NORMAL PHOTOSYNTHESIS BY ISOLATED CHLOROPLASTS

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Numerous attempts have been made to prepare isolated chloroplasts which are capable of carrying on the complete process of photosynthesis at the same rates and with the same products as intact plants. Preparations have been made which evolve O_2 at rates comparable with intact tissues, but which require artificial or added electron acceptors, and for which the products of carbon fixation, if it occurs, are not known (Walker et al. 1967, Arnon et al. 1964). Preparations have been made which fix CO_2 but do not have normal patterns of photosynthetic products or require the addition of cofactors (Walker and Hill, 1967). Two laboratories have reported preparations which produce some of the normal photosynthetic intermediates, but either their rates of photosynthesis are not equal to those of intact tissues, or else they fall off rapidly with time (Everson et al. 1967, Jensen and Bassham, 1966). We wish to report a preparation of chloroplasts which is capable of carrying on photosynthetic gas exchange at rates which may exceed those of intact plants, whose photosynthetic products are essentially identical with those of intact plants and which can maintain maximum rates for periods of several hours.

The organism used is the giant algal cell, Acetabularia mediterranea, which is maintained in laboratory culture by methods similar to those of Keck (1964). The details of the culture technique will be published elsewhere (Shephard, 1968). These cultures are not truly axenic, but strenuous efforts are made to monitor the contamination and keep its level below the limits of error of

the assays performed on the cells. We find no increase in dark respiration of the chloroplast fraction when substrates such as glucose are added, and no unicellular algae are seen during hemacytometer counts of this fraction. Contamination by blue-green algae has been eliminated and contamination by green algae, if it occurs, is at a level below one algal cell per 10,000 chloroplasts.

The cells used are 15 to 25 mm in length and are in the elongation phase of their growth. The fractionation scheme is given in Figure 1. Practically no chlorophyll is lost to the supernatants and the final pellet represents about 50% recovery of the original chlorophyll in intact-appearing (Leech, 1964) chloroplasts. There is little contamination by other cytoplasmic structures except starch grains. The presence of some mitochondria cannot be ruled out but the addition of Krebs' cycle substrates and ADP does not increase dark respiration. The final pellet from 1 g of cells contains about 250 µg chlorophyll as determined photometrically (Arnon, 1949).

Acetabularia is a marine alga and the chloroplast isolation procedure developed for it may not be suitable for use with other plant tissues. The success of this proceedure may depend largely on the fact that scissor mincing cuts each Acetabularia cell into several small fragments from which the cytoplasm flows spontaneously. We were not able to obtain active chloroplasts from pea leaves disrupted with a ground-glass tissue grinder using these media, but the use of such a grinder on uncut Acetabularia cells also results in a major loss of activity. Activity of the chloroplasts is also destroyed by the following: dilution of the medium, centrifugal forces in excess of 2,000 x g., phosphate ion concentration above one millimolar, inadequate pH control, bubbling gas through the suspension, addition of detergent (0.01% triton X-100) or substituting 0.3-0.5 M NaCl for the mannitol. The Acetabularia chloroplasts, therefore, do not appear to be exceptionaly resistant to damage, and phase microscopy shows no unusual features of size or appearance.

Experiments were conducted to compare photosynthesis in cells and chloro-

plasts. Twenty cells (approximately 0.1 gram fresh weight) and a comparable sample of isolated chloroplasts each suspended in 5 ml of the appropriate medium buffered at pH 7.45 containing bicarbonate (1 mM), were placed in 15 ml. centrifuge tubes on a platform rocker. 4.0 μc of $^{14}CO_2$ in 1 cc of N_2 were injected through a serum stopper into each tube, and the tubes were gently rocked under 1500 ft-c illumination (cool white and high-output fluorescent) for 10 minutes. Then the chloroplasts were quickly collected on a membrane filter (Gelman Versipor type 6429) and extracted by washing three times in boiling 85% v/v ethanol. Cells were collected in the same manner, except that they were disrupted in an ice-cold glass homogenizer prior to filtration and alcohol extraction. The homogenization added only 15 seconds to the total time for these samples. The alcohol extracts were adjusted to identical chlorophyll concentrations (optical densities) and the total alcohol soluble radioactivity was determined from an aliquot. The radioactivity of the alcohol insoluble material remaining on the filter was determined after combustion in a Nuclear Chicago Dynacon (Bidwell, 1963). Equal aliquots of the alcohol extracts were

Table 1. Products of 10 minutes photosynthesis in $^{14}CO_2$ by whole plants or isolated chloroplasts of Acetabularia mediterrania.

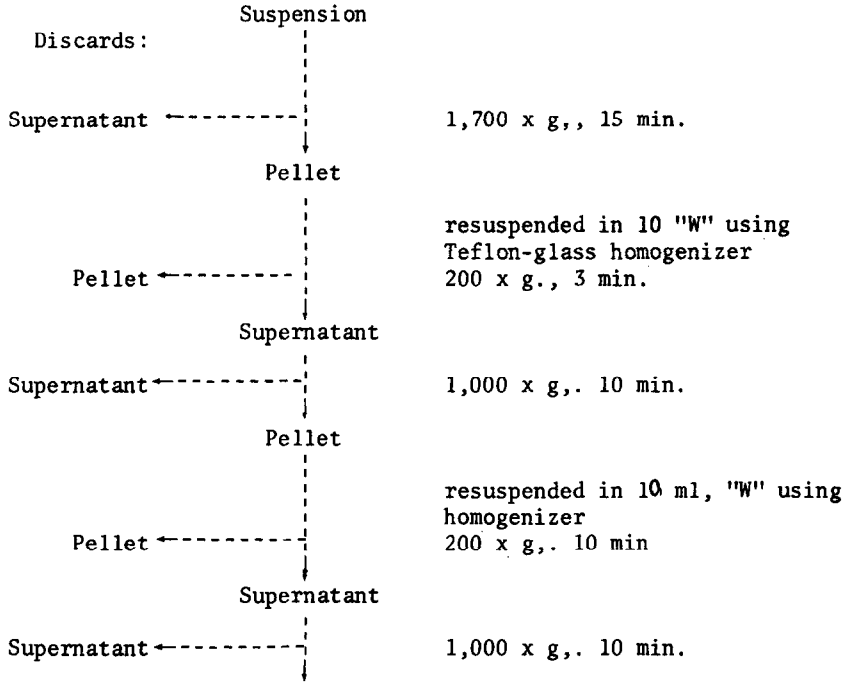
	Whole Cells		Chloroplasts	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
Alcohol Soluble ^{14}C mμc/mg Chl.	264	303	202	261
Alcohol Insoluble ^{14}C mμc/mg Chl.	303	-	285	296
Alcohol Soluble Compounds, as % of total soluble radioactivity:				
Sugar phosphates*	8.3	6.8	5.0	8.6
Phosphoglyceric acid	5.9	4.4	4.5	5.1
Glycollic acid	9.4	7.8	11.0	-
Malic acid	2.6	4.4	3.1	-
Sucrose	31.6	13.7	20.8	31.9
Glucose	14.8	21.9	17.6	13.9
Aspartic acid	8.9	19.0	12.8	12.3
Glutamic acid	5.9	-	2.7	6.7
Alanine	12.6	22.1	22.4	16.5

* Largely hexose monophosphates and ribulose diphosphate

concentrated in vacuo and subjected to paper chromatography and radioautography (Bidwell, 1963), and the radioactivity of the identified spots was measured

Figure 1. Fractionation

Scissor mince 1 g fresh weight of cells in 2 ml homogenizing (H) medium. The resulting slurry is strained through 173 mesh bolting silk and the debris rinsed with 8 ml. washing (W) medium. The suspension is further dispersed with a Teflon-glass homogenizer. All operations are carried out at 4° C.



Composition of Media Used

	Mannitol	EDTA*	BSA*	TES*	DTT*	KCl	$MgCl_2$	KH_2PO_4	pH*
H	.6	10^{-3}	0.1%	10^{-1}	10^{-3}	-	-	-	7.8
W	.6	10^{-3}	0.1%	$5x10^{-3}$	10^{-4}	-	-	-	7.2
A	.6	-	-	$5x10^{-3}$	-	10^{-2}	$5x10^{-3}$	10^{-3}	7.2

* EDTA= disodium ethylenediaminetetearacetate, BSA-bovine serum albumin.

TES=n-tris(hydroxymethyl) methyl-2-aminoethane sulfonic acid,

DTT-dithiothreitol. pH-adjusted with KOH.

(Bidwell, 1961). The radioactivity of individual compounds was calculated as a percentage of the total soluble radioacitvity. All these data presented in Table 1. A typical radioautograph of the products of chloroplast photosynthesis is presented in Figure 2. It may be seen that the total amount of

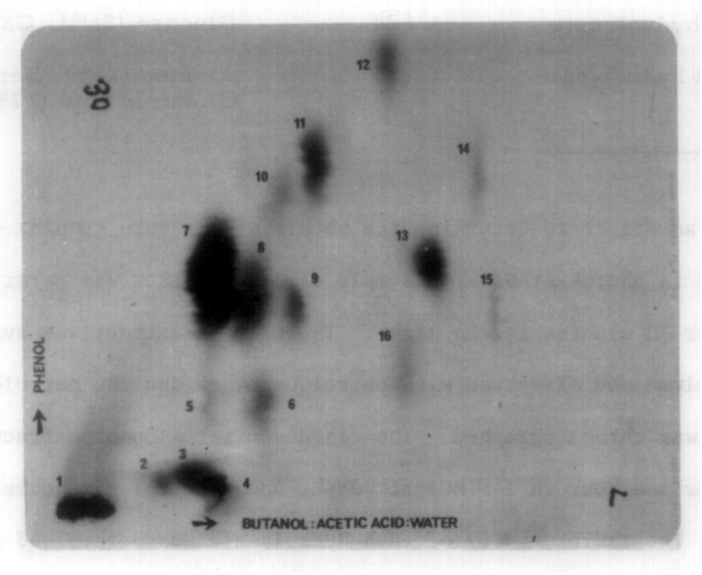


Figure 2. Photosynthetic Products of Isolated Chloroplasts

An aliquot of chloroplast suspension was removed from the incubation medium containing $^{14}CO_2$ after 30 minutes of illumination, applied directly to the paper and exposed to hot ethanol vapors. The chromatogram was run in the indicated solvents and radioautographed.

1. Origin (insoluble material), 2. Ribulose diphosphate, 3. Hexose monophosphate, 4. Phosphoglyceric acid, 5. Unknown, 6. Aspartic acid, 7. Sucrose, 8. Serine and Glycine, 9. Glutamic acid, 10. Unknown, 11. Alanine, 12. Unknown, 13. Glycolic acid, 14. Succinic acid, 15. Fumaric acid, 16. Malic acid.

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^{14}C fixed in the alcohol soluble and insoluble fractions of the cells and chloroplasts were about the same, and the spectra of alcohol soluble products of photosynthesis were essentially identical.

Table 2. Products of photosynthesis for 70 minutes in $^{14}CO_2$ by isolated Chloroplasts of Acetabularia mediterrania.

Fraction	^{14}C, mμc	Major Labelled Components
Alcohol Soluble	3040	organic phosphates (17%), sugars (49%), amino acids (30%), organic acids (11%)
Pet. Ether Soluble	349	mostly chlorophyll
Weak HCl hydrolysate	624	Glucose (56%), Galactose (44%)
Strong HCl hydrolysate	135	Alanine (21%), Serine & Glycine (40%), Glutamic acid (17%), Leucine (22%)

In an effort to determine the nature of the more complex compounds produced in photosynthesis, a sample of chloroplasts was permitted to fix $^{14}CO_2$ for 70 minutes in the light. The alcohol extract was evaporated to a small volume and extracted with petroleum ether and the petroleum ether extract was chromatographed. The residue after alcohol extraction was hydrolyzed for one hour in 1 N HCl at 90° C, and then for six hours in 3 N HCl at 105° C. The radioactivity of each hydrolysate was determined and aliquots were chromatographed. The radioactivity of each fraction is presented in Table 2 together with a list of the major labeled compounds found in them. Since both chlorophyll and chlorophillide or pheophorbide were labeled it is evident that the isolated chloroplasts are capable of synthesizing chlorophyll. The products in the hydrolysates indicate that chloroplasts are able to synthesize both polysaccharides and protein.

Gas exchange measurements are being made as follows: O_2 is measured polarographically (Estabrook, 1967), and CO_2 is measured in a closed circulating system by following the disappearance of $^{14}CO_2$ with a Geiger counter or by using a Beckman infrared CO_2 analyzer (Bidwell et al. 1968). Measurements made on cells and chloroplasts are presented in Table 3, and show that rates of gas exchange are similar. These rates have been observed to continue for six hours, the duration of the longest experiments that have been performed

Table 3. Rates of photosynthetic gas exchange by whole plants and isolated chloroplasts of Acetabularia mediterranea. The rates, presented as µl O_2 or CO_2 per min. per mg chlorophyll, were calculated from the slopes of the linear portions of continuous recordings after the initial several minutes required for equilibration. Individual measurements were continued for periods ranging from 10 minutes to several hours.

		Whole Cells	Chloroplasts
Expt. 1	O_2	13.8	21.4
	CO_2	19.1	16.4,18.7
Expt. 2	O_2	-	15.8
	CO_2	-	11.6
Expt. 3	O_2	10.2	21.7
Expt. 4	O_2	26	17.6

to date. The evolution of O_2 is unaffected by the addition of phosphoglyceric acid, glycolic acid, ribulose diphosphate, fructose diphosphate, ferredoxin, NADP or ADP when adequate bicarbonate is present. In the absence of bicarbonate, added phosphoglycerate will support near maximum rates of O_2 evolution. The rate of O_2 evolution is proportional to the bicarbonate concentration over the range from near 0 to approximately 2 mM at pH 7.2.

Experiments are now in progress on the biochemical and physiological aspects of photosynthesis in these chloroplasts, and on the kinetics of their reaction systems.

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