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PROPERTIES OF ACYL HYDROLASE ENZYMES FROM *PHASEOLUS MULTIFLORUS* LEAVES

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Key Word Index—*Phaseolus multiflorus*; Leguminosae; runner bean leaves; acyl hydrolase; enzyme properties; transmethylation; sulpholipid breakdown.

Abstract—The properties of acyl hydrolase enzymes purified from the leaves of *Phaseolus multiflorus* have been studied. Hydrolase I which deacylates phosphatidylcholine and oleoylglycerol had a pH optimum towards phosphatidylcholine of 5.3. Hydrolase II which deacylates glycosylglycerides and oleoylglycerol showed pH optima of 7.3 (monogalactosyldiglyceride, MGDG) and 4.3 (sulphoquinovosyldiglyceride, SQDG). Both enzymes showed activity peaks towards oleoylglycerol at pH 6.8 and 8.8. Unesterified fatty acids and Triton X-100 inhibited the rate of SQDG hydrolysis while bovine serum albumin increased activity. An apparent K_m for SQDG of 0.15 mM was found. Hydrolase II catalysed transmethylation of liberated fatty acids during the hydrolysis of oleoylglycerol when methanol was included in the assay system. A number of salts inhibited SQDG hydrolysis but their effect on oleoylglycerol was less consistent. The position of ester cleavage of oleoylglycerol was determined by the use of $H_2{}^{18}O$. Cell-free extracts from *P. multiflorus* leaves degraded SQDG as far as sulphoquinovose.

INTRODUCTION

In contrast to animal and microbial phospholipases, which in some cases are quite specific [1], no such property has been clearly demonstrated in plants. For example, 'sulpholipase' was also active with phospholipids [2] and galactolipid-hydrolysing enzymes from *Phaseolus* leaves [3, 4] had higher activities with lysophospholipids and monoacylglycerol [5]. Non-specific acyl hydrolases from potato tubers have been studied in several laboratories (e.g. [5–8]). Similar enzymes have also been reported from *P. vulgaris* leaves [9, 10], rice bran [11], potato leaves [12], spinach leaves [13] and *P. multiflorus* leaves [4, 14–16].

Previous work on acyl hydrolase activity from *P. multiflorus* leaves showed that it was possible to partially purify two enzymes, one of which would preferentially hydrolyse phosphatidylcholine (PC) rather than glycosylglycerides and a second which had the opposite specificity. Both preparations would deacylate oleoylglycerol (OG). The MW of the glycosylglyceride-hydrolysing enzyme was in the range 70–78 000 [16]. Additional experiments had revealed that the enzyme would cleave both positions of the acyl substrates [14], with higher activity towards the monoacyl derivatives [15] in a similar manner to the recently purified enzymes from other plant tissues [10–12]. This property contrasted, therefore, with the step-wise degradation of the plant sulpholipid (diacylsulphoquinovosylglycerol, SQDG) by extracts from *Scenedesmus obliquus* [2] or *Chlorella pyrenoidosa* [17].

The photosynthetic tissues of higher plants are rich in potential acyl lipid substrates for the acyl hydrolases. Characteristic of chloroplast lamellae are the three glycosylglycerides diacylgalactosylglycerol (monogalactosyldiglyceride, MGDG), diacylgalabiosylglycerol (digalactosyldiglyceride, DGDG) and SQDG. In contrast, PC is the major lipid of non-photosynthetic membranes. While the acyl hydrolases play an obvious role during such processes as senescence [18], they are also important in the normal turnover of leaf acyl lipids (c.f. [19]). Furthermore, it has been noted in several systems that the hydrolysis of photosynthetic membranes by acyl hydrolases causes a rapid impairment of their physiological function (e.g. [20]). Accordingly, we have now examined some of the properties of the acyl hydrolase activities from *P. multiflorus* leaves.

RESULTS AND DISCUSSION

A number of important properties for the *P. multiflorus* acyl hydrolases have already been demonstrated. The enzymes are able to cleave both the 1- and 2-positions of acyl lipids and, since monoacyl substrates were apparently hydrolysed faster than the diacyl substrates, no lyso intermediates accumulated during the reaction [14]. This was in contrast to SQDG hydrolysis in *Scenedesmus obliquus* [2] and *Chlorella pyrenoidosa* [17] but agrees with recent data for acyl hydrolases from potato tuber [9] and leaves [12], rice bran [11] and *P. vulgaris* leaves [10]. The enzymes from *P. multiflorus* were relatively unspecific with regard to substrates hydrolysed. Partial separation of two enzymes was achieved [16] with the less hydrophobic enzyme (hydrolase I) hydrolysing OG and PC while the

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other (hydrolase II) hydrolysed OG and glycosylglycerides. Until the final stage of purification (hexyl-agarose chromatography), the acyl hydrolase resembled those from potato [9, 12], rice bran [11] or *P. vulgaris* [10] in that it would hydrolyse a large number of acyl lipids. The relative rate of hydrolysis of these different substrates was very similar for the *P. multiflorus* enzyme when compared with those from a range of plant sources. The general order of hydrolysis was OG > lyso PC > MGDG > DGDG > PC > SQDG > triacylglycerol [15, 19]. The *P. multiflorus* enzyme also cleaved molecular species of SQDG in the order dilinolenoyl- > palmitoyl, linolenoyl- > dipalmitoyl- [14].

The pH profile of the hydrolysis of SQDG was studied. The acyl hydrolase preparation which had been purified up to the hydrophobic chromatography stage had a broad pH optimum of *ca* pH 4.6. A similar broad pH curve was obtained for the hydrolysis of MGDG and DGDG by a crude preparation from runner bean leaves [3]. On further purification, the pH optimum was shifted to *ca* 4.2–4.3. This alteration in pH profile for SQDG hydrolysis may have been due to the removal of a surface-active agent during hydrophobic chromatography since it has been noted with the potato tuber enzyme that the addition of surface-active compounds caused a large shift in the pH optimum [6].

The pH profiles were also determined for the two purified acyl preparations towards OG, PC and MGDG. Hydrolase I which contained phospholipase A rather than glycosylglyceride acyl hydrolase activity showed a pH optimum with PC as substrate of *ca* 5.3. This preparation showed a bimodal curve of hydrolysis towards oleoylglycerol with peaks at pH 6.8 and 8.8. The second acyl hydrolase (hydrolase II) gave a pH optimum for MGDG of 7.3 and a bimodal pH curve profile towards OG which was very similar to hydrolase I. These values can be compared with the recent data from Hirayama's group on purified acyl hydrolases. They found that hydrolysis of MGDG by the rice bran enzyme had a pH optimum of 7.5 [11] and of 5.3 for the enzymes from *P. vulgaris* and potato leaves [10, 12]. The latter enzyme had a pH optimum for PC of 5 [12] whereas the enzyme from *P. vulgaris* had an optimum of 6.5 [10].

It had previously been noted [14] that the rate of SQDG hydrolysis started to fall significantly after 60 min in spite of the fact that only a small percentage of the substrate had been hydrolysed at that point. It was also noted that unsaturated molecular species of SQDG were hydrolysed preferentially. This result was also found with PC: distearoyl-, dipalmitoyl- and dioleoyl-species being hydrolysed at 2.72, 2.66 and 5.52 μmol/hr/mg protein, respectively. These rates were approximately doubled when the substrates were extensively sonicated and it was assumed that the physical size of the micelles caused the different rates of hydrolysis of molecular species.

In an effort to increase the rates of hydrolysis and the linearity of the reaction, detergents were added. None of these were effective; in fact Triton X-100 at 0.3% concentrations caused 52% inhibition (cf. Table 1). The potato lipolytic acyl hydrolase was stimulated by unesterified fatty acids with PC as substrate but was unaffected with OG as substrate [21]. The effect of added linoleic acid or bovine serum albumin (BSA) additions on SQDG hydrolysis is shown in Fig. 1. The results show that unesterified fatty acids cause an inhibition of acyl hydrolase activity and this conclusion is confirmed by the stimulatory effect of BSA. However, it will be seen that addition of BSA failed to increase the linearity of the enzyme reaction which might have been expected if the slowed hydrolysis was due to accumulation of unesterified fatty acid products. Moreover, BSA was not routinely added to the incubation system since it caused difficulties in the quantitative extraction of fatty acid products.

Another possible reason for the non-linearity of substrate hydrolysis might have been the removal of products by the action of lipoxygenase which has been reported in extracts of runner bean leaves [22]. Accordingly, enzyme preparations were monitored for such activity but none was detected.

The rate of hydrolysis of SQDG was followed over a 60 min period at SQDG concentrations varying between 27 and 266 μM. Initial reaction rates were extrapolated and

Table 1. The effect of various reagents on the hydrolysis of SQDG or OG

Addition	Final concn of reagent	SQDG substrate Measured pH	SQDG substrate Activity (μmol/hr/mg protein)	OG substrate Measured pH	OG substrate Activity (μmol/hr/mg protein)
None	—	4.5	1.64	7.0	31.64
$MgCl_2$	10 mM	4.7	0.73	6.9	24.09
$MnCl_2$	10 mM	4.6	0.59	6.4	46.36
$FeSO_4$	10 mM	4.6	0.46	6.6	18.18
Fe_2Cl_3	10 mM	3.8	0.18	5.9	23.64
$CaCl_2$	10 mM	4.6	0.46	6.8	42.27
EDTA	5 mM	4.6	1.55	7.0	29.32
EGTA	5 mM	4.5	1.55	7.0	34.14
o-Phenanthroline	5 mM	4.8	1.50	7.1	31.81
Triton X-100	0.3%	4.7	0.86	—	—
Triton X-100	0.6%	4.7	0.86	—	—
Triton X-100	0.3%	5.7	0.82	—	—
Triton X-100	0.6%	5.7	0.41	—	—

Assays were with the hydrolase II peak from the hexyl-agarose column (see Experimental).

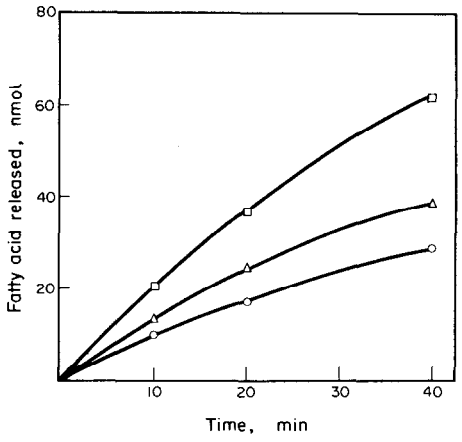

Fig. 1. Effect of fatty acids and bovine serum albumin on SQDG hydrolysis. The enzyme preparation had been purified as far as the hexyl-agarose column. △ — △, Control assay; □ — □, assay in the presence of 0.3 mg bovine serum albumin; ○ — ○, assay in the presence of 0.15 mg linoleic acid.

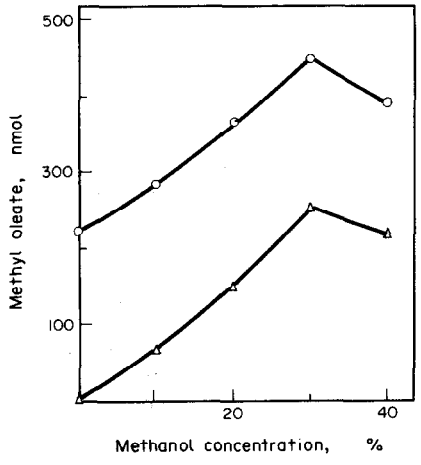

Fig. 2. Effect of methanol on hydrolysis of OG and transmethylation of the fatty acid product. The enzyme preparation contained the combined active fractions after hexyl-agarose chromatography. ○—○, Fatty acid methyl esters after methylation with diazomethane; △—△, fatty acid methyl ester reaction products.

an 'apparent K_m' found to be 0.15 mM. A V_{max} of 1.09 μmol fatty acids released/hr/mg protein was also obtained. These values compare with K_m values of 0.65 and 0.31 mM obtained by Helmsing [4] for MGDG and DGDG and Sastry and Kates [3] found 7.8 and 1.5 mM respectively for these lipids with their preparations of the runner bean enzyme. K_ms for MGDG hydrolysis of 0.34, 0.24, 0.26 and 0.80 mM respectively were obtained for the acyl hydrolases from rice bran [11], *P. vulgaris* [10], potato leaves [12] and potato tubers [6]. Because of the K_m we had determined for SQDG, we used concentrations of 1.5 mM routinely for assay. This was below the concentration of substrate found to be inhibitory for the unspecific acyl hydrolase from potato tubers [22].

Under appropriate conditions, acyl transferase activity would predominate over acyl hydrolysis for the potato enzyme [5]. The existence of such transferase activity was examined using a purified runner bean enzyme. OG was used as a substrate at pH 7 with increasing concentrations of methanol up to 40%. The results (Fig. 2), in agreement with data for the potato enzyme [5], showed that 30% methanol gave optimal rates of acyl transferase activity.

A number of acyl hydrolases from plant tissues are known to be susceptible to changing cation concentrations as well as various sulphydryl reagents. Different salts and chelating agents were tested on the hydrolysis of SQDG and OG (Table 1). The rate of hydrolysis was unaffected by the chelators but was reduced for SQDG by various salts. The inhibition by ferric chloride may, in part, have been due to the pH shift and the cation effects may have been caused by the insolubility of the different salt forms of SQDG. Indeed, it has been reported [23] that sulphoquinovose was precipitated by calcium ions and that SQDG deacylation in *Chlorella* was inhibited by calcium and magnesium ions [17]. Because of the possibility of cation effects on SQDG substrate, the effect of similar additions on OG hydrolysis was also tested. In this case, magnesium chloride, ferric chloride and ferrous sulphate were inhibitory while manganese chloride and calcium chloride were stimulatory. As with SQDG hydrolysis, the addition of chelators had no effect (Table 1). In a similar way, the recently purified acyl hydrolases from *P. vulgaris* and potato leaves and from rice bran [10–12] were variously sensitive to the addition of salts. Whereas PC hydrolysis could be stimulated 40-fold by the addition of Triton X-100 to the potato enzyme [6], the reagent only inhibited SQDG hydrolysis in the present work (Table 1). However, there was some evidence of a pH effect in this inhibition, perhaps due to a shift in the pH optimum because of the inclusion of the surface-active reagent (cf. [6] and previous discussion). Inhibition by Triton X-100 has also been noted for the galactolipase from rice bran [11]. In addition to the compounds shown in Table 1, a number of sulphydryl reagents were tested for inhibition of SQDG hydrolysis. These included sodium arsenite, *p*-chloromercuribenzoate, *N*-ethylmaleimide, iodoacetamide, 5′,5′-dithiobis (2-nitrobenzoic acid) and cysteine. None of the compounds, at the concentrations tested, produced any effect. Although sulphydryl inhibitors were found to be similarly ineffective with two other acyl hydrolase preparations [4, 11] cysteine was found in these cases to be a particularly effective inhibitor. As discussed before [14], it is possible that these inhibitions were due to changes in the pH of the solution on adding cysteine. The galactolipid acyl hydrolase from *P. vulgaris* [24] was

Table 2. MS analysis of the M^+ of oleic acid produced by the hydrolysis of OG in the presence of $H_2{}^{16}O$ or $H_2{}^{18}O$

Isotope of water	M^+ (oleic acid) *m/e*	Intensity of M^+ as percentage of base peak
$H_2{}^{16}O$	282	9.88
$H_2{}^{16}O$	284	0.91
$H_2{}^{18}O$	282	3.25
$H_2{}^{18}O$	284	3.56

$H_2{}^{18}O$ was used at a final enrichment of 48.1% in the medium.

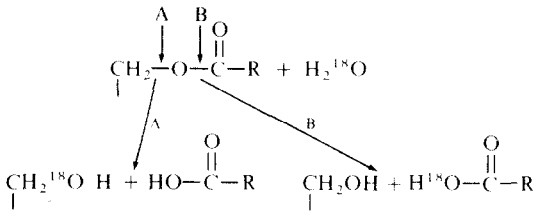

Fig. 3. Possible sites of fatty acyl ester cleavage.

unaffected by cysteine addition in agreement with the present work.

The position of hydrolysis of OG was investigated by the use of $H_2{}^{18}O$. In order to minimize possible exchange between $H_2{}^{18}O$ and the released fatty acids, the incubations were carried out at pH 5 or less. The hydrolysed unesterified fatty acids were isolated and examined by MS. If the hydrolysis took place at position A (Fig. 3) then the glycerol product should have been enriched with ^{18}O and if at position B then the oleic acid product would have been enriched. The original incubation was carried out in 62.5% ^{18}O-enriched H_2O which after dilution by enzyme and buffer was 48.1% enriched.

The results in Table 2 showed that the oleic acid product M^+ was enriched in ^{18}O to a similar level as the water. This clearly showed that OG was cleaved on the fatty acid side of the oxygen ester bond (i.e. mechanism B in Fig. 3).

Further breakdown of sulpholipid

Although the above properties concern acyl hydrolase activity in runner bean leaves, it is of interest to note some observations on the further breakdown of the deacylated product. Incubation of [^{35}S]-SQDG with homogenates of runner bean leaves released water-soluble radioactivity which was separated by TLC (see Experimental). Two radioactive peaks were observed, which corresponded respectively to sulphoquinovose and sulphoquinovosylglycerol. It was apparent, therefore, that the latter was degraded by the cell-free homogenate perhaps by the action of a glucosidase. Sastry and Kates [3] suggested that digalactosylglycerol was degraded by α-galactosidase to galactosylglycerol which in turn was cleaved by β-galactosidase to glycerol and galactose. These enzymes were detected in the soluble fraction from *P. multiflorus*. Lee and Benson [25] studied the metabolism of sulpholipid in the coral tree (*Erythrina crista-galli*) and alfalfa (*Medicago sativa*) leaves. They found breakdown of SQDG via sulphoquinovose to sulpholactate and sulphoacetate. We directly tested a purified α-glucosidase with [^{35}S]-sulphoquinovosylglycerol and [^{35}S]-SQDG but were unable to detect any [^{35}S]-sulphoquinovose under the conditions tested. Runner bean extracts are, therefore, capable of degrading sulpholipid at least as far as sulphoquinovose. The nature of the enzyme cleaving sulphoquinovosylglycerol is, however, unknown at present.

EXPERIMENTAL

Substrates. DGDG, MGDG and SQDG were isolated from Swiss chard or Spring cabbage as previously described [14, 26]. Fatty acid standards and other lipids were obtained from Sigma. Purity of substrates was checked before use by TLC [14].

Enzyme assays. Acyl hydrolase activity was routinely determined using the CH_2N_2 procedure as described in ref. [14]. Quantitation was with an int. standard of pentadecanoic acid. Fatty acid Me esters were separated by GLC on 15% diethylene glycol succinate on Chromosorb W AW or on 15% EGSS-X on Supelcoport columns at 185°. Lipoxygenase activity was assayed with linolenic acid substrate [27] and α-glucosidase (yeast) with maltose [28]. Protein was determined, after precipitation with 10% TCA and solubilization at 100° for 5 min with M NaOH, by the method of ref. [29]. Where the total protein in the sample was less than 10 μg, the method of ref. [30] was used. In both cases, BSA was used as standard.

Enzyme purification. Runner bean seeds (*P. multiflorus* cv Scarlet Emperor) were obtained from Thompson and Morgan. Leaves were harvested from plants grown in John Innes No. 2 compost with normal daylight conditions between 2 and 4 weeks of growth. Leaves were homogenized and the 6.1 g min supernatant prepared as previously described [16]. Acyl hydrolase activity was purified by heat treatment, DEAE-cellulose chromatography, gel filtration and hexyl-agarose hydrophobic chromatography [16]. The last stage resulted in two active fractions. The first (hydrolase I) would deacylate PC and 1-oleoylglycerol (OG) and the second (hydrolase II) would deacylate glycosylglycerides (MGDG, DGDG, SQDG) and OG [16].

Catabolism of [^{35}S]-*SQDG.* [^{35}S]-SQDG was prepared biosynthetically by incubating runner bean leaves with [^{35}S]-sulphate [31]. [^{35}S]-Sulphoquinovose was prepared from [^{35}S]-SQDG by the method of ref. [32] and [^{35}S]-sulphoquinovosylglycerol by mild alkaline digestion and further purification by TLC on Si gel G using $CHCl_3$–MeOH–H_2O (8:5:1). Sulphoquinovose and sulphoquinovosylglycerol had R_fs of *ca* 0.14 and 0.78 in this system. Incubation of [^{35}S]-SQDG was carried out with a cell-free homogenate of runner bean leaves [16] and the reaction products separated by two-phase extraction [33]. $CHCl_3$-soluble products were separated by TLC on Si gel G using $CHCl_3$–MeOH–H_2O (65:25:4) and only [^{35}S]-SQDG was detected. H_2O-soluble products were separated by TLC on Si gel G using $CHCl_3$–MeOH–H_2O (8:5:1).

MS. $H_2{}^{18}O$ (62.5 atom % excess of ^{18}O; British Oxygen) was used in an incubation with purified acyl hydrolase at pH 5 and the samples analysed at 70 eV with a direct insertion probe at 150°.

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REFERENCES

1. Dawson, R. M. C. (1973) in *Form and Function of Phospholipids* (Ansell, G. B., Dawson, R. M. C., Hawthorne, J. N., eds.) pp. 97–116. Elsevier, Amsterdam.
2. Yagi, T. and Benson, A. A. (1962) *Biochim. Biophys. Acta* **57**, 601.
3. Sastry, P. S. and Kates, M. (1964) *Biochemistry* **3**, 1280.
4. Helmsing, P. J. (1969) *Biochim. Biophys. Acta* **178**, 159.
5. Galliard, T. and Dennis, S. (1974) *Phytochemistry* **13**, 1731.
6. Galliard, T. (1971) *Biochem. J.* **121**, 379.
7. Hasson, E. L. and Laties, G. G. (1976) *Plant Physiol.* **57**, 142.
8. Shepard, D. V. and Pitt, D. (1976) *Phytochemistry* **15**, 1471.
9. Galliard, T. (1975) in *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. I., eds.) pp. 319–357. Academic Press, London and New York.
10. Matsuda, H., Tanaka, G., Morita, K. and Hirayama, O. (1979) *Agric. Biol. Chem.* **43**, 563.

11. Matsuda, H. and Hirayama, O. (1979) *Agric. Biol. Chem.* **43**, 697.
12. Matsuda, H. and Hirayama, O. (1979) *Biochim. Biophys. Acta* **573**, 155.
13. Helmsing, P. J. (1967) *Biochim. Biophys. Acta* **144**, 470.
14. Burns, D. D., Galliard, T. and Harwood, J. L. (1977) *Phytochemistry* **16**, 651.
15. Burns, D. D., Galliard, T. and Harwood, J. L. (1977) *Biochem. Soc. Trans.* **5**, 1302.
16. Burns, D. D., Galliard, T. and Harwood, J. L. (1979) *Phytochemistry* **18**, 1793.
17. Wolfersberger, M. G. and Pieringer, R. A. (1974) *J. Lipid Res.* **15**, 1.
18. Draper, S. R. (1969) *Phytochemistry* **8**, 1641.
19. Galliard, T. (1979) in *Advances in the Chemistry and Physiology of Plant Lipids* (Appelqvist, L.-A. and Liljenberg, C., eds.) pp. 121–132. Elsevier, Amsterdam.
20. Okamoto, T., Katoh, S. and Murakami, S. (1977) *Plant Cell Physiol.* **18**, 551.
21. Galliard, T. (1971) *Eur. J. Biochem.* **21**, 90.
22. Wardale, D. A. and Galliard, T. (1975) *Phytochemistry* **14**, 2323.
23. Roughan, P. G. and Batt, R. D. (1968) *Analyt. Biochem.* **22**, 74.
24. Anderson, M. M., McCarty, R. E. and Zimmer, E. A. (1974) *Plant Physiol.* **53**, 699.
25. Lee, R. F. and Benson, A. A. (1972) *Biochim. Biophys. Acta* **261**, 35.
26. O'Brien, J. S. and Benson, A. A. (1964) *J. Lipid Res.* **5**, 432.
27. Galliard, T. and Phillips, D. R. (1971) *Biochem. J.* **124**, 431.
28. Gutmann, I. (1974) in *Methods of Enzymatic Analysis* (Bergmeyer, H. U., ed.) p. 1185. Verlag Chemie, Weinheim and Academic Press, New York.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265.
30. McKnight, G. S. (1977) *Analyt. Biochem.* **78**, 86.
31. Nicholls, R. G. and Harwood, J. L. (1979) *Phytochemistry* **18**, 1151.
32. Davies, W. H., Mercer, E. I. and Goodwin, T. W. (1966) *Biochem. J.* **98**, 369.
33. Garbus, J., DeLuca, H. F., Loomans, M. E. and Strong, F. M. (1963) *J. Biol. Chem.* **238**, 59.