RATES OF PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

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Systematic attempts to reproduce complete photosynthesis outside the living cell began after Büchner's historic discovery in 1897 that fermentation can be carried out by enzymes released from yeast cells.[1] Büchner's discovery was the first demonstration that complex biochemical processes can proceed independently of the degree of cellular organization associated with a living cell. However, the many attempts to reproduce photosynthesis *in vitro*,[2-6] including those in Büchner's own laboratory,[7] met with failure. By 1953, there was no valid evidence to dispute the view that "the photosynthetic process, like certain other groups of reactions in living cells, seems to be bound to the structure of the cell; it cannot be repeated outside that structure."[8]

Although complete photosynthesis, i.e., a light-induced conversion of CO_2 to carbohydrates accompanied by oxygen evolution, was not reproduced by 1953 in a cell-free system, significant progress was made by Hill,[9, 10] who demonstrated that chloroplasts—the organelles that contain the chlorophyll pigments in the cell—could, upon illumination, evolve oxygen when isolated from the cell. In these experiments, isolated chloroplasts could not assimilate carbon dioxide but produced oxygen when carbon dioxide was replaced by ferric oxalate[9] or by other nonphysiological electron acceptors such as benzoquinone or ferricyanide.[11]

The missing link to complete extracellular photosynthesis, the conversion of carbon dioxide to carbohydrates, was reinvestigated when the sensitive $C^{14}O_2$ technique became available, but again the results were negative.[12, 13] Such $C^{14}O_2$ conversion as was observed in cell-free systems did not proceed beyond phosphoglycerate.[14, 15] There was no evidence for a reductive assimilation of CO_2 to the level of carbohydrate.

In 1954, a reinvestigation of this problem by different methods yielded evidence for a light-dependent assimilation of CO_2 by a cell-free system.[16] Chloroplasts isolated from spinach leaves assimilated $C^{14}O_2$ to the level of carbohydrates, including starch, with a simultaneous evolution of oxygen.[17, 18] When the conversion of $C^{14}O_2$ by isolated chloroplasts to sugars and starch was confirmed and extended in other laboratories,[19-23] the capacity of chloroplasts to carry on complete extracellular photosynthesis ceased to be a matter of dispute.

A novel feature of the methods used to obtain extracellular photosynthesis was the isolation of chloroplasts in isotonic sodium chloride solutions[16, 18] rather than, as formerly, in isotonic sugar solutions.[9] The replacement of sugar by salt eliminated from the reaction mixture a possible source of chemical energy and metabolites, thereby strengthening the evidence that radiant energy was the sole source of energy for the total synthesis of carbohydrates from CO_2. The same energy considerations applied to photophosphorylation,[16, 24, 25] a component process of photosynthesis that was also discovered in "saline" chloroplasts. Photophosphorylation, the synthesis of adenosine triphosphate (ATP) by chlorophyll-containing cellular organelles at the expense of radiant energy, proved to be a component of

photosynthesis that is independent from, but a prerequisite for, CO_2 assimilation.[26]

Saline chloroplasts gave low rates of CO_2 assimilation,[18] about 1.5 μmoles CO_2/mg chlorophyll/hour—or about one per cent of the photosynthetic activity expected from the best parent leaf material. These rates were obtained with whole chloroplasts prepared in isotonic salt solutions. Higher rates were obtained later with "broken" chloroplasts, supplemented with fructose 1,6-diphosphate, but even this system gave only a rate of 6 μmoles CO_2/mg chlorophyll/hour.[27] Such low rates of extracellular photosynthesis were not unexpected and were, in fact, similar to rates of other cellular processes that had been reconstructed *in vitro*. For example, the rate of Büchner's cell-free fermentation[1, 28] was only 1.6 to 4.6 per cent of the corresponding rate *in vivo* and even lower relative rates were obtained for protein synthesis[29] and polymerization of deoxyribonucleic acid (DNA)[30] when these were first reproduced in cell-free systems.

As with other cellular processes reconstructed *in vitro*, the primary purpose of investigations of extracellular photosynthesis was not the achievement of high rates but the elucidation of the biochemical and biophysical mechanisms of the process. For this purpose high extracellular rates, although desirable, were not crucial. What was crucial was that chloroplasts *in vitro* yielded the same intermediates and final products[18, 27] as are formed by photosynthesis *in vivo*.[31]

Since photosynthesis *in vivo* is the dominant chemical process for the synthesis of organic compounds on earth, high rates of this process *in vitro* attracted, nevertheless, special interest. Moreover, it became apparent that CO_2 assimilation remained the only extracellular activity of chloroplasts that was not greatly increased by changes in experimental techniques. The initially low rates of photophosphorylation by saline chloroplasts were later increased to a level more than sufficient to sustain maximum rates[32, 33] of photosynthesis *in vivo*. Similarly, the light-induced electron transfer from water to ferredoxin—the reaction that leads to production of the reductant required for CO_2 assimilation and yields oxygen as a by-product—proceeds in saline chloroplasts at rates consistent with maximum rates of photosynthesis.[34]

A possible reason for the low rates of CO_2 assimilation by isolated chloroplasts was revealed when the enzymes catalyzing CO_2 assimilation were found[35] to be present in the water-soluble portion of the chloroplasts (stroma) and were therefore subject to possible loss and dilution during isolation carried out in aqueous media. Isolated chloroplasts that were disrupted by a hypotonic shock lost almost completely their capacity for CO_2 assimilation.[35] This capacity was restored upon the addition of an aqueous extract of chloroplasts and was further enhanced by the addition of ATP and triphosphopyridine nucleotide (TPN).[35] Similar results were reported by Heber and Tyszkiewicz,[36] who supplemented chloroplasts isolated in an aqueous medium with an extract of chloroplasts prepared in a nonaqueous medium. Another investigation showed[37] that when supplied with ATP and reduced TPN, the soluble enzyme fraction of chloroplasts can convert CO_2 to sugars in the dark, even if it is experimentally separated from the insoluble, chlorophyll-containing portion of chloroplasts.

Kahn and von Wettstein[38] showed by electron microscopy that a significant portion of spinach chloroplasts is damaged by isolation in isotonic salt. They found

that about 65 per cent of the isolated chloroplasts retained their structural integrity but about 35 per cent lost their outer membranes and stroma. Walker[39] found with isolated pea chloroplasts that loss of outer membranes reduced their rate of CO_2 assimilation about 90 per cent. Using undamaged pea chloroplasts, supplemented with sugar phosphates, Walker[30, 40] obtained rates of CO_2 assimilation in excess of 30 μmoles/mg chlorophyll/hour. More recently, Walker and associates reported similar rates of CO_2 assimilation for chloroplasts isolated from market spinach and rates of 65–105 μmoles/mg chlorophyll/hour for spinach chloroplasts isolated from freshly gathered, field-grown leaves.[41]

To obtain these high rates of CO_2 assimilation by leaf chloroplasts, Walker[39, 40] made several changes in the methods for isolating chloroplasts and in the conditions under which CO_2 assimilation was measured.[40] Once the full photosynthetic capacity of isolated chloroplasts was established, the avoidance of sugars or related compounds during isolation and assay of chloroplast activity ceased to be an issue. Walker isolated chloroplasts not in isotonic saline but in isotonic sorbitol or sugar solutions and added sugar phosphates to the reaction mixture. He also reduced to a minimum the time for macerating leaves and for other steps in the isolation procedure that might damage the structural integrity of chloroplasts. The superiority of sugar solutions for CO_2 assimilation was also observed by Jeffrey *et al.*[42] with chloroplasts isolated from a marine alga.

A further modification of Walker's method[39, 40] gave Jensen and Bassham[43] a higher rate of CO_2 assimilation by isolated spinach chloroplasts than was previously reported. For the first six minutes of illumination, the isolated chloroplasts fixed CO_2 at a rate of 155 μmoles CO_2/mg chlorophyll/hour, the rate declining at the end of 30 minutes to 40 μmoles/mg chlorophyll/hour. The intact parent spinach leaves fixed CO_2 at a rate of 245 μmoles/mg chlorophyll/hour. Thus, the initial rate of CO_2 fixation by isolated chloroplasts was 63 per cent and, at the end of 30 minutes, 16 per cent of the rate in the parent leaf material. The appearance of the isolated chloroplasts in the electron microscope attested to their structural integrity.

The products of CO_2 assimilation in the experiments of Jensen and Bassham[43] were similar to those obtained with saline chloroplasts,[18, 27] indicating that, despite differences in rates, the general pattern of CO_2 assimilation was essentially the same. The present investigation was undertaken, therefore, to identify the factors in the preparative and assay procedures that influence high rates of extracellular CO_2 assimilation by isolated chloroplasts. Of special interest were the comparison of sodium chloride with sugars and the effect of the two main modifications introduced by Jensen and Bassham into Walker's method: the use of pyrophosphate and of the hydrogen ion buffers of Good *et al.*[44]

Methods.—As in earlier experiments on photosynthesis by isolated chloroplasts,[16, 18, 26] spinach leaves (*Spinacia oleraces*, var. Viroflay) were grown in water culture in a greenhouse, using a synthetic nutrient solution described elsewhere.[45] Mature leaves, about 8 to 10 weeks old, were usually used. In experiments with "young leaves,"[43] leaves from 5-week-old plants were used. The leaves were harvested 1 to 2 hr before each experiment, washed with distilled water, shaken to remove excess liquid, and placed in a plastic bag in the refrigerator to retain their turgidity.

CO_2 assimilation by leaf disks: CO_2 assimilation by parent leaf tissue was measured in circular disks of 1.2 cm diameter that were cut from freshly picked leaves with a cork borer and placed in 1 ml H_2O in a Warburg vessel of about 20 ml capacity (without center well). A sidearm of the vessel contained 10 μmoles $Na_2C^{14}O_3$ (10^6 cpm) and was fitted with a rubber stopper. Vessels were placed in a glass-bottomed constant-temperature water bath and were preilluminated for 5

min prior to the liberation of $C^{14}O_2$ gas from the sidearm by injecting 0.2 ml 9 N H_2SO_4 with a syringe through the rubber stopper (final CO_2 tension was about 1%). Illumination (from below) was by incandescent light of an intensity of about 20,000 lux; the temperature was 18° and the gas phase was air. Under these conditions, $C^{14}O_2$ assimilation by leaf disks progressed linearly for at least 10 min. Rates of CO_2 assimilation by leaf disks were usually measured after 4 min illumination in the presence of $C^{14}O_2$. Photosynthesis was stopped by immersing the leaf disks in boiling 80% ethanol. The radioactivity fixed in the soluble compounds was measured in an aliquot of the ethanol extract. To determine the $C^{14}O_2$ fixed into insoluble compounds, the leaf disk was homogenized in a test tube with a glass stirring rod. Aliquots of the homogenate and of the ethanol extract were analyzed for acid nonvolatile C^{14} with a gas-flow counter, making corrections for self-absorption. Chlorophyll was determined on a duplicate disk from the same leaf after homogenization and extraction with 80% acetone.[45]

Preparation of chloroplasts: Ten gm of fresh leaves (3 to 8 leaves, depending on size) were freed of midribs and were torn into strips of about 1 cm width, mixed with 32 ml of the preparative solution (described below), and disrupted in a Waring Blendor[46] using a Monel container. The leaf strips were packed around the blades with a glass rod. The power was turned on for 1 sec, the strips were repacked around the blades, and the blending was continued for about 5 sec. The slurry was then squeezed through six layers of either cheesecloth or, preferably, filtering silk (Joymar Scientific, Inc., New York, N. Y.). The filtrate was centrifuged for 1 min at 2,500 × g. The supernatant fluid was discarded and the pellet containing whole chloroplasts was resuspended in the preparative solution (described below) with a glass rod. The final concentration of chloroplasts was adjusted to give 1 mg chlorophyll/ml. Chlorophyll was measured as previously described.[45]

Preparative and assay solutions: A simplified isotonic solution for the isolation of chloroplasts (henceforth called the preparative solution) and a solution for assaying chloroplast activity (henceforth called the assay solution) were prepared to include certain components of the corresponding solutions used by Walker[39, 40] and by Jensen and Bassham.[43] The 4 components of the preparative solution (versus 5 of Walker's[40] and 9 of Jensen and Bassham's[43] were: 0.35 M sorbitol; 0.025 M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES buffer),[44] pH 7.6; 0.002 M ethylenediamine tetraacetic acid (EDTA); and 0.002 M isoascorbic acid. The HEPES buffer and EDTA were adjusted to pH 7.6 with NaOH. The 5 components of the assay solution (versus 10 of Walker's[40] and 11 of Jensen and Bassham's[43]) were: 0.35 M sorbitol; 0.05 M HEPES buffer, pH 7.6; 0.004 M pyrophosphate (adjusted to pH 7.6 with NaOH); 1.7×10^{-5} M isoascorbic acid; and 0.0067 M C^{14}-bicarbonate (10^6 cpm/μmole). Final volume was 1.5 ml.

Assays for CO_2 assimilation were carried out in Warburg vessels as described for leaf disks. The bicarbonate was added to a sidearm, and other components were added to the main compartment. The vessels were preilluminated for 3 min prior to adding the C^{14}-bicarbonate from the sidearm. The reaction was stopped with 0.2 ml 1 N HCl. An aliquot of the acidified reaction mixture was analyzed as described for the leaf disks.

$C^{14}O_2$ assimilation progressed linearly for 12 min and was proportional to the amount of added chloroplasts up to 0.4 mg chlorophyll per vessel. For each treatment, the rate of $C^{14}O_2$ assimilation was taken as the rate for 12 min, computed by averaging separately measured rates at the end of 4, 8, and 12 min.

Paper chromatography and radioautography: The products of CO_2 assimilation by isolated chloroplasts and by the parent leaf tissue were analyzed by two-dimensional paper chromatography in propanol-ammonia-H_2O and s-butanol-formic acid-H_2O[47] and by radioautography. The main difference between the carbon products of leaf disks and chloroplasts was the absence of sucrose in the latter. Products common to leaf disks and to chloroplasts included phosphorylated sugars and phosphoglycerate. The pattern of CO_2 assimilation in chloroplasts prepared and assayed by the present techniques was found to be the same as that in chloroplasts prepared and assayed according to Jensen and Bassham[43] and in saline media.[18, 27]

Results and Discussion.—Table 1 shows the superiority of sugars and related compounds over sodium chloride in maintaining the activity of isolated chloroplasts for CO_2 assimilation. Sorbitol, mannitol, inositol, sucrose, and glucose had approximately the same effect. When sodium chloride was used in both the pre-

TABLE 1

SUPERIORITY OF SUGARS AND RELATED COMPOUNDS OVER SODIUM CHLORIDE FOR CO_2 ASSIMILATION BY ISOLATED CHLOROPLASTS

	Relative Rates of CO_2 Assimilation		
	Compound in first column used in both preparative and assay solutions	Compound in first column used in assay solution and sorbitol in preparative solution	Compound in first column used in preparative solution and sorbitol in assay solution
Sodium chloride	26	54	84
Mannitol	100	100	100
Inositol	95	88	85
Sorbitol	92	96	89
Sucrose	92	93	92
Glucose	81	90	96

Each compound in first column was used to give a final concentration of 0.35 *M*. Except for the indicated replacement of sorbitol, the preparative and assay solutions were as described under *Methods*. The rates of CO_2 assimilation in the mannitol treatment were 45.5, 42.9, and 34.6 μmoles/mg chl/hr, respectively, in last three columns.

parative and assay solutions, the rate of CO_2 assimilation was only about a quarter of that obtained with chloroplasts prepared and assayed in sorbitol or mannitol. When chloroplasts were prepared in mannitol or sorbitol but assayed in sodium chloride, their rate of CO_2 assimilation was cut in half. However, chloroplasts prepared in sodium chloride and assayed in sorbitol showed only a small decrease in the rate of CO_2 assimilation. It is evident, therefore, that the use of sodium chloride, selected initially[16] to exclude extraneous sources of energy and metabolites, had a depressing effect on the rates of CO_2 assimilation by isolated chloroplasts.

Another contributing factor to low rates of CO_2 assimilation proved to be the relatively prolonged, old procedure[18, 26] for grinding leaves, washing and centrifuging chloroplasts to separate them from other cellular particles—a safeguard that also ceased to be essential. In one experiment, in which the new sorbitol-containing preparative and assay solutions were used throughout, chloroplasts isolated by the old preparative procedure assimilated CO_2 at a rate of 28.5 μmoles/mg chlorophyll/hour, whereas the rate with the new short preparative procedure was 45.7 μmoles CO_2/mg chlorophyll/hour.

Another factor that limited the rate of CO_2 assimilation by isolated chloroplasts was the use of Tris buffer in the old assay solutions.[18, 26] Table 2 shows a significant increase in the rate of CO_2 assimilation when Tris was replaced in the assay medium by Tricine and particularly by HEPES, two buffers introduced recently by Good *et al.*[44] Varying the pH of the preparative solution between 7.2 and 8.0, and of the assay solution between 7.6 and 8.0, had little effect on CO_2 assimilation. However, greater changes in pH levels were unfavorable.

TABLE 2

EFFECT OF BUFFERS ON THE RATE OF CO_2 ASSIMILATION BY ISOLATED CHLOROPLASTS

Experiment	Buffer in preparative solution	Buffer in assay solution	Rate of CO_2 assimilation (μmoles/mg chl/hr)
A	HEPES	HEPES	52.0
A	HEPES	Tricine	42.0
A	HEPES	Tris	27.0
B	HEPES	HEPES	42.5
B	Tris	HEPES	40.5
B	Tricine	HEPES	40.0

Each buffer was adjusted to pH 7.6: HEPES (N'-2-hydroxyethyl-piperazine-N'-2-ethanesulfonic acid) with NaOH; Tricine (N-tris (hydroxy-methyl)methyl glycine) and Tris (tris-hydroxymethyl-aminomethane) with HCl. Each buffer was added to give a final concentration of 0.025 *M* in the preparative solution and 0.05 *M* in the assay solution.

In experiments carried out to test the efficacy of other components of the preparative and assay solutions, we found that the omission of EDTA or isoascorbate[40] lowered the rate of CO_2 assimilation about 20 per cent.

TABLE 3

EFFECT OF PYROPHOSPHATE ON THE RATE OF CO_2 ASSIMILATION BY ISOLATED CHLOROPLASTS

Treatment	Rate of CO_2 assimilation (μmoles/mg chl/hr)
Complete, A	52.8
Pyrophosphate omitted, A	27.5
Complete, B	55.7

In treatment A, the preparative and assay solutions were as described under *Methods;* in treatment B, as described by Jensen and Bassham.[43]

Table 3 shows that the omission of pyrophosphate, which was included in the reaction mixture of Jensen and Bassham,[43] halved the rate of CO_2 assimilation by chloroplasts that were isolated and assayed as described herein. A possible explanation for this effect of pyrophosphate was our earlier (unpublished) observation that low concentrations of orthophosphate enhanced CO_2 assimilation by chloroplasts, although higher concentrations are inhibitory.[16] On this basis, one possible reason for the stimulatory effect of pyrophosphate was its ability to maintain a low concentration of orthophosphate in the reaction mixture. This explanation is supported by two findings. First, spinach chloroplasts have an active pyrophosphatase (average rate of pyrophosphate hydrolysis[48] was 140 μmoles/mg chl/hr. Second, as shown in Figure 1, orthophosphate at low concentrations is as effective as pyrophosphate in increasing the rate of CO_2 assimilation; at higher concentrations, orthophosphate, unlike pyrophosphate, strongly inhibited.

A comparison of the rate of CO_2 assimilation by isolated chloroplasts with that of the parent leaf tissue is given in Table 4. Chloroplasts from young leaves had a higher activity than chloroplasts from mature leaves, but in each case, the chloroplasts retained a major fraction of the photosynthetic activity of intact leaves. Table 4 shows that under our experimental conditions, the extra components included in the preparative and assay solutions of Jensen and Bassham,[43] but absent from ours, gave no increase in the rate of CO_2 assimilation.

As noted by other investigators,[41] the rates of CO_2 assimilation by isolated spinach chloroplasts may vary from day to day. The highest rate which we obtained was 80 μmoles CO_2/mg chlorophyll/hour (corresponding to 45 per cent of the rate in the parent leaf material), but the rate that was consistently observed was closer to 50 (corresponding to about 30 per cent of the rate in the parent leaf material). The photosynthetic activity of isolated chloroplasts

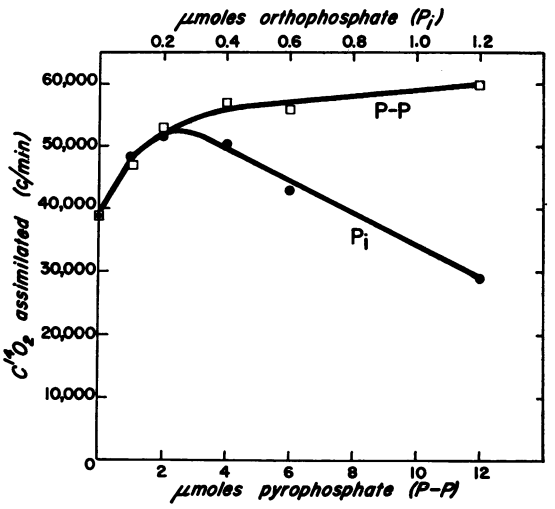

FIG. 1.—Effect of ortho- and pyrophosphate concentration in the assay solution on CO_2 assimilation by isolated chloroplasts (max. rate, 40 μmoles/mg chl/hr). Chloroplasts were prepared and assayed as described under *Methods*, except that pyrophosphate was omitted in the orthophosphate series.

TABLE 4

CO_2 ASSIMILATION BY ISOLATED CHLOROPLASTS AND LEAF DISKS AS INFLUENCED BY AGE OF LEAVES AND STORAGE TIME

	μMoles CO_2/Mg Chl/Hr		Per cent of Original Activity after Mature Leaves Were Stored at 25° — Days			
	Young leaves	Mature leaves	1	2	3	6
Leaf disks	174	136	77	54	33	32
Isolated chloroplasts, A	51.0	43.8	45	58	46	27
Isolated chloroplasts, B	46.5	38.2	—	—	—	—

Leaf disks and chloroplasts A were prepared and assayed as described under *Methods*. For chloroplasts B, the preparative and assay solutions of Jensen and Bassham[43] were used.

varies with the photosynthetic activity of the parent leaf material, which in turn depends on the age of the plant, the length of time that excised leaves are stored (Table 4), and on such internal factors as the carbohydrate reserves within the leaf.[27]

The importance of using freshly harvested leaves to obtain high rates of CO_2 assimilation by chloroplasts is illustrated by the marked decrease in photosynthetic activity that resulted from storage of leaves at 25° (Table 4). Storage of leaves at 0° slowed down but did not prevent the drop in the rate of CO_2 assimilation by isolated chloroplasts and leaf disks. However, to obtain high rates of CO_2 assimilation by isolated chloroplasts, the use of fresh leaves must be combined with the use of appropriate preparative and assay methods. Thus, the rate of CO_2 assimilation by the chloroplasts used in the present investigation was over 30 times greater than the rate of CO_2 assimilation by saline chloroplasts.[16, 18] Yet in both cases, the chloroplasts were isolated from freshly harvested spinach leaves, of the same variety, grown in the same nutrient medium and in the same greenhouse location.

CO_2 assimilation by isolated chloroplasts had an absolute dependence on light. No significant uptake of CO_2 occurred in the dark. The rate of CO_2 assimilation was not enhanced by the addition of ribose-5-phosphate, fructose-1,6-diphosphate, or 3-phosphoglycerate. Under our experimental conditions, it appears that none of these intermediates was limiting the rate of CO_2 assimilation by isolated chloroplasts (cf. Baldry *et al.*[41]).

Concluding Remarks.—The low rates of CO_2 assimilation that first characterized the reconstruction of complete photosynthesis by isolated chloroplasts[16–18] have been traced to elements of the experimental procedure (especially the use of sodium chloride and a relatively long and drastic preparative technique) that were initially introduced to guard against external sources of energy and metabolites and against contaminating cellular components. By replacing sodium chloride with a sugar (or a sugar derivative), using new buffers, inorganic phosphate, and a shorter and milder preparative technique, isolated spinach chloroplasts consistently gave photosynthetic rates of about 50 μmoles CO_2/mg chlorophyll/hour. These rates represent about 30 per cent of the photosynthetic activity of the intact leaf and are about 30 times greater than the first reported rate of CO_2 assimilation by isolated chloroplasts but yield no significant change in products.

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