Cardenolide Biosynthesis in Foxglove[1]

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Abstract: The article reviews the state of knowledge on the biosynthesis of cardenolides in the genus *Digitalis*. It summarizes studies with labelled and unlabelled precursors leading to the formulation of the putative cardenolide pathway. Alternative pathways of cardenolide biosynthesis are discussed as well. Special emphasis is laid on enzymes involved in either pregnane metabolism or the modification of cardenolides. About 20 enzymes which are probably involved in cardenolide formation have been described "downstream" of cholesterol, including various reductases, oxido-reductases, glycosyl transferases and glycosidases as well as acyl transferases, acyl esterases and P450 enzymes. Evidence is accumulating that cardenolides are not assembled on one straight conveyor belt but instead are formed via a complex multidimensional metabolic grid. For example "fucose-type" cardenolides and "digitoxose-type" cardenolides seem to form via different biosynthetic branches and the "norcholanic acid pathway" identified recently seems to be operative only in the formation of fucose-type cardenolides.

Key words: Biosynthesis, cardenolides, cardiac glycosides, glycosidase, glycosyl transferase, enzymes, feeding experiments, *Digitalis*, oxido-reductases, secondary metabolism, Scrophulariaceae, tracer studies.

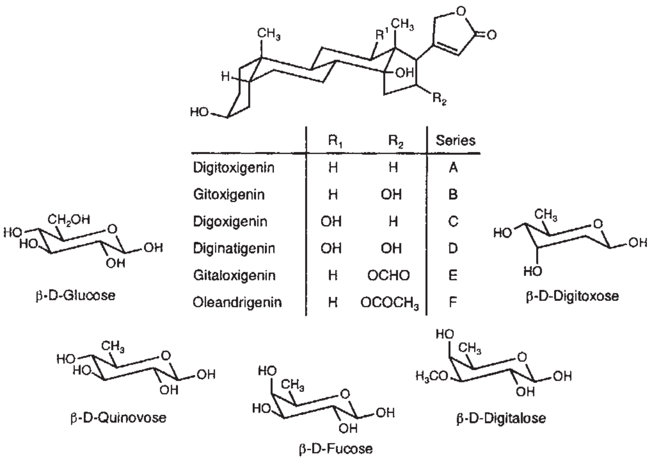
Abbreviations:

GPC:	gel permeation chromatography
SDS-PAGE:	sodium dodecyl sulphate polyacrylamide gel electrophoresis

Introduction

About 80 different glycosides of the cardenolide-type occur in the genus *Digitalis* (1). The *Digitalis* cardenolides are characterized by a steroid nucleus with rings connected *cis-trans-cis*; it has a 14β-hydroxy group, and an unsaturated five-membered lactone ring is substituted at C-17β. A sugar side chain with up to five carbohydrate units containing glucose and various rare 6-deoxy, 2,6-dideoxy and 6-deoxy-3-methoxy sugars, such as D-fucose, D-digitoxose or D-digitalose, is attached at position 3β. According to their genin part they are divided into 6 series, termed A through F (Fig. **1**). Most of the genuine cardiac glycosides present in *Digitalis* species have a terminal glucose; these cardenolides have been termed primary glycosides. After harvest or during the controlled fermentation of dried *Digitalis* leaves most of the primary glycosides are hydrolyzed to yield the so-called secondary glycosides. *Digitalis* cardenolides are valuable drugs in the medication of patients suffering from cardiac insufficiency. In therapy genuine glycosides, such as the lanatosides, are used as well as compounds obtained after enzymatic hydrolysis and chemical saponification, for example digitoxin (**31**) and digoxin, or chemical modification of digoxin, such as metildigoxin. *Digitalis lanata* Ehrh. and *D. purpurea* L. are the major sources of the cardiac glycosides most frequently employed in medicine.

The putative biosynthetic pathway leading to the cardenolides (Fig. **2**) is basically deduced from studies using radiolabelled precursors. For more details, the reader is referred to previous reviews by Grunwald (2) and Schütte (3). The more recent identification and characterization of various enzymes involved in pregnane and cardenolide metabolism have further clarified the pathway and this knowledge may now open up a possible route for manipulating cardenolide biosynthesis in plants. In this review emphasis is laid on cardenolide formation, whereas cardenolide degradation, biotransformation and storage are not discussed in great depth.



	R_1	R_2	Series
Digitoxigenin	H	H	A
Gitoxigenin	H	OH	B
Digoxigenin	OH	H	C
Diginatigenin	OH	OH	D
Gitaloxigenin	H	OCHO	E
Oleandrigenin	H	$OCOCH_3$	F

Fig. 1 The *Digitalis* cardenolides are composed of six different genins and various sugars including 6-deoxy and 2,6-dideoxy sugars.

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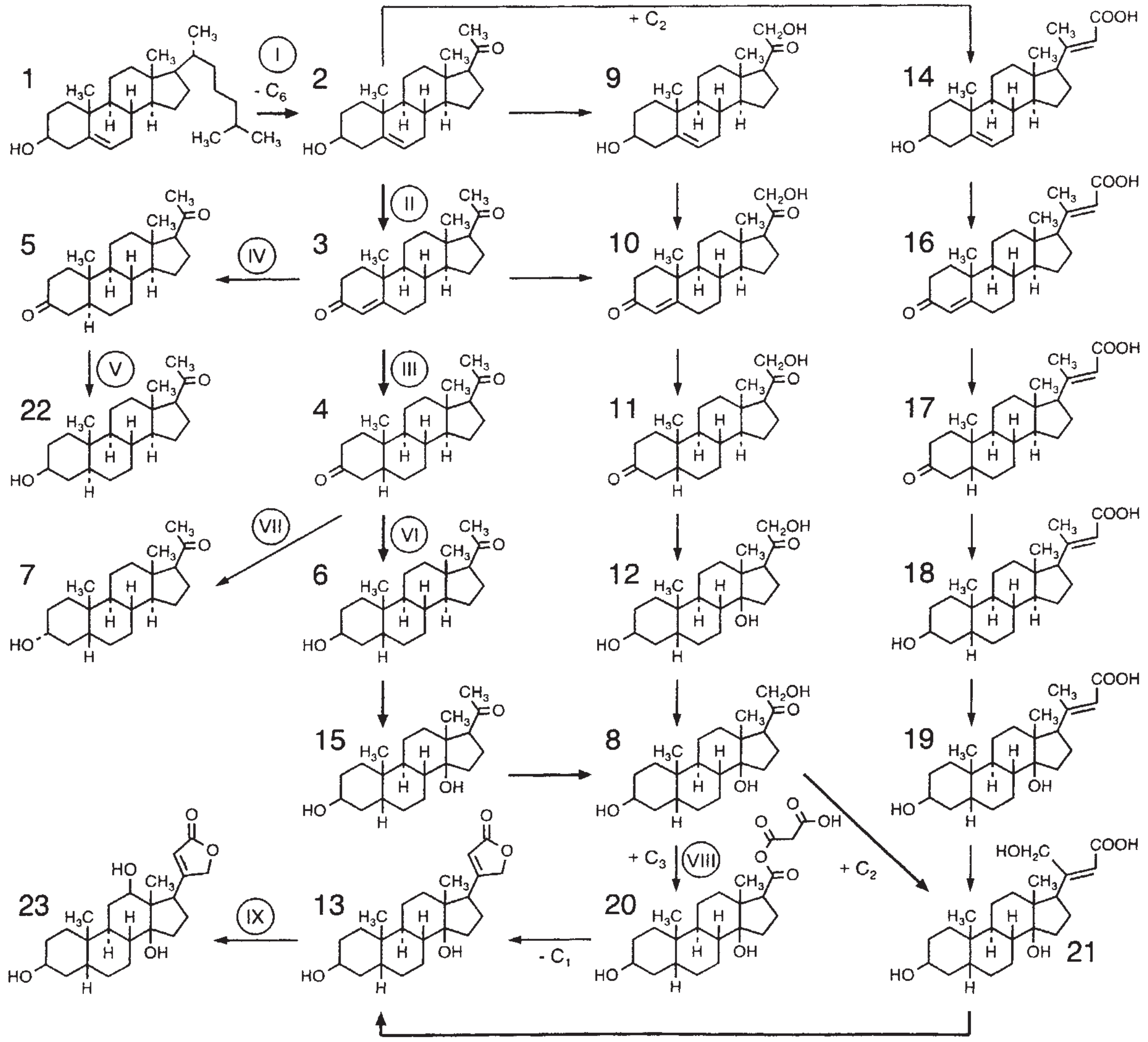

Fig. 2 Routes for cardenolide genin formation in *Digitalis*. The putative cardenolide pathway as depicted in standard text books is traced with bold arrows. However, evidence is accumulating that cardenolides are not assembled on a straight conveyor belt but rather are formed via a complex multidimensional metabolic grid. The scheme shown here does not consider pregnane glycosides which may be obligate intermediates in cardiac glycoside formation (see text). **1**, Cholesterol; **2**, pregnenolone; **3**, progesterone; **4**, 5β-pregnane-3,20-dione; **5**, 5α-pregnane-3,20-dione; **6**, 5β-pregnan-3β-ol-20-one; **7**, 5β-pregnan-3α-ol-20-one; **8**, 5β-pregnane-3β,14β,21-triol-20-one; **9**, pregnen-21-ol-20-one; **10**, cortexone; **11**, 5β-pregnan-3β,21-diol-20-one; **12**, 5β-pregnan-21-ol-3,20-dione; **13**, digitoxigenin; **14**, 23-nor-5,20(22)*E*-choladienic acid-3β-ol; **15**, 5β-pregnane-3β,14β-diol-20-one; **16**, 23-nor-4,20(22)*E*-choladienic acid-3-one; **17**, 23-nor-5β-chol-20(22)*E*-enic acid-3-one; **18**, 23-nor-5β-chol-20(22)*E*-enic acid-3β-ol; **19**, 23-nor-5β-chol-20(22)*E*-enic acid-3β,14β-diol; **20**, 5β-pregnane-3β,14β-diol-21-O-malonyl hemiester; **21**, 23-nor-5β-chol-20(22)*E*-enic acid-3β,14β,21-triol; **22**, 5α-pregnane-3βol-20 one; **23**, digoxigenin. **I**, *Cholesterol side-chain cleaving enzyme (SCCE)*; **II**, *NAD: Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3β-HSD)*; **III**, *NADPH: progesterone 5β-reductase (5β-POR)*; **IV**, *NADPH: progesterone 5α-reductase (5α-POR)*; **V**, *NADPH: 3β-hydroxysteroid 5α-oxidoreductase (3β-HS-5α-OR)*; **VI**, *NADPH: 3β-hydroxysteroid 5β-oxidoreductase (3β–HS-5β-OR)*; **VII**, *NADPH: 3α-hydroxysteroid 5β-oxidoreductase (3α-HS-5β-OR)*; **VIII**, *malonyl-coenzyme A:21-hydroxypregnane 21-O-malonyltransferase (MHPMT)*; **IX**, *Digitoxin 12β-hydroxylase (D12H)*.

Studies with Precursors *in vivo*

The putative pathway of cardenolide formation as found in standard text boods was basically deduced from studies with radiolabelled precursors carried out mainly in the 1960s and 1970s. While summarizing the results from these studies it has to be considered that exogenous substances have to pass various barriers, such as biological membranes, before reaching the site of synthesis. In this respect, sterols seem to be particularly critical compounds, due to their affinity to membranes which may lead to false negative results, since these precursors may simply not enter the cardenolide pathway. In addition, compartmentalization and metabolite channelling have to be taken into consideration. Depending on the tissue investigated and the method and site of precursor application, the compound in question may be modified in various ways prior to reaching the site of cardenolide synthesis. Hence, results obtained in different experimental systems may differ and may sometimes lead to contradictory conclusions.

Cardenolides are steroids and thus supposed to be derived from mevalonic acid via the triterpenoid pathway. As early as 1960, it was found that 2-^{14}C-mevalonic acid is incorporated into the steroid part of **31** (4). Later on, degradation experiments revealed that the label of 2-^{14}C-mevalonic acid appeared in C-1, C-7 and C-15 of the cardenolide genin (5). In similar experiments with 3′-^{14}C-mevalonic acid the label appeared in C-18, C-19 and C-21 (6). Evidence against a route via a C_{20}-steroid and in favour of a route via C_{21}-pregnanes was obtained by feeding 3-^{14}C-mevalonic acid (7) and [1,7,15,22,26-$^{14}C_5$]-cholesterol (**1**) (8). These results are consistent with a biosynthetic route via the mevalonic acid pathway. On the other hand, it was found that the carbon atoms C-22 and C-23 of the butenolide ring of the cardenolides are not derived from mevalonic acid (9). These findings led to the hypothesis that a pregnane has to be condensed with a C_2 donor, such as acetyl CoA or malonyl CoA, to yield the cardenolide genin. In fact, labelled pregnenolone (**2**) accumulated to significant levels when [2-^{14}C]-mevalonic acid was fed to *D. purpurea* plants (10). Since **1** is a major sterol in *Digitalis* plants and cell cultures (11, 12) it was hypothesized that pregnanes used for the formation of cardenolides are derived from **1**. Actually, **2** was identified as the main biotransformation product of **1** in *D. purpurea* plants (13). However, other phytosterols, such as β-sitosterol were also metabolized to yield pregnanes (14). Other steroids, such as smilagenin and sodium glycocholate, may also serve as cardenolide precursors (15). Indirect evidence of a favoured route not involving **1** was provided by studies with a specific inhibitor of 24-alkyl sterol biosynthesis. The feeding of 25-azacycloartanol led to an increase in endogenous **1** in *D. lanata* shoot cultures. Under these conditions the number of 24-alkyl sterols was dramatically reduced, as were the cardenolides (16).

Pregnenolone may be considered as the starting point for cardenolide formation regardless of the assumed sterol precursor. [21-^{14}C]-Pregnenolone glucoside was incorporated into cardenolides (17) and it is interesting to note that small amounts of xysmalogenin, a $\Delta^{5,6}$-unsaturated cardenolide genin not found in *Digitalis* but in the related genus *Isoplexis*, were also detected. The incorporation of **2** into *Digitalis* cardenolides has been demonstrated in several studies (e.g., 18, 19). Compound **2** was incorporated into digitoxigenin (**13**) more efficiently than into digoxigenin (**23**) or gitoxigenin. Since the 3α-H of [3α-^3H, 4-^{14}C]-pregnenolone was lost in cardenolide formation it was inferred that 3-oxosteroids, such as progesterone (**3**), are intermediates of the cardenolide pathway. The conversion of **2** to **3** was actually seen in intact leaves (18) and in cell cultures (20) of the *Digitalis* species. After administration of radiolabelled **3** to *D. lanata* leaves the label was found not only in cardenolides (21) but in **2** as well, indicating that the pregnenolone oxidation/double bond migration is a reversible process (22). Since all *Digitalis* cardenolides are 5βH-configured, the administration of **3** should result in the formation of 5β-pregnanes. However, only small amounts of 5β-pregnane-3,20-dione (**4**) and 5β-pregnan-3β-ol-20-one (**6**) have been detected (22, 23), which may be best explained by the assumption that the stereospecific 5β-reduction of **3** is a rate-limiting step in cardenolide formation; once accomplished the products are rapidly processed and channelled into the cardenolides. In most of the feeding experiments carried out, 5α-pregnanes were the main pregnane products accumulating after the administration of **3** (20, 24, 25). 5α-Pregnanes were the only products formed from exogenous **3** in cardenolide-free suspension cultures and white dark-grown shoot cultures (25, 26). In this context it has to be added that **3** was also transformed to 20-hydroxypregnenes (27) and that the isomerisation of 5α-pregnanes to 5β-pregnanes has never been reported. Compound **4** was converted to **6** and *vice versa* (28) and both were incorporated into **13** by *D. lanata* leaves (28, 29) and shown to stimulate cardenolide production in *D. lanata* shoot cultures (25, 30).

The 14β-hydroxy group is an important structural feature of all cardiac glycosides. Neither 14α-hydroxysteroids (31) nor 5β-pregn-8(14)-en-3,20-dione (29, 32) were incorporated into cardenolides. On the other hand, [8-^3H]-cholesterol was incorporated into cardenolides without loss of radioactivity (31) and it was thus concluded that a route via $\Delta^{8(14)}$- or $\Delta^{8(9)}$-pregnenes or an 8,14-epoxide as once postulated (19) does not seem to be operative. This assumption was further substantiated by the findings that neither 14βH-steroids (33) nor $\Delta^{8(14)}$-cardenolides (34) were incorporated into cardenolides. Direct hydroxylation with a change in configuration at C-14 seems to be the most probable mechanism of 14β-hydroxylation, although according to another hypothesis, which has never been tested in detail, the 14α-hydrogen is replaced by a hydroxy group which is then converted into the 14β-hydroxy via a 14-oxo radical (17).

Since 14β-hydroxyprogesterone was incorporated into cardenolides, it was concluded that 14β-hydroxylation must occur prior to the formation of the butenolide ring. This assumption was recently substantiated by the finding that 5β-pregnane-3β,14β,21-triol-20-one (**8**) increased cardenolide production in *D. lanata* shoot cultures by more than 4-fold (30).

Steroid 12β-hydroxylation and 16β-hydroxylation can occur at the pregnane level, the cardenolide genin and the glycoside level (35, 36, 37). 16β-Hydroxylation is achieved by direct replacement of the 16β-hydrogen (38). There is circumstantial evidence that 16β-hydroxylation is preferably performed at the C_{21}-stage, whereas 12β-hydroxylation occurs mainly at later stages of cardenolide biosynthesis.

According to the putative pathway, **8** is the last C_{21}-intermediate in the cardenolide pathway. Actually, this compound has been shown to be a much better precursor of cardenolides than, for example, 5β-pregnane-3β,21-diol-20-one (**12**) or **9** (30). However, 5β-pregnane-3β,14β-diol-20-one (**15**) has not yet been tested as a substrate and hence it still remains unclear whether pregnane 14β-hydroxylation precedes 21-hydroxylation or *vice versa*.

At this point it can be assumed that **2**, derived from a sterol precursor, is modified in a sequence of stereo- and site-specific modifications, the crucial reactions being progesterone 5β-reduction and 14β-hydroxylation. There is strong evidence, however, that another route of cardenolide genin formation is operative in *Digitalis*. Feeding experiments with labelled C_{23} steroids revealed that 23-norcholanic acids can serve as cardenolide precursors. It has been shown that the radioactivity of side-chain labelled 23-norcholanic acids appears in the butenolide ring, thus indicating the incorporation of the C_{23} steroid without degradation (39). 23-Nor-5-cholenic acids were better precursors than the respective 5β-steroids and the highest incorporation was seen with 23-nor-5,20(22)*E*-

choladienic acid-3*β*-ol (**14**). In a follow-up study (40) 21-[^3H]-3*β*,20ξ-dihydroxy-23-nor-5*β*-cholanic acid was administered together with 21-[^{14}C]-3*β*-hydroxy-5*β*-pregnan-20-one and it was found that the norcholanic acid was preferably incorporated into cardenolides in *D. purpurea*. These results led to the hypothesis of a "norcholanic acid pathway" in cardenolide formation, implying that the formation of a C_{23} intermediate may be a much earlier event in cardenolide biosynthesis than previously assumed. In order to avoid confusion it should be mentioned that the textbook pathway (Fig. **2**) also contains 23-cholanic acid intermediates, but only at the final stages of cardenolide genin formation. Hence, the "norcholanic acid pathway" should not be regarded as an alternative pathway, but rather as the discovery of a new set of tubes of a branched, anastomosing canal system funnelling precursors to the desired end products, the cardenolides.

It has already been mentioned that the carbon atoms C-22 and C-23 of the butenolide ring of the cardenolides are not derived from mevalonic acid. Nucleophilic attack at the C-20 carbonyl of a properly activated acetate or malonate is proposed as one possible mechanism of attaching C-22 and C-23 to the pregnane skeleton. The formation of the butenolide ring system can then be accomplished by formal elimination of water and lactonization. Experimental evidence for these steps is still lacking and a different mechanism of butenolide ring formation has recently been suggested, involving the formation of a pregnane 21-*O*-malonyl hemiester with subsequent intramolecular condensation under decarboxylation and dehydratisation (41).

So far, only a few investigations have focussed on the formation of the sugar side chain of the cardenolides, especially the stage at which the characteristic 2,6-dideoxy sugars are attached to the cardenolide genin. The hypothetical pathway implies that the various sugars are attached at the cardenolide aglycone stage, although it cannot be ruled out that pregnane glycosides are obligate intermediates in cardenolide formation. Some results indicate that digitoxose is formed from glucose without rearrangement of the carbon skeleton (42) and that nucleotide-bound deoxysugars are present in cardenolide-producing plants (43). Recent investigations into cardenolide biosynthesis showed high incorporation of ^{14}C-labelled malonate into cardenolides but one third of the radioactivity disappeared after acid hydrolysis of the cardiac glycosides and was therefore postulated to be incorporated into the carbohydrate side chain (44).

To study cardenolide genin glycosylation in more detail **13** was fed to light-grown and dark-grown *D. lanata* shoot cultures, as well as to suspension-cultured cells (45). In either system the substrate was converted to **23**, digitoxigen-3-one, 3-epidigitoxigenin, digitoxigenin 3-*O*-*β*-D-glucoside (**24**), 3-epidigitoxigenin 3-*O*-*β*-D-glucoside, glucodigifucoside (**26**) and additional cardenolide products. Digitoxosylation was not observed in these studies. Administration of cardenolide mono- and bisdigitoxosides or cardenolide fucosides did not lead to the formation of cardenolide tridigitoxosides either. These results support the hypothesis that cardenolide fucosides and digitoxosides may be formed via different biosynthetic routes and that glycosylation may be an earlier event in cardenolide biosynthesis than previously assumed. Only recently was a set of pregnane and cardenolide fucosides synthesized (46) and it was shown that feeding of the 3-*O*-*β*-D-fucoside of **9** to *D. lanata* shoot cultures leads to a 25-fold increase in the formation of **26** when compared to a control where the respective aglycone was fed (47).

Enzymes Involved in the Formation of the Cardenolide Genins

I. Cholesterol side-chain cleaving enzyme (SCCE)

Compound **1** is supposed to be a precursor of cardenolides (see above) during the formation of which the side chain of **1** has to be cleaved between C-20 and C-22 to yield **2** (Fig. **2**). Analogous to the formation of steroids in animals this reaction is thought to be catalyzed by P450scc ("cholesterol side chain-cleaving enzyme"), however, this enzyme has never been characterized in detail in plants. The enzyme activity was determined by measuring either the decrease in **1** (48), the radioactivity of the C_6 fragment formed from the cleavage of [26-^{14}C]-cholesterol (49) or quantification of the product **2** by a sophisticated HPLC-MS method (50). Lindemann and Luckner (50) found the enzyme associated with mitochondria and microsomal fractions of proembryogenic masses, somatic embryoids and leaves of *D. lanata*. Formation of **2** was highest with sitosterol as the substrate, however, other sterols were also accepted.

II. NAD:Δ^5-3β-hydroxysteroid dehydrogenase/Δ^5-Δ^4-ketosteroid isomerase (3β-HSD)

The conversion of **2** into **3** involves two steps: The first reaction is the NAD-dependent oxidation of the 3-hydroxy group yielding Δ^5-pregnen-3-one catalyzed by the Δ^5-3*β*-hydroxysteroid dehydrogenase. The double-bond is shifted from position 5 to position 4 by the action of Δ^5-Δ^4-ketosteroid isomerase (51). The enzyme system is referred to ***in toto*** as 3*β*-HSD (Fig. **2**). The enzyme exhibited maximal activity at pH 8.0 and around 50 °C.

3*β*-HSD was isolated from phytohormone-habituated *D. lanata* cell suspension cultures as well as from shoot cultures and leaves of *D. lanata* plants. NAD is the preferred proton acceptor. The addition of Triton X-100 (0.1 %) to the extraction buffer resulted in an almost 70 % loss of 3*β*-HSD activity. In addition to the NAD-dependent dehydrogenase, an oxidase requiring only molecular oxygen acts on the substrate. The enzyme was partially purified only recently. The molecular weight as determined by GPC was 80 – 90 kDa (52).

III. NADPH:progesterone 5β-reductase (5β-POR)

The 5*β*-POR catalyzes the transformation of **3** into **4**, i.e., the rings A and B of the steroid are then connected *cis* (Fig. **2**). Hence, one of the important structural characteristics of the *Digitalis* cardenolides seems to be accomplished at this stage. Optimal enzyme activity was seen at 30 °C and pH 8.0. The 5*β*-POR requires NADPH as the co-substrate and **3** was the preferred substrate. The relative conversion rates for other steroids such as testosterone, cortisone and cortisol were much lower. The enzyme was purified 770-fold to homogeneity from the cytosolic fraction of shoot cultures of *D. purpurea*. The molecular weight as determined by GPC was 280 kDA (53).

With the exception of the 5*β*-POR, all known enzymes of the putative biosynthetic pathway were detected in plants, organ cultures and suspension cultures of *D. lanata* (54) supporting the view that 5β-POR is a key enzyme in cardenolide biosynthesis as proposed by Gärtner and Seitz (55). This concept, however, was not accepted by Lindemann and Luckner (50), who found 5β-POR expressed in a cardenolide-free embryogenic cell line of *D. lanata* and they speculated that cardenolide formation is mainly regulated by the availability of **1** and its transport into mitochondria, where the SCEE is assumed to be located.

IV. NADPH:progesterone 5α-reductase (5α-POR)

5α-POR, which catalyzes the reduction of **3** to 5α-pregnane-3,20-dione (**5**), probably in a competitive situation with the 5β-POR, was isolated and characterized from cell cultures of *D. lanata* where it was found to be located in the endoplasmic reticulum (**56**). 5α-POR requires NADPH as a reducing cosubstrate, and optimum conditions for the 5α-POR were at pH 7 and 40 °C. At temperatures below 45 °C, the product of the enzyme reaction, **5**, was enzymatically reduced to 5α-pregnan-3β-ol-20-one (**22**) (see below).

V. NADPH:3β-hydroxysteroid 5α-oxidoreductase (3β-HS-5α-OR)

The enzyme catalyses the conversion of **5** to **22**. 3β-HS-5α-OR was first isolated and characterized in the microsomes of *D. lanata* cell cultures (57). The enzyme worked best at pH 8.0 and 25 °C. Just slightly increasing the temperature to 27 °C resulted in a marked reduction of 3β-HS-5α-OR activity. Both NADPH and NADH were able to provide the necessary reduction equivalents to drive the reaction.

Differential centrifugation as well as linear sucrose density gradient centrifugation revealed that most of the 3β-HS-5α-OR is soluble and it was thus inferred that it is not associated with a specific cell compartment.

VI. NADPH:3β-hydroxysteroid 5β-oxidoreductase (3β-HS-5β-OR)

The 3β-HS-5β-OR catalyzes the conversion of **4** to **6** (Fig. **2**). It was found to be a soluble protein (55). Optimum enzyme activity was found at a pH-value of 6.5 and at around 40 °C. The 3β-HS-5β-OR was catalytically active in the presence of either NADPH or NADH, but NADPH was the preferred co-substrate. The reverse reaction was observed, yielding **4** when using **6** and NADP as a substrate and co-substrate, respectively.

VII. NADPH:3α-hydroxysteroid 5β-oxidoreductase (3α-HS-5β-OR)

This enzyme catalyses the conversion of **4** to 5β-pregnan-3α-ol-20-one (**7**). In a situation similar to that described for the progesterone reductases, the hydroxysteroid 5β-oxidoreductases may compete for 5β-pregnane-3-ones and part of these putative intermediates in the cardenolide pathway will be withdrawn due to the action of the 3α-HS-5β-OR. Actually, 3α-cardenolides have never been described in *D. lanata* and the final products of the 5α-pregnane pathway are not yet known.

Cell-free buffered extracts from light-grown *D. lanata* shoots were shown to reduce **4** almost exclusively to **7** when 0.05 M $MgCl_2$ were present in the incubation mixture (25). These conditions were inhibitory for the formation of **6**. The 3α-HS-5β-OR could be recovered from membrane-free protein extracts. Optimum enzyme activity was observed at pH 7.0 and 42 °C. The enzyme reaction was found to be NAD(P)H-dependent and SH reagents were essential for enzyme activity. The enzyme seems to be specific for 5β-pregnane-3-ones; 5α-pregnane-3-ones or Δ^4/Δ^5-pregnenes were not accepted as substrates.

Pregnane 21-hydroxylation and 14β-hydroxylation: The enzymes involved in pregnane 21-hydroxylation and pregnane 14β-hydroxylation in the course of cardenolide formation have not been described as yet.

VIII. Malonyl-coenzyme A:21-hydroxypregnane 21-O-malonyltransferase (MHPMT)

As far as the formation of the butenolide ring is concerned, it is supposed that the condensation of **8** with a dicarbon unit yields **13** (Fig. **2**). When the 3β-*O*-acetate of **8** was incubated together with malonyl-coenzyme A in a cell-free extract of *D. lanata* leaves, a product was formed which was identified as the malonyl hemiester of the substrate (41). The compound decomposes rapidly at temperatures higher than about 100 °C and during prolonged storage and two products are formed, namely 5β-pregnane-14β-ol-20-one 3β-*O*,21-*O*-diacetate and the 3-*O*-acetate of **13**. The enzyme catalysing the formation of the malonyl hemiester was termed malonyl-coenzyme A:21-hydroxypregnane 21-*O*-malonyltransferase (MHPMT).

The major part of the MHPMT was found to be soluble. Temperature and pH optima were at 50 °C and pH 6.5, respectively. Thiol reagents stimulated MHPMT activity. Malonyl-coenzyme A and acetoacetyl-coenzyme A were accepted as co-substrates. No ester formation was observed when acetyl-CoA or succinyl-CoA were added to the incubation mixture. CoA inhibited the malonylation reaction. Compound **8** and its 3β-*O*-acetate were the most suitable substrates for the transferase reaction. Pregnen-21-ol-20-one **9**, cortexone (**10**), 5β-pregnan-21-ol-3,20-dione (**11**) and **12** were only very poor substrates. The enzyme could so far be detected only in cardenolide-producing plants (41).

IX. Digitoxin 12β-hydroxylase (D12H)

This microsomal cytochrome P-450-dependent monooxygenase is capable of converting digitoxigenin-type cardenolides to their corresponding digoxin-type cardenolides (58). The enzyme was first isolated from cell suspension cultures of *D. lanata*, where the enzyme was found to be located in the endoplasmic reticulum.

The pH optimum of the D12H was at pH 7.5 and the temperature optimum at 20 °C (Table **1**). Compound **31**, β-methyldigitoxin and α-acetyldigitoxin (**33**) as well as digitoxigenin-type cardenolides with shorter or no sugar side chain were hydroxylated (59). Gitoxigenin, k-strophanthin-β and cymarin, on the other hand, were not accepted as substrates. NADPH and O_2 are essential for the catalytic activity and the enzyme reaction is competitively inhibited by $NADP^+$ and cytochrome c. The D12H was inhibited by CO, but illumina-ton with blue light (λ_{Tmax} 450 nm) reversed this inhibition almost totally. KCN stimulated hydroxylation *in* *vitro*, whereas Co^{2+}, Zn^{2+} and Hg^{2+} were strongly inhibitory. After immobilization in alginate the enzyme retained 70% of its original activity. The kinetic data of D12H immobilized in alginate were the same as for the enzyme in freely suspended microsomes (60).

It should be mentioned that digitoxin 16β-hydroxylase (D16H) has been detected in protein extracts prepared from *D. purpurea* cell suspension cultures but the enzyme has not yet been characterized in detail (61).

Enzymes Involved in the Formation of the Sugar Side Chain

X. UDP-glucose:sterol 3-O-glucosyltransferase (SGT)

This enzyme catalyzes the transfer of the sugar moiety of UDP-glucose to a steroid substrate (Fig. **3**). [4-^{14}C]-Epiandrosterone was used as the standard substrate. In the cultured cells the enzyme was not associated with a specific subcellular fraction. However, almost 60% of the enzyme isolated from leaves was associated with the microsomal fraction from which it could be solubilized with 0.1 % sodium deoxycholate (62).

SGT was partially purified from cell cultures and leaves of *D. purpurea*. The purified enzyme had its pH optimum at pH 7.5. All Δ^5-steroids tested were good substrates for the SGT. 5αH-Steroids, such as epiandrosterone and 5α-pregnan-3β-ol-20-one, were better substrates than their corresponding 5βH-analogues. Epiandrosterone (5αH-configured) containing a 3β-hydroxy group was a better substrate than its 3α-hydroxy analogue, whereas of the 5βH-steroids tested, those with a 3α-hydroxy group were better substrates than the respective 3β-hydroxy compounds. Compound **13** was only a poor substrate for the SGT. However, taking into consideration the small amounts of glucodigitoxigenin genuinely found in *Digitalis* it may well be that the sterol glucosyltransferase is involved in the formation of this compound. It should be mentioned that quite large amounts of glucodigitoxigenin were formed when **13** was fed to suspension-cultured cells of *D. purpurea* (36) or shoot cultures of *D. lanata* (63). To summarize, it still remains to be clarified whether sterol glycosyltransferases, like the enzyme isolated from *D. purpurea*, are actually involved in the formation of *Digitalis* glycosides.

XI. UDP-glucose:digitoxin 16′-O-glucosyltransferase (DGT)

The enzymatic glucosylation of secondary glycosides to their respective primary glycosides (Fig. **3**) was first shown by Franz and Meier (64) in particulate preparations from *D. purpurea* leaves and was investigated in more detail in cell cultures of *D. lanata* (65). The DGT requires two substrates: a secondary cardiac glycoside and a sugar nucleotide. Of 6 sugar nucleotides tested only UDP-α-D-glucose served as a glycosyl donor, whereas other glucose nucleotides (65) or UDP-α-D-fucose (66) were not accepted. As far as pH and temperature are concerned, the highest glucosylation rates were found at pH 7.4 and 40 °C, respectively.

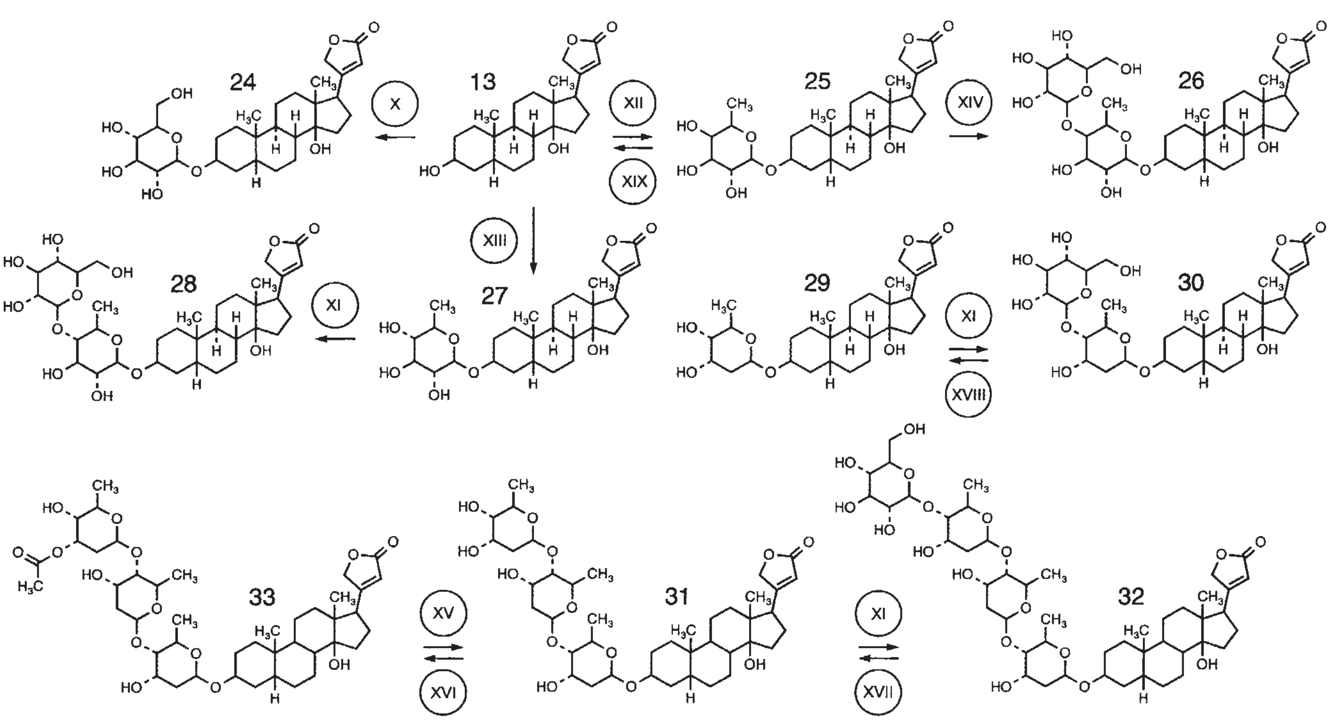

Fig. 3 Enzymes involved in the formation of the sugar side chain of *Digitalis* cardenolides. **13**, digitoxigenin; **24**, digitoxigenin 3-*O*-β-D-glucoside; **25**, digiproside; **26**, glucodigifucoside; **27**, digitoxigenin 3-*O*-β-D-quinovoside; **28**, glucodigitoxigenin 3-*O*-β-D-quinovoside; **29**, evatromonoside; **30**, glucoevatromonoside; **31**, digitoxin; **32**, purpureaglycoside A; **33**, α-acetyldigitoxin. **X**, *UDP-glucose: sterol 3-O-glucosyltransferase (SGT)*; **XI**, *UDP-glucose: digitoxin 16′-O-glucosyltransferase (DGT)*; **XII**, *UDP-fucose:digitoxigenin 3-O-fucosyltransferase (DFT)*; **XIII**, *UDP-quinovose: digitoxigenin 3-O-quinovosyltransferase (DQT)*; **XIV**, *UDP-glucose: digiproside 4′-O-glucosyltransferase (DPGT)*; **XV**, *acetyl coenzyme A: digitoxin 15′-O-acetyltransferase (DAT)*; **XVI**, *lanatoside 15′-O-acetylesterase (LAE)*; **XVII**, *cardenolide 16′-O-glucohydrolase I (CGH I)*; **XVIII**, *cardenolide glucohydrolase II (CGH II)*; **XIX**, *cardenolide β-D-fucohydrolase (CFH)*.

Strong DGT activity was found in buffered extracts from young leaves, roots and flowers of *Digitalis lanata* plants, whereas only weak activity could be detected in stems and mature leaves. DGT was demonstrated in leaves, callus and suspension-cultured cells of *D. lanata*, *D. purpurea* and *D. heywoodii* (67). The DGTs of the three *Digitalis* species examined differed considerably with regard to their substrate preferences. Compound **31** and digoxin were glucosylated much better by cell-free extracts from *D. lanata* than their 15′-*O*-acylated derivatives. Although 15′-*O*-acylated glycosides do not occur in *D. purpurea*, they were glucosylated to their corresponding primary glycosides by enzyme preparations from *D. purpurea* cell cultures (65). Cardenolide monodigitoxosides, such as evatromonoside (**29**) were accepted very well, whereas cardenolide genins or bisdigitoxosides were glucosylated at a much slower rate (68). Digitoxigenin quinovoside (**27**) was glycosylated by partially purified DGT to yield glucodigitoxigenin quinovoside (**28**). Under the same conditions glucosylation was not observed when digiproside (**25**) was tried as the glucosyl acceptor, indicating that DGT accepts only substrates with an equatorial OH group in the 4′ position (66).

XII. UDP-fucose:digitoxigenin 3-O-fucosyltransferase (DFT)

The DFT catalyzes the transfer of the sugar moiety of UDP-α-D-fucose to a cardenolide aglycone, such as **13** or **23** (66) (Fig. **3**). Compound **25**, the product formed in the presence of **13** is a minor glycoside in *D. lanata* (**1**). DFT is a soluble enzyme in *D. lanata* leaves. Fucosylation activity was highest at pH 5.7 and 37 °C. Gitoxigenin and **13** were much better substrates than **23**. The apparent molecular weight of DFT is about 60 kDa, as determined by GPC (69).

XIII. UDP-quinovose:digitoxigenin 3-O-quinovosyltransferase (DQT)

Incubation of crude protein extracts together with **13** and UDP-fucose not only resulted in the formation of **25** but also of **27** (Fig. **3**), the 4′-epimer of **25**, which is a minor glycoside in *D. lanata* (**1**). When UPDF was preincubated with the enzyme extract before adding **13**, the cardenolides **25** and **27** were produced in almost equal amounts, indicating that the sugar is modified at the sugar nucleotide level and not at the glycoside stage and that DQT must be active in these extracts (66). Neither DQT nor epimerase activity were present in the partially purified DFT preparation (see above).

XIV. UDP-glucose:digiproside 4′-O-glucosyltransferase (DPGT)

Glucodigifucoside (**26**) was formed upon incubation at 37 °C and pH 5.7 of a soluble enzyme preparation from young leaves of *D. lanata* in the presence of UDP-α-D-glucose and **25** (66) (Fig. **3**). The enzyme is not identical with the glucosyltransferases described above, but has not been characterized in detail as yet. Compound **26** is a major cardenolide in *D. lanata* leaves during all stages of development and may be regarded as the end-product of the "fucose pathway" (see above).

XV. Acetyl coenzyme A:digitoxin 15′-O-acetyltransferase (DAT)

This soluble, cytosolic enzyme catalyzes the 15′-*O*-acetylation of cardenolide tri- and tetrasaccharides (Fig. **3**). Using acetyl coenzyme A as the acetyl donor, DAT activity was detected in partially purified protein extracts from *D. lanata* and *D. grandiflora*, both known to contain lanatosides (70). The enzyme from either source exhibited its pH optimum at pH 6.0 and about 40 °C.

XVI. Lanatoside 15′-O-acetylesterase (LAE)

An esterase converting acetyldigitoxose-containing cardenolides to their corresponding non-acetylated derivatives (Fig. **3**) was demonstrated in *D. lanata* cell suspension cultures and leaves (71). The LAE was shown to be bound ionically to the cell wall, from which it could be solubilized with 0.1 M sodium citrate buffer, pH 6.0. Citrate is needed for enzyme extraction, but not for the enzyme activity itself. LAE was present in *D. lanata* leaves and cell cultures (71) but was not detectable in cell suspension cultures of *D. grandiflora* and *D. purpurea* (83), and in leaves of *D. purpurea* and *D. heywoodii* (71). The pH optimum of the purified enzyme was at 5.5, the temperature optimum was around 40 °C. The enzyme could not be inhibited by *p*-hydroxymercuribenzoate or eserine. The pI was at pH 8.7, as determined by chromatofocussing. The apparent molecular mass of the LAE, as determined by GPC, was 120 kDa. In SDS-PAGE one dominant protein band was seen at about 50 kDa. It was hence concluded that the LAE is composed of two identical subunits.

Lanatosides as well as their corresponding secondary glycosides were good substrates, α,β-diacetyldigoxin was deacetylated to some extent, yielding small amounts of β-acetyldigoxin but not the respective α-derivative. Apigenin 7-*O*-acetylglucoside was not deacetylated. Hence, LAE seems to be a site-specific cardenolide acetylesterase capable of removing the 15′-acetyl group of lanatosides and their deglucosylated derivatives.

XVII. Cardenolide 16′-O-glucohydrolase I (CGH I)

In 1935, Stoll et al. (73) reported on enzyme activities in *Digitalis* leaves capable of hydrolyzing primary glycosides, such as the purpureaglycosides or the lanatosides (Fig. **3**). These enzymes were called "desmoenzymes" because they could not be extracted from dried pulverized leaves. They are associated with plastids (74) and could be solubilized from leaves of various *Digitalis* species using buffers containing Triton X-100 or other detergents (67). The *Digitalis* species examined differed only slightly with regard to the assay conditions needed for optimum cardenolide glucosidase activity. The pH optimum was at around 4.5 and the highest conversion rates were found to occur at about 50–60 °C. However, considerable variations in substrate preferences were observed among the cardenolide 16′-*O*-glucosidases of the three species. The enzyme of *D. lanata*, termed CGH I, was purified from young leaves. The apparent molecular mass of the CGH I was 154 kDa, as determined by non-denaturating PAGE. Fragments of about 27, 37 and 76 kDa were obtained in SDS-PAGE (75). The pI of CGH I was 5.8, as determined by chromatofocussing.

XVIII. Cardenolide glucohydrolase II (CGH II)

Meanwhile, another cardenolide glucohydrolase, termed CGH II, was isolated from *D. lanata* and *D. heywoodii* leaves and cell cultures. This soluble enzyme hydrolyzes cardenolide disaccharides with a terminal glucose and seems to be quite specific for glucoevatromonoside (**30**) (Fig. **3**), which is supposed to be an intermediate in the formation of the cardenolide tetrasaccharides. The tetrasaccharides deacetyllanatoside C and purpureaglycoside A (**32**), which are rapidly hydrolyzed by CGH I (see above) were very poor substrates for CGH II. The enzyme was purified about 500-fold from leaves of *D. heywoodii*; it exhibited optimum activity at pH 6.0 and 50 °C. The molecular mass of CGH II was determined as 69 kDa by GPC and 65 kDa by SDS-PAGE, the enzyme's pI is at pH 6.2 (76, 77).

XIX. Cardenolide β-D-fucohydrolase (CFH)

A β-D-fucosidase was isolated from the 25% ammonium sulphate precipitate of protein extracts from young *D. lanata* leaves. This soluble enzyme catalyzes the cleavage of **25** and synthetic pregnane 3β-*O*-D-fucosides to D-fucose (6-deoxygalactose) and the respective genin (Fig. **3**). Digitoxigenin 3β-*O*-D-galactoside was not hydrolyzed by the enzyme. It is not identical with the cardenolide glucohydrolases described above which do not accept β-D-fucosides as substrates. Optimal enzymatic hydrolysis was observed at 37 °C and pH 7.0. The enzyme has not yet been characterized in detail or purified further (47).

Compartmentalization of Cardenolide Formation

Digitalis leaves are not only the starting material for the isolation of commercial cardenolides but have also been shown to be the site of cardenolide biosynthesis. Several studies have reported a positive correlation between light, chlorophyll content and cardenolide production (78). However, chloroplast development is not sufficient for expression of the cardenolide pathway. Photomixotropic, chlorophyllous cell cultures are incapable of producing cardenolides (79) whereas cultivated embryoids and morphogenic clumps have been shown to contain cardenolides, albeit at quite low concentrations (80, 81). Cultivated shoots, on the other hand, accumulated significant amounts of cardenolides (82). From these observations it may be concluded that for cardenolide formation tissue differentiation is at least as essential as the presence of active chloroplasts.

Digitalis roots cultivated *in vitro* are not capable of producing cardenolides although they do contain these compounds *in situ*. Suspension-cultured *Digitalis* cells, which do not synthesize cardenolides *de novo* (83), as well as roots or shoots cultivated *in vitro* (45), are able to take up exogenous cardenolides and modify them. It has been demonstrated that cardenolides may enter and leave the cells by diffusion. Only the primary cardenolides, i.e., those containing a terminal glucose, are actively transported across the tonoplast and stored in the vacuole. A model comprising the events leading to cardenolide storage has been proposed (83).

Cardiac glycoside transport was also investigated on the organ and whole plant level. The long-distance transport of primary cardenolides from the leaves to the roots or to etiolated leaves was demonstrated. It was established that the phloem but not the xylem is a transporting tissue for cardenolides (84). To summarize, it seems as if primary cardenolides may serve as both the transport and the storage form of cardenolides. After their synthesis they are either stored in the vacuoles of the source tissue, or loaded into the sieve tubes and transported to various cardenolide sinks, such as roots or flowers.

Concluding Remarks

Taking cholesterol as the starting point, about 20 enzymes which probably affect the formation of cardenolides have been identified in recent years. Among these are enzymes responsible for cardenolide formation and storage, as well as enzymes responsible for removing precursors from the cardenolide pathway. Only a few of them have been purified and just one, namely progesterone 5β-reductase, has been partially sequenced. The discovery of enzymes which have not yet been described but which might catalyze pregnane 14β- and 21β-hydroxylation may help to clarify the cardenolide pathway(s) further. In summary, a more detailed knowledge of the enzymes and genes involved in cardenolide formation is necessary for studying the regulation and engineering of the cardenolide pathway in future.

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