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PROGESTERONE METABOLISM IN *DIGITALIS LANATA*

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Abstract—After the administration of progesterone-4-^{14}C to a *Digitalis lanata* plant, the following radioactive metabolites were isolated: digitoxigenin, gitoxigenin, digoxigenin, 5α-pregnane-3,20-dione, 5β-pregnane-3,20-dione, 5α-pregnan-3β-ol-20-one, and Δ^5-pregnen-3β-ol-20-one. The results suggest that progesterone may be an intermediate in the biosynthesis of cardenolides from pregnenolone.

INTRODUCTION

IN OUR previous studies on steroid biosynthesis in *Digitalis lanata*,[1,2] we found the pregnenolone (I)-4-^{14}C was a precursor not only of the cardenolides but also of progesterone (II). It then became of interest to determine whether progesterone was also an intermediate in cardenolide biosynthesis. Accordingly, we have studied the metabolism of progesterone-4-^{14}C in a *D. lanata* plant.

While this work was in progress, a paper appeared by Caspi and Lewis[3] describing the conversion of pregnenolone-7α-^{3}H to progesterone, and of progesterone-7α-^{3}H to cardenolides, by excised *D. lanata* leaves. The results presented here confirm and extend their findings.

RESULTS

Progesterone-4-^{14}C was administered twice a week for 5 weeks to the leaves of a *D. lanata* plant. The plant was then worked up, and an extract containing the cardenolides, as well as nonpolar materials, was obtained by mild acid hydrolysis. The radioactivity of this extract represented 60 per cent of that originally administered to the plant. Further extraction of the aqueous layer from the hydrolysis gave a glycoside fraction, containing 18 per cent of the original activity.

The scheme used to fractionate the first extract is presented in Fig. 1. A preliminary separation by column chromatography on alumina gave a polar fraction, consisting mainly of the cardenolides, and a nonpolar fraction. The latter, after silica gel column chromatography, yielded radioactive components corresponding chromatographically to 5α-pregnane-3,20-dione (III), 5β-pregnane-3,20-dione (IV), progesterone, and pregnenolone.

* 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] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Phytochem.* **11**, 1521 (1967).
[2] H. H. SAUER, R. D. BENNETT and E. HEFTMANN, *Naturwissenschaften* **54**, 226 (1967).
[3] E. CASPI and D. O. LEWIS, *Science* **156**, 519 (1967).

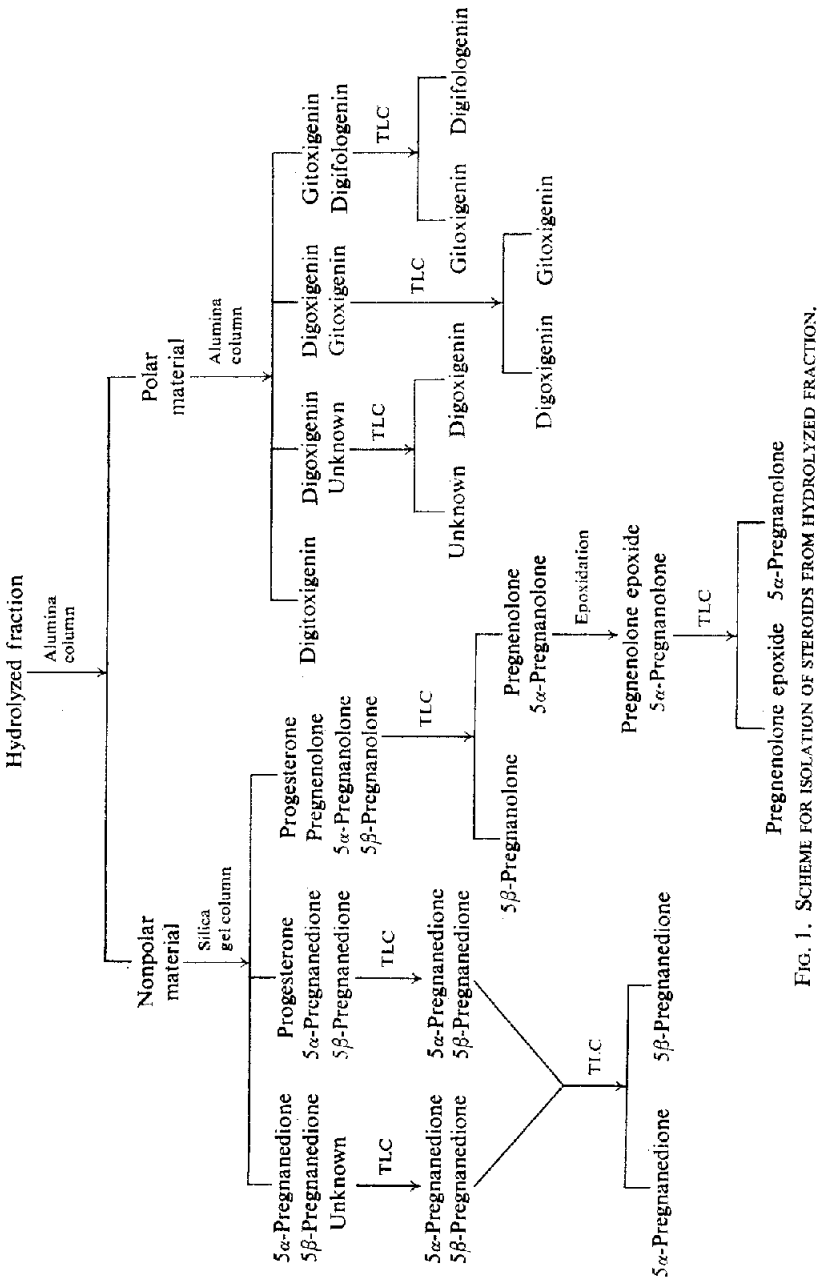


FIG. 1. SCHEME FOR ISOLATION OF STEROIDS FROM HYDROLYZED FRACTION.

The pregnanedione fraction was separated by preparative thin-layer chromatography (TLC) into its 5α-and 5β-components. Each was diluted with carrier material and crystallized to constant specific activity, after which they were reduced with sodium borohydride to 5α-pregnane-3β,20β-diol and 5β-pregnane-3α,20β-diol, respectively. These derivatives were isolated by preparative TLC and crystallized to constant specific activity (Table 1, A and B).

TABLE 1. RECRYSTALLIZATION OF STEROIDS FROM HYDROLYZED FRACTION TO CONSTANT SPECIFIC ACTIVITY*

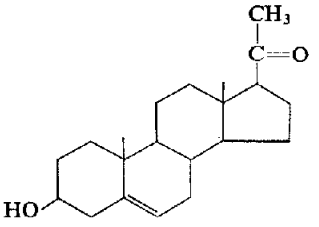
	Compound	Solvent used for crystallization	Counts/min/μMole†
A.	5α-Pregnane-3,20-dione		2,830 ± 70
		Dichloromethane-ether-light petroleum	2,850 ± 70
		Dichloromethane-methanol-water	2,800 ± 70
	5α-Pregnane-3β, 20β-diol	Hexane-acetone	2,550 ±110
		Hexane-acetone	2,590 ±110
		Methanol-water	2,530 ±110
B.	5β-Pregnane-3,20-dione		304 ± 10
		Ether-light petroleum	309 ± 13
		Methanol-water	304 ± 13
	5β-Pregnane-3α, 20β-diol	Ethyl acetate	300 ± 16
		Methanol-acetone	299 ± 16
C.	5α-Pregnan-3β-ol-20-one acetate		879 ± 42
		Hexane	897 ± 42
		Methanol	923 ± 42
	5α-Pregnan-3β-ol-20-one	Hexane-acetone	909 ± 44
		Methanol	944 ± 44
D.	Δ⁵-Pregnen-3β-ol-20-one epoxide acetate		32·3± 1·6
		Methanol	31·7± 1·6
		Hexane-acetone	30·9± 1·6
	Δ⁵-Pregnen-3β-ol-20-one epoxide	Hexane-acetone	30·2± 1·6
		Ethyl acetate	30·5± 1·6

* Portions of 0·2 mg or less were plated from solution 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 1·2 counts/min.

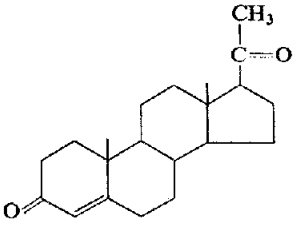
† 90 per cent confidence level.

The pregnenolone fraction contained a small amount of radioactive material corresponding chromatographically to 5β-pregnan-3β-ol-20-one (V). This was isolated by preparative TLC and was shown to have the same mobility as 5β-pregnanolone by TLC in three systems. When it was acetylated, however, about 99 per cent of the radioactivity was separated from 5β-pregnanolone acetate, added as carrier material, by continuous development TLC. Most of the remaining radioactivity was lost when crystallization to constant specific activity was attempted.

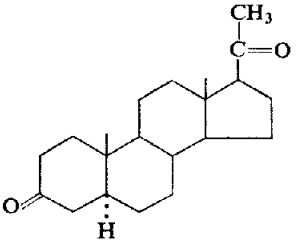
When a portion of the pregnenolone fraction was subjected to continuous development TLC to separate pregnenolone from 5α-pregnan-3β-ol-20-one (VI), it appeared that most of the radioactivity was associated with the latter. The separation between the two, however, was not sufficient for preparative TLC. Accordingly, it was necessary to convert pregnenolone to a derivative before isolating it and determining its radioactivity. This was accomplished by treatment of the fraction with *p*-nitroperbenzoic acid, which reacts with pregnenolone,



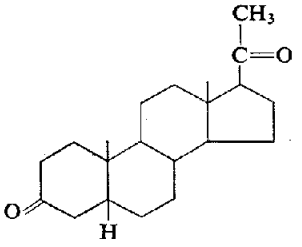
(I) Pregnenolone



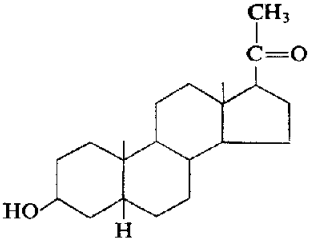
(II) Progesterone



(III) 5α-Pregnanedione



(IV) 5β-Pregnanedione



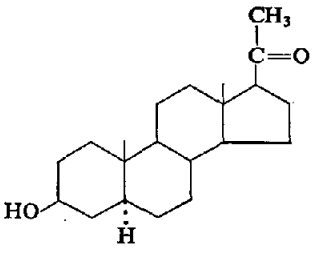
(V) 5β-Pregnanolone

but not with 5α-pregnanolone, to form an epoxide. TLC showed that most of the radioactivity of the product corresponded to 5α-pregnanolone, but a small peak had the same mobility as pregnenolone epoxide. The two were then separated by preparative TLC. The 5α-pregnanolone fraction, after acetylation, was diluted with carrier material, crystallized to constant specific activity, hydrolyzed to 5α-pregnanolone, and further crystallized (Table 1, C).

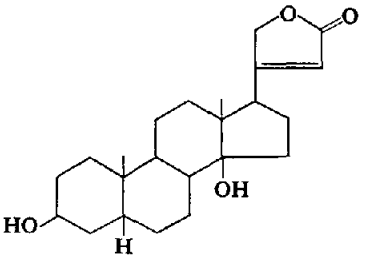
The pregnenolone epoxide fraction was acetylated, and pregnenolone epoxide acetate was isolated by preparative TLC. After addition of carrier material and crystallization to constant specific activity, it was hydrolyzed to pregnenolone epoxide and further crystallized (Table 1, D).

The polar fraction from the alumina column was rechromatographed on a column of the same adsorbent. The three cardenolides, digitoxigenin (VII), gitoxigenin (VIII), and digoxigenin (IX) were isolated from the column fractions and purified by preparative TLC. They were shown to be radiochemically pure by TLC and by crystallization to constant specific activity.

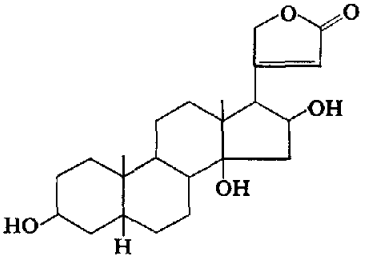
The column fractions also contained several other radioactive components, one of which corresponded chromatographically to digifologenin (X). This material, after isolation by preparative TLC, had the same mobility as digifologenin in two systems. It was then treated



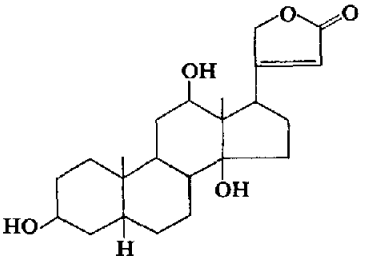
(VI) 5α-Pregnanolone



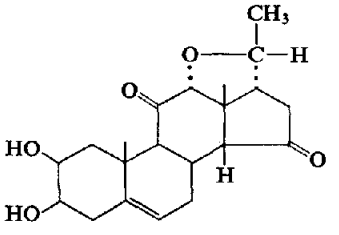
(VII) Digitoxigenin



(VIII) Gitoxigenin



(IX) Digoxigenin



(X) Digifologenin

with sodium borohydride, to give a mixture of reduction products. Although TLC showed zones corresponding to each of the reduction products of authentic digifologenin, none of them were significantly radioactive.

The total radioactivity of each of the steroids isolated from the chloroform extract is presented in Table 2. The values are based upon fractions which were radiochemically

TABLE 2. TOTAL RADIOACTIVITY OF INDIVIDUAL STEROIDS ISOLATED FROM HYDROLYZED FRACTION

Compounds	Counts/min × 10^{-3}	Per cent of original radioactivity
Digitoxigenin	4,600	7·5
Gitoxigenin	2,090	3·4
Digoxigenin	220	0·36
5α-Pregnanedione	263	0·43
5β-Pregnanedione	44	0·072
5α-Pregnanolone	282	0·46
5β-Pregnanolone	<0·2	<0·0006
Pregnenolone	4·6	0·0053

homogeneous by TLC. If the presence of radioactive impurities was demonstrated by a decrease in specific activity during crystallization, an appropriate correction was made.

The glycoside fraction was hydrolyzed by the Mannich-Siewert method.[4] Under the conditions used, the three cardenolides are partially decomposed to less polar products, probably anhydrogenins. TLC showed that all of the radioactive peaks of the hydrolyzate corresponded to the cardenolides or to their decomposition products. Gitoxigenin and digoxigenin were isolated by preparative TLC and shown to be radiochemically homogeneous by TLC.

DISCUSSION

Caspi and Lewis[3] tentatively suggested that progesterone may be an intermediate in the biosynthesis of cardenolides from pregnenolone. If the conversion of pregnenolone to progesterone previously observed in *D. lanata*[1,3] is reversible, it would also be conceivable that the biosynthesis of cardenolides from progesterone proceeds through pregnenolone. Our present finding that *D. lanata* can indeed convert progesterone to pregnenolone would tend to support this alternative, but the following considerations suggest that it is unlikely.

First, the rate of conversion of progesteron-4-^{14}C to the cardenolides was 11·3 per cent, as compared to the rate of 1·3 per cent determined for the incorporation of pregnenolone-4-^{14}C into the cardenolides under similar conditions.[1] This large difference in rates confirms the previous conclusion of Caspi and Lewis[3] that progesterone is a more efficient cardenolide precursor than pregnenolone.

Secondly, the yield of progesterone from pregnenolone-4-^{14}C was 0·4 per cent,[1] while that of pregnenolone from progesterone-4-^{14}C was only 0·0053 per cent. This suggests that the latter conversion may be of minor importance.

Finally, in our previous work pregnenolone-4-^{14}C was converted to digifologenin (X) in 0·1 per cent yield,[1,2] whereas the digifologenin present in the plant treated with progesterone-4-^{14}C was not significantly radioactive. If progesterone were being converted into pregnenolone at a rate sufficient to produce high yields of cardenolides from the latter, then digifologenin should also have been labelled.

Our results are consistent with the hypothesis of Caspi and Lewis[3] that, as in animals, the plant cannot directly saturate the Δ^5-double bond of pregnenolone but must first convert it to the Δ^4-3-keto system of progesterone. We found 5β-pregnane-3,20-dione (IV) as a metabolite both of pregnenolone[1] and progesterone in *D. lanata*, and this compound would be expected to follow progesterone in the biosynthesis of cardenolides if the above hypothesis is correct. On the other hand, digifologenin was formed from pregnenolone but not progesterone. Since the Δ^5-double bond of pregnenolone remains intact in digifologenin, progesterone would not be required as an intermediate in this case. Similarly, we have observed earlier[5] that pregnenolone, but not progesterone, is converted to the *Holarrhena* alkaloids, which are Δ^5-3-aminopregnene derivatives.

To our knowledge, the conversion of progesterone to pregnenolone has never been observed in animals. However, three of the radioactive compounds found in the present experiment, 5β-pregnanedione, 5α-pregnanedione, and 5α-pregnanolone are known to be metabolites of progesterone in animals.[6] It is unlikely that the latter two are involved in

[4] E. HEFTMANN, P. BERNER, A. L. HAYDEN, H. K. MILLER and E. MOSETTIG, *Arch. Biochem. Biophys.* **51**, 329 (1954).

[5] R. D. BENNETT and E. HEFTMANN, *Phytochemistry* **4**, 873 (1965).

[6] R. I. DORFMAN and F. UNGAR, *Metabolism of Steroid Hormones*, Academic Press, New York (1965).

cardenolide biosynthesis, since no 5α-cardenolides have been found in *D. lanata*. The saponins of this plant have the 5α-configuration, but they are C_{27} compounds and are biosynthesized from cholesterol.[7] As expected, they were found to be nonradioactive in the present work.

5α-Pregnanedione and 5α-pregnanolone have also been found as metabolites of progesterone in plant tissue cultures of several species, including *Digitalis purpurea*.[8] The latter tissue culture contained no cardenolides.

Little or no radioactivity was associated with 5β-pregnan-3β-ol-20-one (V), which might be expected to immediately follow 5β-pregnanedione (IV) in the biosynthetic pathway, and the radioactivity of the latter was low. These results could be due to a very rapid turnover of the 5β-pregnane derivatives or to transformations in other parts of the molecule, e.g. position 14, prior to reduction of the Δ^4-double bond or the 3-keto group.

As in our previous work,[1] a glycoside fraction was obtained which was not cleaved by the mild acidic conditions used for hydrolysis of glycosides of 2-desoxysugars. It was necessary to apply the more vigorous Mannich–Siewert method to hydrolyze this fraction. The results of the hydrolysis indicate that most of the radioactivity was associated with cardenolides. However, the latter are partially destroyed under these hydrolytic conditions, so that no quantitative estimate of their original radioactivity was possible. To our knowledge, this is the first indication that the cardiac glycosides in *D. lanata* may contain both 2-hydroxy- and 2-desoxysugars.

EXPERIMENTAL

Methods

Thin-layer chromatographic techniques were as described previously.[9] All chromatograms were run on Silica Gel G plates purchased from Analtech, Inc., Wilmington, Delaware.* For column chromatography, neutral alumina (Woelm, Eschwege, Germany) and silica gel, 0·05–0·2 mm (Brinkmann Instruments, Westbury, New York) were used. Aliquots of radioactive samples were counted on planchets at infinite thinness under a gas-flow detector (see Table 1, legend, for details).

Materials

Progesterone-4-^{14}C, having a specific activity of 57·3 μc/μM, was purchased from New England Nuclear Corporation. *Digitalis lanata* plants were raised from seeds supplied by the Harry E. Saier Seed Company, Dimondale, Michigan.

Administration of Progesterone

Progesterone-4^{14}C was administered in doses of $6{\cdot}14 \times 10^6$ counts/min to the leaves of a potted *D. lanata* plant, 6 months old, by the technique previously described.[10] A total of 10 such treatments were given, twice weekly.

Extraction and Fractionation

Two days after the last treatment, the plant was harvested above the soil line. The fresh plant material, weighing 129 g, was homogenized in a blender with 500 ml of water for 30 min, whereupon 500 ml of ethanol were added. The mixture was refluxed for 5 min and filtered. The filter cake was then extracted by boiling under reflux for 5 min with 500-ml portions of 50, 60, 70, 80, 90, and 100 per cent ethanol in succession. Each alcohol extract was filtered and the filtrates were combined, then concentrated under vacuum to 150 ml and extracted with four 200-ml portions of $CHCl_3$. The $CHCl_3$ extracts were washed successively with 50 ml of

* 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.

[7] R. Tschesche and H. Hulpke, *Z. Naturforsch.* **21b**, 494 (1966).
[8] J. M. H. Graves and W. K. Smith, *Nature* **214**, 1248 (1967).
[9] R. D. Bennett and E. Heftmann, *Phytochemistry* **5**, 747 (1966).
[10] R. D. Bennett and E. Heftmann, *Phytochemistry* **4**, 475 (1965).

water, 50 ml of 10 per cent $KHCO_3$, and 50 ml of water, filtered, combined, and evaporated. The residue was refluxed for 25 min with a mixture of 20 ml of 0·1 N H_2SO_4 and 20 ml of methanol. The methanol was removed under vacuum, and the aqueous residue was extracted with four × 50 ml $CHCl_3$. The $CHCl_3$ extracts were washed successively with 10 ml of water, 10 ml of 10 per cent $KHCO_3$, and 10 ml of water, filtered, combined, and evaporated. The residue was taken up in 60 ml of ethyl acetate and extracted with two 40-ml portions of 0·5 N NaOH and two 40-ml portions of water. The extracts were washed with 20 ml of ethyl acetate, combined, acidified with 12 N HCl, and extracted with three 80-ml portions of CH_2Cl_2. The CH_2Cl_2 extracts were washed with 40 ml of water, filtered, combined, and evaporated (Phenolic Fraction).

The aqueous layer from the first $CHCl_3$ extraction was acidified to pH 1 with 4 N H_2SO_4, diluted with an equal volume of methanol, and refluxed for 25 min. The methanol was removed under vacuum, and the aqueous residue was extracted with four 200-ml portions of $CHCl_3$. The $CHCl_3$ extracts were washed as before, combined with the two ethyl acetate extracts from above, and evaporated (Hydrolyzed Fraction).

The aqueous layer from the last hydrolysis was extracted with three 200-ml portions of $CHCl_3$-ethanol (2:1). The extracts were washed successively as before, filtered, combined, and evaporated (Glycoside Fraction).

Phenolic Fraction: 0·35 g, $1·87 \times 10^6$ counts/min

Hydrolyzed Fraction: 1·98 g, $3·71 \times 10^7$ counts/min

Glycoside Fraction: 0·50 g, $1·10 \times 10^7$ counts/min

Phenolic Fraction

A portion of the phenolic fraction was subjected to TLC with dichloromethane–methanol (23:2) and scanned for radioactivity. The major peak was at the origin, but a smaller peak was observed corresponding in mobility to estriol, a phenolic steroid estrogen. The whole fraction was then chromatographed on a 100-g column of silica gel, which was eluted with dichloromethane–methanol (17:3). The first 200 ml of eluate was discarded. The next 200 ml contained the radioactive material corresponding to estriol (49 mg, $8·97 \times 10^5$ counts/min). This was freed of some less polar radioactive material by preparative TLC with dichloromethane–methanol (22:3), giving 12 mg, $5·36 \times 10^5$ counts/min. When a portion of this material was subjected to TLC with cyclohexane–ethyl acetate (1:9), however, three principal radioactive peaks were observed. All of these radioactive substances ran slower than estriol and none of them had mobilities corresponding to the steroidal estrogens available to us as reference material.

Hydrolyzed Fraction

The hydrolyzed fraction was chromatographed on a 60-g column of alumina, Grade III. Fractions of 400 ml each were collected with the following eluents: 1, 50 per cent benzene in hexane; 2, benzene; 3, 50 per cent $CHCl_3$ in benzene; 4, $CHCl_3$; 5, 5 per cent; and 6, 10 per cent methanol in $CHCl_3$.

Fraction 1 contained 1·18 g of material, but the radioactivity was only $2·2 \times 10^5$ counts/min. This was not investigated further. Fractions 2–4, which contained nonpolar material, were combined (148 mg, $1·28 \times 10^7$ counts/min) and chromatographed on a 140-g column of silica gel, packed as a slurry in CH_2Cl_2–methanol (19:1). Fractions of 10 ml each were collected with this eluent.

Fractions 1–24, which contained material of low specific activity, were discarded. Fractions 25–28 (41 mg, $6·84 \times 10^5$ counts/min) contained, as the major radioactive components, a mixture of 5α-pregnanedione and 5β-pregnanedione. This mixture was isolated by preparative TLC with dichloromethane–methanol (99:1), which yielded 10 mg, $4·20 \times 10^5$ counts/min. Progesterone accounted for most of the radioactivity of fractions 29–32 (26 mg, $8·45 \times 10^6$ counts/min), but they also contained a small amount of the pregnanedione mixture, which was separated by preparative TLC, as above, and weighed 7·2 mg ($5·0 \times 10^4$ counts/min). The two pregnanedione fractions were combined, and 2·5 mg each of authentic 5α-pregnanedione and 5β-pregnanedione were added as carriers. The isomers were separated by preparative TLC with cyclohexane–ethyl acetate (17:3, continuous development[11] for 6 hr).

The less polar 5α-pregnanedione (2·7 mg, $2·91 \times 10^5$ counts/min), which was radiochemically homogenous by TLC in two systems, chloroform–ethyl acetate (4:1) and dichloromethane–methanol (49:1), was diluted with 28·5 mg of carrier material and crystallized (Table 1). The crystals and mother liquors were then combined, dissolved in 2 ml of ethanol, and treated with 30 mg of $NaBH_4$ for 22 hr at 25°. The solution was acidified to pH 3 with 2 N HCl, diluted with an equal volume of water, and extracted with five 6-ml portions of CH_2Cl_2. The extracts were washed successively with 3-ml portions of water, 2 N Na_2CO_3, and water twice more, dried (Na_2SO_4), and evaporated. The residue was subjected to preparative TLC with cyclohexane–ethyl acetate (2:3), and the zone corresponding in mobility to authentic 5α-pregnane-3β, 20β-diol was removed and eluted. This gave 17 mg of material, which was crystallized as shown in Table 1.

The 5β-pregnanedione (2·8 mg, $4·4 \times 10^4$ counts/min), which was homogeneous in the same TLC systems as used for the 5α-isomer, was diluted with 27 mg of carrier material and crystallized (Table 1). The crystals and mother liquor were combined and reduced with $NaBH_4$ as above. The main product, 5β-pregnane-

[11] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **12**, 245 (1963).

3α,20β-diol, was isolated by preparative TLC with cyclohexane–ethyl acetate (2:3). This gave 13 mg of material, which was further crystallized (Table 1).

TLC indicated that fractions 33–36 from the silica gel column above (31 mg, $3{\cdot}26\times10^6$ counts/min) contained progesterone, pregnenolone, and an unidentified, more polar compound as the major radioactive components. This fraction, with 0·5 mg of pregnenolone as carrier, was subjected to preparative TLC with cyclohexane–ethyl acetate (1:1), and a zone containing progesterone and pregnenolone, which do not separate in this system, was removed and eluted. TLC of this material (10 mg, $1{\cdot}17\times10^6$ counts/min) with CH_2Cl_2–methanol (97:3) showed two major peaks, corresponding to progesterone and pregnenolone, and a minor peak which had the same mobility as 5β-pregnan-3β-ol-20-one. By preparative TLC in the same system, the pregnenolone and 5β-pregnanolone zones were isolated.

The latter material (0·4 mg, $9{\cdot}5\times10^4$ counts/min) had the same mobility as authentic 5β-pregnanolone in two TLC systems, cyclohexane–ethyl acetate (1:1) and CH_2Cl_2–acetone (9:1). It was acetylated (acetic anhydride–pyridine, 1:1, 16 hr, 25°), after the addition of 50 μg of 5β-pregnanolone as carrier. The acetate showed only a single peak, corresponding to 5β-pregnanolone acetate, by TLC with dichloromethane–acetone (99:1). However, continuous development TLC with dichloromethane–hexane (4:1) separated most of the radioactivity from the carrier. The 5β-pregnanolone acetate, when isolated by preparative TLC in the same system, gave 0·1 mg, 700 counts/min. This material was diluted with 15 mg of carrier, and crystallization to constant specific activity from methanol was attempted. The specific activity decreased by 60 per cent in the first crystallization, and the low activity remaining made further crystallization impractical.

The pregnenolone zone from above (0·5 mg, $3{\cdot}29\times10^5$ counts/min) was examined by continuous development TLC with benzene–methanol (99:1, 5 hr). This indicated that most of the radioactivity was associated not with pregnenolone, but with the slightly more polar 5α-pregnan-3β-ol-20-one. A portion ($1{\cdot}00\times10^5$ counts/min) of the whole fraction was then combined with 1 mg each of carrier pregnenolone and 5α-pregnanolone, and treated with 10 mg of *p*-nitroperbenzoic acid* in 1 ml of ether and 1 ml of benzene for 1 hr at 25°. The solution was washed with 1 ml of 10 per cent Na_2CO_3, which was backwashed with 1 ml of ether. The organic layers were combined and evaporated, and the residue was subjected to preparative TLC with CH_2Cl_2–acetone (9:1) to separate 5α-pregnanolone from the more polar pregnenolone epoxide.

The 5α-pregnanolone ($8{\cdot}58\times10^4$ counts/min) was acetylated as above, and the acetate was shown to be radiochemically homogeneous by TLC with dichloromethane–acetone (99:1). After dilution with 25 mg of 5α-pregnanolone acetate, it was crystallized twice as shown in Table 1. The crystalline material was refluxed with 1·5 ml of 0·1 N NaOH in 80 per cent methanol for 15 min. Then 0·5 ml of water was added and the methanol was removed by azeotropic distillation with benzene. The benzene layer was separated, and the aqueous layer was extracted with two 1-ml portions of benzene. The benzene solutions were combined and evaporated, and the residue was further crystallized (Table 1).

The pregnenolone epoxide fraction ($8{\cdot}74\times10^3$ counts/min), which was shown to be radiochemically homogeneous by TLC with dichloromethane–methanol (19:1) and cyclohexane–ethyl acetate (2:3), was acetylated as above. The acetate was isolated by preparative TLC with dichloromethane–acetone (97:3), which gave 0·6 mg, $1{\cdot}62\times10^3$ counts/min. This material was diluted with 15·6 mg of authentic pregnenolone epoxide acetate and crystallized (Table 1). The crystals were combined with the mother liquors and hydrolyzed with 0·1 N NaOH, as above, to give pregnenolone epoxide, which was further crystallized (Table 1).

Fractions 5 and 6 from the alumina column were combined (201 mg, $2{\cdot}00\times10^7$ counts/min) and chromatographed on an 8-g column of alumina, Grade II. Fractions of 26 ml each were collected with the following eluents: *1–3*, $CHCl_3$; *4–7*, 0·2 per cent; *8–11*, 0·4 per cent; *12–15*, 0·6 per cent; *16–19*, 2 per cent; *20–23*, 5 per cent; and *24–26*, 9 per cent methanol in $CHCl_3$.

Digitoxigenin was found in Fractions 7–10 and was isolated by preparative TLC with $CHCl_3$–isopropyl alcohol (9:1). This gave 16·2 mg of material, $4{\cdot}60\times10^6$ counts/min, which was shown to be radiochemically pure by TLC with ethyl acetate and with CH_2Cl_2–acetone (4:1). After dilution with carrier material, the specific activity was not changed by crystallization from ether–light petroleum.

Fractions 14–15 contained digoxigenin and a slightly less polar, unknown compound as the major radioactive constituents. They were separated by preparative TLC with ethyl acetate. The unknown material (6·6 mg, $3{\cdot}70\times10^5$ counts/min) absorbed short-wave u.v. light on TLC plates like a Δ^4-3-ketone. It was therefore compared chromatographically to several oxygenated progesterone derivatives. It was similar in mobility to trihydroxy derivatives of progesterone but was not identical with any of the reference compounds available to us.

The digoxigenin (2·4 mg, $3{\cdot}76\times10^5$ counts/min) was further purified by preparative TLC with $CHCl_3$–isopropyl alcohol (9:1). This gave 2·3 mg of material, $1{\cdot}73\times10^5$ counts/min, which was homogeneous by TLC with CH_2Cl_2–acetone (4:1). It was diluted with carrier digoxigenin and recrystallized from methanol–ether–light petroleum without change in specific activity.

Fraction 16 contained a mixture of digoxigenin and gitoxigenin, which was resolved by preparative TLC, first with ethyl acetate and then with $CHCl_3$–isopropyl alcohol (9:1). Both the digoxigenin (1·3 mg, $4{\cdot}7\times10^4$

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counts/min) and the gitoxigenin (2·7 mg, $3·94 \times 10^5$ counts/min) showed only a single peak when subjected to TLC with $CHCl_3$–acetone (4:1).

Fractions 17–20 were combined and subjected to preparative TLC with $CHCl_3$–isopropyl alcohol (9:1). Zones corresponding to digifologenin and gitoxigenin were removed and eluted. The digifologenin fraction (7·1 mg, $1·36 \times 10^5$ counts/min) was then further purified by TLC with ethyl acetate. This gave 1·8 mg of material, $8·2 \times 10^3$ counts/min, which was treated with 20 mg of $NaBH_4$, as above. TLC of the reduction product with $CHCl_3$–methanol (19:1, continuous development[12] for 3 hr) showed four zones which corresponded in mobilities to the four reduction products of authentic digifologenin. However, the major radioactive peak was located between two of these zones, and none of them appeared to be significantly radioactive.

The gitoxigenin fraction (9·8 mg, $1·70 \times 10^6$ counts/min) from Fractions 17–20 was homogeneous in TLC with ethyl acetate and with $CHCl_3$–acetone (4:1). It was combined with the gitoxigenin from Fraction 16. Upon crystallization from hexane-acetone, no loss of specific activity was observed.

Glycoside Fraction

A portion of the glycoside fraction was subjected to the mild acid hydrolysis used previously (0·05 N H_2SO_4, 25 min). Most of the radioactivity could not be extracted with $CHCl_3$, indicating that no hydrolysis had occurred. A second portion was then refluxed for 2 hr with 3 N HCl. Almost all of the radioactivity could then be recovered by chloroform extraction. TLC with cyclohexane–ethyl acetate (1:1) and with dichloromethane–methanol (97:3) showed that the sapogenins gitogenin and digitogenin, were the major constituents of the extract, but that they were not significantly radioactive. The two major radioactive peaks were located at the origin and at about $R_f = 0·5$. The latter material corresponded in mobility to the major product(s) formed by heating a mixture of digitoxin, gitoxin, and digoxin with 3 N HCl for 2 hr.

Another portion ($1·12 \times 10^6$ counts/min) of the glycoside fraction was treated with 40 μl of conc HCl in 4·0 ml of acetone for 2 days at 25°. The acetone was then evaporated under N_2 at 25°. The residue was taken up in 10 ml of water and extracted with three 20-ml portions of $CHCl_3$. The extracts were washed successively with 5 ml of water, 5 ml of 10 per cent $KHCO_3$, and 5 ml of water, filtered, and combined. Evaporation gave a residue of 17·8 mg, $7·98 \times 10^5$ counts/min.

Separate samples of digitoxin, gitoxin, and digoxin were subjected to the same hydrolytic conditions, and the products were examined by TLC with CH_2Cl_2-methanol, 23:2. The glycosides gave products with the following R_f values:

Digitoxin: 0·34 (digitoxigenin), 0·58

Gitoxin: 0·23 (gitoxigenin), 0·34, 0·52

Digoxin: 0·18 (digoxigenin), 0·28, 0·33

A portion of the hydrolyzate of the glycoside fraction was subjected to TLC in the same system and scanned. The major peak was located at R_f 0·34 and thus could have been due to digitoxigenin and/or decomposition products of gitoxigenin and digoxigenin. Peaks were also observed at R_f 0·50–0·60, 0·28, 0·23, and 0·18.

A portion ($5·80 \times 10^5$ counts/min) of the hydrolyzate was subjected to preparative TLC in the same system, and the zones corresponding to gitoxigenin and digoxigenin were removed and eluted. Each was further purified by preparative TLC with ethyl acetate, giving 0·4 mg of gitoxigenin ($1·6 \times 10^4$ counts/min) and 0·2 mg of digoxigenin ($4·7 \times 10^3$ counts/min). Both were homogeneous by TLC with dichloromethane–acetone (4:1). No attempt was made to isolate radioactive digitoxigenin, because of difficulties in separating decomposition products of the other two genins from it.

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[12] R. D. BENNETT and E. HEFTMANN, *J. Chromatog.* **21**, 488 (1966).