# A Protein from Leaves Catalysing the Reduction of Haem-Protein Compounds by Illuminated Chloroplasts

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It was shown previously (Davenport, Hill & Whatley, 1952) that extracts of leaves contain a factor capable of catalysing the reduction of methaemoglobin and metmyoglobin by illuminated chloroplasts. Although this hydrogen-transferring capacity appeared to be analogous to that of ferric oxalate in a similar system (Hill & Scarisbrick, 1940), the naturally occurring substance was shown to be thermolabile, non-diffusible and stable only between the limits pH 5·0–9·0. Attempts at purification by fractional precipitation with ammonium sulphate gave active precipitates at near-saturation with the salt. It was concluded that the methaemoglobin-reducing activity was associated with a protein fraction of the leaf. In the earlier investigation it was found that the preparations of the factor were too unstable to allow a purification sufficient for characterization. It has now been found that if the leaves are held in a frozen state before extraction, the stability of the preparations was considerably improved and a highly purified product could be obtained. More recently (Hill, Northcote & Davenport, 1953) a similar activity was detected in cell-free extracts of the alga *Chlorella*. The present paper is concerned with the further purification of the 'methaemoglobin-reducing factor' and some additional properties are described. A brief account of this work was published previously (Davenport & Hill, 1955).

## EXPERIMENTAL

*Plant materials.* Most of the plant material used in the preparation of leaf-protein fractions and of isolated chloroplasts was grown under ordinary garden conditions near Cambridge. Some species, however, notably *Lamium album*, were collected from their natural habitat as they became seasonally available.

*Metmyoglobin.* Crystalline whale metmyoglobin was prepared from sperm-whale muscle (obtained with the kind co-operation of Dr J. G. Sharpe, Low Temperature Research Station, Cambridge) by a method similar to that of Keilin & Schmidt (1948). The crystals were stored in 3 M-ammonium sulphate solution, and, for use, the ammonium sulphate was removed by dialysis against distilled water. The concentration of metmyoglobin was then estimated as cyan-metmyoglobin. $\epsilon_M^{540}$ was taken as $1{\cdot}11 \times 10^4$ and the stock solution was adjusted to 2 mM.

*Cytochrome* c. Horse-heart cytochrome *c* was prepared by the method of Keilin & Hartree (1945) with the additional purification step using the ion-exchange resin Amberlite IRC 50 (Rohm and Haas Co.) as advocated by Margoliash (1952). In the reduced state $\epsilon_M^{550}$ was assumed to be $2{\cdot}8 \times 10^4$.

*Isolated chloroplasts.* Chloroplasts were prepared from *Pisum sativum*, *Hordeum vulgare*, *Chenopodium bonus-henricus* and *Stellaria media* according to seasonal availability. Leaves were ground in a chilled mortar with about five times their weight of 6% glucose in 0·03 M-phosphate buffer, pH 7·4 (Sörensen, 1912). After coarse filtration through glass wool the larger particles were removed by centrifuging at 500 *g* and 0° for 2 min. The supernatant suspension was then recentrifuged at 2000 *g* for 10 min. to give a pellet consisting essentially of whole chloroplasts. This was rinsed with and resuspended in the cold glucose solution.

*Chlorophyll concentration.* This was estimated by the method of Mackinney (1941).

*Boundary electrophoresis.* For analytical purposes a Perkin–Elmer version of the Tiselius apparatus with a cell of 2 ml. capacity was used. Preparative use was also made of a large Tiselius apparatus of cell capacity 10 ml.

*Paper electrophoresis.* The method was essentially that of Durrum (1950). To minimize pH changes during a run large electrode vessels of capacity 2·5 l. were used. Phosphate buffers (0·066 M; Sörensen, 1912) containing mM-sodium ethylenediaminetetra-acetate proved most satisfactory. Whatman no. 3 papers up to 40 cm. total width could be accommodated. Direct current from a 200 v line was connected to platinum-foil electrodes to give a potential gradient on the paper of 5 v/cm. The apparatus was installed in a cold room at 4° and was enclosed in a glass container to reduce evaporation from the paper. At the end of a run, usually 5 hr., the coloured zones were cut out and the protein was eluted by downward capillary displacement.

*Diffusion coefficient.* For this measurement the Perkin–Elmer Tiselius apparatus was used. The formed boundary was displaced into the right limb of the cell by lowering a glass plunger, operated by clockwork, into the opposite electrode vessel. The schlieren pattern of the boundary was photographed at 1 hr. intervals for 6 hr.

*Sedimentation coefficient.* The observations were made with a Spinco analytical untracentrifuge at the maximum rotor speed of 59 780 rev./min. The schlieren pattern was photographed at intervals of 32 min. during the run.

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*Chloroplast reaction-velocity measurements.* When metmyoglobin is reduced in solutions saturated with air the myoglobin produced is immediately oxygenated to oxymyoglobin. The progress of the reaction was followed spectroscopically by the method previously described (Hill, 1936; Davenport *et al.* 1952). This method was also applied to the measurement of cytochrome *c* reduction, and then reduced and oxidized cytochrome replaced oxymyoglobin and metmyoglobin in the cups of the spectrocolorimeter.

*Protein nitrogen.* This was determined by the Kjeldahl procedure of Chibnall, Rees & Williams (1943).

## RESULTS

### *Purification of the active leaf protein*

The purification procedure to be described has been applied to leaves of *Pisum sativum, Lamium album, Chenopodium bonus-henricus, Aethusa cynapium* and *Stellaria media*. The choice of material was determined not only by the ultimate yield of active material but also by the absence of the tendency, common to plants rich in polyphenol oxidase, towards the production of dark pigments. These pigments may become firmly attached to the various protein fractions. In these respects *Pisum* proved to be the most satisfactory plant and the following method refers specifically to leaves from the garden pea.

Freshly picked leaves (2 kg.) were washed in tap water, drained by manual centrifuging in a muslin bag and rapidly chilled to $-20°$. They were held at this temperature for at least 2 days. When this freezing step was omitted rapid loss of activity occurred during subsequent fractionation. No explanation of this stabilizing effect can yet be suggested.

The frozen leaves were pounded in batches in a large mortar chilled to $-20°$ and accumulated as a frozen leaf powder. To the powder 1700 ml. of saturated ammonium sulphate solution was added with stirring to give a final concentration of the salt around 50% saturation. Stirring was then continued until the preparation thawed ($-3°$). It was then squeezed through two layers of muslin and the fibre discarded. To the press juice a further 1700 ml. of saturated ammonium sulphate solution was then added with stirring and the precipitate was removed by filtration on a Büchner funnel (18·5 c.) with Whatman no. 1 paper and 25 g. of Hyflo Supercel as filter aid. The filter pad was rejected and the filtrate fully saturated with ammonium sulphate by addition of the solid at room temperature. It is of importance at this stage to approach complete saturation as nearly as possible since the small precipitate contains the active material. This precipitate was removed by filtration as before but with 25 g. of Celite analytical-grade filter aid (Johns–Manville). Coarser grades of Celite do not retain the precipitate. The filtrate was rejected and the filter pad eluted with 0·06 M-phosphate, pH 7·0, to give 200 ml. of a brown extract. This extract was fractionated three times with ammonium sulphate. At each stage the precipitate between 80 and 100% saturation was removed by filtration and retained. The final filter pad was extracted with and dialysed against 0·01 M-phosphate, pH 7·0. The contents of the dialysis sac, containing 62 mg. of protein, were freeze-dried and stored over $P_2O_5$, where the activity is retained for at least 1 month.

Samples of the freeze-dried material were subjected to moving-boundary electrophoresis as described. At pH 7·2 ($I$ 0·2) the boundaries of three components could be detected moving towards the anode. Although it was possible to establish that the most mobile component possessed metmyoglobin-reducing activity and that the least mobile component was inactive, the relative mobilities of the three components were too close for complete separation to occur. Since the three protein fractions were all acidic, attention was turned to electrophoresis on paper where the endosmotic flow acting in the opposite direction to electrophoretic migration might enhance the separation. Paper electrophoresis was carried out as described. The freeze-dried preparation was dissolved in a minimum volume of water and streaked on the paper at right angles to the machine direction at the rate of 1 mg./cm. width. At pH 7·2, after the current had passed for 5 hr., complete separation into three coloured zones occurred (Fig. 1).

On the anode side of the point of origin a pink zone ($A$) was clearly demarcated from a less-mobile brown zone ($B$). A yellow zone ($C$) appeared to migrate towards the cathode but by the use of glucose as an uncharged marker this effect was shown to be due to endosmotic flow. The protein nature of the coloured zones and the absence of

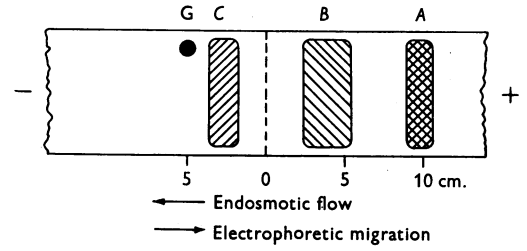


Fig. 1. Diagrammatic representation of the location of coloured protein zones on Whatman no. 3 paper strip after electrophoresis, relative to an uncharged marker (G, glucose). Pea-leaf protein applied at 0 cm. at 1 mg./cm. width; 0·06 M-phosphate, pH 7·2, containing 5 mM-sodium ethylenediaminetetra-acetate; 5 v/cm.; current on 5 hr.

uncoloured protein fractions was confirmed by dyeing a longitudinal strip of the paper in zinc sulphate–acetic acid–bromophenol blue reagent (Jencks, Jetton & Durrum, 1955).

After elution of the coloured zones from the paper it was confirmed that the most mobile component (*A*) was highly active in catalysing the reduction of metmyoglobin in presence of illuminated chloroplasts. Activity was absent from components *B* and *C* but spectroscopic examination of *C* after reduction with $Na_2S_2O_4$ revealed absorption bands at 559 and 530 m$\mu$ indicating the presence of cytochrome $b_3$ (Hill & Scarisbrick, 1951) in this fraction. The protein material from zones *A* and *C* was dialysed against 0·01 M-phosphate, pH 7·0, and accumulated in the freeze-dried state. From 2 kg. of pea leaves 16·6 mg. of protein was recovered in fraction *A*.

*Properties of the purified protein*

*Sedimentation and diffusion measurements.* Fraction *A* protein from pea leaves was examined in a Spinco analytical ultracentrifuge. At the maximum rotor speed of 59 780 rev./min. sedimentation was slow but only one symmetrical boundary could be detected. At pH 7·2 in phosphate buffer (*I* 0·2), $S_{20,w}$ was $1{\cdot}6 \times 10^{-13}$.

The diffusion coefficient was measured on the same sample of protein by the method described above. In phosphate–NaCl (Miller & Golder, 1950) at pH 7·0 (*I* 0·2), $D_{20,w}$ was $7{\cdot}72 \times 10^{-7}$.

From these values, and assuming a partial specific volume of 0·74, the calculated molecular weight ($M_{calc.}$) is 19 000.

*Activity measurements.* It was shown previously (Davenport *et al.* 1952) that the rate of reduction of metmyoglobin in a test system containing isolated chloroplasts, extracts of acetone-dried leaf and metmyoglobin is dependent upon the concentration of chlorophyll and metmyoglobin, the amount of added extract, the pH of the medium and the light intensity. These variables may be standardized but a further variable is the photochemical activity of the chloroplast preparations used. This activity, as measured by the ability of the preparation to reduce 2:6-dichlorophenolindophenol under standard conditions, may show wide variations from one preparation to another. Moreover, chloroplast preparations may lose activity rapidly after extraction (Hill, 1939). It was not therefore possible to devise a completely reproducible test system for the assay of activity of protein fractions isolated from the leaves. A compromise was reached by removing samples at different stages of a preparation and comparing their activity together, with pea chloroplasts where possible. These usually retain their activity unchanged for several hours at 0°.

The test mixture usually contained 0·04 mg. of chlorophyll, 0·4 $\mu$mole of metmyoglobin, the appropriate amount of leaf extract and 0·03 M-phosphate to 2·5 ml. The reaction was followed as described and the reaction rate expressed in terms of chlorophyll. $Q_{O_2}^{chl.}$ is defined as $\mu$l. of oxygen/mg. of chlorophyll/hr., where the volume of oxygen is the calculated stoicheiometric equivalent to the metmyoglobin reduced.

The earlier observations (Davenport *et al.* 1952), with crude leaf extracts, showed that progressive increments in the amount of leaf extract added led to increased reduction rates until the chloroplast system became saturated. Further additions then gave no further increase in reaction rate. Early in the present work it became apparent that purification of the crude extracts resulted in an increase in the $Q_{O_2}^{chl.}$ at saturation. This is illustrated in Fig. 2, where the rate curve for a crude juice expressed from thawed pea leaves is compared with a preparation from the same leaves after fractionation with ammonium sulphate.

The lower saturation rate with the crude material suggested the presence of an inhibitor but no evidence of this could be obtained by addition of the purified material to the crude juice. The saturation level was then that of the purified protein. It was also noticed that some samples of the pure, or partially purified, protein lost activity on storage, particularly in solution. The loss consisted of a progressive flattening of the rate curve until the maximum $Q_{O_2}^{chl.}$ fell to about half the original value. A change of this kind may imply a decrease in the catalytic activity of the protein molecule rather than the complete inactivation of a proportion of the molecules in the solution. No explanation can yet be offered for these fluctuations in maximum

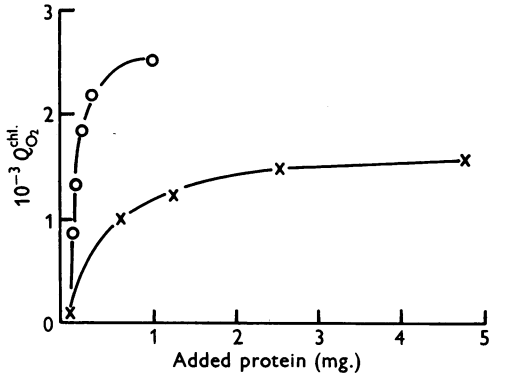


Fig. 2. Rate curves for reduction of metmyoglobin by illuminated pea chloroplasts catalysed by: ×, expressed sap from thawed pea leaves; ○, pea-leaf protein after ammonium sulphate fractionation. Chlorophyll (0·04 mg.) 0·39 $\mu$mole of metmyoglobin; 0·066 M-phosphate buffer, pH 7·4, to 3 ml.

$Q_{O_2}^{chl.}$ but their occurrence made invalid a quantitative assay of recovery of protein, in terms of its activity, at the different steps in the preparation. Nevertheless the activity at saturation of a fresh preparation is of the same order as the most efficient hydrogen acceptors in the Hill reaction yet described. Maximum $Q_{O_2}^{chl.}$ values of 2700 were commonly observed under the assay conditions described.

The efficiency of the plant protein in catalysis is illustrated in Fig. 3. Here the rate curve of reactions with an electrophoretically purified preparation is expressed in terms of m-$\mu$moles of the leaf protein calculated in terms of the mol.wt. of 19 000. When the chloroplast system was 75% saturated, 0·39 $\mu$mole of metmyoglobin underwent reduction in the presence of 3 $\mu$m-moles of leaf protein at a rate corresponding to $Q_{O_2}^{chl.}$ 1550.

*Absorption spectrum.* After paper electrophoresis the active fraction *A* from all the plants examined was pinkish orange in colour. The absorption spectra of preparations from different species resembled each other closely (Fig. 4). In every case absorption in the visible range increased towards the shorter wavelengths with two diffuse absorption bands at 465 and 422 m$\mu$. Denaturation by heating the solution to 60° for 5 min. was accompanied by the disappearance of the two absorption bands to give a clear-yellow solution devoid of metmyoglobin-reducing activity. In a similar way loss of activity occurring spontaneously in stored preparation was always attended by this colour change. It would therefore appear likely that the chromophore, whatever its nature, is not a mere contaminant of the protein.

*Origin of the oxidation–reduction properties.* The high activity of the protein in catalysing hydrogen transport led us to seek evidence for the presence of substances commonly associated with such activity. The method of Crammer (1948) was followed in an attempt to demonstrate flavin. On adjusting a solution containing 5·4 mg. of the electrophoretically purified protein to pH 2·0 the colour was completely discharged. The acidified solution was allowed to stand for 5 min. and the denatured protein removed by centrifuging after addition of solid ammonium sulphate to 70% saturation. The supernatant was extracted with phenol, and, after the addition of water to the separated phenol layer, ether was added to return any flavin present to the aqueous phase. Examination of this phase (1·2 ml. total volume) in ultraviolet light revealed no fluorescence. Crammer (1948) reported that 0·01 $\mu$g. of flavin can be detected by the characteristic green fluorescence. The method is therefore adequately sensitive to detect flavin present in the original material if it occurred as 1 molecule of flavin/molecule of protein (mol.wt. 19 000).

Kearney & Singer (1955), however, were unable to remove flavin from their preparations of succinic dehydrogenase by boiling or cold acid treatment. They had recourse to a preliminary digestion of their material with trypsin and chymotrypsin. This method was followed with the leaf protein but again no fluorescence could be detected in the final aqueous extract. From these observations we con-

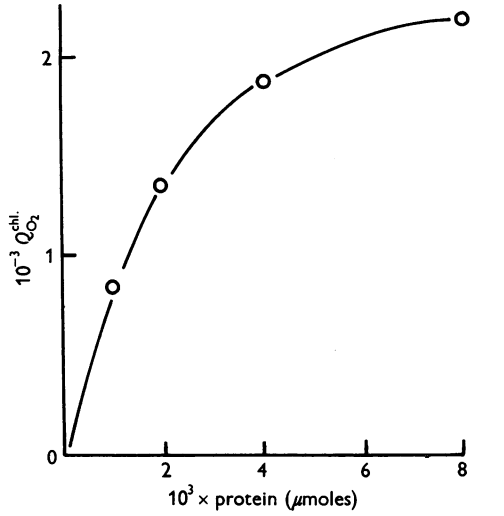


Fig. 3. Rate curve for reduction of metmyoglobin by illuminated pea chloroplasts with an electrophoretically purified preparation of pea-leaf protein as catalyst. Added protein is expressed as $\mu$moles, assuming mol.wt. 19 000; 0·04 mg. of chlorophyll; 0·39 $\mu$mole of metmyoglobin; 0·066 M-phosphate, pH 7·4, to 3 ml. Temp., 15°.

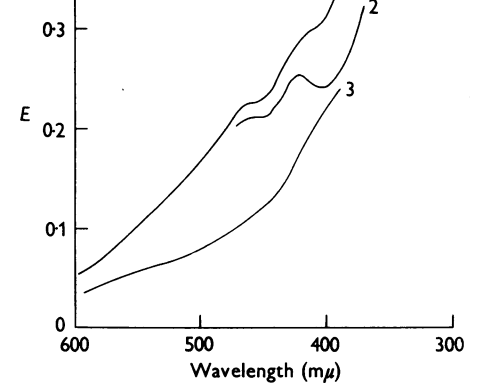


Fig. 4. Absorption spectra of electrophoretically purified leaf protein (fraction *A*). 1, Protein from *Lamium album*; 2, protein from *Pisum sativum*; 3, protein from *L. album* after denaturation at 60° for 5 min.

cluded that either flavin is absent from the protein or it occurs in a tightly bound form.

Attempts were also made to demonstrate the presence of haematin. Impure preparations may contain cytochrome $b_3$, but after electrophoresis no haem could be detected, as pyridine haemochromogen, in any of the active preparations examined. In a more critical observation 7 mg. of the freeze-dried purified protein in a 1 cm. optical cell was dissolved in 0·5 ml. of 30% pyridine in 0·1N-sodium hydroxide. After the addition of a little sodium dithionite the solution was examined with a small-dispersion spectroscope but no haemochromogen bands could be detected. The method will readily detect 0·25 $\mu$mole of haem, and since 7 mg. of the protein corresponds to 0·37 $\mu$mole it was concluded that haem could not account for the oxidation–reduction properties of the protein.

As we have reported above, the colour of an active preparation is altered by denaturation and is discharged at pH 2·0. If the acid solution is adjusted to pH 10·0 colour reappears and the solution is then bright yellow. This reaction points to a flavonoid pigment as a source of the colour in the active material but further work is necessary to establish its nature.

*Inhibitors of metmyoglobin reduction*

It was shown in the earlier work (Davenport *et al.* 1952) that inhibitors of the type of 1:10-phenanthroline and urethane, effective in the Hill reaction with artificial hydrogen acceptors, also inhibit the chloroplast–leaf protein–metmyoglobin reaction. In an attempt to find an inhibitor specific to the leaf-protein part of this reaction we examined the effect of *p*-chloromercuribenzoate and phenylmercuric acetate.

The effect of phenylmercuric acetate on metmyoglobin reduction with a partially purified leaf protein is shown in Fig. 5. Incubation of the protein for 5 min. with 75 $\mu$M-phenylmercuric acetate resulted in a reduction in the reaction rate to 20%. Activity was restored to 70% of the uninhibited rate by a further incubation period of 5 min. in the presence of 750 $\mu$M-cysteine. Similar results were obtained with *p*-chloromercuribenzoate. Although these results suggested that the action of the mercurial compounds is on thiol grouping in the protein, the integrity of these being essential for activity, this interpretation must be accepted with caution since more prolonged incubation of the protein with either phenylmercuric acetate or *p*-chloromercuribenzoate was found to lead to irreversible inactivation.

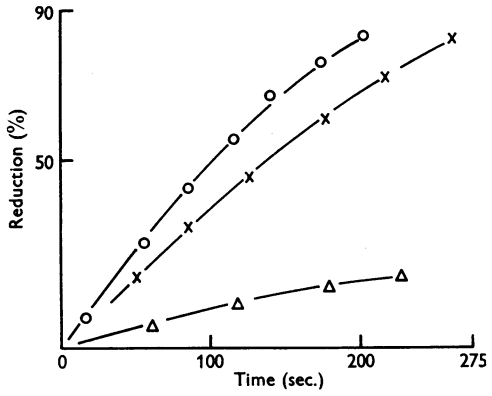


Fig. 5. Reduction of metmyoglobin by illuminated pea chloroplasts in the presence of partially purified pea-leaf protein, showing inhibitory effect of phenylmercuric acetate. ○, No inhibitor; △, 75 $\mu$M-phenylmercuric acetate for 5 min. before illumination; ×, 75 $\mu$M-phenylmercuric acetate for 5 min. followed by 740 $\mu$M-cysteine for a further 5 min. before illumination.

Although we knew of no reports that organic mercurial compounds are effective in inhibiting the Hill reaction it was necessary to confirm this and also to show that the inhibitory effect was not due to a reaction with the metmyoglobin component of our system. Two series of control experiments were therefore carried out. In the first it was found that neither phenylmercuric acetate nor *p*-chloromercuribenzoate affected the rate at which 2:6-dichlorophenolindophenol was reduced by chloroplasts in the light. In the second series ferric oxalate replaced the leaf protein as catalyst of the reduction of metmyoglobin by the illuminated chloroplasts (Hill & Scarisbrick, 1940). Here again neither phenylmercuric acetate nor *p*-chloromercuribenzoate affected the reaction velocity. The effect of the mercurial compounds appeared therefore to be specific for the leaf-protein part of our reaction system.

*Specificity of the hydrogen-transferring activity*

In this and the earlier work metmyoglobin has been used as a convenient indicator of the activity of leaf-protein fractions without reference to other possible hydrogen acceptors, either natural or artificial. We have now examined the reactivity of the 'methaemoglobin-reducing factor' towards a number of other hydrogen acceptors including reagents widely used as oxidants in the Hill reaction.

*Cytochrome* c. Holt (1950) found that cytochrome *c* was reduced by chloroplasts in the light and that reoxidation, mediated by cytochrome oxidase in the chloroplast preparation, occurred in the dark. We have confirmed this observation and have found also that fractions from leaves, active in catalysing the reduction of metmyoglobin, greatly stimulate the rate of reduction of cytochrome *c*. Fig. 6 illustrates a typical experiment with pea

chloroplasts. In the absence of added leaf protein reduction proceeded slowly and was half complete in 200 sec. Further illumination carried the degree of reduction to 75% in 500 sec. In the presence of a crude preparation of the leaf protein reduction was half complete in 50 sec. and continued apparently to completion. Fig. 7 indicates the presence of an active cytochrome oxidase in the pea chloroplasts used. With added leaf protein reduction proceeded almost to completion but in darkness reoxidation occurred. With the comparatively stable chloroplasts from peas this cycle of alternate reduction and oxidation could be repeated many times. The dark oxidation was shown to be inhibited by cyanide. Reduction in light was stimulated by this reagent, showing that the oxidase also operated in the light. In the absence of cyanide the rate of reduction of cytochrome *c* by illuminated pea chloroplasts thus represents a net balance between the forward and backward reactions.

To determine the efficiency of the purified leaf protein in promoting the reduction of cytochrome *c* it was desirable to use a chloroplast preparation substantially free from cytochrome-oxidase activity. Carefully fractionated chloroplasts from leaves of spinach beet were found to meet this requirement. In a typical experiment, illustrated in Fig. 8, reduction without added leaf protein is slow ($Q_{O_2}^{chl.}$ 150). The addition of 2 $\mu$m-moles of a purified pea-leaf protein (fraction *A* after electrophoresis) greatly stimulated the reaction rate to the very high $Q_{O_2}^{chl.}$ value of 3800. This, so far as we are aware, is the highest reaction rate yet reported for a chloroplast reaction. No significant oxidation of the reduced cytochrome took place after 200 sec. darkness, indicating the relative freedom of this preparation of chloroplasts from cytochrome oxidase.

*Cytochrome* $b_3$. The auto-oxidizable cytochrome component $b_3$ was detected by Hill & Scarisbrick (1951) in both the aerial and underground organs of higher plants and it was extracted by them from leaves and partially purified. In the present work a small quantity of the cytochrome was accumulated as a by-product of the electrophoresis separation of the active leaf protein. Freeze-dried preparations of fraction *C* from the paper were dissolved in a little water. The amount available was too small for the setting up of comparison standards of the oxidized and reduced cytochromes in the cups of the spectrocolorimeter. Solutions of globin haemochromogen and globin parahaematin were therefore

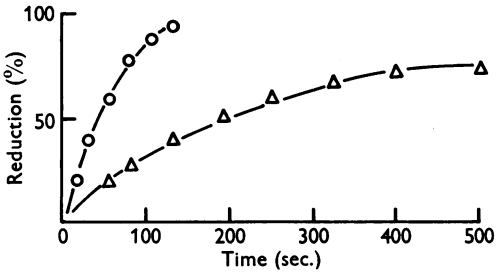


Fig. 6. Reduction of cytochrome *c* by illuminated pea chloroplasts, showing effect of adding partially purified pea-leaf protein. △, No added protein; ○, 0·2 mg. of protein added before illumination. Chlorophyll (0·05 mg.); 0·05 $\mu$mole of cytochrome *c*; 0·06 M-phosphate, pH 7·2, to 3 ml. total vol.

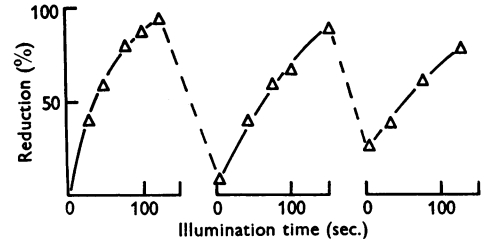


Fig. 7. Reduction of cytochrome *c* by illuminated pea chloroplasts and reoxidation in darkness. Components of the system were as in Fig. 6, with leaf protein added. Each broken portion of the graph represents 15 min. darkness.

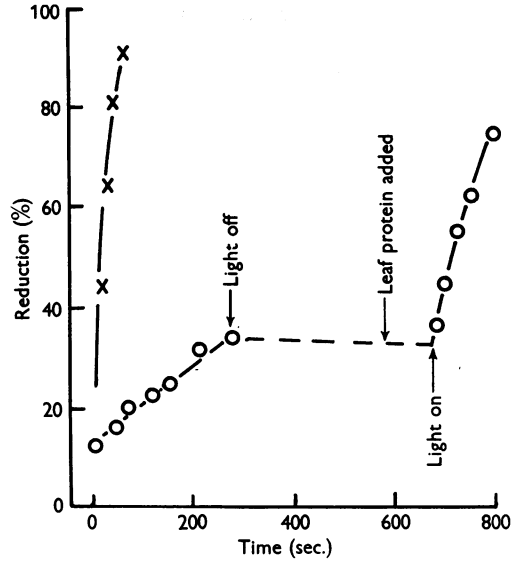


Fig. 8. Reduction of cytochrome *c* by illuminated spinach-beet chloroplasts showing the effect of adding electrophoretically purified (fraction *A*) protein from pea leaves. ×, 2 $\mu$m-moles of added protein; ○, no protein added at start of illumination, 2 $\mu$m-moles was added at time indicated. Chlorophyll (0·02 mg.); $3{\cdot}05 \times 10^{-2}$ $\mu$moles of cytochrome *c*; 0·05 M-tris buffer, pH 7·4, to total vol. 3 ml. Temp., 17°.

used instead. It was found that the spectrum of the haemochromogen was sufficiently similar to that of the reduced cytochrome for valid measurements to be made.

When cytochrome $b_3$ was illuminated in the presence of washed pea chloroplasts no bands of the reduced cytochrome could be detected in 1 min. The addition of 0·1 ml. of an active fraction of the leaf protein resulted in the immediate appearance of the $\alpha$ and $\beta$ bands of the reduced cytochrome at 559 m$\mu$ and 530 m$\mu$ respectively. The reaction proceeded in 20 sec. to 70% completion. In the dark, reoxidation occurred in less than 10 sec. and the cycle of reduction and reoxidation was repeated ten times in a 30 min. period. It was concluded that the 'methaemoglobin-reducing factor' is highly active in catalysing the reduction of cytochrome $b_3$ by illuminated chloroplasts.

*Heart-muscle preparation.* The efficiency of the 'methaemoglobin-reducing factor' in hydrogen transport between the insoluble chloroplast and soluble haem–protein compounds led us to investigate the possibility that similar hydrogen transport could occur between two insoluble systems. A suitable particulate preparation to act as hydrogen acceptor proved to be the standardized heart-muscle preparation of Keilin & Hartree (1947). A preparation of this type (kindly presented to us by Dr E. F. Hartree) represents physically disrupted mitochondria still retaining an active succinoxidase system.

The spectroscopic observations were carried out in tubes of effective light path 1·8 cm. and containing 2 ml. of the heart-muscle preparation and washed pea chloroplasts (0·05 mg. of chlorophyll), made up to 6 ml. with 0·05 M-phosphate, pH 7·5. No reduction of cytochrome could be detected either in the presence or the absence of added leaf protein. This result was expected since the highly active cytochrome oxidase in the preparation would mask a low rate of cytochrome reduction. The cytochrome oxidase was therefore partially inhibited by cyanide at a concentration of 0·16 mM. In the presence of cyanide after 20 sec. illumination of a tube with added leaf protein, the bands of cytochrome *a*, *b* and *c* could be readily observed; cytochrome *b* was reduced to a much smaller extent than cytochrome *c*. In the absence of the active leaf protein no reduction occurred. In darkness reduced cytochrome was reoxidized in less than 10 sec. The 'methaemoglobin-reducing factor' will thus catalyse hydrogen transport between the insoluble chloroplasts and a particulate preparation containing the respiratory cytochrome system.

*Other hydrogen acceptors.* The available information about the specificity of the 'methaemoglobin-reducing factor' towards a variety of hydrogen acceptors is shown in Table 1. All the active acceptors are haem–protein compounds.

*Specificity towards the hydrogen donor.* In all the observations reported above the reducing power of illuminated chloroplasts supplied the necessary potential for active hydrogen transport catalysed by the 'methaemoglobin-reducing factor'. The reactivity of the protein suggests an analogy with the coenzyme-linked cytochrome *c* reductases. We therefore attempted to replace the illuminated chloroplast by reduced coenzymes I and II.

Coenzyme I was reduced by a lactic dehydrogenase preparation from heart muscle (kindly provided by Dr V. Massey). The activity of the enzyme was confirmed by following the appearance of the band at 340 m$\mu$ in the Beckman DU spectrophotometer with a test system containing 3 $\mu$moles of coenzyme I, 75 $\mu$moles of sodium lactate, 0·02 ml. of the enzyme and 0·06 M-phosphate, pH 8·0, to 3 ml. The enzyme was added to start the reaction.

In a similar system, where 0·4 $\mu$mole of metmyoglobin and 10 $\mu$m-moles of active leaf protein replaced part of the phosphate buffer, no reduction of the metmyoglobin occurred. A similar negative result was also obtained with cytochrome *c*. At the end of the observation washed pea chloroplasts were added and the reaction cell was illuminated. Rapid reduction was observed in both haem–proteins.

A parallel experiment was set up with reduced coenzyme II. Here the coenzyme was reduced by purified *iso*citric dehydrogenase from baker's yeast (the gift of Miss J. Moyle). The system contained 0·2 ml. of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) buffer (0·5 M; pH 7·4), 0·1 ml. of $MnCl_2$ (2 mM), 0·2 ml. of coenzyme II (0·125 mM), 0·1 ml. of *iso*citrate (0·01 M) and water to 3 ml. Increments in density at 340 m$\mu$ were observed. Here also the addition of metmyoglobin or cytochrome *c* to the system, either in the presence or the absence of active leaf protein, did not lead to reduction of the haem–protein. Chloroplasts subsequently added

Table 1. *Specificity of the 'methaemoglobin-reducing factor' towards various hydrogen acceptors in the Hill reaction*

| Rate of reduction by an illuminated chloroplast system stimulated by added leaf protein | Rate of reduction by an illuminated chloroplast system not stimulated by added leaf protein |
|---|---|
| Methaemoglobin | Ferricyanide |
| Metmyoglobin | Ferric oxalate + ferricyanide |
| Cytochrome *c* (in solution) | 2:6-Dichlorophenolindophenol |
| Cytochrome $b_3$ (in solution) | Methylene blue |
| Washed heart-muscle prep. (in the presence of $CN^-$): | |
| Cytochrome *a* | |
| Cytochrome *b* | |
| Cytochrome *c* | |

led to a rapid reduction on illumination. The 'methaemoglobin-reducing factor' is thus not able to accept hydrogen from reduced coenzyme in the manner of a coenzyme-linked reductase.

*Location of the 'methaemoglobin-reducing factor' in the cell*

The ease with which 'methaemoglobin-reducing' activity passes into solution when leaves are disrupted would suggest that the activity is not associated with a particle in the cell. However, cytochrome *c* (Hill & Scarisbrick, 1951), presumably arising from leaf mitochondria, and cytochrome $b_3$, stated by Martin & Morton (1957) to be microsomal in origin, may appear in solution under conditions suitable for the extraction of the active leaf protein. A particulate origin for the active protein cannot therefore be excluded. The observation that activity is confined to green tissues (Davenport *et al.* 1952) and the great reactivity of the protein towards chloroplasts led us to look at the possibility that extracts of isolated chloroplasts might possess some activity.

Leaves of *Lamium album* (130 g.) were ground in 500 ml. of 0·5 M-glucose containing 0·03 M-phosphate, pH 7·4. The chloroplasts were obtained by centrifuging and were washed once in the glucose medium and converted into an acetone-dried powder. The powder was extracted with water, filtered and the filtrate diluted to a volume such that 1 ml. was equivalent to 1 g. of leaf, based on the original chlorophyll content. A similar extract was prepared from an acetone-dried powder of *Lamium* leaves and adjusted to the same equivalent concentration. With pea chloroplasts and metmyoglobin, it was found that the reaction rate at saturation with respect to added extract was the same with both preparations ($Q_{O_2}^{chl.}$ 1540), but the volume required to reach saturation was three times as great with the chloroplast extract as with the leaf extract. It was clear from this result that chloroplasts contain a 'methaemoglobin-reducing' activity equal to 30 % of that of the whole leaf. It is not yet possible to decide whether in the intact leaf the chloroplasts contain the whole of the active material.

## DISCUSSION

The observations reported in this paper show that the activity of leaf extracts in promoting the photochemical reduction of a variety of haem–protein compounds by illuminated chloroplasts can be accounted for in terms of a protein of small molecular weight. Purification of the active material was greatly facilitated by the finding that the bulk of soluble leaf protein in all the species examined was precipitated at 50 % saturation with ammonium sulphate. The albumin fraction, precipitated at over 50 % saturation, was invariably small and since this fraction contained all the haem–protein-reducing activity a considerable measure of purification could be achieved by ammonium sulphate fractionation alone.

The high activity of the purified protein in catalysis is most clearly seen when metmyoglobin is used as hydrogen acceptor. This haem–protein was used here as a convenient reagent, which is not reduced by chloroplasts in the absence of added catalyst, but without reference to the nature of possible hydrogen acceptors *in vivo*. On the other hand, it has been known for some time (Hill & Scarisbrick, 1951) that cytochrome *c* is a constituent of green leaves and that it will serve as hydrogen acceptor in the Hill reaction (Holt, 1950). More recently Jagendorf (1956) has measured the rate of reduction of the cytochrome by chloroplasts and has obtained rates corresponding to $Q_{O_2}^{chl.}$ of 314 and 1370. Under our conditions it was shown that addition of catalytic amounts of the purified leaf protein could bring about a stimulation of the reduction rate by as much as 25-fold.

In the catalysed reduction of metmyoglobin and the other haem–proteins by the illuminated particulate system containing chlorophyll it would be natural to assume that the factor undergoes an oxidation–reduction cycle. On this assumption the factor is acting as a hydrogen acceptor for the chloroplast preparation. From the data presented here it can be concluded that both the rate of reduction and the affinity of the factor for the hydrogen-donating 'end' of the chloroplast particulate material are quite large. This would appear to place the factor in a class apart from any other soluble substance so far found in leaf-tissue extracts. In short it would be concluded that the factor could act in an important path for hydrogen transfer in photosynthesis.

When we consider the substances shown to accept hydrogen (or to be reduced) in the presence of the factor, the relation this process might have to photosynthesis is far from clear. The reduction of cytochrome components would be in general even less favourable from the energetic point of view than the reduction of metmyoglobin. Further, the factor will not act as a cytochrome reductase, nor will it catalyse the reduction of dyes in the so-called Hill reaction. The important property is the specific reaction with the chloroplast material.

In the early experiments (Hill, 1939; Davenport, 1949) with extracts of acetone-extracted leaf powder, oxygen production could be observed to take place rapidly in the presence of chloroplasts in the light. The factor itself would have been present in too small a quantity to give a measurable amount of oxygen under these conditions. These earlier experiments, together with the present work, lead

to the conclusion that the factor can catalyse the reduction of soluble material in the leaf extracts. Further work is necessary to trace the paths of hydrogen transport after the reduction of the factor by the illuminated chloroplast and to determine the nature of the group concerned in the oxidation–reduction cycle.

## SUMMARY

1. A protein factor from leaves, previously shown to be active in catalysing the reduction of methaemoglobin and metmyoglobin by illuminated chloroplasts, has been purified by fractionation with ammonium sulphate followed by electrophoretic separation of the active material on paper.

2. The purified protein was found to give a single symmetrical boundary in the ultracentrifuge and in the Tiselius electrophoresis apparatus.

3. The molecular weight of the protein, calculated from sedimentation and diffusion measurements, is 19 000.

4. The purified protein is highly active in catalysing the photochemical reduction of metmyoglobin and also stimulates the reduction of cytochrome *c*, cytochrome $b_3$ and the cytochrome components present in a particulate heart-muscle preparation.

5. The photochemical reduction is inhibited by organic mercury compounds and this inhibition is partially reversed by cysteine.

6. The protein does not catalyse the reduction of haem–proteins when coenzymes served as hydrogen donor. Illuminated chloroplasts were the only effective hydrogen-donating system observed.

7. The chemical nature of the group conferring oxidation–reduction properties to the protein has not yet been determined but neither flavin nor haem could be detected.

8. Metmyoglobin-reducing activity has been observed in extracts of chloroplasts and was shown to represent one-third of the activity extractable from the whole leaf.

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# The Isolation and Properties of a Proteolytic Enzyme, Cathepsin D, from Bovine Spleen

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Interest in the fate of protein antigens in an antibody-forming organ has led us to investigate the proteolytic enzymes of the spleen. The intracellular proteases (cathepsins) have been studied in many animal tissues, though none has been fully characterized (see Smith, 1951). Those of the spleen were studied by Anson (1939), using his haemoglobin assay (Anson, 1938) to follow the activity, and an extensive series of papers by Bergmann, Fruton and co-workers (summarized by Fruton, 1957–58) describe many properties of cathepsins A, B and C which have been obtained from both spleen and

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