PYRIDINE NUCLEOTIDE TRANSHYDROGENASE FROM SPINACH[1]

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During the course of purification of the enzyme, photosynthetic pyridine nucleotide reductase (PPNR), it was observed that the purified enzyme catalyzed the reduction of triphosphopyridine nucleotide (TPN), but not diphosphopyridine nucleotide (DPN), in the presence of illuminated grana; whereas, both TPN and DPN were reduced when a partially purified preparation of the enzyme was used (San Pietro, 1959; San Pietro and Lang, 1958). It was suggested, therefore, that the PPNR is specific for TPN and that DPN was reduced secondarily by virtue of a pyridine nucleotide transhydrogenase (Colowick et al, 1952) which was present in the partially purified enzyme preparation but absent from the purified enzyme. Arnon et al (1957) have also invoked pyridine nucleotide transhydrogenase to explain their observation that DPN was as effective as TPN in increasing photosynthetic phosphorylation provided either a large amount of chloroplast material or an excess of chloroplast extract was used. In this paper, data are presented which demonstrate directly the presence of pyridine nucleotide transhydrogenase activity in a partially purified preparation of PPNR which corresponds to the "extract of acetone precipitate" described by San Pietro and Lang (1958). In addition, the photochemical reduction of DPN in the presence of grana appears to require both enzymatic activities.

The experiments with illuminated grana were performed in the following manner: The reaction mixture is prepared in a glass cuvette and mixed. The optical density at 340 mμ is measured against a blank which contains all the com-

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ponents except enzyme. Both the complete reaction mixture and the blank are placed in a water bath at approx. 20° and illuminated. After illumination, the optical density is again determined and the increase in optical density, obtained from these two measurements, serves as a direct measure of enzymatic activity.

The results of a typical experiment are presented in Table I. It can be seen that both TPN and DPN are reduced when a partially purified preparation of PPNR is used (Exp. 1). Furthermore, the purified PPNR catalyzes the reduction of only TPN in the presence of illuminated grana; DPN is not reduced under these conditions (Exp. 2).

Table I - Reduction of TPN and DPN by Illuminated Grana.

Exp.	Additions	TPN	DPN
1	Partially Purified PPNR	0.91	0.43
2	Purified PPNR	0.88*	0.05
3	Supernatant from Protamine Sulphate Precipitation of PPNR	0.05	0.05
4	As in Expts. 2 + 3	1.16	0.57

* Illumination Time - 2.5 min.

The grana were prepared as follows: 50 g. of spinach leaves were deveined and ground for 3 min. in a Waring Blendor with 100 ml. of cold 0.05 M tris buffer, pH 7.2, containing 0.01 M KCl. After filtering through cheesecloth and glass wool, the filtrate was centrifuged for 2 min. at 4600 g. The supernatant was centrifuged for 20 min. at 18,000 g. and the residue suspended in buffer. Each reaction mixture contained grana equivalent to either 76 micrograms (Exp. 1) or 87 micrograms (Expts. 2-4) of chlorophyll, 60 μmoles tris-HCl of pH 7.5, 200 μmoles of potassium phosphate of pH 7.5, 0.5 μmole of pyridine nucleotide and the additions indicated below. In exp. 1, partially purified PPNR, 2 units; in expts. 2 and 4, purified PPNR, 2 units, and in expts. 3 and 4, 1.4 mg. of protein as supernatant of protamine sulphate precipitation was added. Final volume, 3 ml. Time of illumination was 5 min. The data are presented as μmole of pyridine nucleotide reduced in 10 min. when the reaction mixture contains grana equivalent to 100 micrograms of chlorophyll.

The further purification of the partially purified PPNR is accomplished by the precipitation of the enzyme with protamine sulphate (San Pietro and Lang, 1958). It can be seen that the addition of the supernatant fluid from this precipitation step to a system containing purified PPNR results in the reduction of DPN (Exp. 4). On the other hand, DPN is not reduced when either component is omitted (Expts. 2 and 3).

The simplest interpretation of these data is that PPNR catalyzes the

reduction of only TPN in the presence of illuminated grana and that DPN is subsequently reduced through the action of pyridine nucleotide transhydrogenase. This interpretation is supported by the fact that we have been able to demonstrate certain transhydrogenase activities in the protamine sulphate supernatant fluid by the usual assay procedures (Kaplan, 1955; Stein et al, 1959). It is clear from the results in Table II that the protamine sulphate supernatant fluid contains transhydrogenase(s) which catalyze the oxidation of TPNH by either DPN or APTPN (reactions 1 and 2) but not by APDPN (reaction 3).

TPNH + DPN ——> DPNH + TPN (1)
TPNH + APTPN ——> APTPNH + TPN (2)
TPNH + APDPN ——> APDPNH + TPN (3)

Table II - Transhydrogenase Activities in Protamine Sulphate Supernatant

Exp.	Reductant	Oxidant	ΔOD/3 min.
1	TPNH	DPN	0.25
2	TPNH	APTPN	0.31
3	TPNH	APDPN	0.0

In Exp. 1, the generation of reduced DPN mediated by transhydrogenase in the presence of the isocitric dehydrogenase system and a catalytic amount of TPN was followed at 340 mμ.

In Expts. 2 and 3, reduced TPN (TPNH) was added in substrate quantity and the reduction of the acetylpyridine analogues of TPN and DPN (APTPN and APDPN, respectively) measured at 365 mμ.

In separate experiments, not indicated in Table II, it has been established that DPNH does not serve as the hydrogen donor for reduction of TPN, APTPN or APDPN (reactions 4-6).

DPNH + TPN ——> TPNH + DPN (4)
DPNH + APTPN ——> APTPNH + DPN (5)
DPNH + APDPN ——> APDPNH + DPN (6)

It would thus appear that the transhydrogenase activity described herein may be specific for TPNH as the reductant since all attempts to demonstrate the transhydrogenase activities indicated by reactions 4-6 have been unsuccessful.

The transhydrogenase activity from spinach appears to have different properties from both the Pseudomonas (Colowick et al, 1952; Kaplan et al, 1953) and the mitochondrial enzymes (Stein et al, 1959).

The bacterial enzyme does not catalyze the transfer reaction from TPNH to APDPN or APTPN whereas the spinach enzyme catalyzes transfer to the latter analogue (Weber and Kaplan, 1957). In addition, the enzymes from the two sources are affected very differently by the presence of 2'-adenylic acid. With the bacterial system, reaction 1 is not affected by the mononucleotide when the assay is carried out in tris buffer. Under similar conditions, the mononucleotide is inhibitory in the spinach system. The degree of inhibition caused by the mononucleotide in the spinach system is essentially the same whether the activity is assayed directly (Table II, Exp. 1) or indirectly by measuring the photochemical reduction of DPN (Table I, Exp. 1). Finally, 2'-adenylic acid promotes the reversibility of reaction 1 (i.e., reaction 4) with the Pseudomonas enzyme. All attempts to show reversal of reaction 1 with the spinach system have been uniformly unsuccessful.

With respect to the mitochondrial enzyme, it fails to catalyze reaction 2 but does catalyze reaction 3. The reverse is true for the spinach system. Furthermore, 2'-adenylic acid is without influence on the enzyme from beef heart whereas it has an effect on the spinach enzyme as indicated above.

It should be noted that grana were used in the experiments described in Table I. When chloroplasts were used in place of grana, it was not possible to demonstrate reduction of DPN either with the partially purified PPNR or the reconstituted system. A possible explanation of this observation may be impermeability of the chloroplast to the transhydrogenase.

A second point of interest is the fact that the photochemical reduction of DPN in the presence of grana proceeds in the absence of any added TPN (Table I, Expts. 1 and 4). Furthermore, the addition of catalytic amounts of TPN does not stimulate the rate of photochemical reduction of DPN in this system. This suggests that one of the components of the system, most probably the grana, is providing sufficient TPN to saturate the transhydrogenase.

Finally, in separate experiments, it has been established that the rate of the transhydrogenase reaction is sufficient to account for the rate of photochemical reduction of DPN reported in Table I.

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