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The Warburg Effect in a Chloroplast-Free Preparation from *Euglena gracilis*[1]

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Abstract. A supernatant fraction, free of plastids, was prepared by centrifugation from *Euglena gracilis* and used to ascertain whether or not the inhibition of carbon dioxide fixation by oxygen, known as the Warburg effect, is entirely independent of the light-driven phase of photosynthesis. This fraction exhibited in the dark the main features of the Warburg effect; namely, an inverse relationship between the degree of inhibition by oxygen and bicarbonate concentration, reversibility of the inhibition when the oxygen partial pressure is lowered and an increase in the proportion of 2-carbon compounds. It is proposed, therefore, that the inhibition by oxygen is manifest in the photosynthetic carbon reduction cycle and is independent of photosynthetic electron transport and phosphorylation.

The inhibition of CO_2 fixation by O_2, in isolated spinach chloroplasts has recently been described in detail (3, 6). It was then suggested that this inhibition, termed the Warburg effect, was primarily related to some aspect of the carbon reduction cycle rather than depression of activity of one of the light-driven reactions (photophosphorylation or photoreduction of NADP).

In our experiments, we have used the supernatant fraction from *Euglena gracilis* from which plastids had been removed by centrifugation to obtain data consistent with this proposal. When provided with ribose-5-phosphate, ATP and NADPH, this preparation fixed $^{14}CO_2$ in the dark at reasonable rates and in a linear fashion. Using this system, it therefore seemed probable that any contribution by the light driven phase of photosynthesis towards the mechanism of the Warburg effect could be detected.

Three important characteristics of the Warburg effect are: an inverse relationship between oxygen sensitivity and bicarbonate concentration; a rapid reversal of this inhibition by reduction of the oxygen partial pressure and an increase in the synthesis of 2-carbon compounds (glycolate in isolated chloroplasts and glycolate together with glycine in whole algal cells). The first task was to show if the chloroplast free supernatant exhibited a real Warburg effect as defined by these characteristics and whether the addition of chloroplasts and light altered any of them.

Materials and Methods

Euglena gracilis, "Z" strain were grown autotrophically, in 5 % CO_2 and 95 % N_2, as described by Evans and San Pietro (4). The harvested cells were washed twice in 50 mM tricine-NaOH buffer pH 8.1, by centrifugation and then suspended in 20 ml of a solution containing 330 mM sorbitol, 50 mM tricine-NaOH, pH 8.1 and 2.5 mM $MgCl_2$. The cells were broken in a French Press at 400 psi and the cell suspension was centrifuged at 480*g* for 1 min to remove whole cells and large fragments. An aliquot (10 ml) of the supernatant solution was removed with care to not disturb the pellet. It was centrifuged at 3020*g* for 1 min to collect the intact chloroplast fraction. The resulting supernatant solution was centrifuged at 20,200*g* for 10 min to remove broken chloroplasts and other cellular inclusions. The supernatant fractions from this latter centrifugation was employed in the experiments described.

A 0.5 ml aliquot (containing approximately 8 mg protein per ml) was added to 2.5 ml of a solution containing: 330 mM sorbitol, 50 mM tricine-NaOH, pH 8.1, 2.5 mM $MgCl_2$, 2.5 mM $MnCl_2$ and 2 mM Na phosphate, pH 8.1. Ribose-5-P was added to a final concentration of 0.1 mM; NADPH and ATP to 1 mM concentration. The reactions were performed in test tubes immersed in a water bath held at 20°. Where indicated, illumination was provided by two 300 watt flood lamps. The gas phase was controlled by bubbling continuously through the solution the appropriate gas *via* a plastic drinking straw inserted down the center of the tube. A small amount of silicone anti-foam agent (DOW Corning A) was present to prevent foaming. The reaction was commenced by the addition of $NaH^{14}CO_3$, which was used over a 1 to 20 mM concentration range. The specific radioactivity of the $NaH^{14}CO_3$ was about 2 to 5 μcuries per μmole.

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Measurement of isotope incorporation and separation of photosynthetic intermediates by paper chromatography were performed as previously described (3). Samples of 0.5 ml were removed at the desired time intervals and added to 0.02 ml of 23 N formic acid. To terminate the reaction, a 50 μliter aliquot was placed on a planchet containing a small disc of lens paper. The sample was dried under an infrared lamp and measured in a thin window gas flow automatic counter using a gas mixture of 1.3 % butane and 98.7 % helium. After descending paper chromatography, discs of equal area were cut from the chromatograms and the activity of each disc measured in 10 ml of "liquiflour"-toluene (a mixture of 2,5-diphenyloxazole and 1,4-*bis*-[2-(5-phenyloxazole]-benzene in toluene) in a Nuclear Chicago Unilux II liquid scintillation counter.

Results

The fixation of $^{14}CO_2$ and the influence of R-5-P, ATP, and NADPH on the rate of fixation is shown in Fig. 1. Because of the absence of chlorophyll, there is a problem as how to best express the rate of fixation. From whole cells containing approximately 0.4 mg chlorophyll, 20 ml of solution containing from 150 to 200 mg protein was obtained and 0.5 ml of this solution was added to 2.5 ml of reaction mixture. One may, therefore, express the rate in terms of crude protein or back extrapolate to a chlorophyll basis in the intact cells. In Fig. 1, the maximum rate of CO_2 fixation is equal to 26 μmoles per mg Chl per hr based on chlorophyll equivalence calculated by extrapolation. The chloroplasts isolated by the procedure described are not capable of $^{14}CO_2$ fixation, although they are capable of satisfactory rates of photophosphorylation and NADP photoreduction. The best rates of ATP formation obtained were 45 and 85 μmoles per mg Chl per hr with ferricyanide (with a P/2e ratio approaching unity) and pyocyanin, respectively. In the presence of added spinach ferredoxin the chloroplasts can provide ATP and NADPH for subsequent $^{14}CO_2$ fixation by the supernatant solution (about 8 μmoles CO_2 fixed per mg Chl per hr above the rate in the dark when ATP and NADPH are absent).

Three characteristics of the Warburg effect were considered earlier. In the dark and without added chloroplasts, the supernatant fraction exhibits both the sensitivity to bicarbonate concentration and the rapid reversibility resulting from removal of oxygen (Fig. 2 and 3). While the concentrations of bicarbonate over which this preparation is sensitive are higher than in the case of isolated chloroplasts, the overall kinetics of incorporation of ^{14}C are very similar to those previously reported for isolated spinach chloroplasts (3).

The effect of changing the gas phase from O_2 to N_2, at 1 particular bicarbonate concentration, on the rate of ^{14}C incorporation is shown in Fig. 3. After approximately 1 min, the new rate approximates that

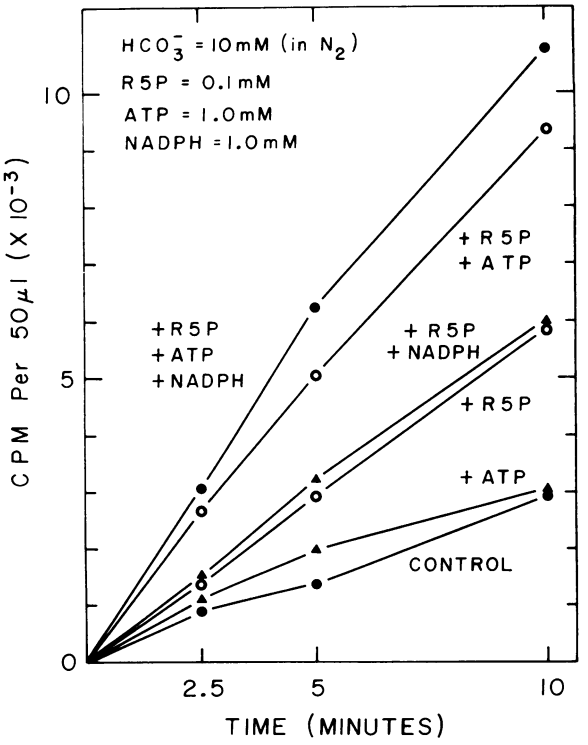

FIG. 1. Influence of ribose-5-P, ATP, and NADPH on $^{14}CO_2$ fixation in the dark. Experimental conditions as described in Methods.

of the control (under N_2). However, as with spinach chloroplasts, the rate and degree of recovery of CO_2 fixation is dependent upon the bicarbonate concentration (Fig. 2).

The distribution of isotope between photosynthetic intermediates is given in tables I and II. Table I contains data obtained from an experiment such as that shown in Fig. 3; that is, the change from O_2 to N_2 is carried out in the dark. Similar kinetic data is obtained when the experiment is performed in the light. For purposes of comparison, the results obtained from side by side light and dark experiments are given in table I. Oxygen causes a marked increase in the proportion of label in glycolate and glycine and this proportion increases with time. However, replacement of O_2 by N_2 after 6 min of photosynthesis results in a marked reduction in the rate of ^{14}C incorporation into these 2-carbon compounds. Although in this experiment no chloroplasts were present, light caused a significant shift in the distribution of ^{14}C between glycolate and glycine. Glycolate is favored in the dark and light promotes a large increase in the proportion of label in glycine.

The synthesis of glycolate is crucially linked with the mechanism of the Warburg effect. Because glycolate synthesis may involve a pathway at least partially independent of the photosynthetic carbon

reduction cycle, it was of interest to see whether the addition of chloroplasts could alter the distribution of isotope amongst the photosynthetic intermediates. The labeling of photosynthetic intermediates by the supernatant in the light, where chloroplasts have or have not been added back, is shown in table II. In all cases ATP, NADPH and ribose-5-P were added to the reaction mixture. Glycerate-3-P is heavily labeled initially and there is a decrease with time as the radioisotope spreads into sugar phosphates (to a greater degree under N_2 than O_2) and 2-carbon compounds (to a greater degree under O_2 than N_2). These trends apply irrespective of whether chloroplasts are present or not. Because the experiment was performed in the light, the balance between the 2-carbon compounds heavily favors glycine. Of particular interest, however, is the great increase in the labeling of glycolate brought about by the addi-

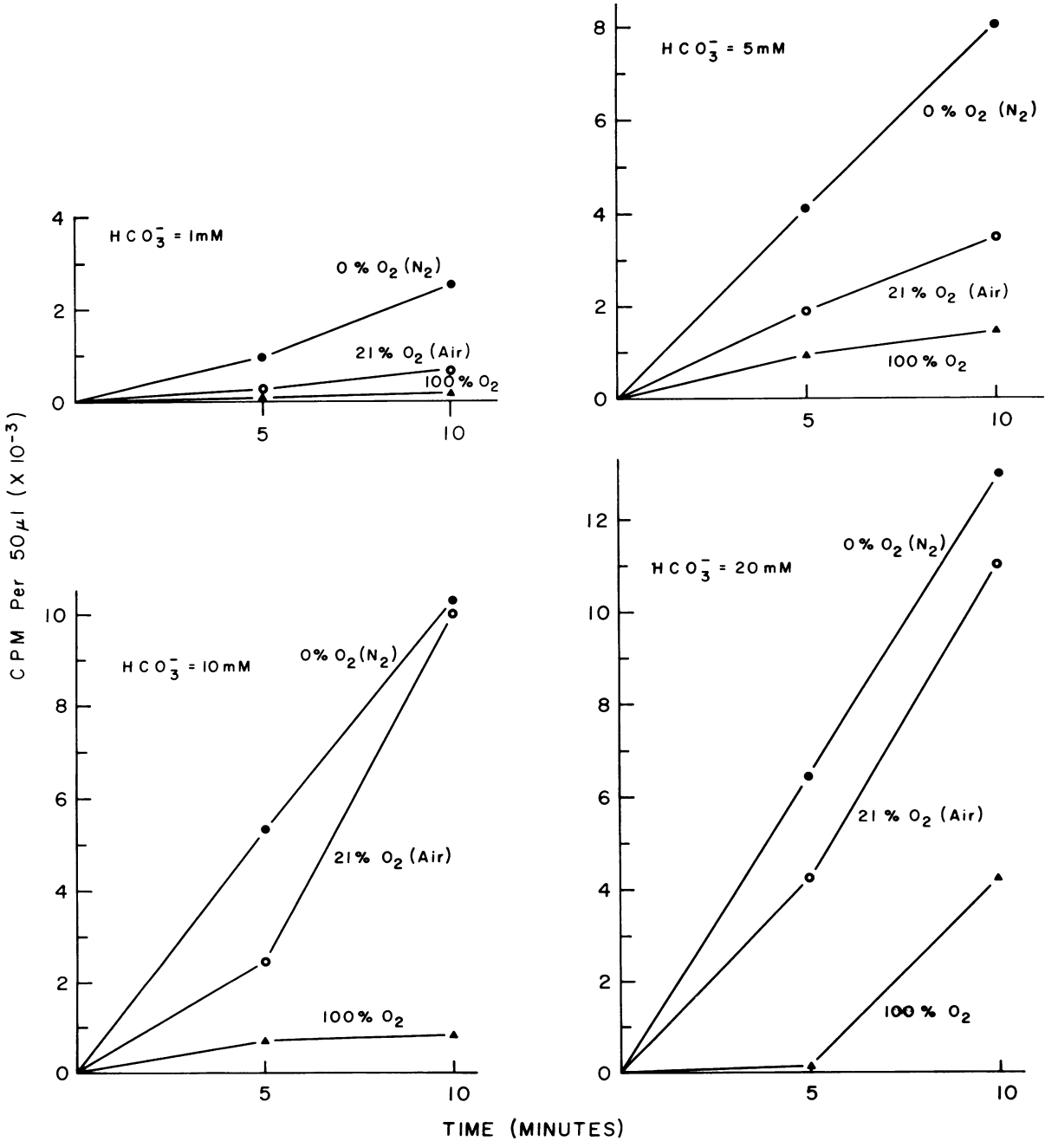

FIG. 2. Kinetics of $^{14}CO_2$ fixation at several concentrations of bicarbonate and oxygen. The same chloroplast-free supernatant solution was used in all the experiments. Experimental procedures were as described in Methods.

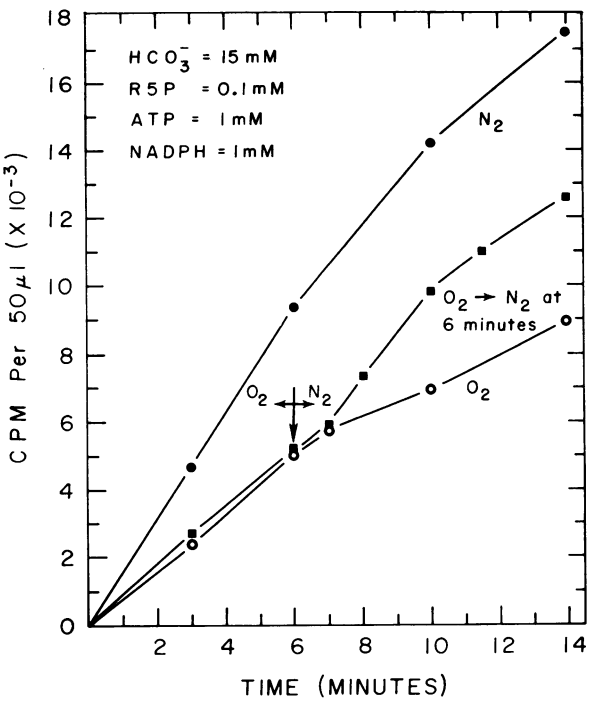

FIG. 3. The reversibility of the Warburg effect. Time zero is the time of addition of $NaH^{14}CO_3$. (—●—●—), Continuous N_2; (—■—■—), Changed from O_2 to N_2 at 6 min; (—○—○—), Continuous O_2.

tion of chloroplasts under both O_2 and N_2 (table II, compare expts. 1 and 2, 3, and 4). This increase occurs without any significant change in the proportion of glycine labeled.

Discussion

The evidence presented here corroborates earlier data which suggested that the Warburg effect is independent of the light driven phases of photosynthesis (3, 5) and represents an abberation of the photosynthetic carbon reduction cycle (1, 2, 3). The 3 important characteristics of the Warburg effect; namely, an inverse relationship between degree of inhibition and bicarbonate concentration, rapid reversibility and an increased accumulation of 2-carbon compounds, can all be observed in the dark when chloroplasts are absent.

Coombs and Whittingham (1, 2) reported that, in whole *Chlorella*, O_2 causes a significant increase in the proportion of glycolate and glycine labeled, and that they are formed at the expense of sugar-P. Ellyard and Gibbs (3) showed that glycolate can be the only product of CO_2 fixation in spinach chloroplasts provided certain combinations of high O_2 and low CO_2 are used. Glycolate accounts for an increasingly larger proportion of the ^{14}C fixed by the chloroplast as either (or both) the bicarbonate concentration is decreased or the O_2 concentration is increased. This also occurs at the expense of sugar-P labeling. Another important fact is that glycolate is exported rather than remetabolized by the intact *Chlorella* cell and the spinach chloroplast. There can be little doubt that the increased synthesis of glycolate is a crucial occurrence associated with the mechanism of the Warburg effect.

With this *Euglena* preparation a change of gas phase from O_2 to N_2 arrests the drainage of carbon from intermediates of the photosynthetic carbon reduction cycle into 2-carbon compounds (table II). This occurs simultaneously with the increase in the rate of CO_2 fixation resulting from the change of gas phase (Fig. 3).

The mechanism of glycolate synthesis, known to be crucially associated with the mechanism of the Warburg effect, has been the object of much speculation. Although chloroplasts isolated as described from *Euglena* are incapable of fixing CO_2, their addition to the chloroplast-free supernatant results in a dramatic increase in the labeling of glycolate but virtually no change in the labeling of glycine. These data support the view that glycolate synthesis involves a light-driven reaction which is partially (or totally) independent of the photosynthetic carbon reduction cycle. However, some cooperative interaction between the photosynthetic electron transport chain and the photosynthetic carbon reduction cycle appears necessary to facilitate glycolate synthesis. One possibility is that photosystem 2 provides a strong oxidant to oxidize the C_2-ThPP-transketolase complex to release glycolate as an end product.

Table I. *Effect of Light and Gas Phase on the Percentage Distribution of ^{14}C in Photosynthetic Intermediates*

Experimental conditions are as described in text and in Fig. 3. $NaHCO_3$ was 5 mM and was added at zero time.

Conditions	Time	PGA + Sugar P	Serine	Glycine	Glycolate
	min		%		
Light N_2	0.15	100.0	...	...	...
	6	65.5	5.5	17.8	12.0
	15	58.0	4.5	22.6	14.0
Light O_2	0.15	100.0	...	...	...
	6	56.1	6.0	22.0	14.8
N_2	15	61.1	4.8	20.4	13.2
Light O_2	0.15	99.0	...	1.0	...
	6	58.5	5.2	24.8	11.8
	15	40.7	5.3	34.0	19.5
Dark N_2	0.15	99.5	...	...	0.5
	6	63.5	6.9	15.0	16.0
	15	48.5	2.8	12.8	25.6
Dark O_2	0.15	98.5	...	...	1.5
	6	54.7	8.3	19.5	18.5
N_2	15	50.4	6.1	23.2	20.9
Dark O_2	0.15	98.0	...	...	2.0
	6	54.0	4.8	20.0	20.0
	15	32.8	5.7	27.5	34.0

Table II. *Percentage Distribution of ^{14}C in Photosynthetic Intermediates After Fixation by* Euglena *Chloroplasts-Free Preparation*

Fixation was measured in the light under N_2 and O_2 in both the absence and presence of added chloroplasts $NaH^{14}CO_3$ was 15 mM and was added at zero time. Chloroplasts added correspond to a final concentration of 35 μg chlorophyll per ml.

Expt.	Conditions	Time	Glycolate	Glycine	Serine	3-PGA	PMP[1]	di(P)[2]	HMP[3]	Malate
No.		*min*				%				
1	N_2	0.15	...	...	...	100.0	...	...	...	...
		5	0.3	3.8	1.0	86.5	2.0	3.4	3.0	...
		10	2.7	12.8	3.8	62.0	7.2	5.2	5.6	...
2	N_2	0.15	...	...	...	100.0	...	...	...	...
	+ chloroplasts[4]	5	3.5	7.0	1.0	78.0	3.4	3.7	2.1	...
		10	11.2	13.8	2.6	52.3	5.9	5.3	6.6	...
3	O_2	0.15	0.4	2.9	...	96.6	...	...	...	...
		5	1.1	8.3	1.4	84.0	2.6	1.7	1.5	...
		10	1.2	17.5	2.1	70.0	3.8	1.4	0.8	2.8
4	O_2	0.15	0.9	2.0	0.8	96.3	...	...	...	...
		5	1.5	11.3	1.2	76.0	2.2	0.8	5.8	1.7
		10	10.0	16.8	0.6	68.0	1.7	0.8	0.8	1.9

[1] Pentose monophosphate.
[2] Sugar diphosphates.
[3] Hexose monophosphates.
[4] Chloroplasts equivalent to 45 μg per ml chlorophyll.

There is data in the literature which can be interpreted to support this suggestion (6, 7, 8, 10, 11).

The proportions of glycolate and glycine labeled in this *Euglena* system (without chloroplasts) is dramatically altered by light. Hess and Tolbert (7) have suggested that glycine synthesis in algae may not occur *via* glycolate. On the other hand, Marker and Whittingham (9) indicate that in *Chlorella* at least glycine and glycolate are on the same biosynthetic pathway. Two distinct pathways involving these 2-carbon compounds may be operative in *Euglena*. One is the synthesis of glycolate which requires cooperative interaction with the photosynthetic electron transport chain (seen here when chloroplasts are present). The other is related to the synthesis of glycolate in the dark by the chloroplast-free preparation and its subsequent oxidation and amination to glycine in the light. From the data available presently, it would seem that the first pathway is insensitive to O_2 while the second is a pathway which is stimulated by O_2.

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