Studies on the Effect of Diphenylcarbazide in Isolated Chloroplasts from Spinach

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The influence of diphenylcarbohydrazide (DPC) on electron transport parameters of chloroplasts was studied through the use of hypotonically disintegrated plastids in various states of membrane disarray.

The observation that DPC acts as donator for PS II when the natural source becomes inefficient was confirmed.

Contrary to prior contentions oxygen evolution is not diminished upon addition of DPC.

A definite though transient influence of DPC on the pigment system was detected. At a certain state of structural disarray DPC increases the quantum yield and electron transport efficiency through PS II, while simultaneously decreasing the activity of PS I. The effect is not based on addition or removal of individual pigments to the pigment complexes. The analysis of light intensity curves and fluorescence kinetics suggest a better migration of trapped light to open reaction centers.

The results are discussed for possible implications on the analysis of the oxygen evolving system as well as on spillover mechanisms.

Introduction

Vernon and Shaw [1, 2] in an extension of the studies of Yamashita and Butler [3] used 1,5-diphenylcarbohydrazide (DPC) as a means of assessing the parameters of photosystem II independently from an intact oxygen evolving system. The possible mechanism of electron donation and the fate of the oxidized donor was studied in more detail by Shneyour and Avron [4]. They showed an involvement of PS I pigments in the process which they interpreted as a catalytic sensitization of the disproportionation of the 1,5-diphenylcarbazone formed. Their observation provided the first evidence that – besides mere electron donation – DPC might influence other photosynthetic activities.

This communication attempts to elucidate further various aspects of the mode of action of DPC. Following its effect on Hill activity, pigment and fluorescence properties in the course of the *in vitro* degradation of hypotonically suspended chloroplasts, the role of DPC as electron donor and its influence on the efficiency of electron transport was assessed and interpreted.

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Material and Methods

Spinach chloroplasts were prepared in the usual manner using 0.4 M sucrose 0.1 M phosphate-buffer pH 6.8 – 1 mg/ml serum albumin as isolation medium. The plastids were harvested at low centrifugal speed and washed once. They were resuspended in 0.1 M phosphate buffer pH 6.8 – 1 mg/ml serum albumin, diluted with the same medium to 250 – 300 μg chlorophyll/ml and stored in the dark at 0 °C. Aliquotes were withdrawn at definite intervals and used in the various assay systems.

The light induced reduction of dichlorophenolindophenol (DPIP) and NADP was continually followed at the appropriate wavelengthes using the spectrophotometric setup of Postius and Jacobi [5]. The assay contained in 3 ml buffer solution chloroplasts equiv. to 20 – 30 μg chlorophyll, 2×10^{-7} mol DPIP, 5×10^{-5} mol NH_4Cl to diminish any effect due to phosphorylation and, if noted, 3×10^{-6} mol DPC. The amounts of reagents in the assay for NADP reduction, using either water or ascorbate/DPIP as electron source, were as described before [6].

Oxygen evolution was followed using both a Clark type electrode (Rank Brothers, Bottisham) or the Warburg method. The manometric determinations were performed under a nitrogen atmosphere. The vessels contained in a total of 2 ml buffer plastids equiv. 500 μg chlorophyll, 2×10^{-4} mol DPIP or 0.2 ml quinone solution (5 mg/ml), 5×10^{-5} mol NH_4Cl and, if noted, variable amounts

of DPC. Red actinic light ($\lambda > 610$ nm) was used in all instances, modifying the beam with Balzers grey filters if necessary. Part of the Warburg experiments were done with white actinic light, no difference in performance being observed.

Fluorescence excitation spectra were taken with a Shimadzu MPS-50 spectrophotometer, the rise kinetics were measured in a fluorescence apparatus as described before [7].

Results

1. *DPC as electron donator for PS II*

Electron donating properties similar to those described by Vernon and Shaw [1] were observed under light saturating, *i. e.* enzyme limiting, conditions in hypotonically suspended plastids. They increasingly manifested themselves in relation to the progressive, hypotonically induced decay of chloroplast structure *in vitro*. The results of such an experiment, using DPIP as electron acceptor, are depicted in Fig. 1. A decrease in rate is noticed, in some experiments after an initial slight increase in activity, which can be partly reversed by addition of DPC. The finding argues – in line with current knowledge – for a gradual loss of oxygen evolving capability during the course of plastid degradation and an increasing substitution of DPC as electron donator.

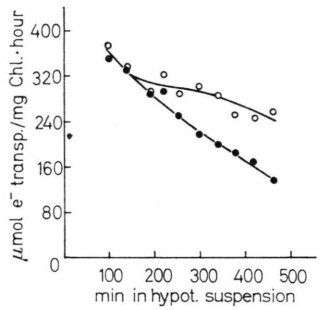

Fig. 1. Effect of DPC on DPIP-Hill activity of disintegrating chloroplasts as a function of time suspended in hypotonic medium. Spinach chloroplasts, suspended in 0.1 M phosphate buffer pH 6.8–1 mg/ml serum albumin, were assayed at the time indicated using red saturating light. ● ,Control; ○, DPC added.

The influence of DPC concentration on the measured rate of DPIP reduction is given in Table I. Maximum stimulation is obtained at 1 mM, the concentration used in the standard assay system. Addition of higher amounts of DPC led to inhibition of activity.

Table I. Rate dependence of the uncoupled DPIP-Hill activity on the concentration of added DPC.

DPC concentration [mM]	Relative activity
Control	100
0.1	114
0.2	129
0.5	139
0.8	152
1	145
2	136
3	106
4	67

The rate was measured 6 hours after preparation and hypotonic suspension of the chloroplasts. Actual rate of control: 290 µmol electrons transported × mg^{-1} chlorophyll × $hour^{-1}$, saturating red light.

2. *Light intensity measurements*

Light intensity curves which mirror the interaction between pigment complex and redox chain provide evidence whether only the electron transport enzymes are involved in the action of DPC.

It was observed, that during hypotonic degradation of chloroplasts the addition of DPC affected first the rate at low light intensities and only later a stimulation at saturating intensities could be noticed. Fig. 2 shows this sequence of light intensity curves taken at various states of plastid decay. The effect is especially pronounced at acidic assay conditions. The data suggest a change in the kinetic mechanism of the DPC response during the slow decay of the suspended chloroplasts.

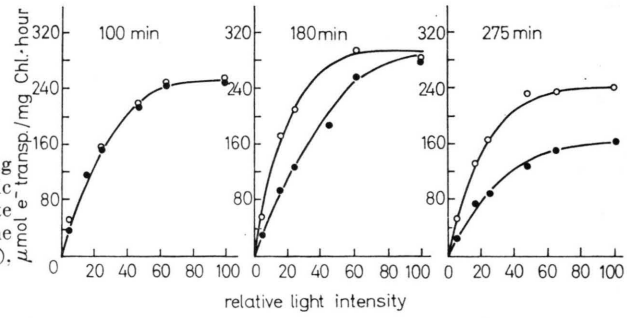

Fig. 2. Light intensity dependences of DPIP reduction at various states of chloroplast decay (given in min of hypotonic exposure) showing the two modes of action of DPC. Closed symbols, control; open symbols, DPC added.

When plotting the light intensity data in a reciprocal way [8–10], *i. e.* rate v against the ratio of rate over intensity v/I, one obtains the relative quantum yield $\alpha\Phi$ as the intercept of the curve with the

v/I axis. In this way an increase in $\alpha\,\Phi$ relative to the control was noticed upon addition of DPC during prolonged hypotonic exposure of the plastids (Fig. 3). It can be interpreted as a reversal by DPC of the decline observed in the control. This notion, however, is only valid if the amount of light absorbed by the pigments remains constant throughout the time course of plastid degradation.

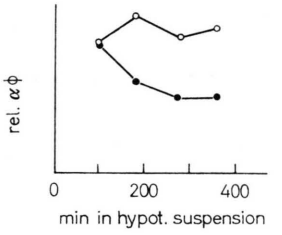

Fig. 3. Alteration with time in hypotonic suspension of the relative quantum yield as determined by the intersects on the v/I axis in reciprocal plots. ●, control; ○, DPC added.

Sorokin [11] published a kinetic expression for the dependence of v on I which allows to calculate the probability L_0 of a quantum being transformed into chemical energy within the photosynthetic unit it excites electronically. The equation provides a means to assess the influence of DPC in more detail. Using his relationship

$$\bar{v} = \frac{I - \left(\frac{v}{I}\right)_{\text{rel}}}{I - L_0\left(\frac{v}{I}\right)_{\text{rel}}}$$

where

$$\bar{v} = \frac{v}{v_{\max}}$$

$$\left(\frac{v}{I}\right)_{\text{rel}} = \frac{v/I}{(v/I)_{v=0}},$$

v = electron transport rate, I = incident light intensity, $v_{\max}$ = maximum attainable v_1 L_0 = maximum probability that a quantum harvested in a specific pigment unit will be transformed into chemical energy within the same unit,

the data of Fig. 2 were replotted in Fig. 4. The

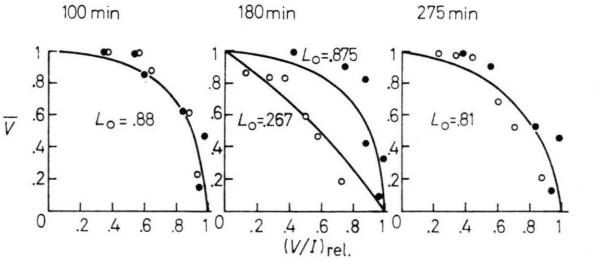

Fig. 4. Plot of the data of Fig. 2 according to Sorokin [14]. Same symbols as before. The lines represent theoretical curves constructed from best fit L_0 values.

curves are drawn using the best fitting L_0 values. Two features can be discerned. Firstly, L_0 in the controls changes very little during plastid degradation. Secondly, addition of DPC lowers the value of L_0, an effect especially pronounced in the early phases of plastid decay. This can be taken as evidence for an increase of the tendency of photon migration between photosynthetic units under the influence of DPC, resulting in higher quantum efficiency. Failure to decrease L_0 significantly in later stages through addition of DPC seems to indicate that the mechanism of DPC action changes, electron donating properties becoming dominant.

3. *Influence of DPC on PS I activity*

In this context additional information can be obtained using the ascorbate/DPIP → NADP reaction. In this type of assay the function of DPC as electron donor is blocked by DCMU. The effect of DPC on this system is summarized in Table II. At low light intensities NADP reduction decreases when a rate increase is noticed in the DPIP Hill reaction under

Table II. Effects of DPC on the reaction rates of various partial chloroplast reactions of hypotonically degrading plastids.

Time in hypotonic suspension [min]	$H_2O \rightarrow$ DPIP control	+DPC	$H_2O \rightarrow$ NADP control	+DPC	Asc./DPIP → NADP control	+DPC
90	79	86	42	28	35	28
160	73	86	32	23	31	23
230	31	92	26	20	33	25
330	29	63	48	48	30	29
465	38	48	28	24	37	37

Activity in μmol electrons transported × mg chlorophyll^{-1} × hour^{-1}; light intensity is 1.1% of lamp output in the red region or about 3% of saturating intensity. In saturating light the activity of the NADP-reductions was not influenced by addition of DPC.

the same conditions and no stimulation is as yet visible at saturating illumination.

It can also be seen that this phenomenon is transitory and cannot be observed after the plastids have passed a certain state of disarray. It is likely that the observed difference in PS I activity is even larger in actuality. This photosystem mediates the formation of diphenylcarbazone ($\varepsilon_{340} = 1.25 \times 10^3$) from DPC and its subsequent disproportionation to DPC and DPC_{ox} ($\varepsilon_{340} = 3.6 \times 10^3$) [4]. Thus a negative interference of these absorbing species, which cannot be fully corrected for, results in apparent activities higher than the actual rate. The $H_2O \rightarrow$ NADP system which behaves similarly to the asc./DPIP $\rightarrow$ NADP reaction upon addition of DPC is also included in the Table.

4. *Effect of DPC on fluorescence parameters*

The previous results again argue for an influence of DPC on the pigment systems of the chloroplasts and should be reflected also in fluorescence parameters which were examined next.

No qualitative difference was discerned in the excitation spectra of the blue region when compared to the untreated control. This indicates that no specific accessory pigment is added or lost in the light harvesting process upon addition of DPC. The chemical changed, however, the variables of the fluorescence rise kinetics (Fig. 5). The steady state

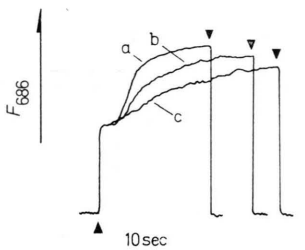

Fig. 5. Kinetics of chloroplast fluorescence in the presence of varying amounts of DPC, 150 min after exposure to hypot. medium. Excitation 650 nm, emission 684 nm. a, Control; b, .5 mM DPC; c, 1 mM DPC.

level F_∞ which indicates the amount of harvested but unused and, therefore, reemitted energy decreased while the rise time increased. Since the area above the induction curve which reflects the amount of primary acceptor Q present, remained relatively constant upon addition of DPC, the increased rise time suggests a better interaction between pigment system and electron carriers, *i. e.*, a higher efficiency.

Actual values of the various fluorescence signals in the presence and absence of DPC are collected in Table III. The data were obtained from plastid preparations which had been kept in hypotonic suspension for 6 hours to insure a maximum DPC effect. The only significant difference, besides the altered rise time, is seen in F_∞ and parameters containing this quantity. The decrease is around 25% as averaged over 5 different experiments.

Table III. Influence of DPC on life-fluorescence parameters of spinach chloroplasts suspended 6 hours in hypotonic medium (0.1 M phosphate buffer pH 6.8 — 1 mg/ml serum albumin).

Parameter	Control	+DPC
F_0	3.88 ± .69	3.63 ± .21
F_∞	7.35 ± .82	4.83 ± .42
F_{DCMU}	10.85 ± .6	9.95 ± .64
$F_\infty - F_0$	3.48 ± .19	1.2 ± .32
$F_{DCMU} - F_\infty$	3.5 ± .47	5.13 ± .35

All data in relative units, averaged from 4—6 consecutive determinations. Excitation at 650 nm, emission at 684 nm. The chlorophyll/Q ratio was 70.5 in either case. The Hill activity under saturating conditions was 138 μmol × mg Chl^{-1} × $hour^{-1}$ in the control and 7 μmol at 2.5% intensity. After addition of DPC the rates were 185 and 26 μmol respectively.

5. *Effect of DPC on oxygen evolution*

Shenyour and Avron [4], using a Clark type oxygen electrode, observed a reduction in oxygen evolution upon addition of DPC. They interpreted this finding as a competition between water and DPC as electron donor for photosystem II. Using their method, similar observations were made in the course of this study.

When, however, the plastids ability to evolve oxygen was assessed in the Warburg apparatus using

Table IV. Influence of added DPC on the rate of oxygen evolution in hypotonically treated spinach chloroplasts.

DPC conc. [mM]	DPIP acceptor rel. units	Quinone acceptor rel. units
control	100	100
.125	138	108
.25	151	—
.6	230	179
1.2	190	145

The actual rate of the controls (average from 3 different preparations and experiments) was 47 μl O_2 evolved × mg^{-1} chlorophyll × $hour^{-1}$ with DPIP, 170 μl O_2 evolved × mg^{-1} chlorophyll × $hour^{-1}$ with quinone as electron acceptor. The measurements were started 150 min after resuspension of the plastids in hypotonic medium. The actinic light used was non saturating.

either DPIP or quinone as electron acceptor, a different result was obtained (Table IV).

It was observed that oxygen evolution is not only undiminished upon addition of DPC but that a stimulation even takes place provided the structural damage has not progressed too far. The information gathered in this manner is, however, severely limited due to the unusually large amounts of plastids and acceptor necessary to sustain an observable reaction for a sufficient length of time. Meaningful $0/2\,e^{\ominus}$ rations which would greatly support the argument could not be obtained due to the same difficulties. The failure to obtain these results with the Clark electrode is likely due to an interference between DPC and the electrode due to their almost identical potential. Checks in the absence of plastids and reaction components showed a change in electrode response and characteristics after addition of DPC.

Discussion

The data measured under light saturating conditions agree with the results of Vernon and Shaw [1, 2] and confirm their interpretation of DPC as an electron donating system for PS II. This notion can be extended, based on the experiments concerning the oxygen evolving system, to include, that DPC provides electrons in addition to water, *i. e.*, it does not inactivate or replace oxygen evolution.

The effects of DPC under light limiting conditions provide further information on the possible arrangement of the different photosystems. The increase in both quantum efficiency and O_2-evolution upon addition of DPC in the early stages are an indication that the decline of activity observed in the controls must be due initially to hypotonically induced membrane disarray, leakage of essential PS II components becoming dominant only at later stages. It is conceivable that DPC migth act *e. g.* as link between the physically separated components bringing them into close contact again by either actually complexing them or by acting as a chemical intermediate.

The observed decrease in PS I activity under light limiting conditions might indicate that light normally harvested by PS II accessory pigments but transferred in some way to PS I (spillover?) is no longer available to the latter. It is conceivable that DPC increases the available number of open trapping centers in PS II so that the harvested light is converted more efficiently into chemical energy, resulting in an improved quantum yield and a decreased fluorescence F_∞. No surplus energy formerly transfered to PS I is available, thus the decrease in NADP reduction. Alternatively, DPC might close some of the traps of PS I, so that energy harvested by common accessory pigments is funneled preferentially to PS II. This notion is not favoured, since it would postulate an increase in F_{DCMU} upon addition of DPC, a feature not observed in actuality. Clearly, further experimentation is needed to resolve this question.

The results have so far been discussed under the aspect of the mechanism of DPC action only. However, the DPC effect is tightly correlated to the structural and functional integrity of the chloroplast, an association amply demonstrated in Fig. 1. Since the parameters of osmotically induced plastid degradation are known in detail [12–14] the described action of DPC can be correlated to the various intermediate states of chloroplast breakdown. It appears that it's action on quantum yield coincides with the early stages of pigment separation [14] while its donor properties become pronounced after transition to class II particles [12].

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