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THE OBLIGATORY INVOLVEMENT OF A C_{21} INTERMEDIATE IN THE BIOSYNTHESIS OF CARDENOLIDES FROM CHOLESTEROL*

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Abstract—Previous studies have indicated that the biosynthesis of the three main cardenolides—digitoxigenin, gitoxigenin and digoxigenin—from preformed C_{27} precursors could proceed by different pathways and that, in contrast to the previous assumptions of other workers, apparently neither pregnenolone nor progesterone are the sole or even the major biosynthetic intermediates in this sequence. Formally, two major biosynthetic routes may be envisaged from C_{27} precursors—one involving "modified" C_{21} intermediates arising from the cleavage of the cholesterol side-chain between C-20 and -22, and the other involving C_{23} intermediates formed by cleavage between C-23 and C-24. Experiments designed to test the operation of these two routes have been carried out, and are described. The administration of doubly labeled 7-^{3}H-(1,7,15,22,26)-^{14}C-cholesterol to a *Digitalis lanata* plant led to the formation of radioactive digitoxigenin, gitoxigenin and digoxigenin. The ^{3}H/^{14}C ratio of these products, relative to the precursor, indicated that only three positions of these cardenolides were labeled with carbon-14. It was shown by chemical degradation that no ^{14}C was present in the lactone ring. Hence, it may be concluded that the carbons 22 and 23 of cardenolides do not originate from the exogenous cholesterol. Similarly, when digitoxigenin was biosynthesized from 4R-4-^{3}H-2-^{14}C-mevalonic acid, the ^{3}H/^{14}C ratio confirmed that the carbons 22 and 23 of the cardenolides do not originate from MVA. The ^{3}H/^{14}C ratio of tigogenin isolated from the same experiment indicated that *only two* positions, *rather than three*, were labeled with tritium. The absence of tritium at C-20 was established. The loss of this tritium atom could have occurred either during the biosynthesis or in the isolation processes.

INTRODUCTION

AT PRESENT it is assumed that the biosynthesis of cardenolides from mevalonic acid (MVA) proceeds via a C_{27} intermediate of the cholesterol type.[1–5] Support for this view was provided by the demonstration that exogenously supplied cholesterol is transformed into cardenolides in the plant.[6] During the biogenetic sequence, such a C_{27} intermediate is thought to undergo initial degradation to a C_{21} C-20 ketone, by cleavage of the side-chain between C-20 and C-22.[1–4] In accordance, the biosynthetic conversion of cholesterol to pregnenolone (I),[7] and the incorporation of pregnenolone into cardenolides was demonstrated.[8–10]

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Subsequently, the conversion of pregnenolone (I) to progesterone (II)[10] and the transformation of the latter into cardenolides was proved.[10,11]

Recently, we presented evidence which *could be viewed* as being indicative of the simultaneous operation of several routes in the biosynthesis of cardenolides from cholesterol.[6] Our observations suggested that the route cholesterol → pregnenolone (I) → progesterone (II) → cardenolides, is not the main pathway involved. In contrast to the previous assumptions of Tschesche[12] and of Leete *et al.*,[3] neither pregnenolone nor progesterone appear to be the sole or even the major biosynthetic intermediates in this sequence. In this context two general biosynthetic pathways may be considered. The steroid nucleus may undergo modification (e.g. by saturation of the Δ^5-double bond and, or, hydroxylations, etc.) prior to cleavage of the cholesterol side-chain between C-20 and -22. Alternatively, C-23 intermediates resulting from the cleavage of the side-chain between C-23 and C-24 may be involved. Consequently, we have undertaken the present work specifically designed to evaluate the feasibility of these possibilities.

Our first approach was to administer to a *Digitalis lanata* plant ^{3}H-^{14}C-cholesterol bearing ^{3}H in the nucleus and a ^{14}C-atom at C-22. From the loss or retention of ^{14}C in the cardenolides, it would be possible to decide unambiguously whether carbons 22 and 23 originate from the exogenous cholesterol. The presence or absence of ^{14}C at C-22 would be clearly reflected in the ^{3}H/^{14}C ratios of the cardenolides and could be confirmed by degradation of the lactone ring. Rather than carry out the long and tedious synthesis of 22-^{14}C-cholesterol, it was more convenient to use biosynthetic (1,7,15,22,26)-^{14}C-cholesterol.[13] The latter was mixed with 7-^{3}H-cholesterol and the doubly labeled composite specimen was employed in our studies.

In a second experiment, 4R-4-$^3H_1$-2-^{14}C-MVA was administered to a *D. lanata* plant. From the ^{3}H/^{14}C ratios of the recovered cardenolides, the presence of one tritium atom (presumably at C-17) and three ^{14}C-atoms (at C-1, -7 and -15) *or* four ^{14}C-atoms (C-1, -7, -15 and -22), could be determined.

RESULTS AND DISCUSSION

30 days after the administration of the mixture of biosynthetic (1,7,15,22,26)-^{14}C-cholesterol (III) ($2{\cdot}23 \times 10^7$ dpm) and 7-^{3}H-cholesterol (^{3}H/^{14}C ratio 17·0) to a *Digitalis lanata* plant, the plant was harvested and processed in the manner described previously.[6,14] Digitoxigenin, digoxigenin and gitoxigenin were isolated from the chloroform extract.[6] The ^{3}H/^{14}C ratio of the purified digitoxigenin (VI) was found to be 29·0. The absence of ^{14}C in the lactone ring was established by degradation of the ^{3}H-^{14}C-digitoxigenin-acetate to 3β-acetoxy-14β,21-dihydroxypregnan-20-one (VII).[14] The ^{3}H/^{14}C ratio, 28·7, of the ketol (VII) was essentially identical to that of the digitoxigenin.

Digoxigenin and gitoxigenin were characterized as their diacetates (VIII) and (IX); in each case the ^{3}H/^{14}C ratio was found to be 28·1. For reference purposes a portion of the original ^{3}H-^{14}C-cholesterol was incubated with a preparation of bovine adrenal mitochondria. Progesterone isolated from the incubate had a ^{3}H/^{14}C ratio of 26·1.

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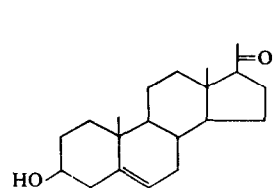

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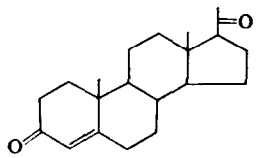

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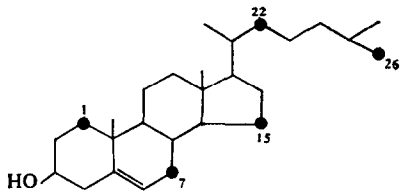

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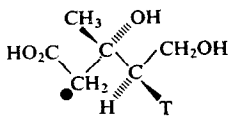

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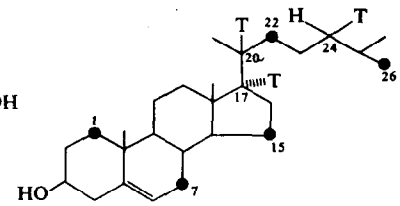

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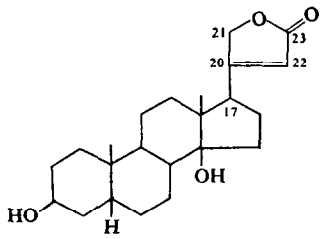

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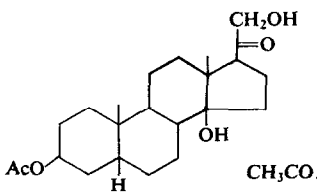

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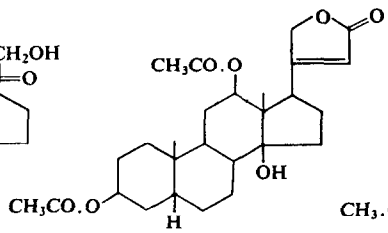

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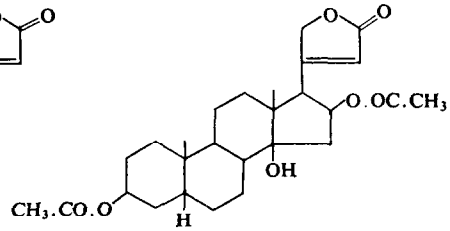

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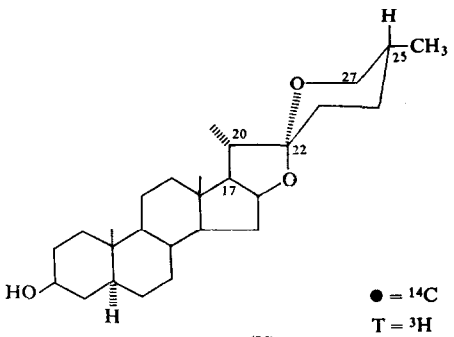

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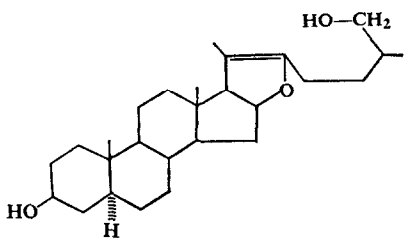

(XI)

The biosynthetic ^{14}C-cholesterol (III) present in the ^{3}H-^{14}C-cholesterol administered to the plant is labeled with ^{14}C at C-1, -7, -15, -22 and -26.[13] If the cardenolides were biosynthesized by pathways involving side-chain cleavage between C-20 and C-22, two ^{14}C-labeled atoms located at C-22 and C-26 would be lost; the $^{3}H/^{14}C$ ratio of the derived

cardenolides would be $17{\cdot}0 \times 5/3 = 28{\cdot}3$. On the other hand, if the biosynthesis involved cleavage of the cholesterol side-chain between C-23 and C-24, only one ^{14}C-labeled atom would be lost. Then the expected $^{3}H/^{14}C$ ratio in the biosynthetic cardenolides would be $17{\cdot}0 \times 5/4 = 21{\cdot}3$. The experimentally determined $^{3}H/^{14}C$ ratios of 29·0, 28·1 and 28·1 obtained for digitoxigenin, digoxigenin and gitoxigenin, respectively, clearly indicate that the biosynthesis of cardenolides from cholesterol proceeds via C_{21} intermediates, probably resulting from cleavage of the side-chain between C-20 and C-22. The demonstration that the $^{3}H/^{14}C$ ratio of the ketone (VII) is identical with that of the digitoxigenin fortifies this deduction.

The progesterone (II) recovered from the incubation of the ^{3}H-^{14}C-cholesterol with a preparation of bovine adrenal mitochondria should show a ratio of 28·3 ($17{\cdot}0 \times 5/3$). The experimentally determined ratio of 26·1 was somewhat lower than anticipated. The reason for this discrepancy is obscure. However, it is unmistakable that the isotopic ratios of the progesterone and of the cardenolides indicate the retention of only three ^{14}C atoms of the cholesterol.

TABLE 1. THE $^{3}H/^{14}C$ RATIOS OF THE PRODUCTS RECOVERED AFTER THE ADMINISTRATION OF 4R-4-$^{3}H_1$-2-^{14}C-MEVALONIC ACID ($^{3}H/^{14}C$ RATIO 4·98) TO A *Digitalis lanata* PLANT

Compound	Crystallization	$^{3}H/^{14}C$ ratio
Digitoxigenin	1st	3·12
	2nd	1·97
	3rd	1·79
	4th	1·78
Tigogenin	2nd	2·06
	3rd	2·05
	4th	2·07
Pseudotigogenin	Crude product	1·92
	1st	1·98

5 weeks after the administration of the mixture of 4R-4-^{3}H-2-^{14}C-mevalonic acid ($^{3}H/^{14}C$ ratio 4·98) (IV) to a *D. lanata* plant, the plant was harvested and processed in the manner referred to above and digitoxigenin (VI) and tigogenin (X) were isolated. The $^{3}H/^{14}C$ ratios of the isolated tigogenin and digitoxigenin were determined (Table 1).

As indicated earlier, the transformation of MVA into cardenolides is assumed to proceed via a C_{27} intermediate of the cholesterol type. Such an intermediate derived from 4R-4-^{3}H-2-^{14}C-mevalonate should have the same distribution of the two isotopes as in the cholesterol (V) derived from the same precursor.[15] Hence it may be inferred that the C_{27} intermediate will be labeled at C-1, -7, -15, -22 and -26 with ^{14}C, and at C-17α, -20R and -24R* with tritium. The expected $^{3}H/^{14}C$ ratio of this intermediate should be $4{\cdot}98 \times 3/5 = 2{\cdot}99$. Consequently, the cardenolides biosynthesized from such a C_{27} intermediate by routes involving cleavage between C-20 and C-22 should have only one ^{3}H atom (at C-17α) and three ^{14}C-labeled atoms (at C-1, -7 and -15). The calculated $^{3}H/^{14}C$ ratio for the resulting products would be: $4{\cdot}98 \times 1/3 = 1{\cdot}66$. On the other hand, if cleavage occurred between C-23 and C-24 with retention of C-22 and C-23, then the cardenolides should possess one ^{3}H-labeled atom

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* We have now proved the 24R-configuration in cholesterol. See CASPI *et al.*, *Chem. Commun.* 45 (1969).

(at C-17α) and four ^{14}C-labeled atoms (at C-1, -7, -15 and -22); the $^{3}H/^{14}C$ ratio would be $4{\cdot}98 \times 1/4 = 1{\cdot}24$. The experimentally determined $^{3}H/^{14}C$ ratio of 1·78 for digitoxigenin, indicates that cleavage occurs between C-20 and C-22.

Thus, the results from both experiments conclusively establish that the biosynthesis of cardenolides from cholesterol proceeds via C_{21} intermediates and that carbons 22 and 23 do not originate from cholesterol.[1–4]

Our previous observations[6] suggested the operation of several pathways leading from cholesterol to the different cardenolides. In view of the present results, it may be concluded that transformations of the steroid nucleus (e.g. hydroxylations and/or saturation of the C-5 double bond, etc.) precede the side-chain scission between C-20 and C-22.

From the experiment with 4R-4-$^{3}H_1$-2-^{14}C-MVA (IV), we also isolated a small amount of ^{3}H-^{14}C-tigogenin which showed an isotopic ratio of 2·07. The biosynthesis of tigogenin from mevalonic acid has been shown to proceed via a C_{27} intermediate, which is converted into the spirostanols.[16] Consequently, the tigogenin derived from the ^{3}H-^{14}C-MVA (IV) should contain three atoms of tritium (at C-17, -20 and -24) and five atoms of ^{14}C (at C-1, -7, -15, -22 and -26), the expected $^{3}H/^{14}C$ ratio being 2·99 ($3/5 \times 4{\cdot}98$).[15] The experimentally determined value of 2·07 for the $^{3}H/^{14}C$ ratio of tigogenin is compatible only with the presence of two tritium atoms in the tigogenin ($4{\cdot}98 \times 2/5 = 1{\cdot}99$).

With a view to determine the position of the ^{3}H atom which was lost, the ^{3}H-^{14}C-tigogenin was converted to pseudotigogenin (XI).[17] The pseudotigogenin was found to have a $^{3}H/^{14}C$ ratio of 1·98, indicating the absence of ^{3}H at C-20. By inference, the two ^{3}H-labeled atoms persisting in tigogenin should be located at C-17 and C-24. Experimental evaluation of this deduction was not possible due to inadequate quantities of ^{3}H-^{14}C-tigogenin.

The loss of the C-20 tritium may have occurred either during the biosynthetic sequence or in the workup involving acid hydrolysis. Reversible, acid-catalyzed, sapogenin–pseudosapogenin transformations are well documented, and may be responsible for the loss of the C-20 tritium.[18–20] Experiments aimed at the elucidation of this problem are in progress.

EXPERIMENTAL

M.ps. were taken on a hot stage and are corrected. I.r. spectra were recorded in KBr wafers. NMR spectra were recorded at 60 Mc/sec, using $CDCl_3$ solutions. Peaks were recorded on a Varian M-66 instrument.

Chromatography

SiO_2 (Merck $HF_{254+366}$) was used for TLC in the indicated solvent systems. Thin-layer plates of silica gel impregnated with A_gNO_3 (20%) were used for the purification of tigogenin.

The specimens of labeled cholesterol employed were tested for chemical and isotopic purity by chromatography in several systems and by co-crystallization with non-radioactive material. Similarly, the chromatographically homogenous products isolated were further checked for purity and identity by co-crystallization to constant specific activity and constant $^{3}H/^{14}C$ ratio.

Counting

Counting was carried out in an automatic liquid scintillation counter. The samples were dissolved in 15 ml of a scintillator solution of toluene containing 4 g of 2,5-diphenyloxazole and 100 mg of *p*-bis-2-(5-phenyloxazolyl)-benzene per 1000 ml.

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Biosynthesis of Cholesterol from 2-^{14}C-*Mevalonic Acid*

The biosynthesis was carried out according to the procedure described by Cornforth *et al.*[13,15] 2-^{14}C-mevalonic acid (102 μc) was incubated aerobically at 37° for 3 hr, with the 10,000 g supernatant of a rat-liver homogenate prepared from 23 g of rat liver. The unsaponifiable residue was fractionated by TLC (ethyl acetate–hexane (3:7)), developed twice. The cholesterol zone was further purified by TLC (benzene–methanol (19:1)). The purified cholesterol contained 8·08 μc of ^{14}C.

Administration of 7-^{3}*H*-(1,7,15,22,26)-^{14}C-*Cholesterol to a* Digitalis lanata *Plant*

A mixture of the biosynthetic (1,7,15,22,26)-^{14}C-cholesterol (III) ($2{\cdot}23 \times 10^7$ dpm) and 7-^{3}H-cholesterol (^{3}H/^{14}C, ratio 17:0) (purchased from New England Nuclear Corp.) was administered to a *Digitalis lanata* plant, via the leaves, in the manner previously described.[6] The plant was illuminated with light of 12,000 lm/m^2 for 16 hr each day, and watered as necessary. The washings of the administration vial were applied in the same manner 6 days later. After 30 days, the plant was harvested and the leaves were rinsed with ethyl acetate to remove unabsorbed radioactive material. The aerial parts were processed as described previously.[6] The distribution of the radioactivity in the various extracts is summarized in Table 2.

TABLE 2. DISTRIBUTION OF RADIOACTIVITY IN EXTRACTS OF *Digitalis lanata* PLANT AFTER ADMINISTRATION OF 7-^{3}H-(1,7,15,22,26)-^{14}C-CHOLESTEROL ($2{\cdot}23 \times 10^7$ dpm OF ^{14}C; ^{3}H/^{14}C, RATIO 17·0)

Extract	Radioactivity ($\times 10^6$ dpm)		^{3}H/^{14}C ratio
	^{3}H	^{14}C	
Leaf washings	116	12·9	8·99
Ligroin extract	5·16	0·67	7·7
Chloroform extract	7·22	0·63	11·5
Chloroform–ethanol (2:1)	0·19	0·016	11·9
Chloroform–ethanol (3:2)	0·088	0·012	7·3

Isolation of Digitoxigenin (VI)

The chloroform extract was evaporated to dryness and the glycosides were hydrolyzed by treatment with aqueous methanolic H_2SO_4 as described previously.[6] The products were fractionated by TLC (ethyl acetate–benzene (4:1)), and the digitoxigenin zone (detected under u.v. light) was subjected to a second TLC (benzene–methanol (9:1)). A single symmetrical radioactive peak coincident with the digitoxigenin zone was observed. The eluate of the zone was diluted with nonradioactive digitoxigenin and crystallized several times from MeOH until the specific activity and the ^{3}H/^{14}C ratio were constant (Table 3).

Ozonolysis of Digitoxigenin Acetate

The ^{3}H-^{14}C-digitoxigenin (5 mg) was converted to the 3β-acetate by treatment with Ac_2O in pyridine. The crude, chromatographically homogenous, crystalline acetate was ozonized according to the method of Euw and Reichstein.[14] The acetate was dissolved in ethyl acetate (3 ml) and ozonized at −80°, until the solution remained faintly blue. After standing at −80° for 20 min, the solution was allowed to warm to 0°, and N_2 was bubbled through until the solution was colorless. Glacial acetic acid (0·3 ml), water (0·3 ml) and Zn dust (50 mg) were added, and the mixture was stirred for 1 hr at 0°. The reaction mixture was filtered and the residue was washed with ethyl acetate. The combined filtrate and washings were evaporated to near dryness. The residue was taken up in $CHCl_3$–ether (1:3) and washed twice with H_2O. The organic phase was then washed with a dil. $NaHCO_3$, H_2O, dried and evaporated. The product was dissolved in MeOH (3 ml) and treated with a solution of $KHCO_3$ (50 mg) in H_2O (1·5 ml). After standing for 19 hr the MeOH was removed *in vacuo* (below room temperature) and the residue was diluted with H_2O (10 ml). The crystalline product was extracted with $CHCl_3$ (3 × 15 ml); the extract was washed with H_2O and dried.

TLC of the crude product in two systems ($CHCl_3$–IsoPrOH [9:1]; and benzene–MeOH [9:1]) showed that it comprised a single major compound. The product was identified as 3β-acetoxy-14β,21-dihydroxy-pregnan-20-one (VII) by comparison with an authentic sample. The latter was prepared in a similar manner from cold digitoxigenin and a sample re-crystallized from acetone–isopropyl ether had a m.p. of 129–131°. The mass spectrum of the ketol (VII) showed prominent peaks at *m/e* 392 (M^+), 364 (M-28), 361 (M-31), 343 (361-18) and 301 (M-(60+31), base peak).

The ^{3}H-^{14}C-ketol (VII) was re-crystallized to constant specific activity and constant ^{3}H/^{14}C ratio (Table 3).

TABLE 3. THE $^3H/^{14}C$ RATIOS OF THE PRODUCTS (AND THEIR DERIVATIVES) RECOVERED AFTER THE ADMINISTRATION OF 7-^{3}H-(1,7,15,22,26)-^{14}C-CHOLESTEROL ($2{\cdot}23 \times 10^7$ dpm OF ^{14}C; $^3H/^{14}C$ RATIO 17·0). (*a*) TO A *Digitalis lanata* PLANT, (*b*) TO A PREPARATION OF BOVINE ADRENAL MITOCHONDRIA

	Crystallization	$^3H/^{14}C$ ratio
(*a*) Digitoxigenin (VI)	1st	28·5
	2nd	29·8
	3rd	29·0
3β-Acetoxy-14β,21-dihydroxypregnan-20-one (VII)	1st	25·8
	2nd	25·3
	3rd	28·9
	4th	28·7
Digoxigenin diacetate (VIII)	2nd	27·4
	3rd	27·6
	4th	28·1
Gitoxigenin diacetate (IX)	2nd	22·3
	3rd	24·1
	4th	25·4
	5th	28·1
(*b*) Progesterone (II)	1st	25·7
	2nd	26·1

Isolation of 3β,12β-*Diacetoxy Digoxigenin* (*VIII*) *and* 3β,16β-*Diacetoxy Gitoxigenin* (*IX*)

The zone containing the digoxigenin and gitoxigenin from TLC (ethyl acetate–benzene [4:1]) of the hydrolyzed $CHCl_3$ extract was subjected to a second TLC (chloroform–isopropyl alcohol [9:1]). The digoxigenin and gitoxigenin zones were located under u.v. light and the areas of radioactivity corresponding to these zones were extracted separately. The digoxigenin and gitoxigenin extracts were diluted with non-radioactive digoxigenin (20 mg) and gitoxigenin (15 mg), respectively, and each was converted to a diacetate by treatment with Ac_2O in pyridine. The diacetates were purified by TLC ($CHCl_3$–IsoPrOH [97:3]), and crystallized from ethyl acetate to constant specific activity and $^3H/^{14}C$ ratio (Table 3).

Incubation of the 7-^{3}*H*-(1,7,15,22,26)-^{14}C-*Cholesterol with a Bovine Adrenal Mitochondrial Preparation*

An acetone powder of bovine adrenal mitochondria (1 g)[21] was stirred with 0·02 M phosphate buffer (pH 7·4, 75 ml) at 0° for 1 hr. The mixture was then blended with a Potter–Elvehjem homogenizer. To this enzyme preparation was added 7-^{3}H-(1,7,15,22,26)-^{14}C-cholesterol ($5{\cdot}86 \times 10^5$ dpm of ^{14}C; $^3H/^{14}C$, ratio 17·0), suspended in water (2 ml) with Triton X-100 (40 mg). The mixture was incubated at 37° for 3 hr after the addition of: (i) NADP (10 mg) and glucose-6-P (160 mg) dissolved in 0·02 M phosphate buffer (pH 7·4, 5 ml); (ii) a solution of 3β-hydroxychol-5-enic acid (1·87 mg) in dimethylformamide (1 ml); (iii) glucose-6-P dehydrogenase (25 enzyme units). After 1 hr an equal portion of glucose-6-P dehydrogenase was added.

The incubate was then extracted several times with Et_2O–benzene (4:1). The combined extracts were dried and evaporated. The product was fractionated by TLC (CH_2Cl_2–MeOH (97:3); developed twice). The progesterone zone was detected under u.v. light and the area of radioactivity coincident with this zone was extracted. The extract was purified by sequential chromatography in the following systems: (i) TLC (benzene–ethyl acetate [1:4]); (ii) paper chromatography (heptane–MeOH–H_2O [20:19:1]) for 3·5 hr. The purified progesterone was mixed with nonradioactive progesterone (10 mg), and crystallized to constant specific activity and constant $^3H/^{14}C$ ratio (Table 3).

Administration of 4*R*-4-$^3H_1$-2-^{14}C-*Mevalonic Acid* (*IV*) *to a* Digitalis lanata *Plant*

(4R-4-$^3H_1$)-Mevalonic acid dibenzylethylenediamine salt (375 μc), prepared according to the procedure of Cornforth *et al.*,[22] was mixed with 2-^{14}C-mevalonic acid (75 μc) ($^3H/^{14}C$, ratio 4·98) and administered to a *D. lanata* plant by the "wick" method.[14] The plant was illuminated with light of 12,000 lm/m^2 for 16 hr each day and watered as necessary.

After 5 weeks the plant was harvested, and processed as previously described.[6,14] The distribution of the isotopes in the various extracts is given in Table 4.

[21] P. R. RAGGATT and M. W. WHITEHOUSE, *Biochem. J.* **101**, 819 (1966).

[22] J. W. CORNFORTH, R. H. CORNFORTH, C. DONNINGER and G. POPJÁK, *Proc. R. Soc.*, B **163**, 492 (1966).

TABLE 4. DISTRIBUTION OF TRACERS IN EXTRACTS OF A *Digitalis lanata* PLANT AFTER THE ADMINISTRATION OF 4R-4-$^3H_1$-2-^{14}C-MEVALONIC ACID (75 μc OF ^{14}C; $^3H/^{14}C$ RATIO 4·98)

Extract	Radioactivity ($\times 10^{-5}$ dpm) ^3H	^{14}C	$^3H/^{14}C$ ratio
Ligroin extract	22·86	1·93	11·81
Chloroform extract	18·70	1·44	12·97
Chloroform–ethanol (2:1)	1·52	0·22	6·93
Chloroform–ethanol (3:2)	2·65	0·45	5·83

Isolation of Digitoxigenin (VI)

The isolation of digitoxigenin was carried out on the hydrolysate of the $CHCl_3$ extract.[6,14] The aglycones were fractionated by TLC (benzene–ethyl acetate (9:1); developed 3×). The digitoxigenin zone was then purified by sequential chromatography (TLC) in: (i) ethyl acetate–benzene (4:1), (ii) benzene–MeOH (9:1).

The purified digitoxigenin was diluted with nonradioactive digitoxigenin (30 mg) and crystallized several times from acetonitrile until the specific activity and $^3H/^{14}C$ ratio were constant (Table 1). On the basis of tritium and ^{14}C present in the digitoxigenin cocrystallized to constant specific activity and constant ratio, the digitoxigenin in the total $CHCl_3$ extract contined $6·13 \times 10^3$ dpm of ^{14}C and $11·0 \times 10^3$ dpm of ^3H.

Isolation of Tigogenin (X)

Tigogenin was isolated from the combined $CHCl_3$–ethanol (2:1) and (3:2) extracts (see Table 3). After evaporation of the solvents, benzene (50 ml) and 3 N EtOH H_2SO_4 (50 ml) were added to the residue and the mixture was refluxed for 4 hr in N_2. H_2O (200 ml) was then added and the phases were separated. The aqueous portion was then extracted with isobutanol (3 × 50 ml). The combined extracts were washed with ice-cold H_2O (2 × 20 ml), 2 N KOH (40 ml) and ice-cold water (2 × 20 ml). The organic phase was dried and evaporated. The product was subjected to TLC (benzene–ethyl acetate (4:1); developed 2×). The tigogenin zone was detected under u.v. light, and the area of radioactivity corresponding to the tigogenin zone was extracted. The extract was then purified by TLC on silica gel impregnated with A_gNO_3 (20%) (benzene–ethyl acetate (9:1); developed 2×). The radioactivity corresponding to the tigogenin zone was extracted. This extract was diluted with nonradioactive tigogenin (20 mg) and crystallized several times from acetone, until the specific activity and $^3H/^{14}C$ ratio were constant (Table 4). On the basis of the ^3H and ^{14}C present in the tigogenin crystallized to constant specific activity and constant ratio, the tigogenin in the total ethanol–$CHCl_3$ extracts contained $3·16 \times 10^3$ dpm of ^{14}C and $6·51 \times 10^3$ dpm of ^3H.

^3H-^{14}C-Pseudotigogenin (XI)

The preparation of pseudotigogenin from tigogenin was carried out essentially as described by Cameron *et al.*[17] The ^3H-^{14}C-tigogenin was further diluted with cold material (15 mg) and treated with *n*-octanoic acid (2 ml). The mixture was refluxed in N_2 in a Dean-Stark apparatus for 4 hr. The reaction mixture was cooled and extracted with Et_2O (2 ml). The Et_2O extract was washed with 2 N NaOH (2 × 3 ml). The washings were re-extracted with Et_2O (2 × 1 ml). The combined Et_2O extracts were washed with H_2O (2 ml), dried and evaporated. The product was treated with 5% KOH (w/v) in MeOH (5 ml) and heated under reflux for 30 min. The reaction mixture was diluted with H_2O and extracted with Et_2O. The Et_2O extract was washed, dried and evaporated. The crude solid product was purified by TLC (benzene–ethyl acetate–methanol, 85:10:5), crystallized from acetonitrile and identified as pseudotigogenin by comparison with an authentic sample. The authentic sample was prepared by the method described above and had a m.p. 184–188° (reported[17] 179–189°); λ_{max}^{KBr} 3350 (OH; broad); 1690 and 1270 (C=C—O—C), and 1060 cm^{-1}; NMR ($CDCl_3$) *ca.* 280 (broad multiplet; 1H(16αH)), *ca.* 213 (broad, partly obscured multiplet; 3αH), 209 (doublet, J=6 cps; 2H, 27-CH_2OH); 96 (21-CH_3), 57 (doublet, J=6 cps, 26-CH_3); 50 (19-CH_3) and 40 (18-CH_3) cps; mass spectrum *m/e* 416 (M^+); 398 (M-18); 347 (M-72; $(CH_3.HOH_2C.C{=}CH_2)^+$); 273 (M-143); 181 ($C_{11}H_{18}O_2$).

The ^3H-^{14}C pseudotigogenin was recrystallized from acetonitrile and counted (Table 1).

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