DISEASE-RESISTANCE FACTORS IN WHEAT

Electrophoretic and Chromatographic Analysis of Protein Extracts of Wheat Seedlings

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Electrophoretic and chromatographic studies of aqueous protein extracts of Pawnee (a disease-resistant wheat) and Michigan Amber (a susceptible wheat) wheat seedlings showed differences in protein characteristics between the healthy and diseased Michigan Amber plants and no protein difference between the healthy and diseased Pawnee plants. The infectious agent used was Race 9 of *Puccinia triticina*. Extracts from the infected Michigan Amber plants showed, by the application of these methods, a lower amino nitrogen and greater carboxyl content of the aqueous protein than was found in the healthy plants. Electrophoretic analysis showed that the aqueous protein extract from diseased Michigan Amber plants had a greater negative mobility than that from the healthy plants. The susceptibility of Michigan Amber wheat seedlings to Race 9 of *Puccinia triticina* may be associated with high content of amino nitrogen and/or low carboxyl content or with a high ratio of amino nitrogen content to carboxyl content.

Many pathogens are able to grow only in intimate association with living tissues of their plant or animal hosts; these are obligate parasites. The chemical nature of obligate parasitism, as in the case of the wheat leaf rust fungus, is not clearly understood.

Several investigators have suggested that resistance and susceptibility to the rust fungi were referable to specific proteins of the host plant. Gassner and Hassebrauk (*7*) observed that the addition of nitrogen compounds, such as asparagine, glycocoll (glycine), and urea to wheat leaves increased susceptibility to the rust fungus, *Puccinia triticina*. The addition of potassium and/or phosphoric acid caused the infection type to shift toward the resistant side. It is generally recognized and accepted today that increases in the nitrogen supply to plants increase their susceptibility to the rust fungi.

These investigations, a necessary preliminary to a study of the protein nutrition of obligate parasites, using radiotracers, were undertaken to determine the protein constituents of rust-susceptible and rust-resistant wheat plants. The investigations were directed to electrophoretic studies of the protein complexes in wheat seedling extracts and chromatographic studies of the amino acids remaining in the extracts after acid hydrolysis. Reports of such investigations by either electrophoresis or chromatography have not been encountered.

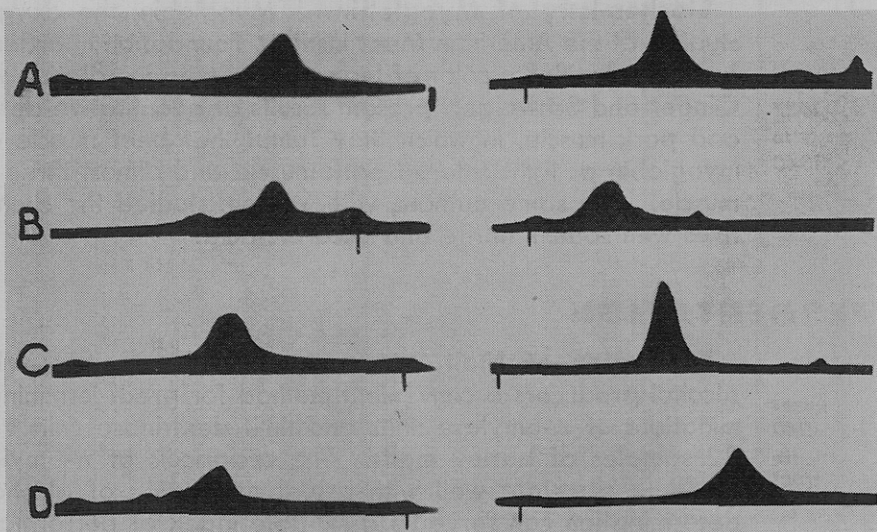
Experimental Methods

Seed of Michigan Amber and Pawnee wheat varieties were planted in ordinary greenhouse soil in 6-inch clay pots; approximately 40 seedlings were grown in each pot. Eight days later, seedlings of both varieties were inoculated with Race 9 of *Puccinia triticina*, to which Michigan Amber is susceptible and Pawnee is resistant. Michigan Amber reacts to infection by producing large quantities of rust uredospores, and no alterations of the infected tissues; Pawnee reacts by production of small necrotic lesions from which few, if any, spores are produced. The same seedlings were inoculated a second time 7 days later, in order to increase the incidence and severity of the rust disease. Extractions were made from the various leaf tissues 27 to 29 days after planting.

Method of Extraction The entire tops of the wheat plants, consisting entirely of leaves, were cut off about 1 inch above the soil line, and cut into lengths of 0.5 inch or less. The leaves were then weighed to the nearest gram. The leaves of the inoculated Michigan Amber plants were severely rusted. Most of the uredospores of *Puccinia triticina* were removed from the

Figure 1. Electrophoretic patterns for protein extracts from wheat seedlings at pH 9.5 and ionic strength 0.10

Descending patterns are on left and ascending patterns on right. Field strength and time for A (Michigan Amber) 6.38 volts per cm., 100 minutes; B (Michigan Amber diseased) 6.53 volts per cm., 40 minutes; C (Pawnee) 6.32 volts per cm., 100 minutes; D (Pawnee diseased) 6.19 volts per cm., 100 minutes



diseased leaves, prior to extraction, by means of a "cyclone-separator" (*4*) connected to a suction pump. The inoculated leaves of the Pawnee plants exhibited considerable flecking and an occasional minute uredospore pustule as a result of the inoculations. In both cases, extracts were obtained only from diseased leaf tissue.

About 20 grams of cut wheat leaves were placed in a colloid mill with 200 ml. sodium carbonate–sodium bicarbonate buffer of pH 9.5, ionic strength 0.5. The colloid mill was operated with a gap clearance of approximately 0.1 inch and for periods of 15 seconds "on" and 15 seconds "off" to minimize local heating. The mill was cooled with circulating ice water and operated for 5 minutes for each 20-gram sample. The resultant extract was filtered under slight vacuum through two thicknesses of cheesecloth. The filtrate was used to extract another 20 grams of leaves. Three to four such extractions were filtered through the same cheesecloth. The total filtrate of each sample was processed through the colloid mill with a gap clearance of approximately 0.009 inch for the same time as outlined above.

Immediately after extraction, the various chlorophyll-green colored filtrates were placed in 2-ounce bottles, frozen, and stored for future use. After thawing, the samples were centrifuged at 25,000 times gravity for 1 hour in preparation for Kjeldahl, chromatographic, and electrophoretic analyses. Kjeldahl analyses showed that the total nitrogen extracts varied from 1.65 to 3.04 mg. of nitrogen per ml. of solution.

Electrophoretic Procedure Electrophoretic measurements were made with the aid of the Tiselius-Klett electrophoresis apparatus, employing the schlieren scanning method described by Longsworth (*2*). A standard 11-ml. cell thermostated at 0.5° C. was used, and mobilities were calculated from the descending boundaries with conductivity measurements made at 0.0° C.

Buffers were made up to 0.10 ionic strength, using a monobasic sodium phosphate–dibasic sodium phosphate system for pH 7.0 and 8.0 and a sodium carbonate–sodium bicarbonate system for pH 9.5. All solutions for electrophoresis were dialyzed against the buffer for 36 to 72 hours with two changes of dialyzate.

Relative areas of electrophoretic patterns were estimated from descending boundaries by placing the patterns on cross-sectional paper and counting the squares.

Acid Hydrolysis The extracted material was prepared for two-dimensional paper chromatography by acid hydrolysis. Hydrochloric acid was added to a dialyzed extract to give a mixture $6N$ with respect to hydrochloric acid, and this mixture was heated in a sealed borosilicate bomb tube at 130° C. for about 22 hours. After removal of the aqueous hydrochloric acid solution, the residue was taken up in 5 ml. of water and filtered through filter paper. The paper was washed with water, and the combined filtrate and washings were again concentrated. The concentrate was taken up in 1 or 2 ml. of 10% aqueous isopropyl alcohol.

Chromatographic Procedure Ascending chromatography using Whatman No. 1 paper was made on known amino acid mixtures as well as acid hydrolyzate extracts. Two-dimensional chromatography was made using phenol and a mixture of 2,4,6-collidine and 2,4-lutidine (collidine-lutidine) as solvents.

One $8^3/_4$ by 10 inch borosilicate glass battery jar was used as the lower half of a container holding the spotted filter paper, and another battery jar was inverted over the first and sealed.

After drying 20 to 24 hours, following removal from the collidine-lutidine, the sheet was trimmed to approximately a 13-inch square and immersed in a

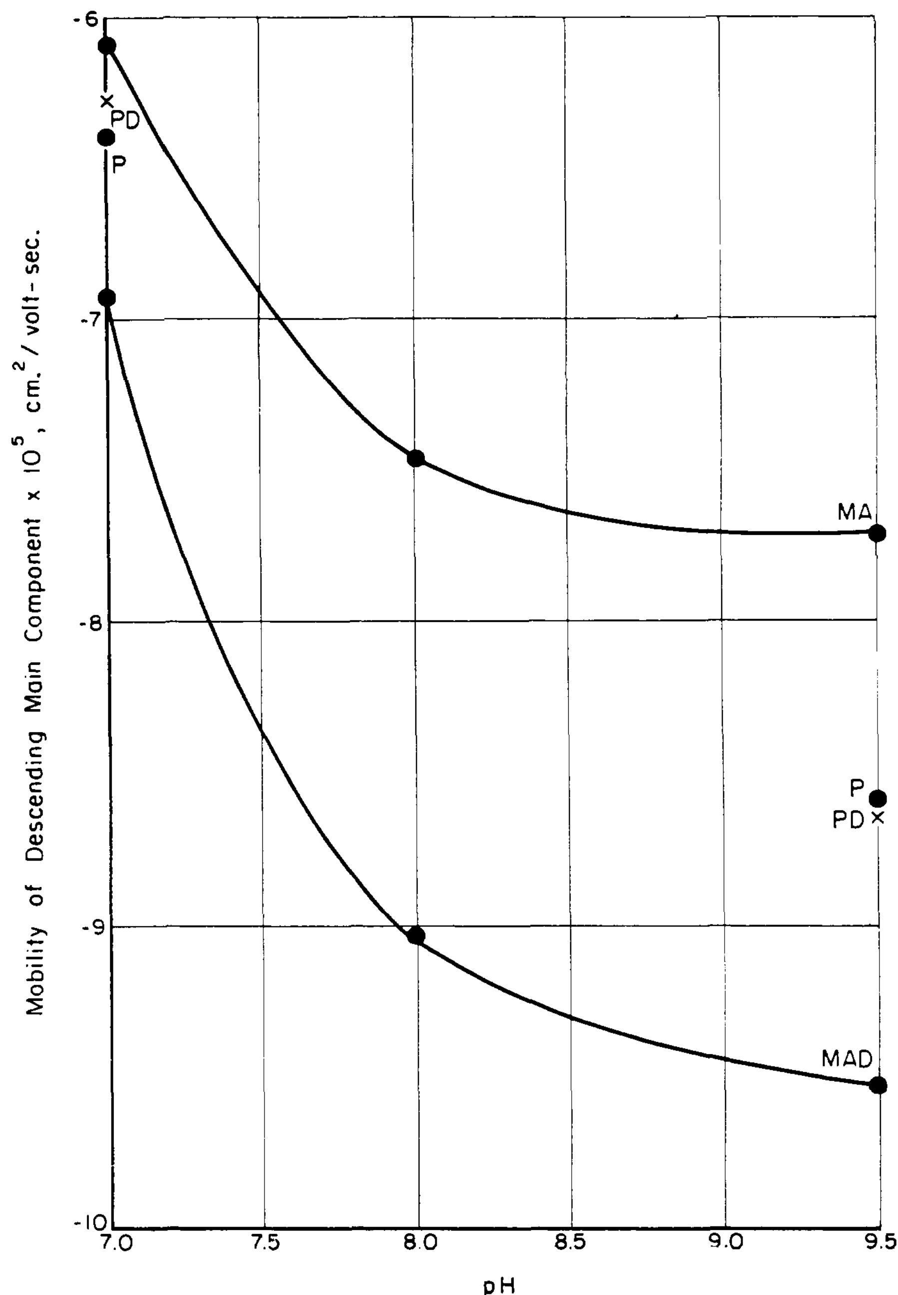


Figure 2. Mobility-pH curves for extracts of wheat seedlings

MA. Michigan Amber
MAD. Michigan Amber diseased
P. Pawnee
PD. Pawnee diseased

Table I. Electrophoretic Properties of Extracts from Wheat Seedlings

Variety of Wheat[a]	pH of Dialysis	Area of Descending Component, Sq. Mm. Main	Minor	Mobility of Main Component $\times 10^5$, Sq. Cm./Volt-Sec. Descending	Ascending
MA	9.5	75	3, 4	−7.71	−7.57
MAD	9.5	52	4, ?	−9.61	−9.61
P	9.5	73	2, 3	−8.61	−9.22
PD	9.5	52	?, ?	−8.63	−9.31
MA	8.0	41	3	−7.47	−8.29
MAD	8.0	24	...	−9.03	−8.83
MA	7.0	58	...	−6.10	−6.43
MAD	7.0	36	...	−6.92	−7.52
P	7.0	75	...	−6.39	−6.58
PD	7.0	52	...	−6.28	−6.41

[a] MA. Michigan Amber.
MAD. Michigan Amber diseased.
P. Pawnee.
PD. Pawnee diseased.

solution of 0.25% ninhydrin (C.P. acetone as solvent) contained in a large photographic tray. The treated paper was then air-dried for about 2 hours, at which time the colored areas representing the respective amino acids were encircled with a pencil and the shades of color noted.

The filter paper employed in this technique was handled at all times with rubber gloves to prevent contamination. All the chromatographic work was conducted in a laboratory where the temperature variations during the course of a run were negligible.

Electrophoretic Studies

Electrophoretic measurements were made on dialyzed extracts from healthy and diseased seedlings of both Michigan Amber and Pawnee wheat. Representative electrophoretic patterns, shown in Figure 1, indicate that the protein found in wheat seedling extracts is composed of one major heterogeneous component. Other electrophoretic properties of the extracts are shown in Table I. Although few differences are indicated between the varieties, there are significant differences between the mobilities of the major components of healthy Michigan Amber and diseased Michigan Amber. These are shown graphically in Figure 2.

Chromatographic Studies

Amino acid positions detected with ninhydrin solution are shown for the acid hydrolyzates in Figure 3. The amino acids identified were aspartic acid, glutamic acid, histidine, leucine and/or isoleucine, lysine, serine, threonine, tyrosine, and valine. The position of an unidentified substance is also shown. The positions were determined by obtaining the mean location from two or more chromatograms of each hydrolyzate. The identification of the amino acids was facilitated by comparison of the spots with those obtained by the addition of known amino acid solutions, and by comparison of the respective R_f values. Known amino acids were also added, in pairs, to the spots of the acid hydrolyzates and the resulting chromatograms compared with the originals. Further verification of the identity of these spots was determined by area enhancement and deepening of color with known amino acids; a new spot, not appearing on the original chromatogram, indicated that the known acid added was not detected in the acid hydrolyzate.

Glycine is probably present in the hydrolyzates. A slight reddish purple background to the light-brown color of the unidentified substance appeared on every chromatogram. This indicates that the glycine spot is present, but obscured by the unidentified substance. The possible presence of alanine is indicated in the hydrolyzates. If so, it is obscured by the threonine spot on the chromatograms; this is believed unlikely. Methionine, if present, would probably be obscured by the leucine-isoleucine spot. Its presence is unlikely, as methionine usually induces a secondary spot, associated with methionine sulfoxide, in the vicinity of histidine, and this was not observed.

Presented in Table II are estimated ratings of the concentrations of acid

Figure 3. Mean locations of amino acid positions on phenol–collidine-lutidine two-dimensional chromatograms

1. Unidentified
2. Serine
3. Threonine
4. Lysine
5. Valine
6. Leucine and/or isoleucine
7. Tyrosine
8. Glutamic acid
9. Aspartic acid
10. Histidine

Tyrosine was not identified on chromatograms of Michigan Amber diseased and Pawnee; glutamic acid and histidine were not identified on chromatographs of Michigan Amber

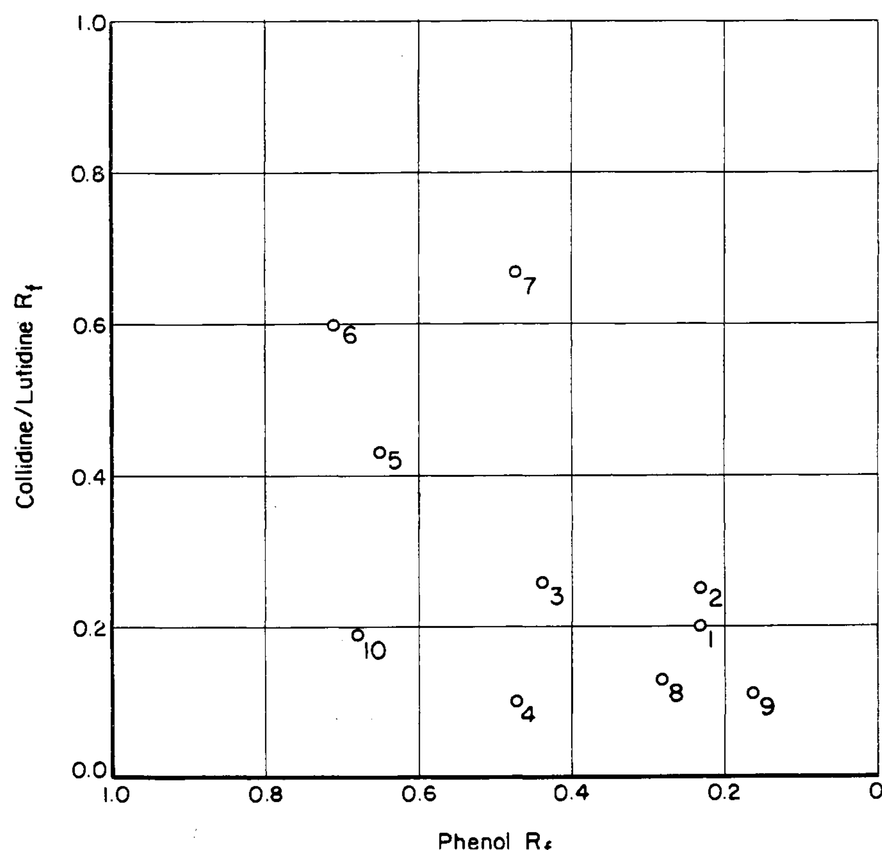


Table II. Relative Amounts of Amino Acids in Hydrolyzates of Wheat Seedling Extracts

	Acid Hydrolyzate[a]			
Amino Acid	Michigan Amber	Michigan Amber diseased	Pawnee	Pawnee diseased
Aspartic acid	0 to +[b]	++	++	++
Glutamic acid	0	+++	+ to ++	+ to ++
Histidine	0	+	0 to +	0 to +
Leucine and/or isoleucine	+++	+++	+++	+++
Lysine	++	0 to +	++	++
Serine	++	++	++	++
Threonine	+++	+++	+++	+++
Tyrosine	+	0	0	+
Valine	+++	+++	+++	+++
Unidentified	+++	+++	+++	+++

[a] +++. Maximum intensity. 0. Amino acid not detected.
[b] Atypical spotting.

spots which appeared on the two-dimensional chromatograms. These ratings were based on the color intensity and spot dimensions of individual amino acids for each of the four hydrolyzates. The differences are readily apparent and are discussed in connection with the electrophoretic studies.

Discussion

Electrophoretic mobility measurements of healthy Michigan Amber wheat extract show that its protein is less negatively charged than healthy Pawnee protein at pH 7.0, 8.0, and 9.5. Upon attack by the rust (susceptible reaction), Michigan Amber protein acquires a much greater negative charge, whereas Pawnee protein is unchanged after attack (resistant reaction) by the rust. The change in mobility of Michigan Amber protein due to attack by the rust may occur by (1) an increase in the $-COO^-$ groups giving a new negative gain or/and (2) a decrease in the $-NH_3^+$ groups giving a net positive loss. (Possible changes in protein size and shape are unknown. Their effects on mobility are probably small, but would require further investigation.)

The principal contribution to the rate of movement of a protein under a given field strength—i.e., mobility—comes from polyfunctional amino acids in the protein molecule. Chain end groups are small in number and tend to cancel each other at the pH under consideration. The most important amino acids influencing mobility at these pH's include aspartic acid, glutamic acid, lysine, cystine, and arginine. The imidazolium group in histidine is ionized very slightly even at pH 7. As two-dimensional chromatography did not show the presence of cystine or arginine, it is believed that aspartic acid, glutamic acid, and lysine are the only ones that need to be considered.

Electrophoretic mobility measurements did not show whether Michigan Amber wheat protein increased in carboxyl groups and/or decreased in amino groups when attacked by *Puccinia triticina*. However, the two-dimensional chromatograms of the acid hydrolyzates indicated some differences in the acidic and basic amino acids of the wheat. These differences, summarized in Table II, indicated that there is a net gain of carboxyl groups in Michigan Amber wheat as a result of attack by the rust fungus. This result is shown below, where the +'s for lysine and for aspartic and glutamic acids in Table II are added together. A value of 0.5 was assigned to 0 to +, 1.0 to +, 1.5 to + to ++, etc.

Variety	Carboxyl (—)[a]	Amino (+)[b]	Net Charge
Pawnee	3.5	2.0	−1.5
Pawnee diseased	3.5	2.0	−1.5
Michigan Amber	0.5	2.0	+1.5
Michigan Amber diseased	5.5	0.5	−5.0

[a] Includes aspartic and glutamic acid, either as free or amide-linked in intact protein.
[b] Includes lysine only, as histidine imide linkage is probably not ionized at pH higher than 7.0, and does not include arginine, as this was not identified on the chromatograms.

There is no change in Pawnee (resistant) after attack by *Puccinia triticina*, but Michigan Amber (susceptible) varies from +1.5 to −5.0 after attack, a net gain of 6.5 negative units. Although some amino groups are lost following infection by the fungus, there is a greater increase in carboxyl groups. This increase must be due to a synthesis of carboxyl groups in the plant protein directly or indirectly by the fungus, as any asparagine or glutamine present was hydrolyzed to aspartic and glutamic acids.

To establish the form for an increase of the new carboxyl groups, a semiquantitative analysis was made of the amide nitrogen in the seedling extracts of healthy and diseased Michigan Amber wheat. For this determination, aliquots of acid hydrolysis extracts were treated with 50% sodium hydroxide in a modified Conway flask. The released ammonia (originating from amide groups in the wheat proteins) was collected in boric acid and titrated with approximately 0.01*N* hydrochloric acid. The results showed that only small amounts of asparagine and glutamine are found in the protein and, therefore, cannot account for the aspartic or glutamic acids indicated on the paper chromatograms. It appears that the increase in new carboxyl groups results from a synthesis by the fungus.

Although further evidence is needed to establish more quantitative relationships among amino acids in wheat seedlings, it is possible that Race 9 of *Puccinia triticina* would attack wheats having proteins that run high in amino or amide nitrogen and/or low in carboxyl groups. Such an attack would result in a decrease in amino or amide nitrogen and an increase in carboxyl groups, giving greater negative mobilities in the pH range 7.0 to 10.0. The susceptibility of wheat to the rust might be associated with a high ratio of amino to carboxyl groups. The absence of detectable glutamic acid in healthy Michigan Amber wheat needs further investigation. The results obtained have further implications as to the effects of soil, fertilizer, and growing conditions on the resistance of the plants to the rust fungus—for example, soils running low in available nitrogen might be expected to decrease the susceptibility of the plants to *triticina*, as they do. Smith and Agiza (*3*) have shown that as growth proceeds in wheat plants, the quantity of acidic amino acids increases and the quantity of basic amino acids decreases. Infection by races of *Puccinia triticina* with a host reaction similar to Race 9 might also affect the metabolism of the host.

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