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METABOLISM OF TRITERPENOIDS IN THE SEEDS OF *CALENDULA OFFICINALIS* GERMINATING

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Abstract—It has been shown that germinating seeds of *Calendula officinalis* possess the ability to synthetize triterpene compounds of the oleanane, ursane and lupane groups. The biosynthesis of the various triterpenes occur in different parts of the seed. In the embryo, only oleanane derivatives are formed, but cotyledons also synthetize compounds belonging to ursane and lupane groups.

INTRODUCTION

IT HAS been shown that the following triterpenoids occur in the seeds[1] and flowers[2] of *Calendula officinalis*: sterols and pentacyclic mono- and dihydroxy triterpenes of the oleanane, ursane and lupane groups. In the oleanane group, β-amyrin derivatives at higher oxidation levels, oleanolic aldehyde and oleanolic acid, are also present. The triterpenoids occur in various forms:[1] mono-alcohols mainly as free compounds, diols almost exclusively in the form of esters and sterols as mixtures of free compounds (90%), glycosides (7%) and esters (2%).

It has been shown[3,4] that the shoots and roots of *Calendula officinalis* contain sterols and triterpenes only of the β-amyrin type. To discover when triterpene alcohols of the ursane and lupane groups disappear from germinating seeds or young seedlings, we determined the level of all triterpenoids during 21 days of development.[1] These determinations showed that up to the fifth day, the level of all types of triterpenoids decreased slightly. The concentration of triterpene alcohols, other than β-amyrin and its congeners, markedly decreased, but after the tenth day the level of sterols and oleanolic acid increased significantly. These results indicate that the ability to synthetize triterpene mono-alcohols and diols of the ursane and lupane groups disappears during the growth of young seedlings and the synthesis of sterols and triterpenes of the β-amyrin type develops.

The aim of the present study was to show in germinating seeds of *Calendula*, how the synthesis of the different triterpene alcohols of various types changes, and whether there is any spatial separation of these processes.

RESULTS AND DISCUSSION

The *Calendula* seeds germinated during 48 hr were administered 1-^{14}C-acetate. The incorporation of radioactivity into triterpenoid compounds was subsequently investigated at various time intervals for 0–15 days.

Incorporation of radioactivity into free triterpene monoalcohols is shown in Fig. 1. Peaks of labelling of all compounds occur after 12 hr, followed by a decrease of radioactivity,

[1] Z. KASPRZYK, J. ŚLIWOWSKI and D. BOLESŁAWSKA-KOKOSZA, *Acta Biochim. Polon.* **17**, 11 (1970).
[2] Z. KASPRZYK and J. PYREK, *Phytochem.* **7**, 1631 (1968).
[3] Z. KASPRZYK and M. FONBERG-BROCZEK, *Physiol. Plantarum* **20**, 321 (1968).
[4] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **8**, 1921 (1969).

which is most rapid in α-amyrin and ψ-taraxasterol (after 5 days the level of radioactivity in these compounds is negligible), and slower in lupeol and taraxasterol. On the other hand, the level of radioactivity in β-amyrin is constant from third day till the end of the experiment.

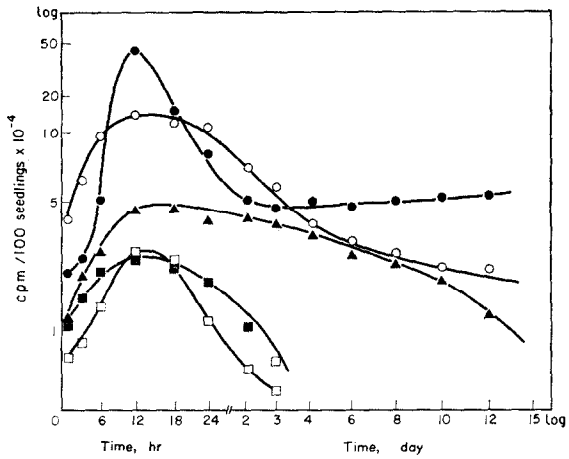

FIG. 1. DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO TRITERPENE MONOLS IN GERMINATING SEEDS OF *Calendula officinalis*.
β-amyrin (●), taraxasterol (○), ψ-taraxasterol (■), α-amyrin (□) and lupeol (▲).

Figure 2 shows incorporation of radioactivity to triterpene diols. Peaks of labelling in the diols occur 6 hr later than the labelling of mono-alcohols. This fact indicates that in germinating seeds diols are formed probably by a process of hydroxylation of the free mono-alcohols identical with that found in the flowers. The radioactivity in erythrodiol remains constant similar to that of β-amyrin after the sixth day till the end of experiment. The radioactivity of brein, ursadiol,[5] faradiol (plus arnidiol) and calenduladiol drops during this period.

No incorporation of radioactive acetate into esters of triterpene mono-alcohols was observed in germinating seeds. On the other hand, radioactivity appears in esters of triterpene diols (Fig. 2B) after 48 hr, reaches a maximum for brein and faradiol (plus arnidiol) after 7–8 days, and for the remaining diols after 10 days, and then decreases. A comparison of the radioactivity level of diol esters and their time of labelling indicate that the process of esterification of these compounds is rather slow in comparison to the synthesis of free diols.

The dynamics of labelling of oleanolic acid is presented in Fig. 3. Radioactivity in this compound increases during the whole period of experiment, reaching values 100 times higher than for other triterpenes and 10 times higher than for sterols.

Figure 4 shows the incorporation of radioactive acetate into free sterols, sterol glycosides and esters. In the case of free sterols, maximum of labelling for Δ^7-sterol, sitosterol, and for stigmasterol occurs after 3, 18 and 72 hr (3 days) respectively, supporting the established sequence of their biosynthesis.[4,6,7] This is followed by a decrease in total radioactivity of both sitosterol and stigmasterol until the 8th day and then increases. The labelling rate of sterol glycosides increases in parallel to the free sterols but instead of

[5] J. ŚLIWOWSKI and Z. KASPRZYK, in press.
[6] D. E. JOHNSON, E. HEFTMANN and G. V. C. HOUGHLAND, *Arch. Biochem. Biophys.* **104**, 102 (1964).
[7] R. D. BENNET, E. HEFTMANN, W. H. RESTON and J. R. HAUN, *Arch. Biochem. Biophys.* **103**, 74 (1963).

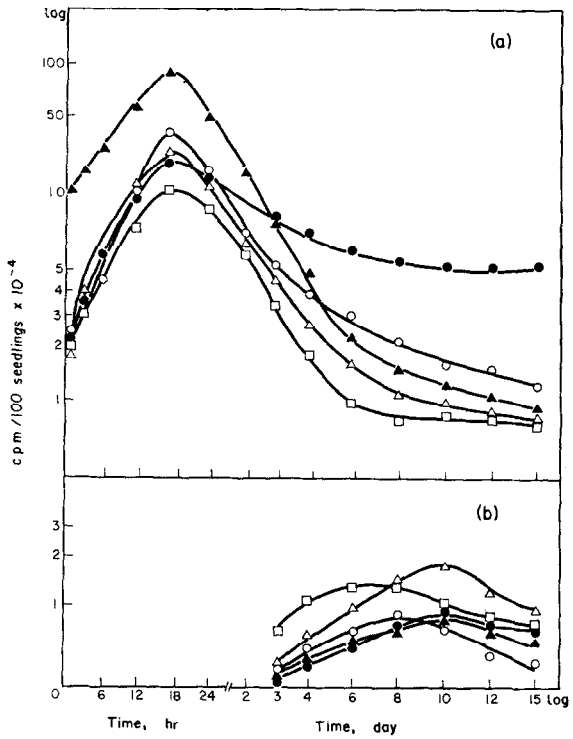

FIG. 2. DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO: (A) TRITERPENE DIOLS; (B) TRITERPENE DIOL ESTERS ERYTHRODIOL (●), FARADIOL WITH ARNIDIOL (○), BREIN (□), URSADIOL (△) AND CALENDULADIOL (▲).

decreasing, is followed by a further increase until near the 15th day. The decrease in the level of radioactivity of free sterols observed at the seventh day can be explained by assuming a higher rate of glycosylation over aglycone synthesis during that period. Further increase of sterol labelling after this time is most probably connected with a reverse of this situation.

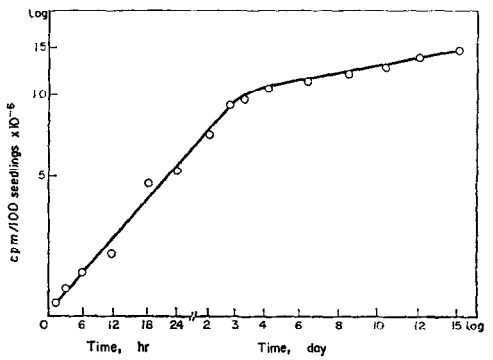

FIG. 3. DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO OLEANOLIC ACID.

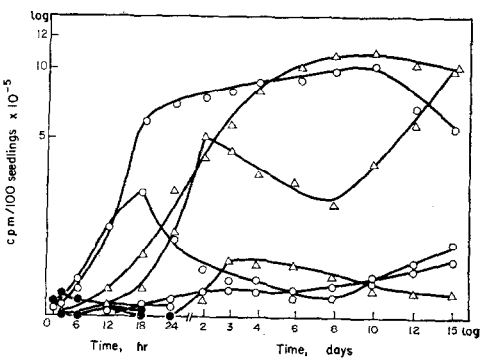

FIG. 4. DYNAMICS OF INCORPORATION OF 1-^{14}C-ACETATE INTO: (1) FREE STEROLS; (2) STEROL GLYCOSIDES; (3) STEROL ESTERS Δ^7-STEROLS (●), SITOSTEROL (□) AND STIGMASTEROL (△).

Labelling of sitosterol and of stigmasterol esters is slower than that of free sterols and sterol glycosides. Esters of Δ^7-sterols are not labelled at all.

The results presented above indicate that germinating *C. officinalis* seeds, like flowers of this plant,[2] have the ability to synthesize various types of triterpene alcohols and to hydroxylate them to the corresponding diols, to oxidize β-amyrin to oleanolic acid, and to synthesize free and bound sterols. The process of glycosylation of sterols and oleanolic acid is particularly intensive in germinating seeds, esterification of triterpene diols and of sterols being much slower.

The experiment carried out indicate that, unlike shoots and roots of older plant, germinating *Calendula* seeds possess the ability to synthesize triterpene alcohols of the ursane and lupane types. In order to show how long this ability persists in developing seedlings, the

TABLE 1. INCORPORATION OF 1-^{14}C-ACETATE INTO TRITERPENES IN THE SEEDLINGS OF *Calendula officinalis*

Compound	Age of seedlings (days)	Radioactivity (cpm/100 seedlings $\times 10^{-3}$)
α-Amyrin and β-amyrin	1	36
	3	54
	6	240
ψ-Taraxasterol and taraxasterol	1	14
	3	168
	6	270
Faradiol and arnidiol	1	101
	3	236
	6	364
Oleanolic acid	1	2609
	3	3271
	6	15 436

following experiment was performed. 1, 3 and 6-day seedlings were administered with 1-^{14}C-acetate and were then analysed after 24 hr. The compounds labelled with highest intensity are shown in Table 1. The results obtained indicate that the synthesis of all types of triterpene alcohols is observed in 6-day-old seedlings. The rate of synthesis increases with the growth of the seedlings. The amount of radioactivity incorporated in 6-day-old seedlings into amyrins and oleanolic acid is 5 times greater than that in 3-day-old ones, whereas the radioactivity incorporated into ψ-taraxasterol and taraxasterol, faradiol and arnidiol is increased only by 1·5 times over this period. This shows that in germinating seeds there is lower increases in synthesis of the various types of triterpene alcohols than in the rate of synthesis of triterpenes of the β-amyrin type and of sterols. This suggests that the metabolic processes might be spatially separated in young *Calendula* seedlings, similarly to that found for sterols in corn seedlings.[8] In order to confirm this suggestion, 5-day-old *Calendula* seedlings were fed 1-^{14}C-acetate, incubated for 24 hr and the radioactivity incorporated

[8] R. J. KEMP, L. J. GOAD and E. J. MERCER, *Phytochem.* **6**, 1609 (1967).

into individual triterpenoids was determined separately in cotyledons and embryos. The results obtained are presented in Table 2. Triterpene alcohols of all types were labelled in cotyledons, but in the embryos activity was found only in β-amyrin and erythrodiol. Oleanolic acid and sterols are labelled more intensively in embryos. This indicates a spatial separation of the biosynthesis of various types of triterpenes in *Calendula* seedlings. The ability to synthesize alcohols of the ursane and lupane types is transferred only to cotyledons and disappears as they are used up. The embryo, from which the plant develops, contains only the enzymatic ability to synthesize compounds of the β-amyrin group, with oleanolic acid as a final product. The renewed ability to synthesize triterpenes of different groups appears only as the flowers develop.

TABLE 2. INCORPORATION OF 1-^{14}C-ACETATE INTO TRITERPENOIDS IN COTYLEDONS AND EMBRYOS OF *Calendula officinalis* SEEDLINGS

Compound	Cotyledons (cpm/100 seedlings × 10^{-2})	Embryos
Amyrins	526	448
ψ-Taraxasterol	120	0
Taraxasterol	197	0
Lupeol	533	0
Erythrodiol	697	755
Brein	684	0
Faradiol with Arnidiol	853	0
Ursadiol	423	0
Calenduladiol	1422	0
Oleanolic acid	19 200	27 500
Sterols	2662	4714

EXPERIMENTAL

Material. Calendula officinalis L. var. Radio, seeds were germinated for 48 hr on cotton wool saturated with tap water in a growth chamber with light of intensity 2500 lx for 14 hr/day. Temperature during the day was 25° and 15° in the night.

Administration of 1-^{14}C-*acetate.* Administration of 1-^{14}C-acetate to germinating seeds and young seedlings was carried out as follows: (*a*) after 48 hr under conditions described above, the seeds were transferred into Petri dishes with filter paper moistened with an aqueous solution of 1-^{14}C-acetate of specific activity 1·13 mCi/mM (1 mCi/100 seeds). The dishes were illuminated with 2500 lx and samples of germinating seeds were collected at intervals during 1–72 hr. The remaining seedlings were transferred to a gauze spread over a cuvette filled with 10-fold diluted nutrient solution.[3] The samples for investigations were then collected from these seedlings at corresponding times 3–15 days. (*b*) 5-Day-old seedlings of *Calendula* grown on gauze were administered with 1-^{14}C-acetate as described above. The seedlings were processed 24 hr after administration of acetate.

Fractionation of the material. The seeds and seedlings were ground with anhydrous Na_2SO_4 (in the case of experiment 3 cotyledons and embryos were treated as separate samples), and the powder was extracted with dry EtOH. An equal volume of H_2O was added, EtOH was removed by distilation and the residue was extracted with Et_2O. Free and esterified triterpenes and sterol glycosides contained in the Et_2O extract were separated by means of TLC on SiO_2 in the system *n*-heptane–$CHCl_3$–MeOH, 20:10:1. Individual compounds were separated with use of TLC: alcohols on SiO_2–$AgNO_3$ in $CHCl_3$; diols as acetates on SiO_2–$AgNO_3$ in benzene and in $CHCl_3$; sterols as acetates on SiO_2–$AgNO_3$ in $CHCl_3$. Esters of triterpenoid compounds were hydrolysed in 10% KOH in MeOH for 3 hr under reflux. Free triterpenoids were extracted from the hydrolysate with Et_2O after addition of the equivalent amount of H_2O and were then separated according

to the method described above for the free compounds. In order to separate α- and β-amyrins and samples were oxidized with SeO_2. It was shown that under the conditions applied (10 mg SeO_2 for 1 mg of α- and β-amyrin mixture dissolved in a mixture benzene–HOAc 1:2, heated in sealed ampoules at 100° during 3 hr), β-amyrin is quantitatively converted into the 9(11), 12-diene. The diene obtained was separated from unoxidized α-amyrin,[9] by chromatography over SiO_2–$AgNO_3$ in $CHCl_3$. Triterpenoid glycosides contained in the aqueous phase and sterol glycosides isolated from the Et_2O extract were hydrolysed with 10% H_2SO_4 for 3 hr under reflux. Oleanolic acid and sterols were extracted from the hydrolysate with Et_2O and separated by means of TLC on SiO_2 in the system $CHCl_3$–MeOH, 19:1. The radioactive compounds were localized by means of autoradiography.

Measurement of radioactivity. The isolated compounds were eluted with Et_2O directly to the scintillation vial, the Et_2O was removed by evaporation and after adding toluene with scintillator, radioactivity was counted in a scintillation counter with an efficiency of 36%.

[9] E. J. COREY and J. URSPRUNG, *J. Am. Chem. Soc.* **78**, 183 (1956).

Key Word Index—Calendula officinalis; Compositae; triterpenes; biosynthesis; metabolism.