Phytochemistry, Vol. 20, No. 6, pp. 1235–1239, 1981.
Printed in Great Britain.

0031-9422/81/061235-05 $02.00/0

ATP CITRATE LYASE FROM *IPOMOEA BATATAS* ROOT TISSUE INFECTED WITH *CERATOCYSTIS FIMBRIATA**

AKIRA TAKEUCHI, MIYUKI YAMAGUCHI and IKUZO URITANI

Laboratory of Biochemistry, Faculty of Agriculture, Nagoya University, Chikusa, Nagoya 464, Japan

(*Received* 8 *September* 1980)

Key Word Index—*Ipomoea batatas; Ceratocystis fimbriata*; Convolvulaceae; ATP citrate lyase; sesquiterpene; ipomeamarone.

Abstract—ATP citrate lyase was detected in sweet potato (*Ipomoea batatas*) root tissue infected with *Ceratocystis fimbriata*. The activity increased strikingly after a 1 day lag in response to infection of the tissue, but hardly appeared in non-infected cut tissue. The time-course pattern of the increased activity in diseased tissue was similar to that of sesquiterpene accumulation, suggesting the participation of the activity in sesquiterpene production. The enzyme from diseased tissue was partially purified by ammonium sulphate fractionation and DEAE-cellulose column chromatography. The latter procedure indicated that the enzyme was composed of two forms. The specific activity of one of the two was raised about 300-fold. Furthermore, it appeared that citrate lyase was also present in diseased tissue.

INTRODUCTION

Large amounts of furanosesquiterpenes, such as ipomeamarone, are accumulated in the infected region of sweet potato (*Ipomoea batatas* Lam.) root tissue infected with *Ceratocystis fimbriata* Ell. and Halst. [1, 2]. Previously, we suggested that ATP citrate lyase (ATP:citrate oxaloacetate lyase, *pro*-3*S*-CH_2COO^- → acetyl-CoA; ATP-dephosphorylating, EC 4.1.3.8) (ACL) might play a role in supplying acetyl-CoA to the sesquiterpene biosynthetic pathway, since radioactivity from ^{14}C-labelled citrate was incorporated into ipomeamarone in the *in vivo* system and the incorporation was markedly inhibited by (−)-hydroxycitrate, a potent inhibitor of ACL [3]. ACL was first found in pigeon liver by Srere and Lipmann [4]. The enzyme is widely distributed and is found in large amounts in various animal organs such as liver, lactating mammary glands, adrenals, adipose tissue and brain [5–7]. ACL in higher plants was first described in ripening mango fruit and next in developing soybean cotyledon by Matoo and Modi [8], and Nelson and Rinne [9], respectively. However, it was later reported that the latter enzyme was not ATP- and CoA-dependent [10]. Recently, Fritsch and Beevers [11] demonstrated the presence of ACL with a strict requirement for ATP and CoA in endosperm tissue of germinating castor bean. They also demonstrated that the enzyme is stabilized by citrate and glycerol [11].

This paper deals with the occurrence of ACL in diseased tissue and the time courses of the appearance of ACL activity and sesquiterpene accumulation in the root tissue of *I. batatas* in response to infection by *C. fimbriata*.

RESULTS

Proof of occurrence of ACL in diseased sweet potato root tissue

ATP, CoA and $MgCl_2$ were required for citrate-dependent NADH oxidation by a crude enzyme extract from diseased tissue (Table 1). This indicated that ACL was present in the crude enzyme extract. The lack of a requirement for added malate dehydrogenase (MDH) was attributed to the presence of endogenous MDH in the extract. The evidence for this is described later in the paper. To demonstrate the enzymatic formation of oxaloacetate, a radioisotopic method was employed. In the presence of a crude enzyme extract, ATP and CoA, radioactivity from [1,5-^{14}C]citrate was recovered in a compound which migrated with the 2,4-dinitrophenylhydrazone of oxaloacetate on paper chromatography (Table 2). However, some enzymic activity was also detected in the absence of ATP and CoA. This was possible due to the presence of citrate lyase (citrate oxaloacetate-lyase, *pro*-3*S*-CH_2COO^- → acetate, EC 4.1.3.6) in the crude enzyme extract. The formation of the 2,4-dinitrophenylhydrazone of ^{14}C-labelled oxaloacetate in the assay was confirmed by the requirement for the addition of 2,4-dinitrophenylhydrazine to the reaction mixture before or after the reaction (Table 3).

Another product of ACL, acetyl-CoA was shown to be formed from citrate by the partially purified ACL sample by using the hydroxamate method (described later in Table 7) and by Huang's method [12] (data not shown).

Stabilization of ACL by citrate

ACL activity in crude enzyme extracts with 10 mM citrate was always higher than in those without citrate when assayed at pH 7.1 and 7.6 (Table 4). These results indicated that citrate had a stabilizing effect on ACL. When the enzyme solution was pre-incubated without citrate, the enzyme activity was very low compared to the complete system (Table 1). This also suggested that ACL was stabilized by citrate.

* Part 138 in the series "Phytopathological Chemistry of Sweet Potato with Black Rot and Injury". This work was supported in part by a Grant for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Table 1. Requirement of ATP, CoA and $MgCl_2$ for citrate-dependent NADH oxidation by a crude enzyme extract from diseased tissue

System	NADH oxidation (nmol/min/g fr. wt)		
	Pre-incubation*	+ CoA†	Addition‡
Complete	9.9	36.1	—
−ATP	6.3	6.3	25.2
−Malate dehydrogenase	8.1	32.5	32.5
$-MgCl_2$	9.0	9.0	16.3
−Citrate	0.0	0.0	0.9

A crude enzyme extract from diseased tissue was prepared using the homogenizing medium and standard buffer which did not contain citrate. The enzyme activity was assayed by the coupled spectrophotometric method.

* Pre-incubation done without CoA and without each component indicated.

† Enzyme reaction started by addition of CoA.

‡ The component omitted from the complete system added.

Table 2. Requirement for ATP plus CoA for the formation of ^{14}C-labelled oxaloacetate from [1,5-^{14}C]citrate by a crude enzyme extract

System	Radioactivity in the position of 2,4-dinitrophenylhydrazone of [^{14}C]oxaloacetate (dpm)
Complete	3060
−(ATP + CoA)	500
0-time	220

The enzyme sample was prepared as described in the footnote to Table 1. 200 μl of crude enzyme extract was used per assay. The enzyme activity was measured by the radioisotopic method after a 10 min incubation period.

Table 3. Proof of enzymatic formation of ^{14}C-labelled oxaloacetate from [1,5-^{14}C]citrate

System	Radioactivity in the position of 2,4-dinitrophenylhydrazone of [^{14}C]oxaloacetate (dpm)
Complete*	12600
−2,4-Dinitrophenylhydrazine†	490
+2,4-Dinitrophenylhydrazine‡ (added after incubation)	14900
0-time	220

The enzyme sample was prepared as described in the footnote to Table 1. The enzyme activity was measured by the radioisotopic method after 60 min incubation.

* Assay conditions were the same as the complete system shown in Table 2, i.e. 2,4-dinitrophenylhydrazine was added at the end of the incubation period.

† All operations were the same as the complete system except for the omission of 2,4-dinitrophenylhydrazine.

‡ 2,4-Dinitrophenylhydrazine was added to an aliquot of the reaction mixture after incubation in the same ratio, as in the case of the complete system.

Effect of Triton X-100 on extraction of ACL

Triton X-100 had no effect on the yield of ACL activity, however, more citrate lyase-like enzyme was extracted when the tissue was homogenized with a medium containing Triton X-100 (Table 5).

DEAE-cellulose column chromatography of ACL

As shown in Fig. 1, DEAE-cellulose column chromatography indicated the occurrence of two forms of ACL in diseased tissue. One form was not adsorbed onto the column whereas the second form was adsorbed and could be eluted by KCl. MDH activity overlapped ACL activity, and indicated the presence of three forms of the enzyme. The presence of MDH in the ACL-containing fractions meant that ACL assay by the coupled spectrophotometric method was independent of added MDH. The ACL activity profile when measured by the hydroxamate method was the same as that when measured by the spectrophotometric method. The degree of purification of each fraction, form I or II, is given in Table 6.

As indicated in the previous section, acetyl-CoA was shown to be produced from citrate using ACL form II, by the hydroxamate method. The formation of a hydroxamate was dependent on the presence of ATP and CoA (Table 7). ^{14}C-labelled acetyl-CoA formation from [1,5-^{14}C]citrate by ACL form II was confirmed as being ATP and CoA-dependent by the method of Huang [12]. The optimal pH of ACL form II was 8.5 and was almost the same as that (pH 8.4) of ACL from rat liver [13] but higher than those (pH 7 and 7.5) of ACL from mango fruit [8] and endosperm of castor bean [11], respectively.

Change in ACL activity after fungal inoculation

As shown in Fig. 2, ACL activity in diseased tissue increased strikingly after a lag of about 1 day but was barely detectable in cut tissue without fungal inoculation, for the 4-day period studied. Sesquiterpene accumulation followed a similar pattern to that of ACL activity.

Table 4. Stabilization of ACL by citrate

pH of reaction mixture	ACL activity (nmol/min/g fr. wt)	
	+Citrate	−Citrate
7.1	5.5	0.92
7.6	11.0	3.70

Enzyme extraction from diseased tissue was carried out in a homogenizing medium containing Triton X-100 and without citrate. The supernatant solution after centrifugation at 300 g for 10 min was divided into two parts. One part was passed through a Sephadex G-25 column pre-equilibrated with 0.1 M Tricine–KOH (pH 7.5) containing 0.2 mM DTT, 10% glycerol and 10 mM K citrate, and the effluent was centrifuged at 20 000 g for 20 min. The other part was treated in the same manner as described above except that no K citrate was present in the pre-equilibration buffer. The supernatant solutions were used as the enzyme samples. ACL activity was assayed by the coupled spectrophotometric method.

Table 5. Effect of Triton X-100 on extraction of ACL and citrate lyase-like enzyme in crude enzyme extract

Homogenizing medium	ACL activity	Citrate lyase-like enzyme activity
	(nmol/min/g of fr. wt)	
+Triton X-100	99.5	8.8
−Triton X-100	94.4	35.9

Enzyme extraction was done as described in the Experimental, i.e. diseased tissue was homogenized in the presence or absence of Triton X-100, and the homogenate was centrifuged at 20 000 g for 20 min. The supernatant solution was passed through a Sephadex G-25 column and the effluent was used as a crude enzyme extract. ACL activity was assayed by the coupled spectrophotometric method. Citrate lyase-like enzyme activity represents the activity of citrate-dependent but ATP- and CoA-independent NADH oxidation.

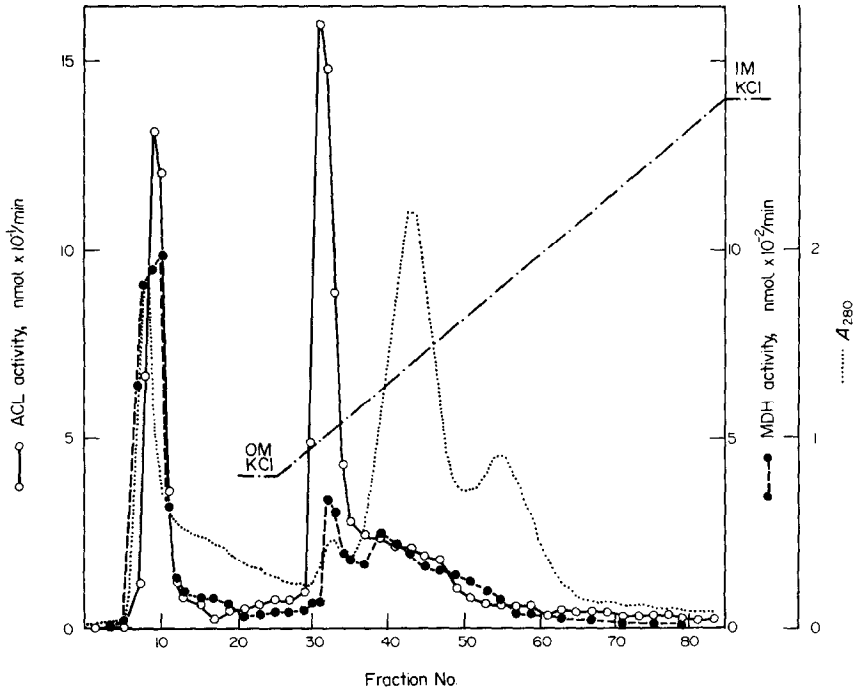

Fig. 1. DEAE-cellulose column chromatography of ACL from diseased tissue. ACL activity was assayed by the coupled spectrophotometric method. Form I, fractions 8–11; form II, fractions 30–34. MDH: malate dehydrogenase.

Table 6. Summary of purification of ACL

Step	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg protein)
Ammonium sulphate precipitate	76	112	1.5
DEAE-cellulose form I	4.26	335	78.5
DEAE-cellulose form II	1.07	489	457

Table 7. Assay of acetyl-CoA formed by ACL by the hydroxamate method

System	A_{520nm}
Complete	0.42
−ATP	0.15
−CoA	0.14
Boiled enzyme	0.14
0-time	0.14

ACL form II (100 μl) was used in the assays. After 120 min of enzyme action in the presence of hydroxylamine, the colouration by $FeCl_3$ was carried out and the A_{520nm} measured.

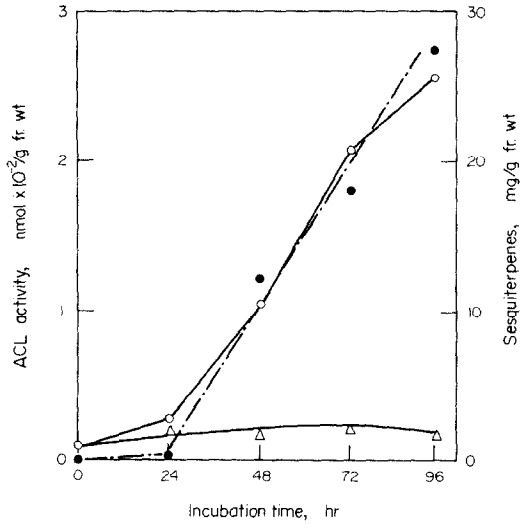

Fig. 2. Time course of ACL activity and sesquiterpene accumulation in response to *C. fimbriata* infection. Crude enzyme extracts from fresh and cut tissues were prepared in the same way as from diseased tissue. ACL activity in the crude enzyme extract was assayed by the coupled spectrophotometric method. ○—○, ACL activity in diseased tissues; △—△, ACL activity in cut tissues; ●———●, sesquiterpene content.

DISCUSSION

The occurrence of ACL in diseased tissue was demonstrated by several experimental methods dealing with the conversion of citrate to oxaloacetate and acetyl-CoA in the presence of ATP and CoA by crude enzyme extracts and partially purified enzyme samples. It appeared that a citrate lyase-like enzyme, which was independent of ATP and CoA and thus similar to a bacterial type citrate cleavage enzyme, also occurred in diseased tissue, as reported by Nelson and Rinne [10] for developing soybean cotyledons.

Fritsch and Beevers [11] reported that ACL from endosperm of germinating castor bean was stabilized by freezing and addition of citrate and glycerol. These stabilizing effects were previously described in animal tissue [5]. This was also the case for ACL in diseased tissue of sweet potato, at least in the case of added citrate. The successful isolation of ACL from diseased tissue was apparently due to the presence of citrate at all stages of the isolation procedure. We initially isolated ACL in the presence of Triton X-100 in the homogenizing medium, according to the method of Fritsch and Beevers [11], in which Triton X-100 was used to destroy plastids where ACL is localized in germinating castor bean endosperm. However, the yield of the ACL (Table 5) and the profile of DEAE-cellulose column chromatogram was independent of the presence or absence of Triton X-100. These results suggest that ACL is present in the cytosol in the case of diseased tissue of sweet potato.

It is of particular interest that ACL activity increased markedly only in diseased tissue, since HMG-CoA reductase [14] and pyrophospho-mevalonate carboxylase [15] behave similarly in the sweet potato root system. If citrate is present in a sufficient concentration in the cytosol of diseased tissue, the activity of ACL would be able to supply 75% of the acetyl-CoA required for sesquiterpene production. According to our *in vivo* experiments [3, 16], pyruvate was a more effective precursor for sesquiterpene biosynthesis than citrate. Thus, we suggested that a cytosol pathway, which utilized the enzymes pyruvate decarboxylase, aldehyde dehydrogenase and acetyl CoA synthetase, might play an important role in the supply of acetyl-CoA for sesquiterpene biosynthesis [17]. Since we have now found that ACL activity is high compared to the activity of this pathway, we shall have to re-investigate more carefully the contribution of both routes in supplying acetyl-CoA for sesquiterpene biosynthesis.

DEAE-cellulose column chromatography indicated that two forms of ACL were present in diseased tissue. Fritsch and Beevers [11] detected two forms of ACL which differed in molecular weight according to agarose gel chromatography. We intend to elucidate the enzymatic nature of the two forms of ACL in diseased tissue in the hope that it will clarify their physiological roles in acetyl-CoA supply to sesquiterpene biosynthesis.

EXPERIMENTAL

Plant material. Sweet potato (*Ipomoea batatas* Lam. cv. Norin 1) roots were harvested in Shizuoka and Aichi in the autumn, and stored at 15° until used.

Chemicals. Sodium [1,5-^{14}C]citrate and sodium [2-^{14}C]acetate were obtained from the Radiochemical Centre, Amersham. Malate dehydrogenase (MDH) and ATP, NADH, CoA and Na oxaloacetate were purchased from Boehringer Mannheim GmbH, Sigma Chemical Company, P-L Biochemicals Inc., and Katayama Chemical Company, respectively. The other reagents used here were either special grade or first grade.

Preparation of fresh, cut and diseased tissue. These were prepared according to the previous paper [18]. In the case of diseased tissue, the incubation (30°) was performed for 48 hr unless otherwise stated.

Preparation of crude enzyme extract from diseased tissue. Non-infected tissue (1–2 mm thick, 40 g) adjacent to the infected region was homogenized with 100 ml 0.1 M Tricine–KOH (pH 7.5) containing 10 mM EDTA, 10 mM 2-mercaptoethanol, 10% glycerol, 10 mM K citrate and 8 g of Polyclar AT (homogenizing medium). Triton X-100 (0.1%) was added to the homogenizing medium in some experiments. The homogenate was filtered through nylon cloth, and the filtrate was centrifuged at 20 000 g for 20 min unless otherwise stated. The supernatant soln was passed through a Sephadex G-25 column which was pre-equilibrated with 20 mM Tricine–KOH (pH 7.5) containing

0.2 mM dithiothreitol (DTT), 10% glycerol and 10 mM K citrate (standard buffer), and the effluent was used as the crude enzyme extract. In some experiments the crude enzyme extract was prepared with no K citrate in either the homogenizing medium or the standard buffer.

ACL assay by the coupled spectrophotometric method. ACL activity was usually assayed by coupled spectrophotometry based on the methods of Takeda *et al.* [19] and Fritsch and Beevers [11] with slight modifications. The assay mixture contained, in a total vol. of 500 μl, 0.1 M Tricine–KOH (pH 8.5), 1 mM DTT, 10 mM K citrate, 2 mM ATP, 4 mM $MgCl_2$, 6 units of MDH and 10–200 μl of enzyme sample. $A_{340\,um}$ was monitored for a 5 min pre-incubation period, then 40 μm CoA was added and the reaction followed for a further 5 min at 25°.

ACL assay by the radioisotopic method. ^{14}C-Labelled oxaloacetate formed from [1,5-^{14}C]citrate by ACL was trapped by 2,4-dinitrophenylhydrazine as its phenylhydrazone by a modified form of the method of Nelson and Rinne [10]. The reaction mixture was composed of 0.1 M Tricine–KOH (pH 7.5), 10 mM DTT, 20 μM K citrate, 18.5 μM Na [1,5-^{14}C]citrate, 5 mM ATP, 4 mM $MgCl_2$, 0.12 μM CoA, 5 mM 2,4-dinitrophenylhydrazine and an aliquot of crude enzyme extract (citrate-free), in a total vol. of 200 μl. The mixture was incubated at 25° for 10 or 60 min, the reaction was stopped by adding 100 μl 2 N HCl satd with 2,4-dinitrophenylhydrazine, and then 3 μl of 1% oxaloacetate soln was added to the mixture, which was left for 30 min. The 2,4-dinitrophenylhydrazone of ^{14}C-labelled oxaloacetate was extracted by a modification of the method of Hawary *et al.* [20], and subjected to PC using *n*-BuOH EtOH–0.5 N NH_3 (5:2:3, v/v) as developing solvent. After the development, the part of the chromatogram corresponding to the R_f value of 2,4-dinitrophenylhydrazone of oxaloacetate, which was located by its particular yellowish colour, was cut out and assayed for radioactivity by liquid scintillation spectrometry.

ACL assay by the hydroxamate method. ACL activity was assayed by the hydroxamate method of Takeda *et al.* [19] with slight modifications. The assay mixture contained 60 mM Tricine–KOH (pH 8.5), 0.2 mM DTT, 20 mM K citrate, 4 mM ATP, 0.4 mM CoA, 4 mM $MgCl_2$, 200 mM hydroxylamine and varying amounts of enzyme soln in a final vol. of 500 μl. The reaction was started by the addition of the enzyme soln and the mixture was incubated at 30° for varying periods, then mixed with 100 μl 10% TCA to stop the reaction. After deproteinization by centrifugation, 300 μl of the supernatant soln was mixed with 50 μl 2 M $FeCl_3$ and the amount of acetohydroxamate formed estimated from the *A* at 520 nm.

Formation of ^{14}C-labelled acetyl-CoA from [1,5-^{14}C]citrate by ACL. The enzyme reaction was performed in a similar way to the radioisotopic method described above with some modifications, i.e. the 0.1 M Tricine–KOH was adjusted to pH 8.5 and 2,4-dinitrophenylhydrazine was omitted. In this case, the partially purified enzyme sample (ACL form II, see Results) was used instead of crude enzyme extract. The reaction was started by adding the enzyme sample, incubated at 30° for 30 min and stopped by the addition of 50 μl of HOAc. Proof of ^{14}C-labelled acetyl-CoA formation followed the method of Huang [12], i.e. after deproteinization by centrifugation, 10 μl of the supernatant soln was chromatographed on Si gel-impregnated glass fibre sheet (I.T.L.C. SA, Gelman Co.) developed with H_2O-satd Et_2O–HCO_2H (7:1, v/v) for 15 min. The radioactivity at the origin was measured, since acetyl-CoA does not migrate in this system whereas [1,5-^{14}C]citrate and ^{14}C-labelled oxaloacetate both move from the origin.

Determination of protein content. Protein content was determined by the method of Lowry *et al.* [21].

Partial purification of ACL from diseased tissue. $(NH_4)_2SO_4$ was added to the crude enzyme extract (equivalent to 40 g of fr. wt) and the ppt. obtained between 20 and 50% satn was suspended in a small vol. of standard buffer and dialysed against standard buffer. The dialysate was applied to a DE-52 column (50 ml bed vol.) pre-equilibrated with standard buffer, and the column was washed with standard buffer. The proteins were eluted with standard buffer containing 0–1 M KCl and 5 ml fractions were collected. ACL activity was determined by both the coupled spectrophotometric method and the hydroxamate method. MDH activity was assayed by measuring the initial rate of oxidation of NADH (oxaloacetate reduction) according to Kitto [22].

Acknowledgements—We wish to express our sincere thanks to Professor Harry Beevers (University of California) for kindly reviewing the manuscript. We are grateful to Dr. K. Oba for her help in performing this work.

REFERENCES

1. Hiura, M. (1943) *Rep. Gifu Agric. Coll.* **50,** 1.
2. Hyodo, H., Uritani, I. and Akai, S. (1969) *Phytopathol. Z.* **65,** 332.
3. Oguni, I. and Uritani, I. (1974) *Plant Cell Physiol.* **15,** 179.
4. Srere, P. A. and Lipmann, F. (1953) *J. Am. Chem. Soc.* **75,** 4874.
5. Mersmann, H. J. and Houk, J. M. (1975) *Int. J. Biochem.* **6,** 85.
6. Srere, P. A. (1959) *J. Biol. Chem.* **234,** 2544.
7. Srere, P. A. (1972) *Curr. Top. Cell Regul.* **5,** 1128.
8. Mattoo, A. K. and Modi, V. V. (1970) *Biochem. Biophys. Res. Commun.* **39,** 895.
9. Nelson, D. R. and Rinne, R. W. (1975) *Plant Physiol.* **55,** 69.
10. Nelson, D. R. and Rinne, R. W. (1977) *Plant Cell Physiol.* **18,** 1021.
11. Fritsch, H. and Beevers, H. (1979) *Plant Physiol.* **63,** 687.
12. Huang, K. P. (1970) *Analyt. Biochem.* **37,** 98.
13. Inoue, H., Suzuki, F., Fukunishi, K., Adachi, K. and Takeda, Y. (1966) *J. Biochem.* **60,** 543.
14. Suzuki, H., Oba, K. and Uritani, I. (1975) *Physiol. Plant Pathol.* **7,** 265.
15. Oba, K., Tatematsu, H., Yamashita, K. and Uritani, I. (1976) *Plant Physiol.* **58,** 51.
16. Oba, K., Shibata, H. and Uritani, I. (1970) *Plant Cell Physiol.* **11,** 507.
17. Takeuchi, A., Scott, K. J., Oba, K. and Uritani, I. (1980) *Plant Cell Physiol.* **21,** (in press).
18. Oba, K. and Uritani, I. (1975) *J. Biochem.* **77,** 1205.
19. Takeda, Y., Suzuki, F. and Inoue, H. (1969) *Methods Enzymol.* **13,** 153.
20. Hawary, M. F. S. El. and Thompson, R. H. S. (1953) *Biochem. J.* **53,** 340.
21. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265.
22. Kitto, G. B. (1969) *Methods Enzymol.* **13,** 106.