ANOTHER INTERMEDIATE IN LEUCINE BIOSYNTHESIS[+]

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The isolation of β-carboxy, β-hydroxyisocaproic acid, its characterization, and its enzymatic synthesis from α-ketoisovalerate and acetyl COA was described in a previous communication (Jungwirth et al, 1961). This note reports the isolation of another compound from culture filtrates of a mutant of Neurospora crassa, its enzymatic conversion to α-ketoisocaproic acid, and leucine by extracts of Salmonella typhimurium and Neurospora. The compound has been tentatively identified as α-hydroxy, β-carboxyisocaproic acid.

METHODS. The mutant organisms used were representative of the mutational alterations in the four known genes controlling leucine biosynthesis in both Neurospora and Salmonella. The mutants of Neurospora were: R59, R156, R86, and 33757 (Barratt et al, 1954, and Gross and Gross, 1961). The Salmonella mutants were: leu-120, leu-128, leu-129, and leu-130 (Margolin, 1959).

Auxanographic tests of culture filtrates of Salmonella were carried out as described previously (Jungwirth et al, 1961). Corresponding auxanographic tests on Neurospora were carried out on sorbose glycerol synthetic medium (Lester and Gross, 1959).

Extracts of Salmonella were prepared from cells grown with a two hour doubling time on limiting leucine (15 μg/ml) in a chemostat. Neurospora extracts were prepared from conidia that were allowed to germinate for 16 hours at 34°C

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in synthetic medium containing 15 μg leucine per ml. In both cases the cells obtained were washed twice with 0.1 M phosphate buffer pH 7.0 and disintegrated ultrasonically (20 KC).

RESULTS AND DISCUSSION. Chromatography of ethyl acetate extracts of filtrates of Neurospora mutant 33757 followed by bioautography against the Salmonella mutants revealed the presence of a compound in addition to β-carboxy, β-hydroxyisocaproic acid, which replaced the leucine requirement of strains leu-120, leu-128, and leu-130 but not of strain leu-129. This compound, like β-carboxy β-hydroxyisocaproic acid, did not replaee the leucine requirement of any of the Neurospora mutants.

The compound was isolated by the following procedure: Strain 33757 was grown in 10 liters of synthetic medium supplemented with 15 μg L-leucine per ml for three days. Thereafter, at two-day intervals, six liters of medium were removed and an equivalent amount of fresh medium replaced until 18 liters of culture filtrate was obtained. The pooled filtrate was evaporated *in vacuo*, adjusted to pH 2 and extracted continuously for twelve hours with ethyl acetate. The extract was evaporated to dryness; the residue resuspended in water, neutralized and placed on a Dowex 1 formate, 8 X, 200-400 mesh 35 x 2.5 cm column. A hyperbolic gradient established with one liter of water in the mixing vessel and 4 M formic acid in the reservoir was employed. Eight ml fractions were collected. The β-carboxy, β-hydroxyisocaproic acid was obtained in fractions 80-100 while the new compound eluted almost quantitatively between fractions 100-120. The latter fractions were pooled and re-chromatographed twice employing the same system. The thrice-chromatographed material was concentrated and crystallized as long, thin needles from a mixture of ethyl acetate and chloroform. The compound had the following properties:

Melting point: 146-147°C; $(\alpha)_D^{24} = -5.2$ (1.5% H_2O)

Analysis:

Calculated for $C_7H_{12}O_5$	-	C: 47.73;	H: 6.82; O: 45.45
Found	-	C: 47.63; 47.81	H: 6.98; O: 45.52 7.05

Neutralization equivalent assuming the compound to be a dicarboxylic acid:

Calculated	- 88.0
Observed	- 87.7

The compound does not form a semi-carbazone or 2,4 dinitrophenylhydrazone. The infra-red absorption spectrum differs from that of the β-carboxy, β-hydroxyisocaproic acid but shares with it the indication of the presence of a hydroxyl group which, in agreement with the analytical evidence, suggests that the compound is a hydroxylated dicarboxylic acid. Extracts of all mutants of Salmonella except leu-129 (the strain that did not respond to the compound) and all mutants of Neurospora except 33757 (the strain which accumulated the compound) contain an enzyme which rapidly converts this compound to α-ketoisocaproic acid. α-Ketoisocaproic acid has been identified as the end-product of the reaction by chromatography of its 2,4 dinitrophenylhydrazone and enzymatic conversion to leucine by a transaminase obtained from Neurospora.

Some properties of an enzyme which converts the new intermediate to α-ketoisocaproic acid are described in Table I. The enzyme preparation derived from mutant leu-120 displayed a strong dependence for DPN as an electron acceptor and a requirement for both magnesium and phosphate ions after dialysis against versene and glutathione in 0.01 M Tris pH 8.0. At most only a slight phosphate requirement could be demonstrated when glycl glycine buffer was used instead of Tris. Mn^{++} could replace the Mg^{++} requirement. In purified preparations, TPN would not act as an electron acceptor. However, in crude preparations, TPN was effective. The nature of the metal requirement is not at all clear. Versene at a concentration of 10^{-4}M doubles the activity of the enzyme in the absence of added Mg^{++} or Mn^{++} although the metal requirement can still be demonstrated. The inhibition of the preparation by a heavy metal contaminant is not excluded although extensive dialysis against versene and glutathione has failed to diminish the observed stimulation by versene. Preliminary investigation of the kinetics of CO_2 production as a function of DPN reduced is indicative of concurrent dehydrogenation and decarboxylation.

Table I

	μMoles DPNH formed - 9 min.	μMoles α-keto-isocaproic acid formed - 9 min.
Complete system	0.25	0.27
Complete system, no Mg^{++}	0.028	0.030
Complete system, no PO_4	0	0
Complete system, with TPN replacing DPN	0	0

Complete system: DPN, 1.4 μmoles; $MgCl_2$, 10 μmoles; KPO_4 buffer pH 8.0, 10 μmoles; Tris-HCl pH 8.0, 200 μmoles; α-hydroxy, β-carboxyisocaproic acid, 2 μmoles; enzyme (from strain leu 120 extract): 1 mg of the 30-75% $(NH_4)_2SO_4$ precipitate dialyzed against 0.01 M Tris-HCl pH 8.0 with 10^{-4}M glutathione and 10^{-4}M versene.

The analytical and enzymatic evidence presented above suggests that the compound is a hydroxylated dicarboxylic acid derivative of isocaproic acid which is readily converted by oxidation and decarboxylation to α-ketoisocaproic acid. It seems therefore compelling to propose that the compound isolated is α-hydroxy, β-carboxyisocaproic acid, a compound proposed by Strassman et al (1956) on the basis of isotope evidence, as an intermediate in leucine biosynthesis derived from β-carboxy, β-hydroxyisocaproic acid. The accumulation patterns demonstrated by mutants of Neurospora, the replacement of the leucine requirement in several of the mutants of Salmonella, and the absence of replacement of the leucine requirements in those mutants of Neurospora and Salmonella shown to lack the enzyme catalyzing the conversion of the compound to α-ketoisocaproic acid strongly implicates this compound as a true intermediate in leucine biosynthesis.

Evidence for still another compound involved in the biosynthesis of leucine has been obtained in experiments on the interconversion of α-hydroxy-β-carboxyisocaproate and its β-hydroxy isomer. Incubation of either compound with extracts of strain leu-120 and strain leu-129 results in an increase in optical density in the 230-240 mμ region. Strains leu-128 and leu-130 do not yield extracts exhibiting such activity. No compound absorbing in this range has been found to accumulate in the culture fluids of any of the mutants examined.

These experiments provide additional support that the pathway of leucine biosynthesis proposed by Strassman et al (1956) is at least generally correct. This pathway consists of the formation of β-carboxy, β-hydroxyisocaproate by the condensation of α-ketoisovalerate and acetyl coenzyme A followed by its conversion to α-ketoisocaproate and leucine in a manner analogous to the conversion of citrate to α-ketoglutarate and glutamate.

References

Barratt, R. W., Newmeyer, D., Perkins, D. D. and Garnjobst, L. Advances in Genetics 6: 1 (1954)
Gross, S. R. and Gross, H. S. Genetics 46: 868 (1961)
Jungwirth, C., Margolin, P., Umbarger, H. E., and Gross, S. R. Biochem. Biophys. Res. Comm. 5 (6): 435 (1961)
Lester, H. and Gross, S. R. Science 129: 572 (1959)
Margolin, P. Genetics 44: 525 (1959)
Strassman, M., Locke, L. A., Thomas, A. J., and Weinhouse, S. J. Am. Chem. Soc. 78: 1599 (1956)