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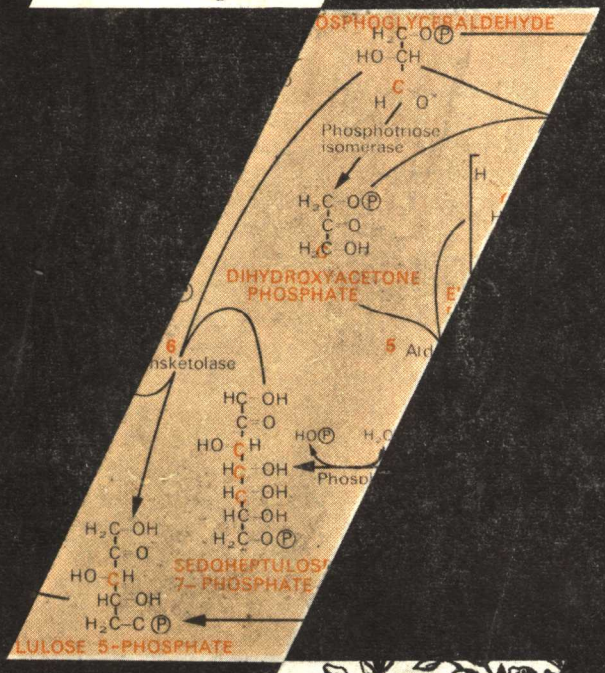
Photosynthesis

C.P. Whittingham



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Introduction

The earliest investigations of the reactions of green plants, beginning with those of Priestley, showed that when they were exposed to light the process of respiration exhibited by all other living organisms was reversed, that is to say, in the light they take up carbon dioxide and evolve oxygen. The carbon taken up is incorporated into organic carbon compounds and in the broadest sense all the organic materials of the green plant may be considered to be the products of photosynthesis. Before biosynthesis can take place, the carbon dioxide must be reduced and this is balanced by a corresponding oxidation in which hydrogen is removed from water and oxygen is evolved. The energy required is provided ultimately from the light energy absorbed by the plant pigments. The considerable energy bound by the process of photosynthesis becomes most apparent in the process of combustion of wood or coal, products of past photosynthesis, but when it is controlled by organized systems of enzymes it powers the respiration and biosynthesis of all the non-photosynthetic organisms. Thus photosynthesis is not only the indirect source of most of the organic matter on earth; it is also the primary source of the energy of all living things.

The earliest investigations included quantitative determinations of the amount of carbon dioxide taken up relative to that of oxygen liberated. Under a wide range of conditions and with many different plants, the volume of oxygen liberated was found to be equal to that of the carbon dioxide absorbed. More recent experiments using water distinguished by isotopic labelling (see Fig. 1) show that the oxygen comes almost wholly from the water, indicating that the equation should more correctly be written:

$$CO_2 + 2H_2O \rightarrow CH_2O + H_2O + O_2$$

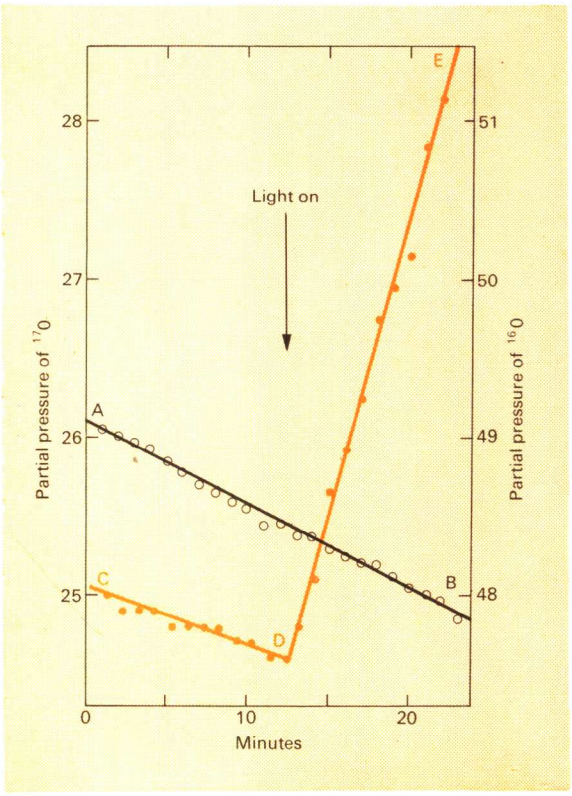
not $CO_2 + H_2O \rightarrow (CH_2O) + O_2$.

Prior to the Second World War there was little definitely known concerning the biochemistry of photosynthesis. Indeed it was generally believed that photosynthesis was a special kind of chemical process entirely peculiar to the green plant.

Although it proved relatively easy to obtain from yeasts cell-free preparations capable of catalysing *in vitro* partial reactions of the process of respiration, by 1937 people had only just begun to obtain preparations from green plants which would effect any part of photosynthesis. At first attention was therefore confined to the study of the kinetics of the process *in vivo*. This led F. F. Blackman in 1905 to postulate a dark thermochemical process, involving the uptake of carbon dioxide, and a separate light reaction, the products of which drive the dark reaction in the direction of synthesis.

The dark reactions include all those concerned with the conversion of carbon dioxide to organic chemical compounds. These have been greatly clarified by the use of radioactive isotopes of carbon, which became generally available only after 1945. The light reactions utilize light energy to provide the necessary chemicals of high energy content which are able to direct the synthesis of organic compounds from carbon dioxide against the natural thermochemical tendency for the reverse to take place. Finally there are the reactions concerned in the evolution of oxygen from water. Even at the present time, our knowledge of this last group of reactions is extremely small.

Consequently this discussion of our present knowledge will fall essentially into two parts: the reactions involving carbon incorporation and reduction, and secondly the reactions involving the utilization of light energy to generate high energy chemical intermediates.



After Van Norman and Brown (1952). *Plant Physiol.* **27**, 691.

FIG. 1. Differentiation between the simultaneous processes of oxygen uptake due to respiration and of oxygen production due to photosynthesis in light, using oxygen isotopes. The isotope ^{17}O was present in the gas phase, and its consumption was proportional to the rate of respiration (line A–B). The isotope ^{16}O was present in both the gas phase and the water. It was consumed in the dark (line C–D) but produced in the light (line D–E) due to photosynthesis.

THERMAL REACTIONS INVOLVING CARBON

The isotope ^{11}C has a half-life of the order of 20 minutes. Ruben, Kamen, and Hassid used it in 1938 to study carbon metabolism in photosynthesis. They showed that almost all the carbon appeared during short-time fixation in the carboxyl (COOH) group of an acid but they could not identify the compound further. After 1945 Calvin was able to use the recently discovered isotope ^{14}C (half-life 5000 years) to study photosynthesis.

The isotope was added to photosynthesizing leaves or algae and the compounds which became radioactive were identified. With both barley leaves and algae during short periods of photosynthesis certain amino acids (alanine, aspartate, glutamate, glutamine, glycine, and serine), carboxylic acids (succinate, fumarate, glycollate, isocitrate, and malate), and sucrose and phosphate esters all became radioactive. There was some indication that barley leaves and *Chlorella* did not behave in exactly the same way. It was decided that *Chlorella* was an easier system to work with and most of the subsequent measurements were made with *Chlorella* grown in the laboratory in air enriched with 5% carbon dioxide.

The algal suspension is placed in a so-called 'lollipop', a vessel made of two sheets of perspex bolted together. Radio-carbon dioxide is injected into this and samples taken after given times of fixation. The cells are normally killed by running them into boiling alcohol.

After only five seconds of photosynthesis, five different compounds became radioactive; alanine, malic acid, sucrose, phosphorylated sugars containing five or seven carbon atoms, and phosphoglyceric acid (PGA), a three-carbon compound. An attempt was made to try and cut the time shorter, and thus to limit radioactivity to only one compound. If the time was short enough the carbon would only have time to enter the first photosynthetic compound and would not get any further; in this way the first formed compound could be identified. In practice this proved to be impossible.

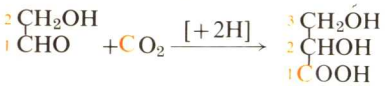
Calvin then noted that in a reaction sequence of the type

$$a + CO_2 \rightarrow b \rightarrow c \rightarrow d$$

in the very first second of adding the radioactivity it will all pass into *b*, but not beyond. Hence *b* has 100% of the radioactivity at zero time. Conversely *c* starts with nothing, because when *b* has 100%, clearly *c* must have zero. The % in *c* begins to rise as the % in *b* falls. If the final products are represented by *d*, then ultimately all the radioactivity will flow through and end up in *d*, and the % in *d* will rise to 100. The experimental data are shown in Fig. 2, from which *b* is identified as phosphoglyceric acid, *c* as the sugar phosphates, and *d* as the amino acid alanine, malic acid, and sucrose (not shown).

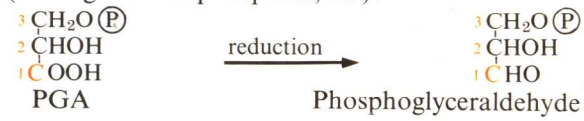
There is a finite limit to the speed with which an experiment can be completed. After only 1·7 s of photosynthesis, over 70% of the carbon incorporated was in PGA. It is conceivable that the radioactivity in another compound could have fallen to zero from 100% even within this short time and that that in PGA had risen from zero. This experiment gives an indication of the sequence of the intermediates but is not a complete proof that PGA is the first formed compound in photosynthesis.

The second line of evidence pursued was to discover which atoms in each molecule were radioactive. By suitable chemical techniques, the three carbon atoms of PGA can be obtained in separate fractions each representing one carbon only. With the shortest times of feeding, that is between 1 and 5 s, all the radioactivity was found in the carbon atom of the carboxyl group C-1. This would be consistent with the formation of PGA by carboxylation of a two-carbon precursor group, e.g. such a reaction as

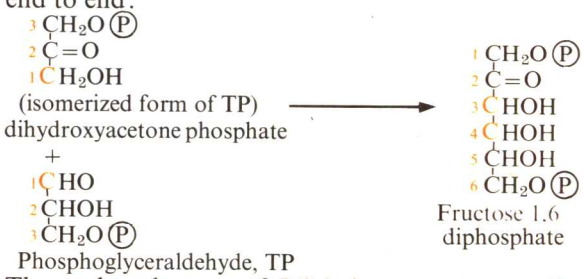


Calvin and his colleagues spent five years looking for such a precursor but without success.

The overall process of photosynthesis is the formation of a sugar containing an aldehyde (−CHO) group rather than an acid containing a carboxyl group. (A sugar is an organic compound containing one or several alcohol groups >CHOH, and either an aldehyde group −CHO (e.g. glucose, ribose), or a ketone group >C=O (e.g. fructose, ribulose)). Note that the numbering of the carbon atoms of sugars and related compounds begins at the most oxidized end of the molecule.) Therefore there must be a reduction reaction to produce the corresponding aldehyde phosphoglyceraldehyde (the sugar triose phosphate, TP):



If the phosphoglyceraldehyde is derived by direct reduction, it follows that the aldehyde group should contain all the radioactivity of the molecule which was previously confined to the carboxyl group. By reversing some of the known reactions of the Embden–Meyerhof–Parnas pathway of respiration two molecules of triose phosphate can form one molecule of fructose 1, 6-disphosphate by joining end to end:



The carboxyl atom of PGA becomes eventually both the C-3 and C-4 atoms of the hexose sugar fructose diphosphate, and initially radioactivity should be totally confined to these atoms. When the fructose sugar formed in a short 5 s period of photosynthesis is degraded, it is indeed found that all the label is in the C-3 and C-4 atoms.

If the reaction is continued for up to 30 s or 1 min, the pattern begins to change. Radioactivity now appears in the other carbon atoms of the phosphoglyceric acid and also in the C-1 and C-6 and the C-2 and C-5 atoms of the fructose. This must mean that if PGA is formed by carboxylating a precursor, the precursor also becomes radioactive after 30 s. Therefore there must be some

feedback from the products to form more precursor molecules.

It was realized by this time that the acceptor for carbon dioxide need not contain only two carbon atoms; it could just as well contain five, and be regarded as a two-carbon moiety joined to a three-carbon moiety. If on carboxylation the product split, we should get one molecule of PGA with new carbon introduced from the CO_2 and a residual three-carbon moiety, unlabelled.

5C precursor $+CO_2 \rightarrow$ PGA + PGA

The five-carbon (5C) sugar ribulose 1,5-diphosphate could serve as such an acceptor molecule. A reaction mechanism for forming this from the products hexose and triose via the pentose phosphates was therefore postulated by Calvin. About the same time the enzymes which Calvin had postulated to be required for such a sequence were discovered by Ochoa, Racker, and others.

The 5C precursor ribulose 1,5-diphosphate is carboxylated giving two molecules of PGA (reaction 1, Fig. 3). With the addition of phosphate bond energy (ATP) and reducing power from $NADPH_2$, both provided by the light reaction of photosynthesis (as explained later), PGA is reduced to the triose sugar 3-phosphoglyceraldehyde phosphate (reaction 2). This then becomes involved in four reactions:

(i) Two molecules of the triose phosphate condense, producing the hexose (6C) sugar fructose (reaction 3).

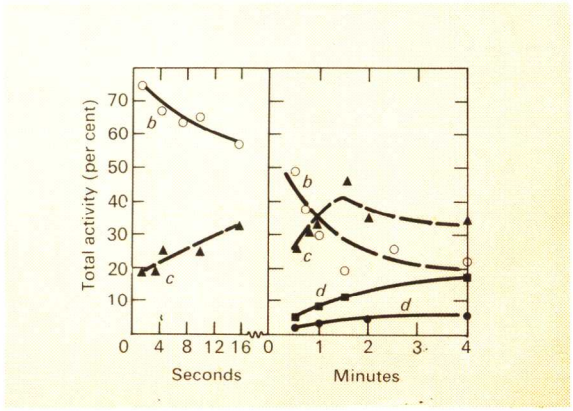
(ii) One molecule of triose phosphate reacts with the fructose 6-phosphate formed in (i), producing the 4C sugar erythrose phosphate and the 5C sugar xylulose phosphate (reaction 4).

(iii) One molecule of the triose phosphate, in its isomeric form of dihydroxyacetone phosphate, condenses with one molecule of erythrose phosphate produced in (ii), giving the 7C sugar sedoheptulose diphosphate (reaction 5).

(iv) One molecule of triose phosphate reacts with one molecule of sedoheptulose phosphate, producing two 5C (pentose) sugars, ribose 5-phosphate and xylulose 5-phosphate (reaction 6). (Xylulose phosphate has also been produced in (ii).)

All the pentose phosphates are converted into the 5C sugar ribulose 5-phosphate (reactions 7 and 8), which, with phosphate donated by ATP, becomes the carbon dioxide-accepting ribulose diphosphate (reaction 9).

Each turn of this cycle requires for an input of one CO_2 molecule the energy from three ATP molecules and the donation of hydrogen from two molecules of $NADPH_2$. Five out of every six PGA molecules produced by one turn are reconverted to pentose in the cycle; the sixth can either be fed into the metabolic pool or upgraded to hexose and polysaccharides (arrow on bottom right of Fig. 3).



After Calvin *et al.* (1951). *Symp. Soc. exp. Biol.* **5**, 284, and Bassham *et al.* (1954). *J. Am. chem. Soc.* **76**, 1760.

FIG. 2. The percentage radioactivity incorporated in different compounds plotted against time, during photosynthesis in the alga *Scenedemus* in the presence of $^{14}CO_2$. ○ = phosphoglyceric acid. ▲ = sugar phosphates. ■ = malic and aspartic acids. ● = alanine. *b*, *c*, and *d* are the products of the reaction $a + CO_2 \rightarrow b \rightarrow c \rightarrow d$.

If sedoheptulose is formed in this way, initially it ought to be equally labelled in its C-3, C-4, and C-5 atoms. Two molecules of the ribose phosphate would be labelled in the C-3 atom only, and one in C-1, C-2, and C-3. The actual patterns of radioactivity within the molecules observed are consistent with this reaction mechanism.

A third line of evidence came from the study of the effect of sudden changes in the experimental conditions in which the reaction was taking place. According to the theory just discussed, carbon dioxide only enters the cycle at one point, the carboxylation of ribulose 1,5-diphosphate to form two molecules of PGA. If the concentration of carbon dioxide were suddenly reduced, the reaction sequence would be broken; ribulose diphosphate would accumulate and PGA decrease. Fig. 4 shows the effect in *Chlorella* when the concentration of carbon dioxide is suddenly decreased from 4% to 0·03%. In about 40 s, the radioactivity of PGA falls and that in ribulose diphospate rises; with longer times more complex changes take place.

A similar situation would apply if the light were suddenly turned off. Since light energy ultimately supplies the material ($NADPH_2$) that reduces

PGA to triose phosphate, if the light intensity were reduced the radioactivity of the sugar phosphates should fall and that of PGA should rise. Again this has been observed experimentally.

ATP and $NADPH_2$ are essential components of the Calvin cycle. The two most important reactions in the cycle are (a) the carboxylation of ribulose 1,5-diphosphate to form PGA, and (b) the reduction step PGA→triose phosphate, which is essentially the reverse of the oxidative reaction which takes place in respiration. It is catalysed by triose phosphate dehydrogenase but whereas in mitochondria the coenzyme is normally NAD, in the chloroplast it is NADP. In respiration the oxidation is coupled to phosphorylation and the generation of ATP; hence in the reverse direction a supply of ATP is required.

Photosynthesis in isolated chloroplasts

Isolated chloroplasts are free of all complications of respiration, and represent the simplest photosynthetic system. The kinetics of the system, e.g. the effect of light intensity or of carbon dioxide concentration on the rate, are most easily observed with chloroplast preparations.

After 1950, chloroplasts were isolated which retained their ability to catalyse certain biochemical processes. Arnon and his collaborators showed that chloroplasts isolated from spinach leaves in aqueous medium would fix carbon dioxide, if the liquid from which they had been separated was concentrated and added to them. Without the concentrate little fixation was observed. If the chloroplasts were first illuminated and then the extract added in the presence of $^{14}CO_2$ in the dark, fixation took place. Thus the reaction between plastid and light was separated from the reactions in which CO_2 was fixed. Further, the light stage could be omitted altogether if ATP and $NADPH_2$ were added to the chloroplasts. This means that light is essential only for the reduction of PGA to phosphoglyceraldehyde (triose phosphate), and plays no subsequent part in the photosynthetic process; and its essential role can be replaced by ATP and $NADPH_2$.

Spinach leaves photosynthesize at approximately 150 μmoles CO_2/mg chlorophyll/h, whereas the best rates of carbon fixation for isolated chloroplasts were at the time less than 1.

Walker came to the conclusion that it was essential to try to keep chloroplasts as intact as possible in order to preserve their biochemical potentialities. To do this it was necessary to use plants from which chloroplasts can be extracted with minimal damage. Chloroplasts are isolated by centrifugation, and when this is done the starch grains inside them act like small bullets, puncturing them. This

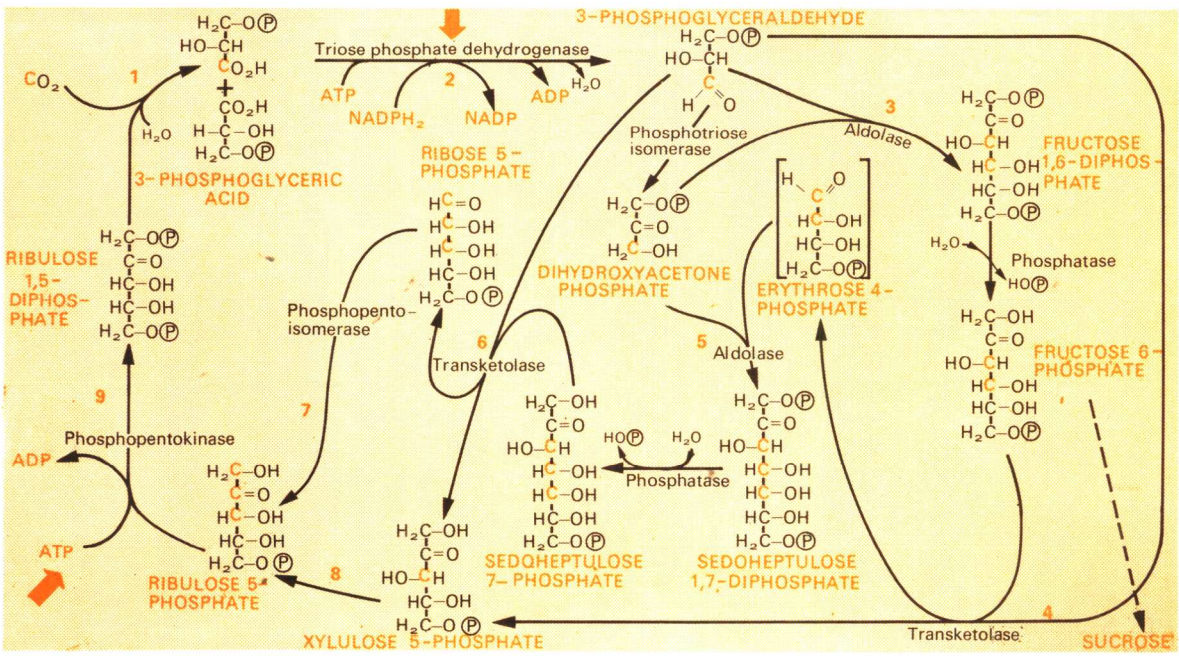


FIG. 3. Photosynthetic carbon reduction cycle (Calvin cycle). ⬆ = drive from light reaction.

difficulty was overcome by using pea plants grown under conditions of low light and low temperature as these are relatively starch-free. Under the light microscope, such preparations showed two kinds of chloroplast, some surrounded by a halo, others in which the internal granular structure is visible. The halo was thought to be due to reflection by the outer membrane; the others had lost their outer membranes. By making different preparations and comparing the proportion of chloroplasts of each type with activity, it was shown that the preparations with the most intact membranes were best able to fix $^{14}CO_2$.

These preparations and similar ones from spinach made by Jensen and Bassham in America showed rates of carbon fixation as good as, and some times in excess of, the rate for intact leaves.

Jensen and Bassham and others compared the products of photosynthesis in whole spinach leaves fed $^{14}CO_2$ with those of chloroplasts isolated from the same leaves. Sucrose, the most abundant product of photosynthesis in the leaf, was completely absent from the chloroplast preparation, as were the amino acids serine and glycine. In fact the chloroplasts manufacture only an isomer of PGA called dihydroxyacetone phosphate, the monophosphates of the hexose sugars, and a small quantity of sugar diphosphates.

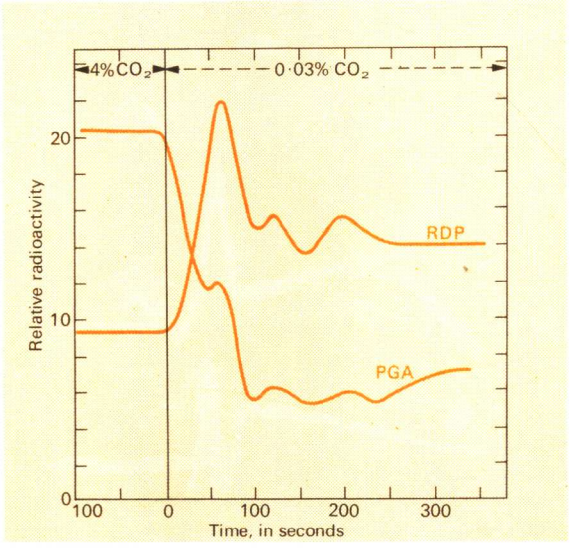


FIG. 4. Changes in the concentration of phosphoglyceric acid (PGA) and ribose diphosphate (RDP), following a reduction of carbon dioxide concentration. (After Wilson and Calvin (1955). *J. Am. chem. Soc.* **77**, 5948.)

This suggests the possibility that in the synthesis of sucrose in the intact leaf, there may be some transport of carbon compounds between chloroplast and cytoplasm.

In some plants the Calvin cycle may not be the whole story

There is increasing evidence that in some plants, a considerable incorporation of radioactivity into other compounds takes place before incorporation into PGA. In sugar cane, Hatch and Slack confirmed that initial fixation was largely into malic acid and aspartic acid:

^4COOH	^4COOH
$^3CH_2$	$^3CH_2$
^2CHOH	$^2CH.NH_2$
^1COOH	^1COOH
Malic acid	Aspartic acid

In one second as much as 93% of the ^{14}C incorporated was in these intermediates; only subsequently did radioactivity appear in PGA. Furthermore this sequence was not greatly affected by appreciable changes in carbon dioxide concentration or of light intensity. The radioactivity was largely incorporated into C-4 of the malate and then later into C-1 of PGA. If the plants were now transferred to ordinary carbon dioxide, the total counts remained almost unchanged, but there was a large decrease in the radioactivity present in

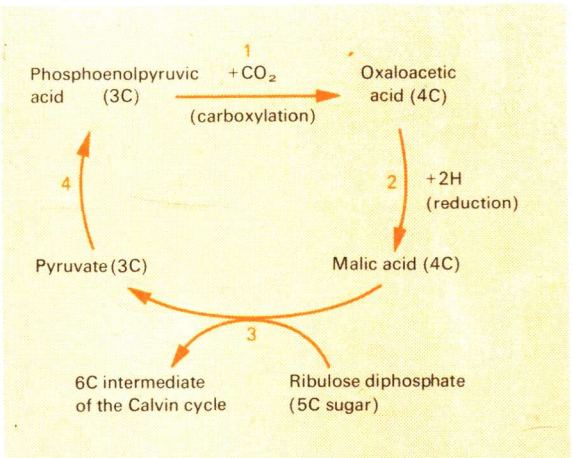


FIG. 5. Accessory cycle to the Calvin cycle in some plants. CO_2 is used to produce oxaloacetic acid from phosphoenolpyruvic acid (reaction 1). In reaction 2, oxaloacetic acid is reduced to malic acid, which reacts with ribulose diphosphate, forming the 6C intermediate of the Calvin cycle (reaction 3). The remaining 3C fragments of the malic acid appear as pyruvic acid which is converted to phosphoenolpyruvic acid (reaction 4), thus completing the accessory cycle.

malate and aspartate and a considerable increase in radioactivity in sugars. Hatch and Slack investigated 32 plant species with respect to their time-course of incorporation of ^{14}C and found that 14 of them followed the sugar cane pathway; these included various varieties of sugar cane, sorghum varieties, maize, and other tropical grasses. In these plants they suggested two cyclic processes linked together by a carbon transfer reaction. The role of the new cycle was to fix carbon dioxide and to provide carboxyl groups to react with the acceptor of the Calvin cycle. A suggested scheme is shown in Fig. 5, but this is hypothetical and likely to be modified.

THE PHOTOCHEMISTRY OF PHOTOSYNTHESIS

Excitation of atoms and molecules: absorption spectra

An atom consists of a nucleus surrounded by electrons. When the electrons are in their lowest orbits the atom is in the *ground state*. If one of the electrons absorbs energy it may be ejected into a different orbit. There is a discrete energy difference between such excited atoms and those in the ground state. Only certain orbits are possible, which means that the energy supplied to the atom must be just sufficient to achieve the transition, otherwise electrons will not be raised to new levels of orbit.

One way for the atom to gain energy is from radiation. Radiation exists in quanta, discrete packets of energy the size of which is related to the wavelength of the radiation by the expression

$$E = h\nu$$

h being Planck's constant, and ν being the frequency, which is inversely proportional to wavelength. For a given transition in a particular atom, there can only be one wavelength of radiation which fits this formula; if the quantum is too small or too large the transition cannot occur. In other words, for a simple atom there is an absolute relationship between the difference in energy content of the excited state and the ground state and the wavelength of radiation which can effect the transfer. If it is a relatively small energy difference infrared radiation will be required; if larger, visible or ultraviolet radiation. A particular atom will selectively absorb the one wavelength which corresponds to the formula, and the resulting absorption spectrum will be a line spectrum.

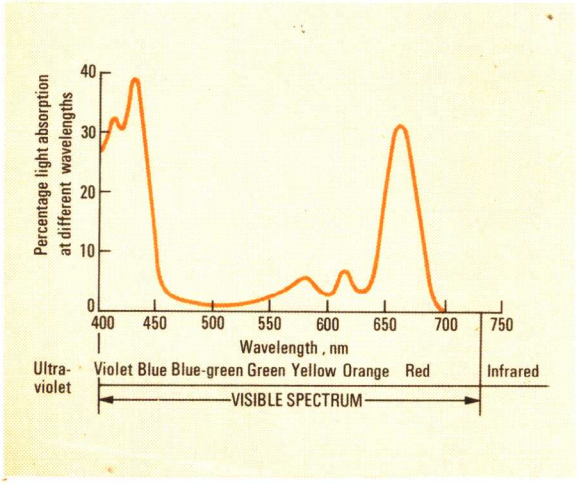


FIG. 6. Absorption spectrum of chlorophyll *a*.

Molecules are more complex than atoms and rotate and vibrate within themselves; superimposed on each electronic state is a range of states of agitation corresponding to slightly different energy states. The vibrational and rotational levels differ little in energy content compared with the difference between the excited and the ground state. The excited state also has an equal number of states of agitation and there are thus a range of wavelengths which can be absorbed. The resulting absorption spectrum is a band instead of a line.

In the case of chlorophyll *a* there are two absorption bands which both fall in the visible spectrum, one with maximum absorption at 660 nm and the other at 430 nm (Fig. 6). Corresponding to these wavelengths the average difference in energy content between the ground state and the excited states is 180 kJ/mole and 251 kJ/mole chlorophyll respectively. The absorption spectrum for chlorophyll *b* shows the two absorption maxima to have moved closer together, because the energy content of the higher excited state is lower and that of the lower higher.

In addition to chlorophylls *a* and *b*, green plants have the accessory pigments carotene and certain xanthophylls. One feature of these molecules is that their absorption bands are between those of chlorophyll; for example carotene and xanthophyll have absorption maxima between 430 and 490 nm. It is thus possible for the plant to absorb quanta of a wider range of energy contents than would be possible with a single pigment. Amongst lower plants, the green algae are similar to higher plants; but the red and blue-green algae also possess the

pigments phycocyanin and phycoerythrin (phycobilins), the proportion varying in different species. These two effectively fill in the region of the visible spectrum near to the long-wavelength chlorophyll bands, and the complete range of photosynthetic pigments effectively absorb visible radiation from the blue through to the red end of the visible spectrum.

The other group of red and yellow pigments in plants, the anthocyanins largely responsible for autumn colours, do not occur in the chloroplast and have no connection with the process of photosynthesis.

Fluorescence and phosphorescence

If the electron in an excited molecule falls back very quickly to its ground-level orbit, the energy it gives out in falling is dissipated as heat. For the energy to be used in a chemical reaction the life time of the excited state must be long enough for something else to collide with the excited molecule and so utilize the energy of excitation. Also, if they remain in the excited state sufficiently long, some of the molecules may return to the ground state spontaneously in a single step, and in doing so will emit radiation as fluorescence. On average, emission occurs from a lower energy level than the level attained immediately after excitation, and the fluorescent light emitted consists of smaller quanta and is of a longer wavelength than the absorbed light.

For chlorophyll *a* there is no fluorescence emission corresponding to the highest excited state, and this state must have a very short life. The electrons fall quickly to the lower excited state, dissipating some energy as heat. Thus although absorption of blue, short-wavelength light initially produces high excitation (level 1 in Fig. 7), this quickly falls to the same level produced by longer, red-wavelength light (level 2 in Fig. 7).

Chlorophyll *a* molecules in the level 2 excited state emit a band of fluorescence with a wavelength maximum at 690–700 nm. This means that the life of this particular excited state is probably sufficient to allow collision with various chemical molecules, thereby initiating chemical reaction. The wavelength of the fluorescent light corresponds to an excited level above the ground state of an average value of 167 kJ/mole (level 3 in Fig. 7). Chlorophyll *b* also emits fluorescence as a single band at a wavelength slightly shorter than that of chlorophyll *a* fluorescence. Carotenes do not emit fluorescence and neither do xanthophylls; the phycobilins have

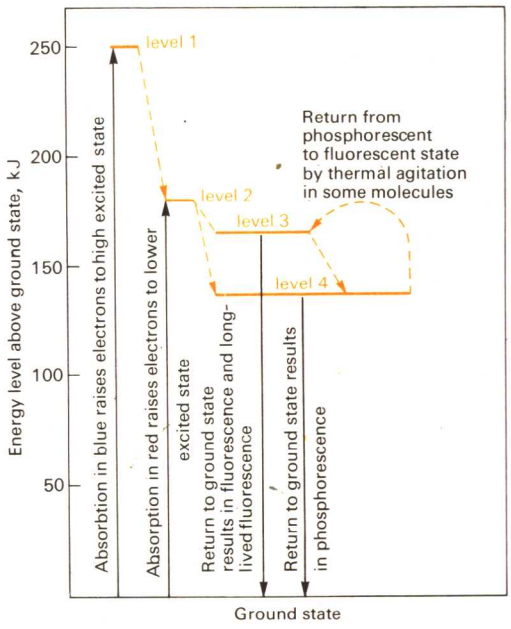


FIG. 7. Energy levels of the chlorophyll molecule.

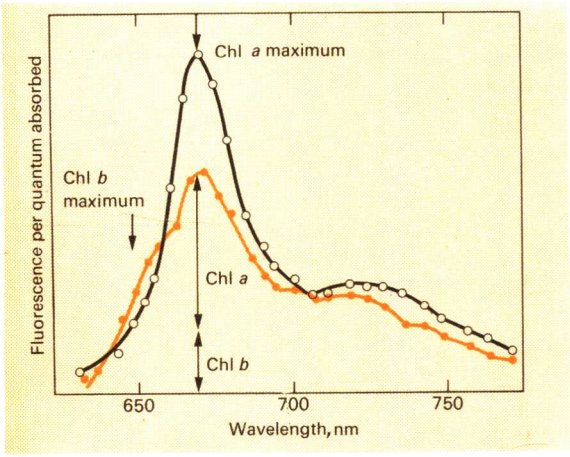


FIG. 8. Energy transfer between pigments in solution. Fluorescence spectra for chlorophylls in ether solution when irradiated with a wavelength of 429 nm (70% absorption due to chlorophyll *a*) and with 453 nm (95% absorption by chlorophyll *b*). The vertical line at 670 nm indicates that in the second case a large portion of the fluorescent light is characteristic of chlorophyll *a*, whereas chlorophyll *b* was the pigment excited: partial transfer of energy from chlorophyll *b* to chlorophyll *a* has taken place. ○ = exciting light 429 nm, ● = exciting light 453 nm.

a higher fluorescence yield than the chlorophylls.

In addition to fluorescence, some excited molecules continue to emit light even after the exciting light has been turned off. This is called *phosphorescence*. In the case of chlorophyll *b* phosphorescence corresponds to an average energy level of 146 kJ/mole above the ground state (level 4 in Fig. 7). The difference between the fluorescent and the phosphorescent states is only 21 kJ/mole and some molecules may be sufficiently energetic to transfer spontaneously by thermal agitation from the phosphorescent to the fluorescent state. When this happens the return to the ground state emits fluorescence and not phosphorescence and is called long-lived fluorescence. This phenomenon has only been demonstrated for chlorophyll *b* and then under very special conditions.

The properties of the pigments discussed so far refer to their behaviour when they are extracted from the plant and are in organic solution, for example in ether or acetone. We assume that there are corresponding states when the pigments are *in vivo*. When the individual absorption spectra of the pigments extracted from a plant are added in appropriate proportions, the total approximates to the absorption spectrum of the plant itself, although the absorption bands are broadened and the maxima are moved to longer wavelengths (e.g. to 680 nm as compared with 660 nm for chlorophyll *a*). However, the energy states in extracted pigments and pigments *in vivo* are probably sufficiently similar to permit qualitative, if not quantitative, comparisons of pigment properties.

The fluorescence of a mixture of two pigments may be different from their separate emissions. Chlorophyll *a* in ether absorbs at 660 and at 429 nm and both excitations cause fluorescence of about 690 nm. Chlorophyll *b* in ether absorbs at 640 and at 453 nm, and both excitations cause fluorescence at 670 nm. Consequently with a dilute solution in ether containing chlorophyll *a* and chlorophyll *b*, excitation at 640 nm absorbed by chlorophyll *b* should give fluorescence only at 670 nm characteristic of chlorophyll *b*. Since no light has been absorbed by chlorophyll *a*, no chlorophyll *a* fluorescence is expected. But with a concentrated mixture of the two pigments, light which excites chlorophyll *b* alone produces fluorescence characteristic of chlorophyll *a*; with the right mixture it is possible completely to suppress all fluorescence due to chlorophyll *b*. This is due to energy transfer between the excited chlorophyll *b* and chlorophyll *a* in the ground state. The fluorescence of the second species is sensitized indirectly from excitation of the first (Fig. 8). The molecule to which energy is to be transferred must have a lower energy content than the molecule from which transfer takes place, that is to say, the fluorescence band of the acceptor molecule must overlap the absorption band of the donor. The greater the overlap of the fluorescence and absorption bands the greater the efficiency of the transfer.

In an intact plant illuminated by monochromatic light of wavelength about 490 nm, which is largely absorbed by xanthophylls, a fluorescence emission which is characteristic of chlorophyll *a* is observed. It can be shown similarly for all the accessory pigments, with the probable exception of β-carotene, that the energy absorbed by them is transferred through the pigment system, ultimately resulting in chlorophyll *a* fluorescence. Thus the role of these other pigments is that of secondary absorbers which transfer the energy they absorb to chlorophyll. The efficiency of transfer has been shown to be very high—in excess of 90% in all the cases so far studied. It involves a special sort of transfer called *resonance transfer*.

Action spectrum

Similar conclusions were reached by comparing the amount of photosynthesis produced in plants excited with equal amounts of energy of different wavelengths. This is called an *action spectrum*. If chlorophyll *a* were the only pigment which could photosensitize photosynthesis and there was no energy transfer, the action spectrum should correspond to the absorption spectrum of chlorophyll *a*, the only useful pigment. If *b* could absorb and transfer its energy to *a*, then the action spectrum would extend to include absorption by both *b* and *a*. Thus a careful comparison of the absorption and action spectra would reveal any ineffective pigments.

For the alga *Chlorella*, there is a marked discrepancy between the absorption spectrum and the action spectrum only in the region where absorption by β-carotene is maximal; absorption by carotene does not, therefore, channel energy into photosynthesis (Fig. 9).

Blinks, working on the California coast, extended such observations to a number of seaweeds. In red seaweeds which contain phycoerythrin and phycocyanin he found little photosynthesis in the region where chlorophyll predominantly absorbs (between 660 and 680 nm). By contrast, where the phycobilins absorb to the greatest extent, maximal

photosynthesis is observed (see Fig. 10).

Blinks also studied the fluorescence spectra and again found evidence for very effective transfer from the phycobilins to chlorophyll: indeed the fluorescence emitted after phycobilin excitation was greater than if the chlorophylls absorbed the same amount of light energy direct. This seems inconsistent with the view that energy is transferred from the phycobilins to the chlorophyll, but fits in with a further observation made on *Chlorella*. This

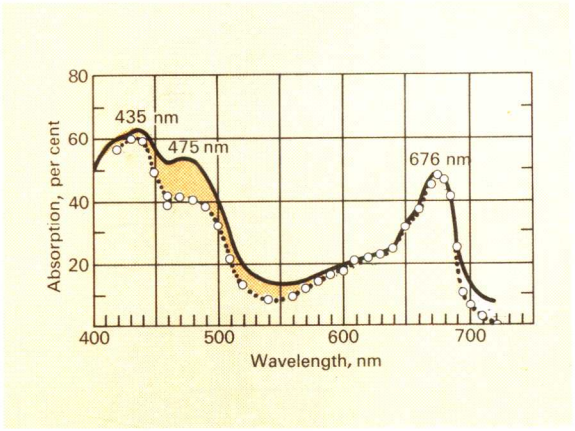


FIG. 9. The absorption spectrum for a cell suspension of *Chlorella pyrenoidosa* (——) compared with the photosynthetic action spectrum (.....). The dark tint shows the region of carotene absorption.

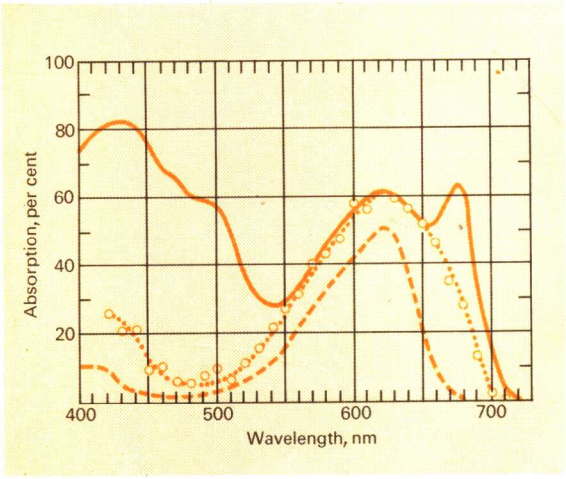


FIG. 10. The absorption spectrum of a cell suspension of *Cyanidium caldarium* (——) compared with the action spectrum for photosynthesis (.....). The absorption due to C-phycocyanin is shown (- - -). The action spectrum resembles the absorption spectrum of C-phycocyanin much more closely than that of intact cells.

revealed that in the far red region (beyond 690 nm) photosynthetic action decreased with increase in wavelength much more sharply than did the absorption, suggesting that there may be an inactive form of chlorophyll which is absorbing at the longer wavelengths. The accessory pigments of *Chlorella* may transfer energy preferentially to the 'active' form of chlorophyll, which is efficient in photosynthesis; the 'inactive' chlorophyll may be less able to accept energy from the accessory pigments or to produce photosynthesis. In a similar way, absorption by 'inactive' chlorophyll could explain the greater fluorescence by 'normal' chlorophyll in red algae after phycobilin excitation.

No one has yet succeeded in extracting two such different forms of chlorophyll with organic solvents; but detailed studies of the absorption spectra of intact plants indicate that different forms exist *in vivo*. One possibility is that part of the chlorophyll is adsorbed on the lipids of the chloroplasts, the rest being more closely associated with the hydrophilic proteins.

Difference spectrum

In this experimental procedure, algae in a container are illuminated by two beams at right angles. The first beam is an alternating one (produced by using a rotating disc), and is used to measure the absorption spectrum. The second is steady. By using appropriate measuring devices, it is possible to measure the absorption of a given wavelength when the system is and is not illuminated by the steady cross beam.

Duysens observed that the difference in spectrum obtained for oxido-reduction of cytochrome *f* in a test tube by addition of suitable chemical reagents corresponded quite closely to the difference spectrum obtained with secondary illumination causing photosynthesis in the red alga *Porphyridium*. He inferred that a change in the oxidation/reduction state of cytochrome *f* was probably a reaction involved in photosynthesis.

In fact *Porphyridium* gives the simplest difference spectrum of all plants so far observed. With *Chlorella* and the leaves of higher plants there is not a simple change of spectrum, but at least five significant regions of change. No one has yet been able to analyse these complex changes in terms of known isolated biochemicals.

Enhancement spectrum

A similar arrangement with one exciting beam in one direction and another exciting beam at right angles can be used in measuring net production of

oxygen by photosynthesis. The second beam would be expected to increase net photosynthesis and produce an action spectrum enhanced to a different extent according to its wavelength. Such a spectrum is called the *enhancement spectrum*. When the wavelength in the first direction is kept constant, say at 690 nm, and that in the second direction is varied, it is found that the enhancement due to the second beam extends to wavelengths at and beyond 690 nm, although these by themselves are incapable of producing photosynthesis.

On the other hand, when the wavelength of the first beam was varied it was found that wavelengths absorbed by any of the accessory pigments were able to enhance the photosynthetic activity due to simultaneous excitation beyond 690 nm. This suggests that two pigment systems might be involved in photosynthesis and that they act sequentially. The pigment absorbing in the far red (first system) is unable to work alone; but the energy absorbed by it becomes effective when the second, shorter wavelength system is also excited. The first system, absorbing at longer wavelengths, must react with some product of the second system. Unfortunately it is theoretically impossible to excite the second system without also exciting the longer wavelength system, since absorption by chlorophyll *a* extends through the visible spectrum into the shorter wavelengths. It is only because the number of pigments absorbing decreases one by one as excitation extends into the far red that the system absorbing furthest into the red can be excited alone. There is evidence that the two systems do not need to be excited simultaneously; they can be excited independently at different times separated by a finite dark interval and enhancement is still observed.

Biochemistry of the light reactions

Whilst the physical approach to a study of the light reactions of photosynthesis was being undertaken by plant physiologists, biochemists were investigating the nature of the intermediates involved. As early as 1937 Hill showed that chloroplasts isolated from green leaves could reduce in the light ferricyanide to ferrocyanide or quinone to hydroquinone, water being simultaneously oxidized to oxygen. This process is photosynthesis in which ferricyanide or quinone act as hydrogen acceptors instead of the normal CO_2.

(It may be useful to remind the reader that reduction is the acquisition of one or more electrons by a molecule. This is often the same thing as addition of hydrogen since the electrons enable the molecule to capture hydrogen ions, which are always available in aqueous medium because of the dissociation of water molecules.)

By contrast, photosynthetic bacteria use a variety of hydrogen donors such as H_2S or H_2 *instead* of water to reduce carbon dioxide in photosynthesis. Certain algae when grown in the absence of oxygen can also acquire the ability to use hydrogen gas as a hydrogen donor and produce a bacterial type of photosynthesis called *photoreduction*. All these observations can be integrated into a generalized concept of photosynthesis. The process requires a hydrogen-donating and a hydrogen-accepting system. Light energy separates hydrogen from the donor and the corresponding oxidized moiety appears as a product of the reaction (e.g. O_2, S); the hydrogen reduces the acceptor and ultimately carbon dioxide (Fig. 11).

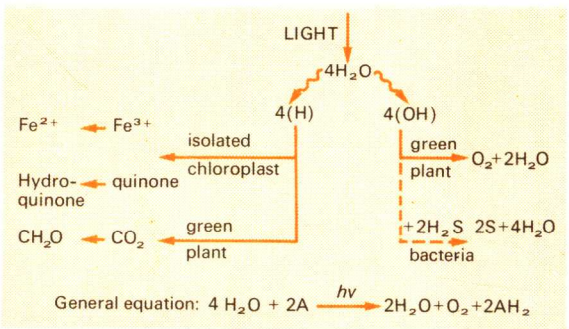


FIG. 11. General mechanism of photosynthesis.

Isolation of components of the light reaction

In the early work of Hill the only substances whose reduction could be demonstrated were chemicals which did not occur naturally in leaves and it took time to find how to modify the system so that it could reduce something of biological significance. For example, the early preparations could not reduce NADP. Davenport and Hill tried to recover some essential component which might have been washed out of the chloroplasts during their separation.

Independently, San Pietro was attempting to isolate a component which he called photosynthetic pyridine nucleotide reductase (PPNR), which would link the Hill reaction in chloroplasts to NADP reduction. Others working with non-photosynthetic bacteria, particularly *Clostridium*, isolated a factor called ferredoxin, an iron-containing protein, which catalysed a reaction between hydrogen gas and NADP (Fig. 12). Arnon showed

FIG. 12.

that if this substance was added to isolated chloroplasts in the light, it was much more effective than either the factor of San Pietro or of Davenport and Hill in reducing NADP (Fig. 13). It turned out

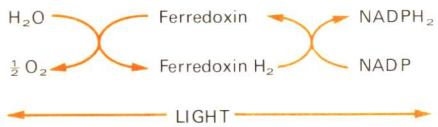


FIG. 13.

that all three factors when sufficiently purified were the same substance, ferredoxin. When purified further, ferredoxin proved unable to catalyse the reaction between chloroplasts and coenzymes without an additional factor, a flavoprotein, NADP reductase. Whereas in the non-photosynthetic bacterium *Clostridium*, hydrogen gas is used to reduce ferredoxin in the dark which then in turn can reduce coenzyme, in the case of chloroplasts, light energy is used to reduce the ferredoxin, because the hydrogen must come from water (Fig. 14).

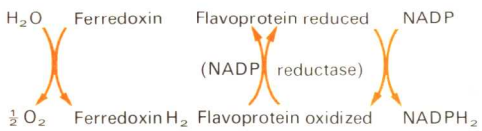


FIG. 14.

Ferredoxin is not specially related to the photosynthetic system; it can be reduced by dark or light reactions. Similarly it has the potentiality not only of reducing the flavoprotein NADP reductase but can also pass on electrons to hydrogen ions forming hydrogen gas, and can reduce molecular nitrogen to ammonia. Hence the fact that the photosynthetic bacteria do all these three things is not very surprising. In them there must be a competition for the reduced ferredoxin by the CO_2 fixing system, by the nitrogen-fixing system, and in certain organisms by the hydrogen-evolving system. In green plants the photosynthetic system cannot be used to evolve hydrogen or fix molecular nitrogen; it is largely directed to CO_2 reduction although it may also be utilized in the reduction of nitrate. Bacteria possess versatility at both ends of the photosynthetic reaction sequence; they can use a variety of hydrogen donors (e.g. H_2, H_2S, succinate). In the green plant water is normally the obligatory hydrogen donor, and at the other end ferredoxin is primarily used for the reduction of carbon dioxide.

Manufacture of ATP by chloroplasts

Whilst this work was in progress, Arnon was attempting to see if light energy could be used by chloroplasts to make ATP. He found a relatively simple system in which ADP was converted by chloroplasts to ATP by the uptake of inorganic phosphate provided that some exogenous carrier was added. The surprising result was that the only product of the light reaction was ATP; there was no reduced product and no oxygen production. The Calvin cycle (Fig. 3) requires reducing power ($NADPH_2$) and phosphate bond energy (ATP) to drive it. In this process, called *cyclic photophosphorylation*, light energy is converted into ATP energy, but no $NADPH_2$ is generated (Fig. 15).

Arnon's chloroplast preparations were also capable of performing the Hill reaction in which oxygen *is* evolved. In this case a stoichiometrical relationship between the amount of ferricyanide reduced and the amount of ATP formed was observed, i.e. the processes of reduction and ATP generation were obligatorily coupled. This process

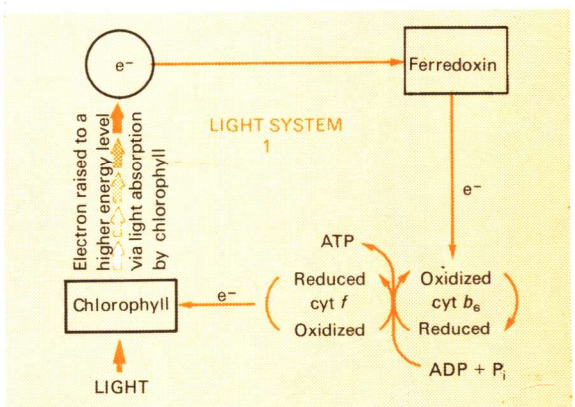


FIG. 15. Cyclic photophosphorylation. Chlorophyll absorbs a photon of light of sufficient energy; this sends an electron into a high energy state. The electron reduces in turn ferredoxin and the cytochrome system. The reproduction of cytochromes is coupled with the phosphorylation of ADP to form ATP. The electron returns, at a lower energy state, to the chlorophyll which had acquired a positive charge after the initial ejection of the electron. No 'external' electron donor is necessary for this process.

was called *non-cyclic phosphorylation*. It is capable of generating both ATP and $NADPH_2$ (Fig. 16).

Role of cytochromes

In respiration two types of cytochrome operate in an electron chain carrying electrons from $NADH_2$ down to cytochrome *b*, thence from *b* to *c*, and from *c* to oxygen through a_3 (Fig. 17). The essential feature is that the fall of electrochemical potential is in this way divided into a series of convenient steps so that the energy liberated can be utilized to convert ADP to ATP. The cytochrome chain channels the energy of oxidation into phosphorylation. It is the passage of electrons from a more reducing cytochrome to a more easily reduced cytochrome which liberates the energy which is trapped in phosphate compounds.

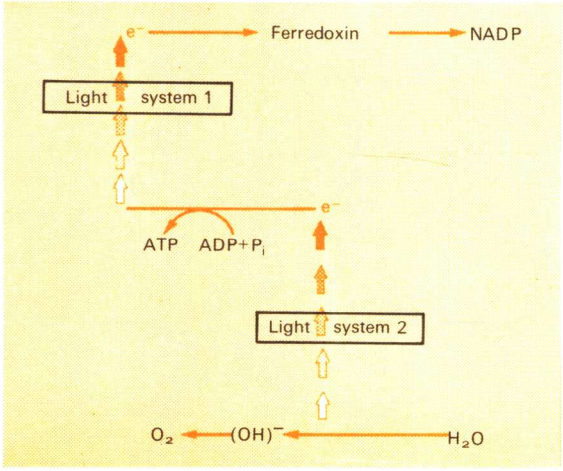


FIG. 16. Non-cyclic photophosphorylation. Unlike the the cyclic system, electrons raised to a high energy state ultimately reduce NADP. The protons necessary for reduction come from the dissociation of water, a process which also results in the production of oxygen, characteristically evolved by green plants.

After the demonstration of photophosphorylation by chloroplasts, it was natural to enquire whether cytochromes are also involved in photosynthesis. The non-green parts of plants contain cytochromes b_3 and *c*. A cytochrome similar to *c*, cytochrome *f*, was found exclusively in chloroplasts. It will be remembered from page 11 that Duysens had found circumstantial evidence from the difference spectrum implying that cytochrome *f* plays a part in photosynthesis. Chloroplasts also contain a special *b* cytochrome, b_6.

If the two types of cytochrome characteristic of photosynthetic tissue (*f* and b_6) were to be used in a similar way to those of non-green tissue in respiration, it would be necessary for cytochrome b_6 to react exothermally with cytochrome *f*, liberating energy to produce ATP. This reaction was the essential feature of the mechanism of photosynthesis proposed by Hill and Bendall. Light energy via chlorophyll raises an electron to a high energy level. If, on its 'downward' journey back to chlorophyll, it reduces cytochrome b_6, which is then oxidized by cytochrome *f* with an accompanying phosphorylation, and nothing else happens, this would constitute the cyclic system: no electron donor is required and no oxygen is evolved.

Effect of wavelength on cyclic and non-cyclic photophosphorylation

One important distinction between cyclic and non-cyclic photophosphorylation in isolated chloro-

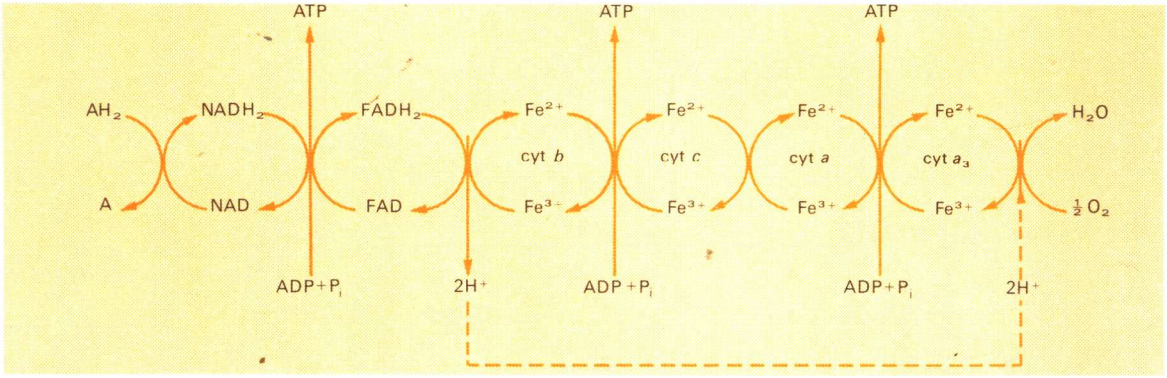


FIG. 17. The respiratory chain. Hydrogen is passed from the donor molecule, *A*, first to NAD and then to FAD. From FAD onwards the hydrogen atom is split into two parts. The positively charged hydrogen ion diffuses out into the solution and the electron flows down over a series of cytochromes to oxygen. The negative charge on oxygen is neutralized by the absorption of a hydrogen ion from solution. The final result is a water molecule.

plasts is the relationship between the wavelength of the exciting light and the rate of reaction. In the critical region around 700 nm cyclic photophosphorylation increases in rate for a given amount of light energy as the wavelength is increased, but the non-cyclic system shows a decrease in rate with increasing wavelengths just like photosynthesis *in vivo*.

Presumably cyclic photophosphorylation requires photosensitization by only one type of chlorophyll, that absorbing near 690 nm. This is the pigment which activates system 1. On the other hand, in non-cyclic photophosphorylation, water must be activated to reduce cytochrome b_6. This reaction is photosensitized by the other chlorophyll forms (system 2 pigments). It will be recalled that enhancement spectra data had already implicated two pigment systems in photosynthesis.

Hill and Bendall suggested that non-cyclic photophosphorylation involved both system 1 and system 2 pigments. Light energy via system 2 is used to split water into H^+ and OH^-. The hydroxyl ion (as at the anode during the electrolysis of water) forms oxygen and high energy electrons:

$$\text{Light energy} + 2OH^- \rightarrow H_2O + \tfrac{1}{2}O_2 + 2e^-$$

The electrons are used to reduce cytochrome b_6, which reacts thermally with cytochrome *f*, generating ATP in the process. The energy level of the electrons is now raised by system 1 pigment, and can then be used to reduce ferredoxin, which in turn reduces NADP via NADP reductase:

$$\text{Ferredoxin} + 2H^+ + 2e^- \rightarrow \text{Ferredoxin } H_2$$

The thermal reaction in between the two light steps is responsible for phosphorylation in all cases (see Fig. 18).

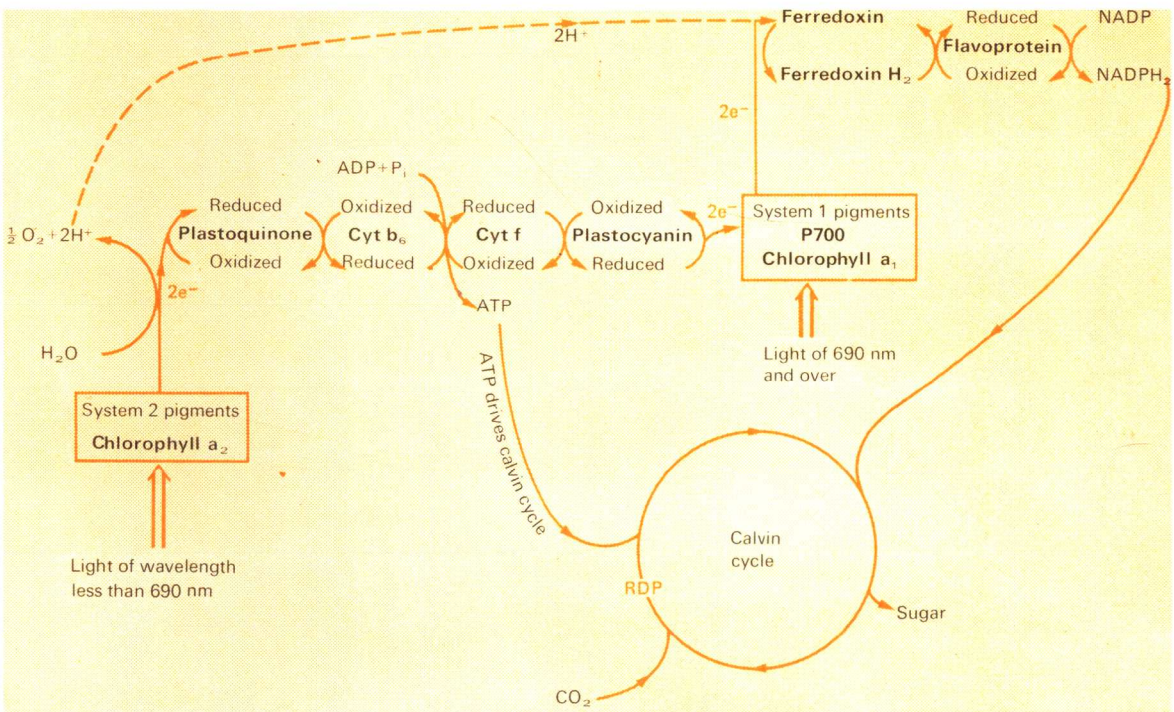
Electron carriers in photosynthesis besides cytochromes

Other natural carriers believed to be involved in the electron flow between the two photochemical systems have now been isolated from chloroplasts.

One is plastoquinone, which has been shown to be oxidized and reduced in the course of photosynthesis. Excess of system 2 light (short wavelengths) reduces it, and excess of system 1 light (long wavelengths) oxidizes it, i.e. it accepts electrons from system 2 and hands them on to system 1 (Fig. 18).

Katoh isolated a copper-containing protein, plastocyanin, from chloroplasts. After it has been extracted, the chloroplast will no longer reduce coenzymes utilizing water, but activity is restored

FIG. 18. A scheme of the photosynthetic mechanism.



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when it is added back. Extraction also inhibits cyclic photophosphorylation. This is typical of the experimental evidence used to establish the part played by carriers in photosynthesis.

The potential of plastocyanin is 0·37V and it is therefore presumed to react at a point near that of cytochrome *f*. Levine has studied mutants of *Chlamydomonas*, some of which are devoid of cytochrome *f*, and some of plastocyanin. Absence of plastocyanin prevented photosynthesis but the loss of cytochrome *f* had little effect. This suggests that cytochrome *f* may represent an extra reservoir for electrons but is not an essential component of the reaction path.

P700 is a pigment identified as responsible for the marked change in the absorption spectrum about 700 nm. It becomes oxidized in long wavelengths and reduced in shorter wavelengths. It may be a form of chlorophyll, the system 1 pigment that donates the electrons (thereby becoming oxidized) for the reduction of ferredoxin (Fig. 18).

Reaction centres in vivo

The abundance of each intermediate carrier relative to chlorophyll has been determined. For each molecule of cytochrome *f*, plastocyanin, and P700 there are about 350 molecules of chlorophyll *a*. A system 1 centre would therefore be expected to contain this grouping.

There are twice as many cytochrome b_6 molecules as cytochrome *f* molecules, and there are three molecules of plastoquinone for each molecule of cytochrome b_6. Hence for each molecule of plastoquinone there are approximately $350/6 \simeq 60$ chlorophyll *a* molecules, and several chlorophyll *a* molecules must be associated with one reaction centre. The possibility exists that the two pigment systems occur as physically discrete entities (reaction centres) within the chloroplast *in vivo*. To try to separate the centres, chloroplasts have been broken up using various detergents or dispersing agents such as digitonin or Triton X100. Using digitonin, Boardman and Anderson obtained two chloroplast fractions, one less dense than the other. The first striking difference between them was the ratio of chlorophyll *a* to chlorophyll *b*. The less dense fraction had a ratio of anything between 4 and 7 to 1; the denser fraction a ratio of 2 : 1. Since the ratio for the whole chloroplast was about 3 : 1, the less dense fraction was relatively enriched in chlorophyll *a*, the denser fraction in chlorophyll *b*. Similarly, the less dense fraction had proportionately more cytochrome *f* than the denser fraction, which possessed relatively more cytochrome b_6. Again another cytochrome, characterized by an absorption maximum at 559 nm, is unequally distributed between the particles. The two types of particle might represent two distinct reaction centres. However, it is just possible that they are an artefact produced by the detergent treatment.

Thus the work on the biochemistry of electron transport in photosynthesis (which has developed particularly from studies of isolated chloroplasts) and of physiological studies on the effect of light of different wavelengths upon photosynthesis in whole plants, can both be interpreted in terms of a model of photosynthesis involving two pigment systems. Recent studies with the electron microscope have attempted to visualize the physical arrangement of these two systems in the chloroplast. The work is still in progress and there is the hope that definite evidence as to the nature of the physical entities involved will be obtained.

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