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CARDENOLIDE AND TRITERPENE SYNTHESIS IN THE LATICIFERS OF ASCLEPIAS CURASSAVICA L.

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SUMMARY

The latex of *Asclepias curassavica* L. was shown to contain 40–60 $mg.ml^{-1}$ cardenolides and 0.4–0.6 $mg.ml^{-1}$ esterified triterpenes. The dynamics of ^{14}C incorporation of D-glucose-U-^{14}C, acetate-1-^{14}C and mevalonate-2-^{14}C into esterified triterpenes and cardenolides in excised stem parts have been studied. The results obtained indicate that glucose is a very effective precursor in latex triterpene synthesis. The three precursors used were only slightly involved in cardenolide synthesis. The results are discussed in relation to the view that both latex constituents probably have different sites of synthesis.

1. INTRODUCTION

Cardenolides occur as glycosides in about 14 plant families (GRUNWALD 1980). They occur in variable quantities in all the plant tissues and are in several cases reported to be latex constituents.

In *Calotropis* both triterpenes and cardenolides are major latex constituents (HESSE et al. 1939, HEGNAUER 1964). In *Asclepias curassavica* these plant products appeared to occur together inside the laticiferous system. The triterpenes in the laticifers of *Hoya* and *Euphorbia* were shown to be mainly synthesised from sugars (GROENEVELD 1977). Acetate and pyruvate appeared to be involved in trace amounts only (GROENEVELD et al. 1982). Up to squalene, cardenolides and triterpenes have a common pathway in biosynthesis from acetyl-CoA (REICHSTEIN 1967; TSCHESCHE 1972). The present paper deals with the possible participation of glucose, acetate and mevalonate in the synthesis of cardenolides and triterpenes in the laticifers of *Asclepias curassavica.*

2. MATERIALS AND METHODS

Asclepias curassavica L. was grown in the greenhouse and actively growing vegetative shoots were used in the incorporation experiments. After incorporation the latex was tapped and diluted 10–20 fold with 0.2 M phosphate buffer pH 7.8. The obtained latex suspension was diluted with an equal volume acetone and extracted with light petroleum. The cardiac glycosides were extracted with chloroform from the remaining mixture. An acetone extract (soxhlet) from plant tissue was evaporated to dryness and redissolved in an ethanol 96%/light petroleum (40–60°) mixture (1/1). Water was added to obtain two layers. The petroleum ether contained all the triterpenoids and the remaining mixture was extracted twice with chloroform to isolate the cardiac glycosides. The triter-

pene extracts were purified and separated into triterpene esters, triterpenols and sterols by alumina absorption chromatography as described by KEMP & MERCER (1968). The water free chloroform extracts were adsorbed on 5 gram Al_2O_3 columns (grade II–III) and eluted with 25 ml portions of $CHCl_3$, ($CHCl_3$-Methanol)/(9–1) and ($CHCl_3$-Methanol)/(1–1). All the cardiac glycosides were eluted with the second fraction which was evaporated to dryness at 45°C and redissolved in 1 ml of chloroform-methanol (2–1). Cardenolides were purified on silicagel-G thin layer plates developed in ethyl acetate-methanol-H_2O (75–10–7.5) (STAHL 1972). After spraying with 3,5-dinitro benzoic acid (2% in 70% ethanol) and subsequently with 10% KOH the cardenolides appeared as red spots after heating for 3 min. at 110°C (DUFFEY & SCUDDER 1972).

The 3,5-dinitro benzoic acid was used in a quantitative colorimetric procedure modified after ROWSON (1952). Aliquots of the cardenolide containing chloroform extracts were mixed with 1 ml 2% 3,5-dinitro-benzoic acid to which 0.5 ml 1 N NaOH was added. The maximum absorption peak occurred at 568 mμ and was measured after 5 min. Digoxin and digitoxin were used as standards. Cardenolides were hydrolysed in 50% methanol containing 0.5 N H_2SO_4 at 70°C during 2 h.

The triterpenols and their esters were separated on silicagel-G thin layer plates developed in cyclohexane-ethyl acetate (5–1). The prepared acetates were separated on silicagel-G thin layer plates impregnated with 15% $AgNO_3$ and developed in benzene-light petroleum (3–2). Spots were detected by spraying with chlorosulphonic acid-glacial acetic acid (1–2) and heating to 120°C during 3 min. Gasliquid chromatography on 3% SE-30 was used for mass determination with 5α-cholestane as internal standard.

Calculation of the triterpene yield from labelled glucose. The glycolytic breakdown of 9 molecules of glucose yields 18 molecules of acetyl-CoA which are subsequently used for the synthesis of one molecule of triterpenol. In this pathway 30 of 54 C-atoms of glucose are incorporated into the triterpenol skeleton, 24 C-atoms disappear as CO_2. The amount of glucose-U-^{14}C directly used in triterpenol synthesis can be calculated with the formula:

$$\frac{\text{dpm}^{14}\text{C-triterpenol}}{2.22 \times 10^6} \times \frac{54}{30} \times \frac{1}{\text{spec.act.glucose}} = \mu\text{mol glucose used.}$$

The triterpenol yield is calculated with the formula:

$$\frac{\mu\text{mol glucose used}}{9} \times 426 = \mu\text{g triterpenol synthesised.}$$

3. RESULTS AND DISCUSSION

Freshly tapped latex was extracted with light petroleum and chloroform to isolate the triterpenoids and the cardenolides respectively. In the petroleum extract only triterpene esters could be detected. After saponification and subsequent acetylation a single spot in argentation TLC was obtained. GLC of this prepared acetate revealed a single peak and with 5α-cholestane as internal

standard a triterpene content of 0.38–0.45 mg.ml^{-1} latex was measured.

The cardenolides in the $CHCl_3$ extract were purified with Al_2O_3 adsorption chromatography and were detected as one Kedde positive spot in TLC with a RRf 1.5 to digoxin and 1.25 to digitoxin. The spectrophotometric Kedde assay (ROWSON 1952) with digoxin as a reference revealed 40–60 mg cardenolide per ml latex. The cardenolide-to-triterpene ratio was consequently 100–130 to 1. Both types of constituents, however, were assayed in a different way. The molecular response of the latex triterpene was not determined in this study and therefore the actual triterpene content of the latex might deviate slightly from the measured value. This is also the case with the cardenolides: using digitoxin as a reference a 15% higher value of cardenolide content was obtained. Nevertheless cardenolides are to be considered as major constituents of this latex, exceeding the esterified triterpene content at least eighty times.

Asclepias curassavica of Brasilian origin was found to contain uzarin, calotropagenin, coroglaucigenin and corotoxigenin (TSCHESCHE 1958). Specimen from India were shown to contain in addition asclepin, calactin and uzarigenin (SINGH & RASTOGI 1969). In the present work no attempts were made to identify the cardenolides and triterpenes in the latex of our plants.

3.1. Incorporation experiments

Within 5 h ^{14}C-glucose, ^{14}C-acetate or ^{14}C-mevalonate containing solutions were absorbed by defoliated 10 cm stem tips. After 52 h incubation at 25°C, small amounts of radioactive latex could be tapped and were extracted as described. The ^{14}C-amounts in cardenolides and triterpenes from the tapped latex as well as from the remaining stem tissue were measured. Results of a typical incorporation experiment, presented in *table 1*, clearly show that the label from acetate, glucose and mevalonate is incorporated into both cardenolides and triterpenes. In these experimental conditions apparently several types of precursors can be taken up and metabolised by the laticiferous system of *Asclepias curassavica*. As far as the triterpenes are concerned, the ^{14}C of all the used precursors was incorporated into the occurring esterified triterpene. The uptake of ^{14}C-mevalonate by laticifers and subsequent conversion to triterpenes was not observed in *Euphorbia lathyris*, but similar results were obtained with labelled glucose and acetate (GROENEVELD et al. 1982).

Not all the ^{14}C-labelled latex is expelled at tapping and a considerable part of the ^{14}C-triterpenes of the latex still remains in the incubated stem tissue. After saponification of the ester fraction extracted from these tissues ^{14}C was detected in the obtained sterols, 4α-methyl sterols and triterpenols (TLC), while varying amounts of ^{14}C co-chromatographed with carotenes. The prepared acetates of the liberated triterpenols revealed a similar ^{14}C-distribution as was found in the corresponding latex fraction. From these data may be concluded that the esterified ^{14}C-triterpenes in the stem tips mainly originated in the laticifers.

The cardenolide fraction isolated from the tapped latex revealed one ^{14}C-peak in TLC, co-chromatographing with a Kedde positive spot. These results

Table 1. Incorporation of ^{14}C from D-glucose-U-^{14}C, acetate-1-^{14}C and mevalonate-2-^{14}C into the triterpenes and cardenolides of *Asclepias curassavica L.* 10 cm stem tips, 25°C, 52 h. inc.

	12.5 μCi D-glucose-U-^{14}C DPM	7.5 μCi acetate-1-^{14}C DPM	2.5 μCi mevalonate-2-^{14}C DPM
LATEX			
total lipid extract	192,240	16,560	115,160
esterified triterpenes	30,160	2,540	50,200
acid moiety of triterpenes	11,420	900	–
cardenolides	7,780	6,060	1,420
cardenolides after hydrolysis	4,460	3,000	1,200
STEM TISSUE			
esterified triterpenes	141,540	159,760	203,780
cardenolides	84,000	117,960	6,720
cardenolides after hydrolysis	47,880	39,320	5,280

Table 2. Incorporation of ^{14}C from 5 μCi D-glucose-U-^{14}C into the esterified triterpenes of *Asclepias curassavica*; 120 μl solutions of labelled glucose were absorbed by 10 cm defoliated stem tips 40 h. incubation, 25°C.

spec. act. (μCi/μmol)	d.p.m. ^{14}C-triterpenes	nanomoles of glucose used in triterpene synthesis	% glucose used	μg ^{14} C-triterpene yield
3.0	58,595	15.8	0.95	0.75
0.7	46,200	54.1	0.75	2.57
0.4	45,315	93.9	0.74	4.47
0.21	42,165	163.4	0.68	7.77
0.14	41,640	236.3	0.67	11.24

were also obtained with the cardenolide fraction of the incubated stem tissues, regardless the precursor used. Acid hydrolysis of these cardenolide fractions produced a chloroform soluble fraction with less radioactive carbon. In the mevalonate incubated tissue nearly all the ^{14}C was recovered in the genin moiety. In the ^{14}C-glucose incubated tissue 57% of the label in the cardenolides was recovered after acid hydrolysis and after ^{14}C-acetate incorporation 33% of the ^{14}C was measured in the genin moiety of the cardenolides.

3.2. Quantitative aspects

The mass ratio between cardenolides and triterpenes is not reflected in the corresponding ^{14}C ratio. As far as the latex is concerned both glucose and mevalonate were more involved in triterpene synthesis. Only acetate was more efficient in cardenolide synthesis, but the obtained ^{14}C-triterpene-to-cardenolide ratio of 0.59 is far from the corresponding mass ratio of 0.02. Apparently in these experimental conditions cardenolide synthesis is less active than triterpene synthesis, notwithstanding a common biochemical pathway up to squalene.

When ^{14}C-glucose is incorporated in the presence of increasing amounts of glucose the final ^{14}C-content of the esterified triterpenes decreases (*table 2*). The amount of triterpene synthesised from this exogenously supplied glucose ranges from 0.75 to 11.24 µg. Without tapping an amount of 20 µg esterified latex triterpene was found in such 10 cm stem parts. Therefore a 35 µmol glucose supply yields about a 50% restauration of the naturally occurring triterpene content. Such a triterpene production in two days may be considered as an enhanced lipid synthesis. In a growing plant this proportion of triterpene is supposed to be synthesised in about 1–1½ week, being the time in which 5 cm stem is formed.

3.3. Different sites of triterpene and cardenolide synthesis in laticifers

The relatively low rate of synthesis of cardenolides compared with the concurrent rate of triterpene production in latex may be caused by different sites of synthesis in the wall-lining cytoplasm, from which both constituents are finally secreted into the large central vacuole. In general, triterpenes and their esters occur as lipid particles in the vacuolar sap of laticifers (as is the case with rubber containing latices). In a particle fraction obtained by gelfiltration of freshly tapped latex (GROENEVELD 1977) only triterpene esters could be detected. The absence of cardenolides in this fraction may be due to their polar nature. Probably the cardenolides occur as separate colloidal aggregates (micelles?) in the aqueous phase of the latex. The differences in the rate of synthesis of the apolar particulate triterpene esters and the polar cardenolides as found in our experiments may point to different sites of synthesis of these two groups of substances in the laticifers.

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