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VARIATION IN CARDENOLIDES AND SAPOGENINS IN *DIGITALIS PURPUREA* DURING GERMINATION

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Key Word Index—*Digitalis purpurea*; scrophulariaceae; cardenolides; sapogenins; variation in seedlings.

Abstract—Variations in the relative proportions and amounts of cardenolides have been estimated during germination of *Digitalis purpurea* seeds. A decrease of cardenolides was observed during germination followed by a rapid increase which coincided with the development of the first true leaves. The proportions of the principal cardenolide, digitoxigenin, increased initially, but decreased later as the proportions of gitoxigenin increased. The amounts of extractable sapogenins reached two maxima during germination, the first peak corresponded with the development of the cotyledons and the second with the development of the first true leaves. The principal sapogenin was digitogenin but the proportions of this steroid decreased as those of gitogenin increased during 1 month of observations.

INTRODUCTION

Digitalis purpurea contains cardenolide[1–5] and sapogenin[6–10] steroids, both of these groups representing compounds of considerable importance to the pharmaceutical industry. Recent work[11–13] has led to a better understanding of pathways of biosynthesis, but their biological role in plants has received little attention. This communication describes a comparative investigation into the variations in concentrations and composition of both groups of steroids during germination of the seeds.

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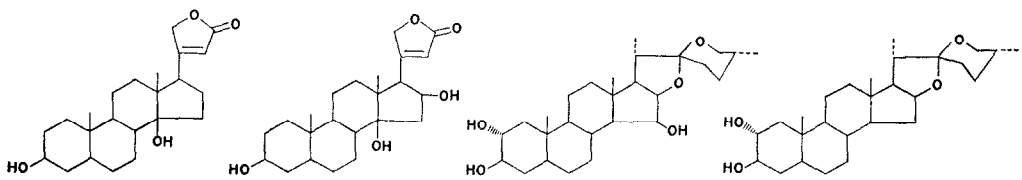
RESULTS AND DISCUSSION

Extraction removed all detectable steroidal material from the seeds and seedlings. The lipid fraction was extracted with light petroleum and the glycosides extracted from the crude extract with chloroform–methanol. This glycoside fraction was divided into two portions for analysis. From the larger portion (60 ml) the saponins were precipitated by the addition of cholesterol, and estimated colorimetrically as digitogenin by the anthrone furfural reaction.[14] The supernatant was hydrolysed and the cardenolides estimated as gitoxigenin by the Kedde reaction.[15] A differential analysis, after further purification was obtained from the second portion of the glycoside fraction for the sapogenin steroids,[16] and from the solution remaining after colorimetry for the cardenolides.[15]



The cardenolides isolated from *D. purpurea* consisted of the dihydroxy compound digitoxigenin (I) and the trihydroxy compound gitoxigenin (II). The C-16 formyl ester gitaloxigenin could not be detected in the hydrolysate, and it is possible that formic acid was removed during the hydrolysis of the digitalose containing glycosides. The gitoxigenin values given in this paper are therefore the sum of the natural gitoxigenin plus a small amount produced by the decomposition of gitaloxigenin during extraction.

The seeds contained large quantities of cardenolides, but these decreased slowly over the germination period (a period of 30 days) from 0·2 mg to 0·042 mg/g fr. wt. If this data is calculated on the basis of mg of cardenolides per 1000 seedlings the quantity of steroids can be seen to decrease from 0·035 mg to 0·023 mg per 1000 seedlings, 15 days from the start of the experiment (Fig. 1). At this stage, which corresponded with the development of chlorophyll in the first true leaves, synthesis of cardenolide kept pace with the growth of the seedlings. The highest level of 0·055 mg per 1000 seedlings was recorded at the end of the experiment after 30 days of growth. This gradual turnover of cardenolides indicates that these compounds are not metabolically inert. The seed stores were utilised during the germination and the levels fell until the green pigments were produced in the leaves, at which stage synthesis commenced. The accumulation of cardenolides in growing tissues has been observed previously,[17] this possibly being explained by the binding of cardenolides in the form of the cardiac glycosides to structural elements in the growing seedlings. It is also evident that young green tissues are required for the production of these compounds, the seeds forming a store during early growth.

A thin-layer-densitometric method was developed for the estimation of the ratios of I

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to II.[15] Prior to the analysis the phenolic pigments were removed[18] and the steroids finally purified by TLC. At all stages of the germination the predominant cardenolide was digitoxigenin. The relative proportions increased by about three times during the first 10 days, this increase being reflected in the increase in the ratio of digitoxigenin to gitoxigenin from 1·88 to 3·59 in the 10 day seedlings. The proportion of digitoxigenin then fell slightly over a period of eleven days, the ratio having decreased to 2·91 at age 21 days. Thereafter it rapidly decreased to level off between 1·49 and 1·43 at 27 to 39 days.

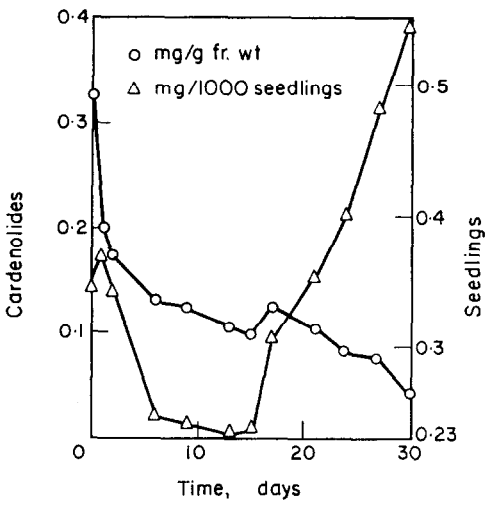

FIG. 1. VARIATION OF THE TOTAL CARDENOLIDES DURING GERMINATION CALCULATED AS DIGITOXIGENIN.

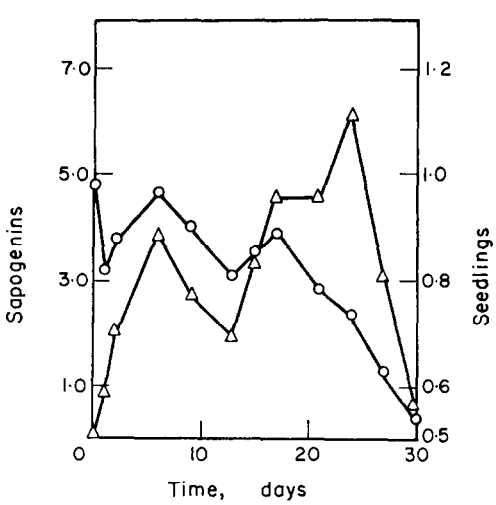

FIG. 2. VARIATION OF THE TOTAL SAPOGENINS DURING GERMINATION CALCULATED AS DIGITOGENIN.

The sapogenin fractions from the seeds and seedlings contained the trihydroxy digitogenin (III) and the dihydroxy gitogenin (IV). As with the cardenolides the seeds contained the largest quantities of sapogenin steroids, but the amounts decreased over the whole germination period from 4·8 mg/g in the seeds to 0·442 mg/g fr. wt in the seedlings at 30 days. The amounts of sapogenins increased in the seeds after moistening for a period of 5–6 days. Then as the cotyledons produced chlorophyll pigments the quantities of steroids decreased over the next 10 days, before increasing once again to reach a second peak after 17 days. This second peak corresponded to the production of the first pair of true leaves (Fig. 2). If the data is re-calculated as before on the basis of the mg of sapogenin per 1000 seedlings this pattern is illustrated even more forcefully (Fig. 2). The rise in the extractable sapogenin before leaf formation was of interest as this could indicate some positive physiological role for these steroids in the plants. It is not clear however, if these increases were due to a biosynthesis of steroids by the seeds or merely due to an increase in the extractable form of the glycosides at certain stages of the germination. It has been suggested by other workers that sapogenins are bound to cell wall structures and released by enzymatic activity during their preparation.[19] The decrease in extractable amounts during leaf development could equally well represent catabolic metabolism or a binding of the sapogenins to

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the cell walls of actively growing tissues. The cholesterol method used for purification does not precipitate furostanol saponins, and if these compounds are produced by *Digitalis* species, their formation could be favoured at this stage. By means of GLC,[16] the ratio of digitogenin to gitogenin was assessed after further TLC purification to remove the sugar decomposition products. Digitogenin (III) formed 74% of the sapogenin mixture from the seeds. During germination the proportion of this constituent decreased considerably, as was shown by the fall in the ratio of the two sapogenins from 2·8 in the seeds to 1·02 in the seedlings after 30 days. During the initial stages of germination, up to 15 days, the decrease in the proportion of digitogenin in the mixture is more rapid, and then the proportion initially rises and then falls again between 24 and 30 days from the start of the experiment.

The production of sapogenins and cardenolides by *Digitalis* species is associated with the further metabolism of cholesterol. Cardenolide production involves oxidation of the side chain to a pregnane derivative[18,20] followed by acetate condensation, whilst the sapogenins are produced by hydroxylation of the side chain followed by cyclisation.[21,22] These diverging biochemical pathways appear to be unconnected in the germinating seeds, the production of sapogenins being independent of the cardenolides. Digitoxigenin, the least hydroxylated compound, formed the bulk of the cardenolide mixture, and increased in the initial stages of germination, whilst in the case of the sapogenins the more highly hydroxylated digitogenin formed the bulk of the mixture, but the proportions decreased during germination.

EXPERIMENTAL

Cultivation. Seeds were evenly spread on sheets of blotting paper, which were placed on sterilized beds of sand and peat mixtures. The beds were lightly sprayed 4 times a day with distilled water. Every 4th day a copper fungicide was applied and on the 7th day a nutrient solution was given.[23] The seeds germination under natural light conditions at an average temp. of 25°.

Collection. The sheets were scraped clean of seedlings and weighed at once to obtain the fr. wt.

Extraction. About 30 g of plant material was homogenized in a blender with 200 ml H_2O for 5 min. The marc was re-homogenized with 50 ml of 50% MeOH, and then extracted in a Soxhlet with MeOH for 6 hr, and further extracted with benzene for 2 hr. The filtrates were bulked and the benzene and methanol removed under reduced pressure. The solution was diluted with an equal volume of 0·5% $(NH_4)_2SO_4$ and the lipids and green pigments removed with 400 ml petroleum–ether (3:2). The aqueous phase was re-extracted successively with 200 ml $CHCl_3$–MeOH (4:1), 100 ml $CHCl_3$–MeOH (2:1) and 100 ml $CHCl_3$–MeOH (3:2). The $CHCl_3$ layers were bulked and the $CHCl_3$ removed under reduced pressure. The crystalline residue was dissolved by treating in 100 ml of 50% EtOH.

Purification. The glycoside solution was divided into two portions.

First portion. (1) *Colorimetric analysis of Sapogenins.* 60 ml of the glycoside solution was used for the precipitation of saponins with cholesterol, and the total sapogenin content was estimated as digitogenin by the anthrone–furfural reaction.[14] $E^{1\%}_{1cm}$ for this assay is 8·7 with a coefficient of variation of 2%.[15] (2) *Colorimetric analysis of cardenolides.* The supernatant from the saponin precipitation was diluted with one-tenth of its vol. of lead subacetate and the cardiac glycosides extracted with $CHCl_3$–MeOH (2:1). The solvent was removed and the digitoxose glycosides hydrolysed by refluxing with 0·1 N H_2SO_4 in 90% EtOH for 15 min. After diluting with an equal vol. of H_2O the cardenolides and digitalose glycosides were recovered with $CHCl_3$; the solution was dried and the solvent removed as before. The residue was dissolved in 10 ml of methanol containing 2% HCl and incubated at 22° for 114 hr. The cardenolides were recovered as above and the residue analysed colorimetrically by a modification of the Kedde reaction.[15] $E^{1\%}_{1cm}$ for this assay was 148 with a coefficient of variation of 2·4%. (3) *Estimation of the ratio of digitoxigenin–Gitoxigenin.* The cardenolides were extracted from the excess solution from the colorimetric assay by partition with EtOAc. The phenolic pigments were removed by partition with alkali at 0–4°[18] and the steroids finally purified by TLC on 500 μ layers of silica gel G activated at 120° for 30 min prior to use. The plates were developed with

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$CHCl_3$–Me_2CO (49:1), the steroid zone located in UV light and the cardenolides removed by elution with 50 ml $CHCl_3$–EtOH (9:1). The residue was dissolved in 1 ml of tetrahydrofuran and 5 μl of solution was applied as a spot of each of three 2·5 × 20 cm glass clates coated with a 300 μ layer of silica gel H. The plates were developed 15 cm with EtOAc and the steroids visualised with 10% $SbCl_3$ in benzene and heating at 120° for 20 min. The peak areas were estimated on the Chromoscan densitometer at 620 nm. The specific response factor for the reaction of digitoxigenin–gitoxigenin with $SbCl_3$ was 0·657 with a coefficient of variation of 4%.[15]

Second portion. The ratio of digitogenin–gitogenin in the second portion of the crude glycoside extract was estimated by GLC after acid hydrolysis as previously described.[16]

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