PHOTOSYNTHETIC PYRIDINE NUCLEOTIDE REDUCTASE*

I. PARTIAL PURIFICATION AND PROPERTIES OF THE ENZYME FROM SPINACH

BY ANTHONY SAN PIETRO AND HELGA M. LANG†

(From the McCollum-Pratt Institute, The Johns Hopkins University, Baltimore, Maryland)

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In 1939, Hill (1) reported the fundamental discovery that isolated chloroplasts possess the ability to affect a portion of the over-all reaction of photosynthesis; namely, the photolysis of water. In the original experiments of Hill, ferric oxalate was used as the hydrogen acceptor. During the past 20 years, a variety of substances has been found to function as oxidant for the Hill reaction (2). Of special interest are the pyridine nucleotides in view of their well known physiological function as coenzymes in numerous enzyme-catalyzed reactions.

The ability of illuminated chloroplasts to reduce pyridine nucleotides was first demonstrated in 1951-52 by Vishniac and Ochoa (3, 4), Tolmach (5), and Arnon (6). In these experiments, the formation of reduced pyridine nucleotides was demonstrated indirectly by coupling the photochemical reaction with a suitable dehydrogenase and measuring the formation of the product of the dehydrogenase system. No directly measurable reduction of pyridine nucleotides was observed in the absence of the coupling system (7). It was, therefore, suggested (4, 7) that the inability of pyridine nucleotides to undergo directly measurable reduction was a consequence of their low oxidation-reduction potential ($E_0^\text{'}$ at pH 7 = −0.32 volt) since most substances that are effective as oxidants in the Hill reaction have high oxidation-reduction potentials ($E_0^\text{'}$ at pH 7 = +0.1 to +0.4 volt).

In 1956, San Pietro and Lang (8) presented evidence that, under the proper conditions, photochemical reduction of pyridine nucleotides can indeed be demonstrated directly. It was shown that when DPN$^1$ is

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† Present address, Nazareth College of Rochester, Rochester, New York.

$^1$ The following abbreviations are used: DPN and DPNH, oxidized and reduced diphosphopyridine nucleotide; TPN and TPNH, oxidized and reduced triphosphopyridine nucleotide; acetylpyridine-DPN and acetylpyridine-TPN, the 3-acetylpyridine analogues of DPN and TPN; NMN, nicotinamide mononucleotide; $\alpha$-DPN, $\alpha$ isomer of DPN; Tris, tris(hydroxymethyl)aminomethane; ATP, adenosine triphosphate.
incubated with chloroplast grana, in the light, either aerobically or anaerobically, DPNH accumulates in the reaction mixture. In these studies, however, it was necessary to employ fairly high concentrations of grana and DPN. At low grana concentrations (less than 60 μg of chlorophyll per ml.), no measurable reduction of DPN occurred. At higher grana concentrations, the amount of DPNH formed was almost directly proportional to the amount of grana initially present. Similar results were obtained when the reaction was carried out either aerobically or anaerobically.

Preliminary results at that time indicated that it was possible to demonstrate reduction of pyridine nucleotides at low grana concentration, provided that a soluble extract of chloroplasts was added (8). This finding allows one to measure directly the initial rate of photochemical reduction of pyridine nucleotides since the reduction can be followed spectrophotometrically by measuring the increase in absorption at 340 μm of the reaction mixture without prior removal of the grana. Previously, we found it feasible only to determine the total amount of reduced nucleotide formed during a certain incubation period.

The present paper is concerned with the isolation of a soluble enzyme from either chloroplasts or whole spinach leaves, which is required in addition to grana for the photochemical reduction of pyridine nucleotides. The enzyme has been partially purified and certain of its properties studied. It will be shown that the enzymatic reaction requires the intact dinucleotide structure, and, in addition, appears to be more active toward TPN than toward DPN. It is tentatively suggested that the enzyme be named "photosynthetic pyridine nucleotide reductase."

Materials and Methods

Preparation of Grana—Spinach leaves, purchased at a local grocery, were depetioled and the midribs removed and ground with 8 times their weight of ice-cold buffer (0.05 M Na₂HPO₄·KH₂PO₄, pH 7, 0.4 M sucrose, and 0.01 M KCl). Grinding was carried out in a Waring blender at 100 volts for 3 minutes. The homogenate was filtered through cheesecloth and glass wool to remove the coarse material and the filtrate was centrifuged for 2 minutes at 4600 × g. The supernatant fluid was centrifuged for 20 minutes at 18,000 × g; the residue from the high speed centrifugation was suspended in buffer and centrifuged again at 18,000 × g. The final residue was made up in 10 ml. of buffer for each 25 gm. of spinach leaves. Chlorophyll concentration was determined by the modification of Arnon (9).

Nucleotides—DPN and TPN were purchased from the Pabst Laboratories. The 3-acetylpyridine analogues of DPN and TPN, which were

Part of the DPN used in these studies was a generous gift of the Pabst Laboratories.
prepared enzymatically by an exchange reaction catalyzed by pig brain DPNase as described by Kaplan and Ciotti (10, 11), were a generous gift of Dr. N. O. Kaplan. NMN and α-DPN were also supplied by Dr. N. O. Kaplan. These two compounds were prepared as described by Kaplan and Stolzenbach (12).

Nucleotide concentration in the stock solutions was determined by the cyanide procedure described by Colowick et al. (13). The value of 7.7 for the millimolar extinction coefficient of the cyanide complex of acetylpyridine-DPN and acetylpyridine-TPN at 340 μm was used. For α-DPN, the value of 5.0 for the millimolar extinction coefficient of the cyanide complex at 332 μm was used. For DPN, TPN, and NMN, the value of 6.3 for the millimolar extinction coefficient of the cyanide complex at 325 μm was used.

When the acetylpyridine analogue of DPN or TPN was used as the acceptor in the Hill reaction, the reduction of analogue was measured at 365 μm. The concentration of reduced analogue was calculated by using the value of 7.7 for the millimolar extinction coefficient of reduced analogue at this wavelength.

**Determination of Activity—** Enzymatic activity was determined by measuring the initial rate of formation of reduced pyridine nucleotides in the Hill reaction. In this procedure, the photochemical reduction process is followed directly by measuring the increase in optical density at 340 μm (or 365 μm) as follows: The reaction mixture, which contains buffer, pyridine nucleotide, enzyme, and grana, is prepared in a glass cuvette3 and mixed. The optical density of the mixture is measured at a given wavelength, as indicated above, against a blank which contains only buffer and grana. The cuvette containing the reaction mixture is partially immersed in a water bath at 12-15° and illuminated for a certain period of time. The light source was either a 75 or 100 watt bulb at a distance of approximately 3 inches. After illumination, the cuvette is returned to the spectrophotometer and the optical density is again measured. The increase in optical density, calculated from the two measurements, serves as a measure of the enzymatic activity. In all the experiments reported, a Beckman model DU spectrophotometer with a photomultiplier attachment was used for the optical measurements.

1 unit of enzyme is defined as that amount which produces a change in optical density of 1.0 in 10 minutes at 340 μm when the reaction mixture contains 100 γ of chlorophyll per 3 ml. This unit of enzyme corresponds to the reduction of 4.8 μmoles of pyridine nucleotide per mg. of chlorophyll in 10 minutes.

3 Purchased from the Kontes Glass Company, Vineland, New Jersey. These cells have a light path of 1 cm. and are 9.0 cm. in length.
Specific activity is expressed as units per mg. of protein. The protein content of the enzyme preparations was determined either by the Folin phenol reagent method of Lowry et al. (14) or by the ultraviolet absorption procedure of Warburg and Christian (15).

**Results**

**Partial Purification of Enzyme from Spinach Leaves**

*Preparation A*—All steps of the purification were carried out at 0-4°C; centrifugations were carried out in the cold.

100 gm. of spinach leaves, purchased at a local grocery, were freed from veins and ground in 130 ml. of cold distilled water in the Waring blender for 5 minutes at 100 volts. The dark green homogenate was filtered through a double layer of cheesecloth and glass wool and the residue discarded. To the dark green filtrate was added sufficient 0.5 M Tris-HCl buffer, pH 8, to give a final concentration of 0.05 M Tris.

Acetone, previously cooled in the deep freeze, was added to the adjusted filtrate slowly and with mechanical stirring to a final concentration of 35 per cent. The preparation was centrifuged at 1000 × g for 15 minutes and the clear yellow-green supernatant fluid, which contains the majority of the activity, was decanted and saved. The dark green residue contains very little of the activity and was discarded.

The activity is precipitated from the supernatant solution by the slow addition of acetone, chilled in the deep freeze to a final concentration of 75 per cent. During the addition, the solution was stirred mechanically. The precipitate is flocculent and settles rapidly when stirring is discontinued. After several minutes, the greater part of the supernatant fluid can be decanted before collection of the precipitate by centrifugation for 5 minutes at 1000 × g. The resulting clear yellow-green supernatant fluid is discarded and the light brown-colored residue suspended thoroughly in 10 ml. of ice-cold 0.005 M Tris, pH 8. The suspension is centrifuged at 18,000 × g for 20 minutes and the residue discarded. The clear brown supernatant solution is dialyzed overnight against 0.005 M Tris, pH 8, in the cold.

Further purification of the enzyme has been accomplished by the use of protamine sulfate. The enzyme is precipitated by the addition of 1 per cent protamine sulfate, pH 6, at a ratio of approximately 6 mg. of protamine sulfate to 100 mg. of protein. The solution is centrifuged for 15 minutes at about 1500 × g and the brown supernatant fluid discarded. The residue is extracted thoroughly with 0.5 M Tris, pH 8, and the resulting suspension centrifuged as above. The recovery of enzyme in the supernatant solution varied between 60 and 70 per cent and represents an increase in specific activity of approximately 4- to 7-fold.
The results of a typical purification are presented in Table I (Preparation A). The recovery varied between 40 and 50 per cent and the range in purification varied between 20- and 35-fold. Many of the experiments reported were carried out with the Tris buffer extract of the acetone precipitate.

Preparation B—The enzyme has also been isolated from chloroplasts by the procedure described by Avron and Jagendorf (16) for the purification of a TPNH diaphorase from chloroplasts. This procedure has been described in detail elsewhere (16) and involves the following steps: (a) isolation of chloroplasts from spinach leaves; (b) extraction of the enzyme from the chloroplasts; (c) precipitation with 3 volumes of acetone and extraction of the precipitate to yield the partially purified enzyme.

The results of a typical purification are presented in Table I (Preparation B). The specific activity of the purified enzyme is 0.62 unit per mg. of protein, and 82 per cent of the total units present in the crude extract are recovered. Some of the experiments reported were performed with this type of enzyme preparation.

It is clear from the results presented in Table I that Preparation A is the preferred method of preparation of the enzyme. Whereas the specific activity of the partially purified enzyme (extract of acetone precipitate) from either preparation is the same, the recovery of enzyme relative to the amount of spinach leaves used is much greater in Preparation A than

\[ \text{It is difficult to determine accurately the purification and recovery achieved in the acetone precipitation step due to the rather low specific activity of the enzyme in the crude homogenate.} \]
in Preparation B. When 1000 gm. of spinach leaves are used as the starting material in Preparation A, 2040 units of enzyme are recovered; in Preparation B, the recovery is 110 units from 1500 gm. of spinach leaves.

Fig. 1. Enzyme concentration versus rate of TPN reduction in the presence of illuminated grana. Each reaction mixture contained 280 μmoles of phosphate buffer, pH 7.1, 0.5 μmole of TPN, enzyme, and grana equivalent to 125 γ of chlorophyll. Final volume, 3 ml. The optical density of each reaction mixture at 340 mμ was determined before and after illumination as described in the text. Time of illumination, 10 minutes. The results are presented as the increase in optical density at 340 mμ calculated from these two measurements.

Properties of Enzyme System

Effect of Enzyme Concentration—Fig. 1 illustrates the relationship of the concentration of enzyme to the amount of reduced pyridine nucleotide formed. In this experiment, TPN served as the Hill oxidant. It can be seen that the test is almost linear for quantities of enzyme which reduce up to 25 per cent of the TPN initially present. Similar results were obtained when DPN was used in place of TPN.

Effect of Anaerobiosis—Although not indicated in Fig. 1, the amount of TPNH formed was the same, whether the reaction was carried out aerobically or anaerobically under an atmosphere of nitrogen. This finding is in contrast to our earlier results on the accumulation of reduced pyridine nucleotides by illuminated grana (8). In our original experi-
ments, more reduced nucleotide was formed under anaerobic conditions than aerobically. However, at that time it was possible to demonstrate accumulation of reduced pyridine nucleotides only at high concentrations of grana and nucleotide. In the present experiments we are using rather low concentrations of grana and nucleotide and, in addition, are providing the enzyme required for the reduction of pyridine nucleotides. This difference in experimental conditions could conceivably account for the lack of dependence on anaerobiosis in the present experiments.

**Light Dependence**—The light dependence of the reaction was determined as follows: Two complete reaction mixtures containing grana, buffer, DPN, and enzyme were prepared and the optical density of each at 340 mμ was determined. One was illuminated and the other kept in the dark by wrapping the cuvette with tinfoil. The increase in optical density was then determined at the end of each of two successive 5 minute intervals of incubation. The results of such an experiment are presented in Table II. It can be seen that no DPNH is formed when the complete reaction mixture is incubated in the dark in contrast to incubation in the light. Identical results were obtained when the reaction was carried out aerobically rather than anaerobically under an atmosphere of nitrogen.

The product of the light reaction in Table II was identified enzymatically as DPNH with acetaldehyde and yeast alcohol dehydrogenase (17). Similarly, when TPN was used as the Hill acceptor, TPNH was identified enzymatically with the glutathione reductase from peas which is specific for reduced TPN (18).

**Nucleotide Specificity**—The nucleotide specificity of the enzyme was determined in the following manner: Each reaction mixture contained

<table>
<thead>
<tr>
<th>Total time of incubation</th>
<th>Total ΔE₄₅₀</th>
<th>Light</th>
<th>Dark</th>
</tr>
</thead>
<tbody>
<tr>
<td>min.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>0.180</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>0.355</td>
<td>0.008</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE II**

*Light Dependence of Pyridine Nucleotide Reduction*

Each reaction mixture contained 250 μmoles of phosphate buffer, pH 7.1, 0.83 μmole of DPN, 500 γ of enzyme, and grana equivalent to 60 γ of chlorophyll. Final volume, 3 ml. Each cuvette was flushed with nitrogen for 2 minutes and stopped. The dark reaction cuvette was wrapped with tinfoil. The optical density of each reaction mixture at 340 mμ was determined before and at the end of each of two successive 5 minute periods of illumination as described in the text.
pyridine nucleotide reductase, buffer, nucleotide, and enzyme. The increase in optical density was measured for a 5 minute period of illumination either at 340 or 365 μm, depending on the absorption properties of the nucleotide used. The results of such an experiment are presented in Table III. It is clear from these data that the enzyme is specific for the intact dinucleotide structure since NMN is not reduced in this reaction. The finding that the enzyme is specific for the intact dinucleotide is in agreement with our earlier results on the accumulation of reduced pyridine nucleotides by illuminated grana (8).

The observation that α-DPN is not reduced, even though it contains the intact dinucleotide structure, is consistent with the findings of Kaplan et al. (19) that α-DPN cannot replace DPN in several DPN-dependent dehydrogenase systems.

Furthermore, of the two natural coenzymes, the enzyme exhibits a greater activity with TPN than with DPN, the initial rate of reduction of DPN being only about 70 per cent of the rate observed with TPN. A typical time-course of the reaction for these two nucleotides is shown in Fig. 2.

It is possible to explain the apparent lack of specificity for either TPN or DPN in one of several ways. In brief, these possibilities are as follows. (a) The reduction of both coenzymes could be catalyzed by a single enzyme which is non-specific with respect to the coenzyme; (b) it is possible that

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**Table III**

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Initial concentration</th>
<th>Amount of reduced nucleotide formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>μmoles per 3 ml.</td>
<td>μmole</td>
</tr>
<tr>
<td>TPN</td>
<td>1.24</td>
<td>0.117</td>
</tr>
<tr>
<td>DPN</td>
<td>1.20</td>
<td>0.085</td>
</tr>
<tr>
<td>Acetylpyridine-TPN</td>
<td>0.82</td>
<td>0.134</td>
</tr>
<tr>
<td>Acetylpyridine-DPN</td>
<td>1.10</td>
<td>0.059</td>
</tr>
<tr>
<td>NMN</td>
<td>0.77</td>
<td>0.014</td>
</tr>
<tr>
<td>α-DPN</td>
<td>0.83</td>
<td>0.015</td>
</tr>
</tbody>
</table>

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* The grana used in this experiment were prepared in 0.01 M phosphate-0.01 M KCl buffer, pH 7, rather than the 0.4 M sucrose-0.05 M phosphate-0.01 M KCl buffer, pH 7. Identical results were obtained with grana prepared in the latter buffer.
the reduction of each coenzyme is catalyzed by a single enzyme which is specific for only one of the coenzymes; (c) it could be that the reduction is catalyzed by a single enzyme, which is specific for only one of the natural coenzymes, and that all of the other nucleotides tested are reduced secondarily by virtue of a transhydrogenase type reaction (20, 21).

It is interesting that, whereas acetylpyridine-TPN is reduced at a rate comparable to that obtained with TPN, acetylpyridine-DPN is reduced at approximately one-half this rate. The finding that the acetylpyridine analogues are reduced is entirely consistent with the observations of Kaplan et al. (22) that the analogues can function as coenzymes in a number of dehydrogenase systems. The mechanism whereby the analogues are reduced is not known at the present time; they may be reduced directly by substituting for the natural coenzyme or through a transhydrogenase type reaction.

**Effect of TPN Concentration**—The relationship between the rate of TPN reduction and the concentration of TPN initially present in the reaction mixture is shown in Fig. 3. It can be seen that, at low concen-
trations of TPN, the rate of TPN reduction is not proportional to the concentration of TPN initially present in the reaction mixture. The reason for the lack of proportionality between these two quantities, at low TPN concentration, is not readily apparent at the present time. It is possible that it is due to a slow reoxidation of the TPNH formed which is independent of the TPNH concentration in the reaction mixture. Alternatively, the nature of the initial portion of the curve presented in Fig. 3 suggests the possibility that 2 molecules of TPN are involved in the reaction sequence.

**pH Optimum**—The pH-dependence of the reaction is shown in Fig. 4. As can be seen, the maximal rate is obtained when the pH of the reaction mixture is 6.9, although the rate does not vary markedly over the pH range tested.
It is important to realize that we are measuring the effect of pH on the total reaction rather than on an isolated portion of the reaction. In the simplest formulation, the complete system can be thought to be composed of at least two components. One of these is the photolytic system which catalyzes the photolysis of water to yield reducing and oxidizing potential. Secondly is a system which is capable of using this reducing potential, produced by the photolysis of water for the formation of reduced pyridine nucleotides. This is very conceivably an oversimplification of the actual process since nothing is known, to date, of the number of intermediate steps required to couple the formation of reducing potential and its eventual utilization for the formation of reduced pyridine nucleotides. In view of these considerations, it is clear that at the present time we can measure only the pH-dependence of the over-all reaction.

Effect of Grana Concentration—The effect of grana concentration on the rate of formation of TPNH was determined at two different concentrations of enzyme. The results are shown in Fig. 5. It can be seen that
at either enzyme concentration the rate of photochemical reduction of TPN is linearly proportional to the grana concentration. This finding can be interpreted in one of several ways. First, it is possible that the grana contain a substance which in the reduced form serves as the substrate for the enzyme; that is, the hydrogen or electron donor for the reduction of pyridine nucleotides. In this case, the rate of pyridine nucleotide reduction could be proportional to the substrate (or grana) concentration, provided that we are operating on the linear portion of the substrate concentration curve for the enzyme. Alternatively, it is possible that the activity requires the formation of a ternary complex between the enzyme, pyridine nucleotide, and a molecule present in the grana, which, in the light, is in an activated or excited state. If the existence of the excited state is transitory,

**Table IV**

*Effect of p-Chloromercuribenzoate on Photochemical Reduction of TPN by Illuminated Grana*

<table>
<thead>
<tr>
<th>Inhibitor concentration</th>
<th>ΔE\text{\textmu}</th>
<th>Per cent inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.407</td>
<td>0</td>
</tr>
<tr>
<td>1.6 × 10^{-5}</td>
<td>0.420</td>
<td>0.6</td>
</tr>
<tr>
<td>4.0 × 10^{-5}</td>
<td>0.368</td>
<td>16.4</td>
</tr>
<tr>
<td>8.0 × 10^{-5}</td>
<td>0.340</td>
<td>50.4</td>
</tr>
<tr>
<td>1.2 × 10^{-5}</td>
<td>0.202</td>
<td>90.5</td>
</tr>
<tr>
<td>1.6 × 10^{-5}</td>
<td>0.039</td>
<td></td>
</tr>
</tbody>
</table>

then the probability of formation of the ternary complex would be increased by increasing the grana concentration. These two explanations are rather similar; the major difference is in the nature of the substance which serves as the substrate for the enzyme.

*Effect of Inhibitors*—The effect of various inhibitors on the reduction of TPN was determined as follows: The complete reaction mixture contained buffer, TPN, enzyme, inhibitor, and grana. The control contained everything but inhibitor. The optical density at 340 m\text{\mu} of each was determined before and after illumination for 10 minutes.

The results of the experiments with p-chloromercuribenzoate are presented in Table IV. It can be seen that p-chloromercuribenzoate, at a concentration of 1.2 × 10^{-5} M, inhibits the reduction of TPN about 50 per cent. Although not indicated in Table IV, the per cent inhibition obtained with p-chloromercuribenzoate was approximately inversely pro-
portional to the enzyme concentration. At a concentration of $1.2 \times 10^{-5}$ M, the inhibition was 50 per cent when the reaction mixture contained 700 $\gamma$ of enzyme and 100 per cent when the reaction mixture contained 280 $\gamma$ of enzyme.

The effect of a variety of other inhibitors on the reduction of TPN was also tested. These include sodium azide, iodoacetate, and cyanide at a concentration of $6 \times 10^{-4}$ M, sodium arsenite $3.3 \times 10^{-4}$ M; and Versene $1 \times 10^{-3}$ M. At these concentrations, each of the inhibitors was without effect on the reduction of TPN.

Stability of Enzyme—The enzyme is stable when stored at $-15^\circ$ for several weeks. The activity is completely lost by keeping the enzyme at $100^\circ$ for 5 minutes. It is interesting that the heated enzyme, which is inactive when tested alone, is inhibitory when added to the complete reaction mixture containing unheated enzyme.

DISCUSSION

The reduction of pyridine nucleotides by illuminated grana requires at least two component systems; namely, the photolytic system (Equation 1) and a system for transferring hydrogen (or electrons) from the photolytic system to the pyridine nucleotides, for example TPN (Equation 2). The (H) and (O) represent reducing and oxidizing potential, respectively,

$$\text{H}_2\text{O} \xrightarrow{\text{light}} 2(\text{H}) + (\text{O}) \quad (1)$$

$$2(\text{H}) + \text{TPN}^+ \rightarrow \text{TPNH} + \text{H}^+ \quad (2)$$

formed by the photolysis of water. It is evident from the data presented above that the enzyme, which has tentatively been named “photosynthetic pyridine nucleotide reductase,” is required in addition to grana for the photochemical reduction of pyridine nucleotides. Our results support the hypothesis that the enzyme catalyzes the transfer of hydrogen (or electrons) from the photolytic system to the pyridine nucleotides (Equation 2). Additional evidence for this hypothesis is provided by the finding that the enzyme is without effect on the photolytic activity of grana when measured either spectrophotometrically with 2,3,6-trichlorophenol indophenol according to the procedure described by Jagendorf (23) or manometrically with ferricyanide.

To date, nothing is known concerning the number of intermediate steps required to transfer hydrogen (or electrons) to the pyridine nucleotides. The catalytic activity of the partially purified enzyme preparation used in these studies could be the result of a single enzyme or a multi-

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enzyme system. Further purification of the enzyme preparation is necessary before an unequivocal answer to this question can be offered.

It should be noted that some 5 years ago Vishniac and Ochoa (4) explained the low rate of pyridine nucleotide reduction observed in their experiments by the statement that "the grana may be deficient in one or more of the factors required to transfer hydrogen from the photolytic system to the pyridine nucleotides." The results of our studies support their explanation. It is clear from the data presented in Fig. 1 that the rate of pyridine nucleotide reduction is proportional to the enzyme concentration. In the absence of enzyme, there is no measurable reduction of pyridine nucleotides. The maximal rate of reduction observed in these experiments was 25 μmoles per hour per mg. of chlorophyll. This rate is much higher than that of 0.03 to 1.6 μmoles per hour per mg. of chlorophyll reported by Vishniac and Ochoa (4). Faster rates of nucleotide reduction have been reported by Hendley and Conn (24), who used chloroplast fragments and the TPN-glutathione reductase system from wheat germ or parsley. The maximal rate obtained by them was 38 μmoles per hour per mg. of chlorophyll. It is possible that the reductase preparations used in their experiments contained the enzyme described herein.

In recent experiments, we have been using whole chloroplasts rather than grana. The chloroplasts are prepared according to the procedure of Arnon et al. (25), with the exception that sucrose-phosphate-KCl buffer is used in place of NaCl. The maximal rate of pyridine nucleotide reduction with whole chloroplasts is approximately 3 times that obtained with grana for an equivalent amount of chlorophyll. In either case the ratio of photolytic activity as measured with dye (23) to TPN reduction is about 4.

The finding that the enzyme can be isolated from chloroplasts (Table I, Preparation B) suggests the possibility that it is essential in photosynthesis. According to present concepts (2), TPNH and ATP are required for the reduction of carbon dioxide to the level of carbohydrate. The TPNH is necessary for the reduction of 1,3-diphosphoglyceric acid to glyceraldehyde-3-phosphate by triosephosphate dehydrogenase. This is the only step in the reduction of carbon dioxide which requires reducing potential. The presence of a TPN-linked triosephosphate dehydrogenase in green leaves has been established by Gibbs (26) and Arnon (27). It seems reasonable, therefore, that the enzyme we have isolated would serve to provide the reduced pyridine nucleotide (TPNH) required in the carbon dioxide reduction pathway. The observation that the enzyme exhibits a greater activity toward TPN than toward DPN is in accord with the requirement of TPNH, not DPNH, for the reduction of carbon dioxide in photosynthesis.

Recently, Whatley et al. (28) have shown that the reduction of carbon
dioxide by broken chloroplasts requires ATP, pyridine nucleotides, and a soluble extract of chloroplasts. In view of the data presented above, it seems reasonable to assume that one of the essential factors present in the chloroplast extract used in their experiments was the enzyme described in the present paper. This hypothesis is based upon the similarity between our observations and those of the Arnon group. First, they found (28) that added TPN exerts a greater stimulatory effect than does added DPN on the fixation of carbon dioxide by broken chloroplasts supplemented with a water extract of chloroplasts. This finding is in agreement with our data on the nucleotide specificity (Table III) of the enzyme described herein. In their experiments and ours, DPN was approximately 70 per cent as effective as TPN. In addition, they reported (25) that 1.5 \times 10^{-4} \text{M} \text{p-chloromercuribenzoate} completely inhibited carbon dioxide fixation by whole chloroplasts. We have observed a similar inhibition of TPN reduction by grana with this inhibitor (Table IV). The effect of this inhibitor on the reduction of TPN by whole chloroplasts is essentially the same as that observed with grana.\textsuperscript{6}

The reduction of pyridine nucleotides by illuminated grana (or chloroplasts) is difficult to interpret in terms of known oxidation-reduction potentials if the reduction is a 1 quantum process. If, for example, the reduction of TPN is pictured as proceeding according to Equation 3, then

\[
\text{TPN}^+ + \text{H}_2\text{O} \rightarrow \text{TPNH} + \text{H}^+ + \frac{1}{4}\text{O}_2
\]  

the energy required for this reaction is approximately +52 kc. per mole. Since the energy of 1 Einstein (6 \times 10^{28} \text{quanta}) of red light is about 44 kc., it is evident that the reduction of pyridine nucleotides would proceed only to a small extent at pH 7. For example, Arnon (29) has calculated that the energy of 1 quantum of red light is sufficient to maintain the ratio of reduced to oxidized pyridine nucleotide at 10^{-5} at pH 7.0 and an oxygen tension of 0.2 atmosphere. The results presented in this paper are incompatible with this calculation. It would appear, therefore, that the reduction of pyridine nucleotides by illuminated grana (or chloroplasts) requires more than 1 quantum of red light. Since the reduction of pyridine nucleotides is a 2 electron process, it is possible that the reaction requires 1 quantum per electron transferred to the pyridine nucleotide. This does not necessarily imply that the reaction proceeds through a free radical intermediate, since Baltrop et al. (30) have presented a mechanism for the reduction of pyridine nucleotides by way of thioctic acid which requires 2 quanta of red light per molecule of reduced pyridine nucleotide formed.

It should be noted, however, that Arnon et al. (25) have suggested that "in photosynthesis by chloroplasts sulfhydryl compounds appear to be concerned not with the early (photolysis) but with the later phases of the
photosynthetic process (photosynthetic phosphorylation and especially carbon dioxide fixation)." This suggestion is based on their finding that iodoacetamide, p-chloromercuribenzoate, and arsenite did not inhibit the photolysis reaction, whereas they did inhibit carbon dioxide fixation. Furthermore, of the three sulfhydryl group inhibitors, only p-chloromercuribenzoate was inhibitory to photosynthetic phosphorylation.

We have investigated the effect of added thioctic acid on the reduction of pyridine nucleotides by illuminated grana. The results of these experiments indicate that the rate of reduction is the same in the presence or absence of thioctic acid. In addition, the enzyme preparation appears to be devoid of lipoic acid dehydrogenase when DPNH was used as substrate.

In view of the energy considerations presented above, it is clear that, if the reduction of pyridine nucleotides is a 1 quantum process, a discrepancy of some 8 kc. (52 minus 44) exists between the energy input and that required for the reduction of pyridine nucleotides. It has been suggested by numerous investigators (see Whatley et al. (28)) that this additional energy could be provided by the pyrophosphate bond energy of ATP. In preliminary experiments with chloroplasts, all attempts to demonstrate the involvement of ATP in the reduction of TPN have been unsuccessful. The various approaches tested include the following: (a) addition of ATP; (b) addition of hexokinase to the complete reaction mixture to remove any ATP present initially or formed during illumination; and (c) supplementation of the complete system with the soluble factor of Avron and Jagendorf (31) which is required for photosynthetic phosphorylation by washed chloroplasts.

The effect of grana concentration on the rate of pyridine nucleotide reduction is not completely understood at the present time. The fact that the photolytic activity, measured by dye reduction, is greater than the rate of pyridine nucleotide reduction implies that the transfer of hydrogen from the photolytic system to the pyridine nucleotides is the rate-limiting step in our experiments. Under these conditions, it is difficult to explain the effect of grana concentration merely in the light of an increased photolytic activity.

It is suggested, therefore, that the grana may provide, in addition to the photolytic system, a substance which is the acceptor of the (H) formed by photolysis and is reduced (Equation 4). In the reduced form,

\[ \text{H}_2\text{O} + X \xrightarrow{\text{light}} \text{XH}_2 + \frac{1}{2}\text{O}_2 \]  
(4)

\[ \text{XH}_2 + \text{TPN}^+ \rightarrow \text{TPNH} + \text{H}^+ + \text{X} \]  
(5)

Sum: \[ \text{H}_2\text{O} + \text{TPN}^+ \xrightarrow{\text{light}} \text{TPNH} + \text{H}^+ + \frac{1}{2}\text{O}_2 \]  
(3)

The hexokinase was a crystalline preparation from yeast and was a generous gift of Dr. S. P. Colowick and Dr. R. A. Darrow.

Kindly supplied by Dr. A. Jagendorf and Dr. M. Avron.
this substance could serve as the hydrogen (or electron) donor for the reduction of dyes, other Hill oxidants, or pyridine nucleotides (Equation 5). The sum of Equations 4 and 5, in which \( X \) and \( XH_2 \) denote the oxidized and reduced forms, respectively, of the postulated substance, is equivalent to Equation 3. In the above scheme, the rate of pyridine nucleotide reduction will be linearly proportional to the grana concentration, provided that the following assumptions are correct. First, the assumption is that essentially all of the \( X \) is present in the reduced form \( (XH_2) \) in illuminated grana. This is reasonable since the rate of photolysis is faster than the rate of pyridine nucleotide reduction by illuminated grana. Secondly, it is necessary to assume in our experiments that we are operating on the linear portion of the substrate saturation curve; i.e., the rate of pyridine nucleotide reduction is linearly proportional to the substrate \( (XH_2) \) concentration. This assumption implies that the concentration of \( X \) (or \( XH_2 \)) in the grana is lower than that required to saturate the enzyme. This could well be the case since only a catalytic amount of \( X \) is required in this scheme. Under these conditions, the rate of pyridine nucleotide reduction will be linearly proportional to the concentration of \( XII_2 \) or the grana concentration.

Alternatively, the grana could contain a molecule which, in the light, is in an activated or excited state (Equation 6). The return of the activated molecule to the non-activated state would then be expected to provide

\[
Y \stackrel{\text{light}}{\longrightarrow} Y^* \quad (6)
\]

\[
Y^* + H_2O + TPN^+ \rightarrow Y + TPNH + H^+ + \frac{1}{2}O_2 \quad (7)
\]

Sum: \( TPN^+ + H_2O \stackrel{\text{light}}{\longrightarrow} TPNH + H^+ + \frac{1}{2}O_2 \quad (3)\]

sufficient energy to couple the protolysis of water with the reduction of pyridine nucleotides (Equation 7). Here, also, the sum of Equations 6 and 7, in which \( Y \) and \( Y^* \) represent the non-activated and activated forms, respectively, of the postulated molecule, is equal to Equation 3.

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**SUMMARY**

1. An enzyme has been isolated from spinach leaves which is required in addition to grana for the photochemical reduction of pyridine nucleotides. The enzyme has been purified some 20- to 35-fold and its possible role in photosynthesis discussed. It has tentatively been suggested that the enzyme be named "photosynthetic pyridine nucleotide reductase."

2. The initial rate of photochemical reduction of pyridine nucleotides is linearly proportional to the amount of enzyme present in the reaction
mixture. No reduction occurs in the absence of enzyme or when the complete reaction mixture is incubated in the dark. Identical results are obtained when the reaction is carried out aerobically rather than anaerobically under an atmosphere of nitrogen.

3. The enzyme is specific for the intact dinucleotide structure since nicotinamide mononucleotide is not reduced in the reaction.

4. Of the two natural coenzymes, the enzyme exhibits greater activity with triphosphopyridine nucleotide (TPN) than with diphosphopyridine nucleotide.

5. The pH optimum of the reaction is about 7.

6. The rate of photochemical reduction of TPN is linearly proportional to the grana concentration under the conditions employed. Two possible interpretations of this finding have been presented.

7. The reaction is inhibited by p-chloromercuribenzoate but is insensitive to azide, iodoacetate, cyanide, arsenite, and Versene.

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Anthony San Pietro and Helga M. Lang


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