Biosynthesis of Plant Steroids. I. The Origin of the Butenolide Ring of Digitoxigenin[1]

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Digitoxin isolated from Digitalis plants which had been fed mevalonic acid-2-C^{14} was radioactive. However, contrary to expectations, no activity was detected in the butenolide ring of the steroidal aglycone digitoxigenin. On the other hand, digitoxigenin obtained from plants which had been fed acetate-1-C^{14} was labeled at C-20 and C-23. Radioactive digitoxigenin derived from mevalonic acid-3'-C^{14} had one-third of its activity located at C-21 and the rest at C-18 and C-19. These results are consistent with the hypothesis that digitoxigenin is formed by the condensation of a pregnane derivative (derived from mevalonic acid via squalene) with one molecule of acetic acid.

It is now well established that the animal steroid cholesterol is produced by the cyclization of squalene which is derived from six molecules of mevalonic acid.[4] When we commenced our work on the biosynthesis of digitoxin (digitoxigenin-3-tridigitoxose), we assumed, as a working hypothesis, that digitoxigenin (III, 3β,14-dihydroxy-5β-card-20(22)-enolide) would be formed in *Digitalis* plants by an analogous series of reactions. Cholesterol (II) derived from mevalonic acid-2-C^{14} (I) is labeled at C-1, C-7, C-15, C-22, and C-26, and we considered that the four carbons of the butenolide ring of digitoxigenin were derived from the C-17 side chain of a steroid or triterpene related to cholesterol, in which the eight-carbon side chain had been cleaved between C-23 and C-24 as illustrated in Figure 1. We tested this hypothesis by feeding DL-mevalonic acid-2-C^{14} to *Digitalis purpurea* and *D. mertonensis* plants by means of a cotton wick inserted into the stems.[5] Digitoxin was isolated from the plants using the procedure developed by Gisvold.[6] Since the digitoxin isolated from *D. purpurea* had a higher specific activity than that isolated from *D. mertonensis*, we have used the former species in all subsequent feeding experiments. The radioactive digitoxin[7] was hydrolyzed in methanol with dilute sulfuric acid yielding digitoxigenin which was degraded by the procedure illustrated in Figure 2. Acetylation afforded 3-acetyldigitoxigenin (IV) which was ozonized and the resultant ozonide reduced with zinc and acetic acid affording the glycolic ester V.[8] Hydrolysis of this ester with potassium bicarbonate yielded glycolic acid, which could be isolated as its *p*-bromophenacyl ester, and 3β-acetoxy-14β,21-dihydroxy-5β-pregnan-20-one (IX). Cleavage of this ketol with periodate yielded formaldehyde and 3β-acetoxy-14β-hydroxy-5β-androstane-17β-carboxylic acid (VIII). This acid was esterified with diazomethane and then dehydrated with phosphorus oxychloride in pyridine. The resultant acid VI was hydrolyzed with ethanolic sodium hydroxide af-

Table I. Administration of Tracers to *Digitalis purpurea* Plants

Expt. no.	Precursor fed	Wt., mg.	Activity, mc.	Method of feeding	Digitoxin Wt., mg.	Digitoxin Activity, d.p.m./mmoles	Incorporation,[a] %
1	Mevalonic acid-2-C^{14}	38.2	0.2	Hydroponics	...	...	0.0015[b]
2	Mevalonic acid-2-C^{14}	19.1	0.1	Wick	...	...	0.030[b]
3	Sodium acetate-1-C^{14}	4.1	0.25	Wick	...	...	0.006[b]
4	Mevalonic acid-2-C^{14}	4.7	0.1	Excised leaves	162	5.25×10^5	0.051
5	Mevalonic acid-2-C^{14}	4.7	0.1	Spraying	20	3.20×10^6	0.038
6	Mevalonic acid-2-C^{14}	4.7	0.1	Spraying	13	5.48×10^6	0.042
7	Mevalonic acid-3'-C^{14}	53	0.023	Spraying	38	5.68×10^5	0.056

[a] Incorporation is defined here as the total radioactivity present in the isolated digitoxin divided by the total activity fed to the plant. [b] In these experiments the crude radioactive digitoxin was diluted with inactive material and hydrolyzed, and the resultant digitoxigenin was purified as its 3-acetyl derivative. The incorporation reported is therefore based on the activity of the 3-acetyldigitoxigenin.

(1) A preliminary account of part of this work has appeared as a communication: H. Gregory and E. Leete, *Chem. Ind.* (London), 1242 (1960). This investigation was supported by a research grant (GM-13246) from the U. S. Public Health Service and in part by a Du Pont grant-in-aid to H. G.

(2) Alfred P. Sloan Foundation Fellow.

(3) Fellow of the Consejo Nacional de Investigaciones Cientificas y Técnicas, Argentina.

(4) Recent reviews: G. Popjak and J. W. Cornforth, *Advan. Enzymol.*, **22**, 281 (1960); E. Staple in "Biogenesis of Natural Compounds," P. Bernfeld, Ed., Pergamon Press, Inc., New York, N. Y., 1963, p. 155; J. H. Richards and J. B. Hendrickson in "The Biosynthesis of Steroids, Terpenes and Acetogenins," W. A. Benjamin, Inc., New York, N. Y., 1964, p. 305.

(5) C. L. Comar in "Radioisotopes in Biology and Agriculture," McGraw-Hill Book Co., Inc., New York, N. Y., 1955, p. 151. Initial feeding experiments were carried out by adding the tracer to the nutrient solution in which the *Digitalis* plants were growing hydroponically; however, the incorporation of tracer into the cardiac glycosides was very low (*cf.* Experimental, Table I). More recently we have sprayed aqueous solutions of labeled mevalonic acid onto the leaves of *Digitalis* plants growing in soil and obtained a satisfactory incorporation of tracer into digitoxigenin.

(6) O. Gisvold, *J. Pharm. Sci.*, **52**, 83 (1963).

(7) E. Ramstad and J. L. Beal, *Chem. Ind.* (London), 177 (1960), and *J. Pharm. Pharmacol.*, **12**, 552 (1960), fed mevalonic acid-2-C^{14} to *Digitalis lanata* plants and obtained radioactive lanatoside-A (digitoxigenin–digitoxose–digitoxose–acetyldigitoxose–glucose) in which all the radioactivity was confined to the digitoxigenin; however, no degradations were carried out to determine the distribution of activity.

(8) M. Zingg and K. Meyer, *Helv. Chim. Acta*, **43**, 145 (1960).

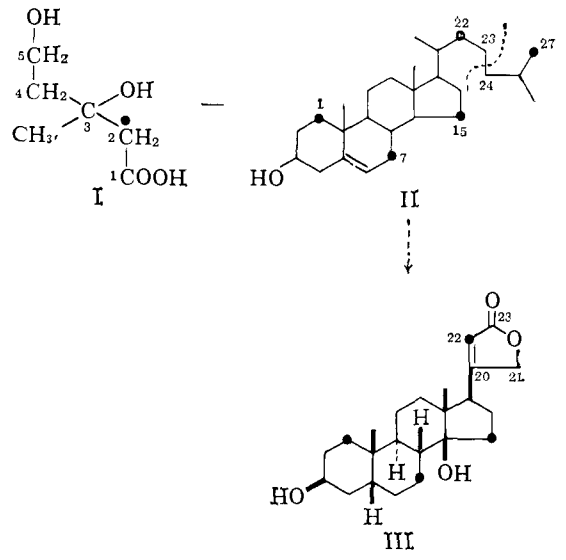

Figure 1. Original hypothesis for the biosynthesis of digitoxigenin.

fording 3β-hydroxy-5β-androst-14(15)-ene-17β-carboxylic acid (VII) which was subjected to a Schmidt reaction with sodium azide and concentrated sulfuric acid. The activities of the degradation products of digitoxigenis derived from meva lonic acid-2-C^{14} are recorded in Table II, and it was found that the ester VI had the same specific activity as 3-acetyldigitoxigenin, indicating that C-21, C-22, and C-23 were devoid of radioactivity. Furthermore the Schmidt reaction on the etianic acid VII yielded inactive carbon dioxide, indicating that there was no activity at C-20 either.

Table II. Degradation Products of Digitoxigenin

	Activity, d.p.m./mmoles	Relative activity
(a) Derived from mevalonic acid-2-C^{14}		
3-Acetyldigitoxigenin (IV)	2.74×10^4	100
3β-Acetoxy-17β-methoxycarbonyl-5β-androst-14(15)-ene (VI)	2.75×10^4	100
Barium carbonate[a]	$<0.01 \times 10^4$	0
(b) Derived from acetate-1-C^{14}		
3-Acetyldigitoxigenin (IV)	1.31×10^4	100
Barium carbonate[b] (C-23)	0.11×10^4	8.5
Formaldehyde dimedone[b] (C-22)	$<0.01 \times 10^4$	0
Barium carbonate[c] (C-20)	0.074×10^4	5.7
Formaldehyde dimedone[d] (C-21)	$<0.01 \times 10^4$	0
(c) Derived from mevalonic acid-3′-C^{14}		
Digitoxigenin (III)	1.87×10^4	100
3-Acetyldigitoxigenin (IV)	1.90×10^4	102
1-Acetamidonaphthalene[e]	0.62×10^4	33
Barium carbonate[f]	$<0.01 \times 10^4$	0
N-Methylbenzamide[f]	0.60×10^4	32
p-Bromophenacyl glycolate	$<0.01 \times 10^4$	0
Formaldehyde dimedone[d]	0.58×10^4	31
3β-Acetoxy-14β-hydroxy-5β-androstane-17β-carboxylic acid (VIII)	1.21×10^4	65

[a] Obtained from a Schmidt reaction on the acid VII. [b] Obtained by the periodate cleavage of glycolic acid. [c] Obtained by a Schmidt reaction on the etianic acid VIII. [d] Obtained by the periodate cleavage of the ketol IX. [e] Derivative of the acetic acid obtained by the Kuhn–Roth oxidation of digitoxigenin. [f] Obtained by a Schmidt reaction on the previously mentioned acetic acid.

This result led us to consider other hypotheses for the origin of the butenolide ring. Tschesche[9] suggested that

(9) R. Tschesche, *Fortschr. Chem. Org. Naturstoff.*, **12**, 131 (1955).

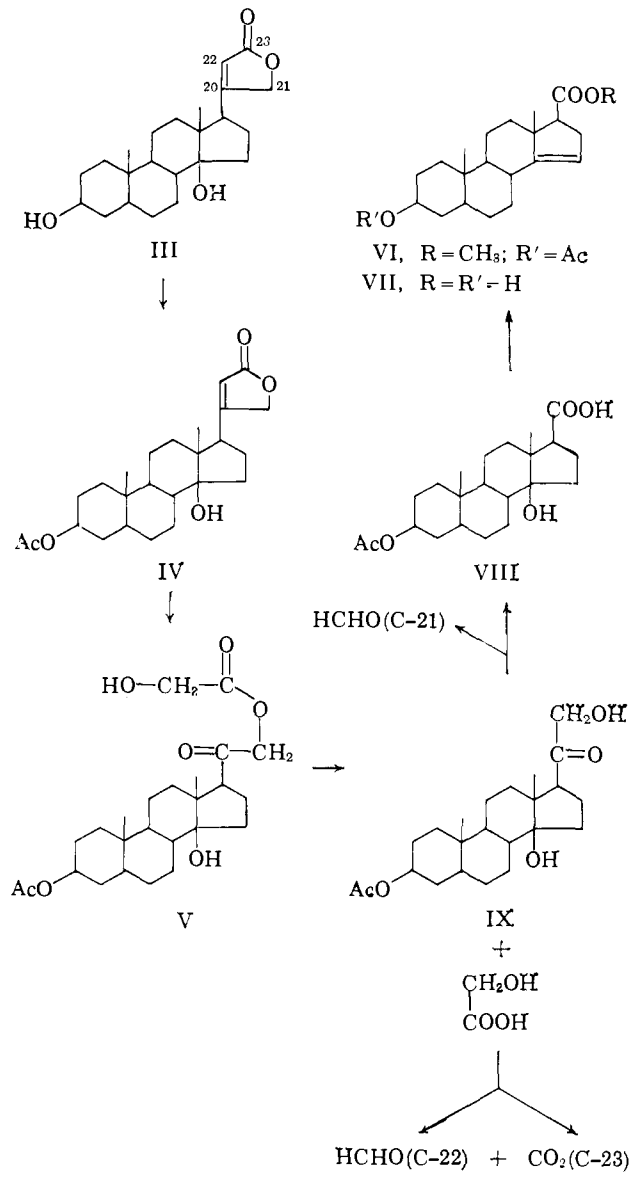

Figure 2. Degradation of digitoxigenin derived from acetate-1-C^{14} and mevalonic acid-2-C^{14}.

the lactone ring is formed from two molecules of acetic acid (presumably activated as acetyl coenzyme A) with an etianic acid as illustrated by means of partial formulas in Figure 3. Reaction of acetyl coenzyme A with the etianic acid X yields a β-keto acid XI which decarboxylates to a pregnane derivative XII. Reaction of this ketone with an additional molecule of acetate affords the β-hydroxy acid XIII. The unsaturated acid XIV obtained on dehydration then undergoes allylic oxidation at the methyl group, and the resultant γ-hydroxy-α,β-unsaturated acid lactonizes to the butenolide XV. To test this hypothesis sodium acetate-1-C^{14} was fed to *D. purpurea* plants. Radioactive digitoxigenin was obtained and the butenolide ring was degraded as before. Labeling of the ring had occurred with significant amounts of activity located at C-20 and C-23 and essentially no activity at C-21 and C-22. There was also considerable labeling of the steroid nucleus, but this was to be expected since acetate is a precursor of mevalonic acid. Our tracer results with acetate-1-C^{14} are thus consistent with Tschesche's hypothesis.

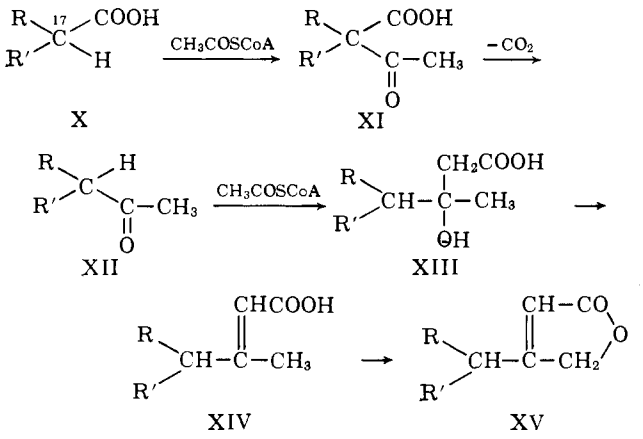

Figure 3. Hypothesis for the formation of the butenolide ring.

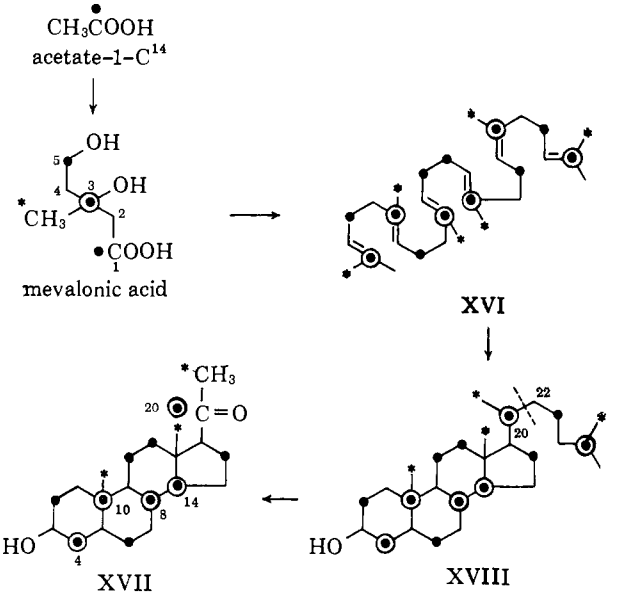

Figure 4.

However, an alternate route to the butenolide ring, also discussed by Tschesche,[10] is illustrated in Figure 4. Mevalonic acid-1,3,5-C^{14} derived from acetate-1-C^{14} is converted to squalene labeled at the positions indicated with heavy dots in formula XVI. The side chain of the resultant steroid XVIII could then be cleaved between C-20 and C-22 to yield a pregnane derivative XVII which would be labeled at C-20. Reaction with one molecule of acetate-1-C^{14} would then yield a butenolide ring labeled at C-20 and C-23. Recently Euw and Reichstein[11] have carried out experiments which favor this latter hypothesis. They fed mevalonic acid-3-C^{14} to *Digitalis lanata* plants and obtained digitoxigenin which had a significant amount of activity located at C-20.[12] We have also carried out independent experiments which favor the intermediate formation of a pregnane derivative. A pregnane derivative formed from mevalonic acid-3′-C^{14} would be labeled at only three positions: C-18, C-19, and C-21 (indicated by asterisks in formula XVII). DL-Mevalonic acid-3′-C^{14} was prepared from ethyl acetate-2-C^{14} using the procedure of Tschesche and Machleidt.[13] This labeled compound was sprayed on the leaves of intact *D. purpurea* plants. The digitoxin isolated 2 weeks later was radioactive and the activities of its degradation products are recorded in Table II. In agreement with theory we found one-third of the activity located at C-21. A Kuhn–Roth oxidation of digitoxigenin yielded acetic acid derived from the angular methyl groups (C-18 and C-19) and their adjacent carbons. We have found that the most reliable way of assaying acetic acid for radioactivity is to convert it to 1-acetamidonaphthalene which is readily soluble in the toluene solution used in liquid scintillation counting. Details for the preparation of this compound from a few milligrams of sodium acetate are recorded in the Experimental section. The acetic acid obtained from digitoxigenin had all its activity located on the methyl group and was one-third that of the steroid, indicating that the suspected pregnane precursor is produced by the same metabolic route as that occurring in animal tissues. Additional data which favors a pregnane intermediate had recently been obtained by Tschesche and Lilienweiss[14] who fed the 3β-D-glucopyranoside of Δ^5-pregnen-3β-ol-20-one-21-C^{14} to *D. lanata* plants and isolated digitoxigenin which contained 2.2% of the administered tracer. Circumstantial evidence favoring a pregnane precursor has also been presented by Euw and Reichstein,[11] who point out that pregnane derivatives have been found in *Digitalis* and related plant species. Our results from the acetate-1-C^{14} feeding experiment are also in agreement with the latter proposed biosynthetic scheme. If this hypothesis is correct we would expect to find the activity at C-23 (formed fairly directly from the carboxyl group of an acetate unit) somewhat higher than the activity at C-20 (formed from acetate *via* mevalonate, squalene, etc.). This, in fact, was found to be the case.

Experimental[15]

DL-*Mevalonic Acid-3′-C^{14}*. Starting with 1.1 g. of ethyl acetate-2-C^{14} (1.6×10^9 d.p.m.),[16] mevalonic acid-3′-C^{14} (112 mg., 1.4×10^8 d.p.m./mmoles) was obtained by the procedure of Tschesche and Machleidt.[13]

Administration of Tracers of Digitalis Plants and Isolation of Digitoxigenin. The *Digitalis* plants were grown in a greenhouse and were between 3 and 4 months old

(10) R. Tschesche, *Angew. Chem.*, 73, 727 (1961).

(11) J. v. Euw and T. Reichstein, *Helv. Chim. Acta*, 47, 711 (1964).

(12) Mevalonic acid-3-C^{14} labels squalene at the positions indicated with open circles [⊙], and the resultant pregnane derivative XVII should have its activity equally divided between C-4, C-8, C-10, C-14, and C-20. Therefore there should theoretically have been 20% of the activity located at C-20 of digitoxigenin. Only 14.4% was detected at this position. This divergence from theory was attributed to two causes. (a) A small amount of radioactivity was detected at C-21, C-22, and C-23 and it was assumed that some randomization of activity had occurred resulting in general labeling. (b) Schmidt reactions on a model substance, cyclohexane-carboxylic acid-7-C^{14} yielded carbon dioxide, collected as barium carbonate, which was less active than expected. It was therefore suggested that the carbon dioxide obtained by a Schmidt reaction on the etianic acid obtained by the degradation of digitoxigenin would be less active than expected.

(13) R. Tschesche and H. Machleidt, *Ann.*, 61, 631 (1960).

(14) R. Tschesche and G. Lilienweiss, *Z. Naturforsch.*, **19b**, 266 (1964).

(15) Some of the radioactive compounds were assayed on aluminum planchets in a Nuclear Chicago Q gas-flow counter, making corrections for self-absorption and geometry. More recently samples have been counted in a Nuclear Chicago Model 724 liquid scintillation spectrometer, using as solvents toluene or dioxane–water with the usual scintillators (*cf.* A. R. Friedman and E. Leete, *J. Am. Chem. Soc.*, 85, 2141 (1963)).

(16) Purchased from Tracerlab, Waltham, Mass.

when fed tracers. The amounts of tracer used in each experiment and the method of feeding is recorded in Table I. In the experiments where tracers were administered by spraying the leaves, an aqueous solution of the tracer was carefully directed onto the center of each leaf from a small chromatographic spray bottle operated with filtered air from the lungs of the senior author. In experiment 1 the plants were grown in soil and then transferred to a hydroponic setup where the roots were washed clean and then placed in a beaker containing aerated nutrient solution[17] to which the tracer was added. In experiment 4 the leaves were cut off with a razor blade and the cut ends were placed in small beakers containing solutions of the tracer. In all the experiments the plants were harvested 2 weeks after the initial feeding of tracer and the digitoxin was isolated by established methods.[6] The radioactive digitoxin was diluted with inactive material and crystallized to constant specific activity prior to the subsequent degradations. The activities recorded in Table II are for the diluted digitoxin and its derivatives.

Degradation of Digitoxin to Determine Activity at C-20, C-21, C-22, and C-23. The diluted digitoxin (1.0 g.) was hydrolyzed by refluxing in a mixture of methanol (50 ml.) and 0.1 *N* sulfuric acid (50 ml.) for 1 hr. The methanol was then removed *in vacuo* and the residual aqueous solution was extracted with chloroform. The chloroform extract, dried over sodium sulfate, was evaporated to yield crude digitoxigenin. After crystallization from ethanol, the digitoxigenin was dissolved in pyridine (12 ml.), and acetic anhydride (6 ml.) was added. After standing for 24 hr. at room temperature the solution was added to water (200 ml.). The precipitated 3-acetyldigitoxigenin was crystallized from a mixture of acetone and ether until material, m.p. 227–228°, of constant specific activity, was obtained (400–450 mg.). The 3-acetyldigitoxigenin was dissolved in propyl chloride (40 ml.) and cooled to −70°, and ozone was passed in for 10 min. when the solution became blue. After standing for an additional 20 min. the solution was treated at 0° with zinc dust (2.0 g.) and 50% aqueous acetic acid (12 ml.). After stirring for 2 hr. the reaction mixture was kept at 0° for 24 hr. The zinc was then filtered off and the filtrate was evaporated to dryness. The residual sirup was dissolved in chloroform, washed with 10% sodium carbonate solution and water, and then dried over magnesium sulfate. The residue obtained on evaporation of the chloroform was dissolved in methanol (12 ml.), and 6 ml. of 10% aqueous potassium bicarbonate was added. After standing at room temperature for 20 hr. water (12 ml.) was added and the methanol was removed *in vacuo*. The ketol XI was extracted from the aqueous solution in chloroform which was then washed with water and dried over magnesium sulfate. The residual aqueous solution was made acidic with hydrochloric acid and extracted with ether in a continuous extractor for 20 hr. In the case of the degradation involving digitoxin derived from mevalonic acid-3′-C^{14} the residue obtained on evaporation of the ether extract was dissolved in water (2 ml.), neutralized with sodium hydroxide, and then added to a solution of *p*-bromophenacyl bromide (50 mg.) in ethanol (15 ml.). The mixture was refluxed for 2 hr., then cooled, and water was added until the solution became turbid. After standing overnight, crystalline *p*-bromophenacyl glycolate separated out (25 mg.) and had m.p. 137–139° after two crystallizations from ethanol.[18]

In the case of the degradation involving digitoxin derived from acetate-1-C^{14} the residue obtained on evaporation of the ether was dissolved in a phosphate buffer having pH 5.8, and all traces of carbon dioxide were swept out of the solution by means of a stream of pure nitrogen. The flow of nitrogen was then stopped and a solution of sodium metaperiodate (120 mg.) in water (2 ml.) was added. After 24 hr. nitrogen was blown through the solution and the evolved carbon dioxide was collected as barium carbonate. The oxidation mixture was then cooled, and 6 *N* sulfuric acid (1 ml.), 20% potassium iodide (1 ml.), and enough sodium arsenite solution to remove the iodine color were added. On addition of an aqueous solution of dimedone (0.4%, 15 ml.), formaldehyde–dimedone derivative crystallized out.

The chloroform solution of the ketol XI was evaporated and the residue was dissolved in methanol (2 ml.). A solution of periodic acid (250 mg.) in water (0.6 ml.) was added and the solution was allowed to stand at 25° for 2 hr. The solution was then evaporated *in vacuo* at 25°, the distillate being collected in a trap cooled to −70°. The contents of the trap was dissolved in water and treated with dimedone solution (0.4%, 15 ml.) when the formaldehyde–dimedone derivative separated out. The residue in the flask was dissolved in ether and dried over magnesium sulfate. The residue obtained on evaporation of the ether was crystallized from a mixture of acetone and petroleum ether yielding 3β-acetoxy-14β-hydroxy-5β-androstane-17β-carboxylic acid (VIII), m.p. 228–230°.

A Schmidt reaction was carried out on this acid as follows. The acid VIII (60 mg.) dissolved in dry benzene (3 ml.) was mixed at 0° with sodium azide (40 mg.) and concentrated sulfuric acid (0.5 ml.). The mixture was then warmed to 45° and the evolved carbon dioxide was collected as barium carbonate (15 mg., 48%).

In the degradation of the digitoxin derived from mevalonic acid-2-C^{14} the acid VIII was treated with an ether solution of diazomethane and the resultant ester was dehydrated with a solution of phosphorus oxychloride in pyridine. After standing overnight, the reaction mixture was added to water and the unsaturated ester was extracted with ether. The residue obtained on evaporation of the dried ether was chromatographed on Woelm alumina (activity II). The ester VI was eluted from the column with petroleum ether (b.p. 60–68°)–benzene (1:1), and after crystallization from aqueous methanol had m.p. 120–121°. This ester (41 mg.) was hydrolyzed by refluxing for 1 hr. with 0.5 *N* ethanolic sodium hydroxide (attempted hydrolysis with methanolic potassium hydroxide failed completely). The solution was acidified and extracted with ether. The dried ether on evaporation yielded the acid VII which was dissolved in chloroform (3 ml.) and treated with sodium azide (40 mg.) and concentrated sulfuric acid (0.5 ml.) at 0°. The mixture was warmed to 45° and the evolved carbon dioxide was collected as barium carbonate (15 mg.).

(17) E. Leete, *J. Am. Chem. Soc.*, 78, 3520 (1956).

(18) The reduction of the ozonide to the glycolic ester V is discussed by M. Zingg and K. Meyer, *Helv. Chim. Acta*, 43, 145 (1960).

Kuhn–Roth Oxidation of Digitoxigenin. Digitoxigenin (77 mg.) was added to a solution of chromium trioxide (5 g.) in 2 *N* sulfuric acid (10 ml.) and the mixture was distilled. Water was added to the distillation flask to maintain the volume at about 10 ml. When about 80 ml. of distillate had been collected it was titrated with 0.1 *N* sodium hydroxide (2.2 ml.) and then evaporated to dryness. The residue was crystallized from ethanol–ether affording sodium acetate (18 mg.).

1-Acetamidonaphthalene. Sodium acetate (2 mg.) and 1-aminonaphthalene hydrochloride (4 mg.) were dissolved in water (1 ml.). 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride[19] (20 mg.) was added. After stirring for a few minutes with a glass rod, the oily precipitate which was first formed became solid and was filtered off after 10 min. The pink precipitate (3–4 mg.) was dried at 60° and then sublimed *in vacuo* (140°, 0.01 mm.). The white sublimate was dissolved in a little boiling benzene. On addition of petroleum ether 1-acetamidonaphthalene separated out as colorless needles (2–3 mg.), m.p. 159–160°.

Acknowledgments. We thank Robert McLeester (Botany Department) and Professor Herbert Jonas (School of Pharmacy) for the cultivation of the *Digitalis* plants. We also thank Professor Ole Gisvold (School of Pharmacy) for providing us with a detailed method for the isolation of digitoxin prior to publication.[6]

(19) J. C. Sheehan, P. A. Cruickshank, and G. L. Boshart, *J. Org. Chem.*, **26**, 2525 (1961). The compound is available from the Ott Chemical Co., Nuskegon, Mich.

Biosynthesis of Plant Steroids. II. The Distribution of Activity in Digitoxigenin Derived from Mevalonic Acid-2-C^{14} [1]

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The steroid moiety of digitoxigenin, the aglycone of the cardiac glycoside digitoxin, has been degraded systematically. It has been established that digitoxigenin, derived from mevalonic acid-2-C^{14}, is labeled only at C-1, C-7, and C-15, and the activity is equally divided between these positions. This result strongly suggests that the plant steroids are produced from squalene by the same series of metabolic reactions as those which lead to animal steroids such as cholesterol.

In the previous paper[4] evidence was obtained which strongly favored the formation of digitoxigenin from a pregnane derivative and one molecule of acetic acid. It was assumed as a working hypothesis that the pregnane was formed in *Digitalis* plants from mevalonic acid, *via* squalene, by the same route as that which leads to the formation of animal steroids. There is now considerable evidence, circumstantial[5] and experimental,[6] which supports this general hypothesis. However, in none of these experiments involving radioactive precursors was the steroid nucleus degraded to determine the complete pattern of labeling. In mammalian tissues the cyclization of squalene derived from mevalonic acid-2-C^{14} leads to a pregnane derivative labeled at C-1, C-7, and C-15. Our degradation of digitoxigenin, illustrated in Figure 1, was thus designed to determine directly the activity as these three positions.

Activity at C-1 was obtained by the following route. Digitoxigenin (VI) was dehydrated with dilute sulfuric acid and the resultant diene was hydrogenated over platinum. The 3-hydroxy group was then oxidized with chromium trioxide yielding 3-oxo-5β-cardanolide (I).[7] This ketone was brominated in acetic acid affording 2,4-dibromo-3-oxo-5β-cardanolide which was dehydrobrominated to 3-oxo-card-1,4-dienolide (II) by refluxing in collidine. This dienone was hydroxylated with 2 moles of osmium tetroxide and the resultant 1,2,4,5-tetrahydroxy-3-oxo-cardanolide oxidized with lead tetraacetate yielding 1,5-dioxo-1,5-seco-A-trisnorcardanolide (III). This β-ketoaldehyde was cleaved on refluxing with ethanolic sodium hydroxide to formic acid and the ketone IV in which the methyl group at C-10 can be expected to have the more stable (equatorial) α-configuration.[8] The formic acid which originated from C-1 of digitoxigenin was collected as its *p*-bromophenacyl ester.

To obtain information on the activity at C-7, digitoxigenin was converted to 7β-hydroxydigitoxigenin (V) by means of the microorganism *Rhizopus nigricans*.[9]

(1) A preliminary account of part of this work has appeared as a communication: E. G. Gros and E. Leete, *Chem. Ind.* (London), 698 (1963), and was also presented at a lecture at the 148th National Meeting of the American Chemical Society, Chicago, Ill., Aug. 30–Sept. 4, 1964, Abstracts, p. 26C. This investigation was supported by a research grant (GM-13246) from the U. S. Public Health Service.

(2) Fellow of the Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina.

(3) Alfred P. Sloan Foundation Fellow.

(4) E. Leete, H. Gregory, and E. G. Gros, *J. Am. Chem. Soc.*, **87**, 3475 (1965).

(5) C. Djerassi in "Biochemistry of Steroids," E. Mosettig, Ed., Pergamon Press, Inc., New York, N. Y., 1958, p. 1.

(6) H. J. Nicholas, *J. Biol. Chem.*, **237**, 1485 (1962); E. Capstack, D. J. Baisted, W. W. Newschwander, G. Blondin, N. L. Rosen, and W. R. Nes, *Biochemistry*, **1**, 1178 (1962); D. J. Baisted and W. R. Nes, *J. Biol. Chem.*, **238**, 1947 (1963); S. Bader, L. Guglielmetti, and D. Arigoni, *Proc. Chem. Soc.* (London), 16 (1964); A. R. Battersby and G. V. Parry, *Tetrahedron Letters*, 787 (1964).

(7) H. M. E. Cardwell and S. Smith, *J. Chem. Soc.*, 2012 (1954).

(8) J. Castells, E. R. H. Jones, G. D. Meakins, and R. W. J. Williams, *ibid.*, 1159 (1959).

(9) We are indebted to Sra. Elba T. Gros for carrying out this microbiological oxidation.