The Biosynthesis of Leucine. III. The Conversion of α-Hydroxy-β-Carboxyisocaproate to α-Ketoisocaproate*

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Examination of the culture fluids of several leucine auxotrophs derived from *Neurospora crassa* revealed that one strain, 33757, accumulated, in addition to β-carboxy-β-hydroxyisocaproate, a second compound which supported the growth of three of four classes of leucine auxotrophs of *Salmonella typhimurium*. This compound was isolated and identified as α-hydroxy-β-carboxyisocaproate by nuclear magnetic resonance spectrum and by elementary analysis. The compound was converted to α-ketoisocaproate and carbon dioxide by an NAD-linked dehydrogenase. The enzyme was partially purified from extracts of *S. typhimurium* by ammonium sulfate precipitation and by chromatography on brushite. The enzyme exhibited a requirement for both mono- and divalent cations for maximal activity. NADP would not substitute for NAD. The optimum *p*H was 9.5. The formation of the dehydrogenase was dependent upon single structural genes in *S. typhimurium* and *N. crassa*.

In the preceding paper the conversion of β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxyisocaproate was shown to be an obligatory step in leucine biosynthesis (Gross *et al.*, 1963). In this paper, the isolation of α-hydroxy-β-carboxyisocaproate is described as well as the reaction by which it is converted to α-ketoisocaproate, the immediate precursor of leucine.

MATERIALS AND METHODS

Organisms and Media.—The organisms employed in this study were wild strains of *Neurospora crassa* and *Salmonella typhimurium* (strain LT-2), and several leucine auxotrophs derived from them. The genetic and some of the biochemical characteristics of the mutants have been previously described (Margolin, 1959; Gross and Gross, 1961; Jungwirth *et al.*, 1963).

Analyses.—The nuclear magnetic resonance spectrum was very kindly prepared and interpreted by Dr. W. M. Ritchey of the Sohio Research Laboratory. The elementary analyses were made by the Galbraith Company. The toluene extraction method of Friedemann and Haugen (1943) was employed for the determination of α-ketoisocaproate. Protein was determined by the method of Lowry *et al.*, (1951).

Chemicals.—Nicotinamide adenine dinucleotide (NAD) and the sodium salt of α-ketoisocaproic acid were obtained from the Sigma Chemical Co. Taraconic acid was synthesized according to the method of Stobbe (1895). Dimethylcitraconic acid was a gift from Dr. C. Jungwirth. Dimethylmesaconic acid and the racemates of synthetic α-hydroxy-β-carboxyisocaproic acid were obtained from Dr. J. Calvo. Brushite was prepared according to the procedure of Tiselius *et al.*, (1956). Other chemicals were obtained from commercial sources.

RESULTS

The Accumulation of Leucine Precursors in N. crassa Culture Fluids.—Upon examining the culture fluids of representatives of the four classes of leucine auxotrophs derived from *N. crassa* it was noted that one strain, *33757*, in addition to β-carboxy-β-hydroxyisocaproate accumulated a second compound capable of replacing the requirement of certain leucine auxotrophs of *S. typhimurium*. Whereas β-carboxy-β-hydroxyisocaproate supported the growth of only class I leucine auxotrophs of *S. typhimurium*, the second compound allowed growth of classes I, III and IV auxotrophs (hereafter designated *leu I*, *III*, *IV*, respectively). Like β-carboxy-β-hydroxyisocaproate, the second compound did not support growth of any *N. crassa* auxotrophs. Curiously, no similar compound could be found in the culture filtrates of *S. typhimurium* auxotrophs which could not respond to this compound (i.e., *leu II* mutants).

Elsewhere evidence has been presented that the four classes of *leu* auxotrophs of *S. typhimurium* are blocked in four different cistrons which occur in a cluster (Margolin, 1963). It has also been established that the *leu I* cistron was concerned with the formation of the first enzyme (Jungwirth *et al.*, 1963). The demonstration of a compound which lay before the block in the *leu II S. typhimurium* mutants and after the blocks in the *leu III* and *leu IV* mutants provided the first indication that the enzyme missing in *leu II* mutants was involved in a step later in the biosynthetic sequence than that controlled by the other three mutant classes. It was therefore possible to conclude that the genetic linkage did not parallel the order of the enzymatic steps they governed.

Isolation of the Active Compound.—Preliminary observations on the unknown compound showing growth factor activity, such as retention by anion but not by cation exchange resins and its solubility under acidic conditions in relatively nonpolar solvents, indicated that the compound was an organic acid. In order to isolate and characterize this acid a large volume of filtrate from a culture of *N. crassa*, strain *33757* was collected.

For this purpose, a twenty-liter carboy with a three-inch bed of clean sand and ten liters of minimal medium supplemented with 15 mg L-leucine per liter was employed. The medium was inoculated with a suspension containing about 10^7 conidia from a mature culture of *N. crassa*, strain *33757*. The culture was incubated at room temperature and aerated vigorously. After three days six liters of culture fluid was withdrawn through

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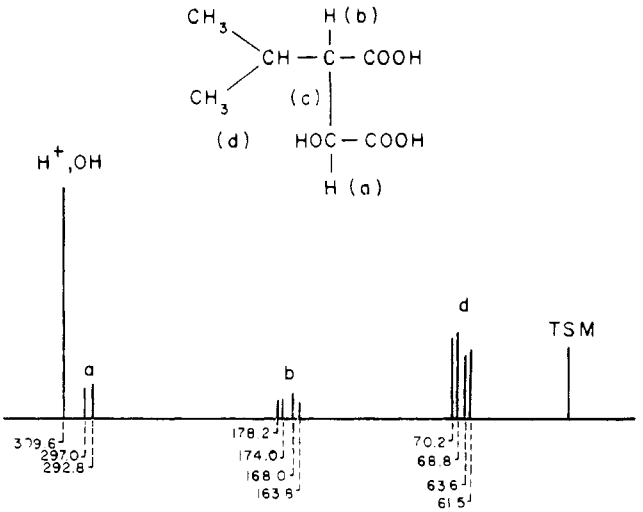

FIG. 1.—The nuclear magnetic resonance spectrum of the *N. crassa* excreted intermediate. The abscissa represents the linear distance in cps/second from the external standard (tetramethyl silane). The signals are represented as lines drawn from the center of each peak and are proportional to the height of each peak. The numbers under the lines are the peak locations (in cps) with respect to the external standard. The spin-spin coupling constants (J) in cps are: J_{ab}, 4.2; J_{bc}, 10.2; J_{bd}, 6.8. The shielding numbers in ppm are: H^+, —OH, 5.16; a, 4.92; b, 2.86; d, 1.12 and 1.08. Precision: cps/s = ± 0.06, J = ± 0.2, shielding number = ± 0.01.

a tube which contained a glass wool plug and had been imbedded within the sand layer. It was thus possible to remove the culture fluid while leaving the entire mycelial mat behind. The medium was replaced with six liters of fresh sterile medium containing five mg L-leucine per liter and the incubation continued. At two-day intervals this procedure was repeated until 18 liters of culture filtrate was obtained.

The culture filtrate was evaporated to dryness *in vacuo* and taken up in 150 ml of water. The *p*H was adjusted to 2.0 with hydrochloric acid and the active material extracted with ethyl acetate for twelve hours in a Kutscher-Steudel apparatus.

The extract thus obtained was evaporated to dryness and the residue dissolved in a minimum of water. The aqueous solution was neutralized and placed directly on a 35 × 2.5 cm column of Dowex-1-formate, (8×, 200–400 mesh). Elution was performed with formic acid, the concentration of which was increased over a hyperbolic gradient by allowing one liter of 4 M formic acid to enter a mixing chamber containing one liter of distilled water. The flow rate was about 25 ml per hour and the size of each fraction collected was 8 ml.

The presence in the fractions of either β-carboxy-β-hydroxyisocaproate or the other active compound was conveniently determined by auxanography. *S. typhimurium* strain *leu* 120 responded to either β-carboxy-β-hydroxyisocaproate or the new compound, whereas *S. typhimurium* strain *leu* 128 responded only to the new compound. For the purpose of the assay one-tenth ml portions of each fraction were placed on filter discs (Whatman 3 MM) in duplicate. The impregnated discs were placed on a stainless steel mesh and autoclaved for ten minutes at 15 pounds psi in order to remove the excess formic acid. One of each pair of discs was then placed on the surface of each of two minimal agar plates which had been seeded with cells of *S. typhimurium* strains *leu* 120 and *leu* 128, respectively. Following incubation of the plates overnight at 37°, growth zones around the discs indicated that tubes 80–100 contained β-carboxy-β-hydroxyisocaproate whereas the unknown compound appeared in tubes 100–200. The contents of these tubes (100–200) were pooled and the chromatography was repeared two times.

The thrice-chromatographed material was dissolved in ethyl acetate. Upon addition of chloroform the compound crystallized as long, thin needles. The active material melted at 146–147° and was levorotatory, $(\alpha)_D^{24} = -5.2$, (1.5% in water).

ANALYSIS

	C	H	O
Calculated for $C_7H_{12}O_5$	47.73	6.82	45.45
Found	47.63	6.98	45.52

Characterization of the Compound as α-Hydroxy-β-Carboxyisocaproic Acid.—The results of the auxanographic analysis mentioned previously indicared that the unknown compound was an intermediate in leucine biosynthesis occurring after β-carboxy-β-hydroxyisocaproate. In accordance with the scheme of leucine biosynthesis proposed by Strassman *et al.* (1956) and based upon its behavior during the course of purification the compound was tentatively identified as α-hydroxy-β-carboxyisocaproic acid. This identification was strengthened by the results of elementary as well as nuclear magnetic resonance analyses of the purified material.

The nuclear magnetic resonance spectrum of the compound was obtained with a Varian Model DP-60 Spectrometer at 60 megacycles and is shown in Figure 1. The purified compound was examined as a D_2O solution at a sweep rate of about one cps per second with tetramethyl silane as an external standard. Although the signals were low owing to the concentration of sample used, the resolution was such as not to affect the interpretation of the spectrum. The field locations of the resonance lines and the spin-spin coupling constants are both consistent with the proposed structure. The spectrum is composed of six groups of signals. The pair of doublets at 61.5–68.4 and 63.6–70.2 cps correspond to the methyl groups of the isopropyl moiety; the signals were split owing to the presence of a tertiary proton on the adjacent carbon. As can be seen with a molecular model, this tertiary proton also causes restricted rotation of the carbon atom to which it is attached. The restricted rotation brings about a nonequivalency of the methyl groups. The signal of the tertiary proton is not discernible in the spectrum primarily because its signal was split into a multiplet of eight peaks by the six protons of the methyl groups and the proton of the β-carbon. These eight peaks are also broadened by the nonequivalency of the methyl groups. The proton of the β-carbon yielded a quartet (163.8–178.2 cps) owing to the presence of the protons on both the α- and γ-carbons. On the other hand, the proton of the α-carbon appeared as a doublet since it has but one neighboring proton. In contrast to the nuclear magnetic resonance spectrum of β-carboxy-β-hydroxyisocaproate (see Jungwirth *et al.*, 1963) the methyl lines in Figure 1 occur somewhat upfield owing to their increased remoteness from the hydroxyl oxygen. The spin-spin coupling constants all fell in the range expected and were characteristic of the types of coupling anticipated for α-hydroxy-β-carboxyisocaproic acid.

Enzymic Conversion of α-Hydroxy-β-Carboxyisocaproate to α-Ketoisocaproate.—It was mentioned previously that the leucine requirement of all but one of the four classes of leucine auxotrophs of *S. typhimurium* was replaced by α-hydroxy-β-carboxyisocaproate. As in the case of β-carboxy-β-hydroxyisocaproate, the iso-

TABLE I

UTILIZATION OF COMPOUNDS APPEARING IN CULTURE FLUID OF *N. crassa* STRAIN 33757

Filter discs which had been impregnated with about 40 μg of purified α-hydroxy-β-carboxyisocaproate were placed on the surface of minimal agar adjusted to *p*H 5.5, which had been seeded with about 5 × 10^8 cells of the strain listed. Growth was observed as a halo around the impregnated discs following overnight incubation of the plates at 37°.

Organism	Cistron Affected	Utilization for Growth: β-Carboxy-β-hydroxy-isocaproate	Utilization for Growth: Second Compound
leu 124	I	+	+
129	II	−	−
126	III	−	+
128	IV	−	+

meric α-hydroxy acid could replace the leucine requirement of responding organisms only if the medium were adjusted to *p*H 5.5. No growth supporting activity was noticeable even after prolonged incubation if medium at *p*H 7.0 were used. Table I shows the growth response of representatives of the four classes of auxotrophs to α-hydroxy-β-carboxyisocaproate. Extracts of those organisms which were able to grow on the α-hydroxy acid were also able to convert this compound to a keto acid as was shown by the extractability under acid conditions into toluene of the 2,4-dinitrophenylhydrazone derivative of the reaction product.

For the determination of the requirements of the enzymatic conversion of α-hydroxy-β-carboxyisocaproate to the α-keto acid an extract of *S. typhimurium*, strain *leu* 128 was treated with charcoal and dialyzed against 200 volumes of Tris-succinate, *p*H 7.0, for 24 hours. An extract thus treated showed optimum formation of the keto acid from α-hydroxy-β-carboxyisocaproate in Tris-HCl, *p*H 8.0, only when NAD. $MnCl_2$, and KCl were added to the reaction mixture. A standard assay was developed in which enzymic activity was followed by determining the amount of keto acid formed. The reaction mixture contained per two ml: Tris-HCl, *p*H 8.0, 300 μmole; $MnCl_2$, 1.0 μmole; KCl, 100 μmole; NAD, 2 μmole; α-hydroxy-β-carboxyisocaproate, 1.0 μmole; and extract containing from 0.02–0.25 units of activity. The mixture was incubated at 37° for ten minutes after which the 2,4-dinitrophenylhydrazine reagent was added to stop the reaction. A unit of activity is defined as the amount of enzyme required to form one μmole of keto acid (measured as α-ketoisocaproate) in the assay. Specific activity is the number of units per mg of protein.

In Table II are shown results obtained in using this assay procedure to examine the α-hydroxy-β-carboxyisocaproate-keto acid reaction in the wild type parent and representatives of the four classes of *leu* auxotrophs of *S. typhimurium*. For this purpose the mutants were grown in minimal medium supplemented with limiting amounts (10 mg per l) of L-leucine and 50 mg L-isoleucine per liter.[1] As shown in the table the enzyme is missing in strain *leu* 129. This strain has been shown by genetic analysis to contain a lesion in the *leu* II cistron (Margolin, 1959). Similar observations with other *leu* II mutants indicate unequivocally that this cistron is specifically concerned with the formation of the enzyme catalyzing the conversion of α-hydroxy-β-carboxyisocaproate to an α-keto acid.

[1] It was observed that addition of isoleucine to the growth medium produced cells with an increased capacity for converting α-hydroxy-β-carboxyisocaproate to the α-keto acid. This effect is probably attributable to the competition between L-isoleucine and L-leucine either for entry into the cell or for occupancy of the amino acid pool (Britten and McClure, 1962), thus causing a limitation of leucine resulting in derepression of the formation of the keto acid forming system.

TABLE II

THE FORMATION OF KETO ACID FROM α-HYDROXY-β-CARBOXYISOCAPROATE BY SONIC EXTRACTS OF VARIOUS *S. Typhimurium* LEUCINE AUXOTROPHS

The organisms were grown as described in the text. The standard assay was used.

Organism	Cistron Affected	Enzymatic Specific Activity
leu 124	I	4.0
129	II	0.0
126	III	6.0
128	IV	8.4
Lt-2	none	0.4

Partial Purification of the Keto Acid Forming Enzyme. —In order to study in greater detail the reaction in which α-hydroxy-β-carboxyisocaproate is converted to a keto acid it was desirable to obtain the enzyme free of NADH oxidase activity. For this purpose, *S. typhimurium* strain *leu* 128 was grown at 37° with aeration in 12 liters of minimal medium supplemented with 5 mg of L-leucine per liter. When growth had stopped owing to exhaustion of the growth factor, an additional 2 mg of L-leucine was added. This step-wise addition of leucine was continued until a total of 21 mg per liter had been added.

The cells in the culture grown in this manner were harvested in the Sharples centrifuge. This and all the other manipulations were performed in a 4° cold room unless otherwise specified. The cell paste was resuspended in 50 ml of 0.05 M potassium phosphate, *p*H 7.2 and the cells were disrupted by means of a French pressure cell. The disrupted preparation was centrifuged at 14,000 rpm in a Servall SS-4 centrifuge for twenty minutes and the supernatant obtained was centrifuged at 90,000 × *g* for one hour in a Model L Spinco preparative centrifuge. This supernatant was diluted with distilled water to give a protein concentration of about 10 mg per ml. After acidification to *p*H 6.0 by the drop-wise addition of 0.5 M acetic acid, the extract was warmed to 15° and the nucleic acids were precipitated by the slow addition of 1.5% protamine sulfate (*p*H 5.5), until 0.015 mg protamine sulfate per mg protein had been added. The nucleate formed was removed by centrifugation and discarded. The protamine treated extract was further fractionated by the addition of solid ammonium sulfate. The protein precipitating between 45–65% saturation was dissolved in a minimal amount of 0.01 M potassium phosphate, *p*H 6.8 and dialyzed against 200 volumes of the same buffer for twelve hours. A calcium phosphate (brushite) column 30 × 2.5 cm was prepared and sufficient pressure applied so that the solids remained stationary upon inversion of the column. The dialyzed ammonium sulfate fraction was added to the column and eluted with a linear concentration gradient of potassium phosphate, *p*H 7.0. The gradient was established by placing 250 ml of distilled water in the mixing chamber and 250 ml of 0.2 M potassium phosphate in the reservoir. The protein eluting between 160–190 ml contained the keto acid forming activity.

The results of the purification procedure presented in Table III illustrate a thirty-fold increase of specific activity over that found in the crude extract. This specific activity corresponds to a 300-fold increase with

TABLE III

PURIFICATION OF THE KETO ACID FORMING SYSTEM

The last column represents units of enzyme other than those retained for further purification.

Step of Purification	Enzyme Units	Protein (mg)	Specific Activity	Other Units
Crude extract	5167	954	5	—
Protamine sulfate				
$(NH_4)_2SO_4$	4015	291	14	1020
Calcium phosphate	2409	14	164	1500

respect to the comparable activity of a wild type culture. Subsequent chromatography of the partially purified preparation on diethylaminoethyl cellulose columns resulted in complete recovery of the enzyme with no significant purification.

The partially purified protein was free of NADH oxidase activity and provided an enzyme preparation suitable for use in determining the stoichiometry of the α-hydroxy-β-carboxyisocaproate degrading reaction.

Reaction Balance and Identification of the Keto Acid.—The partially purified enzyme preparation was incubated with 10 μmoles of α-hydroxy-β-carboxyisocaproate and 20 μmoles of NAD together with the standard assay supplements in a total volume of 3.2 ml. The evolution of CO_2 was determined in a standard Warburg apparatus. An identical reaction mixture was incubated until the reaction was completed. Samples were removed and the keto acid determined as the 2,4-dinitrophenylhydrazone. NADH was measured spectrophotometrically. Table IV shows that the yield of keto acid (as α-ketoisocaproate), CO_2, and NADH occurred in a ratio of 1:1:1, in amounts corresponding to the complete degradation of the substrate. The keto acid formed was converted to the 2,4-dinitrophenylhydrazone derivative for chromatographic comparison with the 2,4-dinitrophenylhydrazone of authentic α-ketoisocaproate. The R_F values on Whatman #1 paper were identical with the three solvent systems employed (butanol:ethanol, 4:1, saturated with water; water-saturated butanol; and butanol saturated with 3% (w/v) ammonia). The keto acid was further characterized by its ability to form leucine when incubated together with glutamic acid and pyridoxal phosphate in the presence of an *Escherichia coli* extract known to have strong transaminase activity. The aminated product was identified as leucine by chromatography with water-saturated phenol.

The name proposed for the enzyme converting α-hydroxy-β-carboxyisocaproate and NAD to α-ketoisocaproate, NADH and CO_2 is α-hydroxy-β-carboxyisocaproate dehydrogenase.

Properties of α-Hydroxy-β-Carboxyisocaproate Dehydrogenase.—As mentioned previously, the dehydrogenase activity demonstrated a strong dependency on

TABLE IV

STOICHIOMETRY OF α-HYDROXY-β-CARBOXYISOCAPROATE DEHYDROGENASE REACTION

The amounts of reaction products shown in the table were obtained from 10 μmoles of α-hydroxy-β-carboxyisocaproate. The details of the experiment are given in the text.

Compound Formed	Amount (μmoles)
α-Ketoisocaproic acid	9.8
Carbon dioxide	9.4
NADH	9.1

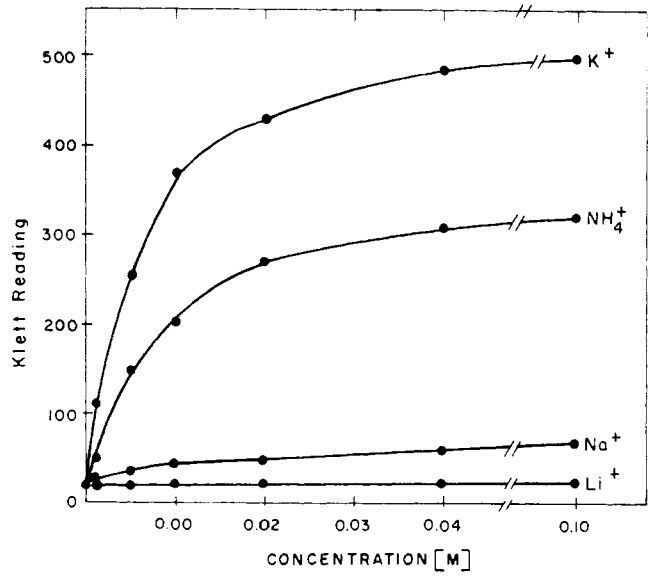

FIG. 2.—The effect of monovalent cations on the reaction rate of α-hydroxy-β-carboxyisocaproate dehydrogenase. The standard assay was used but with the specified concentrations of the cation. The partially purified preparation of the dehydrogenase was dialyzed against 200 volumes of Tris-succinate, *p*H 6.8 for 24 hours before use.

potassium ions. Whereas the requirement for potassium ions could be partially replaced by ammonium ions, sodium ions showed little stimulation of activity and lithium was completely inactive. The effects of the various monovalent ions are shown in Figure 2. Potassium ions were only slightly inhibitory at concentrations in excess of one molar. Typically, the requirement for divalent cations was lower than for the monovalent cations. Table V illustrates the effect of various divalent cations at a concentration of 5×10^{-4} M on the dehydrogenase activity; of the ions tested, barium, zinc, and calcium were inhibitory. This inhibitory effect was less in the presence of those ions which stimulated activity (Mn, Mg, Co), although even these cations were somewhat inhibitory at concentrations above 5×10^{-4} M.

The *p*H optimum for the dehydrogenase reaction as determined in Tris-succinate buffer in the presence of magnesium ions was 9.5, with about eighty per cent of the optimum activity expressed at *p*H 10 and *p*H 9.0. The selection of *p*H 8.0 rather than *p*H 9.5 for the routine analysis of the enzyme was prompted by the insolubility of such ions as manganese and cobalt at the higher value.

α-Hydroxy-β-carboxyisocaproate possesses a high

TABLE V

THE STIMULATION OF α-HYDROXY-β-CARBOXYISOCAPROATE DEHYDROGENASE ACTIVITY BY DIVALENT CATIONS

Addition	Relative Activity
$MnCl_2$	165
$CoCl_2$	82
$MgCl_2$	117
$BaCl_2$	21
$ZnCl_2$	9
$CaCl_2$	17
—	25

A standard reaction mixture was used but with the specified compound in a final concentration of 5×10^{-4} molar. The partially purified preparation of the dehydrogenase employed was dialyzed against 200 volumes of Tris-succinate, *p*H 6.8 for 24 hours.

affinity for the dehydrogenase as evidenced by a K_m for the reaction of 3.8×10^{-6} M. In light of the equilibrium of the isomerase reaction yielding the α-hydroxy acid from the β-hydroxy acid (see accompanying paper) the high affinity of the enzyme for its substrate together with the irreversibility of the reaction can be viewed as the driving force in leucine biosynthesis. The K_m for NAD as determined with a NADH oxidase-free enzyme preparation was 2.5×10^{-4} M.

The effect of temperature on the velocity of the dehydrogenase reaction is shown in Figure 3. The enzymic reaction had a temperature coefficient of about two per ten degrees rise in temperature over the range tested. The activation energy of the reaction as computed from the Arrhenius plot was 2,600 cal per mole.

The Specificity of α-Hydroxy-β-Carboxyisocaproate Dehydrogenase.—The specificity of the reaction was tested by determining the ability of the partially purified dehydrogenase to reduce NAD in the assay in the presence of various compounds. The same compounds were tested for their ability to decrease the rate of reduction of NAD when present with α-hydroxy-β-carboxyisocaproate in the assay. β-Carboxy-β-hydroxyisocaproate, dimethylcitraconate, taraconate, dimethylmesaconate, DL-isocitrate, DL-malate, and isopropylmalonate were added to the reaction mixture in concentrations ten-fold that of α-hydroxy-β-carboxyisocaproate. The reaction was assayed spectrophotometrically by following the appearance of NADH at 340 mμ. None of the compounds tested decreased the rate of reduction of NAD when α-hydroxy-β-carboxyisocaproate was present in the reaction mixture, nor was NAD reduced when any of the compounds was added to a similar mixture from which α-hydroxy-β-carboxyisocaproate was omitted. The two racemic mixtures of synthetic α-hydroxy-β-carboxyisocaproic acid were tested for activity in the assay; these compounds were also tested for inhibition of the oxidation of the compound excreted by *N. crassa*. Neither racemate inhibited the rate of NAD reduction when assayed together with the biologically produced α-hydroxy-β-carboxyisocaproate, and only one of the racemates was oxidized in the enzymatic system. The active racemate is inseparable by chromatography from the biologically derived material (Calvo *et al.*, 1962).

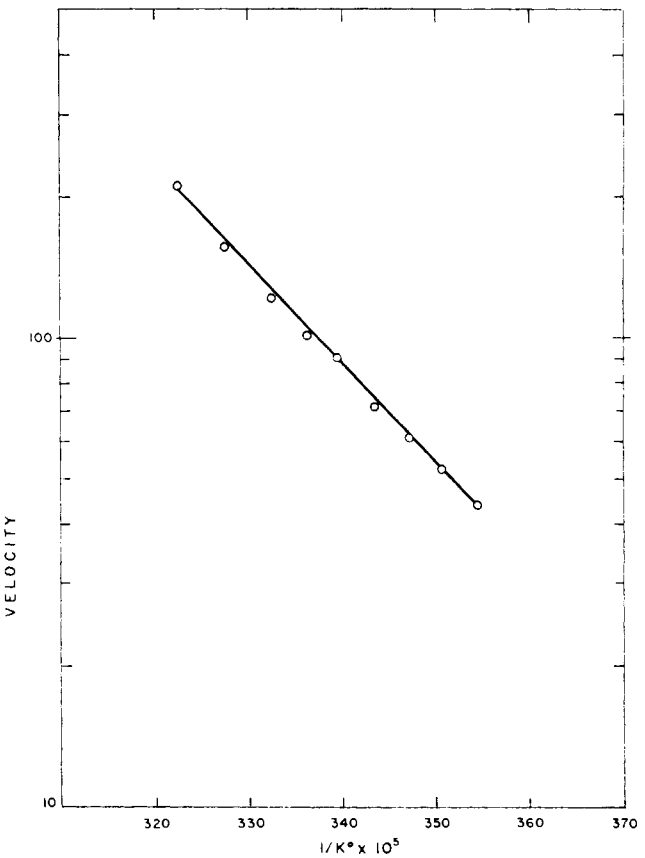

FIG. 3.—The effect of temperature on the rate of α-hydroxy-β-carboxyisocaproate dehydrogenase activity. A partially purified preparation was used. The reaction rate was measured at the temperatures shown.

DISCUSSION

The role of α-hydroxy-β-carboxyisocaproic acid in the biosynthesis of leucine had been suggested previously by Strassman *et al.* (1956) on the basis of isotope incorporation experiments in yeast. The present work amplifies the earlier evidence for the participation of this compound in leucine biosynthesis.

Although synthetic α-hydroxy-β-carboxyisocaproate was not available for comparison with the material excreted by *N. crassa* at the time that the characterization experiments were performed, the analytical data presented point clearly to the identification of the biologically derived material as α-hydroxy-β-carboxyisocaproic acid. Subsequent availability of the synthetic compound corroborated both the identification of the isolated compound as well as its role in the biosynthesis of leucine.

With the description of the last specific step in leucine biosynthesis the similarity of the leucine system to the citrate-α-ketoglutarate system becomes obvious. The condensation of α-ketoisovalerate with acetyl coenzyme A, the isomerization of the resulting β-hydroxy acid to an α-hydroxy acid and the oxidative decarboxylation of this α-hydroxy acid to α-ketoisocaproate are all analogous to the conversion of oxaloacetate to α-ketoglutarate. The single difference in the overall mechanism is that in the citric acid cycle the —OH group is transferred to the carbon distal to the carbons derived from acetate, whereas in leucine biosynthesis the —OH is transferred to the carbon derived from the methyl group of acetate.

The specificity of α-hydroxy-β-carboxyisocaproate dehydrogenase is demonstrated by the inability of various structurally related compounds either to inhibit the conversion of α-hydroxy-β-carboxyisocaproate to α-ketoisocaproate or to compete with the enzyme in this conversion.

Enzymic and genetic data indicate that this reaction proceeds by way of a single enzyme. The integrity of the enzymic activity and its unimodal behavior during the course of the purification procedure, as well as the linearity of the reaction rate with time and protein concentration, all argue for a single enzyme. The continuity of the Arrhenius plot also indicates the action of one enzyme or at least enzymes with similar temperature coefficients (Stearn, 1949). It is also of interest that findings of rather exhaustive genetic analysis have resulted in the conclusion that one cistron controls the formation of α-hydroxy-β-carboxyisocaproate dehydrogenase (Margolin, 1963).

Although the description of leucine biosynthesis is similar in both *N. crassa* and *S. typhimurium*, one dissimilarity is evident. It will be recalled that whereas certain *N. crassa* mutants accumulated α-hydroxy-β-carboxyisocaproate, comparable *S. typhimurium* mutants did not. It may be that this difference will be explained on the basis of mechanisms controlling the formation of the leucine enzymes rather than on the intrinsic nature of the enzymes. For example, it was indicated in the foregoing paper that strains of *N. crassa*

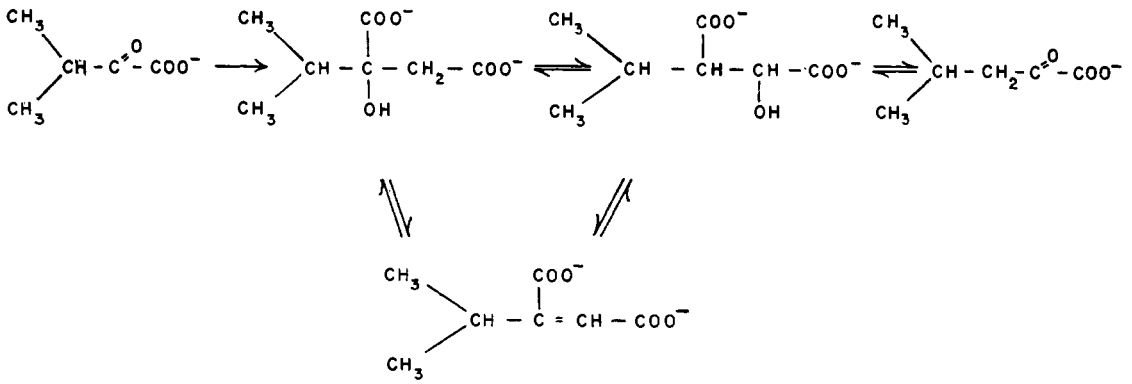

FIG. 4.—The pathway of leucine biosynthesis in *S. typhimurium* and *N. crassa*.

which accumulate α-hydroxy-β-carboxyisocaproate exhibit greatly increased levels of the isomerase. The question of the control of leucine biosynthesis is currently being investigated in these laboratories.

The description of leucine biosynthesis in *N. crassa* and *S. typhimurium* represents the most recent example of the use of mutant methodology for the elucidation of a biosynthetic pathway. Although a general scheme for leucine biosynthesis based on the results of isotopic data was formulated by Strassman *et al.* (1956), it was not until the accumulation patterns of various leucine auxotrophs of *N. crassa* and *S. typhimurium* were examined that the active intermediates in leucine biosynthesis became available. It is also noteworthy that the concepts of enzyme repression aided in the *in vitro* demonstration of the formation and utilization of the intermediates in leucine synthesis. Whereas, at least in the case of *S. typhimurium* and *E. coli* (Burns and Umbarger, unpublished), wild type strains contain detectable activity of the three leucine enzymes, cultures grown under conditions of leucine limitation possessed a manyfold increase of the same activity.

The leucine synthesizing system as described in this and previous papers (Gross *et al.*, 1963; Jungwirth *et al.*, 1962) demonstrates that leucine biosynthesis proceeds by the scheme shown in Figure 4. Of the four reactions in this pathway the last appears to be catalyzed by two rather nonspecific transaminases in *S. typhimurium* that seem to be analogous to transaminase A and transaminase B described by Rudman and Meister (1953) in *E. coli*. Only one of these, transaminase B, has ever been reported missing in a mutant. Such mutants, however, do not require leucine but do require isoleucine for growth since this enzyme offers the only mechanism for the conversion of α-keto-β-methylvalerate to isoleucine. It would thus appear that the leucine-glutamate transaminase activity in transaminase A is sufficient to provide for leucine biosynthesis even in the absence of transaminase B.

The three enzymes catalyzing the steps leading to α-ketoisocaproate, however, appear to be completely specific and, as these results and those in the previous publications have demonstrated, they are controlled by four specific genes in *N. crassa* and *S. typhimurium* (Gross and Gross, 1961; Margolin, 1959). Whereas the four genes in *S. typhimurium* occur in a cluster and constitute a single operon in *S. typhimurium* (Margolin, 1963), this relationship is apparently not an obligatory one since the four corresponding genes in *N. crassa* are distributed on three chromosomes.

The first step in the pathway, the condensation of α-ketoisovalerate and the acetyl group of acetyl coenzyme A which is catalyzed by the β-carboxy-β-hydroxyisocaproate condensing enzyme, has previously been shown to be controlled by the *leu I* gene of *S. typhimurium* and by the *leu 4* gene of *N. crassa*. In the preceding paper, the isomerase converting β-carboxy-β-hydroxyisocaproate to α-hydroxy-β-carboxyisocaproate was described. The exact role of dimethylcitraconate in leucine biosynthesis is not known. However, the isomerase reaction as depicted in Figure 4 is considered to be analogous to the aconitase reaction (Racker, 1950) in that dimethylcitraconate is one of the three species of compounds which can reversibly dissociate from the enzyme. As reported in the previous paper, the isomerase, though apparently a single protein, is formed only in the presence of wild type *leu III* and *leu IV* genes in *S. typhimurium* and wild type *leu 2* and *leu 3* genes in *N. crassa*. Finally, α-hydroxy-β-carboxyisocaproate is converted to α-ketoisocuproate by the enzyme described in this paper, α-hydroxy-β-carboxyisocaproate dehydrogenase, a NAD-linked enzyme. The formation of this enzyme is dependent upon the presence of the wild type *leu II* gene in *S. typhimurium* and the *leu 1* gene in *N. crassa*.

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