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INCORPORATION OF $^{14}CO_2$ INTO CARDENOLIDE AND SAPOGENIN STEROIDS OF *DIGITALIS PURPUREA*

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Abstract—The relative rates of biosynthesis of cardenolide and sapogenin steroids of *Digitalis purpurea* were estimated by their uptake of $^{14}CO_2$. The incorporation of label into both groups, although initially slow, indicated that biosynthesis occurs even at the end of the growing season. The sapogenins were produced more rapidly than the cardenolides at this stage of plant development. Within the group of sapogenins, digitogenin, the trihydroxy compound, was produced at a greater rate than the dihydroxy steroid gitogenin. In the case of the cardenolides, the trihydroxy gitoxigenin was produced at a slower rate than the dihydroxy digitoxigenin.

INTRODUCTION

In DIGITALIS species the cardenolide and sapogenin steroids are produced by the further metabolism of phytosterols,[1,2] the composition and distribution of which have been studied in *Digitalis purpurea*.[3-6] It has been demonstrated that the tissue levels and compositions of both sapogenin and cardenolide steroids vary during germination,[7] and also with the age of the plant or the morphological unit examined.[8] This communication describes an investigation into the relative rates of production of cardenolides and sapogenins in mature *D. purpurea* by exposure to $^{14}CO_2$.

RESULTS

Ten-month-old *Digitalis purpurea* plants were exposed to $^{14}CO_2$ in a growth chamber and harvested at time intervals from 6 hr to 25 days. After drying, the pigments and lipids were removed by Soxhlet extraction with petroleum spirit and the glycosides by extraction

[1] TSCHESCHE, R. and LILIANWEISS, G. (1966) *Z. Natursforsch.* **21,** 494.
[2] TSCHESCHE, R. and HULPKE, E. (1966) *Z. Natursforsch.* **21,** 294.
[3] COWLEY, P. S., EVANS, F. J. and GINMAN, R. F. A. (1971) *Planta Med.* **19,** 249.
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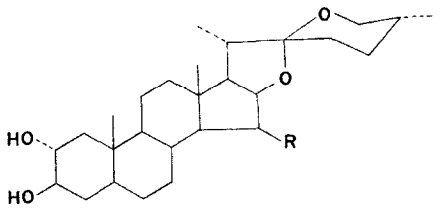
[6] COWLEY, P. S. and EVANS, F. J. (1972) *Planta Med.* **22,** 88.
[7] EVANS, F. J. and COWLEY, P. S. (1972) *Phytochem.* **11,** 2729.
[8] EVANS, F. J. and COWLEY, P. S. (1972) *Phytochem.* **11,** 2971.

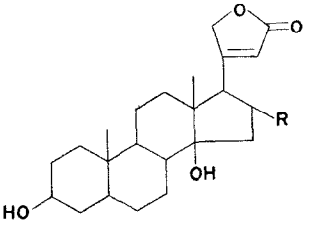
with methanol. The crude glycoside extract was divided into two portions for the isolation of cardenolide and sapogenin steroids.

Sapogenins

The sapogenins were obtained by partition with chloroform–methanol after strong acid hydrolysis.[9] Purification by TLC produced between 40 and 50 mg of pale yellow crystals. These were further separated by TLC into two zones, the lower zone corresponding to digitogenin (II) and the upper zone to gitogenin (I). The acetates of both zones were further purified by TLC and then recrystallized to constant specific activity from ethanol–ether. This normally required 3–4 recrystallizations. The purified acetates had the same R_fs as authentic samples of digitogenin and gitogenin acetates and the liberated alcohols the same R_fs as digitogenin and gitogenin.[10] By means of GLC their TMS–ethers corresponded to digitogenin and gitogenin-TMS.[11] The products had identical IR spectra to authentic samples, the upper zone had an m.p. of 268° (lit. 270°) and the lower zone of 294° (lit. 296°).



(I) R = H-Gitogenin
(II) R = HO-Digitogenin



(III) R = H-Digitoxigenin
(IV) R = HO-Gitoxigenin

Radioactivity was detected in the sapogenins within 12 hr of exposure to $^{14}CO_2$. The trihydroxy digitogenin (II) incorporated the label more rapidly than the dihydroxy gitogenin (I). During the first 48 hr, the activity of gitogenin reached 786 cpm/μmol, whereas digitogenin had an activity of 1660 cpm/μmol within 12 hr and 2560 cpm/μmol within 48 hr (Fig. 1). The rapid uptake of $^{14}CO_2$ by digitogenin continued up to 7 days, reaching a level of 4360 cpm/μmol. Thereafter the rate of increase of $^{14}CO_2$ uptake was slower for 5 days, the rate increasing again to reach a specific activity of 10 133 cpm/μmol after 25 days. The incorporation of $^{14}CO_2$ into gitogenin increased at a slower but steady rate over 25 days to reach a level of 2630 cpm/μmol (Fig. 1).

Cardenolides

The cardiac glycosides were isolated from the second portion of the crude extract by extraction with chloroform–methanol mixtures. After mild hydrolysis[12] the steroids were separated into two zones by TLC. The upper zone which corresponded to digitoxigenin (III) (yield 15–20 mg) and the lower zone gitoxigenin (IV) (10–15 mg) were separately acetylated and purified by TLC with chloroform–isopropanol (97/3) as solvent. The residues were recrystalized from ethanol–ether to constant specific activity as before. The acetates by TLC had identical R_fs to authentic samples of digitoxigen and gitoxigenin acetates, the free

[9] ROTHMAN, E. S., WALL, N. E. and WALENS, H. A. (1952) *J. Am. Chem. Soc.* **74**, 5791.
[10] DUNCAN, C. R. (1962) *J. Chromatog.* **8**, 37.
[11] COWLEY, P. S., EVANS, F. J. and GINMAN, R. F. A. (1971) *J. Chromatog.* **54**, 185.
[12] VOSS, W. and VOGT, G. (1936) *Chem. Ber.* **69**, 2333.

sterols similar R_fs[13] to digitoxigen and gitoxigenin. The IR spectra were identical to authentic samples. Digitoxigenin had an m.p. of 248° (lit. 250°) and gitoxigenin of 232°.

The incorporation of $^{14}CO_2$ into the cardenolides was slow for the first 12 days of exposure, reaching a specific activity of 250 cpm/μmol in gitoxigenin and 634 cpm/μmol in digitoxigenin. From 12 to 25 days exposure to $^{14}CO_2$, the rate of uptake of the label increased to a level of 3451 cpm/μmol in gitoxigenin and 5332 cpm/μmol in digitoxigenin (Fig. 2).

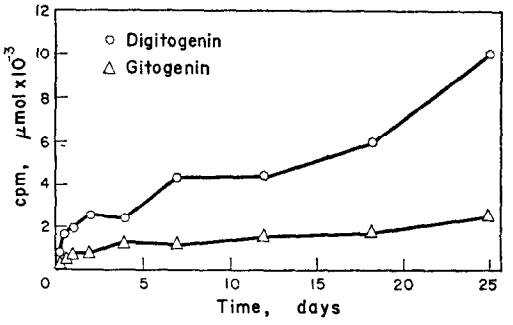


FIG. 1. THE RATE OF UPTAKE OF $^{14}CO_2$ BY THE SAPOGENINS OF *D. purpurea*.

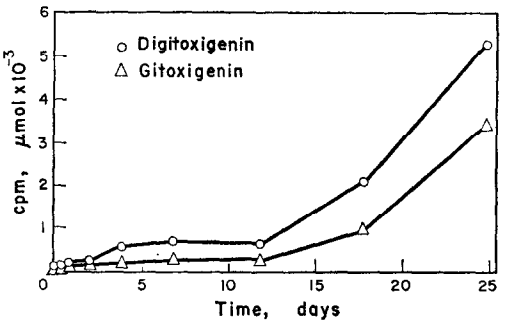


FIG. 2. THE RATE OF UPTAKE OF $^{14}CO_2$ BY THE CARDENOLIDES OF *D. purpurea*.

DISCUSSION

Our previous work[8] had suggested that towards the end of the first year's growth, the steroids of *Digitalis purpurea* were undergoing changes in tissue levels. The concentrations of cardenolides were decreasing, whilst those of the sapogenins were increasing. When plants at this stage of growth were exposed to $^{14}CO_2$, there was a slow but significant uptake of the label in both groups of steroids. Both groups were therefore being biosynthesized in mature 12-month-old plants. A balance must exist in the plant tissue between synthesis and catabolism, the balance being dependent upon the age or ecology of the plant. One question which arises from this observation is whether steroid levels are controlled by the rates of synthesis or removal from plant cells.

The low radioactivity of both groups of compounds compared to the activity of the chamber atmosphere indicates that the pathways of biosynthesis are well removed from the primary biochemical centres of acetate metabolism. Certain fractions from the plants, such as the lipid and water-soluble fractions, were very radioactive, even within 24 hr. As a group, the cardenolides did not take up the $^{14}CO_2$ as rapidly as the sapogenins, thus confirming the observation of Euw and Reichstein[14] that the biosynthesis of the sapogenins is more rapid than that of the cardenolides. Both groups are produced by further metabolism of cholesterol[1,2] by means of diverging biochemical pathways. The cardenolides are produced by side chain oxidation to a pregnane derivative followed by acetate condensation,[15] whilst the sapogenins are produced by hydroxylation of the side chain followed by cyclization.[16,17] Phytosterols are directed into both pathways in the 10-month-old *D. purpurea* plants, the phase of growth governing the relative proportions directed into each pathway.

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[14] EUW, J. V. and REICHSTEIN, T. (1966) *Helv. Chim. Acta* **49**, 1468.
[15] TSCHESCHE, R., HULPKE, H. and SCHOLTEN, H. (1967) *Z. Natursforsch.* **22**, 677.
[16] JOLY, R. A., BONNER, J., BENNETT, R. D. and HEFTMANN, E. (1969) *Phytochem.* **8**, 857.
[17] VARMA, K. R., WICKRAMASINGHE, J. A. F. and CASPI, E. (1969) *J. Biol. Chem.* **244**, 3951.

Both saponins[18] and cardiac glycosides[19] are known to have pronounced activities on plant tissues and it is possible that their preferential production at various phases of growth has a physiological significance.

In the sapogenin fraction digitogenin (II) the trihydroxy steroid took up the $^{14}CO_2$ label more rapidly than the dihydroxy compound gitogenin (I). It is possible that the trihydroxy compound is initially produced and slowly reduced to gitogenin.[20] However, gitogenin does not accumulate in second year *D. purpurea*[8] and it is more likely that gitogenin is rapidly converted to digitogenin at this stage of growth. It is also possible that hydroxylation occurs before spirostanol side chain formation, C-15 hydroxylase activity being reflected in the production of digitogenin. A reverse situation exists for the cardenolides where the ^{14}C label was incorporated more rapidly into the less hydroxylated steroid digitoxigenin (III) than into gitoxigenin (IV), the C-16 hydroxy compound. It is not known if these steroids are interconverted in *Digitalis* species or if they are produced by separate pathways. Recent work[15] suggests that hydroxylation at C-16 precedes lactone ring formation; however, the conversion of ^{14}C-digitoxin to ^{14}C-gitoxin by tissue culture of *D. purpurea* has been reported.[21]

EXPERIMENTAL

Cultivation. Digitalis purpurea plants were raised from seeds obtained from Verenigde Nederlanse Knuiden-Cooperatie. They were placed in individual pots at an age of 10 months and arranged inside the growth chamber.

Exposure to $^{14}CO_2$. Plants were exposed to an atmosphere of 1 mCi of $^{14}CO_2$ in a chamber which contained a double door system for the removal of plants at time intervals with a minimal loss of radioactivity.

Extraction. The plant material was dried at 60° for 12 hr, powdered and about 25 g dry wt extracted and the lipids removed by partition with light petrol.–Et_2O (3:2) as previously described.[6] The aqueous phase containing the steroidal glycosides was divided into equal portions.

Cardenolides. This portion was diluted with one-tenth of its vol. of lead subacetate solution. The filtrate was mixed with an equal volume of 1% ammonium sulphate solution and the cardiac glycosides extracted with 250 ml of $CHCl_3$–MeOH (3:2). After removal of solvent the residue was hydrolysed by mild acid hydrolysis.[12] The cardenolides were extracted with ethyl acetate and the phenolic pigments removed with 2 N NaOH at 0°.[22] The cardenolides were purified by TLC on silica gel G layers (0·5 mm) using $CHCl_3$–acetone (98:2) as solvent. The eluted zone was separated into a digitoxigenin and gitoxigenin fraction by TLC with ethyl acetate as solvent. The acetates of each fraction (Ac_2O–pyridine (2:1)) were purified by TLC with $CHCl_3$–*iso*propanol (97:3) as solvent. The eluted acetates were recrystalized from EtOH–Et_2O (1:5) to constant specific activity. About 0·5 mg samples were taken for liquid scintillation counting on an automatic spectrometer.

Sapogenins. The second portion of original extract was hydrolysed by strong acid hydrolysis[9] and the sapogenins extracted as previously described.[7] By means of TLC on silica gel (0·5 mm layers), developing with $CHCl_3$– acetone (9:1), a sapogenin zone was produced which was further separated into a digitogenin and a gitogenin fraction as before.[7] The prepared acetates (Ac_2O–pyridine, 2:1) were purified by TLC with $CHCl_3$-acetone (96:4) as developing solvent. The eluted acetates were recrystallized from Et_2O to constant specific activity.

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