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Glutamic Acid Decarboxylation in *Chlorella*[1, 2]

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ABSTRACT

The decarboxylation of endogenous free glutamic acid by *Chlorella pyrenoidosa*, Marburg strain, was induced by a variety of metabolic poisons, by anaerobic conditions, and by freezing and thawing the cells. The rate of decarboxylation was proportional to the concentration of inhibitor present. Possible mechanisms which relate the effects of the various conditions on glutamate decarboxylation and oxygen consumption by *Chlorella* are discussed.

It was discovered by Warburg *et al.* (17) that the presence of 0.01 M NaF at low pH induces a rapid enzymic, cyanide-sensitive decarboxylation of the endogenous free glutamic acid of the green alga *Chlorella*. The result is a "CO_2 burst" and a quantitative accumulation of the decarboxylation product γ-aminobutyrate. From further research on this phenomenon, Warburg and Krippahl (19) have concluded that glutamic acid plays an important role in the light reactions of photosynthesis. They envision a cooperative involvement of glutamate with chlorophyll, carbon dioxide, and phosphate in the manganese-catalyzed formation of "photolyte" (21–24). According to their scheme the photolysis of this "activated CO_2" in the chloroplast would result in the evolution of molecular oxygen.

This conclusion regarding the involvement of glutamic acid is based partially on the stoichiometry exhibited in *Chlorella* between glutamate and chlorophyll. It is also based on the correlation between the amount of glutamic acid decarboxylated by the action of fluoride, and the accompanying inhibition of photosynthesis. The work described in this paper was undertaken to provide some insight into the mechanism(s) of endogenous glutamate decarboxylation, and to establish a base from which to test further Warburg's hypothesis of the participation of glutamic acid in photosynthesis. The results indicate that a variety of substances and treatments other than fluoride induce the decarboxylation of glutamate in *Chlorella*, although a distinction between at least two mechanisms is not clear.

MATERIALS AND METHODS

All experiments were carried out with the Marburg strain of *Chlorella pyrenoidosa* (4). This strain is acid-tolerant and, as pointed out by Bishop and Gaffron (4), is particularly useful in experiments with acid poisons, since the pH can be kept low enough to insure maximum entry of the poisons into the cells (11).

The algae were cultured autotrophically, as previously described (11, 12), at 24 C for 72 hr in the inorganic salt medium K of Warburg (20), and harvested and washed by centrifuging at 0 C. The harvested algae were suspended in Warburg's S medium (17), containing, per liter: 5.0 g of $MgSO_4 \cdot 7H_2O$ and 2.5 g of KH_2PO_4. The pH of this working suspension was adjusted to 3.8, and all solutions to be added during the course of an experiment were also adjusted to pH 3.8. (Exceptions were the experiments with α,α′-dipyridyl, in which the pH was 7.5.) The concentration of cells in the working suspensions was usually manipulated such that the final reaction mixtures, after all additions had been made, contained about 100 μl packed cell volume per ml (corresponding to about 30 mg dry wt/ml).

Gas exchange was measured by standard manometric techniques. All experiments were carried out at 20 C, in the dark on a covered Warburg apparatus. Soluble materials were extracted from the cells by pipetting aliquots of reaction mixtures into boiling water, boiling for 2 min, and allowing to cool for 10 min more. The dead cells were removed by centrifuging, and the hot water extracts were concentrated under reduced pressure at about 45 C. The basic fraction (containing the amino acids) was then separated from the rest of the extract by passing it through a column of Dowex 50 resin (hydrogen form) (5). The amino acids were separated by unidimensional multiple paper chromatography (15) with butanol–acetic acid–water (3:1:1, v/v/v) as the solvent system.

Radioactive glutamic acid was isolated from *Chlorella* extracts by the method of Lips and Beevers (7), eluting glutamate from Dowex 1 resin (acetate form) with a linear gradient of 0 to 2 N acetic acid. Radioactive samples were counted on nickel-plated planchets with a thin window continuous gas flow Geiger-Muller tube.

Sodium acetate-2-^{14}C at specific radioactivity of 20.5 mc/mmole was obtained from commercial sources.

RESULTS

The chromatogram pictured in Figure 1 shows the effects of 0.003 M fluoride and 0.01 M fluoride on the free amino acids of *Chlorella*. Under the growth conditions employed, this strain of *Chlorella* contains little or no γ-aminobutyrate (see extracts 1 and 2). Exposing the cells to a low concentration of fluoride (0.003 M) (extracts 3 and 4) resulted in a small amount of glutamate decarboxylation in 5 min, and more in 3 hr, as evidenced by the loss of glutamate (spot B) and the accumulation of γ-aminobutyrate (spot A). A higher concentration of fluoride (0.01 M) (extracts 5 and 6) caused a considerably higher rate of glutamate decarboxylation.

A "CO_2 burst" indicative of a high rate of glutamic acid decarboxylation was elicited from intact *Chlorella* cells by several metabolic poisons besides fluoride (Figure 2). For each substance, chromatography of hot water extracts of cells confirmed

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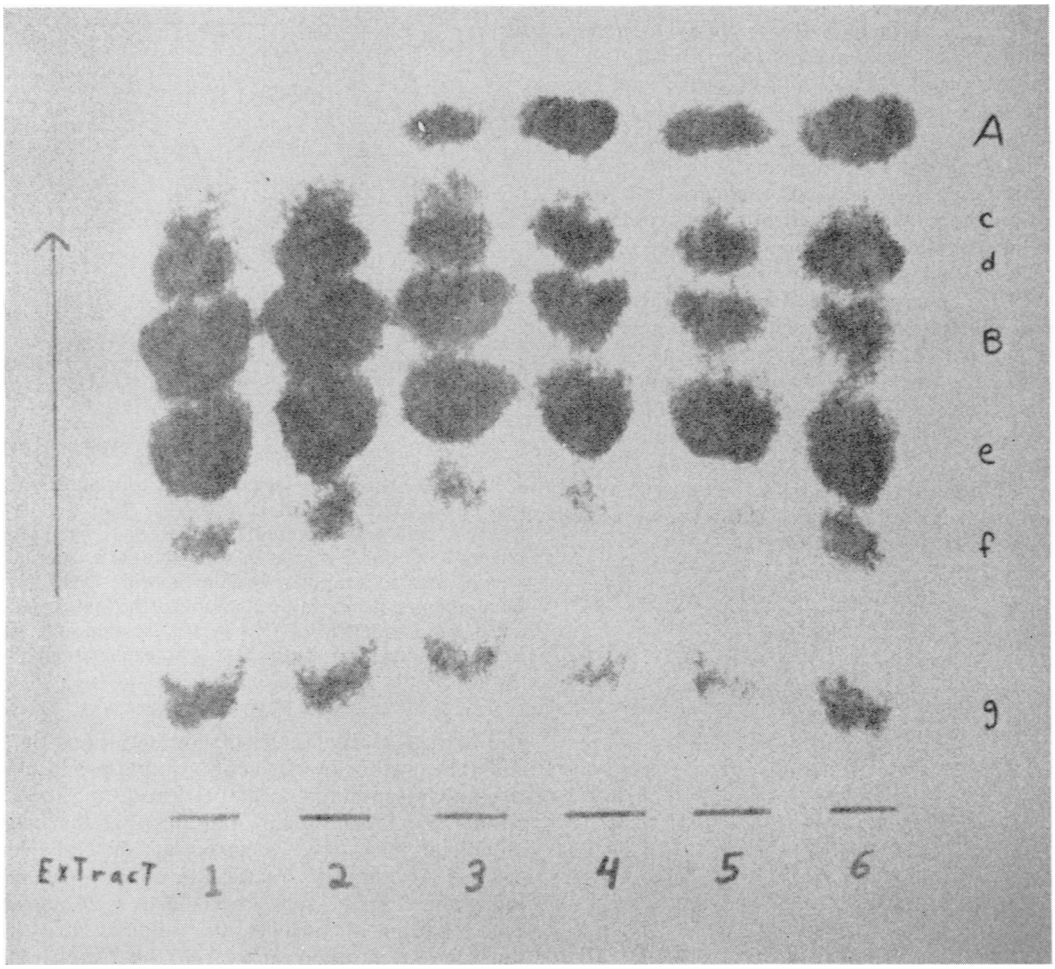

FIG. 1. Paper chromatogram showing the effects of NaF on the free amino acids of *Chlorella*. Chromatogram developed by allowing solvent (butanol–acetic acid–water, 3:1:1, v/v/v) to ascend the length of the paper two different times, the chromatogram being thoroughly dried between solvent passes. Amino acid spots were stained by dipping chromatogram into 0.25% ninhydrin in acetone and drying at 70 C. Amino acid spot A is γ-amino-butyrate, and spot B contains glutamate and threonine. Spots c, d, e, and f represent proline, alanine, glycine-serine-aspartate, and arginine-asparagine-lysine, respectively. The identity of spot g is unknown. Extracts 1 and 2 are from untreated *Chlorella* cells after 5 and 180 min; 3 and 4 from cells treated with 0.003 M fluoride for 5 and 180 min; 5 and 6 from cells treated with 0.01 M fluoride for 5 and 180 min, respectively.

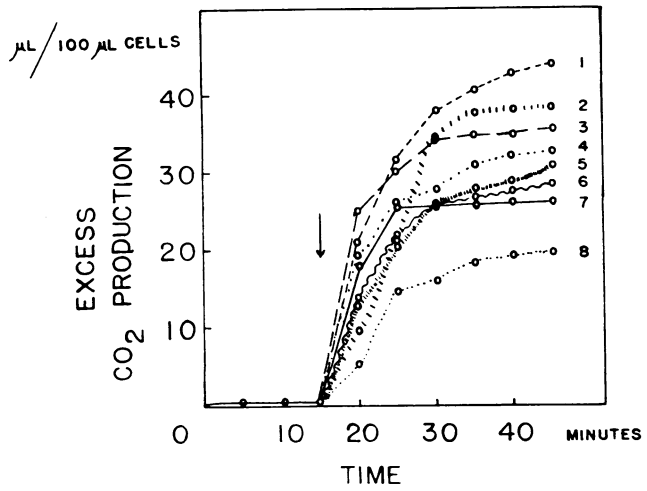

FIG. 2. The carbon dioxide burst in *Chlorella*. Protocol of each experiment was similar to that described in Table I, except that no KOH was present in any vessel. Thus, the positive pressure developed represents CO_2 in excess of that offsetting any O_2 consumption that may have occurred. Contents of main well and side arm were mixed at the time indicated by the arrow, to achieve final concentrations of poisons as follows: curve 1, 0.01 M acetate; curve 2, 0.7 M malonate; curve 3, 0.01 M formate; curve 4, 0.01 M propionate; curve 5, 0.01 M iodoacetate; curve 6, 0.2 M lactate; curve 7, 0.01 M fluoride; curve 8, 0.03 M fluoroacetate. All inhibitors supplied as sodium salts, pH 3.8.

that rapid glutamate decarboxylation accompanied the measured CO_2 burst.

Table I indicates the effects of these various inhibitors on oxygen consumption by *Chlorella*. In each case, the concentration of poison which caused rapid glutamate decarboxylation (Figure 2) inhibited O_2 uptake, while a lower concentration resulted in its stimulation. Parallel chromatographic analysis of hot water extracts showed that the lower concentration of each inhibitor caused a *slow* decarboxylation, as noted in the case of fluoride in Figure 1. Summarizing, each *acid* poison produced two pairs of effects, depending on its concentration. The lower concentration stimulated oxygen consumption and resulted in a slow decarboxylation of glutamate, while the higher concentration strongly inhibited respiration and brought about the CO_2 burst.

The lower concentration of the basic inhibitor α,α'-dipyridyl stimulated O_2 uptake but induced no decarboxylation, while the higher concentration of dipyridyl inhibited respiration and

Table I. *Effect of Inhibitors on Oxygen Consumption by Chlorella*

Oxygen consumption was determined manometrically on a covered Warburg apparatus. The main well of each vessel contained 300 μl of *Chlorella* cells, suspended in S* medium (17), adjusted to pH 3.8, (pH 7.5 in dipyridyl experiments). All inhibitor solutions were adjusted to pH 3.8 (dipyridyl, pH 7.0), and 0.1 to 0.3 ml of an inhibitor was contained in the side arm of a vessel at the beginning of an experiment. A control vessel (containing cells, but 0.2 ml of S* medium in the side arm) was carried through each experiment. The center well of each vessel contained 0.12 ml of 20% KOH absorbed on a small folded square of filter paper. The total volume in all vessels was 3 ml. Oxygen uptake was usually monitored by taking 5-min readings for 15 to 30 min, during which time the rates in all the vessels were about equal. Then each vessel was removed from the bath briefly and tipped such that the contents of each side arm were mixed with the cell suspension in the main well of that vessel, exposing the cells to a final concentration of inhibitor as indicated in the table. The time of tipping is shown in the table as 0 min. Readings were then continued at 5-min intervals for at least 60 min.

Inhibitor[1]	Oxygen Consumption during the Interval			
	−5 to 0 min	10–15 min	30–35 min	60–65 min
	μl O_2 / 100 μl cells			
None	3.8	3.8	3.6	3.4
Fluoride				
0.002 M	3.8	8.5	6.6	6.2
0.01 M	3.8	0.8	0.3	0
Acetate				
0.002 M	3.9	10.5	7.0	4.6
0.01 M	3.8	1.1	0.4	0.4
Iodoacetate				
0.001 M	3.7	5.0	4.9	5.0
0.01 M	3.8	1.1	0.9	0.9
Propionate				
0.002 M[2]	3.9	5.0	5.4	5.2
0.01 M	3.8	1.3	1.2	1.0
Formate, 0.01 M[2]	4.0	1.1	1.0	...[3]
Fluoroacetate				
0.015 M	3.8	4.8	4.7	4.8
0.03 M	3.8	0.5	0.2	0.2
Lactate				
0.1 M	4.1	7.6	5.5	5.5
0.2 M	4.0	1.1	0.2	0
Malonate				
0.2 M	3.7	5.5	3.4	2.0
0.7 M	3.9	1.8	1.1	1.0
α,α′-Dipyridyl				
0.01 M[2]	3.8	5.7	5.4	4.7
0.1 M[4]	6.0	3.3	1.7	1.5

[1] All inhibitors, except α,α′-dipyridyl, supplied as sodium salts.

[2] Values from one experiment only. Other values are averages of at least two different experiments.

[3] Not determined.

[4] 0.1 M dipyridyl dissolved in ethanol; thus 0.3 ml ethanol added to controls.

caused a slow decarboxylation. Anaerobic conditions also resulted in slow decarboxylation of glutamate.

Figure 3 shows that the loss of glutamic acid during treatment with fluoride was proportional to the fluoride concentration. The endogenous glutamate was labeled by exposing the cells to acetate-2-^{14}C for 5 min. The cells were then incubated with appropriate concentrations of fluoride for 5 min before being killed and extracted. The range of concentrations of NaF was from 0.0001 M (well below the concentration producing stimulation of oxygen consumption) to 0.01 M (producing the CO_2 burst). The dashed line on the graph indicates that the most rapid loss of glutamate may have occurred at an NaF concentration of 0.006 M. The linearity of this relationship can be interpreted as indicating that the same mechanism is responsible for the decarboxylation of endogenous glutamic acid over the whole fluoride concentration range, from the relatively slow decarboxylation to the rapid burst.

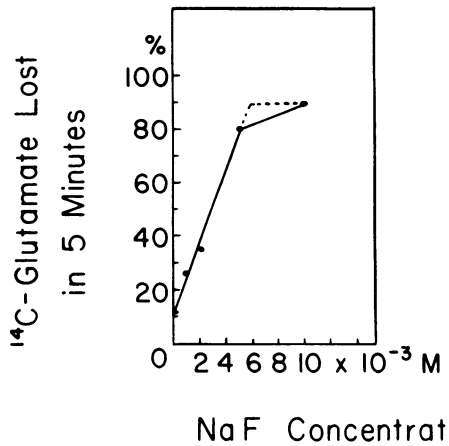

FIG. 3. Relationship between NaF concentration and the rate of loss of ^{14}C-glutamic acid in *Chlorella*. Two hundred microliters of *Chlorella* cells in S* medium (pH 3.8) were incubated with 0.2 μc of acetate-2-^{14}C, for 5 min at 20 C in the dark (specific radioactivity of acetate, 20.5 mc/mmole). Sodium fluoride (pH 3.8) was then added (final volume 3 ml), and cells were further incubated for 5 min, then killed and extracted. Glutamate was isolated and assayed for radioactivity as described in the text. For explanation of dashed line, see the text.

Spontaneous decarboxylation of glutamate occurred in *Chlorella* which had been frozen in liquid nitrogen and thawed. The chromatogram shown in Figure 4 indicates this and shows further that the soluble amino acids were released from the cells into the salt medium after this treatment. Medium 1, from frozen-thawed *Chlorella*, showed the same amino acid complement as hot water extracts of intact cells (Figure 1), while the medium from intact cells showed only a small amount of glutamate, the most abundant free amino acid in this strain of *Chlorella*. Another indication of metabolic disorganization arising from the freeze-thaw treatment is the increased rate of O_2 consumption exhibited by frozen-thawed *Chlorella*, illustrated in Figure 5.

DISCUSSION

The results reported above suggest that the enzymic decarboxylation of endogenous glutamate, by whatever mechanism it occurs, is a generalized, nonspecific effect. Warburg *et al.* (16) called this phenomenon the "acid effect" and explained the decarboxylation occurring under anaerobic conditions as due to the endogenous accumulation of lactic acid during fermentation. The same explanation could apply to the effect of α,α′-dipyridyl, since this basic poison is known to inhibit the tricarboxylic acid cycle at the aconitase step (6); one would expect, then, that this inhibitor could induce aerobic fermentation. These experiments must be carried out at low pH; thus, apparently only the undissociated forms of the acid inhibitors enter the cells (11). At the higher pH inside the cells, the acids would then dissociate, the result being an influx of protons into the cells.

The additional hydrogen ions inside the cells might lower the

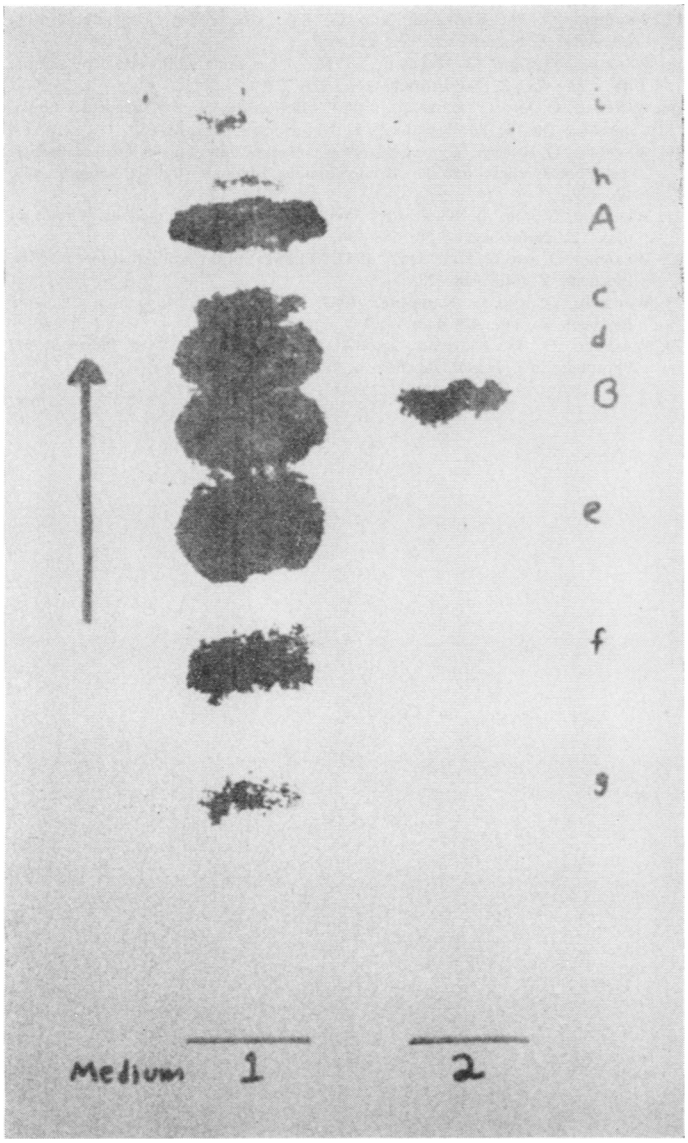

FIG. 4. Accumulation of γ-aminobutyrate by, and loss of free amino acids from frozen-thawed *Chlorella*. Algae were frozen in liquid N_2, thawed quickly, shaken in the dark for 45 min, and then centrifuged from the medium—yielding medium 1. Untreated cells were shaken in the dark for 45 min and centrifuged to yield medium 2. Amino acid spot A is γ-aminobutyrate, and spot B contains glutamate and threonine. Spots c, d, e, f, h, and i represent proline, alanine, glycine-serine-aspartate, arginine-asparagine-lysine, valine, and leucine-isoleucine, respectively. The identity of spot g is unknown.

internal pH, as suggested by Warburg *et al.* (16). This could lead to an increased activity of glutamate decarboxylase, since the optimum pH for this enzyme from numerous sources seems to be well below neutrality (9, 10). In fact, this enzyme from *Chlorella* has highest activity at about pH 5.8 (unpublished results).

The stimulation of respiration by low concentrations of metabolic poisons has been reported many times (1, 2, 14). Barker *et al.* (1, 3) feel that this effect in strawberry leaves, given by fluoride and iodoacetate, is produced by increased accessibility of substrates to enzymes. They concluded that large amounts of sucrose and ascorbate leak out of vacuoles in the presence of one of these metabolic inhibitors, resulting in an increase in the rate of respiration. Uncoupling of respiration, however, is still a very real possibility in their case. An increase in organelle permeability could cause uncoupling according to the chemiosmotic

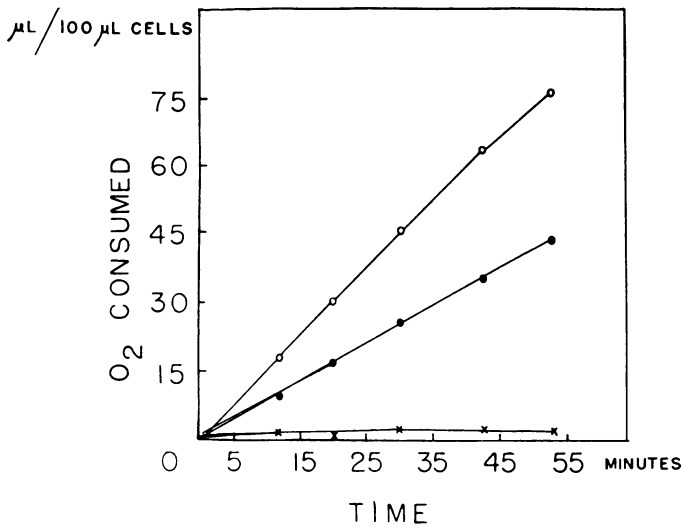

FIG. 5. Oxygen consumption by intact and frozen-thawed *Chlorella*. Oxygen uptake was measured manometrically. Protocol is described in table I. ●: Cells intact; ○: cells frozen in liquid N_2 *once* and thawed; ×: cells frozen and thawed 10 times in succession.

theory of Mitchell (8). The additional protons carried into the cells by the weak acids might in some manner increase the permeabilities of organelles (with or without an actual pH change).

Disruption of cellular membranes certainly occurs in frozen-thawed *Chlorella*, as shown by the leakage of soluble substances from the cells. Thus the increased rate of O_2 uptake in these frozen-thawed cells could be due to increased accessibility of substrates, or to uncoupling caused by disruption of mitochondrial membranes.

The report by Warburg and Krippahl (18) that the cytolytic agent octanol produces the same effect is consistent with the hypothesis that endogenous decarboxylation of glutamic acid in *Chlorella* results from an increase in the accessibility of glutamate to the enzyme glutamate decarboxylase. However, the disruption of cellular membranes might well cause a pH change inside the cell, by releasing substances from vacuoles or by the influence of the outside medium. Thus, a distinction between these two hypotheses cannot yet be made.

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