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PURIFICATION AND CHARACTERIZATION OF AN
α-KETOISOCAPROATE OXIDASE FROM RAT LIVER

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Patrick John Sabourin

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# PURIFICATION AND CHARACTERIZATION OF AN α-KETOISOCAPROATE OXIDASE FROM RAT LIVER

By

Patrick John Sabourin

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ABSTRACT

PURIFICATION AND CHARACTERIZATION OF AN
α-KETOISOCAPROATE OXIDASE FROM RAT LIVER

By

Patrick John Sabourin

Isopycnic sucrose gradient separation of rat liver organelles revealed the presence of two distinct enzymes which decarboxylate α-ketoisocaproate; the mitochondrial branched-chain α-keto acid dehydrogenase and a cytosolic α-ketoisocaproate oxidase. The branched-chain α-keto acid dehydrogenase uses α-ketoisocaproate, α-keto-β-methylvalerate, and α-ketoisovalerate (the α-keto acids of leucine, isoleucine and valine) as substrates and requires CoASH and $NAD^+$ as cofactors. This enzyme has been studied extensively and operates by a mechanism similar to that of pyruvate dehydrogenase. The cytosolic α-ketoisocaproate oxidase uses only α-ketoisocaproate and α-keto-γ-methiolbutyrate (the α-keto acids of leucine and methionine) as substrates and does not require CoASH or $NAD^+$ for activity. The purpose of this study was to further characterize the cytosolic α-ketoisocaproate oxidase activity.

The cytosolic α-ketoisocaproate oxidase activity was partially characterized in 70,000 x g supernatant fractions of rat liver homogenates (cytosol preparation). Oxygen was required for enzymatic activity. Cytosol preparations consumed oxygen when either α-ketoisocaproate or α-keto-γ-methiolbutyrate were added. None of the other α-keto acids tested stimulated oxygen consumption. α-Ketoisovalerate, α-keto-β-methylvalerate, α-ketobutyrate and α-ketononanoate inhibit the α-ketoisocaproate oxidase activity. Phenylpyruvate is a very potent inhibitor. α-Ketoisocaproate oxidase activity was only detected in liver and kidney.

In order to further characterize the cytosolic α-ketoisocaproate oxidase (decarboxylase) activity, it was purified from rat liver. The purified enzyme required $Fe^{2+}$ and a sulfhydryl reducing reagent for optimal activity. Other metal ions tested would not replace $Fe^{2+}$. During purification this enzyme was quite unstable. Inclusion of 5% monothioglycerol in all buffers increased stability. Using a 180 fold purified preparation of the α-ketoisocaproate oxidase, the assay conditions for this enzyme were optimized. Maleate, up to 0.2 M increased the activity. This was not due to an ionic strength effect. Optimal activity was obtained at pH 6.0 in the presence of 1 mM $FeSO_4$, 0.5 mM ascorbate and 1 mM dithiothreitol.

The $K_m$ of the α-ketoisocaproate oxidase for α-keto-γ-methiolbutyrate is about 6 times higher than that for α-ketoisocaproate (1.9 mM vs. 0.3 mM). This enzyme converts α-ketoisocaproate to β-hydroxyisovaleric acid. Isovalerate is not a free intermediate of the reaction. $^{18}O$ incorporation experiments indicate that this enzyme is a dioxygenase, that inserts one O atom of $O_2$ into the carboxyl group and the other O atom of $O_2$ into the β-hydroxyl group of β-hydroxyisovaleric acid.

To Carol Lee

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

## ABBREVIATIONS

| | |
|---|---|
| ADP | adenosine 5'-diphosphate |
| BCAA | branched-chain amino acid(s) |
| BCKA | branched-chain keto acid(s) |
| Buffer A | 20 mM Tris HCl pH 7.8, 1% Isopropanol |
| CoA | coenzyme A |
| CoASH | reduced coenzyme A |
| DEAE | diethylaminoethyl |
| DTNB | 5,5'-dithiobis(2-nitrobenzoic acid) |
| DTT | dithiothreitol |
| EDTA | (ethylenedinitrilo)-tetraacetic acid, disodium salt |
| FAD | flavin adenine dinucleotide |
| Hepes | N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid |
| β-HIVA | β-hydroxyisovaleric acid |
| α-KIC | α-ketoisocaproate |
| α-KγMB | α-keto-γ-methiolbutyrate |
| MES | 2(N-morpholino)ethane sulfonic acid |
| MOPS | morpholinopropane sulfonic acid |
| $NAD^+$ | nicotinamide adenine dinucleotide (oxidized) |
| NADH | nicotinamide adenine dinucleotide (reduced) |
| $NADP^+$ | nicotinamide adenine dinucleotide phosphate (oxidized) |
| NADPH | nicotinamide adenine dinucleotide phosphate (reduced) |
| SDS | sodium dodecyl sulfate |
| TCA | tricarboxylic acid |
| Tris | tris(hydroxymethyl)aminomethane (Trizma base) |

# INTRODUCTION

The branched-chain amino acids, leucine, isoleucine and valine are essential in the diets of all mammals. The initial step in the catabolism of these amino acids is a transamination to produce α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate, respectively. The α-keto acids are then decarboxylated by a branched-chain α-keto acid dehydrogenase and converted to the acyl-CoA. A single enzyme, the branched-chain α-keto acid dehydrogenase (EC 1.2.4.3 and 1.2.4.4), catalyzes the decarboxylation and conversion of all three branched-chain α-keto acids to their respective acyl-CoA's (1-4). However, there are also reports in the literature of dehydrogenases and/or decarboxylases which use only one or two of the three branched-chain α-keto acids as substrates (5-10).

The occurrence of branched-chain acylcarnitines (11,12) and branched-chain carnitine acyltransferase activities (11,13-15) in mammalian tissues indicates that carnitine may be involved in metabolism of the carbon skeletons of branched-chain amino acids. Solberg *et al.* (16) have shown conversion of branched-chain α-keto acids to branched-chain acylcarnitines by mitochondria of various rat tissues. Isobutyrylcarnitine is metabolized by isolated beef and rat liver mitochondria (17).

A role for carnitine in branched-chain amino acid metabolism would be particularly likely if some of the branched-chain acyl-CoA's were formed outside of the matrix of mitochondria. The existence of glyoxylate:

leucine aminotransferase (18), carnitine acetyltransferase (19) and carnitine isobutyryl transferase (13) activities in peroxisomes indicates that the metabolism of the branched-chain acyl residues may occur in more than one cellular compartment. In order to investigate this possibility, the subcellular distribution of the branched-chain α-keto acid decarboxylating activities in rat liver was determined.

The data presented herein demonstrates the presence of at least two branched-chain α-keto acid decarboxylating activities in rat liver: a mitochondrial activity which decarboxylates all three branched-chain α-keto acids, α-ketoisocaproate, α-keto-β-methylvalerate and α-ketoisovalerate and a soluble enzyme which decarboxylates only α-ketoisocaproate and α-keto-γ-methiolbutyrate.

The mitochondrial α-keto acid decarboxylating activity is due to the mitochondrial branched-chain α-keto acid dehydrogenase. This enzyme requires both CoA and $NAD^+$ as cofactors. Recently, this enzyme has been the subject of numerous investigations since it appears to be highly regulated and the rate limiting step of branched-chain amino acid metabolism in most tissues.

The cytosolic α-ketoisocaproate decarboxylating activity is different from the mitochondrial branched-chain α-keto acid dehydrogenase. The cytosolic enzyme does not require CoA or $NAD^+$. Although there are previous indications of a soluble α-ketoisocaproate decarboxylase activity (1,5,7-9), a detailed characterization of this activity is lacking.

The data presented herein shows that the cytosolic enzyme is an oxidase which converts α-ketoisocaproate to β-hydroxyisovalerate. The purification of this enzyme and the determination of some of its physical and kinetic properties are also presented. Speculation concerning the

possible role(s) of this enzyme in branched-chain amino acid metabolism is covered in the discussion.

# LITERATURE REVIEW

## I. Metabolism of the Branched-Chain Amino Acids

### A. Metabolic Pathway

The catabolism of leucine, isoleucine and valine occur via very similar pathways. The amino acid is first transaminated producing the α-keto acid. The α-keto acid is then decarboxylated and converted to the corresponding acyl-CoA by a mitochondrial branched-chain α-keto acid dehydrogenase. Further metabolism of these acyl-CoA groups occurs inside the mitochondria. Leucine is ketogenic since it is metabolized to acetyl-CoA and acetoacetate. Isoleucine is only slightly ketogenic due to acetyl-CoA formation. Both isoleucine and valine are gluconeogenic since they can be metabolized to intermediates of the tricarboxylic acid (TCA) cycle. β-Hydroxy-β-methylglutaryl-CoA, formed during leucine catabolism, may also be used in sterol synthesis (20,21).

Transamination of leucine, isoleucine and valine is catalyzed by a branched-chain amino acid transaminase. Ichihara et al. (22-28) have isolated three isozymes of this enzyme. Isozyme I uses all three branched-chain amino acids (BCAA) as substrates and has been found in several tissues of rat and hog. Isozyme II uses only leucine and methionine as substrates and has only been found in rodent liver. Isozyme III uses all three BCAA as

substrates and has only been found in brain, ovary and placenta (29).

α-Ketoglutarate is the primary acceptor of amino groups from the BCAA. Pyruvate and oxaloacetate are not utilized by the BCAA transaminase purified from hog heart and brain or from rat liver (24,30,31). When rat diaphragm, atria or epididymal fat pad slices are incubated with leucine, production of alanine and glutamate plus glutamine increases (32-34). However, the transaminase in rat skeletal muscle has a much greater affinity for α-ketoglutarate ($K_m$ = 0.10 mM) than for pyruvate ($K_m$ = 3.4 mM) (35). Therefore, production of alanine is probably due to transamination of glutamate with pyruvate. The branched-chain α-keto acids, α-ketoisocaproate (α-KIC), α-ketoisovalerate and α-keto-β-methylvalerate can also serve as amino group acceptors for the BCAA (31).

The branched-chain α-keto acids (BCKA) can be transported into the mitochondria where they are decarboxylated and converted to acyl-CoA's by the mitochondrial BCKA dehydrogenase. This enzyme has been purified from rat liver (3) and from bovine liver and kidney (2,36). It uses all three BCKA as substrates, requires both CoA and $NAD^+$ as cofactors, and appears to operate by a mechanism similar to that of pyruvate dehydrogenase.

Further metabolism of the acyl CoA's produced by the BCKA dehydrogenase occurs in the mitochondria via a pathway very similar to β-oxidation. Little is known about the enzymes involved in this pathway. Recently, a specific isovaleryl-CoA dehydrogenase has been identified and isolated from pig and rat liver

(37-39). This enzyme is different from the butyryl-CoA or general acyl-CoA dehydrogenases involved in β-oxidation. This enzyme, therefore, may be specific for metabolism of isovaleryl-CoA, a metabolite of leucine. Isobutyryl-CoA, a metabolite of valine, is not a substrate for this isovaleryl-CoA dehydrogenase.

B. Regulation and Interorgan Distribution of Branched-Chain Amino Acid Metabolism

The metabolism of the BCAA has been studied in a variety of mammalian tissues both in vivo and in vitro. Most amino acids are taken up and metabolized by the liver (40), however, the BCAA are a notable exception, showing very little uptake by or net transport from the liver (41). Leucine, isoleucine and valine, however, are rapidly taken up and oxidized by skeletal muscle (42,43).

The initial transamination of BCAA occurs mainly in extrahepatic tissues (26,44,45). The tissue distribution of this enzyme has been done in rat. Stomach and pancreas contain the highest transaminase activity (46) followed by kidney, heart, brain, diaphragm, testes and skeletal muscle. Liver has very low transaminase activity (26,44,45). On the other hand, the highest specific activity of the BCKA dehydrogenase in rat, monkey, guinea pig and man occurs in liver and kidney (45,47,48). Skeletal muscle has very little BCKA dehydrogenase activity. These data suggest that the BCAA may be transaminated by peripheral tissues and the resulting α-keto acids transported to the liver for further metabolism.

Although the BCKA dehydrogenase is apparently low in muscle tissue, Goldberg et al. (49) have suggested that this is the major site for leucine catabolism, due to the large total mass of muscle, and the low transaminase activity in liver. Indeed, skeletal muscle is capable of oxidizing leucine completely to $CO_2$ (50,51). However, when rat atria or skeletal muscle are incubated with leucine, approximately 20-40% of the α-KIC produced is released into the media (35,45,50,52,53). The BCKA dehydrogenase, therefore, is probably the rate limiting step in atria and skeletal muscle.

Livesey et al. determined arterio-venous differences of the branched-chain α-keto acids in tissues of both rat (54) and man (55) after protein ingestion or leucine infusion. In both cases there was a net release of the BCKA by muscle and a net uptake by liver. Noda and Ichihara (56) reported a significant depression (14-60%) of total leucine oxidation in rats when the liver or kidney vessels were ligated. These results are again consistent with the involvement of both muscle and liver in BCAA metabolism.

Recent investigations have revealed that the BCKA dehydrogenase is highly regulated (57-67) most likely via a phosphorylation/dephosphorylation mechanism (59,61,62,66). Under the proper assay conditions, the BCKA dehydrogenase activity of heart or muscle can be increased as much as 10 fold over basal levels (60,67). Therefore, previous estimates of the BCKA dehydrogenase activity in various tissues may have been carried out at

suboptimal conditions and must be reinvestigated under both physiological and optimal conditions.

## II. Effect of Carnitine on Branched-Chain Amino Acid Metabolism

Carnitine stimulates the oxidation of BCAA in rat skeletal muscle (68) and heart (69) but not in liver (69,70). The lack of effect of carnitine on the oxidation of BCAA by liver appears to be due to the low level of BCAA transaminase in this tissue. When α-KIC is used as a substrate, carnitine does stimulate oxidation by liver homogenates (70). Van Hinsbergh et al. (71) and May et al. (72) have shown stimulation of BCKA oxidation by carnitine in rat liver, heart, skeletal muscle, and kidney. The effect of carnitine, therefore, must be at or distal to the BCKA dehydrogenase step.

Solberg and Bremer (73) have shown that when various rat or mouse tissues are incubated with the BCKA and L-[methyl-$^{3}$H]carnitine, branched-chain acyl [$^{3}$H-methyl] carnitines are formed. Branched-chain acylcarnitines as well as branched-chain carnitine acyltransferase activities have also been identified in many tissues of beef and rat (11-15,17). Since the branched-chain acyl-CoA's formed by the BCKA dehydrogenase can cause product inhibition of this enzyme (74), carnitine may stimulate BCAA metabolism by removing these acyl-CoA groups.

Two hypotheses were proposed to account for the stimulatory effect of carnitine. The first, proposed by Bieber and Choi (12) is that carnitine may be involved in shuttling of acyl-CoA's produced by the BCKA dehydrogenase across an intracellular permeability barrier for further metabolism. The second hypothesis, proposed by Van

Hinsbergh et al. (69), is that carnitine removes inhibitory branched-chain acyl-CoA groups from the mitochondria and maintains free CoA levels. Both of these effects would favor activation of the BCKA dehydrogenase.

The first hypothesis would be supported if any of the BCKA dehydrogenase were located outside of the mitochondria or even located on the outside of the inner mitochondrial membrane. Indeed, several investigators have reported evidence of extramitochondrial BCKA decarboxylase or oxidase activities (1,5-8). In addition the data of Johnson and Connelly (5) suggests that the BCKA dehydrogenase is located on the outside of the inner mitochondrial membrane.

Data presented herein, however, demonstrates that only one BCKA dehydrogenase could be detected in rat liver and that this was exclusively mitochondrial. Extramitochondrial α-keto acid decarboxylating activities detected by other investigators (1,5-8) may be due to an oxidase similar to the α-ketoisocaproate oxidase described in this paper. Furthermore, Bremer and Davis (75) have shown that the BCKA dehydrogenase must be located on the inside of the inner mitochondrial membrane since addition of CoA and $NAD^+$ are not required for optimal activity in intact mitochondria. Previous experiments by Johnson and Connelly (5) utilized a ferricyanide assay which is quite insensitive and subject to interfering reactions (76).

In light of these results, the second hypothesis seems more attractive. Studies in rat heart (75), liver (72) and skeletal muscle (71) indicate that carnitine stimulates the BCKA dehydrogenase by removing inhibitory branched-chain acyl-CoA's. The addition of isovalerylcarnitine decreases the oxidation of BCKA apparently due to

build up of intramitochondrial isovaleryl-CoA (71-72). Thus carnitine apparently stimulates BCAA metabolism by decreasing intramitochondrial acyl-CoA's and increasing the CoA/acyl-CoA ratio as well as the availability of free CoASH. These affects would favor "activation" of the BCKA dehydrogenase, which is believed to be the rate limiting step in BCAA metabolism.

## III. Specific Effects of Leucine on Metabolism

Leucine and/or its metabolites affect several metabolic processes which are not affected by isoleucine or valine. These include protein synthesis and degradation, insulin secretion, and glucose and pyruvate oxidation. The α-KIC oxidase described herein uses the α-keto analogue of leucine as a substrate, but does not use the α-keto analogues of isoleucine and valine. Therefore, this enzyme may be important in regulation of leucine levels separately from isoleucine or valine. In this section, therefore, I will describe the recent research concerning the specific metabolic effects of leucine.

### A. Effect of Leucine on Protein Turnover

Fulks, Li and Goldberg (77) found that all three BCAA together, leucine alone, or a combination of leucine and isoleucine stimulated protein synthesis and inhibited protein breakdown in rat diaphragm. Buse et al. (78), however, saw no effect of valine or isoleucine alone on rat diaphragm. In heart, both leucine and its metabolites stimulate protein synthesis and inhibit protein degradation (79). In gastrocnemius muscle, however, only

leucine was effective; α-KIC, isovalerate and acetate had no effect. Isoleucine and valine together had no effect on protein turnover in either tissue (79). These results must be interpreted with caution since uptake of leucine metabolites may differ in heart vs. gastrocnemius muscle.

The exact mechanism of leucine's effect on protein turnover is unknown. Goldberg and Tischler (80) found that cycloserine, an inhibitor of leucine transamination, prevents the inhibition of protein breakdown by leucine without affecting the stimulatory effect of leucine on protein synthesis. Therefore, leucine itself may regulate protein synthesis. Morgan et al. (79) found levels of protein synthesis to be approximately proportional to intracellular leucine concentrations in heart and skeletal muscle. Their studies indicate that leucine facilitates peptide chain initiation as measured by a decrease in ribosomal subunits.

In order to exert its inhibitory effect on protein degradation, leucine must be metabolized. As mentioned previously, inhibition of leucine transamination prevents leucine's inhibitory effect on protein degradation. α-KIC mimics the effects of leucine on protein degradation in rat diaphragm and heart (79,80).

The catabolism of muscle protein is a serious problem in patients with sepsis or trauma. Administration of a mixture of BCAA has been found to be useful in preventing muscle wasting and improving nitrogen balance in some of these patients (81).

B. Effect of Leucine on Insulin Secretion

Leucine and α-KIC stimulate insulin secretion by rat pancreatic islets (82-84). Isoleucine, valine and their respective α-keto acids, however, have no effect (82,85). It appears that leucine must be converted to α-KIC in order to stimulate insulin secretion (82) but the mechanism of action is unknown. It should be kept in mind that in all of these studies, leucine or α-KIC levels are above normal physiological values. Approximately 5 mM α-KIC (82,84,86) or leucine (83) is required for significant stimulation of insulin secretion. Normal plasma levels of leucine vary between 0.1 to 0.5 mM and BCKA levels normally do not exceed 0.08 mM (87).

C. Effect of Leucine on Glucose and Pyruvate Metabolism

Leucine inhibits formation of $^{14}CO_2$ from D-[U-$^{14}$C] glucose and inhibits pyruvate oxidation in the fasted rat diaphragm and heart at concentrations between 0.2 and 1.2 mM (50,88). At 0.5 mM, isoleucine and valine had no effect on pyruvate oxidation in these tissues. At higher concentrations, the α-keto derivatives of both leucine and valine inhibit the oxidation of pyruvate and/or α-ketoglutarate in a variety of tissues (89-91).

Aminooxyacetate, an inhibitor of the BCAA transaminase, abolishes leucine's ability to inhibit pyruvate oxidation. α-KIC, however, inhibits pyruvate oxidation in the presence or absence of aminooxyacetate (88). Thus, metabolism of leucine is required for its effect on pyruvate oxidation.

Using a perfused rat heart system, Waymack et al. (60) have shown that 0.5 mM α-KIC causes considerable inactivation of the pyruvate dehydrogenase while activating the BCKA dehydrogenase. The addition of 0.25 mM pyruvate to the perfusate caused appreciable inhibition of the BCKA dehydrogenase and activated the pyruvate dehydrogenase. Thus, these two dehydrogenases appear to be regulated in an independent and reciprocal manner.

## IV. Metabolic Diseases Associated with Branched-Chain Amino Acid Metabolism

Several diseases associated with defects in branched-chain amino acid metabolism have been recognized. These are characterized by the accumulation of one or more of the BCAA and/or their metabolites in blood or urine.

Maple syrup urine disease is a rare (but often misdiagnosed) disease which occurs in early infancy. This disease is characterized by a distinct maple syrup-like odor in the sweat and urine of the child and the accumulation of the BCAA and BCKA in urine and serum (92). This disease leads to mental retardation and usually death within a year. It is unknown whether the BCAA or BCKA are responsible for the clinical abnormalities. α-Ketoisocaproate and α-ketoisovalerate inhibit pyruvate and/or α-ketoglutarate oxidation by mitochondria isolated from rat brain, liver, kidney and heart (89-91). Leucine is known to stimulate insulin secretion and may be responsible for the hypoglycemia seen in these patients. Snyderman (93) has noticed that in all of the maple syrup urine disease patients they studied, leucine was elevated to a much greater degree than the other

branched-chain amino acids. Of the three branched-chain amino acids, leucine and its metabolites appear to be mainly responsible for the observed neurological symptoms (94), hypoglycemia (95) and inhibition of gluconeogenesis (96) seen in untreated cases.

Isovaleric acidemia has been reported in several children (97-99). This disease is characterized by a "cheesy" odor to the breath and body fluids (92) and recurrent episodes of vomiting, acidosis and coma occurring after excessive protein intake or infection. Fibroblasts from a patient showed a deficiency in the isovaleryl-CoA dehydrogenase (100,101) and the accumulation of isovaleric acid and its glycine conjugate in the blood and urine (102). Interestingly, leucine and α-ketoisocaproate do not accumulate (92). This may be due, at least partly, to a loss of regulation and concomitant increase in activity of the BCKA dehydrogenase (103). The isovaleryl-CoA dehydrogenase activity has been shown to be due to a specific enzyme which can be separated from the butyryl-CoA and general acyl-CoA dehydrogenase (37). This enzyme does not use isobutyryl-CoA, a metabolite of valine, as a substrate. Therefore, the metabolic block associated with this disease impairs leucine metabolism without directly affecting isoleucine or valine metabolism.

Other metabolites also have been found to accumulate in patients during isovaleric acidemic episodes. Tanaka et al. (104) detected grossly elevated levels of lactate, acetoacetate, β-hydroxybutyrate and β-hydroxyisovalerate in urine of these patients.

Other metabolites of the branched-chain amino acids have also been found to accumulate in certain metabolic disorders. These disorders are named by the metabolite that accumulates, for example,

β-methylcrotonylglycinuria, propionic acidemia and methylmalonic acidemia (105). In β-methylcrotonylglycinuria, the defect appears to be at the β-methylcrotonyl-CoA carboxylase, which can be due to either a defective enzyme (106) or a biotin deficiency (107). In one patient, with no β-methylcrotonyl-CoA carboxylase activity, large amounts of α-ketoglutarate and β-hydroxyisovalerate as well as β-methylcrotonylglycine were excreted in the urine (106), but no ketosis was present. In a biotin responsive patient, however, β-hydroxybutyric acid, β-hydroxyisovaleric acid, lactic acid, β-hydroxyisobutyric acid and β-hydroxypropionic acid were all elevated along with β-methylcrotonylglycine (105). This is due to the biotin requirement of propionyl-CoA carboxylase and pyruvate carboxylase as well as that of β-methylcrotonyl-CoA carboxylase.

Metabolic disorders of branched-chain amino acid metabolism may also be secondary to other abnormalities. Landaas (108,109) has shown increased levels of β-hydroxyisovaleric acid, β-hydroxyisobutyric acid and 2-methyl-3-hydroxybutyric acid in patients with ketoacidosis from a variety of causes. These β-hydroxy acids are formed from leucine, valine and isoleucine, respectively. A positive correlation was found between urinary levels of β-hydroxybutyric acid and β-hydroxyisovaleric acid (108). Increased levels of all three branched-chain amino acids are also seen in ketoacidotic patients (108,110). The mechanism by which branched-chain amino acids and their β-hydroxy metabolites are elevated is unknown.

V. Evidence for the Existence of an Extramitochondrial Branched-Chain α-Keto Acid Decarboxylase Activity.

The existence of a single mitochondrial BCKA dehydrogenase which utilizes the α-keto acids of leucine, isoleucine and valine as substrates is now widely accepted. However, dehydrogenases or decarboxylases, which use only one or two of the BCKA as substrates, have been reported (5-10).

Wohlhueter and Harper (1) first reported decarboxylation of α-[1-$^{14}$C] KIC by a soluble preparation of rat liver. They recognized that this activity was not due to "leaked" mitochondrial BCKA dehydrogenase, since deliberate attempts to release the enzyme from mitochondria were unsuccessful. Under their assay conditions, only 8% of the total α-[1-$^{14}$C] ketoisocaproate decarboxylating activity was soluble, the rest was mitochondrial.

In bovine liver, Johnson and Connelly (5) identified a small but significant amount of α-[1-$^{14}$C] KIC and α-[1-$^{14}$C] keto-β-methylvalerate decarboxylating activity in the soluble fraction. α-Ketoisovalerate was not decarboxylated by this fraction. This activity did not require the addition of CoA. However, they did not eliminate the possibility of endogenous CoA in their preparation.

Grant and Connelly (7) have shown that α-KIC, but not α-ketoisovalerate or α-keto-β-methylvalerate, is decarboxylated by cytosolic preparations from liver and kidney of mouse, rat, rabbit, guinea pig and beef and from the liver only of chicken. This decarboxylase activity did not require added CoA or $NAD^+$ and showed very little activity with pyruvate, α-ketoglutarate, α-ketoisovalerate, or α-keto-β-methylvalerate as substrates (8).

An α-ketoisocaproate decarboxylase activity has also been isolated from beef liver (9). This activity does not require CoA and $NAD^+$ as substrates and does not use the other two BCKA as substrates. This activity is also insensitive to arsenite, an inhibitor of the mitochondrial BCKA dehydrogenase. Properties of this enzyme are very similar to those of the α-ketoisocaproate oxidase described herein.

The cytosolic α-ketoisocaproate decarboxylating activity appears to be widely distributed among mammals (7). Previous studies indicate that this activity is quite low in comparison to the mitochondrial BCKA dehydrogenase (1,5). However, cytosolic decarboxylation of α-ketoisocaproate has been shown to increase under conditions which cause high circulating levels of the branched-chain amino acids, for example, high protein meal (111) or diabetes (112). This enzyme activity therefore may be important in BCAA metabolism when serum or tissue levels of the BCAA are elevated.

## MATERIALS

The materials used in this study are listed below with the source and catalog number. All other chemicals used were of analytical reagent grade or the finest commercially available.

DEAE (diethylaminoethyl)-cellulose, Whatman DE52, reeve angel, Clifton, NJ

[1-$^{14}$C] sodium isovalerate, ICN Pharmaceuticals Inc., Irvine, CA (Cat. No. 12128)

L-[1-$^{14}$C] Leucine, ICN Pharmaceuticals Inc., Irvine, CA (Cat. No. 10088) or New England Nuclear, Boston, MA (Cat. No. NEC-169)

L-[U-$^{14}$C] Leucine, ICN Pharmaceuticals Inc., Irvine, CA (Cat. No. 10089)

L-[4,5-$^{3}$H] Leucine, ICN Pharmaceuticals Inc., Irvine, CA (Cat. No. 20036) or Schwarz/Mann, Orangeburg, NY (Cat. No. 0332-313344)

L-[1-$^{14}$C] Methionine, Amersham Corp., Arlington Heights, IL (Cat. No. CFA 433)

Oxygen-$^{18}O_2$, Merck and Company Inc., Rahway, NJ (Cat. No. MO-1454) 90 atom % O-18, Lot No. 55-F

Phenyl-sepharose CL-4B, Sigma, St. Louis, MO (Cat. No. P-7892)

Sephacryl S-200 Superfine, Pharmacia, Piscataway, NJ (Cat. No. 17-0871-01)

Sephadex G-150, Pharmacia, Piscataway, NJ (Cat. No. 17-0070-01)

L-[1-$^{14}$C] Valine, New England Nuclear, Boston, MA (Cat. No. NEC-171)

Water-$^{18}$O (normalized) Merck and Company Inc., Rahway, NJ (Cat. No. MO-1670) 97 atom % $^{18}$O, Lot No. 2164-E

# METHODS

## I. Sucrose Gradient Separation of Rat Liver Organelles

Male Sprague-Dawley rats, 300 gm, were fasted 2 days and then sacrificed by decapitation. Subcellular organelles from liver were separated by isopycnic density centrifugation as previously described (113) except that a 1 to 10 (instead of 1 to 20) homogenate of rat liver was used and the buffer for both the grinding medium and the sucrose gradient was 1 mM sodium phosphate, pH 7.5. Organelles were separated on a 600 mL zonal gradient (Figure 1B) or a 60 mL tube gradient (Figure 1A). The zonal gradient was identical to the 60 mL tube gradient except that the volume of all the sucrose solutions and amount of rat liver homogenate applied to the gradient were increased 10 times. Fractions were collected and assayed.

Aliquots for assay of α-KIC decarboxylase activity were stored at -80°C until assayed. α-Ketoisovalerate decarboxylase activity was measured on aliquots that were stored overnight at 4°C. Catalase (114), fumarase (115) or glutamate dehydrogenase (113), and NADPH-cytochrome c reductase (116), the marker enzymes for peroxisomes, mitochondria and microsomes, respectively, were assayed by the cited methods with the following modifications. To rupture the organelles, 0.1% Triton X-100 was added to catalase and fumarase assays. The assay of NADPH-cytochrome c reductase included 0.01 mM rotenone and 0.68 mM sodium azide to inhibit mitochondrial electron transport.

II. Preparation of Radioactively Labeled α-Keto Acids

$^{14}C$ or $^{3}H$ labeled α-keto acids were prepared from the correspondingly labeled L-amino acids and purity determined according to the method of Rudiger et al. (117). Solutions of 1-$^{14}C$-labeled α-keto acids were made up to the desired concentration by addition of unlabeled α-keto acids and stored at -80°C.

α-[4,5-$^{3}H$] KIC was purified prior to use to remove contaminants which eluted in the water wash and with isovaleric acid from Dowex-1 chloride columns. The contaminants amounted to approximately 20% of the total cpm. To purify α-[4,5-$^{3}H$] KIC, peak fractions from the Dowex-$H^{+}$ column (117) containing α-[4,5-$^{3}H$] KIC were neutralized and applied to a 0.5 x 4.5 cm Dowex-1 chloride (100-200 mesh) column. The column was washed with water followed by a 200 mL linear gradient of 0-0.2 N HCl and 5 mL fractions were collected. Fractions containing only α-[4,5-$^{3}H$] KIC were pooled, pH adjusted to 5.0 and lyophilized. In order to remove the large amount of salt present, the residue was resuspended in 0.02 N HCl, saturated with NaCl, and extracted with 1-octanol. Ether was not used for extraction because much of the α-KIC was decarboxylated, probably due to contaminating peroxides in the ether. The octanol layer was re-extracted with water and adjusted to pH 6. The aqueous layer containing the α-[4,5-$^{3}H$] KIC, showed only one peak when rechromatographed on the Dowex-1 chloride column.

No contaminating substances were detected when α-[U-$^{14}C$] KIC was chromatographed on Dowex-1 chloride columns. This compound was therefore used without further purification.

## III. Assay of α-Ketoisocaproate Oxidase Activity

### A. Assay of α-[1-$^{14}$C] Keto Acid Decarboxylating Activities

Assays for determination of α-keto acid decarboxylating activities were carried out in 1.5 cm diameter culture tubes, which were tightly stoppered with a serum cap. A plastic cup (Kontes) hanging from the serum cap contained 0.2 mL Hyamine (methylbenzethonium hydroxide) to trap $^{14}CO_2$ released during the reaction. The reaction conditions were varied throughout this work in order to establish optimal conditions for the assay. Two sets of reaction conditions were widely used and are described below. Decarboxylase assays using other conditions are described in the figure and table legends.

Method A:

Reaction mixture A contained in a final volume of 0.4 mL; 0.2 M Tris base, 0.2 M maleic acid, pH adjusted to 6.5 with NaOH, 1.5 M ammonium sulfate, 1.0 mM $Na_2CO_3$, 1.0 mM α-[1-$^{14}$C] KIC (or other α-keto acid) (50-100 dpm/nmole), and 25 to 100 μL of rat liver cytosol preparation or partially purified α-KIC oxidase. This reaction mixture was used for initial studies of the α-KIC oxidase in crude rat liver preparations and in initial attempts at purification. All components of the reaction mixture except the enzyme and α-[1-$^{14}$C] keto acid were equilibrated at 25°C. The enzyme was then added to all assays approximately 8-15 min before addition of the α-[1-$^{14}$C] keto acid.

All reactions were initiated by the addition of the α-[1-$^{14}$C] keto acid. In some cases the α-keto acid was injected through the serum cap with a syringe. However, usually the α-keto acid was added directly to the reaction mix and then the tube was quickly stoppered with a serum cap. A cup hanging from this cap had previously been filled with 0.2 mL Hyamine.

Incubations were for 60 min (unless noted otherwise) in a 25°C shaking water bath. To terminate the reactions, 0.2 mL of 20% trichloroacetic acid was added and an additional hour with shaking was allowed for collection of $^{14}CO_2$. The cup plus Hyamine was put into a scintillation vial and counted in 10 mL of scintillation fluid (5). Specific activity was determined by releasing all of the $^{14}CO_2$ from the α-[1-$^{14}$C] keto acid using ceric sulfate (118). Nonenzymatic decarboxylation of the α-keto acids was determined by replacing the rat liver preparation with buffer in the assay. The blank value determined in this way was the same as when boiled enzyme preparations were used and is subtracted from all values reported herein.

Method B:

Reaction mixture B contained in a final volume of 0.4 mL; 0.2 M Tris base, 0.2 M maleic acid, pH adjusted to 6.5 with NaOH, 1.0 mM $FeSO_4$, 0.5 mM ascorbic acid, 1.0 mM dithiothreitol, 1.0 mM α-[1-$^{14}$C] KIC (or other α-keto acid) (approximately 100 dpm/nmole), and 5 to 100 μL of the sample to be assayed. A stock solution containing 16 mM $FeSO_4$, 8 mM ascorbic acid, and 16 mM dithiothreitol (cofactor mix) was prepared fresh daily. Addition

of 25 µL of this cofactor mix per assay gave the desired final concentrations of $FeSO_4$, ascorbic acid and dithiothreitol (1.0 mM, 0.5 mM, 1.0 mM). The reaction was preincubated for 1 hour at 25°C (unless noted otherwise) with all components excluding the α-[1-$^{14}$C] keto acid. The α-[1-$^{14}$C] keto acid was then added to initiate the reaction and the assay continued as in Method A.

B. Measurement of Oxygen Consumption in Presence of α-Keto Acids

The reaction mix contained in a final volume of 3.0 mL; 0.2 M Tris, 0.2 M maleate, pH 6.5, 1.5 M ammonium sulfate, 1.0 mM $Na_2CO_3$ and 0.5 mL of rat liver cytosol preparation (see below). Oxygen consumption was measured with a Yellow Springs Instruments oxygen monitor. After establishing a basal rate of $O_2$ consumption the α-keto acid was added to give a final concentration of 1 mM and the change in the rate of $O_2$ consumption was recorded. All reactions were carried out at 25°C.

Oxygen solubility in the reaction mix was determined by the method of Robinson and Cooper (119). A value of 96 ± 10 nmol $O_2$/mL was found. This value was used for all polarographic measurements.

IV. Isolation of Rat Liver Cytosol Preparations

Male Sprague-Dawley rats, 150-200 g, were fasted for 24-48 h and then sacrificed. The livers were homogenized using a Potter-Elvehjem glass homogenizer in 3 vol of 0.25 M sucrose buffer (0.25 M sucrose in 2.5 mM Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic

acid], pH 7.4, 0.25 mM EDTA). The homogenate was centrifuged 12 min at 500 x g and the pellet discarded. The 500 x g supernatant fluid was centrifuged 12 min at 20,200 x g. The 20,200 x g supernatant fluid was centrifuged 1.5 h at 70,000 x g. The supernatant fluid was decanted and the volume reduced by ultrafiltration. The concentrated fluid was dialyzed for 4 h against several changes of 33 mM potassium phosphate buffer, pH 7.4. This preparation is referred to as rat liver cytosol and was stored at -80°C. Protein was determined by the Lowry method (120) as previously modified (121).

V. Purification of Rat Liver Cytosolic α-Ketoisocaproate Oxidase

Several purifications of the rat liver α-ketoisocaproate oxidase were carried out. Those which are pertinent to this work are described below. Protein was determined in purification fractions using the method of Bradford (122). α-KIC oxidase activity was monitored by decarboxylation of α-[1-$^{14}$C] KIC as previously described. All purification steps were carried out at 4°C unless noted otherwise.

A. Purification A: Partial Purification of Rat Liver Cytosolic α-Ketoisocaproate Oxidase

Sprague-Dawley rats were decapitated and the livers removed and homogenized with a Potter-Elvehjem glass homogenizer in 5-10 vol of 0.25 M sucrose. The homogenate was centrifuged 12 min at 500 x g and 12 min at 20,000 x g and the supernatant saved. This was stored at -80°C until used. The 20,000 x g supernatant fraction was thawed and centrifuged at 70,000 x g for 1.5 h. Lipids

were aspirated from the top of the sample and the supernatant fluid was saved. This "70,000 x g supernatant" fraction was stored at -80°C overnight. After thawing, one-tenth volume of cold 2% protamine sulfate was slowly added while stirring at 4°C. After stirring for 15 min, the preparation was centrifuged for 18 min at 13,000 x g. The "0.2% protamine sulfate supernatant" was brought to 35% saturation with the slow addition of powdered ammonium sulfate, stirred for 15 min at 4°C, and centrifuged 18 min at 13,000 x g. The "35% $(NH_4)_2SO_4$ supernatant" was stored at -80°C.

The α-KIC oxidase activity was stable at -80°C for several weeks, but 80% of the activity was lost in 4 days at 4°C. One percent isopropanol completely stabilized the activity in the "35% $(NH_4)_2SO_4$ supernatant fraction" for at least 1 week at 4°C. Up to 1% isopropanol in the assay reaction mix did not affect the α-KIC oxidase activity.

The frozen "35% $(NH_4)_2SO_4$ supernatant" fraction was thawed and dialyzed against 10 liters of 20 mM Tris-HCl, pH 7.8, 1% isopropanol for 48 h, with two changes of buffer. The dialysate was centrifuged 30 min at 13,000 x g to remove precipitated material. The supernatant, "pre-DEAE dialysate" was stored for 4 days at -80°C and then applied to a 4 x 40-cm DEAE-cellulose (Whatman DE52) column, equilibrated with 20 mM Tris-HCl, pH 7.8, 1% isopropanol (Buffer A). This was washed with 2 liters of the equilibration buffer followed by a 2-liter linear gradient of 0 to 0.5 M NaCl in Buffer A. The α-KIC oxidase activity eluted at approximately 0.8 M NaCl and peak fractions were pooled ("DEAE-

cellulose pool"). This was stored 4 days at -80°C and then concentrated to 9.8 mL with an Amicon PM 30 ultrafiltration filter ("concentrated DEAE pool"). The concentrated DEAE pool was centrifuged at 13,000 x g for 10 min to remove precipitated material and applied to a 4.8 x 82-cm Sephadex G150 (40-120 μm) column which was equilibrated with Buffer A containing 0.2 M NaCl. The column was eluted with the same buffer at a flow rate of approximately 40 mL per hour and 9.4-mL fractions were collected. The α-KIC oxidase activity was retained by the column and eluted after catalase activity. The peak fractions were pooled and stored at -80°C. After thawing, the sample was concentrated to 13.6 mL with an Amicon PM 30 filter and again stored at -80°C ("Sephadex G-150 pool").

B. Purification B: Final Purification of α-Ketoisocaproate Oxidase

The 10,000 x g supernatant fractions (in 0.25 M sucrose, 1% isopropanol) from a number of rat liver preparations were stored at -80°C. These are stable for at least two years.

Powdered $(NH_4)_2SO_4$ was slowly added to 7.2 L of the 10,000 x g supernatant fraction while stirring until 45% of saturation was achieved. After stirring for an additional 30 min, the preparation was centrifuged 30 min at 10,000 x g. The "45% $(NH_4)_2SO_4$ supernatant" was brought to 75% saturation with the slow addition of powdered $(NH_4)_2SO_4$, stirred an additional 30 min, and centrifuged 30 min at 10,000 x g. The pellet (45-75% $(NH_4)_2SO_4$ fraction) was resuspended in Buffer A. This fraction was then dialyzed 60 hrs against 10 L of Buffer A,

with 5 changes of buffer and centrifuged 15 min at 8,000 x g to remove precipitated material (Pre-DEAE dialysate).

The "Pre-DEAE dialysate" was applied to a 4.8 x 83 cm DEAE cellulose (Whatman DE52) column, equilibrated with Buffer A. This was eluted with 2.5 L of Buffer A followed by a 9 L linear gradient of 0-0.2 M NaCl in Buffer A. The α-KIC oxidase activity eluted at approximately 0.06 M NaCl and fractions containing activity were pooled ("DEAE cellulose pool"), concentrated to 250 mL using an Amicon PM 10 ultrafiltration membrane ("concentrated DEAE pool"), and stored at -80°C.

In previous purifications (i.e.: Purification A) large losses of α-KIC oxidase activity occurred when using DEAE, phenyl sepharose and sephacryl columns during later stages of the purification. In passage over Sephacryl S-200 only 10% of the applied activity was recovered. Protein containing fractions from the NaCl gradient elution of the initial DEAE column, which eluted before and after the peak of α-KIC oxidase activity, were pooled ("DEAE side fractions"). When columns were pretreated with the "DEAE side fractions", 90% of the applied α-KIC oxidase activity was recovered. Therefore, all columns (except the first DEAE column) were pre-treated with the "DEAE side fractions" and then washed extensively with the elution buffer until no more protein was detected in the elluent.

Stability studies (see Results) also demonstrated that 5% monothioglycerol stabilized the α-KIC oxidase activity. Subsequently, 5% monothioglycerol was included in all buffers.

A portion of the "concentrated DEAE pool" (116 mL) was adjusted to 5% monothioglycerol and 2.5 M NaCl by slowly adding these compounds while stirring (Pre-phenyl sepharose). This was applied to a 4.0 x 40 cm phenyl sepharose CL-4B column (Pharmacia) which had been pretreated with 1.5 L Buffer A, 1.0 L of Buffer A containing 5% monothioglycerol, 2.5 M NaCl, 1.8 L of "DEAE side fractions" containing 2.5 M NaCl, 2 L Buffer A, and equilibrated with 1.5 L Buffer A containing 5% monothioglycerol, 2.5 M NaCl. The α-KIC oxidase activity was eluted with 1.5 L Buffer A containing 5% monothioglycerol, 2.5 M NaCl, followed by a 2 L linear gradient of 2.5-0 M NaCl in Buffer A containing 5% monothioglycerol. Fractions containing α-KIC oxidase activity were pooled and concentrated to 20 mL (Phenyl pool concentrate).

The "phenyl pool concentrate" was then applied to a 4.8 x 82 cm Sephacryl S-200 column which had been pretreated with "DEAE side fractions" and equilibrated with Buffer A containing 5% monothioglycerol, 0.1 M NaCl. The column was eluted with the equilibration buffer at a flow rate of 13.6 ml/hr. Fractions containing α-KIC oxidase activity were pooled (Sephacryl S-200 pool) and stored at -80°C.

Since monothioglycerol at concentrations greater than 0.6% inhibits the α-KIC oxidase activity (see Results), only small aliquots (10-20 μL) of the Sephacryl S-200 pool could be assayed accurately. In some experiments requiring larger amounts of this enzyme, the monothioglycerol was removed from the Sephacryl S-200 pool. This was accomplished by passing 0.5 mL of the Sephacryl S-200 pool over a 0.75 x 14 cm Bio Gel P-6 (50-100 mesh) column

which was pretreated with "DEAE side fractions" and equilibrated with Buffer A containing 0.1 M NaCl. Protein was monitored by adding 50 µL of Coomassie Blue Reagent (122) to 10 µL of each fraction and visually checking for blue color. To monitor monothioglycerol, 1 mL of 0.3 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid) in .25 M glycylglycine pH 8.2, was added to the same assay tubes and yellow color checked visually. Fractions containing protein, but no monothioglycerol, were pooled and stored at -80°C (P-6-Pool). The purified α-KIC oxidase appears to be stable for at least 1 week in the absence of monothioglycerol if kept at -80°C.

C. Purification C

The α-KIC oxidase activity is fairly stable (20% loss in 10 days) in crude preparations, however this enzyme becomes very unstable as it is purified. Several purifications were done as part of a series of stability studies. One of these, which is pertinent to the experiments in this thesis is described here. This purification was attempted before Purification B and is essentially the same except for the following changes.

A portion (200 mL) of the "concentrated DEAE pool" was thawed and centrifuged to remove precipitated material. To this portion, 50 mL of 95% ethanol at -20 to -30°C was slowly added while stirring on an ice bath. The temperature of the solution was maintained below -5°C. After complete addition of ethanol, the solution was stirred an additional 20 min, centrifuged at 10,000 x g and the pellet discarded. To this "25% ethanol supernatant",

150 mL of 95% ethanol (-20 to -40°C) was added slowly. The solution was allowed to stir an additional 20 min after complete addition of ethanol and centrifuged 20 min at 10,000 x g. The pellet was resuspended in Buffer A containing 2.5 M NaCl by stirring overnight at 4°C. A large portion of the pellet did not redissolve. This fraction was then centrifuged 20 min at 10,000 x g and the supernatant (25-100% ethanol fraction) stored at -80°C. The yields of α-KIC oxidase activity from the ethanol precipitation step were variable; therefore this step was omitted in the final purification (Purification B).

The "25-100% ethanol fraction" was thawed and applied to a phenyl sepharose column and the purification continued as in Purification B. Buffers used to elute phenyl sepharose and Sephacryl S-200 columns did not contain 5% monothioglycerol. These columns were pretreated with the "DEAE side fractions".

The "Sephacryl S-200 pool" was dialyzed 35 hr against 400 mL of Buffer A containing 1% monothioglycerol, with 3 changes of buffer. The dialysate was aplied to a 0.8 x 12 cm DEAE (Whatman DE-52) column which had been pre-equilibrated with Buffer A containing 1% thioglycerol. Note that this column was not pretreated with "DEAE side fractions". The column was washed with 60 mL of Buffer A containing 1% monothioglycerol and eluted with a 180 mL linear gradient 0-0.1 M NaCl in the same buffer. Fractions containing α-KIC oxidase activity were stored separately at -80°C ("2nd DEAE column").

VI. Analysis of Reaction Products

The reaction mix used for analysis of α-KIC oxidase reaction products contained 0.2 M Tris, 0.2 M maleate, pH adjusted to 6.5 with NaOH, 1 mM dithiothreitol, 0.2 mM $FeSO_4$, 0.4 mM ascorbic acid, 1 mM $Na_2CO_3$, 1 mM α-KIC, 0.3 μCi α-[4,5-$^3$H] KIC, 0.1 μCi α-[1-$^{14}$C] KIC, and 0.2 mL of the "Sephadex G-150 pool" (Table 5) in a final volume of 0.8 mL. The reaction was followed by measuring the loss of α-[1-$^{14}$C] KIC and was terminated at 4.5 h with 0.4 mL of 20% trichloroacetic acid. The protein was removed by centrifugation and the supernatant fluid was diluted with 20 mL of $H_2O$ and neutralized with NaOH. This was applied to a 0.7 x 37-cm Dowex-1 x 8 chloride (200-400 mesh) column and washed with 70 mL $H_2O$. The column was then eluted with a 300 mL linear gradient of 0 to 0.01 N HCl, 4 mL fractions were collected, and dpm of $^3$H and $^{14}$C were determined by double-label counting techniques. The fractions corresponding to the largest $^3$H peak (peak I, Fig. 29) were pooled, the pH adjusted to 7 and lyophilized. The residue was resuspended in 2 mL of 2 N HCl, saturated with NaCl, and extracted 10 times with 1 mL diethyl ether; 90% of the $^3$H was extracted into the ether layer. The ether was then evaporated to approximately 50 μL under $N_2$. The sample was analyzed on a Hewlett-Packard 5830 A gas chromatograph equipped with a stream splitter which divides the sample eluted from the gas chromatograph column between the flame detector and an external outlet tube. Samples were collected every minute from the outlet tube by condensation in a cold Pasteur pipet. The sample was rinsed from the pipet with 10 mL of scintillation fluid (5) into a scintillation vial and counted. A 6-ft x 2-mm i.d. glass column packed with

15% SP 1220, 1% $H_3PO_4$ on 100/120 Chromosorb WAW, was used with a variable temperature program. The temperature was kept at 110°C for 5 min followed by a 3°C per minute increase up to 140°C. Injection temperaure was 180°C and $N_2$ was the carrier gas. The peak containing radioactivity was identified by using the same gas chromatograph system (except the carrier gas was He) in line with a Hewlett-Packard mass spectrometer. These analyses were performed by the mass spectrometry facilities at Michigan State University, under the supervision of C.C. Sweeley and J. Watson.

VII. Polyacrylamide Gel Electrophoresis Using Denaturing or Non-Denaturing Conditions

SDS (sodium dodecyl sulfate) polyacrylamide gel electrophoresis was carried out on slab gels according to the procedure of Laemmli (123). Gels contained 10% acrylamide. Staining with Coomassie blue dye was carried out according to the procedure of Bonner and Laskey (124).

Tube gels containing 7.5% acrylamide were prepared for native gel electrophoresis as described (123) except SDS was omitted. Gels were pre-run overnight at a constant current of 2 mA per gel. The cathode chamber contained .375 M Tris, 0.08% L-cysteine pH 9.0 and the anode chamber 2.5 mM Tris/glycine pH 8.3, 0.008% L-cysteine during this pre-run. After this pre-run the buffers were replaced with 25 mM Tris/glycine pH 8.3, 0.08% L-cysteine (cathode chamber) and 2.5 mM Tris-glycine pH 8.3, 0.008% L-cysteine (anode chamber). The sample was applied in a solution containing 10% glycerol, 0.003% bromophenol blue. Electrophoresis was carried out at a constant current of 1 mA

per gel for 1 hr, 2 mA per gel for 1 hr, and 3 mA per gel for the final 3 hrs. The gels were stained with Coomassie blue dye as described (124). α-Ketoisocaproate oxidase activity was monitored in some of the gels which were not stained. These gels were sliced into 2 mm sections, each section put into a separate test tube, and 50 μL of 20 mM Tris HCl pH 7.8, 1% isopropanol, 0.1 M NaCl, 5% monothioglycerol added. These were shaken for 2 days at 4°C and then assayed for α-KIC oxidase activity (see Method B).

VIII. Determination of Molecular Weight of the α-Ketoisocaproate Oxidase by Sephacryl S-200 Chromatography

A 1.6 x 63 cm Sephacryl S-200 column was equilibrated with 20 mM Tris HCl pH 7.8, 1% isopropanol, 0.1 M NaCl. The column was then treated with 40 mL of the "DEAE side fractions" (see Section VB, Methods) and washed extensively with equilibration buffer. To calibrate the column, bovine serum albumin, ovalbumin, and cytochrome c were applied separately to the column, eluted with the equilibration buffer and their elution volumes determined by measuring $OD_{280}$ or $OD_{410}$ (cytochrome c). The flow rate of the column was 1.7 ml/hr. In a separate experiment, 0.5 ml of the purified α-KIC oxidase (Sephacryl S-200 pool, Purification B) was applied to the column and eluted with the equilibration buffer containing 5% monothioglycerol. α-KIC oxidase activity was measured in fractions by Method B. The data was plotted and molecular weight determined by the method of Andrews (127).

IX. Enzymatic Incorporation of $^{18}O$ Into β-Hydroxyisovaleric Acid

A. Incubations with $^{18}O_2$

In $^{18}O_2$ incorporation experiments, the reaction mixture contained in a final volume of 0.30 mL; 0.2 M Tris base, 0.2 M maleic acid, pH adjusted to 6.5 with NaOH, 1 mM $FeSO_4$, 0.5 mM ascorbic acid, 1 mM dithiothreitol, 2 mM α-[U-$^{14}C$] KIC (.025 μCi) and 0.1 mL of purified α-KIC oxidase (P-6-Pool, see purification B).

All components except the α-[U-$^{14}C$] KIC were added to a culture tube and frozen in a acetone, dry ice bath. While frozen, the α-[U-$^{14}C$] KIC was added and this was also frozen. The culture tube was then capped with a serum cap and the air removed by evacuation. The tube was flushed with $N_2$, thawed, refrozen, and evacuated again. This process was repeated 4 times to insure all $O_2$ was removed. The culture tube was then filled with 90 atom % $^{18}O_2$ (approximately 15 mL) via a syringe. The contents of the culture tube were then thawed and incubated 3 h at 25°C. At the end of the reaction, β-hydroxyisovaleric acid was isolated and analyzed as described below.

The ratio of $N_2$ to $O_2$ was determined in 1 mL of the gas phase of the reaction vessel at the start and end of the reaction to insure that air was not leaking into the reaction vessel. Oxygen and nitrogen were analyzed on a Varian 3700 gas chromatograph using a 2 mm x 2 m stainless steel column packed with Molecular Sieve 5A. The injection temperature was 40°C and column temperature was 40°C isothermal. The flow rate of helium, the carrier gas, was 30 $cm^3$/min. Detection was achieved with a

thermal conductivity detector at 100°C with a filament temperature of 190°C. The instrument output was calibrated with a standard mixture of 1% $O_2$:99% $N_2$. $^{18}O_2$ dilution by $^{16}O_2$ due to leakage was calculated to be less than 3%.

B. Incubations with $H_2{}^{18}O$

The reaction mixture for $H_2{}^{18}O$ experiments was the same as for $^{18}O_2$ experiments except the reaction contained 93% $H_2{}^{18}O$. In order to reduce $H_2{}^{16}O$, 0.187 mL of 0.32 M Tris, 0.32 M maleic acid pH adjusted to 6.5 with NaOH, 18.7 µL of a solution containing 16 mM $FeSO_4$, 8 mM ascorbic acid, and 16 mM dithiothreitol, and 0.2 mL of purified α-KIC oxidase (P-6-Pool) were combined, frozen and lyophilized. To the lyophilized powder, 300 µL of 97% $H_2{}^{18}O$ and 11 µL of 80 mM α-[U-$^{14}C$] KIC were added and all components mixed well. The tube was then capped with a serum cap, gassed with oxygen for two min and incubated 3 h at 25°C. β-hydroxyisovaleric acid was then isolated and analyzed as described below.

C. Isolation of β-Hydroxyisovaleric Acid

Isotopic oxygen incorporation experiments were terminated by passing the reaction mixture over a 0.5 x 5.0 cm Dowex-1 chloride (200-400 mesh) column. The column was washed with 5 mL $H_2O$ and β-hydroxyisovaleric acid eluted with six 1 mL portions of 0.02 N HCl. The fraction containing β-[U-$^{14}C$] hydroxyisovaleric acid was saturated with NaCl, and extracted three times with an equal volume of diethyl ether. The ether extractions were pooled

and concentrated to approximately 50-100 μL under $N_2$. β-Hydroxyisovaleric acid was analyzed by gas chromatography-mass spectrometry as described in Section VI of Methods except the gas chromatograph column temperature was 150°C, isothermal. β-Hydroxyisovaleric acid had a retention time of 7.7 min under these conditions.

# RESULTS

## I. Subcellular Distribution and Partial Characterization of an α-Keto-isocaproate Oxidase of Rat Liver

### A. Subcellular Distribution of Branched-Chain α-Keto Acid Decarboxylating Activities in Rat Liver

Peroxisomes, mitochondria and microsomes from male rat liver were separated on a sucrose gradient as shown in Figure 1. The subcellular distribution of α-[1-$^{14}$C] ketoisocaproate (α-KIC) and α-[1-$^{14}$C] ketoisovalerate decarboxylase activities in rat liver using assay conditions optimized for the mitochondrial branched-chain α-keto acid dehydrogenase are shown in Figure 1A. All of the α-[1-$^{14}$C] ketoisovalerate decarboxylating activities coincided with the mitochondrial marker, fumarase. None was associated with peroxisomes (catalase marker) or microsomes (NADPH-cytochrome c reductase marker). However, when α-[1-$^{14}$C] KIC was used as a substrate, two peaks of decarboxylating activity were found; one with mitochondria and the other with the cytosol fractions.

Other studies showed that the mitochondrial activity requires CoA and $NAD^+$ (data not shown) and uses all three branched-chain α-keto acids as substrates. This activity is due to the mitochondrial branched-chain α-keto acid dehydrogenase (E.C. 1.2.4.3 and 1.2.4.4).

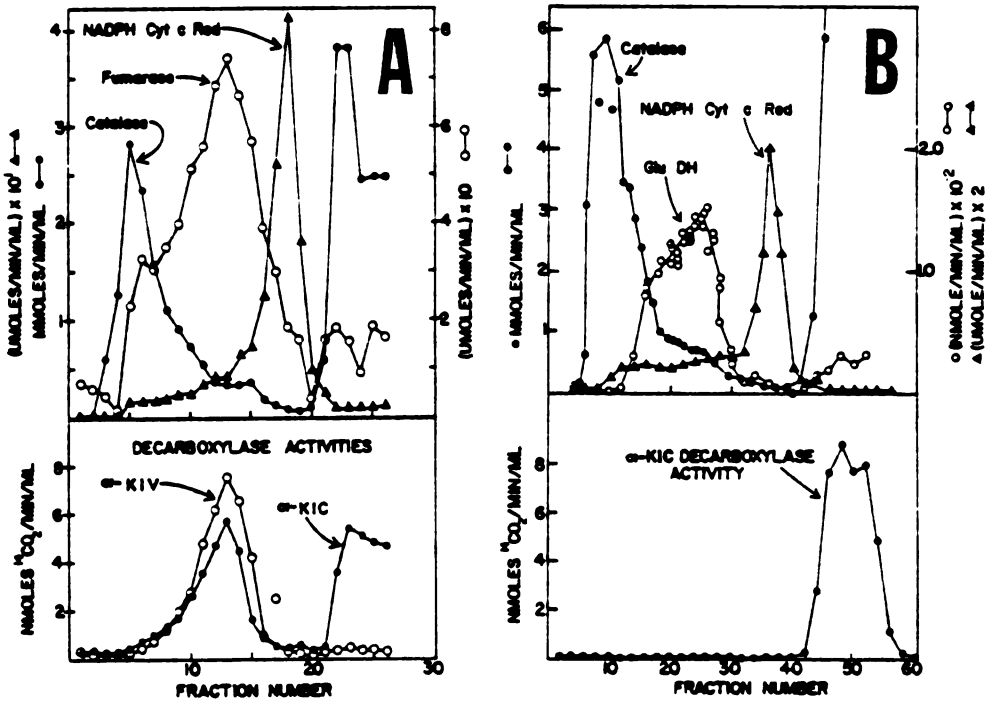



Figure 1. Subcellular Distribution of Branched-Chain α-Keto Acid Decarboxylases in Rat Liver. Marker enzymes for peroxisomes (catalase), mitochondria (fumarase or glutamate dehydrogenase), and microsomes (NADPH cytochrome c reductase) are shown in the upper figures of A and B.

In Figure A, decarboxylase activity was measured using conditions optimized for the mitochondrial branched-chain α-keto acid dehydrogenase (lower figure). Each assay contained in a final volume of 0.4 mL; 33 mM sodium phosphate, pH 7.2, 1 mM $MgCl_2$, 0.25 mM $CaCl_2$, 1 mM $Na_2CO_3$, 0.6 mM CoASH, 1 mM $NAD^+$, either 0.4 mM α-[1-$^{14}$C] ketoisovalerate (α-KIV) or 0.6 mM α-[1-$^{14}$C] ketoisocaproate (α-KIC) (100,000 cpm) and 0.1 mL of the fraction to be assayed.

In Figure B, decarboxylation of α-[1-$^{14}$C] ketoisocaproate was measured using assay conditions optimized for the cytosolic α-ketoisocaproate oxidase activity (see Methods, Method B). The assays also contained 5 mM sodium arsenite.

Abbreviations are Glu DH = glutamate dehydrogenase and NADPH Cyt c Red = NADPH cytochrome c reductase.

The cytosolic α-KIC decarboxylase activity is clearly different from the mitochondrial activity, as will be shown. After optimizing assay conditions for this enzyme, the subcellular distribution was again determined (Figure 1B). Sodium arsenite (5 mM), an inhibitor of the mitochondrial branched-chain α-keto acid dehydrogenase was included in each assay. Again the α-KIC decarboxylase activity was detected only in the soluble fraction.

B. Properties of Rat Liver Cytosolic α-Ketoisocaproate Decarboxylase Activity

Cofactor requirements and other properties of the cytosolic α-KIC decarboxylase activity were investigated after dialysis. Table 1 shows that added $Mg^{2+}$, $Ca^{2+}$, $Na_2CO_3$, phosphate, CoA, $NAD^+$, $NADP^+$, FAD, thiamine pyrophosphate, and lipoic acid did not appreciably affect the activity. Although phosphate was present in all of the assays for Table 1, other experiments (data not shown) using rat liver cytosol prepared in Tris-maleate buffer, showed that phosphate is not required for α-KIC decarboxylase activity.

The assay for the α-KIC decarboxylase activity was optimized for concentration and pH of the buffer. Optimal activity was obtained in 0.2 M Tris, 0.2 M maleate buffer at pH 6.5. Activity at pH 7.2 was approximately 80% of that at pH 6.5.

Connelly et al. (6) reported that bovine liver α-ketoisocaproic:α-keto-β-methylvaleric acid dehydrogenase was activated by ammonium sulfate. When rat liver cytosol was assayed in 0.2 M Tris, 0.2 M maleate, pH 6.5, 1.5 M ammonium sulfate stimulated

TABLE 1

COFACTOR REQUIREMENTS OF RAT LIVER
CYTOSOLIC α-KETOISOCAPROATE DECARBOXYLASE ACTIVITY[a]

| Factor omitted or added | α-KIC decarboxylase activity (% of control) |
|---|---|
| None (control) | (100) |
| - $MgCl_2$ | 102 |
| - $CaCl_2$ | 95 |
| - $Na_2CO_3$ | 111 |
| - $NAD^+$ | 88 |
| - Coenzyme A | 116 |
| - Potassium phosphate, + 33 mM Tris | 120 |
| + 0.5 mM Thiamine pyrophosphate | 110 |
| - $NAD^+$ + 1 mM $NADP^+$ | 100 |
| - $NAD^+$ + 1 mM FAD | 61 |
| + 0.5 mM DL-α-lipoic acid | 86 |
| + 0.5 mM Thiamine pyrophosphate, + 0.5 mM DL-α-lipoic acid | 101 |

[a]The assay conditions were the same as in Fig. 1A except for the additions and deletions shown. Each assay contained 0.1 mL rat liver cytosol preparation. Control activity was 0.55 nmol/min/mg protein.

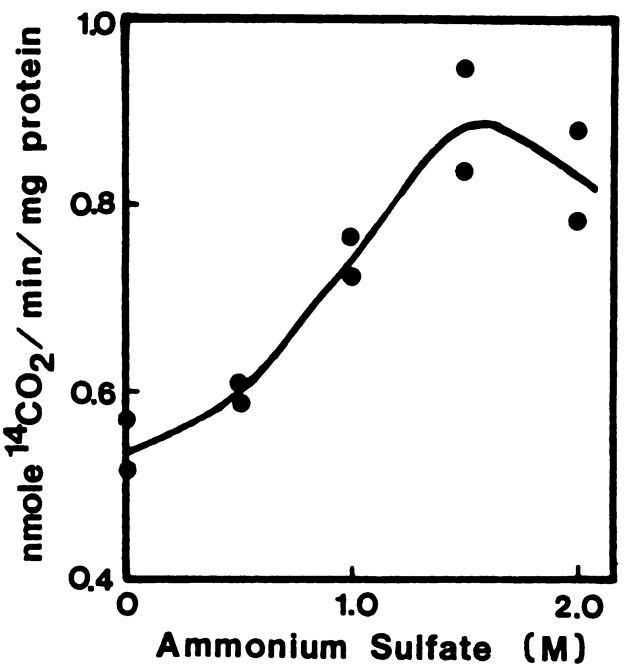



Figure 2. The Effect of Ammonium Sulfate on Cytosolic α-Ketoisocaproate Decarboxylase Activity. α-KIC decarboxylase activity was assayed as in Methods (Method A) except that the ammonium sulfate concentration was varied and the incubation time was 30 min.

the α-KIC decarboxylase activity (Figure 2). This stimulation was seen consistently, but the degree of stimulation was variable in different rat liver cytosol preparations.

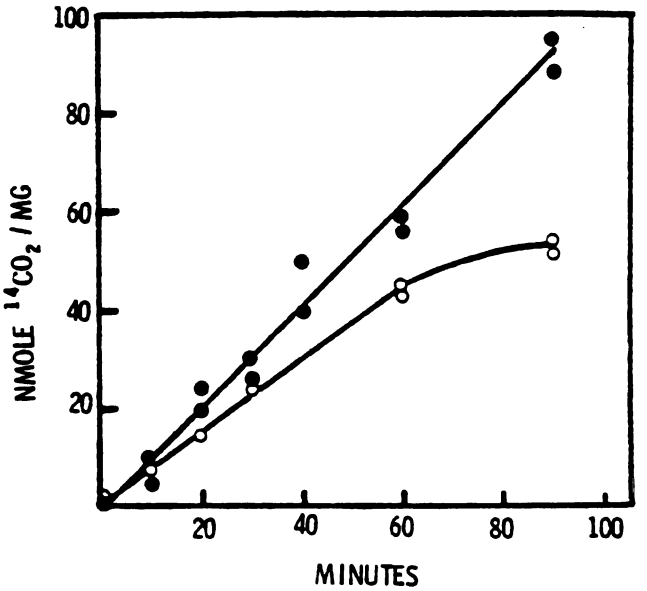
Figure 3 shows that with these assay conditions, the reaction rate was linear for at least 90 min when 1.5 M ammonium sulfate was present. In the absence of ammonium sulfate, decarboxylase activity was always lower and linear for about 60 min. α-KIC decarboxylase activity was linear with protein concentration up to 2.8 mg of rat liver cytosolic protein per assay (the largest amount tested).

The apparent $K_m$ for the rat liver cytosolic α-KIC decarboxylase activity for α-KIC using these improved assay conditions is 0.03 mM (Figure 4A). With the assay buffer used for the sucrose gradient in Figure 1A, which was not at the optimal pH for the cytosolic α-KIC decarboxylase activity and did not include ammonium sulfate, an apparent $K_m$ of 0.2 mM was determined (Figure 4B).

During the course of these experiments, it was noted that there is a slow increase in α-KIC decarboxylase activity with time at 4°C. This is shown in Figure 4B where the two lines represent identical assays done on the same rat liver cytosol preparation but assayed 1 h apart. The apparent $K_m$ value was not affected by this activation of the enzyme. In all experiments, assays were grouped and started within 20-30 min of each other.

The release of $^{14}CO_2$ from α-[1-$^{14}$C] KIC was completely inhibited in the absence of oxygen (Table 2). This indicated that the α-KIC decarboxylase activity uses oxygen as an

Figure 3. Effect of Time and Protein Concentration on Cytosolic α-Keto-isocaproate Decarboxylase Activity. In the upper figure 100 µL of a cytosol preparation (2.8 mg protein) was assayed in the presence (●) or absence (○) of 1.5 M ammonium sulfate. In the lower figure the amount of cytosol preparation was varied.


100
80
60
40
20
NMOLE $^{14}CO_2$ / MG
20
40
60
80
100
MINUTES

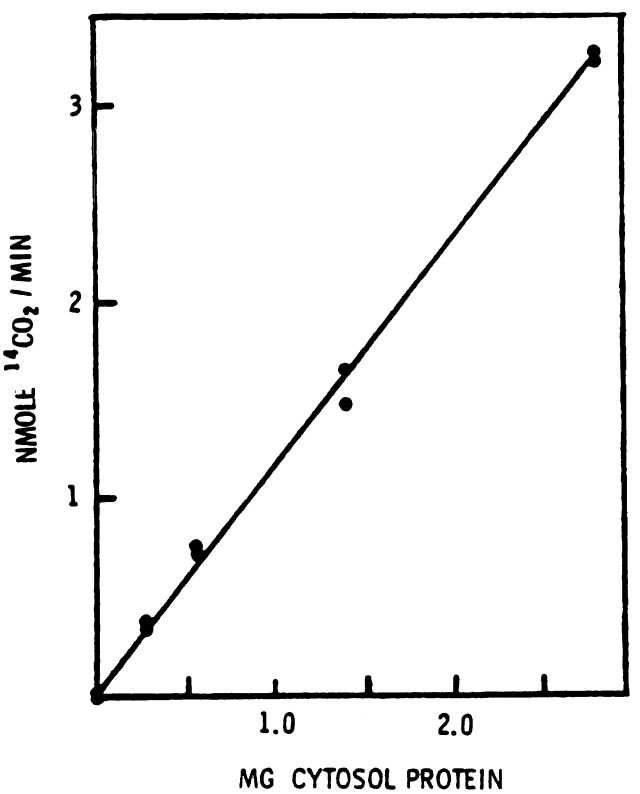

3
2
1
NMOLE $^{14}CO_2$ / MIN
1.0
2.0
MG CYTOSOL PROTEIN

Figure 3

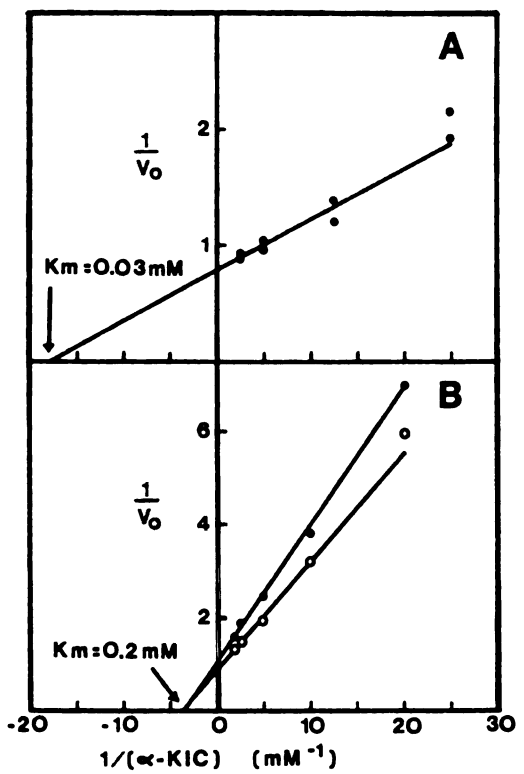



Figure 4. Effect of Substrate Concentration on Cytosolic α-Ketoisocaproate Decarboxylase Activity. α-KIC decarboxylase activity vs. the concentration of α-KIC was determined under two different assay conditions. Assays in A contained in a total volume of 0.4 mL, 0.2 M Tris-maleate pH 6.5, 1.5 M ammonium sulfate, 1 mM $Na_2CO_3$, 0.04-0.40 mM α-[1-$^{14}$C] KIC (630 cpm/nmol) and 1.7 mg/ml cytosolic protein. The incubation time was 15 or 30 min. Assays in B were done in a total volume of 0.4 mL containing 33 mM potassium phosphate, pH 7.2, 1 mM $Na_2CO_3$, 0.05-0.50 mM α-[1-$^{14}$C] KIC (1,100 cpm/nmol), and 2.1 mg/ml cytosolic protein. Incubation time was 15 min. Two identical assays were done approximately 1 h apart; (●) first set of assays, (○) second set of assays. Experimental points in both A and B were plotted and the best-fitting lines drawn by computer analysis (WILKIN 2 PROGRAM, Michigan State University, East Lansing, MI). Initial velocities ($V_o$) are expressed as nmol/min/mg cytosolic protein. $K_m$ and $V_{max}$ were determined by the data weighting procedure suggested by Wilkinson (144).

electron acceptor. The second column of Table 2 shows that rat liver cytosol preparations consume oxygen when α-KIC is added to the assay. In several experiments, using either the rat liver cytosol preparation or the partially purified α-KIC oxidase (Sephadex G-150 pool, see Table 5), 0.7-0.9 molecules of $O_2$ were consumed for each $CO_2$ molecule released from α-KIC.

C. Effect of α-Keto Acids on Cytosolic α-Ketoisocaproate Oxidase Activity

Both assay methods gave similar results when the different α-keto acids were tested as inhibitors of the α-KIC oxidase (Table 2). 1 mM α-Ketoisovalerate, α-keto-β-methylvalerate, α-ketobutyrate, α-ketononanoate, and phenylpyruvate strongly inhibited the decarboxylase and oxidase activity. Phenylpyruvate is an exceptionally strong inhibitor, showing complete inhibition at 0.01 mM. α-Ketoisovalerate showed no inhibition at 0.01 mM. 1 mM α-ketoglutarate, pyruvate, α-keto-γ-methiolbutyrate, and arsenite, an inhibitor of dihydrolipoyl transacetylase, had little effect at a concentration of 1 mM.

The oxidation of various α-keto acids by a rat liver cytosol preparation is shown in Table 3. At a concentration of 1 mM, the two branched-chain α-keto acids, α-ketoisovalerate and α-keto-β-methylvalerate, as well as pyruvate, α-ketobutyrate, and α-ketononanoate caused little $O_2$ consumption. However, α-keto-γ-methiolbutyrate, the keto analog of methionine, was oxidized by rat liver cytosol preparations at a rate approximately 2 times the rate of α-KIC oxidation when both were assayed at a

TABLE 2

EFFECTS OF α-KETO ACIDS, ARSENITE, AND OXYGEN ON α-KETOISOCAPROATE OXIDASE ACTIVITY IN RAT LIVER CYTOSOL PREPARATIONS

| Addition (or deletion) | α-Ketoisocaproate oxidase activity[a] | |
|---|---|---|
| | $^{14}CO_2$ released (% of control) | $O_2$ consumed (% of control) |
| None (control) | 100 (1.0 nmol/min/mg)[b] | 100 (0.87 nmol/min/mg)[b] |
| 1 mM α-Ketoisovalerate | 13 | 9 |
| 0.01 mM α-Ketoisovalerate | 97 | 89 |
| 1 mM α-Keto-β-methylvalerate | 25 | 19 |
| 1 mM Pyruvate | 75 | 70 |
| 1 mM α-Ketoglutarate | 105 | 93 |
| 1 mM β-Phenylpyruvate | 0 | 0 |
| 0.01 mM β-Phenylpyruvate | 0 | 0 |
| 1 mM α-Keto-γ-methiolbutyrate | 90 | - |
| 1 mM α-Ketobutyrate | 30 | 29 |
| 1 mM α-Ketononanoate | 3 | 14 |
| (Minus $O_2$) | 1 | - |
| 1 mM Arsenite | 102 | - |

[a]α-Ketoisocaproate oxidase activity was assayed by $^{14}CO_2$ release (Method A) or $O_2$ consumption as described under Methods. Values are the mean of two replicate assays. Variability between replicates was never more than 5% of the control activity. Assay concentration of α-ketoisocaproate was 1 mM.

[b]Control activities expressed as nmol $^{14}CO_2$ released or $O_2$ consumed/min/mg protein.

TABLE 3

OXYGEN CONSUMPTION BY RAT LIVER CYTOSOL PREPARATIONS USING VARIOUS α-KETO ACIDS AS SUBSTRATES

| Substrate[a] | nmol $O_2$ consumed/min/mg cytosol protein[b] |
|---|---|
| α-Ketoisocaproate | 0.87 |
| α-Keto-γ-methiolbutyrate | 1.87 |
| α-Ketoisocaproate + α-keto-γ-methiolbutyrate | 0.97 |
| α-Ketoisovalerate | 0.03 |
| α-Keto-β-methylvalerate | 0.04 |
| Pyruvate | 0.02 |
| α-Ketoglutarate | 0.02 |
| β-Phenylpyruvate | 0.02 |
| α-Ketobutyrate | 0.02 |
| α-Ketononanoate | 0.07 |

[a]All additions gave a final assay concentration of 1 mM.

[b]Assays were carried out as described under Methods (Method A). Values are the mean of two replicate assays. Deviation between assays was always less than 0.10 nmol $O_2$ consumed/min/mg protein.

concentration of 1 mM. α-[1-$^{14}$C] keto-γ-methiolbutyrate is decarboxylated by rat liver cytosol preparations at a rate about 2 times that of α-KIC (unpublished observation). The addition of both 1 mM α-KIC and 1 mM α-keto-γ-methiolbutyrate to cytosol preparations produced a rate of $O_2$ consumption intermediate between the addition of either substrate alone.

D. Tissue Distribution of α-Ketoisocaproate Oxidase in the Male Rat

Table 4 shows the distribution of the α-KIC oxidase in 27,000 x g supernatant fractions of several rat tissues. The highest specific activity occurs in liver. Activity was also found in the kidney. Activity was not detected in brain, heart, muscle or pancreas. The absence of α-[1-$^{14}$C] ketoisovalerate decarboxylating activity in all tissues indicates that the mitochondrial branched-chain α-keto acid dehydrogenase is not active with the assay conditions used.

II. Purification and Stabilization of the α-Ketoisocaproate Oxidase Activity from Rat Liver

A. Stabilization of and Cofactor Requirements of α-Ketoisocaproate Oxidase Activity

In order to further characterize physical and kinetic properties of the cytosolic α-KIC oxidase activity as well as identify the products of the reaction, purification of this enzyme was necessary.

α-KIC oxidase was partially purified from a 70,000 x g supernatant fraction of a rat liver homogenate as shown in Table 5.

TABLE 4

TISSUE DISTRIBUTION OF α-KETOISOCAPROATE DECARBOXYLASE (OXIDASE) IN THE MALE RAT

| Tissue | α-Keto Acid Decarboxylating Activity (nmole $^{14}CO_2$ produced/min/mg protein) | |
|---|---|---|
| | α-[1-$^{14}$C] Ketoisocaproate | α-[1-$^{14}$C] Ketoisovalerate |
| Liver | 0.39 | 0.02 |
| Kidney | 0.17 | <0.03 |
| Brain | <0.02 | <0.04 |
| Heart | <0.02 | <0.04 |
| Muscle | <0.02 | <0.03 |
| Pancreas | <0.02 | <0.03 |

Each tissue except muscle was homogenized 5 times for 30 seconds in 2 to 3 volumes of .25 M sucrose, with a Polytron homogenizer. This was followed by 2 passes in a Potter Elvehjem glass homogenizer. Muscle was homogenized for 15 sec in a Waring blendor. All treatments were at 4°C. The homogenate was centrifuged at 27,000 x g for 45 min and the pellet discarded. The supernatant was adjusted to 1% isopropanol. Isopropanol stabilizes the α-ketoisocaproate oxidase activity. The 27,000 x g supernatant fluids were assayed as in Methods (Method B), except assay concentrations of $FeSO_4$, dithiothreitol, and ascorbic acid were 2 mM, 2 mM and 1 mM, respectively. Values are mean of duplicate assays.

TABLE 5

PARTIAL PURIFICATION OF α-KETOISOCAPROATE OXIDASE FROM RAT LIVER

| Fraction | Volume (mL) | Total protein (mg) | Total activity (nmol/min)[a] | Specific activity (nmol/min/mg)[a] | Recovery | Fold pure |
|---|---|---|---|---|---|---|
| 1. 70,000 x g supernatant | 465 | 5000 | 1280 | 0.25 | 100 | 1.0 |
| 2. 0.2% Protamine sulfate supernatant | 510 | 3600 | 2240 | 0.62 | 175 | 2.5 |
| 3. 35% $(NH_4)_2SO_4$ supernatant | 550 | 3600 | 1030 | 0.29 | 80 | 1.2 |
| 4. Pre-DEAE dialysate | 790 | 1900 | 770 | 0.40 | 60 | 1.6 |
| 5. DEAE-cellulose pool | 258 | 200 | 400 | 2.01 | 31 | 8.0 |
| 6. Concentrated DEAE pool | 9.8 | 200 | 310 | 1.35 | 24 | 5.4 |
| 7. Sephadex G-150 pool | 13.6 | 60 | 40 | 0.62 | 3 | 2.5 |
| Assayed without $(NH_4)_2SO_4$ | 13.6 | 60 | 84[b] | 1.40[b] | 6[b] | 5.6[b] |
| Assayed without $(NH_4)_2SO_4$, + 1 mM dithiothreitol, + 0.2 mM $FeSO_4$, + 0.4 mM ascorbate | 13.6 | 60 | 270[b] | 4.54[b] | 21[b] | 18.2[b] |

[a]α-KIC oxidase was assayed by measuring α-[1-$^{14}$C] KIC decarboxylation as under Methods (Method A) except the final concentration of α-KIC was 0.5 mM.

[b]α-KIC oxidase was assayed as under Methods (Method A) except for addition or deletions as shown.

The total activity of α-KIC oxidase is doubled by protamine sulfate precipitation. When 35% ammonium sulfate was added to the 0.2% protamine sulfate supernatant, no additional protein was precipitated and there was a large loss of activity.

Column chromatography using DEAE cellulose or Sephadex caused a large loss of activity. This could be due to the removal of low molecular weight materials necessary for enzyme activity. Therefore, various compounds were added to the assay mixture in an effort to restore activity; see Table 6. The activity of the α-KIC oxidase was increased severalfold by the addition of ferrous iron, an electron donor (ascorbate, NADH, or NADPH) and a sulfhydryl reducing compound (CoASH or dithiothreitol). All three factors are required for optimum activity. At higher concentrations of $Fe^{2+}$, ascorbate is not required (see Figure 18). α-Ketoglutarate, a cofactor for some dioxygenases (125), was not required. Catalase, which increases the activity of some oxygenases, also had no effect on the α-KIC decarboxylase activity. However, some catalase is present in the enzyme preparation.

Optimal concentrations of $FeSO_4$, ascorbate and dithiothreitol were found to be 1.0 mM, 0.5 mM and 1.0 mM, respectively when using either the "DEAE-cellulose pool" or "Sephacryl S-200 pool" as a source of α-KIC oxidase. Despite the inclusion of these compounds in all assays, large losses of activity occurred during purification when DEAE-cellulose, phenyl sepharose or sephacryl columns were used. When 0.4 mL of the concentrated DEAE pool (see Table 5) was applied to a 1.3 x 56 cm Sephacryl S-200

TABLE 6

EFFECTS OF POSSIBLE COFACTORS ON α-KETOISOCAPROATE OXIDASE ACTIVITY IN THE PARTIALLY PURIFIED PREPARATION (SEPHADEX G-150 POOL)

| Additions[a] | α-KIC oxidase activity: nmol $^{14}CO_2$/min/mg/protein[b] | α-KIC oxidase activity: Percentage of control |
|---|---|---|
| Expt 1 | | |
| None | 1.24 | 100 |
| CoASH, $Fe^{2+}$, ascorbate, α-KG | 3.45 | 278 |
| CoASH, $Fe^{2+}$, α-KG | 1.35 | 108 |
| CoASH, ascorbate, α-KG | 1.80 | 145 |
| $Fe^{2+}$, ascorbate, α-KG | 1.11 | 89 |
| CoASH, $Fe^{2+}$, ascorbate, α-KG, DTT | 3.56 | 286 |
| $Fe^{2+}$, ascorbate, α-KG, DTT | 4.00 | 322 |
| α-KG | 1.13 | 91 |
| Expt 2 | | |
| None | 1.09 | 100 |
| DTT, $Fe^{2+}$, ascorbate, α-KG | 4.74 | 433 |
| DTT, $Fe^{2+}$, ascorbate | 4.36 | 399 |
| DTT, $Fe^{2+}$, ascorbate, α-KG, catalase | 4.33 | 397 |
| DTT | 0.91 | 83 |
| DTT, $Fe^{2+}$, NADH, α-KG | 2.77 | 254 |
| DTT, $Fe^{2+}$, NADPH, α-KG | 2.86 | 262 |

[a]Concentration of additions were as follows; CoASH, 1 mM; $FeSO_4$ ($Fe^{2+}$), 0.2 mM; ascorbate, 0.4 mM; α-ketoglutarate (α-KG), 0.5 mM; dithiothreitol (DTT), 1 mM; catalase, 100 U/assay; NADH, 0.5 mM; NADPH, 0.5 mM.

[b]Assay of α-[1-$^{14}$C] KIC decarboxylase was carried out as under Materials and Methods (Method A) except the ammonium sulfate was omitted and the concentration of α-ketoisocaproate was 0.5 mM. Each assay included 25 or 50 μL of the "Sephadex G-150 Pool" (Purification A, Table 5). Values are mean of duplicate assays.

column, less than 10% of the applied activity was recovered. Elution with high concentrations of salt removed more protein from the column but did not increase the yield of α-KIC oxidase. Pretreatment of the Sephacryl S-200 column with protein containing fractions recovered from the DEAE-cellulose column (step 5, Table 5), which did not contain α-KIC oxidase activity ("DEAE side fractions") increased recovery to 82%. Kaufman and Fisher (126) used a similar procedure in the purification of phenylalanine hydroxylase from rat liver.

Results of a second attempt to purify rat liver α-KIC oxidase are shown in Table 7. $FeSO_4$, ascorbate, and dithiothreitol were included in all assays (Method B). Phenyl sepharose and Sephacryl S-200 columns were pretreated with "DEAE side fractions".

Ammonium sulfate fractionation resulted in a 2 fold increase in specific activity with a 85% recovery. DEAE cellulose columns did not require pretreatment with protein, due to the large amount of protein applied to the column, and always gave a 5 to 6 fold increase in specific activity. Ethanol fractionation gave a 6 fold increase in specific activity, however, yields were quite variable from one purification to another. Therefore, this step was omitted from later purifications.

Despite pretreatment of phenyl sepharose and sephacryl columns with "DEAE side fractions", large losses of α-KIC oxidase activity were still apparent. Note that 60% of the α-KIC oxidase activity was lost while concentrating the phenyl sepharose pool to 7.2 mL (Pre-Sephacryl S-200); very little activity was lost on

TABLE 7

PURIFICATION OF α-KETOISOCAPROATE OXIDASE FROM RAT LIVER: PURIFICATION C

| | Volume (mL) | Total Protein (mg) | Total Activity (nmole/min)[a] | Specific Activity (nmole/min/mg)[a] | % Recovery | Fold Pure |
|---|---|---|---|---|---|---|
| 1. 100,000 x g supernatant | 9,000 | 117,900 | 60,300 | 0.51 | (100) | (1.0) |
| 2. 45-75% $(NH_4)_2SO_4$ fraction | 1,320 | 52,900 | 51,350 | 0.97 | 85 | 1.9 |
| 3. Pre-DEAE dialysate | 1,730 | 45,000 | 52,800 | 1.17 | 87 | 2.3 |
| 4. DEAE-cellulose pool | 1,740 | 6,260 | 33,900 | 5.42 | 56 | 10.6 |
| 5. Concentrated DEAE Pool | 400 | 7,000 | 36,300 | 5.15 | 60 | 10.1 |
| Only 200 mL of the "concentrated DEAE pool" was carried through the remainder of the purification. | | | | | | |
| 6. 25-100% Ethanol fraction | 93 | 465 | 14,600 | 31.4 | 48[b] | 61.5 |
| 7. Phenyl sepharose pool | 1,800 | 180 | 8,000 | 44.5 | 26[b] | 87.2 |
| 8. Phenyl pool concentrated | 7.2 | 120 | 3,730 | 31.1 | 12[b] | 61 |
| 9. Sephacryl S-200 pool | 41 | 53 | 3,140 | 58.9 | 10[b] | 115 |
| 10. 2nd DEAE column (fractions were not pooled) | - | 45 | 1,756 | 32-76 | 6[b] | 64-152 |

[a]α-Ketoisocaproate oxidase activity was measured as in Methods (Method B).

[b]Recovery is corrected to show expected recovery if all of the "concentrated DEAE pool" had been carried through the purification, i.e.: % Recovery = (Total Activity ÷ 60,300) (400 ÷ 200).

the sephacryl column itself. The α-KIC oxidase activity is fairly stable at 4°C in less pure preparations such as the "concentrated DEAE pool" (9% loss of activity in 4 days, Figure 5). Purified preparations of α-KIC oxidase, however, rapidly lose activity (Figure 6; 50% loss of activity in 4 days). This loss of activity does not appear to be related to the protein concentration, since dilution of the "concentrated DEAE pool" to 1.68 mg protein/mL did not substantially alter stability (Figure 5). The α-KIC oxidase activity was quite stable at -80°C for up to 20 days in all purification fractions (Figure 5 and 6), however, at -20°C or room temperature activity was rapidly lost. α-KIC oxidase activity was more stable at 4°C than at -20°C or room temperature.

The addition of 5% monothioglycerol (0.6 M) stabilized α-KIC oxidase activity at 4°C for at least 6 days (Table 8). One percent monothioglycerol (0.12 M) was not effective in preventing loss of activity. Dithiothreitol at 1 mM or 5 mM and in the presence or absence of 5% glycerol also did not prevent loss of activity. Although monothioglycerol prevents the loss of α-KIC oxidase activity, this compound caused a decrease in the measured initial activity of the α-KIC oxidase (Table 8). Figure 7A shows that α-KIC oxidase activity is inhibited by assay concentrations of monothioglycerol greater than 0.6%. Increasing the amount of $FeSO_4$ in the assay mixture did not prevent this inhibition (Figure 7B). The inhibitory effect of monothioglycerol is most likely due to a depletion of $O_2$ in the assay mixture.

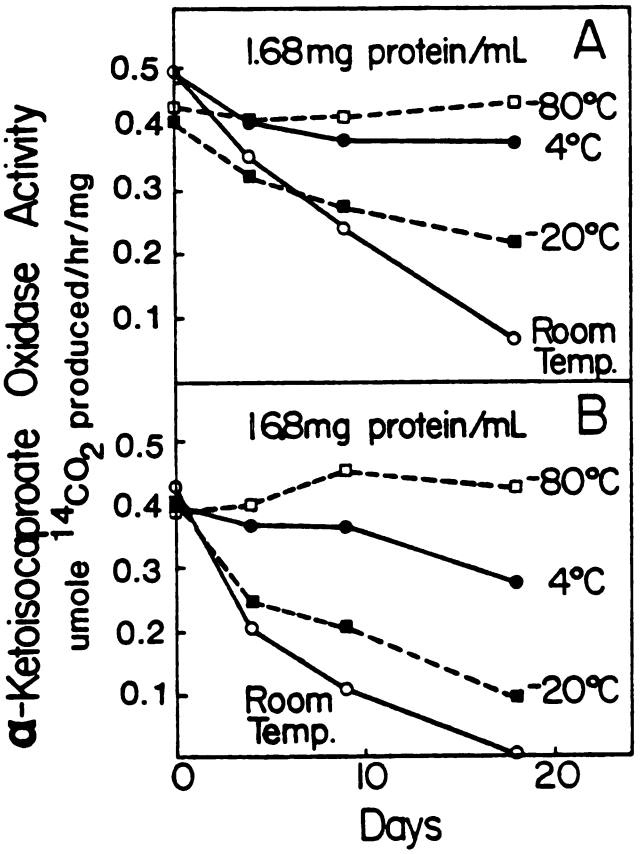



Figure 5. Stability of the α-Ketoisocaproate Oxidase in a 12-Fold Purified Preparation. Aliquots of the "concentrated DEAE Pool" (Purification B) (B) or a 1:10 dilution of the "concentrated DEAE Pool" (A) were stored at the temperatures indicated. α-KIC oxidase activity was monitored as in Methods (Method B). Frozen samples were only thawed once, then discarded. Values are the mean of duplicate assays.

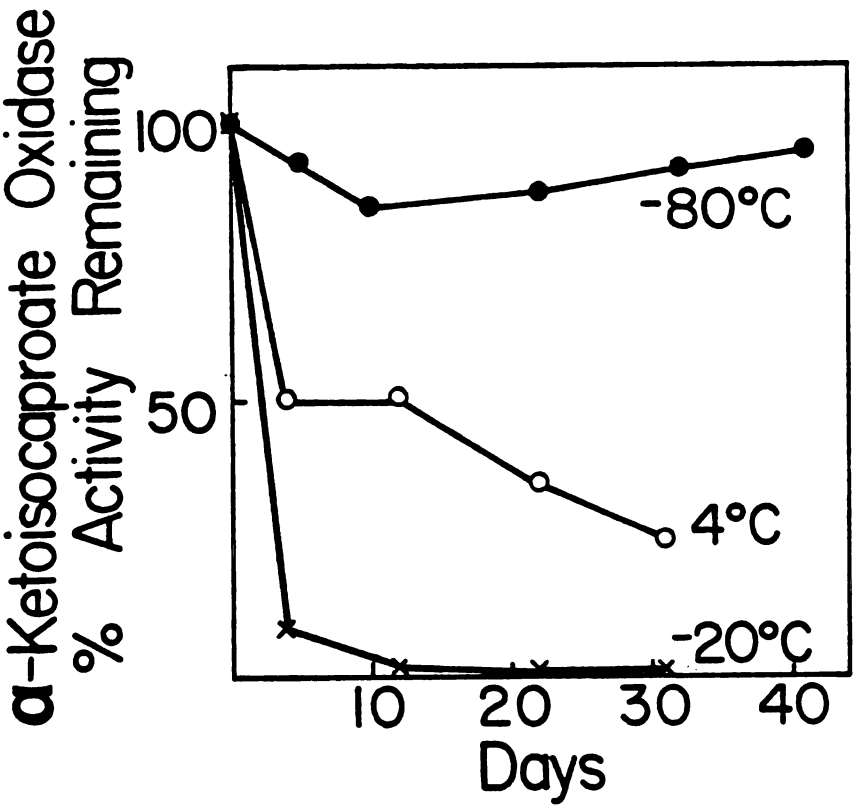



Figure 6. Stability of the α-Ketoisocaproate Oxidase in an 80-Fold Purified Preparation. Aliquots of pooled fractions from the "2nd DEAE column" of Purification C (Table 7) were stored at the temperatures indicated. α-KIC oxidase activity was measured as in Methods (Method B). Frozen samples were thawed only once, then discarded. 100% is 42 nmole/min/mg protein for -80°C experiments and 31.7 nmole/min/mg protein for 4°C and -20°C experiments. Values are mean of duplicate assays.

TABLE 8

STABILITY OF α-KETOISOCAPROATE OXIDASE IN THE PRESENCE OF MONOTHIOGLYCEROL, DITHIOTHREITOL, OR GLYCEROL

| Addition | α-Ketoisocaproate Oxidase Activity nmole $^{14}CO_2$ produced/min/mg protein Initial | Final | Days of Storage at 4°C | % Activity Remaining |
|---|---|---|---|---|
| None | 26.4 | 11.6 | 6 | 44 |
| 1 mM Dithiothreitol | 25.5 | 9.48 | 6 | 37 |
| 5 mM Dithiothreitol | 25.0 | 7.93 | 6 | 32 |
| 1% Monothioglycerol | 24.3 | 7.13 | 6 | 29 |
| 5% Monothioglycerol | 20.9 | 21.1 | 6 | 101 |
| 5% Glycerol + 1 mM Dithiothreitol | 21.7 | 11.6 | 8 | 53 |
| 5% Glycerol + 5 mM Dithiothreitol | 21.1 | 6.43 | 8 | 30 |

Aliquots (0.15 mL) of pooled fractions from the "2nd DEAE column" of Purification C (see Table 7) were incubated at 4°C with the additions shown. All aliquots were adjusted to the same final volume (0.17 mL). α-Ketoisocaproate oxidase activity was measured as in Methods (Method B) immediately (Initial Activity) and after 6 or 8 days storage at 4°C (Final Activity). Results are mean of 2 replicate assays. Each assay contained 25 µL (29 µg protein) of sample.

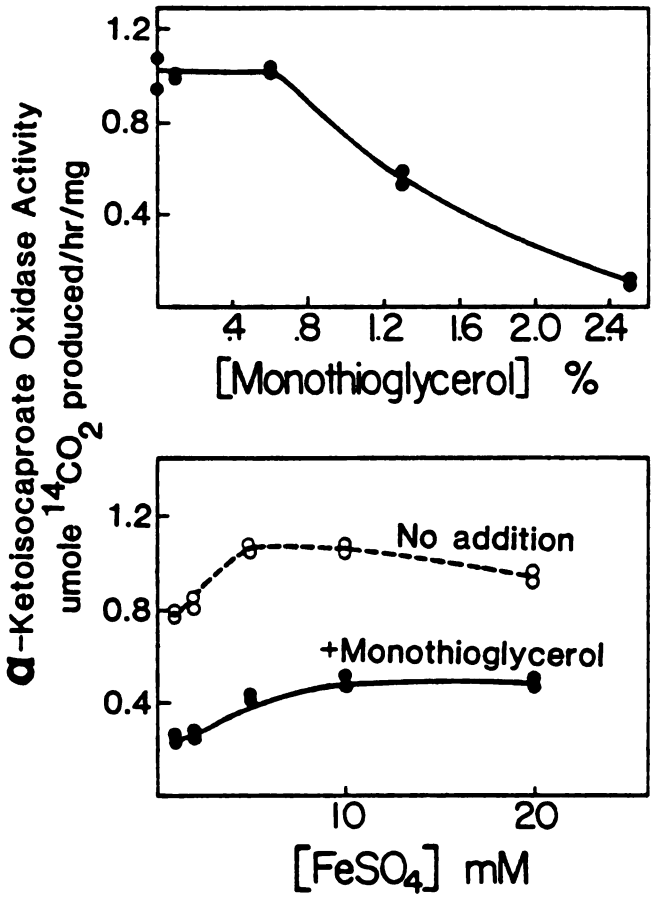



Figure 7. Effect of Monothioglycerol on α-Ketoisocaproate Oxidase. α-KIC oxidase was assayed as in Methods (Method B) with the following modifications. In A, monothioglycerol was added as indicated. In B, the final assay concentration of $FeSO_4$ was varied from 1 to 20 mM and assays were carried out in the presence or absence of 1.25% monothioglycerol as indicated. Each assay contained 50 µL (31 µg protein) of pooled fractions from the "2nd DEAE column" of Purification C (Table 7).

B. Purification of α-Ketoisocaproate Oxidase Activity

Table 9 shows the results of an α-KIC oxidase purification using conditions which stabilize this enzyme. The initial steps in the purification (steps 1-5) did not include monothioglycerol. This may be the basis for the low recovery (50%) in the "concentrated DEAE pool". Phenyl sepharose and sephacryl S-200 columns were pre-treated with "DEAE side fractions" and 5% monothioglycerol was included in all buffers used.

The specific activity of α-KIC oxidase was increased 2.2 fold by 45-75% $(NH_4)_2SO_4$ fractionation with only a 25% loss of total activity. This fraction was then dialysed and applied to a DEAE column (Figure 8). A 15% loss of activity occurred during dialysis. Loss of activity during the DEAE column chromatography was due to pooling of peak fractions off this column.

When applied to phenyl sepharose in a buffer containing 2.5 M NaCl, α-KIC oxidase activity was retarded and elutes just behind the column void volume (Figure 9). A large amount of protein remained bound to the column. The purification at this step may be improved by increasing the concentration of NaCl in the sample and elution buffer to promote a stronger hydrophobic interaction between the α-KIC oxidase and phenyl sepharose.

Sephacryl S-200 chromatography (Figure 10) increased the specific activity of the α-KIC oxidase almost 5 fold. Three peaks of protein were found. The α-KIC oxidase activity migrated with the first of these protein peaks. The overall yield of α-KIC oxidase activity was 27% with a final specific activity of 104 nmole/min/mg protein.

TABLE 9

PURIFICATION OF α-KETOISOCAPROATE OXIDASE FROM RAT LIVER

| | Fraction | Volume (mL) | Total Protein (mg) | Total Activity (nmole/min) | Specific Activity (nmole/min/mg) | % Recovery | Fold Pure |
|---|---|---|---|---|---|---|---|
| 1. | 10,000 x g supernatant | 7,200 | 104,200 | 58,600 | 0.56 | (100) | (1.0) |
| 2. | 45-75% $(NH_4)_2SO_4$ fraction | 1,200 | 34,700 | 43,100 | 1.24 | 74 | 2.2 |
| 3. | Pre-DEAE dialysate | 1,400 | 20,900 | 34,800 | 1.67 | 59 | 3.0 |
| 4. | DEAE-cellulose pool | 1,770 | 4,000 | 30,500 | 7.62 | 52 | 13.6 |
| 5. | Concentrated DEAE pool | 250 | 4,200 | 29,200 | 6.95 | 50 | 12.4 |
| | Only 116 mL of the "concentrated DEAE pool" was carried through the remainder of the purification | | | | | | |
| 6. | Pre-phenyl sepharose | 120 | 1,800 | 12,400 | 6.87 | 45[a] | 12.3 |
| 7. | Phenyl sepharose pool | 675 | 513 | 9,790 | 19.1 | 36[a] | 34.1 |
| 8. | Phenyl pool concentrated | 20 | 474 | 10,700 | 22.6 | 39[a] | 40.3 |
| 9. | Sephacryl S-200 pool | 60 | 71 | 7,450 | 104 | 27[a] | 186 |

α-Ketoisocaproate oxidase activity was purified from rat liver as described in Methods (Purification B) and activity measured by Method B.

[a]Recovery is corrected to show expected recovery if all of the "concentrated DEAE pool" had been carried through the purification. i.e.: % Recovery = (Total Activity ÷ 58,600)(250 ÷ 116).

Figure 8. DEAE-Cellulose Chromatography of α-Ketoisocaproate Oxidase. Crude preparations of α-KIC oxidase obtained by 45-75% ammonium sulfate fractionation were dialyzed and applied to a DEAE cellulose column. α-KIC oxidase activity was eluted with a 0-0.2 M NaCl gradient (see Methods, Purification B) and assayed as in Methods (Method B).

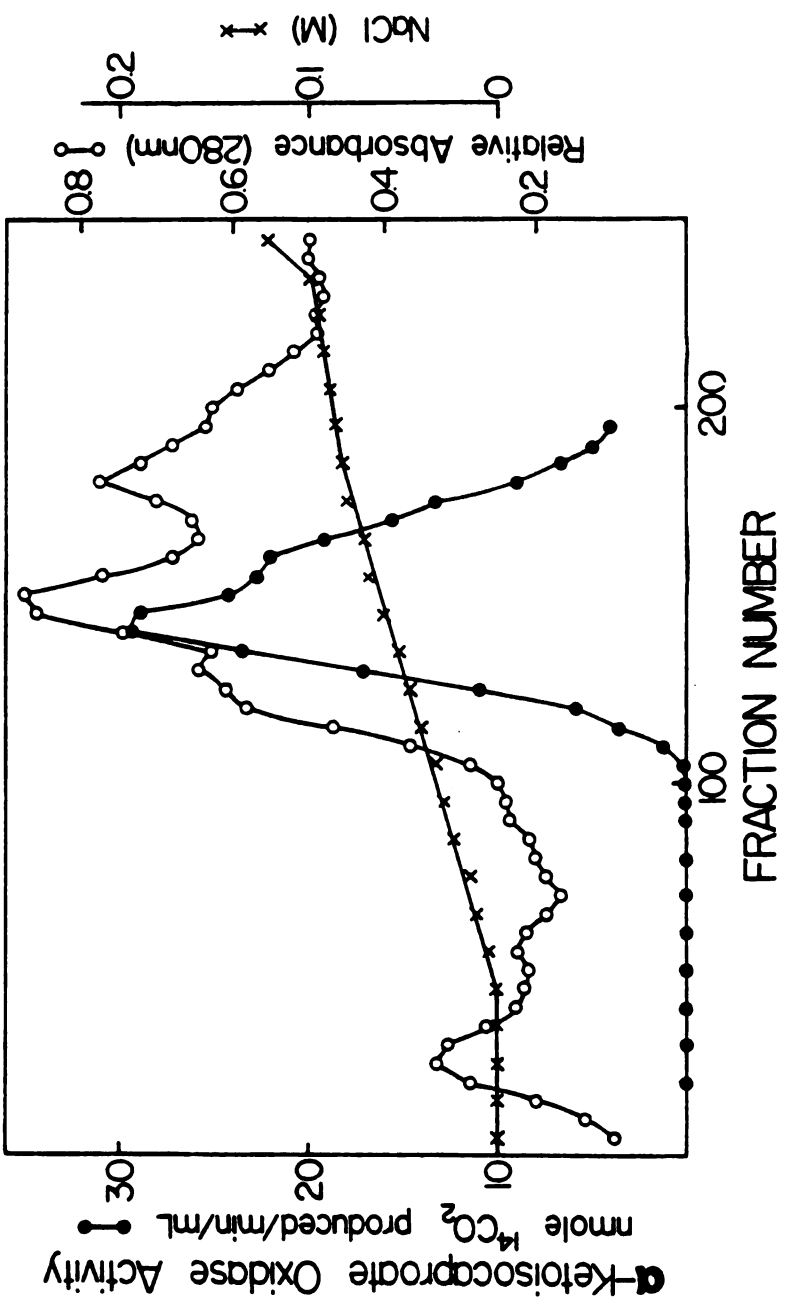

α-Ketoisocaproate Oxidase Activity
nmole $^{14}CO_2$ produced/min/mL
Relative Absorbance (280nm)
NaCl (M)
FRACTION NUMBER
0
10
20
30
0.2
0.4
0.6
0.8
0
10
0.2
100
200

Figure 8

Figure 9. Phenyl-Sepharose Chromatography of α-Ketoisocaproate Oxidase. Peak fractions off the DEAE-cellulose column (Figure 8) were concentrated, made up to 2.5 M NaCl, 5% monothioglycerol, and applied to a phenyl sepharose column (see Methods, Purification B). The column was eluted with 20 mM Tris HCl pH 7.8, 1% isopropanol, 5% monothioglycerol, 2.5 M NaCl. At fraction number 70 a 2 Liter linear gradient of 2.5-0.0 M NaCl in 20 mM Tris HCl pH 7.8, 1% isopropanol, 5% monothioglycerol was started and elution continued. α-KIC oxidase (Method B) and protein were assayed as in Methods.

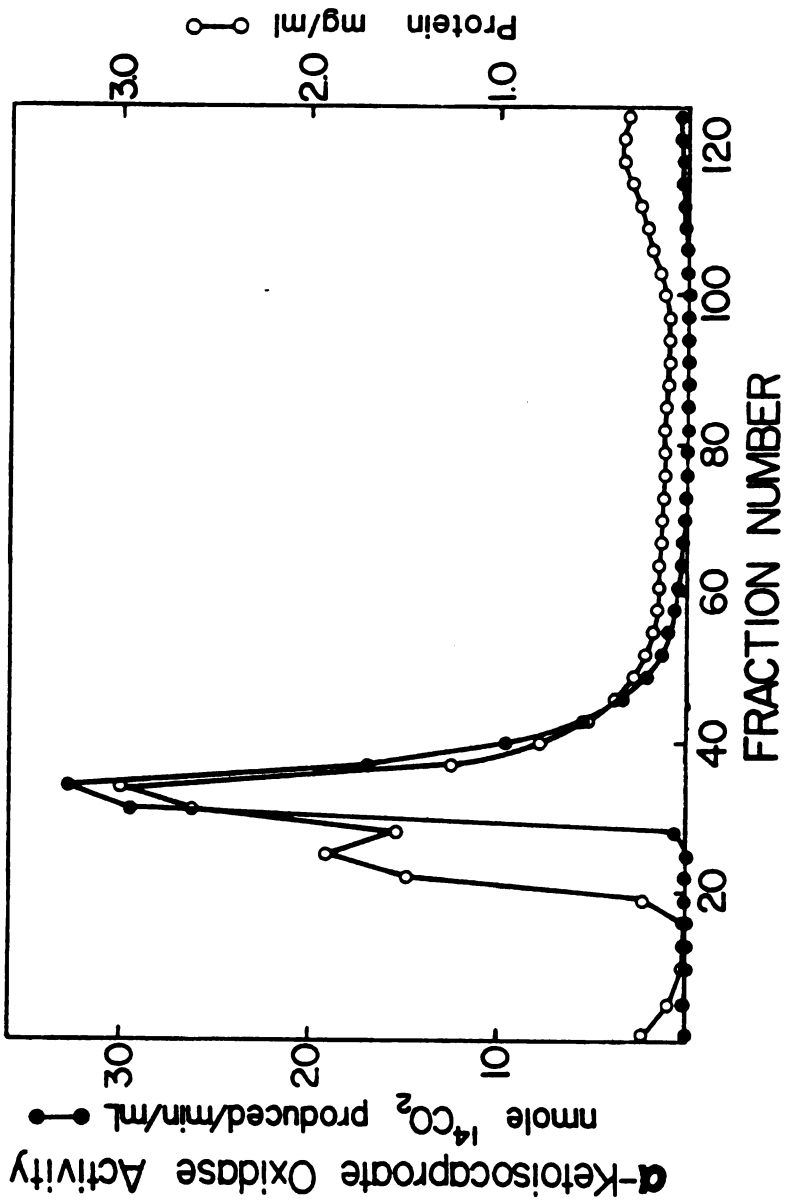

α-Ketoisocaproate Oxidase Activity
nmole $^{14}CO_2$ produced/min/mL
Protein mg/ml
FRACTION NUMBER
10
20
30
1.0
2.0
3.0
20
40
60
80
100
120

Figure 9

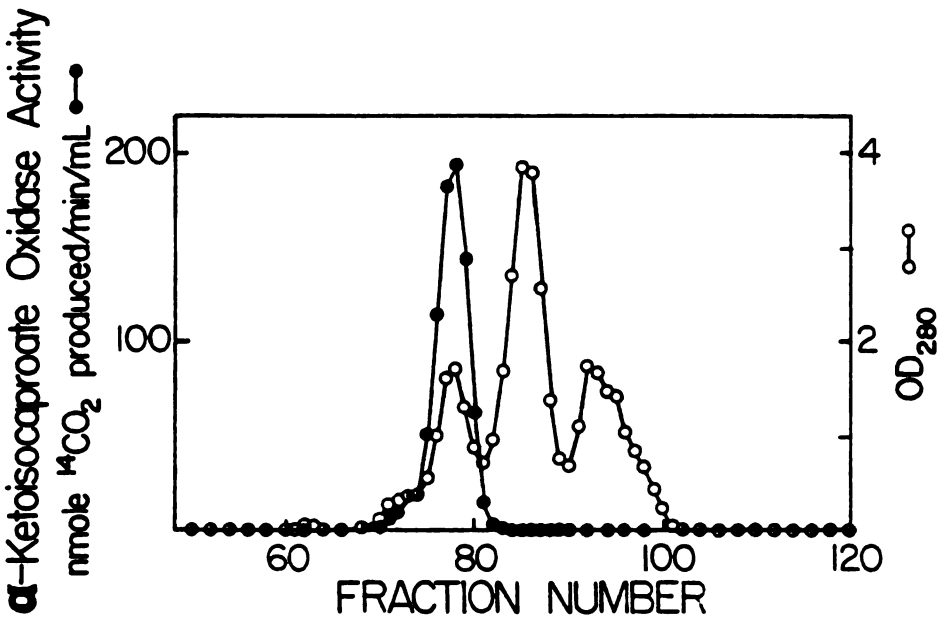



Figure 10. Sephacryl S-200 Chromatography of α-Ketoisocaproate Oxidase. Peak fractions from the phenyl sepharose column (Figure 9) were concentrated, applied to a sephacryl S-200 column and eluted as described in Methods. α-Ketoisocaproate oxidase was measured as in Methods (Method B) and protein monitored by absorbance at 280 nm.

III. Physical Properties of Rat Liver α-Ketoisocaproate Oxidase

SDS gel electrophoresis of the "Sephacryl S-200 pool" (Purification B, Table 9) revealed one major protein band with several minor protein bands (Figure 11). When electrophoresis was carried out under non-denaturing conditions, α-KIC oxidase activity migrated with the major protein band (Figure 12) indicating that this protein is the α-KIC oxidase.

The subunit molecular weight of α-KIC oxidase determined by SDS gel electrophoresis (Figure 11) was 46,000 (Figure 13). Molecular weight of the α-KIC oxidase was also determined under non-denaturing conditions, that is, Sephacryl S-200 chromatography, according to the method of Andrews (127). A molecular weight of 51,000 was obtained by this method (Figure 14). This enzyme appears to be a monomer. The 10% difference in molecular weights determined under denaturing and non-denaturing conditions may be due to assymetry of this protein.

IV. Kinetic and Catalytic Properties of the Purified α-Ketoisocaproate Oxidase

A. Stability of the Purified α-Ketoisocaproate Oxidase

The purified α-KIC oxidase (Sephacryl S-200 pool, Table 9) was quite stable at 4°C in the presence of 5% monothioglycerol. Only 15% of the initial activity was lost over 3 weeks (Figure 15). By 70 days, however, all of the α-KIC oxidase activity was lost. This may be due to the gradual autooxidation of the monothioglycerol which stabilizes the enzyme.


Figure 11. SDS-Polyacrylamide Gel Electrophoresis of α-Ketoisocaproate Oxidase. Gel electrophoresis of 60 µg (A), 24 µg (B) or 12 µg (C) of the purified α-KIC oxidase ("Sephacryl S-200 Pool, Table 9) was carried out as in Methods. Molecular weight standards (column D) are glycogen phosphorylase a (94,000), bovine serum albumin (66,000), ovalbumin (45,000), trypsinogen (24,000) and β-lactoglobulin (18,400).

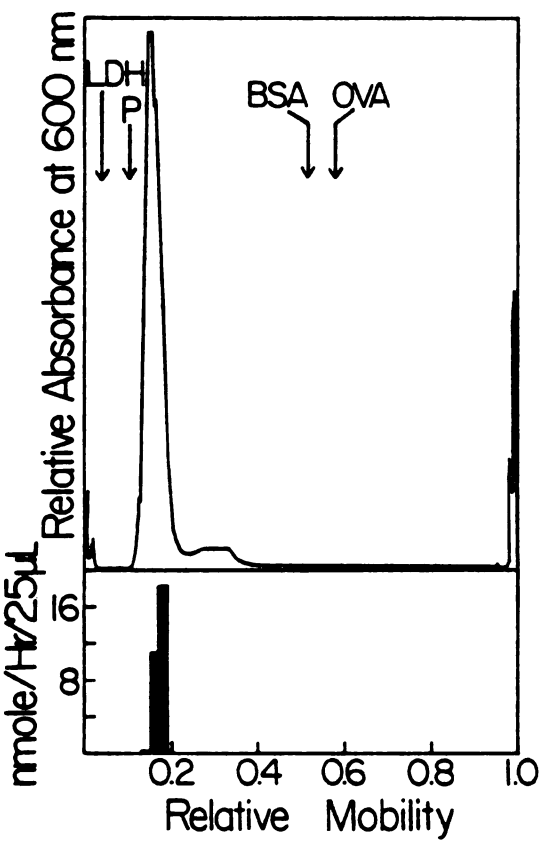



Figure 12. Native Gel Electrophoresis of α-Ketoisocaproate Oxidase. A 30 µL (36 µg protein) aliquot of the purified α-KIC oxidase (Sephacryl S-200 pool, Table 9) was applied to two separate gels and electrophoresis carried out as in Methods. Protein (upper figure) and α-KIC oxidase activity (lower figure) were monitored as in Methods.

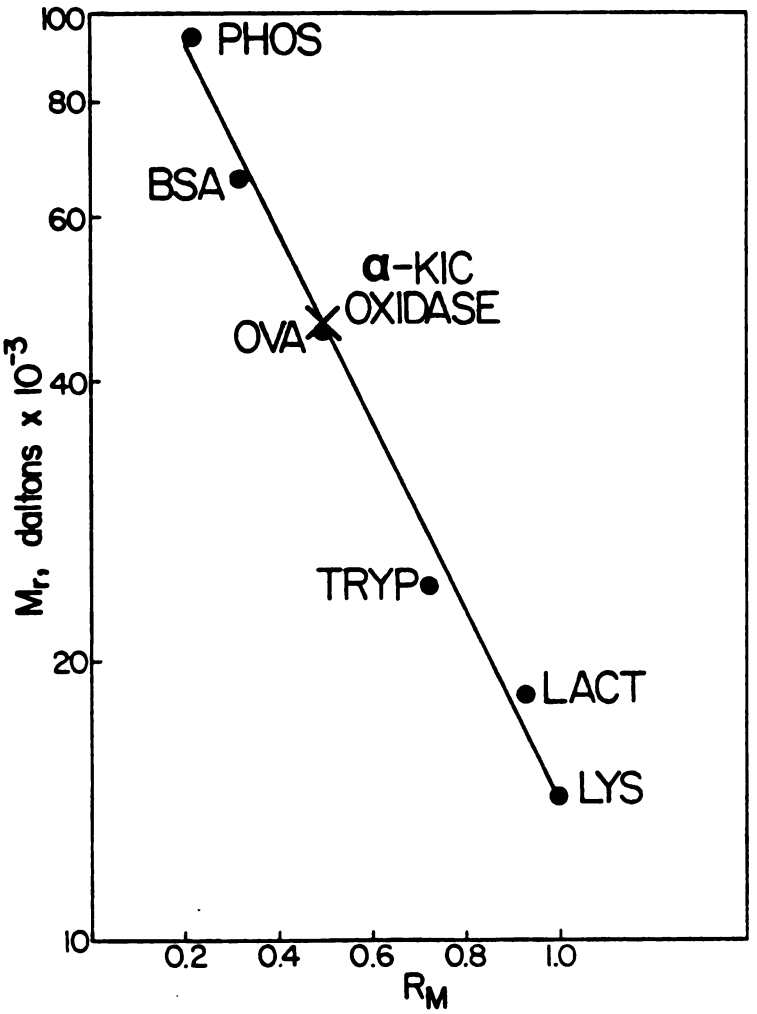



Figure 13. Determination of the Subunit Molecular Weight of α-Ketoisocaproate Oxidase. The data were obtained from the SDS-polyacrylamide gel shown in Figure 11. Relative mobility ($R_m$) was determined by measurement of the migration of the protein relative to migration of bromophenol blue. Abbreviations and molecular weights are PHOS, glycogen phosphorylase a (94,000); BSA, bovine serum albumin (66,000); OVA, ovalbumin (45,000); TRYP, trypsinogen (24,000); LACT, β-lactoglobulin (18,400); LYS, lysozyme (14,300).

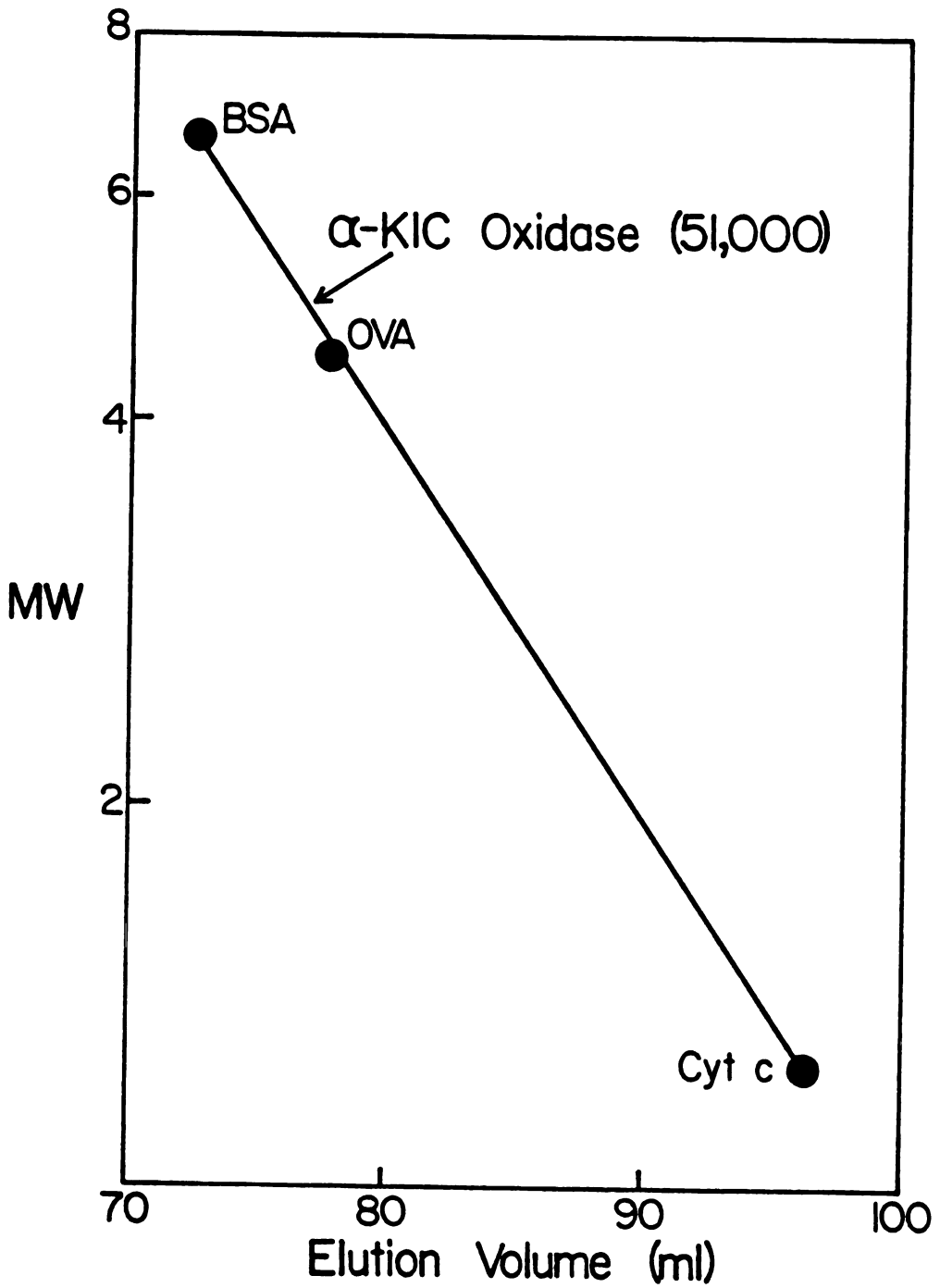



Figure 14. Molecular Weight of the α-Ketoisocaproate Oxidase Determined by Sephacryl S-200 Chromatography. The molecular weight of the purified α-KIC oxidase (Sephacryl S-200 pool, Table 9) was determined by Sephacryl S-200 chromatography as described in Methods.

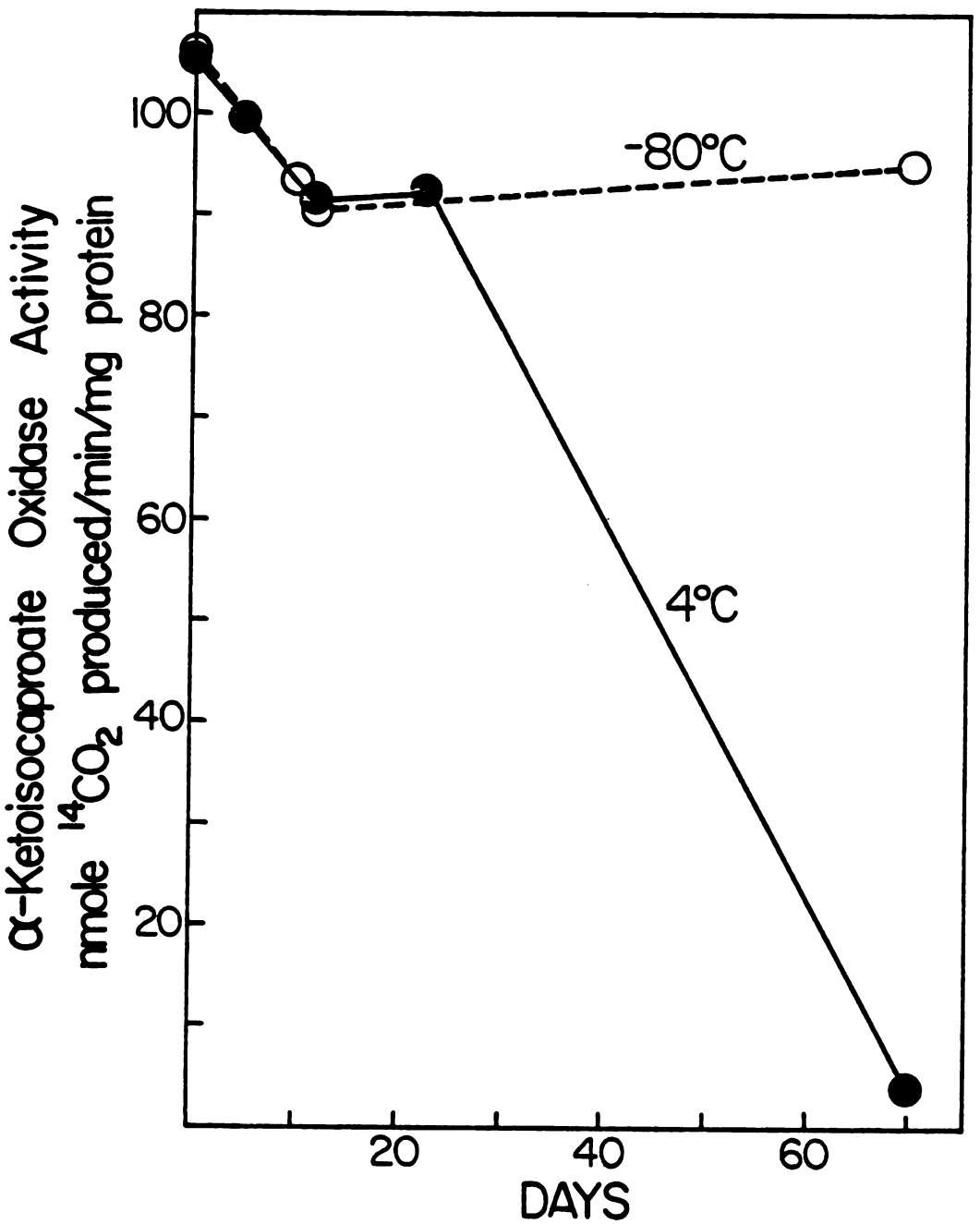



Figure 15. Stability of the Purified α-KIC Oxidase. Aliquots of the purified α-KIC oxidase (Sephacryl S-200 pool, Table 9) were stored at 4°C or -80°C. α-KIC oxidase activity was measured at various time intervals as described in Methods (Method B). Values are mean of duplicate assays.

At -80°C, there is initially a 15% loss of α-KIC oxidase activity, possibly due to freezing and thawing, but then the activity is stable for at least 70 days. Routinely, small aliquots of the purified α-KIC oxidase (Sephacryl S-200 pool) were stored separately at -80°C and thawed immediately prior to use.

B. Metal Requirement of the α-Ketoisocaproate Oxidase

The effect of a wide variety of metal ions on the α-KIC oxidase activity was tested (Table 10). In the presence of ascorbate and dithiothreitol, ferrous iron ($FeSO_4$ and $FeCl_2$) and ferric iron ($FeCl_3$) activated the α-KIC oxidase activity. In the absence of added iron, but with ascorbate and dithiothreitol included in the assay, activity was 25% of that in the presence of iron. If ascorbate and dithiothreitol as well as iron are omitted, very little activity is observed. α-KIC oxidase activity, observed in the presence of ascorbate and dithiothreitol when iron is omitted from the assay, may be due to contaminating iron in the buffers used. o-Phenanthroline, an iron chelator, abolishes most of the α-KIC oxidase activity obtained in the presence of ascorbate and dithiothreitol. A high non-enzymatic decarboxylation of α-KIC (Blank Activity) is observed in the presence of 1 mM o-phenanthroline. This high rate of non-enzymatic decarboxylation occurred only when both o-phenanthroline and ascorbate were included in the assay mixture, but the mechanism is unknown.

$CsCl_2$, $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnCl_2$, $CdCl_2$ and $NiCl_2$ had no effect on α-KIC oxidase activity at a

TABLE 10

EFFECT OF VARIOUS METAL IONS ON α-KETOISOCAPROATE OXIDASE ACTIVITY

| Addition (or deletion) | α-KIC Oxidase Activity nmole/min/mg | Blank Activity nmole/min/mg | % of Control Activity |
|---|---|---|---|
| None | 23.5 | 1.7 | 25 |
| None (-ascorbate, -DTT) | 1.3 | 2.2 | 1 |
| 1 mM o-phenanthroline | 5.1 | 41.1 | 5 |
| 1 mM $FeSO_4$ | 93.1 | 0.6 | (100) |
| 1 mM $FeCl_2$ | 99.3 | 0.6 | 106 |
| 1 mM $FeCl_3$ | 92.7 | 0.6 | 99 |
| 1 mM $CoCl_2$ | 19.8 | 1.8 | 21 |
| 1 mM $HgCl_2$ | 15.1 | 0.8 | 16 |
| 1 mM $CaCl_2$ | 24.8 | 1.5 | 27 |
| 1 mM $CuCl_2$ | 9.4 | 1.5 | 10 |
| 1 mM CuCl | 9.7 | 1.5 | 10 |
| 1 mM $CuSO_4$ | 9.7 | 1.4 | 10 |
| 1 mM $MgCl_2$ | 24.8 | 2.0 | 27 |
| 1 mM $MnCl_2$ | 25.1 | 1.7 | 27 |
| 1 mM $ZnCl_2$ | 27.0 | 1.7 | 29 |
| 1 mM $CdCl_2$ | 26.2 | 1.1 | 28 |
| 1 mM $NiCl_2$ | 20.0 | 2.2 | 21 |

α-Ketoisocaproate oxidase activity was assayed as in Methods (Method B) except $FeSO_4$ was replaced by the addition shown. All assays contained 1 mM dithiothreitol (DTT) and 0.5 mM ascorbate except where indicated otherwise. Each assay contained 10 µL (12 µg protein) of Sephacryl S-200 pool (Purification B). Values are mean of 2 replicate assays. Blank activity was measured in duplicate assays in which the Sephacryl S-200 pool was replaced by 10 µL 20 mM Tris HCl pH 7.8, 1% isopropanol, 0.1 M NaCl, 5% monothioglycerol. Activity is expressed in nmole/min/mg protein.

concentration of 1 mM. $HgCl_2$ and cuprous or cupric chloride were inhibitory. The α-KIC oxidase, therefore, appears quite specific in its requirement for iron.

The ability of ferric iron ($Fe^{3+}$) to replace ferrous iron ($Fe^{2+}$) in the activation of the α-KIC oxidase could be due to reduction of $Fe^{3+}$ to $Fe^{2+}$ by ascorbate. To test this possibility, the effect of ascorbate and dithiothreitol on α-KIC oxidase activity in the presence of $FeSO_4$, $FeCl_3$ or no added iron was determined (Figure 16).

In the presence of ascorbate and dithiothreitol, $FeSO_4$ and $FeCl_3$ produce approximately the same activation of the α-KIC oxidase. In the absence of added iron the activity is 72% lower, as seen previously (Table 10). The omission of dithiothreitol from the assay has no significant affect on these results. If ascorbate is omitted from the assay, activation of the enzyme by $FeSO_4$ decreases slightly (17%), whereas activation by $FeCl_3$ decreases 50%. In the absence of iron very little activity is found. The omission of both ascorbate and dithiothreitol from the assay produce the same results as the omission of ascorbate alone.

Previous studies (see Table 6) indicated that the α-KIC oxidase required a sulfhydryl reducing reagent, such as dithiothreitol, for activation of the α-KIC oxidase activity. No effect of dithiothreitol on the enzyme activity is apparent in Figure 16. The enzyme sample being assayed in Figure 16 contained 0.6 M monothioglycerol. When 10 μL of this sample was assayed, a final concentration of 15 mM monothioglycerol was introduced into the

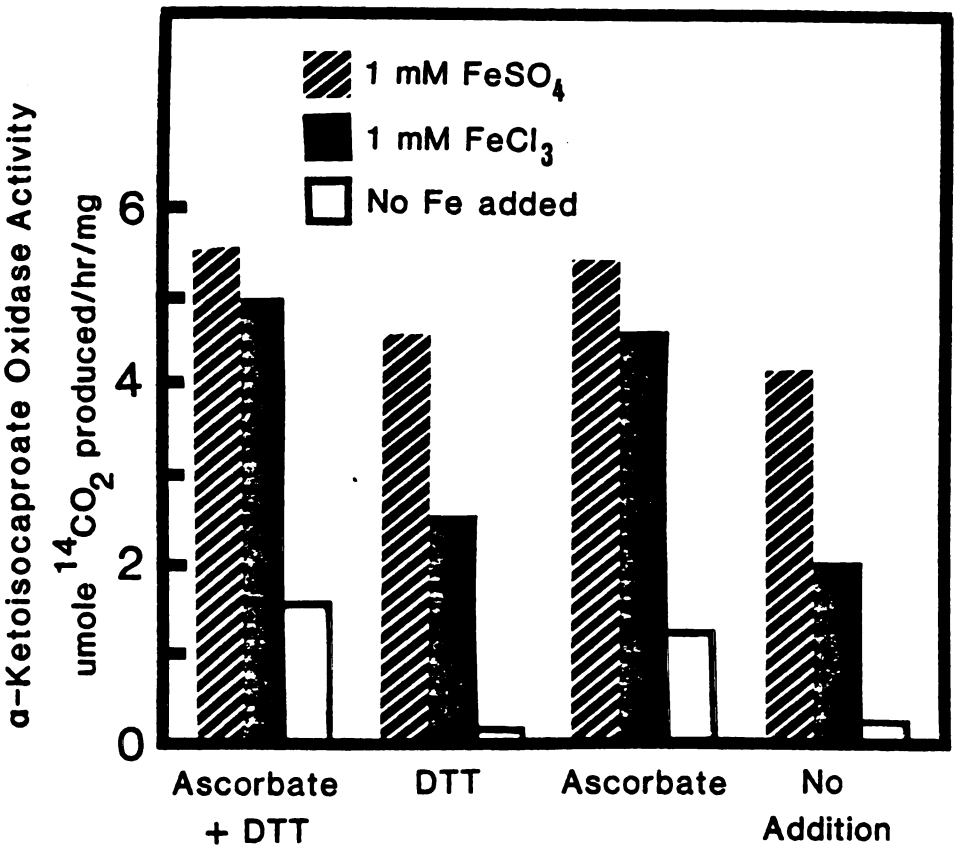



Figure 16. Effect of Dithiothreitol and Ascorbate on Activation of the α-Ketoisocaproate Oxidase by $FeSO_4$ or $FeCl_3$. α-KIC oxidase activity was measured as in Methods (Method B) except $FeSO_4$, ascorbate and dithiothreitol were only added as indicated. Final assay concentrations were: $FeSO_4$, 1 mM; $FeCl_2$, 1 mM; ascorbic acid, 0.5 mM; dithiothreitol (DTT), 1 mM. Each assay contained 10 µL (12 µg protein) of the α-KIC oxidase (Sephacryl S-200 pool; Table 9). The monothioglycerol in the Sephacryl S-200 pool gave a final assay concentration of 0.125% (15 mM) monothioglycerol. Values are mean of duplicate assays.

assay mixture. Therefore, monothioglycerol most likely provided the reduced sulfhydryl groups.

These results suggest that the reduced form of iron, $Fe^{2+}$, is utilized by the enzyme. In the presence of ascorbate, ferric iron ($Fe^{3+}$) may be reduced to supply the enzyme with $Fe^{2+}$. In the absence of ascorbate and dithiothreitol ferric iron still activates the α-KIC oxidase to some extent. The reason for this is presently unclear. Monothioglycerol, which is present in all assays at a concentration of 15 mM due to carry over into the assay with the enzyme, may reduce $Fe^{3+}$ to $Fe^{2+}$.

$FeSO_4$ gave optimal activation of the α-KIC oxidase between 1.0 mM and 5.0 mM (Figure 17). Concentrations above 5 mM became progressively inhibitory.

The effect of ascorbate on α-KIC oxidase activity was tested at suboptimal (0.05 mM) and optimal (2 mM) concentrations of $FeSO_4$ (Figure 18). In the presence of 0.05 mM $FeSO_4$, 1.0 mM ascorbate increased α-KIC oxidase activity 67%. However, in the presence of 2 mM $FeSO_4$, ascorbate had very little effect on α-KIC oxidase activity. The stimulatory effect of ascorbate, therefore, may be solely due to its capacity to keep iron in the reduced, ferrous state. As noted earlier (Table 6), NADH and NADPH can replace ascorbate as a source of reducing equivalents.

C. Activation of the α-Ketoisocaproate Oxidase

In routine assays of α-KIC oxidase activity, the enzyme was preincubated with $FeSO_4$, ascorbate and dithiothreitol in the

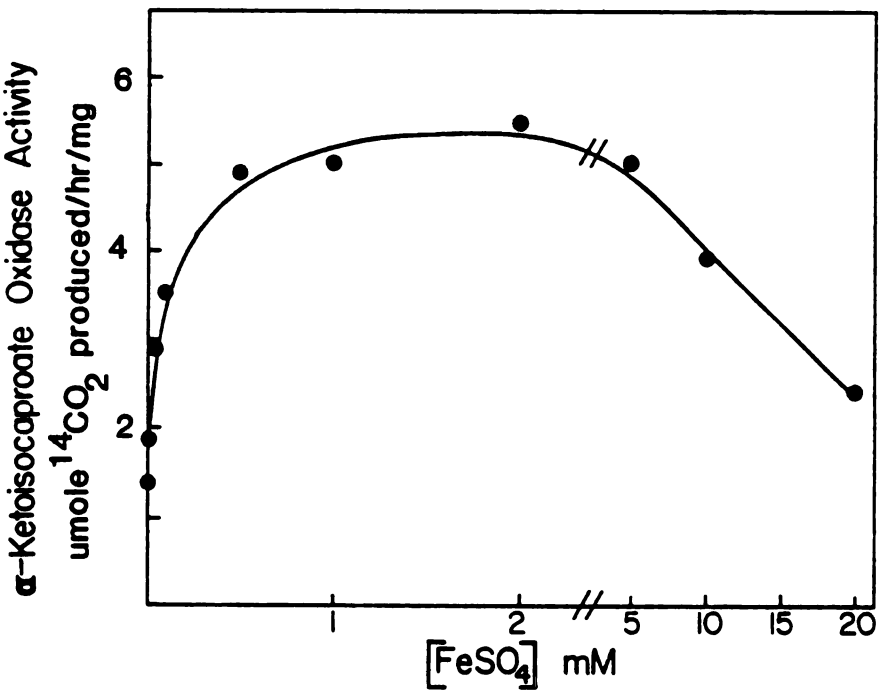



Figure 17. Effect of $FeSO_4$ Concentration on α-Ketoisocaproate Oxidase Activity. α-KIC oxidase activity was measured as in Methods (Method B) except for the variation of the $FeSO_4$ concentration. Each assay contained 10 µL (12 µg protein) of the α-KIC oxidase (Sephacryl S-200 pool; Table 9). Values are mean of duplicate assays.

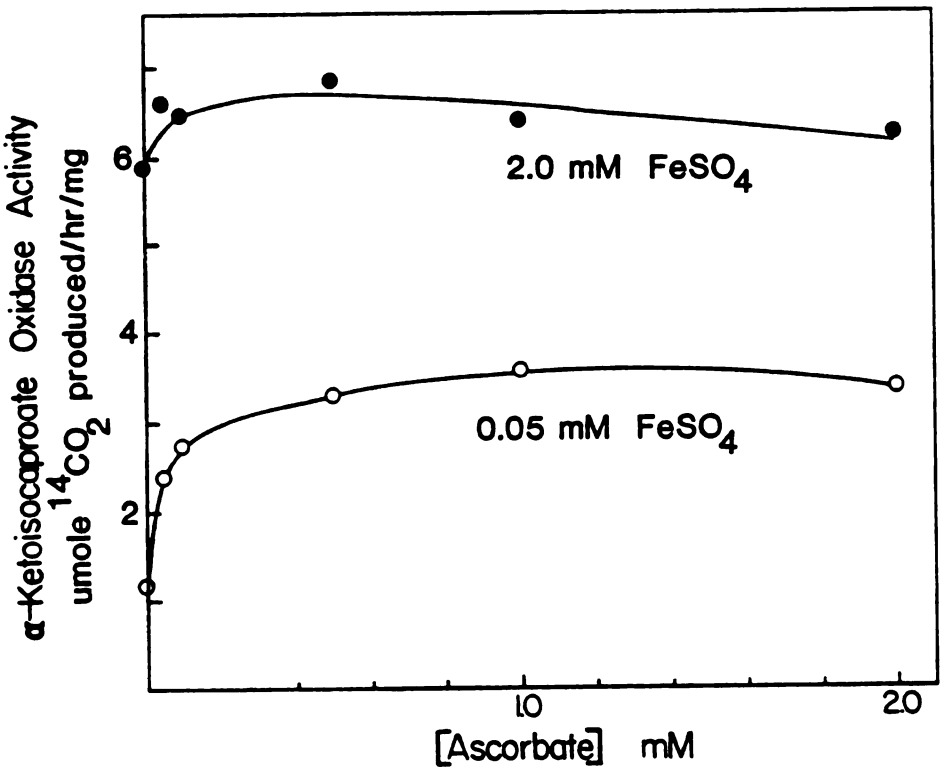



Figure 18. Effect of Ascorbate on α-Ketoisocaproate Oxidase Activity in the Presence of Optimal and Suboptimal Concentrations of $FeSO_4$. α-KIC oxidase activity was measured as in Methods (Method B) except each assay contained either 2 mM (solid circles) or 0.05 mM $FeSO_4$ (open circles) and the concentration of ascorbate was varied. Each assay contained 10 µL (12 µg protein) of the α-KIC oxidase (Sephacryl S-200 pool; Table 9). Values are mean of duplicate assays.

assay mixture for one hour. α-[1-$^{14}$C] KIC was then added to the assay and release of $^{14}CO_2$ monitored. From this type of assay it was not possible to determine whether $FeSO_4$ was only required for activation of the enzyme (i.e. during the pre-incubation) or if $FeSO_4$ was required during the enzymatic reaction. The experiments shown in Table 11 were designed to answer this question.

In these experiments the purified α-KIC oxidase preparation (Sephacryl S-200 pool, Table 9) was incubated at 25°C for 1 hour with or without 0.5 mM $FeSO_4$, 1.0 mM dithiothreitol (activation mixture). Ascorbate was omitted from the reactions to reduce the stimulation of activity caused by carry over of the activation mixture into the assay mixture. After the 1 hr activation period, 10 μL of the activation mixture was assayed for α-KIC oxidase activity in the presence or absence of $FeSO_4$ and dithiothreitol. When $FeSO_4$ and dithiothreitol were omitted from the assay mixture, the assay concentrations of $FeSO_4$ and dithiothreitol were 0.0125 and 0.025 mM, respectively, due to carry over from the "activation mixture".

If the α-KIC oxidase is incubated without $FeSO_4$ and dithiothreitol during the "activation period", very little activity is obtained when these compounds are omitted from the assay mixture (Experiments 6 and 8). The inclusion of $FeSO_4$ and dithiothreitol in the assay mixture increased α-KIC oxidase activity, as expected (Experiments 5 and 7).

When the α-KIC oxidase is incubated with $FeSO_4$ and dithiothreitol during the "activation period", α-KIC oxidase activity

TABLE 11

ACTIVATION OF α-KETOISOCAPROATE OXIDASE BY $FeSO_4$

| Experiment | 0.5 mM $FeSO_4$, 1 mM DTT Present During Activation Period | Final Assay Concentration | | Assay Preincubation Time (min) | α-KIC Oxidase Activity μmole/hr/mg protein |
|---|---|---|---|---|---|
| | | $FeSO_4$ (mM) | DTT (mM) | | |
| 1 | Yes | 0.5 | 1.0 | 4 | 2.92 |
| 2 | Yes | 0.0125 | 0.025 | 4 | 1.37 |
| 3 | Yes | 0.5 | 1.0 | 60 | 5.20 |
| 4 | Yes | 0.0125 | 0.025 | 60 | 2.91 |
| 5 | No | 0.5 | 1.0 | 4 | 1.88 |
| 6 | No | 0.0125 | 0.025 | 4 | 0.08 |
| 7 | No | 0.5 | 1.0 | 60 | 3.91 |
| 8 | No | 0.0125 | 0.025 | 60 | 0.23 |

The "Sephacryl S-200 Pool" (Purification B, Table 9) was incubated for 1 hr at 25°C with or without 0.5 mM $FeSO_4$ and 1.0 mM dithiothreitol (DTT). After this "activation period", 10 μL of the sample was assayed as in Methods (Method B) except ascorbate was omitted, $FeSO_4$ and dithiothreitol were present in the concentrations indicated above, and the preincubation period was varied as shown. $FeSO_4$ and dithiothreitol present in assays of experiments 2 and 4 was due to carry over from the enzyme being assayed. For control purposes, this same amount of $FeSO_4$ and dithiothreitol was added to assays of experiments 6 and 8. Ascorbate was omitted from these experiments, and $FeSO_4$ reduced to 0.5 mM in order to reduce background activity due to carry over from the activation mixture into the assay. Values are mean of duplicate assays.

is significantly elevated compared to the activity when these compounds are omitted during the "activation period" (compare Experiments 1-4 to 5-8). If the α-KIC oxidase activity is measured in the absence of $FeSO_4$ and dithiothreitol very little activity is observed (Experiments 6 and 8). However, if the enzyme is first "activated" with $FeSO_4$ and dithiothreitol a large increase in activity is seen (Experiments 2 and 4). α-KIC oxidase activity is always greater when $FeSO_4$ and dithiothreitol are included in the assay mixture itself (Experiment 1 vs. 2 or 3 vs. 4). Activity was also greater when the enzyme was preincubated in the assay mixture for 60 min as opposed to 4 min.

These experiments indicate that the α-KIC oxidase can be activated by $FeSO_4$ and dithiothreitol prior to the enzymatic decarboxylation of α-KIC. This "activated enzyme" retains its activity even when assayed in the absence of $FeSO_4$ and dithiothreitol (Experiment 2 and 4).

The "activated" α-KIC oxidase has higher activity when 0.5 mM $FeSO_4$ and 1.0 mM dithiothreitol are included in the assay mixture, than in the absence of these compounds. The lower activity in the absence of $FeSO_4$ and dithiothreitol was found to be due to a time dependent loss of α-KIC oxidase activity after removal of these compounds (Figure 19). After activation of the α-KIC oxidase (Sephacryl S-200 Pool, Table 9) with 1 mM $FeSO_4$, 0.5 mM ascorbate, 1 mM dithiothreitol, the activity was monitored vs. time in an assay mixture containing 1 mM $FeSO_4$, 0.5 mM ascorbate, 1 mM dithiothreitol (solid squares), or in an assay mixture without these compounds (solid circles). α-KIC oxidase activity

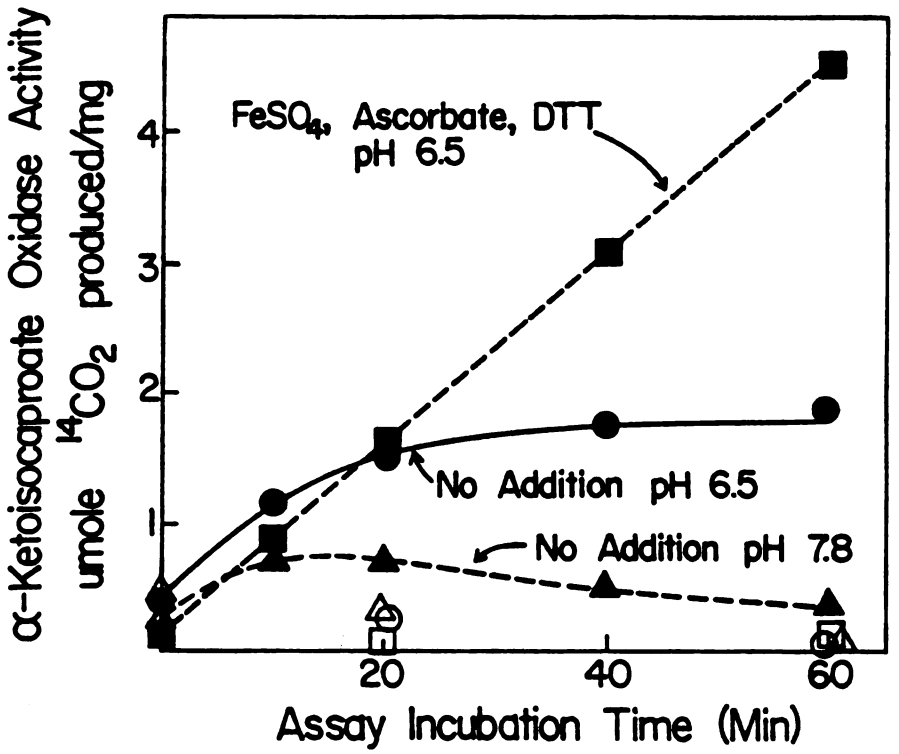



Figure 19. Stability of the "Activated" α-Ketoisocaproate Oxidase in the Presence or Absence of $FeSO_4$, Ascorbate and Dithiothreitol. The α-KIC oxidase was "activated" by incubation for 1 hr at 25°C in a mixture containing in a final volume of 0.25 mL: 0.2 mL (0.24 mg protein) purified α-KIC oxidase (Sephacryl S-200 pool, Table 9; solid symbols) or 0.2 mL buffer (20 mM Tris HCl pH 7.8, 1% isopropanol, 0.1 M NaCl, 5% thioglycerol, open symbols); 1.0 mM $FeSO_4$, 0.5 mM ascorbic acid and 1.0 mM dithiothreitol. Then, 10 µL of this mixture was assayed vs. time as in Methods (Method B) except the preincubation was omitted and the incubation time was varied (solid and open squares). In two sets of experiments, $FeSO_4$, ascorbate and dithiothreitol were omitted from the assay (open and closed circles and triangles) and in one of these experiments, the pH of the Tris-maleate buffer was adjusted to 7.8 with NaOH (open and closed triangles). Open symbols designate the "blank" activity. The blank activity has not been subtracted from total activity (solid symbols). Values are mean of duplicate assays.

was linear vs. time for 60 min when $FeSO_4$, ascorbate, and dithiothreitol were included in the assay mixture. The initial activity of the "activated" α-KIC oxidase in the absence of $FeSO_4$, ascorbate, and dithiothreitol was slightly greater than in the presence of these compounds. However, the activity was rapidly lost when these compounds were omitted from the assay mixture. The α-KIC oxidase activity was even lower when the "activated" enzyme was assayed in the absence of $FeSO_4$, ascorbate and dithiothreitol at pH 7.8 (solid triangles).

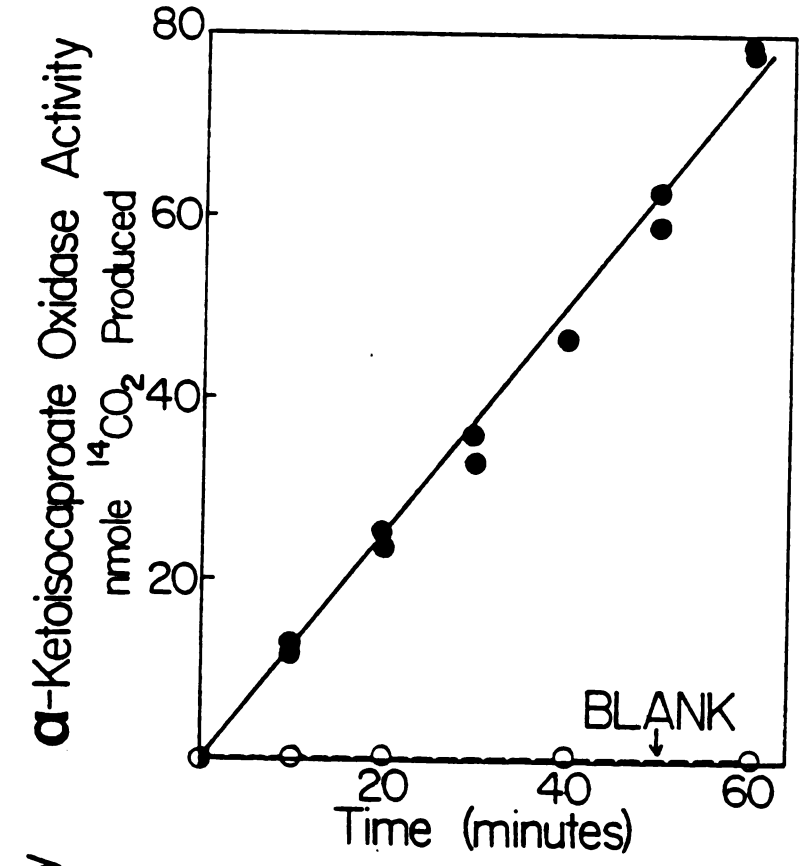
In summary, the α-KIC oxidase is "activated" in the presence of $FeSO_4$, ascorbate and dithiothreitol. After removal of these compounds (i.e. by dilution) the α-KIC oxidase remains active for a short period of time before returning to an inactive state. In order to carry out reactions which are linear with time for up to 1 hr, $FeSO_4$, ascorbate and dithiothreitol must be included in the assay mixture.

D. Optimal Assay Conditions for the α-Ketoisocaproate Oxidase

The α-KIC oxidase activity was linearly related to time (up to 60 minutes) and protein concentration (up to 6 μg); see Figure 20. The α-[1-$^{14}$C] KIC decarboxylating activity in the absence of enzyme (Figure 20; Blank) was negligible compared to the enzymatic activity.

When a large number of assays were undertaken, the apparent α-KIC oxidase activity, as measured by release of $^{14}CO_2$ from α-[1-$^{14}$C] KIC, decreased gradually. Since the purified α-KIC oxidase is stable for several weeks at 4°C, this loss of activity

Figure 20. Effect of Time and Protein Concentration on the α-Ketoisocaproate Oxidase Activity. α-KIC oxidase activity was measured in the Sephacryl S-200 pool (Table 9) as in Methods (Method B) except for the variation of time (upper figure) or protein concentration (lower figure).


α-Ketoisocaproate Oxidase Activity
nmole $^{14}CO_2$ Produced
80
60
40
20
BLANK
20
40
60
Time (minutes)

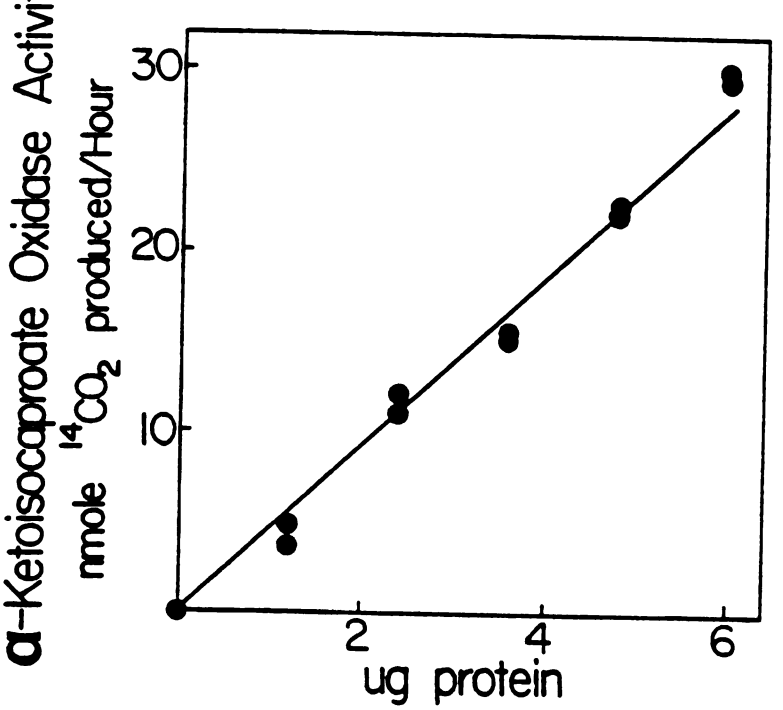

α-Ketoisocaproate Oxidase Activity
nmole $^{14}CO_2$ produced/Hour
30
20
10
2
4
6
ug protein

Figure 20

is most likely not due to instability of the enzyme, but rather instability of some component of the assay mixture. Indeed, when the cofactor mixture (containing $FeSO_4$, ascorbate and dithiothreitol; see Methods) was added to the assay mixture 100 min before addition of the enzyme, a 15% loss of activity was observed (Experiment C, Table 12) compared to when the enzyme was added immediately (Experiment A) or 49 min after addition of the cofactor mixture (Experiment B). The longer the cofactor mixture remained in the Tris, maleate buffer before addition of enzyme, the lower the observed α-KIC oxidase activity (unpublished observation). The cofactor mixture itself was not unstable. The use of cofactor mixture which was set at 4°C for 101 min (Experiment D) gave the same results as fresh cofactor mixture (Experiment A and E). The enzyme (α-KIC oxidase) was also stable during this 120 min period (compare Experiments A and E). When added to the assay buffer, the cofactor mixture may gradually remove a vital component of the α-KIC oxidase reaction or slowly produce an inhibitor. It is quite likely that the cofactor mixture depletes the assay mixture of $O_2$ or a complexed form of $O_2$ which is required by the α-KIC oxidase for activity. When assays are all carried out within 50 min, no apparent loss of α-KIC oxidase activity was seen (Experiment B, Table 12). Therefore, the cofactor mixture could be added to all assays at the same time. However, when a large number of assays were carried out, the cofactor mixture was added to each assay immediately (within 30 sec) before the enzyme.

TABLE 12

STABILITY OF α-KETOISOCAPROATE OXIDASE ASSAY MIXTURE

| Experiment | Time of Addition of Cofactor Mix (min) | Time of Addition of Enzyme (min) | Amount of time between addition of Cofactor Mix and enzyme (min) | α-KIC oxidase activity μmole/hr/mg protein | % of Control |
|---|---|---|---|---|---|
| A | 2 | 2.5 | 0.5 | 6.20 | (100) |
| B | 1 | 50 | 49 | 6.18 | 99 |
| C | 0 | 100 | 100 | 5.27 | 85 |
| D | 101 | 101.5 | 0.5 | 6.55 | 105 |
| E | 120[a] | 120.5 | 0.5 | 6.71 | 108 |

α-Ketoisocaproate oxidase activity was measured as in Methods (Method B) except that the enzyme was added at various times after the addition of 25 μL of Cofactor Mix to the assay mixture. The Cofactor Mix contained 16 mM $FeSO_4$, 8 mM ascorbic acid, and 16 mM dithiothreitol giving a final assay concentration of 1 mM, 0.5 mM and 1 mM, respectively. Values are mean of duplicate assays.

The addition of Cofactor Mix to Experiment C was set as the zero time. All other times are relative to this. Both enzyme (Sephacryl S-200 pool; Table 9) and Cofactor Mix were kept at 4°C before addition to assay mixture. After addition of enzyme, the assays were carried out as described in Methods (Method B).

[a]In experiment E, Cofactor Mix was prepared fresh and used within 5 min, whereas in experiment D, the Cofactor Mix had set on ice for 101 min before use.

The pH optimum of the α-KIC oxidase was determined using several different buffers (Figure 21). The ionic strength was kept constant in these experiments. The pH optimum in a Tris-maleate buffer was at 6.0. α-KIC oxidase activity, however, was much lower when MES, MOPS, or Tris buffers were used. Above pH 7.0 the assay mixtures turned a reddish brown color presumably due to oxidation of $FeSO_4$.

The effect of the concentration of the Tris-maleate buffer and other buffers on α-KIC oxidase activity was determined (Table 13). Increasing concentrations of Tris-maleate caused an increase of α-KIC oxidase activity. When 50 mM MES (2[N-Morpholino]ethylene sulfonic acid) or 50 mM Bis Tris Propane (1,3-bis-[tris(hydroxymethyl)-methylamino]propane) are used as buffer, the α-KIC oxidase activity is lower than in 20 mM Tris, 20 mM maleate buffer. In the presence of phosphate buffer very little α-KIC oxidase activity is observed.

Ionic strength is not responsible for the variable α-KIC oxidase activity in different buffers. Increasing concentrations of NaCl had little effect on α-KIC oxidase activity (Figure 22). However, maleate concentration had a dramatic effect on activity (Figure 23). α-KIC oxidase activity increased 57% when the concentration of maleate was increased from 50 mM to 0.1 M. Optimal activity was obtained at 0.1 to 0.2 M maleate. Concentrations of maleate above 0.4 M were inhibitory. The concentration of Tris base had no effect on α-KIC oxidase activity. α-KIC oxidase in 0.2 M Tris, 0.2 M maleate, pH 6.5 was the same as in 50 mM Tris,

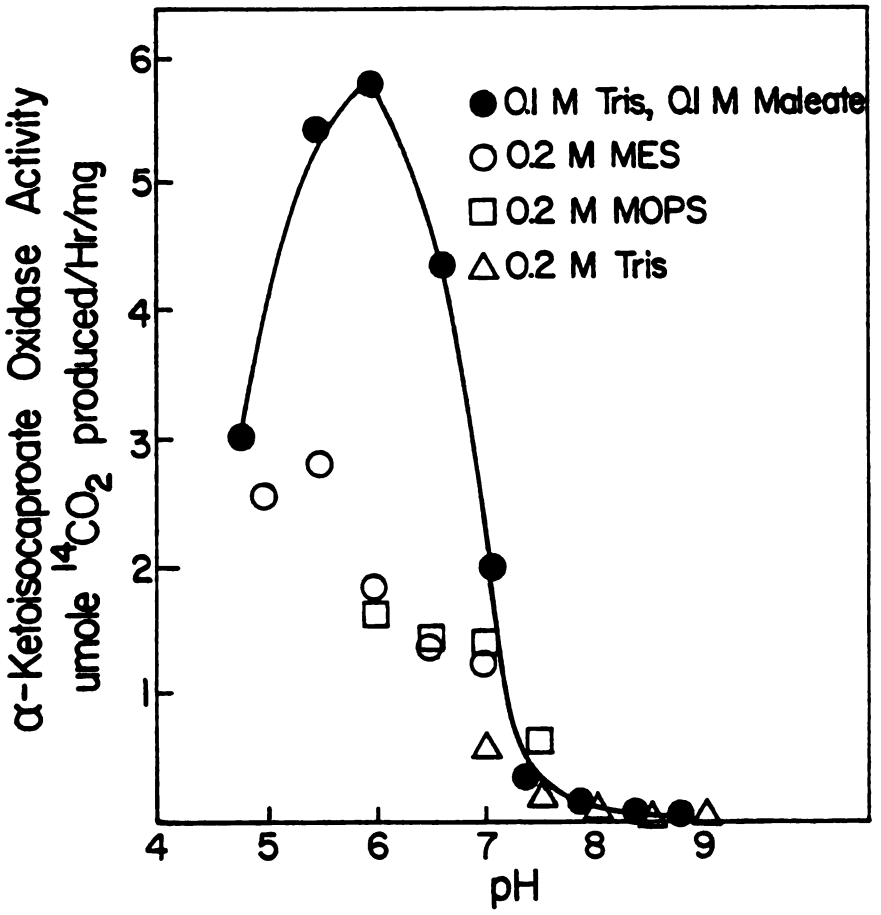



Figure 21. pH Optimum of the α-Ketoisocaproate Oxidase. α-KIC oxidase activity was assayed as in Methods (Method B) except the following buffers were used: 0.1 M Tris, 0.1 M maleic acid (solid circles); 0.2 M MES (open circles): 0.2 M MOPS (open squares); or 0.2 M Tris (open triangles). The pH of the buffers was adjusted with NaOH or HCl. The final ionic strength of all buffers was adjusted to 0.47 with NaCl. Each assay contained 10 µL (12 µg protein) of the Sephacryl S-200 pool (Table 9). Values are mean of duplicate assays.

TABLE 13

EFFECT OF VARIOUS BUFFERS ON α-KETOISOCAPROATE OXIDASE ACTIVITY

| Assay Buffer | α-Ketoisocaproate Oxidase Activity μmole/hr/mg protein |
|---|---|
| 0.2 M Tris, 0.2 M maleic acid pH 6.5 | 5.93 |
| 0.1 M Tris, 0.1 M maleic acid pH 6.5 | 4.40 |
| 50 mM Tris, 50 mM maleic acid pH 6.5 | 2.96 |
| 20 mM Tris, 20 mM maleic acid pH 6.5 | 2.31 |
| 50 mM sodium phosphate pH 6.5 | 0.46 |
| 50 mM MES pH 6.5 | 1.90 |
| 50 mM BisTris propane pH 6.5 | 1.54 |

α-Ketoisocaproate Oxidase activity was assayed as in Methods (Method B) except the buffer was varied as shown above. Each assay contained 10 μL (12 μg protein) of the Sephacryl S-200 Pool (Purification B). Abbreviations are: MES, 2[N-Morpholino]ethane sulfonic acid; BisTris propane, (1,3-bis[tris(hydroxymethyl)-methylamino]-propane. The pH of Tris:maleic acid buffers was adjusted with NaOH. Values are mean of duplicate assays.

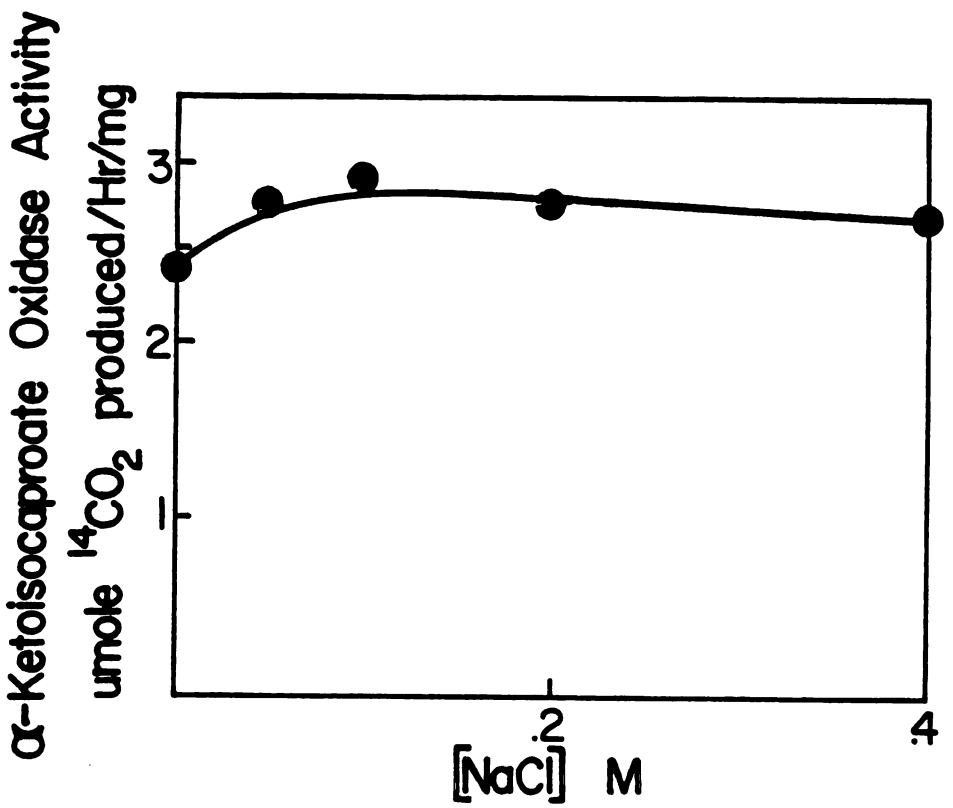



Figure 22. Effect of NaCl Concentration on the α-Ketoisocaproate Oxidase Activity. α-KIC oxidase activity was assayed as in Methods (Method B) except the buffer used was 50 mM Tris, 50 mM maleic acid pH 6.5. The concentration of NaCl in the assays was varied as shown. Each assay contained 10 µL (12 µg protein) of the Sephacryl S-200 pool (Table 9). Values are mean of duplicate asays.

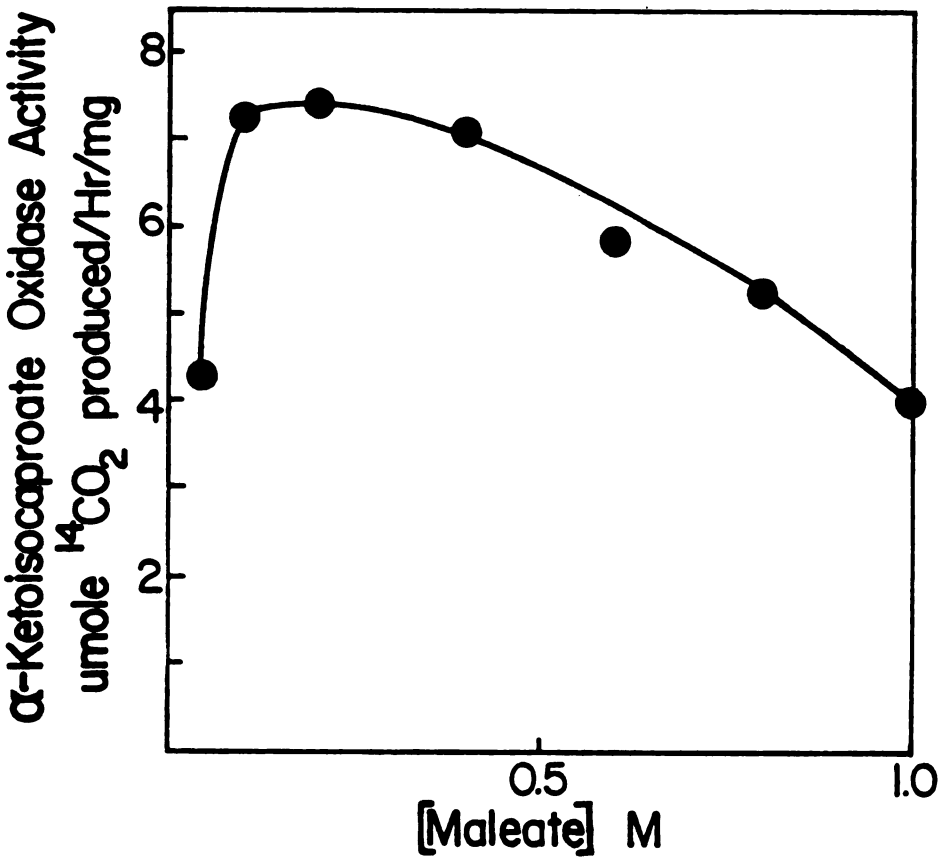



Figure 23. Effect of Maleate Concentration on the α-Ketoisocaproate Oxidase Activity. α-KIC oxidase activity was assayed as in Methods (Method B) except the buffer used was maleic acid adjusted to pH 6.0 with Tris base. The concentration of maleate in the assay was varied as shown. Each assay contained 10 µL (12 µg protein) of the Sephacryl S-200 pool (Table 9). Values are mean of duplicate assays.

TABLE 14

EFFECT OF ADP OR EDTA ON α-KETOISOCAPROATE OXIDASE ACTIVITY

| Addition | α-Ketoisocaproate Oxidase Activity μmole/hr/mg protein |
|---|---|
| None | 1.93 |
| 1 mM ADP | 0.78 |
| 2 mM ADP | 0.58 |
| 5 mM ADP | 0.59 |
| 10 mM ADP | 0.79 |
| 1.1 mM EDTA | 0.10 |
| 2 mM EDTA | 0.11 |
| 5 mM EDTA | 0.09 |

α-Ketoisocaproate oxidase activity was measured as in Methods (Method B) except 50 mM MES pH 6.0 was substituted for 0.2 M Tris, 0.2 M maleate pH 6.5. ADP and EDTA were added as indicated. Each assay contained 10 μL (12 μg protein) of the Sephacryl S-200 pool (Purification B, Table 9). Values are mean of duplicate assays.

0.2 M maleate, pH 6.5 (5.78 vs. 5.75 μmole/hr/mg protein of Sephacryl S-200 pool).

Maleate may activate the α-KIC oxidase by forming a chelate of $Fe^{2+}$ which is favorable for the catalytic reaction. EDTA and ADP, other iron chelators, were tested in the presence of 50 mM MES, a buffer which has very little tendency to bind metal ions (128). These compounds caused considerable inactivation of α-KIC oxidase activity (Table 14). Therefore, the α-KIC oxidase either prefers the Fe-maleate complex or maleate activates this enzyme by some other mechanism.

The effect of time of preincubation of the α-KIC oxidase with $FeSO_4$, ascorbate and dithiothreitol in the assay mixture before initiating the enzymatic reaction is shown in Figure 24. Optimal activation of the α-KIC oxidase was achieved after 60 min. Further preincubation, for up to 200 min, caused very little change in α-KIC oxidase activity.

E. Substrate Specificity of the α-Ketoisocaproate Oxidase

Crude preparations of rat liver cytosol (Table 3) oxidatively decarboxylated both α-KIC and α-keto-γ-methiolbutyrate (α-KγMB). In order to determine whether or not both of these α-keto acids are decarboxylated by the same enzyme, decarboxylation of α-[1-$^{14}$C] KIC and α-[1-$^{14}$C] KγMB were monitored in the various purification fractions (Table 15). The ratio of decarboxylation of α-KIC to that of α-KγMB was approximately 1.0 and did not vary significantly throughout the purification. This indicates that

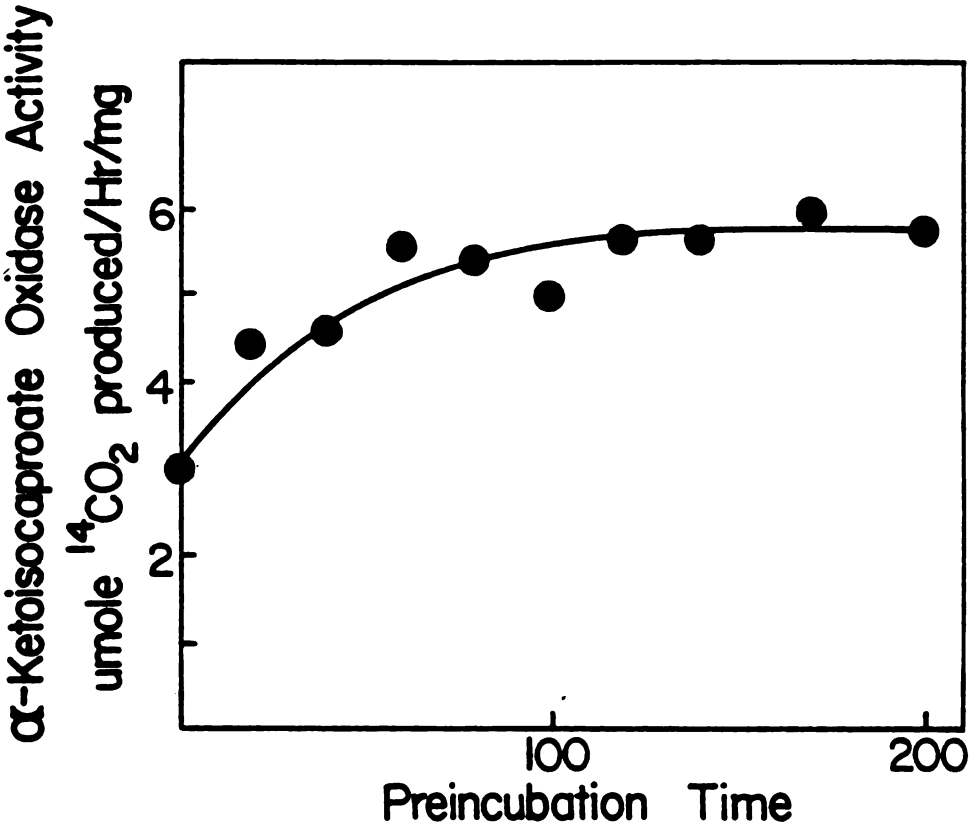



Figure 24. Effect of Preincubation Time on α-Ketoisocaproate Oxidase Activity. α-KIC oxidase activity was assayed as in Methods (Method B) except for variation of the preincubation time. Each assay contained 10 µL (12 µg protein) of the Sephacryl S-200 pool (Table 9). Values are mean of duplicate assays.

TABLE 15

DECARBOXYLATION OF α-KETOISOCAPROATE AND α-KETO-γ-METHIOLBUTYRATE BY PURIFICATION FRACTIONS

| Fraction | nmole $^{14}CO_2$/min/mg protein substrate α-[1-$^{14}$C] KIC | α-[1-$^{14}$C] KγMB | Ratio $\frac{\text{α-KIC}}{\text{α-KγMB}}$ |
|---|---|---|---|
| 10,000 x g supernatant | 0.61 | 0.53 | 1.15 |
| DEAE-cellulose pool | 7.60 | 7.24 | 1.05 |
| Phenyl pool concentrated | 23.4 | 23.4 | 1.00 |
| Sephacryl S-200 pool | 94.5 | 95.2 | 0.99 |

Decarboxylation of α-[1-$^{14}$C] ketoisocaproate (α-KIC) and α-[1-$^{14}$C] keto-γ-methiolbutyrate (α-KγMB) were measured in purification fractions (see Table 9) by Method B. Values are mean of 2 determinations.

one enzyme catalyzes the decarboxylation of both of these substrates.

The apparent $K_m$'s of the α-KIC oxidase for α-KIC and α-KγMB were determined using optimized assay conditions (Figure 25). The apparent $K_m$ for α-KIC was 0.32 ± 0.02 mM. The apparent $K_m$ for α-KγMB, however, was almost 6 times higher, 1.90 ± 0.12 mM. The apparent $V_{max}$ determined in the presence of α-KγMB is higher than when determined in the presence of α-KIC as the substrate (247 ± 6 vs. 130 ± 3 nmole/min/mg protein).

V. Studies on the Mechanism of the α-Ketoisocaproate Oxidase

A. Product Identification

An 18 fold purified preparation of α-KIC oxidase (Sephadex G-150 pool; Purification A) was used to determine the reaction products. α-[4,5-$^3$H] KIC was incubated with the partially purified enzyme and the products were separated by anion exchange chromatography. A profile similar to that shown in Figure 29A was obtained. Some radioactivity, possibly due to $^3H_2O$, eluted in the water wash (not shown in Figure 29A). When α-[4,5-$^3$H] KIC was incubated under identical conditions, but without the enzyme preparation, only one peak cochromatographing with α-KIC was seen (data not shown).

The major product, peak I of Figure 29A, was analyzed by gas-liquid chromatography (Figure 26). One major peak with a retention time of 15.1 min was observed. Greater than 80% of the injected radioactivity was associated with this peak. Using this system, authentic α-hydroxyisovaleric acid, β,β-dimethylacrylic

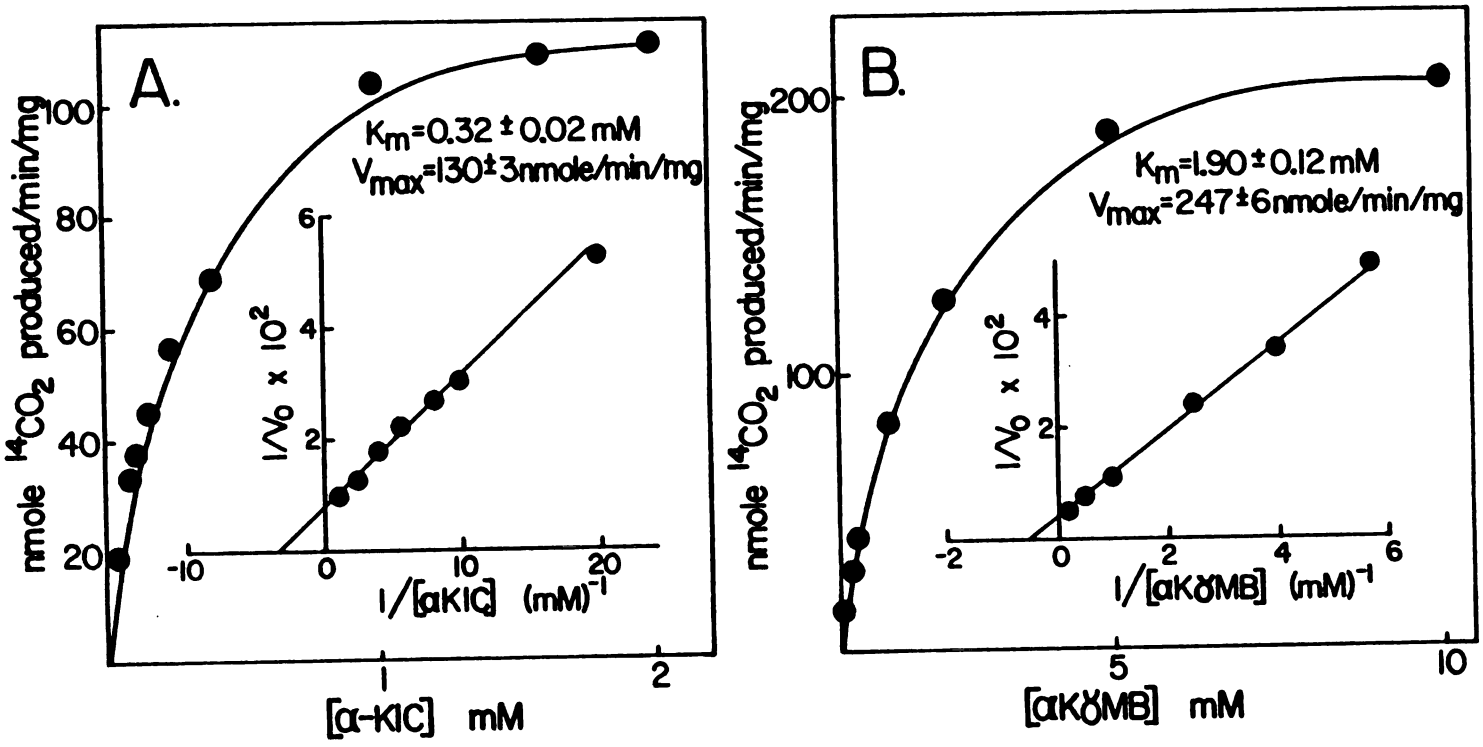



Figure 25. Effect of Substrate Concentration on α-Ketoisocaproate or α-Keto-γ-methiolbutyrate Oxidase Activity. Decarboxylation of α-[1-$^{14}$C] KIC (A) or α-[1-$^{14}$C] KγMB (B) was assayed as in Methods (Method B) with the following modifications. The preincubation time was increased to 2 hrs and the buffer was 0.2 M maleic acid adjusted to pH 6.0 with Tris base. The concentrations of α-[1-$^{14}$C] KIC and α-[1-$^{14}$C] KγMB were varied as shown and the incubation time was varied to insure initial velocity measurements. Each assay contained 5 µL (6 µg protein) of the Sephacryl S-200 pool (Table 9). Values are average of 4 determinations. Values of $K_m$ and $V_{max}$ were determined by computer analysis (WILKIN PROGRAM, Michigan State University, East Lansing, MI) using the data weighting procedure suggested by Wilkinson (144).

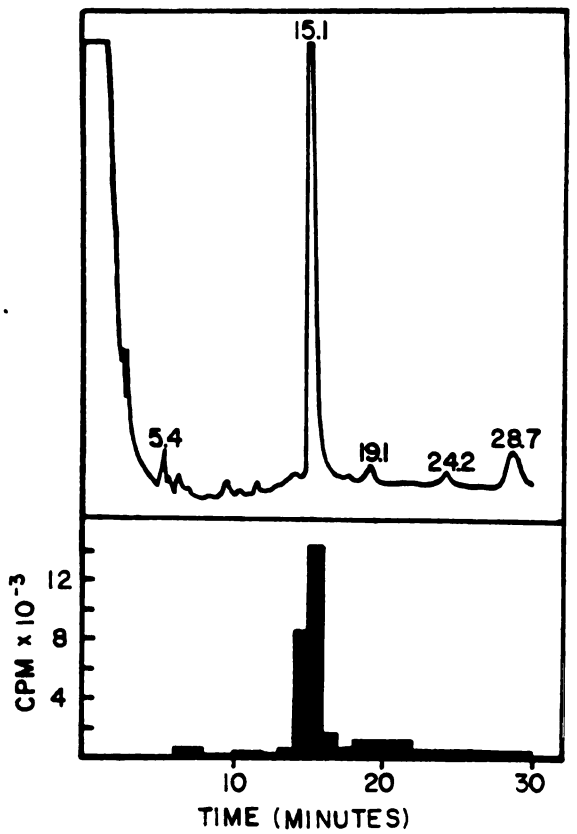



Figure 26. Gas Chromatographic Profile of the Major Product of α-Keto-isocaproate Oxidase. The upper figure shows the gas-chromatographic profile of the major product of α-KIC oxidase isolated as described under Methods. Below this is shown the radioactive profile of the gas chromatograph effluent.

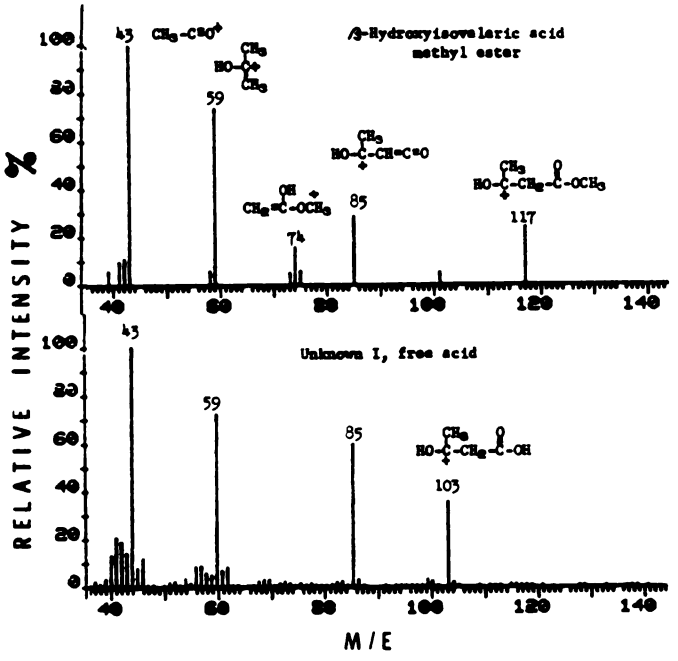



Figure 27. Mass Spectrum of the Major Product of α-Ketoisocaproate Oxidase. The mass spectrum of standard β-hydroxyisovaleric acid, methyl ester (Michigan State University mass spectrum library No. 9052), is shown along with the mass spectrum of the free acid of the unknown compound with a retention time of 15.1 min on the gas chromatograph (Figure 26).

Figure 28. Dowex-1 Chloride Chromatography of the Reaction Products of the Purified α-Ketoisocaproate Oxidase. α-[U-$^{14}$C] Ketoisocaproate was incubated with the purified α-ketoisocaproate oxidase in a reaction mix containing in a final volume of 0.10 mL: 0.2 M Tris, 0.2 M maleic acid pH adjusted to 6.5 with NaOH, 1.0 mM $FeSO_4$, 0.5 mM ascorbic acid, 1.0 mM dithiothreitol, 0.5 mM α-[U-$^{14}$C] ketoisocaproate (0.06 μCi), and 35 μL (14 μg) of "P-6-Pool" (solid line) or boiled "P-6-Pool" (dashed line). The "P-6-Pool" is the "Sephacryl S-200 Pool" (Table 9) preparation of α-KIC oxidase with monothioglycerol removed (see Methods). After a 45 min preincubation of all other components of the reaction mix, the α-[U-$^{14}$C] KIC was added. The tube was then capped with a serum cap which held a hanging plastic cup containing 0.2 mL Hyamine. This was incubated 3 hrs at 25°C. The reaction was terminated by addition of 50 μL of 20% trichloroacetic acid and let set 45 min to trap $^{14}CO_2$ released. The protein precipitate was removed by centrifugation, the pH adjusted to 6.0 and volume adjusted to 3.1 mL. Three mL of this solution was applied to a 0.5 x 22 cm Dowex-1-$Cl^-$ (100-200 mesh) column. The column was eluted with 7 mL $H_2O$, 150 mL of a linear 0-0.02 N HCl gradient, 150 mL of a linear 0.02-0.20 N HCl gradient and 150 mL 0.2 N HCl. Fractions of 1.5 mL were collected and radioactivity determined in a 1 mL aliquot. The number above each peak refers to the percentage of the total $^{14}$C dpm in that peak.

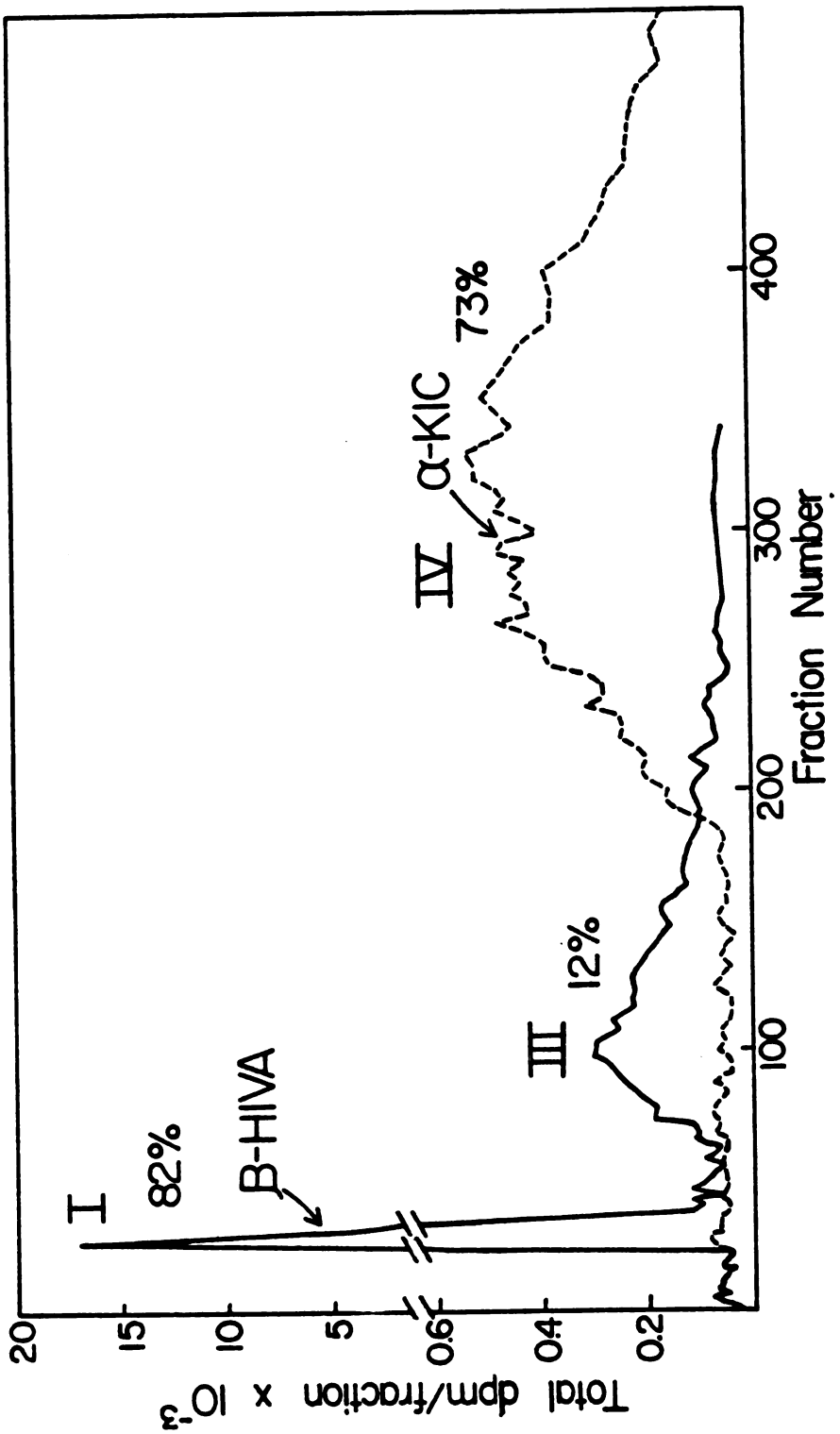

Total dpm/fraction x $10^{-3}$
20
15
10
5
0.6
0.4
0.2
100
200
300
400
Fraction Number
I
82%
B-HIVA
III
12%
IV
α-KIC
73%

Figure 28

acid and isovaleric acid had retention times of 18.4, 6.9 and 3.9 min, respectively.

Mass spectral analysis of the peak at 15.1 min of Figure 28 gave a spectrum which identifies the compound as β-hydroxyisovaleric acid (Figure 27). Comparison of the mass spectrum to that of the methyl ester of authentic β-hydroxyisovaleric acid shows identical peaks at mass 43, 59 and 85. The difference between the peaks at 117 and 103 is 14 mass units, which corresponds to a $CH_2$ group. This is expected since the unknown sample was in the free acid form whereas the standard was methylated.

The reaction products formed from α-[U-$^{14}$C] KIC by the purified α-KIC oxidase (Sephacryl S-200 pool, Table 9) were also analyzed. Dowex-1 chloride chromatography showed the formation of 2 products (Figure 28). No radioactivity was detected in the $H_2O$ wash or at the position of peak II in Figure 29A. Peak I was identified as β-hydroxyisovaleric acid by gas chromatography. Peak III has not been identified, is present in minor amounts and is not produced in the absence of the α-KIC oxidase. This compound migrates similar to isovaleric acid during Dowex-1 chloride chromatography.

B. Evidence That Isovalerate Is Not An Intermediate

Figure 29 shows the Dowex-1 chloride column profile of products obtained from α-KIC oxidase under various conditions. In Figure 29A the partially purified α-KIC oxidase (Sephadex G-150 pool, Purification A) was incubated with α-[4,5-$^{3}$H] KIC. $Fe^{2+}$, ascorbate, and dithiothreitol were omitted from the

reaction mixture. The profile obtained is the same when these compounds are included in the reaction mixture. This experiment has been repeated several times and five peaks of radioactivity are always obtained. One peak is in the $H_2O$ wash (not shown). Peak I has been identified as β-hydroxyisovaleric acid. Peaks II and III have not been identified. Peak II is not found when purified α-KIC oxidase is used (see Figure 28). Peak III is found in variable amounts in different experiments. Up to 30% of the $^{3}H$-labeled products migrate with peak III in some experiments. Peak IV is the unreacted α-[4,5-$^{3}H$] KIC. This experiment has been repeated several times with recovery of 85-100% of the applied radioactivity.

Peak III migrates the same as isovaleric acid on the Dowex-1 chloride column. To determine if isovalerate is a free intermediate in the formation of β-hydroxyisovalerate, the partially purified α-KIC oxidase was incubated simultaneously with α-[4,5-$^{3}H$] KIC and [1-$^{14}C$] isovalerate (Figure 29B). The $^{14}C$ label (dotted line) remained associated with the isovaleric acid peak, whereas 55% of the $^{3}H$ label (solid line) was incorporated into β-hydroxyisovaleric acid. This experiment shows that isovalerate is not a free intermediate in β-hydroxyisovalerate formation from α-KIC.

Another possibility is that peaks II and III arise from the metabolism of β-[4-$^{3}H$] hydroxyisovalerate. When β-[4-$^{3}H$] hydroxyisovalerate was incubated with the partially purified α-KIC oxidase only β-hydroxyisovalerate was found, indicating that it is a terminal product of this system (Figure 29C).

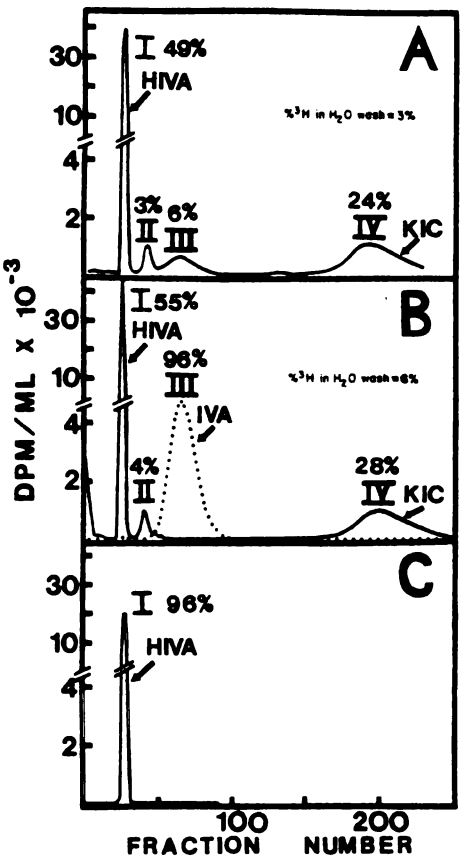



Figure 29. Evidence That Isovalerate Is Not An Intermediate of the α-Ketoisocaproate Oxidase Reaction. Reactions contained 0.2 M Tris-maleate, pH 6.5, 1 mM $Na_2CO_3$, 25 µL of α-KIC oxidase (Sephadex G-150 pool, see Table 5), and 0.1 mM α-[4,5-$^3$H] KIC (A); 0.1 mM α-[4,5-$^3$H] KIC and 0.1 mM [1-$^{14}$C] isovalerate (B); or 1.0 mM β-[4-$^3$H] hydroxyisovalerate (C) in a final volume of 0.1 mL. Experiments B and C also contained 0.2 mM $FeSO_4$, 0.4 mM ascorbate, and 1 mM dithiothreitol. The reactions were incubated for 3 h (A) or 1 h (B and C) at 25°C, then terminated by adding 50 µL 20% trichloroacetic acid. Protein was removed by centrifugation and the supernatant neutralized. This was applied to a 0.5 x 22 cm Dowex-1 x 8 chloride (100-200 mesh) column and washed with 5 mL $H_2O$. The column was then eluted with a 150-mL linear gradient of 0-0.02 N HCl, followed by another 150-mL linear gradient of 0.02-0.20 N HCl, followed by 150 mL of 0.20 N HCl. Fractions of 1.5 mL were collected and the dpm of $^3$H (solid line) or $^{14}$C (dotted line) were determined in every other fraction. The number above each peak refers to the percentage of the total $^3$H or $^{14}$C dpm in that peak. Abbreviations are α-ketoisocaproic acid (KIC), isovaleric acid (IVA), and β-hydroxyisovaleric acid (HIVA). β-[4,5-$^3$H] Hydroxyisovalerate was isolated from a previous reaction of α-KIC oxidase with α-[4,5-$^3$H] KIC.

C. Mechanism of Formation of β-Hydroxyisovaleric Acid by the α-Ketoisocaproate Oxidase

Previous experiments indicated that $O_2$ was required for α-KIC oxidase activity (Table 2). In order to determine the source of the oxygen incorporated into β-hydroxyisovaleric acid, enzymatic incorporation of $^{18}O$ from $^{18}O_2$ or $H_2{}^{18}O$ was determined.

Figure 30A shows the mass spectrum of enzymatically formed β-hydroxyisovaleric acid when no isotopically labeled oxygen (other than natural abundance) is present. The ion at m/e 103 contains both oxygen atoms which are incorporated into the newly formed β-hydroxyisovaleric acid. The removal of one of the carboxyl oxygen atoms, as $H_2O$, leads to the formation of the ion at m/e 85. One of the carboxyl oxygen atoms originates from the substrate, α-KIC, while the other carboxyl oxygen atom is incorporated from $O_2$ or $H_2O$ during the enzymatic reaction. Since the two carboxyl oxygen atoms are equivalent, one half of any $^{18}O$ incorporated into the carboxyl group of A (Table 16); will be lost when fragmented to B (Table 16). $^{18}O$ incorporated into the β-hydroxyl group will not be lost during this fragmentation. Fragment C (ions, m/e 59,61) contains only the hydroxyl oxygen of β-hydroxyisovaleric acid. Therefore, only incorporation of $^{18}O$ into the β-hydroxyl group of β-hydroxyisovaleric acid can lead to an increase in m/e 61. Fragment D (ions, m/e 43, 45) also lacks the carboxyl group, should give identical results as those seen with fragment C, and does.

Figure 30. Enzymatic Incorporation of $^{18}O$ from $^{18}O_2$ or $H_2{}^{18}O$ into β-Hydroxyisovaleric Acid. β-Hydroxyisovaleric acid was produced enzymatically in the presence of no isotopically labeled oxygen (A), 90% $^{18}O_2$ (B) or 93% $H_2{}^{18}O$ (C), isolated, and the mass spectrum determined as described in Methods. In experiment D, β-hydroxyisovalerate was incubated in the complete mixture (minus α-KIC) with 93% $H_2{}^{18}O$.

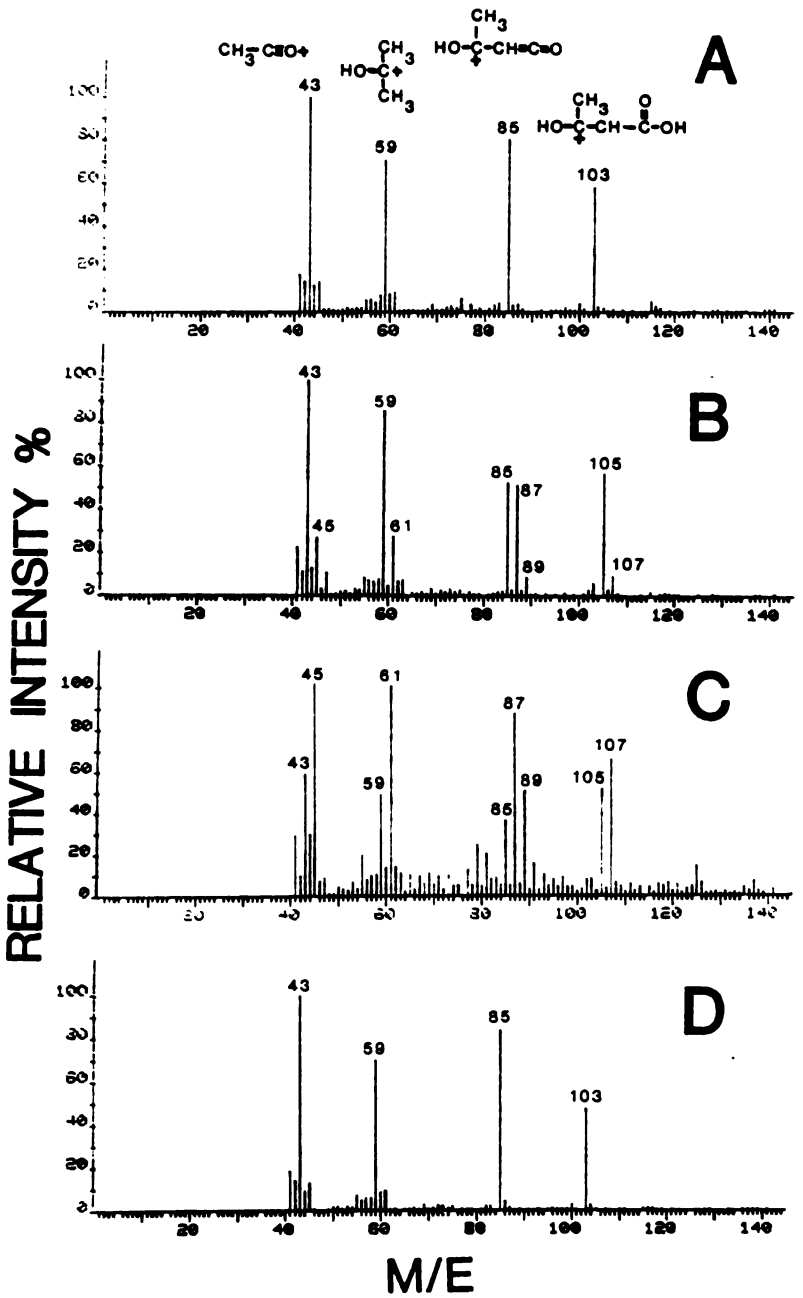



Figure 30

TABLE 16

PRESENCE OF $^{18}O$ IN ENZYMATICALLY FORMED β-HYDROXYISOVALERIC ACID AND ITS FRAGMENTS

| | Fragment | m/e | $^{18}O$ atoms | % abundance | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | $^{18}O_2$ Expt. 1 | $^{18}O_2$ Expt. 2 | $H_2{}^{18}O$ | $^{16}O$ $H_2{}^{16}O$ | Incubation of $H_2{}^{18}O$ with β-hydroxy-isovalerate |
| A. | O<br>C-OH<br>$CH_2$<br>$CH_3$-C-OH | 103 | 0 | 5 | 8 | 6 | 92 | 97 |
| | | 105 | 1 | 81 | 79 | 41 | 5 | 2 |
| | | 107 | 2 | 13 | 13 | 52 | 3 | 1 |
| B. | O<br>C<br>CH<br>$CH_3$-C-OH | 85 | 0 | 45 | 47 | 21 | 94 | 98 |
| | | 87 | 1 | 47 | 46 | 50 | 4 | 2 |
| | | 89 | 2 | 7 | 8 | 29 | 1 | 0 |
| C. | $CH_3$-C-OH<br>$CH_3$ | 59 | 0 | 71 | 71 | 30 | 84 | 88 |
| | | 61 | 1 | 23 | 23 | 63 | 14 | 11 |
| D. | $CH_3$-C $O^+$ | 43 | 0 | 70 | 73 | 35 | 87 | 88 |
| | | 45 | 1 | 22 | 20 | 60 | 13 | 11 |

When β-hydroxyisovaleric acid was produced in the presence of $^{18}O_2$ (Figure 30B), 13% of the molecules incorporated $^{18}O$ into the β-hydroxy position. Greater than 94% of the β-hydroxyisovaleric acid molecules contained at least one $^{18}O$ atom. Since the $^{18}O_2$ used in this experiment contained 10% $^{16}O_2$, the value of 5-8% at m/e 103 indicates that all of the β-hydroxyisovaleric acid was labeled with at least one $^{18}O$ atom.

When produced in the presence of $H_2{}^{18}O$ (Figure 30C), 60% of the β-hydroxyisovaleric acid molecules incorporated $^{18}O$ into the β-hydroxyl group. Virtually all of the molecules contained at least one $^{18}O$ atom, therefore the carboxyl group was almost completely labeled with one $^{18}O$ atom.

When the previously formed product, β-hydroxyisovaleric acid was incubated with $H_2{}^{18}O$ under conditions identical to those used for the enzymatic formation of β-hydroxyisovaleric acid (except α-KIC was omitted), no incorporation of $^{18}O$ into the molecule was detected (Figure 30D).

# DISCUSSION

The results presented herein clearly demonstrate the existence of two separate α-KIC decarboxylating activities in rat liver; one mitochondrial, the other cytosolic. No activity was detected in peroxisomes or microsomes.

The mitochondrial α-KIC decarboxylase activity is due to the branched-chain α-keto acid (BCKA) dehydrogenase (EC 1.2.4.3 and 1.2.4.4) which has recently become the focus of numerous investigations (1-4, 56-67). This enzyme uses all three branched-chain α-keto acids, α-KIC, α-ketoisovalerate and α-keto-β-methylvalerate, as substrates and requires both CoA and $NAD^+$ as cofactors. The mechanism of this enzyme is similar to that of the pyruvate and α-ketoglutarate dehydrogenase complexes (2).

The cytosolic α-KIC decarboxylase activity does not utilize α-ketoisovalerate and α-keto-β-methylvalerate as substrates and does not require CoA or $NAD^+$. This enzyme activity is due to an oxidase which decarboxylates and hydroxylates α-KIC to form β-hydroxyisovalerate (β-HIVA). This enzyme also utilizes α-keto-γ-methiolbutyrate, the α-keto analogue of methionine, as a substrate.

Previous investigations indicated that rat liver cytosolic α-KIC decarboxylating activity was insignificant in comparison to the activity of the mitochondrial BCKA dehydrogenase (1). However, even when using assay conditions which had been optimized for the mitochondrial BCKA

dehydrogenase, almost equal amounts of the mitochondrial and cytosolic α-KIC decarboxylase activities were found (see Figure 1). The cytosolic α-KIC oxidase activity in rat liver is markedly affected by the nutritional and hormonal status of the animal. Dixon and Harper have found that when rats are fed a 50% casein diet for 6 days, cytosolic α-KIC decarboxylase activity increases approximately 3.5 times, whereas the mitochondrial BCKA dehydrogenase activity remains constant (111). Diabetes also elevates rat liver cytosolic α-KIC decarboxylase activity without affecting the mitochondrial BCKA dehydrogenase (112). Therefore it appears that, at least in some situations, the cytosolic α-KIC oxidase may have an important role in leucine metabolism. The purification and characterization of this enzyme as well as the identification of the products of the reaction should serve as a starting point for understanding the function(s) of this enzyme.

The apparent $K_m$ of the purified cytosolic α-KIC oxidase for α-KIC was found to be 0.3 mM when optimal assay conditions were used (Figure 25). This is 20 fold higher than the apparent $K_m$ of the mitochondrial BCKA dehydrogenase for α-KIC, which is 15 μM (3). The $K_m$ of the cytosolic α-KIC oxidase for α-KIC, however, is quite dependent on the assay conditions employed. Using a crude rat liver cytosolic preparation in the presence of 1.5 M ammonium sulfate, the apparent $K_m$ of the α-KIC oxidase for α-KIC was 0.03 mM (see Figure 4). The actual affinity of this enzyme for α-KIC *in vivo*, therefore, may be quite different from that determined *in vitro*.

The relative proportion of α-KIC utilized by the cytosolic α-KIC oxidase as compared to the mitochondrial BCKA dehydrogenase may also be

affected by factors other than $K_m$. May et al. (112) and Dixon and Harper (111) have reported an increase in mitochondrial BCKA dehydrogenase activity after freeze-thawing of mitochondria from rat liver. This may be due to an increase in the activity of BCKA dehydrogenase due to freeze-thawing or to an increase in accessibility of the BCKA dehydrogenase to its substrate. If the latter possibility is true then transport of the α-keto acids into the mitochondria may be rate limiting in some situations. A transporter(s) of the BCKA has been identified in rat liver (129,130) and may be very important in regulation of BCAA metabolism. The $K_m$ for transport of α-KIC into isolated rat liver mitochondria is 0.2-0.5 mM (131), which is several fold greater than the $K_m$ of the mitochondrial BCKA dehydrogenase for α-KIC (3).

The subcellular pool in which α-KIC is produced may also determine which enzyme (α-KIC oxidase or BCKA dehydrogenase) metabolizes this compound. The branched-chain amino acid transaminase is located both in the mitochondrial and cytosolic compartments of rat liver (26). Therefore, BCKA's can be formed in at least two different intracellular pools.

The activation of the α-KIC oxidase by ferrous iron ($Fe^{2+}$), ascorbate (NADH or NADPH), and a sulfhydryl compound is typical of many non-heme iron oxygenases (132). Other metal ions tested were unable to substitute for $Fe^{2+}$. Ferric iron ($Fe^{3+}$), however, was equally effective as $Fe^{2+}$ in activation of the α-KIC oxidase when ascorbate was present. This may be due to the reduction of $Fe^{3+}$ to $Fe^{2+}$ by ascorbate. The stimulatory affect of ascorbate on the α-KIC oxidase appears to be solely due to reduction of iron to the ferrous state

($Fe^{2+}$). In the presence of high concentrations of $Fe^{2+}$, ascorbate has no effect. Other reducing agents such as NADH and NADPH can replace ascorbate.

$FeSO_4$ activation of the α-KIC oxidase persists for up to 10 minutes after removal of $FeSO_4$ by dilution (see Figure 19). However, α-KIC oxidase activity quickly diminishes thereafter if $FeSO_4$ is not present in the assay mixture. This may be explained by a weak association of $Fe^{2+}$ with the enzyme, either at the active site or at another site which may be necessary to provide the proper conformation of the enzyme for catalytic activity.

The α-KIC oxidase activity requires the presence of a reduced sulfhydryl compound, such as dithiothreitol or CoASH. Monothioglycerol at a concentration of 0.6 M (5%) stabilizes the purified enzyme when stored at 4°C. These results indicate that the enzyme contains sulfhydryl groups which must be reduced in order for the enzyme to express catalytic activity. The stabilization of the α-KIC oxidase by monothioglycerol may not be entirely due to its ability to keep enzyme sulfhydryl groups reduced. Monothioglycerol at a concentration of 0.12 M (1%) should be a good sulfhydryl reducing agent, yet does not prevent α-KIC oxidase inactivation. The higher concentration of monothioglycerol (0.61 M) may also protect the enzyme from inactivation by removing oxygen from the solution. Several dioxygenases are known to be inactivated by oxygen (132,133).

Optimal activity of the purified α-KIC oxidase occurs at pH 6.0 in the presence of 0.2 M maleate, 1 mM $FeSO_4$, 0.5 mM ascorbate and 1 mM dithiothreitol. The low pH optimum of this enzyme may be due to the fact that $Fe^{2+}$ is rapidly oxidized at higher pH to ferric hydroxide. In

reactions carried out at pH 7.0 or greater, assay mixtures developed a reddish-brown color. At pH 6.0 or 6.5 this was not detectable.

Increasing maleate concentration from 50 μM to 0.2 M gave almost a 2 fold increase in the α-KIC oxidase activity. This increased activity was not due to ionic strength of the buffer. Maleate may complex with $Fe^{2+}$ in a form which is favorable for catalytic activity. Other metal chelators tested, EDTA and ADP, could not replace maleate and actually inhibited α-KIC oxidase activity.

Our results indicate that the cytosolic α-KIC oxidase consumes 0.7-0.9 mol of $O_2$ per mole of $CO_2$ released from α-KIC (Table 2). This ratio is consistent with the formation of β-hydroxyisovalerate from α-KIC. The expected value of 1.0 was probably not achieved due to the formation of small amounts of products other than β-hydroxyisovalerate (i.e., peaks II and III, Figure 29A). Isovalerate is not a free (undissociated) intermediate in the conversion of α-KIC to β-hydroxyisovalerate. Partially purified α-KIC oxidase preparations converted α-KIC but not isovalerate to β-hydroxyisovalerate. β,β-Dimethylacrylic acid also failed to yield β-hydroxyisovalerate when incubated with the purified α-KIC oxidase (unpublished observation).

In order to determine the enzymatic mechanism of β-hydroxyisovalerate formation, incorporation of $^{18}O$ from $^{18}O_2$ or $H_2{}^{18}O$ was determined (see Figure 31). When α-KIC was incubated in the presence of $^{18}O_2$ gas, greater than 92% of the β-hydroxyisovalerate molecules incorporated one $^{18}O$ atom into the carboxyl group. In 15% of the molecules, one atom of $^{18}O$ was also incorporated into the β-hydroxyl

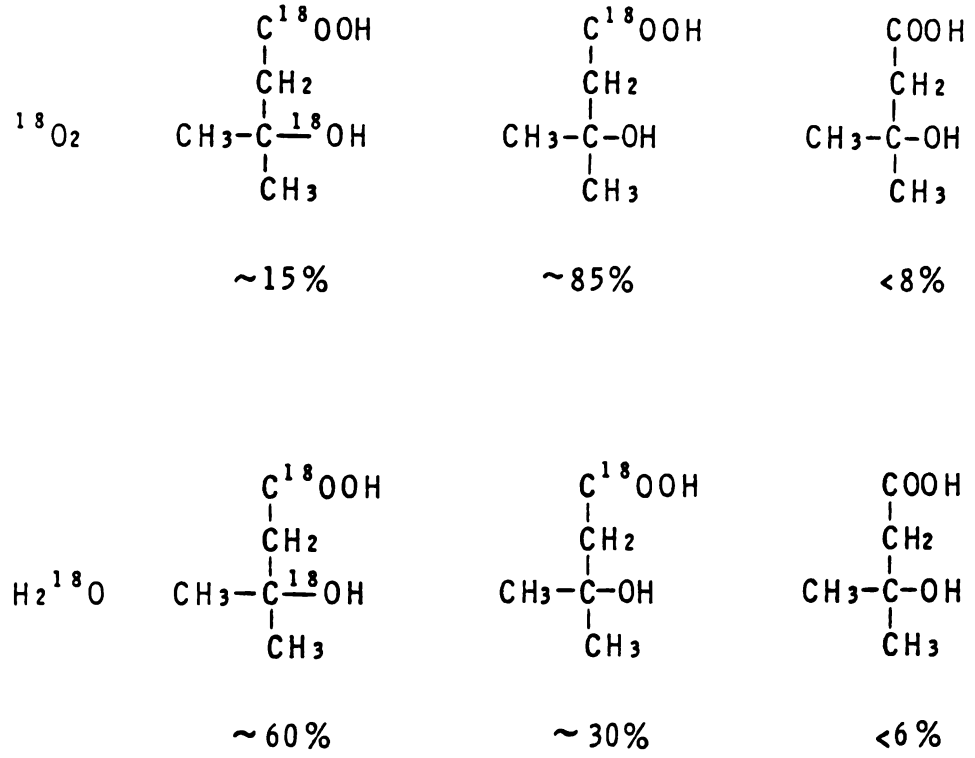



Figure 31. Incorporation of $^{18}O$ from $^{18}O_2$ or $H_2{}^{18}O$ into β-Hydroxyisovaleric Acid.

group. These results indicate that both oxygen atoms incorporated into β-hydroxyisovalerate originate from $O_2$. The incorporation of one $^{18}O$ atom from $H_2{}^{18}O$ into the carboxyl group is not at variance with this proposal, since α-keto acids exchange the ketonic oxygen with water (134). The low incorporation of $^{18}O$ from $^{18}O_2$ into the hydroxyl position of β-HIVA could be explained by exchange with water. This is indeed the case. When α-KIC is incubated with the α-KIC oxidase in the presence of $H_2{}^{18}O$, 60% of the β-HIVA molecules contain one $^{18}O$ atom in the hydroxyl group. $^{18}O$ from gaseous $^{18}O_2$ can not exchange into this hydroxyl position (134), therefore, incorporation of molecular $^{18}O_2$ must be enzymatic.

When β-hydroxyisovalerate is incubated with $H_2{}^{18}O$ under identical reaction conditions used for other $^{18}O$ experiments (minus α-KIC) no incorporation of $^{18}O$ occurs. Therefore, exchange of the hydroxyl oxygen with $H_2O$ must occur with an intermediate of the reaction.

A mechanism for the α-KIC oxidase reaction, consistent with the results is shown in Figure 32. This mechanism was proposed by Hamilton for dioxygenases which require an α-keto acid as a cosubstrate (135,136). In the case of α-KIC an intramolecular reaction mechanism may be involved with the α-keto group of α-KIC itself serving as the cosubstrate. This mechanism involves the formation of a peracid from the reaction of enzyme bound $Fe^{2+}$, $O_2$ and α-KIC, as shown. The peracid could then act to insert an oxenoid oxygen at an unactivated C-H bond in the way that nitrenes and carbenes insert. The products of the reaction would then be β-hydroxyisovalerate, $CO_2$ and the regenerated enzyme-$Fe^{2+}$ complex.

Lindblad *et al.* have proposed a similar mechanism for the enzymatic formation of homogentisate from p-hydroxyphenylpyruvate by

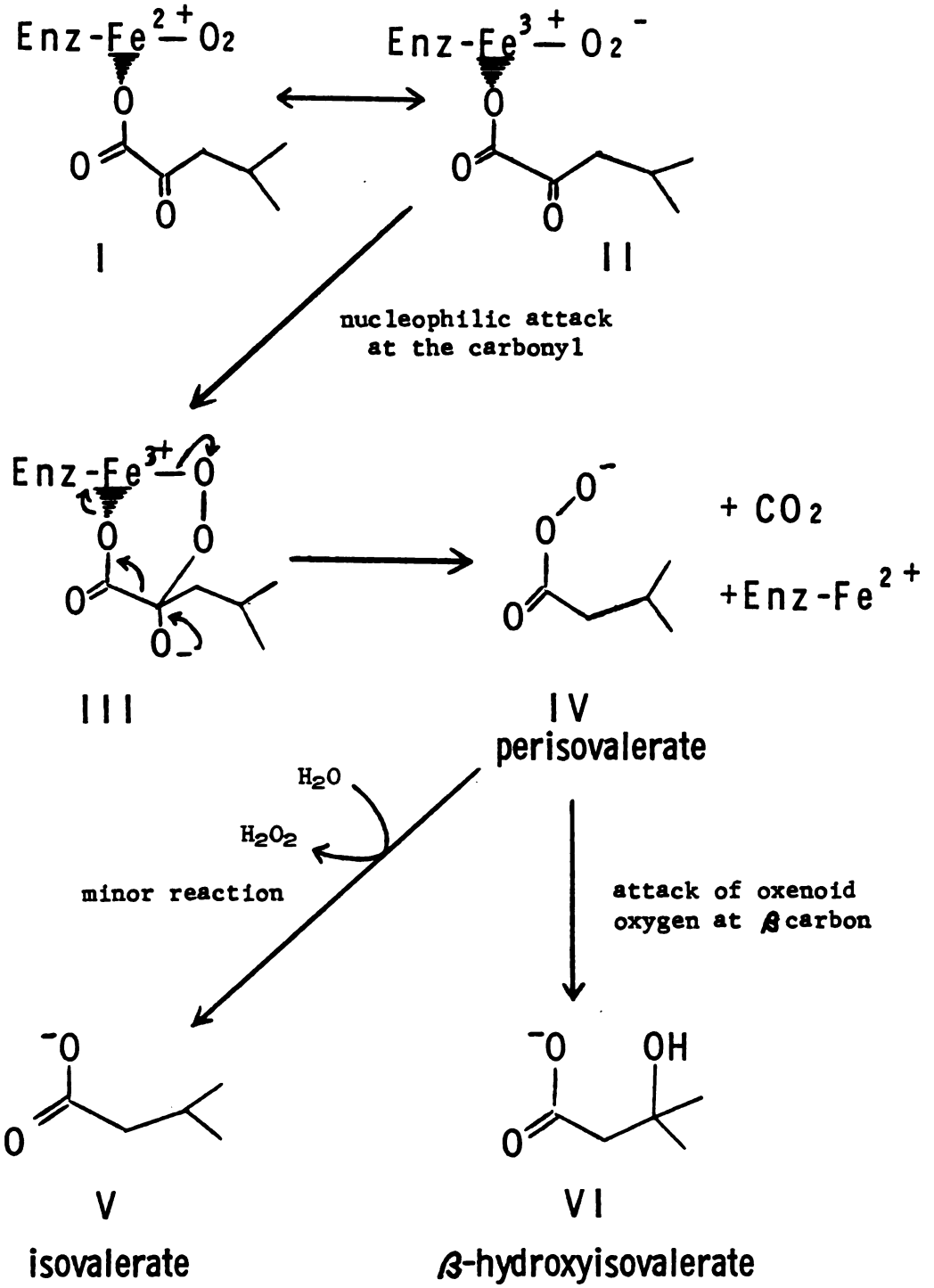



Figure 32. Proposed Mechanism of the α-Ketoisocaproate Oxidase Reaction.

p-hydroxyphenylpyruvate hydroxylase (134). This enzyme also requires $Fe^{2+}$ and ascorbate for maximal activity. The results of their $^{18}O$ incorporation experiments using $^{18}O_2$ and $H_2{}^{18}O$ are very similar to the results reported herein for the α-KIC oxidase.

The regeneration of $Fe^{2+}$ during the enzymatic reaction would explain why the α-KIC oxidase retains activity even after the unbound $Fe^{2+}$ is removed from the assay by dilution (Figure 19). The loss of activity by 20 min may be accounted for by a slow dissociation of iron from the enzyme due to weak binding at the active site.

The enzymatic formation of isovalerate (peak III, Figure 28 and 29) could be explained by the mechanism proposed. The breakdown of perisovalerate before formation of β-HIVA would lead to the formation of isovalerate. This uncoupling of the decarboxylation and hydroxylation reactions has been observed with prolyl hydroxylase (137).

The α-KIC oxidase activity is inhibited by other branched-chain and long-chain α-keto acids. Phenylpyruvate is an especially potent inhibitor of this activity. In patients with phenylketonuria, phenylpyruvate accumulates in the body fluids. An α-KIC oxidase similar to that described herein has also been found in human liver (unpublished observation). Inhibition of this enzyme by phenylpyruvate may adversely affect leucine catabolism in patients with phenylketonuria.

β-Hydroxyisovaleric acid has been identified in the urine of patients with several different types of clinical disorders (104,105,108,109,138). It is currently believed that this compound is produced by hydration of β-methylcrotonyl-CoA (139) followed by hydrolysis of the resulting

β-hydroxyisovaleryl-CoA formed. The cytosolic α-KIC oxidase, however, can catalyze the formation of β-hydroxyisovalerate directly from α-KIC. This enzyme has only been found in the liver and kidney of rats. No activity was detected in brain, heart, skeletal muscle or pancreas. The α-KIC oxidase may function as a "safety valve" to prevent buildup of α-KIC, which is quite toxic. The tissue distribution of this enzyme is consistent with such a function.

Tanaka et al. identified high amounts of β-hydroxyisovaleric acid in the urine of patients with isovaleric acidemia (104). This disease is due to a block at the isovaleryl-CoA dehydrogenase (100), therefore, no β-methylcrotonyl-CoA is formed. Since β-hydroxyisovalerate was presumed to be formed from β-methylcrotonyl-CoA, this observation was puzzling. The presence in humans of the α-KIC oxidase which forms β-hydroxyisovalerate directly from α-KIC could explain this inconsistency.

Landaas (108,109) has identified β-hydroxyisobutyric acid, β-hydroxyisovaleric acid, and α-methyl-β-hydroxybutyric acid in the urine of ketoacidotic patients and has shown that these acids arise from the metabolism of valine, leucine, and isoleucine, respectively. He found a positive correlation between the concentration of these acids and the degree of ketoacidosis in a variety of clinical disorders. The branched-chain amino acids are metabolized similarly via transamination, decarboxylation, and dehydrogenation to form methylacryl-CoA (from valine), β-methylcrotonyl-CoA (from leucine), and tiglyl-CoA (from isoleucine). Hydration of these compounds would yield the β-hydroxy acids, which are seen. However, there are several reasons to question this pathway for formation of the β-hydroxy acids. If the formation of these β-hydroxy acids was due to a block in branched-chain amino acid metabolism, as indicated by

the high serum levels of the branched-chain amino acids, then one would expect other intermediates, such as β-methylcrotonylglycine or isovaleric acid (100,105,138) to accumulate. Landaas notes that although leucine, isoleucine, and valine accumulate in the serum of ketoacidotic patients, other metabolites of branched-chain amino acid metabolism are not seen (109). A cytosolic α-KIC oxidase activity with properties similar to those described for the rat liver enzyme has also been identified in human liver (unpublished observation). This enzyme may be responsible, at least partially, for production of the β-hydroxyisovalerate which accumulates in these patients.

The rat liver cytosolic α-KIC oxidase also uses α-keto-γ-methiolbutyrate (the keto analog of methionine) as a substrate. The affinity of this enzyme for α-keto-γ-methiolbutyrate (apparent $K_m$ = 1.9 mM) is much less than that for α-KIC (apparent $K_m$ = 0.3 mM). The product formed by the α-KIC oxidase from α-keto-γ-methiolbutyrate has not yet been identified, but migrates identically to β-hydroxyisovalerate using Dowex-1 chloride chromatography (unpublished observation; see Figure 29). If the reaction is similar to the α-KIC oxidase reaction, the expected product would be 3-hydroxy-3-methylthiopropionate. The product of this reaction is not 3-methylthiopropionate. 3-Methylthiopropionic acid can be extracted into diethyl ether (140). The product of the α-KIC oxidase reaction using α-keto-γ-methiolbutyrate as a substrate cannot be extracted into diethylether (unpublished observation).

Steele and Benevenga (140) showed that rat liver homogenates can transaminate methionine to form α-keto-γ-methiolbutyrate and decarboxylate α-keto-γ-methiolbutyrate to form 3-methylthiopropionate. In rat liver, 75% of the decarboxylase activity is associated with the

mitochondrial fraction and 15% is cytosolic (141). They reported a high $K_m$ (>1 mM) for the cytosolic α-keto-γ-methiolbutyrate decarboxylase activity. This is consistent with the results presented here and indicates that this activity may only be of importance when methionine or α-keto-γ-methiolbutyrate levels are extremely elevated, as in hypermethioninaemia (142).

The mitochondrial α-keto-γ-methiolbutyrate decarboxylase activity has a $K_m$ of 0.1-0.6 mM for its substrate (141). This activity appears to be due to the BCKA dehydrogenase (143). The utilization of α-keto-γ-methiolbutyrate by the same cytosolic and mitochondrial enzymes that metabolize α-KIC is interesting, especially since rat liver also contains an enzyme that transaminates both leucine and methionine to form these α-keto acids (25).

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