Plant Physiol. (1974) 53, 879–885

The Effect of Light on the Tricarboxylic Acid Cycle in Green Leaves

I. RELATIVE RATES OF THE CYCLE IN THE DARK AND THE LIGHT

Received for publication January 31, 1973 and in revised form June 11, 1973

E. A. CHAPMAN AND D. GRAHAM
Commonwealth Scientific and Industrial Research Organization, Division of Food Research, and School of Biological Sciences, Macquarie University, North Ryde 2113, Sydney, Australia

ABSTRACT

Excised green leaves of mung bean (*Phaseolus aureus* L. var. Mungo) were used to determine the effect of light on the rate of endogenous respiration via the tricarboxylic acid cycle. Illumination with white light at an intensity of 0.043 gram calories $cm^{-2}min^{-1}$ (approximately 8600 lux) of visible radiation (400–700 nm) gave a rate of apparent photosynthesis, measured as net CO_2 uptake, of 21 mg CO_2 $dm^{-2}hr^{-1}$ which was about 11-fold greater than the rate of dark respiration. The feeding of $^{14}CO_2$ or ^{14}C-labeled acids of the tricarboxylic acid cycle in the dark for 2 hours was established as a suitable method for labeling mitochondrial pools of cycle intermediates.

At a concentration of 0.1 mM 3-(3,4-dichlorophenyl)-1,1-dimethylurea, apparent photosynthesis was inhibited 82%, and the refixation of $^{14}CO_2$ derived internally from endogenous respiration was largely prevented. In the presence of this inhibitor endogenous respiration, measured as $^{14}CO_2$ evolution, continued in the light at a rate comparable to that in the dark. Consequently, under these conditions light-induced nonphotosynthetic processes have no significant effect on endogenous dark respiration. Inhibitors of the tricarboxylic acid cycle, malonate and fluoroacetate, were used to determine the relative rates of carbon flux through the cycle in the dark and in the light by measuring the rate of accumulation of ^{14}C in either succinate or citrate. Results were interpreted to indicate that the tricarboxylic acid cycle functions in the light at a rate similar to that in the dark except for a brief initial inhibition on transition from dark to light. Evidence was obtained that succinate dehydrogenase as well as aconitase, was inhibited in the presence of fluoroacetate.

Controversy has surrounded the question of the effect of light on endogenous aerobic respiration via glycolysis and the tricarboxylic acid cycle in algae and green leaves of higher plants. Reports have included stimulation, inhibition, or no effect of light on respiration. For example, physiological studies, using the mass spectrometer to measure respiratory and photosynthetic gas exchange simultaneously, showed that relatively low light intensities had little or no effect on rate of respiratory oxygen uptake in barley leaves or in suspensions of the alga *Chlorella* (8). With the green alga *Ankistrodesmus braunii* (9) or the algal flagellate *Ochromonas malhamensis* (52) it was found that respiratory CO_2 evolution was almost independent of light intensity, and that respiratory oxygen consumption was not affected by low light although it was enhanced at high light intensities. Hoch *et al.* (24) concluded from their results obtained with the mass spectrometer that low light intensities inhibited endogenous respiration in *Anacystis,* a blue-green alga, but not in *Scenedesmus,* a green alga, whereas higher light intensities promoted oxygen uptake in both organisms. The total oxygen consumption or CO_2 evolution cannot be separated, however, into dark endogenous respiratory and light-induced photorespiratory components, and the metabolic system(s) responsible for each can only be inferred from physiological experiments (26, 33). In view of the complex interactions of photosynthesis, photorespiration, and endogenous respiration, an accurate assessment of endogenous respiration in the light is difficult to attain on the basis of physiological measurements of gas exchange.

Early biochemical studies indicated that photosynthetically incorporated $^{14}CO_2$ (4, 7) did not readily enter the tricarboxylic acid cycle intermediates in the light, nor was pyruvate oxidation readily achieved in the light (18, 36). The interconversion of exogenously fed ^{14}C-labeled intermediates of the tricarboxylic acid cycle was shown to occur both in the light and in the dark in leaves of mung bean, and it was suggested that the observed reversal by light of the ratio of ^{14}C in malate-aspartate in the dark was the result of differences in the ratio of NAD/NADH between light and dark (20). This postulate was confirmed and correlated with changes of ^{14}C in intermediates of the tricarboxylic acid cycle during dark-light transition (19). It has been clearly demonstrated that light induces changes in the ratio of ATP/ADP (43) and in the ratio of oxidized-reduced nicotinamide adenine dinucleotides (19, 22, 23, 37).

In *Chlorella,* a light-induced blockage of glycolysis at the triose phosphate dehydrogenase step was proposed (27) which is probably due to the changes in the ratio ATP/ADP (23, 43). It is clear from these various studies that light has marked effects upon the relative levels of cofactors of respiratory metabolism and the pool sizes of respiratory intermediates.

In a different approach from those described above, the determination of the rates of equilibration of ^{14}C in the carboxyl carbons of tricarboxylic acid cycle intermediates in *Scenedesmus* (35) showed that there were no significant differences between those in the dark and in light saturating for photosynthesis. The results were interpreted as showing no difference in rate of the tricarboxylic acid cycle in the two conditions.

Many of the reported variations in the response of respiration to light are doubtless due to the wide variation in experimental regimes, some of which included conditions suboptimal

for photosynthesis. In the experiments to be described here, excised leaves of a higher plant have been used under relatively high light intensities such that photosynthesis is operating at a rate some 10- to 12-fold greater than dark respiration.

In this paper the suitability of the experimental material and experimental design, in particular the use of inhibitors, has been examined. An assessment of the relative fluxes of carbon in the tricarboxylic acid cycle in dark or light has been made using inhibitors of photosynthesis (DCMU) and of the tricarboxylic acid cycle (malonate and fluoroacetate). The results indicate that, apart from an inhibition during the first few minutes of illumination, the tricarboxylic acid cycle operates in the light at a rate comparable to that in the dark.

MATERIALS AND METHODS

Growth of Plants. Mung beans (*Phaseolus aureus* L. var. Mungo, Yates Seeds, Sydney, Australia) were grown from seed for 7 days as described by Graham and Cooper (19).

Experimental Procedures. On the 8th day after planting, seedlings were placed in the dark for 2 hr and then the 1st pair of leaves was harvested under a green safelight (54) by excision at the petiole.

Leaves were fed radioactive isotope in the dark in the following manner.

1. *$^{14}CO_2$.* Twenty leaves (total fresh weight 500 ± 10 mg) were placed on moist filter paper in a 250-ml Petri dish in which $^{14}CO_2$ was liberated by injecting $Na_2{}^{14}CO_3$ into lactic acid. The initial concentration of $^{14}CO_2$ was about 0.2% (v/v).

2. *^{14}C-labeled tricarboxylic acid cycle acids.* Leaves were placed in feeding trays as described previously (20) and 0.4 ml of radioactive isotope solution was fed to five leaves through the transpiration stream.

At the end of 2 hr in the dark and before subsequent illumination, the leaves were removed from the ^{14}C source and placed in water in an air draught for 15 min.

Illumination. Leaf samples were illuminated by a 250 w tungsten-filament reflector spotlight placed at a distance of 35 cm. The samples were maintained at room temperature (20–25 C) by using a water-cooled heat filter (53). Light intensity was measured with a Kipp actinometer and was as follows.

1. *With the Perspex lid in place.* Total radiation was 0.089 g cal $cm^{-2}min^{-1}$ (approximately 17,800 lux). Visible radiation (400–700 nm) was 0.043 g cal $cm^{-2}min^{-1}$ (approximately 8,600 lux).

2. *Without Perspex lid.* Total radiation was 0.095 g cal $cm^{-2}min^{-1}$ (approximately 19,000 lux). Visible radiation was 0.046 g cal $cm^{-2}min^{-1}$ (approximately 9,200 lux).

Measurement of CO_2 Evolution. To collect $^{12}CO_2$ or $^{14}CO_2$ the feeding tray was covered with a gas-tight Perspex lid to give a vessel of 200-ml volume, and was connected to a supply of compressed air flowing at the rate of 400 ± 5 ml min^{-1}. The sample size was 10 leaves. $^{12}CO_2$ evolved was measured using a Beckman infrared gas analyzer (Model 315). $^{14}CO_2$ was trapped in vials, each containing 5.5 ml of methanol-ethanolamine (4:1, v/v) (1), linked in series. At the end of the collection period, 10 ml of toluene scintillator were added to each of these vials.

Extraction of Intermediary Metabolites. After light or dark treatment, intermediary metabolites were extracted from leaves by boiling in 80% aqueous ethanol (v/v) for 3 min followed by two extractions with boiling 20% aqueous ethanol (v/v) (5). The ethanolic extract was dried under reduced pressure at 30 to 35 C, resuspended in 1 ml of 50% ethanol, and washed with 4 ml of chloroform which was separated to give aqueous ethanol-soluble and chloroform-soluble fractions, respectively.

Chloroform-soluble Compounds. Chloroform fractions were dried and resuspended in 1 ml toluene and 0.2-ml samples were counted in toluene fluor (see below).

Ethanol-soluble Compounds. Samples of each fraction were chromatographed on Whatman No. 1 paper using a 2-dimensional system; phenol-water (100 g/39.5 ml) in the first direction and 1-butanol-acetic acid-water (74:19:45) in the second direction (6).

Insoluble Material. Residues of extracted leaves were solubilized by homogenizing them in 2 ml of NCS solubilizer (Nuclear Chicago Corporation, Des Plaines, Illinois, U. S. A.) contained in a 5-ml all-glass homogenizer with a motor-driven plunger. The clear homogenate was made to 5 ml with toluene and 0.1 ml samples were counted in toluene fluor (see below).

Isolation of Intermediates. Chromatographs of ethanol-soluble compounds were radioautographed and preliminary identifications were made from published sources. All radioactive areas were then cut from chromatographs, placed in toluene fluor, and counted. To confirm the identification of most compounds, papers were removed from the scintillant, washed with ethyl ether, eluted with 50% ethanol, and chromatographed on Whatman No. 1 paper with the appropriate known compounds in at least two solvents as follows: amino acids: (*a*) 1-butanol-propionic acid-water (10:5:3) (6); (*b*) phenol-ammonia-water (160 g:0.5 ml:40 ml) (44). Organic acids: (*a*) 95% ethanol-ammonia-water (8:1:1) (41); (*b*) 1-butanol-90% formic acid-water (10:3:10) (41). Phosphate esters: (*a*) isobutyric acid-1 N ammonia-0.1 M EDTA (100:60:1.6) in the first direction, then 1-butanol-propionic acid-water (375:180:245) in the second direction (46), using Whatman No. 4 paper washed in aqueous EDTA (15); (*b*) 1-propanol-ammonia-water (6:3:1) modified after Isherwood and Hanes (25); (*c*) ethyl acetate-acetic acid-water (3:3:1) (5).

Compounds were detected on the chromatographs by spraying with the following locating reagents: amino acids—ninhydrin solution (30); organic acids—bromophenol blue solution (44); phosphate esters—either (*a*) the reagent of Hanes and Isherwood (21) as modified by Bandurski and Axelrod (2), or (*b*) Rosenberg's reagent (42).

Measurement of Radioactivity. Radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, Model 3375, after addition of 15 ml of toluene fluor (3 g of 2,5-diphenyloxazole and 0.2 g of *p*-bis-2,5-diphenyloxazole in 1 litre of toluene) to a glass vial containing ^{14}C-labeled material on paper or in methanol-ethanolamine mixture. Radioactivity in aqueous samples was measured by the addition of 10 ml of mix I (toluene fluor-Triton X-100, 2:1) (38) to 1 ml of sample. Where appropriate, corrections for quenching and efficiency were made.

Uptake of Dye. The rate at which water entered a cut leaf in the dark or in the light was observed by placing the leaf in 0.1% aqueous ethanol containing a small quantity of methyl red, and following its path through the leaf under a microscope.

Stomatal Count. Leaves previously fed ^{14}C-acetate for 2 hr in the dark were excised, and casts were made of both sides of the leaves (in the dark or after exposure to light for 30 and 60 min) using clear nail polish. The numbers of open or closed stomates were counted in the casts using a Reichert-Visopon projection light-microscope.

Radiochemicals. ^{14}C-Labeled compounds were obtained from the Radiochemical Centre, Amersham, England and had the following specific activities: acetate-2-^{14}C, 38 mc/mmole; $^{14}CO_2$ as $Na_2{}^{14}CO_3$, 56 mc/mmole; citrate-1,5-^{14}C, 15.8 mc/mmole; fumarate-2,3-^{14}C, 6.95 mc/mmole; malate-U-^{14}C, 47.2 mc/mmole; and succinate-2,3-^{14}C, 1.45 mc/mmole. One μc was used per leaf in all cases except ^{14}C-

succinate when 0.13 μc per leaf was used. All ^{14}C-substrates with the exception of $^{14}CO_2$ were checked for purity by 2-dimensional paper chromatography (6) and radioautography.

RESULTS AND DISCUSSION

Experimental Condition of Excised Leaves. The results reported in this paper indicate that excised leaves of mung bean are suitable material for investigating the influence of light on endogenous respiratory metabolism via the tricarboxylic acid cycle. All leaf cells showed substantial uptake of the dye, methyl red, within 15 min of application. It is likely, therefore, that rapid distribution of solutes occurs throughout the leaf.

In the dark about 40% of the stomates were closed whereas after illumination for 30 min under the usual experimental conditions only 5% remained closed.

When CO_2-exchange was measured with an infrared gas analyzer, the usual value for dark respiration was about 1.9 mg $dm^{-2}hr^{-1}$ while after 30 min illumination the value for photosynthesis was approximately 11-fold greater (21 mg $dm^{-2}hr^{-1}$).

Uptake and Redistribution of Radioisotopes. The time required for gross uptake by leaves of a radioactive substrate was determined by sampling the external solution during the feeding period in the dark. By measuring $^{14}CO_2$ evolved from the leaves under similar conditions, the time course of metabolism of a substrate was determined. $^{14}CO_2$ evolution reached a maximum rate between 80 and 100 min and thereafter declined very slowly, while uptake of ^{14}C-labeled solution was essentially completed by 120 min. Therefore, in order to achieve maximal uptake of ^{14}C and distribution to active metabolic pools, a standard time of 2 hr was used for the feeding of labeled substrates. Uptake of ^{14}C-substrates varied with each leaf sample and accordingly, to enable comparisons to be made, data are generally expressed in this and the subsequent papers (12, 13) as percentages of the ^{14}C in the total ethanol-soluble fraction.

The percentages of the fed substrates which were recovered from the leaf samples unchanged (but including also ^{14}C metabolized into the corresponding metabolic pool(s)) varied from 4.4% to 20.7% of that taken up. Because no marked utilization of this portion of the substrate occurred during the experimental period, it is concluded that such substrate was not readily available to the active metabolic pools. Further, the total amount of any of the ^{14}C-substrates fed during the 2-hr dark period was approximately equal to the total pool size in the leaf sample (34). Because most of the fed substrate was metabolized during the dark period, it is considered unlikely that the pattern of metabolism of the excised leaves subsequently was greatly disturbed from normal by the feeding of the substrates in the manner indicated.

Table I shows the gross distribution of ^{14}C among three major fractions, ethanol-solubles, chloroform-solubles, and insolubles (for definition of these fractions see "Materials and Methods"). Illumination resulted in relatively minor changes in percentage distribution among the fractions except after ^{14}C-succinate feeding, when a large increase of ^{14}C in the insoluble fraction occurred.

Effect of DCMU. To determine whether or not respiratory $^{14}CO_2$ production is influenced by photosynthesis or by some other light-induced process, photosynthetic electron transport was inhibited by DCMU. The effectiveness of various concentrations of DCMU on $^{12}CO_2$ fixation in the light was determined. At 0.1 mM DCMU, photosynthesis (CO_2 fixation) was inhibited 82% relative to the illuminated control. This concentration of DCMU is high but the ability of the leaves to continue respiring at the same rate in the dark for at least 6 hr indicated that the inhibitor was not deleteriously affecting the respiratory metabolism of the leaves.

The effects of 0.1 mM DCMU on $^{14}CO_2$ evolution in the dark and in the light when $^{14}CO_2$ or citrate-1,5-^{14}C were fed to leaves in the dark are shown in Figure 1, a and b, respectively. In leaves previously fed $^{14}CO_2$ in the dark, there is no effect of the inhibitor on evolution of $^{14}CO_2$ in the dark nor on the initial decrease in $^{14}CO_2$ evolution in the first minutes of illumination. However, after about 5-min illumination, the rate of $^{14}CO_2$ evolution returns to a level similar to that in the previous dark period. Return to darkness after 30-min illumination gave an almost negligible $^{14}CO_2$-burst compared with the control.

These results are consistent with the inhibition of photosynthesis by DCMU and with the partial cessation of the CO_2-evolving mechanism (tricarboxylic acid cycle) in the first minutes of illumination. Subsequently in the light, it is suggested that the rate of respiratory production of $^{14}CO_2$, mainly via the tricarboxylic acid cycle, is similar to that in the dark. Presumably in the light, even when photosynthesis is largely inhibited, a regulatory mechanism still operates to maintain a constant internal $^{14}CO_2$ level.

When citrate-1,5-^{14}C was fed in the dark, the effect of DCMU on $^{14}CO_2$ evolution in the light again supports the conclusion that the rate of endogenous respiration is similar to that in the dark. The initial rapid decrease in $^{14}CO_2$ evolu-

Table I. *Distribution of ^{14}C among Extracted Fractions after Feeding ^{14}C-substrates to Leaves in the Dark*

Substrates	Treatment	Extracted Fraction					
		Ethanol-soluble		Chloroform-soluble		Insoluble	
		cpm × 10^{-5}	% of total extracted	cpm × 10^{-5}	% of total extracted	cpm × 10^{-5}	% of total extracted
$^{14}CO_2$	Dark	2.13	65.0	0.17	5.3	0.97	29.7
	Dark plus 30 min light	2.71	61.0	0.01	0.3	1.50	38.7
Acetate-2-^{14}C	Dark	3.21	38.2	2.53	30.0	2.67	31.8
	Dark plus 30 min light	7.41	45.3	3.88	23.7	5.08	31.0
Succinate-2,3-^{14}C	Dark	4.82	80.8	0.28	4.7	0.86	14.5
	Dark plus 30 min light	1.40	55.6	0.26	10.4	0.86	34.0
Fumarate-2,3-^{14}C	Dark	4.19	72.4	0.45	7.7	0.12	19.9
	Dark plus 10 min light	11.70	83.1	0.68	4.8	2.10	12.1
Citrate-1,5-^{14}C	Dark	1.65	58.0	0.39	13.6	0.81	28.4
	Dark plus 30 min light	3.00	69.6	0.47	11.0	0.84	19.4

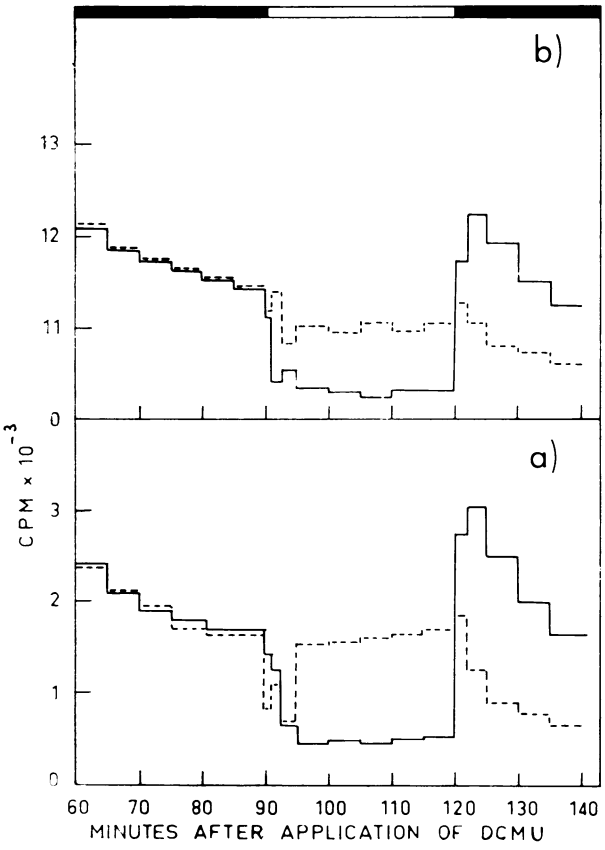

FIG. 1. Effects of illumination on $^{14}CO_2$ evolution by leaves in the presence of 0.1 mM DCMU. Samples of 10 excised leaves were fed (a) $^{14}CO_2$ or (b) citrate-1,5-^{14}C for 2 hr in the dark then were transferred to distilled water, and $^{14}CO_2$ evolution was measured as described in "Materials and Methods." DCMU (in 1% v/v aqueous ethanol) was present in the dark and during illumination. Controls were in 1% (v/v) aqueous ethanol. Control: solid line; DCMU: broken line. Solid bar represents darkness and open bar represents illumination.

tion in the first minutes of illumination is, however, not evident, although the average evolution over the first 5-min period is slightly less than that in the dark or in the subsequent period in the light.

Continued evolution of $^{14}CO_2$ in the light, in the presence of DCMU at rates comparable with those in the dark, indicates that light-induced nonphotosynthetic processes, for example blue light effects (14, 29), have no significant effect on endogenous respiratory processes under the conditions of these experiments.

Determination of Carbon Fluxes in the Tricarboxylic Acid Cycle in the Presence of Inhibitors. In order that the complex data resulting from light-induced changes in intermediary metabolites may be interpreted in terms of control points, recourse will be made in the following paper (12) to the use of the Chance crossover theorem (11). The application of this theorem requires that the relative flux of carbon be known on transition from one condition to another. We postulate here that a change in the rate of accumulation of carbon in an intermediate of the tricarboxylic acid cycle prior to a block by an inhibitor would indicate a change in flux of carbon through the cycle. Accordingly, measurement of carbon flux changes has been attempted in detached leaves on transition from dark to light in the presence of inhibitors of the tricarboxylic acid cycle. The inhibitors chosen were malonate to follow the accumulation of ^{14}C in succinate, and fluoroacetate to follow the ^{14}C accumulation in citrate.

Malonate, at the concentrations used in the present experiments, is a relatively specific inhibitor for succinate dehydrogenase (51). The implication that malonate is an inhibitor of photosynthesis has no sound foundation. Both the findings of Bassham *et al.* (3) showing inhibition of $^{14}CO_2$ entry into malate, then thought to be a primary product of photosynthesis, and the observation of Kortschak *et al.* (28) that malonate inhibits malate formation in sugar-cane leaves may be explained on the basis of an inhibition of the tricarboxylic acid cycle; *e.g.*, see Chapman and Osmond (13).

Fluoroacetate is a competitive inhibitor of mitochondrial aconitase. In plants however, it is not as potent an inhibitor as it is in animals. There are at least three possible reasons for this: (*a*) insensitivity of plant aconitase to fluorocitrate (31, 32, 45, 49, 50); (*b*) inability of either the thiokinase or citrate synthase to accept the fluoroderivative (16); and (*c*) defluorination of fluoroacetate (39, 40, 49, 50). Plants, however, are capable of forming fluorocitrate, (32, 49, 50, 55) and citrate does accumulate though to a lesser extent than in animal tissues (16, 49, and this paper). The concentrations of fluoroacetate necessary to demonstrate aconitase inhibition will vary considerably depending upon the detoxification mechanism in operation. Although fluorocitrate is thought to be specific for mitochondrial aconitase, inhibition of succinate dehydrogenase has been reported (10, 17) and we also found evidence for this (Table II and Fig. 3b). Vickery and Vickery (47) have suggested that fluoroacetate biosynthesis in *Dichapetalum*

Table II. *Effect of Either Fluoroacetate or Malonate on Light-induced Changes in ^{14}C-Labeled Intermediates of the Tricarboxylic Acid Cycle*

After 2 hr feeding of $^{14}CO_2$ in the dark each sample of 5 leaves was removed and placed either in water (control) or in 0.1 M fluoroacetate at pH 4.0 for 10 min in the dark or in 0.01 M malonate for 80 min in the dark. Values represent percentage of distribution of ^{14}C in total ethanol-soluble compounds. Values in brackets represent the difference between light and dark samples.

Intermediate	Control		0.1 M Fluoroacetate		0.01 M Malonate	
	Dark	20 min light	Dark	20 min light	Dark	20 min light
	%					
Aspartate	18.3	4.5 (−13.8)	22.4	14.1 (−8.3)	12.9	8.2 (−4.7)
Citrate	14.6	14.0 (−0.6)	15.4	19.5 (+4.1)	20.3	7.2 (−13.1)
Fumarate	trace	trace	1.4	2.4 (+1.0)	3.7	4.7 (+1.0)
Malate	39.1	53.3 (+14.2)	17.0	19.8 (+2.8)	13.0	20.5 (+7.5)
Succinate	2.0	1.7 (−0.3)	20.2	18.3 (−1.9)	24.9	32.7 (+7.8)
Glutamate	16.8	11.5 (−5.3)	15.6	16.5 (+0.9)	15.2	10.1 (−5.1)
Other organic acids and amino acids	8.0	9.6 (+1.6)	7.2	8.1 (+0.9)	9.4	12.0 (+3.6)
Sugar phosphates	0	0.9 (+0.9)	0	0.3 (+0.3)	0	0.7 (+0.7)
Sucrose	1.2	4.5 (+3.3)	0.8	1.0 (+0.2)	0.6	3.8 (+3.2)

is intimately associated with photosynthesis but there seems to be no evidence to support this. In the present experiments with mung bean leaves neither CO_2 nor $^{14}CO_2$ fixation was inhibited by 0.1M fluoroacetate, compared with controls over a period of 2 hr though in this time ^{14}C accumulated in citrate.

$^{14}CO_2$ evolution in the dark and the accumulation of either ^{14}C-succinate or ^{14}C-citrate were used to determine the lowest concentrations of the two inhibitors effective as partial inhibitors of respiration. When $^{14}CO_2$ was the labeled substrate, $^{14}CO_2$ evolution in the dark was inhibitod 50% by 0.01M malonate and 19% by 0.1M fluoroacetate. ^{14}C-succinate accumulated in the presence of malonate and ^{14}C-citrate accumulated in the presence of fluoroacetate. Lower concentrations, *e.g.*, 5mM malonate or 50mM fluoroacetate, were ineffective even after prolonged periods of exposure. It should be noted that inhibition of the tricarboxylic acid cycle, measured as $^{14}CO_2$ evolution, may well be higher than 50% and 19% since MacLennan *et al.* (34) have shown that at least 50% of the CO_2 evolved from some plant tissues arises from reactions outside the tricarboxylic acid cycle.

Under similar experimental conditions, when acetate-2-^{14}C or fumarate-2,3-^{14}C were fed a stimulation of $^{14}CO_2$ evolution occurred in the presence of either malonate or fluoroacetate. Stimulation was greatest at the lowest concentrations of inhibitors. Stimulatory effects of fluoroacetate on respiration have also been reported for lettuce leaves (48) but no satisfactory explanation of these findings can be offered.

Using $^{14}CO_2$ as the labeled substrate and 0.01M malonate or 0.1M fluoroacetate as the inhibitors, the relative rates of accumulation of ^{14}C in either succinate in the presence of 0.01M malonate, or citrate in the presence of 0.1M fluoroacetate were determined in the dark and in the light.

The results of a representative experiment with malonate are shown in Figure 2. In the presence of malonate in the dark, a considerable accumulation of ^{14}C-succinate was found and accumulation continued during the time course of the experiment (compare with Table I in ref. 12). This result is consistent with inhibition of succinate dehydrogenase in the tricarboxylic acid cycle. Illumination of the leaves resulted in a small but reproducibly consistent decline in the accumulation of succinate during the first 5 min of illumination, which

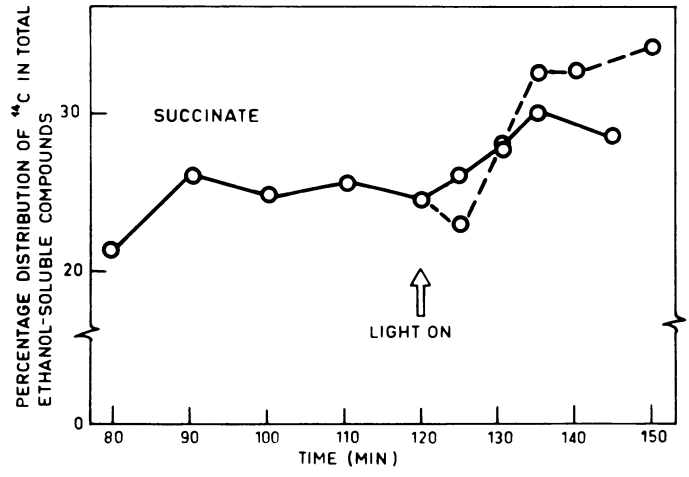

FIG. 2. Effects of illumination on the percentage distribution of ^{14}C in ^{14}C-succinate in the presence of 0.01 M malonate at pH 4.0. $^{14}CO_2$ was fed in the dark to samples of five leaves for 2 hr and the leaves were transferred to the inhibitor in the dark. Sampling in the dark was begun after 80 min and a series of leaf samples was illuminated from 120 min. Dark: solid line; light: broken line.

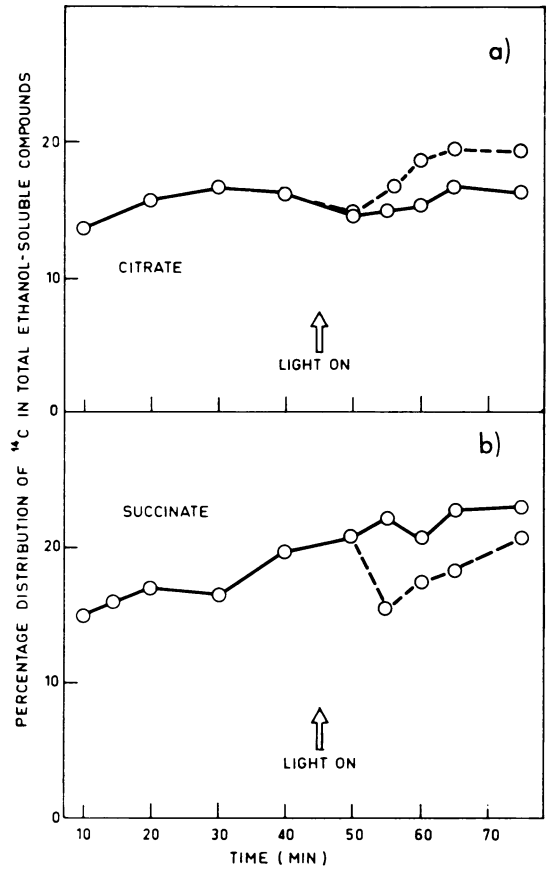

FIG. 3. Effects of illumination on the percentage distribution of ^{14}C in ^{14}C-citrate and ^{14}C-succinate in the presence of 0.1 M fluoroacetate at pH 4.0. Conditions were similar to those described in Figure 2 but sampling began after 10 min and illumination began at 45 min: a: ^{14}C-citrate; b: ^{14}C-succinate. Dark: solid line; light: broken line.

is interpreted as a decline in carbon flux through the cycle. Subsequently in the light, accumulation of ^{14}C-succinate resumed at a rate which was as high or higher than that observed in the dark.

This initial decline in carbon flow through the tricarboxylic acid cycle, although apparently small, was obtained consistently. It was obtained for $^{14}CO_2$ evolution in the presence of DCMU in two separate duplicated experiments with $^{14}CO_2$ as substrate (Fig. 1a), and one duplicated experiment with ^{14}C-citrate as substrate (Fig. 1b). The decline in the percentage of ^{14}C in succinate was obtained in three separate experiments with malonate as inhibitor (Fig. 2), and in two experiments out of three when fluoroacetate was the inhibitor (Table II and Fig. 3).

Changes in the percentage of ^{14}C in citrate in the light paralleled those in the dark control in two experiments (Fig. 3a) with fluoroacetate, while in the third experiment (in which 40% of ^{14}C accumulated in citrate), ^{14}C accumulated more rapidly in the light for 4 min and then paralleled the dark control. The explanation of the ^{14}C-citrate data is necessarily complicated because of the position of citrate at the entry to the cycle and its relation to controls in the cycle. If citrate synthase is inhibited in the light then carbon flow into citrate will be slowed. If, however, isocitrate dehydrogenase is also inhibited in the light then this will tend to inhibit carbon flow out of citrate. The result will depend upon the degree to which citrate synthase and isocitrate dehydrogenase are in-

hibited and upon the temporal relationship of the control by these enzymes. Hence it is likely that when aconitase is only partially inhibited, as in two of our experiments, then the controls at citrate synthase and isocitrate dehydrogenase may result in an imperceptible change in ^{14}C in citrate. When aconitase is more fully inhibited, citrate-^{14}C accumulates faster in the light than in the dark for the first 4 min while at the same time ^{14}C-succinate declines. Such a result is consistent with a slower flow of carbon out of citrate.

Likewise the initial decline in ^{14}C-succinate may be interpreted either as a decreased synthesis or an increased utilization of succinate. The ratio of NAD/NADH is known to decrease in the light (19) thereby restricting the oxidation of isocitrate and malate. Such a situation favors a slower entry of ^{14}C into succinate rather than a faster removal from succinate. In addition, no evidence was obtained of an increased synthesis in the light of Chl, an alternative product of succinyl CoA metabolism.

Therefore, taken together with the results of the experiments with DCMU (Figs. 1a and 1b), it is reasonable to interpret the changes observed on illumination in the presence of malonate (Fig. 2) or fluoroacetate (Fig. 3) as an initial decline in flow of carbon through the tricarboxylic acid cycle, followed by a return to a rate which is about the same or somewhat greater than that occurring in the dark.

In the light, malonate and fluoroacetate markedly inhibited the increase in labeled malate and the decrease in labeled aspartate compared with a control (Table II). Because malonate and fluoroacetate are mitochondrial enzyme inhibitors, such a finding indicates that the labeled malate and aspartate pools are either predominantly mitochondrial or in rapid equilibrium with the mitochondrial pool.

CONCLUSIONS

It has been established that excised mung bean leaves are suitable material in which to measure the effects of moderately high light intensity, which is sufficient to nearly saturate photosynthesis, on endogenous respiratory processes. Use of mitochondrial enzyme inhibitors has established that tricarboxylic acid cycle intermediates labeled with ^{14}C in the dark are in mitochondrial pools or in pools in rapid equilibrium with them. Operation of the tricarboxylic acid cycle was demonstrated both in the dark and the light by the use of inhibitors. Light-induced nonphotosynthetic processes appear not to affect endogenous respiratory metabolism under these conditions.

The relative rates of the tricarboxylic acid cycle during dark-light transition and during the illumination period have been established. The results indicate that for about the first 5 min of illumination there is a decrease in carbon flow through the tricarboxylic acid cycle but that during most of a 30 min period of illumination the rate of carbon flow in the tricarboxylic acid cycle is at least as high as that in the dark (about 1.9 mg CO_2 $dm^{-2}hr^{-1}$) or somewhat higher. This information will be applied in the following paper to the determination of the control points in the tricarboxylic acid cycle under the conditions of these experiments.

Acknowledgments—The work reported in this paper and the following paper was carried out in the laboratories of the CSIRO Division of Food Research Plant Physiology Unit in the School of Biological Sciences, University of Sydney, Sydney, Australia. We are grateful to Professor M. G. Pitman and the staff of the Botany Department for their generous provision of services and facilities. In particular we wish to thank Dr. P. Martin for the use of his infrared gas analyzer and advice on its operation. We wish also to thank Dr. R. J. Mead of the University of Western Australia for his very helpful discussion on the utilization of fluoroacetate by plants.

LITERATURE CITED

1. Baggiolini, M. and M. H. Bickel. 1966. A new type of incubation apparatus for the determination of metabolically produced $^{14}CO_2$. Anal. Biochem. 14: 290–295.
2. Bandurski, R. S. and B. Axelrod. 1951. The chromatographic identification of some biologically important phosphate esters. J. Biol. Chem. 193: 405–410.
3. Bassham, J. A., A. A. Benson, and M. Calvin. 1950. The path of carbon in photosynthesis. VIII. The role of malic acid. J. Biol. Chem. 185: 781–787.
4. Bassham, J. A., K. Shibata, K. Steenberg, J. Bourdon, and M. Calvin. 1956. The photosynthetic cycle and respiration: light-dark transients. J. Amer. Chem. Soc. 78: 4120–4124.
5. Benson, A. A. 1955. Phosphorylated sugars. *In:* K. Paech and M. V. Tracey, eds., Modern Methods of Plant Analysis, Vol. 2. Springer-Verlag, Berlin. p. 113.
6. Benson, A. A., J. A. Bassham, M. Calvin, T. C. Goodale, V. A. Haas, and W. Stepka. 1950. The path of carbon in photosynthesis. V. Paper chromatography and radioautography of the products. J. Amer. Chem. Soc. 72: 1710–1718.
7. Benson, A. A. and M. Calvin. 1950. The path of carbon in photosynthesis. VII. Respiration and photosynthesis. J. Exp. Bot. 1: 63–68.
8. Brown, A. H. 1953. The effects of light on respiration using isotopically enriched oxygen. Amer. J. Bot. 40: 719–729.
9. Brown, A. H. and D. Weis. 1959. Relation between respiration and photosynthesis in the green alga, *Ankistrodesmus braunii*. Plant Physiol. 34: 224–234.
10. Brunt, R. V., R. Eisenthal, and S. A. Symons. 1971. Inhibitory effects of fluorocitrates on yeast mitochondria. FEBS Lett. 13: 89–91.
11. Chance, B., W. Holmes, J. Higgins, and C. M. Connelly. 1958. Localization of interaction sites in multi-component transfer systems: theorems derived from analogues. Nature 182: 1190–1193.
12. Chapman, E. A. and D. Graham. 1974. The effect of light on the tricarboxylic acid cycle in green leaves. II. Intermediary metabolism and the location of control points. Plant Physiol. 53: 886–892.
13. Chapman, E. A. and C. B. Osmond. 1974. The effect of light on the tricarboxylic acid cycle in green leaves. III. A comparison between some C_3 and C_4 plants. Plant Physiol. 53: 893–898.
14. Codd, G. A. 1972. The photoinhibition of malate dehydrogenase. FEBS Lett. 20: 211–214.
15. Eggleston, L. V. and R. Hems. 1952. Separation of adenosine phosphates by paper chromatography and the equilibrium constant of the myokinase system. Biochem. J. 52: 156–160.
16. Eloff, J. N. and B. Von Sydow. 1971. Experiments on the fluoroacetate metabolism of *Dichapetalum cymosum* (Gifblaar). Phytochemistry 10: 1409–1415.
17. Fanshier, D. W., L. K. Gottwald, and E. Kun. 1964. Studies on specific enzyme inhibitors. VI. Characterization and mechanism of action of the enzyme-inhibitory isomer of monofluorocitrate. J. Biol. Chem. 239: 425–434.
18. Gibbs, M. 1953. Effect of light intensity on the distribution of C14 in sunflower leaf metabolites during photosynthesis. Arch. Biochem. Biophys. 45: 156–160.
19. Graham, D. and J. E. Cooper. 1967. Changes in levels of nicotinamide adenine nucleotides and Krebs cycle intermediates in mung bean leaves after illumination. Aust. J. Biol. Sci. 20: 319–327.
20. Graham, D. and D. A. Walker. 1962. Some effects of light on the interconversion of metabolites in green leaves. Biochem. J. 82: 554–560.
21. Hanes, C. S. and F. A. Isherwood. 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164: 1107–1112.
22. Harvey, M. J. and A. P. Brown. 1967. Potassium and nicotinamide coenzyme content of isolated pea chloroplasts. Biochem. J. 105: 30P–31P.
23. Heber, U. W. and K. A. Santarius. 1965. Compartmentation and reduction of pyridine nucleotides in relation to photosynthesis. Biochim. Biophys. Acta 109: 390–408.
24. Hoch, G. O. V., H. Owens, and B. Kok. 1963. Photosynthesis and respiration. Arch. Biochem. Biophys. 101: 171–180.
25. Isherwood, F. A. and C. S. Hanes. 1953. Separation and estimation of organic acids on paper chromatograms. Biochem. J. 55: 824–830.
26. Jackson, W. A. and R. J. Volk. 1970. Photorespiration. Annu. Rev. Plant Physiol. 21: 385–432.
27. Kandler, O. and I. Haberer-Liesenkötter. 1963. Über den Zusammerhang zwischen Phosphathaushalt und Photosynthese. Z. Naturforsch 18b: 718–730.
28. Kortschak, H. P., C. E. Hartt, and G. O. Burr. 1965. Carbon dioxide fixation in sugarcane leaves. Plant Physiol. 40: 209–213.
29. Kowallik, V. and W. Kowallik. 1969. Enhancement of respiration by light in photosynthesizing *Chlorella* and its dependence on the wavelength of light. Planta 84: 141–157.
30. Lee, Y. P. and T. Takahashi. 1966. An improved colourimetric determination of amino acids with the use of ninhydrin. Anal. Biochem. 14: 71–77.
31. Louw, N., O. T. de Villiers, and N. Grobbelaar. 1970. Inhibition of aconitase activity from *Dichapetalum cymosum* by fluorocitrate. S. Afr. J. Sci. 66: 9–11.

32. LOVELACE, C. J., G. W. MILLER, AND G. W. WELKIE. 1968. Accumulation of fluoroacetate and fluorocitrate in forage crops collected near a phosphate plant. Atmos. Environ. 2: 187–190.
33. LUDWIG, L. J. AND D. T. CANVIN. 1971. The rate of photorespiration during photosynthesis and the relationship of the substrate of light respiration to the products of photosynthesis in sunflower leaves. Plant Physiol. 48: 712–719.
34. MACLENNAN, D. H., H. BEEVERS, AND J. L. HARLEY. 1963. Compartmentation of acids in plant tissues. Biochem. J. 89: 316–327.
35. MARSH, H. V., J. M. GALMICHE, AND M. GIBBS. 1965. Effect of light on the tricarboxylic acid cycle in *Scenedesmus*. Plant Physiol. 40: 1013–1022.
36. MILHAUD, G., A. A. BENSON, AND M. CALVIN. 1956. Metabolism of pyruvic acid-2-C^{14} and hydroxypyruvic acid-2-C^{14} in algae. J. Biol. Chem. 218: 599–606.
37. OGREN, W. L. AND D. W. KROGMANN. 1965. Studies on pyridine nucleotides in photosynthetic tissue. Concentrations, interconversions and distribution. J. Biol. Chem. 240: 4603–4608.
38. PATTERSON, M. S. AND R. C. GREENE. 1965. Measurement of low energy beta-emitters in aqueous solution by liquid scintillation counting of emulsions. Anal. Chem. 37: 854–857.
39. PETERS, R. A. AND M. SHORTHOUSE. 1971. Identification of a volatile constituent formed by homogenates of *Acacia georginae* exposed to fluoride. Nature 231: 123–124.
40. PREUSS, P. W. AND L. W. WEINSTEIN. 1969. Fluoroorganic compounds in plants. II. Defluorination of fluoroacetate. Contrib. Boyce Thompson Inst. 24: 151–155.
41. RANSON, S. L. 1955. Nonvolatile mono-, di-, and tricarboxylic acids (Chromatographic and Ion Exchange Methods). *In:* K. Paech and M. V. Tracey, eds., Modern Methods of Plant Analysis, Vol. 2. Springer-Verlag, Berlin. pp. 539–582.
42. ROSENBERG, H. 1959. The detection of phosphates on chromatograms. J. Chromatog. 2: 487–489.
43. SANTARIUS, K. A. AND U. HEBER. 1965. Changes in the intracellular levels of ATP, ADP, AMP, and Pi and regulatory function of the adenylate system in leaf cells during photosynthesis. Biochim. Biophys. Acta 102: 39–54.
44. SMITH, I. 1960. Chromatographic and electrophoretic techniques, vol. 1. William Heinemann, Medical Books Ltd. London. p. 85.
45. TREBLE, D. H., D. T. A. LAMPORT, AND R. A. PETERS. 1962. The inhibition of plant aconitate hydratase (aconitase) by fluorocitrate. Biochem. J. 85: 113–115.
46. TSYKIEWICZ, E. 1962. An improved solvent system for the paper chromatography of phosphate esters. Anal. Biochem. 3: 164–172.
47. VICKERY, B. AND M. L. VICKERY. 1972. Fluoride metabolism in *Dichapetalum toxicarium*. Phytochemistry 11: 1905–1909.
48. WARD, P. F. V. AND N. S. HUSKISSON. 1969. The metabolism of fluoroacetate by plants. Biochem. J. 113: 9P.
49. WARD, P. F. V. AND N. S. HUSKISSON. 1972. The metabolism of fluoroacetate in lettuce. Biochem. J. 130: 575–587.
50. WARD, P. F. V. AND R. A. PETERS. 1961. The chemical and biochemical properties of fluorocitric acid. Biochem. J. 78: 661–668.
51. WEBB, J. L. 1965. Enzyme and Metabolic Inhibitors, Vol. 2. Academic Press, New York.
52. WEIS, D. AND A. H. BROWN. 1959. Kinetic relationships between photosynthesis and respiration in the algal flagellate, *Ochromonas malhamensis*. Plant Physiol. 34: 235–239.
53. WITHROW, R. B. AND L. PRICE. 1953. Filters for the isolation of narrow regions in the visible and near-visible spectrum. Plant Physiol. 28: 105–114.
54. WITHROW, R. B. AND L. PRICE. 1957. A darkroom safelight for research in plant physiology. Plant Physiol. 32: 244–248.
55. YU, M. H. AND G. W. MILLER. 1970. Gas chromatographic identification of fluoroorganic acids. Environ. Sci. Technol. 4: 492–495.