Title

Photosynthesis

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Publication Date

1964-06-01

UCRL-11487

University of California

Ernest O. Lawrence Radiation Laboratory

PHOTOSYNTHESIS

Berkeley, California

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Chapter in Vol. III "Survey of Progress in Chemistry"

UCRL-11487

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Lawrence Radiation Laboratory
Berkeley, California

AEC Contract No. W-7405-eng-48

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J. A. Bassham

June 1964

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By J. A. Bassham

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ABSTRACT

The general properties of photosynthesis are discussed. The possible evolutionary history of photosynthesis in green plants is described. The current status of our knowledge about the photoelectron transport system and the primary quantum conversion acts in photosynthesis are briefly reviewed. The historical development and present status of the carbon reduction pathways of photosynthesis are outlined. A few areas and problems for future investigation are then mentioned.

*This work was sponsored, in part, by the U. S. Atomic Energy Commission.

To be published as a chapter in Volume 3 of "Survey of Progress in Chemistry". Academic Press Inc., 111 Fifth Ave., New York 3, N. Y.

Submitted 6/5/64

TABLE OF CONTENTS

		Page
I.	Introduction	1
	A. General properties of photosynthesis	1
	B. Photosynthetic evolution	2
	C. Historical	6
II.	Recognition of component reactions of photosynthesis	7
	A. Light and dark reactions	7
	B. The Hill reaction	8
	C. Isotopic oxygen experiments	9
	D. Carbon dioxide reduction in the dark	9
III.	Structure of the photosynthetic apparatus	10
	A. Chloroplasts	10
	B. Quantasomes	12
	C. Chemical composition of the photoelectron transport system	13
	D. Evolutionary relation between particles from mitochondria and from chloroplasts	15
	E. Membrane structure	16
	F. Orientation of pigment molecules	17
IV.	Photoelectron transport and photophosphorylation	18
	A. End reactions	18
	B. Two light wavelength effects	23
	C. Quantum conversion	28
V.	The reduction of carbon dioxide in photosynthesis	36
	A. Methods of mapping the path of carbon	36
	B. Experimental results and their interpretation	40
	C. The photosynthetic carbon reduction cycle	49

		Page
VI.	Secondary carbon reduction pathways	52
	A. Carboxylic acids	52
	B. Fatty acids and fats	54
	C. Carbohydrates	55
	D. Amino acids and proteins	56
VII.	Problems for the future	57

PHOTOSYNTHESIS

(Survey of Progress in Chemistry)

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I. Introduction

A. General properties of photosynthesis

Photosynthesis is the absorption of light energy and its conversion to chemical energy through the synthesis of new organic molecules. The product molecules collectively have a smaller (less negative) free energy of formation from the elements than do the reactant molecules. This difference in negative free energies of formation is the energy stored by photosynthesis.

All living cells are characterized at the molecular level by a continuous expenditure of chemical energy. The energy is made available by respiration and fermentation. These processes degrade compounds of higher energy content, such as the products of photosynthesis, to compounds of lower energy content, such as the reactants in photosynthesis. Virtually all substances used for energy release by living cells are derived ultimately from photosynthesis.

One of the most elementary kinds of photosynthesis is the conversion of acetate to larger organic molecules by light energy in certain types of bacteria. The far more common type of photosynthesis that occurs in green plants is also far more complex. Green plants use their absorbed light energy to bring about an oxidation-reduction reaction between water and other inorganic oxides. The energy from the light is used to take

electrons from water, ultimately releasing gaseous oxygen. Some of the electrons are transferred to carbon dioxide, reducing and converting it to organic compounds such as sugars and fats. Other electrons are transferred to nitrate, reducing it to ammonia and water. However, plants can use nitrogen supplied in the form of ammonium ion. Sulfate also is reduced. Reduced carbon, nitrogen and sulfur may be further converted by photosynthesis to amino acids and proteins.

B. Photosynthetic evolution

The complexity of photosynthesis in green plants is a result of evolution. The response of living cells to a changing environment has been the gradual addition of many simple steps to what was probably an uncomplicated process in the beginning. Let us consider a theory of photosynthetic evolution. Such a theory, even though at present incomplete and possibly inaccurate in its details, can form a useful prologue to a discussion of the mechanism of photosynthesis. Hopefully, our understanding of the intricate mechanism which evolved can be aided by even an imperfect recapitulation of its development.

It is one currently popular concept that the primitive prebiotic earth contained in its atmosphere high levels of reduced gases such as ammonia and methane, as well as water vapor. It is very probable that there was little or no gaseous oxygen.

Under solar irradiation and perhaps with other energy sources, these gases and water combined to make simple organic molecules. Since O_2 was absent from the atmosphere, much more ultraviolet (U.V.) radiation reached the earth than at the present time. The greater energy of U.V. radiation made possible many more photochemical reactions than are mediated by visible light.

Polymerization of these simple molecules, partly by photochemical reactions, resulted in the formation of macromolecules from which the first primitive cells evolved. These cells, developing in ponds or seas, were protected from the damaging effects of U.V. by the water in their environment. Presumably, these primitive cells used as an energy source the available complex organic substances, degrading them into simpler organic materials by fermentative processes.

We may suppose that the primitive environment included molecules of pigments which can be formed thermally or photochemically from simpler molecules. Thus, such compounds as acetate and glycine could in time be converted non-biologically to tetrapyroles and ultimately to porphyrins. Eventually, chelation of a magnesium atom would produce chlorophyll.

Once such pigment molecules were incorporated in primitive cells, they became radiation antennae with which the cells could capture electromagnetic energy in the visible region. The first mutant cell to make use of this captured energy probably performed some simple photosensitized oxidation-reduction reaction. Such reactions can be performed today by chemists, using solutions of chlorophyll and suitable electron donors and acceptors.

As the supply of energy-rich substrates diminished, the primitive photosynthetic cells no doubt developed photochemical reactions for storing more of the absorbed energy. A primeval ancestor of the acetate-utilizing photosynthetic bacteria may have evolved. Indeed, the central position in metabolism occupied by acetate may well speak for the importance and abundance of acetate in primitive metabolic evolution.

We may suppose that the photosynthetic organisms learned to recombine products of the photochemical oxidation-reduction reaction in a useful way.

The energy released by this recombination was used to bring about the formation of the biological acid anhydrides, such as adenosine triphosphate (ATP), from inorganic phosphate, and organic phosphates such as adenosine diphosphate (ADP).

The carboxyl groups of organic acids such as acetate could then be activated by reaction with ATP. The resulting acetyl phosphate is much more reactive towards such reactions as carboxylations and condensations.

Because of its low molecular weight, hydrogen gas was continuously escaping from the earth's gravitational field. As a consequence of this escape, the earth's atmosphere became gradually less reducing, and more oxidizing. Increasing amounts of carbon dioxide appeared in the earth's atmosphere as a consequence of the reaction:

(1) $$2H_2O + CH_4 \longrightarrow CO_2 + 4H_2 \nearrow \text{escape}$$

Even though the equilibrium constant at 25° in the presence of liquid water for this reaction is about 10^{-23}, the continual removal of hydrogen gas permitted the slow accumulation of carbon dioxide.

Carbon dioxide provided a source of carbon, but in its lowest level of chemical energy. Chemical energy many have been less a limiting factor in some environments that the supply of water soluble carbon compounds. For example, a small amount of methane could be used together with carbon dioxide to provide both energy and twice as much carbon at the level of carbohydrate as contained in the methane alone:

(2) $$CH_4 + CO_2 \longrightarrow 2\,(CH_2O) + H_2O \qquad \Delta F' = -25 \text{ Kcal}$$

At physiological pH, this reaction goes spontaneously in the direction written, releasing 25 Kcal/mole of chemical free energy.

Thus it may have happened that conditions favored bacterial mutants which had developed carboxylation mechanisms, in which the products of carboxylation were reduced by electrons obtained from organic compounds. When supplies of organic compounds became scarce, photosynthetic organisms developed that were capable of using light energy as an energy source for transferring electrons from less energetic donors, such as H_2S, to co-factors with reducing power sufficient for the reduction of carboxylation products.

The dissociation of water to oxygen and hydrogen also would be aided by the escape of hydrogen gas. In this case, the equilibrium constant is very unfavorable, but the reaction rate was greatly accelerated as a result of the photodissociation of water by the ultraviolet light from the sun. Much of the oxygen produced must have reacted by non-biological processes with the various organic compounds present in the atmosphere, thereby converting them to carbon dioxide and water. Eventually, with the continual escape of hydrogen, enough oxygen would have accumulated to permit its participation in biological reactions.

The eventual appearance of oxygen in significant quantities in the atmosphere made possible the evolution of new types of organisms. These are the aerobic organisms,which are capable of reacting oxygen with organic compounds and utilizing with high efficiency the large release of energy which accompanies this respiration.

The stage was then set for the appearance of the most important type of energy conversion present in today's biosphere. This is photosynthesis by green plants, in which light energy is used to obtain electrons for the reduction of carbon dioxide, by oxidizing water to O_2. Photosynthesis and respiration were mutually enhancing — each process produces the sub-

strates for the other. Other types of metabolism, such as fermentation and bacterial photosynthesis, have been relegated to restricted locations where the "primitive" environment which they require still persists.

Such a theory of metabolic and photosynthetic evolution goes far towards explaining the similarities and differences amongst the various metabolic processes as we know them today. The path of carbon reduction in photosynthesis employs many reactions which are similar to those employed by heterotrophic organisms in the oxidation of carbohydrates. The apparatus for transporting electrons from water to the point of reduction of carbon dioxide in photosynthesis is similar in many aspects to the apparatus used for the transport of electrons from the oxidation of carbohydrate to oxygen in respiration.

C. Historical

The first discoveries concerning the nature of photosynthesis occured at the time of the beginnings of modern chemistry. An interesting account of these early investigations has been written by Rabinowitch (1945). Joseph Priestly found in 1772 that after candles had been allowed to burn out in a closed jar, the air could be "restored" by placing a sprig of mint in the jar for several days. By 1780, Ingen Housz showed that light was necessary for this restoration of the air. Jean Senebier reported in 1782 that "fixed air" was transformed by photosynthesis into "pure air". The meaning of these early findings became clear upon the discovery of oxygen by Lavoisier in 1775, and following his finding that "fixed air" is a compound of carbon and oxygen, in 1781. Somehow the illuminated plants were replacing carbon dioxide in the air with oxygen.

The role of water in photosynthesis was established by de Saussure, in 1808. A meticulous experimenter, de Saussure measured the increase in

dry weight of a plant growing in a pot of earth, and also measured the volume of carbon dioxide taken up by the plant and the volume of oxygen evolved. He confirmed the fact that all of the carbon made by the plant into organic materials comes from carbon dioxide. Moreover, he showed that the increase in dry weight of the plant was greater than the difference in weight between the carbon dioxide taken up and the oxygen evolved. The weight of the soil in the pot did not change significantly. The only other source of weight increase was water; thus, water is a reactant in photosynthesis.

The primary importance of photosynthesis as an energy converting reaction had to await the development of the concept of chemical energy. In 1845, Robert Mayer recognized that the energy of sunlight was converted by photosynthesis to the stored chemical potential of the products. Thus today, we write the equation for the photosynthesis of carbohydrates from water and carbon dioxide:

(3) $$CO_2 + H_2O + \text{light} \longrightarrow [CH_2O] + O_2 \quad \Delta F = +113 \text{ Kcal}$$

We recognize the fact that the conversion of one mole each of water and carbon dioxide to carbohydrate and molecular oxygen stores 113 Kcal of the absorbed light energy in the form of increased chemical free energy of the chemical bonds in the products over that of the reactants.

II. Recognition of component reactions of photosynthesis

A. Light and dark reactions

The first clues to the mechanism of photosynthesis were found in kinetic studies. By 1905, Blackman had discovered that even when the rate of photosynthesis could not be further increased by additional light intensity or increase in

the level of carbon dioxide, an acceleration of the rate of photosynthesis could be accomplished by raising the temperature. These results indicate that photosynthesis includes non-photochemical processes whose rates are thermally controlled. In living cells, such reactions are typically catalyzed by enzymes.

The next couple of decades saw the advancement of many hypotheses and theories, frequently erroneous. However, advances in the characterization of the structure of chlorophyll, the most important photosynthetic pigment, were made during this period.

The beginning of the modern area of rapid advances in the understanding of photosynthesis can be placed at about 1930. Van Niel and others made extensive studies of the stoichiometry of photosynthesis in various photosynthetic bacteria. Van Niel, (1931, 1935), found that in the green sulfur bacteria the photosynthetic equation could be represented by:

(4) $$CO_2 + 2H_2S \longrightarrow [CH_2O] + H_2O + 2S$$

From the nature of this equation and others, Van Niel proposed that there is a common mechanism for CO_2 reduction amongst the various types of photosynthetic organisms. The differences among the organisms were attributed to their means of obtaining electrons for the reduction of carbon dioxide. Thus the general formulation for photosynthesis in all organisms became:

(5) $$CO_2 + 2H_2A + \text{light} \longrightarrow [CH_2O] + 2A + H_2O$$

where H_2A represents the electron donor.

B. The Hill Reaction

In the case of green plants, the electron donor is water, according to Van Niel's theory. This view was supported by the discovery of the Hill

Reaction, (Hill and Scarisbrick, 1940), in which electrons are transferred in the light from water to an artificial electron acceptor by fragments of green cells or chloroplasts which have lost most or all of their capacity for carbon dioxide reduction. Other experiments some years later would show that isolated chloroplasts and even small fragments of chloroplasts could oxidize water to oxygen, and at the same time form natural cofactors required for the reduction of carbon dioxide. (See Sec. IV)

C. Isotopic oxygen experiments

Experiments with isotopic oxygen showed that all of the oxygen evolved during photosynthesis comes instantaneously from water, (Ruben, et.al., 1941). When photosynthesizing plants were supplied with either water or CO_2, in which some of the oxygen atoms were ^{18}O instead of ^{16}O, the oxygen evolved just after the addition of the heavy isotope agreed in isotopic composition with the water. Thus, photosynthesis in green plants should be represented as follows:

(6) $$CO_2 + 2H_2O^* + \text{light} \longrightarrow [CH_2O] + O_2^* + H_2O$$

D. Carbon dioxide reduction in the dark

The first studies of the path of carbon reduction in photosynthesis by Ruben, et.al., (1939-40), showed that the dark fixation of CO_2 by photosynthetic plants is much greater immediately following preillumination than after the plants have been in the dark for several minutes. This is in accord with the concept that CO_2 fixation occurs by "dark reactions" which use relatively stable chemical species formed in the light. Calvin and Benson and their co-workers used the radioisotope tracer method to study carbon reduction in photosynthesis, (Calvin and Benson, 1948, 1949).

They found that if ^{14}C-labeled carbon dioxide is added to the green plant, immediately after the light is turned off some of the labeled carbon is reduced to the level of sugar phosphates. Eventually, Calvin and his co-workers mapped the path of carbon in photosynthesis, (Sec. V), showing that the entire pathway from carbon dioxide to carbohydrate is accomplished by dark reactions mediated by enzymes. Some of these reactions require co-factors derived from the earlier light reactions of photosynthesis.

III. Structure of the photosynthetic apparatus

A. Chloroplasts

The subcellular unit responsible for photosynthesis in green plants is a membrane-enclosed entity which is called the chloroplast. The chloroplast contains a complex structure. Prominent features of this structure are the lipid and protein layers which are called lamellae. These lamellae are large thin sheets which may extend the length and width of the chloroplast. They were revealed originally by techniques of electron microscopy.

If a chloroplast is stained with osmium tetroxide or potassium permanganate, embedded, sectioned and viewed in the electron microscope, alternate dark staining and light layers are revealed, (Steinman and Sjostrand, 1955). These lamellae extend throughout the chloroplast. Such an electron micrograph is shown in Fig. 1. In some chloroplasts, particularly those found in leaf cells, the lamallae are thicker and more closely packed in certain areas than in others. These regions of greater thickness and closer packing are often disk-shaped, and stacks of these disks are sometimes called grana. The grana correspond to the small green areas which are just visible under the light microscope.

Grana appear not to be essential for photosynthesis, since they are not found in unicellular algae such as chlorella. Probably the grana are

useful to some functional specialization in higher plants. For example, leaves of higher plants may convert a large fraction of carbon taken up to sucrose for transport to non-photosynthetic parts of the plant.

The less pigmented region of the chloroplast having fewer lamellae is called the stroma, and the lamellae can be considered as embedded in a stroma matrix, (Park, 1963). The entire chloroplast is usually surrounded by a double membrane. Variations in the structure have been found in a wide variety of organisms with organized chloroplasts. Even in blue-green algae, where the membrane surrounding the chloroplast is lacking, a similar lamellar system is seen, (Ris and Singh, 1961). The repeating interval in the closely stacked lamellae is usually about 160 Å.

Trebst, (1958), as well as Park and Pon, (1961), fragmented chloroplasts from spinach and obtained mixtures of particulate green matter and soluble enzymes, which together were capable of slowly reducing carbon dioxide to sugar in the light. When this preparation was subjected to centrifugation at high speed, the particulate matter containing the chlorophyll and other pigments sediments to the bottom of the centrifuge tube. This material upon resuspension was found to be capable of convérting absorbed light energy to chemical energy. As part of this energy conversion, the green particles transfer electrons from water (liberating molecular oxygen) to electron acceptors. These can be either natural cofactors, or such substances as ferricyanide. Part of the energy conversion results in the formation of a biological acid anhydride, adenosine triphosphate (ATP), about which more will be said later.

The soluble proteins left in the supernatant after the centrifugation appear to include most of the enzymes required for CO_2 fixation during photosynthesis. By themselves, they are capable of very little fixation

and reduction of carbon dioxide. However, when the particulate green material obtained by sedimentation was recombined with the soluble proteins and illuminated in the presence of radioactive carbon dioxide, a significant amount of fixation of carbon dioxide and formation of reduced carbon compounds was observed. Thus, the separation of "light" and "dark" phases of photosynthesis (predicted by Van Niel and confirmed by the experiments of Hill and Ruben) was demonstrated in terms of the physical separation of the "light" and "dark" biochemical machinery.

Under certain conditions, the green particulate material is capable of rates of energy conversion and oxidation-reduction reactions comparable to the rates exhibited by whole cells. However, in the combined system, the rates of carbon dioxide fixation and reduction are, at best, only 1 to 5% of the rates of the intact organism on a unit chlorophyll basis. It is important that this difference be kept in mind when one considers the probable organization and relationships of the component machinery of photosynthesis as it is performed in vivo, (Bassham, 1963).

B. Quantasomes

The particulate material obtained by sedimentation of broken chloroplasts consists of lamellae and lamellar fragments. Park (1963) has suspended this green precipitate in water and then precipitated it according to the critical point method of Williams, (1953). When the precipitated material was dried down on a screen, shadowed with heavy metal for viewing in the electron microscope, and photographed, the material was found to be clearly lamellar in structure, (Fig. 2).

By this method, one observes the horizontal surface of the lamella. There were found to be two types of surface, one smooth and another parti-

culate. It occasionally appears that the smooth layer has been torn away, revealing the particulate layer. This suggests that the smooth layer may in fact form a kind of envelope containing the particulate mat which is almost crystalline in appearance. These particles have been named quantasomes, (Calvin, 1962). An individual quantasome appears to be 185 Å long, 155 Å wide, and 100 Å thick (Park & Biggins, 1964). They are embedded in the enveloping layer, which is composed of the material which stains with osmium tetroxide. Presumably, the conversion of light energy to chemical energy takes place in a system which includes both the oblate spheres and a part of the membrane. A quantasome is thought to be one of these spheres with its attached membrane. It must contain all of the machinery necessary to carry out the photochemical oxidation of water and transfer of electrons to some acceptor. Whether or not an individual quantasome also performs photosynthetic phosphorylation (formation of ATP in the light) is not yet entirely established. Certainly, photosynthetic phosphorylation is a function of the lamellar system.

C. Chemical composition of the photoelectron transport system.

Park (1963) has investigated the chemical nature of the lamellar fragments as they are related to biochemical function. These fragments can be lyophilized and, upon resuspension in water, retain their Hill activity. However, if the lyophilized quantasomes are extracted with organic solvents such as hexane, approximately 50% of the material is soluble and the residue has a nitrogen content of 18%, indicating that what is left is nearly pure protein. Under the electron microscope, the extracted protein globules appear to be about half the size of the original quantasomes. Spectrochemical analysis of the lyophilized material revealed the presence of several metal atoms. The ratio of magnesium:iron:copper:manganese is about

150:6:3:1. However, the calculated weight of the quantasome based on its size, density, etc., is 2,000,000. Therefore, there should be about 300 chlorophyll molecules per quantasome. (Each chlorophyll molecule contains one atom of magnesium.) Each quantasome contains 12 iron atoms (2 in cytochrome), 6 copper atoms, and 2 manganese atoms.

The iron atoms and copper atoms very likely play roles in electron transport. The presence of manganese is of great interest, since it is known to be required for the oxygen evolution in photosynthesis. Presumably the atoms of manganese play some essential role in either the oxidation of water, or in the evolution of oxygen gas from some intermediate oxidation state of oxygen, such as an organic peroxide. The aspect of photosynthesis about which the least is known at the present time is the mechanism of the oxidation of water and the liberation of molecular oxygen.

A number of molecules known to be involved in electron transport and in oxidative phosphorylation reactions in biological systems are found in the lamellar material. One class of these compounds are the cytochromes. These substances are protein molecules which contain iron, chelated in tetrapyrrole pigment structures. These substances are electron carriers in biological systems. Electrons may be accepted by the forms in which the iron is in the +3 state, and donated by forms in which iron is in the +2 state of oxidation. Through variations in the configuration of the protein, cytochromes found in chloroplasts have physiological redox potentials (electromotive force for the reactions in which they accept electrons at pH 7) ranging from that of cytochrome f at +0.365 V to cytochrome b_6 at -0.03 V. By comparison, the potential for hydrogen ion H^+ to accept electrons at pH 7, and thereby be reduced to hydrogen gas, is -0.42

volts.

Protein, which contains iron not bound in heme, is also found in chloroplasts in the lamellae. One such compound which is not tightly bound to the lamellar system is chloroplast ferredoxin, (see Sec. IV). The redox potential of ferridoxin is -0.43 volts. Ferredoxin and cytochrome f represent nearly the extremes of redox potentials associated with substances normally isolated from electron transport systems. Other substances which are stronger reducing agents than reduced ferredoxin, or stronger oxidizing agents than oxidized cytochrome f, may exist in the naturally occurring material, but would be difficult to isolate.

Another class of compounds of importance in electron transport systems, and found in chloroplast material are the quinones. Plastoquinone is thought to play an important role in both photoelectron transport and photophosphorylation, (Crane, et.al., 1960). It has sometimes been suggested that vitamin K and other quinones may be components of the photoelectron transport system, (Kegel, et.al., 1962).

B. Evolutionary relation between particles from mitochondria and from chloroplasts.

There are many similarities between the photoelectron transport particle from photosynthesis, the quantasome, and the basic electron transport particle involved in oxidative phosphorylation in non-photosynthetic cells. The latter particle, found in mitochondria and sometimes called the elementary particle or oxosome, is also probably a high molecular weight particle with dimensions in the order of 100 to 200 Å. It contains a complement of cytochromes, non-heme iron proteins, quinones, etc., and performs the transport of electrons coupled to the formation of ATP. Thus, one can make a strong case for the evolutionary relationship between the basic oxidative electron transport particle and the photoelectron transport

particle. Speculations as to which one came first in evolution are dependent upon what one assumes to have been the environmental conditions under which the evolution occurred.

E. Membrane structure

Little is known about the structure of the continuous layer to which the particles of the quantasomes are attached or embedded. Presumably this lamellar membrane is mostly lipid in character. The major fraction of lipids in the chloroplasts are surfactant molecules which are concentrated in the grana. Of these, galactolipids are predominant, comprising about two-thirds of the chloroplast lipids, (Benson, et.al., 1958). The rapid labeling with carbon-14 of these compounds during photosynthesis in the presence of radioactive carbon dioxide suggests that they may be involved in carbon compound metabolism during photosynthesis. Benson (1961) has suggested that there may be two types of lipid lamellae surfaces: an outer one dominated by galactolipids in the region of carbohydrate synthesis, and an inner one dominated by chlorophyll and the electron transport systems.

This interesting proposal leads one to wonder if the entire process of carbon reduction during photosynthesis could be occurring in enzymes located on the surface of the lamellae. On the other side of the lamellae could be embedded the photoelectron transport particles. A system with the various components of the photosynthetic reaction brought into such close juxtaposition could be highly efficient. For example, electrons with a high reducing potential (generated by the photoelectron transport system) might be conducted by some mechanism directly through the lipid layer to the carbon reduction system. Such suggestions are at present

purely speculative. There is much yet to be learned about the nature of the detailed arrangement of molecules within the chloroplasts in the living cell.

F. Orientation of pigment molecules

As for the photoelectron transport system itself, something is now known about the arrangement of its molecules. It appears that the chlorophyll molecules are to some extent aggregated in the quantasome. This arrangement permits the transfer of energy from one molecule to another. Thus, when a photon is absorbed by the chloroplyll molecule it may migrate through the quantasome by some type of exciton migration. Moreover, certain of the pigment molecules are oriented in a rather specific way. These oriented molecules appear to have a maximum absorption peak at longer wavelengths than ordinary chlorophyll a, (Brody, 1958). Butler (1961) domonstrated that fluorescence at 720 mμ and -196°C is due to a pigment which absorbs at 705 to 710 mμ. Ordinary chlorophyll a absorbs very little in this region. It is also shown that energy absorbed by ordinary chlorophyll could be transferred to this longer wavelength absorbing pigment, at least at -196°. Olson, et.al.(1961) studied the polarization of fluorescence of long wave light emitted by chloroplasts. They concluded that the molecules emitting the long wavelength fluorescence (a) accept energy from other pigment molecules, and (b) are highly oriented.

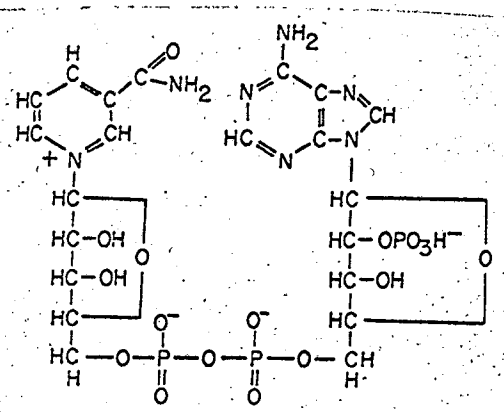
Sauer and Calvin (1962) demonstrated the orientation of some pigment molecules, in spinach quantasomes. These particles exhibit electric birefringence and electric dichroism. Thus the orientation of pigment molecules is not solely related to the whole lamellar system, but is a property of the basic macromolecular particle involved in quantum conversion.

IV. Photoelectron transport and photophosphorylation

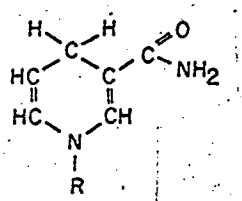
A. End reactions

1. Cofactor requirements for reductions and syntheses

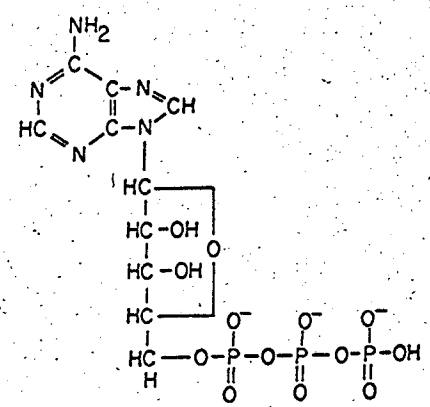
In Section II, the argument was presented that photosynthesis in green plants is the oxidation of water coupled to the reduction of carbon dioxide, nitrate and sulfate together with the syntheses of organic compounds from these inorganic oxides. Such reduction and syntheses require two types of carriers of electrons and chemical energy. Electron carriers must accept the electrons from the oxidation of water, and act in their reduced forms as the reducing agents for the enzyme-mediated reductions of the carbon, nitrogen and sulfur. Electron carrying cofactors such as reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced chloroplast ferredoxin, a non-heme iron protein, function near the terminal stage of the photoelectron transport system. These cofactors are closely associated with the final reactions, in which the reduction of inorganic oxides to organic compounds is accomplished.



Nicotinamide adenine dinucleotide phosphate (oxidized form) ($NADP^+$)



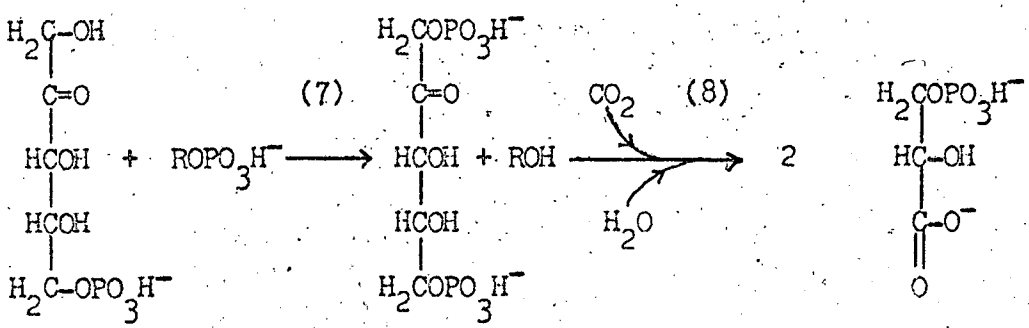
Nicotinamide portion of NADPH (reduced $NADP^+$)



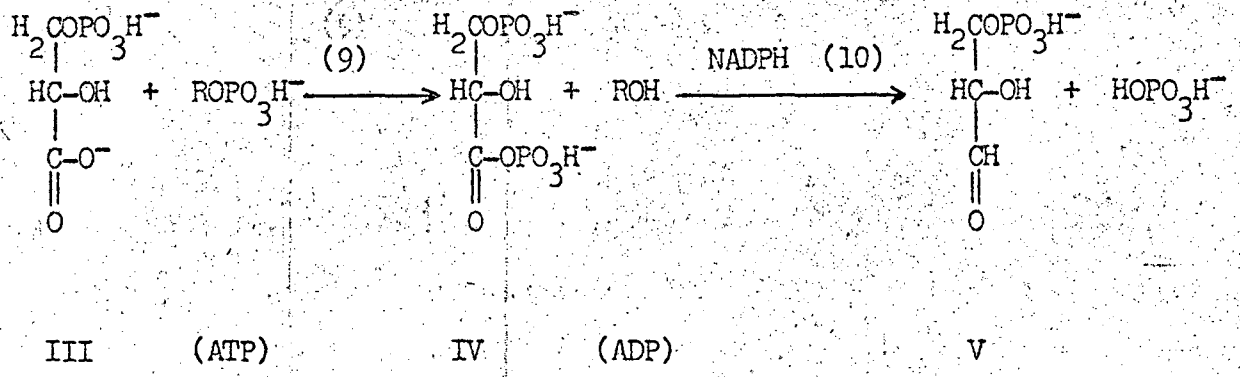
Adenosine triphosphate (ATP)
In adenosine diphosphate (ADP), terminal phosphate is replaced by -OH

The other class of energy carrying cofactors are the acid anhydrides of the type adenosine triphosphate (ATP). These substances are activating reagents. Enzymes utilize these acid anhydrides to convert substrates to forms which are more reactive in subsequent enzymic steps. For example, an enzyme of the type called kinases may replace the hydrogen atom of an alcohol with a phosphate group. This occurs in the photosynthetic carbon reduction cycle. As we shall see, ribulose-5-phosphate (I) is phosphorylated to give ribulose-1,5-diphosphate, (II)., [Reaction (7)]. The resulting sugar diphosphate is a sufficiently reactive molecule to permit its enzymic carboxylation. This is the basic carboxylation step of the carbon reduction cycle of photosynthesis. The product of the carboxylation reaction is 3-phosphoglyceric acid (PGA), (III)., [Reaction (8)].

Another enzyme of the carbon reduction cycle activates PGA, converting it to phosphoryl-3-phosphoglyceric acid (IV). This acid anhydride can then be reduced in a subsequent enzymic step mediated by triose phosphate dehydrogenase. For its reducing agent, this enzyme uses nicotinamide adenine dinucleotide phosphate (NADPH) and thereby converts the carboxylic acid to 3-phosphoglyceraldehyde (V). This resulting triose phosphate is a sugar phosphate. The following sequence of reactions



I (ATP) II (ADP) III



III (ATP) IV (ADP) V

thus uses molecules of ATP and NADPH to accomplish the fixation and reduction of carbon dioxide to the level of sugar.

The reduction of nitrate to ammonia, which occurs through several stages, and the reduction of sulfate to the sulfhydryl groups of amino acids also requires electrons which are supplied by NADPH, reduced ferredoxin, or both. It appears [illegible] that reduced ferredoxin is a branch point from which the electrons derived from the light reactions are distributed to various metabolic pathways for a variety of reductive steps.

The synthetic reactions requiring electrons and ATP are not limited to the initial reduction of the inorganic oxides. Many secondary photosynthetic pathways in the chloroplast convert the products of the primary carbon reduction cycle plus ammonium and sulfhydryl to a host of secondary products. Among these are carbohydrates, fats, proteins, nucleic acids, various coenzymes, and many other substances needed for both the growth and activity of the chloroplasts and for export to other parts of the cell or organism.

2. The production of cofactors by isolated chloroplasts

In experiments with isolated chloroplasts, the photoreduction of $NADP^+$ to NADPH with simultaneous O_2 evolution was demonstrated by Vishniac and Ochoa (1951), Tolmach (1951), and Arnon (1951). Formation of ATP in

the light, called photosynthetic phosphorylation, was discovered by Frenkel (1954) in particles from photosynthetic bacteria, and by Arnon, et.al.(1954) in isolated chloroplasts.

The electromotive force for the reduction of $NADP^+$ to NADPH at physiological conditions is E' = -.324 volts. This means that NADPH is a relatively strong biological reducing agent. However, an even stronger reducing agent is produced in the light reactions of photosynthesis. San Pietro and Lang (1958) discovered an enzyme cofactor in isolated chloroplasts capable of transferring electrons from water to $NADP^+$ in the presence of illuminated chloroplast fragments. They named this enzyme "phosphopyridine nucleotide reductase" (PPNR). It was subsequently shown by Tagawa and Arnon (1962) that PPNR is a non-heme iron protein cofactor. This cofactor is called chloroplast ferredoxin and, as already noted, it has a redox potential of -0.43 volts. This means that in its reduced form it is a strong biological reducing agent, comparable to hydrogen gas at pH 7. Ferredoxin functions in conjunction with a flavoprotein enzyme found in the lamellar material of the chloroplast. Together, enzyme plus ferredoxin facilitate the transfer of electrons from the photoelectron transport system to $NADP^+$. However, ferredoxin can function as a cofactor for the reduction of other substances besides $NADP^+$.

Photophosphorylation by chloroplasts has been classified as cyclic and non-cyclic (Whatley, et.al., 1959). During cyclic photophosphorylation, electrons which have been transferred by a photochemical reaction from a weak reducing agent to a stronger reducing agent, return through electron transport cofactors to the weaker reducing agent. The energy which would be liberated by this chemical reaction is utilized in the formation of ATP from ADP and inorganic phosphate. This utilization of chemical energy from

one chemical reaction to promote another is common to photosynthetic phosphorylation and oxidative phosphorylation. The mechanism of the coupling between the oxidation reduction reaction and the formation of ATP is not precisely known in either case. In cyclic photophosphorylation, since the electrons return to the same cofactor from which they were initially removed, (see Fig. 3) there is no stoichiometric conversion of either electron donors or acceptors. The reaction of cyclic photophosphorylation may be written as follows:

$$(11) \quad ADP^{-3} + HPO_4^{-2} + H^+ \longrightarrow ATP^{-4} + H_2O \quad \Delta F^S = +11 \text{ Kcal}$$

This value for the free energy change is based upon an assumed physiological standard free energy change of +6.9 Kcal when all reactants are at unit activities except for hydrogen ion at 10^{-7} molar. This standard physiological free energy change is then corrected by assuming the activity of inorganic phosphate to be 10^{-3}. The correction in free energy change is then $-RT \ln (10^{-3}) = +4.1$ Kcal.

Arnon (1958) reported a stoichiometric relation between the simultaneous photoelectron transport of electrons from water to $NADP^+$ and photophosphorylation. This combination of reactions which is called non-cyclic photophosphorylation may be expressed by the following equation:

$$(12) \quad NADP^+ + H_2O + HPO_4^{-2} + ADP^{-3} \longrightarrow 1/2\ O_2 + NADPH + ATP^{-4} \quad \Delta F^S = +63.6 \text{ Kcal}$$

Other studies (Turner, et.al., 1962) indicate that the stoichiometric ratio of one for ATP and NADPH production is not realized under many conditions. Thus, either ATP formation can be uncoupled from photoelectron transport, or there are alternate pathways of electron transport, of which only some require coupling to ATP formation.

B. Two light wavelength effects

1. Enhancement

The possiblility that two separate photochemical acts were involved in photoelectron transport was first suggested by experiments of Emerson and co-workers (1956, 1957). These workers studied the yield of oxygen produced by photosynthesis in Chlorella in the presence of only long wavelength light. The production of oxygen at wavelengths of light around 700 mμ is very inefficient in terms of oxygen molecules evolved per quantum absorbed. However, if supplementary light of a shorter wavelength is added to the long wavelength red light, the total evolution of oxygen is now greater than the sum of the oxygen evolved separately by the long wavelength and shorter wavelength light. In fact, the amount of oxygen evolved becomes almost as great as if the long wavelength light were used as efficiently as the shorter wavelength light.

This experimental result strongly suggests that the longer wavelength light (700 mμ) can only accomplish part of the transport of electrons from water to $NADP^+$. Presumably the shorter wavelength light can accomplish the entire process by itself. However, if both kinds of light are present, the shorter wavelength light can be used exclusively to accomplish the more difficult part of the photoelectron transport, leaving the easier part to be accomplished by the long wavelength light. With only short wavelength light, some of the light must be used for each of the two parts of the process. Thus, the addition of some long wavelength light to the shorter wavelength light becomes equivalent to adding more of the shorter wavelength light by itself. Several mechanisms may be proposed to explain this enhancement effect by itself. Many other studies have been required to show that the cooperation between two light wavelengths seemingly operates through a biochemical mechanism.

2. Light wavelength transients

Blinks (1960, 1959) studied the transients in the photosynthetic rate upon a sudden change in the wavelength of incident light. The organisms which he studied include a variety of red, green and brown algae. In his experiments, the incident light was adjusted in intensity so that at each wavelength the same photosynthetic rate was obtained. When the incident wavelength was suddenly changed, transient changes in rate occured. In some of the organisms which he studied, the absorption spectrum of accessory pigments is well separated from the absorption spectrum of chlorophyll a. Thus he was able to demonstrate that the transients observed on switching from one wavelength to another were related to the absorption by different pigment systems.

Myers and French (1960) studied the Blinks effect by observing the transient increase in rate upon changing from 700 mμ light to light of another wavelength. They also studied the enhancement of photosynthesis (the Emerson effect), by adding the other wavelength light to 700 mμ light. When the two effects in Chlorella were plotted on comparable scales as a function of the added wavelength, their action spectrum were found to be identical. Thus, enhancement and chromatic transient effects appear to be manifestations of the same phenomena: there is a possibility in photosynthesis for the products of one photochemical reaction to be used as substrate for a second photochemical reaction to bring about the overall photoelectron transport from water to the cofactors required for the carbon cycle. The light reaction mediated by the longer wavelength light has come to be known as Light Reaction 1, while the other light reaction which requires shorter wavelengths is termed Light Reaction 2.

3. Chemical separation of two light steps

Many experiments have now been performed with chloroplasts and other photosynthetic material in which electrons are injected or withdrawn from the photoelectron transport pathway by the addition of electron donors or acceptors. These studies, together with the enhancement and chromatic transient experiments, have led to the formulation of a currently popular scheme for photoelectron transport, shown in Fig. 3.

According to this scheme, there are two pigment systems. These are labeled Pigment System 1 and Pigment System 2, for the longer wavelength light reaction and the shorter wavelength light reaction respectively. Pigment System 2 is responsible for the absorption of light energy and the conversion of this energy to bring about the oxidation of water, liberating oxygen and transferring electrons to some intermediate cofactor of the electron transport system. Electrons then flow through a sequence of electron transport cofactors, which may include such substances as plastiquinone, cytochrome b, cytochrome f and perhaps others which are not yet known. Light energy absorbed by Pigment System 1 is converted to chemical energy by the oxidation of chlorophyll a. This oxidation results in the donation of electrons to some acceptor which becomes a strong reducing agent. The oxidized chlorophyll a regains electrons by the oxidation of cytochrome f, the terminal electron-carrying cofactor in the intermediate system. The energy liberated through the flow of electrons in the intermediate system is used by means of coupled reactions to convert ADP and inorganic phosphate to ATP.

The strong reducing agent formed as a result of the Pigment System 1 energy conversion reaction then brings about the reduction of ferredoxin, which in turn reduces $NADP^+$ and supplies the cofactors for the carbon reduction cycle. Alternatively, some of the electrons from the strong

reducing agents produced by Pigment System 1 can be shunted back to the start of the intermediate electron transport system. From there, the electrons flow back through the coupled reactions to cytochrome f, allowing the production of ATP. This process is called cyclic photophosphorylation. The transport of electrons from water to $NADP^+$ with the concurrent reduction of ATP is the non-cyclic photophosphorylation.

With this scheme before us, let us consider some of the biochemical evidence in its support. One can choose only a few of the many interesting experiments which have been published. More complete descriptions of these experiments may be found in collections of papers presented at symposia such as that sponsored by the National Academy of Sciences (1963) and the Centre National de la Recherche Scientifique (1963).

Ash, et.al. (1961) first showed that tetrazolium blue and methyl red could be photoreduced in a Hill reaction by spinach chloroplasts. Since no electron donor was added, it was presumed that this reaction was accompanied by the evolution of oxygen. Reduction of both dyes was strongly inhibited by the addition of dichlorophenylmethylurea (DCMU), which is thought to block photosynthetic oxidation of water leading to oxygen evolution (System 2). Reduction of the tetrazolium blue or methyl red was restored upon the addition of ascorbate and dichlorophenol indophenol (DPIP) as electron donors. It was presumed that these electron donors were supplying electrons to the intermediate electron transport system, permitting the transfer of electrons through the light reaction of System 1 and their eventual utilization for the reduction of the dyes. Thus, System-1 was presumed to be operating under artificial conditions, even though System 2 was blocked by the action of DCMU.

Losada, et.al. (1961) reported the separation of the two light reactions

in non-cyclic photophosphorylation and $NADP^+$ reduction in green plants. They blocked System 2, which oxidizes water, by adding the inhibitor DCMU and then adding a dye as an electron donor. Under these conditions they were able to demonstrate the photochemical reduction of $NADP^+$ and the simultaneous non-cyclic photophosphorylation.

Levine and Smillie (1962) used mutants of Chlamydomonas reinhardi to show clearly the separation of photoelectron transport into two phases, each with its own light reaction. Two mutant strains which are incapable of carrying out the Hill reaction are able to photoreduce $NADP^+$ without oxygen evolution, provided they are supplied with electrons from an electron donor. Both mutants had an increased content of cytochrome f and a decreased content of plastiquinone. Presumably they are capable of performing the reactions carried out by System 1 only.

Another mutant studied by Levine and Smillie was able to carry out both oxygen evolution and photoreduction of NADPH, provided that an electron donor was added. This mutant had a normal complement of plastiquinone. In this case, both light systems apparantly are operative, but there is a block somewhere between the plastiquinone and cytochrome f which can be bridged upon the addition of the electron donor.

From these experiments and many others, it seems established that photoelectron transport in green plants can occur via two photochemical steps, together with some intermediate dark steps. In one photochemical step (System 2), plus one or more dark steps, electrons are removed from water and transferred to an electron acceptor which may be plastiquinone. Dark reactions plus a second photochemical step transfer electrons to another electron acceptor, which becomes a reducing agent of at least the strength of hydrogen gas or reduced ferredoxin. Coupled to one or more stages of the

electron transport, ATP formation by non-cyclic photophosphorylation occurs. Upon the addition of cofactors to broken systems, electrons from the strong reducing agent formed by System 1 may be cycled back to an intermediate reducing agent, and in this process cyclic photophosphorylation occurs. Quite possibly the formation of ATP by cyclic photophosphorylation takes place in the identical electron transport step involved in non-cyclic photophosphorylation, though this point remains to be demonstrated.

Even though the scheme for photoelectron transport in photosynthetis shown in Fig. 3 accounts for many experimental observations, and is currently widely accepted as being at least approximately correct, it cannot be considered as proved. In particular, it should be noted that even though some photoelectron transport may proceed via a two-light reaction pathway, there may be other pathways in which electrons can be taken from water to some cofactor such as reduced chloroplast ferredoxin in a single light reaction. In other words, the two-light reaction path may not be the exclusive mechanism of electron transport in photosynthesis in all organisms.

C. Quantum conversion

1. Photoinduced changes in absorption spectra and EPR signals

Two types of experimental observations have been particularly useful in studies of the mechanism of the primary steps of conversion of electromagnetic energy to chemical energy, or quantum conversion. These are the measurement of changes in absorption spectra for visible light, and of electromagnetic energy of the frequency 10^9 cpm (wavelength 3 cm). In both cases, the changes measured are elicited by allowing the plant material to absorb exciting light which may be either a flash or continuous

light. In both cases, the signals observed are by no means limited at room temperature to primary quantum conversion events, but may be associated with other subsequent steps in the photoelectron transport process, or even later metabolic reactions. However, in the cases of those changes which are still observed at very low temperatures, such as the boiling point of liquid N_2, it can be argued that one is dealing with phenomena related only to excited states of the molecules, electron migration, and charge separation. These are acts considered to be involved in the primary quantum conversion steps.

The photoinduced changes in absorption spectra of plant pigments usually are very small compared to total absorption. Thus, measurement requires rather sensitive techniques. Two light beams are used in the experiments. There is an activating beam to cause the change, and a measuring beam which is partially absorbed. It is the amount of absorption of the measuring beam which measures the extent of the photoinduced change. Elegant techniques have been devised for measuring the absorption change for the measuring light without interference from the exciting light, and many studies of the kinetics of the changes in absorption under a variety of conditions have been published. Some of these have been reviewed recently by Rumberg, et.al. (1963), Duysens (1963), Kok (1963), Chance and Bonner (1963).

Electron paramagnetic resonance (EPR) signals are detected by measuring the amount of absorption of electromagnetic energy (wavelength 3 cm) by samples which are in the presence of a strong magnetic field. This field causes a splitting of energy levels of unpaired electrons, permitting energy absorption. Thus, EPR signals indicate the presence in the sample of unpaired electrons. The significance of EPR signals has been discussed in recent

review papers (Calvin and Androes, 1962; Commoner, 1961; Beinert and Kok, 1963).

A negative change in absorption spectrum (bleaching) at around 700 mu has been extensively studied by Kok (1963). This change was observed for the most part in chloroplasts from leaves of plants such as spinach. The bleaching of the pigment can be caused chemically (in the dark), and the bleached form has the properties of a weak oxidant (redox potential +0.43 V). Kok has termed this pigment P700. He has concluded that it is a form of chlorophyll a, and a key substance in Pigment System 1 (see Fig. 3), which mediates the "long wave length" step in photoelectron transport. This step is responsible for the transport of electrons to a high (negagive) redox level, from which they can be used to reduce oxidized chloroplast ferredoxin and subsequently $NADP^+$. This conclusion is based on such evidence as the following.

The reduction of $NADP^+$ was studied as a function of exciting light wavelength both in the presence and absence of the poison DCMU (which blocks the flow of electrons through Pigment System 2), (Hoch and Martin, 1963). When DCMU was present (poisoned chloroplasts), reduced dye was added to supply electrons in place of Pigment System 2. Measurements were made in each case of (a) amount of monochromatic flashing light absorbed (to give the number of quanta used by system), (b) number of equivalents (moles of electrons) transferred to added $NADP^+$, (c) number of moles of P700 bleached (actually the sum of repetitive bleaching) assuming an extinction coefficient equal to that of the red absorption band of chlorophyll a. The following results were reported:

In the unpoisoned chloroplasts, two quanta of 650-680 mu light are required to transfer one electron from water to $NADP^+$. In the poisoned chloro-

plasts, with dye as an electron donor, the quantum requirement is relatively high at shorter wavelengths (some quanta presumed "wasted" in poisoned System 2) but falls with increasing wavelength until it approaches one quantum per electron transferred at long wavelengths (around 700 mu). The number of moles of P700 bleached corresponded to the number of equivalents of electrons transferred to $NADP^+$.

Such measurements are difficult, and different results have at times been reported. Recently, Biggins and Sauer (1964) have made a very extensive examination of the quantum requirements for $NADP^+$ reduction as a function of wavelength with poisoned and unpoisoned chloroplasts. Two respects in which their techniques differ from those of Hoch and Martin have to do with the method of correction for light scattering (a very serious problem in quantum requirement measurements with particulate material) and their method of measurement of $NADP^+$ reduction. It is reassuring to find that while their results differ in detail from those of Hoch and Martin, they do get a quantum requirement of about 2 to 2.5 per electron transferred in the unpoisoned chloroplasts and about 1.0 for the poisoned system plus reduced dye. Thus the role of the long wavelength light reaction of photosynthesis as a one quantum per electron transfer from the intermediate electron transport system to $NADP^+$ seems almost established.

The photochemical transfer of a single electron should result in the formation of species with unpaired electrons. These species can be expected to give EPR signals, and it has been of interest to see if the EPR signals produced by the absorption of light in photosynthetic material can be correlated with the P700 reaction.

Beinert and Kok (1963) compared the number of unpaired electrons (as

indicated by EPR signals) produced in illuminated photosynthetic material of various types with the amount of P700. Both the number of unpaired electrons and the circumstances and kinetics of their appearance and disappearance correlate with the amount of bleached P700, which they write as $P700^+$. They had previously shown that with particles from red algae they could produce corresponding amounts of $P700^+$ and unpaired electrons in the dark by using ferricyanide as an oxidizing agent. All of this does not prove that the species directly responsible for the EPR signals is $P700^+$. Indeed, evidence presented below for the presumed corresponding system in bacterial chlorophyll suggests that the oxidized pigment does not give the EPR signals. Perhaps a more likely candidate is the hypothetical Y^-, which is supposed to accept an electron from the excited molecule of chlorophyll.

Clayton (1962) and Loach, et.al.(1963) demonstrated a relationship between optical changes in absorption spectra and EPR signals with photosynthetic bacteria. In those experiments, light-induced absorption changes and EPR signals could be simulated in the dark by chemical oxidation.

Calvin and Androes (1963) studied the chemically-induced and photo-induced EPR signals in spinach quantasomes and in the photosynthetic bacterial chromatophores from R. rubrum. In both cases, the EPR signals could be produced in the dark by adding oxidizing agents with a potential of +.44 V. alone.
Similar EPR signals were produced by the light. Ruby et.al. (1964) examined the kinetics of the rise and decay of the EPR signals and changes in absorption in chromatophores (the photosynthetic particles from photosynthetic bacteria) from R. rubrum. The molecular species of bacterial chlorophyll which undergoes bleaching (and may correspond in function to P700 in green plants) has its long wavelength bleaching maximum at 865 mμ. It was found

that the kinetics of the decay of the EPR signals corresponded not with the absorption change at 865 mμ, but instead were correlated with a change at 433 mμ. The conclusion is that it is not $BChl^+$ which exhibits the EPR signals, but another substance. Presumably this substance might be the electron acceptor.

2. Mechanism of quantum conversion

Calvin (1958) has proposed essentially the following mechanism for the primary quantum conversion steps of photosynthesis. Following the absorption of light by any of the "antennae" chlorophyll molecules in the quantasome or chromatophore, the excited state of the chlorophyll molecule may be transferred from one pigment molecule to another by a process of resonance transfer or exciton migration. Ultimately the energy is transferred to a set of specialized chlorophyll or bacterial chlorophyll molecules (the long wavelength absorbing species) which are part of an organized, oriented array of pigment molecules in close contact with electron acceptor and donor molecules. The acceptor and donor molecules are required to be separated from each other by a sufficient distance to prevent recombination, however. Once one of these specialized Chl or BChl molecules in contact with an acceptor molecule receives the exciton, its electrons are raised to an excited state from which one of the electrons can be transferred to the acceptor molecule, accomplishing thereby a charge separation. The reduced acceptor, holding a localized, unpaired electron, exhibits EPR. The charge on the Chl^+ or $BChl^+$ is delocalized over several molecules, causing a broadening of the EPR signal to a point where no EPR signal attributable to the oxidized pigment molecules can be detected. In a very short time, this delocalized positive charge or "hole" interacts with an electron donor, such

as cytochrome f, causing oxidation of the donor and neutralization of the pigment. All the processes described in this paragraph can occur at very low temperatures and are not enzymic reactions in the usual sense.

It may be supposed that Pigment System 1 and the bacterial system are both evolved from a common primative photosynthetic system. The fact that bacterial photosynthesis operates at longer wavelengths may be a consequence of photosynthetic bacteria adapting to a world in which green plant photosynthesis has become dominant. One could imagine photosynthetic bacteria living in the shade of green plants and utilizing long wavelength light (710-850 mμ) not absorbed by the plants.

3. Pigment System 2 and O_2 evolution

The photoelectron transport of electrons from water to the intermediate electron transport system (plastoquinones, cytochromes, etc., Fig. 3) has not been resolved into its component reactions. These must include the primary quantum conversion act of Pigment System 2, the oxidation of water, the evolution of O_2 from the immediate product of this oxidation, and the reduction of some stable intermediate electron carrier, such as plastoquinone, by the electrons from the oxidation of water. It is known that the operation of Pigment System 2 becomes very inefficient (in terms of electrons transported per quanta absorbed) at wavelengths longer than about 700 mμ. The evolution of O_2 requires manganese (Kessler, 1957) and is inhibited by such substances as DCMU (Kok and Hoch, 1961). This system is disrupted much more easily by aging or breaking of the particulate system than is Pigment System 1 (Kok and Hoch, 1961).

Rumberg, et.al. (1963) have ascribed photoinduced changes in absorption at 515 mμ and at 254 mμ to the operation of Pigment System 2. The action spectra for these changes (amount of change as a function of wavelength of activation light) is the same as for O_2 evolution and has its peak at 674 mμ.

The change at 254 mμ is presumed to be due to reduction of plastoquinone. Both the 515 mμ and 254 mμ changes can be diminished by partial extraction of the plastoquinone from the plant material (chloroplast fragments) and reappear when the plastoquinone is added back. It seems that the 515 mμ change is due to a substance closely related to plastoquinone in the reactions mediated by Pigment System 2. It could in fact be the substance X (see Fig. 3) which accepts electrons from the quantum conversion reaction.

It is possibly noteworthy that neither EPR nor temperature-independent visible light absorption changes have been related to the operation of Pigment System 2. Thus one is not constrained to assume that the quantum conversion mechanism of Pigment System 2 is similar to that in Pigment System 1, beyond the migration of excitons to the locus of the quantum conversion machinery. Evidence has been given that quantum conversion by System 1 is a one electron transfer mechanism, and that one quantum of light is required per electron. It has been rather widely assumed that the mechanism of System 2 operates in similar fashion and ~~will~~ also requires one quantum per electron. Thus two quanta would be required to transfer each electron from water to $NADP^+$ and ultimately to CO_2. For the reduction of one molecule of CO_2 to carbohydrate (reaction 3), four electrons must be transferred, thus the quantum requirement would be 8 quanta per O_2 molecule evolved.

However, one should not overlook the possibility that Pigment System 2 is capable of transferring 2 electrons from water to X^- or plastoquinone, for example, with only <u>one</u> quantum. In this case, the quantum requirement for transfer of four electrons through the two light reactions would be $4 + 2 = 6$. If we recall that the free energy change corresponding to reaction 3 was 113 Kcal per mole, we can calculate what the efficiency of photosynthesis would be for six or eight quanta per O_2 molecule. Using the relation $E = Nhc$, where N is Avogadros number (6×10^{23}), h is plank's constant,

c the velocity of light, and λ the wavelength of light which we will take as 680 mμ, application of the appropriate units gives a value of about 42 Kcal / einstein (an einstein being a mole of quanta). For an ideal quantum requirement of eight, the efficiency would be 113 / 336 = 34%

Despite a large amount of work from several laboratories, attempts to measure the overall quantum requirement of photosynthesis have not yielded results which could permit one to make a conclusive choice between these mechanisms. One can say that, except for the studies by Warburg and his collegues, measurements of the quantum requirement of photosynthesis have generally given values around eight or more, with a few reported values between seven and eight. (For a review of this subject, see Kok, 1960). The author is among a minority of investigators in this field who believe that the closeness of many experimentally measured quantum requirements to eight, and the fact that some are even less than eight, suggests that the theoretical quantum requirement is in fact below eight, and is probably six or seven. This is another way of saying that less than two quanta may be required to transfer an electron from water to $NADP^+$.

V. The reduction of carbon dioxide in photosynthesis

A. Methods of mapping the path of carbon

1. use of radioisotopes as tracers

Now let us turn our attention to the reductive and synthetic reactions of photosynthesis. The basic carbon reduction cycle by which carbon dioxide is reduced to sugar phosphate involves at least twelve intermediate compounds. Some of these substances are found in very small concentrations. Many similar compounds are also present in the photosynthetic cell. In some cases they are closely linked by metabolism to the intermediates in the carbon reduction cycle. In order to understand the mechanism of photosynthetic carbon reduction,

one must know the identity of the intermediates involved and the sequence in which these substances are made.

These problems resisted scientific investigation until the advent of modern biochemical methods. One of the most valuable of these new methods proved to be the use of radiocarbon as a tracer element to follow the newly incorporated carbon dioxide during photosynthesis in a green plant. Photosynthesizing plants do not discriminate significantly between ordinary carbon dioxide, $^{12}CO_2$, and radioactive carbon dioxide, $^{14}CO_2$. The plants incorporate $^{14}CO_2$ into the intermediates in the carbon reduction pathway.

Ruben, et.al. first used radiocarbon in studies of the path of carbon in photosynthesis (1940). They found that CO_2 fixation in the dark is greater following preillumination. This is in accord with the concept that CO_2 fixation occurs by "dark reactions" which use relatively stable chemical species formed in the light. They discovered that the radioactive product formed from labeled CO_2 after preillumination was a carboxylic acid. These findings suggested that the path of carbon reduction in photosynthesis might very well include CO_2 fixation mechanisms similar to those found in non-photosynthetic plant tissues.

Calvin and Benson and their co-workers used the radioisotope method to study carbon reduction in photosynthesis, (Calvin and Benson, 1948-49). They allowed the plants to photosynthesize intermediate compounds from $^{14}CO_2$ for short periods of time (a few seconds) and under a variety of experimental conditions. The plants were then killed, stopping the biochemical reactions. The radioactive products in the plant material were analyzed and identified. A careful study was made to determine the amounts of radiocarbon incorporated into chemical substances as a function of the experimental conditions. From the results of this study, they were able

to map the path of carbon in photosynthesis.

2. Analysis and identification of intermediate compounds

a. Paper chromatography

The separation and identification of the minute amounts of radioactive organic compounds formed by photosynthesis with $^{14}CO_2$ was a difficult analytical problem. This problem was solved through the use of two-dimensional paper chromatography (Benson, et.al., 1950). In this method, after the plant has photosynthesized organic compounds from $^{14}CO_2$, it is killed and the soluble compounds are extracted with such solvents as alcohol and water. Then one removes the excess solvent by evaporation and dries the concentrated extracts on a large sheet of filter paper near the corner. After that, one allows suitable mixtures of organic solvents wet with water to traverse the paper by capillarity. These solvents dissolve and carry along the various compounds at different rates of travel. The rate for each compound depends on its physical properties.

When this development by a chromatographic solvent is complete, the paper is dried. At this point, the substances have been separated into a row near one edge of the paper. That edge of the paper is then dipped in a second solvent which has different solvent properties from the first one. This time the compounds are carried in a direction at a right angle to the first direction. As a result, the compounds which have been separated in a row following the first development, separate into a two-dimensional pattern over the paper after the second stage of chromatography.

b. Radioautography

The compounds of immediate interest contain atoms of ^{14}C which emit β^- particles. When a sheet of unexposed medical X-ray film is placed in the dark in contact with the dry paper chromatogram, the areas of the

paper holding radioactive compounds are a source of the material which exposes the X-ray film. After a suitable period of exposure, the X-ray film is developed. Dark spots on the film reveal corresponding areas of radioactive substances on the paper chromatogram. Such a radioautograph made from unicellular plants which had photosynthesized with $^{14}CO_2$ for a few seconds is shown in Fig. 4.

c. Identification of radioactive products

Small amounts of many known chemical compounds have been chromatographed one at a time, using the same chromatographic procedure. Chemists found the locations of these substances on the paper chromatograms by spraying the papers with chemical reagents which react with the substance to give colored compounds. The substances are revealed as colored spots on the paper. From the locations of the compounds, a chromatographic map of known compounds was made. This map helped in the identification of the unknown radioactive compounds from the photosynthesis experiments.

Comparison of the location of an unknown radioactive compound on the paper with this map gives a preliminary indication of the possible identity of the substance. One may then elute the radioactive compound from the paper chromatogram with water. Next, one mixes the labeled substance with an unlabeled sample of the suspected compound, and rechromatographs the two together. Following this co-chromatography and radioautography, the paper is sprayed to give a colored spot. If the radioactive spot coincides precisely with the colored spot, the radioactive substance is tentatively identified. Further chemical tests, and chromatography with different solvent systems, verify the identification. In this way the compounds labeled during photosynthesis with $^{14}CO_2$ were discovered (Benson, et.al.,

1950, 1951; Benson, 1951; Buchanan, et.al., 1952).

3. Chemical degradation of labeled products

Information about the derivation of one intermediate substance from another along a metabolic path can be obtained by degrading the substances chemically. One determines the amount of labeling in each carbon atom position of the molecules. For example, Bassham, et.al. (1950) hydrolyzed 3-phosphoglyceric acid and then allowed the glyceric acid to react with periodate to give three different substances, carbon dioxide, formic acid, and formaldehyde, which were derived respectively from the carboxyl group, the alpha carbon atom, and the beta carbon atom of the original molecules. They determined the radioactive content of each of these derivatives. From this content of ^{14}C, the distribution of tracer in the original molecule could be calculated. Comparison of this distribution of label within the molecule with the distribution of label within a sugar molecule such as a molecule of glucose, gave information about the possible biochemical relations between the two substances, (see Fig. 6).

B. Experimental results and their interpretation

1. First labeled products

a. 3-Phosphoglyceric acid

The labeled products of photosynthetic reduction of $^{14}CO_2$ for 60 seconds (Fig. 4) are several sugar phosphates and diphosphates, 3-phosphoglyceric acid, phosphoenolpyruvic acid, and small amounts of other carboxylic acids and amino acids (Calvin and Benson, 1949). At only 2 seconds (Fig. 5) by far the most prominently labeled product is 3-phosphoglyceric acid (PGA) (III).

Calvin, et.al. (1951) degraded labeled PGA formed during 5 seconds' photosynthesis and showed that 95% of the radiocarbon was located in the

carboxyl atom. This finding supported their conclusion that the first reaction in the fixation of carbon dioxide it incorporates into the carboxyl group of PGA. The nature of the substance which supplies the other two carbon atoms of PGA remained for some time unknown.

b. Sugar phosphates

Among the first sugar phosphates identified were fructose-6-phosphate (VI) and glucose-6-phosphate. Soon therafter dihydroxyacetone phosphate (VII) and fructose-1,6-diphosphate (VIII), both of which occur in rather small concentrations, were identified. The finding of these compounds led Calvin and Benson (1948, 1949) to conclude that the path of carbon dioxide reduction in photosynthesis included a reversal of several steps of the glycolytic pathway which leads from hexose phosphate to PGA.

After a time, several other sugar phosphates were identified. Most important among these were the 7-carbon compounds, sedoheptulose-7-phosphate (IX) and sedoheptulose-1,7-diphosphate (SDP) (X), and the 5-carbon compounds, ribulose-1,5-diphosphate (RuDP) (II) and ribose-5-phosphate (XI), xylulose-5-phosphate (XII), and ribulose-5-phosphate (I), (Benson, et.al., 1952). The roles of these compounds in the path of carbon in photosynthesis became more clear after they had been degraded to locate the position of radiocarbon atoms within the individual molecules (Bassham, et.al., 1954).

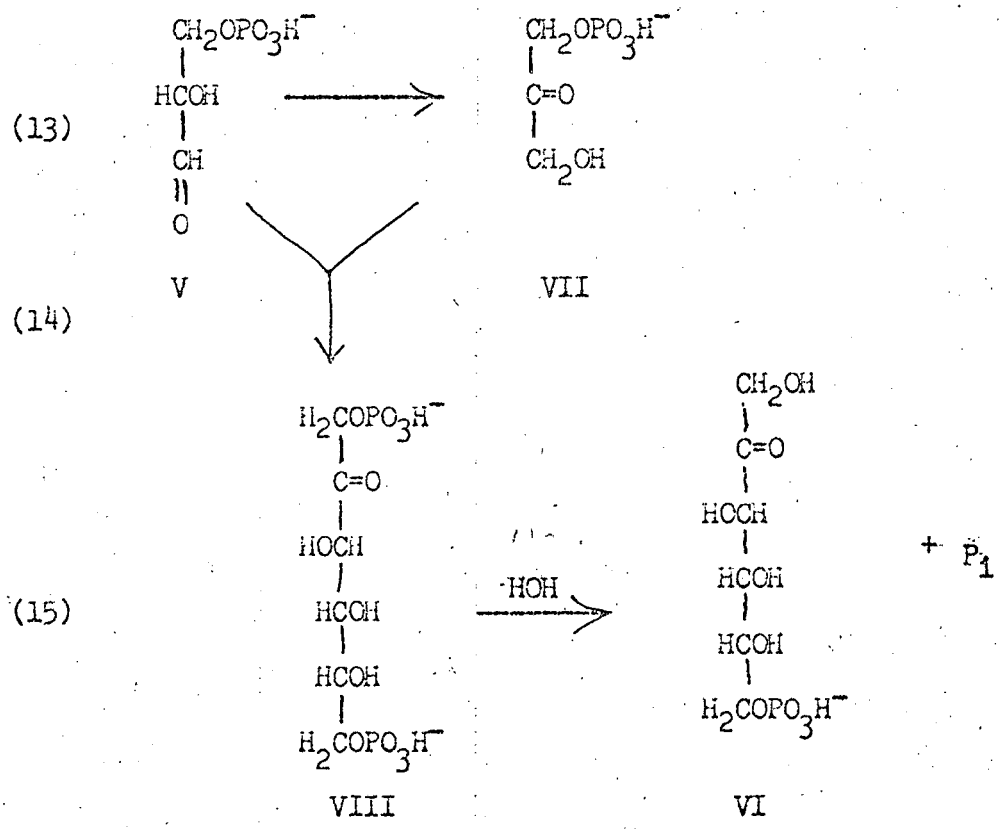
2. Location of radiocarbon within molecules

a. Carbon dioxide to hexose phosphate

As already mentioned, most of the radiocarbon found in PGA was located in the carboxyl carbon following short periods of photosynthesis. The remaining radioactivity was found equally distributed at all times between the two other carbon atoms (denoted α and β). For example, after 5 seconds of photosynthesis with $^{14}CO_2$, the distribution of ^{14}C among

carboxyl α and β carbons respectively was 95%, 2.5%, 2.5%. After 30 seconds' photosynthesis the distribution was 50%, 25%, 25%.

If the formation of hexose phosphates occurred via a reversal of the glycolytic pathway from PGA, both carbon atoms 3 and 4 of the hexose should be formed from the carboxyl of PGA. Carbon atoms 2 and 5 would come from the a carbons of PGA, while carbon atoms 1 and 6 would derive from the β carbons of PGA. When Calvin, et.al. (1951) degraded hexose molecules in such a way as to obtain these pairs of carbon atoms, they found the distribution of radiocarbon in the hexose to be as predicted by this pathway, (see Fig. 6). They concluded that during photosynthesis, the PGA formed by the primary carboxylation reaction is reduced to glyceraldehyde phosphate (V), [Eqs. (9) and (10)], which isomerizes to dihydroxyacetone phosphate (VII). These two triose phosphates then condense end to end to make fructose-1,6-diphosphate (VI), and eventually fructose and glucose monophosphates, (see Fig. 6).



It is possible that these reactions are catalyzed by enzymes similar to those of glycolysis. Many such enzymes have been isolated from plant tissue, including photosynthetic tissue (Peterkofsky and Racker, 1961). Such tissues usually are capable of glycolysis, so that isolation of the enzymes involved does not in itself prove their role in photosynthesis. Some photosynthetic organisms appear to lack sufficient amounts of some of these enzymic activities (as assayed in cell-free systems) to catalyze their assigned step in the path of carbon in photosynthesis (Richter, 1959; Fewson, et al., 1962). This might mean that these organisms perform carbon reduction by a different path. Since there is no direct evidence for such an alternate path, a more likely explanation for the apparent enzyme deficiencies is that the photosynthetic path is mediated by enzymes whose activity is in some way

lost or diminished during the preparation of a cell-free system. Perhaps the photosynthetic carbon reduction cycle enzymes are more active in a particulate, or organized enzyme system in vivo, than they are following isolation.

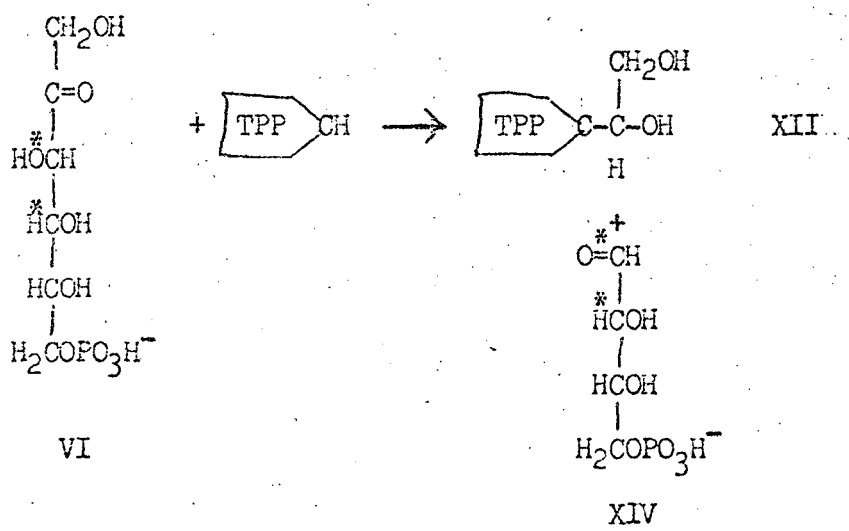
Reactions (9,10,13-15) bring about the conversion of PGA to fructose-6-phosphate (VI). The corresponding glycolytic enzymes would be: (9) phosphoglyceryl kinase; (10) triose phosphate dehydrogenase; (13) triose phosphate isomerase; (14) aldolase. Equation (15) would require a phosphatase.

b. Hexose phosphates to heptose phosphates to pentose phosphates

The 7-carbon sugar phosphate, sedoheptulose-7-phosphate (IX), was isolated from plants which had photosynthesized in $^{14}CO_2$ for a few seconds. Degradation of the sugar revealed that its label of ^{14}C was located in the three middle carbon atoms, numbers 3, 4, and 5 (Bassham, et al., 1954). We have already seen that the 6-carbon sugars were labeled in their two center carbon atoms (numbers 3 and 4) and that the trioses were labeled in their unphosphorylated terminal carbon atom. Condensation of a triose phosphate with either carbon atoms 1-4 or 3-6 of the hexose would give sedoheptulose labeled as found experimentally.

The enzyme transketolase (Racker, et al., 1953; Horecker, et al., 1953) mediates the removal of carbon atoms 1 and 2 of the ketose phosphate such as fructose-6-phosphate, forming a thiamine pyrophosphate-glycolaldehyde compound (XII) with the 2-carbon piece and producing at the same time an aldose phosphate with two fewer carbon atoms in its chain [Reaction (16)].

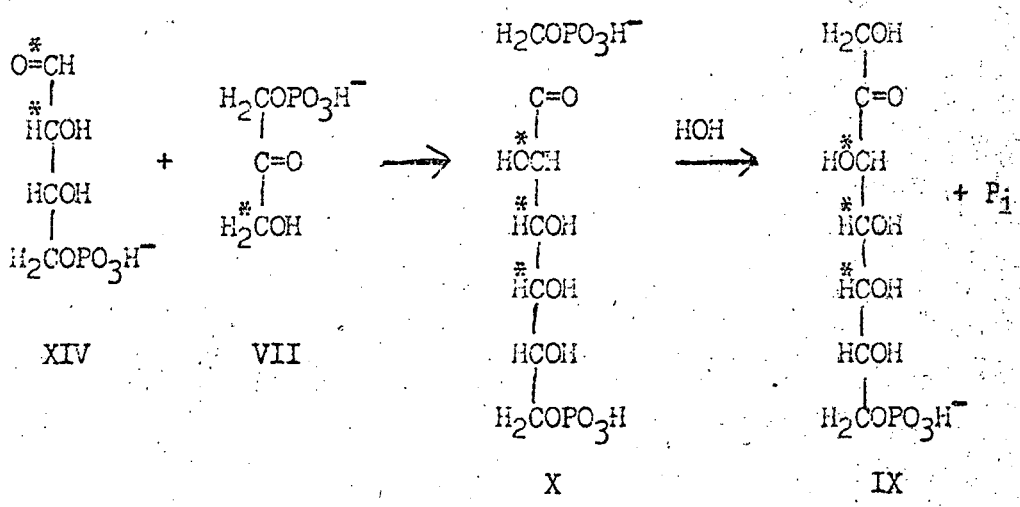
(16)



VI XII XIV

In this case, the resulting aldose phosphate is erythrose-4-phosphate (XIV), labeled in carbon atom positions 1 and 2. When this 4-carbon sugar phosphate is condensed by aldolase with dihydroxyacetone phosphate (VII), Equation (17), the resulting sedoheptulose-1,7-diphosphate (X) is labeled in positions 3, 4, and 5, as found experimentally. Removal of the phosphate [Equation (18)] on the number one carbon atom by a specific phosphatase then gives sedoheptulose-7-phosphate (IX).

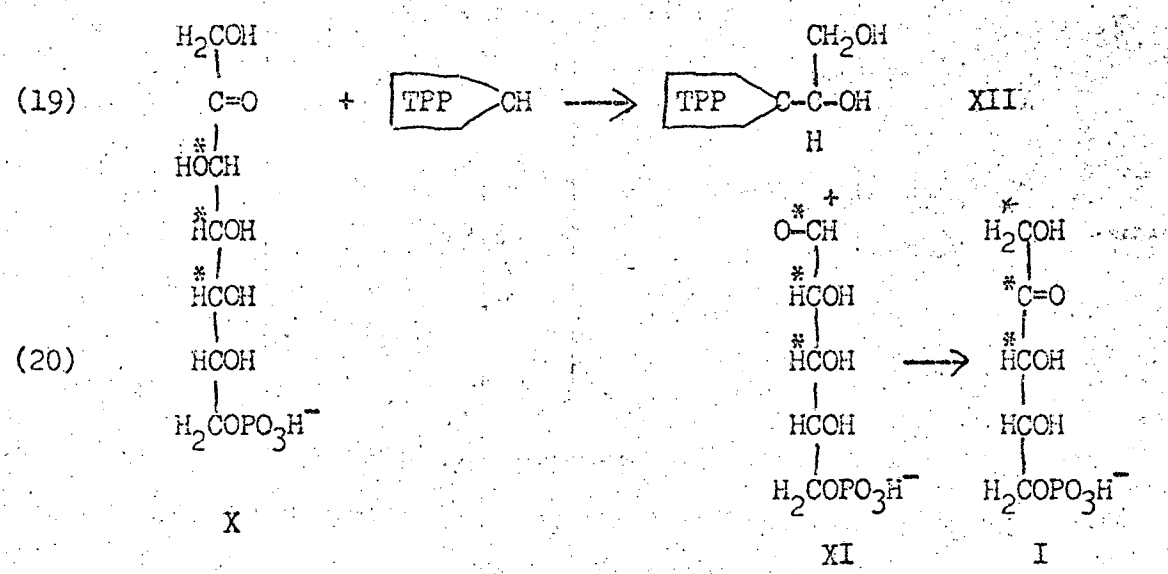
(17)(18)



XIV VII X IX

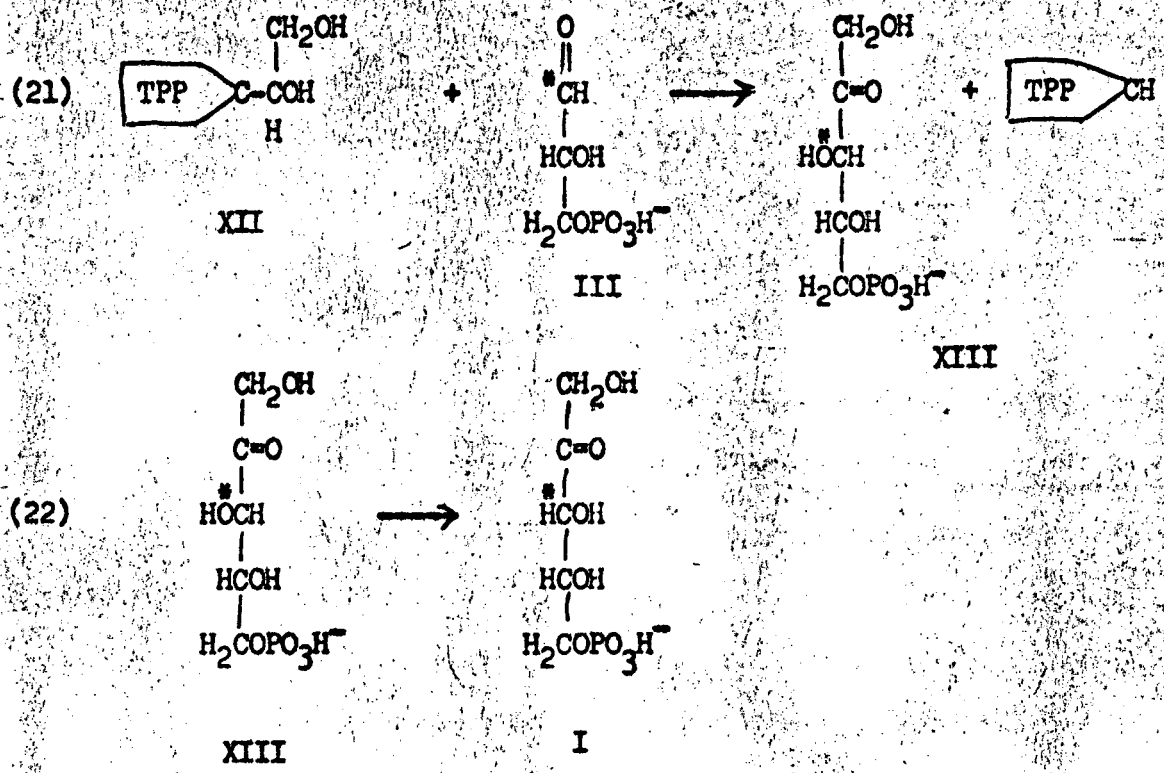
Transketolase catalyzes a reaction [Equation (19)] between thiamine pyrophosphate and sedoheptulose-7-phosphate to produce thiamine pyrophosphate-glycolaldehyde and a 5-carbon compound, ribose-5-phosphate (XI),

labeled in carbon atoms 1, 2, and 3.



Phosphoribose isomerase (Axelrod and Jang, 1954) converts this compound to ribulose-5-phosphate (I) [Eq. (20)].

The two molecules of thiamine pyrophosphate-glycolaldehyde produced by Equations (16) and (19) could react with any of the aldose monophosphates mentioned so far. We shall see in the next section that the sugar phosphate which is used up by the carboxylation is a pentose phosphate. The product of the carboxylation, PGA, is reduced to triose phosphate. Thus, there must be a net flow of carbon from triose to pentose under steady-state conditions of photosynthesis in order to complete the cycle. This flow is accomplished by a net reaction of thiamine pyrophosphate-glycolaldehyde molecules with glyceraldehyde phosphate molecules to produce xylulose-5-phosphate (XIII)[Eq. (21)]. These are converted by the action of ribulose-phosphate-xylulose-phosphate isomerase (Srere, et al., 1955) to ribulose-5-phosphate (I) [Eq. (22)].



The end result of Equations (13-22) is the conversion of five molecules of glyceraldehyde-3-phosphate to three molecules of ribulose-5-phosphate (see Fig. 6). Two of these molecules formed by Equations (21) and (22) are labeled in carbon atom position 3, while the third one, from Equations (19) and (20), is labeled in positions 1, 2, and 3. The resultant average labeling of ribulose phosphate is heavy in position 3 and lighter in positions 1 and 2. When the ribulose molecules, labeled after a few seconds' photosynthesis with $^{14}CO_2$, were degraded (Bassham, et al., 1954), this pattern of labeling was found (see Fig. 6). Thus, the mechanism of the conversion of five molecules of triose phosphate to three molecules of pentose phosphate was established.

3. Light-dark transient changes

Quite a different type of experiment was required to reveal the nature of the reaction which converts ribulose-5-phosphate to ribulose-1,5-diphosphate [Eq. (7)]. Calvin and Massini devised a system which

recirculated a stream of $^{14}CO_2$ through a suspension of photosynthesizing algae. The supply of $^{14}CO_2$ was such that it did not change appreciably during the course of the experiment (Calvin and Massini, 1952). Small aliquot samples of the algae were taken and killed from time to time. Subsequent analysis by paper chromatography and radioautography showed that the ^{14}C content of intermediate compounds in the carbon reduction pathway no longer increased after about 5 minutes of photosynthesis. By this time, enough ^{14}C had passed through these intermediates on the way to end products to "saturate" each carbon atom position with the same degree of labeling (specific radioactivity) as the $^{14}CO_2$. Since both the $^{14}CO_2$ specific radioactivity (S) and the total radioactivity of an intermediate compound (R) could be experimentally determined, Calvin and Massini could calculate the concentration of carbon in the compound as C= R/S.

As long as the algae photosynthesized under constant conditions, the concentrations of intermediate compounds remained constant. Then the light was turned off and more samples were taken. Since certain steps in the path of carbon must require cofactors produced by light, one would expect such steps to be blocked by darkness. Calvin and Massini found that the concentration of PGA rose quickly. This was expected, since light is needed to form the ATP and NADPH required for the reduction of PGA to sugar phosphates. At the same time, the concentrations of ribulose-1,5-diphosphate fell rapidly to zero, indicating that its formation requires ATP produced by light [Eq. (7)]. This reaction is mediated by the enzyme phosphoribulokinase (Hurwitz, et al., 1956).

4. High-low CO_2 transient changes

These studies were continued by Wilson and Calvin (1955), who left the light on but suddenly lowered the tension of CO_2 to .003%. Since PGA is a product of the carboxylation reaction, it was expected that its concentration would fall. This is exactly what happened. At the same time, the concentration of ribulose

diphosphate rose rapidly and then fell. This behavior is to be expected if ribulose diphosphate is a substrate for the carboxylation reaction, since lowering the CO_2 pressure will stop the reaction which uses up RuDP. Thus, the carboxylation of ribulose diphosphate to give PGA as a first step in the carbon reduction pathway was discovered [Eq. (8)].

The carboxylation of ribulose diphosphate by cell-free extracts of Chlorella was demonstrated by Quayle, et al.(1954), who named the enzyme carboxydismutase. The enzyme has been purified from spinach leaves (Weissbach, et al., 1956; Mayaudon, et al., 1957). The purified enzyme mediates the addition of water and CO_2 to ribulose diphosphate, with an intramolecular oxidation-reduction reaction, or dismutation, leading to the formation of two molecules of PGA. In the carboxylation part of the reaction the carbon atom of CO_2 becomes bonded to the Number 2 carbon atom of ribulose-diphosphate.

C. The photosynthetic carbon reduction cycle

1. The complete cycle

The complete photosynthetic carbon reduction cycle is shown in detail in Fig. 6. A somewhat idealized distribution of label found experimentally following a short period of photosynthesis with $^{14}CO_2$ is indicated by the asterisks.

Some details of the distribution of label, not previously discussed, should be mentioned. The concentration of dihydroxyacetone phosphate is considerably greater than that of phospho-glyceraldehyde. When these two similarly but not equally labeled triose phosphates condense the result is a hexose phosphate labeled more heavily in the number 4 carbon atom than in the number 3 carbon atom position. This distribution was observed experimentally by Kandler and Gibbs (1956).

Labeling of sugars is also affected by the fact that the transketolase reactions are highly reversible. A gylcolaldehyde thiamine pyrophosphate molecule has about as good a probability of reacting with the aldose sugar phosphate from which it has just split as it has of reacting with a different aldose sugar phosphate. There is a common pool of glycolaldehyde thiamine pyprophosphate which interacts

almost equally with fructose-6-phosphate, sedoheptulose-7-phosphate, and xylulose-5-phosphate. This permits a feedback of label from the number 1 and 2 carbon atoms of the pentose phosphates to the number 1 and 2 carbon atoms of hexose phosphate (Bassham and Calvin, 1957). Under certain physiological conditions this feedback is sufficiently great to result in greater labeling of carbon atoms 1 and 2 of hexose than the corresponding carbon atoms 5 and 6. This effect was also noted by Kandler and Gibbs (1956).

2. Stoichiometry of the cycle

Under conditions of steady photosynthesis, the concentrations of the intermediates of the carbon reduction cycle remain constant. Suppose that n molecules of CO_2 enter the cycle by the carboxylation reaction. Then n atoms of carbon incorporated into organic compounds are taken from the cycle by secondary reactions. These reactions utilize cycle intermediates as a starting point for the synthesis of various end products.

For example, the hexose phosphates may be converted to sucrose, oligosaccharides, and polysaccharides such as starch and cellulose. Another example is the conversion of 3-phosphoglyceric acid to phosphoenolpyruvic acid and pyruvic acid and thence to alanine, an amino acid.

Consider one complete revolution of the carbon cycle, shown in Fig. 6. Each reaction occurs at least once. Three molecules of pentose diphosphate (15 carbon atoms) react with 3 molecules of carbon dioxide giving 6 molecules of PGA or 18 carbon atoms in all. Of the 18 carbon atoms, 15 are required to regenerate the 3 molecules of pentose phosphate while 3 are used in the formation of various end products. Besides 3 molecules of carbon dioxide, a complete cycle uses 9 molecules of ATP and 6 molecules of NADPH (2 electrons per molecule).

These requirements are for the cycle as written in Fig. 6. It has been suggested that *in vivo*, the carboxylation of ribulose-1,5-diphosphate might be a reductive carboxylation (Wilson and Calvin, 1955). Broken isolated chlorophasts and cell free systems perform only the non-reductive carboxylation of ribulose diphosphate. It is a hypothesis, at present unproved, that *in vivo* enzyme systems capable of using electrons more directly from the light reactions could catalyze reductive carboxylation [Eq. (23)]. Such a system might be disrupted when the chloroplasts are removed from the cells. In a cycle with a reductive carboxylation, the cofactor requirements might be different. For each complete cycle (3 molecules of CO_2 taken up) 3 of the ATP molecules would not be needed if 3 of the $NADPH_2$ molecules could be replaced by molecules of reduced ferredoxin. The total requirement would then be 6 ATP, 3 $NADPH_2$, and 6 reduced ferredoxin molecules, per 3 CO_2 molecules taken up.

3. Quantitative importance of cycle

PGA and the sugar phosphates clearly account for most of the ^{14}C found in individual compounds following a few seconds of photosynthesis with $^{14}CO_2$. Nonetheless, one might ask whether or not other important pathways of CO_2 reduction not involving these compounds have been overlooked. For example, there might be a pathway from CO_2 to sucrose which does not include the intermediate compounds of the carbon reduction cycle. If so, this path would have to include substances which are so small in concentration as not to be seen, or which are so unstable as not to be isolated by the methods of paper chromatography.

These possibilities were tested by Bassham and Kirk (1960), who refined the steady-state photosynthesis studies to permit direct comparison of the externally measured rates of ^{14}C and $^{12}CO_2$ uptake with the rates of appearance of ^{14}C in individual compounds. They demonstrated that in *Chlorella*

the rate of labeling of sucrose (the most rapidly labeled carbohydrate) is only a few percent of the total rate of ^{14}C uptake. In fact, its rate of labeling is no greater than that of some other secondary products such as alanine. The sucrose labeling rate is insignificant during the first few seconds. These experimental results rule out the possibility of significant sucrose formation via a sequence of unknown intermediate compounds, all occurring at very small concentrations.

It was also found that labeling of PGA and the sugar phosphates accounts for a least 70% of the externally measured ^{14}C uptake between 10 and 40 seconds after the introduction of $^{14}CO_2$. The pool size of unstable intermediates preceding these stable compounds was not more than the equivalent of 5 seconds of photosynthesis. It is likely that even this small pool is nothing more than intracellular CO_2 and enzyme-bound CO_2.

It is clear that even if pools of unstable intermediate compounds do exist, they must be far too small to be involved in an unknown path to carbohydrates. This conclusion follows from the fact that such carbohydrates would become labeled much more rapidly than the experiments show if they were formed from ^{14}C via compounds of such small pool sizes.

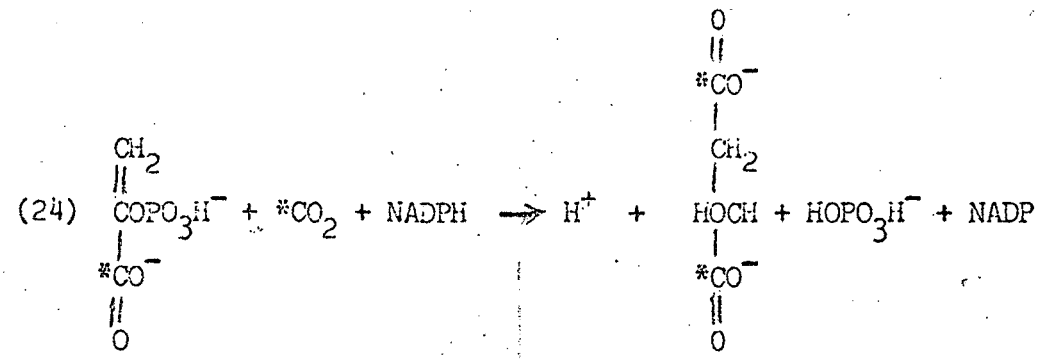
VI. Secondary carbon reduction pathways

A. Carboxylic acids

From the earliest studies of photosynthesis with $^{14}CO_2$ by Calvin and Benson (1948, 1949), it was clear that many substances besides PGA and sugar phosphates were quickly labeled with ^{14}C. Among the more important early products were carboxylic acids, such as malic acid, succinic acid, and glycolic acid. Also labeled at very short times were certain amino

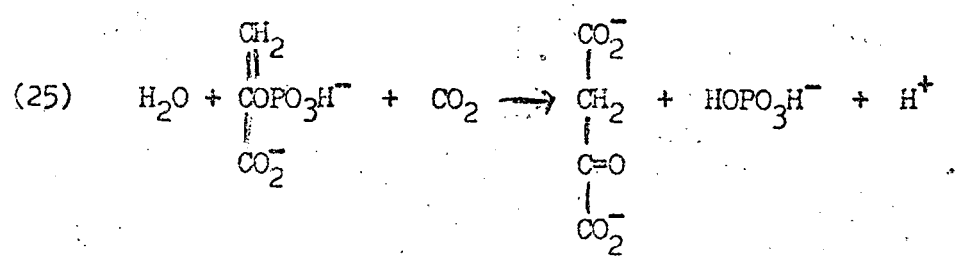
acids, such as alanine, aspartic acid, serine, and glycine (Stepka, et al., 1948). The rate of labeling of such compounds during the first few seconds of photosynthesis with $^{14}CO_2$ is often greater than the labeling rate of carbohydrates such as sucrose. Such findings suggested that these non-charbohydrate secondary compounds are formed directly from intermediates of the carbon reduction cycle rather than from unphosphorylated carbohydrates.

By analogy with known respiratory reactions, malic acid might be formed by reductive carboxylation of phosphoenolpyruvic acid (PEPA) (see Fig. 6) according to Eq.(24).



(24) $\mathrm{CH_2{=}C(OPO_3H^-)(*CO^-){=}O} + {}^*CO_2 + \mathrm{NADPH} \rightarrow H^+ + \mathrm{HOCH(*CO^-){=}O} + HOPO_3H^- + \mathrm{NADP}$

Citric acid is rapidly labeled by plants photosynthesizing in the presence of $^{14}CO_2$. Presumably it is formed by the condensation of acetyl coenzyme A with oxaloacetic acid. The oxaloacetic acid might be formed by the direct carboxylation of PEPA from the carbon reduction cycle [Eq. (25)].



(25) $H_2O + \mathrm{CH_2{=}C(OPO_3H^-)(CO_2^-)} + CO_2 \rightarrow \mathrm{CO_2^-CH_2C(=O)CO_2^-} + HOPO_3H^- + H^+$

The possible source of acetyl coenzyme A in photosynthesis is discussed in the next section.

One of the most interesting and yet incompletely understood aspects of photosynthetic carbon reduction is the synthesis of glycolic acid. The formation of glycolic acid during photosynthesis is favored by low CO_2 pressures (0.1%) slightly greater than those to which plants are exposed under natural environments (Pritchard, et al., 1961). High concentrations of O_2 also increase glycolic acid formation (Bassham and Kirk, 1962). It appears that glycolic acid is formed from carbon atoms 1 and 2 of the sugar phosphates of the carbon reduction cycle. It may be formed by oxidation of glycolaldehyde-thiamine pyrophosphate (Calvin and Bassham, 1962) (see Fig. 6).

B. Fatty acids and fats

If a rapidly growing photosynthetic cell such as an alga cell is exposed to $^{14}CO_2$ for 1 to 2 minutes and then killed, as much as 30% of the radioactive compounds formed behave as lipid-like substances when partitioned between aqueous and organic solvents. A considerable portion of the chloroplast structure consists of lipid materials, and rapid lipid synthesis is required for chloroplast growth and division.

The starting point for synthesis of fatty acids and other lipoid substances such as carotenoids and terpenes is acetyl coenzyme A. Very little labeled acetic acid can be isolated from the photosynthesizing cell. It is presumed that the concentration of acetyl coenzyme A is very small and that the small pool turns over very rapidly.

A plausible pathway to acetyl coenzyme A from the carbon cycle would be via a phosphoroclastic splitting of the thiamine-pyrophosphate glycolaldehyde compound formed by the transketolase reaction. Such a split [Eq. (26)] could be mediated by an enzyme similar in part to phosphoketolase (Heath, et al., 1958).

(26) $$\text{TPP}\!>\!\text{C}-\begin{matrix}\text{CH}_2\text{OH}\\ \text{CHOH}\end{matrix} + \text{HOPO}_3\text{H}^- \longrightarrow \text{TPP}\!>\!\text{CH} + \begin{matrix}\text{CH}_3\\ \text{COPO}_3\text{H}^-\\ \|\\ \text{O}\end{matrix} + \text{H}_2\text{O}$$

The resulting acetyl phosphate could undergo a transacylation reaction with coenzyme A to give inorganic phosphate and acetyl coenzyme A.

Once acetyl coenzyme A has been formed, subsequent reactions leading to fatty acid systhesis are probably similar to those which occur in other biosynthetic systems (Calvin and Bassham, 1962). In the chloroplast these reactions may be photosynthetic reactions in that they employ ATP and reduced pyridine nucleotide cofactors formed by the photochemical reactions of photosynthesis.

The fatty acids thus photosynthesized are then esterified with glycerol or glycerol phosphate formed directly from triose phosphate of the carbon reduction cycle [Eq. (27)]. Calactolipds (Benson, et al.,

(27) $$\begin{array}{l} CH_2OPO_3H^- \\ | \\ CO \\ | \\ H_2COH \end{array} + NADPH \longrightarrow \begin{array}{l} CH_2OPO_3H^- \\ | \\ HCOH \\ | \\ H_2COH \end{array} + NADP^+$$

1958) may be formed by reactions of these compounds with UDPGalactose (Neufeld and Hall, 1964). UDPGalactose in turn is formed photosynthetically in the chloroplast from UDPG (see VII C) by UDPGalactose epimerase.

C. Carbohydrates

Buchanan (1953) reported that sucrose is photosynthesized from fructose-6-phosphate of the carbon reduction cycle. One molecule of fructose-6-phosphate is converted to glucose-6-phosphate, which reacts with uridine triphosphate (UTP) to form uridine diphosphoglucose (UDPG). UDPG is always labeled with ^{14}C during short periods of photosynthesis with $^{14}CO_2$. The UTP was presumed to be formed from uridine diphosphate and photosynthetically produced ATP. UDPG then reacts with fructose monophosphate to produce sucrose phosphate and eventually sucrose. UDPG is very likely involved in the synthesis of other oligosaccharides and polysaccharides. As mentioned earlier, the synthesis of sucrose can account for

nearly all of the uptake of carbon dioxide during photosynthesis in the mature leaves of certain green plants. On the other hand, a rapidly growing and dividing unicellular algae, such as Chlorella pyrenoidosa, may utilize 5% or less of the photosynthetic CO_2 uptake for the synthesis of sucrose.

D. Amino acids and proteins

The earliest separations of the products of photosynthesis of $^{14}CO_2$ by two-dimensional paper chromatography revealed certain amino acids to be rapidly labeled products of $^{14}CO_2$ reduction (Stepka, et al., 1948). Most important among these are alanine, aspartic acid, serine, glutamic acid, and glycine. Using quantitative steady-state tracer studies, Smith, et al. (1961) were able to show that Chlorella pyrenoidosa incorporate as much as 30% of the $^{14}CO_2$ taken up photosynthetically directly into these amino acids. The rate of incorporation of $^{14}CO_2$ into alanine by Chlorella pyrenoidosa may exceed the rate of labeling of sucrose.

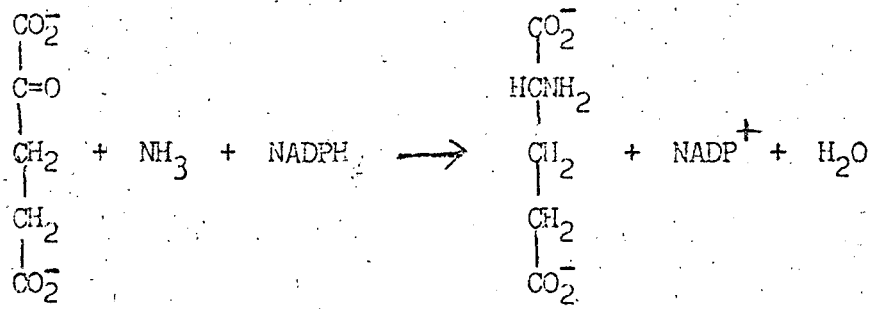
A study of the kinetics of the labeling of alanine show that its rate of labeling reaches a maximum as soon as the intermediates of the carbon reduction cycle are "saturated" with ^{14}C. Since no secondary products of carbon photosynthesis such as sucrose are approaching saturation at this time (3-5 min), it appears that alanine is formed directly from intermediates of the cycle. Presumably, alanine is formed from PGA by the transamination of pyruvic acid derived from phosphoenolpyruvic acid which in turn is derived from PGA [Eq. (28)].

(28) PGA $\longrightarrow$ $\longrightarrow$ PEPA $\xrightarrow{HOH}$ $\begin{matrix} CH_3 \\ | \\ C{=}O \\ | \\ CO_2^- \end{matrix}$ $\xrightarrow{\text{glutamic acid}}$ $\begin{matrix} CH_3 \\ | \\ HCNH_2 \\ | \\ CO_2^- \end{matrix}$ + α-ketoglutaric acid

The photosynthetic formation of glycine appears to depend upon the prior formation of glycolic acid. Presumably, glycolic acid is oxidized to glyoxylic acid, which is then transaminated to give glycine.

Other amino acids such as serine and aspartic acid are presumably photosynthesized from intermediate keto acids derived from the carbon reduction cycle. In each case, transamination by glutamic acid is probably the major synthetic reaction.

Glutamic acid is thus of primary importance in photosynthesis of labeled amino acids. It is probably formed as in non-photosynthetic tissues by reductive amination of α ketoglutaric acid [Eq. (29)].

(29)


In the chloroplast the reductive amination utilizes electrons formed by the light reactions of photosynthesis. The α ketoglutaric acid is formed from citric acid via aconitic and oxalosuccinic acids, (the tricarboxylic acid cycle.)

VII. Problems for the future

As knowledge regarding the process of photosynthesis has accumulated, it has become ever more apparent that the total reaction of photosynthesis is composed of an intricate complex of many sub-reactions. Even in those parts of this complex which have been illuminated by meaningful experimental results, there remain substantial questions about precise mechanisms. Other areas of the complex remain virtually unknown territory.

Least known of all are the reactions by which light energy is used to transfer electrons from water molecules to some acceptor from which they can continue their journey along the photoelectron transport chain which leads ultimately to the reduction of other inorganic oxides. Why is there a require-

ment for manganese for photosynthetic O_2 evolution? Might the several oxidation states of manganese (such as Mn^{+4} and Mn^{+2}) be involved in some kind of oxidation - reduction reaction with water? Presumably manganese could be chelated in some complex, bringing the redox potential of the manganese to the right level for it to accomplish its role.

The simplest assumption to make regarding the quantum conversion act in the oxidation of water would be that Pigment System 2 accomplishes quantum conversion the same way as is postulated for Pigment System 1: by a one electron transfer which requires most of the energy of the excited state of the pigment molecule. However, the possibility that two electrons might be transferred per quantum (see Sec. IV, C, 3) leads one to consider also other mechanisms. For example, the hypothetical chelated manganese molecule just suggested might also be a pigment molecule, capable of accepting an exciton and using its energy to undergo charge separation. If this kind of quantum conversion could result in the transfer of two electrons from Mn^{+2} to an acceptor (such as plastoquinone), the resulting Mn^{+4} might then oxidize water to O_2. Clearly there is a critical need for more information about the chemistry of the photochemical apparatus for oxidizing water.

The mechanism for quantum conversion by Pigment System 1 outlined in Sec. IV, C, 2, seems well supported by experimental evidence. However, considerably more knowledge about the actual physics of the quantum conversion act is called for, and the nature of the chemical species serving as electron donors and acceptors is still mostly unknown. An even more basic question is whether the transfer of electrons from water to ferredoxin must involve exclusively two photochemical steps (Pigment Systems 1

and 2) or possibly could be mediated by an alternative single photochemical act.

Information about the intermediates in photoelectron transport and photophosphorylation is now rapidly accumulating, but the precise sequence of cofactors involved in electron transport, and the mechanism of coupling with phosphorylation remain to be elucidated. Again, the possibility for alternate pathways remains.

The advances in understanding of the structure of the photosynthetic apparatus are particularly encouraging, and we may look forward confidently to a detailed relation of biochemical function to morphoplgical entities in the near future. The recent pictures of quantasomes (Park and Biggins, 1964) seem to reveal sub-structure. Will these sub-units turn out to be Pigment Systems 1 and 2, intermediate electron transport particles, etc.?

As for the carbon reduction cycle, probably the most important unanswered questions have to do with the mechanisms of the carboxylation reaction and other steps in the cycle. Although all of the biochemical evidence from isolated enzyme systems suggests that the carboxylation reaction is a non-reductive carboxylation of ribulose diphosphate leading to the formation of one molecule of PGA, kinetic evidence with whole cells indicates the possibility of a reductive carboxylation leading to the formation of one molecule of PGA and one molecule of triose phosphate. If this reductive carboxylation does occur, it may be that electrons are somehow conveyed directly from the light reaction to the carbon reduction cycle (Bassham, 1964). If there is such a difference between the <u>in vivo</u> system and the <u>in vitro</u> systems this difference may reside in some intricate structural arrangement in the living cell which is easily disrupted.

The finding of multifunctional enzyme systems for biosynthetic pathways, such as fatty acid synthesis, suggests that such organized systems may have importance elsewhere. Whether they exist in photosynthesis and how they operate if they do exist is a very important question for the future. The answer will come from a combination of tracer studies, investigation of the enzymes isolated by a variety of sophisticated techniques, and the gathering of better and more detailed knowledge of the structure of the chloroplast through the application of electron microscopy and various techniques of chemical and physical analysis.

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Fig. 1. Electron Micrograph of Spinach Lamellae with Quantasomes.

Lamellae were prepared from broken spinach chloroplasts according to the method of Park and Pon (1961). After air-drying of the lamellae on a screen, the preparation was chromium-shadowed, and its image in the electron microscope was photographed. The "smooth" layer may be predominantly lipid material on one surface of the top lamellar membrane. Where the top membrane has been torn away, the "inner" side of the next, opposing membrane, consisting of an almost crystalline array of quantasomal particles, is revealed. By close inspection of individual quantasomes, one can see what appears to be sub-structure.

Permission to publish this photograph was kindly granted by Professor Roderic B. Park.

Fig. 2. Electron Micrograph of Part of a Section Through a Spinach Chloroplast, Showing Portion of Chloroplast Membrane and Lamellae in Cross Section.

Spinach chloroplasts were stained with permaganate, embedded, and sectioned. The image from the electron microscope has been photographed and photographically enlarged to the same approximate scale as the electron micrograph shown in Fig. 1. The dark staining layers are presumed to be the lipid layers or possibly the lipid-quantasomal interfaces. The light areas within the lamellar envelope may correspond to the array of quantasomal particles. The closely stacked lamellae are in the grana; the more widely separated lamellae seen in the stroma actually extend from one grana stack to another. Near the top left corner of the picture is seen the

chloroplast membrane, consisting of two unit membranes. Permission to publish this photograph was kindly granted by Professor Roderic B. Park.

Fig. 3. Scheme of Photoelectron Transport in Photosynthesis.
The path of photoelectron flow associated with "non-cyclic photophosphorylation" through the two postulated light reactions mediated by Pigment Systems 1 and 2 is indicated by the heavy lines. Redox potentials of electron-carrying cofactors along this path is indicated by scale on the left. Further explanation in text.

MWB-2915

Fig. 4. Radioautogram of Products of 60 seconds' Photosynthesis with $^{14}CO_2$.
Radioautograph of two-dimensional paper chromatogram of products formed by Chlorella pyrenoidosa during 60 seconds of photosynthesis with $^{14}CO_2$.
Abbreviations: P, PO_3H^-; UDPG, uridine diphosphoglucose; PGA, 3-phosphoglyceric acid; PEPA, phosphoenolpyruvic acid. Sugar diphosphate includes ribulose-1,5-diphosphate, sedoheptulose-1,7-diphosphate, and fructose-1,6-diphosphate.

Biochem p 34

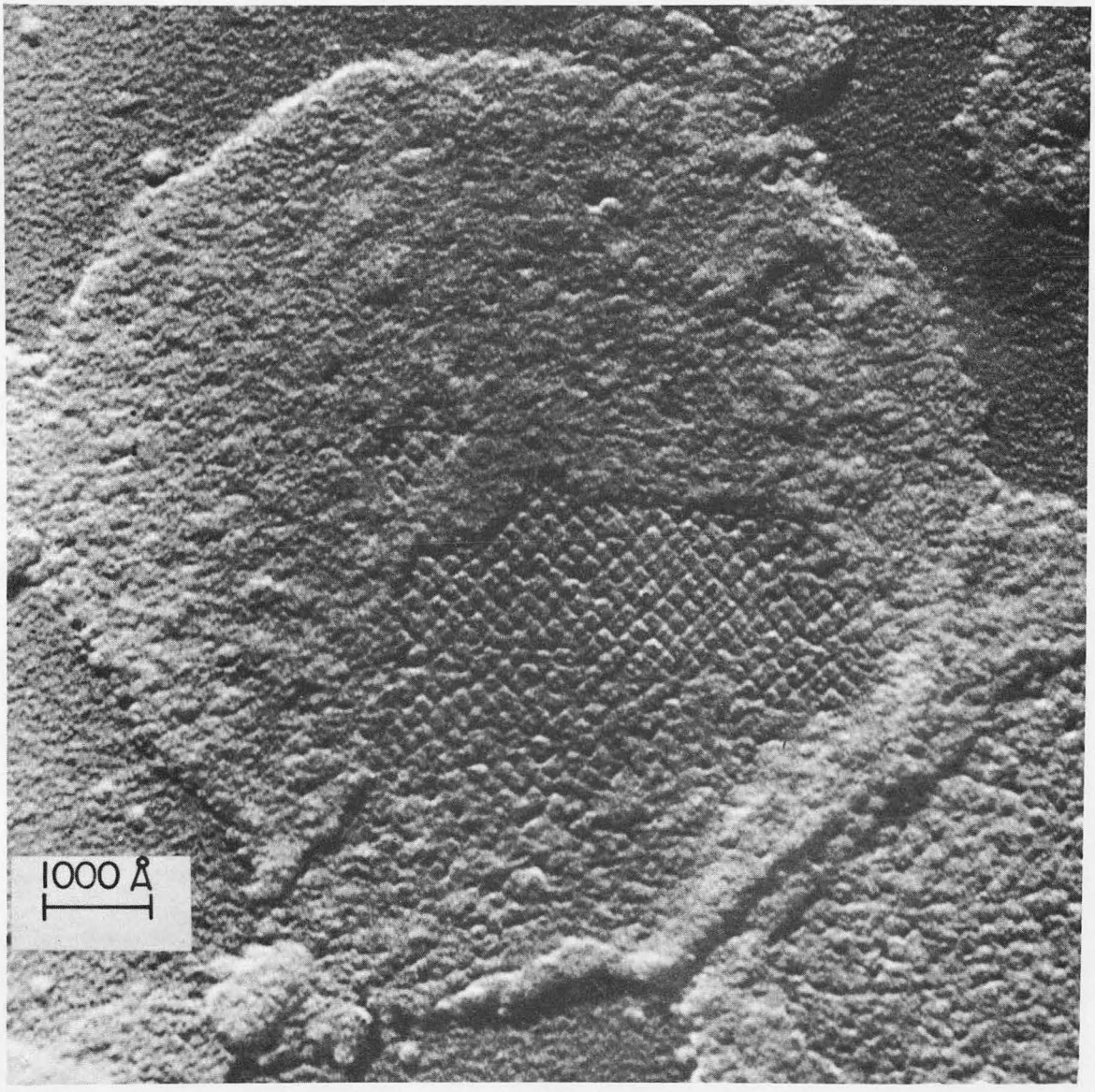
Fig. 5. Radioautograph of Two-dimensional Paper Chromatogram of Products Formed by Chlorella Pyrenoidosa During 2 seconds of Photosynthesis With $^{14}CO_2$.
Abbreviations: Same as for Fig. 4.

Biochem p 32

MWB-2359A

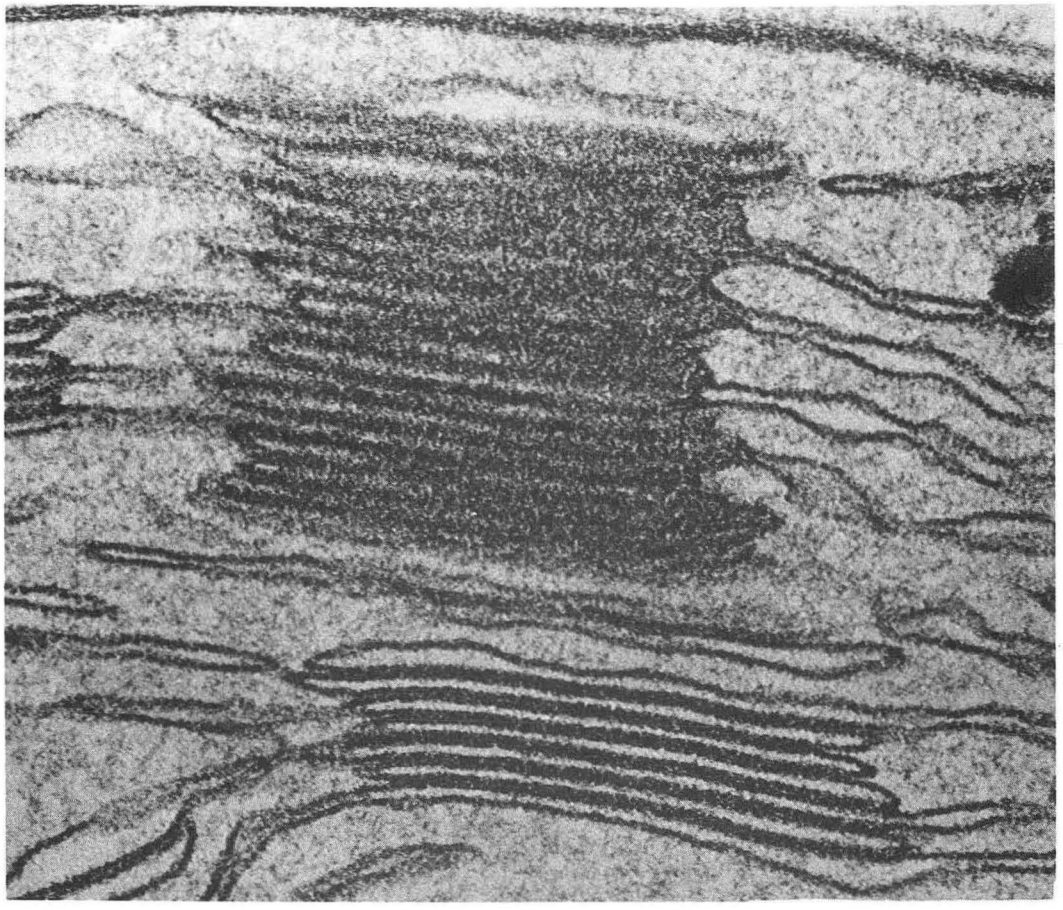
Fig. 6. The Carbon Reduction Cycle of Photosynthesis.
Solid arrows indicate reactions of the carbon reduction cycle as

formulated by Calvin and co-workers. Dashed line represents hypothetical reductive carboxylation reaction discussed in text. Open arrows indicate start of some of the biosynthetic paths leading from intermediate compounds of the cycle. Asterisks indicate approximate relative degree of labeling after a few seconds of photosynthesis. They reflect the results of degradation studies by various workers, as discussed in the text.



ZN-4304

Fig. 1



ZN-4305

Fig. 2

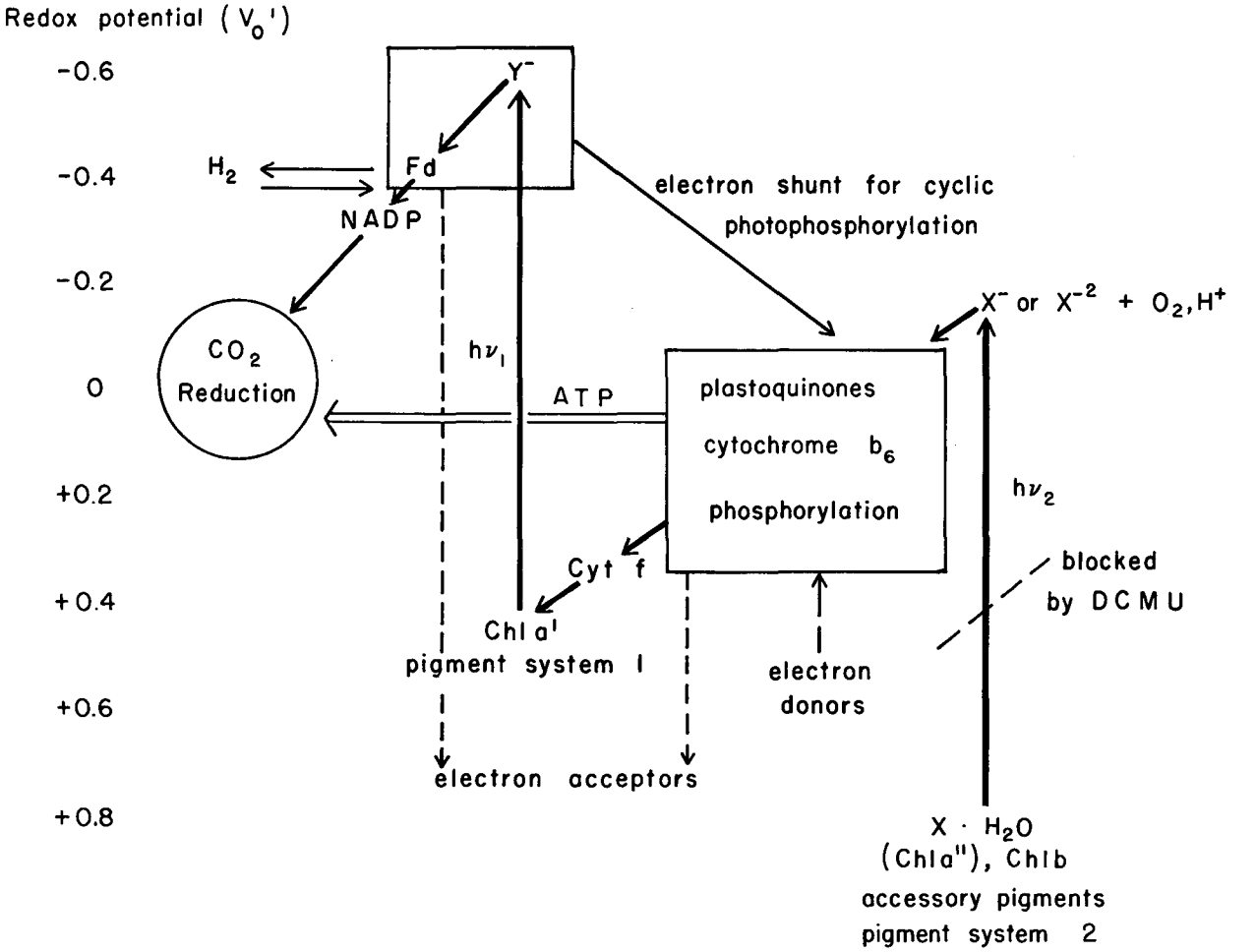
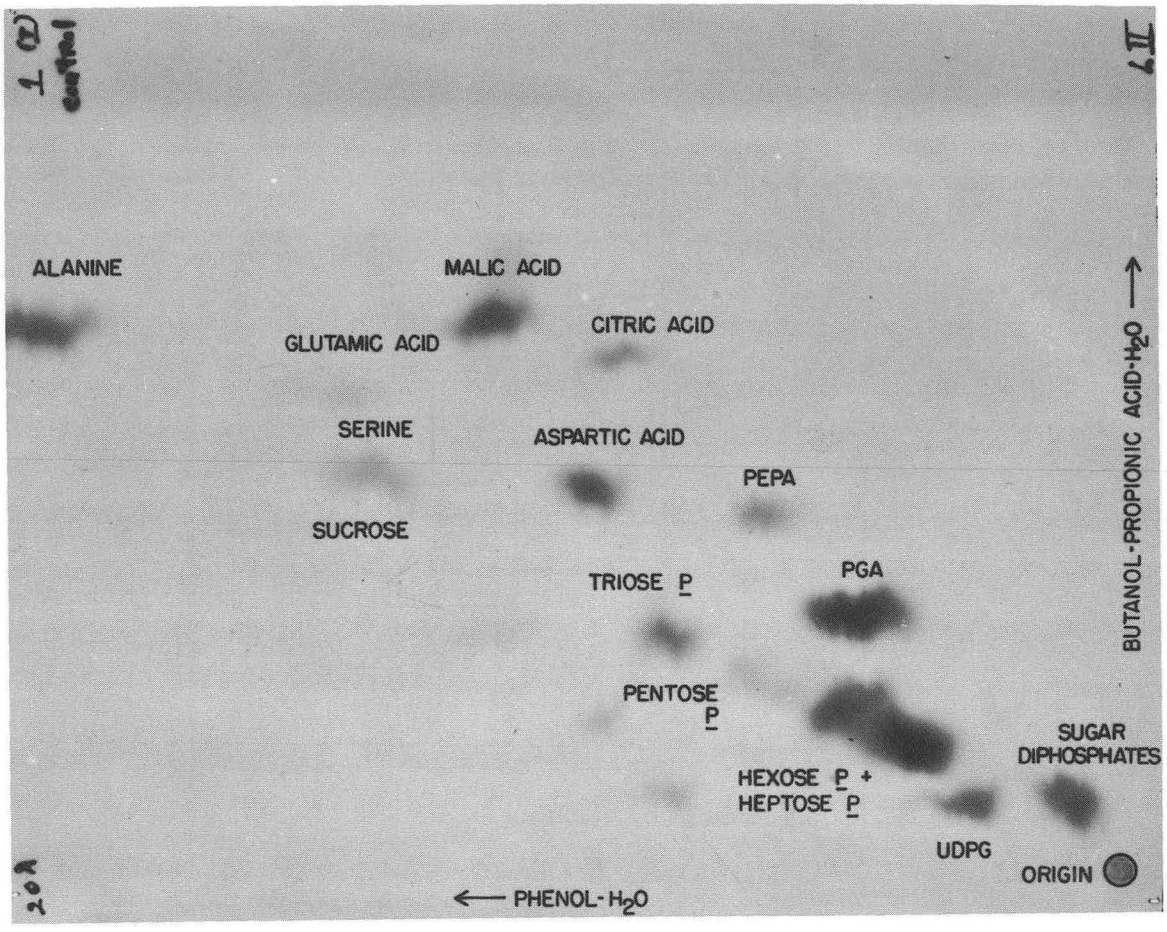
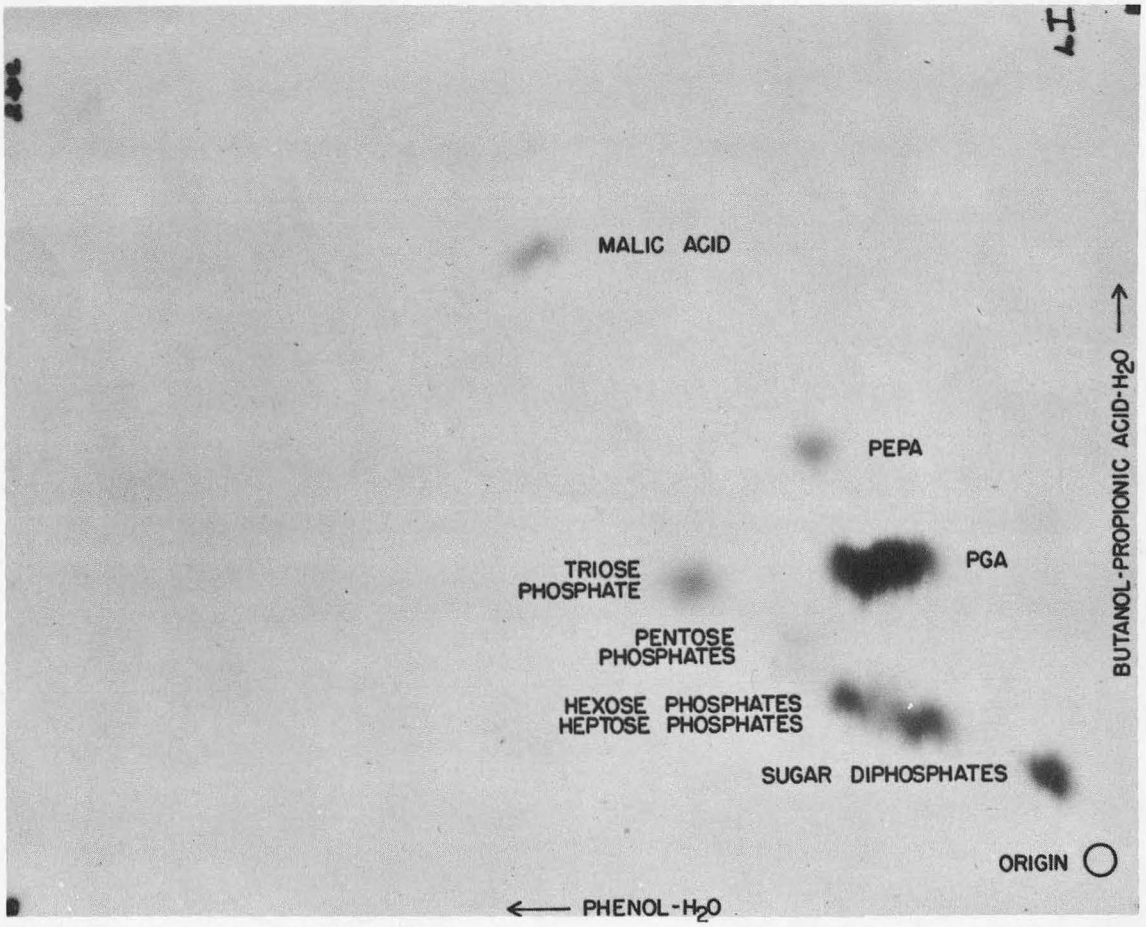

Redox potential (V_0')
-0.6
-0.4
-0.2
0
+0.2
+0.4
+0.6
+0.8
Y⁻
H_2
Fd
NADP
electron shunt for cyclic photophosphorylation
X^- or X^{-2} + O_2,H^+
CO_2 Reduction
$h\nu_1$
ATP
plastoquinones
cytochrome b_6
phosphorylation
$h\nu_2$
Cyt f
blocked by DCMU
Chl a'
pigment system I
electron donors
electron acceptors
X · H_2O
(Chl a''), Chl b
accessory pigments
pigment system 2
MUB-2915

Fig. 3


1 (2) control
ALANINE
MALIC ACID
GLUTAMIC ACID
CITRIC ACID
SERINE
ASPARTIC ACID
PEPA
SUCROSE
TRIOSE P
PGA
PENTOSE P
SUGAR DIPHOSPHATES
HEXOSE P + HEPTOSE P
UDPG
ORIGIN
BUTANOL-PROPIONIC ACID-H2O
PHENOL-H2O
20A

ZN-4303

Fig. 4


MALIC ACID
PEPA
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TRIOSE PHOSPHATE
PENTOSE PHOSPHATES
HEXOSE PHOSPHATES
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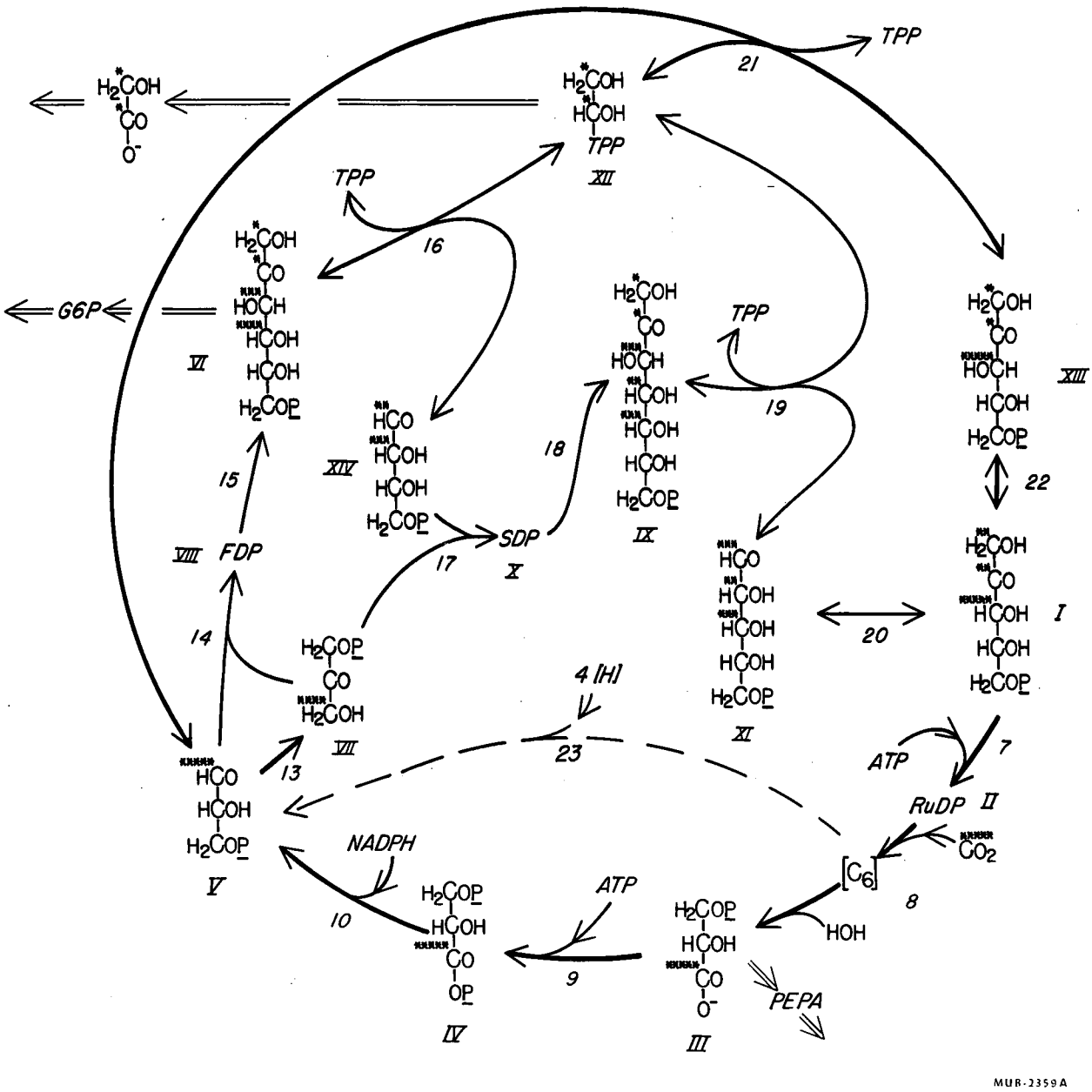
SUGAR DIPHOSPHATES
ORIGIN
BUTANOL-PROPIONIC ACID-H2O
PHENOL-H2O

ZN-4302

Fig. 5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21
TPP
XII
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XIII
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XIV
18
SDP
X
IX
15
VIII
FDP
17
20
I
14
4 [H]
23
ATP
7
VII
13
RuDP II
CO2
NADPH
[C6]
8
ATP
10
HOH
9
PEPA
V
IV
III
MUB-2359A

Fig. 6