The Control of Photosynthetic Carbon Metabolism

Photosynthesis of carbon compounds is regulated to allocate intermediates according to metabolic need.

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Interest in the control of photosynthetic metabolism was stimulated by the mapping of the basic path of carbon reduction in photosynthesis by Calvin and his co-workers 15 years ago (*1*). Before that time it was possible to suppose that photosynthesis produced some single product such as glucose. This substance then could have been used by plant cells in much the same way as an organic substrate is used by nonphotosynthetic cells.

The pathway of carbon dioxide fixation and reduction to sugar phosphates proved to be a cyclic series of reactions (sometimes referred to as the "Calvin cycle" or "reductive pentose phosphate cycle") (Fig. 1). Many of the intermediate compounds are chemically identical to metabolites of the pathway used by nonphotosynthetic cells to break down sugars (glycolysis). This commonality of compounds made it possible that some intermediates in photosynthetic metabolism might flow directly into other metabolic pathways. For example, some molecules of 3-phosphoglyceric acid (PGA) (Fig. 2) might be taken from the photosynthetic cycle and used directly in the synthesis of an amino acid, such as alanine. Quantitative kinetic studies with radioactive carbon showed that this is the case (*2*). Thus, all the carbon taken up by the leaf during photosynthesis is not converted to free sugars before it is used for synthesis of other plant constituents.

A second aspect of control in green cells is the loci of biosynthetic pathways, and the mechanism whereby carbon flows between these loci. Reduction of carbon dioxide to sugar phosphates and carbohydrates occurs within the green subcellular organelle, the chloroplast. For a time it seemed that the chloroplast might be metabolically isolated from the rest of the cell (*3*). Then it was found that a highly selective process allowed the export of specific intermediates from the chloroplasts (*4, 5*).

The regulation of the Calvin cycle and movement of intermediate compounds out of the chloroplast are the principal subjects of this article. Among other important areas of photosynthetic metabolism are "tropical grass" or "C-4" metabolism (*6*) and photorespiration (*7*).

Cell Life Cycle and Regulation

During the life of a green cell in a leaf, the metabolite needs of the cell change drastically. A "young" cell channels a large proportion of the fixed carbon into new materials of which the cell is constructed. In this way the cell can grow and divide. The materials needed include amino acids and proteins to function as enzymes, fatty acids and fats to form membranes, cellulose for cell walls, pigments such as chlorophyll, with which the plant captures light for photosynthesis, and so on. The function of cells within a mature leaf is more specialized. Growth may cease, and the entire output of the leaf may be in the form of compounds such as sucrose which are translocated to other organs of the plant. The signal to the cells for such changes may come in the form of plant hormones or other as yet unknown mechanisms which in some way alter the rates of enzyme synthesis. In turn, the rates of biosynthesis along certain pathways are changed.

An increased flow of carbon from the photosynthetic cycle to the beginning of any biosynthetic pathway requires that some mechanism operates to divert more carbon from the basic carbon reduction cycle of photosynthesis. This is usually accomplished by means of some type of metabolic regulation of the activities of enzymes at or near the point at which carbon is taken from the cycle. In fact, such control may be exerted on both an enzyme in the cycle and an enzyme just a step or so from the cycle along the biosynthetic path.

Many factors may alter the activity of a regulated enzyme and hence change the rate at which it catalyzes the conversion of one metabolite to another (*8*). In feedback control, some compound further along the metabolic pathway binds to the enzyme and changes its shape or other properties so as to alter its activity as a catalyst. If this controlling compound binds at the same place on the enzyme as the substrate (the "active" site), it may interfere with the conversion of the substrate and thereby act as a competitive inhibitor. In other cases, the controlling compound binds at another site in such a way that it alters the shape or physical-chemical properties of the enzyme, causing a change in its activity as a catalyst (*9*). This effect, called allosteric, may either stimulate or inhibit the catalytic activity of the enzyme.

A more general kind of control over enzyme activity can be exerted by the ratio of adenosine triphosphate (ATP) to total adenylates (adenosine tri-, di-, and monophosphate) of the cell. This ratio, designated "energy charge" by Atkinson (*8*) is a measure of available biochemical energy. It regulates key enzymes of glycolysis and of some other biosynthetic pathways. A generalized type of regulation in green plant cells may be mediated in part by variable *p*H and concentrations of other inorganic ions in chloroplasts. Such variations, affecting activities of several enzymes, could play a role in allowing the green plant cell to make the transition from light to dark metabolism when the day ends or when the plant is placed in deep shade.

Photoelectron Transport, Photophosphorylation, and Ion Flux

Photosynthesis is a complex of linked reactions in which the energy of the absorbed light is used to drive ener-

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getically unfavorable oxidation-reduction reactions (*10*). Water is oxidized to molecular oxygen, whereas carbon dioxide, nitrate, and sulfate are reduced to organic compounds. After the absorption of light by chlorophyll and other plant pigments, the energy of the excited pigment molecules is converted to chemical energy by transfers of electrons in such a way as to produce relatively strong oxidants and reductants.

The strongest oxidant becomes reduced again by oxidizing water, thereby liberating oxygen and protons. The strongest reductant is reoxidized by giving up its electrons to ferredoxin, a low-molecular-weight protein that contains iron atoms. Reduced ferredoxin is used either directly or via other electron carriers in enzyme-catalyzed reactions to bring about the reduction of carbon dioxide, nitrate, and sulfate. Each of these inorganic oxides also can be reduced nonphotosynthetically in other parts of the plants, but only at the expense of the release of chemical energy through respiration.

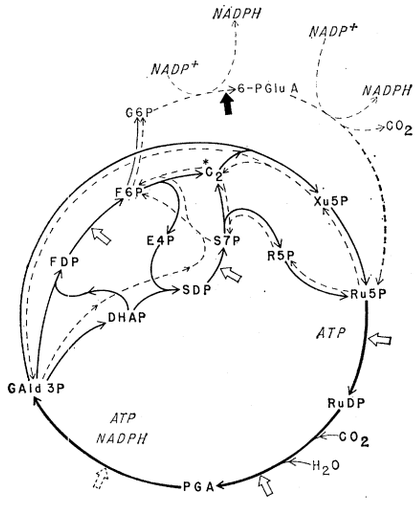

Fig. 1. Photosynthetic metabolism. The reductive pentose phosphate cycle (Calvin cycle). Photosynthetic metabolism in the light is indicated by solid arrows, the oxidative pentose phosphate cycle by dashed lines. White arrows are at sites of metabolic regulation which are active in the light. Dark arrows show reactions which are activated in the dark. The dotted, white arrow indicates a reaction for which evidence of regulation is so far limited to studies of the properties of the isolated enzymes. The $*C_2$ indicated in the cycle is actually enzyme-bound thiamine pyrophosphate–glycolaldehyde, an intermediate in the two reactions mediated by transketolase. PGA, 3-phosphoglycerate; GAld3P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; FDP, fructose-1,6-diphosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; E4P, erythrose-4-phosphate; SDP, sedoheptulose-1,7-diphosphate; S7P, sedoheptulose-7-phosphate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; Xu5P, xylulose-5-phosphate; RuDP, ribulose-1,5-diphosphate; NADPH, $NADP^+$, nicotinamide adenine dinucleotide phosphate, reduced and oxidized forms, respectively; 6PGluA, 6-phosphogluconate; ATP, adenosine triphosphate.

In 1964, Neumann and Jagendorf (*13*) found that isolated, illuminated spinach chloroplasts take up hydrogen ions, and it seems now well established (*14*) that photoelectron transport in chloroplast membranes leads to formation of a *p*H gradient across the membranes with the low *p*H inside the thylakoids. Hind and Jagendorf (*15*) found that isolated chloroplasts phosphorylate ADP in the dark following a sudden imposed rise in the *p*H of the suspending medium. Whether or not transmembrane *p*H gradients and membrane charge are required parts of the coupling mechanism for linking phosphorylation of ATP to electron transport (*16*) is still unresolved (*17*). The mechanism of this energy coupling is not yet clear. In any case, it seems possible that light-induced ion flux through the membranes may be of great importance in regulation of carbon metabolism, since *p*H and the concentrations of other ions, notably magnesium, have large effects on the activities of key enzymes, as discussed later.

Some of the changes in metabolism in the unicellular green alga *Chlorella* seen during the light-to-dark transitions (*18*) seem to be similar to reversible changes seen in light on addition and removal of octanoic acid and certain other fatty acids (*19*). These acids also cause a large and partly reversible change in the difference between 515-nanometer light absorbed by illuminated and dark *Chlorella* cell suspensions (*20*). A change in absorption at 515 nanometers produced in spinach chloroplasts within 20 nanoseconds after the beginning of a light flash has been interpreted (*21*) as an indication of a primary light-produced charge across the membranes. The dark decay of this charge (after a very short light flash) is concurrent with the flux of hydrogen and other ions through the membranes observed with indicators (*22*), and it has been proposed that ion flux is driven by the postulated electrical field across the membrane. Regardless of whether or not all aspects of these proposals prove to be correct, there may be a relation between the action of the fatty acids on the properties of thylakoid mem-

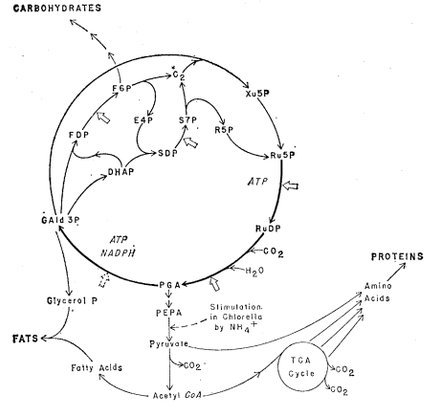

Fig. 2. Suggested metabolic paths from the Calvin cycle to end products. A great many steps, intermediate compounds, and details are omitted from this diagram, which is intended only to give a general view of the connections between the Calvin cycle and biosynthesis of some end products in green cells, and to show the relation of pathways to regulated steps of the cycle and the reaction converting phosphoenolpyruvic acid to pyruvate, which was found to be stimulated, in *Chlorella* by addition of ammonium ion. The line from pyruvate to amino acids denotes the formation of alanine by transamination. Alanine synthesis is rapidly stimulated by addition of ammonium ion. PEPA, phosphoenolpyruvate; CoA, coenzyme A; TCA cycle, tricarboxylic acid cycle (Krebs cycle).

branes (increased change in absorption at 515 nanometers) and ion flux through the membranes.

The enzymes involved in carbon metabolism are commonly thought to be located in the stroma region (although not inside the stroma thylakoids). In considering general regulatory mechanisms for those enzymes, it would be helpful to know what changes in *p*H and concentration of other ions, such as magnesium, occur in the stroma region, but, unfortunately, little is known. It may be inferred that the light-pumping of hydrogen ions into the thylakoids, which is thought to establish a lower steady-state *p*H inside the thylakoids, would also cause the steady-state *p*H in the stroma to be higher in the light than in the dark (*23*). The extent of such a difference would depend on relative volumes of the two spaces, buffering capacity, and other factors. Only a small change, such as half a *p*H unit, would be required to effect considerable change in the activities of some enzymes discussed later.

Reported light-induced efflux of magnesium and potassium ions from broken chloroplasts (*24*) led to a suggestion (*23*) that concentration of magnesium ions in the stroma region might increase in vivo, thus activating enzymes. However, Nobel (*25*) found a light-induced efflux of potassium, magnesium, and other ions from isolated, intact pea chloroplasts which was quantitatively similar to an observed light-induced shrinkage of the "osmotically responding" volume, so that under certain assumed conditions, the internal ion concentrations seemed to remain roughly constant between light and dark. The high internal concentration of magnesium ion, 16 millimoles per liter, reported by Nobel (*25*), would be very important in activation of several of the suspected regulated enzymes below a *p*H of 8.

The initial stages in the reduction of carbon dioxide, nitrate, and sulfate each require reduced nicotinamide adenine dinucleotide phosphate (NADPH) (*26*), a two-electron carrier. Thus, electrons must be transferred from the one-electron carrier, ferredoxin, to $NADP^+$. This is catalyzed by a soluble enzyme, ferredoxin-NADP reductase (*12, 27*). After two electrons have been transferred to nitrate and sulfate, reducing them to nitrite and sulfite, respectively, the further, six-electron reductions to ammonium and sulfhydryl use reduced ferredoxin and particulate systems (*26*).

In the reduction of carbon dioxide by the reductive pentose phosphate cycle, the product of the carboxylation reaction, PGA, is first activated by phosphorylation with ATP and then the resulting acyl phosphate is reduced to triose phosphate by NADPH. The activity of the enzyme involved in transfer of electrons from ferredoxin to $NADP^+$ is affected by the concentration of inorganic pyrophosphate (PP_i) (*28*). Since PP_i concentration in vivo changes with physiological state (*18, 29*) it may be that PP_i concentration in vivo exerts some regulatory control on the rates of utilization of electrons from ferredoxin for reduction reactions. The activity of isolated pyrophosphatase, the enzyme catalyzing the hydrolysis of PP_i, is affected by *p*H and magnesium ion concentration (*30*).

Study of Regulation in vivo

Whereas many enzymes show changes in activity due to the presence of metabolites, changes in *p*H or in ion concentrations, and so forth (*31*), the significance of these effects for regulation in vivo can only be proven by studies with whole cells. The most direct, and therefore the most satisfactory, such evidence is the measurement of changes in metabolite concentrations that occur during the course of such regulation.

Determination of metabolite concentrations and their changes in vivo by isotopic techniques has proved valuable. By observing the responses of metabolite concentrations to external and internal stimuli in vivo we are able to obtain a much clearer picture of the actual control system. Photosynthesis is particularly suited to such a study. Its substrate, carbon dioxide, is a gas which can be easily maintained at constant concentration and specific radioactivity during the course of an experiment. This greatly facilitates subsequent kinetic analysis of the observed changes in labeled metabolites.

The green plants are allowed to take up radioactive carbon dioxide, $^{14}CO_2$, for a long enough time under steady-state conditions to label completely with ^{14}C the intermediate compounds of the primary carbon reduction cycle (*32*). The specific radioactivity of each carbon position in each metabolic pool then is at the same specific radioactivity as the entering $^{14}CO_2$. The ^{14}C content of the pool of the compound in question is subsequently measured. Division of this total amount of radiocarbon by the specific radioactivity and by the number of carbon atoms per molecule gives the size of the pool of the compound in the plants. This concentration may be expressed in terms of plant volume, amount of chlorophyll, or other suitable standard.

After the metabolites are completely labeled, the environment of the plant can be changed. For example, the illumination might be stopped. A series of additional samples are taken with time. When these samples are later analyzed by standard techniques of paper chromatography and radioautography (*18, 19*), the changes in metabolite concentrations and the kinetics of these changes can be determined. It was partly through the application of this technique that the cyclic path of carbon reduction (*1*) and the flow of carbon into secondary biosynthetic paths were originally mapped (*2*).

Additional information is gained by the simultaneous application of $^{14}CO_2$ and phosphate labeled with phosphorus-32 to the photosynthesizing cells (*18, 19*). Both labeled compounds are incorporated by photosynthesis, but only labeled phosphate is incorporated by respiration. Respiration uses endogenous stores of sugars that are mostly unlabeled with carbon-14. The investigator thus can examine relations between photosynthesis and respiration by means of kinetic light-dark experiments (*18*) in the presence of $^{14}CO_2$ and phosphorus-32–labeled monohydrogen phosphate.

Photosynthetic Carbon Reduction, an Oxidative Cycle, and Biosynthesis

Photosynthetic reduction of carbon dioxide in green plants (other than C-4 plants) (*6*) is accomplished by a single metabolic cycle (*1*). The incorporation of $^{14}CO_2$ by means of the carbon reduction cycle of most plants is by carboxylation of ribulose-1,5-diphosphate (RuDP) (Fig. 1) (*1*). The recently incorporated carbon becomes the carboxyl carbon of one of two molecules of PGA.

Some of the molecules of PGA can be used as the starting material for biosynthetic paths leading to amino acids, fatty acids, and so forth. Maintenance of the carbon reduction cycle

requires that most of the PGA be reduced to sugar phosphates, from which the carboxylation substrate, RuDP, is regenerated.

Glucose-6-phosphate (G6P) is rapidly formed from fructose-6-phosphate (F6P), in either light or dark. In the light, these sugar phosphates are converted to carbohydrates (Fig. 3). In the dark, G6P undergoes oxidation to 6-phosphogluconic acid. Further oxidation leads to ribulose-5-phosphate (Ru5P) and carbon dioxide. Three Ru5P molecules are then converted (dashed lines, Fig. 1) to two molecules of F6P, which are recycled, and a net single molecule of glyceraldehyde-3-phosphate.

Most metabolites needed for synthesis of noncarbohydrate end products come from PGA and dihydroxyacetone phosphate (DHAP) (Fig. 2). Both compounds occur in the carbon reduction cycle after the carboxylation reaction and before the diphosphatase reactions.

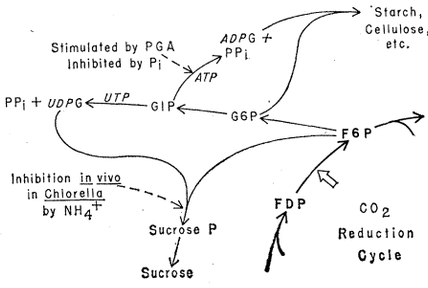

Fig. 3. Metabolic paths from the Calvin cycle to carbohydrates. UDPG, uridine diphosphoglucose; ADPG, adenosine diphosphoglucose; UTP, uridine triphosphate.

Light-Dark-Light Regulation

It is the nature of studies of in vivo metabolism that single experiments are seldom conclusive. One must piece together many indications from the kinetic data to form some kind of consistent hypothesis. Only a few examples of these data can be presented within the scope of this article. Further evidence may be found in the original publications (*4, 18, 19, 23, 30, 33–38*).

The metabolic transients accompanying the light-dark and dark-light transactions in the unicellular alga *Chlorella pyrenoidosa* (*18*) are informative. Of particular interest is the labeling of PGA with phosphorus-32 and carbon-14 (Fig. 4). The light was turned off only after enough photosynthesis with the radioisotopes had occurred to completely label PGA and sugar phosphates (but not free sugars and starch). With darkness, the production of NADPH and ATP in the chloroplasts ceased. The reduction of PGA abruptly stopped. Meanwhile, the carboxylation of RuDP, producing PGA, continued for some seconds, since the supply of RuDP was not immediately exhausted and no cofactors from the light reactions are required. Thus, the amount of PGA labeling with both phosphorus-32 and carbon-14 rose rapidly.

After about 30 seconds, the carboxylation reaction ceased, and the conversion of PGA to products such as amino acids and fatty acids outside the cycle caused the concentration of PGA to fall. After this transition period, the process of glycolysis commenced. Sugars stored in the chloroplasts had not been significantly labeled during the few minutes of photosynthesis with $^{14}CO_2$. These sugars were phosphorylated with ATP to give sugar phosphates, which were split to triose phosphates and then oxidized to form PGA. This process of glycolysis is essentially the reverse of the reduction of PGA to sugars in photosynthesis. Formation of PGA from the only slightly labeled sugars results in dilution of the amount of radiocarbon in the PGA, which consequently declined as PGA was converted to other products.

The ATP used for the phosphorylation of the sugars is labeled with phosphorus-32 whether it is made by photosynthetic phosphorylation in the chloroplasts in the light or by oxidative phosphorylation in the mitochondria in the dark. The phosphorus-32 in the ATP ends up in the phosphate group of PGA in either light or dark. Consequently, there is no dilution of the phosphate label of PGA. The amount of phosphorus-32 in PGA remains a true measure of the PGA concentration in both light and dark. Thus, we can see that after the transitory period the amount of PGA remains as high in the dark as it was in the light.

If the photosynthetic and glycolytic pools of PGA were isolated from each other, only the photosynthetic pool should be affected by the light coming on again, at least in the first seconds. The light would cause a sudden reduction of PGA before the RuDP con-

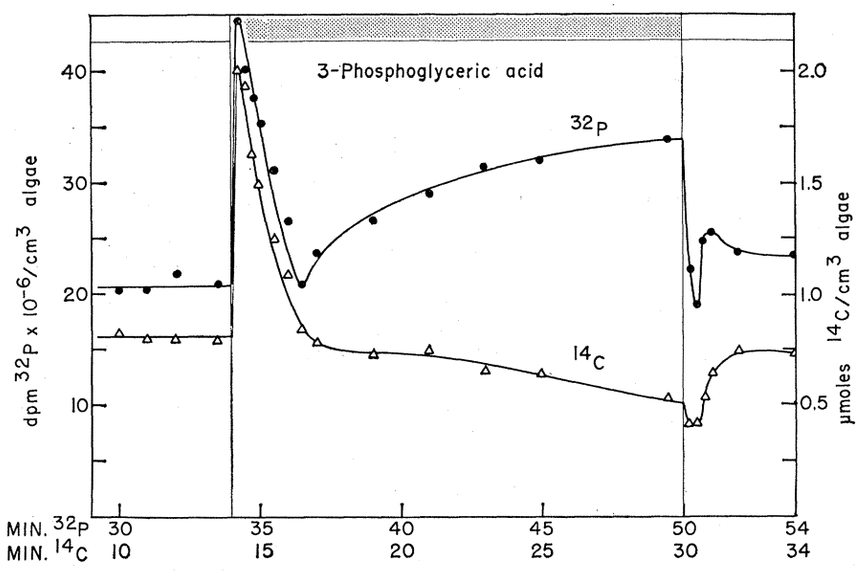

Fig. 4. The effects of light and dark on the amounts of ^{14}C and ^{32}P labeling of 3-phosphoglycerate in *Chlorella pyrenoidosa*. After 30 minutes of photosynthesis under steady-state conditions with unlabeled CO_2, ^{32}P-labeled phosphate was added to the algae, and 20 minutes later, $^{14}CO_2$ was added. These additions were made in such a way that CO_2, phosphate concentration, and specific radioactivities were kept constant during the course of the experiment. At the times indicated, and during the light, dark, and again in the light, samples were taken and cells were killed and analyzed by two-dimensional paper chromatography and radioautography. The amount of radiocarbon and phosphorus-32 in samples taken in the light indicated steady-state concentrations of PGA in the light. In the dark, amount of phosphorus-32 indicates the size of the pool of PGA, whereas the amount of radiocarbon roughly indicates how much of the PGA formed in the light still remains. The large drop in amount of phosphorus-32 when the light is turned on shows that the entire pool of PGA was affected by the light.

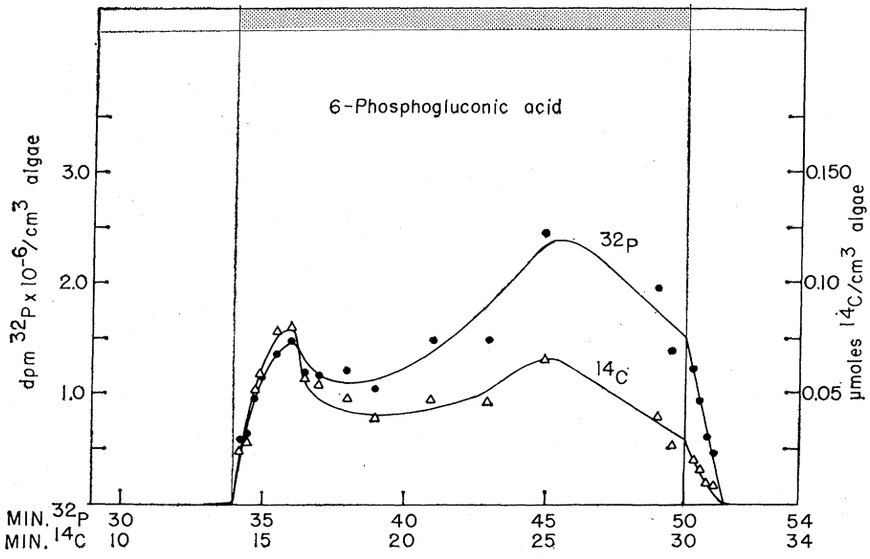

Fig. 5. The effect of light and dark on amount of labeled 6-phosphogluconate. Conditions as given in Fig. 2. 6-Phosphogluconate, a marker for the operation of the oxidative pentose phosphate cycle (hexose phosphate shunt) in the chloroplasts, appears as soon as the light is turned off and disappears shortly after the light is turned on again. It is proposed that the oxidative cycle operates in the chloroplasts in the dark to provide a supply of NADPH for biosynthesis.

centration had risen enough to support carboxylation. Such an isolated photosynthetic pool of PGA should still have the same ratio of phosphorus-32 to carbon-14 as it had during the previous light period. Therefore, the drop in amount of phosphorus-32 label when the light came on again should be no greater than the drop in amount of carbon-14.

The experiment shows that the drop in amount of phosphorus-32 label is as great in proportion to itself as the drop in amount of carbon-14 label is in proportion to itself. This proves that if there are two pools of PGA, they are in rapid equilibration. Thus, there is essentially a single pool of PGA which is immediately affected by the onset of the photochemical generation of NADPH.

Apparently oxidative phosphorylation is sufficient to maintain the concentration of ATP, which was found to be at least as high in the dark as in the light. This suggests that the "energy charge" of the chloroplasts, if defined as the ratio of ATP to total adenylates (*8*), is not the general regulatory factor in this system as it is in some other metabolic systems (*8*). Of course, this finding does not rule out the possible importance of that ratio for regulation in this system under other circumstances in which the concentration of ATP might not be maintained.

These same studies indicated several light-dark changes in the rates of reactions of the reductive and oxidative pentose phosphate cycles. For example, as soon as the light is turned off, 6-phosphogluconate appears (Fig. 5). This shows that the oxidative cycle (see Fig. 1) has commenced.

In the dark the amounts of fructose diphosphate (FDP) and its precursor, DHAP (Fig. 6), fall rapidly because PGA is no longer reduced. In the dark these metabolites are regenerated later by carbon coming from Ru5P formed by the oxidation of G6P. When the light comes on, PGA is reduced and both FDP and DHAP increase quickly. The amount of FDP, in fact, is greater than that present in the steady state. Such changes in metabolite concentrations can be accounted for if the reaction that converts FDP to F6P proceeds more slowly in the dark and remains slow for the first 20 seconds in the light. Later, if this reaction proceeds faster, due to activation of enzymes in the light, the FDP concentration would fall, as is observed. After further oscillations, the concentration of FDP approaches what it was in a steady state in the light.

The enzyme (aldolase) that converts DHAP and 3-phosphoglyceraldehyde to FDP apparently is very active in light and dark, since the interconversion is highly reversible. Transient behavior of the concentration of FDP is reflected back into DHAP concentration. It is clear, nevertheless, that the primary effect is on the concentration of FDP, since the relative magnitude of its change is much greater. The concentration of F6P (not shown) falls when that of FDP is rising, and then rises rapidly after 20 seconds at the time when the concentration of FDP falls.

Light-Dark Effects Compared with Effects of Added Octanoic Acid and Vitamin K_5

The analogous reaction which converts sedoheptulose diphosphate (SDP) to sedoheptulose-7-phosphate (S7P) exhibits the same activation in the light and inactivation in the dark. We were thus very interested to find that both reactions (and other light-dark regulated steps) can be reversibly inactivated in the light by the addition and removal of octanoic acid (*19*). To *Chlorella*, photosynthesizing with $^{14}CO_2$ and phosphorus-32–labeled monohydrogen phosphate in a buffer at *p*H 5, we added this acid, dissolved in a little alcohol. Octanoic acid is partly undissociated at *p*H 5, and it apparently dissolved into the fatty membranes of the chloroplasts. Photosynthesis, as indicated by oxygen evolution and carbon dioxide uptake, completely stopped. Then, after 4 minutes, the *p*H of the medium was raised to 7, at which octanoic acid is completely dissociated. The rate of photosynthesis was restored to 50 percent or more of its original amount. Thus, we had found a way to reversibly stop photosynthesis without turning off the light.

Upon analyzing the metabolic products, we found that the addition of octanoic acid caused a rapid increase in amounts of labeled FDP and SDP. This was interpreted as being due to the inactivation of the respective phosphatase, FDPase and SDPase. When the *p*H of the medium was increased, and photosynthesis recovered, the concentrations returned to normal. Neither alcohol nor *p*H change alone produced any of these effects.

The light-dark studies and the experiments with octanoic acid and with other chemicals indicated several other points of regulation. Many of these results were duplicated in experiments with isolated spinach chloroplasts. The chloroplasts were first separated out of cells from spinach leaves (*39*) and then were allowed to photosynthesize with $^{14}CO_2$ in kinetic experiments similar to those described for algae.

According to our interpretation of

such kinetic data, both darkness (*18*) and the addition of octanoic acid (*19*) cause inactivation of the carboxylation reaction and of the conversions of FDP and SDP to their respective monophosphates. Moreover, addition of the acid inhibits photophosphorylation and possibly oxidative phosphorylation. These effects are reversible.

Because photophosphorylation is thought to be related in some way to the ion fluxes through the membrane, we suggested (*23*) that the connection between effects of fatty acid on photophosphorylation and the effects on carbon metabolism might be found in the concentrations of ions in the stroma region (as discussed earlier). The observation (*20*) that fatty acids reversibly caused an increase in absorption of 515-nanometer light [which has been suggested to indicate an electric field across the membrane (*21*)] raises the possibility that alteration in membrane properties caused by intercalation of fatty acid molecules somehow blocks certain events subsequent to the formation of the electric field, such as ion flux and photophosphorylation.

Addition of vitamin K_5, an electron acceptor which is sufficiently lipid-soluble to penetrate the cell membranes, caused some similar and some different effects. Its addition inactivated conversion of FDP and SDP to their respective monophosphates after several seconds and also stopped the reaction which converts Ru5P to RuDP. There was no evidence of either inhibition or stimulation of photophosphorylation, for the concentration of ATP did not change. Another difference from the action of octanoic acid was the immediate appearance (with vitamin K_5) of 6-phosphogluconic acid. All of these effects were seen both with algae and with isolated spinach chloroplasts. With addition of low concentrations of vitamin K_5, the effects were partially reversed with time.

In the dark, of course, there is neither photoelectron transport nor ion pumping through the thylakoids. The appearance of 6-phosphogluconate upon addition of vitamin K_5 suggests that electrons from photoelectron transport are diverted to the reduction of the air-oxidized form of vitamin K_5 so that reduction of $NADP^+$ ceases. Observed regulatory effects would be due to the increased ratio of oxidized to reduced cofactors. Thus, glucose-6-phosphate dehydrogenase may be activated by the increased amount of $NADP^+$, one of its substrates.

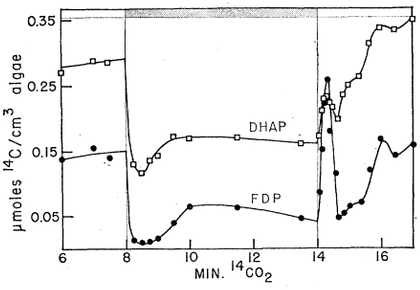

Fig. 6. The effects of light and dark on the amounts of labeled fructose-1,6-diphosphate (FDP) and dihydroxyacetone phosphate (DHAP). Conditions were as given with Fig. 2. When the light is first turned on, FDPase is still inactive, and as PGA is reduced to triose phosphate by cofactors coming from the light reactions, the triose phosphates condense, forming fructose-1,6-diphosphate, which is not converted to fructose-6-phosphate immediately. After about 20 seconds, FDPase becomes activated and the concentration of FDP decreases. Dihydroxyacetone phosphate, in rapid equilibrium with glyceradehyde-3-phosphate and FDP in both light and dark, reflects the FDP transients.

This proposal is supported by the similarity of the effects to those seen with the light-dark transition, and by the fact that vitamin K_5 in air is readily oxidized to a form that can accept electrons from the photoelectron transport system. Thus, added vitamin K_5 can provide an electron "short-circuit" to oxygen. With limited amounts of added vitamin K_5, the effects are reversed after a few minutes, due to the destruction in air and light of the added compound, by further reactions of the oxidized form to give colored compounds.

When nitrite was added to photosynthesizing *Chlorella*, at concentrations where it functioned as an electron acceptor (*40*), a delayed (for 20 seconds) inhibition of the conversions of FDP and SDP to monophosphates occurred. When observed, this effect was compared with octanoate effects, but in retrospect it appears more analogous to the effects of vitamin K_5, in accord with the diversion of electrons just ascribed to vitamin K_5. Finally, phosphoribulokinase, the enzyme which converts Ru5P to RuDP, is activated by sulfhydryl reagents (*41*), so a reducing environment in the chloroplasts may favor its activation.

To summarize, kinetic tracer studies of metabolite concentrations during light-dark and dark-light transitions, and in the light upon addition of inhibitors, indicate regulated carboxylation of RuDP, hydrolysis of FDP and SDP, and phosphorylation, with ATP, of Ru5P, as well as activation of the conversion of G6P to 6-phosphogluconate in the dark. Based upon inferred effects of the added inhibitors (octanoate and vitamin K_5) and upon known properties of the respective enzymes for these reactions, RuDP carboxylase, FDPase and SDPase, and phosphoribulokinase (discussed later), a tentative hypothesis for the regulatory mechanisms can be stated—both ion flux through the lamellae leading to a rise of *p*H in the stroma and electron flow to ferredoxin and $NADP^+$ may be required for activation of FDPase and SDPase. Ion flux may be required for RuDP carboxylase and electron flow for phosphoribulokinase, whereas interruption of electron flow would activate conversion of G6P to 6-phosphogluconate. Of course, other mechanisms are possible, either instead of, or in addition to, these.

Function of Light-Dark Regulation

These sites of metabolic regulation in the cycle are shown in Fig. 1. They operate together in the light-dark transition to activate the photosynthetic carbon reduction cycle in the light and to block it in the dark. They also activate the oxidative pentose phosphate cycle in the dark and block it in the light. We propose that the purpose of the oxidative cycle in the chloroplasts in the dark is to generate NADPH for biosynthesis inside the chloroplasts. This cofactor apparently does not penetrate the outer chloroplast membrane (*42*). There is an adequate supply of ATP which does penetrate this membrane (*43*) and is produced by oxidative phosphorylation in the dark. With both ATP and NADPH, biosynthetic conversions (such as carbohydrates to lipids) can occur in the chloroplasts in the dark.

Biosynthetic conversion of sugars to fats would require reduction of some DHAP to glycerol phosphate (see Fig. 2). Thus, some limitation on the rate of oxidation of triose phosphates to PGA in the dark may be required. More importantly, the operation of the oxidative pentose phosphate cycle (dashed lines in Fig. 1) requires a reaction between 3-phosphoglyceraldehyde and S7P, so that the concentration of the former (usually about 1/20 that of DHAP) must not fall too low. There is enzymic evidence (discussed later) that interconversion between 3-phosphoglyceraldehyde and PGA may be slower in the dark than in the light.

Regulation in the Light

Metabolic regulation controls the transition from light to dark, and this regulation can be mimicked even in the light by adding certain compounds. Are there mechanisms that control the flow of carbon during photosynthesis in the light? How is the flow of carbon from the cycle to various biosynthetic paths altered in response to the physiological needs of the green cells? As it turns out, some of the same points of regulation are involved as were seen in the light-dark-light regulation.

In order to obtain uniform populations of cells in several physiological stages we synchronized cultures of *Chlorella pyrenoidosa* by subjecting them to successive periods of light and dark (*37*). Each of these cell populations was then allowed to photosynthesize under steady-state conditions with $^{14}CO_2$ and phosphorus-32–labeled monohydrogen phosphate. By taking samples for analysis over a period of 30 minutes, we were able to use both the initial labeling rates and the pool sizes at isotopic "saturation" to calculate the rates of flow of carbon through various metabolic steps. From these rates we could see the relative enzymic activities of those steps at different stages of cell growth and division.

Changes in rate of metabolism were correlated with relative shifts between amino acid and protein synthesis (greatest during the fast-growing period and DNA-synthesizing stage) and sucrose synthesis (greatest in the divided cells and cells immediately prior to division). Two classes of effects were identified—those due to the stage of cell growth and division and those due to the dark period which the cells had just experienced. These effects could be separated in part, but to some extent they were additive (*37*).

Specific sites of metabolic regulation in the light, suggested by these studies, include: (i) FDPase, (ii) the carboxylation of RuDP, (iii) the synthesis of sucrose, probably at the reaction between uridine diphosphoglucose and F6P to give sucrose phosphate, (iv) the reduction of nitrate and nitrite, and (v) the conversion of phosphoenolpyruvic acid to pyruvate. The last step, catalyzed by the enzyme, pyruvate kinase, controls the flow of carbon from the photosynthetic carbon reduction cycle to the synthesis of most amino acids (see Fig. 3) and the synthesis of fatty acids.

During the fast-growing stage, and especially during the DNA-synthesizing stage, FDPase activity appeared to be limited. This was indicated by a low ratio of fructose-6-phosphate to fructose diphosphate. Thus, the flow of carbon through the cycle was restricted at a point just after PGA and DHAP, from which fats and proteins are made, and just before F6P, from which carbohydrates are made. This FDPase activity appeared to be greatest immediately before and after division, when sucrose synthesis is rapid, and little amino acid synthesis occurs.

Effect of Ammonium Ion

The newly divided algal cells, which had been in the dark, were nearly unable to reduce nitrate or nitrite (*38*). This led us to suspect that the resulting lack of ammonium ions within the cell might have regulatory effects. Two regulatory effects on carbon metabolism were found when we added ammonium ion (1 millimole per liter) to an unsynchronized population of *Chlorella* cells. The added ammonium ion greatly increased the conversion of phosphoenolpyruvic acid to pyruvate, and it completely stopped sucrose synthesis (*29*). (Starch synthesis decreased only slightly.) The ratio of fructose-6-phosphate to fructose diphosphate decreased about 50 percent. The concentration of PP_i decreased more than 50 percent. Thus, concentration of ammonium ion within the cell may be of primary importance in regulation in photosynthetic cells.

Transport through the Chloroplast Membrane

In 1966, Jensen and Bassham (*39, 44*), by modifying methods developed by Walker (*45*), were able to isolate chloroplasts in such a physiological state that they were able to carry out photosynthesis with carbon dioxide for 15 minutes or longer in an artificial medium at rates comparable to that of photosynthesis in vivo. In some experiments chloroplasts were quickly centrifuged for a few seconds after 3 minutes of photosynthesis, and the pellet and supernatant solution were then biochemically inactivated with methanol (*4*). Some intermediate compounds of the photosynthetic cycle appeared much faster than others in the solution used for suspension of the chloroplasts (*4*). For example, FDP appeared in the suspending medium (S) at a rate 35 times as great as in the chloroplast pellet (P). In contrast, the S/P ratio for F6P was 0.4. Since the ratio of volume of supernatant to chloroplasts was about 100, this is not necessarily "active" transport (energy-requiring), but it is a highly selective diffusion.

Compounds produced after the carboxylation reaction and before the reaction catalyzed by FDPase and SDPase appear rapidly in the medium, whereas those compounds produced after the diphosphatase-mediated reactions and before the carboxylation reaction (except pentose monophosphates) are very well retained in the chloroplasts. Similar differences in movement of intermediate compounds from chloroplasts to cytoplasm had been indicated earlier by Heber and Willenbrink (*5*), who allowed whole leaves to photosynthesize for short periods with $^{14}CO_2$ and then, after the leaves were freeze-dried, isolated chloroplasts by nonaqueous procedures. This pattern of selective diffusion seemed to fit fairly well with the sites of regulation elucidated from kinetic studies. However, since several of the enzymes of the cycle (but not diphosphatases or carboxylase) are present and active in the medium, due to rupture of some chloroplast membranes during the experiments, the actual transport reactions are difficult to pinpoint.

An answer to this question may come from studies of the effects of factors from the leaf juice that affect the rate of photosynthesis by the isolated chloroplasts (*35*). The factors from leaf juice that most affected the $^{14}CO_2$ uptake rate proved to be a purified protein preparation and magnesium ions (*35, 46*). Very small amounts of these factors stimulated CO_2 fixation, whereas only slightly larger amounts greatly inhibited the rate. An enzyme fraction from the leaves and magnesium were synergistic in their inhibitory effects, in one case producing 96 percent inhibition together whereas the same amount of enzyme fraction alone stimulated 90 percent and the magnesium ions alone stimulated 12 percent. These effects occur significantly only in the presence of 1 to 5 m*M* PP_i, yet the inhibition could be completely overcome by higher amounts of PP_i.

Kinetic studies with $^{14}CO_2$ showed that addition of enzyme fraction and magnesium ions to photosynthesizing chloroplasts increased the amount of labeled FDP and decreased the amount

of labeled F6P. Furthermore, the enzyme fraction plus magnesium ions in excess of PP_i decreases the retention in the chloroplasts of nearly all intermediates. By itself, magnesium in excess of PP_i causes many of these effects, whereas added PP_i (5 m*M*) alone increases the retention of those compounds (F6P, G6P, RuDP, and so forth), which the chloroplasts tend to retain anyway. This may be a partial explanation of why PP_i stimulates CO_2 uptake by chloroplasts (*39, 44*). Finally, when the enzyme-fraction preparation, which had already been extensively purified and from which free small molecules had been removed by extensive dialyzing, was denatured by heating, some soluble fraction is released which still affects CO_2 uptake, although the effect is no longer strongly increased by magnesium ion. Obviously, further experimentation will be required to interpret these results. What is presently clear experimentally is that magnesium, PP_i, and enzyme fraction or some factor derived from or bound to it, in amounts which may be present in the cytoplasm of green cells, exert strong effects on both metabolite transport and CO_2 fixation, with collaboration and antagonism among the three factors.

Properties of the Regulated Enzymes

The allosteric and other properties of ribulose diphosphate carboxylase, fructose diphosphate, and other enzymes of the Calvin cycle have been extensively studied and reviewed (*31*). A property of the carboxylase and diphosphatase is that the *p*H optimum shifts from about 8.5 to 9.0 at magnesium concentrations of 1 to 4 moles per liter to around 7.6 at 20 to 40 m*M* magnesium (*34, 47*). A shift from *p*H 7.2 to 7.7 at a magnesium concentration of 10 moles per liter could have a large effect on the activities of the carboxylase (*34*). Very probably other controlling effects are also exerted. Wildner and Criddle (*48*) reported a light-activation factor for the carboxylase. Buchanan *et al.* (*49*) have reported that ferredoxin activates the diphosphatase.

Phosphoribulokinase, catalyzing the conversion of Ru5P to RuDP, may require a "reducing" environment for its activation in the light, since its activity is preserved by sulfhydryl reagents and is decreased by high concentrations of oxygen (*41*).

The two-step conversion of PGA to 3-phosphoglyceraldehyde is mediated by (i) PGA kinase, which phosphorylates the carboxyl group, using the terminal phosphate of ATP, and (ii) glyceraldehyde phosphate dehydrogenase (GPDH), which reduces the intermediate phosphoglyceryl phosphate, using the two electrons from NADPH and liberating P_i. Studies of leaf extracts (*50*), isolated chloroplasts (*51*), and partially purified enzyme preparations (*52*) show a light-enhanced activation of the GPDH (*50–53*). This activation requires the reduced form of the coenzyme, NADPH, which is also its substrate in the reducing reaction (*51*). The enzyme is also activated by ATP and by high concentrations of magnesium (*54*). The dark inactivation of the GPDH specific for $NADP^+$ may be the result of its conversion in the dark to GPDH specific for NAD^+, since light to dark and dark to light shifts in the amounts of these two enzymic activities were found when they were isolated from cells (*53, 55*) and even from isolated chloroplasts where new protein synthesis was unlikely (*53*).

The properties of pyruvate kinase in other systems are known to include activation by ammonium ions (*56*), and the enzyme in green cells might be similarly activated.

Preiss *et al.* (*57*) found that the enzyme catalyzing the reaction of G1P with ATP to give adenosine diphosphoglucose and ADP can be activated by PGA and inhibited by P_i. They proposed that starch synthesis stops in the dark because of decreased amounts of PGA and increased amounts of P_i. In our own experiments we found little change in PGA concentration between light and dark, but it is certainly possible that the concentration of P_i may rise sharply in the chloroplasts in the dark when photophosphorylation stops. Incidentally, the inhibition of carboxylase in the dark could also be due in part to P_i which is known to inhibit the isolated enzyme.

The control of sucrose synthesis seems to be quite different from that of starch. Our kinetic studies of *Chlorella* show clearly that the block which occurs in that organism when ammonium ion is added is beyond uridine diphosphoglucose (Fig. 3), since the concentration of that metabolite rises slightly after addition of ammonium ion. The fact that only a small increase in concentration of uridine diphosphoglucose is seen at the time when sucrose synthesis is completely stopped may be because the reaction between G1P and uridine triphosphate to make uridine diphosphoglucose and PP_i is highly reversible (*58*), and G6P can be converted through other paths (Fig. 3). There is some evidence that sucrose phosphate synthetase from plant tissues exhibits allosteric properties (*59*).

"Irreversibility" of Regulated Reactions

"Irreversibility," meaning a very high ratio of forward to back reaction rate, is a common property of reactions catalyzed by metabolically regulated enzymes (*8, 60*). Although examples of this principle are to be found elsewhere, the photosynthetic metabolism of green cells offers a particularly good system for testing its validity. This is because the steady-state tracer method permits us to measure the concentrations of metabolites in vivo. These are essential to a calculation of negative free-energy change of the steady-state reaction (ΔG^s). The relation of ΔG^s to rates is given by $\ln f/b = \Delta G^s/RT$, where f and b are forward and back reactions rates. Correcting the physiological standard free-energy changes ($\Delta G^{0\prime}$) with the measured metabolite concentrations (C_a,C_b,C_c,C_d) by

$$\Delta G^s = \Delta G^{0\prime} - RT \ln([C_a]^v[C_b]^w[C_c]^x[C_d]^y)$$

we found that for the reactions mediated by FDPase and SDPase, $\Delta G^s = -7$ kilocalories, whereas for the carboxylation reaction, $\Delta G^s = -10$ kilocalories (*36*). For the conversion of Ru5P to RuDP, $\Delta G^s = -4$ kilocalories. Each of the other nine steps of the cycle, which in bright light are not regulated according to our findings, had ΔG^s values between 0 and -2 kilocalories.

Going out of the cycle, we find that the two-step conversion of PGA to phosphoenolpyruvic acid, when corrected for concentrations, had a total ΔG^s of about -2 kilocalories, whereas for the regulated conversion of phosphoenolpyruvic acid to pyruvate $\Delta G^s = -4.5$ kilocalories before addition of ammonium ion and -3.5 kilocalories after its addition (*29*).

These changes in negative free energy represent the loss of chemical potential through its conversion to heat at room temperature. This loss amounts to an unavoidable increase of entropy in the system as a whole. This energy loss is the difference between the en-

ergy that was stored during the generation of ATP, NADPH, and oxygen by the light reactions, and the energy that is eventually stored in the formation of oxygen and the organic end products of photosynthesis. As it turns out, nearly all of this necessarily dissipated potential energy is lost during the course of metabolically controlled reactions. Since the regulation thereby achieved may represent a form of decrease in entropy, this is perhaps another manifestation of the tendency of living systems to minimize increase in entropy.

Conclusions

Strong regulation of photosynthetic metabolism is maintained by controlled key enzymes within the carbon reduction cycle, on biosynthetic paths leading from it, and within the chloroplast membrane. The transition from light to dark metabolism, the allocation of metabolites to biosynthetic paths, and the export of intermediate compounds from the chloroplasts to the cytoplasm are each controlled, and to some extent by the same enzymes. Of these, the most important are ribulose diphosphate carboxylase, controlling the initial entry of carbon dioxide, and fructose diphosphatase, operating as a portal between carbohydrate metabolism and other biosynthesis, and perhaps playing some role in the selective diffusion of sugar phosphates through the outer chloroplast membrane. Intracellular *p*H and concentrations of magnesium ion and inorganic pyrophosphate seem to be candidates for controlling factors in some of these processes, based on preliminary evidence for changes in these factors in vivo or in isolated chloroplasts, and on properties of isolated enzymes which catalyze steps found to be regulated in vivo.

These and other control effects have been demonstrated by determinations of metabolite concentrations in green cells and chloroplasts in vivo by tracer techniques. These quantitative measurements of in vivo metabolite concentrations provide excellent correlative evidence for the "irreversibility" of regulated steps.

Along biosynthetic paths, pyruvate kinase, which controls the conversion of phosphoenolpyruvic acid to pyruvate, and sucrose phosphate synthetase, which controls sucrose synthesis, play important roles. These points of control have been activated artificially in *Chlorella pyrenoidosa* by the addition of ammonium ion, which resulted in increased protein synthesis. It is perhaps not too much to hope that in the future, application of a suitable chemical spray to mature leaves which are producing mostly sucrose may be able to switch their metabolism for a time back to protein production. Since the direct utilization of green leaves by humans may prove to be an efficient agricultural usage in a protein-hungry world (*61*), such an induced switch might prove to be a valuable aid to reducing protein deficiencies in some areas of the world.

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