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METABOLISM OF PHENYLALANINE AND TYROSINE DURING LIGNIFICATION OF BAMBOOS

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Abstract—The content of phenylalanine, tyrosine, methionine and glutamic acid in different parts of a bamboo shoot were determined together with their changes during growth. The content of these amino acids, tyrosine in particular, decreased with growth of the bamboo. Phenylpyruvate and *p*-hydroxyphenylpyruvate when incubated with sliced bamboo tissues were converted to phenylalanine and tyrosine, respectively; at the same time *p*-coumaric acid was found to be synthesized from either. The ability of the sliced tissues from different parts of the shoot to metabolize phenylpyruvate and *p*-hydroxyphenylpyruvate was compared and showed that the tissue from the lower part of the shoot was the most active in accordance with faster lignification in the lower part.

INTRODUCTION

PHENYLALANINE has been known to be a natural intermediate of phenylpropane constituents of lignins in higher plants.[1] Tyrosine has also been shown to be incorporated into lignins of a few families of higher plants such as gramineae and the Compositae.[2,3] Subsequent studies on phenylalanine-[4] and tyrosine-[5] ammonia-lyases have suggested significant roles for these enzymes in the metabolism of phenolic compounds in higher plants.

In previous papers dealing with the lignification of the bamboo (*Phyllostachys pubescens*), the roles of several related enzymes such as peroxidase (EC 1.11.1.7), phenylalanine- and tyrosine ammonia-lyases (EC 4.3.1.5), shikimate:NADP oxidoreductase (EC 1.1.1.25), D-glucose-6-phosphate:NADP and 6-phospho-D-gluconate:NADP oxidoreductase (EC 1.1.1.49) (EC 1.1.1.44), 5-dehydroquinate hydro-lyase (EC 4.2.1.10) and *O*-methyltransferase (EC 2.1.1.6) in lignin metabolism have been reported.[6–11] Minamikawa and Uritani,[12] and Zucker[13] reported also on the changes in activities of phenylalanine ammonia-lyase in relation to biosynthesis of chlorogenic acid.

The present paper describes changes in the content of phenylalanine and tyrosine and in the activities of the transaminase catalysing the conversion of phenylpyruvate and *p*-hydroxyphenylpyruvate to these amino acids during the growth of a bamboo. The results focus on

[1] S. A. BROWN, *Science* **134**, 305 (1961).
[2] S. A. BROWN, *Can. J. Botany* **39**, 253 (1961).
[3] M. R. YOUNG, G. H. N. TOWERS and A. C. NEISH, *Can. J. Botany* **44**, 341 (1966).
[4] J. KOUKOL and E. E. CONN, *J. Biol. Chem.* **236**, 2692 (1961).
[5] A. C. NEISH, *Phytochem.* **1**, 1 (1961).
[6] T. HIGUCHI, I. KAWAMURA, N. YAMAZAKI and I. MORIMOTO, *J. Jap. Forest Soc.* **37**, 502 (1955).
[7] T. HIGUCHI, *Agric. Biol. Chem.* **30**, 667 (1966).
[8] T. HIGUCHI and M. SHIMADA, *Plant Cell Physiol.* **8**, 61 (1967).
[9] T. HIGUCHI and M. SHIMADA, *Plant Cell Physiol.* **8**, 71 (1967).
[10] T. HIGUCHI and M. SHIMADA, *Agric. Biol. Chem.* **31**, 1179 (1967).
[11] T. HIGUCHI, M. SHIMADA and H. OHASHI, *Agric. Biol. Chem.* **31**, 1179 (1967).
[12] T. MINAMIKAWA and I. URITANI, *Arch. Biochem. Biophys.* **108**, 573 (1964).
[13] M. ZUCKER, *Plant Physiol.* **40**, 779 (1965).

the variation of activities of the related enzymes and their roles in the metabolism of phenylalanine and tyrosine in relation to lignification in bamboo.

RESULTS

The free amino acids contained in a young bamboo were examined by paper chromatography and the following were detected: phenylalanine, tyrosine, alanine, leucine, methionine, valine, proline, lysine, serine, glutamic acid, tryptophan, aspartic acid etc. in good accordance with results already reported.[14]

Among these amino acids, phenylalanine, tyrosine, methionine and glutamic acid, which are probably all involved in the formation of lignin precursors, were examined further. Both phenylalanine and tyrosine are efficiently utilized as precursors for lignins in grasses; methionine is known to be a methyl donor for the methoxyl group of lignins,[15–17] glutamic

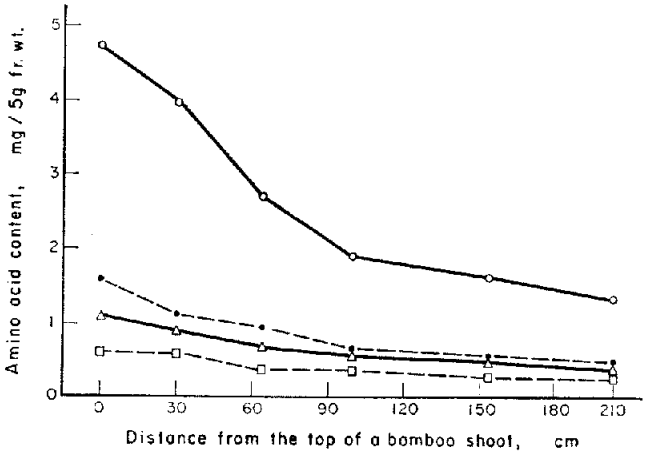

FIG. 1. CHANGES IN AMINO ACID CONTENT WITH GROWTH OF A BAMBOO.
—○—, tyrosine; - - ● - -, phenylalanine;
—△—, methionine; - - □ - -, glutamic acid

acid, which is a main amino donor in transamination reactions in plant tissues, was selected for measurement, although it may not be directly related to lignin formation.

Figure 1 shows the changes in contents of those four amino acids during the growth of a bamboo. Tyrosine was present in the highest amount at any stage of growth, and was highest in the tissue at the top of the bamboo shoot, decreasing rapidly toward lower parts of the shoot. The other acids were present in much smaller amounts and showed less drastic decreases during growth.

Attempts were made to isolate a transaminase from bamboo shoots, but reproducible results could not be obtained. Sliced tissue of the bamboo shoot was therefore used for the estimation of transaminase activity with phenylpyruvate and *p*-hydroxyphenylpyruvate.

Figure 2 shows the time course for the formation of phenylalanine and tyrosine from these two keto acids. The amounts of both phenylalanine and tyrosine reached a maximum

[14] K. KOSHIMIZU and T. MITSUI, *J. Agric. Chem. Soc. Japan* **30**, 63 (1956).
[15] R. U. BYERRUM, J. H. FLOKSTRA, L. J. DEWEY and C. D. BALL, *J. Biol. Chem.* **210**, 633 (1954).
[16] B. J. FINKLE and R. F. NELSON, *Biochim. Biophys. Acta* **78**, 747 (1963).
[17] B. J. FINKLE and M. S. MASRI, *Biochim. Biophys. Acta* **85**, 167 (1964).

after 90 min. Tyrosine was formed in about three times greater amounts than phenylalanine. Figure 3 shows the time course of the formation of *p*-coumaric acid produced via phenylalanine or tyrosine. The results indicate that the formation of *p*-coumaric acid proceeds more or less linearly up to 90 min similar to amino acid formation. On the basis of these results incubations were carried out for 90 min only in all subsequent experiments.

The residual substrates in the reaction mixture using tissue taken from different parts of the shoot, were recovered and estimated (Fig. 4). The results indicate that more phenylpyruvate and *p*-hydroxyphenylpyruvate was recovered from the reaction mixture when

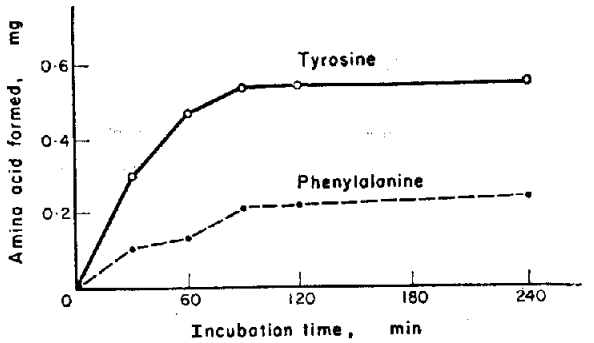

FIG. 2. TIME COURSE OF PHENYLALANINE AND TYROSINE FORMATION BY SLICED TISSUE.

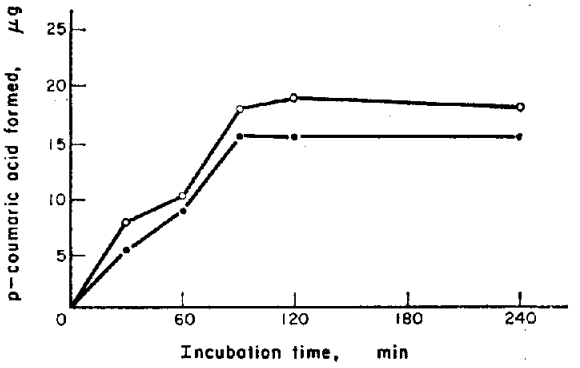

FIG. 3. TIME COURSE OF *p*-COUMARIC ACID FORMATION BY SLICED TISSUE.
—●— *p*-coumaric acid formed from phenylpyruvic acid via phenylalanine;
—○— *p*-coumaric acid formed from *p*-hydroxyphenylpyruvic acid via tyrosine.

tissue slices from the upper part of the plant were used, suggesting that the tissue at the top has a lower metabolic activity than the tissue from lower parts of the shoot.

The patterns of the formation of phenylalanine and tyrosine were determined in the same series of experiments (Fig. 5). The results obtained appear somewhat similar to those shown in Fig. 1. The fact that higher amounts of phenylalanine and tyrosine were obtained from the upper tissues indicates that these tissues cannot convert phenylalanine and tyrosine to other substances, such as cinnamic acids, as rapidly as can lower tissue. The changes in the formation of *p*-coumaric acid from phenylpyruvate and *p*-hydroxyphenylpyruvate (Fig. 6) seem to support this explanation. For *p*-coumaric acid, formed via the corresponding aromatic amino acids, was accumulated in higher amounts using tissue from the lower parts.

In addition, the amount of *p*-coumaric acid formed via phenylalanine was about twice that formed via tyrosine.

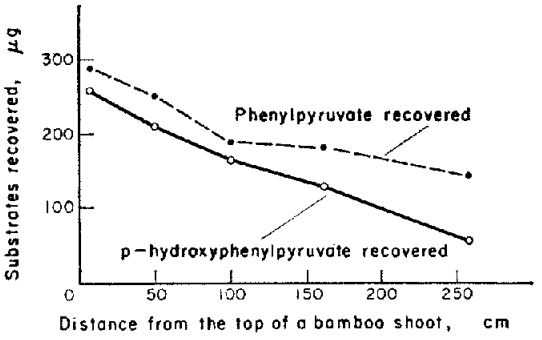

FIG. 4. CONSUMPTION PATTERN OF PHENYLPYRUVATE AND *p*-HYDROXYPHENYLPYRUVATE INCUBATED WITH SLICED TISSUE.

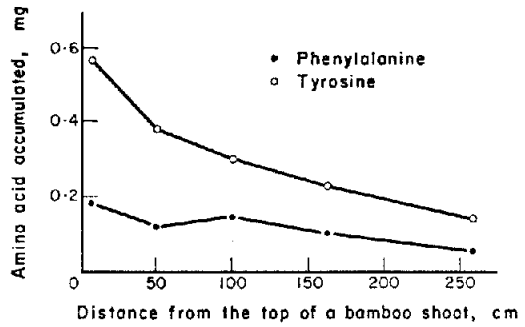

FIG. 5. ACCUMULATION OF PHENYLALANINE AND TYROSINE BY SLICED TISSUE.

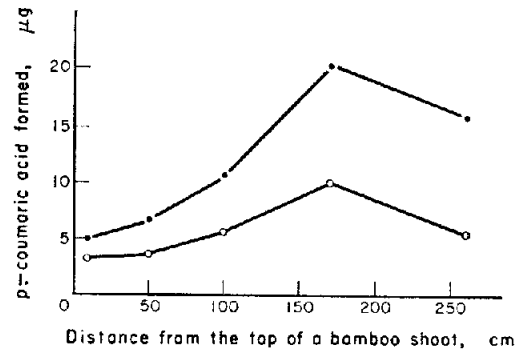

FIG. 6. FORMATION OF *p*-COUMARIC ACID BY SLICED TISSUE.
—●— *p*-coumaric acid formed from phenylpyruvic acid;
—○— *p*-coumaric acid formed from *p*-hydroxyphenylpyruvic acid added as substrate.

Changes in activities of the respiratory enzymes such as citrate (isocitrate) hydro-lyase (EC 4.2.1.3), L-malate hydro-lyase (EC 4.2.1.2), L-malate: NAD oxidoreductase (EC 1.1.1.37) and succinate:(acceptor) oxidoreductase (EC 1.3.99.1) are shown in Figs. 7 and 8, respectively.

Considerably high activities of the enzymes were observed in the upper parts of the shoot. However, all four respiratory enzymes had a tendency to decrease generally in activities from the upper parts to the lower parts of the shoot, indicating gradual decrease of the respiratory activities during maturation of the bamboo.

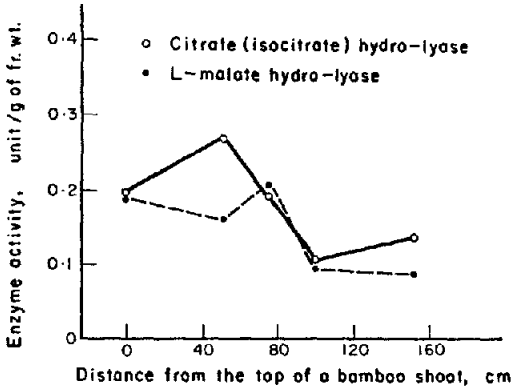

FIG. 7. CHANGES IN ACTIVITIES OF RESPIRATORY ENZYMES WITH GROWTH OF A BAMBOO.

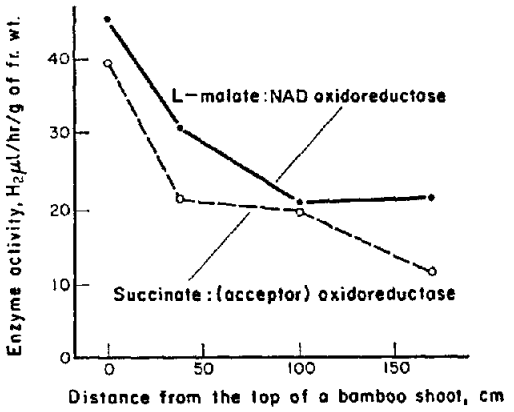

FIG. 8. CHANGES IN ACTIVITIES OF RESPIRATORY ENZYMES WITH GROWTH OF A BAMBOO.

DISCUSSION

Many studies have been reported on the metabolic pathways leading from glucose to lignin polymers. Since both phenylalanine and tyrosine are known to serve as precursors for grass lignins, including bamboos, it might be expected that some variation in the metabolism and amount of these compounds would occur as lignification progresses. During the growth of bamboo, however, marked variations in content were found only for tyrosine (Fig. 1), the amount of which decreased considerably from the top to the basal part of the bamboo shoot. The reason for the accumulation of tyrosine, which mainly takes place in the meristematic tissues of the shoot, has not yet been elucidated. It may be dependent on the balance between the activities of prephenate dehydrogenase, aromatic amino acid transaminase and tyrosine ammonia-lyase during growth.

The time course for the formation of phenylalanine and tyrosine by transamination

shown in Fig. 2 indicates that aromatic amino acid transaminases were active in the excised tissue. The amount of tyrosine formed was about 2·5 times higher than that of phenylalanine but the formation of both compounds stopped after reaching a maximum after 90 min. In contrast to the formation of aromatic amino acids, the amounts of *p*-coumaric acid formed from either of two substrates was more or less the same (Fig. 3). Therefore, it seems probable, in view of the fact that the amount of tyrosine formed was greater than that of phenylalanine, that the activity of phenylalanine ammonia-lyase was greater than tyrosine ammonia-lyase. These results point to differences in the metabolism of phenylpyruvate and *p*-hydroxyphenylpyruvate perhaps being due to the initiation of lignification induced by the formation of the ammonia-lyase.

However, the variation in the activities of transaminases, which have not yet been reported in relation to lignification of woody plants, need also to be considered. Metabolic studies using tissue samples from various parts of the shoot showed that the amounts of residual keto acid substrates were greater when slices of top-tissue were used, which means that the activity of transaminase increased in the lower parts of the shoot. The results suggest that in the later stages of bamboo maturation, transaminases might equally well be related to control of lignification as the other enzymes reported previously.[6–11]

Patterns of formation of phenylalanine and tyrosine by transamination (in Fig. 5) are very similar to the patterns of variations in free amino acids shown in Fig. 1; i.e. tyrosine is formed in larger amounts using top tissue. On the contrary, however, the amount of *p*-coumaric acid formed, either from phenylpyruvate or from *p*-hydroxyphenylpyruvate, was greatest using lower tissue reaction systems (Fig. 6). In addition, it should be noted that more *p*-coumaric acid was formed from phenylpyruvate than from *p*-hydroxyphenylpyruvate. This phenomenon might be explained by more intensive activity of phenylalanine ammonia-lyase compared to tyrosine ammonia-lyase, in accordance with the results previously obtained[7] (Fig. 2).

The results further suggest that the para-hydroxylation to *p*-coumaric acid from cinnamic acid formed by deamination of phenylalanine is mediated in the tissues. Cinnamic acid-4-hydroxylase[18] is presumed to be functioning intensively because cinnamic acid could not be detected as an intermediate between phenylalanine and *p*-coumaric acid by paper chromatography. The possibility of hydroxylation of phenylalanine to tyrosine was ruled out in the present experiment by the negative results obtained from tracer experiments with labelled phenylalanine-G-^{14}C, although Nair and Vining[18] demonstrated phenylalanine hydroxylase in spinach leaves.

From the results of present experiments and the results reported by Gamborg,[19] aromatic amino acid transaminase may contribute to the formation of lignin precursors by supplying phenylalanine as a substrate. Thus, it might be understood that in early stages of the growth, transaminase may participate dominantly in the synthesis of phenylalanine and tyrosine incorporated into protein but with onset of lignification metabolic regulation systems may be transformed to accelerate the synthesis of a series of the related enzymes such as phenylalanine ammonia-lyase, cinnamic acid-4-hydroxylase, *O*-methyltransferase, peroxidase as well as transaminase to supply lignin precursors. However, the physiological nature of the trigger and the physiological situation in which it acts are still obscure. These metabolic controls might be regulated genetically and also by growth regulators such as indole-3-acetic acid.

In relation to this assumption, Minamikawa *et al.*[12] reported that the shikimic acid

[18] P. M. NAIR and L. C. VINING, *Phytochem.* **4**, 401 (1965).
[19] O. L. GAMBORG and L. R. WETTER, *Can. J. Biochem. Physiol.* **41**, 1733 (1963).

pathway was activated to supply phenylalanine required for the biosynthesis of hydroxycinnamic acids. Zucker[13] also reported that phenylalanine ammonia-lyase was induced by light in relation to chlorogenic acid synthesis. Apart from these presumptions, however, there are several complex biochemical reactions between shikimic acid and phenylalanine which still remain to be examined.

In contrast to the lignification enzymes, the activities of enzymes in the TCA cycle such as citrate (isocitrate) hydro-lyase, L-malate hydro-lyase, L-malate:NAD oxidoreductase and succinate:(acceptor) oxidoreductase decreased considerably from the top toward lower parts of a bamboo shoot indicating the decrease of respiratory activity from the top to the lower parts of the shoot (Figs. 7, 8). The results are quite in accordance with the findings that both the amount of carbon dioxide liberated by sliced tissue and C6/C1 ratio with glucose-1-^{14}C and -6-^{14}C decreased toward lower parts of the shoot. Therefore, these respiratory enzymes only appear to be required in quite low levels for the biosynthesis of monomeric substrates for lignin polymers.

However, quantitative interrelationships between activities of enzymes contributing to protein synthesis and those contributing to lignin synthesis still remain to be investigated in relation to the biochemical regulation of wood formation in higher plants.

EXPERIMENTAL

Determination of Free Amino Acids

Young bamboos (*Phyllostachys pubescens*), 2 to 3 m in height, were collected from the experimental farm of Gifu University. Fresh tissue was cut from six parts from the top to the base of the shoot. 10 g of each was homogenized in hot 70% EtOH (100 ml) and the filtrate and washings were evaporated *in vacuo* at about 50°. The amino acids were quantitatively determined by two-dimensional paper chromatography.

Kinetic Experiments

(*a*) 5 g of sliced tissue from young bamboos (*P. reticulata*) (six samples) were infiltrated *in vacuo* with a solution of sodium phenylpyruvate (or *p*-hydroxyphenylpyruvate) (5 μmoles), glutamic acid (5 μmoles), sodium ascorbate (25 μmoles) in H_2O (1·0 ml). The incubation mixtures were kept at 25° and samples taken (Fig. 2), homogenized in hot 70% EtOH (100 ml) and the phenylalanine (or tyrosine) formed determined as described above using suitable controls.

(*b*) *p*-Coumaric acid formed from phenylpyruvate or from *p*-hydroxyphenylpyruvate was also determined in the test solutions used for the estimation of amino acids. The compound was isolated by paper chromatography in toluene–acetic acid–water (4:1:5, v/v organic layer) for 10 hr. The *p*-coumaric acid spot on the chromatogram was then cut out and eluted with 95% EtOH and its absorptivity measured at 310 nm.

Metabolic Activities of Bamboo Tissue

A young bamboo (*P. reticulata*), about 3·0 m in height, was used for these experiments. Five samples of the tissue were prepared from sections 5, 50, 100, 170 and 260 cm from the top. The sliced samples (5 g) were infiltrated with substrates and the reaction systems incubated for 90 min at 25° together with suitable controls. The solutions obtained, after EtOH-extraction, were used for the determination of phenylalanine, tyrosine and *p*-coumaric acid formed as described above. Residual phenylpyruvate and *p*-hydroxyphenylpyruvate were determined by taking the absorbance of the ethanol extracts in 0·1 N NaOH at 320 and 330 nm, respectively, against a suitable blank.

Changes in Activities of Various Enzymes Involved in Respiratory Metabolism

(*a*) Assay of citrate (isocitrate) hydro-lyase (EC 4.2.1.3)[20] and L-malate hydro-lyase (EC 4.2.1.2).[21]

A young bamboo (*P. reticulata*), about 2 m in height, was used for the assay of the enzyme activities in different parts of the bamboo shoot. After the sheath of the shoot was removed, five 20 g samples were cut out from parts 5, 50, 75, 100 and 130 cm from the top of the shoot. Each sample was homogenized with an equal weight of cold 0·05 M potassium phosphate buffer (pH 7·4) containing 0·01 M Na ascorbate. The

[20] C. B. ANFINSEN, *Methods in Enzymology*, Vol. 1, p. 695, Academic Press, London and New York (1955).
[21] V. MASSEY, *Methods in Enzymology*, Vol. 1, p. 729, Academic Press, London and New York (1955).

homogenate was strained through gauze, and the filtrate centrifuged at 10,000 × *g* at 4° for 20 min. $NH_4(SO_4)_2$ was added to the supernatant to 0·7 saturation. The precipitate was dissolved in 5–10 ml of 0·05 M potassium phosphate buffer (pH 7·4) and used for the assay of citrate (isocitrate) hydro-lyase and L-malate hydro-lyase.[20,21] A unit of activity was defined as the amount of fresh weight of tissue producing a change in absorbance of 1·0 per minute.

(*b*) Assay of succinate:(acceptor) oxidoreductase (EC 1.3.99.1)[22] and L-malate:NAD oxidoreductase (EC 1.1.1.37).[23]

A young bamboo (*P. reticulata*), 275 cm in height, was used for the enzyme assay. 10 g fresh tissue was cut from parts at the distance of 5, 35, 100 and 170 cm, respectively, from the top of the shoot. The tissue was homogenized with 2 × 0·2 M potassium phosphate buffer (pH 7·2) in a mortar. After filtering through gauze, the filtrates were used directly for the assay of succinate:(acceptor) oxidoreductase and L-malate:NAD oxidoreductase with Thungberg's method. The reaction mixture contained 1·0 ml of either 0·2 M sodium succinate or 0·2 M sodium malate, 0·5 ml of 0·5 M potassium phosphate buffer (pH 7·0), 0·2 ml of 0·001 M methylene blue and 1·0 ml of enzyme solution. The reaction mixtures were incubated at 30°. The activities of two oxidoreductases were expressed as μl of H^2 transferred to methylene blue per hr per g of fresh weight.

Acknowledgement—The authors wish to thank Mr. E. Hattori of Gifu University for his considerable assistance.

[22] W. D. BONNER, *Methods in Enzymology*, Vol. 1, p. 722, Academic Press, London and New York (1955).
[23] Y. OHTA and Y. YAMAMOTO, in *Methods in Enzyme Chemistry* (edited by S. AKABORI), Vol. 3, p. 670, Asakura, Tokyo (1957).