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Maintenance of High Photosynthetic Rates in Mesophyll Cells Isolated from *Papaver somniferum*[1]

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ABSTRACT

The establishment and maintenance of high rates of photosynthetic CO_2 incorporation in mesophyll cells of *Papaver somniferum* (opium poppy) depend on a regime of dark and light periods immediately following isolation, as well as carefully adjusted conditions of isolation. Analysis of the incorporation pattern of $^{14}CO_2$ by the isolated cells indicates an initial "stress-response" period of approximately 20 hours characterized by increased respiratory-type metabolism and diminished photosynthesis. Under the favorable regime, this period is followed by rapid recovery and the reinstatement of a metabolic state strikingly similar to that of intact leaves in which the initial rate of CO_2 incorporation is between 110 and 175 μmoles CO_2 fixed per mg chlorophyll per hour. The photosynthetic viability of these cells can be maintained for up to 80 hours.

The separation of photosynthetically active cells from leaf tissue has seen notable successes only in recent years (2, 4, 7, 12, 13). The potential usefulness of such systems for studying plant cell metabolism is great since they offer the advantages of a microbiological system with the unique biochemistry of higher plants. In addition, such separated plant cells may eventually offer a means of obtaining autotrophic cells in liquid suspension cultures. Attainment of these cultures will necessarily depend upon the ability of researchers to maintain photosynthetic viability in isolated cells. This latter requirement has been difficult to achieve and reports to date have attained no better than 24 hr of active light-driven CO_2 incorporation (2, 4).

A further problem which has been noted in previous publications (2, 7) is the dissimilarity of CO_2 incorporation products in isolated cells compared to the respective leaf tissue. The degree of dissimilarity may be partly related to the harshness of cell isolation conditions employed. For example, tobacco cells obtained after a 3-hr digestion period (2) showed greater differences from respective leaf tissue than tobacco cells obtained after 1.5 hr of digestion (4). In order to relate modulating influences on isolated cells to the whole leaf, it will be necessaary to obtain control cells which exist in a metabolic state similar to that of the intact tissue.

By modifying the isolation procedures of previous investigators (4, 7, 11) we have obtained mesophyll cells from *Papaver somniferum* (poppy) which meet some of the above criteria. Photosynthetic incorporation rates as well as product distribution have been monitored in these isolated cells for a 72-hr period.

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MATERIALS AND METHODS

Plant Material. *P. somniferum* plants were grown on Hoagland solution in a sandy loam soil mixture. A growth chamber was used with a periodicity of 12 hr light (22 C) and 12 hr dark (20 C). Irradiance from incandescent and fluorescent bulbs was 400 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at plant level. While *P. somniferum* can be grown on a short day periodicity and lower temperature regime, we found that a minimum of 12 hr light/day was necessary to obtain photosynthetically viable cells.

Cell Isolation. Plants were selected in the early stages of maturation (8-10 weeks) for leaf material. Such plants generally consisted of approximately 10 to 15 leaves of which the top third (young, fully expanded leaves) were normally used although older leaves could also be employed sucessfully for cell isolations. Using an apparatus consisting of eight razor blades with spacers, approximately 2 g of leaf material was cut into 1-mm-wide strips and vacuum-infiltrated with 25 ml digestion medium for 1 min (500 mm Hg) in a 125-ml Büchner flask. The vacuum was released slowly to prevent damage to cell ultrastructure. The digestive enzyme used, macerase (Calbiochem), has been used successfully by others (2, 4, 7, 11, 13). The rest of the digestion medium differs in significant aspects from that used by previous investigators and contained the following: 0.35 M sorbitol, 0.2% methylcellulose, 10 mM cellobiose, 0.1% BSA (Sigma fraction V), 20 mM succinic acid (pH 5.7), 1 mM KNO_3, 0.5 mM KH_2PO_4, 0.5 mM $MgSO_4$, and 1 mM EDTA. Sorbitol, which was used to maintain osmotic pressure, was only slightly hypertonic with respect to the plant cells. Methylcellulose was included to prevent clumping of the separated cells. Together with cellobiose, it also protected against action of contaminating cellulases, a problem evident in a portion of the work of Outlaw *et al.* (7). BSA is a well known protector of membrane integrity in organelle isolations; we have included it for the same reason.

Incubation in the digestion medium was carried out on a rotary shaker (80 rotations/min) for 60 min. At the end of the incubation period, cell yield could be increased approximately 4-fold by gently sucking up and releasing the leaf-digestion medium mixture 10 times through a wide mouth pipette. The cells were filtered through a 100-μm nylon screen, collected by centrifugation at 100g (1 min), and washed twice with assay medium. The cell pellet (10 mg Chl/ml packed cells) was finally resuspended in assay medium to a Chl concentration approximating 50 μg/ml.

Assay medium contained 0.3 M sorbitol, 10 mM KNO_3, 0.5 mM KH_2PO_4, 2 mM EDTA, 1 mM $MgSO_4$, 1 mM $MnCl_2$, 1 mM $CaCl_2$, 25 μM Fe-Versenol (Dow Chemical Co.), and 0.5 ml/l Arnon's A-4 (1). Although higher CO_2 incorporation rates could be attained at pH 7, we selected pH 8 because the viability of our cells was maintained longer at the latter pH. Sorbitol (0.3 M) was found to be isotonic with respect to the

cells and was also found to enhance the longevity of cell viability over that of plasmolyzed cells in hypertonic medium.

Isolation of Sterile Cells. Most attempts at obtaining sterile suspensions of leaf cells from *P. somniferum* resulted in a significant loss of photosynthetic viability. Immersion of leaves in traditional plant cell tissue culture milieus (15) always caused severe losses in photosynthetic activity. Inclusion of cephaloridine (300 μg/ml) (4) had a bacteriostatic effect for up to 48 hr but was not bactericidal. While gram-positive bacteria can be easily dealt with through treatment with penicillin (500 μg/ml), antibiotics effective against gram-negative bacteria always adversely affected the isolated cells. Thus streptomycin, gentamycin, and tetracycline which all operate by inhibiting various aspects of procaryotic protein synthesis were also inhibitory to the plant cells, presumably through their effect on organelle ribosomes. Polymyxin E, which damages bacterial membranes, also caused some damage to the isolated cells, although approximately 50% of the original activity could be retained.

The most effective treatment tried, which also resulted in little or no loss of photosynthetic viability, consisted of brief immersions of the leaves (1 min) in dilute solutions of benzalkonium chloride (1:500) just prior to sectioning. While such isolated cells did not always remain free of bacteria (possibly an indication of some sporal resistance), a period of 3 to 4 days generally elapsed before contamination could be detected.

Photosynthetic $H^{14}CO_3^-$ Incorporation in Isolated Cells. Aliquots (800 μl) of the isolated cells (50 μg Chl/ml) were placed in serum-stoppered Micro-Fernbach flasks and assayed at 22 C in a Plexiglas water bath illuminated from below with fluorescent lamps (450 $\mu E \cdot m^{-2} \cdot sec^{-1}$) (3). Cells were incubated in the assay medium described (pH 8) in the presence of 6 mM $NaH^{14}CO_3$ (14.7 μCi/μmol). This level of HCO_3^- was equivalent to a CO_2 concentration of approximately 0.4% under the conditions of our assay (14). Samples were removed at 10-min intervals for up to 60 min and activity was terminated by addition of methanol (85% methanol final concentration). Incorporated $^{14}CO_2$ was determined by removing aliquots from the cell-methanol mixture, acidifying to remove $H^{14}CO_3^-$, and counting by liquid scintillation.

Photosynthetic $^{14}CO_2$ Incorporation in Whole Leaves. In order to be able to compare the metabolism of isolated cells to their counterparts in intact leaves, we have also followed CO_2 incorporation in detached whole leaves of *P. somniferum*. A leaf was detached at the base of the petiole just prior to the measurements and placed in a Plexiglas chamber (the petiole being inserted into a small reservoir of distilled H_2O). The chamber was attached to a circulating reservoir of humidified air containing 550 μl/l CO_2 (6.3 μCi/μmol). Light intensity and temperature were identical to those applied to the isolated leaf cells (450 $\mu E \cdot m^{-2} \cdot sec^{-1}$; 22 C). After 30 min, the leaf was removed and plunged immediately into liquid N_2, ground, and treated as previously described for alfalfa leaflets (10).

Analysis of Photosynthetic Products. Separation of the soluble products of photosythesis was effected by descending paper chromatography and radioautography (3). Portions (100 μl) of each cell-methanol mixture were spotted on Whatman No. 1 paper and developed in two dimensions, first with a phenol-water-acetic acid solvent treated to ensure correct solvent acidity (9), and then in the second dimension with butanol-water-propionic acid (8). The insoluble fraction (obtained from chromatogram origins) was hydrolyzed with trifluoroacetic acid for 1 hr at 100 C and an aliquot chromatographed two-dimensionally for starch glucose analysis (5).

RESULTS

Rates of CO_2 fixation varying between 110 and 175 μmol CO_2/mg Chl·hr were obtained with isolated mesophyll cells from *P. somniferum*. Of greater significance have been our successful efforts of maintaining these high rates of fixation for sustained periods of time (70–80 hr).

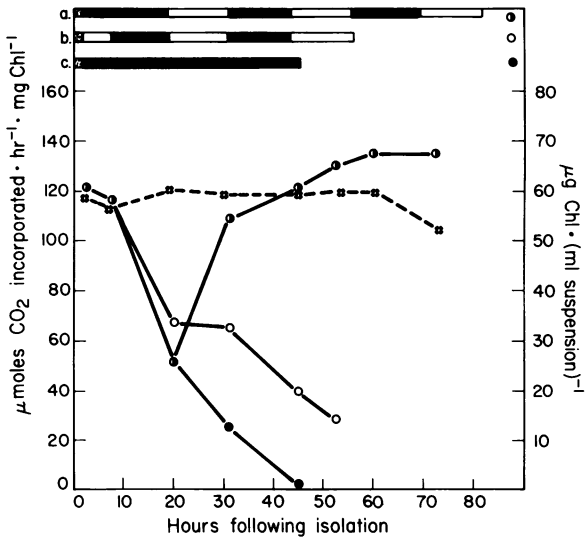

FIG. 1. Effect of varying light/dark periodicity on photosynthetic viability of isolated cells. Aliquots (800 μl) were removed from stock cell suspensions at times indicated and incubated for 30 min in light with $H^{14}CO_3^-$. a: ◑: 18-hr initial dark period followed by intervals of 12 hr light/12 hr dark; b: ○: 5-hr initial light period followed by dark/light periodicity as for a; c: ●: continuous dark period. Temperature regime: 22 C light/8 C dark. ⊠: concentration of Chl in suspended cells.

One of the most crucial parameters with respect to maintaining viability proved to be light/dark periodicity (Fig. 1). It was necessary to include a dark-adaptive period of at least 12 hr before subjecting the isolated cell suspensions to light (Fig. 1a). Including even a brief light period in the hours immediately following cell isolation was detrimental to the long term viability of the cells (Fig. 1b). Although a significant decrease in photosynthetic potential occurred during the first dark period, it was almost completely reversed within 10 hr after the introduction of light. In the absence of a light period, photosynthetic potential and cell viability continued to decline (Fig. 1c).

Coupled with light periodicity but of somewhat less importance was the effect of temperature regimes. Cells maintained at 8 C during the dark periods and at 22 C in the light exhibited a complete recovery and sustained viability while cells kept at a continuous temperature of 22 C did not recover as completely nor did they remain viable as long.

Chl concentrations remained fairly constant during most of the period of high photosynthetic rates. A decline in Chl always preceded (and thus seemed to signal) an impending decline in incorporation rate and over-all loss of cell viability. Thus, with most cell preparations at sometime just prior to 70 hr following isolation, Chl content began to decline and viability loss followed thereafter.

Microscopic Appearance of Cells. Isolated cells from *P. somniferum* were similar in appearance to those isolated from other organisms (2, 7) with the exception that cell plasmolysis was absent in our cells due to the isotonicity of the resuspension medium (Fig. 2). In addition, we found that the more highly differentiated cells seemed to respond better to our medium and isolation conditions than did less differentiated cells originating from the meristematic region of the leaf.

Integrity of the plasmalemma of the isolated cells was demonstrated by measuring leakage of organic radioactive C into the medium. Less than 2% of the fixed C could be detected in the supernatant following a 30-min cell incubation with $H^{14}CO_3^-$.

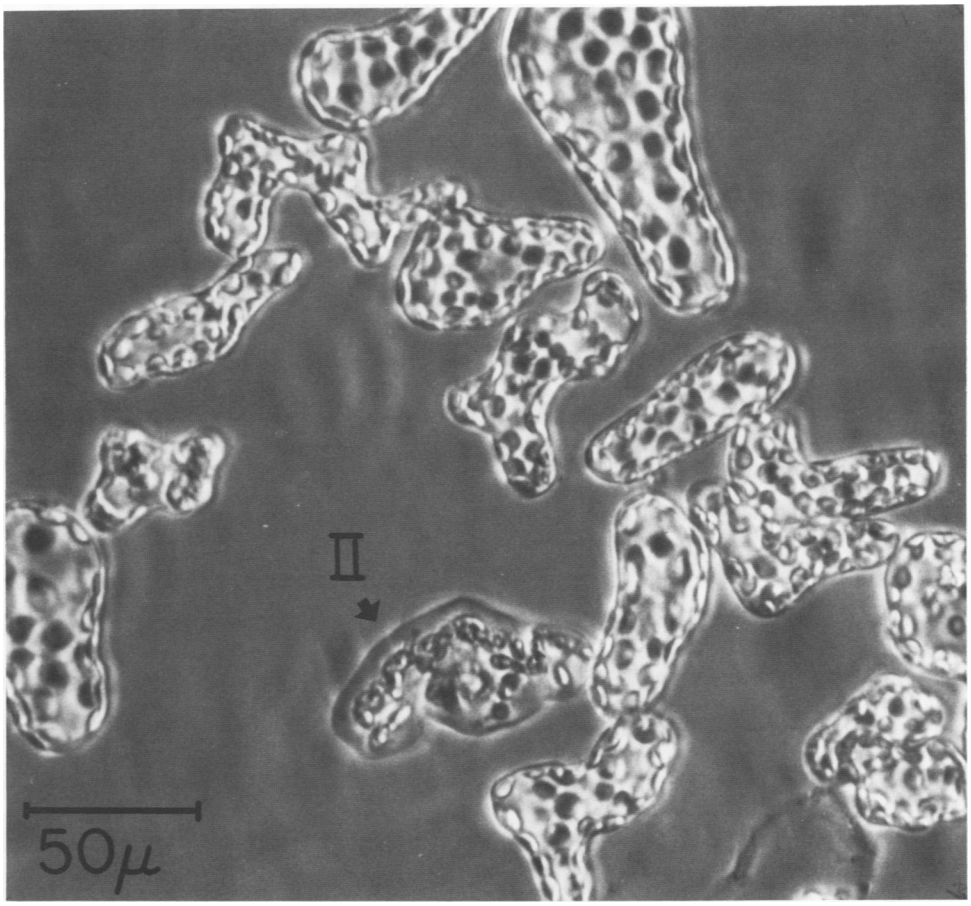

FIG. 2. Poppy cells at 30 hr following isolation. Cells were suspended in isotonic medium (0.3 M sorbitol). Most cells in this picture are class I, possessing an intact tonoplast and peripherally arranged chloroplast. At least one cell (indicated) is of the class II type, having a disrupted tonoplast.

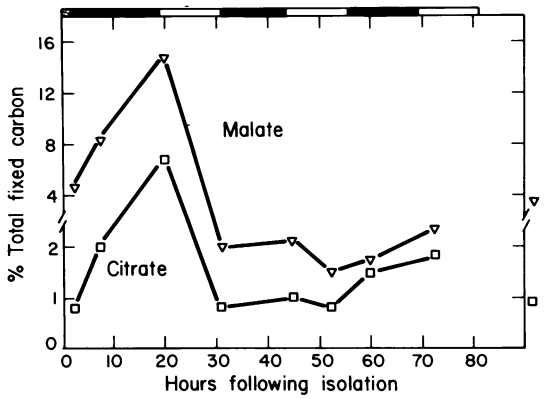

FIG. 3. Variation of flow of carbon into malate and citrate with respect to hr following cell isolation. Treatment of cells was as in Figure 1 with light/dark periodicity as for Figure 1a. Rates of incorporation into malate (▽) and citrate (□) in the intact leaf, incubated under equivalent conditions, are given in right margin of figure.

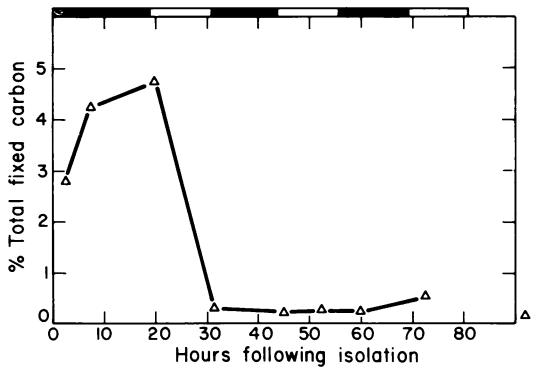

FIG. 4. Variation of the flow of C into glutamine with respect to hr following cell isolation. Treatment of cells was as in Figure 1 with light/dark periodicity as for Figure 1a. Rate of incorporation into glutamine (△) in the intact leaf, incubated under equivalent conditions, is given in right margin of figure.

Adaptive Response of Cells to Liquid Medium. In order to assess the response of the isolated cells' metabolism to the new environment, CO_2 incorporation patterns were followed by removing aliquots from the stock cell suspension at the times indicated in Figure 1a and incubating them with $H^{14}CO_3^-$ in the light for 30 min. The initial metabolic state of the isolated cells is best characterized as a "stress-response" period which lasted for approximately 20 hr. During this time a major portion of the fixed C flowed into citric acid cycle intermediates as evidenced by the high levels of citrate and malate (Fig. 3). This information, taken together with a high incorporation into glutamine also observed during this period (Fig. 4), probably indicates an interval of increased respiratory activity. In contrast, while more C was flowing into respiratory intermediates, less was being deposited in the storage saccharides, sucrose and starch (Fig. 5).

The drop in net CO_2 incorporation (Fig. 1) corresponded exactly with the stress-response period delineated by the incorporation pattern. Similarly, the abrupt reversal in incorporation

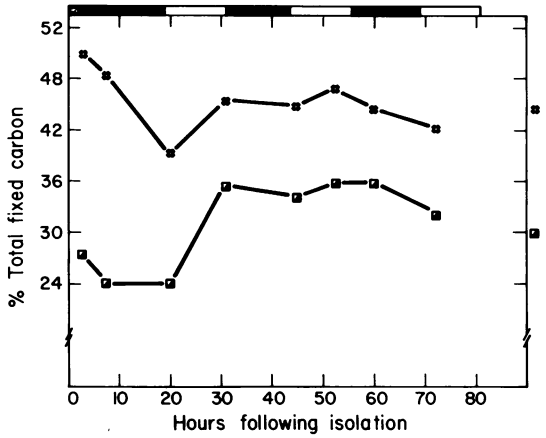

FIG. 5. Variation of the flow of C into sucrose and starch. Treatment of cells was as in Figure 1 with light/dark periodicity as for Figure 1a. Rates of incorporation into sucrose (◪) and starch ( ) in the intact leaf, incubated under equivalent conditions, are given in right margin of figure.

Table I. Comparison of CO_2 Incorporation in Isolated Cells with that in Whole Leaves of P. somniferum

Values are given as the percent of total $^{14}CO_2$ incorporation in a 30 min incubation period. Data for isolated cells were taken on fully recovered cells (45 hr after isolation). Incorporation rate of isolated cells was 125 μmoles/hr/mg Chl and in the intact leaf tissue was 180 μmoles/hr/mg Chl.

Compound	Isolated Cells	Whole Leaves
3-P-Glycerate	1.4	0.8
Hexose monophosphates	1.4	1.0
Citrate	1.0	0.9
Malate	2.2	3.9
Glycerate	1.4	1.7
Maltose	0.7	0.5
Sucrose	33.4	29.5
Aspartate	0.9	1.5
Glutamate	0.6	0.3
Glutamine	0.2	0.1
Alanine	1.3	1.8
Tyrosine	0.6	0.4
Starch	45.0	44.0

rate that occurred between 20 and 30 hr correlated closely with an equally impressive transition in the cells' metabolic state. During this "recovery" period, we found a marked decline in the levels of malate, citrate, and glutamine to values remarkably consistent with those found in whole leaves incubated under similar conditions. At the same time, flow of photosynthate into the storage saccharides increased to levels which were also consistent with values found in whole leaves.

After the cells' metabolic state had stabilized (approximately 30 hr following isolation), light/dark transitions had no further effect on the cells' viability or short term incorporation patterns. Cells from this postrecovery period exhibited an over-all distribution of labeled photosynthate which is comparable to that of the whole leaves (Table I).

DISCUSSION

By carefully adjusting cell isolation conditions and by paying particular attention to physical as well as chemical parameters in the postisolation time period, we have obtained high rates of photosynthetic CO_2 incorporation for sustained time periods in leaf-free cells. The cells remained viable for up to 80 hr before any significant decline in Chl content or CO_2 incorporation could be detected. The facts that light had a detrimental effect on cell viability when applied to freshly isolated cells and that decline in Chl always preceded loss of photosynthetic viability are indications that light absorption is of crucial importance in the viability maintaince of these isolated cells.

The nature of metabolic products observed in the interval following cell isolation is indicative of a stress-response period. The rigors of an enzymic digestion coupled with the radical change in cellular environment are most probably the causal factors of this shift in metabolism. The apparent large pools of malate and citrate observed during this period are indicative of high respiratory rates. Similar increases in glutamine during this time could be symptomatic of an elevated oxidative deamination of amino acids; *i.e.* during periods of high respiratory activity it is quite likely that amino acids are serving as part of the source of metabolic energy. Deamination would result in the release of NH_3, thus favoring the production of glutamine via glutamine synthetase (6).

A light-induced recovery of the cells' photosynthetic viability followed the stress-response period and, at the same time, the CO_2 incorporation pattern returned to that observed in whole leaves of *P. somniferum*. Even in a radically different environment, after adjusting to the stressful conditions of separation from the leaf, highly differentiated mesophyll cells continue to function as mesophyll cells, producing large quantities of carbohydrates (principally sucrose and starch) and incorporating C as they would *in folio*.

Since cells of increased longevity exhibit a metabolism which closely parallels that of the intact leaf, it follows that the effect of a potential modulator upon the isolated cells' metabolic state can be extrapolated to the intact system with a high degree of confidence. Isolated cells of sustained viability should therefore provide an excellent experimental system for the study of plant cell metabolism.

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