

A new method for determining the distribution of allergenic fractions in biological materials: Its application to grass pollen extracts

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Dialyzed whole extracts of seven grass pollens representing six botanical tribes were fractionated with the use of a modified starch gel electrophoretic technique. Without prior elution from the gels, individual protein-containing fractions were analyzed for their allergenic properties by direct skin testing in grass pollen-sensitive individuals and for their antigenic components with the use of immunodiffusion against rabbit antisera to highly purified rye grass fractions and to crude grass pollen extracts. Immunochemically distinct groups of antigens, classified as Groups I, II, and III, were found in most of the grass pollens studied, the principal exception being Bermuda which contained none of these groups. Antigens of a particular group but from different pollens possessed similar allergenic properties in the individuals tested. Each individual could be broadly classified on the basis of his skin reactivity to the different antigenic groups.

Aqueous extracts of pollens from many grass tribes were found to possess several cross-reacting antigens and a few species-specific "minor" antigens, according to immunodiffusion¹⁻³ or agglutination^{4, 5} analysis with the use of rabbit antisera. In man, substantial allergenic cross-reactivity between several grass pollens was observed in neutralization of Prausnitz-Küstner passive al-

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lergic serum transfer experiments^{6, 7}; although with a few sensitive individuals' sera, species-specific allergens were apparently able to be recognized.⁷ Occasional individuals who are skin reactive to the pollen of one or just a few grass pollen species have been detected^{2, 8}; these may well have been due to sensitivities toward "minor" allergens confined to a few species. Among grass pollens important in hay fever, a noted exception to these general observations is Bermuda grass pollen, for which very little antigenic or allergenic cross-reactivity with other grass pollens has been found.^{1, 2, 9}

Previous experiments by one of us identified two highly purified, immunochemically distinct allergenic components (Groups I and II) of perennial rye grass pollen.¹⁰⁻¹⁴ Within a particular group, closely related isoallergenic* variants were found,¹¹ similar to the case observed for ragweed antigen E.¹⁵ Groups I and II showed no evidence of allergenic cross-reactivity by direct skin test¹⁴ or histamine release assay from isolated allergic human leukocytes¹⁶ or of antigenic cross-reactivity by immunodiffusion analysis by using rabbit antisera to Group I or to crude rye grass extract.¹⁰ The rye Group I components (molecular weight about 27,000) were basically protein in nature and appreciably skin reactive in about 90 per cent of 50 grass-sensitive individuals tested¹⁴; corresponding Group II components were also proteins (molecular weight about 11,000) and skin reactive in only about 70 per cent of the people tested. The specific allergenicities of Groups I and II relative to the crude pollen extract varied significantly from individual to individual, although Group I was usually the more active on a weight basis.^{14, 16}

Several observations together make a strong case for the implication of only the protein moieties as significant determinants responsible for immediate hypersensitivity reactions to grass pollens: Augustin¹⁷ destroyed skin reactivity of timothy and orchard pollens by treatment with various proteolytic enzymes but not with certain carbohydrases; Marsh and associates¹⁴ observed for the major rye allergen (Group I) complete loss of skin activity by proteolytic digestion but no detectable loss by demonstrated carbohydrase digestion. More recently, Marsh and co-workers¹⁸ and Haddad and associates¹⁹ showed that mild formalin treatment (which modifies almost exclusively protein components) of rye Group I, or of a crude grass pollen extract (containing rye, fescue, orchard, timothy, and June pollens), was highly effective in almost completely destroying capacity to release histamine from allergic human leukocytes, skin reactivity, and ability to provoke bronchospasm in allergic individuals.†

The purpose of the present study is to define the groups of related proteinaceous antigens of different grass pollens important in human allergy. Particular attention has been paid toward immunological identification of

*For rye grass pollen, the term "isoallergen" has been used to denote a member of a group of allergens which possess experimentally indistinguishable immunological properties and closely related physicochemical structures^{11, 12, 14}; the principal distinctions between the isoallergens of a particular group are differences of electrical charge at alkaline pH, leading to different electrophoretic mobilities of the isoallergens. In this paper, usage of the term will be extended to include the immunologically cross-reacting allergens found in a number of grass pollen species.

†Allergen derivatives which have almost completely lost the original allergenic properties but have retained to a high degree the original antigenic properties of the native molecules are termed "allergoids."¹⁸

Table I. *Grass pollens used for experiments*

<i>Pollen species</i>	<i>Tribe</i>	<i>Symbols</i>	<i>Common in Los Angeles area</i>
Perennial rye (<i>Lolium perenne</i>)	Hordeae	R or Ry	Yes
Meadow fescue (<i>Festuca elatior</i>)	Festuceae	F or Fe	Yes
Orchard (<i>Dactylis glomerata</i>)	Festuceae	O or Or	Yes
Meadow velvet (<i>Holcus lanatus</i>)	Aveneae	V or Ve	No
Sweet vernal (<i>Anthoxanthum odoratum</i>)	Phalarideae	SV	No
Timothy (<i>Phleum pratense</i>)	Agrostideae	T or Ti	No
Bermuda (<i>Cynodon dactylon</i>)	Chlorideae	Be	Yes

components related to rye Groups I and II; a new Group III has also been provisionally defined. Rather than attempt the laborious and complicated column chromatographic purification of the principal components of all the seven grass pollens under study, a simple method was devised to assay qualitatively the allergenicity, in terms of skin reactivity, of a large number of fractions including those exhibiting antigenic relationships with one another.

MATERIALS AND METHODS

Pollen extracts

Seven different grass pollens* from six tribes were studied (Table I). Only the four grasses indicated are commonly found in the Greater Los Angeles area. The pollens were weighed, defatted with peroxide-free dry ether, and extracted for 18 hours at 4° C. with 10 times their weight of 0.125M NH_4HCO_3 at pH 7 to 8 throughout the extraction process. These "10 per cent extracts" were dialyzed in Visking $1\frac{1}{32}$ inch diameter dialysis tubing against several changes of 0.002M NH_4HCO_3 and finally against distilled water over a period of 3 to 4 days at 4° C.¹⁰ The dialyzed extracts were clarified by ultracentrifugation (20 min. at 40,000 to 80,000 g) to remove traces of fat and insoluble material and lyophilized (50 μ Hg pressure or less). This process of dialysis and lyophilization has been shown to affect no significant denaturation or loss of biological activity of grass pollen extracts.^{10, 14, 16}

Allergic individuals

Tests were performed on 10 adults aged 21 to 45 years and on 19 children aged 10 to 15. Seventy per cent of the individuals tested had lived in the Greater Los Angeles area for virtually all of their lives; the remainder (mainly adults) would have received significant exposure (at least one season and usually longer) to tested pollens not commonly found near Los Angeles.

Electrophoretic fractionation

The method used was a development of Smithies²⁰ horizontal starch gel electrophoresis. Since many steps were critical in obtaining a good and uniform separation of the pollen components, the method will be described in detail.

Each dried dialyzed pollen extract was thoroughly mixed with the borate

*The native pollens were generously supplied by Hollister-Stier Laboratories, Spokane, Wash. All samples contained less than 2 per cent moisture and had been stored at room temperature in sealed containers for less than 2 years before used.

gel buffer (pH 8.5) of Smithies at a concentration of 200 mg. of lyophilized solids per milliliter. Although some of the pollen materials were not quite completely soluble, it was necessary to use such high over-all concentrations in order to obtain sufficiently high concentrations of the individual protein components to render them visible in the starch gel following subsequent electrophoretic separation and staining.

Starch gels ($45 \times 10 \times 0.6$ cm.) were prepared with the use of 12 Gm. of hydrolyzed starch (Connaught Laboratories, Toronto, Canada) per 100 ml. of borate gel buffer (pH 8.5, $I = 0.15$) containing 1 Gm. of Merthiolate (Eli Lilly and Co., Indianapolis, Ind.) preservative per 10,000 ml. of buffer. The gels were allowed to set for 16 to 24 hours (no longer) at 4° C. before used. The gels were cut laterally along the "origin" and the two sections of the gel separated to give a slit 2 to 3 mm. wide. Whatman 3 MM filter paper strips (1.5×0.5 cm.) were thoroughly impregnated with a particular pollen extract solution, blotted to remove excess liquid, and carefully inserted into the slit in the gel. The width of the slit, to within 0.5 cm. of each side, was completely filled with strips impregnated with the pollen solution. At the sides, paper squares (0.5 cm.²) impregnated with the gel buffer were inserted. The slit was carefully closed and the surface of the gel blotted sparingly.

Whatman 3 MM filter paper wicks (8×10 cm.) were cut; four thicknesses were used at each end of the gel. The wicks were impregnated with the borate buffer used for the electrophoresis troughs (pH 8.5, $I = 1.85$) and placed on the gel. The gel and wicks were covered with a thin polyvinylchloride sheet (Saran Wrap, Dow Chemical Co., Midland, Mich.), and horizontal electrophoresis was carried out for 7 hours at 4° C. with good ventilation with of 10.5 volts per centimeter gel length and 2.1 to 2.5 mA. per centimeter gel width. Twenty minutes after starting the run, the paper squares which had been used to introduce the pollen solution were removed and the electrophoresis resumed.

The gels were uncovered and carefully blotted. A longitudinal indicator strip (1.0 cm.) was cut through the gel, removed from the gel tray in numbered portions, and stained with a mixture of amido black (0.5 per cent) and Ponceau S (0.5 per cent) in a solution containing the protein precipitants trichloroacetic acid (3 per cent) and sulfosalicylic acid (1 per cent) in distilled water.* The stained portions of the gel were washed in several changes of trichloroacetic acid (3 per cent) to remove background staining (about 12 hr.), blotted, and replaced in their original position in the gel tray.

Sections of the unstained portion of the gel ($4.0 \times 0.5 \times 0.6$ cm.), corresponding to protein-staining bands on the indicator strip (and several unstained controls), were cut out with a stainless steel template. They were transferred to slits of slightly greater size cut in a Plexiglas tray. The top of the tray was sealed with Parafilm (American Can Co., Neenah, Wisc.) and stored at 4° C. until used.

A more conventional electrophoretic experiment, comparing several pollen extracts, was performed essentially according to Smithies'²⁰ procedure; this experiment is shown in Fig. 4, A.

*The conventional water-methanol solvent causes gel shrinkage and is therefore unsuitable for this technique.

Skin testing

Uniform circular scratches were made on the backs of grass pollen-sensitive individuals and nonallergic control subjects with a Center (Center Laboratories Inc., Port Washington, N. Y.) scarifier. As many as 48 scratches were made on the tested subjects. One drop (about 0.1 ml.) of sterile physiologic saline was placed over each scratch. Slices (about 2 mm. thick) were cut from the gel sections containing pollen components (or from negative control sections) and transferred to the scratches, placing the freshly cut areas in contact with the skin.

The gel slices were allowed to remain in position for 20 min., making certain that the regions of contact between the gels and the scarified skin remained wet by sparing addition of more saline where necessary. The gel slices were removed and the skin blotted. Skin reactions were graded as negative or one to five plus, according to the size of the wheal produced and the presence of pseudopodia. Details of this grading are given in the legend to Fig. 1.

At all stages during the handling of the gels great care was taken to avoid possible transfer of material from one fraction to another. The allergenic and antigenic activity of the pollen components within the gel sections has been found to be retained after several months of storage at 4° C.

Purification of antigens

Rye Groups I and II were prepared in high purity according to the method of Johnson and Marsh.¹⁰ In outline, dialyzed extract of rye grass pollen (*Lolium perenne*) was prepared as described under "Pollen extracts" and fractionated successively on DEAE-cellulose and Sephadex G-75 or G-100 columns. The Group I preparation contained mainly the isoallergens I-B and I-C and much smaller amounts of I-A and I-D¹¹; the Group II fraction contained predominantly component II-B.

A third rye allergenic fraction, provisionally termed Group III, was isolated as a mixture of two isoallergenic forms, III-A and III-B, by DEAE-cellulose and repeated Sephadex G-75 chromatography followed by ammonium sulfate precipitation.²¹ A purified timothy grass pollen allergen, antigen B (AgB), was prepared and kindly supplied by Dr. Arthur Malley.^{22, 23} No immunological cross-reactivity was detected between rye Groups I, II, and III^{10, 14, 16, 21} or between any of these rye fractions and timothy AgB.²¹

Preparation of antisera

Dialyzed crude pollen extracts (Table I) or purified Groups I or II, emulsified with complete Freund's adjuvant or adsorbed on alum, were injected into rabbits. Most rabbits received 6 to 8 fortnightly immunizations of 10 mg. crude or 2 mg. purified antigen before bleeding.

Immunodiffusion analyses

The various rabbit antisera were tested initially against solutions of both crude and purified antigens by the double diffusion technique of Ouchterlony in agar gel. Suitable high titer sera were used to investigate the antigenic composition of individual segments cut from the starch gel electrophoretic experiments. Slices of the segments, cut to the size of antigen wells used for normal

immunodiffusion analysis, were placed directly into the antigen wells. Immunodiffusion of the antigenic components against appropriate antisera was allowed to proceed for 2 to 3 days at room temperature until the precipitin lines had fully developed.

Attempts to obtain good precipitating antisera to the rye Group III fraction have failed so far; but it was possible to detect this and related Group III components with the use of a very high titer anti-whole fescue serum (containing some anti-Group III) with the purified rye Group III antigen as a reference. The location of an allergenic fraction of timothy pollen, Malley's AgB, was found in a similar manner with the use of anti-whole timothy serum and purified AgB.

In separate experiments, the fractions of a particular pollen were tested for Group I, II, and III components; the AgB component was sought only in timothy fractions, since other investigations²¹ had revealed that of the pollens studied only timothy possessed appreciable amounts of AgB or AgB-like antigens. After the various antigenic groups had been detected in the fractions from different pollens, fractions of a given type (e.g., Group I) were allowed to diffuse against an appropriate monospecific antiserum (i.e., anti-rye Group I) to determine qualitatively the extent of cross-reactivity between the related components of different pollens. The purity of the fractions examined was tested with the use of antisera toward all the crude pollen extracts.

Radial immunodiffusion. Semiquantitative assessments of the relative concentrations of different antigens related to rye Group I in slices cut from respective sections of the starch gels were made with the use of a modification of the radial immunodiffusion technique of Mancini and associates.²⁴ Anti-rye Group I serum, diluted tenfold, was incorporated into agar gels. Several circular starch gel slices (3 mm. diameter; 2 mm. thick) containing each Group I antigen were placed in wells of the same size previously cut in the agar gels. Ten microliter portions of pure rye Group I solution (80 μ g per milliliter) were placed in other wells as controls. Following incubation of the gels at 32° C. for 30 hours, the diameters of the precipitin rings around the wells were compared with the controls on the same gel.

RESULTS

Electrophoretic fractionation

A comparative routine starch gel electrophoretic separation of components of the grass pollens at pH 8.5 is shown in Fig. 4, A. With the use of the modified electrophoretic procedure described here, uniform and reproducible separations of the protein components of the pollen extracts were obtained. By staining the entire gels, there was no evidence of extensive "tailing" of bands or of differences in their apparent electrophoretic mobilities across the width and depth of the gel except at the edges (which were discarded).

Skin testing: General observations

For a given grass-sensitive individual, characteristic wheal and flare responses were associated only with certain fractions. Nonallergic individuals gave

negative reactions at all test sites. Negative controls cut from virgin starch gel elicited no positive reactions except in one or two instances where dermatographia was evident; such individuals showing nonspecific reactions have been excluded from the analysis of results. Regions of the gels which did not stain for protein, but which may contain carbohydrate components (especially in the cathodic region where much of the uncharged or weakly charged carbohydrate material¹⁰ would be carried by electro-osmosis), gave occasional weakly positive reactions in only one extremely skin-sensitive individual out of 10 persons tested with several such fractions from both rye and fescue pollens.

Individual skin reactions to the pollen fractions in the gel slices (Fig. 1) and average reactions (Fig. 2) are represented by histograms for each pollen. Each protein component is located along the horizontal axis according to its apparent mobility on starch gel electrophoresis at pH 8.5. The electrophoretic pattern for rye grass pollen is included as a reference.

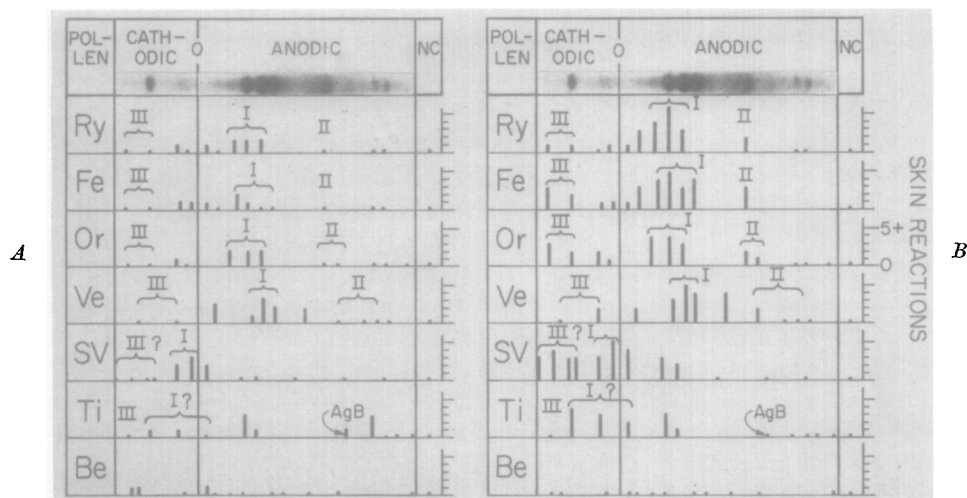
Within the limitations of this skin test assay, the following general observations can be made: (1) Individuals tested gave characteristic and reproducible patterns of reactivity to the fractions of the different pollens. (2) Most individuals reacted to all the pollens tested, including those toward which they had not been significantly exposed. (3) Even though Bermuda is prevalent in the Los Angeles area, 3 grass pollen-allergic individuals (aged 13, 14, and 23* all resident in Los Angeles all of their lives) of the 14 tested with Bermuda pollen were completely unresponsive to whole Bermuda extract and all its fractions; another† of the 14 individuals (aged 23, widely travelled throughout the United States) reacted strongly to most Bermuda fractions and only weakly to very few fractions of other grass pollens. (4) Certain fractions of intermediate positive electrophoretic mobility (later classified as Group I) on the gels of six of the pollens, excluding Bermuda, gave strongly positive reactions in nearly all individuals tested; the fastest migrating fractions of all pollens generally failed to elicit positive skin reactions.

Antigenic classification of allergenic fractions by immunodiffusion analysis.

It was of great interest to correlate, for each sensitive individual, the skin reactions induced by the various fractions (in the same and different pollens) with the antigenic properties of the respective fractions. The allergenic components of Groups I, II, and III and of timothy pollen's AgB were identified (where present) in each pollen by immunodiffusion analysis. Subsequently, the antigenic relationship of fractions within a particular group, but from different pollens, was investigated qualitatively with the use of antisera for the group under test (e.g., Fig. 3). The locations of all Group I, II, and III allergens so far identified are shown diagrammatically on the histograms of skin reactions (Figs. 1 and 2) and on the starch electrophoretic patterns of Fig. 4, *B*. Fractions indicated by arrows on Fig. 4, *B* were used for the particular immunodiffusion experiments illustrated in Fig. 3; comparable precipitin patterns were obtained with other Group I, II, and III antigens of the respective grass pollens.

*Subject H. G. (Fig. 1 and Table III).

†Subject R. Se. (Table III).

**Fig. 1**

Skin reactions of tests with starch gel fractions on 2 grass pollen-sensitive individuals. *A*, Subject E. *B*, Subject H. *G*.: Groups I, II, and III, positive. The fractions are located along the horizontal axis according to their apparent electrophoretic mobility on starch gel at pH 8.5. Protein-stained patterns for dialyzed rye pollen extract are included as references at the top of each diagram. Reactions, shown on the vertical scale, are graded as one to five plus on the following rating: 0: reaction given by negative control with no wheal detectable; 1+: average wheal diameter 3 to 5 mm., with no pseudopods; 2+: average wheal diameter 6 to 8 mm., with no pseudopods; 3+: average wheal diameter 9 to 11 mm., with at least one pseudopod; 4+: average wheal diameter 12 to 15 mm., with more than one pseudopod; 5+: average wheal diameter 16 mm. and over, with multiple pseudopods. *O* = origin, *NC* = negative control (virgin starch gel). A dot on the base line indicates a negative reaction to the fraction tested.

Components of Groups I, II, and III were identified in rye, fescue, orchard, and velvet pollens. For these pollens, all fractions within a particular group showed apparent antigenic identity by immunodiffusion analysis (Fig. 3). In each pollen, the most prominent components of each group, which gave similar intensities of protein staining on the stained indicator strips of the starch gels, were selected for detailed comparison. Semiquantitative determinations by radial immunodiffusion analysis of the relative proportions of Group I antigens in the respective gel slices are summarized in Table II. In calculating these results, it is tacitly assumed that due to their close similarity all such Group I antigens have the same rates of diffusion through the gels, virtually 100 per cent cross-reactivity with rye Group I, and that any losses due to adsorption of antigens on the starch gel were the same. Based on these assumptions, concentrations of the principal Group I antigenic fractions of rye, fescue, and orchard pollens were found to be similar; the concentration of the equivalent velvet fraction was apparently somewhat lower.

Within each antigenic group, the most prominent components showed closely similar patterns of skin reactivity considering the specific sensitivity of each subject separately (Table III and Fig. 1) or taking the population of allergic subjects tested as a whole (Table IV and Fig. 2).

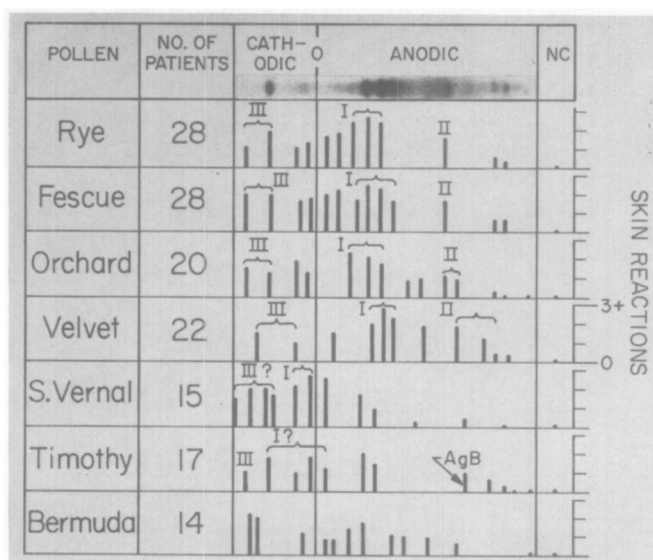


Fig. 2

Average skin reactions for tests with starch gel fractions on grass pollen-sensitive individuals. Grading of reactions and other explanations are given in the legend to Fig. 1.

Table II. Comparison of the apparent concentrations, relative to rye Group I, of the principal Group I antigen starch gel fractions, determined by radial immunodiffusion analysis against anti-rye Group I serum

Rye	Fescue	Orchard	Velvet	Sweet vernal
1.00	1.16	1.06	0.57	0.53

All apparent concentrations were averaged from six determinations for each starch gel fraction containing Group I and indicated by an arrow in Fig. 4, B. Values for the two such fractions of rye and of fescue were averaged. All such averages had a standard deviation of about ± 10 per cent. The relative concentrations quoted here were calculated for each Group I antigen based on a unit value for rye Group I. Timothy Group I concentration proved to be impossible to measure by this technique due to its relatively low degree of cross-reaction with anti-rye Group I (see text).

Sweet vernal pollen was found to possess two Group I fractions, present in different concentrations, which were antigenically similar to those of the aforementioned pollens (Figs. 3, A and 4); analysis of their skin reactivities suggested allergenic similarity with Group I. Group I components of timothy pollen, although well defined on stained indicator strips of the starch gels, showed incomplete antigenic identity with rye Group I (Fig. 3, A; see also Discussion section). For this reason, quantitation of the blurred precipitin circles given by timothy Group I following radial immunodiffusion against anti-rye Group I was not attempted (Table II).

Components related to rye Group II were not located in sweet vernal or timothy in the present study, although traces have been detected in their dialyzed whole extracts in subsequent studies.²¹ A Group III component found

Table III. Skin reactions to the most prominent Group I, Group II, and Group III allergens of four pollens: Rye, fescue, orchard, and velvet

Subject	Group I				Group II				Group III				Classification*
	Ry	Fe	Or	Ve	Ry	Fe	Or	Ve	Ry	Fe	Or	Ve	
G. D.	5	5	4	4	5	5	4	5	3	1	3	3	A
D. S.	3	2	3	3	4	4	2	4	4	2	2	3	A
H. D.	5	5	0	4	4	3	0	1	5	4	4	0	A
H. G.	5	4	4	5	2	3	2	2	1	3	3	0	A
M. H.	4	4	4	3	0	2	2	1	2	4	3	2	A
S. B.	3	1	0	3	2	1	0	0	1	1	2	1	A?
M. W.	2	3	5	3	1	0	1	0	1	0	0	0	B
R. A.	4	1	4	3	0	0	1	0	0	1	0	0	B
E. B.	2	1	2	3	0	0	0	0	0	0	0	0	B
T. H.	3	4	4	0	0	0	1	2	0	2	1	1	B
G. M.	1	0	2	3	1	1?	0	1	0	0	1	0	B
L. L.	0	0	2	1	1	2	1	1	4	3	1	4	C
M. P.	0	0	0	0	0	0	0	0	1	1	1	1	C
R. Sm.	1	2	1	3	3	0	0	0	4	4	0	3	C
R. Sc.	0	0	0	0	0	0	0	0	0	0	0	0	D

The fractions selected here are indicated by arrows in Fig. 4, B. Skin reactions are graded as described in the footnote to Fig. 1. Reactions to the prominent allergenic fractions I-B and I-C of rye and corresponding fractions of fescue were usually very similar; averages of the respective pairs of reactions were used in compiling this table.

*Class A: individuals significantly skin reactive to Groups I, II, and III; Class B: individuals predominantly skin reactive to Group I; Class C: individuals more reactive to Group II and/or III than Group I; Class D: individual unreactive to Groups I, II, and III.

in timothy showed antigenic and allergenic similarity with the aforementioned Group III fractions (Figs. 1, 2, and 3, C and Table IV). Certain components of sweet vernal were probably only partially related antigenically to other Group III fractions, as seen by the apparent spur formation observed in immunodiffusion analysis (Fig. 3, C). The distribution of skin reactivity toward these sweet vernal components was similar to that for Group III allergens in a proportion of the individuals tested, including some who had never received any significant exposure to sweet vernal pollen. No components of Groups I, II, or III were detected in Bermuda pollen, either in the present study or in subsequent studies with the whole pollen extract.²¹

Investigations of the antigenic purity of the various Group I, II, and III fractions tested showed no cross-contamination between one group and another, with the exception of trace contamination between two fractions of I and III in timothy which were close together on starch gel electrophoresis. No cross-contamination by further minor components was observed across the different groups. Certain minor antigens, present in very low concentration and confined to particular fractions, could be detected with the use of highly potent antisera prepared against crude pollen extracts. However, at the present time there is no evidence that such minor antigens play an important role in producing positive skin reactions under the test conditions used.

DISCUSSION

The dialyzed crude extracts of seven grass pollens representing six botanical tribes (Table I) were fractionated by a method which allowed subsequent rapid and extensive immunological assay of almost 100 different pollen components.

Table IV. *Skin reactions to grass pollen allergens*

<i>Pollen</i>	<i>Number of individuals tested</i>	<i>Group I</i>		<i>Group II</i>		<i>Group III</i>	
		<i>% + reactors</i>	<i>Average reaction</i>	<i>% + reactors</i>	<i>Average reaction</i>	<i>% + reactors</i>	<i>Average reaction</i>
Rye	28	85	3.0	63	2.5	63	3.0
Fescue	28	83	2.9	63	2.6	74	2.7
Orchard	20	79	3.0	58	2.0	68	2.2
Velvet	22	90	3.3	52	2.5	62	2.6
Sweet vernal	15	77	3.5	—	—	(64)*	(2.4)*
Timothy	18	(81)*	(1.8)*	—	—	69	1.4
Bermuda	14	← Groups I, II, and III not found in this pollen →					
Averages	—	83	3.1	59	2.4	67	2.4

One individual (R. Sc.), who did not react to Groups I, II, or III but reacted to most Bermuda fractions, is excluded from the percentages. The "average reactions" are based only on those individuals who reacted positively to the respective fractions. Over-all averages (including the negative reactors) may be found in Fig. 2. Values for rye Group I and fescue Group I are averages for the two major Group I fractions (I-B and I-C in each case). All other values are for the principal Group I, Group II, or Group III fractions indicated by arrows on Fig. 4, B.

*Omitted from averages due to the observed lower immunological cross-reactivity between these antigens and other antigens of the respective group (see Fig. 3 and Discussion).

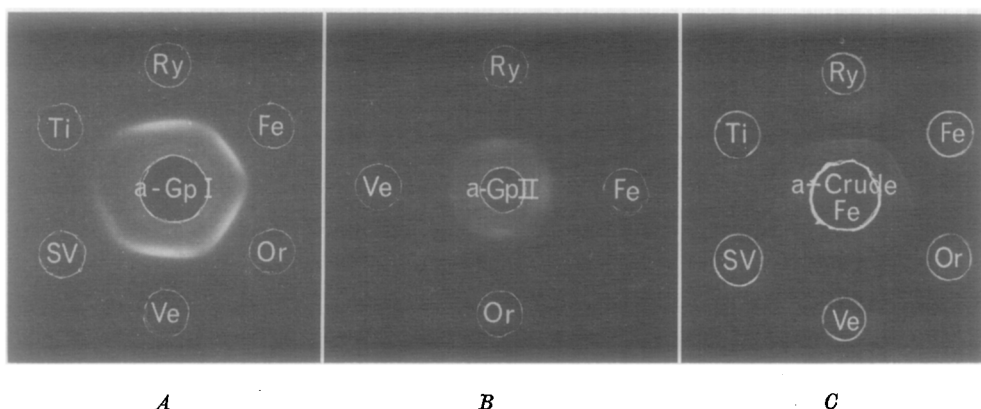
The allergenic properties of each component were investigated by skin tests in a large number of grass pollen-sensitive individuals. Different individuals were found to exhibit differing sensitivities to the different components. Each proteinaceous component was allergenic in a certain percentage of individuals; a few individuals were sensitive to almost every proteinaceous fraction.

The accuracy of the skin test results of the present studies is limited by the well-known errors inherent in the technique and by the possibility that a few individuals may be exquisitely sensitive to some minor components associated with certain allergens. The observed strongly positive skin reactions and the positive immunodiffusion analyses, both employing the starch gel slices directly (without prior elution of the pollen components), clearly show that the various pollen fractions are readily eluted from the starch gels.

Immunologically cross-reacting antigens from the same and different pollens generally exhibited similar allergenic properties when tested in the same individual (Fig. 1 and Table III); the over-all incidence of allergenic activity detectable in our skin tests was also similar for related antigens (Fig. 2 and Table IV). Thus, components which are cross-antigenic in rabbits are generally cross-allergenic in man.

The closest similarity of results was observed for the principal antigens within classified Groups I, II, and III of rye, fescue, orchard, and velvet pollens, where such antigens were well defined on the gels and highly cross-antigenic with one another by precipitin analysis (Fig. 3).^{*} Within a particular group,

^{*}One notable exception to this general observation was Subject H. D. (Table III), who showed significant skin reactions to Group I allergens of rye, fescue, and velvet, but not to the antigenically closely related Group I component of orchard. Unfortunately, Subject H. D. was not available for repeating the skin tests. However, in the light of results in other allergic individuals, we consider that the negative skin reaction to orchard Group I was probably due to an inadvertent error of technique rather than to a lack of cross-allergenicity between orchard and other Group I components.

**Fig. 3**

Identification of antigenic groups in starch gel fractions by immunodiffusion analysis against rabbit antisera. Starch gel slices containing the antigens were placed directly into the antigen wells. The fractions used for this experiment are indicated by arrows in Fig. 4. *B*, *A*, *B*, and *C* show immunodiffusion patterns for the principal fractions of Groups I, II, and III, respectively, in pollens in which these antigens were found in the present study. The antigenic identity of the major precipitin line in *C* with a purified rye Group III preparation was confirmed in an independent experiment.

most of these antigens appeared to be present in similar concentrations in starch gels used for skin testing, according to radial immunodiffusion analysis (Group I) and from the similar intensities of protein staining for Group I, II, and III antigens in the stained indicator strips of the gels. Since Group I components have previously been found to be the major allergens of rye grass pollen,^{10, 14} we conclude that the closely related Group I antigens of fescue, orchard, and velvet pollens are probably also the major allergens in these pollens. Further important allergens in all these four grass pollen species comprise Groups II and III.

Due to the previous immunochemical studies with rye Groups I and II¹⁰⁻¹³ and to the availability of good monospecific antisera against these antigens, we feel confident in the unequivocal identification of the respective groups in other grass pollens. However, we can not yet be quite as certain of the identification of Group III antigens, due to the lack of immunochemical studies of these substances and to the unavailability of monospecific antiGroup III sera.

Most of the antigens of sweet vernal and timothy pollens exhibited less antigenic and allergenic cross-reactivity with Groups I, II, and III of rye than did the respective antigens of fescue, orchard, and velvet. For example, by immunodiffusion analysis against two anti-rye Group I sera (and also an anti-fescue Group I serum), timothy Group I was found to be somewhat less closely related antigenically to rye Group I than were other Group I antigens (note the spur formation between rye and timothy Group I in Fig. 3, *A*). With anti-crude timothy sera, the spur formation between rye and timothy Group I occurred in the converse fashion to that shown in Fig. 3, *A*, confirming that there are some significant differences in antigenic structure between rye and timothy Group I

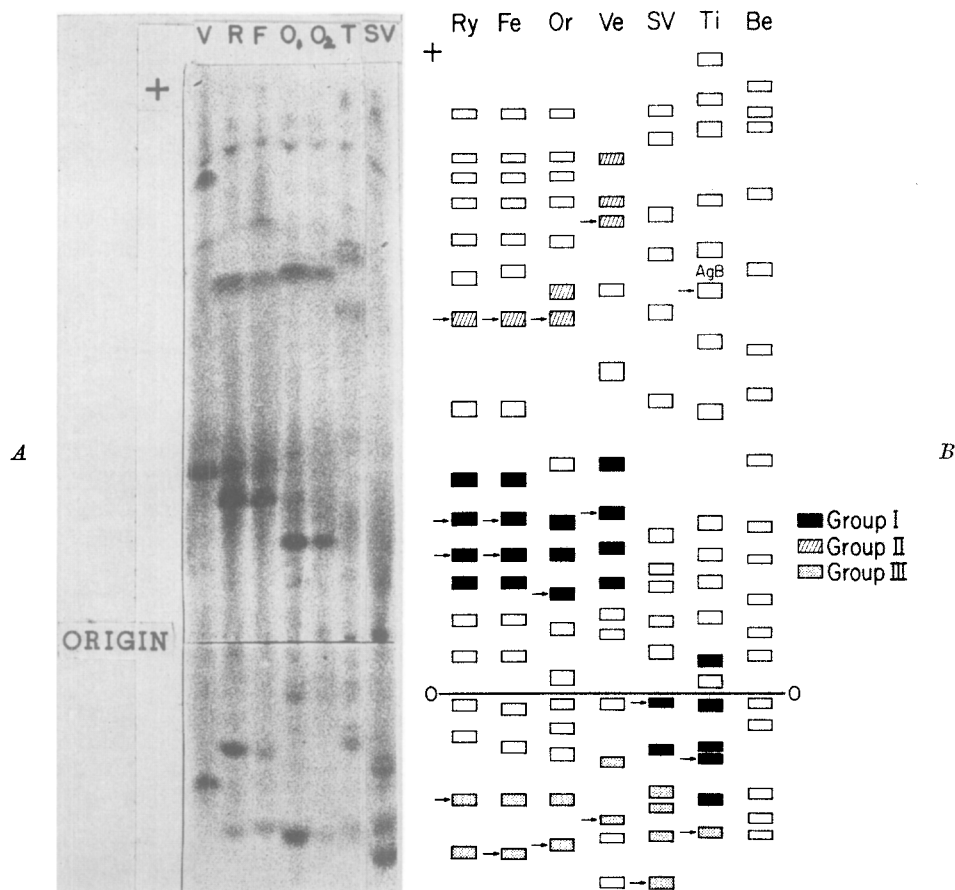


Fig. 4

A, Comparative starch gel electrophoretic patterns for solutions (20 per cent w/v) of dialyzed whole pollen extracts run at pH 8.5; protein components have been stained with amido black. This experiment was performed according to Smithies²⁰ procedure, rather than by the modified electrophoretic method described in detail in the text. The two orchard samples (*O*₁ and *O*₂) are from different batches of orchard pollen and show some variations in the intensities of stained bands, which are typically observed from one batch to another of a particular pollen species. Specimen *O*₁ and the specimens of the other pollens illustrated here were used subsequently for experiments described in the text. *B*, Diagrammatic representation of the distribution of Group I, II, and III allergens in the starch gel electrophoretic separation of pollen components at pH 8.5. The data presented here summarize the results of many starch gel electrophoreses and immunodiffusion analyses, performed as described in the text. Only components which have definitely been established to be Groups I, II, and III by immunodiffusion analysis are identified as such.

antigens. Detailed analysis of our skin test data also suggests weaker allergenic cross-reactivity between rye Group I and timothy Group I than between rye Group I and other Group I antigens. In an independent study (to be published), it has been shown that the chemical structural differences between rye and timothy Group I components are significantly more pronounced than those between rye and fescue Groups I, thus accounting for the observed immunological relationships.

Antigen B was only detected in one fraction of timothy pollen (Fig. 4, *B*). None of the classified Groups was found in Bermuda pollen, although cross-reaction between certain Bermuda allergens and unclassified "minor" allergens of other pollens studied can not be precluded. Our results with Bermuda agree closely with previous observations showing almost complete absence of antigenic and allergenic cross-reactivity with many other grass pollens.^{1, 2, 9}

Variants of related allergenic components, representing each of the three antigenic groups, were found within each pollen studied. Such variants may be classified as isoallergens, by an extension of a term formerly used to describe the allergenic variants of rye Group I, (see footnote, p. 108)¹¹ in which antigenic, allergenic, and chemical relationship has been firmly established.¹⁰⁻¹⁴

Analysis of skin reactivity data for the antigenic fractions of Groups I, II, and III in rye, fescue, orchard, and velvet pollens and of supplementary data from sweet vernal, timothy, and Bermuda suggests a broad classification for grass pollen-allergic individuals, based on the antigenic groups causing appreciable skin reactions. This classification is given in the footnote to Table III, summarizing data from the 15 individuals tested with rye, fescue, orchard, and velvet pollen fractions. Of the total of 29 individuals tested with at least two (and generally four or more) pollens, 12 (41 per cent) were allergic to Groups I, II, and III (Class A), 11 (38 per cent) predominantly to Group I (Class B), 4 (14 per cent) predominantly to Group II and/or III (Class C), and 1 (3 per cent) was unreactive to any of these groups (Class D); a further individual was highly skin sensitive to Groups I and II but not to Group III.

Three immunologically distinct allergenic groups (and probably more groups still to be defined) are to be found in most pollens studied. The degree of cross-reactivity of the isoallergens within a particular group may vary between different pollens, although it is sufficient to make individuals skin reactive to allergens of pollens toward which they had received no significant exposure. The important allergens appear to be essentially protein in nature, since activity is confined to protein-staining regions of the gels. This result is confirmed by studies on rye Group I¹⁴ and on formaldehyde-treated "allergoid" derivatives of Group I and crude mixed-grass extracts.^{18, 19} However, one must still consider the possibility that some (probably relatively unimportant) allergenic determinants of grass pollen components may not be protein in nature.

The present results with rye Groups I and II, showing incidences of skin sensitivity of 85 and 63 per cent, respectively, in 28 individuals allergic to grass pollens, are in good agreement with values of 90 and 67 per cent obtained by previous prick testing with solutions of highly purified allergens in 50 grass pollen-sensitive individuals.¹⁴ The fact that not all grass-sensitive subjects are sensitive to Group II was confirmed by histamine release assays with the use of isolated allergic human leukocyte suspensions.¹⁶

Thus we conclude that the over-all interpretation of our results is correct. Further precision, including the determination of the specific allergenicities of individual components and of quantitative cross-antigenicity and cross-allergenicity between related materials, will be possible only by the isolation of relatively large quantities of homogeneous components by conventional, laborious fractionation procedures.¹⁰

The present studies have considerable relevance in the diagnosis and immunotherapy of grass pollen hay fever. Similar methodology could be usefully employed to study allergenic materials other than from grass pollens.

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