Journal of Natural Products
Vol. 48, No. 2, pp. 210-222, Mar-Apr 1985

METHODS FOR INCREASING DIOSGENIN PRODUCTION BY *DIOSCOREA* CELLS IN SUSPENSION CULTURES[1]

J.S. ROKEM,* B. TAL,[2] and I. GOLDBERG

Department of Applied Microbiology, Hebrew University-Hadassah Medical School, P.O.B. 1172, Jerusalem 91010, Israel

ABSTRACT.—This review describes the application of various methods for the improvement of secondary metabolite (diosgenin) production by *Dioscorea deltoidea* plant cells in suspension cultures.

Initially 1% diosgenin (of dry weight), corresponding to a concentration of 114 mg/liter was obtained after 28 days of incubation. Concentration and rate of diosgenin production were improved to 7.2% (of dry weight) and to a maximal concentration of 230 mg/liter in 12 days.

The interest in growing plant cells in suspension cultures is rapidly expanding because cell suspension cultures are useful in the study of genetics, biochemistry, and physiology of plants as well as for other purposes listed in Table 1. From a biotechnological point of view, the production of valuable metabolites by means of fermentation technology, similar to that in use for microbial metabolites, holds a great potential (1). The required conditions for synthesis of secondary metabolites are not well understood, mainly because different medium constituents and environmental factors influence their synthesis (2-4), and the control of the onset of secondary metabolism is still a main problem under investigation both in microbial (5) and plant systems (6).

TABLE 1. Areas of Research Using Plant Cell Suspension Cultures

1. Asexual multiplication of plants
2. Development of disease free plants (viruses)
3. Fusion of protoplasts to obtain new varieties
4. Biochemical and physiological research:
   a) biochemical pathways
   b) hormone effects
   c) host parasite interactions
   d) embryogenesis
   e) morphogenesis
   f) cell cycle events
5. Genetic research:
   a) improvement of protein quality
   b) increase of nitrogen fixation
   c) increase of photosynthesis
   d) resistance to herbicides
   e) resistance to high salt concentration
6. Biosynthesis of valuable metabolites
7. Biotransformation of chemicals

About 30% of therapeutically active compounds are of plant origin and are presently extracted from various plant tissues (7). There is great interest in the use of plant cell suspensions for the production of such compounds (2-4). In several cases, it has been shown that in plant cell suspension cultures it is possible to obtain concentrations of products similar to, or higher than, those for the whole plant (Table 2). Because sec-

[1]Presented as a plenary lecture at the "Biotechnology in Natural Products Research" Symposium of the 25th Annual Meeting of the American Society of Pharmacognosy at the University of Texas, Austin, Texas, August 19-23, 1984.

[2]Present address; ARCO, Plant Cell Research Institute, Dublin, CA 94556.

TABLE 2. Comparison of Whole Plants with Plant Cell Suspension Cultures for Metabolite Production

Metabolite	Plant species	Per cent of dry weight		References
		Whole plant	Cell suspension culture	
Procyanindin	*Cryptomeria japonica*	17.1 (inner bark)	26.6	(8)
Shikonin	*Lithospermum erythrorhizon*	1-2	12-15	(9)
Saponin	*Panax ginseng*	0.5	2.2	(10)
Catharanthine	*Catharanthus roseus*	0.0017	0.005	(11)
Diosgenin	*Dioscorea deltoidea*	2-4	7.8	(12)
Anthraquinones . . .	*Morinda citrifolia*	2.2	18	(13)
Rosemarinic acid . .	*Coleus blumei*	3	15	(14)

ondary metabolites are usually accumulated in a specific part of the differentiated plant, while plant cells in suspension cultures are undifferentiated cells (15), there are compounds which have, so far, not been found in suspension cultures even though they are present in the whole plant or parts thereof. The best known examples are the antitumor alkaloids, vincristine, and vinblastine, which are present at low concentrations in the whole plant of *Catharanthus roseus* (16), whereas none of these complex alkaloids has been reported to occur in cell suspension cultures of this plant. On the other hand, compounds have been isolated from plant cells in suspension cultures which are not found in the whole plant. One example is the coumarin rutacultin (17), which cannot be found in the plant *Ruta graveolens*. Probably other novel compounds could be found that are not present in the whole plant.

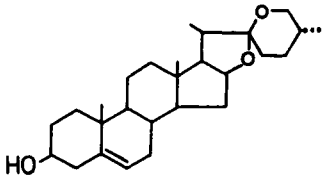
It has been shown by several groups (18, 19) that cells in suspension are totipotent, i.e., have the ability to regenerate the whole plant (20), indicating that the complete genetic makeup of the plant is present also in undifferentiated cells. The main challenge, therefore, is to find the conditions under which a cell suspension culture will produce the desired metabolite. It must be kept in mind that secondary metabolites from plants have a potential industrial value only when the metabolites are not produced by microbial cells since microorganisms usually grow much more rapidly and, in most cases, are better characterized. Various techniques and methods have been described and tested to improve the synthesis of valuable metabolites in plant cell suspension cultures. These include: clone selection, genetics, optimization of media and growth conditions, type and concentration of hormones, physical environment, feeding of precursors, and manipulation of biosynthetic pathways. The methods are similar to the techniques used for the improvement of secondary metabolite production by microbial cells.

We have studied the production of the steroid diosgenin (**1**) by undifferentiated cells of *Dioscorea deltoidea* in suspension cultures. Diosgenin is one of the raw materials required for the manufacturing of pharmaceutically important steroids. The aim of this research has been to improve both rate of synthesis and final diosgenin concentration.

SCREENING OF CULTURES.—With plant cells, the search for a strain with a good production capability can be time consuming. This is due to several factors: (a) the time it usually takes to establish homogeneous suspensions from plants before the strain can be assayed, (b) the limited possibility (due to the character of the compounds of interest) for a rapid screening procedure of a large number of clones once they are established ([recently, screening methods using radioimmunoassay have been developed for a few plant

metabolites (14, 21)], and (c) the growth conditions used in the initial screening are not always optimal for secondary metabolite production.

A number of *Dioscorea* species—including two strains of *Dioscorea composita*, three strains of *D. deltoidea*, and one strain each of *Dioscorea floribunda* and *Dioscorea glanduloza*—were obtained and screened for their ability to produce diosgenin when grown as cell suspension cultures. A hplc method was developed (22) where a time-consuming derivatization of sterols was not necessary. The method was quantitative in the range of 0-40 μg of diosgenin.



1

OPTIMIZATION OF CELL GROWTH AND PRODUCT FORMATION BY CHANGES IN MEDIUM COMPONENTS AND GROWTH CONDITIONS.—Medium constituents and their concentrations are of utmost importance for obtaining high biomass yields and high concentrations of secondary metabolites (23, 24). It should be remembered that optimization of biomass yield is not directly related to secondary product formation. However, with a larger cell mass there is potentially a greater metabolite production.

Two approaches have been taken by various groups to obtain increased product formation: (a) Testing of various media developed for plant suspension cultures and their ability to support product formation (9); (b) taking a known medium and changing concentrations of the medium constituents in order to compare production ability (25). These types of experiments are usually performed in batch cultures (13, 25). The use of the continuous culture technique for medium development was originally utilized for the optimization of biomass production by microorganisms (26, 27).

A combination of continuous culture and batch methods was used (12, 28, 29) to study the influence of medium constituents on biomass and metabolite production. Initialy, batch methods are used to find a medium and a strain that produced diosgenin.

Four different media commonly used for growth of plant cells were tested for their ability to support biomass production by *D. composita* in bath culture (Table 3A). The Murashige & Skoog (MS) medium resulted in the highest biomass yield, but in no case was there any diosgenin produced in the cells. Three media were used for growth of *D. deltoidea*. MS medium (34, 35) gave the highest biomass yield, and 0.9% diosgenin of dry weight was found in the cells after 28 days incubation.

Plant-growth hormones.—To obtain reproducible results when growing plant cells in suspension cultures, it is important to have homogeneous cell suspensions. This can be accomplished by the addition of various plant-growth hormones to the medium (36). The nature of these hormones and their concentration also influence the ability of the cells to produce metabolites. In Table 3B, results are presented for diosgenin production by *D. deltoidea* cells when various combinations of kinetin and 2,4-dichlorophenoxyacetic acid (2,4-D) were added to MS medium. Certain combinations of growth hormones gave a high cell yield, and other combinations resulted in a higher percentage of diosgenin (of dry weight) as well as the total amount of diosgenin (Table 3B).

Continuous culture.—To develop further the MS medium in order to obtain higher concentrations of diosgenin, we chose to utilize the continuous culture technique. It was found that on prolonged storage of the MS medium, precipitates developed. To

avoid this, problem, nitrioltriacetic acid (NTA) (a weak chelating agent) was added to the medium (12). Various NTA concentrations were tested, and 30 mg/liter did not inhibit growth but precipitate formation.

D. deltoidea did not grow in a mechanically stirred fermentor, probably due to the sensititivity of the cells to the shear stress imposed by the impeller. Similar results were obtained with various plant species (7). It was possible to obtain growth and steady states when *D. deltoidea* was grown in a bubble column fermentor of the "Wilson" type (37). At low aeration rates, oxygen limited the growth of the cells, and at 0.3 volumes air per culture volume per minute (vvm) oxygen was no longer limiting (28). A similar value was reported by Zenk (38) as sufficient for growth of plant cells in suspension at the same sugar concentration. Nitrogen sources were also investigated using the continuous culture technique, and both NO_3^- and NH_4^+ were essential for growth of *D. deltoidea* (28).

Analysis of cells from the continuous culture showed low levels of diosgenin (0.1% of dry weight] due to the fact that these cells were continuously growing in the exponential phase where no diosgenin was formed (Figure 1). Continuous culture was used

TABLE 3. Growth and Diosgenin Levels of Various *Dioscorea* Species on Different Media, Hormone Types, and Hormone Concentrations[a]

A. Dry weight of *Dioscorea composita* grown on various media

Day of harvest	Media[b] (g/liter)			
	MS (30)[c]	7IV-10 (31)	LS (32)	4X (33)
1	0.5	0.5	0.8	0.5
6	1.0	1.2	1.6	0.7
13	4.2	2.8	2.5	1.0
21	11.7	10.2	10.2	0.8

B. Growth and diosgenin production by *Dioscorea deltoidea* with various concentrations of kinetin and 2,4-D in MS-medium[d]

2,4-D (mg/liter)	0.1	0.1	1.0	0	0
Kinetin (mg/liter)	0	0.2	0.2	0.2	0
Day of harvest	Dry weight (g/liter)				
7	2.5	1.9	1.3	2.9	2.7
14	6.7	6.1	3.6	6.2	9.6
21	12.9	16.0	8.3	17.7	18.3
28	10.9	11.4	12.6	16.3	16.3
	Diosgenin on day 28				
% of dry weight	0.9	1.0	0.6	0.2	0.25
mg/liter	98	114	76	33	41

[a]Results were kindly provided by Z. Reuveny.

[b]The media contained the following hormones (mg/liter):
MS: 0.1 2,4-dichlorophenoxyacetic acid (2,4-D); 0.2 kinetin
7IV-10: 0.1 2,4-D; 0.25 kinetin; 0.1 α-naphthaleneacetic acid (NAA); 0.1 indoleacetic acid (IAA)
LS: 0.22 2,4-D; 0.186 NAA
4X: 2.0 2,4-D; 0.2 kinetin; 0.5 NAA; 0.5 IAA

[c]Numbers in parentheses refer to reference for medium composition.

[d]Size of inoculum was 0.7 g/liter dry weight.

here to optimize biomass production. In order to investigate the ability of the cells to produce diosgenin, they were further incubated either in a carbon-free medium (to minimize cell growth) under batch conditions or transferred to a second stage fermentor working as a continuous culture (see below).

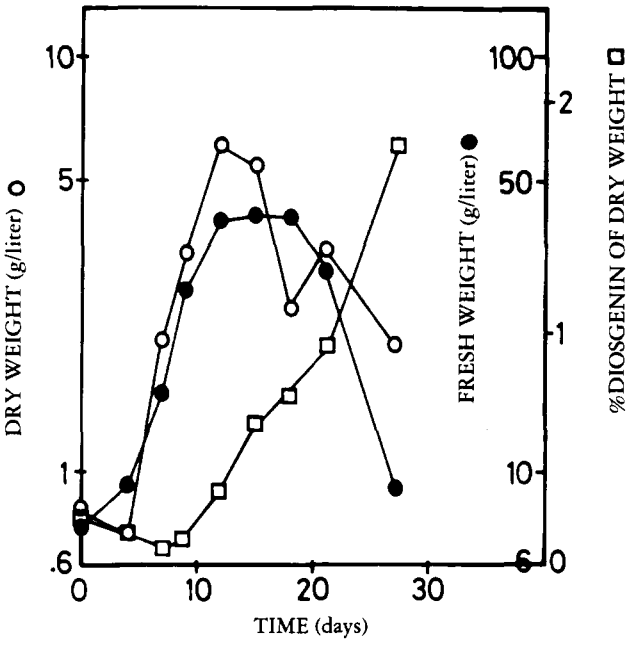


FIGURE 1. Kinetics of growth and diosgenin production by *Dioscorea deltoidea* cells grown in a batch culture on MS 0.1 medium containing 15 g/liter sucrose. "With permission from *Planta Med.*"

Carbon and nitrogen substrates.—Cells taken from the continuous culture were used to investigate the influence of various nitrogen and carbon sources on diosgenin production (Table 4). The nature and concentration of both the nitrogen and carbon sources were important for product formation. Both NH_4^+ and NO_3^- were required at a cer-

TABLE. 4. The Influence of Medium Constituents on Growth and Diosgenin Production by *Dioscorea deltoidea*

Nitrogen source (g/liter)	Carbon source (g/liter)	Dry weight[a] (g/liter)	Diosgenin[b]	
			(% of dry wt)	mg/liter
KNO_3 (1)	Sucrose (30)	1.0	<0.1	
KNO_3 (1.9), NH_4NO_3 (1.65) .	Sucrose (30)	13.0 (18)	1.9 (33)	133
KNO_3 (1.9), NH_4NO_3 (1.65) .	Sucrose (5)	1,75 (16)	1.2 (16)	21
KNO_3 (2.6), NH_4NO_3 (2.4) . .	Sucrose (5)	0.9 (16)	3.1 (21)	12.4
KNO_3 (1.9), NH_4NO_3 (1.65) .	Sucrose (15)	4.7 (16)	3.8 (21)	64.6
KNO_3 (1.9), NH_4NO_3 (1,65) .	Fructose (30)	13.7 (16)	0.1 (16)	13.7
KNO_3 (1.9), NH_4NO_3 (1.65) .	Glucose (30)	12.4 (16)	0.1 (16)	12.4
KNO_3 (1.9), NH_4NO_3 (1,65) .	Lactose (10)	3.4 (16)	1.1 (21)	29.7
KNO_3 (1.9), NH_4NO_3 (1.65) .	Galactose (10)	1.1 (16)	1.8 (21)	18
KNO_3 (1.9), NH_4NO_3 (1.65) .	Starch (30)	7.0 (16)	1.1 (21)	75

[a]Highest dry weight determined (numbers in parenthese=days after inoculation).
[b]Highest diosgenin concentration determined (numbers in parentheses=days after inoculation).

tain ratio (KNO_3=1.9 g/liter and NH_4NO_3=1.65 g/liter) to enable diosgenin production. Some carbon sources (fructose, glucose) supported growth but not product formation whereas other carbon sources (lactose, galactose, and starch) resulted in diosgenin production but in lower amounts than those obtained with sucrose. The initial sucrose concentration influenced the rate of synthesis and final concentration of diosgenin (Table 5). The highest diosgenin concentration (3.8% of dry weight) was obtained with 15 g/liter sucrose whereas the highest productivity (5 mg/liter/day) was obtained with 30 g/liter sucrose.

TABLE 5. Influence of Sucrose Concentration on Growth and Diosgenin Levels in *Dioscorea deltoidea*

Sucrose (g/liter)	Time of growth								
	16 days			21 days			34 days		
	cell dry wt g/liter	Diosgenin		cell dry wt dry wt	Diosgenin		cell dry wt dry wt	Diosgenin	
		%of dry wt	mg/liter		% of dry wt	mg/liter		% of dry wt	mg/liter
5	1.8	1.2	21	0.5	1.2	5	—	—	—
15	4.7	2.2	102	1.6	3.8	62	—	—	—
30	11.3	1.8	203	9.4	1.1	105	6.6	2.0	131
45	18.7	0.1	19	18.0	0.1	18	13.8	0.1	14
60	17.6	0.1	18	20.1	0.1	20	20.8	0.1	21

From the results in Table 5, it can be seen that diosgenin is probably degraded upon further incubation. The total amount of diosgenin decreased from day 16 to day 21.

Magnesium and phosphate.—The influence of magnesium and phosphate concentrations on diosgenin production was investigated (Table 6). Magnesium had no effect on diosgenin levels whereas an increase in the phosphate concentration resulted in a marked increase in diosgenin when expressed both as % of dry weight and total concentration (Table 6).

TABLE 6. Influence of Magnesium and Phosphate Concentration on Diosgenin Production by *Dioscorea deltoidea*

Batch Culture Carbon sourse (g/liter)	$MgSO_4$(g/liter)	KH_2PO_4(g/liter)	Dry weight of cells (g/liter)	Diosgenin	
				% of dry weight	mg/liter
Sucrose 30	0.37	0.17	13(18)[a]	1.9(33)	133
Sucrose 15	0.72	0.08	4.5(18)	2.6(25)	70
Sucrose 15	0.18	0.08	4.5(18)	2.6(25)	70
Sucrose 15	0.37	0.17	4.7(16)	3.8(21)	60
Sucrose 15	0.72	0.25	2.9(18)	7.8(25)	183
Two-stage continuous culture					
First stage					
Sucrose 15	0.37	0.25	7.0	0.05	
Addition to second stage					
—	—	38.2 mg/liter•day	5.5	0.1	

[a]Numbers in parentheses=days after inoculation.

Separation of the growth phase from the production phase.—A combination of continuous culture and batch methods was used in order to study the importance of various nutrients and environmental conditions on diosgenin production when changes were made in growth and production phases. Cells were grown under steady-state conditions in a continuous culture and transferred either to shake flasks or to a second continuous culture, and the diosgenin concentration was measured. It was found that the phosphate concentration during the growth phase had a large influence on the later production of diosgenin whereas if the phosphate was added in the production phase, there was no increase in diosgenin concentration (Table 6).

The sugar concentration in the spent medium of the first stage was very low (50 mg/liter) as compared to the concentration in the medium reservoir (15 g/liter) (12), indicating that no external carbon source was required for biosynthesis of diosgenin in the production phase. Addition of sucrose (2 g/liter/day) directly to the second stage did not have any effect either on biomass or diosgenin level (12). This is similar to the result obtained by Heble and Staba (39) who found that diosgenin was produced mainly by nondividing cells.

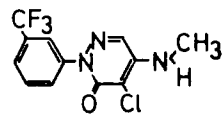
A two-stage continuous culture was one solution for obtaining high concentrations of shikonin in *Lithospermum erythrorhizon* (9). In the first stage, the plant cells were grown in a rich medium that supported biomass production, but no metabolite was formed. These cells were transferred to a second stage where the medium was optimized for shikonin biosynthesis.

As mentioned earlier, cell growth and metabolite production are not conditionally related. The rationale would be first to optimize the medium and growth conditons for biomass yield and then to find the parameters that support high product yield. It might even be, as was shown in the case of shikonin production (9), that for growth and production two completely different media have to be used.

CONTROL OF BIOSYNTHETIC PATHWAYS.—The understanding of the biosynthetic pathways of metabolites of commercial interest has been of great benefit in obtaining high concentrations of these metabolites (40). The biosynthetic pathways of most secondary metabolites are not known in full detail, and the knowledge acquired is based upon the pattern of incorporation of isotopically labelled precursors into intermediary compounds and products, whereas the enzymology is still in its infancy. Our interest is in a steroidal product, and the biosynthesis of sterols has been extensively described (41-44). With this in mind, we used two different approaches to understand better diosgenin formation and to increase the levels of diosgenin.

Change of flow of precursor compounds.—It has been shown that addition of specific inhibitors increased the levels of lycopene in *Citrus* (45, 46) and polyisoprenoids in *Guayule* species (47). Since the early biosynthetic precursors of diosgenin are common to many other metabolites (phytol, carotenoids, chlorophyll, terpenes, etc.) (48), inhibition of the formation of one or more of these metabolites might increase the level of diosgenin (Figure 2). In all experiments the cells of *Dioscorea deltoidea* were grown without illumination. This was done to prevent chlorophyll formation and diversion of metabolites from diosgenin (arrow 2, Figure 3). Specific inhibitors of carotenoid biosynthesis (arrow 1, Figure 3), the phenylpyridazinone herbicides (49-51), were studied for their influence on diosgenin levels. At a concentration of 10^{-6}M, norflurazon (SAN 9789) (**2**) increased the rate production so that 180 mg/liter was obtained after 14 days of incubation as compared to 25 days (Table 6) (52). The effect of norflurazon was greater the earlier in the growth phase it was added to the culture (Figure 2).

Similar uses of specific inhibitors for increasing secondary metabolite levels were recently reported. Various amines were found to increase the formation of indole alkaloids



2

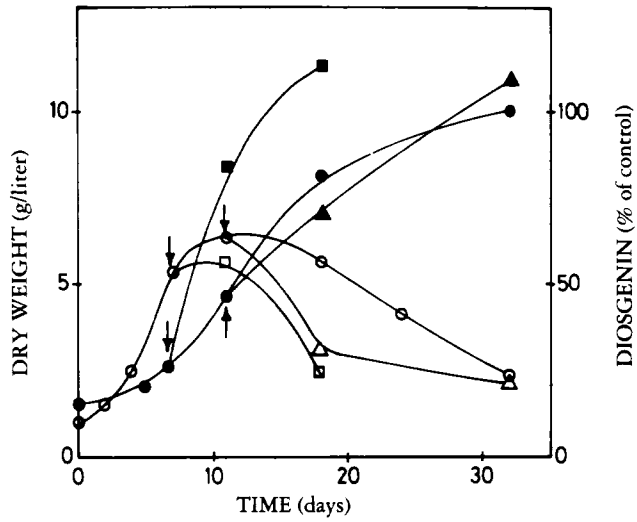


FIGURE 2. The effect of norflurazon (**2**) (1 μM) on growth (open symbols) and diosgenin production (full symbols) by cell suspension cultures of *Dioscorea deltoidea*. Symbols: Control (○,●); addition of norflurazon on day 7 (□,■) and day 11 (△,▲). "With permission from *Phytochemistry*. Published by Pergamon Press."

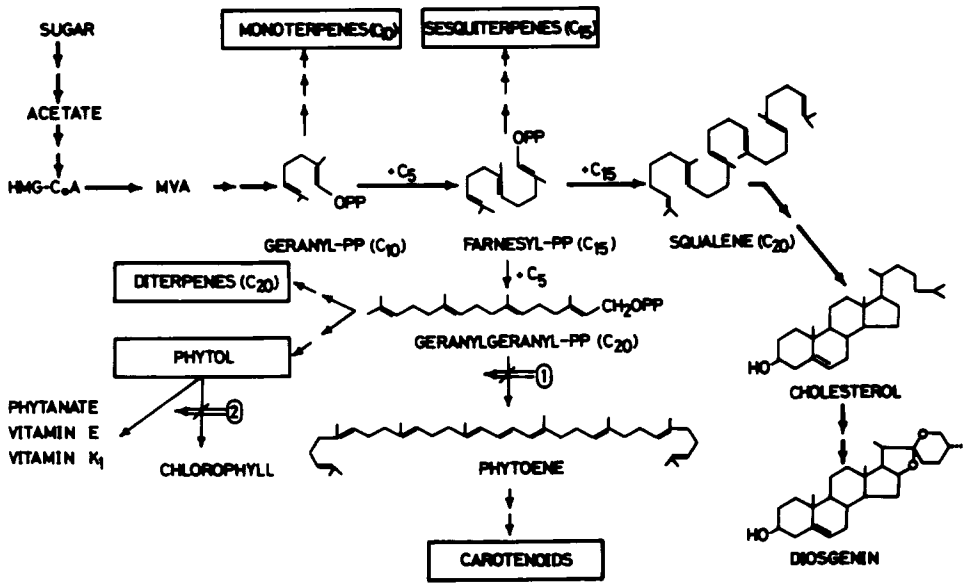


FIGURE 3. Schematic representation of diosgenin biosynthesis. Arrow 1: inhibition of carotenoid formation. Arrow 2: inhibition of chlorophyll formation.

(53) and to increase the sesquiterpenoid biosynthesis rate on account of inhibition of steroids and alkaloids in potato (54).

Kinetics of product formation.—Various metabolic inhibitors, both general and specific, can be used for the study of formation kinetics of secondary metabolites in plant cell suspension culture.

Cycloheximide, a *de novo* protein synthesis inhibitor (55), reduced growth and product accumulation when added at the various stages of growth of *Dioscorea deltoidea* and *Papaver somniferum* (56), indicating the necessity of *de novo* protein synthesis during all stages for product formation.

Compactin, a competitive inhibitor of the enzyme 3-hydroxy-3-methylglutaryl CoA reductase (57), was added at various stages of growth to *D. deltoidea*. It partially inhibited growth. Diosgenin formation was mainly inhibited when compactin was added during the exponential growth phase even though most of the diosgenin was formed after growth had ceased (58). Using (1-^{14}C) acetate, we found that compactin inhibited the incorporation of radiolabel into diosgenin only during the exponential growth phase (58). This observation, and the fact that no carbon source was required for the production of diosgenin in the second stage of the two-stage continuous culture process (Table 6), indicate that diosgenin is synthesized from intermediate metabolites formed during the exponential growth phase.

Intermediate metabolites.—Some metabolites present in the whole plant have not been found in plant cell suspension cultures. The best known example is the two antitumor alkaloids, vincristine and vinblastine (16, 59), found in very low concentration in plants of *Catharanthus roseus*. However, the precursors of vinblastine, vindolinine, and catharanthine, are produced in plant cell suspension cultures, and these can be joined by chemical synthesis to form vinblastine (60). In this way, it might be possible, by combining biotechnology and chemistry, to obtain compounds that are not produced by plant cell suspensions.

In the study of diosgenin production, intermediate metabolites were thought to be formed at a much earlier stage in the growth cycle than diosgenin. An intermediate metabolite (Furostanol I; Figure 4) was isolated and characterized (61). This metabolite was formed concurrently with the growth of *D. deltoidea* (Figure 5). Furostanol I is the

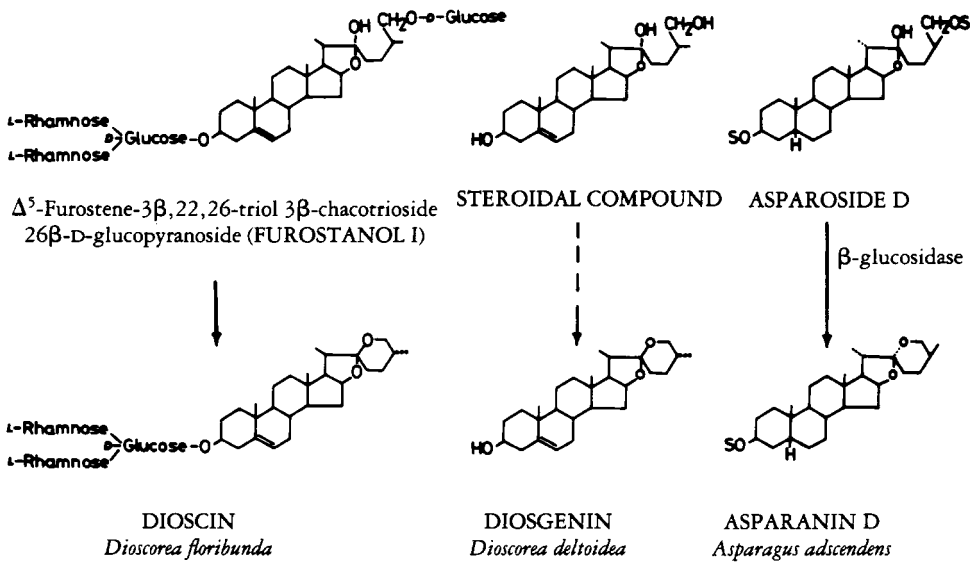


FIGURE 4. Intermediate compounds in sapogenin biosynthesis. "With permission from *Biochem. J.*"

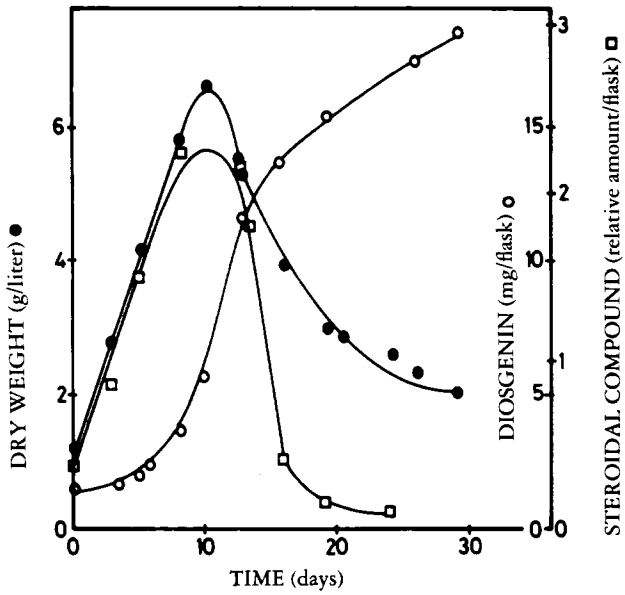


FIGURE 5. Kinetics of growth and product formation by *Dioscorca deltoidea* cells in suspension cultures. "With permission from *Biochem. J.*"

immediate precursor of the end product (dioscin). The last enzyme in the biosynthesis of dioscin is therefore most probably active only in the later stages of growth (Figure 5) (Dioscin, the glycoside of diosgenin, is formed in the cells but since the assay procedure involves a hydrolysis step, diosgenin is obtained). A similar intermediate was described in *Asparagus adscendens*, and it was shown that this intermediate is converted in vitro by a glucosidase to an analogue compound of diosgenin, asparanin D (Figure 4) (62).

It should be possible to shorten diosgenin production considerably by (a) growing the cells in a continuous culture where Furostanol I will be produced, since it is formed concurrently with growth and/or (b) transforming furostanol in vitro to diosgenin using the appropriate enzyme (which could be immobilized).

USE OF STERILIZED FUNGAL MYCELIA AS ENHANCERS OF METABOLITE BIOSYNTHESIS.—Secondary metabolites are formed by many different species both of microbial and plant origin. The function of these metabolites, which are not required in most cases for the growth of the producer strain, is in most instances not known (5). Secondary metabolites are thought to have survival value for the species in which they are produced. We tried to rationalize the presence of dioscin in the tubers of *D. deltoidea*. It has been shown (63) that steroidal saponins are able to inhibit growth of various fungi. Fungi can act as elicitors for the production of these compounds (phytoalexins, 64).

Various fungal mycelia were added after sterilization to plant cell suspension cultures of *D. deltoidea* (Table 7). Some of the fungi increased the levels of diosgenin (expressed as both % of dry weight and final concentration) whereas others decreased levels, indicating that some component in the fungal mycelia enhanced diosgenin production. No such stimulation was found when two known phytoalexin elicitors (laminarin, 65; and arachidonic acid, 66) were added to cell suspensions of *D. deltoidea* (67).

CONCLUSIONS.—The production of metabolites by plant cell suspension cultures can be regarded as a differentiation process on the biochemical level and is dependent both on environmental conditions as well as the genotype of the material in question.

TABLE 7. Levels of Diosgenin in Suspension Cultures of *Dioscorea deltoidea* Cells after Addition of Various Fungal Mycelia[a]

Fungal species	cell dry wt g/liter	Diosgenin	
		mg/liter (% change)	% of dry wt
Control	5.4	134	2.5
Rhizopus arrhizus	3.2	230 (+72)	7.2
Sclerotinia sclerotium	2.7	117 (+32)	6.5
Fusarium oxysporum	3.6	155 (+16)	4.3
Aspergillus niger	3.3	147 (+10)	4.5
Macrophomina phaseolina	4.7	118 (−22)	2.5
Sclerotium rolfsii	4.4	82 (−37)	1.9

[a]Fungal mycelia were sterilized and added (2 ml, 5 μg dry wt/ml) to growing cells (100 ml) of *D. deltoidea* on day 9 of growth. The plant cells were harvested and assayed for diosgenin content on day 12.

The influence of the environmental conditions on diosgenin biosynthesis were investigated. Similar strategies have been described for other secondary metabolites (68), and each plant species requires specific conditions for producing certain compounds. By using microbiological and biochemical techniques, we have increased diosgenin accumulation from 0.9-1% (98-114 mg/liter) of dry weight in 28 days to 7.2% (230 mg/liter) in 12 days. We have as yet not investigated the additive effect of the various treatments described for improving diosgenin production by *D. deltoidea* cells. It might be that this could result in even higher concentrations and production rates of diosgenin.

The efforts for continued research in plant cell suspension cultures for secondary metabolite production should be directed to (a) developing selection methods for promising phenotypes (69) to shorten substantially the time-consuming selection of high-producing clones. For a number of plant metabolites, immunoassays have been described (21). This method allows screening of a large number of isolates where the products are not pigments or show fluorescence; (b) utilizing intermediates produced by the cells in suspension to obtain metabolites that are not synthesized in undifferentiated cultures (e.g., vinblastine) or to shorten the production time by harvesting intermediates, synthesized earlier in the growth phase, that are further treated either chemically or enzymatically; (c) researching methods for obtaining secretion of the plant metabolites into the medium. Microbial metabolites are accumulated in the culture medium at very high concentration when the metabolite is released by the cells (e.g., citric acid, glutamic acid), and a similar system does not, thus far, exist with plant cells where the metabolite remains within the cell.

Attempts have been made to release the metabolite either by using two phase systems (70) or by addition of DMSO.

ACKNOWLEDGMENTS

This research was supported in part by a joint grant from the National Council for Research and Development and the GSF, München, Federal Republic of Germany, by a grant from the Leonard Wolfson Foundation for Scientific Research, Jerusalem, Israel, and by funds granted by the Charles H. Revson Foundation.

We thank Dr. R.I. Mateles and Dr. J. Gressel for their helpful advice during various stages of this project. We also thank Dr. Z. Reuveny for permission to use some of her unpublished results.

LITERATURE CITED

1. W.G.W. Kurz and F. Constabel, in: "Microbial Technology." Ed. by H.J. Peppler and D. Perlman, Vol. 1, New York: Academic Press, 1979, p. 389.
2. E.J. Staba, in: "Plant Cell, Tissue and Organ Culture." Ed. by J. Reinert and Y.P.S. Bajaj, Berlin: Springer-Verlag, 1977, p. 694.

3. D.K. Dougall, in: "Cell Substrates." Ed. by J.C. Petricciani and H.E. Hopps, New York: Plenum, 1979, p. 135.
4. D.K. Dougall, in: "Plant Cell and Tissue Culture— Principles and Application." Ed. by W.R. Sharp, P.D. Larsen, E.F. Paddock and V. Raghaven, Columbus, OH: Ohio State University Press, 1979, p. 727.
5. Y. Aharonowitz and A.L. Demain, *Biotech. Bioeng.*. **22**, Suppl. 1, 5 (1980).
6. K. Mothes, in: "Secondary Plant Products." Ed. by E.A. Bell and B.V. Charlwood, Berlin: Springer-Verlag, 1980, p. 1.
7. M.W. Fowler, *Prog. Ind. Microbiol.*. **16**, 207 (1982).
8. N. Ishikura and S. Teramoto, *Agr. Biol. Chem.*. **47**, 421 (1983).
9. Y. Fujita, M. Tabata, A. Nishi, and Y. Yamada, Proc. 5th Int. Cong. Plant tissue and cell culture, 1982, p. 399.
10. T. Furuya, T. Yoshikawa, Y. Orihara, and H. Oda, *Plant Med.*. **48**, 83 (1983).
11. W.G.W. Kurz, K.B. Chatson, F. Constabel, J.P. Kutney, L.S.C. Choi, P. Kolodzieiczyk, S.K. Sleigh, K.L. Stuart, and B.R. Worth, *Planta Med.*. **42**, 22 (1981).
12. B. Tal, J.S. Rokem, and I. Goldberg, *Plant Cell Rep.*. **2** 219 (1983).
13. M.H. Zenk, H. El-Shagi, and U. Schulte, *Planta Med. Suppl.*. 79 (1975).
14. M.H. Zenk, H. El-Shagi, H. Arens, J. Stockigt, E.W. Weiler, and B. Deus, in: "Plant Tissue Culture and Its Biotechnological Applications." Ed. by W. Barz, E. Reinhard and M.H. Zenk, Berlin: Springer-Verlag, 1977, p. 27.
15. H. Böhm and J. Franke, *Biochem. Physiol. Pflanzen.* **177**, 345 (1982).
16. J.P. Kutney, *Pure and Appl. Chem.*. **54**, 2523 (1982).
17. W. Steck,. B.K. Bailey, J.P. Shyluck, and O.L. Gamborg, *Phytochemistry*. **10**, 191 (1971).
18. J. Reinert and D. Bucks, *Nature*. **220**, 1340 (1968).
19. A.C. Hildebrandt, in: "Control Mechanisms in the Expression of Cellular Phenotypes." Ed. by I.A. Pardykula, New York: Academic Press, 1970, p. 147.
20. M. Mandels, *Adv. Biochem. Eng.*. **2**, 201 (1972).
21. E.W. Weiler, *Biochem. Soc. Trans.*. **11**, 485 (1983).
22. B. Tal and I. Goldberg, *J. Nat. Prod.*. **44**, 750 (1981).
23. T. Matsumoto, K. Nishida, M. Noguchi, and E.E. Tamaki, *Agr. Biol. Chem.*. **37**, 567 (1973).
24. H.V. Amorim, D.K. Dougall, and W.R. Sharp, *Physiol. Plant.*. **39**, 91 (1977).
25. Y. Fujita, Y. Hara, T. Ogino, and C. Suga, *Plant Cell. Rep.*. **1**, 59 (1981).
26. R.I. Mateles and E. Battat, *Appl. Microbiol.*. **28**, 901 (1974).
27. I. Goldberg and Z. Er-el, *Proc. Biochem.*. **10**, 1 (1981).
28. B. Tal and I. Goldberg, *Planta Med.*. **44**, 107 (1982).
29. B. Tal, J. Gressel, and I. Goldberg, *Planta Med.*. **44**, 111 (1982).
30. T. Murashige and F. Skoog, *Physiol. Plant.*. **15**, 473 (1962).
31. I.A. Veliky and D. Rose, *Can. J. Bot.*. **51**, 1837 (1973).
32. E.M. Linsmayer and F. Skoog, *Physiol. Plant.* **18**, 115 (1965).
33. O.L. Gamborg, R.A. Miller, and K. Ojima, *Exp. Cell Res.*. **50**, 151 (1968).
34. J.R. Nitsch and C. Nitsch, *Science*, **163**, 35 (1969).
35. T. Eriksson, *Physiol. Plant.*. **18**, 976 (1965).
36. F. Constabel, J.P. Shyluck, and O.L. Gamborg, *Planta*. **96**, 306 (1971).
37. G. Wilson, *Ann. Botan.*. **40**, 919 (1976).
38. M.H. Zenk, "Proc. Plant Cell Symp.," London, 1982, p. 1.
39. M.R. Heble and E.J. Staba, *Planta Med. Supl.*. 124 (1980).
40. A.L. Demain, in: "Fermentation and Enzyme Technology." Ed. by D.I.C. Wang, C.L. Cooney, A.L. Demain, P. Dunnill, A.E. Humphrey, and M.D. Lilly, New York: John Wiley, 1979, p. 14.
41. W.R. Nes, in: "Advances in Lipid Research." Ed. by R. Paeloetti and D. Kritchevsky, **15**, 233 (1977).
42. T.N. Goodwin, *Biochem. Soc. Trans.*, **5**, 1252 (1977).
43. G.J. Schroepfer, *Ann. Rev. Biochem.*. **50**, 585 (1981).
44. J.W. Porter and S.L. Spurgeon (eds.) "Biosynthesis of Isoprenoid Compounds." New York: John Wiley, 1982.
45. C.W. Coggins, G.L. Henning, and H. Yokoyama, *Science*. **168**, 1589 (1970).
46. H. Yokoyama, C.W. Coggins, and G.L. Hening, *Phytochemistry*. **10** 1831 (1971).
47. H. Yokoyama, E.D. Hyman, J.W. Hsu, and S.M. Poling, *Science*, **197**, 1076 (1977).
48. A.L. Lehninger, in: "Biochemistry." New York, Worth Publishing Co., 1976, p. 680.
49. P.G. Bartels and C.W. Watson, *Weed Sci.*. **26**, 198 (1978).
50. G. Sandman, P.M. Bramley and P. Boger, *Pestic. Biochem. Physiol.*. **14**, 185 (1980).
51. J. St. John, *ACS Symp. Ser.*. **181**, 97 (1982).

52. B. Tal, J.S. Rokem, J. Gressel, and I. Goldberg, *Phytochemistry*, **23**, 1333 (1984).
53. S.L. Lee, K.D. Cheng, and A.I. Scott, *Phytochemistry*, **20**, 1841 (1981).
54. E.C. Tjamos and J.H. Kuc, *Science*, **217**, 542 (1982).
55. D. McMahon, *Plant. Physiol.*, **55**, 815 (1975).
56. A.F. Hsu, *J. Nat. Prod.*, **44**, 408 (1981).
57. A. Endo, *Trends Biochem. Sci.*, **9**, 10 (1982).
58. B. Tal, J.S. Rokem, and I. Goldberg, *Planta Med.*, **50**, 239 (1984).
59. W.G.W. Kurz, K.B. Chatson, F. Constabel, J.P. Kutney, L.S.L. Choi, P. Kolodziejczy, S.L. Sleigh, K.L. Stuart, and B.R. Worth, *Phytochemistry*, **19**, 2583 (1980).
60. M. Lounasmaa and A. Nemes, *Tetrahedron*, **38**, 223 (1982).
61. B. Tal, I. Tamir, J.S. Rokem, and I. Goldberg, *Biochem. J.*, **219**, 619 (1984).
62. S.C. Sharma, R. Chand, B.S. Bhattiand, and P.O. Sati, *Planta Med.*, **46**, 48 (1982).
63. H.U. Luning and A. Schlosser, *Z. Pflanzenkrankh. Pflanzenschutz*, **82**, 699 (1975).
64. J.A. Bailey and J.W. Mansfield (eds.) "Phytoalexins." London: Blackie Glasgow, 1982.
65. P. Albersheim and B.S. Valent, *J. Cell Biol.*, **78**, 627 (1978).
66. R.M. Bostock, J. Kuc, and R.A. Laine, *Science*, **212**, 67 (1981).
67. J.S. Rokem, J. Schwarzberg, and I. Goldberg, *Plant Cell Rep.*, **3**, 159 (1984).
68. E.J. Staba (ed.) "Plant Tissue Culture as a Source of Biochemicals." Boca Raton, FL, CRC Press, 1980.
69. J.M. Widholm, in: "Plant Tissue Culture as a Source of Biochemicals." E.J. Staba (ed.), Boca Raton, FL, CRC Press, 1980, p. 99.
70. W. Bisson, R. Beiderbeck, and J. Reichling, *Planta Med.*, **47**, 164 (1983).