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CARDIAC GLYCOSIDE INTERCONVERSIONS AT THE SUBCELLULAR LEVEL IN *CONVALLARIA MAJAILIS*

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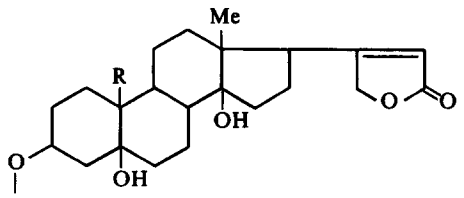
Key Word Index—*Convallaria majalis*; Liliaceae; lily of the valley; cardenolide biogenesis; membrane-bound monooxygenase; alcohol dehydrogenase.

Abstract—Further evidence for the biosynthetic sequence periplorhamnoside → convallatoxol → convallatoxin, proposed after experiments *in vivo*, was obtained with subcellular fractions of *Convallaria majalis* leaves. A NADPH-dependent monooxygenase tentatively localized on mitochondrial membranes converted periplorhamnoside into convallatoxol. In contrast the enzyme transforming convallatoxol into convallatoxin could be detected only in the soluble fraction. The conversion required NAD as a cofactor, thus the specifity observed is that of an alcohol dehydrogenase.

INTRODUCTION

Convallatoxol (**2**) and convallatoxin (**3**) are two of the main cardiac glycosides from *Convallaria majalis* [1]. Previous investigations in our laboratory revealed that labelled convallatoxol (**2**) administered to leaves was converted into convallatoxin (**3**) with high efficiency [2].



rhamnose
1 R = Me
2 R = CH_2OH
3 R = CHO

The reaction in the opposite direction, i.e. reduction of convallatoxin (**3**) to convallatoxol (**2**) could not be detected in subsequent experiments *in vivo* [3]. However, periplorhamnoside (**1**), containing no oxygen function at C-19, was shown to be a precursor of convallatoxol (**2**) and also, to a minor degree, of convallatoxin (**3**). These results point to a biosynthetic sequence periplorhamnoside → convallatoxol → convallatoxin. In the present study subcellular organelles and soluble enzyme preparations from leaves of *Convallaria majalis* were screened for enzyme activities that would hydroxylate the C-19 methyl group of **1**, and oxidize the hydroxymethyl group of **2**, respectively.

RESULTS

When the subcellular location of plant enzymes of secondary metabolism is investigated, only low activities are to be expected. Therefore labelled substrates were chosen for the assays. Hydroxylation was investigated using periplorhamnoside (**1**) tritiated in the lactone ring, whereas convallatoxol (**2**), labelled with tritium at C-19 served as substrate for the postulated dehydrogenase or oxidase reaction. Controls were conducted for both reactions and the enzyme activities measured were corrected for nonenzymic conversions.

For all experiments leaves of plants were taken in the early stage of flowering. At this time the content of the main cardenolides is reaching a maximum [4], and the levels of enzymes performing the final steps in this biosynthetic pathway should be optimal.

Hydroxylation of periplorhamnoside

The subcellular fractions indicated in Table 1 were assayed for their ability to hydroxylate periplorhamnoside. The 10000 *g* mitochondrial pellet was subjected to sucrose density gradient centrifugation in order to remove contaminant thylakoid membranes and light membranes from the endoplasmic reticulum. As shown in Fig. 1 a separation of mitochondria from chloroplast fragments could be achieved. At a density of 40% sucrose contamination by light membrane vesicles was of minor degree only. The ratio of the activities of fumarase and of cytochrome oxidase in the mitochondrial band and on top of the gradient pointed to

Table 1. Hydroxylation of periplorhamnoside-[^{3}H] by subcellular preparations from leaves of *Convallaria majalis* in the presence of a NADPH-regenerating system

Preparation*	Conversion rate pmol convallatoxol-[^{3}H] formed/min/mg protein
Chloroplasts	no detectable activity
10000 *g* pellet	3.0
10000 *g* pellet, NADPH omitted	1.2
Mitochondria	6.3
Microsomes	1.5
Soluble fraction	no detectable activity

*See the Experimental for details.

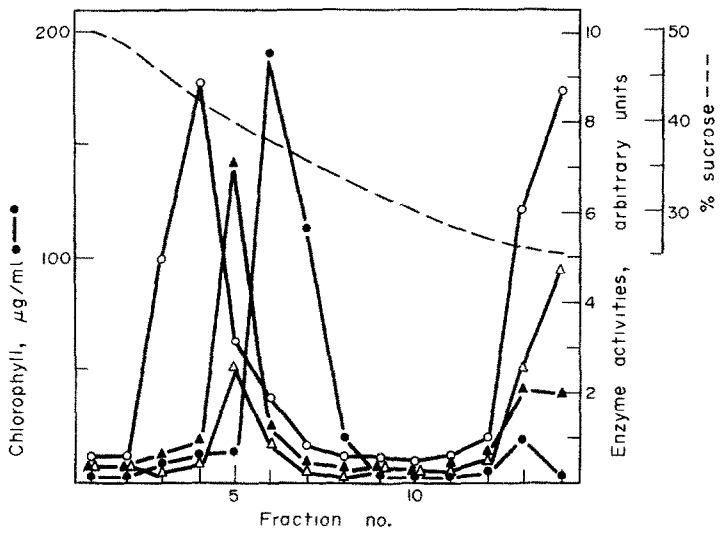


Fig. 1. Sucrose density gradient separation of a 10000 *g* pellet from leaves of *Convallaria majalis*. One arbitrary unit represents the following values for the various enzymes:
▲—▲ cytochrome oxidase: 0,25 µmol/min/ml;
△—△ fumarase: 3 nmol/min/ml;
○—○ catalase: 10 µmol/min/ml

loss of a part of the mitochondrial matrix. The peak of catalase activity was not very distant from the mitochondrial marker enzymes. Thus the pooled mitochondrial fractions (fraction 5 in Fig. 1) contained some microbodies.

The incubation conditions chosen were those for assay of a monooxygenase. A NADPH-regenerating system with glucose-6-phosphate and glucose-6-phosphate-dehydrogenase was used. No activity was found in chloroplasts and soluble supernatant although both contained far more protein than the mitochondrial and microsomal fractions. Mitochondria showed a higher total activity than the microsomes. The conversion found in the latter fraction presumably originated from submitochondrial particles. A comparison of the specific activities of the 10000 *g* pellet and the mitochondrial fraction indicates that contaminating peroxisomes were not responsible for the hydroxylation of periplorhamnoside (**1**). We therefore consider the mitochondria as the site of conversion of **1** into **2**. The mitochondria lost part of their matrix during density gradient centrifugation. Since their specific activity for hydroxylation increased compared to the 10000 *g* pellet, a NADPH-dependent, membrane-bound monooxygenase seemed to be involved. Low yields of labelled products obtained with enzyme preparations from non-sterile plants might cause doubt concerning possible microbial transformations. In our case an involvement of bacterial hydroxylases is unlikely, since they are soluble enzymes [5] and the activity should then be pronounced in the chloroplast and soluble fractions. Furthermore the mitochondrial system appeared to be quite specific. Lokundjoside, the 11α-hydroxylated derivative of periplorhamnoside (**1**), whose formation from that precursor has been observed *in vivo* [3], was not formed by the mitochondrial monooxygenase.

Conversion of convallatoxol (**2**) *into convallatoxin* (**3**)

All the subcellular preparations described above were assayed for oxidation or dehydrogenation of convallatoxol (**2**). No activity was found with cell organelle fractions but, in contrast to the particulate monooxygenase, the enzyme involved was present in the soluble enzyme preparation and the reaction was catalysed by a NAD-dependent dehydrogenase (35.2 pmol convallatoxin-[^{3}H] formed/min/mg protein). NADP could not substitute for NAD. No conversion occurred without added cofactor. Therefore participation of a flavoprotein oxidase was rendered unlikely. The total activity of convallatoxol dehydrogenase was higher by a factor of 20 than that of the periplorhamnoside hydroxylase. This is reflected by the results of previous experiments *in vivo*: 1.7 % of the label from periplorhamnoside (**1**) fed to the leaves was incorporated into convallatoxol (**2**), whereas convallatoxol (**2**) was converted into convallatoxin (**3**) with an efficiency of 6.4 % [2, 3]. Thus C-19 hydroxylation of periplorhamnoside (**1**) might be the rate determining step of the transformations at the monoglycoside level in *Convallaria majalis*.

DISCUSSION

Enzyme activities catalysing each of the two steps in the biosynthetic sequence: periplorhamnoside (**1**) → convallatoxol (**2**) → convallatoxin (**3**) have been detected in subcellular fractions of *Convallaria majalis* leaves. Taken together with evidence obtained from previous experiments *in vivo* [2, 3], this seems to be the logical pathway for the biogenesis of the main cardiac glycosides in *Convallaria majalis*.

Reduction of the C-19 formyl group of cardenolides by rat liver homogenates [6] and in undifferentiated cell suspension cultures of *Digitalis lanata* and *Thevetia neriifolia* [7] has been reported. We believe however, that in the differentiated, compartmentalized leaf cells of *Convallaria majalis* at the stage of active biosynthesis of

cardenolide glycosides, i.e. at the onset of flowering, the biosynthetic sequence as outlined above is operative. Under different metabolic conditions or in other tissue types an interconversion of convallatoxin (3) to convallatoxol (2) might be possible, whereas a reduction of convallatoxol (2) to periplorhamnoside (1) is improbable.

There are several examples of aromatic and aliphatic hydroxylations in higher plants catalysed by membrane-bound monooxygenases. Cinnamic acid hydroxylase activity has been reported for microsomes [8] and chloroplast thylakoid membranes [9] as well as for microbodies [10, 11] and mitochondria [11]. Hydroxylation of kaurene leading to gibberellins takes place in the microsomal fraction [12], and so does N-demethylation of unphysiological substrates [13, 11]. A monoterpene hydroxylase acting on the C-10 methyl groups of geraniol and nerol has recently been demonstrated to be embedded in provacuolar membranes [14]. It appears that mostly light membranes, e.g. those of the endoplasmic reticulum, are involved in hydroxylation reactions. In the case of the hydroxylation of periplorhamnoside (1), however, mitochondria are the predominant if not exclusive site. This is common in animal systems where hydroxylases are often attached to mitochondrial membranes [5, 15]. Furthermore it has to be borne in mind that important steps in the biogenesis of steroids, e.g. the conversion of cholesterol into pregnenolone are performed by mitochondria from animals [16] and higher plants [17]. According to the cofactor requirements the soluble enzyme converting convallatoxol (2) into convallatoxin (3) is a NAD-dependent dehydrogenase. In this respect it reembles an alcohol dehydrogenase from *Thea sinensis* which accepts monoterpenes as substrates [18]. Geranyl dehydrogenase from the orange requires NADP as a cofactor [19]. On the contrary, an alcohol oxidase, probably a flavoprotein, from *Tanacetum vulgare* has been reported [20], that oxidizes geraniol and seems to be involved in the formation of leaf aldehyde (hex-*trans*-2-ene-1-al).

Our results confirm that enzymic interconversions of cardenolides in *Convallaria majalis* leaves indeed occur at the monoglycoside level. Hydroxylation of the C-19 methyl group and the consecutive dehydrogenation of the hydroxymethyl group formed in the first step are performed in different compartments of the leaf cells. Periplorhamnoside (1), the direct progenitor of the predominant cardiac glycosides showing the same hydroxylation pattern in the steroid skeleton, is transformed into convallatoxol (2) by mitochondria. These organelles contain the major part of particle-bound cardenolides in *Convallaria majalis* leaf cells (unpublished results).

A soluble enzyme catalyses the final step (except of further glucosidation) of cardiac glycoside biogenesis in *Convallaria majalis*. This enzyme activity is located either in the cytoplasm or in the vacuole.

EXPERIMENTAL

Plant material. Convallaria majalis plants were grown in the garden of the Institute. Early in May, leaves from plants with developing flower buds were taken and the midrib was cut out.

Organelle preparation. Leaves (31 g) were cut into strips and blended with 200 ml ice cold grinding medium (as described by Walker [21] with the addition of 5 mM dithiothreitol and 10 g/l. of BSA). After blending for 3 × 5 sec the mixture was filtered through a 4-fold layer of miracloth. Chloroplasts were quickly spun down in a Sorvall refrigerated centrifuge by switching off when 7000 rpm (SS 34 rotor) were reached. The pellet was resuspended in grinding medium without BSA and centrifuged at 500 *g* for 5 min to remove the major part of nuclei. Then chloroplasts were pelleted at 2000 *g* for 10 min. After suspending in the appropriate buffer, enzyme assays were performed. The supernatant of the 7000 rpm centrifugation was centrifuged at 10000 g for 15 min. The pellet was suspended in 6 ml 0.06 M Hepes buffer pH 7.5 containing 0.5 M mannitol. Two 1.5 ml portions were further diluted with mannitol buffer to 15 ml each and pelleted at 10000 *g* for 15 min. Both pellets were then assayed for hydroxylation of periplorhamnoside (1) with and without NADPH, respectively. From the remaining 3 ml, aliquots of 1 ml were layered on top of 3 tubes containing 30 ml of a linear sucrose density gradient (25–50% sucrose in 0.06 M Hepes buffer pH 7.5). Centrifugation was performed in a SW 25.1 rotor (Beckman ultracentrifuge L2-65B) for 65 min at 12500 rpm. 2.4 ml fractions were collected from tubes pricked at the bottom and assayed for marker enzymes. Fractions 5 (Fig. 1) corresponding to the maxima for fumarase and cytochrome oxidase were pooled, diluted with Hepes buffer to 0.5 M sucrose and centrifuged at 10000 *g* for 15 min. The pellet was used for the monooxygenase assay. The 10000 *g* supernatant was centrifuged at 100000 *g* for 2 hr. The microsomal pellet was washed once with grinding medium without BSA and then tested for enzyme activities.

Soluble preparation. 13 g Leaves, cut into strips, were ground with quartz sand for 10 min at 4° in a mortar. The grinding medium consisted of 15 ml 0.2 M Hepes buffer pH 7.5, containing 5 mM dithiothreitol and 1 g PVP. The homogenized material was squeezed through a double layer of miracloth and then centrifuged at 50000 *g* for 15 min. The supernatant was put onto a Sephadex G-25 column (25 × 1.5 cm) equilibrated with 0.1 M Na pyrophosphate buffer pH 8.3 and 15 ml collected and concd to 7.5 ml using Aquacide II. Aliquots (2.5 ml) were assayed. The use of Tris buffer pH 7.5 in this procedure resulted in somewhat lower formation of convallatoxin (3) from convallatoxol (2).

Enzyme assays. Hydroxylation of periplorhamnoside (1) was measured by incubating in a total of 2.5 ml; periplorhamnoside-[^{3}H] (0.1 mM), NADPH (tetrasodium salt, 0.15 mM), glucose-6-phosphate (0.3 mM), glucose-6-phosphate dehydrogenase (10 U), $MgCl_2$ (2 mM) and the particulate or soluble preparation in 0.1 M Hepes pH 7.5. After 30 min at 27° the reaction was stopped by the addition of EtOH. Dehydrogenation of convallatoxol (2) was assayed as follows: convallatoxol-[^{3}H] (0.1 mM), NAD (0.2 mM), KCl (1 mM), $MgCl_2$ (2 mM) and the particulate or soluble preparation in 2.5 ml of 0.1 M Na pyrophosphate buffer pH 8.3 were incubated at 27° for 30 min. Again the reaction was stopped by the addition of EtOH. Cytochrome-*c* oxidase was assayed according to Major *et al.* [22], fumarase according to Coope and Beevers [23] and catalase as described by Bergmeyer *et al.* [24].

Other determinations. Protein was measured by the method of Lowry *et al.* [25]. Sucrose concentrations were determined refractometrically.

Labelled compounds. Convallatoxol-[19-^{3}H] (104.8 μCi/mg) was obtained by reduction of convallatoxin (3) with $NaBT_4$ as described previously [2]. Periplorhamnoside-[21,22-^{3}H] (42 μCi/mg) was labelled by catalytic tritium exchange at the lactone ring [26] as described [3].

Chromatographic methods. TLC was carried out using 0.5 mm layers of Si gel (Merck, 60 F_{254}) with solvent system A: $CHCl_3$–MeOH–H_2O (7:3:1), lower phase. Schleicher and Schüll 2043 b Mgl was used for PC with solvent B: PeOH–C_6H_6–H_2O (1:1:2), upper phase, 60% stationary phase; or solvent C: toluene–*n*-BuOH (2:1, saturated with H_2O), 35% stationary phase.

Determination of radioactivity. Radioactive samples were measured in a liquid scintillation counter; PC were scanned with a 4π PC scanner, TLC plates were checked using a thin layer scanner with a 2π proportional chamber.

Identification of products. The extraction of the cardenolide glycosides from the incubation mixture was performed as described previously [2].

Convallatoxol-[^{3}H]. Convallatoxol-[^{3}H] was isolated by PLC in solvent system A. For further purification PC (solvent system B and subsequently system C) was used. Finally the zone containing labelled convallatoxol was cut out and the substance eluted from the paper After addition of unlabelled convallatoxol (100 mg) it was crystallized from MeOH–H_2O (5 ×) to constant sp act.

Convallatoxin-[^{3}H]. Chromatographic purification was as described above. As a final proof 5-fold recrystallisation yielded a product with constant specific radioactivity.

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