Phytochemistry, 1970, Vol. 9, pp. 561 to 564. Pergamon Press. Printed in England.

INCORPORATION OF 1-^{14}C-ACETATE INTO GLYCOSIDES OF OLEANOLIC ACID IN *CALENDULA OFFICINALIS*

ZOFIA KASPRZYK, ZDZISŁAW WOJCIECHOWSKI and WIRGINIA JANISZOWSKA

Department of Biochemistry, University Warszawa, Poland

(*Received* 22 *July* 1969)

Abstract—The rate of formation of the five glycosides of oleanolic acid (*A*, *B*, *C*, *D* and *F*) in shoots of *Calendula officinalis* was determined by incorporation of 1-^{14}C-acetate into the aglycone moiety. It was shown that sugars are probably incorporated into oleanolic acid glycosides in the following sequence: glucuronic acid in position 3 (*F* from oleanolic acid), galactose in position 3′(2′) (*D* from *F*), glucose in position 28 (*C* from *D*) and glucose in position 4′ (*B* from *D* and *A* from *C*).

INTRODUCTION

IT HAS been shown previously,[1] that a series of five glycosides of oleanolic acid occur in flowers of *Calendula officinalis*, namely: 3-monoglucuronoside (*F*), 3-(3′-galactosyl)-glucuronoside (*D*), 3-(3′-galactosyl)glucuronoside, 28 glucoside (*C*), 3-(3′-galactosyl, 4′-glucosyl)glucuronoside (*B*) and 3-(3′-galactosyl,4′-glucosyl)glucuronoside, 28 glucoside (*A*). The same compounds occur in other parts of the plant in variable quantities depending on age and organ.[2] Their structural similarity and, as has been recently shown by us,[3] the metabolic stability of oleanolic acid in shoots allowed us to investigate the sequence of formation of these glycosides.

RESULTS AND DISCUSSION

It has been previously shown[3] that the radioactivity of oleanolic acid (obtained after hydrolysis of the glycoside fraction) increased gradually up to 500 hr after administration of 1-^{14}C-acetate to isolated shoots of calendula. In the present work we have investigated changes in the radioactivity of oleanolic acid obtained from each of the individual glycosides. The glycosides present in the butanolic extract of the plant material could be well separated from each other by preparative thin-layer chromatography on silica gel using a single solvent system.

Each individual glycoside was subjected to hydrolysis and the radioactivity of the oleanolic acid obtained was determined after its purification by means of chromatography on filter paper impregnated with silica gel. This procedure appeared particularly suitable, since incorporation of 1-^{14}C-acetate into the carbohydrate moiety of glycosides would complicate interpretation of the results obtained. The purity of each compound obtained was tested by means of autoradiography. As has been shown previously[4] oleanolic acid purified in this way contains no other radioactive compounds.

---

[1] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **6**, 69 (1967).

[2] Z. KASPRZYK, P. CHOMCZYŃSKI, M. FONBERG and M. KONARSKA, FEBS, Oslo, Abstracts, p. 25 (1967).

[3] Z. KASPRZYK and Z. WOJCIECHOWSKI, *Phytochem.* **8**, 1921 (1969).

[4] Z. KASPRZYK, Z. WOJCIECHOWSKI and K. CZERNIAKOWSKA, *Physiol. Plantarum* **21**, 966 (1968).

The results of one typical experiment carried out with field-grown plants are presented in Fig. 1. The results in three other experiments differed slightly as to the absolute values of incorporation of the radioactive precursor, but the shape of curves obtained was in all cases very similar for individual glycosides. The differences with respect to the absolute values of incorporation in individual series are probably due to differences in the age of the plant and cultivation conditions.

The results presented in Fig. 1 clearly indicate that oleanolic acid in glycoside *F* (3 monoglucuronoside) is labelled the fastest. About 85 per cent of total radioactivity of oleanolic acid is found there 1 hr after administration of 1-^{14}C-acetate. The radioactivity of oleanolic acid in glycoside *F* reaches a maximum after 4 hr followed by a decrease to 50 per cent of the maximal value. In the case of other four glycosides, there is a constant increase in the radioactivity of their oleanolic acid, which in glycosides *C* and *D* reaches a maximum 260 hr after administration of precursor. Radioactivity in glycosides *A* and *B* goes on increasing up to the end of experiment (504 hr).

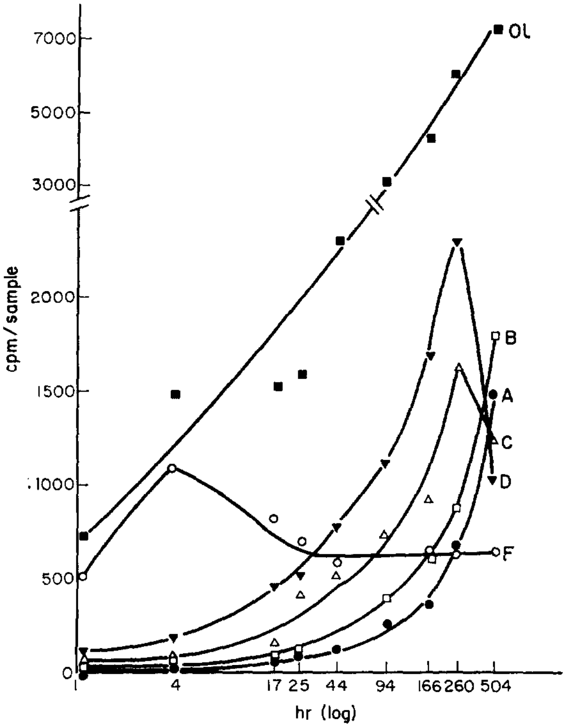

FIG. 1. THE KINETICS OF INCORPORATION OF RADIOACTIVE ACETATE INTO OLEANOLIC ACID DERIVED FROM GLYCOSIDES *A* ●, *B* □, *C* △, *D* ▼, AND *F* ○ AND INTO TOTAL OLEANOLIC ACID (Ol■) IN THE SHOOTS OF CALENDULA GROWN IN THE FIELD.

The variation in the specific activities of oleanolic acid obtained from individual glycosides over the course of the experiment are presented in Table 1. These data indicate that no quantitative changes occur in individual glycosides in the shoots in the course of the experiment. Changes in the radioactivity of oleanolic acid in the individual glycosides are thus related to their mutual transformation. The highest specific activity is found in glycoside *F* and indicates that it is a precursor of the remaining glycosides. Glycoside *D* is presumably formed from *F* as a result of addition of a galactose residue to the position 3 (2) of the glucuronic acid; and glycoside *C*, by subsequent addition of a glucose residue at position 28 of oleanolic acid. The decrease in the radioactivity of the oleanolic acid in both *D* and *C* 260 hr after administration of 1-^{14}C-acetate, together with the simultaneous constant increase

TABLE 1. QUANTITIES OF OLEANOLIC ACID DERIVED FROM INDIVIDUAL GLYCOSIDES AND ITS SPECIFIC ACTIVITY

	Oleanolic acid in glycoside									
	A		*B*		*C*		*D*		*F*	
Time (hr)	μg in a sample	cpm/mg	μg in a sample	cpm/mg	μg in a sample	cpm/mg	μg in a sample	cpm/mg	μg in a sample	cpm/mg
1	11	1,820	23	1,040	36	1,640	60	1,330	6	83,500
4	12	2,570	22	2,220	39	2,590	62	2,920	8	135,750
17	12	3,000	21	4,190	39	4,050	61	3,920	8	102,500
25	12	6,720	24	5,000	41	10,950	60	9,120	7	77,700
44	12	10,000	22	8,520	38	13,790	59	13,270	9	76,560
94	10	22,700	20	20,600	40	17,980	60	18,580	9	77,900
166	15	25,500	21	30,000	41	22,700	58	29,500	8	81,250
260	12	53,200	20	44,000	42	38,800	58	40,000	9	86,700
504	13	113,800	21	85,800	40	31,300	62	17,700	10	75,500

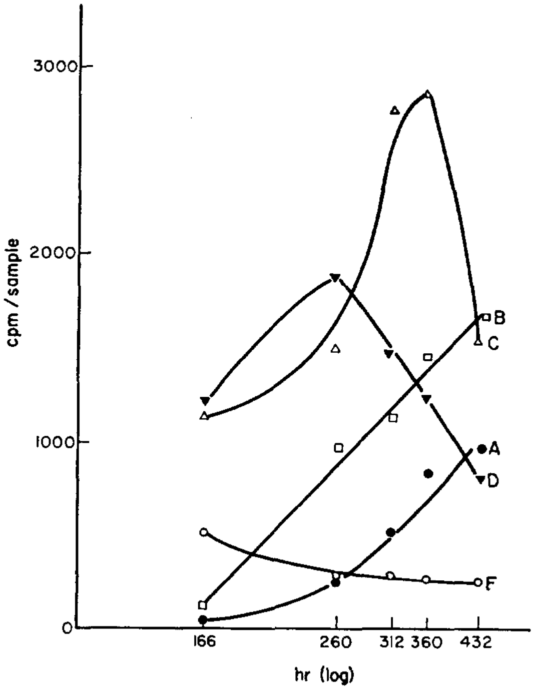

FIG. 2. THE KINETICS OF INCORPORATION OF RADIOACTIVE ACETATE INTO OLEANOLIC ACID DERIVED FROM GLYCOSIDES *A* ●, *B* □, *C* △, *D* ▼, AND *F* ○ IN THE SHOOTS OF CALENDULA GROWN IN A GROWTH CHAMBER.

of radioactivity of oleanolic acid in *B* and *A*, indicates that the latter are probably formed as the result of a further addition of a glucose residue to glycosides *D* or *C*.

In the above experiment the time course of incorporation of ^{14}C into oleanolic acid in the glycosides *C* and *D* was very similar (Fig. 1). A comparison of the structure of these glycosides, however, points to the fact that glycoside *C* could be formed in the plant by addition of glucose to *D* at position 28 of the oleanolic acid moiety. An additional series of experiments was therefore carried out with plants grown in a growth chamber, samples for analysis being taken more frequently during the time when the radioactivity of oleanolic acid in these compounds was maximal. The results, presented in Fig. 2, show that the maximum of radioactivity of oleanolic acid in glycoside *D* is observed earlier (260 hr) than in glycoside *C* (360 hr) and thus support the hypothesis that the latter is formed from *D*.

The results obtained in the present work allow to conclude that glycoside *F* (monoglucuronoside of oleanolic acid) is a precursor of all the other oleanolic acid glycosides in calendula. They are synthesized in the sequence $F \rightarrow D \rightarrow C$. The rapid decrease in the radioactivity of glycosides *D* and *C*, after reaching their maximum, indicates that they are further transformed to glycosides *B* and *A* respectively. It is possible that glycoside *A* may be formed also from glycoside *B* but the fact that, even 500 hr after administration of the precursor, radioactivity of glycoside *B* is still increasing similarly to that of glycoside *A* suggests that *A* is more probably formed from *C*.

MATERIAL AND METHODS

Material

The plants of *Calendula officinalis* var. Radio were cultivated in a growth chamber under conditions described in previous work[3] or in a field.

Administration of 1-^{14}C*-acetate*. Two young shoots (from flowering plants with buds removed) weighing 0·9–1·0 g were fed through their cut ends with 1-$^{14}CH_3COONa$ ($50\ \mu c =$ 0·3 mg in 0·2 ml of H_2O) in light (3000 lux). When the solution was completely absorbed (*ca.* 1 hr), the shoots were transferred to tap water and kept for the duration of the experiment with 8 hr illumination per day.

Extraction. The shoots were ground with an 8-fold amount of anhyd. Na_2SO_4 and extracted with boiling EtOH (30 ml per 1 g dry weight). An equal volume of H_2O was added and the EtOH was distilled off. The aqueous solution was extracted with Et_2O followed by *n*-BuOH.

Preparative chromatography. Glycosides in the butanolic extract were separated by means of TLC on silica-gel. The plates were developed twice in the system *n*-PrOH, 14% NH_4OH (8:2). Individual glycosides localized by comparison with standards were eluted from gel with MeOH, and subjected to acid hydrolysis with a mixture of HCl, AcOH, H_2O (10:35:55) at 100° for 1·5 hr. To the hydrolysate was added a 5-fold volume of H_2O and extracted with Et_2O. The extracts were separated on filter paper impregnated with silica-gel (Whatman SG 81) in the system light petroleum, $CHCl_3$, MeOH (20:10:3). The chromatograms were sprayed with H_2O to localize oleanolic acid, the radioactivity of which was counted directly on the paper with a thin-window counter with efficiency about 6%. The chromatograms were then covered with X-ray film, which were developed after 2 weeks.

Quantitative determination of oleanolic acid. Oleanolic acid was determined colorimetrically as a complex with $CoCl_2$ at 275 nm according to the method described previously.[5]

---

[5] M. FONBERG and Z. KASPRZYK, *Chem. Anal. Warsaw* **10**, 1181 (1965).