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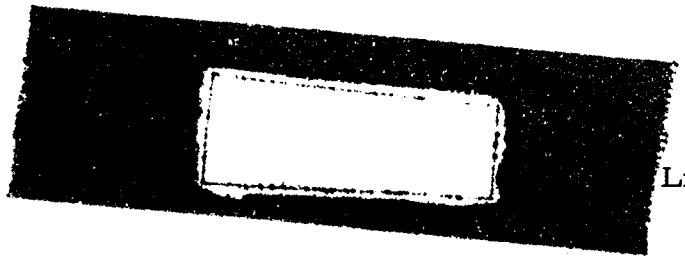

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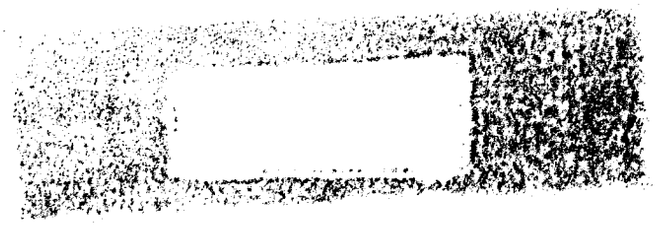
THE PATH OF CARBON IN PHOTOSYNTHESIS

J. A. Bassham and Melvin Calvin

October 1960



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Chapter 1

THE PATH OF CARBON IN PHOTOSYNTHESIS

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Contents

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THE PATH OF CARBON IN PHOTOSYNTHESIS

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INTRODUCTION

Biosynthesis begins with photosynthesis. Green plants and other photosynthetic organisms use the energy of absorbed visible light to make organic compounds from inorganic compounds. These organic compounds are the starting point for all other biosynthetic pathways.

The products of photosynthesis provide not only the substrate material but also chemical energy for all subsequent biosynthesis. For example, nonphotosynthetic organisms making fats from sugars would first break down the sugars to smaller organic molecules. Some of the smaller molecules might be oxidized with O_2 to CO_2 and water. These reactions are accompanied by a release of chemical energy because O_2 and sugar have a high chemical potential energy towards conversion to CO_2 and H_2O. In a biochemical system only part of this energy would be released as heat. The rest would be used to bring about the conversion of certain enzymic cofactors to their more energetic forms. These cofactors would then enter into specific enzymic reactions in such a way as to supply energy to drive reactions in the direction of fat synthesis. Fats would be formed from the small organic molecules resulting from the breakdown of sugars. Thus sugar, a photosynthetic product, can supply both the energy and the material for the biosynthesis of fats.

Photosynthetic organisms achieve energy storage through their ability to convert electromagnetic energy to chemical potential energy. The conversion begins when pigments absorb light energy. The absorbed energy changes the electronic configuration of the pigment molecule (chlorophyll) from its ground energy state to an excited state. The return of the pigment molecule to its ground state energy level is accompanied by some (chemical) reaction which would not proceed without an input of energy. That is, the products of this reaction have a smaller negative free energy of formation from their elements than do the reactants (in the same reaction). Thus some of the light energy is converted to chemical potential.

The detailed mechanism of all of these energy conversion steps is not known. However, the net result is often formulated by two chemical equations. One of these is an oxidation-reduction reaction resulting in the transfer of hydrogen from water to triphosphopyridine nucleotide (TPN):

$$1.\quad HOH + TPN^{+} \xrightarrow{\text{light}} 1/2\ O_2 + TPNH + H^{+} \qquad \Delta F' = +52.6\ \text{Kcal}^{*}$$

The other reaction is the formation of an anhydride, adenosine triphosphate (ATP), from the ions of two phosphoric acids, adenosine diphosphate and ortho-phosphate:

$$2.\quad ADP^{-3} + HPO_4^{-2} \xrightarrow{\text{light}} HOH + ATP^{-4} + H^{+} \qquad \Delta F' = +11\ \text{Kcal}^{*}$$

In each of these reactions some of the light energy is stored as chemical potential as indicated by the positive quantities for free energy change.

The structural formulas of these two cofactors are shown in Figure 1. TPNH and its close relative DPNH (reduced diphosphopyridine nucleotide) serve a double function in photosynthesis and in all biosynthesis. Both

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* Assuming these concentrations: $(TPNH) = (TPN^{+})$, $(ATP^{-4}) = (ADP^{-3})$, $H^{+} = 10^{-7}$ M, $HPO_4^{=} = 10^{-3}$ M.

TPNH and DPNH are reducing agents and carriers of chemical potential, in other words, strong reducing agents. Thus, one of their roles in biochemistry is analogous to that of H_2 in synthetic organic chemistry.

The function of ATP is to carry chemical potential and to act as a powerful phosphorylating agent. In the reduction of an acid to an aldehyde, important in photosynthesis, its role may be compared to that of a mineral acid anhydride in organic synthesis:

Organic Synthesis

Carboxylic Acid — Acid Anhydride — Acyl Derivative — Reducing Agent — Aldehyde Acid

3. $R\text{-}C(=O)O^- + 1/3\ PCl_3 \longrightarrow R\text{-}C(=O)Cl + 1/3\ H_3PO_3$; $R\text{-}C(=O)Cl \xrightarrow[\text{catalyst}]{H_2} R\text{-}C(=O)H + HC$

Biosynthesis

4. $R\text{-}C(=O)O^- + ADP\text{-}O\text{-}PO_3H^- \longrightarrow R\text{-}C(=O)OPO_3H^- + ADP$; $R\text{-}C(=O)OPO_3H^- \xrightarrow[\text{enzyme}]{TPNH} R\text{-}C(=O)O^- + HOPO_3H$

Among the many other reactions of ATP in biosynthesis, one which is of considerable importance in photosynthesis, is in the formation of sugar phosphates from sugars.

5. $H^+ + ROH + ADP\text{-}O\text{-}PO_3H^- \longrightarrow R\text{-}OPO_3H^- + ADP + H_2O$

The only known reactions of the carbon reduction cycle in photosynthesis which would require the use of TPNH and ATP are of the type shown in Eq. 4 and 5. These reactions are the means by which chemical potential, derived from the absorbed light, is used to bring about the reduction and transformation of carbon from CO_2 to organic compounds.

These two cofactors, ATP and TPNH, are at present the only cofactors known to be generated by the light reactions of photosynthesis which at the same time seem to be required for steps in the carbon reduction cycle. The possibility remains, however, that there are other energetic or reduced cofactors acting as carriers of hydrogen and energy from the light reactions to the carbon reduction cycle. Such unknown cofactors might substitute for or replace TPNH or ATP. They could, in fact, be more effective than the known cofactors, particularly in vivo where they might well be built into the highly organized structure of the chloroplast. If such unknown cofactors do exist, they would have to perform essentially the same functions as TPNH and ATP and would presumably be about as effective as carriers of chemical potential. In all discussions of the role of TPNH and ATP, the possibility of their replacement by as yet unidentified cofactors should be kept in mind.

For the purposes of discussion, let us consider the photosynthesis of carbon compounds as an isolated set of reactions. The principal substrates for this set of reactions are CO_2, hydrogen (as TPNH), phosphate (as ATP) and NH_4^+. The ammonium ion may be contained in the plant nutrient or may be derived from the reduction of nitrate. If nitrate reduction is the source of NH_4^+ the energy for the reduction must also come from the light, at least indirectly. Other probable inorganic substrates for photosynthesis of organic compounds include sulfate, magnesium ion, and a number of trace elements. Many of these are required for growth in plants but may or may not be incorporated in organic compounds by photosynthesis.

CARBON REDUCTION CYCLE OF PHOTOSYNTHESIS

We believe the principal pathways for the photosynthesis of simple organic compounds from CO_2 to be those shown in Figure 2 (1,2). The points at which ATP and TPNH act in these pathways are indicated. Kinetic studies (3) show that these pathways account for nearly all of the carbon dioxide reduced during photosynthesis, at least in the unicellular algae, Chlorella pyrenoidosa. From other investigations (4) it appears that the general metabolic sequence is the same in most respects for all photosynthetic organisms. (We shall discuss the recently proposed role of glycolic acid in CO_2 reduction in a later section on Carboxylic Acids.)

The central feature of carbon compound metabolism in photosynthesis is the carbon reduction cycle. Most of the carbon dioxide used is incorporated via this cycle. Pathways lead from intermediates in the cycle to various other important metabolites. A few of these pathways are shown in Figure 2.

The initial step for carbon dioxide incorporation in the cycle is the carboxylation of ribulose-1,5-diphosphate at the number 2 carbon atom of the sugar to give a highly labile β-keto acid. Evidence for the existence of this unstable intermediate has been adduced from in vivo studies (5). It has not been isolated in the in vitro reaction with the enzyme, carboxydismutase. The product of the reaction in vitro is two molecules of 3-phosphoglyceric acid (PGA). The products in intact photosynthesizing cells may be two molecules of PGA or, as kinetic studies indicate (3), one molecule of PGA and one molecule of triose phosphate.

Once formed, the PGA is transformed in two ways. Some molecules are converted to products outside the cycle while the remainder are reduced to 3-phosphoglyceraldehyde via a reaction of the type shown in Eq.4. The enzymes responsible for the two successive steps in the reduction are probably similar to phosphoglycerylkinase (6) and triose phosphate dehydrogenase (7,8,9,10).

The next phase of the carbon reduction cycle is the conversion of five molecules of triose phosphate to three molecules of pentose phosphate by a series of reactions. These reactions include condensations (aldolase), carbon chain length dismutations (transketolase), removal of phosphate groups (phosphatase), and interconversions of different pentose phosphates (isomerase, epimerase). Enzyme systems which catalyze reactions similar to these steps are listed later in Table 2. The sequence of steps may be seen in the cycle diagram (Figure 2).

The various pentose phosphates are converted to ribulose-5-phosphate. The final step is the formation of ribulose diphosphate (RuDP) from ribulose-5-phosphate. This step requires one molecule of ATP (Eq. 5).

In order for every reaction in the cycle to occur at least once (a complete turn of the cycle), the carboxylation reaction must occur three times. The net result of each complete turn of the cycle is the incorporation of three molecules of CO_2 and the production of one three-carbon(or 1/2 six-carbon) organic molecule. Each complete turn of the cycle would require 6 molecules of TPNH or equivalent reducing cofactor (two per CO_2), and 9 molecules of ATP, if each C_6 carboxylation product is split to two molecules of PGA and if all of the PGA is reduced to triose phosphate. If the carboxylation product is reductively split (dashed line in Figure 2) the requirement for TPNH would probably be the same, that is 6 molecules per complete turn of the cycle. In this case, however, the cycle might require either 9 molecules of ATP or only 6.

EVIDENCE FOR THE CARBON REDUCTION CYCLE

The carbon reduction cycle in essentially the form shown in Figure 2 was mapped during the period between 1946 and 1953 (11,12,13,14,15,16,17). The experiments, results, and interpretations leading to its formulation have been extensively discussed elsewhere (2). They will be briefly reviewed here, not necessarily in chronological order.

The carbon which enters the plants' metabolism has been followed through the various intermediate compounds by labeling the carbon dioxide with radiocarbon, C^{14}. The analysis of the labeled compounds has been carried out by paper chromatography and radioautography. The intepretation of results leadhg to the cycle formulation has been based on the kinetics of the appearance of C^{14} in various identified compounds as a function of time of photosynthesis with $C^{14}O_2$ and other variables.

The methods are best described by an illustration. Consider a simple experiment with a suspension of the algae, Chlorella pyrenoidosa, which have been very extensively used in these studies. These green unicellular plants, suspended in water containing the necessary inorganic ions (nitrate, phosphate, etc.) and aerated with a stream of $C^{12}O_2$ (ordinary carbon dioxide), photosynthesize at a rapid rate if illuminated from each side in a thin transparent vessel. The CO_2 is continually taken up from the solution (where it is in equilibrium with bicarbonate ion) and converted by the photosynthetic plant through a series of biochemical intermediates to various organic products.

A solution of radioactive bicarbonate, $HC^{14}O_3^-$, is suddenly introduced into the algae suspension. The plant does not distinguish in any im-

portant way between the C^{12} and C^{14} which are chemically almost identical. Immediately some of the C^{14} is incorporated into the first of the biochemical intermediate compounds. As time passes, the C^{14} gets into subsequent intermediates in the chain. After a few seconds exposure to the $C^{14}O_2$ the suspension of algae is run into methanol to a final concentration of 80% methanol. This treatment denatures all the enzyme instantly and freezes the pattern of C^{14} labeling by preventing further change. Now the dead plant material is analyzed for radioactive compounds to see which are the first stable products of carbon reduction during photosynthesis.

The first step in this analysis is to prepare an extract of the soluble compounds. The early products of carbon reduction have been found to be simple soluble molecules. This extract is then concentrated and analyzed by the method of two-dimensional paper chromatography (12). The importance of the method for these studies stems from the fact that it permits the analysis of a few micrograms or less of dozens of different substances in a single simple operation.

Of these many compounds, those into which the plant incorporates carbon-14 during its few seconds of photosynthesis with $HC^{14}O_3^-$ are radioactive and emit the particles resulting from radioactive decay of the C^{14}. In this case these are β-particles and these may be detected by the fact that they expose x-ray film. Thus, if a sheet of x-ray film is placed in contact with the two-dimensional paper chromatogram, subsequent development of the film will show a black spot on the film corresponding to the exact shape and location of each radioactive compound on the paper. A quantitative determination of the amount of radiocarbon in each compound

may then be made by placing a Geiger-Mueller tube with a very thin window over the radioactive compound on the paper and counting electronically the emitted β-particles.

The next stage in the method of radiochromatographic analysis is the identification of the radioactive compounds. This identification is accomplished in a variety of ways. When a familiar set of chromatographic solvents has been used, the position of an unknown compound compared to the positions of known substances provides a clue to its identity. The next step may be elution or washing of the compound off the paper and the determination of such chemical and physical properties (e.g., the distribution coefficient) of the substance as can be measured with a solution of a few micrograms or less of the material. These properties are then compared with those of known compounds. The final check on the identity of the compound is frequently made by placing on the same spot on filter paper the radioactive compound and 10 to 100 μg of the pure nonradioactive substance with which the radioactive compound is thought to be identical. The new chromatogram is then developed. A radioautograph is prepared to locate the radioactive substance, after which the paper is sprayed with a chemical spray (for example, ninhydrin for amino acids) which produces a color where the carrier compound is located on the paper. Superposition of the paper chromatogram and the radioautograph (x-ray film) will show an exact coincidence between chemically developed color on the paper and the black spot on the film, provided the two substances are identical.

Once the identity of the radioactive compounds formed during a short period of photosynthesis had been established, experiments were performed under a variety of conditions and times of exposure of the algae to radiocarbon.

The radioautogram from the experiment with Chlorella described above is shown in Figure 3 (10 sec PS w/$C^{14}O_2$ Chlorella). Even after only 10 seconds of exposure to C^{14}, a dozen or more compounds are found. Some of these (the sugar phosphates) are not separated from each other by the first chromatography and must be subjected to further analysis. When the sugar monophosphates are hydrolyzed to remove the phosphate groups and rechromatographed, separate spots are found of triose (dihydroxyacetone), tetrose, pentoses (ribulose, xylulose and ribose), hexoses (glucose and fructose), and heptose (sedoheptulose). The radioactive sugar diphosphates area gives free-ribulose, fructose, glucose and sedoheptulose.

After periods of photosynthesis with C^{14} of less than 5 seconds, 3-phosphoglyceric acid (PGA) was found to be the predominant radioactive product. Chemical degradation of this compound showed that the radioactivity first appears in the carboxyl carbon (14). Later kinetic studies showed that the rate of incorporation of C^{14} into PGA at very short times was much greater than the rate of labeling of any other compound (18,1). Therefore, it was concluded that PGA is the first stable product of carbon dioxide fixation during photosynthesis, and, furthermore, that carbon dioxide first enters the carboxyl group of PGA, presumably via a carboxylation reaction.

Further reactions in the photosynthetic sequence were suggested by the already known pathways of the glycolytic breakdown of sugars which lead to PGA as an intermediate. Since the sugar phosphates are important early products of carbon reduction in photosynthesis, it was proposed that they are formed from PGA by a reversal of the glycolytic pathway. Degradation of the radioactive hexoses from short experiments showed that they were labeled in the two center carbon atoms (numbers 3 and 4) just as one would expect if

two molecules of carboxyl-labeled PGA were first reduced to triose and then linked together by the two labeled carbon atoms to give hexose (Figure 4).

The hexose and triose phosphates may be converted by aldolase or transaldolase and transketolase enzymes to pentose and heptose phosphates (Figure 2, Table 2). Degradation of these sugars and comparisons of the labeling patterns within the molecules showed that this conversion did occur and in such a way that five molecules of triose phosphate were ultimately converted to three molecules of pentose phosphate.

Other known metabolic pathways leading from PGA (Figure 4) give rise first to phosphoenolpyruvic acid (PEPA) which then may undergo further transformations including the following: (1) it may be carboxylated and transaminated to give aspartic acid (2) it may be carboxylated and reduced to give malic acid (3) it may be dephosphorylated and transaminated to give alanine. All of these compounds are labeled after short exposures of the algae to $HC^{14}O_3^-$ in the light.

The enzyme system of plants which during respiration brings about the oxidation of triose phosphate to PGA in the glycolytic pathway was known to produce ATP and TPNH (or DPNH). If PGA is to be reduced to triose phosphate during photosynthesis, it follows that ATP and TPNH must be supplied. We have already seen that these two cofactors, and possibly others, are produced as a consequence of the light reaction and the splitting of water. It might be expected that if the light were turned off from plants photosynthesizing in ordinary carbon dioxide at precisely the same time that $C^{14}O_2$ is introduced, PGA would no longer be reduced to sugar phosphates, but would still be formed (if no light-produced cofactors are required for the carboxylation reaction). Moreover, the PGA would still be used in other reactions not

requiring these cofactors. In Figure 5, the radioautograph from just such an experiment, this prediction proves to be correct. Labeled PGA is still formed by the algae from $C^{14}O_2$ during 20 seconds in the dark, but only a very little of the PGA is reduced to sugar phosphates. At the same time, a large amount of alanine is formed from PGA via PEPA in reactions which do not require ATP. The trace of labeled sugar phosphates which does appear may be due to the residual ATP, or some unknown cofactor, which was formed while the light was on but which had not yet been used up when the $C^{14}O_2$ was introduced. The formation of malic acid and of alanine and aspartic acid in the dark, indicate the presence of some reducing cofactors, either remaining from the light or derived from some other metabolic reaction.

Before we discuss the evidence for the remaind r of the carbon reduction cycle, we must describe another type of experiment with $C^{14}O_2$ and photosynthesizing algae. In these experiments, algae are first permitted to photosynthesize for 20 minutes or more in the presence of a constant supply of $C^{14}O_2$. During this time environmental conditions are maintained nearly constant (temperature, CO_2 pessure, light intensity, etc.). After about 10 minutes of exposure to $C^{14}O_2$, so much radiocarbon has passed through the various biochemical intermediate compounds on its way to end products that each carbon atom of each intermediate compounds contains, on the average, the same percentage of carbon-14 atoms as the CO_2 which is being absorbed. In other words, the specific radioactivities of all the carbon atoms of all the early intermediates are the same as the specific radioactivity of the entering radiocarbon, which can be measured.

At this point, samples of the algae are removed without disturbing the rest of the algae and these samples are killed and subsequently analyzed by the methods already described. The <u>total radioactivity</u> of each intermediate

is measured, and when this is divided by the known specific radioactivity of the entering CO_2, the total number of carbon atoms of each intermediate compound in the aample can be calculated. Thus the number of moles per unit volume of algae of the various intermediates of the actively photosynthesizing system may be determined. This number of moles per unit volume of plant material is an average soncentration, since the distribution of molecules in such a heterogeneous system is not homogeneous.

This determination of the concentrations of intermediates in vivo is an extremely valuable tool which has many uses, but let us proceed for the moment with one particular application. Having taken a sample of algae for later determination of the concentrations of compounds, the experimenter turns off the light and proceeds to take a series of samples of the algae as rapidly as possible, which is about every three seconds. When the concentrations of compounds in these samples are determined, any changes resulting from turning off the light will be revealed. The two most striking changes are found to be in the concentration of PGA which increases rapidly and in the concentration of one particular compound, ribulose diphosphate, which drops rapidly to zero (16,20).

The increase in PGA on turning off the light is expected. The cofactors, derived from the light reaction, are necessary for the reduction of PGA. The rapid drop in ribulose diphosphate,taken together with the fact that other sugar phosphates initially do not drop rapidly in concentration, indicates that the formation of ribulose diphosphate from other sugar phosphates requires a light-formed cofactor. This conclusion agrees with the fact that the known enzyme which converts ribulose-5-phosphate to ribulose-1,5-diphosphate (RuDP) requires ATP (Table 2). The drop in ribulose diphos-

phate, alone among the sugar phosphates, means that it is being used up by some reaction which does not require light.

Ribulose diphosphate, then, is used up by some reaction that proceeds in the dark, and PGA continues to be formed in the dark. Could the carboxylation of ribulose diphosphate to form PGA be the first step in carbon dioxide reduction? To answer this question, another experiment similar to the one just described was performed. This time, however, instead of turning off the light, the light was left on and carbon dioxide was suddenly removed (19). The result of this experiment was that the concentration of ribulose diphosphate now rose rapidly while PGA dropped rapidly. Thus the carboxylation of RuDP to give PGA was confirmed.

THE CARBOXYLATION REACTIONS

Thus far we have mentioned two carboxylation reactions in photosynthesis: carboxylation of RuDP (the carbon reduction cycle) and carboxylation of PEPA. When algae have been allowed to photosynthesize for less than a minute, virtually all of the radioactivity found on the chromatogram prepared from the algae is located in compounds apparently derived from these two reactions. There still remained the possibility that other carboxylation reactions might occur which would involve intermediate compounds too unstable or too volatile to be seen on the chromatograms. These possibilities were tested by making a quantitative comparison between the rate of uptake of $C^{14}O_2$ from the medium and the rate of appearance of C^{14} in compounds on the chromatograms (3).

For these experiments, the algae were kept as nearly as possible in steady-state growth in the experimental vessel. Light, temperature, pH and

supply of inorganic nutrients were kept constant. Gas was cimulated through the algae suspension in a closed system by means of a pump. Levels of CO_2, O_2, and, when present, $C^{14}O_2$, were continuously measured and recorded. From the known gas volumes of the system and the recorded rates of changes in gas tensions, we calculated the total change in these gases as a function of time. Then we added $C^{14}O_2$ to the system and took samples of algae every few seconds for the first few minutes and then less frequently up to an hour. Each sample of algae was killed immediately and a portion analyzed as described earlier. A part of each sample was reserved and was dried on a planchet to determine the rate of appearance of C^{14} in all stable nonvolatile compounds. This rate proved to be the same as the externally measured rate of uptake of CO_2 and C^{14} between 20 and 60 seconds after the addition of C^{14}. If unstable or volatile intermediates do precede these stable compounds, they are equivalent in micromoles of carbon to no more than 5 seconds photosynthetic fixation, according to the shape of the fixation curve during the first 20 seconds.

We analyzed each sample by paper chromatography, and determined the radioactivity in each compound in each sample. On the basis of the externally measured uptake rates, at least 85% of the carbon was found to be incorporated into individual compounds on the paper chromatograms during the first 40 seconds. At least 70% of the total carbon uptake rate couldbe accounted for by the appearance of C^{14} in compounds apparently derived from the RuDP carboxylation reaction of the carbon reduction cycle via the pathways shown in Figure 2. Another 5% or more was found to be incorporated via C_1-C_3 carboxylation. About 5% was found in unidentified compounds or in glutamic

acid whose photosynthetic pathway is not definitely known. Of the 15% not accounted for, some may be in nonextractable polysaccharides whose sugar phosphate precursors become labeled very quickly. More of the unaccounted for radiocarbon is undoubtedly in a large number of unmeasured compounds on the chromatograms. Each of these compounds contains by itself too little C^{14} to be readily determined. In any event, it is clear that the known fixation pathways are the only quantitatively important ones unless there are unknown pathways utilizing the same intermediate compounds.

A kinetic analysis of the appearance of C^{14} in PGA and RuDP in this experiment, indicated that the carboxylation reaction results in the formation of only one free molecule of PGA per molecule of CO_2 entering the cycle. The kinetic analysis cannot say what the other three-carbon fragment would be. It might be merely a molecule of PGA bound in some way so that its labeling remains distinct from that of the PGA from the other half of the six-carbon addition product. The only other compounds which seem to satisfy the kinetic requirements and which could readily result from the splitting of the six-carbon addition product are the triose phosphates. The formation of a molecule of triose phosphate in this way would require a reductive split of the addition product, as indicated by the dashed line in Figure 2.

That such a pathway differing from the in vitro reaction may exist seems entirely reasonable since the enzymes of the carbon reduction cycle appear to be closely associated with the molecular structures in which the TPNH is formed in the chloroplast (21). In the intact plant the carboxylation enzyme, as well as the enzyme responsible for the splitting of the product and the enzyme which brings about the reduction of TPN^+ to TPNH, might be part of a structurally organized system. In fact, if a reductive scission of water does occur, the reducing agent could be some substance formed from the oxidation /

and preceding TPNH in the electron transport chain. This substance might never be available in sufficient concentration to be a factor in in vitro systems in which carboxydismutase is coupled with isolated or broken chloroplasts. Such an explanation of the experimentally observed kinetic result is purely hypothetical. We mention it to focus attention on the possibility that a given biosynthetic pathway may follow a different course in an intact cell than that which would be predicted on the basis of studies with fragmented cells or enzymes alone.

In higher plants, much of the product of photosynthesis must be transported to a nonphotosynthetic part of the plant. This requires that higher proportions of easily transported molecules such as sucrose are formed (4). In all higher plants that have been studied, however, there is appreciable direct photosynthesis of amino acids and fats, not just carbohydrates.

BALANCE AMONG SYNTHETIC PATHWAYS

We have seen that in each complete turn of the carbon reduction cycle three molecules of RuDP (15 carbon atoms) are carboxylated by three molecules of CO_2 to give six three-carbon compounds (18 carbon atoms). Thus there is a net gain of three reduced carbon atoms. These atoms are withdrawn from the cycle for further synthesis. They may be withdrawn from the cycle as PGA or as any of the sugar phosphates in the cycle. Before the photosynthetic reactions had been mapped, it was commonly believed that photosynthesis leads first to carbohydrates only and that these carbohydrates are then converted via nonphotosynthetic reactions to other compounds such as amino acids and

fatty acids. We now know that pathways leading from the carbon reduction cycle to amino acids and fatty acids and other substances can be just as important quantitatively as those leading to carbohydrates. This is particularly true in an unicellular algae as exemplified by Chlorella pyrenoidosa, where less than half of the assimilated carbon is directly converted into carbohydrate under some conditions. This carbohydrate synthesis draws its carbon from the cycle in the form of sugar phosphates. Consequently, more than half of the carbon drained from the carbon reduction cycle as PGA or sugar phosphates may be used in fat and protein synthesis.

It is interesting to consider an extreme case in which all of the carbon assimilated by the carbon reduction cycle would be withdrawn from the cycle as PGA, converted to PEPA, and then carboxylated to give four-carbon compounds. In this case, 75% of the assimilated carbon would enter the photosynthetic pathways via the carbon reduction cycle, while the remaining 25% would enter via the carboxylation of PEPA.

With normal conditions of steady-state growth under high light intensity, the ratios of various fixation pathways must be determined to a large extent by the requirements of the plant for the small molecules from which the protein, carbohydrate, fat and other substances of the plant are synthesized.

PHOTOSYNTHESIS VS. OTHER FORMS OF BIOSYNTHESIS

Biosynthetic reactions in plants cannot be classified as photosynthetic or nonphotosynthetic on the basis of direct photochemical action since all reactions in the synthetic pathways are probably 'dark' reactions. However, we can make a classification on the basis of the immediate source of the required cofactors. The conversion of light energy results in the formation of ATP and TPNH and perhaps other unknown cofactors. When these cofactors are formed by the light reaction and are used to bring about the synthesis of carbon compounds, we may consider the reactions to be photosynthetic. Also included in this category would be preliminary steps and intermediate steps such as hydrations, condensations and carboxylations.

It may well be that all photosynthetic reactions, as just defined, occur in the chloroplasts, while the light is on. If this is true, reactions outside the chloroplast would derive their energy from substrate carbon compounds which diffuse from the chloroplast to the extrachloroplastic spaces of the cell. Such an interpretation is suggested by the report by Tolbert (22) who found that chloroplasts isolated from Swiss chard when allowed to photosynthesize with $HC^{14}O_3^-$ excreted mainly glycolic acid into the medium. Phosphate esters, of importance to the carbon reduction cycle, were retained in the chloroplasts. Isolated chloroplasts have a carbon metabolism which is much more limited than photosynthesis in intact cells. This is probably due to loss of enzymic activity by chloroplasts during the isolation process. In all probability the carbon compounds excreted by intact chloroplasts in vivo include other substances besides glycolate.

There is more than a semantic reason for making a distinction between photosynthetic and nonphotosynthetic pathways. The environment of the photo-

synthetic metabolism is unique. There is an abundance of the reduced and energetic form of the coenzymes. Hence synthetic pathways do not require energy derived from degradative reactions such as decarboxylations and oxidations. For example, a well known biosynthetic pathway leading to glutamic acid from acetate includes oxidative and decarboxylation steps. Such a pathway is to be expected in a nonphotosynthetic system where degradation of part of the substrate is the only means of obtaining the energy and reducing power for synthetic reactions. In a photosynthetic system, one might expect instead a pathway involving only condensations, reductions and carboxylations. We cannot say that this difference in type of reaction will always be borne out by the actual mechanisms when they are known. This proposed difference in reaction type may be a useful working hypothesis to those who attempt to map photosynthetic pathways from experimental data.

AMINO ACID SYNTHESIS

Among the first compounds found to be labeled by photosynthesis of $C^{14}O_2$ in algae were alanine, aspartic acid, and several other amino acids (11). These compounds were slowly labeled even in the dark when algae were exposed to $C^{14}O_2$. They and malic acid were much more rapidly labeled if the algae were photosynthesizing, or had been photosynthesizing, just prior to the moment of addition of $C^{14}O_2$. We recognized that these amino acids were therefore products of photosynthetic reduction of CO_2, even though they could also become labeled by reversible respiratory reactions. Accelerated incorporation of C^{14} into amino acids in higher plants during photosynthesis has been noted in this laboratory (23,24) and in many others (25,26,27,28). Nichiporovich (25) has presented and reviewed evidence that synthesis of proteins in the chloro-

plasts of higher plants is greatly accelerated during photosynthesis. This accelerated protein synthesis appears to occur directly from the intermediates of photosynthetic carbon reduction since the proteins were labeled when $C^{14}O_2$ was used but not when C^{14} labeled carbohydrate was administered. Photosynthetically-accelerated synthesis of protein containing N^{15} was also observed when $N^{15}H_4^+$ was administered. Sissakian (29) has reviewed evidence that protein can be synthesized in isolated chloroplasts from nonprotein nitrogen, including peptides.

In experiments in this laboratory (30) it has recently been possible to measure the proportion of the total carbon fixed by Chlorella pyrenoidosa which is directly incorporated into certain key amino acids. These experiments show that during steady-state photosynthesis in bright light with an adequate supply of inorganic nutrients, the synthesis of these amino acids can account for 60% of all the carbon fixed by the algae and 30% of the uptake of NH_4^+ which is also measured. If the light is turned off, the NH_4^+ uptake and C^{14} fixation into amino acids are both accelerated for about 10 minutes and then drop to a very small fraction of the rates in the light. Finally, these experiments indicate clearly that in Chlorella pyrenoidosa there are at least two pools of alanine, glutamic acid, aspartic acid and serine, and probably other amino acids as well. One of these pools, especially in the cases of alanine and aspartic acid, is labeled extremely rapidly after the introduction of $C^{14}O_2$ to the algae. So rapidly are these compounds labeled, in fact, that the site of their synthesis must be freely accessible to their photosynthetically-formed precursors, namely, phosphoenolpyruvic acid and PGA (see Figure 1). The studies of Tolbert (22) and Moses (31) indicate that the photosynthetic pools are isolated from the extrachloroplastic region.

We conclude, therefore, that in Chlorella the more rapidly labeled pools of amino acids are located at the site of photosynthetic carbon reduction, probably in the chloroplast.

The sizes of these pools of amino acids, and their rates of synthesis as determined from kinetic labeling data with Chlorella in a typical experiment are shown in Table 1.

The amino acids shown in Table 1 are those which are most prominently labeled with carbon fourteen during a few minutes of photosynthesis. In addition, a number of other amino acids become labeled as time passes. The rates of labeling seem to indicate that the carbon skeletons of these other acids are probably derived, for the most part, from the listed amino acids. However, the aromatic rings of the amino acids are synthesized by another pathway.

In Table 1 we compare the rates of synthesis of carbon skeletons that have been measured with the rate of uptake of NH_4^+. The rate of synthesis of any given amino acid does not necessarily represent the rate of incorporation of inorganic nitrogen into that amino acid, since it could be formed by transamination from another amino acid. However, the total of the rates of synthesis of all 'primary' amino acids should account for the major fraction of the rate of uptake of ammonia. By 'primary' amino acids we mean those amino acids whose carbon skeletons are not synthesized from some other amino acid. . . Alanine, serine and aspartic acid are clearly 'primary' amino acids since their rates of labeling reach a maximum as soon as the intermediates in the carbon reduction cycle are saturated (about 3 minutes in this experiment) and long before they themselves, or any other amino acids, are saturated with radiocarbon (30). Probably glutamic acid is a 'primary' amino acid also, but kinetic data alone cannot prove this at the moment. Glutamine

is generally supposed to arise from glutamic acid, but there is some evidence to indicate that it may arise as a 'primary' amino acid amide (32,30).

In any event, the rates of synthesis of alanine, serine and aspartic acid in reservoirs which we believe to be closely associated with the chloroplasts in Chlorella are great enough to permit the following conclusions.

1) An appreciable fraction of the carbon assimilated during photosynthesis in Chlorella is used directly in the synthesis of amino acids without the intermediacy of sugars or any other class of compounds except acid phosphates and carboxylic acids.

2) Since this amino acid synthesis accounts for a major portion of the inorganic nitrogen uptake, these amino acids must be used to a large extent in protein synthesis. However, some important amino acids (i.e. glycine) are so slowly labeled that they probably do not supply a major part of the carbon for protein synthesis. Instead, the carbon skeletons corresponding to these amino acids must be incorporated into protein in some form otherthan as the free amino acid.

Before considering synthetic routes to specific amino acids, we wish to reiterate our belief that photosynthetic reactions need not follow the same course as the better known synthetic reactions of other nonphotosynthetic organisms. Also note that few if any enzymes involved in amino acid synthesis have/been isolated from chloroplasts. Thus we are forced to suggest new and untested hypothetical paths. Our guiding principles will be that chemical potential should be used to drive the reactions rapidly in the forward direction and that loss of carbon or reduction level should be avoided wherever possible.

In Figure 6 are shown hypothetical pathways leading from PGA to alanine, serine, aspartic acid and malic acid. These pathways differ somewhat from known enzymatic pathways in that in each step leading to the amino acid, ammonia reacts with a phosphoric acid ester.

The rapid incorporation of inorganic nitrogen into organic compounds would be brought about by the large negative free energy change associated with each of these reactions. Thus, these reactions, and not the reductive amination of ketoglutaric acid alone, would account for a major portion of ammonia incorporation during photosynthesis. This seems entirely reasonable when one considers that PGA is both the immediate precursor in these reactions and the primary product of carbon reduction during photosynthesis. These amino acids could then supply ammonia via transminase reactions for the synthesis of many other amino acids. Holm-Hansen (33) has demonstrated the presence of a transminase activity in spinach chloroplasts which is very effective in the transfer of amino groups from unlabeled alanine to C^{14}-labeled pyruvic acid.

The three-carbon precursors to these amino acids are in rapid equilibrium with PGA. PEPA becomes C^{14}-saturated during photosynthesis in $C^{14}O_2$ in Chlorella almost as soon as PGA itself. The proposed phosphoenoloxalacetate probably does not exist except in enzyme complexes. Thus, by the time the PGA is C^{14}-saturated, these amino acids are being labeled as rapidly as if they were formed directly from $C^{14}O_2$.

It has been suggested that glutamic acid is formed during photosynthesis by a carboxylation of γ-aminobutyric acid (34). Judging by our studies with Chlorella pyrenoidosa during steady-state photosynthesis with $C^{14}O_2$, this reaction apparently does not constitute a source of glutamate since γ-amino-

butyric acid does not become labeled, even by the time the glutamic acid is 50% saturated with C^{14} and long after the rate of labeling of glutamic acid has passed its maximum. Clearly, a compound cannot be a precursor in a steady-state system unless it is itself continuously regenerated. If the reaction does occur at all, the glutamic acid so formed could only be a shuttle for CO_2, regenerating unlabeled γ-aminobutyric acid. Even so, such a carboxylation reaction does not account for more than about 1% of the carbon fixed in our studies of steady-state CO_2 fixation by Chlorella.

One possible route from PGA to glutamic acid would begin with conversion of PGA to PEPA, followed by carboxylation of PEPA to give oxalacetic acid. Condensation of oxalacetic acid with acetyl CoA would give citric acid, thence aconitic acid, thence isocitric acid. Proceeding along the Kreb's cycle, the next two steps are oxidation to oxalosuccinic acid, followed by oxidation and decarboxylation to give α-ketoglutaric acid. Finally, the reductive amination would give glutamic acid. This pathway may be followed in Chlorella pyrenoidosa in the synthesis of glutamic acid, particularly when the light is turned off. We suspect that it is not the principal pathway during photosynthesis for two reasons, one experimental and one theoretical. Experimentally, the rates of labeling of the intermediate compounds such as citric acid and ketoglutaric acid are too slow to permit them to serve as precursors to the more rapidly labeled reservoir of glutamic acid. Theoretically, the pathway is objectionable to us as a photosynthetic route because it involves two oxidations and a decarboxylation

How else might glutamic acid be formed during photosynthesis? the availability of three-carbon and two-carbon compounds suggests the possibility of a simple condensation. Barker and co-workers (35,36,37) found an enzymic pathway in certain microorganisms leading from glutamic acid to pyruvic

acid and acetate via citramalate, mesaconic acid and β-methylaspartate. The reverse of this pathway might operate during photosynthesis also. However, we have been unable so far to find significant amounts of radiocarbon in either β-methylaspartic acid or mesaconic acid in Chlorella which were synthesizing glutamic acid from $C^{14}O_2$. Moreover, a general energy conserving principle would suggest that PEPA and not free pyruvic acid should be the three-carbon compound that combines with the two-carbon fragment. As we shall see in the discussion for the synthesis of aromatic rings, it has been proposed that PEPA can condense with an aldehyde, erythrose phosphate, to give (eventually) phosphoshikimic acid (38). Perhaps a similar reaction between PEPA and glyoxylic acid could lead to a product such as γ-hydroxyglutamic acid which could be subsequently converted to glutamic acid. Dekker (39) has reported the presence of an enzyme in rat liver which converts γ-hydroxyglutamic acid to glyoxylate and some other product which may be alanine. The presence of γ-hydroxyglutamic acid in green leaves has been reported by Virtanen and Hietala (40). The dehydration and reduction of γ-hydroxyglutamic acid to give glutamic acid would be common types of biochemical reactions, analogous to the formation of succinic acid from malic acid. However, we have at present no experimental evidence for such a ptahway.

Threonine does not become labeled as rapidly as the amino acids so far discussed, and it may well be secondary in origin. That is, it may be an example of conversion of 'primary' amino acids (aspartic acid, alanine, serine and glutamic acid) to other amino acids of their respective families which presumably occurs in photosynthesis.

The small amount of labeled glycine which is formed during steady-state photosynthesis may come from either serine or glyoxylic acid.

CARBOXYLIC ACIDS

Malic and Fumaric Acids. Malic acid and fumaric acid are rapidly labeled during steady-state photosynthesis with $C^{14}O_2$. These acids are probably formed by reduction of the product of carboxylation of PEPA. In the steady-state experiment (SS32) which gave the results shown in Table 1, about 5% of the C^{14} uptake rate could be accounted for in the labeling of these two acids. In that experiment, very little of the radioactivity finds its way into succinic acid. It would thus appear that if malic and fumaric acids are labeled by reductive carboxylation of PEPA, either 1) the reaction sequence is highly reversible, leading to exchange labeling, or 2) the malic and succinic acids are converted to other compounds by as yet undetermined paths.

The probability of labeling via exchange 1) may be answered by a thermodynamic argument. Under the conditions existing in the chloroplast during photosynthesis, the actual free energy change accompanying the conversion of PEPA, CO_2, TPNH, and either ADP or IDP to malic acid, TPN^+, and ATP or ITP is probably at least -7 Kcal. The ratio of the forward to back reaction, given by:

$$\Delta F = -RT\ln\left[\frac{\text{forward rate}}{\text{back rate}}\right] = R - RT\ln\left[\frac{\text{back rate plus net rate}}{\text{back rate}}\right]$$

would thus be 10^5 or greater. Since the rate of labeling of malic acid is measurable and gives the net rate by a simple calculation, the back reaction, and hence the exchange labeling, can be shown to be of negligible importance.

This type of calculation is of considerable importance in in vivo steady-state kinetic calculations. Another example is the conversion of malic acid to fumaric acid. In this case, the actual free energy change is small, the two acids are essentially in equilibrium with respect to C^{14}-labeling. Thus, the sum of the pools of the two acids can be treated as a single entity from a labeling standpoint.

In any event, if malic acid is not labeled by exchange and is not converted to succinic acid, yet is being formed at a rapid rate under steady-state conditions, it must undergo some as yet unknown conversion. One possibility might be that it is split to give glyoxylic acid and free acetate. The actual free energy change for such a reaction under steady-state conditions would be negative, whereas the reaction to give glyoxylic acid and acetyl CoA would probably be positive and the latter reaction would not occur. Acetate could be converted to acetyl phosphate with ATP and then to acetyl CoA. The acetyl CoA thus formed could be used in fatty acid synthesis and other biosynthetic reactions. The glyoxylic acid could be used in the synthesis of glycolic acid, glycine, and possibly, as suggested in the previous section, glutamic acid.

The synthesis of labeled malic acid could occur via condensation of glyoxylate with acetyl CoA, provided there is some other route for the labeling of these two-carbon acids (such as are suggested later). It is quite likely that malic acid is so synthesized in the cytoplasm, outside the chloroplasts. Within the chloroplasts, however, the appearance of C^{14} in malic acid in the very shortest exposures to $C^{14}O_2$ and in the pre-illumination experiments (see Figure 5) indicate that it is, in part at least, a product of C_1-C_3 carboxylation and reduction.

Glycolic Acid, Acetic Acid and Glyoxylic Acid. Even if acetate and glyoxylate are formed from malic acid, there are probably other more important synthetic routes from the carbon reduction cycle to these compounds. Benson and Calvin (41) found that barley seedlings subjected to 30 seconds photosynthesis with $C^{14}O_2$ followed by 2 minutes light without CO_2 formed large amounts of C^{14} labeled glycolic acid. Calvin, et al. (14) and Schou, et al. (42) degraded glycolic acid and phosphoglyceric acid obtained from barley leaves and from Scenedesmus which

had photosynthesized for a few seconds in the presence of $C^{14}O_2$ or $HC^{14}O_3^-$. The alpha and beta carbon atoms of PGA were found to be always about equal to each other in radioactivity and always less than the carboxyl carbon until such time (1 to 5 minutes) as all three carbon atoms were completely labeled. The two carbon atoms of glycolic acid were always about equal to each other in labeling. When $C^{14}H_2OH$-COOH was administered to the unicellular algae, Scenedesmus, during 10 minutes photosynthesis with 1/2% CO_2 in air or N_2, a pattern of photosynthetic intermediates was found similar to that obtained during photosynthesis with $C^{14}O_2$. Moreover, upon degradation of the PGA, we found that less than 10% of the radioactivity was in the carboxyl carbon. Clearly, glycolic acid is incorporated for the most part into normal intermediates of the carbon reduction cycle without preliminary conversion to CO_2, since so little C^{14} was found in the carboxyl carbon of PGA. However, alpha and beta carbon atoms of the PGA were found to be equally labeled. Thus the pathway from glycolic acid to the alpha and beta carbon atoms of PGA involves a randomization of the label. This could mean that along this pathway there is a symmetrical intermediate or that an intermediate is in rapid reversible equilibrium with a symmetrical compound. (see below)

When Wilson and Calvin (19) studied the effect of CO_2 depletion following a period of photosynthesis with $C^{14}O_2$ by algae, they found that the lowering of CO_2 pressure resulted in a great increase in the amount of labeled glycolic acid. This increase in labeled glycolic acid was sustained for at least 10 minutes. Upon application of 1% CO_2 again the level of labeled glycolic acid declined.

Tolbert (22) found that glycolic acid formation from $C^{14}O_2$ during 10 minutes photosynthesis in leaves of Sedum alboresum is much higher at very low CO_2 pressure than at high CO_2 pressures. As mentioned earlier, he also found

that glycolic acid is the predominant labeled compound excreted into the medium by chloroplasts from Swiss chard photosynthesizing in the presence of $HC^{14}O_3^-$. He had shown earlier (43) that glycolic labeled with C^{14} is excreted into the medium by Chlorella photosynthesizing in $C^{14}O_2$. He suggested that glycolate may function in ion balance with HCO_3^- between cells and their medium or between chloroplasts and other cell compartments. He also proposed that glycolate might be a carrier of 'carbohydrate reserves' from the chloroplasts to the cytoplasm.

Moses and Calvin (44) exposed photosynthesizing Chlorella pyrenoidosa to tritium-labeled water for various periods from 5 seconds to 3 minutes. Analysis was made by the usual extraction, two-dimensional paper chromatography and radioautography. The greatest darkening of the film by far occurred where it was in contact with the glycolic acid area of the chromatogram. This result, which we will discuss later, seems to agree with Tolbert's suggestion that the glycolic acid acts as a carrier of hydrogen.

During normal photosynthesis (Figure 2), two-carbon moieties (carbon atoms number 1 and 2 from a keto sugar phosphate) are transferred during a reaction similar to that catalyzed by transketolase (45,46) to an aldo-sugar phosphate, producing a new ketose phosphate, two carbon atoms longer than the starting aldose. Other enzymes have been found in nonphotosynthetic organisms which convert the carbon atoms number 1 and 2 of a ketose phosphate to acetyl phosphate, leaving the remainder of the sugar as an aldose phosphate. One of these is phosphoketolase (47), which is specific for xylulose-5-phosphate, while another is fructose-6-phosphate ketolase (48) which can act on either fructose-6-phosphate or xylulose-5-phosphate. These enzymes require thiamine pyrophosphate, inorganic phosphate and, in some cases, Mg^{++}. Stimulation by Mn^{++} or Ca^{++} in place of Mg^{++} could sometimes be observed, while levels of Mn above 10^{-3} were inhibitory.

Breslow has proposed a mechanism for the role of thiamine pyrophosphate in these reactions (49,50). In his mechanism, some of which forms the basis for part of Figure 7, the hydrogen at position 2 of the thiazole ring is an active hydrogen which can dissociate from the acidic carbon at that position to give a carbanion. This carbanion adds to the carbonyl carbon of the ketose (somewhat analogous to cyanhydrin addition). The bond between carbons 2 and 3 of the ketose breaks, with the electron pair going to the reduction of carbon 2 of the ketose, to give a glycolaldehyde-thiamine pyrophosphate. The remainder of the sugar becomes an aldose. Reversal of this reaction path, with a different aldose, completes the transketolase reaction.

Alternatively, glycolaldehyde-thiamine pyrophosphate may eliminate the elements of water (OH^- from the beta carbon and H^+ from the alpha carbon of the glycoladehyde moiety) to give the enol form and thence the keto form of acetyl-ThPP. This compound can undergo phosphoclastic cleavage to give acetyl phosphate and thiamine pyrophosphate (ThPP).

The mechanisms find support in the demonstration by Breslow that the hydrogen atom on the number 2 position of the thiazole ring does exchange rapidly in D_2O (49). In support of an analogous mechanism for the role of ThPP in the oxidation of pyruvate, Krampitz and co-workers (51,52) synthesized the postulated intermediate, an acetaldehyde-ThPP compound with the acetaldehyde bonded to the number 2 carbon atom of the thiazole ring as an alpha hydroxyethyl group. This compound was found to be active in the reactivation of carboxylase and also to be capable of nonenzymatic reaction with acetaldehyde to give acetoin. The postulated mechanism for the oxidation of pyruvic acid thus begins with a reaction between pyruvate and ThPP to give addition of the carbonyl

carbon to the thiazole ring position number 2. Concurrently or immediately following this addition, decarboxylation occurs to give acetaldehyde-ThPP. This compound reacts with oxidized lipoic acid to give acetyl dihydrolipoic acid which, in turns, reacts with Coenzyme A to give dihydrolipoic acid and acetyl Coenzyme A. (53,54,55,56)

Wilson and Calvin (19), following their observation of glycolate accumulation at low CO_2 pressure, suggested that the glycolyl moiety transferred by transketolase is the source of glycolic acid. We would like now to suggest specifically that the glycolaldehyde-ThPP compound formed in the first step of the transketolase or phosphoketolase reactions may undergo oxidation to give glycolyl Coenzyme A and, eventually, glycolate. This oxidation need not follow a pathway exactly analogous to the oxidation of acetaldehyde-ThPP, but we have shown it so in Figure 4. As mentioned earlier, glycolate can be incorporated into the alpha and beta carbon atoms of PGA during photosynthesis, which comes from carbon atoms 1 and 2 of the pentose in the carbon reduction cycle. Thus it appears that the pathway from pentose phosphate to glycolate and glyceraldehyde phosphate should be reversible. The incorporation of glycolate via such a pathway would require an energy input, probably in the form of an activation by ATP. Finally, some state in the incorporation pathway should involve equilibration with a symmetric intermediate because administration of glycolate-2-C^{14} to photosynthesizing plants leads to PGA labeled equally in the alpha and beta carbon atoms. We have indicated one such symmetric compound, and there may be other possibilities.

The formation of glycolyl coenzyme A and reduced lipoic acid as shown in Figure 7 are hypothetical. If glycolyl coenzyme A were formed, then it seems likely that it would be an important intermediate in paths which are as yet unknown. In any event, if there is any conversion of carbon atoms number 1 and

2 of ketose to glycolic acid during photosynthesis, then an oxidation of the glycolyl fragment is required so that some cofactor, though not necessarily lipoic acid, must be reduced.

Let us now attempt to explain the observation that labeled glycolate accumulates during photosynthesis with $C^{14}O_2$ when the CO_2 pressure is reduced.

1. Enzyme systems are present in chloroplasts which can bring about the oxidation of glycolate to glyoxylate with oxygen and the reduction of glyoxylate to glycolate with DPNH . (57) If some steady-state relation between these two acids exists, it might well be shifted towards more glycolate at low CO_2 pressures by the increase in the ratio of $DPNH/DPN^+$ which would result from the decreased utilization of TPNH for the carbon reduction cycle. Moreover, the oxidation of glycolate by O_2 must in fact be limited in rate during photosynthesis, or glycolate would not be seen at all. Possibly glycolate is more effectively oxidized by some intermediate hydroxyl or peroxide involved in the liberation of oxygen following the splitting of water during the primary act in photosynthesis. If so, such an intermediate oxidant may decrease in concentration at low CO_2 pressure due to recombination with primary reductant which would build up, again as a result of decreased utilization by the carbon reduction cycle. A decrease in the oxidant concentration would reduce the oxidation of glycolate.

2. Low CO_2 pressure might result in higher pH inside the chloroplasts. The phosphoketolase reaction, leading to acetyl coenzyme A and involving the removal of OH^- from glycolaldehyde-ThPP, might be blocked, and the oxidation of the glycolaldehyde-ThPP to glycolyl coenzyme A might be favored.

3. If glycolyl coenzyme A is formed and is a biosynthetic intermediate, the reactions in which it is used might require CO_2 analogous to the conversion of acetyl coenzyme A to malonyl coenzyme A in fatty acid biosynthesis.

Low CO_2 pressure could thus lead to an increased concentration of glycoly-CoA and permit its more rapid hydrolysis to glycolate.

Tanner and co-workers (58,59) have recently proposed a direct route from CO_2 to glycolic acid during photosynthesis. According to his scheme, CO_2 is reduced by TPNH and $MnCl^-$ to the radical CHO· . Two of these CHO· radicals are then condensed to give glyoxal, thence glycolic acid. This glycolic acid is then oxidized by two molecules of $MnCl(OH)_2$ (produced in the first step) to give glyoxylic acid. According to Tanner, the greater labeling of glycolic acid at low CO_2 pressure during photosynthesis with $C^{14}O_2$ is due to the first step being first order with respect to the utilization of CO_2 and the production of trivalent manganese, while the second step is second order with respect to the utilization of trivalent manganese.

Whether or not Tanner's suggested route from CO_2 to glycolic acid will be borne out by experiment remains to be seen. In all of our experiments with $C^{14}O_2$, labeled glycolic acid has been a relatively minor product of the photosynthesis, except in those cases where the CO_2 pressure has been permitted to drop to a very low level. Glycolic acid is somewhat volatile, but it is a curious characteristic of this compound on paper chromatograms that although 20 to 85% may evaporate from the paper during development of the chromatogram, the remainder disappears only very slowly from the papers. This statement is based on measurement of radioactivity following chromatography of synthetic C^{14}-labeled glycolic acid. Thus it would seem that if a pathway leading directly from CO_2 to glycolic acid (that is, with no isolable intermediates) were quantitatively important, we should have seen much more labeled glycolic acid following short periods of photosynthesis with $C^{14}O_2$. It could be that under normal conditions of photosynthesis (say with 1% CO_2 in air), the reservoir

size or concentration of glycolic acid is very small so that it would not appear to be strongly labeled even though carbon from $C^{14}O_2$ enters it very rapidly.

However, Moses (44) conducted parallel experiments (3 minutes photosynthesis by Chlorella in the presence of $C^{14}O_2$ in one case, and T_2O in the other). The tritium-labeled glycolic acid accounted for more than 50% of the darkening of the radioautograph in the subsequent analysis by chromatography, while in the parallel experiment, the C^{14} glycolic acid contained less than 5% of all the C^{14} found in compounds on the chromatograph. Thus the incorporation of hydrogen into nonexchangeable positions on glycolic acid seems to occur at ten times or more the rate of incorporation of C^{14} into the same compound. The simplest interpretation is that glycolic acid plays a much more important role in the transport of hydrogen or reducing power than it does as an intermediate in carbon compound formation from CO_2. If any carbon dioxide is reduced directly to glycolic acid during photosynthesis by Chlorella, it would seem to be a minor part of the total.

A special role for glycolic acid in hydrogen transport is suggested by a combination of experimental findings from several laboratories. To Moses' finding of extremely rapid tritium labeling of glycolic acid and Tanner's implication of the role of glycolic acid with the requirement for manganese, we may add Delavin and Benson's report (60) of the light stimulation of the oxidation of glycolic acid with O_2 to glyoxylate and peroxide in isolated chloroplasts. Further, we must mention that manganese is thought by Kessler (61) to play some part in the formation of peroxide or O_2 from water during the early stages of photosynthesis. Some form of peroxide is commonly postulated as an intermediate between water and O_2 during photosynthesis, and it may be that the plant has some mechanism for conserving the chemical potential energy

which would be lost if peroxide were to be permitted to decompose to water and oxygen by a catalse mechanism.

The decrease in labeled glycolate in algae grown in Mn^{++}-deficient media (58,59) may be due to (1) Some increase in the level of an intermediate in the oxygen evolution pathway which is also capable of oxidizing glycolate to glyoxylate. Presumably Mn^{++} might be required for the breakdown of this oxidant to O_2. (2) A decrease in reduced pyridine nucleotide concentration due to impairment of the oxygen evolving pathway; or (3) Some enzymic requirement for Mn^{++} in the biosynthetic pathway from glycolaldehyde-ThPP to glycolate.

Points (1) and (2) are related to the mechanisms suggested earlier for the effedt of low CO_2 pressure on glycolate concentration.

Acetate. As shown in Figure 7, acetyl phosphate can be formed from the carbon reduction cycle via the phosphoketolase pathway. This involves dehydration of the ThPP-acetaldehyde compound derived from carbon atoms 1 and 2 of ketose phosphates. This route is especially attractive as a photosynthetic pathway since it conserves chemical energy and requires no oxidation or decarboxylation. Known enzyme systems would readily convert the acetyl phosphate to acetyl coenzyme A for fatty acid photosynthesis.

Another pathway from the carbon reduction cycle to acetyl CoA could be via oxidative decarboxylation of pyruvic acid. This reaction is of thetype which we have earlier viewed as unlikely in photosynthesizing chloroplasts on ground of economy. However, this economy takes on a different aspect if one considers the rapid formation of alanine, which we believe might be a reductive

amination of phosphoenolpyruvic acid derived from the carbon cycle (30). Our experiments indicate that about one-third of all NH_4^+ uptake occurs via this route. The resulting alanine must be used to a considerable extent in transamination reactions, resulting in the production of pyruvic acid. While pyruvic acid is not labeled soon enough after the introduction of $C^{14}O_2$ to photosynthesizing plants to permit us to consider it a precursor to alanine, it does become slowly labeled at later times. Thus pyruvic acid could be a product of transmination from alanine. The slow labeling of pyruvate may be because alanine has a very large reservoir, which does not saturate with C^{14} for some minutes. Once formed, the pyruvic acid cannot easily be converted back to PEPA. Rather, it must either go to malic acid via reductive carboxylation, or be oxidized to acetyl CoA and CO_2.

The light-dark transient effect in $C^{14}O_2$ uptake during photosynthesis has often been observed (16,20). When the light is turned off, following a period of photosynthesis of algae with $C^{14}O_2$, labeled glutamic acid and citric acid accumulate. One explanation of this effect has been given based on the proposed formation of acetyl coenzyme A by pyruvic acid oxidation. Lipoic acid in its oxidized form is required to accept the electrons in this oxidation. It was suggested that while the light is on this cofactor is kept mostly in its reduced state, dihydrolipoic acid. The reduced cofactor could not promote pyruvic acid oxidation. When the light is turned off and reducing power is no longer generated, the oxidized form of lipoic acid would be made and the oxidation leading to acetyl coenzyme A would occur. Subsequent reactions, via the glyoxylate cycle, would then produce citric and glutamic acids.

However, if acetyl phosphate is formed by phosphoketolase during photosynthesis, a different explanation can be made. If we suppose that acetyl phosphate is still formed via phosphoketolase just after turning off the light, it

will tend to accumulate. No reducing power or ATP is available for synthesis of fatty acids in the dark inside the chloroplasts. Therefore, acetyl phosphate will break down to free acetate which will diffuse out of the chloroplast into the cytoplasm. There it will be used, via the glyoxylate cycle, in the synthesis of glutamic acid (62).

CARBOHYDRATES

Monosaccharides. The carbon reduction cycle (Figure 2) includes as intermediate compounds the following sugar phosphates: 3-phosphoglyceraldehyde, dihydroxyacetone phosphate, fructose-1,6-diphosphate, fructose-6-phosphate, erythrose-4-phosphate, sedoheptulose-1,7-diphosphate, sedoheptulose-7-phosphate, xylulose-5-phosphate, ribulose-5-phosphate, ribose-5-phosphate, and ribulose-1,5-diphosphate. Besides these compounds, glucose phosphates are found to be very rapidly labeled in all plants in which we have studied the photosynthesis of carbon compounds from $C^{14}O_2$. When characterized, both glucose-6-phosphate and glucose-1-phosphate have been found. Other sugars found to be labeled somewhat more slowly in these experiments, and identified as the free sugars following hydrolysis of the sugar monophosphate area, include mannose and galactose.

In virtually all the studies of the labeled products of the photosynthesis of carbon compounds from $C^{14}O_2$ there has been found a striking absence of unphosphorylated monosaccharides (14). This is hardly surprising, since photosyntheisizing chloroplasts form phosphorylated sugars as intermediates in the carbon reduction cycle, since there is an abundance of ATP in the chloroplasts, and since most known transformations of monosaccharides require phosphorylated forms of the sugars. Transformation of the phosphorylated sugars

to the free sugars would for the most part result in a waste of chemical energy, for the sugar would then usually have to be phosphorylated again in reactions requiring ATP or UTP. Only when it becomes necessary to form a molecule which can be transported through the chloroplast membrane is it likely that a free sugar of relatively small molecular weight such as sucrose would be produced.

A listing of various enzyme systems which appear to be responsible for the carbon reduction cycle has been delayed until now, since many of these biochemical steps are of interest in a discussion of carbohydrate synthesis. In Table 2 there are listed the enzymes reported in the literature which appear to be responsible for steps of the carbon reduction cycle (Figure 2). Table 3 lists other enzymes which could account for subsequent steps in the synthesis of carbohydrates found to be labeled following relatively short periods of photosynthesis of algae with $C^{14}O_2$.

We wish to emphasize that the finding of an enzyme in plant tissue does not, of course, prove that that particular reaction goes on in the photosynthesizing chloroplast either at all or in precisely the same way that it has been found to occur *in vitro*. Moreover, we would not consider the isolation of an enzyme with high catalytic activity a necessary condition for believing that a given reaction may occur *in vivo*. The organization of the intact chloroplast inside the living cell and replete with all necessary *natural* cofactors and enzymes is such that some steps which occur *in vivo* may prove extremely difficult to demonstrate in cell free systems. Nonetheless, the isolation of a cell free system, capable of carrying out a reaction which has been suspected on the basis of *in vivo* studies is important corroberative evidence.

The various enzymes listed inTables 2 and 3, if present in chloroplasts, could account for virtually all of the monosaccharide phosphates found to be

significantly labeled with C^{14} following a period of photosynthesis with $C^{14}O_2$ for several minutes in algae. Presumably there is also present another phosphohexose isomerase which catalyzes the conversion of fructose-6-phosphate to mannose-6-phosphate.

Among the enzyme systems listed in Table 3 are several which utilize sugar nucleotides in the biosynthetic conversion of sugars. Such systems have been widely studied and have been discussed and reviewed elsewhere (88,89,90). Hassid and co-workers have widely studied the interconversions of sugars by these systems in higher plants and have summarized the interrelations of many of these systems in plants (91). Certain of these systems, which appear in Table 3, are particularly active in the early labeling of sugars in plants photosynthesizing with $C^{14}O_2$ and must be mentioned here, if only briefly.

Buchanan (15) identified uridine diphosphate glucose (UDPG) and uridine diphosphate galactose (UDPGal) in algae and found that the hexose moieties of these compounds were labeled with C^{14} during short periods of $C^{14}O_2$ photosynthesis even before sucrose. Thus the galactose found to be labeled in some experiments may be formed by the UDPG-UDPGal system.

Disaccharides and Polysaccharides. As already indicated, when Chlorella pyrenoidosa photosynthesize in the presence of $C^{14}O_2$, sucrose is the first free sugar to be labeled to any extent. Benson (92) found that the radiocarbon in the fructose moiety of the sucrose, following photosynthesis of $C^{14}O_2$ by Chlorella, Scenedesmus and soybean leaves, was greater than the radioactivity in the glucose moiety. Moreover, the difference between fructose and glucose became greater as the time of photosynthesis was decreased. The prior labeling of the fructose indicated that the glucose phosphate used in the synthesis of sucrose is formed from fructose phosphate.

A study of the phosphorylated products of short-term photosynthesis in $C^{14}O_2$ led to the discovery of a sucrose phosphate (93). The 'hexose monophosphates' produced during photosynthesis in $C^{14}O_2$ were treated with an invertase-free phosphatase preparation and subjected to paper chromatography. While in most cases there were only minute traces of sucrose formed by this treatment, in sugar beet (5 minutes in $C^{14}O_2$) there was an appreciable quantity. It was identified by cochromatography, and enzymic hydrolysis to glucose and fructose.

When this 'hexose monophosphate' sample was subjected to chromatography in t-butanol:picric acid:water, radioactive areas corresponding to glucose-6-phosphate, fructose-6-phosphate, sedoheptulose and mannose phosphates, and sucrose phosphate were obtained. The sucrose phosphate gave sucrose on phosphatase treatment, and on acid hydrolysis, glucose and fructose phosphate were produced. The latter did not cochromatograph with fructose-6-phosphate.

It appeared that in sucrose synthesis in green plants there are two possible mechanisms. Glucose-1-phosphate might react with fructose-1-phosphate to give sucrose phosphate, which would be dephosphorylated to sucrose. Alternatively, sucrose phosphate synthesis might be envisaged to occur through uridine diphosphate glucose (15) which becomes labeled shortly before sucrose in kinetic experiments with $C^{14}O_2$ (18). The uridine diphosphate glucose may be formed from glucose-1-phosphate by a UDPG pyrophosphorylase (reaction 15, Table 3). This pathway is shown, along with other pathways which may very likely occur during photosynthesis of carbohydrates from CO_2, in Figure 8.

Leloir and Cardini (85) have isolated from wheat germ what appears to be two systems, one which catalyzes the reaction of fructose plus UDPG to give sucrose plus UDP and the other which catalyzes the reaction UDPG plus fructose-6-phosphate to give sucrose phosphate plus UDP. Burma and Mortimer (94) have reported that with excised sugar beet leaves and leaf homogenates radio-

active UDPG and sucrose were formed when radioactive glucose-1-phosphate, fructose-6-phosphate and UTP were added. They propose a mechanism identical to that postulated by Buchanan except in the choice of fructose-6-phosphate as the precursor instead of fructose-1-phosphate.

Not much is known about the formation of other polysaccharides. There is a rapid labeling of unidentified polysaccharides during photosynthesis with $C^{14}O_2$. On the usual two-dimensional chromatogram, developed as described earlier, these compounds form what appears to be a homologous series of polyglucoses extending from the origin nearly to sucrose. The compound of this series closest to sucrose has been hydrolyzed and found to contain only glucose.

FATS

During photosynthesis by unicellular algae, it is not uncommon for as much as 30% of the carbon dioxide taken up to be incorporated into fats. In Scenedesmus, for example, after 5 minutes in light in the presence of C^{14}-labeled carbon dioxide, 30% of the fixed radioactivity is found in lipid materials. This incorporation of radiocarbon is greatly in excess of the rate of any synthesis that could take place in the dark and is an indication of the stimulation of fat production in the light. Fat synthesis requires a greater number of equivalents of reducing agents than does synthesis of carbohydrate or protein. Moreover, the composition of the chloroplasts includes a very high proportion of fat material. Since there is an abundance of reduced cofactors and ATP in the chloroplast, and since the end product, fat, is needed in the chloroplast, it is likely that much fat synthesis takes place in the chloroplast and is therefore to be considered photosynthetic.

Fatty Acids. All the well known biosynthetic pathways to fatty acids require as a starting material acetate or acetyl CoA. We have already suggested under 'Carboxylic Acids' four ways in which acetate, or acetyl CoA, could be made. These were: first, splitting of malic acid to glyoxylate and acetate; second, reduction of glycolic acid to acetate; third, oxidation of pyruvic acid to acetyl CoA; and, fourth, dehydration and phosphoroclastic splitting the postulated glycolyl-enzyme complex from transketolase reaction of the carbon reduction cycle to give acetyl phosphate. We favor the last way as being the most likely. However, if only the first three of these pathways is available, the third is probably the most important.

However the acetate is formed, it is rapidly converted to fats in the light in algae. Experiments with Scenedesmus photosynthesizing in the presence of acetate-1-C^{14} and $C^{12}O_2$ (14) demonstrated a light-accelerated incorporation of acetate into fats. A similar light-enhanced incorporation of acetate-2-C^{14} into lipids by Euglena was found by Lynch and Calvin (95). Sissakian (96) demonstrated the synthesis of higher fatty acids from labeled acetate in chloroplasts from sunflower plants. The utilization of free acetate in the light by chloroplasts is to be expected since there is an abundance of ATP in the photosynthesizing chloroplasts for the conversion of acetate to acetyl phosphate and thence to acetyl CoA.

The scheme of fatty acid synthesis proposed by Wakil and Ganguly (97) for the formation of fatty acids from acetyl CoA in animal tissues has been widely accepted. A similar pathway may exist in photosynthetic tissues. This pathway is incorporated in the hypothetical scheme of fat photosynthesis shown in Figure 9. Wakil (98) and Wakil and Ganguly (99) report that the first step in the synthesis from acetyl CoA is a carboxylation to give malonyl CoA. This step requires biotin and ATP, as well as Mn^{++}. Malonyl CoA and acetyl CoA

then condense to give acetoacetyl CoA which then undergoes a series of reductive steps to give eventually butyryl CoA and carbon dioxide (97).

While the work of Ganguly and Wakil has been with animal tissues, it appears from the studies of Stumpf and co-workers (100,101,102,103) that similar systems of fatty acid synthesis exist in plant tissues. The early stages of fat synthesis may well be similar in photosynthesizing chloroplasts to those known for other plant tissue and animals. The later stages and the fat products formed during photosynthesis in chloroplasts are very likely different, since the chloroplast in all likelihood requires specialized fats for its operation. Benson and co-workers have identified a number of interesting compounds of glycerol phosphate and fatty acids as products of fat formation in green tissues. According to these workers, phosphatidyl glycerol is a major component of plant phospholipids. Moreover, they state that active transphosphatidyl action is observed during photosynthesis (104,105,106).

Glycerol Phosphate. Alpha-D-glyceryl-1-phosphate is presumably formed in chloroplasts during photosynthesis by direct reduction with TPNH of dihydroxyacetone phosphate. This compound could then be further converted to the polyglycerol phosphates reported by Benson. The various glycerol phosphates would then presumably react with fatty acetyl CoA to produce fats. Some of these postulated biosynthetic routes are shown in Figure 9.

AROMATIC NUCLEI

The shikimic acid pathway for the biosynthesis of aromatic compounds, including amino acids, from carbohydrates has been well established by the work of Davis (38) and his collaborators, who used biochemical mutants of E. coli. Without going into the details of this pathway, we may point out that the starting materials are phosphoenolpyruvate, which is readily available as a photosynthetic intermediate, and D-erythrose-4-phosphate, which is also an intermediate of the carbon reduction cycle. Presumably, therefore, the synthesis of aromatic amino acids in photosynthesizing plants would follow a pathway similar to the shikimic acid pathway. The first step in that pathway is the condensation of phosphoenolpyruvate with erythrose-4-phosphate to give a seven-carbon compound which has been identified as 2-keto-3-deoxy-D-arabo-heptonic acid-7-phosphate. This intermediate subsequently undergoes ring closure to give dehydroquinic acid. Rearrangements via a number of additional steps gives eventually phenylalanine and tyrosine. Higuchi (107) has summarized some of the reasons for believing that the shikimic acid pathway does occur in higher plants. For example, shikimic acid is of widespread occurrence and some of the enzymes of the pathway have in fact been found in higher plants. Neish (108) has further reviewed evidence for the shikimic acid pathway in plants.

OTHER BIOSYNTHETIC PRODUCTS

As we learn more about the capabilities of the chloroplast to form compounds from carbon during photosynthesis, we come closer to the conclusion that the chloroplast, as it exists in the living, undisturbed cell, is a self-sufficient factory capable of producing essentially all of the materials required

for its replenishment. Thus it appears to be able to make all kinds of sugars, polysaccharides, protein, fats, pigments, enzymes and cofactors. In addition to this, it produces for export to the cytoplasm, reserves of organic compounds. These are probably sugars, glycolic acid, and other neutral, relatively small molecules, which can be readily transported through the chloroplast membrane. Until more is known about the development and formation of chloroplasts, we cannot say just when it gains this complete synthetic ability. No doubt there are early stages in the development of chloroplasts in which it must be built from cytoplasmic materials derived in turn from already functioning chloroplasts. There is no reason to suppose the chloroplast functions without nuclear control, even though it does not appear to have a nucleus of its own. Presumably it is possible for RNA molecules to move in and out of the chloroplast in some way. It cannot be said at the moment whether or not the chloroplast is capable of synthesizing nuclear material. It would seem likely, however, that the chloroplast can synthesize purines and pyrimidines, coenzymes, nucleotide materials which are needed for the continued functioning of the chloroplast as a self-sufficient biosynthetic factory. If, as we now think, protein synthesis and enzyme synthesis occurs in the chloroplast, then either the chloroplast must obtain a store of RNA molecules at its initial construction, or, else such molecules must be able to travel back and forth from the chloroplast to the cytoplasm.

In conclusion, we would say that the point of view of the ability of the chloroplast to carry out photosynthetic formation of many compounds is a departure from the view held only a few years ago. It was then thought that the primary function of photosynthesis was to form carbohydrate only. This carbohydrate was then thought to be used by the cytoplasm in the synthesis of

all other compounds. Of course, the chloroplast must supply the carbohydrate and reducing power for the cytoplasmic synthesis. It now appears that chloroplasts also synthesize a complete spectrum of biochemical products, all of which might reasonably be considered to be photosynthetic products. Finally, as we learn more about the photosynthetic paths to these products, we are impressed not merely by their complexity but much more by the economy with which both energy and material are utilized.

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Table 1

RATES OF FLOW OF CARBON THROUGH ACTIVE POOLS OF AMINO ACIDS

STEADY STATE EXPERIMENT 32

Compound	Calculated Rate of Synthesis R μmoles of Carbon	Equiv. NH_4^+ Uptake μmoles of NH_4^+
Alanine	2.67	0.89
Serine	0.49	0.16
Aspartic Acid	0.89	0.22
Glutamic Acid	0.98	0.20
Glutamine	0.32	0.13
Glycine*	0.04	0.02
Citrulline**	0.09	0.09
Threonine*	0.20	0.05
Total	5.44	1.69
Externally measured uptake:	17.0	2.6
% of total through these pools:	32	65

* not included in totals

** figures are for carbamyl carbon only

Table 2

PLANT TISSUE ENZYMES WHICH CATALYZE PHOTOSYNTHETIC REACTIONS OR SIMILAR REACTIONS

Enzyme	Number	Reaction	Plant Material	Investigator (1st Author)
Carboxydismutase 'Carboxylating Enzyme'	1	CO_2 + RuDP* + H_2O ---> 2 3-PGA	Chlorella Spinach leaves, etc.	Quayle (63), Mayaudon (64) Weissbach (65,66,67) Jakoby (68), Racker (69)
Phosphoglyceryl kinase	2	3-PGA + ATP ---> 1,3 DPGA	Pea seeds	Axelrod (6)
Triose phosphate dehydrogenase	3	1,3 DPGA + TPNH ---> G13P + TPN^+ + Pi	Sugar beet leaves Several algae and higher plants green tissues	Arnon(7,70), Rosenberg (71) Stumpf (9,10) Fuller (72)
Triose phosphate isomerase	4	G13P ------> DHAP	Pea seeds	Tewfic (73)
Aldolase	5	G13P + DHAP ---> FDP	Pea seeds	Stumpf (9), Tewfic (73)
	6	E4P + DHAP ---> S7P	Pea seeds	Hough (75)
Phosphatase	7	FDP + H_2O ---> F6P + Pi	Spinach	Racker (76,77)
	8	SDP + H_2O ---> S7P + Pi	Spinach	Racker (76,77)
Transketolase	9	F6P + G13P ---> E4P + Xu5P	Spinach	Horecker (45)
	10	S7P + G13P ---> R5P + Xu5P	Spinach	Horecker (45)
Ribulose phosphate-xylulose phosphate isomerase	11	Ru5P ---> Xu5P	Yeast	Srere (78)
Phosphoribulokinase	12	Ru5P + ATP ---> RuDP + ADP	Spinach	Hurwitz (79), Weissbach (80)

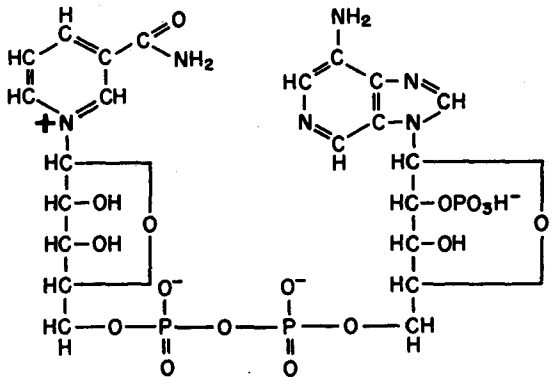
* Abbreviations: DHAP = dihydroxyacetone phosphate, E4P = erythrose-4-phosphate, FDP = fructose-1,6-diphosphate, F6P = fructose-6-phosphate, G13P = glyceraldehyde-3-phosphate, Pi = inorganic phosphate, 1,3DPGA = phosphoglyceryl-3-phosphate, R5P = ribose-5-phosphate, RuDP = ribulose-1,5-diphosphate, Ru5P = ribose-5-phosphate, SDP = sedoheptulose-1,7-diphosphate, S7P = sedoheptulose-7-phosphate, Xu5P = xylulose-5-phosphate.

Table 3

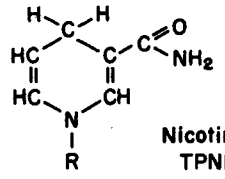
SOME PLANT TISSUE ENZYMES WHICH MAY CATALYZE REACTIONS FOR PHOTOSYNTHETIC FORMATION OF CARBOHYDRATES (BEYOND THE CARBON REDUCTION CYCLE)

Enzyme	Number	Reaction	Plant Material	Investigator (1st Author)
Phosphohexose isomerase	13	F6P ⟶ G6P*	Phaseolus radiatus	Ramasarma (81)
Phosphoglucomutase	14	G6P + Pi-enzyme ⟶ GDP + enzyme GDP ⟶ G1P + Pi-enzyme	Broadbean seeds	Morita (82) Sidbury (83)
UDPG-pyrophosphorylase	15	G1P + UTP ⟶ UDPG + PP	Mung bean seedlings	Ginsberg (84)
UDPG-fructose-6-phosphate transglycosylase	16	UDPG + F6P ⟶ sucrose + UDP	Wheat germ	Leloir (85)
Sucrose phosphatase	17	SuP + H_2O ⟶ sucrose + Pi		
UDPG-4-epimerase (galactowaldenase)	18	UDPG ⟶ UDPGal	Mung bean seedlings	Neufeld (86)
		UTP + sugar-1-phosphate ⟶ UDPSugar + PP	Mung bean seedlings	Neufeld (86) Kalckar (87)

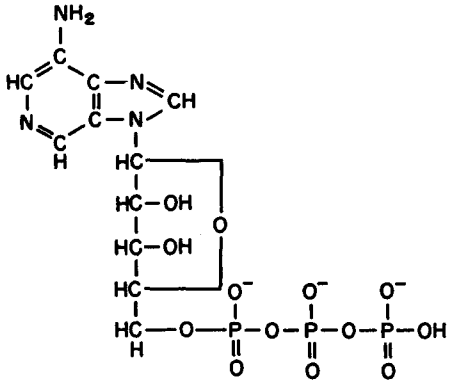
* Abbreviations (see also Table 2): GDP = glucose-1,6-diphosphate, G6P = glucose-6-phosphate, PP = pyrophosphate, SuP = sucrose phosphate, UDPGal = uridine diphosphogalactose, UDPG = uridine diphosphoglucose, UTP = uridine triphosphate.



Triphosphopyridine nucleotide (oxidized form) (TPN^+)



Nicotinamide portion of TPNH (reduced TPN^+)



Adenosine triphosphate (ATP)

In Adenosine diphosphate (ADP), terminal phosphate is replaced by –OH.

MU–17251

Fig. 1. Formulae of TPN and ATP.

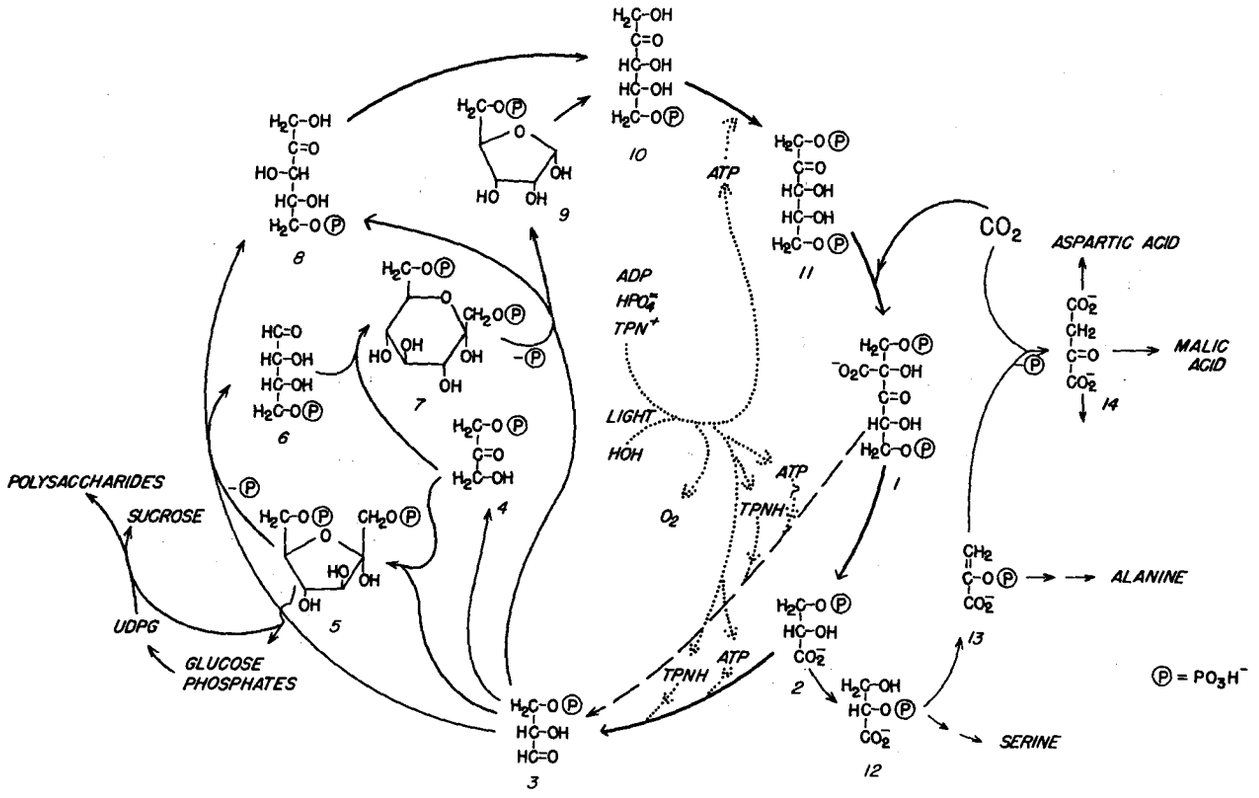
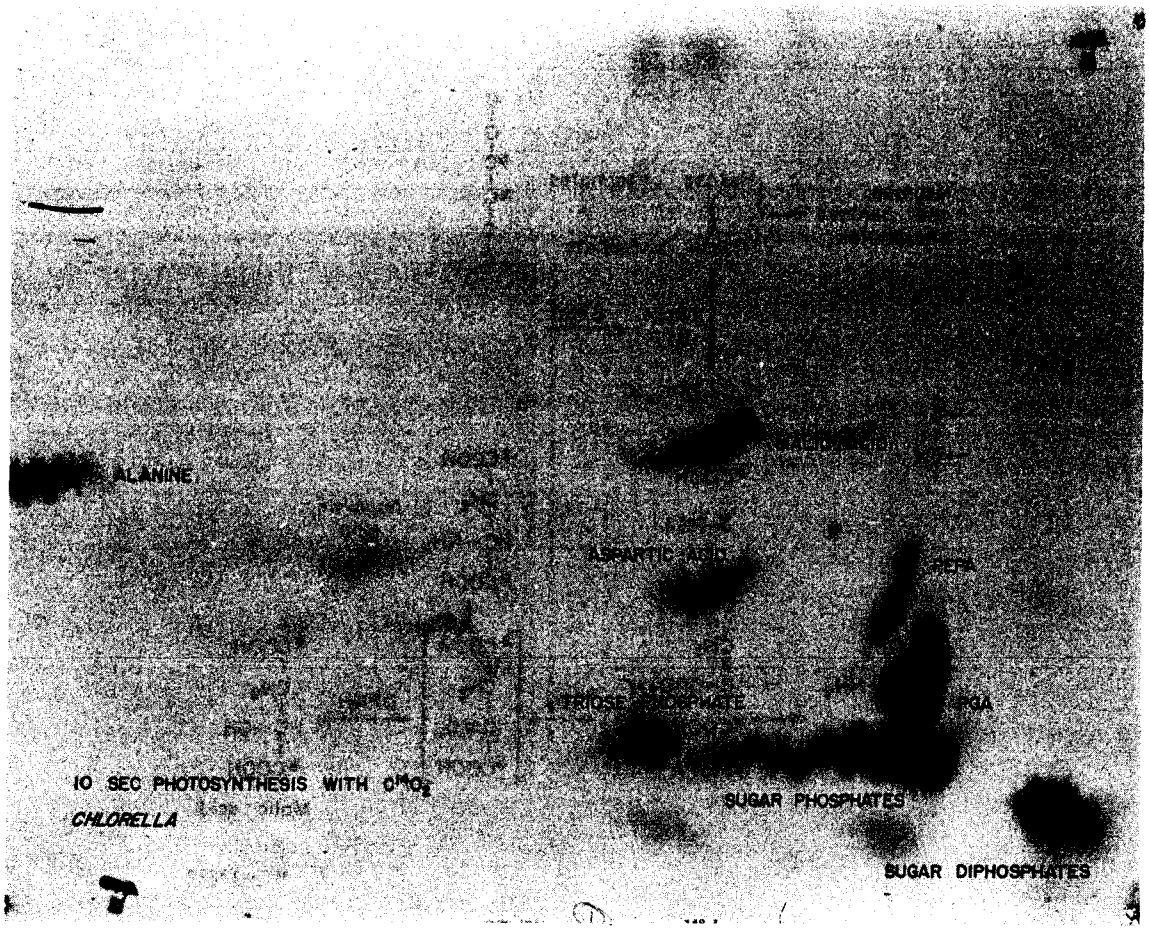


Fig. 2. Carbon reduction pathways in photosynthesis. Compounds: (1) 2-carboxy-3-keto-1, 5-diphosphoribitol, (2) 3-phosphoglyceric acid (3-PGA), (3) glyceraldehyde-3-phosphate, (4) dihydroxyacetone phosphate, (5) fructose-1, 6-diphosphate, (6) erythrose-4-phosphate, (7) sedoheptulose-1, 7-diphosphate, (8) xylulose-5-phosphate, (9) ribose-5-phosphate, (10) ribulose-5-phosphate, (11) ribulose-1, 5-diphosphate, (12) 2-phosphoglyceric acid (2-PGA), (13) phosphoenolpyruvic acid (PEPA), (14) oxalacetic acid.

$\leftarrow$ Ⓟ fructose diphosphate and sedoheptulose diphosphate lose one phosphate group before transketolase reaction occurs.



ZN-2689

Fig. 3. Radioautograph of two-dimensional paper chromatogram. Alcoholic extract of Chlorella pyrenoidosa after 10 seconds photosynthesis with $C^{14}O_2$.

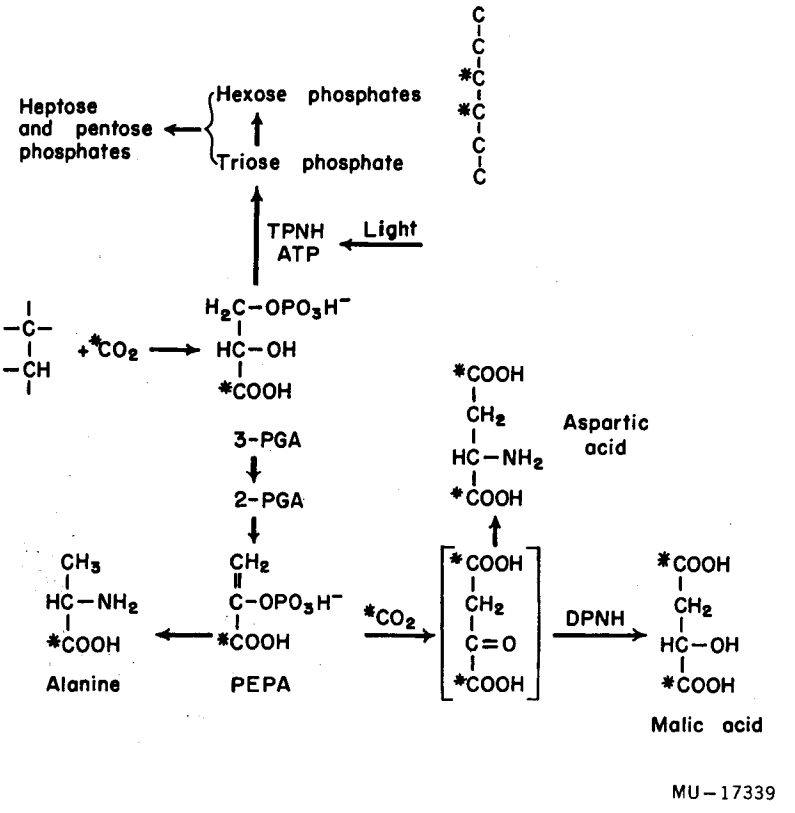
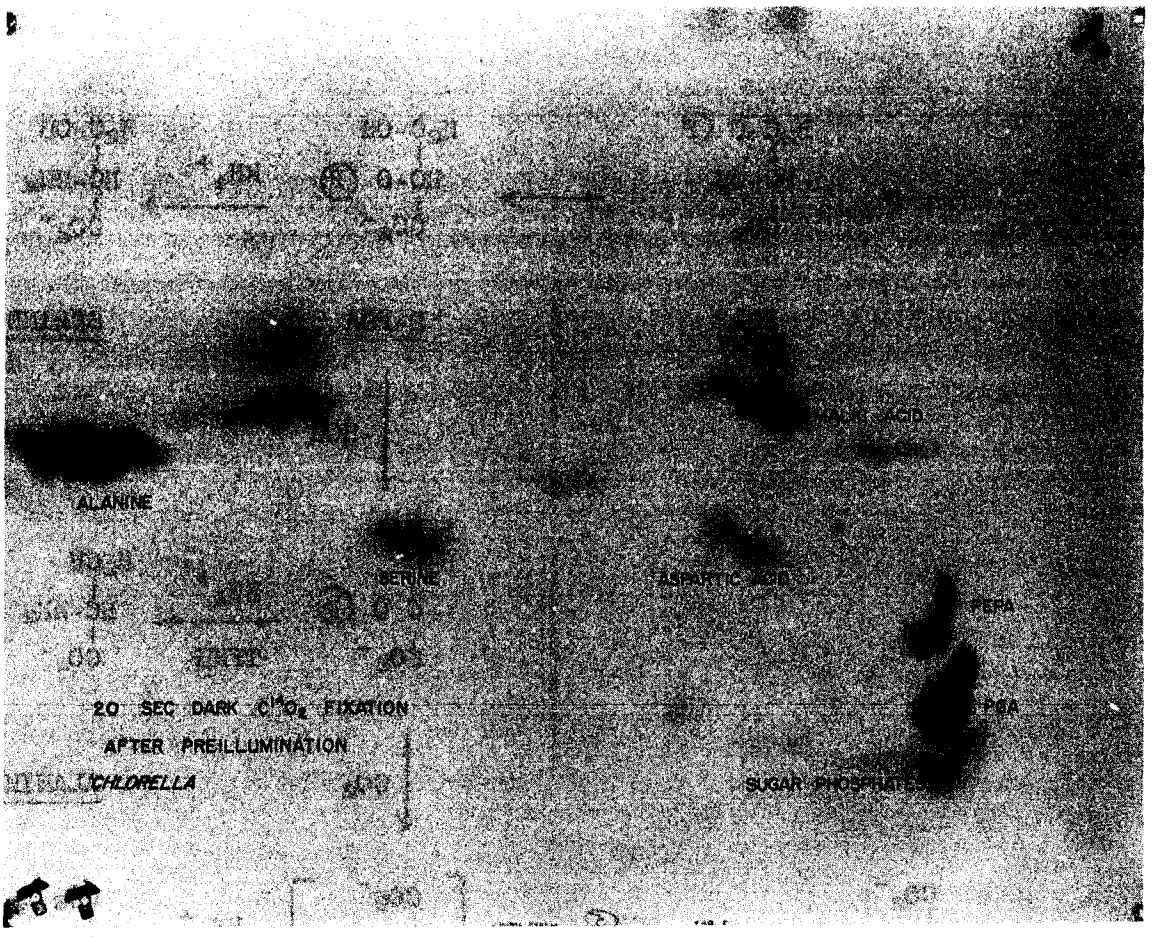


Fig. 4. Labeling of compounds with C^{14} during early steps in carbon dioxide reduction during photosynthesis with $C^{14}O_2$.



ZN-2688

Fig. 5. Radioautograph of chromatogram of products of 20 seconds $C^{14}O_2$ fixation by Chlorella in the dark following a period of photosynthesis.

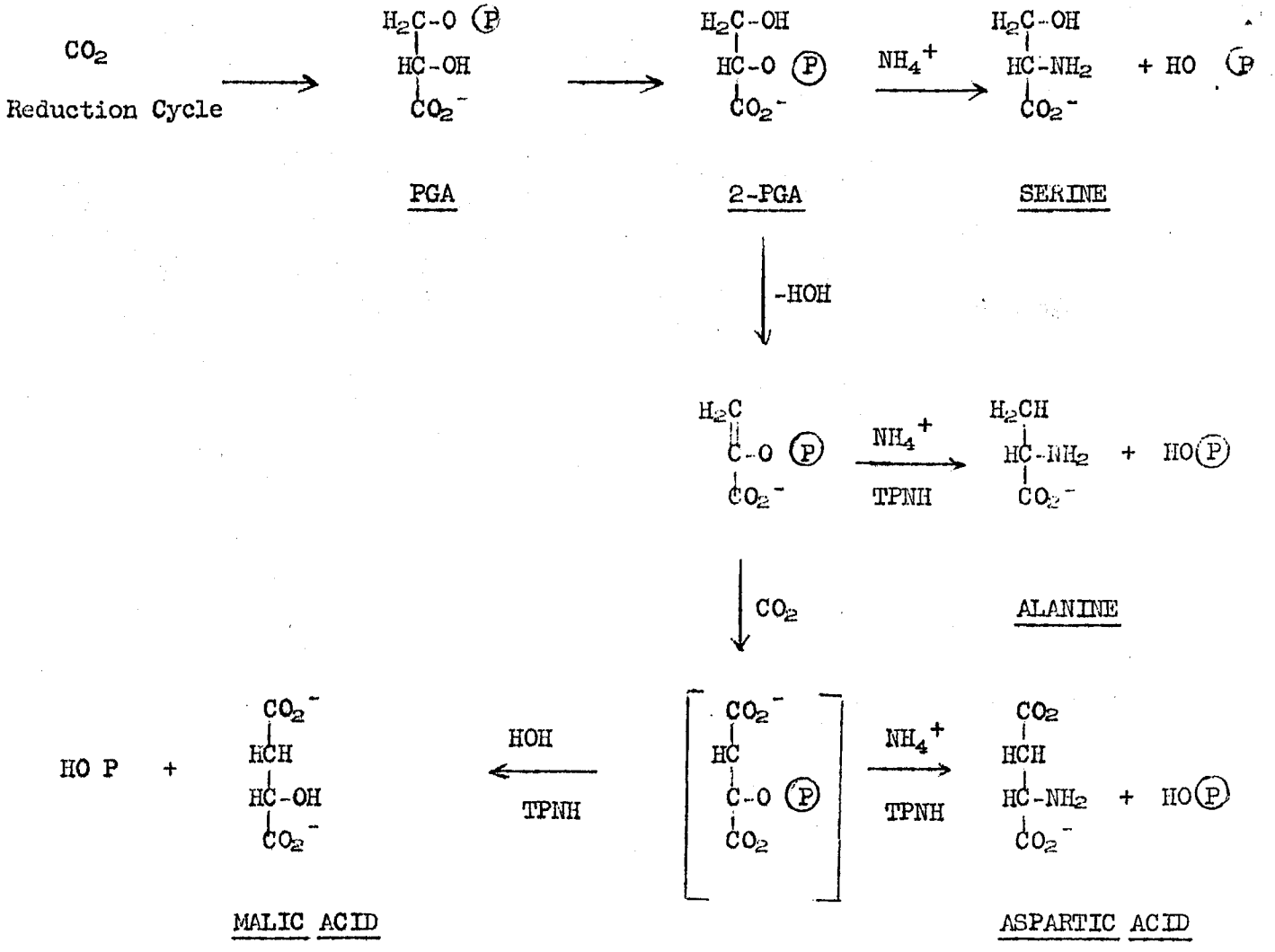


Fig. 6. Hypothetical pathways of photosynthesis of alanine, aspartic acid, serine and malic acid.

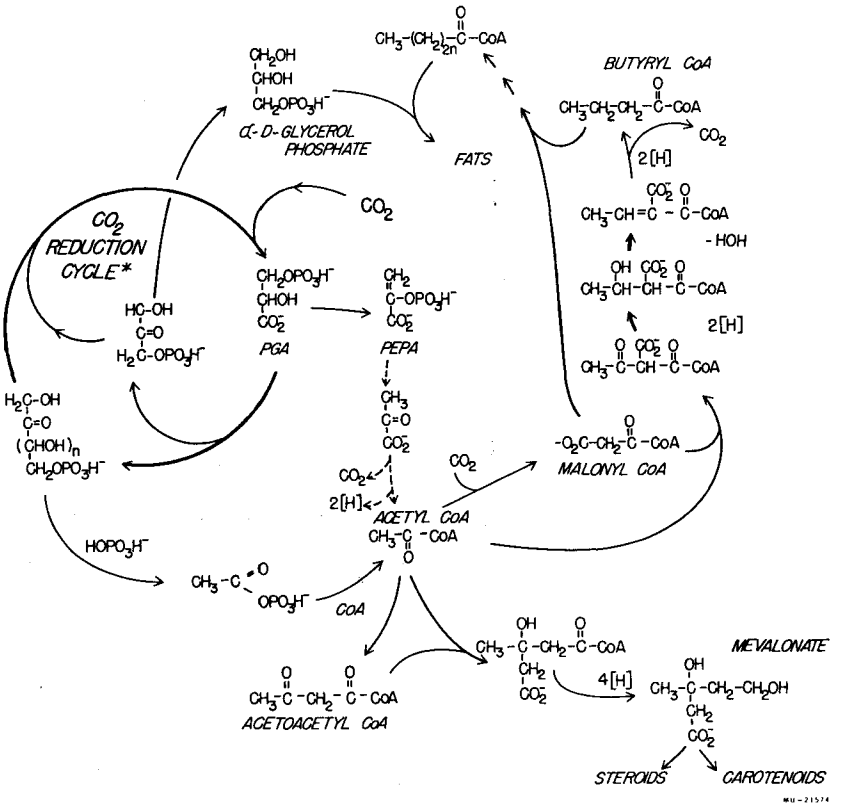


Fig. 7. Pathways from carbon reduction cycle to acetyl phosphate and glycolic acid. For details of the carbon reduction cycle, see Fig. 2.

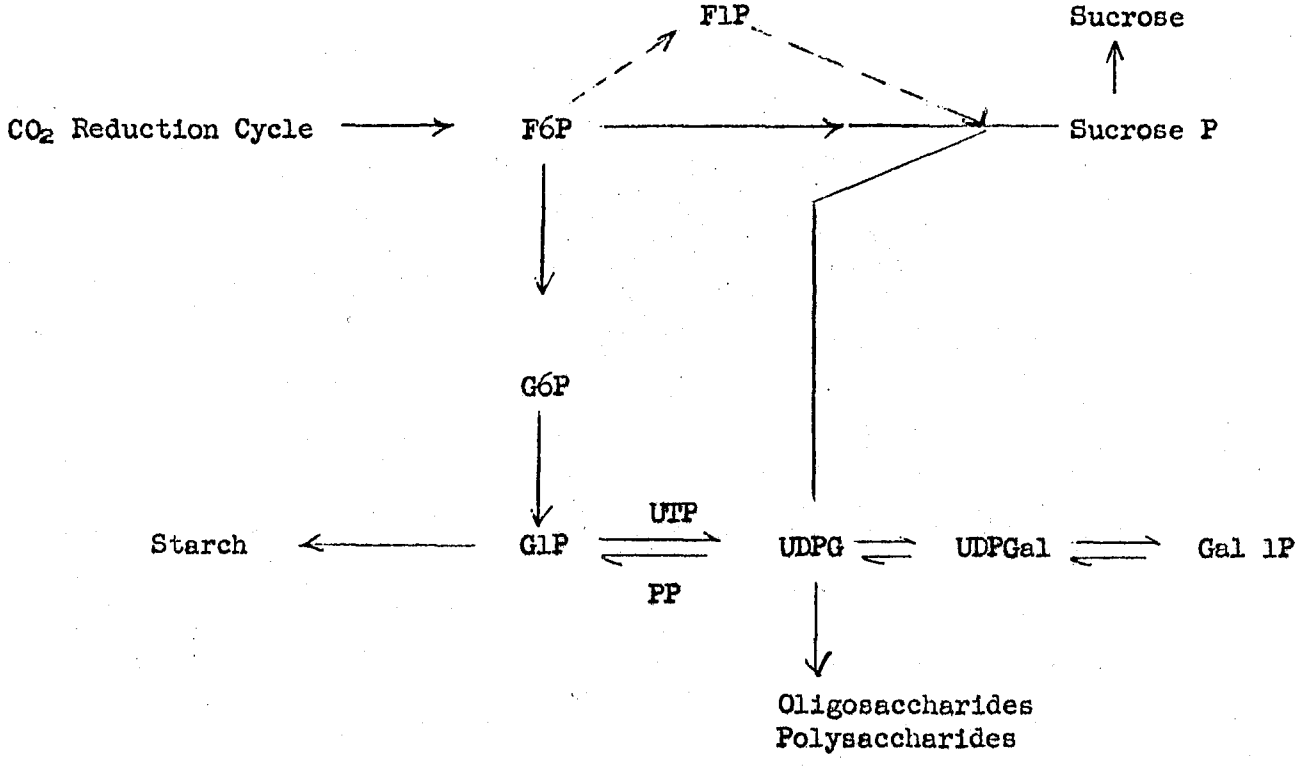


Fig. 8. Biosynthetic pathways for photosynthesis of carbohydrates.

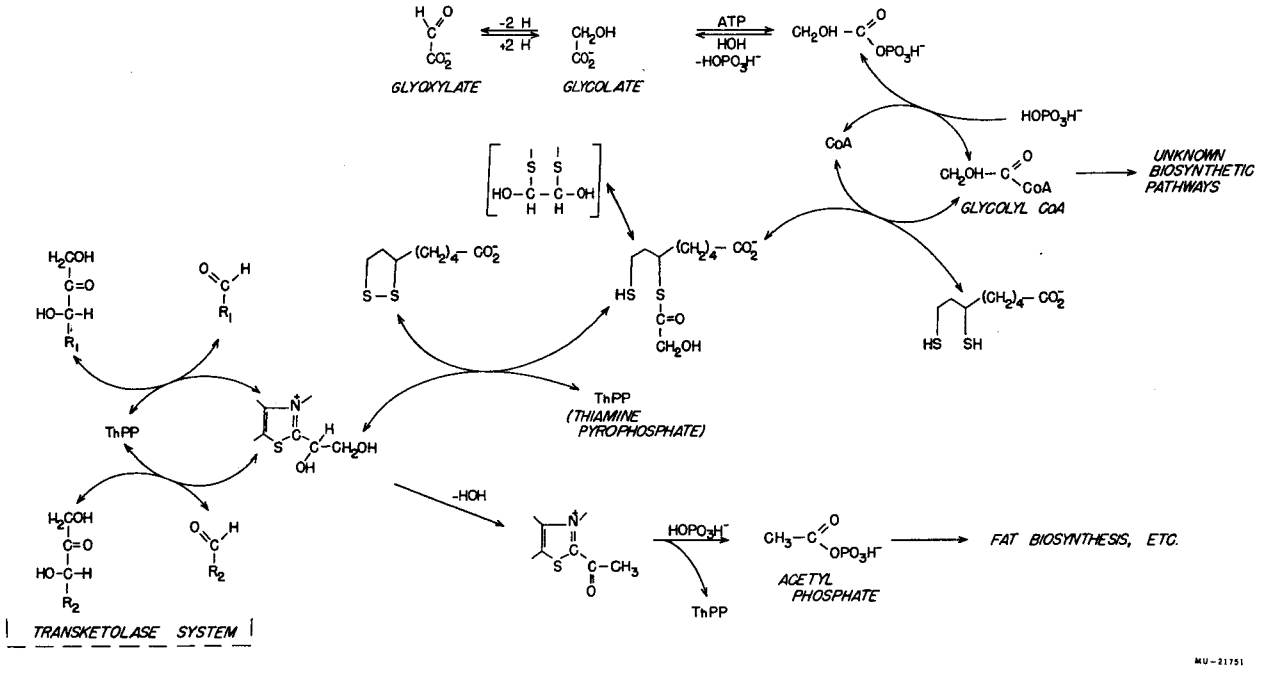


Fig. 9. Some possible pathways of fat photosynthesis from CO_2. For details of the carbon reduction cycle, see Fig. 2.