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PHYTOL IN TISSUE CULTURES OF *KALANCHOË CRENATA*

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Abstract—The production of phytol in greening callus cultures of *Kalanchoë crenata* was studied. There were only trace quantities of phytol in dark-grown callus but the amount of phytol markedly increased on exposure of the callus to light. This increase in phytol occurred before chlorophylls could be detected in the callus and was correlated with plastid maturation. A non-saponifiable component of coconut milk was detected in dark-grown but not in light-grown callus.

INTRODUCTION

PREVIOUS work with callus tissue cultures of *Kalanchoë crenata* has demonstrated that many changes occur in dark-grown callus when it is transferred to the light.[1–4] Chlorophylls were formed and increased in amount over the first few months of growth in the light. The carotenoid composition of the callus changed; the highly oxygenated xanthophylls present in the dark-grown callus were reduced in amount on exposure to light and xanthophylls similar to those found in *Kalanchoë* leaves were produced. The total carotenoid content of green callus was one-third that of *Kalanchoë* leaves. The green callus was capable of a photosynthetic fixation of CO_2 and it was concluded that functional chloroplasts were present.[1] Stobart and Thomas[4] transferred dark-grown callus to the light and in subsequent generations of the callus in the light correlated an increase in chlorophyllase activity with an increase in chlorophyll. This possibly indicated that chlorophyllase was responsible for the *in vivo* synthesis of chlorophyll *a*, a conclusion arrived at by other workers.[5,6] Chlorophyllase catalyses the reaction

$$\text{chlorophyllide } a + \text{phytol} \rightleftharpoons \text{chlorophyll } a$$

Phytol (3,D-7,D-11,15-tetramethylhexadec-*trans*-2-en-1-ol), a C20 diterpenoid alcohol, was first discovered by Willstätter[7] and was shown to be a component of chlorophylls. As the production of chlorophyll and chlorophyllase by the callus had been examined, it was considered worthwhile to investigate the production of phytol in the callus cultures and to correlate the results with the development of chloroplasts.

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RESULTS

The efficiency of the chromatographic techniques for phytol was first assessed by chromatographing known amounts of purified commercial phytol on columns of alumina with increasing concentrations of acetone in petrol. ether (b.p. 40–60°). The amount of phytol in the fractions was estimated by measurement of spot area after chromatography on thin-layer plates of Kieselgel G. The results (Table 1) showed that most of the phytol (*ca.* 90 per cent) was recovered in the 8% (v/v) acetone in petrol. ether fraction. The remaining quantity of phytol was eluted with the 6% and 10% (v/v) acetone in petrol. ether fractions. When purified phytol was subjected to the extraction and saponification procedures before chromatographic separation and quantitative estimation, the recovery of phytol in the 8% (v/v) acetone in petrol. ether fraction was between 76 and 84 per cent.

TABLE 1. RECOVERY OF PHYTOL CHROMATOGRAPHICALLY SEPARATED ON COLUMNS OF BROCKMAN III ALUMINA (SEE EXPERIMENTAL FOR DETAILS OF FRACTIONS)

Phytol added to column (μg)	% recovery					Total % recovery
	Fraction number					
	1	2	3	4*	5	
500	0	0	4·4	88·1	7·2	99·7
500	0	0	2·6	82·2	1·8	86·6
250	0	0	3·6	91·3	4·4	98·3
250	0	0	2·8	90	2·4	95·2

* Fraction 4, 8% (v/v) acetone in petrol. ether.

Callus and *Kalanchoë* leaf extracts provided many unidentified compounds in the fractions off the column. In fractions collected just before and just after the fraction containing phytol were compounds which had R_f values similar to phytol on TLC. Thus, phytol analyses were made routinely only on the 8% (v/v) acetone in petrol. ether fraction. This fraction of *Kalanchoë* leaves and all light-grown callus was subjected to analysis by TLC. A compound of similar R_f to authentic phytol, giving a positive reaction with the location reagents was revealed. Co-chromatography with authentic phytol produced only one spot, R_f 0·23–0·27 on Kieselgel G, ethyl acetate–hexane (15:85 v/v), R_f 0·25–0·3 on Kieselgel G, ethyl acetate–benzene (1:19 v/v) and R_f 0·43 on reverse phase paper chromatography, methanol–water (8:2 v/v). Phytol from extracts of callus grown in the light and from extracts of *Kalanchoë* leaves was purified on a preparative scale on thin-layer plates of Kieselgel G, 500 μ thick. The zone of silica gel containing phytol was scraped off and eluted with acetone. The acetone was evaporated under reduced pressure with a nitrogen bleeder to leave a pale-yellow oil. I.r. spectroscopy showed that the compound obtained from leaves and light-grown callus was identical to purified commercial phytol. The i.r. spectra showed bands for a primary alcohol (3300 cm^{-1}) with C=C bands (1665 cm^{-1}), C—CH_3 groups (1465 cm^{-1} and 1380 cm^{-1}) and C—O (1005 cm^{-1}). These results compare with reported spectra for phytol.[8,9] Although phytol was not detected in the dark-grown callus on employing these techniques, it is of interest to note that a compound, R_f 0·1–0·2 on Kieselgel G, benzene–ethyl acetate (19:1

[8] S. SHIMIZU, H. FUKUSHIMA and E. TAMAKI, *Phytochem.* **3**, 641 (1964).
[9] E. DEMOLE and E. LEDERER, *Bull. Soc. Chim. Fr.* 1128 (1958).

v/v) was present in some quantity in the 8% (v/v) acetone in petrol. ether fraction from the dark-grown callus extracts. It could be judged from the large spot size that this compound was present in a high concentration in the extracts. When the dark-grown callus was exposed to light, this compound decreased markedly and was absent in chlorophyll-containing callus. Non-saponifiable lipid extracts of coconut milk (a constituent of the medium), while not containing phytol, proved to contain this unknown compound. This unknown compound was co-chromatographed with squalene, farnesol, solanesol, borneol, isoborneol, geraniol, frenchol, stigmasterol and ergosterol but no co-incidental spots were obtained. The compound gave a yellow colour on chromatograms sprayed with anisaldehyde. Radioactivity was not incorporated into this compound when dark-grown callus was incubated with 2-^{14}C acetate or DL-2-^{14}C-mevalonic acid for 1 month on the complete growth medium. It would appear that this unknown compound is taken up from the 25 per cent (v/v) coconut milk medium by the dark-grown callus in the course of its growth.

As stated previously, phytol was not detected by chromatographic techniques in dark-grown callus. In an attempt to substantiate this conclusion, dark-grown callus was sub-cultured into ten 100-ml conical flasks each containing 15 ml complete culture medium plus 5 μc 2-^{14}C acetate. After 4 weeks' growth in total darkness the callus was removed, washed thoroughly and the non-saponifiable lipid fraction subjected first to column chromatography and then to TLC. Autoradiographs were prepared from the TLC plates using Ilford X-ray film. The radioactive areas corresponding to phytol were removed and eluted with acetone. The acetone was removed under vacuum. Carrier commercial phytol was added and the azobenzoate derivative of phytol was prepared. The phytol azobenzoate was recrystallized three times and after each recrystallization a weighed amount was counted by the liquid scintillation technique. The amount of phytol azobenzoate added to each counting vial was kept constant so that any differences in count-rate due to quenching were minimized. The specific activity of the phytol azobenzoate was constant after the first crystallization (Table 2).

TABLE 2. SPECIFIC ACTIVITY OF PHYTOL AZOBENZOATE PREPARED FROM EXTRACTS OF DARK-GROWN CALLUS FOLLOWING FEEDING WITH EITHER 2-^{14}C ACETATE FOR 1 MONTH OR D,L-2-^{14}C MEVALONATE FOR 24 hr ON COMPLETE MEDIUM IN THE DARK. SPECIFIC ACTIVITIES ARE EXPRESSED AS cpm PER mg PHYTOL AZOBENZOATE

Phytol azobenzoate assayed after	Callus supplied with	
	2-^{14}C-acetate specific activity of phytol azobenzoate	D,L-2-^{14}C-MVA specific activity of phytol azobenzoate
First crystallization	198	28
Second crystallization	184	23
Third crystallization	182	23

As mevalonic acid is known to be a direct precursor of isoprene compounds such as phytol, the previous experiment was repeated using D,L-2-^{14}C mevalonate instead of 2-^{14}C-acetate. Any incorporation of activity from D,L-2-^{14}C mevalonate would thus be a direct indication of its terpenoid character. 10 g of dark-grown callus tissue was suspended in 15 ml sterile liquid complete medium plus 10 μc D,L-2-^{14}C MVA and the suspension was agitated in the dark at 26° for 24 hr. The callus tissues were washed thoroughly with liquid medium to remove residual isotope. After separation of the non-saponifiable fraction on columns

of alumina, the fractions containing phytol were subjected to TLC. Carrier phytol was added to the eluate of the radioactive phytol areas and the azobenzoate derivative prepared as before, counted by the liquid scintillation technique, and was found to be active. The phytol azobenzoate attained a constant specific activity after the first crystallization (Table 2). Thus, although the phytol was present in extracts of dark-grown callus in insufficient quantities to be detected by chemical techniques, its presence was established from these radioactive tracer experiments. The fact that the specific activity of the phytol azobenzoate was constant after crystallization confirmed that trace amounts of phytol were present in dark-grown callus cultures of *K. crenata.*

It seemed possible that this trace amount of phytol might be located in the plastid precursors, proplastids, known to exist in dark-grown callus.[4,10] Thus, the intracellular distribution of phytol in the dark-grown callus was examined. Dark-grown callus was subcultured on to 15 ml complete medium containing 10 μc D,L-2-^{14}C mevalonic acid and grown on this medium for 4 weeks in total darkness. The callus cultures were washed with complete liquid medium before fractionation in NaCl buffer. The trace quantities of phytol were isolated from each fraction using the procedures normally applied to whole callus. The 8% (v/v) acetone in petrol. ether eluates from the columns of alumina were subjected to TLC. The areas, corresponding on each plate to authentic phytol, were scraped off into a counting vial, covered with scintillation fluid and counted. Most activity was recovered in the 105,000 $\times g$ and 20,000 $\times g$ fractions (Table 3). No activity was detected in the 3000 $\times g$ fraction. Thus, the trace amounts of phytol in dark-grown callus would not seem to be associated with a plastid fraction unless the plastids were extremely fragile and dispersed into very small fragments.

TABLE 3. INTRACELLULAR DISTRIBUTION OF RADIOACTIVITY IN PHYTOL IN DARK-GROWN CALLUS FOLLOWING FEEDING OF D,L-2-^{14}C-MVA FOR 1 MONTH IN THE DARK

Fraction assayed	cpm in phytol*
3000 $\times g$	0
20,000 $\times g$	18 $\pm$ 1·5
105,000 $\times g$	49 $\pm$ 1·6
Supernatant	120 $\pm$ 1·8

* Each count rate is expressed $\pm$ standard deviation.

It is known that chlorophyllase activity developed in *Kalanchoë* callus cultures exposed to the light and that chlorophylls are not detectable in the callus until it has spent some time in the light.[3] In the present series of experiments chlorophyll and phytol determinations were carried out on dark-grown callus subcultured and grown in the light for 4 weeks (first-generation callus), 8 weeks (second-generation callus) and 12 weeks (third-generation callus) (Table 8). The callus was subcultured, as usual, at monthly intervals. Chlorophyll and phytol determinations were also made on the green callus clone and *Kalanchoë* leaves. The results are presented in Table 4. The first- and second-generation callus did not contain detectable quantities of chlorophyll. However, phytol was detected and increased in amount with prolonged exposure to the light, reaching a maximum in the third-generation callus. The level of phytol in the third-generation callus was similar to that in the green callus but the

[10] ANNE T. PICKERING and D. R. THOMAS, *New Phytol.*, in preparation.

chlorophyll content was low in comparison to that of the green callus. Thus, the phytol/chlorophyll molar ratio was high in the third-generation callus whereas the ratio for green callus was between 4 and 5·5 and for *Kalanchoë* leaves approximated unity.

TABLE 4. LEVELS OF PHYTOL AND CHLOROPHYLL IN *Kalanchoë* LEAVES AND IN CALLUS CULTURES

Material analysed	Chlorophyll content (μg per g fresh weight)	Phytol content (μg per g fresh weight)	Molar ratio (phytol/chlorophyll)
Dark-grown callus	0	0	—
First-generation callus	0	5·6	—
	0	4·4	—
	0	8·8	—
Second-generation callus	0	8·9	—
	0	8·4	—
	0	9·1	—
Third-generation callus	1·4	13·5	30·5
	2·6	14·1	17·6
	0·9	12·7	43·2
Green callus	9·6	12·3	4·1
	6·7	11·3	5·3
	11·4	16·7	4·7
Kalanchoë leaf	523	162	1·0
	381	125	1·0
	415	148	1·1

The distribution of phytol and chlorophyll in various fractions of callus cells was examined. The calluses were fractionated in NaCl buffer and the $3000 \times g$, $20,000 \times g$, $105,000 \times g$ fractions and the final supernatants retained for analysis. Chlorophylls were exhaustively extracted from each fraction with methanol. The methanol-extracted pellets were extracted with ethanol and this extract was added to the methanol extract. The bulked extracts were assayed for chlorophyll and phytol by the usual procedures. Phytol but not chlorophyll was present in all fractions of first-generation callus (Table 5). The supernatants of all other material analysed did not contain any detactable chlorophyll although an amount of phytol was present in each supernatant. In all other fractions both chlorophyll and phytol were present. In the fractions prepared from *Kalanchoë* leaves, the phytol/chlorophyll molar ratio approximated unity but in green callus the ratios were higher and increased in successive fractions to attain the maximum value in the $105,000 \times g$ fraction. The molar ratios for each fraction of third-generation callus were greater than those of the green callus, a reflection of the lower chlorophyll content, and reached a maximum value in the $20,000 \times g$ fraction.

A series of experiments was designed to provide further facts concerning the locale of phytol in the cell and also to give some indication of the possible sites of synthesis. First-, second- and third-generation and green callus were grown for 3 weeks on semi-solid complete medium and then transferred to complete liquid medium containing D,L-2-^{14}C mevalonic acid (10 g fresh weight callus supplied 2·5 μc D,L-2-^{14}C mevalonic acid in 15 ml medium) and allowed to complete the normal 4-week growth period in the light in this radioactive medium. The tissues were harvested and washed with liquid medium to remove excess radioactivity. Isolation and determination of phytol was by the usual methods. Finally, the isolated phytol was counted by the liquid scintillation technique. The results (Table 6) showed, as before, that the amount of phytol increased in callus when the callus was exposed to light and attained

TABLE 5. CHLOROPHYLL AND PHYTOL CONTENTS IN FRACTIONS OF *Kalanchoë* LEAVES AND CALLUS

	Fractions											
	3000 × *g*			20,000 × *g*			105,000 × *g*			Supernatant		
Material	C (μg)	P (μg)	Molar ratio (P/C)	C (μg)	P (μg)	Molar ratio (P/C)	C (μg)	P (μg)	Molar ratio (P/C)	C (μg)	P (μg)	Molar ratio (P/C)
First-generation callus	0	28	—	0	53	—	0	48	—	0	27	—
Third-generation callus	19·7	35	5·7	11·7	152	41·5	2·1	19	30·3	0	12	—
Green callus	94·3	91	3·1	67·4	111	5·3	18·6	74	12·7	0	18	—
Kalanchoë leaf	2314	1946	1·2	1946	683	1·1	361	201	1·8	0	Trace	—

In the Table heading, C refers to chlorophyll and P to phytol.

the phytol level of green callus after 3 months in the light. On a fresh weight basis, the most radioactivity incorporated into phytol was obtained with second-generation callus, the least incorporation into phytol occurred in green callus. The specific activity of the phytol was high in first- and second-generation callus, both of which contained no detectable chlorophyll, but was reduced by half in the third-generation callus which contained chlorophyll. The specific activity of phytol from green callus was even less.

TABLE 6. ACTIVITY INCORPORATED FROM D,L-2-^{14}C MEVALONATE INTO PHYTOL BY CALLUS SUPPLIED RADIOACTIVE MEVALONATE FOR THE PENULTIMATE WEEK OF THEIR NORMAL GROWTH PERIOD

Callus	cpm per g* fresh weight	Phytol (μg per g fresh weight)	Specific activity of phytol (cpm per μg)
First-generation	81 ± 1·7	6·3	12·9
Second-generation	134 ± 1·9	9·7	13·8
Third-generation	88 ± 1·7	14·0	6·3
Green callus	49 ± 1·6	12·9	3·8

* Each count rate is expressed ± standard deviation.

The information obtained in the last experiment was elaborated by examination of the activity incorporated from D,L-2-^{14}C MVA into subcellular fractions. First-, second- and third-generation and green callus were transferred after 2 weeks' growth to 15 ml fresh semi-solid complete medium containing 10 μc D,L-2-^{14}C MVA. After 2 weeks' growth on this radioactive medium, the calluses were removed, washed, and the cells fractionated in NaCl-buffer. Phytol was extracted from each fraction, quantitatively determined and counted by the usual methods. Phytol was recovered in all fractions of each type of callus (Table 7). In first- and second-generation callus, which did not contain chlorophyll, most phytol was recovered in the 20,000 × *g* and 105,000 × *g* fractions but in third-generation callus (containing chlorophyll) and in green callus most phytol was recovered in the 3000 × *g* and 20,000 × *g* fractions. In successive generations of callus in the light, successively greater amounts of phytol were

recovered in the 3000 × *g* and 20,000 × *g* fractions. The phytol recovered in the supernatants was similar for all calluses, but varied between 15 and 47 μg for the 105,000 × *g* fraction. There was a low percentage recovery of activity in the 3000 × *g* and supernatant fractions of each callus but higher percentage recoveries were noted for the 20,000 × *g* and 105,000 × *g* fractions. For each callus, the specific activity of phytol increased from a low value in the 3000 × *g* fraction to a high value in the supernatant. In the first-generation callus the specific activity of phytol in the 3000 × *g* and 20,000 × *g* fractions was higher than that of phytol in the same fractions of green callus. Indeed, the specific activity of phytol in these fractions decreased in successive generations of callus in the light to approach the values obtained with green callus. On the other hand, the specific activity of phytol in the 105,000 × *g* fractions and the supernatants was generally high for all callus although some reduction in specific activity was noted in the phytol recovered from the supernatant of the third-generation callus and in the 105,000 × *g* fraction and the supernatant from green callus.

TABLE 7. ACTIVITY INCORPORATED INTO PHYTOL RECOVERED FROM FRACTIONS OF CALLUS SUPPLIED D,L-2-^{14}C MVA DURING THE LAST 2 WEEKS OF GROWTH

Callus and fractions analysed	Phytol (μg)	cpm recovered*	% recovery of activity	Specific activity of phytol (cpm per μg)
First-generation				
3000 × *g*	12·6	73 ± 1·7	8·4	5·8
20,000 × *g*	41·0	285 ± 2·2	29·8	6·3
105,000 × *g*	30·0	372 ± 2·5	43·0	12·4
Supernatant	11·3	162 ± 1·9	18·7	14·3
Second-generation				
3000 × *g*	9·3	20 ± 1·5	1·4	2·1
20,000 × *g*	47·0	606 ± 2·9	41·8	12·9
105,000 × *g*	41·1	600 ± 2·9	41·4	14·6
Supernatant	15·0	224 ± 2·1	15·4	14·9
Third-generation				
3000 × *g*	44·2	22 ± 1·6	6·3	0·5
20,000 × *g*	128·9	90 ± 1·8	25·8	0·7
105,000 × *g*	14·9	180 ± 2·0	51·6	12·1
Supernatant	7·8	57 ± 1·7	16·3	7·3
Green callus				
3000 × *g*	67·0	54 ± 1·6	9·3	0·8
20,000 × *g*	94·1	104 ± 1·9	17·9	1·1
105,000 × *g*	46·9	310 ± 2·3	53·4	6·6
Supernatant	12·4	113 ± 1·9	19·4	9·1

* Each count rate is expressed ± standard deviation.

DISCUSSION

An unidentified compound was detected in the non-saponifiable lipid extracts of dark-grown callus but the experimental evidence suggests that it is not synthesized by the callus, since radioactive acetate and mevalonate were not incorporated into it. The compound was present in coconut milk and was therefore present in the medium supporting the growth of the callus. The unknown compound was not detected in callus grown in the light. Two

possibilities emerge, firstly, uptake might be light inhibited and, secondly, there might be a light-induced utilization of the compound in greening callus, either possibility preventing its accumulation and detection in the light-grown callus.

Phytol was not detected in etiolated shoots of *Phaseolus multiformis*[11] and it was suggested that the synthesis of phytol was light dependent.[12] However, Shlyk and Stanishevskaya[13] showed that phytol synthesis could occur in the dark but took place at a much slower rate than in the light. Certainly in the present work, phytol was not chemically detected in dark-grown callus but radioactivity from ^{14}C acetate and ^{14}C MVA was incorporated into phytol by dark-grown callus and so trace amounts of phytol must be present. Fractionation of the dark-grown callus did not enable the trace amounts of phytol to be assigned clearly to a particulate fraction but it was possible that the proplastids, known to exist in dark-grown callus,[10] were extremely fragile and broke up into fine particles on fractionation, this accounting for most of the radioactivity in the supernatant. Nevertheless, an amount of extraplastid phytol synthesis cannot be ruled out on the basis of this evidence alone. α-Tocopherol, which has a phytol side-chain, has been detected in dark-grown callus and it was possible that during saponification the phytol would be liberated. However, it was reported[14] that there was a 95·4 per cent recovery of α-tocopherol from germinating grain after saponification with a solution containing 160 g KOH per 100 ml water. Also, free phytol was not detected as a product when authentic α-tocopherol underwent the saponification procedure used to provide free phytol. However, if trace amounts of phytol were liberated from callus α-tocopherol during saponification, the small amount of phytol synthesis which occurred in dark-grown callus might well have been located extraplastidically.

An early precursor of phytol is MVA and Goodwin and co-workers[15–17] have demonstrated that chloroplast membranes are impermeable to MVA and proposed that regulation of terpenoid biosynthesis occurred essentially by a compartmentalization allowing two sites of terpenoid synthesis. Goodwin considered that sterols and pentacyclic triterpenes were synthesized outside the plastids but that "photosynthetic terpenoids" were synthesized by the activity of enzymes located in the chloroplast. This evidence would support the view that in the dark-grown callus the trace amounts of phytol formed from ^{14}C-MVA resulted from a cytoplasmic or mitochondrial synthesis as it might be that the proplastid membranes were impermeable to MVA exogenously supplied. The data of Table 7 appear to contradict this view as the specific activity of phytol recovered in the heavier particulate fractions, i.e. the $3000 \times g$ and $20{,}000 \times g$ fractions, was high in first-generation callus and decreased in subsequent generations of callus in the light to approach the values for specific activity of phytol in these fractions from green callus. The larger plastids would be collected in the $3000 \times g$ and $20{,}000 \times g$ fractions of first- and second-generation callus and it is known[10] that many of these plastids possess a primitive arrangement of lamellae into grana but do not contain clearly defined thylakoids. Thus, the plastids in these fractions could still be regarded as juvenile. This evidence alone might suggest that at such a juvenile stage of development the plastid membranes are more permeable to mevalonate than are the membranes of more

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mature chloroplasts or of etioplasts. However, approximately the same order in percentage recovery of activity was obtained for each fraction of the various calluses, that is, a low recovery in the $3000 \times g$ and supernatant fractions and higher activities in the $20,000 \times g$ and $105,000 \times g$ fractions (Table 7). This suggests that "cold" phytol was appearing in all fractions as the callus generations progressed, which was to be expected if two pools of phytol existed in the cells and only the extraplastid pool was accessible to ^{14}C-MVA exogenously supplied. It is known that the activity of mevalonate-activating enzymes increases in illuminated callus[18] and that this is associated with the development of plastids. If the plastid membranes were impermeable to MVA then an increase in enzymes concerned with later reactions in the terpenoid pathway, e.g. phytol synthase, would enable the plastids to synthesize "cold" phytol which on extraction would mix with high specific activity phytol formed from ^{14}C-MVA at extraplastid sites.

Phytol is a component of protochlorophyll, chlorophyll, vitamin K and tocopherols. Protochlorophyll, chlorophyll and vitamin K have not been detected in non-chlorophyllous calluses.[19] Protochlorophyll was not present in green callus and only small quantities of vitamin K were recovered from green callus. α-Tocopherol was present in all calluses but the amounts were small.[19] Extracts were saponified in the isolation of phytol and it was possible, as previously stated, that some phytol was derived from vitamin K and α-tocopherol as well as from chlorophyll. However, it seems likely that the amount of phytol from vitamin K and α-tocopherol would be small in comparison to phytol from chlorophyll and free phytol. The molar ratio, phytol/chlorophyll, was high in third-generation callus but was about five in green callus as opposed to values near unity for *Kalanchoë* leaves. Although no direct estimations were made of free phytol, as the quantity of phytol from vitamin K and α-tocopherol would be small, it is likely that much free phytol was present in the callus to account for the high ratios. It has been considered previously[2] that chlorophyll synthesis was limiting in the callus. It is suggested that there was free phytol in the plastids and that in the reaction converting chlorophyllide *a* to chlorophyll *a*, possibly catalysed by chlorophyllase, the availability of chlorophyllide *a* and not phytol limited the production of chlorophyll. Whilst the phytol/chlorophyll molar ratio for *Kalanchoë* leaf fractions approximated unity, the molar ratio for green callus varied considerably between fractions being about 3 for the $3000 \times g$ fraction increasing to 12 for the $105,000 \times g$ fraction. Proceeding on the assumption that most phytol was located in plastids, this variation in the green callus fractions would appear to reflect the mixed population of plastids in such callus where, although many plastids contain thylakoids stacked into grana, a number of plastids were in a juvenile stage of development.[10]

EXPERIMENTAL

Plant Material

The growth medium and culture conditions for the *Kalanchoë* callus have been reported elsewhere.[1-3] A summary of the types of callus used in the experiments is given in Table 8. Whole plants of *K. crenata* were obtained from the experimental grounds of the Botany Department at Newcastle. Leaves for experimental purposes were removed from the third and fourth nodes and stripped of their midribs before analysis or fractionation. Plant material was fractionated in NaCl buffer by standard procedure.[3]

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TABLE 8. TERMINOLOGY USED TO DESCRIBE *Kalanchoë* CALLUS GROWN FOR VARIOUS PERIODS IN 16 hr DAYS

Terminology	Description
Dark-grown callus	Callus cultures derived from stem cultures and which have been grown for 3 yr in the dark with monthly subculturing
First-generation callus	Callus subcultured from dark-grown callus and then grown in 16-hr days for 4 weeks
Second-generation callus	Callus subcultured from first-generation callus and then grown in 16-hr days for 4 weeks
Third-generation callus	Callus subcultured from second-generation callus and then grown in 16-hr days for 4 weeks
Green callus	Callus grown in 16-hr days for 3 yr with subculturing at monthly intervals

Chemicals

Phytol was obtained commercially and purified by distillation under reduced pressure (198°, 12 mmHg). All solvents were purified before use.

Phytol Extraction Procedure

Leaf and callus tissues were exhaustively extracted with methanol in a M.S.E. blender at full speed. The methanol extracts were filtered and the residue extracted with further quantities of methanol. These extracts were bulked and reduced in volume by vacuum distillation under N_2. A small known volume of the methanol extract was used for chlorophyll determination[2] and the remainder made 2·5% (w/v) with respect to solid KOK and saponified at 70° for 30 min with constant shaking. The free phytol was extracted repeatedly into peroxide-free diethyl ether, the ethereal solution washed with distilled water until neutral and then dried (Na_2SO_4). The ether solution was evaporated to dryness under reduced pressure at 30°. The residue, containing phytol, was redissolved in a small volume of petrol. ether (b.p. 40–60°).

Chromatographic Separation of Phytol

Preliminary purification of phytol from all sources was carried out on columns (14 × 2 cm) of Brockman III Al_2O_3.[20] The petrol. ether extract was added carefully to the top of the column and after penetrating the alumina was chromatographically separated by stepwise gradient elution with petrol. ether (fraction 1) and then with petrol. ether containing respectively 2, 6, 8 and 10 per cent acetone (fractions 2–5). Each fraction was collected and evaporated to dryness. The residues were dissolved in small volumes of peroxide-free acetone.

Thin-Layer and Paper Chromatographic Separation of Phytol

Kieselgel G plates were developed in either benzene–ethyl acetate (19:1 v/v)[8] or *n*-hexane–ethyl acetate (85:15 v/v).[21] Development was usually completed within 30 min. A reverse phase paper chromatographic system also was used to separate phytol.[22] Whatman No. 1 chromatography paper, previously treated with 5% (w/v) liquid paraffin in cyclohexane and then dried, was developed in methanol–water (4:1 v/v).

Location Reagents

0·25 per cent (w/v) $KMnO_4$,[8] a general reagent for unsaturated compounds, was used as a spray for TLC plates and as a dip for paper chromatograms. On TLC plates phytol appeared as a brown spot on a pink background. After dipping, the paper was washed in running tap-water to remove excess permanganate and phytol was visible as a dark-brown area on a light-brown background. Phosphomolybdic acid,[23] 5% (w/v) in ethanol, was used as a spray on TLC plates. The sprayed plates were heated for 10 min at 85° when phytol was located as a dark-blue spot. TLC plates were also sprayed with a mixture of $SbCl_5$–CCl_4 (1:4 w/w) and then heated at 120° until phytol was located as a violet area. Another spray reagent used on TLC plates consisted of 0·5 ml anisaldehyde dissolved in 10 ml HOAc, 85 ml MeOH and 5 ml conc. H_2SO_4.[23] After spraying, the plates were heated at 100° for 10 min when phytol appeared as a blue-violet spot.

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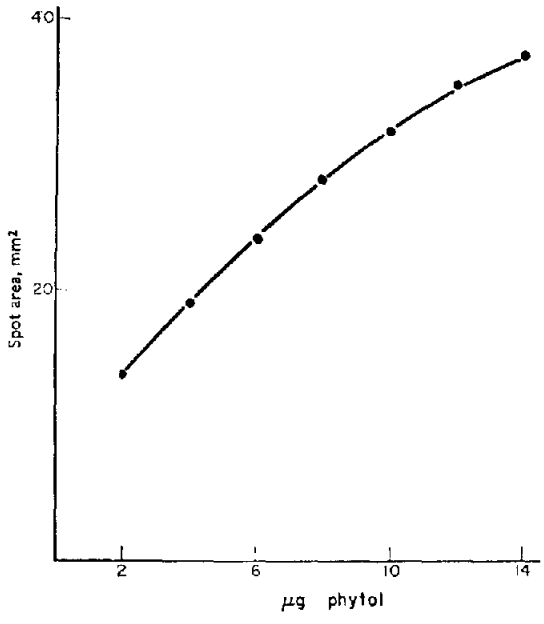
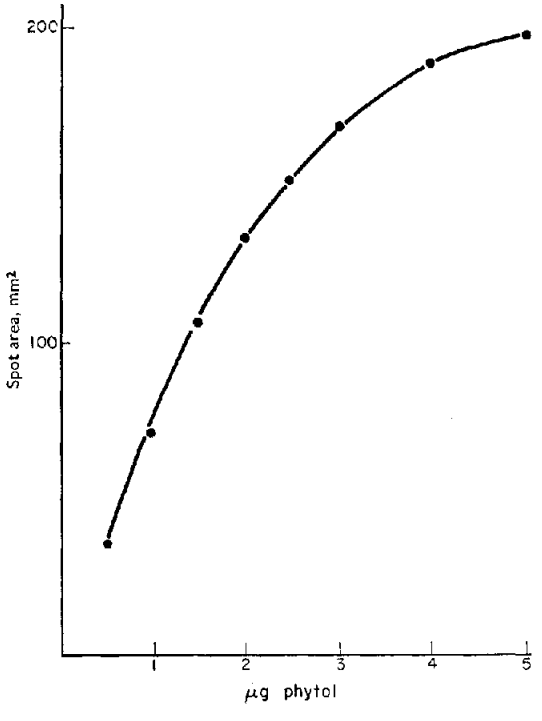


(a)



(b)

FIG. 1. CALIBRATION CURVES FOR PHYTOL DETERMINED BY MEASUREMENT OF SPOT AREA ON TLC PLATES AFTER LOCATION WITH (a) $KMnO_4$ AND (b) ANISALDEHYDE.

Quantitative Estimation of Phytol

The method of Shimizu *et al.*[8] using $KMnO_4$ was first employed but no reproducible calibration curves were obtained with purified samples of commercial phytol, even after much modification. No other convenient colorimetric methods existed in the literature and thus it was decided to employ spot area of phytol separated by TLC as a quantitative estimate. Standard quantities of phytol, over a range of 2–14 μg per spot, were applied to TLC plates of silica gel, 0·25 mm thick, and after development in either of the two solvents (see above) sprayed with $KMnO_4$ solution (10 ml reagent applied per 400 cm^2 plate). Spot area was traced out on paper and this area of paper cut out and weighed. The exact area of the spot was calculated from the weight of the paper. This method provided reproducible calibration curves (error $\pm$ 3 per cent) (Fig. 1a) but even so standard concentrations of authentic phytol were developed on each plate with the unknown samples. Similar calibration curves were prepared for the anisaldehyde reagent (10 ml applied for 400 cm^2 plate) (Fig. 1b). This reagent was more sensitive than $KMnO_4$ and was employed regularly when the phytol content was less than 5 μg (error $\pm$ 3 per cent).

Preparation of Phytol Azobenzoate[9]

Purified commercial phytol plus a quantity of radioactive phytol from callus tissue (total 192 mg) was dissolved in 5 ml dry benzene and 2·5 ml anhydrous pyridine and left for 24 hr at room temperature. 150 ml *n*-pentane was added and the mixture filtered, the residue being well-washed with *n*-pentane, the washings being combined with the filtrate. The *n*-pentane solution was washed thoroughly with N H_2SO_4 and then with 5% (w/v) Na_2CO_3. The *n*-pentane was removed under reduced pressure and the residue was dissolved in benzene and chromatographed on columns of silicic acid (100 mesh, Mallinckrodt) using benzene as the developing solvent. Two orange bands separated almost immediately, development being continued until the first band was eluted from the column. This orange solution was evaporated to a small volume, when phytol azobenzoate separated as crystals after cooling to $-5°$. The crystals were collected in the cold and excess benzene removed *in vacuo*. The crystals melted between $-2·8$ to $-3·2°$ which was in the range given by Demole and Lederer.[9] Chromatography of the dissolved crystals on Kieselgel G with benzene–ethyl acetate (19:1 v/v) revealed a single spot, R_f 0·78. As two bands always were separated on the column of silicic acid it was thought advisable to further establish the identity of the phytol azobenzoate. Its mass spectrum was consistent with the proposed structure and the mole wt was calculated to be within 0·03 per cent of the expected value.

Radioisotope Techniques

2-^{14}C-sodium acetate and DL-2-^{14}C-mevalonic acid lactone were purchased from the Radiochemical Centre, Amersham. DL-2-^{14}C-mevalonic acid was prepared from the lactone by the method of Rogers *et al.*[17] Phytol and phytol azobenzoate dissolved in ethanol were added to 5 ml liquid scintillator fluid consisting of 5 g 2,5-diphenyloxazole and 0·3 g 1,4-*bis*-2-(5-phenyloxazolyl)-benzene in 1 l. toluene. Samples were counted on a Packard Tri-Carb Model 2003 for 100 min or until 10,000 counts were registered. The efficiency of the counting apparatus for ^{14}C was 65 per cent. The standard deviation (σ_s) of the net sample counting rate, i.e. gross sample count rate (*rg*) minus background count rate (*rb*) was calculated[24] from the formula $\sigma_s = \sqrt{(rg/tg + (rb/tb)}$ where *tg* and *tb* are the counting times for the sample and background respectively. Autoradiographs were prepared from TLC plates with Ilford X-ray film.

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