The Use of an Oxygen Sensor and a Carbon Dioxide Electrode to Study the Effects of 2,4-Dichlorophenoxyacetic Acid on Photosynthesis of Isolated Chloroplasts

A Thesis

by

Paul B. Rolfe

Submitted to the Graduate School of Appalachian State University as Partial Fulfillment of the Requirements for the Degree of Master of Science

Approved by:

William L. Haag
Chairman, Advisory Committee

Thomas C. Rhyne
Member

Donald A. Sanders
Member

U. M. Dunlap, assistant
Dean of Graduate School

James W. Bridges
Chairman, Chemistry Department

May 9, 1980

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ABSTRACT

A brief review of the reactions of photosynthesis is presented and it is noted that the current state of knowledge concerning them is incomplete. There are several reasons why the herbicide 2,4-dichlorophenoxyacetic acid, 2,4-D, might be suspected as having an effect on some of these reactions although it may not be the primary mode of action. To study the effect of 2,4-D on photosynthesis, the rate of oxygen evolution and the rate of carbon dioxide fixation by a chloroplast preparation from field pea leaves was determined by using an oxygen sensor and a carbon dioxide electrode. It was found that 2,4-D had no noticeable effect on the light reactions, whereas it appeared to stimulate the fixation of carbon dioxide by 177%. All of the concentrations of 2,4-D used resulted in an increased rate but the concentration where maximum stimulation was found was 5.0×10^{-5} M.

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LIST OF ABBREVIATIONS

NADP	Nicotinamide Adenine Dinucleotide Phosphate
NADPH	Nicotinamide Adenine Dinucleotide Phosphate, reduced form
P700	Special chlorophyll *a* molecule that absorbs radiation of 700nm and is involved in photosystem I
Q	Immediate electron acceptor in photosystem II
FRS	Ferredoxin Reducing Substance, the immediate electron acceptor in photosystem I
ATP	Adenosine TriPhosphate
ADP	Adenosine DiPhosphate
2,4-D	2,4-Dichlorophenoxyacetic acid
2,4,5-T	2,4,5-Trichlorophenoxyacetic acid
IAA	3-IndoleAcetic Acid

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INTRODUCTION

Photosynthesis, the means by which all green plants and some unicellular organisms harness energy from light and convert it into chemical energy to be used in synthetic processes, is a function that is vital to almost all living creatures that inhabit the biosphere. Animals depend on plants not only for oxygen rejuvenation, but also as a primary source of nutrition. An understanding of the biochemical processes that a plant utilizes can help man develop crops with greater yields and higher nutritional value.

Even though a great number of dramatic advances have been made in solving the mystery of photosynthesis, there are still quite a number of pieces to the puzzle yet to be resolved. Over the past 15-20 years, there have been numerous theories proposed to explain certain observations. Controversy surrounding many of these proposals led to the complete disposal of some, the tentative acceptance of others, and the proof of a few following some debate and much experimentation.

A classical example of man's attempts to control plant growth to his advantage has been the development and use of an arsenal of herbicides. At present, there are close to 200 compounds in use as herbicides. By cleaning out unwanted and harmful plant growth, more room was made for crop plants which increased their numbers and yield. Recently, herbicides have been used with great success in laboratories to isolate a particular biochemical pathway so that it may be better understood. Photosynthesis is an example of one of the pathways where much has been learned by fragmenting the pathway with the aid of regulators and studying the role of intermediates, electron transmitters,

enzymes, and the effects of external stimuli such as temperature and light intensity.

What follows is a brief summary of the reactions that are found in the chloroplasts of green plants.(Gregory,1971; San Pietro, 1971; Zelitch, 1979) The reactions of photosynthesis can be divided into two parts, the light and the dark reactions. An oversimplified representation of the combined light and dark reactions can be represented by the following equation:

$$nH_2O + nCO_2 \xrightarrow[\text{chloroplasts}]{h\nu} (CH_2O)_n + nO_2$$

Both reactions occur in a subcellular organelle found in green plants, the chloroplast. The chloroplast is enclosed in a membrane and contains a thylakoid system which is a membrane-bound particle and is the site of the light reactions. The thylakoid system is surrounded by a solution phase, or stroma, which is the site of the dark reactions.

The enzymes for the light reactions are attached to the membrane of the thylakoid system. If the external chloroplast membrane were to rupture, the light reactions would still be active, provided that the proper conditions to maintain viability still existed.

Present theory states that the light reactions are made up of two distinct photosystems. The overall reaction involving both photosystems can be represented by:

$$H_2O + A_{ox} \xrightarrow[\text{chloroplasts}]{h\nu} \tfrac{1}{2}O_2 + A_{red} + 2H^+$$

where A_{ox} and A_{red} are called the electron acceptor and the electron donor respectively. In plant systems electrons are supplied by water

generating oxygen in the process, and received by Nicatinamide Adenine Dinucleotide NADP, to form NADPH. The NADPH is used in the dark reactions to indirectly reduce carbon dioxide; so it can be said that the ultimate electron acceptor is CO_2. Photo-oxidation of water followed by an electron transport which reduces a natural or an artificial electron acceptor is often referred to as the Hill reaction. The term electron transport refers to the electron flow from the oxidation of water to the reduction of an electron acceptor via a number of intermediates.

An example of an artificial electron acceptor is ferricyanide. It has been shown that ferricyanide accepts electrons from photosystem II in broken chloroplasts.(San Pietro, 1971) However, there are theories that it accepts electrons from photosystem I in whole chloroplasts. (San Pietro, 1971)

The photosystems are driven by separate light sensitive pigment systems. Photosystem I has a pigment system that absorbs light of a wavelength of 700nm and the pigment system of photosystem II absorbs at 680nm. Both of the pigment systems contain chlorophyll *a* and *b* along with an assortment of accessory pigments.

In photosystem I there appears to be a "photosynthetic unit" in which chlorophyll and the accessory pigments absorb and channel the energy from light to a specialized chlorophyll molecule (P700) or molecules at a reaction center. The ratio of the pigments making up the unit to those at the reaction center is about 300:1. There are appoximately eight of these systems, or about 2500 chlorophyll molecules, acting together to move four electrons along the electron transport chain which eventually leads to the reduction of a single molecule of CO_2.(Gregory, 1971) The pigment system of photosystem II is more complex and less understood.

The light reactions are usually illustrated by the "Z" diagram shown in Figure 1.

Photosystem II begins with the oxidation of water and ends in the reduction of an electron acceptor labelled Q. The structure of Q has not yet been determined. Q is oxidized by a series of redox intermediates including Plastoquinone, Cytochrome b_3, Cytochrome f and Plastocyanine. Movement along this chain results in a loss of potential but to a level not less than that at the beginning of photosystem II. This series of intermediates leads to the photosystem I oxidant.

Photosystem I is activated by a photon striking its pigment system giving rise to an electron acceptor called Ferredoxin Reducing Substance, FRS. FRS may be a form of enzyme-bound ferredoxin, which is further reduced by a flavoprotein. The electron transport chain is completed with the reduction of NADP.

An alternate pathway that bypasses the production of NADPH is a cyclic flow in which FRS, instead of reducing ferredoxin, passes electrons first to Cytochrome b_6, then into the basal electron flow somewhere after Plastoquinone.

The formation of ATP from ADP in the presence of magnesium ions and inorganic phosphate, is coupled to the electron transport flow, probably between Cytochrome b_3 and Cytochrome f. The mechanism involved in this coupling is not known and there is disagreement as to whether one or two ATP molecules are formed per passage of the basal electron transport. The ATP and NADPH formed in the light reactions are used to supply energy and reducing power for the reduction of CO_2.

ATP formation may be uncoupled from the basal electron transport by the addition of various agents. Uncoupling abolishes photophosphorylation without inhibiting electron transport. In fact, electron trans-

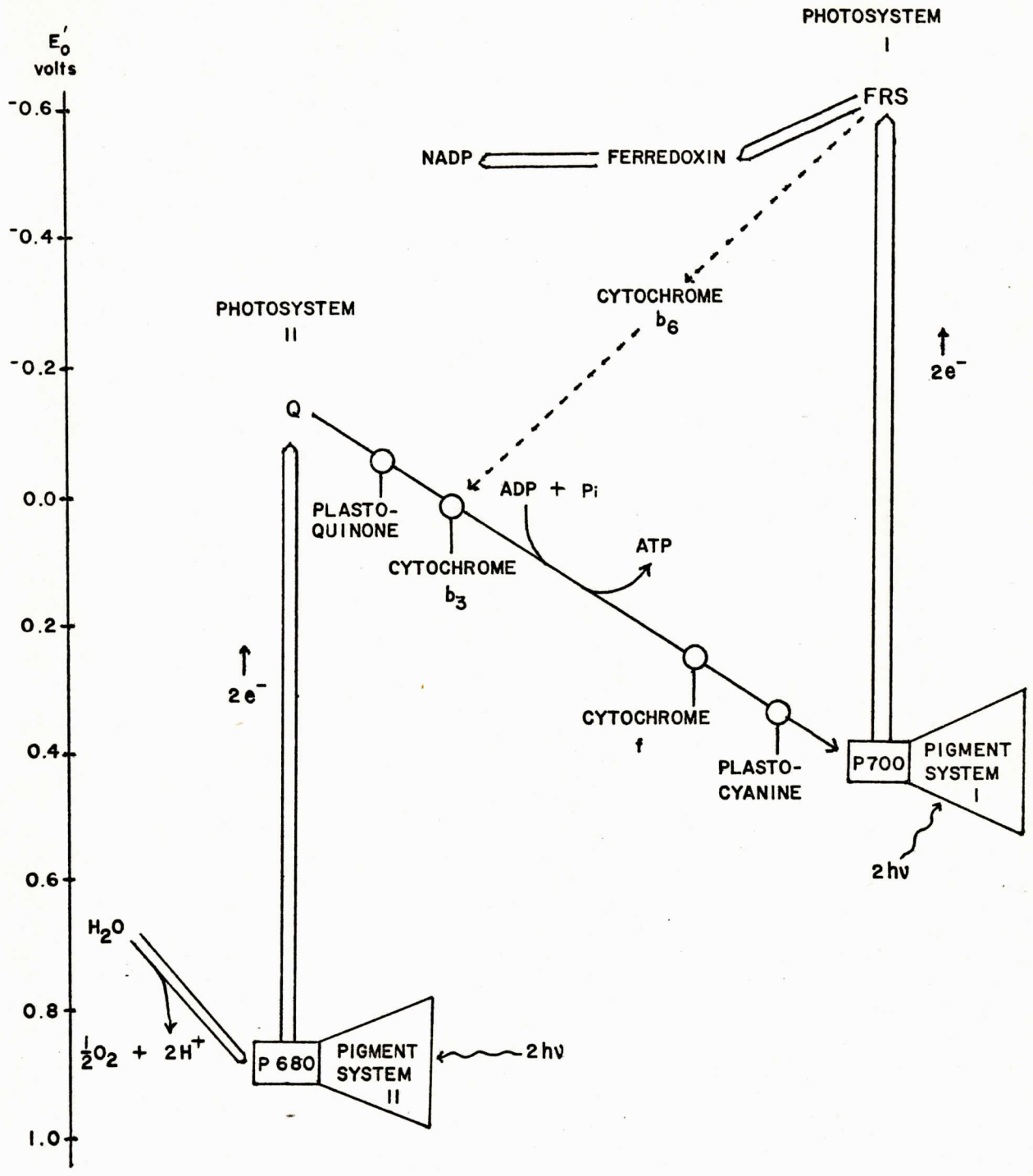


Figure 1. The light reactions of photosynthesis, the "Z" diagram

port, freed from restraints imposed by the coupling mechanism may be greatly accelerated as the phosphorylation diminishes. Ammonium salts are examples of compounds that have been found to reversibly uncouple phosphorylation. (San Pietro, 1971)

The dark reactions, also known as the Calvin cycle after Melvin Calvin who received the Nobel Prize in 1961 for identifying the intermediates involved, occur in the solution phase or stroma portion of the choroplasts. The Calvin cycle is disrupted if the outer chloroplast membrane is ruptured during isolation procedures. However, activity can be restored to broken chloroplasts by the addition of various co-factors. (Jensen and Bassham,1966)

Unlike the energy-harnessing light reactions, the reactions of the Calvin cycle are understood quite well. The chemical form in which carbon is assimilated is free CO_2 rather than HCO_3^- or $CO_3^=$. The Calvin cycle is illustrated in Figure 2.

The first step involved in the cycle is the carboxylation of ribulose-1,5-diphosphate. The product formed is believed to be an enzyme-bound intermediate which breaks down into two molecules of 3-phosphoglycerate (This occurs only in those green plants classified as having C_3 systems. Most plants on this continent have C_3 systems.). The enzyme involved in this step is ribulose-1,5-diphosphate carboxydismutase which is found to be in great supply making up 15% of the total protein within the chloroplast (Lehninger, 1975).

The cycle proceeds through a series of intermediates, eventually returning to ribulose-1,5-diphosphate and completing the cycle. One of the intermediates of the cycle is fructose-6-phosphate which can be converted to other sugars such as glucose. The cycle must be completed six times (six molecules of CO_2 fixed) to produce an adequate excess

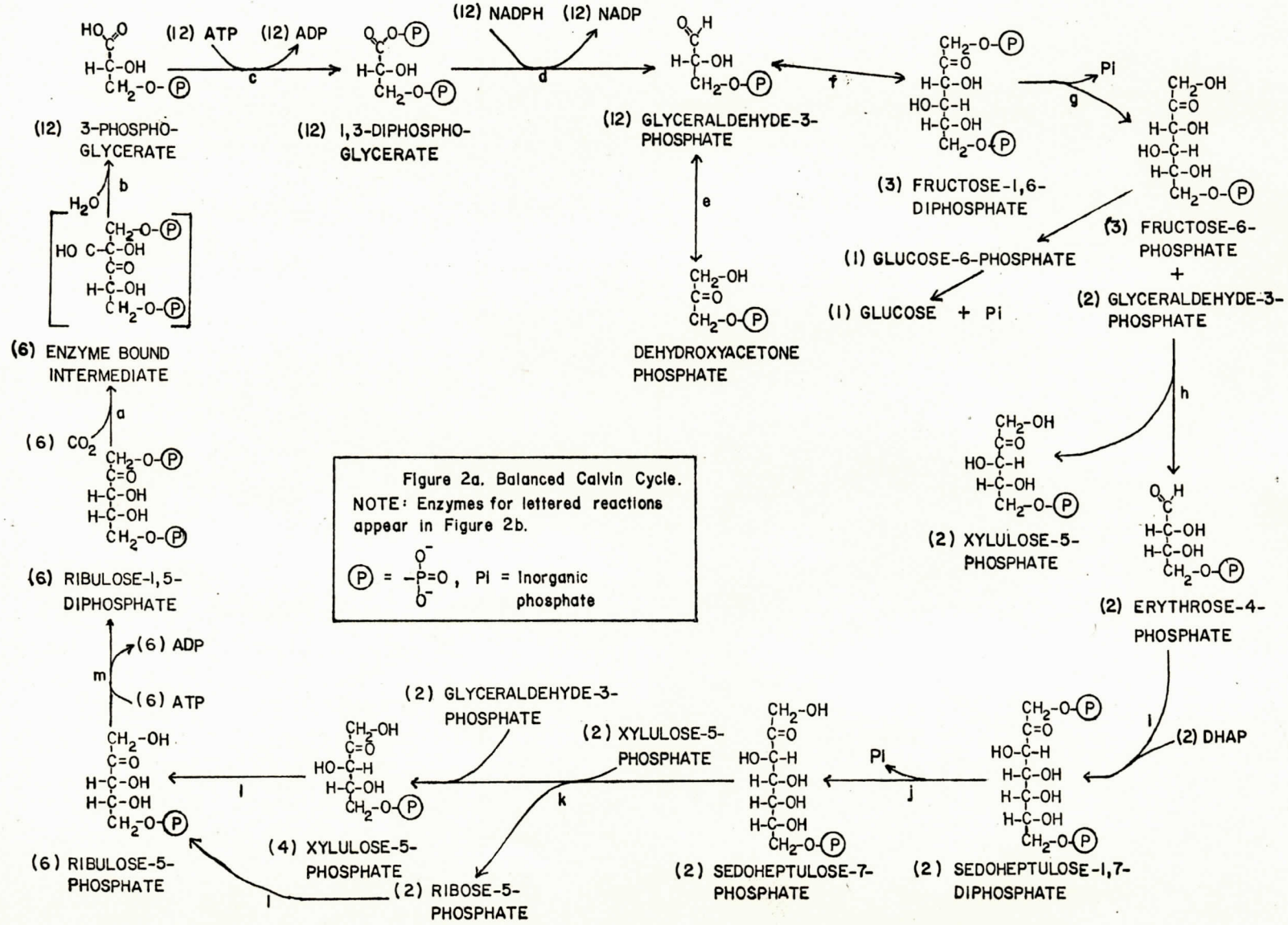


Figure 2a. Balanced Calvin Cycle.
NOTE: Enzymes for lettered reactions appear in Figure 2b.

Ⓟ = $-\overset{O^-}{\underset{O^-}{P}}=O$, Pi = Inorganic phosphate

LETTERED REACTION	ENZYME
a	Ribulosediphosphate carboxydismutase
b	Spontaneous
c	Phosphoglutarate kinase
d	Glyceraldehyde dehydrogenase
e	Triose isomerase
f	Aldolase
g	Fructose-1,6-diphosphate dephosphatase
h	Trans ketolase
i	Aldolase
j	Sedoheptulose-1,7-diphosphate dephosphatase
k	Trans ketolase
l	Phosphopentose isomerase
m	Phosphoribulokinase

Figure 2b. Enzymes of the Calvin Cycle

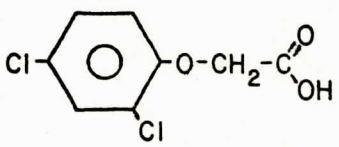
of fructose-6-phosphate (one molecule) for use elsewhere without hindering the flow of the cycle.

The dark reactions are dependent upon the light reactions for energy (ATP) and reducing power (NADPH). There are two energy-requiring steps in the Calvin cycle that utilize ATP. Eighteen moles of ATP are required for every mole of glucose produced.

NADPH is required at only one site in the Calvin cycle, which is at the reduction of 1,3-diphosphoglycerate to glyceraldehyde-3-phosphate. Twelve moles of NADPH are required in the production of one mole of glucose.

The dependency of the Calvin cycle upon the light reactions can be overcome by the addition of ATP and NADPH (Jensen and Bassham, 1966), provided that the outer chloroplast membrane remains intact.

The herbicide used in this work was 2,4-dichlorophenoxyacetic acid, herein referred to by its common abreviation, 2,4-D. 2,4-D, which has the structure,



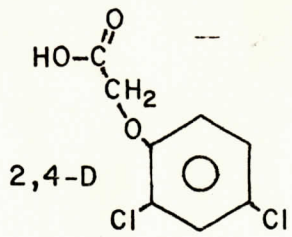
has had wide use as an herbicide since its discovery at the end of World War II. The U.S. production of 2,4-D and 2,4,5-trichlorophenoxyacetic acid, 2,4,5-T, peaked in 1968 at 48397 tons (Ashton and Crafts, 1973) when mixtures of these compounds were used in Viet Nam as the defolient "Agent Orange". Since then its use has steadily declined primarily because of possible health hazards, particularly those associated with 2,4,5-T. The production of 2,4-D and 2,4,5-T in the United States in 1969 was cut nearly in half to 26038 tons (Ashton and Crafts, 1973).

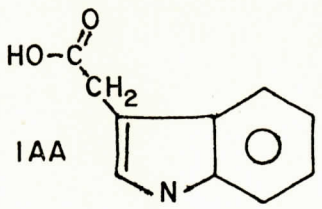
The Environmental Protection Agency suspended all usage of 2,4,5-T in 1979 pending further studies regarding its threat to human health.

2,4-D is a selective herbicide that has damaging or lethal effects on most broadleaf plants. There are several possible explanations for this selectivity. 2,4-D has been found to have profound effects on certain enzymes of susceptible plants. Nonsusceptible plant species may be lacking in these particular enzymes. The action of 2,4-D is primarily on actively growing plant tissues which means that it must be translocated via vascular channels to the parts of the plant which are engaged in rapid growth. Susceptible plant species have vascular systems that are more effecient in this translocation process. Other factors causing greater susceptibility may include a less rapid metabolism and the absence of an agent that alters the 2,4-D molecule and renders it harmless.

A great deal of research has been done on 2,4-D and its effects on plant tissues. It has been determined that there are only a few major biochemical pathways that escape being affected in some way, by some concentration of 2,4-D. Some of these are direct effects and some are indirect. The majority of the sites of action of the herbicide are involved with the growth processes of the plant. Many of these effects have been claimed to be the cause of death of the plant, but the actual cause of death cannot be attributed to any one of them. All of them work together to achieve the final result.

The action of 2,4-D has often been compared to the action of the natural plant auxin 3-indoleacetic acid, IAA. The comparison has been supported by experimental evidence. The plant responses common to 2,4-D and IAA have been attributed to similarities in structure. (Audus, 1976)





However there are several cases where the effects of 2,4-D and IAA diverge.

The gross physiological responses of a plant susceptible to 2,4-D include an initial stimulation of growth which is characterized by massive cell proliferation along the stems and roots. In the vascular tissues, the cell walls soften and stretch allowing the cells to expand. The increased size of the cells leads to narrowing and eventually, complete blockage of the xylem and phloem. The resulting failure of the plant to transport foods, minerals and essential materials to areas of need results in the plant starving to death. In general terms, 2,4-D acts on the vascular tissues causing a disturbance in tissue morphology and in the shape, size and divisions of the cells. Fragmentations and fusions of nuclei, blocked metaphases, enlarged nuclei, multinucleate cells and even prevention of cell divisions also have been observed. (Ashton and Crafts, 1973)

Treatment of the curly dock plant with 2,4-D twelve days before flowering inhibited viable seed formation. Treatment at seven days after flowering produced seeds with embryos in 91% of the seeds but there was a reduction in weight and only 5 to 15% germinated. (Mann and Pu, 1967) Stomatal closure has also been observed in some plants.

It has been reported that 2,4-D has an effect on a great number of processes. As early as 1959 studies were published which reported an increase in protein, nucleic acid and acid soluble nucleotides in response to 2,4-D treatment. Audus (1976), Ashton and Crafts (1973),

and Kearney and Kaufman (1976) provide excellent overviews of the effects of 2,4-D on these processes. 2,4-D stimulates production of all RNA but mostly messenger RNA followed by ribosomal RNA. (Kearney and Kaufman, 1976) Since RNA and protein synthesis in regions of rapid cell proliferation are increased by increased nuclear activity and thus by DNA, it has been proposed that the primary site of action of 2,4-D might be in the nucleus. (Chrispeels and Hanson, 1962) In more recent years, the number of reports concerning increased nucleic acid and protein synthesis as a result of 2,4-D treatment have grown considerably. Each report concerns different plants, different methods of application and methods of analysis but with the same results. One theory for explaining the increase in RNA synthesis is the suggestion that 2,4-D enhances the activity of RNA polymerase. (Audus, 1976 and Moreland, 1967)

The treatment of young grain sorghum plants with 2,4-D has been found to result in a number of chromosome aberrations. (Ashton and Crafts, 1973)

In hemp sesbania plants, 2,4-D at 4.5×10^{-6}M stimulated lipid biosynthesis by 70%. This stimulation decreased to 12% when the 2,4-D concentration was increased to 9.0×10^{-5}M. (Mann and Pu, 1967)

There are a number of reports stating that the phenoxy acid herbicides affect respiration (Lotlikar *et al.*,1965, Wedding and Black, 1961 and Switzer, 1956) and intermediary metabolism (Black and Humphreys, 1961).

Lotlikar *et al.* (1965) used varying concentrations of 2,4-D and studied the response of oxidative phosphorylation in isolated cabbage mitochondria. They reported that 2,4-D at 2.5×10^{-3}M inhibited phosphorylation completely and lowered oxygen uptake by 68%. They also presented evidence that 2,4-D may inhibit electron transport in an

indirect manner. A study of the effect of 2,4-D on ATPase activity and ATP-^{32}P exchange rates suggested that 2,4-D interferes with one or more of the reactions which couple the phosphorylation of ADP to electron transport. 2,4,5-T at 5.0 x 10^{-4}M inhibited oxygen uptake by 17% and phosphorylation by 53%. IAA has been reported to have no affect on oxidative phosphorylation.

Black and Humphreys (1961) studied the effects of 2,4-D at 10^{-5}M on corn seedlings and found that the utilization of glucose via the Pentose Phosphate Pathway, PPP, was increased whereas its utilization via glycolysis was inhibited. This was apparently due to an enhancement in the activity of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, enzymes found in the PPP. At the same time there was a reduction in the activity of the glycolytic enzymes, 6-phosphofructokinase, aldolase, and glyceraldehyde-3-phosphate dehydrogenase.

A secondary effect of 2,4-D treatment is the increased production of ethylene. Ethylene has numerous effects that are either stimulating or inhibiting depending upon its concentration. However it is not considered to be a significant contributor to the cause of death of the plant. (Audus, 1976)

The application of 2,4-D might be expected to have some effects on the many reactions that take place in photosynthesis. Many workers have reported changes in photosynthetic rates as a result of 2,4-D application.

Sasaki and Koylowski (1966) reported an inhibition of carbon dioxide fixation in young pea seedlings *in vivo*. It was suggested that this was the result of an inhibition of the Hill reactions.

2,4,5-T has been shown to inhibit the Hill reactions by 50% at a concentration of 3.6 x 10^{-3}M in isolated chloroplasts from soybean.

Turner and Bidwell (1964) monitored photosynthetic CO_2 uptake in bean leaves, finding an increase in CO_2 fixation as a result of spraying with IAA. They also determined that the increased rate of CO_2 fixation was not due to an effect on the light reactions.

In general, reports indicating a response of the dark reactions to applied 2,4-D have not been frequent. There have been occasional publications that reported an effect of 2,4-D on the light reactions. Reviews and reports listing those herbicides that are photosynthetically active generally do not include 2,4-D. (Ashton and Crafts, 1973, Audus, 1976 and Moreland,1967) Furthermore, 2,4-D is also excluded from lists of those herbicides that do not effect photosynthesis. Table I lists a few examples of photosynthesis-inhibiting herbicides.

The light reactions of photosynthesis may be inhibited (or enhanced) in at least four different ways: 1) electron transport inhibitors; 2) uncouplers of phosphorylation; 3) energy transfer inhibitors; and 4) electron acceptors. (Audus, 1976) Additional effects may be combinations of two or more of these.

One important observation to be noted in studying the effects of an herbicide on the Hill reactions was the ratio of moles of herbicide to moles of chlorophyll. This information may provide a hint as to the site of action of an herbicide. For example, Baldwin *et al.* (1968) found that in the case of the herbicide paraquat, this ratio was approximately 1:200. Paraquat acts by interfering with electron flow but it will also kill the plant very slowly in the dark, which suggests an additional site of action. (Kearney and Kaufman, 1976, b) This extra site has been used to support the findings of Baldwin *et al.* (1968) who should have found a ratio nearer 1:600 which would be equivalent to one mole of herbicide per photosynthetic unit.

The harmful effects on the electron transport reactions of the herbicides listed in Table I can be avoided by a thorough washing of the chloroplasts. This suggests that the herbicides act by forming weak hydrogen bonds or have weak Van der Waal attractions with the electron carriers.

A common structural characteristic of herbicides which inhibit photosynthetic processes is an N-H group attached to a partially positive sp^2 hybridized carbon atom. (Audus, 1976) Although 2,4-D does not have this structural characteristic, there are several reasons why it may be suspected of having herbicidal effects on photosynthesis. The electron transport and coupled phosphorylation reactions that are found in chloroplasts are analogous to the cytochrome system and oxidative phosphorylation found in mitochondria. It would not be unreasonable to predict that the inhibitory and uncoupling action of 2,4-D within the mitochondria would also occur within the chloroplasts. In addition, the growth-stimulating responses due to 2,4-D, such as the stimulation of nucleic acid and protein synthesis, require an accompanying increase in the rates of the energy-yielding processes such as photosynthesis. Therefore 2,4-D might be expected to enhance the production of energy-rich molecules.

Another parallel that would suggest further research on the effects of 2,4-D on photosynthesis can be found in the dark reactions. The glycolysis enzymes previously mentioned as being inhibited by 2,4-D are also found in the Calvin cycle with the exception of phosphofructokinase. It might be suggested that an inhibition of CO_2 fixation would also occur. On the other hand, an increased synthetic activity in response to 2,4-D treatment would require an increased assimilation of carbon from photosynthesis. Thus 2,4-D might also be expected to have a stimulating effect

TABLE I. Photosynthetically-inhibiting herbicides. It should be noted that the herbicides that inhibit the photosystems also indirectly inhibit the fixation of CO_2

CLASS OF HERBICIDES, EXAMPLES	SITE OF INHIBITION	REFERENCES*
Substituted Ureas: diuron, monuron, DCMV	PS II	a, b, c
Triazines: simazine atrazine	PS II	a, b, c
Quaternary Dipyridyl Salts: diquat, paraquat	PS I	a, d
Phenoxy Acetic Acids: 2,4-D, 2,4,5-T	Calvin Cycle and PS II- 2,4,5-T only	b
Substituted Phenols: dinitro and pentachloro phenol	Uncoupler of Phosphorylation	e
Anilides	PS II	f, c
Benznitriles		c
Substituted Uracils		c
N-Phenyl Carbamates		c

*REFERENCES

[a]Black and Myers, 1966

[b]Sasaki and Koylowski, 1966

[c]Moreland, 1967

[d]Baldwin *et. al.*, 1968

[e]Krogmann *et. al.*, 1958

[f]Zweig, 1969

on the dark reactions.

In the past, many methods have been developed for studying the effects of herbicides on photosynthesis. It is difficult to determine the effects of an herbicide on specific biochemical processes of the plant without removing tissues for examination and hindering future growth and development of the plant. As a result, plants were treated with an herbicide and the entire plant or selected parts were broken down and analyzed. An alternate method was to isolate the cellular or subcellular parts in a manner that would not disrupt their normal activity and apply the herbicide to determine its effects *in vitro*. In the case of photosynthesis a chloroplast extraction would be used.

Isolation of an active chloroplast preparation is a technique that has been developed extensively during the past 20 years. Chloroplasts are very fragile; the outer membrane can be ruptured easily if such variables as osmotic pressure, temperature and pH are not regulated. Hill reaction studies can be made with cell-free extracts by rupturing the chloroplast membrane but the system obtained from this procedure would not resemble the *in vivo* system. This rupture is very detrimental to the activity of the dark reactions.(Walker. 1965)

Intact chloroplasts can be distinguished from ruptured ones by the use of a light microscope. Jensen and Bassham (1966) illustrated the observable differences between broken and intact chloroplasts by providing photographs taken through a Zeiss photomicroscope under phase contrast. The broken chloroplasts are dark and appear granulated whereas whole chloroplasts are more rounded and appear opaque.

The isolation procedures developed and reported by Walker (1964 and 1968) and Jensen and Bassham (1966) are the most sucessful and have been adopted, sometimes with slight modifications, by a substantial

number of workers. Stated briefly, their procedure involves a rapid but brief disruption of the cells in a blender or mortar and pestle in a buffer solution. This is followed by a preliminary filtration and then centrifugation of the chloroplasts so that they formed a pellet. The buffer solution was decanted and the pellet broken up and suspended in another buffer solution. Several modifications of this procedure include the use of different buffer systems and occasionally the preliminary filtration was replaced by a preliminary centrifugation at low speed. Rough suspensions, still containing some cell debris, were made by omitting the centrifugation altogether. Once an active chloroplast suspension was obtained, the light and dark reactions could be studied.

An early method for monitoring photosynthetic gas exchange was the use of the Warburg apparatus. This method has been replaced by less cumbersome methods.

A polarographic oxygen sensor has become very popular for monitoring oxygen evolution by illuminated chloroplasts. The theory, construction and application of so-called polarographic oxygen sensors is the subject of a book written by Fatt (1976). The application of an oxygen sensor for determining the rates of photosynthetic oxygen evolution is described by Walker *et al.* (1968) and Shugarman and Appleman (1967). The latter workers describe the use of a chamber, designed and constructed from plexiglass, especially for this purpose.

Another means of determining the rate of the Hill reactions was to follow the rate of disappearance of the natural or an added artificial electron acceptor with visible or ultraviolet light spectroscopy.

The most widely-employed method of evaluating the dark reactions has been to determine the rate of incorporation of carbon dioxide into

the Calvin cycle intermediates. This has been done by chromatographically analyzing the carbohydrates that contain ^{14}C. The ^{14}C is introduced as $^{14}CO_2$ or $NaH^{14}CO_3$. Walker (1964) used this method and the chloroplast isolation procedure mentioned previously to attain the highest carbon dioxide fixation rates yet achieved.

Turner and Bidwell (1964) used $NaH^{14}CO_3$ in a different manner to determine the effects of IAA spraying on CO_2 fixation. They floated an intact, detached leaf on water contained in a beaker. The entire apparatus was placed in a small chamber for gas exchange studies. An attached tube containing a solution of $NaH^{14}CO_3$ was treated with lactic acid to generate $^{14}CO_2$ which diffused into the chamber containing the leaf. The system was sealed and the disappearance of $^{14}CO_2$ from the air above the leaf, determined by a Geiger counter, was taken to be equivalent to the CO_2 fixed by the leaf.

Until now, the use of a CO_2 electrode to monitor the carbon dioxide exchange of isolated chloroplasts has not been reported. The response time of the CO_2 electrode is somewhat slow at low CO_2concentrations. Various factors that affect the response time characteristics of the CO_2 electrode are discussed by Jensen and Rechnitz (1979).

Carbon dioxide fixation and oxygen evolution are most often reported with respect to the milligrams of chlorophyll present in the preparation. This is accomplished by measuring the absorbance at 645 and 663nm of a solution of the chlorophyll in 80% acetone as determined by the absorption spectra of chlorophyll *a* and *b* shown in Figure 3 (Vernon and Seeley, 1966). In 80% acetone, the concentrations of chlorophyll *a* and *b* in mg/mL can be determined by recording the absorbances and substituting into the following equations (Arnon, 1949):

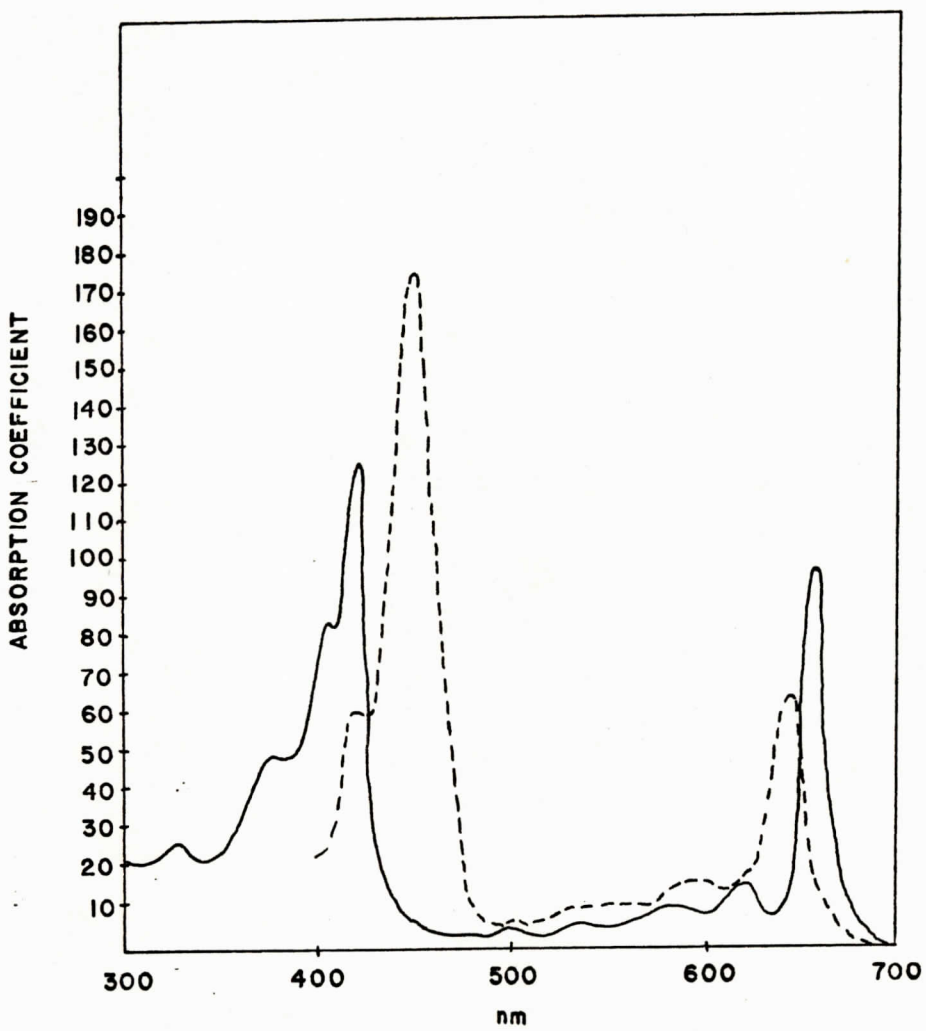


Figure 3. Absorption spectra of chlorophyll *a* (solid line) and chlorophyll *b* (dotted line)

$$C_{chl\ a} = 0.0127(ABS_{663}) - 0.00269(ABS_{645}) \quad (1)$$

$$C_{chl\ b} = 0.0229(ABS_{645}) - 0.00468(ABS_{663}) \quad (2)$$

These equations can only be used when there is a distinct maximum at 645nm representing the absorbance of chlorophyll *b*. In most green plants, the ratio of chorophyll *a* to chlorophyll *b* is approximately 3:1, although variations have been noted.

In the chlorophyll extracts from some plants, there may not be a distinct maximum at 645nm. In this case, the absorbance at 652nm is determined and substituted into the following equation (Arnon,1949):

$$C_{chl} = (ABS_{652})/34.5 \quad (3)$$

The result of using this equation is a value that represents the sum of both types of chlorophyll.

MATERIALS

The chemicals used were obtained from the following suppliers: $NaHSO_3$, NH_4Cl, NaCl, $MgSO_4$, $K_3Fe(CN)_6$, $Na_4P_4O_7 \cdot 10H_2O$, sodium citrate, tris (hydroxymethyl) aminomethane (THAM) and acetone were from Fisher Scientific Company; EDTA-disodium salt, and $NaHCO_3$ were from Matheson, Coleman and Bell; isoascorbic acid from Eastman Kodak Company, and the 2,4-D was from the Sigma Chemical Company. The chemicals contained in Skoogs media were of reagent grade quality and from major suppliers.

The instrumentation and accessories used were the following: 75 watt Plant Light from General Electric; Fisher Accumet Model 320 Expanded Scale Research pH meter; Honeywell dual channel strip chart recorder; Lazar DO-166 Dissolved Oxygen Probe; Orion Research Company CO_2 electrode, Model 95-02; Beckman Model DB-G Grating Spectrophotometer; Sargent Welch Model SRLG Recorder; Light Microscope manufactured by Swift Instruments Internationsl S.A.; Fisher Magnetic Stirring motor and a Servall centrifuge from Ivan Sorvall, Inc..

The plants used were Field Peas, the seeds of which were purchased from a local supermarket. The plants were grown in fine, 100% Vermiculite from Grace Construction Products.

PROCEDURE

The Field Pea seeds were placed approximately one centimeter beneath the surface of a four inch layer of vermiculite contained in 10 ounce waxed paper cups. There were four seeds to each cup. Drainage was provided by piercing the bottoms of the cups with the tip of a knife blade. The cups were placed in tall petri dishes and the entire assembly placed in an aquarium which was used as a growth chamber. The vermiculite was thoroughly soaked with Skoogs growing solution.
Large quantities of the growing solution were prepared each time from stock solutions listed in Table II. These reagents reportedly provide the plants with its required minerals.

A 75 watt plant light was supported about 12 inches above the cups. The light, which also provided some warmth, was controlled by a timer such that the plants would receive about 14 hours of light and 10 hours of darkness in each 24 hour period. On the average, it took the seeds about 72 hours to germinate. This was temperature dependent and the plants were grown at room temperature. The plants were allowed to grow to a height of about 10 inches (the light was moved up to accomodate them) before the leaves were removed. At times during the growing process, when the vermiculite appeared to be drying out, it was remoistened with tap water.

The chloroplast extract was prepared from one large or two small leaves. The amount of leaf material was roughly 0.2 to 0.3 gram. The leaf material was placed in a glass mortar and was ground with a pestle along with about 2 mL of buffer solution. Before filtering, an additional 7 mL of buffer was added and the suspension filtered with suction though

TABLE II: Skoogs growing solution. (Murashige and Skoog, 1962)

MAJOR ELEMENTS	CONCENTRATION, mg/liter
NH_4NO_3	1650
KNO_3	1900
$CaCl_2 \cdot 2H_2O$	440
$MgSO_4 \cdot 7H_2O$	370
KH_2PO_4	170

MINOR ELEMENTS	CONCENTRATION, mg/liter
H_3BO_3	6.2
$MnSO_4 \cdot 4H_2O$	22.3
$ZnSO_4 \cdot 4H_2O$	8.6
KI	0.83
$Na_2MoO_4 \cdot 2H_2O$	0.25
$CuSO_4 \cdot 5H_2O$	0.025
$CoCl_2 \cdot 6H_2O$	0.025

IRON	CONCENTRATION, g/liter
Na_2-EDTA	7.45
$FeSO_4 \cdot 7H_2O$	5.57

three layers of muslin.

The buffer solution maintained a pH of 8.0 and contained the ingredients listed in Table III. Also in Table III are some of the explanations that support the use of the particular solutes.

Part of the chloroplast suspension was examined under a microscope using high magnification and it was noted that it contained some fine particles of cell debris but mostly intact chloroplasts. This microscopic examination was not carried out on each chloroplast preparation. The suspension was used immediately after preparation for either the determination of oxygen evolution or carbon dioxide fixation.

The rate of oxygen evolution was determined using a small tube as a reaction chamber. A #4 rubber stopper, with a large hole bored through it, was placed over the end of the oxygen probe like a sleeve. The size of the stopper was chosen so that the assembled apparatus formed a very tight seal with the reaction chamber. A small magnetic stirring bar was included in the reaction chamber. Completely assembled, the chamber had a volume of 4.88 ±0.03 mL.

Oxygen evolution experiments were started under yellow light to prevent premature reactions. Before assembly, 9.0 mL of a chloroplast suspension was added to the reaction chamber. To this was added 0.5 mL of a supplementary reaction solution which contained enough potassium ferricyanide, ammonium chloride and sodium bicarbonate to give final concentrations of 4.0×10^{-3}, 8.0×10^{-3} and 2.0×10^{-2} molar respectively in the assembled apparatus.

Finally, to those runs where 2,4-D was not to be present, 0.5 mL of water was introduced and for those including 2,4-D, 0.5 mL of a solution of the sodium salt of 2,4-D, which had previously been adjusted to pH 8.0, was used. The concentrations of the 2,4-D solutions were

TABLE III. Solutes present in buffer solution, concentrations and reasons for their use

SOLUTE	MOLAR CONCENTRATION	REASON FOR USE
THAM	2.5×10^{-2}	Buffer
Na_2 - EDTA	2.0×10^{-3}	Chelating agent
Na_2HPO_3	5.0×10^{-4}	Inorganic phosphate is necessary for photophosphorylation.
$Na_2P_2O_7 \cdot 10H_2O$	5.0×10^{-3}	Alleviate the inhibitory effects of orthophosphate[a]*
Isoascorbate	2.0×10^{-3}	Enhance performance of chloroplasts[a]
NaCl	2.5×10^{-2}	Chloride ion is necessary for the oxidation of water in the Hill reactions[b,c]
$MgSO_4$	2.0×10^{-3}	Magnesium ion necessary for photophosphorylation
6N HCl		Sufficient amount was needed to adjust the pH of the final solution to 8.0

*REFERENCES

[a]San Pietro, 1971

[b]Hind, *et. al.*, 1969

[c]Heath and Hind, 1969

varied to obtain a range of final concentrations from 5.0×10^{-4} to 5.0×10^{-2} molar.

After the solutions had mixed thoroughly, the oxygen probe, with the rubber stopper in place, was pushed into the chamber. The excess solution was forced out over the sides of the tube so that once the stopper was firmly in place, there were no air bubbles trapped inside. The apparatus was lowered into a water bath so that the temperature would remain constant at 25° celsius.

The stirring motor, which supported the glass dish containing the water bath, was started. The pH meter was set on an expanded millivolt scale, and the recorder, at a chart speed of 0.25 inch per minute, was turned on.

The initial tracing on the chart was flat but would soon begin to move downscale due to respiratory O_2 uptake. (Mitochondria were not excluded from the preparation.) When the pen reached the lower end of the scale, a light, (the same light used to grow the plants) approximately 10 inches from the reaction chamber, was turned on. The recorder tracing would reverse its direction due to photosynthetic oxygen evolution, and would continue to rise until it gradually reached a maximum, leveled off briefly, and then it would begin to fall again. The light was turned off and the apparatus disassembled.

The amount of chlorophyll present in the suspension was determined by withdrawing a 2.0 mL aliquot and volumetrically diluting to 10 mL with acetone. The acetone caused an osmotic shock which ruptured the chloroplast membranes, releasing the soluble chlorophyll. The debris was removed by transferring the solution to 30 mL centrifuge tubes and centrifuging at 3020 x g for 5-10 minutes. The supernate was scanned in the visible spectrum from 700 to 620nm and the spectra recorded.

No maxima was noted at 645nm so the absorbance at 652nm was used to determine the amount of total chlorophyll present. To account for dilution, equation (3) was changed to:

$$C_{chl} = (ABS_{652}) \times 6.90 \quad (4)$$

The oxygen sensor is sensitive to changes in temperature and pressure and required daily calibration. The oxygen sensor was calibrated according to the procedure in the instruction manual (Lazar). The concentration of dissolved oxygen in a 0.15 M solution of $NaHSO_3$ was taken to be zero. When the probe was placed in this solution, the meter was set to read zero millivolts. Another calibration point was found by holding the probe in the air. The instruction manual included a chart which listed the millivolt readings that would be obtained at a given temperature and atmospheric pressure. The temperature and pressures were widely spaced and values not listed were obtained by extrapolation of the chart data. A portion of this chart including the extrapolated values appears in Table IV. When calibrated in this manner, a change of 50 millivolts represented a change in oxygen concentration of 1.0 ppm.

The dark reactions were monitored with a carbon dioxide electrode and a slightly different apparatus than that used with the oxygen sensor. A full-scale,top-view of the apparatus used in these experiments is shown in Figure 4. The chamber itself was a thick glass dish 6.5 cm in diameter and 2.0 cm deep. A 50 mL beaker was cut so that it was 1.0 cm deep and a round indentation was made in its side. The rounded bottom of a small test tube was flattened so that it would stand upright, and it was cut so that it was also 1.0 cm deep. The small flat-bottomed tube was fitted into the indentation made in the cut-off beaker, and

TABLE IV. Millivolt settings for the calibration of the O_2 probe in air

°C	Pressure, mmHg											
	678	679	680	681	682	683	684	685	686	687	688	689
22	786	787	788	789	790	792	793	794	795	796	798	799
23	772	773	774	775	776	778	779	780	781	782	784	785
24	758	759	760	761	762	764	765	766	767	768	770	771
25	744	745	746	747	748	750	751	752	753	754	756	757
26	731	733	734	735	736	738	739	740	741	742	742	743
27	719	720	721	722	724	725	726	727	728	729	729	730
28	707	708	708	710	711	712	713	714	715	716	716	717

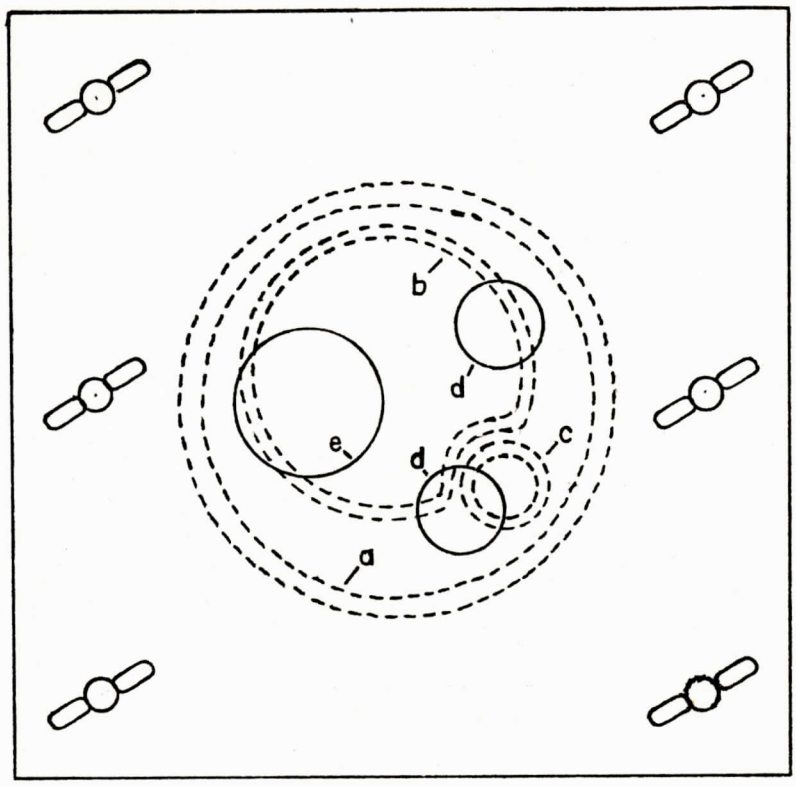


Figure 4. Full scale, top view of CO_2 exchange chamber.

a = Chamber

b = Dish containing chloroplast preparation

c = Tube containing $NaHCO_3$

d = Septums

e = Hole for CO_2 electrode

both fit snugly inside of the larger chamber. A rubber gasket was glued on the top rim of the chamber to form a seal between it and a 4 x 4 x ¼ inch sheet of plexiglass used as a lid. To facilitate the seal, the gasket was greased with silicone stopcock grease. Another sheet of plexiglass of the same dimensions as the lid was placed under the chamber. Both sheets had six holes drilled into them around their perimeter. The holes were to accomodate screws which were passed up through the bottom and through the top and were long enough to permit wing-nuts to be placed on them. When the wing-nuts were tightened by hand, the chamber was sealed.

The top sheet of plexiglass had three additional holes. Two of the holes were for holding septums which would allow the injection of reagents after the chamber was sealed. The septums were made by drilling a hole three-fourths of the way through from the top of a rubber stopper. The stoppers were also coated with silicone grease to facilitate a tight seal. The third hole was for the carbon dioxide electrode. A sleeve was made for the electrode from a rubber stopper as was described for the oxygen probe. The electrode was placed through the hole and into the chamber about 0.5 cm above the top of the cut-off 50 mL beaker.

A chloroplast suspension was prepared as previously described. With all lights off, and the lid removed from the chamber, 8.0 mL of the chloroplast suspension was pipeted into the cut-off beaker. 0.6 mL of 2,4-D solution of varying concentrations were added so that the final range of concentration was 5.0×10^{-6} to 1.0×10^{-2} M. Controls received 0.6 mL of distilled water in place of 2,4-D solution. The small tube received 0.5 mL of a 0.1 M $NaHCO_3$ solution.

Both of the inner containers were supplied with magnetic stirring

bars. The electrode and septums were put in place and the chamber was sealed by tightening the lid with the wing-nuts. The volume of air inside the chamber was 33.6 ±0.5 mL.

The pH meter, once again set to read millivolts on an expanded scale, and the recorder, set at 0.25 inch per minute, was turned on. The recorder pen, which was steady, was allowed to trace a straight line on the chart for 3-4 minutes and then 0.3 mL of dilute HCl was injected with a syringe through one of the septums into the small tube containing $NaHCO_3$. After a lag time of approximately one minute, the recorder pen began to respond to the increasing CO_2 in the chamber. The CO_2 concentration continued to rise until it gradually reached an equilibrium maximum. After a few minutes, the CO_2 concentration started to fall at a very slow rate until it again reached an equilibrium level where it would remain constant. The loss of CO_2 between the two equilibrium levels was taken to be equivalent to the CO_2 fixed by the chloroplasts. The amount of time required from the point of injection of HCl to the place where the recorder tracing ceased to change was usually about one hour.

The electrode and the meter output were calibrated by preparing a calibration curve using standard solutions of sodium bicarbonate to which a standard sodium citrate buffer was added immediately before inserting the electrode into the solution.

RESULTS

In studying the evolution of oxygen due to the light reactions, an attempt was made to obtain a correlation between the amount of oxygen evolved and the concentration of chlorophyll. Thirty-five determinations were made without 2,4-D before any were made with the herbicide present in order to determine the system parameters. This was necessary because of variations found from one determination to another. For each determination, a ratio was determined between the rate of oxygen evolution and the amount of chlorophyll present. The average value of this ratio for the runs without 2,4-D was found to be 11.2. The data are presented in tabular form in Table V.

The concentrations of 2,4-D used in the herbicide runs were 5.0×10^{-4}, 5.0×10^{-3} and 5.0×10^{-2} M and the ratios found for these concentrations were 12.3, 10.4 and 12.6 respectively. Variations between successive determinations were also found when 2,4-D was present but it was not deemed necessary to make a large number of repetitions. The data are included in Table V. The data in Table V are also illustrated graphically in Figure 5.

In monitoring CO_2 fixation due to the dark reactions, the same type of data was collected but different relationships were found. The data collected is tabulated in Table VI. Five identical experiments were carried out without 2,4-D present and also at each 2,4-D concentration. The concentrations of 2,4-D used were 5.0×10^{-6}, 5.0×10^{-5}, 5.0×10^{-4} and 5.0×10^{-3} M. The data in Table VI are illustrated graphically in Figure 6a for each different 2,4-D concentration and altogether in Figure 6b.

TABLE V. Data collected from light reaction experiments

Determination	[2,4-D]	$\frac{ppmO_2}{min}$	$\frac{mgchl}{ml}$	Ratio $\frac{\frac{ppmO_2}{min}}{mgchl/ml}$	Deviation From Mean
1		5.31	0.455	11.7	0.5
2		5.03	0.403	12.5	1.3
3		6.84	0.656	10.4	0.8
4		6.03	0.512	11.8	0.6
5		4.62	0.406	11.4	0.2
6		4.11	0.395	10.4	0.8
7		4.13	0.367	11.3	0.1
8		2.62	0.228	11.5	0.4
9		5.03	0.410	12.3	1.1
10		4.22	0.378	11.2	0
11		3.08	0.280	11.0	0.2
12		3.28	0.256	12.8	1.6
13		3.20	0.300	10.7	0.5
14		4.48	0.353	12.7	1.5
15		3.01	0.397	10.2	1.0
16		2.89	0.273	10.6	0.6
17		1.60	0.145	11.0	0.2
18		1.65	0.156	10.6	0.6
19		1.53	0.155	9.9	1.3
20		1.82	0.171	10.7	0.5
21		1.81	0.181	10.0	0.2
22		5.72	0.795	7.2	4.0

TABLE V., continued

Determination	[2,4-D]	$\frac{ppmO_2}{min}$	$\frac{mgchl}{ml}$	Ratio $\frac{\frac{ppmO_2}{min}}{mgchl/ml}$	Deviation From Mean
23		7.60	0.442	17.2	5.9
24		2.48	0.468	5.3	5.9
25		3.11	0.380	8.2	3.0
26		1.82	0.266	6.84	4.3
27		4.64	0.325	14.3	3.1
28		3.60	0.269	13.3	1.1
29		2.51	0.263	9.5	1.7
30		2.94	0.311	9.5	1.7
31		1.42	0.152	9.3	1.9
32		2.15	0.162	13.2	2.0
33		2.25	0.171	13.2	2.0
34		1.76	0.125	14.1	2.9
35		1.87	0.122	15.34	4.1
1	5×10^{-4}	1.91	0.145	13.2	0.9
2	5×10^{-4}	1.85	0.158	11.7	0.6
3	5×10^{-4}	1.72	0.162	10.6	1.7
4	5×10^{-4}	2.27	0.168	13.5	1.2
1	5×10^{-3}	1.64	0.152	10.8	0.4
2	5×10^{-3}	1.41	0.145	9.7	0.7
3	5×10^{-3}	1.65	0.152	10.8	0.4

TABLE V., continued

Determination	[2,4-D]	$\frac{ppmO_2}{min}$	$\frac{mgchl}{ml}$	Ratio $\frac{\frac{ppmO_2}{min}}{mgchl/ml}$	Deviation From Mean
1	5×10^{-2}	1.70	0.135	12.6	0
2	5×10^{-2}	1.62	0.122	13.3	0.7
3	5×10^{-2}	1.40	0.125	11.9	0.7

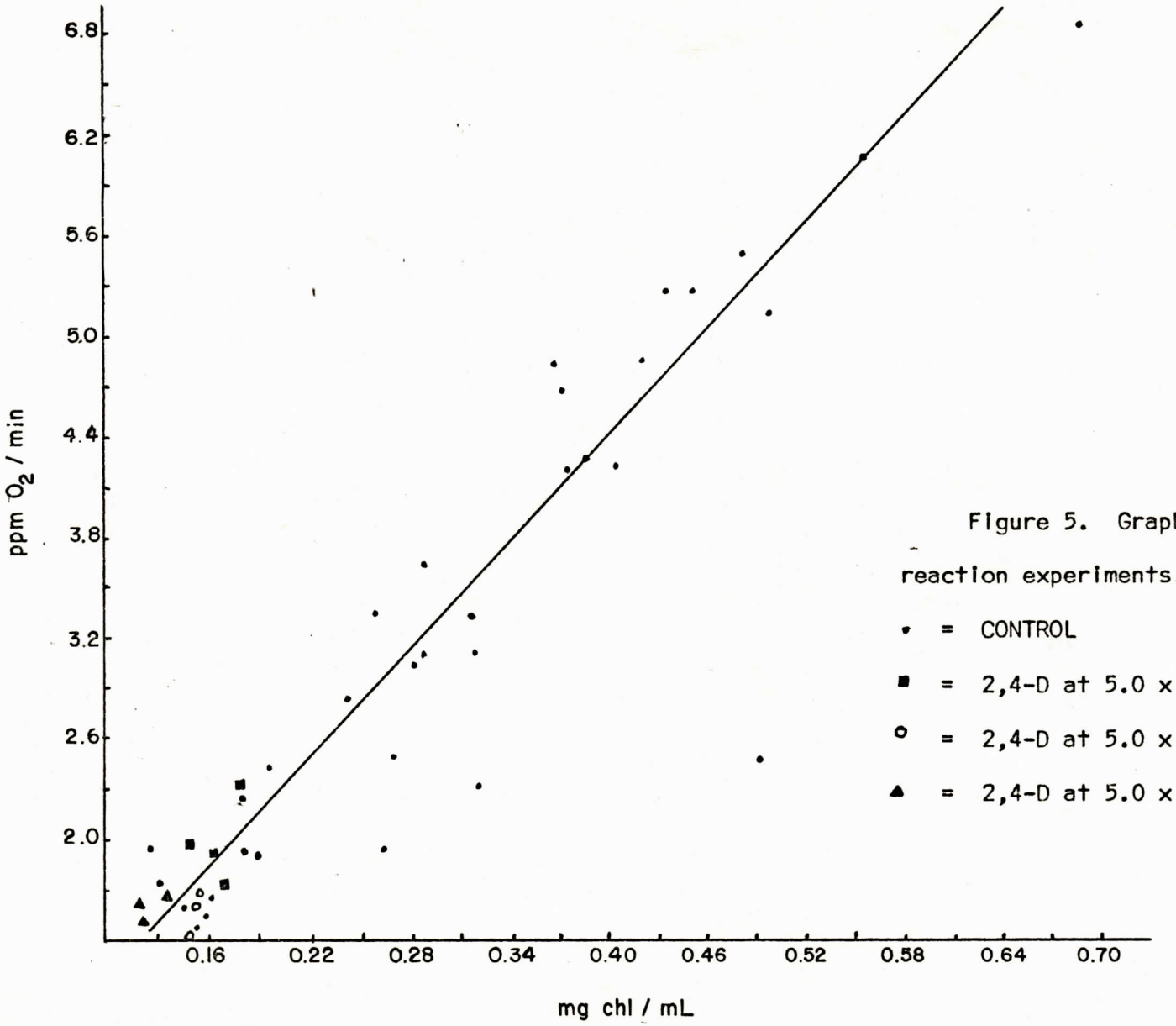


Figure 5. Graph of data from light reaction experiments.

• = CONTROL

■ = 2,4-D at 5.0×10^{-4} M

○ = 2,4-D at 5.0×10^{-3} M

▲ = 2,4-D at 5.0×10^{-2} M

TABLE VI. Data collected from dark reaction experiments. The values presented for CO_2 FIXED and TIME are the numbers of chart grids traversed by the recorder pen.

[2,4-D]	CO_2 FIXED	TIME	$\frac{mgchl}{ml}$	Ratio $\frac{CO_2/TIME}{mgchl/ml}$
0	4.0	35	1.73	0.066
0	6.0	53	1.12	0.101
0	5.1	47	1.56	0.070
0	5.8	63	1.75	0.053
0	6.5	45	1.35	0.106
5×10^{-6}	8.0	58	0.479	0.288
5×10^{-6}	3.4	29	1.05	0.112
5×10^{-6}	4.5	40	1.24	0.091
5×10^{-6}	6.0	37	0.738	0.220
5×10^{-6}	5.5	60	0.707	0.130
5×10^{-5}	6.2	38	0.460	0.355
5×10^{-5}	5.7	38	1.17	0.128
5×10^{-5}	4.7	29	0.633	0.256
5×10^{-5}	7.8	54	0.799	0.181
5×10^{-5}	7.0	42	1.03	0.162
5×10^{-4}	7.7	50	0.682	0.226
5×10^{-4}	6.5	41	0.675	0.235
5×10^{-4}	6.7	42	0.621	0.257
5×10^{-4}	6.5	45	0.765	0.189
5×10^{-3}	6.0	43	0.691	0.202
5×10^{-3}	6.0	51	0.617	0.191
5×10^{-3}	4.1	42	0.902	0.108

TABLE VI., continued

[2,4-D]	CO_2 FIXED	TIME	$\frac{mgchl}{ml}$	Ratio $\frac{CO_2/TIME}{mgchl/ml}$
5×10^{-3}	4.3	36	0.872	0.137
5×10^{-3}	4.2	40	0.819	0.128

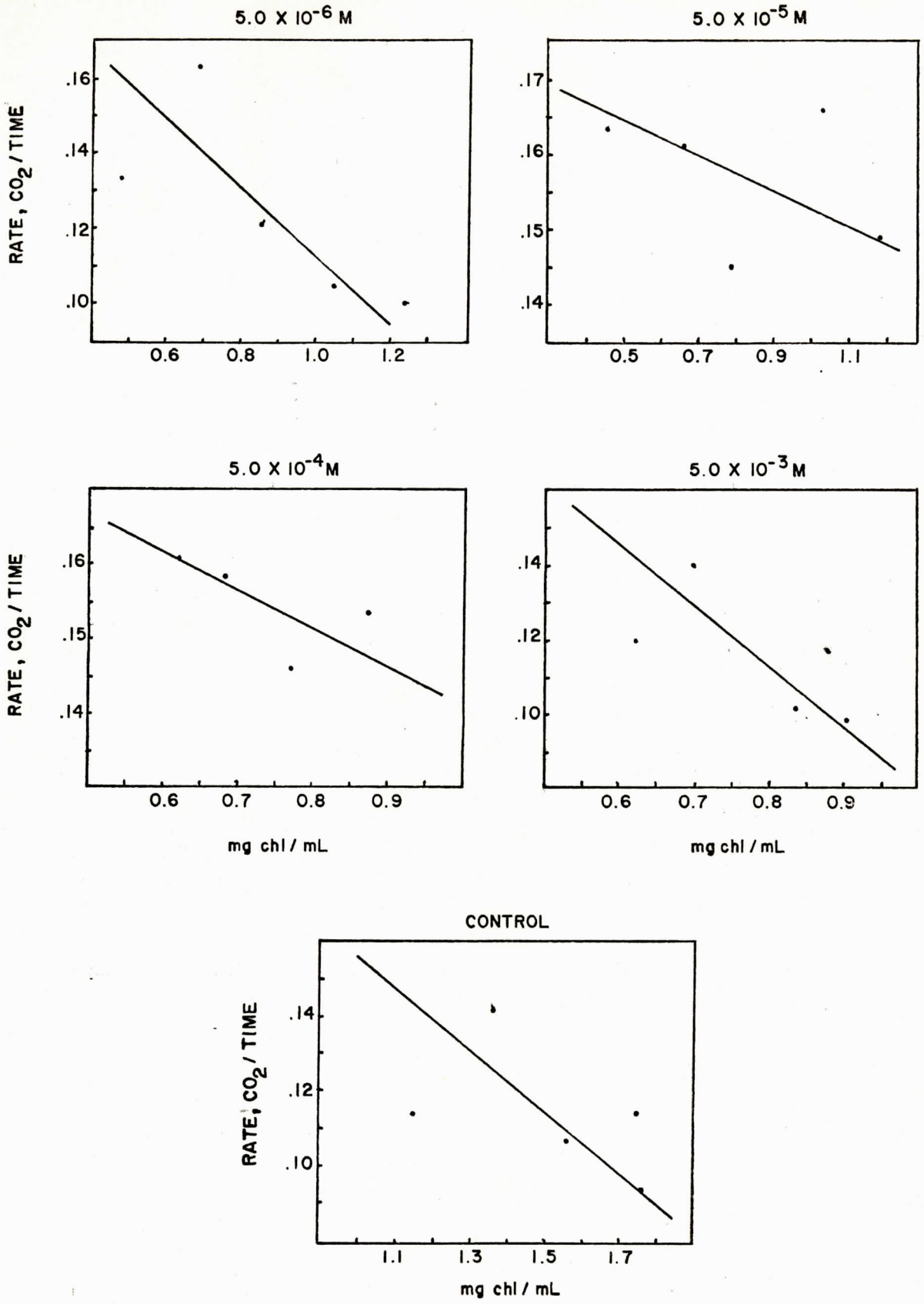


Figure 6a. Graphs of data from dark reaction experiments for each of the 2,4-D concentrations and the control.

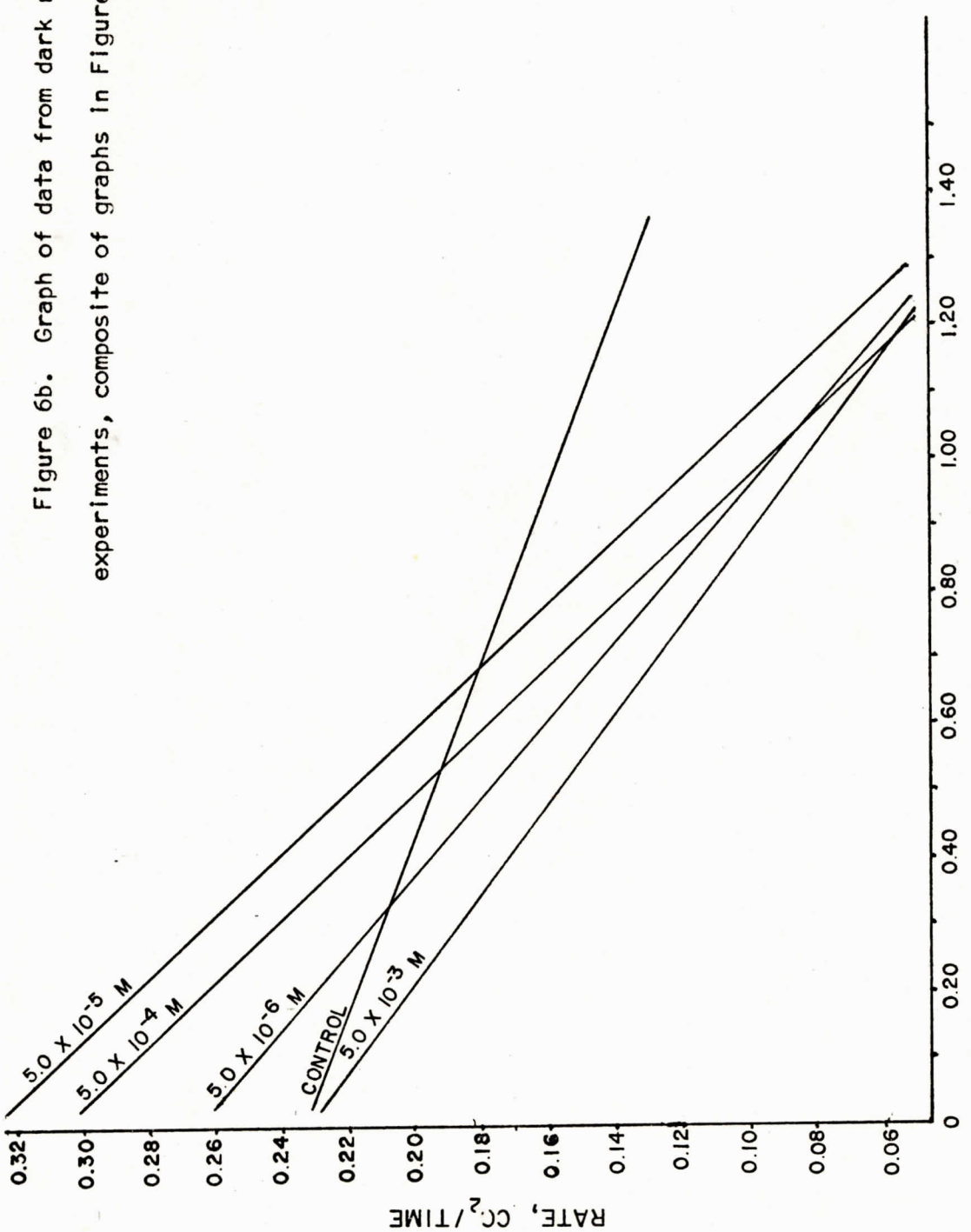


Figure 6b. Graph of data from dark reaction experiments, composite of graphs in Figure 6a.

The data obtained from these experiments indicated a trend in the effects of 2,4-D on the CO_2 fixation rates. For all of the concentrations of 2,4-D tested, there appeared to be a stimulation in the absorption of CO_2 by the chloroplast preparations. At some point between a 2,4-D concentration of 5.0×10^{-5} and 5.0×10^{-4} M the stimulation seemed to reach a maximum. The rate of the reaction per mg of chlorophyll at the maximum is estimated to be 280% of the same rate when 2,4-D was absent. The data are illustrated graphically in Figure 7 and also appear in Table VII. Five identical determinations were made at each 2,4-D concentration and there is some error associated with each set of data. However, even when the error is considered, a trend can be seen.

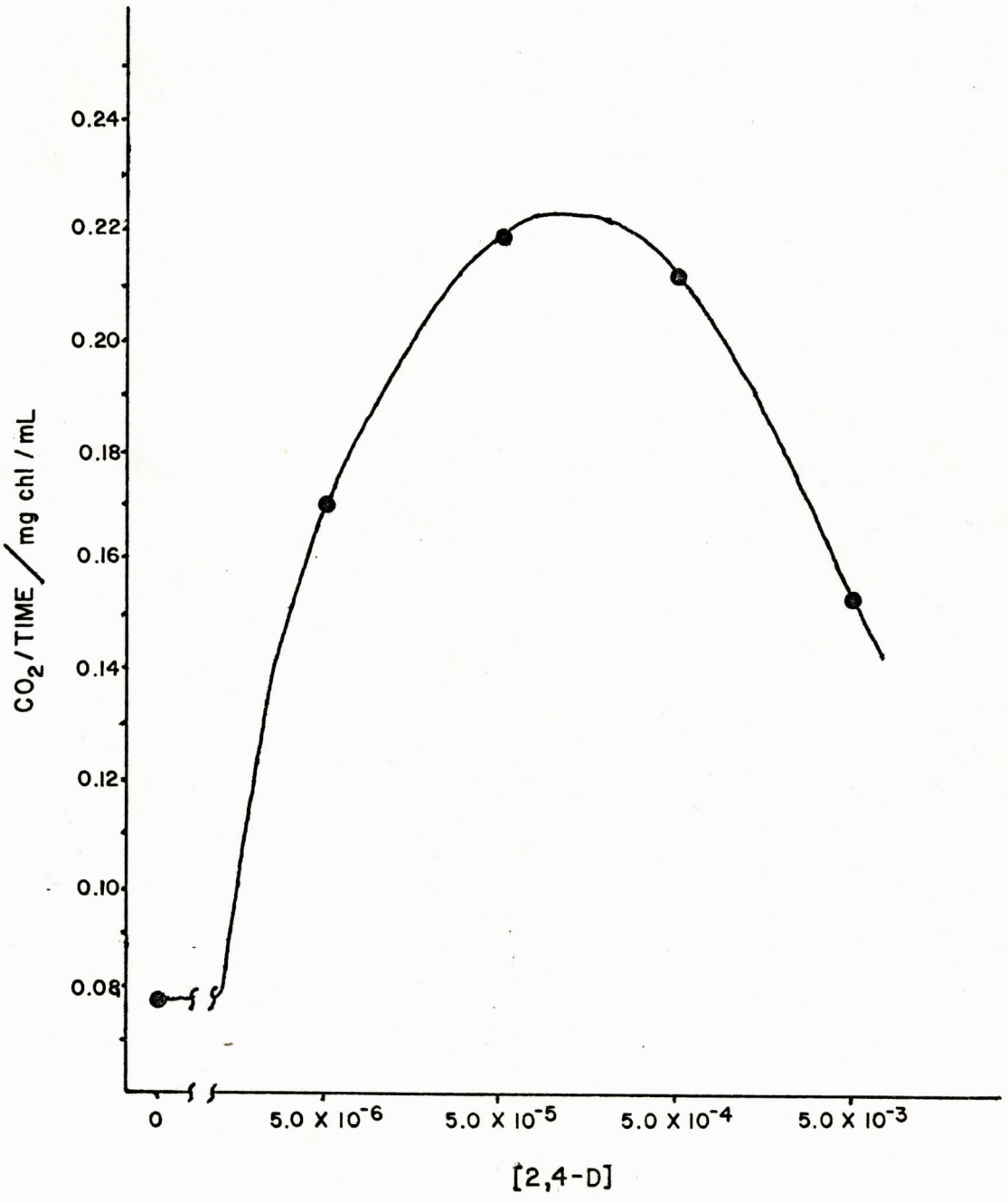


Figure 7. Graph of results from dark reaction experiments.

TABLE VII. Results from dark reaction experiments

[2,4-D]	AVERAGE RATIO	STANDARD DEVIATION	% OF CONTROL
0	0.78	0.019	100
5×10^{-6}	0.168	0.073	215
5×10^{-5}	0.216	0.081	277
5×10^{-4}	0.214	0.034	274
5×10^{-3}	0.153	0.037	196

DISCUSSION

Before starting the experiments it was necessary to determine certain parameters. Most of these were determined by using simple tests and observations. It was necessary to determine if the increase in oxygen concentration in the chloroplast suspension and if the decrease in the carbon dioxide level in the CO_2 chamber was due to the respective photosynthetic processes occurring within the chloroplasts.

The evolution of O_2 by the chloroplast extracts was verified by noting that an increase in the recorded oxygen level was not due to changes in temperature or pressure in the reaction system. It was important to note the possible effect of temperature and pressure since the oxygen probe is sensitive to such changes. These effects were ruled out by noting that the temperature and pressure remained constant throughout the experiments. If the system was kept in the dark, the O_2 concentration would steadily decrease due to respiratory O_2 uptake and eventually level off. It was found that the O_2 concentration would not increase until the chloroplasts were illuminated and the level would decrease if the light was turned off. When the light was turned on, the O_2 concentration would begin to increase. After a limited amount of time the O_2 concentration would no longer respond to the light which was probably due to an exhaustion of the chloroplast enzymes or a saturation of the system. If initially the light was turned on and left on, which was the procedure in these experiments, an exhaustion point was also reached. The amount of time that the chloroplasts were illuminated until exhaustion was reached was found to be approximately the same regardless of whether the chloroplasts were illuminated con-

tinuously or intermittently with darkness.

In the carbon dioxide studies a number of blank determinations were made and it was found that the chloroplasts had to be present in order to get a decrease in CO_2 concentration. This also showed that there were no leaks in the apparatus. When an intact, detached leaf was used instead of a chloroplast suspension, no appreciable activity could be found with or without 2,4-D present.

Another point that needed clarification was to note the effect 2,4-D might have on the measured absorption spectra of chlorophyll at the wavelengths used. A concentrated stock chloroplast suspension was prepared and aliquots were taken from it. Half of these aliquots were volumetrically diluted with water and the other half with an aqueous 2,4-D solution. All of these were made 80% in acetone, centrifuged and the absorption spectra recorded. No significant differences could be detected in the absorption characteristics of chlorophyll in the region from 700.to 620nm.

According to the literature, there is still some doubt as to the exact location of the ferricyanide effect in the Hill reactions. If it accepts electrons from photosystem II, and if 2,4-D had stimulating or inhibiting effects in the electron carrier chain, photophosphorylation or in photosystem I, these effects would not be seen because the entire reaction sequence was not active. However, if ferricyanide accepted electrons from photosystem I, the effects of 2,4-D would probably be seen. This uncertainty could be resolved if NADP, the natural electron acceptor, was used in place of ferricyanide.

From the results in this work, it would be difficult to draw any conclusions about the effects of 2,4-D or the light reactions of photosynthesis. As shown in Table V, there were deviations in the rate of

O_2 evolution per milligram of chlorophyll in the determinations made in the presence of 2,4-D and those made without. However, these deviations do not show a trend, and furthermore, as illustrated in Figure 5 the scattering of the data does not lend itself to any justifiable conclusions, but 2,4-D does not show any significant effect under the conditions used here. Other conditions or more sensitive measurements might show some effect. If 2,4-D does not stimulate the light reactions then the energy for stimulated cellular processes would have to come from the mitochondria. This could explain why the plant outgrows its energy supply.

The apparent stimulation of CO_2 fixation as a result of 2,4-D treatment is best illustrated in Figure 7. The points plotted here represent the slope of the lines in Figure 6b. These slopes were obtained using the method of least squares on the collected data.

Figure 6b shows that the rate of CO_2 fixation was inversely proportional to the concentration of chloroplasts in the preparations. A possible explanation for this involves a variation in the solubility of CO_2 in the buffer solution containing the chloroplasts. The larger the number of chloroplasts suspended in the solution, the higher will be the concentrations of soluble proteins and other soluble cellular materials. This could result in a decrease in the solubility of CO_2 in the chloroplast preparation.

Of particular interest in Figure 6b are the points where the line representing an absence of 2,4-D crosses over the lines representing the individual 2,4-D concentrations. Starting from the chlorophyll concentration that corresponds to a cross over point for a particular 2,4-D concentration it can be seen that if this concentration is lowered, an increase in the rate of CO_2 fixation results, and if it is

increased, the rate decreases. At the 2,4-D and chlorophyll concentrations studied, an increase in the rate of CO_2 fixation per milligram of chlorophyll was noted. This is illustrated in Figure 5 by the greater (more negative) slopes of the lines representing the determinations made with 2,4-D. At chlorophyll concentrations greater than 0.70 mg/mL, the rate of CO_2 absorption into the suspension was greater in the controls than when 2,4-D, at any of the concentrations used, was present. This suggests that at these chlorophyll concentrations, CO_2 cannot dissolve into the suspension in amounts large enough to keep up with the increased rates of the reaction. This observation supports the theory that plants die from an inability to absorb necessary nutrients, such as CO_2, fast enough to support the increased synthetic activity.

A question that remains unanswered concerns the specific site of activity of 2,4-D in the Calvin cycle. To answer this question, the effects of 2,4-D on the activity of the enzymes in the Calvin cycle need to be studied, particularly those enzymes that were found to be affected in glycolysis.

At present, no published evidence has been presented which would indicate whether or not 2,4-D actually diffuses through the chloroplast membrane. From unpublished, earlier work undertaken by this investigator, it was found that 2,4-D may actually bind to the chloroplast membrane. An affinity chromatography column was prepared in which 2,4-D was attached to aminoethylcellulose via an amide bond. A chloroplast extraction from young pea seedlings was eluted through the column and it was found that a large proportion of the green color was held back. A solution of acetic acid and IAA failed to wash this color off, however a solution of 2,4-D succeeded. This indicated there may be a specific receptor site on the chloroplast membrane for 2,4-D, which is not the same as the IAA receptor

site.

Natural hormones act in the body by attaching to specific receptor sites on the membrane of the target cells. The production of a cyclic nucleotide is stimulated which affects various processes within the cell. The 2,4-D may act in an analogous manner except that the receptor site is on the membrane of a subcellular particle, the chloroplast. It has been postulated that 2,4-D does not act on its own in stimulating RNA production but it acts through a mediator, which is thought to be a protein. (Kearney and Kaufman, 1976) This also may explain the action of 2,4-D on the chloroplasts. Since the enzymes that are involved in the dark reactions are attached to the outer chloroplast envelope and the enzymes that are involved in the light reactions are isolated from this region by being enclosed in the thylakoid system, the results of the present study may not be so suprising since the stimulated mediator would not come in contact with the sites of the light reactions.

Turner and Bidwell (1964) studied the effect of spraying IAA solutions on bean leaves. They found CO_2 fixation to be stimulated by a maximum of 28% when the leaves were treated with 5.0×10^{-5} to 3.0×10^{-4} M IAA solutions. The curve they obtained was similar to the one shown in Figure 7, and the concentrations at which maximum stimulation was found were in the same range. The stimulation of CO_2 fixation due to 2,4-D treatment was found to be approximately 177%. This comparison suggests that although the effects of 2,4-D and IAA may be similar, 2,4-D is approximately six times more active in stimulating the Calvin cycle.

There are a number of factors that contributed to the experimental errors reported in this work. In working with biological material, it is to be expected that there will be a scattering of data. A way to partially overcome this scattering is to repeat identical determinations

a large number of times keeping strict control over all variables. The plants should all be of the same genotype and the leaves that are taken for each determination should be the same age, relative size and state of health. The plants used in these experiments were not genetically pure and the age of each leaf used was not the same. Nevertheless, the present study does suggest a starting place for future work.

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