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J. A. Bassham and Martha Kirk

December, 1963

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Lawrence Radiation Laboratory, University of California, Berkeley, Calif. (U.S.A.)

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SUMMARY

Methods have been developed for performing dual tracer experiments with ^{15}N and ^{14}C on the photosynthesis and biosynthesis of amino acids in Chlorella pyrenoidosa. These methods involved the development of apparatus for maintaining algae in a condition of steady-state photosynthesis, including amino acid synthesis, and of adding tracers in a stepwise fashion so that the specific activity of the tracer is maintained at a constant level without alteration of other environmental factors.

This method has been applied, and the kinetics of the labeling of several primary amino acids with ^{14}C and ^{15}N have been studied. The results indicate that the reductive amination to form glutamic acid is the primary route for incorporation of ^{15}N in this organism during photosynthesis of amino acids. Certain relationships between the site of the photosynthesis of carbon skeletons of primary amino acids and the site of ^{15}N incorporation have been proposed and discussed.

INTRODUCTION

We have previously reported the results of studies comparing the externally measured rates of photosynthesis in Chlorella pyrenoidosa with the rates of flow of carbon through individual compounds in the photosynthetic pathways^{1,2}. The methods used depend on the observation of the appearance of radiocarbon in individual compounds as a function of time of exposure of the plant to $^{14}\text{CO}_2$ during steady-state photosynthesis. It was shown that the syntheses of several primary amino

acids, particularly alanine, serine, aspartic acid, and glutamic acid, appeared to utilize intermediates of the carbon reduction cycle and to take place in the chloroplast². These conclusions were based upon the kinetic relationship between the labeling of the intermediates of the carbon reduction cycle and the labeling of these amino acids. Moreover, there appeared to be at least two pools of these amino acids, only one of which was rapidly labeled and "saturated" with carbon 14 during photosynthesis with $^{14}\text{CO}_2$.

Because of the rapidity with which carbon skeletons of certain amino acids such as alanine were labeled with ^{14}C , the question was posed as to whether alanine and perhaps other amino acids besides glutamic acid might be points of primary fixation of ammonium ion into amino groups. In order to gain some evidence on this point, and also to relate ammonium ion incorporation to carbon skeleton synthesis in amino acids, we have performed dual tracer experiments with $^{15}\text{NH}_4^+$ and $^{14}\text{CO}_2$ as substrates for steady-state photosynthesis in Chlorella pyrenoidosa.

EXPERIMENTAL

The experimental methods relating to the maintenance of the algae under steady-state photosynthesis and to the analysis of the labeling of compounds with ^{14}C has been described in this journal^{1,2}. The same methods have been used in these studies, except as noted.

Steady-state apparatus

The steady-state apparatus described in our previous reports^{1,2} has been modified to include both automatic pH control and automatic density control³. The apparatus, as modified, is shown in Fig. 1. A suspension of Chlorella pyrenoidosa (2.5% wet packed volume/suspension

volume, 80 ml) is placed in the illumination vessel of this apparatus. When the algae are first suspended in the nutrient given in Table I, the resulting suspension has a pH of 5.9 after equilibration with 1-2% CO₂ in air. In the experiments described, the pH control was set at 6, and 0.1 N NH₄OH was allowed to flow in automatically until the pH reached the set value, after which time it is added automatically from time to time as the algae photosynthesize, take up NH₄⁺, and tend to become acidic.

The automatic addition of NH₄⁺ is accomplished by means of the pH electrodes (see Fig. 1), the pH meter (1), the control relay (9), and the solenoid-operated valve on the inlet tube from the NH₄⁺ burette (2). All other nutrients are added via an inlet tube from the "medium" burette which passes through a solenoid-operated valve, controlled by the density reading on the recorder. This density reading is derived from the photoresistor (3) which "sees" light transmitted by the algae suspension. The addition medium is the same as that in which the algae were initially suspended. Also, algae suspension is automatically withdrawn from the cell when the volume exceeds a certain amount, due to interruption of light from photocell (5) and consequent signal to the sampler control (10) and solenoid operated valve (6) at the bottom of the illumination vessel. The sampler can also be operated by means of a pushbutton on the sampler control (10).

In the experiments described, the algae were placed in the illumination vessel 24 hrs prior to the commencement of the actual experiment, and allowed to photosynthesize and divide under steady-state conditions. However, it was found to be advantageous for maintaining the algae in an actively photosynthesizing state to turn off the lights

Fig. 1. Apparatus for studies of steady-state photosynthesis. 1. pH electrodes; 2. Inlet tube for $0.1\text{ N } NH_4OH$ solution; 3. Photoresistor for algae suspension density measurement; 4. Inlet tube for diluting medium, containing all ions needed for photosynthesis except NH_4^+ ; 5. Volume (of suspension) sensing light and photoresistor; 6. Solenoid-operated sampling valve; 7. Rubber plug and syringe for adding small amounts of liquid to algae suspension; 8. Connection to thermocouple in algae suspension; 9. pH control relay; 10. Control and push button for sampling valve operation.

TABLE I

NUTRIENT SOLUTION FOR CHLORELLA

KH_2PO_4	1.0 mM
K_2HPO_4	1.0 mM
MgSO_4	2.0 mM
$\text{Ca}(\text{NO}_3)_2$	0.02 mM
FeSO_4 - Versen - ol	0.1 mM in Fe
Modified Arnon's A-4 trace ^a	2 ml/l
NH_4VO_3 (0.023 g/l)	1 ml/l

^aMODIFIED ARNON'S A-4 STOCK SOLUTION

$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.168 mM
H_3BO_3	46.0 mM
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	50.0 mM
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.771 mM
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.316 mM
MoO_3 (99.5%)	0.100 mM

for an 8-hr period ending 10 hrs prior to the commencement of the tracer experiment. At all times before and during the experiment, the temperature of the algae suspension was controlled by means of a water jacket (not shown) on either side of the algae illumination vessel containing circulating water from a thermostated reservoir, the temperature of which was controlled by the signal from a thermocouple in the algae suspension (8).

At the beginning of the experiment, the pH control was turned off and the algae were allowed to photosynthesize for a period of 20-30 min. without addition of NH_4^+ in order to deplete the $^{14}\text{NH}_4^+$ in the medium. It was recognized that this would cause a departure from the steady-state condition of photosynthesis, but it was necessary to avoid isotopic dilution of the $^{15}\text{NH}_4^+$. At the end of this time, the gas circulation system was closed and the stopcock to the 5-liter flask containing a previously prepared mixture of 2% CO_2 and $^{14}\text{CO}_2$ in air (6-7 $\mu\text{C}/\mu\text{m}$) was opened making a large closed system with a total volume of about 6 liters. At the same moment, a solution of 0.4 ml of 0.1 N $^{15}\text{NH}_4^+$ (98.6% ^{15}N) was injected into the algae suspension by means of a syringe (7). Samples of algae were taken by means of the sampler control pushbutton at the times indicated in the figures shown in "Results".

During the experiment, the outputs of instruments measuring oxygen, CO_2 , ^{14}C , pH, and density were recorded on a multipoint recorder. From time to time, the stopcock to the 5-liter flask was closed, causing the algae gas circulating system to have a volume of 310 ml for oxygen and effective volume of 435 ml for CO_2 at pH 6. With this small volume, photosynthesis caused the changes in ^{14}C , CO_2 , and oxygen to be shown

on the recorder as indicated schematically in Fig. 1. Photosynthetic rates given in "Results", were obtained from the slopes of these curves, after correction for the nonlinearity of the CO_2 analyzer. The specific activity of $^{14}\text{CO}_2$ could be calculated at all times from the levels of ^{14}C and CO_2 and did not change significantly during the course of the experiment.

Small injections of 0.1 N $^{15}\text{NH}_4^+$ solution were made from time to time to maintain the pH at 6 during the course of the experiment. The algae suspension was illuminated from one side by an incandescent lamp (est. 13,000 lux incident) and from the other side by a bank of blue and white fluorescent tubes (est. 7,500 lux incident).

Analysis of amino acids

For the analysis of amino acids, 4.0 ml samples of algae suspension were taken. These were extracted successively with 80% alcohol, 20% alcohol and water. The combined extracts were concentrated in vacuo at reduced temperature to approximately 1 ml. Solid material which appeared upon concentration was removed by centrifugation, and the supernatant containing the amino acids and other soluble compounds was applied to a column 4 mm in diameter containing 0.5 cm^3 of an ion exchange resin (Dowex 50, 200-400 mesh, cross link 8) in the H^+ ion form. The sample was washed onto the column with 2.0 ml H_2O . The effluent solution was concentrated and subsequently analyzed by two dimensional paper chromatography, which showed that the amino acids had all been absorbed on the column. The amino acids were then eluted from the column with 2 ml of 4 N NH_4OH .

The eluate was concentrated, and one or more aliquot samples, containing an amount of extract corresponding to about 7.5 mm^3 of

wet packed algae, were applied to two-dimensional paper chromatograms for determination of ^{14}C content and for measurement of the total concentration of individual amino acids. A larger aliquot sample corresponding to about 80 mm³ of wet packed algae was removed and used for the isolation of larger quantities of amino acids for determination of their ^{15}N content.

Chromatographic solvents

The following chromatographic solvents were employed:

1. Phenol:water:acetic acid. Volume ratio 84:16:1, where the 84 parts of "liquified phenol" consisted of 88% phenol, 12% water.
2. "Semi-stench"⁴: EDTA, 1.2 g; 17 N NH_4OH , 100 ml; H_2O , 950 ml; N - propanol, 350 ml; isopropanol, 75 ml; N - butanol, 75 ml; isobutyric acid, 2500 ml. Stir several hours with magnetic stirrer. Do not use earlier than 24 hrs after mixing.
3. n-butanol:propionic acid:water. Made up from equal parts of A, n-butanol:water in the volume ratio 370:25, and B, propionic acid:water in the volume ratio 180:220⁵.
4. Pyridine:water. Volume ratio 80:20.

Procedure for analysis of total amino acid concentration and carbon ^{14}C content

The smaller samples (see above) were chromatographed in two dimensions on Whatman No. 1 or No. 2 filter paper which had been previously washed in 0.5% oxalic acid solution, well rinsed in distilled water, and dried. Chromatography in the first dimension was carried out with solvent 2 and in the second dimension with solvent 3. Most of the amino acids

were separated by this procedure. However, a secondary chromatography employing washed Whatman No. 4 filter paper with solvent 4 in the first dimension and solvent 1 in the second dimension was required to separate glutamine, serine, and glycine, which appeared as a single spot after the first chromatography.

The individual areas of the paper containing amino acids were cut out and the radioactivity in each determined by means of the "automatic spot counter"⁶. As in previous reports^{1,2}, the radio-carbon determinations have been converted to "micromoles of carbon 14" (see "Results") by dividing the radioactivity found in each amino acid by the specific radioactivity of the $^{12}\text{CO}_2$ - $^{14}\text{CO}_2$ used in the experiment. The results have been expressed in terms of μm of ^{14}C found in individual compounds per cm^3 of wet packed algae.

After determination of ^{14}C , the individual amino acids were eluted with water from the paper into 12 ml conical centrifuge tubes to a volume of about 0.6 ml, and 0.1 ml of 0.5 N NaOH was added. The solution in the tube was frozen and taken to dryness in vacuo over H_2SO_4 to remove any free NH_3 . The contents of the tube were then taken up in 0.5 ml H_2O after which 0.1 ml of 0.8 N citric acid was added, giving a buffered pH of 5. Amino acid standards for the colorimetric determination were prepared by adding sodium citrate to solutions of known quantities of the amino acids. The final volume of both standards and samples was 0.6 ml. Both the standards and the samples contained from 0.2 to 1.0 μg of nitrogen.

Ninhydrin solution was prepared 1 to 3 days prior to the amino acid determination by mixing 1 volume of 5% by weight ninhydrin in

methyl cellosolve with 3 volumes of a solution made up by diluting 1 ml of 0.01 molar KCN to 30 ml with methyl cellosolve.

To the tubes containing the buffered samples and standard amino acid solutions were added 0.3 ml of this ninhydrin reagent (total volume 0.9 ml). The tubes were then heated in a boiling water bath for 15-20 min. After the heating the solutions were diluted to 2 ml with 60% isopropanol:water and cooled in an ice water bath to below room temperature. This procedure represented several modifications from the one reported by Yemm and Cocking⁷ and the procedure reported by us previously². The optical density at 570 mμ of the samples and standards was read on a Beckman DU spectrophotometer against blanks prepared in the same manner as the standards except for the omission of amino acid. The sensitivity observed was usually 0.07 optical density units for 0.1 μg of amino nitrogen, when a cuvette with 1.0 cm path length was employed.

Procedure for ¹⁵N analysis

Each larger aliquot portion of the ion exchange column eluate containing amino acids (see above) was applied as a stripe across a sheet of oxalic acid washed Whatman No. 1 or No. 2 filter paper and developed in one dimension with solvent 2. Radioautographs revealed the location of stripes of glutamic acid, alanine, aspartic acid, and a combination of serine, glycine, and glutamine. Strips of the chromatogram containing these radioactive amino acids were eluted. Glutamic acid, alanine, and aspartic acid were further purified by rechromatography in one dimension with solvent 3. The mixture of serine, glutamine, and glycine was rechromatographed by chromatography in pyridine:water, which gave serine as a separate stripe. The mixture

of glutamine and glycine was separated by still another one dimensional chromatography in solvent 1.

The purified amino acids were eluted from the strips of paper. To the eluates, 0.3 ml of 0.5 N NaOH was added. The solution was frozen and taken to dryness in vacuo over H_2SO_4 . The residue was taken up in 0.6 ml of 0.4 N citric acid, giving a buffered pH of about 5. Then 0.3 ml of the ninhydrin-methyl cellosolve solution described above was added and the samples were heated in a boiling water bath for 15-20 min, giving a deep purple color. After the samples were cooled to room temperature, two drops of 6 N NCL were added to decompose the colored ninhydrin complex. The solution was then transferred to one bulb of a reaction flask which consisted of two 5-ml bulbs each connected by 15 cm of tubing to a "y" leading to a standard taper glass joint. This flask was attached temporarily to a vacuum line and most of the methyl cellosolve removed by vacuum distillation. Then the flask was removed from the vacuum line, about 2 ml of NaOBr solution⁸ were added to the other bulb of the flask, and the entire flask (both bulbs) was evacuated until there was some evaporation and degassing of the solutions.

The reaction flask with its contents still under vacuum and an attached stopcock and ground glass joint were transferred to the mass spectrometer (Consolidated Electrodynamics model 21.130), and the contents were frozen by immersion of the flask in liquid nitrogen. The entire system was evacuated. The stopcock between the flask and the spectrometer was closed, the flask contents thawed, and then refrozen and evacuated again, in order to remove as much dissolved air as possible.

Finally, the stopcock was closed again and the contents of the two bulbs of the flask thawed and mixed by pouring the NaOBr solution into the sample solution. The oxidation of ammonium ion to N_2 occurs in a few seconds as indicated by evolution of gas from the solution in vacuo. The reaction flask and stopcock were returned to the mass spectrometer, the contents of the reaction flask frozen in liquid nitrogen, and the system up to the stopcock once again evacuated. The sample of N_2 was then admitted to the mass spectrometer, and analysis of mass peaks from 12 to mass 40 was carried out.

Since it was found that there is always some contamination by air, as revealed by the height of the mass 32 peak, it was necessary to devise a method for calculating the $^{15}N/(^{14}N + ^{15}N)$ ratio that did not depend on the mass 28 peak. For this purpose we used the formula $F=1/(1 + 2r)$, where r is the ratio of the mass 30 peak to the mass 29 peak and F is the fraction of $^{14}N/(^{14}N + ^{15}N)$. The fraction of ^{15}N is then given by $1 - F$. The mass 12 peak was used to calculate the contributions to the mass 28, 29, and 30 peaks due to CO according to the ratio $12/28/29/30 = 1.1/100/3.2/0.25$.

Air correction was made in these peaks by using the mass 32 peak and assuming the ratio $28/29/32 = 100/0.88/18.2$.

It may be noted that the mass 32 peak could include O_2 arising from decomposition of NaOBr. This possibility was obviated by the addition of KI to the NaOBr solution when it was prepared⁸.

Amide nitrogen determination

The chromatographically purified glutamine was hydrolyzed with 1 N HCL for 16 hrs. The hydrolysate was made alkaline with a pellet of NaOH, and the ammonium hydroxide was distilled in vacuo into a

flask containing HCL. The ammonium ion was then assayed for ^{15}N content as described above.

Partial degradation of glutamic acid

The carbon 14 content of the carbon atom No. 1 (carboxyl alpha to the amino group) of glutamic acid was determined by degrading the glutamic acid with ninhydrin according to the published method⁹.

RESULTS

The externally measured photosynthetic rates of CO_2 uptake and oxygen evolution one and one-half hrs before the start of the experiment were 11.2 and 10.0 $\mu\text{moles/min/cm}^3$ respectively, while one hr after the start of the experiment the corresponding rates were 10.6 and 11.3 $\mu\text{moles/min/cm}^3$ respectively. As calculated according to the rate of 0.1 N NH_4OH addition required to maintain the pH at 6, the NH_4^+ ion uptake during the 3-hr period up to 1/2 hr before the start of the experiment was 1.5 $\mu\text{moles/min/cm}^3$ algae. NH_4^+ ion addition was stopped for 30 min preceding the start of the experiment, at which point 40 μmoles of $^{15}\text{NH}_4^+$ ion was added to bring the pH up to 6. The rate of $^{15}\text{NH}_4^+$ ion uptake during the first hour of the experiment was 2.34 $\mu\text{moles/min}$, and during the next 30 min the rate was 1.23 $\mu\text{moles/min}$. It is clear that the 30-min deprivation of NH_4^+ ion, required to avoid dilution of the $^{15}\text{NH}_4^+$ ion, resulted in some non-steady-state fluctuation in rate of NH_4^+ uptake and presumably in amino acid synthesis as well. Probably the period of deprivation caused a build up of keto acid compounds, leading in turn to an accelerated amino acid synthesis upon addition of $^{15}\text{NH}_4^+$, and an overshoot in amino acid concentrations during the subsequent period.

The labeling of glutamic acid, alanine, and aspartic acid with

^{15}N and ^{14}C , as well as the total amino acid concentrations, are shown in Figs. 2 through 4. As in our previous papers^{1,2}, ^{14}C content has been expressed in terms of μmoles of ^{14}C as calculated by dividing the radioactivity of the individual compound by the specific radioactivity of the carbon dioxide used in the experiment. This method of expression permits direct comparison between the rate of uptake of $^{14}\text{CO}_2$ and its appearance in individual compounds. The μmoles of ^{14}C have been plotted on a scale which is equal to the scale for μmoles of compound multiplied by the number of carbon atoms per molecule. A straight line has been drawn through the somewhat scattered values for total amino acid concentration. Total concentrations given by this line have been multiplied by % ^{15}N found to give μmoles of ^{15}N . In addition, the actual percentage of ^{15}N found is shown in numbers by each experimental point.

The ^{14}C content and ^{15}N labeling of serine are given in Table II. Attempted total serine determinations failed in this experiment. Both ^{14}C and ^{15}N -labeled pools of serine show a tendency to saturate similar to that observed with alanine and aspartic acid.

It is apparent that by far the greatest rate of incorporation of ^{15}N is that into glutamic acid. There appears to be an actively-turning-over pool of glutamic acid of approximately 7 μmoles which includes an actively-turning-over smaller pool of glutamic acid carbon skeletons of about 4 μmoles . (20 μmoles ^{14}C). In addition, there is an inactive pool of some 6 or 7 μmoles of glutamic acid. The gradual increase in both ^{14}C and ^{15}N labeling, together with the drop in total concentration shows that this inactive pool of glutamic acid will be gradually labeled with both tracers.

MU-32111

Fig. 2. Free glutamic acid in *Chlorella pyrenoidosa* and its rate of labeling with ^{14}C and with ^{15}N during photosynthesis. Closed circles: total glutamic acid
 Open circles: ^{14}C
 Open triangles: ^{15}N . Numbers are % ^{15}N .

Fig. 3. Free alanine in *Chlorella pyrenoidosa* and its rate of labeling with ^{14}C and with ^{15}N during photosynthesis.

Closed circles: total alanine
 Open circles: ^{14}C
 Open triangles: ^{15}N . Numbers are % ^{15}N .

MU-32109

Fig. 4. Free aspartic acid in *Chlorella pyrenoidosa* and its rate of labeling with ^{14}C and with ^{15}N during photosynthesis.

Closed circles: aspartic acid
 Open circles: ^{14}C
 Open triangles: ^{15}N . Numbers are % ^{15}N .

TABLE II
 ^{15}N and ^{14}C Labeling of Serine

<u>Sample</u>	<u>Time in min</u>	<u>^{14}C $\mu\text{moles}/$ cm^3 algae</u>	<u>% ^{15}N</u>
1	5	0.38	10.2
2	10	0.77	
3	15	1.21	
5	31	1.60	19.3
6	45	1.87	24.0
7	60	1.48	22.4
8	90	2.00	25.4

The maximum slope of the glutamic ^{15}N -labeling curve at roughly 8 min after introduction of the tracer is approximately 0.75 $\mu\text{moles/min}$. Since the actively-turning-over pool (with respect to amino nitrogen) of glutamic acid is approximately 50% labeled (3.5/7) at this time, the rate of flow of nitrogen through the amino group of glutamic acid can be very roughly estimated to be 1.5 $\mu\text{moles/min}$. This would account for a substantial portion of the total uptake of ammonia ion. The actively-turning-over pool of glutamic acid is essentially "saturated" with ^{15}N by 15 min.

The results of a partial degradation of glutamic acid from this experiment are shown in Table III. Of the total ^{14}C labeling in glutamic acid, the amount found in the C_1 carboxyl group is somewhat in excess of the 20% which would be expected with uniform labeling, at all times from 5 min to 90 min.

The ^{15}N labeling of the amide nitrogen of glutamine from the same experiment is shown in Table IV. The percent labeling is quite small for this amide nitrogen compared to the amino nitrogen of glutamic acid, but shows no consistent trend from the shortest to the longest exposure to $^{15}\text{NH}_4^+$, so that there could be a rather small pool of actively-turning-over glutamine which quickly saturates.

DISCUSSION

The relation between the time of maximum labeling of the glutamic acid and the maximum rate of labeling of the other amino acids studied clearly indicates that the amino groups in the other amino acids could all have arisen by transamination of their respective keto acids by

TABLE III

Partial degradation of glutamic acid

<u>Sample</u>	<u>Time, min</u>	<u>Total ^{14}C cpm</u>	<u>C-1 cpm</u>	<u>% of total ^{14}C in C-1</u>
1	5	25,260	6270	25.0
2	10	68,250	19,680	28.3
3	15	118,650	32,370	27.3
5	31	113,500	27,090	23.9
6	45	208,100	50,445	24.5
7	60	60,450	13,205	21.8
8	90	312,250	91,520	29.3

TABLE IV

Labeling of the amide nitrogen of glutamine from

Chlorella following photosynthesis with $^{15}\text{NH}_4^+$

<u>Sample</u>	<u>Time, min</u>	<u>% ^{15}N</u>
1 + 2	7.5 (av)	3.7
3	15	4.0
5 + 6	38 (av)	3.3
7 + 8	75 (av)	2.3

glutamic acid. Thus there is no reason to think that these amino acids other than glutamic acid incorporate ammonia by a direct reductive amination. The rate of labeling of the amino group of glutamic acid according to a rough estimate could account for most of the NH_4^+ uptake in this experiment.

The saturated actively-turning-over pool of ^{15}N -labeled alanine and ^{14}C -labeled alanine are of approximately the same size. This could either be a fortuitous occurrence in which the pools of alanine labeled with the two different tracers are non-identical or it might indicate that the pools of alanine that are actively turning over are one and the same, with both presumably located at the site of photosynthesis. The fact that the maximum rate of labeling of alanine with ^{15}N is approximately the same as the maximum rate of labeling of alanine with ^{14}C , though occurring at a later time, suggests that the two pools are in fact identical. Similar evidence suggests that the aspartic acid becomes ^{15}N -labeled primarily at the photosynthetic site.

In the case of glutamic acid it is clear that there is a concurrent ^{15}N incorporation at both the photosynthetic site and some other site. In this experiment and in past experiments we have noted a tendency for the glutamic acid carbon skeleton also to become labeled at two sites, as indicated by the continued incorporation of ^{14}C into the carbon skeleton of glutamic acid after what appears to be "saturation" of the primarily labeled pool. The degradation of glutamic acid supports this view. We may suppose that at the photosynthetic site ^{14}C is incorporated rather uniformly into the carbon skeleton of glutamic acid. This would produce glutamic acid with 20% of its carbon 14 label in the C_1 carboxyl. Apparently there is an additional synthesis of glutamic acid at another site utilizing some substrate

leading to C₁ carboxyl label and perhaps other unlabeled substrates for other parts of the molecule. According to this view, the principal site of amino acid formation would be at the photosynthetic site, but an additional amount of amino acids, and particularly of glutamic acid, would be formed elsewhere.

From the evidence reported here, the role of glutamine in ammonia incorporation appears to be minor. However, the data did not permit us to preclude the possibility that there is a small actively-turning-over pool of glutamine which saturates very quickly and which accounts for substantial amounts of NH_4^+ incorporation.

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