The Role of Deoxycorticosterone in the Biosynthesis of Cardenolides in *Digitalis lanata*

By E. CASPI, J. A. F. WICKRAMASINGHE AND D. O. LEWIS*
 Worcester Foundation for Experimental Biology, Shrewsbury, Mass. 01545, U.S.A.

(Received 22 January 1968)

The role of deoxycorticosterone in the biosynthesis of digitoxigenin was investigated by the simultaneous administration of deoxy[1,2-³H₂]corticosterone and [4-¹⁴C]progesterone to a *Digitalis lanata* plant. The biosynthetically formed [³H,¹⁴C]digitoxigenin and deoxy[³H,¹⁴C]corticosterone were isolated and the distribution of the two isotopes in these products was determined. The transformation of progesterone into deoxycorticosterone *in vivo* was established. The biosynthetic route from progesterone via deoxycorticosterone to cardenolides was found to be of little significance.

The biosynthesis of sterols in plants is at present assumed to follow a pattern analogous to that in animals (Heftmann, 1967; Frantz & Schroepfer, 1967). This view is fortified by the demonstration of the biosynthesis of cholesterol from mevalonic acid in *Digitalis purpurea* (Jacobsen & Frey, 1967). In accordance with this, the transformation of cholesterol into pregnenolone (I) (Caspi, Lewis, Piatak, Thimann & Winter, 1966) and the conversion of the latter into cardenolides (Tschesche & Lilienweiss, 1964; Tschesche & Brassat, 1966) and bufadienolides (Tschesche & Brassat, 1965) in plants has been demonstrated. Further studies on the metabolism of pregnenolone (I) in *Digitalis lanata* have revealed that this homallylic alcohol is converted into progesterone (II) and that the formation of a 3-ketone is an obligatory step in the biosynthesis of cardenolides (Caspi & Lewis, 1967; Caspi & Hornby, 1968).

The sequence of events leading from the C₂₁ precursors pregnenolone (I) and progesterone (II) to cardenolides must include the introduction of two oxygen functions, namely at C-14 and C-21. Studies on the mechanism of hydroxylation at C-14 are in progress in this Laboratory (Caspi & Lewis, 1968). The present paper deals with the question of C-21 hydroxylation and the possible involvement of deoxycorticosterone (V) in the biosynthesis of cardenolides.

The rationale of our approach was to administer simultaneously a mixture of [4-¹⁴C]progesterone (II) and deoxy[1,2-³H₂]corticosterone (V) to a *Digitalis lanata* plant, and to evaluate the relative extents of incorporation of the two tracers into

digitoxigenin (III). As mentioned above, in the route from pregnenolone to cardenolides progesterone is formed and is incorporated to the extent of about 2% into digitoxigenin (Caspi & Lewis, 1967; Caspi & Hornby, 1968). Therefore, as a first approximation, it was expected that the relative importance of the biosynthetic roles of the two steroids could be defined from changes in the ³H/¹⁴C ratio of the biosynthesized products.

EXPERIMENTAL

Chromatography. Silica gel (Merck HF₂₅₄) was used for thin-layer chromatography in the indicated solvent systems. Strips (40 cm. long) of Whatman paper no. 1 were used for paper chromatography. With 'Bush systems' the papers were pretreated before development (Caspi & Lewis, 1967; Bush & Crowshaw, 1965).

Radioactivity measurement. Counting was carried out in a Nuclear-Chicago automatic liquid-scintillation counter model Mark I. The samples were dissolved in 15 ml. of a scintillator solution of toluene containing 4 g. of 2,5-diphenyloxazole and 100 mg. of 1,4-bis-(5-phenyloxazol-2-yl)benzene/l.

The ³H/¹⁴C ratios were determined by using the following expression:

$$\frac{{}^3\text{H}}{{}^{14}\text{C}} = \left(\frac{A}{B} - X \right) \frac{E_{14c}}{E_{3H}}$$

where *A* is the recorded counts in the ³H channel, *B* is the recorded counts in the ¹⁴C channel, *X* is the fraction of ¹⁴C recorded in the ³H channel (this fraction was determined on the basis of the leak of counts of standard [¹⁴C]toluene from the ¹⁴C channel into the ³H channel; the leak of ³H into the ¹⁴C channel was insignificant and was disregarded), *E*_{3H} is the efficiency of ³H counting in the ³H channel and *E*_{14c} is the efficiency of ¹⁴C counting in the ¹⁴C channel. *E*_{3H} and *E*_{14c} were determined with the use of standard [³H]toluene and [¹⁴C]toluene respectively.

* Present address: Toms River Chem. Co., Toms River, N.J. 08753, U.S.A.

(III) R=H

(IV) R=CH₃·CO

Administration of the mixture of deoxy[1,2-³H₂]corticosterone and [4-¹⁴C]progesterone to *Digitalis lanata*. A mixture of deoxy[1,2-³H₂]corticosterone (V) (190 μc) and [4-¹⁴C]progesterone (17.2 μc of ¹⁴C) (³H/¹⁴C ratio 11.05) was administered to a whole *Digitalis lanata* plant in the manner previously described (Caspi & Lewis, 1967; Caspi & Hornby, 1968; Bennett & Heftmann, 1965). The plant was illuminated with light of 12000 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 4 weeks the plant was harvested, the leaves were rinsed with ethyl acetate to remove unabsorbed radioactive material and then processed as previously described (Caspi

& Lewis, 1967; Caspi & Hornby, 1968; Euv & Reichstein, 1964). The distribution of the isotopes in the various extracts is given in Table 1.

Isolation of digitoxigenin (III). The isolation of digitoxigenin was carried out on portions of the hydrolysates of the chloroform extract (Caspi & Lewis, 1967; Caspi & Hornby, 1968; Euv & Reichstein, 1964) by thin-layer chromatography. A portion of the aglycones (20%) was mixed with non-radioactive digitoxigenin (3 mg.) and fractionated by thin-layer chromatography on silica gel with benzene-ethyl acetate (1:4, v/v). The digitoxigenin was located under ultraviolet light, and the area of radioactivity corresponding to this zone was extracted. The

Table 1. *Distribution of isotopes in extracts of Digitalis lanata plant after the administration of deoxy[1,2-³H₂]corticosterone (190 μ C) and [4-¹⁴C]-progesterone (17.2 μ C) (³H/¹⁴C ratio 11.05)*

Extract	10 ⁻⁷ × Radioactivity (disintegrations/ min.)		³ H/ ¹⁴ C ratio
	³ H	¹⁴ C	
Leaf washings	0.27	0.03	9.04
Light petroleum	0.56	0.02	26
Chloroform	6.40	0.88	7.3
Chloroform-ethanol (2:1)	1.5	0.16	9.45
Chloroform-ethanol (3:2)	1.53	0.04	35.2
Percentage recovery in 'sterol fractions'*	24.04	29.32	

* Corrected for the unabsorbed radioactivity.

extract was then purified by continuous thin-layer chromatography for 3 hr. on silica gel with benzene-methanol (50:1, v/v). A single radioactive symmetrical peak coinciding with the zone of digitoxigenin was observed. The eluate of this zone contained 1.32×10^6 disintegrations/min. of ¹⁴C (equivalent to the incorporation of 3.51% of ¹⁴C) and 6.56×10^6 disintegrations/min. of ³H (equivalent to the incorporation of 1.58% of ³H). The results were corrected for the amounts of the isotopes left in the vial from which the steroids were administered and for those found in the leaf washings. The extract was diluted with non-radioactive digitoxigenin (50 mg.) and crystallized several times from ethyl acetate until the ³H/¹⁴C ratio was constant (Tables 2 and 3).

Digitoxigenin acetate (IV). A sample of the diluted [³H,¹⁴C]digitoxigenin (10 mg.) was converted into the 3 β -acetate, crystallized from ethyl acetate and counted (Table 2).

Oxidation of digitoxigenin to digitoxigenone (VI). The [³H,¹⁴C]digitoxigenin (20 mg.) was further diluted with non-radioactive material (20 mg.), dissolved in acetone (5 ml.) cooled to 10° and treated with Jones's reagent (0.25 ml.). The reaction mixture was stirred for 5 min., then poured into aq. NaHCO₃ soln. and the product isolated with ethyl acetate. The chromatographically homogeneous digitoxigenone (VI) gave an infrared spectrum identical with that of authentic digitoxigenone. Samples of the digitoxigenone were then counted (Table 2).

Isodigitoxigenone (VII). The radioactive digitoxigenone (VI) (33 mg.) was heated under reflux for 16 hr. with 5% (w/v) KOH in aq. 80% (v/v) methanol (1 ml.) under N₂. The reaction mixture was diluted with water and the isodigitoxigenone was recovered with ethyl acetate from the acidified aqueous phase. The product was purified by continuous thin-layer chromatography for 4 hr. on silica gel with ethyl acetate-benzene (2:3, v/v). The isodigitoxigenone (VII) was located under ultraviolet light, extracted and counted. On re-equilibration of this material, the ³H/¹⁴C ratio remained unchanged (Table 2). The twice-equilibrated product was identified as isodigitoxigenone (VII) by its chromatographic behaviour, infrared spectroscopy and mass spectrometry. The mass-spectroscopic

Table 2. *³H/¹⁴C ratio of products recovered after the administration of deoxy[1,2-³H₂]corticosterone (190 μ C) and [4-¹⁴C]progesterone (17.2 μ C) (³H/¹⁴C ratio 11.05) to a Digitalis lanata plant*

³H/¹⁴C ratios are given as the means with individual values in parentheses.

	³ H/ ¹⁴ C ratio
Digitoxigenin (III)	
First crystallization	5.21 (5.19, 5.24)
Second crystallization	4.85 (4.75, 4.95)
Third crystallization	4.81 (4.66, 4.96)
Mother liquor (third crystallization)	4.88
Digitoxigenin acetate (IV)	
First crystallization	4.54 (4.64, 4.85, 4.12)
Digitoxigenone (VI)	4.80 (4.82, 4.79)
Isodigitoxigenone (VII)	2.94 (2.804, 3.086)
Isodigitoxigenone (VII) from re-equilibration	2.82
Deoxycorticosterone	
First crystallization	9.71 (9.60, 9.83)
Second crystallization	10.15 (10.21, 10.1)
17 β -Carboxyandrost- 4-en-3-one	10.27 (10.31, 10.23)

Table 3. *Percentage incorporation of exogeneous deoxy[1,2-³H₂]corticosterone and [4-¹⁴C]progesterone into the biosynthesized products*

	Incorporation (%)			
	Expt. 1		Expt. 2	
	¹⁴ C	³ H	¹⁴ C	³ H
Digitoxigenin (III)	3.51	1.58	3.75	1.59
Deoxycorticosterone (V)	0.076	0.066		

fragmentation pattern was identical with that of authentic isodigitoxigenone: *m/e*: 372 (*M*⁺; 86%); 354 (*M*-H₂O; 9%); 328 (*M*-CO₂; 48%); 288 (*M*-C₄H₄O₂; 22%); 248 (100%).

Isolation of deoxycorticosterone (V). A portion of the hydrolysate of the chloroform extract (25%) was submitted to thin-layer chromatography on silica gel with benzene-ethyl acetate (1:4, v/v). The zone corresponding to deoxycorticosterone (*R_F* 0.77; digitoxigenin *R_F* 0.5) was extracted and rechromatographed on silica gel with benzene-ethyl acetate (1:1, v/v). Unlabelled material (50 μ g.) was added to the extract (to facilitate detection) and the mixture was purified by sequential chromatography on paper in the following systems: (i) light petroleum (b.p. 60-90°)-toluene-methanol-water (33:17:40:10, by vol.) for 3½ hr.; (ii) toluene-propylene glycol for 3½ hr. Each chromatogram was scanned for radioactivity, and the deoxycorticosterone zone was located under ultraviolet light. Only the area of the chromatogram where radioactivity coincided with the deoxycorticosterone zone was extracted.

The purified deoxycorticosterone containing 2.84×10^4 disintegrations/min. of ^{14}C (equivalent to the net incorporation of 0.076% of ^{14}C) and 2.7×10^5 disintegrations/min. of ^3H (equivalent to the net incorporation of 0.066% of ^3H) was mixed with 20mg. of non-radioactive deoxycorticosterone, then crystallized twice from acetone-ethyl acetate and counted (Table 2).

17 β -Carboxyandrost-4-en-3-one (Δ^4 -3-oxoetiocholenic acid) (VIII). The above biosynthetic deoxy[^3H , ^{14}C]corticosterone (16mg.) was dissolved in ethanol (4ml.) and treated with 5.3ml. of 1% (w/v) sodium metaperiodate soln., pH 4 (Caspi, Ungar & Dorfman, 1957). After standing for $2\frac{1}{2}$ hr. at room temperature in the dark, the reaction mixture was diluted with water made basic and the neutral material removed by extraction with ether. The aqueous phase was then acidified and extracted with chloroform. The chloroform extract was washed with saturated Na_2SO_3 soln. and then water, dried and evaporated. The 17 β -carboxyandrost-4-en-3-one (VIII) (13mg.), the infrared spectrum of which was identical with that of authentic material, was crystallized from chloroform-light petroleum. The $^3\text{H}/^{14}\text{C}$ ratio of the acid was determined (Table 2).

Determination of the ^3H content at C-2 of deoxy[1,2- $^3\text{H}_2$]corticosterone. To a solution of deoxy[1,2- $^3\text{H}_2$]corticosterone (130mg.; 2.42×10^5 disintegrations/min./m-mole) in ethanol (24ml.) was added 31ml. of 1% sodium metaperiodate soln., pH 4 (Caspi *et al.* 1957). After standing for $2\frac{1}{2}$ hr. at room temperature in the dark, the reaction mixture was processed as described above to yield the Δ^4 -3-oxoetiocholenic acid (VIII) (106mg.; 2.21×10^5 disintegrations/min./m-mole). The acid (106mg.) was dissolved in methanol (4.8ml.), and 1.2ml. of 0.045N-KOH was added. The reaction mixture was heated under reflux in an atmosphere of N_2 for 4 hr. A sample was removed and the remainder heated for a further 4 hr. The aetio acid (VIII), isolated with chloroform, from the acidified sample had a specific activity of 1.2×10^5 disintegrations/min./m-mole. The specific activity of the acid isolated from the residual reaction mixture heated for 8 hr. remained unchanged (1.2×10^5 disintegrations/min./m-mole).

RESULTS AND DISCUSSION

It is appropriate to point out at the outset that the administration of two different precursors constitutes a complicated biosynthetic model. It is obvious that the relative rates of absorption, transport and metabolism of these exogenously supplied steroids will differ. There is also little doubt that the supply of 'massive amounts' of the two steroids would disturb the existing physiological equilibria. Burstein & Dorfman (1962) and Burstein & Bhavnani (1967) attempted a mathematical analysis of such models and indicated the difficulties encountered in the interpretation of the results. Hence any conclusions drawn from the present studies are subject to such errors incurred, and must be viewed with caution.

At the termination of the experiment the plant was harvested and processed in the manner described earlier (Caspi & Lewis, 1967; Caspi & Hornby, 1968; Euv & Reichstein, 1964). The distribution

of the two isotopes in the various extracts was determined, and considerable variations were observed (Table 1). However, this is not surprising on the basis of the limitations indicated above.

The isolation of digitoxigenin (III) was carried out on the chloroform extract and the isotopic ratio ($^3\text{H}/^{14}\text{C}$) found (4.8; Table 2) was less than half of that (11.05) of the initially administered mixture of steroids. The decrease in the $^3\text{H}/^{14}\text{C}$ ratio could be interpreted either as a consequence of a greater incorporation of ^{14}C into digitoxigenin, or the loss of ^3H from the enolizable C-2 of deoxycorticosterone.

To determine whether ^3H was lost from C-2 during biosynthetic transformations, the [^3H , ^{14}C]digitoxigenin was oxidized to digitoxigenone (VI). The transformation proceeded without loss of ^3H . Equilibration of the digitoxigenone (VI) in aq. methanolic potassium hydroxide (Caspi & Hornby, 1968) gave isodigitoxigenone (VII), which had a $^3\text{H}/^{14}\text{C}$ ratio 2.28, indicating a loss of 42% of ^3H .

It was now considered necessary to evaluate the amount of ^3H at C-2 of the deoxy[1,2- $^3\text{H}_2$]corticosterone administered to the plant. Consequently a sample of the deoxy[1,2- $^3\text{H}_2$]corticosterone (2.42×10^5 disintegrations/min./m-mole) was oxidized with periodic acid (Caspi *et al.* 1957) to the aetio acid (VIII) (2.21×10^5 disintegrations/min./m-mole). The slight loss of the isotope observed is probably the consequence of some acid-catalysed equilibration during the reaction. Base-catalysed equilibration of the acid gave a specimen of the aetio acid (VIII) of specific activity 1.21×10^5 disintegrations/min./m-mole. It is evident that 50% of the ^3H of the deoxy[1,2- $^3\text{H}_2$]corticosterone is located at C-2. Judged from the amount of ^3H detected at C-2 of the biosynthetic digitoxigenone (VI) (42%), about 8% of the ^3H of the administered deoxy[1,2- $^3\text{H}_2$]corticosterone remained unaccounted for. Presumably this loss may be ascribed to certain conditions *in vivo* favouring enolization. A critical review of the biosynthetic results summarized in Tables 2 and 3 indicated, however, that this discrepancy (8%) will not affect (significantly) our deductions and may therefore be disregarded. It may thus be inferred that the observed $^3\text{H}/^{14}\text{C}$ ratio of digitoxigenin (4.8) was indicative of a greater incorporation of the [^{14}C]progesterone. Hence at the end of the experiment the amount of digitoxigenin (III) originating from [^{14}C]progesterone was about 2.3 times that from deoxy[1,2- ^3H]corticosterone. The overall incorporation of ^{14}C into digitoxigenin was about 3.6% and that of ^3H was about 1.6%.

The question arose whether the pathway progesterone (II) $\rightarrow$ deoxycorticosterone (V) $\rightarrow$ digitoxigenin (III) is a major biosynthetic route. Should this be the case the transformation of a considerable

amount of the [4- ^{14}C]progesterone into deoxycorticosterone would be expected. To evaluate the validity of this hypothesis, the isolation of deoxycorticosterone from the plant material was undertaken. The recovered deoxycorticosterone contained both ^3H and ^{14}C in a ratio 10.2. Additional, unambiguous, proof for the incorporation of ^{14}C into the deoxycorticosterone was obtained by converting the latter into the aetio acid (VIII), which had a $^3\text{H}/^{14}\text{C}$ ratio 10.6.

In view of these observations, there is little doubt that the plant has the capacity to convert progesterone into deoxycorticosterone. When the plant was harvested, 0.076% of the exogenous progesterone was 'present' as deoxycorticosterone ($^3\text{H}/^{14}\text{C}$ ratio 10.2). At this point it may be recalled that at 'zero time' the isotope ratio of the deoxycorticosterone was infinite. Consequently, if the route progesterone $\rightarrow$ deoxycorticosterone $\rightarrow$ digitoxigenin was of major importance a relatively high $^3\text{H}/^{14}\text{C}$ ratio would have been expected for the digitoxigenin. Obviously this was not the case, and the relatively low $^3\text{H}/^{14}\text{C}$ ratio (4.8) found for the digitoxigenin tends to indicate that this is not a major biosynthetic pathway. The view is also supported by the aforementioned fact that, though the overall incorporation of [4- ^{14}C]progesterone into digitoxigenin was unusually large (3.6%), that of deoxy[1,2- $^3\text{H}_2$]corticosterone was lower (1.6%). Whether the high incorporation of progesterone, which normally is about 2%, is seasonal (the experiment was carried out in June) or the result of a metabolic 'potentiation effect' caused by the administered deoxycorticosterone cannot be decided on the basis of these results.

In summary, the present observations indicate that the plant has the capacity to introduce a hydroxyl group at C-21 of progesterone and to convert deoxycorticosterone into cardenolides. Neither of these two reactions appear to be on the main biosynthetic route from progesterone to

digitoxigenin. It may be inferred that in the sequence of events leading from progesterone to cardenolides other transformations precede the introduction of a C-21 oxygen function.

This paper constitutes part 6 in a series on the biosynthesis of plant sterols (part 5: Caspi, 1968). This work was supported by U.S. Public Health Grants HE10566 and CA K316614 from the National Institute of Health. D.O.L. was a Post-doctoral Fellow at the Worcester Foundation during 1965-67, and was the holder of a special Post-doctoral Fellowship 1F3-HE344771-01 from the U.S. Public Health Service. J.A.F.W. is currently (since 1967) a Post-doctoral Fellow at the Worcester Foundation.

REFERENCES

- Bennett, R. D. & Heftmann, E. (1965). *Phytochemistry*, **4**, 475.
Burstein, Sh. & Bhavnani, B. (1967). *Endocrinology*, **8**, 351.
Burstein, Sh. & Dorfman, R. I. (1962). *Acta endocr., Kbh.*, **40**, 188.
Bush, I. E. & Crowshaw, K. (1965). *J. Chromat.* **19**, 114.
Caspi, E. (1968). *Proc. Symp. Dtsch. Akad. Wiss. Berlin: Über biochemische Aspekte der Steroidforschung, Berlin* (in the Press).
Caspi, E. & Hornby, G. M. (1968). *Phytochemistry*, **7**, 423.
Caspi, E. & Lewis, D. O. (1967). *Science*, **156**, 519.
Caspi, E. & Lewis, D. O. (1968). *Phytochemistry*, **7**, 683.
Caspi, E., Lewis, D. O., Piatak, D. M., Thimann, K. V. & Winter, A. (1966). *Experientia*, **22**, 506.
Caspi, E., Ungar, F. & Dorfman, R. I. (1957). *J. org. Chem.* **22**, 326.
Euv, J. von & Reichstein, T. (1964). *Helv. chim. Acta*, **47**, 711.
Frantz, I. D. & Schroepfer, G. J., jun. (1967). *Annu. Rev. Biochem.* **36**, 691.
Heftmann, E. (1967). *Lloydia*, **30**, 209.
Jacobsen, G. M. & Frey, M. J. (1967). *J. Amer. chem. Soc.* **89**, 3338.
Tschesche, R. & Brassat, B. (1965). *Z. Naturf.* **20b**, 707.
Tschesche, R. & Brassat, B. (1966). *Z. Naturf.* **21b**, 894.
Tschesche, R. & Lilienweiss, G. (1964). *Z. Naturf.* **19b**, 226.