Photosynthetic Phosphorylation in the Presence of Spinach Phosphodoxin

C. C. Black, A. San Pietro, G. Norris, and D. Limbach
Charles F. Kettering Research Laboratory, Yellow Springs, Ohio

The isolation and purification of a new substance named phosphodoxin from photosynthetic organisms was described recently (7, 8). Phosphodoxin is a naturally occurring thermosetable, water-soluble compound(s) which catalyzes photophosphorylation with both chloroplasts and chromatophores in the absence of exogenous electron acceptors. This report will present further properties of spinach phosphodoxin and the effects of spinach phosphodoxin on some photochemical reactions catalyzed by spinach chloroplast fragments.

Materials and Methods

Components of Reaction Mixture. The standard reaction mixture contained the following components in μmoles: Tris-HCl buffer, pH 7.8, 48; MgCl₂, 2; P₁ + P₃₂ (containing from 0.4 to 1.5 μcuries of P₃₂), 1; ADP, 1; chloroplast fragments (containing from 15 to 40 μgrams of chlorophyll); total volume of 1 ml. Reaction mixtures were illuminated laterally in Beckman cuvettes of 1 cm light path at 2500 ft-c, employing the light source previously described (21). Light intensity was varied by varying the distance of the cuvettes from the light source. Following illumination the reactions were stopped by adding 0.1 ml of 20% trichloroacetic acid and ATP₃₂ determined as previously described (21). When exogenous electron acceptors were included in the reaction mixtures they were added in the following final concentrations: NADP, 10⁻³ M; methyl phenazinium methosulphate (PMS), 5 × 10⁻⁵ M; and potassium ferricyanide, 10⁻³ M. Fresh spinach chloroplast fragments were prepared for each experiment and chlorophyll concentration was determined as previously described (21).

Reagents. Spinach photosynthetic pyridine nucleotide reductase (PPNR), pyridine nucleotide transhydrogenase and the antibody to the transhydrogenase were prepared and assayed by the methods of Keister and San Pietro (14, 16, 20). Phosphodoxin was obtained from spinach leaves (7).

ADP, NADP, PMS, antimycin A, and atebrin were purchased from the Sigma Chemical Co. Solutions of antimycin A were prepared in 95% ethanol. P₃₂ was purchased from Oak Ridge National Laboratory and purified by Dr. S. Hood by boiling in 0.1 N HCl with charcoal prior to use. CdCl₂, NH₄Cl, and arsenic trioxide were purchased from the Mallin- krodt Chemical Works. Fresh solutions of sodium arsenite were prepared from arsenic trioxide and NaOH. The Central Research Department of the E. I. du Pont de Nemours Company, Incorporated, supplied m-chlorocarboxyl cyanide phenylhydrazone (CCCP), 3-(p-chlorophenyl)-1,1-dimethyleurea (CMU) and 3-(3,4 dichlorophenyl)-1,1-dimethyleurea (DCMU). Solutions of CCCP, CMU, and DCMU were prepared in 95% ethanol. The concentration of CCCP was determined by its absorption at 378 mμ in an alkaline solution (13). Dr. J. W. Lighthown furnished the 2-heptyl-4-hydroxyquinoline-N-oxide (heptyl-HQNO) which was prepared in 10⁻³ M NaOH and the concentration determined by absorption at 346 mμ (10). When materials prepared in solutions containing ethanol or NaOH were studied, equivalent amounts of ethanol and NaOH were added to control reaction mixtures. The data given in this paper are corrected for dark controls.

Infrared spectra of phosphodoxin were obtained with a Perkin Elmer, Model 21, double beam infrared spectrophotometer by Dr. H. V. Knorr of this laboratory. The Sadler Corporation of Philadelphia assisted in interpretation of the spectra.

Emission spectrographic analyses for metals were graciously performed by Mr. H. E. Bales of this laboratory.

Results

Linearity of photophosphorylation by spinach chloroplast fragments with spinach phosphodoxin is indicated by the data in figure 1. In most of the sub-

![Graph](https://via.placeholder.com/150)

**FIG. 1.** Photophosphorylation by spinach chloroplast fragments with spinach phosphodoxin.
sequent experiments reported in this paper, the level of 0.1 ml of spinach phosphodoxin per milliliter of reaction mixture was used.

The requirement of spinach phosphodoxin catalyzed photophosphorylation for ADP is given in figure 2. No $^{133}$P was incorporated in the light in the absence of ADP. The optimum concentrations of ADP, $P_i$, and Mg$^{2+}$ are 1 to 3, 2 to 5, and over 1 µmoles per milliliter, respectively (fig 2).

Table 1 illustrates the metal requirement of spinach phosphodoxin. Only Mg$^{2+}$ gives a substantial increase in rate over the endogenous activity. Several metals are definitely inhibitory, including Mn$^{2+}$, Ca$^{2+}$, Zn$^{2+}$, Fe$^{2+}$, Fe$^{3+}$, and Cl$^{-}$ (table 1). Note that in the absence of a metal (fig 2, table 1) the rate of ATP production is only 30 to 40% decreased.

A broad pH optimum of 7.4 to 7.8 was observed in Tris-HCl buffer (fig 3). There is a definite lag in rate at light intensities below 100 ft-c. Above this value, the rate is proportional to intensity and becomes maximal between 1000 and 2000 ft-c (fig 4).

The effect of inhibitors on the spinach phosphodoxin catalyzed reaction is indicated in figures 5 and 6. A brief summary of the effects of various inhibitors is given in table 2. The effects of inhibitors on the spinach chloroplast ATPase activity are shown in figure 7.

Table I

<table>
<thead>
<tr>
<th>Compound</th>
<th>µmoles of metal per ml</th>
<th>Photophosphorylation as % of maximum*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>60</td>
<td></td>
</tr>
<tr>
<td>MgCl$_2$</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>MgCl$_3$</td>
<td>98</td>
<td></td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>Ca(NO$_3$)$_2$ + MgCl$_2$</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>MnCl$_2$</td>
<td>77</td>
<td></td>
</tr>
<tr>
<td>MnCl$_3$</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Zn(C$_2$H$_3$O$_2$)$_2$</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>FeCl$_3$</td>
<td>28</td>
<td></td>
</tr>
<tr>
<td>Co(NO$_3$)$_2$</td>
<td>74</td>
<td></td>
</tr>
<tr>
<td>FeSO$_4$</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td>Na$_2$MoO$_4$</td>
<td>50</td>
<td></td>
</tr>
<tr>
<td>MgSO$_4$</td>
<td>79</td>
<td></td>
</tr>
<tr>
<td>CaCl$_2$</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

* Maximum was 133 µmoles of ATP/mg chlorophyll hour.

Fig. 2. Effect of ADP, $P_i$, and Mg$^{2+}$ on photophosphorylation by spinach chloroplast fragments in the presence of spinach phosphodoxin. When one cofactor was varied the other cofactors were present at the following concentration: ADP, 1; $P_i$, 1; and Mg$^{2+}$, 2.

Fig. 3. Effect of pH, Tris-HCl buffer, on photophosphorylation by spinach chloroplast fragments in the presence of spinach phosphodoxin.

Fig. 4. Effect of light intensity on photophosphorylation by spinach chloroplasts in the presence of spinach phosphodoxin.

Fig. 5. Effect of inhibitors on photophosphorylation by spinach chloroplasts in the presence of spinach phosphodoxin.
Phosphodoxin-catalyzed photophosphorylation was quite sensitive to the action of the substituted ureas, DCMU and CMU, and to CCCP with the 50% inhibition level being $3 \times 10^{-7}$, $6 \times 10^{-7}$ and $8 \times 10^{-7} \text{ M}$, respectively. Heptyl-HQNO and atebrin at $10^{-5} \text{ M}$ were about equally effective and antimycin A at $4 \times 10^{-5} \text{ M}$ was 50% inhibitory. High concentrations of Cd$^{2+}$, $10^{-4} \text{ M}$, and NH$_4^+$, $6 \times 10^{-4} \text{ M}$, were required for 50% inhibition. Arsenite at concentrations as high as $10^{-3} \text{ M}$ did not affect the activity of spinach phosphodoxin.

The addition of transhydrogenase in combination with PPNR or alone did not affect the photochemical activity of spinach phosphodoxin (Table II). Further indication of their lack of involvement was the inactivity of the transhydrogenase antibody toward this phosphorylation.

The effect of purified phosphodoxin on the photochemical production of ATP in the presence of other exogenous electron acceptors is given in Figure 7. A control curve of phosphodoxin alone is given for comparison. Note that ATP production in the presence of NADP and PPNR was not affected, whereas, in sharp contrast, ATP production in the presence of PMS increased at low levels of phosphodoxin. This increase in the initial rate of photophosphorylation with PMS plus phosphodoxin varied from 2- to 4-fold in separate experiments. In the presence of ferricyanide an inhibition was noted at 0.2 ml of phosphodoxin per milliliter of reaction mixture. This inhibition of ferricyanide-supported photophosphorylation varies from 0 to 90% at 0.2 ml of phosphodoxin per milliliter of reaction mixture. In other experiments we have increased the concentration of phosphodoxin and observed an inhibition in the presence of PMS and NADP with a concomitant decrease in the activity of phosphodoxin alone.

**Discussion**

Phosphodoxins, which have been detected in all major groups of photosynthetic organisms (7, 8), are naturally occurring substances which have been shown to catalyze photosynthetic phosphorylation at rates proportionate with other photosynthetic reactions of chloroplasts and chromatophores. The photochemical reaction catalyzed by spinach phosphodoxin in the presence of spinach chloroplast fragments is similar to other photochemical reactions of spinach chloroplasts in the response to variations in experimental conditions. This similarity is illustrated by a comparison of photophosphorylation catalyzed by spinach phosphodoxin with photos phosphorylation in the presence of various exogenous electron acceptors.

The optimal pH of 7.4 to 7.8 for spinach phosphodoxin (fig 3) is similar to photosynthetic phosphorylation in the presence of NADP (15), FMN (4, 19), PPNR (9, 11), and PMS (3). Photosynthetic phosphorylation in the presence of NADP (21), 2,3,6-trichlorophenolindophenol (17), FMN, vitamin $K_3$ (2), and spinach phosphodoxin exhibit similar responses to light intensity.

The effect of Mg$^{++}$ and ADP concentration

---

**Table II**

*Effect of PPNR and Transhydrogenase on Photophosphorylation Catalyzed by Spinach Phosphodoxin*

<table>
<thead>
<tr>
<th>Protein added</th>
<th>Units*</th>
<th>μmoles of ATP/mg chlorophyll hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>...</td>
<td>106</td>
</tr>
<tr>
<td>PPNR</td>
<td>0.16</td>
<td>92</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>91</td>
</tr>
<tr>
<td></td>
<td>1.6</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>4.8</td>
<td>99</td>
</tr>
<tr>
<td>Transhydrogenase</td>
<td>5</td>
<td>103</td>
</tr>
<tr>
<td></td>
<td>25</td>
<td>112</td>
</tr>
<tr>
<td></td>
<td>50</td>
<td>117</td>
</tr>
<tr>
<td>PPNR + transhydrogenase</td>
<td>0.16 + 25</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>0.8 + 5</td>
<td>93</td>
</tr>
<tr>
<td></td>
<td>0.8 + 25</td>
<td>97</td>
</tr>
<tr>
<td>Antibody to transhydrogenase</td>
<td>0.34</td>
<td>97</td>
</tr>
</tbody>
</table>

* Defined in refs. 14, 16, and 20.
(fig 2) on the reaction catalyzed by spinach phospho-
doxin is consistent with other available data
(1, 4, 11, 18). The optimum P1 concentration (fig 2)
is in accord with previously reported values (4, 18);
however, the inhibition by higher concentrations
is at variance with most data (4, 18). The lack
of a specific requirement for a metal (table I, fig 2)
is surprising since most photophosphorylations require
a metal (1, 4, 11, 18). Evidently either the small
metal content of spinach phosphodoxid or of the
chloroplast preparation is sufficient to supply about
60% of the metal requirement of spinach phospho-
doxin.

The inhibitor studies reported in this paper (fig 5, 6)
further correlate the similarity of photophos-
phorylation catalyzed by spinach phosphodoxid to
photophosphorylation in the presence of other cata-
lysts. The inhibition by CMU and DCMU agrees
with other investigations (6, 18) as does that of
heptyl-HQNO (5, 6) and CCCP (6, 13). The inhi-
bition concentration of aternin is the same as re-
ported by Baltscheffsky for FMN, FAD, and PMS
(5). The reaction is relatively insensitive to anti-
mycin A as are other types of photosynthetic phos-
phorylation (6) with the exception of the phos-
phorylation catalyzed by PPNR which is sensitiv-
to antinmycin A (11). The action of Cd++ may
be due to an uncoupling similar to NH4+ but this
appears doubtful since Cd++ at 10^-4 M also inhibits
NADP reduction 50% (M. Gibbs, and C. C. Black.
unpublished data, 1961) whereas NH4+ does not
(15). Arsenite does not affect photophosphorylation
in the presence of PMS, FMN, NADP, vitamin K3
(12) and spinach phosphodioxid.

The variable effects of spinach phosphodoxid on
ATP production in the presence of other electron
acceptors is surprising (fig 7). For example, the
stimulation of the initial rate of ATP formation in
the presence of PMS is the first reported increase of
PMS-catalyzed phosphorylation by a naturally occur-
rning substance. It is also surprising that the phos-
phodioxid catalyzed ATP production is not additive
in the presence of exogenous electron acceptors. The
selective inhibition of ferricyanide-supported ATP
production is another anomalous result. At the pres-
et time the results in the presence of other exo-
genous electron acceptors cannot be clearly explained.

It is clear that spinach phosphodoxid is capable
of catalyzing photophosphorylation at rates com-
patible with the needs of intact leaves for growth.
The chemical structure of spinach phosphodoxid is
currently under investigation. Infrared spectra indi-
cate that spinach phosphodoxid contains an OH
group from a band at 296 μ, a CH2 or CH3 group
from bands at 3.45 and 6.95 μ. Although a broad
band between 8.5 and 10.5 μ was observed which is
indicative of a phosphate group, the phosphate could
not be detected in spinach phosphodoxid by emission
spectrography or by organic or inorganic phosphate
assays.

Metal analysis indicated that spinach phospho-
doxid probably does not contain a functional metal.

Summary

Further properties of a new substance, phos-
dioxid, isolated from spinach have been presented.
Spinach phosphodoxid has been shown to catalyze
adenosine triphosphate formation in the presence
of spinach chloroplast fragments at rates up to
200 μmoles per milligram chlorophyll per hour.
Spinach phosphodoxid contains low quantities of se-
veral metals. The following experimental conditions
are reported: metal requirement, response to pH,
concentration of magnesium chloride, inorganic phos-
phate and adenosine diphosphate; response to light
intensity; effect of inhibitors and uncouplers; effect
of spinach photosynthetic pyridine nucleotide reduc-
and pyridine nucleotide transhydrogenase and the
antibody to the transhydrogenase; and the effect of
spinach phosphodoxid on photophosphorylation in the
presence of nicotinamide adenine dinucleotide, methyl
phenazinium methosulfate and potassium ferri-
cyanaide.

Literature Cited

   In: Brookhaven Symposium in Biology No. 11.
   pp 181-235.
2. ARNON, D. I. 1961. Cell-free photosynthesis and
   the energy conversion process. In: Light and
   Hopkins Press, Baltimore. p 489-564.
3. AYRON, M. 1960. Photophosphorylation by swiss-
chard chloroplasts. Biochim. Biophys. Acta 40:
   257-72.
4. AYRON, M., A. T. JAGENDORF, and M. EVANS.
   1957. Photosynthetic phosphorylation in a partially
   purified system. Biochim. Biophys. Acta 26:
   262-69.
5. BALTSCHEFSKY, H. 1960. Inhibitor studies on
   light-induced phosphorylation in isolated spinach
6. BAMBERGER, E. S., C. C. BLACK, C. A. FEWSON, and
   M. GIBBS. 1963. Inhibitor studies on CO2 fixa-
   tion adenosine triphosphate formation and triphospho-
   pyridine nucleotide reduction by spinach chloroplasts.
7. BLACK, C. C. and A. SAN PIETRO. 1963. Photo-
   synthetic phosphorylation with bacterial chromato-
phores: Catalysis by a naturally occurring factor
   (phosphodoxid). In: Bacterial Photosynthesis H.
   Gest et al., eds. Antioch Press, Yellow Springs,
8. BLACK, C. C., A. SAN PIETRO, D. LIMBACH, and G.
   NORMIS. 1963. Photosynthetic phosphorylation
   catalyzed by factors isolated from photosynthetic
9. BLACK, C. C., C. A. FEWSON, M. GIBBS, D. L. KIELST-
   ER, and A. SAN PIETRO. 1962. Further studies on
   photosynthetic pyridine nucleotide reductase.
   Federation Proc. 21: 398.
10. CORNFORTH, J. W. and A. T. JAMES. 1956. Struc-
   ture of a naturally occurring antagonist of di-
   Further studies on the photochemical production of
   reduced triphosphopyridine nucleotide and adeno-
   sine triphosphate by fragmented spinach chloro-
Test of the Intermediary Role of Nicotine-1′-Oxide in Conversion of Nicotine to Nornicotine

W. Stepka and L. J. Dewey

Department of Research and Development, The American Tobacco Company and The Departments of Physiology and Biochemistry, The Medical College of Virginia, Richmond

In previous studies on the post-harvest conversion of nicotine to nornicotine in the tobacco leaf (7) the present authors found that the methyl group of nicotine was mostly oxidized to CO₂. Attempts to detect likely pyridyl intermediates were unsuccessful. Nicotine-1′-oxide, proposed as an intermediate by Wenkert (8) on theoretical grounds and implicated experimentally by Ergi (4), was one of our prime targets and received particular attention. None was detected. Nor could we associate radioactivity with nicotine-1′-oxide when either randomly or specifically C¹⁴-labeled nicotine served as the substrate.

On the basis of this evidence we were inclined to reject an intermediary role for the oxide (7). However, calculations based on the specific activities of the labeled nicotines used as substrates indicated a remote possibility that the nicotine-1′-oxide could be present below detectable limits. Since the compound represented such an attractive potential for an intermediary role in the conversion, experiments were designed to eliminate the ambiguity. The objective of this paper is to clarify the role of nicotine-1′-oxide in the post-harvest conversion of nicotine to nornicotine.

Materials and Methods

One of the obstacles encountered in our earlier experiments arose from the fact that the experimental leaves already contained normal amounts of unlabeled nicotine and, usually, traces of nornicotine. The radioactive nicotine subsequently introduced thus became diluted by the endogenous pool. Since the endogenous quantity exceeded the quantity of the labeled substrate we could afford to introduce for both physiological and economic reasons, the resulting dilution of the specific activity pressed us close to the calculated limit of detection of the intermediates.

Plant Material. In an attempt to circumvent this problem we grew tobacco plants free of pyridyl alkaloids. To accomplish this we grafted tobacco scions onto tomato rootstocks. Dawson (1) and others (3) have shown that the tobacco shoot developing from such grafts is substantially free of nicotine. The scions were obtained from the same

1 Received July 29, 1963.