BIOCHEMICAL STUDIES OF *PIRICULARIA ORYZAE* BRIOSI ET. CAVARA

By

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Introduction

It has been generally known that the blast disease of rice plant gave serious damage to the rice-crop in Japan. Accordingly, many scientific researches have been made by many investigators, in order to prevent the rice plant from this disease. Then, three parts to be studied seem to be involved in such scientific researches: the first is the study of the blast fungus itself; the second, the physiological and chemical study of host plant; the third, the biochemical study including together the two parts of research just mentioned. Many researches dealing with the first part have been conducted from earlier year, but

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almost all of these researches have been made principally from the phytopathological point of view and the biochemical study of the blast fungus itself, i.e., *Piricularia oryzae*, has been overlooked for longer time. These few years, however, several reports concerning these three parts have come to be presented. LEAVER (1) and TANAKA et al. (2, 3) showed that both biotin and thiamine acted as growth factor towards this microorganism—this discovery was remarkably valuable in that the cultivation of blast fungus was made possible thereafter in the complete synthetic medium—and the dicarboxylic amino acids and polycarboxylic acids were found to be effective for its growth by the latters (4). MORITA (5) recognized glucose as one of the constituents of mycelium and moreover assumed the probable presence of gluco-xylan, polyuronide, pentosan and xylan. Recently, TAMARI et al. (6) separated two toxic organic crystals from the culture medium of this fungus, which were α-picolinic acid and unknown substance respectively. The latter compound was named as piricularin by them and was found to be much more inhibitory for the growth of young rice plant than the fomer compound (6). SHIMOMURA observed the inhibitory effect of the culture filtrate of this fungus for both the germination of unhulled rice and its further growth and in addition, abnormal elongation of the roots caused by the autolyzate of mycelium (these observations seem to be due to the specific physiological action of piricularin described by TAMARI et al. (7)). The relation between the susceptibility of rice plant to the blast disease and the content of silica in host plant was also examined by several investigators (8, 9, 10) and it was concluded that the content of silica in rice plant resistant to blast disease was much higher than in that less resistant and the application of silica was greatly effective in preventing the host plant from the blast disease. Besides these works, the effectiveness of magnesia was also reported by ISHIZUKA et al. (11). The relation of the amount and kind of nitrogen compound in rice plant to its sensibility to blast disease was investigated by OTANI (12, 13, 14) and it was known that the number and area of pathogenic spot on the leaf, which was caused by the invasion of blast fungus, was together increased in the particular rice plant and particular growth period containing the higher amount of water-soluble nitrogen compounds, including acidic amino acids. In accordance with this observation, TANAKA et al. (15) presented that the sensibility to blast disease was dependent upon the accumulation of dicarboxylic amino acids in the host plant and these acidic amino acids

were the good nitrogen source for present fungus.

As is well known, the blast disease is likely to break out in high probability by the application of excessive nitrogen fertilizer, which may lead to the sub-normal metabolic changes in rice plant. It will be possibly assumed that such metabolic unusuality may be favorable for the germination of blast fungus and its further growth. Thus, the biochemical correspondence of this fungus to such physiological and chemical conditions of rice plant just described should be naturally taken into consideration. The studies worked by several researchers mentioned above, at least some parts of these studies, would be made from such point of view. Such biochemical conditions, however, are most complicated and appear as the total sum of so many metabolic systems which are continuously moving under close inter-relation. It is a most difficult to separate any one of these metabolic processes in its original form which are metabolically pooled. Thus the blast fungus itself has been taken as the object of present research, being apart from taking the host plant into account for a while, and its biochemical behaviors towards the nutritional circumstances, especially nitrogenous compounds, have been investigated. Then it is an unquestionable fact that the nitrogen in inorganic form utilized by microorganisms is converted into that of organic and accordingly, the secondary utilization of nitrogen will be conducted through the organic nitrogen compounds. As the organic nitrogen compound most closely relating to living things, protein should be taken into first consideration and in more fundamental meaning, amino acids should be taken. As mentioned above, TANAKA et al. (4) studied the utilization of amino acids by blast fungus. But the aim of their study was to find out the biochemical effect of amino acids as growth-factor-like substance and thus the utilization mechanism of amino acids as general nutrients was not investigated. The effectiveness of several amino acids as nitrogen source was presented by LEAVER (1) and OTANI (16). In these papers, however, the mechanism of utilization was not stated as in the case of TANAKA et al.. In these experiments amino acids were treated as nitrogen source for this fungus, but it is known that amino acid acts not only as nitrogen source but as carbon source. In present study, several amino acids were examined for their roles as carbon source, that is, the conversion of nitrogen compound to non-nitrogen compound, one of the most important metabolic processes in which amino acids participate, and in relation to this, the adaptive formation of some enzymes was

also studied which will give some newer knowledge on the relationship between the blast fungus and its nutritional circumstances, in other word, host plant.

Chapt. I. Utilization Process of Amino Acids by *P. oryzae* (kindly supplied by Dr. Y. OTANI), especially the Formation of α-Keto Analogues.

Part I. Behavior towards several Amino Acids and Identification of α-Keto-Isocaproic Acid from L-Leucine.

As was described in introduction, the investigation of the possibility of utilization of amino acids as carbon source by blast fungus seems to present a progressive step in the study of nitrogen metabolism of present fungus. Part I deals with the examination of such metabolic possibility with several amino acids both in the first and second cultivation and in addition, the separation and identification of α-keto acid from L-leucine, which presents a direct evidence showing the conversion of amino acid to non-nitrogenous compound.

Experimental Results and Discussion

1. Effect of several amino acids in the first cultivation—*Piricularia oryzae* grown at 27–8° for two weeks on potato-agar medium supplemented with sucrose (3 per cent) was incubated in 30 ml. of TOCHINAI's culture solution (17) (Table. 1), which consisted of every 3 ml. of A, B

TABLE 1. TOCHINAI's Culture Solution.

A	KNO_3	2.0/100 (g/ml)
B	KH_2PO_4	1.0/ "
	$MgSO_4 \cdot 7H_2O$	0.5/100
C	Surose	30.0/100 "
D	$CaCl_2 \cdot 2_2H_2O$	0.1/100 "
E	$FeCl_3 \cdot 6H_2O$	3.0/100 "

and D, one drop of E, every 1γ of thiamine and biotin, 50 mg of L-leucine, L-aspartic acid, L-glutamic acid, or L-phenylalanine in place of C, and water amounting to 30 ml. of total volume per flask. The macroscopical observation of its growth on fourteenth day and on twenty first day of cultivation are shown in Table 2.

TABLE 2. Growth Conditions of *P. oryzae* in the Medium containing L-Amino Acids and no Sucrose.

No.	I	II	III	IV	V
after 14 days	++	++ ++	+++ ++	+	+++ ++
after 21 days	++	+++ ++	+++ ++	++	++ ++ ++ ++

I contained 50 mg of L-leucine, no sucrose.
II " " L-glutamic acid, no sucrose.
III " " L-aspartic acid, " .
IV " " L-phenylalanine, " .
V " 900 mg of sucrose, no amino acid.

Experiment No. V in Table 2 is control incubation added with 900 mg of sucrose (3 ml. of C) in place of amino acid. It is known from the results in Table 2 that the maximal growth was attained in the medium containing normal carbon source, i.e., sucrose, but, even in the absence of such carbon source the presence of glutamic acid or aspartic acid brought vivid growth of this fungus, not less than sucrose and, in addition, leucine or phenylalanine is not so effective for its growth, compared to dicarboxylic acids, although some parts of respective amino acid supplemented were distinctly utilized. When these amino acids are utilized as carbon source as was speculated from Table 2, the formation of ammonia resulting from deamination process is naturally expected. In order to obtain one of the evidences meeting such probable expectation, pH value of the medium of respective experiment in Table 2 was determined (Table 3) and it was found that the better the growth was, the higher pH was. The determinations of ammonia in 5 ml. of twenty one-day old culture medium added with amino acid, that is, I, II, III and IV indicated the approximate proportionality of the amount of ammonia-nitrogen produced to pH. The formation of ammonia in V, which contained sucrose and no amino acid, would be perhaps out of consideration. In practical, the amount of ammonia detected in this case was almost negligible (Table 3). It was already observed that in the incubation experiment using such sucrose-containing medium, whose initial pH was about 4.5, pH of the ten to fourteen-day old culture medium increased almost always to about 7.8. The similar increase of pH value was observed also in present experiment employing glutamic acid or aspartic acid in place of sucrose. Accordingly it seems that the nutritional factors relating to the increase of pH in the case added

with amino acids are fundamentally different from those in the case added with sucrose. However, TANAKA et al. (18) reported recently that the increase of pH in the latter case might be due to the selective absorption of nitrate anion by present fuugus.

TABLE 3. Ammonia-Nitrogen in 1.25 ml. of Culture Solution and pH of each Case in Table 2 after 21 days.

No.	I	II	III	IV	V
Nitrogen (mg)	0.113	0.298	0.251	0.018	trace
pH	5.8	7.6~7.7	7.6	5.5	7.7

These results described here indicate that *P. oryzae* is able to grow by its utilization of dicarboxylic amino acids even in the absence of carbon source and moreover, leucine and phenylalanine are available for this fungus, although their availability is less than the acidic amino acids.

2. Utilization of L-leucine by mycelial felts in the presence or absence of carbon source—It is assumed that the employment of mycelial felts grown in normal (sucrose-containing) medium is far favorable, in order to find directly the metabolic behavior of present fungus towards amino acids. In present experiment L-leucine was used as the amino acid to be tested and the utilization of this amino acid by mycelial felts was studied. Mycelial felts of three culture flasks, which were grown in normal medium for two weeks, were washed with sterilized water, pressed and transferred together into the flask containing 60 ml. of culture solution (Table 1) supplemented with 100 mg of L-leucine or the same amount of culture solution added with 6 ml. of water in place of 6 ml. of C in Table 1 and the same amount of this amino acid. Further incubation was made at 26–7° for twelve days and every three days, ammonia- and amino-nitrogen and pH of the medium were determined by usual methods (Table 4 and Fig. 1). In either incubation, further good growth was observed: on second to third day of incubation aerial mycelium was formed and on fifth to sixth day the surface of the culture liquid was nearly covered with aerial mycelium. From these results it was known that the increase of ammonia-nitrogen and the decrease of amino-nitrogen in the medium containing sucrose and leucine were far smaller than respective cnange of either nitrogen-form in the medium added with leucine only. In the incubation added

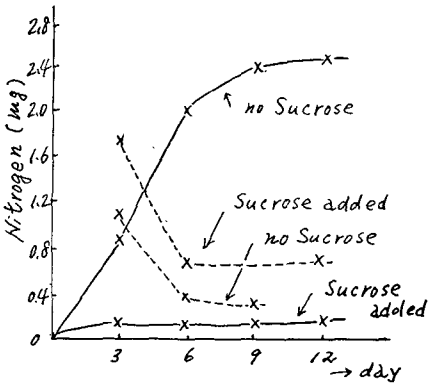
TABLE 4. Change of Ammonia- and Amino-Nitrogen from L-Leucine in the Presence or Absence of Sucrose and of pH of each Case.

Day		3	6	9	12
Sucrose added	pH	5.6	5.8	6.1	6.8
	*ammonia-N.	0.136	0.094	0.133	0.152
	*amino-N.	1.755	0.648	—	0.726
no Sucrose	pH	6.3	7.7	7.7	7.8
	*ammonia-N.	0.852	1.984	2.404	2.508
	*amino-N.	1.130	0.372	0.290	—

* These values are shown in mg-no. of nitrogen contained in 10/60 ml..

with sucrose, the formation of ammonia nearly stopped as far as third day, but, to the contrary, further decrease of amino acid was observed as far as sixth day, although this decrease did not lead to the formation of as much ammonia. This fact seems to show the availability of present amino acid as nitrogen source, as was indicated by LEAVER et al. (1). Comparing to the slower change of pH of the medium added with sucrose and leucine, more steep increase of pH was observed in relatively earlier stage of incubation containing L-leucine only, being followed by almost constant progress thereafter. These findings are in good accordance with the changes of ammonia and amino acid, and thus it may be possible to speculate that the increase of pH is mainly dependent upon ammonia produced, at least in the incubation added with leucine only. At any rate, present experiment seems to indicate the availability of L-leucine as carbon source for blast fungus.

Fig. 1. Change of Ammonia- and Amino-Nitrogen from L-Leucine in the Absence or Presence of Sucrose.



Full line expresses the amount of ammonia-nitrogen in 10 ml. of medium and dotted, the amount of amino-nitrogen.

3. Separation and identification of α-keto-isocaproic acid—In the case of probable utilization of amino acid as carbon source, the detection of the corresponding α-keto acid would present a direct evidence indicating such utilization process. Three mycelial felts were incubated

at 26–7° in the same way as just described in experiment 2, in the medium, which consisted of 90 ml. of TOCHINAI's solution (not containing sucrose), 10 ml. of straw extract as biotin source, 10 γ of thiamine, 150 mg of L-leucine and 80 ml. of water. Good growth was observed and on seventh day the surface of the medium was covered over with white aerial mycelium. On the same day, mycelium was removed by filtration and the filtrate showed strong purple color by the addition of ferric chloride solution, which was characteristic of α-keto acid expected. This filtrate was concentrated to 40 ml. below 40°, acidified with 2 N hydrochloric acid, and extracted with ether for about three hours. The aqueous solution of the ether- extract was treated with 5 ml. of sodium bisulfite solution (20%) and the combined part with bisulfite was decomposed with sulfuric acid, being followed by second extraction with ether. The aqueous solution of present extract was treated with the solution of 2.4-dinitrophenylhydrazine in 2N hydrochloric acid. Soon after milky yellow turbidity was observed, crystalline mass of 2.4-dinitrophenylhydrazone separated out. On being recrystallized from 50 per cent ethanol, it melted at 152–5°. Repeated recrystallization resulted in constant melting point of 156–7°, which was nearly identical with that of 2.4-dinitrophenylhydrazone of α-ketoisocaproic acid reported by KREBS (20). Analysis:

sample, 3.820 mg	H_2O, 1.512 mg ; CO_2 6.503 mg.	
	H, 4.43%	; C, 46.46%
Calcd. for $C_{12}H_{14}O_6N_4$;	H, 4.51%	; C. 46.45%

These results present the direct evidence indicating the formation of α-ketoisocaproic acid from L-leucine by *P. oryzae*.

4. Behavior towards L-glutamic acid and L-aspartic acid in the absence of carbon source—The availability of leucine as carbon source in the absence of sucrose was known from the preceding experiment 1 and 2. The same incubation experiment was conducted with L-glutamic acid and L-aspartic acid, whose nutritional significance has been much more recognized these few years. The probable availability of these two dicarboxylic amino acids as carbon source has been already known from the results in Tab. 2, but present experiment has been undertaken to find the timely sequence of the metabolic change of both amino acids.

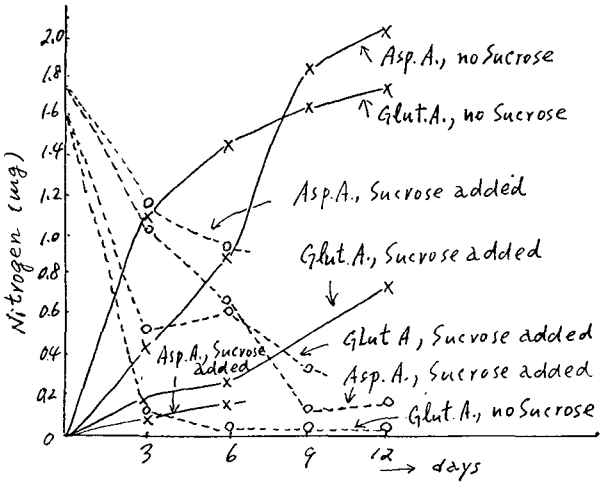
Two mycelial felts grown in normal medium (Tab. 1) for 2 weeks were incubated in the same way as in experiment 2, in 60 ml. of normal

medium containing 100 mg of L-glutamic acid or the same amount of L-aspartic acid, respectively. In the experiment added with no sucrose, 6 ml. of water was used in place of 6 ml. of C in Tab. 1. In both cases, active growth was observed but, on and after ten days of cultivation, the growth appeared to be a little better in the presence of sucrose than in the absence of it. Every three days the same determinations as in experiment 2 were made with each case (Tab. 5 and Fig. 2).

TABLE 5. pH Change of the Culture Solution containing L-Glutamic or L-Aspartic Acid.

Day		3	6	9	12
L-Glutamic	Sucrose added	5.5	5.5	6.7	6.8
	no Sucrose	7.4	7.6	7.8	7.8
L-Aspartic	Sucrose added	5.4	5.5	6.6	6.8
	no Sucrose	6.2	7.0	7.6	7.8

Fig. 2. Change of Ammonia- and Amino-Nitrogen from L-Glutamic and L-Aspartic Acid in the Presence or Absence of Sucrose.

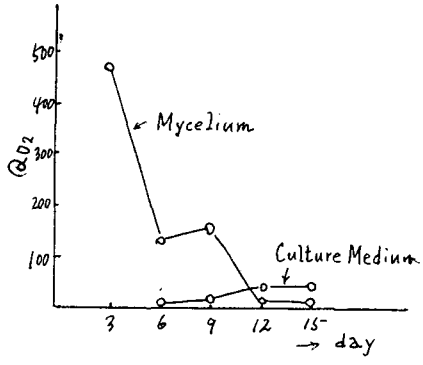


Values indicate the amount of nitrogen in mg contained in 10/60 ml. culture solution. Full line indicates ammonia-N and dotted line, amino-N.

In both cases, similar results as in the case of L-leucine (Fig. 1) were obtained: the increase of ammonia and the decrease of amino acid in either culture medium supplemented with glutamic acid only

or aspartic acid only were far greater than those in each case added with respective amino acid and sucrose. From Fig. 2, it is clearly known that almost all of either amino acid added, that is, glutamic acid or aspartic acid, disappeared during the first ten days of cultivation, but relatively faster utilization of the former amino acid than the latter acid was recognized, from the change of amino-nitrogen of either amino acid. In either incubation added with glutamic acid or aspartic acid only, the amount of ammonia detected was found to be less than that corresponding to the decrease of each amino acid. This fact seems to indicate the possible utilization of amino acid in its original form, perhaps as nitrogen source. Furthermore, it was recognized that the velocity of deamination was much greater in relatively earlier stage of incubation than in the later stage. The similar tendency was also observed in the case of L-leucine. These findings will be additionally supported by the activity curve of L-amino acid oxidase of present fungus (Fig. 3) (presented in details in Chapter II of this series), in which maximal consumption of oxygen for L-leucine as substrate was attained on the third day of cultivation, being followed by gradual decrease of oxygen consumption thereafter. However, the amount of ammonia detected in either twelve day old culture medium added with respective amino acid only was measured to be somewhat more than the theoretical number of nitrogen, i.e., 1.587 mg for glutamic acid and 1.755 mg for aspartic acid, which may be due to the partial autolysis in later stage.

Fig. 3. Oxygen Uptake by L-Amino Acid Oxidase of *P. oryzae* Mycelium grown in no Carbon-Source Medium.



$Q_{O_2} = O_2$ $\mu\ell$/hr./g. or cc. Substrate, L-Leucine.

In the incubation added with both glutamic acid and sucrose, considerably active decrease of amino nitrogen was observed as far as third day, being followed by relatively slower decrease thereafter, but the formation of ammonia, on the other hand, was far less in its amount and its velocity than that corresponding to the decrease of amino acid during the incubation. This finding indicates the availability of glutamic acid as other nutritional source, probably as nitrogen source, rather than as the carbon source, when sufficient amount of proper carbon

source is present. Such interpretation just mentioned seems to be able to explain the growth promoting effect of glutamic acid in the cultivation of this fungus in media containing proper carbon source, reported by TANAKA et al. (4) and LEAVER (1). In addition, it is supposed that the organic nitrogen source such as amino acids, especially glutamic acid or aspartic acid, as well as ordinary inorganic nitrogen source is possibly necessary for the full growth of present fungus. The similar results as in the case of glutamic acid were obtained with aspartic acid, though the degrees of the increase of ammonia and the decrease of amino acid were somewhat lower than those in employment of glutamic acid. Thus, it follows that the same interpretation as in the case of glutamic acid is possibly applicable for aspartic acid, too. The changes of pH in the presence or in the absence of sucrose were observed to be the same as in experiment 2 (Tab. 4 and Tab. 5). Thus the increase of pH value in the absence of surose superior to that in the presence of it seems to be mainly dependent upon the amount of ammonia produced via deamination.

The good availability of these two dicarboxylic amino acids as carbon source, especially in the cultivation supplemented without proper carbon source, seems to suggest the formation of respective α-keto analogue, that is, α-ketoglutaric acid from glutamic acid and oxaloacetic acid from aspartic acid, as was already recognized with L-leucine. By the application of the same procedure as in experiment 2 to either incubation added with glutamic acid or aspartic acid, trace amount of 2, 4-dinitrophenylhydrazone of m.p. 151–2° was obtained from the latter case but not identified at present. In the former case, slight amount of crystalline 2, 4-dinitrophenylhydrazone of m.p. 166° was isolated but it was clearly different from 2, 4-dinitrophenylhydrazone (m.p. 216–8°) of α-ketoglutaric acid. Perhaps it may be 2, 4-dinitrophenylhydrazone of an intermediary metabolite following after α-ketoglutaric acid in the metabolic process of glutamic acid, which would be taken in reasonable consideration in present experiment using intact microorganism. Furthermore, some notice would be paid to the well-known fact that these two keto acids are easily and directly introduced in the metabolic process such as tricarboxylic acid cycle. Accordingly, the employment of a certain inhibitor may be favorable for the separation and identification of keto acids, including the two keto acids just described. When present report has been being written, TANAKA and MORIWAKI (21) presented the formation of α-keto-

glutaric acid by *P. oryzae* from glutamic acid both in the medium deficient in thiamine and in the medium added with arsenite as inhibitor.

Short Summary

It was found that *Piricularia oryzae* could grow in the culture medium containing no proper carbon source in the presence of L-glutamic acid, L-aspartic acid and L-leucine. L-glutamic acid was most effectively utilized among these three amino acids. This fact suggests the conversion of amino acid to ammonia and its corresponding keto analogue. This suggestion was confirmed both by the identification of α-ketoisocaproic acid from L-leucine as its 2, 4-dinitrophenylhydrazone and by the greater activity of L-amino acid oxidase in the earlier stage of cultivation than in the later stage. Thus it was shown that present fungus could utilize amino acid as carbon source via deamination. In addition, even in the presence of proper carbon source and inorganic nitrogen source, some parts of amino acid added was found to be utilized in the form of amino acid itself. The increase of pH value of the culture medium added with amino acid only was thought to be mainly due to the accumulation of ammonia produced and thus to be different from that in either case added with carbon source only or amino acid and carbon source.

Part 2. Formation of Dimethylpyruvic Acid from DL-Valine.

It has been shown by OTANI (13) and TANAKA et al. (15) that rice blast disease is likely to break out in a high probability during the growth period of 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 *Piricularia oryzae*, the pathogenic fungus causing blast disease, towards the biochemical condition of the host plant described above. The present author has already found 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 metabolic

step of its utilization; thus α-ketoisocaproic acid has been proved from L-leucine by the author. For further confirmation of this utilization process, present part 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 Results and Discussion

P. oryzae was grown at 27–8° in 30 ml. of the first culture medium described in part 1 of Chapt. II. After 14 days of incubation, the mycelium of the three culture flasks were transferred together, as was described in preceding part 1, to 90 ml. of the second culture medium and further incubation was made at 27–8°. Of this second culture medium, No. 1. contained three times as much 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 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.

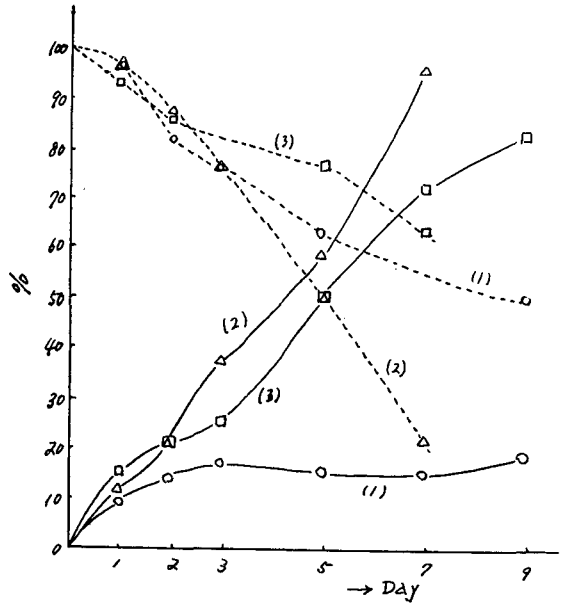
The ammonia- and amino-nitrogen contents of each flask were measured at appropriate intervals by usual methods. The results obtained are given in Table 6 and Fig. 4.

TABLE 6. Change of Ammonia- and Amino-Nitrogen from DL-Valine.

Day	No. 1		No. 2		No. 3	
	NH_3-N	NH_2-N	NH_3-N	NH_2-N	NH_3-N	NH_2-N
1	1.728	17.334	1.953	17.334	2.628	16.686
2	2.484	14.596	3.852	15.520	3.708	15.259
3	3.168	13.707	6.894	13.707	4.536	—
5	2.644	11.246	10.656	8.894	8.928	13.863
7	2.644	—	17.280	3.922	13.052	11.300
9	3.456	8.887	—	—	14.904	8.409

Ammonia was determined by distillation method and amino-N, by VAN SLYKE method. Figures indicate the amount of N of either form in mg in 90 ml. of medium. Other experimental details, see text.

Fig. 4. Ammonia- and Amino-Nitrogen from DL-Valine by *P. oryzae*.



(1), DL-Valine+sucrose; (2), DL-valine only; (3), DL-valine+arsenite. Full lines represent ammonia-N and dotted, amino-N. Values are expressed as percentage to the DL-Valine added.

In the three cases, the velocity of ammonia formation and the amount of ammonia produced 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 source (part 1). 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. (1) and OTANI (16), 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' report (22) that the activity of the keto analogue of valine is only 50% of this amino acid towards 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 acid, namely dimethylpyruvic acid, both the isolation and the 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 reconcentrated at 60–65° 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° 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° and gave following analytical values: sample, 4.381 mg. Found; CO_2, 7.084 mg; H_2O, 1.548 mg.

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 KREBS (20) and MEISTER (23). 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° 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° in the crystals described above and following analytical data: sample, 3.720 mg. Found: CO_2, 6.171 mg; H_2O, 1.361 mg. C (%), 44.78; H, 4.10. Calcd. for $C_{11}H_{12}O_6N_4$: C, 44.59; H, 4.05. These results indicate the possibility of α-keto acid formation from DL-valine in both the absence and presence of a carbon source.

Short Summary

Piricularia oryzae is able to utilize DL-valine for its growth as carbon source in the absence of 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 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.

Part 3. Behavior towards DL-Norleucine and DL-Alanine.

As was described in preceding parts. the study of the biochemical behavior of *Piricularia oryzae* towards nitrogenous compounds, especially amino acids, seems to be of fundamental importance in order to prevent the host plant from the blast disease. From such point of view, the utilization process of several amino acids by this fungus, especially the conversion of the amino acid to its α-keto analogue has been investigated by the author. In current experiment, DL-norleucine and DL-alanine were taken as amino acid to be tested. Already, the six carbon amino

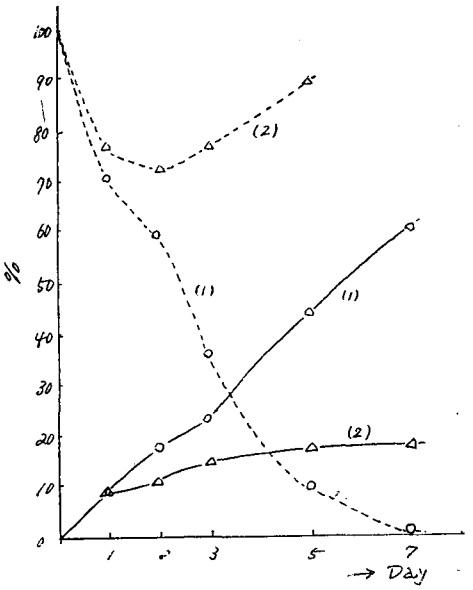
acids such as leucine and isoleucine (part 1 and part 4) were examined for their conversion to respective α-keto acid, and thus the remaining six carbon amino acid, that is, norleucine was naturally taken into consideration as supplement, although its natural occurrence is questionable at present (24), in spite of early works of ABDERHALDEN et al. (25, 26). On the other hand, the general metabolic significance of alanine has been well known and it was reported by TOMIZAWA (27) and OTANI (16) that this amino acid is effective as nitrogen source as much as asparagine, aspartic acid, glutamic acid, and glycine, but the metabolic conversion of these amino acid was not stated at all. TANAKA (28) presented that glutamic acid could be able to be utilized preferentially to proper carbon source by this fungus and that this fact might be interpreted as one of the biochemical mechanisms involved in the invasion of blast fungus in host plant and its further growth. Similar results have been obtained with alanine by the author.

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

Experimental and Results

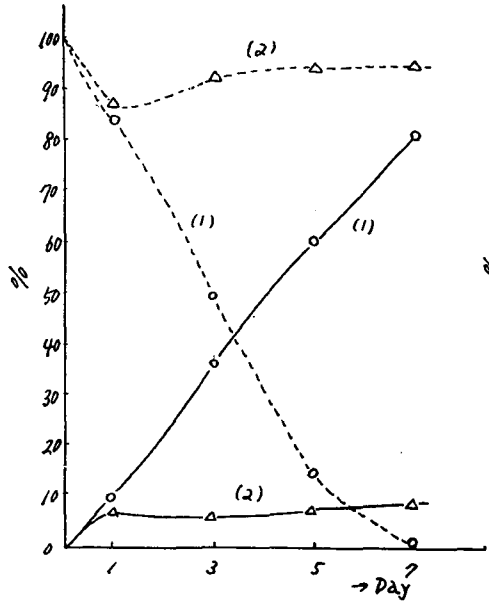
Incubation — Two mycelial felts were incubated at 27–8° in 60 ml. of complete mediun (part 2) containing 100 mg of DL-norleucine or DL-alanine in place of sucrose. In the inhibition experiment, 5 ml. of M/20 potassium arsenite was substituted for 5 ml. of water. Ammonia- and amino-nitrogen in the culture filtrate during the incubation were determined as was previously reported (Fig. 5 and

Fig. 5. Change of Ammonia- and Amino-Nitrogen from DL-Norleucine (complete medium).



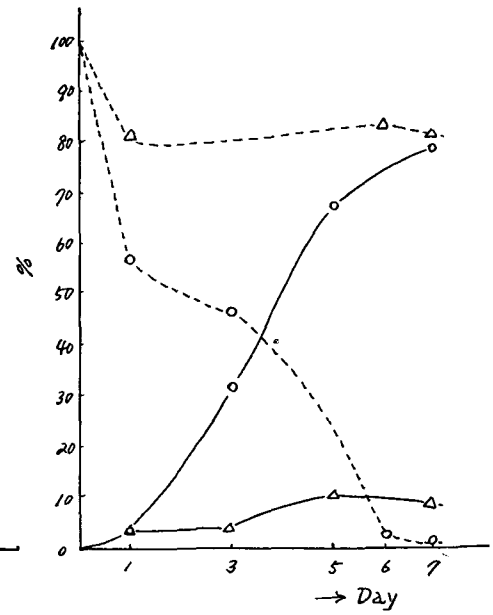
Full line expresses ammonia-nitrogen and dotted, amino-nitrogen. Values are expressed as percentage to the amount of norleucine added. (1), DL-norleucine only; (2), DL-norleucine plus arsenite. Experimental details, see text.

Fig. 6. Change of Ammonia- and Amino-Nitrogen from DL-Alanine (complete medium).



Expressions are the same as in Fig. 5. Experimental conditions, see text.

Fig. 7. Change of Ammonia- and Amino-Nitrogen from DL-Alanine (phosphate buffer medium).

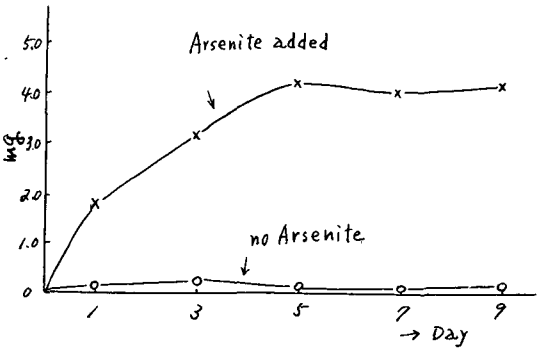


Expressions are the same as in Fig. 5. Experimental conditions, see text.

Fig. 6). The same determinations were made with the culture medium which consisted 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 complete medium just mentioned were obtained (Fig. 7). This fact indicates that it is possible to carry out such experiment in the phosphate buffer medium added with no other inorganic salts. The determination of pyruvic acid accumulated in the phosphate buffer medium was made by a modification of the FRIEDEMAN-HAUGEN method by SHIMIZU (29) (Fig. 8).

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 which contained 150 mg of DL-alanine, 792.5 mg of D-glucose and as much 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 was used as carbon source in normal cultivation. It was already known by OTANI (102) that glucose was equally available for this fungus

Fig. 8. Accumulation of Pyruvic Acid from DL-Alanine in Phosphate Buffer Medium.



The incubation mixture consisted of 100 mg of DL-alanine, 30 ml. of M/15 phosphate buffer (pH 6.9) and 30 ml. of water. In inhibition experiment, 5 ml. of M/20 pot. arsenite was used in place of 5 ml. of water. Values indicate the amount of pyruvic acid in 60 ml. of medium.

TABLE 7. Utilization of D-Glucose and DL-Alanine.

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

Values are expressed as percentage to the amount of glucose added or alanine, except those in the fourth line. Experimental details, see text.

as sucrose. At appropriate intervals, ammonia- and amino-nitrogen, remaining glucose and dry weight of mycelium were estimated. The results are presented in Table 7.

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. 245 ml. of 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 2N hydrochloric acid and reconcentrated under 14 mm Hg at about 45°. The treatment of the distillate with 2, 4-dinitrophenylhydrazine gave yellow crystal mass which melted at about 130°. Recrystallization with acidic dilute ethanol resulted in long yellow needle of M.P. 148–9°. The analytical data of this hydrazone were as follows: sample, 5.297 mg. CO_2, 8.989 mg; H_2O, 2.073. 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 RF=0.81–0.83 was observed. This melting point is in good accordance with that of 2, 4-dinitrophenylhydrazone of α-keto-caproic acid described by KREBS (20). 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 obtained also from the medium without arsenite, although its separation procedure was a little more difficult and its yield was unsatisfactory, compared to the case added with arsenite 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. 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 descrived 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 authentic sample. Yield, ca. 7 mg. In paper chromatography (butanol—3% ammonia solvent; room temperature; ascending method) only one spot of RF= 0.28–0.30, i. e., anti-type of this hydrazone (30) was observed. The separation of this keto acid from the medium without arsenite was unsuccessful, as was expected from the negligible ROTHERA's reaction of the filtrate. This fact is only characteristic of alanine among ten amino acids used as a supplement in this series.

Discussion

The formation of either α-keto acid from leucine and isoleucine (part 4) was already established by the author. This led him to try 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. 5) 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 as far as second day of incubation and 40–47 per cent as far as seventh day. This experimental result is somewhat different from the cases conducted with leucine (part 1) and valine (part 2), in which only 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 was examined by several investigators: growth-promoting effect for hiochi-bacteria (31), brewer's yeast (32), and *Neurospora* mutant (33) and, to the contrary, inhibitory effect for *E. Coli* (34, 35). In addition, its roles in transamination (36, 37, 38) and in oxidation by amino acid oxidase (39) were also observed. Present work seems to show additional 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 day, in spite of the almost constant amount of ammonia produced. The similar tendency was observed in the case of α-amino-butyric acid (part 4) 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 nitrogen source for blast fungus was shown by TOMIZAWA (27), and OTANI (39) reported that amino acid oxidase secreted by present fungus was able to attack alanine. Present investigation was undertaken to examine the metabolic significance as carbon source and thus the conversion of this amino acid to its α-keto analogue. Good utilization of alanine is known from Fig. 6 and the same utilization process was also found in the phosphate buffer medium (Fig. 7). It is known from this result that it is possible to carry out such kind of research 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 third day and fifth day, respectively, but in the presence of arsenite such utilization process was almost negligible, although seven to eight per cent of 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. 8). The similar results were obtained in the investigation of the aerobic degradation of alanine and glucose by *E. coli* and *Clost. perfringens* (40, 41) and of the metabolic behavior of *Asp. Niger* growing in glucose media (42). These facts may probably be based upon the inhibitory effect of arsenite for deamination 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 the similar tendency with that of ammonia produced (Fig. 6 and Fig. 7), 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 potassium arsenite. The other characteristic of alanine is that it is preferentially utilized by present fungus, especially in relatively earlier stage of growth, to proper carbon source such as glucose (Table, 7), as it was recognized with glutamic acid by TANAKA (28). The consumption of alanine was observed to be far superior to that of glucose during the first ten days of growth, thereafter followed by gradual increase of the consumption of glucose, though it did not reach the consumption of alanine. After the twentieth day of incubation, remarkable increase of consumption of glucose was recognized, compared to the relatively slower increase of consumption of alanine. 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 host plant.

Short 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 was identified as its 2, 4-dinitrophenylhydrazone.

2. In the utilization of DL-alanine by *P. oryzae*, some parts of this amino acid was utilized in the form of amino acid itself and the other parts, via prior conversion to pyruvic acid, but the accumulation of present keto acid was observed only in the presence of arsenite, as was already known with other several microorganisms.

3. It was established that alanine was preferentially utilized to

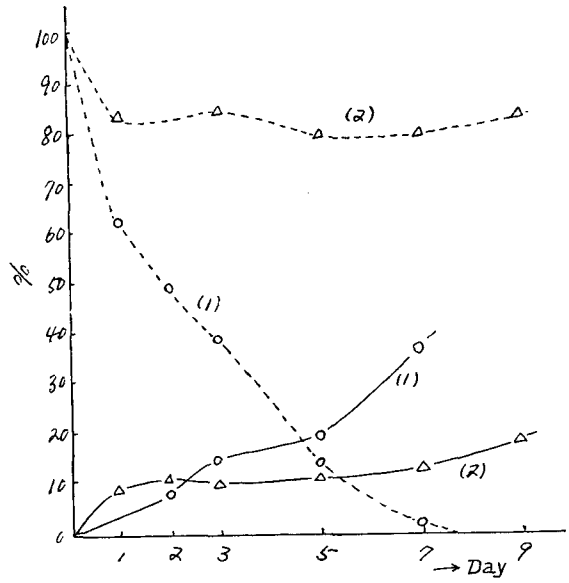
proper carbon source such as D-glucose, as it was recognized with glutamic acid. This may present a probable step towards solving the problem of the biochemical mechanism of the invasion of this fungus and its further growth in host plant.

Part 4. Behavior towards DL-α-Aminobutyric Acid and DL-Isoleucine: Formation of α-keto-β-Methylvaleric Acid from either Amino Acid

In the successive study of the behavior of *Piricularia oryzae* towards amino acids, DL-α-aminobutyric acid was used as the amino acid to be tested. 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 data. In order to obtain the sample comparablewith the 2, 4-dinitrophenylhydrazone from α-aminobutyric acid and also to find the behavior towards isoleucine, similar incubation experiment as was made previously (part 3) was undertaken with DL-isoleucine and the α-keto acid corresponding to this amino acid, i.e., α-keto-β-methylvaleric acid was successfully separated and identified as its 2, 4-dinitrophenylhydrazone. This hydrazone was proved to be identical with that derived from DL-α-aminobutyric acid. This fact indicates the formation of a six carbon compound of isoleucine carbon skeleton from four carbon amino acid. Interest in the biosynthesis of isoleucine and valine increased when BONNER et al. (43, 44) described a *Neurospora* mutant requiring both of these amino acids. In earlier reports, it was demonstrated by UMBARGER, ADELBERG and TATUM (45, 46) that these two amino acids might have their origin in common four carbon compounds metabolically related to threonine. More recently, however, the independence of biosynthetic pathway of two amino acids has been presented by ADELBERG et al. (47, 48, 49) and HIRSCH et al. (50) and, in addition, the experimental results by STRASSMANN et al. (51, 52) and ABELSON (53) seem to be favorable for such an independent biosynthetic route. On the basis of nutritional experiments with induced mutant strains of microorganisms and isotopic competition experiments, four carbon amino acids such as homoserine, threonine and α-aminobutyric acid have been presented in the biosynthetic pathway of isoleucine by several investigators (45, 46, 53, 54,

55, 56). In practical, α, β-dihydroxy-β-methylvaleric acid was separated and identified as the precursor of isoleucine from the culture medium of *Neurospora* mutant fed with either acetate (57) or threonine (48, 49), and WAGNER (58) succeeded in obtaining α-keto-β-methylvaleric acid from the culture filtrate of *Neurospora* mutant fed with threonine. In spite of the nutritional significance of α-aminobutyric acid as an intermediate in the biosynthetic route of isoleucine, the direct separation of isoleucine carbon chain compound, as was observed with threonine, derivable from this amino acid is yet unknown. Present part deals with the formation of α-keto-β-methylvaleric acid by *P. oryzae* from DL-α-aminobutyric acid as well as from DL-isoleucine and, in addition, the close metabolic relationship between these two amino acids, which is suggested by the fact that both pyruvic acid and α-ketoglutaric acid were together recognized in the presence of arsenite in respective experiment added with either α-aminobutyric acid or isoleucine as the supplement.

Fig. 9. Ammonia- and Amino-Nitrogen from DL-Isoleucine.

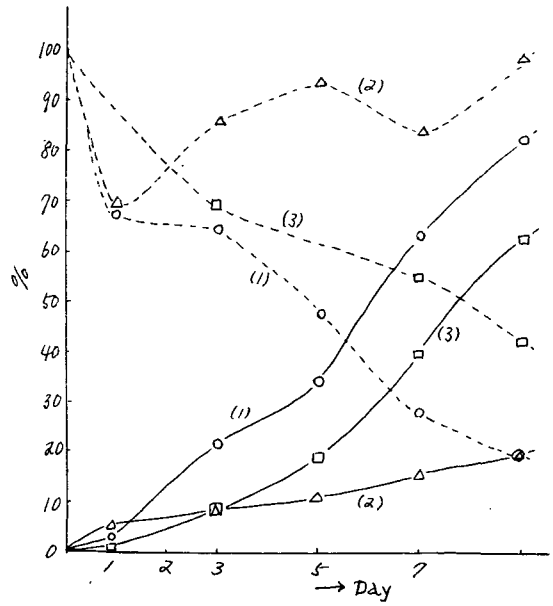


(1), DL-Isoleucine, only; (2) DL-isoleucine+arsenite. Full line expresses ammonia-nitrogen and dotted, amino-nitrogen. Values are expressed as percentage to theoretical value of nitrogen of isoleucine supplemented.

Experimental and Results

Incubation—The general procedure was similar to that described in part 3 of this series: two mycelial felts grown in normal medium (part 2) for 17 days were transferred to the flask containing 60 ml. of second culture medium which consisted of 40 ml. of M/15 phosphate buffer solution (pH 6.9), 20 ml. of water and 100 mg of DL-isoleucine or DL-α-aminobutyric acid and further incubation was made at 27–8°. In the inhibition experiment, 5 ml. of M/20 potassium arsenite was added in place of 5 ml. of water. At appropriate intervals, ammonia- and amino nitrogen contents were determined by usual methods. The same determinations were applied to the filtrate which contained 144 mg of sodium formate besides 100 mg of aminobutyric acid. The results are shown in Fig. 9, Fig. 10, and Fig. 11.

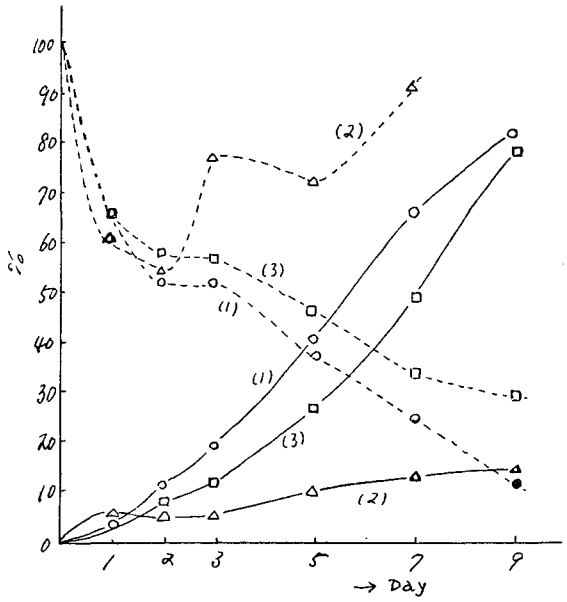
Fig. 10. Ammonia- and Amino-Nitrogen from DL-α-Aminobutyric Acid [I].



(1), DL-α-aminobutyric acid only; (2) aminobutyric acid+arsenite; (3), aminobutyric acid+formate. Expressions are the same as in Fig. 9. Experimental details, see text.

Separation and Identification of Keto Acids—On basis of the results in preceding papers, the separation of keto acids were carried out

Fig. 11. Ammonia- and Amino-Nitrogen from DL-α-Aminobutyric Acid [II].



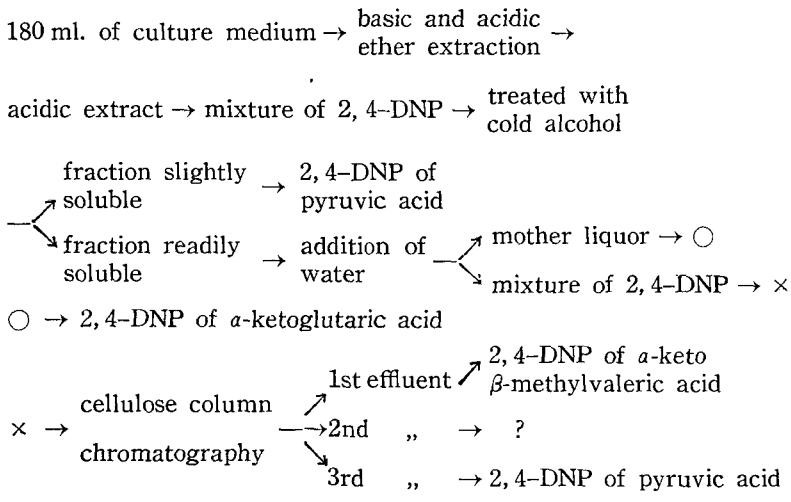
All expressions are the same as in Fig. 10.

mainly with the culture filtrate added with arsenite. In this case, the incubation mixture consisted of 35 ml. of M/15 phosphate buffer solution (pH 6.9), 5 ml. of M/20 potassium arsenite and 50 mg of DL-isoleucine per flask. On the fifth day of incubation at 27–8°, the mycelial felts were removed by filtration and 180 ml. of the filtrate was treated in the same manner as in previous papers (basic ether extraction, 8 hours; acidic ether extraction, 20 hours). The crystalline mass of 2, 4-dinitrophenylhydrazone obtained was divided into two fractions, that is, easily soluble in cold 94 per cent alcohol and slightly soluble in the same alcohol. On being recrystallized with hot alcohol, the latter fraction gave yellow hexagonal plate, which melted at 213–4° and showed no depression in the determination of mixed melting point with authentic pyruvic acid. In the mixed paper chromatography in which n-butanol saturated with water was used as solvent, the present hydrazone of RF = 0.24 corresponded to anti-type of 2, 4-dinitrophenylhydrazone of pyruvic acid. In some cases, dilute spot of syn-type of pyruvic acid 2, 4-dinitrophenylhydrazone (RF = 0.46) was also observed besides the clear spot of anti-type (30). These data indicate that the present

hydrazone is that of pyruvic acid. Yield, ca. 7 mg. Careful addition of water to the former fraction gave rise to the mixture of long needles and fine crystals wnich showed two clear spots on paper strip, RF = 0.24 and 0.85, respectively. It seems that the lower spot is related to the 2, 4-dinitrophenylhydrazone of pyruvic acid and the upper spot, to the 2, 4-dinitrophenylhydrazone of α-keto analogue of isoleucine. After being kept at 0° overnight, the crystalline mass was separated by filtration. The separation of this mixture into respective component was carried out by cellulose column chromatography (59): cellulose powder (Toyo Roshi Kaisha. Ltd.) suspended in n-butanol saturated with water was poured carefully onto a glass-funnel (1×25 cm) to the height of 14 cm without suction and was allowed to stand overnight. The solution of 2, 4-dinitrophenylhydrazone mixture just mentioned in a minimal amount of water-saturated-butanol and alcohol was dropped into the column and successive development with the same solvent was carried out at a flow rate of about 5 ml. per hour. Three yellow bands could be observed on the cellulose column. The effluent of each band was reduced to dryness in vacuo at room temperature and the residual mass was treated with alcohol containing water. From the third effluent yellow plate (m.p., 216°) was obtained in a small quantity, which was identified to be 2, 4-dinitrophenylhydrazone of pyruvic acid in the same manner as that just described. From the second, a small quantity of amorphous substance was separated but not any crystalline substance, except a trace quantity of pyruvic acid 2, 4-dinitrophenylhydrazone contaminated. The repeated recrystallization of the first effluent resulted in a yellow crystal of long needle, which melted at 164–6° and gave following analytical values: sample, 4.214 mg. H_2O, 1.455 mg; CO_2, 7.171 mg. H (%), 3.86%; C, 46.44%. Calcd. for $C_{12}H_{14}O_6N_4$: H, 4.51; C, 46.45. Sample, 1.697 mg. N_2, 0.269 cc (762.0 mm Hg, 24.5°C). N (%), 18.24, Calcd. for $C_{12}H_{14}O_6N_4$: N, 18.07, and showed RF=0.86 in paper chromatography in n-butanol-water solvent. Yield, ca. 0.8 mg. Although these analytical values are in good accord with those of 2, 4-dinitrophenylhydrazone of α-keto analogue of isoleucine, the melting point of the present hydrazone is somewhat lower than 176°, melting point of 2,4-dinitrophenylhydrazone of d- or l-α-keto-β-methylvaleric acid reported by MEISTER (60, 61). In this case, however, some notice must be paid to the fact that the melting point of 2, 4-dinitrophenylhydrazone of racemic mixture of this keto acid is 168–9° (60). The 2, 4-dinitrophenylhydrazone of pure d-α-keto-β-methylvaleric acid (kindly gifted

by Dr. A. MEISTER) was prepared by the author and it was found to melt at 172–3°. In order to obtain 2, 4-dinitrophenylhydrazone of racemic mixture, following procedure was carried on according to MEISTER (60): the aqueous solution of d-α-keto-β-methylvaleric acid was adjusted to pH 9.5 with 0.1 N sodium hydroxide and kept at room temperature for about half an hour, followed by the back-adjustment to acidic condition with 0.1 N hydrochloric acid. The 2, 4-dinitrophenylhydrazone from this solution melted at 164–6° and was proved to be identical with the 2, 4-dinitrophenylhydrazone from the first effluent, by paper chromatography and mixed melting point determination. These data show the formation of racemic α-keto analogue of isoleucine. When the yellow filtrate from which the mixture of 2, 4-dinitrophenylhydrazones of pyruvic acid and α-keto-β-methylvaleric acid was removed, was treated with further addition of water, pale yellow crystal was separated in a slight amount. On being recrystallized with water, it melted at 217–8° and showed RF = 0.07 in paper chromatography in n-butanol-water solvent. This hydrazone was identified to be that of α-ketoglutaric acid by the mixed paper chromatography and mixed melting point determination with an authentic sample. This hydrazone, however, was detected only in rare case.

Above procedure is summerized briefly in the following diagram:



The isolation and identification of keto acids derivable from DL-α-aminobutyric acid was conducted with the culture filtrate of the same incubation conditions as stated above. Many experiments were

conducted in the presence or absence of arsenite. In the absence of arsenite the isolation of 2, 4-dinitrophenylhydrazone of six carbon keto acid was comparatively easy, probably owing to the absence of other keto acids and other unknown carbonyl compounds, though its yield was unsatisfactory. On the other hand, arsenite stimulated the relative accumulation of pyruvic acid, α-ketoglutaric acid and others as well as six carbon keto acid, but the separation procedure was pretty difficult. Some of these experiments are described as follows. A portion of 230 ml. of the filtrate without arsenite on fourth day of incubation was treated in the same way as just described and the yellow mass of 2, 4-dinitrophenylhydrazone was dissolved in 95 per cent alcohol and the insoluble dark red substances were discarded. The yellow solution was added drop-wisely with water till slight turbidity was observed. After being warmed in water bath, the solution was allowed to stand still. A rod-shape-like crystal separated and melted at about 150°. Recrystallization in the same way resulted in a pure yellow crystal, m.p., 165–7°; RF = 0.88–0.91 in butanol-ammonia water solvent and RF = 0.85 in butanol-water solvent (sample 2.699 mg. CO_2, 4.634 mg; H_2O, 1.212 mg. C (%), 46.86; H, 5.02. Calcd. for $C_{12}H_{14}O_6N_4$: C, 46.45; H, 4.51). Yield, ca. 0.8 mg. As six carbon keto acid derivable from six carbon amino acid other than isoleucine, α-ketoisocarproic acid from leucine and α-ketocarproic acid from norleucine may be taken into consideration. The melting point (156–7°) of 2, 4-dinitrophenylhydrazone of the former keto acid (part 1) may be somewhat similar to that (165–7°) of present hydrazone. The determination of mixed melting point with the two hydrazones gave temperature depression by 15 to 20°, which indicate that present hydrazone is not that of α-keto analogue of leucine. The lower melting point (148–9°) of 2, 4-dinitrophenylhydrazone of α-keto analogue of norleucine makes it unnecessary to carry out such determination. Thus, the present 2, 4-dinitrophenylhydrazone was proved to be identical with that of racemic mixture of α-keto analogue of isoleucine by mixed paper chromatography and mixed melting point determination. In the presence of arsenite, 72 ml. of seven-day old culture filtrate was treated in the similar manner as in the case of isoleucine and the mixture of 2, 4-dinitrophenylhydrazones gave two clear spots (RF = 0.04 and 0.22) and one streaming spot with a bigger RF on paper strip (n-butanol-water solvent; ascending method; room temperature). This mixture was subjected to column chromatography just mentioned. On the cellulose column appeared four bands, designated as No. 1, No.

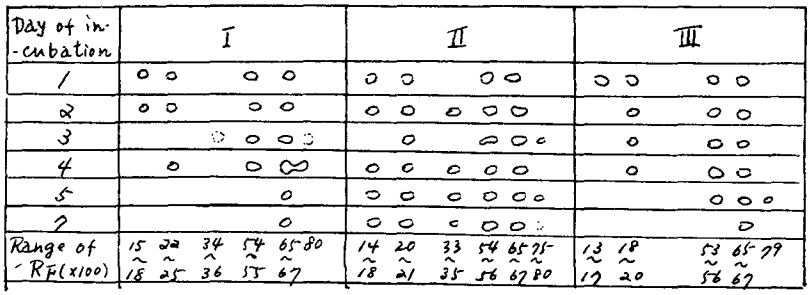
2, No. 3 and No. 4 starting from the lowest band, but the separation between No. 1 and No. 2 was not so distinct between each other. Similar treatment as in the case of isoleucine was applied to each effluent. From No. 4, pale yellow plate, m.p., 217–8°, was obtained and was identified to be 2, 4-dinitrophenylhydrazone of α-ketoglutaric acid by mixed melting point determination and co-chromatography (RF=0.08). Yield, ca. 0.4 mg. Yellow crystal from No. 3 was identified to be 2, 4-dinitrophenylhydrazone of pyruvic acid by the same methods. Yield, ca. 1.5 mg. The mixture of No. 1 and No. 2 gave needle-like crystal, which did not show sharp melting point but softened at about 120–30° and partially liquefied. The application of paper chromatographic technique indicated that this crystalline hydrazone consisted of three components showing RF=0.44, 0.62 and 0.82 respectively. The yellow color of the two lower spots was very weak and also the separation was not clear each other. From the color-intensity and area of each spot it was assumed that a greater part of this hydrazone mixture was occupied by the 2, 4-dinitrophenylhydrazone of RF=0.82 which was recognized to be identical with that of isoleucine keto analogue by parallel paper chromatography. The other two components are not known at present, but the hydrazone of RF=0.44 may be that of α-ketobutyric acid (62), the formation of which would be possibly deduced from several experiments conducted so far by the author and the hydrazone of RF=0.62 might be that of valine keto analogue (58). In addition, 2, 4-dinitrophenylhydrazone of six carbon keto acid was chromatographically separated from the 2, 4-dinitrophenylhydrazones which was prepared directly by the addition of the reagent to the original filtrate and thus about 1 mg of 2, 4-dinitrophenylhydrazone (m.p., 166°) of isoleucine keto analogue was obtained from 40 ml. of four-day old culture medium supplemented with formate, and small quantity of this hydrazone (m.p., 165–6°), from 40 ml. of four-day old filtrate added with arsenite and minimal amount in general, from the filtrate on fifth day added with aminobutyric acid only. In most cases of such experiments, yellow bands of keto acids hydrazones other than the 2, 4-dinitrophenylhydrazone of isoleucine keto analogue were not observed on the column, except unknown faint orange hydrazone. This fact may be due to the presence of keto acids, especially pyruvic acid, in a certain combined form with phosphorus or arsenic. This problem is under investigation.

In general, the isolation of six carbon keto acid as its 2, 4-dinitrophenylhydrazone from the medium supplemented with aminobutyric

acid was far more difficult in procedure and thus more unsatisfactory in yield than from the medium containing isoleucine as supplement. This may suggest the metabolic complexity and variety involved in the conversion process of aminobutyric acid to α-keto analogue of isoleucine. In addition, some notice should be paid to the fact that the employment of the successive culture of *P. oryzae* on agar plate brought the gradual decrease of the yield of this keto acid hydrazone.

Paper chromatography of amino acids—Detection of amino acids in the culture medium was made by paper chromatography (ascending method; phenol-water solvent; room temperature). The condition of incubation was the same as described. Several experiments were conducted repeatedly and the similarity in the appearance and disappearance of ninhydrin-positive spots were observed in either case of three incubation conditions, that is, aminobutyric acid only, the same amino acid plus arsenite and the same amino acid plus formate, although small unconformity was unavoidable owing to the use of intact mycelial felt. An example of these experiments is diagrammatically illustrated in Fig. 12.

Fig. 12. Detection of Amino Acid in the Culture Filtrate by Paper Chromatography.



Experimental conditions, see text. I, indicates the case of aminobutyric acid only; II, the case of aminobutyric acid plus arsenite; III, the case of aminobutyric acid plus formate.

The culture filtrate was extracted with ether at pH 8.5 and successively at pH 4.0 for five hours respectively and the remaining aqueous solution was reduced to dryness in vacuo. The extract of the residue with 70 per cent ethanol was evaporated and dissolved in a small amount of water. Application of paper chromatography to this aqueous solution indicated all other spots on paper strip except the spot of RF=0.15–0.18. In parallel chromatography employing pure amino acids,

spot of RF=0.18–0.25 corresponded to glutamic acid, spot of RF=0.53–0.56 to alanine, spot of RF=0.65–0.67 to aminobutyric acid and spot of RF=0.75–0.80 to valine. The spot indicating the minimum RF is unknown at present.

Discussion

The nutritional significance of isoleucine towards microorganisms has been studied these few years and it has been considered chiefly on the basis of enzymic experiments that the formation of α-keto acid by way of deamination would proceed as one of its metabolic changes. However, the formation of α-keto analogue of isoleucine by intact microorganisms has been seldom observed. In the experiment using an *Escherichia* mutant, paper chromatographic evidence indicating the formation of α-keto analogue of isoleucine in the culture medium supplemented with DL-isoleucine was reported by UMBARGER et al. (63), but the identification of this keto acid was not presented. Recently, WAGNER and BERGQUIST (58) reported the formation of this keto acid (racemic) not from isoleucine but threonine, using an induced strain of *Neurospora*. In present experiment, the formation of racemic α-keto acid analogue from DL-isoleucine by *P. oryzae*, as was observed with other several amino acids (part 1, part 2, part 3), was recognized by the separation and identification of its 2, 4-dinitrophenylhydrazone. These data indicate that the present fungus has the ability to utilize isoleucine as carbon source as well as nitrogen source. It was supposed in previous papers (part 2, part 3) that this fungus was able to deaminate either form of amino acids used, D or L, in accordance with the quantity of ammonia produced. The present identification of racemic mixture of isoleucine keto analogue, especially in the original filtrate untreated in any way, seems to give additional evidence supporting such interpretation. In either incubation added with arsenite or without, the change of the amount of ammonia- and amino-nitrogen in the medium showed the similar tendency as in preceding papers, and in present incubation experiment without arsenite, additional evidence was obtained that some parts of isoleucine added were absorbed and utilized in the form of amino acid itself, that is, 36.3 per cent of isoleucine supplemented on second day of incubation and 66.2 per cent on fifth day, for instance. It is noteworthy that only in the presence of arsenite, pyruvic acid was detectable almost always and also α-ketoglutaric acid rarely. These results can be interpreted to mean that either keto

acids are formed from isoleucine or isoleucine indirectly stimulates the endogenous production of keto acids. If the former relationship holds, this fact will present a new problem in the study of the metabolic fate of isoleucine. In addition, the application of paper chromatography to the culture filtrate containing arsenite (two-day and four-day old) showed spots corresponding to alanine, but rarely to glutamic acid, besides isoleucine. The experimental results that pyruvic acid derivable from alanine was detected only in the presence of arsenite (part 3), (40) may suggest that pyruvic acid recognized in the present incubation is possibly derivable from alanine. COON and associates (64, 65) found the formation of 2-methylbutyrate and propionate as the intermediate in the metabolism of isoleucine and described the probable formation of pyruvate from propionate. The author's present results appear to be favorable for such hypothesis, at least concerning the formation of pyruvate. It is uncertain at present whether isoleucine might be directly convertible to these amino acids or not, but the independence of the amino acids upon autolysis will be presumed from the decrease of their concentration or their disappearance in later stage of incubation and no detection of any amino acid except these two amino acids just described.

When DL-α-aminobutyric acid was used as supplement, a keto acid other than the α-keto analogue of this amino acid was separated as its 2, 4-dinitrophenylhydrazone in either incubation condition. Its analytical values were correspondent to those of 2, 4-dinitrophenylhydrazone of six carbon keto acid, but present hydrazone was distinctly different from that of either α-ketoisocaproic acid or α-ketocaproic acid. At last, it was identified to be that of dl-α-keto-β-methylvaleric acid. This fact indicates the formation of branched six carbon compound of isoleucine carbon skeleton from four carbon amino acid. In addition to this, almost constant formation of pyruvic acid and rare formation of α-ketoglutaric acid were found in the presence of arsenite. On the basis of the nutritional experiments conducted with induced mutant strains of microorganisms such as *Neurospora* or *E. Coli* and isotopic competition experiments, four carbon amino acids such as homoserine, threonine and α-aminobutyric acid were presented as the intermediate in the biosynthetic pathway of isoleucine (45, 46, 53, 54, 55, 56). In pratical, ADELBERG et al. successfully isolated α, β-dihydroxy-β-methylvaleric acid from the culture medium of *Neurospora* strain fed with threonine (48, 49) or acetate (45, 57), besides α,β-dihydroxyisovaleric acid,

the presumable valine precursor and also recognized the effectiveness of aminobutyric acid in the accumulation of α, β-dihydroxy-β-methylvaleric acid. Recently, WAGNER and BERGQUIST (58) identified acetaldehyde, pyruvic acid, α-ketoisovaleric acid and α-keto-β-methylvaleric acid in the culture medium of *Neurospora* strain added with threonine. In spite of these several experiments, the direct separation of a compound of isoleucine carbon chain from α-aminobutyric acid has not been recognized, except the findings of the nutritional effectiveness of this amino acid. The present identification of α-keto analogue of isoleucine will be the direct evidence indicating that α-aminobutyric acid can be included in the biosynthetic route of isoleucine. At the same time, it is suggested by this fact that such biosynthetic route of isoleucine will be probably prevailing at least in microorganisms. In present experiment α-ketobutyric acid was not separated unexpectedly, but it would be rather possible to regard that the deamination of aminobutyric acid would proceed as was already observed with other several amino acids, although in the present case this keto acid was not detected distinctly. In some cases, indeed, a spot presumably corresponding to α-ketobutyric acid was observed in paper chromatography besides α-keto-β-methylvaleric acid. WAGNER et al. (58) described that six carbon compound formed by aldol condensation of acetaldehyde and α-ketobutyric acid which was derivable from threonine (66) was followed by a pinacol type of rearrangement to give dihydroxy precursor of isoleucine, which was then converted to α-keto acid precursor, i. e., α-keto-β-methylvaleric acid, as was already postulated by ADELBERG (47, 49) and STRASSMAN (52) and that pyruvic acid derivable from threonine was related to the formation of valine carbon chain via a similar reaction. However, ADELBERG (48, 49) reported the independent biosynthetic route of isoleucine upon that of valine, based on the use of C^{14}-labeled compound. In addition, WAGNER et al. postulated that pyruvic acid found in their experiment might be derived from serine formed from glycine (67, 68) resulting from the cleavage of threonine to acetaldehyde and glycine (69). In present experiment conducted in the presence of arsenite, pyruvic acid was nearly always identified and, by paper chromatography the appearance of alanine and presumable serine rarely was observed in the culture medium. These results make it possible to consider that pyruvic acid may be derivable from alanine as it was discussed in the case of isoleucine. The interconversion of alanine and serine (70) and the metabolic similarity of both amino acids in the

formation of isoleucine carbon chain (71) seem to be favorable for the derivation of pyruvic acid from alanine. In the discussion of the formation of pyruvic acid from threonine, WAGNER et al. considered the transfer of one carbon unit according to SAKAMI (67), as just described. *P. oryzae* was incubated in the medium supplemented with both aminobutyric acid and sodium formate and the possibility of utilization of formate was recognized by the smaller formation of ammonia and smaller consumption of amino acid than in the case added with aminobutyric acid only (Fig. 10, Fig. 11). Furthermore, it was found that this fungus could utilize 75.2 per cent of sodium formate on third day and 92.9 per cent on seventh day in the incubation experiment in which 75 mg of glycine and 100 mg of formate were used as the supplement in the absence of arsenite. Thus pyruvic acid found in the medium, at least certain parts of this acid, may be formed via such metabolic change, although either glycine or acetaldehyde was not detected in present experiment.

The spot of RF=0.75–0.80, which was detected in relatively later stage of incubation added with either arsenite or formate, was assumed to be correspondent to valine in all likelihood and in most cases it disappeared again thereafter. This may be interpreted to show the additional evidence of the role of aminobutyric acid as a valine percursor. However, the independence of biosynthetic route of valine and isoleucine by ADELBERG (48, 49) must be kept in mind. The examination of carboxylic acid, which has been found recently by the author in the residual ether extract after removal of keto acids, may somewhat be contributed to above interpretation.

The formation of α-ketoglutaric acid and also of glutamic acid is unexplainable at present. It may be only a side reaction deviating from the main pathway of aminobutyric acid. If the following hypothesis is accepted that aminobutyric acid (perhaps threonine also) would act not only as a donor but as an acceptor of one carbon fragment, as it is known with glycine, two moles of aminobutyric acid would yield alanine (pyruvic acid) and valine, and additional incorporation of one mole of aminobutyric acid in the first reaction would lead to alanine and isoleucine, although timely sequence of deamination and transfer of one carbon fragment is out of range of consideration. The contamination of one carbon fragment in the first reaction might result in the formation of glutamic acid and thus α-ketoglutaric acid.

The change of amino nitrogen in the medium added with arsenite

and aminobutyric acid was indeed distinct from those of other experiments: re-increase of amino-nitrogen was determined on three-day old culture and that thereafter. The same incubations were repeated thrice and the same results were obtained (Fig. 10, Fig. 11). The presumable independence of these results on a simple autolysis will be shown by the transparency of the medium and constancy of paper chromatographic spots. The production of endogenous amino acids or the re—amination of keto acids may be possibly taken into consideration, but at present any decisive data are not available.

At any rate, the existence of a close metabolic relationship between aminobutyric acid and isoleucine is suggested by the isolation of the same keto acids from either amino acid used as the supplement.

Short Summary

1. *P. oryzae* is able to utilize DL-isoleucine as carbon source through its conversion to α-keto analogue, i.e., α-keto-β-methylvaleric acid. This keto acid was identified as its 2, 4-dinitrophenylhydrazone (racemic mixture).

2. In the incubation of *P. oryzae* in the medium supplemented with DL-α-aminobutyric acid, the identification of α-ketobutyric acid was unsuccessful, but the formation of α-keto-β-methylvaleric acid was recognized by the separation of its 2, 4-dinitrophenylhydrazone (racemic). This fact presents the direct evidence supporting the nutritional significance of aminobutyric acid as an intermediate in the biosynthetic pathway of isoleucine, in addition to the suggestive prevalency of such biosynthetic route, at least, in microorganisms.

3. The close metabolic relationship between isoleucine and α-aminobutyric acid is suggested by the separation and identification of pyruvic acid, α-ketoglutaric acid and α-keto-β-methylvaleric acid as 2, 4-dinitrophenylhydrazone in either incubation added with isoleucine or α-aminobutyric acid in the presence of arsenite.

4. The availability of formate to this fungus was indicated.

5. The possible mechanism of the formation of these three keto acids was discussed, taking into account the paper chromatographic evidence obtained with the culture medium.

Part 5. Behavior towards L-Phenylalanine.

In preceding parts of this series, amino acids of open chain structure

were used as materials to be tested for the conversion to α-keto acid. Now it is desirable to study the behavior of *P. oryzae* towards amino acid of ring structure and thus L-phenylalanine has been employed as material in present investigation.

Two thirteen-day old mycelium were incubated at 27–8° in the flask containing 40 ml. of M/15 phosphate buffer (pH 6.9) supplemented with 100 mg of L-phenylalanine. In inhibition experiment, 5 ml. of M/20 potassium arsenite was substituted for the same volume of buffer solution. Good further growth was observed in the absence of arsenite: white aerial mycelium began to be observed on the third day of incubation and covered over the liquid surface on the eighth day. On the other hand, it was not the case with inhibition experiment. The isolation of corresponding α-keto acid, i.e., phenylpyruvic acid, was carried out in the same way as in part 2, with about 80 ml. of three-day old culture medium added with arsenite, which was strongly positive for ferric chloride (19, 20) (basic ether extraction, six hours; acidic ether extraction, ten hours). The removal of ether from the final extract resulted in a mixture of oily substance and white crystal. When present mixture was treated with ligroin, oily substance settled down on the bottom of beaker and crystal was suspending in present solvent. Crystal collected by this procedure, though its yield was naturally unsatisfactory, was dissolved in ether and decolorized with charcoal, followed by careful addition of ligroin till slight turbidity was observed and kept overnight at 0°, but no crystal separated. Concentration of this solution in vacuo at room temperature for a short time led to prompt separation of leaflet. This crystal was slightly soluble in water, strongly positive for ferric chloride (dark green) and melted at about 124°, accompanying partial sublimation. Recrystallization from ether-petroleum ether gave a plate of m.p. 127°. The treatment of this crystal with 2, 4-dinitrophenylhydrazine in 2 N HCl resulted in rapid separation of yellow crystalline hydrazone, which, on being recrystallized with dilute ethanol (50%), lost its crystalline form at 144–6° and melted at 186–7°. In paper chromatography using butanol-ammonia solvent only one spot of RF=0.85–0.86 appeared on paper strip. The melting point of this crystal was lower by about 20° than that reported for phenylpyruvic acid by UEMURA (19), but it should be kept in mind that DIECKMANN (72) found 83–84° for this keto acid. The melting point of 2, 4-dinitrophenylhydrazone and its RF-value were almost identical with those described by KREBS (20) and SATAKE et al. (62). Judging from its other several

qualitative properties and, in addition, the behavior of present fungus towards the other amino acids, it is most probable that this crystal is that of phenylpyruvic acid. The application of the same procedure to 240 ml. of four-day old culture filtrate without arsenite gave the same crystal (m.p. 126–7°) as that just mentioned. The aqueous solution of this crystal was treated with sodium bisulfite at 70° for fifteen minutes, cooled and extracted with ether. This ether extract showed negative color reaction with ferric chloride. The residual solution was decomposed with 2–3 ml. of conc. sulfuric acid and successively extracted with ether. From present extract pretty plate, m.p. 126–7°, was separated and it was strongly positive for ferric chloride. On one hand, phenyllactic acid is reported to show similar melting point, 124–6°, (73, 74). It would be not necessarily unreasonable to expect such oxy-acid from phenylalanine, as was already seen with α, β-dihydroxy-acid from threonine (48, 49). However, preceding experimental data indicate that the crystal of m.p. 126–7° obtained in either incubation added with arsenite or without, is not oxy-acid but keto acid, i.e., phenylpyruvic acid in all likelihood.

The change of ammonia- and amino-nitrogen in the presence or absence of carbon source (sucrose) was measured in the same manner as in preceding parts. Ninety ml. of culture solution (Tab. 9) contained 150 mg of L-phenylalanine and 900 mg of sucrose or the same amount of L-phenylalanine only, respectively. The results obtained are presented in Table 8.

TABLE 8. Change of Ammonia- and Amino-Nitrogen in the Medium containing L-Phenylalanine.

Day		2		4		6	
		mg	%	mg	%	mg	%
No. 1	NH_3-N	2.628	20.7	6.624	52.5	13.978	109.8
	NH_2-N	10.391	81.7	7.272	57.1	4.257	33.4
No. 2	NH_3-N	1.818	14.3	2,947	23.1	2.360	18.5
	NH_2-N	11.151	87.6	9.560	75.1	8.514	66.9

No. 1 contained only 150 mg of L-phenylalanine and No. 2 the same amount of amino acid plus 900 mg of sucrose. Figures express mg of nitrogen in 90 ml. of medium and its percentage to theoretical value.

The increase of ammonia and the decrease of amino acid in either

case showed the same tendency as in preceding experiments conducted with several amino acids: smaller increase of ammonia and smaller decrease of amino acid in the presence of sucrose than in the absence of this sugar. On sixth day of incubation added with phenylalanine only, about 33% of amino acid supplemented remained still in the medium. This utilization rate of phenylalanine was the least of several amino acids examined by the author, and in addition, the formation of ammonia exceeding the theoretical amount (12.727 mg N) was also measured. These data seem to suggest that L-phenylalanine may be less available for present fungus than the amino acids of open chain structure. This less availability of phenylalanine may be perhaps due to the minor ability of present fungus to splitt benzene ring.

As was described above, the formation of phenylpyruvic acid can be detected by green color of the culture solution caused by ferric chloride. In some cases, however, ROTHERA'S color reaction (75) was strongly positive (permanganate color), notwithstanding nearly negative ferric chloride color reaction. This datum may suggest the formation of ketone body from phenylalanine. If this is the case, it would be possible to regard that the well-established metabolic pathway of phenylalanine in which p-hydroxyphenylpyruvate and homogentisate are involved as intermediate (76) should exist in present fungus. Further investigation may give some evidences supporting this presumption.

Chapt. II. Enzymes participating in the intermediary Metabolism of Amino Acid

The formation of α-keto acids from several amino acids was described in Chapt. I. There are two general mechanisms for such deamination process. One of these is oxidative deamination catalyzed by amino acid oxidase and the other, transamination catalyzed by transaminase. The latter metabolic process seems to be of great significance in L-specific reamination of α-keto acid derivable from D-amino acid.

The detection of the presence of these two enzymes will be much desirable from the standpoint of the further investingation of the biochemical behavior of present fungus towards amino acids. Thus Chapt. II deals with the detection of two enzymes just mentioned in cell-free state and their some properties.

Part 1. Presence of L-Amino Acid Oxidase

It was shown in Chapt. I of this series that *Piricularia oryzae* is able to utilize L-amino acids as carbon cource in the absence of proper carbon source and that the initial step of this utilization is the conversion of amino acid to corresponding keto analogue. Such interpretation was clearly ascertained by the identification of α-ketoisocaproic acid derivable from leucine, dimethylpyruvic acid from valine, phenylpyruvic acid from phenylalanine, pyruvic acid from alanine, α-ketocaproic acid from norleucine and α-keto-β-methylvaleric acid from isoleucine. In relation to these results, the formation of α-ketoglutaric acid from glutamic acid in the medium deficient in thiamine or containing arsenite as inhibitor was also shown by TANAKA et al. (21). These findings suggest the presence of an enzyme catalyzing such metabolic process of present fungus, that is, amino acid oxidase. The confirmation of its presence will present one of the decisive evidences explaining the utilization process of amino acids by *P. oryzae*.

Since NEUBAUER (77), KNOOP (78) and KREBS (20, 79) recognized the deamination of amino acid to corresponding α-keto acids, so many investigations have been undertaken and now there is little doubt of the existence of such enzyme system, in which L-and D-amino acid oxidase are included. Concerning this oxidase of microorganism, especially of molds, relatively active investigations of D-oxidase were conducted with *Neurospora crassa*, *Asp. niger* and *Penicillium* genus by HOROWITZ (80), and with *Penicillium Chrysogenum* Q 176 by EMERSON et al. (81), owing to relatively smaller sensibility of this enzyme, but the greater sensibility of L-oxidase resulted in later examination of this enzyme in comparison with D-oxidase. These few years, several reports dealing with the existence, the formation and the properties of L-oxidase of molds, in which *Neurospora crassa* (82, 83), *Penicillium* strain and *Asp. niger* (84) are included, have come to be presented. The attempt to find out the existence of this L-oxidase in *P. oryzae* seems to be useful for the investigation of the distribution of this enzyme in mold field as well as for that of the physiological behavior of present fungus.

Experimental results and discussion

TANAKA et al. (85) reported the growth-promoting effect of inorganic microelements for this fungus and thus the complete synthetic medium containing microelements such as iron, manganese and zinc was used

in present experiment (Tab. 9). *P. oryzae* was grown at 26–7° in the first culture medium of Tab. 9 for fourteen to seventeen days. Three mycelial felts were transferred into the flask containing the second culture medium, in the same manner as was described in part 1 of Chapt. I and further incubation was made at the same temperature. At appropriate intervals, the consumption of oxygen by fresh mycelium, the filtrate and enzyme preparation obtained by various treatments was examined manometrically in the atmosphere of oxygen or in the air.

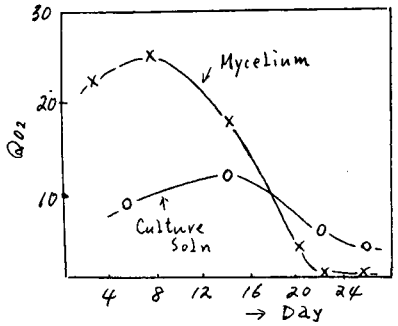
TABLE 9. Culture Solution used in Experiments.

	First Culture (mg)	Second Culture (mg)		First Culture (mg)	Second Culture (mg)
KNO_3	60.0	180.0	$ZnSO_4 \cdot 7H_2O$	0.4	1.2
KH_2PO_4	30.0	90.0	Biotin	1 γ	3 γ
$MgSO_4 \cdot 7H_2O$	15.0	45.0	Thiamine	10 γ	30 γ
$CaCl_2 \cdot 2H_2O$	3.0	9.0	Sucrose	900.0	0
$FeSO_4 \cdot 7H_2O$	0.2	0.6	L-Amino Acid	0	100.0
$MnSO_4 \cdot 4H_2O$	0.05	0.15	Water	30 cc	90 cc

1. On the basis of the capability of present fungus to maintain its growth by utilization of L-leucine in the absence of carbon source, as was described in part 1 of Chapt. I, enzymic conversion of L-leucine was first examined. Fresh mycelium grown in the second culture medium containing 100 mg of L-leucine in place of sucrose and the filtrate were used as material to be tested for the amino acid oxidase activity. The main chamber of Warburg apparatus contained 50–60 mg of fresh mycelium which was thoroughly washed with water and pressed beforehand, or 2 ml. of concentrated filtrate (below 35° in vacuo) and 2 ml. of M/15 phosphate buffer (pH 6.9); the side arm, 0.5 ml. of M/30 L-leucine solution; the center well, 0.2 ml. of 10% sodium hydroxide solution. The reaction temperature was 30° and the gas phase, oxygen. Qo_2 indicates μl of oxygen consumed by 100 mg of mycelium or by 100 ml. of original filtrate per hour. In control experiment, 0.5 ml. of water was substituted for 0.5 ml. of substrate solution. The results are presented in Tab. 10 and Fig. 13.

Rapid increase of enzymic activity of mycelium was observed in the earlier stage of incubation, being followed thereafter by relatively faster decrease of activity. The maximal activity was estimated with

Fig. 13. Oxygen Uptake by Mycelium or Culture Soln. of *P. oryzae* grown in the Medium contg. L-Leucine as Carbon Source.



Estimation was performed manometrically. Main chamder: 50-60 mg of fresh mycelium washed or 2 ml. of concentrated (below 35° in vacuo) culture soln.; side arm: M/30 L-Leucine, 0.5 ml.; center well: 10% NaOH, 0.2 ml.; reaction temperature, 30°; gas phase, oxygen. $Q_{2_2}=O_2\mu\ell/100$ mg (mycelium) or 100 ml. (culture solution, original)/hr. Control values were subtracted.

TABLE 10. Oxygen Uptake by Mycelium and Filtrate

Day	Mycelium	Filtrate
	Q_{O_2}	Q_{O_2}
3	22.1	—
5	—	7.8
7	23.9	—
14	17.6	11.3
20	2.9	—
22	trace	5.2
26	—	3.4

Expressions and experimental conditions are the same as in Fig. 13.

the mycelium grown for seven days in the second culture medium. These observations seem to be in good accordance with those of THAYER and HOROWITZ (82) and BURTON (83) that strong activity of endogenous enzymic activity of *Neurospora crassa* was found only in the mycelium obtained after a shorter period of cultivation. On the other hand, the apparent activity of L-oxidase secreted in the medium was far smaller, compared to the case of mycelium and in addition much slower change of activity was observed. The maximal activity in this case was estimated in far later stage of incubation than in the case of mycelium, that is, in fourteen-day old culture medium, thereafter being followed by slower decrease of activity. This finding seems to be considerably favorable for HOROWITZ's results (82) that it was necessary to continue the cultivation at least for two to three weeks in order to obtain the crude preparation of L-amino acid oxidase from the culture medium of *Neurospora*.

2. Extraction of enzyme and its properties.

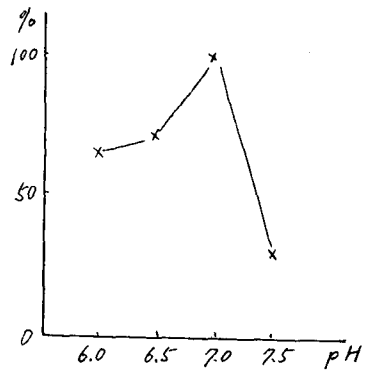
Since the presence of L-oxidase both in mycelium and culture medium was clearly recognized from the experiment just described, the possibility of the extraction and isolation of this enzyme was examined secondly. Starting from two week-old culture filtrate containing amino acid and no sucrose, three samples were obtained: the

precipitate separated at the saturation degree of 0.8 by the addition of solid ammonium sulfate, the supernatant resulting from the preceding procedure and its dialyzate after twenty four hours' dialysis against running water in cellophane bag. Consumption of oxygen by each preparation for L-leucine was examined manometrically in the same way as in experiment 1, but uptake of oxygen was not recognized in any case. This finding, which is quite different from that obtained with *Neurospora* (82), seems to be due to the original lower activity of present amino acid oxidase and its higher sensibility. Accordingly, mycelium grown for five to seven days in the second medium containing 50 mg of L-leucine per flask was employed as material to be examined for the possibility of extraction of this enzyme and for its some properties.

A) Relation to pH.

Fresh mycelium was used as enzyme material. Buffer solution was M/15 phosphate buffer. Oxygen uptake for L-leucine was measured in the same manner as in preceding experiment. The results obtained were expressed as per cent to the value of pH 7.0 (Fig. 14). The decrease of activity was more rapid in alkaline side than in acidic side.

Fig. 14. Influence of pH to Oxygen Uptake by L-Amino Acid Oxidase.



Enzyme material was the fresh mycelium after 5–6 days' cultivation in the medium contg. L-leucine and no sucrose. Other experimental details were the same with those in Fig. 13.

B) Extraction with phosphate buffer.

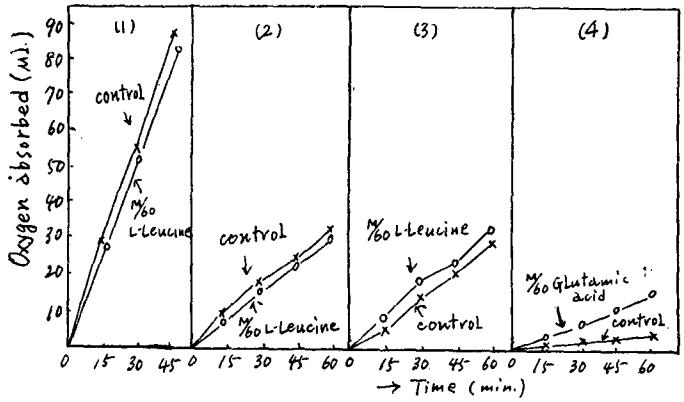
In preceding experiment mentioned above, fresh mycelium was employed as enzyme material, but it is more desirable to find out the enzymic activity in cell-free condition. The possibility of extraction of enzyme from mycelium was thus examined.

Mycelium which was incubated for seven days in the second culture medium containing L-leucine and no sucrose was filtered, washed thoroughly with water and pressed. After the removal of adhering water, mycelium was ground in a mortar with 4 ml. of M/15 phosphate buffer (pH 6.9) per gram of mycelium and extracted for one hour. Manometrical technique was applied to this extract at 30° in the range of pH 5.0 to 8.0 in the air, employing L-leucine or L-glutamic acid as substrate, but no consumption of oxygen was observed at any pH. Time

of extraction was thus prolonged. Mycelium was extracted for twenty four hours at 27° at pH 6.9 in the presence of toluol and centrifuged. The residual material was re-extracted for further twenty four hours with the same buffer solution (1 ml. per gram of residual mycelium). This procedure was repeated further twice. Oxygen uptake of 1 ml. of each extract for L-leucine or L-glutamic acid is presented in Fig. 15. On one hand, each extract was dialyzed in cellophane bag against running water for twenty four hours and the enzymic activity of each resulting dialyzate was determined at pH 6.9 as was just described (Fig. 16).

In spite of the apparent absence of present enzyme activity in either extract obtained after twenty four hours' extraction or forty eight hours', distinct consumption of oxygen for L-leucine or L-glutamic acid by each preparation after seventy two hours' extraction or ninety six hours' was recognized from the results presented in Fig. 15. In addition, it seems that the gradual decrease of oxygen uptake accompanying the time course of extraction is due to the successive removal

Fig. 15. Oxygen Uptake by M/15 Phosphate Buffer Extracts of Mycelium grown in Medium contg. L-Leucine.

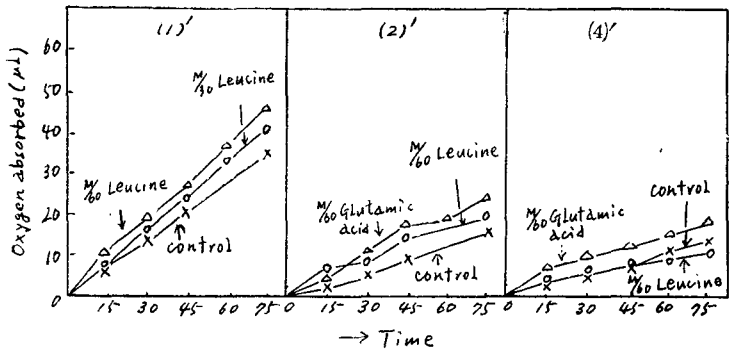


(1), Extraction for 24 hrs; (2), Extraction for 48 hrs; (3), Extraction for 72 hrs; (4), Extraction for 96 hrs.. Enzyme Soln., 1.0 ml. of each extract. Other experimental details were the same with those in Fig. 13.

of various substances consuming oxygen from mycelium. Such interpretation seems to be additionally supported by the results of Fig. 16 in which dialyzed preparation of each extract was used as enzyme material. Thus, the possibility of extraction of present enzyme in

cell-free state was found out, though the time for extraction was comparatively longer. But the enzymic activity measured in present experiments was not satisfactory: in No. 4′ of Fig. 16, the consumption

Fig. 16. Oxygen Uptake by dialyzed Buffer Extract of Mycelium.



Time of dialysis was 24 hrs. Other details were the same as in Fig. 15.

of oxygen for L-glutamic acid during sixty minutes was only correspondent to 11.8 per cent of theoretical value and to 16.1 per cent even after seventy five minutes.

In the experiment employing dialyzed preparation of each extract of Fig. 15, remarkable decrease of oxygen uptake was observed (Fig. 16), compared to the case of nondialyzed extract. Moreover, the distinct consumption of oxygen both for L-leucine and L-glutamic acid was observed in the experiment using each dialyzed extract corresponding to either extract after twenty four hours' extraction or forty eight hours', which did not show the consumption of oxygen for substrate as was known from Fig. 15. On one hand, dialyzed extract after seventy two hours' or longer extraction was found to lost their oxidizing power for L-leucine, though their activity for L-glutamic acid remained still as before. These facts seem to indicate that some parts of endogenous L-amino acid oxidase of *P. oryzae* was able to be extracted during twenty four hours or forty eight hours, but in the practical determination of enzymic activity it was masked by the greater relative amount of various oxygen-consuming substances in each extract and the successive removal of these contaminative substances by repeated extraction (Fig. 15, No. 3) or by dialysis (Fig. 16) brought about the recognizable consumption of oxygen for substrate. In the procedure of present dialysis, average increase of the volume of the content in

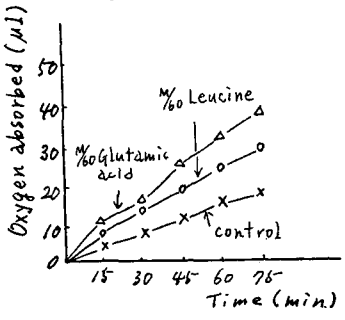
cellophane bag by 9.1% was observed. Taking into consideration this dilution of each extract, the availability of dialysis for detection of enzymic activity, was additionally known. As was indicated in No. 4' of Fig. 16, L-glutamic acid was able to be successively attacked, irrespective of disappearance of enzymic activity for L-leucine. It is uncertain whether this finding is due to the characteristic of present L-amino acid oxidase or to the presumptive existence of glutamic dehydrogenase. Concerning the concentration of amino acid as substrate, M/60 was found to be more effective than M/30, throughout these manometrical experiments.

3. Adaptive formation of present enzyme.

It has been generally known that the addition of a certain substance to a culture medium, which is able to be attcked by a specific enzyme as substrate, resulted in new formation of an enzyme reponsible for this substrate or further increase of its activity. Concerning L-amino acid oxidase of *Neurospora*, THAYER and HOROWITZ (82) reported the adaptive increase of activity of this enzyme for the addition of casein hydrolyzate. It is possible to say that the author's preceding experiments are based upon the adaptive increase of L-oxidase responsible for L-leucine as supplement. However, amino acid in this case was used in a sense of carbon source and in addition, the number of amino acid was only one. On one hand, the availability of some amino acids as nitrogen source for *P. oryzae* was studied by OTANI (16). From these facts, it is desirable to examine whether present fungus possesses the ability to introduce the adaptive increase of enzymic activity in the presence of amino acid mixture (casein hydrolyzate) as nitrogen source.

Three mycelium grown in ordinary medium for fourteen days were incubated at 27–8° in the second culture medium (Tab. 9) containing 900 mg of sucrose and 10ml. of casein hydrolyzate (94 mg of amino-nitrogen) in place of inorganic nitrogen source. After three days, mycelium was collected and treated in the same way as in experiment 2-B (forty five hours for extraction). Oxygen uptake of this preparation for L-leucine or L-glutamic acid was presented in Fig. 17.

Fig. 17. Adaptive Formation of L-Oxidase.



Active consumption of oxygen for either substrate, especially for L-glutamic acid was observed from the initial stage of reaction and larger increase of enzymic activity than in the case of L-leucine only was clearly known in comparison of Fig. 17 with Fig. 16. Thus it seems that present fungus is able to display the increase of enzymic activity, corresponding to the addition of casein hydrolyzate as nitrogen source. However, the kinds of amino acid directly responsible for such increase of activity are not known from this experiment.

Short Summary

When *P. oryzae* was cultured in the medium containing amino acid (L-leucine) in place of sucrose, L-amino acid oxidase activity was recognized both in mycelium and culture solution. Qo_2 per 100 mg of fresh mycelium was far greater than that per 100 ml. of culture solution within 14 days' cultivation. During the initial stage of cultivation (4–6 days) endoenzyme activity was rapidly increased but after 14 days it was also rapidly decreased and not nearly detected after 20 days. In contrast to this, the change of exoenzyme activity was relatively slow. The optimal pH of the endoenzyme was about 7.0. It was found that time necessary for extraction of endoenzyme with M/15 phosphate buffer (pH 6.9) was 24–48 hours and the dialysis of this extract against water was very effective for detection of L-amino acid oxidase activity. In these cases, glutamic acid was found to be more available as substrate than L-leucine, which may suggest the nutritional significance of this acidic amino acid for present fungus. Adaptive formation of endogenous L-oxidase by present fungus was recognized when it was nourished with casein hydrolyzate as nitrogen source.

Part 2. Qualitative Evidence of Transamination

As one of the metabolic processes in which amino acid participates, enzymic transamination was first reported by BRAUNSTEIN and KRITZMANN in 1937 (86). In earlier stage of investigation, only three amino acids, that is, glutamate, aspartate and alanine, were thought to be involved in this reaction, but now subsequent investigations of CAMMARATA et al. (87), FELDMAN et al. (88) and MEISTER et al. (89) indicate that virtually all of the natural amino acids are reactive in enzymic transamination. The ability of *P. oryzae* to form α-keto analogues from several amino acids has been clearly established in this series. These results may

suggest that α-keto acids formed may act as member in transamination reaction as well as as so-called carbon source. In addition, present fungus was found to be able to utilize D-amino acid as well as L-amino acid, from the change of ammonia and amino-nitrogen in the culture medium. The stereoisomeric center of amino acid is eliminated by its conversion to α-keto acid, which may subsequently be converted to L-amino acid via transamination. In practical, MASON et al. (90) reported the cleavage of D-tryptophan into ammonia and indolpyruvic acid by rat kidney slice and subsequent probable conversion of this keto acid to L-tryptophan via transamination. Thus, the investigation of transaminase in present fungus will make appreciable contribution for the explanation of the additional metabolic significance of keto acids just described.

Experimental and Results

Twenty-day old mycelium in ordinary medium was separated by filtration, washed with water thoroughly and pressed with hand. Five g of this pressed mycelium was ground with sand in a mortar containing 20 ml. of M/15 phosphate buffer of pH 7.4, kept at 5° for 16 hours, and filtered with gauze. The milky filtrate was centrifuged for 30 minutes at 0°C (12,000 r.p.m.) and the supernatant (17 ml.) was added with 6.8 g of solid ammonium sulfate. Grayish precipitate was separated and then filtered. This precipitate was treated again with buffer solution and filtered. The filtrate was dialyzed against running water in cellophane bag for 20 hours and the resulting content in the bag was used as enzyme material.

Reaction mixture consisted of 2 ml. of this enzyme solution, 1 ml. of M/100 DL-alanine, 1 ml. of M/100 α-ketoglutarate, 0.5 ml. of pyridoxine (40 mg/100 ml.) and 3.5 ml. of M/15 phosphate buffer (pH 7.4). In control experiment, 2 ml. of water was employed in place of alanine and α-ketoglutarate. Reaction temperature was 38°. After 30 min., 60 min., 90 min, and 170 min. respectively, 1 ml. of reaction mixture was heated for a short time, filtered from coagulated protein and concentrated to a small volume, which was assayed for both amino acid and α-keto acid by paper chromatography (phenol-water solvent for amino acid; n-butanol ammonia solvent for 2, 4-dinitrophenylhydrazone of keto acid). In every determination, two ninhydrin-positive spots of RF=0.20–0.24 and 0.52–0.53 respectively, were observed on a paper strip and the lower spot just corresponded to glutamic acid and the upper spot, to alanine. In control experiment no spot was detected. This observation seems

to indicate the formation of glutamic acid from α-ketoglutarate in the presence of alanine as amino-donor. This interpretation was supported additionally by the paper chromatogram of keto acid: in every case, two yellow spots, which gave RF=0.04–0.06 and 0.21–0.24 respectively, appeared on a paper strip and the upper spot was proved to be correspondent to 2,4-dinitrophenylhydrazone of pyruvic acid. These data show the formation of pyruvic acid from alanine in the presence of α-ketoglutarate as amino-acceptor. Thus, the existence of transaminase, at least glutamate-alanine transaminase, was clearly recognized. With another enzyme preparation (12 ml.), which was obtained from 16 g of pressed mycelium according to the same procedure just described, similar determination was made. In this case, M/100 solution of L-leucine, L-aspartic acid, DL-methionine and DL-alanine was used as amino-donor respectively and it was found that leucine and methionine were inactive in transamination reaction except alanine and aspartic acid. In the presence of aspartic acid, clear spot of glutamic acid was detected chromatographically. This indicates the existence of glutamate-aspartate transaminase besides glutamate-alanine transaminase. It is noteworthy that in the incubation mixture containing alanine, pyruvic acid was not detected in any way in spite of the appearance of the clear spot of glutamic acid. This fact may suggest the existence of an enzyme or enzymes participating in further breakdown of pyruvic acid, for example, decarboxylation. LICHSTEIN et al. (91) already reported the probable breakdown of the reaction product, especially keto acid, by side reaction in microorganisms including *E. Coli*. Reflecting upon the behavior of pyruvic acid in the absence of arsenite, this problem seems to be interesting.

At any rate, the existence of at least two transaminases, that is, glutamate-alanine and glutamate-aspartate transaminase, in *P. oryzae* was clearly known, although other amino acids such as leucine and methionine were inactive in transamination reaction. This finding may suggest the posssible existence of further metabolic process relating to α-keto acid, for example, L-specific reamination by transamination leading to L-amino acid which is generally available for microorganisms.

Chapt. III Production of Amylase by *P. Oryzae* and its some Properties

It is evident that the biochemical behaviors of *P. oryzae* are funda-

mentally attributed to its enzyme systems. Up to the present, however, there have been not so many researches about enzymes of this fungus. Presence of several kinds of enzyme has been reported, such as proteinase (14, 92), dipeptidase (92), polypeptidase (92), xylanase (93), amylase (93, 94) L-amino acid oxidase (Chapt. II), and transaminase (Chapt. I). With amylase among these enzymes, YOSHII (95) studied the effects of cephalothecine to the secretion of amylase by this fungus and TANAKA et al. (94) reported the influence of inorganic nitrogen sources such as nitrate and ammonium salt to the activity of exo- and endo-amylase. In the study of the change of amylase activity during the cultivation of this fungus in which sucrose was used as carbon source, it was demonstrated that the activity was parallel to the weight of mycelium and the sudden decrease of activity in later stage seemed to be due to the decomposition of this polyase by proteinase (93). Moreover, from the comparative study of two carbon sources, glucose and xylose, in the successive preculture, TANAKA and TSUJI (93) regarded this amylase as the constitutive enzyme of this fungus. On the other hand, the metabolic adaptability of this fungus to nutritive circumstances and, in relation to this fact, the adaptive increase of L-amino acid oxidase activity were recognized by the author.

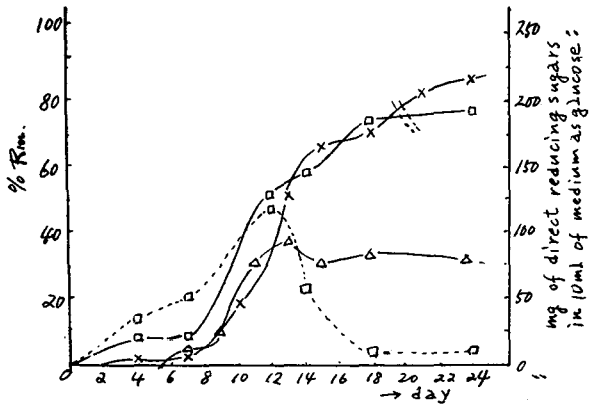
In the biochemical study of blast disease of rice plant, it is most important to know if this fungus possesses the ability to show the adaptive increase of amylase activity as in the case of L-amino acid oxidase. In present paper the change of amylase activity in the presence or absence of starch as substrate, the influences of amino acids to the production of this enzyme, some properties of partially purified enzyme preparation and moreover the crystallization of this amylase are recorded.

Experimental and Results

1. Amylase activity in the presence or absence of starch—*P. oryzae* was grown at 27–8°C in 30 ml. of medium containing 900 mg of sucrose, inorganic salts and growth factors (Chapt. I). At appropriate intervals, the contents of two culture flasks were filtered together and the clear filtrate was subjected to determination of exo-amylase activity and direct reducing sugar. In second experiment sucrose was replaced by 450 mg or 900 mg of soluble starch and the same determinations were made, including the observation of iodine color test. These results are presented in Fig. 18 and Tab. 11.

In the determination of amylase activity in the filtrate, especially

Fig. 18. Changes of Amylase Activity and direct reducing Sugars Amount in Culture Medium containing Starch and Sucrose.



Full line indicates amylase activity and dotted, direct reducing sugar. Reaction mixture consisted of 15 ml. of filtrate, 10 ml. of 1% soluble starch soln.,M/15 phosphate buffer soln., (pH 6.9) and 5 ml. of water; temperature 38–9°. After 120 mins' incubation, increase of reducing value as maltose was determined by Fehling-Lehrman-Schoorl or micro-Bertrand's method. In every determination, control value was subtracted.

TABLE 11. Changes of pH and Iodine Color Test of Culture Medium containing soluble Starch.

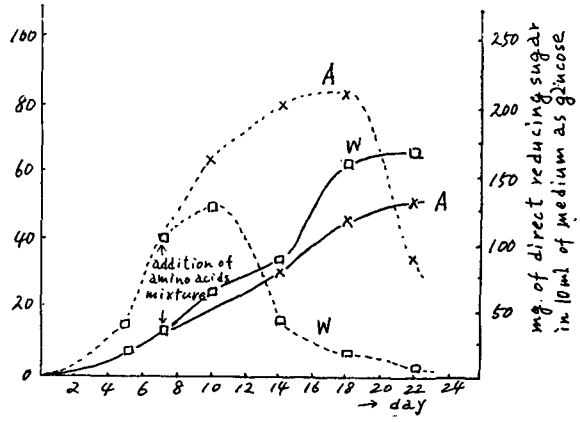
Day		4	7	10	12	13	14	15	18	21	24
Medium contg. 900mg of starch	pH	5.3	5.5		6.2		6.4		7.4		7.4
	Iodine Color	blue	blue		bluish purple		purple		slightly yellow		color-less
Medium contg. 450mg of starch	pH	4.7	6.0	6.8		6.9		7.1	7.3	7.4	7.4
	Iodine Color	blue	blue	purple		reddish purple		color-less			color-less

in that containing soluble starch as inductive substrate, some notice must be paid to the increase of reducing value observed during 120 minutes' incubation without the new addition of starch as substrate. This increase seems to be due to the attack of amylase present to the partially degraded starch remaining in the filtrate. In practical analysis in such a case, it is a most difficult to distinguish the increase of reducing value due to starch newly added from that derived from the

remaining degraded starch. This blank increase during the incubation time, however, reduced to nearly negligible value at least thereafter, when the iodine color test indicated purple-red. Besides this, it was found that amylase possessed much greater affinity towards native starch than degraded starch, when the two kinds of polysaccharide just described were incubated together (96). Accordingly the amylase activity detected in relatively earlier stage of cultivation, which was calculated from the difference between the value obtained in the presence of starch as substrate and that in the absence of starch is not so far from the true activity.

2. Effects of amino acids on amylase activity.—This fungus was grown in each of tweny culture flasks, containing as much the same amount of soluble starch and other salts as mentioned above. On the seventh day of cultivation 20 ml. of the aqueous solution of twenty amino acids was added into the each of ten flasks up to the total volume of 50 ml.. The concentration of single amino acid was M/1,000 and the total concentration of amino acids, M/50. The amino acid mixture consisted of DL-valine, L-leucine, DL-isoleucine DL-threonine, DL-phenylalanine, DL-methionine, L-tryptophan, L-arginine hydrochloride,

Fig. 19. Changes of Amylase Activity and direct reducing Sugar Amount in the Presence or Absence of the Mixture of twenty Amino Acids.



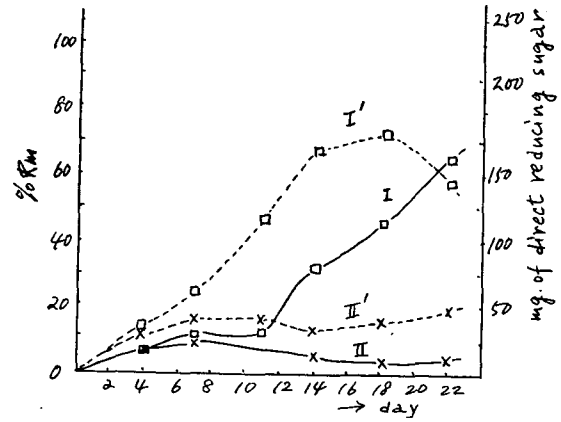
Full line indicates amylase activity as % of hydrolysis and dotted line shows direct reducing sugar. Capital A shows the experiment in which amino acids mixture was used and W, the experiment in which water was used in place of amino acids. Determinations of activity and reducing value were made by the same methods as in Fig. 18.

L-lysine hydrochloride, L-histidine hydrochloride, DL-alanine, DL-serine, L-aspartic acid, L-glutamic acid, L-hydroxyproline, L-tyrosine, L-cysteine hydrochloride, L-taurine, DL-α-amino-n-butyric acid and glycine. In place of amino acids mixture, 20 ml. of water was added into the other ten flasks. The results obtained are presented in Fig. 19 and Tab. 12.

TABLE 12. Changes of pH and Iodine Color Test in the Experiment represented in Fig. 10.

Day	5	7	Addition of Amino Acids	10	14	18	22
pH	5.3	5.8	W	6.1	6.4	7.2	7.6
			A	5.1	4.4	4.7	5.0
Iodine Color	blue	bluish purple	W	reddish purple	yellow	colorless	colorless
			A	brown	yellow	colorless	colorless

Fig. 20. Changes of Amylase Activity and direct reducing Sugar Amount of the Culture Solutions contg. "essential" and "non-essential" Amino Acids.



Determinations and expressions are the same as in Fig. 18. Full line expresses the amylase activity of medium contg. "essential" amino acids and II, that of medium contg. "non-essential"; dotted line I′ shows the direct reducing sugar amount in the medium contg. "essential" amino acids and II′, that of medium contg. "non-essential".

3. Influences of "essential" and "non-essential" amino acids.—These twenty amino acids are divided into two groups, that is, the ten "essential" amino acids and the other ten "non- essential" for the

nutrition of rat (97). The effect of each group to the adaptive production of amylase was examined respectively. In total volume of 30 ml., soluble starch (900 mg), other salts and "essential" or "non-essential" amino acids were contained. The concentration of each amino acid was M/600. At appropriate intervals, enzymic activity and weight of mycelium were measured as described above (Fig. 20 and Tab. 13).

TABLE 13. pH and Iodine Color Reaction of Medium contg. "essential" and "non-essential" Amino Acids and the Weight of Mycelium in each Medium.

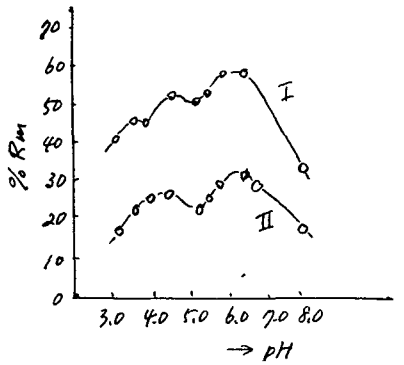
Day		4	7	11	14	18	22
pH	"essential" amino acids	4.7	4.7	4.4	4.6	5.0	5.2
	"non-essential" amino acids	3.4	3.3	3.3	—	3.8	4.4
Iodine Color	"essential"	blue	blue	reddish purple	yellow	colorless	colorless
	"non-essential"	blue	blue	blue	blue	blue	blue
Weight of Mycelium (mg)	"essential"	—	21.3	—	52.8	106.2	113.8
	"non-essential"	—	9.2	19.4	36.4	70.8	80.2

Mycelium was filtered from the medium, washed with water and dried at 100°; values express the average of two mycelium.

4. Partial purification of amylase and its some properties.—Partial purification of amylase was made almost according to NISHIDA (98). This blast disease fungus was cultured for twenty days in the medium containing soluble starch and other salts as mentioned above and to 250 ml. of clear filtrate whose % Rm was 76.0, N-acetic acid was added till the pH-value reached 6.4–6.5 and about 125 ml. of 3% tannin solution was added under vigorous stirring. This mixture was kept at 0°C for twenty hours. Cloudy precipitate was separated by centrifugation, washed with cold acetone in the centrifuge until the supernatant acetone became colorless, dissolved in 10 ml. of distilled water and filtered. 25 ml. of cold acetone was added to this filtrate under stirring and the mixture was kept at 0°C for half an hour. The precipitate separated was washed twice with cold acetone and kept in vacuo. Yield, 5.8 mg of grayish white powder. The enzyme preparation obtained by this

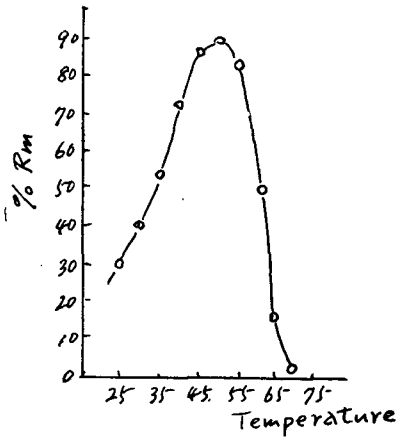
procedure was stable in this state for more than three weeks. This preparation was redissolved in 25 ml. of distilled water and the solution was applied to the determination of optimum temperature and pH. The results are shown in Fig. 21 and Fig. 22.

Fig. 21. Effect of pH on the Activity of partially purified Amylase.



Reaction mixture: 0.5/25 ml. enzyme soln. + buffer soln., 5 ml. + 1% soluble starch soln., 5 ml. + water, 4.5 ml.: temp., 38–9°. I shows % Rm after 60 mins' incubation and II, % Rm after 120 mins.

Fig. 22. Effect of Temperature upon partially purified Amylase Activity.



Experimental conditions are the same as in Fig. 21 except that pH was held at 5.9; time, 120 mins.

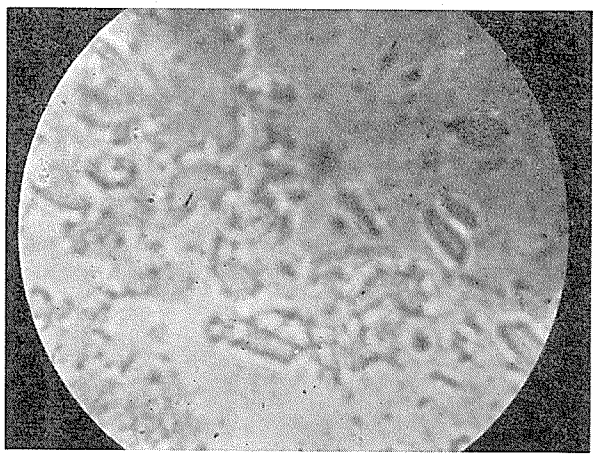
5. Determination of amylase-type.—In this experiment it was examined whether this amylase belonged to α-type or β-type. The dextrine formation power was measured at pH 5.9 according to WOHLGEMUTH (99) (expressed as X) and its modified method (100) (denoted as D). The value of X of the original filtrate whose % Rm was 76.0 after twenty days cultivation was 1.1–1.3 and $D_{120'}^{38°}$ was 1.4–1.7. Moreover, $D_{120'}^{40°}$ of the aqueous solution of partially purified amylase described in experiment 4 was 2.8. The stability of this partially purified amylase preparation towards acid- and heat-treatment was also examined. The amylase preparation used in this test was obtained by the same procedure with that presented in experiment 4 from 225 ml. of the filtrate whose % Rm was 77.3. The enzyme preparation was dissolved in 25 ml. of distilled water. To 2 ml. of this aqueous solution, 8 ml. of water was added and the mixture was kept at 70°C for just fifteen minutes. Immediately after that time, it was cooled with running water to room

temperature and subjected to determination of amylase activity at pH 5.9 and 38°C. During 120 minutes incubation, almost no increase of reducing value was recognized and accordingly the iodine color remained unchanged. In next experiment, 2 ml. of the cold aqueous solution just mentioned was brought to pH 3.4 with ice-cold N-acetic acid and kept at 0°C for fifteen minutes. Then 0.1 N solution of sodium hydroxide was added up to pH 6.4, the original pH of amylase solution. The activity of this amylase solution was measured at pH 5.9 and 38°C in total volume of 30 ml.. 73.5 per cent of the activity of untreated amylase solution was recovered in this test and iodine color remained blue. These data show that the exo-amylase produced by *P. oryzae* is mainly of β-type.

6. Crystallization of amylase.—The general principle of procedure was that according to FUKUMOTO (101). This fungus was grown for 21 days in the medium containing 900 mg of soluble starch per flask. 725 ml. of clear filtrate was concentrated in vacuo below 40°C to a small volume and filtered. This filtrate, 24 ml., was saturated with solid ammonium sulfate (saturation degree, 0.8), allowed to stand overnight and centrifuged. The precipitate collected was dissolved in 5 ml. of distilled water, filtered from insoluble materials, which were washed with water. The amount of filtrate and washing water was 18 ml.. This fractionation procedure was repeated further twice. About 20 ml. of enzyme solution obtained in the third procedure was dialyzed in cellophane bag against running water for seven hours and successively distilled water for sixteen hours. The content of the bag was concentrated to 10 ml. as described above. To this aqueous solution absolute ethanol was added drop by drop under vigorous stirring to the concentration of 60 per cent (v/v). Grayish turbidity was observed. After the mixture was allowed to stand at 0°C for one hour, the precipitate was collected by centrifugation. Furthermore, small amount of enzymatically active precipitate separated out from the supernatant when it was kept at 0°C overnight. The aqueous solution of first and second precipitate was subjected once more to alcohol precipitation procedure just mentioned. In this case, however, the concentration was raised up to 70 per cent. 13 ml. of the aqueous solution of the precipitate obtained was fractionated with ammonium sulfate at the saturation degree, 0.80 and the solution of precipitate obtained was again dialyzed against running water and distilled water for fifteen hours and thirty five hours respectively. 8 ml. of cold acetone was added dropwisely to

12 ml. of cold dialyzed solution, which was found to be strongly active towards soluble starch, under continuous stirring and the mixture was allowed to stand at 0°C. On the next day silky stream of very fine particle was observed on stirring and fine rod-shape-like particles were microscopically found out. This particle, however, lost its shape into liquid when it was allowed to lie on the slide glass for some minutes. After seven days' incubation at 0°C, these particles showed microscopically sharp parallelogram-like shape and the shape changed no longer on being allowed to stand for several hours (Fig. 23). This crystal produced qualitatively remarkable amount of reducing sugar from soluble starch at pH 5.9 and 40°C and yet the iodine color of the reaction mixture remained original blue color. But the yield was a most unsatisfactory and further investigation of this crystalline amylase preparation was not performed, to the author's great regret.

Fig. 23. Crystalline amylase (β-type) of *P. oryzae*.



Discussion

The study of the biochemical behaviors of *P. oryzae* towards the nutritive circumstances is a most important in order to protect rice plant from the blast disease. It was already observed by the author that, when this fungus was nourished with amino acid as carbon source, activity of L-amino acid oxidase was increased. This finding enables us to expect the biochemical adaptability of this fungus to starch, one of the most common constituents of host plant. It was reported that soluble starch was not so good carbon source for *P. oryzae* (102) and

also amylase of this fungus seemed to be constitutive enzyme (93). But the production of amylase by this fungus in the presence of soluble starch was over again examined by the author. The change of amylase activity in the presence of soluble starch as carbon source was compared with that in the presence of sucrose (Fig. 18) and it was found that the activity in soluble starch medium was much higher than that in sucrose medium. In latter medium, maximum % Rm 37.2 was attained on thirteenth day of cultivation, followed by almost constant activity thereafter (% Rm, 30–32). On the other hand, in the former medium containing soluble starch, % Rm 50.8 was measured on twelveth day and continuous increase of activity was found until the maximum % Rm 72.1 was attained on eighteenth day. Thereafter almost no change of activity was observed. These facts indicate that in the presence of soluble starch the production of amylase was raised nearly twice as much, compared to the production of amylase in sucrose medium. Moreover, good growth was observed on eleventh day. These findings represent the biochemical adaptability of this fungus: this blast fungus seems to possess the ability to maintain its growth obstinately, corresponding smoothly to the nutritive conditions of host plant.

The increase of the amount of the direct reducing sugar in the starch medium, calculated as glucose, was approximately correspondent to that of amylase activity as far as twelveth day. Thereafter, sudden decrease of reducing value was measured, in spite of the further steady increase of amylase activity. In later stage of incubation, when the nearly constant amount of reducing sugar, i.e., 8–10 mg per 10 ml. of medium, was measured, the change of amylase activity was no more observed. The same tendency was also observed during the incubation with sucrose: the amount of reducing sugar reached the maximum value, i.e., 242 mg per 10 ml. of medium on nineth day, thereafter followed by the steep decrease of reducing sugar, i.e., 5.8 mg on eighteenth day and the amylase activity showed continuous increase as far as thirteenth day, thereafter followed by nearly constant activity level, though it was much lower than in starch medium. These findings suggest the presence of a certain coupling between the production of amylase and the energy metabolism (103), not to speak of the adaptive formation to α-1.4-glucosidic linkage of starch (104, 105). Even when the value of % Rm showed 72–77 during 120 mins. incubation in the determination of activity, the iodine color of the reaction mixture was

purple~purplish red. This fact may indicate that a greater part of amylase secreted in the medium is of β-type. The difference in the amount of soluble starch added, i. e., 450 mg and 900 mg respectively, did not show so remarkable influences upon amylase activity, change of pH, iodine color and the amount of residual reducing sugar.

HOKIN reported the effectiveness of amino acids, especially "essential" amino acids, upon amylase synthesis and secretion by pigeon pancreas slices (106). The effect of twenty amino acids mixture was examined and it was found that this mixture had not direct effect stimulating the production of amylase and, on the contrary, the activity of amylase was slightly reduced in this experiment, compared with the case of no addition of amino acids. These results are in good accord with those obtained by NAKAMURA and SUGAWARA (104). It is noteworthy that the remarkable increase of residual reducing sugars was detected in this case and the time showing the maximum amount of residual sugars was far prolonged, in comparison with the case of no amino acids. This may be ascribed to the relative increase of residual sugars which is due to the rapid utilization by *P. oryzae* of α-keto acids formed from amino acids added (Chapt. I).

The addition of amino acid mixture disturbed the further development of pH of culture medium as observed in the case of no amino acids and the difference of the pH observed in two cases seems not to have direct influences upon the production of amylase, at least in the present experimental conditions (Fig. 19 and Table 12).

The amino acids mixture just mentioned involved two nutritional groups for the rat, i.e., "essential" amino acids and "non-essential". The effect of each group on the amylase production was examined (Fig. 20). In the case of "essential" amino acids added, the processes of the changes of amylase activity and residual sugars were almost the same as those in Fig. 19. On the other hand, in the case of "non-essential" acids, both the activity of amylase produced and the amount of residual sugars were found in much lower level, especially in the later stage of cultivation. It is evident from the results indicated in Fig. 19 that pH of medium are not so closely related to the amylase production. Accordingly, the much smaller amylase activity in this case may be due to the direct inhibitory effect of "non-essential" amino acids on the amylase production. If this is the case, some inhibition should be recognized in the preceding experiment in which the mixture of twenty amino acids was used. But the fact was found to be the

reverse. One possible explanation of this fact is that the inhibitory effect of "non-essential" amino acids may be covered by "essential" amino acids. Then it may be possible to think about the indirect stimulating effect of "essential" amino acids. At any rate, it was found that amino acids did not take positive part in the production of amylase and it might mainly depend upon the inorganic nitrogen source (103).

The optimum pH-range of partially purified amylase preparation was 5.9–6.5. In the more acidic pH-range this preparation was relatively stable and 76 per cent of the maximum activity was recovered even at pH 3.2. In the more basic range, however, more rapid inactivation was observed. It was already shown that the relatively broad optimum pH range was characteristic of the saccharogenic amylase of *Asp. Oryzae* (107). When this preparation showed % Rm of 60 and 89, the iodine color of reaction mixture remained purple and reddish purple respectively. Optimum temperature was 45–50°C and higher temperature resulted in sudden decrease of activity. During the incubation at 50°C for 120 minutes, % Rm reached 89.1 and yet the iodine color test gave reddish purple color. The very small dextrine formation power of this preparation was concluded from the results in experiment 5. When this preparation was treated at pH 3.4, 73.5 per cent of activity of untreated preparation was recovered, though its whole activity was lost by the heat-treatment at 70°C. These findings seem to indicate that this amylase preparation is of so-called β-type or saccharogenic amylase type. In addition, the supernatant separated by centrifugation from the first precipitate which was caused by addition of tannin was concentrated below 40°C and three times as much volume of cold acetone was added to this concentrated solution. The precipitate obtained was found to be nearly enzymatically inactive. Thus it is most probable that the main part of exo-amylase of *P. oryzae* is of β-type. The determination of true type of this amylase, for instance, β-type, saccharogenic amylase type and gluc-amylase type (108), will be attained by further research.

In order to obtain pure crystal of this amylase, purification procedure was repeated several times and only once parallelogram-like crystal was obtained. Although the very unsatisfactory yield prevented the author from making detailed research of this crystal, qualitative investigation indicated that this crystalline amylase was of so-called β-type.

Short Summary

The adaptive production of amylase by *P. oryzae* was recognized and the amylase activity in the presence of soluble starch was raised nearly twice as much, compared to that in the presence of sucrose as carbon source. The mixture of twenty amino acids which "essential" and "non-essential" amino acids were involved in did not show positive effect upon the production of amylase and it seemed that "non-essential" amino acids inhibited the production of amylase and this inhibitory effect was covered by "essential" amino acids.

Some properties of partially purified amylase preparation were examined and it was concluded that this preparation was of β-type.

Parallelogram-like crystalline amylase was obtained, though its yield was a most unsatisfactory. This crystal appeared to be qualitatively of β-type.

General Discussion

The utilization of amino acids by *P. oryzae* has been studied by several investigators these few years. In almost all of these investigations, however, amino acids were used in the sense of nitrogen source for present fungus and, in addition, their metabolic changes involved in their utilization have not been studied. In present experiment, the significance of several amino acids as carbon source was examined in the absence of proper carbon source and it was found that amino acids were utilized not only as nitrogen source but as carbon source via their prior conversion to respective α-keto acid. Such metabolic change of amino acids used was confirmed by the identification of each α-keto acid from each amino acid. It is noticeable that enzymic activity of L-amino acid oxidase catalyzing deamination process of amino acid ($R \cdot CHNH_2 \cdot COOH + 1/2O_2 \rightarrow R \cdot CO \cdot COOH + NH_3$) was adaptivelly increased by the addition of amino acids mixture. The same result was obtained with exo-amylase of *P. oryzae*, when this fungus was nourished with soluble starch as sole carbon source. These findings indicate the possibility of present fungus to be able to maintain its growth even under nutritionally subnormal environment. The existence of ergothioneine-like substance in *P. oryzae* was found out by the author et al. (109) recently. This compound was reported to be antagonistic against sulfhydryl inhibitor such as cupric ion by MANN et al, (110), which seems to give some suggestions in the investigation of biochemical effect of

copper-containing agricultural chemicals for *P. oryzae*. These facts are most undesirable from the standpoint of practical extirpation of blast disease, but at the same time they will be probably useful in further investigation of complete extirpation of blast disease. As was described above, the formation of α-keto acids from several amino acids was clearly recognized. Then, transamination reaction ($R \cdot CHNH_2 \cdot COOH + R' \cdot CO \cdot COOH \rightarrow R \cdot CO \cdot COOH + R' \cdot CHNH_2 \cdot COOH$) is naturally taken in consideration as one of the metabolic processes in which keto acids participate. Several experiments indicated the existence of two transaminases in *P. oryzae*, that is, glutamate-alanine and glutamate-aspartate transaminase. Present results may suggest the conversion of α-keto acid from D-amino acid to L-amino acid, that is, L-specific reamination, although other transaminases were not detected in present experiment, except the two trnasaminases just mentioned. Preferential utilization of glutamic acid to proper carbon source by *P. oryzae* was already reported by TANAKA (28). The same results were obtained with alanine by the author. It is well known that glutamic acid, alanine, and corresponding keto acids are most closely related to tricarboxylic acid cycle. Taking together into account the nutritional effectiveness of some intermediary organic acids on the cycle by TANAKA et al. (4) and OTANI (102), it seems that present fungus performs the breakdown of carbon source via tricarboxylic acid cycle.

The formation of α-keto acid of isoleucine carbon skeleton from α-aminobutyric acid is most remarkable in the utilization of amino acids by *P. oryzae*. The nutritional sparing effect of this four carbon amino acid for isoleucine was observed with some microorganisms such as *Neurospora* and *E. Coli* and thus these few years aminobutyric acid has been taken as an intermediate in the biosynthetic route of isoleucine. Present results show the direct evidence favorable for such biosynthetic pathway. In addition, the detection of both pyruvic acid and α-ketoglutaric acid in the medium supplemented with either aminobutyric acid or isoleucine suggests the close metabolic relationship between these two amino acids. The good utilization of formate and the formation of alanine from aminobutyric acid make the author speculate the presence of following metabolic process in *P. oryzae*: the metabolic manner of α-aminobutyric acid as both donor and acceptor of one carbon fragment may lead to the formation of six carbon compound of isoleucine carbon skeleton via five carbon compound such as valine or its precursor, as was described in Chapt. I of this series,

although the independent pathway of isoleucine and valine was reported by ADELBERG (48, 49).

At any rate, these findings seem to show the usefulness of present fungus for the study of amino acid metabolism.

Conclusions

1. When *Piricularia oryzae* was incubated in the culture medium containing amino acid as sole organic nutrient, some parts of amino acid were utilized in the form of amino acid itself, that is, probably as nitrogen source and the other parts were utilized through its prior conversion to α-keto analogue, that is, as carbon source. Even in the presence of both amino acid and proper carbon source, a certain part of amino acid was deaminated to its α-keto analogue, though the conversion rate was naturally less than in the presence of sole amino acid.

2. The conversion of several amino acids to respective α-keto acid was demonstrated by the separation and identification of each keto acid as its 2. 4-dinitrophenylhydrazone, that is to say, α-ketoisocaproic acid from leucine, α-ketocaproic acid from norleucine, dimethylpyruvic acid from valine, pyruvic acid from alanine, α-keto-β-methylvaleric acid from either α-aminobutyric acid or isoleucine, and phenylpyruvic acid from phenylalanine.

3. The formation of α-keto-β-methylvaleric acid from α-aminobutyric acid is noteworty from the standpoint of the biosynthesis of isoleucine. The finding presents the direct evidence supporting the metabolic significance of α-aminobutyric acid as an intermediate in the biosynthetic pathway of isoleucine.

4. The formation of both pyruvic acid and α-ketoglutaric acid from either α-aminobutyric acid or isoleucine was demonstrated, which seems to indicate the existence of close metabolic relationship between these two amino acids.

5. Sparing effect of formate for α-aminobutyric acid was recognized and thus present fungus was found to be able to utilize formate, in other words, one carbon fragment. In addition, the degradation of α-aminobutyric acid to alanine was chromatographically recognized. Taking into consideration additional detection of valine in all likelihood, following presumption would be introduced: two moles of α-aminobutyric acid would yield alanine (pyruvate) and valine and further incorporation of one mole of aminobutyric acid in the first reaction would lead to alanine and isoleucine, although timely sequence of deamination and

transfer of one carbon fragment was out of range of consideration. Contamination of one carbon fragment in first reaction might result in the formation of glutamic acid or α-ketoglutarate.

6. Preferential utilization of alanine to proper carbon source was clearly known. This fact presents a probable step towards the investigation of the biochemical mechanism of the invasion of this fungus and its further growth in host plant.

7. Adaptive increase of enzymic activity of endogenous L-amino acid oxidase and exogenous amylase of *P. oryzae* was established in the presence of either amino acids mixture or soluble starch. These facts indicate the ability of *P. oryzae* to maintain its growth obstinately, corresponding smoothly to its nutritional circumstance. The existence of ergothioneine-like substance, an antagonist against sulfhydryl inhibitor, presents additional evidence supporting such interpretation.

8. Exo-amylase of *P. oryzae* was proved to be mainly of β-type. The mixture of twenty amino acids in which "essential" amino acids and "non-essential" amino acids were involved did not show positive effect upon the production of amylase. The inhibitory effect of "non-essential" amino acids was covered by "essential" amino acids. Parallelogram-like crystalline amylase was obtained and it was found to be of β-type.

9. The existence of at least two transaminases, that is, glutamate-alanine and glutamate-aspartate transaminase, was recognized. This finding may suggest the possible existence of further metabolic process relating to α-keto acid, for example, L-specific reamination by transamination leading to L-amino acid, which is favorable for the explanation of utilization of D-amino acid by *P. oryzae*.

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