

A factor in pollen which enhances vascular permeability

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A method is described for the extraction and purification of a permeability-enhancing factor (PEF) from ragweed pollen. PEF is a neutral polysaccharidic compound composed of arabinose and galactose associated with small amounts of pigment and protein. As little as 35 mcg. of PEF, when injected intravenously into mice along with Evans blue dye, will induce blueing in the ears of these animals. Blueing of the ears is likely due to the release of histamine, since the administration of PEF results in the disruption of the mast cells in the ears of mice. In addition, mice injected repeatedly with compound 48/80, a material which depletes tissues of their histamine, are no longer able to respond to PEF with ear blueing. It is suggested that PEF acts by combining with natural antibody present in mouse serum or on mouse mast cells.

Much of the recent work on hypersensitivity to pollens has dealt with the isolation and characterization of the pollen allergens. The presence of biologically active materials in pollen other than allergens which might influence the allergic process has not been emphasized. Several years ago, however, Kind and co-workers¹ found that rye grass pollen extracts contained a material which enhanced vascular permeability when injected intravenously into mice. Increased vascular permeability was demonstrated by a marked blueing of the ears of mice injected with a mixture of Evans blue dye and pollen extract. Following an intravenous injection of a moderate dose of pollen extract (35 to 100 mcg.) and dye, leakage of dye into the tissues was evident only in the ears of mice. After the administration of a larger dose of pollen extract (200 to 500 mcg.) extravasation of dye was also visible on

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the inner surface of the skin of the trunk. No blueing and edema formation of the paws and external genitalia, as reported after the administration of dextran in the rat,² was observed. Blueing of the ears did not occur in mice injected with dye plus one of the following: bradykinin, *Escherichia coli* endotoxin, *Crotalus atrox* venom, or trypsin. Ear blueing did occur in mice sensitized with bovine serum albumin and subsequently challenged with this antigen, as well as in mice injected with dye plus either of the following: serotonin, histamine, or the histamine liberator, compound 48/80. Ear blueing due to pollen extracts was inhibited by the antihistamine, Neoantergan, as well as by the antiserotonin drug, UML 491. The experiments to be reported are a continuation of the work just discussed and are concerned primarily with the isolation and characterization of the permeability-enhancing factor (PEF) from low ragweed. The designation PEF is used primarily for ease of discussion and no implication of any relationship between PEF and known permeability factors is intended. Indeed PEF from low ragweed may well be an antigen which exerts its effect via combination with natural antibody in the mouse.

MATERIALS AND METHODS

Extraction of pollen grains

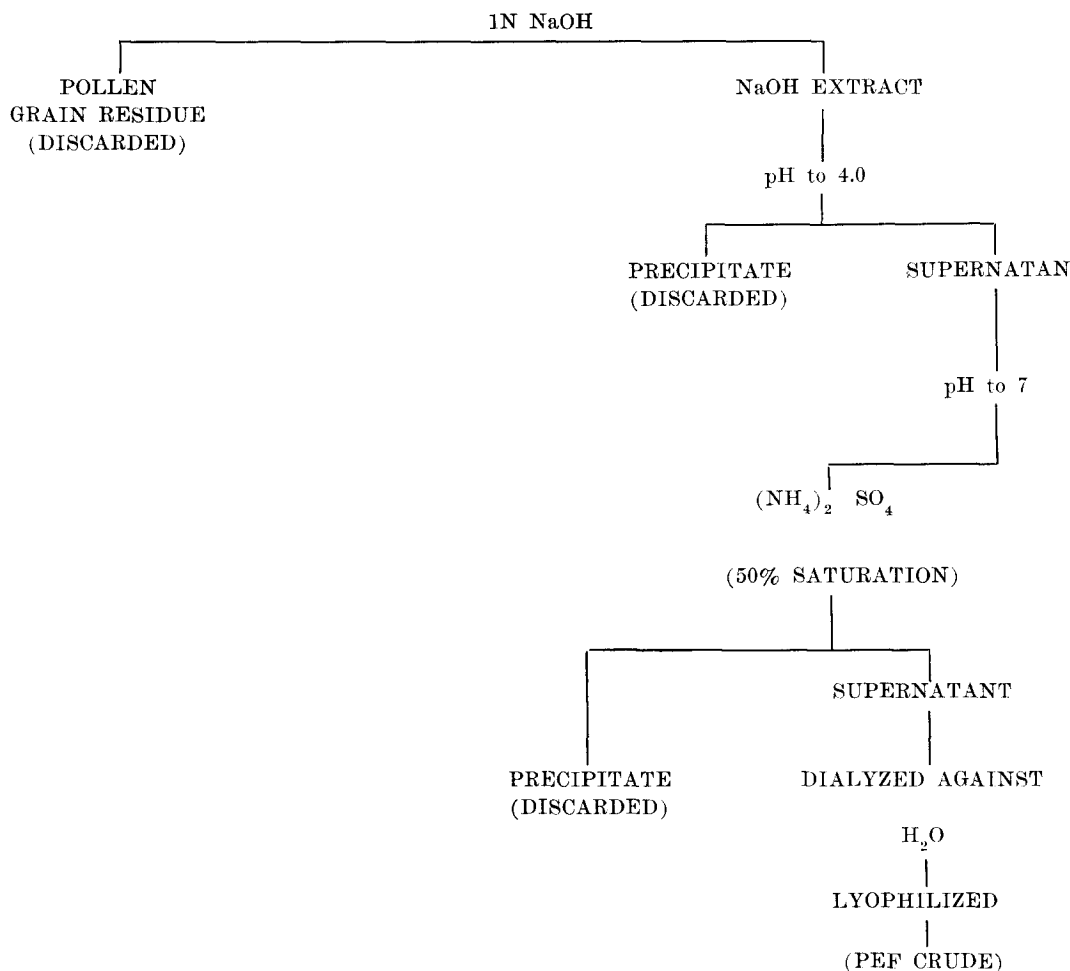
Three hundred grams of low ragweed pollen grains, which had previously been defatted and extracted with Coca's solution, served as the starting material. The extraction procedure is outlined in Table I. The grains were first extracted with 1N sodium hydroxide overnight at 25° C. The pH of this extract was then adjusted with HCl to 4.0 and the copious precipitate which formed was discarded. The supernatant was adjusted to pH 7.0 and additional proteins were precipitated with ammonium sulfate (50 per cent saturation). The supernatant was dialyzed against distilled water for a few days and then lyophilized. One gram of lyophilized material (crude PEF) was obtained from 300 grams of previously extracted ragweed pollen grains. Crude PEF was then further fractionated by zone electrophoresis. Occasionally, if the activity of crude PEF was low, its potency could be enhanced by precipitating it from aqueous solution with 70 per cent alcohol. Fractionation by zone electrophoresis then followed.

Zone electrophoresis

Two hundred milligrams of crude PEF was fractionated by block electrophoresis on Pevikon-Geon according to the method of Fahey and McLaughlin.³ The Pevikon-Geon mixture was suspended in 0.1 M NaOH-glycine buffer and was then poured into a 44 by 9 cm. plexiglass tray. Crude PEF was applied to a trough 14 cm. from the cathodal end and electrophoresis was carried out for 24 hours at 4° C. using a potential gradient of 3.4 v. per centimeter. One centimeter sections were cut and the separated material eluted with 6 ml. of H₂O. Protein distribution in the block fractions was determined by the Folin method⁴ and the polysaccharide distribution, by the orcinol procedure.⁴ Pooled fractions were dialyzed against H₂O, lyophilized, and tested in mice for permeability-enhancing effects. Fifty milligrams of

Table 1. *Extraction of permeability factor from low ragweed pollen grains*

300 GRAMS OF RAGWEED POLLEN GRAINS PREVIOUSLY
EXTRACTED WITH COCA'S SOLUTION



highly active PEF was obtained from 200 mg. of crude PEF. The color produced by the Folin reagent might have been due in part to the presence of phenolic pigments in the various fractions. The nitrogen content of the active material was, therefore, determined by the Nessler procedure.

Analysis of active polysaccharide

The active polysaccharide from low ragweed isolated by block electrophoresis was hydrolyzed in 2N HCl at 100° C. for 16 hours, and the hydrolysate was dried over KOH in vacuo. Descending paper chromatography of the hydrolysate was carried out for 17 hours on Whatman No. 1 paper with use of the solvent systems ethyl acetate: acetic acid: water (volumes 9:2:2) and

ethyl acetate: pyridine: water (volumes 8:2:1).⁵ The spray reagent was o-aminodiphenyl.⁶ Active polysaccharide obtained from giant ragweed and rye grass by the methods described above were analyzed in a similar manner.

Mouse test for activity of PEF

Swiss-Webster 20 gram female mice were injected intravenously with a mixture of 0.1 ml. of a pollen preparation plus 0.2 ml. of 0.5 per cent Evans blue dye in saline. Within 15 minutes, with small doses of PEF (35 mcg.), a few blue spots appeared, usually near the base of the ears; with larger doses of PEF (75 to 200 mcg.), 25 to 100 per cent of each ear would become blue. Control mice injected with dye plus saline showed no blueing of the ears. The median effective dose (ED_{50}) of various preparations was calculated by the method of Litchfield and Wilcoxon.⁷

Mast cell counts

Test mice were injected intravenously with 500 mcg. PEF and control animals were given an equal volume (0.1 ml.) of saline. Five to ten minutes later, the animals were killed by dislocation of the cervical vertebrae, and an ear from each mouse was cut off near the base and fixed in a solution containing 10 per cent formaldehyde, 5 per cent acetic acid, and 50 per cent ethanol. Paraffin sections (6 micra) were stained with 0.1 per cent toluidine blue and the mast cells were observed at a magnification $\times 450$. Data are presented as per cent of mast cells disrupted for 200 mast cells counted.

Desensitization of mice with compound 48/80

Mice were injected intravenously with 20 mcg. of compound 48/80 on each of 4 successive days. Five hours after the last injection the test animals, as well as control mice previously injected with saline, were challenged with 40 mcg. of compound 48/80 plus Evans blue dye or 500 mcg. of arabinogalactan (larch tree) plus dye. Blueing of the ears was noted for 30 minutes.

RESULTS

Zone electrophoresis of crude PEF on Pevikon-Geon yielded two orcinol-positive fractions (elution pattern in Fig. 1), a neutral polysaccharidic compound (Fraction I), and an acidic polysaccharidic compound (Fraction II). Only Fraction I enhanced vascular permeability in mice (ED_{50} , 35 mcg. per mouse). As much as 500 mcg. of Fraction II was completely inactive. Fraction I contained one per cent N which was not due to the presence of hexosamine as determined by the Elson-Morgan procedure.⁴ A small amount of pigment was also present. Hydrolysis of Fraction I and paper chromatography of the hydrolysate (see Materials and Methods) indicated that the active polysaccharide fraction was composed of arabinose and galactose. This finding was of particular interest since many polysaccharides (other than pollen carbohydrates) were tested for permeability-enhancing effects, and the most active (150 mcg. per mouse) was an arabinogalactan* obtained from timber of the western larch tree (Table II).

*Stractan, Stein-Hall, Inc., New York, N. Y.

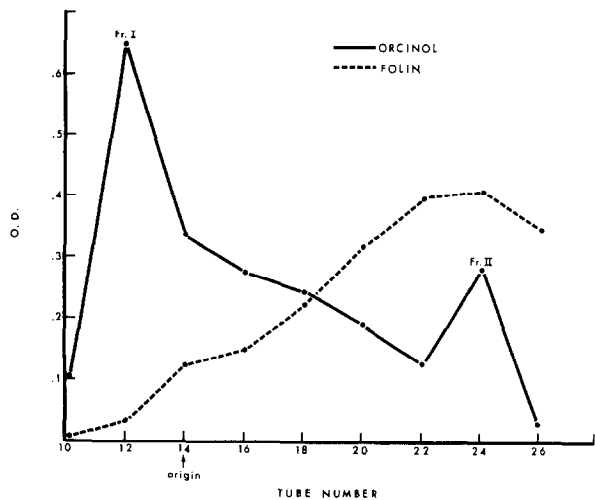


Fig. 1
Pevikon-Geon block electrophoresis of crude PEF. The arrow indicates the segment in which the sample was applied. The unbroken line indicates *orcinol*-positive material (polysaccharide). The broken line represents *Folin*-positive material.

Table II. Ability of various polysaccharides to induce ear blueing in mice

Polysaccharide	ED ₅₀ (mcg.)
Ragweed pollen	35
Arabinogalactan (larch tree)	150
Phosphomannan (yeast)	1,500
Dextran (200,000)	3,000
Carageenan	>500*
Glycogen	>2,500*
Amylopectin	>2,500*

*Maximum dose injected. No ear blueing at this dose.

We have thus far been unable to separate our active pollen polysaccharide from the small amount of pigment and protein with which it is associated. It is unlikely that the pigment has any permeability-enhancing effects since Fraction II contains much pigment and is completely inactive. However, the pigment in Fraction II need not necessarily be identical with that in Fraction I. The protein in Fraction I is likely not responsible for the activity of this fraction, since the data in Table III indicates that only the enzyme, cellulase, and those enzymes present in the gut of the snail, *Helix pomatia*, were able to inactivate PEF. Cellulases have been shown to attack β 1-4 linkages,⁸ and snail gut enzymes have been used to break down cell walls of microorganisms.⁹ Various proteolytic enzymes had no effect on PEF. In addition, the arabinogalactan from the western larch tree, which is quite active, contains only traces of protein (0.06 per cent).

Polysaccharide fractions isolated from giant ragweed and rye grass pollen have also been separated by zone electrophoresis. In both of these pollens, as in low ragweed, permeability-enhancing activity was present in an immo-

Table III. *Effect of various enzymes on permeability-enhancing effect of ragweed pollen*

Pepsin	-
Trypsin	-
Pronase	-
α -amylase	-
Cellulase	+
Enzymes from <i>Helix pomatia</i>	+
DNA-ase	-
RNA-ase	-
Lysozyme	-

bile fraction which contained a polysaccharide composed of arabinose and galactose plus a small amount of pigment and protein.

The mechanism of action of PEF is suggested by the data in Table V. The data indicate that the administration of PEF results in the disruption of the mast cells in the ears of mice. The causal relationship between mast cell disruption and histamine release is well known. The data in Table VI furnish additional evidence that histamine is responsible for the ear blueing which we have observed. It is evident from this table that mice injected repeatedly with compound 48/80, a material which depletes tissues of their histamine, were no longer able to respond to PEF with ear blueing.

DISCUSSION

Evidence has been presented that the permeability factor in low and giant ragweed and rye grass pollen extracts is a neutral polysaccharide(s) composed of the sugars, arabinose and galactose, associated with small amounts of pigment and protein. Lea and Sehon,⁵ using paper electrophoresis, had previously isolated a neutral and an acidic polysaccharide from ragweed pollen; the neutral polysaccharide was composed of arabinose and galactose and was invariably associated with pigment and protein. Augustin¹⁰ had also isolated an arabinogalactan from rye grass pollen. Since polysaccharides similar in composition to PEF have been found in the cell walls of many plants,¹¹ it is probable that PEF from pollens is a cell wall constituent. We have tested eight different pollen extracts for their ability to enhance vascular permeability and all possessed this capacity. A certain amount of PEF is extractable from the pollen grain by aqueous solutions and, as can be seen in Table IV, PEF is present in detectable amounts in some commercial aqueous pollen extracts. We recently found that PEF from ragweed pollen was excluded from Sephadex G-75; such data suggest a molecular weight greater than 50,000. The arabinogalactan from western larch (ED₅₀, 150 mcg. per mouse) is composed primarily of a fraction with an average molecular weight of 100,000.¹²

There seems little doubt that ear blueing produced by the administration of PEF to mice is due to the action of histamine. Evidence for this assumption is the following: (1) PEF disrupts mast cells in the ears of mice (Table V). (2) Antihistamines inhibit ear blueing.¹ (3) The histamine content of

Table IV. *Presence of PEF in aqueous commercial extracts*

<i>Pollen</i>	<i>PNU</i>	<i>Activity</i>
Timothy	12,000	+
Bermuda	12,000	+
Orchard	12,000	+
Low ragweed	8,000	-
Plantain	10,000	-
Sage	17,800	-
Hickory	10,000	-
Oak	12,000	-

Table V. *Effect of PEF on mast cells in mouse ears*

<i>Test animals* (No.)</i>	<i>% disrupted mast cells</i>	<i>Control animals† (No.)</i>	<i>% disrupted mast cells</i>
1	54	6	4
2	48	7	7
3	14	8	4
4	54	9	2
5	15	10	2

*PEF, 500 mcg., intravenously.

†Saline, 0.1 ml., intravenously.

Table VI. *Inhibition of ear blueing by compound 48/80*

<i>Substance used to desensitize mice</i>	<i>Challenge†</i>	<i>No. mice with ear blueing/ Total No. mice challenged</i>
48/80*	500 mcg. arabinogalactan plus Evans blue dye	1/10
Saline	500 mcg. arabinogalactan plus Evans blue dye	10/10
48/80	40 mcg. compound 48/80 plus Evans blue dye	0/10
Saline	40 mcg. compound 48/80 plus Evans blue dye	10/10

*Dose, 20 mcg. a day, intravenously, for 4 days.

†Challenged 5 hours after last injection of compound 48/80.

the ears of mice drops after the administration of PEF.¹ (4) Repeated injections of the histamine liberator, compound 48/80, desensitize mice to the ear-blueing effects of an arabinogalactan from the larch tree (Table VI).

It had been our opinion that PEF induced the disruption of mast cells and subsequent release of histamine by either (1) activating anaphylotoxin, or (2) by combining with natural antibody present in mouse serum or on the surface of the mast cell. Current experiments indicate the latter theory is likely the correct one, and data to support this hypothesis will be presented in a subsequent publication. Our current working hypothesis is that many and perhaps all pollen grains contain in their cell walls an arabinogalactan which will release histamine in mice upon combination with natural antibody

to this antigen. The presence of such a natural antibody in humans and its possible significance in the allergic state is presently under investigation.

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