Branched Chain α-Keto Acid Metabolism

I. ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF BOVINE LIVER α-KETO-ISOCAPROIC-α-KETO-β-METHYLVALERIC ACID DEHYDROGENASE*

(Received for publication, October 19, 1967)

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SUMMARY

A soluble enzyme complex, capable of catalyzing the oxidative decarboxylation of both α-ketoisocaproic and α-keto-β-methylvaleric acids, has been obtained from bovine liver. The purified activity (approximately 70-fold) has a pH optimum of about 7.6 for both substrates and exhibits $K_m$ values of $3.5 \times 10^{-3}$ M and $2.5 \times 10^{-3}$ M for α-ketoisocaproic and α-keto-β-methylvaleric acids, respectively. The enzyme preparation is highly substrate-specific, being most notably inactive with α-ketoisovalerate. Evidence is given for the separation of enzymes active with the latter acid from α-ketoisocaproate dehydrogenase, and the significance of this phenomenon relative to the genetic disease, branched chain ketoaciduria, is discussed.

Prior to the detection and characterization of the genetic anomaly, branched chain ketoaciduria, or maple syrup urine disease, little importance had been placed upon the catabolism of valine, leucine, or isoleucine. These "essential" amino acids, which have in common branched chain aliphatic structure, are not known to play any special roles in mammalian metabolism. They are incorporated into new protein or are metabolized primarily to coenzyme A complexes, which in turn eventually become available to the tricarboxylic acid cycle, or to branched chain fatty acids (3, 4). Although tracer work established the major steps of catabolism of these compounds (5), detailed consideration of the oxidative decarboxylation of the α-keto acid products of deamination was not provided. On the basis of meager experimental evidence, the decarboxylation and transacylation which result in acyl-CoA intermediates are generally considered to be analogous to the well characterized α-ketoglutaric and pyruvic acid dehydrogenase reactions (6). Furthermore, primarily because of the concurrent accumulation of all three branched chain keto acids, as well as their amino acid precursors, in the branched chain ketoaciduria condition, it has been a commonly accepted fact that the transformation of these three keto acids to their respective acyl-CoA derivatives occurs via a common metabolic pathway. Attempts in this laboratory (1, 2) to purify the enzymes involved in the α-keto acid oxidative decarboxylation indicated the occurrence of separate pathways for catabolism of the branched chain keto acids. This work describes (a) the isolation, partial purification, and characterization of enzymes from bovine liver which catalyze specifically the oxidative decarboxylation of α-ketoisocaproic and α-keto-β-methylvaleric acids; and (b) the differentiation of this activity from α-ketoisovaleric acid dehydrogenase.

EXPERIMENTAL PROCEDURE

The complex nature of the oxidative decarboxylase activity presents a number of possibilities for assay methods. Those used included measurement of end product formation (NADH or CO$_2$) or the oxidative decarboxylation of the α-keto acid with ferricyanide as the electron acceptor.

Assay Procedures—α-Keto acid dehydrogenase activity was routinely determined essentially by the spectrophotometric method previously described by Gubler (7). This assay provided a simple and rapid means of following the dehydrogenase activities studied. Direct relationship of K$_3$Fe(CN)$_6$ reduction to dehydrogenase activity was established by concurrent measurement of K$_3$Fe(CN)$_6$ reduction and release of $^{14}$CO$_2$ from C-1-labeled α-keto acid (see Fig. 1). The rate of CO$_2$ production was determined by a modification of the method described by Snyder and Godfrey (8). A reaction mixture consisting of 0.4 ml of saturated (6 M) (NH$_4$)$_2$SO$_4$, 150 μmoles of phosphate

Although (NH$_4$)$_2$SO$_4$ was not routinely used in Fe(CN)$_6$³⁻ assays, in long term measurements (NH$_4$)$_2$SO$_4$ was employed to protect the enzyme activity against the effects of dilution (see text).
buffer (pH 7.4), 10 μmoles of substrate, 0.5 μmole of thiamine pyrophosphate, 3.34 μmoles of K$_3$Fe(CN)$_6$, and enzyme (in sidearms), in a total volume of 3.0 ml, was placed in a standard one-arm Warburg flask. A small tube (10 × 22 mm) containing 0.5 ml of Hyamine was placed on the well, and the flask was capped. After preliminary incubation at 30°C for 10 min, the enzyme was added to the reaction mixture by tipping, and the reaction was allowed to continue for 10 min. Addition of 0.2 ml of 2.0 N H$_2$SO$_4$, by syringe through the cap, stopped the reaction and released the CO$_2$ from solution. Distillation of the CO$_2$ into the Hyamine was allowed to continue for 1 h. The Hyamine tube was transferred to a scintillation vial containing 10 ml of solvent (0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis-2-[5-phenyloxazolyl]-benzene (w/v) in toluene). 4C$_2$O$_4$, samples, in triplicate, were counted for 10 min. The quantity of CO$_2$ evolved, based upon the specific activity of C-1-labeled α-keto acid substrate, was determined.

The K$_3$Fe(CN)$_6$ assay was conducted as follows. Reduction of K$_3$Fe(CN)$_6$ was followed continuously with time at 420 μm with the use of a Cary 15 recording spectrophotometer. The following components were combined in a volume of 7.0 ml: 150 μmoles of sodium-potassium phosphate buffer (pH 7.4), 0.5 μmole of thiamine pyrophosphate, 3.34 μmoles of K$_3$Fe(CN)$_6$, and enzyme. After rapid mixing, the reaction was divided equally between two 1-cm cuvettes, and the instrument was balanced to zero with the cuvettes in position. Substrate, 20 μmoles, was added to the reference cell, the contents were mixed, and the recording was begun immediately. The time elapsed between addition of substrate and start of recording was not more than 10 sec. The rate of change in optical density was linear for 50 to 100 sec. 4E$_{max}$ for K$_3$Fe(CN)$_6$ was experimentally determined to be 1.05 × 10$^6$ μg$^{-1}$ cm$^{-1}$. The stoichiometry between Fe(CN)$_6^{3-}$ reduced and keto acid oxidized, as established by Hager (9) (also see Reference 10 and Fig. 1), is 2:1. Dehydrogenase activity is expressed as micromoles of keto acid decarboxylated per min.

α-Keto acid dehydrogenase activity was determined according to the method of Searls and Sanadi (11). It should be noted that this assay system was not capable of reducing NAD in the absence of Fe(CN)$_6^{3-}$, which acts as an electron acceptor from the α-hydroxyalkylthiamine pyrophosphate in the first reaction step.

**Enzyme Preparation**

**Preparation of Extract**—A summary of purification is shown in Table I. All of the following procedures were carried out within 15 min of slaughter and were immediately packed in ice or in a cold room or in ice. Bovine livers were obtained from C-1-labeled α-ketoisocaproic acid substrate, was determined.

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**Preparation of Extract**—A summary of purification is shown in Table I. All of the following procedures were carried out within 15 min of slaughter and were immediately packed in ice for transport. The tissue (300 g) was ground, mixed with 600 ml of 0.033 M sodium-potassium phosphate buffer, pH 7.4, and homogenized for 5 min in a Waring Blender at high speed, with one-half voltage (60 volts). The resulting homogenate was centrifuged at 28,000 × g for 10 min in an International B-20 rotor. The clear, deep red supernatant fraction was termed PS. 1,045 128 14,560 1.990 71 6.7

**Table I** Summary of enzyme purification

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Recovery of homogenate</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2,000</td>
<td>180 120,000</td>
<td>mg</td>
<td>%</td>
<td>ml</td>
<td>μmoles/ min</td>
</tr>
<tr>
<td>EE</td>
<td>950</td>
<td>128 10,000</td>
<td></td>
<td>0.675</td>
<td>71</td>
<td>4.5</td>
</tr>
<tr>
<td>PE</td>
<td>1,045</td>
<td>128 14,500</td>
<td></td>
<td>1.000</td>
<td>71</td>
<td>0.7</td>
</tr>
<tr>
<td>AS</td>
<td>313</td>
<td>163 7,720</td>
<td></td>
<td>2.075</td>
<td>91</td>
<td>14.0</td>
</tr>
<tr>
<td>EE</td>
<td>235</td>
<td>94 10,000</td>
<td></td>
<td>10.000</td>
<td>52</td>
<td>67.0</td>
</tr>
</tbody>
</table>

a Protein by nitrogen determination.

b Apparent increase in activity was routinely observed in ammonium sulfate step. Levels of activity could be varied directly, within limits, according to ionic strength with either (NH$_4$)$_2$SO$_4$ or NaCl but not ammonium acetate (see text).

**Proteamine Sulfate Fractionation**—EDTA-recrystallized (NH$_4$)$_2$SO$_4$ was slowly added to a stirring solution of PS until 55% saturation was reached (31.5 g/100 ml). After 10 min of additional stirring, the suspension was centrifuged 10 min at 10,000 × g. The green-brown precipitate was dissolved in 0.2 volume (based on the PS) of 0.033 M phosphate buffer, pH 7.4, to produce a brown-yellow solution which is termed AS.

**Ethanol Precipitation and Extraction**—AS, 50 to 200 ml, was cooled to −5°C with stirring, in a salt-ice bath. After addition of 1.25 volumes of cold (−40°C) 95% ethanol (via an insulated pipette), the slurry was stirred for an additional 5 min. Centrifugation at 10,000 × g for 10 min produced a three-layered precipitate (white-green-pink). The precipitate was mashed into a slurry, 50 to 100 ml, was placed on the well, and the flask was capped. After preincubation at 30°C for 10 min, the enzyme was added to the reaction mixture by tipping, and the reaction was allowed to continue for 10 min. Addition of 0.2 ml of 2.0 N H$_2$SO$_4$, by syringe through the cap, stopped the reaction and released the CO$_2$ from solution. Distillation of the CO$_2$ into the Hyamine was allowed to continue for 1 h. The Hyamine tube was transferred to a scintillation vial containing 10 ml of solvent (0.4% 2,5-diphenyloxazole, and 0.01% 1,4-bis-2-[5-phenyloxazolyl]-benzene (w/v) in toluene). 4C$_2$O$_4$, samples, in triplicate, were counted for 10 min. The quantity of CO$_2$ evolved, based upon the specific activity of C-1-labeled α-keto acid substrate, was determined.

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hydrochloric acid. Substrates were stored at −18°C and thawed just prior to use.

Keto acids were adjudged to be free of interfering contaminants by thin layer chromatography of their 2,4-dinitrophenyldrazone derivatives with the method of Dancis, Hutzler, and Levitz (14).

RESULTS

As a preliminary step in the attempt to isolate the enzymes which catalyze the oxidative decarboxylation of branched chain keto acids, a survey was conducted to determine the general distribution of α-ketoisocaproic acid dehydrogenase. The Table II

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Ketoacid dehydrogenase activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Rat</td>
</tr>
<tr>
<td>Liver</td>
<td>0.32</td>
</tr>
<tr>
<td>Kidney</td>
<td>0.15</td>
</tr>
<tr>
<td>Heart</td>
<td>0</td>
</tr>
<tr>
<td>Intestine</td>
<td>0</td>
</tr>
<tr>
<td>Testis</td>
<td>0</td>
</tr>
<tr>
<td>Brain</td>
<td>0</td>
</tr>
<tr>
<td>Spleen</td>
<td>0</td>
</tr>
<tr>
<td>Muscle</td>
<td>0</td>
</tr>
<tr>
<td>Lung</td>
<td>0</td>
</tr>
<tr>
<td>Plasma</td>
<td>0</td>
</tr>
</tbody>
</table>

As expected, the greatest level of activity was found in liver, although relatively high levels were observed in heart and kidney (Table II). Investigation of the intracellular distribution disclose that essentially all activity resided in the mitochondrial fraction. Subsequent to these studies, attempts were made to obtain activity in a soluble form from rat liver mitochondria and also, because of the ready availability of large quantities of tissue, from bovine liver homogenate. All efforts to extract the dehydrogenase activity from rat liver mitochondria failed, owing to lack of solubilization or to complete loss of activity. Techniques employed included simple extraction with buffer, sonic disruption, detergent action, organic solvent extraction, freezing and thawing, hydraulic press, phospholipase A treatment, and various combinations of these.

In the case of bovine liver, however, α-ketoisocaproic acid dehydrogenase activity was obtained by simple extraction of ground and homogenized tissue with a phosphate buffer at pH 7.4. Consequently, this tissue was used as starting material, and a detailed description of the preparation of this enzyme is presented in the "Experimental Procedure." Properties of Dehydrogenase—Linearity of enzyme activity with protein concentration, as assayed both by product (CO₂) formation and ferricyanide reduction, is shown in Fig. 1. The stoichiometry of Fe(CN)₆³⁻/CO₂ is 2.0 as predicted by Hager (9). Furthermore, assay of the preparation for dehydrogenase activity, by following NADH production at 340 nm, indicated that purification through the ethanol extract step did not disrupt the complex.³

Consideration of the possible number of enzymatic steps involved in the transformation of the keto acid to the acyl coenzyme A derivative prompted a variety of studies on the influence of cofactors and, secondly, on the stability of the enzyme. The results of these studies are listed in Table III. It is apparent that the preparation contains sufficient levels of the cofactor, thiamine pyrophosphate, for the ferricyanide assay. It was noted, however, that the addition of thiamine pyrophosphate increased the linearity of activity from 2 to about 5 min. Activity of the complex was essentially unaffected by metals, although Mn⁺⁺ did slightly decrease activity. Furthermore, the lack of significant inhibition by EDTA suggests that labile metals are not required for activity.

The preparation is very sensitive to dilution, and it was not possible to reactivate the dialyzed enzyme activity with thiamine pyrophosphate, lipoic acid, coenzyme A, Mg²⁺, or a combination of these. Furthermore, protection by (NH₄)₆SO₄ against this inactivation supported the possibility that the primary effect of dialysis is related to a decreased salt concentration rather than to a loss of cofactors. Simple dilution of the preparation by buffer also resulted in a decrease in specific activity. The concentrated enzyme is very stable at −18°C, although rapid (days) inactivation occurs at 0°C. Finally, some involvement of sulphydryl groups in either activity or in stability is evidenced by the marked inhibitory effect of p-chloromercuribenzenesulphonate at low concentrations.

Lineweaver-Burk plots of dehydrogenase activity with both active substrates (see Table IV) are shown in Fig. 2. Values

³ Certain subsequent purification steps, not yet incorporated, have produced severalfold increases in purity of the dehydrogenase activity as measured by ferricyanide reduction, whereas dehydrogenase activity measured by NADH formation has been lost.
TABLE III
Cofactor requirement and stability of ethanol extract-fraction activity

<table>
<thead>
<tr>
<th>Condition</th>
<th>Control</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>100</td>
</tr>
<tr>
<td>- Enzyme</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- Substrate</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- Fe(CN)₆⁻³</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>- TPP*</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+TPP (2.8 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+CoA-SH (0.2 mg)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+Lipoic acid (1.4 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+MgCl₂ (2.9 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+MgCl₂ (6 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+MnCl₂ (3 × 10⁻⁴ M)</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>+MnCl₂ (6 × 10⁻⁴ M)</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>+EDTA (2.9 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+EDTA (6 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+CMBS* (1.4 × 10⁻⁴ M)</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>+CMBS (2.8 × 10⁻⁴ M)</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

Dialysis versus

-0.01 M Tris-Cl                                  33
-0.03 M Pi, pH 7.4                               31
-0.03 M Pi, pH 7.4 in 10% (NH₄)₂SO₄              65

Storage

-10°, 7 days                                     25
-10°, 10 days                                    0
-18°, 1 day                                      92
-18°, 100 days                                   90

* TPP, thiamine pyrophosphate; CMBS, p-chloromercuribenzenesulfonate.

The rather narrow substrate specificity of this enzyme is indicated by the data in Table IV. Dehydrogenase activity with α-keto-β-methylvaleric acid is approximately 50% greater, at the concentration used, than that seen with α-ketoisocaproic

![Fig. 2. Double reciprocal plots of dehydrogenase activity versus keto acid concentration. Enzyme activity was measured by Fe(CN)₆⁻³ assay described in “Experimental Procedure.” Substrates employed were α-ketoisocaproic acid (●) and α-keto-β-methylvaleric acid (○). Activity is expressed as micromoles of keto acid decarboxylated per min.](http://www.jbc.org/)

![Fig. 3. pH profile of α-ketoisocaproic acid:α-keto-β-methylvaleric acid dehydrogenase. Activity was measured by the Fe(CN)₆⁻³ assay as described in “Experimental Procedure” except that the buffer used was Tris-maleate. ○—○, activity when α-keto-β-methylvaleric acid is the substrate; ●—●, activity when α-ketoisocaproic acid is the substrate.](http://www.jbc.org/)
were conducted by means of the Fe(CN)₆³⁻ assay indicated in "Experimental Procedure." α-0, α-ketoisocaproic acid dehydrogenase activity; Δ, α-ketoisovaleric acid dehydrogenase activities of bovine liver. Enzyme assays were conducted by means of the Fe(CN)₆³⁻ assay indicated in "Experimental Procedure." O — O, α-ketoisocaproic acid dehydrogenase activity; Δ — Δ, α-ketoisovaleric acid dehydrogenase activity; O — O, total protein.

Fig. 4. Separation of α-ketoisocaproic acid and α-ketoisovaleric acid dehydrogenase activities of bovine liver. Enzyme assays were conducted by means of the Fe(CN)₆³⁻ assay indicated in "Experimental Procedure." O — O, α-ketoisocaproic acid dehydrogenase activity; Δ — Δ, α-ketoisovaleric acid dehydrogenase activity; O — O, total protein.

Fig. 5. Distribution and recovery of soluble keto acid dehydrogenase activities during centrifugation of bovine liver extracts. Each was active for its respective keto acid but inactive with α-ketoisovaleric acid, α-ketoisocaproic acid, α-keto-β-methylvaleric acid, and α-ketobutyric acids. This difference is also noted when NADH formation is followed. In the cases of other α-keto acids tested, the enzyme is essentially inactive. These findings are consistent with the generally narrow substrate specificity observed for α-ketoglutarate and pyruvate oxidative decarboxylase activity (15). However, in view of the widely accepted probability that all branched chain keto acids are catabolized by a single pathway, it was quite unexpected to observe that the crude bovine liver preparation (Fraction S₀), as well as the more purified preparations, was inactive with α-ketoisovaleric acid. This startling observation provided the first definite evidence that this keto acid was catabolized apart from α-ketoisocaproic acid. Separation of these two activities during centrifugation is shown by Fig. 4. It is evident that α-ketoisovaleric acid dehydrogenase remains in the particulate fraction throughout, whereas a major portion (see Fig. 5) of the α-ketoisocaproic acid dehydrogenase activity is soluble. Furthermore, a gross purification of the latter activity is effected by the removal of particulate protein.

In view of the complex nature of the α-ketoglutaric acid dehydrogenase macromolecule, and since it is reasonable to expect that the branched chain keto acid dehydrogenases exist in a similar form, it was considered essential to differentiate between (a) the actual separation of two active enzymes (insoluble α-ketoisovaleric acid and soluble α-ketoisocaproic acid:α-keto-β-methylvaleric acid dehydrogenases) and (b) the isolation of a single nonspecific enzyme the apparent substrate specificity of which resulted from loss of activity toward α-ketoisovaleric acid during preparation. Fig. 5 indicates the distribution of activities between supernatant and sediment during centrifugation. Although assay of the highly particulate samples (low centrifugation fractions) was somewhat inaccurate, it was possible to account for essentially 100% of both activities. Under the conditions of homogenization employed, nearly half of the α-ketoisocaproic acid dehydrogenase activity is obtained in soluble form, while all of the α-ketoisovaleric acid dehydrogenase activity remains particulate. Re-extraction of the sediment by blending solubilized additional α-ketoisocaproic acid dehydrogenase did not influence the insoluble α-ketoisovaleric acid dehydrogenase. This suggests that the former enzyme, rather than being present in two (soluble and particulate) forms is easily solubilized.

DISCUSSION

This work reports the isolation and partial purification and characterization of the enzyme complex which catalyzes the oxidative decarboxylation of two 6-carbon branched chain keto acids, α-ketoisocaproic acid and α-keto-β-methylvaleric acid. Efforts are now being made to determine whether the decarboxylation of α-ketoisocaproic acid and α-keto-β-methylvaleric acid involves a single semispecific enzyme complex or whether the preparation described herein is comprised of two associated dehydrogenases, each specific for a single substrate. Preliminary observations suggest that the former is the case. Results of this work will be the subject of a subsequent publication.

In regard to the question above, a significant consequence of the isolation and purification of the α-ketoisocaproic acid:α-keto-β-methylvaleric acid dehydrogenase is the observation that the enzyme is not active with the third branched chain keto acid, α-ketoisovaleric acid. Contrary to the generally accepted view (18) that oxidative decarboxylation of the keto acids, derived from the branched chain amino acids, is catalyzed by a single high levels of NADH-oxidase activity in the more particulate preparations interfered with the NADH assay but did not influence the ferricyanide reduction.
enzyme complex, the work presented here establishes that at least two distinct dehydrogenases are present in bovine liver. Evidence has also been obtained for the existence of separate branched chain keto acid dehydrogenases in intact rat liver mitochondria (1). A preliminary note reports the existence of separate dehydrogenase mechanisms also in human and bovine leukocytes (19). It appears quite likely, at least among mammals, that independent dehydrogenases occur for the degradation of pyruvic, α-ketoglutaric, α-ketoisovaleric, and α-ketoisocaproic, α-keto-β-methylvaleric acids.

The discovery of at least two separate enzymatic paths for the oxidative decarboxylation of the branched chain α-keto acids lends support to the thesis, proposed by Menkes (20), that accumulation of the three branched chain keto acids in patients with branched chain ketoaciduria results from a genetic lesion in only one of the catabolic paths (probably α-ketoisocaproic acid dehydrogenase). The increased level of the corresponding keto acid acts to inhibit the operation of the other paths. Indeed, Snyderman et al. (21) and Gerritsen have observed clinical conditions which suggest the primary involvement of leucine metabolism and only incidental involvement of valine metabolism. Direct evidence has been obtained for the ability of α-ketoisocaproic acid to inhibit both α-ketoisovaleric acid and α-ketoisocaproic acid dehydrogenases in vitro (1), and for the ability of α-ketoisovaleric acid to inhibit α-ketoisocaproic acid dehydrogenase. Currently in progress are efforts designed to determine the relative inhibitory capacity of the various keto acids in vitro. A more comprehensive consideration of the importance of the separate branched chain keto acid dehydrogenases to this genetic disease will be contained in a subsequent article.

6 T. Gerritsen, personal communication.
7 J. A. Bowden and J. L. Connelly, manuscript in preparation.

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Branched Chain α-Keto Acid Metabolism: I. ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF BOVINE LIVER α-KETOISOCAPROIC:α-KETO-β-METHYLVALERIC ACID DEHYDROGENASE

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