Biochemical Studies of *Piricularia oryzae*

Part III. Formation of Dimethylpyruvic Acid from DL-Valine

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It has been shown that *Piricularia oryzae* could grow in the presence of amino acid, even in the absence of a proper carbon source and that the first step of utilization was the formation of the corresponding α -keto acid by deamination in the medium. For further confirmation of this process, DL-valine was used as the amino acid to be tested in the current experiment. In each of the three cases, that is, DL-valine alone, DL-valine plus arsenite and DL-valine plus sucrose, the dimethylpyruvic acid formed was identified as its 2,4-dinitrophenylhydrazone. Even in the presence of a sufficient amount of sucrose, some parts of DL-valine added were found to be utilized as a carbon source through the conversion to its α -keto analog.

It has been shown by Otani¹⁾ and Tanaka et al.²⁾ that rice blast disease is likely to break out in a high probability during the growth-period of the rice-plant containing in its body a comparatively high amount of water-soluble nitrogen compounds including acidic amino acids, their amides and other amino acids. They have also shown that in any growth-period these nitrogen compounds are found in a far greater amount in host plants sensitive to blast disease than in those which are resistant. These findings seem to require further research on the attitude of *P. oryzae*, the pathogenic fungus causing blast disease, toward the biochemical condition of the host plant described above. The present authors have already found³⁾ that this fungus has the ability to utilize amino acids as a good carbon source and to form the corresponding α -keto acids by deamination as the first

step of its utilization; thus α -keto isocaproic acid has been proved from L-leucine by the authors. For further confirmation of this utilization process, the present communication records the formation of dimethylpyruvic acid from DL-valine in a medium containing both sucrose and amino acid as well as in media containing either amino acid alone or amino acid and arsenite respectively.

Experimental and Results

P. oryzae was grown at 27–8°C in 30 ml. of a medium containing KNO₃, 60 mg; CaCl₂·2H₂O, 3 mg; MgSO₄·7H₂O, 15 mg; KH₂PO₄, 30 mg; MnSO₄·4H₂O, 0.05 mg; ZnSO₄·7H₂O, 0.4 mg; FeSO₄·7H₂O, 0.2 mg; thiamine, 10 γ ; biotin, 1 γ ; sucrose, 900 mg. After 14 days of incubation the mycelium of the three culture flasks were transferred together, as previously described³⁾, to 90 ml. of the second culture medium and further incubation was made at 27–8°C. Of this second culture medium, No. 1, contained three times as much the amount of each material as in the first medium and also 150 mg of DL-valine.; No. 2, only DL-valine 150 mg, in place of sucrose; No. 3, only DL-valine 150 mg, and potassium arsenite, 36 mg. Active growth was observed in both No.

1) Y. Otani, Memoirs Fac. Agr., Hokkaido Univ., **1**, 375, (1952).

2) S. Tanaka and F. Katsuki, *J. Chem. Soc. Japan*, **73**, 303 (1952).

3) Y. Nakamura and T. Shimomura, *J. Agr. Chem. Soc. Japan*, **27**, 694 (1953).

1 and No. 2. After three days, white aerial mycelium was observed, which covered over the liquid surface of the flask within seven days. On the other hand, in No. 3 with arsenite, almost no aerial mycelium was formed and growth appeared to cease within three days.

The ammonia- and amino-nitrogen contents of each flask were measured at appropriate intervals by usual methods. The results obtained are given in the figure. In the three cases, the velocity of ammonia formation and the amount of

Ammonia- and Amino-Nitrogens from DL-Valine by *P. oryzae*.

(1) DL-valine + sucrose; (2) DL-valine only;
(3) DL-valine + arsenite.

— represents ammonia-N, and ---- amino-N. Values are expressed as percentage to the DL-valine added.

ammonia production were the largest in No. 2, followed by No. 3. In No. 1 the amount of ammonia produced was found to be 17% three days later, followed by almost no further increase within the successive nine days. These results are in good accord with those obtained in the experiment, when L-leucine was used as a carbon source³⁾. In each case, the decrease of amino acid by deamination was found to be almost identical in amount up to the first three days but further incubation revealed that the largest decrease of amino acid followed in No. 2, this corresponding to the increase of ammonia.

In spite of the smaller formation of ammonia in No. 1, a relatively larger decrease of amino acid was observed. This fact appears to indicate the occurrence of absorption and utilization of some parts of the amino acid added as a direct nitrogen source, particularly seen in the later stage of incubation. This interpretation seems to be supported by the fact that the total sum of ammonia- and amino nitrogen amounted to 95–96% of the theoretical value during the first three days of incubation but after five days it reduced to 77% and after nine days to 68%. In this case, however, it must be recognized that certain parts of DL-valine added were utilized as a carbon source, especially in the early stage of incubation. This can also be confirmed by the identification of keto acid from DL-valine as described below. It has been learned that even in the presence of sucrose as a carbon source amino acid acted, not only as a nitrogen source, as has been shown by Leaver et al,⁴⁾ and Otani,⁵⁾ but also as a carbon source accompanying the deamination process in the medium itself. The occurrence of such an utilization process may be suggested from Eades' report⁶⁾ that the activity of the keto analog of valine is only 50% of this amino acid toward microorganisms (*Lactobacillus*).

In No. 3, arsenite added, a good correspondence was found between the increase of ammonia- and the decrease of amino-nitrogen during the early stage of incubation. Thereafter, considerable deviation was observed in both nitrogen forms; the decrease of amino nitrogen slowed down and the increase of ammonia became yet more vivid. Taking account of hardly any recognizable growth in this case, the only one possible explanation of this fact is that it should be attributed to partial autolysis caused by the inhibition in the metabolic process, due to arsenite. Among the three cases, the highest accumulation of α -keto acid was qualitatively recognized by the addition of 2,4-dinitrophenylhydrazine to the culture medium. In conformity to the experimental data, and the discussion above, which suggest the formation of α -keto

4) F.W. Leaver, J. Leal and C.R. Brewer, *J. Bact.*, 54, 401 (1947).

5) Y. Otani, *Ann. Phytopath. Soc. Japan*, 17, 9 (1952).

6) C.H. Eades, Jr., *J. Biol. Chem.*, 187, 147 (1950).

acid, namely dimethylpyruvic acid, both the isolation and identification of this acid mentioned above, were carried out here in the three cases respectively.

About 90 ml of culture solution (No. 3) was separated from the mycelium after seven to eight days' incubation and concentrated under diminished pressure at a range of pH 8.0–8.1 to a small volume, ca. 2–3 ml, then acidified with 2 ml of 5% sulfuric acid and reconstituted at 60°–65°C under 10 mm. Hg. This distillation process was repeated thrice and the total distillate was treated with 2,4-dinitrophenylhydrazine solution in 2N-HCl. A yellow crystal mass was isolated, which melted at 191–2°C after being washed with water. After repeated recrystallization from acidic ethanol, by adding water without heating, yellow needles were obtained, which melted at 195–6°C (Found: C, 44.13; H, 3.95. Calcd for $C_{11}H_{12}O_6N_4$: C, 44.59; H, 4.05). This melting point was almost identical with that described by Krebs⁷⁾ and Meister.⁸⁾ Yield, ca. 12 mg as 2,4-dinitrophenylhydrazone. Application of the same operation to No. 2 gave a yellow crystal of 2,4-dinitrophenylhydrazone, 5 mg, which melted at 195° and gave a mixed melting point at 196°C for that obtained from No. 3 (no temperature depression). The identification of this acid in No. 1 was pretty difficult, owing to its low production and also further utilization, but the addition of arsenite to No. 1 brought the same effect to the formation and accumulation of this acid as observed in No. 3. By application of acidic ether extraction or

distillation keto acid was recognized as the same hydrazone which gave a mixed melting point of 196°C in the crystals described above (Found: C, 44.78; H, 4.10). These results indicate the possibility of α -keto acid formation from DL-valine in both the absence and presence of a carbon source.

Further experiments were conducted on α -amino-*n*-butyric acid and phenylalanine as a carbon source. The formation of phenylpyruvic acid from the latter and an unidentified carbonyl compound from the former (its 2,4-dinitrophenylhydrazone, m.p. 153–4°; C, 46.86; H, 5.02; R_F =0.91 to *n*-butanol saturated with 3% ammonia) will be described in our next report.

Summary

P. oryzae is able to grow by its utilization of DL-valine in the absence of a proper carbon source. The formation of α -keto acid, corresponding to the amino acid mentioned, dimethylpyruvic acid is the first step of utilization. This acid (dimethylpyruvic acid) was identified as its 2,4-dinitrophenylhydrazone. A comparatively small amount of this keto acid could be recognized even in the culture medium containing both DL-valine and sucrose. This fact appears to indicate that certain parts of DL-valine, when added to a medium containing sucrose sufficient for growth, were utilized not as a nitrogen source but as a carbon source, especially in the early stage of incubation.

7) H.A. Krebs, *Z. Physiol. Chem.*, **217**, 191 (1933).

8) A. Meister, *J. Biol. Chem.*, **197**, 309 (1932).