PHOTOSYNTHESIS BY PROTOPLASM EXTRUDED FROM CHARA AND NITELLA*

BY N. E. TOLBERT AND L. P. ZILL

(From the Biology Division, Oak Ridge National Laboratory, Oak Ridge)

(Received for publication, December 4, 1953)

Many earlier investigators have attempted to obtain a plant sap or leaf macerate which can utilize light energy for carbon dioxide fixation and reduction. If the cell-free process were like that of the whole plant, it should be able to reduce the CO₂ to a carbohydrate level by normal photosynthetic intermediates. A portion of the whole photosynthetic process has been studied in isolated chloroplasts by Hill and Scarisbrick (1), who observed the photochemical formation of reducing energy and oxygen from water. These observations have been extended to biologically important electron transport systems by several investigators (2, 3) who, by using appropriate coupling enzymes, have observed the resultant fixation in low yields of carbon dioxide into the carboxyl groups of malic and citric acids. The latter work has demonstrated the ability of chloroplasts to form minute amounts of reduced pyridine nucleotides, but the reduction of CO₂ to sucrose by steps characteristic of the path of carbon in photosynthesis, as elucidated by Calvin’s group (4–6), has not been accomplished in a cell-free system. Recently, a cell-free brei from spinach leaf has been reported to be able to fix a small amount of NaHCO₃ in the light over and above the dark fixation (7). This fixation both in the light and in the dark was mainly into phosphoglyceric and pyruvic acids and demonstrates an increase of carbon dioxide fixation in low yields into the early photosynthetic intermediates.

The ability to reduce CO₂ photosynthetically in greater than trace amounts ceases abruptly when plant cells are macerated or otherwise disrupted. Many hypotheses are possible to explain this effect. Among them are dilution of the enzymes of the protoplasm with vacuolar sap, change in pH, and disturbance of the structural arrangement between the enzymes and chloroplasts within the cell. Any one of these possibilities might make it extremely difficult to obtain from a higher plant a cell-free macerate capable of carrying on the complete cycles of CO₂ fixation in photosynthesis. Effort to obtain such a cell-free system seems justified, however, so that a more direct biochemical and biophysical investigation of the individual steps of the process can be made.

* Work performed at the Oak Ridge National Laboratory under Contract No. W-7405-Eng-26 for the Atomic Energy Commission.
PHOTOSYNTHESIS BY EXTRUDED PROTOPLASM

Members of the Characeae family have been used in the past to study mineral uptake in components of plant cells, since each cell is large enough to permit a partial separation into intracellular sap and protoplasm without the aid of microscopic technique. Because of the cell size it also seemed possible that these plants would be ideal for photosynthetic studies, since the protoplasm would suffer a minimum amount of disturbance in separating it from the cell wall.

In this communication will be reported the fixation in the light or dark of NaHCO₃ at appreciable rates, not only by whole or cut *Nitella* or *Chara* cells, but also by the protoplasm extruded from these cells. It has been possible to obtain a marked fixation of C¹⁴ into normal photosynthetic intermediates by the extruded protoplasm at a rate of 12 to 13 per cent of that of the intact cells. Thus the use of these plants has made possible for the first time the observation of photosynthesis at significant rates by protoplasm outside the cell wall.

**Materials**

Considerable information is available on the taxonomy of *Nitella* and *Chara* (8), but no data could be found on culture methods for these algae. It appears that, at present, the most suitable way to obtain the algae is from their natural habitat in ponds. High light intensity, high temperature, and high salt or CO₂ concentrations, as well as distilled water, are all toxic to *Chara* and *Nitella*. The cells, as collected from ponds, can be kept in apparently healthy condition for days in jars of pond water but, as reported in the Results, their photosynthetic capabilities decrease.

The *Chara* was a non-corticated species, *C. braunii* var. *schweinitzii*. They have been grown in balanced aquariums after having been potted at the lake and transported, submerged in water, to the greenhouse. However, the work reported in this paper was all done with *Chara* immediately after harvesting them from the lake.

The *Nitella* flexilis (L.) Ag. was likewise done at the collection site so as to have fresh cells. *Nitella* has been kept alive in aquariums and, although growth has occurred, the algae appeared to be in such an unsatisfactory state that they were not used in any of the experiments reported. *N. davida* was raised in balanced aquariums and used in the experiments reported in this paper. This plant is the most promising because of its very large cells.

The NaHCO₃ was prepared from 25.2 per cent C¹⁴, BaC¹⁴O₃ (89 μc./mg.). Generally, approximately 10,000 to 100,000 counts per second were used, depending on the number of cells in the experiment. This represented from 5 to 50 μl. of our NaHCO₃ preparation. Counting of the C¹⁴ was done in a gas flow proportional counter.

ATP and DPNH₂ were obtained from Sigma Chemical Company and CoA from Pabst Laboratories. Dihydroxyxmalic acid was synthesized according to the directions of Fenton (9). Hydroxypyruvate was prepared from pyruvic acid and the

---

1 Direction from J. S. Karling, Purdue University.
former was decomposed to give a solution of glycolaldehyde (10). Acetyl phosphate, 90 per cent purity, was prepared as its dilithium salt (11). Salts of the compounds were treated with dionex-50 to solubilize them, and the supernatant was adjusted to pH 7.5 with KOH.

Methods

Only the long internode single cells have been utilized. The smaller, greener, and probably more active cells of the whorls have not been used except when grinding up the whole plant for sap or brei. This procedure has been followed since the cells of the whorls are actively dividing, and, unless microscopic techniques were employed, one could not be certain that all whole cells were eliminated. Furthermore, the whorl cells are much shorter and more delicate than the long internode cells and thus harder to handle. The longer of the internode cells (1 to 2 inches in length) were separated individually or in chains of two or three. Cells were always washed with water before use to avoid contamination by small algae.

Three "test systems" have been used in these photosynthesis experiments: (a) whole cells, (b) cells with both ends cut off which have been designated "cut cells," and (c) the protoplasm from the cell.

Whole Cells.—Controls were run with groups of ten whole single cells of about equal length trimmed clean of whorl cells. On each cell remained very small node cells and small pieces of other cells. The cells varied in size, making exact duplication difficult. The results indicate only the correct order of magnitude of C\(^{14}\) fixation. The ten cells were washed with water into the bottom of a test tube whose sides had been flattened together to within about 1 cm. or less. In these tubes the cells could be suspended in a relatively small volume of water. The experiments were run at 23°C in a constant temperature bath with glass sides so that the desired light intensity could be obtained from photoflood bulbs. Substrates, if used, were then added to the test tube and at zero time NaH\(C^{14}\)O\(_3\) was placed in the solution. Dark controls were run by covering the test tubes with aluminum foil.

Cut Cells.—When both ends of a group of long internode cells were cut off, most of the vacuolar sap immediately flowed out, and in the case of Chara, the cells collapsed. This removal of both ends eliminated all node cells but resulted in a 10 to 15 per cent loss of plant material: in most cases twelve cut cells were used for comparison with ten whole cells. The cut cells were transferred immediately to the test tubes by picking them up on the end of the knife blade and washing them into the tube. Unless shaken violently, the protoplasm remained within the cell wall. Photosynthesis experiments, as measured by the C\(^{14}\) fixation, were performed in the same way as those using whole cells.

Experiments were stopped for the whole-cell or cut-cell experiments by adding an excess of 1 N HCl (pH of solution became 1 or less) and immediately plunging the test tube into a boiling water bath. The cooked cells were thoroughly macerated with a glass rod during boiling. Aliquots were counted with a proportional counter for fixed C\(^{14}\) after being completely dried on a glass counting plate in a draft of hot air. The drying conditions in acid and heat were severe enough to destroy labile compounds such as \(\alpha\)-keto acids, but the conditions eliminated the danger of any carbonate remaining on the counting plate.
Extruded Protoplasm.—When one end of the Nitella or Chara cell is cut off the vacuolar sap can easily be squeezed out by applying gentle pressure uniformly over the cell (12). The protoplasm can then be pushed out, using a dull knife blade that does not cut the cell wall, by starting at the uncut end and moving toward the cut end. This is best done on a ground glass surface in order to provide friction for holding the cell in place. In the protoplasm so extruded from Nitella or Chara the chloroplasts tend to clump, and if water is added to the protoplasm, the chloroplasts more rapidly undergo physiological changes such as swelling followed by clumping. This may result from added substrates in the subsequent experiments, contaminating vacuolar sap, or water not completely removed from the outer cell wall.

For this reason, studies with the protoplasm were carried out directly on the glass dish or plate used for counting C14. Reagents were first aliquoted onto the dish and dried in warm air before adding the protoplasm. In a typical experiment 5 μl. of NaHC14O3 (about 10,000 counts per second) and 10 μl. of 0.1 M K2HPO4 were dried on the dish. After cooling the dish, the protoplasm from three to five cells was squeezed directly onto the plate (taking only ~ 5 to 10 seconds) and allowed to stand in the light and in the air without stirring for the allotted time. Experiments were stopped by adding 0.1 ml. of 1 N HCl, and the whole was dried in a hot air current before counting for fixed C14.

The light source was a 200 watt photoflood bulb separated from the photosynthesizing material by a 3 inch thick water bath. Experiments were conducted on the plexiglass top of a second water bath mounted under the light assembly. In long experiments, drying out of the protoplasm was most easily prevented by inverting a small dish over the plate containing the protoplasm. However, even though the protoplasm lost water by evaporation, it generally would continue to fix C14 at an unchanged rate until quite dry.

Dark controls for the protoplasm experiments were run by cutting one end of the cells in the light as previously described, and then quickly moving away from the photoflood light into normal room light and squeezing the protoplasm onto the dish containing the NaHC14O3. The dish was then immediately covered with an aluminum beaker.

Chromatographic Procedures.—Products formed from the fixed C14 were identified by two dimensional descending paper chromatography after the procedure of Benson et al. (5). The solvents were phenol-water in the first direction (from right to left on the figures of the chromatograms) and butanol-propionic acid-water in the second direction (from bottom to top on the figures of the chromatograms). Radioactivity in the spots on the paper was detected by no-screen x-ray film and measured by counting with an end-window Geiger counter. Compounds were identified by their relation (Rf) to a map of known compounds, and by cochromatography with known compounds in which there was coincidence of C14 activity and specific color spray tests for the compound. Spray tests were ninhydrin for amino acids and aniline trichloroacetic acid or aniline phthalate for the sugars (13). Organic acids were eluted from the chromatograms and further identified by cochromatography with unlabeled acids in ether-acetic acid-water solvent using the brom-cresol green spray test (14). Phosphate esters were eluted and rechromatographed with suspected phosphate esters in a solvent of phenol-formic acid-water (336 gm. phenol,
10 gm. formic acid, and 172 gm. water). Their location was determined by treatment with ammonium molybdate spray and ultraviolet light (15). Phosphates were also eluted and treated with a commercial phosphatase, polidase-S, after which the organic moiety of the ester was rechromatographed with known compounds (6).

RESULTS

Rates of C\textsuperscript{14} Fixation

The data in this section are concerned primarily with a comparison of the rate of C\textsuperscript{14} fixation in the light and dark by whole cells, cut cells, and extruded protoplasm.

Fixation by Whole Cells or Cut Cells.—The C\textsuperscript{14} fixed by the cells has been used as a measure of photosynthesis rate. For the whole cells of *Nitella flexilis* this proceeds at a constant rate (line A, Fig. 1). An approximately equal amount of cut cells also fixed C\textsuperscript{14} at about the same rate as the whole cells, if the experiments were run with cells immediately after their removal from the lake (line B, Fig. 1). If the *Nitella* remained overnight in the buckets used to bring them from the lake the capability of the cut cells to photosynthesize dropped (line C, Fig. 1). The nature of this loss of activity is not known, but the suspension was not warmed, and exposure to several hours of light did not restore the lost photosynthetic ability.

In these experiments the dark controls fixed an easily measurable amount of C\textsuperscript{14}, but the amount fixed was very small compared to the amount fixed in the experiments run in the light. Approximately the same dark fixation was obtained for both the whole and cut cells (line D, Fig. 1).

The rate of C\textsuperscript{14} fixation by cut *Chara* cells is shown in Fig. 2. This rate was constant for 8 minutes, and was of the same order of magnitude as that of the whole cells. In this experiment the amount of total C\textsuperscript{14} fixed in the dark decreased with time. There was more fixed C\textsuperscript{14} in the cells after 20 or 40 seconds in the dark than there was after 1 or 2 minutes. This rapid fixation in the dark immediately after the cells had been in the light indicates the utilization of stored reducing energy from the light. However, this fixed C\textsuperscript{14} disappeared shortly thereafter, suggesting that the fixation of the CO\textsubscript{2} during the first seconds in the dark was into very active metabolic compounds and probably into carboxyl groups.

Fixation by Protoplasm.—C\textsuperscript{14} fixation in light and dark by the protoplasm from the *Chara* cells is shown in Fig. 3. Two different experiments reported were run about 1 month apart; they indicate the reproducibility of the results. Experiment 1 was run in the laboratory with *Chara* collected the previous day. Experiment 2 was run with *Chara* taken directly from a lake. The results show a marked effect of light on the fixation of C\textsuperscript{14} by the *Chara* protoplasm.

Substantial C\textsuperscript{14} fixation in the light by the protoplasm of *Nitella flexilis* also can be demonstrated. However, it has not been possible to obtain re-
producible results with the *Nitella* protoplasm. Stimulation of C\(^{14}\) fixation in the light by protoplasm from fresh cells was always observed, but the

![Graph](image)

Fig. 1. Rate of C\(^{14}\)O\(_2\) fixation in the light by whole or cut internode cells of *Nitella flexilis*. Ten cells were placed in a test tube with 5 µl of NaHC\(^{14}\)O\(_3\) solution, 25 µl of 0.1 w K\(_2\)HPO\(_4\), and 12 ml of water, and exposed to about 1500 ft.-c. of light from a 200 watt photoflood lamp for various lengths of time. The cells were killed by adding 0.5 ml of 1 N HCl and placed in a boiling water bath. Work done at the University of Virginia Mountain Lake Biological Station. Curves A, B, and D were run approximately 2 hours after harvesting the algae from the lake; curve C was run 18 hours afterwards.

amount fixed varied greatly for the various lengths of time. Generally constant fixation rates, comparable to the rate obtained with *Chara*, were observed for periods up to about 2 to 4 minutes, but for longer times, results became erratic.
Fixation by Protoplasm Squeezed through Cheese-Cloth.—Another way to separate protoplasm from these large cells is to apply pressure to a large number of them within several layers of cheese-cloth. The cell walls of Chara can be broken with gentle pressure, and the colorless vacuolar sap can be squeezed out through the cloth. Under a substantially higher pressure the protoplasm can also be squeezed from the cell walls and through the cheese-cloth. This material after centrifugation to remove any cell fragments has a dull greenish brown appearance, in contrast to the bright green color of the protoplasm pushed out from individual cells as described previously. The protoplasm squeezed out through cheese-cloth will fix C\textsuperscript{14}, but the results were not consistent, and the highest rates were less than those for the protoplasm pushed from the cells.

Fig. 2. Rate of C\textsuperscript{14}O\textsubscript{2} fixation by cut Chara cells in the light as affected by additional substrates. Ten cut cells were placed in a test tube with 5 μl. of NaHCO\textsubscript{3} solution, 25 μl. of 0.1 M K\textsubscript{2}HPO\textsubscript{4}, milligram to microgram amounts of substrates, and about 2 ml. of water, and exposed to full sunlight for various lengths of time. The cells were killed by adding 0.5 ml. of 1.0 N HCl and placed in a boiling water bath.
PHOTOSYNTHESIS BY EXTRUDED PROTOPLASM

Some Factors Affecting the Fixation Rate

Viscosity of protoplasm from the various species varied from that of Chara, which appeared almost as thin as water, to that from Nitella flexilis, which appeared as a thick gummy paste. Any manipulation of the protoplasm that was squeezed from the cell wall greatly decreased the rate of C¹⁴ fixation. Stirring the protoplasm on the dish reduced the amount of C¹⁴ fixed. It might be postulated that this caused still further disorganization of the protoplasmic structure. Dilution with water also reduced fixation.

K₂HPO₄ was used to buffer the acids of the protoplasm, which had a pH of ~6 after extrusion from the cell wall. Unless phosphate buffer was used, C¹⁴O₂ was rapidly lost when the protoplasm was added to the dish containing NaHCO₃ solution.

---

**Fig. 3.** Fixation of C¹⁴O₂ in light and dark by the protoplasm from Chara cells. Protoplasm from three Chara cells was squeezed into a dish containing 5 μl of NaHCO₃ solution (~6000 counts per second) and 10 μl of 0.1 M K₂HPO₄ previously reduced to dryness. Light intensity was about 1500 ft.-c. from a 200 watt photoflood lamp with a water filter for removing heat. Reaction stopped by addition of 0.1 ml of 1 N HCl and plate dried in hot air before counting.
N. E. TOLBERT AND L. P. ZILL

the radioactive bicarbonate. Activity on the dish was checked with a thin-window laboratory Geiger monitor. By trial and error, an amount of 0.1 M K$_2$HPO$_4$ was added to the reaction sufficient to change the pH of the protoplasm to ~7.5 and to prevent loss of bicarbonate. For three cells, 10 µl was used; larger amounts were used in some experiments. For instance, 25 µl of 0.1 M K$_2$HPO$_4$ was added to the NaHCO$_3$O$_4$ and dried on the dish before the protoplasm from three cells was squeezed onto the plate, but this reduced the bicarbonate fixation rate 50 per cent. It is not known whether this was due to a pH effect, an inhibitory effect of the phosphate ions, or osmotic pressure increase.

So far, all compounds that have been added to the solution containing the cut cells markedly inhibit C$^{14}$ fixation. The amount of C$^{14}$ fixed by cut cells after 4 minutes in the presence of certain of these compounds is shown in Fig. 2. In other experiments it was noted that cysteine, glutathione, glycolaldehyde, and glycolic, hydroxypyruvic, and dihydroxyxymaleic acids likewise did not stimulate C$^{14}$ fixation, but rather inhibited it.

The various compounds tried with the cut cells for stimulating fixation were also inhibitory to the fixation rate for the extruded protoplasm. In this work it was also necessary to add these compounds as dried-down reagents to avoid dilution with water.

McClendon and Blinks (16) have shown that isolation of red algae chloroplasts in a 40 per cent carbowax-4000 solution preserved them in a more normal physiological state than the use of either salt or sugar solution. Therefore, experiments with the protoplasm were tried in which carbowax was already on the reaction dish along with the NaHCO$_3$O$_4$ and the phosphate buffer. It was found necessary in subsequent counting to make a large correction factor of 2 to 4 because of the absorption of the weak C$^{14}$ β-radiation by the carbowax. With the carbowax, the amount of C$^{14}$ fixed by the protoplasm was about as many times greater as was the counting correction factor for absorption. Since there was no counting advantage in the use of carbowax, it was not used as routine. However, this procedure offers a real physiological advantage for obtaining considerably higher rates of photosynthesis by the extruded protoplasm, and is being further investigated. In an attempt to identify by paper chromatography products labelled with C$^{14}$, a way was also sought to remove the carbowax after these experiments. Dialysis of the reaction mixture to remove C$^{14}$-labelled compounds from the carbowax-4000 was not successful, since a considerable amount of the carbowax also passed through the Visking dialyzing tubing. Carbowax-6000 was not tried.

_Fixation Products_

In order to determine whether qualitative as well as quantitative differences exist among the whole cells, cut cells, and the extruded protoplasm, the
extracts prepared as previously described were separated into their components by chromatographic procedures. One marked difference in the distribution and nature of the light fixation products was found between whole and cut cells on one hand and extruded protoplasm on the other hand. From this difference a tentative explanation for the loss of CO₂-fixing ability by the protoplasm from Chara and Nitella can be deduced.

**Products Formed by Whole Cells or Cut Cells.**—Chromatography of whole or cut Chara cells reveals that the C₁⁴ was fixed in 10 minutes into the usual products associated with photosynthesis (4, 5). The main products formed in whole Chara cells were the organic phosphate esters, sucrose, free sugars, alanine, and glyceric and aspartic acids (Fig. 4 a). Similar products were formed by the cut cells of this species (Fig. 4 b). There was little quantitative variation in the amount of C₁⁴ in the products from either cut or whole cell photosynthesis. However, two products present in only trace amounts in the whole Chara cell showed a considerable increase in the C₁⁴ activity in the cut cells. One of these is an unknown in the lower left-hand corner of the chromatogram. The other is an area identical with the green chlorophyll color in the upper left-hand corner.

The composition of the phosphate esters of both whole cells and cut cells was studied chromatographically, as described in the Method section. Treatment with phosphatase showed that glucose, fructose, sedoheptulose, and pentoses were present as labelled phosphate esters, as well as glyceric acid and several unknowns. From the sugar phosphates the most C₁⁴ activity was in glucose. There was also a large amount of activity in fructose, and a substantial amount in sedoheptulose, but only a trace amount of activity in the pentose area. Since some C₁⁴ was also present in the free pentose sugar area of the original chromatogram, the combined free pentose and pentose phosphates were a significant amount.

Cut Nitella flexilis cells also produced the usual C₁⁴-containing compounds during fixation in the light. These products are dominated by the formation of C₁⁴-labelled sucrose, showing that the Nitella cell—even though both ends were cut off—was still able to reduce CO₂ to a carbohydrate level. The unknown in the lower right-hand corner of the chromatogram was formed in small amounts and the chlorophyll area contained a substantial amount of C₁⁴. In the whole Nitella cells the chlorophyll area became labelled with only trace amounts of C₁⁴ activity; the unknown in the lower right was not detectably labelled.

The products formed from whole Nitella clavata in the light are similar to those from N. flexilis or Chara. The cut N. clavata cells were also able to reduce C¹⁴O₂ in the light to sucrose.

A comparison between the products formed in the light and in the dark clearly shows that the cut cells were utilizing light energy for photosynthesis.
Fig. 4. Radiochromatograms obtained after 10 minutes of C\(^{14}\) fixation by \textit{Chara}: (a) by whole cells in the light, (b) by cut cells in the light, (c) by cut cells in the dark, (d) by extruded protoplasm in the light.
Photosynthesis By Extruded Protoplasm

In Fig. 4c are shown the products labelled with C\textsuperscript{14} by cut Chara in the control experiments in the dark. Similar chromatographic pictures were also obtained with both of the Nitella. The main products, malic, fumaric, aspartic, and glutamic acids, and \( \beta \)-alanine and alanine, are associated with dark respiration and, presumably, were primarily labelled in the carboxyl carbons.

Products Formed by Extruded Protoplasm.—The Chara protoplasm prepared as described in the section on Methods, fixed C\textsuperscript{14} also into products associated with the path of carbon in photosynthesis (Fig. 4d). The protoplasm prepared by squeezing Chara in cheese-cloth also produced a similar chromatogram. The C\textsuperscript{14} appeared in as reduced a state as free fructose and sucrose. Sucrose was eluted, hydrolyzed by boiling in dowex-50 for 30 minutes, and rechromatographed with carrier glucose and fructose. The C\textsuperscript{14} activity was about equally divided between the two sugars, which were detected by the aniline phthalate spray. The phosphate area, containing a considerable amount of the C\textsuperscript{14}, was treated with phosphatase and found to consist of 13 per cent phosphoglyceric acid, 67 per cent glucose phosphate, and 20 per cent fructose phosphate. There was no detectable labelled sedoheptulose or pentoses.

The products formed by the protoplasm during dark fixation were not extensively investigated because of the very low specific activity. They appeared to be similar to those found during dark fixation by the cut cells.

Discussion

The object of this work was to obtain a cell-free plant protoplasm which would be able to accomplish many of the enzymatic reactions of the whole cell. One of the first investigations of photosynthesis with C\textsuperscript{14} used cell-free brei prepared by grinding Nitella, but this showed no photosynthetic fixation of radioactive bicarbonate (17). Because of their large size, it is possible to carefully separate individual cells of Chara and Nitella into vacuolar sap, cell wall, and protoplasm. In the present work photosynthesis by these cells continued at an almost unchanged rate, when both ends of the cells were cut off and most of the vacuolar sap had been removed. The products formed by the cut cells, in general, were the same as those formed by whole cells. Thus the vacuolar sap had little or no effect on the immediate photosynthetic rate.

The protoplasm was separated from the cut cells by extrusion with gentle pressure. In the extruded protoplasm the rate of photosynthesis over a period of 8 minutes was approximately 12 to 15 per cent of that for a comparable number of whole cells. The protoplasm could still reduce CO\textsubscript{2} to sucrose and hexose phosphates. However, on a percentage basis, smaller amounts of sucrose were formed and more malic, glyceric, and aspartic acids, and alanine were produced than in the whole or cut cells.
A most pertinent clue about prior difficulties in obtaining cell-free photosynthesis lies in the nature of these products. While the whole or cut cells formed labelled hexoses and sucrose as well as the three-carbon compounds which are the initial site of fixation, these whole cells also produced labelled pentoses and sedoheptulose. In the extruded protoplasm, the sedoheptulose and pentose phosphates or free sugars were either not formed or were present in vanishingly small concentrations. Since evidence is now available that these compounds are involved in the CO₂ acceptor generation sequence of reactions (6), the present data suggest that the most sensitive site on disruption of the cells affects primarily the "C-2 acceptor" cycles and not primarily the fixation or reduction steps.

Dark controls were necessary in this work to differentiate between CO₂ reduction in photosynthesis and CO₂ fixation or exchange in the dark due to enzymatic carboxylation of organic acids. The rate of the dark fixation was found to be much less than that of the light fixation, as was to be expected. The products labelled with C¹⁴ from dark fixation in the whole cells, cut cells, or extruded protoplasm were the organic acids and amino acids associated with respiration.

Investigation of the effect of added substrates and enzyme cofactors on the rate of C¹⁴ fixation by the cut cells or protoplasm has not been extensive. All substances so far tested, however, were found to be inhibitory in the concentrations and under the conditions used.

The possibility existed that contaminating algae on the outside of the large Nitella or Chara cells might account for some of the radioactive bicarbonate fixation. For this reason, cells were always washed before use. That contamination was not significant is indicated by several facts. (a) Chara and Nitella, as used, did not inoculate water baths or aquariums with a load of smaller algae. (b) On a cell volume basis or chlorophyll basis, the fixation by any contamination would have been vanishingly small since no microscopically visible contamination was observed. (c) The bicarbonate fixation by the protoplasm was free of any contamination on the outer cell wall since it was separated from this membrane material.

**SUMMARY**

(a) Photosynthesis with protoplasm isolated from Chara or Nitella as measured by C¹⁴ fixation has been obtained at a rate 12 to 15 per cent of that of the whole cells.

(b) Photosynthesis by cut cells of Chara or Nitella with the vacuolar sap removed was at a rate comparable to that of the whole cells.

(c) Both the protoplasm and the cut cells reduced CO₂ in the light to sucrose and hexose phosphates. Other products formed were also detected by paper chromatography. In contrast, dark controls fixed the C¹⁴ into products associated with plant respiration.
PHOTOSYNTHESIS BY EXTRUDED PROTOPLASM

(d) An important difference in the products from the extruded protoplasm was the absence of C4-labelled pentoses or sedoheptulose which were formed, however, by the whole or cut cells. This suggests that the most sensitive site affected by disruption of the cells may be the steps involved in the regeneration of the "C-2 acceptor" for CO2 fixation in photosynthesis.

The authors wish to acknowledge the capable technical assistance of Mrs. Betty A. Gwin. We are indebted to several people for helping us acquire the plants. Dr. L. R. Blinks supplied us with a start of Nitella clavata. Most of the work with Nitella flexilis was done at the University of Virginia Mountain Lake Biological Station. Dr. Gordon Hunt and Dr. H. Silva aided us in finding and classifying algae.

REFERENCES

   1952, 74, 4477.
   University Press, 1945.
13. Block, R. J., LeStrange, R., and Zweig, G., Paper Chromatography, New York,