COMPARATIVE STUDIES OF PHOTOSYNTHESIS
AND THE HILL REACTION
IN NOSTOC MUSCORUM AND CHLORELLA PYRENOIDOSA

DISSERTATION
Presented in Partial Fulfillment of the Requirements
for the Degree Doctor of Philosophy in the
Graduate School of The Ohio State
University

by
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1954

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Acknowledgements

The author wishes to express his appreciation for the kind cooperation of Dr. Carroll A. Swanson and other members of the Department of Botany and Plant Pathology at the Ohio State University. He wishes to thank the Charles F. Kettering Foundation, in whose laboratories at Yellow Springs, Ohio the work on this dissertation was performed, for the Fellowship which made this study possible. The author especially wishes to express his gratitude to Dr. Kenneth A. Clendenning under whose guidance this investigation was conducted, and to Dr. H. Clyde Eyster for his generous cooperation.
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Introduction

The individual cells of Cyanophyta resemble bacteria in having no visible organization of the cell contents. Plastids, mitochondria, nuclei, and other organelles are invisible under the light microscope. Flagellated forms are unknown. Members of the Cyanophyta may be either unicellular or organized into filamentous or non-filamentous colonies. Sexual reproduction is unknown (72). A gelatinous material is exuded through the cell membrane and accumulates about the cell as a sheath. This sheath may or may not be pigmented, and its thickness varies greatly with the species (73) as well as the conditions of culture.

The pigments of the Cyanophyta usually include c-phycocyanin, c-phycoerythrin, six or more carotinoids (48), and chlorophyll a. Unlike Chlorophyta, Euglenophyta and higher plants, but like the other algal groups, blue-green algae do not contain chlorophyll b (73). The blue chromoprotein phycocyanin usually predominates, and it is from this pigment that the group derives its common as well as its scientific name. However, there are vivid red species in which c-phycoerythrin predominates such as *Trichodesmium erythraeum*, which is responsible for the color of the Red Sea (35). Although blue-green algae do not contain chloroplasts, it has been demonstrated by electron microscopy and fractional centrifugation that their photosynthetic pigments are located in laminated grana which are similar in size and organization to those of higher plant chloroplasts (19). The Cyanophyta resemble photosynthetic
bacterja and differ from all other plants in that they exhibit photosynthesis without possessing chloroplasts which can be seen at the highest magnification of the light microscope, although grana-like structures have been observed (19).

The Cyanophyta usually are found growing independently but a number of species grow symbiotically with fungi in lichens, parasitically in the digestive tract of man and lower animals, and epiphytically on higher plants (73). Members of the Cyanophyta are found as pioneer vegetation on almost all parts of the earth's surface, from alpine heights down to several meters below the surface of fresh and salt water, and in desert, tropical, polar, and temperate regions (72). They have been found living at temperatures as high as 85-90°C, (47) as well as near the freezing point of water.

The metabolism of the Cyanophyta has been investigated along several lines of some interest to the student of photosynthesis, although in comparison to other plant groups and photosynthetic bacteria, photosynthesis itself in blue-green algae has received little attention. The areas of investigation in which extensive work has been done, and on which brief reviews follow are (1) algal "blooms", (2) chromatic adaptation, (3) energy transfer from accessory pigments to chlorophyll, (4) hydrogen adaptation, (5) assimilation of atmospheric nitrogen, (6) phototaxis and other forms of locomotion.
(1) Algal "blooms".

An algal "bloom" is caused by a sudden increase in the algal population, occasioned by optimum conditions for photosynthesis and growth. The accumulated algae quickly decompose and cause an obnoxious contamination of the water (75). It has been reported that poisoning and death of livestock has been caused by water heavily contaminated with blue-green algae (76). Dense populations of Microcystis were found toxic in experimental studies (76). This same poisonous property, whose chemical nature is unknown, may destroy large populations of fish (75). Members of the Cyanophyta of importance largely because of their "blooms" are: Aphanizomenon, Anabaena, Coclosphaerium, Diplocystis, Microcystis, and the red member Trichodesmium of the Red Sea (35, 75). Enormous accumulations of Anabaena and Microcystis occur during July and August in shallow parts of Lake Erie. Members of other algal groups may also form blooms, but those of the Cyanophyta are of greatest importance. Research in this applied field has provided collections of pure strains, as well as information on their culture and control. The strain of Nostoc muscorum used in the present study was originally provided by Dr. Gerloff and Dr. Skoog of the University of Wisconsin, where considerable research was being conducted on algal "blooms".

(2) Chromatic adaptation to light of different colors and intensities.

According to the theory of complementary chromatic adaptation, which was first applied to the Rhodophyta, the color of the pigment
complex is approximately complementary to the color of the light in which the plant is growing. Thus it is green light which reaches the lowest zone of vegetation in the ocean, where the plants are red in color. When the same red algae are grown in red light, they may lose a good deal of their phycoerythrin and gain chlorophyll so that their color changes from red to green (39). Corresponding changes in pigmentation within the Cyanophyta are called "Gaidukov phenomena", and occur in several species, as shown by Dangaerd (22), Gaidukov (36, 37, 38, 39), Klingstedt (50), and Sargent (70).

Both the total amount of photosynthetic pigments and their concentration ratios may also be changed by varying the light intensity (58) and by inducing mineral deficiencies (44, 45). Conflicting evidence concerning the pigmentation in relation to these environmental factors may be due to their simultaneous operation (54). Boresch has concluded that chromatic adaptation is mainly due to changes in the concentration of the accessory pigments (9, 10). Quantitative methods for the determination of phycocyanin and phycoerythrin, however, have not been developed or applied in this field of research.

(3) Energy transfer from accessory pigments to chlorophyll.

If monochromatic light which is absorbed specifically by one pigment such as c-phycocyanin induces fluorescence in a second pigment such as chlorophyll a, and if the reverse phenomenon does not occur, then it may be concluded that light energy absorbed by c-phycocyanin is transferred to chlorophyll a, and that energy transfer does not occur in the reverse direction. If the chlorophyll
fluorescence induced by phycocyanin is as intense as when the same amount of light is absorbed by chlorophyll itself, this would be taken as evidence that the transfer of energy had occurred with 100% efficiency.

Emerson and Lewis (29) studied the action spectra of the unicellular blue-green alga Chroococcus in Warburg's No. 9 buffer, and from their work it appeared that light absorbed by phycocyanin is utilized just as efficiently as light absorbed by chlorophyll. Arnold and Oppenheimer (6) concluded from their related studies of Chroococcus that its phycocyanin-sensitized photosynthesis could be completely accounted for by the transfer of energy to chlorophyll by internal conversion. Wassink (86) has presented a theoretical diagram to illustrate energy transfer within the pigment-protein complex. He and his colleagues investigated energy transfer within members of different algal groups as well as photosynthetic bacteria (86). Duysens (26) conducted the most thorough investigation that has yet been reported on the transfer of energy from accessory pigments to chlorophyll within all of the major groups of photosynthetic organisms. He showed that the phycocyanins, phycoerythrins, and fucoxanthin transfer absorbed light energy to chlorophyll a with approximately 100% efficiency. Carotenoids other than fucoxanthin such as carotenes are much less effective as primary absorbers (26).

Bannister (8) has recently studied the transfer of energy within c-phycocyanin obtained from Synechocystis. He found that ultraviolet light absorbed by the colorless protein of phycocyanin is transferred
with high efficiency to the chromophore, phycocyanobilin. This observation (8) suggests that the colorless protein of chloroplasts and grana may also serve in energy transport, in agreement with Wassink's concept (86).

(4) Hydrogen adaptation in blue-green algae.

When certain algae are incubated in darkness under an atmosphere of hydrogen, they will absorb hydrogen when illuminated with light of moderate intensity and will assimilate carbon dioxide without any accompanying production of oxygen. Such hydrogen-adapted algae possess photosynthetic characteristics resembling those of photosynthetic bacteria (41).

Considerable morphological resemblances exist between some of the filamentous Sulphur- and Iron-Bacteria and certain of the Oscillatoriaceae, the Sulphur-Bacteria having even been classified with Cyanophyta (35). This similarity extends to certain details of structure and method of movement with sulphur droplets being found in the cells of some Oscillatorias and colorless species of this genus being known (35). Since blue-green algae have the same primitive organization as the photosynthetic bacteria, greater similarities in their metabolism might be expected than with seed plants in which hydrogen adaptation is unknown. Hydrogen adaptation has been effected with Synechococcus and Chroococcus (34), but negative results have been reported for Nostoc, Cylindrospermum, and Oscillatoria (34, 68). According to Fogg (32), it appears that blue-green algae which assimilate molecular nitrogen cannot
metabolize molecular hydrogen. There is no evidence that hydrogen adaptation plays any part in the normal process of photosynthesis in algae or higher plants.

(5) Assimilation of atmospheric nitrogen.

The assimilation of atmospheric nitrogen by micro-organisms is an essential part of the nitrogen cycle in nature, and with reference to agriculture it is mainly effected by soil bacteria. Photosynthetic bacteria often possess this property, however, as was shown very clearly by Gest, Kamen and Bregoff (40).

Drewes (25) provided the first evidence for the assimilation of molecular nitrogen by pure cultures of blue-green algae. Recent research on this process has been assisted by the use of N\textsuperscript{15} as a tracer. It has been shown by the tracer method that Nostoc, and Calothrix, are active nitrogen-assimilating organisms (87). According to Fogg (32) there is evidence for nitrogen fixation by at least 21 species of Cyanophyta. So far as is known, no other blue-green alga assimilates molecular nitrogen more rapidly than Nostoc muscorum, which was the principal organism used in the present study of photosynthesis. The detailed mechanism of molecular nitrogen assimilation in bacteria as well as in blue-green algae remains unknown.

Our calculations indicate that nitrogen fixation amounts to less than 0.5% of the accompanying CO\textsubscript{2} assimilation in blue-green algae, so it does not introduce significant errors in measurements of photosynthetic gas exchange. Nevertheless, nitrogen fixation is
an interesting property of Cyanophyta which is of practical importance at least in the rice fields (33).

(6) Phototaxis and motility in blue-green algae.

Primitive flagellated organisms are able to swim actively in their culture media, and the direction in which they swim is often controlled by light. Such phenomena are also shown by higher plants in the form of phototropism (77), as well as chloroplast phototaxis (69). Blue-green algae do not include flagellated, free-swimming cells (35), but most of the filamentous species are able to move spontaneously either by a waving rotation of the filaments in Oscillatoriaceae (35), or by a gliding motion in other families (35). Spontaneous movement has been claimed for Oscillatoria, Spirulina, Anabaena (20), Aphanizomenon (1), Cylindrospermum (43), Synechococcus (66), Gloeotheca (52), Merismopedia (60), Holopedia (55), Isocystis (11), Symplca (71), Nostoc (43), and Tolypothrix (56). Among these, phototaxis is exhibited at least by Symplca (71), Tolypothrix (11), and by many species of Oscillatoria (71), and by Nostoc (43). According to Burkholder (18), rate of movement in Oscillatoriaceae is from 2 to 5 microns per second, but it varies with the species, temperature, pH, and light intensity (17, 18). The most recent work in this field is that by Manten (56) on Tolypothrix, in which it was shown that phototaxis occurs only under conditions favorable for photosynthesis and growth. Recent workers attribute movement of
the Cyanophyta to rhythmic waves along the trichomes (78), but the detailed mechanism remains obscure. The usual ability of blue-green algae to move spontaneously may serve as a means for their dispersal (12).

From these brief reviews it is apparent that several aspects of the physiology of the Cyanophyta have been thoroughly investigated. Surprisingly enough, very little research has been conducted previously on their normal process of photosynthesis. Photosynthesis in leaves has been investigated thoroughly by Willstätter and Stoll (88) and many other workers, and a large amount of information has been provided on photosynthesis in Chlorella by Warburg and other investigators, and on photosynthetic bacteria by Van Niel, Gaffron, and others. With exceptions already noted, corresponding information on photosynthesis in blue-green algae is either meager or entirely lacking. Search of the literature has revealed no instance in which the full rate of photosynthesis has ever been measured on any of the Cyanophyta. Photosynthesis in Oscillatoria geminata in relation to temperature was investigated quantitatively by Bunning and Herdtle (15) but photosynthesis in their experiments was limited by CO₂ supply, and infrared radiation was not removed from their incident light.

The present study is one part of the program of research on blue-green algae which was undertaken several years ago by the Charles F. Kettering Foundation. Aspects which have already been reported are the mineral element requirements (30, 31), and the
assimilation of radioactive acetate (4) and formate (5). The purposes of the present investigation were as follows: (1) the development of manometric methods of general utility in studies of Cyanophyta, (2) the systematic investigation of the factors which control the normal process of photosynthesis in Nostoc muscorum, (3) the investigation of photosynthesis in Nostoc and Chlorella from a comparative standpoint, (4) the classification of some photosynthetic phenomena as attributes of CO₂ assimilation and water photolysis, respectively, through parallel studies of photosynthesis and the quinone reaction, and (5) definition of some of the photosynthetic characteristics of Nostoc during the development of and recovery from potassium deficiency symptoms.
Materials and Methods

Methods of culture

The algae used in this work were:

Nostoc muscorum Born and Flah., a strain obtained from Drs. G. C. Gerloff and Folke Skoog, University of Wisconsin, Madison, Wisconsin.

Chlorella pyrenoidosa, Emerson strain, obtained from Dr. Dean Burk, National Institutes of Health, Bethesda, Maryland.

These algae were collected and maintained on bacteria-free agar slants and in liquid culture for the present and related investigations by Dr. H. C. Eyster and Mrs. L. Birtle, who also harvested the cells and made the packed cell volume and cell count measurements. Descriptions of the methods they used are included for the sake of completeness.

The agar medium for Nostoc contained the inorganic salts used by Emerson and Lewis (28): the agar medium for Chlorella contained Knop's inorganic salts plus glucose and peptone. Eyster's aqueous nutrient solution which supports rapid growth in Nostoc muscorum has the following composition: Solution I, 5.19 gm. NaHCO₃, 0.24 gm. K₂HPO₄ in 4 l.; Solution II, 1.00 gm. MgSO₄·7H₂O, 1.38 gm. Ca(NO₃)₂·4H₂O, plus 8 ml. trace element solution in 4 l.; Solution III, 1.00 gm. FeSO₄·7H₂O in 1 liter; Trace element solution, 126 mgm. Na₂MoO₄·2H₂O, 20 mgm. CoCl₂·6H₂O.
19 mgm. Cu(NO₃)₂·3H₂O, 22 mgm. ZnSO₄·7H₂O, 285 mgm. H₃BO₃, 180 mgm. MnCl₂·4H₂O in 500 ml. Solutions I and II are used in equal amounts, and 7 ml. of solution III are used per liter of complete Nostoc medium. The bicarbonate and dipotassium phosphate keep the pH of Nostoc medium at 7.0 or slightly higher when it is aerated with 5% CO₂ in air. The liquid media used in growing Chlorella were those described by Warburg and his associates (85) and by Emerson and Lewis (28), which had pH values of 4.0 and 6.0, respectively, when in equilibrium with 5% CO₂ in air.

The liquid cultures were grown in the custom-built apparatus illustrated in Figure 1., which was designed by Dr. K. A. Clendenning. This apparatus is similar to that used previously at the University of California, Berkeley, and at the National Research Council Laboratories, Ottawa. It consists of a heavy shaking mechanism for large culture flasks, which is provided with suitable light sources and CO₂ gas train. Provision also is made for approximate temperature control (ca. 25°C) either by keeping the moving flasks partially immersed in a thermostated water bath, or by controlling the temperature of the room with an air-conditioner. The liquid cultures were kept thoroughly mixed by ca. sixty 17 cm. horizontal excursions per minute. The algae were illuminated with ca. 1000 f. c. white light from banks of fluorescent lamps placed above and below the cultures. Carbon dioxide was supplied by a stream of 5% CO₂ in air (usually 35-50 ml./minute).
FIG. 1 ALGAE CULTURING APPARATUS
stemming from a commercial tank source which was regulated with a flow meter. This gas mixture ordinarily passed through several culture flasks in series, and was filtered through cotton plugs to prevent air-borne contamination. It was shown by gas analysis that there was little change in composition of the gas mixture as it passed through several flasks at the adopted flow rate. The custom built Pyrex culture vessels are modified low-form flasks of 3 l. total capacity, having gas inlet and outlet tubes, and additional tubes for withdrawing algal suspension and adding fresh culture solution without opening the flasks. Their all-Pyrex construction allows sterilization by autoclaving, and their design also makes them suitable for use on a continuous culture basis.

To initiate liquid cultures, 100 ml. of liquid medium in sterile 250 ml. Erlenmeyer flasks were inoculated aseptically with cells from agar slants. After several days growth on a wrist-action shaker, when the cell density had attained ca. 1 mm$^3$ packed cells/ml., this "starter" culture was used in inoculating one liter volumes of sterile culture solution in the large culture flasks (Fig. 2). The growth rate of both Nostoc and Chlorella amounted to 200-300% increase per day at ca. 25°C. This rapid growth made it desirable to harvest the cells either daily or on alternate days. Continuous cultures were maintained as at Berkeley by withdrawing 9/10 of the cell suspension and adding back an equal volume of fresh culture solution.
FIG. 2 CULTURE FLASKS
Packed cell volume and cell counts.

Packed cell volume measurements were made on representative samples, before the algal suspensions were used in photosynthesis studies. From 10-20 ml. aliquots, depending on the cell density, were centrifuged in duplicate 50 ml. plastic tubes for five minutes at 2600 g. Most of the clear supernate was discarded. The sedimented cells now were transferred quantitatively with 2-3 ml. culture solution to 3 ml. Exax tapered hematocrit tubes. During 30 minutes' centrifuging at 2600 g., the cells of both Nostoc and Chlorella cultures attained constant "packed cell" volumes. This double centrifuging procedure considerably increased the accuracy with which the final sedimentation volume could be measured. It is necessary to read the packed cell volume of Nostoc pellets shortly after centrifuging because of their "sponginess"; after ten minutes at rest the packed cell volume of Nostoc increases, in contrast to Chlorella sediment which retains a constant volume for a longer period. For this reason, packed cell volumes were always read immediately after the final centrifuging.

The relationship of packed cell volume to numbers of cells was checked periodically with the aid of a haemocytometer. In terms of packed cell volume, the photosynthetic capacity of Nostoc is only 1/2 that of Chlorella. Since however, 1 mm$^3$ packed cells represents $16 \times 10^5$ Nostoc cells and $51 \times 10^5$ Chlorella cells, the photosynthetic capacity of Nostoc exceeds that of Chlorella in terms of cell numbers. The algal suspensions were also examined regularly under the microscope as well as by the lactose broth test
for evidence of contaminating organisms. The cultures used in the following studies occasionally gave a positive broth test for bacteria, but bacterial contamination was never evident under the microscope. Work was included on algal cultures which were kept entirely free of bacteria, without revealing noticeable changes in photosynthetic capacity, respiration rate, or other characteristics. Growth records also were maintained by Dr. Eyster and Mrs. Birtle on the algal populations which were successively harvested almost daily over a period of eighteen months.

**Gas exchange measurements**

Manometric measurements of photosynthesis, respiration, and of the Hill reaction were made in the thermostats shown in Figures 3 and 4. The larger "Emerson type" thermostat and its accessories (Fig. 3) are essentially the same as were installed in Dr. Ciendenning's previous laboratory at the National Research Laboratories, Ottawa, Ontario. The large capacity of the bath allows the temperature to be controlled to ± 0.01°C. even with intermittent use of quite intense light sources. The temperature was regulated by an intermittent immersion-type heater which was controlled by an Aminco mercury thermoregulator and mercury-type relay. A large number of thermoregulators were set for different temperatures, so it was only necessary to change the thermoregulator in order to change the bath temperature. Continuous cooling was provided by circulating ethylene glycol solution of controlled temperature through immersed copper coils, which lined the main
FIG. 3 "EMERSON TYPE" THERMOSTATIC BATH
part of the bath. The glycol solution was refrigerated and circulated by a Mayer refrigeration unit. This unit would maintain temperatures only down to 15°C. at best, so for work at low temperatures it was necessary to adapt a standard refrigerated Warburg apparatus for photosynthesis research.

Light was usually provided in the "Emerson bath" by a set of five closely spaced 150 Watt reflector floodlamps, housed under the Pyrex window which forms the bottom of the overhanging thermostat shelf. A layer of Edmund heat-absorbing glass filters was placed immediately above the lamps to screen out most of the infra-red radiation before light entered the bath. The lamps and heat filters were cooled by forced air circulation, which was controlled by the light switch. Light intensity was varied by inserting neutral wire-screen filters of known transmission above the heat filters. Light of different quality was obtained by placing large plastic or glass selective filters over the Pyrex window of the bath. Light intensity was measured with a thermopile as well as with a General Electric f.c. meter, and spectral distribution was observed with a Zeiss hand spectroscope. For respiration measurements as well as dark equilibration, a heavy black cloth was thrown over the entire unit, and light in the laboratory was also reduced to a minimum.

The temperature of gases used in the Warburg vessels was adjusted by passing them through an immersed coil of copper tubing
and through an immersed gassing manifold before entering the reaction vessels. The smoothest horizontal shaking of the Warburg vessels was obtained with a Craftsman "Vari-Slo" speed control mechanism, which is an adjustable pulley-type control powered by a 1/4 h. p. motor.

The second, smaller bath (Fig. 4) is a standard Aminco refrigerated Warburg apparatus, modified for photosynthesis and Hill reaction studies at low temperatures. Oblique overhead lighting was provided by 300 W and 150 W reflector floodlamps. The light was filtered through two layers of glass heat filters, and both the lamps and the heat filters were cooled by forced air circulation. Much of the low temperature work consisted of comparative studies of photosynthesis and the Hill reaction. For this reason amber glass filters (Corning No. 3482) were inserted below the heat filters to remove wavelengths shorter than 5300 Å which induce an inhibitory side reaction in quinone reaction systems. The filtered light was concentrated with 9" Pyrex condensing lenses on the Warburg vessels and on a waterproofed mirror placed just beneath them. This lighting arrangement provided intense illumination with orange-red light (5300-6800 Å) which saturated the quinone reaction as well as photosynthesis in dilute algal suspensions.

Most of the required gas mixtures could be obtained from commercial sources, and their composition was checked periodically by Orsat gas analysis. Since the purpose of a
FIG. 4 STANDARD WARBURG THERMOSTATIC BATH
nitrogen atmosphere is to provide an inert oxygen-free gas phase, all traces of oxygen had to be removed. This was accomplished by passing the commercial nitrogen first through acetone-washed copper mesh at 500-600°C in a Cenco muffle furnace, and then through a solution of alkaline pyrogallol. A custom-built gas-mixing apparatus was also available for the preparation of special gas mixtures. This apparatus consisted of a heavy, calibrated one-liter gas burette which was connected to a levelling bulb and partially filled with mercury. The position of the levelling bulb was controlled by a hand-wheel. By manipulating the three-way stopcock and hand-wheel, a succession of gases could be drawn into the gas burette and finally expelled into the reaction vessels. To prevent trace contamination of the effluent gas with mercury, it was filtered through a gold leaf tube or through a series of gas washing bottles containing dilute nitric acid and water. Mercury was used in preference to concentrated salt or glycerol solutions in this apparatus because of the insolubility of all gases in mercury.

Manometric methods in general are adequately described in monographs by Dixon (24) and by Umbreit, Burris, and Stauffer (79). They offer the advantage that both the rate and the total exchange of a gas may be observed continuously and quite conveniently, and by using a combination of vessels possessing different gas/liquid ratios, the simultaneous exchange of two gases may also be measured. For these reasons, manometric methods have probably been used in measurements of metabolic and enzyme
activity to a greater extent than all other methods combined. Their main disadvantage is that only pressure changes are observed, so care must be continually exercised to exclude artifacts.

Both single-vessel or direct, and two-vessel or indirect methods were used in the present study, and special conditions had to be provided in each case for applications to blue-green algae. The direct method is simpler and more convenient, but it necessitates the use of alkaline media in which algae will not grow or exhibit their full rate of photosynthesis. The two-vessel method requires more time and greater skill, but under its conditions the cells are in a physiologically favorable environment in which they exhibit their full rate of photosynthesis, and both the absorption of carbon dioxide and evolution of oxygen are measured simultaneously. The general practice followed was to use the direct method in all work of an exploratory nature, and to use the indirect method as the reference procedure.

The direct or single-vessel manometric method

When the single-vessel method is used to measure photosynthesis, the liquid phase must ordinarily be so alkaline that little carbon dioxide remains in the gas phase. Since there is almost no accompanying absorption of gaseous carbon dioxide, oxygen production can be calculated from \( h \times K_{O_2} \). The necessarily low partial pressure of carbon dioxide in the gas phase reduces the concentration of dissolved carbon dioxide to a limiting value, so the cells usually are subjected to sub-optimal \( CO_2 \) concentrations as well as to high \( pH \) values (8.5-9.5) in applications of this method.
Several dozen experiments were conducted before a medium was chosen for direct photosynthesis measurements on *Nostoc* (0.025 M No. 9 bicarbonate buffer 85 Na: 15K, on unwashed cells or the same buffer made up in solution of *Nostoc* macro culture salts when the cells were washed). This medium is about as satisfactory for use with *Nostoc* and other blue-green algae as is 0.1 M No. 9, 85 Na: 15K, for use with *Chlorella*. However, in both cases the rate of photosynthesis in saturating light is at least 30% lower than under conditions of the two-vessel method.

Single-vessel measurements of photosynthesis have occasionally been made in the past in culture media under a saturating concentration of carbon dioxide (Kok's method 4 (51). Here the observed pressure changes result from simultaneously occurring carbon dioxide absorption and oxygen evolution, and small changes in their ratio cause large changes in the flask constant. Since the ratio of the exchange of CO₂ and O₂ cannot be measured with a single vessel, this method is of uncertain accuracy, and for that reason was not used in the present study.

The indirect or two-vessel manometric method

Warburg's indirect or two-vessel manometric method offers the advantage of simultaneous measurements of carbon dioxide and oxygen exchange under physiologically "ideal" conditions, which are not provided by the previously described direct method. Applications of the indirect method in studies of photosynthesis, particularly in quantum efficiency measurements, however, have been criticized (61). Professor J. F. Stauffer, University of Wisconsin, who wrote
part of "Manometric Techniques and Tissue Metabolism" (79) has taken these criticisms sufficiently seriously to decide against including it in future editions of the above volume (personal communication). Therefore, it must be realized that Warburg's indirect method is subject to errors in addition to those which apply to simple manometry, and that applications of the indirect method require much greater attention to details than applications of the direct method.

One serious objection to applications of the two-vessel method, particularly when Chlorella is used at low light intensities, is the "CO₂ gush" and "CO₂ pickup" phenomena which occur when the light is turned on and off, respectively (61). These are sources of large errors, particularly when the two vessels equilibrate at different rates (61). In all of the following applications, errors from these sources were eliminated by basing all measurements on the steady state in continuous light.

A further objection to this method is that equilibration may occur at different rates in the two vessels as mentioned above. This source of error was nullified in the present study by continuing the measurements over a period of hours, during which the pressure changes in each vessel were approximately constant. Extending the time of measurement also increased the pressure increments above those observed in a shorter time with No. 9 bicarbonate buffer, with an attending increase in precision.

The principle on which the indirect method is based is the difference in solubility of carbon dioxide and oxygen in water.
When identical exchange of the two gases occurs in manometer vessels having different gas/liquid ratios, different pressure changes are observed. From the observed differences in pressure change, the carbon dioxide and oxygen exchange is calculated with the following equations (79):

\[
\text{mm}^3 \text{O}_2 = \frac{H \cdot K_{\text{CO}_2} - h \cdot k_{\text{CO}_2}}{K_{\text{CO}_2} - k_{\text{CO}_2}} \quad \text{mm}^3 \text{CO}_2 = \frac{H \cdot K_{\text{O}_2} - h \cdot k_{\text{O}_2}}{K_{\text{O}_2} - k_{\text{O}_2}}
\]

H or h = manometric readings in mm.
K or k = flask constants.

It is customary to use lower case symbols to designate the pressure changes and flask constants for the vessel showing the smaller response.

The first requirement in applications of the two-vessel method in photosynthesis studies is that photosynthetic activity must be the same in each vessel. A simple method which was used to determine whether the algae in each vessel had identical photosynthetic capacities was to recover the cells, resuspend them in alkaline bicarbonate buffer, and then make direct photosynthesis measurements in the same vessels and under the same conditions.

The indirect method ordinarily is based on pressure changes observed in two vessels, but so long as identical rates of photosynthesis are maintained in each vessel, combinations of three or more vessels may be employed (16). As the number of vessels is
increased the number of equations which must be used in calculating CO₂ and O₂ exchange increases, of course, correspondingly.

All previous applications of the indirect method to studies of photosynthesis were made on green algae in acid medium (usually pH 4.5 or lower). Under these circumstances the situation is simplified by the existence of carbon dioxide in only the dissolved and gaseous states. No attempt had been made previously to apply this method to members of the Cyanophyta which require an alkaline medium, in which part of the carbon dioxide is bound as bicarbonate. In applying the indirect method to blue-green algae at and above pH 7.0 care was taken to avoid errors due to changing amounts of chemically bound carbon dioxide.

When photosynthesis is measured at low light intensities as in quantum efficiency investigations, the accompanying rate of respiration used as a correction is usually large in comparison to the photosynthetic rate. The accuracy of the corrected rate of photosynthesis under these conditions thus becomes dependent on the accuracy of the respiration correction, regardless of the method used. This situation is controversial, and may be quite complicated when the supply of respiratory substrates is controlled by photosynthetic activity and when the two sets of reactions are sharing glycolytic enzymes. The accuracy of the photosynthesis rate measurements tends to decrease as the light intensity is progressively reduced, since there is less photosynthesis to measure, and since the respiration correction becomes proportionately larger. These considerations do not apply, however, to the present
studies, which were made on dilute cell suspensions at saturating or approximately saturating light intensities. In this work the respiration correction was sufficiently small as to be essentially insignificant. In all of the experiments reported below on photosynthesis, the conclusion would remain the same with or without a respiration correction. Because of the very low respiratory gas exchange shown by 5-10 mm$^3$ cells/vessel, the quantity of cells was increased to 50-100 mm$^3$ cells/vessel when accurate respiration data were required.

Readings from replicate samples varied only about 1-2 mm. from the mean regardless of magnitude of gas exchange. Overall measurement accuracy was from 3-5% which is well in line with that of other workers (51).

Mention should be made of the fact that the organization of Nostoc cells into short filaments plus their thin gelatinous sheaths introduces difficulties in quantitative work which are not encountered with Chlorella. Nostoc suspensions settle rapidly, and to keep them uniform they were stirred thoroughly during sampling, usually with a magnetic stirrer. The cell suspensions were made sufficiently dilute to require the withdrawal of 5-10 ml. aliquots with a rapid delivery Mohr pipette. This sampling method was easily reproducible to ± 5%. The gelatinous sheaths of blue-green algae also cause the cells to show a greater "creeping" tendency than Chlorella, this being more marked in conical than in
rectangular vessels. For the foregoing reasons, _Nostoc_ and other blue-green algae should be regarded as useful in comparative photosynthetic studies, but their gelatinous filaments make them less suitable for quantitative work than _Chlorella._

Quinone reaction measurements were made on both _Nostoc_ and _Chlorella_ in accordance with the method of Clendenning and Ehrmantraut (21): i.e., cells suspended in M/20 phosphate buffer, pH 7.0, 1.08 mgm. resublimed p-benzoquinone in water placed in a sidearm; 0.2 ml. 10-20% KOH in center well with fluted filter paper; nitrogen atmosphere. Research leading to development of the methods used in the measurement of _Nostoc_ photosynthesis is presented in the Results section.
Results and Discussion

1. Effect of centrifugal force

In preparing algal cells for quantitative measurements of photosynthesis, they are normally centrifuged out of the culture medium, washed one or more times with water or "reaction" medium, and then suspended in fresh medium and transferred to the reaction vessel. This preparatory treatment could conceivably influence photosynthetic activity in more than one way. Upon applying sufficient centrifugal force, the intracellular location of the chloroplasts and other particulates would be altered. If photosynthesis involves the interaction of the chloroplast and surrounding cytoplasm, mechanical displacement might have a considerable effect. It has long been known that photosynthesis is quite sensitive to mechanical injury; e.g.; photosynthesis is essentially abolished when the cell walls are ruptured. Warburg, Burk, and Schade (83) used the mildest centrifugation necessary to effect sedimentation of the cells. At the present time (Clendenning, personal communication) the centrifugation treatment being used in Warburg's laboratory is so mild that incomplete sedimentation occurs and some cells are lost in each decantation.

Since centrifuging treatments have varied from one laboratory to another, it appeared desirable to investigate the effect of centrifugal force by itself, on which information is entirely lacking in the literature. Such information was also needed in studies of cell-washing treatments, which usually involve use of the centrifuge.
To magnify the effects of centrifugal force, much more drastic treatments were included than are normally used. Centrifuging was conducted in a refrigerated Servall at 0°C., using 15 ml. round bottom plastic tubes, and was continued for twenty minutes at the indicated forces unless otherwise noted. Washing effects were excluded in these experiments by carefully resuspending the cell sediment either in the supernatant culture medium or in bicarbonate buffer without preparatory washing. Particular care was taken to avoid injuring the cells mechanically at this stage. In the main experiment, the control cells were transferred directly from freshly harvested suspensions to Warburg vessels without centrifuging, otherwise the control treatment was the minimum centrifugal force which would effect sedimentation (800 g). The photosynthesis measurements were made in saturating white light at 25°C., and 10 mm³ cells were employed in both the single-vessel and two-vessel measurements. The reported rates of photosynthesis are based on readings which were continued for one hour under steady-state conditions (Figure 5).

The results of this work adequately show that the application of centrifugal force by itself has little, if any, effect on the subsequent photosynthetic capacity of Nostoc and Chlorella, measured either in complete culture medium under 5%CO₂ or in bicarbonate buffers. The photosynthetic activity of Nostoc which was centrifuged for two hours at over 20,000 g. remained the same as when the cells were centrifuged briefly at 800 g. The rate of photosynthesis by Chlorella was much higher under the conditions of the two-vessel vs. the
Fig. 5  Photosynthetic Capacity of Nostoc and Chlorella
as a Function of Centrifugal Force.
direct method. This difference was observed consistently on both Chlorella and Nostoc throughout the present studies, and will be discussed further below.

The experiments summarized in Figure 5 justify the conclusion that the large effects of washing described below were not caused by the centrifuging treatments used in their study. Warburg, Burk, and Schade (83) state that angle centrifuging at high speed is to be avoided, nevertheless data presented here refer to angle centrifuging at very much higher speeds than are ordinarily used on algae. Heavily centrifuged cells form hard sedimentation cakes which are difficult to resuspend, and if proper care is not taken, algal cells can be broken mechanically while being resuspended.
2. Effects of washing

The medium which had been used in some previous work on blue-green algae was M/10 No. 9 bicarbonate buffer, (used by Emerson and Lewis on *Chroococcus* (28) and by Frenkel, Gaffron, and Battley on *Nostoc* and other Cyanophyta (34). The usual practice is to wash algal cells once or twice with the solution in which photosynthesis measurements are to be made. Upon washing *Nostoc* cells with M/10 "all sodium" No. 9 buffer and resuspending them in this solution, however, they exhibited little or no photosynthetic activity. This phenomenon was found to be caused by two factors which were operating simultaneously, namely, *Nostoc* cells are sensitive to washing (leaching) treatments, and they also require a more dilute bicarbonate buffer than is used with *Chlorella*. The effects of washing treatments on photosynthesis and the Hill reaction in *Nostoc* and *Chlorella* have therefore been compared, and the chemical element which is mainly responsible for this effect has been identified.

Table I reports comparative data on the influence of washing *Nostoc* and *Chlorella* upon their capacities for photosynthesis and the Hill reaction.

These data show that photosynthesis in *Nostoc* is far more sensitive to preparatory washing than in *Chlorella*. Three 10 ml. washes abolished photosynthesis in 5 mm *Nostoc* cells while reducing photosynthesis in *Chlorella* only by 20%. However, photosynthesis was appreciably reduced by these washing treatments even in *Chlorella*, so this factor is probably of general importance.
Table I. Photosynthetic capacities of Chlorella and Nostoc and Hill reaction capacities of Nostoc as a function of washing with water.

<table>
<thead>
<tr>
<th>Number of 10 ml. water washes</th>
<th>Chlorella</th>
<th>Nostoc</th>
<th>Hill reaction Rate as percent of zero washing</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0</td>
<td>100.0</td>
<td>100.0</td>
</tr>
<tr>
<td>1</td>
<td>82.3</td>
<td>91.8</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td>37.7</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>81.1</td>
<td>0</td>
<td>73.9</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>68.2</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

In this connection, Warburg (84) has recently reported large differences in the photosynthetic activity of Chlorella cells before and after washing with M/5 No. 11 buffer, presumably prepared with sodium bicarbonate and sodium carbonate. In the present work capacity for the Hill reaction remained at 74% of the control in Nostoc cells whose net photosynthetic activity had been abolished by washing. Thus those parts of the photosynthetic apparatus in Nostoc which do not participate in the Hill reaction apparently are the most sensitive to cell-washing treatments. As would be expected, the response to washing varied with the relative amounts of cells and wash liquid used, being most pronounced when a minimum of cells is washed with a large volume of water.
It has already been shown that the harmful effects of washing Nostoc cells were not caused by the centrifuging treatments alone. Reduction of photosynthetic activity in Nostoc occurred to a similar extent upon washing either with distilled water or with up to 0.1 M sodium bicarbonate solution, but no reduction in photosynthetic activity occurred upon washing in the same way with complete culture medium. These and related observations showed that the washing effect was not due to the ionic strength of the fluid but to the absence of one or more components of the complete culture medium.

It was first necessary to determine whether the macro culture salt solution or micro culture salt solution contained the causal factor. After mild washing treatments, which reduced but did not abolish photosynthesis, the rate in 0.01 M No. 9 buffer was unaffected by the presence of all of the micro culture salts (including iron) used in culturing the cells, and at the same concentration as in the culture medium. Upon including the macro culture salts in the wash liquid and in the reaction medium, the photosynthetic activity was unaffected by washing treatments, as was also the case in the continuous presence of complete culture medium. So the next step was to identify which salts in the macroelement solutions for Nostoc were responsible for the washing effect. (NaHCO₃, K₂HPO₄, MgSO₄, Ca(NO₃)₂). Sodium bicarbonate could already be eliminated since it had been regularly included in the wash liquid and reaction medium. Thus it was only necessary to compare the effects of K₂HPO₄, MgSO₄, and Ca(NO₃)₂. To exclude possibility of serious osmotic effects, all test media were adjusted to approximately the same ionic strength (1/4 of complete culture medium).
Table II. Determination of effective cation in photosynthetic measurements with Nostoc.

<table>
<thead>
<tr>
<th>Reaction media</th>
<th>ml. O₂/hr/ml. p. c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M No. 9 buffer</td>
<td>4.38</td>
</tr>
<tr>
<td>&quot; + Ca(NO₃)₂·4H₂O</td>
<td>4.50</td>
</tr>
<tr>
<td>&quot; + MgSO₄·7H₂O</td>
<td>4.13</td>
</tr>
<tr>
<td>&quot; + K₂HPO₄</td>
<td>5.71</td>
</tr>
<tr>
<td>1/4 conc. complete macro culture salts</td>
<td>5.36</td>
</tr>
</tbody>
</table>

Table II, which is based on three closely agreeing experiments, shows that the rate of photosynthesis in Nostoc after mild washing treatments was increased by both K₂HPO₄ and complete macro culture salt solution of the same ionic strength, and that the other salts in the latter were essentially without effect. It was necessary to distinguish the effects of the potassium and phosphate ions provided by the dipotassium hydrogen phosphate. Comparison of the action of dipotassium hydrogen phosphate, disodium hydrogen phosphate, potassium chloride, and complete culture solution are reported in Table III.

Potassium chloride and dipotassium hydrogen phosphate were equally effective, both sustaining higher rates of photosynthesis than complete culture solution of the same total ionic strength. Inclusion of disodium hydrogen phosphate in the wash liquid and reaction medium however, reduced the photosynthetic activity below
Table III. Determination of effective anion in photosynthetic measurements with Nostoc.

<table>
<thead>
<tr>
<th>Reaction media</th>
<th>ml. O₂/hr/ml. p.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.01 M No. 9 buffer</td>
<td>4.61</td>
</tr>
<tr>
<td>&quot; + Na₂HPO₄</td>
<td>3.11</td>
</tr>
<tr>
<td>&quot; + KCl</td>
<td>6.10</td>
</tr>
<tr>
<td>&quot; + K₂HPO₄</td>
<td>6.33</td>
</tr>
<tr>
<td>1/4 conc. complete macro culture salts</td>
<td>5.55</td>
</tr>
</tbody>
</table>

the rate observed in cells washed and suspended in potassium-free 0.01 M bicarbonate-carbonate buffer. Thus the "washing effect" is a "potassium effect" which is caused by leaching of potassium from Nostoc cells. This conclusion is supported by many experiments in addition to the foregoing which all indicate the essentiality of potassium as a constituent of washing and reaction media used in measuring photosynthesis in washed Nostoc cells.

Chloroplasts that have been thoroughly washed with distilled water retain their photochemical activity provided M/100 chloride or related anions are added. They do not require potassium, as described here for Nostoc (21). It has been shown (21, 42) that the Hill reaction in washed chloroplasts is not stimulated by adding potassium either with the phosphate buffer or with the required anion. The present washing effect on photosynthesis in Nostoc is a response to the cation, potassium.

The work of Osterhout (63) indicates that washing of Nitella cells abolishes their differential permeability with regard to
sodium and potassium and also affects the bioelectric properties of the cells. No attempt has been made to assess all of the physiological processes which can be affected by cell-washing, but Osterhout's observations (63) at least indicate that photosynthesis is not the only process which is sensitive to leaching treatments.
3. **Research on methods for direct measurements of photosynthesis.**

   A. **Bicarbonate buffer for Nostoc.**

   The foregoing studies demonstrated that *Nostoc* is more sensitive to washing treatments than *Chlorella*, and that the concentration of potassium in the culture solution should be maintained in washing fluids. Experiments were next conducted in a search for the most suitable bicarbonate buffer for use in direct or single-vessel measurements on *Nostoc* and other blue-green algae. The factors investigated were buffer molarity, bicarbonate-carbonate ratio, Na : K ratio, and effects of culture medium concentration on the bicarbonate concentration requirement.

   The most suitable buffer molarity was selected by comparing the rates of oxygen production by 10 mm$^3$ unwashed *Nostoc* cells in bicarbonate buffers at successively lower concentrations. Upon diluting pure bicarbonate or No. 9 buffer solutions from 0.1 M - 0.025 M, the rate of photosynthesis was usually doubled, and the rate during the first 30-60 minutes remained approximately constant over the range 0.01-0.025 M. The supply of bicarbonate, however, soon became limiting at the lowest concentration. Regardless of the bicarbonate concentration used (over the range 0.01-0.1 M) declining rates of photosynthesis always were observed in *Nostoc* after ca. ninety minutes in saturating light. Thus *Nostoc* is more sensitive to washing, requires a more dilute bicarbonate buffer, and also exhibits steady rates of photosynthesis in bicarbonate solution for a shorter time than in the case of *Chlorella*. *Pratt* (67) and many others have observed that photosynthesis in *Chlorella* may continue at a high rate
for at least a day in M/10 bicarbonate buffers. In these respects, Nostoc shows greater resemblance to blade-type marine algae (Clendenning, K. A. and Haxo, F. T., unpublished).

The quantity of potassium in complete culture solution is much smaller than in "all-potassium" 0.01 M bicarbonate buffer. Use of "all-potassium" bicarbonate buffer was consistently more detrimental over the range 0.025-0.1 M than "all-sodium" buffers on unwashed Nostoc cells.

Table IV. Photosynthetic capacity of Nostoc as a function of sodium and potassium content of buffer.

<table>
<thead>
<tr>
<th>Composition 1/10 M buffer</th>
<th>ml. O₂/hr./ml. p. c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>all sodium</td>
<td>1.61</td>
</tr>
<tr>
<td>85 Na : 15 K</td>
<td>3.01</td>
</tr>
<tr>
<td>15 Na : 85 K</td>
<td>2.47</td>
</tr>
<tr>
<td>all potassium</td>
<td>0.30</td>
</tr>
</tbody>
</table>

As Table IV shows, the most favorable Na : K ratio, of those investigated, in Nostoc bicarbonate buffer as 85 : 15, which is obtained by using sodium bicarbonate and potassium carbonate in preparing the No. 9 buffer. The bicarbonate/carbonate ratio provided by No. 9 buffer (85/15) was found to support as high, if not higher, rates of photosynthesis than with any other possible ratio.

In investigating this point, the bicarbonate/carbonate ratios were varied from 100/0 to 0/100. The most generally useful buffer which was found in this way for direct photosynthesis measurements on
Nostoc was 0.025 M No. 9 buffer in which the Na:K ratio was 85:15. This artificial medium allows photosynthesis to be measured in Nostoc about as satisfactorily and conveniently as when Chlorella photosynthesis is measured in M/10 No. 9 buffer by simple manometry.

Blue-green algae normally are cultured in more dilute solutions than Chlorella. Thus the growth medium for Nostoc has a total salt concentration of less than 0.01 M, whereas Warburg's medium for Chlorella has a total salt concentration of 0.1 M, which is also the molarity of the No. 9 buffer which has long been used on Chlorella. If the culture solution were made more concentrated, would the "adapted" Nostoc cells then tolerate more concentrated bicarbonate buffers? Figure 6 shows that when the total salt concentration in Nostoc growth medium is raised stepwise to 0.1 M (at the expense of reduced growth), photosynthetic activity in 0.1 M as well as 0.025 M No. 9 buffer is greatly increased on a packed cell volume basis. Thus the permissible bicarbonate concentrations vary with the concentration of the culture solution. The bicarbonate buffer which was adopted for Nostoc is the most suitable, however, for cells cultured in the dilute mineral solution in which they grow rapidly.

Acknowledgement should be made here that the investigation of the optimum concentration of "reaction medium" in relation to the concentration of "culture medium" was undertaken at the suggestion of Dr. H. C. Eyster, who also provided the cells used in the photosynthesis measurements.
Figure 6: The effect of culture medium on the rate of plating efficiency. The plating efficiency of normal culture medium = 0.003

Plating efficiency of culture medium

ml. (pH 7.0) N. S. packed cells

with 0.1 ml. N. S. buffer

with 0.025 ml. N. S. buffer

with 0.0 mg. N. S. buffer
B. **Bicarbonate buffer for Chlorella.**

Although *Chlorella* has been used in photosynthesis research for over thirty years, the "artificial" bicarbonate buffers that have been employed in different investigations have varied greatly in composition. Warburg's M/10 No. 9 buffer containing 85 parts bicarbonate and 15 parts carbonate has been used to the greatest extent, but it has been prepared entirely with sodium salts or entirely with potassium salts, as well as with intermediate Na : K ratios. The best Na : K ratio for use with the buffer has remained unsettled. In addition, different bicarbonate/carbonate ratios and buffer molarities have been employed. Thus Myers (57) used "all-potassium" M/10 No. 11 buffer on *Chlorella* in which the bicarbonate/carbonate ratio was 95/5; two years ago Warburg, Geleick, and Breise (84) recommended that all other bicarbonate buffers be dropped in direct measurements on *Chlorella* in favor of M/5 No. 11 buffer containing traces of MgSO₄ and Ca(NO₃)₂ and provided with an atmosphere of 2% CO₂ in air. Despite all of the previous work on *Chlorella* it appeared necessary to test several bicarbonate buffers before selecting one for direct photosynthesis measurements on this reference organism.

Table V shows that the Na : K ratio in the No. 9 buffer has a large effect on photosynthesis in *Chlorella* cells grown as described in the Methods section.
Table V. Photosynthetic activity of *Chlorella* in various bicarbonate buffers.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Buffer</th>
<th>Na : K ratio</th>
<th>Photosynthesis ml. O$_2$/hr/mp.c.</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. 1</td>
<td>0.025 M No. 9</td>
<td>85 : 15</td>
<td>14.3</td>
</tr>
<tr>
<td></td>
<td>0.10 M No. 9</td>
<td>85 : 15</td>
<td>16.9</td>
</tr>
<tr>
<td>No. 2</td>
<td>0.10 M No. 9</td>
<td>100 : 0</td>
<td>10.8</td>
</tr>
<tr>
<td></td>
<td>0.10 M No. 9</td>
<td>85 : 15</td>
<td>18.5</td>
</tr>
<tr>
<td></td>
<td>0.10 M No. 9</td>
<td>15 : 85</td>
<td>8.4</td>
</tr>
<tr>
<td></td>
<td>0.10 M No. 9</td>
<td>0 : 100</td>
<td>12.3</td>
</tr>
<tr>
<td>No. 3</td>
<td>0.10 M No. 9</td>
<td>85 : 15</td>
<td>12.7</td>
</tr>
<tr>
<td></td>
<td>0.20 M No. 11, air + salts</td>
<td>100 : 0</td>
<td>10.4</td>
</tr>
<tr>
<td></td>
<td>0.20 M No. 11, 2% CO$_2$ + salts</td>
<td>100 : 0</td>
<td>9.0</td>
</tr>
<tr>
<td></td>
<td>0.20 M No. 11, 2% CO$_2$</td>
<td>100 : 0</td>
<td>9.4</td>
</tr>
</tbody>
</table>

+ Salts are 50 mg. MgSO$_4$ and 10 mg. Ca(NO$_3$)$_2$ $\cdot$ 4H$_2$O/liter.
"All-sodium" as well as "all-potassium" No. 9 buffers were consistently less satisfactory than No. 9 buffer containing 85 parts sodium: 15 parts potassium. Reversing this ratio had a strongly detrimental effect. The rate of photosynthesis is adversely affected if this buffer is made much more concentrated or much more dilute than the usual strength (0.1 M).

Warburg, Geleick, and Breise (84) have reported that the quantum efficiency or photosynthetic rate at limiting light intensities for Chlorella is approximately twice as high in the new M/5 No. 11 buffer as in M/10 No. 9 buffer. They also reported a detrimental effect of washing Chlorella cells with the new bicarbonate buffer, which did not contain potassium (84). Various cations were tested for use in the M/5 No. 11 buffer (84), and it was stated that cesium and lithium were detrimental, whereas equal rates of photosynthesis were observed when "all-sodium", "all-potassium", or mixtures of sodium and potassium bicarbonates and carbonates were used in preparing this buffer.

The present applications of Warburg's M/5 No. 11 buffer were made on cells grown according to Warburg's directions, but they differ from Warburg's application (84) in that the dilute cell suspensions were subjected to saturating light. At the low light intensities employed in quantum efficiency measurements, the rate of photosynthesis is controlled by the photochemical efficiency of the cells, whereas at the high light intensities used here, the rate is controlled by a limiting dark reaction. Table V shows that the rate of photosynthesis actually was lower with M/5 No. 11 buffer than with the
best of the M/10 M No. 9 buffers. This result was obtained with and without the culture salts recommended by Warburg et al. (84), and also with and without a 2% CO₂ atmosphere. The experiments conducted on M/5 No. 11 buffer thus have not revealed any advantage over M/10 No. 9 buffer, at least for use on dilute suspensions of Chlorella at high light intensities. From the foregoing and related experiments, M/10 No. 9 buffer with a Na:K ratio of 85:15 was selected as the most suitable bicarbonate medium for direct measurements of photosynthesis in Chlorella.

The artificial bicarbonate buffers selected on the basis of the foregoing experiments for use with Chlorella as well as with Nostoc allow photosynthetic activity to be measured conveniently by simple manometry in these organisms. The alkalinity and low CO₂ content of these buffers, however, prevent photosynthesis from proceeding at its maximum rate in saturating light, in contrast to the conditions provided in the more cumbersome two-vessel method, which was used extensively as a reference procedure (see Comparison of Methods).
C. **External diethanolamine buffers.**

Diethanolamine (DEA) is an organic base which combines with carbon dioxide reversibly, and to a greater extent at low than at high temperatures. Use has been made of this property for many years in the industrial production of dry ice (49), and it has also been used to a limited extent in the laboratory as an external CO₂ buffer. Pardee (65) introduced its use as an external CO₂ buffer in manometry. Equilibrium CO₂ concentrations of from 0.1-5% were obtained at 38°C. by incorporating potassium bicarbonate (2M) and different amounts of hydrochloric acid (0-2M) in 3.8M DEA solution. Pardee reported that carbon dioxide concentrations up to 1.5% were satisfactorily buffered at 38°C. by 0.6 ml. DEA solution, which was placed in the center well of a standard vessel along with a large roll of fluted filter paper. Pardee also reported that the buffering action of DEA failed when the gaseous CO₂ concentration exceeded 3%.

Krebs (53) did not find any external CO₂ buffer which was superior to those used by Pardee (65). He showed that the required buffer volume increases with increasing CO₂ concentration, increasing temperature, and increasing flask constant. He also demonstrated that diethanolamine is subject to autoxidation, even after recrystallization. The gradual oxidation of the DEA was reduced but not abolished by 0.1% thiourea. To provide a required CO₂ concentration (e.g. 1%), Krebs preferred to gas 4M DEA solution overnight with air containing the desired CO₂ concentration, at the same temperature as used in the application.
The only reported use that has been made of DEA in photosynthesis research, with algae, is in the quantum efficiency measurements reported by Burk et al (16). In this application, DEA solutions and Chlorella suspensions were placed in separate compartments of specially constructed Warburg vessels. These authors reported that the respiration rates and photosynthesis rates at low light intensity which were observed with the single-vessel-DEA system agreed closely with measurements made by other methods.

External CO₂ buffering appeared to offer the possibility in photosynthesis research of combining the advantages and eliminating the disadvantages of the direct (single-vessel) and two-vessel manometric methods. Thus if diethanolamine were sufficiently efficient as a CO₂ buffer, direct or single-vessel measurements could be made at saturating CO₂ concentrations with the algae suspended in their culture medium, and with the DEA in the side arms or the center well of the same vessel. Because of these potential advantages, considerable research was included in the present investigation on external CO₂ buffering. The results of several dozen experiments, however, have so far revealed more disadvantages than advantages for DEA buffers, and have also placed doubts on their only previous application in photosynthesis research (16).

The topics investigated in this study were (a) DEA autoxidation and its control, (b) Equilibration time, (c) Buffering efficiency, and (d) Comparative rate measurements of photosynthesis, using DEA buffering systems in parallel with other methods. To avoid duplication, topic d has been included in the section on Comparison of Methods.
Autoxidation of diethanolamine.

Krebs (53) has already shown that oxygen absorption by diethanolamine buffers cannot be neglected in manometric applications, at least at high temperatures. Photosynthesizing cells require aerobic conditions, so this property of DEA requires attention in photosynthesis measurements.

Preliminary studies revealed that it is not the diethanolamine itself but the diethanolamine-CO\textsubscript{2} complex which is subject to oxidation at an appreciable rate. Oxidation of pure DEA solutions was scarcely detectable in the absence of CO\textsubscript{2}, even at 40°C. Upon equilibrating the same DEA solutions with 5% carbon dioxide in air, the oxidation rate then corresponded with the data of Krebs (53).

The oxidation rate of DEA-CO\textsubscript{2} complex was next compared in the presence of all the antioxidants which had previously been used with it either in the laboratory or industrially (2, 53). The effect of dithizone (diphenylthiocarbazone) varied markedly with the freshness of the dithizone solution. The freshly prepared 0.02% solution strongly inhibited DEA oxidation, but solutions that had been stored for a day or two actually stimulated the oxidation rate. For this reason, dithizone is judged of doubtful value as an antioxidant for DEA systems. Thiourea and potassium iodide (0.1%) both inhibited the oxidation of the DEA-CO\textsubscript{2} complex by 90-95%, thiourea being slightly superior in this respect. When either of these antioxidants were employed, the rate of oxygen absorption by 1 ml. 4M DEA per vessel under 5% CO\textsubscript{2} in air usually amounted to less than 5 mm\textsuperscript{3} oxygen per hour. The oxidation rate varied with the CO\textsubscript{2}
tension as well as the age of the DEA solution, however, which indicated the need for control vessels in manometric applications. These were therefore included in the investigations described below.

(b) Equilibration time.

When Pardee- or Krebs-type DEA buffers which had been stored overnight at bath temperature (25°C.) were placed in Warburg vessels under air (with or without fluted filter paper), carbon dioxide was regularly evolved from the buffer for more than one hour. This initial equilibration period could be greatly shortened, of course, by providing a CO₂ atmosphere in the vessel which was already in equilibrium with the DEA buffer. The slowness with which equilibrium was otherwise obtained, however, indicates that the equilibration is similarly sluggish in physiological applications.

Burk et al (16) used two "Pardee" mixtures which were shown here by gas analysis to provide 6% and 50% CO₂ in the gas phase, respectively. Thus both of their DEA buffers provided CO₂ concentrations above the level at which DEA exhibits efficient CO₂ buffering (53, 65). With the better of their two buffers, over ninety minutes was required for the attainment of gaseous equilibrium at 25°C. (i.e. when temperature-equilibrated buffer is placed in Warburg vessels under air).

(c) Buffering efficiency.

This property was thoroughly investigated by Krebs (53) who demonstrated the importance of many factors, which even included the size and shape of the manometer vessels. Because of the
suggested effect of flask geometry (53), some tests of buffering efficiency appeared necessary under the conditions which might be used in photosynthesis measurements.

The general method which was used by the author in testing buffering efficiency was to equilibrate the manometric-DEA system, then generate a known volume of carbon dioxide gas separately within the vessel, and observe the pressure changes until a new equilibrium was eventually established. If the DEA buffer was 100% efficient, the position of the manometer meniscus would eventually return to its original level. With decreasing buffering efficiency, the final pressure readings would be progressively higher than before the carbon dioxide was generated.

Figure 7 reports measurements of the buffering efficiency of Kreb's (53) and Pardee's (65) DEA buffers, each of which provided an equilibrium atmosphere of ca. 1% CO₂ in the gas phase. Kreb's buffer was prepared by gassing 4M DEA overnight with 1% CO₂ at 25°C, and Pardee's buffer for 1% CO₂ was prepared by incorporating KHCO₃ and HCl and the DEA solution. An atmosphere of 1% CO₂ was provided in these vessels to shorten the initial equilibration time and to maintain the buffer compositions. In addition to thermobarometer and DEA controls, manometer vessels were included which contained (a) no CO₂ absorbing agents, and (b) 20% KOH. Standard double-sidearm Warburg vessels were used in this experiment with 0.4 ml. DEA or KOH in the center well plus 0.8 ml. in one sidearm; fluted filter paper was included in each of these compartments. In the main
compartment was placed 1.0 ml. M/100 NaHCO₃, and 0.5 ml. M/10 H₂SO₄ was placed in the second sidearm. After the initial equilibration, 224 mm³ CO₂ was generated by acidifying the bicarbonate solution, this being close to the maximum which could be used in this manometric equipment. CO₂ absorption by the DEA and KOH solutions was then observed over a period of several hours (Figure 7).

The "blank" measurement shows how much carbon dioxide actually was generated, and also provides evidence on the magnitude of instrumental "lag" in this experiment. Over 99% of the carbon dioxide was recorded in the first five minute "blank" reading after acidifying the bicarbonate solution. The efficiency of KOH as a CO₂ absorber under the adopted conditions is also shown in this figure: over 98% of the carbon dioxide evolved from the bicarbonate (224 mm³) was absorbed by the KOH within the first five minutes, and over 99% was absorbed within the first ten minutes. The difference between the KOH and DEA solutions with respect to speed of CO₂ absorption was very striking. Both of the diethanolamine buffers required more than one hour to attain a new equilibrium with the gas phase. This new equilibrium was well above the original equilibrium pressure, which was carefully established before generating the extra CO₂. This experiment demonstrates that DEA buffers prepared according to the directions of either Pardee (65) or Krebs (53) have quite similar buffering capacities as well as
equilibration times. Those which provide ca. 1% CO₂ in the gas phase have a buffering efficiency of ca. 90% under the described conditions, but only after a long equilibration period. The present vessel arrangement is very similar to that used by Pardee (65) but his work was done at 38°C. vs. 25°C. This rather high temperature (38°C.) however, is rarely used in photosynthesis research. When the temperature is reduced below 25°C. even longer equilibration times are to be expected than are shown in Figure 7.

The experiment reported in Figure 7 is typical of many which were conducted in the same general way but with different types of vessels, buffer compositions and volumes, and CO₂ atmospheres. In agreement with Pardee (65) and Krebs (53), it was shown by the present method that the buffering efficiency of DEA systems decreases with increasing CO₂ in the atmosphere. In photosynthesis research, CO₂ concentrations above 1% are usually required. When the equilibrium CO₂ concentration is raised above 1%, however, the buffering efficiency of DEA becomes progressively lower than is shown in Figure 7. For this reason, the ability of the Pardee mixtures used by Burk et al (16) to maintain constant CO₂ concentrations in the gas phase is open to question.

The foregoing tests of CO₂ buffering refer to diethanolamine absorption of CO₂ from the gas phase, not to its ability to evolve CO₂, as is required in the maintenance of constant CO₂ tensions over photosynthesizing algae. Lacking a simpler test system which would absorb CO₂ at uniform, known rates from the gas phase, tests of DEA
buffering capacity in the direction of CO₂ liberation were made by measuring apparent rates of photosynthesis by *Chlorella* and *Nostoc* with DEA systems in parallel with measurements by the two-vessel and direct methods. These tests included rates of photosynthetic gas exchange which matched the rates of respiration in Pardee's experiments under very similar conditions except for the use of a lower temperature in the photosynthesis applications. The results of these tests are given in the following section.

After completing the present study, attention was drawn to a second investigation of quantum efficiency in *Chlorella* which was made with the aid of diethanolamine buffer (Kök, B., 1952, Acta Botanica Neerl. 1:445-467). Kok used circular 250 ml. Warburg vessels having 125 ml. *Chlorella* suspension in the main compartment and 3 ml. Kreb's diethanolamine for 5% CO₂ in a separate compartment. In agreement with the present observations, Kok always observed lower rates of photosynthesis with diethanolamine buffers, in saturating light, than by the two-vessel method.

Although the conditions used in the direct measurements on Chlorella and Nostoc in bicarbonate buffers were carefully chosen, several lines of evidence indicated that the full rates of photosynthesis in saturating light are not obtained by this method; (a) the rates observed in culture solution under 5% CO₂ were consistently higher with either Nostoc or Chlorella in this investigation, (b) the rates of net photosynthesis calculated from the growth rate (organic solids accumulation) usually were higher than the photosynthesis rate in bicarbonate buffer at the same temperature, and (c) sub-optimal rates of photosynthesis in No. 9 bicarbonate buffers had been observed in previous studies of Chlorella by Warburg and Burk (83), Kok (51), and Tamiya (74). For these reasons, the full rates of photosynthesis are taken to be those shown under the conditions of the two-vessel method. The buffering efficiency of DEA systems therefore can be assessed by comparing rates of oxygen production by algae in culture solution under CO₂ atmospheres maintained by Pardee's method and under the conditions of the two-vessel method. Photosynthesis measurements were made on both Nostoc and Chlorella with 5% CO₂ gas phase by the Pardee and two-vessel methods, as well as by the direct method involving use of No. 9 buffer. The Pardee buffer compositions which provided 5% and lower CO₂ concentrations at 25°C. were determined by Orsat gas analysis. The Chlorella cells were suspended in their acid culture medium in the two-vessel and Pardee measurements. The Nostoc
cells require a neutral or slightly alkaline medium and the two-vessel and Pardee measurements on Nostoc were made at pH 7.5. This pH was maintained under the 5% CO₂ atmosphere by adding the equilibrium bicarbonate concentration (2.44 x 10⁻² M) which was calculated from the Henderson-Hasselbach equation (79). As a check on the accuracy of the two-vessel measurements, duplicates were included with different volumes of liquid in the vessels (Table VI).

Table VI. Comparison of rates of photosynthesis in Nostoc and Chlorella by different methods.

<table>
<thead>
<tr>
<th></th>
<th>2-vessel method</th>
<th>Single-vessel method</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 ml. liquid phase</td>
<td>3 ml. liquid phase</td>
</tr>
<tr>
<td>CO₂</td>
<td>O₂</td>
<td>CO₂</td>
</tr>
<tr>
<td>Nostoc</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21.8</td>
<td>21.0</td>
<td>20.8</td>
</tr>
<tr>
<td>Chlorella</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40.7</td>
<td>42.0</td>
<td>39.4</td>
</tr>
</tbody>
</table>

The calculated rates of carbon dioxide assimilation and of oxygen evolution agreed closely in the "four-vessel" measurements on both Nostoc and Chlorella. The observed rate under the conditions of the Pardee method was almost as low as with No. 9 buffer. The apparent rate of photosynthesis observed with DEA
buffers, remained lower than that observed by the indirect Warburg method when the CO₂ concentration was reduced to 1%. Many experiments of the type reported in Table VI were conducted, but the results obtained were always as shown: the rates of photosynthesis observed under the conditions of the two-vessel method were always higher than with the Pardee method or with No. 9 bicarbonate buffer. The photosynthetic capacity of Nostoc was also consistently lower than for Chlorella in terms of packed cell volume.

Direct tests of CO₂ buffering by diethanolamine in the direction of CO₂ absorption had revealed that equilibration is slow and that buffering is incomplete after equilibration (Figure 7). Buffering in the reverse direction is required in photosynthesis applications; the apparent rates of photosynthesis at 25°C. by the Pardee method were always below those observed by the two-vessel method, even when the type of vessel, shaking and rate of gas exchange closely matched those in Pardee's experiments at 38°C.

The main points established in the foregoing study are as follows:

1. Antioxidants such as thiourea or potassium iodide should be included in DEA solutions, but inclusion of DEA blank vessels is also desirable.

2. At temperatures of 25°C. and lower, as are usually employed in photosynthesis measurements, equilibration periods of more than one hour are to be expected. The shorter equilibration periods observed by Pardee refer to measurements made at 38°C.
3. Photosynthesis measurements usually require CO$_2$ concentrations above 1%, but the buffering efficiency of DEA solutions decreases with increasing CO$_2$ concentration.

4. The full rate of photosynthesis is not observed with Pardee buffers under conditions essentially similar to those used by Pardee except for light, or under any other sets of conditions which were tested.

Acknowledgement should be made here of assistance provided by Dr. K. A. Clendenning in the research on DEA systems and in the comparisons of manometric methods. The results obtained adequately explain why no use was made of DEA buffers in the experiments which follow.
5. **Cell Suspension Density Requirement.**

If dense cell suspensions are employed in photosynthesis measurements, the light intensity to which individual cells are exposed varies to an extreme extent, due to mutual shading, and "light saturation" is impossible to achieve. Further, if very dilute cell suspensions are employed, mutual shading is eliminated but the gas exchange per vessel may be very small. Thus the ideal cell suspension density for use in photosynthesis measurements at high light intensities is sufficiently dilute to minimize mutual shading effects, yet contains enough cells for accurate photosynthesis measurements.

The cell suspension density requirement varies with the type of vessels used in respect to vessel geometry (the larger the illuminated area the larger the amount of cells which can be used). Figures 8 and 9 illustrate the effects of cell suspension density on the rate of oxygen production per vessel and per unit of cells when standard, conical Warburg vessels are employed. These data show that not more than 10 mm$^3$ cells should be employed per vessel under this experimental arrangement. With standard, rectangular Warburg vessels the permissible cell concentration is approximately doubled.

Further experiments were conducted with *Chlorella* to determine the effect of cell suspension density of the Hill reaction (Figure 10). It is apparent that increasing the number of cells brings about an increased reaction rate per vessel without decreasing the total oxygen yield appreciably. Since the oxygen yield in the quinone reaction was essentially independent of the cell suspension density,
Fig. 8  Photosynthetic Capacity of Nostoc at 30°C, as a Function of Cell Suspension Density.
Fig. 9
Photosynthetic Capacity of Chlorella at 34°C
as a Function of Cell Suspension Density.

ml. O₂/hour/ml. packed cells

mm³ O₂ per vessel

rate per unit of cells

rate per vessel
Fig. 10  Effect of Cell Suspension Density on the Time Course and Yield of Quinone Reaction in Chlorella
dark reduction of quinone to hydroquinone by algal constituents must have been insignificant. The latter possibility thus can be neglected in selecting cell suspension densities for the quinone reaction. The usual practice was to employ the same number of cells per vessel in measurements of both photosynthesis and the quinone reaction.

Although study of the effect of light intensity on photosynthesis and the Hill reaction rates has not been completed, it was found that the saturating light requirement for both Nostoc and Chlorella is similar when the pre-history of the cells is the same.
6. **Storage Characteristics.**

As recently noted by Van Niel, Allen, and Wright (59) one of the difficulties in photosynthesis research is the inconsistent photosynthetic capacity of algae. There are changes during the growth cycle; there is considerable variation between successive daily harvests unless exceptional precautions are taken to control all factors in the growth environment including the inoculum; there are further changes in photosynthetic activity following harvest, whether the cells are stored at room temperature or at 0°C.

The effects of these sources of variation have been circumvented in most of the present experiments by running up to six measurements simultaneously on freshly harvested cells in the large Emerson-type bath. Each experiment under these conditions is complete in itself. However, in lengthy runs involving a succession of manometric experiments, information on the storage characteristics of the cells is required, of which there is none in the literature.

Rather extensive studies of the effect of storage on the photosynthetic and quinone-Hill reaction capacities of both *Nostoc* and *Chlorella* were undertaken. Cells of both organisms were stored in the dark at 0°C. after initial activity measurements had been made on the freshly harvested cells. Under these storage conditions, the cells remain alive for weeks, and yet are as nearly metabolically static as possible. This storage temperature was maintained under dark conditions by storing the cells in stoppered one-liter Erlenmeyer flasks immersed in four-liter beakers containing crushed ice and distilled water. The beakers were masked with aluminum foil and
heavily covered on top with cloth towelling. The ice baths were kept in a cold room at 0-1°C. Aliquots were removed at this temperature, the stored material never leaving the cold room. Light entered the flasks only during sampling.

Packed cell volume determinations made before and after each storage experiment showed that no growth occurred, and that the size of the cells also remained the same during storage. Determinations of pH were made at each sampling. The pH remained constant in the case of Chlorella but rose from about 7.1 to 7.8 during storage of Nostoc, presumably due to a gradual loss of CO₂ from its bicarbonate-enriched culture medium.

The results show that under the selected storage conditions, there were considerable changes in photosynthetic activity measured at 25°C. It can be seen from Figs. 11 and 12, that there is an initial drop in activity during the first day or two. This is followed by a period of several days in which photosynthetic activity in Chlorella remains quite constant, after which it falls abruptly. The "storage plateau" shown by Chlorella was not exhibited by Nostoc, in which there was a continuous decline in photosynthetic activity. Thus there was a constant difference in the storage characteristics of Nostoc and Chlorella with respect to retention of photosynthetic activity. In contrast to photosynthesis, the capacity for the quinone reaction at 10°C. in both these organisms remained quite uniform. This difference signifies that it is not the parts of the photosynthetic
Fig. 11  Effect of Dark Storage at 0°C, on Photosynthetic and Hill Reaction Capacities of Nostoc.
Fig. 12 Effect of Dark Storage at 0°C. on Photosynthetic and Hill Reaction Capacities of Chlorella.
apparatus which participate in both photosynthesis and the Hill reaction, but rather those parts which play no part in the Hill reaction which are most affected by cold storage. The observed changes in photosynthetic activity accompanying the storage of both *Nostoc* and *Chlorella* require consideration in experiments which cannot be conducted immediately after the cells are harvested. When the cells must be stored at low temperature, the present data indicate that experimentation should be postponed until after the initial, rapid decline in photosynthetic activity, and that successive experiments then be conducted in a minimum of time.

A search of the literature revealed only one previous quantitative study of the temperature relations of photosynthesis and respiration in a blue-green alga, namely that by Bunning and Herdtle on Oscillatoria geminata (15). Since it is difficult to obtain uniform samples of this filamentous species, they used the same samples at all temperatures. The temperature history can alter photosynthetic activity at a standard temperature, but no information was provided on this point. The bicarbonate concentration employed by these authors was about the same as employed here in direct measurements on Nostoc. Since infra-red radiation was not removed from their light source, the temperature of the algae would likely be higher than the recorded bath temperatures. Their data indicate equally high rates of photosynthesis at 35° and 40°C, which also represent the optimum temperature range for respiration in this organism (15).

The present study of the temperature relations of photosynthesis, respiration, and the quinone-Hill reaction in green and blue-green algae is believed to be one of the most extensive which has ever been conducted. Although a great deal of work has been done on photosynthesis in Chlorella, complete data on even its temperature relations were lacking (the temperature at which maximum photosynthetic activity is shown by either the Emerson or Burk strains actually was not known). The Emerson and Burk strains of Chlorella pyrenoidosa as well as the Gerloff-Skoog strain of Nostoc muscorum were used in the present studies. Prior to each "temperature series", 
freshly harvested cells were stored overnight at 0°C., after which aliquots were withdrawn successively until the series was completed. To minimize storage changes, the measurements were conducted as a continuous series, one operator adjusting the thermostat to the next temperature while the other prepared a new set of algae samples for measurement at each temperature. Temperature control was maintained in these experiments to ± 0.02°C. by Dr. K. A. Clendenning, but otherwise the experimental work was conducted by the author. Approximately 24 hours of almost continuous laboratory work was required per series. The activity measurements refer to twenty minutes of steady-state activity. It was necessary to limit the photosynthesis measurements to direct measurements in bicarbonate buffer. Vessels used for respiration measurements were carefully masked with tinfoil to exclude light (Figures 13 and 14 with Tables VII and VIII).

There is a general similarity between the temperature curves for photosynthesis in both strains of Chlorella as well as Nostoc. Maximum rates of photosynthesis in the two strains of Chlorella were observed at 37.5°C. (blood temperature), and maximum rates of photosynthesis in Nostoc were observed at 35°C. The optimum temperature range for respiration in these organisms was much broader but otherwise corresponded with the photosynthesis data. Respiration, however, is considerably less sensitive to high temperatures than photosynthesis. The temperature relations of the quinone reaction in these algae are discussed below from a comparative viewpoint.
Table VII. Photosynthesis and respiration in *Nostoc* as a function of temperature, (25 mm$^3$ cells per rectangular vessel for photosynthesis, 100 mm$^3$ cells per vessel for respiration, 20 min. at steady-state).

<table>
<thead>
<tr>
<th>Temp, Deg. C.</th>
<th>ml./hr./ml. p.c.</th>
<th>$Q_{10}$ Respiration</th>
<th>$Q_{10}$ Corrected Photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiration</td>
<td>Net photosynthesis</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.09</td>
<td>0.27</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.12</td>
<td>0.59</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.26</td>
<td>0.73 (0-10°) 2.89</td>
<td>(0-10°) 2.75</td>
</tr>
<tr>
<td>15</td>
<td>0.49</td>
<td>1.58</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>0.73</td>
<td>3.11 (10-20°) 2.81</td>
<td>(10-20°) 3.88</td>
</tr>
<tr>
<td>25</td>
<td>1.21</td>
<td>5.01</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>1.69</td>
<td>9.12 (20-30°) 2.32</td>
<td>(20-30°) 2.82</td>
</tr>
<tr>
<td>32.5</td>
<td>1.41</td>
<td>10.45 $Q_{10}$</td>
<td>$Q_{10}$</td>
</tr>
<tr>
<td>35</td>
<td>1.70</td>
<td>12.82 (30-35°) 1.01</td>
<td>(30-35°) 1.34</td>
</tr>
<tr>
<td>37.5</td>
<td>1.75</td>
<td>11.74</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>1.64</td>
<td>7.44</td>
<td></td>
</tr>
<tr>
<td>45</td>
<td>1.45</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Table VIII. Photosynthesis and respiration in *Chlorella pyrenoidosa* (Emerson's strain) as a function of temperature (20 mm$^3$ cells per rectangular vessel for photosynthesis, 100 mm$^3$ cells per vessel for respiration, 20 min. at steady-state).

<table>
<thead>
<tr>
<th>Temp. Deg. C.</th>
<th>ml./hr./ml. p.c.</th>
<th>Q_{10} Respiration</th>
<th>Q_{40} Corrected Photosynthesis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Respiration</td>
<td>Net photosynthesis</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0.10</td>
<td>1.13</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.24</td>
<td>2.10</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.39</td>
<td>3.45</td>
<td>(0-10°) 3.90</td>
</tr>
<tr>
<td>15</td>
<td>0.61</td>
<td>5.54</td>
<td>(0-10°) 3.16</td>
</tr>
<tr>
<td>20</td>
<td>0.80</td>
<td>8.73</td>
<td>(10-20°) 2.05</td>
</tr>
<tr>
<td>25</td>
<td>0.91</td>
<td>13.52</td>
<td>(10-20°) 2.48</td>
</tr>
<tr>
<td>30</td>
<td>1.11</td>
<td>17.90</td>
<td>(20-30°) 1.39</td>
</tr>
<tr>
<td>32.5</td>
<td>1.05</td>
<td>19.81</td>
<td>(20-30°) 1.89</td>
</tr>
<tr>
<td>35</td>
<td>1.18</td>
<td>20.40</td>
<td></td>
</tr>
<tr>
<td>37.5</td>
<td>1.07</td>
<td>20.86</td>
<td></td>
</tr>
<tr>
<td>40</td>
<td>0.98</td>
<td>16.23</td>
<td></td>
</tr>
<tr>
<td>42.5</td>
<td>0.79</td>
<td>5.04</td>
<td></td>
</tr>
</tbody>
</table>
FIG. 13

Relationship between Temperature and Respiration in Nostoc

Temperature \( ^\circ C \)

m. O₂/hour/m³ packed cells

Respiration

Photosynthesis
Fig. 14  Photosynthesis and Respiration in Chlorella pyrenoidosa. Emerson and Burk Strains, as a Function of Temperature.
The $Q_{10}$ values for photosynthesis and respiration were above 3.0 over the range 0-10°C. in Chlorella, and they declined with increasing temperature, as was observed by Warburg (80) and other investigators (27, 64). The rate of photosynthesis in Nostoc was reduced to a greater extent by low temperature than in Chlorella. The high $Q_{10}$ shown by photosynthesis and other processes at low temperatures has been frequently referred to. It appears to us to be mainly a mathematical artifact. The lower the temperature, the more nearly the rate approaches zero; since the rate at the lowest temperature forms the denominator in the $Q_{10}$ calculation, its approach toward zero necessarily raises the $Q_{10}$ value.

Much higher rates of photosynthesis in No. 9 buffer were consistently shown by Chlorella cultured in Warburg and Burk's medium vs. Emerson and Lewis medium. This proved to be due to the higher salt concentration of the Warburg-Burk medium, not to the strain of Chlorella. Corresponding phenomenon in Nostoc have already been discussed. The differences shown in Figure 14 refer only to measurements made in bicarbonate buffer. When Chlorella cells of either strain cultured in Emerson-Lewis medium were suspended in this medium under 5% CO$_2$, they showed as high rates of photosynthesis by the two-vessel method as Chlorella cells which were cultured and suspended in Warburg-Burk medium for the photosynthesis measurement.
Corresponding temperature relations data for the quinone reaction in Burk's strain of *Chlorella* are shown in Figure 15 and Table IX. Only the rate of quinone reaction changes, the total oxygen yield remains the same.

Table IX. Quinone-Hill reaction in *Chlorella* as a function of temperature.

<table>
<thead>
<tr>
<th>Temp. in Deg. C.</th>
<th>first 5 min reading</th>
<th>20 min. reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ml/O₂/hr/ml.p.c.</td>
<td>ml/O₂/hr/ml.p.c.</td>
</tr>
<tr>
<td>0</td>
<td>7.66</td>
<td>5.88</td>
</tr>
<tr>
<td>5</td>
<td>9.78</td>
<td>(0-10°)</td>
</tr>
<tr>
<td>10</td>
<td>13.08</td>
<td>9.57</td>
</tr>
<tr>
<td>15</td>
<td>15.90</td>
<td>10.46</td>
</tr>
<tr>
<td>20</td>
<td>14.76</td>
<td>8.66</td>
</tr>
</tbody>
</table>

It should be emphasized that, in contrast to all previous studies, consistently higher rates of water photolysis (oxygen production) were observed by the quinone reaction than by photosynthesis with the same cells, over the temperature range 0-15°C. The temperature coefficient for the quinone reaction was lower than for photosynthesis or respiration in the range 0-10°C. The faster oxygen production in the quinone reaction vs. photosynthesis is most striking at the lowest temperatures (0-5°C.) but even at 10°C. the quinone reaction rate is often four times as high as the corresponding rate of photosynthesis (Table X). This new observation applies to
photosynthesis measured in complete culture medium under 5% CO₂ as well as in bicarbonate buffer, and it applies to both the Emerson and Burk strains of Chlorella as well as to Nostoc.

Table X. Rates of photosynthesis and quinone reaction at low temperatures in Nostoc and Chlorella (ml. O₂/hour/ml. packed cells):

<table>
<thead>
<tr>
<th>Temp. Deg. C.</th>
<th>Chlorella</th>
<th>Nostoc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Photosynthesis</td>
<td>Quinone reaction</td>
</tr>
<tr>
<td>0</td>
<td>1.13</td>
<td>7.66</td>
</tr>
<tr>
<td>5</td>
<td>2.10</td>
<td>9.78</td>
</tr>
<tr>
<td>10</td>
<td>3.45</td>
<td>13.08</td>
</tr>
<tr>
<td>15</td>
<td>5.54</td>
<td>15.90</td>
</tr>
</tbody>
</table>

In a related investigation to be reported elsewhere (Clendenning, K. A., and Brown, T. E., unpublished) it was shown that the oxygen yield per 35 micro second flash is higher by the quinone reaction than by photosynthesis in the same cells at 0-10°C., and with dark intervals of 0.01-0.1 seconds. Thus it is now possible to make water photolysis proceed much faster than the full rate of photosynthesis at low temperatures in flashing as well as continuous light. Presumably one or more dark reactions which limit the rate of photosynthesis at low temperatures is excluded in the Hill reaction, allowing the latter to proceed faster.

Use of aliquots of the same cell suspension in simultaneous measurements of photosynthesis and the quinone reaction thus has
brought out striking differences in the temperature relations of these two processes. The optimum temperature for the quinone reaction in *Chlorella* was 20°C. lower than for photosynthesis and respiration in the same cells. The experiments described in the next section were undertaken with the purpose of explaining this large difference.
8. **Thermal inactivation of the Hill reaction in algae by dark exposure to quinone.**

When quinone is added to algae suspensions, their capacity for photosynthesis is lost at once, but a Hill reaction then occurs just as with isolated spinach chloroplasts. The advantage of using algae instead of isolated chloroplasts is that precise comparisons with photosynthesis can be made with aliquots from the same algal suspension. It should be noted that the quinone reaction rates obtained in this study of *Nostoc* are higher than had previously been obtained with blue-green algae; the rate of oxygen production observed with *Cylindrospermum* (21) was only 1/4th of that shown by *Chlorella* on a packed cell volume basis.

Quinone and furfural (21, 82, 89) apparently are the only artificial hydrogen acceptors which have been successfully used on algae, quinone being the more convenient to use. Other Hill reagents apparently fail to penetrate the cells (21). Aqueous quinone solutions undergo a darkening polymerization reaction which is accelerated by the blue light absorbed by quinone as well as by alkalinity. The product of this reaction has a strongly inhibitory effect on the quinone reaction (21, 42). For this reason, the quinone solutions always were freshly prepared in the absence of light, and light absorbed by quinone was excluded with filters in the Hill reaction measurements. As part of the present study, it was shown that the quinone reaction in *Nostoc* and *Chlorella* is unaffected by pH in the range 6.5-7.5.

Additional background information was presented in preceding sections.
The inactivation of photosynthesis which occurs above 40°C resembles protein denaturation and the inactivation of isolated enzymes at similar temperatures. The inactivation of the Hill reaction in algae cells at very much lower temperatures is difficult to explain in terms of enzyme inactivation---optimum temperatures for isolated enzymes usually are similar to those observed here for photosynthesis. Why should the optimum temperature for the quinone reaction in algae be so much lower than that for photosynthesis and respiration in the same algae, or for isolated enzymes? The experiments which follow were conducted with the purpose of explaining this phenomenon.

A series of experiments were conducted on Nostoc and Chlorella to determine the effect of exposing the cells to quinone in darkness for different lengths of time and at different temperatures. Subsequent rates of the quinone reaction as well as total oxygen yields were observed under standard conditions at a temperature of 10°C. The dark exposure treatments could be varied stepwise in this way, while retaining constant conditions in the photochemical activity measurements, (Figs. 16 and 17).

Increasing the dark exposure time at 10°C and higher temperatures reduced the subsequent rate of the quinone reaction at 10°C, and to a greater extent in Nostoc than in Chlorella. The higher the dark temperature and the longer the dark pretreatment, the greater was the resulting reduction in subsequent photochemical activity at 10°C, (Fig. 18). The total yield of oxygen however was substantially unaffected by these dark pretreatments with either Nostoc or Chlorella.
Theoretical yield

Fig. 16. Effect of Dark Pretreatments with Quinones on the Reaction Rate and Oxygen Yield in Nostoc.
Fig. 17 Effect of Dark Pretreatments with Quinone on the Reaction Rate and Oxygen Yield in Chlorella.
Fig. 16 Effect of Dark Exposure Time to Quinone at 10°C and 25°C upon the Subsequent Photochemical Reaction Rate at 10°C in Chlorella.
Upon raising the cell suspension density to 50 mm$^3$ cells per vessel, the effect of dark pretreatment with quinone was lessened. Increasing the amount of cells increases the available photochemical apparatus so that more of it has to be inactivated before the Hill reaction rate is affected. Conversely as the quantity of cells per vessel is reduced, the effect of dark pretreatments with quinone becomes increasingly important.

The present data are the first that have been provided on the effects of dark pretreatments with quinone on the Hill reaction in green and blue-green algae. They show that in all such studies the preceding time of exposure to quinone can have important effects on subsequent photochemical activity. The present data also indicate that photochemical activity in algae which have been exposed to quinone in darkness is about as sensitive to temperature as photochemical activity in isolated chloroplasts. Quinone kills the cells just as effectively as when they are mechanically macerated. The photochemical activity of chloroplasts then becomes very thermolabile, whether or not they are separated from or remain within the cell walls. An earlier study of the temperature relations of the Hill reaction in isolated chloroplasts by Arnon (7) revealed an optimum temperature of ca. 15°C., as was observed here for the quinone reaction in both Chlorella and Nostoc cells.
9. **Photosynthetic Induction.**

There have been so many investigations of induction in photosynthesis that no attempt will be made to review them here. It should at least be noted that the observation of a lengthy photosynthetic induction period in the sea lettuce *Ulva Lactuca* by Osterhout and Haas (62) was the first discovery concerning photosynthesis which was made in this country (Marine Biological Laboratory, Woods Hole, August, 1917). It is agreed that oxygen is required for photosynthesis in plants (with the exception of *Scenedesmus* (3)) and that under anaerobic conditions the induction period is lengthened. The brief but real induction period observed under aerobic conditions or even under an atmosphere of pure oxygen apparently is caused by an initial lack of CO$_2$-acceptor (21). The site of induction phenomena (aerobic and anaerobic) at least is connected with the dark CO$_2$ assimilation reactions rather than with water photolysis and oxygen production, since Hill reactions in whole cells as well as isolated chloroplasts do not exhibit an induction period regardless of the oxidant employed (7, 33, 42).

No studies have been reported on induction in photosynthesis and the Hill reaction in Cyanophyta. This subject therefore has been investigated comparatively in *Nostoc* and *Chlorella*.

Two special methods were used to follow the initial time courses of oxygen production manometrically, both of which essentially
eliminated manometric "lag" due to faulty equilibration. The first of these is the well-known method used by Warburg in his original studies of photosynthetic induction in *Chlorella* (81). Since respiration is almost negligible in comparison to photosynthesis in saturating light, the oxygen produced during a short period in light may be accurately measured by continuing to shake the manometer system for a few minutes in darkness until the full yield is observed. Further, if each illumination period is preceded by a uniform dark period (e.g. 10 min.) comparable oxygen yields for 0.5, 1.0, 1.5, 2.0 minutes, etc. periods of saturating illumination can then be measured with ordinary manometric equipment, but without the usual lag due to slow equilibration of the gas and liquid phases.

The second method is the "boat method" of Brinkman, Margaria, Meldrum and Roughton which has been used for about twenty years in studies of carbon anhydrase (14), but which had not previously been used in studies of photosynthesis. In this method the reaction vessel is shaken at high speed independently of a stationary manometer, to which it is connected with heavy pressure tubing. The thorough shaking of the reaction vessel ensures rapid equilibration, and the stationary manometer allows the pressure changes to be read accurately at half-minute or even shorter time intervals. The suitability of the latter method for studies of photosynthetic induction was indicated by the very similar time courses of oxygen production which were observed with it and with Warburg's method involving intermittent illumination. Warburg's method was used in
comparing photosynthetic induction in Chlorella and Nostoc under atmospheres of air and pure oxygen (Fig. 19). In agreement with earlier studies, the steady rate of photosynthesis was lower under oxygen than under air. In the present studies, Nostoc photosynthesis is shown to be depressed to a greater extent by an atmosphere of oxygen than Chlorella. Brief induction periods of similar duration were observed in both organisms, and under air as well as oxygen. These were similar to those observed by Warburg on Chlorella using the same manometric method, in the recent polarographic studies by Brackett, Olson and Crickard (13) and in the spectroscopic measurements reported by Hill and Whittingham (46). The fact that blue-green and green algae exhibit similar photosynthetic induction periods indirectly supports the view that photosynthesis is basically similar throughout the plant kingdom.

The time courses of photosynthesis and the quinone reaction in Chlorella at 20°C. are shown in Figure 20, as measured by the boat method. To increase the oxygen production per vessel, 50 mm³ Chlorella were employed. The temperature (20°C.) used in this experiment was more favorable for photosynthesis than for the quinone reaction, causing the gradually declining Hill reaction rate which is apparent in Figure 20. The quinone reaction consistently lacked the induction period shown by aliquots of the same cell suspension which were photosynthesizing in bicarbonate buffer. The same characteristics as shown for Chlorella in Figure 20 were observed in corresponding experiments on Nostoc, but the oxygen production per unit of packed Nostoc cells was lower in both reactions.
Fig. 19 Photosynthetic Induction under Air and Oxygen in Nostoc and Chlorella.
Fig. 20. Initial Time Course of Oxygen Production by Photosynthesis and the Hill reaction in Chlorella.

Complete culture solution for Nostoc contains eleven mineral elements in addition to oxygen and carbon; the essentiality of boron, calcium, iron, potassium, magnesium, manganese, molybdenum, nitrogen, phosphorus, sodium, and sulphur for Nostoc has been established by Dr. H. C. Eyster (30, 31). Most of these elements, however, are required by all living cells. Determining the influence of any one of them on photosynthesis necessitates the drawing of distinctions between (a) effects common to all living cells (b) effects due to accelerated synthesis or breakdown of one or more parts of the photosynthetic apparatus and (c) effects due to direct catalysis of or participation in the process of photosynthesis. When an essential element is withheld from algae cultures, growth is diminished and eventually abolished, and the process of photosynthesis is usually affected in a similar way. The fact that photosynthesis is reduced by a mineral deficiency does not in itself allow this effect to be classified under one of the foregoing three categories. A very extensive literature already exists on effects of mineral deficiencies on photosynthesis in algae and higher plants. In all but a few instances however, these earlier investigations do not allow any conclusions to be drawn as to the precise way or ways in which the deficient element affects photosynthesis.

Because of the interesting effects of potassium which were observed in cell washing experiments on Nostoc, effects of withholding potassium from the culture solution were also investigated.
It should be noted that in the complete absence of an essential element, growth ceases and the cells die. An appreciable amount of the deficient element is introduced with the inoculum, however, if it consists of healthy cells. Several growth cycles thus are required to bring the inoculum completely into equilibrium with the deficient medium, so that uniform rates of photosynthesis and growth are observed in successive harvests. It is necessary to establish experimentally the lowest concentration of the deficient element which can be used for the continuous culture of cells at a reduced rate of growth. Growth experiments conducted by Dr. H. C. Eyster and Mrs. L. Birtle showed that a potassium concentration which was 1/6th of the normal level (i.e. 2.25 ppm. vs. 13.5 ppm. K) was the most suitable for the production of potassium deficient Nostoc (the cells died when the potassium concentration was reduced to 1/10th). The cells used in the following photosynthesis experiments accordingly were cultured in a medium modified to contain 1/6th of the normal amount of potassium.

When normal cells were used as the initial inoculum, the rate of photosynthesis dropped off rapidly during the first two or three days of continuous culture (Fig. 21). After three days, the rate of photosynthesis declined more gradually, but a significant decrease in activity occurred between the twenty-second and twenty-ninth day of "continuous culture" in mineral solution of uniform potassium content.
Fig. 2: The progressive decline in *Nostoc* photosynthesis caused by its continuous culture at \(1/6\) of the normal potassium level.
The effect of restoring potassium to Nostoc cells which had been cultured for nine days at 1/6th of the normal potassium level is shown in Fig. 22. Control measurements were included on cells suspended in all-sodium 0.025 M No. 9 buffer, and on cells to which were added all constituents of complete culture medium except potassium. The control activities remained within 10% of the steady-rate observed prior to tipping. When potassium was added, there was little response until after twenty minutes. The rate of photosynthesis then rose gradually and steadily for more than one hour. If potassium were serving simply as a coenzyme of photosynthesis, an instantaneous rather than a gradual response to the added potassium would be expected. Experiments of the same type as shown in Fig. 22 were conducted on Nostoc cells showing widely different degrees of potassium deficiency, but in no case was an instantaneous response or recovery observed. These experiments indicate that when Nostoc cells are grown in potassium deficient media, the situation is more complex than when healthy cells are washed thoroughly with K-free media. Upon adding potassium to potassium deficient cells, recovery of full photosynthetic activity appears to require as much time as recovery of the normal growth rate.
Fig. 22  Time Course of Photosynthesis in Potassium Deficient Nostoc in "all-sodium" No. 9 Buffer with and without Complete Culture Solution and with Potassium-Free Culture Solution.
SUMMARY AND CONCLUSIONS

The blue-green alga *Nostoc muscorum* was studied manometrically with respect to its photosynthesis, respiration and Hill reaction capacities in comparison with *Chlorella pyrenoidosa*.

The most generally useful bicarbonate buffer for direct measurements of photosynthesis in *Nostoc* was found to be 0.025 M No. 9 buffer in which the Na : K ratio is 85 : 15. For *Chlorella* the same buffer at 0.1 M was found to be the most suitable. The alkalinity and low CO₂ content of these buffers, however, prevent photosynthesis from proceeding at its maximum rate in saturating light, in contrast to the conditions provided by the two-vessel, reference method.

By incorporating equilibrium concentrations of sodium bicarbonate in dilute *Nostoc* suspensions under 5% CO₂ in air, the indirect or two-vessel method could be used in measuring the full rate of *Nostoc* photosynthesis in the pH range of its normal culture medium (pH 7.0-7.5). In terms of packed cell volume, the photosynthetic capacity of *Nostoc* is only 1/2 that of *Chlorella*; in terms of cell numbers, however, the photosynthetic capacity of the larger *Nostoc* cells exceeds that of *Chlorella*.

External CO₂ buffering with diethanolamine was found to have the following disadvantages: (a) equilibration occurred slowly; (b) CO₂ buffering was incomplete after equilibrium had been established; (c) the diethanolamine-CO₂ complex was subject to autoxidation even in the presence of antioxidants; and (d) rates of photosynthesis observed in saturating light with diethanolamine CO₂ buffers were
never as high as the full rate observed by the two-vessel method, using either *Nostoc* or *Chlorella*. For these reasons external CO₂ buffering with diethanolamine cannot be recommended, at the present time, for use in photosynthesis measurements.

Photosynthesis was found to be relatively insensitive to extreme centrifuging treatments (up to 2 hours at over 20,000 g) in both *Nostoc* and *Chlorella* provided mechanical injury did not subsequently occur. This applies to photosynthesis measurements made either with the single or two-vessel manometric methods.

*Nostoc* cells are sensitive to washing treatments either with water, bicarbonate buffers of different concentrations, or potassium-free culture medium. *Nostoc* cells are far more sensitive to this treatment than are *Chlorella* cells, although the latter are significantly affected. The fact that the cells are not adversely affected by complete culture medium or by wash liquids containing potassium indicates that the "washing effect" is due to the leaching of potassium from the cells.

*Nostoc* grown under optimum conditions requires a more dilute bicarbonate buffer, and also exhibits a steady rate of photosynthesis in bicarbonate buffer for a shorter time than in the case of *Chlorella*. *Nostoc*, however, can be adapted to concentrated bicarbonate buffers by growing the cells in an equally concentrated culture medium. Much higher rates of photosynthesis in No. 9 buffer were consistently shown by *Chlorella* grown in Warburg and
Burk medium vs. Emerson and Lewis medium. This difference was caused by the higher salt concentration of the Warburg-Burk medium. When Chlorella cells grown in either medium were placed in the same medium under 5% CO₂ (two-vessel method), their rates of photosynthesis then were the same.

The pH relations of Nostoc photosynthesis have not been investigated in detail, but it has been established that Nostoc requires a more alkaline medium for both photosynthesis and growth than Chlorella. The saturating light requirement for Nostoc photosynthesis at 25°C. was very similar to that of Chlorella grown at the same temperature and light intensity, and tested at the same cell suspension density.

Upon storing Nostoc and Chlorella at 0°C., there is an initial drop in photosynthesis activity during the first day or two, which is followed by several days in which photosynthesis in Chlorella remains quite constant, after which it falls abruptly. The "storage plateau" shown by Chlorella was not exhibited by Nostoc. Thus there was a constant difference in the cold storage characteristics of Nostoc and Chlorella with respect to retention of photosynthetic activity.

The optimum temperature for photosynthesis in Nostoc is ca. 35°C., very similar to that of the Emerson and Burk strains of Chlorella in which the optimum temperature was found to be ca. 37.5°C. The optimum temperature range for respiration in both organisms is broader but otherwise corresponds to photosynthesis. Respiration, however, is considerably less sensitive to high temperatures than is photosynthesis. Photosynthesis in Nostoc is inhibited to a greater extent by low temperatures than in Chlorella.
In agreement with earlier studies, the steady rate of photosynthesis in both *Nostoc* and *Chlorella* was lower under oxygen than under air. In the present studies, *Nostoc* photosynthesis was depressed to a greater extent by an atmosphere of oxygen than *Chlorella*. Brief photosynthetic induction periods of similar duration were observed under air and oxygen in both organisms. The fact that blue-green and green algae exhibit similar photosynthetic induction periods indirectly supports the view that photosynthesis is basically similar throughout the plant kingdom.

A high capacity for the quinone reaction was retained in *Nostoc* cells whose capacity for net photosynthesis had been abolished by thorough washing with water. In contrast to photosynthesis, the capacity for the quinone reaction in *Nostoc* and *Chlorella* remained quite uniform during lengthy storage of the cells at 0°C. Thus it is those parts of the photosynthetic apparatus which do not participate in the Hill reaction which are most sensitive to cell washing treatments as well as to cold storage. The total oxygen yield in the quinone reaction is not controlled by the quantity of *Nostoc* or *Chlorella* cells employed, which indicates that the dark reduction of quinone to hydroquinone by algal constituents is of negligible importance.

In contrast to all previous studies, consistently higher rates of oxygen production (or water photolysis) were observed for the quinone reaction than for photosynthesis in *Nostoc* and *Chlorella* cells over the range 0-15°C. This difference in rate of oxygen production
between these two reactions was greatest at the lowest temperature (0°C.) employed in this study. This new observation signifies that rate-limiting processes in photosynthesis can be excluded in the quinone reaction, allowing the latter to proceed more rapidly in the low temperature range. The optimum temperature for the quinone reaction in *Nostoc* and *Chlorella* is in the range 15-20°C., in contrast to 35-40°C. for photosynthesis. Increasing the time of dark exposure of the cells to quinone at 10°C. and higher temperatures reduces the subsequent rate of oxygen production at 10°C. but does not affect the total oxygen yield. The higher the dark temperatures and the longer the pretreatment with quinone, the greater is the reduction in subsequent rate of oxygen production under otherwise standard conditions. The interpretation which is offered for the much lower optimum temperature for the quinone reaction as compared to photosynthesis in the same cells is that quinone kills the cells, and in so doing renders the photochemical apparatus as thermolabile as isolated chloroplasts.

When potassium of the culture solution is reduced to a deficient level, the photosynthetic capacity of the cells (per unit of packed cells) declines rapidly at first and then more slowly during several weeks of continuous or cyclical culture. When the normal concentration of potassium is restored, there is no response for twenty minutes. The rate of photosynthesis then rises steadily for more than one hour. Recovery of full photosynthetic activity in potassium deficient cells appears to require as much time as recovery of the normal growth rate.
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