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ABSTRACT

Using radioactive carbon dioxide, an attempt has been made to distinguish the various pools of intermediary metabolism which may be physically or chemically separate within the cell. Some correlation between the structural elements of the cells and these pools appears possible.

THE RELATIONSHIP BETWEEN THE METABOLIC POOLS OF PHOTOSYNTHETIC AND RESPIRATORY INTERMEDIATES*

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INTRODUCTION

The question of the interrelations at a biochemical level of the processes going on in respiration and photosynthesis has long been of interest. Almost every compound lying in the pathways of the carbon reduction cycle also plays a part in one of the two main routes of respiratory breakdown of sugars: glycolysis and the pentose phosphate cycle. Indeed, part of the carbon reduction cycle involves reactions which are the reverse of those taking place in glycolysis, while the transketolase reactions in the pentose phosphate cycle are the same as those operating in the photosynthetic cycle in the opposite direction.

Once the detailed biochemical reactions included in these cyclic operations became known, the question immediately arose as to what influence these processes had upon each other, and whether identical metabolic reservoirs of the intermediates in the same cell were involved in reactions apparently proceeding to a

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physiologically significant extent in both directions. Brown[1] was able to show that respiration as measured by oxygen absorption could proceed uninterruptedly in green plants in the light, together with photosynthesis. Benson and Calvin[2] found that ^{14}C fixed during short periods of continuous photosynthesis by algae from $^{14}CO_2$ entered many of the phosphorylated intermediates also common to respiration, but was not found in certain compounds of the tricarboxylic acid cycle, such as citric and glutamic acids. Ryther,[3] using a marine flagellate uniformly labelled with ^{14}C, measured the loss of labelled carbon from the cells when they were placed in a ^{12}C environment in the light and dark. After 24 hours, cells in the dark had lost about 20% of their ^{14}C, but cells in the light had lost none. He concluded that respiratory carbon was used in preference to external carbon dioxide for photosynthesis, but was unable to come to any conclusions as to the form in which the carbon is returned to the photosynthetic cycle.

Weigl, Warrington and Calvin,[4] approaching closer to the problem of single or separate reservoirs, found that ^{14}C recently incorporated photosynthetically did not serve as a substrate for respiration in the light, but was evolved as $^{14}CO_2$ when the light was turned off after a short period of photosynthesis (40 min.). After a sufficiently long period in the light (about 2 hours), there was no evolution of $^{14}CO_2$ in the subsequent period of darkness. They concluded that the evolution of $^{14}CO_2$ in the dark after a short light period was due to respiration of labelled photosynthetic intermediates, a process in some way inhibited in the light. However, after longer periods in the light, the cells having exhausted the external supply of carbon dioxide long before the light was extinguished, the incorporated ^{14}C would be found in stable storage materials. Respiration used other (unlabelled) storage materials as substrates, but whether these differed from photosynthetically formed reserve substances in chemical

composition, or solely in physical location, could not be decided.

Direct information as to the identity of reservoirs is difficult to obtain, and in the present communication work, is described using several indirect approaches. None of these alone is conclusive, but taken together they provide evidence strongly suggestive of a physical separation of metabolic pools of chemically identical substances participating in both respiration and photosynthesis in the Chlorella.

METHODS

The green alga, Chlorella pyrenoidosa, was grown for experimental purposes as described earlier.[5] For feeding experiments with labelled glucose or acetate, 1 ml. aliquots of cell suspension in distilled water (containing 50 µl. of wet-packed cells) were pre-adapted for 30 min. in the light or dark with a stream of air containing 1% CO_2, and then incubated for 3 min. in the light or dark with one or more of the following substrates: $NaH^{14}CO_3$ (20 µC.; 1.35 µmoles); glucose-^{14}C (33 µC.; 0.75 µmoles), or an equal quantity of unlabelled glucose; $^{14}CH_3.COONa$ (43 µC.; 21 µmoles), or an equal quantity of unlabelled acetate. Except when $NaH^{14}CO_3$ was used as a substrate, the cell suspensions were flushed during the incubation period with air containing 1% CO_2. At the end of the incubation period the cells were killed by the addition of 4 vols. of ethanol at room temperature. For experiments on the feeding of labelled acetate, the cells were starved before use by maintaining them for 24 hr. in the dark in nutrient solution while they were flushed with air containing 4% CO_2. The techniques used for the exposure of Chlorella to labelled substrates, both in the light and in the dark, are described by Moses and Calvin.[6]

In "steady state" experiments, 50 ml. of cell suspension in a modified nutrient solution (containing 250 µl. of wet-packed cells) were placed in an apparatus modified from that described by Bassham et al.[7] and by Wilson and Calvin.[8] This was a closed system in which the atmosphere was recirculated and bubbled through the algal suspension which was exposed to light from two RS-P-2 spotlights on either side of a "lollipop"; the light intensity was just sufficient to saturate photosynthesis. Continuous automatic measurements were made of the O_2 and CO_2 concentrations and the radioactivity level in the circulating atmosphere, as well as of the pH and temperature of the algal suspension. The medium contained (g./l.): KNO_3, 0.75; $MgSO_4.7H_2O$, 0.10; KH_2PO_4, 0.05; plus the necessary trace elements; pH 6.6-6.9. After flushing the cell suspension with air containing 1.5% CO_2 for 30 min., the system was closed and $^{14}CO_2$ released into the gas space (6 mC.; final specific activity, 2 µC./µmole). Medium was added periodically to keep the cell suspension at a constant density as measured photoelectrically and a volume of cell suspension equal to that of the added medium was withdrawn. The rates of gas exchange during photosynthesis were 36-39 µmoles of O_2 evolved/min./ml. of wet-packed cells, and 34-39 µmoles of CO_2 utilized/min./ml. of wet-packed cells. Samples (1.5 ml.) of the suspension were taken 1.5 hr. and 3 hr. after the addition of $^{14}CO_2$, the cells killed with methanol at room temperature, and subsequently analyzed.

The procedures for the extraction of the cells, chromatography, radioautography, spot identifications, and radioactivity determinations, have been described elsewhere.[5,10]

RESULTS

Feeding experiments with labelled glucose and acetate.

Chlorella was exposed, both in the light and dark, to three substrate

combinations: (i) glucose-^{14}C plus $^{12}CO_2$; (ii) glucose-^{12}C plus $^{14}CO_2$; (iii) $^{14}CO_2$ alone. The patterns of ^{14}C distribution in most of the compounds in each extract are shown in Table I. As the fixation of $^{14}CO_2$ in the dark, with and without glucose, was very low, and label appeared only in substances of the tricarboxylic acid cycle,[11] these values have not been included in Table I.

With acetate feeding, the following substrate combinations and light conditions were employed: (i) $^{14}CH_3.COONa$ plus $^{12}CO_2$ in the dark; (ii) same as (i), but in the light; (iii) $^{12}CH_3.COONa$ plus $^{14}CO_2$ in the light; (iv) $^{14}CO_2$ in the light. Table II shows the distribution of activity in various substances from each of these combinations.

Steady-state experiment.

From a knowledge of the specific activity of the external $^{14}CO_2$ supplied to the cells, it is possible to determine the pool sizes of various compounds in the cells by assuming that these substances have the same specific radioactivity in their carbon atoms as the input $^{14}CO_2$. It is known that the photosynthetic intermediates rapidly acquire the same specific activity as the substrate carbon dioxide.[12] Thus, in the first sample, taken after 1.5 hr., the sizes of the photosynthetic reservoirs (and of any other pools rapidly incorporating labelled carbon) can be determined from the amounts of ^{14}C in them. If the cells contain any other pools of these substances, not in rapid equilibrium with the photosynthetic pools, it would be expected that ^{14}C would enter these other reservoirs more slowly than those of the photosynthetic intermediates. For this reason, the total pools of any such substances measured on the basis of their ^{14}C content, should show a rise in absolute concentration until all the

pools have the same specific activities as the incoming $^{14}CO_2$. Table III shows the apparent concentrations of several substances in the cells, as determined by the quantities of ^{14}C incorporated into them after 1.5 hr. and 3 hr., respectively. As already mentioned, a known volume of medium was added from time to time to the algal suspension, bringing the density (as measured by light absorption) back to the starting level, and an equal volume of cell suspension was removed, thus maintaining the suspension volume at a constant level. However, calculation of the algal growth during the course of the experiment from the rates of CO_2 uptake indicated that, on an increase in weight basis, there was a net increase in algal cell volume. A correction was made for this growth in calculating the apparent concentrations of photosynthetic pools. In calculating the increase in cell volume it was assumed that, (i) specific gravity of the average cell remained constant, (ii) each millimole of CO_2 incorporated resulted in an increase in dry weight of 30 mg. (eq. to CH_2O) and (iii) wet weight equaled five times dry weight. The validity of this calculation is indicated by the fact that rates of CO_2 uptake and O_2 evolution per unit volume of algal cells as determined by this calculation remained constant within 10% during the course of the experiment. It will be noted that without such a correction for growth, the increase in calculated apparent concentrations of metabolic pools would have been greater than the increases shown in Table III.

DISCUSSION

Glucose Feeding.

The pattern of ^{14}C incorporation from labelled glucose in the dark indicates that glucose is metabolized via glycolysis, since label appeared in several glycolytic intermediates (glucose and fructose monophosphates, phosphoglyceric acid, and

fructose diphosphate), and probably also by the pentose phosphate cycle as witnessed by the presence of ^{14}C in sedoheptulose and pentose phosphates. The one compound which plays a role in the photosynthetic carbon cycle but has no part in respiration, ribulose diphosphate, incorporated no radiocarbon from glucose in the dark. Previous studies[7] showed that the concentration of ribulose diphosphate in the photosynthetic pool goes to zero in the dark in Scenedesmus. Tricarboxylic acid cycle activity was also indicated by the presence of ^{14}C in malic and glutamic acids.

If the pools of photosynthetic and respiratory intermediates were not in equilibrium, no label should have been incorporated into ribulose diphosphate in the light unless $^{14}CO_2$ produced by oxidation of glucose were refixed photosynthetically, or unless some glucose itself can reach and be incorporated into the photosynthetic pool. Experimentally, it was found that ribulose diphosphate was labelled in the light when glucose-^{14}C was the substrate (Table I), but it must be borne in mind that respiratory carbon is used in preference to an external source of this substance[3].

A more definite conclusion regarding the separation of the pools may be derived from a consideration of the distribution patterns of ^{14}C from glucose-^{14}C in the light and dark, compared with that from $^{14}CO_2$ in the light. The Q_{O_2} for oxygen evolved during photosynthesis is about 255 (36-39 µmoles of oxygen evolved/min./ml. of wet-packed cells; 1 ml. of wet-packed cells contained about 200 mg. of dry cell material). Millbank[13] has shown that the Q_{O_2} for the aerobic oxidation of glucose by Chlorella vulgaris is 12-13; hence photosynthesis proceeded about 20-21 times faster than respiration.

There was an increase in the percentage ^{14}C fixed from glucose-^{14}C into the sum of the diphosphates in the light compared with the dark, and although label appeared in ribulose diphosphate, the percent activity in fructose diphosphate also

increased some twenty times. That fraction of ^{14}C in the diphosphates which appeared in ribulose diphosphate was much lower when glucose-^{14}C was the substrate in the light (37%), than the typical values for photosynthesis (64-68%). From all these considerations it appears that the ^{14}C found in ribulose diphosphate arrived there by refixation of respiratory $^{14}CO_2$. Thus it is easily possible to account for the amount of labelled ribulose diphosphate (0.4/10 = 1/25) from labelled glucose compared with the ribulose diphosphate derived from labelled carbon dioxide, in terms of the reincorporation of labelled carbon dioxide derived from labelled carbon dioxide, in terms of the reincorporation of labelled carbon dioxide derived from glucose. One would expect that were the photosynthetic and respiratory pools in equilibrium, in the light, virtually the whole of the distribution pattern of ^{14}C should have been typical of photosynthesis. Examination of the data in Table I, however, shows that only a few compounds were affected by switching on the light when glucose-^{14}C was the substrate. The most outstanding effect of light was the rise of the percent of ^{14}C in the sugar monophosphates and the fall of that in sucrose. This may be accounted for by a relative lack of adenosine triphosphate necessary for the conversion of the sugar monophosphates to sucrose (via uridinediphosphoglucose), because of the rapid utilization of the nucleotide in photosynthesis. This might also explain the reduced total utilization of glucose-^{14}C in the light, as the initial step of glucose incorporation also involves a phosphorylation. Although the <u>percentage</u> of ^{14}C found in uridinediphosphoglucose was the same in the light and dark (Table I), the absolute amount was smaller by a factor of about 3.3 in the light in accordance with a corresponding reduction in the total ^{14}C incorporated in the light compared with the dark.

Perhaps most significantly, ^{14}C was found in glutamic acid from glucose-^{14}C even in the light, but not from $^{14}CO_2$. Thus, glucose carbon, even though it passed

through either glycolysis or the pentose phosphate cycle, and hence through compounds also present in the carbon reduction cycle, was able to enter glutamic acid in three minutes, whereas carbon fixed from $^{14}CO_2$ into these same intermediates by photosynthesis was not able to pass into glutamic acid. With photosynthesis proceeding some twenty times faster than respiration, the addition of unlabelled glucose to cells carrying on photosynthesis with $^{14}CO_2$ had, as may be expected, no significant influence on the ^{14}C uptake and distribution.

Acetate feeding. The picture of events obtained from the experiment with the feeding of labelled acetate is in some ways simpler to interpret. Very little label appeared in the phosphorylated compounds from acetate-2-^{14}C in the light, and none in the dark. It therefore seems likely that only a little of the methyl-carbon of acetate was oxidized to carbon dioxide, and that the small labelling in phosphorylated compounds which was actually seen in the light was due to the refixation of this respiratory $^{14}CO_2$. In the tricarboxylic acid cycle, the methyl-carbon of acetate is not released as carbon dioxide during the first turn of the cycle. The pattern of labelling in the intermediates and amino acids of this cycle from acetate-2-^{14}C in both light and dark indicates that very little acetate carbon travels all the way round the cycle as little or no activity was detected in fumaric and malic acids (Table II).

The results with acetate confirm some of the conclusions from glucose studies. In particular, ^{14}C entered glutamic acid in the light from both substrates (but not from $^{14}CO_2$), demonstrating that the formation of this compound may proceed in the light. Other studies on the photosynthetic incorporation of tritium from tritium oxide[6], and the distribution of $^{14}CO_2$ in the presence of ammonia[14], have also shown the formation of glutamic acid in the light, yet cells suspended in distilled water as in the present investigation formed none from $^{14}CO_2$ in three minutes, as was earlier

found by Benson and Calvin[2]. Hence, carbon from photosynthetic intermediates is inhibited from entering tricarboxylic acid cycle substances by light in the absence of ammonia although other carbon in the same chemical compounds is not restricted in this way.

The incorporation of ^{14}C into aspartic acid from acetate-2-^{14}C in the presence of $^{12}CO_2$ was supressed in the light as compared with the dark. The incorporation of ^{14}C into aspartic acid from $^{14}CO_2$ in the light was supressed by the presence of unlabelled acetate. These two effects suggest that a precursor of aspartic acid, malate or oxalacetate, may be synthesized both from acetate (via the glyoxalate cycle[15]) and from photosynthetically incorporated CO_2 (via carboxylation of pyruvate or phosphoenolpyruvate) (Fig. I). In each case, the specific activity of the four-carbon acid would be reduced due to the synthesis from the unlabelled substrate. Consequently, aspartic acid derived from the four-carbon precursor, would have a lower specific activity and consequently a lower total activity, provided that its rate of formation were not increased due to an increased concentration of its four-carbon acid precursor. The reason that the four-carbon acid precursor foes not markedly increase in concentration in any case where acetate is present is that the rate of the condensation reaction between four-carbon acid and acetate is accelerated by acetate, as is seen in the increased labelling of citric acid and glutamic acid in light and $^{14}CO_2$ (in the presence of acetate.). Further indication of the synthesis of malate from acetate via the glyoxylate cycle is to be seen in the labelling of glycollic acid when acetate-2-^{14}C was present.

Steady-State experiments.

The evidence presented above for the existence of separate metabolic pools is further confirmed by the estimate of the pool sizes of three photosynthetic intermediates, and particularly phosphoglyceric acid, made on the basis

of the rapidity with which they incorporate ^{14}C photosynthetically from $^{14}CO_2$. Calvin and Massini[12] showed that in steady-state conditions the quantity of ^{14}C in phosphoglyceric acid rose rapidly for the first five minutes after the introduction of $^{14}CO_2$, and thereafter increased very slowly. This suggested that within five minutes photosynthetic phosphoglycerate had acquired the same specific activity as the external $^{14}CO_2$. The distribution of label in phosphoglyceric acid is known to be uniform after five minutes. However, the size of the labelled pool of this substance in algal cells increased two-fold between 1.5 hours and three hours after labelled carbon dioxide was added (Table III). Thus, there appears to be one or more other reservoirs of phosphoglycerate not in rapid equilibrium with the pool involved in the carbon reduction cycle, and one of these other reservoirs may well be on a respiratory pathway.

Recent work by Tolbert[15] has shown that phosphorylated intermediates are not released from chloroplasts (the site of photosynthetic activity) in the light. Investigations are currently in progress to see whether these various enzymatic activities are separately localized in different subcellular fractions.

TABLE I

Distribution of activity in various compounds after feeding Chlorella labelled glucose and carbon dioxide in different combinations in light and dark. For experimental details see text. Three min. exposure period to labelled substrates. The values given represent the percentage of the activities of the sum of all substances, which appeared in each compound. About 92% of the total soluble activity is accounted for.

Substrates	Glucose-^{14}C + $^{12}CO_2$	Glucose-^{14}C + $^{12}CO_2$	$^{14}CO_2$ + Glucose-^{12}C	$^{14}CO_2$
Light conditions	dark	light	light	light
Sugar diphosphates:				
glucose and sedoheptulose	0.2	1.1	1.8	1.8
fructose	0.04	0.8	1.0	0.6
ribulose	0.0	2.0	9.5	8.0
glyceric acid	0.0	0.2	0.6	0.6
glycollic acid	0.0	0.1	0.1	0.1
2-carboxy-4-ketopentitiol*	0.3	1.2	1.0	1.3
Sugar monophosphates:				
glucose	15.3	23.7	17.3	13.3
fructose	7.8	12.5	8.7	8.0
sedoheptulose	2.7	6.5	10.5	11.3
Pentose phosphate	0.7	1.9	1.1	1.5
Phosphoglyceric acid	6.7	6.7	18.9	26.1
Uridinediphosphoglucose	8.3	8.1	3.9	4.3
Malic acid	0.3	0.0	1.1	1.1
Glyceric acid	1.5	3.0	1.5	2.8
(Aspartic acid)**	(5.4)	(19.2)	(4.7)	(5.2)
Glutamic acid	1.8	3.0	0.0	0.0
Alanine	10.0	9.6	17.8	17.0
Sucrose	44.2	19.2	5.2	2.4
Percent of ribulose in diphosphates	0.0	36.9	68.0	64.4
Total ^{14}C fixed into soluble materials (dis./min./ml. cells x 10^{-6})	66.58	20.17	129.1	127.7

* Identity not definitely established.

TABLE I

**Aspartic acid chromatographed together with a radioactive contaminant in the labelled glucose, and is thus not included in the total; it is also omitted from the total in columns 3 and 4 to provide data comparable with columns 1 and 2.

TABLE II.

Distribution of activity in various compounds after feeding starved Chlorella labelled acetate and carbon dioxide in different combinations in the light and dark. For experimental details see text. Three minutes exposure period to labelled substrates. The values given represent the percentages of the total soluble fixed ^{14}C which appeared in each compound.

Substrates	Acetate-2-^{14}C + $^{12}CO_2$	Acetate-2-^{14}C + $^{12}CO_2$	$^{14}CO_2$ + Acetate-^{12}C	$^{14}CO_2$
Light conditions	dark	light	light	light
Sugar diphosphates	0.0	0.0	21.5	24.0
Sugar monophosphates	0.0	1.2	13.0	16.0
Phosphoglyceric acid	0.0	0.0	2.9	4.3
Phosphoenolpyruvic acid	0.0	0.0	0.5	0.6
Uridinediphosphoglucose	0.0	0.0	2.1	4.4
Phosphoglycollic acid	0.0	0.0	1.5	1.4
Malic acid	2.2	3.5	4.8	7.0
Citric acid	12.1	22.5	3.9	0.1
Fumaric acid	0.0	0.0	1.2	1.1
Succinic acid	11.7	11.7	0.0	0.0
Glyceric acid	4.9	4.8	2.1	0.9
Lactic acid*	3.0	1.4	0.0	0.0
Glycollic acid	4.8	5.5	0.0	0.0
Sucrose	0.0	3.0	21.9	10.8
Aspartic acid	10.6	1.7	4.0	7.3
Glutamic acid	50.4	42.1	3.0	0.3
Serine plus glycine	0.0	0.6	11.6	13.8
Alanine	0.5	1.2	5.9	8.0
Glutamine	0.0	0.7	0.0	0.0
Total ^{14}C fixed into soluble materials (dis./min./ml. cells x 10^{-6})	21.56	23.36	390.3	266.9

* Identity not confirmed by cochromatography

TABLE III.

Pool sizes of three compounds in Chlorella. Pool sizes given after continuous exposure of the alga to $^{14}CO_2$ in the light. Reservoir sizes determined on the basis of the incorporated ^{14}C into each compound; for explantion see text. Concentrations of each substance expressed as μmoles/ml. of wet-packed cells.

Exposure period to $^{14}CO_2$	Substance		
	Phosphoglyceric acid	Hexose and heptose monophosphates	Pentose monophosphates
1.5 hr.	3.0	9.5	0.8
3.0 hr.	6.4	11.4	0.7

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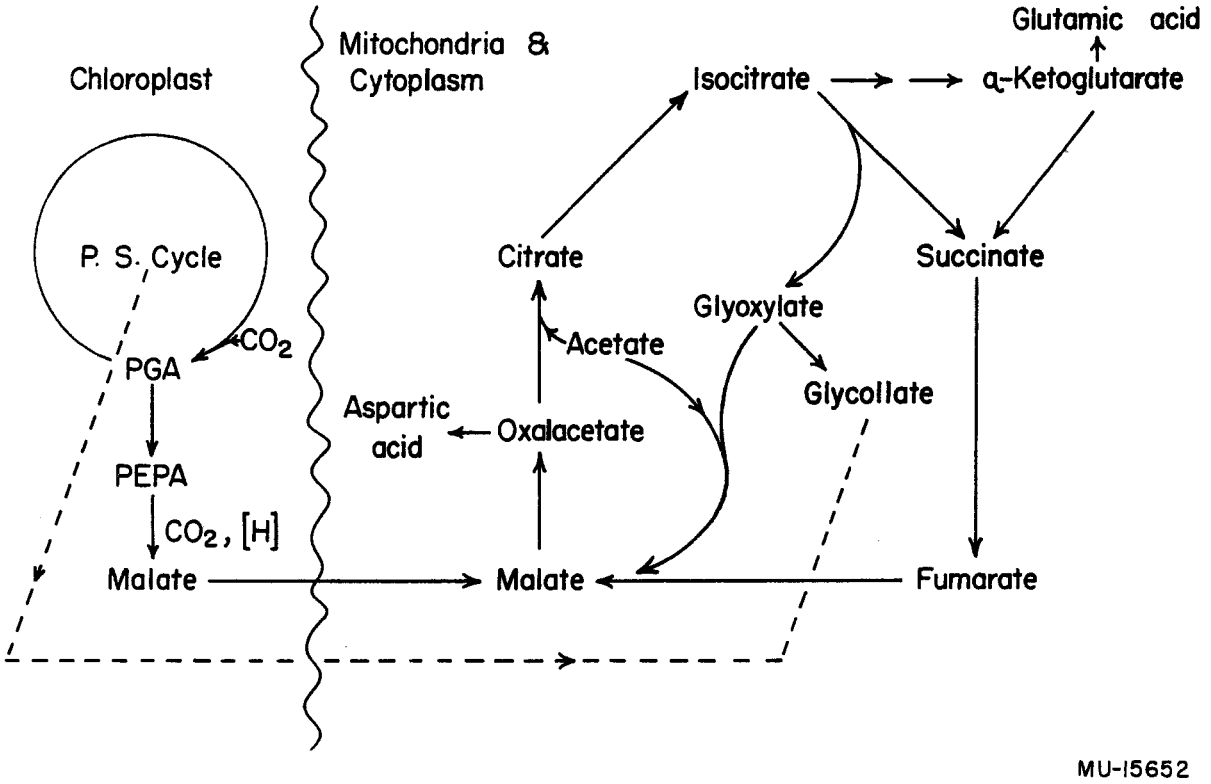

Fig. 1. Relationship between photosynthetic cycle, gloxylate cycle and tricarboxylic cycle.