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Metabolism of Separated Leaf Cells

I. PREPARATION OF PHOTOSYNTHETICALLY ACTIVE CELLS FROM TOBACCO[1]

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ABSTRACT

Suspensions of mesophyll cells, prepared from tobacco leaves by treatment with pectinase, fixed CO_2 by photosynthesis. The products of carbon assimilation were similar for both cells and intact tissue. The cells sustained a constant fixation rate for 20 to 25 hours. For optimal CO_2 fixation, enzymatic maceration of the tissue was accomplished in 0.8 M sorbitol, but photosynthesis was optimal in 0.6 M sorbitol at pH 7 to 7.5. A hypertonic environment during maceration, which results in cell plasmolysis, is essential to maintain intact plasmalemmas and hence photosynthetically active cells. For sustained CO_2 fixation, light intensities below 500 foot-candles were required. Higher light intensities (to 1000 foot-candles) gave high initial rates of CO_2 fixation, but the cells bleached and were inactive on prolonged incubation. At pH 7.0 the bicarbonate concentration at maximal velocity of CO_2 fixation was about 1.5 mM and the apparent Km for bicarbonate was 0.2 mM.

For metabolic studies with photosynthetic tissues of higher plants, it would be convenient to have separated cells in liquid suspension so that they could be handled much like unicellular algae. This would facilitate taking uniform samples by pipette and would make it much simpler to present all of the cells with equivalent quantities of added metabolites and to remove them again. The techniques which have been developed for the separation and culturing of photosynthetically active cells of seed plants in order to obviate these difficulties are quite arduous (6, 12). Recently, however, Takebe *et al.* (10) described a procedure for the preparation of *large quantities* of tobacco mesophyll cells in which they macerated leaf tissue with pectinase in a hypertonic medium. These cells could synthesize tobacco mosaic virus (10). Moreover, when treated with cellulase they yielded protoplasts capable of regenerating their cell walls (11); hence they must have been capable of a wide variety of biosynthetic processes. In this paper we report on an improved method of preparing separated tobacco leaf cells, based on procedures of Takebe *et al.* (10) and Zaitlin (14). These isolated cells are capable of photosynthetic $^{14}CO_2$ fixation and assimilation of the ^{14}C into products similar to those assimilated by intact leaf tissue. We have utilized the rate of CO_2 fixation of the cells as a guide to optimizing conditions of pH, light intensity, and composition of the medium. In an accompanying paper we report the results of studies on the uptake of amino acids and nucleic acid precursors by such cell preparations and incorporation of these precursors into proteins and nucleic acids (1).

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METHODS AND MATERIALS

Plant Material. *Nicotiana tabacum* var. Turkish Samsun plants, 15 to 20 cm tall in 6-inch pots were grown in a greenhouse. Fully expanded leaves (about 10–15 cm) from relatively young plants gave the most consistent CO_2 fixation rates. Plants were often held for as long as a week in a temperature-controlled growth room (at 23 C during a 16-hr, 450 ft-c light period and 20 C during an 8-hr dark period) before use, apparently without affecting fixation rates of the cell preparations. Leaves were normally harvested in the morning.

Source of Chemicals. Macerozyme, a crude polygalacturonase preparation was obtained from Kanematsu-Goshu, Ltd., Tokyo, Japan; as a note of caution, one lot of the enzyme received was almost completely inactive. Potassium dextran sulfate (molecular weight of source dextran 560, sulfur content 17.3%) came from the Meito Sangyo Co., Ltd., Minatoku, Tokyo; cephaloridine (Loridine) from Eli Lilly and Co., Indianapolis; and Rimocidin from Charles Pfizer and Co., Groton, Connecticut. HEPES and MES were obtained from Sigma Chemical Co., St. Louis, Missouri.

Media. Maceration medium contained 0.5% Macerozyme; 0.3% potassium dextran sulfate; 0.8 M sorbitol; and a salt solution containing 1 mM KNO_3, 0.2 mM KH_2PO_4, 0.1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 1 μM KI, and 0.01 μM $CuSO_4$. It was adjusted to pH 5.8 with HCl before use. Medium was always freshly prepared for each experiment, although the salts and sorbitol were taken from boiled, stored concentrated stock solutions. Wash medium contained 0.8 M sorbitol, the salt solution of the maceration medium plus 1 mM $CaCl_2$, 300 μg/ml cephaloridine, 10 μg/ml Rimocidin, 1 μg/ml benzyladenine, and 1 μg/ml 2,4-D. Photosynthetic incubation medium was identical to the wash medium except that it contained 0.6 M sorbitol and was buffered with 0.05 M HEPES adjusted to pH 7.0 with KOH. It also contained 7.5 mM $NaH^{14}CO_3$ (0.4 μc/μmole).

Preparation of Cell Suspensions. Leaves were rinsed with distilled water and blotted, the midribs were removed, and the laminae were cut into strips 1 to 2 mm × 20 to 30 mm. One gram of tissue strips was infiltrated while bathed in maceration medium, by forming and releasing a vacuum three times with a water aspirator in a 50-ml Buchner flask. The macerating

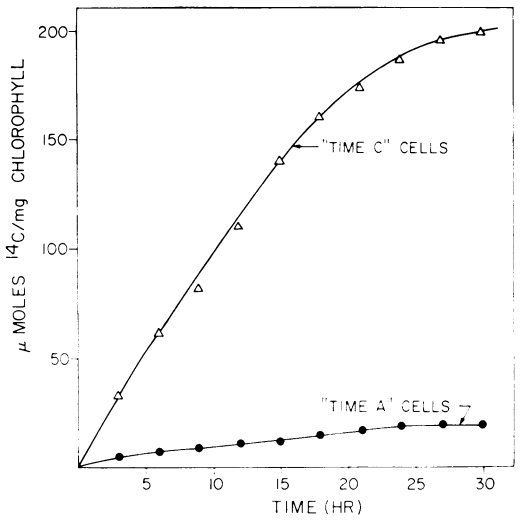

FIG. 1. Time course of photosynthetic CO_2 fixation by separated tobacco leaf cells. Time A cells, released during the first 30 min of maceration, fixed CO_2 initially at 1.5 μmoles/mg chlorophyll per hr. Time C cells, released between 60 and 90 min of maceration, fixed CO_2 initially at 11 μmoles/mg chlorophyll per hr.

medium was poured off, and the tissue was transferred to a 28 × 100 mm Pyrex centrifuge tube containing 20 ml of fresh medium and shaken at room temperature on a reciprocating shaker making about 100 strokes per min. Cells released from the leaf strips were harvested at intervals of 30 min by decantation and filtration through two layers of rinsed cheesecloth into centrifuge tubes, the maceration medium being replaced with 20 ml of fresh medium after each harvest. After three lots of cells were collected, the remaining undigested leaf tissue was discarded, and cells from each harvest were centrifuged at room temperature at 80*g* for 1 min in a swinging bucket rotor. (Normally only cells from the third harvest were used; see "Results".) The supernatant fraction was removed by suction, and the cells were washed by centrifuging as above in 30 ml of wash medium.

CO_2 Fixation. Cells containing a total of 20 to 40 μg of chlorophyll were suspended in 2 ml of incubation medium in 9 × 75 mm test tubes stoppered with a serum cap, and each treatment was run in duplicate. The tubes were placed in a water bath at 25 C and illuminated from below with an intensity of 400 ft-c from fluorescent lamps. To start photosynthetic CO_2 fixation, 50 μl of 0.3 M $NaH^{14}CO_3$ were added through the serum cap with a gas-tight syringe. To determine the amount of CO_2 fixed, 100-μl samples were removed by syringe and were analyzed for radioactivity, after acidification with 10 μl of formic acid, by drying aliquots on lens paper for planchet counting.

The chlorophyll content was determined in 80% acetone-H_2O by the method of Vernon (13). The various ^{14}C-labeled products were determined by two-dimensional paper chromatography and radioautography (4).

RESULTS

Conditions for Preparation of Photosynthetically Active Cells. Initial studies with cells prepared after Takebe *et al.* (10) showed that they fixed CO_2 in the light, but not in the dark. We found, however, that the fixation rate was extremely variable from day to day and that some of our preparations were almost completely inactive. Several factors were instrumental in determining whether a given preparation was photosynthetically active; the most important was to avoid utilizing the first cells which were separated from the tissue strips during the maceration process. The cells released during the first 30 min of maceration ("time A" cells) had little or no CO_2-fixing activity, whereas those released between 30 and 60 min ("time B" cells) were more active. Cells from the third harvest ("time C" cells), released between 60 and 90 min of shaking, were always more active than either time B or time A cells although their activity varied from experiment to experiment. The time course of CO_2 fixation from time A and time C cells prepared from the same leaf tissue is shown in Figure 1. The photosynthetic activity of the cell preparations was also dependent on the amount of tissue macerated in a given volume of medium, on the plant from which the leaf tissue was taken and the age of the leaf, and possibly on the environment during plant growth. One gram of tissue macerated in 20 ml of medium yielded cells with good activity. Although we have not determined the exact conditions under which plants should be grown in order to produce cell suspensions of high photosynthetic activity, it has been our general experience that young plants with only two to four fully expanded leaves are more suitable than older plants.

Takebe *et al.* (10) reported that low molecular weight potassium dextran sulfate was helpful during maceration in order to produce cells which were active in virus multiplication. Our studies indicate that potassium dextran sulfate is not obligatory for photosynthetically active cells (Table I) as it may be partially replaced by K_2SO_4, Na_2SO_4, or KCl. However, the presence of potassium dextran sulfate during maceration does result in more active cells, and we have routinely included it in our maceration medium. It has no effect on CO_2 fixation of cells when present during incubation.

Microscopic Appearance of Separated Cells. Maceration of leaves yielded two distinct kinds of cells as seen by phase contrast microscopy. One cell type (type I, Fig. 2) had yellow-green, highly refractile chloroplasts, and these cells were in a highly plasmolyzed condition, forming small protoplasts inside much larger cell walls. The integrity of the plasmalemma of these cells was demonstrated by the fact that, when transferred to a hypotonic medium, the protoplasts swelled rapidly. The chloroplasts of the other type of cells (type II, Fig. 2) were a darker green, and under high magnification prominent starch grains and grana could be seen. The protoplasts of these

Table I. *Effect of Various Salts in Maceration Medium and Potassium in Incubation Medium on Photosynthetic Activity of Tobacco Leaf Cells*

Tobacco leaves were macerated in 0.8 M sorbitol, 0.5% Macerozyme plus the salts as given below. The incubation medium was similar to that in "Materials or Methods," except that the buffer was neutralized with either KOH or NaOH so that the "high K^+" medium had 12 mM K^+ and no Na^+, the "low K^+" medium 1.2 mM K^+, 11 mM Na^+.

Addition to Maceration Medium	Composition of Incubation Medium			
	High K^+		Low K^+	
	3 hr	21 hr	3 hr	21 hr
	μmoles CO_2 fixed/mg chlorophyll			
None	19.4	25.6	16.9	22.5
0.3% K^+ dextran sulfate[1]	58.0	115.8	45.3	86.3
12.5 mM K_2SO_4	40.3	104.5	39.8	57.7
12.5 mM Na_2SO_4	45.7	85.9	31.3	53.1
25 mM KCl	25.0	42.4	25.4	36.7

[1] Gives 16.7 mM K^+ calculated from analytical data of manufacturer.

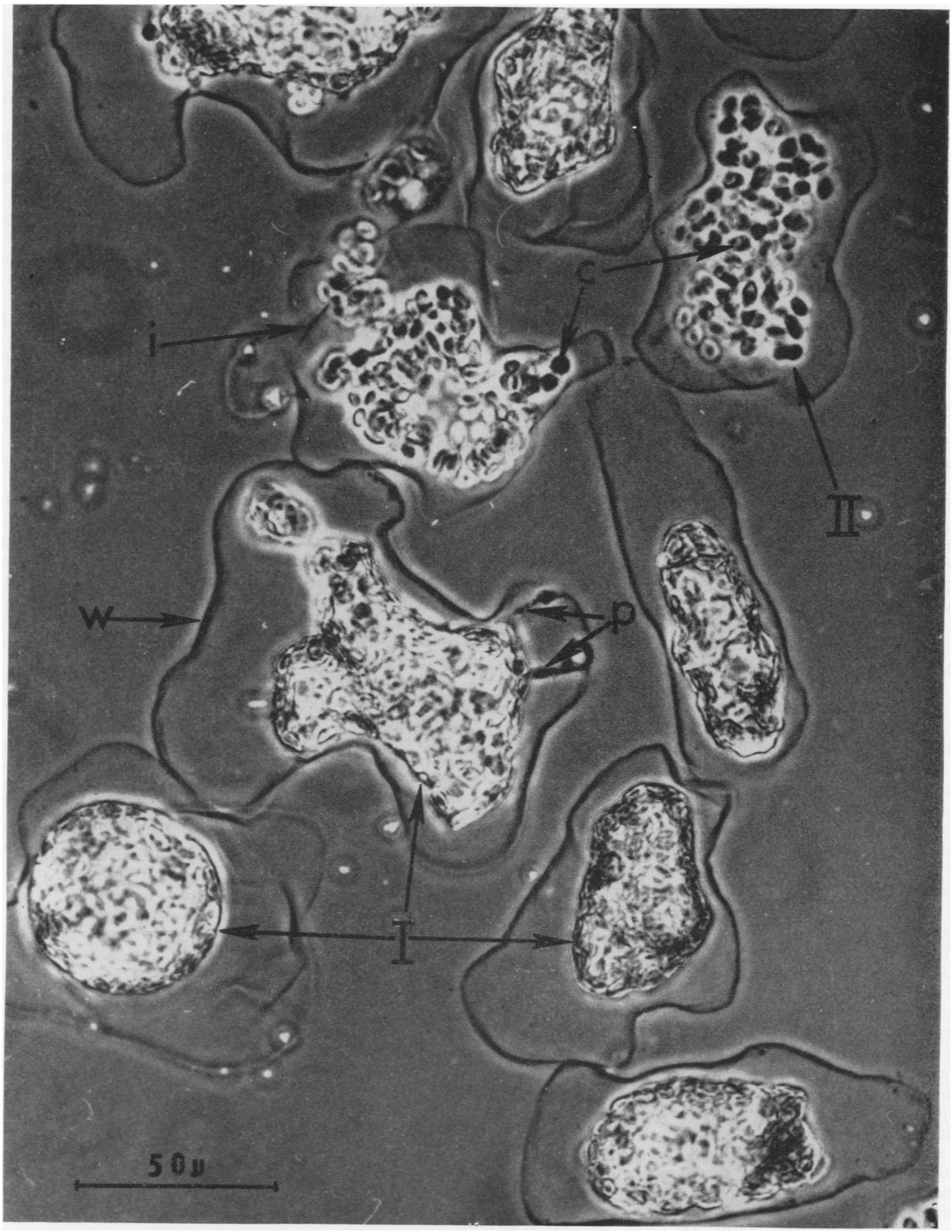

FIG. 2. Freshly prepared, separated tobacco mesophyll cells as seen by phase contrast microscopy. Cells were suspended in the wash medium which contained 0.8 M sorbitol. I: Photosynthetically active type I cells; II, inactive type II cells; i: cell intermediate between type I and type II; w: cell walls; c: chloroplasts; p: plasmadesmatal connections still attached to both cell wall and protoplast. (The depth of focus of these cells prevents all structures from being viewed satisfactorily in the same micrograph. Protoplasts of type I cells were always highly plasmolyzed and appeared stringent. Focusing on the periphery of these protoplasts showed the adjoining yellow green chloroplasts to be orientated in profile. Protoplasts of type II cells were more relaxed, and the darker green chloroplasts were rarely seen in profile. Chloroplasts of cells intermediate between those of type I and type II were similar to those of type I cells, but the protoplasts were much more relaxed (i); these cells converted to type II cells on incubation.)

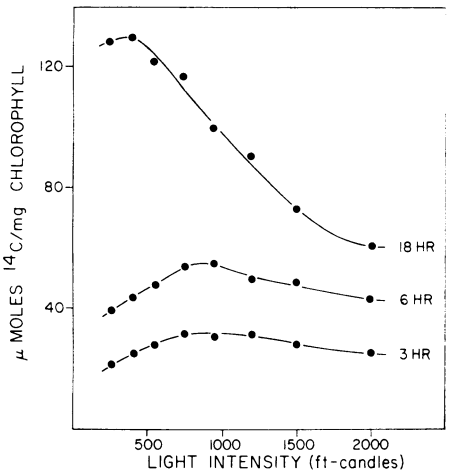

FIG. 3. Effect of light intensity and total time of illumination on photosynthetic CO_2 fixation. Each tube contained 2.0 ml of incubation medium with cells having 30 μg of chlorophyll. Light was provided by a bank of cool white fluorescent lamps. Fixation measurements were made at 3, 6 and 18 hr.

cells did not appear to be constricted, and we conclude that the plasmalemmas may have been damaged during maceration. A small but variable proportion of cells were of an intermediate type (type i, Fig. 2) containing refractile chloroplasts but apparently devoid of an intact restricting plasmalemma. These cells eventually converted to type II cells.

The majority of cells in the photosynthetically active time C cell preparations were type I, whereas those in the inactive time A preparations were type II. The proportion of the different cell types in time B preparations was intermediate between those of time A and time C preparations. In addition, each of the preparations contained small numbers of type i cells and a few epidermal and vascular tissue cells. The photosynthetic activity of tobacco leaf cell preparations was dependent on the presence of type I cells. Preparations with a very high proportion of type II cells (some time A preparations contained nearly all type II cells) failed to fix significant amounts of CO_2 (Fig. 1). Type II cells were probably the result of injuries sustained by the outermost cells of the leaf strips when the leaf was cut. However, there was nothing injurious secreted into the medium by the damaged cells as the macerating medium, from which photosynthetically inactive time A cells and cell fragments had been removed by centrifugation, was as effective as fresh medium in releasing active time C cells.

CO_2 Fixation by Cell Preparations. A time course of CO_2 fixation by time C cells is presented in Figure 1, showing an initial rate of 11 μmoles of CO_2 fixed per mg of chlorophyll per hr, slowing down to about 2 μmoles/mg chlorophyll·hr by 30 hr. In other experiments sustained rates as high as 53 μmoles/mg chlorophyll·hr were observed over the first several hours of incubation.

For maximal photosynthetic activity, tobacco leaf cells were best suspended in 0.8 M sorbitol during maceration and in 0.6 M sorbitol during incubation for photosynthesis. When the cells were transferred from the maceration medium to the incubation medium, they swelled slightly. However, the chloroplast structure appeared similar to that in cells of intact leaves, and after a few hours in this medium cytoplasmic streaming could be observed for at least 48 hr of incubation.

The optimal pH for CO_2 fixation was between 7.0 and 7.5. For sustained CO_2 fixation, light intensities below 500 ft-c were required (Fig. 3). Stronger illumination up to 1000 ft-c, although inducing high initial rates of CO_2 fixation, bleached the cells on prolonged incubation.

The bicarbonate concentration at maximal velocity of CO_2 fixation was about 1.5 mM at pH 7.0 (Fig. 4). The bicarbonate concentration for one-half of the maximal velocity (apparent *Km*) was 0.2 mM.

Only minor changes have been made in the nutrient salt content of our solutions from those used by Takebe *et al.* (10). Concentrations of potassium greater than 5 mM were generally required to sustain prolonged CO_2 fixation (Table I). Potassium may be required to enable bicarbonate uptake in the light, as has been observed in leaf fragments from *Pisum sativum* (7). One millimolar calcium was also required for good photosynthetic activity, but it is inhibitory to the release of cells when present during maceration (15).

The addition of the two antibiotics cephaloridine and Rimocidin did not affect CO_2 fixation but ensured the freedom of the incubated cell preparations from bacterial and fungal contamination for periods in excess of 30 hr, providing that initial contamination of all solutions and apparatus was kept to a minimum. These observations are based on phase contrast microscopic observations and on the maintenance of a linear incorporation of amino acids from the medium over this time period (1).

Products of CO_2 Fixation. The products into which $^{14}CO_2$ was assimilated after fixation in the light were determined for both leaf cell suspensions and leaf tissue. Both cell suspensions and tissue assimilated the ^{14}C into sucrose, oligo- and polysaccharides as well as small amounts of sugar phosphates, glycolic acid, malic acid, serine, aspartic acid, glycine, and alanine. The only striking difference in the product patterns of cells and leaf tissue was the finding of significant amounts of ^{14}C-maltose associated with the cell preparations.

With the cells we also looked for products lost into the medium during incubation. Samples of incubated cells were centrifuged to separate them from the supernatant fraction which contains the products lost to the medium. Only sucrose, maltose, and small amounts of glycolic and malic acids were detected in the supernatant fraction. The amount of these products appearing in the supernatant fraction increased upon incubation but varied among cell preparations. In one experiment, after 3 hr of incubation, 7% of the total ^{14}C incorporated

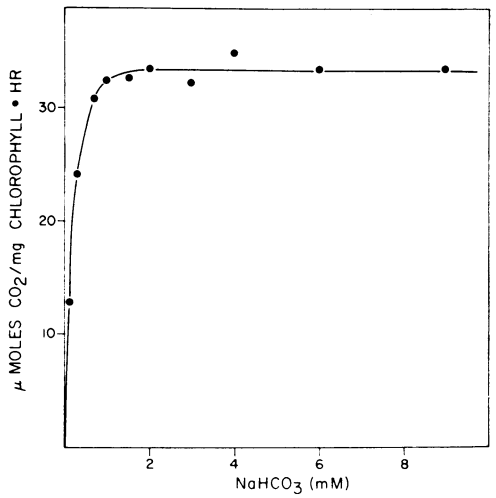

FIG. 4. Dependence of the initial rate of photosynthetic CO_2 fixation on bicarbonate concentration. Tubes contained 1.0 ml of incubation medium, cells having 15 μg of chlorophyll and bicarbonate as given. Rates of CO_2 fixation were calculated from the amount of ^{14}C incorporated within the first 5 min of illumination.

was found in the supernatant fraction; after 24 hr this increased to 40%.

$^{14}CO_2$ was also incorporated into protein. Samples of the cells were pipetted into ice-cold 10% trichloroacetic acid, and the precipitate was washed several times with cold trichloroacetic acid, alcohol, and ether (17). The insoluble precipitate was hydrolyzed *in vacuo* with 6 N HCl for 24 hr at 110 C. After removal of the excess HCl by evacuation, the samples were chromatographed and labeled amino acids were detected by radioautography, indicating that some of the ^{14}C in the trichloroacetic acid-insoluble precipitate was protein.

DISCUSSION

Tobacco leaf cell suspensions prepared and assayed by the method described in this paper are capable of photosynthetic CO_2 fixation at rates about one-fifth of those reported for intact and attached tobacco leaves assayed at 3500 ft-c (16). However, the rates of the cell suspensions are about the same magnitude as those found with tobacco leaf discs (18).

The products of the assimilated carbon were similar with leaf tissue and cells except for the relatively large amounts of maltose found in the separated cells. Maltose was unexpected as a major product of photosynthesis (2). In higher plants it has only been seen in small amounts and its appearance after prolonged exposure to $^{14}CO_2$ is thought to be a product of starch breakdown (5). The finding of maltose with our tobacco cells could be due to the degradation of labeled polysaccharides by residual amylase from crude Macerozyme used in the maceration.

Our procedure for preparing tobacco leaf cells is similar to that reported by Takebe *et al.* (10) but differs in several significant aspects. Takebe *et al.* (10) stripped the epidermis from the leaves prior to maceration, which they regard as essential to obtain a pure population of spongy and palisade parenchyma cells. We find the stripping of the epidermis to be tedious and unnecessary; thus, we use leaf strips for maceration (14). We have replaced mannitol as used by Takebe *et al.* (10) with sorbitol in our media to obtain hypertonic conditions; with sorbitol, cells were capable of higher rates of CO_2 fixation and its higher solubility makes it more convenient to use. We also find that potassium dextran sulfate is useful in the medium during cell maceration (Table I); but it can be partially replaced by potassium or sodium salts. No complete substitute for potassium dextran sulfate has been found, and its function remains obscure. Higher concentrations of potassium than those used by Takebe *et al.* (10) are required during incubation for leaf cells to maintain photosynthesis for long periods.

Takebe *et al.* (10) used the microscopic appearance of the cells as a guide to their metabolic capacity. We have found that measurement of the rate of photosynthetic CO_2 fixation by cell suspensions is a convenient, rapid, and more sensitive method of assessing the effectiveness of various procedures and substances for the preparation of metabolically active cells. It has the unique advantage over other biochemical criteria that the metabolic activities resulting from bacterial contaminations cannot be confused with the photosynthetic activity of the leaf cells. The ability of the cells to fix CO_2 correlated well with their ability to undergo metabolic activities such as uptake and incorporation of amino acids, uracil, and uridine (1).

Another approach to the production of isolated cells has been reported by Gnanam and Kulandaivelu (3). Leaves of certain species of plants release whole cells when carefully ground in a mortar. These workers prepared cells from a number of species of plants and found initial photosynthetic rates which were comparable to those reported here. The two approaches to cell production are not mutually exclusive, however, because each method will not yield cells from all species of plants (3, 8, 14).

Suspensions of isolated leaf cells offer a new approach to the study of the metabolism of photosynthetic tissue. They are useful in relatively short term experiments for up to about 30 hr after preparation. Rates of CO_2 fixation and uptake of metabolites (1) are approximately linear for the first 20 hr or so, but they then tend to decline. The microscopic appearance of the cells does not change markedly upon prolonged incubation, and there is no suggestion of any marked rupture of cells which would explain the decline. The incubation in 0.6 M sorbitol after isolation in 0.8 M sorbitol does cause a slight relaxation and swelling of the cells, but once this has taken place there appears to be no volume increase upon further incubation. It is conceivable that the cells could slowly take up sorbitol which might slow down their metabolism. The cells do not seem to be degrading proteins and nucleic acids rapidly (1), and cytoplasmic streaming was still observed in cells incubated for 3 days. These facts suggest that the process of cell degradation is not very rapid, perhaps because of the cytokinin in the medium which is known to arrest the degradative processes subsequent to leaf excision (9).

With slight modification of the procedures described in this paper, cells prepared from spinach, soybean, alfalfa, and cotton leaves were also photosynthetically active. For example, photosynthesis with separated cotton cells was maximal if the leaf tissue was macerated in 0.6 M sorbitol and incubated in 0.7 M sorbitol (Manandhar and Jensen, unpublished results).

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