Biochimica et Biophysica Acta, 679 (1982) 287–299
Elsevier Biomedical Press

BBA 41024

TRANSPORT AS THE BASIS OF THE KOK EFFECT

LEVELS OF SOME PHOTOSYNTHETIC INTERMEDIATES AND ACTIVATION OF LIGHT-REGULATED ENZYMES DURING PHOTOSYNTHESIS OF CHLOROPLASTS AND GREEN LEAF PROTOPLASTS

U. HEBER *, U. TAKAHAMA **, S. NEIMANIS and M. SHIMIZU-TAKAHAMA

Institute of Botany and Pharmaceutical Biology of the University of Würzburg, D-8700 Würzburg (F.R.G.)

(Received July 16th, 1981)

Key words: NADP level; Photosynthesis; Photophosphorylation potential; Enzyme activation; Kok effect; (Spinach chloroplast, Protoplast)

Experiments were performed with intact chloroplasts and leaf cell protoplasts isolated from spinach. The light-dependent decrease in (H^+) in the chloroplast stroma counteracts carbon reduction and is offset at low light intensities by a large decrease in NADP and a significant increase in [ATP]/[ADP] ratios. Excess accumulation of NADPH and/or ATP permits 3-phosphoglycerate reduction to occur. With increasing light intensity, NADP levels and [ATP]/[ADP] ratios increased. High rates of photosynthesis were observed at high and at low [ATP]/[ADP] ratios. Levels of dihydroxyacetone phosphate were dramatically increased in the light. In chloroplasts, this permitted conversion to ribulose bisphosphate which on carboxylation yields 3-phosphoglycerate. The light-dependent alkalization of the chloroplast stroma is known to be responsible for phosphoglycerate retention in the chloroplasts. A high chloroplast ratio of phosphoglycerate to dihydroxyacetone phosphate aids carbon reduction. Measured ratios of dihydroxyacetone phosphate to phosphoglycerate were averages between low chloroplast ratios and high cytosolic ratios. They were far higher, even under low-intensity illumination, than dark ratios. Since cytosolic NADH levels are known to increase much less in the light than cytosolic dihydroxyacetone phosphate levels, the large increase in the ratio of didydroxyacetone phosphate to phosphoglycerate must considerably increase cytosolic phosphorylation potentials even at very low light intensities. It is proposed that this increase is communicated to the mitochondrial adenylate system, and inhibits dark respiratory activity, giving rise to the Kok effect. The extent of stroma alkalization, the efficiency of metabolite shuttles across the chloroplast envelope, and rates of cytosolic ATP consumption are proposed to be factors determining whether and to what extent the Kok effect can be observed. Light activation of chloroplast enzymes was slow at low and fast at high light intensities. This contrasts to low NADP levels at low and usually higher levels at high light intensities. Maximum enzyme activation was observed far below light saturation of photosynthesis, and light activation of enzymes was often less pronounced at very high than at intermediate light intensities.

Introduction

In a number of plant species, the rate of photosynthesis, when plotted against light intensity, shows a break far below light saturation. It rises more steeply below than above the break. This effect which was first observed by Kok [1] is known as the 'Kok

Abbrevatiation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

* To whom correspondence should be addressed at Lehrstuhl Botanik I der Universität Würzburg, Mittlerer Dallenbergweg 64, D-8700 Würzburg, F.R.G.

** Present address: Kyushu Dental College, Kitakyushu 803, Japan.

effect'. Mass spectrometry experiments suggested that it is caused by a suppression of dark respiration which becomes effective at low light intensities [2]. Inhibition of dark respiration by light has repeatedly, but not always, been observed [3–5]. Since dark respiration is believed to be controlled by the phosphorylation potential [ATP]/[ADP] [P_i] [6], and communication exists between the phosphorylation potentials of plastids, cytosol and mitochondria [7,8], it is tempting to explain the Kok effect by the increase in the ATP/ADP ratios which photophosphorylation can cause not only in chloroplasts, but also in extrachloroplastic leaf cell ecompartments [9,19]. However, at the low light intensities at which the Kok effect is usually observed, the production of NADPH and ATP by the electron transport chain of chloroplasts limits the rate of photosynthesis. There is the question of whether under such limitation ATP can accumulate sufficiently to affect mitochondrial oxidation. ATP and NADPH are both consumed during the reduction of 3-phosphoglycerate to dihydroxyacetone phosphate which is catalyzed in the chloroplast stroma by phosphoglycerate kinase, NADP-dependent glyceraldehydephosphate dehydrogenase and trisephosphate isomerase. As long as metabolic fluxes are small, high enzyme activities ensure that the reaction is not far from thermodynamic equilibrium

$$\frac{[ATP^{4-}][NADPH][H^+][PGA^{3-}]}{[ADP^{3-}][P_i^{2-}][NADP^+][DHAP^{2-}]} = K = 7.25 \cdot 10^{-7} \quad (1)$$

During the transition from dark to light, the proton concentration decreases in the chloroplast stroma [11,12]. This decrease needs only slightly to be overcompensated by an increase in the ratios of other reactants in order to swing the reaction from triosephosphate oxidation in the dark toward phosphoglycerate reduction in the light. From Eqn. 1 it is obvious that phosphoglycerate reduction can be driven in the dark-/light transition either by an increased [NADPH]/NADP] ratio (if this were large enough, phosphorylation potentials could actually drop in the light to below dark values) or by an increased phosphorylation potential, or both.

In order to understand whether control of mitochondrial respiration by a high phosphorylation potential can give rise to the Kok effect, we wanted to know what the concentrations of light products are in chloroplasts and leaf protoplasts during light-limited photosynthesis as compared to light-saturated photosynthesis.

The following experiments show that photosynthesis can proceed efficiently at widely differing ratios of [ATP] to [ADP]. Even when [ATP]/[ADP ratios are low, levels of light products rise much more steeply with light intensity than does the rate of photosynthesis. The accumulation of photoproducts under light which is rate-limiting for photosynthesis appears to be governed mainly by the increase in the stroma pH and by the extent of phosphoglycerate accumulation in the chloroplast stroma.

Materials and Methods

Because there is some physiological variability of adenylate and pyridine nucleotide pool sizes in leaves which can make it difficult to perform comparative experiments using different leaves, we isolated intact protoplasts [13] and chloroplasts [14] from freshly harvested leaves of *Spinacia oleracea*. The percentage of broken chloroplasts contaminating suspensions of intact chloroplasts was routinely determined by the ferricyanide method [9]. Chloroplast preparations used in this work contained more than 80% chloroplasts with functional envelopes. The protoplasts were purified in a discontinuous gradient [15]. Chlorophyll was determined spectrophotometrically [16]. Chloroplast experiments were performed in a reaction medium comprising 330 mM sorbitol/50 mM (Hepes)/ 10 mM NaCl/1 mM $MgCl_2$/1 mM $MnCl_2$/2 mM EDTA/0.5 mM KH_2PO_4/2 mM $NaHCO_3$ (pH 7.6). Catalase (1600 i.u./ml) was also added. The reaction medium for protoplast experiments consisted of 0.5 M sorbitol/30 mM Hepes/1 mM $CaCl_2$/2 mM $NaHCO_3$ (pH 7.6). Oxygen evolution was recorded by a Clark-type electrode. Illumination was provided by a slide projector. Its light was filtered through 30 mm water, a 2 mm Calflex C heat absorbing filter (Balzers, Liechtenstein) and a 3 mm RG 610 cut-off filter of Schott and Gen., Mainz. The half-band width of the resulting red light was between 610 and 770 nm, i.e. part of the incident energy was photosynthetically inactive. Chlorophyll concentrations were between 20 and 50 μg/ml, and the light path was 0.5 or 1 cm. Reactions were terminated during illumination or in the

dark by adding $HClO_4$ to a final concentration of 0.7 M. After neutralization, NADP was determined by enzymic cycling [17] and adenylates by Strehler's method [18]. Depending on concentration, phosphoglycerate and dihydroxyacetone phosphate were determined spectrophotometrically or fluorometrically [19]. The fluorescence of 9-aminoacridine [5 μM] was measured as described previously [20].

For measurements of light activation of enzymes, chloroplasts were illuminated, and samples were withdrawn in the light and injected into hypotonic reaction media in which chloroplasts ruptured [21]. Glyceraldehydephosphate dehydrogenase, phosphoribulokinase and fructose bisphosphatase were immediately measured spectrophotometrically at 340 nm in the following media: glyceralhydephosphate dehydrogenase: 20 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2 mM ATP, 2 mM 3-phosphoglycerate and 0.2 mM NADPH, pH 7.8 (endogenous phosphoglycerate kinase was not rate-limiting in this assay); phosphoribulokinase: 20 mM Hepes, 2 mM phospho*enol*pyruvate, 5 mM $MgCl_2$, 1 mM ATP, 1 mM ribose 5-phosphate, 0.4 mM NADH, 20 μg (= 8 units) pyruvate kinase/ml and 20 μg (= 4 units) lactate dehydrogenase/ml, pH 7.8 (the activity of endogenous ribose-5-phosphate isomerase was not rate-limiting in this assay); fructose bisphosphatase: 20 mM triethanolamine hydrochloride, 5 mM $MgCl_2$, 2.4 mM EDTA, 1 mM fructose bisphosphate, 0.2 mM NADP, 40 μg (= 16 units) glucose-6-phosphate isomerase/ml and 2 μg (= 1 unit) glucose-6-phosphate dehydrogenase/ml, pH 7.8. Reactions were run at 20°C.

Results

(1) NADP levels in cloroplasts and protoplasts from spinach leaves

NADP levels in darkened isolated chloroplasts and in darkened protoplasts were similar. After correction for NADP dissolved in the medium outside the chloroplasts or cells, values ranged between 5 and 15 nmol per mg chlorophyll. NADPH levels were also significant, and in chloroplasts 40–60% of the NADP system was reduced in the dark [22]. The sum of [NADP] and [NADPH] remained essentially constant during 6 min illumination periods [22]. Fig. 1 shows NADP levels in intact chloroplasts and CO_2-dependent oxygen evolution with bicarbonate (2 mM) or phosphoglycerate (0.25 mM) as substrates during illumination with 600 $W \cdot m^{-2}$ red light. Another light intensity used with bicarbonate as substrate was 14 $W \cdot m^{-2}$. At the low light intensity, [NADP] decreased more in the light and remained at a lower level during illumination than at the higher intensity. The rate of photosynthesis was low at the low intensity. At the high intensity, [NADP] increased after a transient decrease which lasted for about 1 min and stabilized at a level which was higher than the original dark level. The same was observed with phosphoglycerate as substrate, and in this case the transient decrease was brief, as was the lag phase of oxygen evolution. Rates of oxygen evolution were high at the high light intensity.

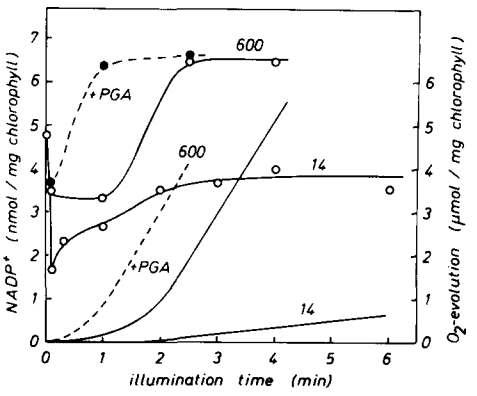

Fig. 1. NADP levels in intact chloroplasts (upper curves) and oxygen evolution (lower curves) from 2 mM bicarbonate (———) or 0.25 mM 3-phosphoglycerate (PGA) during illumination with 14 and 600 $W \cdot m^{-2}$ red light (figures on curves). Addition of phosphoglycerate in the dark increased NADP levels by about 10% (not shown). The rate of CO_2-dependent oxygen evolution was 10 μmol/mg chlorophyll per h at 14 $W \cdot m^{-2}$.

Fig. 2 shows the NADP level in intact chloroplasts after 6 min illumination with 2 mM HCO_3^- as a function of light intensity. Again, NADP levels, which were high in the dark, were lower at low than at high light intensities. Conversely, NADPH levels were higher at low than at high light intensities [22].

In a similar experiment with protoplasts (Fig. 3), NADP levels were higher in the dark than under low intensity illumination. Increasing the light intensity caused no further decrease in NADP levels, but the pronounced shift toward oxidation of the NADP

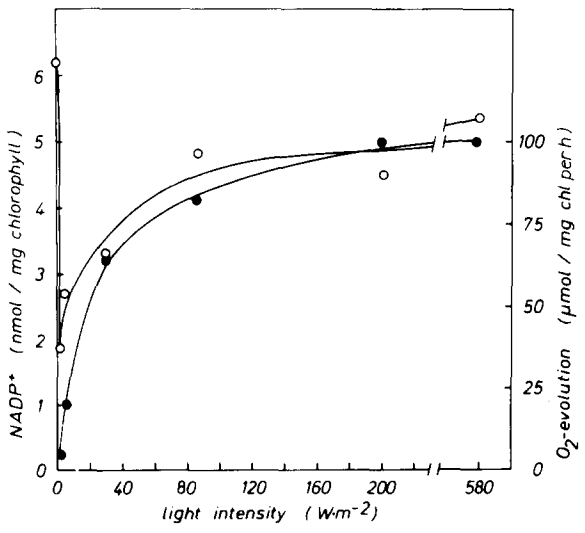

Fig. 2. NADP level (○) in intact chloroplasts after 6 min illumination and rates of CO_2-dependent oxygen evolution (●) as a function of light intensity (illumination with red light).

system seen in isolated chloroplasts (Fig. 2) was absent in the protoplast experiment, perhaps because the protoplast preparation was not very active in photosynthesis. Still, in kinetic protoplast experiments similar to the chloroplast experiment of Fig. 1, a secondary increase in NADP levels was observed after the initial decrease (data not shown). This decrease lasted only a short time, and the lag phase of photosynthesis was also shorter in protoplasts than in intact chloroplasts.

(2) Adenylate levels in chloroplasts and protoplasts from leaves

In the experiment of Fig. 4, chloroplasts were illuminated with two different light intensities and adenylate levels and oxygen evolution were measured as a function of illumination time. Under saturating light (580 W · m^{-2}), the rate of CO_2-dependent oxygen evolution was 222 µmol/mg chlorophyll per h and under light limitation (16 W · m^{-2}) 44 µmol/mg chlorophyll per h. Oxygen evolution started with a lag and accelerated until a steady rate was achieved. ATP levels increased and ADP and AMP levels decreased rapidly on illumination not only with high-intensity but also with low-intensity light. The changes in adenylate pool sizes were somewhat smaller at the low than at the high light intensity. After the initial fast increase, ATP levels decreased slowly during illumination. This has been observed in more detail before [23] and reflects the slow decrease in chloroplast phosphate levels observed during CO_2 assimilation [24]. Thus the slow decrease in chloroplast ATP levels and [ATP]/[ADP] ratios in the light does not necessarily indicate a significant change in the chloroplast phosphorylation potential [ATP]/[ADP] [P_i]. Since under the conditions of our experiments initial stromal phosphate concentrations were

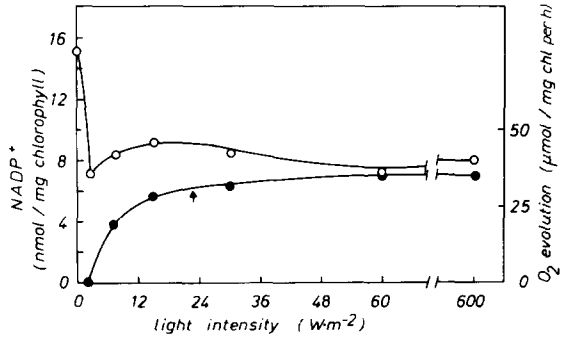

Fig. 3. NADP level (○) in spinach protoplasts after 3 min illumination with 2 mM bicarbonate as a function of light intensity. The absence of a significant secondary increase in NADP such as seen in the chloroplast experiment of Fig. 2 may be due to the low photosynthetic activity of the protoplasts. The lower curve shows CO_2-dependent oxygen evolution (●) in µmol/mg chlorophyll per h.

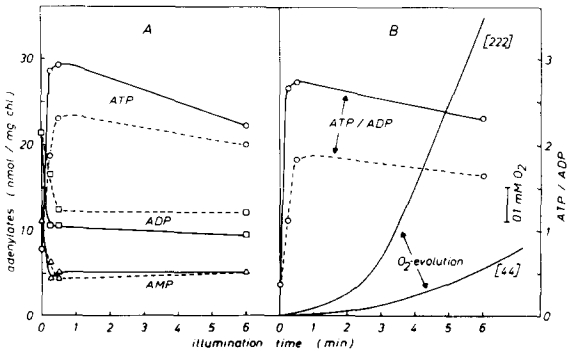

Fig. 4. Adenylate levels (A) and [ATP]/[ADP] ratios (B) in intact chloroplasts and photosynthetic oxygen evolution (B) at two different intensities of red light (16 (– – –) and 580 (———) W · m^{-2}). Numbers in brackets denote maximum rates of photosynthesis in µmol O_2 evolved/mg chlorophyll per h.

close to 12 mM [24] phosphorylation potentials were 30 M^{-1} before illumination and 225 and 150 M^{-1} during light-saturated and light-limited photosynthesis, respectively. The high phosphorylation potentials reported for broken chloroplasts [25,26] were never observed with intact chloroplasts [26].

Fig. 5 shows adenylate levels, [ATP]/[ADP] ratios and rates of photosynthesis after 6 min illumination as a function of light intensity. In this experiment, illumination increased [ATP]/[ADP] ratios up to a maximal value of about 5. The maximum rate of photosynthesis was 150 μmol/mg chlorophyll per hour. In the experiment of Fig. 6, maximal [ATP]/[ADP] ratios remained almost one order of magnitude below the values shown in Fig. 5. Still, the maximum rate of photosynthesis was even higher than in the experiment of Fig. 5. Obviously, high [ATP]/[ADP] ratios are not a necessary requirement for high rates of photosynthesis. Efficient photosynthesis at low [ATP]/[ADP] ratios has also been reported by Lilley et al. [24], and reduction of glycerate via phosphoglycerate has been observed to occur at extremely low ATP levels [23]. In both Figs. 5 and 6, the slope of the increase in [ATP]/[ADP] ratios was steeper than that of photosynthesis. In protoplasts, [ATP]/[ADP] ratios are high already in the dark. Fig. 7 shows [ATP]/[ADP] ratios in spinach protoplasts and photosynthetic oxygen evolution as a function of illumination time. In contrast to the chloroplast experiment shown in Fig. 4, [ATP]/[ADP] ratios were not or not much higher during light-saturated photosynthesis than under illumination with rate-limiting light. The slow decrease in [ATP]/[ADP] ratios following the initial fast increase led to values which, after 6 min illumination, were similar to the initial dark

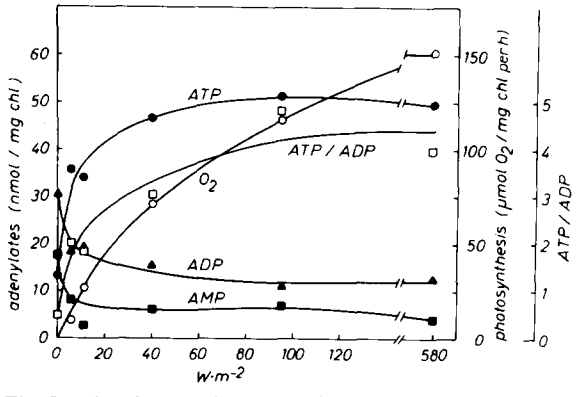

Fig. 5. Adenylate levels and ATP/ADP ratios in intact chloroplasts after 6 min illumination and CO_2-dependent oxygen evolution as a function of the intensity of red light.

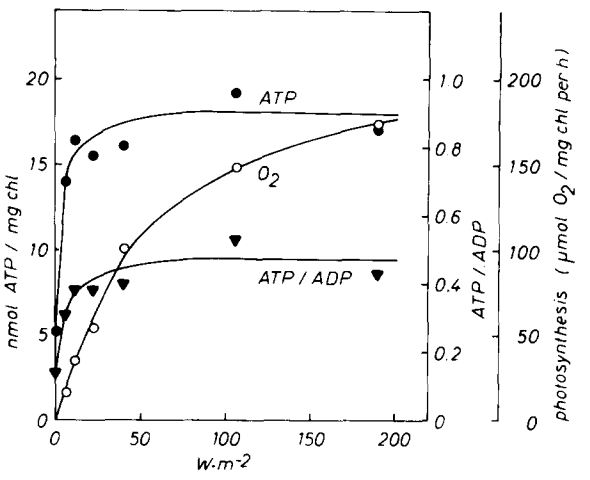

Fig. 6. ATP levels, [ATP]/[ADP] ratios and photosynthesis by intact chloroplasts as a function of light intensity. Compare data with those of Fig. 5.

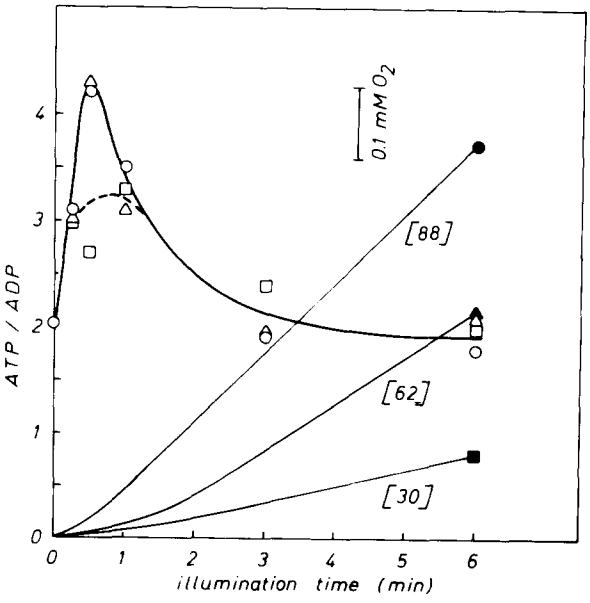

Fig. 7. [ATP]/[ADP] ratios in spinach protoplasts and CO_2-dependent oxygen evolution at three different light intensities. Numbers in brackets denote rates of photosynthesis in μmol/mg chlorophyll per h. Symbols for ATP/ADP ratios at 580 W · m^{-2} (o———o), at 16 W · m^{-2} (Δ———Δ), and at 6.5 W · m^{-2} (□———□).

ratio. The decline in [ATP]/[ADP] is in part based on the conversion of phosphate to phosphate esters as is the decline in [ATP]/[ADP] ratios observed in isolated chloroplasts (Fig. 4). Stitt et al. [27] have recently reported on a decline in chloroplast phosphate levels during prolonged illumination of protoplasts. While the initial increase in protoplast [ATP]/[ADP] ratios on illumination indicates a light-dependent increase in protoplast phosphorylation potential, the following decline therefore does not necessarily suggest a significant subsequent decrease. Since phosphate consumption is slow in the dark/light transition, the increase in [ATP]/[ADP] observed after 15 or 30 s illumination may be used as an approximate measure of the light-dependent increase in the overall protoplast phosphorylation potential. Fig. 7 suggests that it doubles on illumination. Still, the phosphorylation potential cannot be a main driving force for photosynthesis in the experiment of Fig. 7, since [ATP]/[ADP] ratios did not increase with illumination, while photosynthesis did.

Fig. 8 shows [ATP]/[ADP] ratios in two different protoplast preparations as a function of light intensity. In Fig. 8A, adenylate levels were measured after 15 s in Fig. 8B after 6 min illumination. Fig. 8A reveals the light-dependent increase in phosphorylation potential which is also apparent from Fig. 7. It is saturated at surprisingly low light intensities (see also Fig. 7). After 6 min illumination (Fig. 8B), the increase in phosphorylation potential has not become apparent. It is presumably masked by the slow decline in phosphate levels observed by Stitt et al. [27] which must also cause a decline in [ATP]/[ADP] ratios.

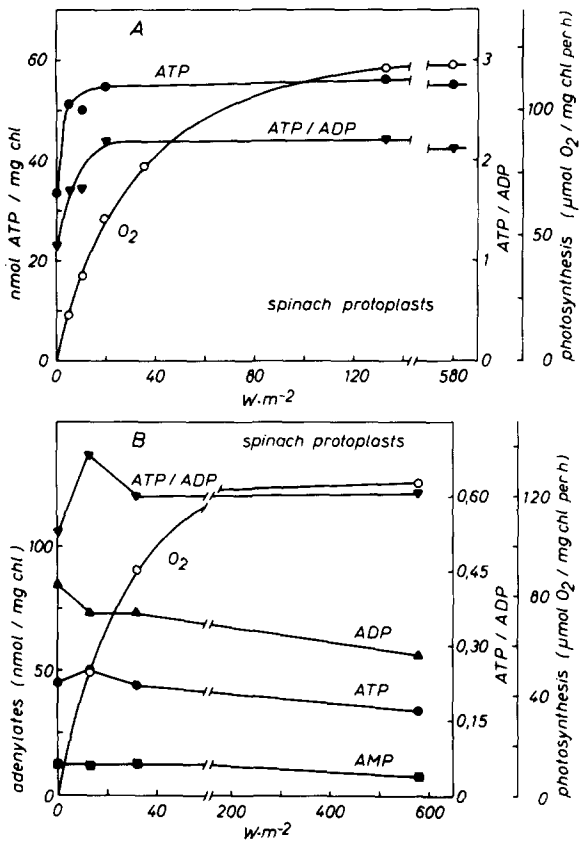

Fig. 8. Adenylates and [ATP]/[ADP] ratios in spinach protoplasts after 30 s. (A) and 6 min (B) illumination and rates of photosynthesis as a function of light intensity. The data in A and B are from two different experiments.

(3) Light-dependent alkalization of the chloroplast stroma

Upon illumination, the H^+ concentration in the chloroplasts stroma decreases. The stroma alkalization which is maintained against passive proton fluxes from the extrachloroplastic space [12,28] is an important factor in the regulation of carbon flux in the Calvin cycle [29–32]. It also affects the steady-state distribution of stromal metabolites (see Eqn. 1). In isolated chloroplasts, the stroma alkalization can be decreased or abolished by the addition of salts of certain weak acids which establish a proton shuttle across the chloroplast envelope without affecting the transthylakoid proton gradient [30,31]. A decrease in the stroma pH can then be seen as a salt-dependent decrease in the intrathylakoid pH. The latter can be monitored by the distribution of a suitable fluorescent amine such as 9-aminoacridine between the medium used for suspending the chloroplasts and the intrathylakoid space. Fig. 9 shows the light-dependent stroma alkalization of isolated chloroplasts as a function of light intensity. It was measured by the increase in 9-aminoacridine flurorescence quenching on addition of 3.6 mM potassium glyoxylate to a suspension of intact chloroplasts. The stroma alkalization was light-saturated at the low intensity of 10 $W \cdot m^{-2}$, i.e., far below light intensities that saturated CO_2 reduction.

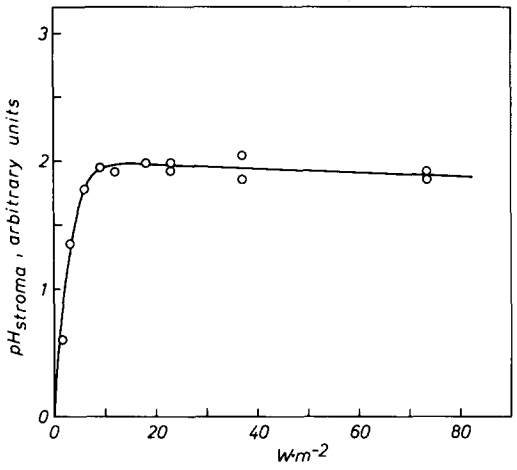

Fig. 9. Light intensity dependence of the alkalization of the chloroplast stroma as measured by the increase in 9-aminoacridine fluorescence quenching on addition of 3.6 mM potassium glyoxylate to intact chloroplasts. Electron acceptor was 2 mM oxaloacetate, concentration of 9-aminoacridine 5 μM, pH 7.6. For the sake of simplicity, the relationship between the increase in fluorescence quenching and the extent of stroma alkalization is assumed to be linear. This assumption is justified for a limited range of pH.

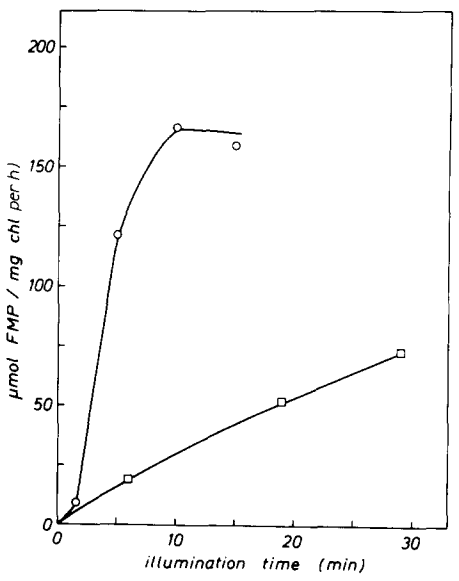

Fig. 10. Activation of fructose bisphosphatase in the stroma of intact chloroplasts at two different light intensities (580 (○) and 6 (□) W · m^{-2}) as a function of time. Activity is expressed as μmol fructose 6-phosphate (FMP) formed/mg chlorophyll per h.

(4) Activation of carbon cycle enzymes in chloroplasts and protoplasts

The light-dependent increase in the chloroplast phosphorylation potential and the decrease in NADP level reveal a considerable accumulation of light products at light intensities which are rate-limiting for photosynthesis. It was therefore of interest to measure the activation state of several carbon cycle enzymes which are known to be light-activated [33]. Fig. 10 shows light-activation of fructose bisphosphatase as a function of illumination time. Chloroplasts were permitted to photoreduce CO_2 at a low and a high light intensity. After different times, they were hypotonically ruptured and assayed at pH 7.8 for fructose biphosphatase activity [21]. Light activation was slow at 6 W · m^{-2} and fast at 580 W · m^{-2} although the NADP system was more reduced at 6 W · m^{-2} than at 580 W · m^{-2} (Fig. 2).

Fig. 11 shows the activity of glyceraldehydephosphate dehydrogenase, phosphoribulokinase and fructose bisphosphatase after 6 min illumination as a function of light intensity. Both phosphoribulokinase and glyceraldehydephosphate dehydrogenase exhibited maximum activity at about 30 W · m^{-2}, i.e. much below light-saturation of photosynthesis. Under the conditions of the test system, both enzymes were active already in the dark. The activation of fructose bisphosphatase which was practically inactive in the dark required somewhat more light than that of the other enzymes. Light-activated fructose bisphosphatase is still subject to pH regulation. It is inactive at pH 7 and highly active above pH 8. Below 10 W · m^{-2}, when considerable activation was already indicated by the assay which was performed at pH 7.8, a suboptimal pH inside the chloroplasts limits activity of the enzyme more than is apparent from the experiment of Fig. 11.

With the exception of fructose bisphosphatase, light activation of enzymes was more pronounced in protoplasts than in chloroplasts, and maximum activity of enzymes was again observed below light saturation of photosynthesis (Fig. 12). In contrast to the chloroplast experiment, the activity of fructose bisphosphatase was significant already in the dark, probably because protoplast extracts contain a cytosolic fructose bisphosphatase [33] which is not subject to

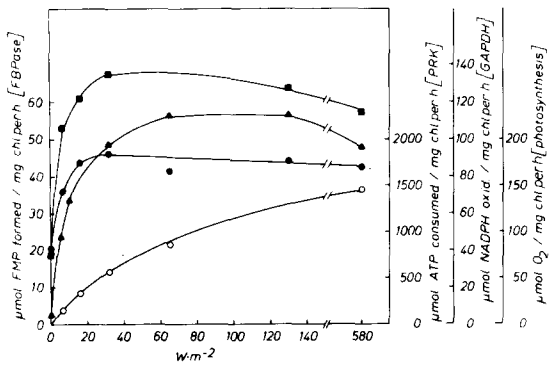

Fig. 11. Activation of glyceraldehydephosphate dehydrogenase (GAPDH) (■), phosphoribulokinase (PRK) (●) and fructose bisphosphatase (FBPase) (▲) in intact chloroplasts after 6 min illumination and rates of photosynthesis (O_2 evolution, ○) as a function of light intensity.

light activation and which is assayed together with the chloroplast enzyme.

It should be noted that light activation of enzymes usually exhibited a maximum and decreased somewhat towards very high light intensities. It is possible that this decrease is related to the increased oxidation of the chloroplast NADP system as shown in Fig. 2.

(5) Levels of 3-phosphoglycerate and dihydroxyacetone phosphate in leaf cell protoplasts

It has been shown above that levels of NADP decrease and ratios of [ATP] to [ADP] increase in chloroplasts on illumination, while the stromal H^+ concentration decreases. Assessment of the state of the reaction reducing phosphoglycerate (Eqn. 1) requires also information on the response of phosphoglycerate and triosephosphate to illumination. Fig. 13A shows the level of 3-phosphoglycerate in protoplasts during illumination with 16 W · m^{-2} red light which does not saturate photosynthesis (cf. Figs. 5, 6, 11 and 12). In the dark, the level of phosphoglycerate was very high. There was a dramatic decrease upon illumination. In another protoplast preparation (Fig. 13B), dark levels of phosphoglycerate were lower by a factor of almost 50 than in the experiment of Fig. 13A. Large variations in pool sizes of phosphoglycerate have also been observed in leaves [34]. Illumination with 18 W · m^{-2} red light again caused a fast decline in phosphoglycerate to a minimum. Subsequently, phosphoglycerate increased and reached a level after 6 min illumination which was higher than the original dark level. The increase in phosphoglycerate coincided with the acceleration of photosynthesis after the initial lag. 7.5 W · m^{-2} red light caused a similar response as 18 W · m^{-2}, but the decrease in phosphoglycerate was somewhat slower, the minimum occurred at a higher phosphoglycerate level and the phosphoglycerate concentration reached after 6 min illumination was lower than at 18 W ·

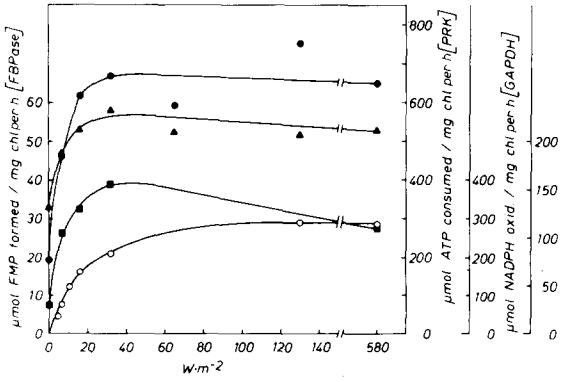

Fig. 12. Activation of glyceraldehydephosphate dehydrogenase (GAPDH), phosphoribulokinase (PRK) and fructose bisphosphatose (FBPase) in spinach protoplasts after 6 min illumination and rates of photosynthesis as a function of light intensity. Symbols as in Fig. 11.

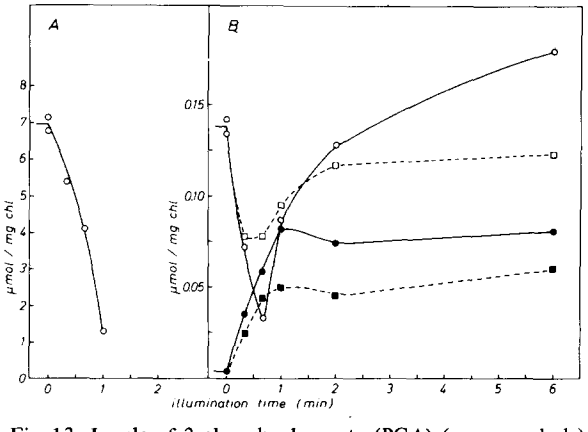

Fig. 13. Levels of 3-phosphoglycerate (PGA) (open symbols) and dihydroxyacetone phosphate (DHAP) (filled symbols) during illumination with low intensities of red light. Note large differences in phosphoglycerate levels in protoplasts from different spinach naterial (Expts. A and B). Light intensities in A: ○———○, 15 W · m^{-2}; in B: □, ■, 7.5 W · m^{-2}, ○, ●, 18 W · m^{-2}.

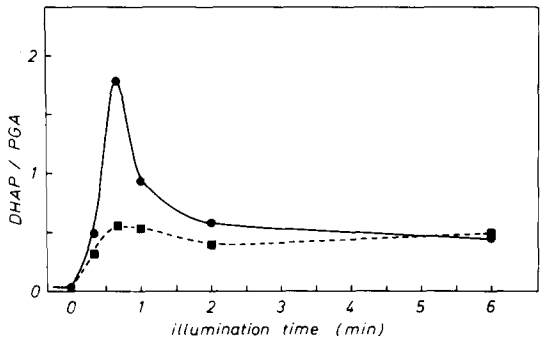

Fig. 14. Ratios of dihydroxyacetone phosphate to 3-phosphoglycerate as a function of illumination time. Data from the experiment shown in Fig. 13B. •——•, 18 W · m^{-2}; ▪- - - - -▪, 7.5 W · m^{-2}.

m^{-2}. The concentration of dihydroxyacetone phosphate was too low in the dark to permit accurate measurements. Measured values ranged between zero and 9 nmol/mg chlorophyll. There was a rapid rise on illumination which coincided with the decrease in phosphoglycerate. The increase in dihydroxyacetone phosphate leveled off after about 1 min illumination. Maximum levels of dihydroxyacetone phosphate were higher under 18 W · m^{-2} than under 7.5 W · m^{-2} red light. Fig. 14 shows ratios of dihydroxyacetone phosphate to phosphoglycerate calculated from the data of Fig. 13B. On illumination, there was a rapid rise in this ratio to a maximum which was larger at the higher than at the lower light intensity. There was a subsequent decline to a ratio which was, after 6 min illumination, not very different at the different light intensities. It is important to note that final ratios observed in the light were far higher than the initial ratio in the dark, which reflects the state of the triosephosphate oxidation system close to equilibrium with the dark phosphorylation potential.

Discussion

(1) The redox state of the chloroplast NADP system

The interpretation of metabolite measurements after acid extraction from organelles or cells is made difficult by the possibility that in situ part of the extracted metabolites is bound to proteins and that measured concentrations therefore do not represent thermodynamically active concentrations. However, previous work on leaf cell energization has shown that mass action ratios of adenylates extracted from chloroplast [23] and leaves (Kobayashi, unpublished results) are, irrespective of the metabolic state of the system, very close to the equilibrium of the adenylate kinase reaction. This argues against significant binding of adenylates to chloroplast or leaf cell components [23]. Also, when measured concentrations of the components of the triosephosphate oxidation system including NADPH and NADP were inserted into Eqn. 1, the resulting mass action ratio was surprisingly close to the equilibrium of the system when metabolic fluxes were small [22]. This suggests that binding of NADP and NADPH to chloroplast components is not very extensive and that NADP measurements reflect within reasonable limits the thermodynamic state of NADP in situ. If this is accepted, the data of Figs. 1–3 show that during photosynthesis the NADP system is never fully reduced, and furthermore, that it is often actually more reduced at low than at high light intensities. Electron transport is known to be subject to photosynthetic control [35]. Increased energization of the thylakoid membrane at high light intensities may therefore be responsible for increased oxidation of the NADP system. In terms of Eqn. 1, increased oxidation of the NADP system at high light intensities may be balanced (or rather overbalanced so as to drive reduction in the chloroplasts) by an increased phosphorylation potential or an increased ratio of phosphoglycerate to dihydroxyacetone phosphate or by both [22].

(2) State of the adenylate system

In the dark, [ATP]/[ADP] ratios were low and variable in isolated chloroplasts (0.4 and 0.1 in the experiments shown in Figs. 4–6; in other experiments, ratios up to 1 were observed [26]) ATP is derived in isolated chlorplasts from the oxidation of triosephosphate which is formed during starch breakdown [12,36]. Chloroplasts in situ exhibit higher [ATP]/[ADP] ratios in the dark than isolated chloroplasts [9] because the chloroplast adenylate system is linked in the leaf in a complex manner [12,32,37] to the adenylate system of the cytosol. Illumination resulted in a large rise of the chloroplast [ATP]/[ADP] ratio (Figs. 4–6). Its extent was a function of light intensity. A comparison of Figs. 5 and 6 with

Fig. 2 reveals that the sensitivity of the adenylate system to light was lower than that of the chloroplast NADP system. It is remarkable that high rates of photosynthesis were observed at very different ratios of [ATP]/[ADP] (Figs. 5 and 6). Robinson and Walker [38] have recently concluded from experiments with a reconstituted chloroplast system that [ATP]/[ADP] ratios play an important regulatory role in photosynthesis. In their system, low [ATP]/[ADP] ratios inhibited photosynthesis, presumably by decreasing the steady state 1,3-diphosphoglycerate concentration. However, in intact chloroplasts enzymes and substrates are not as diluted as in the reconstituted chloroplast system. Chloroplasts appear to be sufficiently flexible to balance low [ATP]/[ADP] ratios by increased NADPH/NADP ratios or an increased phosphoglycerate concentration (see Eqn. 1). Intact chloroplasts have even been observed to photoreduce glycerate via phosphoglycerate to sugarphosphate at high rates at an [ATP]/[ADP] ratio of 0.16 which was much below the dark ratio of [ATP]/[ADP] in the absence of glycerate [23]. These data do not support a major role of changes in the [ATP]/[ADP] ratio in the regulation of photosynthesis.

As in chloroplasts, [ATP]/[ADP] ratios rise on illumination of protoplasts (Figs. 7 and 8). Since phosphate levels do not increase on illumination, the increase in [ATP]/[ADP] ratios indicates an increase in phosphorylation potential. This is not restricted to chloroplasts, but extends to extrachloroplast compartments [9,10]. Experimentally, this is difficult to verify for the cytosol of which the [ATP]/[ADP] ratios are so high already in the dark [38,39] that measurements of a light-dependent increase requires experimental accuracy that cannot presently be attained. Also, separation of chloroplasts and extrachloroplastic material as recently achieved by silicone layer centrifugation techniques is time-consuming [39]. The importance of the time factor is apparent from the fact that at a rate of 200 μmol CO_2 reduction/mg chlorophyll per h the chloroplast pool of ATP turns over about six times per s. Since cytosolic ATP/ADP ratios are high even in the dark, the observed light-dependent increase in extrachloroplast [ATP]/[ADP] ratios must be due in part to a light-dependent increase in mitochondrial [ATP]/[ADP] ratios.

When illumination of protoplasts was prolonged, [ATP]/[ADP] ratios decreased, often below original dark ratios, while photosynthesis remained unaffected (Figs. 7, 8B). This phenomenon has already been discussed for chloroplasts. It does not necessarily indicate a large decrease in protoplast phosphorylation potential in the light.

Occasionally, protoplasts were obtained which differed from other material in having unusually low [ATP]/[ADP] ratios (compare Fig. 8B with Figs. 7 and 8A). It should be noted that these protoplasts did not differ significantly in their photosynthetic capacity from protoplasts which showed much higher [ATP]/[ADP] ratios. For chloroplasts, similar observations have been discussed above.

(3) Regulation of leaf cell metabolism by the redox state of the chloroplast NADP system and by phosphorylation potentials

When the light dependency of the pH rise in the stroma, the redox state of the chloroplast NADP system, [ATP]/[ADP] ratios and activation of enzymes are compared, a hierarchy of events becomes apparent (Fig. 15). Very low light intensities that do not yet cause much stroma alkalization already shift the redox state of the NADP system significantly to the reducing side. While the stroma pH increases, [ATP]/[ADP] ratios also rise and light-regulated enzymes are activated. After reduction of the NADP system, the stroma alkalization appeared to be second in approaching light-saturation. Light-activation of glyceraldehydephosphate dehydrogenase and phosphoribulokinase followed closely in chloroplasts. Fructose bisphosphatase appeared to require somewhat higher light intensities to reach full activation. The response of adenylates to light intensity was found to vary between remarkably wide limits. In isolated chloroplasts, ATP levels sometimes were light-saturated at 10 $W \cdot m^{-2}$, but in other experiments full saturation required up to 100 $W \cdot m^{-2}$. In protoplasts, [ATP]/[ADP] ratios were high already in the dark. The increase observed after short illumination was largely saturated at or below 10 $W \cdot m^{-2}$ (Fig. 8). For both NADPH and ATP it is important to realize that at higher light intensities rather small changes in levels that may be difficult to detect experimentally can produce significant changes in the ratios of [NADPH]/[NADP] or [ATP]/[ADP]. These ratios are more im-

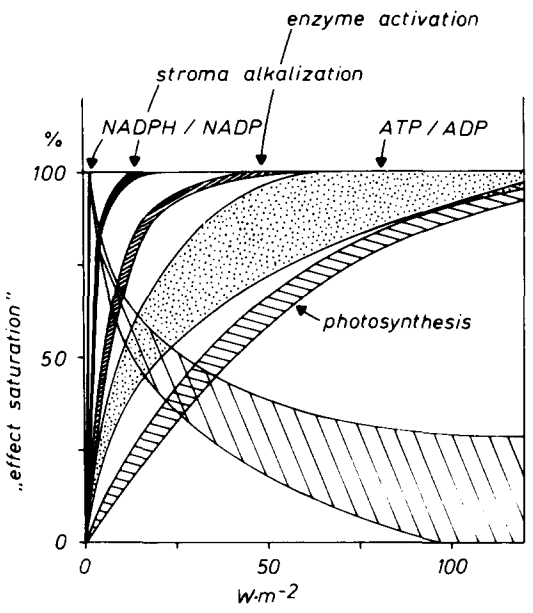

Fig. 15. Schematic representation of the light dependence of different photosynthetic parameters in isolated chloroplasts. 100 denotes saturation of effects (maximum rate of photosynthesis, maximum activity of enzymes, maximum stroma alkalization, highest [ATP]/[ADP] and [NADPH]/[NADP] ratios), and zero denotes dark state.

portant as parameters for phosphoglycerate reduction than pool sizes as can be seen from Eqn. 1. Under rate-limiting light, photosynthesis is, by definition, limited by the availability of products formed by light, i.e., by NADPH and ATP. Yet both photoproducts accumulate while photosynthesis is still far from being light-saturated. On the basis of the data presented above, the following events are envisaged to take place in the dark/light transition when chloroplasts or leaf protoplasts are illuminated with a low light intensity that limits photosynthesis. Even before the pH of the stroma rises significantly, NADP is photoreduced. Since the level of ATP was significant even in the dark, this leads to phosphoglycerate reduction (see Eqn. 1) and the ratio of dihydroxyacetone phosphate to phosphoglycerate increases. A large increase is indeed observed upon illumination (Fig. 14). At its peak, carbon flux is minimal and photosynthesis is still in the lag phase. Dihydroxyacetone phosphate now feeds the reactions leading to ribulose monophosphate. This reacts with ATP to form ribulose bisphosphate which on carboxylation generates phosphoglycerate. The increase in phosphoglycerate (Fig. 13B) decreases the ratio of dihydroxyacetone phosphate to phosphoglycerate (Fig. 14), and accumulation of phosphoglycerate in the chloroplasts makes carbon reduction possible, ending the lag phase of photosynthesis. It should be noted that the level of dihydroxyacetone phosphate (Fig. 13B) which is an important factor in generating ribulose bisphosphate is higher at 18 than at 7.5 $W \cdot m^{-2}$. By regenerating ribulose bisphosphate, dihydroxyacetone phosphate contributes to driving photosynthesis. The trivalent phosphoglycerate anion formed in the ribulose bisphosphate carboxylate reaction is trapped in the alkaline stroma of the chloroplast]24,30]. Actually, the overall ratio of dihydroxyacetone phosphate to phosphoglycerate depicted in Fig. 14 is misleading. This ratio is much lower in the chloroplasts than shown in Fig. 14 because of the trapping of phosphoglycerate, and high chloroplast levels of phosphoglycerate have been shown to be important in driving phosphoglycerate reduction [22].

The ATP needed for reduction and for phosphorylation of ribulose monophosphate appears to be produced in more than one reaction. NADP reduction generates ATP by noncyclic phosphorylation. When the ratio [NADPH]/[NADP] increases, electrons can reduce oxygen or are diverted into the cyclic pathway, and further ATP is made by pseudocyclic or cyclic photophosphorylation [40,41]. The reduction state of NADP thus controls electron transport pathways and the accompanying phosphorylation [22]. From Eqn. 1 it can be seen that either the phosphorylation potential or the ratio of phosphoglycerate to dihydroxyacetone phosphate or both, must increase as the proton concentration of the stroma decreases by proton uptake into thylakoids. Since glyceraldehydephosphate dehydrogenase becomes highly active even under illumination with low intensity light, the reaction reducing phosphoglycerate cannot be far from equilibrium when rates of photosynthesis are low. The accumulation of NADPH and ATP that is seen under these conditions in chloroplasts is balanced by the consumption of protons in the stroma space. The activation state of fructose bisphosphatase controls the levels of 3-phosphoglycerate and dihydroxyacetone phosphate via the level of fructose bisphosphate.

In contrast to the trivalent anion 3-phosphoglyc-

erate, the divalent dihydroxyacetone phosphate anion is not retained in the chloroplasts. It is exported via the phosphate translocator of the chloroplast envelope in exchange for divalent phosphate [12,37]. In consequence, the ratio of dihydroxyacetone phosphate to phosphoglycerate is higher in the cytosol than in the chloroplast [27,42]. It is also higher than the overall ratio shown in Fig. 14. The very low dark ratio reflects the steady state of triosephosphate oxidation which is coupled to phosphorylation. The dramatic light-dependent increase in the ratio of dihydroxyacetone phosphate to phosphoglycerate must lead to an increase in the cytosolic phosphorylation potential, especially as the cytosolic [NADH]/[NAD] ratio does not show a corresponding increase [43]. The extrachloroplastic [ATP]/[ADP] ratio has indeed been observed to increase considerably in the light [12,32,37]. Since, in the dark, respiration is controlled by the phosphorylation potential or the [ATP]/[ADP] ratio, any light-dependent increase in the latter would be expected to decrease mitochondrial respiration. Thus, the stroma alkalization and the activation state of chloroplast fructose bisphosphatase appear to be main factors responsible for the increase of the phorphorylation potential under rate-limiting light that decreases or inhibits respiration and thus gives rise to the Kok effect. It should be noted that prerequisites for the Kok effect are a pH gradient across the chloroplast envelope and metabolite transfer across the chloroplast envelope that must be so efficient that cytosolic ATP production can cope with cytosolic ATP consumption. A high chloroplast [ATP]/[ADP] ratio is not a necessary prerequisite, because a high [NADPH]/[NADP] ratio in the chloroplasts can replace a high [ATP]/[ADP] ratio to ensure efficient photosynthetic carbon reduction, which in turn produces a high cytosolic ratio of dihydroxyacetone phosphate to phosphoglycerate by metabolite exchange across the chloroplast envelope. When cytosolic ATP consumption in the light in reactions such as sucrose synthesis is so fast as to prevent the light-dependent increase in the cytosolic phosphorylation potential, the Kok effect should not be observable. In fact, it has not always been observed, and it is absent in some plant species [44]. It appears that the Kok effect can be used to assess the state of the extrachloroplastic adenylate system in illuminated leaves.

It is appropriate to add a remark on metabolic flexibility. It appears that the chloroplast can, depending on light intensity, place the main burden of carbon reduction on different shoulders (Fig. 15). At very low light intensities, the NADP system may carry the main load. At higher intensities, the phosphorylation potential and the NADP system share the burden. At high light intensities, generation of a high ratio of phosphoglycerate to dihydroxyacetone phosphate helps in driving carbon reduction [22]. This possibility of shifting emphasis may enable the chloroplast to operate with only slight increase in energy cost if coupling of phosphorylation to electron transport is not optimal or is even impaired [41]. A decrease in the phosphorylation potential will then be offset by an increase in reduction of the NADP system which acts to divert electrons into the cyclic pathway. This in turn increases phosphorylation. The system thus automatically adjusts so as to permit photosynthesis at the least cost of light quanta.

(4) Enzyme activation

Light activation of several chloroplast enzymes is mediated in part by thioredoxin which in the light is reduced by ferredoxin [45]. Ferredoxin also reduces NADP. The observation that fructose bisphosphatase is activated more slowly at low light intensities (Fig. 10) when NADP is largely reduced (Fig. 2) than at high light intensities when it is more oxidized raises the question of the redox state of ferredoxin at different light intensities. It appears that electron transfer between ferredoxin and NADP (and vice versa) is under tight control. Activation of ferredoxin-NADP reductase by light has been reported by Satoh and Katoh [46].

Acknowledgments

This work was supported by the Stiftung Volkswagenwerk and by the Deutsche Forschungsgemeinschaft. U.T. wishes to express his gratitude to the Nagamatsu Shogakushikin which made his stay in Würzburg possible by providing partial financial support.

References

1 Kok, B. (1949) Biochim. Biophys. Acta 3, 625–631
2 Hoch, G.O., Von Owens, H.O. and Kok, B. (1963) Arch. Biochem. Biophys. 101, 171–180

3 Graham, D. and Chapman, E.A. (1979) in Encyclopedia of Plant Physiology, New Series, vol. 6, Photosynthesis II (Gibbs, M. and Latzko, E., eds.), pp. 150–162, Springer, Heidelberg

4 Evans, E.H. and Carr, N.G. (1979) in Encyclopedia of Plant Physiology, New Series, vol. 6, Photosynthesis II (Gibbs, M. and Latzko, E., eds.), pp. 163–173, Springer, Heidelberg

5 Ishii, R. and Murata, T. (1978) Japan. J. Crop Sci. 47, 547–550

6 Chance, B. and Williams, G.R. (1956) Adv. Enzymol. 17, 65–98

7 Strotmann, H. and Murakami, S. (1976) in Encyclopedia of Plant Physiology, New Series, vol. 3, Transport in Plants III (Stocking, C.R. and Heber, U., eds.), pp. 398–416, Heidelberg

8 Heber, U. (1974) Annu. Rev. Plant Physiol. 25, 393–421

9 Heber, U. and Santarius, K.A. (1970) Z. Naturforsch. 25b, 718–728

10 Sellami, A. (1976) Biochim. Biophys. Acta 423, 524–539

11 Heldt, H.W., Werdan, K., Milovancev, M. and Geller, G. (1973) Biochim. Biophys. Acta 314, 224–241

12 Heber, U. and Walker, D.A. (1979) Trends Biochem. Sci. 4, 252–256

13 Edwards, G.E., Robinson, S.P., Tyler, N.J.C. and Walker, D.A. (1978) Plant Physiol. 62, 313–319

14 Jensen, R.G. and Bassham, J.A. (1966) Proc. Natl. Acad. Sci. US 56, 1056–1101

15 Kaiser, G. (1979) Masters Thesis, Dipl. Biol., University of Düsseldorf

16 Arnon, D.I. (1949) Plant Physiol. 24, 1–15

17 Slater, T.F. and Sawyer, B. (1962) Nature 193, 454

18 Strehler, B.L. (1974) in Methods of Enzymatic Analysis Bergmeyer, H.U., ed.), pp. 2112–2121, Academic Press, New York

19 Lowry, O.H. and Passoneau, J.V. (1972) A Flexible System of Enzymatic Analysis, Academic Press, New York

20 Tillberg, J., Giersch, Ch. and Heber, U. (1977) Biochim. Biophys. Acta 461, 31–47

21 Heber, U., Enser, U., Weis, E., Ziem, U. and Giersch, Ch. (1979) in Covalent and Non-Covalent Modulation of Protein Function (Atkinson, D.E., ed.), pp. 113–138, Academic Press, New York

22 Takahama, U., Shimizu-Takahama, M. and Heber, U. (1981) Biochim. Biophys. Acta 637, 530–539

23 Kobayashi, Y., Inoue, Y., Furuya, F., Shibata, K. and Heber, U. (1979) Planta 147, 69–75

24 Lilley, R., Chon, C.J., Mosbach, A. and Heldt, H.W. (1977) Biochim. Biophys. Acta 460, 259–272

25 Kraayenhof, R. (1969) Biochim. Biophys. Acta 180, 213–215

26 Giersch, Ch., Heber, U., Kobayashi, Y, Inoue, Y., Shibata, K. and Heldt, H.W. (1980) Biochim. Biophys. Acta 590, 59–73

27 Stitt, M., Wirtz, and Heldt, H.W. (1980) Biochim. Biophys. Acta 593, 85–102

28 Enser, U. and Heber, U. (1980) Proceedings of the 5th International Photosynthesis Congress, Lassandra, Chalkidike, in the press.

29 Werdan, K., Heldt, H.W. and Milovancev, M. (1975) Biochim. Biophys. Acta 396, 276–292

30 Purczeld, P., Chon, C.J., Portis, A.R., Heldt, H.W. and Heber, U. (1978) Biochim. Biophys. Acta 501, 488–498

31 Enser, E. and Heber, U (1980) Biochim. Biophys. Acta 592, 577–591

32 Heber, U. (1980) in Cell Compartmentation and Metabolic Channeling (Nover, L., Lynen, F., and Mothes, K., eds.), pp. 331–344, Fischer, Jena and Elsevier, Amsterdam

33 Kelly, G.J., Latzko, E. and Gibbs, M. (1976) Annu. Rev. Plant Physiol. 27, 181–205

34 Urbach, W., Hudson, M.A., Ullrich, W., Santarius, K.A. and Heber, U. (1965) Z. Naturforsch. 20b, 890–898

35 Hall, D.O. (1976) in The Intact Chloroplast (Barber, J., ed.), pp. 135–170, Elsevier, Amsterdam

36 Stitt, M. and Heldt, H.W. (1981) Plant Physiol., in the press

37 Heber, U. and Heldt, H.W. (1981) Annu. Rev. Plant Physiol. 32, 139–168

38 Robinson, S.P. and Walker, D.A. (1979) Biochim. Biophys. Acta 545, 528–536

39 Hampp, R., Riehl, M. and De Pilippis, L. (1980) Ber. Dtsch. Bot. Ges. 93, 607–618

40 Slovacek, R.E., Crowther, D. and Hind, G. (1980) Biochim. Biophys. Acta 592, 495–505

41 Heber, U., Egneus, H., Hanck, U., Jensen, M. and Köster, S. (1978) Planta 143, 41–49

42 Giersch, Ch., Heber, U., Kaiser, G., Walker D.A. and Robinson, S.P. (1980) Arch. Biochem. Biophys. 205, 246–259

43 Heber, U. and Santarius, K.A. (1965) Biochim. Biophys. Acta 109, 390–408

44 Ishii, R., Shibayama, M. and Murata, Y. (1979) Japan. J. Crop Sci. 48, 52–57

45 Buchanan, B.B. (1980) Annu. Rev. Plant Physiol. 31, 341–374

46 Satoh, K. and Katoh, S. (1980) Plant Cell. Physiol. 21, 907–916