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EFFECT OF AGEING ON STEROL METABOLISM IN POTATO TUBER SLICES

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Key Word Index—*Solanum tuberosum*, solanaceae, potato tuber, effect of ageing, *de novo* synthesis of isofucosterol

Abstract—When fresh potato tuber slices were incubated with [1-^{14}C]-sodium acetate, cycloartenol was heavily labelled but no radioactivity was recovered in 24-methylene cycloartanol and free sterols If potato slices were aged for 0–24 hr before feeding with radioactive acetate, a rapid increase of the label in the sterol precursors and the free sterols was observed The free sterol content was 5× higher after ageing for 24 hr Isofucosterol synthesis was especially stimulated The synthesis of sterols during the ageing process seems to be related to the appearance of a cycloartenol C24-methylase and may be linked to a biogenesis of membranes

Nomenclature (1) 4,4,14α-trimethyl 9β,19β-cyclo-5α-cholest-24-en 3β-ol, (2) 4,4,14α-trimethyl 9β,19β-cyclo-5α-ergost-24(28)-en 3β-ol, (3) 4α,14α-dimethyl 9β,19β-cyclo 5α-ergost 24(28)-en 3β-ol, (4) 4α,14α-dimethyl 5α-ergosta 8.24(28)-dien 3β-ol, (5) 4α-methyl 5α-ergosta 7,24(28)-dien 3β-ol, (6) ergosta 5,24(28)-dien 3β-ol, (7) stigmasta 5,Z-24(28)-dien 3β-ol, (8) (24R)-24 methyl cholest 5-en 3β-ol, (9) (24R)-24 ethyl cholest 5-en 3β-ol, (10) (24S)-24 ethyl cholesta 5,E-22(23)-dien 3β-ol, (11) cholest 5-en 3β-ol

INTRODUCTION

NUMEROUS metabolic changes are initiated by the excision of slices from storage tissue, such as the potato tuber When the slices are aged aerobically, a process here termed "ageing", there is a marked increase in respiration rate,[1,2] synthesis of protein[3] and RNA [4] The activity of a number of enzymes is also stimulated, for instance the enzymes associated with carbohydrate[5,6] and phospholipid[7–9] metabolism. Evidence for *de novo* synthesis of enzymes in ageing slices from storage tissue has been presented by several workers.[10,11] These metabolic events are accompanied or followed by ultrastructural changes in cellular organelles, such as changes in the size of nucleoli[12] and the formation of polyribosomes.[13,14] Willemot and Stumpf[7] postulated that all these events may be associated with

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biogenesis of membranes. Sterols appear to be universal constituents of higher plant membranes[15–17] and, for this reason, may be considered as membrane markers. To contribute to the elucidation of membrane morphogenesis, we studied the effects of ageing on sterol metabolism in potato tuber slices. In the present report, we will show that ageing promotes *de novo* synthesis of isofucosterol. Part of this work has been reported in a preliminary form[18]

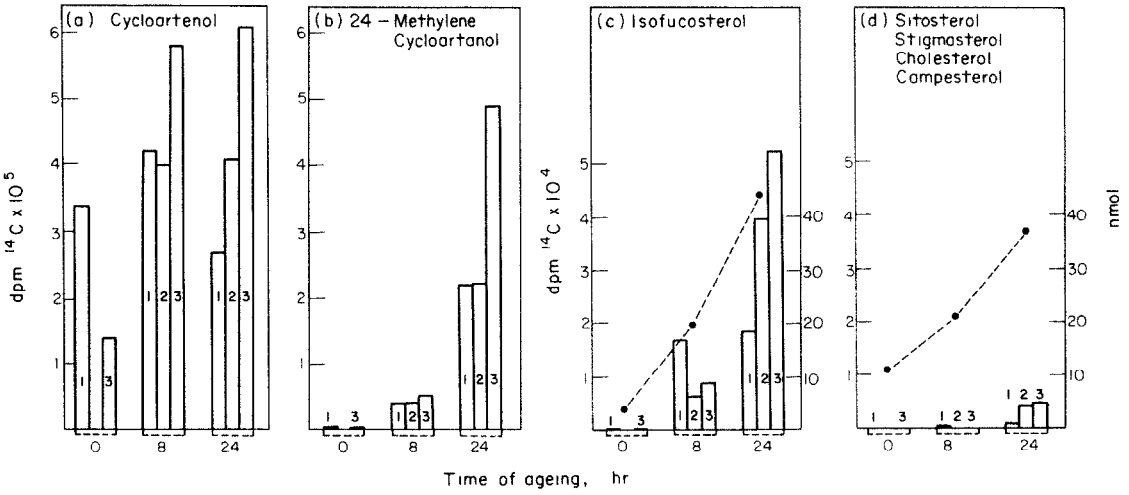

FIG 1 ^{14}C-ACTIVITY OF STEROL PRECURSORS AND FREE STEROLS IN 3 EXPERIMENTS OF AGEING PERFORMED AFTER VARIOUS STORAGE PERIODS OF POTATO TUBERS

In all experiments, slices were fed with [^{14}C]-acetate over a 2 hr period. Expt 1: 5 Dec 1972, expt 2: 16 Jan 1973, expt 3: 11 April 1973. Closed circles: nmol/g dry wt of 4-desmethylsterols (average of the three experiments).

RESULTS

Isolation of sterol precursors and free sterols from non-aged potato tuber slices

The sterols identified in potato tuber are similar to those reported for a large variety of higher plants:[19] cycloartenol (1), 24-methylene cycloartanol (2), cycloeucalenol (3), obtusifoliol (4), 24-methylene lophenol (5), 24-methylene cholesterol (6), isofucosterol (7), campesterol (8), sitosterol (9), stigmasterol (10), cholesterol (11). Von Ardenne *et al*[20] showed the same distribution of these sterols in potato leaves. However 24-ethylidene lophenol which is present in the leaves could not be detected in the tuber. In contrast, isofucosterol, which was not identified in the leaves was found in large quantities in the tuber.

Effect of ageing on free sterol biosynthesis

Potato tuber slices were aged for 0–32 hr at 25°. After various periods of ageing, the slices were incubated in [1-^{14}C]-sodium acetate at 25° for 2 hr. It is assumed that the ^{14}C-activity corresponds to sterol biosynthesis during these 2 hr. The steroidal content which occurred in tuber slices after each ageing period is expressed in nmol. Figure 1 gives the

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incorporation of the ^{14}C-label into free sterols and their precursors found in three experiments which were performed after various periods of tuber storage. The quantitative results for experiment 1 (5 Dec. 1972) correspond to the data presented in Table 1.

TABLE 1 ^{14}C-ACTIVITIES AND QUANTITIES OF STEROL PRECURSORS AND FREE STEROLS DURING AGEING OF POTATO TUBER SLICES (EXPT 1)

Time of ageing		0	4	(hr) 8	24	32
Cycloartenol	dpm ^{14}C	340000	440000	420000	270000	220000
	nmol†	7 5	14	16	5	3
24-Methylene cycloartanol	dpm ^{14}C	3000	26000	40000	222000	120000
	nmol	2	2	4 5	11 5	5
Cycloeucalenol	dpm ^{14}C	0	11800	12500	7800	5300
	nmol	—	0 5	1 5	0 6	0 6
Obtusifoliol	dpm ^{14}C	0	9300	8200	6700	4400
	nmol	—	0 3	0 6	0 3	0 2
Isofucosterol	dpm ^{14}C	0	10400	11700	17900	33000
	nmol	6 5	12	23	34	41
(Sit, Stig, Chol, Camp)*	dpm ^{14}C	0	0	200	800	2300
	nmol	13	15	21	25	35

* Sit = sitosterol, Stig = stigmasterol, Chol = cholesterol, Camp = campesterol
† nmol/g dry wt These values are essentially relative because they do not take into account the losses during the purification procedure

In non-aged slices (time 0), it was observed that only cycloartenol was radioactively labelled The 24-methylene cycloartanol was not labelled. Also no radioactivity was recovered in 4α-methyl sterols and in 4-desmethylsterols (Table 1, Fig. 1A and B). Therefore biosynthesis of sterols seems to be blocked after cycloartenol synthesis After only 4 hr of ageing, ^{14}C-activity appeared in 24-methylene cycloartanol. The radioactivity increased and was maximal after 24 hr of ageing. The high specific radioactivity of 4,4-dimethylsterols during ageing is probably due to the rapid turnover of these products which is in agreement with their role as precursors in the biosynthesis of phytosterols.[19] The appearance of the ^{14}C-label in 24-methylene cycloartanol coincides with the appearance of ^{14}C-radioactivity in the 4α-methylsterols (cycloeucalenol and obtusifoliol) and in the 4-desmethylsterols (isofucosterol).

Isofucosterol was not labelled in non-aged potato tuber slices (time 0) Table 1 and Fig. 1C. The label occurred exclusively in aged slices and increased with time of ageing. The results also indicate a parallel increase in the isofucosterol content during ageing and this increase seems to be dependent on the storage time (Fig. 1). The other 4-desmethylsterols (sitosterol, stigmasterol, cholesterol and campesterol) were much less labelled than isofucosterol (Table 1, Fig. 1D). However, an increase in their quantities was observed (Table 1 and Fig. 1).

Table 2 gives the relative percentages of the 4-desmethylsterols during ageing. The percentage of isofucosterol increases significantly. In non-aged potato tuber slices, isofucosterol constituted 30% of the total 4-desmethylsterol fraction and after 32 hr of ageing, it accounted for 60%. During the same period the percentages of sitosterol and cholesterol decreased and those of stigmasterol and campesterol remained constant.

The distribution of ^{14}C-activity was further determined. Table 3 gives the ^{14}C-activity and the specific radioactivity of the individual 4-desmethylsterols after 24 and 32 hr of age-

ing. The highest ^{14}C-activity was associated with cholesterol, a component which represented only 3% of the sterol fraction The specific radioactivity of cholesterol (1 5 μCi/μmol) was 10× higher than the specific radioactivity of sitosterol (0 13 μCi/μmol) or stigmasterol

TABLE 2 RELATIVE PERCENTAGES OF FREE 4-DESMETHYLSTEROLS IN AGEING POTATO SLICES

Time of ageing	(hr) 0	8	24	32
Isofucosterol	30	43	51	60
Sitosterol	40	33 5	28	17
Stigmasterol	10	19	15	16
Cholesterol	17	3 5	4	3 5
Campesterol + 24-methylene cholesterol	1 1	1 0	1 5	2 5

Percentages were determined by measuring GLC peak areas as described in Experimental Standard error ca 5% (two experiments)

Effect of ageing on sterol ester biosynthesis (*Table* 4)

In non-aged tissue the sterol ester content (17 4 nmol) was very similar to the free sterol content (13 4 nmol) The sterol ester content decreased greatly after 8 hr of ageing and as compared to free sterols remained very low. After 24 hr of ageing, ^{14}C-activity was recovered in the sterol esters but it was much lower than that of the free sterols.

TABLE 3 ^{14}C-ACTIVITY AND SPECIFIC RADIOACTIVITY OF INDIVIDUAL 4-DESMETHYLSTEROLS IN POTATO TUBER SLICES AFTER 24 AND 32 HR OF AGING (EXPT 3)

	24 hr		32 hr	
	dpm ^{14}C	(μCi/μmol)	dpm ^{14}C	(μCi/μmol)
Cholesterol	1120	0 30	2400	1 50
Sitosterol	700	0 04	900	0 10
Stigmasterol	380	0 04	460	0 06
Isofucosterol	52 100	0 5	50 300	0 80

Specific radioactivity of isofucosteryl acetate was determined after $AgNO_3$–TLC The other 4-desmethylsteryl acetates were further analysed by GLC The individual steryl acetates were recovered using an effluent splitter as described in Experimental and their specific radioactivity was determined

DISCUSSION

In ageing potato tuber slices, there was a strong stimulation of the biosynthesis of sterol precursors and free sterols and this biosynthesis occurred for more than 24 hr Ageing seems to act particularly on isofucosterol biosynthesis Isofucosterol did not seem to be synthesised in non-aged potato tuber slices in which it constituted 30% of the 4-desmethylsterol fraction But quantitatively it became the most important sterol during ageing and its amount was 10× higher after 24 hr of ageing. The incorporation of ^{14}C-acetate into the other 4-desmethylsterols (sitosterol, stigmasterol, cholesterol and campesterol) was much lower. However, a 3–5× increase in their content occurred during the ageing process. What is the reason for the very low ^{14}C-activity in these sterols during early ageing when, in contrast, their absolute content increased? During ageing large quantities of isofucosterol were formed in potato slices It has been postulated that in higher plants isofuco-

TABLE 4 ^{14}C-ACTIVITIES AND QUANTITIES OF 4-DESMETHYLSTEROL ESTERS COMPARED TO THOSE OF FREE 4-DESMETHYLSTEROLS (EXPT 3)

			(hr) 0	8	24
Free sterols	Isofucosterol	dpm ^{14}C	0	8300	52100
		nmol†	3	17	50
	(Sit stig, chol, camp)*	dpm ^{14}C	0	0	4600
		nmol	10	23	47
Sterol esters	4-desmethyl-sterols	dpm ^{14}C	0	0	500
		nmol	17	5	8

The 4-desmethylsterol esters were not separated by $AgNO_3$–TLC
* Sit = sitosterol, Stig = stigmasterol, chol = cholesterol, camp = campesterol
† nmol/g Dry wt

sterol is the precursor of sitosterol and stigmasterol.[21,22] If the proposed pathway occurs in potato tuber, then it may be assumed that *de novo* synthesised isofucosterol is diluted in the large precursor pool in turn decreasing the ^{14}C-radioactivity which is recovered from sitosterol and stigmasterol The hypothesis that some of the 4-desmethylsterols (sitosterol, stigmasterol, cholesterol and campesterol) are not synthesised *de novo* but come from bound sterols (esters, glycosides and acylglycosides) cannot be excluded. The decrease of sterol esters and their possible conversion to free sterols during the first 8 hr may explain the initial rapid increase of free sterols, but the large increase over a 24 hr period is difficult to explain in this manner because the steryl esters were not highly labelled The steryl acylglycosides which occur in large quantities in potato tuber[23,24] would be another possibility as a free sterol source.

The distribution of radioactivity in the individual 4-desmethylsterols after 24 and 32 hr of ageing showed the occurrence of a relatively high ^{14}C-activity in cholesterol. However cholesterol constituted only about 5% of the 4-desmethylsterols. These results can be taken as evidence that cholesterol is synthesised *de novo* in ageing potato tuber slices. Its high specific radioactivity may have resulted from a rapid turnover of cholesterol.

Further ageing of potato tuber slices seems to act specifically on the transformation of cycloartenol into 24-methylene cycloartanol. The latter compound which was not synthesised in non-aged potato slices was formed after only 4 hr of ageing The formation of 24-methylene cycloartanol from cycloartenol would involve the participation of a C24-methyltransferase, a key enzyme in sterol biosynthesis. Indeed all the 4-desmethylsterols occurring in potato tuber, with the exception of cholesterol, have one or two supplementary carbon atoms in their C17-side chain. These carbon atoms result from a transmethylation reaction with the participation of *S*-adenosylmethionine Our results suggest a regulation in the sterol biosynthesis at the C24-methyltransferase level.

It should be noted that Ben Abdelkader *et al.*[25] observed changes in phospholipid metabolism with the same material and under the same conditions of ageing, for instance the induction of oleylcoenzyme A desaturase synthesis.

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The metabolic behaviour of potato tuber slices during ageing is commonly viewed as a rejuvenation phenomenon[26] involving a derepression of the genome and the initiation of protein synthesis. Ben Abdelkader and Auderset[14] observed that these changes during ageing were associated with the appearance of polyribosomes which were attached to endoplasmic reticulum. Castelfranco *et al*.[27] found that if potato tuber slices were aged for 24 hr and then incubated with radioactive choline, most of the radioactivity was recovered in a fraction enriched in dictyosomal fragments. All these results suggest that ageing seems to be associated with a biogenesis of membranes. Therefore we think that potato tuber slices constitute good material to study the role of sterols in membrane morphogenesis.

EXPERIMENTAL

Preparation of potato slices and ageing conditions. Cylinders 9 mm dia were cut from potato tubers, *Solanum tuberosum*, ev Bintje. Slices 2 mm thick were prepared, washed several times in cold H_2O and then aged for various times at 25° on a shaker in the dark. 10 g samples of fr. tissue were shaken in 10 ml of 0·1 mM $CaSO_4$. The ageing medium was changed every 4 hr and the operation was performed under aseptic conditions. The aged tuber slices were incubated for 2 hr in 10 ml of 0·1 mM $CaSO_4$ containing 1-^{14}C NaOAc (5 μCi/ml). After the incubation the discs were washed, frozen and lyophilized.

Isolation of sterols. The lyophilized slices were extracted 3 × with 50 ml $CHCl_3$–MeOH (2:1). The extracts were bulked, evaporated to dryness, dissolved in basic MeOH–H_2O (1:1) to remove free fatty acids. Under these conditions sterol esters were not hydrolyzed. The free sterols and sterol esters were extracted from the mixture with light petrol and were separated on TLC with cyclohexane–EtOAc (9:1) as the solvent (2 runs). Four groups of products were separated: 4-desmethylsterols, 4α-methylsterols, 4,4-dimethylsterols and squalene mixed with esters. Each band was scraped off and eluted. Sterol esters were hydrolyzed by refluxing in MeOH containing 6% KOH for 30 min under N_2 and the non-saponifiable lipids were extracted with light petrol., then chromatographed as described above. Labelled [^{14}C]-4,4-dimethylsterols, 4α-methylsterols and 4-desmethylsterols were acetylated with Ac_2O-[^{3}H]. The crude acetates were first separated by TLC with cyclohexane–EtOAc (9:1) as solvent then continuously for 18 hr by $AgNO_3$–TLC with the solvent cyclohexane–C_6H_6 (7:3). With this system, the cycloartenyl acetate was separated from the 24-methylene cycloartanyl acetate, the acetates of obtusifolyl and cycloeucalenyl were separated from the 24-methylene lophenyl acetate, the acetates of 24-methylene cholesteryl, isofucosteryl and 3rd band consisting of a mixture of 4-desmethylsterol acetates (sitosterol, stigmasterol, cholesterol and campesterol) were separated. The 4-desmethylsterol acetates were analysed by GLC equipped with a FID before and after $AgNO_3$–TLC. Glass columns packed with 1% OV-17 and 1% SE-30 on chromosorb W-HP were used at 270°. Quantitative determinations were made by measuring peak areas which were compared with a standard n-$C_{32}H_{66}$ included with the test sample. An effluent splitter was used to study the radioactivity distribution and samples were trapped in glass tubes at room temp. The radioactivity associated with each component was assayed using a liquid-scintillation spectrometer. Radioactivities of ^{14}C and ^{3}H were measured at each step of the purification procedure. Specific radioactivities of the isolated products were calculated as described previously.[28] Radioactivities of ^{3}H were also used to calculate the quantities of each compound.

Identification of isofucosterol. After $AgNO_3$–TLC, the band corresponding to isofucosteryl acetate was analysed by GC–MS using a column packed with 1% Dexsil at 270°. Only one peak was obtained and corresponded in relative retention time to an authentic sample of isofucosteryl acetate. The MS showed a fragmentation pattern identical to that of the data in the literature.[29] *m/e* (relative intensity): 394 (16) [M$^+$–MeCOOH]$^+$, 296 (100) [M$^+$–MeCOOH–C_7H_{14}]$^+$, 255 (3,8) [M$^+$–MeCOOH–$C_{10}H_{19}$]$^+$, 213 (14,8) [M$^+$–MeCOOH–$C_{13}H_{25}$]$^+$. The presence of a strong peak at *m/e* 296 (base peak corresponding to a MacLafferty type rearrangement) was evidence of a Z configuration.[29]

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