Allergen Purification by Discontinuity Gel Electrophoresis

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■ *Discontinuity gel electrophoresis can be used to prepare several allergens from purified grass pollen and house dust allergens. It is suggested as a general technique for the purification of allergens.*

IN THE PAST, methods of allergen purification have been adapted in many laboratories from the standard preparative methods of protein chemistry. However the purest protein or carbohydrate preparations resulting from application of these techniques have been demonstrated to be nonhomogenous.[7] For further purification of these allergens the disc electrophoresis technique of Davis and Ornstein[2] can be adapted to large scale preparations.

Materials and Methods

Preparation of Allergens

House dust and timothy pollen allergens were prepared as previously described.[4,7]

Orchard grass pollen was prepared by extracting the dried and defatted pollen (600 gm) with 3 liters of glucose-phosphate buffer[7] and the residue re-extracted with 500 ml of the same buffer. The extracts were combined, dialyzed against distilled water, made 0.15M with sodium chloride and adjusted to pH 10 with 0.15M sodium hydroxide. The preparation was clarified by centrifugation at 2500 rpm for 30 minutes. Ammonium sulfate (1,125 gm) was added to 3 liters of this solution and the resulting precipitate dissolved in and dialyzed against de-ionized water. As a result of dialysis a new precipitate formed. This precipitate was dissolved in 0.15M NaCl and adjusted to pH 10 with 0.15M NaOH. The final volume of solution was 102 ml and contained 2.5 gm of protein. The solution was brought up to 200 ml with 0.15M NaCl and 200 ml of ethanol at −15° C added. Following centrifugation at −10° C the supernatant was dialyzed against distilled water, lyophilized and dissolved in 0.15M NaCl to make a solution containing 0.9 per cent protein. The ethanol precipitate had considerable allergenic activity but less than the supernatant. Gel filtration was performed on 10 ml of the ethanol supernatant, using a column of Sephadex* G-75M (1.6 × 130 cm) and eluting with isotonic saline solution. Two peaks and a shoulder following the first peak were detected by spectrophotometry of the eluates at 280 millimicrons (Figure 1). The eluates in the tubes corresponding to each of the three fractions were pooled, dialyzed against de-ionized water, and lyophilized.

Preparation of Gels

Stock solutions were made up as follows:

	*A Electrophoresis Gel Solution**	*B Spacer Gel Solution†*
Acrylamide	286.00 gm	150.00 gm
N,N′-methylenebisacrylamide	1.87 gm	4.00 gm
12 N HCl	5.72 ml	10.00 ml
Tris-hydroxymethylaminomethane	51.90 gm	91.60 gm
N,N,N′,N′-tetraethylmethylenediamine	0.66 ml	1.15 ml
Potassium ferricyanide	40.00 mg	75.00 mg
Distilled water to make	1.00 liter	1.00 liter

*Cross-linked polydextran gel manufactured by Pharmacia Fine Chemicals, Inc., Upsala, Sweden.

†Polymerization initiator: 0.56 gm ammonium persulfate in 100 ml of water (Solution C).

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These studies were supported by a grant from the Hartford Foundation, New York, and United States Public Health Service Grant No. E-4412.

Presented before the Section on Allergy at the 93rd Annual Session of the California Medical Association, Los Angeles, March 22-25, 1964.

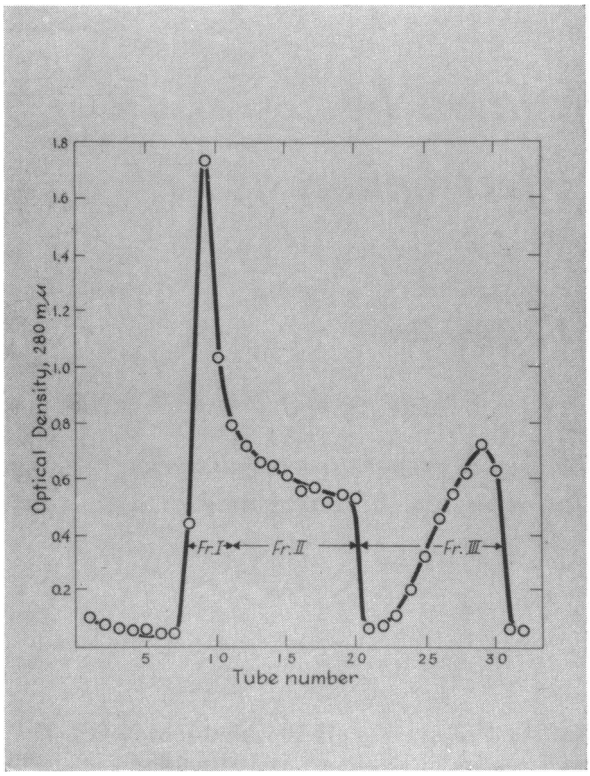

Figure 1.—Chromatography of ethanol soluble orchard grass allergen on Sephadex G-75M.

Electrophoresis (Figure 2)

A rubber sleeve about 2 inches long is fitted on the bottom of a glass tube 2.2 cm in diameter and 40 cm long so that 1½ inch of rubber tubing protrudes. A rubber stopper is inserted into the sleeve to within ½ inch of the end of the tube. This will result finally in a knob of gel hanging below the tube. Hard gel, containing 40 ml of solution A, 30 ml water and 10 ml of solution C is poured into the tube and allowed to harden. After the hard gel has set, the soft gel containing 6.7 ml of solution B, 10.8 ml of water and 2.5 ml of solution C is poured on top of the hard gel and allowed to harden.

The rubber sleeve and stopper are removed from the bottom of the tube. A rubber sleeve ½ inch long is then fitted over the top of the tube. This sleeve must make a tight seal with the hole in the bottom of the upper buffer vessel (fabricated from a plastic refrigerator jar (Figure 2)). The tube is immersed in a lower buffer vessel (a chromatography cylinder 14 cm × 45 cm) filled with undiluted Davis and Ornstein's buffer.[2] This immersion aids in cooling the gel. The upper buffer vessel is then filled with the same buffer, and the allergen solution, made dense by the addition of about 10 per cent glucose, is layered on top of the spacer gel, below the buffer (Figure 2). Platinum electrodes are inserted as shown. Current is maintained at 20 milliamperes per tube but no more than 400 volts should be employed. As electrophoresis proceeds, the allergen components form into thin discs and separate. Good separation can be obtained for samples as large as 100 mg protein.

After maximum separation has been attained, the gel is removed from the tube by pulling from the lower end. Each fraction is sliced out with a razor blade. Each slice is macerated with the help of a garlic press and the allergen is extracted by soaking the macerate in 0.15M NaCl.

Results

Typical patterns obtained by electrophoresis of house dust allergen and orchard grass allergen are shown in Figures 3 and 4. In Figure 3 the results of electrophoretic patterns of four different house dust allergen preparations run simultaneously are shown: (1) allergen prepared by the method of Berrens and Young[1] (B-Y); (2) house dust insoluble in 54.5 per cent ethanol at −15° C but soluble at 0° C; (3) and (4) cold ethanol soluble allergens prepared from the second and eighteenth extracts of the same batch of dust. Since the ethanol-soluble

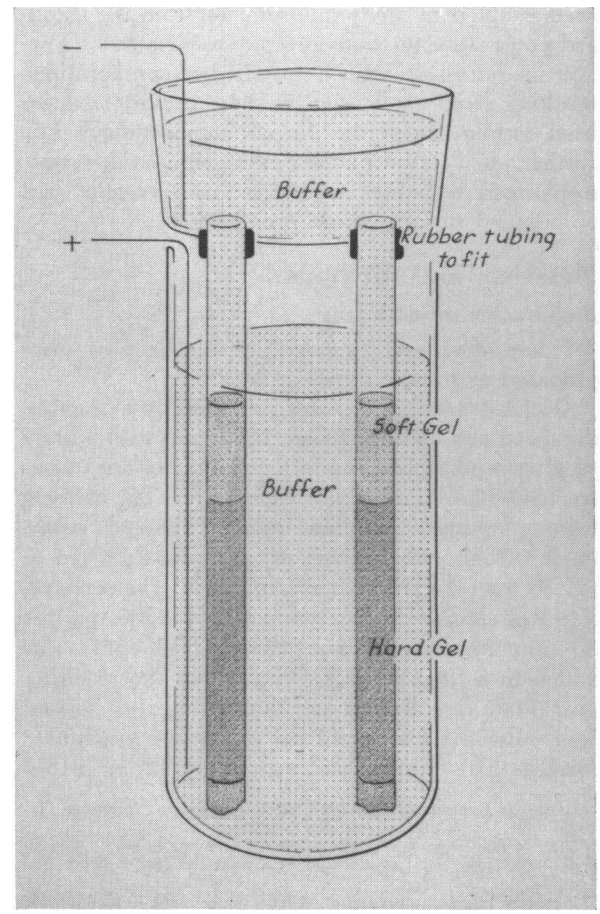

Figure 2.—Construction of electrophoresis cell.

allergens moved faster than the others, to obtain all patterns on the same scale for this figure the ethanol soluble allergens were run on a special hard gel containing 50 per cent additional monomer. When these allergens were run on the standard gel, the second disc split into five components. In Figure 4, the three fractions of orchard grass allergen obtained by Sephadex chromatography were each analyzed separately by electrophoresis. The dark band moving farthest toward the anode in each was colored blue by the bromphenol blue tracking dye present in the buffer. Anodic to it were several light yellow bands and above it one or two dark brown bands. In a similar fashion, ethanol-soluble timothy pollen allergen[7] produced six yellow bands on electrophoresis.

All the fractions of house dust allergen and all six fractions of timothy pollen allergen had allergenic activity. Quantitatively, different patients reacted differently to each fraction. However, no fraction was consistently most active or least active in all patients.[5] The orchard grass pollen allergens were tested by a passive transfer technique. Twenty-four hours before the test, serum from a patient with high sensitivity to grass pollens was injected intradermally (0.3 ml) into sites on the skin of a volunteer who does not give immediate skin reactions to grass allergen. Each colored disc from the orchard grass pollen allergen electrophoresis gels was extracted with 0.15M NaCl, dialyzed against distilled water, lyophilized and dissolved in 100 ml of 0.15M

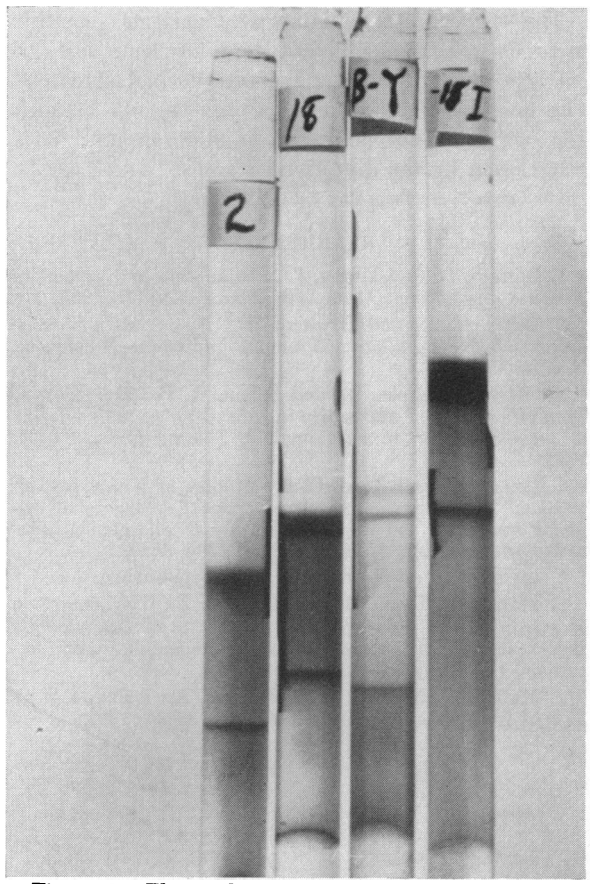

Figure 3.—Electropherograms of house dust allergens. Anode end on top. First and second tubes, using gels made from 50 per cent additional monomer, contain ethanol soluble allergen made from the second and eighteenth extracts of the same batch of crude dust. The third tube contains allergen prepared according to Berrens and Young,[1] the fourth house dust allergen soluble in ethanol but insoluble in cold ethanol.

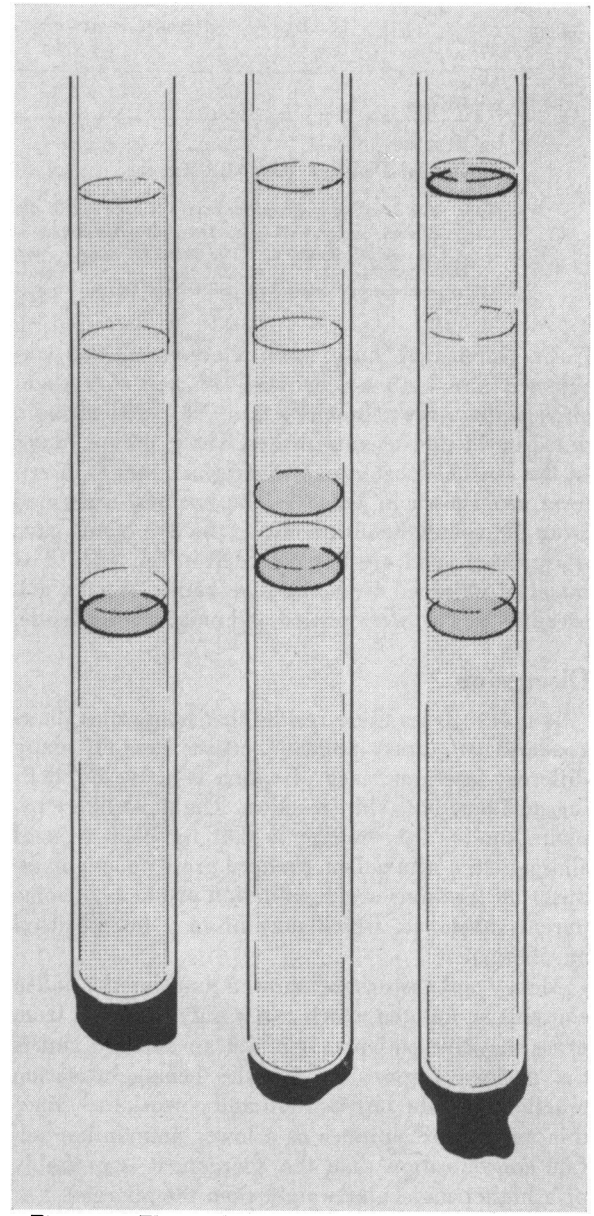

Figure 4.—Electropherograms of orchard grass allergens. Cathode end on top. The first, second and third tubes contain ethanol-soluble fractions III, II, and I respectively, from Sephadex G-75M. The first light line below the top at the same place in all three tubes represents the interface between the gels.

TABLE 1.—*Allergenic Activity of Orchard Grass Fractions Separated by Disc* Electrophoresis*

Fraction	*I-1*†	*I-2*	*II-1*	*II-2*	*III-1*	*I,II,III-3 (pooled)*
Reaction	+3	+	+2	+2	+2	+
Optical Density‡ (280 millimicrons)	0.081	0.012	0.094	0.104	0.012	0.084

*The discs are numbered from the cathode end of the cell downward.

†Roman numeral=Fraction from chromatography on Sephadex (see Figure 1). Arabic numeral=Band number.

‡An optical density of 0.100 corresponds to a concentration of ethanol-soluble orchard grass allergen of 70 micrograms per ml.

NaCl. One-fiftieth ml of each allergen solution was injected into both a sensitized site and a non-sensitized site in the volunteer's skin. No reactions were noted in the non-sensitized sites. The reactions found in the sensitized sites, as evaluated by standard criteria, are shown in Table 1. The material separated from the yellow bands anodic to the dye bands gave no reactions and are not recorded in the table. The material obtained from the blue bands of each gel, ascribed to dye, were pooled and only one test made.

Discussion

It is seen from these results that house dust allergen and two grass pollen allergens contain many different fractions, each of which is active in eliciting an immediate skin reaction. The most likely explanation for this finding is that for each type of allergen (timothy pollen, orchard grass pollen, house dust) all fractions are degradation products of some parent substance, which may or may not be itself an allergen.

Malley and coworkers[6] showed that timothy pollen contains an antigen which reacts only with sera from grass-sensitive patients. It is not an allergen and is the antigen responsible for the hemagglutination reaction described by Gordon and coworkers.[3] Since this antigen precipitates at a lower ammonium sulfate concentration than the allergen, it is probably of a higher molecular weight than the allergen. For this reason it has been suggested[4] that the hemagglutinating antigen is the parent form of the allergen and is degraded to it by enzymatic processes in the pollen. By this concept, a susceptible person who at first is not sensitive becomes immunized to the hemagglutinating antigen and then, upon subsequent exposure to the degradation product of the hemagglutinating antigen, responds by making an incomplete antibody, reagin.

The theory has been tested by injecting a house dust preparation which did not contain any detectable allergen but could be degraded to allergen[4] into patients sensitive to grass pollen but not sensitive to house dust.[4] As predicted, these patients acquired skin sensitivity to house dust.

The theory also explains why patients sensitive to foods will frequently not show any sensitivity to extracts of the foods by the intradermal skin test. The undigested food contains only the parent antigen, which is not degraded to allergen until it is acted upon by the digestive enzymes.

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