Ferredoxin and Photosynthesis

An iron-containing protein is a key factor in energy transfer during photosynthesis.

Daniel I. Arnon

Ferredoxin is the name given by Mortenson, Valentine, and Carnahan (*1*) to a protein containing iron which is neither a heme protein nor a flavoprotein. Mortenson *et al.* isolated this protein from *Clostridium pasteurianum*, a nonphotosynthetic, anaerobic bacterium which normally lives in the soil without any exposure to light. In this, and in other nonphotosynthetic, obligately anaerobic bacteria where ferredoxin was later found (*2–4*), it appeared to function as a link between the enzyme hydrogenase and different electron donors and acceptors. Thus, the distribution of ferredoxin seemed likely to be limited to those obligately anaerobic bacteria that contain an active hydrogenase system.

When ferredoxin was isolated there was nothing to indicate that it 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 (*5*). In fact, it was recognized (*5*) that, between 1952 and 1958, 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" (*6*), "TPN-reducing factor" (*7*), "photosynthetic pyridine nucleotide reductase" (PPNR) (*8*), and "haem-reducing factor" (*9*). All these terms are now known to be synonymous; they have been replaced by the term ferredoxin (*5, 10*). It also became apparent that the "red enzyme" isolated in 1962 in Warburg's laboratory is analogous to ferredoxin (*11, 12*). Adoption of the new terminology in 1962 and recognition of the crucial role of ferredoxin in photosynthesis rest on several developments.

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Definition of Ferredoxin

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 triphosphopyridine nucleotide (TPN) (*13*). But in that year K. Tagawa and M. Nozaki (*14*) and Losada *et al.* (*15*) 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 TPN and the evolution of oxygen by chloroplasts, although *Chromatium* cells are incapable of evolving oxygen in light. This work indicated that proteins similar to those functioning in the TPN-reducing apparatus of chloroplasts are also present in photosynthetic bacteria devoid of chloroplasts.

As long as all the proteins that catalyze the photoreduction of TPN by chloroplasts were derived from green plants and photosynthetic bacteria, there was no basis to suppose that similar proteins were present in nonphotosynthetic cells. However, their presence in such cells became apparent when Tagawa and I (*5*) crystallized ferredoxin from *Clostridium pasteurianum* (Fig. 1) and found that it was able to replace the native chloroplast protein in the photoreduction of TPN. The same investigation led also to other findings. (i) The chloroplast protein, like ferredoxin of *C. pasteurianum*, contained iron (*16*) and was reversibly oxidized and reduced, with characteristic changes in its absorption spectrum. (ii) Crystalline ferredoxin from *C. pasteurianum* had a remarkably low oxidation-reduction potential ($E'_0 = -417$ mv at *p*H 7.55), close to the potential of hydrogen gas and about 100 mv more electronegative than the oxidation-reduction potential of pyridine nucleotides. (iii) The oxidation-reduction potential of the spinach chloroplast protein was also strongly electronegative ($E'_0 = -432$ mv at *p*H 7.55).

These similarities led us 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 are, at least in part, functionally interchangeable in the photoreduction of TPN by isolated chloroplasts. Thus, 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 TPN by washed chloroplasts is included provisionally in the definition of ferredoxins because all the ferredoxins that have been tested so far in our laboratory exhibit this property. By contrast, the replaceability of different ferredoxins in other enzyme reactions is less consistent.

This "working" definition allows for dissimilarities of some properties among different ferredoxins. For example, the absorption spectra of ferredoxins from bacterial cells, whether photosynthetic or nonphotosynthetic, 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 distinguish, on the basis of spectral characteristics, between 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 proteins

which function as electron carriers on the "hydrogen side" of pyridine nucleotides. This definition stresses the 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.

Ferredoxins and TPN Reduction

To test the effectiveness of different ferredoxins in catalyzing the photoreduction of TPN, we have crystallized several ferredoxins from organisms other than *Clostridium pasteurianum*. Crystalline ferredoxin from spinach chloroplasts is shown in Fig 2. Figure 3 shows crystalline ferredoxin from the blue-green alga *Nostoc*, and Fig. 4 shows crystalline ferredoxin from the photosynthetic bacterium *Chromatium*. Despite the diversity of sources, all these ferredoxins are effective as substitutes for the native spinach ferredoxin in catalyzing the reduction of TPN by illuminated spinach chloroplasts. Figure 5 shows results obtained when the ferredoxin of spinach chloroplasts is replaced by varying amounts of crystalline *Chromatium* ferredoxin —an effect which, as previously mentioned, had been observed with crude *Chromatium* ferredoxin when it was still called "pyridine nucleotide reductase" (*15*).

Spectra and Redox Potentials

Unlike cytochromes, which exhibit well-defined absorption peaks when they are in the reduced state, ferredoxins have distinctive absorption peaks when in the oxidized state; on reduction, these peaks disappear.

The first ferredoxin to be crystallized, that of *Clostridium pasteurianum*, exhibited in its oxidized state a distinctive spectrum with peaks in the visible and ultraviolet at 390, 300, and 280 mμ (*5*). The crystalline preparation gave an absorption ratio of 390 mμ/280 mμ = 0.79 (*5*). These spectral characteristics of ferredoxin from *C. pasteurianum* were confirmed and extended by Buchanan, Lovenberg, and Rabinowitz (*3, 4*) to ferredoxins of other species of *Clostridium*, which they prepared in crystalline form.

Figure 6 shows that the absorption spectrum of ferredoxin of the photosynthetic bacterium *Chromatium* (*17*) closely resembles that of ferredoxin from the nonphotosynthetic *Clostridium* species. In the oxidized state, the absorption spectrum of *Chromatium* ferredoxin exhibits a flat peak at 385 mμ, a shoulder at 300 mμ, and a peak at 280 mμ. In our purest preparation, the ratio of optical density, 385 mμ/280 mμ, was 0.74 (*17*).

As shown in Fig. 6, *Chromatium* ferredoxin was reduced by three methods: (i) hydrogen gas in the dark, in the presence of a hydrogenase preparation from *Clostridium pasteurianum*; (ii) sodium dithionite, in the dark; and (iii) photochemically, with a heated preparation of spinach chloroplasts and with reduced dichlorophenol indophenol as the electron donor.

Complete reduction of *Chromatium* ferredoxin was obtained only photo-

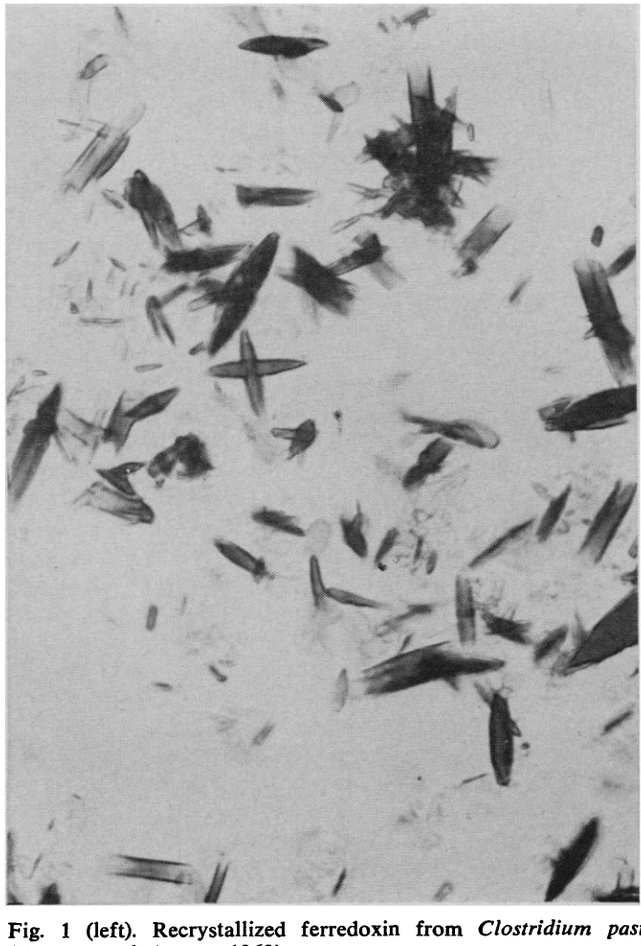


Fig. 1 (left). Recrystallized ferredoxin from *Clostridium pasteurianum* (*5*). (Tagawa and Arnon, 1963).

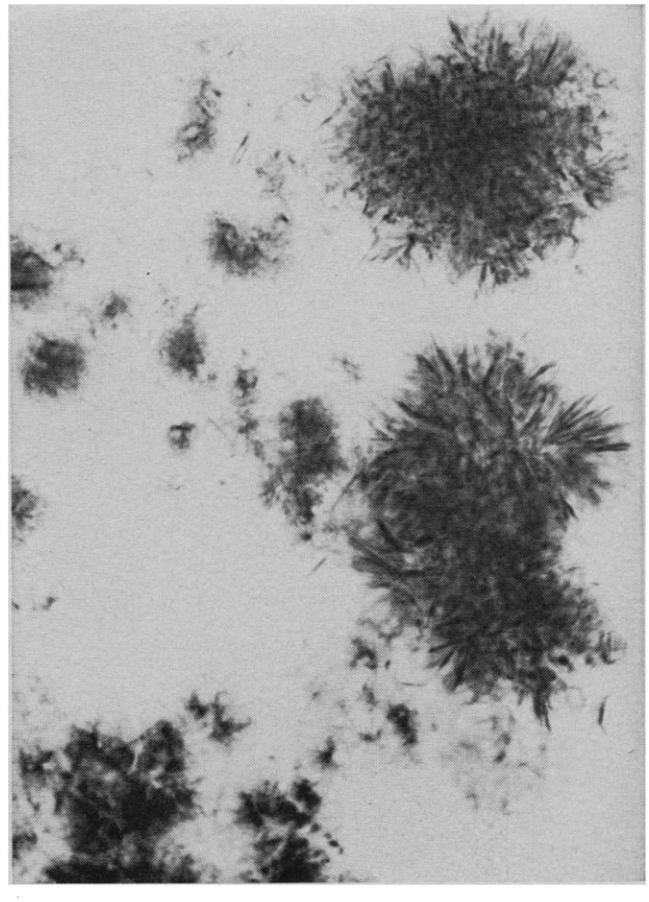


Fig. 2 (right). Crystalline spinach ferredoxin

chemically. Taking the reduction in this system as 100 percent, the reduction by the H_2-hydrogenase system was 24 percent. The reduction by the dithionite system was intermediate between these two. From these data the oxidation-reduction potential of *Chromatium* (at *p*H 7) was calculated (*17*) to be about −490 mv—that is, it was considerably more electronegative than that of the spinach chloroplast ferredoxin or that of the *Clostridium* ferredoxin (*5*). It remains to be seen whether the ferredoxins from other photosynthetic bacteria will also prove to be as strongly reducing as *Chromatium* ferredoxin.

Figure 7 shows that the absorption spectrum of ferredoxin (oxidized state) from the blue-green alga *Nostoc* is of the chloroplast type (*18*). The spectrum closely resembles 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 visible and in ultraviolet light are 470, 423, 331, and 276 mμ, as compared with 463, 420, 325, and 274 mμ for spinach ferredoxin (*5*). Our purest preparation of *Nostoc* ferredoxin had a ratio of optical density, 423 mμ/276 mμ, of 0.57 (*18*).

The similarity between the ferredoxins 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 photosynthesis in bacteria, photosynthesis in blue-green algae is accompanied by evolution of oxygen. Whether the occurrence of the chloroplast type of ferredoxin in blue-green algae is related to the types of photosynthetic pigment system and oxygen evolution that distinguish algal photosynthesis from bacterial photosynthesis has not been determined.

A preliminary determination of the oxidation-reduction potential of *Nostoc* ferredoxin gave a value of $E'_0 = -405$ mv at *p*H 7.5 (*18*).

Photoproduction of Hydrogen Gas

Photoproduction of hydrogen gas by photosynthetic cells was first observed by Gaffron and Rubin in the green alga *Scenedesmus* (*19*) and by Gest and Kamen in photosynthetic bacteria (*20*). The nature of this phenomenon was obscure. In photosynthetic bacteria, photoproduction of hydrogen gas was thought to depend on the presence of exogenous organic acids and carbon dioxide (*20–26*). According to one idea, the evolved hydrogen came from the decomposition of α-ketoglutaric acid (*24*).

In more recent experiments, we found that *Chromatium* cells produced hydrogen gas in light solely at the expense of a simple inorganic oxidizable substrate, thiosulfate (*27*). These findings demonstrated that the bacterial photoproduction of hydrogen gas is basically independent of carbon dioxide assimilation or of the decomposition of organic acids and is probably a variant of the noncyclic electron transport in chloroplasts—that is, it is a case in which the bacterial cells use the energy of captured photons to drive an electron

Fig. 3 (left). Crystalline ferredoxin from *Nostoc* (*18*).

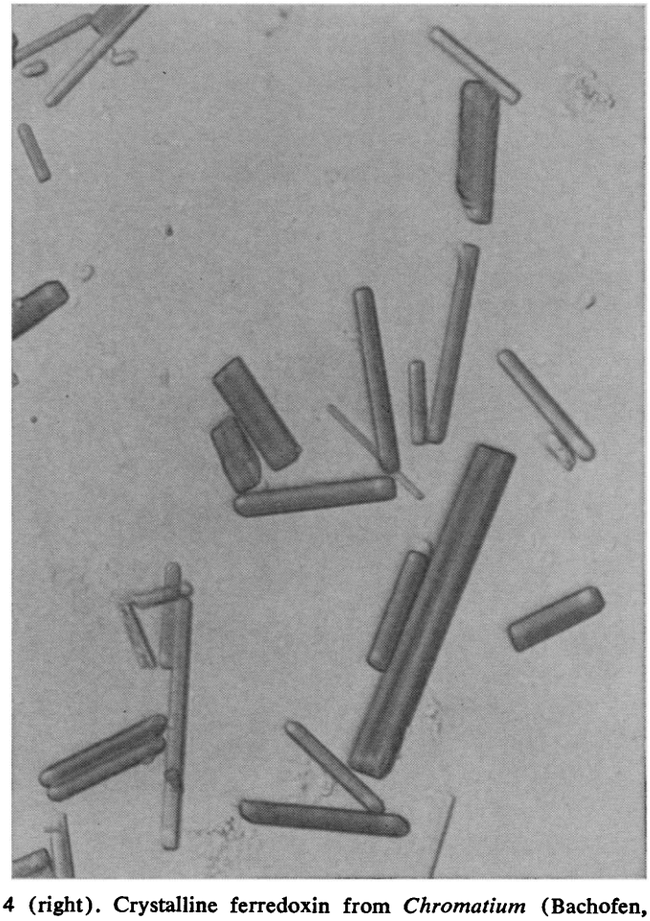


Fig. 4 (right). Crystalline ferredoxin from *Chromatium* (Bachofen, Oda, and Arnon, 1963).

flow "uphill" against the thermodynamic gradient, from thiosulfate to a hydrogenase system (*27*). Hydrogenase, which is a normal constituent of *Chromatium* cells, would then catalyze the terminal reaction in the photoproduction of hydrogen gas: $2H^+ + 2e^- \rightarrow H_2$.

Photoproduction of hydrogen gas by chloroplasts or by a cell-free bacterial system had never been observed. However, according to the electron flow theory in photosynthesis (*28*), photoproduction of hydrogen gas should occur in a photosynthetic system in which an "open" or noncyclic electron flow is photochemically induced between an electron donor system and hydrogenase as the electron acceptor system. Isolated chloroplasts readily exhibit noncyclic electron flow but lack the enzyme hydrogenase, which, however, can be prepared from bacteria. Thus, if the noncyclic electron flow theory is correct, isolated chloroplasts coupled to a bacterial hydrogenase should give a cell-free system that will produce hydrogen gas.

These considerations were experimentally verified. When photoproduction of oxygen was suppressed (oxygen is deleterious to hydrogenase activity) and when cystein or reduced dichlorophenol indophenol was used instead of water as the electron donor, spinach chloroplasts, supplemented with a bacterial hydrogenase, evolved hydrogen gas in light (*29–31*).

Figure 8 shows photoproduction of hydrogen gas by isolated chloroplasts coupled to a hydrogenase isolated from the nonphotosynthetic obligate anaerobe *Desulfovibrio desulfuricans*. A similar photoproduction of hydrogen gas occurred in a system in which isolated chloroplasts were supplemented with a hydrogenase isolated from *Chromatium* cells. The identity of the gas evolved was determined by adsorption on palladium asbestos.

Equivalence of Light and Hydrogen

The photoproduction of hydrogen gas shown in Fig. 8 depended on the addition of a nonphysiological electron carrier, methyl or benzyl viologen, which linked the electron donor system with hydrogenase (*32*). However, no addition of a viologen dye was required for photoproduction of hydrogen gas when a crude extract of *Clostridium pasteurianum* was used as the source of hydrogenase. It soon became apparent that the extract of *C. pasteurianum* supplied not only hydrogenase but also ferredoxin and that it was ferredoxin which acted as the electron carrier between the illuminated chloroplasts and the hydrogenase of *C. pasteurianum* (*5*).

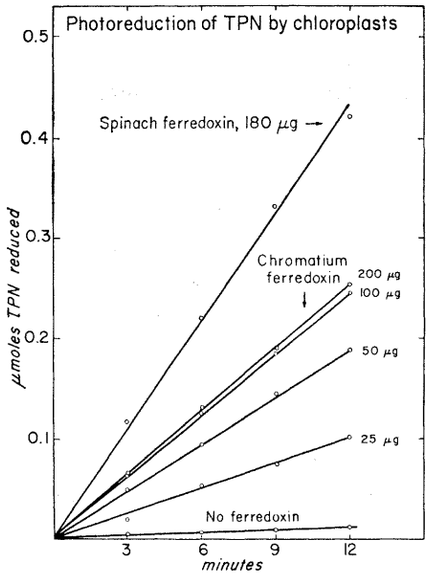


Fig. 5. Substitution of *Chromatium* ferredoxin for spinach ferredoxin in the reduction of TPN by illuminated spinach chloroplasts (*17*).

With the recognition of ferredoxin as an electron carrier between hydrogenase and chloroplasts, it became possible to design experiments in which the enzymic apparatus of chloroplasts reduced TPN in the dark, in a system in which hydrogen gas and hydrogenase replaced light as a source of energy.

In the reduction of TPN by hydrogen gas in the dark the chlorophyll pigments made no contribution whatsoever and were, in fact, omitted in later experiments. The essential contribution of chloroplasts was limited to the flavoprotein fraction, which evidently contained the enzyme that catalyzed the reduction of TPN. The replacement of light by H_2-hydrogenase as the source of energy for TPN reduction was useful in later experiments aimed at the elucidation of the mechanism of pyridine nucleotide reduction in chloroplasts.

The evidence from these and related experiments on the role of ferredoxins in photosynthetic electron transport and in biological utilization and production of hydrogen is diagrammatically summarized in Fig. 9.

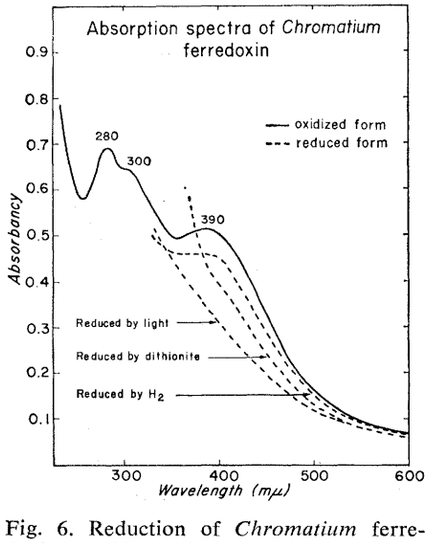


Fig. 6. Reduction of *Chromatium* ferredoxin by H_2, sodium dithionite, and by illuminated spinach chloroplasts (*17*).

Crucial Electron Acceptor

Recognition of the role of ferredoxin in reduction of TPN by chloroplasts made it clear that ferredoxin and not TPN is the crucial electron acceptor in the photochemical reactions of chloroplasts. This change extends further the experimentally established value of the reducing potential that is generated by chloroplasts during photosynthesis. Before 1951, isolated chloroplasts were known to reduce only nonphysiological electron acceptors (Hill reagents) with strongly electropositive oxidation-reduction potentials—for example, 0.43 volt for ferricyanide and 0.28 volt for benzoquinone (*p*H 7). This and other evidence led to generalizations that illuminated chloroplasts could not form a reductant which had an oxidation-reduction potential more reducing than 0 volt (*33*, *34*).

In 1951 Vishniac and Ochoa (*35*), Tolmach (*36*), and I (*37*) independently found that illuminated chloroplasts are capable of reducing pyridine nucleotides and thereby established the first link between photon capture by chloroplast pigments and the reduction of a physiological electron carrier with a strongly electronegative oxidation-reduction potential ($E'_0 = -320$ mv). The photoreduction of ferredoxin demonstrates that isolated chloroplasts can generate a reductant with a oxidation-reduction potential about 100 mv more electronegative than that of pyridine nucleotides.

The emphasis in this discussion is not on a theoretical reducing potential that can be generated by illuminated chloroplasts, or on evidence that chloro-

Table 1. Stoichiometry of photoreduction of spinach ferredoxin (Fd) and its subsequent reoxidation by TPN in the dark (after Whatley, Tagawa, and Arnon, *38*). The ratio of Fd to TPN is 2.17.

Reaction	Amount (μmoles)
Fd photoreduced	0.102
Fd reoxidized by TPN in the dark	.106
TPN reduced	.047

plasts can reduce nonphysiological agents with strongly reducing potentials, but on the experimental isolation and characterization of the strongest known reductant, *native to photosynthetic cells*, that is formed by the photochemical act of photosynthesis. The wavelengths of light absorbed by chloroplasts, even those with the least energy, can theoretically generate stronger reductants than reduced ferredoxin. For example, 1 einstein of red light ($\lambda = 663$ mμ) is equivalent to 1.87 electron volts and has more than enough energy to transfer electrons from water to ferredoxin. However, all such possibilities must remain speculative without evidence that photosynthetic cells contain reductants stronger than ferredoxin.

Stoichiometry

Normally the role of ferredoxin in TPN reduction is catalytic. The photoreduced ferredoxin is promptly reoxidized by TPN, and the accumulation of reduced TPN is measured spectrophotometrically by the increase in absorption at 340 mμ. However, by using "substrate" amounts of ferredoxin in the absence of TPN, it was possible (*38*) to reduce spinach chloroplast ferredoxin quantitatively with an illuminated preparation of chloroplasts. The progressive photoreduction of ferredoxin by illuminated chloroplasts was followed by measuring its spectral changes in a recording spectrophotometer.

The ferredoxin reduced by light was stable under the strictly anaerobic conditions of the experiments and was not spontaneously reoxidized in a subsequent dark period. However, the reduced ferredoxin became quickly and completely reoxidized when TPN was added. The results, which showed that 1 mole of TPN reoxidized 2 moles of ferredoxin (Table 1), established that the reduction and oxidation of one molecule of ferredoxin involves a transfer of one electron. This stoichiometry between ferredoxin and TPN was confirmed by Horio and San Pietro (*39*).

Table 2. Some properties of bacterial and spinach ferredoxin.

Clostridium pasteurianum	*Chromatium*	Spinach
Iron content (atoms per molecule of protein)		
7	3	2
Inorganic sulfide content (moles per mole of protein)		
7	3	2
Molecular weight (approx.)		
6,000	6,000	13,000
Redox potential (mv at pH 7.55)		
−417	−490*	−432

* Approximate.

Some Chemical Properties

Apart from iron, ferredoxins of chloroplasts and bacteria are noted for containing "labile sulfide," an inorganic sulfide group which is equimolar with iron. This was first observed in spinach ferredoxin by Fry and San Pietro (*40*) and independently in Warburg's laboratory in the red enzyme of *Chlorella* (*11, 12*). Buchanan, Lovenberg, and Rabinowitz (*3, 4*) 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 characteristic absorption spectrum and also its biochemical activity (*3, 4, 40*).

Ferredoxins are small molecules. The bacterial ferredoxin, first estimated to have a molecular weight of around 12,000 (5), is now known to have a molecular weight of around 6000 (*4*). The chloroplast ferredoxin is estimated to have a molecular weight of approximately 13,000 (*38*). 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 (*17*) and that of *Clostridium* has seven (*4*).

A summary of some chemical properties of several ferredoxins is given in Table 2. From the standpoint of their cellular function as electron carriers, the most striking property of ferredoxins is their strongly electronegative oxidation-reduction potentials (Table 2).

Mechanism of TPN Reduction

Elucidation of the nature of ferredoxin as an electron carrier led to the resolution of the mechanism of TPN reduction by chloroplasts into three steps: (i) a photochemical reduction of ferredoxin; (ii) reoxidation of ferredoxin by a flavoprotein enzyme, ferredoxin-TPN reductase; and (iii) reoxidation of the reduced ferredoxin-TPN reductase by TPN (*41*).

Elucidation of the mechanism of photoreduction of TPN by chloroplasts was favored by several developments. The recognition that chloroplast ferredoxin cannot transfer electrons to TPN directly but only indirectly through the flavoprotein fraction of chloroplasts (*5*) led to the crystallization of the specific flavoprotein involved, which was named ferredoxin-TPN reductase (Fig. 10) (*42*). The crystalline enzyme acted primarily as a reductase but also had secondary activities that correspond to the TPNH diaphorase of Avron and Jagendorf (*43, 44*) and to the transhydrogenase

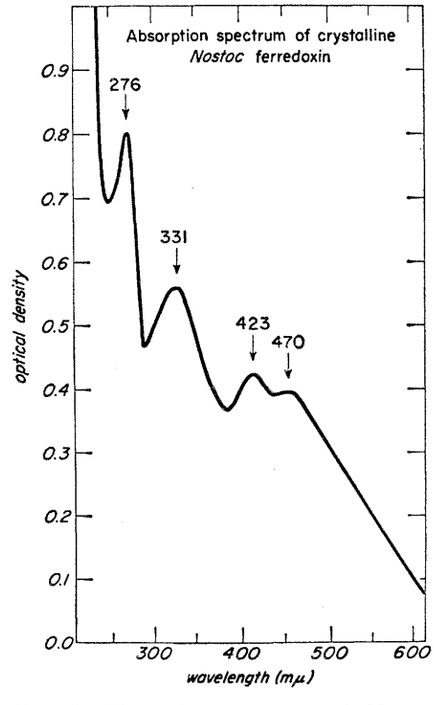


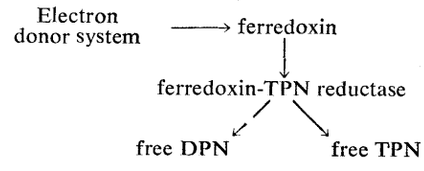
Fig. 7. Absorption spectrum of *Nostoc* ferredoxin in the oxidized state (*18*).

of Keister *et al.* (*45*). The absorption spectrum of the ferredoxin-TPN reductase showed a typical flavoprotein absorption spectrum with peaks at 275, 385, and 456 mμ and minima at 321 and 410 mμ.

Crystalline spinach ferredoxin and crystalline spinach ferredoxin-TPN reductase were used to reduce TPN in a system in which hydrogen gas and hydrogenase replaced illuminated chloroplast fragments as a source of reducing power for the reduction of ferredoxin (*41*). This "dark" method was preferable to the photochemical method in investigating the mechanism of TPN reduction. First, it eliminated the interference of chloroplast pigments and of actinic light in the spectrophotometric assay of TPN reduction at 340 mμ. Second, it made it possible to replace the grana-bound ferredoxin-TPN reductase (*42*) with the crystalline enzyme, the concentration of which could be varied independently of the reducing power generated photochemically.

The same mechanism also operated in the reduction of DPN by ferredoxin-TPN reductase, except that the affinity of the enzyme for TPN is much greater than for DPN. The Michaelis constant K_m for DPN was found to be 3.75×10^{-3} mole per liter, which was about 400 times greater than the value for TPN (9.78×10^{-6} mole per liter). The great difference between the affinities of ferredoxin-TPN reductase for TPN and DPN accounts for the apparent specificity of the pure enzyme for TPN. Under physiological conditions DPN would hardly be reduced by ferredoxin-TPN reductase. This would result from the combination of a low affinity of the enzyme for DPN, the approximately equal concentrations of DPN and TPN in the cell (*46*), and the competition between DPN and TPN (*41*).

The evidence for the mechanism of TPN reduction by chloroplasts (and its comparison with other schemes in the literature) is presented in detail elsewhere (*41*). Under physiological conditions (solid lines in the scheme below), the electron flow in the reduction of pyridine nucleotides by chloroplasts can be summarized as follows:



Ferredoxin and Photophosphorylation

The elucidation of the detailed mechanism of TPN reduction by chloroplasts strengthened the view that photoreduction of ferredoxin is the key step in the physiological electron transport induced in chloroplasts by light. A corollary of this conclusion is that the photoreduction of ferredoxin must be linked with the other photochemical events in chloroplasts: cyclic and noncyclic photophosphorylation (*28*) and evolution of oxygen. Cyclic and noncyclic photophosphorylations are subdivisions of photosynthetic phosphorylation, a process for the conversion and storage of radiant energy into the pyrophosphate bonds of adenosine triphosphate (ATP), which my associates, M. B. Allen and F. R. Whatley, and I discovered in 1954 (*47*).

A puzzling feature of cyclic photophosphorylation in chloroplasts, a feature which distinguishes it from cyclic photophosphorylation in chlorophyll-containing particles from photosynthetic bacteria, was dependence on an added electron carrier, such as menadione or phenazine methosulfate. A possible, though heretofore experimentally unsupported, explanation of this difference was that chloroplasts, but not the bacterial particles, automatically lose a soluble constituent in the process of isolation. Recent findings point to ferredoxin as the endogenous, water-soluble catalyst of cyclic photophosphorylation in chloroplasts which is, at least in part, lost from chloroplasts when they are removed from the cell.

Evidence for a ferredoxin-catalyzed cyclic photophosphorylation which proceeds anaerobically without the addition of other cofactors was obtained after the experimental conditions under which this type of photophosphorylation can be observed had been established (*48*). These conditions include a relatively low light intensity when a broad spectral band of light is used (illumination higher than 10,000 lux was inhibitory), an optimum *p*H of about 8.2, and the use of *p*-chlorophenyldimethyl urea (CMU) as an inhibitor of oxygen evolution (*49*).

Cyclic photophosphorylation catalyzed by ferredoxin not only was resistant to inhibition by CMU but was, in fact, markedly enhanced in the presence of this inhibitor (*48*). It appears that ferredoxin-catalyzed cyclic photophosphorylation is so strictly anaerobic that it is impeded by traces of oxygen

Table 3. Effect of ferredoxin on cyclic photophosphorylation in an atmosphere of argon (*48*).

Ferredoxin added (μg)	ATP formed	
	Counts per minute	Micromoles
0	23	0.00
30	146	.02
75	410	.07
150	789	.14
300	2,109	.37
750	7,404	1.29
1,500	10,816	1.88

that might be produced by illuminated chloroplasts in the absence of CMU. The effect of ferredoxin on anaerobic cyclic photophosphorylation in the absence of other catalysts of photophosphorylation is shown in Table 3.

A notable feature of ferredoxin-catalyzed cyclic photophosphorylation was its sensitivity to antimycin A, a sensitivity which was apparent over a wide range of chlorophyll concentrations. Antimycin A inhibits the endogenous anaerobic cyclic photophosphorylation by chromatophores of the purple bacteria, *Rhodospirillum rubrum* (*50, 51*) and *Chromatium* (*52*). The inhibition of cyclic photophosphorylation in bacterial chromatophores does not occur in the presence of catalytic amounts of phenazine methosulfate (*50, 53*) which evidently serve as a by-pass around the site of antimycin A inhibition. Likewise, the usual type of cyclic photophosphorylation in chloroplasts, the photophosphorylation that is measured in the presence of catalysts other than ferredoxin, is also resis-

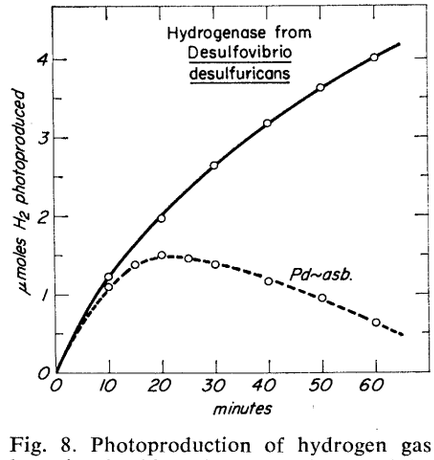


Fig. 8. Photoproduction of hydrogen gas by spinach chloroplasts, supplemented by a hydrogenase from *Desulfovibrio desulfuricans*. H_2 was identified by adsorption on palladium asbestos (Pd~asb.) (*30*).

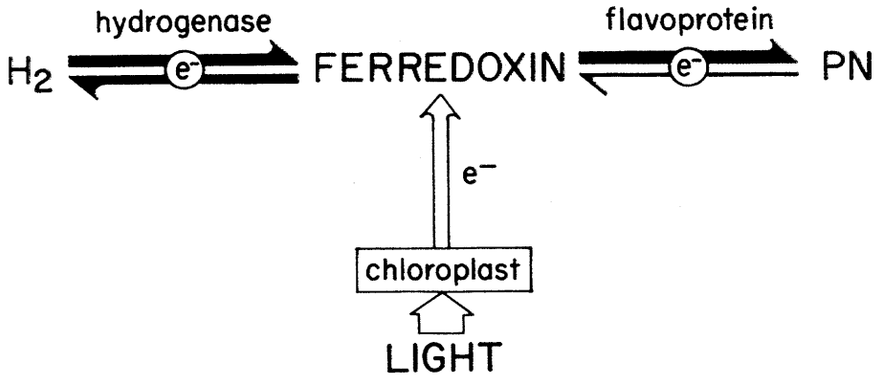


Fig. 9. Diagrammatic representation of the role of ferredoxin in photochemical reduction of pyridine nucleotides (PN) and in the biological production and consumption of H_2 (*5*).

tant to inhibition by antimycin A (*54*).

Inhibition by antimycin A of oxidative phosphorylation in mitochondria is considered to be indicative of the participation of cytochrome *b* in electron transport (*55*). It is possible, therefore, that the cytochrome *b* component of chloroplasts (*56, 57*) participates in, and is responsible for, the sensitivity to antimycin A of the cyclic photophosphorylation catalyzed by ferredoxin. There is no evidence of any kind that chloroplast cytochrome *b* is involved in the cyclic photophosphorylation that is catalyzed by such exogenous catalysts as phenazine methosulfate.

Figure 11 illustrates a differential sensitivity to inhibition by antimycin A of cyclic photophosphorylation catalyzed by ferredoxin, menadione, or phenazine methosulfate; it also illustrates that under the very low light intensity under which these experiments were carried out, ferredoxin is by far the most effective catalyst for the conversion of radiant energy into the pyrophosphate bond energy of ATP. Phenazine methosulfate, which is widely used as a catalyst of cyclic photophosphorylation at high light intensity, was the least effective catalyst at low light intensity. The catalytic effect of menadione in cyclic photophosphorylation was intermediate between that of ferredoxin and phenazine methosulfate.

Heretofore, a notable distinction between oxidative phosphorylation by mitochondria and photosynthetic phosphorylation by chloroplasts was the insensitivity of the latter to low concentrations ($5 \times 10^{-5} M$) of 2,4-dinitrophenol (see review, *58*). It was interesting to find, therefore, that the ferredoxin-dependent cyclic photophosphorylation is distinct from all other types of photophosphorylation in chloroplasts, but similar to oxidative phosphorylation in mitochondria, in being strongly inhibited by low concentrations of 2,4-dinitrophenol (50-percent inhibition was observed when the concentration was $3 \times 10^{-5} M$).

In addition to the anaerobic cyclic photophosphorylation, ferredoxin catalyzes pseudocyclic photophosphorylation (*59*). Aerobic, ferredoxin-dependent pseudocyclic photophosphorylation is distinguished from anaerobic, ferredoxin-catalyzed cyclic photophosphorylation by its sensitivity to CMU and by its insensitivity to inhibition by antimycin A.

Cyclic and pseudocyclic photophosphorylations produce only ATP, whereas in noncyclic photophosphorylation, the formation of ATP is stoichiometrically linked with the photoreduction of the electron acceptor. When noncyclic photophosphorylation was discovered, the formation of ATP was linked with the photoreduction of TPN or a nonphysiological substitute, such as ferricyanide (*60*). When the photoreduction of TPN was recently found to be a dark reaction that follows the photoreduction of ferredoxin, it became important to determine whether ATP formation of the noncyclic type is also stoichiometrically coupled with photoreduction of ferredoxin in the absence of TPN, when ferredoxin itself is made to serve as a terminal electron acceptor in this type of photophosphorylation.

To measure the stoichiometry between ATP and ferredoxin, it was necessary first to suppress cyclic photophosphorylation by antimycin A and pseudocyclic phosphorylation by cystein (*61*). In the presence of both cystein and antimycin A, ATP was formed photochemically only by noncyclic photophosphorylation. The amount of ATP formed was proportional to the amount of ferredoxin reduced, in a ratio of approximately 1 mole of ATP to 2 moles of ferredoxin ($P/2e = 1$). This ratio (Table 4) provides additional evidence that the oxidation-reduction of ferredoxin involves a transfer of one electron (*62*).

Thus, evidence has been obtained that ferredoxin catalyzes cyclic, pseudocyclic, and noncyclic photophosphorylation. When ferredoxin is the stoichiometric electron acceptor in noncyclic photophosphorylation, it is reduced in a ratio of 2 moles of ferredoxin to 1 mole of ATP formed.

Table 4. Stoichiometry of noncyclic photophosphorylation with various amounts of ferredoxin (*62*).

Time (min)	ATP formed (μmole/ml) after addition of ferrodoxin			
	Ferredoxin (μmole/ml)			
	0	0.20	0.40	0.60
2.5	0.00	0.13	0.13	0.08
5	.00	.13	.21	.16
10	.00	.12	.21	.27
15	.00	.11	.21	.27
	*Ratio of Fd:ATP**			
		1.5	1.9	2.2

* Based on the highest values of ATP for each concentration of ferredoxin.

Ferredoxin and Oxygen

As already stated, when photoreduction of ferredoxin is the terminal photochemical event in noncyclic photophosphorylation by chloroplasts, it must be linked not only with photophosphorylation but also with oxygen evolution. The evolution of oxygen by chloroplasts is uniquely dependent on light, and it

Table 5. Stoichiometry between oxygen evolution and photoreduction of ferredoxin (Fd) by isolated chloroplasts (*62*).

Test No.	Amount (mμmole)		Ratio of Fd added: O_2 produced
	Fd added	O_2 produced	
	Experiment D		
1	110	26	4.2
2	110	27	4.1
3	110	27	4.1
	Experiment E		
1	128	32	4.0
2	128	33	3.9
3	128	32	4.0
	Experiment F		
1	154	37	4.2
2	154	42	3.7
3	154	39	3.9

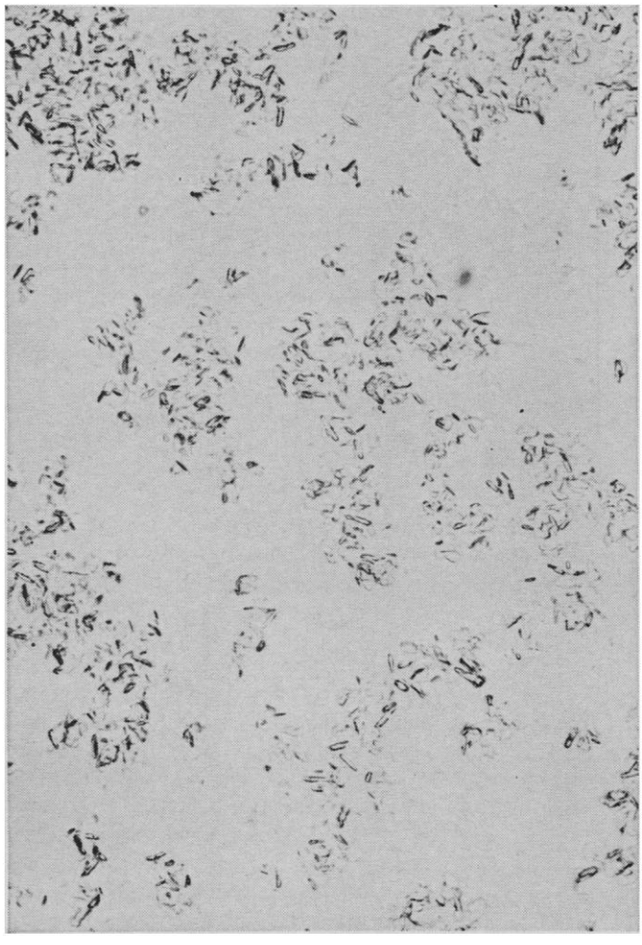
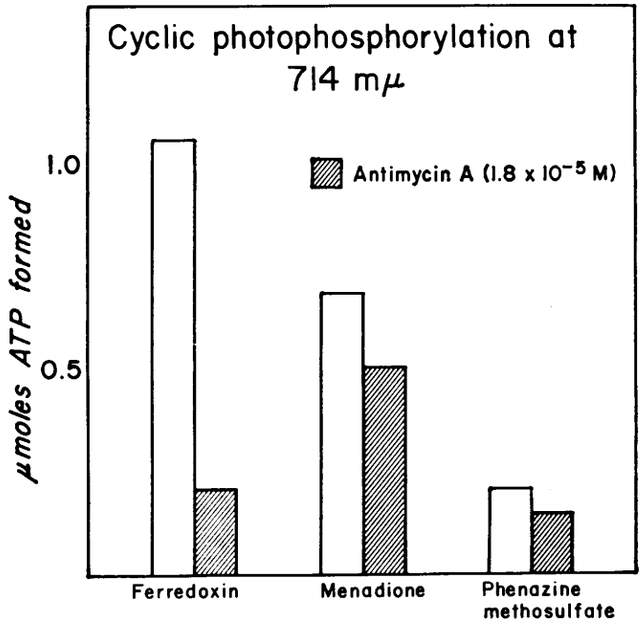


Fig. 10 (left). Crystalline ferredoxin-TPN reductase (*42*).

Fig. 11 (above). Differential effect of antimycin A on cyclic photophosphorylation catalyzed by ferredoxin, menadione, and phenazine methosulfate. Illumination: monochromatic lights, 714 mμ Arnon, Tsujimoto, and McSwain, 1964).



occurs only in the presence of a proper electron acceptor. Thus, photoproduction of oxygen by chloroplasts should accompany 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 polarographically and determining the photoreduction of ferredoxin by the decrease in optical density at 420 mμ (*62*). Oxygen was rigidly excluded until the light was turned on. Under these conditions, chloroplasts reduced ferredoxin completely. When the light was turned off, ferredoxin was reoxidized. The amount of ferredoxin reduced in the light was equal to the amount of ferredoxin reoxidized in the dark.

The photoreduction of ferredoxin was accompanied by evolution of oxygen. No oxygen was evolved without addition of ferredoxin. When the light was turned off, the oxygen that had evolved during the preceding illumination period was consumed. The successive evolution and consumption of oxygen paralleled the photoreduction and reoxidation of ferredoxin. Of special interest was the stoichiometry between the ferredoxin reduced and the oxygen produced. Table 5 shows that the stoichiometry between ferredoxin reduced and oxygen produced was 4 to 1 and remained the same when different amounts of ferredoxin were added (*62*). This result substantiates the conclusion that the photoreduction of ferredoxin involves transfer of one electron.

The reactions of ferredoxin (Fd) may be summarized as follows:

1) Photoreduction of ferredoxin accompanied by stoichiometric evolution of oxygen:

$$4Fd_{ox} + 2H_2O \xrightarrow{h\nu} 4Fd_{red} + O_2 + 4H^+$$

2) Stoichiometric reoxidation of reduced ferredoxin in the dark:

$$4Fd_{red} + O_2 + 4H^+ \longrightarrow 4Fd_{ox} + 2H_2O$$

3) Ferredoxin-catalyzed cyclic photophosphorylation (P_i represents orthophosphate):

$$ADP + P_i \xrightarrow[Fd]{h\nu} ATP$$

4) Ferredoxin-catalyzed pseudocyclic photophosphorylation:

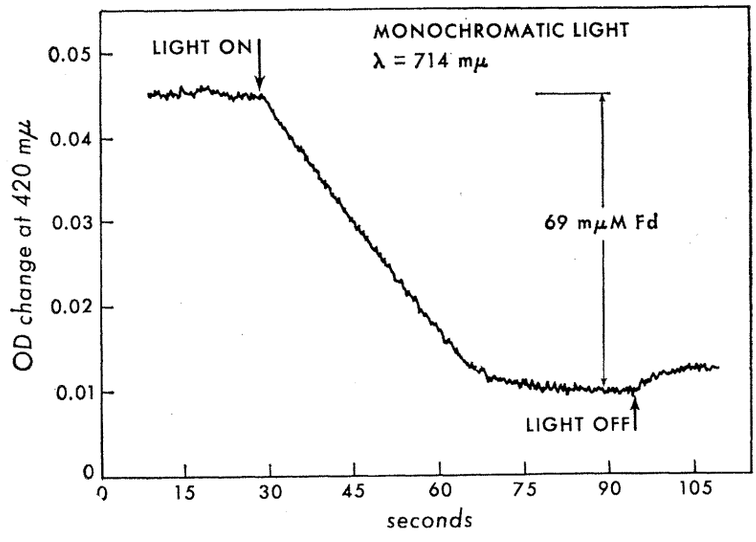
$$ADP + P_i \xrightarrow[O_2,\ Fd]{h\nu} ATP$$

5) Ferredoxin-dependent noncyclic photophosphorylation:

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

Separation of Oxygen Evolution from Photophosphorylation

I have previously suggested that cyclic photophosphorylation is fundamentally independent of oxygen and is basically the same in the oxygen-evolving green plants and in the strictly anaerobic photosynthetic bacteria, the photosynthesis of which is never accompanied by evolution of oxygen



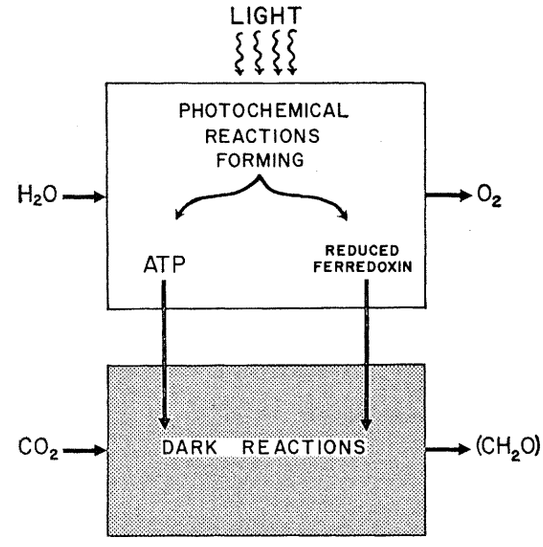


Fig. 12 (left). Photoreduction of ferredoxin (Fd) by isolated chloroplasts illuminated with monochromatic light at 714 mμ (*62*). Fig. 13 (right). Diagrammatic representation of the light and dark reactions of photosynthesis in chloroplasts.

(*63*). Oxygen evolution is, in this view, a consequence of removing electrons from water—a reaction that occurs in algae and green plants but not in photosynthetic bacteria.

The finding that the photoreduction of ferredoxin is linked both with oxygen evolution and with cyclic photophosphorylation opened the possibility of experimentally separating, in chloroplasts, ferredoxin-dependent oxygen evolution from cyclic photophosphorylation. This was done (*64*) by using monochromatic light of a far-red wavelength that would not support oxygen evolution but would still support the ferredoxin-dependent cyclic photophosphorylation in the presence of an artificial electron donor system (ascorbate-dichlorophenol indophenol).

In photosynthesis by intact cells, far-red monochromatic light above 700 mμ does not support oxygen evolution (*65*). This is also the case with isolated chloroplasts. Significant oxygen evolution occurred at wavelengths below, but not above, 700 mμ. However, ferredoxin-dependent cyclic photophosphorylation occurred at 714 mμ. The cyclic nature of this type of photophosphorylation was confirmed by its sensitivity to inhibition by antimycin A (*64*).

Direct evidence for the photoreduction of ferredoxin at 714 mμ is shown in Fig. 12. Since no oxygen is produced by chloroplasts at this wavelength, very little reduced ferredoxin was reoxidized when the light was turned off. To insure the completeness of the photoreduction of ferredoxin at 714 mμ, antimycin A was added to inhibit the light-induced cyclic electron flow which reoxidizes reduced ferredoxin under anaerobic conditions. The completeness of the photoreduction of ferredoxin at 714 mμ was established with different amounts of added ferredoxin (*62*).

Ferredoxin and Energy Conversion

These results indicate that the conversion of radiant energy to chemical energy in photosynthesis by chloroplasts yields three products simultaneously: reduced ferredoxin, ATP, and O_2. Contrary to some speculations, the formation of such a strong reductant as ferredoxin by illuminated chloroplasts is accompanied by formation and not by consumption of ATP. Concurrent formation of ATP, a strong reductant, and oxygen was first noted when noncyclic photophosphorylation was discovered (*60*). The change now concerns the identity of the reductant. Instead of reduced TPN, the strongest photochemically formed reductant, native to chloroplasts, is now known to be reduced ferredoxin, whose reducing power is no less than that of molecular hydrogen.

Of the products of noncyclic photophosphorylation, oxygen escapes, whereas the ATP and the reduced ferredoxin which remain jointly constitute the assimilatory power that drives photosynthetic carbon assimulation (Fig. 13). Since water is the source of electrons in photosynthesis of green plants, noncyclic photophosphorylation—which generates reduced ferredoxin, ATP, and O_2—appears to be the main pathway of photosynthetic energy conversion in chloroplasts as long as CO_2 assimilation proceeds. The ATP that is needed for CO_2 assimilation in excess of the amount supplied by noncyclic photophosphorylation can be provided by a cyclic photophosphorylation catalyzed by ferredoxin. The regulatory mechanism used by chloroplasts to switch from noncyclic to cyclic photophosphorylaton is unknown, but one possibility is the availability of oxidized TPN to accept electrons from reduced ferredoxin (*48*). When all the TPN is reduced, a cyclic electron flow, with a coupled phosphorylation, would ensue.

Ferredoxin and Carbon Assimilation

When the oxidation-reduction potential of ferredoxins was found to be about 100 mv more electronegative than that of pyridine nucleotides (*5*), it became a matter of conjecture whether ferredoxins could participate directly as reductants in carbon assimilation instead of participating indirectly, by way of pyridine nucleotides, with an attendant drop of about 100 mv in reducing potential. Until recently, however, there was no experimental evidence for the direct participation of ferredoxin as a reductant in any enzymic reaction concerned with carbon assimilation.

An enzymic CO_2 fixation reaction,

driven by reduced ferredoxin, was recently found by Bachofen *et al.* (*66*) and Buchanan *et al.* (*67*). Reduced ferredoxin is the electron donor in the reductive synthesis of pyruvate from CO_2 and acetyl coenzyme A (Eq. 1).

$$CO_2 + \text{acetyl-CoA} + Fd_{red} \longrightarrow \text{pyruvate} + CoA + Fd_{ox} \quad (1)$$

The reaction was first found in extracts of the nonphotosynthetic bacterium *Clostridium pasteurianum* (*66*), but it has now been found also in the photosynthetic bacteria *Chromatium* (*67*) and *Chlorobium thiosulfatophilum* (*68*). The method of partial purification of this enzyme, tentatively named pyruvate synthase, and further discussion are given elsewhere (*68*). This enzyme may be significant in bacterial photosynthesis.

The pyruvate synthase reaction constitutes a new primary CO_2 fixation reaction (as defined by Wood and Stjernholm, *69*) that is consistent with previously reported carbon labeling patterns of amino acids isolated from bacterial cells that were supplied with labeled CO_2 or acetate. Over a decade ago, Ehrensvärd and associates (*70*), working with the photosynthetic bacterium *Rhodospirillum rubrum*, and Tomlinson (*71*), working with the nonphotosynthetic anaerobe *Clostridium kluyveri*, obtained labeling data which suggested the operation of some unknown enzymic mechanism for the condensation of CO_2 with acetate (or a derivative thereof) to give a C_3 compound (for example, pyruvate) that was then used for the synthesis of amino acids. More recently, similar reactions were indicated by the work of Sadler and Stanier (*72*) and Hoare (*73*) on the photoassimilation of acetate and CO_2 by intact cells of *Chlorobium limicola* and *Rhodospirillum rubrum*, respectively (see also reviews by Wood and Stjernholm, *69*; Krampitz, *74*; and Elsden, *75, 76*).

Prior to the recognition of the pyruvate synthase reaction, it was generally considered (*77, 78*) that, as in other photosynthetic or chemosynthetic organisms, the only primary CO_2 fixation in photosynthetic bacteria which leads to the formation of a C_3 compound is the carboxylation of ribulose diphosphate followed by its split into two molecules of phosphoglycerate. The pyruvate synthase reaction constitutes an alternative mechanism for the synthesis of a C_3 compound from CO_2 and acetyl coenzyme A, which photosynthetic bacteria can form from acetate (*67, 68*). Pyruvate leads to the formation of the amino acids which are known to be the primary products of CO_2 assimilation by photosynthetic bacteria (*77, 78*). Aspartate, glutamate, and alanine, but not phosphoglycerate or phosphorylated sugars, are the main soluble products of CO_2 assimilation in bacterial photosynthesis.

The pyruvate synthase system provides directly the C_3 carbon skeleton for alanine. The C_4 and C_5 carbon skeletons needed for aspartate and glutamate are readily supplied by the previously demonstrated (*77*) reactions in *Chromatium* extracts—that is, by a carboxylation of phosphoenolpyruvate (or of pyruvate in the presence of ATP) to oxalacetate and the condensations of acetyl coenzyme A with either pyruvate or oxalacetate.

Concluding Remarks

Research in the last 3 years has uncovered evidence that the reduction of ferredoxin is the key photochemical event in photosynthesis by chloroplasts. The photoreduction of ferredoxin is coupled with oxygen evolution and with photosynthetic phosphorylation. The mechanism of what was previously called the photoreduction of TPN by illuminated chloroplasts was found to involve (i) a photoreduction of ferredoxin, (ii) a reduction, in the dark, of ferredoxin-TPN reductase by reduced ferredoxin, and (iii) reoxidation in the dark of reduced ferredoxin-TPN reductase by TPN.

The direct participation of reduced ferredoxin as a reductant in photosynthetic CO_2 assimilation is theoretically possible and is attractive from the standpoint of the quantum efficiency of the energy conversion process. However, an enzymic reaction for a direct involvement of ferredoxin in CO_2 fixation has been uncovered so far only in photosynthetic bacteria, where reduced ferredoxin drives the pyruvate synthase reaction. Whether the reducing potential of ferredoxin is used directly by some enzyme systems for a primary CO_2 fixation reaction in photosynthesis of green plants is a matter to be decided by further experimentation.

Although ferredoxin has been found in every species of photosynthetic bacteria that has been examined, there was until recently no experimental evidence that bacterial ferredoxin is photochemically reduced by the chlorophyll system of photosynthetic bacteria in a manner analogous to photoreduction of ferredoxin by chloroplasts. Evidence for photoreduction of ferredoxin by the chlorophyll system of photosynthetic bacteria has now been obtained by Evans and Buchanan (*68*). Evidence for the participation of bacterial ferredoxin in bacterial photophosphorylation is being actively sought.

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The Kinetics and Analysis of Very Fast Chemical Reactions

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The reactions about which I propose to speak are considered fast because they take place in times measured by microseconds and milliseconds. They comprise adiabatic processes such as explosive and pyrolytic reactions which themselves are made up of intermediate reactions involving transients of very short life, and also the isothermal reactions of atoms and radicals in both gaseous and liquid media. Reactions in this category, whose half-lives vary between 10^{-6} and 10^{-2} seconds, are not the fastest known processes: they are exceeded by ionic processes in solution, by the reactions of many electronically excited species and by the relaxation of rotational energy of molecules. They comprise, however, a very large class which until recently it had not been possible to follow kinetically or to analyse objectively. Very much had indeed been achieved by the study of the overall kinetics and stoichiometry of explosive and photochemical processes, and the various patterns of chain reactions and radical reactions which have emerged constitute an impressive inductive achievement. That the new methods of kinetic spectroscopy have confirmed in no small measure the modern deductions from reaction theory is satisfactory, but I hope to show here that they have also contributed much in their own right, and have elucidated mechanisms of chain reactions, radical processes and relaxation phenomena which were hitherto obscure.

The methods of flash photolysis and kinetic spectroscopy which were discovered in our laboratory at Cambridge and developed in the beginning by Porter are well known (*1, 2*), so I shall refer only to the general principles involved. When a powerful flash created by a discharge of 2000–4000 joules through an inert gas is applied to a photochemically responsive system, very high momentary concentrations of atoms and free radicals are produced. The concentrations produced are in certain cases so high that radical-radical reactions are more important than radical-molecule reactions. They are also so high as to make possible the photography of their absorption spectra by a second flash-lamp triggered electronically by the first flash at specific intervals of 0–10^{4} microseconds after the first. Thus by a series of experiments the growth and decay of transient species in the reacting system may be observed. The first flash is called the photoflash; the second, which is of much lower intensity and results from a discharge of about 100 joules through inert gas, is called the specflash. The general plan of the apparatus is shown in Fig. 1. The success of the method depends upon making the duration of the photoflash and specflash for a given output of

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