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The composition of phytosterols in first and second year *Digitalis purpurea* L

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The phytosterols of *Digitalis purpurea* occur as lipids and as glucosides. Gas-liquid chromatography and mass-spectrometry indicated that both fractions contained sitosterol as the major component, and also varying proportions of stigmasterol, campesterol and cholesterol. Certain of the lipid fractions contained 24-methylenecholesterol. The phytosterol concentrations and relative proportions were determined at monthly intervals during the first year of growth, and also in various morphological units of second year plants. The composition of the phytosterol fractions depended upon the plant organ examined and also upon the phase of growth of the plant.

The phytosterols of *Digitalis purpurea* seed oil are composed of a complex mixture of 4,4′-dimethyl, 4α-methyl and 4-des-methylsterols (Evans, 1972). The latter group are known as the true phytosterols; other compounds of this fraction may be regarded as biosynthetic precursors (Williams, Goad & Goodwin, 1967). Previous work has demonstrated that the true phytosterols are present in *D. purpurea* as either lipids (free sterols and steryl esters), or as glucosides (Cowley, Evans & Ginman, 1971). Variation in the concentrations and relative proportions of sterols during germination of *D. purpurea* seeds has been reported by Cowley & Evans (1972). I have investigated the composition and distribution of phytosterols in mature second year plants, and also the variations in concentrations and proportions during the first year of growth and development.

METHODS AND RESULTS

Cultivation. *Digitalis purpurea* seeds were germinated in sterile soil and separated into individual pots after one month. The aerial parts were collected at monthly intervals for twelve months. The second year plants were harvested at one time and divided into distinct morphological units (Table 2). About 100 g fresh weight of each sample was ground with an equal weight of ammonium sulphate immediately after collection and then deep frozen.

Estimation of the total phytosterols. Samples were extracted and separated into glucoside and lipid fractions for colorimetric analysis as described by Cowley & others (1971). The 5-ene sterols of both fractions were estimated as sitosterol.

First year plants. Little variation was found in the concentrations of total lipid phytosterols during the first year, a fluctuation of between 50–58 μg/g fresh weight being recorded (Fig. 1). However, the concentration of glucoside sterols increased from 20 to 42 μg/g fresh weight during the first four months when the seedlings were rapidly growing. Thereafter concentrations decreased to 27 μg/g after twelve months of growth.

Second year plants. The leaves were collected (a) from the basal rosette, (b) from

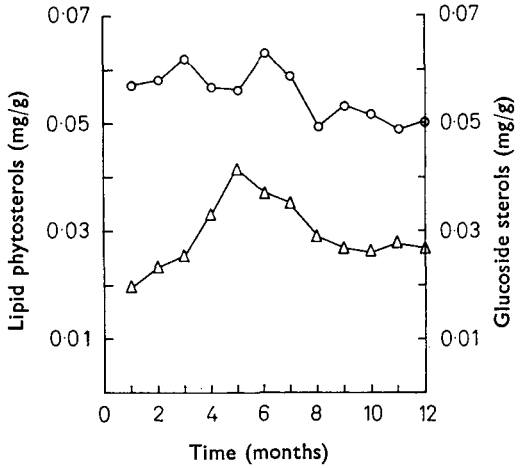

FIG. 1. Variation in the concentrations of phytosterols in the lipid and glucoside fractions during one year of growth. ○ Lipids. △ Glucosides.

the mid-portion of the stem and (c) from immediately beneath the influorescence. All leaf samples contained sterols of both lipid and glucoside-types. Highest concentrations of lipid fraction sterols were obtained from leaves of the basal rosette (61 μg/g) and the lowest from young leaves beneath the influorescence (19 μg/g) (Fig. 2). The sterols of the glucoside fraction were isolated in largest concentrations from the leaves at the top of the plant (31 μg/g), the old leaves from the basal rosette contained only 16 μg/g fresh weight (Fig. 2). Root sample (d) contained about four times as much lipid as glucoside fraction sterols, but in the stem (l), similar concentrations of both fractions were detected. The influorescence was separated into flower buds (e), mature flower buds (f), newly opened flowers (g), mature

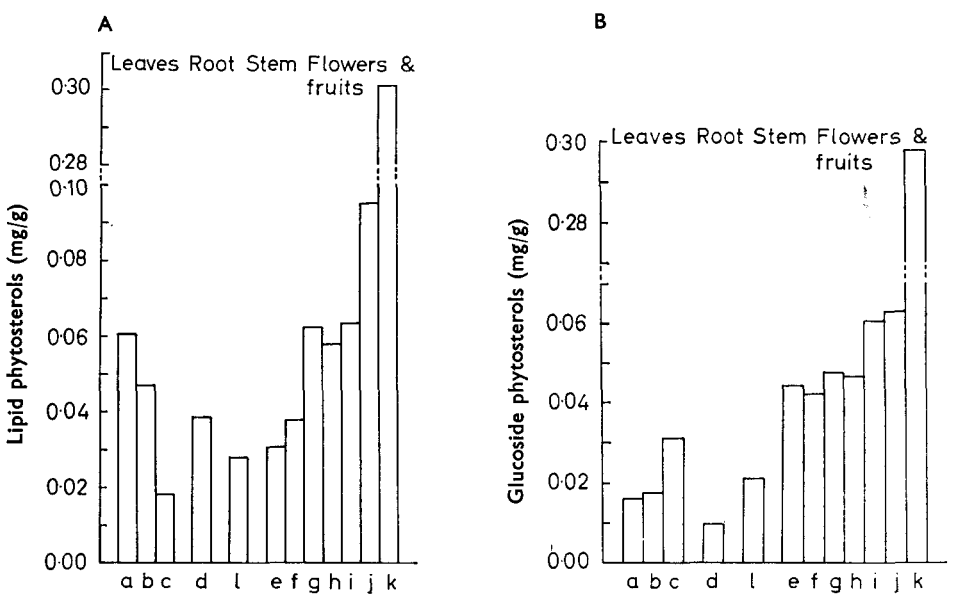

FIG. 2. The distribution of (A) lipid fraction and (B) glucoside fraction phytosterols in 2nd year *D. purpurea*.

flowers (h), immature green fruits (i), mature green fruits (j) and ripe dry fruits (k). In the developing flowers large quantities of both lipid and glucoside sterols were isolated. The lipid fraction phytosterols slowly increased from 31 in the flower buds to 58 μg/g fresh weight in the mature flowers. Concentrations increased slowly during maturation of the green fruits (95 μg/g) and then more rapidly during fruit ripening (307 μg/g). Although the flowers contained higher concentrations of glucoside sterols than the leaves, the levels increased but little during development of the flowers. As before, the concentrations increased during development and ripening of the fruits to reach a level of 297 μg/g. Part of this apparent increase in both types of sterols may be explained by a loss of moisture during fruit ripening.

Composition of the phytosterol fraction. The phytosterols were recovered from the glacial acetic acid solution left from colorimetry by extraction with dichloromethane (10 ml), made neutral with 2N NaOH and diluted with an equal volume of distilled water. The extracts were dried (anhydrous sodium sulphate) and the lipid fraction sterols purified by preparative layer chromatography (p.l.c) (Silica gel G 1 mm, activated 120° 1 h, developed with chloroform–isopropanol (97:3)). The acetates of both lipid and glucoside fractions (acetic anhydride–pyridine 1:1) were purified by p.l.c. (Hexane–dichloromethane 2:3). Both groups of acetates gave a single spot (R_F 0·72) by analytical t.l.c. in the same system as above. G.l.c. produced four peaks for these zones and five for some of the lipid fractions (Table 1). The first

Table 1. *The composition of glucoside and lipid sterol mixtures during the first years growth of* Digitalis purpurea *L.*

	Percentage of phytosterol mixture									
	Cholesterol		24-Methylene-cholesterol		Campesterol		Stigmasterol		Sitosterol	
Time in months	L	G	L	G	L	G	L	G	L	G
1	14·3	5·3	—	—	11·2	7·2	33·9	34·9	40·6	52·6
2	15·9	11·3	—	—	11·8	9·6	31·5	25·2	40·8	53·9
3	16·3	9·7	—	—	12·6	9·9	26·90	24·9	44·20	55·5
4	14·02	11·2	—	—	11·43	20·3	22·26	24·1	52·27	54·4
5	16·05	10·2	—	—	4·52	9·8	23·96	25·8	55·21	54·2
6	18·47	12·7	—	—	5·83	10·5	19·72	22·4	55·96	54·4
7	18·02	15·5	—	—	12·6	8·8	16·10	23·4	53·26	52·3
8	9·44	12·7	6·60	—	8·69	9·4	22·82	23·5	52·35	54·4
9	7·95	9·8	11·53	—	12·12	9·5	20·48	20·9	53·10	59·8
10	5·32	10·2	12·10	—	10·00	9·2	21·52	20·9	51·06	59·7
11	9·50	10·8	11·00	—	9·00	8·1	23·50	20·1	48·00	61·0
12	11·52	11·0	10·02	—	8·40	6·7	24·49	20·2	45·77	62·1

L = Lipid fraction.

G = Glucoside fraction.

G.l.c. was carried out on a Marryat g.l.c. unit fitted with a glass 9 ft × ¼ in column (1·5% SE30 on Chromosorb W (100–120) AW–DMCS) at 240° and 30 ml/min of N_2. The peak areas were calculated by the ratio of the height and width at half height and corrected by multiplying by the specific response (Evans, 1971, 1972).

peak had the same retention time as cholesteryl acetate (Rt 2·78, relative to cholestane). The mass spectrum of a condensed sample (10 μg) had a molecular ion at *m/e* 428 and significant fragmentations ions at *m/e* 413, 368, 353, 315, 173, 255 and 213, a spectrum similar to authentic cholesteryl acetate. The second peak (Rt 3·61) had the same retention time as campesteryl acetate. The mass spectrum had a molecular ion at *m/e* 442 and significant fragmentations at *m/e* 427, 382, 367, 315, 273, 255, 213 and 145. This was identical to the mass spectrum of campesteryl acetate. The third peak (Rt 3·92) had significant ions in the mass spectrum at *m/e* 394, 379, 315, 273, 255, 213 and 145, indicating the presence of stigmasteryl acetate. The final peak (Rt 4·61) had an identical mass spectrum to sitosteryl acetate (a

molecular ion at *m/e* 456, and significant fragment ions at *m/e* 441, 396, 381, 315, 273, 255 and 213). Certain of the lipid fractions (Table 1) contained an extra peak (Rt 3·15) with a similar retention time to 24-methylene-cholesteryl acetate. The mass spectrum of a condensed sample had a molecular ion at *m/e* 440 and significant fragment ions at *m/e* 425, 380, 365, 258, 313, 296, 281, 253 and 213. This is identical to the mass spectrum of 24-methlyene-cholesteryl acetate (Knights, 1968; Evans, 1972).

Variation in sterol composition during growth

First year plants. Sitosterol was at all times the major sterol of the lipid fractions. It increased in proportion during plant growth from about 40% of the phytosterol mixture in the seedlings to 56% in the 6-month old plants. During this period there occurs a fall in the proportion of stigmasterol from 34 to 16%. Initially, campesterol formed 11% of the mixture, but the proportion decreased over one year to 8·4%. 24-Methylene-cholesterol appeared as a component of the 8-month sample, and proportions increased to a level of 12·1% of the sterol mixture in the 12-month sample. Cholesterol levels increased initially during plant growth, but later decreased to 5·3% of the mixture in the 10-month old plants. The glucoside fraction did not contain 24-methylene cholesterol. Sitosterol was, as before, the major component and its proportions varied but little during 12 months growth, increasing gradually from 53 to 62% of the sterol mixture. Stigmasterol over the same period decreased in proportion from 35 to 20%. Campesterol proportions were almost unchanged during plant growth, whilst cholesterol increased from 5 to 15·5% within seven months, thereafter decreasing to 11% at the end of the growing season (Table 1).

Second year plants. Sitosterol was the major component of the lipid fractions from the second year plants. It formed about 50% of the mixtures from the leaves and root samples (Table 2). Higher proportions (60–67%) were obtained from the developing fruits and stem samples, whereas the proportion of the mixture of the flowers contained only 43%. Stigmasterol accumulated in roots, stems, immature flowers and young leaves. During fruit ripening the proportions of this sterol decreased to 7%. 24-Methylenecholesterol was absent from leaf, root and stem samples, but appeared as a constituent of the sterol mixtures from the flowers and

Table 2. *The composition and distribution of glucoside and lipid phytosterols in second year* Digitalis purpurea *L.*

		Percentage of phytosterol mixture									
		Cholesterol		24-Methylene cholesterol		Campesterol		Stigmasterol		β-Sitosterol	
	Morphological unit	L	G	L	G	L	G	L	G	L	G
a	Leaves of basal rosette ..	14·6	—	—	—	10·7	—	25·1	23·13	49·6	76·87
b	Leaves from mid-portion of stem	12·4	8·50	—	—	9·0	9·20	24·2	21·0	54·4	61·30
c	Leaves beneath influorescence	5·7	7·46	—	—	8·9	8·67	33·2	39·33	52·2	44·54
d	Roots	4·6	6·12	—	—	16·7	7·45	27·9	17·23	51·2	69·20
e	Immature flower buds ..	10·3	10·50	—	—	19·8	11·20	26·8	21·78	43·10	56·52
f	Mature flower buds	10·4	12·21	4·8	—	16·9	11·24	21·6	22·98	46·3	53·57
g	Newly opened flowers ..	10·2	11·60	4·2	—	13·4	11·51	20·7	21·88	47·5	55·01
h	Mature flowers	10·9	15·35	4·95	—	15·0	12·69	25·3	26·57	43·8	45·39
i	Immature green fruits ..	6·5	8·08	2·2	—	6·4	11·31	17·7	25·54	67·3	55·07
	Mature green fruits	10·8	7·46	3·1	—	11·5	9·74	14·5	23·60	60·1	59·20
k	Ripe fruits	4·9	10·47	10·98	—	15·8	10·33	7·3	15·33	61·1	63·87
l	Stems	6·1	8·20	—	—	9·3	10·0	22·0	22·10	62·2	59·70

L = Lipid fraction.
G = Glucoside fraction.
G.l.c. was carried out on a Pye 104 fitted with a glass 13 ft × ¼ in column (1·5% SE30 on Chromosorb W (100–120 AW-DMCS) at 235° and 40 ml/min. The peak areas were calculated by the ratio of the height and width at half height and corrected by multiplying by the specific response (Evans, 1971b).

fruits. The proportions of campesterol were lowest in the young leaves and stems, and higher in the sterol fraction of the flowers, fruits and roots (15–19·8%). Cholesterol, the C-27 sterol, occurred in highest proportions in old leaves (14·6%) and developing flowers. The roots, stems and fruit proportions of this compound were relatively low (Table 2). The glucoside fraction from the second year was of a similar composition to the first year plants. The proportions of sitosterol were greatest in the older tissues such as mature leaves (76·8%) and ripe fruits (63·87%). Stigmasterol accumulated in young leaves (39·33%) and developing flowers (Table 2). Campesterol was evenly distributed throughout the plant, slightly higher proportions being isolated from the flowers. Cholesterol levels varied widely in the plant. High proportions were found in developing flowers (10·5–15·35%) and in ripe fruits (10·47%). Both cholesterol and campesterol were absent from the basal rosette leaves.

DISCUSSION

Lipid and glucoside phytosterols were isolated from all parts of *D. purpurea*. The lipid fraction contained 5-ene sterols together with traces of their 7-ene biosynthetic precursors (Williams & others, 1967), indicating that phytosterols are produced to some extent by all tissues. The relatively large quantities of 7-ene and 5-ene sterols isolated from the mature leaves of the basal rosette and from the flowers and fruits indicates that these are the principal sites of biosynthesis. Concentrations of both types of sterols increased in the fruits as the ovules accumulated sterol stores. During germination these compounds are required for the production of new tissues before leaf formation (Cowley & Evans, 1972).

The 5-ene and 7-ene compounds of the lipid fraction were present as esters and in the free form (Evans, 1971). In the glucoside fraction, however, only 5-ene sterols were detected. It is evident that biosynthesis of sterols occurs in the free form, and that either glucoside or ester formation occurs at a stage when biosynthesis is complete. The enzymatic selection of certain sterols for complex formation suggests a positive segregation of biological roles. Phytosterols in the free form have been implicated in membrane structures (Ansell & Hawthorne, 1964). The general distribution of these compounds in first and second year *D. purpurea* supports this suggestion. Sterol esters are thought to be the form involved in intracellular sterol transportation (Kemp, Goad & Mercer, 1967). It is possible that glucoside sterols represent intercellular transportation forms from areas of synthesis (mature leaves) to areas of growth (immature leaves, flower buds and developing fruits), where sterols are required for membrane expansion.

Both the lipid and glucoside fractions consisted of sitosterol, stigmasterol, campesterol and cholesterol. Certain lipid fractions contained 14-methylene-cholesterol. Sitosterol, at all times the predominant sterol, tended to accumulate in fruits and mature leaves, stigmasterol the C-22 unsaturated isomer, in young leaves and flower buds. In the first year plants the ratio of these isomers varied throughout the growing season. The conversion of sitosterol to stigmasterol has been demonstrated in a *Digitalis* species (Bennett & Heftmann, 1969). The extent of this conversion is dependent upon the morphological unit examined and also upon the phase of plant growth. 24-Methylenecholesterol occurred in leaf samples towards the end of the first year, and also in flower and fruit samples. This compound has been isolated as a constituent of flower and fruit extracts of certain of the Cruciferae (Ingram

Knights & others, 1968), but has not previously been isolated from the aerial parts of a *Digitalis* species. Cholesterol is an active metabolite in *Digitalis* producing cardenolides (Caspi, Lewis & others, 1966), and the sapogenins (Tschesche & Hulpke, 1966). The proportions of cholesterol glucoside in *D. purpurea* were highest in young organs such as the immature leaves and flower buds, and it is possible that these tissues are the sites of sterol metabolism to secondary metabolites. This view is supported by the work of Lemli (1962) on *D. purpurea* seedlings.

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