Biochimica et Biophysica Acta, 440 (1976) 461–475

BBA 47160

MULTI-TEMPERATURE EFFECTS ON HILL REACTION ACTIVITY OF BARLEY CHLOROPLASTS

WILLIAM G. NOLAN and ROBERT M. SMILLIE

Plant Physiology Unit, C.S.I.R.O. Division of Food Research and School of Biological Sciences, Macquarie University, North Ryde 2113, Sydney (Australia)

(Received April 6th, 1976)

SUMMARY

1. The relationship between temperature and Hill reaction activity has been investigated in chloroplasts isolated from barley (*Hordeum vulgare* L. cv. Abyssinian).

2. An Arrhenius plot of the photoreduction of 2,6-dichlorophenolindophenol (DCIP) showed no change in slope over the temperature range 2–38 °C. The apparent Arrhenius activation energy (E_a) for the reaction was 48.1 kJ/mol.

3. In the presence of an uncoupler of photophosphorylation, methylamine, the E_a for DCIP photoreduction went through a series of changes as the temperature was increased. Changes were found at 9, 20, 29 and 36 °C. The E_a was highest below 9 °C at 63.7 kJ/mol. Between 9 and 20 °C the E_a decreased to 40.4 kJ/mol and again to 20.2 kJ/mol between 20 and 29 °C. Between 29 and 36 °C there was no further increase in activity with increasing temperature. The temperature-induced changes at 9, 20 and 29 °C were reversible. At temperatures above 36 °C (2 min) a thermal and largely irreversible inactivation of the Hill reaction occurred.

4. Temperature-induced changes in E_a were also found when ferricyanide was substituted for DCIP or gramicidin D for methylamine. The addition of an uncoupler of photophosphorylation was not required to demonstrate temperature-induced changes in DCIP photoreduction following the exposure of the chloroplasts to a low concentration of cations.

5. The photoreduction of the lipophilic acceptor, oxidized 2, 3, 5, 6-tetramethyl-*p*-phenylenediamine, also showed changes in E_a in the absence of an uncoupler.

6. The temperature-induced changes in Hill activity at 9 and 29 °C coincided with temperature-induced changes in the fluidity of chloroplast thylakoid membranes as detected by measurements of electron spin resonance spectra. It is suggested that the temperature-induced changes in the properties and activity of chloroplast membranes are part of a control mechanism for regulation of chloroplast development and photosynthesis by temperature.

Abbreviations: DAD_{ox}, oxidized 2,3,5,6-tetramethyl-*p*-phenylenediamine; DCIP, 2,6-dichlorophenolindophenol; 16NS, 3-oxazolidenyloxy-2-(14-carbmethoxytetradecyl)-2-ethyl-4,4-dimethyl; E_a, Arrhenius activation energy.

INTRODUCTION

The early conceptual development of photosynthesis was advanced by studying the process at various temperatures [1, 2]. In recent years measurements of reactions at different temperatures have been employed to study dynamic properties of membranes and membrane-associated enzymes in a variety of organisms. The effect of temperature on the physical state of membrane lipids and the activation energy of membrane enzymes has received much attention [3], and in plants, temperature-induced changes in the properties and activities of mitochondrial and chloroplast membranes have been linked to the phenomenon of chilling sensitivity [4, 5].

Shneyour et al. [6] have reported that chloroplasts isolated from the chilling-sensitive plants bean and tomato show a marked increase in Arrhenius activation energy (E_a) for $NADP^+$ photoreduction at low temperatures. The temperature-induced increase in E_a was reversible and occurred at temperatures lower than 12 °C, a temperature below which the plants develop symptoms of chilling injury. This response of $NADP^+$ photoreduction to temperatures below 12 °C was attributed to a temperature-induced change in membrane components associated with the enzyme ferredoxin-$NADP^+$ reductase (EC 1.6.99.4) [3, 6]. When the Hill reaction was assayed using either 2,6-dichlorophenolindophenol (DCIP) or diquat instead of $NADP^+$ as the acceptor, there was no change in E_a over the range of 4 °C to about 25 °C. Chloroplasts isolated from the chilling-resistant plants, lettuce and pea, showed a linear Arrhenius plot for Hill reaction activity within the same temperature range even when $NADP^+$ was used as the oxidant.

In this report, we present data demonstrating the existence of temperature-induced, reversible changes in E_a for Hill reaction activity using DCIP, oxidized 2,3,5,6-tetramethyl-*p*-phenylenediamine (DAD_{ox}) and ferricyanide as Hill oxidants. Further, these temperature-induced changes are shown to occur at physiological growth temperatures in chloroplasts from barley, a chilling-resistant plant.

MATERIALS AND METHODS

Plant growth. Barley plants (*Hordeum vulgare* L. cv. Abyssinian) were grown in vermiculite in a constant temperature room at 22 °C. A photoperiod of 16 h was used and light of 2200 lux was obtained from an array of Grow-lux fluorescent tubes and incandescent bulbs. The leaf material used for chloroplast isolation was the terminal 5 cm region of leaves from 6- to 11-day-old plants.

Chloroplast isolation. Chloroplasts were isolated (at 0–4 °C) in a medium of 0.05 M Sørensen's phosphate buffer (pH 7.5), 0.05 M NaCl and 0.5 % (w/v) bovine serum albumin. Leaf segments were blended in a Sorvall Omnimix operated at 0.75 of line voltage for four periods of 5 s each. The homogenate was filtered through one layer of Miracloth and the filtrate centrifuged at $200 \times g$ for 90 s. Chloroplasts were isolated from the supernatant by centrifuging for 10 min at $1000 \times g$. The chloroplasts were then washed twice in the blending medium with 15 min centrifugations at $12\,000 \times g$ and resuspended in the same medium. Morphologically, the preparation consists of loosely connected intact thylakoids with a small percentage of the lamellae remaining closely appressed in grana-like structures. The usual practice of including sucrose in the isolation medium and at a reduced concentration in the

assay medium was avoided to eliminate possible time and temperature-dependent osmotic effects during the assay of Hill activity.

Low salt treatment of chloroplasts. Chloroplasts were isolated as above but washed only once in the blending buffer and then suspended in distilled water (pH 7.6) at a chlorophyll concentration of approx. 75 μg/ml. The chloroplasts were centrifuged at 14 500 × *g* for 15 min, resuspended in distilled water at the same chlorophyll concentration, and allowed to stand for 15 min at 0 °C prior to centrifugation at 14 500 × *g* for 15 min. The chloroplasts were washed twice in the blending medium with 15 min centrifugations at 12 000 × *g*, resuspended in the same medium, and kept at 0 °C for a further 30 min.

Assays. Photochemical reactions were measured in an Aminco-Chance dual wavelength spectrophotometer equipped with a temperature-controlled cuvette compartment as previously described [6]. The cuvette compartment was continuously flushed with dry N_2 gas to prevent condensation. The wavelengths used were 575 nm minus 550 nm for DCIP reduction and 430 nm minus 450 nm for ferricyanide or DAD plus ferricyanide reduction. The basic reaction mixture (0.75 ml) consisted of 45 mM Sørensen's phosphate buffer (pH 7.5), 45 mM NaCl, 0.045 % (w/v) bovine serum albumin and 3 μg chlorophyll. The electron acceptors used were 21 μM DCIP, 340 μM $K_3[Fe(CN)_6]$, or 0.61 mM DAD plus 1.52 mM ferricyanide. The uncouplers when included were 74 mM methylamine (pH 7.6) or 4 μg/ml gramicidin D. The reaction mixture (minus chloroplasts) was pre-equilibrated to the required temperature. The chloroplasts, stored at 0 °C, were added in a small volume (10 μl) to the reaction mixture. The cuvette containing the reaction mixture was then placed in the cell compartment and after a further equilibration period of 2 min the chloroplasts were assayed for photochemical activity. The temperature of the reaction mixture was measured with a calibrated thermocouple just before and immediately after the completion of the photochemical assay. The variation between the two temperature readings was small (±0.2 °C or less) and the average value was used in the calculations.

The activities were calculated from steady-state rates, i.e. from rates linear with time after any initial transient, except for the DAD plus ferricyanide reactions and for most of the reactions at temperatures higher than 35 °C. Reactions involving DAD were slightly biphasic and the initial linear portion of the recorder traces were used in the calculations of the data presented. The same temperature effects were seen when the steady-state values were used. The activities for uncoupled reactions at temperatures higher than 35 °C were estimated because of a decline in the rates with time. In the case of reactions assayed in the absence of an uncoupler the rate did not decline with time until temperatures in excess of 37 °C were reached. A dark oxidation was sometimes observed at temperatures higher than 30 °C and the rate of reduction in the light was corrected for this. The $\Delta\varepsilon$ (mM) for ferricyanide decreased by approx. 1 % per 5 °C increase in temperature and rates for reactions involving ferricyanide were accordingly corrected. Lines of best fit through the data points, correlation coefficients, and *t*-test values were determined as described [7]. The correlation coefficients (r) for the lines drawn were in all cases greater than 0.97 and in most cases greater than 0.99. The P values from the *t*-test determinations are given in the figure legends.

Electron spin resonance spectra using the spin label 3-oxazolidenyloxy-2-

(14-carbmethoxytetradecyl)-2-ethyl-4,4-dimethyl (16NS) were recorded as described by Raison and Chapman [7]. Chlorophyll was determined according to Arnon [8].

RESULTS

Temperature dependence of the Hill reaction activity of barley chloroplasts

Fig. 1 shows the relationship between temperature and the Hill activity of isolated barley chloroplasts as measured by the photoreduction of DCIP. In this and the experiments which follow the results are presented as an Arrhenius plot, that is, the logarithm of the rate of Hill reaction activity (μmol DCIP or ferricyanide reduced · mg chlorophyll^{-1} · h^{-1}) is shown as a function of the reciprocal of the absolute temperature. The results obtained for the barley chloroplasts agree with results previously published for pea, bean and maize chloroplasts which showed that a linear relationship existed between the logarithm of the rate of DCIP photoreduction and the absolute temperature in the temperature range of 4 °C to about 25 °C [6]. In the case of the barley chloroplasts this range can be extended to 38 °C (Fig. 1). The E_a was 48.1 kJ/mol. Above 38 °C Hill activity declined rapidly with time and after the 2 min incubation in the dark at 42 °C, only a small activity could be detected and this rapidly declined to zero within the first min of illumination.

The use of spin-labeled probes has, at least in chloroplasts isolated from chilling-sensitive plants, pointed to a decrease in chloroplast membrane fluidity at chilling temperatures [3]. The observed effect of low temperature on the E_a of $NADP^+$ photoreduction by bean and tomato chloroplasts [6] was not considered to be a specific one on the enzyme ferredoxin-$NADP^+$ reductase, but rather a more

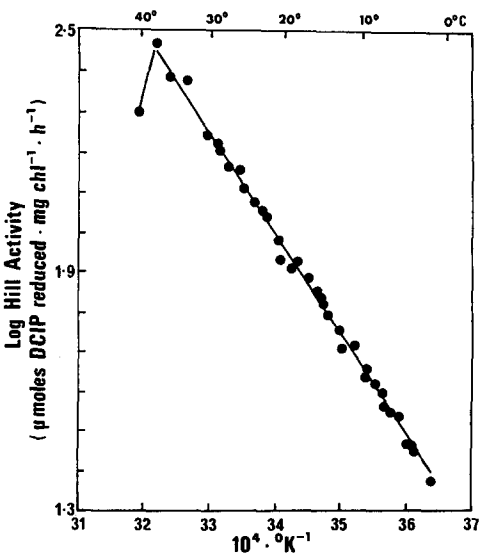

Fig. 1. Arrhenius plot of DCIP reduction. The results from two chloroplast preparations have been normalized and corrections for fall-off in activity of the preparations with time have been made (see text).

generalized action of temperature on the properties of chloroplast membranes. Thus temperature-induced effects might possibly have been expected for photosynthetic electron transport reactions other than those involving ferredoxin-$NADP^+$ reductase. While the earlier results [6] and those shown in Fig. 1 gave no indication of this, it was possible that the photoreduction of DCIP was limited by a reaction showing a constant E_a for the temperature range used in these experiments. Eliminating or circumventing this step might then reveal temperature-induced effects on photosynthetic electron transport if they exist. For these reasons the experiment shown in Fig. 1 was modified by subjecting the barley chloroplasts to treatments known to increase markedly the rate of the Hill reaction.

The inclusion of methylamine in the reaction mixture resulted in a 4–6-fold increase in Hill reaction activity with either DCIP or ferricyanide as the acceptor. Fig. 2 shows an Arrhenius plot for DCIP photoreduction by barley chloroplasts in the presence of methylamine. Four effects of temperature on DCIP photoreduction, at around 9, 20, 29 and 36 °C, can be seen. Below 9 °C the Arrhenius activation energy of the reaction was 63.7 kJ/mol. Above 9 °C the E_a decreased to 40.4 kJ/mol and above 20 °C the E_a decreased further to 20.2 kJ/mol. Between 29 and 36 °C the Hill activity steadily declined with increasing temperature. In this temperature range the values for Hill activity on an Arrhenius plot fitted either a straight line or else a slightly curved one. Since there is some uncertainty as to the nature of the line which should be fitted, no line has been drawn for the activity data presented in this temperature range. Above about 36 °C the rate declined precipitously apparently due to thermal inactivation of the Hill activity. With uncoupled chloroplasts the onset of this decline in activity was always seen at a few degrees lower than in the absence of

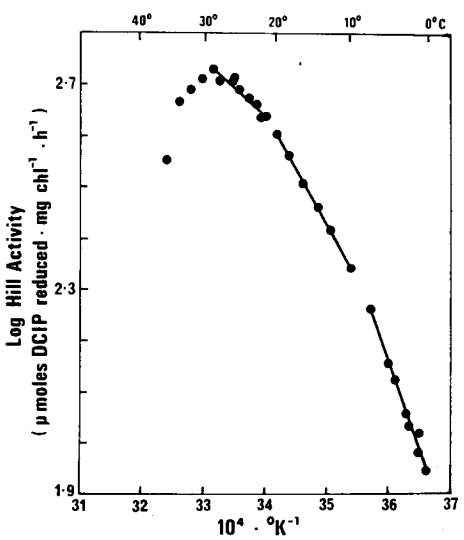

Fig. 2. Arrhenius plot of DCIP reduction in the presence of methylamine. The results are the normalized data for two chloroplast preparations and corrections for fall-off in activity of the preparations with time have been made (see text). The changes in slope at 9, 20 and 29 °C are significant at $P < 0.005$.

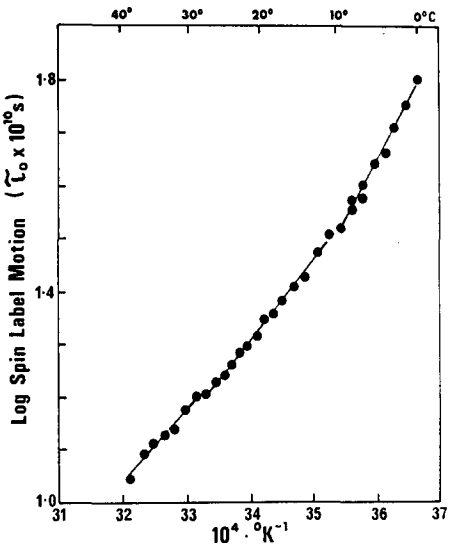

Fig. 3. Arrhenius plot of spin label motion (τ_0) of 16NS. The changes in slope at 10 and 28 °C are significant at $P < 0.005$ and $P < 0.05$, respectively.

uncoupler (Fig. 1). If chloroplasts were assayed after incubation at 27, 33 and 37 °C for times varying from 35 s to 9 min (data not shown), those incubated at 27 °C still retained 98 % of their activity after 4 min and 96 % after 9 min. Chloroplasts incubated at 33 °C retained 97 % of their activity after 4 min and 84 % after 9 min. Thus within the 2 min incubation and subsequent assay time intervals (1–2 min) there was no significant time-temperature factor in the results presented. However, the chloroplasts showed a rapid decrease in their ability to reduce DCIP after even the shortest periods of incubation at 37 °C.

Fig. 3 shows that the temperature-induced changes in DCIP photoreduction at 9 and 29 °C corresponded with temperature-induced changes in membrane fluidity. An Arrhenius plot of motion of the spin label probe 16NS, (plotted as the logarithm of the correlation time τ_0 against the reciprocal of the absolute temperature) incorporated into chloroplast membranes showed changes in slope around 10 °C and again at 28 °C. Thus changes in the kinetics of DCIP photoreduction at around 9 and 29 °C can be correlated with changes in the physical properties of the membranes which occurred at approximately the same temperatures.

The change in slope at 20 °C shown in Fig. 2 has been repeatedly observed in our experiments. Apparently any change at this temperature in the ESR measurements was not large enough to be easily detected, at least with 16 NS as the spin label. In the experiments which follow, the change at 20 °C is not shown since two chloroplast preparations were used to cover the entire temperature range studied (2–42 °C) and there are insufficient points on both sides of 20 °C from any one chloroplast preparation. However, a comparison of the E_a values in the following figures (see below) for temperatures between 9 and 20 °C from the experiments covering the lower range of temperatures and between 20 and 29 °C from the experiments covering the higher range of temperatures confirms the existence of the change at 20 °C.

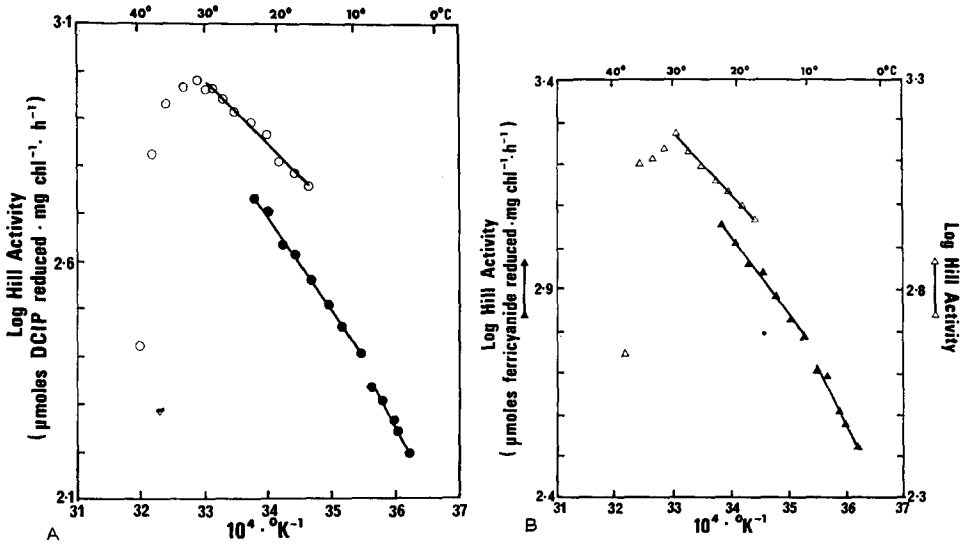

Fig. 4. Arrhenius plot of photochemical activity in the presence of gramicidin D with (A) DCIP or (B) ferricyanide as electron acceptor. The data for each figure was obtained from two experiments indicated by the open and solid symbols. The changes in slope at 9 and 29 °C are significant at $P < 0.05$ and $P < 0.005$ in A and at $P < 0.025$ and $P < 0.005$ in B.

Two separate preparations were used in the experiments which follow since a fall-off in activity of the stock chloroplast suspension (stored at 0 °C) was noticeable during the time taken to cover the 40 °C span of temperatures and accordingly it became necessary to correct the activities. However, when the temperature span was divided into two regions (approx. 2–27 °C and 17–42 °C) the time required for an experiment was appreciably reduced and no correction was made for the fall-off in activity which was 10 % or less of the original activity.

The addition of methylamine results in an increased ionic strength of the reaction medium and it is known that the addition of this uncoupler at the concentration employed results in structural changes to the barley chloroplasts (Smillie, R. M., unpublished results). The temperature-induced changes in photoreduction of DCIP in the presence of methylamine are not due to increased ionic strength of the medium, since results similar to those shown in Fig. 2 were obtained using another uncoupler of photophosphorylation, gramicidin D, at a concentration of 4 μg/ml (Fig. 4). This figure also shows that temperature-induced changes in Hill activity did not depend on using DCIP as the electron acceptor since the photoreduction of ferricyanide also showed changes in the Arrhenius activation energy around 9 and 29 °C in the presence of gramicidin D.

The effect of temperature on DCIP photoreduction by chloroplasts exposed to a low concentration of cations

The results with the uncouplers suggested that a reaction related to the mechanism coupling electron transport to phosphorylation is the rate-limiting step for DCIP photoreduction in the isolated chloroplasts. Barley chloroplasts exposed to a low concentration of cations behave as uncoupled chloroplasts with high photo-

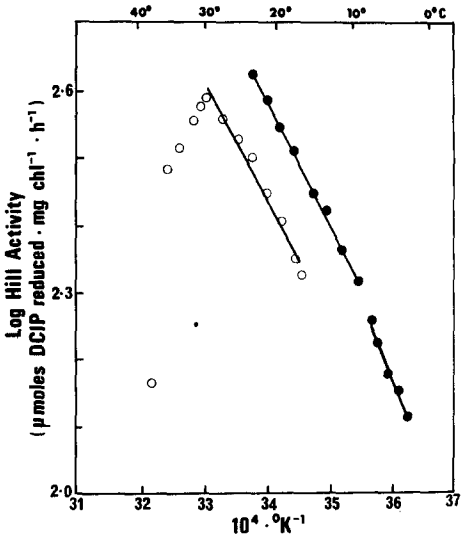

Fig. 5. Arrhenius plot of DCIP reduction, in the absence of uncoupler, by distilled water-washed chloroplasts. The data are from two separate experiments. The changes in slope at 9 and 29 °C are significant at $P < 0.005$.

reductive activities and lack the ability to accumulate protons in the light (Smillie, R. M., unpublished results). Fig. 5 shows the photoreduction of DCIP as a function of temperature by water-washed barley chloroplasts. Temperature-induced changes around 9 and 29 °C are now apparent even though an uncoupler was not present in the reaction mixtures. Barley chloroplasts treated with EDTA (1 mM, pH 8.0) gave results identical to those in Fig. 5. It is not known whether EDTA removes the coupling factor from the thylakoids of barley as it does from spinach thylakoids [9].

The effect of the temperature on the photoreduction of a lipophilic acceptor

The photoreduction of lipophilic electron acceptors is very rapid when compared to DCIP or ferricyanide reduction assayed in the absence of an uncoupler (see ref. 10; compare also Figs. 1 and 6). In order to confirm the results presented in Fig. 5 that the addition of an uncoupler is not obligatory to demonstrate the temperature-induced effects and at the same time to avoid any pre-treatments we have utilized the lipophilic electron acceptor DAD_{ox} (Fig. 6). Changes in slope of the Arrhenius plot occurred at around 9 and 29 °C and the photoreductive activity was rapidly inactivated at temperatures above 37 °C. It was not necessary to add an uncoupler of photophosphorylation in order to demonstrate temperature-induced changes at 9 and 29 °C using DAD_{ox} as the electron acceptor.

The activation energies for the DAD plus ferricyanide reaction for temperatures up to 29 °C were considerably lower than for the DCIP or ferricyanide Hill reactions. From the photochemical data presented in Figs. 2, 4 and 5, the average E_a was below 9 °C : 52.8±6 kJ/mol, between 9 and 20 °C : 37.2±1.6 kJ/mol, and between 20 and 29 °C : 24.5±4.3 kJ/mol. However, the E_a for photoreduction of DAD plus ferricyanide was consistently lower for each corresponding temperature

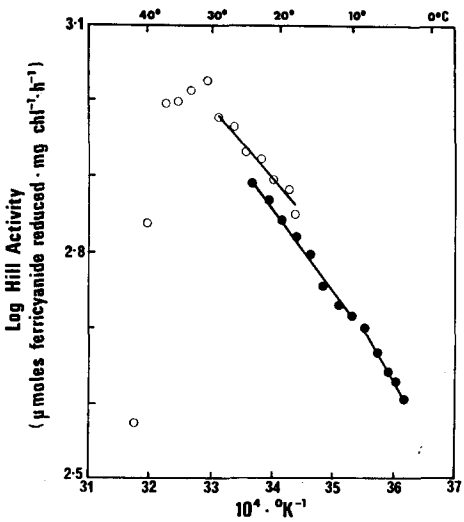

Fig. 6. Arrhenius plot for reduction using the DAD plus ferricyanide reaction in the absence of uncoupler. The data are from two separate experiments. The changes in slope at 9 and 29 °C are significant at $P < 0.025$ and $P < 0.005$, respectively.

TABLE I

REVERSIBILITY OF TEMPERATURE-INDUCED EFFECTS ON PHOTOREDUCTION OF DCIP

In Experiments 1 and 2 chloroplasts were added to a cuvette containing the basic reaction mixture minus DCIP and incubated at the various treatment temperatures for 2 min. The preparation was then brought to the assay temperature by placing the cuvette in a water bath for 3 min, DCIP was added and activity immediately assayed. In Exp. 3 a small aliquot of chloroplasts (300 μg/ml chlorophyll) was incubated at the treatment temperature for 2 min, a 10 μl sample was then added to a reaction mixture containing methylamine and further incubated for 2 min at the assay temperature before a determination of activity was made. Controls were preparations which were processed similarly except that the treatment temperature was the same as the assay temperature. The activity is expressed as percent of control rate.

Assay temperature	Treatment temperature (°C)							
	8	21	26.5	32	36	41	43	46
Exp. 1								
5 °C*	106	101		99	100	48		
5 °C	98	108						
Exp. 2								
21 °C				105	108	111	88	24
Exp. 3								
14.5 °C			101					

* Contained 4 μg/ml gramicidin D.

range. Using the data in Fig. 6, the E_a was, below 9 °C: 27.9 kJ/mol, between 9 and 20 °C: 22 kJ/mol, and between 20 and 29 °C: 16 kJ/mol. This suggests that E_a values may be useful in distinguishing between various types of electron acceptors. DAD_{ox} owing to its lipid solubility is apparently capable of by-passing a reaction with high E_a which is involved in both DCIP and ferricyanide reduction.

Reversibility of temperature-induced changes in photoreduction of DCIP

The temperature-induced changes at around 9, 20 and 29 °C in DCIP photoreduction are apparently reversible in that incubation for short periods above each of these temperatures does not appreciably affect the activity of the chloroplasts when returned to and assayed at a lower temperature. This is indicated by the data shown in Table I. Barley chloroplasts were incubated for 2 min at various temperatures and then returned to a lower temperature for assay. These temperature treatments did not change the rate of DCIP photoreduction measured at 5 °C indicating that the effects of the temperature at 9 and 29 °C were reversible. Similarly, it is shown that the temperature change around 20 °C is reversible (Exp. 3, Table I). Incubation of the chloroplasts at 41 °C in the presence of gramicidin and at 43 °C in its absence resulted in loss of activity of the chloroplasts when assayed at lower temperatures. Hence at these high temperatures the effect of temperature on DCIP photoreduction is not reversible, at least after a 2 min heat treatment. As was observed in the previous experiments, inactivation of Hill reaction activity by high temperatures became evident 2–3 °C lower in the presence of an uncoupler than in its absence. There was no suggestion though of a significant heat-induced uncoupling of chloroplasts preceding the thermal inactivation of Hill reaction activity, since there was no appreciable increase in DCIP photoreduction (assayed at 21 °C in the absence of uncoupler) following the 2 min incubation period at temperatures just below the inactivating temperature.

DISCUSSION

When DCIP was used to assay Hill reaction activity of the isolated barley chloroplasts, the reaction showed a constant E_a between 2 and 38 °C (Fig. 1). However, under a variety of other assay conditions distinct and reversible changes in E_a occurred at specific temperatures within this range. These conditions were obtained by adding the uncouplers of photophosphorylation, methylamine or gramicidin D, exposing the chloroplasts to a low cation concentration, or employing a lipophilic acceptor. All of these procedures have in common the fact that they very significantly increased the rate of photosynthetic electron transport reactions and presumably by eliminating or circumventing some rate-limiting process with a constant E_a allowed the temperature-induced changes in Hill reaction activity to be seen. The Hill reagents used in this investigation can accept electrons to varying degrees from both photosystems and it is conceivable that the temperature effects observed reflect changes in their sites of electron acceptance. Even if this were the case, it would still indicate a temperature-induced membrane change. The only other possibility is a change in reactivity of the acceptor itself. This is unlikely since a constant E_a was observed in Fig. 1 and experiments with chloroplasts from other plants show changes at temperatures different to those for barley. Since these temperature-induced changes were

accompanied by changes in E_a for the motion of the spin-labelled compound, 16NS, incorporated into chloroplast membranes (Fig. 3), and by inference a change in membrane fluidity [3, 11], a variety of chloroplast membrane properties and functions would almost certainly have been affected likewise.

In the past little attention has been given to the actual assay temperature employed when comparing results of different investigators and one implication of our results is that chloroplast membranes may assume different configurations and conformations depending upon the temperature. This view is substantiated by other reports of non-linear Arrhenius plots for various parameters of chloroplast function. Cox [12] has recently reported an increase in E_a below 3 °C for the photoreduction of DAD_{ox} and an increase in E_a below −15 °C for methyl purple photoreduction by spinach chloroplasts suspended in a medium containing 50 % (v/v) ethyleneglycol. These measurements were also carried out in the presence of the uncoupler NH_4Cl. Although the photoreduction of DAD_{ox} had a constant E_a between 3 and 25 °C, other measurements have indicated that spinach chloroplasts show an additional temperature-induced change around 18 °C. Studies with a spinach subchloroplast fraction, prepared by treating chloroplasts with detergent, have disclosed a change in slope at around 18 °C for Arrhenius plots of ferricyanide photoreduction and *N*-methylphenazonium methosulphate cyclic photophosphorylation [13]. With isolated spinach chloroplasts, changes in slope around 18 °C have been found for the decay of light-induced quenching of atebrin fluorescence [14], the decay of the electrochromic absorption change at 515 nm [15], and spin label motion [16]. Jursinic and Govindjee [17] have also reported a deviation from a linear Arrhenius relationship between 10 and 15 °C for measurements based on delayed light emission by cells of *Chlorella*.

In the case of the barley chloroplasts, it is possible that additional changes in E_a of the Hill reaction occur very close to 0 °C or at sub-zero temperatures as in the case of spinach chloroplasts. At physiological temperatures there could also be additional and more subtle temperature-induced changes in E_a of the Hill reaction, similar to those reported here, which were not detected by the methods employed.

It is to be noted that while the temperature-induced changes at 9 and 20 °C resulted in a decrease in the rate of increase of Hill activity with increasing temperature, at temperatures above 29 °C the activity actually showed a slight decline with increasing temperature. This could be due to some thermal inactivation or to a reversible thermal effect on the membrane system resulting in a small decrease in activity. While the exact nature of the change in E_a above 29 °C is not clear, the major contributing factor is probably a temperature-induced membrane effect similar to those at 9 and 20 °C. While the onset of a thermal inactivation process may account for part of the change seen above 29 °C, it is not the main cause of the change in E_a at 29 °C; otherwise deviation from the slope of the Arrhenius plot at 29 °C should not be so marked, but rather a more gradual deviation might be expected, increasing in magnitude as the temperature is further increased. Further, the effect of temperatures higher than 29 °C are reversible in the sense that incubation of chloroplasts for a few min above 29 °C did not affect the activity measured at a temperature below 29 °C (Table I). Since the ESR data indicated also a change in membrane fluidity in the vicinity of 29 °C (Fig. 3), the changes seen at 9, 20 and 29 °C are all reversible temperature-induced changes in the properties of the chloroplast mem-

branes, but at 29 °C there is a superimposition of the beginnings of some thermal inactivation process.

The effect of temperature on the Hill reaction activity at temperatures higher than 36 °C is different again from that at 29 °C, or at 9 and 20 °C. At temperatures just below 40 °C the activity declined with time and it was not regained by decreasing the temperature to below 36 °C. The change in activity was therefore not reversible under the assay conditions used, although the possibility that the process may still have been reversible after extremely short periods of heating was not investigated. The sensitivity of Photosystem II to thermal inactivation compared with that of Photosystem I is well known. Temperatures in excess of 50 °C have usually been used to inactivate Photosystem II [18] but as shown by Emmett and Walker [19] and Santarius [20], temperatures around 40 °C are sufficient to inactivate the Hill reaction in isolated spinach chloroplasts. The same holds for barley chloroplasts. Nevertheless, as with the temperature-induced changes at 9, 20 and 29 °C, the change brought about by temperatures in excess of 36 °C was probably not a specific effect of the temperature on the Hill reaction but instead reflected some temperature-induced change in the physical state of certain thylakoid membrane components which in turn affected several of the functions of the membrane system. A thermal inactivation of photophosphorylation occurred at around the same temperature which inactivated Hill activity in spinach chloroplasts [19, 20] and the rate of chlorophyll synthesis in barley leaves decreased at temperatures above about 35 °C (unpublished results).

A comparison of the results in Figs. 1 and 2 and Table I suggests an apparently greater resistance of chloroplasts in the absence of an uncoupler compared with uncoupled chloroplasts to inactivation of Hill activity by temperatures approaching 40 °C. Assuming that the reaction which limits the rate of DCIP photoreduction in chloroplasts (assayed in the absence of uncoupler) is not inactivated at these temperatures, then a thermal inactivation of DCIP photoreduction would not become apparent in such chloroplasts until the rate of the temperature-sensitive step of electron transport activity had declined to below that of the original rate-limiting step. It is likely then that the lowest temperature causing an irreversible decrease in Hill activity in uncoupled chloroplasts, i.e. around 36 °C (Fig. 2), represents the temperature above which the potential for electron transport activity begins to decline in both the presence or absence of an uncoupler.

It is important to consider whether or not the temperature-induced changes observed by us have practical implications for studies of the growth and metabolism of the intact plant. It might be argued that since the temperature-induced changes in activity were observed only under conditions which allowed a maximum rate of photochemical activity, e.g. uncoupled chloroplasts, metabolic processes in the intact plant tissue such as photosynthesis would not be affected to the same extent. However, if one accepts a value of 200 μmol $CO_2 \cdot$ mg chlorophyll$^{-1} \cdot h^{-1}$ as reasonable for CO_2 fixation by barley in vivo [21] then the rate of electron transport in vivo must approximately equal that observed for uncoupled chloroplasts in vitro. Previously, temperature-induced changes in the enzymic activity and fluidity of plant membranes have been interpreted in terms of the sensitivity of plants to chilling temperatures since such changes in higher plants appeared to be restricted to chilling-sensitive plants [3–5]. Clearly, the temperature-induced changes in E_a of DCIP photoreduction cannot be considered in terms of chilling sensitivity since barley is a plant which is not

particularly susceptible to damage by chilling temperatures. Further, similar temperature-induced changes in the E_a of this Hill reaction activity have been found using chloroplasts from pea, another chilling-resistant plant, as well as chloroplasts from two chilling-sensitive plants, mung bean and maize (manuscript in preparation). A number of considerations lead us to propose instead that the multi-temperature effects on chloroplast activity are related to and indeed reflect a temperature-regulated control of both growth and metabolism over the entire physiological temperature range for growth. The interaction of temperature with chloroplast membrane components, while causing changes in E_a of the Hill reaction, can also similarly affect both chloroplast development and photosynthesis. In a separate study the relationship between temperature and chlorophyll synthesis in detached barley leaves has been studied and temperature-induced changes in the slope of the Arrhenius plot for chlorophyll production were found at 9, 19 and 28 °C and a peak of chlorophyll production was found at 35 °C (Smillie, R. M., unpublished experiments). Thus there was a good correlation in the response to temperature between chlorophyll synthesis in greening leaves and the Hill activity of chloroplasts isolated from fully greened leaves.

Several published studies have indicated that there is also a non-linear relationship between the logarithm of the maximum rate of photosynthesis and $°K^{-1}$ in both intact cells [22, 23] and isolated whole chloroplasts [24]. In the low temperature range, that is below about 10 °C, the E_a (or Q_{10}) for photosynthesis increased markedly. The results have usually been interpreted as a gradual deviation from a linear Arrhenius relation over a relatively large temperature range, rather than as a distinct shift to a new E_a over a very narrow temperature span as is indicated by our measurements of Hill activity. The average E_a for temperatures from 20 to 30 °C is in general lower than for the range 10–20 °C and in the case of isolated pea chloroplasts this decline above 20 °C has been attributed to heat inactivation of photosynthetic CO_2 fixation [24]. As in the case of chlorophyll synthesis, both the high E_a in the low temperature range and the low E_a in the high temperature range for photosynthesis could be largely explained in terms of reversible temperature-induced changes in chloroplast membrane properties.

The temperature-induced changes found at 9, 20 and 29 °C in barley chloroplasts could be due to either a direct effect of the temperature on the conformation of membrane proteins or alternatively, to a temperature-induced change in rotational freedom of certain membrane lipids. The observed changes in membrane fluidity using spin-labelled probes which coincide with the changes in E_a of membrane enzymic activities, and more especially the demonstration of similar temperature-induced changes in lipids extracted from the membranes [3] favours the latter mechanism. Reversible temperature-induced changes in membrane fluidity could induce secondary changes in enzyme function by altering, for example, protein conformation of membrane-bound enzymes [3] or interactions between proteins [25]. The temperature-induced change in Hill activity at temperatures above 36 °C are of a more destructive nature and may be due to a temperature-dependent conformational change in the membrane proteins, or to a temperature-dependent dissociation of lipid-protein complexes in the membrane, e.g. the chlorophyll-protein complexes [26].

ADDENDUM

The computer data analysis procedure used in determining changes in slope of the Arrhenius plots presented in this paper is based on the assumption that straight lines fit the data points. The point at which a change in slope occurs is determined by where a minimum in the sums of the residual sums of squares for all possible straight lines through the data points occurs (see ref. 7). We have examined in detail by computer whether all the points below 29 °C are best fit by various curves. The correlation coefficients for linear regression analysis, exponential, power and logarithmic curve fits are not sufficiently different to allow selection of any of these and in addition their correlation coefficients do not vary significantly from those of the lines of varying slope presented (the values are generally greater than 0.99 with variation in the third or fourth place). Polynomial curve fitting (of first through fifth order) has also been performed. However, this did not alter our conclusion that the experimental points can be fitted to straight lines as drawn in the figures.

ACKNOWLEDGEMENTS

The authors wish to thank Dr. J. K. Raison for providing the electron spin resonance data used in Fig. 3, and Dr. A. Reisner for performing the curve fitting analyses. Dr. P. Y. White kindly synthesized and provided the sample of DAD. The technical assistance of Mrs. C. Turner is gratefully acknowledged.

REFERENCES

1 Blackman, F. F. (1905) Ann. Bot. Lond. 19, 281–295
2 Emerson, R. and Arnold, W. (1932) J. Gen. Physiol. 15, 391–420
3 Raison, J. K. (1973) Symp. Soc. Exp. Biol. 27, 485–512
4 Raison, J. K. (1974) in Mechanisms of Regulation of Plant Growth (Bieleski, R. L., Ferguson, A. R. and Cresswell, M. M., eds.), Bulletin 12, pp. 487–497, The Royal Society of New Zealand, Wellington
5 Lyons, J. M. (1973) Annu. Rev. Plant Physiol. 24, 445–466
6 Shneyour, A., Raison, J. K. and Smillie, R. M. (1973) Biochim. Biophys. Acta 292, 152–161
7 Raison, J. K. and Chapman, E. A. (1976) Aust. J. Plant Physiol. 3, 291–299
8 Arnon, D. I. (1949) Plant Physiol. 24, 1–15
9 Howell, S. H. and Moudrianakis, E. N. (1967) J. Mol. Biol. 27, 323–333
10 Saha, S., Ouitrakul, R., Izawa, S. and Good, N. E. (1971) J. Biol. Chem. 246, 3204–3209
11 Mehlhorn, R. J. and Keith, A. D. (1972) in Membrane Molecular Biology (Fox, C. F. and Keith, A. D., eds.), pp. 192–227, Sinauer Assoc., Stamford
12 Cox, R. P. (1975) Biochim. Biophys. Acta 387, 588–598
13 McEvoy, F. A. and Lynn, W. S. (1972) Arch. Biochem. Biophys. 150, 632–635
14 Kraayenhof, R., Katan, M. B. and Grunwald, T. (1971) FEBS Lett. 19, 5–10
15 Graeber, P. and Witt, H. T. (1974) in Proceedings of the 3rd International Congress on Photosynthesis (Avron, M., ed.), Vol. 2, pp. 951–956, Elsevier, Amsterdam
16 Torres-Pereira, J., Mehlhorn, R., Keith, A. D. and Packer, L. (1974) Arch. Biochem. Biophys. 160, 90–99
17 Jursinic, P. and Govindjee, (1972) Photochem. Photobiol. 15, 331–348
18 Arnon, D. I. and Whatley, F. R. (1949) Arch. Biochem. Biophys. 23, 141–156
19 Emmett, J. M. and Walker, D. A. (1973) Arch. Biochem. Biophys. 157, 106–113
20 Santarius, K. A. (1973) Planta 113, 105–114
21 Apel, P. and Lehmann, C. O. (1969) Photosynthetica 3, 255–262

22 Warburg, O. (1919) Biochem. Z. 100, 230–270
23 Emerson, R. (1929) J. Gen. Physiol. 12, 623–639
24 Baldry, C. W., Bucke, C. and Walker, D. A. (1966) Biochim. Biophys. Acta 126, 207–213
25 Vessey, D. A. and Zakim, D. (1971) J. Biol. Chem. 246, 4649–4656
26 Thornber, J. P. (1975) Annu. Rev. Plant Physiol. 26, 127–158