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Light activation of fructose bisphosphatase in photosynthetically competent pea chloroplasts

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The fructose bisphosphatase (EC 3.1.3.11) activity of type A chloroplasts isolated from young (9-day-old) pea (*Pisum sativum* var. Progress no. 9) plants, assayed at physiological pH, substrate and Mg^{2+} concentrations, increased rapidly on illumination. The enzyme activity detected was more than sufficient to account for observed rates of CO_2 fixation both during the induction period and during steady-state CO_2 fixation, whether or not dihydroxyacetone phosphate had been added to the preparation. Omission of catalase from the suspension medium had no effect. On switching off the light, CO_2 fixation by the chloroplasts ceased at once, yet fructose bisphosphatase activity decreased much more slowly. Changes in enzyme activity were much less marked if assays were conducted at 3 mM substrate and 10 mM-Mg^{2+}. Chloroplasts from older (13–20-day-old) peas only fixed CO_2 rapidly if catalase was present in the assay medium. The fructose bisphosphatase activity detected under physiological assay conditions was again more than sufficient to account for observed rates of CO_2 fixation. In the presence of added dihydroxyacetone phosphate, however, the rate of CO_2 fixation appeared to be determined by the rate of light activation of fructose bisphosphatase. In general, the rates of CO_2 fixation and enzyme activation, and the final enzyme activity achieved, decreased markedly with increasing age of the plants. The role of light activation of fructose bisphosphatase as a means of controlling the rate of CO_2 fixation in pea chloroplasts is discussed.

Most of the work to date on CO_2 fixation and the enzymes of the Calvin cycle has been carried out with chloroplasts from mature spinach (*Spinacia oleracea*) leaves, although, more recently, wheat chloroplasts have been studied (Leegood & Walker, 1980*a*,*b*). Work by Robinson & Wiskich (1976, 1977) and Stankovic & Walker (1977) has shown that chloroplasts isolated from young pea (*Pisum sativum*) leaves have markedly different properties in relation to their CO_2 fixation ability. For instance, adenine nucleotides cross the mature spinach chloroplast envelope at only very low rates (Heldt, 1969), but they are apparently able to cross the envelope of young pea chloroplasts rapidly. In fact, both adenine nucleotides and pyrophosphate are required by young pea chloroplasts for optimum rates of CO_2 fixation apparently because of an unidentified 'ATP-consuming reaction' that occurs in the stroma at high rates (Robinson & Wiskich, 1976). It was also shown that chloroplasts isolated from 'older' peas show significantly lower rates of CO_2 fixation and less stimulation by adenine nucleotides and pyrophosphate.

Abbreviation used: FBPase, fructose bisphosphatase (EC 3.1.3.11).

Light activation of FBPase in isolated spinach (Robinson & Walker, 1980; Charles & Halliwell, 1981*a*) and wheat (Leegood & Walker, 1980*a*) chloroplasts is too rapid and great in extent to account for the induction period (Walker, 1976) of CO_2 fixation. Light activation of the spinach enzyme is brought about by the generation of the reduced form of the protein thioredoxin, via a reduced ferredoxin:thioredoxin reductase enzyme (Buchanan *et al.*, 1979). Reduced thioredoxin acts on spinach chloroplast FBPase so as to decrease its K_m values for Mg^{2+} and substrate, enabling the enzyme to achieve close to its maximum velocity at physiological concentrations of substrate and Mg^{2+} (Charles & Halliwell, 1981*a*). The thioredoxin system is presumably also responsible for the light activation of FBPase in wheat chloroplasts (Leegood & Walker, 1980*b*).

By contrast, Anderson *et al.* (1979) have suggested that light activation of FBPase in pea

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chloroplasts occurs by a completely different mechanism from that in spinach chloroplasts, in that it involves a thylakoid-bound 'light-effect mediator' that is close to ferredoxin in the electron-transport chain, rather than a soluble thioredoxin molecule. A thioredoxin has recently been detected in pea chloroplast fractions, however (Scheibe & Anderson, 1981). Anderson has also claimed that the rate of activation of FBPase by the light-effect mediator in pea chloroplasts controls the rate of CO_2 fixation. Unfortunately, the chloroplasts used in these experiments were prepared by methods unlikely to yield a significant percentage of type A chloroplasts. A proper evaluation of this claim requires a comparison of the rates of CO_2 fixation and FBPase activity along the lines of the experiments carried out with spinach and wheat chloroplasts.

In the present paper, we report studies of the light activation of FBPase in type A (Hall, 1972) pea chloroplasts obtained from plants of well-defined ages and able to fix CO_2 at significant rates.

Materials and methods

Materials

All reagents were purchased from Sigma Chemical Co., Poole, Dorset, U.K. Pea (*Pisum sativum* var. Progress no. 9) seeds were purchased from Suttons Seeds, London, U.K., and soaked overnight in tap water at room temperature before being sown in vermiculite, moistened with tap water, the following morning. The day of sowing constituted day 1 in our representation of plant age. After sowing, the seeds were germinated in a greenhouse where the temperature was thermostatically maintained at 20–25°C. Supplementary light was supplied from 12 200 W tungsten bulbs, placed 1 m from the surface of the vermiculite and at $\frac{1}{3}$m centres for 12h periods (06:00–18:00h) separated by 12h of darkness. Shoot tips had usually appeared 3 days after sowing. In the present experiments plants were used 9, 13 and 20 days after sowing.

Preparation of intact chloroplasts

Pea seedlings (20g) were harvested after 2–4h in the light and homogenized for two 1s bursts in a Polytron homogenizer at speed setting 6 in 80ml of a partially frozen solution containing 330mM-betaine, 50mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/KOH buffer (pH7.6), 2mM-EDTA, 1mM-$MgCl_2$ and 1mM-$MnCl_2$. The resultant brei was filtered through a layer of cotton wool sandwiched between two layers of muslin into a 100ml centrifuge tube and centrifuged in an MSE Super Minor centrifuge equipped with a swing-out rotor. The maximum speed was 2000 ***g*** and the total centrifugation time 60s. The supernatant was decanted and the pellet was carefully rinsed with homogenizing medium. The pellet was finally resuspended by careful stroking with a paint brush in 2ml of homogenizing medium. All operations were performed as near to 0°C as possible. Chloroplasts used in the present study were at least 70% intact as determined by the ferricyanide test (Lilley *et al.*, 1975). Chlorophyll was determined by the method of Arnon (1949).

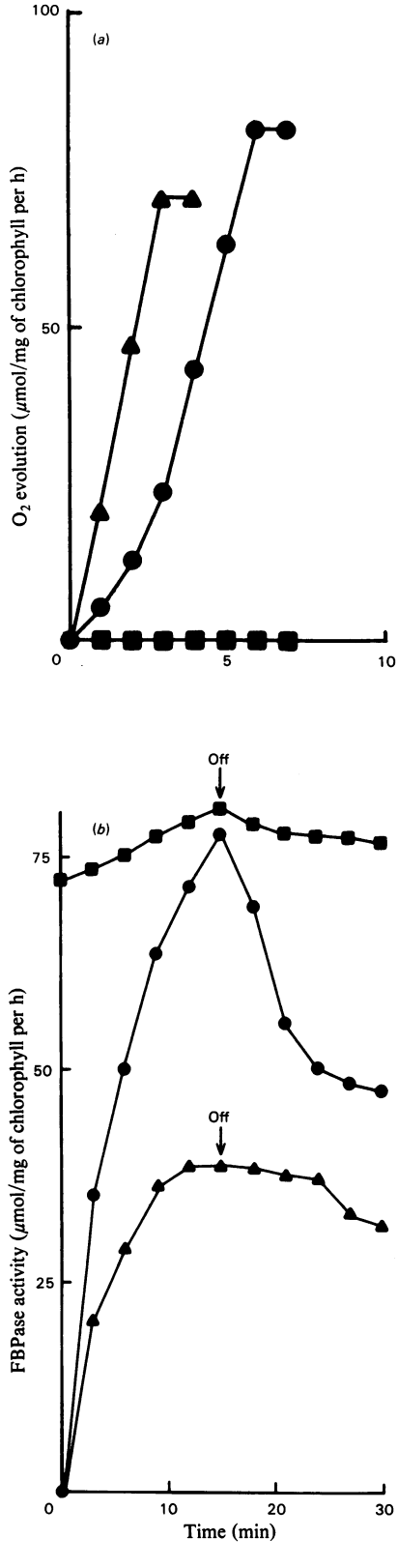
Measurement of CO_2-dependent O_2 evolution

CO_2-dependent O_2 evolution was measured at 25°C using an O_2 electrode supplied by Hansatech, Norfolk, U.K. The reaction mixtures (2ml) were illuminated with a 250-W slide projector equipped with a tungsten light source and a red filter with little transmittance below 600nm. The light was passed through a spherical water-filled flask, which acted as an additional lens and also as a heat filter. The mixtures contained 330mM-betaine, 50mM-Hepes/KOH buffer (pH8.0), 2mM-EDTA, 1mM-$MgCl_2$, 1mM-$MnCl_2$, 0.2mM-Na_2HPO_4, 5mM-$Na_4P_2O_7$, 0.2mM-ATP, 10mM-$NaHCO_3$ and 2000 units of catalase (EC 1.11.1.6) per ml. The chlorophyll concentration was 50μg/ml.

Determination of FBPase activity

Reaction mixtures, at 25°C, contained, in a total volume of 1ml, 100mM-Tris/HCl buffer (pH8.0), 0.5mM-EDTA, 5mM-$MgCl_2$, 0.3mM-$NADP^+$, 3 units of glucose phosphate isomerase (EC 5.3.1.9), 1 unit of glucose 6-phosphate dehydrogenase (EC 1.1.1.49), 0.02% Triton X-100 and 0.1mM-fructose 1,6-bisphosphate. The reaction was started by the addition of 100μl (equivalent to 5μg of chlorophyll) from the O_2 electrode and monitored by the change in A_{340} due to reduction of $NADP^+$ in a Pye–Unicam SP8-100 u.v. spectrophotometer. The change in absorbance was proportional to the amount of extract added over the range used in this study.

Recent estimates of the concentration of fructose 1,6-bisphosphate in isolated chloroplasts range from 0.1 to 0.4mM (Lilley *et al.*, 1977; Heldt *et al.*, 1978, 1980; Kaiser & Bassham, 1979). The lowest concentration, and incidentally the most recent obtained using very sensitive techniques (Heldt *et al.*, 1980), was used, as the object of the present experiments was to assess the importance of the increase in FBPase activity in controlling the rate of CO_2 fixation. The basal concentration of Mg^{2+} in isolated chloroplasts has recently been assessed as 1–3mM (Portis, 1981) and to increase by approx. 2mM in the light (Portis & Heldt, 1976; Krause, 1977). The assumption was therefore made that the assay conditions used approximated physiological conditions.



Results

Experiments were conducted on freshly isolated type A chloroplasts from 9-, 13- and 20-day-old pea seedlings grown as described in the Materials and methods section. Fig. 1(*a*) shows the rate of CO_2 fixation by a typical preparation from 9-day-old plants, measured as CO_2-dependent O_2 evolution. Increasing the concentration of inorganic phosphate in the reaction mixture from 0.2 mM (which gave maximum rates of photosynthesis) to 5 mM-inhibited CO_2 fixation completely, as did omission of ATP and pyrophosphate from the reaction mixture. Surprisingly, omission of catalase had no effect on the induction period and caused only a 10% inhibition of the final rate of CO_2 fixation (results not shown). This may be contrasted with results obtained with isolated spinach chloroplasts, where a 40% inhibition of CO_2 fixation on omission of catalase is not uncommon (Charles & Halliwell, 1981*a*). Addition of 0.1 mM-dihydroxyacetone phosphate to the pea chloroplast reaction mixture shortened the induction period as expected.

At intervals samples of the reaction mixture were withdrawn, lysed by osmotic shock and exposure to Triton X-100 and immediately assayed for FBPase activity using physiological conditions (see the Materials and methods section) (Fig. 1*b*). No activity was detected in freshly isolated chloroplasts kept in the dark, but illumination induced a rapid rise in activity. Omission of catalase, increasing the concentration of P_i to 5 mM or addition of 0.1 mM-dihydroxyacetone phosphate all had no effect on enzyme activation. These observations are in complete contrast with results obtained with mature spinach chloroplasts (Charles & Halliwell, 1981*a*,*b*). However, omission of ATP and pyrophosphate

Fig. 1. *CO_2 fixation (a) and effect of light on the activity of FBPase (b) in type A pea chloroplasts isolated from 9 day old plants*

(*a*) CO_2-dependent O_2 evolution was measured using an O_2 electrode as described in the Materials and methods section. Illumination was started at 0 min in the presence or absence of various substrates as shown. Symbols: ●, control; ▲, +0.1 mM-dihydroxyacetone phosphate; ■, +5 mM-P_i or −0.2 mM-ATP and 5 mM-pyrophosphate. (*b*) CO_2 fixation was carried out in an O_2 electrode as described in the Materials and methods section or in the absence of 0.2 mM-ATP and 5 mM-pyrophosphate as shown. Illumination was commenced at 0 min and continued for 15 min and then stopped as indicated by the arrows. Portions were removed at the times indicated and assayed for enzyme activity as described in the Materials and methods section unless otherwise indicated. Symbols: ●, control; ■, control (activity determined using 3 mM substrate and 10 mM-Mg^{2+}); ▲, −0.2 mM-ATP and 5 mM-pyrophosphate.

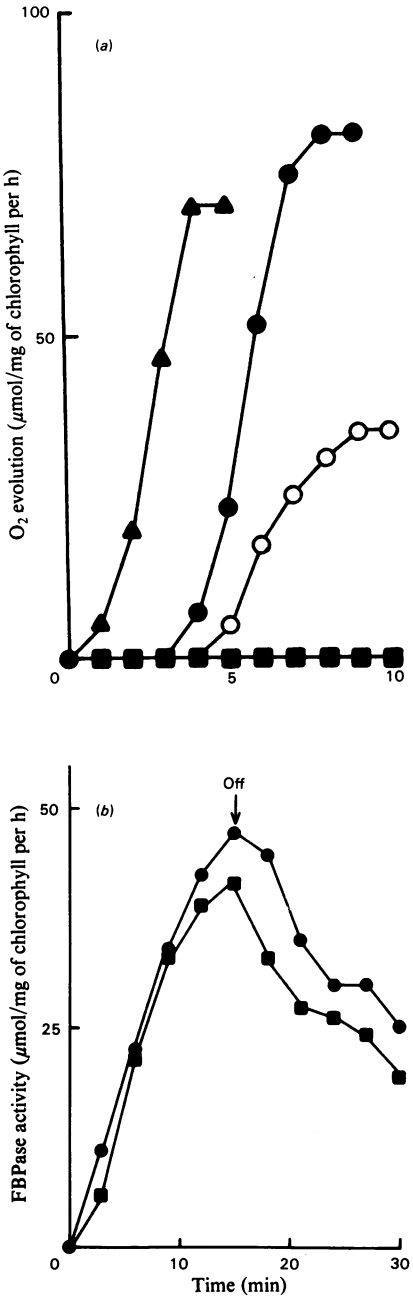


Fig. 2. *CO_2 fixation (a) and effect of light on the activity of FBPase (b) in type A pea chloroplasts isolated from 13-day-old plants*

(*a*) CO_2-dependent O_2 evolution was measured using an O_2 electrode as described in the Materials and methods section. Illumination was started at 0 min in the presence or absence of various substrates and catalase as shown. Symbols: ●, control; ▲, +0.1 mM-dihydroxyacetone phosphate; ○, −catalase; ■, +5 mM-P_i or −0.2 mM-ATP and 5 mM-pyrophosphate. (*b*) CO_2 fixation was carried out in an O_2 electrode as described in the Materials and methods section or in the absence of catalase as shown. Illumination was commenced at 0 min and continued for 15 min and then stopped as indicated by the arrow. Portions were removed at the times indicated and assayed for enzyme activity as described in the Materials and methods section. Symbols: ●, control; ■, −catalase.

approximately halved the rate and extent of activation (Fig. 1*b*). It should be noted that increasing the substrate concentration in the assay from 0.1 to 0.4 mM (the highest recent estimate; Kaiser & Bassham, 1979) gave essentially the same results.

Switching off the light caused an immediate cessation of CO_2 fixation (results not shown) in agreement with results obtained with mature spinach chloroplasts (Charles & Halliwell, 1981*a*). However, enzyme activity measured under physiological conditions decreased by only 18–39% after 15 min in the dark (Fig. 1*b*). Fig. 1(*b*) also shows the effect of assaying FBPase at pH 8.0 with 3 mM substrate and 10 mM-Mg^{2+}. Under these assay conditions illumination had little effect on FBPase activity. It seems, then, that the effect of illumination on the pea chloroplast enzyme is not to change its $V_{max.}$, but to decrease its K_m values for fructose 1,6-bisphosphate and Mg^{2+} from high concentrations to the physiological range, as happens in illuminated spinach chloroplasts (Charles & Halliwell, 1981*a*).

Similar experiments were performed on chloroplasts isolated from 13-day-old and 20-day-old pea seedlings. Fig. 2(*a*) shows CO_2 fixation by chloroplasts isolated from 13-day-old peas. The induction period was much longer and under standard conditions (see the Materials and methods section) no CO_2 fixation occurred for 3 min, although the final rate was the same as in the chloroplasts isolated from 9-day-old peas (a little over 80 μmol/mg of chlorophyll per h). Addition of 0.1 mM-dihydroxyacetone phosphate or elevated concentrations of P_i had the same effect as with the younger chloroplasts and similarly no CO_2 fixation occurred in the absence of ATP and pyrophosphate. However, by contrast with chloroplasts from the younger plants, omission of catalase lengthened the induction period and suppressed the final rate of CO_2 fixation by 57%, an effect similar to that seen in chloroplasts isolated from mature spinach leaves.

The light activation of FBPase assayed using physiological conditions in chloroplasts isolated from 13-day-old pea seedlings is shown in Fig. 2(*b*). Comparison with Fig. 1(*b*) shows that the rate of activation was much slower and the maximum enzyme activity achieved was just over half that observed in chloroplasts from 9-day-old plants. The effects of adding 0.1 mM-dihydroxyacetone phos-

phate, elevated concentrations of P_i or omission of ATP and pyrophosphate all gave similar results to the effects seen in the younger chloroplasts. However, despite the drastic effect of omitting catalase on CO_2 fixation (Fig. 2*a*) the rate and extent of activation of FBPase were only slightly inhibited (Fig. 2*b*), again in sharp contrast with the results obtained with mature spinach chloroplasts (Charles & Halliwell, 1981*a*). Switching off the light gave similar results to those seen with the younger pea chloroplasts (cf. Figs. 1*b* and 2*b*). Again, similar results were obtained if the concentration of substrate in the assay was raised to 0.4 mM.

Chloroplasts prepared from 20-day-old pea seedlings had similar properties to those from 13-day-old plants, except that they showed a marked deterioration in all properties tested. Using the chloroplast isolation procedure detailed in the Materials and methods section, typically only 50% of the organelles obtained were intact, as opposed to at least 70% in preparations from younger plants. CO_2 fixation exhibited an induction period of 13 min and reached a final rate of only 12 μmol/mg of chlorophyll per h (results not shown). FBPase activity, assayed using physiological conditions, reached a maximum of approx. 7 μmol/mg of chlorophyll per h after 12 min illumination (results not shown). Assays performed at pH 8.0 with 3 mM substrate and 10 mM-Mg^{2+} again showed little alteration in activity on illumination, but the amount of activity was only approx. 15 μmol/mg of chlorophyll per h (results not shown), compared with about 80 μmol/mg of chlorophyll per h for chloroplasts from 9-day-old pea seedlings (Fig. 1*b*).

Before an activation system can be classed as being physiologically relevant in controlling CO_2 fixation, a correlation must be made between the enzyme activity generated and the rate of CO_2 fixation observed. To do this it is necessary to calculate the theoretical rate of CO_2 fixation that the measured enzyme activity could support. For this purpose we have assumed that isolated chloroplasts produce and export triose phosphates as products, in exchange for external P_i, rather than produce starch as a product. Accordingly, from the stoichiometry of the Calvin cycle, 33 μmol/mg of chlorophyll per h of FBPase activity would be required to support a CO_2 fixation rate of 100 μmol/mg of chlorophyll per h.

Table 1. *Observed rates of CO_2* fixation by type A pea chloroplasts isolated from 9- and 13-day-old plants in relation *to their FBPase activity*

CO_2 fixation, in some cases in the presence of 0.1 mM-dihydroxyacetone phosphate as shown, and FBPase activity were measured as described in the Materials and methods section.

	Plant age (days)	Reaction conditions	Illumination time (min)	Enzyme activity (μmol/mg of chlorophyll per h)	Maximum theoretical rate of CO_2 fixation (μmol/mg of chlorophyll per h)	Observed rate of CO_2 fixation (μmol/mg of chlorophyll per h)
(*a*)	9	Control	0	0	0	0
			3	35	106	24
			6	50	152	81
			9	64	194	81
			12	71	215	81
			15	77	233	81
		+0.1 mM-Dihydroxyacetone phosphate	0	0	0	0
			3	39	118	70
			6	53	161	70
			9	63	191	70
			12	74	224	70
			15	77	233	70
(*b*)	13	Control	0	0	0	0
			3	11	33	0
			6	23	70	52
			9	34	103	81
			12	43	130	81
			15	47	142	81
		+0.1 mM-Dihydroxyacetone phosphate	0	0	0	0
			3	12	36	47
			6	23	70	70
			9	35	106	70
			12	43	130	70
			15	47	142	70

These values were calculated for the enzyme activations seen in the chloroplasts from 9- and 13-day-old peas, under standard (see the Materials and methods section) and optimum (+0.1 mM-dihydroxyacetone phosphate) conditions of CO_2 fixation. The results are shown in Table 1. The chloroplasts from 9-day-old peas contained more than enough enzyme activity (measured at physiological pH, substrate and Mg^{2+} concentration) to support the rates of CO_2 fixation observed at all times in the presence or absence of 0.1 mM-dihydroxyacetone phosphate (Table 1*a*). The rate and extent of enzyme activation observed in chloroplasts isolated from 13-day-old peas fixing CO_2 under standard conditions was again more than sufficient to account for the rates of CO_2 fixation observed (Table 1*b*). However, in the presence of 0.1 mM-dihydroxyacetone phosphate the rate of enzyme activation appeared to be controlling CO_2 fixation during the induction period (cf. Table 1*b* and Fig. 2*a*), but became more than adequate subsequently. Again, this result is in contrast with results from similar experiments conducted on mature spinach chloroplasts (Charles & Halliwell, 1981*b*). Similar experiments conducted on chloroplasts isolated from 20-day-old pea plants (results not shown) gave essentially the same results as the chloroplasts isolated from 13-day-old peas, except, of course, the activities were much lower, as already mentioned.

Discussion

The results presented in this paper are, as far as we are aware, the first to demonstrate CO_2 fixation and enzyme activation in the same isolated type A pea chloroplasts and they have therefore enabled us to assess the relative importance of the light activation of FBPase (Anderson *et al.*, 1979) in regulation of CO_2 fixation in pea chloroplasts. The results shown in Figs. 1(*a*) and 1(*b*) and Table 1(*a*) clearly show that the light activation of FBPase in young pea chloroplasts (isolated from 9-day-old pea seedlings) is too rapid and too great in extent to account for the observed induction period of CO_2 fixation. It follows that the induction period is due to factors other than light activation of FBPase and probably reflects the time taken to increase the concentration of Calvin-cycle intermediates at the onset of illumination (Walker, 1976). On switching off the light, CO_2 fixation ceases abruptly, and yet enough enzyme activity remains even after 15 min in the dark to support more than the maximum rate of CO_2 fixation observed (Fig. 1*b*). Obviously, dark 'de-activation', presumably by a reversal of the light-activation system, is not a mechanism by which FBPase is prevented from functioning in the dark. Changes in stromal pH and cation concentrations are much more significant control mechanisms (Charles & Halliwell, 1980*a*,*b*). We have reached a similar conclusion using mature spinach chloroplasts (Charles & Halliwell, 1981*a*). By contrast, de-activation is apparently quicker in wheat chloroplasts, although sufficient FBPase activity remains after 5 min of darkness to support a CO_2 fixation rate of about 120 μmol/mg of chlorophyll per h (Leegood & Walker, 1980*b*).

Experiments conducted on chloroplasts isolated from 13- and 20-day-old peas gave essentially the same results and we would, therefore, draw similar conclusions from them. However, the light activation of FBPase in the presence of catalytic amounts of a Calvin-cycle intermediate (0.1 mM-dihydroxyacetone phosphate) appeared to be controlling CO_2 fixation during the induction period in chloroplasts from 13-day-old peas (Figs. 2*a* and 2*b* and Table 1*b*) and 20-day-old peas (results not shown). This observation gives support to Walker's explanation of the induction period of photosynthesis, i.e. a need for an autocatalytic build-up of Calvin-cyle intermediates rather than light activation of catalysts. Light activation in our chloroplasts only became limiting during the induction period if the concentration of Calvin-cycle intermediates was increased artificially by adding one that was able to enter the chloroplast.

What, then, is the function of the light-activation system in pea chloroplasts? Obviously, the system is required in the first instance to generate the physiologically active form of the enzyme, i.e. the one that will work at near maximal velocity at stromal concentrations of substrate and Mg^{2+}. In spinach chloroplasts, the physiologically active form is converted into a form with much higher K_m values for substrate and Mg^{2+} by H_2O_2, which is generated in the light in intact chloroplasts (Charles & Halliwell, 1980*c*). The catalase requirement for CO_2 fixation and enzyme activation in pea chloroplasts becomes greater with increasing physiological age (cf. Figs. 1 and 2 and the text). Chloroplasts contain no catalase activity (Halliwell, 1978) but *in vivo* they are closely associated with peroxisomes to remove any H_2O_2 that is generated. Also Foyer & Halliwell (1976) proposed that (in spinach chloroplasts) NADPH, reduced glutathione and ascorbate act via glutathione reductase (EC 1.6.4.2) (Halliwell & Foyer, 1978) and ascorbate peroxidase (Groden & Beck, 1979) to remove H_2O_2 in the stroma. In addition, we have suggested that the light-activation system (in spinach) exists to protect the physiologically active forms of chloroplast enzymes from the effects of H_2O_2 (Charles & Halliwell, 1981*a*). Our results for chloroplasts from 9-day-old peas, which do not require catalase for enzyme activation of CO_2 fixation (Figs. 1*a* and 1*b* and the text), suggest that a rather efficient mechanism for the removal of H_2O_2 exists in the stroma of these

chloroplasts or else that little H_2O_2 is generated. The decreased enzyme activation in the older chloroplasts in the absence of catalase presumably reflects the inability of the activation system to keep up with the effect of H_2O_2.

Our chloroplasts, from all physiological ages of plants, were unable to fix CO_2 unless ATP and pyrophosphate were present. It seems that adenine nucleotides in the chloroplasts had diminished to a very low level either during the isolation procedure or before. As the light activation of FBPase is dependent on electron transport, which is to some extent dependent on the presence of adenine nucleotides, it is not surprising that addition of ATP gave rise to an increase in the rate of activation, and doubled the final enzyme activity achieved (Fig. 1*b*).

Throughout the present investigation we have noticed a gradual decline, with increasing physiological age, in the efficiency of all the systems we have looked at. The rate and extent of the light activation of FBPase decrease with age. Also, the total amount of enzyme activity (measured at 3mM substrate and 10mM-Mg^{2+}; see Charles & Halliwell, 1980*a*, 1981*a*) declined from approx. 80μmol/mg of chlorophyll per h in 9-day-old peas (Fig. 1*b*) to approx. 14μmol/mg of chlorophyll per h in 20-day-old peas (results not shown). Therefore 20-day-old peas would be theoretically capable of fixing CO_2 at a maximum rate of only 40μmol/mg of chlorophyll per h. The observed rate of CO_2 fixation for 9-day-old peas was approx. 80μmol/mg of chlorophyll per h and so the decrease in total and physiological enzyme activity was at least partly responsible for the decrease in CO_2 fixation.

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