

CHLOROPLAST STRUCTURE AND FUNCTION
IN *ac-20*, A MUTANT
STRAIN OF *CHLAMYDOMONAS REINHARDI*

I. CO₂ Fixation and
Ribulose-1,5-Diphosphate
Carboxylase Synthesis

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ABSTRACT

A mutant strain of the green alga *Chlamydomonas reinhardi*, *ac-20*, is described in which both the rate of CO₂ fixation by whole cells and the rate of carboxylation of ribulose-1,5-diphosphate in cell-free extracts are reduced, particularly when sodium acetate is present in the growth medium. Of the enzymes of the reductive pentose phosphate cycle tested, only ribulose-1,5-diphosphate carboxylase activity is reduced in the mutant strain, and it appears that the low carboxylase activity limits the strain's rate of photosynthetic carbon metabolism. Evidence is presented to show that the fluctuation in the level of the enzyme activity in the presence or absence of acetate results from the fluctuation in the level of some factor(s) limiting the rate of synthesis of the protein.

INTRODUCTION

This paper is the first of a series (12, 19) that presents the photosynthetic and structural properties of *ac-20*, a mutant strain of the unicellular green alga, *Chlamydomonas reinhardi*. The *ac-20* mutation apparently affects a single nuclear gene that has been mapped to linkage group XIII of the alga's genome (15), and yet the biochemical and morphological consequences of the mutation are complex. The effect of the mutation on the strain's ribulose-1,5-diphosphate (RuDP) carboxylase activity was the first to be described (20). The present paper extends these initial observations and describes more recent experiments, in which it is demonstrated that the synthesis of RuDP carbox-

ylase in the *ac-20* strain is specifically sensitive to growth in the presence of sodium acetate. The second paper in the series (19) describes a similar acetate sensitivity exhibited by certain components of the mutant strain's photosynthetic electron-transport chain, components that are closely associated with Photochemical System II. The third paper (12) describes the fine structure of the *ac-20* chloroplast in the presence and absence of acetate in the growth medium. It is shown that chloroplast membrane organization, pyrenoid formation, and chloroplast ribosome formation are all defective in the acetate-grown cells. Possible interpretations of the various *ac-20* phenomena

are reserved for the final paper, where it is proposed that the initial *ac-20* lesion somehow affects the formation of chloroplast ribosomes.

MATERIALS AND METHODS

Organisms and Culture Conditions

Organisms used in these experiments were the wild-type strain 137c of *C. reinhardi* and the mutant strains *ac-20*, *ac-141*, and *ac-206*, derived from the wild type by ultraviolet irradiation followed by a screening test for $^{14}\text{CO}_2$ fixation (17). A double mutant strain *arg-2 ac-20* was obtained by crossing an arginine auxotroph, *arg-2*, with *ac-20*. A diploid strain carrying both *ac-20* and wild-type alleles for the *ac-20* locus was obtained by crossing an arginine auxotroph, *arg-7*, with the double mutant *arg-2 ac-20* and selecting for colonies growing on non-arginine-supplemented acetate plates (7).

Liquid cultures of these strains were maintained at 25°C and agitated on rotary shakers. The culture medium was either a minimal inorganic salt medium (26) or the same minimal medium supplemented with 0.2% (w/v) sodium acetate. Cells grown in the light (4000 lux from daylight fluorescent lamps) on minimal medium are referred to in the text as phototrophic cells. Cells grown in the light on the acetate medium are referred to as mixotrophic cells. Cells grown in the dark on acetate medium are referred to as heterotrophic cells.

CO₂ Fixation

The rate of $^{14}\text{CO}_2$ fixation by whole cells was measured in an apparatus that consisted of a series of test tubes connected to a Vortex mixer (Scientific Industries, Inc., Springfield, Mass.) through a metal bar, such that vibrations transmitted to the bar effected a mixing of the cell suspensions within the test tubes. The lower ends of the tubes were immersed in a 25°C water bath. The tubes were illuminated from below, through a water-cooled heat filter, by a tungsten flood lamp that provided an incident light intensity of 40,000 lux.

1 ml of a cell suspension in minimal medium that contained $1-3 \times 10^6$ cells was placed in each tube and shaken in the light for 5 min. At this time, a $\text{NaH}^{14}\text{CO}_3$ solution (0.1 ml containing 5 μmoles and 2.5 μCi) was injected into the cell suspension. At prescribed time intervals, the reaction was stopped by turning off the light and by injecting glacial acetic acid (0.5 ml) into the tubes. Aliquots of 0.1 ml were plated on aluminum planchets, dried under a lamp, and counted with the aid of a gas flow counter. Tubes wrapped in double layers of aluminum foil and treated as above served as dark controls.

Light intensity and $\text{NaH}^{14}\text{CO}_3$ concentrations

were above saturation levels in these experiments, and the rate of $^{14}\text{CO}_2$ fixation was proportional to chlorophyll concentration and to the time of exposure of cells to $\text{NaH}^{14}\text{CO}_3$, up to 15 min. Routinely, $^{14}\text{CO}_2$ fixed in the light for the first 5 min, corrected for the dark rate, was used as the measure of the rate of $^{14}\text{CO}_2$ fixation.

Enzyme Activity

Cell-free extracts for enzyme assays were prepared by suspending washed cells in Tris-HCl buffer (pH 7.5, 0.005 M) and subjecting them to sonication at 0°C for 90 sec with a Mullard 20 KC ultrasonic disintegrator (Measuring and Scientific Equipment Ltd., London, England). The preparations of broken cells were centrifuged at 30,000 *g* for 40 min at 0°C, and the pale-green supernate was used as a crude cell-free extract.

RuDP carboxylase activity was measured as $^{14}\text{CO}_2$ fixed by an extract in the presence and absence of RuDP, as described previously (20). Glycerate-3-P or phosphoenolpyruvate were substituted for RuDP to check the secondary carboxylation reactions. These reactions were found to be negligible under the experimental conditions used.

Phosphoribulokinase was assayed according to the method of Fuller et al. (10). Phosphoriboisomerase was assayed according to the method of Axelrod (3). Phosphoglycerate kinase was assayed in the forward direction by the method of Bücher (4). Triosephosphate dehydrogenase, coupled to NADP and NAD, was assayed by the method of Gibbs (11). Triosephosphate isomerase was assayed by the method of Cooper et al. (5). Fructose-1, 6-diphosphate aldolase was assayed according to the method of Wu and Racker (27).

Protein was determined by the Folin method (21). Chlorophyll was determined by a modification (2) of the procedure of Mackinney (22).

Gel Electrophoresis

Cell extracts were prepared as above and subjected to polyacrylamide gel electrophoresis according to the method of Davis (6). After electrophoresis, each of the gel samples being compared was placed on a flat surface, and all were aligned by the position of a marker dye. One of the sample columns was then stained to serve as the reference for the position of the protein bands, and each of the remaining columns was cut into 10 5-mm sections. Comparable sections from each column were placed in a small test tube where they were dispersed with a glass rod. They were extracted for 30 min in 2 ml of 0.1 M Tris-HCl buffer, pH 7.5. Following extraction, the gel particles were allowed to settle to the bottom of each tube, and the supernates were tested for RuDP carboxylase activity.

TABLE I
Photosynthetic CO₂ Fixation and RuDP Carboxylase Activity in Wild-Type and Mutant Strains of *C. Reinhardi*

Strain	Growth condition	Chlorophyll/ cell	CO ₂ fixation		RuDP carboxylase activity		
			μmoles CO ₂ fixed/		μmoles CO ₂ fixed/		
			μg/10 ⁶ cells	10 ⁸ cells per hr	10 ⁸ cells per hr	mg chlorophyll per hr	mg protein per hr
Wild type	Phototrophic	2.4	28.8	120	3.95	16.5	1.49
	Mixotrophic	3.5	35.5	100	3.00	8.5	0.90
	Heterotrophic	1.1	7.7	70	2.10	19.0	1.25
<i>ac-141</i>	Mixotrophic	2.0	—	1	3.24	16.2	0.84
<i>ac-206</i>	Mixotrophic	1.8	—	<1	3.55	19.7	0.90
<i>ac-20</i>	Phototrophic	1.2	7.2	60	0.44	3.7	0.15
	Mixotrophic	2.4	0.7	3	0.05	0.2	0.03
	Heterotrophic	0.7	0.3	4	0.04	0.6	0.03

RESULTS

Table I shows rates of CO₂ fixation and RuDP carboxylase activity for wild-type and several mutant strains of *C. reinhardi* under various growth conditions. The amount of chlorophyll per cell and the size of cells in *C. reinhardi* cultures often varies appreciably from one strain to another and even from one culture to the next for a given strain. For this reason, reaction rates are given on a chlorophyll, cell, and protein basis in Table I. It is seen that RuDP carboxylase activity is consistently somewhat higher in phototrophic wild-type cells than in mixotrophic wild-type cells, indicating that the presence of sodium acetate in the growth medium has a slight inhibitory effect. Mixotrophic and heterotrophic wild-type cells exhibit similar RuDP carboxylase activities on a protein basis, indicating that the presence of light is not required for the synthesis of this enzyme in *C. reinhardi*.

The RuDP carboxylase activity of two mutant strains of *C. reinhardi*, *ac-141* and *ac-206*, was also examined and was found to be similar to that of the phototrophic wild type on a chlorophyll, cell, and protein basis (Table I). Thus, the slight inhibitory effect of mixotrophic growth on wild-type RuDP carboxylase activity is not observed with these mutant strains; no explanation can be offered for this repeatedly observed discrepancy. The *ac-141* and *ac-206* strains carry mutations that completely block photosynthetic electron transport (13, 18). Their capacity to fix CO₂ is therefore nil, but their levels of RuDP carboxylase are nevertheless normal, indicating that the absence of an

intact photosynthetic electron-transport chain has no effect on the level of this enzyme.

RuDP Carboxylase Activity: ac-20

Rates of CO₂ fixation and RuDP carboxylase activity for phototrophic cells of *ac-20* are substantially lower than for the phototrophic wild type (Table I). CO₂ fixation is reduced 4-fold on a cell basis, and RuDP carboxylase activity is reduced 10-fold on a protein basis. This slow rate of CO₂ fixation is evidently sufficient to support the slow (doubling time of 18 hr) phototrophic growth of *ac-20*.

Mixotrophic cells of *ac-20* contain more chlorophyll than phototrophic cells (Table I) and are capable of much more rapid growth (doubling time of 10 hr). However, their capacity to fix CO₂ is drastically reduced (Table I). This reduction can be attributed, at least in part, to a similarly drastic reduction in RuDP carboxylase activity. Compared with phototrophic *ac-20*, the enzyme activity of mixotrophic *ac-20* is reduced 10-fold, and compared with mixotrophic wild-type cells the enzyme activity is reduced 40-fold (Table I).

Some component enzymes of the reductive pentose phosphate cycle were tested to determine whether their levels were also affected in the *ac-20* strain grown in the presence of acetate. When wild-type and mixotrophic *ac-20* cells are compared (Table II), it is seen that only RuDP carboxylase activity is sufficiently reduced to account for the 30-fold reduction in CO₂ fixation rates exhibited by the mutant strain. All of the other enzymes tested show comparable activities for both wild type and *ac-20*.

TABLE II
Specific Activity of Some of the Enzymes of the Reductive Pentose Phosphate Cycle in Mixotrophically-Grown Wild Type and *ac-20*

Enzyme	Activity	
	wild type	<i>ac-20</i>
	μmoles/hr per mg protein	
Phosphoriboisomerase	123	158
Phosphoribulokinase	97	72
RuDP carboxylase	0.9	0.03
3-PGA kinase	472	480
G-3-P dehydrogenase (NAD)	45	40
G-3-P dehydrogenase (NADP)	43	74
Triosephosphate isomerase	120	95
FDP aldolase	89	75

Transfer Experiments

The selective reduction in the activity of RuDP carboxylase in mixotrophic *ac-20* cells could result either from a reduction in the total amount of enzyme present or, alternatively, from an inhibition of the enzyme's activity. For example, acetate or some product(s) of acetate metabolism either could inhibit the synthesis of the protein or could directly inhibit the activity of the enzyme.

Three different kinds of experimental approaches were used to distinguish between these two alternatives. Two approaches, to be described in the following sections, involved *transfer experiments*. A transfer experiment is performed as follows: a culture of mixotrophic cells is washed with minimal medium and resuspended in two flasks of minimal medium; one flask is returned immediately to the light (a *light-to-light* transfer), and the other is aerated in the dark for 12 or 16 hr (a *light-to-dark* transfer) before being exposed to light. Cells maintained in the dark on minimal medium are in effect being starved, for *C. reinhardi* is apparently incapable of utilizing its starch reserves in the dark (12).

The transfer of mixotrophic cells to minimal medium in the light permits them to "recover" from their acetate-induced photosynthetic disabilities and to acquire the photosynthetic capacity of phototrophic cells. The kinetics of this recovery in a light-to-light transfer experiment are indicated by the open circles in Fig. 1 (CO₂ fixation) and Fig. 2 (RuDP carboxylase activity). It is seen that a lag of ~8 hr occurs before either activity begins to show a substantial recovery, and that the

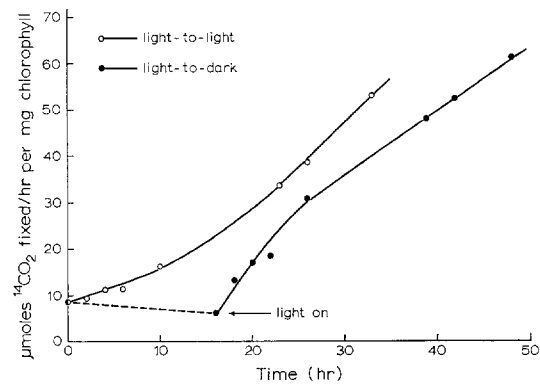


FIGURE 1 Recovery of CO₂ fixation by whole cells following the transfer of mixotrophic *ac-20* cells to minimal medium at 0 hr in concurrent light-to-light and light-to-dark transfer experiments.

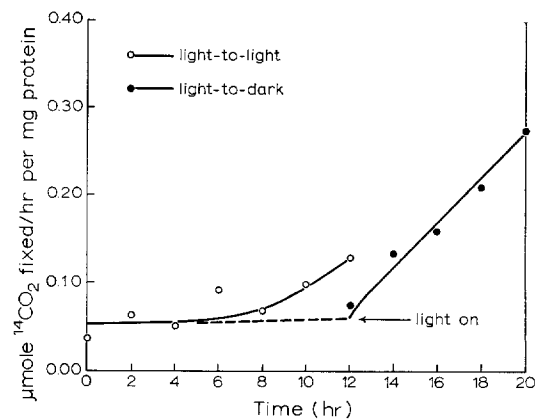


FIGURE 2 Recovery of RuDP carboxylase activity in cell-free extracts following the transfer of mixotrophic *ac-20* cells to minimal medium at 0 hr in concurrent light-to-light and light-to-dark transfer experiments.

rate of increase of CO₂ fixation rates does not reach a maximum before 12–16 hr after transfer.

At the end of a 12 or 16 hr dark incubation following a light-to-dark transfer, on the other hand, no change in either CO₂ fixation rates or carboxylase activity occurs (Figs. 1 and 2), closed circles). When these dark-incubated cells are returned to the light, however, both photosynthetic activities begin an immediate and linear increase (Figs. 1 and 2, closed circles). The lag observed in the light-to-light transfer is abolished by the dark incubation, and the initial rate of recovery is equivalent to the maximum rate attained by unstarved cells.

The *ac-20* recovery process is not accompanied by any substantial increase in chlorophyll, in con-

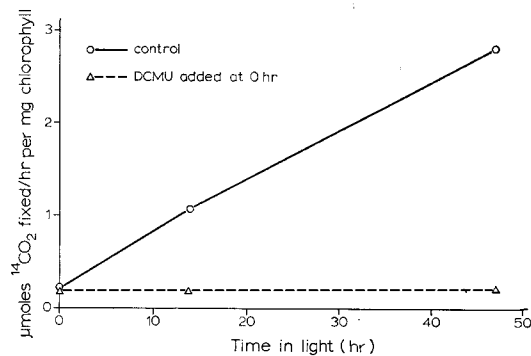


FIGURE 3 Effect of DCMU (10^{-5} M) upon the recovery of RuDP carboxylase activity in cell-free extracts from *ac-20* cells. Mixotrophic *ac-20* cells were subjected to a light-to-dark transfer for 16 hr. Just prior to exposing the cells to light (0 hr), DCMU was added to one flask.

trast to classical "regreening" systems. The chlorophyll content of a culture subjected to a transfer does not change during the course of the experiment. However, a cell division occurs during the first 6 hr of a light-to-light transfer such that the chlorophyll content *per cell* is reduced to the level of phototrophic cells (about half of that of mixotrophic cells, as seen in Table I).

These transfer experiments suggest that when mixotrophic cells are transferred to minimal medium, some light-independent process must occur before substantial RuDP-carboxylase recovery can begin. The recovery of enzyme activity itself, however, appears to be light-dependent. In accordance with this conclusion, it is found that if 1×10^{-5} M 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) is administered to a culture that has undergone a 16 hr light-to-dark transfer and the culture is then returned to the light, no increase in RuDP carboxylase activity occurs (Fig. 3). Since DCMU inhibits photosynthetic electron transport, at least a part of the light-dependent process apparently requires the full operation of the photosynthetic electron-transport chain.

Requirement for Protein Synthesis: Double Mutant Experiment

To determine whether the recovery of RuDP carboxylase in a transfer experiment is dependent on *de novo* protein synthesis or on enzyme activa-

tion, transfer experiments were performed with a double mutant strain, *arg-2 ac-20*. Cells of this strain were grown in the light in an arginine-supplemented acetate medium and were then subjected to a light-to-dark transfer to unsupplemented minimal medium. After a 16 hr dark incubation, the culture was divided into two flasks; arginine was added to one flask, and both were placed in the light. At intervals, RuDP carboxylase activity was measured.

Cells from both cultures showed a rapid initial increase in enzyme activity, probably reflecting the utilization of an endogenous pool of arginine. After a brief lag, enzyme activity in the arginine-supplemented cells increased linearly up to 48 hr (Fig. 4, curve A), whereas no further increase was observed in the arginine-starved cells (Fig. 4, curve B). If, after 48 hr, arginine was added to the arginine-starved culture, enzyme synthesis began, and after another 48 hr (96 hr after the start of the experiment), enzyme activity was found to be comparable to the level found in the arginine-supplemented cells after 48 hr (Fig. 4). Thus, the increase in enzyme activity was found to require arginine, suggesting that *de novo* protein synthesis and not a simple enzyme activation is involved in the process.

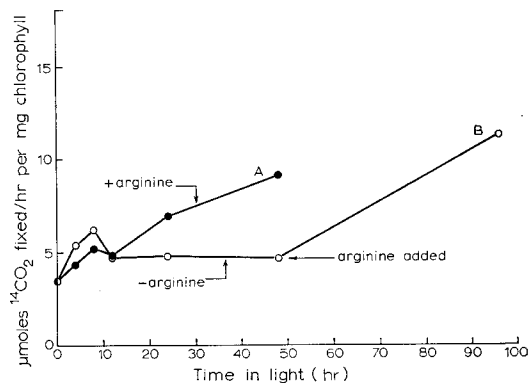


FIGURE 4 Effect of arginine deprivation upon the recovery of RuDP carboxylase activity in cell-free extracts from *arg-2 ac-20* double mutant cells. Cells were grown in arginine-supplemented (0.48 mM) acetate medium, washed, and transferred to minimal medium, and incubated in the dark for 16 hr (light-to-dark transfer). Arginine (final concentration 0.48 mM) was added to one flask just before the onset of illumination at 0 hr (curve A) and to the second flask after 48 hr of illumination (curve B).

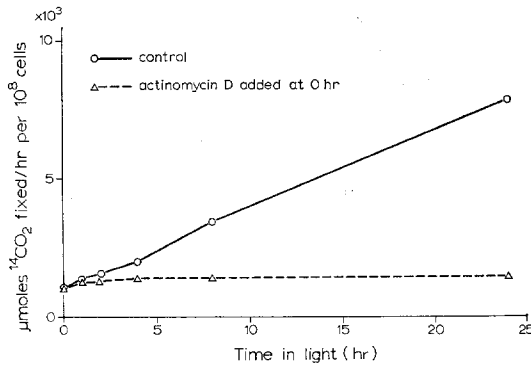


FIGURE 5 Effect of actinomycin D upon recovery of CO₂ fixation in whole cells of *ac-20*. Mixotrophic *ac-20* cells were subjected to a light-to-dark transfer for 16 hr. Just prior to exposing the cells to light (0 hr), actinomycin D (30 μg/ml of culture) was added to one flask.

Requirement for Protein Synthesis: Actinomycin D

A second demonstration that *de novo* protein synthesis is involved in RuDP carboxylase recovery was made with the use of actinomycin D, an inhibitor of RNA synthesis and hence of protein synthesis. A light-to-dark transfer experiment was performed. Following the 16 hr dark incubation period, actinomycin D (30 μg/ml) was added to the culture, and the culture was then exposed to light. The normal increase in the rate of CO₂ fixation in the subsequent light period was almost completely eliminated (Fig. 5). Similar results have been obtained with inhibitors of the translational steps of protein synthesis (experiments to be reported elsewhere).

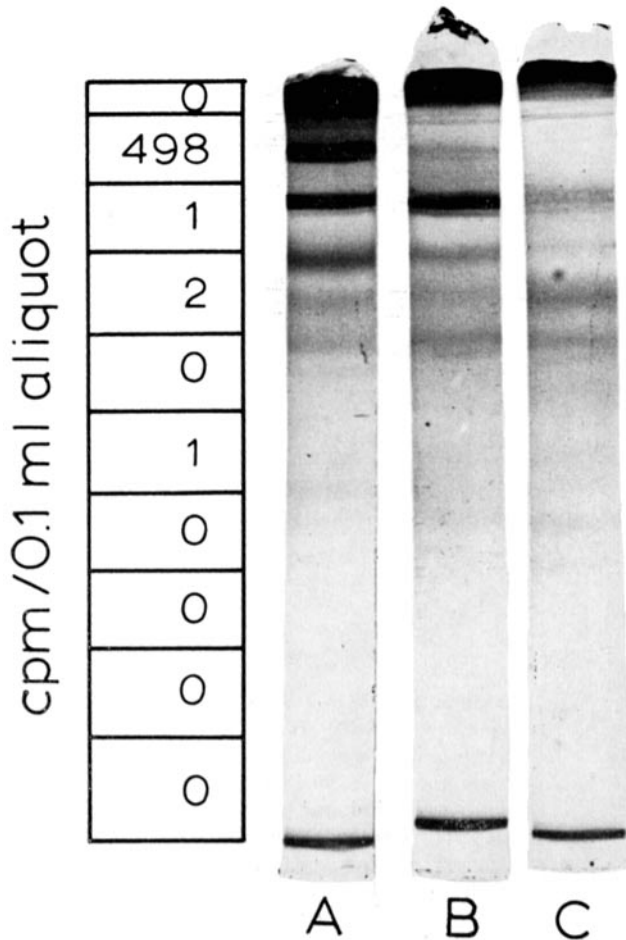


FIGURE 6 Polyacrylamide gel electrophoresis patterns of cell-free extracts from phototrophic cells of wild type (A), and *ac-20* (B), and mixotrophic cells of *ac-20* (C). The distribution of RuDP carboxylase activity along the gel column is indicated.

TABLE III
 $^{14}\text{CO}_2$ Fixation by *ac-20* Wild-Type Diploid Cells

Cell type	Growth condition	CO_2 fixation	
		$\mu\text{moles}/$ <i>mg</i> <i>chlorophyll</i> <i>per hr</i>	$\mu\text{moles}/$ <i>10⁸ cells</i> <i>per hr</i>
<i>arg-1 ac-20</i> × <i>arg-7</i> isolate 576.1	phototrophic	149	40
	mixotrophic	138	50
	phototrophic	196	69
	mixotrophic	92	80
<i>arg-2 ac-20</i> × <i>arg 7</i> isolate 578.2	phototrophic	188	59
	mixotrophic	100	30
	phototrophic	150	38
	mixotrophic	150	47

Absence of the Protein: Gel Electrophoresis

The RuDP carboxylase protein is present in large amounts in plant cells and can be readily visualized by gel electrophoresis. Extracts of *ac-20* and phototrophic wild-type cells were therefore compared by gel electrophoresis to determine whether the fluctuations in carboxylase activity in the mutant cells could be seen as fluctuations in the level of the protein itself.

The results of these experiments are shown in Fig. 6. The band corresponding to RuDP carboxylase in the wild-type gel (A) is identified by its carboxylase activity (see Methods). A faint carboxylase band is found in the gel obtained from phototrophic *ac-20* cells (B), and no band corresponding to the enzyme can be detected in the gel obtained from mixotrophic *ac-20* cells (C). These results again indicate that fluctuation in the activity of the enzyme is the consequence of fluctuation in the total amount of enzyme present rather than in its effective catalytic activity.

The Recessive Nature of the *ac-20* Mutation

The observations presented above suggested that in the mutant strain, the synthesis of the carboxylase enzyme is subject to regulation, control being more relaxed in the absence of acetate (or some acetate product) than in its presence. To test the possibility that the *ac-20* mutation might be a mutation to a regulatory state, vegetative diploid strains (7) were made that carry both *ac-20* and its wild-type allele. As seen in Table III, none of the four isolates of the diploid strain exhibits a

great reduction in CO_2 fixation when grown mixotrophically rather than phototrophically. This apparent dominance of the wild-type over the mutant phenotype is not consistent with a classical regulatory-gene hypothesis, for if *ac-20* cells produce repressor molecules that limit the rate of synthesis of RuDP carboxylase whereas wild-type cells do not, then the diploid cells should also produce repressor molecules, and the mutant phenotype should be dominant and not recessive. Other regulatory gene models could be proposed, but the hypothesis is inherently a complex one, for it is difficult to visualize how the unregulated synthesis of an enzyme could come under regulatory control as the consequence of single mutation.

DISCUSSION

Various environmental factors, such as the presence of an organic carbon source in the growth medium or the presence or absence of light, have been reported to affect the level of activity of RuDP carboxylase in photosynthetic and chemosynthetic bacteria (1, 16) and in green algae (10, 24). In most cases, the change in the level of activity of this enzyme was part of an over-all change in the photosynthetic apparatus, and in particular, changes in the amount of chlorophyll. (9). However, Russell and Gibbs (24) have shown that in *Chlamydomonas mundana* the presence of acetate in the medium reduces the level of activity of RuDP carboxylase and several other enzymes of the reductive pentose phosphate cycle without affecting chlorophyll content or the activity of the photosynthetic electron-transport chain. Similarly, Anderson and Fuller (1) have shown that the level of RuDP carboxylase and several other enzymes of the reductive pentose phosphate cycle in *Rhodospirillum rubrum* can be altered by the presence of acetate or malate in the growth medium.

The *ac-20* phenomenon in *C. reinhardi* is probably unrelated to either of these reports, for among the enzymes of the reductive pentose phosphate cycle tested, RuDP carboxylase is the only enzyme to be drastically affected. Moreover, photosynthetic electron transport (19) and chloroplast structure (12) are also affected by the mutation.

The experiments with *ac-20* reported in this paper demonstrate that if acetate is removed from the medium, RuDP carboxylase activity is partially, although never completely, restored. The restoration of activity is seen to involve two distinct

processes. The first process requires neither light nor active metabolism, for it occurs in starving cells that are maintained in the dark. Experiments to be reported in the third paper of this series (12) indicate that one of the events that occurs during this process is the assembly of chloroplast ribosomes. The second process, which proceeds only after the first is underway, is the *de novo* synthesis of the RuDP carboxylase protein. This synthesis appears to require light and an active photosynthetic electron-transport chain.

The requirement for light and photosynthetic electron transport for carboxylase synthesis in *ac-20* is not readily understood. In regreening *Chlorella* (23) and *Euglena* (25), DCMU does not prevent the recovery of the cells' capacity for photosynthetic CO₂ fixation and, therefore, by implication, for the synthesis of RuDP carboxylase. Moreover, etiolated pea (14) and bean (8) seedlings synthesize RuDP carboxylase in the dark following a brief exposure to red light. Finally, and most significantly, wild-type *C. reinhardi* does not require light for carboxylase synthesis (Ta-

ble I), and two mutant strains of *C. reinhardi*, *ac-141* and *ac-206*, are unable to carry out photosynthetic electron transport and yet synthesize the enzyme in normal amounts if provided with a carbon source (Table I). The observed requirement for light and photosynthetic electron transport possibly reflects a large energy requirement for the recovery process, one that cannot be met by endogenous respiration. This possibility is currently being studied.

Abbreviated reports of this research have appeared earlier in abstract form (*Plant Physiol.*, 1966, **41**: S-1vii; 1967, **42**: S-47).

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