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Changes in Succinyl CoA Synthetase Activity in Etiolated Bean Leaves Caused by Illumination[1, 2]

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Abstract. The illumination of etiolated bean leaves (*Phaseolus vulgaris*) causes an increase in the activity of succinyl coenzyme A synthetase. Continuous white light or short periods of red or blue light followed by darkness will induce an increase with the highest activity at about 6 hr after the onset of illumination. Thereafter the activity decreases so that at 12 hr it is the same as the initial dark activity. Treatment with cycloheximide before illumination prevents the increase in activity. A number of other enzymes have been studied in an attempt to determine the significance of the transient nature of the changes in succinyl CoA synthetase activity.

Succinyl coenzyme A has been shown to be a precursor of porphyrins in animals and bacteria (1, 6). The tracer studies of Shemin and Kumin (11) showed that duck erythrocyte preparations would incorporate label from the carboxyl carbons of succinate into heme. Tricarboxylic acid cycle activity could not be involved in this system since carboxyl carbons would be lost as CO_2 before reaching α-ketoglutarate dehydrogenase. Using malonate inhibition of the tricarboxylic acid cycle they concluded that 30 to 50 % of succinyl CoA could be derived directly from succinate, presumably *via* succinyl CoA synthetase. In guinea pig liver mitochondria (2) 30 to 60 % of succinyl CoA was estimated as coming from succinate directly. Incorporation of ^{14}C from carboxyl labeled succinate into chlorophyll has been demonstrated in greening barley leaves (14) so that succinyl CoA synthetase could be involved in porphyrin biosynthesis in leaves. For these reasons it seemed that succinyl CoA synthetase could be important during the greening of etiolated leaves and a study of the activity of the enzyme before and during illumination would provide information on the mechanisms controlling light development of the photosynthetic apparatus.

The previous studies of succinyl CoA synthetase (E.C. 6.2.1.5) from plant sources have been concerned with the preparation of the isolated enzyme, its substrate and cofactor requirements and the reaction mechanism (4, 5, 10). In this study we report upon a light induction of an increase in activity of succinyl CoA synthetase and the light systems involved during greening of etiolated bean plants.

Materials and Methods

Phaseolus vulgaris var. Black Valentine (Charter Seed Company, Twin Falls, Idaho. Crop No. 1-04502) was the experimental material. For some of the later experiments the variety Resistant Asgrow Valentine (Crop No. 1-5269) were used. Seeds were germinated in the dark at 22° in vermiculite and the plants used on the sixth or seventh day after sowing. All treatments were performed on intact plants which were dehusked before use. Dark handling was performed with the minimum exposure to dim green safety lights.

Cool white fluorescent and incandescent lamps in a 26° growth chamber of continuous illumination gave 900 ft-c at the level of the cotyledons. Colored light sources used Rohm and Haas cast plexiglass sheets: blue No. 2424 with white fluorescent lamps (400–550 mμ, peak at 470 mμ); red No. 2444 with red fluorescent lamps (610–720 mμ) and far-red No. V-58015 with incandescent lamps and a 10 cm barrier of water (710–1000 mμ) (3, 17). The incident energies of the various systems were measured with a standardized Eppley Thermopile attached to a Vistion-Becker D.C. Breaker Amplifier (Beckmann Instruments) and are reported in the Results section.

For the enzyme assay 30 to 35 leaf pairs were ground in a chilled mortar with 15 mM tris·HCl buffer pH 7.2 and 1 μmole 2-mercaptoethanol per ml. For fully greened leaves the value of buffer was increased. The homogenate was strained through 8 layers of cheesecloth and the residue extracted with a second volume of solution. This extraction procedure left negligible succinyl CoA synthetase (SCS) activity in the residue. The filtrate was used for the enzyme assay without further treatment.

The assay was based on the hydroxamate method

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of Kaufmann (4). In 2 ml total volume the reagents were: 60 μmoles tris•HCl buffer pH 7.2; 10 μmoles $MgCl_2$; 10 μmoles 2-mercaptoethanol; 980 μmoles neutralized hydroxylamine hydrochloride; 20 μmoles sodium succinate; 5 μmoles ATP; 0.13 μmole coenzyme A and 0.5 ml enzyme (equivalent to about 1.5 leaf pairs). The reaction was started by the addition of the enzyme and was carried out at 25° for 30 min; it was linear over this period. The reaction rate was linear within 3-fold increases in the homogenate volume used from both dark and light grown leaves. Mixed homogenates from dark and red light treated leaves gave an SCS activity equal to the sum of the activities of the individual components: there was neither activation nor inhibition. The reaction was stopped by the addition of 2 ml of the ferric chloride reagent of Lipmann and Tuttle (8); 5% $FeCl_3$: 12% trichloroacetic acid: 3 N HCl (1:1:1 v/v). Zero time and succinate-less blanks were used. After removal of the precipitated protein by centrifugation the optical density at 540 mμ of the supernatant was recorded and converted to nmoles of succinyl CoA by using a standard curve.

Protein was measured using the Folin phenol reagent (9).

When plants were treated with cycloheximide, 15 ml of solution (300 μg/ml) were sprayed on to the plants of 1 pan (about 200 seedlings) with an atomizer.

Other enzymes were assayed by measuring the change in optical density at 340 mμ in a Gilford multiple sample absorbance recorder attached to a Beckman DU monochromator with sample temperature regulated at 25°:

Glutamate-oxaloacetate Transaminase. 150 μmoles potassium phosphate buffer (pH 7.5); 60 μmoles potassium aspartate; 20 μmoles potassium α-ketoglutarate; 0.33 μmole NADH; 5 μmoles NaEDTA; 1.3 units malate dehydrogenase (Sigma); and enzyme extract; in a total volume of 1 ml.

NADP-isocitrate Dehydrogenase. 30 μmoles potassium phosphate buffer (pH 7.5); 0.6 μmole sodium isocitrate; 0.3 μmole NADP; 0.6 μmole $MnCl_2$; and enzyme extract; in total volume of 1 ml.

Malate Dehydrogenase. 225 μmoles potassium phosphate buffer (pH 7.5); 20 μmoles oxaloacetic acid; 0.33 μmole NADH; and enzyme in a total volume of 1 ml.

Glucose 6-P Dehydrogenase. 2 μmoles glucose 6-P; 1 μmole glycylglycine buffer (pH 7.5); 1 μmole NADP; 10 μmoles $MgCl_2$; and enzyme in a total volume of 1 ml.

Metabolism of added succinyl CoA in the absence of added cofactors by the homogenate was measured by following the change in optical density at 232 mμ in the following system: 30 μmoles tris•HCl buffer (pH 7.2); approximately 2 μmoles succinyl CoA (prepared by the method of Simon and Shemin (12); 4 μmoles $MgCl_2$; 4 μmoles 2-mercaptoethanol; and enzyme in a total volume of 1 ml.

The remaining assays were modifications of the hydroxamate method: α-ketoglutarate dehydrogenase. 100 μmoles tris•HCl (pH 7.2); 20 μmoles potassium α-ketoglutarate; 0.13 μmole coenzyme A; 1 μmole NAD; 900 μmoles neutralized hydroxylamine; 10 μmoles 2-mercaptoethanol; and enzyme in a total of 2 ml.

Pyruvate Dehydrogenase System. 100 μmoles tris•HCl (pH 7.2); 50 μmoles potassium pyruvate; 0.13 μmole coenzyme A; 1 μmole NAD; 450 μmoles neutralized hydroxylamine; 6 μmoles $MnCl_2$; 10 μmoles 2-mercaptoethanol; 0.2 μmole thiamine pyrophosphate; and enzyme in a total of 2 ml.

Acetyl CoA Synthetase. 100 μmoles tris•HCl buffer (pH 7.8); 20 μmoles sodium acetate; 0.13 μmole coenzyme A; 10 μmoles ATP; 450 μmoles neutralized hydroxylamine; 10 μmoles $MgCl_2$; 10 μmoles 2-mercaptoethanol; and enzyme in a total of 2 ml.

These assays were carried out at 25° and were stopped after 30 min with the $FeCl_3$ reagent of Lipmann and Tuttle (8) and measured as described above for SCS using the appropriate standard curve. In the α-ketoglutarate dehydrogenase and pyruvate dehydrogenase assays NAD was not limiting since an active NADH oxidase was present in the homogenate.

In all assays, blanks lacking substrate were run in parallel and the readings substracted from the experimental reading.

Results and Discussion

Activity in Dark and Illuminated Leaves. The activity of succinyl CoA synthetase in dark grown leaves increased per leaf pair up to the sixth day after sowing, thereafter it decreased slowly (Fig. 1). This normal dark decrease was less than that in plants treated with cycloheximide. The change in activity in untreated plants after the sixth day was −1 enzyme unit per hr per leaf pair (here for ease of expression an enzyme unit is equal to the production of 1 nmole succinyl CoA/hr). Cycloheximide treated plants showed a change of −7 units per hr per leaf pair suggesting that in the dark after 6 days enzyme synthesis was occurring but that breakdown was proceeding at a higher rate.

When dark grown plants were transferred to continuous white light SCS activity increased rapidly to a maximum at 5 to 6 hr followed by a decrease so that at 12 hr the activity was about the same as the initial dark level (Fig. 1). At 20 to 24 hr activity began to increase linearly, concurrent with the rapid growth of the leaves and continued for at least 40 hr. During this period the specific activity (activity/mg protein) remained more or less constant. (See Fig. 7).

Activity After Brief Irradiations With Colored Light. In attempts to characterize the light reactions involved in the changes in enzyme activity

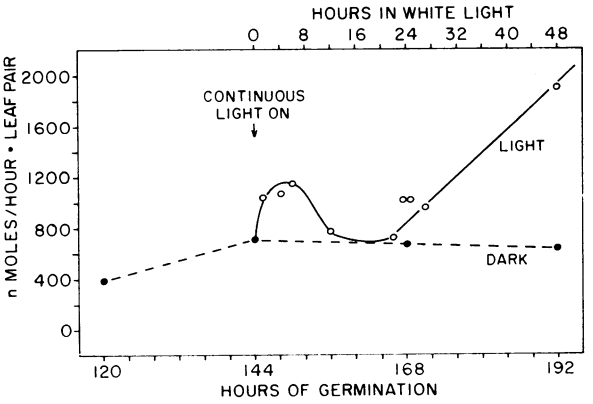

FIG. 1. Succinyl CoA synthetase activity in dark grown leaves and during continuous white illumination of etiolated leaves.

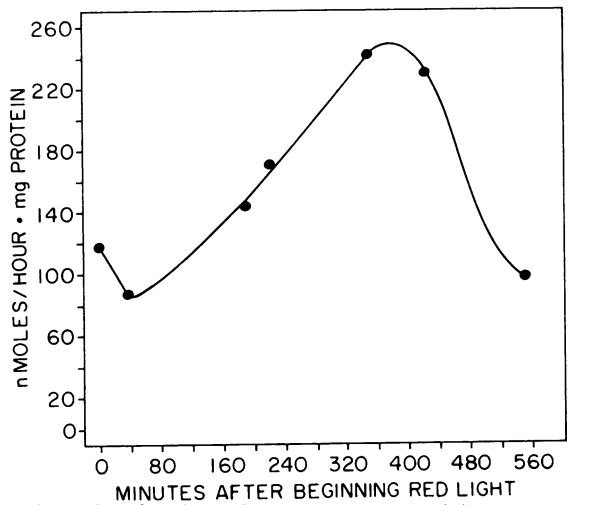

FIG. 2. Succinyl CoA synthetase activity per mg protein after 10 min red irradiation followed by darkness.

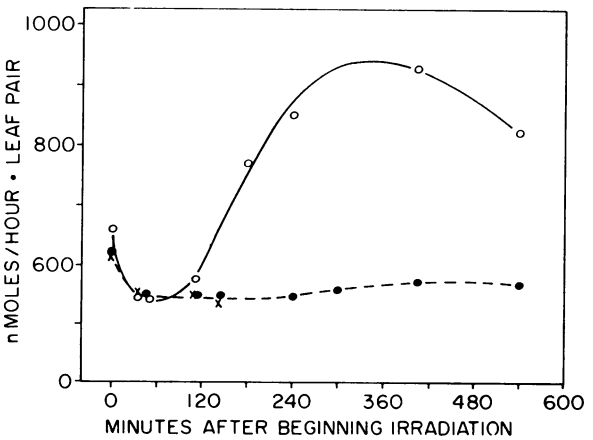

FIG. 3. Succinyl CoA synthetase activity after 10 min red irradiation followed by darkness. ○——○ normal; • x-----x, ●-----● sprayed with cycloheximide (300 μg per ml) 4 hr before irradiation.

during the first 12 hr of illumination, further experiments were done in which the continuous white light was replaced by short exposures to colored light. The activity after 10 min of red light (1160 Kergs/cm²) followed by darkness is shown in Fig. 2. The data fall into 3 phases: an initial decrease in activity followed by an increase, in turn followed at 6 hr by another decrease to below the initial dark level (see also Fig. 7). At 6 hr, the enzyme activity equal to that induced by illumination declined leaving a base level represented by a specific activity of about 90 nmoles per hr per leaf pair; a similar amount to that after the initial decrease. A similar resistant activity is seen in Fig. 3 where cycloheximide treated plants maintained a level of enzyme activity for 9 hr equivalent to the post initial decrease activity in control plants. Cycloheximide treatment abolished the light induced increase in enzyme activity.

The initial decrease in activity promoted by red irradiation showed a limited reversal by far-red light (Fig. 4). Far-red subsequent to red light produced enzyme activity only marginally lower than the dark activity after 2 hr. Further irradiations did not alter the activity from that level; a limited red/far-red reversal was seen.

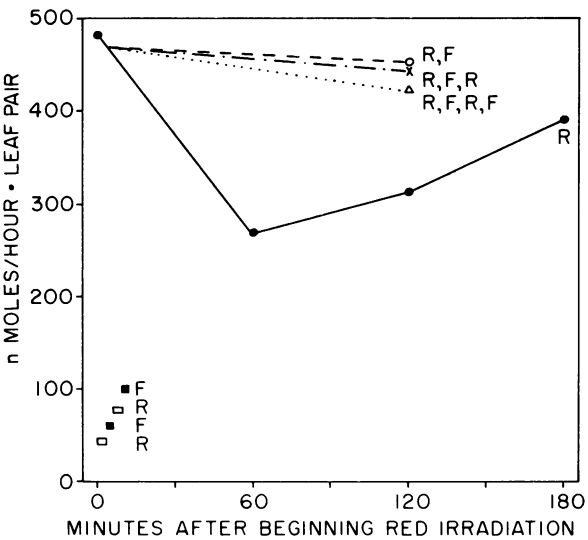

FIG. 4. Far-red reversal of the red mediated initial decrease in succinyl CoA synthetase activity in leaves. Red (R) irradiation was of 3 min duration, far-red (F) of 2 min.

The energy relationships of the initial decrease phase are shown in Fig. 5 expressed as activity per leaf pair rather than protein so that changes in protein induced by the different illumination periods would not obscure activity changes. The activity measured at 20 min after the beginning of illumination shows that there was a linear response to irradiation of up to 5 min duration. Periods above 5 min invoked no greater response. Other experiments showed that the decrease had the same rate

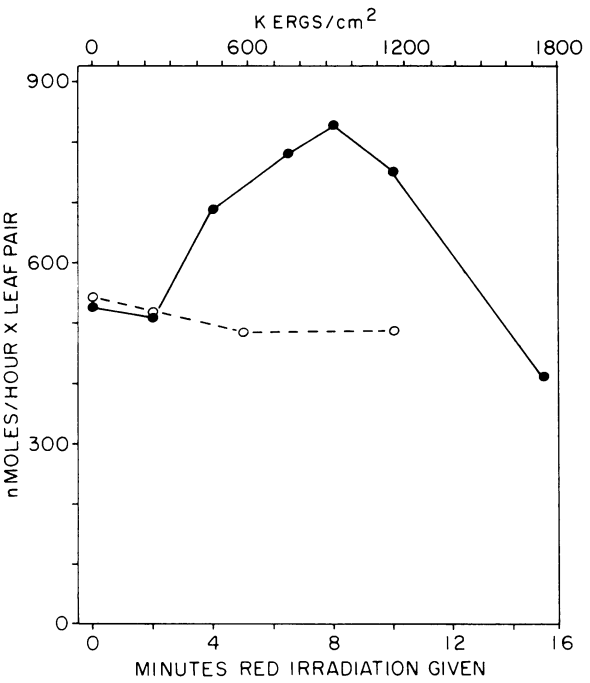

FIG. 5. Succinyl CoA synthetase activity as a function of the amount of red irradiation given. ○-----○ activity assayed 20 min after the beginning of irradiation. ●————● activity assayed 6 hr after the beginning of irradiation.

at 5° as at 22°; it appeared to be temperature independent within that range.

For comparison Fig. 5 includes the response of the red induced increase when SCS activity was assayed at 6 hr after exposure. At low energies this system showed no response and had a peak after 8 min (930 Kergs/cm²) irradiation. Illumination periods of 15 min resulted in activity after 6 hr being lower than the initial dark level. The time course of SCS activity after 15 min red irradiation showed an initial decrease followed by an increase to about the dark level at 90 min and then a further gradual decrease so that by 6 hr the activity was less than the initial dark activity. This, together with the depressant effects on SCS activity of second red light pulses, suggested that the decrease in activity after 6 hr may also be effected by light. No further evidence was collected on this phase.

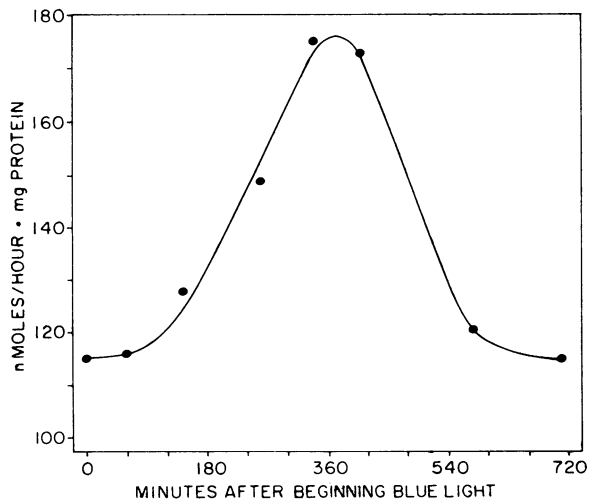

FIG. 6. Succinyl CoA synthetase activity after 15 min blue irradiation followed by darkness.

Exposure to 15 min of far-red light induced a small increase in activity (at a rate of 6.5 nmoles per hr per mg protein) reaching a maximum at 5 hr. During the phase of increasing SCS activity the red/far-red relationship was complex but it seemed that the rate of increase was that induced by the light to which the seedlings were last exposed. This requires more experimental evidence.

In response to blue illumination the enzyme activity showed an increase also (Fig. 6). As after red the activity had a maximum at 6 hr and at 12 hr the activity had fallen to a level equivalent to the initial dark activity. Far-red subsequent to blue did not alter the enzyme activity during the 60 min lag phase. Table I illustrates the energy relationships of the blue induction system. One hundred and thirty-three Kergs per cm² did not produce a response as measured as activity at 5 hr but quantities above that and at least up to 1000 Kergs per cm² produced a small but increasing magnitude of response. Using the far-red filter (V-58015) with the white fluorescent lamps used for the blue source no charge in SCS activity was observed. The blue induction was not likely due to irradiation with wavelengths between 400 and 550 mμ.

While 10 min red irradiation induced hypocotyl hook opening and leaf growth in these experiments neither 15 min far-red nor 15 min blue induced these changes; morphologically the plants remained in the dark-grown condition.

In a single experiment with etiolated oat leaves 10 min of red light induced the same trend in changes in SCS activity as in bean leaves but the changes were very small. With pea apices 10 min red light induced a doubling of activity by 5 hr followed by a decrease, but in this tissue the initial red decrease phase did not appear to be present.

Table I. *Succinyl CoA Synthetase Activity After Different Amounts of Blue Irradiation*

Enzyme activity was assayed 5 hr after the beginning of irradiation.

Irradiation time	Irradiation units	Activity
min	*kergs/cm²*	*nmoles/hr × leaf pair*
0	0	626
2	133	611
5	333	673
10	666	690
15	1000	716

Table II. *Enzyme Activity of Subcellular Fractions After Far Red Irradiation*

Frozen leaves were homogenized in a blendor with a solution containing 700 mM mannitol; 1 mM Na-EDTA; 2 mM cysteine; 1 mg/ml bovine serum albumin; 10 mM K phosphate buffer (pH 6.5). The homogenate was centrifuged 4000*g* for 45 sec to precipitate debris. The supernatant was centrifuged at 15,000*g* for 20 min. The pellet was washed once with the mannitol medium and was designated the mitochondrial fraction. The supernatant was assayed without further treatment. Assay methods are described in the Methods section.

Enzyme	Fraction	Min after 15 min of far-red		
		0	69	190
		nmoles per hr per mg protein		
NADP-isocitrate dehydrogenase	Mitochondria	0	115	0
	Supernatant	1664	2070	1411
Glucose-6-P dehydrogenase	Mitochondria	0	77	0
	Supernatant	...	1110	1185
Glutamic-oxaloacetate transaminase	Mitochondria	863	1156	875
	Supernatant	9084	6701	4828
Succinyl CoA synthetase	Mitochondria	123	106	285
	Supernatant	119	147	238

Changes in Other Systems. The significance of the changes in SCS activity is not readily apparent. The rapid and short-lived increase did not correlate with known changes in the leaf: For example, with the rate of chlorophyll synthesis. In the hope that a correlation with other systems could be found a number of enzymes were assayed after treatments that induced an increase in SCS activity: α-ketoglutarate dehydrogenase as an enzyme also producing succinyl CoA; succinyl CoA consumption in the absence of added cofactors, possibly catalyzed by succinyl CoA deacylase; acetyl CoA synthetase and pyruvate dehydrogenase complex as systems also involved in coenzyme A metabolism; glutamate-oxaloacetate transaminase as a mitochondrial enzyme; NADP-mediated isocitrate dehydrogenase as a tricarboxylic acid cycle enzyme and glucose 6-P dehydrogenase as a cytoplasmic enzyme. None of those assayed showed an increase in activity during the first 6 hr. Only one, glutamate-oxaloacetate transaminase, showed any rapid change at all: a fall in supernatant activity after 15 min of far-red irradiation (table II). This table shows also that increased SCS activity was apparent in both particulate and supernatant fractions of leaf preparations. After 10 min of red irradiation glutamate-oxaloacetate transaminase, NADP-isocitrate dehydrogenase and malate dehydrogenase showed no change in activity during the subsequent 6 hr. This was also true for α-ketoglutarate dehydrogenase and the pyruvate dehydrogenase complex during the first 6 hr of continuous white illumination but acetyl CoA synthetase showed a decrease between 4 and 6 hr. Assay of succinyl CoA consumption by the crude homogenate without added cofactors showed no change after 15 min of far-red irradiation.

Fig. 7 shows the specific activity expressed as a percentage of the initial dark activity of a number of enzymes during 72 hr of continuous white illumination. Four have the same pattern; glutamate-oxaloacetate transaminase, NADP-isocitrate dehydrogenase, α-ketoglutarate dehydrogenase and the pyruvate dehydrogenase system. All are concerned in respiratory metabolism. δ-Aminolevulinic acid dehydrase has been included as an enzyme apparently closely linked with chloroplast development (15). Both SCS and acetyl CoA synthetase show patterns of activity different from these 2 groups and from each other. Apparently, SCS is not solely involved with the citric acid cycle nor does it have a close correlation with chloroplast organization as manifest by δ-aminolevulinic acid dehydrase activity.

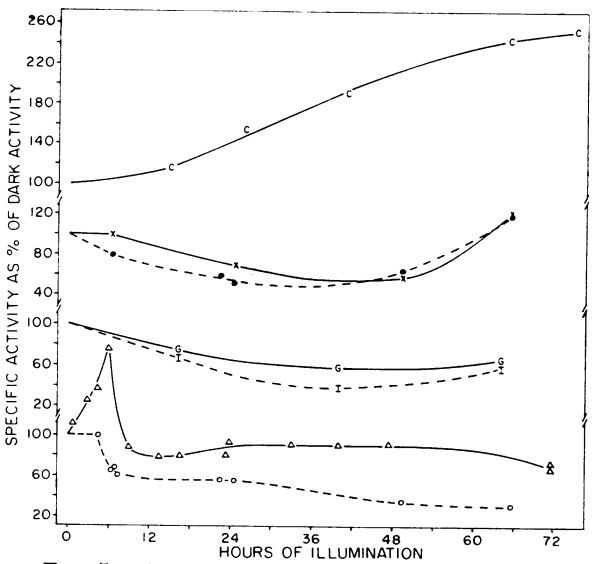

FIG. 7. Activity per mg protein of a number of enzymes during continuous white illumination of leaves expressed as a percentage of the initial dark activity. ○-----○ acetyl CoA synthetase; △————△ succinyl CoA synthetase; I— — —I NADP isocitrate dehydrogenase; G———G glutamate -oxaloacetate transaminase; ●--------● pyruvate dehydrogenase system; x———x α-ketoglutarate dehydrogenase; C———C delta-amino-levulinic acid dehydrase.

Enhancement of respiratory gas exchanges by light treatments were always small and the patterns of response to light was complicated by rapidly increasing respiration rate of dark control leaves. For these reasons it was difficult to correlate SCS activity and respiratory activity. A 2-fold increase of SCS activity was never reflected in a 2-fold increase of respiration rate. At all times the respiratory quotient of the bean leaves remained close to unity.

Chlorophyll synthesis in these leaves exhibited a lag phase of 2 to 3 hr. Thereafter synthesis continued at a linear rate for 22 hr. During this phase it is likely that photosynthetic activity commenced (13, 16) and photosynthetically fixed carbon utilized in chlorophyll synthesis. It is interesting to note that only after 5 hr illumination of bean leaves did the photosynthetic electron pathway inhibitor, 3-(4-chlorophenyl)-1-dimethylurea depress chlorophyll synthesis (7). Before that time the carbon skeletons for chlorophyll must have derived from elsewhere. It is possible that the increased succinyl CoA synthetase activity during the first few hr of illumination supplies succinyl CoA for chlorophyll synthesis by the mobilization of a succinate pool. Later the precursors are from photosynthetically fixed carbon but this hypothesis remains to be tested. Certainly the significance of the transient nature of the changes in SCS activity await further study.

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