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STEROL COMPOSITION AND SYNTHESIS IN POTATO TUBER DISCS IN RELATION TO GLYCOALKALOID SYNTHESIS

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Key Word Index—*Solanum tuberosum*; Solanaceae; potato; biosynthesis; phytosterols; glycoalkaloids; ethephon; tridemorph.

Abstract—The synthesis of sterols and sterol precursors was analysed in potato tuber discs, which accumulate glycoalkaloids, and in discs where this accumulation was inhibited by the addition of ethephon or tridemorph. In the 4,4-dimethylsterol fraction and the 4α-methylsterol fraction, only compounds with a nonalkylated side-chain were found. The 4-desmethylsterols synthesized *de novo* were, in tridemorph-treated discs, pollinastanol and 5α-cholest-8-en-3β-ol; in ethephon-treated discs, isofucosterol; and in control discs, isofucosterol and cholesterol. The cholesterol concentration decreased concurrently with the accumulation of glycoalkaloids. The results show that cholesterol synthesis is stimulated in potato discs and indicate that cholesterol is a precursor of glycoalkaloids in potato.

INTRODUCTION

Potato (*Solanum tuberosum* L.) tubers normally contain low levels of the toxic glycoalkaloids α-solanine and α-chaconine. The amounts of glycoalkaloids in tubers increase, sometimes to unsafe levels for consumption, if the tubers are exposed to light or if they are wounded. Very little is known about the biosynthetic pathway leading to the formation of solanidine (**14**) (Fig. 1), the aglycone of α-solanine and α-chaconine, but it has been proposed that solanidine is synthesized from cholesterol (**13**) via several as yet hypothetical intermediates [1–2].

We have studied the regulation of glycoalkaloid accumulation in tuber discs, in which glycoalkaloids start to accumulate *ca* 20 hr after slicing [3]. It was found that this accumulation was inhibited by additions of the sterol biosynthesis inhibitor tridemorph or the ethylene-releasing compound ethephon [4]. Tridemorph is known to inhibit the opening of the 9β,19-cyclopropyl ring of sterol precursors as well as the Δ^8-Δ^7-sterol isomerase step [5–7], and we concluded therefore that *de novo* synthesis of sterols was necessary for glycoalkaloid formation. The effect of ethephon is still unexplained but we have suggested that it is a consequence of the observed increase in the activity of *S*-adenosyl-L-methionine:sterol C-24 methyltransferase (SMT) in the presence of ethephon [4]. Regulation of sterol biosynthesis at the SMT level was suggested by Hartmann and Benveniste [8], who reported that 2-hr incubations with [^{14}C]acetate resulted in heavy labelling of cycloartenol (**1**) in fresh potato discs, but occurred in 24-methylenecycloartanol (**7**) only if the discs had been aged before feeding with a labelled acetate. An increased level of SMT activity could cause a channelling of the sterol precursor cycloartenol (**1**) towards sterols with an alkylated side-chain (Fig. 1), thereby reducing the amount of sterols with a nonalkylated side-chain, such as cholesterol (**13**), the proposed precursor of solanidine (**14**).

In higher plants, sitosterol (**17**) and stigmasterol (**18**) often constitute more than 70% of the total sterols. The biosynthetic pathway of these major phytosterols and the properties of the enzymes involved have been studied [5, 9–12], and thereby the main pathway of sterol biosynthesis (Fig 1, right) has been established. The pathway to cholesterol (**13**) has not yet been established, but it appears likely that this minor plant sterol is formed via a parallel route without the side-chain methylation (Fig. 1, left). The intermediates of such a parallel pathway accumulated in a bramble cell suspension culture grown in the presence of 25-azacycloartanol, which is a strong inhibitor of the C-24 and C-28 methyltransferases and a weak inhibitor of the Δ^{24}-reductase [13].

With the aim of unravelling parts of the biosynthetic pathway to glycoalkaloids, we have examined the sterol composition and synthesis in potato tuber discs aged in the absence and presence of the glycoalkaloid synthesis inhibitors, ethephon and tridemorph.

RESULTS AND DISCUSSION

At time zero the concentration of glycoalkaloids was *c* 0.2 mg g^{-1} dry wt. In control discs, glycoalkaloids accumulated to 0.5–1.0 mg g^{-1} dry wt after 24 hr and 2.5–4.7 mg g^{-1} dry wt after 48 hr incubation. With tridemorph no accumulation was seen after 24 hr, and after 48 hr only a slight increase was found. Ethephon additions completely inhibited the glycoalkaloid accumulation.

Sterols occur as free sterols (FS), steryl esters (SE), steryl glucosides (SG) and acylated steryl glucosides (ASG). Studies performed with yeast indicate that the pools of FS and SE are freely interconvertible [14, 15], and we therefore analysed FS and SE together. SG and ASG were also pooled.

4,4-*Dimethylsterols and* 4α-*methylsterols in FS and SE*

Control discs. The 4,4-dimethylsterols found were cycloartenol (**1**) and 24,25-dihydrocycloartenol (cycloartanol, **2**). The relative amounts of the two sterols differed between samples, but the total amount in all samples were *c.* 15 μg g^{-1} dry wt. The reported accumulation of cycloartenol (**1**) and 24-methylenecycloartanol (**7**) in ageing potato discs [8] was not found; in fact, no 24-methylenecycloartanol (**7**), cycloeucalenol (**8**) nor obtusifoliol (**9**) was found, and only occasionally trace amounts of 24-methylenelophenol (**10**) or 24-ethylidenelophenol (**11**). The 4α-methylsterols we found were 29-norcycloartenol (**3**), 29-norcycloartanol (**4**), 29-norlanosterol (**5**) and 24,25-dihydro-29-norlanosterol (**6**).

In Fig. 1 these intermediate sterols are arranged in a proposed pathway, according to the available information about the enzymes participating in plant sterol biosynthesis. The reduction of the double bond at C-24 has been demonstrated and, to some extent, characterized in rat liver microsomes [16, 17]. The sterol Δ^{24}-reductase could use both lanosterol and desmosterol as substrates, and no reversibility of the enzyme was found. Such a sterol Δ^{24}-reductase activity has not yet been demonstrated in plant extracts. Therefore, the suggested reduction of one of the substrates cycloartenol (**1**), 29-norcycloartenol (**3**) or 29-norlanosterol (**5**) has not been shown.

The 4-demethylation has been extensively studied in microsomes from maize embroys [12]. The first 4-demethylation requires a substrate with a 9β,19-cyclopropyl ring and is inhibited by the presence of a Δ^{24} double bond, which would exclude cycloartenol (**1**) as a substrate. To account for the formation of 29-norcycloartenol (**3**) and 29-norlanosterol (**5**), we therefore have to speculate that the 4-demethylase in potato does accept cycloartenol as substrate or perhaps at a very low rate; or that the reductase reaction is reversible, such that 29-norcycloartenol (**3**) and 29-norlanosterol (**5**) are formed from 29-norcycloartanol (**4**) and 24,25-dihydro-29-norlanosterol (**6**), respectively.

Ethephon-treated discs. In ethephon-treated discs the same 4,4-dimethylsterols, i.e. cycloartenol (**1**) and cycloartanol (**2**), were found as in control discs and in the same total amounts. In contrast, we found differences in the 4α-methylsterols between control discs and ethephon-treated ones, both with regard to the amounts and the incorporation of [^{14}C]mevalonate (Table 1). The amounts of 4α-methylsterols increased with time in the control discs, whereas no such increase was seen in the ethephon-treated discs. Moreover, the incorporation of [^{14}C]mevalonate into 4α-methylsterols reached a maximum after 24 hr in the controls, but not until 48 hr in ethephon-treated discs, indicating a faster turnover in the former discs.

Fig. 2 shows the composition of the 4α-methylsterols in control and ethephon-treated discs. The main difference is the absence of 24,25-dihydro-29-norlanosterol (**6**) and the much lesser amounts of 29-norlanosterol (**5**) in the discs treated with ethephon. This difference might be explained by the higher activity of SMT in the ethephon-treated discs. When examined in bramble cells [18] and sunflower [10], 29-norcycloartenol (**3**) has been shown to be a relatively good substrate for this enzyme, and might therefore be methylated to yield cycloeucalenol (**8**). However, the differences might also be explained by effects on the sterol Δ^{24}-reductase and the 4-demethylase.

Tridemorph-treated discs. We found the same 4,4-dimethylsterols, and in the same amounts, as in the controls when tridemorph was added to the potato discs. However, tridemorph had a substantial effect on the 4α-methylsterols. As much as 225 μg g^{-1} dry wt of 4α-methylsterols was found after 24 hr incubation, compared with 6 μg g^{-1} dry wt in control discs. The main increase was in 29-norcycloartanol (**4**) and 29-norcycloartenol (**3**). We also found minor amounts of unidentified sterols. These findings are as expected, because tridemorph inhibits the opening of the 9β,19-cyclopropyl ring. No cycloeucalenol (**8**) was found, the main 4α-methylsterol in other plants treated with tridemorph [19–21], consistent with the fact that in the potato discs no 24-methylenecycloartanol (**7**) was synthesized.

4-*Desmethylsterols in FS and SE*

Control discs. The composition of 4-desmethylsterols in control discs and the incorporation of [^{14}C] mevalonate into 4-desmethylsterols after various times is shown in Fig. 3.

Sitosterol (**17**) was the main sterol in the potato tubers, and remained so during the time-course of the experiment (Fig. 3A). However, the relative contribution of sitosterol (**17**) decreased, mainly in favor of cholesterol (**13**) and 5α-cholest-7-en-3β-ol (**12**), the immediate precursor of cholesterol (**13**) (Fig. 1). The pattern of incorporation (Fig. 3B) showed that, during the first 24 hr, mainly cholesterol (**13**) and isofucosterol (**16**) were synthesized. After 24 hr, the radioactivity in cholesterol (**13**) had decreased, whereas that in isofucosterol (**16**) remained stable, and that in sitosterol (**17**) showed a steady increase. This finding is in accord with the idea that glycoalkaloids, which start to appear after *c.* 24 hr, are formed from cholesterol (**13**). We have no explanation for the relative increase of isofucosterol (**16**). An accumulation of

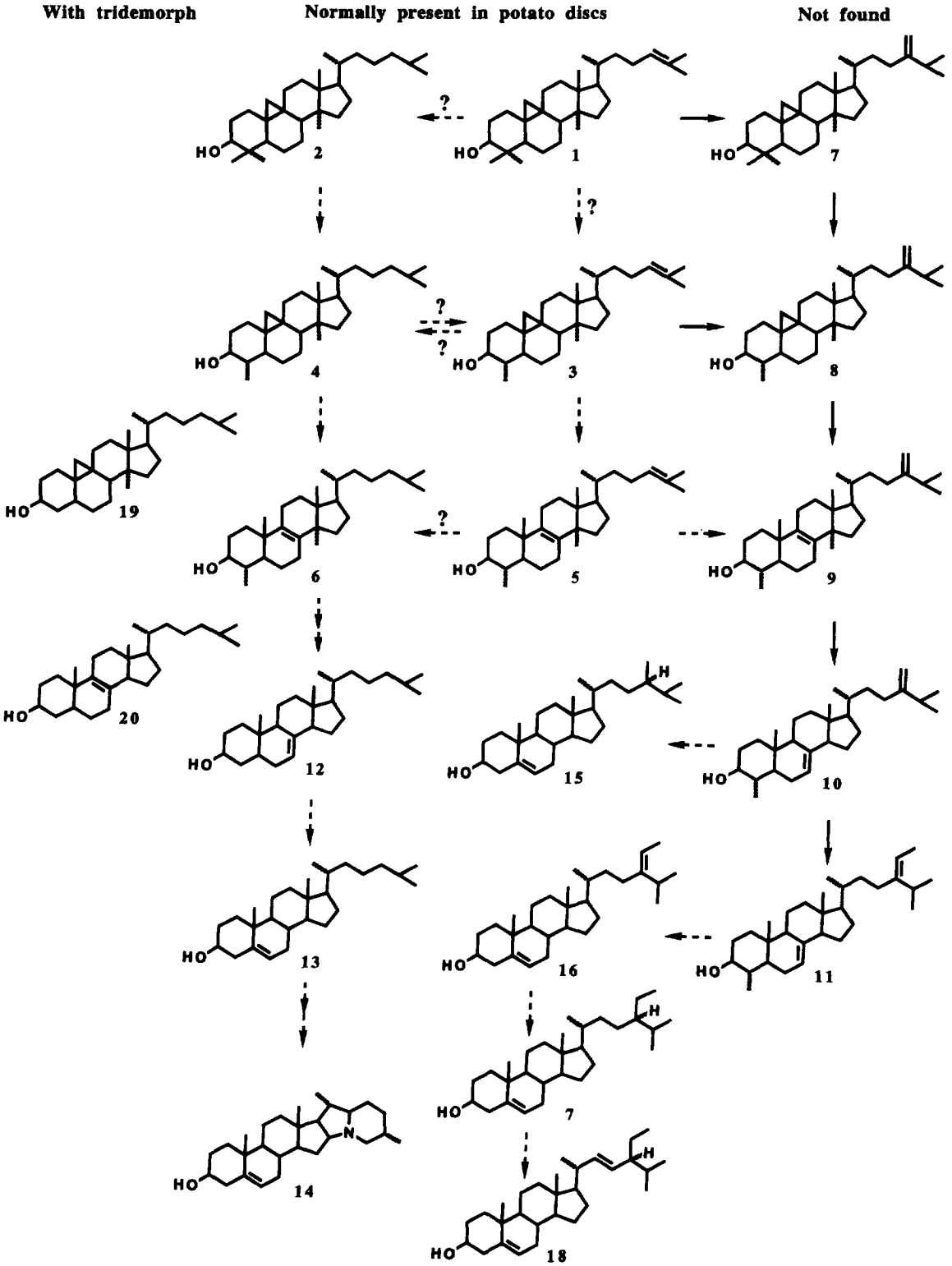

Fig. 1. Sterols found in potato discs and the biosynthetic pathway leading to the main membrane sterols: **1** = cycloartenol, **2** = cycloartanol, **3** = 29-norcycloartenol, **4** = 29-norcycloartanol, **5** = 29-norlanosterol, **6** = 24,25-dihydro-29-norlanosterol, **7** = 24-methylenecycloartanol, **8** = cycloeucalenol, **9** = obtusifoliol, **10** = 24-methylenelophenol, **11** = 24-ethylidenelophenol, **12** = 5α-cholest-7-en-3β-ol, **13** = cholesterol, **14** = solanidine, **15** = campesterol, **16** = isofucosterol, **17** = sitosterol, **18** = stigmasterol, **19** = pollinastanol, **20** = 5α-cholest-8-en-3β-ol.

Table 1. The total amount of and the incorporation of [^{14}C]mevalonate into 4α-methylsterols in potato discs after different times

	Total amount ($\mu g\ g^{-1}$ dry wt)*		Incorporation (d p m g^{-1} dry wt)*	
Time (hr)	Control	Ethephon	Control	Ethephon
2	1.6	1.6	400	400
9	2.4	1.5	12400	7900
24	6.3	1.4	14200	10900
48	3.9	1.8	6200	20100
70	4.0	2.0	3400	8400

*The experiment was performed twice with similar results.

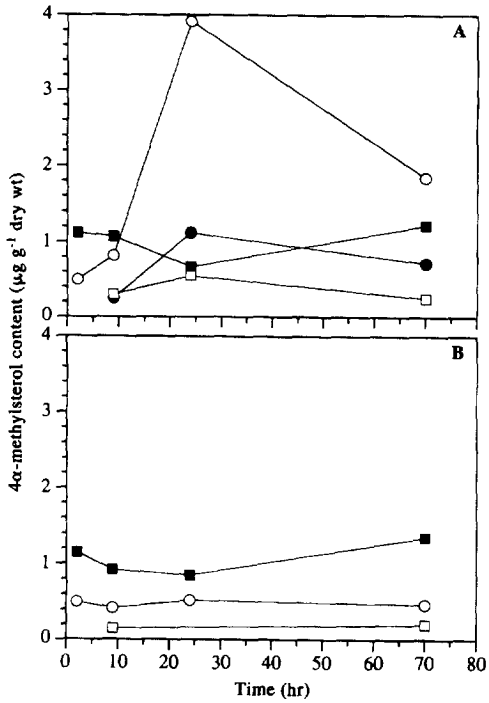

Fig. 2. Effect of ethephon on the amount of 4α-methylsterols found in discs after different times of incubation. Control (A); 5 mM ethephon (B). 29-norlanosterol (○) 24,25-dihydro-29-norlanosterol (●), 29-norcycloartenol (□) and 29-norcycloartanol (■). Data are from one of two separate experiments showing similar results.

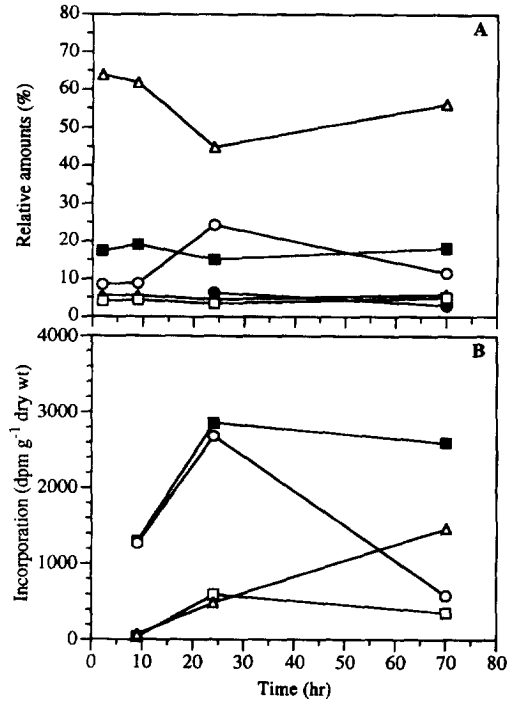

Fig. 3. The relative amounts of 4-desmethylsterols in control discs after different times of incubation (A), and the incorporation of [^{14}C]mevalonate into 4-demethylsterols in these discs (B). Cholesterol (○) 5α-cholest-7-en-3β-ol (●), campesterol (▲), stigmasterol (□), sitosterol (△) and isofucosterol (■). Data are from one of four separate experiments showing similar results.

this sterol in potato discs was found by Hartmann and Benveniste [8] and has recently been found to constitute the main sterol in the steryl glucoside fraction in potato cell cultures [22].

Ethephon-treated discs. The 4-desmethylsterol composition of ethephon-treated discs and the incorporation of [^{14}C]mevalonate into 4-desmethylsterols is shown in Fig. 4. Here, we do not find any increase in cholesterol (**13**), and only a slight increase in the relative amount of isofucosterol (**16**). The incorporation was mainly localized to isofucosterol (**16**) (Fig. 4B), with hardly any incorporation into cholesterol (**13**).

Table 2 shows a higher rate of incorporation of [^{14}C]mevalonate into 4-desmethylsterols in ethephon-treated discs than in control discs, but without any increase of the total amount of sterols. This might be explained by the earlier observation that FS and SE are more rapidly converted into SG and ASG in ethephon-treated discs [4].

Tridemorph-treated discs. In tridemorph-treated discs, the total amount of 4-desmethylsterols was doubled after 24 hr and remained at this high level of *c.* 120 $\mu g\ g^{-1}$ dry wt. Fig. 5 shows the composition of 4-desmethylsterol fraction. After 24 hr, two new sterols appeared,

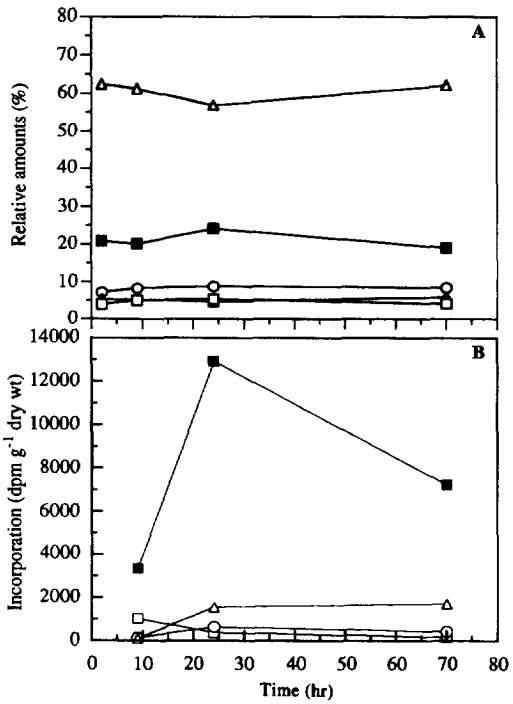

Fig. 4. The relative amounts of 4-desmethylsterols in ethephon-treated discs after different times of incubation (A), and the incorporation of [^{14}C]mevalonate into 4-demethylsterols in these discs (B). Cholesterol (○), campesterol (▲), stigmasterol (□), sitosterol (△) and isofucosterol (■). Data are from one of four separate experiments showing similar results.

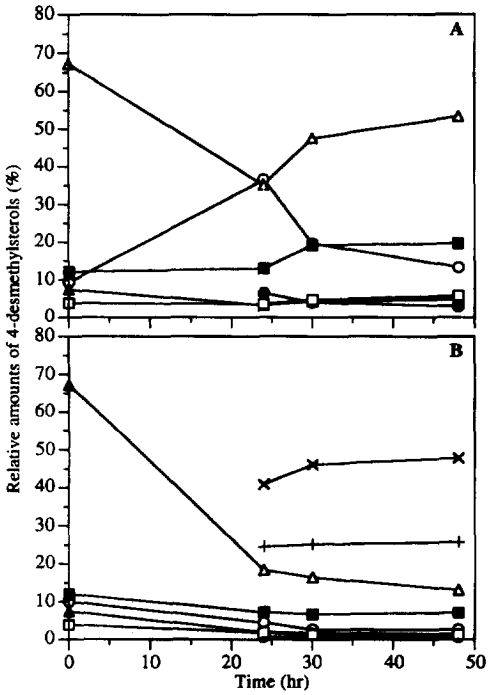

Fig. 5. Effect of tridemorph on the relative amounts of 4-desmethylsterols in discs after different times of incubation. Control (A); 20 μg ml^{-1} tridemorph (B). Cholesterol (○), 5α-cholest-7-en-3β-ol (●), campesterol (▲), stigmasterol (□), sitosterol (△), isofucosterol (■), pollinastanol (+) and 5α-cholest-8-en-3β-ol (×). Data are from one of four separate experiments showing similar results.

pollinastanol (**19**) and 5α-cholest-8-en-3β-ol (**20**). Their total contribution was more than 50% and they accounted for all the increase in the total amounts of 4-desmethylsterols. The relative and absolute amounts of sitosterol (**17**) decreased, whereas those of the other sterols remained constant. The results are in agreement with earlier findings that tridemorph inhibits both the opening of the 9β,19-cyclopropyl ring and the Δ^8–Δ^7-isomerase reaction. The amounts of tridemorph added were obviously not enough to completely inhibit the ring-opening enzyme, as further processing and accumulation of 5α-cholest-8-en-3β-ol (**20**) occurred. Further, the results establish that side-chain methylation of newly formed sterol precursors is of minor importance in potato discs.

Sterol moieties in SG and ASG

The sterol moieties liberated from SG and ASG were cholesterol (**13**), campesterol (**15**), stigmasterol (**18**) and sitosterol (**17**). The relative amounts were similar to the proportions in the FS and SE fractions, but there were no differences found between control discs and

Table 2. The incorporation of [^{14}C]mevalonate into 4α-desmethylsterols and the concentration of 4-desmethylsterols found in potato discs after different times

	Total amount (μg g^{-1} dry wt)*		Incorporation (d.p.m. g^{-1} dry wt)†	
Time (hr)	Control	Ethephon	Control	Ethephon
2	51	56	200	400
9	46	37	8300	9300
24	67	38	21400	33700
48	65	58	nd‡	nd
70	50	54	11300	24200

*The experiment was performed four times with similar results.
†The experiment was performed twice with similar results.
‡nd, not determined.

ethephon-treated discs and no increase in cholesterol (**13**) or isofucosterol (**16**) (not shown). None of the new sterols that accumulated in discs after tridemorph treatment appeared in the SG or ASG. Instead, the same sterol moieties were found as in controls and in the same proportions. This corresponds well with the results from a sterol-overproducing tobacco calli mutant, where the extra amount of sterol intermediates was found in the SE fraction [23, 24].

In conclusion, the results indicate that in the ageing potato discs the preferred pathways of sterol synthesis does not include the methylation of cycloartenol (**1**), and instead results in the formation of C_8 side-chain sterols such as cholesterol (**13**). Furthermore, the available evidence supports the early suggestions by Tschesche and Hulpke [25] that glycoalkaloids are formed from cholesterol (**13**). As for the effects of ethephon, we conclude that cholesterol formation is inhibited in the presence of ethephon. As we cannot deduce any inhibitory effect on any of the enzyme steps leading to the formation of cholesterol (**13**), we have no evidence to refute our previous hypothesis that ethephon mainly acts by a stimulation of the SMT.

EXPERIMENTAL

Radiochemicals and chemicals. Tridemorph (trade name Calixin) was a gift from BASF Svenska AB (Solna, Sweden).

Plant material. Potato tubers (*S. tuberosum*) cv. Bintje were purchased from a local supermarket and stored at 6° in darkness. Potato tuber discs were incubated with RS-[2-^{14}C]mevalonate (185 kBq) and 5 mM ethephon in 50 mM potassium phosphate buffer pH 7.5, as described in [4]. In experiments with tridemorph, 0.25 ml of 20 μg ml^{-1} tridemorph solubilized with 1 mg ml^{-1} Tween 80 was added. Controls received only buffer or only Tween 80 solution.

Glycoalkaloid extraction and quantification. Glycoalkaloid (α-solanine and α-chaconine) analyses were done according to ref. [3].

Extraction and separation of sterol classes. Free and conjugated sterols were extracted from 1 g of lyophilized and homogenized potato with a mixture of $CHCl_3$ MeOH (2:1) at 90° for 1 hr × 3. For isolation of FS and SE, the residue was dried and saponified for 1 hr at 90° in methanolic KOH (6%, w/v) and the FS extracted × 3 with *n*-hexane. For isolation of SG and ASG, the residue was separated by TLC with a mixture of CH_2Cl_2 : MeOH : H_2O (85:15:0.5). Sterols were visualized with 0.1% berberine in EtOH, and the bands corresponding to SG (R_f 0.35) and ASG (R_f 0.55) [26] were eluted with CH_2Cl_2 MeOH (2:1) and hydrolysed with 10% H_2SO_4 in EtOH at 90° for 3 hr. FS were then extracted with *n*-hexane. The sterols and standards were subjected to TLC (two runs) with hexane EtOAc (85:15) as developing solvent. Sterols were visualized as above and the bands corresponding to 4,4-dimethylsterols, 4α-methylsterols and 4-desmethylsterols were eluted with CH_2Cl_2.

Analyses of sterols. For determination of incorporation of [2-^{14}C]mevalonate into sterols, they were separated by HPLC, pooled and the radioactivity measured using a scintillation counter. A 150 × 4.6 mm Nucleosil 100–5 μm C18 column was used, and isocratic elution was carried out at room temp with 1 ml min^{-1} MeOH: H_2O (97:3, v/v). UV-detection was 205 nm. For separation of isofucosterol (**16**), stigmasterol (**18**) and cholesterol (**13**), which coelute under these conditions, the pooled sterols and standards were acetylated and then separated on $AgNO_3$-silica gel plates, developed with cyclohexane–toluene (60:40) visualized as above and the bands scraped off the plates. GC-analysis of free sterols was performed on a DB-1 WCOT capillary column, 30 m × 0.32 mm, with splitless injection of 1–2 μl sample. He was used as carrier gas with a flow rate of 34 cm s^{-1}. Injector and detector (FID) temperatures were 310° and 300°, respectively; oven programmed from 60° (2 min), then 30° min^{-1} to 240°, 2° min^{-1} to 280° and 280° for 10 min. GC-MS performed with free sterols on a DB-5 WCOT capillary column, with splitless injection of 4 μl sample. He used as carrier gas with a linear flow velocity of 30 cm sec^{-1}. Injector and detector temperatures 250° and 300°, respectively, oven programmed from 70° (3 min), then 30° min^{-1} to 260°, 4° min^{-1} to 300° and 300° for 10 min. The electron energy at ionization 70 eV. Sterols identified by RR_t (HPLC and GC) and by GC-MS. GC-MS spectra were compared to standards or to literature data [27, 28].

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