Effect of Puccinia graminis tritici on Organic Acid Content of Wheat Leaves

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Introduction

Although greatly increased rates of oxidative metabolism result from infection of higher plants by rust fungi (1, 6), the significance of the high rates for the development of the rust pathogen is not understood. Previous work has indicated that the increase in respiratory rates may be correlated at least partially with the development of the parasite (5, 6). At the flecking stage, when visible symptoms of infection are first noticed and only vegetative mycelium is present, there is a moderate increase in rates without any detectable change in the nature of the respiratory pathways. The subsequent larger increases in rates associated with the process of sporulation are characterized by a decline in \( C_6/C_1 \) ratios. This latter feature of the metabolic alterations induced by all rust infections studied suggests that an oxidative pentose, or similar, pathway may be of importance in obligate parasitism. Shu and Ledingham (23) have shown that the germinating spore of the wheat rust fungus has all the enzymes of the pentose pathway and the respiration is characterized by low \( C_6/C_1 \) ratios. Consequently, if the mycelium of the parasite is largely responsible for the increased rates of respiration via a pathway requiring a \( C_1 \) decarboxylation (6), major changes should be apparent in the amounts or activity of metabolic components either directly in, or closely related to, the pathways of glucose catabolism. In contrast to other tissues in which low \( C_6/C_1 \) ratios have been induced (2, 17), data to be published on rust-infected plants do show increases in carbohydrate compounds which arise after decarboxylation of the aldehydic group of glucose.

The effects of sodium fluoride on the respiration of safflower hypocotyls (8) suggested the cyclic (3) operation of the pentose pathway and suggest also that the tricarboxylic acid cycle (TCA) would not necessarily function at rates appreciably different from those in uninfected tissue. Similar inhibitor experiments with wheat (7, 21) and bean (7) rust-affected plants were not consistent. However, results obtained by Farkas and Kiraly (10) and Bauermeister (4) with malonic and fluoroacetate acids have been interpreted as indicating a change in respiratory pathways, presumably TCA cycle reactions, during disease development. In both instances rust-affected wheat plants were less sensitive to these inhibitors than normal tissue, but the competitive nature of malonate inhibition presents kinetic difficulties (7).

A quantitative study of organic acid concentrations in wheat infected with the leaf rust fungus was made by Staples (24) while Bauermeister (4) noted changes in size of spots of organic acids separated by paper chromatography. In each instance moderate increases in malic and citric acids were observed after infections were well established and the major respiratory changes had occurred. The significant unexplained exception noted by both workers wasaconitic acid which actually declined 3 to 6 days after infection.

The anomalies of the changes among TCA cycle acids and the detection of moderate increases only when infections were well advanced might be explained by a reduction in TCA activity during disease development followed by an active translocation of acids from other leaves as respiration rates increased. Previous work (20) has indicated a capacity of infected tissue to accumulate exogenous metabolites.

The data to be presented are part of a comparative study of organic acid changes in wheat and bean tissue infected by rust fungi in which a stage characterized by vegetative growth of the parasites (fleck stage) was compared to the sporulating stage of development. Particular attention was paid to variables such as infection intensity, ontogenic host drifts in metabolism, diurnal changes in metabolism, and environmental control, aspects which often are not assessed adequately. Since wheat plants under our conditions showed characteristics which normal bean tissue did not possess, more extensive data on bean rust infections will be discussed elsewhere.

Materials & Methods

Growth and inoculation of wheat was as described previously (6, 7, 8). The first leaf of Little Club wheat (Triticum aestivum L.) was inoculated approximately one week after planting with Puccinia graminis tritici (Eriks & Henn.) race 56. A tem-
temperature of 21 ± 1°C and a photoperiod of 1,200 ft·c from cool white fluorescent lights for 14 hours was maintained during subsequent development of rust.

There are reports of diurnal fluctuations in organic acid content of wheat (24). Since diurnal changes in Rf \( Q \) have been demonstrated for both healthy and rust-affected wheat leaves under these growth conditions (7), preliminary experiments were carried out to establish the magnitude of the possible changes in organic acids. It was found that malic acid content particularly was altered, in some instances increasing by approximately 50% after 7 hours of exposure of plants to light. For this reason samples were collected shortly before or at the onset of the light period.

The flecking stage in these experiments occurred on the 4th and 5th days after inoculation. Samples obtained at sporulation were collected 4 days later.

The procedures for extraction and analysis were essentially the same as those of Staples (24) and Palmer (14). Usually 200 leaves, representing 12 to 15 g of tissue, constituted a sample for analysis. After weighing, the sample was blended for 1 to 2 minutes in 100 ml 80% ethanol. The extract was filtered through cheesecloth and centrifuged. The residues were blended twice again in 100 ml of 80% ethanol and the alcohol extracts combined for passage (1–2 ml per min under pressure) through a 10 × 0.8 cm column of Dowex-1X (200–400 mesh) in the formate form.

Gradient elution of the acids (14) with 6N formic acid with an automatic fraction collector was standardized so that reproducible recovery patterns of the acids were obtained. Fractions, 2 ml, were dried at 46°C by blowing a stream of air into the collection tubes for 2 to 3 hours. Distilled water, 2 ml, was added and the fractions titrated with 0.01 NaOH delivered from a microburette. Phenol Red was the indicator. The order and fraction number for recovery of acids was: Di-carboxylic amino 12-20; succinic 28-35; malic 38-45; citric 66-80; fumaric 110-120; phosphoric 130-160; aconitic 160-190.

The individual titration values for each fraction were summed and the concentrations in \( \mu \)M of acid for each peak were calculated. The peaks were distinct except between phosphate and aconitate, and occasionally between malic and succinic acids when either of the latter peak was in high concentration. Even in such cases the peaks were sufficiently separate so that a reasonably accurate estimate of amounts of acid was obtained from the shape of the elution curves. If care is exercised in the drying process, recovery of acids is 90% or greater.

In experiments involving incorporation of isotopic carbon from glucose, tissue collected at the start of the light period was cut into sections approximately 0.5 cm in length and floated on 0.1 M phosphate buffer (pH 4.6) containing 1 mg/liter ml of uniformly labeled glucose-C\(^{14}\) (activity equaled \( 10^6 \) cpm/mg). The glucose U-C\(^{14}\) was purchased from Volk Radiochemical Co. Tissue, 2 g, was placed on 20 ml of isotope solution in standard petri plates for 5 hours in the dark. After collection of each 2 ml fraction, an aliquot was transferred to a stainless steel planchet and dried. Activity then was measured in a Tracerlab automatic flow-counter. In all cases, radioactivity in the elution curve corresponded to titration values, indicating that the radioactivity was present only in recoverable organic acids and not in residues formed possibly from labile acids such as oxalacetic or glyoxylic (14).

The identity and purity of the individual acid peaks after elution was determined by paper chromatography in two solvents: Ethyl ether, formic acid, water (5:2:1); n-butanol, benzyl alcohol, formic acid, water (43:43:5:9).

The location of acid spots was determined with brom phenol blue indicator spray while ninhydrin was employed for amino acids. When radioactive samples were chromatographed, activity was located with a Tracerlab chromatogram scanner. Except in sporulating tissue, the amino acid peak consisted of glutamic and aspartic acids. At sporulation two additional barely detectable spots with higher Rf values were evident, but no radioactivity was noted. The succinic, malic, and aconitic peaks were pure.

It has been shown (14) that fractions corresponding to citric acid (66-80 in our experiments) may contain citric, isocitric, pyruvic, malonic (23), and a phosphate compound (25), perhaps a derivative of citric. Pyruvic and malonic acids were not detected in wheat although they may have existed in small amounts.

Since citrate and isocitrate have identical Rf values in all solvents employed, it was necessary to check for the presence of isocitric acid by formation of isocitric lactone. The lactone has an Rf significantly higher than either citric or isocitric acids. Formation of the lactone was accomplished by evaporating to dryness a solution of combined fractions 60 to 80 in 1 N HCl and heating for 2 hours. The resulting material was suspended in water and chromatographed. Good conversion of known isocitrate was obtained, but healthy or diseased tissue extracts did not form isocitric lactone.

Radioactivity data indicated a second component of the citric acid peak which remained at the origin when chromatographed. It was thought first that since citric acid is a known chelating agent, it was possible for citric acid to complex with ions present in the stainless steel planchets; but pure citric 5-C\(^{14}\) carried through the same procedures did not result in a spot at the origin. Neither did pyruvic U-C\(^{14}\).

Vickery and Palmer have reported phosphoric acid in the citric acid fraction of tobacco (24). However, the presence of C\(^{14}\) and the low Rf in the solvents employed is indicative of an organic phosphate. Mr. Marlin Bolar has shown that hexose monophosphates are eluted in the same fractions. The presence of such esters would not alter the data obtained on acid concentrations in leaves since the dissociation constants of the second and third hydrogens are low.
In the data for radioactivity, the unknown is treated separately until its identity is known. The nature of the results will not be altered appreciably. The evidence from chromatograms indicates that the only titrable acid was citric.

The concentrations of organic acids in leaves is presented in tables 1 to 4 as $\mu$M acids per 10 g leaf tissue. The use of $\mu$M, rather than milliequivalents (24) or mg, of acid appears more suitable for expressing the relationships among the acids which differ in numbers of carboxyl groups and molecular weights. The radioactivity data are expressed in cpm. In the case of the amino and citric acid peaks, the relative percentage of radioactivity in each of the materials present in the peaks was determined either by a chromatogram scanner or by cutting out the acid spots from the chromatogram and counting the activity of each spot with the flow counter. From this data, the absolute level of radioactivity in each component then was calculated from the previously determined total activity of the peak.

Results

Concentration of Acids in Healthy & Infected Leaves. Tables I and II record the amounts of organic acids obtained when strong flecks were apparent and 4 days later when 100% of the infection sites were sporulating. Levels of acids in healthy wheat were much higher in the experiment of table I than in the experiment of table II, yet the development of rust and general appearance of the plants were the same. Although no critical experiments were performed, it is possible this difference resides in the nutritional status of the plants. Plants used for experiments of tables I and III were grown on soil which had been sterilized and re-used several times, while the data for tables II and IV were obtained on soil recently composted. This situation was desirable since it permits an evaluation of possibly significant trends in disease development in host tissue of different acid status.

From the data of table I, with an infection level of 125 pustules per leaf at flecking, when respiratory rates are only slightly elevated (6), it can be seen that the levels of all components, except aconitic, were higher in rusted than in healthy leaves. Note, however, that the increases are not uniform. Malic acid increased most markedly on a molecular basis (approximately 40 $\mu$M/10 g). On a percentage basis, the difference was 232% while the other acids were: amino 125; succinic 157; citric 136. Aconitate levels were only 40% of the control tissue.

At sporulation the differences in all acids were more pronounced and in diseased tissue fumaric acid was detected. Although aconitic acid concentrations were below the control plants, there was a significant (approximately 30%) increase in the diseased plants when compared to tissue collected 4 days previously.

<table>
<thead>
<tr>
<th>Acid</th>
<th>Flecking</th>
<th>Sporulating**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H 75 p/l***</td>
<td>200 p/l</td>
</tr>
<tr>
<td>Amino</td>
<td>12.1</td>
<td>8.6</td>
</tr>
<tr>
<td>Succinic</td>
<td>1.4</td>
<td>1.5</td>
</tr>
<tr>
<td>Malic</td>
<td>4.3</td>
<td>5.8</td>
</tr>
<tr>
<td>Citric</td>
<td>2.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Fumaric</td>
<td>3.5</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* $\mu$M per 10 g tissue.  
** 4 Days after flecking sample.  
*** Pustules per leaf.

The increases in citric and malic acids, as well as the amino acids, were approximately the same at this stage of disease development.

The data of table II show that when the acid levels are low in host tissue, different effects are observed in the amino and aconitic fractions. These data also show that the level of infection has a pronounced bearing on the magnitude of the effects observed. Although 75 pustules per leaf appears visually to be a heavy infection and is appreciably higher than has been reported in some experiments, it is obvious that levels of infection may not be sufficient to permit detection of changes early in the development of rust.

In contrast to the data of table I, at the flecking stage the dicarboxylic amino acids are lower in concentration and the difference is a function of the level of infection. Of equal importance is the fact that aconitic acid levels are the same with moderate infections but are increased with heavy infections. Despite the differences in these components, at flecking there was a marked increase in malic acid at this stage of the heavy infection.

At sporulation, both intensities of infection produced significant increases in levels of all components but the effect with high intensities is more marked. In agreement with the data of table I, the differences between healthy and diseased tissue in citric acid at
metabolism. The data indicate that despite changes in the pathways of glucose catabolism in diseased tissue (1.6, 5 hours), the incorporation of C\(^{14}\) into individual acids is similar to the changes observed in the concentrations found in intact tissue. It should be noticed that, at flecking, the greatest percentage increase of incorporation into Kreb's cycle intermediates was in the citric acid pool, and the other 6-carbon acids, aconitic and succinic, when compared to the control tissue. At sporulation, however, malic was no less markedly different as at flecking.

The total amount of radioactivity in the citric acid fraction was actually lower in diseased tissue at flecking, but the decrease appears to be due largely to a difference in the unknown material present in the peak. The amount of radioactive label in citric acid appears to be the same. The other 6-carbon acids, aconitic, also had approximately the same rate of incorporation. At sporulation, however, both citric and aconitic acids had much higher levels of activity in diseased tissue when compared to healthy.

The pattern of labelling in aspartic and glutamic acids at the two stages of disease development reflected, in general, the distribution of label in organic acids. At flecking, no radioactivity was detected in glutamic acid separated from aspartic acid by chromatography. Visual estimation of the size of the glutamic acid spot of the chromatogram indicated that the concentration of glutamic acid was only one-third the concentration of aspartic acid. In the later stages of infection the reverse situation was found. Approximately two-thirds of the total incorporation into dicarboxylic amino acids was associated with glutamic acid.

### Discussion

The data indicate that despite changes in the pathways of glucose catabolism in diseased tissue (1.6, 5 hours), the incorporation of C\(^{14}\) into individual acids is similar to the changes observed in the concentrations found in intact tissue. It should be noticed that, at flecking, the greatest percentage increase of incorporation into Kreb's cycle intermediates was in the citric acid pool, and the other 6-carbon acids, aconitic, also had approximately the same rate of incorporation. At sporulation, however, both citric and aconitic acids had much higher levels of activity in diseased tissue when compared to healthy.

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### Table III

<table>
<thead>
<tr>
<th>Acid</th>
<th>Healthy 70% Sporulation 115 hr</th>
<th>Sporulating 70% 115 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 hr</td>
<td>5 hr</td>
</tr>
<tr>
<td>Amino</td>
<td>22</td>
<td>32</td>
</tr>
<tr>
<td>Succinic</td>
<td>5.1</td>
<td>4.5</td>
</tr>
<tr>
<td>Malic</td>
<td>38</td>
<td>65</td>
</tr>
<tr>
<td>Citric</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Aconitic</td>
<td>146</td>
<td>195</td>
</tr>
</tbody>
</table>

* 200 Leaves placed with cut ends immersed in 10 ml of 0.1 m KH\(_2\)PO\(_4\) containing 1 mg glucose per ml.

### Table IV

<table>
<thead>
<tr>
<th>Acid</th>
<th>Flecking 115 hr</th>
<th>Sporulating 115 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amino**</td>
<td>63.9 ± 3.8</td>
<td>12.2 ± 4.9</td>
</tr>
<tr>
<td>Aspartic</td>
<td>16.0 ± 2.0</td>
<td>12.2 ± 4.9</td>
</tr>
<tr>
<td>Glutamic</td>
<td>47.9 ± 2.0</td>
<td>75.4 ± 2.0</td>
</tr>
<tr>
<td>Succinic</td>
<td>4.9 ± 0.7</td>
<td>6.6 ± 0.7</td>
</tr>
<tr>
<td>Malic</td>
<td>12.1 ± 2.0</td>
<td>19.6 ± 2.0</td>
</tr>
<tr>
<td>Citric</td>
<td>50.6 ± 2.0</td>
<td>38.5 ± 2.0</td>
</tr>
<tr>
<td>Origin</td>
<td>29.6 ± 2.0</td>
<td>17.2 ± 2.0</td>
</tr>
<tr>
<td>Citric</td>
<td>21.0 ± 2.0</td>
<td>21.0 ± 2.0</td>
</tr>
<tr>
<td>Aconitic</td>
<td>0.75 ± 0.70</td>
<td>1.0 ± 0.5</td>
</tr>
</tbody>
</table>

* 2 g of tissue comparable to that of Table II were floated for 4 hours on 20 ml of 0.1 m KH\(_2\)PO\(_4\) containing 1 mg/ml of glucose, U-C\(^{14}\), cpm equals 10\(^{5}\) per ml.

** At sporulation four ninhydrin-reacting compounds were observed, but only aspartic and glutamic had detectable radioactivity.
8, 21), acids associated with the TCA cycle are synthesized and accumulate in rust-affected wheat leaves. The observed changes in wheat are much greater than suggested by previous results (4, 24) and have been found consistently in experiments not described here. It is apparent that levels of infection are of importance in determining the magnitudes of induced alterations in acid concentrations. The relatively slight changes observed by other workers may be a consequence of low infection intensities. The decline in aconitic acid (4, 24) is not a unique feature of infection by rust fungi, but is associated with the initial levels of acids present during establishment of the fungus.

The large increases resulting from infection may help to interpret several aspects of the literature on obligate parasitism. The failure to obtain (4, 10) as much inhibition of respiration of diseased tissue with malonate or fluoroacetate is explained most readily by the size of substrate pools. The influence of environment, particularly light intensity, on the degree of inhibition by malonate during growth (4, 10) may be a reflection of light-controlled diurnal fluctuations of acid content.

The possibility that differences in enzyme activity in extracts prepared from healthy and rust-affected tissue (12, 15) may be a function of different pH or redox values during preparation or assay must also be considered. Kaul and Shaw (11) have reported marked changes in redox potentials in wheat sap as a result of infection. Attempts to correlate (11) such phenomena with enzyme functions in vivo seem premature until the contributions of acids (probably located in the vacuole) to the measured potentials can be determined.

Although greater amounts of acids in diseased tissue can account for the insensitivity to competitive inhibitors such as malonate, the absence of sound kinetic data or labelling patterns preclude a decision as to whether the Kreb's cycle is operating at higher or even at the same levels in diseased tissue when compared to healthy tissues. A solution for this basic question has been attempted for various tissues including filamentous fungi (13, 22) and wheat (5) but kinetic uncertainties make any estimate of TCA cycling difficult. The conspicuous changes in malate, measured either on a molecular basis or by incorporation data, at the flecking stage of development may be of considerable significance. The recent recognition of auxiliary mechanisms [malate synthetase (9), carbon dioxide fixation (18)] in formation of malate might suggest that the initial changes in acids, when rates of respiration are beginning to rise, occur by reactions other than the formation of citrate via condensing enzyme. It might be argued, however, that failure to detect increases in citric or aconitic acids at this stage is only a reflection of greater utilization of these acids when compared to malate at this stage.

This concept is supported by the pronounced decline in aconitic in tissues initially high in organic acids and by the absence of an appreciable pool of glutamic acid in diseased, but not healthy, wheat at flecking. Utilization of glutamic acid for synthesis of components not detected in this study would account for such results. Glutamine, for example, has been demonstrated to be involved in reactions leading to the formation of glucosamine (16), an integral unit of fungal cell walls. In a kinetic situation, where α-ketoglutaric acid was continually siphoned from TCA cycling, additional non-cycle reactions leading to malate formation would be of considerable importance in maintaining steady-state conditions for citrate formation. In this respect the observation of Sen Gupta and Sen (19) that indole acetic acid increases CO₂ fixation into malate may be pertinent, since infection by rust fungi increases indole acetic acid levels (7).

The marked accumulation of citric and glutamic acids at sporulation also has been found consistently. At present the functional significance of this accumulation is not understood nor is the site (host versus parasite) of the accumulation known. Since spores of the fungus contain relatively large amounts of lipoidal materials, it is possible that the transition from active vegetative growth to sporulation results in a relative decrease in metabolic demands for amino acids furnished by Kreb's cycle intermediates. Since respiration continues to increase during sporulation, the accumulation of citric and glutamic acids might be expected. A separate more detailed comparison of infected bean tissue and germinating bean rust spores has shown the same general trends with infected tissue as those described above. It would appear that the initial increase in malate at flecking followed by citrate and glutamate accumulation at sporulation is characteristic of infection by rust fungi. Until reaction pathways are determined, the correlation of such changes with metabolic alterations previously found cannot be evaluated.

Summary

Healthy and rust-affected first leaves of wheat have been examined for changes in organic acids associated with the TCA cycle during the vegetative (flecking) and sporulating stages of parasite development. Although large increases in total acid are found, the acid status of the host plant and the amount of infection by rust fungi determine the degree of differences for individual acids. In all instances, however, an increase was found in the malic and succinic acids during vegetative development. Citric acid accumulates to a greater extent at sporulation. Increases in other acids are not so pronounced. Aconitic acid actually decreases if present originally in large amounts in normal host tissue.

The significance of the results for obligate parasitism is discussed, particularly the possibility of ancillary formation of malate by reactions normally not considered as part of the Kreb's cycle.
Literature Cited


