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Progesterone: Its Possible Role in the Biosynthesis of Cardenolides in Digitalis lanata

Abstract. *The incorporation of progestrone-7α-^{3}H and pregnenolone-7α-^{3}H into digitoxigenin, gitoxigenin, and digoxigenin in isolated, surviving leaves of* Digitalis lanata *was demonstrated. In addition, the conversion of pregnenolone to progesterone in the same system was proved. The results tend to indicate that progesterone is as good a precursor of cardenolides as pregnenolone. It is suggested that the biosynthesis of cardenolides might proceed through the intermediacy of progesterone.*

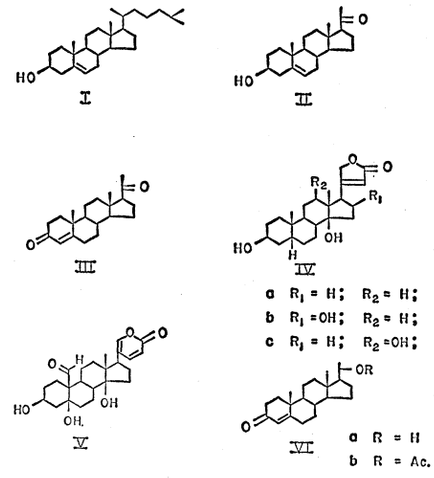
The transformation of cholesterol (I) to pregnenolone (II) in *Digitalis purpurea* (*1*) and in *Haplopappus heterophyllus* (*2*) has been described. The role of pregnenolone in the formation of steroid hormones from cholesterol in animals is well documented (*3*, p. 125). Thus pregnenolone (or its analog) may have an analogous role in the biosynthesis of certain plant sterols, such as cardenolides, bufadienolides, and others. This is supported by the observation that carbons No. 22 and No. 23 of cardenolides do not originate from mevalonic acid (MVA) (*4*), and that pregnenolone may be a precursor of digitoxigenin (IVa) (*5*) and bufadienolides (hellebrin V) in plants (*6*).

If it is tentatively assumed that pregnenolone (II) is a key intermediate in the biosynthesis of cardenolides, the question arises as to what is the mechanism of reduction of its C-5 double bond. In animals the saturation of the homo-allylic alcohol (Δ^5-3β-OH) proceeds by way of the oxidation of the 3β-hydroxyl to a 3-ketone and isomerization of the olefinic bond from C-5 to C-4 to yield a Δ^4-3-ketone moiety (*3*, p. 310, references in tables 17–19; *7*). Subsequently, the conjugated double bond is reduced to either a *cis*-(5β-H) or *trans*-(5α-H) junction between rings A and B (*8*; also *3*, p. 406); and this is followed by the reduction of the C-3 ketone.

If a mechanism analogous to that described for animals operates in *Digitalis* plants, two conditions must first be satisfied. The plant should be able to convert pregnenolone (II) to progesterone (III) and to utilize the latter in the biosynthesis of cardenolides (IV). We now offer proof of these transformations in isolated *Digitalis lanata* leaves.

One average-size leaf was cut with its stem from a *Digitalis lanata* plant, and its surface was rinsed with acetone. An acetone solution of pregnenolone-7α-^{3}H (*9*) (9.0×10^8 dpm, disintegrations per minute) was then applied to the surface of the leaf; and, after evaporation of the acetone, the leaf was sprayed with a 10 percent solution of silicon oil DC 200 (*10*) in ligroin (b.p. 60° to 90°C) (*11*). The leaf was then suspended in approximately 40 ml of a modified Hoagland's medium containing the requisite amounts of micrometabolic elements (*12*) and continuously illuminated with light of 12,000 lu/m^2. The nutrient medium was changed at 48-hour intervals, and the experiment was terminated after 10 days. The leaf was rinsed with ethyl acetate to remove the unabsorbed pregnenolone (4.8×10^7 dpm), then macerated with a razor blade. The products were recovered exactly as described by von Euw and Reichstein (*13*). An analogous experiment was carried out with progesterone-7α-^{3}H (9.65×10^8 dpm), and 3.5×10^8 dpm were found in the ethyl acetate washings of the leaf at the end of the experiment (Table 1).

We found 19.2 percent of the radioactivity of pregnenolone and 25.6 percent of that of progesterone in the fractions containing "plant sterols." The bulk of cardenolides (IV) is recovered with chloroform (*14*), and, consequently, we have undertaken the



processing of this extract. The results of both experiments reported here refer to products from this fraction. The glycosides were hydrolyzed by refluxing with a mixture (8 ml) of methanol and of 0.1*N* sulfuric acid (1 : 1), and the aglycones were recovered with ethyl acetate. The aglycones originating from progesterone (III) contained 8.6×10^7 dpm, and those from pregnenolone (II) 12.88×10^7 dpm. The isolation of digitoxigenin (IVa) [systems A, B(6 hours), C], gitoxigenin (IVb) [systems A, B(6 hours), D], and digoxigenin (IVc) [systems A, B(6 hours), B(19 hours)] was carried out on portions of the aglycones by chromatography (*15*). When systems B, C, and D were used the paper was treated as suggested by Bush and Crowshaw (*16*).

Table 1. Radioactivity in the "sterol fractions" recovered from isolated leaves of *Digitalis lanata* after the application of pregnenolone-7α-^{3}H (9.0×10^8 dpm) and progesterone-7α-^{3}H (9.65×10^8 dpm). The counting was carried out in a scintillation counter with 15 ml of scintillating fluid consisting of 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-[2-(5-phenyloxazolyl)]-benzene dissolved in 1000 ml of toluene.

Extract	Radioactivity (10^7 dpm)	
	Pregnenolone-7α-^{3}H	Progesterone-7α-^{3}H
Ligroin	2.98	0.48
Chloroform	8.6	14.43
Chloroform-ethanol (2:1)	3.7	0.62
Chloroform-ethanol (3:2)	0.62	0.85
Unabsorbed radioactivity*	4.8	35.0
*Percentage recovery in "sterol fractions"**	19.2	25.6

*The percentage recovery is corrected for the unabsorbed radioactivity present in the ethyl acetate washings of the leaves at the termination of the experiment.

Table 2. Amounts of cardenolides formed from pregnenolone-7α-^{3}H and progesterone-7α-^{3}H in isolated leaves of *Digitalis lanata*.

Metabolite*	Pregnenolone-7α-^{3}H (8.52×10^6 dpm)†		Progesterone-7α-^{3}H (6.15×10^8 dpm)†	
	(10^6 dpm)	(% conversion)	(10^6 dpm)	(% conversion)
Digitoxigenin	10.57	1.24	12.40	2.02
Gitoxigenin	3.18	0.37	1.87	0.30
Digoxigenin	2.10	0.25	1.49	0.24

*The counting was carried out in the scintillation liquid described in Table 1. †This amount is corrected for the radioactivity recovered from the surface of the leaf at the termination of the experiment (Table 1).

To facilitate the detection of a product on paper, 40 μg of the appropriate unlabeled material was added to the extract prior to chromatography. The chromatogram was scanned for radioactivity, and the cardenolide was located by its color with 3,5-dinitrobenzoic acid on a test strip 1.5 mm wide cut from the center of the paper. Only the area of the chromatogram in which the radioactivity coincided with the color spot was extracted, and the extract was further purified. Each aglycone was purified until it showed, in two consecutive chromatograms, a single symmetrical peak of radioactivity coinciding with a positive color test.

The amounts of aglycones produced from the administered precursors are given in Table 2. The results represent the radioactivity recovered from the chromatogram in which the product appeared for the first time as a single symmetrical zone. A portion of the metabolite recovered from the last chromatography was then further diluted with nonradioactive material (20 to 30 mg) and crystallized to constant specific activity as the free alcohol and as the acetate or diacetate, as the case might be. At least two isolation and identification experiments were performed for each product.

Crystallization to constant specific activity was carried out for digitoxigenin (IVa), gitoxigenin (IVb), and digoxigenin (IVc) derived from progesterone as a precursor. In the experiment with pregnenolone (II), digitoxigenin (IVa) was processed in the same way; but gitoxigenin (IVb) and digoxigenin (IVc) were identified by chromatography only. In most instances, some radioactive impurity accompanied the isolated metabolites and was removed by cocrystallization with cold material. Generally, the contamination was small (less than 5 percent), and this amount does not significantly influence the calculated conversions shown in Table 2.

We then turned our attention to the conversion in the leaf of pregnenolone (II) to progesterone (III). A portion (20 percent) of the steroids derived from the chloroform extract was fractionated by sequential chromatography on paper as follows: (i) hexane and propylene glycol for 6 hours and (ii) heptane, methanol, and water (20:19:1) for 3 hours. The recovered progesterone was then purified by continuous (3 hours) thin-layer chromatography [silica gel–benzene : methanol (99 : 1)] (*17*). The product (1.81×10^5 dpm) was diluted with 95 mg of unlabeled progesterone (III) and crystallized to constant specific activity (4.2×10^5 dpm/mmole). This material was then reduced with sodium borohydride and the mixture of the allylic alcohols (Δ^4-3ξ-OH) was reoxidized with dichlorodicyanoquinone (*18*). The 20β-alcohol (VIa) obtained was acetylated to yield 20β-acetoxypregn-4-en-3-one (VIb), which showed the same specific activity (4.25×10^5 dpm/mmole) as the progesterone. The evidence presented constitutes proof that the exogenously supplied pregnenolone (II) was metabolized to progesterone (III) in the surviving leaf of *Digitalis lanata* (*19*). In addition, the pregnenolone (II) was also incorporated into digitoxigenin (IVa), gitoxigenin (IVb), and digoxigenin (IVc) (*20*).

Similarly, externally fed progesterone was transformed into the same three cardenolides under analogous experimental conditions. Thus, both pregnenolone and progesterone served as precursors of the cardenolides. Though it might be premature to draw conclusions concerning the relative merits of the two steroids as precursors of cardiac aglycones, results in Table 2 tend to indicate a somewhat better incorporation of progesterone (III) than of pregnenolone (II).

In view of the above results, it would seem likely that pregnenolone (II) is first converted to progesterone (III) which is further utilized in the biosynthetic processes. Such a pathway is not inconsistent with the existence in the leaves of an enzymatic system for the saturation of the C-5 double bond by a mechanism similar to that in animals (*3, 7, 8*). Possibly the amount of progesterone in the plant (9.05×10^5 dpm, equivalent to 0.106 percent of the pregnenolone) represents the steady-state concentration. These considerations should be viewed critically since the experimental conditions were not physiological.

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