

Studies on Wheat Stem Rust Resistance Controlled at the Sr6 Locus.

II. Peroxidase Activities

Paul M. Seevers and J. M. Daly

Department of Biochemistry and Nutrition, University of Nebraska, Lincoln 68503.

Published with approval of the Director, Nebraska Agricultural Experiment Station, as Journal Series Paper No. 2768.

Supported by ARS, USDA Grant No. 12-14-100-7658(38) and, in part, by a grant from the Nebraska Wheat Commission. We thank W. Q. Loegering for his critical reading of the manuscript, and especially Paul Ludden for his excellent technical assistance in many phases of this work.

Accepted for publication 15 June 1970.

ABSTRACT

Peroxidase activity was measured in leaves of paired near-isogenic healthy or inoculated lines of wheat carrying the *Sr6* allele for resistance or the *sr6* allele imparting susceptibility to race 56 of *Puccinia graminis tritici*. At 20 C, infected leaves of both lines showed similar increases in peroxidase levels during the 1st and 2nd days after infection. Subsequently, leaves showing a resistant reaction type continued to increase markedly in peroxidase activity, while the susceptible leaves increased only slightly, if at all. When inoculated resistant lines

with increased peroxidase activity were transferred to 26 C, reversion to a susceptible infection type occurred but peroxidase activity did not decrease significantly during the reversion process. It was observed, however, that pustule numbers were decreased. The peroxidase activity induced by disease does not appear to be correlated with resistance or susceptibility as measured by infection type. It is possible that a second type of resistance mechanism which limits pustule numbers may involve peroxidase. *Phytopathology* 60:1642-1647.

Additional key words: *Triticum*, obligate parasitism.

In the first paper of this series, a quantitative and qualitative study of phenolic compounds in healthy and rust-affected wheat was made (14). The study was designed to establish whether aromatic compounds were involved in the marked increase in decarboxylation of indoleacetic acid (IAA) which occurs during the development of race 56 of *Puccinia graminis* Pers. f. sp. *tritici* Eriks. & E. Henn. in leaves of wheat carrying the homozygous *Sr6* allele for resistance (1). One possible interpretation (14) of the relationship between IAA decarboxylation and rust resistance was that concn of phenolic compounds were increased. The increased levels of phenolic compounds might then serve to inhibit pathogen development and, as only an incidental effect, simultaneously to stimulate IAA decarboxylation through their action on peroxidative enzymes (1, 14). No significant differences in types or quantities of phenols were observed, however, among healthy and infected, resistant or susceptible leaves at any stage of infection.

In the absence of an increase in aromatic compounds, the increase in IAA decarboxylation might result from greater synthesis or activity of peroxidases in infected tissue. In diseases caused by facultative parasites, peroxidase activity generally is higher in diseased tissues than in healthy tissues, especially in resistant reactions (4, 16, 19). While our study was underway, Macko et al. (10) reported greater peroxidase activity in resistant Khapli wheat infected with race 56 of *Puccinia graminis tritici* than in susceptible Little Club wheat. The change occurred at a time after infection corresponding to the increases observed in IAA decarboxylation (1).

It is generally assumed that peroxidase activity in infected tissues is a reflection of a metabolic demand for hydroxylation reactions in the synthesis of aromatic

compounds. In view of previous results on soluble phenols (14), it is difficult to describe a role for peroxidase, per se, in resistance, although Macko et al. (10) did report effects of peroxidase on germination and germ tube elongation of rust fungi. The present study was designed to see if more definitive correlations could be obtained among IAA decarboxylation, peroxidase activity, and resistance, and to establish whether there is a mechanism involving peroxidase which can explain, rather than merely correlate with, resistance.

MATERIALS AND METHODS.—The growth, inoculation, and estimation of disease reaction of wheat are described in detail elsewhere (14). The wheat lines, rust race, temp, and light conditions were the same as in the previous report (14).

Peroxidase extraction and assay.—The distal 5 inches of freshly harvested primary leaves were extracted in 0.1 M phosphate buffer, pH 4.6, containing 0.25 M sucrose. Tissue maceration, by steel balls, of a known number of leaves was carried out in a high-speed oscillating shaker for one min. After straining the brei through cheesecloth, the resulting suspension was centrifuged for 20 min at approx 20,000 g. Appropriate dilution of an aliquot of the supernatant was made to use for colorimetric assay of peroxidase activity. The enzyme preparation was kept at 2-5 C at all times, starting with the maceration process. To check on completeness of extraction, residues were re-extracted in the presence of 2 M sodium chloride, but only an additional 5% of enzyme was obtained.

The peroxidase assay used, with diphenylamine as substrate, was essentially that of Lück (9) except that the hydrogen peroxide stock was used undiluted, buffer volume was 2.0 ml, and sample volume was 0.1 ml. The change in OD at 485 nm was recorded for 5 min

at 25 C using a Beckman DB spectrophotometer and attached recorder, and calculated as Δ OD/min. Distilled water replaced hydrogen peroxide in the reference cell.

IAA oxidase extraction and assay.—Approximately half of the supernatant from the peroxidase preparation was used for assay of IAA oxidase. To remove inhibitors, the supernatant was dialyzed overnight against at least 100 volumes of the extraction medium, with one change of dialyzing buffer. The dialyzed preparation was then centrifuged at 20,000 g for 10 min.

IAA oxidase activity was assayed at 25 C by the procedure of Sequeira & Mineo (15). The reaction cuvette contained 1.8 ml 0.1 M KH_2PO_4 , pH 4.6, 0.1 ml 3×10^{-2} M MnCl_2 , 0.1 ml 1.5×10^{-2} M 2,4-dichlorophenol, 1.0 ml IAA in 0.1 M KH_2PO_4 (containing 125 μg IAA), and 0.1 ml enzyme preparation. Reference cuvette contents were equivalent except that 1.0 ml buffer was substituted for the IAA solution. The reaction was followed at 247 nm, and rates were calculated during the 5- to 8-min interval after the start of the reaction. This was necessary because of a variable lag period at the onset.

Expression of data.—Protein was determined on all enzyme preparations by the method of Lowry et al. (8). The fresh wt of a known number of leaves was measured; they were then dried in an oven at 80 C for 2 hr. After room-temp equilibration in a desiccator, dry wt were recorded.

With the sampling procedures employed, enzyme activity could be expressed in units of fresh wt, dry wt, protein, or per leaf. All methods of expression showed essentially the same relationship, but we have presented the data on a leaf basis for several reasons. During the later stages of infection there are substantial differences in both fresh and dry wt between inoculated resistant and susceptible leaves, due largely to sporulation of the fungus. Expression of the data on a protein basis was somewhat variable because the procedure contained two sources of error, one due to protein determination and the other to enzyme assay. Furthermore, the results of Johnson et al. (7) suggest that the use of the Lowry method with undialyzed extracts from tissues infected with obligate parasites may not represent actual protein concn. Expression of data on the basis of an infected organ appears appropriate, as it can be more directly related to severity of disease, especially infection sites per leaf.

Control treatments.—In preliminary experiments, it was found that increases in enzyme activity occurred almost immediately after inoculation. It was necessary to determine whether these effects were due to the environmental conditions of the dew chamber during inoculation or to the infection process. Plants were dusted as usual with talc and inoculum in the dew chamber, but some pots were immediately returned to growth chambers. Simultaneously, undusted plants were incubated in the chamber for the same length of time as inoculated plants. Assay of all treatments for peroxidase and IAA oxidase were made on the following 2

days. Neither the dusted, nonincubated leaves nor the incubated, undusted leaves showed any difference in activity when compared to undusted plants left in the growth chambers during the same period. Infected plants showed characteristic changes in enzyme activity. Consequently, undusted plants left in the growth chambers were used as controls in order to avoid any infections that might occur from air-borne contamination if plants were exposed to dew chamber conditions.

Variability of data.—For the enzyme assays, 20 randomly selected leaves supplied the most convenient amount of tissue where a limited amount of tissue was available. Twenty leaves provided suitable enzyme activity, and the variability in activity from day to day was not unusual for healthy tissue or for tissues with low rates of activity. For leaves with high induced enzyme activity, the variability was somewhat greater because of variation in the infection density as well as normal leaf variation.

RESULTS.—The upper section of Table 1 presents data on peroxidase activity per leaf for healthy and inoculated, resistant and susceptible lines of wheat grown continuously at 20 C. At this temp, susceptible leaves supported an average of 187 lesions which, by day 10, showed 96% sporulating pustules of reaction type 3⁺. Resistant leaves had 91 lesions/leaf, none of which showed visible sporulation (type 0;).

In the experiment of Table 1, it was necessary to harvest leaves from susceptible or resistant plant on alternate days. The data show clearly, however, that there was a marked difference in peroxidase levels when resistant and susceptible reactions were compared. On day 4, when the first faint flecks were observed, tissue from the inoculated resistant line had

TABLE 1. Peroxidase activity in extracts of healthy or rust-affected wheat leaves showing resistant or susceptible infection types

Day	Δ OD/min per leaf					
	Kept at 20 C				Transferred to 26 C on day 6	
	S ^a	SI	R	RI	R	RI
1	6.0	4.5				
2			3.2	7.5		
3	4.0	6.2				
4(F) ^b			5.7	13.0		
5	6.5	8.9				
6(S)			8.9	16.2		
7					8.0	24.1
8	9.5	8.0	9.8	37.0	10.2	27.9
9	12.5	8.9	13.2	36.0	10.2	24.2
10					12.4	32.0
15			10.5	45.0	11.4	20.0

^a S = Healthy susceptible leaves; SI = inoculated susceptible leaves. Infection intensity for SI was 187 pustules of type 3⁺/leaf. R = healthy resistant leaves; RI = inoculated resistant leaves. For RI at 20 C, the infection intensity was 91 lesions of type 0; At 26 C, the infection intensity was 49 pustules of type 3⁺ and some flecks.

^b (F) = appearance of flecks on SI and RI at 20 C; (S) = sporulation on SI.

more than doubled in peroxidase activity, while tissue from the inoculated susceptible line showed an increase of 50% on day 3 and less than this on succeeding days. By day 8, when 60% sporulation was evident in the susceptible reaction, the amt of peroxidase extracted was less than that for the healthy controls. In contrast, the higher peroxidase levels in tissues from resistant reactions continued to increase, and on day 9 the levels were 3-fold higher than healthy tissue and 4-fold higher than inoculated susceptible leaves. The difference between inoculated and healthy tissue on day 2 for the resistant line was confirmed in subsequent experiments, but increases at this stage were found also with susceptible reactions.

One experimental advantage in the use of the *Sr6* allele for studying mechanisms of resistance is the fact that it is temp-sensitive. Mechanisms responsible for resistance, therefore, may be induced and then "challenged" for their effects by transfer to higher temp where susceptibility is expected. In contrast to challenge infections with other diseases, the same inoculation is used for both induction and challenge; thus there are no complications due to variations in inoculum level, genetic differences in the pathogens employed, or nonspecific damage caused by two successive inoculations. With the host-parasite combination employed, the approx time necessary for expression of resistance appears to be 3 days (1), and transfers to 26 C were made at appropriate periods after these events.

In the experiment of Table 1, sporulating pustules were evident on susceptible plants on day 6. The resistant plants at 20 C showed development of characteristic macroscopic symptoms of resistance on that day. The number of visible infection sites does not normally increase significantly beyond that time, and there was no sporulation evident on resistant plants kept at 20 C on day 10 when lesions were counted. On day 6, groups of healthy and inoculated plants were transferred to 26 C. Four days later, the fleck lesions of the resistant reaction had changed, and 60% of the visible sites on plants that had been transferred were classified as susceptible reactions (type 3⁺). The remaining 40% showed a resumption of parasite development as evidenced by enlargement of flecks and incipient sporulation. On day 15, all visible sites were in the susceptible reaction class.

Despite the reversion in reaction type, the differences in peroxidase activity between healthy and inoculated leaves increased on the 1st day after transfer and then remained essentially unchanged. On day 10, both resistant leaves at 20 C and reverted leaves at 26 C had levels of peroxidase approximately 3 times greater than noninoculated controls. On day 15, when infected tissue was showing desiccation and other senescence effects, the peroxidase levels were reduced. It should be noted, however, that the activity of peroxidase at that time was still higher than the activity of resistant tissue at the time of transfer on day 6. Healthy and inoculated plants of the susceptible near-isogenic line were also

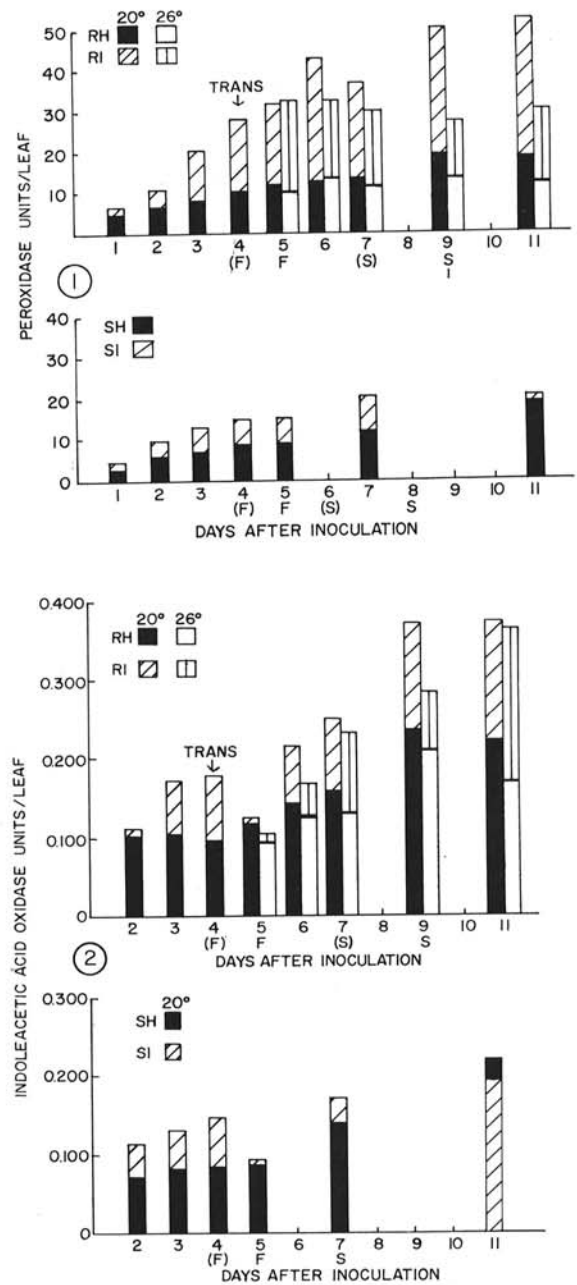


Fig. 1-2. 1) Peroxidase activity (Δ OD/min per leaf) in healthy susceptible (S) and resistant (R) or inoculated (SI and RI) lines of wheat. All plants were kept at 20 C until the 4th day after inoculation; then plants of R and RI were transferred to 26 C. On day 10, transferred RI plants were classified as having susceptible reactions, while RI plants held at 20 C had resistant infection types. The total height of the bar represents the activity of infected tissue; the lower portion of the bar represents the activity of the healthy noninoculated control leaves. 2) Changes in IAA oxidase units (Δ OD/min per leaf) during infection. A portion of the same extracts used for peroxidase assay was dialyzed overnight and assayed for IAA degradation.

transferred to 26 C, but the peroxidase levels on days 7, 8, and 9 were not different from plants retained at 20 C.

Figure 1 presents results from a similar experiment, except that the transfer from 20 to 26 C was made on day 4. During the first 2 days at 20 C, there was no significant difference in peroxidase activity between infected resistant or susceptible leaves. Both tissues, however, responded to infection by an increase in activity. By the 3rd day, inoculated resistant tissue at 20 C showed significant increases when compared to susceptible infected tissues, and the difference magnified with time.

As in the previous experiment, resistant wheat, when transferred to 26 C, maintained essentially the same total peroxidase activity as was measured at the time of transfer from 20 C, even though the tissue was undergoing a reversion from a resistant to a susceptible reaction. Figure 1 compares total peroxidase per leaf for resistant and reverted leaves. The presentation is somewhat misleading, since the healthy control tissue and, possibly, the uninvaded tissue of infected leaves at 20 C increased in peroxidase activity with age while the transferred leaves did not. A more direct comparison is the comparison of the increments in peroxidase induced by infection (Table 2).

Despite the fact that transfer to 26 C was made 2 days earlier than the first experiment, reversion from a resistant to susceptible reaction was somewhat slower. Sporulation was underway by day 8. On day 10, 55% of the pustules were classified as infection type 3, with the remainder showing incipient sporulation.

IAA oxidase also was measured in this experiment, and the absolute values are shown in Fig. 2. As with peroxidase, tissue with a susceptible reaction at 20 C had higher activity during the first few days, but since the enzyme activity in healthy tissue increased with age, the differences disappeared. Tissue exhibiting a resistant reaction maintained a greater capacity to oxidize IAA, even in the later stages of the experiment (days 7-11).

Tissue reverting from a resistant reaction to suscep-

tibility at 26 C continued to show a significant increase in activity, but the data were more variable than with peroxidase. At the late stages of infection (days 7-11), however, the disease-induced increases of IAA oxidase in tissue undergoing reversion approached or exceeded those measured in resistant tissue at 20 C.

Although the resistant leaves developed a susceptible infection type when transferred to 26 C, the total number of visible infection sites were either equal to, or lower than, the number of sites of resistant lines kept continuously at 20 C (Tables 1, 2). Furthermore, the number of sites in both cases was less than the number observed on the susceptible lines of wheat. These results were not expected in view of the data of Antonelli & Daly (1), who observed no difference in levels of infection when resistant lines grown continuously from inoculation at 26 C were compared with susceptible lines grown continuously either at 26 or 20 C. Resistant lines maintained at 20 C did have fewer visible sites, presumably since resistant reactions limited development to less than a visible stage in at least a portion of the total infected sites (1, 14). An attempt was made to determine if pustule numbers were affected by the induction of resistance mechanisms related to infection type.

Wheat was germinated and inoculated at 20 C and, starting about 1 hr after removal from the inoculation chamber (day 1), three pots of the resistant line were transferred daily from 20 C to 26 C. Lesions were estimated on days 11, 13, 15, and 20, as well as the percentage sporulation (Table 3). Starting on day 3, transfer from 20 C to 26 C resulted in a decrease in observable lesions. The decline resulted not only in fewer pustules relative to susceptible tissue kept at 20 or 26 C, but also relative to leaves with resistant reactions kept at 20 C. In agreement with Antonelli & Daly's observation (1), resistant lines transferred to

TABLE 3. The effect of temp changes on the number and sporulation of infected sites developed on wheat carrying the *Sr6* allele

Tissue ^a	Sites per leaf	S.D. ^b	Per cent sporulating on day			
			11	13	15	20
<i>Temp 20 C</i>						
SIC	167	20	99			
RIC	143	19	0			
<i>Temp 26 C</i>						
SIC	174	12	99			
RI1	162	8	99	100	99	97
RI2	163	16	82	98	97	97
RI3	115	11	61	90	92	90
RI4	90	2	18	59	78	92
RI5	89	11	38	61	79	88
RI6	70	4	6	30	52	81
RI7	100	11	1	20	55	80

^a R = Resistant leaves; S = susceptible leaves; I = inoculated leaves; C = constant temp of 20 or 26 C; numerals = the day following inoculation when transferred from 20 to 26 C.

^b Standard deviation of the mean.

TABLE 2. Diseased-induced peroxidase activity in wheat leaves showing resistant or susceptible reactions to *Puccinia graminis tritici*^a

Tissue ^b	No. of visible sites ^c	Δ OD/min per leaf on day									
		1	2	3	4	5	6	7	9	11	
<i>Held continuously at 20 C</i>											
SI	226	2	4	6	6	7		8		1	
RI	136	1	5	12	19	20	30	22	31	32	
<i>Transferred to 26 C on day 4</i>											
RI	135					22	20	19	15	18	

^a Peroxidase activity of infected leaves minus peroxidase activity of healthy control leaves.

^b R = Resistant leaves; S = susceptible leaves; I = inoculated leaves.

^c SI pustules were of type 3; RI showed type 0; at 20 C; RI at 26 C showed type 3 with some flecking.

TABLE 4. The effect of temp changes on the number of infection sites developed on wheat carrying the *sr6* allele

	20 C		26 C		
	SIC ^a	SI2	SI3	SI4	SI6
Pustules/leaf	146	156	143	150	161
S.D. ^b	21	27	20	18	25

^a C = constant temp of 20 C; numerals = the day after inoculation when transferred from 20 C to 26 C.

^b Standard deviation of the mean.

26 C immediately after inoculation are not statistically different in pustule numbers from susceptible leaves at either temp.

A similar, but not as extensive, separate test was carried out with the susceptible line of wheat. No significant effects induced by temp changes were observed (Table 4).

DISCUSSION.—Our results suggest that the previously described (1) biochemical lesion involving IAA decarboxylation is only a reflection of high peroxidase activity. The results do not eliminate the possibility that the high rates of IAA decarboxylation are mediated by a specific IAA "oxidase", or that the rates were controlled by mechanisms concerned with IAA uptake (6). The decarboxylation of IAA by tissue slices (1), IAA degradation measured by procedures applicable to horseradish peroxidase (Fig. 2), and total peroxidase (Fig. 1), however, are reasonably well related.

The data obtained here suggest that peroxidase activities measured *in vitro* cannot be related directly to resistance or susceptibility as measured by infection types. In all tests made, it has been possible to obtain reversion of resistant reactions to susceptible reactions after induction of high levels of peroxidase activity. Despite the variability encountered in high induced peroxidase activity, the values for total peroxidase on any day after transfer to 26 C did not deviate by more than 15% of the average value during the reversion process. When transfer was made on day 6 (Table 1), at a time when susceptible leaves at 20 C were sporulating but resistant leaves showed visible signs of resistant reactions, the average peroxidase levels during reversion were 3 times those of healthy plants. Transfers at day 4 (Fig. 1, Table 2) resulted in maintenance of peroxidase activity equivalent to that occurring at 20 C, but the activity did not reach the values observed subsequently at 20 C. It should be noted, however, that resistance becomes fixed or operative no earlier than the 3rd day. Therefore, chemical reactions associated with resistance should be evident but not necessarily maximal.

Furthermore, in several experiments there did not appear to be a critical level of peroxidase activity associated with resistance. For example, the average peroxidase activity for tissue undergoing reversion at 26 C in one experiment (Fig. 1) was 31 peroxidase units/leaf from days 5 through 11, while the max in resistant reactions obtained at 20 C in another experiment (Table 1) was 37 units. Despite this relatively small difference, the pathogen was suppressed in one instance

but resumed normal development in the other. This would not be expected if peroxidase activity *per se* was a requirement for expression of resistance. The finding that transfer of inoculated lines showing resistance at 20 C to higher temp resulted in fewer numbers of observable infection sites, however, raises several questions about evaluation of resistance in plants and the associated biochemical responses to infection. There are several alternatives to explain the data.

It should be pointed out first that the numerical relationship of pustules among resistant and susceptible lines and lines transferred to other temp is not constant. A review of published (1, 14) and unpublished data shows that in some cases the ratio of infection sites in resistant reactions to sites developed from the same inoculation of susceptible leaves may be close to unity, while in others it may only be 30% of those on susceptible leaves. The ratio appears to vary with temp and inoculum load. As discussed previously (14), there are difficulties in obtaining accurate estimations of infection levels, especially in heavy infections, but the differences reported here (Table 3) are so consistent as to rule out statistical variation.

The reversion process itself might cause the observed number of pustules to be lower than the initial number of discrete infected sites. The resistant infection sites obtained in our studies are small but quite distinct even when they occur in close juxtaposition, as with heavy infection levels. During reversion, the sites enlarge and become diffuse, resembling flecks in the early stages of sporulation. At this stage in reversion, it becomes difficult to estimate numbers. It is evident from the data of Table 3 that all sites do not resume development at the same speed. This could result in the spread of more rapidly growing mycelium into areas where slower growing colonies exist. Coalescence of sites or anastomosing of hyphae thus would lower the number of visible sites below that of resistant plants kept continually at 20 C.

A second alternative is that at 20 C the induction of resistance on the 3rd or 4th day after infection places the infected sites in a new metabolic condition which is sensitive to sudden temp changes. An abrupt increase of 5 C might then result in damage to some sites leading to death of the pathogen. The lack of development of the pathogen in such a situation would not be comparable to normal resistance mechanisms at 20 C.

Finally, there is the possibility that the lower number of pustules, either on resistant plants at 20 C or on plants after transfer to 26 C, is a manifestation of a second resistance mechanism. In common with other diseases, the susceptibility or resistance scale (17) for stem rust diseases is a measure of development of individual colonies of the pathogen, but there may be separate reactions which determine whether or not a colony is established at all. Previous reports by Brown & Shipton (2, 3) and Daly (5) with other races of rust and different wheat varieties suggest that the number of infection sites is at least partially under host control and is independent of reaction class. Schein (13) reported, for bean rust infections, that the number, not

type, of infections per unit area is a function of tissue age. In general, these studies indicate that the efficiency of penetration and colony establishment is not high. The fact that the reduction in pustule numbers is first manifested on the 3rd day after infection, when peroxidase levels are high, may be further indication of a second type of resistance.

We have not been able to devise procedures to experimentally evaluate the three major alternatives given above. Consequently, it has not been possible by direct means to eliminate peroxidase activity as a factor in some form of resistance. It may be involved in limitation of pustule numbers rather than reaction type. If so, this would imply localized effects, perhaps through extremely high activities in certain cells.

Previous work on peroxidase and phenols in disease resistance does not offer any parallel to the situation that our results suggest. The designation of resistance in most, if not all, instances of plant disease is an arbitrary designation. The distinction between resistance in terms of lesion type versus lesion number generally is not recognized. Furthermore, with rare exceptions, even a reaction type or lesion graded as highly susceptible has biochemical elements of resistance, as evidenced by the fact that the lesion eventually is restricted in some manner.

Such considerations assume importance in attempting to sort out the mechanisms responsible for restriction of pathogen development in disease resistance. In any host-parasite interaction, several different biochemical mechanisms may operate at different stages of the infection process, leading to different expressions of resistance. In the case of rust diseases, past emphasis has been placed on only one possible expression of resistance (infection type), and the genetic basis for this type of resistance is well known. Masri & Ellingboe (11) and McCoy & Ellingboe (12) attempted to define genetic control in terms of stages in parasite development and number of cells involved, but their approach with powdery mildew fungi has not been applied to other systems.

In any event, it is necessary to define more clearly the nature of the response to infection if precise biochemical or physiological interpretations are to be obtained. With the possible exception of the data of Tomiyama (18), quantitative measurements of resistance itself, rather than the biochemical events associated with resistance, are not available. In most instances, the nature of the resistance response appears to be analogous to the reaction classes of rust diseases. In no instance, however, is it possible to clearly decide if peroxidase activities or the concn of chemical toxicants are a determinant or a consequence of the disease response observed. Subsequent papers will be concerned with this relationship.

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