Some Effects of Light on the Interconversion of Metabolites in Green Leaves

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When *Chlorella* is allowed to photosynthesize for 30 sec. in the presence of labelled carbon dioxide it does not incorporate radioactivity into glutamate and isocitrate. After the removal of $^{14}\text{CO}_2$, appreciable activity does appear in these compounds if the algae are then kept for a further 150 sec. in the dark but not if they are maintained for the same length of time in the light. From these results (Calvin, 1949) and similar results (Benson & Calvin, 1950; Calvin & Massini, 1952) it was concluded that products newly formed in photosynthesis could not readily enter Krebs-cycle reactions in the light. Calvin & Massini (1952) attributed this to an inhibition of pyruvate oxidation.

Experiments in which pyruvic acid was fed to *Scenedesmus* led to a partial modification of this thesis and to the notion that light might not affect the oxidation of pyruvate at sites spatially removed from the chloroplast (Milhaud, Benson & Calvin, 1956). The findings of these workers that light greatly enhances lipid formation in *Chlorella* is itself not compatible with a light-inhibition of pyruvate oxidation unless there is some source of acetyl-coenzyme A other than pyruvate. More recent work has established that considerable quantities of radioactivity from $^{14}\text{CO}_2$ can pass into Krebs-cycle intermediates in the light (Moses, Holm-Hansen, Bassham & Calvin, 1959; Bassham & Kirk, 1960) and these workers clearly no longer regard the first suggestion as wholly tenable (Smith, Bassham & Kirk, 1961). Outright rejection of light-inhibition of pyruvate oxidation would, of course, leave the original observations unexplained unless a satisfactory alternative were forthcoming. The present work was undertaken to assess the validity of such an alternative.

MATERIALS AND METHODS

Mung-bean seeds (*Phaseolus mungo*) were germinated in vermiculite in covered trays at $35 \pm 1^\circ$ with continuous illumination (two Mazda 80 w., 5 ft. 'daylight' fluorescent tubes at 20 cm.) for 6 days (the light intensity is not critical and could be varied by at least 50%). The first pair of leaves were then fully expanded and were used for experiment on the sixth or seventh day.

Chenopodium bonus henricus and *Vicia sepium* were collected in the field.

Substrates. $[4-^{14}\text{C}]$ Aspartate was prepared enzymically from phosphoenolpyruvate, $^{14}\text{CO}_2$ and glutamate according to the method of Dearing & Walker (1960). $[4-^{14}\text{C}]$ Oxaloacetate and $[4-^{14}\text{C}]$ malate were prepared in a similar way in reaction mixtures from which glutamate was either omitted or replaced by NADH_2 . The aspartate and malate were purified chromatographically but the solution containing oxaloacetate (which is readily broken down to pyruvate and $^{14}\text{CO}_2$) was acidified to precipitate the enzyme protein, filtered, neutralized and then used immediately. The solution containing phosphoenolpyruvate carboxylase, glutamate-aspartate transaminase and malic dehydrogenase required for these syntheses was prepared from *Kalanchoë crenata* (Walker, 1957).

Uniformly labelled mono-ammonium $[^{14}\text{C}]$ glutamate, $[2,3-^{14}\text{C}_2]$ fumaric acid, $[2,3-^{14}\text{C}]$ succinic acid and sodium $[^{14}\text{C}]$ carbonate were obtained from The Radiochemical Centre, Amersham, Bucks. $[1,5-^{14}\text{C}_2]$ Citric acid was obtained from California Biochemical Corp.

Feeding with labelled intermediates. In our first experiments we fed intermediates individually to pairs of leaves through the cut petiole, which was placed in a small glass tube (capacity approx. 0.1 ml.). In later experiments we obtained better uptake if the intermediate was introduced through the cut base of the leaf. Leaves were cut at right angles to the petiole about 2 mm. above the base of the leaf.

The detached laminae were then placed in a shallow circular trough cut into the surface of a Perspex block. The trough was 2 mm. wide and 5 mm. deep and the external diameter of its circle was 11.5 cm. The side walls of the trough were parallel and inclined outwards at an angle of 45° to the surface of the block so that leaves standing in the trough projected from it at this angle to the vertical. This arrangement permitted equal illumination of the leaves from a point above the centre of the block and ensured that the leaves presented the maximum surface area to the light source. Illumination was provided by a 250 w Mazda reflector spotlight 35 cm. above the centre of the block and the light was also reflected from the lower surface of the block by a white reflector. A 3 cm. depth of ferrous ammonium sulphate solution (3%, w/v, in 1%, v/v, H_2SO_4), cooled by running tap water, was used as a heat filter. The filter transmitted a minimum of 90% of the incident light at wavelengths between 380 and 740 μ . In preliminary experiments it was found that the light-intensity could be varied by at least $\pm 25\%$ without appreciably affecting the results. The solution to be fed (usually 0.4 ml.) was introduced into the appropriate part of the trough and the leaves were added immediately they were cut from the plant. A second identical block was used in the dark room.

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Extraction procedure. Leaves were quickly washed in deionized water before being placed in boiling 80% (v/v) ethanol (100 ml.). After boiling for 3 min. and standing for about 30 min. the supernatant was decanted and two further extractions of the leaves were made with boiling 20% (v/v) ethanol (50 ml.). The combined extracts were evaporated to dryness *in vacuo* at 30°. The residues were dissolved in 80% (v/v) ethanol (1 ml.) and portions (25 μ l.) of this solution were used for chromatography.

Chromatography. Two-dimensional paper chromatograms were produced on Whatman no. 1 paper with phenol-water and butan-1-ol-acetic acid-water as solvents (Benson *et al.* 1950). Chromatograms were dried in still air. Radioautographs were prepared with Ilfex No-Screen X-ray film.

Mitochondria. These were separated by centrifuging extracts from the endosperm of castor-bean seedlings which had been ground in a pestle and mortar with 0.5 M-sucrose-0.1 M-phosphate, pH 7.1 (Beevers & Walker, 1956). The mitochondria were washed twice in grinding medium and used immediately. Reactions were terminated by addition of boiling ethanol (4 vol.).

Counting. Radioactivity on chromatograms was counted directly on the paper. Spots were located by radioautography and then cut out and fastened to a paper strip which was fed continuously (12 in./hr.) into a semi-automatic recording scintillation counting assembly (Isotopes Developments Ltd., Reading). About one-third of the counts obtained in this way were checked with conventional scintillation and thin end-window Geiger-Müller counters. Additional counts were made on intermediates eluted from chromatograms and dried uniformly on tissue paper supported by aluminium planchets. The various methods gave figures which were in good agreement when compared on the same basis. In the Results section we have expressed all the counts in terms of Geiger-Müller counts/min.

RESULTS

Experiments with $^{14}\text{CO}_2$

Short-term photosynthesis followed by light and dark conditions. Leaves that were put into the dark after photosynthesis (2 min.) in the presence of $^{14}\text{CO}_2$ accumulated radioactivity in glutamate and citrate more rapidly than those that were kept in the light (Fig. 1). Malate and aspartate became labelled during the first 2 min. and the amount of radioactivity in malate was greater than that in aspartate. These proportions were maintained if the leaves were illuminated for the next 5 min., but if instead they were kept for 5 min. in the dark this ratio was reversed and more activity was found in aspartate than malate (see also results of feeding experiments). The decline in malate in the dark was greater than the total increase in aspartate, citrate and glutamate.

Fixation of $^{14}\text{CO}_2$ in light and dark conditions. The rate and extent of dark fixation of $^{14}\text{CO}_2$ by mung beans were determined because this would affect the interpretation of our results. Table 1 records the amounts of radioactivity which were detected in various intermediates (Materials and Methods section) after exposure of comparable samples of

Fig. 1. Effects of light or dark conditions on the distribution of radioactivity in citrate (a), glutamate (b), aspartate (c) and malate (d) after short-term photosynthesis in the presence of $^{14}\text{CO}_2$. Excised mung-bean leaves (21) were pre-illuminated for 3 min. in a closed Petri dish containing about 4 mg. of $\text{Ba}^{14}\text{CO}_3$ (approx. 400 μC) in a shallow dish. Lactic acid (0.5 ml.) was then injected through a rubber cap in the lid of the Petri dish into the ^{14}C carbonate, thus releasing $^{14}\text{CO}_2$. After illumination for a further 2 min. the lid was removed and the labelled CO_2 blown off in an air stream. One sample of seven leaves was killed at this time, a second was removed to the dark and a third left in the light. After a further 5 min. the second and third samples were killed and extracted (see Materials and Methods section). $\circ$, Samples in the light; $\bullet$, samples in the dark.

leaves to $^{14}\text{CO}_2$ for 10 min. in the light or in the dark. Despite the uniformity of the samples these figures do not permit a direct comparison of the rates of light and dark fixation since stomatal closure is not taken into account. However, in young leaves such as these, with thin cuticles, much of the CO_2 will pass directly through the cuticle (Freeland, 1948) and therefore it is unlikely that true dark fixation could be much greater than the apparent value for dark fixation which we recorded in this experiment (0.7% of that in the light). It will be seen that some label had appeared in glutamate and citrate after photosynthesis for 10 min. (cf. Fig. 1). Again a high ratio of labelled malate/labelled aspartate was characteristic of light-metabolism whereas in the dark this ratio was lower.

Dark fixation followed by light or dark conditions. Table 2 shows the changes that occurred in the amounts of radioactivity in a number of intermediates when dark fixation of $^{14}\text{CO}_2$ was followed by exposure of leaves to light and then dark conditions in air. The ratio malate/aspartate, which was observed to be low in the dark (see above), was

increased in passing from dark to light and again decreased after the leaves had been returned to the dark.

Table 1. *Major products of fixation of $^{14}\text{CO}_2$ by mung-bean leaves in light and in the dark*

Ten pairs of mung-bean leaves were divided into two samples (fresh wt. 305.6 and 305.3 mg.) by taking one leaf from each pair. One sample was illuminated for 10 min. in a closed Petri dish containing a shallow dish of lactic acid. A solution (0.4 ml.) of $\text{Na}_2^{14}\text{CO}_3$ (2.25 mg., 500 μC) was then injected through a rubber cap in the lid into the acid, thus generating $^{14}\text{CO}_2$. After illumination for a further 10 min. the leaves were killed and extracted (see Materials and Methods section). The second sample of leaves was treated in exactly the same way except that it was kept in the dark both before and after the addition of the $\text{Na}_2^{14}\text{CO}_3$. Tr., trace.

Labelled compound	Radioactivity (counts/min.)	
	Light	Dark
Aspartate	768	252
Alanine	243	41
Citrate	304	237
Fumarate	101	—
Glutamate	2 015	44
Glutamine	662	—
Glycine	16 317	—
Glycerate	37 660	209
Glycollate	1 458	83
Malate	2 605	188
Phosphate esters	15 336	258
Serine	16 519	155
Succinate	1 031	Tr.
Sucrose	201 000	—

Feeding experiments with Krebs-cycle and associated intermediates

^{14}C -Labelled citrate, glutamate, succinate, fumarate and malate. Table 3 shows the distribution of radioactivity amongst a number of compounds after these radioactive metabolites had been fed to excised mung-bean leaves in the light and in the dark. Radioactivity spread from each of these metabolites to the other major intermediates of the Krebs cycle both in the light and in the dark. In part this spread of radioactivity must be attributed to release and refixation of $^{14}\text{CO}_2$. Dark fixation of CO_2 (see Table 1) occurs readily in mung-bean leaves but at a rate which is probably never more than a very small fraction of that in the light. Refixation of $^{14}\text{CO}_2$ in the light would largely account for the appearance of radioactivity in sucrose, sugar phosphates, glycine, serine and other compounds that are typical of photosynthesis. It is notable that this occurred most markedly when the metabolite fed was a compound, such as [4- ^{14}C]-malate, which would give rise to $^{14}\text{CO}_2$ most readily. If the amount of label in sucrose, sugar phosphates, etc., in these instances is accepted as an indication of the maximum extent of refixation then it can be concluded that refixation in these experiments was relatively small. Even had it been considerably larger these results would still clearly indicate the operation of the Krebs cycle in the light as well as in the dark. (It should perhaps be emphasized that in the experiments detailed in Table 3 we sought only to determine the effect of light on the distribu-

Table 2. *Changes in labelling in light and in the dark after dark fixation of $^{14}\text{CO}_2$*

Mung-bean leaves (15) were exposed to $^{14}\text{CO}_2$ in the dark, in a closed Petri dish (diam. 9 cm.) by injecting 0.8 ml. of lactic acid into $\text{Ba}^{14}\text{CO}_3$ (approx. 0.75 mg., 75 μC). After 2 hr. the $^{14}\text{CO}_2$ was blown off in an air stream (for 15 min.) and five leaves were killed and extracted. The remainder were illuminated in air for a further 30 min. and a further sample was taken. The remaining five leaves were returned to the dark for 30 min., at the end of which time the last sample was taken and extracted. Tr., trace.

Labelled compound	Radioactivity (counts/min.)		
	Dark (2 hr. 15 min.)	Dark (2 hr. 15 min.) followed by light (30 min.)	
		Dark (2 hr. 15 min.) followed by light (30 min.)	Dark (2 hr. 15 min.) followed by light (30 min.)
Arginine (?)	990	986	942
Aspartate	2351	415	1023
Alanine	134	203	406
Citrate	276	86	306
Citrulline	Tr.	Tr.	Tr.
Fumarate	734	186	942
Glutamate	1644	1224	1879
Glutamine	Tr.	272	274
Glycine and serine	—	1215	—
Glycerate	—	191	140
Glycollate	—	140	311
Malate	738	637	910
Phosphate esters	Tr.	1431	767
Serine	744	—	844
Succinate	Tr.	Tr.	88
Sucrose	—	1117	2080

tion of radioactivity between the various compounds listed. Individual figures in adjoining columns in Table 3 are not strictly comparable because it was not possible to ensure that the labelled intermediate fed to the leaves was taken up completely, or at precisely the same rate, by both samples of leaves.)

In examining the radioautographs of two-dimensional chromatograms of leaf extracts (see Materials and Methods section) obtained in these experiments the most striking quantitative difference observed was in the distribution of label

between malate and aspartate (see Table 3, values in bold type). In the dark the amount of radioactivity in malate was consistently low (relative to aspartate). In the light the activity in malate was greater and that in aspartate was less so that the position was reversed. This relationship was so marked that it could be detected even in experiments in which labelled malate and labelled aspartate (see next section) were fed and where the figures must necessarily include some proportion of these compounds which would be taken up by the leaves but not metabolized. The same relationship

Table 3. *Distribution of radioactivity after feeding of labelled metabolites to mung-bean leaves in the light and in the dark*

In experiments A to E the labelled intermediate was fed individually to five pairs of leaves, through the petiole, for 4 hr.: 30–40% was taken up in the dark and 40–50% in the light. The intermediates fed were (A) 3.75 μC of [4- ^{14}C]aspartate, 22 $\mu\text{C}/\mu\text{mole}$; (B) 4 μC of uniformly labelled ammonium [^{14}C]glutamate, 3.84 $\mu\text{C}/\mu\text{mole}$; (C) 3.75 μC of [4- $^{14}\text{C}_2$]malate, 22 $\mu\text{C}/\mu\text{mole}$; (D) 3.75 μC of [2,3- $^{14}\text{C}_2$]fumarate, 1.6 $\mu\text{C}/\mu\text{mole}$; (E) 3.75 μC of [2,3- $^{14}\text{C}_2$]succinate, 13.9 $\mu\text{C}/\mu\text{mole}$. In experiment (F) the [4- ^{14}C]oxaloacetate (approx. 10 μC at approx. 4 $\mu\text{C}/\mu\text{mole}$) was fed through the cut ends of five excised leaves for 30 min.: 10–15% was taken up in the dark and 15–20% in the light. In experiments G to I the labelled intermediates were fed to five excised leaves for 2 hr. with 90–100% uptake in the light and in the dark: (G) 1 μC of ammonium [4- ^{14}C]aspartate, 22 $\mu\text{C}/\mu\text{mole}$; (H) 6 μC of [2,3- $^{14}\text{C}_2$]fumarate, 1.6 $\mu\text{C}/\mu\text{mole}$; (I) 6 μC of [1,5- $^{14}\text{C}_2$]citrate, 2.6 $\mu\text{C}/\mu\text{mole}$. Tr., trace.

		Radioactivity (counts/min.)									
Metabolite fed	...	(A) Aspartate		(B) Glutamate		(C) Malate		(D) Fumarate		(E) Succinic	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
Labelled compounds											
Aspartate		1304*	2763*	552	2395	934	2988	344	2117	196	1406
Alanine		Tr.	—	Tr.	—	Tr.	Tr.	Tr.	Tr.	Tr.	Tr.
Citrate		199	249	48	318	201	390	153	361	86	306
Fumarate		Tr.	Tr.	Tr.	Tr.	Tr.	Tr.	23451*	Lost	138	Tr.
Glutamate		510	631	8749*	6014*	570	801	953	1420	357	1259
Glutamine		2038	535	471	165	535	611	155	190	155	Tr.
Glycine and serine		211	—	—	—	Tr.	—	Tr.	—	—	—
Glycerate		—	—	195	Tr.	—	—	Tr.	—	—	—
Glycollate		—	—	Tr.	Tr.	—	—	—	—	—	194
Malate		2880	791	2182	879	2185*	1614*	1532	727	671	268
Oxoglutarate		—	—	Tr.	Tr.	Tr.	—	Tr.	Tr.	Tr.	Tr.
Phosphate esters		Tr.	Tr.	Tr.	Tr.	—	—	Tr.	—	—	—
Succinate		342	Tr.	1005	505	—	—	Tr.	260	4253*	2120*
Sucrose		3233	—	498	—	1892	—	317	Tr.	210	Tr.
Threonine		Tr.	—	Tr.	—	Tr.	—	Tr.	Tr.	Tr.	Tr.

Radioactivity (counts/min.)

		Radioactivity (counts/min.)							
Metabolite fed	...	(F) Oxaloacetate		(G) Aspartate		(H) Fumarate		(I) Citrate	
		Light	Dark	Light	Dark	Light	Dark	Light	Dark
Labelled compounds									
Aspartate		268	740	502*	1048*	236	1841	66	266
Alanine		Tr.	—	Tr.	Tr.	156	192	Tr.	69
Citrate		142	119	Tr.	124	Tr.	180	716*	900*
Fumarate		—	—	Tr.	—	8062*	6687*	165	214
Glutamate		122	134	99	129	487	582	3760	1440
Glutamine		Tr.	—	221	182	51	133	101	54
Glycine and serine		207	—	Tr.	—	Tr.	—	173	—
Glycerate		142	—	Tr.	Tr.	Tr.	Tr.	78	—
Glycollate		Tr.	—	—	—	—	—	59	Tr.
Malate		1481	295	469	75	405	220	164	83
Oxoglutarate		—	—	—	—	Tr.	Tr.	—	—
Phosphate esters		Tr.	—	Tr.	—	Tr.	Tr.	Tr.	—
Succinate		Tr.	Tr.	—	—	119	165	212	244
Sucrose		155	—	395	Tr.	76	Tr.	398	—
Threonine		—	—	Tr.	Tr.	Tr.	Tr.	Tr.	—

* These values are for radioactive metabolites fed to leaves and recovered unchanged.

(Table 4) was observed when $[2,3-^{14}\text{C}]$ succinate was fed to leaves of two other species chosen at random. It is also implicit in results that Naylor, Rabson & Tolbert (1958) obtained when they fed labelled aspartate to barley, wheat, tomato and lupin.

If an intermediate accumulates, it follows that its production must exceed its consumption. Accumulation may therefore be attributed to increased production, diminished consumption, or both. If it is accepted (see section on light and dark fixation of $^{14}\text{CO}_2$, and above) that refixation of $^{14}\text{CO}_2$ accounts for only a relatively small proportion of the label which finds its way into various intermediates then the accumulation of malate in the light (Tables 3 and 4) could be a direct consequence of retarded conversion of malate into oxaloacetate together with continued production of malate in the Krebs cycle. Unchanged production and retarded consumption are suggested particularly by the results obtained with $[2,3-^{14}\text{C}_2]$ fumarate. This intermediate would not yield $^{14}\text{CO}_2$ for refixation as readily as a carboxyl-labelled acid. Almost all the labelled malate formed from $[2,3-^{14}\text{C}_2]$ fumarate is therefore probably produced by the direct hydration catalysed by fumarase. It is difficult to visualize a direct accelerating effect of light on this reaction and the most likely alternative explanation is a retarding effect of light on malate consumption. Undoubtedly, however, the aspartate-feeding experiments (see below) imply that the conversion of aspartate into malate is enhanced in the light and diminished in the dark (cf. Naylor *et al.* 1958) and this must have an important bearing on the concentration of malate.

$[4-^{14}\text{C}]$ Aspartate. Radioactivity was detected in Krebs-cycle and other intermediates after excised

mung-bean leaves had been allowed to take up labelled aspartate in the light and in the dark. Table 3 shows that malate was the major product and that the conversion of aspartate into malate was greater in the light than in the dark. These results are in accord with earlier findings (Naylor *et al.* 1958). Normal or exchange transamination (Hiller & Walker, 1961) could account for the conversion of aspartate into oxaloacetate, which could then be reduced to malate. Oxaloacetate readily undergoes β -decarboxylation (loss of C-4) and the relatively high degree of refixation of $^{14}\text{CO}_2$ in the light which was observed is therefore consistent with the view that oxaloacetate is an intermediate in the conversion of aspartate into malate. Further support for this view was provided by experiments in which oxaloacetate was fed to leaves (see below).

$[4-^{14}\text{C}]$ Oxaloacetate. In a typical feeding experiment labelled oxaloacetate gave malate (72%) rather than aspartate (28%) in the light and aspartate (85%) rather than malate (15%) in the dark (Table 3). The reactions involved are most probably those catalysed by malic dehydrogenase and glutamic-aspartic transaminase. If this is so these figures would represent percentage reduction or percentage transamination. The proportions of malate and aspartate formed in such a system would be largely governed by the amount of NADH_2 available. This result would then indicate a higher amount of NADH_2 in the light than in the dark.

Experiments with isolated mitochondria

Pyruvate oxidation by *Ricinus* mitochondria is known to be limited if CoA and cocarboxylase are omitted from the reaction media (Walker & Beevers, 1956). Experiments were carried out with these mitochondria in order to determine the effects of these omissions on the ratio labelled malate/labelled aspartate. To a 15 ml. flask were added: potassium malate, $8.7 \mu\text{moles}$, 0.1 ml. ; potassium aspartate, $8.7 \mu\text{moles}$, 0.1 ml. ; $[2,3-^{14}\text{C}_2]$ -fumaric acid, $2 \mu\text{C}$, $1.24 \mu\text{moles}$, 0.1 ml. ; NAD, 0.1 mg. , 0.025 ml. ; ATP, 0.1 mg. , 0.025 ml. ; *Ricinus* mitochondria, 0.6 ml. ; final volume was 1 ml. Two controls were also prepared, containing, in addition to the above-named reactants, CoA (0.01 mg.) and cocarboxylase (0.05 mg.). All three flasks were incubated at 28° but one of the controls was strongly illuminated and the other kept in the dark. After 30 min. the reactions were stopped by the addition of boiling ethanol (4 vol.). Chromatography and counting showed a marked diminution in the citrate and glutamate formed (50% of that in the controls) in the reaction mixtures from which the CoA and cocarboxylase were omitted. The ratio of labelled aspartate (6625 counts/min., 66%) to labelled malate (3366 counts/min., 34%) was

Table 4. *Distribution of radioactivity between various intermediates in the leaves of two species after feeding with $[2,3-^{14}\text{C}_2]$ succinate in the light and in the dark*

	Radioactivity(counts/min.)			
	<i>Chenopodium bonus henricus</i>		<i>Vicia sepium</i>	
	Light	Dark	Light	Dark
Aspartate	290	505	79	70
Alanine	22	57	40	Tr.
Citrate	41	39	Tr.	Tr.
Fumarate	Tr.	Tr.	Tr.	Tr.
Glutamate	803	1132	327	192
Glutamine	283	327	92	50
Glycine and serine	Tr.	Tr.	—	—
Glycerate	Tr.	—	Tr.	—
Glycollate	Tr.	Tr.	—	—
Malate	1238	148	259	51
Oxoglutarate	—	—	—	—
Phosphate esters	—	—	—	—
Serine	—	—	—	—
Succinate	6438	8952	3911	8696
Sucrose	334	—	231	—
Threonine	—	—	Tr.	—

unchanged, however, when compared with the dark control (aspartate 5698 counts/min., 66%; malate 2970 counts/min., 34%). The corresponding figures for the illuminated control were also similar (aspartate, 5676 counts/min., 69%; malate 2500 counts/min., 31%).

A marked effect on the ratio of labelled aspartate to labelled malate was produced by exposure to anaerobic conditions. A reaction mixture was prepared to contain three times the contents of the controls described above, and in addition approx. 0.5 mg. of NADH₂. A sample (1 ml.) was taken (and added to boiling ethanol) after incubation for 30 min. under N₂ (in the dark at 28°). At this time the N₂ was flushed out with air and the incubation prolonged for a further 30 min., when a further sample (1 ml.) was taken. After chromatography the ratio aspartate/malate in the first sample (taken after 30 min. in N₂) was 15:85 (1014 counts/min.: 5767 counts/min.). In the second sample (taken after a further 30 min. in air) the ratio was 65:35 (6265 counts/min.: 3441 counts/min.).

DISCUSSION

We have been able to confirm with leaves of *Phaseolus mungo* that ¹⁴CO₂ fixed in photosynthesis passes to glutamate and citrate more readily in the dark than in the light. The effect was less pronounced than in algae but the algae used in the first experiments (Calvin, 1949) were relatively deficient in nitrogen and this can influence the spread of radioactivity to the Krebs cycle (Holm-Hansen, Nishida, Moses & Calvin, 1959). The effect of light on the subsequent utilization of photosynthetic products has been attributed to an inhibition of pyruvate oxidation (Calvin & Massini, 1952). Oxidized lipoic acid is needed for the enzymic oxidation of pyruvate and in the light this was thought to be held in the reduced state either by a direct transfer of electrons from chlorophyll or by a coenzyme-mediated photoreduction (Calvin, 1956). Both NAD and NADP can be reduced by chloroplasts in the light (Das & Crane, 1961), but if these were the coenzymes involved we might expect reactions other than pyruvate oxidation to be affected, particularly those in which the equilibrium position does not favour oxidation. The equilibrium position of the reaction catalysed by malic dehydrogenase is so very unfavourable ($K = [\text{oxaloacetate}][\text{NADH}_2]/[\text{L-malate}][\text{NAD}]$

$$= 2.3 \times 10^{-5};$$

Stern, Ochoa & Lynen, 1952) that an effect of light on the conversion of malate into oxaloacetate would seem to be inevitable if photoreduction were sufficiently active to maintain much of the coenzyme in the reduced condition. Our findings provide experimental evidence for such an effect. For example, in feeding experiments, a greater

proportion of oxaloacetate is reduced in the light than in the dark. This implies a balance of reduced to oxidized NAD in the light such that oxaloacetate reduction is enhanced and malate oxidation is diminished. Such an increase in the ratio NADH₂/NAD in the light has been observed in *Chlorella* (Oh-hama & Miyachi, 1960).

The increase in the conversion of aspartate into malate in the light which we observed (cf. Naylor *et al.* 1958) could be explained in the same terms. Labelled aspartate can be converted into labelled malate either by normal or exchange transamination (Hiller & Walker, 1961), followed by a reduction which would be favoured by an increased amount of NADH₂. Similarly, citrate, glutamate, succinate and fumarate all give rise to more malate than aspartate in the light and to less malate than aspartate in the dark. However, our results might be explained, at least in part, by an effect of light on the oxidation of pyruvate. Thus if pyruvate oxidation were inhibited, oxaloacetate could not condense with acetyl-CoA and accumulating oxaloacetate might retard the reaction catalysed by malic dehydrogenase. In order to test this possibility we carried out experiments with mitochondria in which pyruvate oxidation is known to be limited if CoA and cocarboxylase are omitted from the reaction mixture (Walker & Beevers, 1956). The low ratio malate/aspartate, which is characteristic of leaves in the dark, was also observed in these experiments in which mitochondria were fed with radioactive fumarate in the presence of a full complement of cofactors. This ratio remained unchanged, however, when CoA and cocarboxylase were omitted, though a marked diminution in the citrate and glutamate formed (50% of that in the controls) indicated that the omission of these cofactors had achieved the expected effect of inhibiting pyruvate oxidation. In contrast we observed a marked increase in the ratio malate/aspartate when mitochondria were mixed with [¹⁴C]fumarate as before but were then incubated in nitrogen rather than air and a little NADH₂ was added at the outset. In experiments with mitochondria therefore we have been able to simulate the effect which we observed in leaves on passing from light to dark (or dark to light) by changing the oxidation status of NAD but not by inhibiting pyruvate oxidation.

On the basis of the present results we would therefore reinterpret Calvin's findings as follows. During short-term photosynthesis phosphoenolpyruvate would be carboxylated to yield oxaloacetate and a relatively high amount of NADH₂ produced by photoreduction (see Das & Crane, 1961; Oh-hama & Miyachi, 1960) would deflect this towards malate (Fig. 2B). Only when a new steady-state equilibrium had been established would the spread of radioactivity into the Krebs

cycle, beyond malate into citrate and glutamate, reach its maximum rate. In the dark a lower amount of NADH_2 would permit the conversion of malate into oxaloacetate or the direct utilization in the Krebs cycle of oxaloacetate produced by carboxylation (Fig. 2A). This would resolve the apparently contradictory findings that citrate production is slowed in the light whereas lipid formation is enhanced. Such an explanation would not require an effect of light on the oxidation status of lipoic acid or for the production of acetyl-CoA from some source other than pyruvate.

If the integrated reactions of the Krebs cycle are restricted to the mitochondria and if succinic oxidase is specifically absent from the chloroplast then many of our results would call for a fairly rapid two-way movement of substrates or co-enzymes between mitochondrion and chloroplast. The distribution of label between malate and aspartate is the same in feeding experiments with $[2,3-^{14}\text{C}]$ succinate as it is in experiments with other metabolites. If this distribution is controlled by the oxidation status of NAD (as we suggest), then it would follow that metabolites derived from succinate must migrate from the mitochondrion to the chloroplast or that coenzymes must migrate from the chloroplast to the mitochondrion. Our data do not at present permit us to distinguish between these possibilities.

Our results also support the view (for a recent review see Hackett, 1959) that the Krebs cycle occurs in green leaves in the light as well as in the dark.

Fig. 2. Major changes in movement of ^{14}C between Krebs-cycle and associated intermediates in passing (A) from light to the dark and (B) from the dark to light.

SUMMARY

1. Evidence is presented that the Krebs cycle occurs in green leaves in the light as well as in the dark.

2. When leaves are fed with various labelled metabolites (including glutamate, aspartate, the major Krebs-cycle intermediates and carbon dioxide) the ratio labelled malate/labelled aspartate is high in the light and low in the dark.

3. When leaves are fed with labelled oxaloacetate more of this intermediate is reduced to malate in the light than in the dark.

4. A high ratio of labelled malate/labelled aspartate can be induced in mitochondria by exposure to reducing conditions but not by inhibition of pyruvate oxidation.

5. These findings provide an alternative explanation to the postulated light-inhibition of pyruvate oxidation in green plants.

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