Origin of Cardenolides in Rhizomes and Roots of *Convallaria majalis* L. Biogenesis *in situ* or Transport from Leaves?

Roswitha Schrutka-Rechtenstamm, Brigitte Kopp

Institut für Pharmakognosie der Universität Wien, Währinger Straße 25, A-1090 Wien

and

Wolfgang Löffelhardt

Institut für Allgemeine Biochemie der Universität Wien und Ludwig-Boltzmann-Forschungsstelle für Biochemie, Währinger Straße 38, A-1090 Wien

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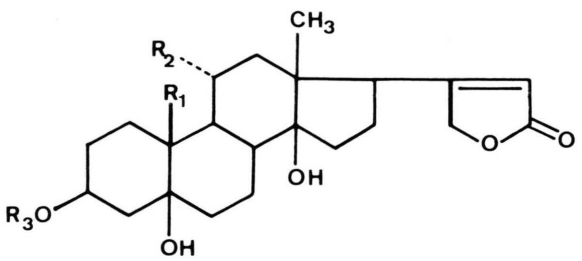
After administration of sodium[1-^{14}C]acetate to rhizome or root cuttings of *Convallaria majalis* L. two of the main cardiac glycosides were isolated and examined for incorporated radioactivity. With a detection limit of 0.0003% of incorporated label it can be assumed, that the subterranean organs of *C. majalis* are not capable of cardenolide biogenesis *de novo*. Interconversions on the cardenolide level do not take place, too, as was proved by application of [U-^{14}C]convallatoxol and examination of convallatoxin, the main metabolite in leaves. Therefore the cardenolide content of the subterranean parts of *C. majalis* (0.2%) must have its origin in the leaves, from where the cardiac glycosides are translocated to rhizomes and roots, which only fulfil a storage function in this respect.

Introduction

Convallaria majalis L. contains cardiac glycosides (Fig. 1) in all tissues except of the ripe berries without seeds. Previous investigations showed, that the total cardenolide content in relation to dry weight is variable throughout the vegetation period in the surface organs, whereas the content in the subterranean parts, *i.e.* rhizomes and roots, remains nearly constant [1, 2]. Furthermore, the cardenolide pattern is different in above and underground organs of *Convallaria majalis* towards the end of the vegetation period [2].

The capacity of cardenolide biosynthesis of the green leaves has already been proved [3, 4]. For *de novo* biosynthesis in subterranean organs no conclusive evidence has been obtained till now. When plants were allowed to photoassimilate $^{14}CO_2$, labelled cardenolides were also found in rhizomes and roots [4]. Application of [4-^{14}C]progesterone, a known precursor of cardiac glycosides, which is present in leaves as well as in subterranean organs [5], to whole plants via the leaf epidermis led to incorporation of radioactivity into cardiac glycosides in underground organs, too [3]. When progesterone was injected into rhizome discs, no labelled cardenolides could be detected [3].

In the latter case, permeability problems might have caused the lack of incorporation. Alcohol



	R_1	R_2	R_3
Convallatoxin	–CHO	–H	RH–
Convalloside	–CHO	–H	GLU–RH–
Convallatoxol	$-CH_2OH$	–H	RH–
Lokundjoside	$-CH_3$	–OH	RH–

Fig. 1. Structure of some of the main cardenolides in *Convallaria majalis* L.; RH = 6-deoxy-α-L-mannopyranosyl-; GLU = β-D-glucopyranosyl-.

Reprint requests to Univ.-Doz. Dr. B. Kopp.

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dissolved progesterone administered to the stalks of intact leaves yielded almost no detectable radioactivity in the cardenolide fraction [3]. An autoradiography of the leaf showed, that most of the administered activity (95%) was located in the stalk and on the beginning of the main veins. The precursor actually had not been transported to the site of biogenesis [3].

On the other hand, the positive results obtained with whole plants might reflect transport to, rather than biosynthesis within the underground parts of *Convallaria majalis*. The fact, that even strophanthidol, an aglycone, could be found in underground parts after application to the leaves [6], supports this possibility.

In order to make a definite decision, we administered sodium[1–^{14}C]acetate to isolated root and rhizome cuttings. This precursor is well soluble in water, so it should easily reach the sites of cardenolide synthesis in the tissue. In addition we administered [U–^{14}C]convallatoxol to rhizomes to get information about conversions at the cardenolide level.

Material and Methods

Plant material

Subterranean parts of *Convallaria majalis* L., grown in the garden of the institute, were separated into roots and rhizomes. They were cleaned mechanically and surface sterilized by immersing in 5% sodium hypochlorite solution for 40 minutes. Then the material was washed with sterilized water to pH 7.

Chemicals

Sodium[1–^{14}C]acetate (specific activity: 684 μCi/mg) was obtained from Radiochemical Center, AMERSHAM. [U–^{14}C]convallatoxol was isolated from leaves of *Convallaria majalis* after photoassimilation of $^{14}CO_2$ [8]; the specific activity was 4.9×10^5 dpm/mg. Floxapen^R and Clamoxyl^R (BEECHAM, active principles: flucloxacillin and amoxycillin, respectively) served as antibiotics. All other chemicals were of analytical grade.

Incubation of plant material with sodium [1–^{14}C] acetate

Rhizomes and roots were separately transferred to buffer solution of the following composition: 40 mM potassium phosphate buffer, pH 7, 0.0025% (= 25 μg/ml) flucloxacillin and amoxycillin. In the buffer solution 7.5 g of the rhizomes and 2.0 g of the roots were cut into pieces of 3 to 5 mm length and the wet cuttings were quickly put into a Petri dish containing 0.33 mg precursor (228 μCi) dissolved in 0.5 ml buffer. The dishes were closed and kept in complete darkness for 48 hours at room temperature.

Incubation of rhizome cuttings with [U–^{14}C]convallatoxol

15.0 g of the rhizomes were incubated with 0.44 mg precursor (2.15×10^5 dpm) in the same way as described above.

Extraction of cardenolides

After incubation the cuttings were washed carefully to remove non-absorbed radioactivity. Then the samples were freeze-dried and pulverized under liquid nitrogen. Extraction was performed twice with boiling 70% ethanol. The solvent was evaporated under reduced pressure. For elimination of non-polar substances the residue was extracted repeatedly with *n*-hexane and *n*-hexane/chloroform (1 + 1). Afterwards the cardenolide fraction was obtained by extraction with chloroform/methanol (1 + 1). For further cleanup the residue of the chloroform/methanol extract was redissolved in water, then the aqueous solution was extracted with chloroform/*n*-butanol (2 + 1). The resulting organic phase contained the cardiac glycosides.

Separation of the cardenolides

Preparative TLC (silica gel 60 F_{254} MERCK; mobile phase: chloroform/methanol/water 7 + 3 + 1, lower phase). The radioactive zones were scraped off and eluted with methanol/water (95 + 5). The resulting 5 fractions were analyzed by HPLC [7] and then the separated glycosides were collected from the columns in preparative scale [8].

Measurement of radioactivity

Each individual cardenolide sample was divided into 3 parts: Two of them were chromatographed in different TLC systems (silica gel 60 F_{254} MERCK; ethylmethylketone/toluene/water/methanol/glacial acetic acid: 40 + 5 + 3 + 2.5 + 1; ethylmethylketone, water saturated). The TLCs were examined for

radioactivity using a 2π-TLC scanner (BERTHOLD) followed by detection of the glycosides by spraying with vanilline sulfuric acid [9]. To the third part a scintillation cocktail (0.5% butyl-PBD in toluene) was added and the absolute radioactivity was determined in a liquid scintillation counter (BECKMAN LS-230).

Results and Discussion

To root and rhizome cuttings of *Convallaria majalis* L. we administered sodium[1-^{14}C]acetate, separately. Antibiotics were added for exclusion of microbial conversions.

Extraction of the two samples with solvents of increasing polarity yielded three fractions containing labelled metabolites (Table 1).

Further fractionation of the chloroform/methanol extract led to the cardenolide fraction, which contained 4.5×10^6 dpm in roots and 22.0×10^6 dpm in rhizomes. These values were comparable to the radioactivity found in the cardenolide fractions from leaves after photosynthesis experiments with $^{14}CO_2$, where significant incorporation of label into individual cardiac glycosides could be detected [4, 8]. From the cardenolide fractions of the subterranean organs the two main glycosides, convalloside and lokundjoside, were purified by TLC and HPLC. The total counts of the four samples were between 1500 and 2000 dpm (as compared to about 3×10^5 dpm for the photosynthesis experiments [8]). The low radioactivity observed was obviously due to background contamination, as could be shown by additional examination of the 4 cardenolide samples by TLC in two different solvent systems. The lokundjoside zones never did coincide with a peak of radioactivity. In the case of convalloside comigration was observed with one solvent system, but not with the other one (Fig. 2).

Table I. Distribution of incorporated radioactivity between phases of different polarity, obtained from rhizomes and roots of *Convallaria majalis* L., respectively.

	Rhizomes dpm × 10^6	Roots dpm × 10^6
Administered radioactivity of sodium[1-^{14}C]acetate	506.0	506.0
Extract (70% alcohol)	495.0	498.0
Elution of the alcoholic extract with:		
n-hexane and *n*-hexane/chloroform (1 + 1 v/v)	67.0	38.0
chloroform/methanol (1 + 1 v/v) (contains the cardenolides)	39.0	23.0
water	389.0	437.0

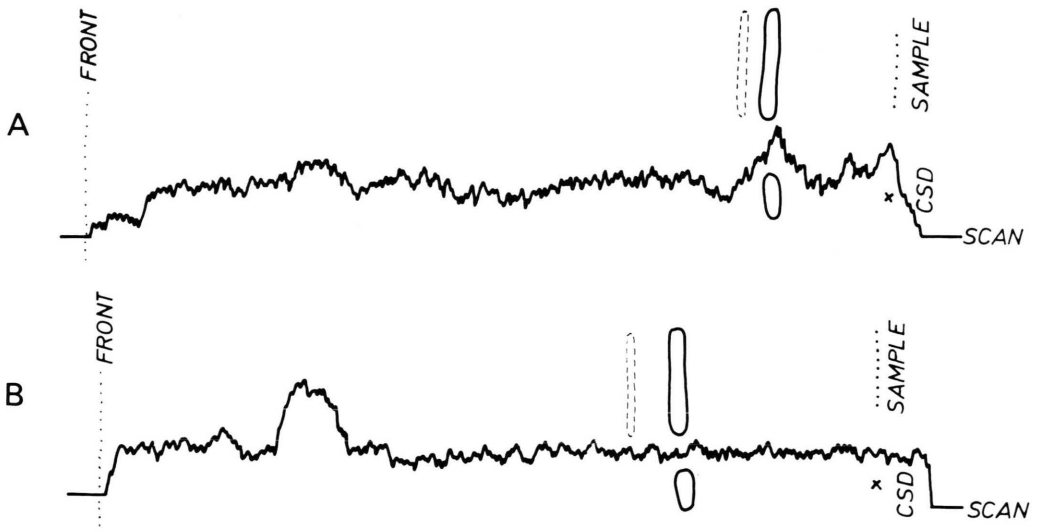

Fig. 2. TLC examination and scan of the convalloside (CSD) fraction obtained from roots in two different solvent systems: A – ethylmethylketone/toluene/water/methanol/glacial acetic acid (40 + 5 + 3 + 2.5 + 1 v/v); B – ethylmethylketone, water saturated.

The detection limit of the used TLC scanner is about 80 dpm for one peak. Thus our conclusion is as follows: if there is any incorporation of acetate into cardenolides in rhizomes or roots, it must be lower than 0.0003%. This value certainly cannot account for an even moderate *de novo* biogenesis of cardenolides in the subterranean organs of *Convallaria majalis*. Therefore the cardiac glycosides comprising about 0.2% of the root and rhizome dry weight must have been synthesized in the photosynthetically active organs and then transported to subterraneous parts.

The rhizome cuttings, which were incubated with [U-^{14}C]convallatoxol, were extracted in the same way. Convallatoxin, the main metabolite in leaves [10, 11], was isolated and examined for radioactivity incorporated. This was below the detection limit, corresponding to a conversion $<0.1\%$ (calculated for 8.10^5 dpm of precursor taken up), whereas the incorporation rate in leaves is about 6% [10].

Since we have not been able to detect one of the most important steps among the cardenolide interconversions in rhizomes, one might assume that after the transport from leaves no further biosynthesis reactions involving cardenolides take place in the subterranean organs of *Convallaria majalis*, which then in this context would function as storage organs only.

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