

- Horecker, B. L., and Kornberg, A. (1948), *J. Biol. Chem.* 175, 385.
- Horio, T., and Kamen, M. D. (1960), *Biochim. Biophys. Acta* 43, 382.
- Horio, T., and Kamen, M. D. (1961), *Biochim. Biophys. Acta* 48, 266.
- Horio, T., and Kamen, M. D. (1962), *Biochemistry* 1, 144.
- Horio, T., Taylor, C. P. S., and Chance, B. (1962), in press.
- Kamen, M. D. (1961a), in *Structure and Function, Symposium of the International Union of Biochemists*, Stockholm, 1960, London, Academic Press, Inc., p. 277.
- Kamen, M. D. (1961b), in *Light and Life*, McElroy, W. D., and Glass, B., editors, Baltimore, Johns Hopkins Press, p. 483.
- Kamen, M. D., and Vernon, L. P. (1953), *Biochim. Biophys. Acta* 17, 10.
- Kamen, M. D., and Vernon, L. P. (1954), *J. Biol. Chem.* 211, 633.
- Kato, S. (1961), *J. Biochem. (Japan)* 49, 126.
- Krasnovsky, A. A., and Voinovskaya, K. K. (1957), *Doklady Akad. Nauk., S. S. S. R.* 112, 911.
- Morita, S. (1955), *J. Biochem. (Japan)* 42, 533.
- Nakamura, H. (1937), *Acta Phytochim. (Tokyo)* 9, 189.
- Newton, J. W. (1960), *Biochim. Biophys. Acta* 42, 34.
- Nozaki, M., Yamanaka, T., Horio, T., and Okunuki, K. (1957), *J. Biochem. (Japan)* 44, 453.
- Olson, J. M., and Chance, B. (1960), *Arch. Biochem. Biophys.* 88, 26, 40.
- Rudney, H. (1961), *J. Biol. Chem.* 236, PC39.
- Singer, T. P., and Kearney, E. B. (1950), *J. Biol. Chem.* 183, 409.
- Smith, L., and Ramirez, J. (1959), *Arch. Biochem. Biophys.* 79, 233.
- Smith, L., and Baltscheffsky, M. (1959), *J. Biol. Chem.* 234, 1575.
- Teale, F. W., quoted by Weber, G. (1961), in *Comparative Biochemistry of Photoreactive Systems*, Allen, M. B., editor, New York, Academic Press, Inc., p. 386.
- Van Niel, C. B. (1941), *Adv. Enzymol.* 1, 263.
- Van Niel, C. B. (1944), *Bacteriol. Rev.* 8, 1.
- Vernon, L. P., and Kamen, M. D. (1953), *Arch. Biochem. Biophys.* 44, 298.
- Vernon, L. P., and Kamen, M. D. (1954), *J. Biol. Chem.* 211, 643.
- Yagi, K., Matsuoka, Y., Koyama, S., and Tada, M. (1956), *J. Biochem. (Japan)* 43, 93.
- Yang, C. C., and Legallais, V. (1954), *Rev. Sci. Instruments* 25, 801.

2-Isopropylmalate and 3-Isopropylmalate as Intermediates in Leucine Biosynthesis*

JOSEPH M. CALVO,† M. G. KALYANPUR, AND CARL M. STEVENS

From the Department of Chemistry, Washington State University, Pullman, Washington

Received June 18, 1962

2-Isopropylmalic acid and 3-isopropylmalic acid have been synthesized and identified as excretion products of a leucine-requiring mutant of *Neurospora crassa*. Homogenates of *Escherichia coli* form 2-isopropylmalic acid from valine.

It is known that in some microorganisms the carbon skeleton of leucine is derived from acetate and the isobutyl moiety of valine (Strassman *et al.*, 1956; these authors also present a good review of the literature). A biosynthetic pathway to leucine was proposed by Strassman *et al.* involving the condensation of acetate with α -ketoisovalerate followed by a sequence of reactions, analogous to the Krebs cycle, leading to α -ketoisocaproate. The synthesis of 2-isopropylmalic acid and 3-isopropylmalic acid, two of the intermediates in this proposed pathway, and their identification in culture media of a mutant of *Neurospora crassa*, is described in this report. During the progress of this work, Jungwirth *et al.* (1961) have reported that extracts of *Salmonella typhimurium* form 2-isopropylmalic acid from valine and acetyl coenzyme A and that leucine-less mutants of this

organism excrete 2-isopropylmalic acid into the culture medium. Also, Gross *et al.* (1962) have tentatively identified a *N. crassa* metabolite as 3-isopropylmalic acid by its neutralization equivalent, by elementary analysis, and by the fact that extracts of *S. typhimurium* and *N. crassa* convert it to α -ketoisocaproate.

EXPERIMENTAL RESULTS

2-Isopropylmalic Acid.—The synthesis of 2-isopropylmalic acid, developed independently in this laboratory, differed from that reported by Yamashita (1958) in that ethyl isobutyrylacetate cyanohydrin was isolated from the reaction of ethyl isobutyrylacetate, NaCN, and NaHSO₃. Yield, 53%; b.p. 118–120°/10 mm.

Anal. Calcd. for C₈H₁₃NO₃: C, 58.36; H, 8.16; N, 7.56. Found: C, 58.45; H, 8.24; N, 7.46.

Acid hydrolysis of ethyl isobutyrylacetate cyanohydrin gave 2-isopropylmalic acid in the yield reported.

* This work was supported in part by Research Grant E-1111 from the National Institutes of Health.

† National Science Foundation Fellow (1959–1961).

tion of acids. Elution was carried out with CHCl_3 containing 6% *n*-butanol or 50% 4-methyl-2-pentanone (v/v) or with a gradient employing 20 ml of CHCl_3 in the column, 250 ml of CHCl_3 in the mixing vessel, and CHCl_3 -*t*-amyl alcohol (1:1 v/v) containing 1% water and 0.8% ethanol in the reservoir. All solvents were equilibrated with 10% of their volume of 0.05 *N* H_2SO_4 . Eluted acids were determined by titration, and radioactivity was measured by drying the fractions in steel planchets or in glass vials and counting in a Geiger counter or a liquid scintillation counter, respectively. In double labeling experiments, the procedure of Okita *et al.* (1957) was used to calculate the activity of H^3 and C^{14} .

Microorganisms.—*Escherichia coli* used in these studies was isolated from sewage by Mr. Richard Green, Washington State University. The organism was grown at 30° on a medium which had the following composition in grams per liter: $(\text{NH}_4)_2\text{SO}_4$, 1.5; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5; NaCl, 1.0; sodium citrate, 0.5; K_2HPO_4 , 5.4; glucose, 2; concentrated HCl, 1.0. *N. crassa* 14079, a leucine-less mutant obtained from the American Type Culture Collection, was grown on a minimal medium supplemented with 0.1 mg/ml of L-leucine at 25° (Regnery, 1944).

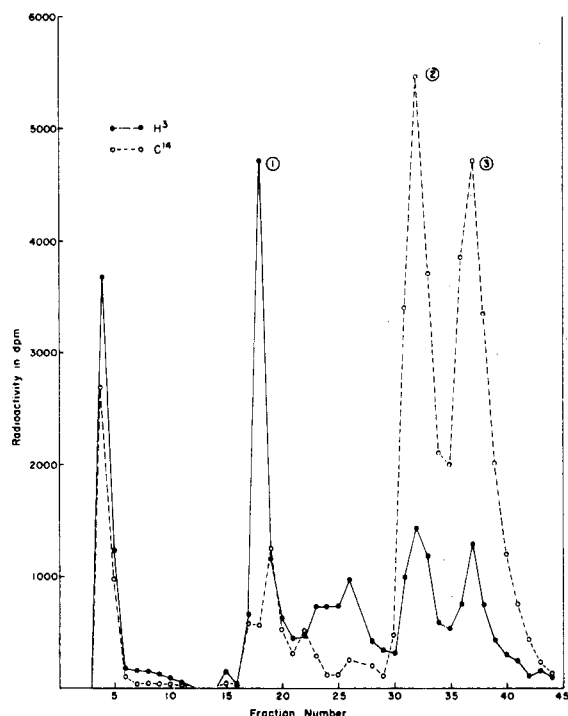


FIG. 2. Silicic acid chromatogram of an extract of the culture medium of *N. crassa* 14079 incubated with DL-valine-1- C^{14} and acetate- H^3 . Column diameter, 1 cm; silicic acid, 6 g; stationary phase, 3.69 ml 0.05 *N* H_2SO_4 ; fraction volume, 2 ml; elution, CHCl_3 -6% *n*-butanol; radioassay, Tri-Carb liquid scintillation spectrometer.

Excretion of Leucine Precursors by *N. crassa* 14079.—A 200 ml culture of *N. crassa* 14079, which had depleted the supply of exogenous leucine after 12 days' growth, was incubated for 4 days with 1.66 μmoles (5 μC) of DL-valine-1- C^{14} . In double labeling experiments, a similar culture was incubated for 4 days with 4.98 μmoles (15 μC) of DL-valine-1- C^{14} and 0.78 μmoles (78 μC) of acetate- H^3 . The mycelium was removed by filtration and the filtrate was acidified and evaporated to dryness *in vacuo*. The residue was extracted three times under reflux with 75-ml portions of ether and the ether extract was extracted with dilute NH_4OH . The NH_4OH extract was concentrated *in vacuo* and aliquots were chromatographed on silicic acid.

The results of the double labeling experiment are shown in Figure 2. Peak 1, identified by its retention value, is almost certainly acetic acid. The doubly labeled peaks 2 and 3 were tentatively identified as 2-isopropylmalate and 3-isopropylmalate (m.p. 119–119.5°) by co-chromatographing material from these peaks with the synthetic acids. When the racemate of 3-isopropylmalate melting at 122–122.3° was co-chromatographed with the material from peak 3, a wide separation of acidity from radioactivity was obtained. The material from peak 2 of Figure 2 was diluted with unlabeled 2-isopropylmalic acid and was recrystallized from ethyl acetate-ligroin to a constant specific activity. The material from peak 3 of Figure 2 was diluted with synthetic 3-isopropylmalic acid (m.p. 119–119.5°) and was recrystallized from ether-ligroin to a constant specific activity. The H^3 C^{14} ratio of thrice-recrystallized 2-isopropylmalate was 0.236 ± 0.024 :

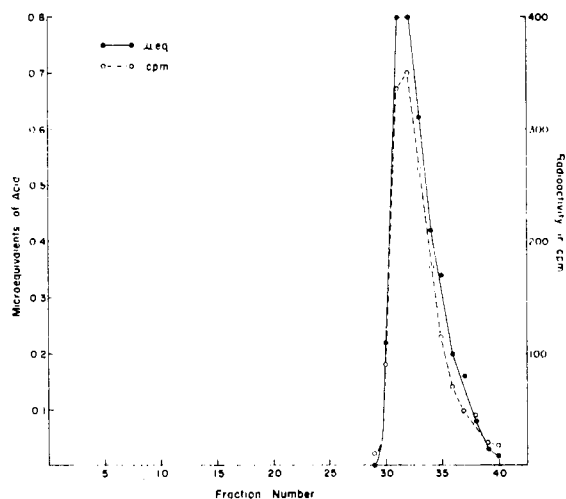


FIG. 3. Silicic acid chromatogram identifying 2-isopropylmalate as an excretion product of *N. crassa* 14079. Sample: 0.5 mg of product obtained from the repeated recrystallization of synthetic 2-isopropylmalate with the material from peak 2 of Figure 2. Chromatographic conditions are the same as those shown in Figure 2.

that of thrice-recrystallized 3-isopropylmalate, 0.255 ± 0.026 . The recrystallized acids were chromatographed on silicic acid (see Fig. 3 and 4); in each case, a single peak was obtained with a constant specific activity (C^{14} cpm/ μ eq) in each fraction. Similar results were obtained when the acids were eluted with $CHCl_3$ -4-methyl-2-pentanone (peak tube number: 2-isopropylmalate, 43; 3-isopropylmalate, 49) or with $CHCl_3$ -*t*-amyl alcohol (2-isopropylmalate, 36; 3-isopropylmalate, 38).

Enzymatic Formation of 2-Isopropylmalic Acid.—The cells from a 1-liter culture of *E. coli* (Klett value, 165) were taken up in 30 ml of 0.05 M phosphate buffer, pH 7, containing 0.12 mmoles of glutathione, and were treated for 15 minutes at 0.9 amp in a 10-kc sonic oscillator. Ten milliliters of the crude homogenate was incubated for 2 hours at 30° with 32 μ moles (4.87 μ C) of L-valine- $U-C^{14}$, 20 mg of glucose, 80 μ moles of glutathione, 64 μ moles of α -ketoglutarate, 0.32 μ moles of pyridoxal phosphate, 1 μ mole of acetyl coenzyme A (Ochoa, 1957), 0.4 μ moles of coenzyme A, 20 μ moles of adenosine triphosphate, and 5 μ moles of $MgCl_2 \cdot 6H_2O$. After incubation, the reaction mixture was acidified to pH 1.0 and the water was removed *in vacuo*. The residue was refluxed three times with 50-ml portions of ether and the ether extracts were concentrated to a small volume *in vacuo*. An aliquot of this extract was co-chromatographed with synthetic 2-isopropylmalic acid; the results are shown in Figure 5. The radioactivity in 2-isopropylmalic

acid was 0.9% of the total activity added to the reaction mixture.

DISCUSSION

The biosynthetic pathway to leucine proposed by Strassman *et al.* (1956) appears to be essentially correct. The studies presented here and those of Jungwirth *et al.* (1961), Gross *et al.* (1962), and Strassman and Ceci (1962) suggest that this pathway operates in *N. crassa*, *S. typhimurium*, *E. coli*, and *Saccharomyces cerevisiae*. There is evidence, however, that a different biosynthetic pathway to leucine operates in *Ruminococcus flavefaciens*. Allison *et al.* (1962) have reported that isovalerate-1- C^{14} is converted readily to leucine by *R. flavefaciens*, the resulting amino acid being labeled in the 2 position.

It has been shown that 2-isopropylmalate is formed by homogenates of several microorganisms and that 2-isopropylmalate and 3-isopropylmalate are excreted by a leucine-requiring mutant of *N. crassa*; however, the interconversion of these two compounds has not yet been demonstrated. The presumed enzymatic isomerization of 2-isopropylmalate to 3-isopropylmalate has an analogy in the citrate-isocitrate conversion as catalyzed by aconitase. The mechanism of the latter reaction is thought to involve a carbonium ion intermediate and an intramolecular hydride shift (Speyer and Dickman, 1956). In the double labeling experiment in which *N. crassa* 14079 was incubated with valine-1- C^{14} and acetate- H^3 , 2-isopropylmalate and 3-isopropylmalate isolated from the culture medium contained essentially the same ratio of tritium to carbon-14 (0.236 ± 0.024 , 0.255 ± 0.026). If it is assumed that 3-isopropylmalate is derived only from 2-isopropyl-

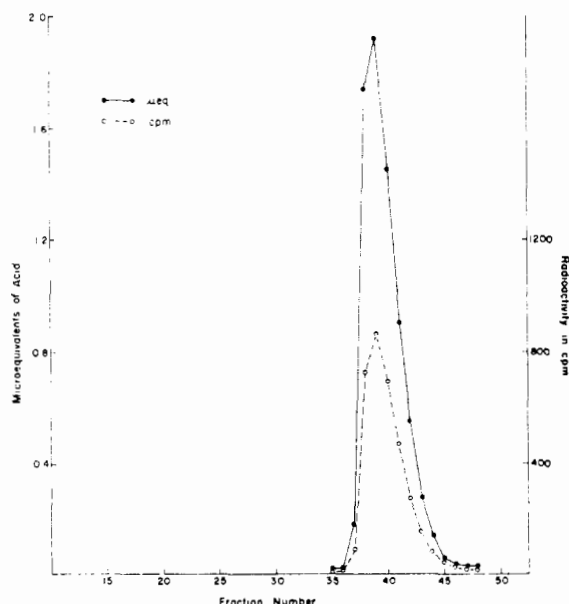


FIG. 4.—Silicic acid chromatogram identifying 3-isopropylmalate as an excretion product of *N. crassa* 14079. Sample: 1.1 mg of product obtained from the repeated recrystallization of synthetic 3-isopropylmalate (m.p. 119–119.5°) with the material from peak 3 of Figure 2. Chromatographic conditions are the same as those shown in Figure 2.

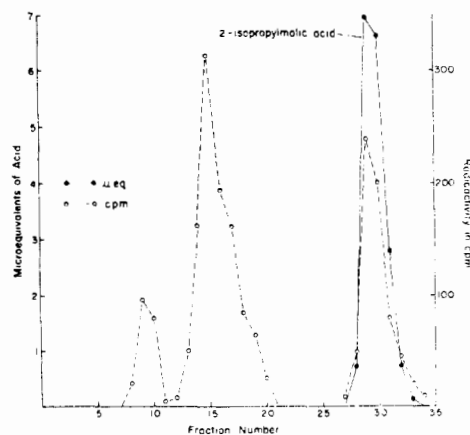


FIG. 5.—Silicic acid chromatogram identifying 2-isopropylmalate formed by extracts of *E. coli*. Sample: ether extract obtained from the incubation of L-valine- $U-C^{14}$ with an *E. coli* homogenate co-chromatographed with 15 μ eq of synthetic 2-isopropylmalate. Chromatographic conditions are the same as those shown in Figure 2. Radioassay, Nuclear-Chicago Model D-47 gas flow detector.

malate in this organism, then the simplest interpretation of the results above is that an intramolecular hydride shift is involved in the interconversion of these compounds.

The series of reactions leading to the synthesis of α -ketoglutarate and α -ketoisocaproate involves the conversion of a α -keto acid to a homologous α -keto acid containing one more methylene group. An identical pathway has been proposed for the synthesis of α -ketoadipate from α -ketoglutarate (Strassman and Weinhouse, 1953) and for the synthesis of α -ketobutyrate, α -keto-*n*-valerate, and α -keto-*n*-caproate from pyruvate, α -ketobutyrate, and α -keto-*n*-valerate, respectively (Ingraham *et al.*, 1961). It will be interesting to see whether further studies bear out the generality of this pathway.

ACKNOWLEDGMENT

The authors are grateful for the capable technical assistance of Miss M. Cathleen Bates.

REFERENCES

- Allison, M. J., Bryant, M. P., and Doetsch, R. N. (1962), *J. Bact.* 83, 523.
- Gross, S. R., Jungwirth, C., and Umbarger, E. (1962), *Biochem. Biophys. Research Commun.* 7, 5.
- Ingraham, J. L., Guyman, J. F., and Crowell, E. A. (1961), *Arch. Biochem. Biophys.* 95, 169.
- Jungwirth, C., Margolin, P., Umbarger, T. E., and Gross, S. R. (1961), *Biochem. Biophys. Research Commun.* 5, 435.
- Kinnory, D. S., Takeda, Y., and Greenberg, D. M. (1955), *J. Biol. Chem.* 212, 379.
- Miyazaki, M., Mizuno, C., and Umio, S. (1957), *Yakugaku Zasshi* 77, 415 (cf. *Chem. Abstr.* 51, 12068c).
- Ochoa, S. (1957), *Biochem. Preparations* 5, 27.
- Okita, G. T., Kabara, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics* 15, 111.
- Regnery, D. C. (1944), *J. Biol. Chem.* 154, 151.
- Speyer, J. F., and Dickman, S. R. (1956), *J. Biol. Chem.* 220, 193.
- Strassman, M., and Ceci, L. N. (1962), *Federation Proc.* 21, 10.
- Strassman, M., Locke, L. A., Thomas, A. J., and Weinhouse, S. (1956), *J. Am. Chem. Soc.* 78, 1599.
- Strassman, M., and Weinhouse, S. (1953), *J. Am. Chem. Soc.* 75, 1680.
- Yamashita, M. (1958), *J. Org. Chem.* 23, 835.

The Biological Transformation of Xanthopterin by a Bacterium Isolated from Soil*

CARL C. LEVY† AND WALTER S. McNUTT

*From the Department of Pharmacology, Tufts University School of Medicine,
Boston 11, Massachusetts*

Received June 19, 1962

The utilization of the naturally occurring pteridine, xanthopterin, by a bacterium isolated from soil, has been studied in the hope that some of the intermediates produced might be related to intermediates in the normal biogenesis of pteridines. The following products have been isolated from cultures of this organism and identified by ultraviolet absorption spectra, infrared absorption spectra, and paper chromatography: 6-oxylumazine, 6,7-dioxylumazine, and leucopterin. Enzymes have been obtained from this organism which catalyze (1) the deamination of xanthopterin and isoxanthopterin to 6-oxylumazine and 7-oxylumazine respectively, (2) the oxidation of 6-oxylumazine, but not 7-oxylumazine, to 6,7-dioxylumazine, and (3) the transformation of 6,7-dioxylumazine to an unidentified compound.

The relationship between purines and pteridines has been the subject of a number of investigations during the past several years. Plaut (1954), in his studies with the yeast *Ashbya gossypii*, found that the metabolites glycine, CO₂, and formic acid were incorporated into riboflavin in a pattern similar to that of their incorporation into purines. Somewhat later, a radioactive lumazine was isolated from the mycelia of this organism

during further studies of the incorporation of formate C¹⁴ into riboflavin (Maley and Plaut, 1959a.) These authors believe that the lumazine is, in fact, an intermediate in the biogenesis of riboflavin (Maley and Plaut, 1959b). Evidence to the contrary, however, has been presented by others (Korte and Aldag, 1959). In another yeast, *Eremothecium ashbyii*, McNutt (1956, 1961) demonstrated that all of the atoms of the purine ring except the carbon atom in position 8 were incorporated as a unit into the pteridine portion of riboflavin. A lumazine derivative, also present in this organism, was similarly labeled (McNutt

* Supported by U.S.P.H.S. Grant A-3675.

† Present address: Dermatology Branch, National Cancer Institute, National Institutes of Health, Bethesda 14, Md.