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The Photosynthetic Energy Conversion Process in Isolated Chloroplasts*

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When EMIL FISCHER[1] discussed photosynthesis at the beginning of this century, he predicted that the elucidation of 'the precise nature of the assimilation (photosynthesis) process . . . will only be accomplished when biological research, aided by improved analytic methods, has succeeded in following the changes which take place in the actual chlorophyll granules (chloroplasts)'.

Much progress has been made in recent years toward realizing EMIL FISCHER's prophecy. Investigations with whole cells have been mainly concerned with assimilation of carbon dioxide[2,3]. On the other hand, investigations with chloroplasts have been mainly concerned with the identification, isolation and characterization of those photochemical reactions of photosynthesis which generate the first chemically defined, energy-rich products that are formed *prior* to, and are essential for, the conversion of CO_2 into organic compounds.

The advantages of isolated chloroplasts for investigations of this aspect of photosynthesis are substantial. Chloroplasts cannot respire – they lack the terminal respiration enzyme (Atmungsferment), cytochrome oxidase[4–6]. This feature insures that the early products of photosynthesis in chloroplasts would not be confused with intermediate products of respiration (including ATP) – a possibility that cannot be excluded with certainty in intact cells and which, therefore, has been the subject of much controversy in research on photosynthesis. Furthermore, isolated and fragmented chloroplasts, unlike whole cells, do not have permeability barriers to the entry of such key intermediates as nucleotides, particularly the adenosine phosphates (AMP, ADP), and, as will be shown later, small protein molecules. Thus, working with isolated chloroplasts, it is possible to supply these normally catalytic substances in substrate amounts and thereby follow stoichiometrically their conversion, under the influence of light, to products that are richer in energy.

These features of isolated chloroplasts and the use of radioactive phosphorus proved to be great experimental advantages in the discovery of photosynthetic phosphorylation, a process which generates ATP and a strong reductant, reduced ferredoxin (ferredoxin is an iron-containing protein which will be described later). Regarding ATP, other investigators, working with whole cells, had already proposed that a portion of light energy captured during photosynthesis is transformed into ATP prior to CO_2 assimilation, but the evidence for this was at best suggestive (see review[7]). It remained for experiments with isolated chloroplasts to uncover a new major cellular site of ATP formation that is peculiar to photosynthesis in that it is always associated with the chlorophyll pigments and is independent of ATP formation by respiration or fermentation. Photosynthetic phosphorylation (photophosphorylation) by chloroplasts is subdivided into cyclic photophosphorylation, a reaction which produces only ATP, and non-cyclic photophosphorylation, in which the production of ATP is stoichiometrically coupled with electron transfer that results in the reduction of ferredoxin and the evolution of oxygen.

Cyclic and non-cyclic photophosphorylation jointly constitute the process of conversion of radiant energy into chemical energy by chloroplasts. From this viewpoint of the energetics of photosynthesis, the final products of CO_2 assimilation, starch and other carbohydrates, are storage depots that preserve in a stable form the chemical energy that is liberated by the quantum conversion act and is trapped in the course of photosynthetic phosphorylation as ATP and reduced ferredoxin.

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No attempt will be made here to deal exhaustively with the very extensive literature of photosynthetic activity by isolated chloroplasts. Several comprehensive reviews are available elsewhere[8–11]. The principal aim of this article is to summarize the work of our laboratory, placing special emphasis on recent developments.

Photosynthetic capacity of isolated chloroplasts

Biochemical research on photosynthesis by isolated chloroplasts rests on the premise that in photosynthesis, as was the case earlier in fermentation and respiration, the elucidation of the constituent reactions and their mechanisms would most likely come when the process is reconstructed outside the intact cell. Since, within photosynthetic cells, chloroplasts contain all the photosynthetic pigments and were observed, almost a century ago[12–14], to produce starch and oxygen on illumination, it was thought for many years that photosynthesis in green plants begins and ends in chloroplasts.

It is often difficult for the student of photosynthesis today to realize that this view was never supported by critical experimental evidence and was largely abandoned after HILL[15,16] showed that isolated chloroplasts could evolve oxygen but could not assimilate carbon dioxide. In this reaction, which became known as the Hill reaction, isolated chloroplasts evolved oxygen when carbon dioxide was replaced by ferric oxalate or, as found later by WARBURG[17], by other non-physiological electron acceptors, benzoquinone and ferricyanide. The ability of isolated chloroplasts to assimilate CO_2 was reinvestigated when the very sensitive $C^{14}O_2$ technique became available, but here again the results were negative[18,19]. Such $C^{14}O_2$ fixation as was observed was limited in scope. Thus, FAGER[20,21] found no fixation of $C^{14}O_2$ by chloroplasts but by a protein preparation ('enzyme') from spinach leaves. The fixation of CO_2 by the 'enzyme' was enhanced in the presence of the illuminated chloroplast preparation but did not proceed beyond phosphoglycerate. There was no evidence for a reductive assimilation of CO_2 to the level of carbohydrate.

Without proof that isolated chloroplasts were the site of total photoassimilation of carbon dioxide, photosynthesis in the early 1950's came to be regarded, like fermentation in the days of PASTEUR, as a process that cannot be separated from the structural and functional complexity of whole cells[22]. Nevertheless, the possibility remained that the observed restricted photosynthetic capacity of isolated chloroplasts was merely a consequence of inappropriate experimental methods that were used in different laboratories, including our own. This proved to be the case. By changing our experimental methods, we found in 1954 that isolated spinach chloroplasts, unaided by other cellular particles or enzyme systems, reduce CO_2 to the level of carbohydrates, including starch, with a simultaneous evolution of oxygen at physiological temperatures and with no energy supply except visible light[23–25]. By using the new experimental methods, or modifications thereof, the conversion of $C^{14}O_2$ to phosphorylated sugars and starch by isolated chloroplasts was confirmed and extended in other laboratories[26–30]; compare also[31–33]. Like most cellular processes that are reconstructed in vitro, the rates of this extracellular CO_2 assimilation were low. Nevertheless, they provided reproducible biochemical evidence which has finally documented the frequently asserted, but never before proved, thesis that chloroplasts are the cytoplasmic structures in which the complete photosynthetic process takes place. Once the complete photosynthetic capacity of isolated chloroplasts was experimentally established, it was possible to concentrate with confidence on them rather than on whole cells in the search

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[21] E. W. FAGER, Biochem. J. *57*, 264 (1954).
[22] To illustrate: In 1953, RABINOWITCH (Sci. Am., Nov. 1953, p. 80) wrote that 'the task of separating it [photosynthesis] from other life processes in the cell and analyzing it into its essential chemical reactions has proved to be more difficult than was anticipated. The photosynthetic process, like certain other groups of reactions in living cells, seems to be bound to the structure of the cell; it cannot be repeated outside that structure'. In a review in 1954, LUMRY et al. (R. LUMRY, J. D. SPIKES, and H. EYRING, A. Rev. Pl. Physiol. *5*, 271, 1954) summarized the many investigations with isolated chloroplasts as pointing to the conclusion that the chloroplast was 'a system much simpler than that required for photosynthesis', and was the site of only 'the light-absorbing and water-splitting reactions of the over-all photosynthetic process'.
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for those photochemical reactions that precede the conversion of CO_2 into organic compounds.

Discovery of photophosphorylation

The first experiments with the sensitive P^{32} technique to test the ability of isolated chloroplasts to form, on illumination, ATP gave negative results[34]. The most plausible model for ATP formation in photosynthesis became one that envisaged a collaboration between chloroplasts and mitochondria. Chloroplasts would, in that scheme, reduce NAD photochemically and mitochondria would reoxidize it with oxygen and form ATP via oxidative phosphorylation[35]. This model posed a serious physiological problem. Photosynthesis in saturating light can proceed at a rate almost thirty times greater than the rate of respiration. It was difficult to see, therefore, how the respiratory mechanisms of mitochondria could cope with the ATP requirement in photosynthesis.

In 1954, ARNON, ALLEN and WHATLEY[23] discovered a light-induced ATP formation by isolated spinach chloroplasts unaided by mitochondria. This process, which they named photosynthetic phosphorylation (photophosphorylation) to distinguish it from the respiratory (oxidative) phosphorylation by mitochondria, was independent of CO_2 assimilation. ATP was formed under conditions when no CO_2 was supplied to the reaction mixture and the reaction vessels contained KOH in the center well. The possibility cannot be excluded that, even under these conditions, residual CO_2 may have had a catalytic function on the photochemical reaction, as observed by WARBURG et al.[36] and STERN and VENNESLAND[37] for the photoproduction of oxygen by chloroplasts. What can be excluded is that substrate amounts of carbon compound(s) were first synthesized in the light from exogenous CO_2 and were then used as electron donors for the formation of ATP.

Several unique features distinguished this type of photophosphorylation from ATP formation in fermentation (substrate level phosphorylation) or in oxidative phosphorylation[23,38]: (1) ATP was formed only in chlorophyll-containing structures and was independent of any other organelles or enzyme systems; (2) no oxygen was consumed or produced; (3) no energy-rich chemical substrate was consumed, the only source of energy being that of the absorbed photons; (4) ATP formation was not accompanied by a coupled oxidation-reduction involving an external electron donor and acceptor (equation 1).

$$n \cdot \mathrm{ADP} + n \cdot P_i \xrightarrow{h\nu} n \cdot \mathrm{ATP} \qquad (1)$$

The discovery of photosynthetic phosphorylation was confirmed and extended to other photosynthetic organisms. Photosynthetic phosphorylation in cell-free preparations of photosynthetic bacteria was observed by FRENKEL[39] and later by WILLIAMS[40], GELLER and GREGORY[41], KAMEN and NEWTON[42], and ANDERSON and FULLER[43]; in algae by THOMAS and HAANS[44], and PETRACK and LIPMANN[45]; and in isolated chloroplasts by AVRON and JAGENDORF[46], WESSELS[47] and many others. It was thus established that in all photosynthetic cells a major phosphorylating site is always associated with the chlorophyll pigments and supplies, independently of respiration or fermentation, the ATP needed in photosynthesis.

Soon after the demonstration of photosynthetic phosphorylation in isolated chloroplasts, attempts were made to compare its rate with that of CO_2 assimilation by illuminated whole cells. Since, as with most newly discovered cell-free reactions, the rates of photosynthetic phosphorylation were rather low, there was little inclination at first to accord this process quantitative importance[48] as a mechanism for converting light into chemical energy.

With further improvement in experimental methods, we obtained rates of photosynthetic phosphorylation up to 170 times higher[49] than those originally described[38] and even these high rates were exceeded by JAGENDORF and AVRON[50]. The improved rates of photosynthetic phosphorylation were equal to or greater than the maximum known rates of carbon assimilation in intact leaves. It appeared, therefore, that isolated chloroplasts retain, without substantial loss, the enzymatic apparatus for photosynthetic phosphorylation – a conclusion which is in harmony with evidence that the phosphorylating system is tightly bound in the water-insoluble grana portion of the chloroplasts.

When photophosphorylation was first extended from chloroplasts to photosynthetic bacteria, a question arose whether these two processes were funda-

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[49] M. B. ALLEN, F. R. WHATLEY, and D. I. ARNON, Biochim. biophys. Acta *27*, 16 (1958).
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mentally similar in not requiring a chemical substrate. FRENKEL[39] found that photophosphorylation in a cell-free preparation from *Rhodospirillum rubrum* became dependent on a substrate (α-ketoglutarate) when the chlorophyll-containing particles were washed. However, in later experiments, FRENKEL[51] and other investigators[42,43,52] found that the role of α-ketoglutarate and other organic acids in the bacterial system was catalytic and regulatory and not that of a substrate. When this basic point was clarified, the fundamental similarity of cyclic photophosphorylation in chloroplasts and in bacterial systems was no longer in doubt.

Once the main facts of photophosphorylation were firmly established, the next objective was to explain its mechanism. All known cellular phosphorylations occur at the expense of free energy liberated during electron transport from a high-energy electron donor to an electron acceptor, but there was no direct evidence for this in photophosphorylation. After early attempts to link photophosphorylation with photochemical splitting of water[23,24], we postulated that ATP formation was coupled to a special type of electron flow that is induced by light but is hidden in the structure of the chloroplast. The hypothesis was[8,53] that a chlorophyll molecule, on absorbing a quantum of light, becomes excited and promotes an electron to an outer orbital with a higher energy level. This high-energy electron is then transferred to an adjacent electron acceptor molecule with a strongly electronegative oxidation-reduction potential. The transfer of an electron from excited chlorophyll to an adjacent electron acceptor molecule, present in chloroplasts, is the energy conversion step proper. It transforms a flow of photons into a flow of electrons; that is, it constitutes a mechanism for generating a strong reductant at the expense of the excitation energy of chlorophyll. Once the strong reductant is formed, the subsequent electron transfer steps are exergonic. In subsequent reactions within the chloroplast, an electron is transferred, without any additional input of radiant energy, to a second electron acceptor with a more electropositive oxidation-reduction potential, from the second to the third and so on.

The number of these exergonic electron transfer steps in this type of photophosphorylation is still under investigation. We envisage that such an electron 'cascade' liberates free energy that is used to form one or more ATP's from ADP and orthophosphate. In the end, the electron originally emitted by the excited chlorophyll molecule returns to the electron-deficient chlorophyll molecule and the quantum absorption process is repeated. Because of the envisaged cyclic pathway traversed by the emitted electron, we named the process *cyclic photophosphorylation*[8,53].

A puzzling feature of cyclic photophosphorylation in chloroplasts, a feature which distinguished it from cyclic photophosphorylation in bacterial systems, was a dependence on an added catalyst or a primary electron acceptor – a function fulfilled by many different substances of a physiological or non-physiological character. An example of the former is menadione[54] and of the latter, phenazine methosulfate[50]. However, as discussed later, recent evidence points to ferredoxin, an iron-containing protein native to chloroplasts, as being the endogenous primary electron acceptor in cyclic photophosphorylation by chloroplasts.

Non-cyclic photophosphorylation

In 1957 ARNON et al.[55] discovered a second type of photophosphorylation which provided the first direct experimental evidence for a coupling between light-induced electron transport and the synthesis of ATP. Here, in contrast to cyclic photophosphorylation, ATP formation was stoichiometrically coupled with a light-driven transfer of electrons from water to NADP (or to a non-physiological electron acceptor such as ferricyanide) and a concomitant evolution of oxygen. Moreover, ATP formation in this coupled system greatly increased the rate of electron transfer from water to ferricyanide[55–57] or to NADP[58] and the rate of the concomitant oxygen evolution. It thus became apparent that the electron transport system of chloroplasts functions more effectively when it is coupled, as it would be under physiological conditions, to the synthesis of ATP. The conventional HILL reaction[15,17] could thus be viewed as an electron transport system that is uncoupled from photophosphorylation[55].

In extending the electron flow concept to this new reaction, we envisaged that a chlorophyll molecule excited by a captured photon transfers an electron to NADP (or to ferricyanide). Electrons thus removed from chlorophyll are replaced by electrons from water (OH^- at pH 7) with a resultant evolution of oxygen. In this manner, light induces an electron flow from OH^- to NADP and a coupled phosphorylation. Because of the unidirectional or non-cyclic nature of this electron flow, we have named this process *non-cyclic photophosphorylation*[53,55].

More recent evidence has established that illuminated chloroplasts do not react directly with NADP but react with ferredoxin[59]. As will be discussed later,

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the capture of photons by chloroplasts induces a non-cyclic electron flow to ferredoxin with a coupled phosphorylation and a concomitant evolution of oxygen. Reduced ferredoxin in turn reduces NADP by a mechanism that is independent of light.

Ferredoxin has thus emerged as a key substance in cyclic and non-cyclic photophosphorylation. Since the properties of ferredoxin and its role in the energy conversion process of photosynthesis have only recently been recognized, the pertinent evidence will now be reviewed in some detail.

Ferredoxins in bacteria and green plants

Prior to 1961, there was no evidence to challenge the view that chloroplasts, and only chloroplasts, contain a protein factor or an enzyme that catalyzes the photochemical reduction of NADP. But in that year K. TAGAWA and M. NOZAKI (unpublished data from this laboratory) and LOSADA et al.[60] isolated a 'pyridine nucleotide reductase' from an organism devoid of chloroplasts, the photosynthetic bacterium, *Chromatium*. The bacterial protein was able to replace the native chloroplast protein in mediating the photoreduction of NADP and the evolution of oxygen by chloroplasts, although *Chromatium* cells, from which this protein was isolated, are incapable of evolving oxygen in light. This finding indicated that proteins similar to those functioning in the NADP reducing apparatus of chloroplasts were also present in photosynthetic bacteria devoid of chloroplasts, but the full significance of this observation was understood a year later when TAGAWA and ARNON[59] obtained the same effect with a protein, ferredoxin, from a non-photosynthetic organism.

Ferredoxin is the name given by MORTENSON et al.[61] to a protein containing iron which is neither a heme protein nor a flavin protein. MORTENSON et al.[61] isolated this protein from *Clostridium pasteurianum*, a non-photosynthetic anaerobic bacterium which normally lives in the soil without any exposure to light. In this, and in other non-photosynthetic, obligately anaerobic bacteria where ferredoxin was later found, it appeared to function as a link between the enzyme hydrogenase and different electron donors and acceptors[62,63]. Thus, the distribution of ferredoxin seemed likely to be limited to those obligately anaerobic, non-photosynthetic bacteria that contain an active hydrogenase system. There was nothing to indicate that ferredoxin was in any way linked with photosynthesis.

It soon became clear, however, that ferredoxin-like proteins are present in all photosynthetic cells and play a key role in the energy transfer mechanisms of photosynthesis. In fact, TAGAWA and ARNON[59] recognized that, between 1952 and 1960, proteins which we now call ferredoxins had been isolated from chloroplasts of several species of green plants and had been assigned various functions under such different names as 'methaemoglobin-reducing factor'[64], 'TPN-reducing factor'[65], 'photosynthetic pyridine nucleotide reductase' (PPNR)[66], and the 'haem-reducing factor'[67]. All these terms are now known to be synonymous and have been replaced by the term ferredoxin. It also became apparent that the 'red enzyme' isolated in 1962 in WARBURG's laboratory is analogous to ferredoxin[68,69].

Definition of ferredoxin

TAGAWA and ARNON[59] crystallized ferredoxin from the non-photosynthetic bacterium *C. pasteurianum* and found that it was also able to replace the native chloroplast protein in the photoreduction of NADP. The same investigation led also to other findings: (a) The chloroplast protein, like ferredoxin of *C. pasteurianum*, contained iron and was reversibly oxidized and reduced with characteristic changes in its absorption spectrum [the presence of iron in the chloroplast protein ('PPNR') was also independently observed by FRY and SAN PIETRO[70], HORIO and YAMASHITA[71], KATOH and TAKAMIYA[72], and GEWITZ and VOELKER[69]]; (b) crystalline ferredoxin from *C. pasteurianum* has a remarkably low oxidation-reduction potential ($E'_0 = -417$ mV, at pH 7.55), close to the potential of hydrogen gas and about 100 mV more electronegative than the oxidation-reduction potential of pyridine nucleotides; and (c) the oxidation-reduction potential of the spinach chloroplast protein was also strongly electronegative ($E'_0 = -432$ mV, at pH 7.55).

These similarities led TAGAWA and ARNON[59] to extend the name ferredoxin to the chloroplast protein and to other iron-containing proteins of photosynthetic cells and anaerobic bacteria that have an oxidation-reduction potential close to that of hydrogen gas and

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are, at least in part, functionally interchangeable in the photoreduction of NADP by isolated chloroplasts. In the new terminology, the family of ferredoxins would include those non-heme, non-flavin proteins that transfer to appropriate enzyme systems some of the most 'reducing' electrons in cellular metabolism – electrons released by the photochemical apparatus of photosynthesis or by the H_2-hydrogenase system. Ability to catalyze the photoreduction of NADP by washed chloroplasts was included provisionally in the definition of ferredoxins because, in the experience of this laboratory, all the ferredoxins that were tested so far exhibit this property. By contrast, the replaceability of different ferredoxins in other enzymic reactions is less consistent.

It is to be noted that this provisional definition allows for dissimilarities of some properties among different ferredoxins. For example, the absorption spectra of ferredoxins from bacterial cells, whether photosynthetic or non-photosynthetic, resemble each other but differ significantly from the type of spectrum common to ferredoxins from algae and from chloroplasts of higher plants. In fact, we now distinguish, on the basis of spectral characteristics, two types of ferredoxins: the bacterial type and the chloroplast type.

A definitive characterization of ferredoxins as a group of electron carriers must await the isolation of a common prosthetic group in ferredoxins of different species. Pending the isolation of a common prosthetic group, it seems useful to retain the tentative definition of ferredoxins as iron-containing proteins which function as electron carriers on the 'hydrogen side' of pyridine nucleotides. This definition stresses the present distinction between ferredoxins and all the heme or non-heme iron proteins (including flavoproteins) with more electropositive oxidation-reduction potentials that serve as electron carriers on the 'oxygen side' of pyridine nucleotides.

Spectral characteristics and oxidation-reduction potentials

Unlike cytochromes, which exhibit well-defined absorption peaks in the reduced state, ferredoxins have distinctive absorption peaks in the oxidized state. On reduction, the absorption peaks of ferredoxins disappear.

The first bacterial ferredoxin to be crystallized, that of *C. pasteurianum*, exhibited in its oxidized state a distinctive spectrum with peaks in the visible and ultraviolet at 390, 300 and 280 nm[59]. The crystalline preparation gave an absorption ratio of 390/280 nm = 0.79. These spectral characteristics of ferredoxin from *C. pasteurianum* were confirmed and extended by BUCHANAN et al.[63] and LOVENBERG et al.[73] to ferredoxins of other species of *Clostridium*, which they prepared in crystalline form.

Figure 1 shows that the absorption spectrum of ferredoxin of the photosynthetic bacterium *Chromatium* closely resembles that of ferredoxin from the non-photosynthetic *Clostridium* species. In the oxidized state, the absorption spectrum of *Chromatium* ferredoxin exhibits a flat peak at 385 nm, a shoulder at 300 nm and a peak at 280 nm. In our purest preparation the ratio of optical density, 385/280 nm, was 0.74[74].

As shown in Figure 1, *Chromatium* ferredoxin was reduced by three methods: (a) H_2 gas in the dark, in the presence of a hydrogenase preparation from *C. pasteurianum*; (b) sodium dithionite, in the dark; and (c) photochemically, using a heated preparation of spinach chloroplasts and reduced dichlorophenol indophenol as the electron donor. Complete reduction of *Chromatium* ferredoxin was obtained only photochemically.

Figure 2 shows that the absorption spectrum of ferredoxin (oxidized state) from the blue-green alga *Nostoc* is of the chloroplast type[75]. It resembles closely the absorption spectrum of ferredoxin from spinach chloroplasts and is different from the absorption spectrum of bacterial ferredoxins. The absorption peaks of *Nostoc* ferredoxin in the visible and in the ultraviolet are 470, 423, 331 and 276 nm, as compared with 463, 420, 325 and 274 nm for spinach ferredoxin. Our purest preparation of *Nostoc* ferredoxin[75] had a ratio of optical density, 423/276 nm, of 0.57. A preliminary determination of the oxidation-reduction potential of *Nostoc* ferredoxin gave a value of $E_0' = -405$ mV, at pH 7.55[75].

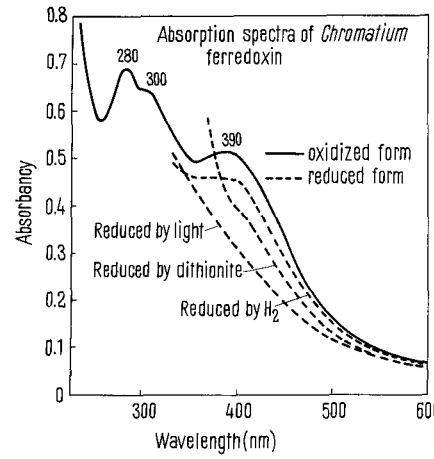


Fig. 1. Reduction of *Chromatium* ferredoxin by H_2, sodium dithionite and by illuminated spinach chloroplasts[74].

[73] W. LOVENBERG, B. B. BUCHANAN, and J. C. RABINOWITZ, J. biol. Chem. *238*, 3899 (1963).
[74] R. BACHOFEN and D. I. ARNON, Biochim. biophys. Acta, in print.
[75] A. MITSUI and D. I. ARNON, unpublished data (1963).

The similarity between the ferredoxin of *Nostoc* and spinach is interesting from an evolutionary point of view. Blue-green algae are considered to be the most primitive algae, not too distant on the evolutionary scale from photosynthetic bacteria. They reproduce vegetatively and, like photosynthetic bacteria, do not have their photosynthetic pigments localized in chloroplasts but distributed throughout the outer part of the cell. However, unlike photosynthetic bacteria, the photosynthesis of blue-green algae is accompanied by the evolution of oxygen. It is an interesting question whether the occurrence of the chloroplast type rather than of the bacterial type of ferredoxin in blue-green algae is related to the type of the photosynthetic pigment system and oxygen evolution that distinguish algal photosynthesis from bacterial photosynthesis.

Some chemical properties of ferredoxin

Apart from iron, ferredoxins of chloroplasts and bacteria are noted for containing 'labile sulfide', i.e. an inorganic sulfide group which is equimolar with iron. This was first observed in spinach ferredoxin by FRY and SAN PIETRO[70] and independently in WARBURG's laboratory in the 'red enzyme' (or ferredoxin) of *Chlorella*[68,69]. BUCHANAN et al.[63] found inorganic sulfide in bacterial ferredoxins. The inorganic sulfur in ferredoxin is liberated as hydrogen sulfide upon acidification. Both iron and inorganic sulfide are loosely bound to the protein and the removal of one is accompanied by the removal of the other. Upon the loss of iron or labile sulfur, ferredoxin loses its spectral characteristics and also its biochemical activity.

Ferredoxins are small molecules. The bacterial ferredoxin, first estimated to have a molecular weight of around 12.000[59], is now known to have a molecular weight of around 6.000[73]. The chloroplast ferredoxin is estimated to have a molecular weight of approximately 13.000[76]. The iron content of bacterial and chloroplast ferredoxin varies. Thus the chloroplast ferredoxin of spinach has, on a molar basis, two atoms of iron per molecule, whereas the bacterial ferredoxin of *Chromatium* has three and that of *Clostridium* has seven. A summary of some chemical properties of several ferredoxins is given in Table I.

Ferredoxins and NADP reduction

To test the effectiveness of different ferredoxins in catalyzing the photoreduction of NADP, we have crystallized several ferredoxins from organisms other than *C. pasteurianum*. Crystalline ferredoxin from spinach[77] chloroplasts is shown in Figure 3. Figure 4 shows crystalline ferredoxin from the blue-green alga *Nostoc*[75] and Figure 5 shows crystalline ferredoxin from the photosynthetic bacterium *Chromatium*[64]. Despite the diversity of source, all these ferredoxins were effective as substitutes for the native spinach ferredoxin in catalyzing the reduction of NADP by illuminated spinach chloroplasts.

Mechanism of NADP reduction by chloroplasts

The elucidation of the nature of ferredoxin as an electron carrier led to the resolution of the mechanism of NADP reduction by chloroplasts into three steps: (a) a photochemical reduction of ferredoxin; (b) reoxidation of ferredoxin by a flavoprotein enzyme, ferredoxin-NADP reductase; and (c) reoxidation of the reduced ferredoxin-NADP reductase by NADP[78].

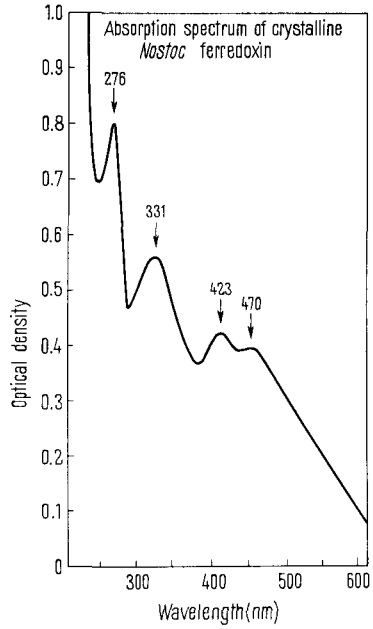


Fig. 2. Absorption spectrum of *Nostoc* ferredoxin in the oxidized state[75].

Table I. Some properties of bacterial and spinach ferredoxins[93]

	Clostridium pasteurianum	*Chromatium*	Spinach
Iron (atoms/molecule protein)	5	3	2
Inorganic sulfide (moles/mole protein)	5	3	2
Molecular weight (approx.)	6,000	6,000	13,000
Redox potential (mV at pH 7.55)	− 417	− 490 (approx.)	− 432

[76] F. R. WHATLEY, K. TAGAWA, and D. I. ARNON, Proc. Natn Acad. Sci. U.S.A. *49*, 266 (1963).
[77] K. TAGAWA and D. I. ARNON, unpublished data (1963).
[78] M. SHIN and D. I. ARNON, J. biol. Chem. *240*, 1405 (1965).

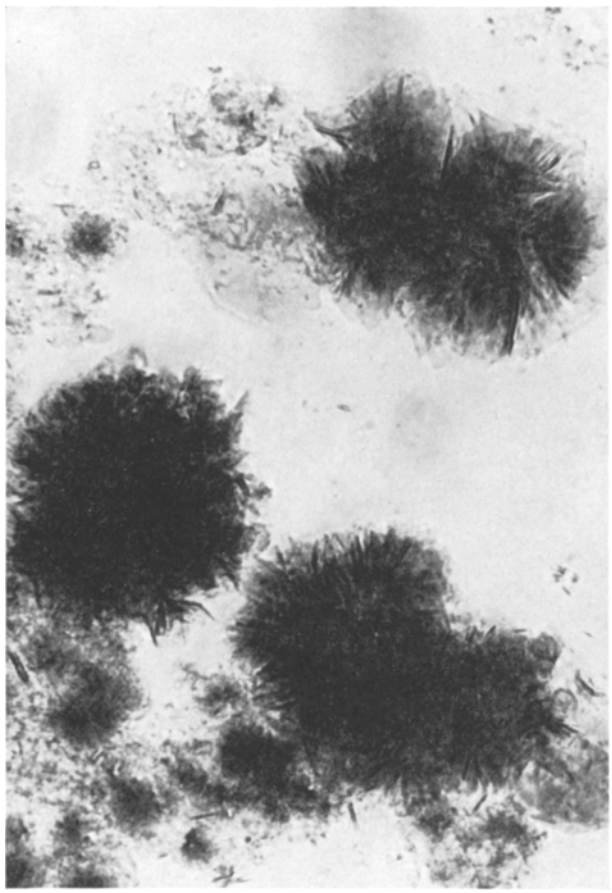


Fig. 3. Crystalline spinach ferredoxin[77].

Fig. 4. Crystalline ferredoxin from *Nostoc*[75].

Crystalline ferredoxin-NADP reductase isolated from spinach chloroplasts[79] is shown in Figure 6. Its absorption spectrum shows a typical flavoprotein absorption spectrum with peaks at 275, 385 and 456 nm and minima at 321 and 410 nm (Figure 7). Figure 8 shows ferredoxin-NADP reductase after it was first reduced (E_{red}) by reduced ferredoxin and then reoxidized by the addition of NADP (E_{red} + NADP). Thus, the oxidation-reduction of the flavin component of ferredoxin-NADP reductase was shown to be an intermediate step in the transfer of electrons from reduced ferredoxin to NADP.

Ferredoxin-NADP reductase catalyzed directly the reduction of either NADP or NAD but its affinity for NADP was very much greater[78]. The Michaelis constant for NAD was found to be $3.75 \cdot 10^{-3} M$, which was about 400 times greater than the K_m found for NADP ($9.78 \cdot 10^{-6} M$). The great difference between the affinities of ferredoxin-NADP reductase for NADP and NAD, the approximately equal concentrations of NAD and NADP in the cell[80], and the competition between NAD and NADP[78] account for the apparent specificity of the pure enzyme for NADP.

The reduction of NAD and NADP by the reduced ferredoxin-NADP reductase is reversible. This reversibility of the enzyme's action accounts for its apparent secondary function as diaphorase and as a transhydrogenase[81,82] which had been reported before its primary function as a NADP reductase was recognized. The reported specificity of its diaphorase and transhydrogenase action for $NADPH_2$ can now be explained by the low affinity of the enzyme for $NADH_2$.

The experiments which have shown that reduced ferredoxin does not transfer electrons directly to NADP have yielded no evidence for the existence of a 'bound' NADP[83,84]. Our evidence indicates that the electron transfer in the reduction of NADP by chloroplasts is not by a transhydrogenation reaction from a reduced 'bound' NADP but by direct reduction of free NADP by reduced ferredoxin-NADP reductase. This is discussed in greater detail elsewhere[78].

[79] M. SHIN, K. TAGAWA, and D. I. ARNON, Biochem. Z. *338*, 84 (1963).
[80] D. G. ANDERSON and B. VENNESLAND, J. biol. Chem. *207*, 613 (1954).
[81] M. AVRON and A. T. JAGENDORF, Archs Biochem. Biophys. *65*, 475 (1956).
[82] M. AVRON and A. T. JAGENDORF, Nature *179*, 428 (1957).
[83] D. L. KEISTER, A. SAN PIETRO, and F. E. STOLZENBACH, J. biol. Chem. *235*, 2989 (1960).
[84] D. L. KEISTER, A. SAN PIETRO, and F. E. STOLZENBACH, Archs Biochem. Biophys. *98*, 235 (1962).

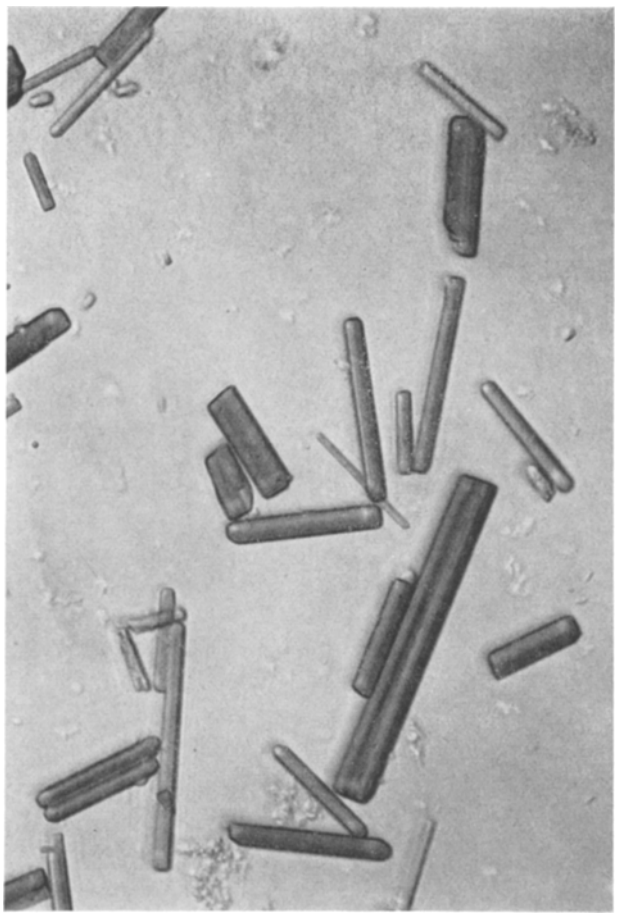


Fig. 5. Crystalline ferredoxin from *Chromatium*[74].

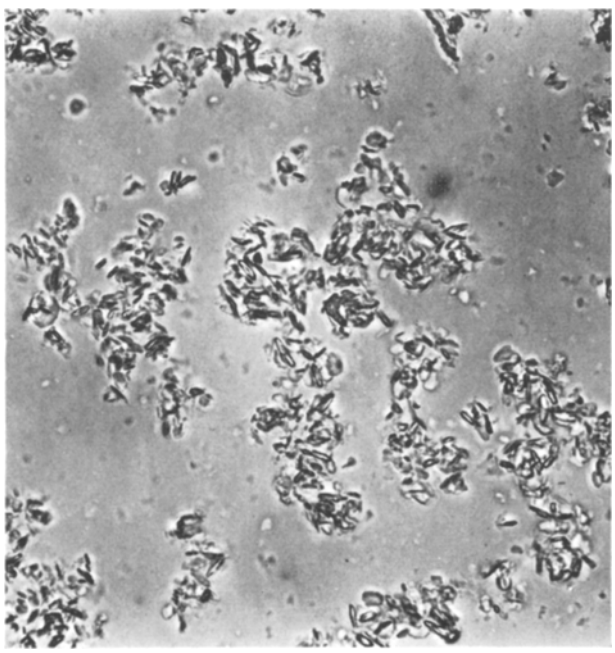
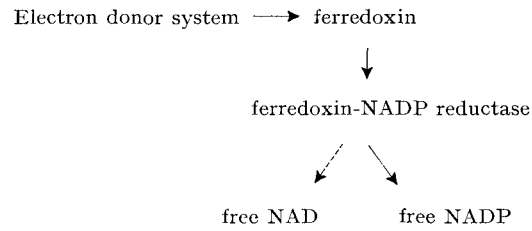


Fig. 6. Crystalline ferredoxin-NADP reductase[79].

Under physiological conditions (solid lines in the scheme below), the electron flow in the reduction of pyridine nucleotides by chloroplasts can now be summarized as follows:



Since ferredoxin and not NADP is the electron acceptor in the photochemical reactions of chloroplasts, the experimentally established reducing potential that is generated by chloroplasts in the course of photophosphorylation is extended by over 100 mV. It should be pointed out that the emphasis here is not on a theoretical reducing potential that can be generated by chloroplasts but on the experimental isolation and characterization of a reductant, native to photosynthetic cells, that is formed by the photochemical act of photosynthesis. It is theoretically possible for illuminated chloroplasts to generate stronger reductants

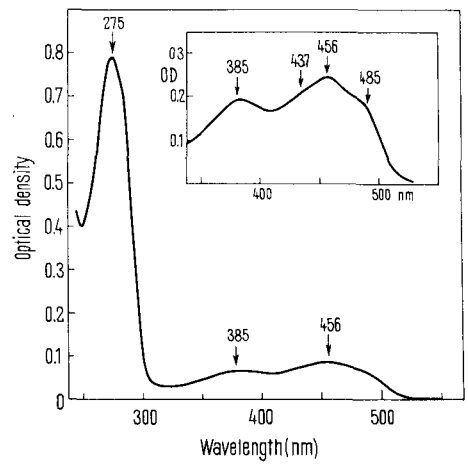


Fig. 7. Absorption spectra of ferredoxin-NADP reductase[79].

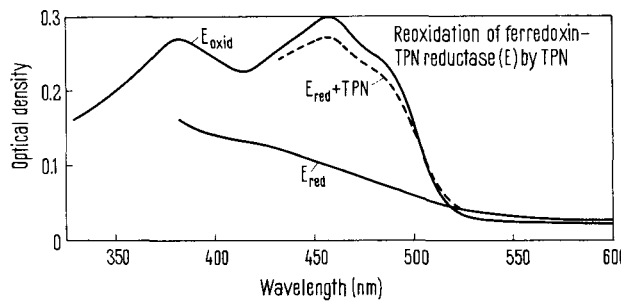


Fig. 8. Reduction of ferredoxin-NADP reductase by H_2 (E_{oxid} $\rightarrow$ E_{red}) and reoxidation of the reduced enzyme (E_{red}) by NADP[78].

than reduced ferredoxin: one einstein of red light ($\lambda = 663$ nm) is equivalent to 43 Kcal or to 1.87 eV. However, all such possibilities must remain speculative without evidence that photosynthetic cells contain reductants stronger than ferredoxin.

Photoreduction of ferredoxin coupled with photoproduction of oxygen

The key role assigned to ferredoxin in the photosynthetic electron transport is subject to a rigid test. The evolution of oxygen by chloroplasts is uniquely dependent on light, and it occurs only in the presence of a proper electron acceptor. Thus, photoproduction of oxygen by chloroplasts should accompany the photoreduction of ferredoxin. Such direct demonstration, however, was technically difficult because reduced ferredoxin is readily oxidized by oxygen. However, when the rapid back reaction between reduced ferredoxin and evolved oxygen was impeded, the stoichiometry between the photoreduction of ferredoxin and photoproduction of oxygen became measurable.

The techniques used involved measuring oxygen evolution polarigraphically and determining the photoreduction of ferredoxin by the decrease in optical density at 420 nm[85]. Traces of oxygen were rigidly excluded prior to turning on the light.

Figure 9 shows the photoreduction of added ferredoxin by chloroplasts under these experimental conditions. On turning off the light, ferredoxin was reoxidized. The amount of ferredoxin reduced in the light was equal to the amount of ferredoxin reoxidized in the dark. The sequence of photoreduction followed by dark reoxidation was reproducible at least three consecutive times. The added ferredoxin was completely photoreduced.

The photoproduction of oxygen, when ferredoxin was the terminal electron acceptor, is shown in Figure 10. No oxygen was evolved without the addition of ferredoxin. On turning off the light, the oxygen evolved during the preceding illumination period was consumed. The successive evolution and consumption of oxygen parallels the photoreduction and reoxidation of ferredoxin shown in Figure 9. The sequence of oxygen production in the light and consumption in the dark was also reproducible several times in succession. Of special interest is the stoichiometry between the ferredoxin added and oxygen produced. Table II shows that the stoichiometry between ferredoxin added and oxygen produced was 4 to 1 and remained the same with different amounts of added ferredoxin. This substantiates the conclusion, based on the stoichiometry of NADP reduction[76,86], that the photoreduction of ferredoxin involves a transfer of one electron.

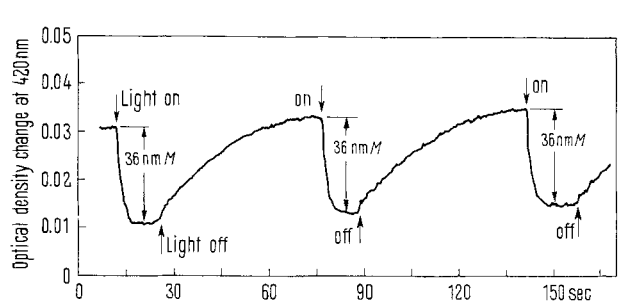


Fig. 9. Quantitative photoreduction of ferredoxin (Fd) by spinach chloroplasts and its reoxidation in the dark[85].

Non-cyclic photophosphorylation with ferredoxin

The stoichiometric evolution of oxygen, coupled with the photoreduction of ferredoxin, was also accompanied by a stoichiometric ATP formation[85]. The amount of ATP formed was proportional to the amount of ferredoxin added in a molar ratio of approximately 1 ATP to 2 ferredoxins (P:2e = 1). This ratio is consistent with the other evidence that the oxidation-

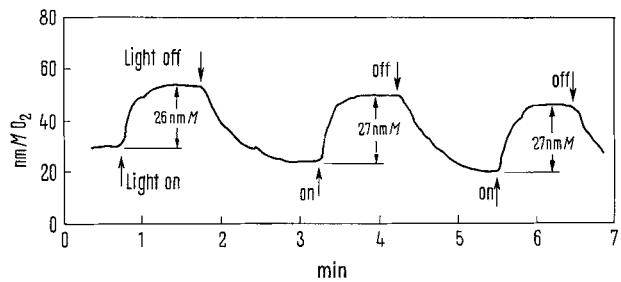


Fig. 10. Ferredoxin-linked oxygen evolution in the light and oxygen consumption in the dark[85].

Table II. Stoichiometry between oxygen evolution and photoreduction of ferredoxin by isolated chloroplasts[85] (nm$M$)

Experiment	Test No.	Ferredoxin added	O_2 produced	Ferredoxin added / O_2 produced
A	1	110	26	4.2
	2	110	27	4.1
	3	110	27	4.1
B	1	128	32	4.0
	2	128	33	3.9
	3	128	32	4.0
C	1	154	37	4.2
	2	154	42	3.7
	3	154	39	3.9

[85] D. I. ARNON, H. Y. TSUJIMOTO, and B. D. MCSWAIN, Proc. Natn Acad. Sci. U.S.A. *51*, 1274 (1964).
[86] T. HORIO and A. SAN PIETRO, Proc. Natn Acad. Sci. U.S.A. *51*, 1226 (1964).

reduction of ferredoxin involves a transfer of one electron. Thus, non-cyclic photophosphorylation can now be summarized by equation 2.

$$4\,Fd_{ox} + 2\,ADP + 2\,P_i + 2\,H_2O \xrightarrow{h\nu} 4\,Fd_{red} + 2\,ATP + O_2 + 4\,H^+ \quad (2)$$

In experiments with isolated chloroplasts, it is usually more convenient to measure non-cyclic photophosphorylation by using catalytic amounts of ferredoxin and stoichiometric amounts of NADP, which, unlike chloroplast ferredoxin, is commercially available and relatively stable to oxygen. However, this is merely an operational convenience which must not obscure the key role of ferredoxin in this type of photophosphorylation.

Cyclic photophosphorylation with ferredoxin

As already mentioned, apart from non-cyclic photophosphorylation, ferredoxin was also found to catalyze cyclic photophosphorylation. Evidence for a ferredoxin-catalyzed cyclic photophosphorylation which proceeds anaerobically without the addition of other cofactors was obtained after the experimental conditions for this type of photophosphorylation had been established[87]. These conditions include the use of an effective inhibitor of the electron flow from OH^- which results in oxygen evolution. It thus became clear that ferredoxin-catalyzed cyclic photophosphorylation and non-cyclic photophosphorylation are mutually exclusive. Cyclic photophosphorylation catalyzed by ferredoxin can be unmasked only when non-cyclic photophosphorylation is stopped.

Another way to demonstrate this mutually exclusive relation between cyclic and non-cyclic photophosphorylation is to use monochromatic light above 700 nm[87]. Chloroplasts illuminated in this region of far-red light cannot produce oxygen; that is, they cannot remove electrons from water but can still carry on cyclic photophosphorylation catalyzed by ferredoxin. Here no inhibitor of photoproduction of oxygen is necessary since the far-red light serves as a physical equivalent of a chemical inhibitor: it allows cyclic photophosphorylation to proceed and makes photoproduction of oxygen impossible[88].

Cyclic and non-cyclic photophosphorylation are also sharply distinguished by their differential sensitivity to several inhibitors. Low concentrations of antimycin A, 2,4-dinitrophenol and desaspidin, a phlorobutyrophenone derivative, inhibit cyclic but do not inhibit non-cyclic photophosphorylation[87,88,88a].

Mechanisms of cyclic and non-cyclic photophosphorylation

The basic facts of cyclic and non-cyclic photophosphorylation are now well established, having been confirmed and extended by many laboratories. However, the theoretical concepts invoked to explain them are subject to change as greater refinements of experimental technique bring us closer to the understanding of the detailed mechanisms involved. Our present hypothesis envisages that, in chloroplasts, the electron transport chain and sites of cyclic photophosphorylation are distinct from those of non-cyclic photophosphorylation. This conclusion is based on the fact that ferredoxin-catalyzed cyclic photophosphorylation will occur only under conditions when non-cyclic photophosphorylation is excluded. We consider cyclic photophosphorylation in chloroplasts as including, under physiological conditions, phosphorylations that are coupled with a flow of electrons from excited chlorophyll to ferredoxin, and then from reduced ferredoxin to cytochromes b_6 and f and back to chlorophyll (Figure 11). We include the chloroplast cytochromes[89,90] b_6 and f as electron carriers in cyclic but not in non-cyclic photophosphorylation although, so far as we are aware, there is so far no direct evidence for the involvement of chloroplast cytochromes in either type of photophosphorylation. Indirect evidence comes from inhibition of ferredoxin-catalyzed cyclic photophosphorylation by antimycin A and 2,4-dinitrophenol, two well-known inhibitors of oxidative phosphorylation where the direct participation of cytochromes is

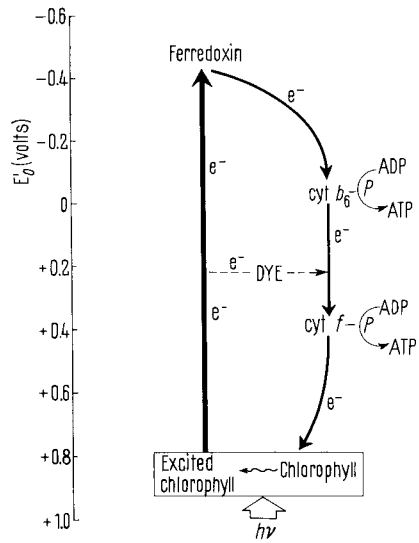


Fig. 11. Scheme for cyclic photophosphorylation[95].

[87] K. TAGAWA, H. Y. TSUJIMOTO, and D. I. ARNON, Proc. Natn Acad. Sci. U.S.A. *49*, 567 (1963).

[88] K. TAGAWA, H. Y. TSUJIMOTO, and D. I. ARNON, Proc. Natn Acad. Sci. U.S.A. *50*, 544 (1963).

[88a] H. BALTSCHEFFSKY and D. Y. KIEWIET, Acta chem. scand. *18*, 2406 (1964); Z. GROMET-ELHANAN and D. I. ARNON, Plant Physiol. *40*, 1060 (1965).

[89] R. HILL, Nature *174*, 501 (1954).

[90] H. E. DAVENPORT, Nature *170*, 1112 (1952).

well documented. Antimycin A inhibition of oxidative phosphorylation is considered to be indicative of the participation of cytochrome *b* in electron transport[91,92]. It is likely that participation of cytochrome b_6 accounts for the sensitivity of ferredoxin-catalyzed cyclic photophosphorylation to antimycin A. As for cytochrome *f*, its joint participation in an electron transport chain with cytochrome b_6 of chloroplasts is considered likely by analogy with oxidative phosphorylation in mitochondria.

The span between the redox potentials of cytochromes b_6 and *f* (– 0.06 V and 0.365 V, respectively) is large enough to accommodate a phosphorylation. Likewise, the span between the redox potentials of ferredoxin (– 0.43 V and cytochrome b_6 (– 0.06 V) is large enough to accommodate at least one phosphorylation in this segment of the cyclic chain. We specify tentatively two phosphorylation sites in this cyclic electron transport chain but this does not exclude the possibility of additional phosphorylation sites.

As discussed elsewhere[88,93], we consider that in chloroplasts the ferredoxin-catalyzed cyclic photophosphorylation is the physiological one. However, experimentally, cyclic photophosphorylation proceeds readily without ferredoxin when catalyzed by one of several dyes or other artificial cofactors. Since such cyclic photophosphorylations are resistant to inhibition by antimycin A, it seems reasonable to conclude that they bypass the cytochrome b_6 site (see dotted line in Figure 11).

Our present concept of non-cyclic photophosphorylation in plants is shown in Figure 12. The phosphorylation is envisaged as being coupled to the oxidation of OH^-, a coupling that would account for the consistent stoichiometry, $P/2e = 1$, between oxygen evolution and ATP formation. We consider that an electron from OH^- is transferred via chlorophyll to ferredoxin in a single light reaction. One quantum of light at 680 nm – the longest wavelength that can still support maximum efficiency of plant photosynthesis[94] – contains sufficient energy for the transfer of an electron from OH^- to ferredoxin and the coupled phosphorylation (Table III). In isolated chloroplasts, ferredoxin may be replaced by non-physiological electron acceptors (HILL reagents) with an attendant drop in the light-generated reducing potential. Figure 12 (dotted lines) illustrates this for ferricyanide and benzoquinone (BQ).

The 'chlorophyll' in Figures 11 and 12 represents the complex of chlorophyll *a* and *b* pigments in their various forms and includes those accessory pigments which are involved in light absorption by chloroplasts[11,96]. We assume that excitation energy is transferred among the chloroplast pigments with the great efficiency that is well documented in algal cells[94].

The electron pathway from OH^- to chlorophyll is the least understood part in the mechanism of photosynthesis and its elucidation must be left to future research. Only a few of the cofactors and catalysts involved therein are now known: chloride ions[17,97], manganese[98,99], and plastoquinone[100–102] (plastoquinone is also involved in cyclic photophosphorylation in chloroplasts[101,103]). The nature of the linkage between

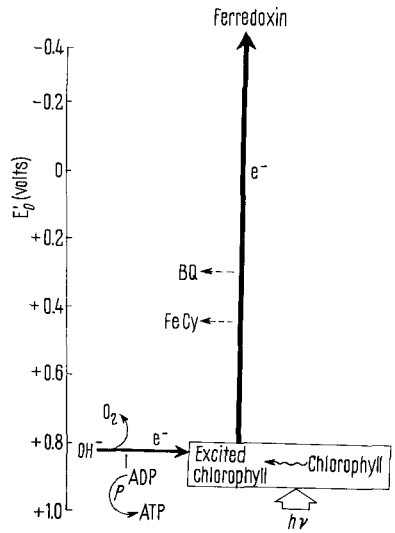


Fig. 12. Scheme for non-cyclic photophosphorylation of the plant type[95].

Table III. Energy balance of non-cyclic photophosphorylation of the plant type

Energy output of 1 Einstein, λ = 680 nm, is 42 Kcal.
$Ferredoxin_{ox} = Ferredoxin_{red} + e^-$; $E'_0 = -0.43$ V (pH 7).
$^1/_2\ H_2O = {}^1/_4\ O_2 + H^+ + e^-$; $E'_0 = +0.82$ V (pH 7).
Potential span between O_2 and $ferredoxin_{red}$ is 1.25 eV = 1.25 · 23.06 = 28.8 Kcal.
Energy requirement (per 1 electron) to form 1 ATP: 10 Kcal/2 = 5 Kcal.
Excess of energy input over output: 42 – (28.8 + 5) = 8.2 Kcal.

[91] B. CHANCE and C. R. WILLIAMS, Adv. Enzymol. *17*, 65 (1956).
[92] E. RACKER, Adv. Enzymol. *23*, 323 (1961).
[93] D. I. ARNON, Science *149*, 1460 (1965).
[94] L. N. M. DUYSENS, Prog. Biophys. biophys. Chem. *14*, 1 (1964).
[95] D. I. ARNON, H. Y. TSUJIMOTO, and B. D. MCSWAIN, Nature *207*, 1367 (1965).
[96] J. H. C. SMITH and C. S. FRENCH, A. Rev. Pl. Physiol. *14*, 181 (1963).
[97] J. M. BOVÉ, C. BOVÉ, F. R. WHATLEY, and D. I. ARNON, Z. Naturforsch. *18b*, 683 (1963).
[98] A. PIRSON, Z. Botan. *31*, 193 (1937).
[99] E. KESSLER, Archs Biochem. Biophys. *59*, 527 (1955).
[100] N. I. BISHOP, Proc. Natn Acad. Sci. U.S.A. *45*, 1696 (1959).
[101] D. W. KROGMANN, Biochem. biophys. Res. Commun. *4*, 275 (1961).
[102] D. I. ARNON and A. A. HORTON, Acta chem. scand. *17*, S135 (1963).
[103] F. R. WHATLEY and A. A. HORTON, Acta chem. scand. *17*, S140 (1963).

electron flow from OH^- and phosphorylation remains obscure but there is good reason to believe that the two are closely linked since, as already mentioned, the rate of electron flow from OH^- is greatly increased by the concurrent phosphorylation[55–58].

The flow of electrons from OH^- to ferredoxin and the resultant oxygen evolution are easily susceptible to damage when chloroplasts are removed from intact cells. It is not surprising, therefore, that a requirement of one quantum per electron transferred to ferredoxin (or NADP) has not been obtained in investigations with isolated chloroplasts[104].

How can ferredoxin participate in both cyclic and non-cyclic photophosphorylation? We have already stressed the fact that in isolated chloroplasts cyclic photophosphorylation occurs only when non-cyclic photophosphorylation (coupled to oxygen evolution) is stopped. The regulatory mechanism(s) in the cell for switching from non-cyclic to cyclic photophosphorylation is unknown, but one possibility has experimental support: the availability of NADP in the oxidized form[87]. As long as oxidized NADP is available, we envisage that electrons will flow from water to ferredoxin and thence (via ferredoxin-NADP reductase) to NADP. However, when CO_2 assimilation temporarily ceases for lack of ATP, NADP accumulates in the reduced state and electrons from reduced ferredoxin begin to 'cycle' within the chloroplasts, giving rise to cyclic photophosphorylation. The additional ATP thus generated would re-establish CO_2 assimilation, which, in turn, would restore $NADPH_2$ to its oxidized form and thereby re-establish non-cyclic photophosphorylation.

Cyclic and non-cyclic photophosphorylation as the two light reactions of photosynthesis: A working hypothesis

Our hypothesis identifies cyclic and non-cyclic photophosphorylation as two complementary and parallel pathways of energy conversion which jointly generate the assimilatory power required tor carbon assimilation in green plants. In monochromatic light non-cyclic photophosphorylation occurs only at 'short wavelengths' (< 700 nm). Cyclic photophosphorylation, on the other hand, occurs at wavelengths shorter and longer than 700 nm (< 730 nm). The overall efficiency of photosynthesis would depend on the efficient functioning of both these parallel photochemical processes.

This hypothesis explains some of the observations that are now used to support the concept of two light reactions working in series (see reviews[11,94,96]). Thus, in our scheme 'red drop' (that is, the drop in quantum efficiency of photosynthesis of intact plant cells in monochromatic light longer than 685 nm) would mean that at the longer wavelengths of light non-cyclic photophosphorylation becomes limiting, thereby reducing the availability of $NADPH_2$ and decreasing the overall efficiency of the process. 'Enhancement' (that is, the synergistic increase in the quantum efficiency of photosynthesis at long wavelengths of light that results from the addition of shorter wavelengths) would mean that the addition of light of a shorter wavelength restores non-cyclic photophosphorylation, removes the shortage of reductant and thereby makes for an increase in the overall efficiency of photosynthesis.

Among the other observations that would appear to fit the present hypothesis is the oxidation of chloroplast cytochromes in longer wavelengths of light and their reduction in shorter wavelengths (see reviews cited above). Our hypothesis envisages that at the shorter wavelengths ferredoxin is reduced by electrons from water and reduced ferredoxin in turn reduces the chloroplast cytochromes. They would remain in the reduced state as long as non-cyclic photophosphorylation is in operation. Cytochromes would become oxidized during cyclic photophosphorylation, which, under laboratory conditions, is established either by long-wavelength illumination or by short-wavelength illumination in the presence of inhibitors of oxygen evolution. Further experiments are now in progress to test this hypothesis.

We have not discussed so far whether cyclic photophosphorylation is essential for photosynthesis in plants; that is, whether the ATP that it supplies to supplement that produced by non-cyclic photophosphorylation is required for carbon assimilation. $2M$ of reduced ferredoxin are required to give $1M$ of $NADPH_2$. Thus, non-cyclic photophosphorylation gives rise to $NADPH_2$ and ATP in a ratio of 1:1 (equation 2). However, if one computes the energy requirement for CO_2 assimilation on the basis of the carbon reduction cycle of CALVIN[2], one obtains a requirement of 2 $NADPH_2$ and 3 ATP per $1M$ of CO_2 assimilated to the level of glucose. Even if this requirement were reduced to 2 ATP and 2 $NADPH_2$, additional ATP would still be necessary to form starch, the main product of photosynthesis in leaves, because ATP is expended in the formation of ADP-glucose from which the glucosyl moiety is transferred to a starch primer[105]. Moreover, as was pointed out elsewhere[55], cyclic photophosphorylation may be an important mechanism for providing the large supplies of ATP that are required for protein synthesis and for other endergonic processes in the cell.

[104] K. SAUER and J. BIGGINS, Biochim. biophys. Acta *102*, 55 (1965).
[105] E. RECONDO and L. F. LELOIR, Biochem. biophys. Ress. Comm. *6*, 85 (1961); H. P. GOSH and J. PREISS, Biochemistry *4*, 1354 (1965).

Quenching of chloroplast fluorescence by cyclic and non-cyclic photophosphorylation

Fluorescence of chloroplasts represents that portion of absorbed radiant energy which is not converted into chemical energy (or heat) but is re-emitted as radiation. If our thesis is correct that photosynthetic phosphorylation constitutes the prime energy conversion process of photosynthesis, then it should act as a quencher of chloroplast fluorescence.

Figure 13 shows a marked quenching of chloroplast fluorescence by the addition of ferredoxin[106]. This result is consistent with our hypothesis that ferredoxin establishes a cyclic electron flow: the degradation of the energy of a molecule excited by photon capture can occur by electron transfer to an appropriate electron acceptor molecule. An additional quenching of chloroplast fluorescence was observed upon adding ADP and inorganic phosphate (Figure 13) – an observation which supports the idea that the cyclic electron flow catalyzed by ferredoxin is accelerated by concomitant phosphorylation.

Quenching of chloroplast fluorescence by non-cyclic photophosphorylation is shown in Figure 14. The addition of ferredoxin and TPN gave a pronounced quenching effect. The addition of ADP and inorganic phosphate to the ferredoxin-TPN system gave a marked additional quenching effect. These results are consistent with earlier findings that the rate of non-cyclic electron flow is markedly increased by a concomitant phosphorylation.

The quenching of chloroplast fluorescence by cyclic and non-cyclic photophosphorylation supports their characterization as the primary photochemical reactions in photosynthesis[8]. The energy of captured photons may be dissipated as fluorescence or may generate an electron flow which yields the chemical energy stored in the pyrophosphate bonds of ATP and in the reducing potential of ferredoxin.

Concluding remarks

This brief survey of photosynthesis by isolated chloroplasts would be incomplete without stressing the dependence of the progress made on at least two 'improved analytical methods' that fulfilled the prophecy of EMIL FISCHER[1], made 60 years ago. Photophosphorylation and the synthesis of carbohydrates by isolated chloroplasts were discovered with the aid of the labelled isotope technique introduced into biology by VON HEVESY[107] and by the development of paper partition chromatography by MARTIN and SYNGE[108].

The concept of photosynthesis that emerged from the work with isolated chloroplasts differs from the conventional view of photosynthesis as a process of CO_2 assimilation. Photosynthesis appears to be first and foremost a process for converting the radiant energy of sunlight into chemical energy. This conversion is more directly linked to the assimilation of phosphorus and the reduction of the iron protein, ferredoxin, than to the assimilation of carbon dioxide.

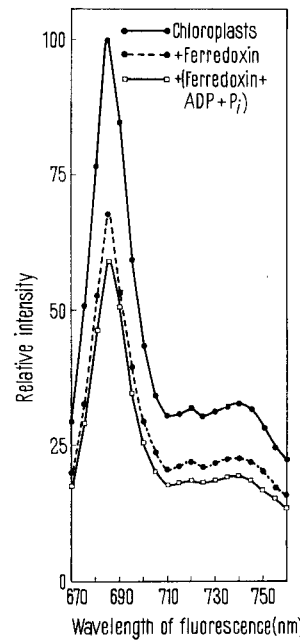


Fig. 13. Quenching of chloroplast fluorescence by ferredoxin, ADP and inorganic phosphate[106].

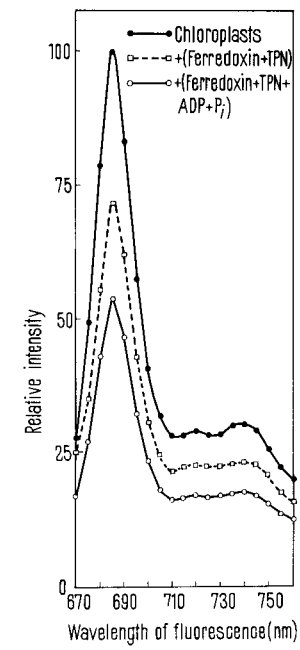


Fig. 14. Quenching of chloroplast fluorescence by NADP (TPN), ferredoxin, ADP and inorganic phosphate[106].

[106] D. I. ARNON, H. Y. TSUJIMOTO, and B. D. MCSWAIN, Proc. Natn Acad. Sci. U.S.A. *54*, 927 (1965).
[107] G. DE HEVESY, Les Prix Nobel en 1944.
[108] A. J. P. MARTIN, Les Prix Nobel en 1952. – R. L. M. SYNGE, Les Prix Nobel en 1952.

The first, chemically defined products of the energy conversion process in photosynthesis proved to be not intermediates of carbon assimilation but ATP and reduced ferredoxin.

Zusammenfassung. Dieser kurze Überblick über das Gebiet der Photosynthese in isolierten Chloroplasten wäre unvollständig, würde nicht die Abhängigkeit des erzielten Fortschrittes von mindestens zwei «verbesserten analytischen Methoden» betont, welche EMIL FISCHERS[1] 60 Jahre zurückliegende Prophezeiung erfüllen. Zur Entdeckung der Photophosphorylierung und der Kohlenhydratsynthese durch isolierte Chloroplasten war die Einführung der Isotopen-Technik in die Biologie durch VON HEVESY[107] und die Entwicklung der Papierchromatographie durch MARTIN und SYNGE[108] von besonderer Bedeutung.

Untersuchungen an isolierten Chloroplasten führten zu einer Auffassung der Photosynthese, die sich von der herkömmlichen Ansicht - als eines CO_2-Assimilationsprozesses – unterscheidet: Die Photosynthese scheint im wesentlichen ein Prozess zur Umwandlung der Strahlungsenergie des Sonnenlichtes in chemische Energie zu sein. Diese Umwandlung steht in engerer Beziehung zur Phosphorassimilation und zur Reduktion des Eisenproteids Ferredoxin als zur Kohlensäureassimilation. Die ersten, chemisch definierten Produkte dieser Energieumwandlungsreaktion in der Photosynthese erwiesen sich nicht als Zwischenprodukte der Kohlenstoffassimilation, sondern als ATP und reduziertes Ferredoxin.

SPECIALIA

Chemical Investigations of *Alangium lamarckii* II. Isolation of Choline from the Leaves

In a recent communication[1], a new crystalline phenolic alkaloid, Ankorine, $C_{19}H_{29}O_4N$, m.p. 174–176 °C, possessing hypotensive action[2] of prolonged duration, was isolated from the chloroform-soluble tertiary alkaloids obtained from the alcoholic extract of the leaves of *Alangium lamarckii* Thw. (N.O. *Alangiacea*). On preliminary pharmacological studies carried out by SANYAL et al.[3], the total tertiary alkaloidal fraction, a mixture of at least five alkaloids of very close Rf values including ankorine, obtained from the alcoholic extract of the leaves possessed mild adrenolytic, hypotensive, non-specific antispasmodic and anticholinesterase activity. It was previously shown[1] that the aqueous solution left after removal of the tertiary alkaloids by filtration and subsequent repeated extraction with chloroform gave a positive test with Mayer's reagent, indicating the possible presence of some water-soluble quaternary bases. A preliminary pharmacological study[4] showed that this aqueous solution possessed acetylcholine-like spasmogenic activity. Attempts were therefore made to isolate the water-soluble quaternary bases from the aqueous solution (D)[1] which might constitute the cholinergic principle present in the leaves of the plant. The water-soluble quaternary base was precipitated with ammonium reineckate, and the separated base reineckate was decomposed with silver sulphate. The base sulphate was converted into base chloride with barium chloride. The quaternary base chloride was dissolved in absolute alcohol and freed from traces of dissolved barium chloride by passage through Brockman aluminium oxide for chromatography. The eluants with absolute alcohol gave on removal of solvent a highly hygroscopic crystalline substance which possessed acetylcholine-like activity, though much less potent quantitatively compared with acetylcholine, when tested pharmacologically[4]. This diminished acetylcholine-like activity of the isolated quaternary base suggested that it might be choline, a decomposition product of acetylcholine, and a much less potent compound than acetylcholine. A paper chromatographic study showed that the isolated quaternary base gave a pink spot (Rf 0.36) on paper similar to choline chloride, and different from the spot of acetylcholine chloride (Rf 0.42) having a yellow shade changing gradually to brown. Paper chromatography was done on Whatman No. 1 paper with a mixture of ethyl acetate:pyridine:water (10:6:3) as the eluant. After drying the paper, Dragendorff's reagent was

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[2] A. K. SANYAL, B. DASGUPTA, and P. K. DAS, unpublished report.
[3] A. K. SANYAL, B. DASGUPTA, and P. K. DAS, Indian J. med. Res. *53*, 1072 (1965).
[4] A. K. SANYAL, B. DASGUPTA, S. P. SEN, S. S. GAMBHIR, and P. K. DAS, Indian J. Physiol. Pharmac., in press.