[CONTRIBUTION FROM THE RADIATION LABORATORY AND DEPARTMENT OF CHEMISTRY, UNIVERSITY OF CALIFORNIA, BERKELEY]

The Photosynthetic Cycle and Respiration: Light-dark Transients[1]

BY J. A. BASSHAM, K. SHIBATA, K. STEENBERG,[2] J. BOURDON[3] AND M. CALVIN

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Studies of the transient changes in radiocarbon found in various photosynthetic and respiratory intermediates in *Scenedesmus* which result when changing from a condition of steady state photosynthesis in the light to dark and then back to light again indicate the following metabolic mechanisms: (1) The carboxylation step in the carbon reduction cycle of photosynthesis results in the formation of two molecules of 3-PGA from one RuDP molecule, one CO_2 and one H_2O. (2) This carboxylation reaction proceeds for about 30 seconds in the dark after turning off the light, its rate being proportional to the falling concentration of RuDP, and stops when the latter concentration falls to zero. (3) Turning off the light results in the transfer of radiocarbon from PGA to citric acid, and glutamic acid. Turning on the light results in a decrease in radiocarbon in citric acid. These results provide new evidence for the theory that the oxidation of pyruvic acid to acetyl CoA and CO_2 with a subsequent condensation of acetyl CoA with oxaloacetic acid to give citric acid is blocked in the light by reduction of a cofactor, which may be thioctic acid, required for pyruvic acid oxidation. (4) These transients in radioactivity found in Krebs cycle acids are taken as evidence for the association with the chloroplast of enzymes and intermediates of the Krebs cycle.

The carboxylation reaction of photosynthesis was first postulated as the carboxylation of a molecule of ribulose-1,5-diphosphate (RuDP) to produce two molecules of 3-phosphoglyceric acid (PGA) by Calvin and Massini[4] following studies of the steady state in photosynthesis and changes in concentrations of photosynthetic intermediates on cessation of light. This carboxylation was incorporated as a key step in the photosynthetic carbon reduction cycle postulated by Bassham, *et al.*[5] The techniques of kinetic experiments to determine transient changes and changes in steady state levels of various intermediate carbon compounds involved in photosynthesis were improved by Wilson[6,7] who studied these changes as a function of changing carbon dioxide concentration.

In the meantime, partial purifications of the carboxylation enzyme, carboxydismutase, were reported[8-11] and it was established *in vitro* that the carboxylation of ribulose diphosphate results in the formation of only PGA.

The improved techniques of Wilson have now been employed in a new light–dark kinetic experiment for the following purposes: 1. To demonstrate if possible, the *in vivo* formation of *two* molecules of PGA from RuDP and CO_2. 2. To determine the concentration to which RuDP falls in the dark and thus to determine whether or not it is necessary to postulate any special mechanism, other than a negligible concentration of the substrate, RuDP, for the cessation of the carboxylation reaction in the dark. 3. To gain further information regarding the conversion of photosynthetic intermediates to tricarboxylic acid cycle (Krebs cycle) intermediates on turning off the light.

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(2) On leave from Isotope Laboratory, Agricultural College of Norway, Vollebekk, Norway.

(3) Fulbright Fellow, 1954–1956.

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(6) Alexander Thomas Wilson, Thesis, University of California, 1954.

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(8) J. R. Quayle, R. C. Fuller, A. A. Benson and M. Calvin, *ibid.*, **76**, 3610 (1954).

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(10) M. Calvin, R. Quayle, R. C. Fuller, M. Mayaudon, A. A. Benson and J. A. Bassham, *Federation Proc.*, **14**, 188 (March, 1955).

(11) A. Weissbach and B. L. Horecker, *ibid.*, **14**, 302 (March, 1955).

Experimental Part

The techniques employed were similar to those of Wilson[7] except where the system was modified to accommodate the light–dark transient instead of changing CO_2 pressure. A small gas pump forced 1% CO_2 labeled with $C^{14}O_2$ through 79 cc. of suspension of 0.5% (wet-packed volume/suspension volume) *Scenedesmus* in weak phosphate buffer (*p*H 7) in a transparent cell 8.4 mm. thick and 125 mm. in diameter. The gas passed through an ionization chamber with a vibrating-reed electrometer and a Liston–Becker CO_2 analyzer. The signals from these instruments were continuously recorded. The gas circulating system was provided with a large vessel which could be by-passed by means of a four-way stopcock making the measuring system small (for determining the rate of photosynthesis) or could be left in the system to provide a large reservoir of CO_2 and $C^{14}O_2$ compared to that used by photosynthesis during the experiment. The concentration of CO_2 and $C^{14}O_2$ was thus allowed to change only 10% during the entire course of the experiment. The algae cell was illuminated with two 7-inch diameter white fluorescent spiral lights. The cell was equipped with a stopcock for rapidly taking out small aliquots of the algae suspension.

The algae were allowed to photosynthesize for one hour in 1% CO_2 (unlabeled) after which they were quickly flushed with air for 5 minutes. The system was then closed, and the mixture of $C^{14}O_2$ in 1% CO_2 was added. Another 30 minutes was allowed for the algae to photosynthesize with the labeled carbon dioxide in order to saturate all photosynthetic intermediates with C^{14} and to reach a steady state of photosynthesis. The small system (420 cc.) was then employed for a few minutes to determine the rate of uptake of CO_2 and $C^{14}O_2$. The large system (6420 cc.) was again employed and another 20 minutes allowed to re-establish steady state. Aliquots of the algae suspension were then taken at 100-second intervals for 500 seconds. The light was turned off and the aliquots were taken as rapidly as possible (at about 2-second intervals for 30 seconds, then more infrequently for 1500 seconds). The light was then turned on again and aliquots were again taken rapidly.

As soon as each aliquot was taken it was run into methanol for quick killing of the algae. Sample tubes were weighed before and after taking each aliquot to determine its size.

Aliquot samples were subsequently extracted with 80% methanol in water, 20% methanol in water at 70°, and water. Extracts of each sample were concentrated and analyzed by paper chromatography and radioautography. A radioautograph from a light steady state sample and one from a dark steady state sample are shown in Figs. 1 and 2.

Radioactivities of individual compounds were determined by counting with a large-area G.M. tube placed directly on the paper chromatogram in the positions indicated by the radioautograph. When corrected for absorption of radiation by the paper, for size of aliquot and for the specific activity of the carbon dioxide employed, these counts gave

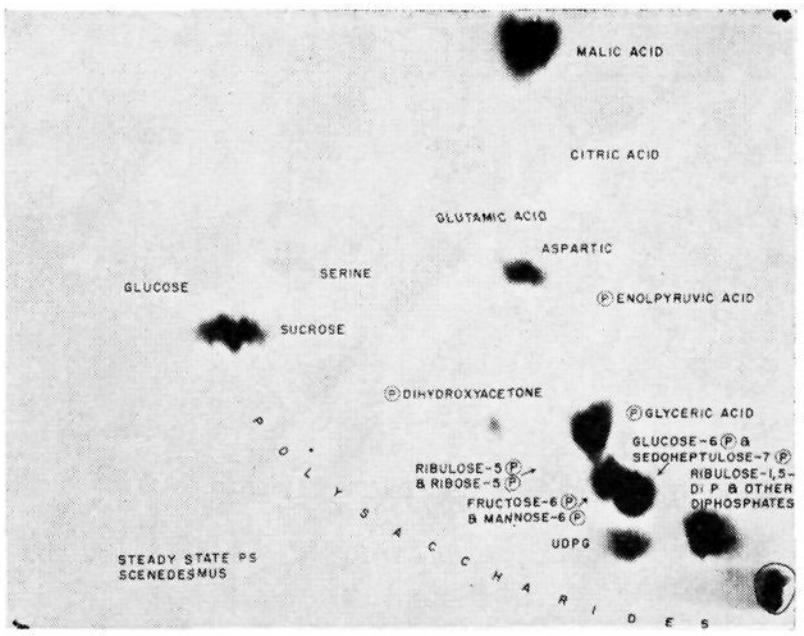


Fig. 1.—Steady state PS *Scenedesmus*.

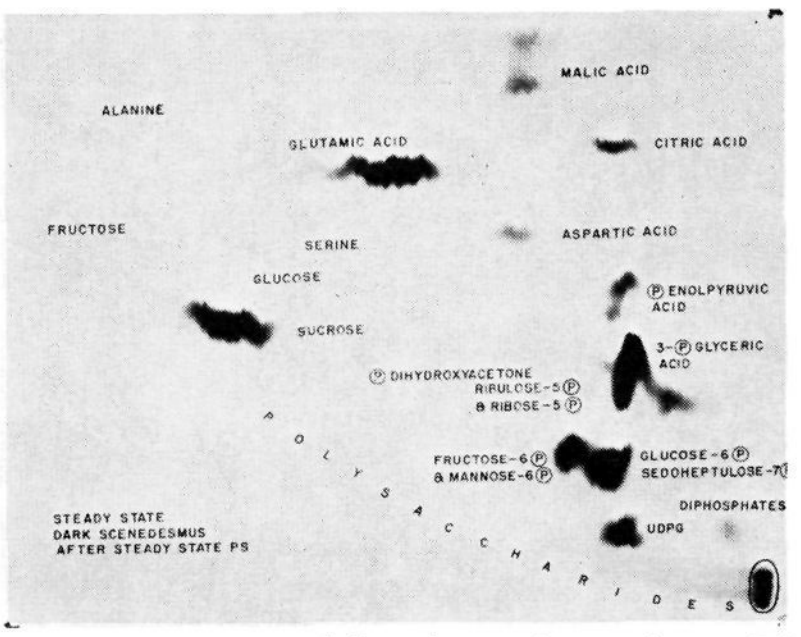


Fig. 2.—Steady state dark *Scenedesmus* after steady state PS.

the concentration of various photosynthetic intermediates in μmoles/cc. of wet-packed algae.

The area of the chromatogram occupied by the sugar diphosphates was eluted and the eluate subjected to enzymatic hydrolysis by phosphatase (purified Polidase-S). The resulting free sugars were then separated by two dimensional paper chromatographs using the same solvent system as for the original plant extract. The resulting ribulose, fructose and sedoheptulose spots were then counted separately.

Results

The steady state concentrations of PGA, RuDP and some other compounds involved in the carbon reduction cycle in photosynthesis are given in Table I. The concentration of ribulose diphosphate drops below a detectable amount in the dark (less than 30 cts./min. in the dark as compared with 5700 cts./min. in the light).

TABLE I

STEADY STATE CONCENTRATIONS OF PHOTOSYNTHETIC INTERMEDIATES AND RELATED HEXOSE PHOSPHATES IN μMOLES/CC. OF ALGAE

Compound	Light	Dark	Change
PGA	1.63	2.42	+0.79
RuDP	0.51	<0.0005	−0.51
Ru5P + Ri5P	.17	.08	−0.09
SDP	.006	.006	0
FDP	.004	.001	−0.003
F6P	.12	.12	0
GDP	.003	.003	0
G6P	.33	.37	+0.04
DHAP + CAl3P	.21	.12	−0.09

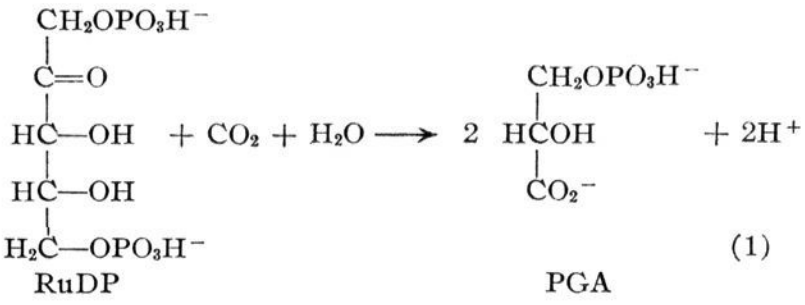
The changes in PGA and RuDP radioactivity (proportional to concentration) are shown in Fig. 3. As can be seen the concentration of PGA rises very rapidly during the first minute and then drops slowly, reaching eventually a steady state dark value somewhat higher than its steady state concentration in the light. The concentration of RuDP drops below measurable limits in about 30 seconds.

The change in PGA concentration on an expanded time scale is shown in Fig. 4. The circles are the first 6 experimental points obtained after turning off the light, while the solid straight lines represent the theoretical rates of change in PGA concentration if its rate of reduction stopped instantly, and if its rate of formation (in moles per minute) were 1.5, 2.0 or 2.5 times the rate of carbon dioxide uptake during steady state photosynthesis.

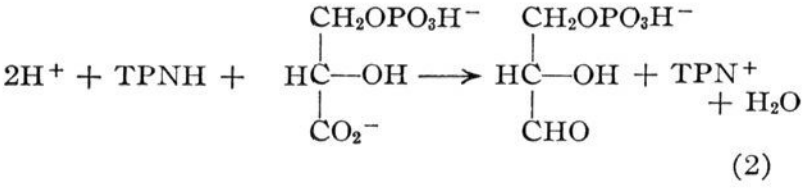
Figure 5 shows the change in radiocarbon in PGA, citric acid and glutamic acid in the same experiment. The radiocarbon found in citric acid and glutamic acid may not be taken as measures of the concentrations of these metabolites since these compounds are not necessarily saturated with radiocarbon during the course of the experiment. The labeling of malic acid (not shown) continued to rise at an essentially unchanged rate on turning the light both off and on.

Discussion

The carboxylation reaction of the carbon reduction cycle in photosynthesis has been written as[4]



and the reduction of PGA as



Reaction 2 is accompanied by the conversion of one ATP molecule to ADP and inorganic phosphate for each molecule of PGA reduced. In addition both TPNH and ATP are described as formed from products of the photolysis of water by the light energy of photosynthesis plus, of course, TPN, ADP and inorganic phosphate, respectively. If the concentrations of TPNH, ATP and the prior compounds formed from the light reaction are small compared to the rate of reduction of PGA, then the reduction of PGA may be expected to stop very quickly when the light is turned off. In contrast, reaction 1 which requires no reducing agents or energy-carrying compounds but is de-

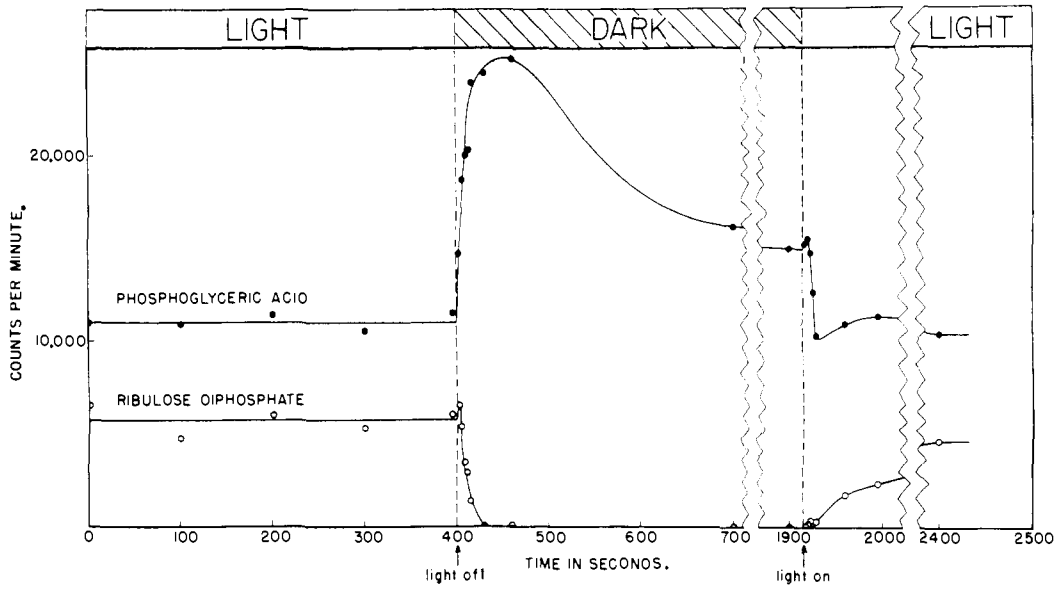


Fig. 3.—Light–dark transients in phosphoglyceric acid and ribulose diphosphate concentrations.

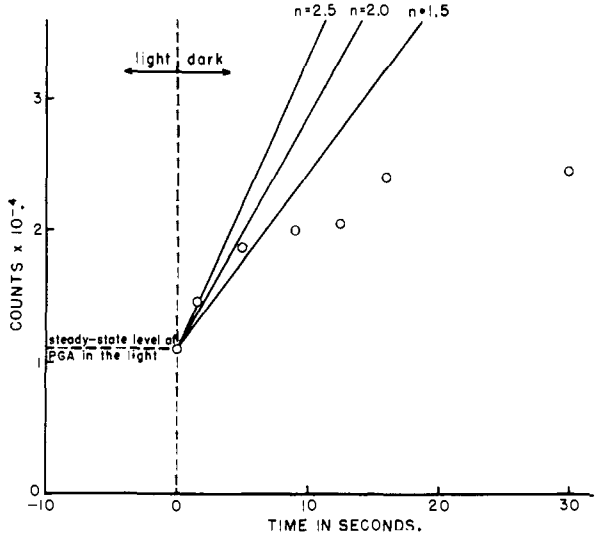


Fig. 4.—Light–dark transients in phosphoglyceric acid concentration (expanded scale).

pendent only on the concentration of RuDP and CO_2, may be expected to continue for a few seconds at its normal rate during photosynthesis even though the light is turned off. The rate of reaction 1 will decrease after a few seconds owing to the decrease in concentration of RuDP. The decrease in RuDP concentration is the net result of its continued carboxylation and diminished rate of formation.

The rate of change of PGA concentration on turning off the light is the difference between the rates of reactions 1 and 2. If reaction 2 stops almost immediately, PGA concentration will increase at a rate which is directly proportional to reaction 1.

Experimentally, the rate of change of PGA on turning off the lights is about twice the rate of CO_2 fixation in the light and this may be taken as evidence that *in vivo* two molecules of PGA are indeed formed for each molecule of CO_2 entering the cycle *via* the photosynthetic carboxylation reaction. The decrease in the rate of change of PGA concentration which follows during the next few seconds is comparable with the drop in RuDP concentration, and after about 30 seconds the RuDP concentration drops to zero, the carboxylation stops, and PGA concentration stops rising.

The subsequent drop in PGA concentration can be explained in terms of the oxidation of PGA *via* pyruvic acid to CO_2 and acetyl CoA. This reaction results in the formation of Krebs cycle intermediates and a rapid rise in radiocarbon in citric acid, which is the first product of the condensation of oxaloacetic acid with acetyl CoA.

The radiocarbon found in citric acid is never very high, and it appears that most of the radiocarbon transferred from the PGA pool finds its way into glutamic acid, which may be thought of as a storage reservoir for the Krebs cycle. In fact, the increase in radiocarbon found in glutamic acid in the dark is roughly equivalent to the decrease in PGA radioactivity from its peak transient value (after turning off the light) to its steady state dark value. No final conclusion can be drawn from this relationship since one-third of the PGA radiocarbon is lost from the system in the oxidation of pyruvate to acetyl CoA and CO_2 and since new radiocarbon may be introduced into the system by carboxylation of pyruvic acid at an accelerated rate during the transient period. None the less, the appearance in the dark of radiocarbon in Krebs cycle intermediates at the expense of a decrease in radiocarbon in photosynthetic intermediates strongly indicates once again that some mechanism for the prevention of such conversion exists during illumination and is altered to permit conversion when the illumination is stopped. In order to understand what this control mechanism might be it is necessary to consider photosynthesis—respiration relations. Some of these possible relations are indicated in Fig. 6.

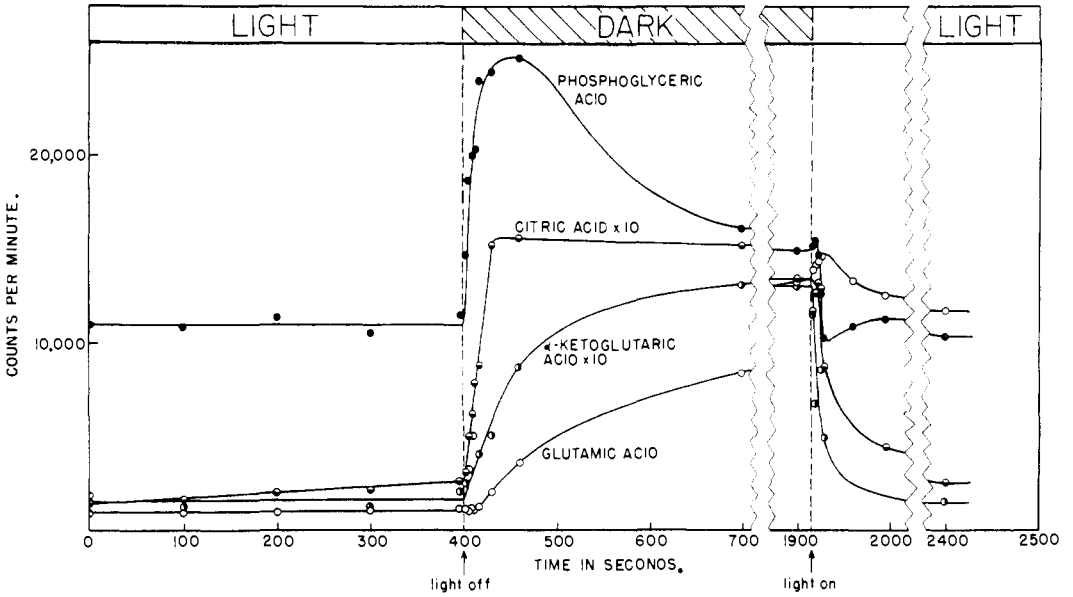


Fig. 5.--Light-dark transients in phosphoglyceric acid, citric acid and glutamic acid, C^{14}-labeling.

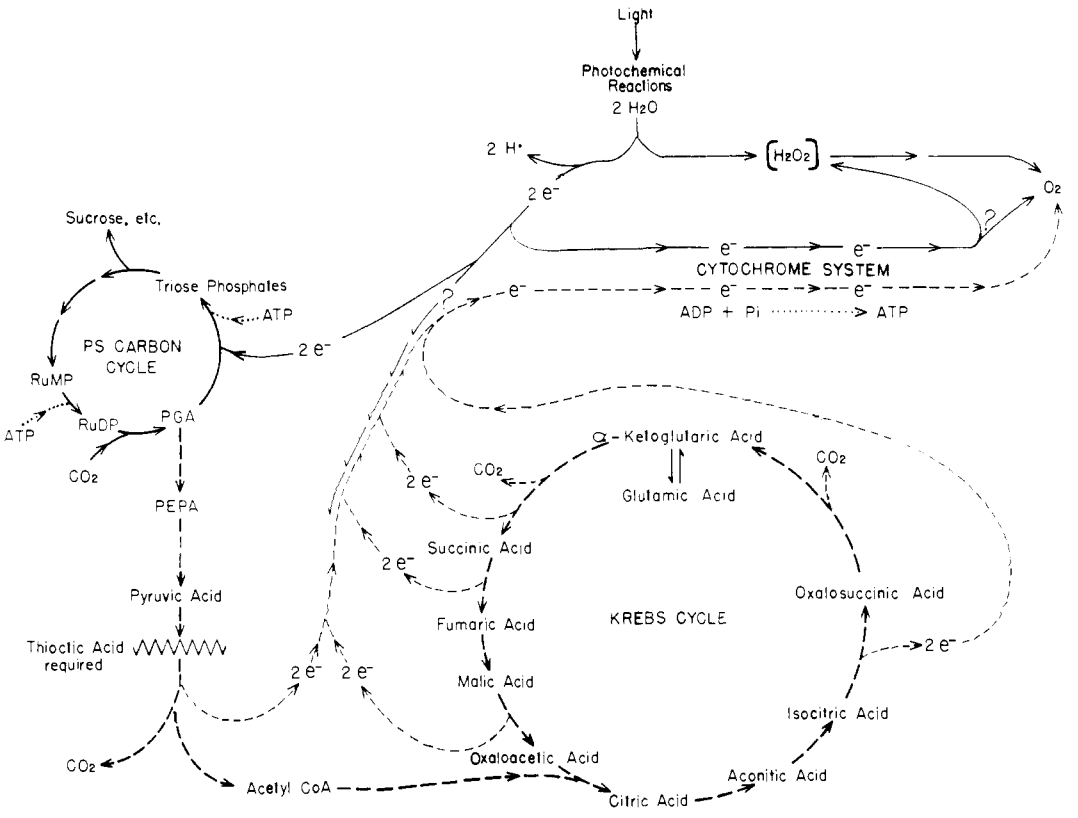


Fig. 6.—Relations between photosynthetic and respiratory metabolism.

The important investigation by Brown,[12] who employed O-18 in studies of photosynthesis and respiration rates, demonstrated that over-all respiration of chlorella was constant during alternate 15- and 20-minute periods of light and dark, over a wide range of light intensities. It may be inferred from this finding that oxidation of hexoses *via* the normal glycolytic pathway through triose, PGA and pyruvic acid, and, thence, *via* acetyl CoA and the Krebs cycle continues at very nearly the normal rate in the light, at least in the cellular space outside the chloroplast. We can also state the premise, now fairly well established experimentally, that the enzymes of the carbon reduction cycle and the reservoirs associated with this cycle

(12) A. H. Brown, *Proc. J. Botany*, **40**, 719 (1953).

are contained in or on the chloroplast and are more or less isolated from the metabolism of the cell outside the chloroplast.[13] With this arrangement in mind one might suggest the following ways in which light could inhibit transformation of "Photosynthetic" carbon to "respiratory" carbon:

(A) Light might produce a general inhibition of the Krebs cycle by supplying to the electron transport system (cytochrome, etc.) electrons from the primary photochemical reductant (formed by photolysis of water) in place of electrons normally obtained from the various oxidative steps of the Krebs cycle. Since we know there is no general inhibition of respiration in the light, this would require a special set of Krebs cycle enzymes and intermediates within the chloroplast, with a contribution of respiration insignificant compared with the cell's total respiration.

(B) A light–inhibition of the oxidation of pyruvic acid derived from the photosynthetic pool. The mechanism of this inhibition might be a substitution of other electrons from the photochemical reaction for those derived from an oxidation of pyruvic acid as suggested in (A) above. More particularly, a specific cofactor, active only in its oxidized state, might be converted wholly to a reduced form in the light. Thioctic acid (pyruvic acid oxidase) has been suggested as the specific cofactor involved in this way.[14]

(C) Light may inhibit the diffusion of photosynthetic intermediates from within the chloroplast to the cellular space outside, where they are converted to Krebs cycle intermediates by normal respiration.

A specific mechanism for this inhibition (C) might be a lowered rate of diffusion of PGA out of the photosynthetic pool in the light due to the smaller concentration of PGA in the light as compared with its concentration in the dark. As can be seen from Fig. 5, however, the concentration of PGA in the photosynthetic pool in the dark becomes at most only about twice its concentration in the light, whereas the rate of increase of citric acid concentration just after turning off the light is some twenty times the rate of its increase in the light. Consequently the possible change in concentration gradient of PGA between photosynthesis and other pools is too small by at least a factor of ten to account for the accelerated rate of labeling of citric acid on a basis of diffusion alone.

Furthermore, from the observation that citric acid and glutamic acid decrease in concentration when the light is turned on again we can eliminate (C), since it is hardly conceivable that light could convert Krebs cycle intermediates outside the chloroplasts back into photosynthetic ones in view of the lack of effect of light on the respiration rate.

(D) An increase in pyruvic acid concentration resulting from an increase in PGA concentration or turning off the light might result in a greater rate of $C_3 + C_1$ carboxylation reaction to give oxalacetic acid and/or malic acid. The same arguments can be used against this possibility as in the case of (C). The concentration of pyruvic acid and hence the rate of the $C_3 + C_1$ carboxylation reaction should not increase by more than the increase in PGA concentration: namely, twofold. Moreover, in the case of malic acid, the carboxylation reaction is reductive and might be expected to decrease in rate if affected at all by turning off the light (if we hold to our assumption of a greater supply of reductive electrons in the photosynthetic apparatus in the light than in the dark). Finally the pronounced effects on labeling of Krebs cycle intermediates on turning the light on and off were found in citric acid and glutamic acid but not in malic acid. It seems reasonable to suppose that the $C_1 + C_3$ carboxylation reaction is indeed accelerated, but as a secondary effect which maintains the malic acid level as malic acid is oxidized to oxalacetic acid at an increased rate to supply the four-carbon fragment for the dark-stimulated $C_2 + C_4$ condensation.

A tentative choice may be made between (A) and (B) on the basis of the decrease in labeling of citric acid on turning on the light again. If there were a general blocking of the Krebs cycle in the light owing to the photochemical supply of electrons to the electron transport system, then the oxidation of citric acid should be blocked on turning on the light again and we should not see the rapid decrease in labeling of citric acid which then occurs. The more probable mechanism for light inhibition of conversion of PGA to Krebs cycle intermediates thus appears to be (B), in which the formation of acetyl CoA from pyruvic acid is blocked. Moreover, this block is most likely accomplished by reduction of a specific cofactor (*i.e.*, thiotic acid) rather than by a general competition for the electron transport system by electrons supplied by photochemical reaction since the latter mechanism again is not consistent with the decrease in radioactivity in citric acid when the light is turned on.

Finally, the fact that the amount of radiocarbon in the Krebs cycle acids goes down when the light is turned on as well as up when the light is turned off indicates that there is a complete system of Krebs cycle enzymes and reservoirs associated with the chloroplast.

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(13) R. C. Fuller, University of California Radiation Laboratory Report, U.C.R.L. 2932, March 30, 1955.

(14) M. Calvin and J. A. Barltrop, THIS JOURNAL, **74**, 6153 (1952).