THE EFFECT OF MEDIUM MODIFICATION AND SELECTED PRECURSORS ON STEROL PRODUCTION BY SHORT-TERM CALLUS CULTURES OF *EUPHORBIA TIRUCALLI*

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ABSTRACT.—Callus tissues of *E. tirucalli* produce the 4,4'-dimethyl sterols, euphol and tirucallol. Gas-liquid chromatography was utilized to compare euphol/tirucallol ratios in latex exudate, stem explants and callus tissue. Sterol profiles show that euphol is present in high amounts in latex, but tirucallol predominates in higher amounts in explants and callus indicating synthesis and/or accumulation of tirucallol by cells other than the laticifer cell. Sterol production was significantly enhanced by certain nutrient media, as well as indole-3-acetic acid, and depressed by benzyl adenine. Precursor stimulation of product synthesis was successful only with squalene, which promoted sterol production at 1.0 mg/liter but inhibited cell growth at higher concentrations. DL-mevalonic acid and lanosterol promoted neither growth nor sterol production. DL-[2^{14}C] mevalonate was used to confirm biosynthesis of sterols in both latex and callus cultures.

Serious consideration is being given to the utilization of green plants as sources of biomass for conversion into energy intensive chemicals or direct production of materials such as natural rubber. Plant species belonging to the genus *Euphorbia* appear to be promising sources for such a crop species (1). Latex from these plants contains steroidal alcohols (triterpenes) that are high in energy, having a carbon to oxygen ratio of at least 10 to 1, and, therefore, equal or exceed gasoline in energy content (2). Establishment of sterol-producing cultures would be useful toward the understanding of environmental factors controlling accumulation of these potentially economically important compounds. We have found that static and suspension cultures of *E. tirucalli*, an arborescent succulent native to tropical eastern and southern Africa, produce the 4,4'-dimethyl sterolic isomers euphol and tirucallol.

Both common and uncommon sterols and triterpenes have been detected and identified in a large number of tissue cultures of diverse genera (3), including *Euphorbia* (4). But the influence of medium, growth factors, or biosynthetic precursors on sterol production in cell culture is not very well known. The purposes of this study were to: 1) quantitatively and qualitatively compare the sterol spectrum of latex, parent plant tissue, and the cultured cells; 2) determine the influence of varied concentrations of auxins and cytokinins in a defined medium upon growth and sterol production; and 3) determine the effect of selected precursors upon growth and sterol production in callus tissues.

METHODS AND MATERIALS

PLANTS.—Latex or stem explants from slender terminal branches of *E. tirucalli* L. were collected from several large (2–3 m) greenhouse-grown plants. Latex was obtained by scarification of stems, usually at a nodal region; the exudate was collected directly into clean test tubes. Explants (10–15 cm) were sterilized by immersion into 70% ethanol for 2 minutes, rinsed once with sterile deionized water, and placed into a 5% solution of commercial bleach (5.25% sodium hypochlorite) for 25 minutes. After three rinses in sterile water, the stems were cut into 1–2 cm segments and placed horizontally on the nutrient medium.

CULTURE MEDIUM AND GROWTH CONDITIONS.—The defined medium used in most experiments was that of Murashige and Skoog (5) supplemented with 1.0 g/liter casein hydrolysate and 2% sucrose. Medium pH was adjusted to pH 5.7–5.8 with 0.1 *N* NaOH prior to autoclaving. Other media used in cell yield and production formation experiments are listed in table 3. All static cultures were solidified with 0.9% agar. Suspension cultures (50 ml) were

used to study the kinetics of growth and sterol production and were initiated from existing solid callus (ca. 2.0 g fresh weight). Culture flasks (125 ml) were covered with sterile, polypropylene and placed or shaken in the dark at 25±1°C. Cultures were maintained on the defined medium by transferring about 5 mg (dry weight) of callus pieces to fresh media every 28 days. In every case, two replicate flasks were harvested, and the data were recorded for the third passage. Fresh weight (fwt—final wet weight minus initial wet weight) and dry weight (dwt—final lyophilized weight minus initial lyophilized weight) were employed as growth parameters.

MEDIUM MODIFICATIONS.—Hormonal additions to the basal medium of Murashige and Skoog (5) included the auxins IAA (indole-3-acetic acid), 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (α-naphthaleneacetic acid), IBA (indole-3-butyric acid), and the cytokinins KIN (kinetin) BA (benzyl adenine), ZEA (zeatin), and 2iP (N^6—(Δ^2-isopenenyl)-adenine). Auxins and cytokinins were tested for their effects on sterol synthesis in the range of 1×10^{-7} M to 1×10^{-5} M and 1×10^{-7} M to 1×10^{-4} M, respectively. Auxins and cytokinins are required for growth of *E. tirucalli* callus; in the auxin experiments kinetin was added to the medium at 5.0×10^{-6} M; and in cytokinin experiments, IAA was added at 1×10^{-6}M. Hormonal additions to the various other basal media listed in table 3 included kinetin at 5.0×10^{-6} M and IAA at 1×10^{-6} M. The sterol precursors, mevalonic acid lactone and squalene, were added to liquid medium in concentrations of 1.0–10.0 mg/liter.

INCUBATION WITH RADIOACTIVE SUBSTRATE.—To verify the synthesis of sterols in tissue culture, radioactive DL-[2-^{14}C] mevalonic acid lactone (specific activity, 27.3 mc/μ mole, New England Nuclear, Boston, Mass.) was added to the medium. The acid lactone was dissolved in water and converted into the potassium salt by the addition of the required amount of K_2CO_3 before use in incubations. Two microcuries were added to each milliliter of medium. Cultures were extracted and analyzed after two and four weeks of growth.

A water solution containing 20 μc of the [2-^{14}C] potassium mevalonate was also added to 2.0 ml of fresh latex to determine whether the laticifer cell functions in biosynthesis or only in the storage of triterpenes. Duplicate incubations were prepared and kept in darkness at 25°. Samples (0.1 ml) were withdrawn periodically, and enzymatic activity was terminated by the addition of 5.0 ml of hot ethanol.

EXTRACTION OF STEROLS.—Both latex samples and callus were saponified by refluxing in a Soxhlet apparatus for one hour with 10 ml ethanol, 2 ml water, and 2 g KOH. The non-saponifiable lipid was extracted into diethyl ether and dried with a stream of nitrogen. The non-saponifiable lipid was dissolved in a small volume of petroleum ether (bp 30–60°) and chromatographed on 5 g of deactivated (Brockman grade III) alumina (6). In brief, the column was developed by elution with increasing percentages of diethyl ether in petroleum ether. Fraction 3 (6%, v/v diethyl ether in petroleum ether) contained the dimethyl sterols. Fractions were evaporated to dryness with a stream of nitrogen and subjected to thin-layer chromatography on silica gel plates (Eastman 13179). The developing solvent was 35:65 diethyl ether-petroleum ether. A marker spot was located by use of iodine vapor or 3% cupric acetate in 8% phosphoric acid, which gives a blue color on heating (7). An upper spot corresponding to squalene was eluted with chloroform and rechromatographed on plates developed with hexane. After location, spots were scraped off the thin-layer plates and placed into 10 ml of toluene-based scintilation fluid (Omnifluor, New England Nuclear, Boston, Mass.) before being counted in a Beckman LS-100A scintillation spectrometer.

Non-labeled sterol fractions were isolated by refluxing in hot acetone for 30 minutes, evaporated to dryness with N_2, redissolved in acetone, and injected directly into the gas chromatograph for quantification.

GAS-LIQUID CHROMATOGRAPHY (GLC).—All isolated fractions were evaporated to dryness with N_2, dissolved in a small volume of acetone or chloroform, and were usually chromatographed as free sterols or, occasionally, as acetates after acetylation in acetic anhydride-pyridine (4:1) prior to injection. Analyses were performed with a Hewlett-Packard 5710A instrument equipped with a flame ionization detector operated by programming from 220–310° at 4 C/min. Nitrogen (20 ml/min) was used as the carrier gas. The injection port and detector temperatures were 250° and 300°, respectively. Glass columns were pretreated with dimethydichlorosilane in toluene (10%, v/v). Two stationary phases were employed: 3% OV-1 Supelcoport (80/100 mesh) or 3% OV-17 Supelcoport (80/100 mesh). Quantitative data were determined with a Hewlett-Packard 3380A integrator.

RESULTS

No distinction will be made between the terms tetracyclic triterpenes and sterols. The tetracyclic triterpenes which occur in *Euphorbia* are C_{30} compounds that are oxygenated at C-3 of the steroid A ring as a hydroxyl. In this regard, the triterpenes euphol and tirucallol are 3-β-alcohols and may be referred to as steroidal alcohols or sterols.

Although difficult to separate on thin layer plates because of their chemical similarity ($R_f = 0.40$), euphol and tirucallol are easily separated by glc. Relative retention times (to eicosane) for glc of the free sterols were 7.02 and 7.31 minutes on 3% OV-1; 10.02 and 10.36 minutes on 3% OV-17 for euphol and tirucallol, respectively. The mass spectra for these two compounds were identical and had peaks at *m/e* 426 (1.6) M^+, 412 (30), 411 (90), 393 (58), 69 (100), 55 (30), 41 (25), which are in agreement for identification of these compounds as euphol and tirucallol (Biesboer, unpublished).

Squalene, an acyclic precursor of sterol biosynthesis, was observed to occur in latex and callus tissue (<0.03 mg/g dwt.). Retention times (relative to eicosane) were 5.90 on 3% OV-1 and 8.42 on 3% OV-17. The mass spectrum was identical to the fragmentation pattern of squalene (8).

TABLE 1. Ratios of euphol to tirucallol in latex exudate, stem explants, and callus tissues of *E. tirucalli*.

Source of sterols	Specific sterols	Concentration (mg/g dwt.)	Total concentration both sterols (mg/g tissue)	Ratio of euphol: tirucallol
Whole latex[a]	Euphol	368.3	—	3.65/1
	Tirucallol	102.0		
Whole latex[b]	Euphol	393.5	—	4.36/1
	Tirucallol	91.5		
Whole latex[c]	Euphol	371.6	—	4.74/1
	Tirucallol	78.4		
Explants	Euphol	1.87	4.19	1/1.24
	Tirucallol	2.32		
Callus (4 weeks)	Euphol	0.20	1.59	1/6.78
	Tirucallol	0.80		
Callus (8 weeks)	Euphol	0.27	1.07	1/2.93
	Tirucallol	0.80		
Callus (12 weeks)	Euphol	0.16	1.00	1/5.21
	Tirucallol	0.84		
Callus (16 weeks)	Euphol	0.20	1.02	1/4.06
	Tirucallol	0.82		

[a]Greenhouse plant, source unknown.
[b]Greenhouse plant, started from seed, 1966, Botanischer Garten, Berlin, Germany.
[c]Huntington Botanical Gardens, Pasadena, CA.

CALLUS TISSUE.—Table 1 compares the sterol composition of freshly exuded latex, explants from the parent plant, and callus derived from these explants. Sterols comprised approximately 47% of the total dry weight of fresh latex and exhibited a high euphol-to-tirucallol ratio. On the other hand, when stem explants or callus tissue were extracted, the euphol/tirucallol ratio reversed, and tirucallol existed as the predominant isomer, especially in cultured cells. There appeared to be no correlation between the age of the callus and sterol concentration, with ratios of tirucallol to euphol varying from 1.24 to 6.78. The highest concentration of tirucallol occurred at the end of the second passage. A comparison of total sterol concentration between explants and callus showed approximately a four-fold decrease in sterol production between parent plant and cultured cells.

INCORPORATION OF LABELED [2-^{14}C] MEVALONATE.—After incubation of whole latex or callus tissue with [2-^{14}C] mevalonate, the non-saponifiable lipid was extracted, the third column fraction collected, and the eluent separated by thin layer chromatography. Results are given in table 2. In both cases synthesis of

triterpenes was confirmed. Incorporation of mevalonate into both squalene and triterpenes by fresh latex exudate occurred rapidly during the first 24 hours of incubation. Confirmation of sterol biosynthesis was also observed in tissue culture. [2-^{14}C] Mevalonate was incorporated into sterols by callus at 3940 and 6205 cpm/500 mg. dwt after 2 and 4 weeks, respectively.

TABLE 2. The incorporation of [2-^{14}C] mevalonate into sterols and squalene by whole latex.

Time (h)	Sterols cpm/0.1 ml	Squalene cpm/0.1 ml
4	1,916	359
8	11,600	984
12	21,422	1,591
24	52,915	1,866
36	57,078	2,360
48	68,666	1,853

INFLUENCE OF MEDIA COMPOSITION ON STEROL FORMATION.—The cell yield and product formation potential of various culture media were examined by use of various published formulations. As shown in table 3, growth occurred in most of the media. However, wide fluctuations in total sterol production occurred depending upon the medium used. Almost half of the tested media produced nondetectable or very low levels of sterols. Attempts to culture roots which produce sterols in the intact plant were not successful. Other media, especially that of

TABLE 3. The influence of various basal media on growth and sterol production by callus cultures of *E. tirucalli*.

Basal medium[a]	Explant	Cell yield g dwt/culture	Sterols mg/g dwt	Sterols % dwt
White (after Street, 9)	root	0.00	0.00	0.00
White (after Street, 9)	stem	slight	0.00	0.00
Nitsch & Nitsch (10)	stem	0.15	0.00	0.00
Ericksson (11)	stem	0.41	0.12	0.03
Heller (12)	stem	0.32	0.32	0.10
Gamborg B5 (13)	stem	0.42	0.54	0.12
Gamborg B5+2,4-D[b] (13)	stem	0.59	0.86	0.15
White (14)	stem	0.38	0.96	0.25
Murashige & Skoog (5)	stem	0.51	1.68	0.33

[a]Hormonal additions include 1 x 10^{-6} IAA and 5 x 10^{-6} KIN.
[b]2.0 mg/L 2,4-dichlorophenoxyacetic acid.

White (14) and Murashige and Skoog (5), produced high amounts of sterols. However, triterpene production in culture was significantly less than amounts produced by the intact plant on a dry weight basis. Extraction of portions of the stem axis yielded 4.19 mg sterols/g dwt., whereas the various cultures yielded 0.12 to 1.68 mg sterols/g dwt. for a percentage yield of 2.8 to 40%. The transformation of *E. tirucalli* explants to the state of callus tissue is accompanied by a concomitant loss or decrease in the capacity to produce secondary steroidal metabolites.

Figure 1 illustrates the variation in growth with differing concentrations of IAA, 2,4-D, and NAA. The most effective growth regulator was 2,4-D at 1 x 10^{-6} M. Most auxins promoted growth at concentrations between 0.5 x 10^{-6} and 0.5 x 10^{-5} M and inhibited growth at higher and lower concentrations. At optimal growth-stimulating concentrations, the synthetic auxin 2,4-D promoted an 18% dwt. increase over IAA, a 39% dwt. increase over NAA, and a 49% dwt. increase over IBA (see fig. 1).

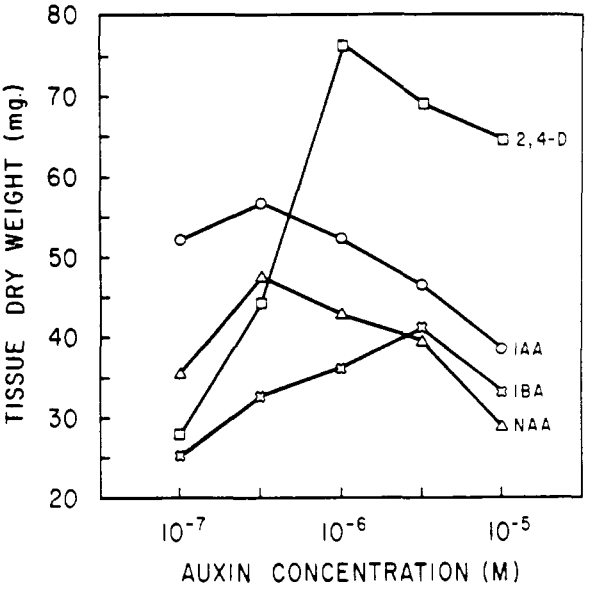

FIG. 1. Growth response of *E. tirucalli* callus tissue to selected auxins. The concentration of kinetin used was 5.0 x 10^{-6} M. Data points represent the average of duplicate assays.

Figure 2 depicts the effects of cytokinins on callus growth. The greatest effect in growth stimulation occurred at high physiological concentrations. The most effective cytokinin was kinetin at 1 x 10^{-5} M, although 0.5 x 10^{-4} M also promoted excellent growth. Other tested cytokinins stimulated cell yield at either 0.5 x 10^{-5} M or 1 x 10^{-5} M. At optimal cell-yield concentrations, kinetin was 13% more effective than 2iP, 15% more effective than ZEA, and 40% more effective than BA in stimulating growth (see fig. 2).

Table 4 illustrates the concentration of hormones which gave both optimal cell yield and optimal sterol production. Clearly, IAA is the growth substance which gave both highest cell and sterol yields. Benzyl adenine (BA) appears to suppress both cell yield and sterol synthesis. Further examination of table 4 suggests that a high correlation exists between optimal cell yield and optimal sterol production. Thus, concentrations which stimulated increased cell growth also stimulated the greatest amounts of sterol biosynthesis. This relationship becomes evident in the linear regression of callus weight plotted against sterol concentration (fig. 3). IAA clearly caused a marked enhancement of sterol synthesis whereas BA definitely depressed synthesis of these compounds. Variations of hormonal concentration which effect an increase or decrease in growth are ac-

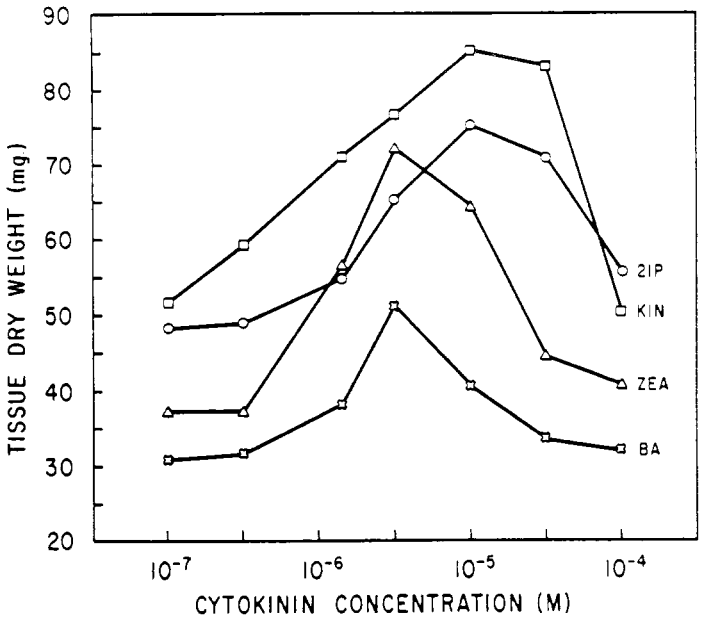

FIG. 2. Growth response of *E. tirucalli* callus tissue to selected cytokinins. The concentration of indole-3-acetic acid used was 1 x 10^{-6} M. Data points represent the average of duplicate assays.

companied by a simultaneous increase or decrease in sterol biosynthesis; therefore, sterol production appears to be directly proportional to total cell mass.

Figure 4 shows this same relationship for growth and sterol synthesis during one cell cycle. Total sterol concentration closely paralleled cell yield. Plots for the individual sterols euphol and tirucallol also generally paralleled cell growth, but, as shown in table 1, much variability existed in the ratios of these two compounds. As observed in other cultures, tirucallol predominated as the major triterpene.

TABLE 4. The effect of various plant hormones on growth and sterol production by callus cultures of *E. tirucalli*.

Hormone[a]	Optimal cell yield			Optimal sterol yield		
	Conc. (M)	g dwt	sterols % dwt	Conc. (M)	g dwt	sterols % dwt
IAA[b]	5 x 10^{-6}	56.7	0.21	5 x 10^{-6}	56.7	0.21
2,4-D[b]	1 x 10^{-6}	76.2	0.15	1 x 10^{-6}	76.2	0.15
IBA[b]	5 x 10^{-7}	41.1	0.16	5 x 10^{-7}	41.1	0.16
NAA[b]	5 x 10^{-6}	47.6	0.15	5 x 10^{-6}	47.6	0.15
2iP[c]	1 x 10^{-5}	75.2	0.14	5 x 10^{-6}	65.3	0.17
KIN[c]	1 x 10^{-5}	88.2	0.13	5 x 10^{-6}	83.1	0.16
BA[c]	5 x 10^{-6}	51.2	0.10	5 x 10^{-6}	51.2	0.10
ZEA	5 x 10^{-6}	72.3	0.16	5 x 10^{-6}	72.3	0.16

[a]Basal medium of Murashige and Skoog (1962).
[b]Supplemented with 5 x 10^{-6} KIN.
[c]Supplemented with 1 x 10^{-6} IAA.

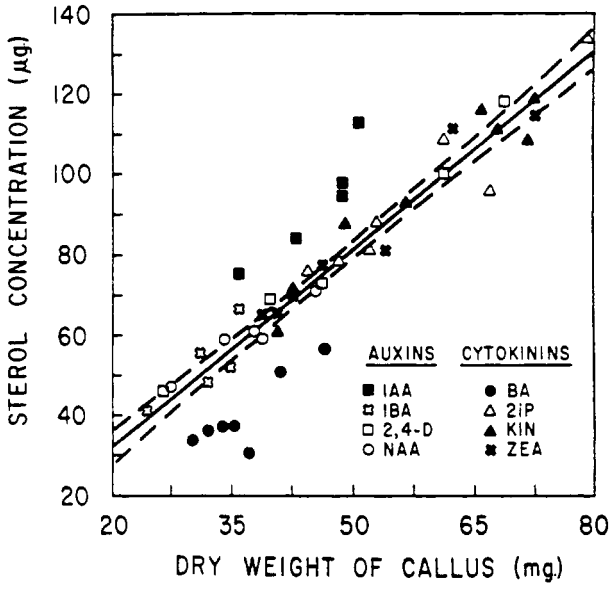

FIG. 3. Effect of various auxins and cytokinins on total sterol production by callus cultures of *E. tirucalli*. The solid line is the linear regression of sterol concentration vs. callus dry weight. The dotted lines delimit the 95% confidence interval.

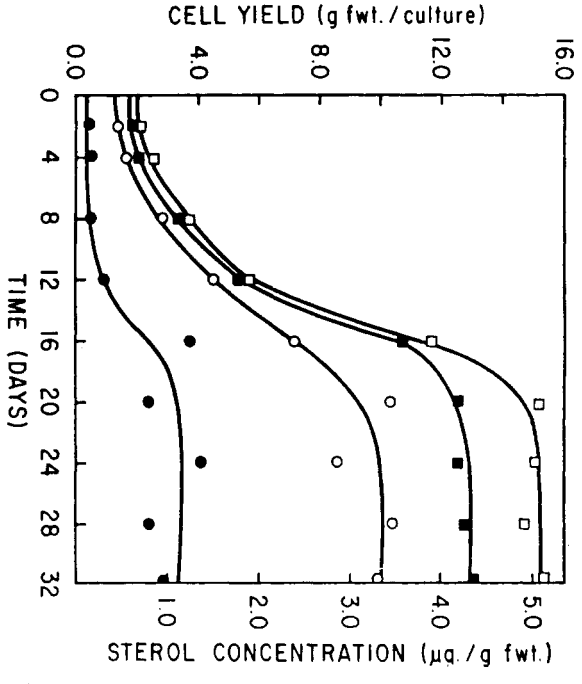

FIG. 4. Kinetics of growth and sterol production by *E. tirucalli* callus cultures. (■)—cell yield; (□)—total sterol yield; (○)—tirucallol; (●)—euphol. Note the variation in ratio of tirucallol/euphol.

EFFECT OF PRECURSORS ON STEROL PRODUCTION.—Mevalonic acid lactone, squalene, and lanosterol were added to liquid basal medium in the concentration range of 1.0 to 10.0 mg/liter in 2.5 mg increments. The results are illustrated in table 5. Mevalonic acid lactone or lanosterol did not enhance sterol formation at any of the experimental concentrations. Lanosterol appeared to cause some inhibition of growth and sterol yield at concentrations greater than 2.5 mg/liter. The most marked effects were shown by squalene at low concentrations of 1.0 mg/liter. Sterol yield was approximately 24% higher than that of the control group. Further increase in squalene concentration caused a rapid decrease in both cell and sterol yield. Some growth would occur, but the callus tissue would become compact and very dark in appearance. Squalene is nearly insoluble in aqueous media, and growth was probably retarded because of the deposition of a layer of this precursor on the cells. This effect was also observed for lanosterol but not to the same extent as for squalene.

TABLE 5. The effect of precursors on the biosynthesis of sterols in callus cultures of *E. tirucalli*.

Precursor concentration mg/liter	Mevalonic acid lactone		Squalene		Lanosterol	
	Cell yield[a]	Sterol yield[b]	Cell yield	Sterol yield	Cell yield	Sterol yield
1.0	90.0	94.9	88.9	124.7	94.0	91.0
2.5	94.6	100.0	76.6	38.0	99.0	87.6
5.0	100.2	92.6	45.6	11.8	76.1	69.0
7.5	91.1	92.1	37.2	10.1	66.2	66.8
10.0	85.3	96.0	5.3	0.0	64.1	52.8

[a]As percent of control.
[b]As percent of control.

CELLULAR COMPOSITION OF CALLUS TISSUES.—Microscopic examination of callus tissues did not reveal the presence of laticifer cells or laticifer-like elements. Meristemoids, groups of smaller meristematic cells, would arise in scattered positions throughout the callus mass, but differentiation was limited to the production of a small number of lignified tracheid-like elements. In one case, out of several hundred experimental flasks, a few roots developed in a superficial position on callus growing in static culture. Callus tissue was usually yellowish-white or yellowish-brown in appearance and somewhat friable.

DISCUSSION

The 4,4'-dimethyl sterols euphol and tirucallol are isomeric compounds differing only in the stereochemical configuration of the hydrogen found at C–20 of the 8 carbon steroid side chain. Euphol is characterized by a β-hydrogen at C–20, whereas tirucallol has an α-hydrogen at this same position.

Comparison of euphol/tirucallol ratios in latex, stem tissue, and callus cultures indicates that these phytosterol isomers do not appear to be specialized products of the non-articulated laticifer cell in *E. tirucalli*. Euphol is the principal terpene accumulated in latex, whereas tirucallol predominates in callus and intact parental tissues. Apparently the absolute configuration of the hydrogen at C–20 is determined by divergent enzymatic mechanisms with the 20 α-H and 20 β-H isomers not merely existing in physical equilibrium. It is not known whether

transport of sterols occurs from these non-laticiferous sites of synthesis into the laticiferous system. If transport does occur, it may account for the extremely high sterol content of latex (approximately 47% sterols dwt. in *E. tirucalli;* see also reference 1). It has not been possible , as yet, to further pinpoint sites of synthesis because of the difficulty of obtaining laticifer-free parent plant tissue for comparison with latex exudate.

In vitro incorporation of [^{14}C]-mevalonate into sterols and squalene by latex of *E. tirucalli* was in close accord with observation of the incorporation of [^{14}C]-mevalonate and [^{14}C]-acetate into latex terpenes of *E. helioscopia* and *E. cyparissias* (15). Triterpenes were also effectively labeled with [^{14}C]-mevalonate in *E. tirucalli* callus cultures. Except for small amounts of squalene, no biosynthetic precursors to euphol and tirucallol were observed in cell cultures or exuded latex. Euphol and tirucallol appear to be the only sterols synthesized in latex. Other more common 4-methyl sterols and 4-desmethyl sterols were not detectable.

Sterol production in *E. tirucalli* cultures was enhanced by the incorporation of low levels of squalene (1.0 mg/liter) into the culture medium suggesting that the availability of squalene may be a limiting step in the biosynthesis of euphol and tirucallol. Higher amounts of squalene (<1.0 mg/liter) inhibited cell growth, probably due to its insolubility in aqueous medium and subsequent coating of callus cells with a layer of this precursor. Mevalonate and lanosterol did not increase sterol production in callus. Mevalonate also did not appreciably inhibit cell growth, although lanosterol caused a decline in cell and sterol yield which was not as marked as in squalene-containing cultures. No unusual metabolites or other sterols were detected by glc after incubation with these selected substrates.

The central role of mevalonic acid and squalene have been well established as early precursors in the biosynthetic route to triterpenes (16, 17). Squalene, although rapidly turned over (18), is found in both higher plants and tissue cultures (15, 19, 20), whereas lanosterol appears to occur infrequently in intact plants and cultures (3, 21). Lanosterol does not appear to be a direct intermediate in sterol biosynthesis (6, 16) and, therefore, may not be expected to be transformed in culture to other sterols. Triterpenes appear to be synthesized via several intermediates in a non-specific process involving cycloartenol as the first cyclized product (22, 23, 24). On the other hand, [^{14}C]-lanosterol has been shown to be incorporated into a wide variety of sterols in *Nicotiana tabacum* cultures (25) and illustrates the capacity of triterpene-synthesizing enzyme systems to utilize these various metabolites. The presence of lanosterol in latex of euphorbiaceous and other plants may result from the enzymatic modification of cycloartenol (26), and provides additional evidence that lanosterol is probably not directly involved in the synthesis of euphol and tirucallol.

As in tissue cultures of *E. esula* and *E. cyparissias* grown on the same medium (5), cultures of *E. tirucalli* required a continuous supply of exogenous auxin for growth (4). But unlike *E. tirucalli* cultures which responded well to both IAA and 2,4-D, *E. esula* and *E. cyparissias* exhibited greater growth with NAA. Exogenous cytokinins are also required by *E. tirucalli* and *E. cyparissias* for growth in contrast to *E. esula* callus and proliferated embryos of *E. marginata* (27). For best growth, *E. tirucalli* cultures required hyper-physiological cytokinin concentrations of 0.5×10^{-5} M to 0.5×10^{-4} M and, in this respect, are similar to callus cultures of *E. pulcherrima* (28).

Plant growth substances play an important role in the induction and repression of biosynthetic pathways leading to the formation of secondary metabolites, although no general pattern for the efficacy of hormones to stimulate secondary

product formation has emerged. This is also true for the nutritional requirements of various callus and suspension cultures. For *E. tirucalli*, the medium of Murashige and Skoog (5) appears to promote growth and sterol production to a greater extent than other defined media. Sterol production is especially enhanced by the addition to the medium of a combination of IAA and kinetin. It is not yet known whether these growth regulators directly affect the production of these sterols through their influence on the enzymes related to sterol synthesis, or whether the effect of growth regulators is indirect through their stimulation of callus growth or differentiation of meristemoids with a related enhanced production of sterols.

The production of euphol and tirucallol in cultured cells indicates that cells other than laticifers can synthesize these compounds in *Euphorbia*. Both cultured cells and laticifers produce the same sterols which characterized this species, although quantitative differences occurred for the two cell systems (29, 30). It is very probable, therefore, that the diverse number of steroidal compounds produced by and characterizing other euphorbiaceous species, as indicated from analyses of latex in a recent glc survey of this genus (31), may be produced in cultured cells from other species. Thus, tissue culture may be a useful technique for the investigation of the biosynthetic and genetic mechanisms of sterol formation in *Euphorbia*.

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