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# PREGNENOLONE METABOLISM IN *DIGITALIS LANATA*

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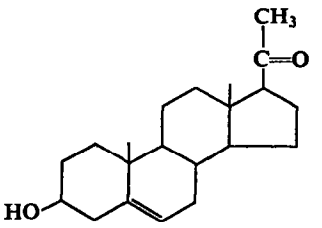
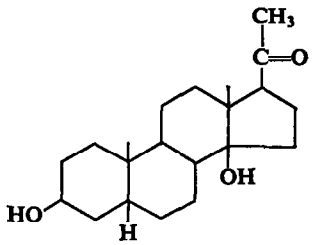
**Abstract**—After the administration of pregnenolone-4-$^{14}$C to a *Digitalis lanata* plant, the following radioactive metabolites were isolated: digitoxigenin, gitoxigenin, digifologenin, 5$\beta$-pregnane-3,20-dione, and progesterone.

## INTRODUCTION

IN 1964 Tschesche and Lilienweiss reported that pregnenolone (I)-21-$^{14}$C glucoside was converted to cardenolides by leaves of *Digitalis lanata*.[1] More recently, the same group has sought to clarify the intermediary steps in this transformation. They found that labelled 5$\beta$-pregnane-3$\beta$,14$\beta$-diol-20-one (II) was converted to the cardenolides, but $\Delta^5$-14-desoxy-digitoxigenin and 14-anhydrodigitoxigenin were not.[2]




(I) Pregnenolone




(II) 5$\beta$-Pregnane-3$\beta$,14$\beta$-diol-20-one

We have approached this problem by making a study of the metabolites of pregnenolone-4-$^{14}$C in *D. lanata*, in an attempt to find cardenolide precursors. A preliminary report of part of this work has appeared elsewhere.[3]

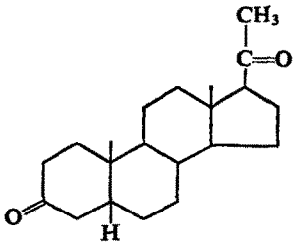
After this work was submitted for publication, Caspi and Lewis[4] reported the conversion of pregnenolone-7$\alpha$-$^3$H to progesterone, and of progesterone-7$\alpha$-$^3$H to cardenolides, by excised *D. lanata* leaves.

## RESULTS

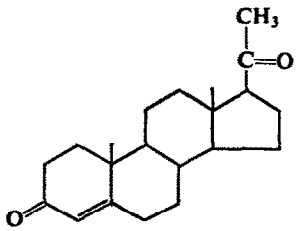
Pregnenolone-4-$^{14}$C was administered once a week for 9 weeks to the leaves of a *Digitalis lanata* plant. The plant was then worked up and fractionated into light petroleum, chloroform, and chloroform–ethanol extracts. The latter two contained most of the radioactivity.

* A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture. Work conducted under a co-operative agreement with the California Institute of Technology.

[1] R. TSCHESCHE and G. LILIENWEISS, *Z. Naturforsch.* **19b**, 265 (1964).
[2] R. TSCHESCHE, H. HULPKE and H. SCHOLTEN, *Z. Naturforsch.* **226**, 677 (1967).
[3] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Naturwissenschaften* **54**, 226 (1967).
[4] E. CASPI and D. O. LEWIS, *Science* **156**, 519 (1967).



(III) 5β-Pregnane-3,20-dione



(IV) Progesterone

The major radioactive constituents of the light petroleum extract were unchanged pregnenolone (I), 5β-pregnane-3,20-dione (III), and progesterone (IV). The latter two were isolated and purified by preparative thin-layer chromatography (TLC), diluted with carrier material, and crystallized to constant specific activity (Tables 1 and 2). They were then reduced with sodium borohydride, to 5β-pregnane-3α,20β-diol and $\Delta^4$-pregnene-3β,20β-diol, respectively. These derivatives were isolated by preparative TLC and recrystallized to constant specific activity (Tables 1 and 2).

TABLE 1. PURIFICATION OF 5β-PREGNANE-3,20-DIONE*

| Compound | Solvent used for crystallization | Counts/ min/μmole† |
|---|---|---|
| 5β-Pregnane-3,20-dione | | 65·3 ± 4·0 |
| | Ether–light petroleum | 61·5 ± 4·0 |
| | Ether–light petroleum | 64·5 ± 5·0 |
| | Methanol–water | 55·3 ± 5·0 |
| | Methanol–water | 50·3 ± 4·0 |
| | Methanol–water | 48·6 ± 5·4 |
| 5β-Pregnane-3α,20β-diol | Ether–light petroleum | 46·2 ± 5·0 |
| | Methanol–water | 46·2 ± 5·0 |

* Aliquots of less than 0·2 mg were plated from dichloromethane solutions on ringed planchets over an area of 12·7 cm² and counted in duplicate on a Beckman Widebeta II instrument. Counter efficiency was 34 per cent and background was 3 counts/min.

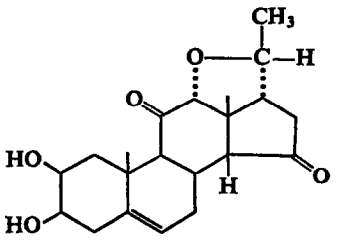
† 90 per cent confidence level. Corrected where necessary for dilution with additional carrier material.

TABLE 2. PURIFICATION OF PROGESTERONE*

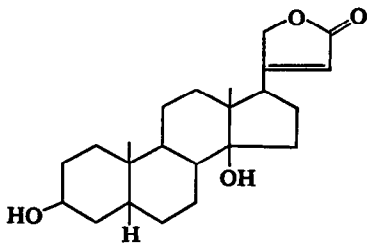
| Compound | Solvent used for crystallizations | Counts/ min/μmole |
|---|---|---|
| Progesterone | | 181 ± 5 |
| | Ether–light petroleum | 133 ± 7 |
| | Ether–light petroleum | 128 ± 10 |
| | Methanol–water | 122 ± 7 |
| $\Delta^4$-Pregnene-3β,20β-diol | Ether–light petroleum | 115 ± 7 |
| | Methanol–water | 118 ± 7 |

* Conditions as in Table 1.

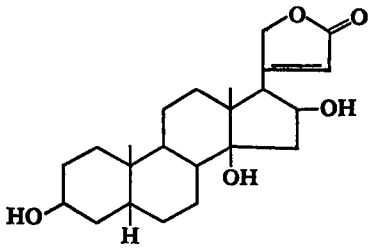
The chloroform extract was fractionated by chromatography on an alumina column. The less polar fractions, after acid hydrolysis, yielded more radioactive progesterone, 5β-pregnanedione and pregnenolone, as well as the digitanol digifologenin (V).[3] A radioactive component slightly less polar than pregnenolone was also observed. This material cochromatographed with a possible intermediate in cardenolide biosynthesis, 5β-pregnan-3β-ol-20-one. The latter was added as carrier, and the radioactive material was subjected to preparative TLC in two systems to separate it from pregnenolone. Final removal of the latter was achieved by reaction with *p*-nitroperbenzoic acid. 5β-Pregnan-3β-ol-20-one does not react with this reagent, but pregnenolone is converted to the more polar pregnenolone epoxide, which is easily separated by chromatography. Although during these purification steps the 5β-pregnanolone remained radioactive, it rapidly lost its activity when crystallization to constant specific activity was attempted.



(V) Digifologenin



(VI) Digitoxigenin



(VII) Gitoxigenin

The more polar fractions from the alumina column consisted mainly of radioactive digitoxin and gitoxin,[3] which yielded digitoxigenin (VI) and gitoxigenin (VII) on acid hydrolysis. Smaller amounts of the latter two cardenolides were also isolated from the chloroform–ethanol extract after mild acid hydrolysis. However, most of the radioactivity of this extract was not released by the hydrolytic conditions used, which cause cleavage of glycosides of 2-desoxysugars but not those of 2-oxysugars.

## DISCUSSION

Tschesche and Lilienweiss immersed isolated *Digitalis lanata* leaves in an aqueous solution of pregnenolone-21-$^{14}$C glucoside and observed incorporation of radioactivity into the cardenolides.[1] The present work demonstrates that foliar application[5] of free pregnenolone to intact plants is also suitable for biosynthetic experiments.

[5] R. D. Bennett and E. Heftmann, *Phytochem.* 4, 475 (1965).

Caspi and Lewis[4] suggested that the $\Delta^4$-3-ketone, progesterone, may be an intermediate in the conversion of the $\Delta^5$-double bond of pregnenolone to the saturated 5$\beta$-configuration of the cardenolides. Our present finding of 5$\beta$-pregnanedione as a metabolite of pregnenolone lends support to this theory.

Most of the radioactivity of the light petroleum and chloroform extracts was associated with unchanged pregnenolone and the five pregnenolone metabolites whose isolation we have reported here. In the chloroform–ethanol extract, however, glycosides of 2-oxysugars apparently accounted for most of the radioactivity. In view of the difficulties involved in the hydrolysis of such glycosides, this extract was not investigated further.

## EXPERIMENTAL

### *Methods*

Thin-layer chromatographic techniques were as described previously.[6] All chromatograms were run on Silica Gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details).

### *Materials*

Pregnenolone-4-$^{14}$C, having a specific activity of 56 $\mu$c/$\mu$M, was purchased from New England Nuclear Corporation. *Digitalis lanata* plants were raised from seeds obtained from the Harry E. Saier Seed Company, Dimondale, Michigan.

### *Administration of Pregnenolone*

Pregnenolone-4-$^{14}$C was administered in a dose of $7{\cdot}65 \times 10^6$ counts/min to the leaves of a *D. lanata* plant, 7 months old, by the technique previously described.[5] A total of nine such treatments were given, once a week.

### *Extraction and Fractionation*

One week after the last treatment, the plant was harvested above the soil line, frozen in liquid nitrogen, and lyophilized. The dried plant (94·8 g) was homogenized in a blender with 500 ml of water. The homogenate was covered with 5 ml of toluene and kept at 25° for 23 hr, to permit hydrolysis by the plant enzymes. After addition of 700 ml of ethanol, the mixture was heated at 60° for 1 hr and then filtered. The filter cake was extracted with 500 ml of 60 per cent ethanol and five 500-ml portions of 70 per cent ethanol. The combined extracts were concentrated in vacuum to 400 ml and thoroughly shaken with an ethanolic suspension of lead hydroxide[7] for 10 min. The mixture was filtered, and the filtrate was concentrated in vacuum to 300 ml, diluted with 900 ml of ethanol, and extracted with three 300-ml portions of light petroleum. The extracts were washed twice with 100 ml of water, combined, dried ($Na_2SO_4$), and evaporated.

The aqueous-alcoholic solution was concentrated in vacuum to 300 ml and extracted with four 400-ml portions of $CHCl_3$. The extracts were washed successively with 50 ml of water, two 50-ml portions of 2 N $Na_2CO_3$, and two 100-ml portions of water, after which they were combined, dried ($Na_2SO_4$), and evaporated.

The aqueous solution was then extracted with four 400-ml portions of chloroform–ethanol (2:1). The extracts were washed, dried, and evaporated in the same way as the chloroform extracts.

Light petroleum extract: 0·27 g, $1{\cdot}53 \times 10^5$ counts/min.

Chloroform extract: 0·96 g, $1{\cdot}08 \times 10^7$ counts/min.

Chloroform–ethanol extract: 2·77 g, $1{\cdot}72 \times 10^7$ counts/min.

### *Investigation of Light Petroleum Extract*

The extract was chromatographed on an 80-g column of Merck silica gel (0·05–0·2 mm), packed in dichloromethane. The earlier fractions from the column, eluted with 750 ml of dichloromethane–methanol (99:1) and 180 ml of dichloromethane–methanol (19:1), contained nonpolar material of low radioactivity. Elution with 100 ml of dichloromethane–methanol (9:1) then gave the major radioactive fraction (38·7 mg, $1{\cdot}05 \times 10^5$ counts/min). Later fractions consisted of highly polar material of low specific activity and were not investigated further.

* Reference to a company or product name does not imply approval or recommendation of the product by the U.S. Department of Agriculture to the exclusion of others that may be suitable.

[6] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **5**, 747 (1966).

[7] J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).

An aliquot of the major radioactive fraction was subjected to TLC with dichloromethane–methanol (49:1) and scanned for radioactivity. Peaks were observed corresponding to cochromatographed standards of 5β-pregnane-3,20-dione, progesterone, and pregnenolone. A fourth radioactive material near the origin was not identified. The entire fraction was then subjected to preparative TLC, and zones corresponding to the three standards cited above were removed and eluted.

The 5β-pregnanedione zone (9·3 mg, $1{\cdot}1 \times 10^4$ counts/min) was further purified by preparative TLC with cyclohexane–ethyl acetate (3:2) and then with chloroform–ethyl acetate (4:1), giving 1·3 mg, $5{\cdot}1 \times 10^3$ counts/min. This material was diluted with 20 mg of authentic 5β-pregnane-3,20-dione and crystallized to constant specific activity as shown in Table 1. More carrier was added as necessary. The material from the final crystallization (11·5 mg) was dissolved in 1 ml of ethanol, 15 mg of $NaBH_4$ were added, and the solution was kept at 25° for 22 hr. It was then diluted with 1 ml of water and acidified to pH 3 with 0·2 N $H_2SO_4$. The mixture was extracted with four 3-ml portions of dichloromethane, and the extracts were passed through 1 ml of 10% $KHCO_3$ and two 1-ml portions of $H_2O$. The combined extracts were evaporated, and the residue was subjected to preparative TLC with chloroform–methanol (24:1). The zone corresponding to 5β-pregnane-3α,20β-diol was removed and eluted (11·1 mg). This material was further crystallized as shown in Table 1.

The progesterone zone from above (5·0 mg, $1{\cdot}9 \times 10^4$ counts/min) was subjected to preparative TLC with cyclohexane–ethyl acetate (3:2), giving 2·0 mg, $1{\cdot}3 \times 10^4$ counts/min. This material was diluted with 16·8 mg of authentic progesterone and crystallized to constant specific activity as shown in Table 2. The material from the final crystallization was diluted with more carrier progesterone (total weight, 17·2 mg) and reduced with 26 mg of $NaBH_4$ as above, except that the reaction time was 4 hr. The product (17·2 mg) was subjected to preparative TLC with chloroform–methanol (24:1). The zone corresponding to $\Delta^4$-pregnene-3β,20β-diol was removed and eluted (13·5 mg). This material was further crystallized as shown in Table 2.

TLC of the pregnenolone zone from above, by developing three times with chloroform–methanol (99:1), showed that no radioactive 5β-pregnan-3β-ol-20-one was present.

*Investigation of Chloroform Extract*

The extract was chromatographed on a 30-g column of neutral alumina,* Grade II–III, packed in benzene. Fractions of 20 ml each were collected, using the following eluents: Fractions 1–2, 45% chloroform in benzene; 3–6, chloroform; 7–8, 1%; 9–10, 3%; 11–13, 5%; and 14–17, 8% methanol in chloroform.

Fractions 1–10 were combined (43 mg, $9{\cdot}42 \times 10^5$ counts/min) and refluxed with a mixture of 4 ml of methanol and 4 ml of 0·1 N $H_2SO_4$ for 25 min. After addition of 5 ml $H_2O$, the methanol was removed in vacuum, and the aqueous residue was extracted with four 15-ml portions of dichloromethane. The extracts were passed successively through 5 ml of water, 3 ml of 10% $KHCO_3$, and two 5-ml portions $H_2O$, combined, dried ($Na_2SO_4$), and evaporated, giving 30 mg, $8{\cdot}8 \times 10^5$ counts/min. TLC with chloroform–methanol (24:1) showed six radioactive peaks. Two of the three major peaks corresponded to standards of progesterone and pregnenolone, and the third was slightly more polar than pregnenolone. The entire fraction was then subjected to preparative TLC with the same system, and the zones corresponding to progesterone and pregnenolone were removed and eluted.

A radiochromatogram of the progesterone zone (14 mg, $3{\cdot}74 \times 10^5$ counts/min) with chloroform–ethyl acetate (4:1) showed a major peak corresponding to progesterone and two much smaller peaks, one of which corresponded to 5β-pregnane-3,20-dione.

TLC of the pregnenolone zone (9·7 mg, $1{\cdot}70 \times 10^5$ counts/min) with chloroform–methanol, 99:1, developed three times, and with chloroform–ethyl acetate (4:1) indicated the possible presence of radioactive 5β-pregnan-3β-ol-20-one. After addition of 1·5 mg of the latter as carrier, this material was subjected to preparative TLC, first with chloroform–ethyl acetate (4:1) and then with chloroform–isopropyl alcohol (19:1). In each case the 5β-pregnanolone zone was removed and eluted. The purified material (1·2 mg, $5{\cdot}0 \times 10^3$ counts/min) was freed of some remaining pregnenolone by treatment with 5 mg of *p*-nitroperbenzoic acid† in 2 ml of benzene–ether (1:1) for 1 hr at 25°. After addition of 2 ml of ether, the solution was washed with 4 ml of 10% $Na_2CO_3$ and four 4-ml portions $H_2O$. The aqueous washes were backwashed with two 4-ml portions of ether. The organic solutions were combined, dried ($Na_2SO_4$), and evaporated. The residue was subjected to preparative TLC with chloroform–ethyl acetate (4:1), and the 5β-pregnanolone, which was well separated from the more polar pregnenolone epoxide, was removed and eluted ($2{\cdot}4 \times 10^3$ counts/min). Carrier 5β-pregnanolone was added and crystallization to constant specific activity, with ether–light petroleum, was attempted. The specific activity decreased by 80 per cent in the first two crystallizations, which indicated that the radioactivity was not associated with 5β-pregnanolone.

Fractions 11–16 from the alumina column yielded radioactive digifologenin, digitoxin, and gitoxin, as described previously.[3]

* Woelm, Eschwege, Germany.

† K and K Laboratories, Hollywood, California.

*Investigation of Chloroform–Ethanol Extract*

A 200-mg portion of the extract was refluxed with a mixture of 5 ml of methanol and 5 ml of 0·1 N $H_2SO_4$ for 30 min. The methanol was removed in vacuum, and the residue was heated at 60° for 30 min. It was then extracted with four 15-ml portions of dichloromethane. The extracts were washed with 3 ml of 10% $KHCO_3$ and two 5-ml portions $H_2O$, combined dried ($Na_2$ $SO_4$), and evaporated, giving 12 mg. TLC showed that the radioactivity was almost entirely associated with digitoxigenin and gitoxigenin.

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