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STEROL CONJUGATE INTERCONVERSIONS DURING GERMINATION OF WHITE MUSTARD (*SINAPIS ALBA*)

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Abstract—Changes in the content of free sterols (FS), steryl esters (SE), steryl glucosides (SG) and acylated steryl glucosides (ASG) in germinating seeds of white mustard (*Sinapis alba*) were studied together with parallel changes in specific activities of some enzymes involved in sterol conjugate transformations It has been found that a distinct increase in the net SE content and a similar, but less pronounced, increase in SG content at the beginning of germination can be correlated with a distinctly earlier appearance of SE and SG synthesizing enzymes, i e triacylglycerol sterol acyltransferase and UDPG sterol glucosyltransferase in comparison with hydrolytic activities, i e SE hydrolase and SG hydrolase Our results suggest that metabolism of SG and ASG takes place mainly in the cotyledons while SE metabolism takes place mainly in the roots

INTRODUCTION

Sterols (FS) play two essential functions in plants firstly, they are structural components of the lipid core of cell membranes, secondly, they are biogenetic precursors of numerous secondary metabolites including plant steroid hormones [1] Much less is known about the biological functions of steryl conjugates such as fatty acid esters (SE), β-D-monoglucosides (SG) or 6′-*O*-acyl-β-D-monoglucosides (ASG) which commonly occur in plants [2–4] The importance of these conjugates as sterol storage forms, as structural components of cell membranes, together with free sterols or in intra- or intercellular transport of sterols has been suggested (for reviews see refs [2–4]) However, none of these suggestions have been sufficiently substantiated by experimental data It can be expected that a better knowledge of sterol interconversion during various physiological processes will contribute to a better understanding of the biological functions of steryl conjugates

Substantial progress has been made recently in our understanding of the biosynthetic pathways of steryl conjugates in higher plants (for reviews see refs [2–5]) Our *in vitro* studies on enzymatic transformations of free and bound sterols in white mustard (*Sinapis alba* L) have shown that the following enzymes activities can be detected in this plant UDPG sterol glucosyltransferase [6], triacylglycerol sterol acyltransferase [7, 8], galacto-(phospho)glyceride SG acyltransferase [9], SG hydrolase [10] and SE hydrolase [11] All these enzymes were partially purified and characterized [6–11]

The aim of this work was to compare changes in the content of various sterol forms, i e FS, SE, SG and ASG with changes in activities of the above listed enzymes during germination of *S alba* seeds and initial stages of seedling development We wished to answer the following question Is it possible to correlate the observed changes in the amount of the individual sterol derivatives with the observed changes in the activities of the corresponding enzymes measured *in vitro*?

RESULTS AND DISCUSSION

Changes in free and bound sterols in germinating S alba

Changes in free sterols (FS) as well as in sterols bound in steryl esters (SE), steryl monoglucosides (SG) and steryl 6′-*O*-acylmonoglucosides (ASG) during germination of *S alba* were followed quantitatively using dry seeds and 3, 7, 9, 11, 14, 18, 20 and 22-day-old whole seedlings (for details see the Experimental) Figure 1 shows changes in total sterols (FS plus sterols from SE, SG and ASG) per plant and per g fresh or dry weight Although, from day 7 of germination, the total sterol content remains relatively constant, on a dry weight basis, a distinct increase (about 2 8-fold) is observed in total sterols present per plant

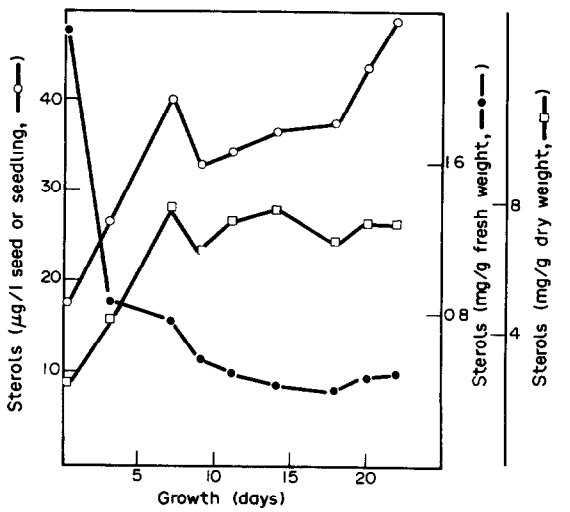

Fig. 1 Changes in total sterol content in germinating seeds of *Sinapis alba* Mean values from three independent experiments are given

These results demonstrate clearly that sterol synthesis starts very early and, from day 7, proceeds approximately parallel to the increase of plant dry weight This rapid accumulation of sterols in growing seedlings probably reflects the requirement for sterols for the formation of membrane structures in developing tissues A similar increase in the content of total sterols was observed also in germinating seeds of *Digitalis purpurea* [12], *Nicotiana tabacum* [13] and *Raphanus sativus* [14]

Changes in concentration of the individual sterol forms are given in Fig 2 The content of FS gradually increases during the whole investigated period The level of FS in 22-day-old seedlings is about 6 5-times higher than in dry seeds Sterols from SE, after an initial increase from 8 0 to 21 0 μg per plant during the first 7 days of germination, markedly decrease during the subsequent period to about 2 4 μg per plant The initial increase in SE was observed in three independent series of experiments This indicates that during the first few days of germination the synthesis of SE takes place and that in this period the esterification predominates over the possible degradation of SE An unquestionable degradation of SE takes place later, from day 7 of germination The amounts of SG and ASG are

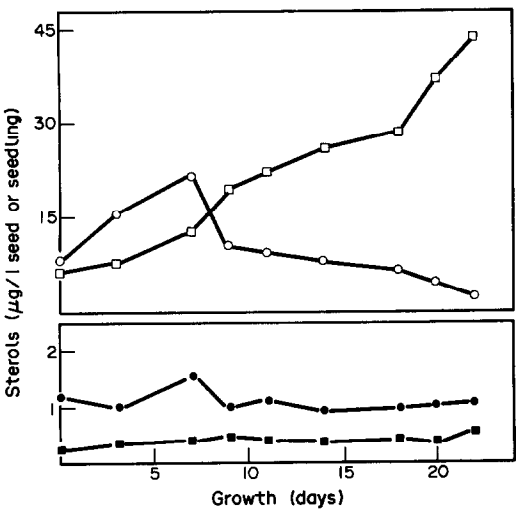

Fig 2 Changes in free sterols (□), sterols from esters (○), from glucosides (●) and from 6'-*O*-acylglucosides (■) during germination of *S alba* Mean values from three independent series of experiments are given

relatively constant during the whole period studied suggesting either a negligible turnover of SG and ASG or an equilibrium between processes of synthesis and degradation of these compounds Our earlier studies [15], in which an intense labelling of SG and ASG was observed in *S alba* seedlings after exposure *in vivo* to $^{14}CO_2$ or [^{3}H]mevalonate, suggest rather the second possibility A relatively small but significant rise ($\sim$ 45%) in SG content is observed only between day 3 and day 7 of germination This indicates that, as in the case of SE, the synthesis of SG seems to be prevailing over their degradation during the first few days of germination

Data on sterol composition of the individual fractions obtained from 7-day-old seedlings are summarized in Table 1 Both free and bound sterol fractions contain mainly 24-ethylcholest-5-en-3β-ol (sitosterol), 24-methylcholest-5-en-3β-ol (campesterol), 24-methylcholesta-5,22-dien-3β-ol (brassicasterol) and cholest-5-en-3β-ol (cholesterol) All fractions contain additionally traces (less than 1 0% of total sterols present in a given fraction) of C_{27}–C_{29} fully saturated sterols, i e stanols (mainly 24-ethylcholestan-3β-ol), C_{27}–C_{29} 7-monoenes (mainly 24-ethylcholest-7-en-3β-ol), 24-ethylcholesta-5,22-dien-3β-ol (stigmasterol), 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol) and 24-ethylcholesta-5,24(28)-dien-3β-ol (isofucosterol) Our results are essentially in accordance with data published by Knights and Berrie [16] and Appelqvist *et al* [17] for sterols isolated from *S alba* seed oil after saponification (free sterols and sterols from SE) However, we were able to detect only traces (less than 1 0% of total sterols) of isofucosterol (Δ^5-avenasterol) which was reported by the above authors as one of the main sterols present in *S alba* seeds The additional detection of small amounts of stanols, 7-monoenes, stigmasterol and 24-methylenecholesterol in this work results, most probably, from the preliminary separation of sterols differing in the number and/or position of double bonds by the use of TLC on silver nitrate–silica gel [18, 19] prior to GLC analyses (see the Experimental)

Some differences can be observed in sterol composition of the individual sterol fractions from 7-day-old seedlings (see Table 1) For example, sterols from SE are distinguished from FS and sterols present in glycoside fractions for having a relatively high content of campesterol On the other hand, sterols from ASG contain much less campesterol than all other fractions A markedly higher amount of brassicasterol is found in FS than in conjugated sterol fractions

The percentage composition of the individual sterol

Table 1 Main sterol components of free sterol (FS), steryl ester (SE), steryl monoglucoside (SG) and steryl 6'-*O*-acylglucoside (ASG) fractions from 7-day-old *Sinapis alba*

	Sterol composition (%)			
Fraction	Sitosterol	Campesterol	Brassicasterol	Cholesterol
FS	66 (±4)	24 (±1)	7 (±2)	2 (±0 5)
SE	61 (±2)	28 (±3)	5 (±1)	5 (±1)
SG	73 (±2)	20 (±3)	4 (±1)	2 (±1)
ASG	78 (±5)	15 (±5)	5 (±3)	0 5 (±0 5)

Data in parentheses represent fluctuations observed for younger or older seedlings

Table 2 Changes in the activity of some enzymes involved in sterol interconversions in *Sinapis alba*

Enzyme	Specific activity (pmol/mg protein/hr)				
	Dry seeds	Seedlings			
		3-day-old	7-day-old	11-day-old	22-day-old
UDPG sterol glucosyltransferase	2 5	6 0	12 0	11 0	11 0
Triacylglycerol sterol acyltransferase	0 0	10 0	100 0	110 0	120 0
Monogalactosyl diacylglycerol steryl glucoside acyltransferase	0 0	6 4	53 0	83 0	85 0
Sterol-ester hydrolase	0 0	0 0	128 0	185 0	190 0
Sterol-glucoside hydrolase	0 0	30 0	260 0	280 0	290 0

Mean values from at least three independent determinations of activity are given

fractions does not change much during germination Some fluctuations observed for younger or older seedlings (see Table 1, data given in parentheses) had an irregular character and fell within the limits of experimental error The only exception was stigmasterol which could not be detected in dry seeds or in seedlings younger than 7 days old Its concentration increased gradually, especially in the free sterol fraction, up to about 2% of total sterols in 22-day-old seedlings

Activity of some enzymes involved in sterol conjugate transformations during germination

Parallel *in vitro* measurements of the activities of some enzymes involved in sterol transformations, i e UDPG sterol glucosyltransferase, triacylglycerol sterol acyltransferase, galacto(phospho)glyceride steryl glucoside acyltransferase, sterol-glucoside hydrolase and sterol-ester hydrolase were made using crude homogenates from dry seeds and 3, 7, 11 and 22-day-old whole seedlings (for details see the Experimental) The results are summarized in Table 2

The following conclusions can be drawn from these experiments (i) only the activity of UDPG sterol glucosyltransferase is present in dry seeds, (ii) the remaining activities appear only during germination and can be detected in 3-day-old seedlings (sterol-glucoside hydrolase, triacylglycerol sterol acyltransferase and galacto(phospho)glyceride steryl glucoside acyltransferase) or after the seventh day of germination (sterol-ester hydrolase), (iii) in 11-day-old and older seedlings the specific activities of the individual enzymes become more or less constant

It seems that the results of the above described enzyme determinations can be, at least to some measure, correlated to the changes in the content of the individual sterol fractions occurring during germination (Fig 2) It is possible that the observed initial increase of SE content during the first seven days of germination, and the subsequent rapid decline between day 7 and day 22 can be explained by a much earlier appearance of steryl ester synthesizing enzyme, i e triacylglycerol sterol acyltransferase in comparison with the activity of sterol-ester hydrolase However, similar, but much less pronounced, initial increase in SG (day 7 of germination) can be explained by the earlier appearance of UDPG sterol glucosyltransferase as compared to sterol-glucoside hydrolase and galacto(phospho)glyceride steryl glucoside acyltransferase activities A more quantitative interpretation of the results of *in vitro* measurements of enzymatic activities in connection with the changes in sterol content is not possible as it is not known to what extent enzyme activities measured *in vitro* correspond to those *in vivo* For example, we do not know what are the physiological levels of endogenous substrates, activators, inhibitors etc

Another important aspect is illustrated by data given in Table 3 Comparison of the activities of the individual enzymes involved in sterol conjugate transformations separately in roots and cotyledons of 7-day-old *S alba* seedlings leads to the following conclusions Although all measured activities can be detected both in roots and cotyledons, the specific activities (per mg protein) of the enzymes involved in the synthesis and degradation of SG and ASG, i e UDPG sterol glucosyltransferase, galacto(phospho)glyceride steryl glucoside acyltransferase and sterol glucoside hydrolase are 3 5 to 9 0-fold higher in cotyledons than in roots On the other hand the specific activities of the enzymes participating in SE synthesis and degradation are distinctly higher in roots These results suggest that synthesis and degradation of SG and ASG takes place mainly in cotyledons while roots are the main site of SE metabolism

Our earlier *in vivo* studies [15] on the dynamics of labelling of FS, SG and ASG in *S alba* seedlings with use of $^{14}CO_2$ or a mixture of [^{3}H]mevalonate and [^{14}C]glucose as precursors have indicated that both the

Table 3 Distribution of some enzymatic activities involved in sterol transformations between roots and cotyledons of 7-day-old seedlings of *Sinapis alba*

Enzyme	Specific activity (pmol/mg protein/hr)	
	Cotyledons	Roots
UDPG sterol glucosyltransferase	18 2	5 2
Triacylglycerol sterol acyltransferase	10 0	190 0
Monogalactosyldiacylglycerol steryl glucoside acyltransferase	89 0	17 0
Sterol-ester hydrolase	18 0	240 0
Sterol-glucoside hydrolase	481 0	53 0

newly synthesized steryl glucosides and their acyl derivatives undergo rapid deacylation and deglucosylation suggesting that even though endogenous amounts of sterol derivatives are relatively steady a considerable degradation and resynthesis may take place at the same time The present results confirm this suggestion It seems that actual levels of steryl glucosides or steryl esters should be considered as the result of a dynamic equilibrium between the processes of their synthesis and degradation Net changes in the content of various sterol conjugates are probably due to changes in equilibrium between enzymes catalysing their synthesis and degradation

EXPERIMENTAL

Plant material Seeds of white mustard were germinated on 4 layers of wet cheese-cloth, at 20°, under 16 hr/day illumination

Isolation of free and bound sterols Samples (10–100 g) of seeds or whole seedlings of different age were homogenized with MeOH Insoluble residue was extracted several times with $CHCl_3$–MeOH (2 1) and components of the combined MeOH and $CHCl_3$–MeOH extracts (crude lipid fraction) were then separated by preparative TLC on silica gel with $CHCl_3$–MeOH (9 1) as the solvent yielding steryl esters, free sterols, steryl acylmonoglucosides and steryl glucosides The purified fractions containing bound sterols were subsequently hydrolysed as described previously [19] Yields of sterols isolated from individual sterol fractions after solvent extraction, TLC separation and hydrolysis were determined in parallel experiments in which precisely known amounts of [4-^{14}C]cholesterol, [4-^{14}C]cholesteryl palmitate, [4-^{14}C]cholesteryl β-D-glucoside and [4-^{14}C]cholesteryl 6′-*O*-acyl-β-D-glucoside (see below) were added to the homogenates, sterols isolated according to the above procedure and assayed by liquid scintillation counting Necessary corrections were made in all further calculations

Sterol analyses Mixtures of free sterols obtained as above were acetylated [19] and separated by means of TLC on $AgNO_3$ impregnated silica gel [18, 19] into seven bands corresponding to the acetates of C_{27}–C_{29} saturated sterols, C_{27}–C_{29} 7-monoenes, C_{27}–C_{29} 5-monoenes, 24-ethylcholesta-5,22-dien-3β-ol, 24-methylcholesta-5,22-dien-3β-ol, 24-ethylcholesta-5,24(28)-dien-3β-ol and 24-methylcholesta-5,24(28)-dien-3β-ol Individual steryl acetate fractions were quantitatively analysed by GLC using free cholesterol as internal standard GLC analyses were performed at 240° on 150 cm all-glass columns filled with 1% SE-30 on Gas-Chrom Q (100–120 mesh) Individual sterols were identified on the basis of TLC and GLC comparison with authentic standards The identity of 24-ethylcholesta-5,24(28)-dien-3β-ol (isofucosterol), 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol) and 24-methylcholesta-5,22-dien-3β-ol (brassicasterol) was additionally confirmed by MS [20] Brassicasteryl acetate, *m/z* (rel int) 440 $[M]^+$ (2), 380 (100), 365 (19), 337 (10), 282 (12), 267 (4), 255 (66), 253 (13), 228 (12), 213 (16), 24-methylenecholesteryl acetate, *m/z* (rel int) 440 $[M]^+$ (2), 380 (100), 365 (20), 296 (55), 283 (56), 281 (26), 259 (15), 255 (26), 253 (56), 228 (15), 213 (28), isofucosteryl acetate, *m/z* (rel int) 394 (52), 379 (7), 296 (100), 282 (8), 255 (16), 253 (16), 228 (14), 213 (16)

Enzyme assays Crude homogenates prepared in an appropriate buffer (0 1 M Tris–HCl, pH 7 3 for UDPG sterol glucosyltransferase and sterol-ester hydrolase, 0 05 M Tris–maleate, pH 5 8 for triacylglycerol sterol acyltransferase, 0 1 M succinate, pH 5 2 for sterol-glucoside hydrolase or 0 1 M Tris–HCl, pH 8 0 for monogalactosyldiacylglycerol steryl glucoside acyltransferase) were used as enzyme preparations UDPG sterol glucosyltransferase assays with [U-^{14}C]UDPG (sp act 280 mCi/mmol) and unlabelled cholesterol were performed as described earlier [6] Enzymatic hydrolysis of steryl glucosides and steryl esters was measured with [4-^{14}C]cholesteryl β-D-glucoside or [4-^{14}C]cholesteryl palmitate, respectively [10, 11] Triacylglycerol sterol acyltransferase was assayed with [4-^{14}C]cholesterol (sp act 47 mCi/mmol) and unlabelled tripalmitoylglycerol as acyl donor according to ref [7] Enzymatic acylation of steryl glucoside was determined as described earlier [9] using [4-^{14}C]cholesteryl β-D-glucoside as acyl acceptor and monogalactosyldiacylglycerol as acyl donor It has been checked that under incubation conditions applied in all enzymatic tests the formation of the reaction product was proportional with time and protein concns in the incubation mixtures

Other methods [4-^{14}C]Cholesteryl palmitate (sp act 47 mCi/mmol) was obtained as described earlier [10] [4-^{14}C]Cholesteryl β-D-glucoside and 6′-*O*-acyl-β-D-glucoside (sp act as above) were prepared enzymatically [6, 9] Protein was determined according to Lowry *et al* [21] with bovine serum albumin as standard

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