

ISOLATION OF ALLERGENS FROM TIMOTHY POLLEN

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MUCH of the modern efforts at isolation and characterization of allergens have been devoted to ragweed pollen. We elected to investigate timothy grass pollen for two reasons. First, grasses, rather than ragweed, are the major pollen allergens of the Pacific Northwest and, second, previous investigations by others had indicated that grass pollen might not have the allergenic complexity of ragweed. Blackley¹ was the first to suggest that chemical investigation of grass pollen would be fruitful, but it was not until the present century that this investigation was productive. The studies prior to 1952 were extensively reviewed by Augustin-Friedman² and will not be reviewed here. In summary, they indicated that the allergenic activity was associated with a protein fraction precipitated by saturated ammonium sulfate, and that proteolytic enzymes did not destroy the activity. The allergen was stable to freeze drying but was denatured by heating and was on the border line of dialyzability through semipermeable membranes.

Augustin²⁻⁹ has reported the results of her own investigations on the allergenic material of timothy pollen. With paper chromatography of whole pollen extracts developed with butanol/acetic acid/water mixtures, she demonstrated the existence of free amino acids, peptides, carbohydrates, proteins, and a number of pigments. The biologically active material remained at the base line. After hydrolysis this protein mixture contained at least 15 amino acids. By a technique of isoelectric precipitation followed by sodium chloride fractionation, she obtained a strongly pigmented carbohydrate-rich fraction which could be separated from a colorless carbohydrate-poor fraction. Both of these fractions, however, proved to be about equally active on a weight basis. From later studies, Augustin concluded that the allergens are primarily protein in nature and that carbohydrates are inactive. Membrane diffusion and ultrafiltration

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experiments indicated that the allergens of timothy grass pollen are nondialyzable and indicated an average molecular weight of about 14,000 and an elongated molecular shape. She demonstrated that the biologic activity was destroyed by pepsin and trypsin but not by autoclaving 15 minutes at 20 atmospheres. P. Johnson and co-workers¹⁰⁻¹⁴ have studied rye grass pollen by various physicochemical techniques. They report that the active material in crude extracts is not dialyzable but that considerable loss through dialysis membranes occurs during electrophoresis convection. Fractionation was attempted by several techniques and highest specific activity was obtained in the fraction precipitated by 100 per cent saturation with ammonium sulfate but was not precipitated by 50 per cent saturation. Some concentration was also achieved by adsorption to calcium phosphate gels. Electrophoresis of these various fractions indicated that they contained a mixture of proteins. From tests on a large number of patients they concluded that there is more than one allergen in rye grass pollen extracts.

Moore and Moore¹⁵ reported isolation of a flavonoid from timothy pollen, which they named dactylin. M. C. Johnson and associates¹⁶ found that a minority of patients gave a positive skin reaction to this flavonoid but Augustin reported that it was inactive.

During the purification of an allergen from timothy pollen, the removal of inactive material must lead to an increase in the specific biologic activity of the purified fraction compared to whole pollen extract. A failure to observe this increase in specific activity means that no purification has been achieved or that denaturation has occurred. It must also be kept in mind that patients may be sensitive to differing proteins in the crude pollen extract so that the relative activities of different fractions may be different in some patients than in the majority. Therefore a number of patients must be tested. These principles have guided our isolation of allergens from timothy pollen.

With this background, we began the isolation of a biologically active allergen from timothy pollen. As a working hypothesis we assumed that the biologically active material was a protein, and we used the standard methods of protein fractionation. An essential feature of our plan of isolation was a comparison of fractions obtained by each procedure with each other and with the whole pollen extract by testing them in the skin of timothy-sensitive volunteers. Only those procedures which yielded significant increase in activity will be described.

METHODS

In the early stages of allergen purification we did not wish to overlook the possibility that an allergen might be some substance other than protein, and we measured the concentrations of the fractions for skin testing by the dichromate reduction method of M. J. Johnson¹⁷ which measures the total organic solid content. However, as our work progressed, it became evident that the allergens were largely polypeptide in composition, and we began using the biuret reaction¹⁸ to estimate protein content of each fraction. The fractions obtained at each time were compared to the whole pollen extract (WP) and to each other by direct skin testing. All fractions were tested in the same patient

at the same time. Since the threshold varies between patients and since patients might vary in their sensitivity to the fraction in question, several patients were tested and the results averaged. These individuals were selected from a group of timothy-sensitive patients. Most had received injection treatment. A solution containing 0.1 mg. of protein per milliliter of buffered isotonic saline at pH 7.4 was sterilized by Seitz filtration and decimal dilutions were prepared. Each dilution was tested in the skin of sensitive patients by intradermal injection of 0.02 ml. The activity of each fraction was expressed as the most dilute solution evoking a threshold response.

ISOLATION PROCEDURE

Timothy (*Phleum pratense*) pollen was obtained commercially* and defatted by ether extraction in a Soxhlet apparatus for 24 hours, dried at low pressure, and stored under low pressure at -15° C. until needed for further use. One hundred grams of dried defatted timothy pollen were extracted with 300 ml. of 0.127 M phosphate buffer and pH 10.4. The solution contained 10 per cent glucose. The suspension was continually agitated by magnetic stirring for 24 hours at 2° to 4° C. and the residue removed by filtration through a Buchner funnel. The pH of the filtrate (WP) was adjusted to 10.0 by the slow addition of 0.1 N sodium hydroxide.

TABLE I. AMMONIUM SULFATE FRACTIONATION PROCEDURE FOR TIMOTHY POLLEN

	Defatted Pollen (100 Gm.)
	Extract with 300 ml. of 0.127 M phosphate buffer, pH 10.4, containing 10% glucose. Filter suspension through a Buchner funnel
Residue	Filtrate (250 ml.) WP Adjust pH to 10.0 and add 28 Gm. ammonium sulfate per 100 ml.
Residue 0-48% saturated ammonium sulfate fraction	Supernatant Add 5 Gm. ammonium sulfate per 100 ml.
Residue 49-55% saturated ammonium sulfate fraction	Supernatant (265 ml.) Add 5 Gm. ammonium sulfate per 100 ml.
Residue 55-62% saturated ammonium sulfate fraction. Dissolved in phosphate buffer (20 ml.) and any 49-55% fraction present reprecipitated.	Supernatant discarded

For preliminary fractionation we used salt precipitation (Table I). Twenty-eight grams of ammonium sulfate were added for every 100 ml. of the filtrate. The precipitate, which contains the hemagglutinating antigen,¹⁹ was removed by centrifugation at 3,000 r.p.m. at 4° C. To the supernatant of this fraction, an additional 5 Gm. of ammonium sulfate per 100 ml. of solution was added.

*Sharp and Sharp Company, Everett, Washington.

The precipitate (49 to 55 per cent saturated ammonium sulfate) was collected by centrifugation at 3,000 r.p.m. at 4° C. and represented 7.5 per cent of the protein in the whole pollen extract. There was a significant increase in biologic activity as a result of the ammonium sulfate fractionation (Table II). The remaining material was discarded because it possessed little or no biologic activity. Each precipitate was dissolved in a minimum of phosphate buffer at pH 10.4, reprecipitated, redissolved in phosphate buffer, and dialyzed against running tap water to remove the ammonium sulfate. The samples were concentrated by pervaporation.

TABLE II. A COMPARISON OF THE TOTAL ORGANIC SOLIDS, TOTAL PROTEIN, AND BIOLOGIC ACTIVITY OF VARIOUS FRACTIONS OF TIMOTHY GRASS POLLEN

FRACTION	TOTAL ORGANIC SOLIDS (GM.)	TOTAL PROTEIN (GM.)	PER CENT PROTEIN	BIOLOGIC ACTIVITY* (MG./ML.)
Whole pollen	38.4	3.1	8.1	10 ⁻²
49-55% sat. ammonium sulfate	0.69	0.233	34.0	10 ⁻³
40% ethanol	0.271	0.158	58.0	10 ⁻⁵

*Represents the mean of the maximum dilution evoking a threshold response.

The 49 to 55 per cent saturated ammonium sulfate fraction was diluted with distilled water to make a 2 per cent protein solution and different volumes of ethanol containing 10 per cent glucose and 0.02 M barium acetate added (Table III). Each precipitate was centrifuged at a temperature slightly above the freezing point, dissolved in chilled water, dialyzed against running tap water, and concentrated by pervaporation. The fraction which possessed the majority of the biologic activity was found to be soluble in 40 per cent ethanol, 10 per cent glucose, and 0.02 M barium acetate at pH 9.0. This fraction contained 5 per cent of the protein of the whole pollen extract and was considerably more active than the 49 to 55 per cent ammonium sulfate fraction (Tables II and IV).

TABLE III. ETHANOL FRACTIONATION PROCEDURE FOR TIMOTHY GRASS POLLEN

	49-55% Saturated ammonium sulfate fraction (30 ml.) Add 30 ml. of 30% ethanol, 10% glucose, and 0.02 M barium acetate, pH 7.0
Residue (15% ethanol)	Supernatant (60 ml.) Add 30 ml. of 60% ethanol, 10% glucose, and 0.02 M barium acetate, pH 9.0
Residue (30% ethanol) discard	Supernatant (90 ml.) Add 30 ml. of 70% ethanol, 10% glucose, and 0.02 M barium acetate, pH 9.0
Residue (30-40% ethanol) discard	Supernatant (120 ml.) over 40% ethanol fraction allergen

At several stages in the purification of timothy pollen allergens we attempted to take advantage of electrophoretic properties of proteins to achieve additional purification. We tried electrophoresis on cellulose filter paper, glass fiber filter paper, and starch blocks, and moving boundary electrophoresis. Each

of these methods proved undesirable because of loss of activity or inability to remove protein from the medium or lack of adequate separation of the proteins. Moving boundary electrophoresis demonstrated that the fraction soluble in 40 per cent ethanol apparently possessed at least three components (Fig. 1) and that their mobilities in the pH range of 6.0 to 9.0 were very similar. In the course of these experiments we found that the allergen was stable over a pH range of 4.0 to 10.5. These proteins had very little mobility in the pH range of 6.0 to 7.25 but migrated slightly toward the anode. This suggests that the negatively charged groups present in these molecules are strongly acidic.

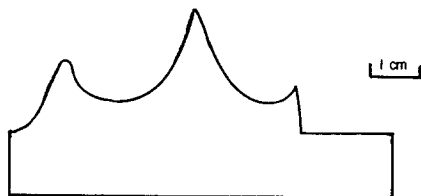


Fig. 1.—Pattern from the timothy pollen fraction soluble in 40 per cent ethanol after 147 minutes of Tiselius electrophoresis.

Further purification of the allergens of timothy pollen was achieved by Sephadex column chromatography. Three hundred and sixty milligrams of the proteins soluble in 40 per cent ethanol was dissolved in 3 ml. of 0.9 per cent saline and placed on a Sephadex G-75 column $1\frac{5}{16}$ inches in diameter and 30 inches in length. The column was developed in the cold laboratory at 4° C. by elution with 0.9 per cent sodium chloride. Protein concentration was determined by absorption at 400 m μ on each sample obtained over the next 26 hours (Fig. 2). Two peaks were obtained. However, examination of the major peak in the Spinco Model H electrophoresis apparatus in a 0.05 M citrate buffer at

TABLE IV. END-POINT DILUTIONS OF TIMOTHY POLLEN FRACTIONS (MG./ML.)

PATIENT	WHOLE POLLEN	AMMONIUM SULFATE	ETOH PRECIPITATES	ETOH SUPERNATANT	BATCH NUMBER
N. R.	10 ⁻⁴	10 ⁻³	10 ⁻³	10 ⁻⁴	I
H. C.	10 ⁻²	10 ⁻¹	10 ⁻²	10 ⁻¹	
H. G.*	10 ⁻¹	10 ⁻¹	10 ⁻²	10 ⁻³	
L. J.*	10 ⁻³	10 ⁻²	10 ⁻²	10 ⁻³	
G. F.*	none	10 ⁻²	none	10 ⁻²	II
D. W.*	10 ⁻²	10 ⁻²	none	10 ⁻⁴	
R. DiO.*	10 ⁻²	10 ⁻²	none	10 ⁻³	
Z. N.*	10 ⁻²	10 ⁻¹	—	10 ⁻⁷	III
G. F.	10 ⁻³	10 ⁻³	10 ⁻³	10 ⁻²	IV
L. A.*	10 ⁻⁴	10 ⁻⁵	10 ⁻⁵	10 ⁻⁷	
C. F.*	10 ⁻⁴	10 ⁻⁵	10 ⁻⁴	10 ⁻⁷	
E. F.	10 ⁻³	10 ⁻²	10 ⁻²	10 ⁻²	
D. St.*	10 ⁻³	10 ⁻³	10 ⁻⁴	10 ⁻⁶	V
D. St. (same as above)	10 ⁻³	10 ⁻³	10 ⁻³	10 ⁻³	V aged 6 days at room tempera- ture

*Assays showing definite increase in activity in the ethanol supernatant fraction.

pH 8.0 showed that this peak contained two overlapping components. Similar examination of the material in single tubes of the ascending and descending limbs of the curve (tubes 15 and 25) indicated the presence of only one protein in each tube while the material in tube 20 contained both proteins, the major component in tube 20 corresponding to the sole material in tube 15 and the minor component in tube 20 corresponding to the sole material in tube 25.

A series of patients were tested with tubes 16 and 26 (Table V). It can be seen that in 6 of the 12 patients the material in tube 16 was more active than that in tube 26 by a factor (geometric mean) of eighteen. The material in tube 26 was more active in 3 patients, by a factor of ten. Three more patients reacted equally to both tubes. It is noteworthy that we found no patients who reacted to only one of the tubes. These results imply that the two proteins have slightly different specificity but cross react. Definitive clarification of this point must await passive transfer neutralization testing.

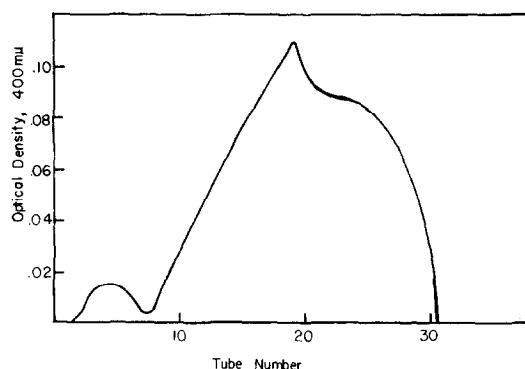


Fig. 2.—Elution of timothy pollen allergen from Sephadex G-75M. A nonabsorbing peak with no allergenic activity and no absorption at 400 $m\mu$ was found centred on tube 36. An optical density of 0.1 corresponds closely to 1 mEq.

TABLE V. RELATIVE SKIN TEST ACTIVITIES OF TUBES 16 AND 26

PATIENT	END-POINT DILUTION, 16 (MG./ML.)	END-POINT DILUTION, 26 (MG./ML.)
M. U.	10 ⁻⁴	10 ⁻⁵
B. T.	10 ⁻¹	10 ⁻²
D. So.	10 ⁻⁵	10 ⁻³
D. Wa.	10 ⁻³	10 ⁻³
P. T.	10 ⁻²	10 ⁻³
J. M.	10 ⁻¹	10 ⁻¹
S. C.	10 ⁻¹	10 ⁻⁵
W. S.	10 ⁻³	10 ⁻¹
P. J.	10 ⁻⁴	10 ⁻⁴
F. L.	10 ⁻¹	10 ⁻³
E. E.	10 ⁻³	10 ⁻²
G. W.	10 ⁻²	10 ⁻¹

A nonprotein high molecular weight material, which was not allergenically active and could be detected by its adsorption maximum at 276 $m\mu$ as well as by its tendency to foam when shaken, came off the Sephadex column at about tube 36. Thus the fraction soluble in 40 per cent alcohol contained at least three major components separable by molecular exclusion chromatography, two of them allergens and one of them inert.

The results of the electrophoresis experiments suggest that these two proteins are of quite high purity but it remains for further investigation to establish their homogeneity.

Some of the characteristic properties of the allergen became evident during isolation procedures. In one experiment the fraction soluble in 40 per cent ethanol was dialyzed against 0.02 M tris-acetate buffer at pH 9.0 and Tiselius electrophoresis was carried out 8 hours. Using the technique of compensation, the faster moving components were isolated in the ascending limb of a Perkin-Elmer standard electrophoresis cell and then examined in the Spinco ultracentrifuge in the same buffer at 6° C. at a speed setting of 59,780 r.p.m. for 3 hours. The protein did not pull away from the initial boundary and consequently no sedimentation coefficient or molecular weight could be calculated. This result suggested, however, that the molecular weight is less than 15,000 and that no higher molecular weight impurities were present.

A molecular weight of 15,000 suggested the possibility that the allergen might pass through a dialysis membrane. Fifty milligrams of the protein fraction soluble in 40 per cent ethanol dissolved in distilled water was placed in Visking No. 24 dialysis tubing and dialyzed against distilled water for 7 days at 2° to 4° C. The dialysate was changed daily, concentrated by lyophilization, and redissolved in 3 ml. of distilled water. Assays of protein concentration and biologic activity were made on a portion of each fraction. None of the seven dialysates showed any protein or any biologic activity. We also detected no loss of protein or of biologic activity from the material in the dialysis bag. We concluded from this that these allergens of timothy pollen do not pass through Visking No. 24 dialysis tubing.

The highly purified allergen is quite unstable in dilute solutions, particularly at room temperature although both the whole pollen extract and the 49 to 55 per cent ammonium fraction showed considerably more stability (see Table IV). Concentrated solutions of the purified allergen are stable for periods up to several weeks when stored in a refrigerator and for longer periods when stored at -15° C. after lyophilization. The loss of activity in dilute solutions and even concentrated solutions during electrophoresis on cellulose or glass fiber paper may be the result of denaturation. However, we have found that the purified pollen protein tagged with radioiodine is firmly adsorbed to cellulose and glass and, therefore, the loss of activity may be due to adsorption. The adsorption to glass is prevented by a silicone coating.

Daetylin was obtained by lowering the pH of the whole pollen extract to 3.0 with citric acid buffer. The crystals were dissolved in sodium hydroxide and recrystallized with the citric acid buffer. A saturated solution of it was prepared in buffered saline, pH 7.4, and tested intradermally. No positive reactions were obtained. Mixtures of daetylin and pollen protein fractions had no greater activity than the protein fractions alone.

Three milliliters of 40 per cent ethanol soluble material were examined in a spectrofluorimeter, using excitation energies of 260 m μ and 300 m μ . A large fluorescent peak was observed at 450 m μ , even using filters to remove excitation wave lengths above 440 m μ .

DISCUSSION

Although the question of the homogeneity of these allergens of timothy grass pollen has not been answered fully, considerable progress in this direction has been made. By ammonium sulfate and ethanol fractionation we have repeatedly obtained a fraction which is more active than whole pollen extract. Further purification by means of various electrophoretic techniques were, in general, unsatisfactory. However, Sephadex G-75 column chromatography appears to have provided a separation of the remaining components of the fraction soluble in 40 per cent ethanol. The results of electrophoresis of the fractions collected from the sephadex column in citrate phosphate buffer at pH 8.0 suggests that there are two highly active allergens of approximately the same size and charge, possibly differing only slightly in their composition.

If the whole pollen extract contains several different allergens and patients vary in their sensitivity to them, one would predict that, as purification progresses fewer of the total population of grass-sensitive individuals would react to the purified allergen. For example, it will be noted on Table IV that 2 patients (E. F. and H. C.) were more sensitive to the crude extract than to any of the purified materials. It is likely that they are allergic to some substance in the pollen other than the common allergens with which we are working. In calculating the increase in specific activity of the purified fraction, it, therefore, seems proper to consider only those patients showing a definite increase in activity, particularly since high dilutions of the purified allergen rapidly lose activity on storage.

It can be seen from Table IV that if only those assays* giving a clearly higher activity for the ethanol supernatant are selected and the increase in activity is expressed as the end-point dilution of starting material divided by the end-point dilution of the purified fraction, then the geometric mean of these figures shows the product to be eighty-eight times as active as the active ammonium sulfate fraction. However, the ethanol soluble fraction contains a full two thirds of the protein in the ammonium sulfate product. Thus, the activity has increased far more than the amount of purification would explain, and there may be some substance among the impurities removed by ethanol fractionation which blocks antibody combining sites on the allergen molecule. This hypothesis was tested by mixing the fractions precipitated by 5, 15, and 30 per cent ethanol with the fraction soluble in 40 per cent ethanol. Assay of these mixtures showed that the activity of the purified fraction had been reduced ten to one hundred times. It, therefore, seems likely that protein interactions of crude pollen extracts block some of the allergenically active sites of the allergen molecule.

One of the purposes of the purification of allergen is to have available a reagent for the study of the immunochemistry of pollen allergy. The feasibility of radioiodine labelling of the molecule opens up an important series of future experiments. Another interesting possibility presents itself from the

*Marked with an asterisk in the table.

spectrum of timothy pollen allergen. In addition to the usual protein absorption maximum at 280 $m\mu$, we found another maximum at 400 $m\mu$. It would seem possible, therefore, to follow the interactions of allergen and timothy antibodies using spectrofluorometric methods. Tryptophane possesses a fluorescent peak at 350 $m\mu$ and its emission can be quenched by dipole-dipole interaction with another molecule possessing an absorption maximum in the region between 300 and 400 $m\mu$. Since the purified timothy pollen allergen satisfies this criterion, we examined it for its fluorescent character. The existence of fluorescent emission had previously been reported in timothy pollen by Inglett and associates²⁰ and was attributed to the daetylin component. It, therefore, seems that spectrofluorometric analysis of reactions between timothy allergen and reagin is feasible and may provide information concerning the equilibrium constants, number of combining sites, and reaction kinetics of these antigen-antibody reactions.

One of our results that has immediate practical application is the confirmation of Hjorth's^{21, 22} observations that the allergen is strongly adsorbed to glass. This adsorption becomes especially significant at high dilutions. Our finding that it can be greatly reduced by silicone coating of the glassware leads to the recommendation that glassware used for storage of dilute solutions of grass allergens be silicone coated.

SUMMARY

Two highly purified allergens have been isolated from timothy pollen. They are low molecular weight proteins, very similar in their specificity and physico-chemical properties. They are very strongly adsorbed to cellulose and glass. The adsorption to glass can be prevented by silicone coating.

ADDENDUM

Studies with rabbit anti-timothy precipitins and chromatography with a longer Sephadex column both indicate that tubes 10-20 contain at least three components. There is no evidence for more than one component in tubes 22 and over.

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