

6. The protease attacks only  $\alpha$ -benzoyl-L-argininamide among the several peptides tested, which shows that it is of the trypsin type.

7. The enzyme is practically unaffected by any of a variety of compounds tested. It is not a sulphhydryl enzyme and does not require metal ions for activity and is inhibited by soya-bean trypsin inhibitor.

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## A Protein from Leaves Catalysing the Reduction of Metmyoglobin and Triphosphopyridine Nucleotide by Illuminated Chloroplasts

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In his early experiments with isolated chloroplasts Hill (1939) was able to show that when these are illuminated in the presence of an extract of acetone-dried leaf, oxygen is evolved. The extracts therefore contained soluble material in substrate amounts capable of accepting hydrogen in the photochemical system. Although the pathway of hydrogen transport in this reaction has not yet been determined, two leaf-protein fractions have since been shown to be active catalysts of hydrogen transport from illuminated chloroplasts.

One of these, occurring in acetone-dried leaf powders, was first observed to catalyse the reduction of metmyoglobin (Davenport, Hill & Whatley, 1952). The active protein has since been obtained

from pea leaves in a highly purified state, homogeneous both electrophoretically and in the ultracentrifuge (Davenport & Hill, 1960) and was shown also to promote the reduction of other haem-protein compounds including cytochrome *c* and cytochrome  $b_3$ .

Meanwhile San Pietro & Lang (1958) isolated a spinach-leaf-protein fraction active in catalysing the photochemical reduction of triphosphopyridine nucleotide. This also has since been purified (San Pietro, 1958) and named 'photosynthetic pyridine nucleotide reductase'.

During some experiments aimed at confirming the observations of San Pietro & Lang (1958) it was noticed that the capacity of leaf-protein fractions to

promote the reduction of triphosphopyridine nucleotide was always accompanied by activity towards metmyoglobin. Attempts to separate the two activities have been unsuccessful. Similarly, the 'haem-protein-reducing factor' in spite of the very different method of preparation, is active towards triphosphopyridine nucleotide. In this material also, attempts to separate the two activities have been unsuccessful. The object of the present paper is to compare metmyoglobin and triphosphopyridine nucleotide as hydrogen acceptors in the photochemical reductions, catalysed by leaf-protein fractions prepared by a variety of methods. This work has been briefly reported (Davenport, 1959*b*).

### EXPERIMENTAL

*Plant materials.* Seedlings of *Pisum sativum*, *Spinacea oleracea* and *Beta vulgaris* (spinach beet) were raised in a cool greenhouse from seed sown in sand and irrigated with a complete nutrient solution (Hewitt, 1952). Leaves intended for protein preparations were either used immediately after harvesting or stored in sealed boxes at  $-20^{\circ}$  until required.

*Chloroplast preparations.* Chloroplasts were prepared from fresh leaves of *S. oleracea*. They were ground in a chilled mortar with about 5 times their weight of an ice-cold solution containing 0.4M-sucrose, 0.01M-NaCl and 0.05M-2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer, pH 7.5. After filtration through a layer of muslin, the filtrate was centrifuged at 500g and  $0^{\circ}$  for 2 min. and the pellet discarded. The supernatant fluid was centrifuged at 2000g for 10 min. to give a pellet consisting largely of whole chloroplasts. This was resuspended in the cold sucrose solution. Where broken chloroplasts (grana) were required the supernatant fluid was again centrifuged at 18 000g for 20 min. and the pellet resuspended as before. The suspensions of grana and chloroplasts were adjusted to a chlorophyll (a + b) concentration of about 0.6 mg./ml.

*Chlorophyll concentration.* This was determined in acetone extracts by the method of Mackinney (1941).

*Metmyoglobin.* Crystalline, sperm-whale metmyoglobin, which had been stored in 3M-(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> solution at  $0^{\circ}$  was prepared for use and the haematin concentration estimated as described by Davenport & Hill (1960).

*Other materials.* Triphosphopyridine nucleotide (TPN), diphosphopyridine nucleotide (DPN), adenosine triphosphate (ATP) and adenosine 5'-phosphate (AMP) were products of Sigma Chemical Co., St Louis, Mo., U.S.A. Adenosine diphosphate (ADP) was obtained from this source and also from Boehringer und Söhne, Mannheim, Germany.

*Protein determination.* Protein contents of leaf preparations were estimated by the Folin-phenol-reagent method of Lowry, Rosebrough, Farr & Randall (1951). This method was calibrated against the Kjeldahl procedure of Chibnall, Rees & Williams (1943).

*Photochemical-activity measurements.* The photochemical reactions were carried out aerobically in silica cells of 1 cm. light path and 3 ml. capacity. Reduction of TPN or of metmyoglobin was followed in an Optica CF4 spectrophotometer as an increase in extinction at 340 m $\mu$  or 582 m $\mu$  respectively. The blank cell usually contained all the ingredients of the reaction mixture except the catalytically

active leaf protein. Reaction and blank cells were illuminated by a 500 w projector lamp at 36 cm. The incident light was filtered through heat-absorbing glass (Chance ON 20) and a 1 cm. layer of 5% (w/v) cupric sulphate and the cells were immersed during illumination in a glass-sided water bath at  $17^{\circ}$ . The time course of a reduction was obtained by alternating periods of illumination with measurements of extinction.

With TPN,  $\epsilon_{340}^{340\text{m}\mu}$  was taken as  $6.25 \times 10^3$  (Colowick, Kaplan & Ciotti, 1951). Under the experimental conditions described an increase in extinction of 0.1 is equivalent to the reduction of 0.048  $\mu$ -mole of TPN, independent of pH.

The increase in extinction at 582 m $\mu$ , occurring during metmyoglobin reduction, is the result of the rapid oxygenation of myoglobin produced in the reaction to oxymyoglobin ( $\alpha$ -band maximum at 582 m $\mu$ ). Since the extinction of metmyoglobin at this wavelength varies with pH (Bowen, 1949) and that of oxymyoglobin does not, the relationship between the observed increase in extinction and  $\mu$ mole of metmyoglobin reduced will also vary with pH. The relationship was therefore determined in each experiment by allowing the reaction to run to completion and relating the overall increase in extinction to the initial concentration of metmyoglobin determined spectrophotometrically as cyanmetmyoglobin (Davenport & Hill, 1960). The validity of this procedure depends on the absence of back reactions leading to the reoxidation of oxymyoglobin and these were never observed. Under the experimental conditions and at pH 7.7, the pH most commonly used, an increase in extinction of 0.1 was found to be equivalent to the reduction of 0.0255  $\mu$ mole of metmyoglobin. This is in good agreement with the value calculated from the spectrophotometric data of Bowen (1949) for horse-heart myoglobin.

*Specific activity of the leaf protein.* San Pietro & Lang (1958) have defined one unit of enzyme as the amount which produces an increase in extinction of TPN of 1.0 in 10 min. at 340 m $\mu$  when the reaction mixture contains 100  $\mu$ g. of chlorophyll in 3 ml. This definition has been adopted in the present work in the absence of a workable method of compensating for the variability observed in the photochemical activity of different chloroplast preparations.

## RESULTS

### Leaf-protein preparations

*Type I.* The preparation of the haem-protein reducing factor from pea leaves by ammonium sulphate fractionation of the expressed leaf juice followed by electrophoresis on paper has been described in detail (Davenport & Hill, 1960). The procedure has now also been applied, without modification, to leaves of spinach and spinach beet. Preparations made in this way will be referred to as type I.

*Type II.* For purposes of comparison the method used by San Pietro & Lang (1958) for the preparation of 'photosynthetic pyridine nucleotide reductase' has also been followed with leaves of pea, spinach and spinach beet. In this procedure a protein fraction precipitated from a leaf juice by cold acetone is redissolved, dialysed against dilute tris buffer, pH 8, and treated with protamine

sulphate. Active material is removed from the insoluble protamine complex by exhaustive extraction with more concentrated tris buffer. Preparations made in this way will be referred to as type II.

A number of combinations of these two preparative methods have also been examined. When type II preparations were subjected to electrophoresis on paper the material active towards metmyoglobin and TPN migrated in the manner described for preparations obtained by ammonium sulphate fractionation (Davenport & Hill, 1960). Similarly, it was found possible to effect a purification of type I preparations before electrophoresis, when these were subjected to the protamine sulphate treatment. When attempts were made to substitute ammonium sulphate fractionation for the protamine treatment with preparations from pea leaves started according to the method of San Pietro & Lang, activity towards TPN was found to be confined to the fraction precipitated above 70% saturation with respect to the salt. The activity in this fraction was rapidly lost when freshly harvested leaves were used as the starting material. By holding the leaves at  $-20^{\circ}$  for at least 2 days this loss of activity was found to be greatly minimized and further fractionation was then possible. This observation is exactly similar to that reported in connexion with the metmyoglobin-reducing activity (Davenport & Hill, 1960) and provided the first indication that the two activities might be properties of a single protein fraction. Modifications of the preparative methods will be referred to at the appropriate point in the text.

#### *Reduction of triphosphopyrinate nucleotide*

Preparations of type I at all stages of purification were active in catalysing the reduction of TPN by illuminated chloroplasts but all were completely inactive towards DPN. The effect of increasing concentration of added leaf protein, before and after electrophoresis, upon the rate of this reaction is shown in Fig. 1 where the final preparative step gave a ninefold increase in specific activity to the high concentration of 83 units/mg. of protein. With both the crude and purified protein the reduction rate was linearly related to the concentration of added protein until the particulate photochemical system became saturated at a maximum reduction rate, in this experiment, of  $184 \mu\text{moles}$  of TPN/mg. of chlorophyll/hr. Half-maximal reduction rate was here obtained by the addition of  $40 \mu\text{g.}$  of the purified protein. This, on the basis of a calculated molecular weight of 19 000 (Davenport & Hill, 1960), is equivalent to  $2 \mu\text{m-moles}$  of the protein.

The highest reduction rate for TPN so far observed is  $270 \mu\text{moles/mg.}$  of chlorophyll/hr.

*Effect of a phosphate acceptor system.* In the

experiment shown in Fig. 1 the reaction mixture was supplemented by ADP, orthophosphate and magnesium chloride since it had been found that all these are necessary for maximal activity. Arnon, Whatley & Allen (1958) have shown that the photochemical reduction of TPN catalysed by a chloroplast extract can be coupled to phosphorylation by including the above phosphate-accepting system in the reaction mixture. Inorganic phosphate was then shown to be incorporated into ATP. There have been no reports that this phosphorylation leads to an increase in the reduction rate. By contrast Arnon *et al.* (1958) found ferricyanide to be reduced more rapidly by illuminated chloroplasts when this reaction, which does not need a catalyst, is coupled to phosphorylation, and this observation has been confirmed and extended by Avron, Krogmann & Jagendorf (1958).

In the present work a stimulation of TPN reduction in the presence of the phosphate-accepting system could always be obtained with preparations of type I or II. The effect of adding the ingredients of this system either separately or in all possible combinations is shown in Table 1. Although ADP alone, or in combination with either orthophosphate or  $\text{Mg}^{2+}$  ions, gave marked stimulation, the maximum rate could only be achieved when all three were present. The table also shows that arsenate is as effective as phosphate in stimulating the reduction.

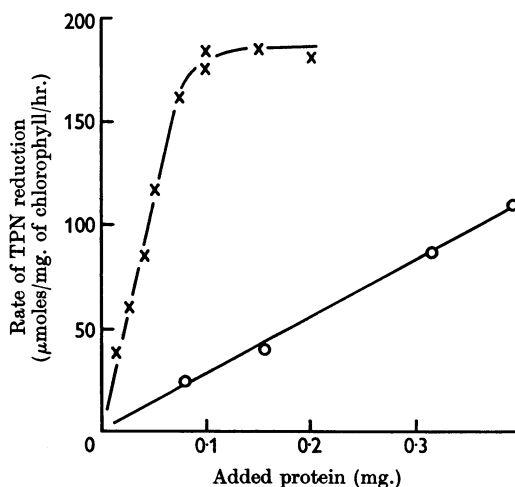


Fig. 1. Rate curves for the reduction of TPN by spinach chloroplasts catalysed by pea-leaf protein (type I). ○, Before electrophoresis; ×, after electrophoresis. Reaction mixtures (3 ml.) contained added protein as indicated, chlorophyll, 0.03 mg. and the following (in  $\mu\text{moles}$ ): TPN, 0.4; ADP, 0.5;  $\text{MgCl}_2$ , 20;  $\text{Na}_2\text{HPO}_4$ , 10; tris-HCl buffer, pH 7.7, 150; NaCl, 40. Leaf protein was omitted from the blank cell.

In the experiment shown in Table 1 the photochemical system was saturated with respect to added protein. It is in this saturation range that the stimulating effect of the phosphate-accepting

Table 1. *Effect of ingredients of a phosphate-accepting system on the rate of reduction of triphosphopyridine nucleotide*

Each reaction mixture contained, in a total volume of 3 ml., spinach chloroplasts (chlorophyll content 0.029 mg.), pea-leaf protein (type I) 0.13 mg. and (in  $\mu$ moles) TPN, 0.4; NaCl, 40; tris-HCl buffer, pH 8.0, 150. Where indicated the following additions were made ( $\mu$ moles): ADP, 0.5; MgCl<sub>2</sub>, 15; Na<sub>2</sub>HPO<sub>4</sub>, 8; Na<sub>2</sub>HAsO<sub>4</sub>, 8. The blank cell contained all the above ingredients except leaf protein and arsenate.

Additions	TPN reduced ( $\mu$ moles/mg. of chlorophyll/hr.)	Percentage of control rate
None	74	100
Mg <sup>2+</sup>	84	114
PO <sub>4</sub> <sup>3-</sup>	76	103
ADP	120	162
Mg <sup>2+</sup> + PO <sub>4</sub> <sup>3-</sup>	90	120
ADP + PO <sub>4</sub> <sup>3-</sup>	130	176
ADP + Mg <sup>2+</sup>	130	176
ADP + PO <sub>4</sub> <sup>3-</sup> + Mg <sup>2+</sup>	191	259
ADP + AsO <sub>4</sub> <sup>3-</sup> + Mg <sup>2+</sup>	195	264

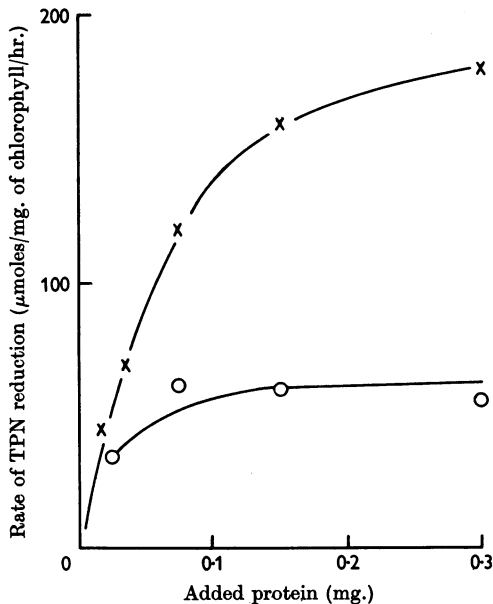


Fig. 2. Effect of a phosphate-acceptor system on TPN reduction catalysed by pea-leaf protein (type II). All reaction mixtures contained (in 3 ml.) leaf protein as indicated, spinach chloroplasts (chlorophyll content 0.016 mg.) and (in  $\mu$ moles) NaCl, 40; TPN, 0.4; tris-HCl buffer, pH 7.0, 150. x, Reaction mixtures contained in addition, ADP, 0.5; Na<sub>2</sub>HPO<sub>4</sub>, 15; and MgCl<sub>2</sub>, 20. The blank cell contained all ingredients except leaf protein. Illumination time, 2 min.

system was found to be most marked. This is illustrated by the experiment shown in Fig. 2 in which a threefold stimulation of the reduction rate occurred in the presence of 0.3 mg. of protein, twofold with 0.05 mg. and no stimulation with 0.02 mg.

Of the adenosine compounds tested in the presence of orthophosphate and Mg<sup>2+</sup> ions only ADP was found to be effective alone. Neither AMP nor ATP could be substituted for ADP in producing an increase in the reduction rate. When, however, AMP was supplemented by a catalytic amount of either ADP or ATP, marked stimulation occurred although the magnitude of the effect was not as great as with ADP alone (Fig. 3). This observation agrees with the finding (Mazelis, 1956) that spinach chloroplasts contain an active adenylate kinase.

#### *Reduction of metmyoglobin*

The activity of preparations of type I towards metmyoglobin and other haem-protein compounds as hydrogen acceptors has already been described in detail (Davenport & Hill, 1960). In the present work this activity has been compared directly with the activity towards TPN with leaf-protein preparations of both types. One comparative study of this kind, with a type II preparation from pea leaves further purified by electrophoresis on paper (Davenport & Hill, 1960), is shown in Fig. 4. Here the

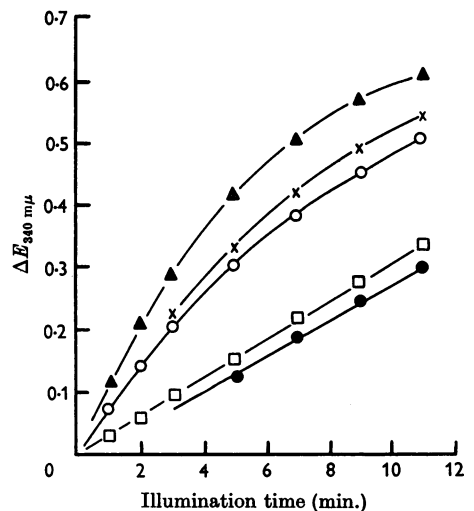


Fig. 3. Effect of adenosine derivatives on the rate of TPN reduction catalysed by pea-leaf protein (type I). All reaction mixtures contained (in 3 ml.) 0.08 mg. of leaf protein, spinach chloroplasts (chlorophyll 0.02 mg.) and (in  $\mu$ moles) TPN, 0.4; tris-HCl buffer, pH 7.0, 150; NaCl, 40; Na<sub>2</sub>HPO<sub>4</sub>, 10; MgCl<sub>2</sub>, 15, with the following additions ( $\mu$ moles) ●, ATP, 0.5; ○, AMP, 0.5; □, AMP, 0.5; x, AMP, 0.5, ADP, 0.05; ▲, ADP, 0.5. Leaf protein and the additional ingredients were omitted from the blank cell.

effect of increasing amounts of the leaf protein on the rate of reduction of the two hydrogen acceptors is shown. At low concentrations of the leaf protein the reaction rates, expressed as  $\mu$ moles of TPN or metmyoglobin reduced in unit time, are equal. Two hydrogen equivalents, however, are required in TPN reduction whereas metmyoglobin requires only one. When the reaction rates are limited by low leaf-protein concentration, therefore, TPN was twice as effective as metmyoglobin as a hydrogen acceptor in the chloroplast reaction. This relationship was found with all the active protein preparations used in making this comparison. When the reaction rates were increased by addition of more leaf protein and the photochemical system became saturated, TPN was then 1.3 times as effective (Fig. 4). This ratio varied in different experiments between 1.2 and 1.7 and this variability appeared to be determined, at least in part, by the chloroplasts used in the reaction. Thus a pea-leaf preparation of type I, assayed with spinach chloroplasts, gave a ratio of 1.3, but this was increased to 1.65 when spinach grana were substituted.

*Effect of a phosphate-acceptor system.* When metmyoglobin served as hydrogen acceptor in assay systems containing protein preparations of type I or II from all the plant species examined, the inclusion

of ADP, orthophosphate and  $Mg^{2+}$  ions had no significant effect upon the reduction rate (Fig. 5).

*Effect of TPN.* With purified preparations of types I and II the rate of metmyoglobin reduction was found to be inhibited by the addition of TPN to the reaction mixture (Fig. 5). No inhibition was observed with DPN. Inhibition was further increased when the system containing metmyoglobin and TPN was supplemented by ADP, orthophosphate and  $Mg^{2+}$  ions. This result suggested that TPN was acting as a competitive inhibitor of metmyoglobin reduction. In similar experiments with crude-pea or spinach-leaf preparations (type II before protamine sulphate treatment) the opposite result was obtained, and here the addition of TPN was found to enhance the rate of metmyoglobin reduction. These divergent results were reconciled when it was found that the crude leaf extract contained a thermolabile system capable of reducing metmyoglobin when reduced TPN served as hydrogen donor. If, in the photochemical reaction, reduced TPN was produced concomitantly with the reduction of metmyoglobin, this reduced TPN could, in the presence of the 'metmyoglobin reductase', reduce a further amount of metmyoglobin to give an apparent stimulation of the rate of metmyoglobin

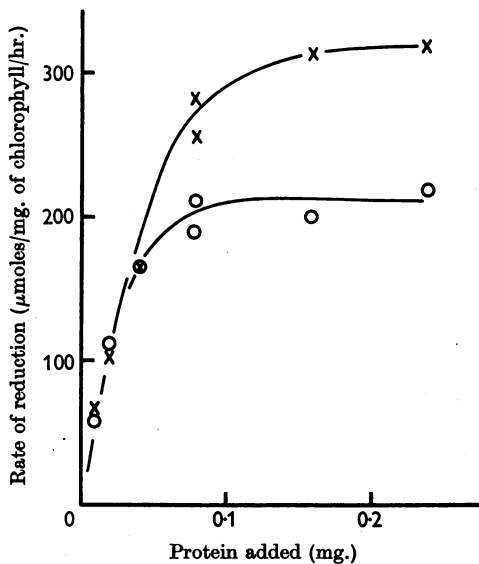


Fig. 4. Comparison of the activities of a pea-leaf-protein preparation (type II after electrophoresis) in catalysing the reduction of TPN and metmyoglobin. Reaction cell contained pea-leaf protein as indicated, spinach chloroplasts (chlorophyll, 0.015 mg.) and (in  $\mu$ moles) phosphate buffer, pH 7.7 ( $K_2HPO_4$ ,  $Na_2H_2PO_4$ ); NaCl, 40 and the following: O, TPN, 0.4;  $MgCl_2$ , 15; ADP, 0.5; x, metmyoglobin, 0.26. Leaf protein was omitted from the blank cells.

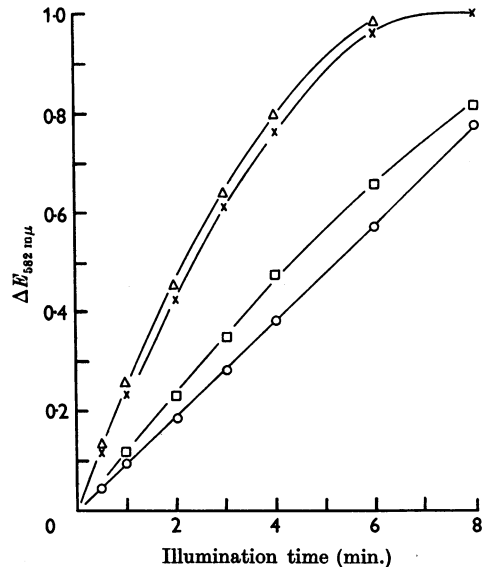


Fig. 5. Effect of TPN upon the time course of metmyoglobin reduction. Reaction mixtures contained (in 3 ml.) pea-leaf protein (type I) 0.12 mg.; spinach chloroplasts (chlorophyll, 0.031 mg.) and (in  $\mu$ moles) metmyoglobin, 0.255; phosphate buffer, pH 7.7 ( $K_2HPO_4$ ,  $Na_2H_2PO_4$ ); NaCl, 40;  $MgCl_2$ , 20; with the following additions: x, none;  $\Delta$ , ADP, 0.5;  $\square$ , TPN, 0.4;  $\circ$ , TPN, 0.4, ADP, 0.5. The blank cell contained neither leaf protein nor the additional ingredients.

reduction. In support of this interpretation was the observation that when the photochemical reduction in this system was stopped by turning off the light the increase in extinction at  $582\text{ m}\mu$  continued for a time in darkness. An attempt was made to use this 'metmyoglobin reductase' to measure the rate of reduction of TPN in the presence of metmyoglobin but it was found that the crude leaf extracts could also oxidize reduced TPN by pathways other than that where metmyoglobin was the hydrogen acceptor (Davenport, 1959*a*). The capacity to oxidize reduced TPN was found to be absent from the purified preparations of type I or II.

#### Absorption spectra

The absorption spectra of preparations of type I have already been described (Davenport & Hill, 1960). The reddish-brown colour of the protein solution is the result of increased absorption in the blue region of the spectrum together with the occurrence of two diffuse absorption bands at  $465$  and  $422\text{ m}\mu$ . In Fig. 6 this spectrum of a preparation from pea leaves is compared with that of a type II preparation, also from pea leaves, which had been further purified by electrophoresis on paper. This was the preparation used in obtaining the data for Fig. 4. In order to facilitate comparison the curves shown in Fig. 6 are 'characteristic absorption spectra' with log extinctions as ordinates. Plotted in this way the shape of the curve is characteristic

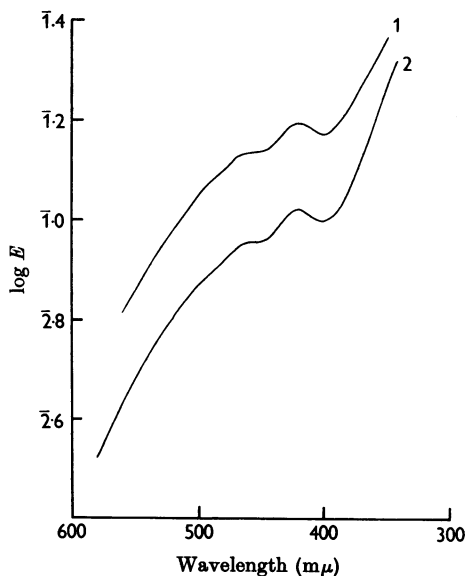


Fig. 6. Characteristic absorption spectra of pea-leaf-protein fractions active in catalysing TPN and metmyoglobin reduction. Curve 1, type II preparation further purified by electrophoresis on paper; curve 2, type I preparation.

of the absorbing substance independently of concentration. The shapes of the curves obtained with protein preparations of types I and II show only minor differences.

Both types of preparation when stored at  $0^\circ$  progressively lost activity towards both TPN and metmyoglobin and this fall in activity was always accompanied by bleaching of the characteristic colour. There appeared to be a direct relationship between colour and activity but insufficient purified material has been available for this to be tested quantitatively.

#### DISCUSSION

It was stressed by Davenport & Hill (1960) that the important property of the 'haem-protein-reducing factor' is its specificity towards illuminated chloroplasts as donors of hydrogen. When metmyoglobin or cytochrome *c* were used as hydrogen acceptors in the photochemical reaction, they were regarded as convenient reagents for demonstrating the high activity of the leaf protein in catalysing hydrogen transport without reference to the nature of natural hydrogen acceptors in the green cell. The finding that TPN is even more effective than metmyoglobin as a hydrogen acceptor adds support to the suggestion (Davenport & Hill, 1960) that the protein 'could act as an important path for hydrogen transport in photosynthesis'. The important role of reduced TPN in carbon fixation is now well established and Arnon *et al.* (1958) go so far as to consider the light reaction in photosynthesis to consist essentially of the conversion of light energy into 'assimilatory power' in the form of reduced TPN and ATP.

From the observations reported above it would appear that, in spite of the very different fractionation procedures, the 'haem-protein-reducing factor' of Davenport & Hill (1960) is a more highly purified form of the 'photosynthetic pyridine nucleotide reductase' of San Pietro & Lang (1958). San Pietro (1958) has reported a further purification of his material from spinach leaves by using a Dowex-bentonite suspension to remove impurities from the protein recovered from the protamine complex. The final specific activity, with reference to TPN reduction, achieved by this step was 33.6 units/mg. of protein. In the present work, with pea leaves, the substitution of electrophoresis on paper for this final purification stage gave preparations having a specific activity up to 90 units/mg. of protein. Moreover, this product is apparently identical, both in activity towards TPN and metmyoglobin and in spectroscopic properties, with the protein obtained by the procedure of Davenport & Hill (1960).

An additional observation in the present work is the effect of a phosphate-acceptor system on the rates of TPN reduction which can be attained by

the use of increasing amounts of the active leaf protein of either type I or II. Rates of reduction up to 270  $\mu$ moles/mg. of chlorophyll/hr. have been obtained by including the complete phosphate-acceptor system. Calculated as the stoichiometric equivalent amount of  $O_2$  this value would correspond to  $Q_{O_2}^{chl}$  of 3000  $\mu$ l. of  $O_2$ /mg. of chlorophyll/hr. This is equal to the maximum rate of photosynthesis for *Chlorella* reported by Hill & Whittingham (1955). In the absence of the phosphate-acceptor system the corresponding value of  $Q_{O_2}^{chl}$  did not exceed 800.

These results are in contrast to the report by Jagendorf (1959) that neither he, nor San Pietro, nor Arnon had been able to observe a stimulation of this kind. A possible explanation of these failures could be the use of an insufficient quantity of the catalytically active protein to raise the reduction rate to a level where the stimulatory effect can be observed (Fig. 2). Occasional failures to obtain the stimulation in the present work were traced to this cause and they usually arose with preparations of low specific activity when it was necessary to add relatively large volumes of the preparation in order to approach saturation of the particulate chloroplast system.

When this stimulation is considered, together with reports of Arnon *et al.* (1958) and San Pietro (1958) that TPN reduction can be coupled to photosynthetic phosphorylation by including the phosphate-acceptor system in the reaction mixture, the pattern of the reaction becomes analogous to that reported for ferricyanide reduction (Arnon *et al.* 1958, Avron *et al.* 1958). Here also the presence of a phosphate-acceptor system strongly stimulates hydrogen transport and concomitantly inorganic phosphate is incorporated into ATP. It therefore seems no longer necessary to postulate (Jagendorf, 1959) that, whereas ferricyanide reduction is fairly tightly coupled to phosphorylation, the coupling in TPN reduction is a loose one.

The capacity of a protein fraction to catalyse the reduction of some haem-protein compounds and TPN, but not DPN, introduces an unusual pattern of specificity if it is assumed that the purified material is a single homogeneous protein. No evidence of heterogeneity was obtained by boundary electrophoresis or in the ultracentrifuge (Davenport & Hill, 1960) and all subsequent attempts to separate the two activities by the more usual methods of protein fractionation have failed. The possibility, however, still remains that the purified material contains two proteins closely similar in their physical properties.

## SUMMARY

1. A purified protein fraction from leaves, previously shown to be active in catalysing the reduction of some haem-protein compounds by illuminated chloroplasts (Davenport & Hill, 1960), will also catalyse the reduction of triphosphopyridine nucleotide (TPN) but not diphosphopyridine nucleotide.

2. Under appropriate conditions the rate of reduction of TPN but not that of metmyoglobin is stimulated up to threefold by including adenosine diphosphate, orthophosphate and magnesium ions in the reaction mixture. Arsenate will replace phosphate in producing this stimulation.

3. The reduction of metmyoglobin is progressively inhibited by TPN alone and by TPN with the phosphate-acceptor system present.

4. The protein obtained by Davenport & Hill (1960) has the properties of a more highly purified form of the photosynthetic pyridine nucleotide reductase of San Pietro & Lang (1958). It was found that the latter preparation is active in catalysing the reduction of metmyoglobin and could be further purified by electrophoresis on paper to give a product indistinguishable from that obtained by Davenport & Hill (1960).

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