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Rapid Isolation of Mesophyll Cells from Leaves of Soybean for Photosynthetic Studies

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ABSTRACT

Mesophyll cells were rapidly isolated from soybean (*Glycine max* [L.]) leaves using a combined Macerase enzyme-stirring technique. About 50% to 70% of the leaf cells on a chlorophyll basis from 3 grams of leaves could be isolated in 15 minutes. The cells obtained by this method were capable of high rates of photosynthesis even after storage in the dark for periods of up to 9 hours. The CO_2-saturated rate of photosynthesis increased from 5 μm CO_2/mg Chl·hour at 5 C to 170 μm CO_2/mg Chl·hour at 40 C. At atmospheric CO_2 concentration, the rate varied from 5 to 55 μm CO_2/mg Chl·hour over this temperature range. The reduced temperature response of photosynthesis at low CO_2 concentration was due to an increased $Km(CO_2)$ of the cells with increasing temperature. The products of photosynthesis in the isolated cells were similar to the products of leaf photosynthesis.

Using enzyme digestion techniques, leaf mesophyll cells and protoplasts have been isolated from a number of C_3 and C_4 plant species (11-13, 19, 21). Isolated cells and protoplasts appear to be ideal material for studying photosynthesis, photorespiration, and their regulation, for unlike chloroplasts and algae, leaf mesophyll cells from C_3 plants contain the complete pathway of photorespiration as well as the photosynthetic cycle.

Present plant cell isolation procedures (6, 12, 19, 21) are not entirely satisfactory in that they require a high osmotic concentration, and 1 to 3 hr are required for high cell yields. A method is described here for the rapid isolation, in high yield, of photosynthetically active mesophyll cells from soybean in the presence of a physiological concentration of osmoticum. Photosynthesis in these cells has been characterized to determine the similarity to leaf photosynthesis and the usefulness of these preparations for studying the kinetics and biochemistry of photosynthesis and photorespiration.

MATERIALS AND METHODS

Plant Growth Conditions. Soybeans (*Glycine max*[L.] Merr. cv. "Wayne") were grown in vermiculite and subirrigated with Hoagland II solution (9) (pH 4.8) supplemented with three times the amount of iron (Sequestrene 330[2]). Plants were grown at 35 klux in a chamber with a 16-hr photoperiod (30 C light/20 C dark) for 3 weeks, and then transferred to a chamber having a 6-hr photoperiod. After 1 week of short day treatment, a mature leaf, usually from the third to fifth emergent trifoliate, was shaded with aluminum foil for 1 to 2 days. On the day of cell isolation, the leaf was illuminated for 1 hr and detached from the plant. At the time of leaf removal, leaf area was about 0.6 dm^2, specific leaf weight was 1.7 to 2 g fresh wt/dm^2, and the leaf contained 3.5 to 4 mg Chl/g fresh wt.

Cell Isolation. The detached leaf was rinsed with distilled H_2O and the midrib was removed. The leaf was placed in a Petri dish containing 20 ml of infiltration medium consisting of 20 mM MES (pH 5.8), 12.5 mM K_2SO_4, 2% (w/v) PVP-40, and 3% (w/v) Macerase (Calbiochem), and cut into strips (1 mm × 1 cm). The Macerase was purified prior to use by $(NH_4)_2SO_4$ precipitation and passage through Sephadex G-25. The leaf strips were vacuum-infiltrated for 15 sec (12), and the infiltration medium discarded. The leaf strips and a stirring bar were placed in the maceration chamber of the cell isolation apparatus (Fig. 1). The maceration chamber was constructed from Plexiglas tubing (2.5 cm long × 3.8 cm i.d. × 6 mm thick), closed at the bottom with Plexiglas sheet and covered at the top with a stainless steel wire net (420-μm mesh opening). The maceration chamber was surrounded by another Plexiglas tube (9.4 cm i.d.) through which circulated cool tap water.

To initiate cell release, 50 ml of maceration medium (0.3 M sorbitol, 20 mM MES [pH 5.8], 12.5 mM K_2SO_4, 2% [w/v] PVP-40, and 3% [w/v] Macerase) was placed in an inverted 50-ml flask (Fig. 1), which acted as a reservoir and debubbler. The medium was circulated through the maceration chamber at the rate of 10 ml/min by a tubing pump. The leaf strips were agitated with a magnetic stirrer placed under the maceration chamber assembly (Fig. 1). As individual cells were released, they were carried by the solution through the wire net, which retained the leaf strips, and collected on a 4.8-cm nylon net (20-μm mesh opening, Nitex) housed in a filter unit (Swinnex-47, Millipore). After 15 min, circulation was stopped and the cells were removed from the nylon net with a gentle stream of wash medium consisting of 50 mM tris-Cl (pH 7.8), 0.2 M sorbitol, 5 mM KNO_3, 2 mM $Ca(NO_3)_2$, and 1 mM $MgCl_2$. Cells were centrifuged at low speed in a clinical centrifuge, washed a second time, and then resuspended in wash solution plus 5 mM DTT.[3] The cell preparation contained mostly separate mesophyll cells, with little contamination by chloroplasts or

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[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

[3] Abbreviation: DTT:dithiothreitol.

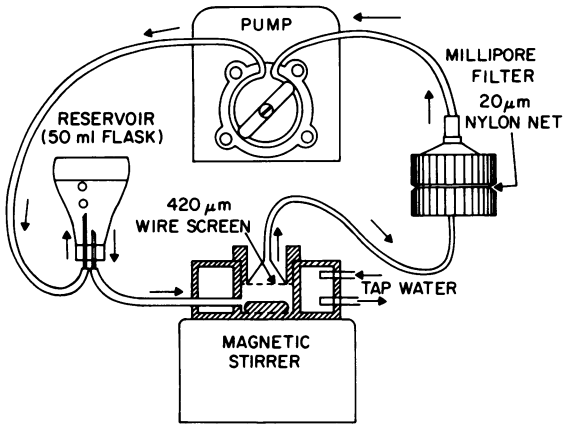

FIG. 1. Schematic diagram of the cell isolation apparatus. Additional details in text.

epidermal cells. Phase microscopy showed that cells were bright green in color, indicating class I type cells (12), and were not significantly plasmolyzed. About 90% of the cells contained intact plasmalemma as evidenced by their failure to be stained by Evan's blue (13). Prior to assay, the cells were illuminated for 30 to 60 min in a shaker bath at 12 klux and 25 C.

Photosynthesis Assay. Cell photosynthesis was conducted in 20-ml glass scintillation vials fitted with serum caps. The assay medium, adapted from Rehfeld and Jensen (19), contained 50 mM tris-Cl (pH 7.8), 0.2 M sorbitol, 5 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $MgCl_2$, $KH^{14}CO_3$ as indicated (1 μCi), and cells. Buffers were prepared under N_2 and stored CO_2-free. Before adding buffer, cells, and bicarbonate, the assay solutions were shaken and flushed for 15 min with N_2/O_2 gas mixtures at pH 5 to remove dissolved CO_2 and obtain the appropriate O_2 concentration in the vials. Reactions were initiated by addition of cells to the vials in the light. Cells were added by syringe (No. 19 needle) through the serum cap. Assay mixtures were shaken at a rate sufficient to keep cells suspended. Assays were terminated by the addition of 0.1 ml of 2 N HCl or 6 N acetic acid. After drying the samples at 60 C, 0.5 ml of water was added to dissolve the residue, followed by addition of scintillation solvent (1). Incorporated ^{14}C was determined by scintillation spectroscopy.

Chl was extracted with methanol and concentration calculated from *A* at 650 and 665 nm (16).

Conversion from HCO_3^- concentration to CO_2 concentration was made using the ionization constant of Gibbons and Edsall (8) and changes in the ionization constant with temperature were made according to Hodgman *et al.* (10).

Separation of Photosynthetic Products. The products of photosynthesis were separated into insoluble, neutral, basic, acid-1 and acid-2 fractions by a procedure similar to that of Atkins and Canvin (2). Acetic acid-terminated samples were combined and centrifuged to remove the insoluble material. The washed insoluble fraction was dried and chemically digested (17) or enzymically digested for 12 hr at 37 C with amyloglucosidase (Sigma).

The soluble extract was passed through a 3-ml column of Dowex 50-X8, H^+ form, to remove basic compounds, and then through Dowex 1-X8, acetate form, to remove acidic compounds. Neutral compounds were washed through both columns. Basic compounds were eluted from the Dowex 50 with 10 ml of 2 N NH_4OH. Acid-1 compounds were eluted from the Dowex 1 with 10 ml of 4 N acetic acid and acid-2 compounds were eluted with 10 ml of 2 N HCl. Aliquots of the various fractions were lyophilized, and subjected to one-dimensional, descending chromatography on sheets of Whatman No. 1 chromatography paper. Amino acids were separated in 1-butanol-acetone-water-dimethylamine (20:20:10:3) (7); organic acids in acid-1 and acid-2 in 1-pentanol-5 N formic acid (1:1) (4); phosphorylated compounds on oxalic acid-washed paper in 1-butanol-propionic acid-water (10:7:3); and 0.1% (w/v) EDTA and sugars using 1-butanol-acetic acid-water (3:1:1) (20). Authentic standards were co-chromatographed and located by color development. Amino acids were detected by the ninhydrin reaction, organic acids by bromphenol blue (4), phosphorylated compounds with ammonium molybdate (3), and sugars with aniline-diphenylamine-phosphoric acid (5). Radioactive areas on the chromatograms were located with a radiochromatogram scanner, cut out, and radioactivity determined.

RESULTS AND DISCUSSION

Cell Isolation. The procedure developed for the isolation of cells from soybean leaves was adapted from that developed for cotton (19). Stirring of leaf sections in the maceration medium was found necessary for the release of cells. Poor cell yields and activity were obtained by shaking the leaf sections for 1 to 2 hr in maceration medium. Removal of cells from the maceration medium by circulation and collection of cells on a nylon net removed from the stirring action greatly increased the rate at which cells were released, total yield of cells, and photosynthesis rate. This system was found to be much more efficient for isolating cells from soybean than the concentric cup apparatus developed for cotton (19). A short day treatment of leaves prior to isolation (19) was found to be necessary for high rates of cell photosynthesis. The highest yield of cells and the highest photosynthesis rates were obtained from young, just fully expanded leaves, as was found for tobacco (6).

The method described here for the isolation of leaf cells from soybean routinely yielded from 50 to 70% of mesophyll cells from up to 3 g of leaves in 15 min. This is considerable improvement over previous methods, which generally require from 1.5 to 3 hr (6, 12, 19, 21). In contrast to procedures for other plants in which 0.6 to 0.8 M osmoticum was used (6, 12, 19, 21), maximum yield and activity were obtained with 0.3 M sorbitol in the maceration medium.

General Photosynthetic Characteristics. Rates of photosynthesis were highest when cells were assayed in 0 to 0.3 M sorbitol, and were inhibited by sorbitol concentrations greater than 0.3 M (Fig. 2). Sucrose could replace sorbitol with little change in activity (Fig. 2). Takebe *et al.* (21) found 0.6 M sorbitol to be essential for the release of mesophyll cells from

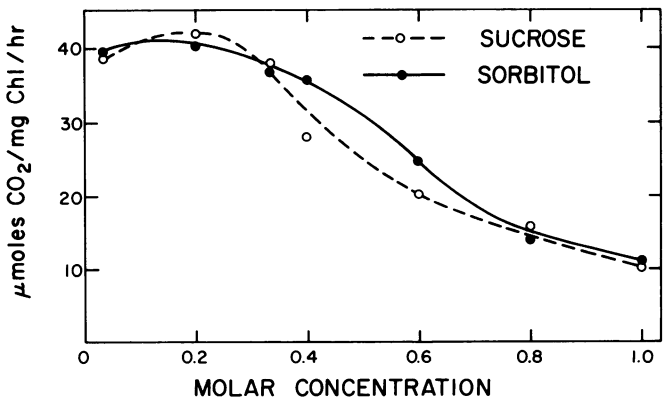

FIG. 2. Effect of osmotic concentration on photosynthesis. Cells were isolated in standard medium. Cells were incubated for 30 min in the assay medium with varying concentrations of sorbitol or sucrose. Prior to assay, vials were flushed in the light for 5 min with 21% O_2 and sealed. Reactions were initiated with 5 mM bicarbonate and terminated after 10 min with 0.2 N HCl.

tobacco. Subsequent investigations with plant cells have also used high sorbitol concentrations during maceration and photosynthetic measurements even though severe plasmolysis occurs (12). It was not determined whether the low sorbitol requirement for soybean cell isolation and activity was a true species difference or whether it was a function of the apparatus used for cell isolation (Fig. 1).

On storage in the dark at 25 C, photosynthetic activity increased over the first 2 hr after isolation and then remained relatively constant or declined, depending on the storage conditions (Fig. 3). Greatest stability occurred at pH 7.8 in the presence of 5 mM DTT. The cells were less stable when stored at pH 5.8 in the absence of DTT. Other experiments, not shown, demonstrated that 70% of the maximum activity was still present after 9 hr of storage in the dark (Fig. 3).

Light saturation of photosynthesis in 10 mM bicarbonate occurred at about 6 klux. This saturation illuminance is similar to that observed for cells isolated from tobacco (6) and cotton (19). Assays were routinely conducted at 12 klux.

Kinetics of CO_2 Fixation. The CO_2 dependence of photosynthesis was determined by assaying $^{14}CO_2$ fixation for 10 min in the presence of varying concentrations of bicarbonate. A plot of bicarbonate concentration *versus* photosynthesis in 2% O_2, 25 C, pH 7.8 and saturating light intensity (12 klux) is seen in Figure 4. Photosynthesis rate increased with increasing bicarbonate concentration and eventually reached a saturated rate. A double reciprocal plot of bicarbonate concentration *versus* photosynthesis (Fig. 4, inset) was linear at low bicarbonate concentrations, but deviated from linearity at high bicarbonate concentration. This deviation has previously been shown with spinach chloroplasts (15). This deviation of photosynthesis from enzyme kinetics with respect to CO_2 may indicate that CO_2 fixation is limited by the regeneration of RuDP by photosynthetic electron transport (15). Thus the extrapolated V_{max} for cell photosynthesis, 111 μmol CO_2/mg Chl·hr, is greater than the CO_2-saturated rate, 85 μmol CO_2/mg Chl·hr. The $Km(CO_2)$ for cell photosynthesis was about 20 μM CO_2 at pH 7.8 and 25 C.

Temperature Dependence of Photosynthesis. The effect of temperature on cell photosynthesis is shown in Figure 5. The $Km(CO_2)$ and V_{max} for cell photosynthesis were measured at each temperature from double reciprocal plots of photosynthesis at four bicarbonate concentrations (0.25, 0.5, 1, and 10 mM). The extrapolated V_{max} (Fig. 5, curve A) increased from 8 μmol CO_2/mg Chl·hr at 5 C to 295 μmol CO_2/mg Chl·hr at 40 C. The CO_2-saturated rate (10 mM bicarbonate) (Fig. 5, curve B) was considerably less than the V_{max} but also showed a strong temperature dependence between 5 and 40 C, increasing from 5 to 170 μmol CO_2/mg Chl·hr.

At low CO_2 concentrations, the stimulation of photosynthesis by increasing temperature was much less dramatic. At

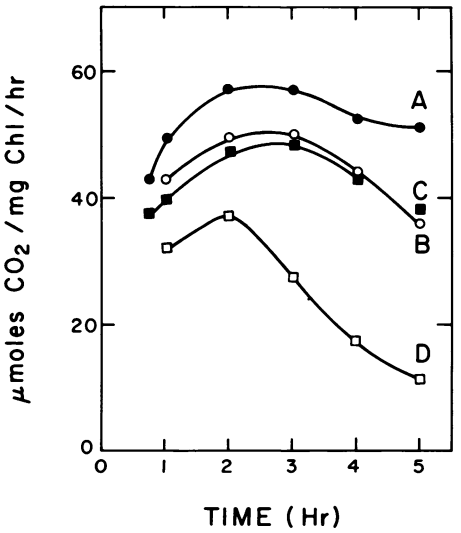

FIG. 3. Effect of pH and DTT on stability of photosynthetic activity. Cells were resuspended in photosynthesis medium without buffer (pH 7.8), and incubated in the dark in photosynthesis medium plus (A) 50 mM tris-Cl (pH 7.8), and 5 mM DTT; (B) 50 mM MES (pH 5.8), and 5 mM DTT; (C) 50 mM tris-Cl (pH 7.8); (D) 50 mM MES (pH 5.8). After the specified interval of time, vials were flushed with 2% O_2 for 5 min in the light and sealed. Reactions were initiated with 10 mM bicarbonate and terminated after 10 min with 0.2 N HCl.

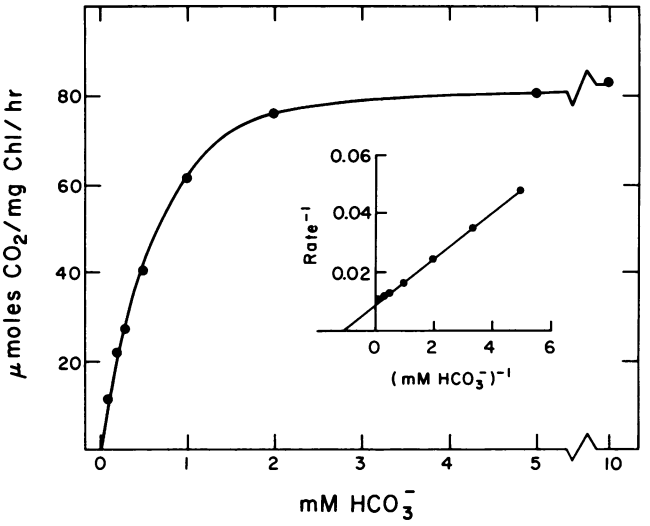

FIG. 4. Bicarbonate dependence of photosynthesis in soybean cells. Assays were initiated with cells in the presence of the indicated bicarbonate concentrations after flushing with 2% O_2. Reactions were terminated after 10 min with 0.2 N HCl. Inset: double reciprocal plot of cell photosynthesis *versus* bicarbonate concentration.

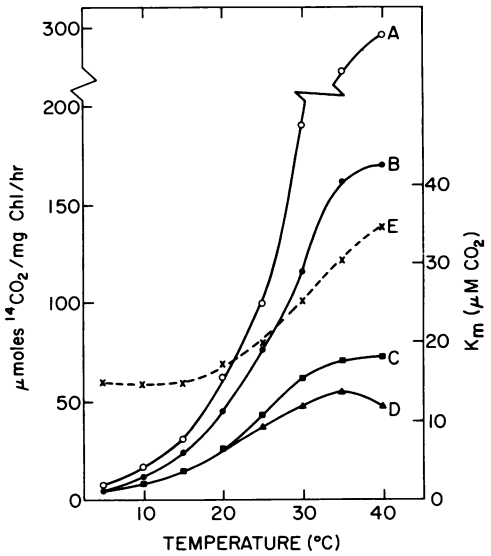

FIG. 5. Photosynthetic rates and $Km(CO_2)$ in soybean cells as a function of temperature. After flushing reaction vessels with 2% O_2 in the light at the indicated temperature for 5 min, bicarbonate was added (B: ●——●, 10 mM; C: ■——■, 0.5 mM). Reactions were initiated by the addition of cells and terminated after 10 min by the addition of 0.2 *N* HCl. V_{max} (A: O——O) and $Km(CO_2)$ (E: ×- -×) were calculated from double reciprocal plots. Rate of photosynthesis at 300 μl/l CO_2 (D: ▲——▲) was calculated as explained in the text.

0.5 mM bicarbonate, raising the temperature from 5 to 40 C increased the rate from 4 to 72 μmol CO_2/mg Chl·hr (Fig. 5, curve C). The difference between the temperature response of cells at high and low CO_2 concentrations is apparently due to a reduced affinity of the cells for CO_2 as the temperature is increased. The $Km(CO_2)$ was found to increase from 17 μM CO_2 at 5 C to 35 μM CO_2 at 40 C (Fig. 5, curve E). From the $Km(CO_2)$ and V_{max} values and knowing the solubility of CO_2 in water at each temperature (10), the photosynthesis rate for cells in an atmosphere of 300 μl/l CO_2 was calculated (Fig. 5, curve D). This curve would be analogous to the intact leaf situation and shows a similarity to the temperature response of the intact leaf (14). The rate varied from 5 μmol CO_2/mg Chl·hr at 5 C to a maximum of 55 μmol CO_2/mg Chl·hr at 35 C and then decreased at 40 C. This latter rate, when expressed on a leaf area basis, is about 20 mg $CO_2/dm^2 \cdot hr$.

The velocity of an enzyme-catalyzed reaction is a function of the V_{max}, Km, and substrate concentration (S), $v = V_{max}\ S/(Km + S)$. Thus, depending on the temperature response of the two enzyme variables, V_{max} and $Km(CO_2)$ and the concentration of CO_2, which in the leaf situation is a function of the solubility of CO_2 in water, the rate of photosynthesis will increase, decrease, or be unchanged by temperature changes. During cell photosynthesis, the V_{max} increased between 35 and 40 C, but the increase in $Km(CO_2)$ and the reduced solubility of CO_2 in water more than offset the increase in V_{max} so the rate of CO_2 fixation at 300 μl/l CO_2 decreased. The affinity of soybean leaves for CO_2 in photosynthesis is also reduced at higher temperature and has been attributed to a temperature-induced increase in the $Km(CO_2)$ of RuDP carboxylase (14). The temperature optimum at 300 μl/l CO_2 in leaves is likely determined in the same manner as described above for cells.

Products of Photosynthesis. The distribution of label in products of soybean cell photosynthesis at atmospheric concentrations of CO_2 and O_2, pH 7.8, was similar to that found in whole leaf photosynthesis (2, 18). About 45% of the label was incorporated into the insoluble fraction as starch, since enzymic digestion of this fraction yielded only glucose. The neutral fraction contained about 20% of the label and was composed primarily of sucrose. Activity in the basic fraction (about 14% of total) was found predominantly in glycine and serine. The acid-1 and acid-2 fractions contained organic acids and phosphorylated compounds which comprised about 10% and 11% of the total label, respectively.

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