Enzymatic Formation of $\alpha$-Isopropylmalic Acid, an Intermediate in Leucine Biosynthesis*

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Until recently, essentially all of our information concerning the biosynthesis of leucine stemmed from experiments with C14-labeled intermediates. Ehrensvärd et al. (1), in experiments with yeast grown on C14-labeled acetate as the sole carbon source, showed that the carboxyl carbon of leucine is probably derived from the carboxyl of acetic acid. In isotope competition experiments, Abelson and Vogel (2) found that valine, $\alpha$-ketoisovaleric acid, acetic acid, and pyruvic acid all dilute the carbon of leucine in *Escherichia coli* grown on uniformly labeled glucose. They suggested a condensation of $\alpha$-ketoisovaleric acid and a 2 carbon compound, probably acetate, followed by decarboxylation to yield the carbon skeleton of leucine. Confirmation of such a reaction course was obtained in experiments reported by us several years ago (3). When the distribution of activity was determined in leucine samples isolated from yeast grown on various C14-labeled acetates and lactates, it was found that both carbons of acetate and the $\alpha$ and $\beta$ carbons of lactate gave rise to highly labeled leucine.

Chemical degradation of these samples revealed that practically all of the activity from the acetate carboxyl carbon was present in the leucine carboxyl, and nearly all of the acetate methyl carbon was incorporated into the $\alpha$ carbon of leucine, thus clearly indicating that these first 2 carbons of leucine are derived from the corresponding carbons of acetic acid. Leucine produced from carboxyl-labeled lactate had low activity, indicating that this carbon of lactic acid does not contribute directly to leucine formation. However, the lactate $\alpha$ and $\beta$ carbons were readily incorporated into the carboxyl and $\alpha$ carbons of leucine, respectively. This result was expected since these 2 carbons are readily converted metabolically via pyruvate to acetic acid. However, the $\alpha$ carbon of lactate was also readily incorporated into the adjacent carbon atoms 3 and 4 of leucine in equal amounts, and the methyl carbon of lactate was extensively incorporated into the methyl carbons of leucine. This pattern of labeling in the isobutyl moiety of leucine was very similar to the lactate labeling in the isobutyl portion of valine observed in earlier experiments (4), indicating that these carbons of the two amino acids probably originate from a common source.

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Based on these findings, we suggested the mechanism shown in Fig. 1. Acetyl coenzyme A (acetyl-CoA) was assumed to condense with the valine precursor, $\alpha$-ketoisovaleric acid, to yield $\alpha$-isopropylmalic acid\(^1\) in a manner analogous to the condensation of acetyl-CoA and oxaloacetate to yield citric acid. By the same series of reactions undergone by citric acid to yield $\alpha$-ketoglutaric acid, this dicarboxylic acid could be converted to $\alpha$-ketoisocaproic acid, which, by transamination with glutamic acid, would yield leucine.

In agreement with this mechanism, Jungwirth, Margolin, and Umbarger (6) recently reported the isolation and identification of $\alpha$-isopropylmalate from the medium of leucine auxotrophs of *Salmonella typhimurium*, and showed that this compound was formed from valine and acetyl-CoA in cell-free extracts of this mutant. In another communication (7) this group reported the isolation of a second compound produced by a leucine auxotroph of *Neurospora crassa*, tentatively identified as $\beta$-isopropylmalate, another intermediate in the proposed pathway for leucine biosynthesis. They showed that this intermediate is oxidatively decarboxylated, as would be expected according to the mechanism. While this manuscript was in preparation, Calvo, Kalyanpur, and Stevens (8) reported the isolation of $\alpha$-isopropylmalic acid and $\beta$-isopropylmalic acid from the media of leucine-requiring mutants of *Neurospora crassa*. These workers also showed the conversion of valine to $\alpha$-isopropylmalate in broken cell preparations of *Escherichia coli*. We found that incubation of yeast extracts with carboxyl-labeled acetate and unlabeled $\alpha$-ketoisovalerate, in the presence of CoA and adenine triphosphate, yielded highly radioactive $\alpha$-isopropylmalic acid, which, as expected, contained all of the acetate carboxyl activity in its $\beta$-carboxyl carbon. To study further the enzymatic formation of $\alpha$-isopropylmalate, we have developed a fluorometric assay procedure for determining this compound in microgram quantities. This procedure is reported in the present study, together with experiments dealing with the production of $\alpha$-isopropylmalic acid has been chosen because of its simplicity as well as the fact that this designation corresponds to the generalized mechanism (Fig. 9), presented under “Discussion,” which portrays the product of the condensation of acetate and $\alpha$-keto acids as an $\alpha$-derivative of maleic acid. The designated $\alpha$ position corresponds to the maleate carbon holding the hydroxyl group. It should be pointed out that $\alpha$-isopropylmalic acid has also been named $\alpha$-hydroxy-$\alpha$-isopropylsuccinic acid by Dr. M. Yamashita (5) and by the authors in previous publications (3) and that the name $\beta$-carboxy-$\beta$-hydroxyisocaproic acid has been adopted by Jungwirth et al. (6) and Gross et al. (7) in describing their work on leucine biosynthesis.
&-carboxyl group were determined by means of the procedures
pmole of CoA; and 0.2 ml of dialyzed yeast extract (12 mg of pro-
p-Carboxyl, observed
p-Carboxyl, calculated (7 times over-all)
mann) was disrupted by means of a high speed refrigerated
detail in an earlier paper (11), were dialyzed against 0.02 M
The concentration of protein in a typical preparation was 65 mg
potassium phosphate buffer, pH 7.2, for 18 to 20 hours at 3".
ether extraction, diluted with inactive synthetic or-isopropylmalic
activity of the cu-isopropylmalic acid (average specific activity for
50 pmoles of potassium ol-ketoisovalerate; 20 pmoles of ATP; 0.8
phosphate buffer, pH 7.2; 25 pmoles of potassium acetate-l-Cl4 (10 PC);
The complete system contained 50 µmoles of potassium phos-
From chetoisovalerate and acetate-l -Cl4
The mixture was aerated at room temperature for 4 hour and
a micro decarboxylation apparatus described in a previous pub-
labeled. To 42 mg of acid with an activity of 64.6 c.p.m. (as
The radioactive spot did not appear unless C¹⁴-labeled acetate
Enzymatic Formation of α-Isopropylmalate
5-oxazolone, as described by Cornforth (13). The sodium α-ketoisovalerate was prepared by neutralizing a cold solution
addition of sufficient acetone to initiate precipitation of the salt. Acetate-1-C¹⁴ was obtained from Nuclear-Chicago Corporation. Coenzyme A and adenosine
tryptophane were purchased from Pabst Laboratories, and
cysteine hydrochloride from Merck and Company. The
product of the reaction, α-isopropylmalic acid, was prepared
by Dr. H. E. Umbarger.
Isolation of Labeled α-Isopropylmalate, Formed Enzymatically
from α-Ketoisovalerate and Acetate-1-C¹⁴—The enzyme reaction
was carried out as outlined in Table I. The reaction mixture
was then deproteinized by addition of tungstic acid as described
(11). After addition of 3 ml of 1 N H₂SO₄, the solution was
extracted continuously overnight with ether. The residue
obtained by evaporation of the ether extracts was dissolved in a
small amount of water and spotted on paper. The solvent
system used for developing the paper chromatogram contained
glacial acetic acid, water, and ether, 3:1:13. By the use of this
solvent system, unreacted radioactive acetic acid was diluted
with a large excess of unlabeled acetic acid and thereby removed
when the chromatogram was dried in air. As a result, spots
containing enzymatically produced acids were free of radioactive
acetate. In this experiment essentially all of the radioactivity
was confined to a single spot having the same Rᵢ value as syn-
thetically produced α-isopropylmalic acid. Elution of the materi-
al in this spot and rechromatographing it in other solvent
systems gave Rᵢ values identical with those of the synthetic
compound. Radioactive spots were identified with the use of a
Tracerlab monitor and an Atomic Accessories, Inc. paper strip
scanner.
The radioactive spot did not appear unless C¹⁴-labeled acetate
was incubated in the presence of α-ketoisovalerate, indicating
a condensation between acetate and α-ketoisovalerate. Elution
of the radioactive spot from a typical chromatogram gave 1.7
mg of impure undiluted radioactive material. Dilution with 135
mg of carrier synthetic α-isopropylmalate and recrystallization
from ethyl acetate gave material having an activity as the free
acid of 485 c.p.m. A sample of this material was completely
oxidized with persulfate to CO₂, which was trapped in 0.5 N
sodium hydroxide and converted to BaCO₃ as previously de-
scribed (4). An average specific activity of 65.3 c.p.m. per
plate, Table I, was observed for the BaCO₃. Subsequent re-
crystallization as shown in Table I produced no loss in activity,
thus further indicating the identity between labeled and un-
labeled acids.
Degradation of Active α-Isopropylmalate—The recrystallized active α-isopropylmalate was degraded chemically, as shown in
the reactions in Fig. 2, to determine which carbon atoms were
labeled. To 42 mg of acid with an activity of 64.6 c.p.m. (as
BaCO₃) there was added 0.3 ml of concentrated sulfuric acid in
a micro decarboxylation apparatus described in a previous pub-
llication (14). During addition of the sulfuric acid, the reaction
flask was cooled in ice. As the solution was allowed to warm
to room temperature, a vigorous evolution of gas occurred.
The mixture was aerated at room temperature for 3 1/2 hour and
the gas was passed into 10 ml of 0.5 N NaOH as described
previously (14). Addition of 10 ml of 20% BaCl₂ to the NaOH
solution gave no precipitate of BaCO₃. It was therefore assumed

Fig. 1. Proposed mechanism for leucine biosynthesis

TABLE I

<table>
<thead>
<tr>
<th>Location of labeling</th>
<th>Times recrystallised</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-Isopropylmalate, over-all</td>
<td>1</td>
<td>65.3</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>68.7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>64.6</td>
</tr>
<tr>
<td>β-Carboxyl, calculated (7 times over-all)</td>
<td>452</td>
<td>1412</td>
</tr>
</tbody>
</table>

α-isopropylmalate in a cell-free yeast extract. A preliminary
report of these findings was presented previously (9).

EXPERIMENTAL PROCEDURE AND RESULTS

Preparation of Yeast Extract—Fresh bakers' yeast (Fleisch-
mann) was disrupted by means of a high speed refrigerated
centrifuge shaker, described by Shockman, Kolb, and Toennies
(10). Extracts from the broken cells, prepared as described in
detail in an earlier paper (11), were dialyzed against 0.02 M
potassium phosphate buffer, pH 7.2, for 18 to 20 hours at 3°.
The concentration of protein in a typical preparation was 65 mg
per ml determined by ultraviolet absorption according to the
procedure of Waddell (12).

Substrates and Cofactors—The substrate, α-ketoisovaleric acid,
was prepared by acid hydrolysis of its 2-phenyl-4-isopropylidene-
that the gas evolved was carbon monoxide, as would be expected in the decarboxylation of α-hydroxy acids in the presence of concentrated sulfuric acid. An example of such a reaction is the conversion of citric acid to acetonedicarboxylic acid in the presence of cold concentrated sulfuric acid (15). In this type of reaction, the α carbon carrying the carboxyl and hydroxyl groups becomes a carbonyl carbon. As shown in Fig. 2, the absence of carbon dioxide during this reaction indicates that the β-carboxyl group of α-isopropylmalic acid was left intact and that β-ketoisocaproic acid was formed during the reaction. Without isolation of this intermediate, the acid solution was diluted by addition of 3.0 ml of water. Since β-keto acids are readily decarboxylated by heating, to yield CO₂, raising the temperature of the reaction mixture to 100°C gave a rapid evolution of gas as expected. This was collected in 0.5 N NaOH, and on addition of 3.0 ml of water. Since α-keto acids are readily decarboxylated to yield CO₂, raising the temperature of the reaction mixture to 100°C gave a rapid evolution of gas as expected. This was collected in 0.5 N NaOH, and on addition of 3.0 ml of water.

The CO₂ formed in this second decarboxylation reaction represents the β-carboxyl of α-isopropylmalate. Since this β-carboxyl is derived from the carboxyl of acetate via condensation of acetate and α-ketoisovalerate, it should contain all of the activity derived from carboxyl-labeled acetate and should therefore have a specific activity 7 times the average over-all specific activity of the whole molecule. This calculated value, 452 c.p.m., shown in Table I, compared quite well with the observed value of 412 c.p.m. determined by the decarboxylation procedure.

**Fluorometric Assay of α-Isopropylmalate**—Attempts to assay α-isopropylmalate by means of fluorometric assay procedures used for malic acid (16, 17) failed. The formation of β-ketoisocaproic acid in the above degradation reactions suggested a modification of a fluorometric assay method used by Leonardi and Glasenapp (18) for the determination of acetoadic acid in biological fluids. The fluorescence produced in this reaction is due to the condensation of a β-keto acid and resorcinol to form a derivative of umbelliflorone as shown in Fig. 2. This procedure as outlined below proved favorable for determination of microgram quantities of α-isopropylmalate and was therefore applicable to enzyme assay.

The enzyme assay mixture is patterned on the one used by Novelli and Lipmann (19) for studying the condensation of acetate and oxaloacetate. The components of the mixture, shown in Table II, were placed in 12-ml heavy wall conical centrifuge tubes and incubated for 1 hour at 37°C. In each assay, after termination of the enzyme reaction by deproteinization with tungstic acid as previously described (11), the mixture was continuously extracted with ether for 20 hours as described. The residues, obtained by evaporation of the ether extracts, were dissolved in 2 ml of anhydrous ether. Aliquots of 0.1 to 0.2 ml were pipetted into 15-ml tapered glass-stoppered centrifuge tubes, and the solutions were evaporated to dryness by placing the tubes in warm water and blowing air over them. The residues were carefully dissolved by addition of 0.4 ml of 98% sulfuric acid, and the solutions were allowed to stand at room temperature for 2 hour. After addition of 0.5 ml of a solution of 1.0 g of resorcinol in 10 ml of water, with shaking, 1.0 ml of concentrated hydrochloric acid was added. The tubes were allowed to stand in the dark for 18 to 20 hours and 7.0 ml of 30% sodium carbonate solution was added with care, since vigorous effervescence took place. The solutions were placed in 20-ml glass-stoppered tubes and 3.5 ml of borate buffer, pH 8.8 (7.32 g of boric acid dissolved in 100 ml of 1.0 N Na₂CO₃ and diluted to 1 liter), were added. The mixtures were shaken, and the fluorescence of each solution was determined with a Turner fluorometer. A standard curve was obtained, in which the intensity of fluorescence was directly proportional to the added

![Fig. 2. Reactions for degradation and assay of α-isopropylmalic acid](image-url)
FIG. 3. Formation of α-isopropylmalate with time. Conditions for enzyme assay are described in Table II, except that 0.1 ml of dialyzed yeast extract was added and cysteine was omitted.

FIG. 4. Effect of enzyme concentration on yield of α-isopropylmalate. Assay conditions were the same as described in Table II, except for variations in enzyme concentration and omission of cysteine. Reaction time was 1 hour.

FIG. 5. Effect of substrate concentration on yield of α-isopropylmalate. Enzyme assay conditions were as described in Table II, except for variation in α-ketoisovalerate concentration as shown in the top curve, variation in acetate concentration as shown in the bottom curve, and addition of 0.05 ml of dialyzed yeast extract. Duration of the reaction was 1 hour, and cysteine was omitted. In this and following figures, IPM denotes α-isopropylmalic acid.

Requirements for Enzymatic Production of α-Isopropylmalate—The results of a typical experiment are shown in Table II. A good yield of α-isopropylmalate, 3.98 μmoles, was obtained when both acetate and α-ketoisovalerate were incubated under nitrogen in the presence of dialyzed yeast extract, potassium phosphate buffer (pH 7.2), Mg++ ions, ATP, CoA, and cysteine. If acetate, α-ketoisovalerate, or enzyme was omitted, poor or no yields of product were obtained. The omission of either nitrogen or cysteine had little effect on the yield; however, if ATP or CoA was omitted, poor yields of α-isopropylmalate were obtained. Omission of Mg++ ion reduced the production of α-isopropylmalate by almost one-half. As shown, doubling the concentrations of ATP and CoA did not increase the yield of product. The reaction mixture shown in Table II, without cysteine, was used in subsequent experiments.

The production of α-isopropylmalate in the presence of 0.1 ml of yeast extract at 37° was determined over a period of 90 minutes. As shown in Fig. 3, the yield of product increases almost linearly with time over this period. An incubation of 1 hour was chosen as a convenient length of time for the enzyme assay.

Effect of Enzyme and Substrate Concentrations—Under the conditions described, the yields of α-isopropylmalate, as shown in Fig. 4, were proportional to the amount of added yeast extract, up to 0.1 ml. With 0.05 ml of extract per tube, it was found that approximately 10 μmoles of acetate, 10 μmoles of α-ketoisovalerate, and 0.02 μmole of CoA per tube constituted the optimal conditions for the reaction. Concentrations in excess of these did not increase the yields of approximately 1.5 μmoles of α-isopropylmalate obtained in 1 hour in the presence of this amount of enzyme.

The effect of concentration of each substrate upon the yield of product in 1 hour was determined more precisely as shown in Fig. 5. From these results it is evident that an initial concentration of 0.003 mM α-ketoisovalerate and 0.0015 mM acetate, respectively, will saturate 0.05 ml of dialyzed yeast extract. From these curves, an approximate Michaelis constant, K_m, for
α-ketoisovalerate is 1.0 × 10^{-3} M, and for acetate, the \(K_m\) value is 6.0 × 10^{-4} M. These values were checked by means of a double reciprocal plot of the data according to the method of Lineweaver and Burk (20).

Yields of α-isopropylmalate appeared to be directly proportional to the amount of added ATP as shown in Fig. 6. The requirement of a large excess of ATP, approximately 10 μmoles per μmole of α-isopropylmalate formed, may be due to the occurrence of side reactions in our crude enzyme system that required ATP.

α-Isopropylmalate Dehydrase—In the mechanism proposed, α-isopropylmalate is presumed to be converted to an α,β-unsaturated derivative via a dehydration reaction similar to the conversion of citrate to cis-aconitic acid. The occurrence of such an intermediate can be determined since α,β-unsaturated acids absorb strongly at 240 nm, the wave length used by Racker (21) for determining fumarate and cis-aconitate. As shown in Fig. 7, a mixture of citrate and yeast extract in phosphate buffer gave a rapid increase in absorbancy at this wavelength. When synthetic dl-α-isopropylmalate was treated with yeast extract, no reaction occurred, as shown in the bottom curve. However, when natural α-isopropylmalate, isolated from Salmonella mutants, was used, a slow but steady increase in absorbancy was found, indicating operation of the α-isopropylmalate dehydration step in the leucine biosynthesis pathway shown in Fig. 1. The lack of enzyme activity in the case of synthetic α-isopropylmalate could conceivably be due to an inhibition by the unnatural optical isomer present.

Role of Cofactors The requirement of CoA and ATP shown in Table II indicates a possible conversion of acetate to acetyl-CoA, which in turn condenses with α-ketoisovalerate to form α-isopropylmalate. To test this possibility, synthetic acetyl coenzyme A was substituted in place of CoA and ATP in the reaction mixture. Acetyl-CoA was prepared according to the method of Simon and Shemin (22). The amount of coenzyme A used in this preparation was checked with the use of a molar absorbancy index of 14.6 × 10^3 at 260 nm (23). The complete conversion of coenzyme A to acetyl-CoA was determined by testing the disappearance of thiol groups with nitroprusside indicator (24). As shown in Table III, α-isopropylmalate was obtained in the absence of ATP, indicating the need for ATP only in the formation of acetyl-CoA and not in the condensation step. The incomplete conversion of acetyl-CoA to α-isopropylmalate observed in this experiment is probably due to cleavage of acetyl-CoA by a phosphotransacetylase occurring in our crude extract. In order to avoid the possibility of this reaction, which requires the presence of phosphate ions, the remaining experiments were carried out with Tris HCl buffer.

Acetyl Coenzyme A Utilization as Compared to α-Isopropylmalate Production—The condensation of acetyl-CoA and α-ketoisovalerate results in the cleavage of the thioester bond of acetyl-CoA. The reaction was therefore further studied by measuring the decrease in optical density at 232 nm, associated with carbon-sulfur bond cleavage, according to the method described by Dixon and Kornberg (25) for determining the condensation of acetyl-CoA and glyoxylate.

Into each of two quartz cuvettes (1-cm light path, 3.0-ml volume) was placed 2.5 ml of the following assay mixture: 1.0 ml of 0.5 M Tris-HCl buffer, pH 8.5; 4.0 ml of water; 0.2 ml of acetyl-CoA solution containing approximately 7 μmoles per ml (22); and 0.15 ml of 0.1 M MgCl\(_2\). The cuvettes were placed...

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**Table III**

<table>
<thead>
<tr>
<th>Omitted</th>
<th>α-Isopropylmalate formed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.00</td>
</tr>
<tr>
<td>α-Ketoisovalerate</td>
<td>0.00</td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>0.00</td>
</tr>
<tr>
<td>Extract</td>
<td>0.02</td>
</tr>
</tbody>
</table>
indicated in Fig. 8. The enzyme assay mixture, in the presence and absence of α-ketoisovalerate, is described under "Experimental Procedures and Results."

![Graph](image-url)

**Fig. 8.** Effect of α-ketoisovalerate on the rate of acetyl-CoA disappearance as measured by decrease in absorbancy at 232 m\(\nu\) with a Cary spectrophotometer. Change in absorbancy was converted into micromoles of acetyl-CoA utilized based on the molar absorbancy index of 4.5 \(\times\) 10\(^3\). The enzyme assay mixture, in the presence and absence of α-ketoisovalerate, is described under "Experimental Procedures and Results."

### Table IV

**Acetyl-CoA disappearance** compared with α-isopropylmalate formation

<table>
<thead>
<tr>
<th>Time</th>
<th>α-Isopropylmalate formation</th>
<th>Acetyl-CoA disappearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>min</td>
<td>µmole</td>
<td>µmole</td>
</tr>
<tr>
<td>Experiment 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>0.135</td>
<td>0.140</td>
</tr>
<tr>
<td>30</td>
<td>0.380</td>
<td>0.260</td>
</tr>
<tr>
<td>45</td>
<td>0.380</td>
<td>0.300</td>
</tr>
<tr>
<td>60</td>
<td>0.308</td>
<td>0.400</td>
</tr>
<tr>
<td>Experiment 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>0.073</td>
<td>0.072</td>
</tr>
<tr>
<td>20</td>
<td>0.162</td>
<td>0.136</td>
</tr>
<tr>
<td>30</td>
<td>0.212</td>
<td>0.104</td>
</tr>
</tbody>
</table>

* Disappearance of acetyl-CoA refers to its utilization by condensation with α-ketoisovalerate and is corrected for hydrolysis of acetyl-CoA in the absence of α-keto acid. This correction is explained under "Experimental Procedures and Results."

**Discussion**

Results of the experiments with yeast cell-free extracts reported here in which the formation of α-isopropylmalate is shown to occur via a condensation of the methyl carbon of acetate and the carbonyl carbon of α-ketoisovalerate offer additional evidence for the scheme for leucine biosynthesis as indicated in Fig. 1. Further evidence for this mechanism has been supplied by Jungwirth, Margolin, and Umbarger (6), who reported the accumulation of α-isopropylmalate in leucine-requiring mutants of *Salmonella typhimurium* and tested the formation of this compound from valine and acetate in extracts of this organism. Although evidence for the second step in the proposed mechanism is still indiscernible, the conversion of α-isopropylmalate to an α,β-unsaturated acid, as suggested by Dixon and Kornberg (25), is consistent with the occurrence of a dehydrase reaction. Finally, the third step in the proposed scheme is revealed by the isolation of β-isopropylmalate from leucine-requiring yeast-induced extracts and by the demonstration that it is a precursor of α-isopropylmalate.
mutants of Neurospora crassa by Gross, Jungwirth, and Umbarger (7). Thus good evidence has now been presented for the involvement of reactions paralleling those of the citric acid cycle in the formation of leucine in yeasts, fungi, and bacteria.

A similar mechanism involving reactions of the citric acid cycle type was also previously suggested on the basis of C14 tracer studies for the biosynthesis of lysine in yeast (14). Results of experiments now in progress in our laboratory substantiate the occurrence of these reactions. Although the results will be reported in detail later, it might be mentioned here that we have found that acetate-2-C14, when incubated in the presence of unlabeled α-ketoglutarate, CoA, ATP, and yeast extract, gives rise to highly labeled β-carboxy-β-hydroxyadipic acid, homocitric acid, whose formation was suggested as a precursor of lysine in yeast (14). This of course represents the first step in the citric acid cycle-like reactions for lysine production.

A generalized scheme for the biosynthesis of the keto acid precursors of leucine and lysine based on the classical formation of α-ketoglutaric acid is presented in Fig. 9. Such a generalized citric acid cycle series of reactions brings into focus the similarity in biosynthetic pathways of these three α-keto acids. It also points out the wider application of this series of reactions involving the condensation of an α-keto acid with acetate to produce an α-substituted derivative of malic acid whose subsequent conversion via the reactions pictured gives rise to an α-keto acid homologue of the original keto acid. This generalization was also pointed out by Calvo, Kalyanpur, and Stevens (8), and additional evidence for such a scheme, leading to compounds of increased carbon chain length, was reported by Ingraham, Guymon, and Crowell (27) for the synthesis in yeast of α-keto-butyrate, α-ketovalerate, and α-ketocaprate from the corresponding lower keto acid analogues.

**SUMMARY**

The present report demonstrates the occurrence in yeast of an enzyme that brings about the condensation of the methyl group of acetate and the carbonyl group of α-ketoisovalerate to produce α-isopropylmalic acid, a previously suggested precursor for leucine synthesis in yeast. The enzyme reaction, studied in detail by means of a fluorometric assay procedure for determination of α-substituted malic acid derivatives, requires coenzyme A (CoA), adenosine triphosphate, and Mg+2 ions in addition to the substrates. Substitution of acetyl-CoA for CoA, adenosine triphosphate, and acetic thiaminephosphate demonstrates that the condensation occurred between acetyl-CoA and α-ketoisovalerate. Rates of acetyl-CoA disappearance in the presence of α-ketoisovalerate and enzyme, measured by decrease in absorbancy with a Cary spectrophotometer, showed that acetyl-CoA net utilization and α-isopropylmalate formation occur simultaneously. In light of these findings, it is felt that an appropriate name for this enzyme would be α-isopropylmalate synthetase.

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2 After submission of this paper there appeared another article by Jungwirth et al. (29), which presents in greater detail the results described above (6, 7). In addition, evidence was presented for the formation of α-ketoisocaproic acid from α-isopropylmalate in the presence of Salmonella extracts, a transformation requiring three steps, isomerization, oxidation, and decarboxylation, in the leucine biosynthetic scheme. Also mentioned briefly was their interesting finding that the cis isomer, isopropylmalic acid, supports growth of leucine requiring Salmonella mutants whereas the trans isomer, isopropylfumaric acid, was inactive.

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**REFERENCES**

Enzymatic Formation of $\alpha$-Isopropylmalic Acid, an Intermediate in Leucine Biosynthesis

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