Phytochemistry, Vol. 21, No. 2, pp. 261–265, 1982.
Printed in Great Britain.

0031-9422/82/020261-05$03.00/0

PEROXIDASE-CATALYSED OXIDATION OF CHLOROPHYLL BY HYDROGEN PEROXIDE

ALBERT HUFF

Plant Sciences Department, University of Arizona, Tucson, AZ 85721, U.S.A.

(*Revised received* 8 *June* 1981)

Key Word Index—*Citrus sinensis*; Rutaceae; orange; catabolism; chlorophyll; peroxidase; chlorophyll: hydrogen peroxide oxidoreductase.

Abstract—Chlorophyll is effectively bleached by H_2O_2 in the presence of certain phenols and peroxidase (EC 1.11.1.7) extracted from acetone powders of orange flavedo (*Citrus sinensis*). Optimal conditions for chlorophyll: hydrogen peroxide oxidoreductase include: pH, 5.9; [H_2O_2], 222 μM; ionic strength 0.11. A phenol is required and resorcinol is the most effective. Catechol and hydroquinone are inhibitory. Chlorophyll *a*, chlorophyllide *a*, and chlorophyll *b* all have similar V_{max}, but K_m for chlorophyll *a* is about one-third that of chlorophyll *b*, while the K_m for chlorophyllide *a* is about one-half that of chlorophyll *a*. Pheophytin *a* was much less reactive than chlorophyll *a*, and Mg^{2+} included in the reaction system did not affect rates of pheophytin destruction.

INTRODUCTION

Little is known of the degradation of chlorophylls during senescence or fruit ripening. While chlorophyll can be bleached by products of lipoxidase-catalysed oxidation of certain fatty acids [1, 2], involvement of this system *in vivo* is more presumed than demonstrated. For example, Walker [3] demonstrated that homogenates from fresh beans could catalyse bleaching of chlorophyll when linoleic acid was included, but he found no lipoxidase activity in commercially frozen beans that, based upon a concurrent accumulation of fat peroxides, were presumed to lose chlorophyll by reactions with intermediates of fat peroxidation. An unknown source of fat peroxides was invoked. And while added lipoxidase will enhance chlorophyll loss from pea homogenates, so will peroxidase and catalase [4].

H_2O_2 has also been implicated in the bleaching of chlorophyll during senescence or fruit ripening. Chlorophyll is bleached during oxidation of glycolic acid catalysed by α-hydroxy acid dehydrogenase [5–7] elevated levels of H_2O_2 have been reported during ripening of pears [8], and infiltration of pears by glycolate or xanthine increased both peroxide levels and rates of ripening. Inhibition of catalase promoted ripening, while inhibition of glycolate oxidase delayed ripening and reduced H_2O_2 levels. Catalase activities reportedly decline during senescence of tobacco [9–11] and rice [12] leaves, and during degreening of 'Shamouti' orange flavedo [13], but not during senescence of wheat or barley leaves [9, 10].

Recently it has been reported that chlorophyll is effectively bleached by H_2O_2 when catalysed by peroxidase in the presence of certain phenols [14, 15]; a common oxidative system in plants. This study is a detailed investigation of chlorophyll catabolism catalysed by a peroxidase extracted from peels of 'Valencia' oranges.

RESULTS

When crude extracts of acetone powders are used as a source of enzyme the reaction in the absence of a phenol proceeds as illustrated by the central curve (c) of Fig. 1. A lag phase is followed by a period of rapid bleaching of chlorophyll, followed in turn by a period of relatively slow bleaching. If H_2O_2 is replenished at this point (break in line) rapid bleaching resumes without an additional lag phase.

Lines a and b in Fig. 1 are representative results obtained using crude extracts that have been heated at 100° for 15 min (a) or dialysed for 48 hr against 12 l. of deionized water at 4° in three changes. Activity lost during dialysis was regained by including 2,4-

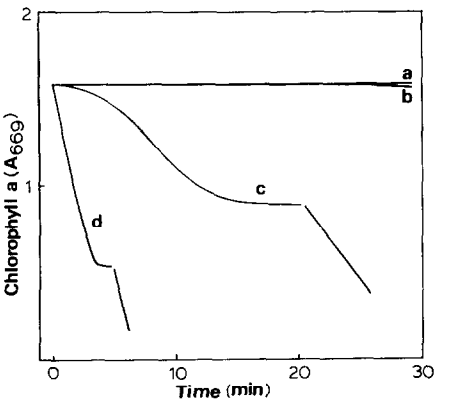

Fig. 1. Chlorophyll bleaching in presence of H_2O_2 and (a) boiled extract; (b) dialysed extract; (c) crude extract; (d) Me_2CO precipitated enzyme with 2,4-dichlorophenol included in reaction medium. Fresh H_2O_2 was added during breaks in lines c and d.

dichlorophenol in the reaction medium. Bleaching then proceeded much as with crude extracts.

If the enzyme was precipitated from crude extracts by addition of 3 vols of Me_2CO, redissolved in buffer, and then used with 2,4-dichlorophenol there was no lag phase and bleaching was nearly linear until coming to an abrupt halt (curve c, Fig. 1). Here again, replenishing H_2O_2 restored bleaching.

Crude extracts of the enzyme from Me_2CO powders of citrus flavedo apparently contain a dialysable promotor or cofactor that can be replaced by some phenols and a nondialysable inhibitor that can be separated from the enzyme by precipitating the latter with Me_2CO.

The inhibitor causes a lag in chlorophyll *a* bleaching as well as reductions in the rate and extent of bleaching, and in part appears to act by destroying H_2O_2. Bleaching using the partially purified enzyme proceeded without a lag phase, but as inhibitor (boiled extract) was added at 15, 40, 60, and 80% that of the crude extracts, the lag phase lengthened, the slope of reaction at its maximum became smaller, and the extent of reaction became less. After bleaching of chlorophyll had ceased, fresh H_2O_2 would restore activity at a similar rate, but without a lag phase. Thus, the reaction ceased mainly because H_2O_2 was depleted and as more inhibitor was added there was progressively less chlorophyll *a* destroyed by the H_2O_2 consumed.

The following experiments characterizing features of the reaction were done using Me_2CO-precipitated enzyme or enzyme that had been further purified by passage through a column of Sephadex G-75. Enzyme activity eluted between 39 and 66 ml ($V_e/V_0 = 1.46$, MW *ca* 40000), and the fraction from 42 to 62 ml was used in the following experiments. Sp. acts. increased *ca* 11-fold after Me_2CO precipitation and doubled again after passage through the column (Table 1).

Chlorophyll peroxidase activity is low at low ionic strengths (Fig. 3) but increases five-fold as ionic strength increases to an optimum at 0.11 ± 0.01. At higher ionic strengths, activity is increasingly depressed. Similarly, H_2O_2 concentration is optimal at $222 \pm 15\ \mu M$ (Fig. 4), higher concentrations are inhibitory and activity falls off sharply to zero at lower concentrations. Optimum pH for the reaction was 5.9 ± 0.1.

As previously mentioned, enzyme preparations lost activity during dialysis and this activity could be regained by inclusion of certain phenols. Phenol, resorcinol and 2,4-dichlorophenol were active in promoting chlorophyll bleaching (Table 2) while hydroquinone and catechol were not. Hydroquinone and catechol also inhibited bleaching in the presence of resorcinol. In addition, catechol caused the absorbance of chlorophyll *a* to increase when mixed with resorcinol. This enhanced absorption was more pronounced in the blue region than red.

Using substrate concentrations between 0.2 and 14 μM, K_m and V_{max} were determined for chlorophyll *a*, chlorophyllide *a*, pheophytin *a*, and chlorophyll *b* (Table 3). At chlorophyll *a* and *b* concentrations greater than 10 μM, reaction rates were somewhat

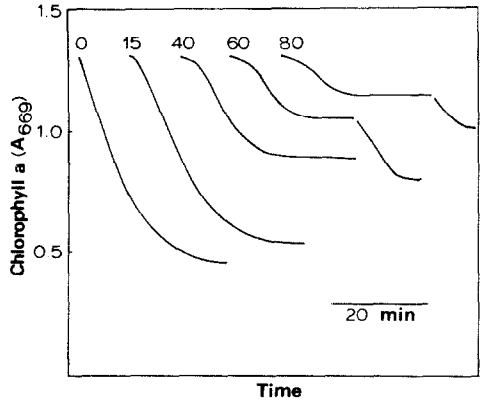

Fig. 2. Inhibition of chlorophyll bleaching by naturally occurring inhibitors. A crude extract was held at 100° for 15 min to inactivate chlorophyll peroxidase and then added to purified preparations of the enzyme so that inhibitors were present at 15, 40, 60, and 80% of their concentrations in crude extracts. The concentration of active enzyme was held constant.

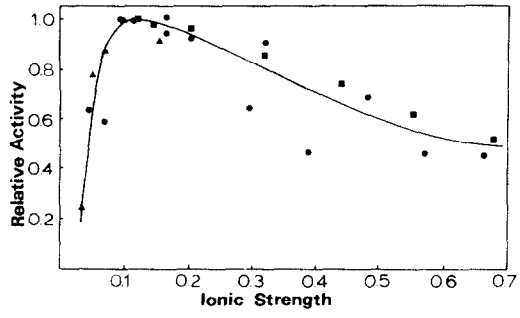

Fig. 3. Relative activity of chlorophyll peroxidase at varying ionic strengths, using K-Pi (●), sodium citrate (▲), and 44.4 mM NaCl with sodium citrate (■).

Table 1. Purification of chlorophyll: H_2O_2 oxidoreductase from *Citrus* peel

Sample	Volume (ml)	Protein (mg)	Activity ($-\Delta A_{669}$/hr)	Recovery (%)	Sp. act. ($-\Delta A_{669}$/hr mg protein)
Crude extract*	100	288	399	100	1.4
Me_2CO precipitate	19	40	606	152	15.2
Sephadex G-75	15	6.9	223	37	32.3

*Extracted from 2.67 g Me_2CO powder.

Table 2. Chlorophyll: H_2O_2 oxidoreductase activity in presence of selected phenols

Phenol	Activity ($-\Delta A_{669}$/hr)* Concentration of phenol 5×10^{-5}	5×10^{-4}	$5\times10^{-4}+5\times10^{-4}$ resorcinol
Phenol	0.16	0.89	3.18
Catechol	0.00	0.00	−0.15†
Resorcinol	0.85	3.33	—
Hydroquinone	0.00	0.00	0.00
2,4-Dichloro-phenol	0.65	2.19	4.36

*Each reaction mixture contained 138 μg protein.
†A at 669 nm increased 4.6%. A at 428 nm increased 36%.

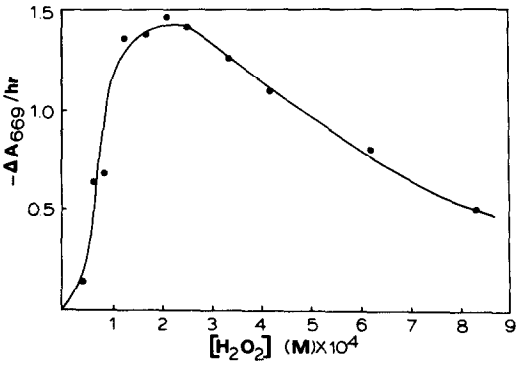

Fig. 4. H_2O_2 concentration effects on chlorophyll peroxidase activity.

Table 3. Apparent kinetic constants for chlorophyll: H_2O_2 oxidoreductase substrates*

Substrate	K_m (μM)	V_{max} (μM/hr per mg enzyme)
Chlorophyll *a*	2.1 ± 0.4	93 ± 15
Chlorophyllide *a*	0.9 ± 0.5	95 ± 15
Pheophytin *a*	18.5 ± 3.1	37 ± 5
Chlorophyll *b*	6.2 ± 0.9	86 ± 15

*Constants ± s.e. were estimated by the method of least squares using enzyme purified through the gel filtration step. Each reaction mixture contained 230 μg protein.

reduced and a slight lag period appeared, indicative of substrate inhibition. Consequently, values for K_m and V_{max} for chlorophylls *a* and *b* and chlorophyllide *a* were estimated using concentrations less than 9 μM. Pheophytin at concentrations up to 14 μM did not exhibit a similar behavior.

Chlorophyll *a*, chlorophyllide *a* and chlorophyll *b* have similar V_{max} in the reaction, but the K_m is somewhat higher for chlorophyll *b* than chlorophyll *a*, and when mixed preparations were used as substrates (Chl *a*/Chl *b* = 1.6) initial rates of chlorophyll *a* loss were from 6 to 8 times greater than those of chlorophyll *b* (Table 4). Chlorophyll *a* continued to be lost at a faster rate than chlorophyll *b* after chlorophyll *a* concentrations became lower than chlorophyll *b* concentrations. Chlorophyllide *a* behaved similarly to chlorophyll *a*. Pheophytin *a* was a much poorer substrate than any of the other three, with a larger K_m and a V_{max} only 41–45% those of chlorophyll *a* and *b* and chlorophyllide *a*. Magnesium (5 mM) in the reaction mixture had no effect on the rate of pheophytin destruction (data not shown).

DISCUSSION

Chlorophyll peroxidase extracted from flavedo of oranges catalyses destruction of chlorophyll by H_2O_2 in a reaction apparently coupled with peroxidation of certain phenols. Reactions of this type catalysed by horseradish peroxidase are well known [16] and the flavedo enzyme is similar to horseradish peroxidase in its MW, pH optimum, and response to excess H_2O_2. In addition, horseradish peroxidase and extracts of several other tissues are highly active in catalysing chlorophyll peroxidation when used in place of flavedo extracts (data not shown). Thus, the flavedo enzyme should be considered a typical peroxidase and chlorophyll peroxidation added to the number of reactions catalysed by the peroxidase system.

It is likely that the inhibitors extracted with the

Table 4. Preferential destruction of chlorophyll *a* in presence of chlorophyll *b*

Time (min)	[Chl *a*] (μM)	[Chl *b*] (μM)	[Chl *a*]/[Chl *b*]
0	5.85	3.77	1.55
10	4.23	3.48	1.22
20	3.35	3.23	1.04
73	2.09	3.02	0.69

enzyme are polymeric polyphenols of the kind inhibiting peroxidase-catalysed peroxidation of indole-3-acetic acid [17, 18], as chlorophyll peroxidation is inhibited by *o*- and *p*-dihydric phenols while promoted by monophenols and *m*-dihydric phenols, and because the inhibitors are not separated from the enzyme by dialysis. The inhibitors apparently consume H_2O_2 before it can act on chlorophyll, either directly or indirectly. Replenishing H_2O_2 re-initiates the destruction of chlorophyll without a lag phase, but since the extent of reaction with a fresh charge of H_2O_2 is not much altered and the rate of bleaching is only slightly more rapid, it does not appear that the inhibitor is appreciably consumed along with H_2O_2.

Peroxidation of pheophorbides was not investigated; however, it is anticipated that the reactions would proceed at rates similar to the corresponding pheophytins since the phytol substituent has little effect on the apparent K_m or V_{max}. In contrast, magnesium coordinated with the tetrapyrole ring markedly facilitates the reaction, reducing K_m for chlorophyll *a* to one-tenth the value for pheophytin *a*, and increasing V_{max} 2.3 times. When chlorophyll is lost during senescence, chlorophyll *a* always decreases more rapidly than chlorophyll *b* [2]. This is also true of degreening oranges [19] and would be readily explained by the observed substrate specificities. The more rapid destruction of magnesium-containing pigments compared with those lacking magnesium may serve as another useful criterion in evaluating any role for peroxidase-catalysed peroxidation of chlorophylls during ripening or senescence.

At the present time, there is no direct evidence that a reaction of this type participates in the destruction of chlorophyll during senescence or ripening, but some results and observations suggest that possibility. For example, when pears ripen, both peroxide levels [8] and particulate bound peroxidase levels [20] have been observed to increase, and factors affecting H_2O_2 production by pear tissues correspondingly affect pear ripening [8]. Similarly, catalase levels are often observed to decline during senescence, although not universally [11, 12, 21], and during color development of 'Shamouti' oranges, catalase levels in flavedo decline along with chlorophyll [13]. Possibly as cytoplasmic catalase levels decline, more H_2O_2 penetrates the chloroplasts and participates in chlorophyll degradation. As pointed out by Robinson *et al.* [22], the ability of H_2O_2 to enter the chloroplast is demonstrated by the effects exogenous H_2O_2 has on photosynthetic carbon metabolism [23–26]. In addition, H_2O_2 is generated within the chloroplast by reduction of O_2 under certain conditions [22, 26–31].

Both peroxidase [32, 33] and phenols [34] are also reportedly found within the chloroplast. Peroxidase activity has been observed apparently bound to internal membranes while simple phenols and flavanoids are located in the stroma. Thus, the necessary elements for peroxidase-catalysed oxidation of chlorophyll by H_2O_2 appear to be present within the chloroplast at one time or another. Whether or not they come together in appropriate fashion during senescence or ripening remains to be demonstrated, but this does seem to warrant further study.

EXPERIMENTAL

Me_2CO powders were prepared from flavedo of 'Valencia' oranges, and chlorophyll peroxidase (chlorophyll: hydrogen peroxide oxidoreductase, EC 1.11.1.7) extracted from powders with 100 mM K-Pi buffer (pH 7) containing 0.5% Triton X-100. After precipitating the enzyme by adding 3 vols of Me_2CO and centrifuging 10 min at 10 000 rpm and 0°, the pellet was redissolved in 100 mM K-Pi buffer (pH 5.9) and passed through a 20 mm × 330 mm column of Sephadex G-75.

Chlorophyll was extracted from fresh spinach leaves with MeOH and separated from carotenoids by precipitation with dioxane as described in ref. [35]. After redissolving chlorophylls in 92% MeOH, chlorophylls *a* and *b* were separated by partitioning between petrol and 92% MeOH until petrol solns of chlorophyll *a* with less than 9% chlorophyll *b* were obtained. These solns were dried (Na_2SO_4) and stored at −20°.

For determining substrate specificities, chlorophyll *b* in the MeOH phase was transferred to petrol after adjusting the H_2O content to greater than 20%. Both chlorophyll preps were then further purified by passage through a powdered sucrose column using 0.5% *iso*PrOH in petrol as solvent [36].

Pheophytin *a* was prepared by washing an Et_2O soln of purified chlorophyll *a* with 6 M HCl. The Et_2O soln was then washed ×4 with salted H_2O, dried (Na_2SO_4) and purified by passage through a powdered sucrose column using 0.5% *iso*PrOH in petrol.

Chlorophyllide *a* was prepared from purified chlorophyll *a* prepns by solubilizing in 100 mM K-Pi buffer (pH 7) containing 0.5% Triton X-100, adding chlorophyllase, and after 16-hr incubation in the dark removing unreacted chlorophyll *a* by extraction with a 1:2 mixture of Me_2CO and hexane. Chlorophyllide *a* was then extracted from the reaction buffer with Et_2O, which was then washed several times with salted H_2O, dried (Na_2SO_4) and stored at −20°.

After transfer to Et_2O, the purity and amounts of each substrate prepared were estimated using spectra and specific absorptions as described in ref. [37].

Chlorophyllase was extracted from Me_2CO powders of orange flavedo with buffer (100 mM K-Pi, 0.50 M NaCl, 0.5% Triton X-100, pH 7) and the supernatant collected after centrifugation at 10 000 rpm for 10 min at 0°. Chlorophyllase was then partially purified by making the supernatant 50% satd with $(NH_4)_2SO_4$. After centrifugation for 10 min at 10 000 rpm at 0°, chlorophyllase was recovered as a yellow flotation layer, resolubilized in buffer and reprecipitated once. The second flotation layer was used for prepn of chlorophyllide *a* after resolubilizing in buffer.

Aq. solns of substrates were prepared by layering an Et_2O soln over appropriate buffers containing 0.25% Triton X-100, then removing Et_2O by vacuum while gently agitating to facilitate substrate solubilization. Final assay conditions chosen were *ca* 16.5 μM chlorophyll in either 45 mM Na succinate, or 100 mM K-Pi buffer at pH 5.9 containing 0.25% Triton X-100 and 316 μM 2,4-dichlorophenol.

Peroxidation of substrates was done by adding 0.5 ml of 100 mM K-Pi buffer (pH 5.9) containing enzyme to 3 ml of buffer containing substrate followed by addition of 1 ml of 0.97 mM H_2O_2 to start the reaction. Progress was generally followed by recording substrate *A* in 1-cm cells at their

longest *A* maximum (chlorophyll *a*, 669 nm). Simultaneous destruction of chlorophylls *a* and *b* in mixed solns was followed by periodically withdrawing samples from the reaction medium. Unreacted chlorophylls were extracted into Et_2O and estimated spectrophotometrically [38]. Apparent kinetic constants were estimated by the method of least squares [39].

Acknowledgements—Janice Palma, Girija Kuttan and Lynda Delph provided valuable technical assistance that is greatly appreciated.

REFERENCES

1. Schobert, B. and Elstner, E. F. (1980) *Plant Physiol.* **66**, 215.
2. Simpson, K. L., Lee, T., Rodriguez, D. B. and Chichester, C. O. (1976) in *Chemistry and Biochemistry of Plant Pigments* (Goodwin, T. W., ed.) Vol. 1, pp. 779–843, Academic Press, New York.
3. Walker, G. C. (1964) *J. Food Sci.* **29**, 383.
4. Wagenknecht, A. C. and Lee, F. A. (1958) *Food. Res.* **23**, 25.
5. Kolesnikov, P. A. (1948) *Dokl. Akad. Nauk SSSR* **60**, 1353.
6. Kolesnikov, P. (1949) *Biokhimiya* **14**, 124.
7. Tolbert, N. E. and Burris, R. H. (1950) *J. Biol. Chem.* **186**, 791.
8. Brennan, T. and Frenkel, C. (1977) *Plant Physiol.* **59**, 411.
9. Farkas, G. L., Dezsi, L., Horvath, M., Kisban, K. and Udvardy, J. (1964) *Phytopathol. Z.* **49**, 343.
10. Kisban, K., Horvath, M., Dezsi, L., Udvardy, J. and Farkas, G. L. (1964) *Acta. Bot. Acad. Sci. Hung.* **10**, 275.
11. Parish, R. W. (1968) *Planta* **82**, 1.
12. Kar, M. and Mishra, D. (1976) *Plant Physiol.* **57**, 315.
13. Monselise, S. P. and Goren, R. (1965) *Phyton* **22**, 61.
14. Aljuburi, H., Huff, A. and Hshieh, M. (1979) *Plant Physiol.* **63**(Suppl.), 73.
15. Matile, Ph. (1980) *Z. Pflanzenphysiol.* **99S**, 475.
16. Saunders, B. C., Holmes-Siedle, A. G. and Stark, B. P. (1964) *Peroxidase.* Butterworths, London.
17. Cassidy, H. G. (1966) *Am. Sci.* **54**, 184.
18. Stonier, T., Singer, R. W. and Yang, H. (1970) *Plant Physiol.* **46**, 454.
19. Jahn, O. L. and Young, R. (1976) *J. Am. Soc. Hortic. Sci.* **101**, 416.
20. Haard, N. F. (1973) *Phytochemistry* **12**, 555.
21. Mattoo, A. K. and Modi, V. V. (1969) *Plant Physiol.* **44**, 308.
22. Robinson, J. M., Smith, M. G. and Gibbs, M. (1980) *Plant Physiol.* **65**, 755.
23. Allen, J. F. (1978) *Plant Sci. Letters* **12**, 151.
24. Allen, J. F. (1978) *Plant Sci. Letters* **12**, 161.
25. Heldt, H. W., Chon, C. J., Lilley R. Mc. C., Portis, A. (1978) in *Proc. 4th Int. Cong. Photosynthesis* (Hall, D. O., Coombs, J. and Goodwin, T. W., eds.) p. 469. Biochemical Society, London.
26. Kaiser, W. (1976) *Biochim. Biophys. Acta* **440**, 476.
27. Egneus, H., Heber, U., Matthiesen, U. and Kirk, M. (1975) *Biochim. Biophys. Acta* **408**, 252.
28. Forti, G. and Gerola, P. (1977) *Plant Physiol.* **59**, 859.
29. Heber, U. and French, C. S. (1968) *Planta* **79**, 99.
30. Huber, S. and Edwards G. (1975) *Biochem. Biophys. Res. Commun.* **67**, 28.
31. Marsho, T. V., Behrens, P. W. and Radmer, R. J. (1979) *Plant Physiol.* **64**, 656.
32. Henry, E. (1975) *J. Ultrastruct.* **52**, 289.
33. Hurkman, W. J. and Kennedy, G. S. (1977) *Am. J. Botany* **64**, 86.
34. Kirk, J. T. O. and Tilney-Bassett, R. A. E. (1978) *The Plastids: Their Chemistry, Structure, Growth and Inheritance.* Elsevier–North-Holland Biomedical Press, New York.
35. Iriyama, K., Ogura, N. and Takamiya, A. (1974) *J. Biochem.* **76**, 901.
36. Perkins, H. J. and Roberts, D. W. A. (1962) *Biochim. Biophys. Acta* **58**, 486.
37. Zscheile, F. P. and Comar, C. L. (1941) *Bot. Gaz.* **102**, 463.
38. Strain, H. H. and Svec, W. A. (1966) *The Chlorophylls* (Vernon, L. P. and Seely G. R., eds.) pp. 21–66. Academic Press, New York.
39. Wilkinson, G. N. (1961) *Biochem. J.* **80**, 324.