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Title THE ENZYMATIC MEASUREMENT OF PRIMARY PRODUCTION

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The purpose of this investigation was to determine and measure some enzymes directly related to photosynthetic and respiratory pathways in marine phytoplankton. The quantitative measure of these enzymes could be used to give a feasible and practical index of primary production.

Enzyme activity from TPN-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPD) in Skeletonema costatum was determined. This enzyme and another, phosphoglycerate kinase (PGK) are enzymes in the reductive phase of the pentose phosphate pathway which is the point of convergence for the "light" and "dark" reactions of photosynthesis. The quantitative measurement of GAPD was found to be low when compared with oxygen data. Using commercial PGK, only DPN-GAPD had high enough activity to account for the oxygen produced. This indicated the possible role of DPN-GAPD and/or TPN-GAPD in the reductive phase of the pentose phosphate pathway.

Malic dehydrogenase, the enzyme used to measure respiratory potential, was found to have higher enzymatic rates than the amount of oxygen respired. This suggested the possibility of a

second route of carbon dioxide fixation.

At the present time, it appears that the enzymatic measurement of primary production will not be feasible until the basic biochemical pathways are definitely resolved in the marine phytoplankton.

THE ENZYMATIC MEASUREMENT OF PRIMARY PRODUCTION

by

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THE ENZYMATIC MEASUREMENT OF PRIMARY PRODUCTION

INTRODUCTION

The measurement of marine phytoplankton production is of prime importance to biological oceanographers, for this is the basis for understanding the mechanisms by which the foundation of nearly all the biological food chains in the sea are formed. Strickland (18, p. 1) states that the photosynthetic fixation of carbon dioxide to form plant material is probably the most important single factor governing the productivity of any sea region; therefore, the quantitative measurement of this fixation is of the greatest importance to fisheries. Steele (17, p. 519) adds that the measurement of plant carbon dioxide fixation is not merely a measure of primary productivity but is a means to explain further not only the physiology of the plants themselves, but their reactions to the physical conditions and interactions with the other organisms in the sea.

Strickland (18, p. 61-100) summarized and discussed the methods currently used for production studies. In his survey it becomes apparent that each of these methods seems to have certain limitations in regard to accuracy and/or utilization at sea. Since the "ultimate" method in primary productivity measurement has not as yet been achieved, it is necessary to investigate other possibilities.

It was mentioned by Strickland (18, p. 49) that if certain key enzymes involved in photosynthesis and/or respiratory cycles

could be measured quantitatively, a reasonable index for both primary production and respiration in plants, and perhaps secondary in animals, could be developed. The measurement of the enzymatic rate under the conditions of the experiment would give the potential rate of the system being measured. For example, if a photosynthetic enzyme were measured the potential or maximal rate of plant production would be obtained. These potential rates would have to be converted to actual rates by consideration of the natural environmental factors. Enzymatic measurement already has become an important biochemical tool in many fields such as botany, food chemistry, agriculture chemistry, and in medicine for the diagnosis of many diseases (5, p. 651-723).

There is an enzyme system which appears to be characteristic to most photosynthetic organisms. Arnon and Hageman (11, p. 421-423) found that the oxidation of glyceraldehyde-3-phosphate (GAP) in green leaves was catalyzed by three enzymes. These were: 1) the "classical" glyceraldehyde-3-phosphate dehydrogenase with the cofactor DPN, as found in non-photosynthetic tissues, 2) an enzyme with characteristics similar to the classical GAP but utilizing the cofactor TPN, and 3) a second TPN-specific enzyme which he called glyceraldehyde-3-phosphate TPN-reductase (GTR). The last one differed from the others in enzymatic properties (14). The pyridine nucleotide dependence of the GAP system was investigated throughout the life cycle of pea plants from seed to seed. It was established that the GAP system changed cyclically from obligate DPN

dependence in the seed to a multi-component system in the shoot, functional with either DPN or TPN, and back to obligate DPN dependence in the newly formed seed. The light-induced emergence of TPN activity localized in photosynthetic tissue, its disappearance in newly formed seeds, and its absence from roots suggested that these species of GAP enzymes are associated in some way with photosynthesis.

Fuller and Gibbs (10) experimented on a number of organisms, both plants and bacteria, and found that all the autotrophs investigated had the TPN-linked GAP dehydrogenase, while the heterotrophs lacked this enzyme. However, a genetic albino barley mutant produced by x-ray irradiation which possessed the TPN-dependent dehydrogenase lacked the carboxylating enzyme for carbon fixation. Benedict and Beevers (4) also have discovered a TPN-linked GAP dehydrogenase in the colorless endosperm tissue of some germinating fatty seedlings. Similie and Fuller (16) state that since the DPN-GAP dehydrogenase is associated with both the chloroplasts and cytoplasm in pea leaves, this enzyme may participate actively in photosynthesis. In anaerobic photosynthetic bacteria, DPN-GAP dehydrogenase appears to be the only one operating in photosynthesis. Further, Similie and Fuller (16) state that the TPN-dependent GAP dehydrogenase, which has been found in the lamallae of chloroplasts, probably does function in the photosynthesis of plants and algae.

In species related to those normally found in the marine

phytoplankton, Richter (13) found the presence of a TPN-dependent GAP dehydrogenase in two photosynthetic algae, Anacystis nidulans and Chlorella pyrenoidosa. Brawermin and Konigsberg (6) traced the formation of a TPN-requiring GAP dehydrogenase during the production of chlorophyll in Euglena gracilis. Their work indicated that green Euglena cells possess a TPN-requiring enzyme, while dark-grown, colorless cells contain only the DPN enzyme. In resting cells exposed to light, the rate of chlorophyll formation is followed by an increase in TPN-enzyme production. If light is removed during this process, it results in the cessation of both TPN enzyme and chlorophyll synthesis. Culturing cells at 34°C in the light produced inhibition of chloroplast synthesis. Return of the cells to a lower temperature brought about a resumption of chloroplast synthesis after a lag of one or two days. Production of TPN followed chloroplast production very closely during both inhibiton and recovery. On the other hand, the synthesis of the DPN-GAP dehydrogenase was linked to the multiplication of the cells during this process.

It can be concluded that the TPN-dependent GAP dehydrogenase has a role intimately connected to photosynthesis. The enzyme has been found in a majority of photosynthetic organisms investigated with the one major exception of anaerobic photosynthetic bacteria (16). Although the enzyme has been shown to be present in two non-photosynthetic tissues, these occurrences appear to be unique exceptions. On the premise that these are exceptions, the

quantitative measurement of TPN-GAP dehydrogenase activity should constitute a specific means of determining potential photosynthesis. In this study potential photosynthesis is defined as the maximal rate of enzymatic activity in the reductive phase of the pentose phosphate pathway, which is described below.

The major photosynthetic and respiratory pathways are represented diagramatically in Figure 1. The path of carbon in the "dark reaction" of photosynthesis has been described by Calvin and Bassham (7, p.8-20), and according to Calvin and Bassham (3, p. 294) accounts for at least 70% of carbon dioxide fixation in the cell. The major portion of the pathway which was under investigation in the present study is referred to by Calvin as the reductive phase of the pentose phosphate pathway. This part of the cycle begins after the initial incorporation of carbon dioxide by ribulose-1,5-diphosphate; that is, with the first stable intermediate, phosphoglyceric acid (PGA). Phosphoglycerate is converted to 1,3-diphosphoglycerate by the enzyme phosphoglycerate kinase (PGK), and this in turn is transformed to glyceraldehyde-3-phosphate by the enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPD). As elucidated by Arnon et al. (1, p. 16-17), the energy used to drive this reaction comes ultimately from the light reaction of photosynthesis in plants and algae via the chemical energy donors ATP and TPNH. Consequently the reductive phase of the pentose phosphate pathway is the point of convergence for the "light" and "dark" reactions of photosynthesis, and if measured could give a representative index

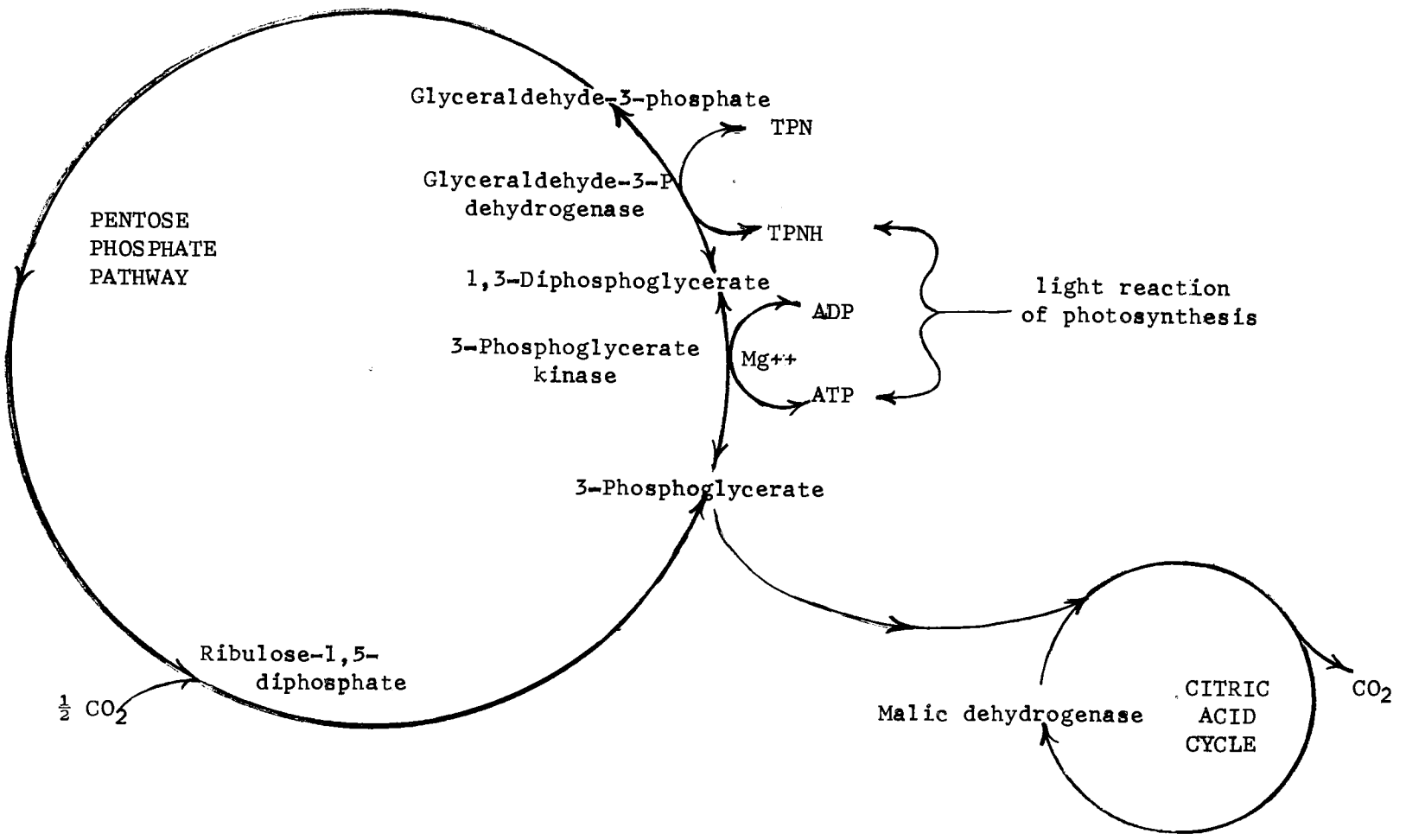

Figure 1: Major photosynthetic and respiratory pathways

of photosynthetic potential.

The respiratory cycle, or citric acid cycle (Figure 1), has a number of enzymes, the activity of which could give a respiratory potential. The enzyme chosen for measurement in this work was malic dehydrogenase.

It was the purpose of this investigation to determine whether or not the measurement of the enzyme systems described would be feasible and practical for the measurement of potential primary productivity.

METHODS AND MATERIALS

The majority of the work was performed upon two marine diatoms, Skeletonema costatum (Grenville) Cleve and Nitzschia closterium (Ehrenberg) W. Smith. Other species included a Chlorella sp., a mixed culture of Euglena sp. and Chaetoceros sp., and a mixed population of marine phytoplankton cultured directly from a sea water sample. All these organisms with the exception of the latter were taken from stock cultures which had been isolated from samples taken at sea off the Oregon coast.

The culture medium consisted of either membrane-filtered natural or autoclaved seawater plus the following additions:

Chemicals

Major constituents	gram/100 ml
$NaNO_3$	0.015
$NaH_2PO_4 \cdot 7H_2O$	0.001
Fe · sequestrene (Fe · EDTA)	0.001
$NaSiO_3 \cdot 9\ H_2O$	0.005
$NaHCO_3$	0.01
Tris (hydroxymethyl) amino-methane	0.05
Trace metals	**µg/100 ml**
$CuSO_4 \cdot 5\ H_2O$	0.96
$ZnSO_4 \cdot 7\ H_2O$	4.40
$CoCl_2 \cdot 6\ H_2O$	2.20
$MnCl_2 \cdot 4\ H_2O$	36.00
$NaMoO_4 \cdot 2\ H_2O$	3.00

Vitamins	µg/100 ml
Thiamine	20.00
Biotin	0.10
B_{12}	0.10

These additions were added on the basis of a single dosage for natural populations and a double dosage for the single species cultures. Culture medium was innoculated with 100 ml of cells in exponential growth phase. Cell counts with a Fuchs-Rosenthal chamber were employed to determine when an exponential growth rate was reached. Usually from three to five days were required, depending upon the species grown. The cells had been grown in the same medium used for culture. This prevented a significant lag phase in growth. The cultures were illuminated at 120 ft-candles with "deluxe warm white" fluorescent lights. The cultures were kept at 18°C and constantly bubbled with atmospheric air.

Before the cultures were harvested oxygen and total carbon data were taken for later comparison with enzymatic studies. Sixty milliter light and dark bottles (18, p. 75) were filled with cell culture and incubated in the light for either two or four hours, after which the oxygen gain and loss was determined by the Winkler-O_2 method (18, p. 75-81). Three 100 ml aliquots of cells were filtered on glass-fiber filters for total carbon analysis in an induction furnace using a procedure described by Curl (8).

The cells were harvested by continuous flow centrifugation in a refrigerated centrifuge at 18°C, 8700 g, and a flow rate of

approximately 200 ml/min. During the centrifugation process the culture flask was bubbled with air to maintain a fairly homogenous cell suspension. The collected cells then were washed and recentrifuged at 8700 g and 18°C. The excess media was siphoned off and replaced in the centrifuge tube by the following: 1) 2 mls 0.2 M Tris (hydroxymethyl) amino-methane buffer plus 0.001 M EDTA (ethylene-diamine-tetra-acetic acid); 2) 40 µmoles of freshly prepared L-cysteine ·HCl neutralized to pH 7; 3) 2 mls of 0.2 mm diameter glass beads; 4) a small addition of Dow Corning antifoam 'A' agent. The tube was then placed in an ice bath and the contents homogenized in a Servall Omni-Mixer for 45 seconds at 6000 rpm followed by an increase to 9000 rpm for 15 seconds. The homogenate was transferred to glass tubes and centrifuged at 3020 g for 5 minutes to remove glass beads and cell debris. The supernate was used immediately for enzyme analysis.

Acetone powders of the various phytoplankton populations were prepared to determine whether or not enzyme activity could be preserved. The preparation of acetone powders (12) was carried out in a cold room at 5°C. The excess medium was siphoned from the collected cells, and the cells from each centrifuge tube were poured directly into a flask containing 75 mls of 90% glass-distilled acetone at -15°C. The acetone-treated cells were kept at -15°C for fifteen minutes before filtering onto a paper filter. The precipitate was washed with 25 ml of acetone and suction was maintained for at least five minutes afterwards to remove as much

moisture as possible. The powdered cells were stored at 5°C inside a vacuum dessicator containing silica gel dessicant. Enzyme preparations were made by weighing 20 to 50 milligrams of dried acetone powder into a dry test tube. After the powder was ground with a glass rod, 2 mls of 0.05 M Tris buffer plus 0.001 M EDTA and 40 µmoles of freshly prepared L-cysteine neutralized to pH 7 were added. The contents were stirred well with a glass rod and then centrifuged at 3020 g for five minutes at 18°C. The supernate was then used for enzyme assays. Ten milligram amounts of the various acetone powders were also weighed for total carbon analysis.

The method of enzyme analysis is based on the following principles. The pyridine nucleotides, TPN and DPN, which react with various dehydrogenase enzymes in organisms, have a maximum absorption of 260 mµ. In the reduced state (DPNH and TPNH) the maximum absorption shifts to 340 mµ. A spectrophotometer can be used to measure the reduction of DPN or TPN by following the increase in absorbance of 340 mµ with time. This gives a relative measure of the activity of a dehydrogenase enzyme or an enzyme coupled to the dehydrogenase present in a sample. The selection of a specific enzyme may be determined by choice of substrate (5, p. 4-7). All enzyme measurements were made on a Beckman D-B recording spectrophotometer at 340 mµ and 17.5°C, utilizing test and control cuvettes of 1 cm light path.

For glyceraldehyde-3-phosphate dehydrogenase the Sigma assay

(15) was used but revised to include various aliquots of enzyme extract. Into each of the two cuvettes (except where noted) the following were pipetted:

1.50 to 1.70 ml of Tris buffer, 0.2 M and 0.001 M EDTA buffered at pH 8.5 for 18°C

0.30 ml Na_2HAsO_4, 0.17 M

0.05 ml L-cysteine · HCl, 0.2 M, pH 7

0.60 ml NaF, 0.1 M

0.05 ml DPN or TPN, 0.02 M

0.05 to 0.25 ml cell or acetone powder extract

0.25 ml distilled water to control cuvette only

0.25 ml glyceraldehyde-3-phosphate (approximately 1 μmole) to the test cuvette only, to start the reaction.

The GAP solution was prepared from the diethylacetal (barium salt) according to Sigma Technical Bulletin No. 10 (15).

The assay for phosphoglycerate kinase was modified from Bergmeyer's (5, p. 224-228 and 539-543) phosphoglycerate and ATP analysis as follows:

To the test cuvette were added:

2.30 ml Tris buffer 0.05 M and 0.001 M EDTA buffered at pH 7.55 for 18°C

0.05 ml L-cysteine · HCl, 0.2 M, pH 7

0.25 ml acetone powder extract

0.05 ml $MgCl_2$, 0.1 M

0.10 ml ATP, 0.01 M

0.05 ml DPNH or TPNH, 0.005 M

0.20 ml phosphoglycerate, 0.05 M

The PGA solution was prepared in a similar manner to that of glyceraldehyde-3-phosphate from the diethylacetyl (barium salt). All chemicals were added to the control cuvette with the exception of either PGA or pyridine nucleotide, which were replaced by an equal volume of buffer.

The assay for malic dehydrogenase was adapted from Bergmeyer (5, p. 757-760). To the test cuvette was added:

2.80 ml of Tris 0.05 M plus 0.001 M EDTA buffered at pH 7.55 for 18°C

0.05 ml cell or acetone powder extract

0.05 ml oxaloacetic acid, 0.05 M

0.05 ml DPNH to start the reaction

The control contained all of the above with the exception of DPNH, which was replaced by an equal amount of buffer.

The biochemicals DPN, TPN, GAP, PGA, L-cysteine, and GAPD were obtained from Sigma Chemical Company of St. Louis, Missouri; DPNH, TPNH, PGK, and ATP from Calbiochem, Los Angeles, California; and oxaloacetic acid from Nutritional Biochemical of Cleveland, Ohio.

RESULTS

Considerable time was spent attempting to obtain reproducible enzyme activity from glyceraldehyde-3-phosphate dehydrogenase in *Skeletonema costatum*. The initial experiments involved the culturing of one liter of *S. costatum* and the homogenization of the cells in 0.1 M Tris buffer. Using glyceraldehyde-3-phosphate obtained from Nutritional Biochemicals and prepared by treatment with neutralized Dowex-50x8 ion exchange resin, some extremely weak results of GAPD were observed with DPN. Chard (*Beta* sp.) chloroplasts prepared according to Avron *et al.* (2) for comparison with *S. costatum* also yielded very low GAPD activity. When a commercial GAPD solution purchased from Sigma was tested with the previously used GAP solution, results were extremely low. Therefore, a new solution of GAP was made using Sigma chemicals and directions. The Sigma GAP yielded excellent activity with commercial GAPD; however, a freshly made homogenate extract of *S. costatum* yielded no results with DPN or TPN. If commercial GAPD was homogenized in the same manner as *S. costatum*, high activity was obtained. When commercial GAPD was added to *S. costatum* cells and homogenized no activity was lost, indicating that the *S. costatum* extract was not inhibiting the reaction. These results suggested that *S. costatum* chloroplasts did not liberate the supposedly soluble GAPD (14) during homogenization, and when centrifuged from the supernate the GAPD could not react.

To test this hypothesis, aliquots of fresh S. costatum homogenates were added directly to complete reaction mixtures and allowed to react for ten minutes before activity was measured. This method appeared to yield very low GAPD activity for both DPN and TPN. In order to increase the measured activity by the above method, the amount of cells was increased from one to ten liters, and a timed series of reactions was attempted. The reaction was stopped after various time intervals by centrifugation or heating. However, activity rates obtained by this technique were not reproducible.

The homogenization technique was thought to be either too damaging to the cells or not efficient enough to release GAPD from the chloroplasts, and consequently was abandoned. Grinding the S. costatum cells in a mortar and pestle, using glass beads or alumina powder as an abrasive, proved to be no more effective than the homogenization method. A "sonicated" extract of S. costatum was prepared by Dr. R. Y. Morita of the Departments of Oceanography and Microbiology at Oregon State University. His associate, Sheril Burton, was able to demonstrate the presence of malic dehydrogenase in the cell extract. This indicated that the cells had at least some definite enzyme activity. The presence of malic dehydrogenase then was demonstrated in an acetone powder prepared by Dr. H. Curl, Jr. GAPD activity was sought and found in the acetone powder , but this activity could not be reproduced at later times.

Dr. Morita suggested the addition of cysteine to the cells before homogenization or elution of acetone powders. This step produced substantial GAPD activity. Glyceraldehyde-3-phosphate dehydrogenase is an enzyme that requires a prosthetic group such as cysteine to activate the enzyme (5, p. 979). When cells from S. costatum were subjected to homogenization or the drying process in acetone powder formation, the prosthetic groups were probably oxidized and consequantly no GAPD activity was found. Even though cysteine had always been supplied to the reaction mixture during all earlier measurements, there was probably not enough time for GAPD to become "activated" by cysteine. It was found absolutely necessary to add cysteine to the cells before homogenization or to the acetone powders during elution in order to obtain activity.

Substantial GAPD activity with both TPN and DPN was subsequently found in both homogenized preparations and acetone powders of S. costatum. The acetone powder had several advantages over homogenates. The weighing of the powders allowed enzyme activity to be put on a total carbon basis for comparison with oxygen results. Possible interference by the color of the dark green homogenate was avoided. The acetone powders of most species, if protected from moisture, appeared to maintain enzyme activity for at least two months.

When the definite presence of glyceraldehyde-3-phosphate dehydrogenase had been demonstrated in acetone powders of S. costatum, the next step was to measure activity of this enzyme

quantitatively.

Attempts were made to measure the activity of GAPD directly, as was done by Brawerin and Konigsberg (6). It was not possible to obtain a first order reaction with either DPN or TPN (Figure 2). A first order reaction, which is generally necessary for enzymatic measurement (5, p. 8-13) is indicated by a linear relationship between absorbance, a logarithmic function, and time. Lack of linearity on the semi-log plot suggested that some step was rate-limiting to the total reaction rate. However, when the concentrations of substrate or cofactors were increased and/or the concentration of enzyme extract was decreased no linear relationship could be established. Only approximations of enzyme activity could be obtained which were based on the appearance of linearity during the first 30 seconds of the reaction. The approximate reaction rate for DPN-dependent GAPD was at least six times that of the TPN-dependent GAPD (Figure 2).

In order to obtain first-order reactions it was decided to reverse the direction of the reaction using the substrate phosphoglyceric acid (PGA). This reaction measures the reductive phase of the pentose phosphate pathway in which glyceraldehyde-3-phosphate dehydrogenase is used as an indicator enzyme with the cofactors TPNH or DPNH, coupled to the reaction enzyme phosphoglycerate kinase (PGK) and cofactor ATP.

Initially, when TPNH alone (i.e., with no PGA substrate) was added to the test reaction mixture and "blanked" against a mixture

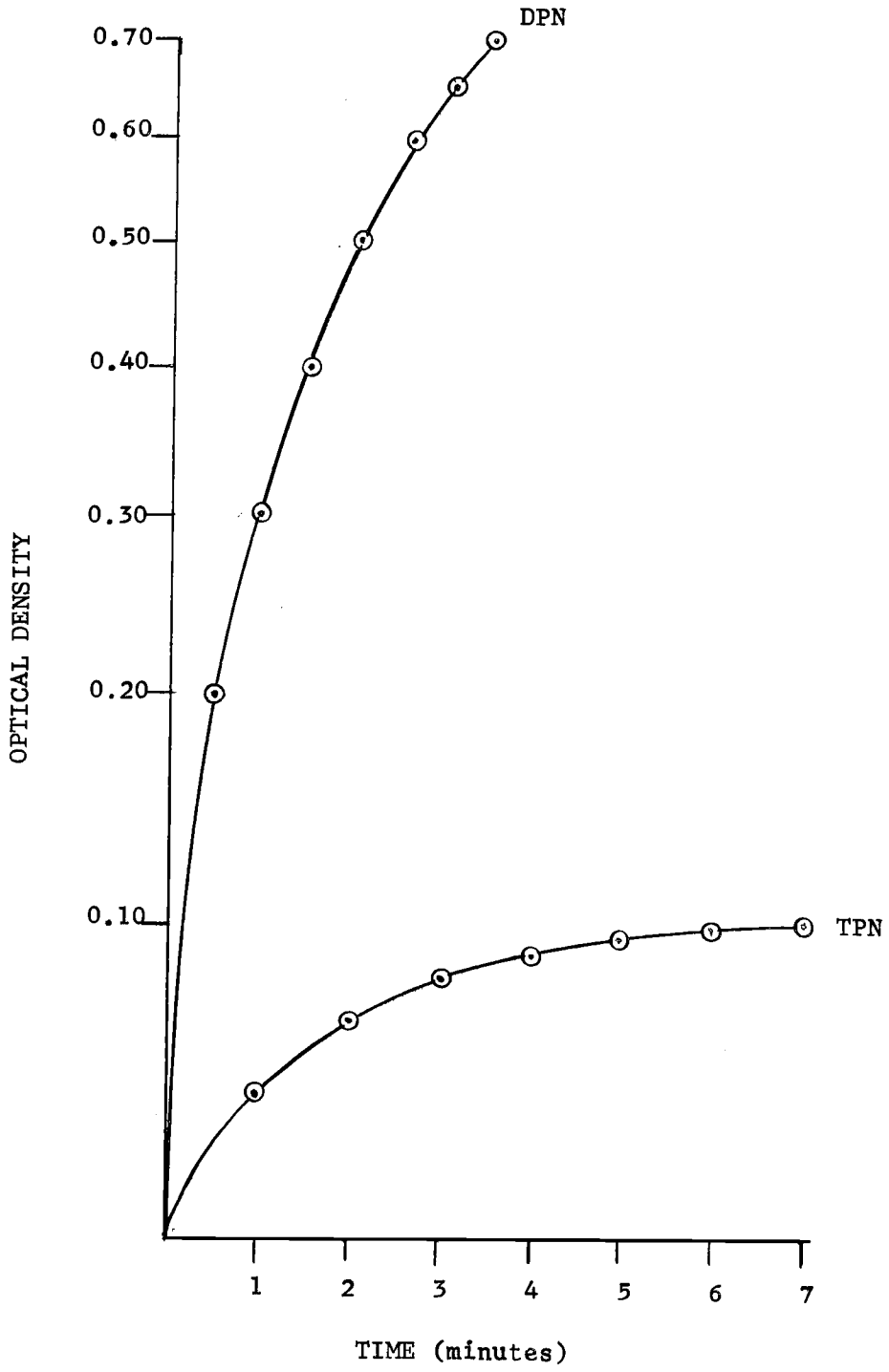

Figure 2: The enzymatic measurement of DPN and TPN dependent glyceraldehyde-3-phosphate dehydrogenase in Skeletonema costatum

without TPNH, a first-order reaction occurred (A, Figure 3). When PGA was added later to both cuvettes the reaction rate doubled but still maintained its first-order characteristics (B, Figure 3). In another trial, TPNH was added to both cuvettes and PGA only to the test cuvette to determine whether the initial reaction was due to endogenous substrate. The reaction was found to be of the first order (C, Figure 3) but only about one half that of the TPNH reaction shown in B, Figure 3. Apparently two reactions rather than one were taking place, suggesting that TPNH must be "blanked" in order to obtain accurate measurement of phosphoglycerate kinase. A second reaction would also account for the curvilinear relationship found in the direct GAPD measurement using TPN (Figure 2). Any TPNH produced could by utilized by a second enzyme and oxidized immediately back to TPN. This would cause the appearance of the observed rate-limiting reaction. When cysteine was replaced by glutathione an enhancement of the TPNH "unblanked" reaction occurred (D, Figure 3), which could be interpreted that glutathione reductase is the second enzyme.

If TPNH was replaced by DPNH for the reaction involving phosphoglycerate kinase, the rate obtained was equal to that found for the TPNH reaction (Table II, B. 2 and 3). PGK, in contrast to GAPD, could be rate limiting for both the DPNH and TPNH cofactors. A lag-phase of up to three minutes, depending upon the concentration of the acetone powder extract, was noted in the TPNH reaction. No lag was observed when DPNH was used. Lack of a lag could be attributed to DPN-dependent GAPD, which is

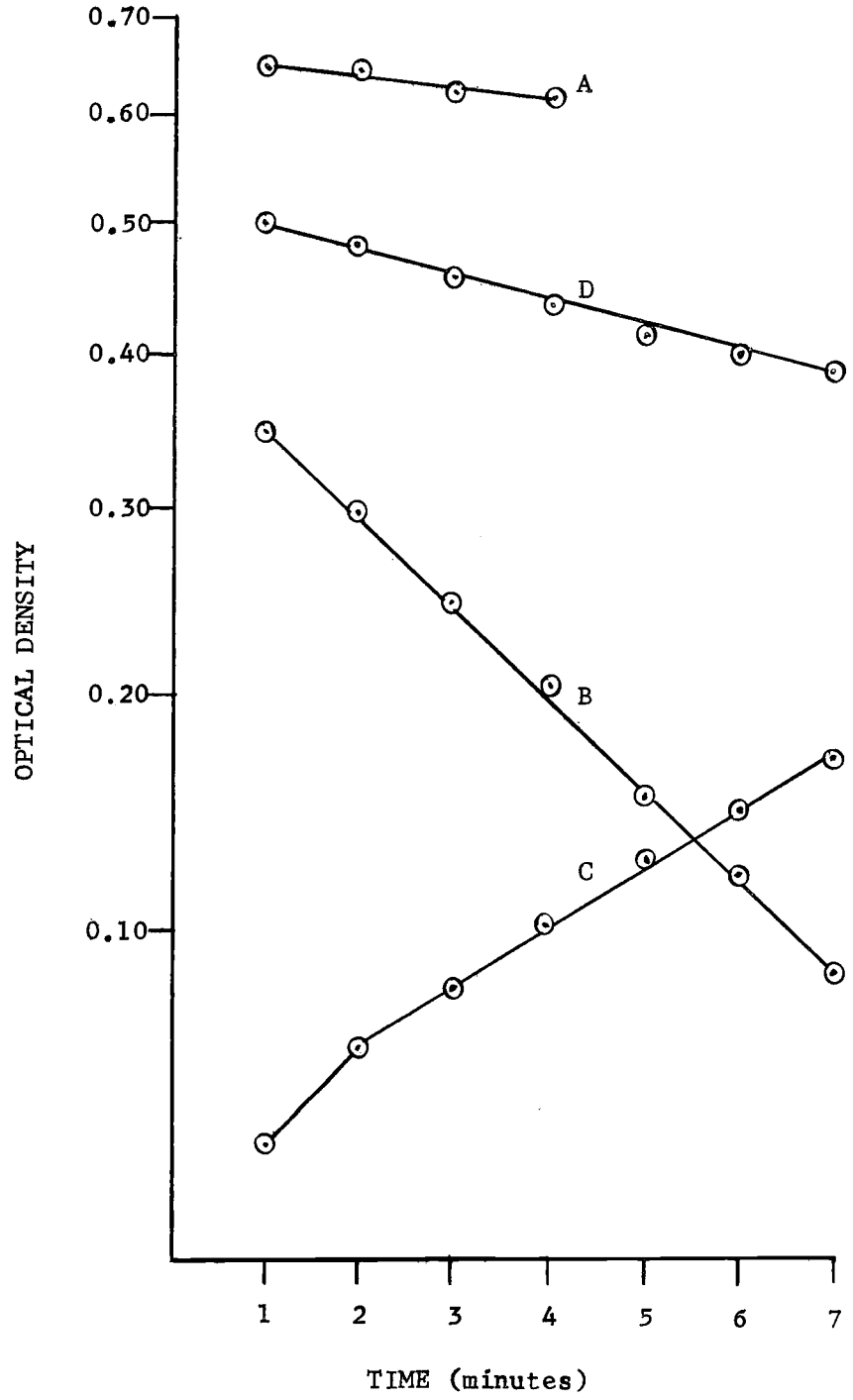

Figure 3: The enzymatic measurement of phosphoglycerate kinase and glutathione reductase with TPNH in Skeletonema costatum

significantly more active than TPN-dependent GAPD (Table II, B. 6 and 7). DPN-dependent GAPD could initially convert the 1,3-diphosphoglycerate product of the phosphoglycerate kinase reaction to glyceraldehyde-3-phosphate at a faster rate than the TPN-dependent GAPD. These same results were obtained for acetone extracts from Nitzschia closterium as well as Skeletonema costatum.

Commercial phosphoglycerate kinase (from Calbiochem) was added to a reaction mixture containing phosphoglycerate and DPNH in order to determine whether or not PGK in acetone powder preparations was rate limiting to the DPN-dependent glyceraldehyde-3-phosphate dehydrogenase. It was found that commercial PGK increased the reaction rate (Table II, B. 3 and 5) approximately 20 fold for S. costatum and 3 fold for N. closterium. This indicates that the PGK enzyme in the acetone powder preparations definitely decreases the maximum rate of GAPD activity.

Malic dehydrogenase (MDH) was the assay enzyme used for the measurement of "potential respiration". First order reactions were obtained for MDH in all organisms assayed, using the substrate oxaloacetic acid and cofactor DPNH (Table II, B. 1). High rates of enzyme activity were obtained with MDH with no observable interference from other enzymes.

Table I is a compilation of data taken in an attempt to derive a relationship between standard primary productivity measurements and enzymatic activities. The data from Table I have been converted to comparable relationships in Table II by the following

TABLE I

Summary of cell counts, oxygen, total carbon and enzyme measurements of three phytoplankton populations.

Species	Skeletonema costatum	Nitzschia closterium	Mixed Population
Cell Counts			
(cells/mm^3)	270	650	700
Oxygen (μg-at O_2/l/hr)			
Gain of O_2 in light bottles	277	264	129
Loss of O_2 in dark bottles	33	26	22
Total Carbon (C_T)			
mg C_T/100 ml culture	2.46	2.95	1.88
mg C_T/10 mg acetone powder (AP)	2.54	3.55	3.14
Enzymatic Rates (ΔE=change in optical density per minute for mg of acetone powder)			
Malic dehydrogenase (MDH)	ΔE = 0.036 for 1.25 mg	ΔE = 0.042 for 0.50 mg	ΔE = 0.040 for 1.25 mg
Phosphoglycerate kinase (PGK) plus TPNH	ΔE = 0.021 for 6.25 mg	ΔE = 0.041 for 2.50 mg	ΔE = 0.009 for 6.25 mg

	Skeletonema costatum	Nitzschia closterium	Mixed Population
Phosphoglycerate kinase (PGK) plus DPNH	$\Delta E = 0.018$ for 6.25 mg	$\Delta E = 0.046$ for 2.50 mg	
Glyceraldehyde-3-phosphate dehydrogenase (GAPD) plus TPN	$\Delta E = 0.072$ for 2.50 mg		
Glyceraldehyde-3-phosphate dehydrogenase (GAPD) plus DPN	$\Delta E = 0.104$ for 0.50 mg		
GAPD plus 0.02 mg of commercial PGK and TPNH	$\Delta E = 0.004$ for 2.5 mg	$\Delta E = 0.015$ for 2.50 mg	
GAPD plus 0.02 mg of commercial PGK and DPNH	$\Delta E = 0.022$ for 0.25 mg	$\Delta E = 0.060$ for 1.00 mg	

TABLE II

Comparable oxygen and enzymatic data for three phytoplankton populations

Species	Skeletonema costatum	Nitzschia closterium	Mixed Population
A. Oxygen in units of µg-at O_2/min/mg C_T			
1. Gain of O_2 due to photosynthesis	0.187	0.149	0.115
2. Loss of O_2 due to respiration	0.022	0.015	0.020
B. Enzymatic Rates in units of umole TPNH or DPNH reduced or oxidized/min/mg C_T			
1. MDH	0.078	0.114	0.049
2. PGK plus TPNH	0.007	0.022	0.002
3. PGK plus DPNH	0.007	0.025	
4. GAPD plus commercial PGK and TPNH	0.003*	0.008*	
5. GAPD plus commercial PGK and DPNH	0.158*	0.082*	
6. GAPD plus TPN	0.052**		
7. GAPD plus DPN	0.374**		

* These reactions were run several days after the preparation of the acetone powder; consequently, these rates may be lower due to loss of enzyme activity with time in the powders.

** These reaction rates were approximated from the first minute of reaction time and may be different than the actual rate.

calculations:

1. Oxygen data are converted to µg-atoms O_2 per minute per milligram of total carbon (C_T). A sample calculation is shown for the light bottle oxygen production of S. costatum.

a. Gain of O_2 due to photosynthesis = 277 µg-at O_2/l/hr (Table I)
= 0.46 µg-at O_2/100 ml/min

b. 100 ml of culture = 2.46 mg total carbon (C_T)

c. Gain of O_2 due to photosynthesis = 0.187 µg-at O_2/mg C_T/min

2. Enzymatic rates are converted to umole of TPNH or DPNH per minute per mg C_T. Bergmeyer (5, p. 36-37) gives an equation for the concentration of pyridine nucleotides per ml.

$$C = \frac{\Delta E \times V}{a_s \times d \times v}$$

where:

C = concentration in µmole/ml

ΔE = change in optical density/min

V = volume size of cuvette (3 ml)

a_s = extinction coefficient, (6.22 $\frac{cm^2}{\mu mole}$ for both TPNH and DPNH at 340 mµ)

d = length of light path (1 cm)

v = volume of sample

In this study, S. costatum had a ΔE = 0.021 for PGK plus TPNH, using 0.25 ml of 50 mg AP/2 ml which is equivalent to 6.25 mg AP. Concentration of TPNH is then calculated.

a. $C = \frac{(0.021 \text{ change in optical density/min})\ (3 \text{ ml})}{(6.22\ \frac{\text{cm}^2}{\mu\text{mole}})\ (1 \text{ cm})\ (6.25 \text{ mg AP})}$

$= 0.0162$ µmole TPNH oxidized/mg AP/min

b. 10 mg of acetone powder (AP) = 2.40 mg C_T

c. $C = 0.0068$ µmole TPNH/mg C_T/min

TABLE III

The amount of enzyme activity maintained over a period of two months. All units are expressed in µmole TPNH or DPNH/mg C_T/min

Species	Initial PGK activity	PGK activity after 2 months	% activity retained
S. costatum	0.006 µmole TPNH	0.000 µmole TPNH	0
N. closterium	0.021 µmole TPNH	0.021 µmole TPNH	100
Chlorella sp.	0.017 µmole TPNH	0.012 µmole TPNH	69
	Initial MDH activity	MDH activity after 2 months	% activity retained
S. costatum	0.047 µmole DPNH	0.047 µmole DPNH	100
N. closterium	0.174 µmole DPNH	0.163 µmole DPNH	94

TABLE IV

The relative enzyme activities for MDH and PGK of various phytoplankton species. All activities are given in µmole TPNH or DPNH/min/mg C_T

Species	PGK plus TPNH	MDH plus DPNH
Chlorella sp.	0.017	0.519
Euglena sp.	0.095	0.315
N. closterium	0.022	0.114
S. costatum	0.007	0.078
Mixed population	0.002	0.049

DISCUSSION AND CONCLUSIONS

The results indicated that the direct measurement of GAPD in S. costatum and N. closterium is complicated by the interference of another enzyme, probably glutathione reductase (Figure 2 and 3). Also, PGK is much lower in activity than the DPN-dependent dehydrogenase (Table II). Therefore, to determine which enzymatic method measures more accurately the rate of the reductive phase of the pentose phosphate pathway, it is necessary to compare the enzyme results with another standard technique.

The oxygen "light and dark bottle" method is a standard technique for measuring both photosynthesis and respiration; however, this measurement can be complicated by oxygen bubble formation and absorption of iodine by phytoplankton oils (18, p. 75-77). In this investigation all samples were run in triplicate and averaged to reduce overall experimental errors including bubble formation. The iodine absorption should be constant in light, initial, and dark bottles during a particular experiment and consequently should not affect the results. The results of the oxygen measurements are given in Table I.

In the light reaction of photosynthesis, the amount of oxygen produced is related to the moles of TPNH formed and later oxidized in the dark reaction of photosynthesis according to the Hill reaction (9, p. 554):

$$TPN + H_2O \xrightarrow{light} TPNH + H^+ + \tfrac{1}{2} O_2$$

For every 0.5 mole of O_2 (i.e., gram-atom of oxygen) formed, an equivalent mole of $TPNH_2$ is also produced. For purposes of comparison, the results for oxygen in Table II in µg-at O_2/mg C_T/min are equivalent to µmole TPN(H) or DPN(H) oxidized or reduced/mg C_T/min.

The oxygen evolved in the light reaction is a measure of the actual photosynthetic rate, whereas the enzymatic measurement of TPN-dependent GAPD is a measure of photosynthetic potential as defined earlier on page 5. A value for photosynthetic potential, because it is by definition a measure of the maximum amount of substrate converted per unit time, would be expected to be somewhat higher than the amount of O_2 produced. However, the results presented in Table II reveal that no TPN(H)-GAPD measurement was high enough to account for the amount of oxygen produced by photosynthesis. It should be noted that TPN-GAPD can lose enzyme activity with time as shown by S. costatum in Table III, but even fresh enzyme preparations have low TPN-GAPD activity in comparison with the much higher results obtained for DPN-GAPD.

It appears, from Table II, that the measurement of PGK is also too low to account for the oxygen produced (Table II, B. 2 and 3). Whether this lower PGK activity is a natural occurence in the organisms or is due to the manner in which cell preparations were made is a question which awaits further investigation. Additions of commercial PGK (Table II, B. 5) greatly enhance the measurement of DPN-dependent glyceraldehyde-3-phosphate dehydrogenase and yielded a linear relationship which approximates the

the oxygen produced in photosynthesis. This method of measuring GAPD removes the interference found when measuring GAPD directly using DPN, and it appears to be the most representative value of potential photosynthesis; however, the question concerning the low activity of PGK still must be resolved.

Because some DPN-GAPD values (Table II, B. 5) do approach the amount of oxygen produced, DPNH, in addition to TPNH, may be utilized in the reductive phase of the pentose phosphate pathway of the organisms investigated. It was noted on page 19 that there was a definite lag phase when TPNH reacted with GAPD; however, no lag was observed when DPNH was used. This could indicate further that DPNH may be used preferentially in the pathway. If this is the case, there should be an enzyme capable of converting the TPNH formed in the light reaction of photosynthesis to DPNH. A second possibility is that the DPNH could be produced directly from the light reaction of photosynthesis, as in anaerobic photosynthetic bacteria (16).

A comparison of the rates of loss of oxygen due to respiration and malic dehydrogenase (Table II, A. 2 and B. 1) indicate that the rate of MDH activity is two to ten times greater than the O_2 respired. This difference is magnified if one considers that MDH is only one of five possible sites of oxygen uptake in the citric acid cycle (9, p. 522). The high activity of MDH might suggest its participation in another carbon dioxide fixation pathway as suggested by Walker (19, p. 232-238).

It is conceivable that one of the two moles of phosphoglycerate (PGA) formed after the initial incorporation of one mole of carbon dioxide may proceed to malic dehydrogenase instead of recycling through the pentose phosphate pathway. This route could possibly proceed through "malic enzyme" (9, p. 512-513). This enzyme incorporates carbon dioxide into pyruvate, previously converted from PGA, and utilizes TPNH which could be derived from the photosynthetic light reaction. Calvin (3, p. 294) states that at least 70% of carbon dioxide is fixed via the pentose phosphate pathway in Chlorella sp.. However, if "malic enzyme" or others similar to it are present in marine phytoplankton, it would be necessary for photosynthetic potential measurement to establish what portion of CO_2 is fixed by these enzymes.

Table IV lists several species that were assayed during this investigation. Chlorella sp. and Euglena sp. have very high enzyme activities but generally are not predominate species in the marine environment. S. costatum, an ubiquitous, neritic, marine diatom, has been found to be a species with low enzymatic rates and one in which it is difficult to preserve enzyme activity, especially for TPN-GAPD (Table III). Nitzschia closterium, another common marine diatom, has moderate enzymatic activities which are preserved well in acetone powders. Therefore, N. closterium would be a suitable species for future investigations into biochemical pathways of marine phytoplankton.

The results that have been obtained in this investigation

indicate that the enzymatic measurement of potential primary productivity is not feasible at present for several reasons. First, large amounts of material are necessary for enzymatic assays. Natural standing crops at sea are relatively low. Strickland (18, p. 56-59) lists selected examples of crop measurements which vary from 0.001 to 3.1 mg C/l. The amounts of acetone powder used in the various enzymatic measurements varied from 0.25 to 6.25 mg AP, which is equivalent to 0.06 to 15.0 mg C_T/l. This comparison indicates that it might be feasible to obtain enough material from one liter of sea water with a standing crop of 0.1 mg C/l to measure the more active DPN enzymes (MDH and DPN-dependent GAPD). It would not be possible to measure the less active TPN-dependent GAPD with the methods employed in this investigation.

Even though the preparation of acetone powders could be simply adapted for operation at sea, the preservation of enzymatic activity works well in some species, but it does not in others (Tables II and III).

It is often difficult to obtain reproducible enzymatic measurements unless several variables are controlled. The acetone powders must be protected from moisture to prevent significant loss of enzyme activity. Biochemicals used in assays should be made freshly in order to prevent biochemical decay. Enzyme rates are dependent upon temperature; therefore, the temperature should be kept constant not only from the time the acetone powder is eluted to the time it is measured but also from one experiment to the

next. Other sources of errors include the weighing of biochemicals and pipetting of solution.

The results have raised questions regarding the actual role of TPNH and DPNH in the reductive phase of the pentose phosphate pathway. Another question raised is the possibility of a second major site of carbon dioxide fixation involving light energy from photosynthesis. Until the actual relationship of enzyme activity can be related correctly to the overall reactions of photosynthesis and respiration, potential primary productivity cannot be interpreted by means of enzymatic measurement.

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