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《Original》 The Metabolism of (2-^{14}C) Mevalonic Acid on Photoperiodic Induction in Grafted Solanum andigena

Moo Bae and E.I. Mercer*

Korea Institute of Science and Technology. Seoul, Korea

Department of Bischemistry, University Colledge of Wales, Aberystwyth. U.K.

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Abstract

The metabolism of sterol precurosor in leaves of *Solanum andigena* grafted between photoinduced and noninduced plant was investigated with the use of (2-^{14}C) mevalonic acid. By the technique of the preparative gas-liquid chromatography, radioactive compounds of squalene, 4,4'-dimethylsterols and 4-demethylsterol were isolated and determined quantitatively. When labeled mevalonic acid was applied to leaves radioactivity was extensively incorporated into non-saponifiable materials of lipid fraction and aqueous fraction (ethanol-water fraction). Radioactivity of ^{14}C derived from (2-^{14}C) mevalonic acid was transmissible from photoinduced plant to non-induced plant across the graft union, as tuberization hormone was, and incorporated into the sterols of the non-induced plant. Inhibitors of sterol biosynthesis, SK & F 7997 and nicotinic acid, are effective suppressors of tuber growing, if applied to leaves during photoinduction period. The experimental results suggest that certain substance containing isoprene unit, or sterol-like compound may participate in tuber growing.

요 약

Solanum andigena의 塊莖生成형상과 스테롤의 관계를 구명하기 위하여 방사성 메바론산을 이용하여 대사를 연구하였다. 塊莖生成에 短日光周性誘導를 요하는 短日植物과 塊莖을 생성치 않는 長日植物을 접목 시켰을때 長日植物이 塊莖生成 홀몬을 받아 塊莖을 生成하는 現象을 스테로이드와 결부시켜 구명할려고한 것이다.

이 연구에서 각종 방사성 스테롤이 特殊裝置 한 까스 크로마토 그라피로서 분리 측정되었다. 여기서 분리된 각종 스테롤이 직접 塊莖生成홀몬이라는 근거는 찾을 수 없으며, 이들이 塊莖의 肥大生長에 필요한 인자로서 관여하고 있는 것으로 논의되었다.

Introduction

Tuberization of *Solanum andigena* is markedly influenced by the length of the photoperiod to which the plant is exposed. Obviously the locus of a photoperiodic reaction is the leaves, in which tuberization hormone produced infl-

uences the development of underground organs by communication in some manner. Gregory[1] postulated the formation of a tuber forming stimulus with short-day condition (8 hr.) and the photoperiod-temperature theory. Okazawa and Chapman[2] suggested that the interaction of the tuber forming stimulus with the tuber inhibiting substances, gibberellin-like materials, should be considered in tuberization. The grafting experiment of non-induced plant to photo-induced plant indicated that the tuber-inducing stimulus (tuberization hormone) is a transmissible factor.[3] Thus, tuberization has been postulated in terms of a hormonal mechanism. On the other hand, the sterol requirement for the reproduction of *phytophthora coctorum* has been established by Elliott[4]. Bonner et al succeeded in inhibiting cocklebur and Pharbitis plants flowering with the inhibitors of steriod biosynthesis. And they concluded that sterols are substances necessary for flowering[5]. Sterols in some species of Solanum plant have been isolated and investigated[6,7,8] and although the role of such compounds in plant tissue is being studied it is not thoroughly understood. We have recently studied that the remarkable changes occur in the levels of free sterols, their esters and glycosides in the leaves of *Solanum andigena* during photoperiod[10]. We wished to demonstrate the relation between transmissible factor and sterols by administering radicactive mevalonic acid to the leaves of photoinduced plants grafted. And another aim of the present work was to determine incorporation of (2-^{14}C) mevalonic acid into the free and esterified sterols in *Solanum andigena* during photopericdism.

Materials and Methods

Plant materials. *Solanum andigena,* which require light periods of short-day cycles for tuberization, were grown to cause tuber formation on photoperiods of 8 hours for 25 days. On the other hand, same number of the plants were exposed to long-day cycles, in which the plants do not initiate tuberization. (2—^{14}C) mevalonic acid (MVA) was purchased from the Radiochemical Centre, Amersham, England.

1. Grafting

At the end of photoperiods for 25 days in short-day condition approach grafts were carried out between the stems of photo-induced plants and those of non-induced plants. At the end of grafting period graft unions were examined microscopically for vascular connection, and to make sure further the translocation of tuberization hormone from photo-induced plants to non-induced plants, cuttings, consisting of two nodes each, were prepared from basal region of both non-induced plants and photo-induced plants and repotted. On two weeks after repotting, cutting culture was completed and examined for tuber formation.

2. Exposure of S. andigenum to(2-^{14}C)MVA

(2—^{14}C) MVA was introduced into plants by painting the photo-induced leaves of grafted plants in two different ways.

Group A: (2—^{14}C) MVA was applied to the leaves after grafting

Group B: (2—^{14}C) MVA was applied to the leaves before grafting

In group A, one week after grafting 25 μc of (2—^{14}C) MVA in 10 ml. of water was applied to the leaves of 10 photoinduced plants grafted on non-induced plants by painting with a small brush. On two days after painting of (2—^{14}C) MVA the leaves of photo-induced plants grafted were treated with another 25 μc of (2—^{14}C) MVA in 10 ml. of water. They were then allowed to introduce the label into both plants (from photoinduced plants to non-induced plants through graft unions) for five

days under long-day condition in the greenhouse.

In group B, 50 μc of (2—^{14}C) MVA solution was applied to the leaves of 10 photo-induced plants before grafting. On three days after painting of MVA, approach grafts were carried out between the stems of photo-induced and non-induced plants in the same way as group A. They were allowed to introduce the label from photo-induced plants across graft unions into non-induced plants for 14 days under long-day condition. On the other hand, 5 photo-induced plants and 5 non-induced plants were treated with 10 μc of (2—^{14}C) MVA separately for a week for the controls of grafted plants.

On harvesting grafted plants of each group were separated into eight groups as follows:

Photo-induced plants: (short-day: S D-)	1. SD-leaves	2. SD-stems
	3. SD-cuttings	4. SD-tubers & stolons
Non-induced plants: (long-day: L D-)	1. LD-leaves	2. LD-stems
	3. LD-cuttings	4. LD-stolons

The tubers and stolons formed from the cuttings of non-induced plants grafted on photoinduced plants were combined to the sample of LD-stolons for the extraction. The tubers and stolons formed from SD-cuttings were also combined to the sample of SD-tubers and stolons.

Table 1. Description of the plant material (group A) harvested and analysed.

	Non-induced plants fresh weight	Photo-induced plants (fresh weight)
Leaves including petioles	66.1 g	79.1 g
Stems inclucing buds	67.3 g	27.9 g
Stolons and tubers	5.2 g	23.5 g

3. Steroid Inhibitors

SK & F 7997[26] (tris-(2-diethylaminoethyl)-phosphate trihydro chloride) obtained from Smith, Kline & French Laboratories, Philadelphia, vanadyl sulphate and nicotinic acid,[25] which are known to be inhibitors of sterol biosynthesis, were tested for their inhibiting effects on photopericdic tuber induction of *Solanum andigena.*

Four groups of 5 Solanum plants were grown from cuttings taken from long-day plants for 2 weeks under long-day condition. They were then transfererd to short-day conditions. Three groups of 5 plants each were referred to each inhibitor experiment and one group of 5 plants to control experiment.

The inhibitors were dissolved in distilled water containing 0.01% of the non-ionic detergent, nonidet, and applied every morning by spraying both sides of leaves with microsprayers over a period of 3 weeks in the short-day condition. After a short-day period, tubers formed were determined.

4. Extraction of lipid.

On the seventh day after(2—^{14}C) MVA had been applied each group of photo-induced and non-induced plants was harvested separately as described above and rinsed thoroughly with deionized water. Plant materials were stored in deep freezer until they were chopped into small strips and homogenized in a waring blender at high speed with 200 ml. of 95% ethanol. The homogenateswere filtered through sintered glass funnel, and the residues re-extracted with 200 ml. of 95% ethanol. The ethanolic solution was concentrated under vacuum at 50–60°C up to about 50 ml. of the solution, 30 ml. of water added and extracted with two 100 ml. portions of ethyl acetate. The extract was washed with a small amount of water. The combined aqueous solution was re-extracted with 100 ml. of ethyl ether (water soluble fractions). The ether

extract was evaporated and combined to ethyl acetate solution.

The residue was homogenized and reextracted further twice with ethyl acetate. The extract was separated from the debris through sintered glass funnel, combined with the ethyl acetate solution extracted from ethanolic solution, dried over andydrous Na_2SO_4 and evaporated (total lipid fraction).

5. Preliminary separation of total lipid.

Aliquots of the extracts were chromatographed on columns of Brockmann grade III neutral alumina developing by step-wise elution with (1) petroleum ether (B.R. 40–60°C), (2) 2% v/v diethyl ether in petroleum ether (2% E/P), (3) 4% E/P, (4) 6% E/P, (5) 10% E/P, (6) 20% E/P, (7) 60% E/P, (8) ether, (9) 95% ethanol. Each fraction volume was 100 ml. for 10 g. of alumina. The fractions eluted were evaporated to dryness, and dissolved in diethyl ether. An aliquot of each fraction was determined for radioactivity of ^{14}C. β-Carotene and squalene were eluted by petroleum ether. 4,4'-dimethyl sterols were eluted in fractions 4% and 6% E/P, and 4α-methyl sterols and 4-demethyl sterols in fraction 6%, 10% and 20% E/P.

6. Purification and Identification.

Purification of β-Carotene and squalene.

Column fraction eluted with petroleum ether in which β-carotene and squalene are contained, was purified on thin-layers of Kieselegel G impregnated with Rhodamine 6G [9], developed with petroleum ether (b. range 40–60°C). Authentic sample of squalene was chromatographed simultaneously as a reference compound. (Kieselgel G; E. Merk A. G., Darmstadt Germany).

7. Purification and Identification of 4, 4'-dimethyl sterols and 4-demethyl sterols.

Column fractions eluted with 4% and 6% ether in petroleum ether were chromatographed on thin-layers of Kieselgel G impregnated with Rhodamine 6G with chloroform as the developing solvent. The main band containing 4,4'-dimethyl sterol was eluted out, and then purified further by repeated thin-layer chromatography with Methanol-Benzene (10:90) as the developing solvent. Authentic samples of 4,4'-dimethyl sterols (lanosterol, β-amyrin) were chromatographed together as marker compounds.

Column fractions eluted with 10% and 20% ether in petroleum ether were chromatographed in the same way as 4,4'-dimethyl sterols and purified further by repeated thin-layer chromatography with methanol-benzene (10:90). Authentic samples of sterols (cholesterol, β-sitosterol stigmasterol) were chromatographed together as marker compounds. Thin-layers were scanned for radioactive zones with a radioactive chromatogram scanner.

These 4,4'-dimethyl sterols and 4-demethyl-sterols were then analysed by gas-liquid chromatography.

8. Gas-liquid chromatography and Preparative gas-liquid chromatography.

The apparatus used in the experiments was Packard gas-chromatograph, equipped with hydrogen-flame ionization detectors and recorder model 840.(hydrogen flow rate, 40 ml./min.). The carrier gas was argon at a flow rate 28 ml./min. The pyrex glass column (length 194 cm., diameter 2.5 mm.) filled with 100–120 mesh. gas-chrom Q coated with 3% OV-1 was

used. The temperature of the injector, column and detector were kept at 255 , 230 and 245°C respectively. Preparative gas-liquid chromatography was fitted with an effluent splitter (1:7) between detector and column, and each of compounds eluted from column were collected in glass U tube cooled in ice. Cholestane was used as a standard sample for determining relative retention data. To determine percentages of free sterols in the mixture areas under peaks by gas-liquid chromatography were measured by triangulation. Standard curves were drawn for the quantitative determination of sterols.

9. Radioassay

(a) Scintillation counting. Samples were assayed in a Packard TriCarb Liquid Scintillation Spectrometer Series 314E. Samples were dissolved in 5 ml. of toluene and added in 5 ml. of double strength scintillator containing 10.0 g. of 2,5-diphenyloxazole, 0.6 g. of Dimethyl POPOP 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene and 11. of toluene. Aqueous samples (1 ml.) were dissolved in 10 ml. of Bray's scintillator containing 60 g. of naphthanlene, 4 g. of 2,5-diphenylozazole, 0.2 g. of dimethyl POPOP-1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene, 100 ml. of methanol, 20 ml. of ethylene glycol and 1.4-dioxane to give volume of 1 1. Coloured samples were decolorized under u.v. light (Hanovia 125 W ultraviolet lamp) before addition of scintillator. All counts were corrected for background.

(b) Scanning of thin-layer chromatograms. Thin-layers were scanned for radioactive zones with a Desaga-Radio active chromatogram scanner 12–2.

(c) Radioautography. Radioautographs were prepared with Kodak X-ray film. (Kodirex).

10. Solvents

Petroleum ether (b.r. 40–60°C) and diethyl ether were dried over sodium wire and distilled over reduced iron powder immediately before use. Ethyl acetate and toluene were of A.R. grade.

10. Result

Solanum andigena is a wild species of plants sensitive to photoperiods for tuberization. When the stems of non-induced plants, which do not initiate tuber formation, were grafted to those of photo-induced short-day plants, the long-day plants formed tubers in underground organ within two weeks and subsequently cuttings prepared from the stems of the long-day plants formed tubers, whilst the grafting of long-day stems to long-day stems did not result in tuber formation. This grafting experiments indicate that certain chemical substances including tuberization hormone translocate across graft unions from photoinduced plants to non-induced plants. As pilot experiment was carried out, it was found that (2-^{14}C) MVA was incorporated into the various lipid fractions of the leaves and stems of this Solanum plants. To investigate the hypothesis that certain metabolites are exported by leaves under short-day condition and that if tuberization hormone is exported by leaves the compound might be derived from ^{14}C compound such as mevalonic acid in photoinduced leaves, (2- ^{14}C) MVA was fed to the photoinduced leaves of grafted plants. It was found in both groups of this experiment that ^{14}C radioactivity from (2- ^{14}C) MVA painted to the leaves of photoinduced plants was significantly transported to non-induced plant across graft union and incorporated into the various lipids of non-induced plant, as well as into aqueous phase, as shown in Table 2 and 3. Radioactivities of aqueous phase would appear to be mainly derived from sterol glycosides and intact MVA taken into the plants. There seems to be no remarkable

difference between group A and B of grafting experiment in the translocation and incorporation, so that the results concern with the Group A of the plants in this work, unless otherwise stated.

Investigation of labelled compounds in petroleum ehter and 2% E/P fractions of alumina columns.

Photoinduced plants of group A containing 10 plants, received 50 μc (2- ^{14}C) MVA through the leaves, and the plants were harvested separately, extracted and the total lipids chromatographed as deseribed in the Experimental Section. Petroleum ether fraction of alumina column contained the greatest amounts of radioactivity in nine fractions: that is, in the group A of the grafted plants 46% of total radioactivity of column chromatography incorporated in SD-leaves fraction, 35% in SD-stems 12% in LD-leaves (main radioactivies fraction 2% E/P) and 53% in LD-stems, respectively. In the column, squalene, β-carotene and some of sterolesters were eluted out with petroleum ether and reminders of sterclesters with 2% ether in petroleum ether.

These petroleum ether and 2% E/P fractions were chromatographed on thin-layers of Kieselgel G impregnated Rhodamin 6G with petrole-

Table 2. Incorporation of radioactivity into Solanum andigena.

	Radioactivity incroporated (counts/min.)	
	Aqueous phase	Lipid phase
LD-leaves	20, 680	19, 560
LD-stems	7, 230	6, 430
LD-stolons	2, 090	620
SD-leaves	804, 900	1, 497, 550
SD-stems	124, 075	17, 530
SD-stolons & tuber	28, 035	8, 950

50μc of (2-^{14}C) MVA was applied to the leaves of the photoinduced *Solanum andigena.*

Tablel 3. Incorporation of (2-^{14}C) MVA into lipids of *Solanum andigenum*

Column fraction	Radioactvity (counts/min.) Grafted plants				Non-grafted plants		
	LD-leaves	LD-stems	SD-leaves	SD-stems	SD-leaves	LD-leaves	Terpenoid constituents
			10^{-3}x^{14}C		10^{-3}x^{14}C	10^{-3}x^{14}C	
P.E.*	2, 100	3, 110	582. 4	5, 280	828. 4	418. 2	squalene, β-carotene sterol esters
2% E/P	7, 920	88	91. 4	916	26. 7	20. 7	
4% E/P	1, 740	160	15917	904	137. 0	115. 0	4, 4'-dimethyl-sterols, 4α-methyle-sterols
6% E/P	3, 160	788	210. 6	2'650	260. 5	48. 1	
10% E/P	890	840	119. 6	2, 032	161. 6	58. 6	4-methyl-sterols
20% E/P	204	290	81. 7	1, 477	25. 5	21. 5	some of sterols,
60% E/P	190	138	35. 2	628	53. 8	34. 1	Carotenoids
Ether	40	0	6. 4	201	5. 1	0. 3	
95% ethanol	110	433	470	940	35. 6	21. 7	Carotenoids

In grafted plants of group A, 50 μc of (2- ^{14}C) MVA was applied to the photoinduced leaves. In non-grafted plants, photoinduced plants (5) were treated with (2-^{14}C) MVA (10 μc) immediately after a photoperiod of 3 weeks in short-day cycle, and on the other hand, the leaves of non-induced plants (5) of the same ages were treated with the same amounts of (2-^{14}C) MVA for a week. At the end of this period the total lipid was extracted with 95% ethanol and then ethyl acetate. Ethanol extracts were concentrated and separated into ethyl acetate soluble fraction and water soluble fraction. Total lipid extracts were chromatographed on columns of Brockmann grade 3 neutral alumina. *P.E.; petroleum ether. E/P; diethylether in petroleum ether, v/v.

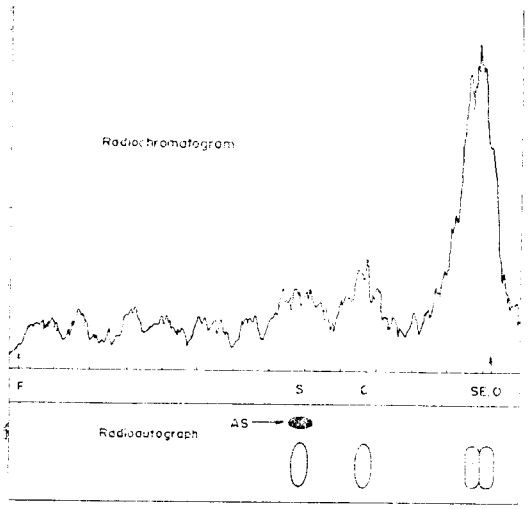

Fig. 1, Radiochromatogram of Petroleum ether fraction containing 2% E/P column fraction from *Solanum andigena.*

A slica gel G plate impregnated with Rhodamin 6G was developed with Petroleumether (B.R. 40-60°C) and scanned with a Desaga Radiocative chromatogram scanner 12-2.

O. Origin: SE. Sterol esters: C. β-Carotene: S. Squalene: F'Solvent Fornt; As, Authentic sample

um ether as the solvent. Thin-layers were then scanned for ^{14}C radioactivity and prepared for radioautography. The radioscannings and radioautographies of thin-layers (Fig. 1) showed that highly radioactive zone was at Rf. 0.06 in both petroleum ether and 2% E/P fractions, and that minor radioactive zones were the Rf. values identical with those of β-carotene (Rf. 0.28) and squalene (Rf. 0.41) Absorption maxima of the pigment in visible light was 424, 450 and 475 mμ in petroleum ether, which was the same spectral shape as β-carotene. Gas-chromatography indicated that radioactive matter identical with the Rf. value of squalene completely cochromatographed with authentic sample of squalene.

The main radioactive matter (sterolesters) was eluted out of thin-layer and hydrolysed with ethanolic solution containing KOH added to the extent of 1 ml. of KOH (60% w/v) per 10 ml. of ethanol for 90 min. on steam bath, extracted three times with diethyl ether and washed alkali-free. The ether extract was dried over anhydrous Na_2SO_4 and evaporated. The extracts were separated into the three consistuent fractions, 4,4'-dimethylsterol, 4α-methyl sterol and 4-demethyl sterol by thin-layer chromatography with chloroform, and analysed by gas-chromatography. As the result of hydrolysis and the quantitative analysis of the esters by gas-chromatography, the 4,4'-demethyl sterol esters were shown to consist of predominantly cycloartenol ester (aproximetely 97%) in the leaves of short-day and long-day plants. And the major component of the 4-demethylsterol esters was β-sitosterol ester (82% of 4-demethyl sterol esters). Cholesterol ester constituted 13% of total 4-demethylsterol ester detected by gas-chromatography, whilst radioactivity incorporated into cholesterol ester was much higher and the characteristic, as given in Table 4. The quantity of stigmasterol ester was much lower (4.9%) than those of others isolated. Gas-chromatography also showed that there are no significant differences between short-day plant and long-day plant in the component of 4-demethylsterol esters. The percentage of stigmesterol ester in total amount of sterol esters was lower than that of stigmasterol in total free sterols in the leaves of Solanum plants. The radioactive cycloartenol derived from cycloartenolester was isolated by preparative gas-chromatography. It was confirmed that the incorporation of ^{14}C radioactivity into cycloartenol ester was the greatest amount in the sterol esters of this plant, as given in Table 4, and more than twice as much as radioactivity incorporated into unesterified cycloartenol. The incorporation of (2- ^{14}C) MVA into squalene and β-carotene are present in Table 5. The radioautography of thin-layer clearly showed that (2- ^{14}C) MVA applied to the

Table 4. Incorporation of (2-^{14}C) MVA into esterified sterols and triterpenoids of Solanum plants grafted between photoinduced and non-induced plants (Group A)

	Radioactivities incorporated (counts/min)			
	LD-leaves	LD-stems	SD-leaves	SD-stems
4, 4'-dimethylsterolesters	2, 160	946	409, 520	1, 810
cycloartenolester	1, 640	—	354, 200	—
4-demethlsterolesters	460	530	81, 530	740
Cholesterol ester	N. D.		23, 470	
stigmasterol ester			5, 330	N.D.
β-sitosterol ester			29, 800	

Conditions same as Table 3.

Petroleum ether fraction and 2% E/P fraction were hydrolysed and ether extracts separated into 4,4'-dimethylsterols and 4-demethylsterols by thin-layer chromatography. Each sterol was trapped by preparative gas-liquid chromatography. (N.D.; not determined) Radioactivities of sterol esters indicate those of free sterols derived from their esters by hydrolysis.

photoinduced leaves was also transported and incorporated into squalene and β-carotene of the non-induced plants grafted.

Purification and Identification of 4, 4-dimethylsterols and 4-demethyl sterols.

The fractions eluted from alumina columns with 4% and 6% E/P were highly radioactive, which contained approximately 30% of total radioactivity incorporated in SD-leaves and LD-leaves of grafted plants, 16% in LD-stems, and 23% in SD-stems, respectively. These fractions contained mainly 4,4'-dimethylsterol and 4α-methylsterol, and were chromatographed on thin-layers of Kieselgel G impregnated Rhodamin 6G with chloroform as the solvent. Thin-layers were then scanned for ^{14}C radioactivity. It was shown that a major radioactive zone of 4% E/P fraction was at 0.43 of Rf. value by radioscanning and had the Rf. value identical with authentic 4,4'-dimethylsterols (β-amyrin and cycloartenol) on thin-layers with both systems of chloroform (Fig. 2) and methanol-Benzen (10:90). On the other hand, thin-layer radioscannings showed

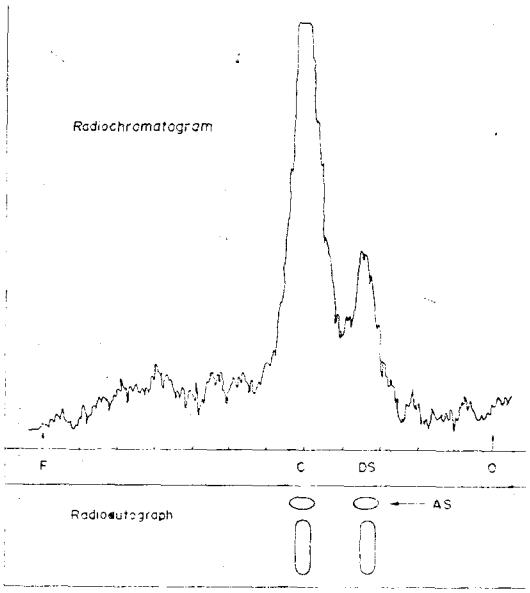

Fig. 2. Radiochromatogram of 6% E/P column fraction from *Solanum andigena*.

A silica gel G plate impregnated with Rhodamin 6G was developed with chloroform
O, Origin; F, Solvent Front; C, Cycloartenol; DS, 4-Demethylsterols; AS, Authentic samples.

that there were two radioactive zones in 6% E/P fraction of alumina column. The major radioactive band (Rf. 0.43) corresponded to 4,4'-dimethylsterols, whereas the second band (Rf. 0.29) corresponded to 4-demethylsterols.

The second band was eluted out and combined to 10% E/P fraction for purification. Gas-chromatography of the 4,4'-dimethylsterols showed the presences of compounds having the same relative retention data as β-amyrin (3.17), cyclosterol (3.60) and 24-methylene cycloartenol (4.19) in both photoinduced and non-incuded plants. The amount of unesterified cycloartenol was shown to be much lower than that of esterified cycloartenol in the both photoinduced and non-induced plants, as indicated in the previous paper (10).

The fractions eluted from alumina column with 10% and 20% E/P contained considerable amount of ratioactivity in both photoinduced and non-induced plants, as shown in Table 2. These fractions were chromatographed on thin-layers of Kieselgel G impregnated Rhodamin 6G with chloroform, and the radioactive band (Rf. 0.32) was eluted out and further purified by thin-layer chromatography with methanol-benzen system as described above. The major radioactive band cochromatographed with authentic samples of 4-demethyl sterols, β-sitosterol and stigmasterol, and the minor radioactive band corresponded in mobility on thin-layer to 4,4'-dimethyl sterols. The major radioactive band was then analysed by gas-liquid chromatography and found to have retention data identical with those of β-sitosterol (3.15), stigmasterol (2.67), campesterol (2.41), brassicasterol (2.13) and cholesterol (1.89). Gas-chromatography suggested that although β-sitosterol is quantitatively a predominant sterol in the plants, small amounts of campesterol and brassicasterol were also present in both photoinduced and non-induced leaves of *S. andigenum*. The amounts of sterols in non-induced plants were comparatively higher than those in photoinduced plants as shown in the previous work (10). Radioactivities incorporated into unesterified stigmasterol and β-sitosterol were in proportion to their contents, as compared with the their esters.

Table 5. Incorporation of (2-^{14}C) MVAinto terpenoids and free sterols of *Solanum andigenum* graftd.

		Radioactivity (counts/min)	
	R.R.D	SD-leaves	LD-leaves
cholesterol	18.9	26.300	658
stigmasterol	2.67	34.500	615
β-sitosterol	3.15	94.500	1076
β-amyrin	3.17	47.100	880
cycloartenol	3.60	162,600	1470
24-methylene-cycloartenol	4.19	56,000	940
squalene	0.99	66,930	*
β-carotene		32,490	*

R.R.D., relative retention data for cholestane (1.0)

Squalene and β-carotene were purified by thin-layer chromatography and sterols were trapped by preparative gas-liquid chromatography. Data of sterols were obtained from mean of three experiments.

*Radioactivities of squalene and β-carotnee of LD-plants grafted to SD-plants (group A) were detected by radioautography.

Effect of inhibitors of steroid biosynthesis on tuber induction.

Inhibitors of sterol biosynthesis were applied to Solanum leaves over the photoinduction period of 3 weeks. Inhibitor concentration of 2mg. SK & F7997 per ml. inhibited growth, and the leaflet became slightly yellow after two weeks of spraying. The leaves treated with nicotinic acid solution in the concentration of 4×10^{-2} M or with vanadyl sulphate solution in the concentration of 1×10^{-3} M showed no symptoms for the same period. None of the inhibitors of sterol biosynthesis used were inhibitory to tuber induction if applied in a concentration of 2 mg. SK & F 7997 per ml. or of 4×10^{-2} M nicotinic acid, but interesting results were obtained with the inhibitors, SK & F 7997 and

Table 6. Effects of Inhibitors of sterol biosynthesis on tuberization.

Inhibitors used	Concentration	No. of plants	Wt. of Tubers harvested
Experiment I			
SK& F 7997	2mg/ml	5	9.6 g
	1mg/ml	5	9.9 g
Nicotinic acid	4×10^{-2}M	5	10,4 g
	2×10^{-2}M	5	21.1 g
$VOSO_4\cdot2H_20$	1×10^{-3}M	5	19.0 g
	5×10^{-4}M	5	22.8 g
None	—	5	25.5 g
Experiment			
SK & F 7997	2mg/ml	5	19.0 g
Nicotinic acid	4×10^{-2}	5	18,5
None	—	5	41,3

In Experiment I, tubers were harvested immediately after a shortday period of 25 days.

In Experiment II, tubers were harvested on a week after a shortday period of 25 days.

nicotic acid. Although the plants initiated tuber formation, the development of tubers formed was retarded by these inhibitors. These results indicate that sterol inhibitor is inhibitory in the process developing tubers. Since tubers were produced by the plants occuring inhibition the tuberization hormone must be produced even in the presence of the sterol inhibitors.

Discussion

When the stems of non-induced plants were grafted to those of photo-induced plants, it was shown that (2- ^{14}C) MVA applied to photo-induced leaves was transported and incorporated into the terpenoids and sterols of non-induced plants across graft unions. In this grafting experiment, tubers were formed in the underground organ of non-induced plants. In this result tuber formation of non-induced plants indicated that certain tuber forming stimulus (tuberization hormone) was translocated into non-induced plant from photo-induced plant as well as (2- ^{14}C) MVA was translocated. However, it remain unexplained whether tuberization hormone is derived from ^{14}C compound such as mevalonic acid.

(2- ^{14}C) MVA was shown to be effectively incorporated into sterol esters of non-induced Solanum plants from photo-induced plants as well as into those of photo-induced plants. And sterol esters greatly predominate for the incorporation of radioactivity in this plant.

The occurrences of terpenoids and sterols in Solanum tuberosum have been demonstrated by previous investigators.[6,7,8] And it has been suggested that the product of squalene cyclization is not lanosterol which is produced in animal,[11,12] but cycloartenol in phytosterol biosynthesis in Solanum tuberosum, We have studied that the remarkable changes occur in the levels of free sterol, their esters and glycosides in the leaves of *Solanum andigena,* when the plants are transferred from long-day to short-day. This transfer caused an initial sharp decrease in the levels of β-sitosterol and cycloartenol.

This result suggest that the high amounts of esterified and unesterified cycloartenol in this plant support the theory that this compound may play an important role in the formation of phytosterols.[6,7] It has been shown by Eichenberger & Menke[13] that the leaves of Anthirrinum (spinach) contain sterol esters, which is 10–22% of the total amount of sterols, and that the binding partner in the sterol esters is palmitic acid. Kasprzyk[14] has indicated that in *Calendula Officinalis* the period of germination is characterized by hydrolysis of esters to free sterols, and the period of flowering by the accumulation of sterols and sterol esters.

The relatively small amount of radioactivity located in squalene may be due to a limited ability of this plant to accumulate this compound. It is assumed that the conversion of MVA into sterols and triterpenoids by this plant is independent of photoperiodism. How-

ever, when the same amounts of (2- ^{14}C) MVA (10 uc) were applied to non-induced and photo-induced plants (five plants per each group) of the same ages, separately, after a photo-period the incorporation of (2- ^{14}C) MVA into lipids of photo-induced plants was much higher than that of non-induced plants.

This result suggest that, if photo-induced plants are transferred to long-day condition after a short-day photo-period of 25 days, the ability of sterol biosynthesis in the leaves of the plants. seems to be rapidly increased. The occurence of esterified and unesterified cholesterol in this plant would suggest that cholesterol may a part of role in the formation of sapogenins[18] and steroidal alkaloids.[19] On investigation of 4α-methyl sterols, 24-ethylidenelophenol (relative retention data; 4.52 on OV-1 column) was detected by gas-liquid chromatography from the non-induced leaves of this plants. The failure in detection of other 4-methyl sterols (24-methylenelophenol lophenol cyclocucalenol and 4 ,14-dimethyloholcsta-8.24-diene-3-ol)[7.8.16] may be due to very low concentration in the small amount of samples examined in this work.

There seem to be apparent differences in sterol synthesis between the photoinduced and non-induced plants, when presumed from their incorporation of radioactivity; the changes in sterol levels during the photo-periodic induction would agree with this. It was suggested that the metabolism of some sterol in the photoinduced plants must be greater than that of the non-induced plants.

An increase was observed in the level of cholesterol in the leaves of the plants during the first photoinduction period in short-day condition; however, after 3 weeks the levels started to decrease rapidly. Accordingly it was thought that the changes in the level of cholesterol in the leaves during photoperiod could be related to its conversion to sterolal alkaroids[21] or to tuber formation of this plants.

In the experiment with the inhibitor of sterol biosynthesis, the plants initiated tuberization and the number of tubers formed was about the same in each case, usually two to three tubers per plant. But interesting result was that the size of tubers was apparently different between the inhibited plants and normal induced plants which were not sprayed with the inhibitor. With the result it is possible that the tuberization hormone must be produced even in the presence of the sterol inhibitors used. And the difference in size of tubers may be due to either (a) not so much tuber hormone produced in the inhibited plants, or (b) the inhibitor of sterol biosynthesis does not affect hormone production, but inhibits the process developing and expanding tuber. The experimental results show that an isopreniod or a compound derived from isopreniod particiapte in the process that tuber develop and grow.

We have recently proved[21] that cholesterol is converted into the steriod-glycoalkariod, solanine, which play an important role in growing of tuber and in disease resistance of potato tuber.[24]

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References

1) Gregory, L. E.; Amer. Jour. Bot *43*, 281 (1956)
2) Okazawa, Y., and H. W. Chapman; Physiol. Plant. *15*, 413 (1962)

3) Chapman H. W.; Physiologia Plantarum *11*, 215 (1958)
4) Elliott, C. G., M. R. Hendrie and B. A. Knights; J. Gen. Microbiol. *42*, 425 (1966)
5) Bonner, J., E. Heftmann, and J. A. D. Zeevaart;, Plant Physiol. *38*, 81 (1963)
6) Johnson, D. F., R. D. Bennett, and E. Heftmann; Science *140*, 198 (1963)
7) M. van Ardenne, G. Osske, and K. Schreiber, K. Sterinfelder, R. Tummler; Kulturflanze *13*, 101 (1965)
8) Schreiber, K. and G. Osske; Kulturphlanze *10*, 372 (1962)
9) Avegan, J., D. S. Goodman and D. Steringerg; J. Lipid Res. *4*, 100 (1963)
10) Bae Moo and E. I. Mercer; Phytochemistry *9*, 63 (1970)
11) Woodward, R. B., and K. Block; J. Amer. Chem. Soc. *75*, 2023 (1953)
12) Maudgal, R. K., T. T. Tchen and K. Block; J. Amer. Chem. Soc. *80*, 2589 (1958)
13) Eichenberger, W. and W. Menke; Z. Naturforsch, *21* 859 (1966)
14) Kasprzyk, Z., J. Pyrek and G. Turoswka; Acta. Biochem. Polonica, Vol. *15*, 150 (1968)
15) Holmes, W. C., and N. W. Ditullio; Am. J. Clim. Nutr. *10*, 310 (1962)
16) Parups E. V. and I. Hoffman; American Potato Journal *44*, 277 (1967)
17) Bowen D. M. and R. E. Olson; Nature, *203*, 762 (1964)
18) Bennett R. D. and E. Heftmann; Phytochemistry *4*, 577 (1965)
19) E. Heftmann, E. R. Lieber and R. D. Bennett:: Phytochemistry, *6*, 255 (1967)
20) Rees, H. H., L. J. Goad and T. W. Goodwin; Phytochemistry, 7, 1875 (1968)
21) Bae Moo and E. I. Mercer; 1970 in preparation
22) Kuc, J., R. E. Henze, A. J. Ullsprup and F. W. Quackenbush; J. Amer. Chem. Soc., *78*, 3123 (1956)
23) Kuc, J., A. J. Ullstrup and F. W. Quackenbush; Science, Vol. 122, 1186 (1955)
24) Allen E. H and J. Kuc; Phytopathology, *58*, 776 (1968).
25) Bowen D.M. and R.E. Olson Nature 203; 762(1964)
26) Holmes, W.L. and N.W. DiTullio Am. J. Clin. Nutr. 10, 310 (1962)