Phytochemistry, Vol. 28, No. 11, pp. 2931–2935, 1989.
Printed in Great Britain.

0031-9422/89 $3.00+0.00

REVERSIBLE ENZYMATIC TRANSESTERIFICATION BETWEEN WAX ESTERS AND STEROLS IN *SINAPIS ALBA* ROOTS

MAŁGORZATA KALINOWSKA, JAN ZIMOWSKI, MARIA BUCHOLC and ZDZISŁAW A. WOJCIECHOWSKI

Department of Biochemistry, Warsaw University, 02-089 Warszawa, Al. Żwirki i Wigury 93, Poland

(*Received* 17 *February* 1989)

Key Word Index—*Sinapis alba*; Cruciferae; white mustard; steryl esters; wax esters; enzymatic transesterification.

Abstract—Young roots of mustard (*Sinapis alba*) contain a membrane-bound acyltransferase which catalyses esterification of cholesterol by direct transfer of acyl groups from fatty acid esters of long-chain primary alcohols (wax esters) or esterification of *n*-hexadecanol with the use of steryl esters as fatty acid sources. Acyl transfer from cholesteryl esters to free cholesterol or from *n*-hexadecanyl esters to *n*-hexadecanol can be also observed with this enzyme. The acyltransferase is located in the membranous structures sedimenting at 16 000 *g* but can be solubilized by acetone treatment and extraction with Tris–maleate, pH 7.3. With the solubilized enzyme the esterification of cholesterol, with *n*-hexadecanyl palmitate as the acyl source, showed two pH optima—at pH 6.4 and 8.4. In this reaction the best acyl donor among *n*-hexadecanyl esters varying in the fatty acid moiety was *n*-hexadecanyl palmitate. Our results suggest that the 'low energy' exchange of acyl groups may be of importance for steryl ester or/and wax ester synthesis in higher plants.

INTRODUCTION

Steryl esters and wax esters (esters of long-chain primary alcohols) are well established constituents of various tissues from many higher plants. However, the information available on their biosynthesis is still somewhat fragmentary.

In animal tissues sterols are esterified by two main pathways: (i) a 'high energy' acyl transfer from acyl-CoA or (ii) a 'low energy' acyl transfer from phosphatidylcholine [1, 2]. Neither of these reactions is evident in higher plants. *In vitro* studies with cell-free preparations obtained from spinach leaves [3–6], white mustard roots [7–9] and corn roots [10] have shown that various acylglycerols (glycerides) can be efficient acyl donors for sterol esterification. In all these plants the activity of acylglycerol:sterol acyltransferase was found to be associated with membranous fractions. However, some differences in the specificity of the enzyme preparations from individual plants were reported. The enzyme from spinach leaves [5, 6] preferentially required 1,2-diacylglycerols as acyl donors for sterol esterification while the enzyme from white mustard roots [8, 9] showed the highest activity with triacylglycerols; di- and monoacylglycerols were considerably less active. The acyltransferase present in corn roots [10] utilized tri-, 1,2-di- and 1,3-diacylglycerols as acyl sources.

Little is also known about biosynthetic pathways leading to the formation of wax esters. The results of experiments with acetone-delipidated enzyme preparations from broccoli leaves [11–13] indicated that wax esters can be synthesized by three independent mechanisms: (i) direct esterification of a fatty alcohol with fatty acid catalysed by the reversal of an esterase-type reaction; (ii) acyl transfer from acyl-CoA and (iii) acyl transfer from phosphatidylcholine or from some other phospholipids. More recently the first two of the above mechanisms were observed in a cell-free system from barley primary leaves [14]. In the course of our investigations on sterol esterification by enzyme preparations from white mustard roots we have found that under similar experimental conditions triacylglycerols (and, to a lower extent, diacylglycerols) could also serve as efficient acyl donors for esterification of fatty alcohols such as for example *n*-hexadecanol [15]. These results suggested that in white mustard roots mechanisms of biosynthesis of steryl esters and wax esters are similar, i.e. that both these ester types can be formed by acyl transfer from triacylglycerols.

In the present paper we describe some experiments which show that delipidated membranous fractions from white mustard roots can, in addition to their ability to catalyze the synthesis of steryl and wax esters with triacylglycerols as acyl sources, also catalyse reversible transesterification between wax esters and sterols.

RESULTS AND DISCUSSION

Our earlier studies [7–9] have shown that lipid-depleted membranous fractions obtained from mustard roots effectively catalysed esterification of cholesterol or some typical phytosterols such as sitosterol in the presence of a crude fraction of neutral lipids isolated from the same plant. With those enzyme preparations various natural and synthetic acylglycerols, preferentially triacylglycerols containing fatty acids of medium chain-length (C_{14}–C_{18}), were found to be efficient acyl donors for sterol esterification [9]. Many polar lipids such as various phospho- or glycoglycerolipids could not serve as acyl sources in this reaction [7, 8].

In an extension of those studies we have found that under similar experimental conditions acetone powder preparations from a 300–16 000 *g* membranous fraction obtained from roots of 10-day-old mustard seedlings catalyse esterification of [4-^{14}C]cholesterol in the presence of *n*-hexadecanyl palmitate at a quite high rate

Table 1. Esterification of [4-^{14}C]cholesterol by an acetone powder preparation from mustard roots in the presence of various acyl sources

Acyl donor added	Ester synthesis (cpm × 10^{-3})
None (endogenous donors only)	0.29
Palmitate	0.35
Palmitate (+ATP, +CoA)	0.39
Tripalmitoylglycerol	8.33
n-Hexadecanyl palmitate	4.67
n-Hexadecanyl palmitate (boiled enzyme control)	0.21
Cholesteryl palmitate	1.12

The reaction mixture contained [4-^{14}C] cholesterol (4.2 nmol/sample, *ca* 4.4 × 10^5dpm) and an unlabelled acyl donor (32 nmol/sample). ATP and CoA (10 μg/sample each) were added when indicated. The incubation time was 60 min. For other details see the Experimental.

which reaches 60% of the esterification rate with tripalmitoylglycerol as the acyl source. Results presented in Table 1 clearly indicate that a direct acyl transfer from *n*-hexadecanyl palmitate to cholesterol takes place. Free palmitate, at a concentration identical to that of *n*-hexadecanyl palmitate, stimulates esterification of [4-^{14}C] cholesterol at a very low rate, even in the presence of ATP and CoA in the incubation medium. This makes it unlikely that the stimulation of [4-^{14}C]cholesteryl palmitate formation is preceded by hydrolysis of *n*-hexadecanyl palmitate to yield free palmitate with possible subsequent activation of the latter compound to palmitoyl-CoA. Moreover, under the experimental conditions used in our experiments hydrolysis of *n*-hexadecanyl palmitate to free palmitate was hardly detectable.

Data presented in Table 1 indicates that an acetone powder preparation from mustard roots can also catalyse fatty acid transfer from cholesteryl esters to free cholesterol. This reaction, however, proceeds at a much lower rate amounting to only *ca* 1/5 or 1/10 of the reaction rates found with *n*-hexadecanyl palmitate or tripalmitoylglycerol, respectively. Autoradiographical analysis of the labelled ester formed from [4-^{14}C]cholesterol, in the presence of both *n*-hexadecanyl palmitate or cholesteryl palmitate as acyl donors, reveals that the product has a chromatographic mobility in several solvent systems identical to that of cholesteryl palmitate. Alkali-labile, labelled product with the chromatographic properties of cholesteryl palmitate was also formed when another incubation variant was applied, i.e. incubation of the enzyme preparation with unlabelled cholesterol and *n*-hexadecanyl [1-^{14}C] palmitate (variant B – see the Experimental). The latter incubation variant, however, was less suitable for studying the acyltransferase preparation from mustard roots because of a substantial incorporation of the [1-^{14}C] palmitoyl moiety into compound(s) with chromatographic properties of steryl palmitate, even in the absence of any exogenous acyl acceptor. This can be explained by a relatively high content of endogenous free sterols in the enzyme preparations which could not be completely removed during preparation of the acetone powder from the membranous fraction of mustard roots. It is possible that these endogenous sterols are preferentially esterified. When incubations are carried out with [4-^{14}C]cholesterol in the absence of an exogenous acyl source (Table 1) very slight synthesis of labelled ester takes place which indicates that in this case no appreciable amount of endogenous acyl sources is present in the enzyme preparation. For the above reason the incubation variant with labelled acyl acceptor was used in most of the subsequent experiments.

Enzymatic transfer of acyl groups from *n*-hexadecanyl ester to free sterol is clearly a reversible process (Fig. 1). Incubation of the enzyme preparation with [1-^{14}C]*n*-hexadecanol and unlabelled cholesteryl palmitate leads to the formation of labelled, alkali-labile compound with chromatographic properties of *n*-hexadecanyl palmitate. Again, an identical reaction product resulted from incubation of the enzyme preparation with unlabelled *n*-hexadecanol and cholesteryl [1-^{14}C] palmitate. It should be noted that the synthesis of labelled *n*-hexadecanyl palmitate occurs also during incubation of the enzyme preparation from mustard roots with [1-^{14}C]*n*-hexadecanol and unlabelled *n*-hexadecanyl palmitate. In this case the rate of esterification is *ca* 6-times higher than

Table 2. Specificity of transesterification reactions catalysed by the enzyme preparation from mustard roots for the fatty acid moiety

Reaction studied	Esterification of [4-^{14}C]cholesterol or [1-^{14}C] *n*-hexadecanol with cholesteryl or *n*-hexadecanyl ester containing fatty acid of the given chain-length (cpm × 10^{-3})					
	$C_{2:0}$	$C_{4:0}$	$C_{12:0}$	$C_{16:0}$	$C_{18:0}$	$C_{18:1}$
[4-^{14}C] Cholesterol	1.14	0.76	4.04	4.52	2.81	3.52
+ *n*-hexadecanyl esters	(25.2)*	(16.8)	(89.4)	(100.0)	(62.2)	(77.9)
[4-^{14}C] Cholesterol	1.25	0.25	0.49	1.21	0.50	0.32
+ cholesteryl esters	(103.3)	(20.7)	(40.5)	(100.0)	(41.3)	(26.5)
[1-^{14}C] *n*-Hexadecanol	5.64	12.78	12.15	14.03	5.89	6.51
+ cholesteryl esters	(40.2)	(91.1)	(86.6)	(100.0)	(42.0)	(46.4)
[1-^{14}C] *n*-Hexadecanol	7.71	8.82	21.21	83.22	39.10	21.54
+ *n*-hexadecanyl esters	(9.3)	(10.6)	(25.5)	(100.0)	(47.0)	(25.9)

*Relative activities (activity with cholesteryl or *n*-hexadecanyl palmitate = 100.0) are given in parentheses.

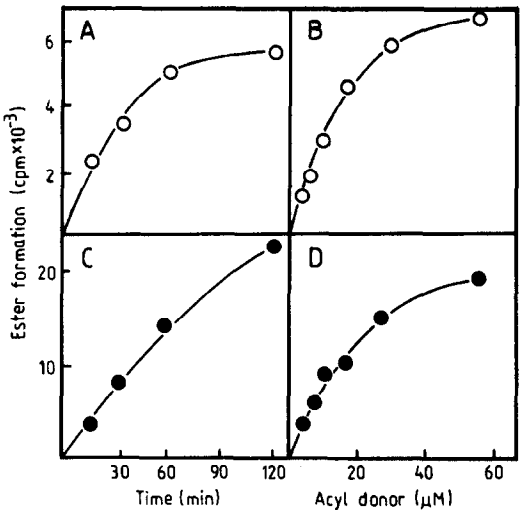

Fig. 1. Effect of incubation time (A, C) and acyl donor concentration (B, D) on esterification of [4-^{14}C] cholesterol in the presence of *n*-hexadecanyl palmitate (–○–) or on esterification of [1-^{14}C] *n*-hexadecanol in the presence of cholesteryl palmitate (–●–). In A and C acyl donor conc. was 32 nmol/sample (*ca* 25 μM). In B and D incubation time was 60 min. For other details see the Experimental.

that with cholesteryl palmitate as the fatty acid source (Table 2). As can be seen in Fig. 1 esterification of [4-^{14}C] cholesterol in the presence of *n*-hexadecanyl palmitate, as well as esterification of [1-^{14}C] *n*-hexadecanol in the presence of cholesteryl palmitate, show dependence on incubation time and acyl donor concentration which is typical for enzymatic processes. For both reactions the formation of labelled ester is proportional with time up to *ca* 60 min and is linear with increasing concentration of ester used as acyl source up to 32 nmol/sample, i.e. up to *ca* 25 μM concentration.

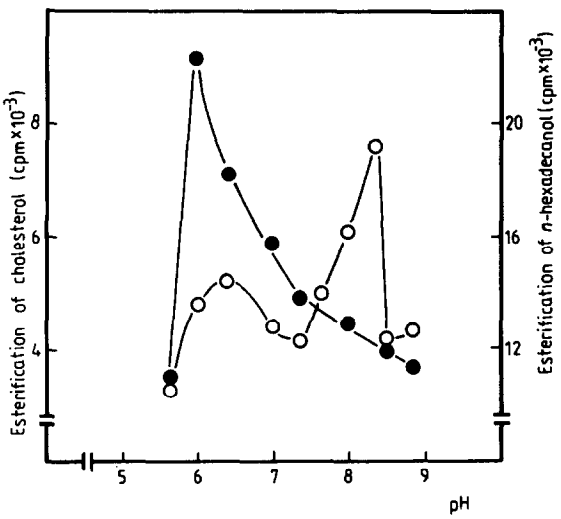

Fig. 2. Effect of pH on enzymatic transfer of palmitoyl group from *n*-hexadecanyl palmitate to [4-^{14}C] cholesterol (–○–) or from cholesteryl palmitate to [1-^{14}C] *n*-hexadecanol (–●–). 0.1 M Tris–maleate buffers were used.

The effect of pH on the esterification rate of [4-^{14}C] cholesterol or [1-^{14}C] *n*-hexadecanol in the presence of *n*-hexadecanyl palmitate or cholesteryl palmitate, respectively, is shown in Fig. 2. For both of the above reactions high activities are observed over a fairly wide pH range but exact pH profiles are clearly different. Esterification of [4-^{14}C] cholesterol with *n*-hexadecanyl palmitate as acyl source shows double pH optima at pH 6.4 and 8.4 while a single activity peak at pH 6.0 is found for esterification of [1-^{14}C] *n*-hexadecanol in the presence of cholesteryl palmitate. As we have reported earlier [9] the optimum pH value of 5.8 was observed for esterification of cholesterol in the presence of tripalmitoylglycerol by the enzyme preparation from mustard roots. A similar pH optimum was reported by other authors [3] for the enzyme isolated from spinach leaves which catalysed esterification of sterols with use of 1,2-diacylglycerols as fatty acid donors.

The rates of all transacylation reactions described in this paper are markedly influenced by the type of fatty acid moiety present in the ester serving as acyl donor (Table 2). Generally, among esters of medium-chain acids the best acyl sources are palmitates ($C_{16:0}$). Esters containing lauric acid ($C_{12:0}$) or stearic acid ($C_{18:0}$) are less effective. Esters of oleic acid ($C_{18:1}$) are also worse acyl donors than palmitates. Unexpectedly, some marked differences were found when esters of short-chain acids, such as acetates ($C_{2:0}$) or butyrates ($C_{4:0}$) were tested. *n*-Hexadecanyl acetate or butyrate are poor acyl sources for esterification of [4-^{14}C] cholesterol or [1-^{14}C] *n*-hexadecanol. However, cholesteryl acetate is equally as active as cholesteryl palmitate in esterification of cholesterol. For esterification of *n*-hexadecanol with cholesteryl esters as acyl donors both butyrate and acetate are relatively good acyl sources (90 or 40% of the activity measured with cholesteryl palmitate, respectively). All labelled esters formed in the experiments summarized in Table 2 had chromatographic properties identical to these of the authentic standards of the expected reaction products. This proves that there are no chemical changes in the transferred acyl groups.

As we have reported in our previous papers [7–9] mustard roots contain an acyltransferase which catalyses the formation of steryl esters using triacylglycerols as the preferred acyl donors. We have suggested that such a 'low energy' acyl transfer may be of importance for the biosynthesis of steryl esters in mustard roots *in vivo*. We have also presented some evidence indicating that a similar mechanism may be involved in the synthesis of esters of long-chain, primary alcohols, i.e. wax esters in mustard roots [15].

Our present results demonstrate that, apart from the above mentioned acyl transfer from acylglycerols to sterol or wax alcohols, lipid-depleted particulate fractions obtained from mustard roots are able to catalyse reversible acyl exchange between wax esters and sterols according to the following equation:

$$\text{wax ester} + \text{sterol} \rightleftharpoons \text{steryl ester} + \text{wax alcohol}$$

It cannot be ruled out that this reaction may also have some significance in the biosynthesis of steryl esters or wax esters in mustard roots. *In vitro* this reaction can be easily measured at relatively low concentrations of the

substrates. It seems possible that in intact cells, especially in some cell structures, substrate levels may be sufficiently high for effective transesterification. Irrespective of the possible role of this reaction in the biosynthesis of steryl esters (or wax esters) our results indicate that data on acyl composition of steryl and wax ester fractions isolated from plant material must be interpreted with caution. It cannot be excluded that in some cases when time-consuming procedures precede isolation of ester fractions (e.g. fractionation and purification of intracellular structures) some modification of their acyl composition may take place due to the action of the acyltransferase activities described in this paper.

The question of the relationship between acylglycerol:sterol acyltransferase described earlier by us in mustard plants [7–9] as well as by other authors [3–6, 10] in some other plants and acyltransferase catalysing reversible esterification of sterols with use of wax esters as acyl donors is still open. In mustard roots these acyltransferases share some common features: (i) they are present exclusively in heavy membrane fractions sedimenting at 16000 *g*; (ii) they can be solubilized by treatment of the particulate fractions with acetone followed by buffer extraction. On the other hand there are also some marked differences: (i) their pH profiles are clearly distinct; (ii) their specificities with respect to acyl groups are different. For sterol esterification with triacylglycerols as acyl sources trioleylglycerol is several-fold better acyl donor than tripalmitoylglycerol [9] but for sterol esterification with wax esters oleate is a less effective fatty acid source than palmitate. We hope that our current studies aimed at purification of acyltransferase activities present in mustard roots will solve this problem.

EXPERIMENTAL

Enzyme preparations. Fresh roots of 10-day-old white mustard (*Sinapis alba* L.) seedlings (75 g), grown as described earlier [1], were homogenized in a blender (5 × 20 sec) with 150 ml cold 0.05 M Tris–maleate, pH 7.3. The homogenate was filtered through 3 layers of cheese-cloth and then centrifuged at 16000 *g* for 20 min. The 16000 *g* pellet was resuspended in 20 ml of the buffer with a Potter–Elvehjem homogenizer and the suspension was added slowly, under vigorous stirring, to 200 ml cold (−15°) Me_2CO. The precipitating protein was allowed to settle for 15 min before centrifuging at 10000 *g* for 10 min. The supernatant was discarded, the precipitate was resuspended in cold, dry Me_2CO and recentrifuged at 3000 *g* for 5 min. The washing with Me_2CO was repeated × 3. The washed ppt. was than dried in a vaccum dessicator at room temp. for 30 min. The dry preparation was ground to uniformity and stored at −20° until needed. No loss in enzymatic activity was noted for storage up to 3–4 weeks. Immediately before use this acetone powder preparation was suspended in cold 0.05 M Tris–maleate, pH 7.3 (0.8 mg/1.0 ml) with a Potter–Elvehjem homogenizer, allowed to stand, with occasional shaking, at 4° for 30 min and centrifuged at 16000 *g* for 15 min. The supernatant was used for enzyme activity assays.

Enzyme incubations. Enzymatic esterification of cholesterol or *n*-hexadecanol was studied using two incubation variants. In the variant A the standard reaction mixture contained 1 ml enzyme preparation in 0.05 M Tris–maleate, pH 7.3 (see above); [4-^{14}C]cholesterol (4.2 nmol, *ca* 4.4×10^5 dpm) or [1-^{14}C]*n*-hexadecanol (9.1 nmol, *ca* 2.2×10^5 dpm) in 10 μl Me_2CO and unlabelled *n*-hexadecanyl (or cholesteryl) palmitate (32 nmol) in 200 μl Me_2CO. In some experiments unlabelled palmitic acid, tripalmitoylglycerol and various esters of *n*-hexadecanol or cholesterol were tested as acyl donors. In the variant B the standard reaction mixture contained 1 ml enzyme (as above); unlabelled cholesterol or *n*-hexadecanol (64 nmol in 110 μl Me_2CO) and *n*-hexadecanyl [1-^{14}C] palmitate or cholesteryl [1-^{14}C]palmitate (32 nmol, *ca* 4.8×10^5 dpm) in 100 μl Me_2CO. Incubations were carried out at 40°, usually for 60 min.

Isolation of labelled products. The enzymatic reaction was terminated by an addition of appropriate, unlabelled cholesteryl or *n*-hexadecanyl ester as the carrier (0.05 mg in 2 ml MeOH) and boiling on a water-bath for 5 min. Subsequently 4 ml $CHCl_3$ and 4 ml H_2O were added to each sample, the $CHCl_3$–MeOH layer was carefully removed and the residue extracted again with $CHCl_3$–MeOH (2:1). The combined extracts were washed with H_2O and evapd to dryness. The labelled reaction products were then isolated by TLC. Labelled cholesteryl or *n*-hexadecanyl esters were separated from labelled cholesterol or *n*-hexadecanol on silica gel using *n*-hexane–C_6H_6 (6:4) as the solvent. Labelled cholesteryl palmitate was separated from labelled *n*-hexadecanyl palmitate on silica gel containing 5% $AgNO_3$ with *n*-hexane–Et_2O (49:1) or *n*-hexane–$CHCl_3$ (3:2) as the solvents. Rhodamine 6G in Me_2CO was used for localization of esters on the plates. In some experiments separation was also checked by autoradiography. Ester bands were scraped off from the plates, eluted with dry Et_2O and radioactivity counted as described earlier [2].

Other methods. Cholesteryl or *n*-hexadecanyl palmitates labelled in the acyl moiety were obtained by heating [1-^{14}C] palmitoyl chloride (14 μmol, sp. act. 252 MBq/mmol) and 1 mmol cholesterol or *n*-hexadecanol in 1 ml dry C_6H_6–pyridine (19:1) at 80° for 3 hr. The labelled esters were then purified by prep. TLC and their radiochemical purity checked by analytical TLC in several solvent systems and autoradiography. Enzymatic hydrolysis of cholesteryl or *n*-hexadecanyl esters was assyed as described earlier [3, 4].

Acknowledgement—This work was supported by Polish Ministry of National Education (Grant CPBP 05.02.1.04).

REFERENCES

1. Chung, J., Albano, D. A., Fless, G. H. and Scance, A. M. (1979) *J. Biol. Chem.* **254**, 7456.
2. Suckling, K. E. and Strange, E. F. (1985) *J. Lipid Res.* **26**, 647.
3. Garcia, R. E. and Mudd, J. B. (1978) *Plant Physiol.* **61**, 354.
4. Garcia, R. E. and Mudd, J. B. (1978) *Plant Physiol.* **61**, 357.
5. Garcia, R. E. and Mudd, J. B. (1978) *Arch. Biochem. Biophys.* **190**, 315.
6. Garcia, R. E. and Mudd, J. B. (1978) *Plant Physiol.* **62**, 348.
7. Zimowski, J. and Wojciechowski, Z. A. (1980) *Bull. Acad. Polon. Sci., Ser. Sci. Biol.* **28**, 203.
8. Zimowski, J. and Wojciechowski, Z. A. (1981) *Phytochemistry* **20**, 1795.
9. Zimowski, J. and Wojciechowski, Z. A. (1981) *Phytochemistry* **20**, 1799.
10. Goad, L. J., Zimowski, J., Evershed, R. P. and Male, V. L. (1987) *The Metabolism, Structure and Function of Plant Lipids* (Stumpf, P. K., Mudd, J. B. and Nes, W. D., eds) pp. 95–102. Plenum Press, New York.
11. Kolattukudy, P. E. (1967) *Biochemistry* **6**, 2705.
12. Kolattukudy, P. E. (1970) *Lipids* **5**, 259.

13. Kolattukudy, P. E. (1975) *Recent Advances in the Chemistry and Biochemistry of Plant Lipids* (Galliard, T. and Mercer, E. J., eds) pp. 203–246. Academic Press, London.
14. Avato, P. (1984) *Planta* **162**, 487.
15. Zimowski, J., Paterczyk, J. and Wojciechowski Z. A. (1982) *Acta Biochim. Polon.* **29**, 27.
16. Wojciechowski, Z. A., Zimowski, J. and Zieleńska, M. (1976) *Phytochemistry* **15**, 1681.
17. Wojciechowski, Z. A., Zimowski, J., Zimowski, J. G. and Łyżnik, A. (1979) *Biochim. Biophys. Acta* **570**, 363.
18. Kalinowska, M. and Wojciechowski, Z. A. (1983) *Phytochemistry* **22**, 59.
19. Kalinowska, M. and Wojciechowski, Z. A. (1985) *Acta Biochim. Polon.* **32**, 259.