-^{14}C. Results are expressed in units, defined as nanomoles of CO_2 per minute; specific enzyme activity is expressed as units per mg of protein. Production of CO_2-^{14}C was linear with respect to incubation time and protein concentrations for all reported experimental conditions.

B. Succinate dehydrogenase (EC 1.3.99.1) was measured according to the method of Bonner (1955) by following ferricyanide reduction at 412 nm.

C. Glycerol-1-phosphate dehydrogenase (EC 1.1.99.5) activity of washed preparations of liver mitochondria was determined by the method of Klingenberg (1970a).

D. Glucose-6-phosphate phosphohydrolase (EC 3.1.3.9) activities were determined by the revised method of Nordlie and Arion (1966).

"Ferricyanide Localization" of Enzyme Activity. Klingenberg (1970a) has shown that some mitochondrial enzymes (*e.g.*, α-glycerophosphate dehydrogenase) are directly accessible to ferricyanide as an electron acceptor, while others (*e.g.*, succinate dehydrogenase) are not. It was also shown that ferricyanide does not penetrate the intact inner membrane. Techniques used to establish the intraorganelle location of the branched-chain α-keto acid dehydrogenase activities of bovine liver mitochondria are essentially those employed previously by Klingenberg (1970a) for other dehydrogenases. Spectrophotometric recordings of optical density (420 nm) changes due to ferricyanide reduction were carried out in a Cary Model 15 dual beam recording spectrophotometer.

A typical reaction mixture contained the following components, in micromoles, in a calibrated mixing tube: triethanolamine hydrochloride buffer, pH 7.2, 75; ATP, 6.0; EDTA, 7.0; $MgSO_4$, 20; TPP, 0.5; $K_3Fe(CN)_6$, 3.34; a suitable amount of enzyme preparation; and 0.3 M mannitol to a final volume of 6.8 ml. These constituents were mixed by inversion and divided equally between two cuvets. The spectrophotometer was balanced to zero with the cuvets in position, at which time the substrate in a volume of 0.1 ml was added to the reference cell, mixed rapidly by inversion, and recording begun immediately. For this purpose mitochondria were suspended in a 0.3 M mannitol medium at a relatively low concentration (0.5 to 1.0 mg of protein per ml) with additions, substrates, and antimycin A as indicated in the appropriate legends. Activity is expressed as micromoles of substrate oxidized per minute per mg of protein.

Enzyme Preparations. Bovine liver mitochondria were prepared by a modification of the procedures utilized by Lusty and Singer (1964).

Mannitol (0.3 M), containing 10^{-4} M EDTA, was substituted for 0.25 M sucrose as the suspending medium for all cell homogenates and mitochondrial preparations. The protein concentration of the washed preparations of mitochondria varied from 23 to 28 mg per ml. The supernatant from the mitochondrial fraction was centrifuged at 80,000*g* for 1 hr. The precipitate of this spin was termed the microsome fraction, and the supernatant was denoted as the soluble or "supernatant" fraction.

α-Ketoglutarate Dehydrogenase. Purified α-ketoglutarate dehydrogenase (EC 1.2.4.2) was prepared from pig heart muscle by the methods of Sanadi and Littlefield (1952).

Protein Determinations. The standard protein assay method used for these studies was the Biuret method (Layne, 1957) with bovine serum albumin (Armour Standard) as the protein standard.

TABLE I: Subcellular Distribution of Bovine Liver Branched-Chain α-Keto Acid Dehydrogenase.

Fraction[a]	α-Ketoisovalerate Dehydrogenase		α-Ketoisocaproate Dehydrogenase		α-Keto-β-methylvalerate Dehydrogenase		Succinate Dehydrogenase		Glucose-6-phosphatase	
	% Homogenate[b]	Sp Act.[d]	% Homogenate	Sp Act.[d]	% Homogenate	Sp Act.[d]	% Homogenate	Sp Act.[d]	% Homogenate	Sp Act.[d]
Nuclear	8.0	0.24	6.1	0.16	9.3	0.21	8.7	0.56	ND[c]	ND
Mitochondria	88.2	5.54	86.8	2.98	88.7	3.51	84.6	3.60	0.72	0.002
Microsomes	2.1	0.05	0.8	0.02	0.9	0.02	5.3	0.18	83.2	0.212
Soluble	1.7	0.06	2.4	0.17	3.2	0.26	1.8	0.06	ND	ND
% Recovery	95.3		98.9		99.3		100.4		ND	

[a] Subcellular fractions were prepared as described in the methods section. [b] Per cent homogenate refers to the amount of activity present in a fraction compared to the total activity for that substrate in the whole homogenate. [c] Not determined. [d] Units per mg of protein (see methods section).

Results and Discussion

Subcellular Distribution of Bovine Liver Branched-Chain α-Keto Acid Dehydrogenase Activity. Among the principal objectives of this study was the elucidation of the cellular localization of the branched-chain keto acid dehydrogenase activities. Table I shows the distribution of each branched-chain α-keto acid dehydrogenase activity in the subcellular fractions. Succinate dehydrogenase (EC 1.3.99.1) was employed as a mitochondrial marker enzyme (Bonner, 1955), and glucose-6-phosphatase (EC 3.1.3.9) served to identify the microsome fraction (Nordlie and Arion, 1966). The major portion of the α-keto acid dehydrogenase was found in the fraction which contained the mitochondria. Minor amounts of activity observed in the nuclear and microsome fractions likely result from some slight contamination with mitochondria.

The origin of the smaller amount of activity located in the supernatant (soluble) fraction is in doubt, since some of the characteristics of this activity appear to differ from those of the mitochondrial branched-chain α-keto acid dehydrogenase. The ratios of the activities with KIV, KMV, and KIC are different, with KIV showing greatest activity in the particulate but least in the soluble fraction. (See also Table IV.) Furthermore, as will be seen, there appears to be dissimilar coenzyme requirements for soluble and particulate activities. These variances, which suggest the possibility of separate activities, will receive further consideration below.

In order to localize the particulate activities within the organelle, mitochondria were treated with a 1% digitonin solution to remove the outer membrane and intramembranal proteins (Morton, 1955). Subsequent to this procedure, activity with all three branched-chain α-keto acids as well as with pyruvate and α-ketoglutarate remained with the inner membrane–matrix complex.

Table II shows the results of studies employed to determine the position of the dehydrogenases relative to the inner membrane. Observations with α-glycerol phosphate agree with those obtained by Klingenberg (1970a), who postulated that the mitochondrial α-glycerophosphate dehydrogenase faces the intermembranal space. With succinate or α-ketoglutarate as substrate, antimycin A considerably decreases the transfer of electrons to ferricyanide from cytochrome *c* (see also von Jagow and Klingenberg, 1970). Antimycin A also has a significant inhibitory effect upon reduction by pyruvate. However, the antimycin A block causes insignificant diminution of the rate of ferricyanide reduction with α-glycerophosphate, KIC, KMV, or KIV as the oxidizable substrate. These data indicate that ferricyanide can apparently interact directly with the enzymes catalyzing oxidation of the branched-chain α-keto acids, an observation which locates the sites of these dehydrogenase activities on the outside of the inner membrane. Such a situation obviates the need for these compounds to cross the inner membrane for oxidation, thus likely excluding membrane permeability to KIC, KIV, and KMV as a limiting factor in the control of branched-chain α-keto acid metabolism. In contrast, the blocking of electron transport from succinate or α-keto-

TABLE II: Sensitivity of Ferricyanide Reduction to Antimycin A.

Substrate	[S], mM	Enzyme Activity[a] (μmole of Substrate/min per mg of Protein) w/o Anti A	+ Anti A	% Inhibition
α-Glycerol-P	5.7	0.20 ± 0.02	0.22 ± 0.04	0
Succinate	5.7	0.02 ± 0.003	0.006 ± 0.003	70
Pyruvate	5.7	0.100 ± 0.01	0.04 ± 0.01	60
α-Ketoglutarate	2.85	0.065 ± 0.01	0.01 ± 0.005	86
α-Ketoisocaproate	7.1	0.03 ± 0.01	0.03 ± 0.01	0
α-Keto-β-methylvalerate	5.7	0.05 ± 0.01	0.05 ± 0.01	0
α-Ketoisovalerate	7.1	0.05 ± 0.01	0.048 ± 0.005	4

[a] Enzyme activities were estimated by spectrophotometric measurement as described in the methods section. Antimycin A (2 μg/ml) was added to assay mixtures where indicated.

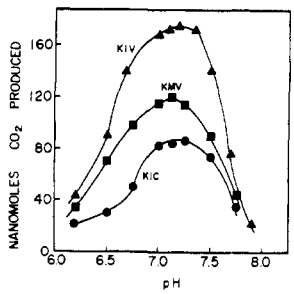

FIGURE 1: pH curves for CO_2-^{14}C assay of branched-chain α-keto acid dehydrogenase activities of bovine liver mitochondria. Assays were conducted at the pH values indicated in Figure 1. α-Keto acid dehydrogenase activities were measured with the CO_2-^{14}C assay. Other reaction components were as stated in the legend of Table III with the exception of α-keto acid substrate. Initial α-keto acid concentration in each assay was 2.0 mM.

glutarate to ferricyanide by antimycin A is in agreement with earlier evidence supporting the supposition that the dehydrogenase enzymes for these compounds are located on the inside face of the inner membrane or in the matrix (Chappell, 1968). In this regard the "mixed" type inhibition of α-ketoglutarate dehydrogenase activity by α-ketoisocaproic acid (Johnson and Connelly, 1971) could reflect dual inhibitory effects of the branched-chain α-keto acids at an α-ketoglutarate translocase (Chappel, 1968) site, and at a catalytic site on the enzyme within the mitochondrial inner membrane.

The case for pyruvate appears to be slightly different. Although the localization studies predict that pyruvate dehydrogenase faces the matrix side of the inner membrane, earlier studies have demonstrated that pyruvate can transverse the inner membrane with little difficulty (Klingenberg, 1970b).

pH Optima. Rates of oxidative decarboxylation of KIV, KIC, KMV, ketoglutarate, and pyruvate were measured at various pH values to determine the value for optimal assay conditions. The results of the effect of pH of the reaction medium upon the rate of CO_2-^{14}C production with carboxyl-labeled KIC, KMV, and KIC as substrates are shown in Figure 1. The optimum pH for enzyme reaction with these substrates is in a range of 6.8–7.4 for bovine liver mitochondria preparations. Maximal reaction rates with α-ketoglutarate and pyruvate were obtained at slightly lower pH values (6.7–7.2). Kanzaki *et al.* (1969) reported maxima of 8.2 and 7.0 for purified pig heart pyruvate and α-ketoglutarate dehydrogenase, respectively. A pH value of 7.2 was chosen for all subsequent assays, since at this hydrogen ion concentration the rate of nonenzymic decarboxylation was minimal and the enzymic activity was satisfactory with each of the α-keto acid substrates.

Cofactor Requirements. The coenzymes NAD^+, CoA, TPP, FAD, and lipoic acid have been shown to be essential in the oxidation of pyruvate and α-ketoglutarate by their respective enzyme complexes (Sanadi and Littlefield, 1952; Hayakawa *et al.*, 1964; Koike *et al.*, 1963). Table III shows the relative effect of these and several other cofactors on the decarboxylation of α-ketoisocaproate by washed preparations of bovine liver mitochondria. The activity of the mitochondrial enzyme which catalyzes this reaction is largely dependent upon the presence of added NAD^+ and CoA. Other components of the standard assay medium which appear to promote optimal reaction rates are Mg^{2+}, Ca^{2+}, and inorganic phosphate (see Figure 5). Attempts to demonstrate a direct

TABLE III: Effects of Several Cofactors on α-Ketoisocaproate Dehydrogenase Reaction.[a]

Reaction Medium	Relative Activity (%)	Specific Activity (nmoles of CO_2/min per mg of Protein)
Complete[b]	100.0	3.72
Minus NAD^+	11.7	0.43
Minus CoA	8.6	0.32
Minus $MgCl_2$	69.0	2.56
Minus $CaCl_2$	12.0	0.44
Minus P_i	2.9	0.11
Minus NAD^+, plus 0.5 μmole of $NADP^+$	8.3	0.31
Plus 0.05 μmole of lipoate	103.0	3.83
Plus 0.5 μmole of TPP	92.6	3.46
Plus 2.0 μmoles of ATP	29.0	1.08
Plus 0.005 μmole of FAD	95.0	3.53
Plus 0.005 μmole of FMN	89.0	3.31
Plus 1.0 μmole of $CdCl_2$	2.3	0.07

[a] KIC dehydrogenase activity was measured as described in the methods. [b] The *complete* reaction mixture contained, in a final volume of 1.0 ml, the following, in micromoles: mannitol, 150; potassium phosphate buffer, pH 7.2, 33; NAD, 1.0; Li_3CoA, 0.6; $MgCl_2$, 1.0; $CaCl_2$, 1.0; Na_2CO_3, 1.0; KIC-*1*-^{14}C, 2.0; and 0.1 ml of a washed preparation of bovine liver mitochondria. Deletions or additions to the complete media were as indicated in the table.

stoichiometric relationship between reduction of NAD^+ and α-keto acid decarboxylation have been thwarted by the generally high degree of opacity (at 340 nm) of reaction mixtures containing the particulate enzyme preparations, and by the variance of reoxidation of NADH by the crude preparations. It is apparent that $NADP^+$ can not replace NAD^+ as an electron acceptor in this reaction system. Addition of other presumptive cofactors such as TPP, lipoate, and flavin derivatives produced no significant effect upon KIC oxidation. However, stimulation of the enzyme activity by these coenzymes would not be expected in consideration of the generally tightly bound nature of these prosthetic groups with their protein apoenzyme moieties. Addition of 1.0 mM $CdCl_2$ resulted in nearly a complete loss of KIC oxidative capacity.

An observation which was not entirely expected was that of the marked reduction of catalytic activity in the presence of ATP (2 mM). This apparent inhibitory effect and the stimulatory nature of inorganic phosphate in this system represents a potential control process, particularly in relation to the report by Linn *et al.* (1969), who have observed that mammalian pyruvate dehydrogenase is inactivated by ATP in the presence of a specific kinase enzyme, and is reactivated by a phosphatase, in the presence of Mg^{2+}.

The results summarized in Table IV verify that both NAD^+ and CoA are essential to maintain maximal branched-chain α-keto acid oxidative capacity with washed preparations of mitochondria. On the other hand, the soluble de-

TABLE IV: Effects of Added NAD^+ and Coenzyme A on Branched-Chain α-Keto Acid Dehydrogenase Activities of Mitochondria and Supernatant Fractions of Bovine Liver.[a]

Enzyme Preparation	Reaction Mixture (nmoles of CO_2/min per mg of protein)			
	Complete[b]	− CoA	− NAD^+	− NAD^+, − CoA
α-Ketoisocaproate dehydrogenase				
Mitochondria	1.74	0.16	0.26	0.06
Supernatant	0.11	0.12	0.05	0.05
α-Keto-β-methylvalerate dehydrogenase				
Mitochondria	3.20	0.29	0.39	0.14
Supernatant	0.29	0.23	0.17	0.13
α-Ketoisovalerate dehydrogenase				
Mitochondria	5.63	0.47	0.66	0.39
Supernatant	0.03	0.03	0.01	0.01

[a] Branched-chain α-keto acid dehydrogenase activity was measured as described in the methods. [b] The complete reaction mixture is as noted for Table III, with 2 μmoles of the appropriate carboxyl-labeled α-keto acid substrate.

carboxylase activity showed no apparent dependence on added CoA in the reaction medium. The existence of an acyl-CoA hydrolase in the soluble fraction, similar to that demonstrated by Namba *et al.* (1969) in *B. subtilus* preparations, conceivably could lessen the apparent need for exogenous CoA by allowing for the continued regeneration of catalytic amounts of the free coenzyme from acyl-CoA compounds present as contaminants in the enzyme preparation. A second possibility is that the supernatant may contain a decarboxylase, specific for the branched-chain α-keto acids, which does not require coenzyme A.

The data of Table IV also disclose that the supernatant enzyme activity is significantly higher with KMV and KIC as substrates, than for KIV. This represents a decided variance from the activity ratios observed for the mitochondrial enzyme activities, where the rate of decarboxylation was greatest with KIV as the α-keto acid substrate. This latter finding parallels the observed properties of the soluble KIC–KMV dehydrogenase of bovine liver (Connelly *et al.*, 1968).

These findings deserve comment relative to the observed absence of branched-chain keto acid dehydrogenase activity in branched-chain α-ketoaciduria. If two types of branched-chain keto acid dehydrogenase exist, one localized in the mitochondria and another in the soluble phase of the cell, they are evidently closely related at the genetic level. Loss of these enzymic capabilities, through an inherited genetic lesion, would then result in total inability (in the classic branched-chain ketoaciduria) to catabolize these keto acids.

Detailed studies were carried out in an attempt to elucidate the extent to which the rate of KIC decarboxylation by mitochondria was affected by varied concentrations of NAD^+ and CoA. An apparent Michaelis constant for NAD^+ of 3.2×10^{-5} M was calculated from the reciprocal plots shown

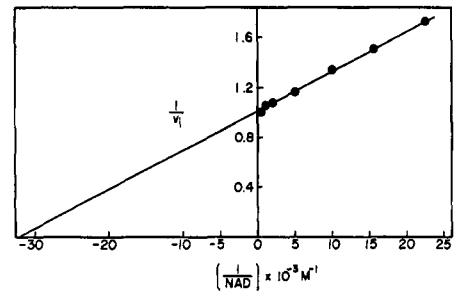

FIGURE 2: Effect of varied NAD concentration on KIC dehydrogenase activity. KIC dehydrogenase activity was measured by the CO_2-^{14}C assay as described in the methods section. Reaction components and experimental conditions were as stated in Table III, except that NAD concentrations were varied as indicated (v_i, nanomoles of CO_2 produced per minute per mg of protein).

in Figure 2. The Lineweaver–Burk plots in Figure 3 illustrate the effect of varied CoA concentrations on KIC oxidation. Apparent inhibition by CoA was observed with concentrations above 10^{-3} M, with a calculated K_m value for coenzyme A of 6.4×10^{-5} M.

Preliminary observations by these authors (Johnson and Connelly, 1971) and prior investigation by Hayakawa *et al.* (1966), Lusty and Singer (1964), and Wohlhueter and Harper (1970) had indicated that the divalent cations Ca^{2+} or Mg^{2+}, and at times both, appeared to modify significantly the α-keto acid dehydrogenase activities. As is shown in Table V, the dehydrogenase activities with each of the three branched-chain α-keto acids were strongly stimulated by added Ca^{2+}, and to a lesser extent by exogenous Mg^{2+}. The effects of Mg^{2+} and Ca^{2+} appear to be additive, suggesting that the activation by these two cations occurs by separate mechanisms. In this regard it should be noted that Mn^{2+} could apparently replace the Mg^{2+} requirement to a limited extent, but could not substitute for Ca^{2+}. The relative rates of oxidation of KIC, KMV, and KIV were decreased by nearly identical amounts when exogenous Mg^{2+}, Ca^{2+}, or both cations were omitted from the reaction medium.

The apparent dependence of branched-chain α-keto acid oxidative decarboxylation on Ca^{2+} and inorganic phosphate prompted further study of their effects. Reciprocal plots illustrate the influence of varying amounts of added Ca^{2+} (Figure 4) and P_i (Figure 5). Apparent affinity constants of the order 1.0 mM for Ca^{2+} and 20 mM for P_i can be estimated from these plots. The dramatic stimulation of branched-chain α-keto acid dehydrogenase activity of whole mitochondria

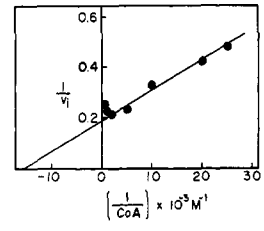

FIGURE 3: Lineweaver–Burk plot of the activity of α-ketoisocaproate dehydrogenase activity as a function of CoA concentration. KIC dehydrogenase activity was measured by the CO_2-^{14}C assay. All components of the reaction medium and experimental conditions were the same as for Table III, except that CoA concentration was varied as indicated (v_i, nanomoles of CO_2 produced per minute per mg of protein).

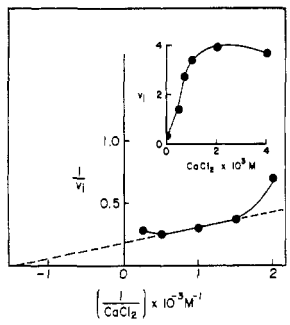

FIGURE 4: Effects of added $CaCl_2$ on the KIV dehydrogenase activity of bovine liver mitochondria. KIV dehydrogenase activity was measured by the CO_2-^{14}C assay as described in the Methods. Reaction mixtures and other experimental conditions were as noted in Table III, except that the α-keto acid substrate was KIV-*1*-^{14}C (2.0 moles, 3500 cpm per μmole) and the amounts of $CaCl_2$ were varied as indicated (v_i, nanomoles of CO_2 produced per minute per mg of protein).

by Ca^{2+} and inorganic phosphate and the nonlinearity of the plots are not understood. A similar effect of Ca^{2+} on this activity was noted by Wohlhueter and Harper (1970) in rat liver mitochondria. It is of some general interest to note that these values approach the physiological levels found for Ca^{2+} in mammalian liver mitochondria (Price, 1956; Long, 1961) and those calculated for P_i in whole liver homogenates (Sacks, 1953; Vallee, 1960). However, since inorganic phosphate (Raaflaub, 1953) and Ca^{2+} (Tapley, 1956) are known to be potent mitochondrial swelling agents at the concentrations utilized in these studies, and since ATP has been shown to reverse the swelling process or cause mitochondria to contract (Neubert and Lehninger, 1962; Aldridge and Street, 1964), the possibility that changes in the structural state of mitochondria give rise to the observed enzymic changes can not be disregarded.

The preceding studies are significant in at least two respects. First, the location of the branched-chain α-keto acid dehydrogenases makes these enzymes immediately accessible to their substrates, which are generated largely outside of the mitochondrion (Wohlhueter and Harper, 1970). Thus, the initial steps in the catabolism of the branched-chain amino acids proceed without a requirement for substrate translocation. Second, a number of the essential components of the enzymic apparatus for oxidation of KIC, KMV, and KIV have been

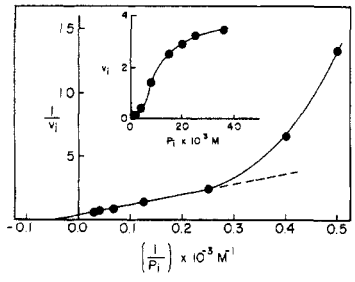

FIGURE 5: Effect of changes in P_i concentration on the KIV dehydrogenase activity of bovine liver mitochondria. Reaction mixtures, experimental conditions, and assay procedures were as stated for Table III, except that P_i concentrations were varied, 0.01 M triethanolamine hydrochloride, pH 7.2, was used to maintain buffering capacity, and the α-keto acid substrate was KIV-*1*-^{14}C (2.0 moles, 3500 cpm per μmole) (v_i, nanomoles per minute per mg of protein).

TABLE V: Effects of Added $MgCl_2$, $CaCl_2$, and $MnCl_2$ in the Reaction Medium on the Branched-Chain α-Keto Acid Dehydrogenase Activities of Bovine Liver Mitochondria Preparations.[a]

Enzyme and Additions[b]	Sp Act. (nmoles of CO_2/min per mg of Protein)	Relative Act. (%)
α-Ketoisocaproate dehydrogenase		
Ca^{2+}, Mg^{2+}	2.72	100
Ca^{2+}	1.88	69
Mg^{2+}	0.16	6
Mn^{2+}	0.13	5
Ca^{2+}, Mg^{2+}, Mn^{2+}	2.56	94
Ca^{2+}, Mn^{2+}	2.24	82
Mg^{2+}, Mn^{2+}	0.18	7
None	0.11	4
α-Ketoisovalerate dehydrogenase		
Ca^{2+}, Mg^{2+}	3.40	100
Ca^{2+}	2.51	74
Mg^{2+}	0.35	11
None	0.21	6
α-Keto-β-methylvalerate dehydrogenase		
Ca^{2+}, Mg^{2+}	3.31	100
Ca^{2+}	2.46	74
Mg^{2+}	0.29	9
None	0.16	5

[a] Branched-chain α-keto acid dehydrogenase activity was measured as described in the methods. [b] Each reaction mixture contained the following, in micromoles: mannitol, 150; NAD, 1.0; Li_3CoA, 0.6; Na_2CO_3, 1.0; potassium phosphate buffer, pH 7.2, 33; carboxyl-labeled α-keto acid, 2.0; 0.1 ml of a washed preparation of bovine liver mitochondria, and 1.0 μmole of each divalent cation where indicated, in a final volume of 1.0 ml.

characterized. These findings strengthen the possibility that interaction of these cofactors, activators, and inhibitors, for example the opposing effects noted for ATP and P_i plus Ca^{2+}, may provide a substantial regulatory influence upon the metabolism of α-keto acids in mitochondria of mammalian liver.

Finally, the possibility of a second, soluble branched-chain keto acid dehydrogenase is intriguing. This could add one more species to the list of enzyme activities which exist both in and outside the mitochondria. However, the final differentiation of these activities and the significance of this possibility will require more complete purification and characterization.

References

Aldridge, W. M., and Street, B. W. (1964), *Biochem. J. 91*, 287.
Bonner, W. D. (1955), *Methods Enzymol. 1*, 722.
Bowden, J. A. (1965), Master's Thesis, University of North Dakota, Grand Forks, N. Dak.

Chappell, J. B. (1968), *Brit. Med. Bull. 24* (2), 150.
Connelly, J. L., Danner, D. J., and Bowden, J. A. (1968), *J. Biol. Chem. 243*, 1198.
Coon, M. J., Robinson, W. G., and Bachhawat, B. K. (1955), *in* Amino Acid Metabolism, McElroy, W. D., and Glass, H. B., Ed., Baltimore, Md., John Hopkins Press, pp 431–441.
Dancis, J., Hutzler, J., and Levitz, M. (1963), *Biochim. Biophys. Acta 78*, 85.
Gunsalus, I. C. (1954), *in* The Mechanism of Enzyme Action, McElroy, W. D., and Glass, H. B., Ed., Baltimore, Md., John Hopkins Press, p 545.
Hayakawa, T., Hirashima, M., Hamada, M., and Koike, M. (1966), *Biochim. Biophys. Acta 128*, 574.
Hayakawa, T., Mutz, H., Hirashima, M., Ide, S., Okabe, K., and Koike, M. (1964), *Biochim. Biophys. Res. Commun. 17*, 51.
Johnson, W. A., and Connelly, J. L. (1971), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 30*, 1256.
Kanzaki, T., Hayakawa, T., Hamada, M., Fukoyoshi, Y., and Koike, M. (1969), *J. Biol. Chem. 244*, 1183.
Klingenberg, M. (1970a), *Eur. J. Biochem. 13*, 247.
Klingenberg, M. (1970b), *in* Essays in Biochemistry, Campbell, P. M., and Dickens, F., Ed., Vol. I, New York, N. Y., Academic Press, p 119.
Koike, M., Reed, L. J., and Carroll, W. R. (1963), *J. Biol. Chem. 238*, 30.
Layne, E. (1957), *Methods Enzymol. 3*, 450.
Linn, T. C., Pettit, F. H., and Reed, L. J. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 234.
Long, C., Ed. (1961), Biochemists Handbook, Princeton, N. J., Van Nostrand, p 681.
Lusty, C. J., and Singer, T. P. (1964), *J. Biol. Chem. 239*, 3733.
Meister, A. (1951), *J. Biol. Chem. 190*, 269.
Morton, R. K. (1955), *Methods Enzymol. 1*, 34.
Namba, Y., Yashizawa, K., Ejima, A., Hayashi, T., and Kaneda, T. (1969), *J. Biol. Chem. 244*, 4437.
Neubert, O., and Lehninger, A. L. (1962), *Biochem. Biophys. Acta 62*, 552.
Nordlie, R. C., and Arion, W. J. (1966), *Methods Enzymol. 9*, 619.
Price, C. A. (1956), *Biochem. J. 64*, 754.
Raaflaub, J. (1953), *Helv. Physiol. Pharmacol. Acta 11*, 142.
Reed, L. J. (1960), *Enzymes 2*, 195.
Sacks, J. (1953), *Amer. J. Physiol. 172*, 93.
Sanadi, D. R., and Littlefield, J. W. (1952), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 11*, 280.
Snyder, F., and Godfrey, P. (1961), *J. Lipid Res. 2*, 195.
Tapley, D. F. (1956), *J. Biol. Chem. 222*, 325.
Vallee, B. L. (1960), *Enzymes 3*, 272.
von Jagow, G., and Klingenberg, M. (1970), *Eur. J. Biochem. 12*, 583.
Wohlhueter, R. M., and Harper, A. E. (1970), *J. Biol. Chem. 245*, 2391.

A Biologically Active Amino-Terminal Fragment of Bovine Parathyroid Hormone Prepared by Dilute Acid Hydrolysis†

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ABSTRACT: Early work with bovine parathyroid hormone extracted from gland tissue with hot hydrochloric acid had shown evidence for a number of small, biologically active fragments arising from cleavages within the peptide chain during extraction. Introduction of improved extraction methods and purification of the 84-amino acid native parathyroid polypeptide has provided the opportunity to investigate these smaller active peptides under controlled conditions. Recovery of biological activity was studied in preparations of hormone treated with 0.03 N HCl at 110° for varying time periods; hydrolysis times of 6 hr or less provided satisfactory yields of active peptides. Native hormone was then treated on a preparative scale for 4 hr and the resulting peptides were identified; most arose from cleavages of the molecule at the 6 aspartic acid residues. A fragment comprising residues 1–29 of the parathyroid hormone sequence, generated by cleavage of aspartic acid at position 30, was isolated from the reaction product by gel filtration and ion-exchange chromatography. This amino-terminal peptide possessed significant biological potency by both *in vivo* and *in vitro* assay methods, and represents the shortest biologically active fragment thus far obtained from the parathyroid hormone molecule.

Parathyroid hormone (BPTH)[1] was first extracted from bovine parathyroid glands by Collip (1925) by a technique using hot hydrochloric acid. Evidence rapidly accumulated, however, that use of these conditions for extraction was accompanied by considerable damage to the extracted peptide, in part through cleavages within the polypeptide chain (Handler *et al.*, 1954; Aurbach *et al.*, 1958).

Ultimately, effective extraction methods, including use of phenol (Aurbach, 1959a) and, later, urea–cysteine–hydrochloric acid (Rasmussen *et al.*, 1964), were developed which employed milder conditions than the hot acid method. Application of these extraction procedures avoided the problem of cleavages during extraction and permitted the eventual isolation of the intact 84-residue parathyroid polypeptide.

Meanwhile, the earlier evidence for the presence of smaller,

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[1] Abbreviations used are: BPTH, bovine parathyroid hormone; CMC, carboxymethylcellulose.