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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**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 γ-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 complexes of coenzyme A, 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 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\(_2\)Fe(CN)\(_6\) reduction to dehydrogenase activity was established by concurrent measurement of K\(_2\)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\(_4\), \(2 \times 150\mu\) moles of phosphate

\[\text{NADH} + \text{Fe}^{3+} \rightarrow \text{NAD}^+ + \text{Fe}^{2+} + 2e^-\]

Although (NH\(_4\))\(_2\)SO\(_4\) was not routinely used in Fe(CN)\(_6\) assays, in long term measurements (NH\(_4\))\(_2\)SO\(_4\) was employed to protect the enzyme activity against the effects of dilution (see text).

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buffer (pH 7.4), 10 μmoles of substrate, 0.5 μmole of thiamine pyrophosphate, 3.34 μmole of K$_2$Fe(CN)$_6$, and enzyme (in sidearm), 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° 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 hour. 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-phenyloxazoyl]-benzene (w/v) in toluene). 4C$_3$O$_3$ 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$_2$Fe(CN)$_6$ assay was conducted as follows. Reduction of K$_2$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 μmole of K$_2$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. $E_{max}$ for K$_2$Fe(CN)$_6$ was experimentally determined to be $1.05 \times 10^8$ M$^{-1}$ 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-Ketoo acid dehydrogenase activity was determined according to the method of Sears and Sanadi (11). It should be noted that this assay system was not capable of reducing NAD in the presence 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 either in a cold room or in ice. Bovine livers were obtained from C-1-labeled α-ketoisocaproic acid was prepared and used as a standard. α-Keto-β-methylvaleric acid was prepared for N-α-hisoleucine and N-α-homo-α-amino acid oxidase, and C-1-labeled α-ketoisocaproic acid was prepared from C-1-labeled α-ketoisocaproic acid oxidase, according to the method of Meister (13).

Materials—Coenzyme A, keto acids, ω-leucine, ω-α-amino acid oxidase, NAD, purified L-α-amino acid oxidase, N-α-amino acid oxidase, and thiamine-PP were purchased from the Sigma Chemical Company. Salmine protein sulfamate and EDTA-recrystallized (NH$_4$)$_2$SO$_4$ were obtained from Mann Research Laboratories, Inc. 14C-labeled ω-leucine was obtained from New England Nuclear. All water was once distilled and passed through an Amberlite resin. Reactants and substrates were adjusted to approximately pH 7.0 with either potassium hydroxide or
hydrochloric acid. Substrates were stored at -18° and thawed just prior to use.

Keto acids were adjudged to be free of interfering contaminants by thin layer chromatography of their 2,4-dinitrophenylhydroxylamine 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 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, protection by (NH₄)₂SO₄ 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°, although rapid inactivation occurs at 0°. Finally, some involvement of sulfhydryl groups in either activity or in stability is evidenced by the marked inhibitory effect of p-chloromercuribenzenesulfonate at low concentrations.

![Fig. 1](https://www.jbc.org/)

**Fig. 1. Linearity of α-ketoisocaproic acid dehydrogenase activity as assayed by Fe(CN)₆³⁻ reduction and CO₂ production.** The reaction mixture contained 76 μmoles of phosphate buffer, pH 7.4; 3.34 μmoles of K₃Fe(CN)₆; 0.5 μ mole of thiamine pyrophosphate; 0.4 ml of saturated (NH₄)₂SO₄; 10 μmoles of 1-¹⁴C-labeled α-ketoisocaproic acid (189,000 cpm); and enzyme as indicated. Total volume was 3.0 ml. △μoles/min/mg protein X 10²

<table>
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<th>Rat</th>
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<th>Pig</th>
<th>Rabbit</th>
<th>Mouse</th>
<th>Beef</th>
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**TABLE II**

**Distribution of α-ketoisocaproic acid dehydrogenase in various species and organs**

Homogenates were prepared in 0.25 m sucrose, 1 g of tissue/10 ml, with a Potter-Elvehjem homogenizer. Activity was measured with the Fe(CN)₆³⁻ assay method described in the “Experimental Procedure.”

Table: 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 μm, indicated that purification through the ethanol extract step did not disrupt the complex. 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, Mg++, or a combination of these. Furthermore, protection by (NH₄)₂SO₄ 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°, although rapid (days) inactivation occurs at 0°. Finally, some involvement of sulfhydryl groups in either activity or in stability is evidenced by the marked inhibitory effect of p-chloromercuribenzenesulfonate at low concentrations.

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

1 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
Enzymatic activity of the ethanol extract fraction was determined by the Fe(CN)$_6^{3-}$/assay, with the medium described in "Experimental Procedure." Dialysis was conducted for 1 hour.

<table>
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<th>Condition</th>
<th>Control</th>
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<td>- Enzyme</td>
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</tr>
<tr>
<td>- Substrate</td>
<td>0</td>
</tr>
<tr>
<td>- Fe(CN)$_6^{3-}$/</td>
<td>0</td>
</tr>
<tr>
<td>- TPP</td>
<td>100</td>
</tr>
<tr>
<td>+TPP ($2.8 \times 10^{-4}$ M)</td>
<td>100</td>
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<tr>
<td>+CoA-SH (0.2 mg)</td>
<td>100</td>
</tr>
<tr>
<td>+Lipoic acid ($1.4 \times 10^{-4}$ M)</td>
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</tr>
<tr>
<td>+MgCl$_2$ ($2.9 \times 10^{-4}$ M)</td>
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</tr>
<tr>
<td>+MgCl$_2$ ($6 \times 10^{-4}$ M)</td>
<td>100</td>
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<tr>
<td>+MnCl$_2$ ($3 \times 10^{-4}$ M)</td>
<td>82</td>
</tr>
<tr>
<td>+MnCl$_2$ ($6 \times 10^{-4}$ M)</td>
<td>111</td>
</tr>
<tr>
<td>+EDTA ($2.8 \times 10^{-4}$ M)</td>
<td>100</td>
</tr>
<tr>
<td>+EDTA ($6 \times 10^{-4}$ M)</td>
<td>100</td>
</tr>
<tr>
<td>+CMBS ($1.4 \times 10^{-4}$ M)</td>
<td>100</td>
</tr>
<tr>
<td>+CMBS ($2.8 \times 10^{-4}$ M)</td>
<td>20</td>
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</table>

Dialysis versus
0.01 M Tris-Cl                  33
0.03 M P$_i$, pH 7.4             31
0.03 M P$_i$, pH 7.4 in 10% (NH$_4$)$_2$SO$_4$ 65

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

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

TABLE IV
Specificity of α-ketoisocaproic acid:α-keto-β-methylvaleric acid dehydrogenase
Enzyme assays were conducted as described in "Experimental Procedure." Specific activity is defined as micromoles of substrate utilized per min per mg of protein.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Specific activity $\times 10^4$</th>
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<tr>
<td>Fe(CN)$_6^{3-}$/assay</td>
<td>Relative activity</td>
</tr>
<tr>
<td>α-Ketoisocaproic, $5.7 \times 10^{-4}$ M</td>
<td>35.0</td>
</tr>
<tr>
<td>α-Keto-β-methylvaleric, $3.7 \times 10^{-4}$ M</td>
<td>50.0</td>
</tr>
<tr>
<td>α-Ketoisovaleric, $1.3 \times 10^{-4}$ M</td>
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<td>α-Ketobutyric, $1.3 \times 10^{-4}$ M</td>
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<tr>
<td>α-Ketoglutaric, $1.3 \times 10^{-4}$ M</td>
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</tr>
<tr>
<td>α-Ketovaleric, $1.3 \times 10^{-4}$ M</td>
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</tr>
<tr>
<td>Pyruvic, $1.3 \times 10^{-4}$ M</td>
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</table>

determined for $K_m$ are $3.5 \times 10^{-4}$ M and $2.5 \times 10^{-4}$ M for α-ketoisocaproic acid and α-keto-β-methylvaleric acid, respectively. The slight difference in $V_{max}$ values is apparently attributable to steric factors relative to the slight difference in structures of the keto acids. This consideration may be responsible for the small difference in pH optima exhibited by the enzyme with these substrates (Fig. 3). Nevertheless, the over-all pH-activity curves are identical in shape, which suggests that the optima observed reflect contributions made primarily by the enzyme.

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 acid.

![Fig. 2. Double reciprocal plots of dehydrogenase activity versus keto acid concentration. Enzyme activity was measured by Fe(CN)$_6^{3-}$/assay described in the "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. O——O, 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." O-0, 0-ketoisocaproic acid dehydrogenase activity; A, a-ketoisovaleric acid dehydrogenase activities of bovine liver. Enzyme assay was performed by means of the Fe(CN)₆³⁻ assay described in "Experimental Procedure." [O]—[O], 0-ketoisocaproic acid dehydrogenase activity; [A]—[A], a-ketoisovaleric acid dehydrogenase activity; [O]—[O], total protein.

In view of the complex nature of the a-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 a-ketoisovaleric acid and soluble a-ketoisocaproic acid dehydrogenases) and (b) the isolation of a single nonspecific enzyme the apparent substrate specificity of which resulted from loss of activity toward a-ketoisovaleric acid during preparation. Fig. 5 indicates the distribution of activities during centrifugation of the particulate and sediment fractions. 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 a-ketoisocaproic acid dehydrogenase activity remains particulate. Re-extraction of the sediment by blending and solubilization did not influence the insoluble a-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, a-ketoisocaproic acid and a-keto-β-methylvaleric acid. Efforts are now being made to determine whether the decarboxylation of a-ketoisocaproic acid and a-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 a-ketoisocaproic acid dehydrogenase is the observation that the enzyme is not active with the third branched chain keto acid, a-keto-β-methylvaleric 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 a-ketoisocaproic acid dehydrogenase, 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.

Branch chain keto acids are catabolized by a single pathway, it was quite unexpected to observe that the crude bovine liver preparation (Fraction S₉₀), as well as the more purified preparations, was inactive with a-ketoisovaleric acid. This startling observation provided the first definite evidence that this keto acid was catabolized apart from a-ketoisocaproic acid. Separation of these two activities during centrifugation is shown by Fig. 4. It is evident that a-ketoisovaleric acid dehydrogenase remains in the particulate fraction throughout, whereas a major portion (see Fig. 5) of the a-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 a-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 a-ketoisovaleric acid and soluble a-ketoisocaproic acid: a-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 a-ketoisovaleric acid during preparation. Fig. 5 indicates the distribution of activities during centrifugation of the particulate and sediment fractions. 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 a-ketoisocaproic acid dehydrogenase activity remains particulate. Re-extraction of the sediment by blending and solubilization did not influence the insoluble a-ketoisovaleric acid dehydrogenase. This suggests that the former enzyme, rather than being present in two (soluble and particulate) forms is easily solubilized.

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Preliminary studies with agarose gel filtration indicate that the a-ketoisocaproic acid: a-keto-β-methylvaleric acid enzyme complex has a molecular weight of the same order of magnitude as that reported for other purified keto acid dehydrogenases.

Branch chain keto acids are catabolized by a single pathway, it was quite unexpected to observe that the crude bovine liver preparation (Fraction S₉₀), as well as the more purified preparations, was inactive with a-ketoisovaleric acid. This startling observation provided the first definite evidence that this keto acid was catabolized apart from a-ketoisocaproic acid. Separation of these two activities during centrifugation is shown by Fig. 4. It is evident that a-ketoisovaleric acid dehydrogenase remains in the particulate fraction throughout, whereas a major portion (see Fig. 5) of the a-ketoisocaproic acid dehydrogenase activity is soluble. Furthermore, a gross purification of the latter activity is effected by the removal of particulate protein.

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**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, a-ketoisocaproic acid and a-keto-β-methylvaleric acid. Efforts are now being made to determine whether the decarboxylation of a-ketoisocaproic acid and a-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 a-ketoisocaproic acid: a-keto-β-methylvaleric acid dehydrogenase is the observation that the enzyme is not active with the third branched chain keto acid, a-keto-β-methylvaleric 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 a-ketoisocaproic acid dehydrogenase, 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.

3 Both a-ketoglutaric acid dehydrogenase prepared from pig heart according to Sanadi, Littlefield, and Bock (16) and pigeon breast muscle pyruvate oxidase prepared by the method of Jagannathan and Schweet (17) were tested for substrate specificity in this laboratory. Each was active for its respective keto acid but inactive with a-ketoisovaleric acid, a-ketoisocaproic acid, a-keto-β-methylvaleric acid, and a-ketoisocaproic, a-ketovaleric, and a-ketobutyric acids.

4 Preliminary studies with agarose gel filtration indicate that the a-ketoisocaproic acid: a-keto-β-methylvaleric acid enzyme complex has a molecular weight of the same order of magnitude as that reported for other purified keto acid dehydrogenases.

5 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 Gerritsen6 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. Current efforts are 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.

REFERENCES
Branched Chain \( \alpha \)-Keto Acid Metabolism: I. ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF BOVINE LIVER \( \alpha \)-KETOISOCAPROIC:\( \alpha \)-KETO-\( \beta \)-METHYLVALERIC ACID DEHYDROGENASE

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