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Photosynthetic Studies With Leaf Cell Suspensions From Higher Plants[1]

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Abstract. A technique for obtaining intact mesophyll cell suspensions derived from higher plant leaves is described. A large number of taxonomically unrelated plants were found suitable for cell 'extraction' including several plant species from monocotyledonous group.

The absorption spectra of leaf cells in suspension differed only slightly from that of *Chlorella* cells. The higher plant cells respired and photosynthesized in aqueous solutions very much like algal cell suspensions. Only osmotically intact cells photosynthesized maximally and their activity was stimulated by 2 to several fold by the addition of bicarbonate to the medium. The isolated cells when stored at low temperature with proper aeration maintained their initial activity for a minimum of 9 hr. Cells stored at the room temperature (27 ± 2°) lost their activity rather rapidly. The isolated cells were physiologically intact as tested by their metabolic response to diverse inhibiting chemicals and growth regulating substances.

Most of our present day knowledge of *in vivo* photosynthetic processes, especially the dark fixation of CO_2 have been derived from studies employing *Chlorella* cells (1). The technical problem of obtaining enough quantities of higher plant leaf cells in suspensions has contributed to the continued use of algae in such studies as substitutes except where photosynthesis and related processes were studied with subcellular *in vitro* systems. Though in recent years attempts have been made to overcome this problem through tissue culture techniques (2, 3), successful reports with reference to the culture of mesophyll cells are not many (4, 5). Even in most of these reports, cells of angiosperms from mechanically separated callus of multicellular origin were used for culture and hence the developed cells are patently of artificial origin, environment and physiology (6). Earlier attempts to obtain cell suspensions from higher plants include mild grinding of leaf tissue for cell preparations (7) and the use of different enzymes particularly pectinases for separating the cells (8). In this we report a simple method of obtaining large quantities of isolated mesophyll cells from fresh leaves of higher plants by mild grinding followed by fractional centrifugation at low speed. In addition, some of the photosynthetic and respiratory characteristics of the cells thus obtained from higher plant leaves are presented.

Materials and Methods

The plant materials used in this study included cultivated and weed plants grown in the botanic garden under normal conditions. Although several plant species have been found useful for cell isolation only the leaves from the cultivated plants of *Dolichos lab lab* var. *Lignosis* Prain (Bean) were used extensively for photosynthetic and other metabolic studies. Ten grams of fresh leaves were chilled and ground in a smooth surfaced porcelain pestle and mortar with 40 ml of grinding media containing sucrose 400 μmoles, $MgCl_2$ 10 μmoles, tris-HCl buffer 20 μmoles pH 7.8. The homogenate was filtered through 2 layers of fine muslin cloth to separate the large debris. The filtrate was centrifuged at 200*g* for 30 sec. The supernatant was slowly decanted, discarding the pellet which essentially contained large aggregates of mesophyll cells. (Should the final yield of isolated cells be low, this pellet can further be mildly ground and further purified). The supernatant was centrifuged again at 400*g* for 30 sec and the pellets containing isolated cells were pooled. They were resuspended in large amount of chilled grinding media and centrifuged to wash away the broken particles. This procedure was repeated thrice or more using everytime fresh

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media. The final pellet was suspended in 5 to 10 ml of grinding medium and used as cell suspension for further studies. All the procedures were carried out between 0 to 4°.

Chlorella sp. was axenically grown at room temperature in a modified Cho-10 medium (9) in 250 Erlenmeyer flasks, each containing 100 ml of medium on a rotary shaker under saturating light intensities from fluorescent day-light lamps. The cells were harvested at the end of their log phase of growth and washed with 0.02 M NaCl solution before use. The absorption spectra of cell suspensions were measured using a Bausch and Lomb Spectronic-20 spectrophotometer at 5 mμ intervals between 400 and 740 mμ without correcting for light scattering and also with an opal glass plate inserted between the sample tube and the phototube as described by Shibata (10). The photosynthetic evolution of O_2 and respiration of the cell suspensions were measured manometrically in a Warburg apparatus (11). The light was provided by a bank of tungsten lamps placed under the glass bottom of the bath. All photosynthetic studies were made at saturating light intensities and at cell concentrations where the light was not rate limiting. When simultaneous respiration rates were measured, blackened Warburg vessels were employed with 0.5 ml of 20 % KOH in the central well. The photosynthetic evolution of O_2 was measured in a final reaction volume of 3 ml containing 200 μmoles of sucrose, 100 μmoles of sodium bicarbonate, 10 μmoles of $MgCl_2$, 20 μmoles of tris-HCl buffer pH 7.8 unless otherwise indicated. The bicarbonate was omitted from the reaction mixture while respiration was measured. All measurements were carried out at 24°.

The cell number in suspensions was determined by direct microscopic counting with Neubauer improved double Haemocytometer and were later standardized with absorbancy at 540 mμ for each plant investigated. The chlorophyll content of the cells was determined by the method described by Arnon (12) and the protein by Lowry method (13). The nucleic acid contents of the cells were determined spectrophotometrically by orcinol and diphenylamine reactions as described by Volkin (14). All the chemicals used in this investigation were Analar grade supplied by either Sarabhai Merck Limited, India or by British Drug Houses (India) Private, Ltd.

Results

From a random exploratory survey, a large number of plants belonging to different families were found to yield intact mesophyll cell suspensions on mild grinding including many monocots (table I). However, *Cajanus indicus* Spr., *Clitoria ternata* L., *Crotalaria verrucosa* L., *Alysicarpus monilifer* DC. (all of Papilionaceae), *Samanea Saman* Prain. (Mimoseae), *Tridax procumbens* L. (Compositae), *Ruellia prostrata* Poir. (Acanthaceae), and *Rauwolfia tetraphylla* L. (Apocyanaceae) were some of the plant species from which the isolation of intact leaf cells was found either impossible or very difficult.

On light microscopic examination, the washed cells were found to be morphologically heterogeneous but intact with varying number of chloroplasts per cell depending upon the plant species without any cellular debris or tissue contamination. *Dolichos lab lab* var. *lignosis* cells contain 9 to 11 well developed chloroplasts in each with an occasional rod-shaped ergastic material and *Phaseolus* cells contain 28 to 31 chloroplasts per cell with an occasional cell containing pinkish anthocyanin instead of chloroplasts. Other plant materials differed in the number and size of chloroplasts per cell with or without ergastic crystals. The presence of mucilage in the leaves did not interfere in the extraction of intact cells. However, in practice it was found that the extent of breakage of cells depends not only on the severity of grinding but also on the plant species chosen.

The absorption spectra measured, with and without an opal glass, of cell suspensions obtained from the leaves of different plant species did not reveal any marked difference in their absorption characteristics and were very much similar to that of *Chlorella* cells.

The leaf cells of *Dolichos lab lab* var. *Lignosis* were found to have 14 μg of DNA, 29 μg of RNA, 8600 μg of protein and 158 μg of chlorophyll per 10^7 cells. The photosynthetic and respiration rates of

Table I. *List of Plant Species That Were Found to Yield Intact Mesophyll Cell Suspensions on Mild Grinding*

Plant name	Family
Erythrina indica Lam.	Papilionaceae
E. suberosa Roxb.	"
Crotalaria laburnifolia L.	"
Abrus precatorius L.	"
Phaseolus mungo L.	"
P. radiatus L.	"
Dolichos lab lab var. *typicus* L.	"
Canavelia ensiformis D.C.	"
Leucaena glauca Benth.	Mimoseae
Ipomoea hederacea Jacq.	Convolvulaceae
I. reptans Poir.	"
Leptadenia reticulata W. & A.	Asclepaedaceae
Morinda tinctoria Roxb.	Rubiaceae
Acalypha indica L.	Euphorbiaceae
Ionidium suffruticosum Ging.	Violaceae
Turnera ulmifolia L.	Turneriaceae
Tabebuia pentaphylla Willd.	Bignoniaceae
Schleichera trijuga Wild.	Sapindaceae
Guazuma tomentosa Kunth.	Sterculiaceae
Thunbergia grandiflora Roxb.	Acanthaceae
Asparagus racemosus Willd.	Liliaceae
Musa paradisiaca Linn.	Musaceae
Canna indica Linn. var. *orientalis*	Cannaceae
Maranta arundinacea Linn.	Marantaceae
Grasses	Graminae

Table II. *Photosynthetic and Respiration Rates of Isolated Mesophyll Cells From Different Plant Species*

Plant species	Family	"Apparent photosynthesis"		Respiration
		µl O_2/mg chl•hr	*µl O_2/10^7 cells•hr*	*µl O_2/10^7 cells•hr*
Dolichos lab lab var. *lignosis*	Papilionaceae	768	143	22
Antigonon leptopus	Polygonaceae	817	94	26
Cissampelos pariera	Menispermaceae	1026	233	67
Mussaenda frondosa	Rubiaceae	1340	170	44
Thunbergia grandiflora	Acanthaceae	231	45	6
Cretaeva religiosa	Capparidaceae	927	139	42

isolated cell suspensions obtained from a few plants are given in table II. The cells of different origin have variable photosynthetic rates both on the basis of chlorophyll concentration and on the basis of cell number. Likewise, the respiration rate was also found to vary from 6 to 67 µl per 10^7 cells per hr possibly due to the age, ecology and 'intrinsic' capabilities as no control was exercised on the growth of the plants from which the cells were collected.

The photosynthetic evolution of O_2 by isolated cells was found to depend on the addition of bicarbonate in the reaction media to varying degree. Though occasionally an absolute dependance on bicarbonate was seen in the rate of O_2 evolution, there was a consistent 2 to several fold enhancement of the rate upon addition of bicarbonate. At equimolar concentration, potassium salt of bicarbonate was found to promote O_2 evolution better than the sodium salt (table III). Furthermore, for maximal photosynthesis, it was necessary to maintain the cells throughout in solutions of near isotonic concentration. The cells maintained at hypertonic conditions (0.8 M sucrose) showed only very slightly depressed rate below that of control (maintained at 0.4 M sucrose or its equivalent concentration). But the cells maintained at hypotonic conditions showed marked decrease in their rate of O_2 evolution (table IV). Isolated cells from bean leaves had a pH optimum of 7.0 for maximal photosynthetic activity (Fig. 1).

Table III. *Effect of Bicarbonate on the Rate of Photosynthetic Evolution of O_2 by Leaf Cell Suspensions of* Dolichos lab lab *var.* lignosis

Concn	None	$NaHCO_3$	$KHCO_3$
	µl O_2 evolved/10^7 cells•hr		
1.[1] 100 µmoles	5	73	...
2. 100 µmoles	6	134	...
3. 10 µmoles	24	81	...
4. 100 µmoles	32	70	...
10 µmoles	32	67	...
5. 100 µmoles	...	160	205
6. 100 µmoles	55	112	137
10 µmoles	55	102	128

[1] The numbers indicate different experiments. The indicated concentrations are in the final 3 ml volume of the reaction mixture.

Table IV. *Effect of Varying Osmotic Concentration of the Media of Cell Preparation on the Rate of Photosynthesis by Isolated Leaf Cells of* Dolichos lab lab *Var.* lignosis

Expt. No.	Osmotic substance	Concentration 0.8M	0.4M	0.2M	None
		µl O_2 evolved/10^7 cells•hr			
1.	Sucrose	65	74	...	17
2.	Sucrose	...	160	...	...
	Potassium chloride	...	...	190	...
3.	Sucrose	...	144	...	89

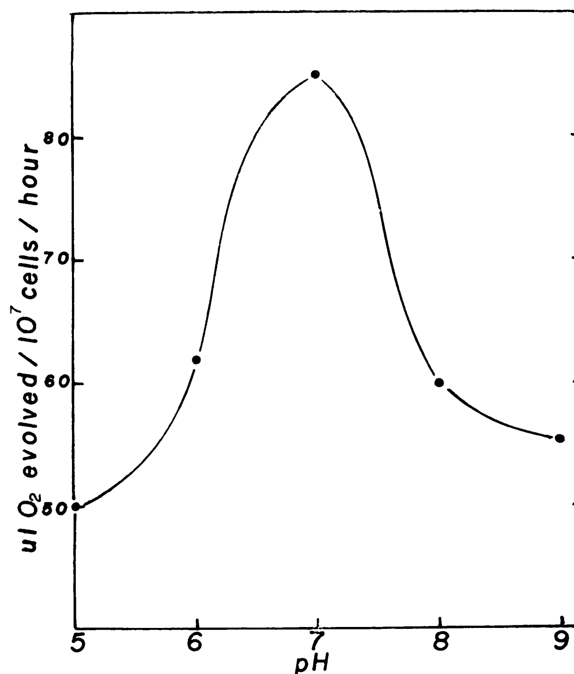

FIG. 1. Effect of pH on the photosynthetic evolution of O_2 by bean leaf cell suspensions. For this study, the cells were prepared in an unbuffered sucrose medium and aliquots were resuspended in a number of phosphate buffer medium of varying pH and the appropriate buffers were used in the reaction mixture during the measurement of O_2 evolution.

Table V. *Effect of Temperature and Aeration on the Stability of Isolated Leaf Cells With Reference to Their Photosynthetic and Respiratory Function*

Washed and resuspended cells were kept either in ice bucket or at room temperature.

Time	0 – 4° Aerated	0 – 4° Non-aerated	27 ± 2° Aerated	27 ± 2° Non-aerated
hr				
	(Photosynthesis µl $O_2/10^7$ cells·hr)			
0	143	143	143	143
3	205	157	144	51
6	177	150	117	24
9	136	120	86	13
	(Respiration µl $O_2/10^7$ cells·hr)			
0	19	19	19	19
3	19	19	44	25
6	38	30	111	55
9	52	41	142	76
	(Respiration µl $O_2/10^7$ cells·hr)[1]			
0	...	24	...	24
3	...	26	...	27
6	...	36	...	31
9	...	30	...	35

[1] The stored cells were washed at 500*g* with fresh grinding media to wash away the bacteria that would not sediment at this speed just before the measurement of respiration.

The isolated cells maintained their initial photosynthetic activity for a minimum of 9 hr, if stored in ice. The cells that were stored in low temperature with constant aeration showed a 50 % increase in their activity after 3 hr and declined slowly to their initial level thereafter. The non-aerated cells which were kept in ice followed the same pattern in their activity levels but to a lesser extent. However, the activity of the cells maintained at room temperature decayed rather dramatically if they were not aerated and lost most of their activity by 9 hr. The respiration rate was found to increase practically in logarithmic fashion. The increment in the rate was most pronounced in the samples kept at room temperature with aeration and was least in samples kept non-aerated in the cold. This pattern of increment in respiratory activity was seen, though to a lesser extent, even when the stored cells were washed at 500*g* with fresh grinding media (to wash away the bacteria that would not sediment at this speed) just before the measurement of respiration. Apparently all the increase in the respiration rate cannot be ascribed solely to bacterial contamination (table V). Storing the cells in the diffuse room light or in complete darkness did not make any difference in their photosynthetic and respiratory activity.

The effect of various chemicals on the rate of photosynthesis and respiration of isolated *Dolichos* leaf cells is summarized in the table VI. At low concentrations, dinitrophenol, potassium cyanide, mercuric chloride, sodium arsenate and sodium azide are all found to inhibit both processes to varying extent. For instance the damage to respiration by cyanide and sodium azide was less as compared to the damage to photosynthesis. Both the processes were inhibited rather drastically by hydroxylamine-HCl and mercuric chloride at low concentrations. Uniformly at low concentrations growth regulating substances like indoleacetic acid (0.57 µmole), 2,4-dichlorophenoxyacetic acid (0.45 and 0.9 µmole) and indolebutyric acid (0.98 µmole) all stimulated

Table VI. *Effect of Various Substances on the Photosynthetic and Respiration Rates of Isolated Bean Leaf Cells*

	Treatment	Apparent photosynthesis O_2 evolved	Apparent photosynthesis Control	Respiration O_2 uptake	Respiration Control
		µl/10⁷ cells·hr	%	*µl/10⁷ cells·hr*	%
1.[1]	None	130	...	28	...
	Dinitrophenol (5 µmole)	101	78	19	68
2.	None	121	...	44	...
	Hydroxylamine-HCl (10 µmole)	51	42	15	34
3.	None	161	...	113	...
	Mercuric chloride (1 µmole)	—10	00	41	36
	Sodium azide (1 µmole)	110	68	109	97
4.	None	130	...	28	...
	Potassium cyanide (1 µmole)	63	46	25	89
	Sodium arsenate (1 µmole)	48	37	...	...
5.	None	106	...	28	...
	Indoleacetate (0.57 µmole)	113	106	35	125
6.	None	130	...	28	...
	Indoleacetate (1.14 µmole)	108	83	37	132
7.	None	73	...	...	...
	Indolebutyrate (0.98 µmole)	100	136	...	...
	2,4-Dichlorophenoxy acetate (0.9 µmole)	97	133	...	...
	Glucose (11 µmole)	108	148	...	...
8.	None	81	...	...	...
	2,4-Dichlorophenoxy acetate (0.45 µmole)	113	140	...	...

[1] The numbers indicate different experiments. All additions were made just before the transfer of cells to Warburg vessels. Concentrations indicated are in the final reaction medium of 3 ml containing 2.5 to 3 × 10⁶ cells.

photosynthesis. Indoleacetic acid at 1.14 μmole concentration inhibited photosynthetic evolution of O_2 by 17 % while promoting the respiration by 32 %. Addition of 11 μmoles of glucose to the reaction medium enhanced the photosynthetic rate. However, all growth regulating substances referred to in the table VI inhibited photosynthesis of isolated cells to greater extent at higher concentrations of 20 μmoles per ml and above probably indicating their herbicidal action at higher concentrations.

Discussion

Cell suspensions have many advantages over higher plant tissue systems with reference to quick administration and removal of diverse chemicals and radioactive substances, besides avoiding the frustrating limitations of permeability barriers, diffusion gradient and wound phenomena inherent in using tissue systems. This is clearly evident in considering the past successes achieved in understanding intermediary metabolism using bacterial, yeast and algal cell suspensions. The method described here for obtaining large quantities of intact leaf cells is the routine procedure followed in many laboratories for obtaining subcellular organelles by grinding in a suitable medium and fractional centrifugation. Emphasis is placed on getting intact leaf cells rather than cell constituents. From cursory survey in the literature, it can be seen that it has been the experience of many who have chosen to work with subcellular systems, particularly chloroplasts from higher plant leaves, to come across problems of whole-cell 'contamination' in their chloroplast preparations. They have been conveniently washed out by repeated low speed centrifugation and naturally over-looked the prospect of separating intact cells probably as the interest was in other fractions. Similar separation of intact cells have also been noticed in soybean leaves by A. T. Jagendorf (personal communication). Earlier, Racusen and Aronoff (7) have also reported that soybean leaf cells could be separated by mild grinding. As the mesophyll cells in the leaves are loosely aggregated rather than compactly associated with a common middle lamella for adjoining cells as in other tissues (15, 16), it is not surprising that they separate from one another so readily.

The absorption spectra of *Dolichos* leaf and *Chlorella* cells do not differ markedly except for the minor variations in the blue end of the spectrum. These variations may probably be due to the differential light scattering by these cells as even a single chloroplast within the bean cells was found to be larger than an entire *Chlorella* cell. The tight packing of chloroplasts and their superimposing orientation might change the extent of light scattering in bean cells as compared to *Chlorella* suspension.

The isolated mesophyll cells from higher plants respired and photosynthesized in suspensions very much like algae. This was found to be true of every plant cell thus far isolated. It was found necessary to keep them in isotonic conditions for maximal activity. Their photosynthetic activity was linear with time for the tested period of 1 hr and also was found directly related to the concentration of cells in the suspension up to 3×10^7 cells per 3 ml of reaction medium. The photosynthetic rates as measured by the amount of O_2 evolution, though very much less than that of a normal leaf on the basis of chlorophyll concentration, compare favorably with earlier reported rates determined with $^{14}CO_2$ fixation by isolated leaf cells (7). The photosynthetic rates, measured at light saturation, of cells isolated from different plant species showed marked variation. This may be due to the differences in the absolute capacities of plant materials differing in type and origin. Reports of such variations in the rates of various terrestrial and aquatic plants have been published (17). Similar variations are found even in the rates of Hill reaction with isolated chloroplasts obtained from different plant species (18). The narrow pH range for optimal photosynthetic activity observed with *Dolichos* leaf cells is rather strange as compared to some algae such as *Chlorella* which are found capable of efficient photosynthesis over a wide range of pH from 4.5 to 10 (19). Perhaps the hydrogen ion concentration of cell sap which is known to vary in higher plants may determine the optimal pH for a plant species.

The response of these cell preparations to diverse chemicals and growth regulating substances are only indicative of their physiological intactness. This, coupled with their ability to maintain their activity for a minimum period of 9 hr confers a great technical advantage in using them not only in studies of different biochemical and metabolic processes directly with higher plant cells but also in studies pertaining to their responses to various external factors such as herbicidal chemicals and growth stimulants.

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