# THE INITIAL STEP IN LEUCINE BIOSYNTHESIS.*

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Isotope incorporation studies with microorganisms have indicated that leucine carbons 3,4,5 and 5' are derived from carbons 2,3,4 and 4', respectively, of $\alpha$-ketoisovalerate and that leucine carbons 1 and 2 are derived from acetate carbons 1 and 2, respectively (Strassman et al., 1956; Roberts et al., 1955; Rafelson, 1957). The widespread occurrence of leucine-glutamate transaminase activity and the frequent isolation of bacterial mutants responding equally well to L-leucine or to $\alpha$-ketoisocaproate, have suggested that leucine biosynthesis proceeds via the corresponding $\alpha$-keto acid. Strassman et al. (1956) have proposed a pathway from $\alpha$-ketoisovalerate to $\alpha$-keto-isocaproate in which the first step is the condensation of $\alpha$-ketoisovalerate with the acetyl group of acetyl-coenzyme A to yield $\beta$-carboxy-$\beta$-hydroxyisocaproate.

Evidence has now been obtained that this compound is, in fact, an obligatory precursor of leucine. In brief, the evidence includes: (a) utilization of the compound by one (leu I) of the four classes of leucine auxotrophs identified in Salmonella typhimurium by abortive transduction (Margolin, 1959), (b) the appearance of the compound in the culture fluids of the three remaining classes (leu II, leu III and leu IV), and (c) the absence in extracts prepared from a leu I mutant of the enzyme catalyzing the synthesis of the compound and its presence in other extracts examined.

Methods. The organisms used in the experiments reported below were two leucine auxotrophs derived from the LT-2 strain of S. typhimurium. One,

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strain leu 120, belongs to class leu I while the second, strain leu 39[1], is a multi-site mutant blocked in the II, III and IV complementation groups of the leu region (Margolin, unpublished observations).

In tests for cross-feeding and in auxanographic tests, a minimal medium was employed which contained in 1 l.: 0.5 gm $K_2HPO_4$, 8.7 gm. $KH_2PO_4$, 1.0 gm $(NH_4)_2\ SO_4$, 0.1 gm $MgSO_4 \cdot 7H_2O$, 5.0 gm dextrose and 15 gm agar. For the cross-feeding tests, this medium was supplemented with 1 mg of L-leucine per l. For liquid cultures the medium used was that of Davis and Mingioli (1950) modified by the omission of citrate and by increasing the dextrose to 5.0 gm per l. A New Brunswick continuous fermenter was employed for the production of a large amount of culture fluid containing the accumulated compound as well as for growing cells used in preparing the extract.

The extract was prepared from cells washed twice in M/20 potassium phosphate buffer, resuspended in buffer (8.0 ml per gm wet cells) and disrupted in a French pressure cell. The protein content was 15 mgm per ml.

Results and discussion. Preliminary experiments using solid medium revealed that mutants of groups II, III and IV consistently excreted into the agar a material which supported the growth of any group I mutant which had been streaked nearby. Strain leu 120 was chosen for its marked ability to respond to the material. The excreting strain, leu 39 was chosen because of the genetic stability of its block.

To isolate the active material a 5 l-culture of strain leu 39 was cultivated continuously with a doubling time of 6 hours and with the density of the culture limited to 0.15 mg dry wet per ml by the level of L-leucine (15 mg/l) in the medium. When 20 l of culture were collected, the cells were removed by centrifugation in a Sharples motor-driven centrifuge and the culture fluid was concentrated in vacuo.

After adjusting the pH to 1 the active material was extracted from a portion of the syrupy, salt-laden concentrate by shaking with ethyl acetate.

[1] Kindly supplied to one of the authors (P.M.) by Dr. M. Demerec

The ethyl acetate extraction was repeated, and the extracts were combined and evaporated to dryness. The residue was dissolved in a small quantity of water, treated with Norite A and passed through a Dowex-50 column ($H^+$ form). After again concentrating and acidifying, the active material was extracted with small quantities of ethyl acetate and precipitated by adding ligroin. The material was recrystallized three times from ethyl acetate-ligroin mixtures.

The isolated compound migrated with synthetic $\beta$-carboxy- $\beta$-hydroxy-isocaproate[2] on paper chromatograms in six different solvent systems and on cellulose acetate strips during ionophoresis. Infrared spectra of the two compounds revealed identical bands. There were marked differences, however, in the melting points (synthetic compound, 146°; isolated compound, 166° to 167°) and in the biological activity as indicated by the growth response of leu 120 in auxanographic tests. The synthetic compound was less active than would be expected on the basis of its racemic nature.

Analysis:

Calculated for $C_7 H_{12} O_5$.....C, 47.73; H, 6.82.

Found.....C, 47.92; H, 7.11.

Table 1 gives preliminary evidence for the enzymatic formation of the compound with an extract prepared from cells of leu 39 grown in the continuous fermenter with a 2 hour mass doubling time. In this experiment, labeled $\alpha$-ketoisovalerate was generated by a transamination reaction between L-valine U-$C^{14}$ and $\alpha$-ketoglutarate catalyzed by the extract. The only appreciably labeled compound formed was $\beta$-carboxy- $\beta$-hydroxyisocaproate. When labeled acetate, ATP and coenzyme A replaced acetyl coenzyme A, small amounts of other labeled compounds were also formed.

[2] Kindly supplied by Dr. M. Strassman.

Table I

The Enzymatic Synthesis of β-Carboxy-β-hydroxyisocaproate

| | β-Carboxy-β-hydroxyisocaproate formed counts/min. |
|---|---|
| Complete system | $2.9 \times 10^4$ |
| Acetyl Co A omitted | $6 \times 10^2$ |
| Complete system but enzyme boiled | 0.00 |

The complete system contained in 2.0 ml: potassium phosphate pH7.6, 100μ moles; L-valine-U-$C^{14}$, 1.1 μ moles containing $5.74 \times 10^4$ cpm; acetyl coenzyme A, ca. 3.2 μ moles; potassium α-ketoglutarate, 15 μ moles; magnesium chloride, 10 μ moles; and 0.2 ml of extract of S. typhimurium strain leu 39. After 80 minutes, the reaction was stopped by mixing with 6.0 ml of ethanol. The mixtures were evaporated to dryness in vacuo. The residues were acidified and extracted with ethyl acetate. To the extracted residue 500 mg of unlabeled β-carboxy-β-hydroxyisocaproate were added and the extraction repeated two more times. The extracts were combined, evaporated to dryness and taken up in 0.5 ml of water. Samples of 0.1 ml were chromatographed in ethanol-15% aqueous ammonia (4:1). The product was located by radio autography and by indicator spray, eluted and the radioactivity determined.

That the role of this compound in leucine biosynthesis is not restricted to bacteria was indicated by the accumulation of the same coupound by three of the four genetically distinct groups of leucine auxotrophs (Barratt et. al.,1954; Gross and Gross, in press) of Neurospora crassa. The other group is comparable to S. typhimurium strain leu 120. Unlike the latter, however, the corresponding N. crassa auxotroph is unable to utilize the compound for growth.

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