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.
Katoh, 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. Biophys. Biochem. 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*

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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.

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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 $NaHSO_3$. Yield, 53%; b.p. 118–120°/10 mm.

Anal. Calcd. for $C_9H_{15}NO_3$: 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.

3-Isopropylmalic Acid.—The synthesis of this compound was afforded by the following sequence of reactions:

$$(CH_3)_2CHCHBrCO_2C_2H_5 + HC(OC_2H_5)_3 \xrightarrow{Zn} (CH_3)_2CHCHCO_2C_2H_5 \quad (CH(OC_2H_5)_2)$$

$$\xrightarrow{HCl,\ HOAc} (CH_3)_2CHCHCO_2C_2H_5 \quad (CHO)$$

$$\xrightarrow[NaCN]{NaHSO_3} (CH_3)_2CHCHCO_2C_2H_5 \quad (CHOH)\ (CN)$$

$$\xrightarrow{HCl} (CH_3)_2CHCHCO_2H \quad (CHOH)\ (CO_2H)$$

Ethyl α-formylisovalerate diethyl acetal, b.p. 103–105°/13 mm, was synthesized in 52% yield by the procedure of Miyazaki *et al.* (1957). Hydrolysis of this compound to ethyl α-formylisovalerate was accomplished by shaking 15 g of the acetal with 15 ml of acetic acid and 30 ml of 2 N HCl for 18 hours at room temperature. The two phases were separated and the organic phase was washed with portions of cold 5% $KHCO_3$ until the washings were basic to litmus. The yield of crude material was 10.15 g. The 2,4-dinitrophenylhydrazone, prepared from this material in 81% yield, melted at 123.5–124.5° after three recrystallizations from ethanol.

Anal. Calcd. for $C_{14}H_{18}N_4O_6$: C, 49.70; H, 5.36; N, 16.56. Found: C, 49.92; H, 5.47; N, 16.61.

To form the cyanohydrin, 9.65 g of crude ethyl α-formylisovalerate, 6.35 g of $NaHSO_3$, and 22.4 ml of H_2O were cooled in an ice bath and 3.15 g of 95% NaCN in 4.8 ml of H_2O was added with stirring and cooling over a period of 1 hour. The reaction mixture was stirred for an additional 30 minutes, and, after separation of the two phases, the aqueous phase was washed once with 25 ml of ether. The combined ether-organic phase, after drying over anhydrous Na_2SO_4, was evaporated *in vacuo* to yield 10.35 g of crude ethyl α-formylisovalerate cyanohydrin.

To convert the cyanohydrin to the dicarboxylic acid, 6.95 g of crude cyanohydrin was allowed to stand with 12 ml of concentrated HCl for 1 hour and then the solution was heated on a steam bath for 1 hour. The reaction mixture was diluted with 25 ml of H_2O and heating was continued for an additional 3 hours. After cooling, the solution was brought to *p*H 1.5 with NaOH and the water was removed *in vacuo*. The residue was refluxed three times with 75-ml portions of ether. Concentration of the ether extracts yielded 5.39 g of an oil which did not solidify after standing for several days.

This crude material contains, in addition to side-products, the two racemates of 3-isopropylmalic acid. These were isolated from the mixture by chromatographing a portion of the product on silicic acid. The separation of the two isomers and the conditions employed in chromatography are shown in Figure 1. The substance represented by the first peak is probably α-formylisovaleric acid cyanohydrin; this compound does not show characteristic amide absorption in the infrared, and when it was rehydrolyzed and rechromatographed it yielded products corresponding to peaks 2 and 3 shown in Figure 1.

The fractions from peak 2 were pooled and the solvent was removed *in vacuo*. The addition of ligroin precipitated 155 mg (10%, based upon ethyl α-formylisovalerate cyanohydrin) of one racemate, m.p. 112–114°. The product was recrystallized twice by dissolving it in ether, adding ligroin, and then allowing the ether to evaporate slowly at room temperature: melting point, 119–119.5°; neutralization equivalent, 87.2 (theoretical, 88).

Anal. Calcd. for $C_7H_{12}O_5$: C, 47.73; H, 6.87. Found: C, 47.63; H, 6.92.

The fractions from peak 3 were pooled and the solvent was removed *in vacuo*. The remaining oil was dissolved in 10 ml of hot benzene and upon cooling, 193 mg (13%, based upon ethyl α-formylisovalerate cyanohydrin) of the second racemate crystallized. Two recrystallizations from benzene gave 144 mg of product; m.p. 122–122.3°; neutralization equivalent, 88.6.

Anal. Calcd. for $C_7H_{12}O_5$: C, 47.73; H, 6.87. Found: C, 47.55; H, 6.69.

Silicic Acid Chromatography.—The procedure of Kinnory *et al.* (1955) was used for the separa-

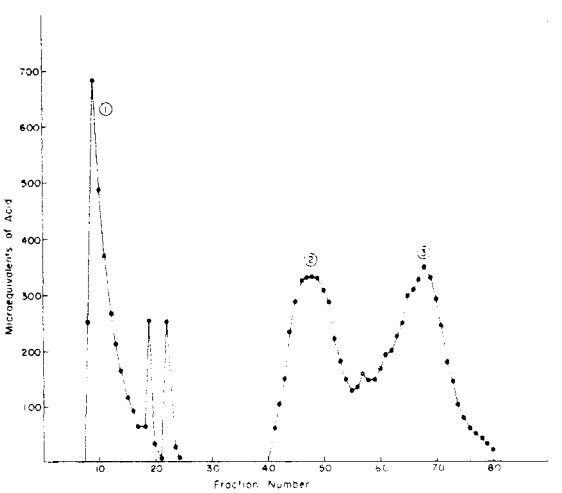

FIG. 1. Separation of the two racemates of 3-isopropylmalate. Sample: 1.4 g of a crude product resulting from the hydrolysis of ethyl α-formylisovalerate cyanohydrin. Column diameter, 2 cm; silicic acid, 20 g; stationary phase, 12.3 ml of 0.05 N H_2SO_4; fraction volume, 3 ml. Elution: fractions 1–15, $CHCl_3$; fractions 16–80, $CHCl_3$–10% *n*-butanol.

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: $(NH_4)_2SO_4$, 1.5; $MgCl_2 \cdot 6H_2O$, 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).

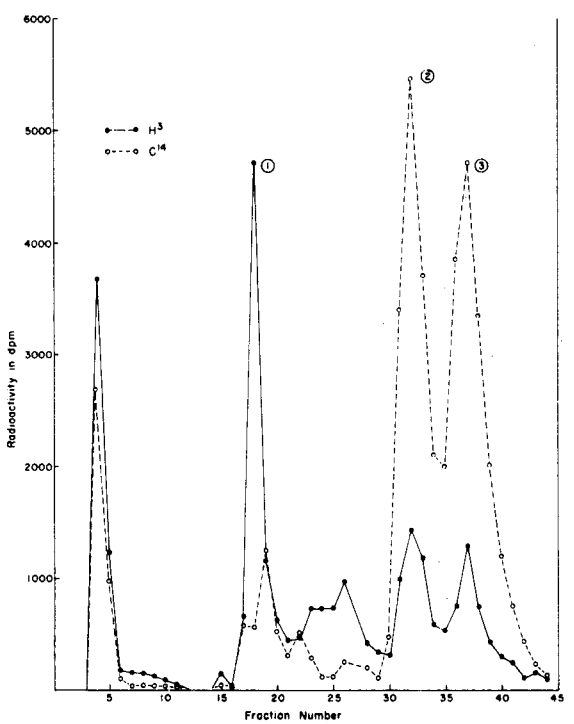

FIG. 2. Silicic acid chromatogram of an extract of the culture medium of *N. crassa* 14079 incubated with DL-valine-1-C^{14} and acetate-H^3. Column diameter, 1 cm; silicic acid, 6 g; stationary phase, 3.69 ml 0.05 N H_2SO_4; fraction volume, 2 ml; elution, $CHCl_3$–6% *n*-butanol; radioassay, Tri-Carb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

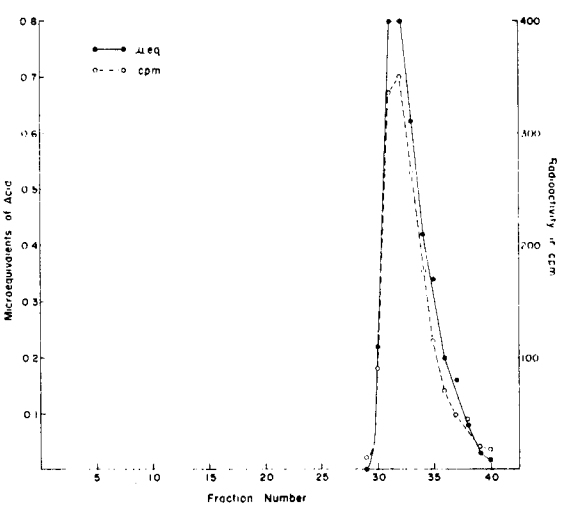

FIG. 3.—Silicic acid chromatogram identifying 2-isopropylmalate as an excretion product of *N. crassa* 14079. Sample: 0.5 mg of product obtained from the repeated recrystallization of synthetic 2-isopropyl malate with the material from peak 2 of Figure 2. Chromatographic conditions are the same as those 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/μeq) 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, *p*H 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 μmoles (4.87 μc) of L-valine-U-C^{14}, 20 mg of glucose, 80 μmoles of glutathione, 64 μmoles of α-ketoglutarate, 0.32 μmoles of pyridoxal phosphate, 1 μmole of acetyl coenzyme A (Ochoa, 1957), 0.4 μmoles of coenzyme A, 20 μmoles of adenosine triphosphate, and 5 μmoles of $MgCl_2 \cdot 6H_2O$. After incubation, the reaction mixture was acidified to *p*H 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.

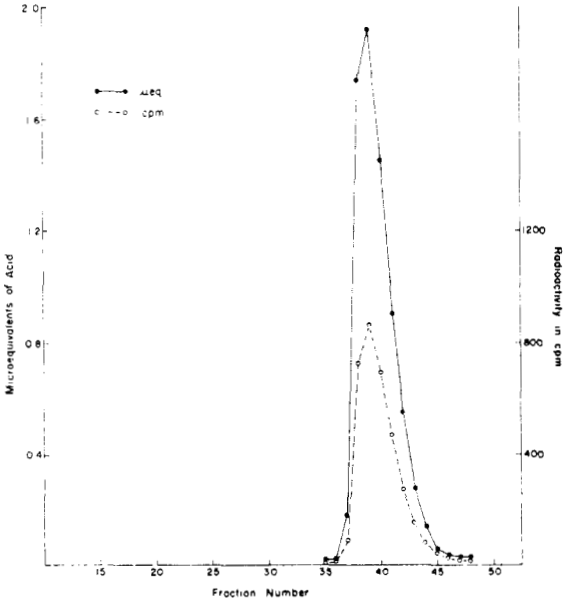

FIG. 4.—Silicic acid chromatogram identifying 3-isopropylmalate as an excretion product of *N. crassa* 14079. Sample: 1.1 mg of product obtained from the repeated recrystallization of synthetic 3-isopropylmalate (m.p. 119–119.5°) with the material from peak 3 of Figure 2. Chromatographic conditions are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

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-

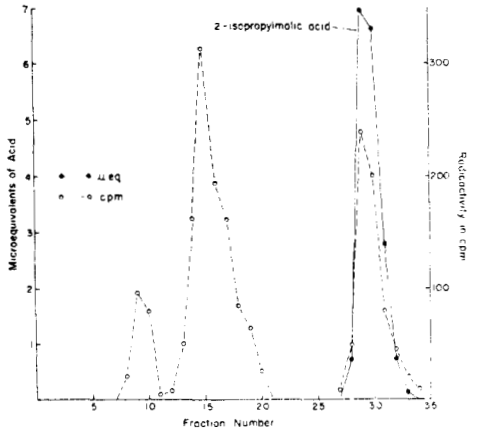

FIG. 5.—Silicic acid chromatogram identifying 2-isopropylmalate formed by extracts of *E. coli*. Sample: ether extract obtained from the incubation of L-valine-U-C^{14} with an *E. coli* homogenate co-chromatographed with 15 μeq of synthetic 2-isopropylmalate. Chromatographic conditions are the same 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.

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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 Communs. 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 Communs. 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*

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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, CO_2, 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 C^{14} 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

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