hytochemistry 1969, Vol. 8, pp. 1921 to 1926. Pergamon Press. Printed in England.

INCORPORATION OF 1-W-ACETATE INTO TRITERPENOIDS IN *CALENDULA OFFICINALIS*

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(Received 7 March 1969, *in revised form* 1 *May* 1969)

Abstract-A dynamics of incorporation of I-W-acetate into sterols, pentacyclic triterpenic alcohols and into oleanolic acid has been studied in ***Calendula oficinalis.*** The results obtained indicate that sterols undergo the following transformations: Δ^7 sterol → *As* sterol → $\Delta^{5,22}$ sterol. Triterpenic diols are formed as the result of hydroxylation of corresponding triterpenic monols and oleanolic acid is produced by stepwise oxidation according to the scheme: β-amyrin → erythrodiol → oleanolic aldehyde → oleanolic acid.

A NUMBER of triterpenoids has been isolated from ***Calendula oficinalis.*** Oleanolic acid glycosides [1,2] as well as sterols,[3,4] β-sitosterol, stigmasterol and an unidentified Δ^7-sterol, were found to occur in all organs during the whole period of vegetation of the plant.[5] Moreover, considerable amounts of mono- and dihydroxy triterpenic alcohols belonging to five classes of pentacyclic triterpene, and the hydroxyaldehyde, oleanolic aldehyde,[6] were detected in the flowers.

The purpose of the present work was to investigate, by means of radioisotope technique, the rate of incorporation of labelled acetate into individual triterpenoids in various organs of *Calendula*. It was expected that comparison of the dynamics of incorporation of labelled precursor might indicate how the more oxidized triterpenes are formed from the corresponding monohydroxy triterpene alcohols, produced in plants by cyclization of squalene.

RESULTS AND DISCUSSION

In order to determine the site of biosynthesis of triterpenoids in ***Calendula oficinalis,*** 1-^{14}C-acetate was administrated to separate parts of the plant. The results (Table 1) indicate that sterols as well as pentacyclic triterpenes are synthesized in all organs. However, there were significant differences in the rates of incorporation. Total incorporation of ^{14}C into the triterpenoid fraction 48 hr after administration of the precursor ranged from 0·023 per cent in the root to 0·14 per cent in the leaves and 0·51 per cent in the flowers. Incorporation of ^{14}C into oleanolic acid was highest in flowers and young leaves (0·02 per cent of administrated 1-^{14}C-acetate) and was significantly lower (by a factor of 30) in the root. Incorporation of activity into sterols in root, leaves and flowers was 0·02, 0·09 and 0·34 per cent respectively. Incorporation of the precursor after 48 hr into sterols was always higher than into oleanolic acid, despite the fact that during the flowering the level of sterols in all organs of *Calendula*

[1] A. WINTERSTEIN and G. STEIN, *Z. Physiol. Chem. 64,199 (1931).*
[2] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* 6, 69 *(1967).*
[3] Z. KASPRZYK and J. PYREK, *Roczniki Chemii* 41, 201 (1967).
[4] Z. KASPRZYK and G. TUROWSKA, *Bull. Acad.* Polon. *Sci.*, in press.
[5] Z. KASPRZYK and M. FONBERG-BROCZEK, *Physiol. Plantarum. 20,321 (1967).*
[6] Z. KASPRZYK and J. PYREK, Phytochem. 7, 1631 (1968).

is several times lower[5] than the level of oleanolic acid. A high incorporation of ^{14}C into pentacyclic triterpenic alcohols was observed only in flowers. Similar significant differences in biosynthetic activity in various organs were observed by Nicholas [7] who has studied incorporation of 2-^{14}C-mevalonate into oleanolic acid and sterols in *Saivia officinalis*.

TABLE 1. INCORPORATION OF 1-W-ACETATE INTO TRITERPENOIDS IN ISOLATED ORGANS OF *Calendula officinalis*

		Pentacyclic triterpenes					
		Monols		Diols			
Plant organ	Sterols	β-Amyrin	Others	Erythrodiol	Others	Oleanolic aldehyde	Oleanolic acid
Tip leaves	11,400	3600	—	80	—	+	2400
Basal leaves	9800	3200	—	90	—	+	1680
Ligulate flowers	42,300	18,900	11,200	220	5,700	+	2120
Tubular flowers	4900	3600	3900	90	500		1730
Calyx	4100	1600	—	+		—	210
Receptacle	9800	500	—	—	—	—	490
Stem	7200	290	—	—		—	180
Root	2700	120	—	—	—	—	80

0·1 mc of 1-^{14}C-acetate was given per 1 g of fresh weight of plant material. The radioactivity incorporated was measured 48 hr after supplying the precursor. The results are presented in cpm/g of fresh weight.

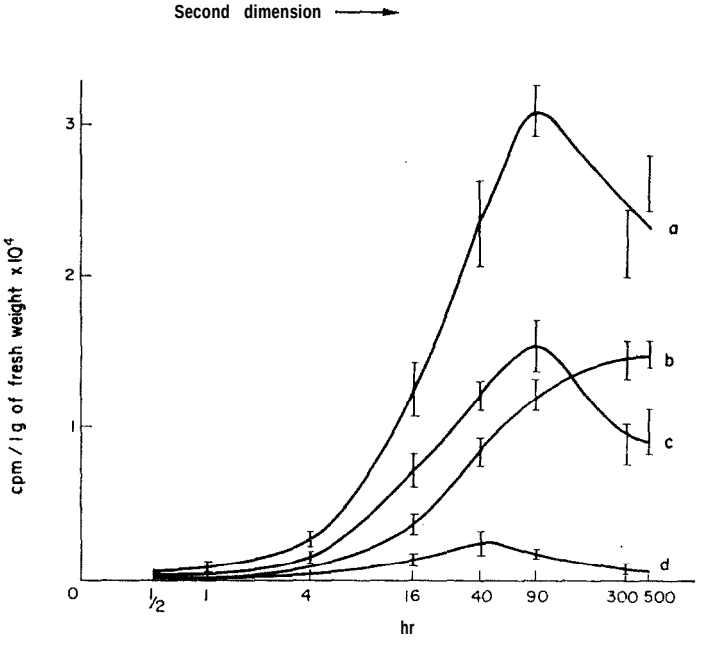


FIG. 1. THE INCORPORATION OF 1-^{14}C-ACETATE INTO FRACTIONS OF (a) STEROLS AND INTO INDIVIDUAL STEROLS IN THE YOUNG SHOOTS OF [redacted] (b) [redacted] (c) [redacted]

[redacted] H. J. NICHOLAS, [redacted] *Chem. 237, 1476* (1963).

The relative high rate of uptake of 1-^{14}C-acetate in isolated shoots and flowers led to their use for further detailed investigations. Studies on isolated shoots were carried out during 0·5–500 hr from the time of administration of the precursor, and on isolated flowers during 6-144 hr. Parallel series of investigations performed with shoots and flowers gave the same results.

The incorporation of ^{14}C in young shoots into total sterol and into individual sterols is presented in Fig. 1. The maximal radioactivity in β-sitosterol was observed 90 hr after administration of the precursor; by comparison, incorporation into stigmasterol was considerably delayed. A similar slow rate of biosynthesis of stigmasterol, compared with β-sitosterol, was observed by Johnson [8] in *Solanum tuberosum* and by Bennett [9] in *Dioscorea spiculiflora*. These authors considered it moved the formation of stigmasterol by dehydrogenation of the side chain of β-sitosterol at position 22; the results now obtained seem to confirm this hypothesis. It is noteworthy that high metabolic activity is possessed by the

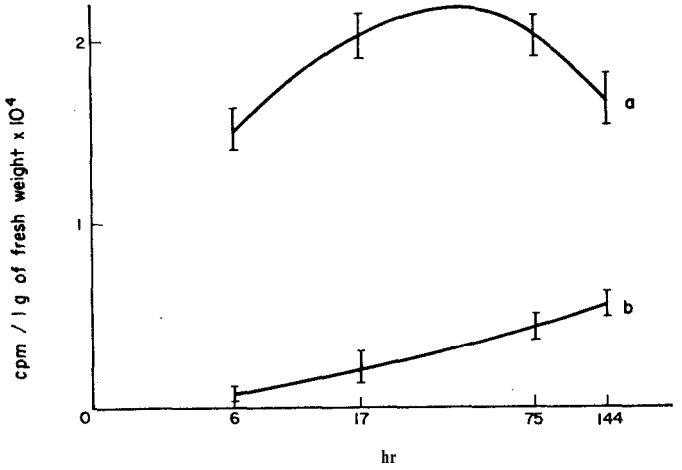


FIG. 2. THE INCORPORATION OF 1-^{14}C-ACETATE INTO FRACTIONS OF TRITERPENIC MONOLS (a) AND DIOLS (b) IN THE ISOLATED LIGULATE FLOWERS OF *C. officinalis*.

sterol with the double bond at position 7. Therefore, maximal radioactivity appears earlier in this compound than in other sterols and indicated that Δ^7-sterols participate in the biosynthesis of Δ^5-plant sterols.[10]

Figure 2 shows the incorporation of 1-^{14}C-acetate into mono- and dihydroxytriterpenic alcohols in isolated flowers of C. *officinalis*. The curves obtained show, on one hand, the distinctive metabolic activity of monols and, on the other, the considerably slower rate of synthesis of triterpenic diols. Similar results were obtained with the incorporation of 1-^{14}C-acetate into the individual compounds, ψ-taraxasterol (a monol) and faradiol (Fig. 3). These compounds differ only in the number of hydroxyl groups, faradiol possessing an additional OH at position 12. These results suggest that faradiol is produced in C. *officinalis* by secondary oxidation of the previously formed ψ-taraxasterol.

[8] D. J. JOHNSON, E. HEFTMANN and G. V. C. HOUGHLAND, *Archs Biochem. Biophys.* 104, 102 (1964).
[9] R. D. BENNETT, E. HEFTMANN, W. H. RESTON and J. R. HAUN, *Archs Biochem. Biophys.* **103**, 74 *(1963)*.
[10] M. DEVYS and M. BARBIER, *Bull. Soc. Chim. Biol. 49,865 (1967)*.

Organs synthesizing oleanolic acid also produced a labelled compound with chromatographic properties similar to β-amyrin and small amounts of labelled compounds with properties of erythrodiol and oleanolic aldehyde (Table 1). These three compounds are known to occur in small quantities in ***Calendula*** flowers,[6] but it is not known whether they are also in the leaf. Therefore, the non-saponifiable compounds (6.4 g) obtained from 8-kg leaves were fractionated and the fractions with the polarity of β-amyrin, oleanolic aldehyde and of erythrodiol obtained by separation on a SiO_2–$AgNO_3$ column, were further investigated. From the fraction of polarity of β-amyrin was isolated, by means of TLC on silica-gel impregnated with $AgNO_3$, 32 mg of substance, which was identified as β-amyrin on the basis of its m.p. (found 190–195°, reported[11] 194-200") and by comparison of i.r. spectrum with authentic material. 5 mg of substance identical in i.r. spectrum with erythrodiol diacetate (m.p. 166–178°, reported[11] 182-188") was also obtained. The oleanolic aldehyde fraction was reduced with $NaBH_4$ and acetylated, giving, after purification, 0·5 mg of erythrodiol diacetate (m.p. and mixed m.p. 176-l 85°). Thus small amounts of β-amyrin, erythrodiol and oleanolic aldehyde do occur in C. *officinalis* leaves.

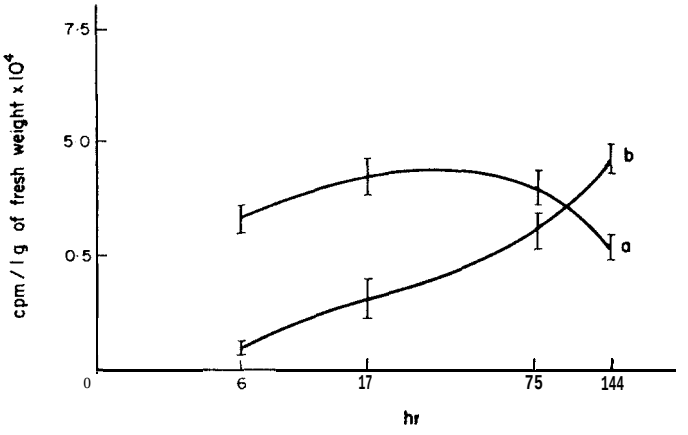


FIG. 3. THE INCORPORATION OF 1-^{14}C-ACETATE INTO ψ-TARAXASTEROL (a) AND FARADIOL (b) IN THE ISOLATED LIGULATE FLOWERS OF *C. officinalis*.

The dynamics of incorporation of 1-^{14}C-acetate into β-amyrin and oleanolic acid in young leaves of *Calendula* is presented in Fig. 4. The maximal radioactivity in β-amyrin is observed 16 hr after administration and is followed by a rapid and considerable decrease, indicating high metabolic activity. By contrast, radioactivity of oleanolic acid increased during the whole period of experiment (up to 500 hr) indicating the low rate of synthesis with respect to β-amyrin and its metabolic stability. Similar results were obtained with isolated flowers. All these data agree with the theory of Rettit[12] and Maglahaes,[13] that oleanolic acid is formed by stepwise oxidation of β-amyrin at position 17. The insignificant labelling of erythrodiol and eolanolic aldehyde observed in the present work indicates, however, that oxidation of β-amyrin to oleanolic acid proceeds without dissociation from the enzyme surface.

[11] P. BOITEAU, B. PASICH and A. RAKOTO RATSIMAMANGA, *Les Triterpenoides*, Gauthier-Villars, Paris (1964).
[12] G. R. PETTIT, H. KLINGER, N. OTTO, N. JORGENSEN and J. OCCOLOWITZ, *Phytochem.* **5**, 301 (1966).
[13] H. MAGALHAES, A. V. H. ARNDT, W. D. OLLIS, W. B. EYTON and W. Z. MAGALHAES, *Phytochem.* **5**, 1327 (1966).

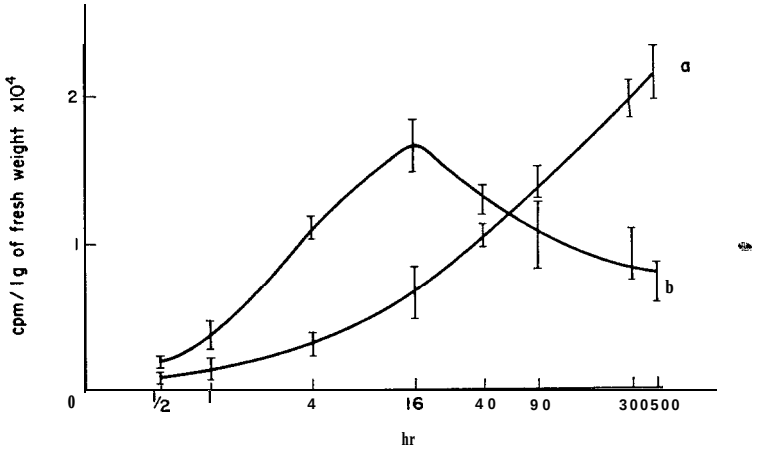


FIG. 4. THE INCORPORATION OF 1-^{14}C-ACETATE INTO β-AMYRIN (a) AND OLEANOLIC ACID (b) IN ISOLATED YOUNG SHOOTS OF *C. officinalis*.

EXPERIMENTAL

Material

Plants of ***Calendula officinalis cv.*** Radio were cultivated in a lumistat under stabilized light conditions (3000 lux, 16-hr a day) and temperature (24" during the day and 16" during the night).[14]

Administration of 1-^{14}C-acetate

Isolated young shoots of flowering plants (with inflorescence buds removed) weighing 1·3 g ± 10 per cent were placed in small tubes containing aqueous solution of 1-^{14}C-acetate (100 μc = 164 mg CH_3COONa in 0·1 ml per 1 g of fresh material) and were then illuminated with light intensity of 3000 lux. When the solution was completely absorbed (about 30 min), the shoots were transferred into tap water and kept for the stated period of time with illumination every 8 hr per day as indicated above. The labelled acetate was administrated to other isolated organs in a similar manner. In order to obtain comparable results, the concentration of administered solution was chosen so that complete absorption took place within 6 hr.

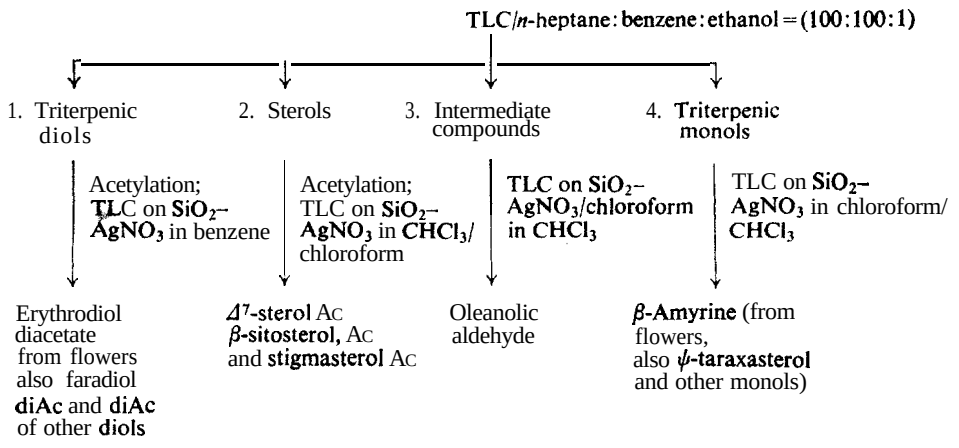
Fractionation of the Material

After incubation with the precursor, fresh material was homogenized with an g-fold amount of anhydrous Na_2SO_4. The powder obtained was then extracted with absolute ethanol. The extract was taken to dryness and hydrolysed with 10% KOH in ethanol at the b.p. for 3 hr. To the hydrolysate was added an equal volume of water, alcohol was removed and the residue was extracted with petrol ether (b.p. 40–60°). In order to remove fatty acids, the aqueous residue was extracted with ether after acidification to pH 4. To the aqueous phase containing oleanolic acid glycosides was added an equal volume of 20% methanolic H_2SO_4 and the mixture was heated at b.p. for 3 hr. Oleanolic acid was extracted from the hydrolysate with ether, after dilution with water.

Further purification was carried out by TLC. The compounds of the unsaponifiable fraction were then subjected to TLC on Al_2O_3 and on SiO_2–$AgNO_3$ as shown on p. 1926.

The samples of β-amyrin obtained in such a way from flowers contain considerable amounts of a-amyrin.6 In order to separate α- and β-amyrins, the samples were oxidized with SeO_2 and subjected to TLC on silica-gel/$CHCl_3$ which separated the oxidized β-amyrin. The radioactivity of the material resistant to oxidation (i.e. a-amyrin) was subtracted from the radioactivity of the whole sample, thus giving the radioactivity of β-amyrin. Oleanolic acid was isolated from the fraction obtained after acid hydrolysis by means of TLC on silica-gel in $CHCl_3$–MeOH (19: 1). Oleanolic aldehyde was separated from other radioactive monomethyl sterols on SiO_2–$AgNO_3$ and its uniformity proved by reduction to erythrodiol using $NaBH_4$.[15] β-Amyrin was separated on SiO_2–$AgNO_3$ from cycloartenol, methylenecycloartenol and lanosterol and then crystallized to constant specific activity after admixture with non-radioactive material. Oleanolic and was similarly treated.

[14] Z. KASPRZYK, Z. WOJCIECHOWSKI and K. CZERNIAKOWSKA, *Physiol. Plantarum 21,966 (1968).*
[15] *M.* J. BERNARD and W. W. REID, *Chemy & Ind.* 997 (1967).



Radioactivity Counting

Radioactivity was measured from the aluminium planchets using thin-window counter with efficiency 6 per cent.