Plant Physiol. (1976) 57, 766-774

Host-Pathogen Interactions

XI. COMPOSITION AND STRUCTURE OF WALL-RELEASED ELICITOR FRACTIONS[1]

Received for publication September 26, 1976 and in revised form January 12, 1976

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ABSTRACT

The structures of the four wall-released elicitor fractions isolated from the *Phytophthora megasperma* var. *sojae* mycelial walls have been examined. The results demonstrate that fraction I is primarily composed of a branched β-1,3-glucan, similar in structure to the extracellular elicitors described previously (Ayers, A., J. Ebel, F. Finelli, N. Burger, and P. Albersheim. 1976. Plant Physiol. 57: 751-759). Fractions II and IV are primarily composed of a highly branched mannan-containing glycoprotein, with fraction IV richer in protein than fraction II. Fraction III contains, attached to protein, a mixture of the two polysaccharide types found in fraction I and in fractions II and IV. The structural data presented here, in concert with the biological data presented in the previous two papers (Ayers *et al.* 1976. Plant Physiol. 57: 751-759; 760-765), demonstrate that the only compound produced by *P. megasperma* var. *sojae* which contains elicitor activity is the glucan. Evidence is presented that the terminal glycosyl residues of the glucan are required for elicitor activity. In addition, it is demonstrated that 90% of the glucan can be removed enzymically without any loss of biological activity. The active residue of the enzymic digestion is a highly branched 3- and 3,6-linked glucan containing about 4% mannosyl residues. The results presented suggest that the mannosyl residues of the glucan, which represent only about 1% of the undegraded glucan, are likely to participate in the active site of this molecule. The role of elicitors and phytoalexins in host-pathogen interactions is discussed. Evidence for the existence of and possible identity of another factor, which determines race specificity of host-pathogen interactions, is summarized.

The Pms[5] wall-released elicitor described in the previous paper (5) is the first example of a pathogen wall component which has been demonstrated to have elicitor activity. The composition and structure of this component was investigated to determine the chemical moiety responsible for elicitor activity and also to determine how these polysaccharides relate to wall polysaccharides isolated in other systems.

[1] Research was supported by the Energy Research and Development Administration E(11-1)-1426, the Herman Frasch Foundation, New York City, a University of Colorado Faculty Fellowship to P. A., and a Deutsche Forschungsgemeinschaft Fellowship to J. E.

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[5] Abbreviation: Pms: *Phytophthora megasperma* var. *sojae*.

MATERIALS AND METHODS

The fractionation of elicitor preparations from the mycelial walls of Pms grown in liquid culture has been described (5).

Sugar Composition and Linkage Determinations. Neutral and amino sugar compositions were determined by the method of Jones and Albersheim (14).

Methylation of elicitor samples was performed according to the general method of Hakomori (13) as adapted by Sandford and Conrad (22), but modified as described below to ensure complete methylation. The elicitor samples (100 μg in 1 ml of H_2O) were lyophilized and then dried over P_2O_5 for a minimum of 10 hr at 60 C *in vacuo*. The samples were dissolved in 100 μl of dry dimethyl sulfoxide (24) by sonication for 12 hr at 60 C. Ten μl of 2 M dimethylsulfinyl anion solution prepared according to Sandford and Conrad (22) were added to each sample, and the samples were sonicated for 1 hr at 60 C. Methyl iodide (1.2 μl, 1.05 eq based on the amount of the dimethylsulfinyl anion) was added to each sample at room temperature, and the solutions were stirred for 1 hr. Two more methylation cycles were performed by sequential additions of dimethylsulfinyl anion and methyl iodide. The samples were mixed for 12 hr at room temperature after the last addition of methyl iodide and two more methylation cycles were performed. The last methyl iodide addition was 5 μl rather than 1.2 μl, and the samples were stirred for 4 hr at room temperature.

The methylated polysaccharides were separated from dimethyl sulfoxide and other reagents by molecular sieving chromatography. The samples were applied to a column (0.5 × 37 cm) of Sephadex LH-20 (Pharmacia) equilibrated with chloroform-methanol (1:2, v/v). Fractions (0.5 ml) of the effluent were collected, and the void fractions (fractions 4-9) were combined. The solvent was removed by evaporation at 50 C under a stream of filtered air.

The fully methylated polysaccharides were hydrolyzed, reduced, and acetylated by the following procedure to yield the corresponding partially methylated alditol acetates. Hydrolysis of the methylated polysaccharides was accomplished in sealed test tubes (13 × 100 mm) with 0.5 ml of 2 M trifluoroacetic acid for 2 hr at 121 C. The acid and water were subsequently removed by evaporation at 50 C with a stream of filtered air.

The partially methylated aldoses, produced by the acid hydrolysis, were reduced to the corresponding alditols by incubation for 2 hr at room temperature in 250 μl of 1 M ammonia containing 2.5 mg of sodium borodeuteride. Any sodium borodeuteride remaining after the reduction reaction was decomposed by dropwise addition of glacial acetic acid until the evolution of gas ceased. The solutions were then evaporated at 50 C with filtered air to remove ammonia and water. The borate remaining in the reaction tubes was removed as the volatile methyl borate as described (2).

Acetylation of the partially methylated alditols produced by reduction was accomplished in a sealed tube with 100 μl of acetic anhydride for 3 hr at 121 C. After the acetylation reaction, the remaining acetic anhydride was hydrolyzed to acetic acid by addition of 1 ml of a saturated aqueous solution of sodium carbonate and the addition of 50 mg aliquots of solid sodium carbonate until CO_2 evolution ceased.

The partially methylated alditol acetates were separated from sodium acetate and other reaction products by extraction into chloroform. Chloroform (250 μl) was added to each reaction tube and the tubes were agitated. The aqueous phase was removed and discarded. The chloroform phase, containing the partially methylated alditol acetates, was transferred by a Pasteur pipette to a culture tube (6 × 50 mm) and was evaporated to dryness under a stream of N_2. The samples were stored in the dry form until used for GLC.

The partially methylated alditol acetates were dissolved in 20 μl of chloroform and from 1 to 3 μl were used for GLC analysis. The partially methylated alditol acetates were identified by comparison of their gas chromatographic retention times with the retention times of standards and by mass spectrometric analysis of the effluent from the gas chromatograph (26). Gas chromatograph detector peaks were integrated (unpublished technique of H. Albert and P. Albersheim) and the integration values were divided by the calculated response factor for each partially methylated alditol acetate derivative (25). The mole per cent of each linkage isomer of a glycosyl residue in a sample was determined by dividing each corrected integration value by the sum of the corrected integration values for the entire sample.

RESULTS

Neutral and Amino Sugar Composition of Wall-released Elicitor Fractions. The neutral and amino sugar compositions of the four fractions of the Pms mycelial wall-released elicitor were determined by GLC of the alditol acetate derivatives. The three sugars which comprise essentially all of the carbohydrate portions of the four elicitor Fractions are glucose, mannose, and glucosamine (Table I). Other sugars account for less than 1% of the carbohydrate in each fraction.

Structural Analysis of Wall-rêleased Elicitor Fractions. The partially methylated alditol acetates of the four fractions of wall-released elicitor from each of the three races of Pms were synthesized. These volatile derivatives were separated and identified by combined gas chromatography-mass spectrometry. Structural assignments were made as described by Björndal *et al.* (12) and McNeil *et al.* (19). Chromatograms of the partially methylated alditol acetate derivatives of the four fractions of wall-released elicitor from the three races of Pms are presented grouped according to fraction in Figures 1 to 4. The chromatograms in these figures clearly demonstrate that the quantitatively major components (particular linkage isomers of a glycosyl residue) represent similar proportions of the same fraction derived from the three races of Pms. These chromatograms also indicate that there are two predominant types of polysaccharides in the four fractions. Fraction I is a glucan composed predominantly of 3-linked glucosyl residues, with the same amount of 3,6-linked branched glucosyl residues as terminal glucosyl residues. The polysaccharides of fractions II and IV, on the other hand, are predominantly mannans with unbranched portions of the molecules composed of approximately equal amounts of 2- and 3-linked mannosyl residues. The mannans contain a variety of branched residues with 2,3-, 2,4-, 2,6-, and 3,6-linked mannosyl residues predominating, and an amount of terminal mannosyl residues equal to the sum of the branched mannosyl residues. The polysaccharide of fraction III consists of a combination of the glycosyl residues present in the glucan and the mannan.

Table I. *Sugar Composition of Pms Wall-released Elicitor Fractions*

The sugar composition of each fraction was determined by alditol acetate analysis.

Fraction	Race	Sugar Composition of the Elicitors		
		Mannose	Glucose	Glucosamine
		mole %		
I	1	6	94	0
	2	5	95	0
	3	6	94	0
II	1	83	13	5
	2	84	12	5
	3	82	14	5
III	1	63	31	6
	2	64	27	9
	3	62	31	7
IV	1	82	11	6
	2	82	11	7
	3	85	9	6

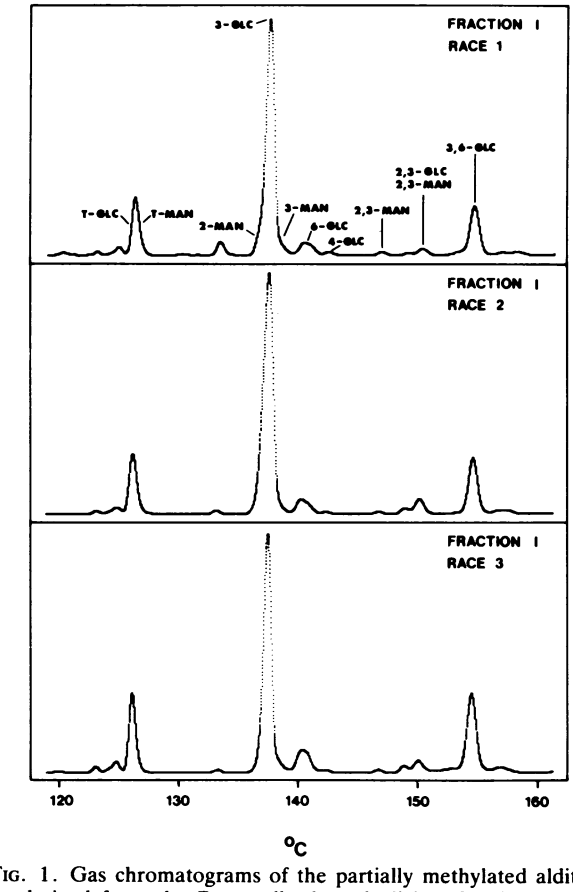

FIG. 1. Gas chromatograms of the partially methylated alditol acetates derived from the Pms wall-released elicitor fraction I. The gas chromatographic separations were performed on a Hewlett Packard model 7620 A gas chromatograph using a 120 × 0.3 cm (o.d.) copper column containing a mixture of 0.2% poly (ethylene glycol adipate), 0.2% poly (ethylene glycol succinate), and 0.4% XF-1150 on Gas Chrom P (100–120 mesh). Chromatography was performed using a helium flow rate of approximately 6 ml/min. Gas chromatograph electrometer settings were range 1 attenuation 2. The 1- to 3-μl sample was injected at a column temperature of 110 C, and after 2 min the temperature was raised at 1 C/min to 190 C. The linkages to each sugar derivative are indicated by numerical prefixes: thus, 3,6-Glc indicates that other glycosyl residues are glycosidically linked in the polysaccharides to the 3 and the 6 carbons of these glucosyl residues. Terminal residues are indicated by T- (*e.g.* T-Glc).

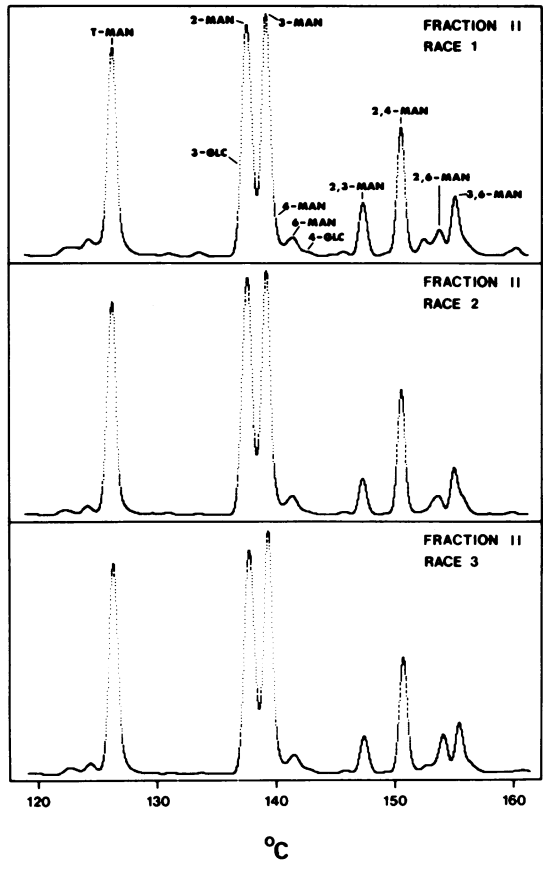

FIG. 2. Gas chromatograms of the partially methylated alditol acetates derived from the Pms wall-released elicitor fraction II. Chromatograms of fraction II from the three races of Pms are compared.

The glycosyl linkage composition (in terms of the percentage of the total glycosyl residues represented by each linkage isomer of a glycosyl residue) of the four elicitor fractions from each of the three races of Pms is presented in Table II. Fraction I is predominantly the glucan which is composed of 56% 3-linked glucosyl residues and, in addition, has 13% each of terminal and of 3,6-linked glucosyl residues. Fraction I also contains a small amount of the mannan, which is made most apparent by the presence of 2,3-linked mannosyl residues. Terminal, 2- and 3-linked mannosyl residues can be detected in this fraction but cannot be sufficiently resolved from other derivatives to permit quantitation.

Wall-released elicitor fractions II and IV are very similar to each other in their glycosyl constituents. The major components in both fractions are 2- and 3-linked mannosyl residues. The sum of the percentages of the branched residues (2,3-, 2,4-, 2,6-, and 3,6-linked mannosyl residues) is 22% and is essentially equal to the percentage of the polysaccharide accounted for by terminal mannosyl residues (23%). The preponderance of branched mannosyl residues indicates that the polymer is a highly branched structure with one branched and one terminal glycosyl residue for every two nonbranched residues. The 3-linked glucosyl residues associated with the glucan can also be detected in these fractions.

Fraction III represents a combination of the predominant glycosyl residues present in fractions I and II. The polysaccharides of fraction III have mannosyl residues with the same linkage distribution as the mannosyl residues of fraction II (or IV) and glucosyl residues with the same linkage distribution as those found in fraction I.

Partially methylated alditol acetate derivatives of aminoglycosyl residues are not observed in this study because under the acid hydrolysis conditions employed the glycosidic bonds between methylated aminoglycosyl residues and other components (usually methylated glycosyl residues) are not broken. The derivatives finally made from these partially methylated di- or oligosaccharides are not suficiently volatile to be analyzed by GLC.

Effect of Exposure to Periodate on Activity of Wall-released Elicitor Fraction I. Neighboring carbon atoms with hydroxyl groups (vicinyl hydroxyls) are oxidized by sodium metaperiodate to yield the corresponding dialdehydes. Mycelial wall-released elicitor fraction I is a 3-linked glucan with branches on carbon 6 of some of the 3-linked glucosyl residues. The 3- and 3,6-linked glucosyl residues of this polysaccharide would be expected to be resistant to periodate oxidation for these glycosyl residues lack vicinyl hydroxyls. Terminal glycosyl residues, on the other hand, have vicinyl hydroxyls on carbons 2 and 3 as well as on carbons 3 and 4 and should be sensitive to periodate oxidation.

The sensitivity of fraction I elicitor to periodate was tested by the following procedure. A sample (130 μg of carbohydrate) of fraction I elicitor was dissolved in 0.5 ml of 15 mM sodium metaperiodate and allowed to react at 20 C for 36 hr. After the reaction, any remaining periodate was consumed by the addition of 100 μl of ethylene glycol. The activity of the periodate-treated elicitor was compared to the activity of elicitor which had been incubated under the same conditions except that ethylene glycol was added to consume the periodate prior to adding the elicitor (Fig. 5). Another control sample which contained periodate pretreated with ethylene glycol but which lacked elicitor was also assayed for activity. Periodate treatment of fraction I reduced the elicitor activity by more than 90%.

Digestion of Fraction I with β-1,3-Exoglucanase. Mycelial

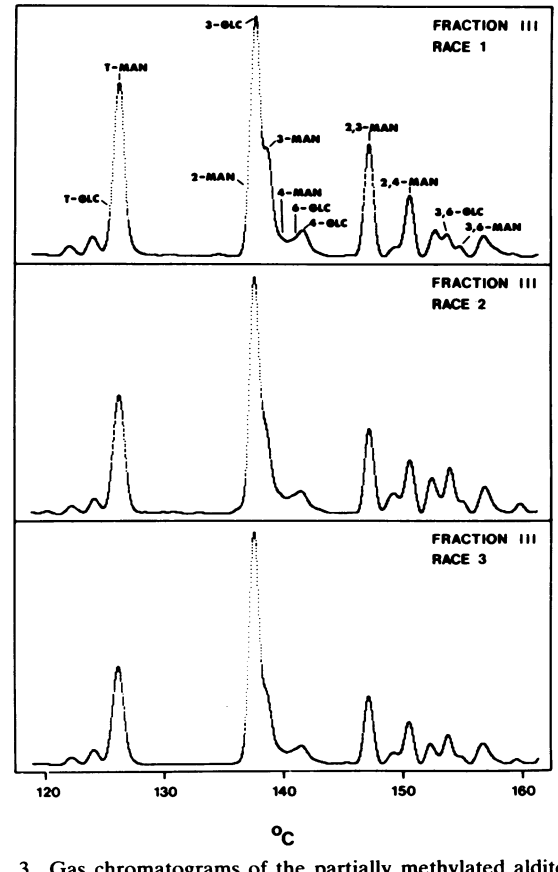

FIG. 3. Gas chromatograms of the partially methylated alditol acetates derived from the Pms wall-released elicitor fraction III. Chromatograms of fraction III from the three races of Pms are compared.

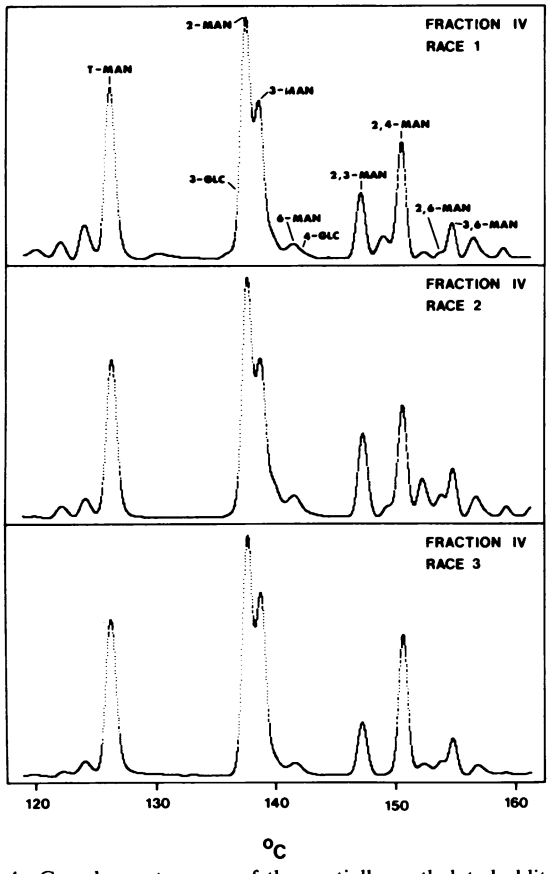

FIG. 4. Gas chromatograms of the partially methylated alditol acetates derived from the Pms wall-released elicitor fraction IV. Chromatograms of fraction IV from the three races of Pms are compared.

wall-released elicitor fraction I contains 13% terminal glucosyl residues. This fraction does not bind to concanavalin A; concanavalin A has an affinity for α-linked terminal glucosyl and mannosyl residues. Therefore, the terminal residues are likely to be linked in the β configuration. Fraction I also contains approximately 56% 3-linked glucosyl residues. If the 3-linked glucosyl residues are in the β configuration, a β-1,3-exoglucanase should hydrolyze the bonds between 3-linked glucosyl residues sequentially exposed to the enzyme from the nonreducing terminals.

A β-1,3-exoglucanase from *Euglena gracilis* var. *bacillaris* was isolated and purified by the procedure of Barras and Stone (8). A sample (1 mg of carbohydrate) of fraction I was treated with this enzyme using conditions which exhaustively digest laminarin, a β-1,3-glucan. The enzyme digestion of fraction I was monitored by assaying for the production of reducing sugars using the method described by Somogyi (23). The β-1,3-exoglucanase cleaved 70% of the glucosidic bonds in fraction I resulting in the production of oligosaccharides and glucose representing 90% of the glycosyl residues of the polysaccharides. Samples of fraction I which had been exhaustively treated with β-1,3-exoglucanase had the same elicitor activity as corresponding untreated samples of fraction I. Removal of small oligosaccharides and glucose from β-1,3-exoglucanase-treated samples of fraction I by molecular sieving on Bio-Gel P-2 (Bio-Rad) yielded polysaccharides with 4.5 times the specific activity of untreated samples of fraction I in both the cotyledon and hypocotyl assays.

The size distribution of the polysaccharides in untreated and β-1,3-exoglucanase-digested samples of fraction I was determined by molecular sieving chromatography on Bio-Gel A-0.5m (fractionation range for dextrans of 1,000 to 100,000 daltons, Bio-Rad). A sample (1 mg of carbohydrate) of fraction I was applied in 0.5 ml of 100 mM potassium phosphate, pH 7.2, to a column (1.5 × 62 cm) of Bio-Gel A-0.5m. The column was eluted with 100 mM potassium phosphate, pH 7.2, and the effluent was assayed for carbohydrate (Fig. 6) and elicitor activ-

Table II. *Glycosyl Linkage Composition of Wall-released Elicitor Polysaccharides*

The partially methylated alditol acetate derivatives were formed from the four wall-released elicitor fractions obtained from each of the three races of Pms. These derivatives were separated and quantitated using GLC as described in Fig. 1 and in "Materials and Methods." The glycosidic linkages to each sugar derivative are indicated by numerical prefixes: thus, 3,6-Glc indicates that other glycosyl residues are glycosidically linked in the polysaccharides to the 3 and 6 carbons of these glucosyl residues. Terminal residues are indicated by T- (*e.g.* T-Glc). The figures presented are the mole per cent of each glycosyl residue found in the polysaccharide of a fraction. Plus (+) indicates that a derivative was detected by mass spectrometry but could not be quantitated due to co-chromatography with another derivative; minus (−) indicates that a derivative was not detected.

Glycosyl Residues & Linkages	% OF TOTAL GLYCOSYL RESIDUES											
	FRACTION I			FRACTION II			FRACTION III			FRACTION IV		
	race			race			race			race		
	1	2	3	1	2	3	1	2	3	1	2	3
t-Glc	12.7	12.7	14.3	-	-	-	+	+	+	-	-	-
t-Man	+	+	+	23.0	23.6	23.4	24.8	19.3	19.0	22.5	20.1	20.3
3-Glc	57.0	61.0	48.7	+	+	+	19.2	19.7	25.0	7.9	5.2	5.5
2-Man	+	+	+	24.2	26.5	25.4	11.0	11.0	12.0	21.5	20.2	22.5
3-Man	+	+	+	23.8	25.3	25.0	11.3	11.5	13.1	19.2	19.6	21.8
4-Man	-	-	-	+	+	+	+	+	+	+	+	+
6-Man	-	-	-	2.8	2.9	2.9	-	-	-	2.7	3.6	2.2
6-Glc	4.7	4.8	7.3	-	-	-	+	+	+	-	-	-
4-Glc	0.9	0.5	0.6	+	+	+	5.2	5.5	5.1	+	+	+
2,3-Man	0.9	0.5	0.6	4.2	3.0	3.1	11.5	9.5	9.1	6.5	8.1	5.5
2,3-Glc	1.6	3.3	2.9	-	-	-	-	-	-	-	-	-
2,4-Man	+	+	+	10.3	10.2	10.0	6.4	6.2	5.8	11.5	11.4	14.0
2,6-Man	-	-	-	2.5	2.5	3.8	2.2	5.3	4.0	0.4	1.7	1.1
3,6-Glc	12.5	12.1	19.0	-	-	-	+	+	+	-	-	-
3,6-Man	-	-	-	6.1	5.0	5.1	0.8	0.8	0.6	3.3	4.1	3.5

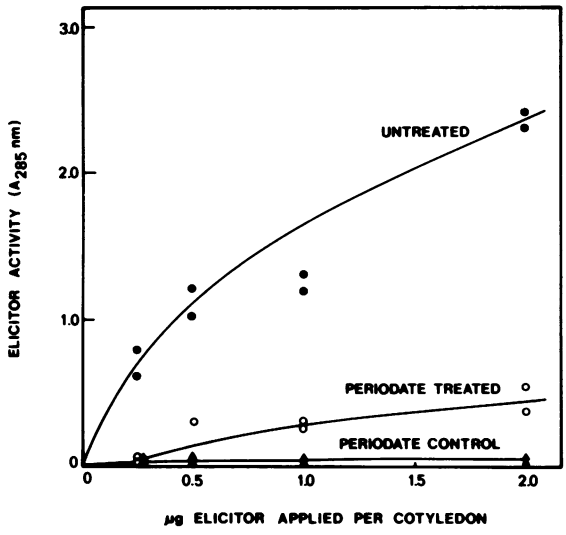

FIG. 5. Effect of periodate treatment on the activity (cotyledon assay) of Pms wall-released elicitor fraction I. Untreated fraction I (●) was compared in elicitor activity to fraction I which had been pretreated with periodate (○). A control reaction which contained periodate but no fraction I was also tested for elicitor activity (▲).

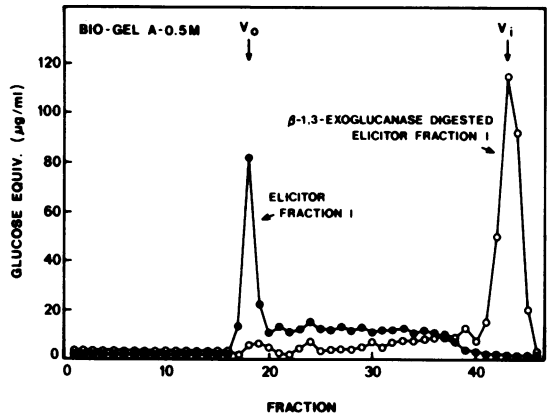

FIG. 6. β-1,3-Exoglucanase digestion of Pms wall-released elicitor fraction I. Untreated (●) and β-1,3-exoglucanase digested (○) fraction I elicitor were each applied to a column (1.5 × 62 cm) of Bio-Gel A-0.5 m. The column was eluted with 100 mM potassium phosphate, pH 7.2, and the distribution of carbohydrate determined. Fractions were 2 ml. The void volume (V_0) was 36 ml and the inclusion volume (V_i) was 86 ml.

ity (Fig. 7). The polysaccharides present in untreated fraction I are heterogeneous in size ranging from 1,000 to greater than 100,000 daltons (based on the fractionation range of dextrans on Bio-Gel A-0.5m). Approximately 30% of the polysaccharide material is excluded from Bio-Gel A-0.5m indicating that these molecules have mol wt in excess of 100,000. The elicitor content of effluent fractions from untreated Fraction I is proportional to the carbohydrate content of these fractions, except in the void fractions (16–18). The high mol wt elicitor present in the void fractions is of lower specific activity (approximately 1/3) than the elicitor which is partially included.

A sample (1 mg of carbohydrate) of β-1,3-exoglucanase digested fraction I was chromatographed on Bio-Gel A-0.5m under the same conditions used for chromatography of the sample of untreated fraction I. The carbohydrate (Fig. 6) and elicitor content (Fig. 7) of the effluent fractions was determined. The glucose and oligosaccharides resulting from β-1,3-exoglucanase digestion of Fraction I are completely included on Bio-Gel A-0.5m (fractions 41–45, Fig. 6). The polysaccharides remaining after β-1,3-exoglucanase digestion are found distributed throughout the fractionation range of the column. The percentage of the carbohydrate in fraction I which has a mol wt greater than 100,000 was reduced from 30% to 4% by β-1,3-exoglucanase digestion. Except for the included region of the column effluent, the only portion of the column effluent which increased in carbohydrate content as a result of β-1,3-exoglucanase digestion was fractions 36–39, containing molecules of approximately 10,000 daltons. The size distribution of molecules with elicitor activity in fraction I after β-1,3-exoglucanase digestion shifted to lower mol wt (Fig. 7) in parallel with the shift in the mol wt of the polysaccharides (Fig. 6). The 10,000 mol wt region of the column was particularly enriched in elicitor activity.

Inhibition of Elicitor Activity by α-Methyl-D-mannopyranoside. There are many examples of recognition phenomena mediated by carbohydrates (1, 18). This recognition can be inhibited in many of these systems by the addition of sugar or glycosides which compete for binding sites with the molecules mediating the recognition phenomena. The Pms elicitor is a carbohydrate which may mediate a recognition phenomenon. It is possible, that elicitor-stimulated accumulation of glyceollin may be blocked by sugars or glycosides. Several sugars and glycosides were tested at 20 μg/cotyledon for their ability to block 0.1 μg of Pms fraction I elicitor activity on soybean cotyledons. α-Methyl-D-mannopyranoside inhibited 70% of the elicitor activity, the α- and β-methyl-D-galactopyranosides inhibited 10% of the elicitor activity, while the α- and β-methyl-D-glucopyranosides did not measurably inhibit the elicitor.

The effectiveness of α-methyl-D-mannopyranoside in blocking elicitor activity was tested by applying fraction I elicitor (1 μg of carbohydrate/cotyledon) simultaneously with an amount of α-methyl-D-mannopyranoside varying from 1 to 100 μg/cotyledon (Fig. 8). The response of cotyledons to 1 μg of elicitor in the presence of 100 μg of α-methyl-D-mannopyranoside was equivalent to the response of 0.15 μg of elicitor without inhibition. α-Methyl-D-mannopyranoside has a similar inhibitory effect on the elicitor activity of fraction II and also inhibits the ability of fraction I to stimulate glyceollin accumulation in a soybean cultivar (Harosoy) which has no known resistance to Pms.

α-Methyl-D-mannopyranoside can inhibit the activity of fraction I elicitor by 85%. The remaining 15% of the activity may result from a second type of component. If there is a second

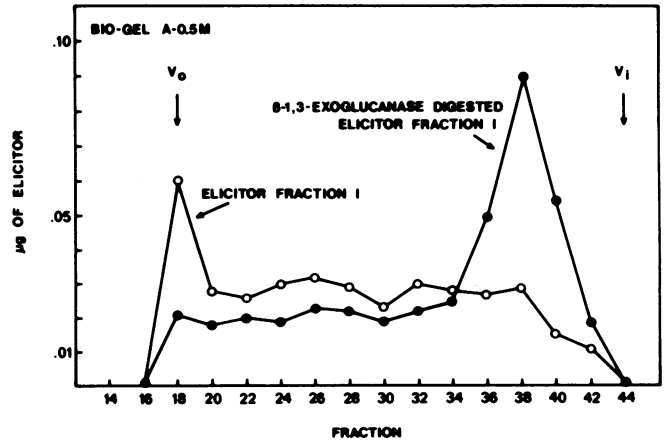

FIG. 7. Size distribution of molecules with elicitor activity in fraction I elicitor before and after digestion with β-1,3-exoglucanase. Untreated (○) and β-1,3-exoglucanase digested (●) Pms wall-released elicitor fraction I were chromatographed on Bio-Gel A-0.5 m (Fig. 6). The equivalent of 2.5 μl of a fraction was applied/cotyledon for measurement of elicitor activity. The A_{285} values for the resulting wound-droplet solutions were converted to μg of elicitor using a standard response curve for elicitor fraction I in the cotyledon assay (4).

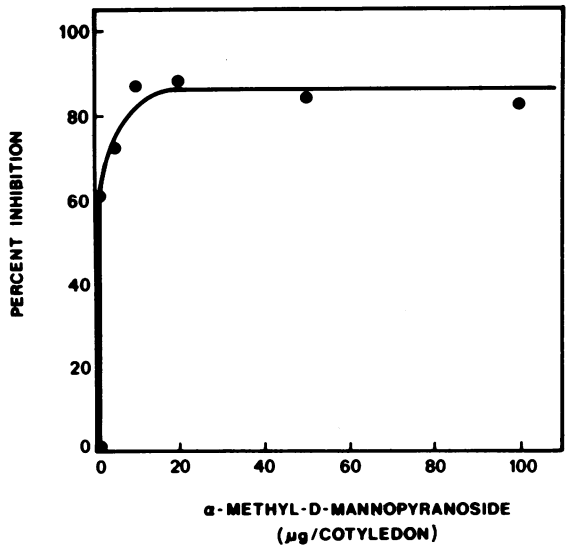

FIG. 8. Inhibition of the response of soybean cotyledons to elicitor by α-methyl-D-mannopyranoside. Fraction I elicitor (1 μg of carbohydrate/cotyledon) was applied with varying concentrations of α-methyl-D-mannopyranoside to wounded cotyledons and the response (A_{285}) was measured. The A_{285} values for the resulting wound-droplet solutions were converted to μg of elicitor using a standard response curve for elicitor fraction I in the cotyledon assay (4). The data are presented as the per cent inhibition compared to untreated elicitor.

component, this component might be inhibited by a glycoside other than α-methyl-D-mannopyranoside. This hypothesis was tested by assaying the elicitor activity of a combination of fraction I elicitor (0.5 μg of carbohydrate/cotyledon), α-methyl-D-mannopyranoside (20 μg/cotyledon), and another methyl glycoside (20 μg/cotyledon). The elicitor activity remaining in the presence of α-methyl-D-mannopyranoside could not be further reduced by any of the α- or β-methyl-D-glycopyranosides of galactose or glucose nor by N-acetylgalactosamine or N-acetylglucosamine. Surprisingly, α-methyl-D-glucopyranoside has the opposite effect. This glycoside eliminates the inhibition of elicitor activity due to the presence of α-methyl-D-mannopyranoside.

DISCUSSION

The similarity in composition and structure of the corresponding wall-released elicitor fractions of the three races of Pms confirms that the procedures described in the previous paper (5) yield highly reproducible fractions. This close similarity also demonstrates that any differences in the polysaccharides of the wall-released elicitors from the three races of Pms are quantitatively minor. Race-specific structural differences would not be expected to be quantitatively major differences. The surface antigens of yeast cells, for example, are responsible for species-specific variations. Yet the structural differences in these antigens, which vary from species to species, result from differences of less than 5% in the glycosyl linkage composition of these molecules (7). Differences of this magnitude are difficult to detect by analysis of the glycosyl linkage composition of an entire polysaccharide. The small differences in the glycosyl linkage composition of the corresponding fractions of wall-released elicitor from the three races of Pms may reflect important differences characteristic of a particular race of Pms or they may result from slight variations in the culture conditions of the three races of Pms. The glycosyl linkage determinations have been duplicated, but variations due to the variable growth rates of the Pms races may give reproducible variations. The significance of the differences observed will await more detailed studies of these fractions.

The fraction I polysaccharide, which does not bind to DEAE-cellulose or to concanavalin A-Sepharose, is similar in chemical properties, composition, and structure to the extracellular elicitor from Pms cultures (4). The fraction I elicitor is a 3-linked glucan. The glycosidic linkages are in the β configuration since β-1,3-exoglucanase digests 90% of the fraction I polysaccharide to glucose and small oligosaccharides. This enzyme digestion decreased the average mol wt of the fraction I elicitor molecules from about 100,000 to approximately 10,000 (Fig. 6) without significantly decreasing the elicitor activity of these molecules. Clearly, portions of the elicitor molecule are composed of β-1,3-linked glucosyl residues, but the active moieties are not digested by this enzyme treatment.

The ability of the *Euglena gracilis* var. *bacillaris* β-1,3-exoglucanase to hydrolyze the glycosidic bond of glucosyl residues which have 6-linked glucosyl residues attached (8) is responsible for the high degree of digestion of fraction I elicitor; fraction I elicitor has 13% 3,6-linked glucosyl residues (Table II). However, the enzyme digested elicitor is more highly branched than the untreated elicitor for the ratio of terminal, 3-linked, and 3,6-linked glucosyl residues in the elicitor-active exoglucanase-digested polysaccharide is 1:1:1 (unpublished results of B. Valent and P. Albersheim). Thus, the exoglucanase preferentially removes 3-linked glucosyl residues, while also removing 3,6-linked residues as gentiobiose. The moieties responsible for elicitor activity are contained within that portion of the polysaccharide which has been enriched in 3,6-linked glucosyl residues by the β-1,3-exoglucanase digestion.

Fraction I elicitor is inactivated by sodium metaperiodate. The branched 3,6-linked and the 3-linked glucosyl residues in this elicitor are resistant to periodate oxidation. The other glycosyl residues can theoretically be oxidized by periodate. The fact that fraction I elicitor is destroyed by periodate indicates that the elicitor is a carbohydrate and that periodate sensitive glycosyl residues, such as the terminal glycosyl residues, are required for elicitor activity.

α-Methyl-D-mannopyranoside inhibits the accumulation of glyceollin in soybean cotyledons treated wih fraction I elicitor. One likely explanation of this inhibition is that α-methyl-D-mannopyranoside interferes with the specific binding of the elicitor to a soybean receptor molecule. This suggests that the active moiety of the fraction I elicitor is an α-linked terminal mannosyl residue. Terminal mannosyl residues are present in this Fraction, accounting for an estimated 10% of the terminal residues. It is difficult to reconcile this observation with the fact that fraction I elicitor does not exhibit an affinity for concanavalin A-Sepharose. A possible explanation for the presence of α-linked terminal mannosyl residues without concanavalin A binding may be that other portions of the elicitor molecule block the binding of α-linked terminal mannosyl residues to concanavalin A. No explanation is offered for the observed reversal of α-methyl-D-mannopyranoside inhibition of elicitor activity by α-methyl-D-glucopyranoside.

The elicitor released from purified mycelial walls of Pms was divided into four fractions based on the chemical characteristics of the molecules with elicitor activity. The finding that molecules with different characteristics all have elicitor activity makes it likely that each of these molecules has the same moiety responsible for activity and that other portions of these molecules provide the chemical characteristics used in the fractionation. This explanation predicts that all of the elicitors should share a common component. There is evidence that this common component is the glucan in fraction I. A comparison of the relative activity of each of the four wall-released elicitor fractions with the sugar composition of these fractions suggests that the activity of a fraction (5) is proportional to its glucose content (Table III). That there is a single moiety responsible for elicitor activity in the wall-released elicitor fractions is substantiated by the finding

Table III. *Comparison of Relative Elicitor Activity and Glucose Content of Wall-released Elicitor Fractions*

The "average relative elicitor activity" is the average of the relative elicitor activities of a fraction measured in the cotyledon and hypocotyl, and, where appropriate, in the cell suspension culture assays.

Fraction	Average Relative Elicitor Activity	% Glucose
I	100	95
II	16	13
III	25	30
IV	7	10

that the two fractions which differ most in sugar composition, fraction I (6% mannose, 94% glucose) and fraction II (83% mannose, 13% glucose) are both inhibited to the same extent (85%) by α-methyl-D-mannopyranoside. This inhibition implies that the moieties responsible for elicitor activity in each fraction are binding to the same sites on soybean cells and, consequently, that the active moieties in each fraction are the same.

GENERAL DISCUSSION

The molecule of pathogen origin which stimulates the accumulation of a phytoalexin in host tissues and is the focus of this study has been termed an "elicitor" (17). We now wish to review briefly the major findings of this study of the Pms elicitor and to speculate on other related aspects of plant disease resistance.

Assays of Elicitor Activity. The development of reliable assays for the Pms elicitor resulted in several significant findings concerning the recognition of an elicitor by plant cells. First, soybean seedlings are very sensitive to the Pms elicitor. Less than 0.01 nmole (2 μg of carbohydrate) of the Pms elicitor applied to a single soybean hypocotyl results in the accumulation of 150 nmoles of glyceollin. This response is a 15,000-fold amplification of the elicitor molecules applied. Second, the reaction of soybean cells to elicitor is highly specific. Several compounds closely related in composition and structure to the Pms elicitor were tested for elicitor activity, but none of these compounds was an active elicitor in even 1,000 times the amount needed to give a response with the Pms elicitor (4). On the other hand, soybean cotyledons, hypocotyls, and cell suspension cultures give equivalent responses to Pms elicitor (5) indicating that the response to the Pms elicitor is a general feature of soybean cells.

Extracellular Elicitor of Pms – A Mycelial Wall Component. The cotyledon assay for elicitor activity was used to purify the extracellular elicitor from Pms cultures (4). The comparison of the composition and structure of this elicitor to the polysaccharide components of the mycelial wall of Pms (Fig. 12 and Table I of ref. 4) provided the evidence which suggested that pathogen wall components are elicitors. Subsequenty, elicitor was released from purified mycelial walls of Pms using a technique originally designed to remove the surface antigens from another fungus, the yeast *Saccharomyces cerevisiae* (21). Comparison of the composition of this elicitor-containing fraction of the mycelial walls to that of the total purified mycelial walls of Pms demonstrated that the wall-released material is a minor, discrete portion of the mycelial wall; this fraction accounts for only 8.9% of the walls but contains much of the mannose, glucosamine and protein present in the purified walls of Pms.

Covalent Attachment of Elicitor to Other Macromolecular Wall Components. The wall-released elicitor was separated into four active fractions on the basis of the affinity of the elicitor molecules for DEAE-cellulose and for concanavalin A-Sepharose. This fractionation demonstrates that elicitor molecules can have different chemical characteristics. This observation, supported by additional data, suggests that the moiety responsible for elicitor activity is covalently linked to several other types of molecules.

Are Elicitors Responsible for Race-specific Resistance? The elicitor activities of the four wall-released elicitor fractions were carefully examined using three separate biological assays; the cotyledon assay (Fig. 3 of ref. 5), the hypocotyl assay (Fig. 4 of ref. 5) and the cell suspension culture assay. All three assays gave the same result: the activities of the elicitors from different races are identical. This finding is in direct contradiction to a preliminary report by Keen (15) on the extracellular elicitor of Pms cultures. He claimed that the elicitor from race I of Pms stimulates a greater accumulation of glyceollin in Harosoy 63 (incompatible with race I of Pms) than in Harosoy (compatible with race I of Pms). Our experience with the elicitor of Pms indicates several major objections to Keen's work. First, the experiments reported were performed using relatively impure preparations of elicitor. The biological assays for elicitor activity are sensitive to many of the compounds present in culture filtrates, making the interpretation of experiments performed with impure elicitor difficult to assess. Second, a comparison of elicitor activity based on the differential response of two different cultivars of soybean is difficult to perform. The elicitor stimulated accumulation of glyceollin in soybean organs varies with the age of the seedlings, with the storage conditions of the soybean seed prior to germination, and with variations in the growth conditions of the seedlings. Any and all of these factors can change the capacity of soybean tissues to accumulate glyceollin. These parameters can be effectively controlled only if plants grown from the same lots of seed under the same conditions are used for a comparison of elicitor activities. These difficulties are evident in the data presented by Keen (15). There is as large a difference in the accumulation of glyceollin stimulated by elicitors from Pms races 1 and 3 on Harosoy, which is compatible with both race 1 and race 3, as on Harosoy 63, which is incompatible with race 1 and compatible with race 3. These considerations essentially preclude experiments based on glyceollin accumulation in two different soybean cultivars. We have avoided this problem in the work presented in these papers by comparing, on the same soybean cultivar, the effect of elicitors isolated from three different Pms races.

Keen also used no direct measure of the amount of his elicitor preparation applied. Instead, a fraction of the culture filtrate was applied to wounded hypocotyls and glyceollin accumulation was determined. Comparisons of activity based on a fraction of a total culture filtrate, *e.g.* one-tenth of the elicitor in 500 ml of culture filtrate is not valid because the rates of growth of the several races of Pms are quite different. The amounts of mycelia and of elicitor present in the filtrates of cultures which had grown at different rates and to different extents vary considerably. The existence of a "specific elicitor" as claimed by Keen (15) requires substantiation with data derived from experiments using known quantities of purified elicitors from different races of Pms assayed on the same cultivar of soybean. Without this substantiation, there is no evidence for race-specific elicitors.

Structure of Pms Elicitor – A Substituted Glucan. The elicitor from Pms was demonstrated to be a polysaccharide by several criteria. The stability of elicitor activity to extremes of pH, heat (121 C for 3 hr), and pronase essentially eliminate the possibility of the Pms elicitor being a protein. The lack of affinity of the extracellular elicitor for most ion exchangers at the pH extremes which maximize binding of proteins and nucleic acids also makes these two biopolymers highly unlikely candidates for the elicitor. The elicitor is a macromolecule which is heterogeneous in size, another characteristic of polysaccharides and not of proteins. Elicitor is inactivated by sodium metaperiodate; carbohydrates are particularly sensitive to this chemical. The elicitor is digested by a purified polysaccharide degrading enzyme, a β-1,3-exoglucanase. This degradation decreases the mol wt of the elicitor. Elicitor activity is specifically inhibited by α-methyl-D-manno-

pyranoside. All of this evidence is consistent with a polysaccharide being the major component of the Pms elicitor.

The correlation of the glucose content of each wall-released elicitor fraction with the elicitor activity of the fractions (Table III) supports the hypothesis that there is only a single elicitor in Pms walls and that this elicitor is a glucan. The ability of α-methyl-D-mannopyranoside to inhibit elicitor activity suggests that the glycosyl residues which are responsible for the elicitor activity of the glucan are α-linked mannosyl residues. The presence of terminal mannosyl residues (approximately 10% of the terminal glycosyl residues) in the fraction I glucan has been demonstrated by methylation analysis. It is likely that the Pms elicitor is a glucan with terminal mannosyl residues. This study resulted in the purification, in the characterization, and in a proposed partial structure of the Pms elicitor.

Glyceollin Accumulation—Necessary but Not Sufficient for Race-specific Resistance. Elicitors with the same biological activities can be isolated from three Pms races which differ in their abilities to grow on the same host. The observation indicates that the accumulation of glyceollin in host tissue stimulated by the Pms elicitor is not sufficient for resistance to an incompatible race of Pms. Glyceollin accumulation is most likely necessary for limiting the spread of the pathogen, but a second defense mechanism is required for race-specific resistance as demonstrated by the inability of elicitor to stop the spread of a compatible race of Pms when the elicitor was added, simultaneously with the pathogen, to wounded hypocotyls (5). This result shows that the presence of elicitor at the onset of an infection does not in itself determine the outcome of that infection. It is probable that elicitor is present in both compatible and incompatible interactions during the early stages of infection and that the presence of elicitor leads to essentially equivalent levels of glyceollin accumulation in both cases (Fig. 5 of ref. 5).

The distinction between compatible and incompatible races of Pms is most likely to be in the rate of colonization of host tissue. The pathogen continually colonizes new tissue stimulating the accumulation of glyceollin. In a compatible interaction, before the glyceollin concentration reaches a level toxic to Pms, the mycelia of the compatible race has penetrated new tissue devoid of glyceollin. Any condition which slows the spread of the pathogen will tend to eliminate the lead that the compatible pathogen has over the accumulation of glyceollin and result in the restriction of the pathogen.

Inhibitors, Phytoalexins, Race-specific Resistance and Specificity Factor Theory. There is evidence in the literature (6, 9, 20, 31) that a normally compatible host-pathogen interaction can be made incompatible by inhibiting the rate of growth of the pathogen. A similar mechanism may be responsible for natural resistance to an incompatible pathogen, *i.e.* race-specific resistance. If an incompatible pathogen triggers a host response which decreases the rate of growth of the pathogen, then the spread of the pathogen will be stopped by the accumulation of phytoalexin (Fig. 9). The compatible pathogen may avoid triggering the inhibitory response and as a result avoid the toxic effects of the phytoalexin (Fig. 9). The triggering of the inhibitory response would, in this model, be the basis of race-specific resistance. The compound of pathogen origin which triggers this inhibitory response will be called a "specificity factor."

The model in Figure 9, which incorporates the use of elicitor, phytoalexin, specificity factor, and inhibitors, is the basis of our proposed specificity factor theory for race-specific resistance. Race-specific resistance may result from any relatively rapid host response which is triggered by an invading pathogen and which is inhibitory to or slows the growth of the pathogen. The most obvious feature of the host reaction to an incompatible pathogen early in infection is the disruption of the membranes of the host cells responding hypersensitively (27, 28). The rapid necrosis of these cells may result from the disruption of lysosomes leading to

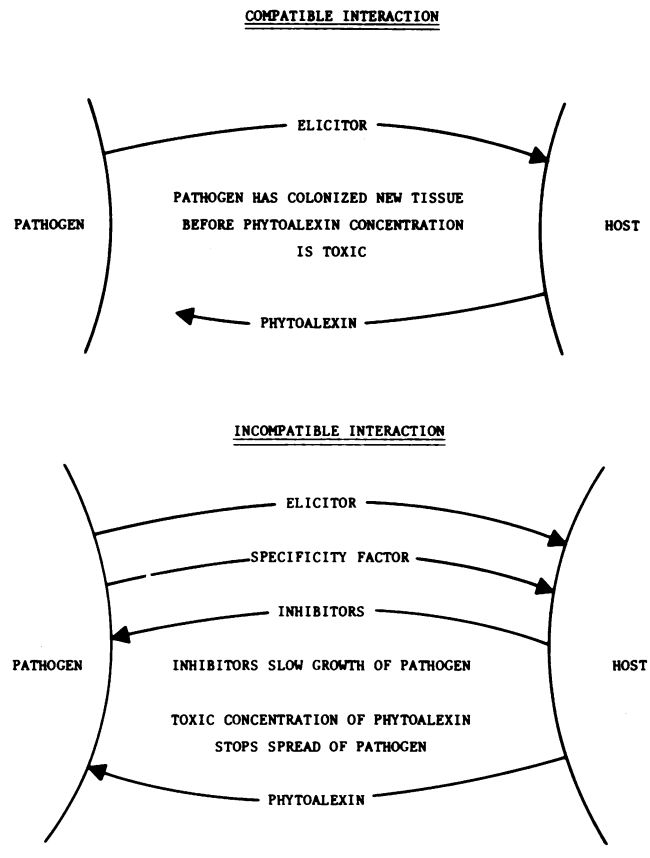

FIG. 9. Elicitor specificity factor model of race-specific resistance.

host cell death by a form of autolysis (30). The disruption of lysosomes may also result in the release of enzymes which digest wall components of the invading pathogen (30) or are in some other way inhibitory to pathogen growth. The early morphological changes which characterize an incompatible host-pathogen interaction may reflect the biochemical events which eventually lead to restriction of the pathogen.

Experiments with the tomato pathogen *Cladosporium fulvum* offer some support for the hypothesis that the hypersensitive disruption of host cell membranes inhibits the spread of an incompatible pathogen. Van Dijkman and Sijpesteijn (29) claim that the macromolecular fraction from cultures of incompatible races of *C. fulvum* increases the loss of electrolytes from tomato cells, whereas equivalent fractions from cultures of compatible races of *C. fulvum* have no effect (29). The conclusion from these experiments was that in each case in which increased leakage occurred, the plant cells had specific receptors in their cell membranes which interacted with specific compounds of the pathogen leading to increased leakage (29). If either the plant or the pathogen lacked the correct component, the interaction was compatible. The active components of the macromolecular fraction of pathogen cultures may in fact be the specificity factors of *C. fulvum*. Unfortunately, these experiments are not completely convincing because the effects observed were small compared to the background and because crude preparations were used.

The *C. lindemuthianum-P. vulgaris* system provides further evidence for the involvement of specific inhibition in addition to phytoalexin accumulation as the basis for race-specific resistance. An elicitor has been obtained from the culture filtrate and from the mycelial walls of *C. lindemuthianum* (3). There are no apparent differences in the elicitors from different races of *C. lindemuthianum* (3). The elicitors from *C. lindemuthianum* are, therefore, not likely to be specific in their effects on *P. vulgaris*, requiring a second factor which is responsible for race specificity.

Bernard *et al.* (10, 11) reported that *C. lindemuthianum* produces "protection factors" which are capable of protecting certain varieties of *P. vulgaris* from infection by normally compatible races of *C. lindemuthianum*. The factors will protect only plant varieties which are normally incompatible with the race of *C. lindemuthianum* which produced the factors (11). This specificity of protection is a characteristic of our proposed specificity factor (Fig. 9).

Many pathogens are observed to have a limited range of hosts. The plant species on which a particular pathogen is not able to grow are said to have a general resistance to that pathogen. The model we are proposing for race-specific resistance(Fig. 9) can also be used to explain some types of general resistance. If it is assumed that a pathogen will grow relatively slowly on a host which is outside of its host range, then the growth of this pathogen is likely to become restricted by phytoalexin accumulation. This explanation is especially attractive because it eliminates the need to hypothesize specific recognition of each pathogen stopped by general resistance, for elicitors are likely to be wall components common to many pathogens.

Yeast Mannan as Model for Pms Specificity Factors. The glucan responsible for elicitor activity in Pms is but one of many examples of the involvement of polysaccharides or oligosaccharides in recognition phenomena (1). It is likely that the specificity factors responsible for race-specific resistance are also polysaccharides. The surface antigens of yeast provide a detailed model of molecules which may be similar to the specificity factors of Pms. The yeast surface antigens are the immunodominant antigens of yeast cells and are the features which distinguish different strains and mating types of yeast. These molecules are involved in the highly specific intercellular interactions of yeast. The specific features of Pms which are involved in host-pathogen interactions may also be surface components with characteristics similar to those of the yeast surface antigens.

The yeast surface antigen is a highly branched mannan-containing glycoprotein (7, 32) which is released from the cell walls of yeast by a heat treatment. A molecule with characteristics very similar to the yeast surface antigen is present in purified walls of Pms. This molecule is released from Pms walls by the same heat treatment and is fraction IV of the wall-released elicitor. Fraction IV is a highly branched mannan-containing glycoprotein. Both the yeast and the Pms molecules have the same quantitatively important terminal and nonbranched glycosyl residues. These similarities suggest that the mannan-proteins of Pms fraction IV should be the primary candidates for the specificity factors of this pathogen.

Acknowledgments—The authors wish to acknowledge the fine technical assistance of S. Martin. They wish to thank H. Albert for integrating the gas chromatographic peaks, M. McNeil for carrying out the mass spectrometric identifications, and S. Markey of the Colorado Medical Center in whose laboratory all of the combined gas chromatography-mass spectrometry was performed.

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