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The Biosynthesis of Leucine. I. The Accumulation of β-Carboxy-β-Hydroxyisocaproate by Leucine Auxotrophs of *Salmonella typhimurium* and *Neurospora crassa**

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A dicarboxylic acid was found in the culture fluids of three of four classes of leucine-requiring mutants (auxotrophs) of *Neurospora crassa* and in the fluids of three of four classes of similar mutants of *Salmonella typhimurium*. This compound was isolated and identified as β-carboxy-β-hydroxyisocaproic acid. It supported the growth of the class of *S. typhimurium* auxotroph which failed to accumulate the compound. The compound was formed from α-ketoisovalerate and acetyl coenzyme A by extracts of *N. crassa* and of *S. typhimurium*. In the presence of DPN the compound was converted to α-ketoisocaproic acid, the immediate precursor of leucine. Evidence was obtained that this conversion occurred via the isomerization of β-carboxy-β-hydroxyisocaproic acid to α-hydroxy-β-carboxyisocaproic acid. The experiments provided support for the general features of a scheme for the biosynthesis of leucine proposed in 1956 by Strassman and co-workers. The steps in the proposed pathway were correlated with the genetic lesions in the leucine auxotrophs.

Studies with biochemically deficient (auxotrophic) mutants, isotopically labeled compounds, and enzyme preparations have made it possible to outline, in a general way at least, most of the enzymatic steps required for the biosynthesis of nearly all of the naturally occurring amino acids. The biosynthesis of leucine, however, has until now been refractory to enzymatic analysis, in spite of much isotopic evidence (Gilvarg and Bloch, 1951; Reiss and Bloch, 1955; Roberts *et al.*, 1955; Rafelson, 1957) supporting a scheme proposed by Strassman *et al.* (1956). One reason for this delay and one which emphasizes the importance of mutant methodology to the elucidation of biosynthetic pathways appears to be that, although leucine auxotrophs were frequently isolated, they all responded to L-leucine or to α-ketoisocaproate but to no other compounds tested as possible intermediates. Furthermore, unlike the auxotrophs often encountered in other pathways, there was no cross-feeding observed among the various strains as would have been expected if a strain blocked late in the pathway accumulated a compound to which a strain blocked earlier could respond.

Recently, a large number of leucine auxotrophs of *Neurospora crassa* and *Salmonella typhimurium* were subjected to an intensive study as a collaborative effort in these two laboratories. The mutants employed were known to represent several distinct loci on the basis of genetic evidence (Barrat *et al.*, 1954; Margolin, 1959; Gross and Gross, 1961). In the early phase of this study, some of the auxotrophs of both species were shown to accumulate a compound which supported the growth of certain *S. typhimurium* auxotrophs. This paper describes the production of the compound, and its isolation and subsequent identification as β-carboxy-β-hydroxyisocaproate, an early intermediate in the scheme proposed by Strassman *et al.* In addition, its formation and its conversion to α-ketoisocaproate are briefly described.

EXPERIMENTAL

Organisms.—Six leucine auxotrophs derived from *S. typhimurium* were employed. They were designated strains *leu*-39, *leu*-120, *leu*-124, *leu*-128, *leu*-129, and *leu*-130. These strains were representative of four groups of leucine auxotrophs in which the mutational sites had previously been shown to lie in four distinct cistrons (designated I–IV) on the basis of complementation analysis by abortive transduction using phage PLT22 (Margolin, 1959). Thus, strains *leu*-120 and *leu*-124 exhibited lesions in the *leu*-I cistron, while strains *leu*-129, *leu*-130, and *leu*-128 contained lesions in the *leu*-II, *leu*-III, and *leu*-IV loci, respectively. Strain *leu*-39 exhibited a multisite lesion in cistrons II, III, and IV. In addition, recombination mapping (by the three-point test of Demerec and Hartman, 1956) revealed that all four *leu* cistrons occur in a cluster

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close enough to the arabinose region of the chromosome to be carried jointly with an arabinose marker in transduction tests.

The *N. crassa* mutants have also been shown to have resulted from mutational events occurring in four genes. In *N. crassa*, however, these genes were unlinked and were distributed on three chromosomes (Barrat *et al.*, 1954; Gross and Gross, 1961). Thus strains 33757, R86, R156, and R59 exhibited lesions in genes *leu*-1, *leu*-2, *leu*-3, and *leu*-4, respectively. These mutants and those derived from *S. typhimurium* are listed in Table I.

TABLE I
SITES OF GENETIC LESIONS IN LEUCINE AUXOTROPHS

S. typhimurium Strain	Gene Affected	*N. crassa* Strain	Gene Affected
leu-120	*leu*-I	33757	*leu*-1
leu-124	*leu*-I	R86	*leu*-2
leu-129	*leu*-II	R156	*leu*-3
leu-130	*leu*-III	R59	*leu*-4
leu-128	*leu*-IV		
leu-39	*leu*-II, III, IV		

Medium.—The minimal medium of Davis and Mingioli (1950) modified by using 5 g of glucose per liter and by omitting citrate was usually employed for the bacterial mutants. It was supplemented as indicated in the text. Liquid cultures were grown either in Erlenmeyer flasks with rotary shaking or in a New Brunswick continuous fermenter at 37°. For the auxanographic tests for the accumulated compound the medium contained in 1 liter: K_2HPO_4, 0.5 g; KH_2PO_4, 8.7 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; dextrose, 5.0 g; and agar, 15 g; (final *p*H 5.5).

For the growth of large quantities of *N. crassa* the medium of Vogel (1956) was employed half-strength and supplemented with 1% sucrose and 15 mg L-leucine per liter.

Preparation of Extracts.—The bacteria were grown in the continuous fermentor employing a minimal medium supplemented with 15 mg of L-leucine per liter. The doubling time was limited by continuously replacing the growth medium over a 4-hour period. The culture volume was kept constant by continuously withdrawing from the culture at the same rate. The harvested material was chilled immediately. All subsequent operations were performed at between 0 and 4°. The cells were collected by centrifugation in a motor-driven Sharples centrifuge and washed by resuspension in one tenth the original volume of 0.01 M phosphate buffer, *p*H 7.6, and centrifuged at 13,000 $\times$ *g* in a refrigerated centrifuge. The washing was repeated and the cell paste was suspended in 0.07 M phosphate buffer (1 g wet cells in 8 ml). The cells were disrupted by means of a French pressure cell or by ultrasonic oscillation. The treated suspension was clarified by centrifugation at 28,000 $\times$ *g* for 20 minutes. The protein content of the extract was determined by the method of Lowry *et al.* (1951).

Analyses.—Radioactivity measurements were made on planchets at infinite thinness with a Tracerlab gas flow Geiger counter. The elementary analyses were performed by Schwarzkopf Laboratories and Galbraith Laboratories.

The infrared spectra were made and interpreted by Dr. H. Jaffe of the Rockefeller Institute for Medical Research and by Dr. E. Davidson of Duke University. The nuclear magnetic resonance spectra were made and interpreted by Dr. W. M. Ritchey of the Research Department of the Standard Oil Company, Cleveland, Ohio.

Cross-feeding Between leu Mutants of S. typhimurium.—When the various *leu* mutants of *S. typhimurium* were streaked on the surface of agar plates containing the usual minimal medium adjusted to *p*H 7.0, no cross-feeding (syntrophism) was observed. When medium adjusted to *p*H 5.5 was employed, it was evident that strains *leu*-129, *leu*-130, and *leu*-128 excreted a material into the medium which supported the growth of the two *leu*-I mutants, *leu*-120 and *leu*-124. Similarly, strain *leu*-39, the multisite mutant in which only the *leu*-I locus was intact, also fed *leu*-120 and *leu*-124. In accord with the experience of others (Davis, 1950), clearer results were obtained in tests for syntrophism if the medium was enriched with a small amount of growth factor, either 100 mg of dehydrated nutrient broth (Difco) or 1 mg of L-leucine per liter.

That reducing the *p*H of the medium was essential only for the utilization of the accumulated material rather than its production was indicated by the fact that any of the strains shown to be "feeders" produced the active material when grown in the usual minimal medium supplemented with limiting L-leucine (15 mg per liter). The material was conveniently assayed by impregnating filter paper disks with concentrates of the culture fluids and placing them on the surface of the assay medium (at *p*H 5.5) seeded with strain *leu*-120 or strain *leu*-124. The pattern of excretion and response is shown in Table II. No activity could be detected with the usual minimal medium (at *p*H 7.0) used for assay.

TABLE II
CROSS-FEEDING BETWEEN LEUCINE AUXOTROPHS

Organism	Behavior in Feeding Test
S. typhimurium	
leu-120	Responded to excreted material
leu-124	Responded to excreted material
leu-129	Excreted active material
leu-130	Excreted active material
leu-128	Excreted active material
leu-39	Excreted active material
N. crassa	
33757[a]	Excreted active material
R86	Excreted active material
R156	Excreted active material
R59	No response; failed to excrete

[a] This mutant, strain 33757, has also been noted to excrete a compound in addition to β-carboxy-β-hydroxyisocaproate which supported the growth of *S. typhimurium* strains *leu*-128 and *leu*-130. A preliminary report presenting evidence that this compound was α-hydroxy-β-carboxyisocaproic acid has been published (Gross *et al.*, 1962).

Parallel experiments with the leucine auxotrophs of *N. crassa* failed to reveal any syntrophism, even though the *p*H of the medium was 5.5. Similarly, none of the *N. crassa* mutants responded to the material excreted into the medium by the *S. typhimurium* mutants. However, as shown in Table II the culture fluids of all the *N. crassa* mutants except strain R59 supported the growth of the *leu*-I *S. typhimurium* mutants.

Isolation of the Active Material.—With the above assay for the active material in culture fluids of the multisite mutant of *S. typhimurium*, strain *leu*-39, the patterns of adsorption on and elution from ion exchange resins and of solvent extraction indicated the growth factor to be acidic. Examination of varying cultural conditions revealed that it was produced in appreciable

amounts only after leucine disappeared from the medium; this indicated that leucine, by repression or end-product inhibition or both, caused the same pattern of precursor accumulation observed in other biosynthetic pathways (Umbarger, 1961). Accordingly, for the production of large amounts of the material, strain *leu*-39 was cultivated at 37° with forced aeration in a New Brunswick continuous fermentor under conditions in which growth was continuously limited by the amount of growth factor (L-leucine) provided.

The culture vessel, containing 4 liters of minimal medium supplemented with 15 mg of L-leucine per liter, was inoculated with a 1-liter culture of strain *leu*-39. When maximum growth in the culture had been achieved, fresh medium was added continuously at the rate of about 0.8 liter per hour (in order to permit a doubling in cell number in about 6 hours). The volume of the culture was kept at 5 liters by simultaneous withdrawal from the culture. The harvested culture was chilled until 16 liters had been collected.

The cells were removed by centrifugation in the cold with use of a motor-driven Sharples centrifuge. The culture fluid was concentrated *in vacuo* and acidified with hydrochloric acid. The active material was extracted several times with ethyl acetate. The extracts were combined and evaporated to dryness. The glassy residue was dissolved in water and decolorized by heating with charcoal. The solution was filtered and passed through a column of Dowex-50 (8X, 200–400 mesh) in the hydrogen form. The effluent solution was evaporated to a semisolid mass, acidified with 10 N HCl, and extracted several times with ethyl acetate. The ethyl acetate extracts were combined and dried over sodium sulfate. The active material was obtained as a white, crystalline compound by the addition of ligroin to the ethyl acetate extract. The crystals were collected on a Buchner funnel, washed with ligroin, and dried *in vacuo* over calcium chloride. Another crop of crystals was obtained from the mother liquor and combined with the first. The compound was recrystallized three times from ethyl acetate–ligroin mixtures.

The active material produced by *N. crassa* strain R86 was obtained in a similar way after removal of the mycelial mat from a 2-liter culture grown with forced aeration at room temperature.

Analysis

	C	H	O
Calculated for $C_7H_{12}O_5$	47.73	6.82	45.45
Found, compound isolated *from S. typhimurium*, strain *leu*-39, culture	47.92	7.11	45.26

Identification of the Compound as β-Carboxy-β-hydroxyisocaproate.—The compound was found to migrate with synthetic β-carboxy-β-hydroxyisocaproic acid (kindly supplied by Dr. Murray Strassman) when chromatographed ascendingly on Whatman No. 1 paper with the solvents listed in Table III. The melting point of the isolated compound was 166–167° (corr.), whereas that of the synthetic compound was 146–147° (corr.). This difference is related to the fact that the isolated compound was levorotatory (α_D^{24} = −4.0 ± 1.0°, 1% in methanol), whereas the synthetic compound is a racemic mixture.

The infrared spectrum of the crystalline material isolated from the culture fluid was identical to that of the synthetic compound. Figure 1 shows the spectra of the compound isolated from *S. typhimurium* strain 39 and of the synthetic compound. A less highly resolved spectrum of the compound isolated from *N crassa* mutant R86 revealed its identity with the compound produced by *S. typhimurium* strain *leu*-39.

TABLE III

R_F VALUES FOR β-CARBOXY-β-HYDROXYISOCAPROIC ACID IN SEVERAL SOLVENT SYSTEMS

Solvent	Composition	R_F
A	Ethanol–7.5 N ammonia (8:2)	0.32
B	Xylene–phenol–88% (w/v) formic acid (5:5:2)	0.49
C	Propanol–15 N ammonia (6:4)	0.52
D	Butanol–88% (w/v) formic acid–water (90:10:24)	0.85
E	Mesityloxide–88% (w/v) formic acid–water (75:75:36)	0.78

The identity of the isolated and synthetic compounds was further supported by their nuclear magnetic resonance spectra (Fig. 2) obtained with a Varian model DP-60 Spectrometer at 60 megacycles. The compounds were examined as 10% solutions in D_2O with a sweep rate of about 1 cps per second. Tetramethyl silane was used as an external reference. The spectrum of the compound isolated from the culture fluid of *S. typhimurium leu*-39 was identical to the spectrum of the synthetic compound. Furthermore, the spectra strongly supported the assumed structure. The field locations of the resonance lines were as expected, and more definite confirmation was provided by the spin-spin coupling. The proton on the γ carbon was resolved as a slightly broadened quartet (123 to 144 cps) with a shielding number of 2.225 and a coupling constant of about 12 cps. The splitting here was due only to the methyl protons, indicating the absence of a proton on the β carbon. The pair of doublets representing the methyl groups (69 to 78.3 cps) indicates a slight non-equivalence of the two groups which can easily be seen with a molecular model. This non-equivalence was the cause of the proton appearing as a broadened quartet rather than a septet. The protons on the methylene (α) carbon displayed non-equivalence and produced a pair of doublets (an AB system) in the region 174 to 212 cps.

The Enzymatic Formation of β-Carboxy-β-hydroxyisocaproate.—For the demonstration of the enzymatic formation of the isolated compound, crude extracts prepared from cells of the various *S. typhimurium* strains were incubated with uniformly labeled α-ketoisovalerate and acetyl coenzyme A in the system described in Table IV. The reaction was stopped by heating. After removal of the coagulated protein by centrifugation a sample was evaporated to dryness, acidified, and extracted with ethyl acetate. One-half milligram of unlabeled β-carboxy-β-hydroxyisocaproic acid was added to the residue and the extraction repeated. The extracts were combined and evaporated to dryness. The resulting residue was dissolved in 0.5 ml of water.

In experiment I, portions of these solutions were applied to Whatman No. 3 MM paper and subjected to electrophoresis at 1700 volts for 2 hours with a 0.5 M pyridine-acetate buffer at *p*H 5.0. In experiment II, the samples were applied to Whatman No. 1 paper and chromotographed with solvent A in Table III. The product was located by radioautography and by spraying with indicator. The major portion of the radioactivity was located in the position of the carrier β-carboxy-β-hydroxyisocaproic acid, since most of the remaining α-ketoisovaleric acid was lost during the evaporation of the ethyl acetate extracts. The compound was eluted and placed on planchets for counting.

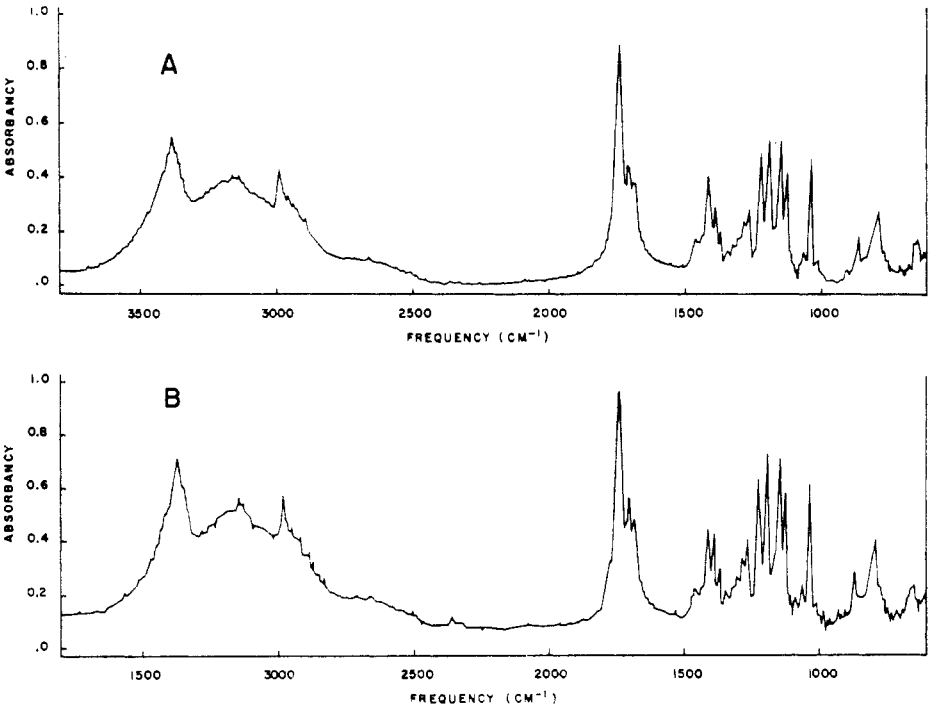

FIG. 1.—The infrared spectra of synthetic (*A*) and isolated (*B*) β-carboxy-β-hydroxyisocaproic acid. The samples (about 0.56 mg) were examined in KBr pellets (250 mg) about 0.7 mm thick.

As shown in Table IV, all the extracts tested except that prepared from strain *leu*-124 converted a considerable portion of the substrate to β-carboxy-β-hydroxycaproate. Similar experiments with the *N. crassa* mutants indicated that all strains except strain R59 could form β-carboxy-β-hydroxyisocaproate from α-ketoisovalerate and acetyl coenzyme A.

The Formation of α-Ketoisocaproate from β-Carboxy-β-hydroxyisocaproate.—For the enzymatic conversion of β-carboxy-β-hydroxyisocaproate to α-ketoisocaproate, a fresh extract prepared from strain *leu*-124 was incubated for one hour at 37° in the system shown in Table V. The product of the reaction was identified as α-ketoisocaproic acid by converting it to the 2,4-dinitrophenylhydrazone. The derivative was extracted with toluene, reextracted with sodium carbonate, and, after acidification, again extracted in toluene. By chromatography on Whatman No. 1 paper in butanol saturated with 3% (w/v) aqueous ammonia, the 2,4-dinitrophenylhydrazone prepared from the enzymatically formed compound was indistinguishable from that prepared from known α-ketoisocaproate.

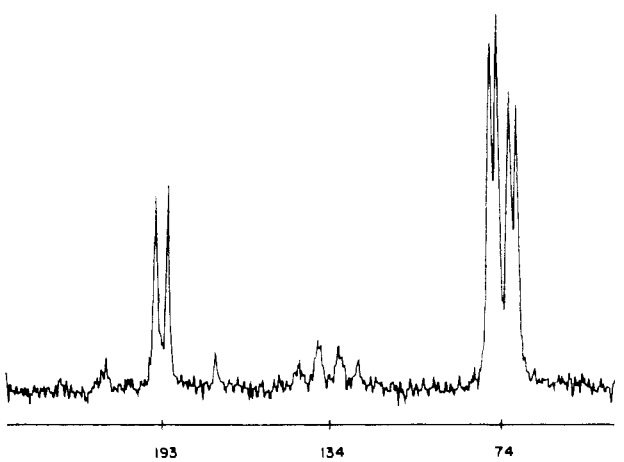

FIG. 2.—Nuclear magnetic resonance spectrum of β-carboxy-β-hydroxyisocaproic acid at 60 mc. The abscissa is expressed in cps from the peak produced by an external standard of tetramethylsilane.

Of the other strains of *S. typhimurium* tested only strain 120 yielded extracts capable of catalyzing the over-all conversion of β-carboxy-β-hydroxyisocaproate to α-ketoisocaproate. Among the *N. crassa* mutants only strain R59 (which did not accumulate the first intermediate) exhibited this property.

DISCUSSION

The finding that three of four genetically distinct classes of leucine auxotrophs derived from *S. typhimurium* as well as from *N. crassa* accumulated β-carboxy-β-hydroxyisocaproate and that one class derived from *S. typhimurium* responded to it provides strong support

TABLE IV

ENZYMATIC FORMATION OF β-CARBOXY-β-HYDROXYISOCAPROIC ACID BY *S. typhimurium* MUTANTS

The complete system in Exp. I contained in 1.0 ml: 100 μmoles of potassium phosphate, *p*H 7.6; 1.2 μmoles of acetyl coenzyme A; 0.123 μmoles of sodium α-ketoisovalerate (1.13×10^4 cpm); and crude extract containing 1.5 mg of protein. In Exp. II the complete system contained in 2.0 ml: 100 μmoles of potassium phosphate; 3.6 μmoles of acetylcoenzyme A; 1.3 μmoles of sodium α-ketoisovalerate (1.2×10^5 cpm); and crude extract containing 3.0 mg of protein. Both experiments incubated 80 minutes at 37°. Samples treated as described in text.

Strain from Which Extract Prepared	Complete System (cpm)	Acetyl Coenzyme A Omitted (cpm)
Exp. I		
leu-124	200	65
leu-39	3,400	30
Exp. II		
leu-129	52,000	180
leu-130	53,000	200
leu-128	50,000	430

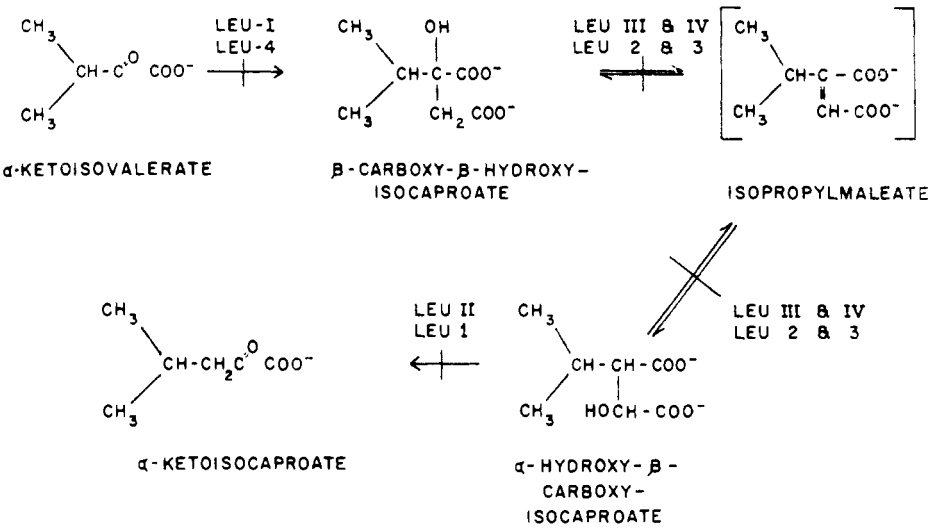

FIG. 3.—Reactions in the biosynthesis of the α-ketoacid precursor of leucine. The *leu* cistrons which appear to control the structure of the corresponding enzymes are given above each reaction. *N. crassa* cistrons are indicated by Arabic numerals, *S. typhimurium* cistrons are indicated by Roman numerals.

for the scheme proposed by Strassman *et al.* (1956). However, proof for the series of steps in their scheme has been obtained only for the first reaction, in that the condensation of α-ketoisovalerate with the acetyl group of acetyl coenzyme A (reaction 1, Fig. 3) can be demonstrated in those leucine auxotrophs which accumulate the compound, whereas those mutants which fail to accumulate it (*leu*-4 mutants of *N. crassa* and *leu*-I mutants of *S. typhimurium*) are deficient in this reaction.

The second enzymatic conversion which has been demonstrated is the formation of α-ketoisocaproate. Presumably this conversion consists of an isomerization, an oxidation, and a decarboxylation.

That the oxidation and decarboxylation of α-hydroxy-β-carboxyisocaproate are catalyzed by a single enzyme is suggested by the observation that the reaction is catalyzed by extracts of all strains tested except *N. crassa* strain 33757, the organism which accumulates α-hydroxy-β-carboxyisocaproate, and *S. typhimurium* strain *leu*-129. Observations on the kinetics of the reaction also support this view. Therefore, the reaction is shown in Figure 3 to yield α-ketoisocaproate directly and to be controlled by *N. crassa* gene *leu*-1 and by *S. typhimurium* gene *leu*-II.

In Figure 3, the isomerization is viewed as a reversible reaction proceeding via the intermediary formation of an unsaturated compound. Evidence for such a compound is presently only indirect. For example, extracts of *leu*-1 *N. crassa* and *leu*-II *S. typhimurium* mutants catalyzed the formation of a compound absorbing in the 230–240 mμ region when incubated with either β-carboxy-β-hydroxyisocaproate or α-hydroxy-β-carboxyisocaproate. Furthermore, the *cis* isomer with that structure, isopropylmaleic acid, supports the growth of *S. typhimurium leu*-I mutants. Isopropylfumaric acid (kindly furnished by Dr. Joseph Calvo), however, is inactive. It is therefore tentatively assumed that if an unsaturated compound occurs as an intermediate, it is isopropylmaleic acid.

Current studies in both laboratories indicate that the isomerization step is catalyzed by a single enzyme the structure of which is controlled by two genes. Thus in the case of *S. typhimurium* neither the *leu*-III mutant nor the *leu*-IV mutant are able to form the unsaturated compound from either direction. Leu-2 and leu-3 *N. crassa* mutants are also deficient in both activities. Furthermore, a recent analysis of the heterokaryotic behavior of the *N. crassa* mutants has provided strong evidence that the isomerase molecule consists of at least two polypeptide chains, one controlled by the *leu*-2 gene and the other controlled by the *leu*-3 gene (Gross, 1962).

It is clear that the earlier attempts to demonstrate cross-feeding between leucine auxotrophs of bacteria were unsuccessful because glucose-mineral salts media are usually buffered near neutrality. The finding that the accumulating compound, β-carboxy-β-hydroxyisocaproic acid, supported the growth of the appropriate mutant only at reduced *p*H, however, is not without precedent. For example, Adelberg (1951) showed that a reduced *p*H was required for the effective utilization of the dihydroxy acid precursors of valine and isoleucine. Indeed, the greater penetration of cells by organic acids at lower *p*H values has often been noted. This factor, however, does not account for the failure of the corresponding *N. crassa* mutant strain to respond to β-carboxy-β-hydroxyisocaproic acid, since the *p*H of the Neurospora medium is routinely adjusted to *p*H 5.5. Attempts to alter the growth conditions in a way which would permit the compound to support the growth of the *N. crassa* mutants have thus far been unsuccessful.

TABLE V

THE CONVERSION OF β-CARBOXY-β-HYDROXYISOCAPROIC ACID TO α-KETOISOCAPROIC ACID

The complete system contained in a final volume of 1 ml: 100 μmoles of potassium phosphate, *p*H 7.6; 1.0 μmole of β-carboxy-β-hydroxyisocaproic acid; 1.0 μmole of DPN; 1.0 μmole of magnesium chloride; and extract of *S. typhimurium* containing 3.0 mg of protein. The reaction mixtures were incubated for 1 hour at 37°. The reaction was stopped by heating. The protein was removed by centrifugation. A portion of the reaction mixture was used for the determination of α-ketoisocaproic acid by the indirect method of Friedemann and Haugen (1943), with toluene used as the solvent.

	α-Ketoacid Formed (μmoles)
Complete system	0.33
Substrate omitted	0.05
DPN omitted	0.09
Extract omitted	0.0

As mentioned above, there was a striking difference in the melting points of the synthetic (racemic) compound and of the natural (levorotatory) one. One further difference in the two compounds, and one which contributed to the delay in verifying the biosynthetic scheme of Strassman and co-workers, was the difference observed in the ability of the two compounds to support growth of *S. typhimurium*, strain *leu*-120. In the auxanographic tests using minimal agar seeded with this strain, the racemic compound appeared to be only about one eighth as active as the naturally occurring compound. Preliminary experiments indicate that the enzymatic basis for this difference may be accounted for by the fact that the unsaturated isomer is an inhibitor of the second (isomerization) step in the pathway. For example, when isomerase activity in an *S. typhimurium* extract was measured with a mixture of the racemic and the levorotatory compound as the substrate, the rate was only 30% of the rate observed when the levorotatory compound alone was used as the substrate. This phenomenon as well as the subsequent steps in the pathway are currently being further investigated in both laboratories.

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The α-Amino-ω-Hydroxamino Acids*

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The DL-α-amino-ω-hydroxamino acids corresponding to ornithine and lysine, DL-2-amino-5-hydroxaminopentanoic acid and DL-2-amino-6-hydroxaminohexanoic acid, respectively, have been prepared from the 5-(ω-bromoalkyl)-hydantoins *via* the corresponding nitro- and hydroxaminohydantoins. A procedure is reported for the isolation of L-2-amino-5-hydroxaminopentanoic acid from ferrichrome A. All three amino acids were obtained as the crystalline mono-2-nitro-1,3-indanedione salts. A new synthesis of DL-2-amino-5-hydroxypentanoic acid based on lithium borohydride reduction of glutamic hydantoin-γ-methyl ester has been described.

The two α-amino-ω-hydroxamino acids corresponding to ornithine and lysine occur in nature. In a pioneering investigation, Snow (1954) isolated and characterized the lysine analog from the growth factor mycobactin. Recently, δ-*N*-hydroxyornithine (L-2-amino-5-hydroxaminopentanoic acid) has been found in the ferrichrome series (Emery and Neilands, 1961) and in the antibiotic albomycin (Turková *et al.*, 1962). In each instance the amino acid was found to be of the L configuration.

It will be the purpose of the present communication to describe the chemical synthesis of the ω-*N*-hydroxy analogs of lysine and ornithine, namely, DL-2-amino-6-hydroxaminohexanoic acid and DL-2-amino-5-hydroxaminopentanoic acid respectively. The preparation of the former compound in microgram amounts has already been reported (Emery and Neilands, 1961).

While it might seem feasible to approach the synthesis of these substances by direct, selective oxidation of the parent diamino acids, no general method has been described that would enable such a reaction. In fact, inspection of the literature reveals that virtually all successful syntheses of alkyl hydroxylamines have involved reduction of the analogous nitro compounds with zinc dust in water. The nitro compounds can be obtained from the corresponding bromo compounds, but in order to apply this reaction in the present instance it would be necessary to block the α-amino function so as to avoid cyclization. Fortunately, two appropriately substituted bromo compounds have already been reported. In 1948 Gaudry described the preparation of 5-(4′-bromobutyl)-hydantoin from 2,3-dihydropyran and subsequently the same author (Gaudry, 1951) reported the synthesis of 5-(3′-bromopropyl)-hydantoin from 2,3-dihydrofuran. We have found it possible to convert these bromo compounds into the desired products in modest yield *via* the route shown in Figure 1. The nitro compounds, Va, Vb, and hydrochlorides of the end-products, VIIa, VIIb, could not be crystallized. The latter, however, crystallized readily as the mono-

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