I

AECU-3378

THE MECHANISM OF THE
PHOTOCHEMICAL ACTIVITY OF
ISOLATED CHLOROPLASTS

3. LIGHT INTENSITY DEPENDENCE
4. EFFECT OF pH
5. EFFECT OF HEAVY WATER

by

JOHN S. RIESKE

JOHN D. SPIKES

and

RUFUS LUMRY

TECHNICAL REPORT NO. 21

15 July 1956

CONTRACT NO. AT (11-1)-82

PROJECT NO. 4

Department of Experimental Biology
University of Utah
Salt Lake City

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

TABLE OF CONTENTS

	Page
I. INTRODUCTION	1
II. HISTORY	4
III. STATEMENT OF PROBLEM	7
IV. MATERIALS AND METHODS	9
A. Preparation of Chloroplast Fragments	9
B. Apparatus	10
1. Reaction Cell Assembly	10
2. Reaction Chamber	14
3. Light Source	15
4. Apparatus for the Measurement of Light Intensity	16
5. Temperature Control	17
6. Potential Recording Apparatus	17
C. Measurements	18
1. Preparation of Reaction Mixture	18
2. Rate Determination	19
3. Light Intensity Measurements	20
4. Precision of the Measurements	26
V. RESULTS	27
PART A. CONTROL EXPERIMENTS	27
1. Electrode System	27
2. Stirring	28
3. Chloroplast Concentration	28
4. Ferricyanide Concentration	28
5. Oxygen Concentration	29
PART B. LIGHT INTENSITY DEPENDENCE IN THE HILL REACTION--RESULTS AND DISCUSSION	30
1. Introduction	30
2. Effects of Non-uniform Illumination Upon Experimental Light Curves	32
3. Statistical Study of the Rate-Light Intensity Relationship of the Hill Reaction	37
PART C. THE EFFECT OF TEMPERATURE UPON THE HILL REACTION LIGHT CURVE--FORMULATION OF THE FUNDAMENTAL RATE EQUATION FOR THE HILL REACTION	50

PART D.	THE EFFECT OF LIGHT WAVELENGTH UPON THE HILL REACTION RATE PARAMETERS k_D AND k_L -- RESULTS AND DISCUSSION	Page 56
PART E.	THE EFFECT OF pH ON THE HILL REACTION-- RESULTS AND DISCUSSION	64
1.	Introduction	64
2.	pH Dependence of k_D and k_L	65
3.	The Effects of Temperature upon the Hill Reaction pH Dependence	67
4.	The Effect of High pH's on the Light Curve--A Possible Light Inhibition	72
PART F.	KINETICS OF THE HILL REACTION IN HEAVY WATER-- RESULTS AND DISCUSSION	77
1.	Introduction	77
2.	The Effect of Heavy Water upon the Hill Reaction Rate Parameters k_D and k_L	79
3.	Progressive Nature of the Heavy Water "Inhibition".	81
4.	Reversibility Test of the Heavy Water Inhibition	85
5.	The Effect of Deuterium Oxide on the Apparent Activation Energy, μ , of k_D	87
6.	Discussion	91
IV.	GENERAL DISCUSSION	93
VII.	SUMMARY	106
VIII.	BIBLIOGRAPHY	108

I. INTRODUCTION

Extensive studies have been made of the kinetics of photosynthesis as carried out by intact plant cells. These studies have included leaf plants under field conditions, marine plants, green, blue-green, and brown algae, and even the photosynthetic bacteria. Especially notable work has been accomplished with the unicellular green algae. However, the range of kinetic data obtainable with intact cell systems is limited. The complexity of the system is so great that only the interrelationships of the most obvious processes have been exploited to any extent. Kinetic schemes, although based only upon these obvious interrelationships, yield such complex expressions as to make more detailed studies with the complete system prohibitive. The problem then resolves itself into finding a way to break the photosynthetic system into parts which can be studied individually, without interference from the other related processes. The closest development to date toward the realization of this ideal situation was the comparatively recent discovery that cell-free chloroplast preparations, in conjunction with a suitable electron acceptor (oxidant), retained the ability to decompose water with oxygen evolution, when illuminated. This reaction in recognition of its discoverer is commonly called the Hill reaction. Although sufficient evidence has accumulated to link this process directly to the overall photosynthetic process, comparatively few careful kinetic studies have been reported on it to date.

The overall process of photosynthesis may be represented by the relationship:

The discovery of the Hill reaction allows the overall process to be modified into two distinct processes; the photolytic oxidation of water and the reduction of carbon dioxide.

In this scheme, A represents some electron transfer system connecting the photolytic site to the carbon dioxide reduction site. Although a limited carbon dioxide fixation can occur in the isolated chloroplast or even in the chloroplast fragment (Arnon, *et. al.*, 1954; Arnon, 1955), this process is essentially bypassed in the presence of a highly efficient electron acceptor as found in the functioning Hill reaction system. Thus, in order for the Hill reaction to function an electron acceptor must be present to replace A or CO₂ in the above scheme. Leaf extracts, ferric oxalate, ferricyanide, quinone, and certain redox dyes have been commonly used for this purpose. The Hill reaction employing ferricyanide as an oxidant (electron acceptor) can then be written stoichiometrically as:

This reaction offers a very convenient tool for the kinetic study of the photolytic process. Not only can the reaction rate be followed by a measurement of oxygen evolution, as with intact cell systems, but it can also be followed by measurement of hydrogen ion production, disappearance of oxidant, appearance of reduced oxidant, and several modifications of these methods.

This thesis outlines a preliminary kinetic study of the Hill reaction based upon the potentiometric measurement of oxidant reduction. Emphasis has been placed upon the development of techniques necessary for quantitative kinetic measurements with this reaction.

II. HISTORY

It was a common belief among early investigators in photosynthesis that once the chlorophyll-containing plant cell was broken the production of oxygen ceased forever. Observations by many workers indicated that freezing, drying, boiling or poisoning ended photosynthesis immediately. However, Haberlandt in 1888 and Ewart in 1896 observed that upon exposure to light, a small quantity of oxygen was evolved from isolated chloroplasts prepared by grinding leaves under water. Molisch in 1904 and 1925 observed oxygen evolution when dried leaf powders were suspended in water and exposed to light. Inman (1935, 1938), in a series of experiments, confirmed the results of Ewart and Molisch. In all of these experiments, however, the amount of oxygen released was so minute that it could only be detected by the luminescence of Beijerinck's bacteria. Yamafuji and coworkers (1938, 1939) ascribed this oxygen evolution to the photodecomposition of peroxide, traces of which were detected in dried plant and animal tissues.

The first indication that cell-free preparations could effect a portion of the overall process of photosynthesis came as a result of R. Hill's experiments (1937, 1939, 1940). In his early experiments, Hill found that an aqueous suspension of leaf chloroplasts evolved considerable amounts of oxygen when illuminated, provided that an aqueous leaf or yeast extract was present. The oxygen evolution was measured by spectrophotometrically following the conversion of hemoglobin to oxyhemoglobin. After observing that the efficiency of the yeast extracts depended upon the quantity of organic iron compounds present, he substituted ferric potassium oxalate for the extract and found this to be efficacious in the evolution of oxygen. Simultaneous with the

evolution of oxygen, the ferric salts were reduced to ferrous salts. Later, he found that by employing potassium ferricyanide in conjunction with ferric oxalate enough oxygen was evolved to be measured by manometric methods. (Hill and Scarisbrick, 1940).

In the decade following Hill's discovery most of the research was concerned with attempts to characterize the optimum conditions for the reaction with respect to selection, preparation, and storage of the chloroplasts, techniques of measurement, type of oxidant, and the external conditions such as temperature, pH, etc. Of significant importance during this period were the findings that several oxidants would serve as electron acceptors in the Hill reaction, and that chloroplast suspensions could be stabilized for long periods of time at temperatures below -40°C .

Also of great importance to the kinetic study of the Hill reaction has been the development of precise techniques for the measurement of the reaction velocity. As was previously mentioned, the Hill reaction velocity can be measured by techniques based upon oxygen evolution, hydrogen ion production, disappearance of oxidant, and the appearance of reduced oxidant. After Hill's initial use of hemoglobin for the measurement of oxygen evolution the manometric method has been widely used for this purpose. Holt and French (1946) as well as Clendenning and Gorham (1950, 1952) have employed a titration of the hydrogen ions produced in the Hill reaction. Holt, Smith and French (1950) followed by several others have employed a redox dye as the oxidant, thus measuring the rate by spectrophotometrically following the dye color change upon reduction. Spikes and coworkers (1950) developed a method of following oxidant reduction by potentiometric methods, employing a platinum and calomel

electrode system immersed in the chloroplast-oxidant-buffer mixture. Details of this method will be discussed later as it was used in the present work.

Although the Hill reaction was suspected of being an integral part of the general photosynthesis process, the work of several investigators has uncovered sufficient evidence to remove most doubt as to this relationship. The evidence that oxygen evolution in the Hill reaction system takes place by the same mechanism as oxygen evolution in photosynthesis of the intact plant can be summarized as follows:

1. The action spectrum for the Hill reaction with chloroplast fragments closely corresponds to that for whole cell photosynthesis. (Chen, 1952)

2. The rate vs. light intensity curves obtained with chloroplast preparations roughly corresponds to those obtained with whole photosynthesizing cells, both showing light limiting and light saturation phenomena.

3. The quantum yields obtained with the Hill reaction are comparable to photosynthesis values. (French and Rabideau, 1945; Erhmantraut and Rabino-
witch, 1951; Wayrynen, 1952)

4. Tracer studies using O^{18} enriched water indicated that the oxygen evolved by the Hill reaction originates from the water. (Holt and French, 1948) This is in agreement with similar studies with whole cell systems. (Ruben, Randall, Kamen, and Hyde, 1941)

5. The observation that certain unicellular algae (Chlorella pyrenoidosa) will evolve oxygen in the presence of certain Hill reaction oxidants in the absence of carbon dioxide. (Warburg and Luttgens, 1946)

6. The similar effects of certain inhibitors upon both the Hill reaction and photosynthesis.

III. STATEMENT OF PROBLEM

Much of the published kinetic data on the Hill reaction either is in disagreement with the data of other workers or is not precise enough to warrant a quantitative kinetic analysis. These difficulties can be attributed in large part to the lack of control of the inherent variables either because of ignorance as to their existence or because of a lack of sufficient refinement in the rate measurements. These two factors are aggravated by the large number of interdependent variables present with the Hill reaction system.

Most experimental relationships are expressed in terms of only two variables. If, as is usually the case, these variables are a function of a third variable then the functional relationship between the two initial variables will depend upon the value of the third. Thus, if the third variable is poorly controlled because of either of the two reasons given above, the agreement between independent, supposedly duplicate, experiments will be poor. This is especially the case in the Hill reaction variables such as chloroplast source and preparation, light intensity, oxidant concentration, pH, temperature, and chloroplast instability. All other kinetic effects will have to be measured as a function of these variables in order that an unambiguous concept of their mechanism of action can be obtained.

The research group of which the author of this study is a member has in the past few years attempted a critical analysis of the above listed factors and their relationship to the Hill reaction. This dissertation will report on the results of a study of a few of the listed variables

and their effects upon the velocity-light intensity relationship of the Hill reaction. This study was considered to be of importance since light must be considered as the fundamental substrate of photosynthesis, and as such will of necessity be intimately involved in any kinetic expression based upon the photosynthetic mechanism. Light can be considered as the fundamental substrate of photosynthesis, therefore, the light intensity must be intimately involved in any kinetic expression based upon the photosynthesis mechanism. Because of the difficulties encountered to date in experimentally determining the true rate-light intensity relationship in photosynthesis, and the relationship of the Hill reaction to photosynthesis, a critical study of the rate-light intensity relationship in the Hill reaction should give valuable information as to the mechanism of photosynthesis.

IV. MATERIALS AND METHODS

A. Preparation of Chloroplast Fragments

The source of the chloroplasts used in this study were the leaves of either Swiss chard (Beta vulgaris L. var. Fordhook) or rhubarb chard (Beta vulgaris L.). These plants were obtained from an outside plot in the summer months and from a greenhouse plot during the winter. In most cases, however, the summer plants were used since these produced much more active chloroplast fragments than plants grown during the winter. All the chloroplast preparations were performed in a walk-in cold room kept at 2° C.

In general the following procedure was used for the preparation of the chloroplast fragments: About 100 grams of the washed leaves were blended for about three minutes in 100 ml. of a 0.5 M sucrose-0.03 M potassium chloride solution in a Waring blender. Frozen cubes of the sucrose solution were added at regular intervals during the blending to keep the temperature of the blendate below 5.0°C. The blendate was then strained through four layers of cheesecloth to remove the fibrous residue. It was then centrifuged at about 1000 x g. in order to separate the large particulate matter. The supernatant, which consisted of a chloroplast fragment suspension was then centrifuged at about 20,000 x g. for fifteen minutes in order to sediment the fragments. With the acquisition of a Spinco Model L. preparative ultracentrifuge this sedimentation was performed at about 144,000 x g. at -2°C. for fifteen minutes. After discarding the supernatant, the packed chloroplast fragments were rinsed off with a portion of the sucrose-KCl solution then resuspended in a small

quantity of the solution by the use of a small blender operated at low speed. After determining the chlorophyll concentration, the resuspended chloroplast suspension was diluted to a chlorophyll concentration of about 300 mg/l for use as the stock suspension. This suspension was then frozen and stored at -36°C . until used.

The chlorophyll concentration was determined by comparing the optical density at 665 m μ of an acetone extract of the chloroplast suspension with a chlorophyll concentration-optical density curve prepared from the purified chlorophylls a and b. For routine work the optical density at 675 m μ of a water suspension of the chloroplast fragments was determined and compared to a chlorophyll concentration--optical density curve prepared from an acetone extraction of the standard chloroplast suspension. A Coleman Junior Spectrophotometer was used in all the chlorophyll determinations.

B. Apparatus

The apparatus was specifically designed for the control and accurate measurement of the light intensity. As is illustrated in Figure 1. the apparatus consists of a light source, a collimating lense system, water filter, intensity and color control filter system, shutter, reaction chamber in a thermostated water bath, and a thermopile or vacuum thermocouple arrangement for light intensity measurements.

1. Reaction Cell Assembly

The reaction cell assembly as illustrated in Figure 2 consisted of a plastic cell consisting of two $1 \times 1\frac{1}{2} \times 1/10$ inches (25.4 x 38.1 x 2.54 mm) clear lucite plates, between which was sandwiched a $1/16$ inch (1.57 mm) opaque lucite sheet with a $1 \times \frac{1}{2}$ inch (25.4 x 12.7 mm) cut to form the

Figure 1. Hill Reaction Apparatus

A. Gas outlet; B. Thermopile electrical connections (2); C. Gas outlet; D. Calomel and platinum electrode connectors; E. Thermopile; F. Reaction cell assembly; G. Cover to reaction chamber; H. Lucite window; I. Shutter; J. Filter holder; K. Water filter; L. Condensing lens; M. Projection lamp; N. Spherical mirror.

Figure 2. Reaction Cell Assembly

1. Calomel electrode; 2. Platinum electrode; 3. Stirrer coil; 4. Handle; 5. Vibrating wire stirrer; 6. Fronts piece clamping nuts; 7. Hole in cell holder for light passage; 8. Perspective drawing of plastic reaction cell; 9. Clear plastic of reaction cell; 10. Opaque plastic inner sandwich; 11. Reaction cell holder frontpiece.

Figure 3. Perspective drawing of cell assembly in reaction chamber (cover not shown).

A. Brass reaction chamber; B. Gasket flange for chamber cover; C. Front flange for gasket against lucite window; D. Reaction Cell holder w/o front plate; E. Brass partition supporting cell holder and stirrer; F. Thermopile; G. Platinum electrode; H. Plastic reaction cell; I. Stirrer coil; J. Calomel electrode (sat. KCl); K. Stirrer wire.

reaction chamber. A well was drilled into the top of the cell to accommodate the calomel electrode. This cell was placed in a machined aluminum holder which consisted of the main block with a groove to hold the plastic cell in place and a front plate which was bolted into place so as to apply pressure to the plastic cell. This was necessary to prevent leakage if the plastic cell was held together with stopcock grease. A hole was drilled in the main block behind the plastic cell in order to form a passage for the light to the thermopile or thermocouple. A black velvet strip was glued to the block behind the illumination area of the cell to prevent reflection of the transmitted light back through the reaction mixture.

Stirring of the reaction mixture was provided by an electromagnetic arrangement consisting of a magnetically actuated vibrating wire which was immersed into the reaction mixture. The electromagnet was connected to a low voltage rectifier (battery charger) in series with a 10 ohm variable resistance. The resistance was adjusted such that good stirring with a minimum of heating was obtained. The stirrer was attached to the reaction cell holder by two pins which fitted two holes drilled in the holder.

2. Reaction Chamber

The reaction cell assembly fitted into a heavy brass chamber as illustrated in Figures 1 and 3. The chamber was made of $\frac{1}{4}$ inch sheet brass and was equipped with a close fitting cover. This cover contained the necessary electrical connections for the electrodes and the thermopile. It also was fitted with gas connections in case that the chamber was to be flushed with nitrogen. For temperature control the lower half of the chamber was immersed in the thermostated water bath. The close fit of the

reaction cell assembly in the chamber as well as the heaviness of the chamber was designed to give the optimum thermal transfer to the water bath. For admission of light the front of the chamber was attached to a plastic window in the side of the water bath tank.

3. Light Source

The light source was a projection lamp mounted such that its filament was at the focus of a five inch condensing lens system. A mask with a three inch aperture was placed on the second lens of the condensing system to eliminate aberration from the edges of the lenses. The collimation was good considering the filament size, crudeness of the lens system, and the intensity obtained.

The light beam was passed through a five-inch water filter to remove the infrared light of wave lengths longer than 1000 μ , then through a filter system designed to regulate the color and intensity. In most of the experiments reported in this thesis, interference filters were employed to give light of a high spectral purity. These filters were obtained from Baird Associates Inc.; Cambridge, Mass., and had a fifty per cent transmission band width of approximately 12 μ . The intensity of the light was varied over wide limits by the use of neutral density filters consisting of either wire screens or Wratten gelatin types. The gelatin neutral-density filters were obtained from the Eastman Kodak Co. Their light transmission values were determined, at the light wavelength employed, in a Beckman DU spectrophotometer. The per cent transmission values obtained by this method gave very good agreement with values obtained with the apparatus.

The intensity of the lamp was held constant by the manual control of

a variable transformer connected in parallel with a voltmeter and the lamp. Light admission into the reaction chamber was controlled by a leaf-type shutter.

4. Apparatus for the Measurement of light Intensity

Three photosensitive instruments have been used in this investigation for the measurement of light intensity. Preliminary measurements of a relative nature were made with a Weston "Photronic" selenium, barrier-layer-type photocell. This cell although suited for relative light intensity measurements was unsuited for absolute measurements because of its non-uniform spectral sensitivity, especially in the region of maximum sensitivity of the Hill reaction system to light. Because of its high sensitivity and large surface area, however, it was used for the determination of the degree of scattering of light in the chloroplast suspension. This will be discussed later.

With essentially monochromatic light, either a compensated thermopile or a vacuum thermocouple was routinely used for light measurements. The thermopile was obtained from the Eppley Laboratory Inc., Newport, R. I. and was a surface type, bismuth-silver junction, compensated instrument. This instrument, however, had such a low sensitivity that with the available galvanometer only readings at the maximum intensities could be accurately made. For recent experiments a commercial vacuum thermocouple has been used with good results. This instrument was a General Electric radiation type ZA-4 thermocouple and had about six times the sensitivity of the thermopile.

The photosensitive instrument was connected to a Leeds and Northrup

D'Arsonval type galvanometer from which the light intensity readings were made. For varying the sensitivity of the galvanometer a decade box was employed as a shunt. The shunt was only required in conjunction with the photocell.

5. Temperature Control

Originally, the apparatus was located inside a walk-in coldroom kept at a temperature of 2°C . With this setup the water bath was maintained at the desired temperature by a 300 watt coil type immersion heater which was regulated by a sensitive mercury relay-mercury regulator control system. With this arrangement the temperature of the water bath never varied more than 0.05°C . More recently, the apparatus was moved outside the cold room which necessitated a modification of the temperature control arrangement. In this case the water bath was cooled by circulating the water through a copper coil immersed in a ten gallon reservoir kept cold by a refrigeration compressor unit. Opposing this was a 150 watt knife type heater. A relay system was arranged such that when the mercury regulator was shorted the heater would be turned off and the circulating pump turned on. The reverse occurred when the mercury regulator circuit was open. By carefully controlling the rate of water circulation in the coils, the temperature could be controlled to about 0.2°C . as measured in the reaction cell by an iron-constantan thermocouple connected to the D'Arsonval galvanometer.

6. Potential Recording Apparatus

The redox potential from the calomel-platinum electrode system was routinely recorded with a Brown "Elektronik" Single-Record Strip Chart Recorder (Series 153 x 11). Between the recorder and the electrode system

was an impedance matching circuit, since a high impedance was necessary to prevent polarization of the electrodes. Details of this circuit are given elsewhere. (Spikes, et al., 1954)

C. Measurements

1. Preparation of Reaction Mixture

The reaction mixture was prepared in general by the following procedure: A tube of frozen stock chloroplast suspension was removed from the deep freeze and thawed under the cold water faucet. In the cold, under dim light, one volume of this suspension was added to one volume each of the oxidant and the buffer solutions.

Samples sufficiently small for individual runs were prepared by quickly freezing in a dry ice-acetone bath, tubes containing 0.5 ml. aliquots of the chloroplast-oxidant-buffer mixture. These tubes were stored until needed either in the deep freeze or in wide mouthed Dewar flask over a pellet of dry ice. Since the activity of the chloroplast fragments in the presence of oxidant and buffer decreased quite rapidly even at the temperature of dry ice, the runs were made within twelve hours after preparation. In this time no noticeable inactivation occurred. Prior to a run, the 0.5 ml. aliquot was quickly thawed by shaking in a flask of water at room temperature. About thirty seconds were required for complete thawing with this procedure. Immediately after thawing 0.3 ml of the mixture was pipetted into the reaction cell. From four to six minutes after thawing was allowed for temperature equilibration before the run was begun.

The buffer employed in this study was a phosphate buffer prepared by

titrating to the desired pH a solution of KH_2PO_4 with a solution of K_2HPO_4 . The buffer concentration in the final reaction mixture was either 0.05 or 0.1M.

The oxidant employed was potassium ferricyanide made up to give an initial concentration in the reaction mixture of between 0.0001 and 0.0005 M. The concentration chosen depended upon the nature of the experiment. The effect of the oxidant concentration upon the Hill reaction will be discussed in more detail later on.

2. Rate Determination

The reaction rate was readily obtained from the potential-time curve traced on the recorder chart during illumination of the reaction mixture. This curve was a typical potentiometric titration curve. The inflection point of the curve corresponded to the E_0 of the ferricyanide-ferrocyanide redox couple so it was assumed that the electrode potential was controlled by the ferricyanide-ferrocyanide ratio. The potential-time curve was converted to a per cent ferricyanide reduced-time plot by the application of the Nernst equation. For ease of calculation, a template, which could be placed over the potential-time curve was constructed out of transparent plastic. Lines were scratched on the plastic corresponding to certain potential intervals and were expressed in terms of per cent ferricyanide reduced, thus both the time and the per cent ferricyanide reduced could be read directly at the points where the potential-time curve intersected the lines of the template. From the slope of the percent ferricyanide reduced, thus both the time and the per cent ferricyanide reduced could be read directly at the points where the potential-time curve intersected the

lines of the template. From the slope of the per cent ferricyanide reduced versus time plot the rate could easily be calculated. All the rate values, in this study are reported in units of moles ferricyanide reduced per mole chlorophyll-min.

3. Light Intensity Measurements

In order for the light intensity to be measured in absolute units it was necessary to calibrate the thermopile and the thermocouple-galvanometer arrangement with a standard radiation source. For this purpose a standard radiation source in the form of a carbon filament lamp was obtained from the National Bureau of Standards. It was discovered, however, that the thermocouple sensitivity was not quite linear with respect to light wavelength, especially in the far infrared. This nonlinearity was probably due to a nonuniform reflection of light from the brass case in which the thermocouple was mounted. The thermocouple was therefore calibrated against the thermopile in the visible region of the spectrum. In this region the nonlinearity amounted to only a 2.5 per cent change in sensitivity from 460 mμ to 675 mμ. It was also necessary to calibrate both the thermopile and the vacuum thermocouple as a function of temperature since their sensitivity was found to vary with temperature. The calibration curves are illustrated in Figure 4.

In making the light-intensity determinations two light intensity measurements were made; that of the light transmitted through the chloroplast suspension, and the transmitted light with the cell full of water. At the concentrations used the absorption of the ferricyanide was found to be negligible. Due to the fact that the receivers of the thermopile and the thermocouple only intercept a small fraction of the light beam, several

Figure 4. Calibration curves of the thermopile and thermocouple. Instruments were calibrated with a standard lamp calibrated by the National Bureau of Standards.

619 024

corrections were necessary in order that the light intensity readings made with these instruments behind the reaction cell could be converted to the value of the intensity in the reaction cell itself. Three corrections were routinely applied in this study. These were for the light divergence due to the slightly nonparallel nature of the light beam, intensity losses due to the reflection on the cell walls, and scattering losses in the chloroplast suspension.

The correction factor for light divergence was determined by placing the thermopile or the thermocouple in position such that the receivers corresponded to the position of the reaction cell. With the light intensity kept constant the galvanometer deflection was taken in this position and again when the light sensitive instrument was in its normal position. The ratio of the galvanometer deflections represented the divergence factor.

The correction factor for the reflection losses can be determined approximately by the relationship:

$$f_r = \frac{1}{1 - a} \quad (\text{IV-1})$$

where $a = \frac{(\text{Gal. Def. with Water Filled Cell})}{2(\text{Gal. Def. without cell})}$

The reflection factor can also be calculated from the refractive indices of the plastic and water.

Because of the optical planarity of the reaction cell, the scattering correction was determined by a very simple procedure. Galvanometer deflection readings were taken with a photocell under the following conditions:

1. The cell filled with water
2. The cell filled with the chloroplast suspension

3. The above readings repeated with a mask over the face of the photocell in which an aperture of the same size and position of the receivers of the thermopile was cut.

The above procedure is based on the assumption that both the transmitted and the scattered light is picked up by the large surface of the photocell, whereas with the limited area of the aperture the photocell only picks up the light normally received by the thermopile. Calculations then indicated that the scattering factor would be:

$$f_s = \frac{W(w/o)}{W(w)} \cdot \frac{Chl(w)}{Chl(w/o)} \quad (IV-2)$$

where: $W(w)$ = Galvanometer deflection with water in the cell and mask in front of the photocell.

$W(w/o)$ = Deflection with water in the cell and photocell without mask.

$Chl(w)$ = Deflection with chloroplast suspension in the cell and mask in front of photocell.

$Chl(w/o)$ = Deflection with chloroplast suspension in the cell and without the mask in front of the photocell.

Table I gives some representative values of these correction factors as determined for the apparatus and preparations used in this study. The significantly smaller scattering factor for the thermocouple as compared to the thermopile can be attributed to its closer proximity to the reaction cell. The magnitude of the scattering factor was a function of the light wavelength as well as the concentration of the chloroplast fragments. The effects of light wavelength and chloroplast concentration upon the scattering factor are given in Table II. The linearity of the scattering factor with respect to the chloroplast concentration is illustrated in Figure 5.

TABLE I

Representative Values of Correction Factors as
Applied to Light Intensity Measurements

Instrument	Divergence f_D	Reflection f_R	Scattering * f_S
Thermopile	1.06	1.05	1.30
Vacuum Thermocouple	1.09	1.05	1.10

*Chloroplast suspension containing 100 mg. chlorophyll/l. = 675 mp

TABLE II**

Effects of Chloroplast Concentration and Light
Color on the Magnitude of the Scattering Factor

Light Wavelength (mp)	Chloroplast* Concentration	f_S
675	100	1.10
675	200	1.18
675	300	1.28
560	100	1.38
457.5	100	1.15

*mg. chlorophyll/l.

**These values were determined for the thermocouple.

Figure 5. The effect of chloroplast concentration upon the measured scattering factor. This plot was made from the data in Table II to illustrate the linear relationship between the scattering factor and the chloroplast concentration.

Since most of the reflected light from the rear plastic wall would be absorbed by the chloroplast suspension, the reflection correction was only applied to the intensity reading with water in the cell. Obviously, the scattering factor was only applied to the intensity readings made with chloroplasts in the cell.

4. Precision of the Measurements

One of the principal problems related to the measurements associated with this study has been a lack of reproducibility of the rate measurement precision. Wide variations in the precision of measurement has been encountered with different chloroplast preparations even though very little difference existed between the preparations in the activity and the conditions of measurement. Much of this difficulty might be due to differences in stability between chloroplast preparations (see Bishop, et. al., 1955). The precision also varied widely with the external conditions such as pH, temperature, type of buffer, etc. However, considering previous measurements with photosynthesizing systems, and early Hill reaction studies, the precision obtained in this study has been remarkably good. Vary rarely did the standard deviation between duplicate rate measurements exceed four per cent.

The uncertainty of the light intensity measurements considering lamp fluctuations, thermocouple-galvanometer drifts, and changes in density filter calibration was around three per cent. The uncertainty in the absolute accuracy of the light-intensity measurements would probably be ten per cent since the available voltmeter-ammeter setup for calibration with the standard lamp did not give readings in exact agreement with the Bureau of Standards instruction sheet.

V. RESULTS

PART A. CONTROL EXPERIMENTS

Before commencing the kinetic study it was necessary to determine what effect, if any, the conditions imposed by the rate measurement system had upon the rate.

1. Electrode System

The electrode system consisted of a homemade capillary-type calomel electrode and a platinum wire. Although the potential of the calomel electrodes varied somewhat from electrode to electrode this merely shifted the whole potential axis without changing the potential-time relationship. The platinum electrodes, however, had to be chosen with care. It was found that the surface conditions of the electrode did influence the sensitivity of the system to back oxidation by oxygen. Contamination of the platinum surface also had the same effect; whether this effect has any detrimental effects upon the measured rate has not been definitely established. It was found, however, that no significant difference in rate resulted when the air in a gas-tight reaction vessel was replaced with nitrogen even though the nitrogen did lower the degree of back oxidation. This finding is supported by calculations made from the standard electrode potential of the reaction:

In water solution with 0.2 atmosphere of oxygen in the gas phase, calculations

indicated that the equilibrium potential of the above reaction, under conditions approximating those of routine rate determinations, was far below the E_0 of the ferricyanide-ferrocyanide couple. Since the rate was measured about the inflection point of the potential-time curve which corresponds to the E_0 , back oxidation by air should have a negligible effect upon the measured Hill reaction rate.

2. Stirring

It was definitely established that stirring was necessary for accurate rate measurements, especially at low light intensities where the rate was highly dependent upon the light intensity. Under these conditions the rate in the unstirred mixture was as much as fifty per cent slower than the stirred control, possibly because of a lack of equilibrium between the illuminated and shaded portions of the suspension.

3. Chloroplast Concentration

The independence of the absolute reaction rate upon the chloroplast concentration was previously established (Spikes, *et. al.*, 1950). However, this was rechecked and the results confirmed the earlier findings.

4. Ferricyanide Concentration

Since potassium ferricyanide was used as the electron acceptor in this study, it was necessary to determine the effect which the initial ferricyanide concentration had on the photolytic activity of the chloroplast fragments. Although studies conducted in this laboratory by other personnel (Spikes, *et. al.*, personal communication) have established that ferricyanide has an activating effect in the 0.003 M to the 0.008 M concentra-

tion range, the effects in the 0.00001 M to the 0.001 M range have been contradictory. Although most of the data indicate that the rate is independent of the ferricyanide concentration in this region, certain experiments have indicated that an activation by increasing ferricyanide can also occur. Apparently this activation, the so called oxidant effect, occurs as a matter of degree, the magnitude and concentration range depending upon the particular chloroplast preparation. Therefore, most of the experiments reported in this study were performed at a constant ferricyanide concentration. In cases where it was necessary to vary the ferricyanide concentration during an experiment it was first determined whether the rate was independent of the oxidant concentration.

5. Oxygen Concentration

Although the oxygen effect upon the electrode-redox system has been discussed, the possible effect of oxygen upon the photolytic process itself was not studied. It is known from previous studies that oxygen can serve as a Hill reaction electron acceptor (Mehler,^{a,b} 1951; Mehler and Brown, 1952; Brown and Good, 1955; Good and Hill, 1955). However, the control experiment, which was previously mentioned, indicated that under ordinary conditions of measurement the oxygen in concentrations at equilibrium with the air has a negligible competition with ferricyanide as an electron acceptor. It was thus considered unnecessary to make the runs in an inert atmosphere.

PART B. LIGHT INTENSITY DEPENDENCE IN THE HILL REACTION

RESULTS AND DISCUSSION

1. Introduction

Of fundamental importance in the Hill reaction is the relationship between the rate and the light intensity. Any mechanism to be correct must first satisfy the requirements of the rate-light intensity kinetic relationship. Since the Hill reaction probably involves the photolytic, oxygen evolution apparatus of photosynthesis, the light relationship of the Hill reaction will probably represent the true relationship in photosynthesis.

This fact can especially be appreciated when one considers the inherent difficulties involved in the measurement of the rate-light intensity relationship in whole cell systems. The rate measurements are severely restricted in their accuracy by carbon dioxide uptake and respiration superimposed upon the oxygen evolution as well as other transient phenomena such as oxygen bursts, induction phenomena, etc. The effective light intensity to give a certain rate is difficult to determine because of the non-uniformity of illumination brought about by the high optical density of even single algal cells, not to mention the optical density of algal suspensions. The geometrical irregularities of the reaction vessels used for manometric measurements also complicate the light intensity measurements. A detailed discussion of these and other factors is given by Rabinowitch, (1951). Many of the above-mentioned difficulties are non-existent or can easily be eliminated in the Hill system, therefore it is ideal for this type of study.

The relation between photosynthesis and the amount of light available to

the plants was first investigated in 1866 by the Russian botanist Volkov (Wolkoff). He counted the number of oxygen bubbles evolved in a given time from submerged aquatic plants when placed at varying distances from a sun illuminated frosted window. He found that the rate of oxygen evolution was proportional to the light intensity. In 1883, Reinke in Germany extended similar measurements to stronger light intensities and found that as the intensity approached that of full sunlight the oxygen evolution rate increase became smaller and finally disappeared. He thus discovered the phenomenon of light saturation. Since then, light curves have been determined for a large variety of plants under both field and laboratory conditions.

The interpretation of these light curves has engendered some controversy both as to the mechanism of photosynthesis and as to the true mathematical relationship between photosynthetic rate and the light intensity. Blackman in the early years of the twentieth century explained the light curve on the basis of his theory of "limiting factors" which would imply that the light curve actually consisted of two intersecting straight lines; a diagonal line at light intensities below saturation and a horizontal line in the saturating range. Although this interpretation has been generally discredited, there is still no general agreement as to the correct formulation of the light curve in photosynthesis. Several workers have proposed a hyperbolic relationship between the rate and the light intensity, based upon kinetic analysis of postulated mechanisms. Although most of these proposed relationships are non-rectangular hyperbolae (eg. Franck and Herzfeld, 1937, 1941), other schemes resulting in rectangular hyperbolic relationships have been suggested. (Burk and Lineweaver, 1935; Robinowitch, 1951) Non-hyperbolic empirical functions have also been proposed. (Brackett, 1935; Smith, 1936, 1937, 1938).

Since the discovery of the Hill reaction and its subsequent investigation numerous studies on its rate as a function of light intensity have been made. However, in all of these cases the light intensity relationship was merely incidental to the investigation of other factors such as inhibitors, activators, quantum yield, etc. No attempt has been made to determine quantitatively the light dependence in the Hill reaction even though it is a problem of fundamental importance to an understanding of the mechanism of photosynthesis.

2. Effects of Non-uniform Illumination Upon Experimental Light Curves

Preliminary experiments with the Hill reaction gave light curves closely approximating rectangular hyperbolae when the rate was plotted as a function of the incident light intensity. However, since our thinnest reaction cell (1.57 mm) absorbed seventy per cent of the incident light at 675 m μ it was necessary to determine if the rectangular hyperbolic relationship was maintained to infinitely thin suspensions. Since it was experimentally impractical to employ thinner or less dense suspensions than those routinely used, another approach was necessary. This approach consisted of deriving an integral light intensity-velocity expression for the thick reaction cell with the assumption that the differential light curve was a rectangular hyperbolic function. The integral expression could then be compared with a rectangular hyperbola to determine the degree of perturbation by the non-uniform illumination.

If the electrode system records only the average of all the differential velocities then the experimentally determined velocity, $\bar{V}$ can be

expressed in terms of the differential velocities by:

$$\bar{V} = \frac{\int_0^a V_x dx}{\int_0^a dx} \quad (V-1)$$

where: dx is an increment of suspension thickness

a is the thickness of the suspension

V_x is the differential velocity at thickness x

If the velocity of the reaction for a given value of x is a rectangular hyperbolic function of the light intensity at this thickness then:

$$V_x = \frac{k I_x}{K + I_x} \quad (V-2)$$

Assuming the validity of Lamberts law the light intensity at the depth x in the reaction suspension will have the following relationship to the incident intensity, I_0 :

$$I_x = I_0 \exp(-\alpha x) \quad (V-3)$$

where α is the absorption coefficient. By differentiation with respect to x one obtains the following expression for dx .

$$dx = 1/\alpha \ dI_x/I_x \quad (V-4)$$

By substituting expressions 2, 3, and 4, into equation 1 and inte-

grating, the following expression is obtained for the average velocity:

$$\bar{V} = \frac{k}{\alpha a} \log_e \frac{K + I_0}{K + I_0 \exp(-\alpha a)} \quad (V-5)$$

Equation (V-5) is not a rectangular hyperbola for any value of αa greater than zero. However, in order to determine the degree of deviation from a rectangular hyperbola in the suspensions routinely used in this study it can be compared with another expression based upon the average light intensity through the chloroplast suspension rather than the average velocity. The average light intensity can be obtained by evaluating the expression:

$$\bar{I} = \frac{\int_0^a I_x dx}{\int_0^a dx} \quad (V-6)$$

By substituting equation (V-3) into the above expression and integrating the following equation can be obtained:

$$\bar{I} = (I_0 / \alpha a) (1 - \exp(-\alpha a)) \quad (V-7a)$$

$$= \frac{I_0 - I_x}{\alpha a} \quad (V-7b)$$

$$= I_A / \alpha a \quad (V-7c)$$

where I_A is the absorbed light in the chloroplast suspension.

The velocity resulting from the average light intensity will then be expressed by the equation:

$$\bar{V}_I = \frac{k I_0 [1 - \exp(-\alpha a)] / \alpha a}{K + I_0 [1 - \exp(-\alpha a)] / \alpha a} \quad (V-8a)$$

$$= \frac{k (I_A / \alpha a)}{K + (I_A / \alpha a)} \quad (V-8b)$$

Since the quantity $[1 - \exp(-\alpha a)]$ is constant for a suspension of a given optical density $[\alpha a = 2.303 \times (\text{optical density})]$, expression (V-8) will be a rectangular hyperbola for all values of αa . For purposes of comparison, equations (V-5) and (V-8a) are expanded in power series of αa , giving:

$$\bar{V}_V = \frac{k I_0}{K + I_0} \left[1 - \frac{K \alpha a}{2!(K + I_0)} + \frac{K(K - I_0) \alpha a^2}{3!(K + I_0)^2} - \frac{K(K^2 - 4 K I_0 + I_0^2) \alpha a^3}{4!(K + I_0)^3} + \dots \right] \quad (V-9)$$

$$\bar{V}_I = \frac{k I_0}{K + I_0} \left[1 - \frac{K \alpha a}{2!(K + I_0)} + \frac{K(2K - I_0) \alpha a^2}{2.3!(K + I_0)^2} - \frac{K(K^2 - 2K I_0) \alpha a^3}{4!(K + I_0)^3} + \dots \right] \quad (V-10)$$

In comparing equations 9 and 10 it can be seen that appreciable divergence only occurs past the third term of the series expansion. Also the rapidity of convergence in both series is dependent upon the magnitudes of K , I_0 , and αa . From inspection it can be seen that the two series become identical at zero light intensity. In both expressions the rate approaches the value k asymptotically as the light intensity approaches infinity. Of course, both equations give the differential light curve expression when the optical density approaches zero.

By calculating $\bar{V}_V$ and $\bar{V}_I$ for several average light intensities in the experimental range with substituted typical values of k , K , and αa the more exact velocity averaged expression (V-5) can be compared to the light in-

tensity averaged expression (V-8) which is rectangular hyperbolic for all optical densities. The results are shown in Table III.

TABLE III

Numerical Comparison between Velocity Averaged and Intensity Averaged Rate Expressions

Av. Light Intensity (quanta/cm ² - sec)	$\bar{V}_I$	$\bar{V}_V$		
		$\alpha a = 0.1$	$\alpha a = 1.0$	$\alpha a = 1.5$
100.0 x 10 ¹⁴	.992	.992	.992	.990
80.0	.988	.988	.987	.986
50.0	.981	.981	.980	.978
10.0	.910	.910	.905	.897
5.0	.833	.833	.826	.810
3.0	.750	.750	.738	.724
1.0	.500	.500	.491	.480
0.5	.333	.333	.329	.321
K = 1.0 x 10 ¹⁴		V _{max} [*] = k = 1.0		

* In units of Moles Ferricyanide reduced / Mole Chlorophyll - min.

As can be seen by the calculated values, the maximum deviation of $\bar{V}_V$ from $\bar{V}_I$ even at $\alpha a = 1.50$ or O.D. = 0.65 is only 4%, which is about the degree of confidence obtainable for routine experimental rate measurements. We can assume therefore that if equation V-5 represents the Hill reaction light curve at finite optical density of the medium then the experimental velocity-light curve will be experimentally indistinguishable from a rectangular hyperbola even if only 22% of the incident light is transmitted. Conversely, if a rectangular hyperbolic light curve is experimentally obtained at optical densities less than 0.6 then the diff-

erential light curve will also be rectangular hyperbolic.

3. Statistical Study of the Rate-Light Intensity Relationship of the Hill Reaction

Although, as mentioned previously, preliminary experiments indicated that the Hill reaction light curves gave a good fit to a rectangular hyperbola it was felt that a critical statistical study would be desirable for establishing this fact. Initial studies indicated that certain chloroplast preparations, especially those harvested in the late summer or autumn, did not give a rectangular hyperbolic light curve in the very low light intensity region. In these cases the measured rate in this intensity region was somewhat slower than that required for a rectangular hyperbolic relationship. Since a statistical study, to be valid, would have to include rate measurements in this region it was necessary to determine whether these deviations were a kinetic phenomenon or an artifact in the measurements. The possibility of an artifact was large since the measured rates in this low intensity region were several fold slower than the rates in the light saturation region. In this case, a progressive inactivation would become significant during the slow runs, as would be found at the low light intensities. This inactivation would not be apparent in the per cent ferricyanide reduced versus time plot, since it was found that linear plots were obtained even though as much as a twenty per cent loss in activity occurred during the run. This apparent anomaly resulted from the manner in which the potential-time curve is affected by a progressive slowing of rate. The inflection point is shifted along the potential-time curve to a lower potential and a larger time interval. If this inflection

point is employed as the E_0 , the curve will be symmetrical about this point, therefore giving a linear relationship between the % ferricyanide reduced and the time interval. Thus, no progressive inactivation will be apparent even though the measured rate will be slowed. Since the zero setting was frequently shifted in the recorder during switching, the apparent E_0 shift during progressive inactivation would not be noticeable.

To test this possibility several approaches were tried. The first approach was an attempt to obtain the true % ferricyanide reduced-time plot by employing a constant E_0 value instead of the inflection points of the potential-time curve. The initial slope of these plots would then represent the true non-inactivated rate. In several cases when no zero shift occurred during the time necessary to complete enough runs for a light curve, this method did give rectangular-hyperbolic light curves even at low light intensities. However, due to the uncertainty of the zero and the inaccuracies involved in obtaining the initial slope of a curved ferricyanide reduced-time plot, this method was not suitable for use in a statistical study.

The second procedure involved the measurement of the inactivation curve by waiting varying lengths of time after placing the reaction mixture in the reaction cell before turning on the light. In this case a progressive inactivation of about fifteen per cent was observed during the first ten minutes after thawing of the chloroplast preparation. This inactivation was sufficient to account for the slowing of the rates at low light intensities. Although this experiment further verified the cause of the low intensity deviation, its application as a method for correcting for this inactivation was limited, since different samples of even the same chloroplast preparation

varied in their sensitivity to inactivation.

A more desirable procedure would be to eliminate this inactivation. It was discovered that the low intensity deviation could be lessened or even eliminated by lowering the temperature at which the runs were made. This deviation could also be eliminated by starting the run in the linear inflection portion of the potential-time curve thus establishing the rate measurement before inactivation occurred. These procedures were used in making the statistical study.

In obtaining the data for the statistical study it was necessary to select the chloroplast preparation with care. In order to obtain measurable rates at very low light intensities and low temperatures a very active preparation was required. The preparation must also possess a good degree of stability in order to avoid inactivation artifacts. After testing several preparations for the above qualifications, a suitable preparation was obtained from greenhouse grown, spring, rhubarb chard leaves. In order to insure uniformity between duplicate light curve determinations, aliquots were taken from the stock chloroplast preparation, immediately frozen, then stored at -35°C . until used. Each aliquot contained enough suspension for a light curve determination. The individual run samples were prepared and used as previously described.

The light intensity was monitored by taking light-intensity readings every few runs. Any drift in light intensity output of the lamp was compensated by adjusting the voltage to the lamp. The light intensity was attenuated by calibrated neutral-density, gelatin filters as previously described. A check of the transmission values in the apparatus agreed within one per cent of those determined in the Beckmann Model DU spectrophotometer.

All of the runs were completed within four days, during which time no noticeable change in the chloroplast stored activity occurred. The runs were made at a temperature of 2.5°C. to further insure a minimum of inactivation effects. The initial ferricyanide concentration was 0.00025 M. The rate values as a function of light intensity are given in Table IV.

TABLE IV

Rate Values of The Hill Reaction as a Function of the Light Intensity

No.	Average Light Intensity*	Velocity** At % of Average Light Intensity							
		100	56.5	25.6	10.0	7.7	4.75	2.56	1.20
1.	9.2 x 10 ³	.435	.418	.380	.283	.258	.188	.126	.070
2.	"	.438	.424	.377	.296	.240	.188	.122	.070
3.	"	.438	.424	.367	.283	.249	.186	.124	.0706
4.	"	.438	.428	.377	.282	.263	.195	.128	.0718
5.	"	.438	.415	.374	.270	.246	.191	.120	.0707
6.	"	.449	.432	.367	.283	.250	.185	.129	.0725
7.	"	.438	.414	.371	.270	.249	.191	.129	.0706
8.	"	.452	.415	.377	.274	.266	.189	.126	.0680
Mean		.441	.421	.374	.280	.253	.189	.126	.0705
Standard Deviation		.0062	.0064	.0049	.0086	.0089	.0032	.0033	.0013
% of Mean		1.40	1.52	1.32	3.08	3.51	1.68	2.64	1.90

* Average light intensity in suspension in ergs/cm²-sec.
= 675 mu

** Velocity in units of moles ferricyanide reduced
mole chlorophyll-min.

In order to test the above data as to its goodness of fit to a rectangular hyperbola the general expression for a rectangular hyperbola, as given in equation V-2, is algebraically transformed into the linear form:

$$I/V = (1/k)I + K/k \quad (V-11)$$

where I/V is the dependent variable and the light intensity I is the independent variable. A good fit would be indicated if the velocities, as calculated from the best statistical values of the slope $1/k$ and the intercept K/k , are within the standard deviation from the mean of the experimental rates.

To determine the best statistical values of the slope and the intercept of equation V-11 the method of least squares was employed. In choosing the least squares equation most applicable to the data in this study two factors were to be considered.

Since the simple least-squares equation is derived upon the assumption that only the dependent variable has an error in measurement, the use of this form would imply that the light intensity measurements were error free. In this study the standard deviation of nine light intensity measurements distributed throughout the time of the experiment was 0.4 per cent of the mean intensity. The relationship of the deviations in the light intensity measurements to the deviations in the light intensity reaching the reaction mixture is of course unknown, since it was not possible to differentiate by light intensity measurements between light intensity fluctuations and errors involved in the measuring apparatus. Any fluctuation in the intensity would be expressed as a corresponding deviation in the rate. Therefore, a significant fluctuation in the light intensity would tend to increase the deviation of the measured rates as the intensity is lowered and the rate becomes more sensitive to light intensity changes. Since, as can be seen in Table IV, the standard deviation shows no apparent percentage trend even at very low

light intensities, it was assumed that no significant deviations occurred in the light intensity during the time of the experiment.

The second factor to be considered is the weighting of the dependent variable residuals. The common least squares formula was derived with the assumption of a fixed error in the dependent variable. However, referring again to Table IV it is apparent that the absolute error in the velocities and therefore the I/V values were proportional to the magnitude of the velocity or the value of I/V. Therefore, it was necessary to derive a least squares expression which included the proper weighting.

The unweighted residual of a function of the form $y = ax + b$ is expressed by:

$$R = y_i - (ax_i + b) \quad (V-12)$$

This equation assumes that the standard deviation of y_i is constant or $\sigma_{y_i} = \text{constant}$. With equation V-11 however, the standard deviation of y_i the dependent variable is proportional to the value of y_i . For this case the residual R can be expressed by:

$$R = 1 - \frac{(ax_i + b)}{y_i} \quad (V-13)$$

By employing the usual procedure of differentiating $\sum R^2$ with respect to a and b, equating to zero, then solving for a and b the following expressions were obtained for a and b:

$$a = \frac{\sum (x_i/y_i) \sum (1/y_i)^2 - \sum (x_i/y_i^2) \sum (1/y_i)}{\sum (x_i/y_i)^2 \sum (1/y_i)^2 - [\sum (x_i/y_i^2)]^2} \quad (V-14)$$

$$b = \frac{\sum (x_i/y_i)^2 \sum (1/y_i) - \sum (x_i/y_i) \sum (x_i/y_i^2)}{\sum (x_i/y_i)^2 \sum (1/y_i)^2 - [\sum (x_i/y_i^2)]^2} \quad (V-15)$$

By making the substitutions $y_i = 1/V$, $x_i = I$, $a = \text{slope} = 1/k$ and $b = \text{intercept} = K/k$ the above equations become the least squares equations used in this experiment.

$$1/k = \frac{\sum V \sum (V/I)^2 - \sum (V^2/I) \sum (V/I)}{\sum V^2 \sum (V/I)^2 - [\sum (V^2/I)]^2} \quad (V-16)$$

$$K/k = \frac{\sum V^2 \sum (V/I) - \sum V \sum (V^2/I)}{\sum V^2 \sum (V/I)^2 - [\sum (V^2/I)]^2} \quad (V-17)$$

The slopes and intercepts for equation V-11 as calculated from the Data in Table IV by use of the above equations are given in Table V.

The light intensities used in these calculations are expressed as the relative values which are identical to the percentage of the maximum intensity value. The intercept as calculated with these units can be converted to absolute units merely by multiplying by the appropriate factor. For purposes of this experiment it was not necessary to employ absolute light intensity units.

The Hill reaction rates as calculated with the above values of slope and intercept substituted into equation V-11 are compared to the experimental rates in Table VI. As can readily be seen, the deviation of the mean rate from the calculated rates, based upon a rectangular hyperbolic relationship, are well within the standard deviation of the experimental rates.

TABLE V

Slopes and Intercepts Calculated from Data in Table IV Applied to Equation V-11* by the Method of Least Squares

Curve No.	Slope (1/k)	Intercept (K/k)
1	2.108	14.640
2	2.090	14.990
3	2.120	14.790
4	2.100	14.219
5	2.137	14.938
6	2.105	14.549
7	2.155	14.489
8	2.078	14.902
Mean	2.112	14.690
Standard Dev.	0.0239	0.265
% of Mean	1.13	1.80
n = 64	2.111	14.680

* $I/V = I/k + K/k$

TABLE VI

Comparison of Calculated and Experimental Hill Reaction Rates as a Function of the Light Intensity

	Per Cent or Relative Light Intensity							
	100	56.5	25.6	10.0	7.7	4.75	2.56	1.20
Calculated V*	.443	.422	.373	.279	.249	.192	.127	.0697
Mean experimental V	.441	.421	.374	.280	.253	.189	.126	.0705
Deviation of mean V from Calculated V	.002	.001	.001	.001	.004	.003	.001	.0008
Standard dev. of exp. V	.006	.006	.005	.009	.009	.003	.003	.0013

* V calculated from $V = \frac{0.4737 I}{6.954 - I}$

619 047

Since the data in Table IV were obtained in a cell of finite depth ($\alpha a = 1.385$) it was necessary to determine the effect of non-uniform illumination upon the data. Although the previous discussion indicated that in the analysis of routine light curves the effect of non-uniform illumination in suspensions of moderate absorbency was comparatively minor, it was necessary to consider this problem in relation to a statistical study. In order to test the experimental validity of equation V-5 it was necessary to convert the incident light intensity I_0 to a factor involving the absorbed light intensity I_A after which equation V-5 is expressed as:

$$V_v = \frac{k}{\alpha a} \log \frac{K(\exp \alpha a - 1) + I_A \exp \alpha a}{K(\exp \alpha a - 1) + I_A} \quad (V-18)$$

If the above equation is valid in the Hill reaction, it should give a better fit to the data than equation V-8b at all but vanishingly small absorbencies. Since equation V-18 was not readily applicable to least squares treatment a trial and error method was employed to determine the value of K giving the best fit to the data in Table IV. The relative intensity values in Table IV were converted to absorbed energy, I_A , expressed in ergs/cm²-sec. Equations V-8b and V-18 were employed, with the best respective values of K to calculate V_v and V_I at the various I_A values. These calculated rates were then compared to the experimental rates. This procedure was employed with the suspension used for the statistical study as well as a suspension of almost twice the absorbency. The data are summarized in Table VII.

It is readily apparent that the intensity-averaged rate expression gives a much better fit to the data than the velocity averaged rate equation.

TABLE VII

Comparison of V_v and V_I to the experimental Rates at Different Light Intensities and at Two Different Suspension Thicknesses

$\alpha a = 1.385$				$\alpha a = 2.54$			
$I_A \times 10^{-3}$	V_v^*	V_I^*	V_{exp}	$I_A \times 10^{-3}$	V_v	V_I	V_{exp}
12.97	.442	.443	.441	15.80	.422	.430	.430
7.33	.420	.422	.421	8.94	.385	.401	.411
3.32	.370	.373	.374	4.05	.316	.338	.339
1.297	.278	.279	.280	1.58	.217	.234	.238
0.998	.249	.249	.253	1.215	.189	.203	.206
0.616	.195	.192	.189	0.750	.142	.149	.147
0.332	.132	.127	.126				
0.156	.0735	.0697	.0705				

* I_A = Absorbed light in ergs/cm²-sec.

K for V_v = 590

K for V_I = 640

This is even more apparent in the highly absorbent chloroplast suspension. Two possibilities exist as to the cause of this apparent discrepancy; the failure to obey the Beer-Lambert law or the presence of a time lag between light exposure and the oxidant reduction. Either one of these factors could invalidate equation V-18, even if the differential light curve were a rectangular hyperbola.

It was experimentally found that the αa versus chloroplast concentration plot was linear, hence obeying the Beer-Lambert law, to as large a values of αa as 2.4 which corresponds to more than ninety per cent light absorption. At higher values of αa , however, the curve deviated from linearity, due probably to the large spectral band width of the light

source as compared to the spectral width of the chloroplast red absorption peak. However, this deviation was not sufficient in the region of measurement to appreciably change the rates calculated by use of equations V-8b and V-18. It was thus felt that this was not the cause of the non-validity of equation V-18.

If an appreciable time lag occurred between the exposure to light of a given intensity and the reduction of oxidant corresponding to this intensity, the velocity averaging method would only be valid if every chloroplast fragment remained in a fixed position. With stirring, however, each fragment passes through the full range of light intensities in a small fraction of a second. If the time required to traverse the range of light intensities is short compared to the time for the Hill reaction to go to completion, then the measured rate would be a function of the average light intensity in the suspension. (ref. Rabinowitch, II,1 page 1011) This theory is supported by several pieces of evidence.

It was observed that a lag of two or three seconds occurred between the time the chloroplasts were illuminated and the ferricyanide reduction was measureable by the potentiometric technique. Flashing light experiments with the Hill Reaction performed in this laboratory indicated that dark times in excess of 0.16 seconds at 15.6°C. were required for the yield per flash to reach a maximum. Limitations of the apparatus and the technique prevented the determination of the dark time required for the maximum yield per flash, however, the maximum was only slowly being approached at a dark time of 0.16 seconds. (Gilmour, 1953) At 2.5°C., the temperature of measurement in this study, the time would probably be doubled which would give a conservative estimate of 0.5 seconds for the Hill reaction to go to

completion. Recent flashing light experiments with Chlorella have indicated that times in the order of seconds are required between illumination and the completion of oxygen evolution. (Kok, 1955)

Since equation V-8b, which is a rectangular hyperbola at all absorbency values, gives a good fit to the data over a wide range of absorbencies it was assumed that the differential light curve of the Hill reaction offers as good a fit to a rectangular hyperbola as can be experimentally obtained. This fit of the experimental data to a rectangular hyperbola is illustrated in Figure 6 in which I/V is plotted against I for the mean velocities given in Table IV.

It must be emphasized, however, that the rectangular hyperbola need not be a unique solution to the light curve data of the Hill reaction. By a proper choice of arbitrary constants, in both number and magnitude, any number of equations may give a good fit to the data in the experimental range of light intensities. Of course, any correct choice among the possible equations will have to satisfy the requirements of a plausible kinetic mechanism.

619 051

Figure 6. Rectangular hyperbolic nature of the Hill reaction light curve. The curves were plotted from the averaged data in Table IV. The reaction temperature was 2.5°C. The initial ferricyanide concentration was 0.00025 M. The rates are expressed as moles ferricyanide reduced/mole chlorophyll-min.

PART C. THE EFFECT OF TEMPERATURE UPON THE HILL REACTION
LIGHT CURVE. FORMULATION OF THE FUNDAMENTAL RATE
EQUATION FOR THE HILL REACTION.

An inspection of the Hill reaction light curve indicates that two fundamental processes are occurring, a photo process which is rate limiting at low light intensities and a non-photo process which becomes rate limiting at high light intensities, the so called light-saturation region. Since photo processes are in general temperature independent, it was felt that a study of the temperature effect upon the light curve would be valuable in elucidating the kinetic nature of the constants k and K in equation V-8b.

The procedure in this experiment employs the techniques previously described. The chloroplast suspension was prepared from outdoor-grown rhubarb chard by the usual procedure. Runs were made as a function of the light intensity at 6.3° , 11.0° , 16.0° and 21°C . The Lineweaver-Burk type plots of this data are illustrated in Figure 7.

It is readily apparent that the slopes, which represent the values of $1/k$ are highly temperature dependent whereas the intercepts which represent the value of K/k are independent of the temperature. In order to satisfy this relationship, K must be a composite function of a temperature independent parameter and the constant k , in which case the k is able to cancel out the k in the denominator of the intercept. Thus, the intercept could be represented by:

$$\text{intercept} = \left[k \cdot F(k_L) \right] / k = K/k \quad (\text{V-19})$$

where k_L represents a temperature independent photolytic parameter. Be-

Figure 7. Lineweaver-Burk plots of the Hill reaction rate-light intensity data for different temperatures. This plot was made to illustrate the contrasting temperature effects upon the slope and intercept of this plot. The initial ferricyanide concentration was 0.00025 M.

cause an increase in the intercept could only represent a decreased quantum efficiency at vanishing light intensities,¹ the photolytic parameter function in equation V-19 must involve a reciprocal, viz.

$F(k_L) = 1/k_L$. It can also readily be shown that the constant k is a measure of the Hill reaction rate at infinite light intensity and therefore is independent of light. This fact, together with its temperature dependence allows one to assign a dark, enzymic process to this constant, therefore, for convenience it is represented by the notation k_D . Thus, the nature of the constants k and K can be assigned as follows:

$$\begin{aligned} K &= k_D/k_L \\ k &= k_D \end{aligned} \quad (V-20)$$

in which case the fundamental expression for the Hill reaction is:

$$V = \frac{k_D I}{k_D/k_L + I} \quad (V-21)$$

where k_D is the rate parameter of the dark, enzymic processes and k_L is the rate parameter of the photolytic processes. These parameters are probably not true rate constants but are combinations of rate constants and concentration factors. The remaining parts of this thesis are devoted to the study of how various conditions and factors affect these parameters. By this approach it was hoped that further information about the mechanism relationships involved in these parameters might be obtained.

¹The intercept is expressed in the same units as I/V .

The values of k_D and k_L as calculated by least squares (Equations V-16 and V-17) from the rate data plotted in Figure 7 are given in Table VIII. It is to be noted that with the linear form of equation V-21 the reciprocal slopes of the I/V versus I plots as given in Figure 7 give the values of k_D whereas the reciprocal intercepts give the values of k_L .

TABLE VIII

The Effect of Temperature upon the Hill
Reaction Rate Parameters k_D and k_L

temperature °C.	k_D	$k_L \times 10^{14}$
6.3	0.531	0.228
11.0	0.754	0.209
16.0	1.11	0.212
21.0	1.67	0.210

An Arrhenius plot of the k_D values given in Table VIII is shown in Figure 8. The Arrhenius plot gives a linear plot and yields an apparent activation energy of the rate limiting step in the dark process of 11.8 ± 1 k cal/mole. A more thorough study of the effect of temperature on the Hill reaction rate at saturating light intensities has been made by Bishop. (Bishop, et. al., 1955) Under these conditions the rate was an approximation of k_p . Over a wide range of preparations and reaction conditions he obtained an activation energy of about 10 kcal. Other workers have obtained values ranging from 7.5 to 21 kcal.

Figure 8. Arrhenius plot of the limiting dark parameter, k_D , as calculated by the method of least squares from the data plotted in Figure 7. and given in Table VIII. The apparent activation energy, μ , as determined from this plot is 12.5 ± 1.0 kcal/mole.

It is apparent from the wide variations in the experimental activation energies that either a large variation in the catalytic efficiency of the dark enzyme system exists or that some essential variable or variables are yet uncontrolled. The variation of the catalytic efficiency could easily be due to shifts in rate limiting steps, due to active enzyme concentration changes brought about by a variety of factors.

PART D. THE EFFECT OF LIGHT WAVELENGTH UPON THE HILL

REACTION RATE PARAMETERS k_D AND k_L .

RESULTS AND DISCUSSION

The analysis of the Hill reaction rate parameters lends itself very effectively to the determination of the relative efficiency of different light wavelengths in the Hill reaction. Since k_L is expressed in units of V/I_{ave} where I_{ave} is in intensity units, k_L will be dependent only upon light intensity, not upon light absorbed. Therefore, any non-productive light absorber whether a natural pigment or an inert substance will merely act to decrease the light intensity, thus behaving like a neutral density filter. Thus, the value of k_L is a measure of the efficiency of the light utilization system which actually participates in the Hill reaction. The ideal action spectrum measurements could be made by determining k_L as a function of wavelength since k_L represents the maximum yield for a given light intensity that can be obtained.

To test the effect of wavelength upon k_D and k_L , light curves were made at three representative wavelengths, these being at 675 mμ, 560 mμ, and at 457.5 mμ. These wavelengths correspond to the maximum absorption peak of the chloroplasts in the red, the minimum absorption region in the green, and close to the maximum peak in the blue, respectively. The transmission bands of the interference filters used in this experiment as compared to the chloroplast transmission curve are illustrated in Figure 9. The light curves and the Lineweaver-Burk plots of the rates for the three light wavelengths are given in Figures 10 and 11, respectively. The parameters k_D and k_L as calculated by the least squares equations V-16

Figure 9. Transmission characteristics of chloroplast fragments and interference filters.

-----Chloroplast fragment suspension; 100 mgm. chlorophyll/l; suspension 1.57 mm thick.

A. & C. Baird Interference filter No.7-2780A

B. Baird Multilayer Interference filter No.7-5041-1

Figure 10. Light curves of the Hill reaction as a function of wavelength. The light color was obtained by the use of the interference filters, the transmission spectra of which were illustrated in Figure 9.

Figure 11. Lineweaver-Burk plots of the same data as plotted in Figure 10. The temperature was 6.3°C. The initial ferricyanide concentration was 0.00025 M.

and V-17 are given in Table IX. Also given are the "natural absorbencies", αa , and the calculated quantum yields for the respective wavelengths. The quantum yield values represent values extrapolated to zero light intensity.

TABLE IX

The Effects of the Light Wavelength upon the Hill Reaction
Parameters k_D and k_L .

Wavelength (mu)	k_D	$k_L \times 10^{14}$	αa	$(0.436 \times 10^{14})(k_L / \alpha a)$ (quantum yield)*
675	0.838	0.287	1.35	0.093
560	0.831	0.037	0.223	0.072
457.5	0.852	0.300	1.85	0.071

*molecules O_2 produced/quanta absorbed

$$\alpha a = \log_e (I_0/I)$$

Because of the large magnitude of scattering of the green light by the chloroplast fragment suspension, the scattering factor for this color was determined by the method of Haxo and Blinks (1950). This method essentially consisted of placing the chloroplast suspension against the face of a photronic cell which was in turn covered with a magnesia-coated hemisphere. A small slit in the hemisphere admitted the light to the suspension. Since any scattered or reflected light from the suspension would be reflected back to the photocell surface, the ratio of the photocell output with water in the cell to the output with the chloroplast suspension will represent the ratio of the incident intensity to the

transmitted intensity. Comparison of this ratio with that obtained with the thermocouple in the normal setup, allows a very simple calculation of the scattering factor. The value of the factor as determined by this method was 1.48 for green light as compared to 1.38 as obtained by the method previously described in this thesis. The difference in values probably represent light lost by lateral scattering and reflection from the chloroplast fragments, which the previous method could not detect. This method was not used for the red and blue wavelengths because of the large sensitivity changes of the photocell in these regions and also because of the relatively small degree of scattering found for these wavelengths.

As can be seen from the above table the value of k_D was, as expected, independent of the light color. The value of k_L , however, was highly wavelength dependent. The utilization of the incident light in the red and blue regions of the spectrum was seven fold more efficient than in the green. At the wavelengths used, the red and the blue are equally efficient although it is probable that at 440 mμ, the maximum absorption peak, the blue would be more efficient than the red.

As was mentioned earlier, the parameter k_L represents the maximum efficiency of the utilization of the incident light. It would be useful to compare this with the maximum efficiency of utilization of the absorbed light at different wavelengths. This can be done by dividing the k_L values by $\alpha a = \log_e(I_0/I)$ at the respective wavelengths. It is noted that these values are proportional to the quantum yields as given in Table IX. The quantum yield values indicate that the utilization of the absorbed light is less efficient in the blue and green regions of the spectrum than in the red absorption peak, being only 75 per cent of that value. Comparison of the

quantum yields with the k_L values indicate that although the k_L values in the red and blue are essentially equal, the quantum efficiency in the blue is lower. It is apparent that some pigment, either not as efficient as chlorophyll in photosynthesis or totally inactive, is absorbing light in this region. At 560 m μ , the small value of k_L can be mostly attributed to the lowered absorption. Again, as in the blue, the lowered quantum efficiency possibly indicates the presence of accessory pigment absorption.

The results obtained in this experiment, in general, agree with the analogous results obtained by others with the Hill reaction and photosynthesis. It must be emphasized, however, that no absolute standard of comparison exists, since the wavelength dependence of the quantum yield as well as the action spectra are somewhat dependent upon plant species.

Chen (1952) found a very close correspondence between the action spectrum of the Hill reaction and the light absorption in both the red and the green regions of the spectrum. This would indicate an equality of the quantum efficiencies in the red and the green spectral regions. The lack of lowered quantum efficiency in the green can, however, be readily explained by an examination of the techniques used by Chen and the nature of the Hill reaction light curves. He used as his photochemical effectiveness values, the rate-incident light intensity ratios at a fixed light intensity in the light limiting region of the light curve. Because of the difference in absorption at different wave lengths for a given incident intensity, the relationship between the absorption spectrum and the action spectrum will depend somewhat upon the choice of intensity. In Figure 11, for example, the green-red ratio of quantum yield can be varied between 0.775 and 1.15 merely by varying the average incident light intensity from zero to 2×10^{14} quanta/cm²-sec, an intensity well below the saturating value. It is thus evident that a

true action spectrum can only be obtained by an extrapolation to zero light intensity, especially for suspensions of appreciable light absorption.

Warburg (1952) studied the effect of light wavelength upon the quantum efficiency of isolated spinach grana suspended in a quinone solution. He found that the quantum requirement per molecule of oxygen produced varied from 65 at 366 mμ to 100 at 644 mμ. His results were noteworthy in the very large quantum requirement (small quantum yield) compared to photosynthesis and the high relative yield with wavelengths as short as 366 mμ. With the low activity and the wavelengths employed it is difficult to compare Warburg's data with the data obtained in this study.

Another study of the Hill reaction as a function of wavelength was made in this laboratory by Wayrynen (1952). His data indicated a maximum in the quantum yield at 675 mμ, a prominent minimum at about 580 mμ, and another maximum of lower yield than in the red between 450 and 500 mμ. However, his data were not corrected for the greater scattering in the green, so the quantum yield values in this region are probably low. With this correction, his data would in general agree with the results reported in this thesis.

With photosynthesis in Chlorella, Emerson and Lewis (1943) noted only a slight drop in the quantum efficiency from 680 mμ to 560 mμ. Tanada (1951) found no decrease in quantum efficiency of the brown algae Navicula minima from 680 mμ to 530 mμ. However, the action spectra and the quantum yield data on several species of multicellular algae as determined by Haxo and Blinks (1950), and Yocum and Blinks (1954), indicated a significant drop in the quantum efficiencies at 560 mμ. It is apparent that the differences between plant species as to the wavelength dependence of photosynthesis may be attributed to differences in activity and distribution of accessory pigments.

PART E. THE EFFECT OF pH ON THE HILL REACTION

RESULTS AND DISCUSSION

1. Introduction

One of the features of the Hill reaction which is different than in vivo photosynthesis is a marked sensitivity to pH. This is probably due to a lack in the Hill reaction system of the cellular pH regulatory mechanisms normally found in intact biological systems. In this respect, the chloroplast fragment suspension is analogous to an in vitro enzyme solution. Because of the involvement of hydrogen ions as a product in the Hill reaction as well as the large dependency of activity upon pH, it was felt that a preliminary study of the effect of pH upon the rate parameters k_D and k_L was not only justified, but essential.

R. Hill (1940) in his original experiments found that the reaction was highly pH dependent, the optimum activity being at pH 8.0. Subsequent experiments, by other investigators indicated that the pH dependence was a highly variable property, apparently dependent upon such diverse factors as chloroplast origin and preparation, type of oxidant, method of measurement, and ionic composition.

Holt and French (1946) studied the effects of the method of measurement and the temperature upon the pH dependence. They found a pH optimum of 7.0 at 15°C. They also measured the pH dependence with chromate as an oxidant and found the optimum pH to be 7.3. Spikes, et. al. (1950) with the potentiometric technique of rate measurement and ferricyanide as the oxidant found a pH optimum of 6.85 at 4°C. It must be pointed out that although the

above mentioned experiments were all performed with spinach, different preparations were involved for each experiment. Therefore, it is difficult to determine whether the pH optima differences were due to differences in preparative techniques or to measurement techniques.

Gorham and Clendenning (1952) experimenting with chloroplast preparations washed free of chloride ions found that the addition of chloride ions shifted the pH optimum of the Hill reactions from 6.5 to 7.1 with ferricyanide-ferric oxalate as the oxidant and from 6.5 to 7.5 with quinone as the oxidant. The pH shift resembled an extension of the acidic segment of the pH-activity curve to higher pH's and activities before being inhibited or inactivated in the alkaline pH region.

2. pH Dependence of k_D and k_L .

The above mentioned experiments were all performed at presumably saturating light intensities, therefore, the rates would be an approximate measure of k_D only. The experiments as described in this thesis consist of measurements of both k_D and k_L as a function of pH. Therefore, the Hill reaction rate was measured as a function of both light intensity and pH. Summer rhubarb chard was employed as the source of chloroplasts. The pH was fixed by employing 0.1 M phosphate buffer. No change in pH could be detected during the time of the run. The data for a typical pH relationship for k_D and k_L are given in Table X and plotted graphically in Figure 12.

As can be seen from Figure 12, both the k_D and k_L parameters possess separate and distinct pH optima, the optimum for k_D being at pH 7.2 and that for k_L being at pH 6.3. Of all the chloroplast preparations tested

Figure 12. pH Dependence of the k_D and k_L rate parameters of the Hill Reaction as plotted from the data given in Table X, Rhubarb chard. Phosphate buffer, 0.1 M. Initial ferricyanide concentration, 0.0003 M.

TABLE X

The Effect of pH upon the Hill Reaction
Rate Parameters k_D and k_L at 10°C.

pH	k_D	$k_L \times 10^{14}$
5.70	0.21	0.20
5.90	0.26	0.34
6.20	0.42	0.50
6.45	0.52	0.50
6.70	0.61	0.40
6.97	0.74	0.29
7.22	0.79	0.20
7.40	0.73	0.154
7.67	0.57	—
7.75	0.55	0.046
7.95	0.44	—

the general relationship between the k_D and k_L optima was preserved, although variations existed in the shape of the pH curves. (Refer to Figures 13 and 17)

3. The Effects of Temperature upon the Hill Reaction pH Dependence

The effect of temperature upon the Hill reaction pH curve was checked with two purposes in mind, to determine the effect of temperature upon the pH optimum and also to determine the effect of pH on the temperature coefficient. For this experiment, rhubarb chard chloroplast fragments prepared by the usual method were used. The values of k_D and k_L were determined as a function of pH for the temperatures 2.5°C. and 11.5°C. It was not possible to obtain data for more than the two temperatures because of a change in the chloroplast activity before the completion of the pH curve at the third temperature. The values of k_D and k_L as

obtained in this experiment are plotted graphically in Figure 13.

It is noted that the k_D -pH optimum as well as the k_L -pH curve are shifted approximately 0.1 pH units in the acid direction by the 9° rise in temperature. This shift approximately corresponded to the shift of the phosphate buffer pK for the same temperature change.

The relationship between the pH and the apparent activation energy of k_D was calculated from the data plotted in Figure 13. For comparison of the k_D values at the two temperatures, it was decided that the k_D values of equal reference to their respective pH optima would most represent identical ionic states of the enzyme systems involved. In this case, this would amount to comparing k_D values obtained with the same buffer mixture at the two temperatures. In Figure 14, the apparent activation energy as calculated from the k_D values at 2.5°C. and 11.5°C. is plotted as a function of the pH at 11.5°C. As can be seen, there is an apparent effect of pH upon the activation energy. It is possible, however, that this effect is more apparent than real because of the possible errors in the determination of the k_D values together with the relatively small changes involved in the activation energy. However, the k_D values used in the activation energy determinations probably represent the best determinations and although the activation energy change is small, its trend with pH appears to be too ordered to be caused by random errors. It is also noteworthy, that the pH giving the minimum apparent activation energy corresponds closely to that for a maximum value of k_D . On the basis of these facts one would hesitate to ignore the possibility of this effect being real without a more extensive investigation.

In examining the pH data presented here as well as that found in the

Figure 13. The effect of temperature upon the k_D and k_L pH curves of the Hill Reaction. Rhubarb chard. Phosphate buffer, 0.1 M. Initial ferri-cyanide concentration, 0.00025 M.

○ k_D , 2.5°C; ● k_D , 11.5°C; + k_L , 2.5°C; × k_L , 11.5°C.

Figure 14. The effect of pH upon the apparent activation energy of k_D in the Hill reaction. The apparent activation energy values were calculated from Figure 13 by application of the Arrhenius equation. The k_D values at the two temperatures were compared with the pH optimum at the respective temperature as the reference point. The pH axis is based upon the pH values at 11.5°C. The half closed circles are values calculated from the smoothed curves given in Figure 13.

literature, it is evident that the effect of pH upon the Hill reaction is very complex. Although this complexity prohibits any quantitative interpretation of the pH dependence, much useful information can be gained from a qualitative standpoint.

The wide variations of pH optima noted in the literature can probably be attributed to the inherent differences between chloroplast preparations, as well as stability variations due to oxidant and temperature differences. This stability loss especially seems to be the case with the low ionic strength preparations of Gorham and Glendenning (1952). Their findings that the pH optimum was shifted to higher pH's by the additions of chloride can be readily explained by the stabilizing effect of chloride ions to inactivation, especially at neutral or alkaline pH's. Because of the inherent stability of the chloroplast preparations used in pH experiments reported here and the precautions taken to avoid inactivation errors, it was felt that the true, reversible, pH effects data are represented in this thesis.

The pH data for k_D and k_L as plotted in Figures 12 and 13 indicates that much of the pH dependence of these parameters could be explained by changes in the ionic nature of the enzymes and proteins intimately associated with the photolytic and dark processes in the Hill reaction. Evidence for this argument is found in the pH shift of both the k_D and k_L pH curves with temperature. The shift has the magnitude and direction one would expect on the basis of the temperature coefficient of the dissociation of protein acidic groups. The general shape of the pH curves are also characteristic of enzyme system pH-activity curves. If this is the case, the variation in the shape of the pH curves could be attributed to a variation in the relative concentrations of the various enzymes and proteins concerned with the

Hill reaction activity.

Of possible significance from a kinetic sense, are the distinct pH curves of the Hill reaction parameters k_D and k_L . If these curves represent composite enzyme activity curves, then a possibility exists that enzymic processes are involved in the photolytic process as well as the dark process.

The significance of the change of activation energy of k_D with pH will of course have to wait for further verification. However, it is possible to explain an apparent change in the activation energy on a basis of enzyme concentration changes, since k_D is not a pure rate constant but a composite function of rate constants and concentration terms.

4. The Effect of High pH's on the Light Curve

A Possible Light Inhibition.

It was often noted that with pH's above 7.6 the light curves no longer displayed a simple rectangular hyperbolic relationship, but instead demonstrated an apparent light inhibition. The Lineweaver-Burk plots of two typical curves of this type are illustrated in Figure 15. This phenomenon was observed for Swiss Chard, rhubarb chard, and sugar beet preparations, although the pH at which it occurred varied somewhat from preparation to preparation. More detailed studies of this phenomenon have to date been impractical because of the inherent difficulties in obtaining precise data at the high pH's required.

The possibility that a true light inhibition exists is supported kinetically by a simple analysis analogous to classical enzyme inhibition. A simple scheme which kinetically would give the observed results can be

Figure 15. Lineweaver-Burk plots of two representative Hill reaction light curves illustrating the deviation from a rectangular hyperbola found at high pH's. Swiss chard. Phosphate buffer, Temperature, 10°C. Initial ferricyanide conc., 0.0003 M.

briefly outlined as follows: The high pH alters the photosensitizing pigment system such that a "short circuit" exists between absorption sites, either in the same molecule or adjacent molecules. If photoactivation first occurs at the non-productive site then the productive absorption site is no longer photochemically active. This scheme would be analogous to a non-competitive enzyme inhibition and would have the following kinetic equation:

$$V' = \frac{V}{1 + AI^n} \quad (V-22)$$

where V' = inhibited reaction rate

V = non-inhibited reaction rate

A = a constant consisting of rate constants

I = light intensity

n = order of inhibition

A fit of equation V-22 would be indicated if a straight line were obtained by plotting $\log(V/V' - 1)$ against $\log I$. The slope would be equal to the order of inhibition, n . By assuming that with the two curves plotted in Figure 15, the rates are uninhibited as the light intensity approaches zero, then the uninhibited rates at all light intensities can be obtained by taking points on a line drawn tangent to $I = 0$. These data applied to equation V-22 are plotted in the linear form in Figure 16. Linear plots are obtained for both cases with an inhibition order of two.

Although equation V-22 seems to give a good fit to the "light inhibited" light curves, more experimentation will be necessary to eliminate the possibility of artifacts such as photo-inactivation which has been re-

Figure 16. Plots of data taken from curves in Figure 15 based on the assumption that the non-linearity of the Lineweaver-Burk plots were caused by a light inhibition. The order of inhibition, n , as determined from the slopes of the plots was found to be 2.

ported to occur in the Hill reaction as well as in certain green algae (Arnon and Whatley, 1949; Myers and Burr, 1940), and ordinary temperature inactivation, accelerated by the high pH and aggravated by the low activity of the chloroplast preparations at these pH's.

619 079

PART F. KINETICS OF THE HILL REACTION IN HEAVY WATER

RESULTS AND DISCUSSION

1. Introduction

Of interest in a kinetic study of photosynthesis or the Hill reaction is the role of water as a primary substrate in the process. Although it is accepted as the origin of oxygen in both photosynthesis and the Hill reaction, the process in which water participates is as yet unknown. That difficulties might arise in performing kinetic experiments involving water in biological systems is understandable when one considers the fact that in these systems water functions as the supporting liquid phase. It is therefore impossible to drastically change the water activity without destroying the structural integrity of the proteins and enzymes concerned with the biological function.

The discovery of the heavy isotope of hydrogen, deuterium, placed in the hands of the chemist a possible tool for the kinetic study of the function of water in biological systems, especially the photosynthetic process in which water is actually oxidized to oxygen. Because of the significant differences in zero point energies of deuterium and hydrogen as well as their mass difference, the rate of a reaction involving water as a reactant might be altered significantly by substituting deuterium oxide for water. Ideally, the substitution of heavy water for water in photosynthesis should affect the rate of the process in which water is used as a substrate.

The first use of heavy water in the study of photosynthesis was made by Reitz and Bonhoeffer (1935) who found that deuterium was utilized by

algae grown in mixtures of ordinary and heavy water at a rate of 2.3 times slower than the utilization of ordinary hydrogen. Curry and Trelease (1935) found that the rate of Chlorella photosynthesis when in pure deuterium oxide was 2.5 times slower than when in ordinary water. Shibata and Watanabe (1936) however, found only a 20% difference with Chlorella ellipsoidea. Craig and Trelease (1937) extended the heavy-water experiments to include water-deuterium oxide mixtures and photosynthesis as a function of light intensity and temperature in both heavy and ordinary water. They found that in water-deuterium oxide mixtures, the rate of photosynthesis varied smoothly from 40% to 100% of the rate in ordinary water as the mole fraction of heavy water was varied from one to zero. The rate of photosynthesis was inhibited at high light intensities, the inhibition disappearing in weak light intensities. Arrhenius plots of the deuterium oxide inhibited rates as well as the water control rates gave almost identical values for the apparent activation energies at temperatures between 10°C. and 30°C. Pratt, Craig and Trelease (1937), and Pratt and Trelease (1938) by the use of flashing light techniques found that heavy water inhibited the flash yield with short dark periods, but the inhibition declined to zero as the flash period was lengthened. They interpreted this to mean that heavy water only inhibited the dark catalytic reaction in photosynthesis.

Horwitz (1954) studied the effects of deuterium oxide on the quinone Hill reaction of Chlorella pyrenoidosa. He found, contrary to photosynthesis, that the oxygen evolution was inhibited by heavy water at all light intensities at pH 6.6 as well as pH 7.43. Further differences were noted in the heavy water inhibition when previously frozen cells were employed, the heavy water inhibition exhibiting a pronounced progressive

effect. Horwitz interpreted this data as a possible effect of the heavy water on the quinone, which was used as the oxidant.

Krasnovskii and Breen (1954) studied the effect of deuterium oxide on the Hill reaction with cucumber leaf preparations using phenol indophenol as a colorimetric oxidant. They found that the rate of dye reduction at 20°C. was twice as slow in heavy water than in ordinary water whereas at 30°C. the rate in heavy water was only 1.3 times slower than in water. On this basis, the temperature coefficient of the Hill reaction in heavy water was 2.1 compared to 1.3 for the reaction in ordinary water. They also found a linear relationship between the percentage inhibition and the concentration of heavy water in the reaction medium. On the basis of their data they concluded that deuterium oxide inhibited the Hill reaction by increasing the activation energy of the hydrogen or proton transfer reactions in the dark process.

In spite of the extensive published data with respect to photosynthesis and the Hill reaction in heavy water, it was felt that an application of the techniques developed in this laboratory to the problem might reconcile the differences in deuterium oxide inhibition between photosynthesis and the Hill reaction.

2. The Effect of Heavy Water upon the Hill Reaction Rate

Parameters k_D and k_L

Preliminary experiments with the Hill reaction in heavy water independently confirm the findings of Horwitz, that the reaction was inhibited at all light intensities by heavy water. They also indicated that the degree of inhibition was a function of the pH. It was thus necessary to

determine the values of both k_D and k_L in deuterium oxide and ordinary water as a function of pH.

Rhubarb-chard chloroplast fragments prepared in the usual way were used for this experiment. The chloroplast suspensions were prepared by suspending half of the sedimented fragments in a 0.5 M sucrose-0.01 M water solution and the other half in a similar solution with heavy water instead of ordinary water. Before suspending in the heavy water, the chloroplasts were rinsed several times with the heavy water solution. About one hour elapsed between the time of resuspension and storage at -35°C . Due to the possibility of deuterium-hydrogen exchange with the 4 moles/liter of sucrose hydroxyl groups, the sucrose was dissolved in heavy water, and after time for exchange equilibrium to take place was then precipitated by the addition of dry dioxane, washed with anhydrous ether, and dried. The phosphate buffer-ferricyanide solution in heavy water was prepared by adding the requisite amount of heavy water to a buffer-ferricyanide residue left by the evaporation to dryness of an aliquot of the appropriate water solution. The heavy water was obtained from the Stuart Oxygen Company by authorization of the U. S. Atomic Energy Commission. It was used without further purification as it was specified to be at least 99.8 per cent D_2O .

The rate determinations were made by the usual procedure as previously described. The pH and the pD of the respective reaction mixtures were determined with the Beckman Model G pH meter. The pD of the heavy water solutions was obtained from the pH meter reading by the addition of 0.4 to the reading. (Hart, 1949; Lumry, et. al., 1951) Six to eight different light intensities were employed for each light curve in order to obtain

an accurate k_D and k_L value. The values of k_D and k_L obtained in deuterium oxide as compared to those in water are given in Table XI. These data are plotted in Figure 17.

TABLE XI
The Effect of D_2O upon the Hill Reaction
Rate Parameters k_D and k_L

H ₂ O control			*	In D ₂ O		
pH	k_D	$k_L \times 10^{14}$	*	pD	k_D	$k_L \times 10^{14}$
5.75	.40	.54 ± .05	*	6.15	.22	.18 ± .05
6.25	.59	.60 ± .10	*	6.60	.40	.20 ± .02
6.75	.81	.45 ± .10	*	7.00	.52	.17 ± .02
7.20	.95	.29 ± .03	*	7.40	.56	.14 ± .02
7.35	.97	.23 ± .02	*	7.60	.62	.14 ± .02
7.65	1.00	.14 ± .01	*	7.80	.57	.09 ± .01
---	---	---	*	8.30	.52	.08 ± .005

As can be seen from Figure 17 both the light limiting parameter k_L and the dark parameter k_D undergo an apparent inhibition by heavy water over wide ranges of pH. Only at high pH's where the value of k_L becomes small does the apparent inhibition by heavy water vanish.

3. Progressive Nature of the Heavy Water "Inhibition"

It was noticed, during a preliminary experiment with water-heavy water mixtures that the difference in activity between chloroplasts suspended in water the heavy water increased even during storage at $-35^\circ C$.

Figure 17. pH plots of the Hill reaction parameters k_D and k_L in H_2O and in D_2O , pD determined by the relationship: $pD = pH$ reading with Beckman glass electrode + 0.4; Rhubarb chard; Phosphate buffer, 0.1 M; Temperature, $10^\circ C$; Initial ferricyanide conc., 0.0003 M.

Also, no immediate reversal of the heavy water inhibition occurred when water was added with the buffer-oxidant solution prior to a run. An experiment was therefore devised to test the apparent progressive nature of the heavy water inhibition.

In order to obtain rate data with heavy water chloroplast suspensions as quickly as possible after the addition of the heavy water, the following procedure was employed: Ten ml. aliquots of the chloroplast suspension were pipetted into each of two 10 ml. plastic centrifuge tubes. These were then centrifuged for 15 minutes at 35,000 rpm in a Spinco preparative ultracentrifuge. With this procedure the chloroplast fragments were sufficiently packed such that no loss occurred when the clear supernatant was poured off and the packed chloroplasts rinsed with the heavy water suspending solution. Exactly 10 ml. of the heavy water-sucrose suspending solution was added to one of the tubes whereas to the second tube was added 10 ml. of the regular water solution for control purposes. The packed chloroplast fragments were suspended in the respective solutions by the use of a steel shaft with a flattened tip revolved at high speed by a small hand grinding tool. The resulting suspensions were then incubated at 2.0°C . during which time one ml. aliquots were removed at measured time intervals and snap-frozen in a dry ice-acetone mixture. (These were stored at -35°C . until used.) Each one ml. aliquot was sufficient for six individual rate determinations after the addition of the buffer-ferricyanide solution. The values of k_D and k_L for each medium as a function of the incubation time are given in Table XII. The respective k_D values are plotted as a function of time in Figure 18. The k_D and k_L values were obtained by the visual examination of the Lineweaver-

84

Burk plots of the light curves rather than by the method of least squares since the objective was qualitative rather than quantitative.

TABLE XII
Progressive Nature of the D₂O "Inhibition" of the Hill Reaction

In D ₂ O			*	H ₂ O control		
Time*(min)	k _D	k _L x 10 ¹⁴	*	Time*(min)	k _D	k _L x 10 ¹⁴
9	.685	.21 ± .01	*	20	.88	.24 ± .01
15	.625	.21 ± .01	*	31	.87	.24 ± .01
20	.60	.22 ± .01	*	42	.86	.24 ± .02
27	.56	.22 ± .01	*	53	.82	.24 ± .01
35	.505	.21 ± .01	*	68	.82	.25 ± .01
47	.46	.21 ± .01	*			
70	.43	.20 ± .01	*			
pD = 6.8			*	pH = 6.7		

*Time of incubation at 2°C.
Runs made at 6.5°C.

Examination of these data indicates that heavy water inhibition of the k_D rate parameter is a progressive nature. Although, as illustrated in Figure 17, the k_D values when extrapolated to zero time still show about 16% inhibition in heavy water it cannot be assumed that this represents a true instantaneous inhibition since it has not been possible to choose the proper pH and pD for making a comparison. If the Hill reaction rate parameters are compared on the basis of pH = pD for the two media, the ionic charge distribution on the proteins and enzymes will be different. However, if the comparison is made on the basis of the

Figure 18. The progressive nature of the heavy water inhibition of the Hill reaction. The D₂O-chloroplast suspension was prepared from rhubarb chard chloroplast fragments; 0.1 M phosphate buffer; Initial ferricyanide conc., 0.00025 M; Temperature of incubation, 2°C.; Temperature of run, 6.5°C.

same ionic states, the hydrogen and deuterium ion activities will be different. Thus, the 16% difference in the initial k_D values could very well represent the differences in activity caused by differences in ionic states.

It is noteworthy that the k_L values in heavy water did not show a progressive effect as did the k_D values. The small difference between the D_2O and H_2O values of k_L is probably due to a compensating pH effect, since a similar experiment with a different chloroplast preparation and at a different pH gave almost a two-fold lowering of the k_L value.

4. Reversibility Test of the Heavy Water Inhibition

Because of the progressive nature of the heavy water inhibition of k_D , it was considered necessary to test the possibility that heavy water was merely accelerating a normal inactivation process rather than giving a true inhibition. Since an inhibition would be reversible whereas an inactivation would be non-reversible, the replacement of the heavy water in an inhibited system by ordinary water would lead to an activation if an inhibition existed.

The D_2O inhibited chloroplast preparation used in this experiment was the identical preparation described in the previous section. The heavy water was replaced by water by the same technique. Immediately after re-suspending the chloroplast fragments an aliquot was withdrawn and frozen. Another aliquot was withdrawn about one hour later in order that sufficient time was allowed for any reversal. Light curves were made at pH 6.7 and at $6.5^\circ C$. by the usual procedure. The results are given in Table XIII

TABLE XIII

Test of the Reversibility of D_2O Inhibition of
the Hill Reaction

Appx. Time after H_2O Addition	* previously in D_2O		* H_2O control		* $k_D(D_2O)$		* $k_L(D_2O)$	
	* k_D	* $k_L \times 10^{14}$	* k_D	* $k_L \times 10^{14}$	* $k_D(H_2O)$	* $k_D(H_2O)$	* $k_L(H_2O)$	* $k_L(H_2O)$
10 min	* 0.52	* 0.12	* 0.71	* 0.21	* 0.73	* 0.73	* 0.57	* 0.57
75 min	* 0.44	* 0.11	* 0.61	* 0.19	* 0.72	* 0.72	* 0.58	* 0.58

From the data in Table XIII it is seen that if any activation by water of the D_2O inhibited k_D occurred it could have occurred only during the first ten minutes after addition of the water, since there was no change in the relative value of k_D from 10 to 75 minutes after the addition of water. It is also noted that even 75 minutes after replacing D_2O by water the value of k_D , of the suspension which was previously in D_2O , is 30 per cent smaller than the water control. There also seems to be no reversal of the k_L inhibition, since the D_2O preparation gave a k_L value smaller by 40 per cent than the water control, even after equilibration with water. It is therefore apparent, that if any reversible inhibition by heavy water existed in the Hill reaction, it would have been masked by a non reversible inhibition or inactivation. However, from the comparison of the data plotted in Figure 17 and the data in Table XIII, which are from the same chloroplast preparation, it must be concluded that if a reversible inhibition of k_D exists, its magnitude could not be much larger than 15 per cent.

5. The Effect of Deuterium Oxide on the Apparent Activation Energy, μ , of k_D .

Further insight into the action of heavy water on the Hill reaction can be obtained by measuring the apparent activation energy of the Hill reaction in heavy water. Any difference in the rate of the water splitting reaction in photosynthesis or the Hill reaction due to the substitution of D_2O for water would probably be due to a change in the activation energy. If this reaction can be made the rate limiting process, then under these circumstances the rate parameter which incorporates this reaction will be affected. Thus, a test of whether D_2O inhibits by this mechanism would be to test for a change in the apparent activation energy of the parameter involving water as a substrate.

Although the data of the previous section indicated, at most, only a minor reversible inhibition by D_2O it was felt that a temperature study would be useful in further evaluation of the effects of heavy water on the Hill reaction. Also of interest in this respect is the confirmation or rejection of Krasnovskii's and Breen's findings of a significantly higher temperature coefficient of the Hill reaction in heavy water than in ordinary water, especially in view of the findings of Craig and Trelease which indicated no difference in the apparent activation energy of the Chlorella dark reaction under the two conditions.

The Hill reaction system in heavy water was prepared by the method as described in the previous section. Light curves for this D_2O system as well as the identically treated water control system were made at temperatures of $6.3^\circ C$., $11^\circ C$., $16^\circ C$., and $21^\circ C$. The Lineweaver-Burk type plots of these

89

light curves in D_2O are illustrated in Figure 19. The k_D and k_L parameter values as calculated from the data illustrated in Figure 19 and the water control are given in Table XIV.

TABLE XIV
Effect of Temperature on the Hill Reaction in
Heavy Water

Temperature (° C.)	In D_2O		H_2O control	
	k_D	$k_L \times 10^{14}$	k_D	$k_L \times 10^{14}$
6.3	0.31	$0.12 \pm .01$	0.53	$0.23 \pm .01$
11.0	0.46	$0.12 \pm .01$	0.75	$0.21 \pm .01$
16.0	0.65	$0.12 \pm .01$	1.11	$0.21 \pm .01$
21.0	0.95	$0.12 \pm .01$	1.67	$0.21 \pm .01$

It is noted that the k_L parameter is independent of temperature in D_2O as well as in water. The k_D parameter from both the D_2O suspension and the water control are expressed as Arrhenius plots in Figure 20. The apparent activation energy, μ for k_D in D_2O as well as in water is determined from these plots. In heavy water μ was 12.5 kcal./mole whereas in ordinary water μ was 12.6 kcal/mole. It is thus apparent from the data in this experiment that the apparent activation energy of k_D is unchanged by the addition of heavy water. This finding, in this respect, is in agreement with the findings of Craig and Trelease with Chlorella photosynthesis.

Figure 19. Lineweaver-Burk plots of the Hill reaction light curve in heavy water at different temperatures. In this case sufficient time was allowed for a significant inhibition or inactivation to occur. The conditions and chloroplast preparation were the same as given in Figure 18.

Figure 20. Arrhenius plots of k_D for a D_2O and a water control chloroplast suspension. The conditions, and the chloroplast preparation were the same as given in Figure 18. $\mu_{H_2O} = 12.6$ kcal.; $\mu_{D_2O} = 12.5$ kcal.

6. Discussion

It is apparent from these heavy water studies, that an unequivocal evaluation of heavy water kinetic data would be very difficult to obtain with the systems and methods available at the present time. Of possible significance are the widely divergent differences in the effects of heavy water on the Hill reaction and on Chlorella photosynthesis.

The essential lack of a reversible inhibition of k_D in the Hill reaction as contrasted to about a 60 per cent reversible inhibition of the dark process in Chlorella indicates that different inhibition mechanisms are involved in the two cases. This difference could be caused by the presence of a D_2O sensitive reaction in the Chlorella dark process which is absent in the Hill reaction. If it is assumed that the water splitting and oxygen evolution in the Hill reaction occurs by the same mechanism as in photosynthesis, then it would be reasonable to postulate that the D_2O inhibition of the dark process in Chlorella does not occur in the water splitting reaction. The D_2O inhibited reaction in Chlorella photosynthesis could be in the reactions connecting the water splitting reactions to the carbon dioxide fixation reactions or in the carbon dioxide fixation itself. This is possible since experimental evidence indicates that enzymes not even involving water as a substrate can be inhibited by heavy water. (Shibata and Watanabe, 1949)

A possible alternative explanation could be that different reactions are rate limiting in the dark processes of the Hill reaction and Chlorella photosynthesis, respectively. In this case, only the step which is rate limiting in Chlorella photosynthesis is inhibited by heavy water. This

scheme would account for the lack of a D_2O effect on the apparent activation energy of the dark process as observed by Craig and Trelease, provided that the rate limiting step is different than that involving water as a substrate. This would also explain the much higher values of the apparent activation energy, (20,500 kcal/mole, for Chlorella photosynthesis as determined by Craig and Trelease, 1937) than for the Hill reaction, (7,000-12,500 kcal/mole as determined in this laboratory).

The progressive nature of the D_2O effect upon k_D in the Hill reaction, as found in this study, as well as the non-reversible character of both the k_D and the k_I "inhibition", indicates an inactivation or denaturation process as the most plausible explanation. This theory has support by the findings that the stability of certain enzymes are drastically changed when heavy water is substituted for ordinary water in the enzyme solution. (Caldwell, et. al., 1936; Caldwell, et. al., 1939) Horwitz's observation of a progressive inhibition in heavy water of the Hill reaction with previously frozen Chlorella cells also gives support to this explanation. It is also apparent that this rapid inactivation of the Hill reaction in heavy water could be interpreted as an inhibition in experiments where an appreciable time was required to prepare the D_2O suspension.

A possible cause for the instability of the Hill system in heavy water could be a protein denaturing effect by heavy water. This denaturation could be caused by the difference between D_2O and H_2O in the energies of secondary "hydrogen" bonding between the D_2O or H_2O molecule and the nucleophilic protein groups.

VI. GENERAL DISCUSSION

In general, the results of this study has indicated that the Hill reaction involves a system of some complexity. This is especially apparent from the wide quantitative variation, from preparation to preparation, in many of the kinetic properties investigated. It has not been possible, to date, to determine whether this variation results from structural or chemical factors, although it is probable that both play an important role in the chloroplast. Only when these variations are defined and controlled will it be possible to obtain from the quantitative kinetic data unequivocal interpretations as to mechanism. This is especially the case with the pH-rate relationships and the apparent activation energy measurements. However, many valuable hints concerning the mechanism of the photolytic activity of the chloroplast material can be obtained from reproducible qualitative kinetic relationships.

The rectangular hyperbolic nature of the Hill reaction rate-light intensity relationship offers a significant framework upon which to build an overall kinetic scheme, since this relationship remained reproducible under a wide variation of experimental conditions. Gilmour (1953), has discussed the kinetic implications of the rectangular hyperbolic light curve relationship. However, in order to discuss clearly the mechanism implications of the data presented in this study it will be necessary to present a discussion correlating reaction mechanism to the rectangular hyperbolic rate-light intensity relationship. This discussion will, in general, utilize kinetic schemes already discussed by Gilmour, although

with several modifications in development.

The simplest kinetic scheme yielding a rectangular hyperbola for the Hill reaction light curve can be written as a first order photo-excitation followed by a first order product yielding reaction of the excited intermediate:

where P and P* represent the non-activated and activated forms, respectively, of the fundamental photolytic unit involved in the Hill reaction. Application of equations VI-1 and VI-2 to elementary steady state treatment yields as the rate law:

$$V = \frac{k_2 p I}{k_2/k_1 + I} \quad (\text{VI-3})$$

where $p = (P) + (P^*)$. Comparison of this expression with the empirical equation given by equation V-21 indicates that the light parameter, k_L , and the dark parameter, k_D , of equation V-21 would be in terms of the above expression:

$$k_D = k_2 p ; k_L = k_1 p \quad (\text{VI-4})$$

Inspection of the above simplified scheme in light of the kinetic behavior of k_D and k_L indicates its inadequacy. With only one concentration term, p, present in both parameters it would be difficult to explain

the highly differential effects of pH, heavy water, γ radiation, and many inhibitors on k_D and k_L , assuming that these effects are mediated through effective changes in the values of concentration factors. It is therefore obvious that at least two distinct concentration factors are required to supply the necessary kinetic latitude. This can be done by the introduction of an additional intermediate reactant in the proposed kinetic scheme. However, under ordinary circumstances this additional reaction yields a scheme which cannot be mathematically expressed as a rectangular hyperbolic function of the rate and the light intensity. Equations VI-1 and VI-2 indicate that the fundamental kinetic requirement for a rectangular hyperbola consists of a bimolecular formation of a transient intermediate from reactant and substrate, followed by a unimolecular decomposition of the intermediate to form products and regenerate the reactant. It is thus apparent that even with the presence of intermediate reactions between the initial light absorption and the dark, enzymic process, these reactions must be so arranged that the overall process behaves kinetically as a simple two step reaction.

A reaction model satisfying the above requirements can be formulated on the basis of already existing kinetic and morphological information. This model is based on the concept of a "photosynthetic unit", in which a large number of chlorophyll molecules feed the photoenergy to a single "dark" or Blackman enzyme system. This concept has support from the data of several investigators who found that at least 200 chlorophyll molecules were required to effectively absorb and utilize one quanta of light (Emerson and Arnold, 1932, Gaffron and Wohl, 1936; Tamiya, 1949; Kohn, 1936; and Thomas, et. al., 1953). Also of importance in formulating

a reaction mechanism are the electron microscope studies of the chloroplast which show that the individual grana possess a dense lamellar structure. This indicates a somewhat ordered arrangement for the chlorophyll molecules and other components in the grana. On the basis of this information a tentative model of a photolytic unit can be roughly diagrammed as follows:

The converging arrowed paths represent the energy transfer paths from the absorbing pigments to the dark enzyme system. These could be other chlorophyll molecules or possibly a series of electron transfer systems.

If as in scheme VI-1 and VI-2 the entire photolytic unit as diagrammed above is represented by the symbol P, then the productive light absorption and transfer reaction can be represented by:

The apparent rate constant for the formation of P^* will incorporate a true rate constant and a concentration term, C , expressing the relative

number of light energy absorption and transfer systems, since any condition which blocks the transfer of absorbed energy to the enzyme will have the same effect on the formation of P^* as if the energy were not absorbed.

Equation VI-5 implies that only the nonactivated photolytic unit, P , absorbs light, however, we know that placing the enzyme of the unit in a reactive state, which is equivalent to P^* , will not prevent further light absorption by the pigment system. Therefore, in order that the photolytic unit behaves kinetically as expressed in VI-5 the condition must be imposed that any light energy absorbed by P^* is wasted as heat.

Since equation VI-5 implicitly contains an assumption kinetically equivalent to equation VI-6, equation VI-6 will not affect the overall kinetic development.

The final reactions would consist of the formation of products from P^* by a series of light independent enzymic reactions involving water as a substrate. It has not been determined as yet whether the water is split at the chlorophyll or the enzyme portions of the photolytic unit. However, this is unimportant with respect to this kinetic development since if chlorophyll is involved in the splitting of water the molecule or molecules involved would merely behave as the dark enzyme. The Blackman or dark enzymic reactions can then tentatively be written as;

The rate constant, k_d , for the process will of course represent an overall constant and will probably be dominated by the constant of the slowest

reaction in a sequence of reactions. The production of free oxygen has not been given as the products in VI-7 since the above reactions are limited to the processing of one quanta of light energy, which is insufficient for the production of an oxygen molecule from two water molecules.

Development of the overall rate equation for reactions VI-5 and VI-7 by the usual steady state derivation gives:

$$v = \frac{k_d p I}{k_d / k_1 c + I} \quad (\text{VI-8})$$

where c and p have the same significance as previously mentioned.

A more detailed derivation of equation VI-8 can be made involving the intermediate reactions including the energy transfer reactions and the coupling reaction with the dark enzyme. Accordingly, the mechanism of the reactions involved in a single photolytic unit can tentatively be written as:

In the above scheme the entire sequence of reactions is for the useful processing of a single photon of light, since the dark enzyme molecule

can only handle the energy from a single photon per reaction cycle, any excess present being immediately dissipated as heat or fluorescence. Thus, essentially no photo energy is carried over between cycles of the dark enzyme.

The above scheme is incomplete since it does not consider the mechanism of the waste reactions. At the present time, the nature of these reactions can only be speculative since no definite correlation between fluorescence, internal conversion, and useful energy absorption have been experimentally obtained. Possible waste reactions, for example, can be written as:

where chl_i refers to an arbitrary chlorophyll or other active pigment molecule, and E^* the active dark enzyme molecule. It would be desirable to be able to derive a suitable overall rate equation in which the nature of the waste reaction equations would automatically impose the rectangular hyperbolic conditions upon the overall rate equation. However, because of the lack of information concerning these waste reactions, only the useful light energy utilization process as illustrated in equations VI-9 to VI-12 will be considered in the derivation of the overall rate equation.

The derivation of the rate equation for the above reaction sequence cannot be carried out by methods employing the usual mass law and steady

state kinetics because of the single molecular nature of the intermediates involved in the reaction sequence. However, for a reaction involving a single molecule, the mean time required for the reaction to take place can be expressed by the reciprocal of the rate constant. Therefore for the above reaction sequence, the total time required between the initial quanta absorption and the formation of products will be the sum of the individual reaction times for the reactions involved, or the sum of the reciprocal rate constants.

$$T = \frac{1}{k_1 c I} + \frac{1}{k_2} + \dots + \frac{1}{k_n} + \frac{1}{k_d} \quad (\text{VI-13})$$

The average time for the absorption of a single photon will be a function of the rate constant for this absorption, the number of absorbing sites, and the incident light intensity. The average time for the transference of this quantum of energy to the dark enzyme will then be the sum of the reciprocal rate constants for these transfer steps. In this scheme, chl_n , the chlorophyll molecule in contact with the dark enzyme, is considered as a collecting point for all the transfer systems. The rate at which a single photolytic unit releases products will be the reciprocal of the total turnover time as expressed by equation VI-13. After rearrangement, the rate is expressed by equation VI-14 as:

$$v = 1/T = \frac{k_d I}{\frac{k_d}{k_1 c} + \left[k_d \left(\frac{1}{k_2} + \dots + \frac{1}{k_n} \right) + 1 \right] I} \quad (\text{VI-14})$$

With the reasonable assumption that the energy transfer and enzyme

coupling reactions are very fast compared to the dark reaction, then

$$1/k_d \gg \left(\frac{1}{k_2} + \dots + \frac{1}{k_n} \right) \quad (\text{VI-15})$$

therefore:

$$1 \gg k_d \left(\frac{1}{k_2} + \dots + \frac{1}{k_n} \right) \quad (\text{VI-16})$$

and:

$$v = \frac{k_d I}{\frac{k_d}{k_1 c} + I} \quad (\text{VI-17})$$

The overall rate per unit chlorophyll concentration will not be changed for a large system of photolytic units provided that every dark enzyme system is active. However, the overall rate expression must include a factor to correct for inactive dark enzyme and therefore inactive photolytic units, whether inhibited or denatured. By assigning p as a symbol of this factor which will have the same dimensions as mole fractions of active photolytic units, the expression for the Hill reaction rate becomes:

$$v = \frac{k_d p I}{\frac{k_d}{k_1 c} + I} \quad (\text{VI-18})$$

which is identical to equation VI-8. In terms of the above equation k_D and k_L , the dark and light rate parameters, respectively, can be expressed as:

$$k_D = k_d p \quad ; \quad k_L = k_1 c p \quad (\text{VI-19})$$

The scheme as outlined above will explain most of the kinetic data obtained in this study as well as data obtained by other studies. Inhibition studies have indicated three possible types of inhibition: inhibition of k_D only, k_L only, and equal inhibition of both k_D and k_L . Inspection of the rate parameters expressed as functions of the rate constants as given in equations VI-19, indicates that anything which affects the activity of the dark enzyme, as given by the symbol p , will affect both k_D and k_L equally whereas if c , the relative number of absorption and energy transfer systems, is affected only k_L will be changed. The inhibition of k_D only, can only be obtained, according to the above mechanism, when P^* is inhibited. The kinetics for this type of inhibition can be expressed as:

The equilibrium constant for this type of inhibition will be:

$$K = \frac{(P^*I_n)}{(P^*)(I)^n} \quad (VI-21)$$

where (I) is the inhibitor concentration and (P^*I_n) the inhibitor- P^* complex concentration. Introduction of equation VI-21 into the derivation of the rate equation from expressions VI-5 and VI-7 yields as the inhibited rate:

$$V = \frac{k_d p I}{\frac{k_d}{k_L c} + I (1 + K I^n)} \quad (VI-22)$$

From this inhibited rate equation the expressions for k_D and k_L can be

represented as:

$$k_D = \frac{k_d p}{1 + k_d p} \quad ; \quad k_L = k_L c p \quad (\text{VI-23})$$

It is thus apparent the k_D only will be affected by an inhibition of P^* .

Temperature studies of k_D and k_L in general support the proposed Hill reaction mechanism. The temperature independence of k_L indicate that the value of k_L is determined by the rate constant of a primary photochemical reaction. Inspection of equation VI-14 which includes the possibly temperature dependent energy transfer and coupling reactions indicates that the rate constants for these reactions occur only in k_D , only the primary photon absorption process constant being involved in k_L . The observed variation in the k_D temperature dependence between various Hill reaction preparations as well as between Chlorella photosynthesis and the Hill reaction, indicates the necessity that k_D be a composite rate constant of several successive reactions. The temperature dependence would then vary as the rate limiting reaction. In view of this concept, however, it is puzzling that in all cases examined an Arrhenius plot of k_D yielded a linear relationship.

The separate pH dependence of k_D and k_L can be explained by the separate pH effects on c and p in equation VI-18. Since in all probability, the values of c and p are each dependent upon at least several factors one could understand the difficulty in obtaining quantitative reproducibility in the respective pH curves. The same arguments can be applied to the differential effects of heavy water, ageing and ionizing radiation on k_D and k_L .

The presence of a common concentration factor p in both k_D and k_L is supported by the salt inhibition data in which it was found that both parameters were inhibited equally. This is further supported by the room temperature thermal inactivation studies of Bishop (1955) in which he found that the inactivation rate was identical for both parameters, and the ultraviolet radiation inactivation studies in which the degree of inactivation of photosynthesis was independent of the light intensity used for photosynthesis. (Holt, et. al., 1951; Redford and Meyers, 1951) The same results were obtained in this laboratory with the ultra violet radiation of the Hill reaction system. (Anderson and Spikes, 1954)

The presence of c , the light energy absorption and transferring system concentration factor, in k_L only, agrees with the finding that the saturating light photosynthesis rate (a measure of k_D) of Aurea leaves, which contain a low chlorophyll content, is the same as that of normal leaves (Rabinowitch, 1951). Thus the value of k_D is independent of the chlorophyll concentration.

It must be realized, however, that even though the mechanism as postulated in this study gives a good fit to the available experimental data, it is only presumptive evidence for the correctness of the mechanism. There is always the possibility that another scheme will give an equally good fit. Only additional favorable data will reduce the number of alternate schemes. A valuable study in this respect would be a critical determination of the relationships, if any, between fluorescence, and other waste processes and the rate parameters of photosynthesis and the Hill reaction. A study of this type is now being undertaken in this laboratory.

If the kinetic and morphological features of photosynthesis and the Hill reaction, as proposed in this study, are essentially correct, it will mean great difficulty for the detailed elucidation of the individual reactions involved in this system. A dense, somewhat ordered structure in the grana would prohibit its reconstruction from individual components, even if one is successful in isolating these components. The true characterization of the functions of individual systems is somewhat dependent upon the reconstruction of a functioning intact system from these component parts. Therefore, until the development of techniques here-to-fore not used most of the information concerning the photolytic process in photosynthesis probably will be obtained by the application of kinetics to an intact functioning system.

The kinetic techniques introduced and utilized in this study are the nearest approach to a quantitative kinetic procedure yet developed in the study of photosynthesis. As such, an extension of the techniques developed here should yield considerable information concerning the photochemical mechanism of photosynthesis. Even the development of more direct experimental approaches to this problem will be dependent upon accurate kinetic information as a guide.

VII. SUMMARY

1. The rate-light intensity relationship of the Hill reaction was found to give an excellent fit to a rectangular hyperbola through a wide range of light intensities and reaction conditions. From this relationship it was possible to experimentally distinguish between two fundamental rate processes; a temperature dependent "dark" process which is rate limiting at high light intensities and a temperature independent "light" process which is rate limiting at low light intensities.

2. The apparent activation energy of the "dark" process was found to vary between 9 kcal. and 12 kcal., in general confirming the results obtained previously in this laboratory in an independent study.

3. The "dark" process was found to be independent of the light wavelength, however the "light" process was strongly dependent upon wavelength, exhibiting maxima in the red and the blue regions and a minimum in the green region of the spectrum.

4. Both the "light" process and the "dark" process were highly dependent upon pH. It is significant that these dependencies were different, the optimum for the "dark" process being around pH 7.3 and the optimum for the "light" process being around pH 6.3. Although the general relationship of the two optima was reproducible, the general shape of the pH curves was different for different chloroplast preparations. Apparent activation energies of the "dark" process as calculated from pH curves determined at two different temperatures indicate the possibility that the apparent activation energy of the "dark" process is pH dependent.

Also observed was an apparent inhibition of the rate by light at high pH's.

5. Contrary to previous studies with Chlorella photosynthesis and the Hill reaction, the effect of heavy water on the Hill reaction was found to be a rapid irreversible inactivation rather than an inhibition. This finding necessitates a re-evaluation of the interpretations based upon the previous findings.

6. The rectangular hyperbolic nature of the Hill reaction light curve as well as the other data can be interpreted in terms of a reaction mechanism involving as the fundamental kinetic unit an energy collecting and transferring system connected to an energy trapping, "dark" enzyme. In order that the mechanism fit the rectangular hyperbolic nature of the light curve, the condition must be imposed that any photo-energy absorbed while the "dark" enzyme is in the energetic form is dissipated in a waste reaction.

VIII BIBLIOGRAPHY

- Anderson, D. R., and Spikes, J. D., Proc. of the Utah Academy of Sciences, Arts and Letters, 31, 112 (1954)
- Arnon, D. I., Allen, M. B., and Whatley, F. R., Nature, 174, 394 (1954)
- Arnon, D. I., Science, 122, 9 (1955)
- Bishop, N. I., Lumry, R., and Spikes, J. D., Arch. Biochem. Biophys., 58, 1 (1955)
- Bräckett, F. S., Cold Spring Harbor Symposia Quant. Biol., 3, 117 (1935)
- Brown, A. H., and Good, N., Arch. Biochem. Biophys., 57, 340 (1955)
- Burk, D., and Lineweaver, H., Cold Spring Harbor Symposia Quant. Biol., 3, 165 (1935)
- Caldwell, M. L., Doebbeling, S. E., and Manian, S. H., J. Am. Chem. Soc., 58, 84 (1936)
- Caldwell, M. L., Doebbeling, S. E., and Wicklen, F. C. von, J. Am. Chem. Soc., 61, 122 (1939)
- Chen, S. L., Plant Physiol., 27, 462 (1951)
- Clendenning, K. A., and Gorham, P. R., Can. Jour. Research, 28, 114 (1950)
- Craig, F. N., and Trelease, S. F., Am. J. Botany, 24, 332 (1937)
- Curry, J., and Trelease, S. F., Science, 82, 18 (1935)
- Ehrmantraut, H., and Rabinowitch, E., Arch. Biochem. Biophys., 38, 67 (1952)
- Emerson, R., and Arnold, W. J., J. Gen. Physiol., 15, 391 (1932)
- Emerson, R., and Lewis, C., Am. J. Botany 30, 165 (1943)
- Ewart, A. J., J. Linnean Soc. London J. Botany, 31, 423 (1896)
- Franck, J., and Herzfeld, K. F., J. Chem. Phys., 5, 237 (1937)
- Franck, J., and Herzfeld, K. F., J. Chem. Phys., 45, 978 (1941)
- French, C. S., and Rabideau, G. S., J. Gen. Physiol., 28, 329 (1945)
- Gaffron, H., and Wohl, K., Naturwiss., 24, 81 (1936)

- Gilmour, H. S. A., Dissertation, Univ. of Utah, Salt Lake City, Utah (1953)
- Good, N., and Hill, R., Arch. Biochem. Biophys., 57, 355 (1955)
- Gorham, P. R., and Clendenning, K. A., Arch. Biochem. Biophys., 37, 199 (1952)
- Haberlandt, G., Flora, 71, 291 (1888)
- Hart, R. G., Nat'l. Res. Council Can., Atomic Energy Project N. R. C. No 2385, (1949)
- Haxo, F. T., and Blinks, L. R., J. Gen. Physiol., 33, 389 (1950)
- Hill, R., Nature, 139, 881 (1937)
- Hill, R., Proc. Royal Soc. (London), 127, 192 (1939)
- Hill, R., and Scarisbrick, R., Proc. Royal Soc. (London), 129, 255 (1940)
- Holt, A. S., and French, C. S., Arch. Biochem., 9, 25 (1946)
- Holt, A. S., and French, C. S., Arch. Biochem., 19, 429 (1948)
- Holt, A. S., Smith, R. F., and French, C. S., Plant Physiol., 26, 164 (1950)
- Horwitz, L., Plant Physiol., 29, 215 (1954)
- Inman, O. L., Cold Spring Harbor Symposia Quant. Biol., 3, 184 (1935)
- Inman, O. L., Science, 88, 544 (1938)
- Kok, B., Private Communication (1955)
- Kohn, H. J., Nature, 137, 706 (1936)
- Krasnovskii, A. A., and Breen, G. P., Doklady Akad. Nauk. S.S.S.R., 96, 1025 (1954)
- Lumry, R., Smith, E. L., and Glantz, R. R., J. Am. Chem. Soc., 73, 4330 (1951)
- (a) Mehler, A. H., Arch. Biochem. Biophys., 33, 65 (1951)
- (b) Mehler, A. H., Arch. Biochem. Biophys., 34, 339 (1951)
- Mehler, A. H., and Brown, A. H., Arch. Biochem. Biophys., 38, 365 (1952)
- Molisch, H., Bot. Z., 62, 1 (1904)
- Molisch, H., Z. Botan., 17, 577 (1925)

- Pratt, R., Craig, F. N., and Trelease, S. F., *Science*, 85, 271 (1937)
- Pratt, R., and Trelease, S. F., *Am. J. Botany*, 25, 133 (1938)
- Rabinowitch, E., "Photosynthesis and Related Processes" Vol II Part I, Interscience Publishers Inc., New York, N. Y., 1951
- Redford, E. L., and Meyers, J., *J. Cellular Comp. Physiol.*, 38, 217 (1951)
- Reinke, J., *Botan. Z.*, 41, 697, 713, 732, (1883)
- Reitz, O., and Bonhoeffler, K. F., *Z. Physik, Chem.*, A 172, 369 (1935)
- Ruben, S., Randall, M., Kamen, M., and Hyde, J. L., *J. Am. Chem. Soc.*, 63, 877 (1941)
- Shibata, K., and Watanabe, A., *Acta Phytochim. Japan*, 9, 107 (1936)
- Shibata, K., and Watanabe, A., *Acta Phytochim. Japan*, 15, 169 (1949)
- Smith, E. L., *Proc. Nat. Acad. Sci. U. S.*, 22, 509 (1936)
- Smith, E. L., *J. Gen. Physiol.*, 20, 807 (1937)
- Smith, E. L., *J. Gen. Physiol.*, 22, 21 (1938)
- Spikes, J. D., Lumry, R., Eyring, H., and Wayrynen, R. E., *Arch Biochem.*, 28, 48 (1950)
- Spikes, J. D., Rieske, J. S., and Marcus, R. J., *Plant Physiol.*, 29, 161 (1954)
- Tamiya, H., and Chiba, Y., *Studies Tokugawa Inst.*, VI, No.2 (1949)
- Tanada, T., *Am. J. Botany*, 38, 276 (1951)
- Thomas, J. B., Blaauw, O. H., and Duysens, L. N. M., *Biochim. et Biophys. Acta*, 10, 230 (1953)
- Volkov (Wolkoff), A., *Jahrb. wiss. Botan.*, 5, 1 (1866)
- Warburg, O., *Z. Naturforsch.*, 7b, 443 (1952)
- Warburg, O., and Luttgens, H., *Biokhimiya*, 11, 303 (1946)
- Wayrynen, R. E., *Dissertation, Univ. of Utah, Salt Lake City, Utah* (1952)
- Yamafuji, K., Fujii, M., and Nishioeda, M., *Biochem. Z.*, 296, 348 (1938)
- Yamafuji, K., Nishioeda, M., and So, K., *Biochem. Z.*, 298, 293 (1938)
- Yamafuji, K., So, K., and Tin, H., *Biochem. Z.*, 300, 414 (1939)

Yamafuji, K., Nishioeda, N., and Imagawa, H., Biochem. Z., 304, 404 (1939)

Yocum, C. S., and Blinks, L. R., J. Gen. Physiol., 38, 1 (1954)

Zill, L. P., and Tolbert, N. E., "The Effects of Ionizing and Non-ionizing Radiation on Photosynthesis by Plants." Abstract: Plant Physiol. 30 (Supplement) xiv, (1955) Reported: The American Soc. of Plant Physiologists and the Physiological Section of the Botanical Soc. of America. Michigan State Univ., East Lansing Mich. Sept. 5-8, 1955

I-III AND

1-112

THIS PAGE
WAS INTENTIONALLY
LEFT BLANK