description of this Table, it seems most probable that this substance is distinct from Factor-E. The former may be either a diastereoisomer or a polymer of the latter. Consequently, Factor-E has been proved to be the new diketopiperazine having the above mentioned structure.

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Biochemical Studies of *Piricularia oryzae*

Part V. Behavior towards DL-Norleucine and DL-Alanine.*

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In the current experiment, the conversion of DL-norleucine and DL-alanine to their respective α -keto analogue by *Piricularia oryzae* was confirmed by the separation of 2,4-dinitrophenylhydrazone of either keto acid. Although the formation of keto acid from norleucine was distinctly recognized both in the presence and the absence of arsenite, the accumulation of pyruvic acid from alanine was only found in the presence of arsenite, as it has been observed with some micro-organisms. In addition, the preferential utilization of alanine to a proper carbon source was identified, which may provide a step towards the investigation of the nutritional relationship between the blast fungus and the host plant.

It is apparent that the study of the biochemical behavior of *Piricularia oryzae* towards nitrogenous compounds, especially amino acids, is of fundamental importance in order to prevent the host plant from the blast disease. From such a point of view, the utilization process of several amino acids by this fungus has been investigated by the

authors^{1,2,3,4,5)} and the conversion of the amino

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acid to respective α -keto analogue was recognized. In current experiments, DL-norleucine and DL-alanine were taken as the amino acid to be tested. As already reported, the six carbon amino acids such as leucine¹⁾ and isoleucine⁵⁾ have been examined in regard of their conversion to respective α -keto acid and thus the remaining six carbon amino acid, that is, norleucine was naturally taken into question as the supplement, although its natural occurrence is still questionable at present⁶⁾, in spite of the earlier works of Abderhalden et al. On the other hand, the general metabolic significance of alanine has been well known and it was reported by Tomizawa⁷⁾ and Otani⁸⁾ that this amino acid is effective as a nitrogen source in the same extent as asparagine, aspartic acid, glutamic acid, and glycine, but metabolic conversion of these amino acids was unstated. Tanaka⁹⁾, reported that glutamic acid could be utilized preferentially to a proper carbon source by this fungus and proceeded that this fact might be interpreted as one of the biochemical mechanisms involved in the invasion of blast fungus in the host plant and its further growth. Similar results have been obtained with alanine by the authors.

The present communication records the utilization process of both amino acids just mentioned, the separation and identification of either keto acid and the preferential utilization of alanine to a proper carbon source, D-glucose.

EXPERIMENTAL AND RESULTS

Incubation—Two mycelial felts were incubated at 27–8° in 60 ml of complete medium³⁾ containing 100 mg of DL-norleucine or DL-alanine in place of sucrose. In the inhibition experiment, 5 ml of M/20 potassium arsenite was substituted for 5 ml of water. Ammonia- and amino-nitrogen in the culture filtrate during the

FIG. 1. Ammonia- and Amino-Nitrogen from DL-Norleucine by *P. oryzae*.

(1), DL-norleucine only; (2), DL-norleucine+arsenite. Full line expresses ammonia-nitrogen and dotted, amino-nitrogen. Values are indicated as percentage to theoretical amount of nitrogen of norleucine added as supplement. Incubation condition, see text.

incubation were determined as previously reported¹⁾ (Fig. 1 and Fig. 2). The same determinations were made with the culture medium consisting of 30 ml of M/15 phosphate buffer (pH 6.9), 100 mg of DL-alanine and 30 ml of water and similar results as in the complete medium just mentioned were obtained (Fig. 3). This fact indicates that it is possible to carry out such experiment in a phosphate buffer medium without the addition of other inorganic salt. The determination of pyruvic acid accumulated in the phosphate buffer medium was made by a modification of the Friedemann-Haughen method by Shimizu¹⁰⁾ (Fig. 4).

In order to ascertain whether *P. oryzae* is able to utilize preferentially alanine to normal carbon source, this fungus was cultured at 27–8° in 30 ml of the medium containing 150 ml of DL-alanine, 792.5 mg of D-glucose and the same amount of inorganic salts and growth-factors as in the complete medium. The amount of carbon involved in present medium corresponded to that of 900 mg of sucrose which had been used as carbon source in normal cultivation. It was

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FIG. 2. Ammonia- and Amino-Nitrogen from DL-Alanine (in complete medium).

(1), DL-alanine only; (2), DL-alanine + arsenite. Expressions are the same as in Fig. 1.

already observed by Otani¹¹⁾ that glucose was equally available for this fungus as sucrose. At appropriate intervals, ammonia- and amino-nitrogen, remaining glucose and the dry weight of mycelium were estimated. The decrease of the amount of detectable amino-nitrogen was expressed in per cent as alanine consumed. The decrease of the amount of alanine due to deamination was also involved in the data, but the formation of ammonia was far smaller throughout the present incubation, that is, within the range of 0.69% to 2.52%. The results are presented in Table I.

Separation and Identification of Keto Acids—

Two mycelial felts were incubated in 40 ml of the medium which consisted of 50 mg of DL-norleucine, 20 ml of M/15 phosphate buffer (pH 6.9), 5 ml of M/20 potassium arsenite and 15 ml of distilled water. Two hundred and forty-five millilitre of a four-day old culture filtrate was concentrated under diminished pressure at pH 8.0~8.2 and extracted with ether for three hours, followed by acidic ether extraction for five hours. The aqueous solution of the residual oily substance from the latter ether-extract was added with a small amount of 2 N hydrochloric acid and recon-

centrated under 14 mm Hg at about 45°. The treatment of the distillate with 2,4-dinitrophenylhydrazine gave a yellow crystal mass which melted at about 130°. Recrystallization with acidic dilute ethanol resulted in a long yellow needle of m.p. 148~9° (Found: C, 46.31; H, 4.38. Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H, 4.51.). In paper chromatography using butanol-3% ammonia solvent, only one spot of $R_F=0.81\sim0.83$ was observed. This melting point is in good accordance with that of 2,4-dinitrophenylhydrazone of α -keto-caproic acid as

FIG. 3. Ammonia- and Amino-Nitrogen from DL-Alanine (in phosphate buffer medium, pH 6.9).

Expressions are the same as in Fig. 2.

FIG. 4. Accumulation of Pyruvic Acid from DL-Alanine.

Values express the amount of pyruvic acid in 60 ml of phosphate buffer medium. (1), indicates the incubation added with no arsenite, and (2), added with arsenite.

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TABLE I. UTILIZATION OF ALANINE AND GLUCOSE

Day	1	3	5	7	10	12	14	17	20	22
Alanine consumed (%)	3.69	13.46	18.77	27.09	24.53	26.15	28.57	34.52	33.06	45.74
Glucose consumed (%)	3.02	5.01	5.01	8.61	7.37	17.46	21.87	26.50	44.09	82.36
Dry weight of mycelium (mg)	—	5.0	11.9	32.1	48.6	84.0	83.6	98.0	137.4	174.6

Present medium contained 150 mg of DL-alanine and 792.5 mg of D-glucose per flask. Figures express the average value of two flasks.

described by Krebs¹²⁾. Yield, Ca. 2.5 mg. From the residual solution remaining in the distillation flask a small amount of the same hydrazone was recovered. The same 2,4-dinitrophenylhydrazone was also obtained from the medium without arsenite, although the separation procedure was a little more difficult and its yield was unsatisfactory, compared to the case in which arsenite was added as just mentioned.

The separation and identification of pyruvic acid derivable from alanine was far easier, compared to the separation experiments conducted so far with other several amino acids. The condition of incubation was the same as in the case of norleucine. An amount of 110 ml of the filtrate on the fifth day of incubation, which was strongly positive for Rothera's test, was treated in the same way as just described above. The 2,4-dinitrophenylhydrazone prepared from the acidic ether extract melted at 216~7°, on being recrystallized from ethanol solution and gave no depression in mixed melting point determination with an authentic sample. Yield, Ca. 7 mg. In paper chromatography (butanol-3% ammonia solvent; room temperature; ascending method) only one spot of $R_F=0.28\sim0.30$, i.e., anti-type of this hydrazone¹³⁾ was observed. The separation of this keto acid from the medium without arsenite resulted in fail, as it was expected from the negligible Rothera's reaction of the filtrate. This fact is only characteristic of alanine among the ten amino acids used, that is, L-leucine, DL-valine, L-aspartic acid, L-phenylalanine, DL-methionine, glycine, DL-norleucine, DL-isoleucine, DL-alanine and DL- α -aminobutyric acid, as a supplement in this series.

DISCUSSION

The formation of either α -keto acid from leucine¹⁾ and isoleucine⁵⁾ by *Piricularia oryzae* has already been established by the authors. This led us to attempt to confirm such metabolic conversion with the remaining six carbon amino acid, that is, norleucine and

thus, the formation of α -ketocaproic acid from this amino acid was successfully recognized both in the presence and absence of arsenite. In the absence of arsenite this amino acid was easily deaminated (Fig. 1) as was observed with other natural amino acids and, in addition, norleucine was found to be utilized in the form of free amino acid itself, that is, 20~23 per cent of the amino acid added up to the second day of incubation and 40~47 per cent up to the seventh day. This experimental result is somewhat different from the cases conducted with leucine¹⁾ and valine³⁾, in which only a slight amount of each amino acid was utilized, not via its conversion to corresponding keto acid. In spite of uncertain natural occurrence of norleucine, the nutritional significance for some microorganisms has been examined by several investigators: growth-promoting effect for *hiochi-bacteria*¹⁴⁾, brewer's yeast¹⁵⁾, and *Neurospora* mutant¹⁶⁾ and, to the contrary, inhibitory effect for *E. coli*^{17,18)}. In addition, its role in transamination^{19,20,21)} and in oxidation by amino acid oxidase²²⁾ was also observed. The present work seems to support further evidence that this amino acid is as much available as other natural amino acids, at least for some microorganisms. The amino nitrogen in the culture filtrate added with arsenite, however, was observed to increase again after the third

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day, in spite of the almost constant amount of ammonia produced. A similar tendency was observed in the case of α -aminobutyric acid²³⁾ and rarely in the case of alanine as a supplement. This may be due to the production of endogenous amino acids, but at present no any data explaining thoroughly such observation are available.

The nutritional availability of alanine as a nitrogen source for blast fungus was shown by Tomizawa⁷⁾, and Otani²²⁾ has reported that amino acid oxidase secreted by present fungus is able to attack alanine. The present investigation was undertaken to examine the metabolic significance as a carbon source and thus the conversion of this amino acid to its α -keto analogue. Sufficient utilization of alanine is proved from Fig. 2, and the same utilization process was also found in the phosphate buffer medium (Fig. 3). From this result it is established that it is possible to carry out such an experiment just mentioned, in the phosphate buffer, if necessary. The absorption and utilization in the form of alanine itself was 14.8 per cent and 25.4 per cent on the third and the fifth day, respectively, but in the presence of arsenite such utilization process was almost negligible, although seven to eight per cent ammonia was produced up to the third day. It is noteworthy that the accumulation of pyruvic acid was detected only in the presence of arsenite and that in the absence of this inhibitor approximate one-fortieth of the amount in the former case was estimated (Fig. 4). Similar results were obtained in the investigation of the aerobic degradation of alanine and glucose by *E. coli* and *Clostr. Perfringens*^{23, 24)} and of the metabolic behavior of *Asp. niger* growing in glucose media²⁵⁾. These facts may probably be based upon the inhibitory effect of arsenite for the deami-

nation of alanine and further metabolic process of pyruvic acid such as decarboxylation. The curve indicating the amount of pyruvic acid in the medium added with arsenite showed a similar tendency with that of ammonia produced (Fig. 2, Fig. 3), although the amount of pyruvic acid detected was somewhat lower than that calculated on the basis of the amount of ammonia liberated, which will show the possibility of utilization of some parts of pyruvic acid formed even in the presence of 5 ml of M/20 arsenite. The other characteristic of alanine is that it is preferentially utilized by the present fungus, especially in a relatively earlier-stage of growth, to proper carbon source such as glucose (Table I), as it was recognized with glutamic acid by Tanaka⁹⁾. The consumption of alanine was observed to be far superior to that of glucose during the first ten days of growth, thereafter, followed by a gradual increase of the consumption of glucose, though it did not reach the consumption of alanine. After the twentieth day of incubation, a remarkable increase of consumption of glucose, compared to the relatively slower increase of consumption of alanine, was recognized. This characteristic behavior of *P. oryzae* towards both glutamic acid and alanine may present one hopeful step towards the investigation of the biochemical relationship existing between this fungus and the host plant.

SUMMARY

(1) *Piricularia oryzae* is able to utilize DL-norleucine through its conversion to α -ketocaproic acid, although, the natural occurrence of this amino acid still remains questionable. This keto acid is identified as its 2,4-dinitrophenylhydrazone.

(2) In the utilization of DL-alanine by *P. oryzae* some parts of this amino acid were utilized in the form of amino acid itself and other parts via prior conversion to pyruvic acid, but the accumulation of the present

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keto acid was observed only in the presence of arsenite, as was already recognized with other several microorganisms.

(3) It was established that alanine is preferentially utilized to a proper carbon source

such as glucose, as it was recognized with glutamic acid. This may present a probable step towards solving the problem of biochemical mechanism of invasion of this fungus and its further growth in the host plant.

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Biochemical Studies of *Piricularia oryzae*

Part VI. Behavior towards DL- α -Aminobutyric Acid and DL-Isoleucine : Formation of α -keto- β -Methylvaleric Acid from either Amino Acid.

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When DL- α -aminobutyric acid was used as a supplement in successive studies on the behavior of *Piricularia oryzae* towards amino acids, a keto acid differing from the α -keto analogue of this amino acid was isolated as its 2,4-dinitrophenylhydrazone upon which this hydrazone was found to be identical with the 2,4-dinitrophenylhydrazone of α -keto- β -methylvaleric acid which was formed from DL-isoleucine in another experiment. This finding indicates the formation of a six carbon compound from four carbon amino acid. The probable role of α -aminobutyric acid as an intermediate in the biosynthetic pathway of isoleucine has been discussed by several investigators, mainly based on the biochemically deficient mutant strain method and more recently on the isotopic competition method. Our present data seem to demonstrate direct evidence supporting the biosynthetic route of isoleucine in which α -aminobutyric acid or its α -keto analogue is included as a precursor of isoleucine. In addition, the presence of a close metabolic relationship between α -aminobutyric acid and isoleucine may be suggested from the fact that pyruvic acid and α -ketoglutaric acid were recognized together in respective experiment in which either α -aminobutyric acid or isoleucine was used as the supplement in the presence of arsenite.

In the successive study of the behavior of *Piricularia oryzae* towards amino acids, DL- α -aminobutyric acid was employed as the amino acid to be investigated. In this experiment a keto acid differing from the α -keto analogue of this amino acid was isolated as its 2,4-dinitrophenylhydrazone from the culture medium, which was assumed to be the 2,4-dinitrophenylhydrazone of a six carbon keto

acid, especially of α -keto analogue of isoleucine, from some experimental data¹⁾. In order to obtain the sample comparable with the 2,4-dinitrophenylhydrazone from α -aminobutyric acid just mentioned, and also in order to find the behavior towards isoleucine, a similar incubation experiment as previous²⁾

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2) T. Shimomura and Y. Nakamura, part V of this series.