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CARDENOLIDES AND SPIROSTANOLS IN *DIGITALIS PURPUREA* AT VARIOUS STAGES OF DEVELOPMENT

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Abstract—The concentrations of cardenolides and spirostanol steroids from first and second year *Digitalis purpurea* have been determined. In the first year the highest concentrations of cardenolides occurred between six and seven months after germination, whilst spirostanols accumulated towards the end of the first year. Mature second year leaves contained relatively high concentrations of spirostanols but young leaves accumulated cardenolides. The developing inflorescence and fruits were rich in both groups of steroids. Digitoxigenin was the major component of the cardenolide fractions with the exception of the inflorescence and roots, which contained principally gitoxigenin. In the spirostanol fractions the proportions of gitogenin were greatest in the young leaves, stems, roots, flower buds and growing plants. Digitogenin accumulated in mature flowers, fruits and old leaves.

INTRODUCTION

THE STEROIDS of *Digitalis* are classified as cardenolides,[1] spirostanols,[2,3] digitenolides[4,5] and phytosterols.[6,7] The latter group are thought to provide biosynthetic precursors for the production of cardenolides[8] and spirostanols.[9] The phytosterol composition,[7] distribution[10] and variations during germination[11] have been studied. It also has been demonstrated that the concentrations and compositions of cardenolides and spirostanols vary during the germination[12] of *D. purpurea* seeds. This communication describes the distribution and composition of these steroids during the vegetative cycle of *D. purpurea*.

RESULTS AND DISCUSSION

Total Steroid Concentrations

About 100 g fr. wt of plant material was collected on each occasion and extracted with petroleum to remove lipids and pigments. The steroidal glycosides were isolated and estimated colorimetrically as previously described.[12] Samples were collected from the first

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year plants at monthly intervals of 12 months. The leaves from the second year plants were collected from three positions on the stems. Sample (*A*) consisted of leaves from the basal rosette, (*B*) from the mid portion of the stem, and (*C*) from immediately beneath the inflorescence. Root samples (*D*) and stem samples (*L*) were also collected. The inflorescence was separated into flower buds (*E*), mature flower buds (*F*), newly opened flowers (*G*), mature flowers (*H*), immature green fruits (*I*), mature green fruits (*J*) and ripe dry fruits (*K*).

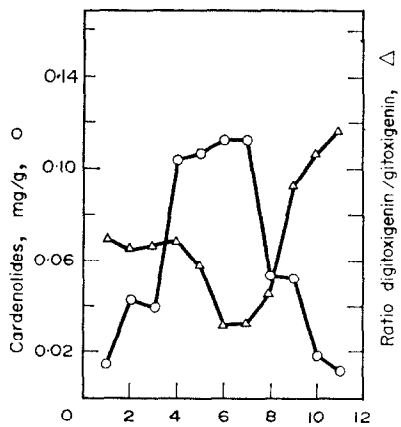

FIG. 1. VARIATION IN THE LEVELS OF CARDENOLIDES DURING FIRST YEAR'S GROWTH.

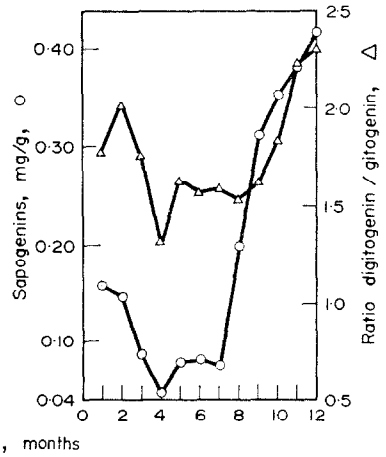

FIG. 2. VARIATIONS IN THE LEVELS OF SPIROSTANOLS DURING THE FIRST YEAR'S GROWTH.

Cardenolides. The concentrations of cardenolide steroids in the first year plants increased from 14·1 to 115·6 μg/g fr. wt during the first 7 months. This was a period of rapid plant growth. During the next 3 months little growth had taken place, and the cardenolide concentrations decreased to 11·0 μg/g fr. wt in the 11-month sample, only to increase again slightly in the final sample as the aerial stems began to form (Fig. 1). In the second year plants the age of the leaves examined had a great effect upon the amounts of cardenolides detected. Concentrations of 130 μg/g of fr. wt were isolated from young leaves from the top of the stem, and these concentrations were similar to the high levels detected in 6–7-month-old plants. The mature leaves from the basal rosette of the second year plants contained only 51 μg/g, less than half that in the young leaves (Table 1). Cardenolides were detected in all parts of the plant including the roots and the stems. The concentrations in these organs were relatively low being 20 and 30 μg/g fr. wt respectively. Cardenolide concentrations were high at all stages of development in the inflorescence. Largest amounts were found in the fully mature flowers (108 μg/g), this concentration being similar to the young leaves and immature plants. In the green fruits the concentrations increased from 75 μg/g fr. wt in the immature fruits to 480 μg/g dry wt of ripe fruit (Table 1).

Cardenolides were generally distributed throughout the second year plants, the highest concentrations occurring in young organs such as flower buds, green fruits and young leaves. This trend was reflected also in the first year plants where actively growing leaves accumulated cardenolides, confirming the reports of other workers.[13,14]

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TABLE 1. THE DISTRIBUTION AND COMPOSITION OF CARDENOLIDES AND SPIROSTANOL STEROIDS IN MATURE SECOND YEAR *D. purpurea*

Morphological unit	Cardenolides μg/g fr. wt calc. as gitoxigenin	Ratio of digitoxigenin gitoxigenin	Spirostanols μg/g fr. wt calc. as digitogenin	Ratio of digitogenin/ gitogenin
A Leaves from basal rosette	51·3	1·16	238·5	2·38
B Leaves from mid portion of stem	55·0	1·82	160·2	2·27
C Leaves from beneath the inflorescence	131·0	2·02	59·1	1·16
D Roots	19·9	0·43	62·5	0·59
E Flower buds from tip of inflorescence	98·5	0·51	77·6	0·892
F Mature flower buds	93·0	0·51	243·4	1·53
G Newly opened flowers	83·5	0·55	583·0	1·50
H Mature flowers	107·5	0·63	507·0	1·65
I Fruits immediately corolla has fallen	90·5	0·74	394·9	1·83
J Mature green fruits	75·3	1·00	469·8	1·77
K Ripe dry fruits	480·0	1·41	3767·0	2·32
L Stems	30·0	0·48	63·6	0·75

Spirostanols. The concentrations of total spirostanols were estimated as digitogenin after cholesterol precipitation from acrude extract. This has resulted in the loss of furostanol saponin forms. Spirostanol concentrations decreased in the leaves of the first year plants during the period of active growth from 158 to 50 μg/g after 4 months. Concentrations remained constant between 70 and 80 μg/g fr. wt for the succeeding 3 months, but then rose significantly to 418 μg/g after 12 months (Fig. 2). The leaves of the last sample were large, dark green and turning yellow at the margins. In the leaves of the second year plants the highest concentrations of spirostanols were also obtained from leaves of the basal rosette, 238 μg/g, as compared to only 59 μg/g fr. wt from the immature leaves beneath the inflorescence (Table 1). Similarly the concentrations from the roots and stems were small, being 62 and 63 μg/g fr. wt respectively. Concentrations were also small in the immature flower buds, but increased gradually during maturation of the flowers from 78 to 583 μg/g fr. wt of mature flowers. After corolla fall the amounts in the green fruits were less than those of the flowers. However, concentrations of spirostanols increased during fruit development to 470 μg/g fr. wt in the mature green fruits. As before, with the cardenolides, spirostanol concentrations apparently increased during fruit ripening to 3767 μg/g dry wt of fruits (Table 1). Sapogenin steroids are thought to be biosynthesised by young tissues of the plant,[15–17] the high concentrations in older organs being explained by the release of sapogenins from bound forms by an endogenous enzyme system.[18] Baker *et al.*[19] have suggested that after biosynthesis in shoot systems sapogenins are translocated to other areas. It seems likely therefore that accumulation of spirostanols in mature plants, old tissues, mature flowers and ripe fruits represents increased enzymatic activity in transforming non-cholesterol

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precipitable furostanol forms into cholesterol precipitable spirostanol forms, by the removal of the side chain sugar moiety. It is possible that this increased activity of mature tissues has a physiological significance, particularly as saponins are known to have pronounced biological effects[20–22] on plant tissues.

Both the cardenolides and sapogenin steroids are phytosterol metabolites, and although they occur together in all parts of the plant, it was only in the flowers and fruits that concentrations of both groups of sterols were higher. Normally organs which were rich in one group were deficient in the other and vice versa. The phase of growth evidently influences the route of cholesterol metabolism to either the sapogenins, by side chain and nuclear hydroxylation, or to the cardenolides by side chain oxidation, rings *C–D* inversion and C14 hydroxylation.

Individual Steroids

Cardenolides. After hydrolysis and purification,[12] the cardenolide steroids consisted of digitoxigenin and gitoxigenin. In the first year plants digitoxigenin initially formed a high proportion of the cardenolide fractions as demonstrated by the ratios of digitoxigenin/gitoxigenin of 1·75–1·74 during the first 4 months growth. Between 4 and 6 months the proportion of digitoxigenin decreased to a ratio of 0·802, but then rapidly increased again in the older plants to a ratio of 2·8–2·9 at 11–12 months (Fig. 1). In the leaves of the second year plants, digitoxigenin was again the major component. Largest quantities were found in the young leaves, as was reflected in the ratio of 2·02, compared to 1·16 from the leaves of the basal rosette (Table 1). The root and stem samples contained mainly gitoxigenin as illustrated by steroid ratios of 0·4 and 0·5 respectively. Gitoxigenin was also the major component of the cardenolides from the inflorescence. During development of the flowers its proportions gradually decreased as shown by the increase in the digitoxigenin/gitoxigenin ratio from 0·5 in the flower buds to 0·63 in the mature flowers. Digitoxigenin proportions continued to increase in the green fruits to reach a maximum of 1·41 in the ripe dry fruits. It has been suggested[23] that C-14 and C-16 hydroxylation of pregenolone precedes the lactone ring formation during cardenolide biosynthesis. The accumulation of either steroid therefore reflects the activity of pregenolone C-16 hydroxylase at various stages of plant growth and in different plant organs.

Spirostanols. The spirostanol fractions consisted of gitogenin and digitogenin. The latter compound is a predominant constituent of the seeds, but has previously been isolated from the aerial parts of a *Digitalis* species.[24,25] The ratio of these two compounds was estimated, after purification, by GLC.[26] In the first year plants the proportion of gitogenin in the sapogenin fractions increased during the months of growth, but decreased at the end of the year in the mature leaves (Table 1). In the leaves of the second year plants gitogenin proportions were higher in the young leaves from the top of the aerial stem (Table 1), whereas in the old yellowing leaves from the basal rosette the proportions of digitogenin were considerably higher. The roots and stems contained mainly the dihydroxy steroid

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gitogenin as indicated by the digitogenin/gitogenin ratio of 0·59 and 0·75 respectively. In the flower buds of the second year plants the principal sapogenin was also gitogenin. During flower development the proportions of digitogenin in the mixture increased as shown by the increase in the ratio from 0·89 to 1·65 in the mature flowers (Table 1). The ratio of this steroid continued to increase in the green fruits to 1·77 and during ripening to 2·32 in the dry fruits.

In *D. purpurea* the mature tissues such as basal leaves of both the first and second year plants, mature flowers and ripening fruits accumulated digitogenin, whilst seedlings, young leaves, flower buds, stems and roots accumulated gitogenin. It has been suggested[27] that polyhydroxy sapogenins are initially produced by plants and are deoxygenated during plant growth. It is not clear, however, whether nuclear hydroxylations during biosynthesis, like those of the cardenolides, precede side chain furostanol formation. The accumulation of gitogenin or digitogenin in an organ would then reflect the activity of a cholesterol nuclear hydroxylase at different stages in the life cycle.

EXPERIMENTAL

Collection of plant material. Plants were germinated from seed obtained from Verenigde Nederlandse Kruiden Cooperatie. About 100 g fr. wt were collected on each occasion and deep frozen till required for analysis. 1st yr plants were harvested at monthly intervals, 2nd yr plants were collected at the same time and divided into distinct morphological units.

Extraction. The steroids were extracted with a graded series of solvents[12] by Soxhlet extraction. The pigments were removed by partition with petrol.–Et_2O (3:2) and the steroidal glycosides extracted with a series of $CHCl_3$–MeOH mixtures as described previously.[12]

Estimation of total steroids. The glycoside samples were separated into cardenolides and spirostanol fractions by cholesterol precipitation.[28] The spirostanols were estimated by colorimetric analysis as digitogenin,[12] and the cardenolides as gitoxigenin.[12,29]

Estimation of individual steroides. The ratio of digitoxigenin/gitoxigenin was obtained, after hydrolysis of the cardiac glycosides, by a TLC-densitometric analysis as previously described.[12] Ratios of digitogenin/gitogenin were obtained by a GLC analysis.[26] Further authentication of these steroids was carried out from a large sample of mature fruits, and the four steroids had identical m.ps, IR spectra, TLC and GLC constants to authentic samples of digitoxigenin, gitoxigenin, digitogenin and gitogenin.

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