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CELL CLONES CONTRASTED IN GROWTH, MORPHOLOGY AND PIGMENTATION ISOLATED FROM A CALLUS CULTURE OF *ATROPA BELLADONNA* VAR. *LUTEA*

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Abstract—Three cell clones differing from one another in growth rate and growth pattern in suspension culture, in nutrient requirement, in callus and cell morphology, in cellular fine structure and in pigmentation have been isolated from a callus of *Atropa belladonna* var. *lutea* Döll of seedling root origin. Two of the cell clones (HC and FC) became green when cultured in light and developed a higher content of chloroplast pigments than the original stock callus, whereas the third clone (W) was of very low pigment content and developed only amyloplasts. The structure of the chloroplasts and the contents of individual chloroplast pigments in the green clones are compared with those of leaf cells of the species. Preliminary work on the photosynthetic activity of the green clone HC is reported. The influence of sucrose supply and of the level and kind of auxin (NAA or 2,4-D) on growth and pigmentation of the green clones is described. The sequential changes in plastid structure and in chloroplast pigment contents which occur when dark-grown clones HC and FC are transferred to light or returned to darkness after growth in light have been followed.

INTRODUCTION

MANY plant tissue cultures develop chloroplast pigments when cultured in light although none capable of continuous autotrophic growth has yet been described.[1-3] The greening of such cultures when transferred from darkness to light proceeds very slowly and often unevenly compared with that of etiolated plant organs.[4,5]

Whenever established callus cultures have been used to initiate clones of single cell origin, some of these clones have been shown to differ from the parent callus and from one another in growth rate, colour and morphology.[6-9] Whether the distinctions between these clones is the outcome of stable states of differentiation or of differences in their genetic constitution has, however, not been critically examined.

For the study of one of the most important aspects of plant cell differentiation, namely the development of photosynthetically active chloroplasts, such greening tissue cultures have certain attractive features. They may be readily grown under a wide range of cultural conditions. The greening process proceeds slowly. The technique of single-cell cloning might be expected to yield cultures which would green more uniformly and intensely than

[1] A. C. HILDEBRANDT, J. C. WILMAR, H. JOHNS and A. J. RIKER, *Am. J. Bot.* **50**, 248 (1963).
[2] J. C. WILMAR, A. C. HILDEBRANDT and A. J. RIKER, *Nature, Lond.* **202**, 1235 (1964).
[3] V. VASIL and A. C. HILDEBRANDT, *Science* **150**, 889 (1965).
[4] N. SUNDERLAND, *Ann. Bot.* **30**, 253 (1966).
[5] A. K. STOBART, I. MCLAREN and D. R. THOMAS, *Phytochem.* **6**, 1467 (1967).
[6] H. C. ARYA, A. C. HILDEBRANDT and A. J. RIKER, *Plant Physiol.* (Lancaster) **37**, 387 (1962).
[7] L. M. BLAKELY and F. C. STEWARD, *Am. J. Botany* **51**, 809, (1964).
[8] T. MURASHIGE and R. NAKANO, *Am. J. Botany* **52**, 819 (1965).
[9] W. H. MUIR, in *Proc. Intern. Conf. Plant Tissue Culture* (edited by P. R. WHITE and A. R. GROVE), p. 485, McCuthan, Berkeley, California (1965).

calluses long-maintained in culture or initiated from multicellular explants. The potentialities of this approach are examined in the present paper using as a starting point an established rapidly-growing callus of *Atropa belladonna* var. *lutea* Döll.[10]

RESULTS

The Growth of Cell Suspensions Derived from the Stock Callus

Stock suspensions were initiated as described under 'Cultural procedures' and serially subcultured every 21–24 days from an initial cell density of 200–250 $\times$ 10^3 cells/ml. Growth data from experimental cultures established in the 5th serial passage are presented in Fig. 1.

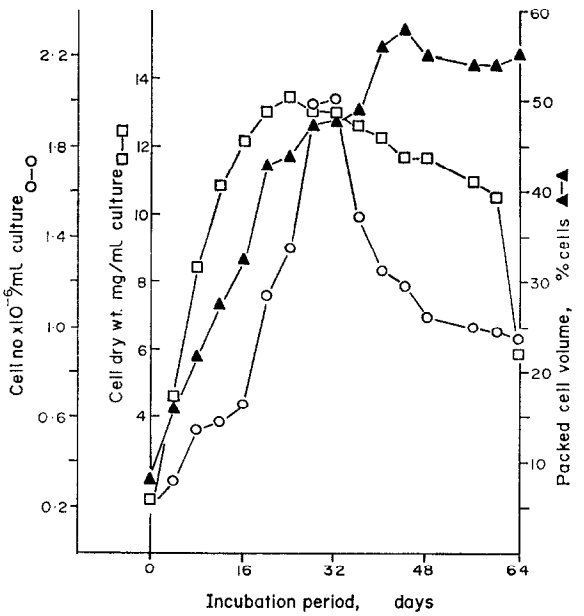

FIG. 1. GROWTH OF A CELL SUSPENSION CULTURE DERIVED FROM THE STOCK CALLUS OF *Atropa belladonna* VAR. *lutea*. GROWTH MEASUREMENTS RELATE TO THE 5TH SERIAL PASSAGE OF THE SUSPENSION.

More frequent cell counts during the first few days of incubation have confirmed the indication from this experiment that there is no lag phase occasioned by subculture (contrast the growth of *Acer pseudoplatanus* cell suspensions).[11] The rapid decline in cell number following the achievement of maximum cell density is interpreted in this and later growth experiments as indicative of cell lysis but the alternative explanation that, with increasing age, an increasing proportion of the cells become susceptible to destruction by the chromic acid treatment used to achieve cell separation before counting cannot be rigorously excluded. During this period of falling cell number, the surviving cells continue to increase in size but it has not been satisfactorily demonstrated that this cell expansion is sufficient to account for the maintenance of the packed cell volume of the culture during this period.

[10] RAJ S. B. BANDARY, H. A. COLLINS, E. THOMAS and H. E. STREET, *Ann. Bot.* **33**, 647 (1969).
[11] S. B. WILSON, P. J. KING and H. E. STREET, *J. Exp. Bot.* **22**, 177 (1971).

Isolation of Clones by the Bergmann[12] Plating Technique

The stock callus when grown in light showed irregularity of growth and greening, often displaying completely colourless segments. This suggested that it might comprise a heterogenous cell population from which single cell clones differing in growth and in capacity to develop chloroplast pigments could be isolated.

Twenty-one-day-old suspension cultures (Fig. 1), filtered through nylon bolting cloth (pore size 250 μm^2) were used to set up Bergmann plates[12] at a plating density of 10–15 $\times$ 10^3 cells/ml. Colony formation was allowed to proceed for 30 days in the dark; under these conditions each plate developed up to 2 $\times$ 10^3 colonies. Illumination during incubation reduced the number of colonies at least by a factor of 10; introduction into the dishes of a 'nurse callus' did not significantly increase colony numbers.

By subculture from discrete colonies on such plates some 2000 clones were established and grown in light. The origin of these clones was not traced back to single cells or even to single small aggregates in the plated suspension culture. Three clones differing from one another and from the parent callus were selected for further study:

Clone W. Almost white (hence W), soft and moist texture, tendency to central necrosis after 4 weeks in culture. Readily disperses (2 days) in liquid medium to form a well dispersed suspension; aggregates if present open. Cells in suspension average dia. 70–100 μm, some cells 25–30 μm dia. Cell walls thin. Plastids refractive. Dry wt. as % fr. wt. = 1·8–2·7.

Clone HC. Fast growing, green (C for chlorophyllous), hard (H) callus irregular in outline. Only slowly (after 2–3 weeks) dispersed when agitated in liquid medium. Cells in suspension average dia. 80–160 μm, some isodiametric, many irregular in shape, giant cells up to 600 μm length present in older suspensions. Aggregates present, cells closely packed. Cell walls thick with internal thickenings (particularly evident in sections of callus). Intercellular spaces in callus packed with anastomosing fibrils. Plastids smaller than in Clone W and green. Dry wt. as % fr. wt. = 3·4–5·3.

Clone FC. Friable (F) green (C) callus forming regular oval colonies. Portions of callus in contact with medium usually yellow. Cell walls intermediate in thickness, resembling those of the stock callus. Disperses in liquid medium at a rate intermediate between Clones W and HC. Cells smaller in size than those of Clone HC, less irregular and giant cells present. Chloroplasts more numerous per cell than in Clone HC. Dry wt. as % fr. wt. = 2·6–4·7.

After these clones had been maintained as suspension cultures through ten subcultures they were replated and 1000 colonies from each clone grown in agar. All colonies had the morphology and pigmentation characteristic of their clone. This indicated the stability of these clones and argues strongly for their single cell origin.

The Growth of Cell Suspensions Derived from the Cell Clones

The cell clones could be serially propagated in suspension culture although the growth obtained with Clone W was occasionally variable. Growth data from experimental suspension cultures over a 30-day incubation period are presented in Fig. 2. The clones resembled one another in the patterns of increase in cell dry weight and packed cell volume (Fig. 2, b and c) but the cell densities achieved in the Clone W cultures were much higher than in the cultures of Clones HC and FC. All cultures showed an immediate fall in cell density after reaching a maximum; this fall was particularly marked with Clone W where

[12] L. BERGMANN, *J. gen. Physiol.* **43**, 841 (1960).

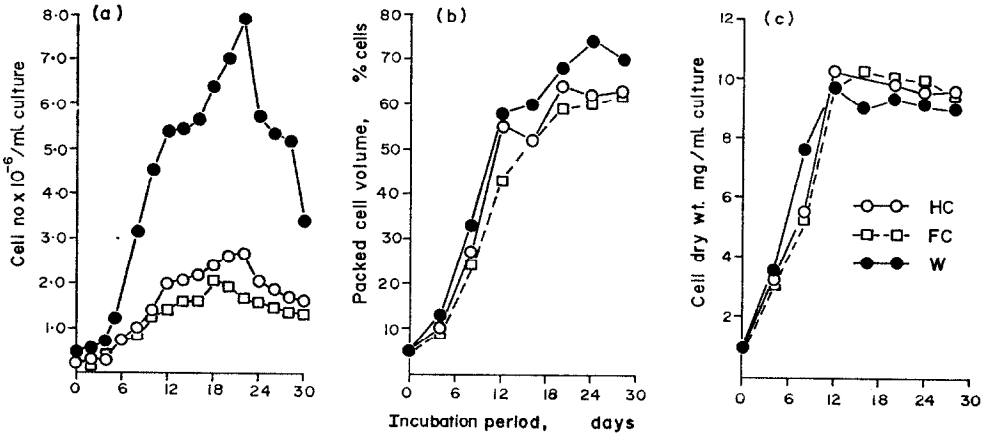

FIG. 2. GROWTH OF CELL SUSPENSION CULTURES DERIVED FROM THE CELL CLONES (W, HC AND FC). GROWTH MEASUREMENTS RELATE TO THE SECOND PASSAGE OF THE SUSPENSIONS. KEY IN FIG. 2(c) APPLIES ALSO TO (a) AND (b).

the density on day 30 was less than half that achieved on day 20 of incubation. At the time of maximum cell density the cultures of Clone W contained many small densely cytoplasmic cells. Despite the evidence of rapid cell lysis after the peak of cell density, the cultures retained their viability for long periods without subculture; the surviving enlarged vacuolated cells retained their capacity to divide when transferred to new medium in cultures of Clone FC up to 20 weeks old and in cultures of Clone W up to 7 weeks old. Cultures initiated from such stock cultures did not show an extended lag phase.

The clones differed strikingly in the minimum effective inoculum density[13] needed for successful growth in suspension culture. The minimum effective density for Clone W was 150×10^3 cells/ml whereas for Clones HC and FC it was 20×10^3 cells/ml. Adjustment of the initial pH of the culture medium to 7·0 (from the standard pH of 5·2) lowered the minimum effective density with Clones HC and FC to 5×10^3 cells/ml but did not reduce the density required for Clone W. By using a medium conditioned by an undiluted stationary phase culture, 21 days old (the most effective ratio, during conditioning of stationary phase culture to medium being conditioned was 1:8 with a conditioning period of 4 days)[13] the minimum effective density for Clone W could be reduced to 5×10^3 cells/ml. These observations strongly suggest that Clone W has more exacting nutritional requirements for growth than Clones HC and FC.

The optimum α-naphthalene acetic acid (NAA) concentration for all 3 clones was for maximum cell density 1·5 mg/l. and for cell dry weight yield 0·5 mg/l. However, when 2,4-dichlorophenoxyacetic acid (2,4-D) was used instead of NAA as auxin, the responses of the green clones differed from that of Clone W in that their growth was strongly inhibited at 1·5 and 2·0 mg/l. (Data for Clone W and Clone FC is shown in Fig. 3a.) These studies also showed that 2,4-D was, compared with NAA, strongly inhibitory to chlorophyll formation particularly in the green clones and that the optimum concentration of NAA for greening was 0·25 mg/l., (Fig. 3b). In auxin-free medium and in presence of 0·25 mg/l. NAA the cultures were most aggregated and showed root initiation from aggregates.[14] The

[13] R. STUART and H. E. STREET, *J. exp. Bot.* **20**, 556 (1969).
[14] E. THOMAS and H. E. STREET, *Ann. Botany* **34**, 657 (1970).

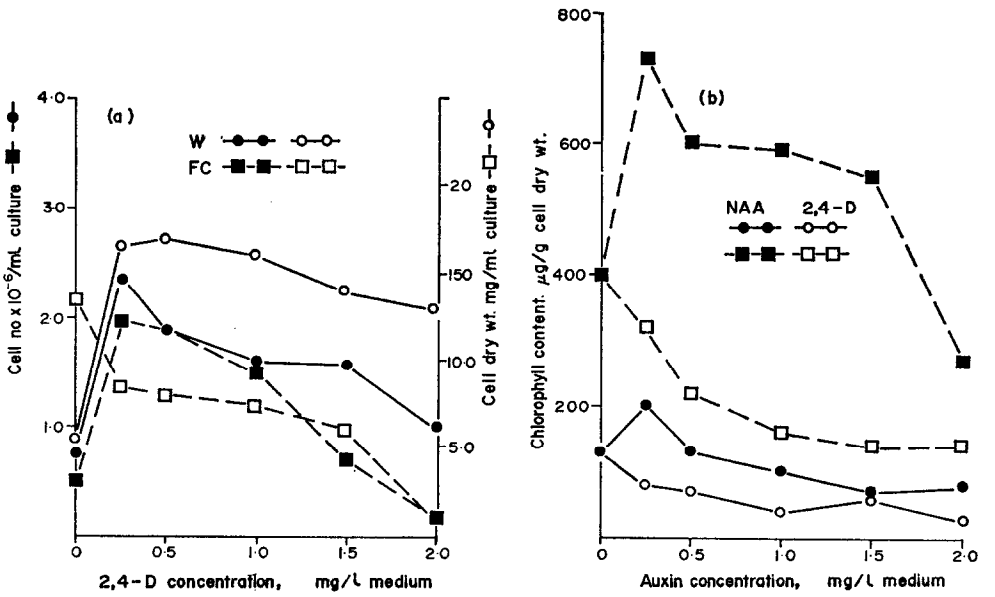

FIG. 3(a). GROWTH OF CELL SUSPENSION CULTURES DERIVED FROM THE CELL CLONES W AND FC IN MEDIUM CONTAINING 2,4-D AS AUXIN. DURATION OF INCUBATION 28 DAYS. DATA FROM THE 3rd SERIAL PASSAGE.

3(b). TOTAL CHLOROPHYLL CONTENT OF CELL SUSPENSION CULTURES (END OF 3rd SERIAL PASSAGE OF 28 DAYS) OF CELL CLONES W AND FC GROWN IN MEDIA CONTAINING VARIOUS CONCENTRATIONS OF 2,4-D AND NAA. (Key to Clones as in Fig. 3a)

inhibitory effect on chlorophyll formation of 2,4-D compared with NAA has been previously reported by Bergmann[15] working with *Nicotiana tabacum* and by Simpkins[16] working with *Acer pseudoplatanus* tissue cultures.

A study with Clone FC of the development of total chlorophyll content in suspension culture, using a culture initiated from 21-day-old cells, is shown in Fig. 4. There was an initial drop in chlorophyll content during the first 4 days of culture. From then onwards chlorophyll steadily accumulated up to day 56 of incubation despite the achievement of maximum cell dry weight by day 24. The chlorophyll *a*:*b* ratio was at a minimum (1·67) on day 8 of culture and reached its maximum value (3·24) by day 40.

The Growth of the Cell Clones on Bergmann Plates

Using standard medium, the minimum inoculum densities resulting in colony formation within 30 days of dark incubation was for the Clones HC and FC, 20×10^3 cells/ml and for Clone W, 150×10^3 cells/ml. Addition of nurse callus did not reduce these critical initial densities although it did increase the number of colonies developing on plates seeded with the critical inoculum in the case of Clones HC and FC. Using conditioned medium (prepared at the most effective stationary suspension: medium-being-conditioned ratio) the minimum inoculum density of the green clones was reduced to 15×10^3 cells/ml; conditioning was particularly effective in increasing the number of colonies developed

[15] L. BERGMANN, *Planta* 74, 243 (1967).
[16] I. SIMPKINS, Ph.D. Thesis, Univ. Wales (1970).

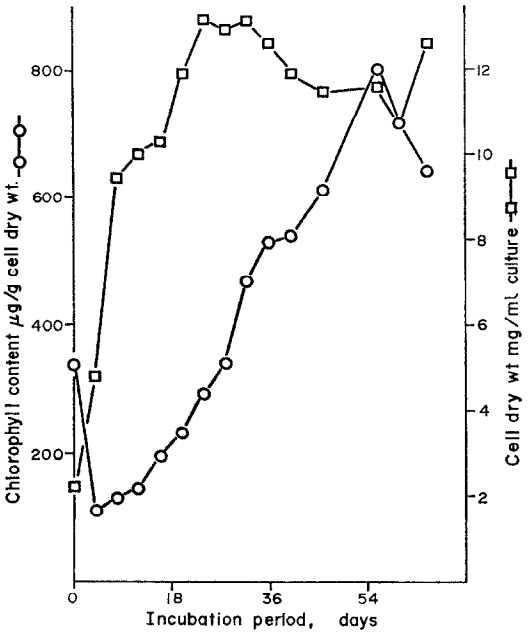

FIG. 4. THE PROGRESS OF CHANGE IN CELL dry wt./ml CULTURE AND IN TOTAL CHLOROPHYLL CONTENT/UNIT CELL dry wt. DURING THE GROWTH IN LIGHT OF A CELL SUSPENSION CULTURE OF THE CELL CLONE FC.

from low density plating of Clone HC. In presence of conditioned medium the minimum effective density for Clone W was reduced to 60×10^3 cells/ml. Cross-conditioning (using conditioned medium prepared from a different clone) was never more effective than self-conditioning and the Clone W only responded to self-conditioning.

It is of interest that the minimum effective densities for plating are much higher than for growth in suspension, $15\times$ as against 5×10^3 for the green clones, and $60\times$ as against 5×10^3 for Clone W. When clones were isolated by plating from suspensions of the stock callus many colonies indistinguishable from Clones HC and FC were obtained, but Clone W was exceptional and similar colonies could not subsequently be detected. This was most probably due to the exacting conditions for colony formation from such cells in the parent callus rather than an indication of the sparcity of such cells. Until plating techniques are improved to the point where single cells can survive and develop into colonies on their own, this technique will fail to reveal the range of variation within plant tissue cultures.

The Chloroplasts of Belladonna *Leaves and of the Callus Cultures Grown in Light*

The structures of immature and mature chloroplasts from the youngest fully expanded leaf of a 5-week-old plant of *Atropa belladonna* var. *lutea* grown in the University Botanic Garden are shown in Fig. 5,A and B. The vesicles and unassociated thylakoids of the immature chloroplast are thought to arise from invaginations of the inner membrane of the plastid envelope (Fig. 5A). Such vesicles and thylakoids are absent from mature chloroplasts. Osmiophilic droplets occur in the stroma and starch grains are normally present. In any one leaf cell the chloroplasts are in a similar stage of differentiation.

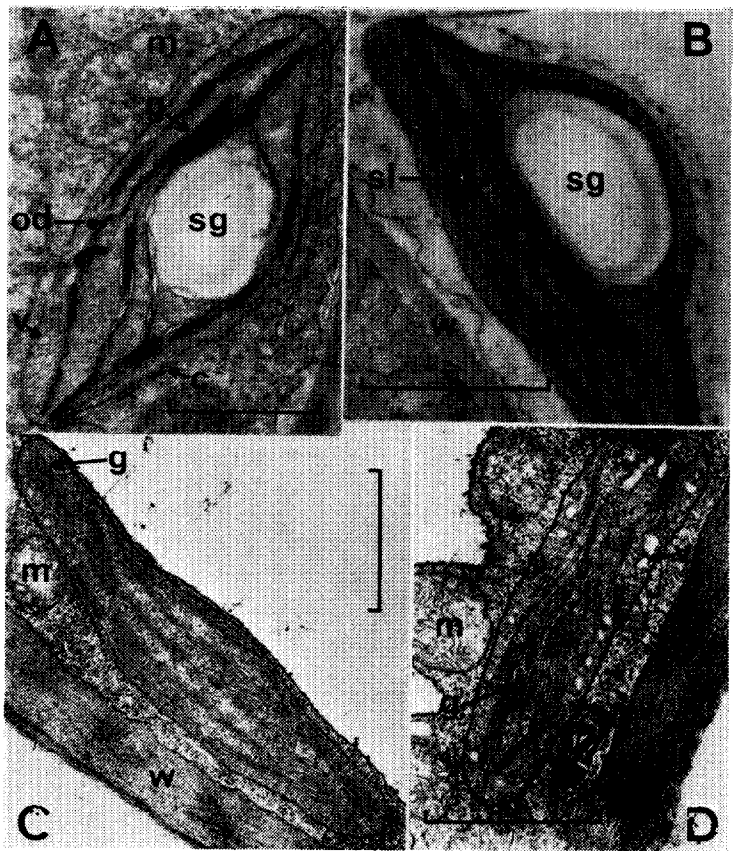

FIG. 5. A–D

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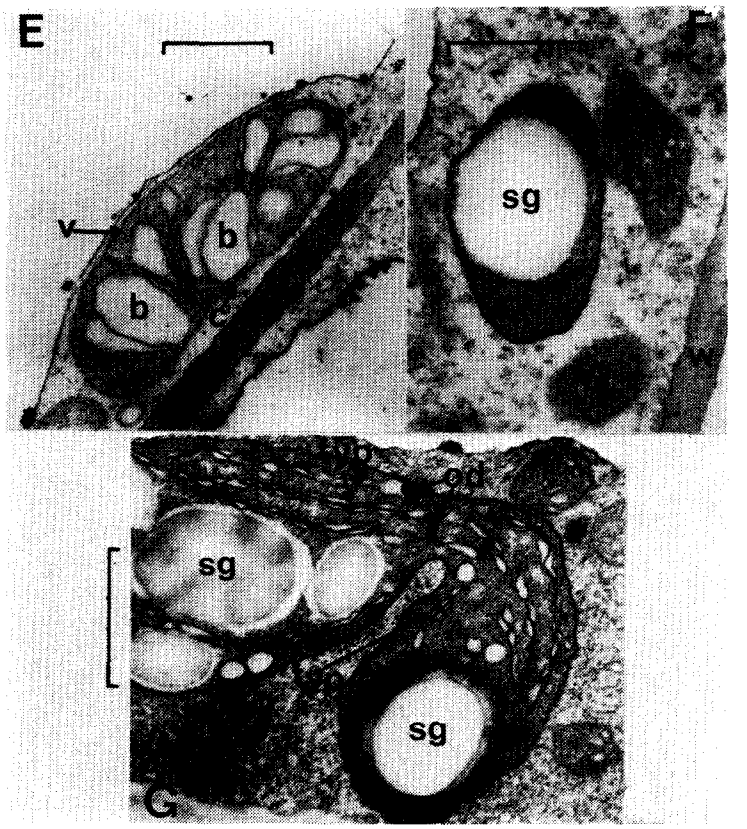

FIG. 5. E–G

FIG. 5. ELECTRONMICROGRAPHS SHOWING ASPECTS OF CHLOROPLAST STRUCTURE. (A) CHLOROPLAST IN AN IMMATURE MESOPHYLL CELL OF THE BELLADONNA LEAF. (B) CHLOROPLAST IN A MATURE PALISADE CELL OF THE LEAF. (C) DIFFERENTIATED CHLOROPLAST FROM A LIGHT-GROWN CALLUS OF THE GREEN CELL CLONE HC. (D) INCOMPLETELY DIFFERENTIATED CHLOROPLAST FROM A SIMILAR CALLUS TO C. THIS CHLOROPLAST RESEMBLES CHLOROPLASTS OBSERVED IN MERISTEMATIC LEAF CELLS. (E) CHLOROPLAST SHOWING THYLAKOIDS SWOLLEN INTO BALLOON-LIKE OR ANGULAR CONFIGURATIONS. FROM A CALLUS SIMILAR TO C. (F) AN AMYLOPLAST FROM A LIGHT-GROWN CALLUS OF CELL CLONE W. (G) A CHLOROPLAST FROM A DARK-GROWN CALLUS OF FC AFTER THE CALLUS HAD BEEN EXPOSED TO LIGHT FOR 7 DAYS.

Key: Scales on each Fig. = 1 μm. b = swollen thylakoids; c = double membraned envelope; g = granum; m = mitochondrion; od = osmiophilic droplet; typ = thylakoid pairs leading to grana development; sg = starch grain; sl = stroma lamellae; v = vesicular ingrowths of the inner membrane of the envelope; w = cell wall.

The cells of the Clones HC and FC contain chloroplasts similar in size and shape to leaf cells; sections of cells of Clone HC contain 4–9 and sections of cells of Clone FC 3–5 chloroplasts. These chloroplasts when examined in the electron microscope are more variable in structure than observed in leaf cells; within the same cell, chloroplasts differ in structure indicating lack of synchrony in their development. Some chloroplasts closely approximate in structure to those of mature leaf chloroplasts in the development of the thylakoid system (Fig. 5C) and in containing starch grains, although osmiophilic or other opaque stroma globules are absent. Others resemble the immature chloroplasts of meristematic leaf cells (Fig. 5D). Many of the chloroplasts, however, show swollen thylakoids leading to balloon-like or angular configurations (Fig. 5E). Membranes enclosing these angular configurations appear to interact at various points and to be there giving rise to granal membranes. These chloroplasts closely resemble those described by Heltne and Bonnett[17] from their studies of the conversion of leucoplasts into chloroplasts in cultured excised roots of *Convolvulus arvensis* exposed to light, and interpreted by them as a normal stage in chloroplast development. Such chloroplasts were always present in the green calluses (in both young and old cultures) but were not observed in leaf cells. It is not clear, therefore, whether such chloroplasts represent an arrested stage in normal development or abnormal structures consequent upon some deficiency associated with the conditions of culture.

Clone W contained only plastids rich in starch and with a poorly developed membrane system (Fig. 5F). Some of these plastids contained osmiophilic droplets resembling those of leaf chloroplasts.

Chloroplast Pigments Present in Leaves and in the Callus Cultures

Chlorophylls *a* and *b* were detected in all calluses and identified by their absorption spectra, the absorption spectra of their phaeophytin derivatives, by the Molisch phase test and by their TLC behaviour compared with authentic chlorophylls isolated from the leaf extracts.

β-carotene, flavochrome, lutein, auroxanthin and neoxanthin were detected and identified in callus extracts by their absorption spectra and chromatographic behaviour. Leaf extracts contained isolutein and violaxanthin not detected in the callus extracts. Callus and leaf extracts contained an unidentified pigment in the carotenoid fraction, properties of which are given in Fig. 6.

The concentrations of these pigments (μg pigment/g dry weight of tissue) in the leaf and callus samples and in suspensions of Clones FC and W are shown in Table 1. The chlorophylls are present at far lower concentrations in the calluses than in leaves; the leaf tissue has 28× the chlorophyll content of the callus Clone HC and 264× that of Clone W. The chlorophyll *a*:*b* ratios of the isolated clones are similar to that in the leaf tissue.

The levels of carotenoids were lower in the calluses than in the leaves although the calluses were richer in carotenoids relative to chlorophylls. Although as in leaves β-carotene and lutein were the most abundant carotenoids in the calluses, their levels of lutein relative to β-carotene were lower. The callus Clones HC and FC were particularly rich in auroxanthin; the auroxanthin level in Clone HC was equal to that in the leaf material. Clone HC had a higher content of carotenoids than FC and a relatively high β-carotene content.

The analytical data in Table 1 show that the green clones HC and FC are significantly richer in chloroplast pigments than the parent stock callus and that Clone W contains all the same pigments but at much lower concentrations.

[17] J. Heltne and H. T. Bonnett, *Planta* **92**, 1 (1970).

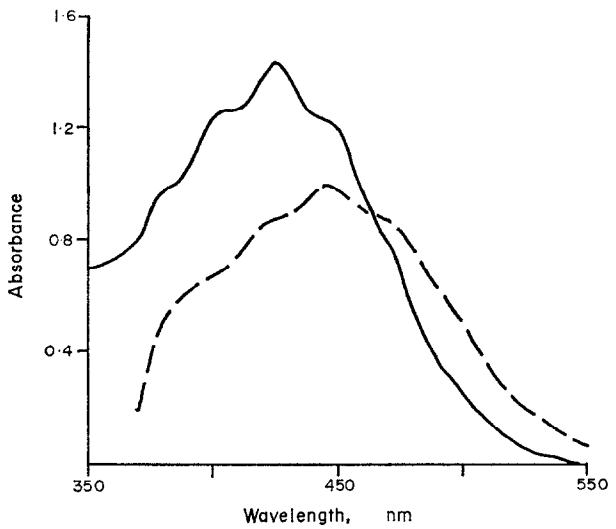

Fig. 6. Absorption spectra of an unidentified pigment eluted within the fraction mobilized by 10% Et_2O in petrol. (60–80°) and coming off between the flavochrome and lutein bands when the carotenoid extract was applied to an aluminium oxide DS-5 column with Ca_2SO_4 binder. Solid line in petrol; broken line in CS_2. Occasionally two separate bands (one pink and one orange in colour) could be distinguished within this zone of unidentified pigment. The pink component had absorption maxima at 400, 419 and 440 nm in petrol., and 422, 443 and 470 nm in CS_2. The corresponding maxima for the orange component were at 400, 421 and 442 and at 420, 446 and 470 respectively.

Table 1. Content of pigments of the expanded leaves of flowering plants of *Atropa belladonna* var. *lutea* and of 4-week-old cultures of light-grown calluses and suspension cultures of the stock callus and of the cell clones HC, FC and W derived from this stock callus

	Pigment concentration μg/g dry weight of tissue								
		Callus cultures				Suspension cultures			
Pigment	Leaf	Stock	HC	FC	W	Stock	HC	FC	W
Total chlorophyll	15,810	252	571	375	61	233	280	294	73
Chlorophyll *a*	11,700	193	416	273	45	164	175	215	49
Chlorophyll *b*	4110	59	155	102	16	69	105	79	24
Ratio *a*:*b*	2·84	3·27	2·68	2·68	2·81	2·38	1·67	2·72	2·04
Total carotenoids	87·3	8·8	30·7	17·8	4·1	25·35	not determined	26·1	6·25
β-carotene	18·86	2·97	10·38	4·51	1·13	6·23		8·58	1·66
Flavochrome	0·55	0·16	0·27	0·48	0·20	0·18		0·39	0·06
Unidentified	5·06	0·67	0·99	1·30	0·42	0·27		0·81	0·09
Lutein	39·20	2·87	10·71	5·88	0·99	12·4		10·58	1·93
Isolutein	12·25	nil	nil	nil	nil	nil		nil	nil
Auroxanthin	6·38	1·62	6·76	4·22	1·16	4·76		4·66	2·13
Violoxanthin	2·78	nil	nil	nil	nil	nil		nil	nil
Neoxanthin	4·23	0·53	1·57	1·42	0·23	1·49		1·01	0·38

The Effect of Sucrose Concentration in the Culture Medium on the Chlorophyll Content in the Calluses of Cell Clones HC and FC

Calluses of these clones were subcultured over three passages of 28 days with various levels of sucrose, transferring 0·4 g fr. wt. of callus at each subculture. Cell dry weight yields and total chlorophyll content recorded at the end of the third culture passage are shown in Fig. 7. In the case of sucrose-omitted medium, growth did not occur during the second passage and, for each clone, the third passage in this medium represents transfer of the whole callus piece from the second passage. The explant from Clone HC remained green in the sucrose-omitted medium.

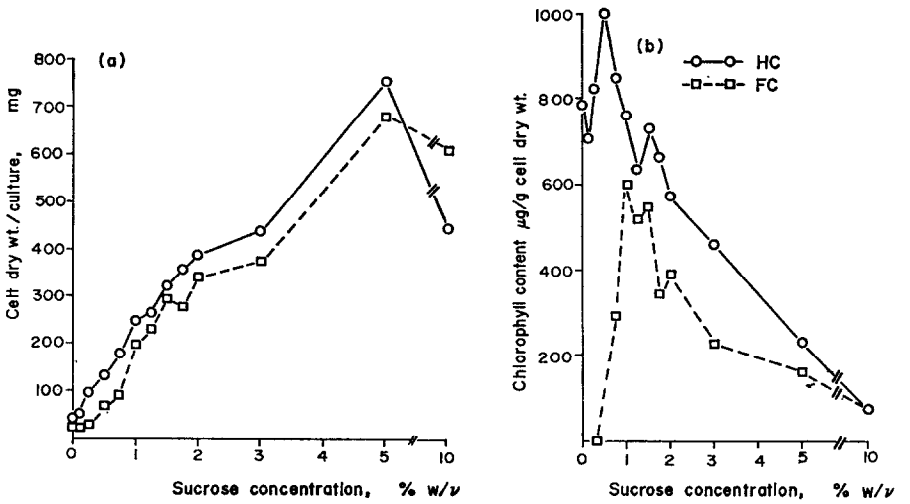

FIG. 7. GROWTH (EXPRESSED AS CULTURE dry wt.) (a) AND CHLOROPHYLL CONTENT (b) (EXPRESSED AS CHLOROPHYLL/UNIT TISSUE dry wt.) OF CALLUS CULTURES OF THE GREEN CELL CLONES (HC AND FC) USING SOLIDIFIED MEDIA CONTAINING VARIOUS CONCENTRATIONS OF SUCROSE. DATA FOR THE 3rd PASSAGE OF 28 DAYS.

The cultures of both clones gave increasing yields of cell dry wt. with increase in sucrose concentration up to 5% (Fig. 7a), although the peak of cell fr. wt. was, for both clones, at 2% sucrose. Further subculture of the cultures in the low sucrose media showed that the minimum effective sucrose concentration for continuous culture was 0·5% for Clone HC and 1·0% for Clone FC and that at these levels chlorophyll content/g cell dry wt. was maximal. Fukami and Hildebrandt,[18] working with cultures of a number of species, have also reported enhanced chlorophyll formation at reduced carbohydrate levels.

Pigment Development Following Exposure of the Dark-grown Cell Clones to Light

The calluses were grown in darkness for 6 months. For studies on chlorophyll accumulation new cultures were established and after 7 days further growth in darkness were transferred to light (6000 lux), allowed to grow for 3 weeks, subcultured, and grown for a further 4 weeks. The changing total chlorophyll content following exposure to light is

[18] T. FUKAMI and A. D. HILDEBRANDT, *Bot. Mag. Tokyo* **80**, 199 (1967).

shown in Fig. 8 and compared with the chlorophyll content of calluses continuously cultured in light. The temporary decline in chlorophyll content at week 4 harvest was associated with subculture 7 days earlier. After 6 weeks growth in light following 6 months darkness all Clones achieve a chlorophyll content similar to that of the cultures maintained continuously in light. The chlorophyll *a*:*b* ratio was high after the first 7 days of illumination and also showed a sharp rise following subculture (i.e. at week 4 harvest).

The changes in total carotenoid content during the first 14 days of exposure to light are shown in Fig. 9. The dark-grown calluses contained detectable quantities of β-carotene, auroxanthin, neoxanthin and the unidentified pigment; oxygenated xanthophylls constituted more than 50 per cent of the total. Their total levels of carotenoids in μg/g dry wt. were for Clone HC 0·53, for Clone FC 0·83 and for Clone W 0·50. Within 7 days of illumination flavochrome and lutein could also be detected in all clones. After 14 days illumination the total carotenoid levels in FC and W cultures were similar to those of cultures continuously maintained in light although in the case of Clone FC the level of β-carotene (2·9 μg/g dry wt.) was lower and the level of auroxanthin higher (8·5 μg/g) than in the light-grown cultures and in the case of Clone W the level of β-carotene was also lower (0·59 μg/g) and the level of neoxanthin higher (0·9 μg/g) than in light-grown cultures (see Table 1). Clone HC cultures after 14 days illumination had only about half the total carotenoid content of light-grown cultures being deficient in β-carotene (1·9 μg/g) and lutein (4·2 μg/g). The relatively high levels in dark-grown and early greening calluses of auroxanthin and neoxanthin agrees with observations on *Kalanchöe* callus.[5] Formation of flavochrome and lutein, presumably via α-carotene, appears to be light dependent. The delay in achieving the full β-carotene level and the early accumulations of auroxanthin and neoxanthin are of interest if the former is regarded as the parent compound of the latter.[19]

The plastids of the dark-grown callus Clones HC and FC resembled those of Clone W in being rich in starch and having a poorly developed membrane system. When such cultures were exposed to light, chains of lamellae consisting of thylakoid pairs were present and membranes were apparently associating into grana (Fig. 5G). As the number of thylakoids increased (apparently predominantly by multiplication of thylakoids already present) they became parallel and more uniform in width with stroma lamellae connecting the developing grana. Some profiles provided evidence of invaginations of the inner membrane of the envelope contributing to the growth of the lamellae. The cells after 4 weeks light exposure contained some chloroplasts resembling those of leaf cells and others not fully differentiated. Although these incompletely differentiated chloroplasts showed swollen membrane profiles the extent of the swelling was never as great as that observed in the cultures grown in continuous light (Fig. 5E) suggesting that such 'abnormal' plastids may only arise after prolonged culture in light. These studies of the differentiation of 'etioplasts' into chloroplasts gave no evidence of the occurrence of a prolamellar body.[20, 21]

Pigment Decline Following Transfer of Light-grown Cell Clones to Darkness

New cultures of Clones HC and FC were established in light and then transferred to darkness and their pigments estimated after 3 weeks growth (Table 2). Chlorophyll and

[19] T. W. GOODWIN, in *Chemistry and Biochemistry of Plant Pigments* (edited by T. W. GOODWIN), p. 127, Academic Press, New York (1965).
[20] D. VON WETTSTEIN, *Brookhaven Sym. Quart. Biol.* **11**, 138 (1959).
[21] B. E. S. GUNNING, *Protoplasma* **55**, 111 (1965).

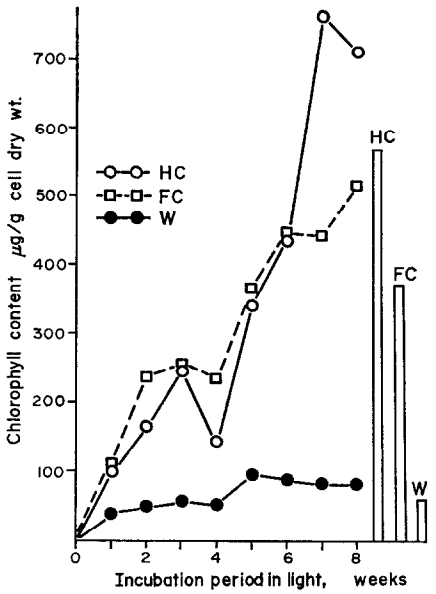

FIG. 8. CHANGES IN TOTAL CHLOROPHYLL CONTENT WHEN DARK-GROWN CALLUSES OF THE CELL CLONES ARE EXPOSED TO LIGHT. HISTOGRAMS INDICATE CHLOROPHYLL CONTENT OF CULTURES GROWN CONTINUOUSLY IN LIGHT (4-WEEK-OLD CULTURES ANALYSED).

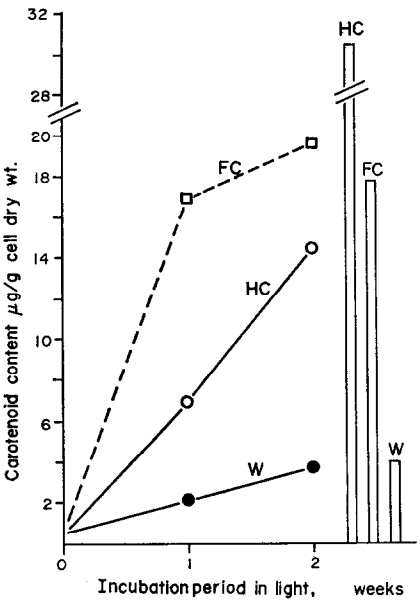

FIG. 9. CHANGES IN TOTAL CAROTENOID CONTENT WHEN DARK-GROWN CALLUSES EXPOSED TO LIGHT FOR 2 WEEKS. HISTOGRAMS INDICATE CAROTENOID CONTENT OF CULTURES GROWN IN CONTINUOUS LIGHT (4-WEEK-OLD CULTURES ANALYSED).

TABLE 2. CONTENT OF PIGMENTS OF CALLUSES OF CLONES HC AND FC, 3 WEEKS AFTER TRANSFERANCE TO DARKNESS

(Compare data with that in Table 1)

Pigment	Chlorophyll μg/g dry wt.			
	HC	% decrease	FC	% decrease
Total chlorophyll	383	33	150	59·5
Chlorophyll *a*	283		115	
Chlorophyll *b*	90		35	
Ratio *a*:*b*	3·26		3·29	
	Carotenoid μg/g dry wt.			
Total carotenoids	25·1	18	11·5	36
β-carotene	4·4	57	2·3	49
Flavochrome	0·32	nil	0·20	59
Unidentified	0·95	4	0·56	57
Lutein	9·1	15	3·5	40
Isolutein	3·8	*	1·2	*
Auroxanthin	5·5	20	3·3	21
Neoxanthin	1·0	38	0·45	68

* Not present in light-grown cultures.

carotenoid breakdown was more rapid in Clones FC than in HC. In both clones chlorophyll *b* declined more rapidly than chlorophyll *a* so that the *a*:*b* ratio rose from 2·68 (Table 1) to 3·26 (HC) and 3·29 (FC). In both clones lutein became the major carotenoid and iso-lutein (an oxidation product of lutein), not detected in light-grown callus, accumulated to a significant content. Decline in β-carotene was particularly marked in Clone HC; loss of β-carotene (6·3 μg/g) exceeded loss of total carotenoid (4·3 μg/g). β-carotene, which only slowly reaches its final level on exposure of dark-grown callus to light, rapidly declines when callus is transferred to darkness.

Photosynthetic Activity of the Callus of the Cell Clone HC

5·0 g portions of dissected callus and of young expanded leaves were fed $^{14}CO_2$ for 15 min, one callus portion and the leaf material at a light intensity of 20,000 lx, the other callus portion in the dark. 50 μl aliquots of tissue extract (0·5 ml ≡ 5 g fr. wt.) in a scintillation spectrometer gave the following disintegrations per minute; leaf extract = 18,214; illuminated callus = 8846; callus in darkness = 644. The extract of the illuminated callus when submitted to two dimensional paper chromatography showed radioactivity in positions possibly corresponding to sucrose, sugar phosphates and certain amino acids but the intensity of labelling was low and the radioactive compounds were not identified.

Chloroplasts prepared from a 4-week-old culture of Clone HC when illuminated in a sucrose suspending medium decolorized 2,4-dichlorophenolindophenol (DCPIP) (Fig. 10). Chloroplasts suspended in a sorbitol-containing buffer, when placed in the chamber of the oxygen electrode, did not evolve O_2 in response to addition of bicarbonate, or ribose-5-phosphate or 3-phosphoglycerate, but O_2 evolution was initiated by addition of potassium ferricyanide, particularly if DCPIP was added before the ferricyanide (Fig. 11).

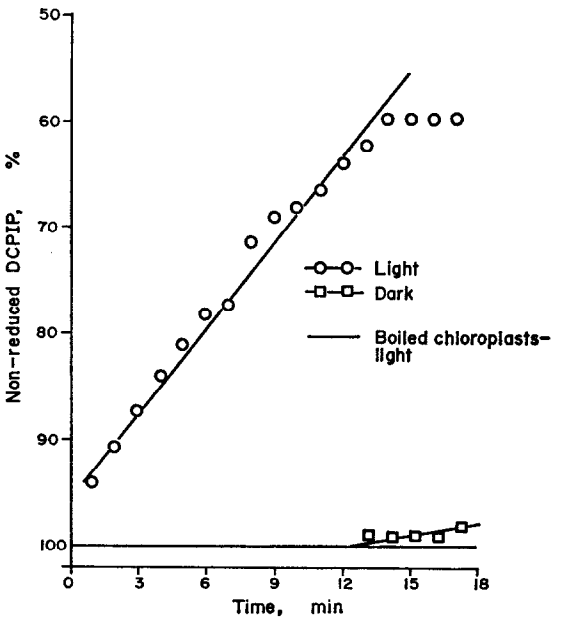

FIG. 10. REDUCTION OF 2,6,-DICHLOROPHENOLINDOPHENOL (DCPIP) BY CHLOROPLASTS ISOLATED FROM A 4-WEEK-OLD CALLUS CULTURE OF CELL CLONE HC AND SUSPENDED IN SORBITOL BUFFER.

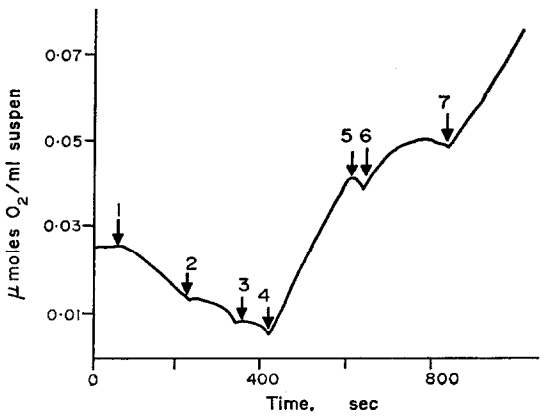

FIG. 11. GILSON OXYGRAPH TRACE OF THE O_2 CONTENT OF A CHLOROPLAST SUSPENSION AS RECORDED BY A CLARKE-TYPE ELECTRODE. SUSPENSION PREPARED FROM A CALLUS OF CELL CLONE HC. Key: 1 = light on; 2 = addition of 0·2 μmole DCPIP; 3 = addition of 0·4 μmole DCPIP; 4 = addition of 0·8 μmole potassium ferricyanide ($K_3Fe(CN)_6$); 5 = light off; 6 = light on; 7 = addition of 0·8 μmole $K_3Fe(CN)_6$.

These very preliminary experiments on photosynthetic activity are sufficiently positive to suggest that a more detailed study of photosynthesis by such green cell clones should be an essential part of future work.

DISCUSSION

The stability of the cell clones isolated from the stock callus suggests that they differ genetically from one another and that the stock callus is genetically heterogeneous either

because of its multicellular origin from the seedling root explant or because of the persistence of somatic mutations arising during prolonged growth in culture. The extent of this heterogeneity cannot be assessed while the special difficulty persists that we cannot grow single cells or even small populations of cells in culture media capable of supporting active growth from a sufficiently large initial population. Improved culture media which will allow of the isolation of low frequency but competent variants may also be expected to permit the growth of at least some mutants of more restricted biosynthetic activity than the parent culture.

The presence in cells of the green clones of some chloroplasts resembling those of leaf cells along with others either arrested in development or abnormal in structure suggests that the cultural conditions are suboptimal for chloroplast differentiation. This view is also supported by the slow rate of greening when dark-grown calluses are exposed to light. Clearly more critical attention should be directed to the light regimes used in such studies. However the importance of the kind and concentration of externally applied auxin on the greening process emphasises that composition of the culture medium may be equally important. Callus and suspension cultures of a number of species, some of which show little or no greening when grown in light, are capable, under appropriate stimuli, of giving rise to shoot buds or embryoids and when this occurs normal green leaves are developed. This is the case with cultures of both *Atropa belladonna* L. and *Atropa belladonna* var. *lutea* Döll.[10,14] The photosynthetic activities of the callus and suspension cultures in such cases are inhibited either directly by nutritional factors or indirectly by such factors by virtue of the absence of organized structure (and thus of division of labour between different tissue cells).

The long periods over which cells of both the green cell clones retained their viability after culture growth had ceased and the survival of callus pieces of the Clone HC on sugar-free and low sugar media are of considerable interest. This is particularly so in view of the evidence of an antagonism between active growth by cell division and the concentration of chloroplast pigments and the observation that chloroplast pigment content continues to rise after growth (both cell number and cell dry matter increase) has ceased. The maintenance of such stationary phase suspension cultures in continuous culture equipment where nutrient depletion can be monitored and checked and hormone and other treatments applied may therefore open the way to the establishment of non-growing but autotrophic cultures ('mesophyll' cultures). Such cultures would be valuable for studies on the photosynthesis of higher plants and, by using alternation of light and dark periods, could permit of critical studies of the reversible chloroplast–etioplast change and the associated synthesis and degradation of chloroplast pigments.

EXPERIMENTAL

Cultural Procedures

The stock callus was established from seedling roots of *Atropa belladonna* var. *lutea* Döll.[10] It was maintained by transferring every 4–6 weeks, 0·25–0·5 g callus to 20 ml synthetic medium (pH 5·2) containing 0·7% Oxoid Ionagar No. 2, 2% sucrose, the inorganic salts of White[22] and Wood and Braun,[23] ferric ethylenediamine-tetra acetate as the source of Fe,[24] the vitamins of White[22] and the growth factors: 100 mg/l. *meso*-inositol, 2 mg/l. α-napthaleneacetic acid (NAA) and 0·5 mg/l. kinetin. Incubation was at 25° and at a light intensity of 6000 lux from Mazda Universal warm white fluorescent tubes. When dark-grown callus

[22] P. R. WHITE, *A Handbook of Plant Tissue Culture*. Jacques Cathell, Lancaster, Pennsylvania (1943).
[23] H. N. WOOD and A. C. BRAUN, *Proc. Natl. Acad. Sci. Wash.* **47**, 1907, (1961).
[24] D. E. G. SHEAT, B. H. FLETCHER and H. E. STREET, *New Phytol.* **58**, 128 (1959).

was required the culture vessels (100-ml Erlenmeyer flasks) were wrapped in aluminium foil and black polythene. The cell clones were similarly maintained as calluses by incubation in light.

Suspension cultures were initiated by transference of 5·0 g callus to 250-ml Erlenmeyer flasks containing 60 ml liquid medium and agitation, during incubation, on a horizontal platform shaker (120 rev/min, horizontal throw 2 in.). Incubation was at 25° and at a light intensity at the platform surface of 1000 lx. Suspensions were subcultured by transference of 10 ml suspension to 50 ml new medium every 3–4 weeks. The basic culture medium was as described above under 'Stock callus' except that agar was omitted and the kinetin concentration reduced to 0·1 mg/l.

Experimental suspension cultures were grown in 100-ml Erlenmeyer flasks containing 18 ml new medium and 2 ml appropriately standardized stock suspension.

Cell clones were isolated by the Bergmann plating technique[12] using 9 cm petri dishes closed by 1 in. Sellotape and containing 14 ml medium solidified with 0·6% agar. Suspensions for plating were prepared aseptically by passage of stock suspensions through nylon bolting cloth of mean pore size 250 μm^2 supported on a Hartley Quickfit funnel FG75, collection of the cell material settling out from the filtrate, suspension in new liquid medium, cell counting and adjustment with medium to the required cell density.[25] 2 ml of the plating suspension was added to 12 ml agar-containing medium initially at 40°, mixed and immediately poured and swirled in the petri dish till setting commenced. These Bergmann plates were incubated, under conditions described above for 'stock callus', for 4–6 weeks. Where a 'nurse' callus[26] was also included this was planted centrally on the plate and confined by a glass ring (0·3 cm high × 1·0 cm dia.) with 4 indentations at its base to allow diffusion of substances from the 'nurse' callus into the surrounding agar. 'Conditioned' medium for incorporation into the Bergmann plate medium was prepared as described by Stuart and Street[13] using their Apparatus B and a high density suspension of either the stock or clonal cultures.

Leaves from 5 week old plants grown in a Saxcil Cabinet at 25° and a 16-hr day were used to investigate the fine structure of fully differentiated chloroplasts. Leaves were harvested from mature garden grown plants to determine their content of chloroplast pigments.

Growth Measurements

Packed cell volume (ml cells/ml culture), cell number/ml and cell fresh and dry weights/ml were determined on suspension cultures as previously described.[27] Colonies in Bergmann plates were counted using a colony counter (as Baird & Tatlock (London) Ltd. 402/0034) with markounter (402/0036). In some experiments the total number of cells on Bergmann plates was determined after incubation by removing the agar medium with cells from each petri dish into 20 ml H_2O, adding 2·0 g chromium trioxide and then proceeding as by the normal cell counting procedure.

Electron Microscopy

Cells were collected and handled in 'transfer tubes'[28] during the preparative stages. They were either embedded in araldite[28] or in methacrylate-styrene.[29] Sections were prepared on a Tesla Ultramicrotome BS490 with glass knives, collected on celloidin-coated Polaron C200 copper grids, stained with Reynolds[30] lead citrate for 5 min and examined in a Siemens Elmiskop 1A electron microscope, using an accelerating voltage of 80 kV, 200 μm condenser aperture and a 50 μm objective aperture.

Separation, Characterization and Estimation of Chlorophylls and Carotenoids from Leaves, Callus and Suspension Cultures: Extraction and Purification of Pigments

Organic solvents were purified by standard techniques before use. Extraction and subsequent manipulations were carried out at 4° in dim light. Extracts were maintained at −20° under N_2 when stored.

Leaf tissue was extracted directly, but callus tissue was freeze dried and ground to a fine powder prior to extraction. Pigments were extracted in 80% (v/v) acetone–H_2O in a Polytron homogenizer. The homogenate was filtered under vacuum and the residue re-extracted with 80% (v/v) acetone–H_2O. Successive extracts were combined and then partitioned with Et_2O. The ether extract was then used for subsequent pigment analysis.

Separation of Chlorophylls

The ether extract was washed with H_2O to remove traces of acetone, and then dried for 5 hr with Na_2SO_4. The solution was filtered, the Na_2SO_4 washed with Et_2O until colourless, and the washings added to the

[25] H. E. Street and G. G. Henshaw, in *Cells and Tissues in Culture* (edited by E. N. Willmer), Vol. 3, p. 459, Academic Press, London (1966).
[26] H. E. Street, in *Les Cultures de tissus de plantes*, p. 177, C.N.R.S., Paris (1968).
[27] G. G. Henshaw, K. K. Jha, A. R. Mehta, D. J. Shakesahft and H. E. Street, *J. exp. Bot.* **17**, 362 (1966).
[28] B. Sutton-Jones and H. E. Street, *J. exp. Bot.* **19**, 114 (1968).
[29] M. R. Davey and H. E. Street, *J. exp. Bot.* **22**, 90 (1971).
[30] E. S. Reynolds, *J. cell. Biol.* **17**, 208 (1963).

filtrate. Absorption spectra and extinction values at 645, 649, 663 and 652 nm of chlorophylls *a* and *b* were recorded. Amounts of chlorophyll present were calculated using three different methods.[31-33] Confirmation of identity and routine separations of chlorophylls *a* and *b* were carried out using a TLC system of Keiselguhr G-1% (v/v) *n*-PrOH in petrol. (b.p. 60–80°).

Separation of Carotenoids

The extraction procedure was similar to that described by Stobart *et al.*,[5] except that, following centrifugation to remove sterols, and reduction in volume to 2 ml, the extract was placed on an Al_2O_3 DS-5 column containing $CaSO_4$ binder. The column had been previously washed in Et_2O and then equilibrated in petrol. (b.p. 60–80°). Carotenoids were chromatographed on and eluted from the column by gradually increasing concentrations of Et_2O in petrol. (b.p. 60–80°) and finally Et_2O containing 2% (v/v) ethanol. The carotenoid fractions were reduced to dryness, re-dissolved in a small volume of petrol. (b.p. 60–80°) and purified on an icing-sugar column. Purified pigments were evaporated to dryness and their absorption spectra and extinction maxima recorded in petrol. (b.p. 40–60°) and CS_2. Amounts of identified and unidentified carotenoids were calculated by the methods of Goodwin[34,35] and Davies.[36]

$^{14}CO_2$ Fixation by Green Callus

$^{14}CO_2$ was generated by addition of 2·5 ml of 88% (v/v) lactate–H_2O to $Na_2{}^{14}CO_3$ (specific activity 55 mc/mM, Radiochemical Centre, Amersham). Callus tissue (5 g) was exposed to $^{14}CO_2$ either in the dark or light (20,000 lx, tungsten filament lamp) for 15 min.

Following exposure the tissue was killed in boiling 80% (v/v) EtOH–H_2O for 5 min. Further extraction of the tissue was carried out similarly to the method of Fowler and ap Rees.[37]

Chromatography of Tissue Extracts

Labelled compounds were separated by 2 dimensional chromatography on acid washed (1% v/v HOAc–H_2O containing 0·01% w/v NaEDTA–H_2O, followed by distilled water) Whatman No. 1 chromatography paper. The solvent systems were phenol (280 g)–HOAc (3·5 ml, glacial)–H_2O (8·8 ml)–NaEDTA (0·33 ml, 1·0 M, pH 7·0) and butanol–propionic acid–H_2O (123:60:81 by vol.). Sugars were detected by spraying with phthalic acid reagent.[38] Amino acids were detected with 1% (w/v) ninhydrin in acetone containing a trace of 2,4,6-collidine. Autoradiographs were prepared on Kodak Auto-Process X-ray film.

Isolation of Chloroplasts

Callus tissue (100 g) was homogenized in a Polytron homogenizer at full speed for 5 sec in a semi-frozen medium containing either 0·38 M sucrose, 1 mM KCl and 6·7 mM KH_2PO_4 at pH 7·5, or 0·33 M sorbitol, 5 mM $MgCl_2$, 2 mM sodium isoascorbate and 10 mM $Na_4P_2O_7 \times 10\ H_2O$ adjusted to pH 6·5 at 0° with HCl.[39] The homogenate was filtered through muslin and the supernatant centrifuged at 500 *g*, 2° for 3 min. The pellet was discarded and the supernatant centrifuged at 4000 *g*, 2° for 2·5 min. Finally the supernatant was discarded and the pellet resuspended in ice-cold solutions containing either 0·38 M sucrose, 10 mM KCl and 6·7 mM KH_2PO_4 at pH 7·0; or 0·33 M sorbitol, 1·0 mM $MgCl_2$, 2 mM EDTA and 50 mM HEPES pH 7·6.

An aliquot of the chloroplast preparation was extracted in 80% (v/v) acetone–H_2O, filtered and the amount of chlorophyll present calculated from the expression: concentration (mg/l.) = $27{\cdot}8D_{652}$ after measuring the extinction value at 652 nm.

Measurement of Chloroplast Activity

Reduction of 2,6-dichlorophenolindophenol was assayed spectrophotometrically at 585 nm and 25° (light intensity 20,000 lx, tungsten filament lamp). Oxygen evolution was estimated polarographically using a basal Clark-type electrode (platinum-KCl silver) separated from the stirred assay mixture by a Teflon membrane. The chloroplasts were illuminated at a saturating intensity by a 150 W quartz-iodide slide

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[32] S. MACLACHLAN and S. ZALIK, *Canad. J. Bot.* **42,** 1053 (1963).
[33] L. P. VERNON, *Anal. Chem.* **32,** 1144 (1960).
[34] T. W. GOODWIN, *Biochem. J.* **58,** 90 (1954).
[35] T. W. GOODWIN, in *Modern Methods of Plant Analysis* (edited by K. PAECH and M. V. TRACEY), Vol. III, p. 272, Springer-Verlag, Berlin (1955).
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[37] M. W. FOWLER and T. AP REES, *Biochem. Biophys. Acta* **201,** 33 (1970).
[38] S. M. PARTRIDGE, *Nature, Lond.* **164,** 443 (1949).
[39] W. COCKBURN, D. A. WALKER and C. W. BALDRY, *Plant Physiol.* (Lancaster) **43,** 1415 (1968).

projector, coupled to water filter (14 cm) and a Calflex C Tempax heat filter. Output was recorded using a Gilson oxygraph. The difference between the output of the electrode system in air saturated water at 20° and water containing sodium dithionite was taken as 0·28 μmole O_2/ml.

Acknowledgements—Grateful acknowledgement is made to Dr. W. Cockburn for expert help in the measurement of oxygen evolution by chloroplasts, to Dr. A. J. Rowe for the use of electron microscope facilities and to the Science Research Council for a studentship which enabled one of us (M.R.D.) to participate in this work.