Protein decomposition products in the soil
by Carol Shafer

A THESIS Submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree
of Master of Science in Chemistry
Montana State University
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Abstract:
A study was made of the response of tomato plants to treatment of the soil with skim milk, with the
filtrates from an aerobic culture and an anaerobic culture of milk inoculated with soil, and with a
solution of nitrogen plus phosphorus in amounts equivalent to that found in milk.

The weights and measurements of the plants produced were compared. The various treatments gave
comparable values, all of which were much larger than the controls.

Then an investigation was made of the amino acids found in the soil following the application of milk
and of nitrogen plus phosphorus solution as compared to controls. The milk treated soils had much
larger amino acid concentrations, and the plants grown in these soils showed larger yields. The nitrogen
plus phosphorus treated soils had about the same amino acid concentration as the control soils, but the
plants grown therein showed larger yields than the control plants. The possible effects of free amino
acids in the soil upon the growth of tomato plants is discussed.

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CAROL SHAFER

A THESIS
Submitted to the Graduate Faculty
in
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Master of Science in Chemistry
at
Montana State College

Approved:

Head, Major Department

Chairman, Examining Committee

Dean, Graduate Division

Bozeman, Montana
June, 1952
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I. ABSTRACT

A study was made of the response of tomato plants to treatment of the soil with skim milk, with the filtrates from an aerobic culture and an anaerobic culture of milk inoculated with soil, and with a solution of nitrogen plus phosphorus in amounts equivalent to that found in milk. The weights and measurements of the plants produced were compared. The various treatments gave comparable values, all of which were much larger than the controls.

Then an investigation was made of the amino acids found in the soil following the application of milk and of nitrogen plus phosphorus solution as compared to controls. The milk treated soils had much larger amino acid concentrations, and the plants grown in these soils showed larger yields. The nitrogen plus phosphorus treated soils had about the same amino acid concentration as the control soils, but the plants grown therein showed larger yields than the control plants. The possible effects of free amino acids in the soil upon the growth of tomato plants is discussed.
II. INTRODUCTION

The chemical analysis of milk indicated that this material might have some value as a fertilizing material. In 1946 V. E. Iverson of the Department of Horticulture of the Montana Agricultural Experiment Station conducted preliminary experiments to determine the effects of skim milk applied to the soil upon the growth of Bonny Best tomato plants. The results of these experiments and of more extensive experiments conducted by V. E. Iverson (1947, 1948) indicated that the plants had larger and more fibrous root systems, larger and taller stems with greater leaf areas, and increased early and total yields. These preliminary studies also suggested that applications of milk to the soil resulted in some changes in the chemical composition of the soil, and a substantial increase in the biological activity of the soil.

Further experiments were conducted in 1950 by F. C. Dawson to investigate the effect of other protein materials on the growth of tomato plants. The results (Dawson, 1951) showed that the growth was greatly enhanced by gelatin, egg albumin, lactalbumin, casein, wheat starch and gluten.

As a continuation of the previous work, experiments were set up to investigate the decomposition products of milk found in the soil which might be responsible, at least in part, for the accelerated growth of plants. Two separate experiments were conducted. In the first one, a comparison was made of the plant growth after five weekly applications of the following treatments:

1. No treatment - used as a control group.

2. Skim milk.
3. The filtrate from an aerobic milk culture inoculated with soil.
4. The filtrate from an anaerobic milk culture inoculated with soil.
5. A solution of inorganic nitrogen and phosphorus in the same concentrations as found in milk, which will be referred to as N + P.

The second experiment was an attempt to separate and identify the amino acids found present in soil as a result of the decomposition of milk, or from synthesis by soil microorganisms.
III. EXPERIMENTAL PROCEDURE AND RESULTS

PART A - PLANT GROWTH EXPERIMENTS

Into each of two six liter Erlenmeyer flasks were put 4.5 liters of pasteurized skim milk. Each was inoculated with a small amount of soil from a previous experiment in which milk had been applied to the soil at weekly intervals for a period of five weeks. Anaerobic conditions were provided in one culture by placing about a quarter of an inch layer of mineral oil on top of the milk. The flask was stoppered with a cotton plug so that any gases produced could escape. In order to produce maximum aerobic conditions in the other culture, air was continuously bubbled through it. The air was filtered through 2.5 feet of cotton in a piece of two inch glass tubing to remove any air-borne contamination, then bubbled through water to saturate it, and thus decrease the evaporation of the milk. To disperse the air through the milk into numerous small bubbles, it was passed through a two foot length of rubber tubing with small holes along the entire length; this tube was coiled on the bottom of the flask, and held down by tying to it several glass stoppers.

After six days, the two cultures were filtered to remove the coagulated material. The filtrate was then autoclaved to destroy the microbiological activity, and to coagulate the protein materials left in the culture filtrate. The flasks were stoppered with sterile cotton plugs, sealed with parofilm, and stored in a dark place until used, which was about a month. Just before use, the culture filtrates were filtered again to remove the material which had coagulated during autoclaving and during the period of storage. Nitrogen and phosphorus analyses were run on skim
milk, and on both the anaerobic and aerobic culture filtrates, which will henceforth be referred to as the anaerobic and aerobic media. It is acknowledged here that the anaerobic culture was not strictly anaerobic, because no attempt was made to remove the oxygen previously dissolved in the milk.

All solutions were then adjusted to the same nitrogen and phosphorus concentrations as skim milk by adding $\text{NH}_4\text{H}_2\text{PO}_4$ and $\text{NH}_4\text{NO}_3$. Table I, page 8, shows the nitrogen and phosphorus concentration of each solution, and the amounts of $\text{NH}_4\text{H}_2\text{PO}_4$ and $\text{NH}_4\text{NO}_3$ added to each. Since the solutions were acidic, the pH was adjusted to 7 (the pH of skim milk was 7), by the addition of 10 N KOH.

Nitrogen was determined by the Kjeldahl-Wilfarth-Gunning Method as described in the Official Methods of the A.O.A.C. (1950). Phosphorus was determined colorimetrically by the method of Allen (1940).

The soil used in this experiment was a stock soil consisting of 25 per cent sand, 25 per cent well rotted manure, and 50 per cent loam. The plants were grown in the greenhouse. The Bonny Best variety of tomato plants was used. The tomato plants were transplanted on July 17, 1951, ten days after seeding. Transplants were made into four-inch pots, one plant per pot. Treatments with the various solutions were begun the following day, and were as follows: control, skim milk, aerobic medium, anaerobic medium and nitrogen plus phosphorus solution. There were fifteen plants in each treatment, giving a total of seventy-five plants in the experiment.

Treatments were administered to the plants at one week intervals over a period of five weeks. A total of 150 ml was applied to each plant, in increments of 10 ml the first week, 20 ml the second week, 30 ml the third
<table>
<thead>
<tr>
<th>MEDIA</th>
<th>DENSITY</th>
<th>PER CENT NITROGEN</th>
<th>GRAMS OF NITROGEN*</th>
<th>PER CENT PHOSPHORUS</th>
<th>GRAMS OF PHOSPHORUS*</th>
<th>$\text{NH}_4\text{H}_2\text{PO}_4^{**}$</th>
<th>$\text{NH}_4\text{NO}_3^{**}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skim milk</td>
<td>1.027</td>
<td>0.584</td>
<td>6.00</td>
<td>0.099</td>
<td>1.020</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Aerobic</td>
<td>1.015</td>
<td>0.153</td>
<td>1.55</td>
<td>0.061</td>
<td>0.615</td>
<td>1.464</td>
<td>13.220</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>1.019</td>
<td>0.076</td>
<td>0.78</td>
<td>0.037</td>
<td>0.374</td>
<td>2.434</td>
<td>14.086</td>
</tr>
<tr>
<td>N + P</td>
<td></td>
<td></td>
<td>6.00</td>
<td></td>
<td>1.020</td>
<td>3.784</td>
<td>15.828</td>
</tr>
</tbody>
</table>

*Expressed as grams per liter of solution.

**Grams added per liter of solution.
week, 40 ml the fourth week, and 50 ml the fifth week. The positions of
the plants were rotated once a week, to insure similar conditions for all
plants during the course of the experiment.

On August 29, 42 days after the first treatment, the plants were har­
vested. They had been allowed to grow two weeks after the last treatment
so the differences in size between the various treatments would become more
evident.

The plants and soil were removed from the pots, then the soil was wash­
ed from the roots. The height of each plant and the circumference at the
base of the stem of each plant were measured, and the number of branches on
each plant was counted. Then the plants were divided into two portions;
the roots and the tops. Each part was wrapped in cheese cloth, dried, and
weighed.

All the results from this experiment are summarized in the tables and
graphs which follow. Table II on page 10, shows the average weights of the
roots and tops, and the total weights of the plants in each treatment. In­
cluded in this table are the extreme weights found in each treatment. Fig­
ure I, page 11, is a graphic representation of these data. Table III, page
12, sets forth the average plant height, the circumference at the base of
the stem, and the number of branches on each plant, including again the ex­
tremes in each treatment.
<table>
<thead>
<tr>
<th>MEDIA</th>
<th>ROOTS</th>
<th>TOPS</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Variation</td>
<td>Average</td>
<td>Variation</td>
</tr>
<tr>
<td>Control</td>
<td>0.38-0.84</td>
<td>0.55</td>
<td>1.42-2.96</td>
</tr>
<tr>
<td>Milk</td>
<td>0.78-1.68</td>
<td>1.03</td>
<td>5.21-6.52</td>
</tr>
<tr>
<td>Aerobic</td>
<td>0.91-1.36</td>
<td>1.10</td>
<td>5.44-6.60</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>0.87-1.33</td>
<td>1.00</td>
<td>5.35-6.99</td>
</tr>
<tr>
<td>N + P</td>
<td>0.89-1.32</td>
<td>1.06</td>
<td>5.09-7.13</td>
</tr>
</tbody>
</table>

*All weights are in grams
Figure 2

DRY WEIGHTS OF ROOTS AND TOPS, AND TOTAL DRY WEIGHTS OF PLANTS
### TABLE III

MEASUREMENTS AND NUMBER OF BRANCHES ON PLANTS

<table>
<thead>
<tr>
<th>MEDIA</th>
<th>PLANT HEIGHT* Variation</th>
<th>Average</th>
<th>CIRCUMFERENCE AT BASE* Variation</th>
<th>Average</th>
<th>NUMBER OF BRANCHES Variation</th>
<th>Average</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5-9</td>
<td>6.9</td>
<td>0.8-1.0</td>
<td>0.9</td>
<td>7-11</td>
<td>8</td>
</tr>
<tr>
<td>Milk</td>
<td>9-12</td>
<td>10.8</td>
<td>1.0-1.3</td>
<td>1.1</td>
<td>8-13</td>
<td>11</td>
</tr>
<tr>
<td>Aerobic</td>
<td>10-13</td>
<td>10.4</td>
<td>1.1-1.3</td>
<td>1.1</td>
<td>11-14</td>
<td>13</td>
</tr>
<tr>
<td>Anaerobic</td>
<td>10.5-13</td>
<td>11.7</td>
<td>1.0-1.3</td>
<td>1.1</td>
<td>11-15</td>
<td>13</td>
</tr>
<tr>
<td>N + P</td>
<td>9.5-13</td>
<td>10.9</td>
<td>1.0-1.3</td>
<td>1.1</td>
<td>9-13</td>
<td>12</td>
</tr>
</tbody>
</table>

*Expressed in inches
PART B - FREE AMINO ACIDS IN THE SOIL

The second experiment was set up to study the amino acids found in the soil after different treatments. Tomato plants were allowed to grow in the soils because the presence or absence of root systems might influence the activity in the soil. The soil used in this experiment was the same as that used previously, and the tomato plants were again the Bonny Best variety. There were three treatments: controls, milk treated plants, and plants treated with the nitrogen plus phosphorus solution. The nitrogen plus phosphorus solution was the same as that used in the first experiment. It contained $\text{NH}_4\text{H}_2\text{PO}_4$ and $\text{NH}_4\text{NO}_3$; the amounts are given in Table I, page 8.

Each plant received a total of 150 ml of solution in increments of 10 ml the first week, 20 ml the second, 30, 40, and 50 ml the third, fourth, and fifth weeks, respectively. On November 16, 1951, about 12 days after seeding, 150 plants were transplanted, 50 plants in each of the three treatments. The next day they received the first application. Two days after application three samples of soil were taken from each treatment. In removing the plants from the soil, care was taken to remove all the roots from the soil. Per cent moisture was determined for each soil sample. Then the soils were extracted in the following manner. One hundred grams of each soil were weighed out, mixed with 30 ml of water and filtered with suction. After filtration an additional 20 ml of water were added and the mixture was filtered again; this was repeated a third time. The combined filtrates were evaporated to dryness on the sand bath at 100°C, taken up in 10 ml of 10 per cent isopropyl alcohol and transferred to small vials, then evaporated to dryness again. The residues were taken up in one ml of
10 per cent isopropyl alcohol per 70 grams of dry soil. These final extracts were stored in the refrigerator. The remainder of the soil was air dried and stored, in the event that more tests should be necessary.

Six days after application (the day preceding the next application) a second set of three samples was taken from each treatment, and handled in the same manner as the preceding one. This was repeated each week during the five weeks of treatment. Two additional samples from each treatment were taken the sixth week and two more the eighth week.

On January 17, 1952, eight plants from each treatment were transplanted into eight-inch pots. They were all in full bloom, and a few of those from the milk and nitrogen plus phosphorus treatments had small fruit on them. These twenty-four plants were allowed to grow through fruition. As the fruit ripened it was harvested and a record kept of the day it was picked, which plant it was from, and the weight of each tomato. Table IV, page 15, gives a summary of these data. It is also illustrated graphically in Figure 2, page 16. The nitrogen plus phosphorus treated plants produced a 22 per cent larger yield than the control, while the milk treated plants had a 43 per cent larger yield than the control. The milk treated plants produced a 17 per cent larger yield than the nitrogen plus phosphorus treated ones.

After all the soil samples had been collected and extracted, work was begun on the separation and identification of the amino acids present in each extract. Paper chromatography was used. The extracts did not show any amino acids. Since some of them had been stored for over two months, it is possible the amino acids were destroyed in some way. Therefore, samples of the soil were extracted again just before running chromatograms
| Days After First Picking | CONTROL | | | | MILK |
|-------------------------|---------|---------|---------|---------|
|                         | Number* | Weight* in grams | Number* | Weight* in grams | Number* | Weight* in grams |
| 1                       | 1       | 79      | 2       | 189      | 3       | 253      |
| 4                       | 4       | 249     | 15      | 1,314    | 13      | 1,129    |
| 7                       | 5       | 336     | 16      | 1,431    | 25      | 2,241    |
| 12                      | 10      | 832     | 21      | 1,827    | 28      | 2,195    |
| 14                      | 15      | 1,489   | 28      | 2,632    | 37      | 3,560    |
| 16                      | 20      | 2,059   | 31      | 2,918    | 47      | 4,662    |
| 18                      | 25      | 2,647   | 38      | 3,597    | 53      | 5,386    |
| 21                      | 33      | 3,660   | 40      | 3,917    | 63      | 6,786    |
| 23                      | 40      | 4,616   | 47      | 4,694    | 78      | 8,504    |
| 26                      | 52      | 6,106   | 60      | 6,092    | 88      | 9,717    |
| 28                      | 56      | 6,583   | 68      | 6,733    | 91      | 10,022   |
| 30                      | 58      | 6,899   | 70      | 7,052    | 96      | 10,718   |
| 32                      | 64      | 7,555   | 77      | 7,956    | 100     | 11,257   |
| 34                      | 69      | 8,234   | 83      | 8,512    | 105     | 11,679   |
| 37                      | 76      | 9,662   | 100     | 10,085   | 124     | 14,471   |
| 39                      | 84      | 10,389  | 117     | 13,266   | 136     | 16,039   |
| 41                      | 92      | 11,149  | 123     | 14,097   | 144     | 16,822   |
| 45                      | 98      | 11,840  | 127     | 14,465   | 146     | 16,950   |

*All figures are cumulative values.
FRUIT HARVESTED FROM TOMATO PLANTS

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Tomatoes</th>
<th>Kilograms of Tomatoes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>N + P Milk</td>
<td>200</td>
<td>15</td>
</tr>
<tr>
<td>Milk</td>
<td>250</td>
<td>20</td>
</tr>
</tbody>
</table>

Number of Tomatoes

Kilograms of Tomatoes
on them. They were extracted in the same manner as the first time, the only change being that warm water was used instead of water at room temperature.

Chromatographic analysis depends upon the difference in the partition coefficients of the components of a mixture between the liquid or mobile phase, and the non-liquid or stationary phase. The mixture to be resolved is absorbed at the edge of a sheet of filter paper. The components of the mixture migrate up the paper with the fresh solvent or mixture of solvents which rise by capillary action. Depending upon their partition coefficients between the two phases, the solutes migrate upward at different rates, and separate one from another. Part of the solute is absorbed on the stationary phase and part is dispersed in the liquid phase; there is an equilibrium of the partition of solutes between the two phases. (Strain, 1950). Once the components are resolved, they can be identified by their individual $R_f$ values. The $R_f$ value is the ratio of the distance traversed by the solute to the distance traversed by the solvent. With a given solvent each substance has a specific $R_f$ value, the values varying with different solvents and conditions. Solvents must be chosen so that the solute with the largest $R_f$ value is not so large that this component will be off the chromatogram before the components with smaller $R_f$ values are separated sufficiently. (Clegg, 1950; Longenecker, 1949; Strain, 1950). Often several solvents, or mixtures of solvents, on different chromatograms are necessary for the complete separation and identification of all the components of a mixture. It has been found possible to separate some amino acids, otherwise difficult to separate, by employing solvents buffered at
different pH's. The filter paper used for the chromatogram is also buffered at the selected pH (McFarren, 1951).

Since amino acids are colorless, in order to visually locate the positions of the amino acids on the chromatogram it is necessary to develop it, i.e., treat it with some substance which will react with the amino acids and produce a color. Ninhydrin is the substance used.

Considerable research has been done, trying to determine the mechanism of the reaction between ninhydrin (triketohydrindene hydrate) and amino acids or proteins. Copley (1941) suggested that the ninhydrin (I) is reduced to 2-hydroxydiketohydrindene (1,2-diketohydrindol)(II).

\[
\begin{align*}
\text{I} & \quad \text{II} \\
\end{align*}
\]

He is of the opinion that the reaction proceeds as Ruhemann (1911) suggested. The 2-hydroxydiketohydrindene (II) reacts with a molecule of ammonia and one of unreduced ninhydrin to give diketoindanyldene-diketoindanylamine (III).

\[
\begin{align*}
\text{II} & \quad \text{I} & \quad \text{III} \\
\end{align*}
\]

The violet color is due to the diketoindanyldene-diketoindanylamine (III). Harding and MacLean (1916) believe the reaction proceeds a little differently giving, finally, the same products. They believe the amino acid dissociates to give ammonia and the corresponding glyoxal.
The glyoxal reduces ninhydrin to 2-hydroxydiketohydrindene which condenses with the ammonia to give 1,2-diketohydrindamine and water. This combines with another molecule of ninhydrin to give diketoindanylidene-diketoindanylamine, as Ruhemann proposed.

This may be part of the explanation, but it hardly seems to be the complete reaction. According to this, if it is only the ammonia which goes to help form the color, all amino acids should give the same color—the blue-violet color of diketoindanylidene-diketoindanylamine. Schönberg, Moubasher, and Mostafa (1940) suggest a possible mechanism in which the amino acids are further involved in the reaction.

Each of the compounds IV-VII could be a color contributor. The reaction could then proceed as follows: 2-hydroxydiketohydrindene (VII) could react with ninhydrin to yield hydrindantine (VIII) which by further reaction with \( \alpha \)-amino acids could form bis-1,3-diketoindanyl (IX). Hydrindantine and bis-1,3-diketoindanyl have both been isolated from the ninhydrin reaction (Schönberg, 1948).
It has been reported (Moubasher and Othman, 1950) that when ninhydrin is treated with primary amines, an intense blue coloration is developed. An interpretation of this reaction is based on the mechanism just described. It is believed these primary amines react in a manner similar to \(\alpha\)-amino acids, first with ninhydrin to give 2-hydroxydiketohydridene, which again reacts with ninhydrin to give hydrindantine. The intense blue coloration is due to the double salt formed by the action of the amine present in
excess with the reduction product of ninhydrin, the hydrindantin. Abderhalden (1930) did a great deal of experimental work with the reactions of ninhydrin with various amino acids. He reports that regardless of the amino acid allowed to react with ninhydrin, he was able to isolate hydrindantine or a compound which was like it in all the properties he checked and which had the same percentage composition.

The procedure used for setting up the chromatograms, except for a few variations, was very similar to that described by Block (1950). With each solvent or mixture of solvents, standards were run to determine $R_f$ values of each amino acid. The chromatograms were resolved in air tight glass cabinets made from regular aquaria, 27 cm x 31 cm x 51 cm, covered with a glass plate with sponge rubber gaskets around the edge. Two glass rods running the length of the cabinet were fastened with rubber suction cups near the top of the cabinet and about 4 cm out from the side walls. The solvent was placed in glass troughs about 48 cm long. See Figure 3, page 22. For the chromatograms, S&S No. 589 Green Label filter paper was used. The sheets were cut into strips about 25 by 58 cm. The soil extract to be resolved was placed 5 cm from the end, the drops usually being about 2.5 cm apart. The drops were applied with a microburette, uncalibrated since this was not quantitative work, but graduated so that equal amounts of the different extracts could be applied. After the spots were dry, the end of the paper to which the drops were applied was placed in the trough containing the solvent, and held down with mercury-filled glass rods. Care was taken that the area where the extracts were applied was not submerged in solvent. The other end of the paper was hung over the rod near the top
Figure 3

GLASS CABINET USED FOR RESOLUTION OF CHROMATOGRAMS
of the cabinet, and weighted down with Castaloy spring clamps. Sufficient solvent was placed in the trough to keep the atmosphere in the cabinet saturated with respect to the solvent. Since the solvents were all saturated with water (or buffer) excess water and solvent was poured on the floor of the cabinet, and the walls of the cabinet were lined with paper towels, the edges of which were allowed to touch the bottom, so the solvent and water there would rise up the paper. This was to insure complete and rapid saturation of the atmosphere with respect to water and the solvent. The cabinets were kept in a constant temperature box at 20°C during the run to provide uniform conditions.

Four different solvents were used to resolve the chromatograms. Thereby a complete separation of several amino acids was effected. The solvents used were prepared and employed as follows:

1. Phenol, unbuffered.

Water and melted phenol were mixed in the ratio 1:4. A small beaker containing sodium cyanide solution was placed in the cabinets. The sodium cyanide inactivates those substances which might cause oxidation of the phenol, and consequent darkening of the chromatogram.

2. Phenol, pH 12

Melted phenol was saturated with buffer at pH 12. The buffer was made up by mixing one part of .067 M Na₂HPO₄ and one part .067 M NaOH. The papers used for the chromatograms were also treated with the same buffer. This was accomplished by dipping the paper in the buffer, then drying it before applying the extract. A
small beaker containing sodium cyanide solution was placed in the cabinet.

3. m-Cresol

The m-Cresol was saturated with water before use.


This was a mixture of one part benzyl alcohol and one part n-butyl alcohol. The solvent was again saturated with water before use.

The length of time required for the resolution of the chromatogram depends upon the solvent used. For each of the four solvents used in this experiment about 24 hours was required. The chromatogram was allowed to run until the solvent had nearly reached the far end of the paper. The solvent was completely evaporated from the chromatograms before developing, since some solvents will give color reactions with ninhydrin. The ninhydrin was dissolved in acetone, in a concentration of approximately 25 per cent. The chromatogram was dipped in this solution, then heated slightly for five minutes. When buffered phenol was used as the solvent, and the paper was buffered, a slight change was made in the ninhydrin solution. It was made up in methyl cellulose, the same concentration as in acetone, with about one per cent acetic acid added. If the ninhydrin solution was not acidic, some of the basic amino acid failed to develop any color.

Chromatograms were run on all the extracts immediately after extraction. Four different chromatograms were run on each extract using the four solvents described above. As soon as the chromatograms were developed a rough comparison was made between the concentrations of the various amino
acids in the different treatments. It was necessary to do this soon after
developing, because upon prolonged standing, the color fades and finally
disappears entirely.

The results of the chromatographic separations are illustrated in the
pictures and diagrams which follow.

Figures 4, 5, and 6 on pages 26, 27, and 28 are photographs of typical
chromatograms with benzyl-butyl alcohol, m-cresol, and unbuffered phenol as
solvents.

Figures 7, 8, 9, and 10 on pages 29, 30, 31 and 32 are diagrams of
representative chromatograms with each of the four solvents or solvent mix­
tures used throughout. Included on these diagrams is a comparison of the
concentrations of the amino acids within the treatments, and also between
the three treatments. The amount of an individual amino acid or a mixture
of amino acids present on a chromatogram is determined by observing the size
of the colored spot formed by reaction with ninhydrin, and the intensity of
the color produced. These chromatograms indicate that the concentrations
of amino acids in the nitrogen plus phosphorus treated soil, and in the
control soils were about the same. This was true except for the samples
taken during the fourth and fifth weeks of treatment. In these, the con­
centrations of amino acids in the nitrogen plus phosphorus treated soil
was slightly greater than in the controls. However, in the samples taken
the week following the final treatment, the amino acid concentrations were
again about the same in the nitrogen plus phosphorus treated soil as in
the controls. The highest concentrations of all amino acids observed were
noted in extracts from the milk treated soils.
POSITIONS OF AMINO ACIDS ON CHROMATOGRAMS:
1. Arginine, Histidine, Lysine
2. Unidentified
3. Aspartic, Glutamic, Glycine, Threonine, Serine
4. Alanine
5. Tyrosine, Methionine, Valine
6. Leucine, Iso-leucine, Phenyl alanine, Tryptophane

Figure 4
PHOTOGRAPH OF CHROMATOGRAM
SOLVENT IS BENZYL-BUTYL ALCOHOL
Positions of Amino Acids on Chromatograms:

1. Unidentified
2. Arginine, Aspartic, Lysine
3. Glutamic, Serine, Glycine
4. Alanine
5. Tyrosine
6. Valine
7. Methionine
8. Leucine, Isolecine, Phenylalanine, Tryptophane

Figure 5

Photograph of Chromatogram

Solvent is m-Cresol
POSITIONS OF AMINO ACIDS ON CHROMATOGRAMS:

1. Unidentified
2. Unidentified
3. Unidentified
4. Aspartic
5. Arginine, Lysine, Serine
6. Glycine, Glutamic, Threonine
7. Alanine, Tyrosine
8. Leucine, Isoleucine, Phenylalanine, Tryptophane, Valine

Figure 6
PHOTOGRAPH OF CHROMATOGRAM
SOLVENT IS UNBUFFERED PHENOL
LEGEND:
Pinkish-red  
Medium Blue  
Light Blue  
Very Faint Blue

POSITIONS OF AMINO ACIDS ON CHROMATOGRAMS:
1. Arginine, Histidine, Lysine
2. Unidentified
3. Aspartic, Glutamic, Glycine, Threonine, Serine
4. Alanine
5. Tyrosine, Methionine, Valine
6. Leucine, Iso-leucine, Phenyl alanine, Tryptophane

Figure 7
DIAGRAMATIC REPRESENTATION OF A CHROMATOGRAM WITH BENZYL-BUTYL ALCOHOL AS THE SOLVENT
### Positions of Amino Acids on Chromatograms:

1. Unidentified
2. Arginine, Aspartic, Lysine
3. Glutamic, Serine, Glycine
4. Alanine
5. Tyrosine
6. Valine
7. Methionine
8. Leucine, Iso-leucine, Phenyl alanine, Tryptophane

### Legend:
- Dark Red
- Pink
- Medium Blue
- Light Blue
- Very Faint Blue

---

**Figure 8**

**Diagramatic Representation of a Chromatogram with m-Cresol as the Solvent**
LEGEND:
Red
Pink
Dark Blue
Medium Blue
Light Blue

POsITIONS OF AMINO ACIDS ON CHROMATOGRAMS:
1. Unidentified
2. Unidentified
3. Unidentified
4. Aspartic
5. Arginine, Lysine, Serine
6. Glycine, Glutamic Threonine
7. Alanine, Tyrosine
8. Leucine, Iso-leucine, Phenyl Alanine, Tryptophane, Valine

Figure 9
DIAGRAMATIC REPRESENTATION OF A CHROMATOGRAM WITH UNBUFFERED PHENOL AS THE SOLVENT
LEGEND:
- Very Dark Blue
- Dark Blue
- Medium Blue
- Light Blue

POSITIONS OF AMINO ACIDS ON CHROMATOGRAMS:
1. Unidentified
2. Aspartic
3. Glutamic, Serine
4. Glycine
5. Alanine, Threonine
6. Tyrosine, Lysine
7. Arginine, Leucine, Iso-leucine, Phenyl alanine, Methionine, Histidine, Valine

Figure 10
DIAGRAMATIC REPRESENTATION OF A CHROMATOGRAM WITH PHENOL, pH 12, AS THE SOLVENT
Figures 6, 7, 8, and 9 also include the identification of the amino acids by their positions on the chromatograms. Chromatograms were run with known amino acids to identify the positions.
IV. DISCUSSION OF RESULTS

PART A

The average weights and measurements of the plants from each treatment were compared. The only significant differences were observed between the controls and the other treatments. The controls weighed less than half as much as the others, and were smaller plants in every way. The differences in weights and measurements observed between the plants of the various treatments were not great enough to be significant. However, since the aerobic culture filtrate and anaerobic culture filtrate produced plants of a size comparable to those produced by the milk, it seemed that the decomposition products in these media might play an important role in the growth acceleration. Had the plants been allowed to grow through fruition, some more significant differences might have been noted.

PART B

Six amino acids were completely separated by means of the chromatograms. They were alanine, aspartic acid, glycine, methionine, tyrosine and valine. Glutamic acid and serine both appeared to be present, but were not completely separated. On the chromatograms with benzyl-butyl alcohol as the solvent, there appeared a spot which may have been a mixture of arginine, histidine, and lysine, or any one or two of the three. Regardless of the solvent used, there always appeared a spot which may have been leucine, iso-leucine, phenyl alanine, and tryptophane, all of them or any combination of them. Besides these amino acids not completely separated and identified, there appeared in the extracts from the milk treated and the nitrogen plus phosphorus treated soils a red or pinkish-red spot, which
never was identified. When the solvent for resolving the chromatogram was
phenol, pH 12, it appeared as a blue spot. The substance was never en­
countered in the extracts from the control soils. The \( R_f \) value of this
unidentified substance was smaller than that of any of the amino acids,
except on chromatograms with benzyl-butyl alcohol as solvent. On those,
three basic amino acids (arginine, histidine and lysine) did not migrate
upward at all. The unidentified substance may have been a di-, tri- or
polypeptide.

Since free amino acids were definitely demonstrated to be present in
the soil, two possible sources may be postulated. They may have come from
decomposition of organic materials in the soil, or they may have been
synthesized by microorganisms in the soil from the inorganic and organic
materials present. It seems likely that both play a part. The fact that
there was a slightly greater concentration of amino acids in the nitrogen
plus phosphorus treated soils than in the controls during the fourth and
fifth weeks of treatment was probably due to increased activity of micro­
organisms.

It is evident from the illustrations given that the milk treated soils
always had the greatest concentration of amino acids. This was to be ex­
pected, since amino acids are the hydrolytic products of proteins, and milk
contains considerable protein material. Enzymes from the microorganisms
in the soil probably hydrolyzed the proteins into their constituent amino
acids. However, it is not those amino acids appearing in large concentra­
tions which alone present a point of interest. Those amino acids which
appeared in only small concentrations or not at all, but which, from a
consideration of the composition of the proteins found in milk, should have been present in very large concentrations provide considerable opportunity for speculation. The protein fraction of milk is about 0.1 per cent lactoglobulin, 16 per cent lactalbumin, and the remainder casein (Jacobs, 1944). From a study of the amino acid composition of the milk proteins listed in Table V, page 37, (Block and Bolling, 1945) there should have been a very large concentration of glutamic acid and of leucine and iso-leucine present in the soil extracts. This was not found to be true. There did not seem to be as great amounts of these amino acids as there were of aspartic acid and alanine, which are present in much smaller concentrations in the milk proteins. It is possible that the amino acids found in lesser quantities than was expected had been absorbed by the plant roots. Bonner (1947) reports that when excised sections of Avena coleoptile were placed in solution of arginine, methionine, or glutamic acid, the growth was increased. White (1937), in a study of nutrition of excised tomato roots, found that histidine, phenylalanine, lysine, leucine, iso-leucine, valine, glutamic acid, proline, and serine were either essential for, or beneficial to tomato roots. In view of these reports, it seems quite likely that the tomato plants use the amino acids produced in the soil. Bonner's and White's investigations were all carried out in sterile cultures. In contrast, these experiments were conducted in soil where the microorganisms present in all probability played an important role. Therefore, in addition to direct utilization by the plant, the amino acids from the milk proteins may have been transformed (deaminated, decarboxylated, or transaminated) or subjected to a complete resynthesis into other materials by the microorganisms of the soil.
### TABLE V*

**APPROXIMATE PERCENTAGE OF AMINO ACIDS IN MILK PROTEINS**

**CALCULATED TO 16 PER CENT NITROGEN**

<table>
<thead>
<tr>
<th>Amino Acids</th>
<th>Casein</th>
<th>Lactalbumin</th>
<th>Lactoglobulin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>4.1</td>
<td>3.5</td>
<td>3.1</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.5</td>
<td>2.0</td>
<td>1.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>6.9</td>
<td>8.0</td>
<td>10.4</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>6.4</td>
<td>5.3</td>
<td>4.3</td>
</tr>
<tr>
<td>Tryptophane</td>
<td>1.8</td>
<td>2.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>5.2</td>
<td>5.6</td>
<td>5.3</td>
</tr>
<tr>
<td>Cystine</td>
<td>0.36</td>
<td>3.0</td>
<td>3.6</td>
</tr>
<tr>
<td>Methionine</td>
<td>3.5</td>
<td>2.8</td>
<td>3.6</td>
</tr>
<tr>
<td>Serine</td>
<td>6.7</td>
<td>4.9</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>3.9</td>
<td>5.3</td>
<td>6.0</td>
</tr>
<tr>
<td>Leucine</td>
<td>12.1</td>
<td>15.**</td>
<td>17.7</td>
</tr>
<tr>
<td>Iso-leucine</td>
<td>6.5</td>
<td></td>
<td>6.6</td>
</tr>
<tr>
<td>Valine</td>
<td>7.0</td>
<td>4.</td>
<td>7.9</td>
</tr>
<tr>
<td>Glutamic Acid</td>
<td>22.8</td>
<td></td>
<td>22.1</td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>6.3</td>
<td></td>
<td>10.1</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alanine</td>
<td>5.6</td>
<td>0-1</td>
<td></td>
</tr>
<tr>
<td>Proline</td>
<td>8.2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Block and Bolling (1945)

**Leucine and iso-leucine**
It seems quite possible that there may have been some direct utilization of the amino acids from the hydrolysis of the milk proteins because the plants growing in the milk treated soil showed a 17 per cent larger yield than the plants in the nitrogen plus phosphorus treated soil.

This coincides with Bonner's reports (1949) that Avena coleoptiles showed increased growth with arginine. He studied the effects of ammonia and nitrate, and found that they were ineffective in replacing arginine. With glutamic acid and methionine the results were duplicated in part, but the increased growth was only 50 per cent as great as with arginine.

While not conclusive, there is considerable evidence that amino acids in the soil may, in part, account for the growth stimulation and the increased yield observed.
V. SUMMARY

It had been observed in the past that skim milk applied to the soil in which tomatoes were grown, accelerated the growth, gave larger plants, and increased yields. Experiments were set up to investigate the decomposition products of milk in the soil which might be responsible for the accelerated growth of plants.

In the first experiment a comparison of plant growth was made after five treatments were carried out: control; skim milk; filtrate from an aerobic, soil inoculated milk culture; filtrate from an anaerobic, soil-inoculated milk culture; and a solution of \( \text{NH}_4\text{NO}_3 \) and \( \text{NH}_4\text{H}_2\text{PO}_4 \). All solutions were adjusted to the same nitrogen and phosphorus concentration as milk.

The soil used was a stock soil consisting of 25 per cent sand, 25 per cent well rotted manure, and 50 per cent loam. The Bonny Best variety of tomatoes was used. A total of 150 ml of media was applied to the soil for each plant.

It was found that the weights and measurements of the control plants were much less than the plants from the other treatments. There were no significant differences between the other treatments. However, since the aerobic and anaerobic media produced plants of about the same size as the milk, the hydrolytic products of milk in these media might play an important part in growth acceleration.

The second experiment was a study of amino acids found present in the soil following various treatments. Three treatments were continued: milk, nitrogen plus phosphorus, and controls. The procedure was the same as for the first experiment.
Soil samples were taken the day following treatment, and six days following treatment. These samples were extracted with water. By means of paper chromatography the amino acids were partially separated and identified, and a rough quantitative comparison made between the treatments. The milk treated soil always contained the greatest concentration of amino acids, while the nitrogen plus phosphorus treated soil and the control soil had about the same amino acid concentration.

Eight plants from each treatment were allowed to grow through fruition. The plants in the milk treated soil showed a 43 per cent larger yield than the controls; the plants in the nitrogen plus phosphorus treated soil showed a 22 per cent larger yield than the controls.

It is thought that the accelerated growth and increased yield of the plants in the milk treated soil are due, in part, to utilization of amino acids present in the soil as a result of micro-biological decomposition of the milk proteins.
ACKNOWLEDGEMENTS

The author wishes to take this opportunity to express her appreciation and thanks to Dr. Leon H. Johnson for his help and suggestions; also to Marion Breeden for his aid in the care of the plants.
LITERATURE CITED

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