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STUDY OF INHIBITION OF AZASERINE AND DIAZA-OXO-NORLEUCINE(DON)
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Petronella Y. F. van der Meulen, and James A. Bassham
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STUDY OF INHIBITION OF AZASERINE AND DIAZA-OXO-NORLEUCINE (DON)
ON THE ALGAE SCENEDESMUS AND CHLORELLA

Petronella Y. F. van der Meulen,\(^1\) and James A. Bassham

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August 1958

Abstract

The effects of azaserine and DON (diaza-oxo-norleucine) on the metabolism of the algae Scenedesmus and Chlorella during photosynthesis with \(^{14}\text{O}_2\) are presented and found to be largely the same for both inhibitors.

DON appears to inhibit the formation of FGAM (\(\alpha\)-N-formylglycinamidine ribotide) from FGAR (\(\alpha\)-N-formylglycinamide ribotide), as is known for azaserine. FGAR and glutamine accumulate with both inhibitors, being absent from the control experiment. A proposal is presented that a possible site of inhibition is the synthesis of glutamic acid from \(\alpha\)-ketoglutaric acid. The relationship between glutamine and glutamic acid is discussed and the suggestion made that there may be a biosynthetic route to glutamine not involving glutamic acid as an intermediate. Indications for a still wider interference of the inhibitors are seen.

Introduction

In studies of the effect of azaserine on Scenedesmus during photosynthesis with \(^{14}\text{O}_2\) the inhibitor was found to cause many changes in the labeling of metabolic intermediates with \(^{14}\text{O}_3\). The transamination reactions were given as possible sites of inhibition. This work has been extended, and in this report a study on the
inhibition effects of both azaserine and diaza-oxo-norleucine (DON) on the metabolism of the algae Scenedesmus and Chlorella is reported.

Experimental Procedures

The experiments were of two types: Type A, in which first inhibitor and then, after 5 minutes, $^{14}O_2$ is added to the algae suspension; and Type B in which the algae are continuously in contact with $^{14}O_2$, before, during and after the addition of inhibitor.

Experiments of Type A show the effect of the presence of inhibitor for a fixed time on the distribution of radioactivity among the metabolic intermediates after 5 minutes photosynthesis. In experiments of Type B the distribution of radioactivity among metabolites following introduction of the inhibitor can be followed over a longer period of time.

A brief description of experiments of Type A and B with experimental details is given below, followed by tables and charts of the results of those experiments. Any deviation from the standard procedure is noted under the experiment concerned. Further experimental details have been published elsewhere.

Experiment of Type A. Freshly harvested algae (Scenedesmus obliquus or Chlorella pyrenoidosa) were centrifuged at 2000 rpm for 7 minutes, washed with distilled water and centrifuged again. They were then suspended to a 1% suspension (1 ml of wet-packed algae per 100 ml) in distilled water, and 1 ml KH$_2$PO$_4$ buffer (pH 6.8, 2.5 x $10^{-3}$ M) was added per 100 ml suspension. Of this suspension, 20 ml was placed in each of two thin illumination vessels (lollipops), arranged next to each other and immersed in a water bath to keep them at the same temperature of 20 to 22°C and to prevent heating by the lights. A stream of 2% or 4% CO$_2$-in-air was introduced into the suspension through a capillary. The lights (reflector flood, 150 watt, on each side at a distance of 20 cm from the lollipops) were turned on,
and the algae were allowed to photosynthesize for 15 minutes. Then a known amount of inhibitor, dissolved in a small amount of water, was added to one of the lollipops, the same volume of distilled water being added to the other. After the time chosen for the inhibitor to effect its influence, 500 μl of bicarbonate-$^{14}C$ (0.036 μCi, 400 μCi per ml) was added at the same time to each lollipop, and the lollipops were immediately closed and shaken in the light for 5 minutes. The suspension was then poured rapidly into 80 ml of boiling alcohol. The resulting 80% ethanol suspension was again brought momentarily to the boiling point, and after cooling, centrifuged at 2000 rpm for 15 minutes. The precipitate was re-extracted with 20% ethanol, and the extracts combined. By evaporating small aliquots of the 80% ethanol suspension and of the combined extracts on aluminum plates, removing excess bicarbonate with acetic acid, and measuring the radiation from the plates, the total radioactivity fixed by the algae, as well as the radioactivity in the soluble compounds, was calculated.

The combined extracts were then concentrated under vacuum and analyzed by the usual methods of paper chromatography and radioautography.5,6 Labeling with $^{14}C$ of individual compounds was determined by direct counting on the paper chromatogram.

**Experiments of Type B.** A suspension of algae (0.5 or 1.0%) was prepared as in experiment A. Sixty ml of this suspension was placed in a thin illumination vessel in a 'steady state' apparatus similar to that described in previous studies.6,7 After about 15 minutes illumination of the algae, a check was made of the rate of photosynthesis. The system was then filled with air and 0.7 to 0.8% $^{14}CO_2$ containing 2.4 μCi of $^{14}C$. At a given time, after 20 to 40 minutes of photosynthesis with $^{14}CO_2$, the inhibitor dissolved in a small amount of water was injected into the suspension by means of a needle through a rubber cap on an opening of the vessel. Samples of
the suspension were taken at different times before and after addition of inhibitor, from 0.5 minutes up to 3 hours.

During the entire period of the experiment, the CO₂ content of the gas changed from 0.8% to between 0.3% and 0.4%, and no significant change in the rate of photosynthesis could be detected.

The samples, each about 1 ml, were run into 5 ml of cold methanol and their sizes were determined by weighing. When necessary, more methanol was added to make an 80% suspension. After boiling for 1 minute the aliquots were centrifuged, the residue re-extracted with 20% methanol, concentrated under vacuum, and the total extract analyzed by paper chromatography and radioautography as in experiments of Type A. The different compounds were counted on the paper and the activity (in cpm on paper) per extract of 1 ml suspension was calculated.

Whereas the photosynthetic intermediates reach a steady state with respect to radioactivity in about 10 minutes, and their radiocarbon content is a measure of their concentration, the amino acids and keto acids are still gaining in radioactivity even after 40 minutes. Thus, the amount of radioactivity is not a measure of the concentration in the case of the latter substances.

Estimation of Amino Acids. In some of the experiments a semiquantitative determination of the amino acids, especially glutamic acid and glutamine, was made. This was accomplished by spraying the papers with ninhydrin solution (0.5 gm ninhydrin in 100 ml of 71% ethanol) and heating for 22 minutes at 65°C. The spots were cut out, cut into small pieces, and then placed in a tube with 3 ml of 71% ethanol and shaken a few minutes. The paper was then removed by centrifugation and the absorption at 575 μ of the supernatant solution was determined.

A standard curve was made by chromatographing known amounts of amino acids and determining the absorption at 575 μ in the same way. It was found that with amounts between 2 μg and 10 μg, an accuracy of 10% could be obtained.
Identification of FGAR. With both inhibitors an apparently new radioactive compound, absent in the control, was found on the chromatogram. This compound was found in the area of the chromatogram where glucose cyclic phosphate is often found. When eluted from the paper, treated with a phosphatase preparation, and then rechromatographed, the compound then moved a little faster than ribose in phenol-water and a little slower than ribose in butanol-propionic acid. This derived spot co-chromatographed exactly with the compound obtained by treatment with phosphatase of formylglycinamide ribotide which Professor J. M. Buchanan kindly provided.

Experiments and Results

A large number of experiments have been carried out. Of these, a few are presented in this report to illustrate the principal results. Results of three experiments of Type A are given in Tables I, II, and III.

Steady State Scenedesmus -- Experiment B-1

A 'steady state' C\(^{14}O\(_2\) photosynthesis experiment with Scenedesmus and azaserine (250 µg per ml suspension) as inhibitor was performed. The experiment was carried out as described under B, with a 0.5% suspension of Scenedesmus. The radioactivity found in various compounds as a function of time is shown in Figures 1 and 2.

Steady State Chlorella -- Experiment B-2

A 'steady state' C\(^{14}O\(_2\) photosynthesis experiment with Chlorella and with DON (15 µg per ml) as inhibitor was performed. A 1% Chlorella suspension was used. Four samples were taken before injection of the DON and several samples were taken from 0.5 minutes up to 2 hours after administration of DON. These samples were analyzed and the radioactivities in individual compounds determined as in experiment B-1. The resulting labeling of various compounds as a function of time before and
TABLE I

Radioactivity in various compounds in Scenedesmus extracts after 5 minutes of photosynthesis with bicarbonate-$\text{C}^{14}$ with DON.

Counts per minute (sensitivity about 1 cpm per 7 dpm)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Inhibited</th>
<th>Ratio: I/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartic acid</td>
<td>5582</td>
<td>485</td>
<td>.087</td>
</tr>
<tr>
<td>serine</td>
<td>3458</td>
<td>384</td>
<td>.11</td>
</tr>
<tr>
<td>glycine</td>
<td>1151</td>
<td>383</td>
<td>.33</td>
</tr>
<tr>
<td>threonine</td>
<td>408</td>
<td>204</td>
<td>.50</td>
</tr>
<tr>
<td>alanine</td>
<td>1689</td>
<td>531</td>
<td>.317</td>
</tr>
<tr>
<td>glutamine</td>
<td>192</td>
<td>2284</td>
<td>12</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>1975</td>
<td>777</td>
<td>.39</td>
</tr>
<tr>
<td>$\alpha$-ketoglutaric acid</td>
<td>219</td>
<td>1055</td>
<td>4.8</td>
</tr>
<tr>
<td>citric acid</td>
<td>1250</td>
<td>1715</td>
<td>1.4</td>
</tr>
<tr>
<td>malic acid</td>
<td>24500</td>
<td>25200</td>
<td>1.0</td>
</tr>
<tr>
<td>sucrose</td>
<td>4014</td>
<td>8093</td>
<td>2.0</td>
</tr>
<tr>
<td>diphosphates</td>
<td>5000</td>
<td>2400</td>
<td>.48</td>
</tr>
</tbody>
</table>

Total Fixation $\quad 23.1 \times 10^6 \quad 21.7 \times 10^6$

Total C$^{14}$ in Extract $\quad 18.4 \times 10^6 \quad 17.1 \times 10^6$

Conditions: 1% suspension of Scenedesmus; inhibitor DON, 100 µg per ml suspension. One hour after adding inhibitor the algae suspension was flushed with air for 1 minute; then 300 µl of bicarbonate-$\text{C}^{14}$ (120 µc) was added and photosynthesis with HC$^{14}$O$_3$ allowed to proceed for 5 minutes. Equal amounts of radioactivity were chromatographed, requiring, respectively, 1/17 and 1/18th of the extracts.
TABLE II

Radioactivity of Various compounds in Chlorella extracts after 5 minutes photosynthesis with bicarbonate-C¹⁴ with DON.

Counts per Minute

<table>
<thead>
<tr>
<th>Compound</th>
<th>Control</th>
<th>Inhibited</th>
<th>Ratio: I/C</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartic acid</td>
<td>1648</td>
<td>760</td>
<td>.46</td>
</tr>
<tr>
<td>serine-glycine*</td>
<td>5811</td>
<td>77-3</td>
<td>--</td>
</tr>
<tr>
<td>alanine</td>
<td>21375</td>
<td>9479</td>
<td>.45</td>
</tr>
<tr>
<td>glutamine</td>
<td>602</td>
<td>1385</td>
<td>2.27</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>9662</td>
<td>9468</td>
<td>--</td>
</tr>
<tr>
<td>α-ketoglutaric acid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>citric acid</td>
<td>406</td>
<td>648</td>
<td>1.6</td>
</tr>
<tr>
<td>malic acid</td>
<td>1172</td>
<td>2140</td>
<td>1.8</td>
</tr>
<tr>
<td>glyceric acid</td>
<td>1519</td>
<td>578</td>
<td>.38</td>
</tr>
<tr>
<td>glycolic acid</td>
<td>140</td>
<td>261</td>
<td>1.9</td>
</tr>
<tr>
<td>fumaric acid</td>
<td>326</td>
<td>426</td>
<td>1.3</td>
</tr>
<tr>
<td>lactic acid</td>
<td>410</td>
<td>1911</td>
<td>4.7</td>
</tr>
<tr>
<td>citrulline</td>
<td>154</td>
<td>489</td>
<td>3.2</td>
</tr>
<tr>
<td>sucrose</td>
<td>57568</td>
<td>32056</td>
<td>.56</td>
</tr>
<tr>
<td>unknown spot 1</td>
<td>719</td>
<td>3142</td>
<td>4.3</td>
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<tr>
<td>unknown spot 2</td>
<td>1464</td>
<td>2978</td>
<td>2.0</td>
</tr>
</tbody>
</table>

*These compounds did not separate well and have been counted together.

Total fixation in cpm 23.4 x 10⁶ 40.5 x 10⁶

cpm in extract 16.5 x 10⁶ 26 x 10⁶

Conditions: 1% suspension of Chlorella; inhibitor DON, 200 μg per ml suspension. After one hour in the presence of inhibitor, 1 minute of air flushing followed by 5 minutes of photosynthesis with bicarbonate-C¹⁴. Equal amounts of radioactivity were chromatographed, corresponding to 1/26th and 1/16th of the extracts (which were the extracts of 8 mg, and 12.5 mg wet-packed algae, respectively).
<table>
<thead>
<tr>
<th>Compound</th>
<th>cpm Control</th>
<th>cpm Inhibitor</th>
<th>Ratio: I/C</th>
<th>µg in C</th>
<th>µg in I</th>
<th>µg I</th>
<th>sp. act. C in cpm/µg</th>
<th>sp. act. I in cpm/µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>aspartic acid</td>
<td>2010</td>
<td>110</td>
<td>.055</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>serine</td>
<td>3540</td>
<td>1850</td>
<td>.54</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>alanine</td>
<td>6130</td>
<td>505</td>
<td>.082</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>glutamine</td>
<td></td>
<td>1380</td>
<td>4.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>310</td>
</tr>
<tr>
<td>glutamic acid</td>
<td>1430</td>
<td>1710</td>
<td>1.2</td>
<td>8.1</td>
<td>0.4</td>
<td>0.05</td>
<td>180</td>
<td>4200</td>
</tr>
</tbody>
</table>

| cpm in extract  | $18 \times 10^6$ | $20 \times 10^6$ |

**Conditions:** 1% suspension of *Chlorella*; inhibitor DON, 10 µg per ml suspension. One hour of photosynthesis in presence of inhibitor, then 5 minutes of photosynthesis with bicarbonate-\(^{14}\)C. Of each, an amount of extract containing $10^6$ cpm was chromatographed and the specific activity of the amino acids determined.
after injection of inhibitor are shown in Figure 3 and 4. The specific activities of glutamic acid and glutamine were also determined; see Figure 5. After a longer time of inhibition some as yet unidentified compounds became labeled, most of them moving quite fast in both directions. They are represented in Figure 4 by the letters R, S, T, X and Y.

Discussion

Four combinations have been investigated:

*Scenedesmus* and azaserine inhibition

*Scenedesmus* and DON inhibition

*Chlorella* and azaserine inhibition

*Chlorella* and DON inhibition.

On *Scenedesmus*, the effects of azaserine and DON are similar. With each organism, there occurs a decrease of labeling of amino acids (e.g., aspartic acid, glutamic acid, alanine, serine), and an increase in labeling of glutamine and the acids: \( \alpha \)-ketoglutaric, citric, malic, lactic, fumaric and succinic. In addition a new radioactive spot, tentatively identified as \( \alpha \)-N-formylglycinamide ribotide, was found in the presence of each inhibitor.

No inhibition was found with azaserine in *Chlorella*.\(^3\) It seems likely that azaserine did not penetrate the cells. The DON apparently penetrates only slowly into the cells of *Chlorella* with the result that a period of time, which depends on the outside concentration, is required before a sufficiently high level of DON within the cells is reached to effect the inhibition.\(^4\) At very low concentration of DON within the *Chlorella* cells, brought about by low concentrations of inhibitor outside or, alternatively, by short-time exposure to a higher concentration outside, an increased total radioactivity was found in glutamic acid in *Chlorella*. The effect of DON on *Chlorella* is, in general, similar to its effect on *Scenedesmus* except that FCAR (\( \alpha \)-N-formylglycinamide ribotide) could not be detected in *Chlorella*.
Fig. 1. Radioactivity in glutamine before and after injection of azaserine in Scenedesmus photosynthesizing in the presence of C\textsubscript{14}O\textsubscript{2} (Exp. B-1). Time of injection of inhibitor is given as time zero.
Fig. 2. Radiocarbon found in aspartic and glutamic acids per extract of 1 ml suspension of 0.5% Scenedesmus suspension during "steady state" $^{14}C$ photosynthesis with azaserine inhibition. (Exp. B-1).
Fig. 3. Activity in cpm on paper/extract of 1 ml suspension of different compounds in samples of 1% Chlorella suspension, taken during a long-term $C^{14}O_2$ photosynthesis with DON (15 $\mu$g/ml suspension). (Exp. B-2)
Fig. 4. Activity in cpm on paper/extract of 1 ml suspension of different compounds in samples of 1% Chlorella suspension, taken during a long-term $^{14}\text{CO}_2$ photosynthesis with DON (15 $\mu$g/ml suspension). Compounds indicated by letters R, S, T, etc. were not identified. (Exp. B-2)
Fig. 5. Specific activity of glutamine and glutamic acid in samples of a 1% Chlorella suspension, taking during a long-term $^{14}$O$_2$ photosynthesis with DON. (Exp. B-2)
Azaserine has been shown to inhibit the formation of α-N-formylglycinamidine ribotide (FGAM) from α-N-formylglycinamide ribotide (FGAR). DON appears to inhibit the same reaction, since, with both inhibitors, FGAR has been found (in Scenedesmus), being absent from the control experiments, and with both inhibitors, the glutamine builds up (in both organisms).

Besides inhibition of this reaction and possibly of other transamidations, the inhibitors appear to affect the metabolism in other points. The more pronounced effects may be summarized as follows:

1) After there has been time for the inhibitor to penetrate the cells (5 to 10 minutes), total radioactivity in glutamic acid may rise for a few minutes and then fall rapidly (Figure 3) or may fall immediately (Figure 2). After a half hour's exposure of the cells to inhibitor, glutamic acid radioactivity may rise slowly (Figure 2). The concentration of glutamic acid falls from the beginning of inhibition while its specific activity increases continuously, (Figure 5).

2) Aspartic acid, serine and alanine appear to decrease in radioactivity from the beginning of inhibition (Figure 3). After an hour's exposure to inhibitor there is very much less radioactivity in these amino acids and in glycine and threonine than is found with noninhibited algae (Table I). Inhibition of radio-carbon found in these amino acids (with the possible exception of aspartic acid) appears never to be greater than the decrease in glutamic acid concentration (Table III).

3) Glutamine increases in radioactivity steadily from the beginning of the inhibition (Figures 1 and 3) and may reach a very high level. Its specific activity, which initially may be higher than that of glutamic acid, increases steadily but falls behind that of glutamic acid. Its concentration increases many-fold during one hour's inhibition in Chlorella.
4) Acids of the tricarboxylic acid cycle, as well as lactic acid, increase in radioactivity following inhibition (Figure 4). In particular, C\(^{14}\) in \(\alpha\)-ketoglutaric acid increases remarkably, especially in the steady-state Chlorella experiment (Figure 3).

It is generally accepted that an important route for the entry of nitrogen into the amino acids in plants involves the conversion of \(\alpha\)-ketoglutaric acid to glutamic acid, presumably via reductive amination with glutamic dehydrogenase. Other amino acids are then formed by transamination of the appropriate \(\alpha\)-keto acid by glutamic acid. Glutamine is formed from glutamic acid and ammonia via a reversible reaction requiring ATP.

Glutamine has been found to donate its amide group in several transamination reactions. In these reactions glutamic acid is formed. Inhibition of such transamination reactions may account for the observed increase in glutamine, and for the decrease in the amount of glutamic acid.

The decrease in radioactive amino acids may be brought about, as a secondary effect, by a decrease in concentration of glutamic acid. Although it is not possible from our data to decide between a real inhibition of transamination by DCM and azaserine\(^3\) and a decrease of transamination as a secondary effect, the latter seems more likely as the decrease in radioactive amino acids never appears to be greater than the decrease in glutamic acid (except possibly for aspartic acid) and the extents of both decreases run parallel. Moreover, upon inhibition of transamination, glutamic acid would be expected to accumulate and less \(\alpha\)-ketoglutaric acid to be formed, while exactly the opposite effects have been found.

Inhibition of transamination, either directly or indirectly, may account for the increase in radioactivity of the Krebs cycle acids. The steep increase in labeled lactic acid may have resulted from reduction of pyruvic acid when
transamination of the latter to alanine no longer takes place.

The uninhibited cells of Chlorella were found to contain a fairly large amount of glutamic acid (6 µg per ml 1% suspension). The amino acids: aspartic acid, serine and alanine were present in amounts of the order of 1 µg per ml 1% suspension. Although glutamine can hardly be detected under normal conditions, it apparently is actively involved in the metabolism of the cells. In the presence of the inhibitor glutamine rapidly accumulated, at a rate of 1-2 µg per ml 1% suspension per 10 minutes. Glutamic acid disappeared at about the same rate.

So far a reasonable interpretation of the inhibitory effects is possible in terms of known metabolic pathways and may be summarized as follows: On inhibition of some transamidation reaction(s), glutamine will accumulate and glutamic acid (a reaction product of transamidations in which glutamine donates its amide group) will no longer be regenerated. The amount of glutamic acid decreases and consequently the synthesis of amino acids from the corresponding α-keto acids by transamination with glutamic acid may be reduced, which, in its turn, may cause an accumulation of Krebs cycle acids.

Some of the other results obtained in these experiments require further explanation. In particular, the increase in total radioactivity in glutamic acid following addition of inhibitor in some experiments is difficult to explain in terms of an inhibition of a transamidation reaction only. The problem becomes more complex when it is remembered that at the beginning of inhibition the specific activity of glutamic acid is very low (even after 40 minutes of photosynthesis with C\(^{14}\)O\(_2\)) and that, at the same time, glutamine specific activity may be higher than that of glutamic acid (Figure 5). Another difficulty is the fact that both concentration and total radioactivity of glutamine continue to increase at a constant high rate, even when the level of glutamic acid has fallen from an initial 6 µg per ml 1% suspension to less than 0.2 µg per ml.
In order to explain these results on the basis of a synthesis of glutamine by way of a reductive amination of \( \alpha \)-ketoglutaric acid to glutamic acid followed by amidation to give glutamine, and by no other pathway, it is necessary to call on a separation of the total reservoir of glutamic acid into two more or less isolated reservoirs, capable of possessing different specific activities. The glutamic acid involved in the transamidation cycle with glutamine could have initially a much higher specific activity than the remaining glutamic acid pool and thus the glutamine could have a higher specific activity than the total glutamic acid reservoir. On inhibition of transamidation, glutamine concentration and total radioactivity would rise while the glutamic acid pool of higher specific activity would decrease in concentration. The decrease in concentration of this glutamic acid pool might result in an accelerated rate of reductive amination of \( \alpha \)-ketoglutaric acid. Since the \( \alpha \)-ketoglutaric acid is presumed to be nearer the point of introduction of \( ^{14} \text{C} \) and, consequently, much higher in specific activity, the specific activity of the glutamic acid pool of the transamidation cycle might increase sharply.

The total radioactivity in glutamic acid, which is, of course, the product of the concentration and the specific activity, might increase for a time until the increase in specific activity was no longer enough to counteract the drop in concentration, after which time the total radioactivity in glutamic acid might be expected to fall. A slow equilibration of the transamidation pool of glutamic acid with other pools of glutamic acid of much lower initial specific activity could easily account, in part, for the eventual rise in total glutamic acid radioactivity which is seen after an hour or more. This slow rise in total glutamic acid radioactivity may also result from the greatly increased reservoir of glutamine which eventually could have the effect of decreasing the rate of the amidation of glutamic acid.
As an alternative to the above explanation of the experimentally observed results, one might suspect the existence of a different route for the synthesis of glutamine, not involving glutamic acid as an intermediate. While no such route has been reported for plants, one might speculate that such a pathway may exist. If, for example, α-ketoglutaric acid were to undergo an amidation in the presence of ammonia and adenosine triphosphate, analogous to the amidation of glutamic acid, the resulting compound would be a α-ketoglutaramic acid, a substance which has been reported as the product of transamination reactions of glutamine in liver.\textsuperscript{13,14,15} Reductive amination of α-ketoglutaramic acid, analogous to the reductive amination of α-ketoglutaric acid would lead to the formation of glutamine.

Thus, glutamine would have been synthesized by a route not involving glutamic acid. An inhibition of transamidation reactions by which glutamine is converted to
glutamic acid would then account for, 1) the continuous increase of radioactivity and concentration of glutamine, 2) the drop in concentration of glutamic acid, 3) a rise in glutamic acid specific activity as more glutamic acid was synthesized directly from α-ketoglutaric acid to replenish its reservoir, and 4) a fluctuation in total glutamic acid radioactivity—first up, then down as its specific activity increased while its concentration was decreasing.
REFERENCES

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9. Dr. J. M. Buchanan, Department of Biochemistry, Massachusetts Institute of Technology, Cambridge, Massachusetts.


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