Changes in Amino Acid Content of Excised Leaves During Incubation
I. The Effect of Water Content of Leaves and Atmospheric Oxygen Level

John F. Thompson, Cecil R. Stewart, and Clayton J. Morris

U. S. Plant, Soil and Nutrition Laboratory, Soil and Water Conservation Division, Agricultural Research Service, United States Department of Agriculture, Tower Road, Ithaca, N. Y. 14850
and
Department of Agronomy, College of Agriculture, Cornell University, Ithaca, New York 14850

Received June 6, 1966.

Summary. Excised leaves were incubated at various water contents to determine the effect of water status on amino acid composition. Considerable proteolysis took place during incubation with a resultant increase in each amino acid in the non-protein fraction. However, serine, proline, $\gamma$-aminobutyric acid and methyleysteine sulfoxide were the only amino acids in which there was an accumulation (i.e., net synthesis). Serine showed a small but consistent accumulation lasting for 6 days. Proline showed a greater accumulation but this ceased after 2 days.

To learn more about the control of the proline accumulation during wilting, turgid and wilted leaves were incubated under aerobic and anaerobic conditions. The amino acid analyses showed that turgid leaves did not accumulate proline and that proline and methyleysteine sulfoxide accumulation was abolished by anaerobiosis. With other amino acids, relative concentration changes between wilted and non-wilted leaves were less striking than the difference between aerobic and anaerobic conditions.

Under anaerobic conditions there was an increase in alanine and a large increase in $\gamma$-aminobutyric acid which were not evident in air. Serine, aspartic acid, glutamic acid, and glutamine disappeared more rapidly and glycine disappeared less rapidly under anaerobic than under aerobic conditions.

On the basis of these results, several pathways of amino acid degradation were suggested.

A serious restriction on plant growth and hence food production in many parts of the world is an insufficiency of water. This insufficiency varies from permanent (desert), to medium long (drought) to temporary (diurnal). Water insufficiency in a growing plant results in wilting which has profound effects (9, 10, 38) on photosynthesis (47), respiration (23), nucleic acids (8, 29) and on nitrogen metabolism (2, 3, 13, 26, 40, 49). In order to understand and possibly control the effects of water deficiency on a plant, more needs to be known about the effects of dehydration at the cellular and subcellular level. With the latter thought in mind, it was decided to investigate the changes in protein-bound and uncombined amino acids during incubation of wilted leaves. An excised leaf system was employed so that it would be possible not only to compare the changes in an individual amino acid in the protein and non-protein fractions, but also to compare the changes of the different amino acids within the non-protein fraction. The ultimate aim was to understand the biochemical basis for the effects of wilting on nitrogen metabolism.

Excised leaves in the normal or wilted condition were incubated for varying lengths of time. Protein-bound and uncombined amino acids were determined in addition to sugar, chlorophyll and ammonia. Proteolysis and a concomitant increase in uncombined amino acids was observed, and this was expected (4, 20, 24, 26). There was a wide variation in the rate at which the uncombined amino acids decreased and there was a net increase (i.e., accumulation) in proline and serine. These observations led to an examination of the effects of anaerobiosis on amino acid contents in excised leaves. Anaerobiosis abolished the accumulation of proline in wilted leaves and produced other significant changes in amino acid content which were different from those obtained under aerobic conditions.

1 Predoctoral fellow, National Institutes of Health, 1965-1966. Present address: Department of Biological Sciences, Purdue University, Lafayette, Indiana.
Materials and Methods

Leaves from greenhouse-grown turnips (Brassica rapa L. var. Shogoin) were used for most experiments. The leaves were excised and the midribs removed. One-half of each of 6 leaves were killed immediately after cutting to serve as controls. The other halves of these 6 leaves were incubated in the various treatments. Half leaf samples obtained in this way were similar in composition when compared on a fresh weight basis. The total amino nitrogen values were equivalent (on a fr wt basis) between paired half leaf samples to an average of 3.0 % (6 pairs), whereas the total uncombined amino nitrogen values agreed to an average of 2.5 %. The total amount of individual amino acids in comparable samples were equal to within ±5 %.

Levels of individual uncombined amino acids in one half leaf sample were within ±10 % of the level in the other half leaf sample, except for valine and tyrosine which were equal to ±15 % and asparagine to within ±30 %. Although these differences are relatively small, consideration of the effects of treatment has been restricted to changes of at least 20 % for total amino acids and of 100 % for uncombined amino acids.

In the first experiment, leaves were dried in light (200 ft-c) at 20° until they had lost 50 % of their fresh weight (1–2 hrs). The leaf samples (6 half leaves) were then rolled loosely and placed in 100 ml bottles which were closed to prevent further water loss. The bottles were opened and aerated daily. The treated leaves were incubated in the dark at 20° for varying lengths of time up to 164 hours with no sign of fungal infection.

In later experiments, leaves were incubated in dark at 20° on moist paper towels in 4-liter polyethylene bags which were inflated and sealed. These were opened daily and reinflated to insure adequate aeration. Also a wider range of conditions was employed. Leaves were incubated at 100, 75 and 50 % of original fresh weight and some leaves were incubated anaerobically. In the latter case, leaves were placed in a large test tube which was evacuated and refilled with nitrogen. In all cases, water content was constant (±2 %) throughout incubation.

Extraction and Hydrolysis. Leaf samples were killed by addition of 95 % ethanol. After standing overnight, the leaves were thoroughly extracted in dim light with 75 % (v/v) ethanol after comminution in a Waring Blendor. The residues (from the extraction) were dried in vacuo at 110° and acid hydrolyzed (35).

Analytical Procedures. Chlorophyll was determined colorimetrically on the alcohol extract (1). Sugar determinations were made by a procedure (Clapp and Dawson, personal communication) analogous to the colorimetric anthrone method (28), but which is more reproducible and sensitive.

Aqueous samples (1 ml) containing 0 to 25 μg of sugar were warmed in test tubes in a water bath to 40°, then thoroughly mixed with 4 ml of concentrated H₂SO₄. After 5 minutes in the water bath, 0.5 ml of a 2 % solution of α-naphthol in 95 % ethanol was added. After allowing 10 minutes for color development at room temperature, absorbance was determined at 570 mμ and compared to blank samples prepared either without sugar solution or without α-naphthol.

Total amino nitrogen in either non-protein fraction or protein fraction was determined by reaction with ninhydrin (19) after removal of ammonia by drying an aliquot with an alkaline buffer (5). Ammonia values were obtained by subtracting the total amino nitrogen value from the total ninhydrin reactive material without removal of ammonia.

Amino acids were purified with ion-exchange resins (34) and analyzed colorimetrically by the ninhydrin reaction after separation by 2-dimensional paper chromatography (33). The basic amino acids were analyzed on a 15-cm column according to the method of Moore, et al. (18).

The data are expressed as a ratio of the content (on a fr wt basis) in the treated sample to the content in its control sample to permit comparison between samples with varying initial composition.

Results and Discussion

In the first experiment, turnip leaves were dried until 50 % of the fresh weight was lost and then incubated at this water content for 0, 2, 5, 10, 20, 45, 70, 117, and 164 hours.

The changes in various leaf constituents with time are shown in figure 1. The marked decrease in protein has often been observed in excised leaves whether wilted (13, 40) or turgid (41, 42, 46, 48). The concomitant decline in chlorophyll is undoubtedly
related to the protein decrease (46). After an initial rapid decline in sugar level, there was a cessation of this decline, indicating that the remaining sugars were less metabolically active. The significant point is that as the rapid disappearance of sugar ceases, there is a rapid rise in ammonia, indicating that deamination of amino acids provides oxidizable substrates for respiration as metabolizable sugar is depleted (41).

If one assumes that the initial levels of uncombined amino acids reflect a balance between formation and metabolism in a normal leaf, it is not surprising that there is a marked increase in the uncombined amino acids (table 1) because of proteolysis (fig 1). Also, if the disappearance of amino acids is largely due to their deamination and subsequent oxidation of resultant keto acids, a difference in the rate of disappearance of the various amino acids would be expected. This is indeed what was found (table 1). Uncombined alanine, arginine, glycine and threonine increased relatively little in comparison to asparagine, isoleucine, phenylalanine, valine, serine, and proline. The former group of acids was probably rapidly degraded while the latter group was slowly degraded although formation must be a significant factor for proline and serine and may be for some others.

The acidic amino acids and their corresponding amides require special discussion. The amide content of proteins is difficult to determine and this was not done. However, it was found that the ammonia released from hydrolysis of the protein was equivalent (±5%) to the total dicarboxylic amino acid content, indicating that the acidic amino acids occur in the proteins only as amides. Calculations in table 1 were based on this assumption. Even if aspartic and glutamic acids are not released by proteolysis, they could be formed by deamination of released amides. The small increase in uncombined aspartic and glutamic acids as compared to other acids in the non-protein fraction indicates a rapid metabolism, as would be expected.

The much greater increase in uncombined asparagine as compared to glutamine (table 1) is correlated with the rapid rate of disappearance of total glutamine (60% after 70 hrs) rather than asparagine (10% after 70 hrs). This marked behavioral difference between these 2 amides has often been noted in plants (4, 17,31) and is striking in excised leaves (13, 40, 42, 46, 48) and in water deficient roots (49). In the wilted turnip leaves there was no net synthesis of asparagine (see last 3 columns of table 1) which is different from the conclusion of Vickery and Meiss (40) based on reasonable indirect evidence that there was a net synthesis of asparagine during the curing of tobacco leaves. The difference between the observations with turnip (table 1) and tobacco (39, 40) may be the result of species or environmental variations that affect asparagine synthesis in excised leaves.

Table I. The Relative Content of Various Amino Acids in Wilted Turnip Leaves as Compared to Initial Content

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Avg initial content in non-protein fraction</th>
<th>Amt* in non-protein fraction relative to initial value</th>
<th>Avg total initial content</th>
<th>Total amount* in sample relative to initial value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(μmoles/g*)</td>
<td>20 hrs</td>
<td>70 hrs</td>
<td>20 hrs</td>
</tr>
<tr>
<td>Alanine</td>
<td>1.7</td>
<td>1.8</td>
<td>2.5</td>
<td>22.9</td>
</tr>
<tr>
<td>γ-Aminobutyric acid</td>
<td>0.31</td>
<td>1.35</td>
<td>3.25</td>
<td>0.31</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.35</td>
<td>3.0</td>
<td>1.5</td>
<td>5.6</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.4</td>
<td>4.4</td>
<td>14.8</td>
<td>25.1**</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.2</td>
<td>1.0</td>
<td>2.7</td>
<td>**</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>2.5</td>
<td>1.1</td>
<td>2.0</td>
<td>**</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5.0</td>
<td>1.7</td>
<td>0.28</td>
<td>25.5**</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.52</td>
<td>1.2</td>
<td>2.1</td>
<td>23.0</td>
</tr>
<tr>
<td>Histidine</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>4.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.25</td>
<td>4.9</td>
<td>10.2</td>
<td>12.7</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.11</td>
<td>3.6</td>
<td>6.1</td>
<td>21.9</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.22</td>
<td>1.7</td>
<td>8.9</td>
<td>4.8</td>
</tr>
<tr>
<td>Methionine</td>
<td>...</td>
<td>...</td>
<td>...</td>
<td>4.8</td>
</tr>
<tr>
<td>Methylycysteine sulfoxide</td>
<td>1.3</td>
<td>2.4</td>
<td>3.2</td>
<td>...</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.19</td>
<td>7.1</td>
<td>32.0</td>
<td>11.4</td>
</tr>
<tr>
<td>Proline</td>
<td>0.23</td>
<td>18.1</td>
<td>10.4</td>
<td>13.9</td>
</tr>
<tr>
<td>Serine</td>
<td>0.71</td>
<td>5.2</td>
<td>17.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.82</td>
<td>3.2</td>
<td>3.1</td>
<td>12.5</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.12</td>
<td>3.1</td>
<td>9.6</td>
<td>7.1</td>
</tr>
<tr>
<td>Valine</td>
<td>0.52</td>
<td>5.9</td>
<td>15.6</td>
<td>17.7</td>
</tr>
</tbody>
</table>

* Amount on initial fresh weight basis. "Total" means amino acids in non-protein fraction plus those in the protein fraction.

** Calculations were based on the assumption that all the aspartic acid and glutamic acid in the protein hydrolysate were as amides (see text).
Some of this difference may be a consequence of 2 pathways of asparagine formation: A) amidation of aspartic acid (44); and B) hydration of \( \beta \)-cyanoalanine (25). However, the rates of disappearance of the amides may also be involved.

Table I also indicates that there was no net synthesis of glutamine which is not in agreement with the findings of Yemm (48) in unwilted barley. This disagreement could be the result of differences in rates of synthesis and further metabolism. Since the increase in uncombined glutamic acid accounted for only 11% of the glutamine lost, a rapid metabolism of glutamine by deamidation and further deamination or transamination of the resultant glutamic acid is reasonable.

Total serine and total proline each showed an increase during the incubation period (fig 2), but they behaved differently. Serine increased more slowly and declined more slowly than proline. The opposite behavior of glycine and serine (fig 2) and their close metabolic relationship (45) could be interpreted to mean that glycine is converted to serine in wilted turnip leaves as it is in wheat (16, 43).

Proline showed a rapid increase and equally rapid decline that started within 24 hours. The coincidence of the proline decrease and sugar decline (fig 1) indicates a relationship (32). In wilted perennial ryegrass, Kemble and Macpherson (13) also observed a proline accumulation but this accumulation persisted for 6 days. The protracted proline accumulation in ryegrass in contrast to the rapid disappearance of proline in turnips was probably the result of the low water content of ryegrass that markedly reduced enzymatic activity.

The accumulation of \( \gamma \)-aminobutyric acid has been observed before (15) but the accumulation of methylcysteine sulfoxide has not. Possible explanations are discussed below.

Since a prominent feature of wilting is stomatal closure (10, 38), a possible explanation of the wilting effects described above could be a result of a lack of oxygen. Consequently, further experiments were carried out under both aerobic and anaerobic conditions. Turnip leaves with high sugar content (32) were incubated at 75% and 100% of their normal fresh weight at 20° in the dark for 6 time periods, both in air and in nitrogen. The changes in selected amino acids of turnip leaves at different moisture levels and in atmospheres of different oxygen tension during a 24-hour incubation period are presented in table II.

In this experiment, an accumulation of serine was observed only in unwilted leaves in air, and this was consistently shown at other incubation times. In the earlier experiments, accumulation occurred in wilted leaves, and since this was observed in several samples, it is believed to be correct. However, subsequent attempts to demonstrate accumulation of serine in wilted leaves have been unsuccessful, presumably due to failure to repeat earlier conditions. It is significant that there is very little or no loss of serine in wilted leaves in air. Anaerobiosis not only abolished the accumulation of serine in unwilted leaves but produced greater serine disappearance than in wilted leaves.

In contrast to serine, the effect of oxygen supply on glycine level was greater in wilted leaves. Furthermore, anaerobiosis reduced glycine disappearance. Oxygen may accelerate glycine breakdown because oxygen is a substrate for glycolic acid oxidase (37) which oxidizes glyoxylic acid, a transamination product of glycine. It is difficult to understand why a similar effect should not pertain to unwilted leaves. As above, a possible glycine-serine interconversion may be involved in these changes, but this area is still one of confusion (43). Also both glycine and serine are metabolized in other ways (e.g., transamination).

There was a net increase in alanine under anaerobic conditions that did not occur in air (12). This can be interpreted as reflecting an increase in pyruvate resulting from decreased oxidation via the Krebs cycle and accelerated glycolysis (Pasteur effect). Since the amount of aspartic acid was reduced under anaerobic conditions and there was no evidence for \( \beta \)-alanine, a \( \beta \)-decarboxylation of aspartic acid also may account for part of the alanine increase. Naylor et al. (22) recovered a high percentage of \(^{14}\)C from aspartic acid in alanine under anaerobic conditions in the light. However, since aspartic acid \( \beta \)-decarboxylase has not been demonstrated in plants, the decrease in aspartic acid is probably explained better by a deceleration of the Krebs cycle and reduced oxaloacetate formation.

There was an accumulation of methylcysteine sulfoxide in air but not in nitrogen. This is consistent with a need for oxygen in the biosynthesis of ATP required for S-adenosylmethionine formation, since methylcysteine is formed by methyl-ation of cysteine (36).

![Fig. 2. The effect of incubation time on serine, glycine and proline content of wilted leaves.](https://www.plantphysiol.org/)

---

Downloaded from on September 22, 2017 - Published by www.plantphysiol.org  
Copyright © 1966 American Society of Plant Biologists. All rights reserved.
There was no accumulation of proline in unwilted leaves and the accumulation in wilted leaves required oxygen. The oxygen requirement was probably related to its role in α-ketoglutarate formation from sugar (32) which is necessary for glutamic acid and proline formation.

γ-Aminobutyric acid increased under all conditions during the first 24 hours; however, the increase was much greater under anaerobic conditions. Macpherson and Slater (15) observed an increase in the percentage of total nitrogen in γ-aminobutyric acid in wilted grass in air and under mercury seal, and in silage, but no increase in unwilted grass as found in turnip leaves. γ-Aminobutyric acid is formed by decarboxylation of glutamic acid in plants (27) and anaerobiosis increases the recovery of labeled γ-aminobutyric acid from labeled glutamic acid (21). The much greater loss of glutamic acid under anaerobic conditions may be associated with the greater increase of γ-aminobutyric acid. The accumulation of γ-aminobutyric acid under anaerobic conditions is consistent with a reduction in the rate of transamination of γ-aminobutyric acid, caused by an accumulation of succinic semialdehyde (6) which is metabolized by the Krebs cycle in the presence of oxygen (11). The amount of glutamic acid that disappeared was much greater than the γ-aminobutyric acid formed, demonstrating that glutamic acid is metabolized in other ways; e.g., glutamic acid may be converted to α-ketoglutaric acid by aminotransferases and by glutamic dehydrogenase. Furthermore, anaerobiosis would reduce the rate of the Krebs cycle reactions and hence the production of α-ketoglutarate which is a substrate for glutamic acid synthesis.

The increase in uncombined glutamine in aerobically incubated wilted leaves (table II) is different from the results shown in table I. This difference is thought to be a result of a limiting oxygen supply in the experiments reported in table I, a conclusion which is substantiated by the fact that there was no increase in uncombined glutamine under anaerobic conditions (table II). The greater the oxygen supply, the greater would be a supply of ATP and ammonia (from oxidative deamination) (7, 30) for glutamine synthesis (14). The low levels of glutamic acid in anaerobically incubated leaves further substantiates the idea that the disappearance of glutamine is not due to just deamination to glutamic acid but is also the result of metabolism to such compounds as γ-aminobutyric acid and α-ketoglutaric acid.

The fact that uncombined asparagine increased under all conditions (table II) whereas uncombined glutamine increased only in wilted leaves under aerobic conditions is further indication of the differences in the metabolism of these compounds (see above discussion). In tobacco, the increase in asparagine has been thought to be due to de novo synthesis associated with ammonia released by oxidative deamination (40, 41) and the same explanation may be applicable to glutamine increase in barley (48). In other reports, the data on protein amino acids are insufficient to eliminate the possibility that the amides were derived by proteolysis. In the turnip leaves, there was no net synthesis of amides (last 2 lines of table II). Consequently, the differences in behavior of uncombined asparagine and glutamine may be explained by different rates of degradation. On this premise, glutamine is more rapidly degraded than asparagine.

Although the effects of incubating excised leaves under various conditions on the amino acid

![Table II. The Relative Content of Certain Amino Acids in Turnip Leaves Subjected to Various Treatments](image-url)
composition are profound, these data cannot provide biochemical explanations. Tracer and enzymological studies are in progress to elucidate the mechanisms.

**Literature Cited**


