

# Determination of Progesterone in Vegetative Organs and Cell Organelles of *Convallaria majalis* L. by Radioimmunoassay

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Progesterone was extracted from leaves and subterranean parts of *Convallaria majalis* L. and quantified by radioimmunoassay. During the stage of flowering the content of progesterone in rhizomes was by a factor of four higher than in leaves and by a factor of ten higher than in roots, whereas five weeks later the level of progesterone was unchanged in rhizomes and roots but was elevated in the leaves. The change in progesterone distribution is discussed in relation to the development of the plants. At the subcellular level the mitochondrial and microsomal fractions contained the largest quantity of progesterone in leaf cells. However, significant amounts were also found in the  $2000\times g$  fraction comprising nuclei and chloroplasts.

## Introduction

The ability to synthesize progesterone from cholesterol or pregnenolone is rather widespread among plants [1–6]. Thus minute amounts of progesterone were assumed to exist in many plants [7], but so far it has been isolated only from *Holarrhena floribunda* [8] and apple seeds [9], and was recently detected in pollen of *Pinus nigra* [10].

Previous investigations indicated that one of the most important functions of progesterone in plants is to serve as precursor for the synthesis of different steroids [11–13], alkalamines [1, 8] and cardiac glycosides [14–20]. Numerous publications deal with the role of progesterone as key substance in the biosynthesis of cardenolides [14–20], therefore it is surprising that progesterone itself never has been isolated from cardenolide producing plants. This communication provides information on the content and distribution of progesterone in organs and subcellular fractions of *Convallaria majalis* L. As we expected only small amounts of progesterone, the quantitative determination was performed by radioimmunoassay (RIA) for the advantage of extreme sensitivity and high specificity.

## Materials and Methods

### Plant material

*Convallaria majalis* plants were pot-grown in the greenhouse of the institute and were harvested at the stage of flowering and five weeks later, respectively.

### Materials

Progesterone RIA kit was obtained from Wien Laboratories Inc. (New Jersey); progesterone, pregnenolone and polyvinylpyrrolidone (PVP) were purchased from Sigma. All other reagents were of analytical grade.

### Organelle preparations

Leaves (23 g) taken from plants with developing flower buds were cut into strips and blended  $3\times 5$  sec with an Ultra-Turrax at full speed. 200 ml Semi-frozen grinding medium according to ref. [21] with the addition of 5 mM dithiothreitol and 2 g PVP were used. After filtration through a fourfold layer of Miracloth consecutive centrifugations at  $2000\times g$  (10 minutes),  $10000\times g$  (20 minutes) and  $100000\times g$  (120 minutes) were carried out. Each pellet was resuspended in 35 ml grinding medium without PVP and centrifuged again.

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### Extraction procedure

Leaf slices (10 g fresh weight) were successively extracted with 50 ml boiling 96% ethanol for 60 minutes and with 50 ml dichloromethane for 15 minutes. The combined extracts were evaporated to dryness, resuspended in 20 ml 50% ethanol and extracted several times with petrol ether (bp. = 45 ° – 60 °C).

The ketonic fraction was separated by Girard's reagent T according to ref. [8] and subjected to TLC in solvent system A. The zone containing progesterone and possibly pregnenolone was eluted with dichloromethane-methanol-water (80:19:1) and rechromatographed in system B where the separation of progesterone (hRf = 90) from pregnenolone (hRf = 65) was achieved. The zone corresponding to progesterone was once more eluted with dichloromethane-methanol-water (80:19:1) and further purified by one additional run on a column of Sephadex LH 20.

Rhizomes and roots were first washed thoroughly to remove adhering soil particles and then extracted as described above.

Each subcellular fraction was extracted with 10 ml boiling methanol for 30 minutes and with 10 ml petrolether for 10 minutes and then worked up as described above.

### Chromatographic methods

TLC was carried out using 0.5 mm layers of silicagel (Merck, 60 F<sub>254</sub>) with solvent system A: *n*-hexane-ethylacetate (5:2) or solvent system B: dichloromethane-methanol (97:3). For column chromatography Sephadex LH 20 was used (column: 8 × 50 mm), the solvent system was dichloromethane-methanol (1:1).

### Radioimmunoassay procedure

Samples in an appropriate petrolether dilution and reagent blanks (0.1 ml dilution solvent), respectively, were pipetted into test tubes and evaporated to dryness with the aid of a stream of nitrogen. 0, 10, 25, 50 and 100 µl of progesterone standard solution equivalent to 0, 100, 250, 500 and 1000 pg per tube, respectively, were transferred to test tubes and evaporated to dryness, too.

At room temperature 50 µl of [1,2-<sup>3</sup>H]progesterone solution (11 500 dpm, spec. activity = 90 Ci/mmol) was added to each tube and mixed well to

dissolve. Then 0.8 ml of 0.01 M phosphate buffer pH = 7.4, containing 0.06% bovine serum albumine, was pipetted into the tubes; after mixing on a vortex mixer, 100 µl of progesterone antibody solution was added. The samples were cooled in an ice bath for 60 minutes and 0.5 ml of a cold suspension of dextran-coated charcoal was added, the contact time being 5 minutes at 0 °C. Separation of free and antibody-bound haptene was achieved by centrifugation for 10 minutes at 2000×*g* at 0 °C and the supernatant, containing the bound progesterone, was decanted into a scintillation vial containing 10 ml scintillation cocktail (667 ml toluene, 337 ml Triton X-100, 5.5 g PPO and 0.1 g POPOP) and counted in a Beckman LS-230 liquid scintillation counter at 2% error.

### Other determinations

Protein was determined by the method of Lowry *et al.* [22].

## Results

### Distribution of progesterone in organs of *Convallaria majalis*

All extracts were subjected to TLC to separate progesterone from other ketonic steroids, especially from pregnenolone in order to avoid cross reactions. For further purification the progesterone solution obtained was put onto a Sephadex LH 20 column, thus minimizing the possibility of nonspecific interactions with the assay system.

For the first experiments leaves, rhizomes and roots were taken from plants in the early stage of flowering; at this time the content of cardenolides is reaching a maximum [23]. As shown in Table I a considerable amount of progesterone was observed in rhizomes. The progesterone content of leaves was

Table I. Distribution of progesterone in organs of *Convallaria majalis* in the early stage of flowering.

Organ	µg/100 g fr.wt.	% of total progesterone content of the plant
leaves	15.7	17.4
rhizomes	81.0	75.2
roots	6.7	7.4

Table II. Distribution of progesterone in organs of *Convallaria majalis* five weeks after flowering period.

Organ	$\mu\text{g}/100 \text{ g fr. wt.}$	% of total progesterone content of the plant	
leaf laminae	57.4	25.5	} 39.9
leaf stalks	49.3	14.4	
rhizomes	99.3	56.1	
roots	6.0	4.0	

lower by a factor of 5, whereas only a small amount of progesterone was present in roots.

Five weeks after the stage of flowering leaves, rhizomes and roots were assayed again for their progesterone content. The results are summarized in Table II. The subterranean organs showed almost the same progesterone concentration as at the onset of flowering, whereas in leaves a significant increase was observed. In order to investigate the distribution of progesterone within the leaves, laminae and stalks were analysed separately. The results pointed out that progesterone is equally distributed between laminae and stalks, whereas the cardenolide content of laminae is markedly increased [23].

#### Intracellular localization of progesterone

Crude cell organelle fractions obtained by differential centrifugation were also assayed for their progesterone content using the RIA method.

The results presented in Table III demonstrate that more than 90% of cellular progesterone was located in cell particles. Chloroplasts and nuclei contained about 25% of total progesterone, but on a protein basis this was by far the smallest amount

among the particulate fractions. The bulk was found in the microsomal fraction and in the fraction containing mitochondria and microbodies, respectively. As to be expected from reason of solubility, the  $100\,000\times g$  supernatant was virtually devoid of progesterone.

#### Discussion

Our results demonstrate that progesterone is present in significant amounts in leaves as well as in subterranean parts of *Convallaria majalis*. The appearance of progesterone in leaves is not particularly surprising since its role as precursor of cardenolide glycosides in this organ has been demonstrated recently [14]. Preliminary studies on the biosynthesis of cardenolides in *Convallaria majalis* pointed out that [ $^{14}\text{C}$ ]progesterone injected into rhizome cuttings was not incorporated into cardiac glycosides. According to these results presently there is no explanation for the comparatively high content of progesterone in rhizomes. In contrast to these observations Lui and Staba [24] reported an increase in the cardenolide content of root cultures from *Digitalis lanata* after administration of progesterone. It is noteworthy that at the onset of flowering and after the flowering stage, respectively, the progesterone content of rhizomes was approximately the same. However, in leaves progesterone accumulated after the flowering period, whereas the cardenolide content decreased concomitantly. Translocation from rhizomes does not appear to be an explanation for the differences in progesterone content since there is no decrease in the subterranean organ. Furthermore vascular transport of progesterone is hampered by its low solubili-

Table III. Progesterone content of subcellular fractions obtained from leaves (23 g fr. wt.) of *Convallaria majalis*.

Preparation	Progesterone [ng]	% Distribution in subcellular fractions	Protein [mg]	Progesterone [ng] / Protein [mg]
$2000\times g$ pellet (nuclei and chloroplasts)	324	25.7	19.2	17
$10\,000\times g$ pellet (mitochondria, microbodies)	525	41.6	8.4	62
$100\,000\times g$ pellet (microsomes)	400	26.9	6.0	67
$100\,000\times g$ supernatant	74	5.8	7.5	1

ty [14]. A possible explanation is that a part of leaf progesterone is used for cardenolide production at the stage of maximum activity of the enzymes of this biosynthetic pathway. This would implicate that only leaves of *Convallaria majalis* are capable of cardiac glycoside biosynthesis.

It appears that the rate of progesterone biogenesis is not immediately correlated with cardenolide production. Therefore, progesterone besides being a precursor of cardenolides may have other functions in plant cells, too.

At the subcellular level the bulk of progesterone content is found in organelles. Mitochondria and microsomes were richest in progesterone, but a considerable amount could also be detected in the 2000×g fraction, containing nuclei and chloroplasts. According to present knowledge progesterone is not an integral component, neither of membranes of organelles nor of plasma membranes; therefore its localization in subcellular compartments could be interpreted as to reflect its site of biosynthesis or metabolism or biological function. We may assume that in plant cells the biosynthesis of progesterone is organized in a similar way as has been found for animal systems. There the conversion of pregneno-

lone to progesterone has been reported to occur both in the mitochondrial and microsomal fractions [25]. As shown by Stohs, metabolism, *e. g.* conversion of progesterone to 5- $\alpha$ -pregnan-3,20-dion, takes place in microsomes of *Dioscorea deltoidea* [26]. Since there is no indication that chloroplasts are involved in progesterone biosynthesis or metabolism, we suggest that the progesterone accumulated in the 2000×g fraction is predominantly bound to nuclei. This suggestion may be supported by the fact that steroids could act directly or indirectly as hormones [27] and that a hormonal control mechanism similar to those found in animal systems or in fungi [28] could also exist in cells of higher plants [29].

The appearance of progesterone in the 2000×g fraction might lead to the speculation that progesterone acts on plant chromosomes the same way it does on animal chromosomes [30–32]. Even though the general biosynthetic sequence for progesterone is well defined, its physiological role in plants is far less understood.

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