Light and Dark $C^{14}O_2$ Fixation by Spinach Leaf Systems [1, 2]

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Despite tremendous progress during the last decade in formulating the pathways of CO_2 fixation, both in light and dark, and in detailing the reactions whereby light energy is converted to reducing power in the form of pyridine nucleotides or chemical energy as ATP, several essential pieces to this fascinating puzzle are still missing. Particularly, it is difficult to explain why an intact leaf in the dark is unable to incorporate $C^{14}O_2$ into sugars or their phosphate esters, or to form appreciable amounts of labelled glycerate-3-P (PGA) (16, 17, 18). If one examines nonphotosynthetic tissue such as mammalian liver or muscle or heterotrophic bacteria, there is a rapid incorporation of $C^{14}O_2$ into carbohydrates in the absence of net synthesis (21).

The photosynthetic experiments with spinach macerates by Fager (9) demonstrated the $C^{14}O_2$ could be fixed into PGA and pyruvate but no activity was detected in the carbohydrate fraction. These experiments were followed by the series of experiments of Allen et al. (1), using isolated washed spinach chloroplasts with which they were able to demonstrate photosynthetic CO_2 fixation into hexose phosphates but not into sucrose, the major product in the long-term whole leaf experiment. Since it seemed likely that there was an intimate biochemical interplay between the chloroplasts and the cytoplasmic organelles and soluble enzyme systems, Holm-Hansen et al. (13) investigated the possibility of reconstructing the biochemical environment by mixing particulate and soluble fractions from spinach leaves. Not only did they observe an enhancement of total $C^{14}O_2$ fixation, but the pattern of labelling in the various compounds revealed by paper chromatography and radioautography more closely resembled the pattern of the intact leaf. However, very little sucrose became radioactive.

Kandler (15) has expressed concern that some essential component of the photosynthetic process might be lost from the chloroplasts during the trauma of isolation. Experimental evidence for this point of view was provided by Smilie and Fuller (30) who observed diminution of 90 % of ribulose-diP carboxylase from plastids. Possibly other enzymes or cofactors could have been lost. Zucker and Stinson (34) have shown that the amount and physical state of proteins associated with isolated chloroplasts from several plants are greatly influenced by the conditions of extraction and preparation.

Another approach to the relation of integrity to function was used by Havir and Gibbs (11) who studied the distribution of label in sugar phosphates formed during photosynthetic fixation of $C^{14}O_2$ by chloroplast preparations as a function of the osmotic environment. They found little migration of tracer from C-3 and C-4 of the hexose into the other positions of the molecule in disintegrated chloroplast preparations. Spread of label into positions 1, 2, 5 and 6 was observed when special care was used to keep the chloroplasts intact. It was concluded that the pathway of CO_2 fixation operative in intact leaves had been disrupted and the broken chloroplast system no longer was representative of the biochemical events of photosynthesis.

A primary goal of this research was to correlate chloroplast ultrastructure with biochemical function. To this end we endeavored to obtain a cell-free preparation from spinach leaf which would fix $C^{14}O_2$ at an appreciable rate both in light and dark by the pathways employed by the intact cell. The principal criterion for comparison was the distribution of label in 80 % ethanol soluble compounds from intact leaf with those of the homogenates. This paper presents a method for the preparation of a cell-free system whose patterns of labelling are similar to that of the whole leaf. A correlation of chloroplast ultrastructure with biochemical function has been made.

Materials and Methods

Fresh spinach leaves (*Spinacia oleracea*) were obtained in a local market. Young leaves were washed in cold distilled water and blotted to remove excess moisture, then exposed to 1000 ft-c for 30 minutes. Petioles and midribs were removed, and only laminae were used. All operations were carried out in a cold room, and all glassware and other apparatus were precooled. There was considerable variability in the photosynthetic effectiveness of leaves from day to day. Chlorophyll content was measured in 80 % acetone (2).

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[1] Received June 29, 1964.

[2] This research was supported by grants from the United States Atomic Energy Commission and the C. F. Kettering Foundation.

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[4] Research Career Development Awardee of the United States Public Health Service.

CO_2 Fixation by Intact Spinach Leaves in Dark and Light. Approximately 4.0 g of 1 cm^2 sections of leaves were placed in a light tight apparatus devised in this laboratory for studying $C^{14}O_2$ fixation in succulents (27). A partial vacuum was created in the chamber by means of an aspirator, and 50 μc of $C^{14}O_2$ (0.08 % CO_2 by volume), previously generated from $BaC^{14}O_3$ and collected in the trap, was drawn into the chamber. The reaction was terminated by homogenizing in boiling 80 % (v/v) ethanol. The extract was filtered and concentrated in the manner described below.

Photosynthesis was studied by the procedure of Smilie and Fuller (30) for intact pea leaves. One g of spinach leaf sections (1 cm^2) were floated adaxial surface down in 0.01 M Tris, pH 7.4 in an Erlenmeyer flask; $KHC^{14}O_3$ containing 90 μc in 4.4 μmoles was added to start the reaction. Illumination was achieved with a 375 w photoflood bulb placed 95 cm above the surface of the leaves. Measured illumination (Weston Meter #756) was 4000 ft-c. Temperature was maintained at 20°. The reaction was terminated by grinding the leaves in boiling 80 % ethanol in a mortar. The extraction and concentration was carried out as described below.

Preparation of Cell-free Homogenate in NaCl-Tris. This method is similar to that described by Allen, et al. for the preparation of chloroplasts (1, 33). The pH of the homogenate was maintained at 7.4.

Preparation of Cell-free Extract with no Added NaCl and Minimal Dilution. Fifty g of fresh cut blades were ground with a pestle in a chilled mortar without any added water, buffer or sand. The slurry formed after 10 minutes was then squeezed through 4 layers of damp cheesecloth previously soaked in ice-cold 0.05 M Tris, pH 7.4. The extract was then centrifuged for 5 minutes, 200 × g at 0° to remove cell debris and whole cells. About 20 ml of liquid could be obtained from 50 g of leaves. The pH of the homogenate was usually 6.9 to 7.0. It was adjusted to 7.4 by adding approximately 1 to 2 ml of 0.10 M Tris, pH 7.8. In later experiments 5.0 ml of 0.05 M Tris-HCl, 0.05 M phosphate, 10^{-4} M EDTA, and 10^{-4} M GSH, pH 7.5 were added before grinding to provide a pH of 7.4 to the slurry throughout the procedure. The homogenate containing chloroplasts, mitochondria, and other particulates and soluble cytoplasm was used for the light and dark reactions.

$C^{14}O_2$ Fixation by Homogenates. Both dark and light reactions were carried out in 30 ml screw-top glass bottles. For the dark experiments, bottles were carefully covered with aluminum foil and black tape. Each bottle contained 5.0 ml of homogenate and 0.2 ml of $KHC^{14}O_3$ containing 90 μc in 4.4 μmoles. The reaction was carried out at pH 7.4. The temperature was maintained at 20° in a Dubnoff shaking incubator. The bottles were 2 cm below the surface of the water bath in order to absorb some of the heat. Light was supplied by two 375 w photoflood lamps 95 cm above the surface of the reaction mixture. Measured illumination was 4000 ft-c. These homogenates are very dense and the shape of the reaction vessels with the volumes used, presented a thin film of well-stirred homogenate to the light. The reaction was terminated by addition of boiling ethanol to provide a final concentration of 80 %. The extract was centrifuged, and the precipitate washed with 20 % ethanol for 5 minutes at 50°. Cofactors were added to the reaction mixtures as indicated in the experiments.

Determination of Total C^{14} Incorporation. The activity in the ethanol soluble fraction was determined using 100 μl aliquots of the combined extracts after centrifugation to remove insoluble materials. Liquid scintillation techniques were employed (26, 31). The scintillation medium was prepared by dissolving 5 g PPO (2,5 diphenyloxazole), 100 mg of dimethyl POPOP (1,4 bis-2-(-4 methyl-5-phenyloxazolyl)-benzene) and 100 ml of hydroxide of Hyamine per liter of 30 % methanol-70 % toluene. The insoluble fraction was hydrolyzed in 1 N HCl for 36 hours at 100° in a sealed vial. Aliquots were taken directly into the scintillator solution and counted as above. Values reported for total incorporation are the sum of 80 % ethanol soluble plus insoluble fractions.

Chromatographic and Radioautographic Method. The combined ethanolic extracts were concentrated under reduced pressure at 40° and adjusted to a final volume of 3.0 ml. If required, the concentrated extract was electrolytically desalted (Kensington Scientific Corporation) to obtain better separation of compounds on paper chromatograms. Aliquots of the concentrated ethanolic extracts were chromatographed on Whatman No. 1 filter paper (46 cm × 56 cm), by descending chromatography. The first solvent was phenol (80) : water (20), (w/w). The second solvent was n-butanol (80) : acetic acid (22) : water (50), (v/v/v). The compounds were located by radioautography using Kodak single coated blue sensitive X-ray film. Activity was determined by cutting out the appropriate area on the paper and counting with a liquid scintillation technique (6, 20).

Authentic samples of suspected compounds were cochromatographed with the extract on replicate papers and identification made by superposition. Amino acids were detected by spraying with 0.1 % (w/v) ninhydrin in 95 % ethanol, and heating the chromatograms at 100° for 5 minutes (8). Sugars were detected by spraying the chromatogram with aniline phthalate and ammonium molybdate (25). Sugar phosphates were detected with molybdate perchloric acid spray (3). Organic acids were detected with 0.1 N $AgNO_3$ followed by a 50: 50 mixture of 80 % phenol and 0.05 N NaOH (7). In many experiments the identities of sugars and sugar phosphates were further confirmed by passing the extract through a column of Amberlite 1R-120 H^+ form and subsequently rechromatographing the sugars and sugar phosphates on paper.

Electron Microscopy of Chloroplasts. Chloroplasts were precipitated by centrifuging the cell-free preparation at 1000 × g for 10 minutes. The result-

ing pellet was fixed in 1 % buffered osmium tetroxide, pH 7.4, for 30 minutes (22, 23). The fixative was removed, and the pellet dehydrated with 35 % ethanol then 70 %, 95 % and finished with absolute ethanol for a total of 3 hours. A 3: 1 mixture of *n*-butyl-methacrylate and methyl methacrylate with 0.2 % benzoyl peroxide catalyst was used to impregnate the specimens for 2 hours. The same methacrylate mixture, partly polymerized, was used for further impregnation and embedding in gelatin capsules. Polymerization using a long wave UV lamp at room temperature required 24 to 48 hours. Thin sections were cut on a Porter-Blum Ultra-microtome and examined with an RCA-3F electron microscope. Sections of intact leaves were fixed in 1 mm^2 sections in the same manner. These were used as a basis of comparison.

Results

CO_2 Fixation. A comparison of the rates of photosynthetic $C^{14}O_2$ incorporation (μmoles CO_2 per mg chlorophyll per hr) and the distribution of radioactivity in the various metabolites for intact leaf, the 0.35 M NaCl-Tris homogenate, and the NaCl-free preparation, with and without supplements of ADP and $NADP^+$ is presented in table I. The cell-free system prepared in the absence of NaCl and with minimal dilution shows a 10-fold greater rate of CO_2 fixation than the NaCl-Tris homogenate. The rate is now 11 % that of the intact leaf, and significantly higher than previously reported for unsupplemented preparations (1, 5, 13, 33). The addition of ADP and $NADP^+$ further stimulated incorporation to 15 % of the leaf.

The distribution of radioactive products in this NaCl-free preparation more closely resembles that of the intact tissue than does the conventional homogenate. In order to minimize the complexity of data presentation only the most significant compounds are indicated individually, and values of other compounds are included in the summation of each group. Although the relative activity in sucrose is less than that found in the leaf, synthesis of free sugars, for the most part glucose, is enhanced. Addition of ADP and $NADP^+$ shifts the label into maltose which can account for as much as 11 % of the activity in free sugar fraction. The relative levels of P-enolpyruvate and glycerate-3-P, sugar phosphate and amino acids are comparable to the leaf. Organic acid synthesis is stimulated, particularly glycerate. The presence of this acid may reflect an enhanced activity of the ribulose-diP carboxylase enzyme and subsequent phosphatase activity. Addition of flavine mononucleotides and vitamin K_3 had no appreciable effect on either the rate or distribution of $C^{14}O_2$ fixation.

The NaCl-free, undiluted preparation, hereafter referred to as the homogenate, was compared with intact leaves with respect to its ability to carry out

Table I. *Rate and Distribution of Radioactivity Following 1 hour Photosynthetic Assimilation of $HC^{14}O_3^-$ by Various Spinach Leaf Preparations*

Each reaction mixture contained 5.0 ml of homogenate for experiments in cell-free systems (columns 2–4). The concentration of $HC^{14}O_3$ was 8×10^{-4} M. Values are given as percent total activity found on the chromatogram.

	Intact Leaf	Homogenate 0.35 M NaCl	Homogenate 0.03 M Tris	Homogenate* 0.03 M Tris ADP + $NADP^+$
μmoles $C^{14}O_2$/mg chlorophyll/hr	17.5	0.3	2.0	2.6
Compounds				
Sucrose	44.0	1.1	8.3	8.0
Glucose	3.0	6.0	14.3	14.6
ΣFree sugars	49.8	7.1	27.7	36.5
Sugar diP	6.0	13.0	2.4	0.9
Sugar monoP	2.5	26.0	1.3	2.5
ΣSugar P	9.6	44.5	3.7	3.4
Glycerate-3-P	0.5	5.2	0.4	0.2
Σ P-enolpyruvate + glycerate-3-P	0.6	6.2	0.5	0.2
Aspartate	6.6	26.5	23.4	3.7
Glutamate	4.5	2.0	5.1	2.0
Glycine-serine	1.0	2.0	3.9	4.5
Alanine	2.5	2.5	2.3	5.6
ΣAmino acids	27.3	34.5	38.9	24.6
Citrate	2.2	0.0	1.4	3.0
Malate	0.6	0.0	7.9	4.5
Glycerate	1.5	4.5	14.2	21.4
Glycolate	7.0	2.5	1.3	5.2
ΣOrganic acids	11.3	7.6	28.5	35.1

* ADP and $NADP^+$ were 4×10^{-4} M.

dark CO_2 fixation reactions. These results are presented in columns [2] and [4] of table II. As had been previously observed (18), in the absence of light no label is found in sugars or sugar phosphates in the intact leaf. The homogenate did incorporate a trace, 0.07 % of the total activity on the paper, in the sugar phosphate fraction. The relative distribution of label in leaf and homogenate was comparable in both light and dark. There appeared to be some alterations in the activity of the enzymes of the citric acid cycle or in the regulation of organic acid storage indicated by the predominance of citrate over malate in the homogenate. The increase of aspartate at the apparent expense of glutamate should be noted.

The ability of ATP, NADH, NADPH, and ribulose-diP to promote the incorporation of label from $C^{14}O_2$ into sugars or sugar phosphates was tested. Results are presented in table II for a series of dark experiments involving these intermediates in various combinations. Clearly, a comparison between [3] and [5] indicates that ATP and NADPH do not replace the activity of light. There is a 2-fold increase in the amount of dark CO_2 incorporation, but the distribution of label is essentially the same as for the dark control [4], with some shift of activity to sugar phosphate and a great increase of glycerate-3-P. The added compounds which most effectively lead to a photosynthetic-like CO_2 fixation in the dark based both on rate and synthesis of carbohydrates were ATP and ribulose-diP [7]. Not only was uptake of the CO_2 stimulated, but radioactivity in sugar phosphates increased significantly. Activity in free sugars did not approach the magnitude observed in the light homogenates.

Ribulose-diP alone [6] is not able to stimulate incorporation into sugars. It appears to serve merely as a substrate for the ribulose-diP carboxylase as indicated by the increase of activity in glycerate-3-P. Addition of ribulose-diP and NADPH [8] is not as effective in both the relative and absolute amounts of carbohydrate synthesized as ribulose-diP and ATP [7]. Supplementation of ribulose-diP with ATP and NADPH [9] increases activity in free sugars, but the rate of fixation and the total activity in sugars and sugar phosphates is less than observed with ribulose-diP and ATP. Particularly noteworthy is the difference in the sugar fraction when NADH replaced NADPH (compare [9] and [10]). There is a marked shift of activity into glycerate-3-P and a drop in the carbohydrates.

The rate of photosynthetic CO_2 fixation by intact leaves can be achieved in the homogenate by supplementation with ribulose-diP, ATP, NADPH, and NADH [11]. However, the distribution of label

Table II. *Effect of Cofactors on Rate and Distribution of $HC^{14}O_3^-$ Assimilation of Spinach Homogenates in Light and Dark*

The reaction mixture consisted of 5.0 ml homogenate, 0.51 mg chlorophyll, including 3.4×10^{-4} M $MgCl_2$, 0.02 M K-phosphate, 2×10^{-5} M GSH, 2×10^{-5} M EDTA, 0.02 M Tris, and 8×10^{-4} M $KHC^{14}O_3$. All other compounds were added at a concentration of 1.8×10^{-4} M. The time of the experiment was 30 min. Values given as percent total activity found on chromatogram.

	Whole leaf		Homogenate									
	[1]	[2]	[3]	[4]	[5]	[6]	[7]	[8]	[9]	[10]	[11]	[12]
	Light	Dark	Light no cofactors	Dark no cofactors	Dark ATP NADPH	Dark RuDP	Dark RuDP ATP	Dark RuDP NADPH	Dark RuDP ATP NADPH	Dark RuDP ATP NADH	Dark RuDP ATP NADPH NADH	Light RuDP ADP NADP+
μmoles $C^{14}O_2$/mg chlorophyll/hr	17.2	0.85	2.4	0.9	1.5	1.6	7.0	3.0	5.6	6.5	17.2	7.6
Compounds												
Sucrose	16.0	0.0	0.4	0.0	0.0	0.0	0.4	0.0	2.5	0.04	0.02	1.8
Glucose	8.8	0.0	3.2	0.0	0.2	0.0	0.2	1.6	2.6	0.03	0.02	4.6
ΣSugars	25.8	0.0	3.6	0.0	0.2	0.0	0.65	1.6	5.1	0.07	0.04	6.4
Sugar diP	3.8	0.0	2.1	0.02	0.1	0.0	0.2	0.8	0.1	0.2	0.1	4.6
Sugar monoP	3.1	0.0	16.2	0.05	1.4	0.7	14.7	4.7	2.1	0.5	0.1	26.4
ΣSugarsP	7.7	0.0	20.0	0.07	1.5	0.7	14.9	7.6	2.4	0.7	0.2	33.8
Glycerate-3-P	1.8	0.0	23.8	0.6	30.4	38.7	14.9	31.8	39.5	73.5	63.7	30.8
Aspartate	16.3	32.2	33.2	65.0	40.5	2.7	10.0	30.2	3.1	3.4	14.0	3.1
Glutamate	4.5	10.1	0.4	1.79	0.2	0.0	0.1	3.1	0.11	0.07	0.01	0.3
Glycine-serine	7.8	9.3	1.3	0.5	4.6	5.2	0.2	4.8	2.2	0.05	1.5	0.6
Alanine	3.0	1.6	3.4	0.63	4.7	10.2	3.1	1.4	10.3	0.5	0.4	4.2
ΣAmino acids	33.3	60.0	40.4	69.4	50.0	18.1	13.5	39.6	16.5	4.1	16.3	11.3
Citrate	3.9	8.6	4.4	17.5	3.1	0.1	0.4	5.6	3.1	0.2	2.52	3.1
Malate	20.4	29.2	3.5	8.23	3.4	0.4	2.4	2.6	0.01	0.7	3.45	4.8
Glycerate	0.3	0.0	1.8	0.0	2.7	40.3	40.0	9.2	17.3	2.3	1.4	2.3
Glycolate	0.5	0.0	1.6	0.0	6.2	0.0	12.0	3.5	10.0	13.5	7.7	1.8
ΣOrganic acids	26.0	39.2	10.7	30.1	15.8	40.8	54.5	22.0	30.8	20.6	18.0	14.0

bears little similarity to that of photosynthesizing leaf. The metabolic pathways have been fundamentally altered by the addition of these cofactors together. The addition of ribulose-diP, ADP and $NADP^+$ to a photosynthesizing homogenate [12], triples the rate of CO_2 incorporation (compare with [3]), and at the same time maintains a similar distribution of activity in the various fractions.

Ultrastructure of Chloroplasts from Homogenates. Electron micrographs of chloroplasts from the intact leaf, the 0.35 M NaCl-Tris, and the NaCl-free homogenates are presented in figure 1. The chloroplast, in situ, is distinguished by a distinct outer limiting membrane, osmiophilic ground substance or stroma, and grana in a characteristic lamellar configuration (fig 1a). Isolation of chloroplasts in 0.35 M NaCl results in rupture of the limiting membrane, removal of the ground substance, and swelling and breaking of the lamellae and grana (fig 1b). We have estimated that 80 % or more of the grana are disrupted or destroyed in such a preparation. In our homogenate, we have maintained better than 90 % of the grana intact. The chloroplast membrane has been destroyed with the loss of much of the stroma. However, the intergrana lamellae are not appreciably altered (fig 1c).

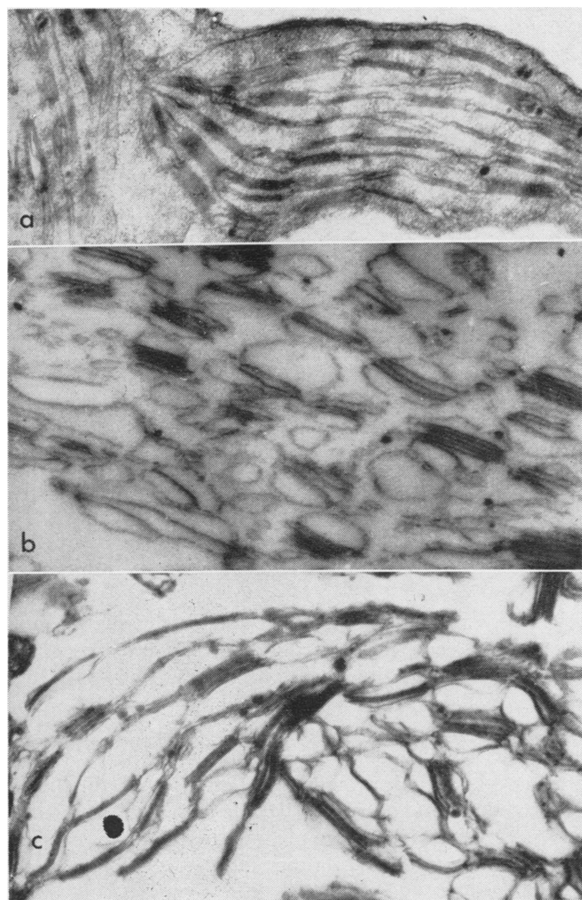

FIG. 1. Electron micrographs, at 40,000 X, of spinach leaf chloroplasts. a, Intact chloroplast in situ. Note limiting membrane and denser ground substance. b, Isolated chloroplasts prepared in 0.35 M NaCl-Tris. Note loss of limiting membrane, ground substance and intergrana lamellae. Grana integrity is distorted or destroyed. c, Isolated chloroplasts prepared in absence of NaCl or dilution. Note loss of limiting membrane and ground substances but retention of lamellar and grana configuration.

Discussion

The work presented here will be considered within the framework of 3 aspects of our total knowledge of photosynthesis. Of primary importance is the consideration of the integrity of the organelle system. Chloroplasts appear to maintain their own particular functions in photosynthesis only when they exist in the same condition as within the cell. The second concept which emerges from these experiments is that the ribulose-diP occupies a unique role as an intermediate in the photosynthetic process. Its interaction with ATP and reducing power serves as a biochemical switching mechanism to lead the CO_2 into the carbohydrates and sugar phosphates. The third aspect is the subcellular localization of ATP and reduced pyridine nucleotides and the concomitant problem of the flux of these important intermediates across subcellular membrane boundaries.

Much of our knowledge concerning mechanisms of photophosphorylation and photoreduction have been obtained using isolated chloroplast preparations in salt solutions and with extensive dilution (1, 9, 10, 13, 32, 33). It has been tacitly assumed that such chloroplasts were representative of the photosynthesis systems operative in the intact cell. Admittedly rates of CO_2 fixation were extremely low, and no attempt was made to evaluate the distribution of the isotope activity into compounds other than the carbohydrates and their derivatives (1, 32, 33). In no case was a careful comparison made between the distribution of CO_2 in these chloroplast preparations and that found in the intact leaf.

Kahn and Von Wettstein (14), using phase contrast and electron microscopy, examined the salt prepared chloroplast. Although they were able to find a few chloroplasts which seemed intact they did not directly correlate their preparations with a simultaneous study of the biochemical properties. Examination of our electron microscope plates revealed the striking changes obtained when a leaf is ground in a buffer and NaCl solution in a fashion which heretofore had been considered to yield optimal chloroplasts. There is not only a complete rupture of the outer membrane of the chloroplast, but a total leaching of the ground substance material. Thylakoids are swollen and broken. Expected changes in permeability properties of these thylakoids and/or loss of enzymatic activity would be serious. In NaCl-free homogenates prepared by the technique described here, there is still considerable loss of ground substance. The membrane is ruptured, but the integrity of the thylakoids and the lamellae are relatively unchanged.

The description of the NaCl preparation is in accord with the data of others (12, 24, 30). These investigators had shown considerable loss of critical enzymatic activities in the chloroplasts upon preparation by the usual techniques.

Considerable changes are observed in the distribution of activity following fixation of $C^{14}O_2$ in the presence of high NaCl concentrations (18). A comparison of the NaCl and NaCl-free systems (table I) shows a 10-fold increase in the rate of CO_2 fixation by the NaCl-free preparation as well as an 8-fold increase of the label incorporation into sucrose.

The addition of ribulose-diP, ADP and $NADP^+$ in the light increases the rate of photosynthesis of the homogenate to 44 % of the intact leaf. This increase is accomplished with label moving out of glycerate-3-P into a pattern similar to the intact leaf. The addition of ribulose-diP alone increases the photosynthetic activity to 90 % of the intact leaf, however 60 % of the label remains in glycerate-3-P (unpublished experiments from this laboratory).

It has been patently accepted that the sole function of the light is to generate ATP and reduce pyridine nucleotide (5, 33). Given these 2 intermediates, all other aspects of the photosynthetic carbon cycle were considered to be metabolic mapping. It is clear from the results with our preparation, which in the presence of light carries out a photosynthetic-like fixation, that ATP and NADPH alone are insufficient to promote dark CO_2 incorporation into carbohydrates. Although glycerate-3-P is synthesized, apparently by the pathways involving ribulose-diP, the major fraction of the activity is in malate and related compounds. The reducing power from the pyridine nucleotides will not support the incorporation of CO_2 into carbohydrate. In experiments where ribose-5-P replaces ribulose-diP a pattern essentially equivalent to that of ATP and NADPH is found. Of a wide variety of sugars and sugar phosphates tested, only ribulose-diP serves as a precursor to the photosynthetic-like CO_2 fixation in the dark. This is particularly important to note in light of the experiments of Calvin and his colleagues (4), where the most dramatic shift from dark to light was the synthesis of the ribulose-diP. When the light was shut off, the ribulose-diP levels fell precipitously and glycerate-3-P was formed as a product. We interpret these results as indicating that the synthesis of ribulose-diP is carried out primarily within the thylakoids. The presence of ATP and ribose-5-P is not equivalent to ribulose-diP.

It is difficult to interpret the role of ATP in generating reducing power. A comparison of columns [6] and [7] in table II reveals a 4-fold increase in the rate of CO_2 fixation, but seems to prevent the reduction of glycerate-3-P to sugars. It is possible that the ribulose-diP serves as a direct donor for electrons in this process or that high concentration of NADPH are inhibitory. It is also possible that the ATP can give rise directly to reducing power by reversal of pathways of oxidative phosphorylation. A comparison of columns [7] and [12] gives some indirect evidence. In the light, 33 % of the label is in sugar phosphates; in the dark ribulose-diP and ATP yield 15 % of the label in sugar phosphates.

These experiments emphasize some basic problems with respect to the subcellular localization of ATP and reduced pyridine nucleotide. The total concentration of ATP and reduced pyridine nucleotide in the light-dark shift within intact leaves are relatively small. However, the compartmentation of these important nucleotides might well change. ATP produced within the thylakoids might be used preferentially at the site of its synthesis by enzymic systems which have no access to cytoplasmic ATP. Little is known about the transport of nucleotide triphosphates across membrane barriers. The recent experiments of Santarius et al. (28) suggest rapid movement of ATP across the chloroplast membrane. The prompt stimulation of active transport of ions by light in *Ulva* (29), assumed to occur via the utilization of ATP produced in the chloroplast by photophosphorylation, supports this view.

Striking differences in the mode of action of NADPH and NADH are seen in comparing columns [9] and [10] of table II. Although there is a slight enhancement of total CO_2 fixation with NADH, there is a ten-fold enhancement of the activity in the sugar fraction by the NADPH. It seems quite clear that in this system, as has been observed in several other metabolic pathways, the NADPH is operative in the anabolic system while the NADH operates on the catabolic side. Where both pyridine nucleotides are added simultaneously (column [11]), there is a vigorous enhancement of CO_2 fixing rate, but profound inhibitions in the incorporation of activity into the sugar fractions.

The homogenate preparation developed in this laboratory constitutes a useful system with which to study the synthetic relationships of photosynthesis, particularly with respect to understanding the products of the light reaction as they directly affect the incorporation of $C^{14}O_2$ into the various metabolic products. The results confirm and extend the elegant studies of Leech (19) on the importance of integrity in the phosphorylation reaction, and of Gibbs (10, 11) and his collaborators with respect to the distribution of label in the various compounds produced in a so-called whole chloroplast and reconstituted broken preparations. Our results also imply that the pyridine nucleotides may not be the essential reducing system for the carbon dioxide. Other electron carrier systems may be functioning in the intact lamellae membrane.

Summary

The preparation of a cell-free homogenate from spinach leaf able to incorporate $C^{14}O_2$ in both light and dark in a fashion which resembles the distribution of label as seen in the intact leaf is described. The rates observed for CO_2 fixation in the light in the cell-free homogenate without added cofactors are 11 % that of the intact leaf. The addition of ribulose-

diphosphate, adenosine diphosphate, and triphosphopyridine nucleotide increase this to 44 % of the intact leaf. Important features in the method of preparation involve avoiding both dilution during grinding and the addition of sodium chloride, commonly added, to regulate osmolarity.

Several cofactors were added to this system in the dark in an attempt to define intermediates produced during the light reaction which might be directly involved in CO_2 reduction. Ribulose diphosphate and adenosine triphosphate were found to be the most effective. Both the rate and the distribution of activity paralleled the results observed for the homogenate in the light. Other sugars and sugar phosphates could not replace ribulose diphosphate in this activity. Reduced tri- and diphosphopyridine nucleotides seem to act by quite different mechanisms. Reduced triphosphopyridine nucleotide is more effective for incorporation of activity into the free sugar fraction. Reduced diphosphopyridine nucleotide seems to block incorporation of activity into carbohydrates of any kind.

The morphology of chloroplasts prepared by this procedure and of those isolated in 0.35 M NaCl has been examined and compared using electron microscopy. The integrity of subcellular structure is radically altered in the presence of NaCl and dilution but, in a large part, is retained by the NaCl-free preparation.

Acknowledgments

The authors wish to thank Drs. Arvan L. Fluharty and A. A. Benson for their critical reviews and discussion of the experiments, the results, and their interpretation.

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Changes in Abscission Processes with Aging[1, 2]

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As leaves grow older they show increasing tendencies for abscission. With aging there are changes in the substances which can regulate abscission, such as a decline in the auxin level (9, 15), increases in the amino acids (17, 19, 11), and increases in extractable abscission-stimulating factors (10, 3, 4, 16). More recently the physiological processes leading to bean leaf abscission have been separated into 2 successive stages (13) and the present study aims to analyse the increasing tendencies for abscission as they may relate to the completion of the 2 successive stages.

Materials and Methods

The abscission tests were carried out in the manner described by Rubinstein and Leopold (13) and Chatterjee and Leopold (5). Seedlings of *Phaseolus vulgaris* cultivar red kidney were grown in vermiculite under controlled environment condition of 2000 ft-c, 23 ± 2° and 16-hour photoperiod for different durations of time. In order to compare leaves of different ages, primary leaves were selected from seedlings 9 to 10 days old (designated as A stage), 15 to 16 days old (B stage), 21 to 22 days old (C stage), and 28 to 29 days old (D stage). As the primary leaves expand at about 7 days from sowing, the actual leaf ages are about 3, 9, 15 and 22 days for the stages A, B, C, and D respectively.

For the explant abscission test, 1 cm petiole pieces were cut from leaves of A, B, and C stages, consisting of 5 mm of tissue on each side of the upper abscission zone. Substances to be tested were combined with 1 % agar solution and poured into petri dishes to a depth of 4 mm. The proximal ends of 10 petiole explants were then inserted into the agar and the dishes returned to the controlled environment chamber at a light intensity of 400 ft-c.

For the intact petiole abscission test, stem cuttings were taken from the kidney bean plants of the 4 different ages (A, B, C, and D), the hypocotyls were trimmed to a length of 10 cm and the basal end placed in distilled water. The primary leaves were cut off leaving approximately 2.8 cm of the petiole and the lower abscission zone intact on the plant. Substances were applied to the cut surface of the petiole as lanolin pastes. The plants were kept in the controlled environment chamber (400 ft-c) during the course of the experiments.

Abscission was determined visually as the development of a ring of cell separation at the abscission zone. Readings were taken every 12 hours and the time required for abscission of 50 % of the 10 pieces was taken as the experimental result. The least significant difference (LSD 5 %) between treatments in the abscission test varied from 19 to 21 hours. All experiments reported here were repeated at least 3 times with consistent results.

[1] Revised manuscript received April 28, 1964.
[2] Journal paper 2155, Agricultural Experiment Station, Purdue University, Lafayette, Indiana.