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SUMMARY

Further kinetic studies have been made on the rates of appearance of C^{14} in individual compounds formed by Chlorella pyrenoidosa during steady state photosynthesis with $C^{14}O_2$. Total and "active" pools of several amino acids have been determined. The effects of adding unlabelled acetate and of turning off the light have been studied in this system.

The following results and conclusions were obtained:

1. From the rates of appearance of C^{14} in the compounds and from the size of the "active" pools, the rates of synthesis of alanine, serine, aspartic acid, glutamic acid, glutamine and several other amino acids have been calculated.

2. The total of the carbon fixed into these amino acids and into the carbamyl carbon of citrulline is about 30 % of the externally measured rate of uptake of CO_2 during steady state photosynthesis. If certain assumptions are made, this total carbon fixation into these amino acids is equivalent to about 60% of the externally measured rate of ammonium ion uptake during these experiments. From this it is concluded that the greater part of carbon used in protein synthesis passes through these amino acids as intermediates.

3. Comparison of "active" pools with measured total pools in the cases of alanine, serine, glutamic acid, and aspartic acid shows that the "active" pool is usually less than half the total pool, and thus indicates that there are at least two pools of these and probably of other amino acids.

4. The early attainment of maximum labelling rate of alanine and of aspartic acid as compared with the time of saturation of compounds of the carbon reduction cycle and of secondary intermediates of carbon fixation (such as sucrose) indicates that the "active" pools of these and probably some other amino acids are formed directly from intermediates of the carbon reduction cycle, without the intermediacy of secondary fixation products. Since the carbon reduction cycle intermediates are believed to be formed and utilized in the chloroplasts, it follows that the "active" pools and the site of

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Abbreviations: PGA or 3-PGA, 3-phosphoglyceric acid; PEPA, phosphoenolpyruvic acid; ATP, adenosine triphosphate; TPNH, reduced triphosphopyridine nucleotide.

synthesis of these amino acids are within the chloroplasts as well. The pools which are less actively turning over during photosynthesis are considered to be in the extra-chloroplastic space, or cytoplasm. The latter pools become labelled very slowly. Thus, during photosynthesis, the greater part of protein synthesis occurs in the chloroplasts.

5. Upon addition of unlabelled acetate to the algal suspension during photosynthesis, there is a rapid synthesis of unlabelled glutamic acid, but no effect on the rate of formation of labelled glutamic acid. This is taken as an indication that the added acetate is used in the synthesis of glutamic acid outside the space occupied by the active pools.

6. When the light is turned off, following a long period of photosynthesis with $C^{14}O_2$, there is first an increase in pools of unlabelled amino acids, followed by an increase in pools of labelled amino acids. A tentative explanation of this coupling effect is given.

7. New, and as yet hypothetical, pathways are proposed for the rather direct conversion of the carbon reduction cycle intermediate, 3-phosphoglyceric acid, to alanine, serine, aspartic acid, glutamic acid, and glutamine.

INTRODUCTION

Methods have been developed which permit comparison of externally measured rates of photosynthesis in Chlorella pyrenoidosa with the rates of flow through individual compounds in the biosynthetic pathways. These methods depend on the observation of appearance of radiocarbon in individual compounds as a function of time of exposure of the plant to $C^{14}O_2$ during steady state photosynthesis.[1] It was found that the carbon reduction cycle[2,3] and carboxyllation of phosphoenolpyruvic acid account for at least 73% and probably much more of the total carbon assimilation. In the present report we will describe measurements of the flow of carbon into several amino acids which are among the more important secondary intermediates in the photosynthesis of carbon compounds.

In the early studies of photosynthesis in algae with $C^{14}O_2$, alanine, aspartic acid, and several other amino acids were identified as being among the first compounds to become labelled.[4] These compounds, as well as malic acid, were slowly labelled even in the dark when the algae were exposed to $C^{14}O_2$. They were labelled much more rapidly if the algae were photosynthesizing or were in the light until the moment of addition of radiocarbon.[5] It was recognized that these compounds were products of photosynthetic reduction of $C^{14}O_2$, even though they could also be slowly labelled by reversible decarboxyllation reactions of respiration.

Accelerated incorporation of $C^{14}O_2$ into the amino acids of higher plants during photosynthesis has been noted in this laboratory[6,7] and in many others.[8,9,10,11] Some workers have suggested that certain amino acids such as glutamic acid[12] and aspartic acid[11] are primary products of CO_2 incorporation during photosynthesis.

Nichiporovitch[8] has presented and reviewed evidence that the synthesis of proteins in the chloroplasts of higher plants is greatly accelerated during photosynthesis. This accelerated protein synthesis appears to utilize intermediates of photosynthetic carbon reduction since the proteins were labelled when $C^{14}O_2$ was administered but not when C^{14}-labelled carbohydrates were supplied.[13] Sissakian[13] has reported and reviewed experiments that show that protein can be synthesized in isolated chloroplasts from non-protein nitrogen, including peptides.

Holm-Hansen[14] found that the incorporation of $C^{14}O_2$ into amino acids was considerably increased in algae when they were supplied with ammonia. This increase was very marked in the case of glutamic acid. It had previously been found that algae photosynthesizing in distilled water made very little labelled glutamic acid until the light was turned off[15,16], whereupon there was a large increase in the labelling of both glutamic acid and citric acid, but no notable increase in labelled alanine.

In the study reported here, we have sought answers to the following questions:

1. What are the rates of flow of carbon through amino acids during photosynthesis and what portion of the total carbon uptake can be ascribed to amino acid synthesis?

2. Is there more than one reservoir of each amino acid? If so, what are the concentrations of the reservoirs directly involved in photosynthesis?

3. What are the metabolic pathways leading from primary products of photosynthesis to these amino acids, and are any of these amino acids themselves primary products of carbon reduction?

4. What is the explanation of light-dark transient effects on the rates of synthesis of various amino acids?

EXPERIMENTAL

The experimental methods relating to maintenance of the algae (Chlorella pyrenoidosa) under steady state photosynthesis and the analysis of the labelling of compounds with C^{14} has been described in this journal.[1] The same methods have been used in these studies except as noted.

1. Nutrient solution. The nutrient solutions used earlier, while adequate for the purposes of those experiments, were insufficient to maintain the levels of amino acids and their rates of formation constant over several hours. We have therefore experimented with various nutrient solutions, testing for the maintenance of steady state photosynthesis as well as for the effect on paper chromatography. We have now found two solutions which appear to satisfy our requirements. One of these is a "starting" medium (Table I) which is used for suspension of the algae at the start of the experiment. The other is the "adding" medium (Table II) which is added automatically to the algal vessel as the pH of the suspension changes in response to mineral uptake by the algae.

In order to assure solution of all the elements in the "adding" medium it is necessary to dissolve the ammonium carbonate in about 80% of the water and to bubble a mixture of 2% CO_2 in air through it until a constant pH is obtained. All the other elements, except the $FeCl_3$, are dissolved in the remaining 20% of water. These two parts are then mixed and the proper amount of a concentrated solution of $FeCl_3$ is added. Even so, when the $FeCl_3$ solution is added to the rest of the "adding" medium, some cloudiness occurs. However, this precipitate does not settle out during the course of the experiment and it is presumed that the chemicals contained in the fine suspension are available to the algae once it is added to the more acidic algal solution.

Table I

STARTING MEDIUM

KH_2PO_4	0.20 mM
$MgSO_4 \cdot 7H_2O$	0.20 mM
$MgCl_2$	0.20 mM
K_2CO_3	0.10 mM
$Ca(NO_3)_2$	0.02 mM
KNO_3	0.05 mM
A solution of trace elements plus $CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3 (15 mg/l)	1 ml/liter
NH_4VO_3 (0.023 g/l)	1 ml/liter
$(NH_4)_2CO_3$	0.15 mM

Saturate with 2% CO_2 by bubbling mixture of 2% CO_2-in-air through solution until pH reaches a constant level.

Add:

$FeCl_3$	0.02 mM

Table II

ADDING MEDIUM

$(NH_4)_2CO_3$	6.75 mM
$NH_4H_2PO_4$	1.10 mM
KH_2PO_4	0.40 mM
$MgSO_4 \cdot 7H_2O$	1.5 mM
$MgCl_2$	1.5 mM
KNO_3	0.05 mM
$Ca(NO_3)_2$	0.05 mM
A solution of trace elements plus $CoCl_2 \cdot 6H_2O$ (40 mg/l) and MoO_3 (15 mg/l)	1 ml/liter
NH_4VO_3 (0.023 g/l)	1 ml/liter
$FeCl_3$	0.1 mM

When the algae are first suspended in the "starting" medium the pH is above 6. After 2% CO_2-in-air has been passing through the algal suspension for several minutes, the pH drops to about 5.8. The lights are then turned on to illuminate the algae cell and photosynthesis begins. For a few minutes the pH rises to about 6.2. When the pH begins to fall due to NH_4^+ uptake, the pH control unit is set at pH = 6 and is then turned on. Occasionally it is necessary to inject a small amount of 0.1 N HCl to bring the pH down to 6.0. The rate of addition of "adding" medium is then found to be fairly constant for several hours. The density of the algae suspension also remains constant.

Table III (see RESULTS) shows the rates of uptake of ammonia and CO_2 along with the rate of oxygen evolution when the above media are used over a period of time to maintain the continuous growth of a 1% suspension of algae.

The rate of NH_4^+ uptake is calculated from the rate of addition of the "adding" medium and the known concentration of NH_4^+ in the "adding" medium. Since some NH_4^+ ion is removed when samples of algae suspension are withdrawn, this calculation gives an upper limit to the actual NH_4^+ uptake rate.

Administration of $C^{14}O_2$ and $HC^{14}O_3^-$.

Administration of $C^{14}O_2$ and $HC^{14}O_3^-$ were made by the same method as described in the previous paper.[1] Specific radioactivities of from 6 to 7 μcuries per μmole of carbon were used in each experiment.

Analysis of Samples.

The analyses were carried out as previously reported.[1] Approximately one ml samples were taken into weighted tubes containing 4 ml of methanol. Samples were then extracted and analyzed by two dimensional paper chromatography. Radioautographs of the chromatograms were prepared and radioactivity of each compound from each sample was determined.

Amino Acid Determination.

a. Carbon labelling.

As indicated in the earlier work[1], the concentration of carbon in a pool which is rapidly turning over may be measured by allowing it to reach a constant level of labelling with carbon 14. At this time the specific radioactivity of this pool of the given compound is the same as that of the carbon dioxide which is administered to the plant. Such a pool will be referred to as the "active" pool. We desired to measure the concentration of amino acids in the "active" pools and to compare them with the total amino acid concentrations. As before, we shall express as "micromoles of C^{14}" the amount of C^{12} and C^{14} in micromoles which corresponds to the measured amount of radioactivity, using the specific radioactivity of the administered $C^{(14+12)}O_2$. In other words, if S = specific radioactivity of the CO_2 (in μcuries/μmoles) and A = radioactivity (in μcuries) found in a given compound from one cc of wet packed algae, then A/S = μmoles of "C^{14}" in one cc of algae.

When the radioactivity of a given metabolic pool of a compound no longer increases as a function of the time of exposure of the photosynthesizing algae to $C^{14}O_2$, the pool is said to be "saturated" with C^{14}, and the μmoles of "C^{14}" as defined above is considered to be equal to the steady state concentration of the metabolic pool. A given metabolic pool is usually only one of several pools of the same compound in the plant. Therefore the total cellular concentration of a given compound is usually greater than the measured "active" pool.

In the case of the sugar phosphates of the carbon reduction cycle, and of 3-phosphoglyceric acid (PGA), the "active" pools saturate in 3 to 5 minutes after the introduction of $C^{14}O_2$, and there is seldom any problem in deciding what the "active" pool concentration is.[1,16] In the present study we found that the "active" pools of some amino acids saturated quickly and thus the levels could be easily determined. In the case of other amino acids, slowness of the "active" pools to saturate, coupled with appreciable rates of labelling of other pools of the same compound, have made measurement of the "active" pool size difficult or at times impossible. Thus some "active" pool sizes are given as approximations or as a range of values.

b. Ninhydrin estimation.

In order to provide a comparison between "active" pool sizes and total concentrations a method was developed to estimate the concentrations of amino acids in spots on the chromatograms. In these studies it was necessary to chromatograph the extract from no more than 5 mg of algae, due to the necessarily high salt content of the extracts. The method of colorimetric estimation of amino acids previously reported from this laboratory[17] did not afford sufficient sensitivity and accuracy for this purpose.

Methods involving ninhydrin are generally the most satisfactory for estimating small amounts of amino acids, but the major difficulty of using these methods on chromatograms or their eluates is that the paper may give a high and variable background color. This is presumably due mainly to traces of ammonia absorbed from the atmosphere or contained in the chromatographic solvents. It is particularly troublesome when the ninhydrin is in the reduced and more sensitive form necessary for the estimation of small amounts of amino acids.

It was found that this background color could be reduced to a low and constant value if the eluates are made alkaline and then scrubbed as described below. Since accurate estimates with reduced ninhydrin must be made under buffered conditions, it is possible to use the alkaline component of the buffer system for scrubbing of the eluates, and then the acid component can be added afterwards.

After location of the amino acid spots on the chromatograms by radioautography, they are cut out and each eluted with about 0.3 ml of water into graduated centrifuge tubes. Then 0.1 ml of 0.5 N NaOH is added and the solution is scrubbed with NH_3-free air under partial vacuum for 15 minutes at room temperature. Next 0.1 ml of a 0.8 M citric acid solution is added to bring the pH to 5.0, followed by the addition of 0.2 ml of a 1.66% solution of ninhydrin in methoxyethanol. The total volume is made up to 0.9 ml with water and after mixing well, the solution is heated for 15 minutes in a boiling water bath (the mouths of the reaction tubes being stoppered with glass marbles to prevent loss [illegible]). After cooling on ice [illegible] 60% ethanol is [illegible]

1 μg of glutamic acid is equivalent to an optical density change of .075. There is a linear relationship between optical density and amount of amino acid over the range 0.1 to 1.5 μg amino nitrogen.

The background values for blank areas of chromatograms are determined and appropriate corrections applied to the amino acid values, depending upon the areas of the spots. (A typical area of about 30 cm^2 of paper weighing 300 mg would have a blank of about 0.1 optical density units.) The radioactivity of the papers after elution is also checked. Usually 97% to 98.5% of the activity has been eluted. Since fingerprints leave ninhydrin positive marks on chromatogram papers, rubber gloves should be worn at all stages of handling the paper chromatograms.

The ninhydrin method described above was modified from that described by Yemm and Cocking[18] by changing the volumes and concentrations of the reagents in order to increase the sensitivity and by including the method of scrubbing the eluates.

Acetate Feeding Experiments.

In experiment SS 31, after the algae had been photosynthesizing for 60 minutes in $C^{14}O_2$ under steady state conditions, 80 μl unlabelled 1.0 N ammonium acetate was injected into the algal suspension (volume 80 ml) to give an initial concentration of 10^{-3} M acetate. After 10 minutes and again after another ten minutes, similar additions were made. All other environmental factors were maintained constant, including pH.

Light-Dark Experiments.

In experiments SS 30 and SS 32, after the algae had been photosynthesizing in $C^{14}O_2$ under steady state conditions for 60 and 104 minutes, respectively, the light was turned off, all other conditions being maintained constant. Samples were taken for another 10 minutes. After analysis by chromatography, the radioactivities of certain compounds were measured to study the effect of the light-dark transient period. During this time the rate of addition of nutrient was still followed.

RESULTS AND DISCUSSION

"Active" Pool Sizes and Rates of Photosynthesis.

In Table III, the rates of CO_2 and NH_4^+ uptake and of O_2 evolution during the course of three steady state experiments are shown. The times given are from the beginning of illumination of algae in the apparatus, the administration of $C^{14}O_2$ being about 1 to 1-1/2 hours after the start of the experiment. The rate of oxygen evolution is the best maintained of the three. The calculated rate of NH_4^+ uptake is also reasonably constant in the last two experiments. There is a tendency for the CO_2 uptake rate to decrease about 25% after a time. Since the algae in the steady state apparatus are exposed to greater illumination than in their growing tubes, this decrease in CO_2 fixation rate may indicate some quantitative change in the overall metabolism. For example, the amount of fatty acid synthesis may have increased

Table III

Rates of NH_4^+ and CO_2 Uptake and O_2 Evolution of Chlorella Grown in Described Media

	Time	Rate		
	Hours after lights on	μmoles/minute/gm wet-packed algae		
		O_2	CO_2	NH_4^+ (upper limit)
Experiment 30	0.5	20.4	22.2	
	1.0			2.40
	2.0	19.8	21.1	3.26
	2.75	20.8	19.3	
Experiment 31	0.5	16.6	19.4	2.3
	1.0	18.2	18.1	2.8
	1.5			2.6
	2.0	18.0	14.8	
Experiment 32	0.5	19.0	19.3	
	1.0			3.0
	2.0			2.6
	2.5	19.6	15.7	2.5
	3.0			2.5
	0 - 8 min. dark			4.3

Thus, a perfect "steady state" has not been achieved in these experiments. Nonetheless, the approach to the true steady state in these experiments is sufficiently close to permit us to measure a number of properties of the system.

If one takes the average rate of NH_4^+ uptake to be 2.5 μmoles per minute per cc of algae, then amino acid synthesis appears to account for about half of the total synthesis of carbon compounds (protein being assumed to be composed of 3.2 atoms of carbon per atom of nitrogen).

The appearance of C^{14} in alanine and in glutamic acid in SS 32 is shown in Fig. 1. These curves illustrate two extreme cases observed with the rapidly labelled amino acids during photosynthesis. In contrast to the glutamic acid pool, the "active" pool of alanine is clearly saturated after 30 minutes and its size can be determined to be 20 μmoles of carbon per cc of algae. Glutamic acid continues to increase in labelling for the entire 100 minutes in SS 32 (Fig. 1.) It is impossible from these data to determine accurately the size of its most "active" pool. Nonetheless, if one fits an exponential curve to the experimental labelling curve, an approximate "active" pool size of about 35 μmoles can be determined. Since the total pool of glutamic acid in SS 32 was found to be about 77 μmoles, it is apparent that there is more than one pool of glutamic acid.

The labelling curve of glutamic acid is further complicated by the indication that a less "active" or secondary pool of glutamic acid is being slowly labelled from 30 minutes onward. This can be seen more clearly in Fig. 2 (SS 30) where the curve for the specific radioactivity of the total glutamic acid pool has followed an exponential course until after 20 minutes and then begins to rise more steeply again.

Aspartic acid (Fig. 3) becomes labelled in a manner kinetically similar to alanine labelling and its "active" pool is nearly saturated after one hour. Serine (not shown) is intermediate between alanine and glutamic acid in the shape of its labelling curve, its secondary pool(s) apparently being slowly labelled during photosynthesis.

The carbon labelling curve of glutamine (Fig. 4) is similar to that of glutamic acid. In fact the curves for these two compounds, if divided by their respective labels at 100 minutes, give curves that are essentially superimposable. From this fact, we can say that no major pool of glutamic acid provides the precursor for glutamine synthesis. Rather, glutamine and glutamic acid must come from a common precursor. Of course, all such kinetic arguments must be qualified by the fact that the common precursor could be a complex, such as an enzyme complex, of one of the two compounds. There is no reason to assume that the precursor is such a complex, and we are inclined to think that the precursor is a biochemically identifiable compound. Earlier work from this laboratory, in which inhibitors of amino acid formation were employed[17, 19] indicated the possibility that glutamine could be formed in photosynthesizing Chlorella pyrenoidosa without the prior formation of glutamic acid. For these reasons we shall regard the incorporation of NH_4^+ and carbon into glutamine as not part of that incorporated into glutamic acid.

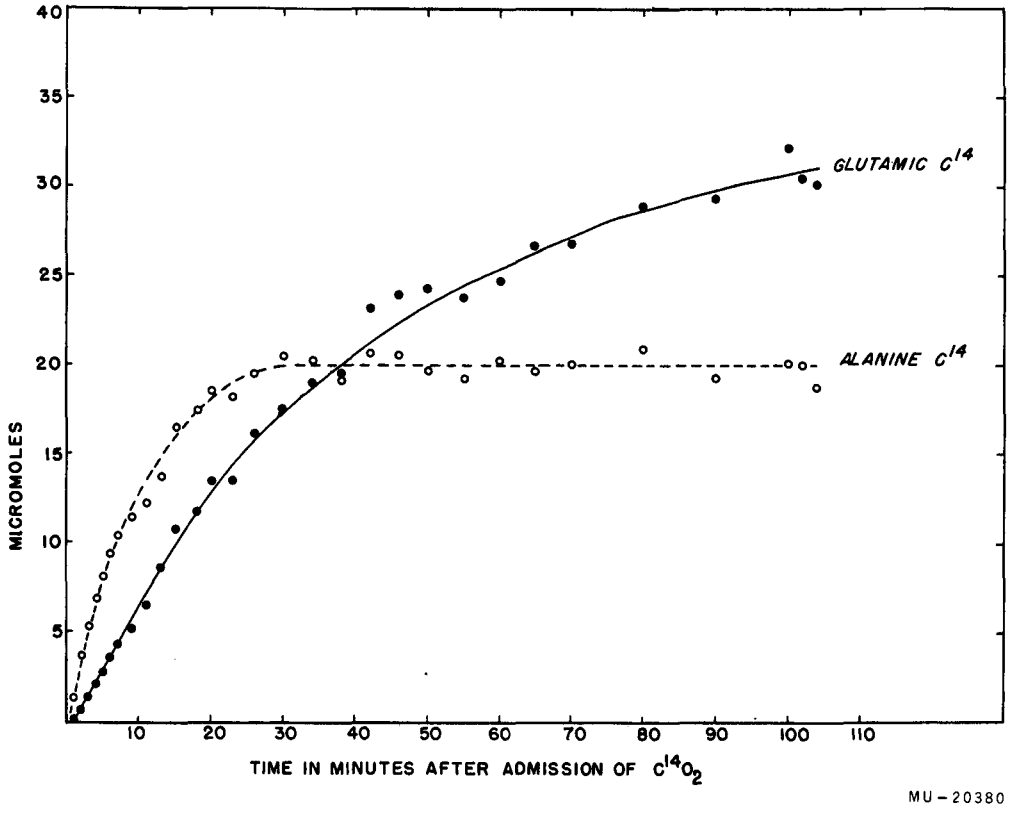

Fig. 1. Labelling of Glutamic Acid and Alanine in Steady State Experiment 32.

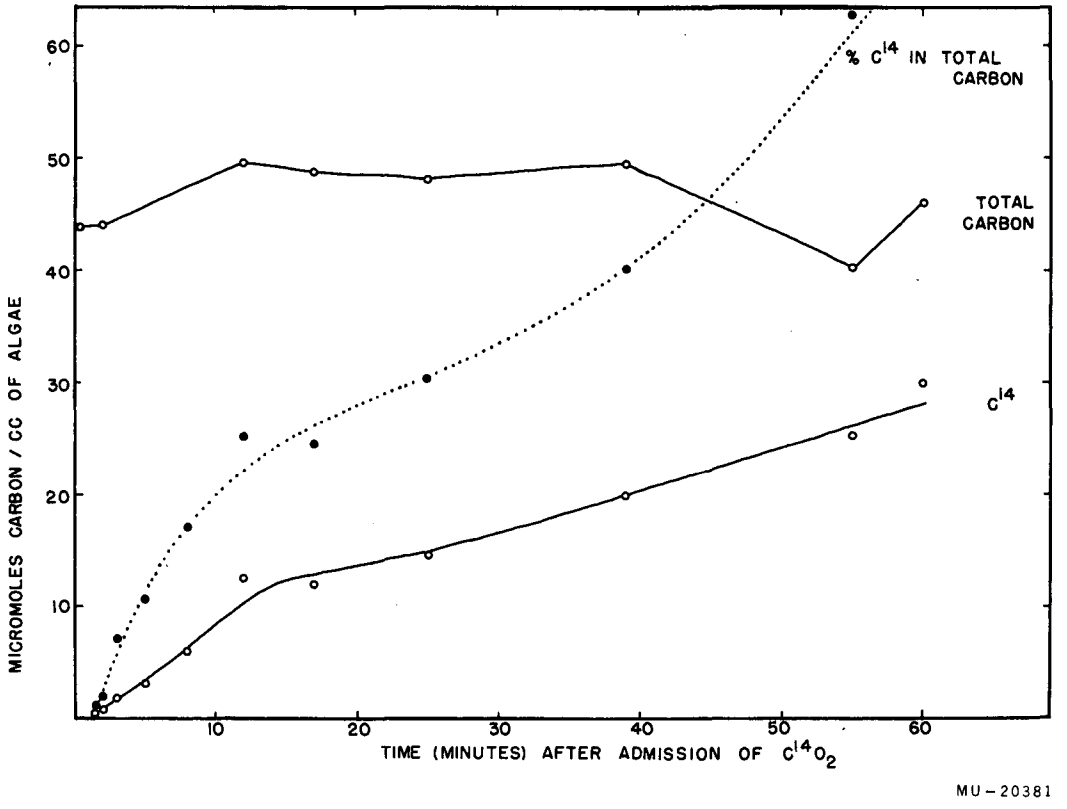

Fig. 2. Total Carbon, Labelled Carbon and Overall Specific Activity of Glutamic Acid in Steady State Experiment 30.

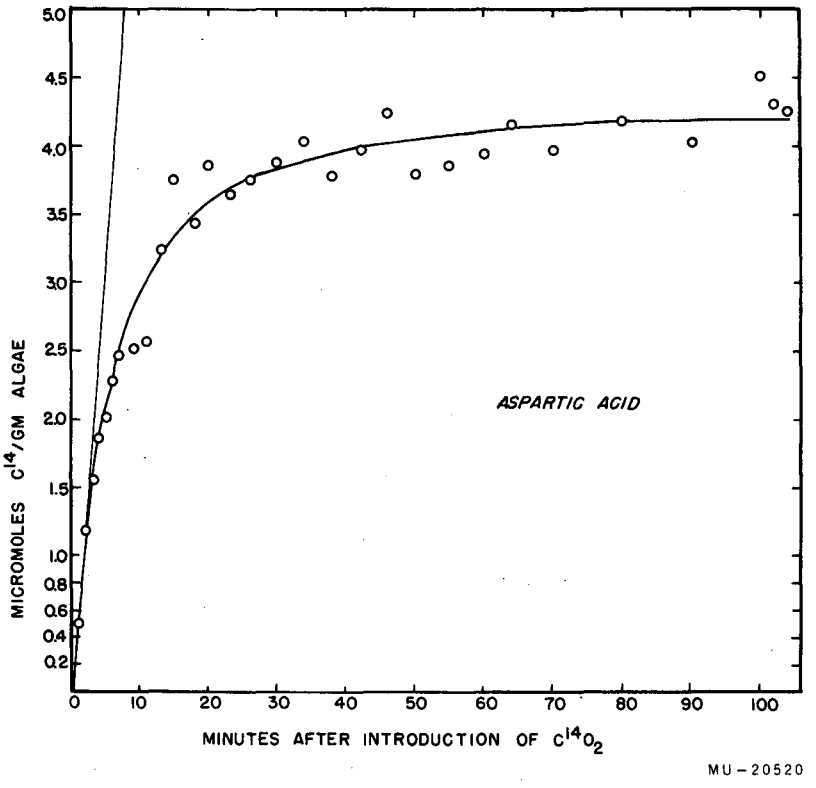

Fig. 3. Appearance of C^{14} in Aspartic Acid in Steady State Experiment 32.

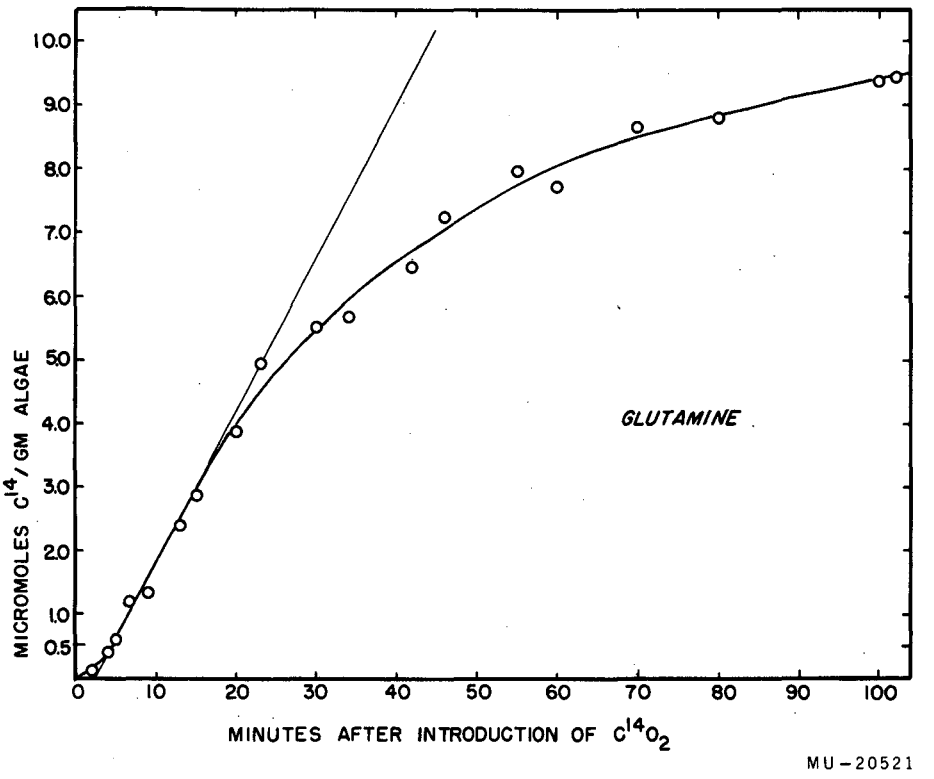

Fig. 4. Appearance of C^{14} in Glutamine in Steady State Experiment 32.

In Table 4, the sizes of such "active" pools and of the totals of all pools are given for the four most rapidly labelled amino acids. In experiments SS 30, SS 31, and SS 32 the "active" pool size is taken as the saturation level of an exponential curve fitted to the first 20 minutes of the experimental curve for glutamic acid and serine. The differences in total concentrations of amino acids between the experiments shown in Table 4 presumably reflect changes in the stock algal cultures during the six month period of the experiments. The sizes of the "active" pools are generally less than half of those of the total pools. In each case there are pools other than the one which is so rapidly labelled during photosynthesis.

In order to calculate the rates of flow of carbon through the "active" pools of several primary amino acids, we determined the maximum slope of the labelling curves as shown in Figures 3 and 4 by the thin line. This slope, N, is the net rate of increase in C^{14} at a given time after the introduction of $C^{14}O_2$. If the actual rate of flow of carbon of both isotopes through the pool is R, and if the precursor is saturated with C^{14}, the rate of flow of C^{14} into the pool is also R. If the degree of saturation of the pool is x, at the time where N is measured, the rate of flow of C^{14} out of the pool is Rx, and $R = N/(1-x)$. The calculated rates are given in Table 5. A range of pool sizes of glutamic acid (the most uncertain case) from 25 moles to 50 micromoles would cause a range in calculated synthesis rates from 1.04 to 0.92 moles/min cc algae; that is, ±7% of the reported value. The slopes are measured at times from 5 to 16 minutes from the introduction of $C^{14}O_2$, by which time the known precursors (PGA and phosphoenolpyruvic acid) are saturated. The slower achievement of maximum labelling rates (measured at 16 minutes) in glutamic acid and glutamine indicates that these amino acids have additional, unidentified precursors. It is possible that unstable, volatile, or non-extractable precursors (which we would not see) are not saturated by 16 minutes and that the measured and calculated synthetic rates are therefore too low. In any event, no large pools of unknown radioactive compounds are seen during this period.

The total of the carbon dioxide and ammonium ion used in the synthesis of these principal amino acids as calculated in Table 5 represents about 60% of all the amino acid synthesis as calculated from the externally measured ammonium ion uptake rate and about 3[illegible]% of the total carbon dioxide uptake. Other amino acids are labelled at appreciable rates and no doubt account for some of the remaining ammonium ion uptake. In addition, amination of some amino acid moieties of proteins may not take place until the carbon chain has been incorporated into the protein or peptide chain. In particular the very small rate of labelling of glycine as compared with the abundance of glycine moieties in plant protein may indicate that free glycine is not an important intermediate in protein synthesis in these organisms. Amination of the two carbon skeleton to give glycine moieties probably would account for at least 10% of the NH_4 fixation.

The results and the conclusions so far may be summarized as follows:

1. The "active" pools of several amino acids have been measured by the C^{14} saturation technique, and are found to represent only 20 to 50% of the total pools of these amino acids in the cases of alanine, aspartic acid, glutamic acid and serine. Therefore two or more pools of each of these amino acids exist in the algal cells

Table IV

AMINO ACID LEVELS IN STEADY STATE EXPERIMENTS

A summary of amino acid concentrations (expressed in micromoles carbon per gm wet packed algae) in 5 steady state experiments. C^{14} pools measured at time when "active" pools of amino acids become saturated. (For explanation see text.)

	GLUTAMIC		ALANINE		ASPARTIC		SERINE		
	μM total carbon	"active" pool μmoles C^{14}	μmoles total carbon	"active" pool μmoles C^{14}	μmoles total carbon	"active" pool μmoles C^{14}	μmoles total carbon	"active" pool μmoles C^{14}	Time at which values measured for C^{14} content.
SS 26	44.7	18			12.3	5.5			20 min.
SS DS1	41.2	17			9.5	5.3			20 min.
SS 30	47.5	15 - 20	41.0	15	6.4	3.0	6.3	3.8 - 4.2	*
SS 31	43.4	15 - 20	32.0		7.1	2	6.7		*
SS 32	77.5	25 - 40	42.3	20.0	19.5	4.2	20.2	3.2 - 4.0	*

*Value estimated on basic of extrapolation of exponential curves for glutamic acid and serine, levelling off of experimental curve for alanine and aspartic acid.

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Table V

Rates of Flow of Carbon Through "Active" Pools of Amino Acids Steady State Experiment 32

Compound	Net Rate in N μmoles of C^{14}	x Degree of saturation	Calculated Rate of Synthesis R μmoles of Carbon	Equiv. NH_4^+ uptake μmoles of NH_4^+
Alanine	2.00	.25	2.67	0.89
Serine	0.42	.15	0.49	0.16
Aspartic Acid	0.67	.25	0.89	0.22
Glutamic Acid	0.73	.25	0.98	0.20
Glutamine	0.23	.25	0.32	0.13
Glycine*	0.034	.1	0.04	0.02
Citrulline**	0.09	-	0.09	0.09
Threonine*	0.10	.5	0.20	0.05
Total			5.44	1.51
Externally measured uptake:			17.0	2.6
% of total through these pools:			32%	58%

*not included in totals

**figures are for carbamyl carbon only

2. The rates of synthesis of these amino acids from carbon dioxide have been calculated, and are found to account for at least 30% of all carbon fixation during steady state photosynthesis under the chosen environmental conditions. If certain assumptions are correct, the synthesis of these amino acids also accounts for about 60% of all the uptake of ammonium ion, which is the sole source of nitrogen in these experiments.

3. The net increase of C^{14} in alanine, serine, and aspartic acid reaches maximum rates by five minutes after the introduction of $C^{14}O_2$, at the same time that intermediates of the carbon reduction cycle of photosynthesis have become saturated with C^{14}, but long before such secondary products of carbon reduction as sucrose and these amino acids themselves have become saturated with C^{14}. This indicates that the immediate precursors of these amino acids are intermediates in the carbon reduction cycle, or are in isotopic equilibrium with intermediates of the cycle (e.g. phosphoenolpyruvic acid).

In isolated chloroplasts from Swiss chard, Tolbert[20] has shown that intermediates of the carbon reduction cycle and related compounds (phosphate esters of sugars and carboxyllic acids) do not diffuse out of the chloroplast. He found, instead, that C^{14}-labelled compounds in the supernatant fluid following $C^{14}O_2$ fixation in the chloroplasts were glycollic acid and sucrose, and to a lesser extent, some free carboxyllic acids, and serine and glycine. It is also noteworthy that alanine and aspartic acid became labelled in the chloroplasts but did not diffuse to the supernatant fluid. If we may assume that chloroplasts from Chlorella and Swiss chard are similar with respect to formation and retention of these compounds, we are led to the conclusion that:

4. The rapid synthesis of the amino acids found in the "active" pools in our experiments takes place within the chloroplasts.

5. Since carbon is not only going into these pools during steady state photosynthesis but is also passing through them; and since the less "active" pools of the same amino acids (presumed to be outside the chloroplasts) are not becoming labelled at an appreciable rate, it follows that more than 60% of the protein synthesis in Chlorella under these conditions of photosynthesis takes place in the chloroplasts, via free amino acids.

6. The slowness of glycine synthesis in these experiments, coupled with the relative frequency of glycine moieties in most plant proteins suggests that the glycine moieties of the chloroplastic protein are either supplied by extra-chloroplastic synthesis from unlabelled substrates, or more likely, are incorporated into the protein chain as some other two carbon compound, such as glyoxyllic acid. This finding would seem to be consistent with the observations of Sisakyan[13] who found that C^{14}-labelled glycine administered to various fragments of tobacco leaf cells was incorporated much more readily into the mitochondrial protein than into the chloroplastic protein. In either case, incorporation of glycine was inhibited by the supernatant solution. Perhaps the supernatant solution contains glyoxlyllate which is used preferentially for protein synthesis, especially in chloroplasts, or contains other factors favoring the formation of glyoxyllate from endogenous substances.

Feeding Unlabelled Acetate.

Figure 5 illustrates the results of the steady state experiment in which unlabelled acetate was supplied to the external medium at intervals beginning 60 minutes after the introduction of $C^{14}O_2$ to the system. The first addition of acetate results in a large and immédiate rise in the total amount of glutamic acid, the second addition causes a smaller rise and the third addition has no significant effect. However, none of the additions of acetate causes any significant change in the concentration of C^{14}-labelled glutamic, so that its specific activity drops sharply after the first two additions. These results complement those of Moses, Holm-Hansen, Bassham and Calvin,[21] who found that when labelled acetate was supplied to Chlorella there was a much greater incorporation of C^{14} into glutamic acid after three minutes than when the label was supplied as $C^{14}O_2$. In the experiment illustrated in Figure 5, addition of acetate caused no appreciable change in the concentration of either total or C^{14}-labelled alanine; this result again complements those of the above authors.

Two main conclusions are suggested by the results of this experiment: (1) the rapidity with which externally supplied acetate is incorporated into glutamic acid, and not into alanine, suggests that the latter may be synthesized entirely from the acetate, perhaps by the glyoxyllate pathway described by H. L. Kornberg [22,23]; and (2) the fact that addition of acetate causes no appreciable change in the concentration of C^{14}-labelled glutamic acid is further evidence for the existence of separate pools of glutamic acid. The rapidity with which the externally supplied acetate enters the "inactive" pool suggests that it may be located outside the chloroplast, while the "active" pool is located in the chloroplast.

It should be mentioned that acetate was added as ammonium acetate and that the results of addition could conceivably be caused by the increase in ammonium ion concentration. However, glutamic acid alone of the amino acids, and only the unlabelled glutamic acid, was significantly affected. Considering previously reported synthesis of glutamic acid from labelled acetate in Chlorella, it seems likely that the observed results are caused by the added acetate, not the additional ammonium ion.

Light-Dark Transient Experiments.

If Scenedesmus in distilled water in the light is supplied with $C^{14}O_2$, very little labelled carbon appears in glutamic acid, but if the light is turned off shortly after admission of $C^{14}O_2$, then labelled carbon appears rapidly in this compound.[15] When Scenedesmus (or Chlorella) is in an ammonia medium in the light, then appreciable amounts of labelled carbon will be incorporated into glutamic acid from $C^{14}O_2$;[14] but again, if the light is turned off shortly after admission of $C^{14}O_2$, then there is a rapid rise in the amount of C^{14} in glutamic acid. In both these kinds of experiments, the light-dark transient effects were observed before the pools of photosynthetic intermediates had become saturated with C^{14}. It was therefore of interest to see if these effects still occurred when the light was turned off some time after saturation of the pools of intermediates, and to determine if they were correlated with changes in the total amount of glutamic acid.

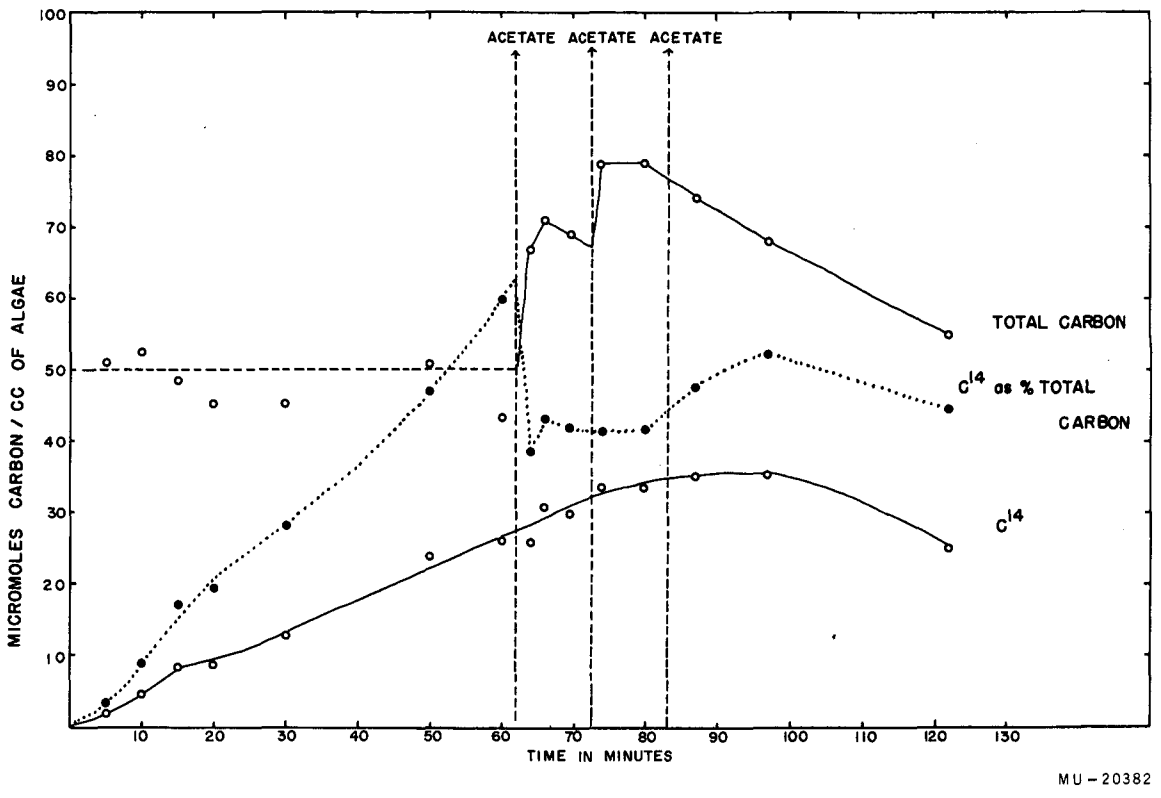


Fig. 5. Total Pool Size and Radiocarbon in Glutamic Acid During Steady State Experiment 32.

Acetate additions were each 80 μmoles to 80 ml algae suspension.

Figures 6 and 7 illustrate the results of this kind of steady state experiment in which the light was turned off some 60 or 100 minutes after the admission of $C^{14}O_2$. Several effects occur when the light is turned off: the total amounts of glutamic acid and alanine immediately begin to rise, while the amount of C^{14} falls somewhat- but rises again shortly afterwards. The total amount of the acids then falls off, followed by a fall in the amount of C^{14}. During the first 8 minutes of darkness, the rate of ammonia uptake increases. (Table III).

The explanation of these effects is complex. The initial drop in C^{14} during the first 30 seconds of darkness might result from the cessation of CO_2 fixation and reduction. The initial rise in the total amount of the amino acids, the subsequent rise in the amount of C^{14} and the increased rate of ammonia uptake all imply that turning off the light has in some way increased the total rate of combination of ammonia into amino acids. Light-dark transient effects have been previously explained in terms of a release of photoinhibition of the decarboxyllation of pyruvic acid, [15, 16]. This would not be a satisfactory explanation for the rise in the pool of "inactive" amino acids observed here, since such an explanation would require that either the "inactive" pools are in the chloroplast, or that the photoinhibition is operative in the cytoplasm. More probably, switching off the light causes a change in the oxidation-reduction state of the cytoplasm, permitting increased oxidation of glucose. Such a change would be transmitted from the chloroplast to the cytoplasm by the diffusion of some substance. For example, it is known that glycollic acid can diffuse out of chloroplasts, [20] and this may be involved in such a mechanism.

The small rise in C^{14}-labelled amino acids that begins about 60 seconds after the light is turned off presumably corresponds to that previously reported. These transient increases in C^{14}-labelling may be due to a release of a photoinhibition. They could also be the result of the diffusion out of the chloroplast of labelled photosynthetic intermediates. For example, it is known that mitochondrial membranes are permeable in the oxidized state, but not in the reduced state. [24] Such a change in permeability might also occur in the chloroplast membrane when the light is turned off.

Photosynthetic Pathways to Amino Acids.

If, as the evidence indicates, the principal site of amino acid photosynthesis is in the chloroplasts, one might expect the environment within the chloroplasts to influence the nature of the biochemical pathways of amino acid synthesis. This environment includes abundant supplies of reduced triphosphopyridine nucleotide, adenosine triphosphate and carbon incorporated into phosphoglyceric acid, phosphoenolpyruvic acid, and various sugar phosphates. It would not be surprising if under these conditions there are pathways different from those commonly encountered in non-photosynthetic systems.

For example, alanine accounts for about half of all the synthesis from carbon of the amino acids studied, and its synthesis requires twice as much ammonium ion fixation as does the synthesis of glutamic acid and glutamine combined. It is clear that however glutamic acid is formed, it does not provide all the nitrogen, via transamination, for the synthesis of alanine. It seems more likely that alanine is synthesized from intermediates of the carbon reduction cycle directly by reductive amination.

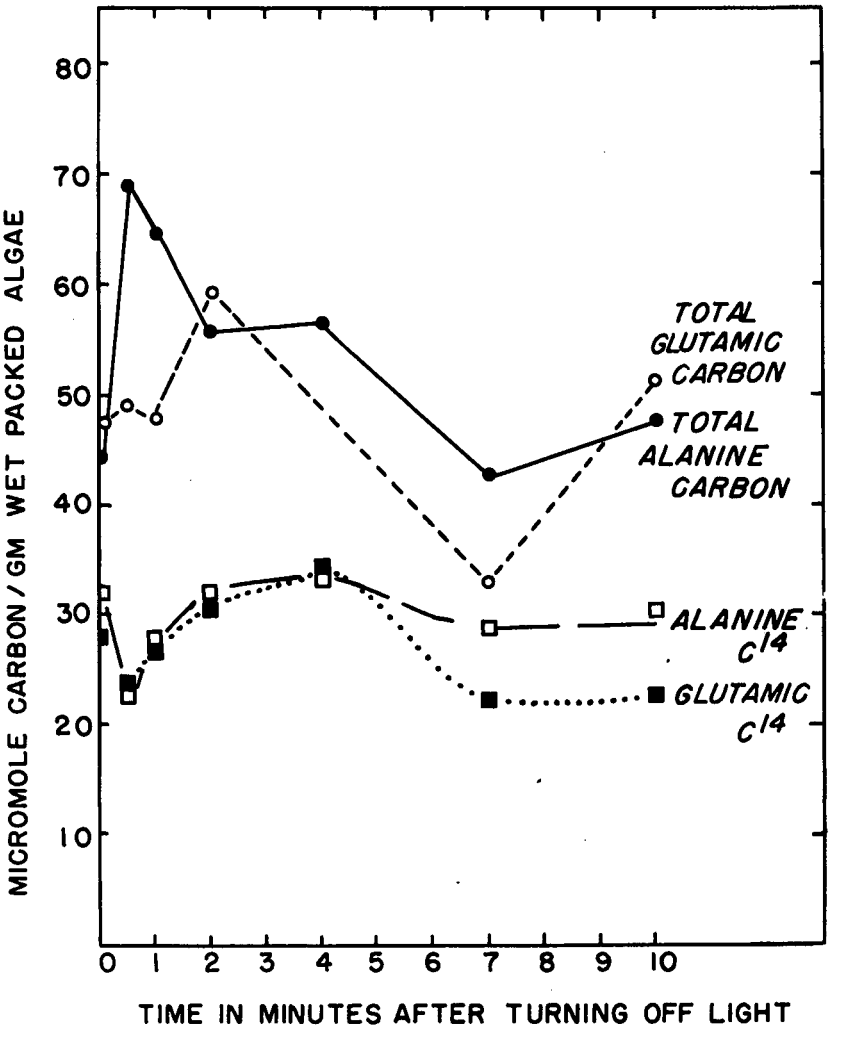


Fig. 6. Changes in Total and C^{14}-labelled Glutamic Acid and Alanine when Light is Turned Off (SS 32).

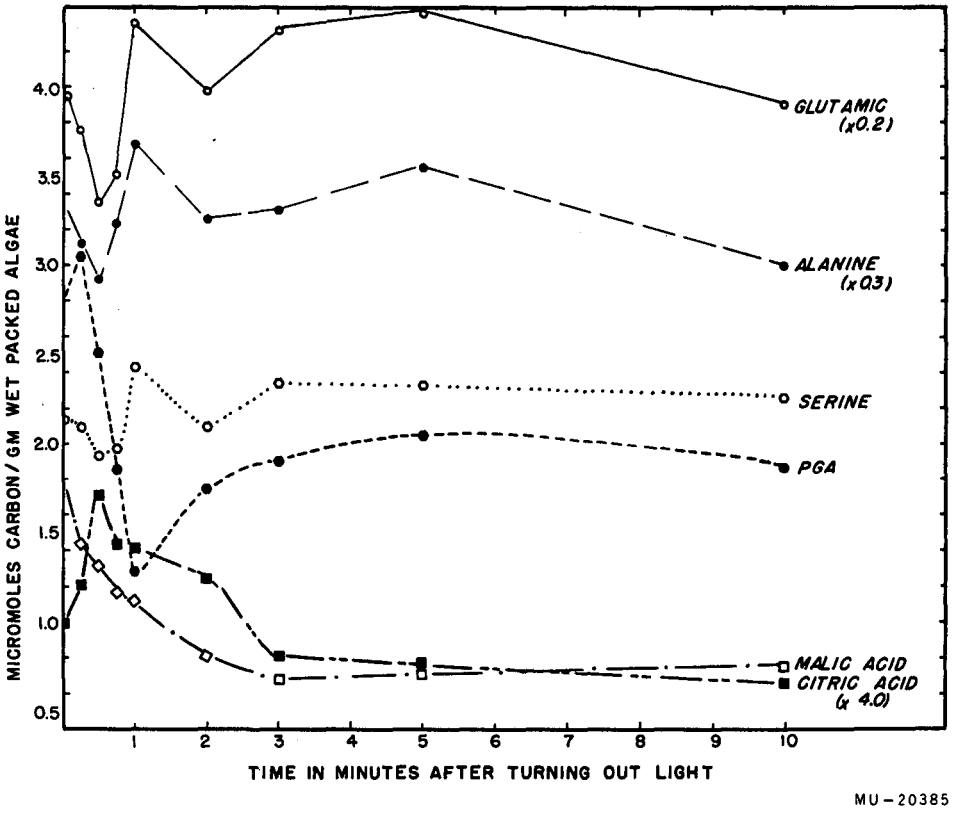
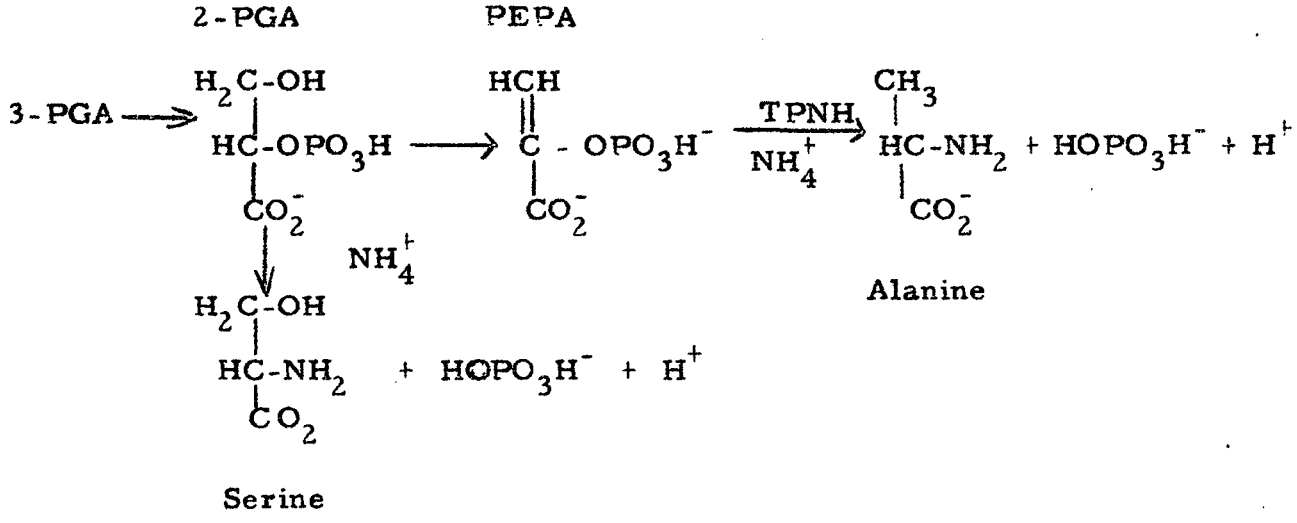


Fig. 7. Changes in C^{14} Content of Several Compounds when Light is Turned Off (SS 30).

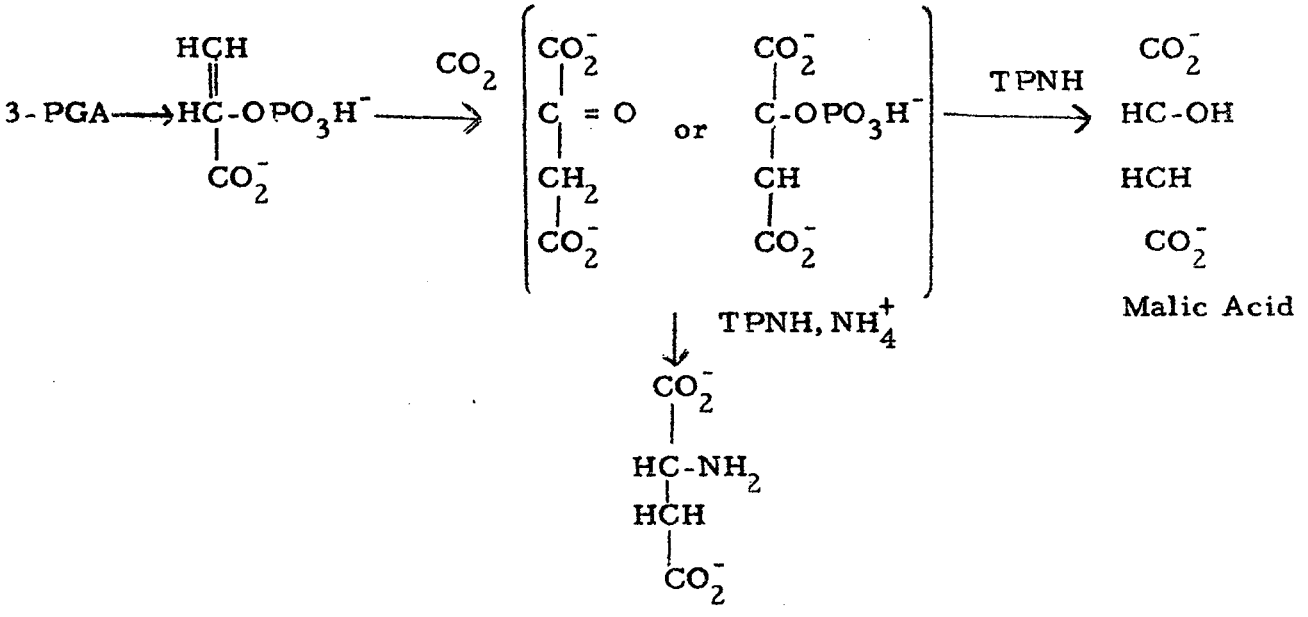
While the light is on, no C^{14} is found in free pyruvic acid upon subsequent analysis by paper chromatography of the products of photosynthesis of $C^{14}O_2$ until long after the "active" alanine pool is saturated (Steady state experiment 32). We have verified the fact that one can see labelled pyruvic acid by radioautography of chromatograms by hydrolyzing carbon-labelled phosphoenolpyruvic acid from our chromatograms and rechromatographing it. Free pyruvic acid does not seem to be an intermediate between the carbon reduction and alanine.

If we tentatively eliminate transamination and reductive amination of pyruvic acid as routes to the synthesis of alanine, we are left with previously undocumented, hypothetical routes. Of these, the one which seems to us the most probable is a reduction and amination of phosphoenolpyruvic acid to give alanine.



Serine might be formed by a similar, but non-reductive amination of 2-phosphoglyceric acid.

The rapid labelling of aspartic acid and malic acid[1] is considered to be evidence for carboxylation of phosphoenolpyruvic acid.



Aspartic Acid

Subsequent reduction of the carboxylation product would give malic acid, while reductive amination would give aspartic acid. An enzyme capable of bringing about the carboxyllation of phosphoenolpyruvic acid has been isolated from green leaves by Bandurski and Griener.[25] Since the carboxylation product is not seen by our method of analysis, malic acid and aspartic acid are, in this sense, the first stable, isolable products of this carboxylation. With certain plants and under suitable physiological conditions, it is possible that either or both compounds might appear more prominently labelled than phosphoglyceric acid. Thus Nezgovorova's[11] observation of aspartic acid as a first stable carbon dioxide fixation product is not contradictory to our results.

We should like to suggest that glutamic acid and glutamine are formed in the chloroplast independently from a common precursor which is in turn formed by condensation and reduction of two carbon and three carbon compounds derived directly from the carbon reduction cycle. It is likely that the two carbon compound would be acetate, glyoxylate or glycolate, while the three carbon compound would be phosphoenolpyruvate, phosphoglycerate, or even alanine. Various possible reactions suggest themselves, but there is little evidence for any of them in green plants at the moment.

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