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LIGHT-INDUCED FORMATION OF ENZYMES OF THE C_4-DICARBOXYLIC ACID PATHWAY OF PHOTOSYNTHESIS IN DETACHED LEAVES

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Abstract—A comparison was made of the light-induced formation of enzymes of photosynthetic CO_2-fixation in two plants, maize and sorghum, with the C_4-dicarboxylic acid pathway and two, wheat and oats, with the Calvin cycle. Enzymes of both pathways were examined in the four species. Illumination of detached, etiolated leaves of these plants resulted in increases of chlorophyll and Calvin cycle enzymes, but not of respiratory enzymes. In maize and sorghum, enzymes of the C_4-dicarboxylic pathway, pyruvate, P_i dikinase, phosphopyruvate carboxylase and adenylate kinase also increased, but light had little effect on the activity of phosphopyruvate carboxylase in wheat and oats. When etiolated leaves of maize were detached and illuminated, there was parallel development of enzymes unique to the C_4-dicarboxylic acid pathway, Calvin cycle enzymes common to both pathways and also ribulose-1,5-diphosphate carboxylase. The light-induced increases in activity of enzymes of both the C_4-dicarboxylic acid pathway and the Calvin cycle were inhibited by chloramphenicol. This was consistent with the hypothesis that the increases reflected synthesis of new enzyme on the 70S chloroplast ribosomes. The concentration of chloramphenicol required for inhibition varied with the different plants, wheat being the most sensitive and maize the least sensitive. Cycloheximide at concentrations of around 1 μg/ml also inhibited. This suggested that chloroplast development in leaves involves a close integration with cytoplasmic protein synthesis. Continuous light was not essential for synthesis of enzymes of the C_4-dicarboxylic acid pathway. Irradiation of dark-grown maize with red light (660 nm) of low intensity for 3 min daily on five successive days increased the activity of pyruvate, P_i dikinase by 10·4-fold and phosphopyruvate carboxylase by 7·5-fold compared with non-irradiated plants.

INTRODUCTION

THE SYNTHESIS of chloroplast proteins including Calvin cycle enzymes has been studied in several recent investigations on organelle biogenesis (reviews[1,2]). A number of tropical grasses and other plants contain an alternative pathway to the Calvin cycle for assimilating CO_2 photosynthetically.[3–6] Studies relating to this pathway, termed the C_4-dicarboxylic acid pathway, have been recently reviewed.[7] The enzyme fixing CO_2 in the Calvin cycle, ribulose-1,5-diphosphate (RDP) carboxylase, is not involved in this pathway. However,

[1] J. T. O. KIRK and R. A. E. TILNEY-BASSETT, in *The Plastids: Their Chemistry, Structure, Growth and Inheritance*, W. H. Freeman, London and San Francisco (1967).
[2] R. M. SMILLIE and N. S. SCOTT, "Organelle biogenesis: the chloroplast", in *Progress in Molecular and Subcellular Biology* (edited by F. E. HAHN), Vol. I, In press, Springer-Verlag, Berlin (1969).
[3] H. P. KORTSCHAK, C. E. HARTT and G. O. BURR, *Plant Physiol.* **40**, 209 (1965).
[4] M. D. HATCH and C. R. SLACK, *Biochem. J.* **101**, 103 (1966).
[5] M. D. HATCH, C. R. SLACK and H. S. JOHNSON, *Biochem. J.* **102**, 417 (1967).
[6] H. S. JOHNSON and M. D. HATCH, *Phytochem.* **7**, 375 (1968).
[7] M. D. HATCH and C. R. SLACK, in *Progress in Phytochemistry* (edited by L. REINHOLD and Y. LIWSCHITZ), Vol. 2, In press, Interscience, London (1970).

chloroplasts in these plants contain several enzymes including pyruvate, P_i dikinase, phosphopyruvate (PEP) carboxylase and adenylate kinase[8,9] that either occur in much lower quantities or are absent from plants with the Calvin cycle.[10-12]

The activity of some of the enzymes operative in the C_4-dicarboxylic acid pathway increases, together with chlorophyll, when etiolated maize plants are transferred to the light.[12] However, no information is available about the nature of this light effect. It is not known whether these increases are due to enzyme synthesis, nor where the sites of such synthesis are located. In this paper we show that light induces the synthesis of enzymes of both the C_4-dicarboxylic acid pathway and Calvin cycle in detached leaves. The effects of inhibitors of protein synthesis and the regulatory role of light on the formation of these enzymes are reported.

RESULTS

Light-Induced Increase in Activities of Enzymes of the C_4-Dicarboxylic Acid Pathway and Calvin Cycle in Detached Etiolated Leaves

All enzymes of the Calvin cycle except RDP carboxylase may possibly operate in the C_4-dicarboxylic acid pathway of photosynthesis.[7] For convenience these enzymes will be referred to as Calvin cycle enzymes irrespective of their pathway of operation. Pyruvate, P_i dikinase, PEP carboxylase and adenylate kinase will be referred to as enzymes of the C_4-dicarboxylic acid pathway.

Table 1 compares the enzymic activities of etiolated and greening detached leaves from four grasses, maize and sorghum in which the C_4-dicarboxylic acid pathway operates and wheat and oats with an operational Calvin cycle.[5,10] The etiolated leaves of all the plants contained substantial amounts of the Calvin cycle enzymes RDP carboxylase, NADP-glyceraldehyde-3-phosphate dehydrogenase and alkaline fructose-1,6-diphosphatase, and the activities in illuminated leaves were between 2- and 20-fold higher than in etiolated leaves. In contrast, illumination did not bring about increases in the activities of non-chloroplast enzymes, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase.

The enzymes PEP carboxylase and adenylate kinase may function in several metabolic pathways in leaves, but in leaves containing the C_4-dicarboxylic acid pathway, most of the activity of these enzymes is believed to function in photosynthesis and to be localized in chloroplasts of the mesophyll cells.[8,9] Both enzymes were much more active in extracts of green maize and sorghum leaves compared with the corresponding etiolated leaves. In contrast, the PEP carboxylase activity of green leaves of wheat was similar to that of the etiolated leaves and in the case of oats the activity was only slightly greater in the green leaves. Another enzyme of the C_4-dicarboxylic acid pathway, pyruvate, P_i dikinase was undetectable in wheat and oat leaves. It was present in low amounts in etiolated maize and sorghum leaves, but increased 15-fold in illuminated maize leaves and 8-fold in illuminated sorghum leaves. Separate experiments showed that no light-mediated activation of pyruvate, P_i dikinase occurs when etiolated leaves are exposed to the light as happens upon illumination of green leaves after a period of darkness.[13]

[8] C. R. SLACK, *Phytochem.* **8**, 1387 (1969).
[9] C. R. SLACK, M. D. HATCH and D. J. GOODCHILD, *Biochem. J.* **114**, 489 (1969).
[10] C. R. SLACK and M. D. HATCH, *Biochem. J.* **103**, 660 (1967).
[11] M. D. HATCH and C. R. SLACK, *Biochem. J.* **106**, 141 (1968).
[12] M. D. HATCH, C. R. SLACK and T. A. BULL, *Phytochem.* **8**, 697 (1969).
[13] M. D. HATCH and C. R. SLACK, *Biochem. J.* **112**, 549 (1969).

TABLE 1. COMPARISON OF ENZYMIC ACTIVITIES DURING CHLOROPLAST DEVELOPMENT IN DETACHED LEAVES OF GRASSES CONTAINING THE C_4-DICARBOXYLIC ACID PATHWAY WITH THOSE CONTAINING ONLY THE CALVIN CYCLE*

Plants	Light intensity (lux)	Conditions	Chlorophyll (μg/g fresh wt.)	Enzymic activity (μmoles/min/g fresh wt.) Pyruvate, P_i dikinase	PEP carboxylase	Adenylate kinase	RDP carboxylase	Glyceraldehyde-3-P dehydrogenase (NADP)	Alkaline fructose 1,6-diphosphatase	Isocitrate dehydrogenase (NADP)	Glucose-6-P dehydrogenase
Maize	9,000	Dark	—	0·03	0·42	8·9	0·24	2·62	0·35	0·45	0·50
		Light	220	0·44	3·15	30·8	0·57	16·6	1·28	0·33	0·60
		Light + CAP† (2 mg/ml)	43	0·13	1·33	21·9	0·15	5·2	0·67	0·45	0·57
Sorghum	20,000	Dark	—	0·03	1·02	10·1	0·60	0·25	0·13	0·93	0·42
		Light	184	0·24	4·20	42·5	1·60	4·0	0·38	0·69	0·41
		Light + CAP (0·5 mg/ml)	34	0·11	1·73	35·0	0·46	1·5	0·32	0·59	0·30
		Light + cycloheximide (1 μg/ml)	84	0·16	1·72	23·5	1·28	2·05	0·23	0·69	0·34
Wheat	9,000	Dark	—	0	0·42	—	3·06	10·9	4·3	0·66	0·52
		Light	640	0	0·38	—	6·17	36·0	9·6	0·65	0·51
		Light + CAP (0·4 mg/ml)	55	0	0·45	—	4·10	21·0	8·6	0·47	0·67
Oats	9,000	Dark	—	0	0·14	—	1·14	11·9	1·5	0·28	0·21
		Light	475	0	0·30	—	2·72	18·9	3·8	0·34	0·19
		Light + CAP (1 mg/ml)	1	0	0·14	—	1·46	16·9	3·5	0·32	0·17

* Maize, wheat and oat plants were grown from seed for 7 days in the dark and the upper 1½ in. (maize) or 3 in. (wheat and oats) of the primary leaf was detached and floated on water or a solution of inhibitor contained in a Petri dish for 4 hr at 22°. Some of the dishes were placed in light for a further 35 hr at 22°. All leaves were then weighed and analyzed for chlorophyll and enzymic activity. Sorghum was grown for 9 days, the leaves were detached and floated for 6 hr before illumination for an additional 32 hr.

† CAP = chloramphenicol.

Effects of Chloramphenicol and Cycloheximide

Chloramphenicol, which inhibits the synthesis of protein on the 70S ribosomes of chloroplasts,[2, 14] inhibited the light-induced increases of chlorophyll and of enzymes of both the C_4-dicarboxylic acid pathway and the Calvin cycle, although, with wheat and oats, enzyme synthesis was affected less than chlorophyll synthesis. Preliminary experiments were carried out to determine the effectiveness of chloramphenicol in inhibiting chlorophyll synthesis in the detached leaves and this was found to vary considerably depending on the plant tested. Chloramphenicol at 0·4 mg/ml produced over 90 per cent inhibition of chlorophyll synthesis in wheat, whereas 2–3 mg/ml were required to achieve the same degree of inhibition in maize. Intermediate concentrations (0·5–1·0 mg/ml) were required for sorghum and oats. Lower concentrations of chloramphenicol suffice at light intensities below those used in these studies (unpublished results).

In contrast to chloramphenicol, cycloheximide inhibits cytoplasmic protein synthesis involving 80S ribosome and it does not inhibit the incorporation of amino acids into protein by isolated chloroplasts of wheat (B. J. Reger, R. M. Smillie and R. C. Fuller, unpublished results). The action of cycloheximide on maize is examined in greater detail below, but it can be seen from Table 1 that it inhibited the synthesis of both chlorophyll and photosynthetic enzymes in sorghum at a concentration which is sufficient to inhibit cytoplasmic protein synthesis in most higher organisms. Neither inhibitor showed much effect on the level of the respiratory enzymes.

The experiments with maize were carried out by cutting off the top 1½ in. of the primary leaf and floating the leaf section on water. Other experiments were carried out at a higher light intensity (22,000 lux), or on floated sections of the secondary leaves, or on whole detached primary or secondary leaves with the cut ends immersed in water. In these experiments results very similar to those shown for maize in Table 1 were obtained.

Chloroplast Development in Detached Maize Leaves: Time-Course of the Increase in Activities of Enzymes of Photosynthesis and the Effect of Inhibitors of Protein Synthesis

The data shown in Table 1 established the feasibility of studying chloroplast development in detached etiolated leaves of the four species of plants tested, since substantial increases in chlorophyll and enzymes of the C_4-dicarboxylic and Calvin cycle pathways of photosynthesis occurred upon illuminating the detached leaves. Figure 1 shows the development of chlorophyll and three enzymes of the C_4-dicarboxylic acid pathway in the detached primary leaf of etiolated maize seedlings. The leaves were floated on water or solutions of chloramphenicol or cycloheximide for 5·25 hr in the dark and then illuminated continuously with white light. Other leaves were floated on water and kept in darkness. The activities of pyruvate, P_i dikinase, PEP carboxylase and adenylate kinase in the illuminated leaves increased rapidly during the initial 24 hr of illumination, after which the rate of increase was less. Likewise, most of the synthesis of chlorophyll occurred during the first 24 hr. In contrast, the activities in detached leaves kept in darkness did not change significantly. Chloramphenicol inhibited the increases in chlorophyll, the pyruvate, P_i dikinase, PEP carboxylase and, to a lesser extent, adenylate kinase. A feature of the inhibition by chloramphenicol was that only partial inhibition was observed during the first 17 hr, but that after this period further chloro-

[14] R. M. SMILLIE, N. S. SCOTT, and D. GRAHAM, Biogenesis of chloroplasts: Functional roles for chloroplast DNA and chloroplast ribosomes, in *Comparative Biochemistry and Biophysics of Photosynthesis* (edited by K. SHIBATA, A. TAKAMIYA, A. T. JAGENDORF and R. C. FULLER), p. 332. University of Tokyo Press, Tokyo (1968).

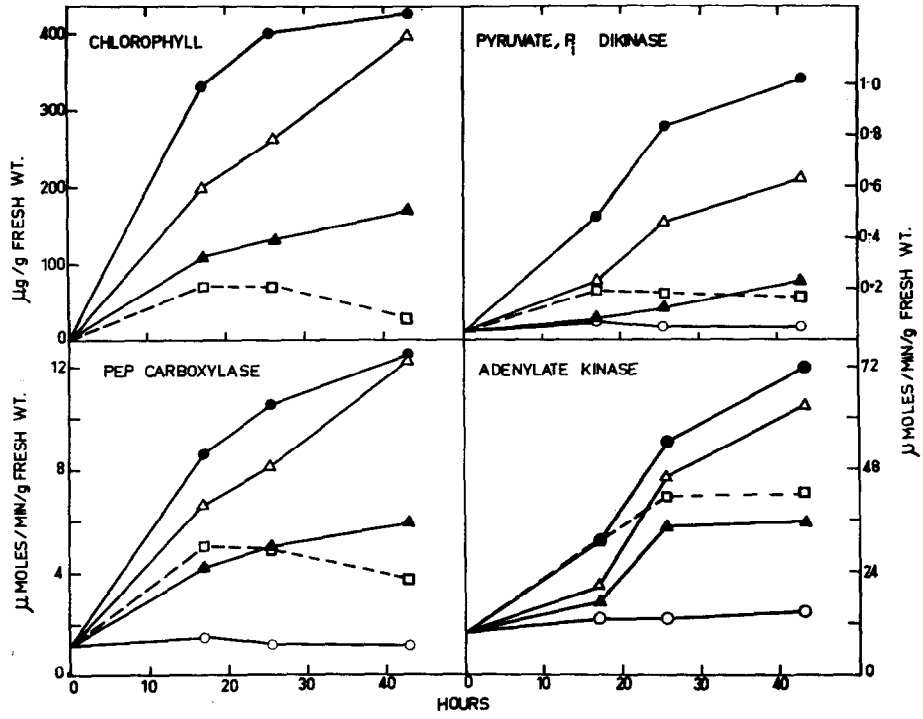

FIG. 1. CHANGES IN CHLOROPHYLL AND ACTIVITIES OF ENZYMES OF THE C_4-DICARBOXYLIC ACID PATHWAY IN DETACHED MAIZE LEAVES.

●, light (20,000 lux); ○, dark; □, light + chloramphenicol (2 mg/ml); ▲, light + cycloheximide (0·5 µg/ml); △, light + cycloheximide (2 µg/ml). Seedlings were grown in the dark for 7 days at 22°. The primary leaf and the surrounding cotyledons were detached under a green safe-light and floated in water or a solution of inhibitor in Petri dishes for 5·25 hr in darkness. Some of the dishes were then placed in light also at 22°. Samples of leaves were harvested just prior to placing the dishes in light and again after 17, 25·5 and 43 hr of illumination. Cotyledon tissue was removed from the leaves which were then blotted dry, weighed and analyzed for chlorophyll and enzymic activities. In some experiments bacterial growth was observed during the latter part of the experiment in dishes containing cycloheximide. This was prevented by transferring the leaves to fresh solutions of cycloheximide at intervals of approximately 12 hr.

phyll and enzyme formation was totally inhibited. Cycloheximide at 0·5 and 2·0 µg/ml inhibited chlorophyll synthesis and the increases in all three enzymes.

Figure 2 shows corresponding data for four Calvin cycle enzymes. Again light induced a substantial increase in the activities of these enzymes, while the activities in the dark, with the exception of RDP carboxylase which showed some increase, remained unchanged. Alkaline fructose-1,6-diphosphatase was assayed only after 25·5 hr and 43 hr of illumination and by these times activities in the illuminated leaves had risen to 3- to 4-fold above those of leaves kept in darkness. Again chloramphenicol inhibited the increases in activities of these enzymes, especially that of RDP carboxylase which was inhibited more by chloramphenicol than any of the other enzymes tested. The greater effectiveness of chloramphenicol after the first 15 hr was again evident. Cycloheximide also inhibited at 2·0 µg/ml, but at 0·5 µg/ml it had essentially no effect on RDP carboxylase and alkaline fructose-1,6-diphosphatase activities and little effect on the other enzymes.

As shown in Fig. 3, acid phosphatase showed little response to light during the first 24 hr

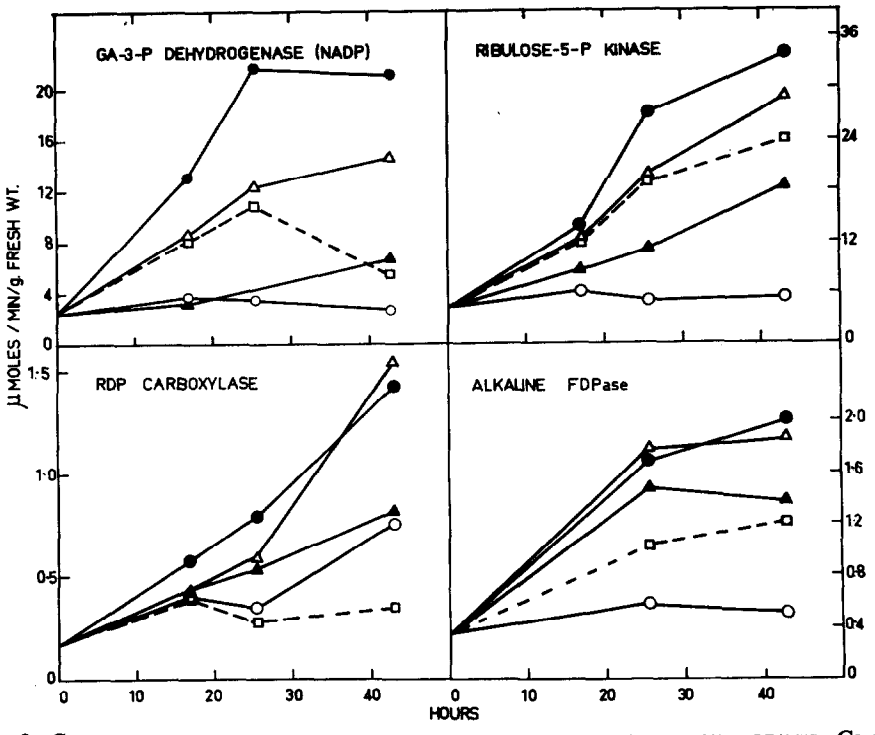

FIG. 2. CHANGES IN ACTIVITIES OF THE CALVIN CYCLE ENZYMES IN DETACHED MAIZE LEAVES. CONDITIONS WERE AS GIVEN IN FIG. 1.

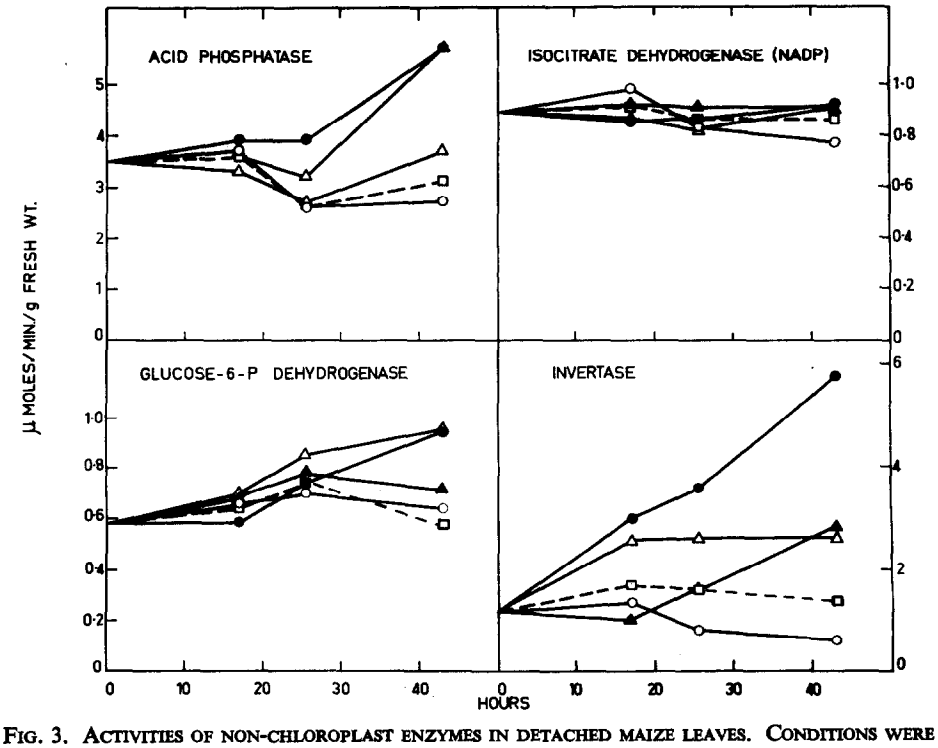

FIG. 3. ACTIVITIES OF NON-CHLOROPLAST ENZYMES IN DETACHED MAIZE LEAVES. CONDITIONS WERE AS GIVEN IN FIG. 1.

of illumination although there was some increase by 43 hr. The activities of isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase showed comparatively small changes in response to either light or inhibitors. Invertase, on the other hand, exhibited a marked response to light and by 43 hr the activity of illuminated leaves was more than 10-fold higher than the activity of the leaves kept in darkness and about 4-fold higher than the activity at zero time. Both chloramphenicol and cycloheximide inhibited the light-induced increase in invertase.

Induction of Photosynthetic Enzymes in Etiolated Leaves by Brief Exposures to Red Light of Low Intensity

Figure 4 shows the activities of several photosynthetic and respiratory enzymes in leaves of etiolated maize seedlings which had been exposed for 3 min to daily irradiation with red light for five consecutive days. Compared with non-irradiated seedlings of the same age, the activities of Calvin cycle enzyme and enzymes of the C_4-dicarboxylic acid pathway had increased substantially, although there was no measurable synthesis of chlorophyll. Of the C_4-dicarboxylic acid pathway enzymes, pyruvate, P_i dikinase increased 10·4-fold, PEP carboxylase 7·5-fold, adenylate kinase 3·9-fold and for the Calvin cycle enzymes RDP carboxylase, NADP-glyceraldehyde-3-phosphate dehydrogenase and alkaline fructose-1,6-

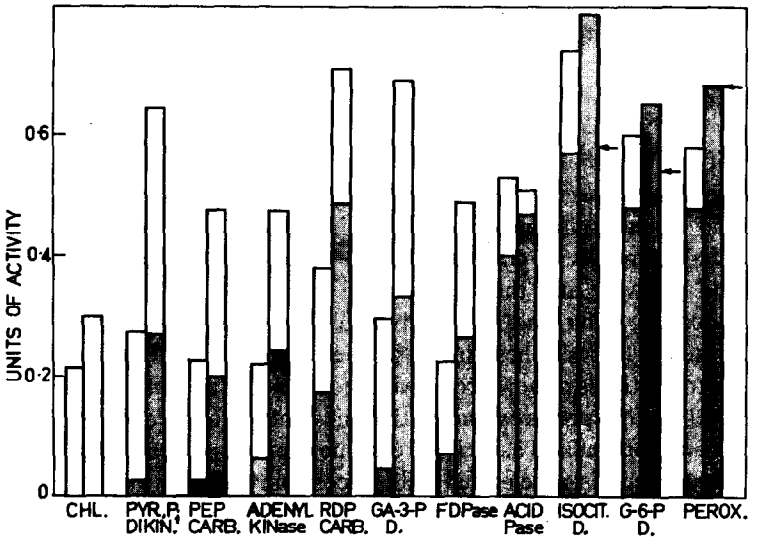

FIG. 4. THE EFFECT OF BRIEF IRRADIATIONS WITH RED (660 nm) LIGHT OR CONTINUOUS WHITE LIGHT ON ENZYMIC ACTIVITIES OF ETIOLATED MAIZE LEAVES.

Plants were germinated and grown in the dark for 8 days at 22°. Some of the plants were irradiated once each day with red light (72 μWatt/cm^2) for 3 min and returned to the dark. After 5 days some of these plants, together with some of the plants grown in continuous darkness, were illuminated for 24 hr with white light (9000 lux) at 22°. The secondary leaves from plants receiving the various treatments were harvested (i.e. 14 days after germination), analyzed for fresh weight, chlorophyll content and enzymic activity. For each set of analyses the shaded areas indicate values for plants receiving no irradiation (left) or irradiated with red light only (right). The white areas show the increments obtained when these plants were exposed to white light for 24 hr prior to harvesting. Where there was no increase as the result of exposure to white light, the value found is indicated by an arrow. Units for chlorophyll are mg chlorophyll per g fresh wt. of leaf. Units for the enzymic activities (μmole substrate converted per min per g fresh wt. of leaf) are ×0·01 for adenylate kinase, ×0·02 for glyceraldehyde-3-phosphate dehydrogenase, ×0·05 for PEP carboxylase and acid phosphatase and ×0·2 for RDP carboxylase, alkaline fructose-1,6-diphosphatase and peroxidase.

dehydrogenase, the increases were 2·8, 7·1 and 3·8-fold, respectively. Respiratory enzymes also showed increases, but the magnitudes of the increases were much smaller (1·2 to 1·5-fold).

When either dark-grown or red-light treated plants were exposed to white light for 24 hr further increases in the activities of the photosynthetic enzymes were obtained (Fig. 4). The net increases for both types of plant were comparable, although slightly greater in the case of the plants treated with red light. The levels of activity reached in plants treated with red light only were 42–68 per cent of the values attained in plants receiving in addition 24 hr of illumination with white light.

The non-chloroplast enzymes acid phosphatase, NADP-isocitrate dehydrogenase, glucose-6-phosphate dehydrogenase and peroxidase, showed small increases in activity when dark-grown plants were illuminated with continuous white light. When the plants treated with red light were similarly illuminated, the activities of these enzymes remained about the same or decreased (Fig. 4).

All the analyses reported in Fig. 4 were made following the white-light treatments (14 days after germination). Samples of leaves from dark-grown plants and plants treated with red light were also analyzed just prior to commencing the 24 hr illumination. The enzymic activities of leaves from the dark-grown plants were the same as those shown for dark-grown plants in Fig. 4 and for plants treated with red light only, the activities of the photosynthetic enzymes were 10–20 per cent lower than the corresponding values shown in Fig. 4. The various light regimes used had little effect on the fresh weight per leaf.

DISCUSSION

The effect of inhibitors of protein synthesis on the appearance of chlorophyll and two Calvin cycle enzymes has been followed previously in detached etiolated leaves, although in these studies the increases in enzymic activity induced by light were small.[15,16] In our studies substantial increases in chlorophyll and photosynthetic enzymes of both the C_4-dicarboxylic acid pathway and the Calvin cycle were observed when detached, etiolated leaves were floated on water and illuminated. When water was replaced by solutions of inhibitors of protein synthesis these increases were inhibited. This suggested that the increases were due to synthesis of new protein and not to activation of existing enzymes. These studies established for the species examined the suitability of using detached leaves for studying chloroplast development and associated enzyme synthesis. Generally, when green leaves are detached, senescence is accelerated with accompanying loss of chlorophyll and leaf proteins. However, the immediate effects of accelerated senescence on chloroplast development following detachment of etiolated leaves may be minimized in many grasses because of the advanced stage of development attained by the etiolated plastids of these plants. Thus the ribosomal RNA content[17] and the capacity for protein synthesis of the wheat etioplast is comparable with that of the mature chloroplast.

The use of detached leaves offers many advantages for experiments involving uptake of isotopes, or non-radioactive metabolities and inhibitors. The effect of chloramphenicol on chloroplast development in bean leaves has been investigated by spraying solutions of the inhibitor on the leaves.[18] While the synthesis of some chloroplast proteins was inhibited

[15] S. CHEN, D. MCMAHON and L. BOGORAD, *Plant Physiol.* **42**, 1 (1967).
[16] C. J. KELLER and R. C. HUFFAKER, *Plant Physiol.* **42**, 1277 (1967).
[17] N. S. SCOTT and H. NAIR, *Abstr. Australian Plant Physiol. Soc. Meetings, Adelaide* (1969).
[18] M. MARGULIES, *Plant Physiol.* **39**, 579 (1964).

there was only a slight inhibition of chlorophyll synthesis, whereas in our experiments using detached leaves floated on solutions of chloramphenicol, considerable inhibition of chlorophyll production was evident and it was concentration dependent.

Illumination of detached etiolated leaves of plants containing the C_4-dicarboxylic acid pathway resulted in increases in the activities of enzymes of this pathway and Calvin cycle enzymes. The percentage increases in activity varied with the different enzymes. For some of the enzymes, such as PEP carboxylase, adenylate kinase and fructose-1,6-diphosphatase, the real percentage increase in enzymic activity in the chloroplasts would have been greater since part of the activity measured in dark-grown plants could be attributed to non-photosynthetic components. However, it is clear that in maize, Calvin cycle enzymes and enzymes of the C_4-dicarboxylic acid pathway show increases which are comparable in magnitude (Figs. 1 and 2). Since the enzymes specific to the C_4-dicarboxylic acid pathway are located in the mesophyll chloroplasts, while those common to both this pathway and the Calvin cycle are located in the chloroplasts of the cells of the parenchyma sheath,[8, 10] it would appear that the two types of chloroplast show parallel development in response to light.

Chloramphenicol and cycloheximide inhibited the light-induced synthesis of enzymes of both the Calvin cycle and the C_4-dicarboxylic acid pathway. In contrast, neither light nor inhibitors significantly altered the levels of cytoplasmic enzymes such as acid phosphatase, isocitrate dehydrogenase and glucose-6-phosphate dehydrogenase. The effects of these inhibitors on protein synthesis, at least in cell-free systems, are highly specific. Cycloheximide at about 1 μg/ml is a potent inhibitor of cytoplasmic protein synthesis in higher organisms,[14] but concentrations as high as 200 μg/ml fail to inhibit the incorporation of amino acid into protein by isolated wheat chloroplasts (B. J. Reger, R. M. Smillie and R. C. Fuller, unpublished experiments). In the same system, chloramphenicol inhibits at low concentrations (50 per cent inhibition at 5 μg/ml). The inhibition of the increases in activities of enzymes of the Calvin cycle and C_4-dicarboxylic acid pathway by chloramphenicol is consistent with their synthesis on the chloroplast 70S ribosomes which are chloramphenicol-sensitive. Although the effects of only one concentration of chloramphenicol are shown in Figs. 1—3, more than one concentration was used and the diminished rates of increase of enzymic activities obtained were dependent on the concentration of inhibitor. Different concentrations were also required to achieve the same level of effect in different species (Table 1) and, within the one tissue, the effectiveness of a particular concentration varied with different enzymes. Chloramphenicol at 2 mg/ml effectively blocked the appearance of RDP carboxylase in maize while producing only moderate effects on ribulose-5-phosphate kinase (Fig. 1). Some of these effects may have been due to differential rates of uptake by chloramphenicol by the different plant tissues, but this would not explain variations in sensitivity towards chloramphenicol shown by individual enzymes within the same tissue. The precise mechanism of chloramphenicol inhibition is not known, but it appears to bind to ribosomes and block the transfer of amino acid from transfer RNA to the growing polypeptide chain.[19] Inhibition by a given concentration of chloramphenicol could conceivably be influenced by the relative concentrations of components of the ribosomal complex. Some of these components, such as messenger and transfer ribonucleic acids, may be produced in different amounts for different enzymes and the rate of production may be dependent upon the light intensity and other environmental factors. A correlation between chloramphenicol inhibition of chlorophyll synthesis and light intensity has been noted in other studies (unpublished experiments).

[19] D. VAZQUEZ, *Symp. Soc. Gen. Microbiol.* **16**, 169 (1966).

The effects of cycloheximide on the synthesis of photosynthetic enzymes in greening leaves may be contrasted with earlier studies with *Euglena*.[2] In *Euglena*, even at a high concentration of cycloheximide (15 μg/ml), synthesis of RDP carboxylase was not inhibited although chlorophyll formation was greatly reduced, suggesting that synthesis of this enzyme does not involve cytoplasmic protein synthesis. The present results and those of others[16] show that in leaves synthesis of photosynthetic enzymes is much more sensitive to the inhibitor than in *Euglena*. Decreased synthesis of photosynthetic enzymes in leaves in the presence of low concentrations of cycloheximide could be explained in several ways. Two of the most attractive are that, in leaves, proteins synthesized in the cytoplasm are essential for chloroplast development and, alternatively, inhibition of cytoplasmic protein synthesis may so alter normal regulatory mechanisms in the cell that chloroplast development is repressed.

In studies such as those reported here on the differential effects of inhibitors of protein synthesis on the synthesis of chloroplast and cytoplasmic proteins, it would be advantageous to be able to follow simultaneously the induction of a cytoplasmic enzyme. Unfortunately, very few inducible cytoplasmic enzymes are known in higher plants, but one such enzyme is invertase. This enzyme was induced when detached leaves of etiolated maize were illuminated (Fig. 3), but both chloramphenicol and cycloheximide diminished the induced increase. This suggested that the synthesis of the light-induced invertase was either localized within the chloroplasts, although Bird *et al.*[20] were unable to find invertase (β-fructofuranosidase) activity in tobacco chloroplasts, or that its synthesis in the cytoplasm was dependent upon photosynthesis or its products.

Although continuous light absorbed by protochlorophyll is required for the synthesis of chlorophyll,[21] continuous light is not essential for the synthesis of at least some chloroplast proteins in higher plants. Some production of Calvin cycle enzymes occurs in dark-grown plants and further synthesis in the dark can be induced by a brief exposure of the plants to red light of low intensity.[2] The photoacceptor involved is phytochrome. Thus, short exposures to red light stimulated the synthesis of Calvin cycle enzymes in bean,[22] pea[23] and rye[24] and these effects of red light were largely negated by a subsequent short exposure to far-red light. Daily exposures of dark-grown maize seedlings to red light for 3 min resulted not only in increases in the activities of Calvin cycle enzymes, but also in large increases in the enzymes of the C_4-dicarboxylic acid pathway when compared with non-irradiated plants (Fig. 4). Increases in cytoplasmic enzymes in irradiated plants were slight. Although the treatment with red light enhanced the rate of synthesis of plastid protein in etiolated seedlings, the potential capacity of the plants for this synthesis was much less affected since, as can be seen in Fig. 4, the enzymic activities in both dark-grown and red-light treated plants were increased by comparable amounts following 24 hr of illumination with white light. The net increases in activity were only slightly greater in plants treated with red light. Pretreatment with red light is known to reduce or eliminate the lag in chlorophyll synthesis which is evident when dark-grown plants are first exposed to light.[25, 26]

Continuous light, then, is not essential for the production of enzymes of the C_4-dicarb-

[20] I. F. BIRD, H. K. PORTER and C. R. STOCKING, *Biochim. Biophys. Acta* (*Amst.*) **100**, 366 (1965).
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oxylic acid pathway in maize. Instead, their appearance seems to be integrated with developmental processes which respond to brief irradiation of the plant with red light. Although the identity of the photoacceptor in maize was not investigated, comparison with the photoregulation of the synthesis of Calvin cycle enzymes in other plants implicates phytochrome as the photoacceptor. The enzyme pyruvate, P_i dikinase is activated by light in green leaves of maize,[13] but this phenomenon is distinct from the red-light induced increase in activity in etiolated leaves reported here.

EXPERIMENTAL

Plant Material

Seeds of maize (*Zea mays* var. DS 606A), hybrid sorghum (*Sorghum* var. Rio), wheat (*Triticum sativum* var. Gala) and oats (*Avena sativa* var. Benton) were soaked for 24 hr in running water and were then sown in a mixture of vermiculite and perlite (3:1). Seedlings were grown in constant-temperature rooms in the dark at 22° and were watered daily.

In order to follow the light-induced development of chloroplasts, leaves or leaf sections were cut from etiolated plants and floated on 15 ml of water in a Petri dish. Illumination was provided by a green safe-light.[27] Some of the dishes were placed in white light (400 watt HPL lamps supplemented by tungsten lamps) in a constant-temperature room and the rest were returned to darkness. Light intensity was measured as previously described.[4] Where inhibitors were used, leaves were floated on a solution of inhibitor for 4–6 hr in darkness before being illuminated and were then maintained in these solutions during periods of illumination.

Leaf Extracts

Leaves (about 1 g fresh wt.) were washed in a sieve under running distilled water, blotted dry and weighed. The samples were then ground with sand (approximately half the weight of the tissue) in a mortar for 45 sec with 3–4 volumes (w/v) of 0·1 M tris-HCl, pH 8·3, 2 mM dithiothreitol, 10 mM mercaptoethanol, 6 mM $MgCl_2$ and 1 mM ethylenediamine tetraacetate. The homogenate was filtered through "Mira-cloth" and 1 ml samples were run through a column of Sephadex G-25 (6 ml) previously equilibrated with a solution of 10 mM tris-HCl, pH 8·3, 2 mM dithiothreitol and 4 mM $MgCl_2$. The mortar and grinding solution were chilled, but otherwise the extraction was done at room temperature. Samples of extract were removed for assay of pyruvate, P_i dikinase and PEP carboxylase and the remainder of the extract was cooled to 0° and stored in ice. Extracts used for direct spectrophotometric assays were centrifuged at 10,000 *g* for 10 min.

Assays

For assays using radioactive $NaHCO_3$, the reaction was stopped with ½ volume of 20% (w/v) trichloroacetic acid. Portions of the acidified mixture were spotted on filter paper, dried and counted with a Geiger-Müller tube.[10]

PEP carboxylase (EC 4.1.1.31) and pyruvate, P_i dikinase[11] and RDP carboxylase (EC 4.1.1.39)[10] were assayed as previously described[10,11] except that dithiothreitol was used instead of 2-mercaptoethanol.

Ribulose-5-phosphate kinase (EC 2.7.1.19) was assayed in a reaction mixture (final volume 0·12 ml) containing extract, 50 mM tris-HCl, pH 8·3, 10 mM dithiothreitol, 20 mM $MgCl_2$, 1 mM K_2HPO_4, 1 mM KF, 5 mM ribose 5-phosphate, 13 mM $NaH^{14}CO_3$ (5×10^5 dpm), 0·05 unit of ribose-5-phosphate isomerase, 0·15 unit of RDP carboxylase and 5 mM ATP. All except the last two components were mixed and, after 10 min, RDP carboxylase was added. After a further 10 min to allow for activation of the carboxylase, the reaction was started by adding ATP. The reaction was stopped by acidification and the radioactivity determined as described above.

The following enzymes were assayed spectrophotometrically at either 340 nm or 470 nm (peroxidase only) using the following reaction mixtures.

Adenylate kinase (EC 2.7.4.3): 20 mM tris-HCl buffer, pH 7·6, 0·5 mM ATP, 0·5 mM AMP, 2 mM $MgCl_2$, 50 mM KCl, 5 mM PEP, 0·2 mM $NADH_2$ and approx. 1 unit each of pyruvate kinase and lactate dehydrogenase (Sigma Chemical Co., St. Louis, Mo., U.S.A.). NADP-glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.13): 33 mM tris-HCl, pH 8·3, 17 mM sodium arsenate, 20 mM KF, 5 mM dithiothreitol, 2 mM glyceraldehyde 3-phosphate and 0·15 mM NADP. NADP-isocitrate dehydrogenase (EC 1.1.1.42): 33 mM tris-HCl, pH 7·6, 1 mM $MnCl_2$, 1 mM *dl*-isocitrate, 0·1 mM NADP. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49): 33 mM tris-HCl, pH 7·6, 3 mM $MgCl_2$, 2 mM glucose 6-phosphate, 0·1 mM NADP. Peroxidase (EC 1.11.1.7): 30 mM sodium acetate, pH 5·0, 0·3 mM guaicol, 1 mM H_2O_2.[28]

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Acid phosphatase (EC 3.1.3.2) was assayed using a reaction mixture (0·7 ml) containing extract, 110 mM sodium acetate, pH 5·3, and 1·4 mM *p*-nitrophenylphosphate. The reaction was stopped by adding 3 ml of 100 mM NaOH and the released *p*-nitrophenol determined by measuring the increase in optical density at 400 nm.

Fructose 1,6-diphosphatase (EC 3.1.3.11) was assayed by measuring the phosphate released from fructose 1,6-diphosphate.[29] The reaction mixture (0·5 ml) contained 100 mM tris-HCl, pH 8·5, 5 mM $MgCl_2$, 1·6 mM ethylenediamine tetraacetate and 5 mM fructose 1,6-diphosphate. The reaction was stopped by adding 0·5 ml of 10% perchloric acid.

Invertase (EC 3.2.1.26) was assayed as described previously[30] and chlorophyll according to Arnon.[31]

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