Effects of Mineral Salts, Initial pH and Precursors on Digitoxin Formation by Shoot-forming Cultures of *Digitalis purpurea* L. Grown in Liquid Media[†]

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Shoot-forming cultures of *Digitalis purpurea* L. were grown in various modifications of Murashige-Skoog medium. The effects of CaCl_2 , MgSO_4 , FeSO_4 coupled with Na_2 -EDTA, minor elements, initial pH of the medium, some organic solvents as precursor solvents, and precursors on their growth and digitoxin formation were investigated. Reduction of the basal CaCl_2 concentration by two-thirds promoted both digitoxin formation and shoot-differentiation while a threefold increase repressed these. Ca^{++} proved to be responsible for this phenomenon. A threefold increase in the MgSO_4 concentration improved digitoxin formation, Mg^{++} being responsible for this improvement. The optimal concentration of FeSO_4 coupled with Na_2 -EDTA was similar to that in the MS basal medium. A threefold increase in the concentration of either KI, Na_2MoO_4 or CuSO_4 improved digitoxin formation, the optimal pH of the medium before autoclaving being 6. Among methanol, ethanol, propylene glycol, dimethyl sulfoxide and acetone as the precursor solvents, methanol and acetone were relatively the least inhibitive at 2%. Progesterone at 0.1 mg/100 ml medium improved the digitoxin content per flask to 180% of that of the control cultures, whereas no promotive effect from cholesterol was observed.

Digitalis cardenolides, especially digitoxin and digoxin, are important in medicine, and cardenolide production by cultured cells of *Digitalis* is a very attractive method. We have already reported that of the green shoot-forming cultures, white shoot-forming cultures, undifferentiated green cells and undifferentiated white cells of *Digitalis purpurea* L., the green shoot-forming cultures accumulated the largest amounts of digitoxin.¹⁾ We next examined the effects of cultural conditions to determine the best for digitoxin formation by the green shoot-forming cultures. We have reported the effects of carbon and nitrogen sources, phosphate, vitamins, natural extracts and some plant growth substances previously,²⁾ and we report here the effects of the other major and minor elements of the Murashige-Skoog basal medium used, the initial pH of the medium and precursors of the digitoxin biosynthesis.

Cardenolides are formed by condensation of the C_{21} pregnane derivative with acetate.^{3~5)} Progesterone is assumed to be a precursor of cardenolides and it has been well established that *Digitalis* plants are capable of transforming progesterone into cardenolides.^{6~8)} Cholesterol is also transformed into cardenolides by *Digitalis* plants and assumed to be a more distant precursor of cardenolides.^{9,10)} We consequently examined the effects of progesterone and cholesterol. As a prelude to the precursor experiments, we investigated the effects of several water-soluble organic solvents in order to select the best solvent in which the precursors should be dissolved and added to the medium.

MATERIALS AND METHODS

Shoot-forming cultures. The green shoot-forming cultures of *Digitalis purpurea* L. were established and sub-

[†] Studies on the Production of *Digitalis* Cardenolides by Plant Tissue Culture. Part IV. See refs. 1, 2, 12.

TABLE I. COMPOSITION OF THE BASAL MEDIUM

A. Mineral salts			
Salts	mg/liter	Salts	mg/liter
NH ₄ NO ₃	1,650	H ₃ BO ₃	6.2
KNO ₃	1,900	MnSO ₄ ·4H ₂ O	22.3
CaCl ₂ ·2H ₂ O	440	ZnSO ₄ ·4H ₂ O	8.6
MgSO ₄ ·7H ₂ O	370	KI	0.83
KH ₂ PO ₄	170	Na ₂ MoO ₄ ·2H ₂ O	0.25
Na ₂ ·EDTA	37.3	CuSO ₄ ·5H ₂ O	0.025
FeSO ₄ ·7H ₂ O	27.8	CoCl ₂ ·6H ₂ O	0.025
B. Organic constituents			
	mg/liter		mg/liter
Sucrose	30,000	myo-inositol	100
Glycine	2.0	Nicotinic acid	0.5
Indoleacetic acid	1.0	Pyridoxine·HCl	0.5
Benzyladenine	1.0	Thiamine·HCl	1.0

cultured every 3-weeks as described previously¹¹ to be used as the stock culture.

Culture conditions. Murashige-Skoog medium¹¹) containing 1 mg/liter of thiamine·HCl, 1 mg/liter of benzyladenine and 1 mg/liter of IAA was used as the basal medium (Table I). Approximately 1.5 g fresh weight of cells was inoculated into a 500 ml Erlenmeyer flask containing 100 ml of liquid medium, after which the cells were cultured in continuous light (fluorescent lamp: *ca.* 4×10^5 erg/s·cm²) at 28°C on a reciprocal shaker (100 s.p.m.: 4 cm long) for 3 weeks.

Measurement of growth. Harvested cells were lyophilized and their dry weight determined.

Assay for digitoxin. The digitoxin concentration was determined by radioimmunoassays, as described previously.¹²⁾

Extraction from cells. The lyophilized shoots and undifferentiated cells in each flask were homogenized with 50 ml of ethanol in a glass homogenizer. This homogenate was then heated at 74°C for 4 hr and filtered. The filtrate was dried *in vacuo*, its residue mixed with 2 ml of ethanol and diluted with 18 ml of H₂O. When necessary, the extract was diluted further with H₂O to the desired concentration. The extract was then submitted to radioimmunoassays. In the precursor experiments, the filtrate was mixed with an equal volume of 0.1 N H₂SO₄, heated at 70°C for 30 min and then extracted three times with 20 ml of chloroform. The extracts were combined, dehydrated with anhydrous Na₂SO₄ and filtered. The filtrate was evaporated to dryness and the residue was dissolved in 1 ml of chloroform-methanol (1:1, v/v) mix-

ture. This solution was kept for spotting.

Extraction from medium. The medium collected by filtration of each culture broth was mixed with an equal volume of 0.1 N H₂SO₄, heated, extracted with chloroform, dehydrated, evaporated, and re-extracted with 1 ml of chloroform-methanol (1:1, v/v).

Preparative TLC. Fifty μ l of the sample prepared as described above was spotted on a silica gel GF₂₅₄ plate (type 60, Merck, 0.5 mm thick) activated at 120°C for 2 hr. On both margins of the plate, a mixture of authentic digitoxigenin and either progesterone or cholesterol was spotted as an index. The plate was developed at room temperature to a distance of 18 cm using a hexane-ethylacetate (1:1, v/v) solvent system. After development and drying, the sample region of the plate was covered with vinyl film, leaving both margins open, and the plate was sprayed successively with 10% *m*-dinitrobenzene in benzene and 10% potassium hydroxide in methanol. The *R_f* values of digitoxigenine, progesterone and cholesterol were 0.4, 0.8 and 0.9, respectively. The area from *R_f* 0 to *R_f* 0.45 was removed by scraping from the plate and then eluted with methanol. The eluate was dried *in vacuo*, and the residue was dissolved in 0.5 ml of ethanol, diluted with 4.5 ml or more of H₂O and submitted to radioimmunoassays.

RESULTS AND DISCUSSION

Control cultures

The control cultures were carried out under standard conditions, and cell yield and digitoxin content of these were *ca.* 1.7 g dry weight and *ca.* 90 μ g/flask, respectively.

FIG. 1. Effect of CaCl_2 Concentration on Growth and Digitoxin Content.

A: The concentration of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ was varied from one-tenth to threefold the value in the basal medium; 1/10, 0.3 mM; 1/3, 1 mM; 1, 3 mM; 3, 9 mM.

B: The concentration of Ca^{++} or Cl^- was changed separately; 1/3 $\text{Ca}^{++} \cdot 1 \text{ Cl}^-$ contains 1 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 4 mM KCl; 3 $\text{Cl}^- \cdot 1 \text{ Ca}^{++}$ contains 3 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 12 mM NaCl. The thin bars represent the standard errors calculated from six replicates.

CaCl₂ concentration

The concentration of CaCl_2 in the medium was varied from one-tenth to threefold the value in the basal medium (Fig. 1). Digitoxin content was highest at one-third CaCl_2 and lowest at threefold CaCl_2 , although the growth of the cultures as indicated by dry cell yield was not much affected by the CaCl_2 concentration. At one-third and one-tenth CaCl_2 concentrations, shoot differentiation was apparently promoted while at threefold, it was repressed (Fig. 1A). To determine whether Ca^{++} or Cl^- was responsible for these effects, the concentrations of these ions were varied separately. When Cl^- was increased threefold by the addition of 12 mM NaCl, the digitoxin content was increased. On the other hand, when Ca^{++} was reduced to one-third, keeping the Cl^- concentration the same as in the basal medium by the addition of 4 mM KCl in place of CaCl_2 , the digitoxin content increased similarly to the case with one-third CaCl_2 (Fig. 1B). These results clearly indicate that Ca^{++} is responsible for the phenomenon. A high con-

centration of Ca^{++} seems to repress shoot differentiation which in turn results in a decrease in the digitoxin content. In plant cells, the bulk of Ca^{++} is outside the protoplast, on the exterior surface of the plasmalemma and in the wall, affecting the nature of cell surface.¹³⁾ During shoot differentiation, intercellular connection is considered to play an important role, and such connection must be affected by the nature of the cell surface. So, it seems that Ca^{++} affects shoot differentiation by changing the nature of cell surface. This is the first report to our knowledge that the Ca^{++} concentration in the medium regulates the shoot-differentiation of cultured plant cells.

MgSO₄ Concentration

MgSO_4 Concentration was varied from one-tenth to tenfold (Fig. 2A). The digitoxin content was highest at threefold MgSO_4 (ca. 120% of the control), and decreased with both lower concentrations and at tenfold MgSO_4 . Growth was not particularly affected. At one-tenth MgSO_4 , the cultures became brownish in

FIG. 2. Effect of MgSO_4 Concentration on Growth and Digitoxin Content.

A: The concentration of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ was varied from one-tenth to tenfold the value in the basal medium; 1/10, 0.15 mM; 1/3, 0.5 mM; 1, 1.5 mM; 3, 4.5 mM; 10, 15 mM.

B: The concentration of Mg^{++} or SO_4^{--} was changed separately; 3 Mg^{++} · 1 SO_4^{--} contains 1.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 3 mM MgCl_2 ; 3 SO_4^{--} · 1 Mg^{++} contains 1.5 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 3 mM Na_2SO_4 . The thin bars represent the standard errors calculated from six replicates.

color, and at one-third MgSO_4 , the cultures turned yellowish green. At tenfold MgSO_4 , shoot differentiation was repressed. To determine whether Mg^{++} or SO_4^{--} was responsible for the promotion at threefold MgSO_4 , the concentration of each ion was separately increased to threefold using MgCl_2 or Na_2SO_4 . When Mg^{++} was increased, the digitoxin content increased by about 40%. On the other hand, when SO_4^{--} was increased, the digitoxin content decreased by about 30% (Fig. 2B).

There is a long list of enzymatic reactions which require or are promoted by Mg^{++} . Furthermore, Mg^{++} is an indispensable component of chlorophyll and its deficiency results in the degradation of the inner structure of the chloroplast. The fact that a two-thirds decrease of MgSO_4 resulted in fading of the green color and holding growth at the normal level indicates that the threshold point of Mg concentration for maintenance of chlorophyll is higher than that for maintenance of the other enzymatic activities necessary for growth. Therefore, the threefold increase of Mg might

promote digitoxin formation indirectly by maintaining chlorophyll or chloroplast better than the normal level of Mg. Tsai *et al.*¹⁴⁾ have reported that increasing $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and KH_2PO_4 concentrations promoted shoot differentiation, but no apparent promotion of shoot differentiation was observed in our cultures at threefold or higher concentrations of MgSO_4 .

FeSO₄ plus Na₂-EDTA Concentration

The concentration of FeSO_4 coupled with Na_2 -EDTA was varied from one-tenth to tenfold (Fig. 3). Maximal digitoxin formation as well as maximal growth took place at the same concentration as in the basal medium.

Minor Elements

The concentration of each of the minor elements of the Murashige-Skoog medium *i.e.*, H_3BO_3 , MnSO_4 , ZnSO_4 , KI , Na_2MoO_4 , CuSO_4 and CoCl_2 , was separately increased to threefold. Growth was not affected in any case, whereas the digitoxin content was slightly increased by a threefold increase in the con-

centration of KI, Na_2MoO_4 or CuSO_4 , and the slightly reduced by ZnSO_4 .

Quite how these elements affect the digitoxin formation is open to discussion. The effect of iodine on plant cells is obscure; Murashige and Skoog decided KI level in their medium arbitrarily.¹¹⁾ The concentration of Cu^{++} in the Murashige-Skoog medium is insufficient in some cases. Ikeda *et al.*¹⁵⁾ have reported that a tenfold increase in Cu^{++} concentration promoted growth and cytochrome aa_3 formation in tobacco cultured cells. Whether this cytochrome aa_3 content affects digitoxin content

or not is unknown. There is a report which showed that seed or plant treatments with an Mo solution increased the content of cardiac glycosides of leaves and flowers of *Digitalis lanata*.¹⁶⁾ Nitrate reductase is the only established Mo-enzyme of the plant.¹³⁾ But it is not believed that a change in the nitrate reductase level might promote digitoxin formation.

Initial pH

The pH of the medium was adjusted to 4, 5, 6, 7 or 8 before autoclaving which modified to 4, 4.5, 5.5, 6.5 and 7.6, respectively, after autoclaving. The pH of the basal medium was adjusted to 6 before autoclaving. The optimal pH of the medium for digitoxin formation before autoclaving was 6, whereas growth was not particularly affected. The pH of the medium at harvest was in a relatively narrow range (5.1~5.4), likewise with many other plant cell cultures^{17,18)} (Table II).

Precursors

Organic solvents. Two ml of each of the following five water-soluble solvents, *i.e.*, methanol, ethanol, propylene glycol, dimethyl sulfoxide and acetone, was added to 100 ml of the medium and the effect on growth and digitoxin content was examined (Fig. 4). Additionally, the digitoxin concentration in the culture medium after 3 weeks of culture was determined. Growth was severely repressed by ethanol, promoted slightly by both propylene glycol and dimethyl sulfoxide, and not affected by methanol and acetone. On the other hand, digitoxin production was decreased by all the solvents, most severely by

FIG. 3. Effect of FeSO_4 and $\text{Na}_2\text{-EDTA}$ Concentration on Growth and Digitoxin Content.

The concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Na}_2\text{-EDTA}$ was varied together from 0 to tenfold the value in the basal medium. The concentration of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ and $\text{Na}_2\text{-EDTA}$ is at 0, 0 and 0; at 1/10, 0.01 mM and 0.02 mM; at 1, 0.1 mM and 0.2 mM; at 3, 0.3 mM and 0.6 mM; and at 10, 1.0 mM and 2.0 mM, respectively. The thin bars represent the standard errors calculated from six replicates.

TABLE II. EFFECT OF INITIAL pH OF THE MEDIUM ON GROWTH AND DIGITOXIN CONTENT

pH before autoclaving	pH after autoclaving	pH at harvest	Dry cell yield/flask (% control)	Digitoxin content/flask (% control)
4	4	5.1 ± 0.6	104 ± 2	89 ± 3
5	4.5	5.2 ± 0.2	103 ± 3	94 ± 18
6	5.5	5.1 ± 0.1	100 ± 1	100 ± 23
7	6.5	5.1 ± 0.1	98 ± 2	72 ± 28
8	7.6	5.4 ± 0.1	99 ± 3	67 ± 2

FIG. 4. Effect of Organic Solvents on Growth and Digitoxin Content.

Two ml of each of the following solvents, *i.e.*, methanol (MeOH), ethanol (EtOH), propylene glycol (PG), dimethyl sulfoxide (DMSO) and acetone, was added separately to the medium. The thin bars represent the standard errors calculated from six replicates.

FIG. 5. Effect of Progesterone on Growth and Digitoxin Content.

Zero to 50 mg of progesterone dissolved in 2 ml of methanol was added to 100 ml of medium. The thin bars represent the standard errors calculated from six replicates.

FIG. 6. Effect of Cholesterol on Growth and Digitoxin Content.

Zero to 50 mg of cholesterol dissolved or suspended in 2 ml of methanol was added to 100 ml of medium. The thin bars represent the standard errors calculated from six replicates.

ethanol followed by dimethyl sulfoxide, propylene glycol, methanol and acetone in that order. We chose methanol as the solvent for precursors, because the degree of inhibition is more reproducible ($64 \pm 3\%$ of the control) than that by acetone ($79 \pm 21\%$). In any case, the digitoxin content in the culture medium was *ca.* 1% of the total digitoxin content in each flask.

Progesterone. From 0.1 to 50 mg of progesterone were added to 100 ml of the medium using 2 ml of methanol as the solvent, and their effects were examined (Fig. 5). Growth was not repressed by 1 mg or less of progesterone, although 10 mg or more did repress growth. Digitoxin content was considerably promoted (183% of the control) by 0.1 mg of progesterone. The digitoxin content in the culture medium was *ca.* 1% of the total digitoxin in each flask, except in the case of 50 mg of progesterone when it was *ca.* 5%.

Cholesterol. Growth was only slightly affected even at 50 mg of cholesterol per 100 ml of medium, but digitoxin formation was repressed by cholesterol at 0.1 mg or more (Fig. 6). The digitoxin content in the medium did not exceed 1% of the total digitoxin.

Lui and Staba¹⁹⁾ have reported that the concentration of digoxin in leaf and root cultures was increased by adding some precursors, including progesterone and cholesteryl acetate. However, in this study, cholesterol was ineffective. The added cholesterol may not have reached the biosynthetic site of the cardenolides in the cell. An increase in digitoxin content by the addition of progesterone suggests that the added progesterone was able to get to the site of cardenolide synthesis and thereafter was transformed into digitoxin. Permeability of the plasma membrane of plant cells can be changed by some organic solvents. Delmer has reported that 5~10% dimethyl sulfoxide increased the permeability of the plasma membrane of cultured tobacco cells to small molecules.²⁰⁾ Alfermann *et al.* have reported that suspension cultured cells of *Digitalis lanata* transformed β -methyl-digitoxin, which was added in a methanolic solution into β -methyl-digoxin, and accumulated the latter in the culture medium.²¹⁾ In this case, the methanol concentration was as high as 8%. Their result suggests that β -methyl-digitoxin and β -methyl-digoxin both passed through the plasma membrane, although they did not confirm the site of the hydroxylation to be in the cell. Under the condition of our

experiments, no significant release of digitoxin into the medium was observed. When the ideal condition which permits the release of digitoxin is established, our shoot-forming cultures would be a useful system for the biotransformation of progesterone or more inexpensive precursors into digitoxin.

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