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STUDIES OF PLASTOQUINONE—2. OXIDATION-REDUCTION REACTIONS OF PLASTOQUINONE IN ISOLATED CHLOROPLASTS

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Abstract—After illumination of isolated sugar-beet chloroplasts, there was a reduction of the endogenous plastoquinone measured as a fall in the oxidation level of the quinone. The value of the photo-reduction varied between 10 and 30 per cent in different preparations of chloroplasts. The reduction was inhibited by either *o*-phenanthroline or DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea). Addition of ascorbic acid plus 2,6-dichlorophenol indophenol stimulated the photo-reduction and the stimulation was not appreciably affected by either *o*-phenanthroline or DCMU. These results are interpreted on the assumption that plastoquinone photo-reduction occurs via the light reaction involved in water photolysis. The possible site of action of plastoquinone in the photosynthetic electron transport system is discussed in relation to these and other experimental results reported.

INTRODUCTION

THE PREVIOUS paper in the series[1] described a method for measuring the concentration and oxidation–reduction state of plastoquinone in isolated chloroplasts.

This method has now been used to measure the changes in oxidation–reduction level of plastoquinone after illumination, and the effects of inhibitors on such changes in an attempt to implicate plastoquinone as a member of the photosynthetic electron transport chain. Preliminary reports of some of these results have been published previously.[2,3]

RESULTS AND DISCUSSION

Illumination of the chloroplast suspension resulted in a reduction of endogenous plastoquinone, shown by a drop in the oxidation level (Table 1). The new level was attained within the first minute and remained steady during continuous illumination for 10 min. In further experiments, the change in oxidation–reduction level was measured after a fixed time interval.

In the experiment reported in Table 1, 20 per cent of the total plastoquinone present became reduced on illumination. In other preparations the reduction of plastoquinone varied and from 10 to 25 per cent of the total quinone present was reduced. Some of these results are listed in Table 2. Crane *et al.*[4] found in preparations of isolated chloroplasts from spinach, 80 per cent of the total endogenous plastoquinone was reduced after 5 min illumination. No suitable explanation can be suggested at present for this discrepancy.

[1] E. R. REDFEARN and J. FRIEND, *Phytochemistry* **1**, 147 (1962).
[2] E. R. REDFEARN and J. FRIEND, *Nature* **191**, 806 (1961).
[3] J. FRIEND and E. R. REDFEARN, *Biochem. J.* **82**, 13P (1962).
[4] F. L. CRANE, B. EHRLICH and L. P. KEGEL, *Biochem. Biophys. Research Communs.* **3**, 37 (1960).

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The extent of the reductions shown in Table 2 could not be correlated with the initial oxidation–reduction level of plastoquinone which, as we have previously reported,[1] showed considerable variation in chloroplasts prepared from different batches of leaves. The Hill reaction activities of the chloroplasts also varied and although a few chloroplast preparations which had low activity in the Hill reaction had high initial oxidation levels of quinone, there was, in general, little correlation between the Hill reaction activity and the oxidation–reduction level of plastoquinone in the chloroplasts.

The oxidation–reduction state of plastoquinone may depend upon the metabolic status of the leaves prior to isolation of the chloroplasts since, as will be shown later, plastoquinone can be reduced by various compounds in dark reactions. The oxidation level of plastoquinone

TABLE 1. CHANGE IN OXIDATION–REDUCTION LEVEL OF ENDOGENOUS PLASTOQUINONE IN ISOLATED CHLOROPLASTS

Time (min)	Percentage oxidation of quinone	
	Dark	Light
0	53·5	53·5
1	52·7	35·8
2	51·2	35·8
3	50·0	33·6
5	48·9	33·8
10	48·9	35·1

1 ml chloroplast suspension containing 1·01 mg chlorophyll and 1·5 ml 0·05 M pH 7·0 phosphate buffer containing 0·01 M KCl. Illumination conditions described in text.

may therefore reflect the general oxidizing or reducing level in the chloroplasts and presumably in the leaf prior to isolation.

Since it was difficult to achieve close environmental control over the plants used in these experiments, it was expected that there would be variations in the metabolic status of different batches of leaves.

Effect of $NADPH_2$

Incubation of the chloroplast preparation with $NADPH_2$ in the dark at 20° resulted in a reduction of 20 per cent of the plastoquinone (Table 3) although there was only 10 per cent reduction of the quinone when such a mixture was illuminated.

Using a system which was presumed to produce $NADPH_2$ by a photochemical reaction, a stimulated photo-reduction of plastoquinone was found. In these experiments the chloroplasts were incubated with NADP and a chloroplast extract containing photosynthetic pyridine nucleotide reductase.[5] Without any addition to the chloroplast suspension there was a reduction of 14 per cent of the plastoquinone after 5 min illumination. With the additions already described there was a drop in oxidation level of 7 per cent after 15 min incubation in the dark but there was a reduction of 28·5 per cent after 5 min illumination.

[5] F. R. WHATLEY, M. B. ALLEN and D. I. ARNON, *Biochim. Biophys. Acta* 32, 32 (1959).

Since chloroplasts contain an enzyme having $NADPH_2$ diaphorase activity which will reduce quinones such as menadione,[6,7] it seems likely that the action of such an enzyme could account for the reduction of plastoquinone both by added $NADPH_2$ and by photochemically-formed $NADPH_2$. The smaller reduction obtained upon illumination of chloroplasts to which $NADPH_2$ was added may be explained on the basis of a photo-oxidation of $NADPH_2$ similar to the one observed by Jagendorf,[8] which would effectively lower the concentration

TABLE 2. EFFECT OF LIGHT ON THE OXIDATION–REDUCTION STATE OF ENDOGENOUS PLASTOQUINONE IN ISOLATED SUGAR-BEET CHLOROPLASTS

Experiment	Treatment: Time (min)	Treatment: Light or dark	Plastoquinone (per cent in oxidized form)	Hill reaction activity (μM dye reduced/ mg/chlorophyll/hr	Chlorophyll concentration (mg/ml of suspension)
1.	15	dark	50·5	135	0·28
	15	light	40·8		
2.	0	dark	96·6	99	0·38
	5	light	74·9		
3.	0	dark	60·0	134	0·79
	10	light	44·5		
4.	0	dark	75·7	116	0·69
	5	light	54·8		
5.	0	dark	85·2	123	0·65
	5	light	60·5		
6.	0	dark	62·1	91	0·95
	5	light	49·7		
7.	0	dark	74·0	130	0·91
	5	light	42·3		
8.	0	dark	64·0	158	0·80
	5	light	47·0		
9.	0	dark	61·2	144	0·87
	5	light	42·5		
10.	0	dark	68·8	164	0·77
	5	light	52·0		
11.	0	dark	69·5	175	0·57
	5	light	46·8		

Experimental conditions as described in the text.
1 ml chloroplast suspension and 1 ml 0·05 M pH 7·0 phosphate buffer containing 0·01 M KCl.

of $NADPH_2$ available for reaction with plastoquinone. In this experiment there was no additional chloroplast extract which could catalyze the photochemical resynthesis of $NADPH_2$.

Effects of Inhibitors

The effects of two inhibitors of the photosynthetic electron transport system, *o*-phenanthroline and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) on the light-catalysed reduction of plastoquinone were examined. As shown in Table 4 both these compounds inhibited the light-catalysed reduction of endogenous plastoquinone, thus providing additional evidence

[6] M. AVRON and A. T. JAGENDORF, *Arch. Biochem. Biophys.* **65**, 475 (1956).
[7] D. L. KEISTER, A. SAN PIETRO and F. E. STOLZENBACH, *J. Biol. Chem.* **235**, 2989 (1960).
[8] A. T. JAGENDORF, *Arch. Biochem. Biophys.* **62**, 141 (1956).

that the light-catalysed reduction is associated with photosynthetic electron transport. *o*-Phenanthroline actually raises the oxidation level of plastoquinone and it is this raised level which is unaffected by light. Wessels has previously shown that addition of *o*-phenanthroline to chloroplasts which were reducing toluylene blue raises the redox potential of the system.[9]

Vernon and Zaugg found that the inhibition of photo-reduction by DCMU could be overcome by addition of ascorbic acid and indophenol.[10] Therefore the effect of these two compounds together was examined. When ascorbic acid and 2,6-dichlorophenol indophenol were added together to chloroplasts they stimulated the photo-reduction of endogenous

TABLE 3. EFFECT OF $NADPH_2$ ON THE OXIDATION–REDUCTION STATE OF ENDOGENOUS PLASTOQUINONE IN SUGAR-BEET CHLOROPLASTS

Addition	Treatment: Time (min)	Treatment: Light or dark	Plastoquinone (per cent in oxidized form)
None*	0	dark	45·8
$NADPH_2$*	5	dark	26·0
$NADPH_2$*	5	light	34·3
None†	0	dark	87·5
None†	5	light	73·5
NADP + chloroplast extract†	15	dark	80·5
NADP + chloroplast extract†	15	light	59·0

Reaction mixtures were as follows: Chloroplast suspensions containing 0·44* and 0·84† mg chlorophyll per ml respectively (1 ml), chloroplast extract (equivalent to 5 g fresh wt. leaves per ml) or water (1 ml). NADP or $NADPH_2$ (0·625 mM). Total volume 2·2 ml.

plastoquinone (Table 4). Addition of DCMU did not inhibit this accelerated photo-reduction. *o*-Phenanthroline appeared to inhibit this reaction to some extent; however, closer examination of the results shows that the difference in oxidation level of plastoquinone between chloroplasts plus phenanthroline in the dark, and chloroplasts plus ascorbate and indophenol and phenanthroline in the light is 44 per cent. This is almost identical with the difference between chloroplasts in the dark and chloroplasts plus ascorbate and indophenol in the light (42 per cent).

Thus in these experiments the light-catalysed reduction of endogenous plastoquinone is following a similar pattern to the photo-reduction of added co-factors by isolated chloroplasts. It has been found that photo-reductions of added NADP, NAD, flavin mononucleotide and menadione were inhibited by DCMU and that the inhibition was relieved by addition of ascorbate and 2,6-dichlorophenol.[10] Since DCMU blocks reactions involved in the evolution of oxygen,[11] it has been assumed that any photo-reduction which is inhibited by DCMU must be mediated through that light reaction concerned in photolysis of water and oxygen evolution. The action of ascorbate and indophenol is to provide an alternative source of electrons (or reducing equivalents).[10]

We may therefore postulate that plastoquinone is reduced by the photochemical system

[9] J. S. C. WESSELS, Thesis, University of Leiden (1954).
[10] L. P. VERNON and W. S. ZAUGG, *J. Biol. Chem.* **235**, 2726 (1960).
[11] N. I. BISHOP, *Biochim. Biophys. Acta* **27**, 505 (1958).

involved in water photolysis, and that when this system is inhibited, plastoquinone can be photo-reduced by electrons provided by ascorbate and indophenol.

The results in Table 4 also show that there is an enhanced photo-reduction of plastoquinone in the presence of ascorbate and indophenol. This additional reduction is apparently due partly to a reduction which does not involve a light reaction, since incubation of chloroplasts in the dark with ascorbate and indophenol lowers the oxidation level of plastoquinone

TABLE 4. EFFECT OF INHIBITORS ON THE LIGHT-CATALYSED REDUCTION OF ENDOGENOUS PLASTOQUINONE OF ISOLATED SUGAR-BEET CHLOROPLASTS

Compound added	Treatment: Time (min)	Treatment: Light or dark	Plastoquinone (per cent in oxidized form): (a)	Plastoquinone (per cent in oxidized form): (b)
None	0	dark	85·5	88·7
None	5	light	75·4	61·7
o-Phenanthroline	0	dark	96·5	
o-Phenanthroline	5	light	98·5	
DCMU	0	dark		87·3
DCMU	5	light		88·7
Ascorbic acid + 2,6-dichlorophenol indophenol	5	light	43·3	33·5
o-Phenanthroline + ascorbic acid + 2,6-dichlorophenol indophenol	5	light	52·5	
DCMU + ascorbic acid + 2,6-dichlorophenol indophenol	5	light		33·5

o-Phenanthroline and DCMU were added in 0·05 ml ethanol to give a final concentration of $5{\cdot}9 \times 10^{-4}$ M of each compound. 0·05 Ml ethanol was added to those tubes without inhibitor. The final concentrations of ascorbic acid and 2,6-dichlorophenol indophenol were $1{\cdot}6 \times 10^{-2}$ and 8×10^{-4} M respectively. The chlorophyll concentrations in the chloroplast suspension used were (a) 0·97 mg/ml, (b) 0·85 mg/ml.

(Table 5). The plastoquinone became more reduced when such a reaction mixture was illuminated; DCMU did not affect the dark reduction but in this later series of experiments the light reduction catalysed by ascorbate plus indophenol appeared to be slightly stimulated by DCMU (7 per cent stimulation).

Two sites at which ascorbate plus indophenol could react with the photosynthetic electron transport system have been proposed by Witt *et al.*[12] One reaction is with one of the reactants in the photochemical system concerned in photolysis of water and the other is with the cytochrome situated between the two light reactions. We should agree that one site of action must involve a light reaction at the site suggested by Witt[12] but our results raise the possibility of a more direct dark reaction between ascorbate plus indophenol and plastoquinone.

The effect of other inhibitors of either photosynthetic electron transport or photophos-

[12] H. T. WITT, A. MÜLLER and B. RUMBERG, *Nature* **192**, 976 (1961).

phorylation was examined. It was found (Table 6) that addition of ammonia caused the plastoquinone to become more oxidized in the light than in the dark; in other words, it appeared to catalyse a photo-oxidation of plastoquinone as opposed to the more usual photoreduction found when chloroplasts were illuminated on their own. Methylamine hydrochloride caused a very slight inhibition of photo-reduction.

Addition of either of the respiratory inhibitors potassium cyanide or sodium azide caused an increase in photo-reduction of the endogenous plastoquinone.

TABLE 5. EFFECT OF ASCORBATE PLUS INDOPHENOL ON THE OXIDATION LEVEL OF ENDOGENOUS PLASTOQUINONE IN ISOLATED SUGAR-BEET CHLOROPLASTS

Compound added	Incubation (Light or dark)	Plastoquinone (per cent in oxidized form)
None	dark	58·0
None	light	47·5
Ascorbic acid + 2,6-dichloro-phenol indophenol	dark	45·0
Ascorbic acid + 2,6-dichloro-phenol indophenol	light	32·0
DCMU	dark	61·0
DCMU	light	59·0
Ascorbic acid + 2,6-dichloro-phenol indophenol + DCMU	dark	47·5
Ascorbic acid + 2,6-dichloro-phenol indophenol + DCMU	light	30·0

1 ml chloroplast suspension containing 0·56 mg chlorophyll and 1·5 ml 0·05 M pH 7·0 phosphate buffer containing 0·01 M KCl and the appropriate additions. The incubation time was 5 min in all experiments. The final concentrations of ascorbic acid and 2,6-dichlorophenol indophenol were $1{\cdot}6 \times 10^{-2}$ M and 8×10^{-4} M respectively. DCMU was added in 0·05 ml ethanol to give a final concentration of $5{\cdot}9 \times 10^{-4}$ M; 0·05 ml of ethanol was added to those tubes which did not contain DCMU.

One explanation of these results is based upon the assumption that the measurements we are making are of steady-state levels of endogenous plastoquinone. The steady-state level will be controlled by a balance between oxidizing and reducing reactions, thus in any situation where the steady-state level of oxidation falls, the reducing reactions are more rapid than the oxidizing ones and vice-versa. On this basis, ammonia and methylamine are either stimulating the re-oxidation of plastoquinone, or blocking the photo-reduction of the quinone, whereas hydroxylamine hydrochloride, cyanide and azide are either inhibiting the re-oxidation of the quinol or stimulating the reduction of quinone.

Ammonia is known to stimulate the Hill reaction and uncouple photophosphorylation [13] at about the same concentration as has been used in our experiments. It could conceivably act by stimulating electron flow at one point in the photosynthetic electron transport system; stimulation after the site of action of plastoquinone would thus account for the observed

[13] D. W. KROGMANN, A. T. JAGENDORF and M. AVRON, *Plant Physiol.* **34**, 272 (1959).

photo-oxidation of plastoquinone. A similar explanation would hold for the action of methylamine hydrochloride since methylamine also stimulates the Hill reaction.[14]

Cyanide and azide could act by inhibiting the re-oxidation of plastoquinol, if this were mediated through a cytochrome or other metalloenzyme system, and hydroxylamine hydrochloride, which has been found to inhibit the photo-reduction of added FMN by chloroplasts,[10] would act by inhibiting the photo-reduction of plastoquinone.

TABLE 6. EFFECT OF VARIOUS COMPOUNDS ON THE OXIDATION–REDUCTION STATE OF PLASTOQUINONE IN ISOLATED SUGAR-BEET CHLOROPLASTS

Compound added and its final conc.	Incubation: Time (min)	Incubation: Light or dark	Plastoquinone (per cent in oxidized form) (a)	(b)	(c)	Per cent stimulation of inhibition of the photo-reduction without additions
None	0	dark	57·4	61·2	87·5	
None	0	light	36·7	42·5	75·5	
$NH_2OH.HCl$ ($2·4 \times 10^{-3}$ M)	5	light	34·5			+10·4
$MeNH_2.HCl$ ($2·4 \times 10^{-3}$ M)	5	light	39·3			−12·5
NH_4OH ($2·4 \times 10^{-3}$ M)	5	light	77·1			photo-oxidation*
KCN (2×10^{-2} M)	5	light		36·4		+33
NaN_3 (10^{-3} M)	5	light			65·0	+88·5

* Ammonia stimulates a photo-oxidation of plastoquinone; after illumination in the presence of ammonia, the plastoquinone became 34·6 per cent more oxidized.

1 ml chloroplast suspension containing (a) 1·02 mg chlorophyll/ml, (b) 0·87 mg chlorophyll/ml, (c) 0·97 mg chlorophyll/ml; 1·5 ml 0·05 M pH 7·0 phosphate buffer containing 0·01 M KCl and the appropriate compound.

Concluding Discussion

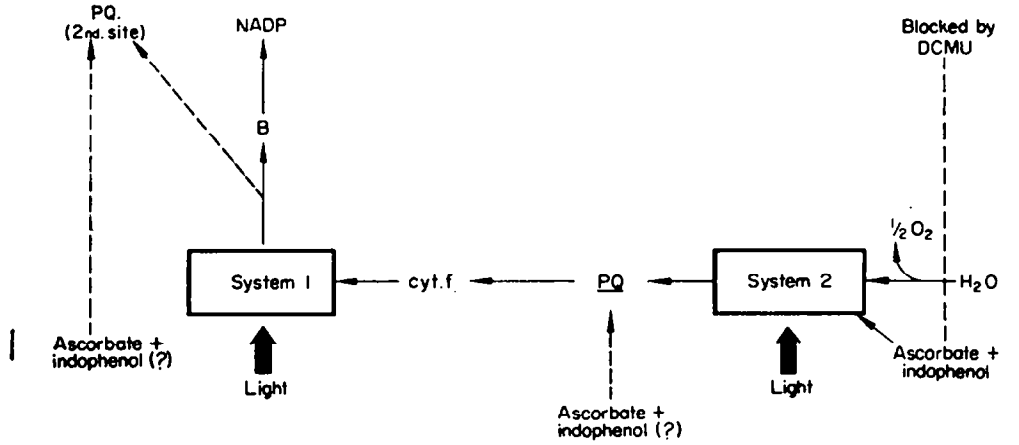
The first suggestion that plastoquinone functions in photosynthetic electron transport was made by Bishop.[15] He found that the Hill reaction of lyophilized isolated chloroplasts was inhibited by extraction of plastoquinone and that activity was restored by addition of plastoquinone back to the extracted chloroplasts.

We have endeavoured, by measuring the oxidation–reduction state of endogenous plastoquinone before and after illumination, to show the place of plastoquinone in the photosynthetic electron transport system. The oxidation–reduction level of endogenous plastoquinone varies in freshly isolated chloroplasts; the reasons for this variation are probably complex, but we can surmise that one factor controlling the variation is the metabolic status of the leaf prior to isolation of the chloroplasts. Despite the variations of initial oxidation level, when isolated chloroplasts are illuminated, about 25 per cent of the endogenous plastoquinone becomes reduced. This reduction involves the light reaction involved in photolysis of water (system 2 of Duysens *et al.*[16, 17]) because it is inhibited by DCMU and the inhibition is released by addition of ascorbate and indophenol. As mentioned earlier, in these reactions the reduction of endogenous plastoquinone is similar to the reduction of added co-factors.

[14] N. E. GOOD, *Biochim. Biophys. Acta* **40**, 502 (1960).
[15] N. I. BISHOP, *Proc. Nat. Acad. Sci. U.S.* **45**, 1696 (1959).
[16] L. N. M. DUYSENS, J. AMESZ and B. KAMP, *Nature* **190**, 510 (1961).
[17] L. N. M. DUYSENS and J. AMESZ, *Biochim. Biophys. Acta* **64**, 243 (1962).

Since under certain circumstances we have observed a photo-oxidation of plastoquinone (addition of NH_4^+ ions; occasional experiments with phenanthroline and DCMU), there is a strong possibility that plastoquinol is re-oxidized by a second light reaction (system 1 of Duysens *et al.*[16,17]). Furthermore, Whittingham and Bishop have shown that NH_4^+ ions accelerate the reaction between the two light reactions.[18] If our postulates are correct, plastoquinone would be situated between the two light reactions, and NH_4^+ ions would accelerate reactions after plastoquinone; that is, the reactions involved in the re-oxidation of plastoquinol.

The position of plastoquinone in the scheme postulated independently by Duysens[16] and by Arnon[19] would be at the positions outlined in Fig. 1. This site of action of plastoquinone has also been indicated by the flash-photometric experiments of Witt *et al.*[20] The situation of plastoquinone before the cytochrome is then analogous to the location of ubiquinone in the mitochondrial electron transport chain.[21]



Both Witt *et al.*[20] and Trebst[22] have suggested that plastoquinone may be involved at another site in the photo-reduction of ferricyanide which occurs after the light reaction of pigment system 1. We have placed this site before B in Fig. 1. The measurements made by our method are of the overall oxidation–reduction level of plastoquinone in the isolated chloroplasts. Since we only find changes of about 20–30 per cent of the total quinone it is possible that part of the plastoquinone is in a storage site as suggested by Trebst and does not show much change in the light under the experimental conditions we have used.

If there is a large excess of plastoquinone in the inactive site, the oxidation level at this site would be the major factor in determining the overall oxidation level in freshly isolated chloroplasts. This may be why we can find no correlation between the initial oxidation level of the plastoquinone and the change in oxidation level on illumination.

Two positions of plastoquinone could also be invoked to explain the enhanced photo-reduction of plastoquinone by ascorbate plus indophenol. It has been pointed out that this consists of both a light catalysed reduction and a dark reduction of plastoquinone, and these two reductions could conceivably occur separately at the two sites.

[18] C. P. WHITTINGHAM and P. M. BISHOP, *Nature* **192**, 426 (1961).
[19] M. LOSADA, F. R. WHATLEY and D. I. ARNON, *Nature* **190**, 601 (1961).
[20] H. T. WITT, A. MÜLLER and B. RUMBERG, *Nature* **197**, 987 (1963).
[21] E. R. REDFEARN, in Ciba Foundation Symposium on *Quinones in Electron Transport*, eds. G. E. W. WOLSTENHOLME and C. M. O'CONNOR. J. & A. Churchill, London (1961).
[22] A. V. TREBST, *Proc. Roy. Soc.* (*London*) **B157**, 355 (1963).

EXPERIMENTAL

Chloroplasts

Chloroplasts were isolated from leaves of sugar-beet plants by the method given previously.[1]

Illumination of Chloroplast Suspensions and Determination of the Oxidation–Reduction state of Plastoquinone

The reaction mixture, consisting of 1 ml chloroplast suspension in 0·05 M pH 7·0 Na/K phosphate buffer containing 0·01 M KCl and the appropriate additions was pipetted into a test-tube with a standard C14 taper. The tube was illuminated from each of two opposite sides using 500-W tungsten lamps as light sources; the lights were focused with condensers made from 500-ml round-bottom flasks filled with water. These also acted as heat filters. The reaction was terminated by the addition of 4 ml of a solution of pyrogallol in methanol (0·1 % w/v), which had been cooled to $-20°$. The tube was then removed from the light, 4 ml light petroleum added as quickly as possible and the tube was shaken.

The plastoquinone was extracted and its oxidation–reduction state determined by the method already described.[1] The differences between replicate determinations did not exceed 1·6 per cent of the estimated oxidation–reduction level.

Hill Reaction Measurements

Hill reaction was assayed using 2,6-dichlorophenolindophenol as electron acceptor. Assays were made by illuminating a cuvette containing 2·9 ml of a solution of 0·01 M KCl in 0·05 M Na/K phosphate buffer pH 7·0, 0·1 ml $1{\cdot}6 \times 10^{-3}$ M 2,6-dichlorophenolindophenol solution, and 0·1 ml chloroplast suspension containing 0·04–0·08 mg chlorophyll. The difference in absorbance was measured after 30 sec. Compensation for any possible dark reduction of indophenol was made by measuring the absorbance change in a cuvette containing an identical mixture which was kept in the dark. In the spectrophotometric blank, 0·1 ml of the phosphate–KCl solution replaced the dye solution.

The chlorophyll concentration used in these assays was higher than that used by others (e.g. Jagendorf[6]). Although higher rates of activity were obtained when a more dilute chloroplast suspension was used, it was routinely more convenient to use the same concentration of chloroplasts which was used in the plastoquinone assay.

Reagents

All chemicals were of the highest grade of purity commercially available.