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Calvin-Cycle Intermediates in Relation to Induction Phenomena in Photosynthetic Carbon Dioxide Fixation by Isolated Chloroplasts

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1. Induction periods in carbon dioxide fixation by isolated pea chloroplasts were shortened by small quantities of Calvin-cycle intermediates. The additional fixation was larger than that which would have followed direct stoicheiometric conversion into ribulose 1,5-diphosphate. 2. When chloroplasts were illuminated in the absence of added substrates (other than carbon dioxide) soluble products were formed in the medium that stimulated fixation by fresh chloroplasts. 3. The induction periods were lengthened by washing the chloroplasts. Addition of catalytic quantities of Calvin-cycle intermediates then decreased the induction periods to their previous values. 4. The induction period was extended by a decrease in temperature but was largely unaffected by a decrease in light-intensity that was sufficient to decrease the maximum rate. 5. It is concluded that the lag periods are a consequence of the loss of Calvin-cycle intermediates, such as sugar phosphates, through the intact chloroplast envelope and that these losses can be made good by new synthesis from carbon dioxide in the reactions of the Calvin cycle.

Since the first demonstration of photosynthetic carbon dioxide fixation by isolated chloroplasts (Arnon, Allen & Whatley, 1954) attempts have been made to determine whether chloroplasts retain the ability to support the full sequence of the Calvin cycle (e.g. see Calvin & Bassham, 1962). Important evidence was obtained by Havir & Gibbs (1963), who showed that, in experiments with 'whole' chloroplasts, radioactivity introduced into C-1 of 3-phosphoglycerate was rapidly distributed between the other carbon atoms of this compound as it is *in vivo*. Further support was provided by the finding that 'whole' chloroplasts will commence to fix carbon dioxide, after a prolonged lag, in the absence of added protein fractions, added cofactors or added substrates other than carbon dioxide (Bamberger & Gibbs, 1965; Walker, 1965*b*).

The present paper provides further evidence that the induction phase or 'lag period' is related to depletion of sugar phosphates. It also shows that small quantities of sugar phosphates will act as catalysts of the Calvin cycle in chloroplasts, in much the same way as certain organic acids promote pyruvate oxidation by isolated mitochondria.

MATERIALS AND METHODS

Chloroplasts were isolated and CO_2 fixation was measured as described in the preceding paper (Bucke, Walker & Baldry, 1966). The chloroplasts were prepared entirely from pea leaves (*Pisum sativum* var. Laxton Superb). Reaction mixtures were incubated in saturating red light at 20° except where indicated. An illuminated bath, with compartments in which temperature and light-intensity could be varied independently and simultaneously (Delieu & Walker, 1966), was used for the experiment illustrated in Fig. 6 and in this experiment sucrose rather than sorbitol was used to maintain the osmotic pressure.

RESULTS

When small quantities of certain intermediates of the Calvin cycle (or related compounds) were incubated with chloroplasts they brought about a response that is consistent with a catalytic role. As Figs. 1 and 2 show, the lag period was shortened and in some instances the maximum rate was increased. In Figs. 1 and 2 an arrow indicates the point at which carbon dioxide fixation would have ceased had it been solely dependent on the stoicheiometric conversion of the additive into ribulose 1,5-diphosphate. In fact fixation invariably continued at an unchanged rate well beyond this point.

Addition of photosynthetic products. It could be inferred from the above results that the chloroplasts are at first deficient in Calvin-cycle intermediates, that these deficiencies are overcome by new synthesis and that this latter process is hastened by any additive that can feed into the Calvin cycle. This interpretation is supported by the results shown in Fig. 3. In this experiment chloroplasts were allowed to photosynthesize in the presence of non-radio-

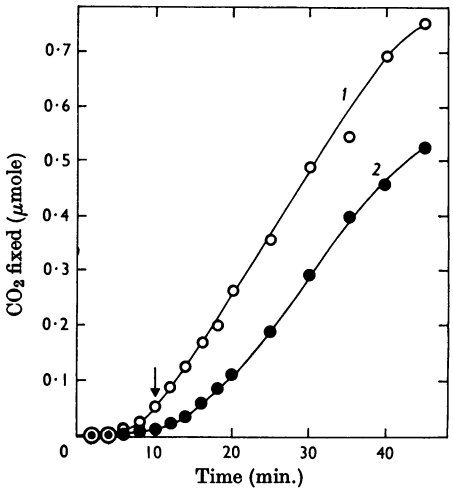

Fig. 1. Progress curve of $^{14}CO_2$ incorporation by isolated pea chloroplasts in the presence of catalytic amounts of triose phosphate. Curve *1* (○), 0·1 μmole of dihydroxyacetone phosphate added; curve *2* (●), no addition. The chlorophyll concentration was 125 μg./reaction mixture (see Materials and Methods section).

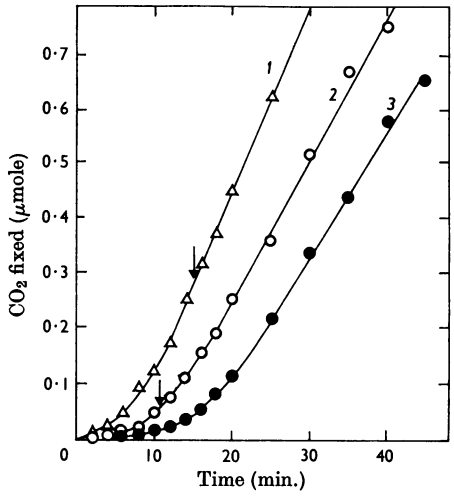

Fig. 2. Progress curves of $^{14}CO_2$ incorporation by isolated pea chloroplasts in the presence of catalytic amounts of sugar phosphates. Curve *1* (△), 0·1 μmole of fructose 1,6-diphosphate + 0·1 μmole of ribose 5-phosphate + 0·1 μmole of dihydroxyacetone phosphate added; curve *2* (○), 0·1 μmole of 3-phosphoglycerate added; curve *3* (●), no addition. The chlorophyll concentration was 164 μg./reaction mixture.

active carbonate (without other added substrate) until well after the normal lag period. They were then centrifuged out of the reaction mixture and the

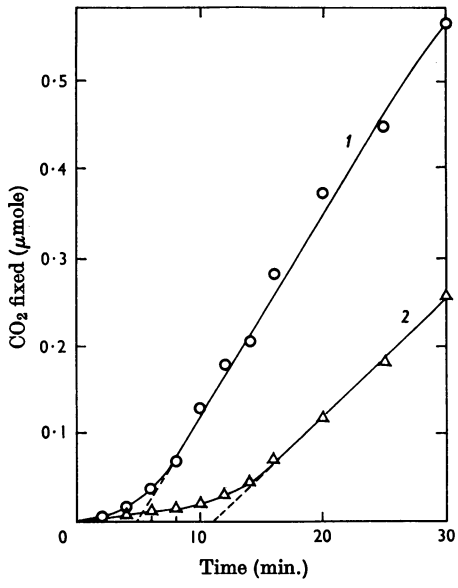

Fig. 3. Progress curves of $^{14}CO_2$ incorporation by isolated pea chloroplasts in the presence of the medium in which pea chloroplasts had been illuminated for 60 min. Curve *1* (○), chloroplasts in the medium; curve *2* (△), dark control (chloroplasts in medium in which chloroplasts had been suspended in the dark for 30 min.). The chlorophyll concentration was 42 μg./reaction mixture.

supernatant was reincubated with fresh chloroplasts and radioactive carbonate. In the control, exactly the same procedure was followed except that the first batch of chloroplasts was not illuminated. Fig. 3 therefore shows that soluble products were lost from illuminated chloroplasts to the medium and that these, when added back to fresh illuminated chloroplasts, caused the same sort of response as added Calvin-cycle intermediates.

Chromatography showed that in other experiments (in which radioactive carbonate was used) the products that were present in the supernatant were not materially different from those in the whole extract. These products were largely sugar phosphates (Walker, 1964, 1965*b*; Baldry, Bucke & Walker, 1966).

Depletion of intermediates. Chloroplasts prepared from pea seedlings grown under low light-intensity and short days (see the Materials and Methods section) contain no detectable starch reserves (iodine test). It might be supposed therefore that, if the remaining reserves were largely soluble and able to diffuse through the chloroplast envelope, these would be depleted by stirring isolated chloroplasts in a large excess of medium. The effect of this treatment is shown in Figs. 4 and 5. Chloroplasts were

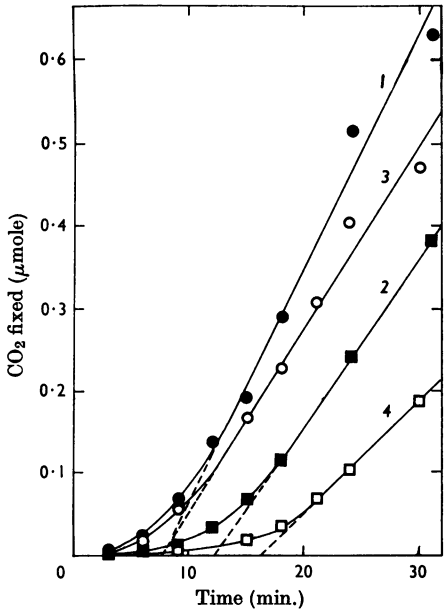

Fig. 4. Progress curves of $^{14}CO_2$ incorporation by isolated pea chloroplasts depleted (by stirring in a large excess of medium) and undepleted. Curve *1* (●), undepleted + 0·1 μmole of ribose 5-phosphate added; curve *2* (■), undepleted without additions; curve *3* (○), depleted + 0·1 μmole of ribose 5-phosphate added; curve *4* (□), depleted without additions. The chlorophyll concentration was 92 μg./reaction mixture (undepleted) or 69 μg./reaction mixture (depleted). The values actually plotted in curves *3* and *4* were multiplied by 92/69 (i.e. corrected for chlorophyll) to permit direct comparison.

prepared normally (see the Materials and Methods section) and half were assayed immediately as before in the presence (Fig. 4, curve *1*) and absence (Fig. 4, curve *2*) of a catalytic quantity of ribose 5-phosphate. The remaining chloroplasts were resuspended in an excess (about 40 ml.) of medium (see the Materials and Methods section) and kept in the cold with occasional gentle stirring. They were then collected by centrifugation and resuspended in the normal small (2·5 ml.) volume of medium and assayed. Not all the chlorophyll was recovered after washing so that in Fig. 4 the rates obtained with the washed chloroplasts (curves *3* and *4*) have been expressed as μmoles of carbon dioxide fixed/mg. of chlorophyll to permit direct comparison. Fig. 4 (curve *4*) shows that washing lengthened the lag by about 4 min. when the chloroplasts were assayed in a reaction mixture that contained no added sugar phosphate. The maximum rate was also depressed by about 35%. Addition of a catalytic quantity of ribose 5-phosphate (Fig. 4, curve *3*) completely eliminated the extension of the induction period seen in its absence and the maximum rate was restored to 78% of the value obtained before washing. In this experiment the standard procedure was followed so that chloroplasts were prepared from leaves that had been in the dark for the preceding 12 hr.

When this was repeated with leaves that had been preilluminated for 2 hr. (1000 ft.-candles, 20–25°), the overall pattern was the same (Figs. 5*c* and 5*d*). All the lag periods were about 2 min. shorter than before, but again washing (Fig. 5*d*) extended the induction period in the control (no added substrate) by about 4 min. (cf. Fig. 5*c*, curve *2*, and Fig. 5*d*, curve *2*), and again the addition of a catalytic quantity of ribose 5-phosphate (Fig. 5*d*, curve *1*) gave the same shorter induction period observed in its presence before washing (Fig. 5*c*, curve *1*). When catalytic quantities of fructose 1,6-diphosphate were used (Figs. 5*a* and 5*b*) the induction period was the same as with ribose 5-phosphate (cf. Fig. 5*a*, curve *1*, and Fig. 5*c*, curve *1*), and again this remained unchanged (cf. Fig. 5*a*, curve *1*, and Fig. 5*b*, curve *1*) after washing, although the maximum rate actually increased.

Effects of temperature and light-intensity. The above results show that the lag period and the maximum rate are not necessarily governed by the same factor. This is also apparent in Fig. 6, which compares the effects of two different temperatures and two different light-intensities. At the higher temperature a decrease in light-intensity (which was sufficient to decrease the maximum rate by one-third) did not produce a measurable effect on the lag period. Conversely, at the higher light-intensity a decrease in temperature (which decreased the maximum rate by two-thirds) more than doubled the length of the lag. At the lower temperature, decreased light-intensity extended the lag by about 1 min. This relationship is complex and requires further investigation, but it does seem clear that in saturating light-intensity the length of the lag period is relatively dependent on temperature and that at high temperatures it is relatively independent of light-intensity.

DISCUSSION

Our results confirm and extend previous observations that led to the proposal that the lag period in carbon dioxide fixation by isolated chloroplasts is brought about by deficiencies in Calvin-cycle intermediates and terminated by their addition or by new synthesis from carbon dioxide. The evidence is as follows:

(1) The lag period could be lengthened by washing, or shortened by the addition of Calvin-cycle intermediates or related compounds.

(2) The effect of small quantities of added

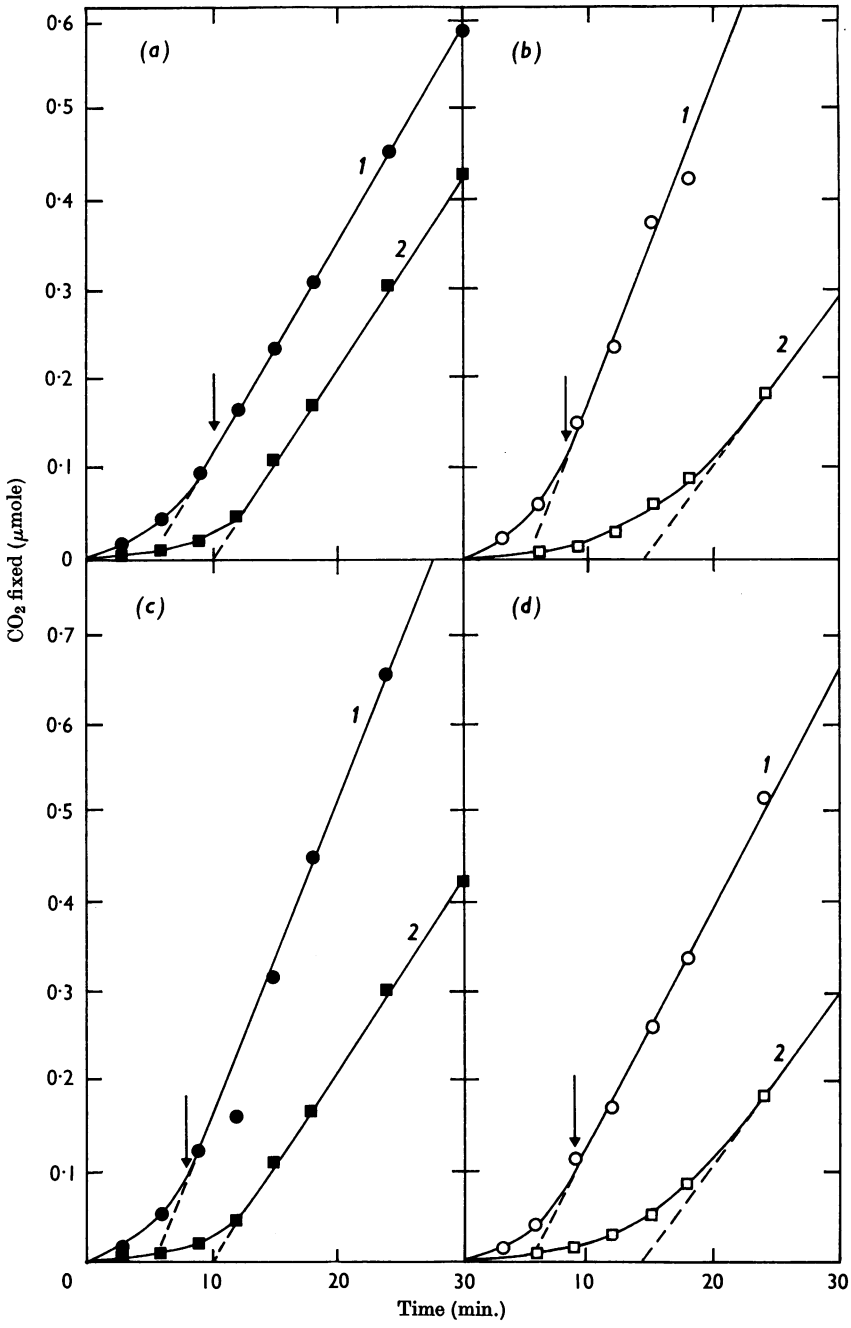

Fig. 5. Progress curves of $^{14}CO_2$ incorporation by chloroplasts prepared from illuminated pea leaves (2 hr. in white light at 1000 ft.-candles). (*a*) Curve *1* (●), undepleted chloroplasts + 0·1 μmole of fructose 1,6-diphosphate added; curve *2* (■), undepleted chloroplasts without additions. (*b*) Curve *1* (○), depleted chloroplasts + 0·1 μmole of fructose 1,6-diphosphate added; curve *2* (□), depleted chloroplasts without additions. (*c*) Curve *1* (●), undepleted chloroplasts + 0·1 μmole of ribose 5-phosphate added; curve *2* (■), undepleted chloroplasts without additions (same as curve *2* in *a*). (*d*) Curve *1* (○), depleted chloroplasts + 0·1 μmole of ribose 5-phosphate added; curve *2* (□), depleted chloroplasts without additions (same as curve *2* in *b*). The chlorophyll concentration was 89 μg./reaction mixture (*a* and *c*) or 59 μg./reaction mixture (*b* and *d*). The values actually plotted in (*b*) and (*d*) were multiplied by 89/59 (i.e. corrected for chlorophyll) to permit direct comparison. The arrows indicate the points at which CO_2 fixation would have ceased had it been solely dependent on the stoicheiometric conversion of the additive into ribulose 1,5-diphosphate.

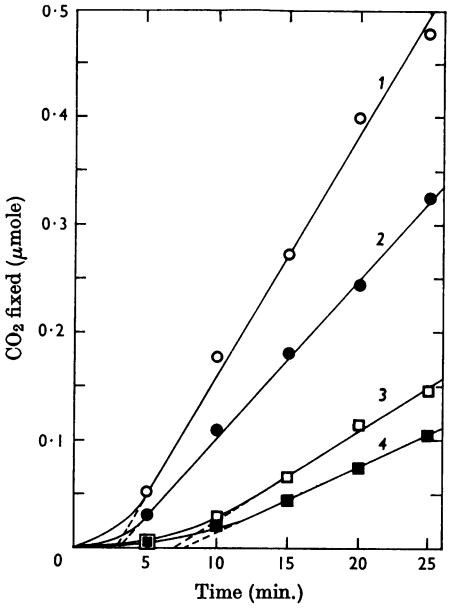

Fig. 6. Progress curves of $^{14}CO_2$ incorporation by isolated pea chloroplasts at combinations of two light-intensities and two temperatures. Curve *1* (○), approx. 10000ft.-candles and 25°; curve *2* (●), 110ft.-candles and 25°; curve *3* (□), approx. 10000ft.-candles and 10°; curve *4* (■), 110ft.-candles and 10°. (Light measurements were made before insertion of red Perspex filters.) The chlorophyll concentration was 25μg./reaction mixture.

intermediates was catalytic. Such an effect is consistent with the operation of an integrated Calvin cycle in which intermediates are interconvertible and in which carbon dioxide leads to a progressive acceleration as the intermediates are built up by a positive-feedback mechanism.

(3) The medium in which chloroplasts were illuminated shortened the lag period when added back to fresh chloroplasts. Since the number of chloroplasts without envelopes does not appreciably decrease during illumination (Walker, 1965*b*) and since the bulk of the fixation is attributable to chloroplasts with apparently intact envelopes (Walker, 1965*a*,*b*) it may be concluded that Calvin-cycle intermediates diffuse both into and out of the intact chloroplast.

(4) The lag period was appreciably lengthened by a decrease in temperature but was less affected by a decrease in light-intensity that was sufficiently large to depress the maximum rate. This would be expected if the length of the lag period was primarily governed by dark rather than photochemical reactions. It strengthens the view (cf. Fig. 6) that the length of the induction period is not wholly dependent on the maximum rate, though factors that tend to diminish the lag also tend to increase the maximum rate.

As we have indicated, it is difficult to escape the conclusion that Calvin-cycle intermediates may diffuse into and out of apparently intact isolated chloroplasts (see also Bamberger & Gibbs, 1956; Walker, 1965*a*,*b*; Bucke *et al.* 1966). It does not necessarily follow that the chloroplast envelopes that are retained are undamaged or that their characteristics *in vivo* are unchanged. Induction phenomena in photosynthesis are well known in intact organisms (for review see Rabinowitch, 1956) and it is noteworthy that lags in carbon dioxide fixation have been observed with chloroplasts in whole leaves (Heber & Willenbrink, 1964; Heber & Santarius, 1965). If Calvin-cycle intermediates, in addition to compounds that might be regarded as an end product of photosynthesis, are free to diffuse from the chloroplast this might well be more pronounced *in vitro*. Such intermediates would presumably diffuse more readily into a relatively large volume of medium that initially contained none. Increased losses could then lead to exaggerated lags. During illumination, steady-state conditions (governed by other limiting factors) would only be reached when the losses were offset by sufficient synthesis to saturate the enzymes of the Calvin cycle.

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