In recent years comparative studies have been made between chlorotic plants and healthy plants to determine if any consistent distortion of the normal metabolic pattern occurs irrespective of the cause of chlorosis. Experiments by Iljin (6) indicated a greater amino acid and organic acid content in chlorotic leaves from grape plants grown on calcareous soil than in normal green leaves. DeKock et al (4) also found that significantly larger amounts of free amino acids and organic acids were associated with tissue in which chlorosis was present. Rhodes et al (11) observed that aspartic and glutamic acids which are formed directly from organic acids were present in high concentrations in lime-induced chlorotic bean leaves.

From earlier studies we may assume that the disorders in metabolism associated with chlorosis in higher plants, such as blocks in the pathways for synthesizing amino acids and proteins from organic acids, or abnormal operations of the TCA cycle are either causative factors or the result of chlorosis. In this study, in order to gain more evidence for the mechanisms by which chlorosis is induced, comparative studies on the organic acid fractions between chlorotic and healthy leaves were conducted. Chlorosis was induced in the experimental plants by high phosphorous, manganese, bicarbonate, or iron deficiency.

### Materials & Methods

**Experimental Plants.** *Glycine max* (L.) Merr. variety PI-54619-5-1 (PI) soybean was selected as an experimental plant because of its sensitivity to iron chlorosis by mineral deficiencies or excesses. Hawkeye (HA) soybean was also used in one experiment to compare with the less resistant PI variety.

The soybeans were germinated on cheesecloth in half strength Hoagland’s nutrient solution (5), and then transferred to containers having the same nutrient composition. In one experiment the plants were transferred to pots containing pearlite (expanded pumice) and irrigated daily with nutrient solution. When seedlings were about ten days old, they were transplanted to aerated nutrient solutions in growth chambers where light intensity, day length, and temperature were controlled. Plants growing in perlite were not transplanted but irrigated daily with the treatment nutrient solution. Leaves from the plants were collected between 2 and 3 PM on the day of harvest to reduce environmental influences of organic acid content.

**Culture Methods.** *Iron deficiency induced chlorosis:* The nutrient solution consisted of the following concentration of minerals: (meq/l) Ca 2, Mg 1, K 2.2, NH₄ 0.5, NO₃ 5.2, SO₄ 0.5; (ppm) P 6.0, Mn 0.08, B 0.04, Zn 0.03, Cu 0.01, Mo 0.01, and Cl 5.0. Iron was added to the control plants at the rate of 7.5 ppm and omitted from the iron deficiency treatments; the solution was maintained at pH 5.5 by additions of NaOH or HCl when required. PI soybeans were grown in pearlite (expanded pumice) in liter plastic containers and irrigated daily with nutrient solution as indicated above. Half of the plants received complete solution and half received solutions deficient in iron. Plants were harvested after 6 weeks when chlorosis first became evident. Apparently pearlite contained iron contaminations which delayed the onset of symptoms. Organic acid determinations from this experiment are denoted in table I as sample A.

### Table I

| Organic Acids in Leaves From Normal & Iron-Deficient Chlorotic Plants |
|-----------------------------|------------------------|------------------------|------------------------|
| Sample treatment            | A*                     | B**                    |
| fr wt basis (meq/g of leaf) | Normal Chorotic        | Normal Chorotic        |
| Fumaric acid                | 8.8                    | 4.9                    | 3.3                    | 2.4                  |
| Succinic                    | 4.9                    | 2.3                    | 19.6***                | 5.6                  |
| Malonic                     | 36.3                   | 16.6                   |                        |                      |
| Malic                       | 29.3                   | 17.4                   | 7.4                    | 6.6                  |
| Citric                      | 37.5                   | 43.8                   | 24.2                   | 30.6                 |
| Total                       | 116.8                  | 85.0                   | 54.5                   | 46.2                 |
| ppm Iron in leaves          | 54                     | 40                     | 62                     | 44                   |

* Plants were grown in pearlite and irrigated daily with nutrient solution containing 7.5 ppm Fe for normal plants and no added Fe for chlorotic plants.
** Plants were grown in nutrient solutions containing 7.5 ppm Fe for normal plants and no added Fe for chlorotic plants.
*** Combined concentrations of succinic and malonic acids.

---

1 Received December 12, 1960.
2 This work was supported in part by National Science Foundation grant G-5891. Approved as Utah State Agricultural Experiment Station Journal Paper no. 159.
Plants were also grown in nutrient solution but received the same nutrient solution treatment as sample A. Chlorosis occurred after 3 weeks, at which time the plants were harvested. Experiments from this treatment are denoted as sample B in Table I.

Bicarbonate-induced chlorosis: The nutrient solution consisted of the same concentration of minerals as listed for the iron deficiency experiment with the exception of Fe. Iron was added at the rate of 3.5 ppm. For bicarbonate treatments 10 meq/l of NaHCO₃ were added and controls contained 10 meq/l of NaCl. In the bicarbonate treatment pH 7.8 was maintained by aerating with 1% CO₂ in air; controls were maintained at pH 7.8 by daily additions of NaOH. PI soybeans were grown in nutrient solution with bicarbonate treatments as listed above. The organic acid analyses of the leaves of the PI soybeans subjected to the bicarbonate treatment for 10 and 14 days in two separate experiments are found in Table II, as samples A and B. Hawkeye soybeans, a more chlorosis resistant soybean than PI, were subjected to the same treatment conditions for 11 days. These results are found under column C in Table II.

High phosphorus induced chlorosis: The nutrient solution consisted of the same concentration of minerals as listed for iron deficiency experiments except for Fe and P concentrations. High phosphorous treatments received 10 ppm P and 3.5 ppm Fe; controls contained 0.05 ppm P and 3.5 ppm Fe. The pH of the nutrient solution was maintained at 5.5. Chlorosis was induced in PI soybeans by the high phosphorous treatment. Plants were harvested 20 days after treatment initiation when chlorotic symptoms first became evident. Organic acid analyses are found in Table III.

High manganese induced chlorosis: The nutrient solution consisted of the following nutrients: (meq/l) Ca 10, Mg 4, K 5, NO₃ 10, SO₄ 4; (ppm) P 16, B 0.25, Zn 0.01, Cu 0.01, Mo 0.015, Fe 3.5 and Cl 5. High Mn treatments contained 10 ppm P and the controls received 0.25 ppm Mn; pH of the solution culture was maintained at 5.5. Leaves from PI soybeans were harvested 8 and 12 days after treatment initiation in two separate experiments. Organic analyses of these experiments are reported in Table IV, under samples A (8 day) and B (12 day).

Table III
Organic Acids in Leaves From Normal & High Phosphorus Chlorotic Plants

<table>
<thead>
<tr>
<th>Sample treatment</th>
<th>Acids</th>
<th>(meq/g of leaf)</th>
<th>fr wt basis</th>
<th>Normal</th>
<th>Chlorotic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fumaric</td>
<td>1.0</td>
<td>3.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinic</td>
<td>8.1**</td>
<td>6.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malic</td>
<td>3.7</td>
<td>5.2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Malic</td>
<td>17.7</td>
<td>28.4</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>30.5</td>
<td>45.7</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ppm Iron in leaves</td>
<td>100</td>
<td>32</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Chlorosis was induced by growth in nutrient solution containing 10 ppm P at pH 5.5. Normal plants were grown in solutions containing 0.05 ppm P in the growing medium. Nutrient solutions were changed every 5 days.
** Combined concentrations of succinic and malonic acids.
The extract was passed through a permutit Q column (28 cm × 1.5 cm) in which the amino acids were absorbed. The effluent from the permutit Q column was run through an Amberlite IR4B column, which absorbed the organic acids. The effluent was discarded. The organic acids were eluted from the Amberlite column with 200 ml of a n-NH₃ solution, and the eluate was concentrated to a small volume in vacuo at 50°C and made up to 10 ml. A portion of this was used for partition chromatography analysis.

Silica gel partition chromatography with a gradient elution technique was used for separation of organic acids in leaf extracts. The concentration of organic acids eluted was determined by titration with standard alkali. Silica gel was prepared from reagent grade sodium metasilicate essentially as described by Neish (10). However, to obtain sufficiently high flow rates on the column it was necessary to remove the finer particles by suspending the material repeatedly in water and decanting the upper liquid after the coarse material had settled. In this experiment the final pH of the washings was about 8.0.

The silica gel column was contained in a glass tube 25 cm long and 1.2 cm internal diameter. Four grams of dry silica gel were mixed with 3.5 ml of 1.0 N H₂SO₄ and slurred with 20 ml of CHCl₃. The slurry of silica gel was poured in gradually and transferred to the chromatographic tube in five or six portions so that about a four centimeter section of the bed was formed at one time. The gel was packed by inserting a circle of filter paper, pressed with glass tubing and adjusted to a final height of 18 cm.

The solvent mixtures employed were 2% and 50% (v/v) reagent grade tert-amyl alcohol in CHCl₃ (afterwards referred to as CA2 & CA50, resp.) The CHCl₃ was washed with distilled water to remove ethanol. The mixtures of CA2 and CA50 were equilibrated with 0.1 N H₂SO₄ and passed through filter paper to remove suspended water droplets.

Two milliliters of the sample solution were made alkaline to thymol blue and evaporated to dryness on a boiling water bath. The residue was cooled and dissolved in 1.5 N H₂SO₄ (about 1.3 ml) to adjust the pH 1 to 2 with pH paper; 0.7 g of silica gel was mixed with the sample to give a free-floating powder. This was transferred to the top of the column, washed down and slurried with a small volume (5 ml) of CHCl₃ and packed under a filter paper disk. The tube was then filled with solvent mixture CA2.

The simple arrangement used for gradient elution consisted of two reservoirs. The recipient was a glass bottle of 250 ml capacity. The top of this bottle was connected to a 500 ml separatory funnel by a rubber cork. The recipient bottle contained 235 ml of CA2 and the funnel contained 450 ml of CA50. The column was fed from the recipient reservoir, which was stirred magnetically. Such a system delivered solvent continuously changing in concentration. By mounting the reservoirs about 80 cm above the column sufficient pressure was obtained to give a flow rate of approximately 18 ml/hour. The effluent was collected in 3.5 ml fractions by a drop counter fraction collector (fig 1).

The 3.5 ml effluent collected in each fraction was transferred to a 24 ml Ehrlenmeyer flask. Five milliliters of boiled distilled water were added and

---

**Table IV**

<table>
<thead>
<tr>
<th>Sample Treatment</th>
<th>Acid</th>
<th>A*</th>
<th>B*</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mg/g of leaf)</td>
<td>Normal</td>
<td>Chlorotic</td>
<td>Normal</td>
</tr>
<tr>
<td>Fr wt basis</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fumaric</td>
<td>9.2</td>
<td>7.4</td>
<td>7.4</td>
</tr>
<tr>
<td>Succinic</td>
<td>32.6**</td>
<td>14.3**</td>
<td>1.0</td>
</tr>
<tr>
<td>Malonic</td>
<td>19.5</td>
<td>15.8</td>
<td>14.0</td>
</tr>
<tr>
<td>Malic</td>
<td>36.2</td>
<td>45.6</td>
<td>21.6</td>
</tr>
<tr>
<td>Total</td>
<td>97.5</td>
<td>83.1</td>
<td>68.9</td>
</tr>
</tbody>
</table>

*PI soybeans were grown in two separate experiments in nutrient solution containing 10 ppm Mn (chlorotic treatment) and 0.25 ppm Mn (normal treatment) at a constant level of 3.5 ppm Fe.

**Combined concentrations of succinic and malonic acids.
Fig. 2. Organic acid separation of a known mixture. Acids were separated using gradient elution of a silica gel column.

The sample was titrated with 0.01 n NaOH using thymol blue as indicator.

Results obtained from the separation of a synthetic mixture of acids are shown in figure 2. The titre for each fraction was plotted against the fraction number. This method gave good separation with well-defined peaks of fumaric, malic, and citric acids. Some overlapping of succinic and malonic acids occurred under these conditions. Better resolution was obtained if the recipient reservoir had a 300 ml capacity. In the experiments, overlapping of malonic and succinic acids occurred but it was often possible to separate them. Recovery of individual acids exceeded 95%.

After titration of each fraction, the fractions within the same peak were combined. The water layer was separated and concentrated to about one milliliter. This solution was subjected to qualitative descending paper chromatography. The general techniques followed were those of Buch, Montgomery, and Porter.

The acid test solution and known acid solutions were spotted on Whatman no. 1 filter paper (45 × 19 cm) using a micro-fine pipette prepared for this purpose. The spot diameters were approximately one centimeter.

The solvent mixture employed was 1 pentanol and 5 m aqueous formic acid, prepared at least 3 hours before by combining equal volumes of the two components. The filter paper was equilibrated for 3 hours and then developed with the organic phase of the solvent system until the solvent front had moved 35 to 40 cm.

After development of the chromatograms, the sheets were removed from the tank, the solvent front was marked, and the solvents were removed by hanging the paper at room temperature for more than 2 hours in a stream of air in a chamber.

The spot locations were identified by spraying the sheet with bromophenol blue or with ammoniacal silver nitrate solution. The acidic spots could be seen immediately after spraying with the former reagent.

The content of iron was determined in the leaves of chlorotic and normal plants by Saywell's colorimetric o-phenanthroline method (12). One gram of leaves was used throughout the experiments (tables I, II, & III). Iron was not determined in the Mn treatments due to loss of samples.

Organic acid determinations from each plant sample were replicated three times. Variation was less than 5%. The reported values are means of the three individual values. For all treatments except high phosphorus, organic acids were determined from two or more separate experiments in which the treatments were identical or similar. These organic acid determinations from different experiments of the same treatments are noted as sample A, B, or C.

**RESULTS**

**Iron Deficiency Chlorosis.** Organic acid analyses of the iron-deficiency experiments are shown in table I. The leaves in sample A contained more total organic acids than in sample B, which was probably related to the difference in age of plants. Normal plants contained greater total organic acid concentrations than chlorotic plants, which was a reflection of the higher concentration in the malonate and succinate fractions. Only the citrate fraction showed greater accumulation in the chlorotic than in the normal plants. Iron content of the chlorotic leaves was slightly lower than that of the normal leaves in both samples A and B.

**Bicarbonate Induced Chlorosis.** As shown in table II, organic acid concentration was greater in chlorotic samples than in the normal. In chlorotic PI soybean tissue, malonate and citrate accumulated, whereas in chlorotic tissue of HA soybean, malate accumulated in addition to citrate and malonate. Total organic acid differences between chlorotic and normal tissues were greater in the HA experiment than in the PI experiment.

**High Phosphorous-Induced Chlorosis.** The relationship between phosphorous-induced chlorosis and organic acid composition of the leaves is shown in table III. As noted in bicarbonate-induced chlorosis the total organic acid concentration was higher in chlorotic plants than in normal plants. Higher concentrations of organic acids were found in the chlorotic plants in the fumarate, malate, and citrate fractions. Citrate was the predominant acid in chlorotic and normal leaves and accounted for most of the increase under chlorotic conditions.

**High Manganese-Induced Chlorosis.** Comparison of the organic acid composition of chlorotic and normal leaves is shown in table IV. The results are similar to those observed with iron deficient treatments. Little difference was noted in total organic acid concentrations between chlorotic and normal leaf tissue. The succinate-malate fraction was greater in normal than chlorotic tissue. Only the citrate concentration increased under chlorotic conditions.
**DISCUSSION**

Less iron was found in the chlorotic leaves than leaves from comparable control plants. It was also evident that increases in the citric acid content occurred in chlorotic tissue regardless of how chlorosis was induced. Citric acid existed in largest amounts in foliage that was most markedly chlorotic. Similar findings were observed earlier by Iljin (6, 7, 8) and Schander (13, 14, 15) in lime-induced chlorotic tissue. Other acids isolated were fumarate, succinate, malonate, and malate. These observations indicated the presence of four of the Kreb’s cycle acids and suggested the existence of the Kreb’s cycle oxidation scheme in PI and HA soybean leaves. The accumulation of citric acid in chlorotic leaves may be due to a block in the pathway of amino acid and protein synthesis from organic acids, or abnormal operation of the Kreb’s cycle.

Wadleigh and Brown (16) and Lindsey and Thorne (9) concluded that lime-induced chlorosis symptoms were similar to iron deficiency chlorosis in many respects, and suggested that bicarbonate-induced chlorosis symptoms were analogous to lime-induced chlorosis symptoms. The present data indicated that iron deficiency-induced chlorosis was not synonymous with bicarbonate or lime-induced chlorosis with respect to organic acid changes observed in chlorotic tissue.

Striking differences in malonate accumulation could be found in chlorotic tissues in comparison between iron deficiency chlorosis and bicarbonate-induced chlorosis. In bicarbonate-induced chlorotic leaves the malonic acid concentration was much higher than in control leaves. Conversely, in chlorotic plants growing in the low iron solutions the malonic acid concentration was higher in the control plants. Little difference could be found in total organic acid concentration between chlorotic and normal tissue in the iron deficiency experiments, but marked differences were observed between chlorotic and normal plants in total organic acid concentration in the bicarbonate experiments. The difference in pH values of the nutrient solutions of the two treatments (5.5 for iron deficiency & 7.8 for bicarbonate) may account for some of the differences observed between treatments. Each treatment was compared to a control where pH was not a variable which indicated an effect of bicarbonate and minus iron on organic acid metabolism independent of pH. High phosphorus-induced chlorosis was similar to bicarbonate-induced chlorosis with respect to organic acid composition of chlorotic leaves. This observation is interesting in view of the experiments in which Brown et al (2) suggested that the effect of HCO₃⁻ on chlorosis was indirect and linked to its effect on phosphorous solubility. Brown (1) suggested further that the bicarbonate ion was a factor contributing to the development of iron-deficiency chlorosis through its indirect action on entry and activity of iron. Though it is not clear whether malonate metabolism is related to the development of bicarbonate chlorosis, the present data indicate that bicarbonate-induced chlorosis or high phosphorous-induced chlorosis was not similar to iron deficiency chlorosis in respect to malonate metabolism and total organic acid concentration.

In the high manganese-induced chlorosis experiments, malonate concentration was much higher in control leaves than in chlorotic leaves, but there was no difference in total organic acid concentration. The organic acid composition of chlorotic leaves from the iron deficiency and high manganese experiments shows a similar pattern. Direct comparison cannot be made since the nutrient solutions of the two treatments differed in mineral concentration. It is postulated, however, that high manganese in the nutrient solution competed with iron absorption and upset the metabolism in a way similar to that in iron deficiency experiments.

**SUMMARY**

Experiments were conducted on iron chlorosis in higher plants as related to organic acid content. The *Glycine max* variety PI–54619–5–1 (PI) soybean was grown in growth chambers where light intensity, day length, and temperature were controlled.

Four types of iron chlorosis were induced: A, iron deficiency induced chlorosis; B, bicarbonate induced chlorosis; C, high phosphorus induced chlorosis; D, high manganese induced chlorosis.

Soybeans were harvested and 25 g of leaf tissues were analyzed qualitatively and quantitatively for organic acid by silica gel partition chromatography.

Chlorotic leaves contained less iron than control leaves in three treatments.

Significant increases in citric acid content were found in the leaves of chlorotic plants when compared to the leaves of control plants regardless of how chlorosis was induced. The leaves of plants grown in iron-deficient nutrient mediums or in solutions containing high phosphorous concentrations developed chlorosis and contained much less malonic acid, when compared to control plants for each treatment. When chlorosis was induced in plants by bicarbonate or high manganese treatments, the malonic acid concentration was markedly higher in leaves from the chlorotic plants than from comparable control plants.

**LITERATURE CITED**

5. **HOAGLAND, D. R., & D. I. ARNON.** 1950. Water

DISTRIBUTION OF BORON IN LEAVES

HARRY C. KOHL, JR. & J. J. OERTLI

University of California at Los Angeles

The boron content of plant tissues has been investigated extensively by Eaton (2). From his data it can be readily ascertained that when the boron supply of the plant is adequate or excessive the leaves contain consistently higher concentrations of boron than do other plant parts. For instance, he has shown that the roots of sugar beets showing symptoms of boron excess contained 28 to 52 ppm of boron while the leaves contained 495 to 1,008 ppm. The foliage of grapes showing neither deficiency nor toxicity symptoms was found to contain 250 to 267 ppm while the stem contained only 50 ppm. Thus, it has been shown that boron tends to collect in the leaves which are, as well, the endpoint for the transpiration stream of the plant. Furthermore, Eaton (2) and Oertli (4) have both shown that the concentration of boron is highest in the marginal, necrotic areas of the lemon leaf from a plant to which excess boron was applied and lowest at the base of the mid-vein. Such a situation can be explained by assuming that boron is passively carried within the plant in the transpiration stream.

The objective of the authors in performing the experiments reported in this paper was to further investigate this matter of the passive transport of boron in the transpiration stream by studying the distribution of boron in some leaves with simpler venation than that of lemon leaves.

MATERIALS AND METHODS

For the most part materials and methods will be described briefly with the report of the results of each experiment. However, the method of boron analysis is common to all experiments and is, therefore, described here.

Plant tissues after being dried and weighed were ashed in cylindrical 20 ml crucibles. Then, after cooling, 0.5 ml of 0.1 N HCl and 2.0 ml of eucuminoxalyl acid reagent (1) were added to each sample. After drying at 55°C the residue was dissolved in 10 ml of ethyl alcohol. Finally, the optical density of the solution was determined at 5,500 A with the aid of a Beckman spectrophotometer (Model B). Possible interference by a calcium oxalate precipitate was

---

1 Received for publication December 19, 1960.
2 Department of Floriculture and Ornamental Horticulture and Department of Soil Science, respectively.
3 Unless otherwise stated, concentrations are expressed on a dry weight basis.