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BIOSYNTHESIS OF CARDENOLIDES IN *DIGITALIS LANATA*

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Key Word Index—*Digitalis lanata;* biosynthesis; cardenolides; 14β hydroxylation; cholesterol as precursor.

Abstract—[8-^{3}H]-Cholesterol was synthesized. A doubly labelled sample of [8-^{3}H, 4-^{14}C]-cholesterol was administered to *Digitalis lanata* plants and the cardenolides were isolated. Biosynthesized digitoxigenin and digoxigenin retained all the tritium. Barring the migration of the tritium in biosynthesis the results are interpreted as indicative that neither intermediates with Δ^7, Δ^8 or $\Delta^{8(14)}$ are participating in the elaboration of cardenolides.

INTRODUCTION

THE GROSS aspects of enzymatic hydroxylation of nonactivated secondary carbons of the steroidal nucleus are now fairly well understood. In animal tissues,[1] microorganisms,[2] and plants,[3] the incoming hydroxyl assumes the stereochemistry of the removed hydrogen. In contrast, the mechanism of hydroxylation of nonactivated *tertiary carbons* is not clear and in some instances the hydroxylation occurs with retention and in others with inversion of configuration. Thus, the conversion of cholesterol to 20*S*-cholest-5-en-3β,20-diol (20α-hydroxycholesterol) in adrenal tissue,[4] and the 14α-hydroxylation of C_{19} and C_{21} steroids by certain microorganisms,[5] are examples of hydroxylation with retention of configuration. On the other hand, the introduction of the 14β-hydroxyl of cardenolides, biosynthesized from cholesterol,[6] pregnenolone,[7,8] progesterone[8] and deoxycorticosterone[9] proceeds with inversion of configuration at C-14. The latter process cannot be rationalized in terms of the proven mechanism of hydroxylation at nonactivated secondary carbons. Frequently, and without supporting evidence, it is assumed that the 14β-hydroxylation is formally analogous to a Walden inversion reaction. Although ultimately this hypothesis may prove correct,

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[1] HAYANO, M. (1962) in *Oxygenases* (HAYAISHI, O., ed.), p. 182. Academic Press, New York.
[2] CHARNEY, W. and HERZOG, H. L. (1967) *Microbial Transformations of Steroids*, p. 18, Academic Press, New York.
[3] VARMA, K. R. and CASPI, E. (1970) *Phytochemistry* **9**, 1539.
[4] ROBERTS, K. D., BANDY, L. and LIEBERMAN, S. (1969) *Biochemistry* **8**, 1259.
[5] CHARNEY, W. and HERZOG, H. L. (1967) *Microbial Transformations of Steroids*, pp. 30, 31, Academic Press, New York.
[6] WICKRAMASINGHE, J. A. F., HIRSCH, P. C., MUNAVALLI, S. M. and CASPI, E. (1968) *Biochemistry* **7**, 3248.
[7] TSCHESCHE R. and LILIENWEISS, G. (1964) *Naturforsch.* **20b**, 894.
[8] CASPI, E. and LEWIS, D. O. (1967) *Science* **156**, 519.

the concept will be difficult to evaluate experimentally without isolating the 14β-hydroxylase.

Under the circumstances, several years ago we outlined three mechanisms, amenable to experimental testing, which could provide a rationalization of the observed reaction consistent with present chemical concepts.[10] One of the pathways involved the initial formation of a 14α-hydroxylated intermediate which could be converted via a 14-oxo radical to a 14β-hydroxylated analogue. We also considered the possible participation of compounds with an 8(14) or 14(15) double bond. The olefins could yield the 14β-hydroxylated product through a β-face epoxide, hydration, etc. The unsaturated precursor could be formed by dehydrogenation of 14α-H sterols or dehydration of, e.g. 14α-hydroxylated intermediates.

We then showed that 14α-hydroxy progesterone was not incorporated into cardenolides.[10] Canonica *et al.* have demonstrated that both C-15 protons of progesterone administered to *Digitalis lanata* plants are retained in cardenolides.[11] This result is in agreement with the observation of Tschesche *et al.*, who proved that progesta-4,14-diene-3,20-dione is not utilized as a biosynthetic precursor of cardenolides.[12] This effectively excludes the participation of intermediates with a hydroxyl, epoxide or double bond, centered around C-15, in the elaboration of cardenolides from progesterone. There remained then the possibility of the involvement of a Δ^7, $\Delta^{8(9)}$ or $\Delta^{8(14)}$-precursor.

Our interest in exploring this route was enhanced by our observation that Δ^7-22-hydroxy steroids cyclize to give 14β,22-ethers, which can be used in the preparation of 14β-hydroxylated steroids.[13] The testing of the possible involvement of an olefin (or olefins) encompassing C-8 of cholesterol is described in this paper.

RESULTS AND DISCUSSION

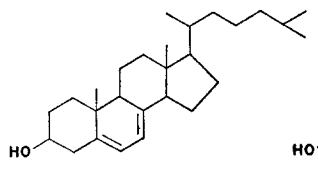
Our approach to the problem of the mechanism of 14β-hydroxylation of steroids was based on the fact that an involvement of a Δ^7, $\Delta^{8(9)}$ or a $\Delta^{8(14)}$—steroidal intermediate in the biosynthetic elaboration of cardenolides by necessity entails the loss of the C-8 hydrogen from the precursors, e.g. pregnenolone, progesterone, cholesterol (or their analogues). Thus administration of a [8-^{3}H, 4-^{14}C]-steroidal precursor to *D. lanata* and isolation of the resulting cardenolides could provide the desired answer. Should the formed cardenolide contain ^{14}C and be devoid of tritium, this would be an indication of the participation of the C-8 hydrogen in the process of introduction of the 14β-hydroxyl. On the other hand, barring C-8 proton migration, retention of the tritium would exclude this possibility.

The approach we chose was to administer [8β-^{3}H, 4-^{14}C]-cholesterol to *D. lanata* and define the [^{3}H, ^{14}C] content in the resulting cardenolides. Although cholesterol is not the most efficient precursor of cardenolides, we decided in its favor over pregnenolone and progesterone because successful tritiation at the C-8 position could be more readily accomplished.

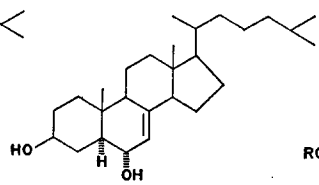
As mentioned earlier, ultimately the interpretation of the biosynthetic results would depend on the retention or loss of tritium in the biosynthesized cardenolides. Therefore, in devising the synthetic route, we wished to select a set of conditions which would result in the introduction of a tritium atom exclusively at C-8. The route we adopted was to start

[9] CASPI, E., WICKRAMASINGHE, J. A. F. and LEWIS, D. O. (1968) *Biochem. J.* **108,** 499.
[10] CASPI, E. and LEWIS, D. O. (1968) *Phytochemistry* **7,** 683.
[11] CANONICA, L., RONCHETTI, F. and RUSSO, G. (1970) *Chem. Commun.* 1675.
[12] TSCHESCHE, R., HOMBACH, R., SCHOLTEN, H. and PETERS, M. (1970) *Phytochemistry.* **9,** 1505.
[13] ABERHART, D. J. and CASPI, E. (1971) *J. Chem. Soc.* 2069.

with cholesta-5,7-dien-3β-ol (I) which was converted via hydroboration[14] to the 5α(H)-3β,6α-diol (II). The assignment of configuration to the hydrogen at C-5 and hydroxyl at C-6 rests on the known mechanism of hydroboration of Δ^5-steroids. The diol [II] was then oxidized with DDQ and acetylated to yield the 3-acetoxy-6-ketone (IV). Lithium—in tritiated ammonia reduction of the conjugated ketone (IV) resulted in 5α-cholestane-3β,6α-diol (VII) and in small amounts of (V) and (VI). The NH_3-^3H was prepared by direct exchange of liquid ammonia with carrier-free ^3H_2O prepared from $^3H_2$ gas. The exchange of hydrogens between ammonia and water is known to be extremely fast in alkaline media.[15]



(I)



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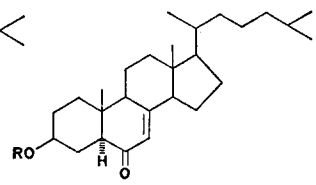

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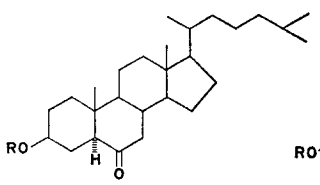
(IV) R = Ac



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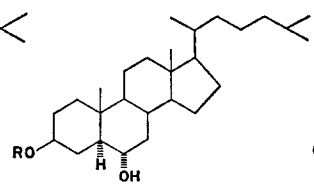
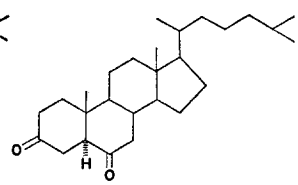


(VII) R = Ac
(VIII) R = H



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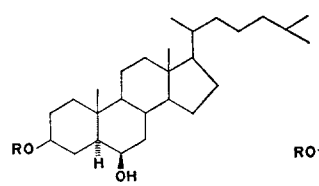
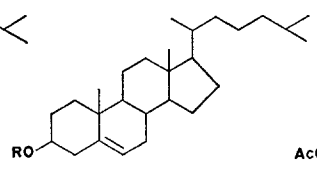
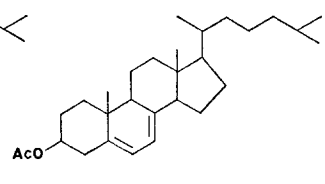


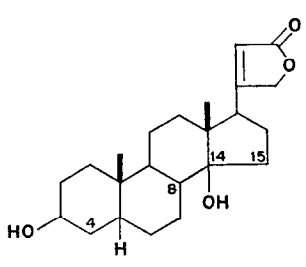
(X) R = H
(XI) R = Ac



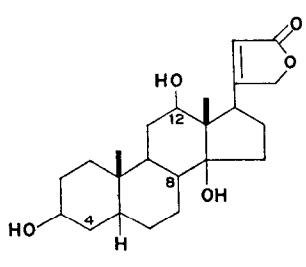
(XII) R = Ac
(XIII) R = H



(XIV)



(XV) Digitoxigenin



(XVI) Digoxigenin

The crude reaction mixture was saponified and oxidized to the diketone (IX). The diketone was then equilibrated with base in an atmosphere of nitrogen to a constant specific activity

[14] CAGLIOTI, L., CAINELLI, G. and MAINA, G. (1963) *Tetrahedron* **19**, 1057.
[15] BRODSKII, A. I. and SULIMA, L. V. (1950) *Dokl. Akaf Nauk SSSR* **74**, 513; *idem.* (1951) *Chem. Abstr.* **45**, 424a.

of ^3H. Unfortunately the equilibration was accompanied by a considerable decomposition of the material.

However, the equilibration removed labile tritium atoms located at C-2, 4, 5 and 7, if present, leaving the tritium atom at the 8β-position. The dione (IX) was reduced to the 5α(H)-3β, 6β-diol (X) and acetylated selectively at the C-3 position. Treatment of the obtained 5α-cholesta-3β,6β-diol-3-acetate (X) with phosphorous oxychloride in pyridine gave cholesteryl acetate (XII) which was converted to [8-^3H]-cholesterol (XIII) by reduction with $LiAlH_4$. To define the location of the tritium a portion of the cholesteryl acetate (XII) was converted to cholesta-5,7-diene-3β-ol acetate (XIV).[16] The transformation was accompanied by the loss of more than 99% of the tritium present in cholesteryl acetate. Since tritium atoms were removed by equilibration from C-2, 4, 5 and 7 it follows that all the isotopic hydrogen was located at C-8.

The [8-^3H]-cholesterol was mixed with [4-^{14}C]-cholesterol (^3H:^{14}C ratio 12·8); and the solution of the doubly labelled sample was spread on the surface of the leaves of 2-month-old *D. lanata* plants as previously described.[6] The plants were exposed to sunlight for 16 hr/day. When necessary the illumination was supplemented by irradiation with fluorescent light for 16 hr/day. After 37 days the plants were harvested, frozen in liquid nitrogen, pulverized and stored at −20°. Subsequently the plant tissue was processed[6] and the distribution of the radioactivity in the obtained extracts is given in Table 1.

TABLE 1. DISTRIBUTION OF TRACERS IN EXTRACTS OF *Digitalis lanata* PLANTS AFTER ADMINISTRATION OF [8-^3H]-CHOLESTEROL AND [4-^{14}C]-CHOLESTEROL ($1·42 \times 10^7$ dpm of ^{14}C); (^3H:^{14}C = 12·8)

Extract	Radioactivity $\times 10^5$ dpm	^3H:^{14}C
Ligroin extract	0·604	18·8
Chloroform extract	1·360	17·0
Chloroform ethanol (2:1)	0·433	12·2
Chloroform ethanol (3:2)	0·170	11·9

Digitoxigenin (XV) and digoxigenin (XVI) were isolated from the chloroform extract by TLC. Unfortunately in this experiment the amount of the [8-^3H, 4-^{14}C]-cholesterol metabolized to cardenolides was smaller than anticipated. In any event, the residues corresponding to digitoxigenin and digoxigenin were diluted with cold authentic samples and were extensively purified by TLC and were crystallized to constant specific activity of ^{14}C and constant [^3H:^{14}C] ratio. The results are summarized in Table 2. It is evident that the biosynthetic conversion of [8-^3H]-cholesterol into digitoxigenin and digoxigenin did not involve loss of tritum. It may thus be inferred that the introduction of the 14β-hydroxyl of cardenolides proceeded without the loss of the C-8 hydrogen of cholesterol. This observation eliminates the possible intermediacy of Δ^7, Δ^8, and $\Delta^{8(14)}$-precursors in the biosynthetic elaboration of cardenolides in *D. lanata*. The conclusion is only valid if no migration of the tritium atom takes place in the biosynthetic processes.

[16] AKHTAR, M. and GIBBONS, C. J. (1965) *J. Chem. Soc.* 5964.

TABLE 2. THE ^3H:^{14}C RATIOS OF THE ADMINISTERED CHOLESTEROL AND THE ISOLATED DIGITOXIGENIN AND DIGOXIGENIN FROM *Digitalis lanata* PLANTS ADMINISTERED [8-^3H, 4-^{14}C]-CHOLESTEROL

	Specific activity ^{14}C dpm/mg	^3H:^{14}C
Cholesterol		
1st crystallization	—	12·8
2nd crystallization	—	12·5
Digitoxigenin		
1st crystallization	55·3	12·8
2nd crystallization	49·4	13·9
3rd crystallization	55·5	12·5
Digoxigenin		
1st crystallization	47·2	13·1

EXPERIMENTAL

TLC was on Silica gel (HF 254 + 366) (Merck AG) in the indicated solvent systems. NMR spectra were recorded in $CDCl_3$ solutions containing $(CH_3)_4Si$ as an internal standard on a Varian Associates model DA60 Spectrometer at 60 MHz. Peaks are quoted in Hertz downfield from tetramethylsilane. IR spectra were recorded as KBr micro discs.[17] MS were obtained on a Varian Associates M-66 or DuPont 21–491 instruments. M.ps were recorded on a hot stage and are corrected.

Counting. Samples were counted on a Nuclear Chicago Mark 1 automatic liquid scintillation counter. The samples were dissolved in 15 ml of a scintillation solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis[2-(5-phenyloxazolyl)]-benzene per 1000 ml.

5α-Cholest-7-en-3β,6α-diol (II). Cholesta-5,7-dien-3β-ol (I) (7-dehydrocholesterol) (Aldrich, 5·0 g) in 50 ml anhyd. tetrahydrofuran (THF) was treated with $NaBH_4$ (450 mg) and freshly distilled BF_3-etherate (2·5 ml) was added from a syringe over 15 min.[14] The mixture was stirred 2 hr at room temp., then H_2O was added to destroy the excess hydride. NaOH (2 N, 40 ml), and 30% H_2O_2 (20 ml) were added and the mixture stirred (no external cooling) for ½ hr. The THF layer was separated and the aqueous phase washed with Et_2O. The combined organic phases were washed with 5% $FeSO_4$, and saturated NaCl, dried (Na_2SO_4) and evaporated The crude product was chromatographed on a column of 300 g silica gel (Will, grade 950, 60–200 mesh, prepared with (EtOAc–hexane, 1:1). Elution with the same solvent gave, after an impure forerun, the diol, 3·25 g, part of which was crystallized from EtOAc–MeOH, m.p. 114°; IR ν_{max} 3340, 1650, 1450, 1370, 1030, 1015, 970 cm^{-1} (KBr). NMR bands at (Hz) 33 (13-Me), 49·5 (10-Me), 52 (9H, *d*, *J* 6Hz, sec. methyls), 170 (2H, –OH, D_2O exchangeable), 224 (2H, *m*, CH–OH), 310 (1H, *m*, vinylic). MS *m/e* 402 (M^+); 384 (M-18); 369 (384–15); 351 (369–18); 325; 271 (possibly $C_{19}H_{27}O$; 253.

3β-Acetoxy-cholest-7-en-6-one (IV). 5α-Cholest-7-en-3β,6α-diol (II) (3·0 g) in dry C_6H_6 (120 ml) was stirred with 2,3-dichloro-5,6-dicyano-1,4-benzoquinone (5·5 g) at room temp. for 48 hr.[18] The volume was reduced to 20 ml *in vacuo*, then $CHCl_3$ was added, and the solution extracted with 5% NaOH. The $CHCl_3$ extract was dried (Na_2SO_4) and evaporated to give crude III. A small amount of (III) was purified by preparative TLC, m.p. 180–184°; IR ν_{max} 3460, 1665, 1615, 825 cm^{-1} (KBr); UV, λ_{max} 245 nm (ε 12 400, MeOH). MS *m/e* 400 (M^+); 385 (M-15) 367 (385–18) 287 (possibly $C_{19}H_{27}O_2$). The remainder of the crude III (*ca.* 3·5 g) was treated with Ac_2O (20 ml) and pyridine (10 ml) at 100° for 1 hr. The volatile components were evaporated *in vacuo*. The acetate was purified by preparative TLC (system: 20% EtOAc–hexane, R_f 0·5) giving 3·32 g of the acetate IV, crystallized from $CHCl_3$–MeOH in prisms, m.p. 149–151°; IR, ν_{max} 1725, 1665, 1230, 850, 820 cm^{-1} (KBr); NMR ($CDCl_3$), bands at (Hz) 36·5 (13-Me) 52 (6H, *d*, *J* ~ 6Hz), 54 (3H, *d*, *J* ~ 6Hz), 56 (10-Me) 122 (OAc), 282 (1H, *m*, CH–OAc), 343 (1H, *m*, vinylic H). MS *m/e* 442 (M^+); 427 (M-15); 382 (M-60) 367 (382–15); 329 ($C_{21}H_{29}O_3$; 314 (329–15); 269 (329–60).

Reduction of IV *with Lithium and* NH_3-^3H: THF (3 ml, freshly distilled from $LiAlH_4$) was stirred with 167 mg of PtO_2 (Engelhard) and carrier-free ^3H gas (20 Ci, New England Nuclear) in a 25 ml flask equipped with a sidearm having a stopcock. The system was evacuated before admitting ^3H. After stirring at room temp. for 36 hr, the THF containing T_2O was distilled, by passing dry N_2 via the sidearm over the solvent, into a

[17] CASPI, E. and SCRIMSHAW, G. F. (1967) in *Steroid Hormone Analysis* (CARTSENSON, H., ed.), Vol. 1, p. 63, Marcel Dekker, New York.

[18] BURN, D., PETROW, V. and WESTON, G. O. (1960) *Tetrahedron Letters* 14.

20 ml long-necked reaction flask which contained 302 mg of IV and was cooled in liquid N_2. The delivery tube was inserted to just above the bottom of the flask, the THF was distilled to dryness. Then 3·0 ml of liquid NH_3 was distilled into the flask, the reaction flask still being cooled in liquid N_2. Li (120 mg) was dropped in, then the mixture was warmed up to −30°, and the reaction was allowed to proceed at the b.p. of NH_3 with a dry ice–acetone condenser.[15,19] After refluxing 7 hr, the NH_3 was evaporated and trapped in dil. HCl. MeOH (5 ml) was added dropwise, and then H_2O (100 ml) was added. The mixture was extracted with Et_2O (2 × 100 ml), the extract washed with sat. NaCl, dried (Na_2SO_4) and evaporated, giving 250 mg of a crude product, 7·53 × 10^{10} dpm ^3H. TLC of the residue showed that the major product was VII, with smaller quantities of V and VI being present.

[8-^{3}H] 5α-Cholestane-3β,6α-*diol* (VIII). The crude Li–NH_3 reduction product was refluxed 5 hr under N_2 with 5% KOH–MeOH (50 ml) and dioxane (20 ml). H_2O (100 ml) was added, and the product extracted with Et_2O. The extract was washed with dil. NaCl, sat. NaCl, dried (Na_2SO_4) and evaporated, giving 258 mg of product, 28 × 10^9 dpm ^3H. TLC indicated that the major radioactive component was VIII.

[8-^{3}H] 5α-Cholesta-3,6-*dione* (IX). The crude VIII in acetone (25 ml) was treated with Jones reagent (1 ml) at room temp. for 10 min. Aqueous $NaHSO_3$ was added to reduce the excess oxidant and the mixture was dil. with H_2O (100 ml). The product was extracted with $CHCl_3$, the extract dried (Na_2SO_4), and evaporated. The crude mixture (220 mg), was fractionated by preparative TLC (EtOAc–hexane, 1:3) and the product (R_f 0·5) extracted with EtOAc in a Soxhlet for 24 hr, giving IX, 170 mg, 3·2 × 10^9 dpm ^3H. This product was refluxed for 12 hr under a slight positive pressure of N_2 with 5% KOH–MeOH (50 ml). The solution was diluted with H_2O (100 ml), and extracted with Et_2O. The extract was washed with H_2O, sat. NaCl, dried (Na_2SO_4), and evaporated to give 160 mg of IX, 4·68 × 10^8 dpm ^3H. The equilibration was repeated in an identical manner. The recovered IX was purified as before by TLC, giving pure IX, 67·1 mg, 8·25 × 10^8 dpm ^3H. It was noticed that considerable amounts of the dione (IX) are destroyed during equilibration. To avoid further decomposition of the valuable labelled product, the extent of the equilibration was checked as follows: A sample (200 000 cpm) of the purified IX was diluted with cold IX (100 mg), and recrystallized from $CHCl_3$–MeOH, m.p. 162° (corr.): 1st crystallization 2180 cpm ^3H/mg: 2nd, 2120. The recrystallized material was then equilibrated once as above, and the isolated product recrystallized and counted: 1st crystallization 2190, cpm ^3H/mg; 2nd, 2210. The equilibration of IX was therefore considered to be complete. MS of cold material, *m/e* 400 (M^+); 385 (M-15); 372 (M-28) 287 (possibly $C_{19}H_{27}O_2$) 260; 245.

[8-^{3}H]-5α-Cholestane-3β,6β-*diol* (X). The diketone, IX (67·1 mg) in Et_2O (50 ml) was refluxed with $LiAlH_4$ for 2 hr. EtOAc was added to destroy the excess hydride, and then H_2O was added dropwise to form a white, granular precipitate, which was filtered and washed with Et_2O. The Et_2O solution was dried (Na_2SO_4) and evaporated, giving X, 76 mg, 8·0 × 10^8 dpm ^3H.

[8-^{3}H]-3β-Acetoxy-5α-Cholestan-6β-ol (XI). Diol X (76 mg) in dry pyridine (1 ml) was treated with (Ac_2O) (0·02 ml) and kept at room temp. After 2 hr, additional Ac_2O (0·02 ml) was added, and after 1 more hr 0·02 ml more Ac_2O was added. After a further hr, at room temp., MeOH (1 ml) was added, and the solution was evaporated to dryness. The required compound XI was isolated by preparative TLC and extracted with EtOAc, giving 26·0 mg of XI. In addition, 35 mg of a mixture of starting material and other products was recovered. The recovered mixture (*ca.* 35 mg) was treated with $LiAlH_4$ (100 mg) in Et_2O (25 ml) at reflux for 2 hr. Workup in the usual way gave 38 mg of crude diol X. Reacetylation of this, as above, gave an additional 15 mg of the monoacetate XI. MS of cold (XI) (m.p. 152–156°) *m/e* 446 (M^+); 428 (M-H_2O); 404 (M-42); 387 (M-59); 368 (428–60); 353, 296, 274.

[8-^{3}H]-*Cholesteryl acetate* (XII). The monoacetate XI (40 mg) in dry pyridine (1 ml) was treated with $POCl_3$ (0·30 ml) at room temp. for 20 hr.[20] The solution was added dropwise to a mixture of dil. HCl, ice and Et_2O. The Et_2O extract was washed with dil. HCl, dil. $NaHCO_3$, sat. NaCl, dried (Na_2SO_4), and evaporated giving pure cholesteryl acetate, XII, 30 mg, 4·0 × 10^8 dpm ^3H total.

Conversion of [8-^{3}*H*]-*cholesteryl acetate into cholesta*-5,7-*dien*-3β-*ol*-3-*acetate* (XIV) (XX). [8-^{3}H]-Cholesteryl acetate (*ca*, 200 000 cpm ^3H) was diluted with cold cholesteryl acetate (200 mg) and recrystallized from $CHCl_3$—MeOH, and counted: 1st crystallization, 1240 cpm ^3H/mg; 2nd, 1270; 3nd, 1270. The diluted [8-^{3}H]-cholesteryl acetate (including mother liquors) was dissolved in purified light petrol. (10 ml, b.p. 60–80°). (The petrol. was purified by washing with conc. H_2SO_4, aq. Na_2CO_3, dried ($CaCl_2$,) and filtered through neutral Al_2O_3 if activity I.) The solution was heated to reflux, and then *N*-bromosuccinimide (150 mg, recryst. from H_2O was added, followed immediately by Br_2 (0·01 ml) in purified light petroleum (1 ml). The mixture was refluxed for 30 min, cooled and diluted with hexane, washed with H_2O, dried (Na_2SO_4) and evaporated *in vacuo* keeping the temp. at *ca.* 10°. A solution of *s*-collidine (0·40 ml) in xylene (10 ml) was heated to reflux, and the bromination product in xylene (3 ml) was added rapidly. The solution was refluxed for 20 min, cooled and diluted with hexane. The hexane solution was washed with dil. HCl, dil. $NaHCO_3$, H_2O, dried (Na_2SO_4) and evaporated *in vacuo* without heating to yield an oily product. A band corresponding to cholesta-5,7-dien-3β-ol-3-acetate was isolated by preparative TLC (10% $AgNO_3$-silica gel; EtOAc–

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hexane (1:9)). The diene (R_f 0·4) was eluted with EtOAc, giving 51 mg of pure XIV. The product was recrystallized from $CHCl_3$–MeOH and counted: 1st crystallization, 10, cpm ^3H/mg; 2nd 9; 3rd 9. Thus, apparently more than 99% of the ^3H in the cholesteryl acetate is shown to be located at the 8-position.

[8-^{3}H]-Cholesterol (XIII). The [8-^{3}H]-cholesteryl acetate (XII) (30 mg) in Et_2O (25 ml) was treated with $LiAlH_4$ (100 mg) at reflux for 2 hr. Workup in the usual way gave [8-^{3}H]-cholesterol ((XIII), 21·5 mg, $2{\cdot}4 \times 10^8$ dpm ^3H.

[8-^{3}H, ^{14}C]-Cholesterol. [8-^{3}H]-Cholesterol was mixed with [4-^{14}C]-cholesterol (New England Nuclear ($1{\cdot}4 \times 10^7$ dpm of ^{14}C). A sample (0·4% of total) was diluted with nonradioactive cholesterol, and recrystallized from $CHCl_3$–MeOH to a constant activity of 765 dpm ^{14}C/mg, and ^3H:^{14}C = 12·8. The diluted cholesterol was then purified via the dibromide in the usual manner, and the product was recrystallized from $CHCl_3$–MeOH to constant activity of 784 dpm ^{14}C/mg; ^3H:^{14}C = 12·9 (see Table 2).

Administration of [8-^{3}*H*, 4-^{14}*C*]-*Cholesterol to* Digitalis lanata *plants*. The experiment was conducted in May and June, using 2-month-old *Digitalis Lanata* plants. The leaf wax was removed from the upper surface of the leaves of 8 plants by wiping gently with cotton wool moistened with EtOAc. The undiluted [8-^{3}H, 4^{14}C] cholesterol was dissolved in hexane (1/2 ml) and applied to the upper surface of the leaves with a glass rod. After evaporation of the hexane, the leaves were lightly sprayed with a 1% solution of Dow-Corning silicone oil DC 200 in hexane. After a few days, some of the leaves showed brown spots as a result of the toxicity of hexane, but most of the leaves grew normally. The test-tube washings (dissolved in 0·1 ml EtOAc, which gave no toxic reaction) were applied to two separate plants 1 week later. The plants were watered as necessary, and either placed in sunlight or were irradiated with fluorescent light (*ca.* 10 000 lm/m^2) for 16 hr per day for 37 days. At the termination of the experiment the plants were harvested, quickly frozen in liquid N_2, crushed in a steel mortar, and the powder was stored at −20° until processed.

Processing of the Digitalis lanata *Plants*. The powdered plant material suspended in 50 ml H_2O and 3 drops of toluene was stored at 23° for 40 hr. 20 ml EtOH was then added and the mixture was warmed to 70° and filtered. The residue was suspended in 50% EtOH (60 ml) and homogenized. The resulting homogenate was again warmed to 70° and filtered. The residue was reextracted with 7× 60 ml EtOH, the concentration of EtOH being increased stepwise from 55 to 90%. The combined extracts were reduced *in vacuo* to about 30 ml and then treated with a suspension of $Pb(OH)_2$ in EtOH (30 ml). The mixture was shaken vigorously for 10 min, and filtered through celite. The clear brownish filtrate was treated with dil. H_2SO_4 to pH 6 and concentrated *in vacuo* to 25 ml. The pH was checked at intervals and maintained at 6. The white precipitate was filtered, and the filtrate (A) extracted with ligroin (b.p. 60–90°); 3 × 50 ml. The combined ligroin extracts were washed 2× 75% EtOH(B), dried and evaporated.

The aqueous phase (A) and the above aqueous EtOH washings (B) were combined and concentrated *in vacuo* to about 50 ml. This was extracted with $CHCl_3$, 5 × 50 ml. The combined $CHCl_3$ extracts were washed with H_2O (10 ml), 2 N NaOH (10 ml), and H_2O (10 ml). The extract was then dried and evaporated. The aqueous phase was combined with the first H_2O washings, and the total was extracted with $CHCl_3$–EtOH (2:1), 3 × 50 ml. The combined organic phase was washed with H_2O (10 ml), 2 N NaOH (10 ml), H_2O (10 ml), and the extract was then dried and evaporated. The aqueous phase was again combined with the first H_2O washing and the total extract concentrated *in vacuo* to about 35 ml, anhyd. Na_2SO_4 (3·5 g) added and extracted with $CHCl_3$–EtOH (3:2), 4 × 50 ml. The combined organic phase was washed with aq. Na_2SO_4 (20%, 20 ml), 2 N NaOH, and aq. Na_2SO_4, (20%, 20 ml). The extract was dried and evaporated. The distribution of the isotopes in the various extracts is given in Table 1.

Isolation of digitoxigenin and digoxigenin. An aliquot of the $CHCl_3$ extract (50%) was dissolved in MeOH (4 ml) and treated with 0·1 N H_2SO_4 (4 ml). The mixture was refluxed under N_2 for 20 min. The MeOH was removed *in vacuo* and the aqueous residue was heated at 70° for a further 20 min. After cooling, the mixture was diluted to 30 ml and extracted with EtOAc, 4 × 25 ml. The combined extracts were washed with ice H_2O (2 × 15 ml), ice-cold 2 N NaOH (15 ml) and finally with ice H_2O (2 × 15 ml). The extract was then dried and evaporated. Nonradioactive digitoxigenin (5 mg) was added and the mixture was fractionated on TLC (silica gel,–benzene–EtOAc (1:4)). The digitoxigenin was located under UV light and was extracted. A more polar zone than digitoxigenin was also detected. The digitoxigenin zone containing *ca.* $7{\cdot}7 \times 10^3$ dpm of ^{14}C and *ca.* $1{\cdot}4 \times 10^5$ dpm of ^{3}H was purified further by TLC (silica gel; benzene–MeOH (9:1) developed twice). The single radioactive peak corresponding to 'digitoxigenin was extracted, diluted with 10 mg of cold material, and crystallized from EtOAc. The ^3H:^{14}C ratios are summarized in Table 2. The zone more polar than digitoxigenin containing $6{\cdot}7 \times 10^3$ dpm of ^{14}C and $1{\cdot}18 \times 10^5$ dpm of ^{3}H after the addition of cold digoxigenin (0·3 mg) and gitoxigenin (0·3 mg) was purified by sequential TLC in two different solvent systems (1) $CHCl_3$–isopropanol (9:1); and (2) EtOAc–MeOH (98:2) (developed 3×). The digoxigenin and gitoxigenin zones were located with UV light and extracted with EtOAc. The isolated digoxigenin zone contained *ca.* $4{\cdot}8 \times 10^2$ dpm of ^{14}C and *ca.* $6{\cdot}4 \times 10^3$ dpm of ^{3}H, while the gitoxigenin zone contained very little ^{14}C and ^{3}H. The purified digoxigenin zone was mixed with cold digoxigenin (3 mg) and recrystallized. The ^3H:^{14}C ratio is summarized in Table 2.