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THE REDUCTION OF ARTIFICIAL ELECTRON ACCEPTORS AT SUB-ZERO TEMPERATURES BY CHLOROPLASTS SUSPENDED IN FLUID MEDIA

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SUMMARY

1. Chloroplasts can be suspended in aqueous/organic mixtures which are liquid at sub-zero temperatures with a good retention of the ability to reduce artificial electron acceptors. The reduction of ferricyanide and 2,6-dichlorophenolindophenol at temperatures above 0 °C is about 50 % inhibited by 50 % (v/v) ethylene glycol. Higher concentrations cause more extensive inhibition.

2. Different solvents were compared on the basis of their ability to cause a given depression of the freezing point of an aqueous solution. Ethylene glycol caused less inhibition of electron transport than glycerol, which in its turn was found to be superior to methanol.

3. The reduction of oxidised 2,3,5,6-tetramethyl-*p*-phenylenediamine could be measured at −25 °C in 40 % (v/v) ethylene glycol. Using an acceptor with a high extinction coefficient, methyl purple (a derivative of 2,6-dichlorophenolindophenol) it was possible to observe electron flow at temperatures as low as −40 °C in 50 % (v/v) ethylene glycol.

4. From studies of the effects of the inhibitors 3(3,4-dichlorophenyl)-1,1-dimethylurea and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone it is suggested that electron flow from the donor side of Photosystem II to the acceptor side of Photosystem I can occur at temperatures at least as low as −25 °C. The ultimate electron donor is presumably water but it was not possible to demonstrate this directly.

INTRODUCTION

One of the most powerful techniques for the study of biochemical reactions is the investigation of the effect of lowering the temperature. This is of particular interest in the case of a complex sequence of reactions such as the electron transport

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Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCMU, 3(3,4-dichlorophenyl)-1,1-dimethylurea; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DMSO, dimethylsulphoxide.

chain of chloroplasts. At temperatures between −60 and 0 °C, one should observe not only the primary photochemical reactions, but also the secondary reactions, to an extent which should increase as the temperature is raised. Experiments at such temperatures can be carried out in liquid media as well as in the frozen state.

Until recently the investigation of biochemical reactions in fluid media at sub-zero temperatures was hindered by a lack of data on the physico-chemical properties on the aqueous/organic solvent mixtures necessary. Such data has now been obtained in this laboratory [1, 2] together with experience with a variety of isolated enzymes [2] which leads to the conclusion that conditions can be found under which the activity of most enzymes can be maintained in aqueous/organic mixtures below 0 °C.

The use of a liquid medium has obvious advantages in the study of chloroplast reactions in avoiding the changes associated with freezing and in allowing experiments involving the diffusion of small molecules to the chloroplasts. The possibility of studying chloroplast reactions in media with freezing points below that of water is thus potentially of great interest.

The advantages of a liquid medium cannot be obtained without the possible problems associated with the addition of high concentrations of organic solvents. However, Inoué and Nishimura [3] showed that chloroplasts could be suspended in 50 % (v/v) ethylene glycol with at least 50 % retention of the ability to photoreduce the electron acceptor DCIP.

A number of reports of experiments to investigate the behaviour of the chloroplast electron transport chain at sub-zero temperatures have appeared. Most of these have concerned the behaviour of chloroplasts in frozen suspensions, or in glasses or extremely viscous solutions such as those obtained with glycerol at low temperatures. The only experiments in reasonably fluid media are those of Amesz et al. [4] who recently reported light-induced absorbance changes at −40 °C in chloroplasts suspended in 50 % (v/v) ethylene glycol. The viscosity of solutions containing ethylene glycol is considerably less than those containing glycerol.

The results reported in this paper are an investigation of the photoreduction of artificial electron acceptors by chloroplasts suspended in aqueous/organic mixtures at sub-zero temperatures. They were performed in order to investigate the possibilities of applying this technique to the study of photosynthetic electron transport, and to provide some information about the effect of solvents and temperature on chloroplast reactions under these conditions. Most of the previous experiments involving chloroplasts suspended in high concentrations of polyols seem to have been motivated by the desire to obtain an optically clear medium. The present experiments were rather concerned with exploiting to the full the advantages of a liquid medium in extending the range of possible types of experiment.

MATERIALS AND METHODS

Chloroplast preparation

Plants of spinach (*Spinacea oleracea*) and lettuce (*Lactuca sativa*, "Cos" variety) were obtained from local markets. Chloroplasts were prepared by the method of Avron [5] and stored frozen at −25 °C in the grinding medium (without ascorbate) to which 5 % (v/v) DMSO had been added.

Aqueous/organic mixtures

The chloroplasts were generally suspended in a medium containing ethylene glycol (E. Merck, Darmstadt), and potassium phosphate buffer. The buffer was adjusted to give an "effective" pH (pH*) of pH* 7.6 or pH* 7.9 at 20 °C at each concentration of solvent using the data given in ref. 1 and extrapolating where necessary. The effects of glycerol on the buffer were assumed to be similar to those of ethylene glycol. All concentrations given are final concentrations after the addition of the organic solvent.

The aqueous/organic mixture was precooled to 0 °C in an ice bath before the addition of chloroplasts. A few minutes were allowed for equilibration before the temperature was lowered to below 0 °C.

Measurement of the reduction of artificial acceptors

The reduction of artificial electron acceptors was measured spectrophotometrically using Cary 15 and Aminco-Chance spectrophotometers fitted with an attachment enabling the temperature of the sample to be varied [6] and equipped for side illumination. Light from a 450 W Xenon arc lamp was passed through 15 mm water and a Balzers K7 Broad band interference filter. The photomultiplier was protected with an 8-mm thickness of saturated $CuSO_4$ solution. The optical path length was 1 cm. The light intensity used was saturating for the reduction of DCIP at room temperature.

The reduction of artificial electron acceptors was always measured in the presence of NH_4Cl to eliminate any effects due to uncoupling by the organic solvent.

The reduction of ferricyanide was measured at 400 nm. The reduction of DAD was measured as the reduction of excess ferricyanide. The reaction medium at pH* 7.6 contained 25 mM potassium phosphate, 25 mM NaCl, 0.8 mM $K_3Fe(CN)_6$, 0.2 mM DAD (when present), 1.0 mM NH_4Cl and chloroplasts at a concentration equivalent to 10 μg chlorophyll/ml.

"Methyl purple" was the kind gift of Dr R. Hill. It is a methyleneoxyindophenol (5, 6′) with one methyl group on the methylene carbon (structure XV in ref. 7 with $R_1 = CH_3$, $R_2 = H$). Its reduction was followed at 592 nm (the reference wavelength for the Aminco-Chance in the dual wavelength mode was 530 nm). The reaction medium at pH* 7.9 contained 0.2 M sucrose, 25 mM potassium phosphate, 10 mM NaCl, 2.5 mM $MgCl_2$, 12 μM methyl purple and 3.3 mM NH_4Cl, and chloroplasts at a concentration equivalent to 60 μg chlorophyll/ml.

The reduction of DCIP was measured at 590 nm. The medium contained 20 mM potassium phosphate pH* 7.0 and 10 mM NaCl. The reaction mixture contained 40 μM DCIP and chloroplasts equivalent to a chlorophyll concentration of 20 μg/ml.

The difference extinction coefficient of methyl purple, $\Delta E_{592-530\ \mathrm{nm}}$, in aqueous alkaline solution is $37\ \mathrm{cm}^{-1} \cdot \mathrm{mM}^{-1}$. The hyperchromic effect observed on the addition of ethylene glycol was partially compensated by a shift of the absorption maximum towards the blue and the results are not corrected for the effect of solvent on the extinction coefficient. The pK of methyl purple is 6.5, such that it is necessary to work at a fairly alkaline pH if the maximum absorption change is to be seen.

For the measurement of the spectrum of the absorption changes attributed to methyl purple at wavelengths greater than 590 nm, the chloroplasts were illuminated with light passed through a 425-nm broad band filter (Intervex DA, MTO) and a piece of blue glass which had similar absorption properties to $CuSO_4$. The photomultiplier was protected with a Pyrex No. 3482 filter and the fluorescence artefact reduced by placing 2 mm of saturated $CuSO_4$ solution between the sample and the photomultiplier. The reference wavelength in this case was 550 nm.

Chlorophyll was measured by the method of Arnon [8].

RESULTS

Fig. 1 shows a comparison of the effects of various solvents on the reduction of DAD in the presence of excess ferricyanide. For experiments in mixed solvents at low temperatures, the important parameter is the extent to which the freezing point can be depressed. Fig. 1 shows that for a given depression of freezing point ethylene glycol is superior to glycerol or methanol. Similar results were obtained when comparing the effects of ethylene glycol and glycerol on the reduction of DCIP (data not shown).

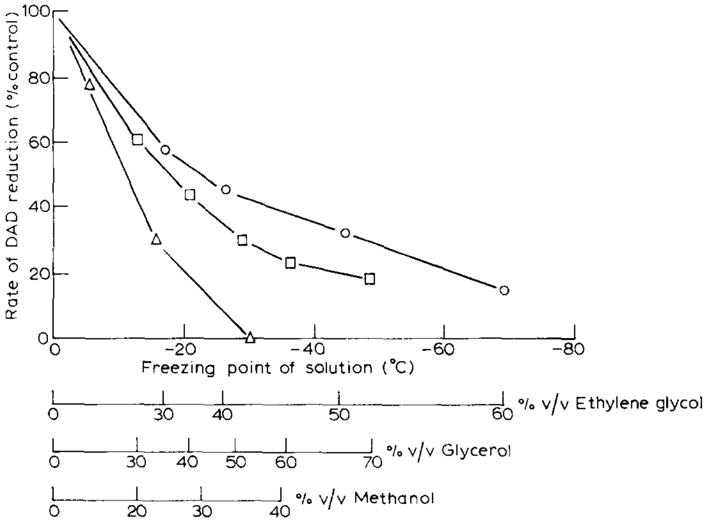

Fig. 1. Comparison of the inhibition of the reduction of DAD by spinach chloroplasts with the depression of the freezing point for three different solvents. DAD reduction was measured at 3 °C as described in Materials and Methods. ○, ethylene glycol; □, glycerol; △, methanol. The absolute activity of the control chloroplasts was 150 μmol ferricyanide reduced /mg chlorophyll per h at +3 °C.

The effect of ethylene glycol on the reduction of artificial electron acceptors by chloroplast preparations was found to vary somewhat between different batches of chloroplasts (Table I). The use of methyl purple was introduced by Hill [7]. It has a higher extinction coefficient than DCIP and an absorption spectrum which overlaps less with that of chlorophyll. At room temperature, in the absence of organic solvents, the reduction of methyl purple was at least 90 % inhibited by 5 μM DBMIB; this suggests that reduction is mostly after Photosystem I since its potential is too low for it to be reduced by components between the site of action of DBMIB and P700.

TABLE I

THE EFFECT OF DIFFERENT CONCENTRATIONS OF ETHYLENE GLYCOL ON THE REDUCTION OF ARTIFICIAL ELECTRON ACCEPTORS BY SPINACH CHLOROPLASTS

The reduction of methyl purple and ferricyanide was measured in a medium containing 0.2 M sucrose, 25 mM potassium phosphate buffer, pH* 7.6, 25 mM NaCl and 1 mM $MgCl_2$ (final concentrations) plus varying amounts of ethylene glycol. The reduction of DCIP was measured at 20 °C; the reduction of the other acceptors was measured at 3 °C. Other details were as given in Materials and Methods. Each value represents the mean of at least three separate experiments except for the values in parentheses which are the means of two experiments only. The values given are percentages of the control rate in the absence of ethylene glycol.

	Concentration ethylene glycol (%, v/v)					
	0	30	40	50	60	70
Freezing point of solution:	0 °C	−17 °C	−26 °C	−44 °C	−69 °C	< −100 °C
Photosystem II Acceptor						
DAD	100	67	47	35	51	
Photosystem I Acceptors						
DCIP	100		63	55	(31)	
Methyl purple	100		54	49	26	(1)
Ferricyanide	100			54	(23)	
All Photosystem I Acceptors						
Range			46–67	36–71	9–39	
Numbers of experiments			6	14	7	

Table I suggests that the inhibition of the reduction of oxidised DAD is greater than the inhibition with other acceptors. Table II shows that the reduction of ferricyanide is stimulated by DAD, and the effect is proportionately less in the presence of ethylene glycol. DAD was placed in Class III in the classification of Saha et al. [9] that is, it was a lipid-soluble compound which accepted electrons from a site between the two photosystems, presumably from the plastoquinone pool.

TABLE II

EFFECT OF ETHYLENE GLYCOL ON FERRICYANIDE REDUCTION IN THE PRESENCE OR ABSENCE OF DIAMINODURENE

Spinach chloroplasts (9 μg chlorophyll/ml) were suspended in a medium containing 25 mM potassium phosphate buffer, pH* 7.6, and 25 mM NaCl. Ferricyanide reduction in the presence of 1 mM NH_4Cl was measured as described in Materials and Methods.

Concentration of ethylene glycol (% v/v)	Rate of ferricyanide reduction at +3 °C (μmol/mg chlorophyll per h)	
	No DAD	+0.2 mM DAD
0	120	200
40	83	93
50	53	62

The data suggest that DAD allows the normal rate-limiting step for the reduction of ferricyanide after Photosystem I [10] (i.e. the step following plastoquinone [11]) to be bypassed. This effect is apparently reduced by the solvent.

The inhibitory effects of high concentrations of ethylene glycol could be at least partially reversed by the addition of water to lower the organic solvent concentration, in agreement with the results of Inoué and Nishimura [3].

There was no evidence that the optimum pH* for the reduction of ferricyanide in the presence of ethylene glycol was significantly different from the optimum pH in the absence of solvent.

The effects of the solvent seemed to depend relatively little on the actual conditions used. No better results were obtained with more complex media containing sucrose or $MgCl_2$ than in a simple potassium phosphate buffer and NaCl medium. There was also no significant effect of changing the experimental protocol so that cold ethylene glycol (0 °C) was very slowly added to an aqueous chloroplast suspension maintained in an ice bath. This procedure should eliminate effects caused by the chloroplasts being transiently subjected to a medium of high effective osmotic strength due to the membrane being less permeable to ethylene glycol than to water, as has been suggested [12]. Chloroplasts lost about 15 % of their ability to reduce DCIP after 1 h of storage at 0 °C in 50 % (v/v) ethylene glycol. The effect of 50 % (v/v) glycerol was similar.

Fig. 2 shows the effect of temperature on the reduction of DAD in the presence of excess ferricyanide. Above 0 °C the apparent activation energy in the presence and absence of 40 % (v/v) ethylene glycol is the same (4.2 kcal/mol). At sub-zero temper-

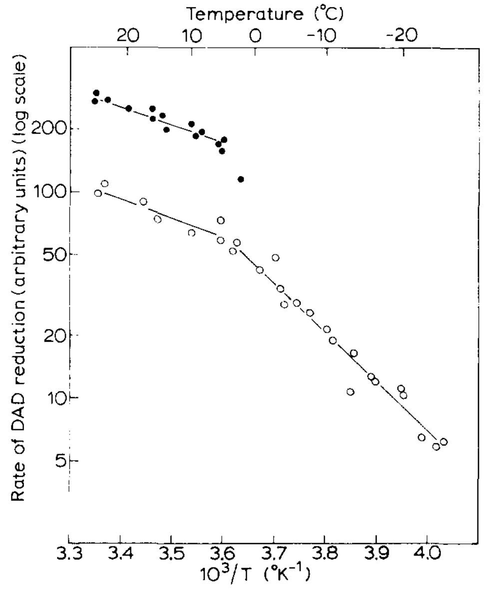

Fig. 2. Arrhenius plot for the reduction of DAD by spinach chloroplasts suspended in a medium containing 40 % (v/v) ethylene glycol (○) and in an aqueous medium (●). The experimental details are described in Materials and Methods. The results from several batches of chloroplasts have been normalised.

atures, there is an increase in the slope of the Arrhenius plot (apparent activation energy, 11.5 kcal/mol) but the reduction of the acceptor can be observed at temperatures as low as −25 °C. Higher concentrations of DCMU than those normally effective were required for inhibition at low temperatures in the presence of ethylene glycol. The reaction at −18 °C was 50 % inhibited by 10 μM DCMU and completely inhibited by 100 μM DCMU. (Control chloroplasts in the absence of the solvent at room temperature were completely inhibited by 10 μM DCMU, the lowest concentration tested).

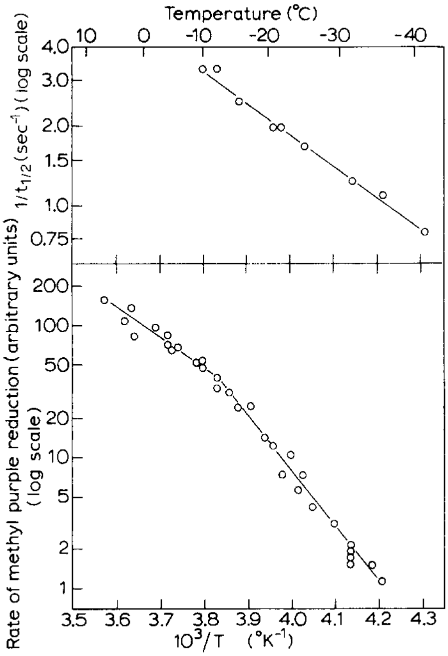

Fig. 3. Arrhenius plot for the reduction of methyl purple by spinach chloroplasts suspended in a medium containing 50 % (v/v) ethylene glycol. Upper plot, fast phase. Lower plot, linear phase. The experimental details are described in Materials and Methods.

The measurement of the rate of DAD reduction at low temperatures is limited by the low extinction coefficient of the final acceptor, ferricyanide. By using methyl purple as the acceptor it was possible to observe photoreduction at temperatures as low as −40 °C in 50 % (v/v) ethylene glycol. The reduction of methyl purple is clearly biphasic at lower temperatures, a rapid, first-order phase being followed by a slower linear phase. The fast phase seems to have a lower activation energy than the linear phase, resulting in an improved resolution as the temperature is lowered. The actual values of the measured activation energies varied from sample to sample; for one sample of spinach chloroplasts the activation energy of the fast phase was 5.5 kcal/mol whilst the Arrhenius plot for the slow phase was complex showing an apparent activation energy of 10.5 kcal/mol down to about −10 °C and 20 kcal/mol between −15 and −35 °C (Fig. 3).

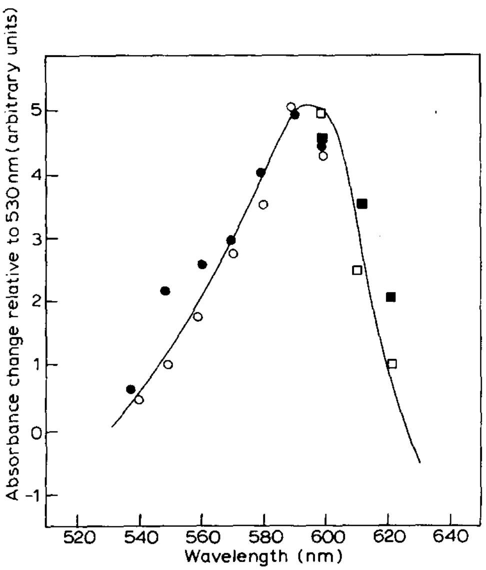

Fig. 4. Comparison of spectra of the fast phase (●, ■) and linear phase (○, □) of the absorbance changes observed on the illumination of chloroplasts in the presence of methyl purple, with the difference spectrum of chloroplasts with and without methyl purple under the same conditions (solid line). Lettuce chloroplasts were suspended at −32 °C in the medium described in Materials and ethylene glycol. ○, ●, change on illumination with red light; □, ■, change on illumination with blue Methods containing 50 % (v/v) light. Further details are given in Materials and Methods.

In Fig. 4, the spectra of the fast and slow phases are compared with the absorption spectrum of methyl purple under the same conditions. Apart from a shoulder in the cytochrome α-band region of the spectrum in the fast phase, both phases have similar spectra in agreement with those expected for methyl purple reduction.

In the temperature range investigated (down to −10 °C) the apparent activation energy for the reduction of DCIP was similar to that obtained for methyl purple. This was the case in both 50 % and 40 % (v/v) ethylene glycol and 40 % (v/v) glycerol and for aqueous buffer above 0 °C. In all these cases, the values ranged from 10 to 12 kcal/mol. The temperature dependence of the reaction is thus independent of the solvent, and the conclusions drawn above about the superiority of ethylene glycol at +3 °C are valid at lower temperatures.

Under suitable conditions, the slow phase, but not the fast phase, of methyl purple reduction can be abolished in the presence of DCMU (Fig. 5). This effect is seen most clearly following a period of illumination in the presence of DCMU. It is noteworthy that there is a reoxidation of methyl purple in the dark under these conditions; the extent of this seems comparable to the fast phase of reduction. The experiments shown in Fig. 5 were performed at −10 and −20 °C. The results at −30 °C seemed basically similar but the dark reoxidation was much slower. The need for a preillumination in the presence of DCMU before the slow phase is abolished is probably due to the plastoquinone pool remaining reduced in the dark at low temperatures, as suggested by Thorne and Boardman [13] on the basis of fluorescence experiments.

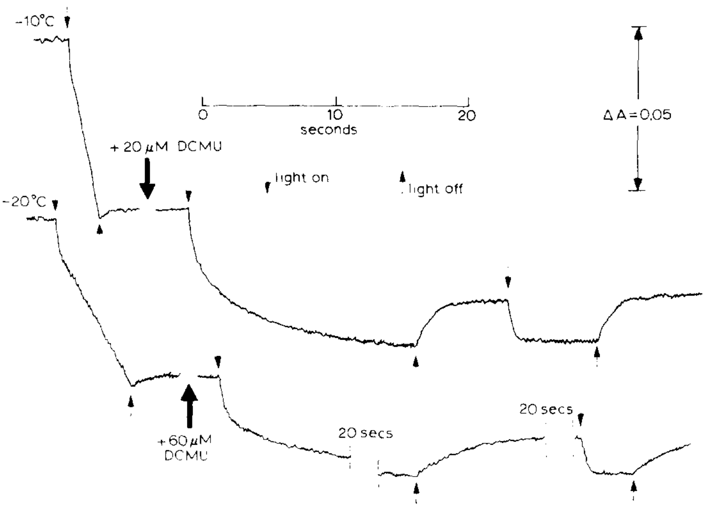

Fig. 5. Absorbance changes (592 – 530 nm) caused by illumination of spinach chloroplasts suspended in a medium containing 50 % (v/v) ethylene glycol and methyl purple. Experimental conditions for the measurement of methyl purple reduction are described in Materials and Methods.

As was the case with DCMU, higher concentrations of DBMIB were required for inhibition in ethylene glycol/water mixtures at low temperatures than in aqueous media. This problem limits the value of DBMIB as an inhibitor under these conditions because of the possibility of the quinone acting as an acceptor in its own right, at the concentrations needed for a high degree of inhibition. However 50 % inhibition of methyl purple reduction was produced by 5 μM DBMIB in chloroplasts suspended in 50 % (v/v) ethylene glycol medium at −25 °C.

Kok et al. [14] and Ke [15] investigated the kinetics of reduction of the acceptor DCIP at room temperature using rapid reaction techniques and found evidence for a fast initial reduction as well as a slower continuous phase. This fast phase was interpreted as being the result of the reaction of DCIP with a component on the reducing side of Photosystem I. The result described here for methyl purple at sub-zero temperatures would support a similar explanation. The rapid change represents the oxidation of a small pool on the donor side of Photosystem I after the rate-limiting step and the site of action of DCMU. The size of this pool corresponds to about 1 equivalent for each 400–500 molecules of chlorophyll. The linear phase of methyl purple reduction probably represents the flow of electrons from the donor through the rate-limiting step to Photosystem I, though a contribution of unknown size may be due to direct reduction of the acceptor from a site between the two photosystems. However, if the interpretation of the results in Fig. 5 given above is correct, the observation that the pool remains reduced in the dark in the presence of methyl purple makes it unlikely that a significant proportion of the slow phase is due to reduction from the pool, and the potential of methyl purple is too low for reduction by components on the oxidising side of Photosystem I.

DISCUSSION

The results reported in this paper show that experiments performed in liquid media below 0 °C are a possible addition to the available techniques for the study of chloroplasts. It appears that the chloroplast electron transport chain is relatively resistant to high concentrations of polyols. Electron flow can be observed at temperatures as low as −40 °C in 50 % (v/v) ethylene glycol. It cannot be stated definitely that water is the electron donor in this case. However, the results of Joliot [17] who observed that continuous illumination at −30 °C caused 6–7 reducing equivalents to be transferred into the plastoquinone pool, provide additional indirect evidence that the water-oxidizing system is able to turnover at surprisingly low temperatures.

In any case, on the basis of the evidence presented in this paper, it seems reasonable to suppose that electron transport from the donor side of Photosystem II to the acceptor side of Photosystem I can occur in chloroplasts suspended in 50 % (v/v) ethylene glycol at temperatures at least as low as −25 °C. Evidence for donation before Photosystem II is provided by inhibition of methyl purple reduction by DCMU and by the reduction of DAD; evidence for reduction after Photosystem I is provided by the biphasic kinetics of methyl purple reduction and the effect of DCMU on this, and also by the inhibition of methyl purple reduction by DBMIB.

The inhibition caused by the solvent seems more likely to be due to a general alteration of the membrane structure than to inhibition at a specific site. The degree of inhibition is less than might be expected for a system involving a number of proteins reacting to the presence of the solvent in different ways. Possibly the small effect is due to many of the components being in a hydrophobic environment in the interior of the membrane and thus more protected from the medium than isolated enzymes.

The "activation energies" for the various reactions obtained from the Arrhenius plots were found to be rather variable from sample to sample, and there is anyway probably little significance in the actual values in such a complicated situation. Apparent "break points" in the Arrhenius plots similar to those observed here were reported by Shneyour et al. [17] for the reduction of various acceptors above 0 °C, and interpreted as the result of phase changes in the membrane lipids.

It is interesting to observe that the concentrations of both DCMU and DBMIB required for inhibition were much greater in the presence of 50 % (v/v) ethylene glycol than they were in aqueous solutions. This may be due to effects on the partition of the inhibitor between the solvent and the hydrophobic phase of the membrane.

The observation of the biphasic kinetics of methyl purple reduction at low temperatures provides an excellent illustration of the potential value of the technique. A phenomenon which could previously only be observed by fast reaction techniques has now been brought into the time range where it can be studied in an ordinary spectrophotometer. The way is now open for the study of the behaviour of individual chloroplast components using this technique.

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