Production of Cardiac Glycosides by Plant Tissue Cultures IV

Biotransformation of Digitoxigenin and Related Substances

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Selected substrates were incubated for periods of 2, 7, and 16 days with spearmint and various digitalis liquid suspension tissue cultures. Concentrated chloroform extracts were prepared following the incubations. Thin-layer chromatography was used for the identification, isolation, and purification of conversion products. Two major conversion products of digitoxigenin were obtained, neither of which was glycosidic.

ARTICLES HAVE been published on potential applications of plant tissue cultures (1, 2), including the release of enzymes (3). Nickell (1) suggested that one practical application of tissue culture enzymes would be biological transformation of alkaloids, antibiotics, steroids, or other physiologically active compounds. Based on these considerations, the biotransformation by selected plant tissue cultures of digitoxigenin and related substances was studied.

The biochemistry of plant steroids has been reviewed (4), and the biogenesis of cardiac glycosides has been studied (5, 6). The formations of both hetero- and hologlycosides are believed to require various cofactors; among them are UTP and ATP.[1]

Although the bioconversions of cardiac aglycones by fungi often have been studied (7–9), only one publication, which considered the microbial transformation of glycosides (10), is known to the authors. Hydroxylations in the 1β, 2β, 7β positions, or dihydroxylations of cardiac aglycones occur most frequently.

MATERIALS AND METHODS

Tissue Cultures—Tissue callus cultures from *Mentha spicata* L. leaf and *Digitalis lanata* Ehrh., *D. purpurea* L., and *D. mertonensis* seeds were used. These cultures were grown on a modification of Murashige's and Skoog's tobacco medium (11). *D. lanata* root suspension cultures were also studied. The media for all tissues contained 1.0 p.p.m. 2,4-D, except for *M. spicata* callus and *D. lanata* root cultures which contained 0.1 p.p.m. 2,4-D. *M. spicata* was studied to determine if digitalis suspension cells retained a specificity greater than mentha for the substrates studied.

Seed-derived callus cultures were established by the procedure described by Staba (12) and divided and subcultured to freshly prepared medium every 5 to 6 weeks. *D. lanata* root suspension cultures were grown from *D. lanata* seed callus by reduction of the 2,4-D concentration from 1 p.p.m. to 0.1 p.p.m. (11). *M. spicata* leaf callus cultures were derived from seedlings grown aseptically from seeds sterilized as previously described (13). Leaves from the aseptic seedling plants were removed and transformed into callus tissue by placing them on medium containing 2,4-D (0.1 p.p.m.) and agar (0.7%).

Prior to their experimental use for transformation studies, all callus tissues were grown in liquid suspension for 7 to 10 days. Five to seven grams of 35- to 40-day-old callus were transferred aseptically to 250-ml. conical flasks containing 50 ml. of liquid medium. The flasks were placed on a reciprocating shaker operating at 88 c.p.m., with a stroke of 9 cm., and at room temperature (26–31.5°) and room light.

Inoculum Preparation and Addition.—The various suspension tissue cultures were incubated with selected substrates for periods of 2, 7, and 16 days. For all inocula, 7- to 10-day-old suspension cultures from four 250-ml. conical flasks were centrifuged at 15,000 r.p.m. for 5 minutes and the medium decanted.

For the 2-day studies, the centrifuged cells were washed twice with 150 ml. of pH 6.0 0.1 *M* phosphate buffer and resuspended in 20 ml. of sterile 0.4% methylcellulose[2] (MC) to insure a more uniform inoculum. The MC cell suspensions from the several centrifuge tubes were combined. For the 7- and 16-day studies, the cells were not washed but were suspended directly in 0.4% MC following centrifugation and decantation of the medium.

Four or five transfers of the MC-suspended cells were added to each test flask. A constant number of transfers were made with a sieved nichrome transfer cup with about 1 ml. vol. This resulted in about 65 mg. of inoculum tissue (dry weight) per 25-ml. test flask and 85 mg. of inoculum tissue (dry weight) per 250-ml. test flask.

Composition of Substrates, Additives, and Cofactors.—The composition and concentration of all substrates[3] are given in Table I. Digitoxose (0.04%) was added to flasks containing digitoxigenin, litho-

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[1] Adenosine triphosphate, ATP; 2,4-dichlorophenoxyacetic acid, 2,4-D; methylcellulose, MC; nicotinamide adenine dinucleotide, NAD; nicotinamide adenine dinucleotide phosphate, NADP; uridine triphosphate, UTP.

[2] Methocel (4,000 cps.), Dow Chemical Co., Midland, Mich.

[3] All substrates, additives, and cofactors were obtained either from Nutritional Biochemicals Corp., Cleveland, Ohio, or General Biochemicals (GBI), Chagrin Falls, Ohio.

TABLE I.—DIGITALIS AND MENTHA TISSUE CULTURE SUBSTRATES

No.	Substrate	Medium Concn. (w/v), %
1	Digitoxigenin	0.02
2	Digitoxin	0.02
3	Lithocholic acid	0.02
4	Squalene	0.02
5	Digitoxigenin + cofactors	0.02
6	Mevalonic acid + cofactors	0.05
7	Hydroquinone	0.20

TABLE II.—SOLVENT SYSTEMS USED TO RESOLVE DIGITALIS AND MENTHA INCUBATION MIXTURE EXTRACTS

System	Compn.	Components Resolved	Ref.
1	Methylene chloride–methanol–formamide (95:5:1)	Digitoxigenin	(16)
2	Methylene chloride–methanol–formamide (90:9:1)	Digitoxin	(16)
3	*n*-Butanol–acetic acid–water (4:1:1)	Squalene, mevalonic acid	(17)
4	Amyl acetate–isopropyl ether–carbon tetrachloride–propyl alcohol–benzene–acetic acid(40:30:20:10:10:5)	Lithocholic acid	(18)

cholic acid, or squalene, and to those that were incubated for either 2 or 7 days. In addition, glucose-1-phosphate (0.05%) was added to flasks containing the three substrates incubated for only 2 days.

The following cofactors and their concentrations in the 2- and 7-day studies were modified from the studies by Loomis and Battaile (14, 15) (concentrations in mg./10 ml. incubation solution): NAD, 2.1; NADP, 1.9; glutathione (reduced), 15.36; ATP, 50.0; coenzyme A, 0.3; UTP, 2.12; and uridine, 4.88. Uridine and UTP were used as cofactors only in the 7-day incubations with *D. lanata* and *D. purpurea* suspension cultures.

Chromatography and Substrate Extraction Procedures.—Thin-layer chromatography[4] was used for comparative studies of the incubation mixture extracts. The thin-layer adsorbent[5] thickness was 250 μ, except that an adsorbent thickness of 400 to 500 μ was used to isolate and purify the transformation products.

For the 2- and 7-day studies, 30 μl. of a concentrated chloroform extract was applied to thin-layer plates. For isolation and purification, 50 μl. was applied to thick-layer plates. The solvent systems are given in Table II (16–18). The detecting reagents were phosphomolybdic acid reagent (18), anisaldehyde reagent (19), and a modified Kedde reagent.[6] Paper chromatography (20) was used only for the identification of hydroquinone and its glycosides.

Following incubation, the filtrates, washed cells, and control solutions were extracted with three 10-ml. chloroform fractions for each incubation mixture of 10 ml. and four 15-ml. fractions for each incubation mixture of 50 ml. The chloroform fractions were combined, partially dried over anhydrous sulfate, and evaporated to dryness in a vacuum desiccator. The residues were dissolved in 1.5 ml. of chloroform for the 10-ml. incubation mixtures and 2.5 ml. of chloroform for the 50-ml. incubation mixtures.

For the substrate, hydroquinone, the incubation filtrate was extracted with ethanol–chloroform (1:4) and the tissue with 95% ethanol.

EXPERIMENTAL

All experiments consisted of tissue controls (tissue suspension without substrate), substrate controls (substrate without tissue), and the incubation mixtures (substrate solution and tissue).

Two-Day Studies.—*D. purpurea* callus was incubated with substrates *1–6* and *D. mertonensis* with substrates *1–4* (Table I). Each 25-ml. conical flask contained 10 ml. of phosphate buffer solution and one substrate. Incubation mixtures were examined in triplicate, while all substrate and control flasks were duplicated. All flasks were incubated on a constant temperature shaker[7] operating at 120 c.p.m. under room light conditions and a temperature of 28 ±0.2°.

Seven-Day Studies.—*D. lanata* callus, *D. purpurea* callus, and *D. lanata* root cultures were incubated with substrates *1–6* under aseptic conditions for 7 days. *M. spicata* callus and *D. mertonensis* callus cultures were incubated with substrates *1–4*. *D. lanata* and *D. purpurea* callus suspensions were also incubated with substrate 7. Each 250-ml. conical flask contained 50 ml. of the appropriate incubation mixture. Both control and incubation mixture flasks were duplicated.

Sixteen-Day Studies.—*D. lanata*, *D. purpurea*, and *M. spicata* callus tissues were incubated with digitoxigenin for 16 days. Twenty 250-ml. conical flasks were prepared for each tissue-incubation mixture so that a sufficient quantity of the metabolites might be produced for isolation and identification purposes.

RESULTS

Two-Day Studies.—No conversion products were detected in *D. mertonensis* or *D. purpurea* callus tissue incubation mixtures.

Seven-Day Studies.—No metabolites of lithocholic or mevalonic acids were detected in the incubation mixtures for the 7-day bioconversion studies. Squalene produced a single conversion product with *M. spicata* callus. Both root and callus cultures of *D. lanata* gave rise to the same metabolite from digitoxin. The metabolite from both sources gave identical color reactions and *Rs* (digitoxin) values. Digitoxigenin with cofactors in the presence of *D. lanata* root cultures yielded three products which had *Rs* (digitoxigenin) values of 0.17, 1.60,

[4] DeSaga thin-layer chromatography apparatus, Brinkman Instruments, Inc., Great Neck, N. Y.

[5] Adsorbosil-1, Applied Science Laboratories, Inc., State College, Pa.

[6] One gram of 3,5-dinitrobenzoic acid in 50 ml. of methanol, 5.6 Gm. of potassium hydroxide in 50 ml. of water; chill the two solutions and mix immediately prior to use as a spray reagent.

[7] Shaker model 2156, Research Specialties Co., Richmond, Calif.

TABLE III.—KEDDE-POSITIVE BIOTRANSFORMATION COMPOUNDS FROM DIGITOXIGENIN INCUBATION MIXTURES WITHOUT COFACTORS

Incubation Period, Days	Tissue	Conversion Products, No.	*Rs* Values
2	*D. mertonensis* callus	...	...
	D. purpurea callus	...	...
7	*D. lanata* callus	1	1.75
	D. mertonensis callus	1	1.75
	D. purpurea callus	1	1.75
	M. spicata callus	2	1.75, 1.45
	D. lanata roots	3	1.75, 1.45, 0.33
16	*D. lanata* callus	2	1.75, 1.45
	D. purpurea callus	3	1.75, 0.45, 0.18
	M. spicata callus	2	1.75, 1.45

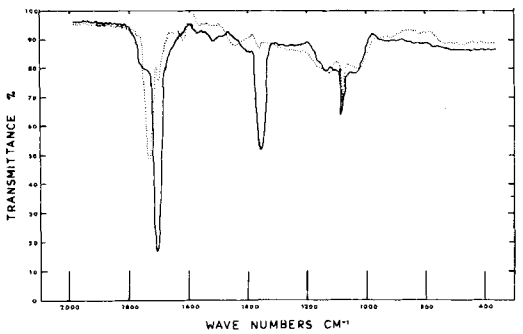

Fig. 1.—Infrared absorption spectrum of digitoxigenin ------- and digitoxigenin conversion product ———.

and 1.85. All transformation products were anisaldehyde-positive, but only the product with an *Rs* of 1.60 was Kedde positive. The most pronounced Kedde-positive conversions were observed in digitoxigenin incubation mixtures without cofactors, (Table III). No conversion products with hydroquinone were observed. Glycoside formation with digitoxigenin, lithocholic acid, mevalonic acid, and squalene substrates was not observed with *D. lanata*, *D. mertonenis*, *D. purpurea*, or *M. spicata* tissues.

Sixteen-Day Studies.—Similar Kedde-positive conversion products of digitoxigenin occurred from both 7- and 16-day incubation studies. In addition, *D. purpurea* callus produced small amounts of two additional Kedde-positive metabolites, while *D. lanata* callus produced one additional Kedde-positive product.

DISCUSSION

The failure to detect conversion products from the 2-day-old incubation mixtures may be attributed to (*a*) the small initial amount of substrate (10 mg.), (*b*) the failure of tissue survival under the experimental conditions, or (*c*) the short incubation period.

Büchner and Staba (21) reported the presence of Kedde-positive substances (cardenolides) in the medium and cells of *D. lanata* and *D. purpurea* tissue cultures. Similar compounds were not observed in these studies. Probably this difference arises from the quantity of tissue extracted. In this study, not more than 100 mg. of tissue (dry weight) was used, while in the work of Büchner and Staba from 2.6 to 7.9 Gm. (dry weight) was used.

Under the conditions described, *D. lanata* and *D. purpurea* callus suspension cultures did not produce glycosides of hydroquinone, although many different plant tissues are known to have this capability (20).

The presence of conversion products in the 7- and 16-day-old incubation mixtures tends to indicate that active extracellular enzymes were released by the plant tissue cultures. A degree of substrate specificity was observed since some substrates were acted on by only one or two tissues. As the transformation products were demonstrable for digitalis and *M. spicata* tissue cultures, similar or the same enzymes were released.

When large amounts (70 ml.) of the chloroform extract of the digitoxigenin control were developed on thick-layer plates and sprayed with Kedde detecting reagent, a faint spot appeared with an *Rs* value corresponding to that of the primary metabolite (*Rs* = 1.45 digitoxigenin). This suggests that the product may be converted quantitatively, with the cellular enzymes catalyzing the spontaneous conversion of digitoxigenin to a degradation product.

Upon purification of the primary Kedde-positive conversion product on thick-layer chromatography plates (*Rs* = 1.45 digitoxigenin), the product was unstable to mild heat and gave rise to two additional products, neither of which were Kedde-positive. The infrared spectra of the primary conversion product indicates that keto group(s) had been added to the molecule (Fig. 1). Work is in progress to isolate and characterize the transformation products produced by digitalis tissue cultures.

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