Phytochemistry, 1969, Vol. 8, pp. 1849 to 1857. Pergamon Press. Printed in England.

# THE EFFECT OF ULTRASONIC AND HEAT TREATMENT ON SOME CHLOROPLAST REACTIONS

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(*Received* 7 *March* 1969)

**Abstract**—Ultrasonication of isolated *Pisum sativum* chloroplasts removed ferredoxin, ferredoxin–$NADP^+$ reductase and plastocyanin. Photoreduction of $NADP^+$, with the ascorbate-DCIP couple as electron donor, was restored on the addition of these three proteins. When oxygen replaced $NADP^+$ as the ultimate electron acceptor in this reaction, plastocyanin alone was required. The requirement for plastocyanin in ultrasonically treated chloroplasts appeared to be inversely related to the requirement for DCIP in mediating ascorbate oxidation. Heating fresh chloroplasts to 55° made the addition of DCIP and plastocyanin unnecessary in this reaction. This need for plastocyanin was induced by ultrasonic treatment of these heated chloroplasts. The results are interpreted as further evidence for the role of plastocyanin in photochemical electron transport.

## INTRODUCTION

THE HILL reaction, whereby illuminated chloroplasts utilize electrons derived from water to to bring about the reduction of $NADP^+$, is now generally considered to represent, at a subcellular level, the conservation of reducing potential required for higher plant photosynthesis. In this reaction the electronegative electron carrier ferredoxin reduced photochemically in the presence of chloroplasts requires the flavoprotein, ferredoxin–$NADP^+$ reductase (EC 1.6.99.4) for the transfer of electrons to $NADP^+$.[1] By appropriate treatments chloroplasts can be depleted of these two proteins, and although active in Hill reactions with artificial oxidants, are incapable of reducing $NADP^+$ until both extracted proteins are restored.[2]

This experimental approach, the depletion and restoration of soluble protein, accompanied by loss and recovery of photochemical activity, has been less successful in resolving the catalytic activities of other known redox proteins of the chloroplast, which are more or less rigidly bound in the lamellae. An exception is the copper protein plastocyanin which has been shown in a reconstituted system to be involved in the electron transport processes leading to the reduction of $NADP^+$.[3–5] The observations reported here provide some additional evidence for this catalytic activity and they extend an earlier brief report.[6]

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[1] K. TAGAWA and D. I. ARNON, *Nature* **195**, 537 (1962).
[2] H. E. DAVENPORT, *Nature* **199**, 151 (1963).
[3] S. KATOH and A. TAKAMIYA, *Biochem. Biophys. Acta* **99**, 156 (1965).
[4] J. S. C. WESSELS, *Biochem. Biophys. Acta* **109**, 614 (1965).
[5] S. KATOH and A. SAN PIETRO, in *Biochemistry of Copper* (edited by J. PEISACH, P. AISEN and W. E. BLUMBERG), Academic Press, New York (1966).
[6] H. E. DAVENPORT, in *Non-heme Iron Proteins, Role in Energy Conversion* (edited by A. SAN PIETRO), Antioch Press, Yellow Springs, Ohio (1965).

## RESULTS

### (*i*) *Ultrasonic Treatment and NADP Reduction*

In a previous study,[2] chloroplasts of the garden pea (*Pisum sativum*) were selected as favourable material from which ferredoxin and ferredoxin–$NADP^+$ reductase could be extracted by repeated washing in a hypotonic medium. Fractionation of protein in the washings from a more extended treatment produced no evidence that appreciable plastocyanin had been extracted; neither was there any indication that the resulting chloroplast fragments supplemented by ferredoxin and ferredoxin $NADP^+$ reductase, required added plastocyanin as an essential factor in restoring $NADP^+$ photoreduction with water as the electron donor.

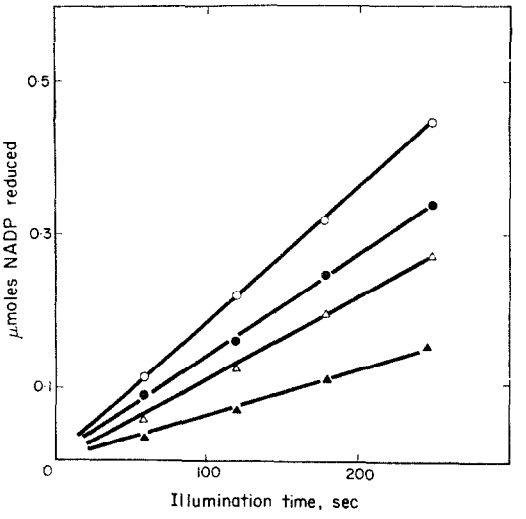


FIG. 1. INHIBITING EFFECT OF PLASTOCYANIN ON $NADP^+$ REDUCTION BY 4× WASHED *Pisum* CHLOROPLASTS.

Reaction mixtures of 3 ml contained (in μmoles) Tris-HCl buffer, pH 8, 150; $NADP^+$ 0·5; chloroplasts containing 0·045 mg chlorophyll, plus 10 mμ mole plastocyanin and saturating amounts of ferredoxin and ferredoxin $NADP^+$ reductase, as indicated. ○ Ferredoxin; ferredoxin $NADP^+$ reductase. ● Ferredoxin; ferredoxin $NADP^+$ reductase; plastocyanin. △ Ferredoxin; ▲ Ferredoxin; plastocyanin. $NADP^+$ reduction was followed as an increase in OD at 340 mμ, after illumination in saturating light at 20°.

Indeed, in assays of this kind the presence of added plastocyanin was found to be markedly inhibitory both in the complete system and where no soluble ferredoxin–$NADP^+$ reductase was added (Fig. 1).

Since hypotonic extraction had proved ineffective in releasing bound plastocyanin, attention was turned to ultrasonic treatment. With *Pisum* chloroplasts fragmented by ultrasonic treatment, the rate of the Hill reaction with $NADP^+$ as the oxidant diminished rapidly with increasing treatment time, even though supplemented with ferredoxin, ferredoxin–$NADP^+$ reductase and plastocyanin (Fig. 2). When water as the electron donor was inhibited by *p*-chlorophenyl-1,1-dimethylurea (C.M.U.) and replaced by the ascorbate-2,6-dichlorophenol indophenol (DCIP) couple,[7] ultrasonic treatment for up to 10 min was without effect on the rate of reaction when assayed in the presence of the three added proteins (Fig. 2).

[7] L. P. VERNON and W. ZAUGG, *J. Biol. Chem.* **235**, 2728 (1960).

That plastocyanin is functional in maintaining electron transport in the latter reaction is shown in Fig. 3 where addition of the copper protein stimulated the reduction rate considerably over that produced by ferredoxin supplemented with the ferredoxin $NADP^+$ reductase. It also shows unequivocally that under these conditions the three chloroplast proteins are required together for the restoration of $NADP^+$ photoreduction. It was noted however, that ultrasonic treatment for periods up to 30 min followed by washing in the centrifuge to eliminate solubilized protein, did not completely abolish $NADP^+$ reduction in the absence of added plastocyanin. This is illustrated in Fig. 4, where the rate of $NADP^+$ reduction in the

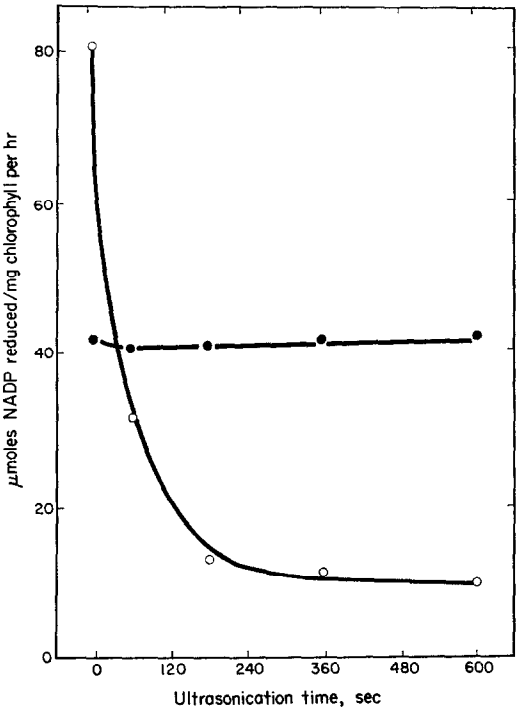


FIG. 2. TIME COURSE OF ULTRASONICATION AND $NADP^+$ REDUCTION.

Reaction mixture of 3 ml contained (in μmoles) Tris-HCl buffer, pH 8·0, 150; $NADP^+$ 0·5; saturating ferredoxin and ferredoxin $NADP^+$ reductase and 10mμmoles plastocyanin. Illumination time: 2 min. ○ No additions. ● C.M.U. 0·15; Ascorbate 20; DCIP 0·5. Reaction conditions as Fig. 1.

absence of added plastocyanin was a third of that observed with saturating amounts of the protein. This observation made desirable an estimate of the effectiveness of ultrasonic treatment in bringing bound plastocyanin into solution. The supernatant fluid obtained by ultracentrifugation of chloroplasts treated ultrasonically for 30 min was fractionated with ammonium sulphate, and the fraction precipitated between 66 and 100 per cent saturation was redissolved, oxidized by the addition of sufficient potassium ferricyanide, and passed through a column of Sephadex G75. The separated blue plastocyanin zone was collected, and by using the molar extinction coefficient of Katoh *et al.*[8] the molar ratio of plastocyanin to chlorophyll was established and found to be consistently near 1:300. This ratio, similar to that reported

[8] S. KATOH, S. IKUKO, I. SHIRATORI and A. TAKAMIYA, *Arch. Biochem. Biophys.* **94**, 136 (1961).

by Katoh *et al.*[8] for spinach chloroplasts, suggested that extraction of the copper protein was substantially complete.

Application of the spectroscopic methods described by Hill and Scarisbrick[9] to the residual pellet of chloroplast fragments obtained by ultracentrifugation after ultrasonic treatment, provided no evidence that the treatment had altered the concentration of the bound cytochromes $b_6$ and f (E.C. cyt $c_6$), relative to total chlorophyll, and compared with untreated chloroplasts. It was not unexpected, therefore, that attempts to detect a catalytic role for added purified cytochrome f[10] supplementing or replacing plastocyanin in experiments where the latter protein proved to be active, were uniformly unsuccessful.

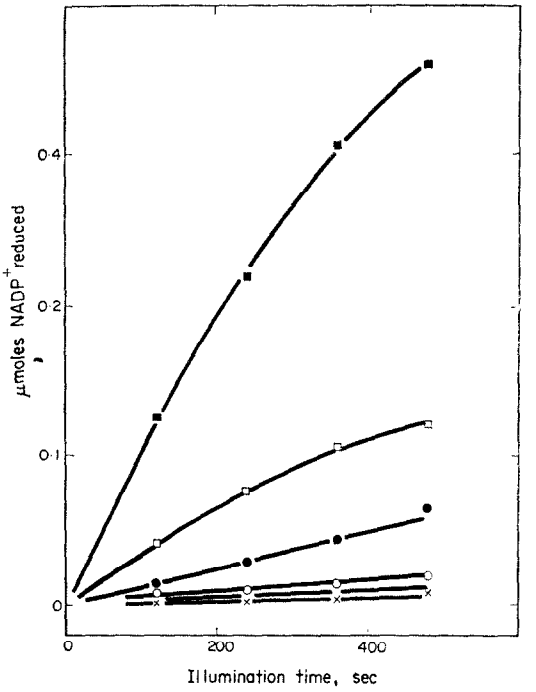


FIG. 3. REDUCTION OF NADP+ BY ULTRASONICATED CHLOROPLASTS: ADDITION OF PROTEIN COFACTORS. Reaction mixtures of 3 ml contained (in µmoles) Tris-HCl buffer, pH 8·0, 150; NADP+ 0·5; Ascorbate 20; DCIP 0·5; C.M.U. 0·15: chloroplasts containing 0·065 mg chlorophyll. Sonication time: 10 min. Addition of protein cofactors in concentrations of Fig. 1. ■ Ferredoxin; ferredoxin NADP+ reductase; plastocyanin. □ Ferredoxin; ferredoxin NADP+ reductase. ● Ferredoxin; plastocyanin. ○ Ferredoxin NADP+ reductase; plastocyanin. × Plastocyanin, × ferredoxin. Reaction conditions as Fig. 1.

### *(ii) DCIP and Plastocyanin as Mediators of Ascorbate Photoxidation*

Vernon and Zaugg[7] observed that with chloroplast fragments the rate of NADP+ reduction with ascorbate as electron donor was markedly stimulated by DCIP. With chloroplast fragments not subjected to ultrasonic treatment, this requirement for a mediator between ascorbate and the particulate chloroplast material was confirmed, and no evidence that plastocyanin could be substituted for DCIP was obtained. Catalytic amounts of DCIP were therefore routinely included in the earlier assay mixtures. It was then observed that ultra-

[9] R. HILL and R. SCARISBRICK, *New Phytol.* **50**, 98 (1951).
[10] H. E. DAVENPORT and R. HILL, *Proc. R. Soc. B.* **139**, 327 (1952).

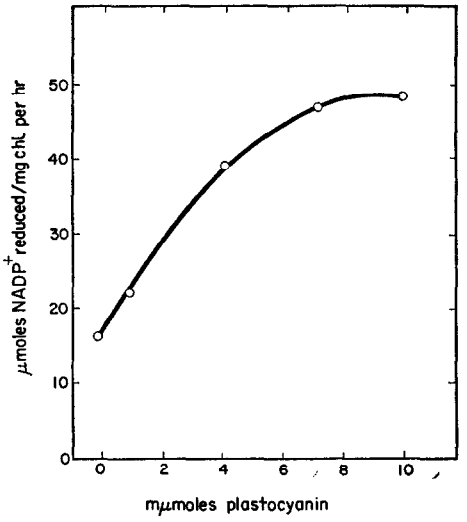


FIG. 4. ABILITY OF PLASTOCYANIN TO RESTORE $NADP^+$ REDUCING ACTIVITY OF ULTRASONICATED CHLOROPLASTS IN PRESENCE OF SATURATING AMOUNTS OF FERREDOXIN AND FERREDOXIN $NADP^+$ REDUCTASE.

Reaction mixtures and conditions as Fig. 3; each tube contained 0·053 mg chlorophyll. Sonication time: 30 min.

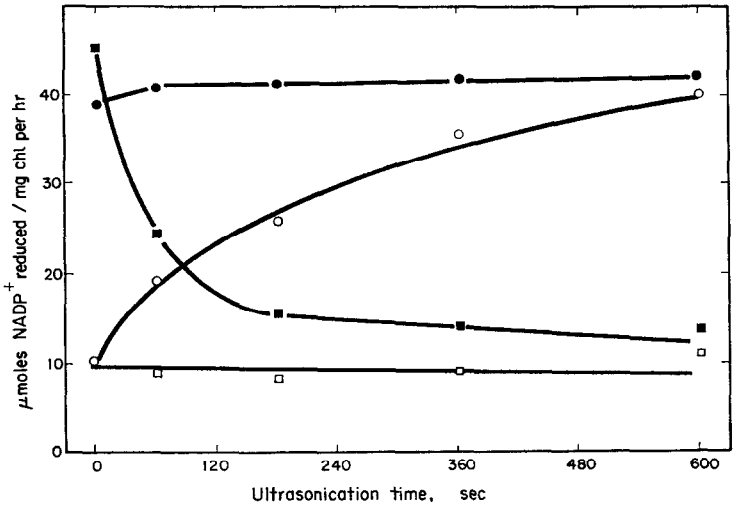


FIG. 5. TIME COURSE OF ULTRASONICATION AND $NADP^+$ REDUCTION: A REQUIREMENT FOR PLASTOCYANIN AND DCIP.

Reaction mixtures of 3 ml contained (in μmoles) Tris-HCl buffer, pH 8·0, 150; NADP 0·5; CMU 0·015; chloroplasts containing 0·067 mg chlorophyll. Saturating ferredoxin and ferredoxin $NADP^+$ reductase. Illumination time: 2 min. Reaction conditions and further additions in concentrations of Fig. 2. ● Ascorbate; DCIP; plastocyanin. ■ Ascorbate; DCIP. ○ Ascorbate; plastocyanin. □ Ascorbate.

sonic treatment for increasing time led to a progressive decrease in the effectiveness of DCIP as mediator until, after treatment for 10 min, the ascorbate DCIP couple was no more effective than ascorbate alone (Fig. 5). In parallel with this change, and following a similar time course, a progressive increase occurred in the activity of plastocyanin as an alternative mediator of the reaction. Figure 5 also illustrates that during this transition from DCIP to plastocyanin, their catalytic activities appeared to be additive, so that no change in the rate of $NADP^+$ reduction occurred with the increasing time of ultrasonic treatment, both were included in the reaction assay mixture. That this effect is a general one, and not peculiar to chloroplast preparations from *Pisum*, is shown by the data in Table 1, where results with this material are compared with those from chloroplast material from other genera.

In the experiments described so far, the need to restore three separate purified protein components to the reaction mixture introduced considerable experimental complexity. In a simpler system, plastocyanin was shown to be the only soluble protein component needed to be added to promote the photoreduction of the electronegative artificial oxidants diquat

TABLE 1. REDUCTION OF NADP BY ULTRASONICATED CHLOROPLASTS FROM VARIOUS PLANTS

| | $\mu$moles $NADP^+$ reduced/mg chlorophyll/hr | | | |
|---|---|---|---|---|
| | | Additions | | |
| Plant material | Ascorbate | Ascorbate DCIP | Ascorbate + plastocyanin | Ascorbate DCIP plastocyanin |
| *Pisum sativum* | 8·4 | 9·7 | 35·5 | 40·5 |
| *Brassica oleracea* | 12·01 | 14·14 | 65 | 59·4 |
| *Spinacea oleracea* | 9·61 | 9·90 | 94 | 91 |
| *Chenopodium bonushenricus* | 7·35 | 8·63 | 32 | 33·5 |

Reaction conditions and additions as for Fig. 5.

(1,1′-ethylene-2-2′-dipyridilium dibromide, $E'_0 = -349$ mV) and paraquat (1,1′-dimethyl-4-4′-dipyridilium dichloride, $E'_0 = -446$ mV) by ultrasonically treated chloroplasts using ascorbate as electron donor. These autoxidizable oxidants have been shown to be effective in catalysing the transfer of electrons from water to molecular oxygen with the generation of hydrogen peroxide in the variant of the Hill reaction observed by Mehler[12] and interpreted by Good and Hill.[13] Since brief periods of ultrasonic treatment had been found to abolish the capacity of illuminated chloroplast fragments to utilize water as electron donor, ascorbate was therefore substituted in manometric measurements where ethanol was oxidized by hydrogen peroxide in the presence of catalase, giving a net oxygen uptake. In the experiment illustrated in Fig. 6, where diquat served as catalyst, ultrasonic treatment of the chloroplasts for 10 min essentially abolished the reaction until a catalytic amount of plastocyanin was added. In this type of experiment, as in the observations with $NADP^+$, plastocyanin could not be replaced by DCIP and it was confirmed that here also the development of a requirement for plastocyanin with increasing time of ultrasonic treatment followed the same time course as the decline in effectiveness of DCIP as mediator of the reaction.

[11] H. E. DAVENPORT, *Proc. R. Soc. B.* **157**, 332 (1963).
[12] A. M. MEHLER, *Archs. Biochem.* **33**, 65 (1951).
[13] N. GOOD and R. HILL, *Archs. Biochem.* **57**, 355 (1955).

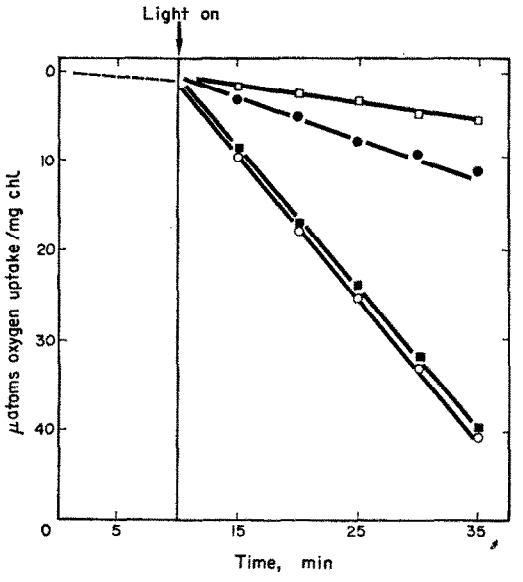


FIG. 6. OXYGEN UPTAKE DURING PHOTOXIDATION OF ASCORBATE BY CHLOROPLASTS ULTRASONICATED FOR 15 min.

Reactions carried out in Warburg flasks at 15° contained (in μmoles) in 3 ml Tris-HCl buffer, pH 8·0, 150; diquat 0·01; ethanol 60; CMU 0·015; catalase (0·02 mg haematin); ascorbate 20: chloroplasts containing 0·2684 mg chlorophyll: further additions in concentrations of Fig. 2. □ None. ● DCIP. ■ Plastocyanin. ○ DCIP; plastocyanin.

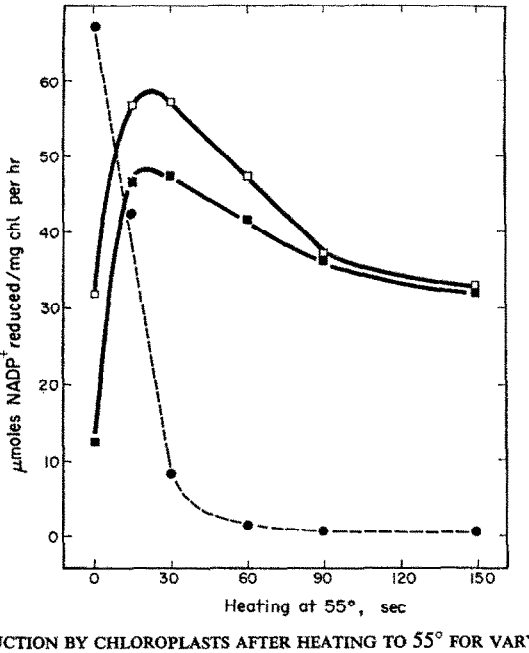


FIG. 7. NADP+ REDUCTION BY CHLOROPLASTS AFTER HEATING TO 55° FOR VARYING TIME INTERVALS.

Reaction conditions contents and concentrations as for Fig. 2, but without ferredoxin NADP+ reductase: chloroplasts contained 0·052 mg chlorophyll. Illumination time: 2 min. Additions ● None. □ Ascorbate; DCIP; CMU. ■ Ascorbate; CMU.

*(iii) Heat and Ultrasonic Treatment*

Wessels[14] demonstrated that chloroplasts heated to 55° lost their ability to evolve oxygen while still retaining a capacity to photo-oxidize ascorbate in the presence of DCIP, $NADP^+$ has been shown to serve as electron acceptor in this reaction when catalytic quantities of ferredoxin are added.[15] Some of the effects of heat and ultrasonic treatment are now briefly compared.

The experiments summarized in Fig. 7 confirmed that heating to 55° for 30 sec essentially abolished the Hill reaction. With ascorbate as electron donor both in the presence and absence of a catalytic amount of DCIP, this short period of heat treatment led to a rapid initial rise in the rate of $NADP^+$ photoreduction, followed by a slower decrease to a stable rate at 150 sec. It is of interest that the catalytic activity of DCIP in stimulating the reaction, observable before and during the initial period of heat treatment, progressively diminished until it could not be observed when the stable reaction rate was attained after 150 sec heating.

Although the only protein catalyst which had to be added to the heated chloroplasts to obtain maximal rates of $NADP^+$ reduction was ferredoxin, a further requirement for both plastocyanin and ferredoxin–$NADP^+$ reductase could be elicited by subsequent ultrasonic treatment. With saturating amounts of the three proteins, activity was then restored to a level similar to that observed with the heated chloroplasts before ultrasonic treatment (Table 2).

TABLE 2. EFFECT OF HEATING FOLLOWED BY ULTRASONICATION ON $NADP^+$ REDUCTION

| | $\mu$moles $NADP^+$ reduced/mg chlorophyll/hr | | |
|---|---|---|---|
| | Additions | | |
| Chloroplast treatment | Ascorbate | Ascorbate + DCIP | Ascorbate plastocyanin |
| Heated | 80·0 | 84·0 | 79·0 |
| Heated sonicated | 4·7 | 7·0 | 74·0 |

Reaction conditions and additions as Fig. 5.

## DISCUSSION

The observations reported here are complementary to a more extensive study carried out simultaneously by Katoh and San Pietro.[5] Unlike these workers, and Katoh and Takamiya,[2] however, we were unsuccessful in obtaining chloroplast fragments to which Hill reaction activity could be significantly restored by the restoration of plastocyanin. This negative finding could well be due to the choice of *Pisum* rather than *Spinacea* as our principal source of chloroplast material. Previous experience had shown that *Pisum* chloroplasts, although highly active in promoting the Hill reaction when freshly prepared, undergo a more rapid inactivation of their oxygen evolving capacity than do chloroplasts from *Spinacea*.

The effects of brief periods of heat treatment on chloroplasts may usefully be compared with those of ultrasonic treatment. Both result in a rapid loss in the ability of the chloroplasts to utilize water as electron donor. With the heated chloroplasts the linear phase of this loss

[14] J. S. C. WESSELS, *Rec. Trav. Chim.* **74**, 832 (1955).
[15] M. SHIN, K. TAGAWA and D. I. ARNON, *Biochem. Z.* **338**, 84 (1963).

is accompanied by a rapid rise in the effectiveness of ascorbate as alternative electron donor both with or without DCIP as mediator of the reaction (Fig. 7). Thereafter, the requirement for DCIP declines. These observations could well be explained as a conformational change induced by heat which results on the one hand in the loss of the power to evolve oxygen, and on the other in an increase in accessibility of a site to which ascorbate can donate electrons directly. That this site could be bound plastocyanin is further suggested by the appearance of a requirement for added plastocyanin when heated chloroplasts are subsequently disrupted by ultrasonic treatment. It is suggested therefore, that the apparent replacement of DCIP by plastocyanin to mediate ascorbate photoxidation with either $NADP^+$ or oxygen as ultimate electron acceptor, is incidental. Thus when heated chloroplasts are subjected to subsequent ultrasonic treatment a progressive unmasking of the plastocyanin requirement was demonstrated, uncomplicated by the diminishing response to DCIP, observed with fresh chloroplasts.

## EXPERIMENTAL

### *Plant Material*

*Petroselinum sativum, Brassica oleracea, Chenopodium bonus-henricus* and *Spinacea oleracea* were grown in garden conditions and harvested as required. *Pisum sativum* plants were grown under greenhouse conditions in John Innes No. 1 compost and harvested after 3–4 weeks.

### *Chloroplast Isolation and Reaction Conditions*

As described by Davenport.[16] Unless otherwise stated, *Pisum* leaves were used.

### *Ultrasonic Treatment*

Chloroplast suspensions were subjected to ultrasonic vibrations by means of an M.S.E. ultrasonic probe (20 KHz/sec, 60 W). The chloroplast suspension, about 10 ml, was contained in a double-walled glass vessel cooled by circulating ice water, and constantly stirred by means of a magnetic stirrer. The probe was dipped into the suspension ($\frac{1}{8}$ in.) and operated at maximum power for the required time.

### *Chlorophyll Content*

The chlorophyll content of chloroplast preparations was estimated by the method of MacKinney.[17]

### *Leaf Protein Preparations*

Frozen *Petroselinum* leaves were ground in a "Hobart" food mincer and the frozen homogenate thawed out in 0·05 M Tris/HCl buffer at pH 8·0, in the ratio of 1000 g of leaves to 800 ml of buffer. After filtration through muslin, the filtrate was treated by the acetone precipitation method of San Pietro and Lang.[18] The crude acetone precipitate so obtained was dialysed overnight, and further purified on a DEAE-cellulose column (Whatman Chromedia DE 11) by the method of Tagawa and Arnon.[1] The fraction eluted from the column with 0·2 N NaCl contained ferredoxin–$NADP^+$ reductase and plastocyanin, and was further purified by $(NH_4)_2SO_4$ precipitation, the reductase being precipitated between 50 and 66% saturation, and the plastocyanin between 66 and 100%. The fraction eluted from the DEAE column with 0·8 N NaCl contained ferredoxin. Ferredoxin and plastocyanin extracts were routinely tested for contaminating ferredoxin–$NADP^+$ reductase by assaying NADPH diaphorase activity. Further purification of ferredoxin was achieved by passing the extract through a column of Sephadex G.50, equilibrated with 0·001 M Tris/HCl buffer at pH 7·3. Saturating levels of ferredoxin and ferredoxin–$NADP^+$ reductase were established by using the experimental technique of Davenport.[2] Further purification of the plastocyanin extract was achieved by re-fractionation with $(NH_4)_2SO_4$, and the concentration estimated by the method of Katoh *et al.*[8]

*Acknowledgements*—A. D. D. wishes to thank the Bath University of Technology for a Research Fellowship which enabled him to work at Long Ashton Research Station for 1 year. The technical assistance of Miss Valerie Hart is gratefully acknowledged.

[16] H. E. Davenport, *Biochem. J.* **77**, 471 (1960).
[17] G. MacKinney, *J. Biol. Chem.* **140**, 315 (1941).
[18] A. San Pietro and H. M. Lang, *J. Biol. Chem.* **231**, 211 (1958).