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Publication Date
1952-10-01

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Radiation Laboratory

Contract No. W-7405-eng-48

CO_2 FIXATION BY EUGLENA

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October 13, 1952

Berkeley, California

CO_2 FIXATION BY EUGLENA*

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During a comparative study of the dark CO_2 fixation by microorganisms one organism was found to differ greatly from all others investigated.[1] This microorganism was the green flagellate, *Euglena gracilis*.

Euglena has long been a controversial organism. It is claimed both by the botanists and the zoologists since it has many of the characteristics of both a plant and an animal and is often placed in the phylogenetic tree on the fork at which the plant and animal kingdoms are conceived to have separated. The biochemist has been interested in its metabolism because of its ready utilization of acetate and its apparent inability to utilize sugars.** Although *Euglena gracilis* does not use sugars, Albaum, *et al.*[2] has shown that the phosphorylated compounds of the glycolytic cycle are present in *Euglena*.

In order to extend our knowledge of some of the metabolic reactions of *Euglena*, a brief survey using radioactive tracer techniques was undertaken.

(*) The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

(**) Recent work by Cramer and Myers has indicated that *Euglena gracilis* can be trained to utilize glucose.

Methods

One organism used in this investigation was Euglena gracilis var. bacillaris obtained from Dr. S. H. Hutner of the Haskins Laboratories. The following growth medium was used: 5 gm. Difco tryptone, 5 gm. sodium acetate · 3 H_2O, 0.5 gm. NH_4NO_3, 0.5 gm. $MgSO_4$ · 7 H_2O, 0.5 gm. K_2HPO_4, 1 ml. of an iron solution containing 0.5% $FeSO_4$ · 7 H_2O and 0.2% tartaric acid, and 1 liter distilled water. The pH was adjusted to 7.0 with NaOH. After inoculation the culture was placed under constant illumination and shaken occasionally. The cells were harvested after growth periods of 3 to 7 days.***

Cell suspensions for use in the radioactive carbon experiments were prepared in the following manner. The cells were removed from the growth medium by centrifugation and washed once with distilled water. A 1% cell suspension was prepared by suspending 1 ml. of these packed cells in 100 ml. of distilled water.

Dark CO_2 Fixation. - 50 ml. of a 1% cell suspension were placed in 125 ml. glass stoppered Erlenmeyer flasks. The flasks were wrapped with aluminum foil and placed in a dark room. All subsequent operations were performed using a yellow safety light. Radioactive carbon was added to the cell suspensions as a sodium bicarbonate solution containing 0.0248 ml. M $NaHCO_3$ and 200 μc. C^{14} per ml. 0.1 cc. of this bicarbonate solution was the amount usually added per experiment. The flasks were then stoppered, shaken occasionally during the exposure period and the cells were killed by dumping into a volume of boiling absolute alcohol to give a final concentration of 80% ethanol.

(***) The authors wish to thank Mrs. Louisa Norris for her assistance in growing these organisms.

Photosynthetic experiments. - 1% cell suspensions were placed in a lollipop[3] and exposed to a light intensity of 7,400 lux. Air was bubbled through for 15 min. before addition of the radioactive bicarbonate solution. The lollipop was stoppered, the suspension constantly shaken in the light beam for a measured time period and then rapidly poured into boiling ethanol to give a final concentration of 80% ethanol.

Radioactivity measurements. - All radioactive experiments were analyzed in the following manner. Immediately after killing in boiling ethanol the suspensions were cooled, the volume determined and an aliquot of the entire suspension plated on aluminum plates and the activity counted using a Geiger-Muller counting tube. In this manner the total amount of radioactivity incorporated into non-volatile, stable compounds was determined. The cell extracts were acid enough so that all unchanged carbon dioxide was removed during the plating procedure. The cell suspension was then filtered through Celite and the activity of the filtrate determined in the same manner as for total activity. This value is reported as the soluble activity or the 80% alcohol soluble activity.

Location and Identification of Products. - Paper chromatography of the 80% alcohol extract was employed to determine the individual compounds which contained radioactivity. The alcohol extract was concentrated *in vacuo* at $< 40^{\circ}$ to a small volume ($\sim$2 ml.) and then aliquots placed on Whatman paper No. 1.

Two-dimensional chromatograms using water-saturated phenol as the first solvent and butanol (37.5 cc.) -propionic acid (17.6 cc.) -water (24.9 cc.) as the second solvent were run and the radioactive areas of the paper located by use of X-ray films. Each radioactive area was identified either by co-chromatography with a known compound or by chemical treatment of the areas after elution from the paper and identification of the resulting products.

The amount of radioactivity in each spot was determined by direct counting on the original chromatogram with a thin-window Geiger-Muller counting tube.[1]

Experimental and Results

Dark CO_2 Fixation. - The radioactive products formed during dark CO_2 fixation by Euglena gracilis were strikingly different from those of any other microorganism, either photosynthetic or non-photosynthetic, that has been investigated.[1] (Figure 1.) Not only were the characteristic dark CO_2 fixation products, malic, citric, aspartic and glutamic acids, formed, but many of the phosphorylated compounds typical of CO_2 fixation in the light were observed. See Table I.

The normal procedure for a dark CO_2 fixation experiment is to add the radioactive bicarbonate solution to the cell suspension immediately after placing the cells in darkness. When Chlorella or Scenedesmus in the absence of CO_2 are exposed to high light intensities and then immediately placed in the dark and CO_2 added, they are capable of fixing an appreciable amount of CO_2 into phosphorylated compounds, the "pre-illumination effect."[4] The compounds formed are qualitatively the same as those produced by photosynthesis. To test whether the dark fixation of CO_2 into phosphorylated compounds by Euglena might also be a "pre-illumination effect," the following experiment was performed. The washed cells were resuspended in distilled water and air bubbled through the suspension while kept in the dark for 16 hours. After this time, radioactive CO_2 was added while the suspension was still in the dark and the cells killed after 40 minutes. All operations were carried out in total darkness. Chromatograms from this experiment again showed that phosphorylated compounds, as well as the usual organic acids and amino acids,

Table I

Radioactive Products Formed During a

40 min. Dark CO_2 Fixation by *Euglena gracilis*

Product	% of 80% alcohol soluble* activity	
	No dark adaptation	16 hrs. dark adaptation in air
Fumaric	1.2	0.8
Succinic	21.3	41.2
Malic	4.8	3.4
Citric	5.0	2.7
Glutamic	6.5	3.4
Aspartic	2.7	4.6
Alanine	2.7	10.5
Phosphopyruvic	1.1	0.3
Serine and glycine	12.4	9.6
Phosphoglyceric	4.6	6.0
Sugar monophosphates	19.8	16.0
Nucleotides	5.4	—

(*) % of total fixed activity soluble in 80% alcohol approx. 50%

had become radioactive (Table I, column 2). Therefore, the dark fixation of CO_2 into phosphorylated compounds by Euglena does not involve the use of energy stored in the cells from a previous exposure to light as in "pre-illumination."

The radioactive phosphates were identified by chromatographic position of the phosphate and of the carbon compound obtained after enzymatic hydrolysis with Polidase-S. The same radioactive phosphates were found to be present after dark CO_2 fixation as are formed during photosynthesis. Even sedoheptulose phosphate, identified by conversion of sedoheptulose into sedoheptulosan by acid treatment, was found to be radioactive.

Phosphoglyceric acid accounted for 5% of the total soluble activity and the sugar monophosphates for 25% of the soluble activity. The organic acids and amino acids represented 43% of the fixed soluble activity. Serine and glycine, formed only in trace amounts in most dark fixation experiments with other organisms, accounted for 12% of the activity.

Several experiments were conducted in an effort to determine whether CO_2 might be entering the metabolic pathways of Euglena via two distinct routes or whether carbon dioxide appearing in sugars had first been fixed into organic compounds via reversal of the tricarboxylic acid cycle.

The rate at which CO_2 was fixed in the dark was determined over the time interval of 5 to 40 minutes. The amount of activity fixed during this time period is shown in Figure 2. Both the total amount of activity fixed and the activity fixed in 80% alcohol soluble compounds are plotted. The total amount of activity increases steadily with time although at a decreasing rate. The alcohol soluble compounds increase rapidly for the first 10 minutes with a very slow increase at later time. The two different fixation rates particularly in the soluble components suggest two simultaneous different routes of CO_2 incorporation.

Paper chromatography was used to separate and identify the compounds present in the alcohol-soluble fraction. The amount of radioactivity in each compound was also determined by direct counting of the compounds on the paper chromatograms. Therefore, the amount of activity in each compound as a function of time could be determined and the results are plotted in Figure 3.

The sugar monophosphates, giving primarily glucose, fructose and sedoheptulose on hydrolysis, contain the largest amount of radioactivity at the shortest time tested. Their activity continues to remain the major fraction at all of the longer time periods investigated. A nucleotide giving glucose as the principle radioactive compound on phosphatase treatment also contains a large fraction of the activity. This has been shown to consist largely of uridine diphosphate glucose.[5,6] Phosphoglyceric acid contains considerable activity but less than the sugar phosphates even at the five-minute interval. Of the non-phosphorylated compounds, the combined activity of glutamic acid and glutamine has the highest value. Aspartic and malic acids have very low activities.

Another experiment designed to elucidate the manner in which CO_2 is incorporated into metabolic pathways in the dark involved treatment of the cells with malonate to inhibit succinic dehydrogenase. Euglena cells were suspended in 0.05 M malonate at pH 4.3 for 105 minutes and then radioactive carbon dioxide added for 15 minutes. Under these conditions, malonate alters the fixation pattern of Scenedesmus.[7] After this treatment the cells were filtered rapidly through celite and killed by pouring boiling 80% ethanol through the algae. A control experiment, identical except for the omission of malonate was also carried out. The amount of alcohol-soluble radioactivity fixed was the same

in both samples. Determination of the activity of individual compounds also shows that the malonate treatment had no significant effect on the distribution of activity within the individual compounds (Table II).

Table II

Effect of Malonate on 15 min. Dark CO_2 Fixation by Euglena

	Control % of soluble activity	Malonate treated % of soluble activity
Succinic	9.6	8.2
Fumaric	1.5	---
Malic	3.4	4.6
Citric	2.7	4.0
Glutamic	10.6	11.5
Glutamine	3.4	3.2
Alanine	10.3	9.3
Aspartic	6.4	6.9
Phosphates	43.6	43.4

When radioactive carbon dioxide is administered to photosynthetic or non-photosynthetic tissues, radioactive sugars may be found. However, the distribution of activity in the individual carbons of the sugar skeleton is strikingly different for the two types of reactions. The radioactive glucose obtained by hydrolysis of the phosphates formed by Euglena during a 40-minute carbon dioxide fixation in the dark was oxidized with periodate.[****] Only the carbon of the primary alcohol group of glucose will be

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(****) We wish to thank Dr. J. A. Bassham and Mrs. A. Harris for performing these oxidations.

oxidized to formaldehyde by this treatment. This formaldehyde was trapped with dimedon and its activity determined. The formaldimedon contained very little radioactivity (less than 0.1%). The activity of the other carbon atoms was not determined. If carbon dioxide were fixed in glucose by the same reactions as occur during photosynthesis, the number 6 carbon of glucose so produced would be radioactive. In order to estimate just how much radioactivity one might expect to find in this carbon atom, it is necessary to determine approximately what fraction of the labeled glucose found in a 40-minute dark fixation might have been formed by a route corresponding to that occurring during photosynthesis. This may be estimated as that corresponding to the rapid rise in the soluble curve of Figure 2, which would be about 8×10^4 c./m./gm. cells. This is an amount of soluble activity that would correspond to about 20 seconds of photosynthesis as may be seen from Table III.

Table III

Amount of $C^{14}O_2$ Fixed by one gram of *Euglena* Cells

	1 min. PS c/m/gm. cells	5 min. PS c/m/gm.	5 min. dark c/m/gm.	10 min. dark c/m/gm.	20 min. dark c/m/gm.	40 min. dark c/m/gm.
Total activity	387,000	1,960,000	118,000	224,000	406,000	620,000
Soluble activity 80% alcohol	290,000	877,000	73,000	102,000	132,000	196,000

The shortest photosynthesis experiments available for *Euglena* was 1 minute and degradation of some glucose from these gave C_6 ~13% and $C_4 + C_5$ ~40%. This is approximately the same distribution as was obtained in a similar experiment with *Scenedesmus*, so that presumably the rate of

photosynthesis labeling in the two organisms are comparable. Hexose from 30 sec. photosynthetic experiments with _Scenedesmus_ shows C_6 ~3%. If this figure is extrapolated back to ~20 sec. of photosynthesis and then diluted by an equal amount of glucose formed by reversible terminal carboxylation reactions and labeled only in C_3 and C_4, it is clear that the existing data does not eliminate the possibility of an appreciable fraction of the glucose found in 40 min. dark fixation by _Euglena_ having been formed by a mechanism similar to that used in the light reaction. Of course, the possibility of still another mode of CO_2 fixation being available in _Euglena_ is very likely.

Photosynthesis Experiments. - Photosynthetic experiments of one and five minutes exposure to radioactive CO_2 were performed. The majority of activity, 84% in one minute, 70% in 5 minutes, is found in phosphorylated compounds. (Table IV.) The general photosynthetic pattern for _Euglena_ is similar to that of other photosynthetic organisms.[8]

Euglena gracilis is known as one of the acetate flagellates, preferring acids and alcohols to sugars as a carbon substrate. The organisms used in our laboratory are grown in the presence of both light and acetate. Therefore, it was of interest for several reasons to investigate the utilization of acetate by resting cells in the presence or absence of light. One mg. of sodium acetate-2-C^{14} was added to a suspension of 500 mg. wet weight of _Euglena_ cells in 100 cc. of distilled water. Four per cent CO_2 in air was continuously bubbled through the suspension to remove any carbon dioxide which might be formed by decomposition of the acetate. Determinations of the total activity at the end of both the light and dark experiments (after 12 minutes) showed that none of the radioactivity had been lost during the experiment. Therefore, the methyl carbon of the acetic acid was not oxidized to carbon dioxide, or, if converted

Table IV

$C^{14}O_2$ Fixation During 1 min. and 5 min.
Photosynthesis by Euglena gracilis

	% soluble activity	
	1 min.	5 min.
Succinic	—	0.4
Fumaric	0.4	2.2
Malic	1.2	—
Citric	0.5	0.3
Aspartic	9.3	8.5
Phosphopyruvic	0.9	0.8
Phosphoglyceric	16.0	7.6
Sugar monophosphates	54.7	42.5
Nucleotide phosphate	12.5	19.6
Dextrins	1.1	4.1
Glutamic	0.1	1.5
Serine	0.6	4.1
Glycine	0.4	5.4
Alanine	0.5	0.6
% Insolubles	25%	55%

to CO_2, it was reutilized by the cells before liberation into the external medium for the cells.

The total amount of acetate radioactivity converted to stable, non-volatile compounds was determined by plating and flooding the plate with glacial acetic acid. This last step was necessary to insure complete removal of unchanged acetic acid. The 80% alcohol soluble activity was determined in an identical manner. The results are shown in Table V.

Table V

C^*H_3COOH Utilization (in 12 minutes) by Euglena

	Activity Fed cts./m	Activity Fixed cts./m	
		Total	80% Alcohol Soluble
Light	8,200,000	1,100,000	230,000
Dark	8,200,000	400,000	250,000

These results show that a considerable amount, 13.4%, of the acetate fed was converted to other organic compounds during a twelve-minute period of photosynthesis. In the light there is a greater utilization of acetate than in the dark. Although more acetate was utilized in the light, the same amount of activity was found in the 80% alcohol-soluble fractions of the light and dark experiments. Chromatograms of the alcohol-soluble materials indicate that in the light the greatest fraction (47%) of this activity was in the lipid and phospholipid fraction. In the dark only 18% of the alcohol extractable incorporated radioactivity is found in the fat fraction with 50% of the fixed activity present as

phosphorylated compounds (Table VI). This enhanced incorporation of acetate into lipid under the influence of light is similar to that found earlier for green algae[4] (Scenedesmus) and purple bacteria[9,*****] (Rhodospirillum rubrum).

Table VI

Acetate-2-C^{14} Feeding Experiment

	% of 80% alcohol soluble activity	
	Light*	Dark*
Aspartic	8.1	5.2
Glutamic	4.9	7.8
Glutamine	3.1	5.8
Alanine	4.8	5.5
Phosphates	20.7	49.3
Dextrins	7.3	3.7
Lipids and phospholipids	47.4	17.3

(*) 12 min. exposure to acetate-2-C^{14}

Discussion

That characteristic of the dark fixation of carbon dioxide by Euglena which is different from that in all other organisms so far examined is most clearly represented in Figure 1. It is clear from this that the pattern of compounds into which carbon dioxide is fixed in the dark by Euglena is very similar to that of photosynthetic carbon dioxide fixation. This is not true of any of the other tissues so far examined in which the animal kingdom is represented by liver slices and a colorless protozoon, and the plant kingdom

(*****) Unpublished observations in this laboratory.

represented by yeast and water molds, a variety of aerobic and anaerobic bacteria, green algae, and leaves from higher plants. Although the data available to us at present regarding the distribution of the labeled carbon in compounds produced by the dark fixation does not permit us to say unequivocally that they have been formed by routes identical with or closely parallel to those used in photosynthetic fixation, there is some indication that this may be so. In addition to the degradation data on glucose already discussed, there is one other bit of information on the malic acid formed during 10 minute and 40 minute dark fixations by *Euglena*.[******] The 10 minute malic acid shows approximately 18% of its radioactivity in the α-carboxyl carbon with all of the remaining activity (approximately 72%) found in the β-carboxyl carbon. On the other hand, the 40 minute malic acid shows 25% in the α-carboxyl and about 65% in the β-carboxyl, leaving approximately 15-25% in the two central carbon atoms of malic acid. This distribution would indicate that a small amount of this malic acid had been formed by a reaction similar to those by which it is formed in the light. In contrast to this, radioactive malic acid obtained from *Chlorella* which have fixed radioactive carbon for 40 minutes in the dark shows approximately 50% of the radioactivity in the α-carboxyl carbon and the remaining 50% in the β-carboxyl carbon.

Even if it is not possible for us to say precisely by what mechanism the carbon dioxide finds its way into the photosynthetic intermediates in the dark, it is quite clear that the coupling of energy, made available by respiration and/or fermentation, with carbon dioxide reduction, exists in *Euglena* to an

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(******) These degradations were performed by Dr. P. K. Christensen, Fellow of the Norwegian Council on Scientific Research, to whom we here express our thanks.

extent not found in any other tissue examined, either plant or animal. A further evidence of this close connection between dark and photosynthetic metabolic routes in Euglena may be seen in the behavior of labeled acetate. In Euglena, labeled acetate in the dark finds its way not only into tricarboxylic acid intermediates but into all of the compounds normally found as intermediates of photosynthetic CO_2 assimilation. This is in contrast to the situation in Scenedesmus in which acetate carbon in the dark finds its way only into the tricarboxylic acid intermediates.

Assuming for the moment that the mechanism for carbon dioxide incorporation in the dark includes metabolic pathways very similar to those used in the light, one can represent the relationships between the Euglena studied in this work with green plants as represented by Chlorella and animal organisms represented by Tetrahymena as shown on the chart (Figure 4). Here it is clear that the ability to utilize chemical energy for carbon dioxide fixation in a manner similar to that by which photochemical energy is used is limited to Euglena. It is capable of carrying out all three types of metabolic sequence in contrast to the other two types of organisms represented by Tetrahymena or Chlorella.

It is interesting to speculate upon the possible significance of these facts with respect to the phylogenetic relationships of Euglena.[10] They could be used to confirm the old notion based upon morphology and gross physiology that Euglena represents the type of parent at which the separation of the plant and animal kingdom took place. We could suggest that the evolution toward the animal kingdom involved the loss of not only the photochemical apparatus but the ability to reduce carbon dioxide by the path of carbon dioxide fixation. This does not imply that all the animal kingdom as we know it today must have passed through photosynthetic organisms in their evolution. It is

quite conceivable and altogether likely that many of them have evolved directly from the primitive heterotrophic, dependent reproductive units. The distinction between those which have and those which have not passed through a photosynthetic stage in their evolution may eventually be found on the biochemical level in terms of carbon dioxide fixation patterns or some similar criterion.

References

(1) Lynch, V. H., and M. Calvin
1952. J. Bact., 63: 525.

(2) Albaum, H. G., A. Schatz, S. H. Hutner and Alta Hirshfeld.
1950. Arch. Biochem. 29: 210.

(3) Benson, A. A., et al.
1950. J. Am. Chem. Soc. 72: 1710.

(4) Calvin, M., J. A. Bassham, A. A. Benson, V. H. Lynch, C. Ouellet, L. Schou, W. Stepka and N. E. Tolbert
1951. Symp. Soc. Exper. Biol., No. V. CO_2 Fixation and Photosynthesis.

(5) Buchanan, J. G., et al.
1952. Phosphate Metabolism, II, Johns Hopkins Press, in press.

(6) Buchanan, J. G., M. Calvin, V. Lynch, A. A. Benson and D. Bradley
J. Chem. Soc., in press.

(7) Bassham, J. A., A. A. Benson and M. Calvin
1950, J. Biol. Chem. 185: 781.

(8) Calvin, M.
1950-1951. The Harvey Lecutres, Series XLVI, Charles C. Thomas, Springfield, Ill.

(9) Glover, J., M. D. Kamen and H. Van Genderen
1952. Arch. Biochem. Biophys., 35: 384.

(10) Hutner, S. A. and Luigi Provasoli,
1951. Biochemistry and Physiology of Protozoa, A. Lwoff, Academic Press, New York. Vol. 1.

Captions to Figures

Figure 1 - Patterns of light and dark CO_2 fixation by _Euglena_ compared to dark fixation by two other organisms.

Figure 2 - Time course of dark CO_2 fixation by _Euglena_.

Figure 3 - Time course of dark CO_2 fixation in various compounds by _Euglena_.

Figure 4 - Diagrammatic representation of possible enzymatic interrelationships among several organisms.

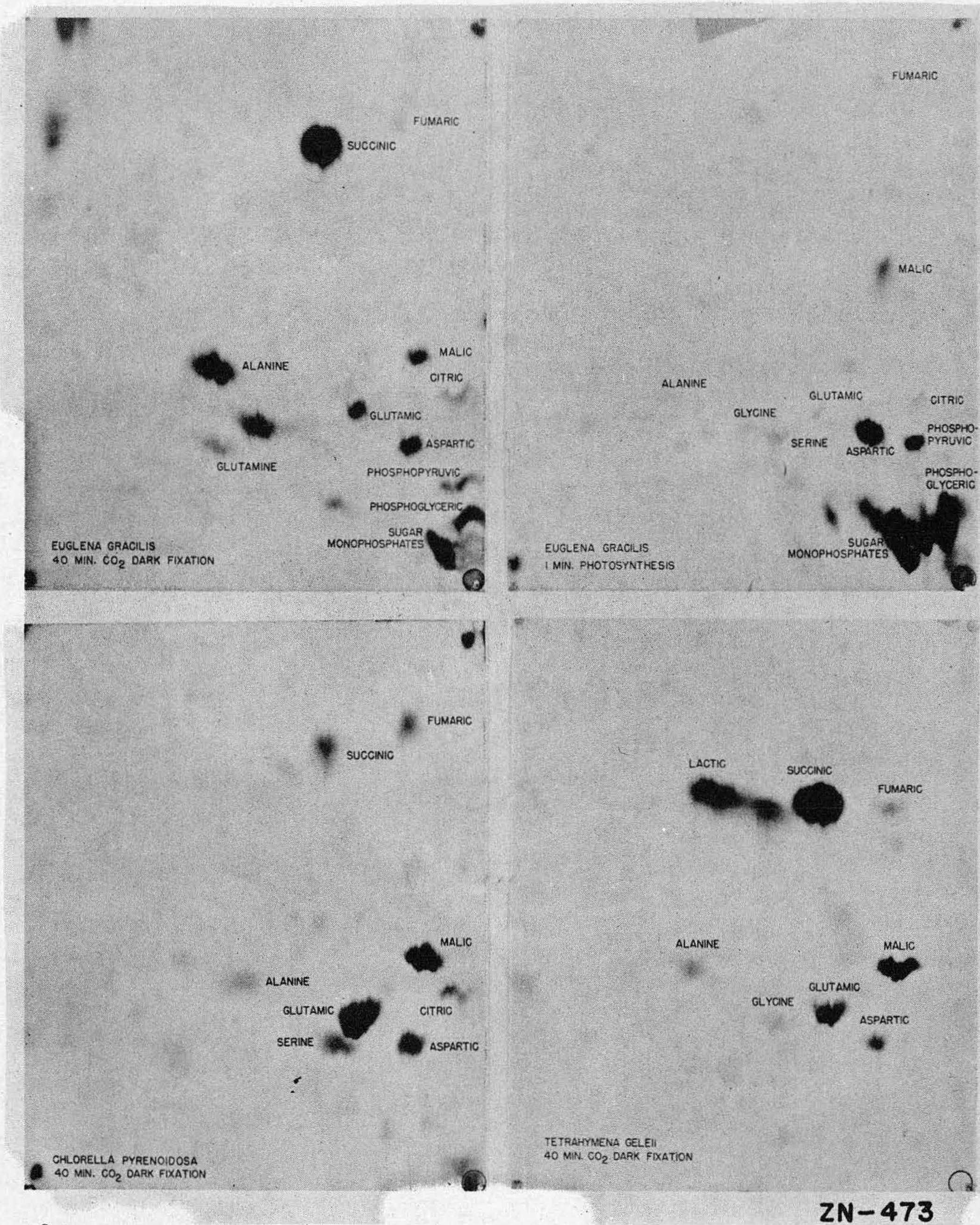
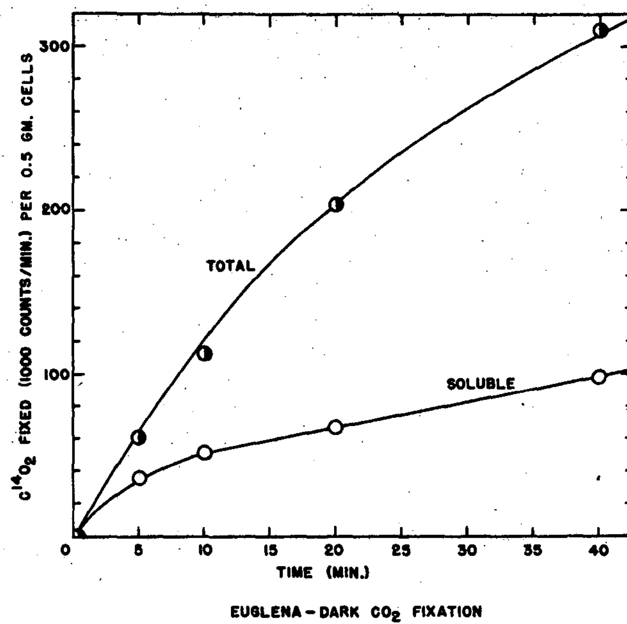


Fig.1



EUGLENA - DARK CO_2 FIXATION

Fig.2

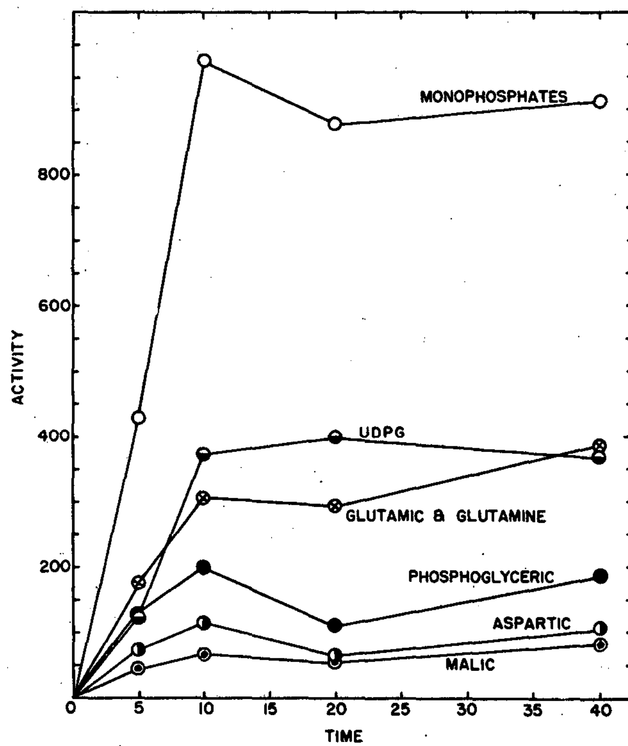


Fig.3

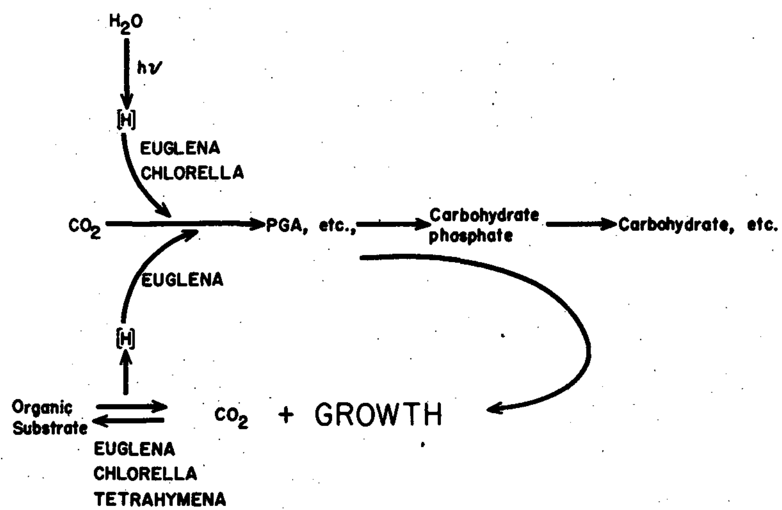


Fig.4