

# The Role of Peroxidase Isozymes in Resistance to Wheat Stem Rust Disease<sup>1</sup>

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## ABSTRACT

In common with other disease situations, rust-resistant wheat leaves show a large increase in peroxidase activity during infection. Peroxidase isozymes from healthy or infected lines of wheat (*Triticum aestivum* L.) near isogenic for resistance and susceptibility to race 56 of *Puccinia graminis tritici* were separated by gel electrophoresis and the activity of each was estimated by photometric scanning. In order to ensure that the activity of isozymes observed on gels reflected the changes found in peroxidase enzymes assayed spectrophotometrically in extracts, a study was made of extraction procedures, substrates, and reaction conditions for both types of enzyme measurements. Of the 14 isozymes detected in both healthy and infected leaves, increases in only 1 (isozyme 9) were associated consistently with the development of resistant disease reaction at 20 C. Additional evidence was obtained to show that this isozyme can account for the increased peroxidase activity observed in extracts from resistant plants. When plants with high induced peroxidase activity due to resistance at 20 C were treated with ethylene or transferred to 25 C, they reverted to complete susceptibility. However, the disease-induced activity of isozyme 9 did not fall. The data suggest that, in this case, the association of peroxidase with resistance was a consequence of, not a determinant in, resistance.

The existence of multiple forms of peroxidase in plants (34) has been known for a number of years, but the relationship of individual isozymes to specific biological functions is not clear. Increases in total peroxidase activity are often found during infection of higher plants by pathogens (1, 8, 27, 41), with the greatest increases associated with a host response classified as resistance. Such increases appear to be caused by the activity of only certain isozymes as reflected by staining intensity in polyacrylamide gels (8, 41). It has been suggested that the peroxidase isozymes which increase in activity during infection function to inhibit pathogen growth, perhaps through participation in biosynthesis of phenolic compounds (4, 30) or by direct inhibition of fungal growth (24), for example, through reactions involving inorganic ions (18, 19).

Previous studies (2) with near isogenic lines of wheat containing the *Sr6* alleles for disease reaction to the wheat stem

rust organism (*Puccinia graminis* Pers. f. sp. *tritici* Eriks and E. Henn.) have shown that there is a metabolic lesion causing increased rates of decarboxylation of IAA by tissue slices of lines carrying the dominant allele for resistance. The increased decarboxylation rate correlated chronologically with the establishment of resistance during infection (2) and appeared to be mediated by peroxidase (33), acting as an IAA oxidase (9, 14).

The dominant *Sr6* allele for resistance to wheat stem rust is effective at 20 C, but at 25 C plants carrying this allele are susceptible (2, 21). When infected resistant plants with high disease-induced peroxidase levels were transferred from 20 to 25 C, peroxidase levels were maintained despite reversion to susceptibility (33). In other experiments (6), increase in total peroxidase activity was induced at 20 C by ethylene treatment of both resistant and susceptible infected lines of wheat. Susceptible lines retained their normal disease reaction but, surprisingly, ethylene induced a reversion of resistant lines to susceptibility at 20 C, despite very high peroxidase levels caused by infection and by ethylene treatment (6).

Although the data suggested that peroxidase is not causally involved in resistance, the results still are ambiguous. It is possible, for example, that a specific peroxidase isozyme is induced during resistant reactions but, during reversion to susceptibility caused by ethylene or by higher temperatures, it is repressed and replaced by different isozymes not related to resistance.

Solution of this problem requires sequential quantitative measurement of concentration or activity of individual isozymes during the development of the biochemical reactions of resistance and particularly during the reversion from resistance to susceptibility. Qualitative isozyme changes are easily observed in gels after electrophoresis. Quantitative comparisons by densitometry of photographs of isozymes have been attempted, but direct photometric scanning of activity in gels would be more accurate. Quantitative analysis by scanning requires substrates which result in the formation of stable insoluble or nondiffusible products. In conventional colorimetric assays for peroxidase, we previously used substrates which give soluble products, formed at a linear rate, for accurate spectrophotometric assay (33). In view of the number of isoperoxidases (34), as well as their variations in substrate preference and optimal conditions for activity (17, 23, 26), some parameters of both types of assays were examined in order to select conditions for the best correlation between them.

## MATERIALS AND METHODS

Wheat (*Triticum aestivum* L.) was grown and inoculated with race 56 of *Puccinia graminis* Pers. f. sp. *tritici* Eriks and E. Henn. under constant environmental conditions at 20 C as described previously (2, 31). The near isogenic wheat lines were the resistant *Sr6* (CI 14163) and susceptible *sr6* (CI

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14164) lines described by Loegering and Harmon (21). On the designated days after inoculation, primary leaves were harvested, weighed, cut into 2- to 3-cm sections, and extracted in a rapidly oscillating stainless steel cylinder which contained one-eighth-inch stainless steel balls. Twenty or 40 leaves, along with extraction buffer (20 to 23 ml), were placed in the cylinder (excluding air during sealing) and oscillated for 1 min. The resulting brei was strained through cheesecloth and centrifuged for 20 min at 20,000g. The volume of the supernatant solution was recorded for calculations of peroxidase activity on a leaf basis. A portion of the crude preparation was used immediately in disc gel electrophoresis or in colorimetric determination of peroxidase activity. However, to ensure accurate comparisons of peroxidase from tissue harvested on different days it was desirable to subject all samples to electrophoresis at the same time. For this purpose, extracts were divided into small volumes (1 to 5 ml) and frozen at  $-20^{\circ}\text{C}$  for subsequent use. With any set of plants, extractions made on succeeding days were carried out on tissues collected at the same time during the day, usually 1 or 2 hr after the start of the light period.

The extraction media used were (a) the medium of Staples and Stahmann (38), 0.1 M tris, pH 8.0, containing 17% sucrose, 0.1% ascorbic acid, and 0.1% cysteine hydrochloride; (b) 0.1 M potassium phosphate, pH 4.7, containing 0.25 M sucrose; and (c) 0.1 M potassium phosphate, pH 8.0, containing 0.25 M sucrose. Several other types of media at various pH values and ionic strengths and with other addenda were investigated but had no advantages over the above solutions.

The electrophoresis system was modified from that of Davis (7). Changes were: solution A, 0.46 ml of N,N,N',N'-tetramethylethylenediamine; solution C, 37.3 g of acrylamide monomer and 1.8 g of N,N-methylenebisacrylamide; solution F, 30% sucrose. A 7% acrylamide gel was routinely used and was composed of 2.0 parts solution A, 3.0 parts solution C, 3.2 parts solution F, 5.8 parts distilled  $\text{H}_2\text{O}$ , and 2.0 parts 0.14% ammonium persulfate. The amount (30 to 50  $\mu\text{l}$ ) of sample applied directly on the top of the running gel was calculated so as to provide equivalent amounts of the original leaf material in each tube. Because of extended running times, gel tubes 10 cm in length were used in place of standard 7-cm tubes. A constant current of 3 milliamperes per tube was applied for 80 min during separation of the faster moving components or for 2 hr in the case of slower moving components.

*p*-Phenylenediamine, guaiacol, benzidine, and 3,3'-dimethoxybenzidine (*o*-dianisidine) were tested as substrates for isozyme activity in electrophoretic gels. With benzidine substrate, gels were incubated at  $25^{\circ}\text{C}$  in 0.2 M sodium acetate, pH 5.0, containing 1.3 mM benzidine and fresh 1.3 mM  $\text{H}_2\text{O}_2$ . Dissolution of the benzidine in the acetate buffer was aided by heat and constant stirring. Hydrogen peroxide was added to the benzidine solution just prior to placing the gels in the incubating solution. After 4 hr, the gels were removed from the solution, rinsed with distilled water, and placed in 0.2 M sodium acetate, pH 5.0, until they were scanned at 340 nm in a Gilford automatic scanning spectrophotometer.

An aliquot of 1% *o*-dianisidine in absolute methanol was added to 0.2 M sodium acetate, pH 5.0, to give a final substrate concentration of 1 mM for the incubating solution. After 1 hr the gels were taken from the incubating solution and rinsed in distilled water. They were then incubated in 0.2 M sodium acetate, pH 5.0, containing fresh 1.3 mM  $\text{H}_2\text{O}_2$  for 90 min. After removal from this solution and rinsing, they were placed in 0.2 M sodium acetate, pH 5.0, and scanned at 470 nm.

All scans were made at a speed of 1 cm/min and the absorbance was recorded with an expanded scale of 2.5 absorbance units. Relative peak areas were determined by tracing

the peaks on bond paper and weighing the tracings. Peak area data are an average of two and, in some cases, three determinations.

Colorimetric assays of total peroxidase in extracts were carried out at  $25^{\circ}\text{C}$  using *p*-phenylenediamine, guaiacol, benzidine, and *o*-dianisidine as substrates. The assay with *p*-phenylenediamine was the same as described in earlier papers (6, 31). Guaiacol was routinely utilized in a reaction mixture containing 1.5 ml of 20 mM sodium acetate, pH 5.0; 0.05 ml of 2 mM guaiacol; 0.2 ml of 30 mM  $\text{H}_2\text{O}_2$ ; and 0.1 ml of enzyme solution. The benzidine reaction mixture consisted of 1.9 ml of 20 mM sodium acetate, pH 5.0, 0.4 ml of 1.3 mM benzidine in 0.2 M sodium acetate, pH 5.0, 0.1 ml of 1% aqueous ammonium molybdate; 0.1 ml of 30 mM  $\text{H}_2\text{O}_2$ ; and 0.1 ml of enzyme solution. The reaction mixture for *o*-dianisidine consisted of 1.8 ml of 20 mM sodium acetate, pH 5.0; 0.05 ml of *o*-dianisidine (1% in absolute methanol); 0.2 ml of 30 mM  $\text{H}_2\text{O}_2$ ; and 0.1 ml of enzyme solution. In all assays, the enzyme solution consisted of the crude extract diluted 1:10 with distilled water. An equivalent volume of buffer was substituted for  $\text{H}_2\text{O}_2$  in the reference cuvette. Absorbance was recorded for 5 min with a recording Beckman DB spectrophotometer. The wavelengths used were 470 nm for guaiacol and *o*-dianisidine, 334 nm for benzidine, and 485 nm for *p*-phenylenediamine. Maximal initial slope of the recorded curves was used for calculation of activity per leaf.

Treatment with ethylene was accomplished by sealing plants in clear Plexiglas chambers (125-liter capacity) approximately 6 hr after the start of a light period.  $\text{CO}_2$  was generated from  $\text{NaHCO}_3$  with 3 N  $\text{H}_2\text{SO}_4$  to give a final concentration of 0.09%. Enough Matheson reagent grade ethylene was injected to provide 80  $\mu\text{l}$ /liter. Approximately 22 hr later the chambers were opened for 2 to 3 hr because continuous enclosure of plants in a sealed container results in abnormal spore development (5). This procedure was followed daily and resulted in visually normal plant and disease development.

Disease reaction was measured by the infection types of Stakman *et al.* (37). Plants classified as resistant usually showed infection type zero fleck (0); or occasionally an infection type 1 pustule. Susceptible plants showed infection types 3 and 4.

## RESULTS

**Comparisons of Extraction Procedures and Substrates.** In previous studies of changes in peroxidase activity during infection, the tissues were extracted with 0.1 M phosphate buffer, pH 4.7, which contained 0.25 M sucrose (medium B), and were assayed at pH 7.0 in 67 mM phosphate buffer with *p*-phenylenediamine as substrate. The procedures were selected because they resulted in linear reaction rates in the spectrophotometric assay of extracts. In assays of isozymes separated by electrophoresis in gels, however, the use of *p*-phenylenediamine as a substrate resulted in the formation of several bands of transitory intensity which faded within 15 to 60 min, some of them completely. The fading was due to diffusion of soluble products from the gel, as evidenced by the color of the incubation medium. In contrast, the use of benzidine as substrate resulted in stable bands with only a relatively small amount of flocculent precipitate in the incubation solutions. Instability of bands was also experienced by Farkas and Stahmann (8) using several substrates. It was necessary to examine different extraction procedures and assay conditions both for total soluble peroxidase in extracts and peroxidase activity on gels in order to ensure that the two types of assays provided equivalent information.

Healthy and infected leaves of wheat carrying the *Sr6* allele were extracted 6 days after inoculation with medium A, B, or

C. Each extract was assayed with each of four substrates at several pH values from 4.7 to 8.0 in phosphate and in acetate buffers.

Only the reaction on *p*-phenylenediamine with extracts prepared with phosphate at pH 4.7 and assayed at pH 7.0 have linear kinetics; other extracting media and higher or lower pH values during assay with this substrate resulted in departure from linearity. All other substrates exhibited biphasic curves, presumably due to the formation of products of enzyme action which initially are soluble and which then polymerize. To obtain reliable data with benzidine as substrate, ammonium molybdate was added in order to slow the conversion of the initial blue products to insoluble secondary products (20). With preparations of varying enzyme concentrations, calculation of both the initial and final slopes in all cases indicated that the two slopes were related proportionally, but the data presented here are given only for the initial slopes. The extraction medium of Staples and Stahman (38) (medium A) consistently gave higher activity of peroxidase when compared with other media on a common substrate, especially with non-infected leaves. This caused the relative magnitude of the peroxidase response during infection to be somewhat less than that observed previously (33).

Increased numbers of isozymes have been reported for flax rust disease (1). With our procedures, 14 distinct bands of peroxidase activity could be detected in gels after electrophoretic separation of extracts from both healthy and infected tissue. Four bands of low activity (bands 1–4) which moved the most rapidly toward the anode during 1.25 hr of electrophoresis could be detected in samples containing approximately 125  $\mu$ g of protein per disc gel. This amount of protein represented 4% of an entire leaf. Under conditions where bands 1 to 4 could be measured, the slower moving, more active bands separated poorly. Comparisons of several lots of plants showed that there was no correlation between the activity of bands 1 to 4 and resistance or susceptibility. To obtain resolution of the

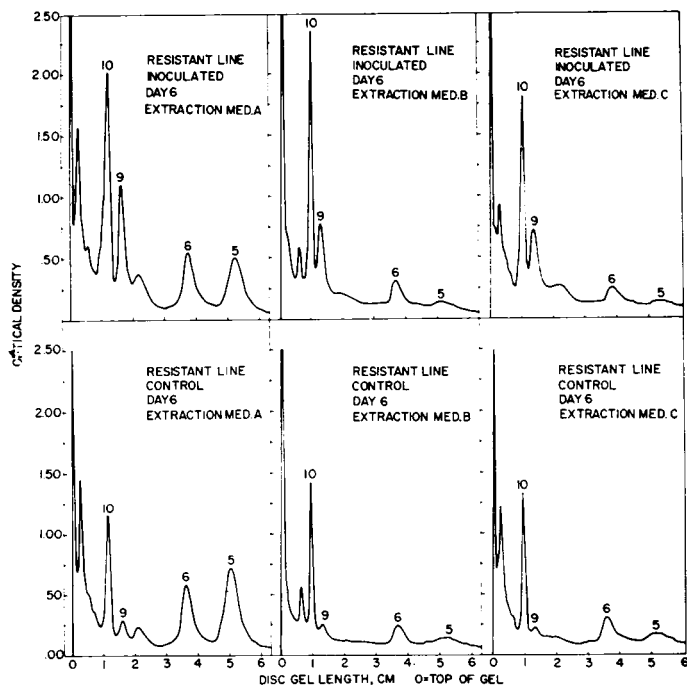


FIG. 1. The influence of extracting media on electrophoretic gel patterns obtained with resistant healthy or diseased primary leaves of wheat. Electrophoresis was for 2 hr; bands 1 to 4 migrated out of the gel. Benzidine was the substrate.

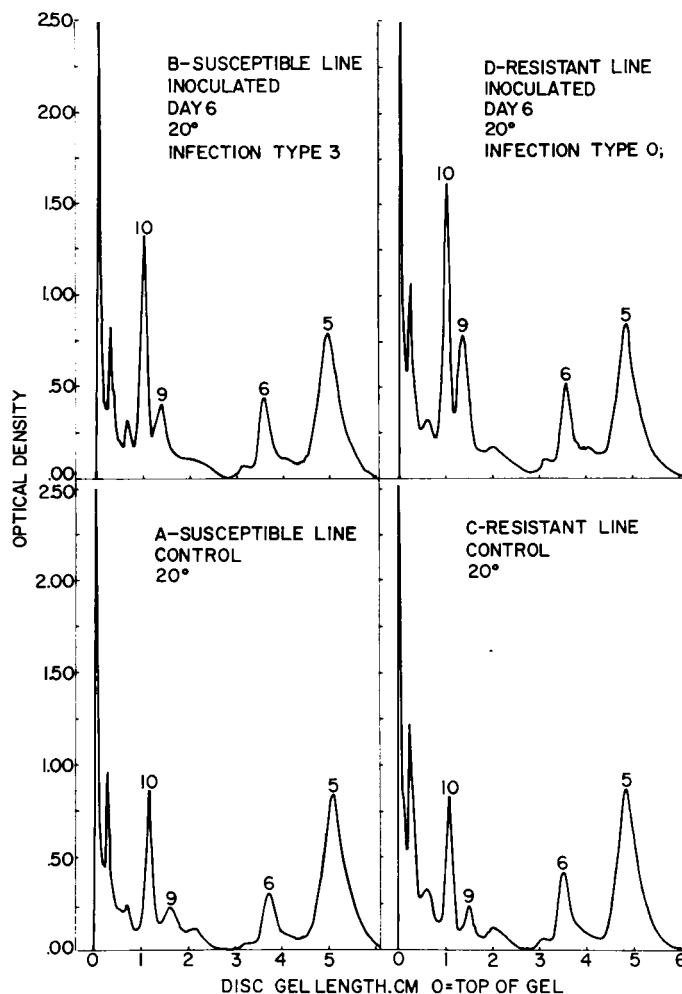


FIG. 2. Isoperoxidase gel patterns (6 days after inoculation) of healthy and diseased resistant and susceptible lines of wheat inoculated with race 56 of *Puccinia graminis tritici*. By day 6, visible expression of either resistance or susceptibility was evident.

bands with major activity less extract was applied (0.005 leaf equivalents per sample) and electrophoresis continued an additional 0.75 hr. Although bands 1 to 4 migrated from the gels, the procedure provided reproducible separation and quantitative measurement of most of the remaining isozymes (Figs. 1 and 2).

Figure 1 indicates the differences observed in total activity because of extraction media. Either the use of phosphate ions or the absence of reducing agents (media B and C) resulted in an appreciable loss of activity in bands 5 and 6. Of the identifiable bands, band 10 appears to be preferentially extracted by medium B and is least affected by extraction procedures. Table I shows that the nature of the buffer ions, the pH, and the presence or absence of reducing agents contribute to the final patterns. The data were obtained for resistant, infected tissue 5 days after inoculation.

Comparisons of various combinations of buffers at different pH values with benzidine, *o*-dianisidine, guaiacol, and *p*-phenylenediamine as substrates showed that the same bands were evident with each substrate but that only the first two substrates, at pH 5.0 and 7.0, gave bands stable for long time periods. However, phosphate buffers at either pH 5.0 or 7.0 markedly reduced color intensity in most, but not all, bands. Acetate buffer at pH 5.0 with benzidine as substrate produced the most intense stains for all bands. A comparison of peroxi-

Table I. *Relative Peak Areas of Four Isozymes Extracted from Rusted Wheat Leaves with Different Media*

Areas were measured by weighing method from spectrophotometric scans of gels with benzidine as substrate. Concentrations of components were: Tris, 0.1 M; phosphate, 0.1 M; sucrose, 17%; ascorbate, 0.1%; cysteine, 0.1%.

Extracting Medium	Isozyme Band			
	5	6	9	10
Tris, pH 8.0, + sucrose, ascorbate, cysteine	230	127	43	69
Phosphate, pH 4.7, + sucrose	63	92	28	68
Phosphate, pH 4.7, + sucrose, ascorbate, cysteine	85	75	19	58
Phosphate, pH 8.0, + sucrose	68	86	25	50
Phosphate, pH 8.0, + sucrose, ascorbate, cysteine	172	103	31	68

Table II. *Peroxidase Activities of Healthy and Infected Lines of Wheat Resistant or Susceptible to Race 56 of Puccinia graminis tritici at 20 C at Various Days after Inoculation*

Tissue <sup>1</sup>	Days after Inoculation			
	2	4	6	8
<i>p</i> -Phenylenediamine substrate in 67 mM phosphate buffer, pH 7.0				
	$\Delta A/\text{min}\cdot\text{leaf}$			
R	8.9	9.0	14.2	15.0
RI	11.0	14.9	26.7	32.9
S	9.7	11.0	17.8	16.8
SI	10.7	13.0	21.3	22.0
<i>Benzidine</i> substrate in 20 mM acetate buffer, pH 5.0				
	$\Delta A/\text{min}\cdot\text{leaf}$			
R	5.6	7.6	14.2	16.6
RI	11.8	15.0	34.0	36.4
S	8.4	10.6	14.0	15.6
SI	11.2	12.0	19.4	21.0

<sup>1</sup> R and S refer to resistant and susceptible healthy leaves; I refers to corresponding infected tissue.

dase activity in extracts (Table II) by the assay used in previous studies and by the assay developed for gels in the present study showed that the latter reflected the general changes in peroxidase activity induced by disease. With both assays, resistant infected tissue showed an earlier and greater increase in peroxidase activity than did susceptible tissues.

It also was necessary to determine whether the absorbance measured in gels could be related to the concentration of each isozyme. A dilution series, with total protein concentrations from 60 to 5  $\mu\text{g}$  per electrophoresis tube, was assayed with benzidine as the substrate. Although the slopes varied slightly for each isozyme, the peak areas for all isozymes increased linearly with concentrations up to 50  $\mu\text{g}$  of protein. Since the amount of protein usually applied was 20 to 30  $\mu\text{g}$  and peroxidase activity in extracts from diseased plants was only three to four times greater than healthy tissue, the procedures for peroxidase assay in gels were capable of measuring the expected quantitative changes in individual isozymes due to disease.

**Changes in Peroxidase Isozymes during Infection.** Figure 2 shows typical patterns of the most active isoperoxidases obtained in studies of several different lots of healthy and inoculated primary leaves. Diseased leaves contained qualitatively the same isozyme components as healthy leaves. Only those bands determined subsequently to be involved in disease reaction or useful in comparisons are labeled. Although peaks are designated as individual isozymes, partial purification of at least one of them (band 6) indicates that it consists of several components. Some peroxidase did not migrate into the gel under these conditions. The use of 4 and 5% gels and reverse polarity permitted partial analysis of these components, but they did not appear to be involved with disease reaction.

Visually, isozyme bands 9 and 10 consistently showed the greatest increase in absorbance as a consequence of disease. In the experiment shown in Figure 2, bands 5 and 6 did not appear to be affected by infection. Variability in the relationship of absorbance to infection for bands 5 and 6 was observed in a number of separate comparisons of infected and healthy tissues. A difficulty in relating activity in bands 5 and 6 to the infection process was a tendency for these bands to decline in activity, sometimes abruptly, with age of healthy tissues. They often did not show significant change with infection (Fig. 2) or were decreased (Fig. 1), but on occasion they were greater than in the control tissue during the later stages of infection (Table III). The slowest migrating bands (11–14) were not routinely separated because significant differences were not observed between resistant and susceptible reactions, even when better enzyme migration was obtained through the use of 4%, rather than 7%, gels.

Figure 3 summarizes changes during the course of infection in activity of bands 9 and 10. No visible symptoms were observed until the 4th day after inoculation when white flecks occurred on both resistant and susceptible leaves. By day 6, susceptible leaves were producing uredospores while the resistant lines showed typical necrotic, light gray lesions. Significant differences between resistant and susceptible plants occurred in band 9 from the 2nd or 3rd day after infection, a time when other evidence suggests that resistance mechanisms are becoming active (2).

The data of Figure 3 are expressed as percentages of increase in infected tissue when compared to the corresponding non-inoculated control leaves. Although useful for evaluating the consequences of infection in either resistant or susceptible tissues, they do not permit comparison of the distribution of activity among individual isozymes or a quantitative comparison

Table III. *Comparison of Relative Activities, Measured as Peak Areas, of Four Peroxidase Isozymes of Healthy and Infected Wheat Leaves*

Tissue <sup>1</sup>	Area of Isozyme Band			
	5	6	9	10
R <sub>6</sub>	11.6	15.0	7.5	20.7
RI <sub>6</sub>	14.5	28.9	25.1	33.1
S <sub>7</sub>	10.4	12.7	9.9	19.7
SI <sub>7</sub>	16.8	26.5	14.8	29.7
R <sub>8</sub>	12.0	9.6	10.2	22.5
RI <sub>8</sub>	22.6	30.3	28.6	40.9
S <sub>9</sub>	12.6	13.2	13.7	23.4
SI <sub>9</sub>	17.9	21.9	19.2	33.4

<sup>1</sup> R, RI, S, and SI are as given in Table II. The subscript refers to the day after inoculation when tissue was extracted.

of possible differences in concentration of individual isozymes among tissues.

Table III provides data, measured as peak areas, for a different lot of infected plants at several stages after resistance or susceptibility had been established. With this measure for activity, band 10 always contributed more to the total activity on the gels than did band 9 and in this series it was the most pronounced of the four bands measured. In different tissues or with frozen extracts, bands 5 and 6 appear to equal or surpass band 10 in concentration. As in all other instances, band 9 showed the largest percentage of increase in activity in resistant infected tissue.

**Relationship of Individual Isozymes to Total Activity of Extracts.** It is commonly assumed that all isozymes are active in assays of extracts and that the intensity of staining among isozymes detected on gels represents their relative contribution to total activity measured in extracts. Certain observations in the present study suggest that it is not necessarily true for peroxidases. In some cases there were significant (75–100%) increases over healthy tissues in the apparent concentration or activity of individual isozymes from susceptible infected plants, although the increase in total activity of extracts with the same substrate rarely exceeded 40 to 50% of the control value. Furthermore, when extracts from infected resistant and susceptible leaves were compared for total activity there were considerable differences between them which were reflected primarily in the relative peak areas measured for isozyme bands 9 and 10, particularly for the former isozyme. Yet, in most instances, isozyme band 9 made a minor contribution to the total activity when total activity was estimated by the absorbance of all peaks. The apparent discrepancies undoubtedly arise from the fact that different kinetic parameters are involved in the two assays, but the observed discrepancies posed an additional requirement for evidence to show that band 9 is the major enzyme activated in resistant reactions.

One line of evidence was the frequent observation that, in gel assays, only bands 9 and 10 were influenced by infection (Fig. 1 and Table IV). In Table IV, the same extracts were used as those in the measurement of total activity for resistant plants given in Table II and the increases shown in Table II can be attributed only to changes in concentration of isozyme bands 9 and 10.

More convincing lines of evidence were obtained during

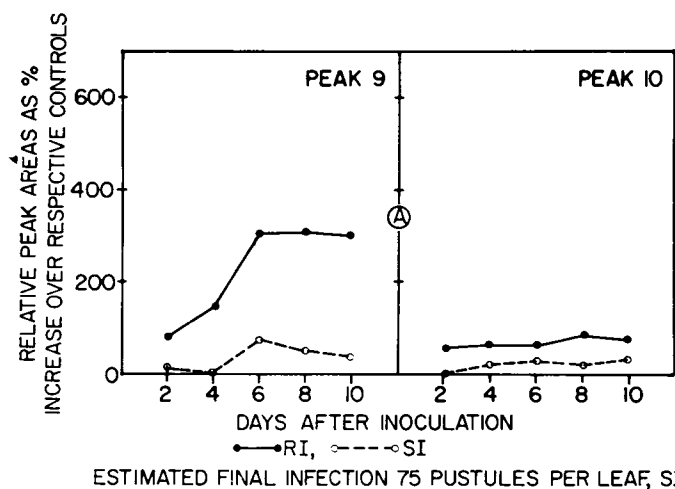


FIG. 3. Percentage of change in peak areas for isozyme bands 9 and 10 in infected wheat leaves when compared to healthy control tissue of the same age. RI: resistant infected leaves; SI: susceptible infected leaves.

Table IV. Relative Activities, as Measured by Peak Area, of Four Major Peroxidase Isozymes Extracted in Different Media

Tissue <sup>1</sup>	Band No.			
	5	6	9	10
Extraction medium A <sup>2</sup> with benzidine substrate				
R <sub>6</sub>	47.0	28.6	6.0	49.4
RI <sub>6</sub>	41.0	31.4	40.0	90.5
Extraction medium B <sup>3</sup> with benzidine substrate				
R <sub>6</sub>	8.4	10.5	6.4	24.4
RI <sub>6</sub>	6.3	11.8	25.0	41.7

<sup>1</sup> Same extracts as in Table II, obtained by resistant healthy or infected tissue on day 6 after inoculation.

<sup>2</sup> Medium contained 0.1 M tris, pH 8.0, 17% sucrose, 0.1% ascorbic acid, and 0.1% cysteine HCl.

<sup>3</sup> Medium contained 0.1 M potassium phosphate, pH 4.7, 0.25 M sucrose.

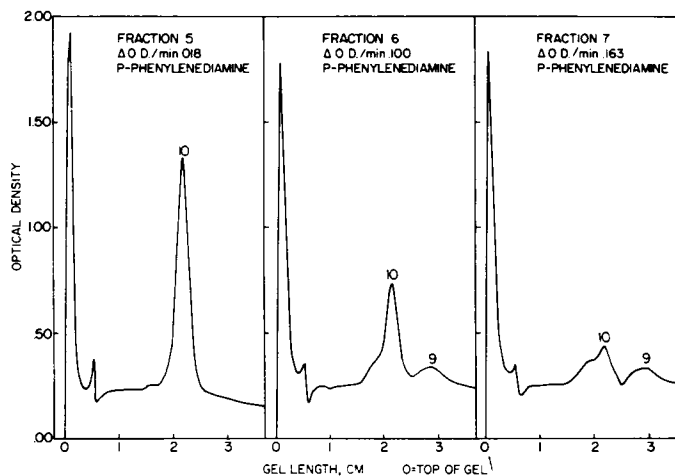


FIG. 4. Gel patterns with benzidine substrate and enzyme activity with *p*-phenylenediamine substrate for consecutive fractions eluted from a Sephadex G-200 column. The numerical data for each fraction are changes in absorbance for 0.1 ml with *p*-phenylenediamine as substrate. The same volume was used for electrophoresis and the bands were identified by comparison with the electrophoretic patterns of the original extract. Electrophoresis was for 2.5 hr. Fraction 5 contained only peroxidase band 10 while the other two fractions contained bands 9 and 10.

preliminary attempts to purify individual isozymes by methods to be described elsewhere. In Figure 4 are shown gel patterns (benzidine substrate) and rates of enzyme activity ( $\Delta A/\text{min}$ ) in solutions with *p*-phenylenediamine as substrate for three consecutive fractions eluted from a Sephadex G-200 column with 5 mM tris, pH 8.0. Fraction 5 shows a substantial peak, with benzidine as substrate, of only band 10, yet there was relatively low activity with phenylenediamine substrate. Fractions 6 and 7 contained small amounts of peak 9 but have high activity towards phenylenediamine.

In Table V are given rates of reaction per milligram of protein for several other fractions isolated by a different procedure involving preparative gel electrophoresis. After isolation, fractions were resubjected to disc gel electrophoresis for identification and were simultaneously measured in con-

ventional colorimetric assays at pH 5.0 and 7.0 with four substrates. Fraction D contained principally band 10 with a small amount of band 9. In view of the data shown in Figure 4, most of the activity with phenylenediamine, and perhaps other substrates, was due to band 9. Fractions E and F contained predominantly band 9 as measured by the staining intensity of the isozymes on gels. Fraction F also contained isozyme 8 (see Fig. 2). Fractions H and I consisted only of bands 6 and 5, respectively. There appears to be some specificity among the isozymes in substrate preference, but in all cases fractions containing band 9 show much higher specific activity on three of the four substrates.

**Role of Individual Isozymes in Disease Resistance.** In all gel patterns examined, isozyme 9 consistently increased on the 3rd or 4th day after inoculation in resistant lines of wheat grown at 20 C. This time period is associated with the development of reactions governing resistance or susceptibility (2). Lines of wheat resistant at 20 C are completely susceptible when grown at 25 C. With continuous growth at 25 C from time of inoculation, the isoperoxidase gel patterns of resistant lines were nearly identical to those of the normally susceptible nearly isogenic line of wheat, with no significant increase in the activity of band 9. This result provided additional evidence that increased activity in band 9 is not a spurious effect of infection on lines containing the *Sr6* allele for resistance but is specifically associated with resistance. It does not, however, provide evidence showing whether the association is determinative in resistance or an end result of resistance.

For determination of whether isozyme 9 was a causal factor in restriction of the pathogen at 20 C, increased activity in this isozyme was induced by maintaining infected plants at 20 C for at least 4 days after inoculation. The infected plants then

were either transferred to 25 C or else treated with ethylene at 20 C in order to cause reversion to a susceptible infection type. Under these conditions, it would be expected that isozymes induced by resistant reactions at 20 C would decrease as susceptibility became apparent.

The results during reversion, due either to higher temperature or ethylene treatments, were similar but reversion was much more rapid with ethylene (80  $\mu$ l/liter) treatment. Table VI shows data on the peak area for isozymes 6, 9, and 10, when daily ethylene treatments were initiated 5 (columns headed B) or 8 (columns headed C) days after inoculation. Tissues from nontreated but infected leaves as well as the appropriate ethylene-treated leaves were extracted on days 5, 8, 10, and 12 after inoculation. When ethylene treatment was started on day 5, there was essentially complete reversion of disease reaction by day 12. Approximately 65% of the infected sites were producing spores; the rest showed incipient sporulation. Treatment started on day 8 also initiated rapid reversion with 25% sporulation 4 days later when the experiment was terminated.

On day 5, when ethylene treatments were started, the values of peak areas for isozymes 6, 9, and 10 in healthy leaves not exposed to ethylene were 80.4, 7.3, and 29.6, respectively. At that time, infection had raised the activity of isozyme 9 about 300% (Table VI), and it continued to increase in untreated, infected, resistant leaves. Disease reversion induced by ethylene applied starting on either day 5 or 8 was not accompanied by a significant reduction in either isozyme 9 or 10. As found in other experiments, the disappearance of band 6 and of band 5 (not shown) with age of tissue (Table VI) appeared to be accelerated by ethylene.

## DISCUSSION

In the present study, qualitatively similar gel patterns have been obtained in a total of 10 different lots of healthy wheat plants under conditions of constant environment. In any given set of plants, reproducible quantitation of isozyme activity was possible but our experience suggests that there are unknown factors which cause variation in the relative distribution of activity among isozymes between sets of plants. This was most noticeable with isozyme bands 5 and 6. In successive extractions of any given lot of plants over a period of 2 weeks, the relative activity between these isozymes remained essentially constant. In some lots, the activities were approximately equal (Table III), while in other sets with the same extraction medium the ratio of activity was quite unequal (Table IV). Since isozymes 5 and 6 appear less active with increasing age of tissue, the differences in relative activity might be due to

Table V. Enzyme Activity in Fractions of Partially Purified Isoperoxidases Extracted from Resistant Lines of Wheat Infected with Wheat Stem Rust Fungus

Fraction	Isozyme $\Delta A$ per min per mg of protein in Phosphate Buffer, pH 7.0 <sup>1</sup>				
	Components	Guaiacol	<i>o</i> -Dianisidine	Phenylene-diamine	Benzidine
D	10 + trace 9	4.2	3.1	3.9	46.4
E	9 + trace 10	3.6	5.5	6.6	76.6
F	9 + 8	2.2	9.0	13.0	85.0
H	6	...	...	0.6	61.7
I	5	...	...	0.4	51.0

<sup>1</sup> Initial slopes.

Table VI. Effects of Ethylene (80  $\mu$ l/liter) on Disease Development and the Activities of Peroxidase Isozymes Induced by Resistant Disease Reaction

Tissue Extracted on Day:	Disease Reaction <sup>1</sup>			Peak Area of Band <sup>2</sup>								
				6			9			10		
	A <sup>3</sup>	B	C	A	B	C	A	B	C	A	B	C
5	R			70.4			25.0			41.5		
8	R	F		63.3	56.9		33.0	21.5		48.0	46.8	
10	R	40% S	10% S	61.6	43.5	52.0	37.6	22.0	31.6	50.0	58.0	48.8
12	R	65% S	25% S	44.6	36.2	41.0	39.0	24.8	27.5	53.2	61.6	57.3

<sup>1</sup> R: resistant infection type; F: flecks of susceptible infection type; S preceded by percentage: percentage of infection sites showing sporulation.

<sup>2</sup> On day 5, the values for noninoculated, untreated healthy leaves for bands 6, 9, and 10 were 80.5, 7.3, and 29.6, respectively.

<sup>3</sup> Column A: nontreated leaves; column B: ethylene treatments started on day 5; column C: ethylene treatment started on day 8.

intrinsic differences in enzyme concentration in the leaves as a result of minor variations in growth patterns (25, 29). These two isozymes also are sensitive to conditions during extraction (Fig. 2 and Table II). Rather than intrinsic differences in enzyme concentrations, differences in other tissue components could influence the solubility of peroxidase isozymes.

Despite the difficulties in relating the activity of specific isozymes to changes in total peroxidase, the evidence implicates isozyme 9 as the peroxidase chiefly, if not exclusively, responsible for the increases induced by resistant reactions (33). The high activity of isozyme 9 with phenylenediamine as substrate is in accord with previous data (33). The fact that the extracting medium used in previous studies resulted in incomplete extraction of isozyme bands 5 and 6 and thus favored extraction of isozyme band 10 (with relatively low activity on phenylenediamine) also supports a major role for isozyme 9. The results suggest some caution in placing complete reliance on gel patterns in attempting to compare enzyme behavior among tissues.

The difficulties in relating the two assays arise from several factors. Conventional colorimetric assay measures an initial rapid reaction in extracts which may contain nonprotein components affecting or participating in the reaction. The well documented behavior of peroxidase acting as an IAA oxidase illustrates this point. Phenolic cofactors causing inhibition or stimulation occur in extracts (11, 13), but the initial variable lag periods (31) and light stimulations (12) frequently observed for this enzyme in extracts are still not completely understood. Stonier (39) has reported high molecular weight inhibitors of IAA oxidase, but their mode of action is not known. Although dialysis would not remove such inhibitors, most likely electrophoresis would. The rate of reaction in crude extracts may also be affected by subsequent action on an initial product by other enzymes, including other isoperoxidases.

In contrast, a gel assay with the same substrate involves secondary reactions under conditions which minimize contributions of extraneous components of the extracts. We attempted to correlate both assays by determining initial rates of reaction through repetitive scanning of gels during 2 hr of reaction. Isozyme bands 9 and 10 showed a more rapid development of products but the differences from other isozymes were not great enough to assign to them a primary kinetic role in short term measurements. True kinetic constants for gel reactions appear unobtainable due to diffusion problems and the cross sectional distribution of enzyme.

When ethylene is applied to resistant infected leaves after an increase in isozyme 9 at 20 C, there is no further increase in this isozyme. Yet, in both situations total peroxidase does increase (6), apparently as a function of isozyme 10. The response to ethylene is interesting and is in accord with specific isozyme responses to hormonal treatment in other instances (15, 16, 36).

There is one puzzling aspect of the role of ethylene in disease reaction and in peroxidase activation. Susceptible infected leaves produce much more ethylene than do resistant infected leaves (6), yet susceptible plants have lower total peroxidase activity (6, 33). In some instances, peroxidase activity actually is less in extracts from susceptible infected leaves than in the uninfected control tissue (33) despite 10- to 20-fold increases in rates of ethylene production (6). The much greater production of ethylene by susceptible infected plants is in accord with the biological effects of external ethylene on resistant plants and it has been pointed out that ethylene may be involved in the development of the parasite in normally susceptible tissue (6). Beyer and Morgan (3) indicate that tissue concentrations of ethylene appear to be correlated with rates of emanation of ethylene by some tissues. In view of this observation, the rela-

tionship between ethylene production and peroxidase activity in susceptible reactions is obscure because ambient ethylene does stimulate additional peroxidase activity even with susceptible infection types.

The fact that isozyme 9 ceases to increase during reversion is additional evidence for its selective increase with normal resistant reactions, but this fact appears to rule out peroxidase as a factor responsible for resistance. It can be argued (32) that the temperature-sensitive *Sr6* allele is biochemically unique and that results obtained with it cannot be applied to other combinations of wheat and stem rust fungi. Studies of the *Sr11* allele for stem rust resistance revealed the development of identical isozyme patterns during infection (22). Because the *Sr11* gene is not influenced by temperature or ethylene, the development of isozyme 9 with both alleles may be a nonspecific biochemical response during development of resistance due to cellular irritation of invaded tissues.

Increases in peroxidase may be viewed only as a biochemical symptom of other and earlier events which actually cause resistance. The fact that a specific peroxidase is increased, however, may provide some information about biochemical events preceding or associated with the development of resistance. In view of the evidence for variations in substrate specificity, optimal conditions for activity (23, 26), and changes in isozyme patterns during growth or hormonal treatment (28, 40), each isozyme may have specific biological functions or unique cellular locations. Examination of isozyme band 9 in connection with biochemical reactions suggested to be mediated by peroxidase *in vivo* (lignin synthesis [10, 35], aromatic hydroxylation [4], ethylene formation [42]) can lead to a more precise description of the changes induced in resistant hosts by pathogens.

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#### LITERATURE CITED

- ANDREEV, L. AND M. SHAW. 1965. A note on the effect of rust infection on peroxidase isozymes in flax. *Can. J. Bot.* 43: 1479-1483.
- ANTONELLI, E. AND J. M. DALY. 1966. Decarboxylation of indoleacetic acid by near-isogenic lines of wheat resistant or susceptible to *Puccinia graminis* f. sp. *tritici*. *Phytopathology* 56: 610-618.
- BEYER, E. M., JR. AND P. W. MORGAN. 1970. A method for determining the concentration of ethylene in the gas phase of vegetative plant tissue. *Plant Physiol.* 46: 352-354.
- BUHLER, D. R. AND H. S. MASON. 1961. Hydroxylation catalyzed by peroxidase. *Arch. Biochem. Biophys.* 92: 427-437.
- DALY, J. M., H. W. KNOCH, AND M. V. WEISE. 1967. Carbohydrate and lipid metabolism during germination of uredospores of *Puccinia graminis* *tritici*. *Plant Physiol.* 42: 1633-1642.
- DALY, J. M., P. M. SEEVERS, AND P. LUDDEN. 1970. Studies on wheat stem rust resistance controlled at the *Sr6* locus. III. Ethylene and disease reaction. *Phytopathology* 60: 1648-1652.
- DAVIS, B. J. 1964. Dis electrophoresis. II. Method and application to human serum proteins. *Ann. N. Y. Acad. Sci.* 121: 404-427.
- FARKAS, G. L. AND M. A. STAHMANN. 1966. On the nature of changes in peroxidase isozymes in bean leaves infected by Southern Bean Mosaic Virus. *Phytopathology* 56: 669-677.
- FOX, L. R., W. K. PURVES, AND H. I. NAKADA. 1965. The role of horseradish peroxidase in indole-3-acetic acid oxidation. *Biochemistry* 4: 2754-2763.
- FREUDENBERG, K. 1959. Biosynthesis and constitution of lignin. *Nature* 183: 1152-1155.
- FURUYA, M., A. W. GALSTON, AND B. B. STOWE. 1962. Isolation from peas of cofactors and inhibitors of indolyl-3-acetic acid oxidase. *Nature* 193: 456-457.
- GALSTON, A. W. AND R. S. BAKER. 1951. Studies on the physiology of light action. III. Light activation of a flavoprotein enzyme by reversal of a naturally occurring inhibition. *Amer. J. Bot.* 38: 190-195.
- GORTNER, W. A. AND M. J. KENT. 1958. The coenzyme requirement and enzyme inhibitors of pineapple indoleacetic acid oxidase. *J. Biol. Chem.* 233: 731-735.
- HARE, R. C. 1964. Indoleacetic acid oxidase. *Bot. Rev.* 30: 129-165.
- IMASEKI, H. 1970. Induction of peroxidase activity by ethylene in sweet potato. *Plant Physiol.* 46: 172-174.

16. IMASEKI, H., M. UCHIYAMA, AND I. URITANI. 1968. Effect of ethylene on the inductive increase in metabolic activities in sliced sweet potato roots. *Agr. Biol. Chem.* 32: 387-389.
17. KAY, E., L. M. SHANNON, AND J. Y. LEW. 1967. Peroxidase isozymes from horseradish roots. II. Catalytic properties. *J. Biol. Chem.* 242: 2470-2473.
18. KLEBANOFF, S. J. 1968. Myeloperoxidase-halide-hydrogen peroxide antibacterial system. *J. Bacteriol.* 95: 2131-2138.
19. LEHRER, R. I. 1969. Antifungal effects of peroxidase systems. *J. Bacteriol.* 99: 361-365.
20. LILLIE, R. D. 1954. *Histopathologic Technic and Practical Histochemistry*. The Blakiston Co., Inc., New York.
21. LOEGERING, W. Q. AND D. L. HARMON. 1969. Wheat lines near-isogenic for reaction to *Puccinia graminis tritici*. *Phytopathology* 59: 456-459.
22. LUDDEN, P. AND J. M. DALY. 1970. Certain biochemical comparisons in wheat stem rust disease reactions controlled either at the *Sr6* or *Sr11* loci (abstr.). *Phytopathology* 60: 1301.
23. MACKO, V. AND A. NOVACKY. 1966. Contribution to the study of plant peroxidase isozymes by means of disc electrophoresis on acrylamide gel. *Biologia* 21: 128-132.
24. MACKO, V., W. WOODBURY, AND M. A. STAHMANN. 1968. The effect of peroxidase on the germination and growth on mycelium of *Puccinia graminis f. sp. tritici*. *Phytopathology* 58: 1250-1254.
25. MACNICOL, P. K. 1966. Peroxidases of the Alaska pea (*Pisum sativum* L.). Enzymic properties and distribution within the plant. *Arch. Biochem. Biophys.* 117: 347-356.
26. NOVACKY, A. AND R. E. HAMPTON. 1968. The effect of substrate concentration on the visualization of isoperoxidases in disc electrophoresis. *Phytochemistry* 7: 1143-1145.
27. NOVACKY, A. AND R. E. HAMPTON. 1968. Peroxidase isozymes in virus-infected plants. *Phytopathology* 58: 301-305.
28. OCKERSE, R., B. Z. SIEGEL, AND A. W. GALSTON. 1966. Hormone-induced repression of a peroxidase isozyme in plant tissue. *Science* 151: 452-453.
29. RACUSEN, D. AND M. FOOTE. 1966. Peroxidase isozymes in bean leaves by preparative disc electrophoresis. *Can. J. Bot.* 44: 1633-1638.
30. ROHRINGER, R. AND D. J. SAMBORSKI. 1967. Aromatic compounds in the host-parasite interaction. *Annu. Rev. Phytopathol.* 5: 77-86.
31. SCHAEFFER, G. W., J. G. BUTA, AND F. SHARPE. 1967. Scopoletin and polyphenol-induced lag in peroxidase catalysed oxidation of indole-3-acetic acid. *Physiol. Plant.* 20: 342-347.
32. SEEVERS, P. M. AND J. M. DALY. 1970. Studies on wheat stem rust resistance controlled at the *Sr6* locus. I. The role of phenolic compounds. *Phytopathology* 60: 1322-1328.
33. SEEVERS, P. M. AND J. M. DALY. 1970. Studies on wheat stem rust resistance controlled at the *Sr6* locus. II. Peroxidase activities. *Phytopathology* 60: 1642-1647.
34. SHANNON, L. M. 1948. Plant isozymes. *Annu. Rev. Plant. Physiol.* 19: 187-210.
35. SIEGEL, S. M. 1953. On the biosynthesis of lignins. *Physiol. Plant.* 6: 134-139.
36. STAHMANN, M. A., A. B. CLARE, AND W. WOODBURY. 1966. Increased disease resistance and enzyme activity induced by ethylene and ethylene production by black rot infected sweet potato tissue. *Plant Physiol.* 41: 1505-1512.
37. STAKMAN, E. C., D. M. STEWART, AND W. Q. LOEGERING. 1962. Identification of physiologic races of *Puccinia graminis* var. *tritici*. *U. S. Dep. Agr. Agr. Res. Serv. Bull.* E617.
38. STAPLES, R. C. AND M. A. STAHMANN. 1964. Changes in protein and several enzymes in susceptible bean leaves after infection by the bean rust fungus. *Phytopathology* 54: 760-764.
39. STONIER, T. 1969. Studies on auxin protectors. VII. Association of auxin protectors with crown gall development in sunflower stems. *Plant Physiol.* 44: 1169-1174.
40. STUBER, C. W. AND C. S. LEVINGS III. 1969. Auxin induction and repression of peroxidase isozymes. *Crop Sci.* 9: 415-416.
41. URITANI, I. AND M. A. STAHMANN. 1961. Changes in nitrogen metabolism in sweet potato with black rot. *Plant. Physiol.* 36: 770-782.
43. YANG, S. F. 1967. Biosynthesis of ethylene. Ethylene formation from methional by horseradish peroxidase. *Arch. Biochem. Biophys.* 122: 481-487.