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BIOSYNTHESIS OF PLANT STEROLS—IV.

AN INVESTIGATION OF A POSSIBLE MODE OF 14β-HYDROXYLATION IN DIGITOXIGENIN[1a,b]

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Abstract—Hypothetical biosynthetic routes for the introduction of 14β-hydroxyls into C/D trans (14α-H) steroids are discussed. Several theoretical possibilities based on established chemical processes, which are susceptible to experimental evaluation, are forwarded. In the light of these hypotheses the role of 14α-hydroxy progesterone, one of the theoretically possible intermediates in the biosynthesis of cardenolides, was explored. It was shown that under the experimental conditions employed the 1-^{3}H-14α-hydroxy progesterone was not incorporated into digitoxigenin in *Digitalis lanata*. The synthesis of the tritiated precursor is described and it was proved that *ca.* 80 per cent of the tritium had the 1α-stereochemistry and a minimum of *ca.* 15 per cent had the 1β-stereochemistry.

ONE of the characteristic features of the cardenolide molecules is the presence of an hydroxyl group at C-14 having the β configuration. This is of special interest since hydroxylation of steroids at non-vinylogous carbon atoms occurs by a direct displacement of a hydrogen; the incoming hydroxyl assuming the stereochemistry of the removed proton.[3]

Evidence gathered in recent years indicates that cholesterol,[4] pregnenolone[5,6] and progesterone,[6] all of which have a trans junction between rings C and D (14α-H), are incorporated into cardenolides. Hence if the hydroxylation proceeds via the known mechanism a product having a 14α-hydroxyl, instead of the 14β-hydroxyl, would be expected. Frequently the contradiction is circumvented by invoking a new, yet unknown, mechanism of this hydroxylation. In such instances an ionic reaction similar in principle to a Walden inversion is tacitly inferred. Postulating novel hypotheses can be fruitful in solving problems, provided the hypotheses are amenable to experimental scrutiny. Though it is not unreasonable to assume a Walden-type mechanism for the 14β-hydroxylation, a critical evaluation of the concept is difficult and at least at present the idea cannot be properly appraised. It is conceivable that eventually the actual mechanism will be proved or disproved by the elimination of *other posibilities* and/or by studies with the pure 14β-hydroxylase.

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[1b] The results were incorporated in a paper entitled "Biosynthesis of Plant Sterols" given at the Symposium "Uber Biochemische Aspekte Der Steroidforschung" of the Deutsche Akademie Der Wissenschaften zu Berlin, held in Jena (D.D.R.) September 18–21 (1967).

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[3] M. HAYANO, in *Oxygenases* (edited by O. HAYAISHI), p. 182. Academic Press, N.Y. (1962).

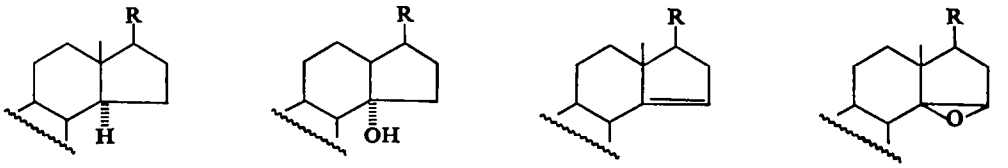
[4] E. CASPI, D. O. LEWIS, D. M. PIATAK, K. V. THIMANN and A. WINTER, *Experientia* **22**, 506 (1966).

[5] R. TSCHESCHE and G. LILIENWEISS. *Z. Naturforsch.* **19b**, 226 (1964); R. TSCHESCHE and B. BRASSAT, *ibid.* **20b**, 894 (1966).

[6] E. CASPI and D. O. LEWIS, *Science* **156**, 519 (1967).

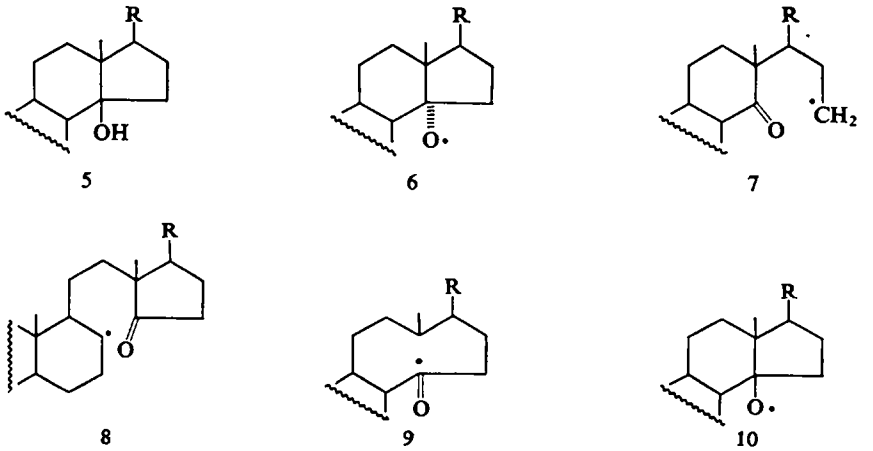
Because of the limitations of the experimental evaluation of the concept of the "14β-hydroxylase," we have considered several alternative possibilities of explaining the reaction. The approach we chose was to attempt to explain the reaction on the basis of acceptable chemical transformations which are amenable to testing. Our assumption was that a 14α-H-steroidal precursor of cardenolides could undergo a normal hydroxylation and then this hydroxyl could be transposed into a 14β-alcohol. To illustrate the point, several such possibilities will be mentioned.

One might first assume that the sequence of events is initiated by the displacement of the 14α-hydrogen in **1** and formation of a 14α-hydroxy intermediate (**2**). The feasibility of this reaction is supported by the isolation of 14α-hydroxylated steroids from plant sources.[7,8] The tertiary alcohol (**2**) on dehydration could give the 14,15 olefin (**3**) which upon β-face oxidation would yield a 14β,15β-epoxide (**4**). Again the presence of a 14β,15β-epoxy moiety in bufadienolides lends some indirect support for this view. Finally reductive opening of the epoxide (**4**) by an attack at C-15 could provide the 14β-alcohol. If the dehydration of **2** yields the 8,14 double bond (not shown in the flow sheet) instead of 14(15), this would not alter the principle of the concept. The olefin could give an 8β,14β epoxide which upon reductive attack at C-8 will yield the 14β-alcohol.



1 2 3 4

The transformation of the 14α-alcohol does not necessarily have to be ionic. In essence, a free radical process is equally possible, particularly in view of the fact that the biosynthesis of cardenolides occurs in the leaves.[5,6] Under the influence of sunlight, the 14α-alcohol could give an oxy-radical (**6**). Such radicals are known to rearrange into keto radicals[9] of the type **7**, **8** and **9**. Reclosure of the ring may proceed with inversion to yield the 14β-oxy radical (**10**)



5 6 7

8 9 10

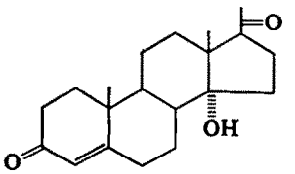
[7] K. NAKANISHI, M. KOREEDA, S. SASAKI, M. L. CHANG and H. Y. HSU, *Chem. Commun.* 915 (1966).
[8] M. N. GALBRAITH and D. H. S. HORN, *Chem. Commun.* 905 (1966).
[9] K. HEUSLER, J. KALVODA, G. ANNER and A. WETTSTEIN, *Helv. Chim. Acta* **46**, 352 (1963).

and finally the alcohol (**5**). The operation of this type of mechanism was demonstrated by Heusler *et al.*[9] in the free radical transformations of 11β- and 4β-hydroxy steroids of the 5α-H series. On treatment with lead tetraacetate the 11β-alcohol gave the 1β,11α-oxido-5α-H product while the 4β-alcohol provided the 4α,9α-oxido-5β-H compound. For the present argument, it is immaterial which bond is actually broken and which keto radical (**7**, **8** or **9**) is formed. All three intermediates could reclose to **10** and eventually give **5**.

The initial hydroxylation does not have to be limited to carbon 14 and may just as well take place at C-15, 8, etc. Such hydroxy compounds could be transformed into the 14β-alcohols either via an olefin or a radical mechanism similar to those described.

We considered it worth while to explore several such possibilities and we first tested the role, if any, of 14α-hydroxy progesterone in the biosynthesis of cardenolides. The projected route was to administer simultaneously 4-^{14}C-progesterone and 1-^{3}H-14α-hydroxy progesterone to a *Digitalis lanata* plant and to establish whether both isotopes or only ^{14}C is incorporated into digitoxigenin. The experimental design was based on the observation that the progesterone which is incorporated into cardiac aglycones (*ca.* 2 per cent) will serve as an internal control.

The initial problem with which we were faced was the preparation of 1-^{3}H-14α-hydroxy progesterone. A convenient starting material was 14α-hydroxy progesterone[10] (**11**) which



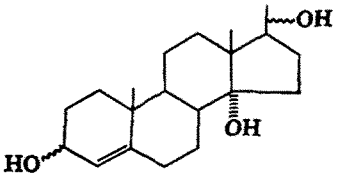
11

could be dehydrogenated with 2,3-dichloro-5,6-dicyanobenzoquinone (D.D.Q.) to the 1,4-dien-3-one. Selective catalytic tritiation would then lead to the 1,2-ditritiated product. To avoid possible interpretational complications, it was considered advisable to remove the labile tritium from C-2 and it was clear that under the condition of base catalyzed equilibration partial epimerization at C-17 would take place. Consequently the approach was slightly modified in that the 14α-hydroxy progesterone (**11**) was first reduced with sodium borohydride to **12** and then the C-3 allylic moiety was reoxidized with D.D.Q. to yield **13a**. The acetate (**13b**) was transformed to the dien-one (**14**) which was then selectively tritiated in the presence of tris-triphenylphosphine rhodium chloride.[11] The C-2 tritium was removed by equilibration with aqueous methanolic potassium hydroxide and the resulting compound (**16**) was rigorously purified by thin-layer and paper chromatography. The purified **16**, which gave a single radioactive zone on TLC, was oxidized (chromium trioxide-pyridine) to **17**. The 1-^{3}H-14α-hydroxy progesterone gave a single radioactive spot on TLC and the specific activity of a sample diluted with cold material remained unchanged in several crystallizations (see Experimental part).

Though by this criteria the product (**17**) appeared homogeneous, it was considered desirable to test further its purity by preparing a derivative. Consequently a diluted sample of **17** ($3{\cdot}86 \times 10^{6}$ dpm/m-mole) was converted to the 14-dehydrodiketal (**18**) by treatment

[10] We thank Dr. J. A. Hogg, The Upjohn Co., Kalamazoo, Michigan, for a generous gift of this compound.
[11] A. J. BIRCH and K. A. M. WALKER, *J. Chem. Soc.* C 1894 (1966).

with ethylene glycol and *p*-toluenesulfonic acid. At this point, rather unexpectedly, **18** showed a 10 per cent increase in specific activity (4.24×10^6 dpm/m-mole). Conversion of the diketal (**18**) to the monoketal (**19**) did not further change the specific activity. A comparable increase in specific activity (14 per cent) was observed when **17** ($6{\cdot}87 \times 10^6$ dpm/m-mole) was treated with chloranil to yield the 4,6-dien-3-one[12] (**20**) ($7{\cdot}83 \times 10^6$ dpm/m-mole).



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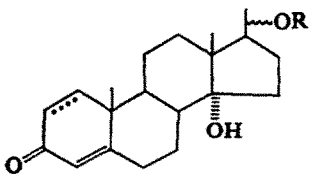
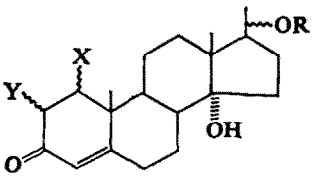
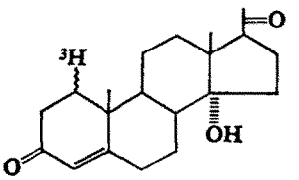


13a; R=H
b; R=Ac
14Δ^1; R=Ac



15 X=^{3}H; Y=^{3}H; R=Ac
16 X=^{3}H; Y=H; R=H



17

In view of these results the 1-^{3}H-14α-hydroxy progesterone (**17**) was further purified by TLC but the specific activity of diluted samples remained unchanged. Consequently the observations were viewed as involving retained solvent (of crystallization) in **17** and this product was used in the biosynthetic investigations.

To determine the sterochemistry of the isotopic hydrogen at C-1 two sets of experiments were performed. Ringold *et al.*[13] have shown that introduction of a C-1 double bond by dehydrogenation of a 3-ketone with D.D.Q. proceeds via the trans diaxial elimination of the 1α and 2β hydrogens. The 1,4,6-trien-3-ones are known to rearrange in the presence of acids to 1-methyl-3-hydroxy benzenes.[14,15] This rearrangement proceeds by the migration of the C-10 methyl to C-1 and elimination of the C-1 hydrogen.[14–16]

Treatment of the 4,6-dien-3,20-dione (**20**) with D.D.Q. in refluxing dioxan yielded the 1,4,6-trien-3,20-dione (**21**) which retained 22 per cent of tritium. On the other hand when the trien-dione (**21**) was exposed to acetic anhydride and *p*-toluenesulfonic acid a mixture of aromatic products was formed as evidenced by TLC and NMR spectroscopy. The formation in this instance of a mixture of aromatic products can probably be traced to the 14α-alcohol. Presumably in addition to the $\Delta^{8,14}$- and Δ^{15}-aromatic products, the 14α-acetoxy and other phenols were formed. In any event the aromatic mixture retained *ca.* 5·5 per cent of the tritium initially present in **20**. Based on these results it may be concluded that *ca.* 78–80 per cent of

[12] E. J. AGNELLO and G. D. LAUBACH, *J. Am. Chem. Soc.* **82**, 4293 (1960); **79**, 1257 (1959).
[13] H. J. RINGOLD and A. TURNER, *Chem. & Ind.* 211 (1962); H. J. RINGOLD, M. HAYANO and V. STEFANOVIC, *J. Biol. Chem.* **238**, 1960 (1963).
[14] E. CASPI, P. K. GROVER and Y. SHIMIZU, *J. Am. Chem. Soc.* **86**, 2463 (1964).
[15] E. CASPI, *Rev. Port. Quimica* **6**, 145 (1964).
[16] H. J. BRODIE, M. HAYANO and M. GUT, *J. Am. Chem. Soc.* **84**, 3766 (1962).

the tritium in 17 has the 1α-stereochemistry and a minimum of *ca.* 15 per cent has the 1β-orientation.

A mixture of the 1-^{3}H-14α-hydroxy progesterone (154 μc) and 4-^{14}C-progesterone (18·9 μc) (^{3}H/^{14}C ratio 8·1) was then administered to a *D. lanata* plant.[6,17] After 2 weeks the plant was harvested and processed as previously described.[6,18] The distribution of the tracers in the various fractions is summarized in Table 1. It is evident that the ^{3}H/^{14}C ratios of the ligroin and chloroform extracts approach that of the administered mixture of "precursors". On the other hand, proportionally much larger amounts of tritiated products were present in the remaining fractions. The fact that considerably larger amounts of tritiated materials were found in the washings of the leaf could indicate a "better" absorption of the 4-^{14}C progesterone or its transformation products. Processing of the plant led to the net recovery of *ca.* 40·2 and 32·5 per cent of the administered tritium and ^{14}C respectively.

TABLE 1. RADIOACTIVITY IN THE FRACTIONS OBTAINED BY PROCESSING* OF THE *Digitalis lanata* PLANT AFTER THE APPLICATION OF 1-^{3}H-14α-HYDROXY PROGESTERONE (154 μc) AND 4-^{14}C-PROGESTERONE (18·9 μc) (^{3}H/^{14}C 8·1)

Extract	^{3}H (× 10^{7} dpm)	^{14}C (× 10^{6} dpm)	^{3}H/^{14}C ratio
Leaf washings (unabsorbed radioact.)	3·49	1·17	30·0
Ligroin	0·78	0·20	8·8
Chloroform	7·17	9·85	7·29
Chloroform/ethanol (2:1)	2·62	1·18	22·2
Chloroform/ethanol (3:2)	2·4	2·02	11·9
Corrected recovery† (%)	40·2	32·5	
Acid hydrolysis of chloroform extract*	6·67	9·34	7·14

* For the description of the fractions see Refs. 6 and 18.
† Corrected for unabsorbed radioactivity found in the leaf washings.

As in the previous instance the isolation of digitoxigenin was carried out on the chloroform extract.[6] Acid hydrolysis of the glycosides (^{3}H/^{14}C 7·29) in this fraction did not influence the ^{3}H/^{14}C ratio of the aglycones (7·14). On the other hand, upon chromatographic fractionation of the aglycones, the ^{3}H/^{14}C ratio of the products in the "digitoxigenin" zone fell off rapidly (Table 2). After four chromatographic separations a small amount of tritiated material was still associated with the area corresponding to digitoxigenin (^{3}H/^{14}C ratio 0·45). The triated impurity was finally removed by co-crystallization with cold material whereby only ^{14}C-digitoxigenin was obtained. If the reasonable assumption is made that the ^{14}C recovered from the last chromatography was indeed incorporated into digitoxigenin then *ca.* 1·88 per cent of the absorbed 4-^{14}C-progesterone was metabolized to digitoxigenin. The usual transformation of progesterone into digitoxigenin is of the order of 2 per cent. Hence it may be concluded that as far as progesterone is concerned its metabolism was not impaired.

In analyzing the present results certain inherent fundamental limitations must be kept in mind. From the outset it was obvious that the two different exogeneously supplied steroids will be absorbed, transported and metabolized at different rates. Burstein *et al.*[19] provided

[17] R. D. BENNETT and E. HEFTMANN, *Phytochem.* **4**, 475 (1965).
[18] J. VON EUW and T. REICHSTEIN, *Helv. Chim. Acta* **47**, 711 (1964).
[19] S. BURSTEIN and B. BHAVNANI, *Endocrinology* **8**, 351 (1967); S. BURSTEIN and R. I. DORFMAN, *Acta Endocrinol.* **40**, 188 (1962).

a mathematical treatment of several biological models of this type and pointed out the difficulties and limitations of interpreting such results. Hence any conclusions derived from the present investigation should only be viewed "as a first approximation" of the events. With this limititation in mind, it may be stated that 14α-hydroxy progesterone *is not* a precursor of cardenolides. In reaching this conclusion it is assumed that the metabolism of the 14α-hydroxy progesterone was not blocked *totally* by the simultaneously administered progesterone. Differently stated, the observed lack of incorporation of isotopic hydrogen into digitoxigenin is inherent in the nature of the tritiated steroid and is not due to the interference of progesterone. On the other hand it is evident that the progesterone was incorporated in nearly normal amounts into digitoxigenin.

TABLE 2. CHROMATOGRAPHIC ISOLATION OF DIGITOXIGENIN FROM THE ACID-HYDROLYZED CHLOROFORM FRACTION* ($^3H/^{14}C$ 7·14)

Chromatographic system†	Digitoxigenin zone $^3H/^{14}C$
TLC-Benzene/ethyl-acetate (1:4)	4·44
Ligroin/Toluene/*t*.BuOH/MeOH/H_2O (5:5:3:4:6)	1·51
Benzene/MeOH/H_2O (2:1:1)	0·76
Toluene-propylene glycol	0·45

* For description of the fraction see Table 1 and Refs. 6 and 18.

† The systems were used sequentially. TLC was carried out on silica gel HF_{254} (Merck A. G.), paper chromatography on Whatman No. 1 filter paper. Solvents—in volumes.

Co-crystallization of material, from last chromatography with cold digitoxigenin.

Crop 1	$3·47 \times 10^5$ dpm/m-mole-^{14}C	No ^3H
Crop 1 recryst.	$3·48 \times 10^5$ dpm/m-mole-^{14}C	No ^3H

Whether other 14α-hydroxy intermediates are involved in the formation of cardenolides can obviously not be answered at this point. It is conceivable, for example, that the "early" introduction of the 14α-hydroxyl inhibited the metabolic transformations in the direction of digitoxigenin. The role of 14α-hydroxy intermediates, if any at all, in the biosynthesis of cardenolides requires further investigation.

EXPERIMENTAL

I.r. spectra were taken on solids incorporated in KBr. NMR spectra were obtained at 60 Mc on $CDCl_3$ solutions containing tetramethylsilane as internal standard in deuterated chloroform on a Varian Associates DA 60 spectrometer. The chemical shifts are expressed in cycles per second. M.ps. were recorded on a hot stage and are corrected. Analyses were performed by I. Beetz, Kronach, Germany. Mass spectra were obtained on a Varian Associates M 66 instrument. Silica gel HF_{254} produced by Merck A. G., Darmstadt, Germany, was used for TLC. The samples were counted in a Nuclear Chicago Model 6860 Scintillation Counter. The scintillation fluid consisted of 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-[2-(5-phenyl-oxazolyl)]-benzene dissolved in 1000 ml of toluene.

14α,20β-Dihydroxypregn-4-en-3-one (13a)

To 100 mg 14α-hydroxy progesterone dissolved in 5 ml methanol was added 25 mg $NaBH_4$. The reaction was shown to be completed after 0·5 hr (TLC). Water was added to the reaction mixture and the steroid triol extracted with ether. The combined extracts were washed with water and dried, and upon removal of the solvent the triol (12) was obtained (102 mg). The i.r. was devoid of carbonyl absorption and showed a broad hydroxyl band at *ca.* 3400 cm^{-1}.

A mixture of the above triol (**12**) (102 mg), D.D.Q. (70 mg), and dry dioxan (10 ml) was allowed to stand at room temperature for 16 hr. Then ether was added and the organic solution was washed thoroughly with a $Na_2S_2O_5$ solution, water and dried. Removal of solvent yielded 86·2 mg of 14α,20β-dihydroxypregn-4-en-3-one, m.p. 148–151°. ν_{max}^{KBr} 3470 (hydroxyl), 1665, 1610 (4-en-3-one) cm^{-1}. NMR 342·5 (1H; C-4) 70 (doublet $J=4$; 3H; 21 CH_3) 72 (19 CH_3); 56·6 (18 CH_3). Mass spectrum m/e 332 (M^+) 314 (M-18); 296 (314–18); 299 (314–15).

20β-Acetoxy-14α-Hydroxypregn-4-en-3-one (**13b**)

The alcohol **13a** was acetylated in acetic anhydride–pyridine in the usual manner to give **13b**, m.p. 253–255°. ν_{max}^{KBr} 3500, 1725, 1665, 1615 cm^{-1}. NMR 343 (1H; C-4) 120 (CH_3COO), 71 (3H, doublet $J=6$; 21 CH_3) 71 (19 CH_3) 49 (18 CH_3); m/e 374 (M^+); 356 (M-18), 314 (M-60). (Found: C, 73·30, H, 8·84. Calc. for $C_{23}H_{34}O_4$: C, 73·76; H, 9·15%.)

20β-Acetoxy-14α-Hydroxypregn-1,4-dien-3-one (**14**)

A mixture of **13b** (108 mg), D.D.Q. (150 mg) and dry dioxan (10 ml) was refluxed overnight. At the termination of the experiment the dioxan was removed at reduced pressure and then benzene (20 ml) was added. The organic solution was washed with a saturated solution of sodium sulfite, water, dried and, upon removal of solvent, **14** was obtained. Crystallization from ethyl acetate afforded 40 mg of 20β-acetoxy-14α-hydroxypregn-1,4-dien-3-one (**14**); m.p. 258–262°, ν_{max}^{KBr} 3500, 1725, 1660, 1620 and 1000 cm^{-1}. NMR 422 (1H; doublet $J=10$; C-1H) *ca.* 372 (1H; quartet; C-2). 365 (1H; C-4H); 67·5 (half of doublet; other half obscured by the 19 CH_3; 21 CH_3); 73·5 (19 CH_3) 50 (18 CH_3). (Found: C, 74·02; H, 8·53. Calc. for $C_{23}H_{32}O_4$: C, 74·16; H, 8·66%.)

1,2-^{3}*H-20β-Acetoxy-14α-Hydroxypregn-4-en-3-one* (**15**)

The 20β-acetoxy-14α-hydroxypregn-1,4-dien-3-one (**14**) (10 mg) in 2·5 ml ethyl acetate and 2·5 ml dioxan was tritiated for 3·5 hr in the usual manner employing tristriphenylphosphine rhodium chloride[11] (25 mg) as catalyst. The tritium gas was then removed and the reduction completed with H_2. The solvent was distilled under nitrogen and the residue was fractionated on TLC (ethyl acetate/benzene, 1:1). The appropriate zone was eluted and further purified by continuous TLC (benzene/methanol (99–1; 22 hr)). Subsequently the material was again chromatographed in the first system and a single zone was detected.

1-^{3}*H-14α,20β-Dihydroxypregn-4-en-3-one* (**16**)

The crude chromatographed di-tritiated material (**15**) was dissolved in 15 ml of 2 per cent KOH in methanol and the solution was flushed with N_2 for half an hour. The mixture was refluxed under N_2 and aliquots of the solution were removed at intervals for counting. When the count was constant (4 hr) the refluxing was terminated and the majority of the methanol was removed under reduced pressure. The residue was diluted with water and the steroid was recovered with ethyl acetate.

The residue, after removal of solvent, was chromatographed on TLC (ethyl acetate/benzene, 3:1). The zone corresponding to **16** was rechromatographed on paper in benzene/methanol/water, 2:1:1 ($R_f=0·8$). An aliquot of the diol extract from this paper, when again rechromatographed in the same system, showed a single symmetrical radioactive peak corresponding to **16**. A second equilibration was carried out under analogous conditions and proceeded without loss of tracer.

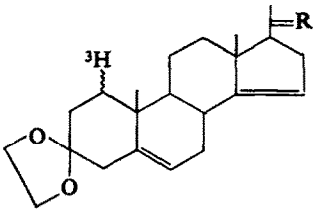
1-^{3}*H-14α-Hydroxy Progesterone* (**17**)

The 1-^{3}H-14α,20β-dihydroxypregn-4-en-3-one (**16**) was dissolved in dry pyridine (1 ml) and CrO_3 (5 mg) was added. The mixture was stored at room temperature for 5 hr then hot benzene was added and the solid was removed by filtration through Celite. The filtrate was washed thoroughly with water and dried. The residue, after removal of solvent, was purified by TLC (benzene/ethyl acetate, 1:1). The 14α-hydroxy progesterone zone was extracted from this plate and diluted with 5 mg of "cold" material and crystallized from ethyl acetate to give a first crop of 2·0 mg. An aliquot of this material was further diluted with 14α-hydroxy progesterone and recrystallized and the following crops were counted: crude material 4854 c/m/mg; first crop, 4863 c/m/mg; second crop, 4886 c/m/mg; first crop recrystallized, 4910 c/m/mg. Rechromatography of this material on TLC and on paper did not change its specific activity. The "once diluted" first crop described above was subsequently administered to the plant.

1-^{3}*H-Pregna-5,14-dien-3,20-bis-ethylene dioxide* (**18**)

A sample of 1-^{3}H-14α-hydroxy progesterone (100 mg; sp. act $3·86 \times 10^6$. dpm/m-mole) was suspended in ethylene glycol (75 ml) and 20 ml of the glycol was removed at 80° under reduced pressure. A catalytic amount of *p*-toluenesulfonic acid was then added and the mixture slowly distilled under vacuum (0·5 hr). After the addition of a few drops of pyridine, the cooled mixture was poured into ice-water. The product was extracted

with ether and washed with saline to yield upon removal of solvent a white solid. Recrystallization from methanol/methylene chloride gave the diketal (70 mg), m.p. 161–162°, of constant specific activity ($\times 10^6$ dpm/m-mole); crude 4·18; 1st cryst. 4·34; 2nd cryst. 4·32; ν_{max}^{KBr} no carbonyl absorption; NMR ($CDCl_3$) 324 (1H; C6-H); 312·5 (1H; C15-H); 232 (8H; C3, 20 ketals); 81·5 (21 CH_3); 62·5 (6H; 18 and 19-CH_3); e/m 400 (M^+); 99 ($O—CH_2—CH_2—O=C—CH=CH_2$)$^+$; 87 ($O—CH_2—CH_2—O=C—CH_3$)$^+$. (Found: C, 74·96; H, 9·06. Calc. for $C_{25}H_{36}O_4$: C, 74·66; H, 8·97%).



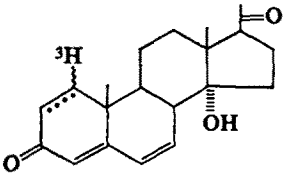
18 R=—OCH_2CH_2O—
19 R=O

1-^3H-*Pregna*-5,14-*dien*-20-*one*-3-*Ethylene Dioxide* (19)

To a solution of Δ^{14}-progesterone diketal (50 mg; sp. act. $4·32 \times 10^6$ dpm/m-mole) in 5 ml acetone was added 3 mg of *p*-toluenesulfonic acid. After standing at room temperature for 0·5 hr a few drops of pyridine were added and the acetone was removed in a stream of nitrogen. Dilute $NaHCO_3$ was added to the residue and the product filtered off. Recrystallization from ethyl acetate yielded the monoketal (31 mg), m.p. 197–203°. Specific activity $4·37 \times 10^6$ dpm/m-mole; ν_{max}^{KBr} 1710 cm^{-1}. NMR ($CDCl_3$) 323 (1H; C6-H); 310 (1H; C15-H); 237 (4H; 3-ketal); 129 (CH_3CO); 62·5 (19 CH_3); 52·5 (18 CH_3); e/m 356 (M^+), 99. Found: C, 77·32; H, 8·98. Calc. for $C_{23}H_{32}O_3$: C, 77·49; H, 9·05%.

1-^3H-14α-*Hydroxypregna*-4,6-*dien*-3,20-*dione* (20)

To a solution of 1-^{3}H-14α-hydroxy progesterone (50 mg; sp. act. $7·87 \times 10^6$ dpm/m-mole) in redistilled *t*-butanol (5 ml), chloranil (137 mg) was added and the mixture was refluxed for 3 hr. Most of the *t*-butanol was distilled off; then $CHCl_3$ was added and the solution was washed with water (3×6 ml), 5% NaOH (3×6 ml), and water (3×6 ml). The isolated product (47·3 mg) was recrystallized from ethyl acetate to give 20 (25 mg), m.p. 180°. Specific activity ($\times 10^6$ dpm/m-mole): 1st cryst. 7·87; 2nd cryst. 7·80; ν_{max}^{KBr} 3410; 1705 (C-20 carbonyl), 1660, 1650 and 1620 (4,6-dien-3-one) cm^{-1}. NMR ($CDCl_3$) 373 (2H; C-6 and C-7); 343 (1H; C-4); 128 (CH_3CO); 68 (19 CH_3); 50·5 (18 CH_3); e/m 328 (M^+) 310 (M-18).



20 NO Δ^1; (^{3}H—100%)
21 Δ^1—; (^{3}H—22%)

1-^3H-14α-*Hydroxypregna*-1,4,6-*trien*-3,20-*dione* (21)

To a solution of 1-^{3}H-14α-hydroxypregn-4,6-dien-3,20-dione (20) (25 mg; sp. act. $7·83 \times 10^6$ dpm/m-mole) in dry dioxan was added D.D.Q. (60 mg), and the mixture was refluxed for 18 hr. A further 60 mg of D.D.Q. was then added and after another 6 hr reflux the dioxan was removed under reduced pressure.

$CHCl_3$ was added, and the solution was washed with water ($\times 3$), a 5% NaOH ($\times 3$) and again with water ($\times 3$). After drying, the solvent was removed to leave a residue which was crystallized from ethyl acetate to give 21 (13·1 mg). M.p. 244–246°; specific activity ($\times 10^6$ dpm/m-mole): 1st cryst. 1·79; 2nd cryst. 1·79. ν_{max}^{KBr} 3360, 1700, 1645, 1620 cm^{-1}. NMR ($CDCl_3$) 424 (1H; doublet $J \simeq 10$; C-1); 380–360 multiplet (*ca.* 4H; C-2, 4, 6 and 7); 127·5 (CH_3CO); 72 (C-19 CH_3); e/m 326 (M^+); 308 (M-18); 293 (308–15); 265 (308–43).

Dienone-phenol Rearrangement of **21**

A sample of **21** (20 mg; sp. act. $1{\cdot}79 \times 10^6$ dpm/m-mole) was dissolved in acetic anhydride (1 ml) containing *p*-toluenesulfonic acid (6 mg) and the mixture was warmed on a water bath (*ca.* 90°) for 3 hr. The solution was stored for 3 hr at ambient temperature and then poured into a solution of $NaHCO_3$ and water. The products were extracted with $CHCl_3$ and the organic solution was washed with water, dried and concentrated to yield a yellow gum (25·2 mg). TLC revealed the presence of three major products and several minor ones. The NMR indicated the presence of aromatic protons, olefinic protons, and acetates. Because of the scarcity of material no further purification was undertaken and the crude gum was counted as such. For computation of specific activity on arbitrary molecular weight of 352 corresponding to 3-acetoxy-1-methyl-19-nor-pregna-1,3,5 (10), 14-tetraen-20-one was chosen. The arbitrary specific activity of this mixture was $4{\cdot}38 \times 10^5$ dpm/m-mole.

Administration of the Steroid to Digitalis lanata *and Processing of the Plant Material*

The leaves were washed with ethyl acetate and a mixture of 1-^{3}H-14α-hydroxy progesterone (154 μc) and 4-^{14}C-progesterone (18·9 μc) (^{3}H/^{14}C 8·1) was deposited on their surface from an acetone solution. Subsequently the leaves were sprayed with a 10% solution of silicon oil[20] DC 200 in ligroin (60–90°). The washings of the container were applied in the same manner after 7 days. The plant was illuminated for 16 hr daily with light of 12×10^3 lu/m^2 and watered as necessary. Arfter 14 days the leaves were harvested and processed as previously described.[6,18] The distribution of the tracers in the fractions is summarized in Table 1. The chromatographic method of isolation and the final recrystallization of the digitoxigenin is presented in Table 2.

[20] We thank the Dow Corning Company for a gift of the silicon oil.