Phytochemistry, 1972, Vol. 11, pp. 3003 to 3005. Pergamon Press. Printed in England.

BIOSYNTHESIS OF STEROLS IN JERUSALEM ARTICHOKE TUBER TISSUE

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(*Received* 3 *December* 1971. *Accepted* 18 *May* 1972)

Key Word Index—*Helianthus tuberosus*; Compositae; biosynthesis; sterols; far red light.

Abstract—Tissues of Jerusalem Artichoke grown *in vitro* were incubated in the presence of sodium acetate 1-^{14}C in the dark and under far red light. Phytosterols were isolated and purified and their specific radioactivities measured. A reproducible increase of the specific radioactivity of sterols was observed for tissues subjected to far red light.

INTRODUCTION

STEROLS are universal constituents of eucaryotes.[1] In animal tissues cholesterol is generally present, but in higher plants, a mixture of sterols (phytosterols) are found,[2] among which sitosterol (I), campesterol (II) and stigmasterol (III) are the most frequently encountered.

The regulation of cholesterol biosynthesis has been recently studied in the hepatic cell.[3,4] By contrast very little work has been done on regulation of plant sterol biosynthesis. Light is one of the most important factors involved in the regulation of metabolism in plant. We have studied the influence of light on phytosterol biosynthesis in etiolated plant tissues, using Jerusalem Artichoke tubers grown *in vitro*.

RESULTS

The following 4,4-dimethyl sterols and 4-desmethyl sterols have been identified in tissues of Jerusalem Artichoke: cycloartenol (IV), 24-methylene cycloartanol (V) and the mixture habitually designed as 'phytosterols'. Phytosterols are present in important amounts in their free form and have been quantitatively and qualitatively analysed. Sitosterol (I) (330 μg/g dry wt of Jerusalem Artichoke tissues, 65% of total sterols) is the most abundant sterol but campesterol (II) (60 μg, 12%), stigmasterol (III) (60 μg, 12%), 24-ethylidene cholesterol (VII) (50 μg, 9%) and 24-methylene cholesterol (VI) (10 μg, 2%) are also present in lower amounts.

(I), (24R)-24-Ethyl cholest-5en-3β-ol; (II), (24R)-24-methyl cholest-5en-3β-ol; (III), (24R)-24-ethyl cholest-5,Z-22-dien-3β-ol; (IV), 9β,19β-cyclolanost-24-en-3β-ol; (V), 24-methylene-9β,19β cyclolanost-3β-ol; (VI), 24-methylene cholest-5-en-3β-ol; (VII), 24-ethylidene cholest-5-en-3β-ol.

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The tissues were continuously fed with ^{14}C-acetate for 12 or 24 hr. Specific radioactivities of identified products were measured by acetylation with ^{3}H acetic anhydride of the biosynthetically labelled ^{14}C alcohols. A doubly labelled acetate was obtained: ^{14}C radioactivity represents biosynthetic label (molecules synthesized *de novo*) and ^{3}H radioactivity represents the mass (endogenous molecules). The ratio $^{14}C/^{3}H$ determined directly by the liquid scintillation spectrometer is proportional to the specific radioactivity of the biosynthesized product.[5] The use of the specific radioactivities obtained presumes that the absorption rate of the labelled precursor remains constant during the incubating period.[6] The change in the concentration of sodium acetate in the growth medium during the incubation period was measured: the absorption rate was essentially constant and even after 24 hr incubation, less than a half of the radioactive precursor was absorbed.

TABLE 1. SPECIFIC ACTIVITY OF 4-DESMETHYL STEROLS IN JERUSALEM ARTICHOKE TUBER TISSUE FOLLOWING INCUBATION WITH 1-^{14}C SODIUM ACETATE

Sterol	Light	Expt I[a] A^S (μCi/μmol)	Expt I[a] difference (%)	Expt II[b] A^S (μCi/μmol)	Expt II[b] difference (%)	Expt III[b] A^S (μCi/μmol)	Expt III[b] difference (%)
24-Methylene-Cholesterol	D	—	—	10 ± 2	n.s.	6·5 ± 1·3	0
	FR	—	—	12 ± 2		6·5 ± 1·3	
24-Ethylidene-cholesterol	D	—	—	8 ± 1·5	0	5·0 ± 1	n.s.
	FR	—	—	8 ± 1·5		6·5 ± 1	
Sitosterol + Campesterol + Stigmasterol	D	1·65 ± 0·20	25	2·50 ± 0·30	35	1·80 ± 0·20	45
	FR	2·05 ± 0·20		3·40 ± 0·30		2·60 ± 0·20	

(a), 12 hr incubation; (b), 24 hr incubation; D, dark; FR, far-red; n.s., not significant. Sterols were extracted from two batches of cylinders of tuber tissue (100 mg dry wt/batch). Specific activity of acetate was 12 μCi/μmol (Expt I) and 6 μCi/μmol (Expts II and III).

Specific radioactivities of sterols (on their purified epoxi-acetate form) extracted from two batches of Jerusalem Artichokes explants fed with sodium acetate 1-^{14}C in the dark and under far red light are shown in Table 1. Specific radioactivities of 24-methylene and 24-ethylidene cholesterol were much higher than the specific radioactivity of the sitosterol, campesterol and stigmasterol mixture and this observation suggests that the former are precursors of the latter.[6] Sterol biosynthesis (sitosterol, campesterol and stigmasterol) was stimulated in tissues exposed to far red light. The stimulation was about 25% after 12 hr reached 35–45% after 24 hr and was essentially reproducible. The constituents of the sterol mixture have not yet been separated and it is not inconceivable that the differences in the specific radioactivities observed under the influences of far red light may be differently partitioned between sitosterol and campesterol on the one hand and stigmasterol on the other, and we intend to check this point. No significant differences could be observed in the specific radioactivity of 24-methylene cholesterol (VI) and 24-ethylidene cholesterol (VII). The difference observed in the case of (VII) (Expt III) was not reproducible.

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DISCUSSION

The observed effect (stimulation of sterol biosynthesis under the influence of far red light) may be interpreted in terms of a specific action on a factor involved in the regulation of one or many sterol biosynthetic enzymes. The fact that we have used an etiolated tissue for these experiments and have observed an effect under the influence of far red light would be in agreement with a phytochrome mediated process. It has been shown[7] that continuous far red light maintains a low but relatively constant concentration of P_{730}. The occurrence of phytochrome in Jerusalem Artichoke tubers has been demonstrated by *in vivo* differential spectrophotometric analysis.[8] This hypothesis of a phytochrome mediated synthesis of sterols needs to be checked by reversion experiments.

EXPERIMENTAL

Incubation techniques. Small cylinders of Jerusalem Artichoke tubers were grown on a liquid medium for 36 hr at 25° in the dark as previously described.[9] To each flask containing 30 cylinders in 30 ml of medium, sodium acetate 1-^{14}C (50 μCi) was added. The final concentration of acetate in the medium was $2{\cdot}7 \times 10^{-4}$ M. A batch of 60 samples was exposed to far red light (730 $\pm$ 50 nm, 3500 erg $\times$ $cm^{-2} \times sec^{-1}$). Another batch was maintained in the dark as a control. After 24 hr of incubation, the tissues were washed, frozen and lyophilized.

Analytical procedures. Lyophilized tissues were sonicated in the presence of light petrol. The organic phase was washed with 50% EtOH and dried over Na_2SO_4. The solvent was evaporated under vacuum and the residue run on TLC plates (solvent: CH_2Cl_2, 2 runs). 5 groups of products were separated, 4-desmethyl sterols, 4-methyl sterols, 4,4-dimethyl sterols, squalene-2,3 oxide and squalene mixed with esters. The 4-desmethyl sterols were acetylated in the presence of pyridine with ^{3}H Ac_2O (20% of anhydride in C_6H_6. Specific radioactivity: 15 μCi/μmol) as previously described.[5] The crude acetates were first chromatographed on plates of ordinary silica (cyclohexane–AcOEt (19:1), one run) and secondly on $AgNO_3$ containing silica (cyclohexane–C_6H_6 (3:2) continuously during 8 hr). By this procedure, acetates of phytosterols (I, II, III) (R_f 0·65), of 24-methylene cholesterol (VI) (R_f 0·30) and of 24-ethylidene cholesterol (VII) (R_f 0·48) were separated. Purified acetates were finally treated with *p*-nitro perbenzoic acid[10] and the epoxi-acetates obtained were chromatographed on silica (cyclohexane–AcOEt (9:1) 2 runs). At each step of the purification procedure, radioactivities of ^{14}C and ^{3}H were measured. Measurements of the ratio $^{14}C/^3H$ were made with a liquid scintillation spectrometer Intertechnique SL-40. Specific radioactivities of the isolated products were calculated with the formula:

$$A^S = 1/2 \times {}^{14}C/{}^3H \times A^S$$

with A^S = specific radioactivity of the biosynthesized product; A^S = specific radioactivity of the ^{3}H acetic anhydride.

Identification of products. Structures of the products were determined by GLC using a Varian Aerograph model 1740 chromatograph fitted with dual flame ionization detectors on a 1% OV-17 packed glass column (1·5 m $\times$ 3 mm) at 250° (nitrogen flow rate 27 ml/min) and by mass spectrometry using a Thomson–Houston THN 208 mass spectrometer at 12 and 70 eV (direct inlet system at 170°). 24-Methylene cholesterol *m/e* (rel. intensity) 440 (4) $[M]^+$, 380 (100) $[M-CH_3COOH]^+$, 296 (34) $[M-60-C_6H_{12}]$, 255 (6) [M-60–lateral chain]. 24-Ethylidene cholesterol acetate 454 (5) $[M]^+$, 394 (30) $[M-CH_3COOH]^+$, 296 (100) $[M-60-C_6H_{12}]$, 255 (4) [M-60–lateral chain].

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