Phytochemistry, 1978 Vol. 17, pp 1511–1516 © Pergamon Press Ltd. Printed in England. 0031-9422/78/0901-1511$02.00/0

CINNAMIC ACID 4-HYDROXYLASE FROM GHERKIN TISSUES

E. ELLEN BILLETT and HARRY SMITH

Department of Physiology and Environmental Studies, University of Nottingham, School of Agriculture, Sutton Bonington, Loughborough, LE12 5RD, Leicestershire, U.K.

(*Revised received* 3 *April* 1978)

Key Word Index—*Cucumis sativus*, Cucurbitaceae; gherkin; endoplasmic reticulum; mixed function oxygenase; properties.

Abstract—Homogenates from 4-day-old gherkin cotyledons and hypocotyls fractionated by sucrose density gradient centrifugation contain cinnamic acid 4-hydroxylase activity, the activity being highest in the endoplasmic reticulum fractions. These fractions also contain very low concentrations of cytochrome P_{450}. Hydroxylase activity is dependent on NADPH and on molecular oxygen, is optimal at pH 7.5 and is inhibited by carbon monoxide. The enzyme is very sensitive to inhibition by 2-mercaptoethanol, but it is not inhibited by the product, *p*-coumaric acid. Further, its responses to various potential inhibitors are fairly typical of mixed function oxidases from other sources.

INTRODUCTION

The hydroxylation of *trans*-cinnamic acid by the mixed function oxygenase *trans*-cinnamic 4-hydroxylase (EC 1.14.13.11) (CA4Hase) is a key reaction in the biosynthesis of a large number of phenolic compounds in higher plants including tannins, flavonoids and phenolic acids [1]. It has been detected in a wide range of cell-free extracts of plant tissues [2–4] and also in parsley cell suspension cultures [5]. Evidence suggests that cytochrome P_{450} is involved in the hydroxylation [1, 6–8] but the exact mechanism of the reaction is not certain. In this paper we report the subcellular localization and properties of gherkin CA4Hase.

RESULTS

CA4Hase activity was detected in both dark grown gherkin cotyledons and hypocotyls, with twice as much activity in the hypocotyls. Blue light increases CA4Hase activity in cotyledons, but not in hypocotyls (unpublished observations) and the results described below refer to CA4Hase extracted from dark-grown gherkin cotyledons after 4 hr blue light treatment, unless otherwise stated.

Subcellular localization of CA4Hase in gherkin cotyledons and hypocotyls

Fractionation of a crude 270 *g* supernatant of cotyledon tissue on a 20–60% linear sucrose gradient gave two

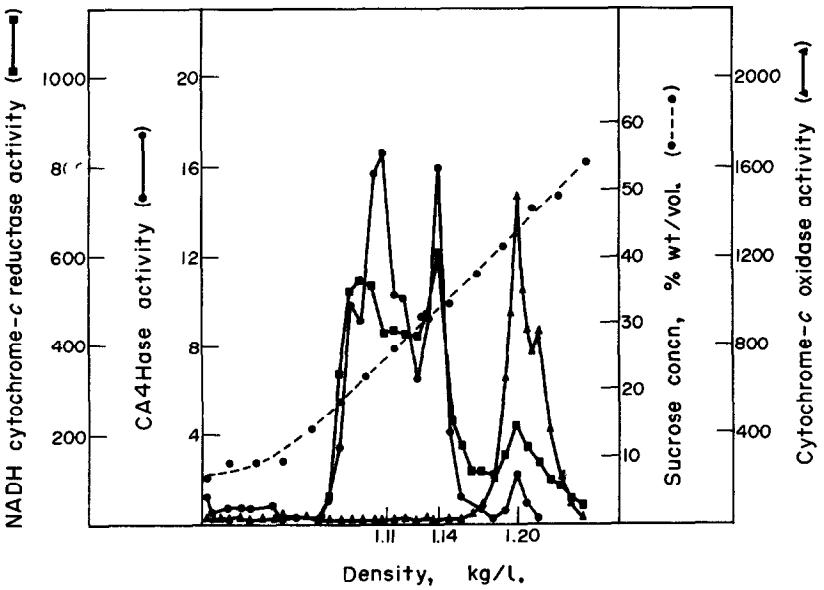

Fig. 1. Localisation of CA4Hase activity after sucrose density centrifugation of a 270 *g* supernatant from cotyledon tissue. Enzyme activities are all expressed per ml of gradient; CA4Hase activity is in pmol *p*-coumarate/min × 10^{-1}, NADH–ctyochrome *c* reductase and cytochrome oxidase activities are in nmol/min.

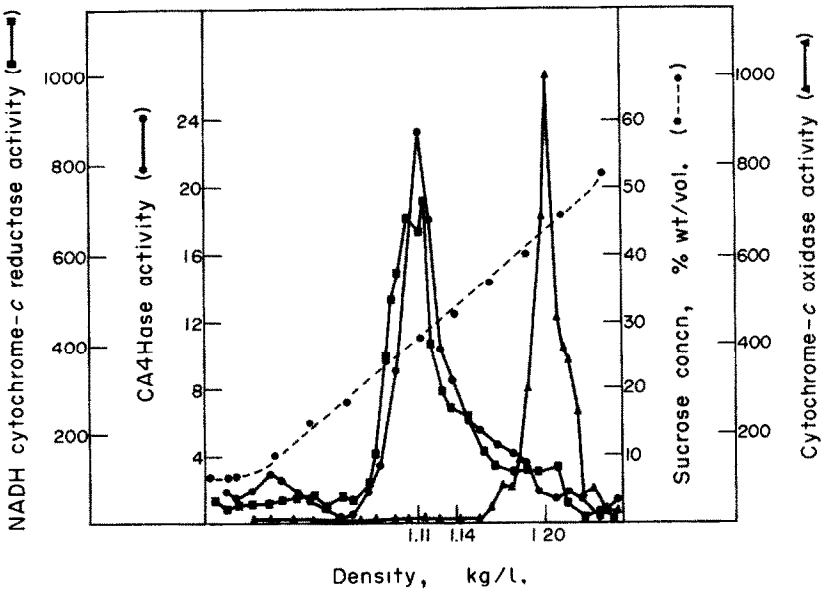

Fig. 2. Localization of CA4Hase activity after sucrose density centrifugation of a 270 *g* supernatant from hypocotyl tissue. Other details as in Fig. 1.

major bands of CA4Hase activity (sucrose density 1.1 and 1.14 kg/l.) and one minor band (density 1.2 kg/l.) (Fig. 1). The major bands are coincident with antimycin A insensitive NADH–cytochrome *c* reductase (EC 1.6.99.3), a marker for endoplasmic reticulum (ER) [9]. The minor band is coincident with cytochrome oxidase (EC 1.9.3.1.) and antimycin A sensitive NADH–cytochrome *c* reductase, markers for mitochondria [9, 10]. Most of the CA4Hase activity of hypocotyl tissue stabilizes at a density of 1.11 kg/l., again coincident with antimycin A insensitive NADH-cytochrome *c* reductase (Fig. 2).

When hypocotyl or cotyledon homogenates are subjected to differential centrifugation all the CA4Hase activity sediments between 21000 *g* and 106000 *g*. Fractionation of the 106000 *g* pellet (microsomes) from cotyledon tissue on a 25–45% linear sucrose gradient results in three bands of CA4Hase activity (Fig. 3). The two lighter bands (1.09 and 1.11 kg/l.) are well separated from the third, heavier band (1.15 kg/l.) but only the third band is coincident with antimycin A insensitive NADH–cytochrome *c* reductase. Mitochondrial contamination of the microsome fraction is small, as indicated by the low cytochrome oxidase activities. Since the microsome fraction is slightly greenish-yellow in colour, however, the pellet must contain some chloro-

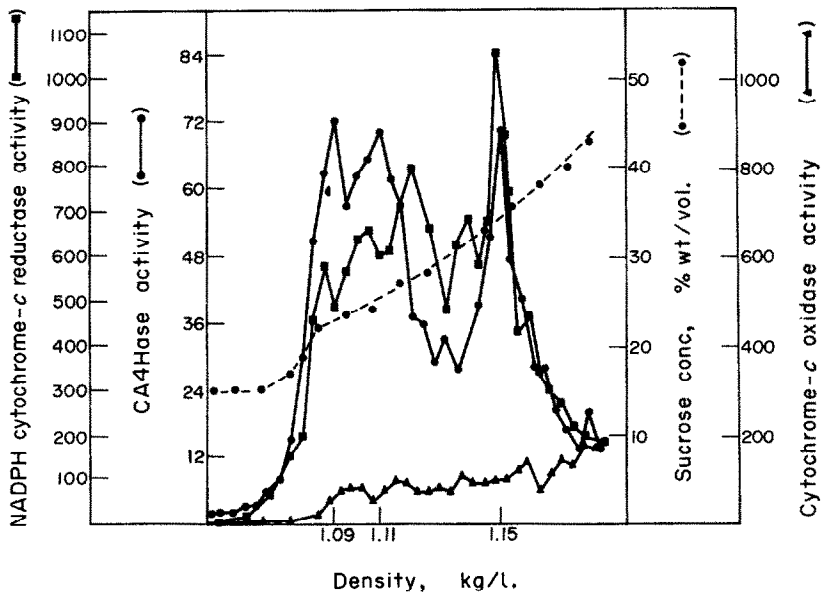

Fig. 3. Localization of CA4Hase activity after sucrose density centrifugation of a 106000 *g* pellet (microsomes) from cotyledon tissue. Other details as in Fig. 1.

plast fragments. The band in the region 1.14–1.15 kg/l. contains measureable amounts of RNA (400 μg/ml) whilst the 1.09–1.11 kg/l. band does not. It is thought that the heavier band is rough ER whilst the lighter band is smooth ER; these densities would agree with previous observations by Lord *et al.* [9]. Thus, cotyledons contain CA4Hase activity which is associated with both rough and smooth ER. In these cotyledons at least some ribosomes remain attached to the ER, despite extraction of tissue with EDTA, and in the absence of Mg^{2+}, and its subsequent fractionation on sucrose gradients in the absence of Mg^{2+}, unlike the situation in castor bean [4]. However, on extraction of gherkin hypocotyls under similar conditions, only smooth ER is recovered on the sucrose gradients, and this has the majority of the CA4Hase activity associated with it.

General properties of CA4Hase in gherkin microsomes

Oxygen is essential for activity and the product was shown by PC (Whatman No. 3 MM) using C_6H_6–HOAc–H_2O (2:2:1, upper), to be solely 4-hydroxycinnamic acid. At 25° with saturating concentrations of cofactors (see below) the rate of hydroxylation is constant for 20 min and then declines. Microsome preparations contain small amounts of cytochrome P_{450}, as deduced from spectral changes resulting from carbon monoxide binding to dithionite-reduced pigment in a frozen sample (Fig. 4), and carbon monoxide inhibits CA4Hase activity, suggesting the involvement of cytochrome P_{450} in the hydroxylation. However, the sensitivity of the spectrophotometric assay for cytochrome P_{450} is too low to allow confirmation of cinnamic acid binding to reduced cytochrome P_{450} [8]. Microsomes also contain cytochrome P_{420}, the denaturation product of cytochrome P_{450} (Fig. 4).

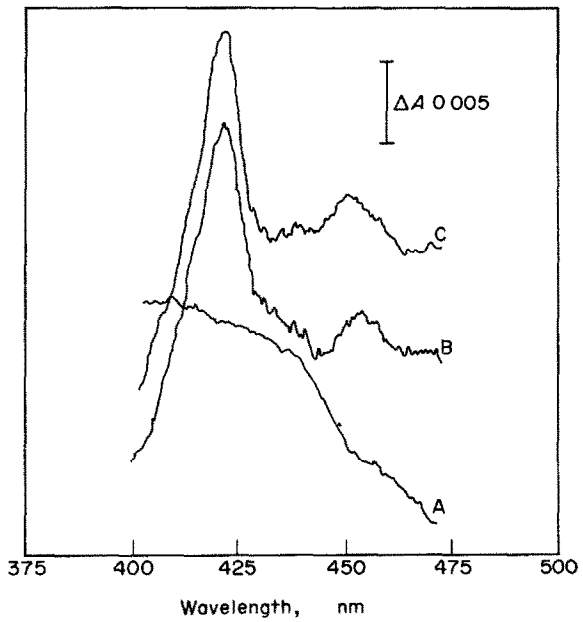

Fig. 4. Difference spectra of carbon monoxide binding to sodium dithionite reduced microsomes. Microsomes were placed in 0.6 M sucrose, 5 mM *N*-Tris(hydroxymethyl)methyglycine and 1 mM EDTA at 4 mg/ml protein and pH 7.5. The preparation was made anaerobic by incubation with *Aerobacterium* mitochondria in the presence of ATP and succinate, before the addition of sodium dithionite to both reference (A) and sample (B, C) cuvettes. Sample cuvettes were subsequently made anaerobic with carbon monoxide in the dark (B) or following light treatment (C). The photodissociation of cytochrome a_3–CO complex is more sensitive to light than that of cytochrome P_{450}–CO and thus, by manipulating the light treatment, it is possible to reverse carbon monoxide binding to cytochrome a_3 but not cytochrome P_{450}. Profile C therefore accurately demonstrates the presence of cytochrome P_{450}. All observations were made in samples frozen in liquid N_2.

Table 1. Effect of various thiol reagents on CA4Hase activity in gherkin microsomes

Reagent	CA4Hase activity 0.1 mM	(pmol product/min) 2.0 mM
None	42.1	
Glutathione	56.6	41.2
Ascorbic acid	49.0	25.8
Dithiothreitol	46.7	15.6
β-Mercaptoethanol	46.7	11.4

The thiol reagents were added at a final concentration of either 0.1 mM or 2 mM. The incubation time was 15 min.

2-Mercaptoethanol inhibits at much lower concentrations (>0.4 mM) than those reported for other sources of the enzyme [4, 6], although at 0.1 mM it stimulates CA4Hase activity. Of the other thiol reagents tried, glutathione is the most stimulatory at low concentrations and least inhibitory at high concentrations (Table 1). The rate of 4-hydroxylation of *trans*-cinnamic acid in the presence of 20 μM cinnamic acid and 100 μM NADPH is optimal at pH 7.5 and half optimal at pH 5.5 and 8.8.

Without the addition of external cofactors, gherkin microsomes do not catalyse the hydroxylation of *trans*-cinnamic acid. NADPH is the most effective cofactor; hydroxylation is not supported by NADH or ascorbic acid (Table 2). However, NADH has a synergistic effect on NADPH-supported hydroxylation at both non-saturating (0.1 mM) and near-saturating (1 mM) concentrations of NADPH (Table 2). The synergistic effect of NADH with NADPH is not due to trans-hydro-

Table 2. Effect of various cofactors on CA4Hase activity in gherkin microsomes

Cofactor	Final concentration (mM)	(pmol product/min)
None	—	0
Ascorbic acid	0.5	0.9
NADPH	0.1	31.5
	1.0	60.3
NADH	0.1	1.8
	1.0	4.2
NADPH+ NADH	0.1 1.0	65.3
NADPH+ NADH	0.1 0.1	55.0
NADPH+ NADH	1.0 1.0	80.2

All nucleotides were supplied in the absence of a regenerating system.

Table 3. Possible transfer of H from NADH to NADPH

Cofactor	Final concentration (mM)	(pmol product/min)
NADPH	0.2	42.8
NADP	0.2	1.5
NADPH+ NADP	0.2 0.2	30.7
NADP+ NADH	0.2 0.4	2.9
NADP+ NADH+ ATP	0.2 0.4 0.4	6.7
NADP+ ATP	0.2 0.4	2.9
NADH+ ATP	0.4 0.4	6.8

All nucleotides were supplied in the absence of a regenerating system.

Table 4. Effect of various additions on CA4Hase activity in gherkin microsomes

Compound tested	Concentration (mM)	Relative activity
None		100
Caffeic acid	0.1	103
Chlorogenic acid	0.1	91
Gallic acid	0.1	80
EDTA	1.0	82
$CuSO_4$	1 0	0
$MnCl_2$	1.0	73
Antimycin A	0.005	84
2,4-Dinitrophenol	0.1	17
Sodium azide	10	36
Menadione	0.1	8
FAD	1	27
	0.1	75
FMN	1	25
	0.1	57
	0 001	83
KCN	10	79
	1	125
KCl	1000	7
	500	31
	200	61

genation from NADH to NADP since CA4Hase activity is not supported by a mixture of NADH and NADP (Table 3). NADH with ATP (±NADP) allows some hydroxylation of *trans*-cinnamic acid but the reason for this is not clear. NADP does not consistently affect hydroxylation; with some microsome preparations NADP inhibits hydroxylation in a competitive manner, as reported previously for CA4Hase of *Helianthus tuberosus* [7] and geraniol hydroxylase of *Vinca rosea* [11].

The CA4Hase activity of gherkin cotyledons follows Michaelis–Menten kinetics at pH 7.5 with respect to cinnamic acid concentration, with an apparent K_m of 2.5 μM. Positive co-operativity is displayed with respect to NADPH (Fig. 5) with an inflexion point at *ca* 125 μM; saturation with NADPH is not achieved even at 1 mM. Gherkin microsomal NADPH–cytochrome *c* reductase, on the other hand, displays negative co-operativity with respect to NADPH (Fig. 5). The activity of P_{450}-linked NADPH–cytochrome *c* reductase could not be measured, due to the low levels of P_{450} relative to the concentration of pigments.

CA4Hase activity in microsome preparations is not inhibited by up to 0.1 mM *p*-coumaric acid or caffeic acid and only slightly by 0.1 mM chlorogenic acid or gallic acid (Table 4). The enzyme responds to various potential inhibitors in a manner fairly typical of mixed function oxygenases from other plant and animal tissues

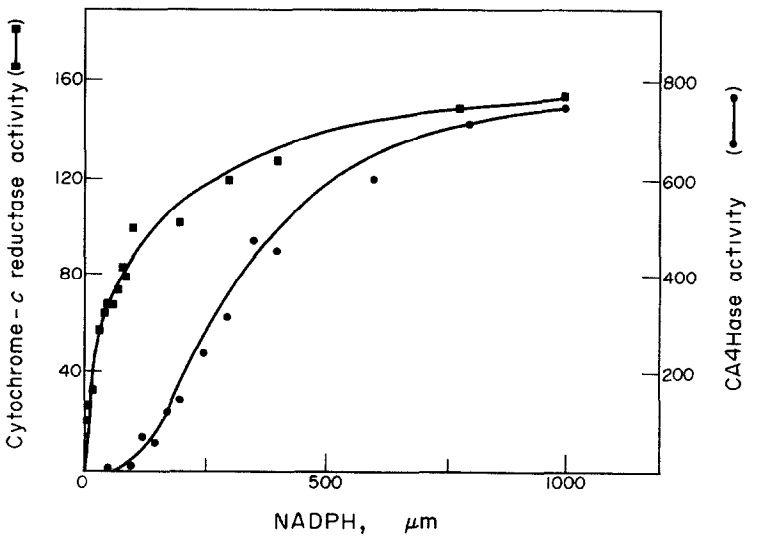

Fig. 5. Relationship between NADPH concentration and the rates of CA4Hase and cytochrome c reductase activities Enzyme activities are expressed per ml of microsomal preparation; CA4Hase is in pmol/min, cytochrome *c* reductase in nmol/min.

[4]. It is strongly inhibited by the electron acceptor menadione and is completely inhibited by cupric ions.

Manganese ions and EDTA have only a small inhibitory effect whilst the flavine nucleotides (FMN and FAD), sodium azide and KCl strongly inhibit. The mitochondrial electron transport inhibitor 2,4-dinitrophenol strongly inhibits, contrary to reports for this enzyme from other sources [6], but antimycin A has little effect. Potassium cyanide at 1 mM stimulates hydroxylation.

Attempts to use the spectrophotometric assay of Lamb and Rubery [2] were unsuccessful. Under optimum conditions of assay, gherkin CA4Hase activity was more than an order of magnitude too low to be satisfactorily detected by the spectrophotometric assay.

DISCUSSION

The demonstration of CA4Hase activity in extracts of gherkin hypocotyls and cotyledons confirms the detection of its activity *in vivo* by Engelsma [12]. Localization of most of the CA4Hase activity in the endoplasmic reticulum is consistent with its localization in other plant tissues [1–9]. On extraction of gherkin cotyledons in magnesium-free buffer, some CA4Hase activity is associated with rough endoplasmic reticulum whilst the rest is associated with smooth endoplasmic reticulum; on extraction of hypocotyls in the same buffer all the activity is associated with smooth endoplasmic reticulum. This result may be due to differential dissociability of ribosomes from the endoplasmic reticulum in the cotyledons and the hypocotyls.

The low concentrations of P_{450} relative to interfering pigments in the extracts did not allow a study of the role of P_{450} in the hydroxylation of *trans*-cinnamic acid by gherkin microsomes, a common finding with plant microsomes [8]. Resolution and partial purification of geraniol hydroxylase from *Vinca rosea* by Madyastha and Coscia [11] has demonstrated that a phospholipid fraction, a reductase fraction and a cytochrome P_{450} fraction are required for maximum activity. This and other evidence suggests that plant mixed function oxidases are organized like the well-studied mixed function oxidases of animal systems, so that it is very likely that the P_{450} present in gherkin microsomes is involved in cinnamate (and possibly other) hydroxylation(s).

The properties of gherkin CA4Hase appear to be similar to those reported for CA4Hase from other sources. It requires O_2 and NADPH, is inhibited by CO, and NADH has a synergistic effect in the presence of NADH. The enzyme is insensitive to inhibitors of mitochondrial electron transport and to the product, *p*-coumaric acid. It is strongly inhibited by FMN and FAD, as is swede CA4Hase, but not others [6], and is stimulated by cyanide. However, it is much more sensitive to thiol reagents, particularly 2-mercaptoethanol, than other sources of the enzyme [4, 6]. Gherkin CA4Hase also displays positive co-operativity with respect to NADPH; the only other source of enzyme with such kinetics is castor bean endosperm [4]. Gherkin NADPH–cytochrome *c* reductase, on the other hand, which is often presumed to be equivalent to NADPH–cytochrome P_{450} reductase, displays negative co-operativity with respect to NADPH. Provided that the observed differences in kinetics signify differences between CA4Hase and its P_{450}-linked NADPH–cytochrome *c* reductase component, they may be important from a regulatory viewpoint.

EXPERIMENTAL

Gherkin seedlings (*Cucumis sativus* cv Venlo Pickling) were grown in darkness at 25° ± 1° for 4 days and pretreated with blue light (70 W/m^2) for 4 hr before extraction. In general cotyledons were used as a source of CA4Hase but in one case (Fig. 1) the upper 2 cm of hypocotyl including plumular hook was used.

Preparation of subcellular fractions. All operations were performed at 4°. Tissue was homogenized using a pestle and mortar in 50 mM tricine, 1 mM EDTA, 0.3 M mannitol, 0.1% BSA and 10% (w/w, fr. wt) PVP (pH 7.5). The ratio of tissue to grinding medium was *ca* 1 g/1.5 ml. After filtration through one layer of Miracloth the homogenate was subjected to differential centrifugation for the prepn of microsomes or to sucrose density centrifugation for the separation of endoplasmic reticulum membranes from other organelles. Differential centrifugation was at 270 *g* for 15 min, then 21000 *g* for 15 min, the pellets and lipid layer being discarded. The supernatant was centrifuged at 106000 *g* in the Ti 60 rotor of the Beckman L2-65B for 60min; the microsomal pellet was suspended to *ca* 15 mg/ml protein in 0.3 M mannitol, 10 mM KPi, 5 mM $MgCl_2$, 10 mM KCl and 15% glycerol (pH 7.5).

Linear sucrose gradients were prepared in a manner similar to that described in ref. [4] for CA4Hase. The homogenate was centrifuged at 270 *g* for 15 min, the supernatant dialysed against 50 mM Tricine, 1 mM EDTA, 10 mM KCl (pH 7.5) (3 changes in 90 min, each 1 l./30 g fr. wt. tissue), and 12 ml of the dialysed supernatant soln was layered on gradients in 38 ml polyallomer tubes (5 ml 20% sucrose, 25 ml gradient). Microsome prepns were layered on the gradients without dialysis (9 ml microsome prepn on a 32 ml gradient). After centrifugation at 20000 rpm for 4 hr in a SW 27 rotor (Beckman) 0.5 ml fractions were collected and analysed.

Enzyme assays. Cinnamic acid 4-hydroxylase was assayed as described previously [14], with the assay mixture containing 0.1 mM glutathione, 22 μM [3-^{14}C] *trans*-cinnamic acid (50 Ci/mol, C.E.N.S., France) and 50 μl microsomal prepn, unless otherwise stated. The NADPH regenerating system consisted of 1.5 mM NADP, 2 mM glucose-6-phosphate and 0.1 units glucose-6-phosphate dehydrogenase.

NADH–cytochrome *c* reductase was assayed by following the increase in A_{550}, as described in ref. [15]; in some cases antimycin A was added to the reaction mixture. Cytochrome oxidase was similarly assayed, but using reduced cytochrome *c* [15].

Cytochrome P_{450} was demonstrated by Dr D. S. Bendall and Mrs M. T. R. S. F. Moreira essentially according to the method of ref. [16] using a Johnson Research Foundation split-beam spectrophotometer, and as described in the legend for Fig. 4.

Other assays. RNA was determined by the orcinol method [17] using ribose as a standard. Aliquots of 0.3 ml from each gradient fraction were precipitated with 0.5 ml 20% TCA at 4°. The ppt was collected by centrifugation, washed with 5% (w/v) TCA, and assayed for RNA following alkaline hydrolysis. Protein was determined according to the method of ref. [18] with BSA as standard. Sucrose concns were determined by refractometry.

Acknowledgements—The authors are particularly grateful to Mr Mark Foulstone for excellent technical assistance and to Dr D. S. Bendall and Mrs M. T. R. S. F. Moreira, Department of Biochemistry, University of Cambridge, for preparing the difference spectra for carbon monoxide binding to cytochrome P_{450}. The support of the SRC in providing a Research Grant to H.S. is gratefully acknowledged.

REFERENCES

1. Rowell, J., Potts, M., Weklych, R. and Conn, E. E. (1974) *J. Biol. Chem.* **249**, 5019.
2. Lamb, C. and Rubery, P. H. (1975) *Analyt. Biochem.* **68**, 554.
3. Nair, P. M. and Vining, L. C. (1965) *Phytochemistry* **4**, 161.
4. Young, O. and Beevers, H. (1976) *Phytochemistry* **15**, 379.
5. Hahlbrock, K., Ebel, J., Ortmann, R., Sutter, A., Wellman, E. and Grisebach, H. (1971) *Biochim. Biophys. Acta* **244**, 7.
6. Hill, A. C. and Rhodes, M. J. C. (1975) *Phytochemistry* **14**, 2387.
7. Benveniste, I., Salaun, J. and Durst, F. (1977) *Phytochemistry* **16**, 69.
8. Rich, P. R. and Lamb, C. J. (1977) *Eur. J. Biochem.* **72**, 353.
9. Lord, J. M., Kagawa, T., Moore, T. S. and Beevers, H (1973) *J. Cell. Biol.* **57**, 659.
10. Dickerson, R. E. and Timkovitch. R. (1975) in *The Enzymes* (Boyer, P. D., ed.) p. 397. Academic Press, New York.
11. Madyastha, K. M., Ridgway, J. E., Dwyer, J. G. and Coscia, C. J. (1977) *J. Cell Biol.* **72**, 302.
12. Engelsma, G. (1966) *Acta Bot. Neerl.* **15**, 394.
13. Vance, C. P., Nambudiri, A. M. D. and Towers, G. H. N. (1973) *Can. J. Biochem.* **51**, 731.
14. Billett, E. E., Wallace, W. and Smith, H. (1978) *Biochim. Biophys. Acta*, in press.
15. Wallace, W. and Johnson, C. B. (1978) *Plant Physiol.*, in press.
16. Omura, T. and Sato, R. (1964) *J. Biol. Chem.* **239**, 2370.
17. Ceriotti, G. (1955) *J. Biol. Chem* 214, 59.