Hydrogen Transport with Chloroplasts*

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Dedicated to Professor Dr. O. WARBURG on the occasion of his 80th birthday

A biochemical approach to the study of photosynthesis in terms of hydrogen transport is described. In particular, the system concerned with reduction of the coenzymes has been found to depend on two proteins; a non haem iron protein closely connected with a photochemical reaction and a flavoprotein which acts as a reductase. The iron protein, previously known as "methaemoglobin reducing factor" and as "photosynthetic pyridine nucleotide reductase", is recognised as a member of a class of proteins now called ferredoxin.

Hydrogen transport

There are many approaches to the study of the process of photosynthesis in green plants. One of the final objectives is to be able to give to a particular organism a complete quantitative and qualitative description which is in accord with the disciplines of physics and of chemistry. Green plants in light are able to reverse the effects of respiration. They indeed can convert the products of the complete burning of the grass back in to the living plant. While the over all effect is simply described as reversal of respiration, it seemed also that even in detail the process could be regarded in the same way. For example, in respiration, when oxygen is consumed hydrogen is added to it to give water. From the green plant a partial system may be studied where oxygen is liberated in the light while some substance is reduced in equivalent amount. It would seem that there is a transfer of hydrogen or of electrons from water to the substance which undergoes reduction. For example, fragments of the chloroplasts from a green plant may be shown to photocatalyse the reduction of ferrioxalate, ferri-cyanide or p-benzoquinone the reactions being respectively as follows:

\[
\begin{align*}
2 \text{Fe(C}_{2}\text{O}_{4})_{3}^{2-} + \text{H}_{2}\text{O} = 2 \text{Fe(C}_{2}\text{O}_{4})_{2}^{3-} + 2 \text{H}^{2+} + \frac{1}{2} \text{O}_{2} ; AG'_{298} &= +33,000 \text{ cal} \\
2 \text{Fe(CN)}_{6}^{3-} + \text{H}_{2}\text{O} + 2 \text{Fe(CN)}_{6}^{4-} + 2 \text{H}^{2+} + \frac{1}{2} \text{O}_{2} ; AG'_{298} &= +16,000 \text{ cal}
\end{align*}
\]

where \(AG'_{298}\) refers to the free energy change calculated for the concentrations obtaining in the experiments.

One approach, therefore, to the study of partial systems relating to photosynthesis, is the consideration of transport of hydrogen or of electrons. In respiration the process as a whole conforms with reactions in the sense of the thermochemical gradient, as defined in the dark. In photosynthesis the process as a whole results in a storage of energy in chemical form resulting from the absorption of the light; the over all transport of hydrogen or of electrons would then be in the opposite sense to the thermochemical gradient as defined in the dark.

A variety of chemical processes can be catalysed by means of the absorption of light. So far, relatively few simple cases have been studied where there is a storage of energy as in the process of photosynthesis. Indeed, in these, the fraction of the light energy stored chemically is extremely small as compared with the process in the green plant. This may be explained in a simple case by the reversal, in dark, of an effect produced in light. With reference to living cells, the coenzymes DPN and TPN have in their reduced states the property of not being reoxidized directly by molecular oxygen at an appreciable rate. This property of these pyridine nucleotides (PN) has an important significance in connection with

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\begin{align*}
\text{C}_{6}\text{H}_{4}\text{O}_{2} + \text{H}_{2}\text{O} &= \text{C}_{6}\text{H}_{4}\text{O}_{3} + \frac{1}{2} \text{O}_{2} ; AG'_{298} &= +23,000 \text{ cal}
\end{align*}
\]

* The following abbreviations were used: DPN = nicotinamide adenine dinucleotide; TPN = nicotinamide adenine dinucleotide phosphate; PPNR = photosynthetic pyridine nucleotide reductase.

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the study of a partial system derived from the green plant. Provided that two specific protein catalysts are present, the chloroplast fragments will catalyse the following reaction with the absorbed light:

\[ \text{TPN} + \text{H}_2\text{O} = \text{TPNH}_2 + \frac{1}{2}\text{O}_2 \quad \Delta G_{298}^\circ = +49,000 \text{ cal.} \]

The storage of energy in a chemical form is here on a larger scale than in the three cases mentioned previously where an “unspecific” hydrogen acceptor is taking part.

It has happened that the investigations leading to the isolation and characterisation of these two catalytic proteins have extended over a number of years and in a number of different laboratories. This has unavoidably lead to a synonymy and we attempt later to show how it came about. In the present connection we are concerned with the identification of components in the green plant which become reduced when light is absorbed by the chlorophyll containing moiety. We have therefore to characterise both the individual catalytic proteins and the metabolic intermediates. Then we have to relate their oxidation and reduction properties to the natural chemical and physical environment. If any one substance becomes reduced something else becomes oxidized when light is absorbed and the final product to be observed in this direction is molecular oxygen itself. The production of molecular oxygen from water would involve two molecules of the latter and the transfer of four equivalents of hydrogen or electrons. Thus there have to be several oxidation reduction steps. At each step there is the possibility of the reverse reaction occurring corresponding with a dark process. The present inquiry then becomes intimately concerned with how the so called “back reactions” are minimised. In particular, for the green plant, the back reactions involving the oxygen molecule are important.

Another approach is to regard the system in the plant as being somehow analogous to a rectifying barrier photo cell. This places emphasis on the significance of structure above the molecular level. It may even lack haem-iron. Thus the study of a natural chlorophyll containing system from the green plant, has come to have a wider significance.

**Pyridine nucleotide reduction**

Some years ago San Pietro and Lang undertook to determine the stereospecificity, if any existed, of the reduction of pyridine nucleotides by illuminated grana with deuterium as a tracer. They demonstrated that deuterium from the medium was incorporated into the latter and the transfer of four equivalents of hydrogen or electrons. Thus there have to be several oxidation reduction steps. At each step there is the possibility of the reverse reaction occurring corresponding with a dark process. The present inquiry then becomes intimately concerned with how the so called “back reactions” are minimised. In particular, for the green plant, the back reactions involving the oxygen molecule are important.

Other proteins which have recently been given the name of ferredoxins places biological importance on the iron atom in a new direction. They form a class of non-haem iron proteins. So far the emphasis has been mainly on the iron-porphyrin containing proteins and now the non-haem iron in organisms has a more definite place. We can see, for example, the necessity for iron in the nutrition of anaerobic organisms which may even lack haem-iron. Thus the study of a natural hydrogen acceptor originally known as the methaemoglobin reducing factor, for the illuminated chlorophyll containing system from the green plant, has come to have a wider significance.

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*The authors have not attempted to review completely the research relating to the reduction of pyridine nucleotides by illuminated chloroplasts (or grana).*
During the course of this study it was observed that reduced pyridine nucleotide accumulated when the reaction mixture was incubated under nitrogen rather than aerobically. This chance observation prompted San Pietro and Lang to investigate the conditions under which the photochemical reduction of pyridine nucleotides could be demonstrated directly. It was shown that when either DPN or TPN is incubated with chlorophyll grana in the light, either aerobically or anaerobically, reduced pyridine nucleotide accumulates in the medium. In these studies, however, it was necessary to employ fairly high concentrations of grana and pyridine nucleotides. More significant in this connection was the relationship between the grana concentration and the amount of pyridine nucleotide reduced. At low grana concentration there was no reduction of pyridine nucleotide; at higher grana concentrations there was a linear relationship between the grana concentration and the amount of reduced pyridine nucleotide formed. This is shown graphically in Fig. 1. These results suggested that a naturally occurring factor or catalyst was required for the reduction of pyridine nucleotides. To test this hypothesis, a soluble extract of chloroplasts was prepared. The addition of the soluble extract to a system containing a low concentration of grana restored the pyridine nucleotide reduction activity.

In 1958 San Pietro and Lang reported the isolation and partial purification of a soluble protein which catalyzed the reduction of pyridine nucleotides by illuminated chloroplasts or grana. They suggested that this protein be named photosynthetic pyridine nucleotide reductase (PPNR). The first demonstration that a soluble factor can be added back to chloroplasts to reconstitute their over-all electron transport reaction must be attributed to Davenport, Hill and Whatley. The following statement taken from Hill summarizes their findings. "We have found that untreated chloroplast suspensions from many plants show an active capacity for reducing muscle methaemoglobin in light. When the chloroplast suspension is diluted, the activity (in terms of chlorophyll present) falls off in a way showing the presence of a soluble factor. The washed chloroplasts were incapable of reducing methaemoglobin in light, but addition of the soluble fraction of the leaf juice restored the activity. The soluble methaemoglobin reduction factor is thermostable." San Pietro and Lang were unaware of the prior work on the methaemoglobin reducing factor when they reported their observations in 1956. PPNR and the methaemoglobin reducing factor were isolated and purified independently in our two laboratories from different plant sources. It appears that these two proteins are identical except for minor differences in the amino acid composition which can be referred to the fact that different plants were used. Recently, Tagawa and Arnon have described this protein under the name of spinach (or chloroplast) ferredoxin. This change in nomenclature was based on the chemical and functional similarities of the proteins isolated from plants and bacteria. Bacterial ferredoxin was discovered and isolated first by Mortenson, Valentine and Carnahan. It is interesting that in spite of the similarities, the proteins isolated from these two sources differ in their absorption spectra, in their iron contents, in their molecular weights, and in their amino acid analyses. The redox potentials, however, of the plant and bacterial proteins are similar.

San Pietro and Lang found that the partially purified PPNR catalyzed the reduction of both TPN and DPN. In these studies the initial rate of re-

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Fig. 1. Relationship between chlorophyll concentration and accumulation of DPNH. Experimental conditions as described in L. E. Davenport and R. Hill, Biochem. J. 74, 493 [1960].


duction of DPN was only about 70% of the rate observed with TPN. However, when PPNR was further purified it catalyzed the reduction of only TPN; DPN was not reduced. These observations were explained on the basis that the PPNR is specific for TPN; the reduction of DPN was thought to require a second protein in addition to PPNR. It was suggested that this second protein which was present in the partially purified PPNR preparation, but absent from the purified PPNR, would exhibit pyridine nucleotide transhydrogenase activity. Arn

non, Whatley, and Allen explained their observation that DPN was as effective as TPN in stimulating photosynthetic phosphorylation, provided either a large amount of chloroplast material or an excess of chloroplast extract was used, in a similar manner. However, at that time the presence of pyridine nucleotide transhydrogenase in spinach had not been demonstrated. Avron and Jagendorf reported the isolation and purification of a TPNH-diaphorase from washed spinach chloroplasts. In the discussion of this paper they state that the diaphorase preparation catalyzed the reduction of the acetylpyridine analog of TPN by TPNH. The similar analog of DPN did not serve as a hydrogen acceptor. The significance of this observation was recognized when the pyridine nucleotide transhydrogenase from spinach was purified and shown to be identical to the TPNH-diaphorase of Avron and Jagendorf.

The occurrence of pyridine nucleotide transhydrogenase activity in a partially purified preparation of PPNR was reported by Keister and San Pietro. The further purification of the partially purified PPNR is accomplished by the precipitation of the enzyme with protamine sulfate. It is during this purification step that the transhydrogenase and PPNR activities separate and that the specificity of PPNR for TPN reduction becomes apparent. The transhydrogenase has been purified by Keister, San Pietro, and Stolzenbach and certain of its properties studied. The enzyme is TPN specific with respect to the hydrogen donor and acceptor and the absorption spectrum of the purified enzyme is typical of a flavin absorption spectrum. In addition, the flavin constituent of the enzyme has been shown to be flavin adenine dinucleotide. This protein has recently been obtained in crystalline form (Shin, Tagawa and Arnon) from spinach under the name of TPN reductase.

It has been found that the reduction of TPN, as well as DPN, by illuminated chloroplasts requires both PPNR and transhydrogenase. The experimental approach used to demonstrate the requirement for transhydrogenase was to prepare antibody to the purified protein. The antibody inhibited the reduction of both pyridine nucleotides by illuminated chloroplasts and the inhibitory effect of the antibody could be alleviated by the addition of purified transhydrogenase.

Using the antibody technique it was possible to estimate that chloroplasts equivalent to one milligram of chlorophyll contain sufficient transhydrogenase to catalyze the reduction of 120 μmoles of TPN per hour. It is understandable, therefore, that the photochemical reduction of TPN could be observed in the absence of added transhydrogenase and was thought originally to require only PPNR. Vernon had shown that hematophorphyrin could catalyze the photoreduction of TPN provided PPNR was present. In these experiments, a partially purified preparation of PPNR had been used. Under similar experimental conditions, there is no reduction of TPN in the presence of the purified PPNR. When both purified PPNR and transhydrogenase are present, the reduction of TPN is observed (San Pietro, Vernon and Limbach, unpublished results).

Tagawa and Arnon and Shin, Tagawa and Arnon reported similar results using two different experimental approaches. First, they reasoned that it should be possible to use hydrogen and hydrogenase in place of illuminated chloroplasts to provide the reducing potential necessary for the reduction of TPN. According to expectation, the reduction of TPN by hydrogen (and hydrogenase) was shown to occur only when both spinach ferredoxin and TPN-reductase were present. The second approach used by them was to extract the TPN-reduc-

16 L. P. Vernon, Acta chem. scand. 15, 1651 [1961].
The extracted chloroplasts were unable to photoreduce TPN in the presence of only added spinach ferredoxin; TPNH formation was observed upon the subsequent addition of TPN-reductase. It should be noted that Davenport has also used the chloroplast extraction technique and obtained evidence for the involvement of both PPNR and a flavoprotein in the photochemical reduction of TPN.

In a recent publication from Professor Warburg’s laboratory by Gewitz and Völk, the purification from Chlorella of a red protein (whose spectrum is similar to those shown in Figure 2; see also Table 2) and a flavoprotein is described. They showed that if grana are illuminated in the presence of TPN and a catalytic amount of the red enzyme, the amount of oxygen evolved is equivalent to the TPN content. Furthermore, the rate of oxygen evolution was increased by the addition of the flavoprotein to a system containing a small amount of the red enzyme.

### Table 1. Comparison of PPNR from spinach and methaemoglobin reducing factor from parsley.

<table>
<thead>
<tr>
<th></th>
<th>Methaemoglobin Reducing Factor</th>
<th>PPNR</th>
</tr>
</thead>
<tbody>
<tr>
<td>λ₃₃₀/λ₂₷₇</td>
<td>0.810</td>
<td>0.65</td>
</tr>
<tr>
<td>λ₄₂₀/λ₂₷₇</td>
<td>0.616</td>
<td>0.49</td>
</tr>
<tr>
<td>λ₄₆₅/λ₂₷₇</td>
<td>0.574</td>
<td>0.44</td>
</tr>
</tbody>
</table>

Fig. 2. Absorption spectra of methaemoglobin reducing factor from parsley and PPNR from spinach. Upper curve: Methaemoglobin Reducing Factor, 0.77 mg of protein per ml; Lower curve: PPNR, concentration equivalent to 0.081 micromole of iron per ml.

### Non-heme iron proteins

The methaemoglobin reducing factor, and PPNR have been extensively purified and shown to be homogeneous, both electrophoretically and in the ultracentrifuge. The absorption spectra of PPNR from spinach and of the methaemoglobin reducing factor from parsley are shown in Figure 2. It is clear that these two spectra are very similar; the major difference between them is the relationship of the absorption in the visible region to that in the ultra-violet. This is shown in Table 1. The values for the methaemoglobin reducing factor are in each case about 1.27 times the corresponding value for PPNR. This difference is most probably due to the fact that the methaemoglobin reducing factor is devoid of tryptophan (Hill, unpublished data).

Whereas PPNR contains one mole of tryptophan (Fry and San Pietro, unpublished data). The presence of tryptophan in PPNR is indicated by the shoulder in the absorption spectrum at 290 mµ which is absent from the absorption spectrum of the methaemoglobin factor. Furthermore, there is a shift in the position of the trough from 295 mµ in the spectrum of the methaemoglobin reducing factor to 305 mµ in the PPNR spectrum.

In view of this difference in tryptophan content a comparison of the ultra-violet absorption spectra of these proteins per unit of protein will not be meaningful. However, if one assumes that the iron in these proteins is an essential constituent of the chromophoric group(s), then the absorption in the visible region per unit of iron should be the same if the chromophoric group(s) in these proteins are the same. The optical density per micromole of iron per ml at the wavelengths of the absorption maxima in the visible region is presented in Table 2. For the purposes of comparison, similar data for spinach ferredoxin and the red enzyme from Chlorella are included. It is clear that the values at each wavelength for these proteins are essentially the same. These data support the idea that the chromophoric group(s) responsible for the visible absorption of these proteins is the same.

The non-heme iron proteins listed in Table 2 have all been found to contain “labile sulfide” in an amount stoichiometric with their iron content. Fry

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and San Pietro reported the presence of “labile sulfide” in PPNR which is liberated as hydrogen sulfide upon acidification. Similar results have been reported subsequently for the red protein of Chlorella, for spinach ferredoxin and for the crystalline clostridial ferredoxins. It is interesting that the three iron flavoproteins, dihydro-orotic dehydrogenase, aldehyde oxidase and xanthine oxidase also contain an amount of “labile sulfide” equivalent to the iron content (P. Handler, personal communication). It is tempting to speculate that this is a property common to all non-heme iron proteins which undergo reversible oxidation-reduction. It is most likely that the iron is involved in electron transport catalyzed by these proteins and undergoes a reversible change in valence state. The redox potentials of spinach ferredoxin, of clostridial ferredoxin and of PPNR (Hill and San Pietro, unpublished data) are similar and close to that of the hydrogen electrode at neutral pH. It seems reasonable to assume that there is a correlation between the low redox potential of these proteins and the fact that they contain non-heme iron and “labile sulfide” in equivalent amounts.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Source</th>
<th>Wavelength [mμ]</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPNR</td>
<td>Spinach</td>
<td>6.70 4.95 4.45</td>
</tr>
<tr>
<td>Red Enzyme</td>
<td>Chlorella</td>
<td>7.37 5.29 4.89</td>
</tr>
<tr>
<td>Spinach</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ferredoxin b</td>
<td>Spinach</td>
<td>6.95 5.16 4.65</td>
</tr>
<tr>
<td>Methaemoglobin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reducing Factor c</td>
<td>Parsley</td>
<td>6.83 5.18 4.67</td>
</tr>
</tbody>
</table>

Table 2. Correlation of absorbance in the visible region and iron content. The values are presented as the absorbance of a solution of protein containing one micromole of iron per milliliter. a Calculated from the data in l.c. b Calculated from the data in l.c. c Calculated from the data in l.c.

21 K. Tagawa, personal communication.
23 J. E. Carnahan, personal communication.