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THE CONTROL OF PHOTOSYNTHETIC CARBON METABOLISM

James A. Bassham

Interest in the control of photosynthetic metabolism was stimulated by the mapping of the basic path of carbon reduction in photosynthesis by Calvin and co-workers fifteen years ago (1,2). Before that time it was possible to suppose that the photochemical process of 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") (Figure 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) (Figure 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 (3). Thus, all the carbon taken up by the leaf during photosynthesis is not first 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 (4). Then it was found that a highly selective process allowed the export of specific intermediates from the chloroplasts (5,6).

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 (7-11) and photorespiration (12-18).

Cell Life Cycle and Regulation

During the life cycle of a green plant 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 the 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 mechanism is usually 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.

Enzyme Regulation

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 (19). In feedback control, some compound farther along the metabolic pathway binds to the enzyme and so changes its shape or other properties 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 (20). 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 ATP to total adenylates (ATP + ADP + AMP) of the cell. This ratio, designated "energy charge" by Atkinson (19) is a measure of available biochemical energy. It has been found to regulate 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 levels of pH and

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 energetically unfavorable oxidation-reduction reactions (21). Water is oxidized to molecular oxygen, whereas CO_2, nitrate and sulfate are reduced to organic compounds. Following 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 O_2 and protons. The strongest reductant is reoxidized by giving up its electrons to ferredoxin, a low-molecular-weight protein which contains iron atoms. Reduced ferredoxin is used either directly or via other electron carriers in enzymically catalyzed reactions to bring about the reduction of CO_2, 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.

The absorption of light, the oxidation of water, and the reduction of ferredoxin all take place in or on the membranes (called lamellae) of the chloroplasts. Pairs of these lamellae are joined together at the edges to form sack-like ("thylakoid") closed systems (22,23). In most higher plants, many thylakoids are tightly appressed in stacks which

are called grana. Some thylakoids extend through the stroma region, the space outside the grana but inside the double membrane surrounding the entire chloroplast. Within these membrane systems, some of the photochemically produced oxidants and reductants recombine in such a way that a part of the energy released is stored as chemical energy. This storage is achieved by the conversion of adenosine diphosphate (ADP) and inorganic phosphate (P_i) to their anhydride, adenosine triphosphate (ATP). Properties of the membrane may be involved in this process, called photophosphorylation (24). Associated with electron transport from water to ferredoxin and perhaps with photophosphorylation as well is a movement of ions through the membrane.

In 1964, Neumann and Jagendorf (25) found that isolated, illuminated spinach chloroplasts take up H^+ ions, and it seems now well established from many studies [recently reviewed (26,27)] that photoelectron transport in chloroplast membranes leads to formation of a pH gradient across the membranes with the low pH inside the thylakoids. Hind and Jagendorf (28) found that isolated chloroplasts phosphorylate ADP in the dark following a sudden imposed pH rise in the suspending medium. Whether or not trans-membrane pH gradients and membrane charge are required parts of the coupling mechanism for linking phosphorylation of ATP to electron transport (29) is still unresolved (30,31). 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 pH and the concentrations of other ions, notably Mg^{++}, have large effects on the activities of key enzymes, as discussed later.

Some of the changes in metabolism in Chlorella seen during the light-to-dark transitions (32) seem to be similar to reversible changes seen in light on addition and removal of octanoic acid and certain other fatty acids (33). These acids also cause a large and partly reversible change in the difference between 515 nm light absorbed by illuminated and dark Chlorella cell suspensions (34). A 515 nm absorption change produced in spinach chloroplasts within 20 nsec after the beginning of a light flash has been interpreted by Witt and co-workers (35,36) 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 H^+ and other ions through the membranes observed with indicators (37,38), 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 membranes (increased 515 nmeter absorption change) and ion flux through the membranes.

The enzymes involved in carbon metabolism are commonly thought to be located in the stroma region (though not inside the stroma thylakoids). In considering general regulatory mechanisms for those enzymes, it would be helpful to know what changes in pH and concentration of other ions, such as Mg^{++}, occur in the stroma region, but, unfortunately, little is known. It may be inferred that the light-pumping of H^+ into the thylakoids, which is thought to establish a lower steady-state pH inside the thylakoids, would also cause the steady-state pH in the

stroma to be higher in the light than in the dark (39). 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 pH unit, would be required to effect considerable change in the activities of some enzymes discussed later.

Reported light-induced efflux of Mg^{++} and K^{+} ions from broken chloroplasts (40) led to a suggestion (39) that Mg^{++} concentration in the stroma region might increase *in vivo*, thus activating enzymes. However, Nobel (41) found a light-induced efflux of K^{+}, Mg^{++} 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 assumptions, the internal ion concentrations seemed to remain roughly constant between light and dark. The high internal concentration of Mg^{++} ion, 16 millimolar, reported by Nobel (41), would be very important in activation of several of the suspected regulated enzymes at pH levels below 8.

The initial stages in the reduction of CO_2, NO_3^-, and SO_4^{-2} each require reduced nicotine adenine dinucleotide phosphate (NADPH) (42), 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 (24,43). After two electrons have been transferred to NO_3^- and SO_4^{-2}, reducing them to the level of nitrite and sulfite respectively, the further, six-electron reductions to the level of NH_4^+ and SH^- use reduced ferredoxin and particulate systems (42).

In the reduction of CO_2 by the reductive pentose phosphate

cycle, the product of the carboxylation reaction, 3-phosphoglycerate (PGA) is first activated by phosphorylation with ATP and then the resulting acyl phosphate is reduced to triose phosphate by NADPH. The activities of the enzymes involved in transfer of electrons from ferredoxin to $NADP^+$ is affected by the level of inorganic pyrophosphate (PP_i) (44). Since PP_i level *in vivo* changes with physiological state (32,33,45) it may be that PP_i level *in vivo* exerts some regulatory control on the rates of utilization of electrons from ferredoxin for reductive reactions. The activity of isolated pyrophosphatase, the enzyme catalyzing the hydrolysis of PP_i is affected by pH and Mg^{++} ion concentrations (46).

Study of Regulation In Vivo

While many enzymes show changes in activity due to the presence of metabolites, changes in pH or in ion concentrations, etc. (47), 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 occurring 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, CO_2, 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 (48). 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 ^{14}C by the specific radioactivity and by the number of carbon atoms per molecule gives the actual pool size 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 (32,33), 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 (1,2) and the flow of carbon into secondary biosynthetic paths were originally mapped (3).

Additional information is gained by the simultaneous application of $^{14}CO_2$ and ^{32}P-labeled phosphate to the photosynthesizing cells (32,33). Both $^{14}CO_2$ and ^{32}P-labeled P_i are incorporated by photosynthesis, but only labeled phosphate is incorporated by respiration. Respiration uses endogenous stores of sugars that are mostly unlabeled with ^{14}C. The investigator thus can examine relations between photosynthesis and respiration by means of kinetic light-dark experiments (32) in the presence of $^{14}CO_2$ and $H^{32}PO_4^{-2}$.

Photosynthetic Carbon Reduction, an Oxidative Cycle, and Biosynthesis

Photosynthetic reduction of carbon dioxide on green plants (other than C-4 plants) (7-11) is accomplished by a single metabolic cycle (1,2). The incorporation of $^{14}CO_2$ via the carbon reduction cycle of most plants is by carboxylation of ribulose-1,5-diphosphate (RuDP) (Figure 1) (1,2). The recently incorporated carbon becomes the carboxyl carbon of one of two PGA molecules.

Some of the molecules of PGA can be used as the starting material for biosynthetic paths leading to amino acids, fatty acids, etc. 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 F6P, in either light or dark. In the light, these sugar phosphates are converted to carbohydrates (Figure 3). In the dark, G6P undergoes oxidation to 6-phosphogluconic acid. Further oxidation leads to Ru5P and CO_2. Three Ru5P molecules are then converted (dashed lines, Figure 1) to two molecules of F6P, which are recycled, and a net single molecule of GAl3P.

Most metabolites needed for synthesis of non-carbohydrate end products come from PGA and DHAP (Figure 2). Both compounds occur in the carbon reduction cycle after the carboxylation reaction and before the diphosphatase reactions.

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 (5,32,33,39,46,49-56).

The metabolic transients accompanying the light-dark and dark-light transactions in the unicellular algae, Chlorella pyrenoidosa (32), are informative. Of particular interest is the ^{32}P and ^{14}C labeling of PGA (Figure 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 ceases. 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 level of PGA labeling with both ^{32}P and ^{14}C rose rapidly.

After about 30 sec, the carboxylation reaction ceased, and the conversion of PGA to products such as amino acids and fatty acids outside the cycle caused the level 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 ^{14}C label 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 ^{32}P whether it is made by photosynthetic phosphorylation in the chloroplasts in the light or by oxidative phosphorylation in the mitochondria in the dark. The ^{32}P 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 ^{32}P label of PGA remains a true measure of the PGA concentration in both light and dark. Thus we can see that after the transitory period the level 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 at a time when the RuDP concentration hadn't risen enough to support carboxylation. Such an isolated photosynthetic pool of PGA should still have the same $^{32}P/^{14}C$ ratio it had during the previous light period. Therefore, the drop in ^{32}P label when the light came on again should be no greater than the drop in ^{14}C level.

The experiment shows that the drop in the level of ^{32}P label is as great in proportion to ^{32}P label as the drop in ^{14}C label is in proportion to ^{14}C label. 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 level 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 ATP/(ATP + ADP + AMP) (19), is not

the general regulatory factor in this system as it is in some other metabolic systems (19). 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 level 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 (Figure 5). This shows that the oxidative cycle (see Figure 1) has commenced.

In the dark the levels of FDP and its precursor, DHAP (Figure 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 level of FDP, in fact, passes its steady-state level. Such changes in metabolite level 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 sec in the light. Later, if this reaction proceeds faster, due to activation of enzymes in the light, the FDP level would fall, as is observed. After further oscillations, the concentration of FDP approaches its former light steady-state level.

The enzyme (aldolase) that converts DHAP and GALP to FDP apparently is very active in light and dark, since the interconversion is highly reversible. Transient behavior of FDP level is reflected back into DHAP level. It is clear, nevertheless, that the primary effect is on the level of FDP, since the relative magnitude of its change is much greater. The level of F6P (not shown) falls when the level of FDP is

rising, and then rises rapidly after 20 sec at the time when the level of FDP falls.

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

The analogous reaction which converts SDP to 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 (33). To Chlorella, photosynthesizing with $^{14}CO_2$ and $H^{32}PO_4^{-2}$ in a buffer at pH 5, we added this acid, dissolved in a little alcohol. Octanoic acid is partly undissociated at pH 5, and it apparently dissolved into the fatty membranes of the chloroplasts. Photosynthesis, as indicated by O_2 evolution and CO_2 uptake, completely stopped. Then, after 4 min, the pH of the medium was raised to 7, at which pH octanoic acid is completely dissociated. The rate of photosynthesis was restored to 50 percent or more of its original level. Thus, we had found a way of reversibly stopping photosynthesis without turning off the light.

Upon analyzing the metabolic products, we found that the addition of octanoic acid caused the levels of labeled FDP and SDP to increase rapidly. This was interpreted as being due to the inactivation of FDPase and SDPase. When the pH of the medium was increased, and photosynthesis recovered, the levels returned to normal. Neither alcohol nor pH 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 (57) 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 (32) and the addition of octanoic acid (33) 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.

Since photophosphorylation is thought to be related in some way to the ion fluxes through the membrane, we suggested (39) that the connection between fatty acid effects on photophosphorylation and the effects on carbon metabolism might be found in the levels of ions in the stroma region (as discussed earlier). The observation (34) that fatty acids reversibly caused an increase in the 515 nm absorption charge [which has been suggested to indicate an electric field across the membrane (35,36)] 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 monophosphates after several seconds and also stopped the reaction which converts Ru5P to RuDP (33). There was no evidence of either inhibition or

stimulation of photophosphorylation, for the level 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 low levels of added 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 with vitamin K_5 addition, 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 level of $NADP^+$, one of its substrates.

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 levels where it functioned as an electron acceptor (58), a delayed (for 20 sec) 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 (59), so a reducing environment in the chloroplasts may favor its activation.

To summarize, kinetic tracer studies of metabolite levels during light-dark and dark-light transitions, and in the light upon addition

of inhibitors, indicate regulated reactions at the carboxylation of RuDP, the hydrolysis of FDP and SDP, and the 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 tenatative hypothesis for the regulatory mechanisms can be stated: Both ion flux through the lamellae leading to a pH rise 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, while 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 Figure 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 (60). There is an adequate supply of ATP which does penetrate this membrane (61) 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 Figure 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 Figure 1) requires a reaction between GAl3P and S7P, so that the level of GAl3P (usually about 1/20 that of DHAP) must not fall too low. There is enzymic evidence (discussed later) that interconversion between GAl3P 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 (55). Each of these cell populations was then allowed to photosynthesize under steady-state conditions with $^{14}CO_2$ and $H^{32}PO_4^{-2}$. By taking samples for analysis over a period of 30 min, 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 activites of those steps at different stages of cell growth and division.

Metabolic rate changes 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 predivision cells). 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 (55).

Specific sites of metabolic regulation in the light, suggested by these studies, include: 1) FDPase, 2) the carboxylation of RuDP, 3) the synthesis of sucrose, probably at the reaction between UDPG and F6P to give sucrose phosphate, 4) the reduction of nitrate and nitrite, and 5) the conversion of PEPA 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 Figure 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 F6P/FDP ratio. 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 maximal during the predivision and postdivision stages, where sucrose synthesis is rapid, and little amino acid synthesis occurs.

The Effect of Ammonium Ion

The newly divided algae cells, which had been in the dark, were nearly unable to reduce nitrate or nitrite (56). This led us to suspect

that the resulting lack of intracellular NH_4^+ might have regulatory effects. Two regulatory effects on carbon metabolism were found when we added millimolar NH_4^+ to an unsynchronized population of Chlorella cells. The added NH_4^+ greatly increased the conversion of PEPA to pyruvate, and it completely stopped sucrose synthesis (45). (Starch synthesis decreased only slightly.) The ratio F6P/FDP decreased about 50 percent. The level of pyrophosphate (PP_i) decreased more than 50 percent. Thus, intracellular NH_4^+ level may be of primary importance in regulation in photosynthetic cells.

Transport Through the Chloroplast Membrane

In 1966, Jensen and Bassham (57,62) by modifying methods developed by Walker (63) were able to isolate chloroplasts in such a physiological state that they were able to carry out photosynthesis with CO_2 for 15 min 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 following 3 min photosynthesis, and the pellet and supernatant solution were then biochemically inactivated with methanol (5). Some intermediate compounds of the photosynthetic cycle appeared much faster than others in the solution used for suspension of the chloroplasts (5). 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 volume ratio of supernatant to chloroplasts was about 100, this is not necessarily "active" transport (energy-requiring), but it is a highly selective diffusion.

Compounds following the carboxylation reaction and preceding the reactions catalyzed by FDPase and SDPase appear rapidly in the medium,

whereas those compounds following the diphosphatase-mediated reactions and preceding 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 (6), who allowed whole leaves to photosynthesize for short periods with $^{14}CO_2$ and then, after freeze-drying, isolated chloroplasts by non-aqueous 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 (52). The factors from leaf juice that most affected the $^{14}CO_2$ uptake rate proved to be a purified FDPase preparation and Mg^{++} ions (52,64). Very small amounts of these factors stimulated the CO_2 fixation rate, whereas only slightly larger amounts greatly inhibited the rate. FDPase and Mg^{++} were synergistic in their inhibitory effects, in one case producing 96 percent inhibition together when the same amount of FDPase alone stimulated 90 percent and the Mg^{++} stimulated 12 percent. These effects occur significantly only in the presence of 1 to 5 mM PP_i, yet the inhibition could be completely overcome by higher levels of PP_i.

Kinetic studies with $^{14}CO_2$ showed that addition of FDPase and Mg^{++} to already photosynthesizing chloroplasts increased the amount of labelled

FDP and decreased the amount of labelled F6P. Furthermore, FDPase plus Mg^{++} in excess of PP_i decreases the retention in the chloroplasts of nearly all intermediates. By itself, Mg^{++} in excess of PP_i causes many of these effects, whereas added PP_i (5 mM) alone increases the retention of those compounds (F6P, G6P, RuDP, etc.), which the chloroplasts tend to retain anyway. This may be a partial explanation of why PP_i stimulates CO_2 uptake by chloroplasts (57,62). Finally, when the FDPase 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, though the effect is no longer strongly increased by Mg^{++} ion. Obviously, further experimentation will be required to interpret these results. What is presently clear experimentally is that Mg^{++}, PP_i, and FDPase, 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 diphosphatase, and other enzymes of the Calvin cycle have been extensively studied and are reviewed elsewhere (47). A property of the carboxylase and diphosphatase is that the pH optimum shifts from about 8.5 to 9.0 at Mg^{++} concentrations of 1 to 4 mM to around 7.6 at 20 to 40 mM Mg^{++} (50,65). A shift from pH 7.2 to 7.7 at a Mg^{++} concentration of 10 mM could have a large effect on the activities of the carboxylase (50). Very probably other controlling effects are also exerted. Wildner and Criddle (66) reported a light-activation factor for the carboxylase. Buchanan *et al.* (67) have reported that reduced ferredoxin activates the diphosphatase.

Phosphoribulokinase, catalyzing the Ru5P to RuDP conversion, may require a "reducing" environment for its activation in the light, since its activity is preserved by sulfhydryl reagents and is decreased by high O_2 levels (59).

The two-step conversion of PGA to GALP is mediated by 1) PGA kinase, which phosphorylates the carboxyl group, using the terminal phosphate of ATP, and 2) glyceraldehyde phosphatedehydrogenase (GPDH), which reduces the intermediate phosphoglyceryl phosphate, using the two electrons from NADPH and liberating P_i. Studies of leaf extracts (68), isolated chloroplasts (69), and partially-purified enzyme preparations (70) show a light-enhanced activation of the GDPH (68-71). This activation requires the reduced form of the coenzyme, NADPH, which is also its substrate in the reducing reaction (69). The enzyme is also activated by ATP and by high Mg^{++} levels (72). The dark inactivation of the GPDH specific for $NADP^+$ may be the result of its conversion in the dark to GDPH 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 (71,73) and even from isolated chloroplasts where new protein synthesis was unlikely (71).

The properties of pyruvate kinase in other systems are known to include activation by NH_4^+ (74,75), and the enzyme in green cells might be similarly activated.

Preiss et al. (76) found that the enzyme catalyzing the reaction of G6P with ATP to give ADPG and ADP can be activated by PGA and inhibited by inorganic phosphate (P_i). They proposed that starch synthesis stops in the dark because of decreased PGA and increased P_i.

In our own experiments we found little change in PGA level between light and dark, but it is certainly possible that the level of P_i may rise sharply in the chloroplasts in the dark when photophosphorylation stops. Incidentally, the dark inhibition of the carboxylase could also be due in part to P_i, which is known to inhibit the isolated enzyme.

The control of sucrose synthsis seems to be quite different from that of starch. Our kinetic evidence with Chlorella shows clearly that the block which occurs in that organism when NH_4^+ is added is beyond UDPG (Figure 3), since the level of UDPG rises slightly after NH_4^+ addition. The fact that only a small increase in UDPG level is seen at the time when sucrose synthesis is completely stopped may be because the reaction between G6P and UTP to make UDPG and PP_i is highly reversible (77), and G6P can be converted via other paths (Figure 3). There is some evidence that sucrose phosphate synthetase from plant tissues exhibits allosteric properties (78).

"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 (19,79,80). 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 methods permit us to measure the in vivo metabolite concentrations. 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 ($\Delta G^{O'}$) with the measured metabolite concentrations (C_a, C_b, C_c-) by $\Delta G^S = \Delta G^{O'} - RT \ln[C_a]^v[C_b]^w[C_c]^x[C_d]^y$, we

found that for the reactions mediated by FDPase and SDPase, ΔG^S = -7 kcal, whereas for the carboxylation reaction, ΔG^S = -10 kcal (53). For the conversion of Ru5P to RuDP, ΔG^S = -4 kcal. Each of the other 9 steps of the cycle, which in bright light are not regulated according to our findings, had ΔG^S values between 0 and -2 kcal.

Going out of the cycle, we find that the two-step conversion of 3-PGA to PEPA, when corrected for concentrations, had a total ΔG^S of about -2 kcal, whereas for the regulated conversion of PEPA to pyruvate ΔG^S = -4.5 kcal before addition of NH_4^+ and -3.5 kcal after its addition (45).

These negative free energy changes represent the loss of chemical potential through its conversion to heat at room temperature. This loss amounts to an unavoidable entropy increase in the system as a whole. This energy loss is the difference between the energy that was stored during the generation of ATP, NADPH, and O_2 by the light reactions, and the energy that is eventually stored in the formation of O_2 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 entropy decrease, this is perhaps another manifestation of the tendency of living systems to minimize entropy increase.

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, but to a great extent by the same enzymes. Of these, the most important are ribulose diphosphate carboxylase, controlling the initial entry of CO_2, 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 pH and concentrations of Mg^{++} and PP_i 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 enzymeswhich catalyze steps found to be regulated *in vivo*.

These and other control effects have been demonstrated by determinations of metabolite levels *in vivo* in green cells and chloroplasts 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, controlling the conversion of PEPA to pyruvate, and sucrose phosphate synthetase, controlling 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 (81), such an induced switch might prove to be a valuable aid to reducing protein deficiencies in some areas of the world.

References and Notes

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Figure Captions

Figure 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. Early stages of some biosynthetic paths leading from the photosynthetic carbon cycle to end products are indicated. Open arrows are at sites of metabolic regulation which are active in the light. Wide, dark arrows show reactions which are activated in the dark. The dotted, open 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.

Abbreviations

PGA = 3-phosphoglycerate; PEPA = phosphoenolpyruvate; 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; $NADP^+$ = nicotinamide adenine dinucleotide phosphate, reduced and oxidized forms respectively; 6PGluA = 6-phosphogluconate; ATP = adenosine triphosphate.

Figure 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

Figure Captions (Cont.)

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 PEPA to pyruvate, which was found to be stimulated, in Chlorella by addition of NH_4^+. The line from pyruvate to amino acids denotes the formation of alanine by transamination. Alanine synthesis is rapidly stimulated by NH_4^+ addition.

Abbreviations

PEPA = phosphoenolpyruvate; CoA = coenzyme A; TCA cycle = tricarboxylic acid cycle (Krebs Cycle).

Figure 3. Metabolic Paths from the Calvin Cycle to Carbohydrates.

Abbreviations

UDPG = uridine diphosphoglucose; ADPG = adenosine diphosphoglucose; UTP = uridine triphosphate.

Figure 4. The effects of light and dark on the levels of ^{14}C and ^{32}P labeling of 3-phosphoglycerate in Chlorella pyrenoidosa.

Following 30-min photosynthesis under steady-state conditions with unlabeled CO_2, ^{32}P-labeled phosphate was added to the algae, and 20 min 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, killed, and analyzed by two-dimensional paper chromatography and radioautography. The ^{32}P and ^{14}C labels in the light indicated steady-state concentrations of PGA in the light. In the dark, the ^{32}P level indicates the total pool size, whereas the

Figure Captions (Cont.)

^{14}C label roughly indicates how much of the PGA formed in the light still remains. The large drop in ^{32}P label when the light is turned on shows that the entire pool of PGA was affected by the light.

Figure 5. The effect of light and dark on level of labeled 6-phosphogluconate.

Conditions as given in Figure 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.

Figure 6. The effects of light and dark on the levels of labeled fructose-1,6-diphosphate and dihydroxyacetone phosphate.

Conditions were as given with Figure 2. When the light is first turned on, FDPase is still inactive, and as 3-phosphoglycerate 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 sec, FDPase becomes activated and the level of FDP falls. DHAP, in rapid equilibrium with GALP and FDP in both light and dark, reflects the FDP transients.

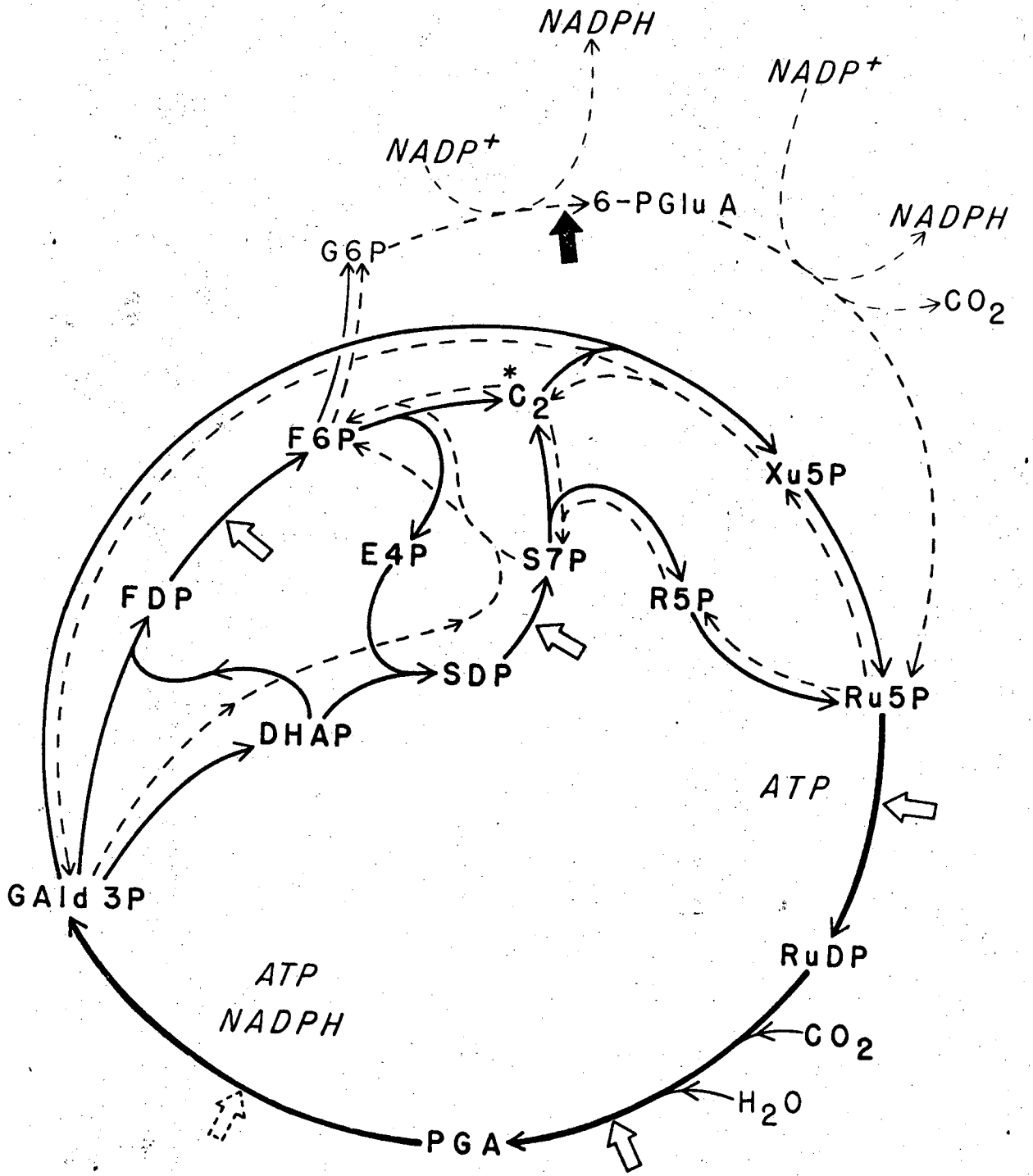

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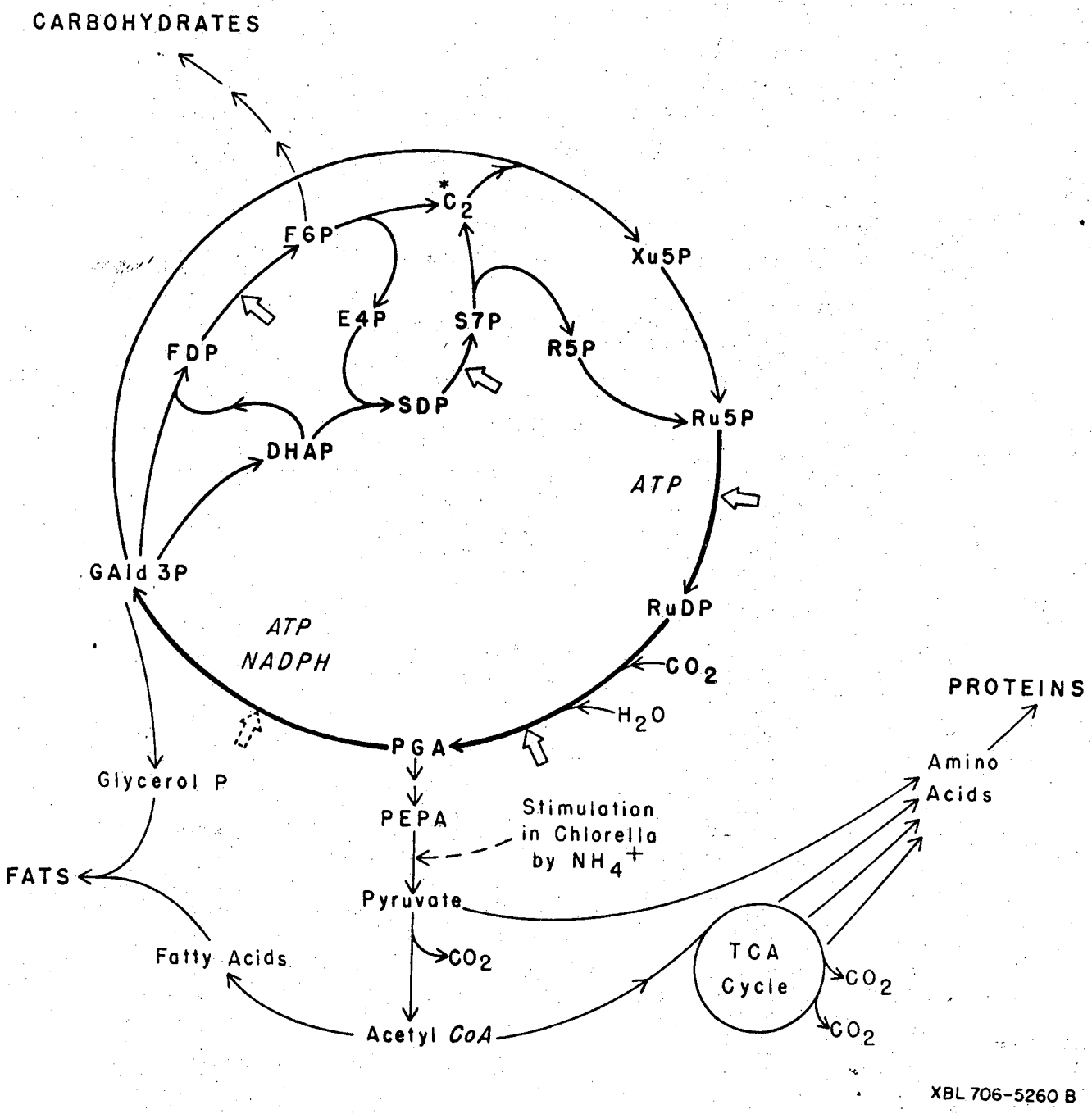
Fig. 1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F6P
Xu5P
E4P
S7P
R5P
FDP
SDP
Ru5P
DHAP
ATP
GAld 3P
RuDP
ATP
NADPH
CO2
H2O
PROTEINS
PGA
Amino Acids
Glycerol P
PEPA
Stimulation in Chlorella by NH4+
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CO2
CO2
Acetyl CoA
XBL 706-5260 B

Fig. 2

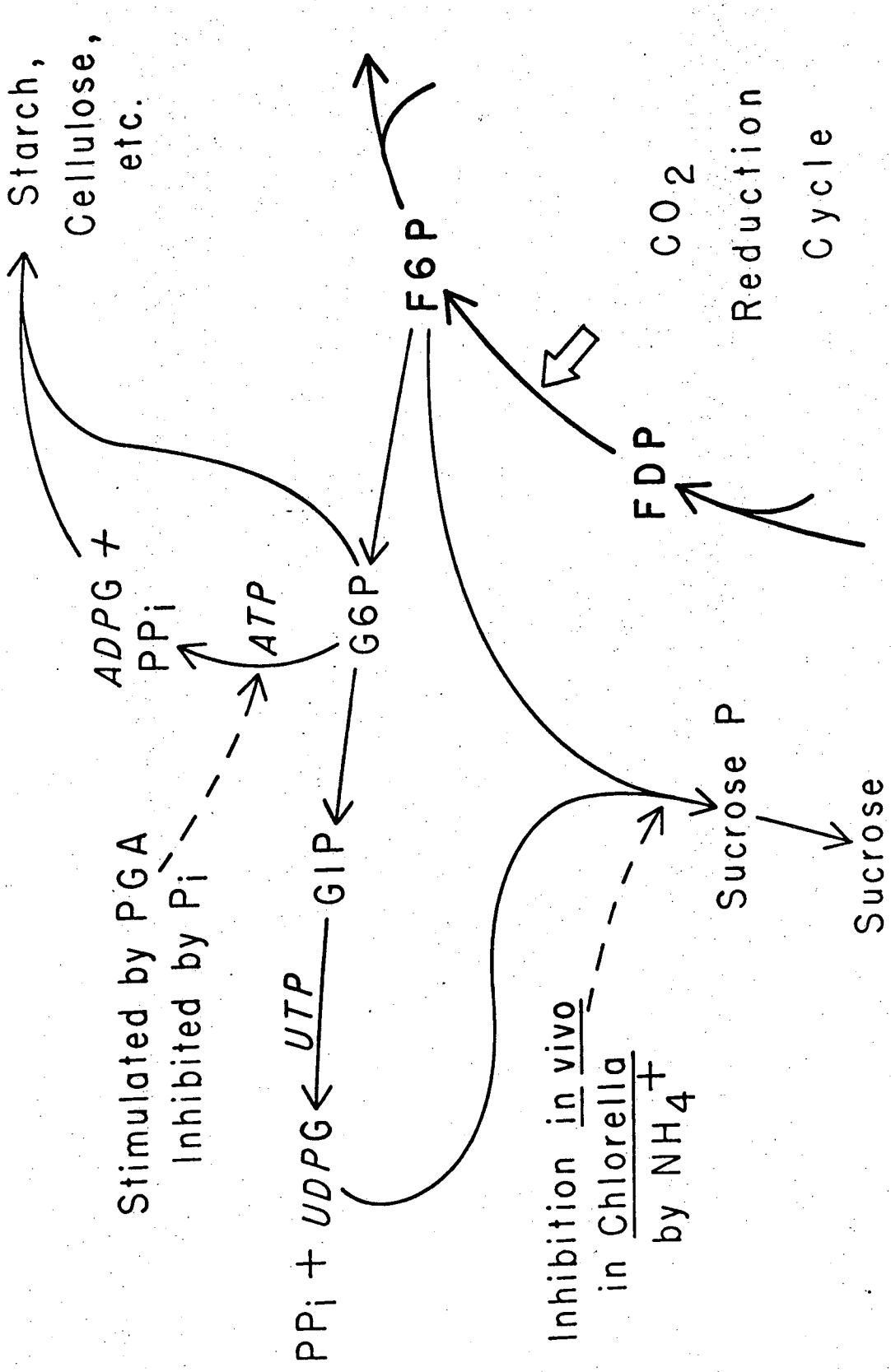

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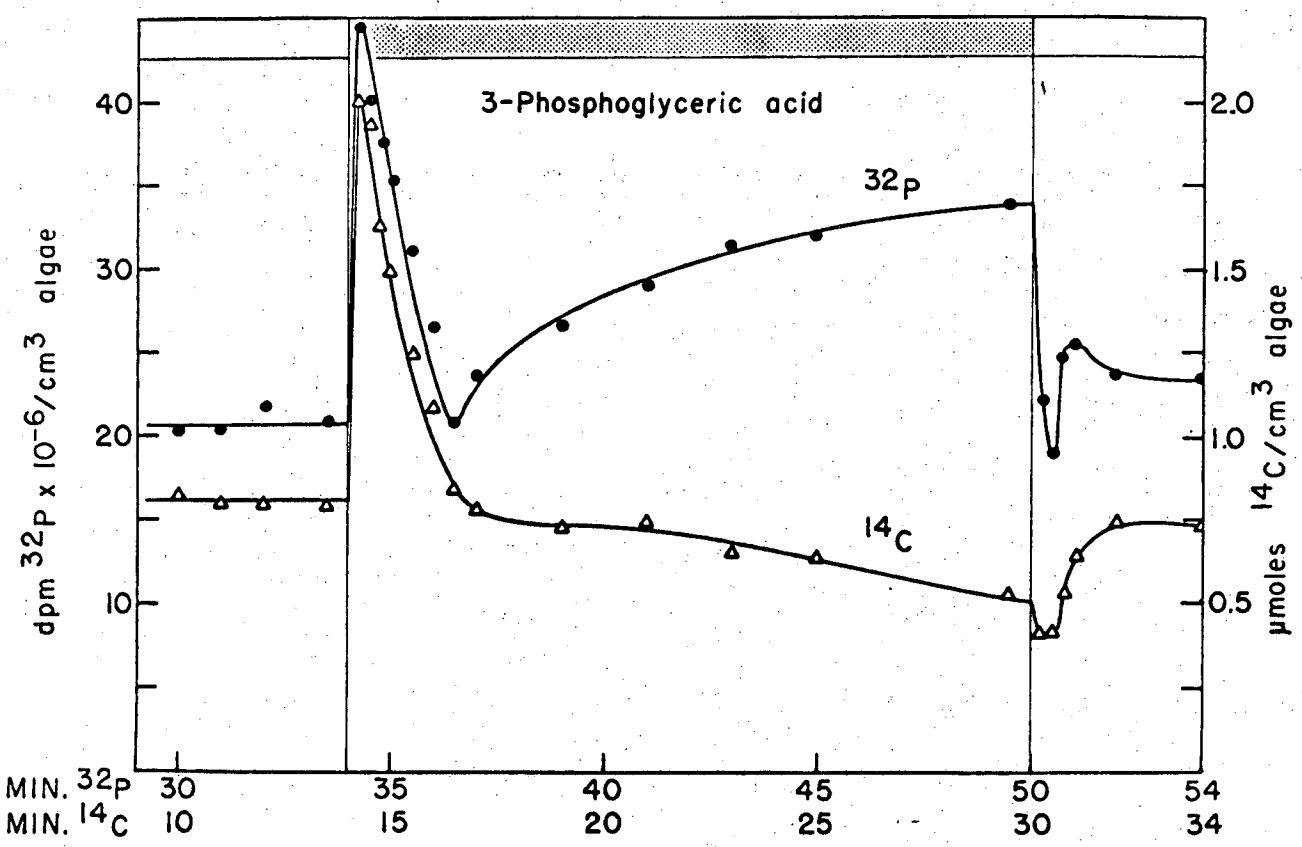
Fig. 3


3-Phosphoglyceric acid
32P
14C
dpm 32P x 10-6/cm3 algae
μmoles 14C/cm3 algae
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30
20
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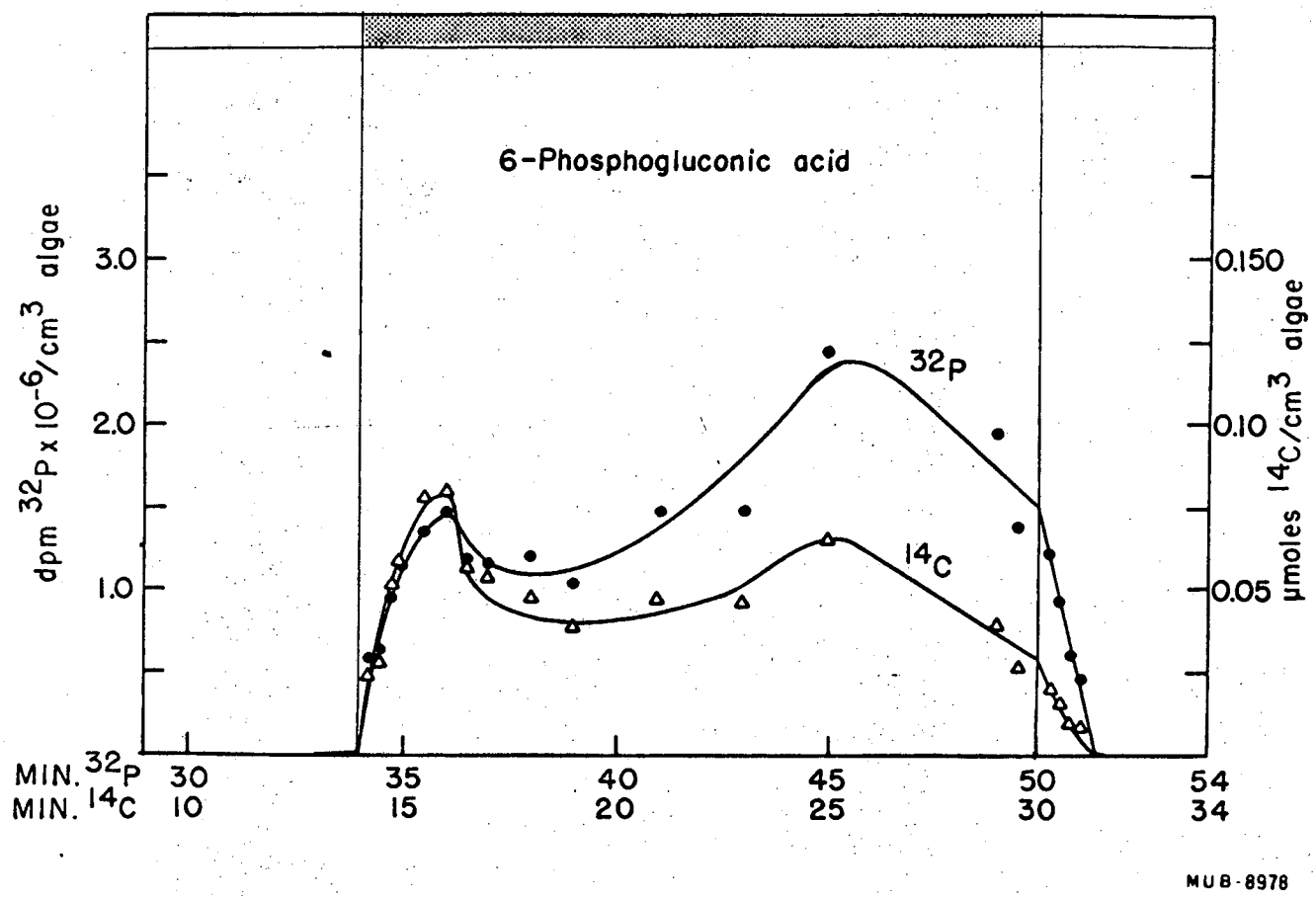
1.0
0.5
MIN. 32P 30 35 40 45 50 54
MIN. 14C 10 15 20 25 30 34
MUB-8974

Fig. 4


6-Phosphogluconic acid
dpm $^{32}P \times 10^{-6}/cm^3$ algae
3.0
2.0
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0.150
0.10
0.05
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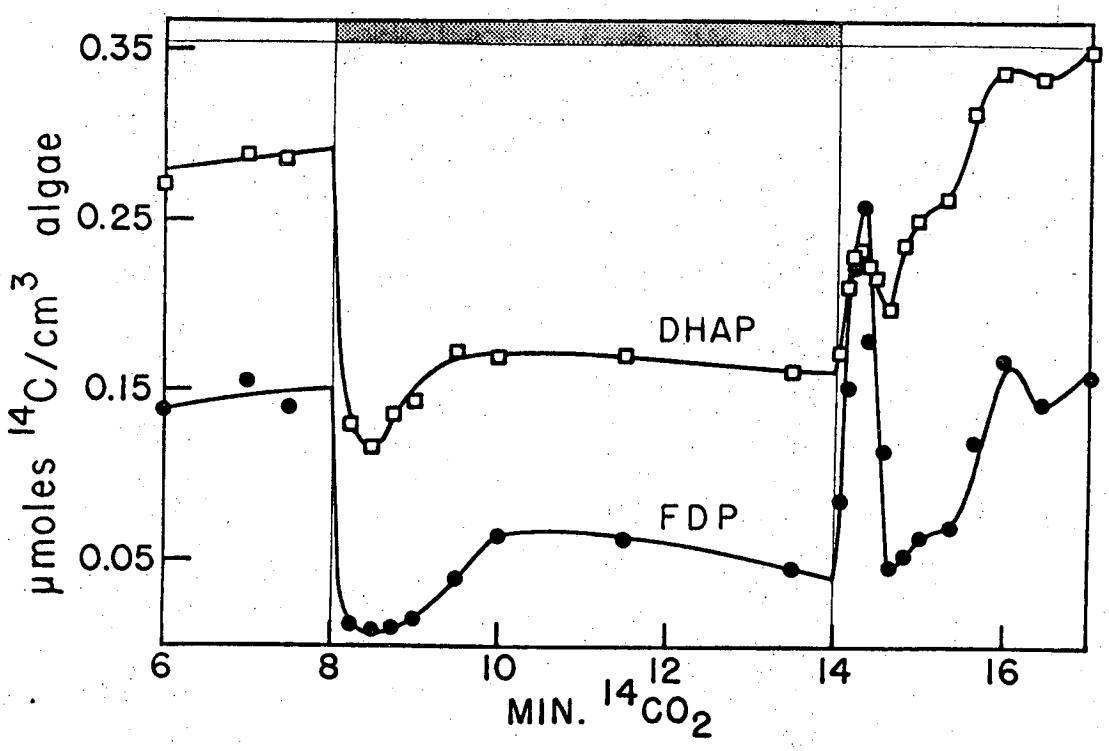
MIN. ^{32}P 30 35 40 45 50 54
MIN. ^{14}C 10 15 20 25 30 34
MUB-8978

Fig. 5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μmoles $^{14}C/cm^3$ algae
DHAP
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10
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14
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MIN. $^{14}CO_2$
XBL 674-1041

Fig. 6

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