

THE FREE AMINO GROUPS OF AMBROSIA ELATIOR POLLEN PROTEINS

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FROM the accumulated data of numerous investigators studying the allergenic material of low ragweed pollen, it is evident that a number of different components are responsible for this activity.¹⁻¹⁹ Although it has been shown that different proteins from ragweed pollen will provoke reactions in the hypersensitive individual, no explanation has been given for this cross reactivity.

A study has been made of the free amino groups of the different ragweed pollen proteins to test the hypothesis that these different proteins react in the same manner because they all have identical configurations at the free amino positions in their molecular structures.

This possibility was suggested by the work of Porter and Sanger¹⁹ who showed that the immunologic cross reactivity of hemoglobin from various animal species could be correlated with the amino acids occupying the terminal position of the peptide chains.

Ragweed pollen protein fractions were prepared by a variety of procedures. These preparations were arylated at room temperature and pH 8.0 with 2,4-dinitrofluorobenzene. Following the hydrolysis of the dinitrophenyl proteins, the resulting dinitrophenyl amino acids were isolated and identified by means of paper chromatography, characterization being made by comparison with synthetic dinitrophenyl amino acids. The results obtained were confirmed by counter-current distribution studies.

EXPERIMENTAL PROCEDURES AND RESULTS

Preparation of Protein Extract.—Low ragweed pollen* *Ambrosia elatior* was extracted with diethyl ether and petroleum ether (B.P. 30° C.-60° C.) in a Soxhlet apparatus until no more color appeared in the extract. The defatted pollen was extracted with shaking at 4° C. for twenty-four hours with ten times its weight of a solution of the following composition:

Sodium chloride	5.0	Gm.
Monobasic potassium phosphate	0.36	Gm.
Dibasic sodium phosphate	7.0	Gm.
Distilled water to make	1,000	ml.

The mixture was filtered first through coarse paper, then through a Berkefeld candle to remove all pollen grains. The resulting clear yellow solution

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was dialyzed at 4° C. against successive lots of distilled water for twenty-eight days. This prolonged dialysis ensured the removal of difficultly diffusible polypeptides.^{16, 17}

After this period a precipitate of euglobulin appeared in the dialysis sack. This was dissolved by dialyzing against 10 per cent sodium chloride solution. It is interesting to note that Dankner and associates found no euglobulin in their extracts.¹⁶

Preparation of Mixed Dinitrophenyl Proteins.—In order to determine all the different kinds of free amino groups in all the ragweed pollen proteins, the mixed proteins were first reacted with 2,4-dinitrofluorobenzene. To do this, 100 ml. of the mixed protein solution containing 14 mg. N, as determined by micro Kjeldahl, was concentrated in vacuo at 30° C. to a volume of 5 ml. and dialyzed against a 10 per cent solution of sodium bicarbonate until no more chloride ion appeared in the dialysate. A solution of 0.4 Gm. (0.002 mol) of 2,4-dinitrofluorobenzene in 10 ml. of absolute ethanol was added to the protein solution and the mixture shaken mechanically for two hours. The mixture was then acidified with 2 N hydrochloric acid and extracted with diethyl ether in a liquid-liquid extractor to remove the ethanol and excess 2,4-dinitrofluorobenzene as well as any 2,4-dinitrophenol formed during the reaction by the hydrolysis of 2,4-dinitrofluorobenzene.

Identification of 2,4-Dinitrophenyl Amino Acids.—The mixed dinitrophenyl proteins were hydrolyzed by refluxing for twenty-four hours with 20.5 per cent hydrochloric acid. This procedure breaks the dinitrophenyl proteins down to their constituent amino acids, those with free amino groups retaining the 2,4-dinitrophenyl substituents. The hydrolysate was diluted to 50 ml. with distilled water and extracted with diethyl ether until no more color appeared in the extract. This procedure removes the ether soluble dinitrophenyl amino acids leaving the unsubstituted amino acids and certain ether insoluble dinitrophenyl amino acids in the aqueous phase.

Both the ether and aqueous phases were evaporated to dryness in vacuo and the residues taken up in a sufficient amount of butanol to make the final concentration 1.0 mg. in 1.0 ml. These solutions were chromatogrammed on Whatman No. 1 filter paper sheets using both the ascending²⁰ and descending²¹ techniques. The following solvent systems were regularly employed:

- a. Liquefied phenol with 0.1 per cent ammonium hydroxide.
- b. Propanol: 0.8 N ammonium hydroxide; (3:1).
- c. Propanol: 4 N acetic acid; (3:1).
- d. Tertiary amyl alcohol saturated with phthalate buffer pH 5.9 on paper previously buffered at the same pH according to the technique of Blackburn.²²

The chromatography solvents were replaced at weekly intervals and the jars insulated to maintain as constant a temperature as possible.

The ether insoluble fraction yielded a single spot in each of the solvent systems. The ether soluble material showed two distinct spots in all systems except propanol: acetic acid where the spots overlapped.

Dinitrophenyl derivatives of all the common amino acids including α -aminobutyric acid were synthesized according to the published procedures of Abderhalden,²³ Porter and Sanger,^{19, 24} and du Vigneaud and associates.^{25*}

These derivatives were chromatogrammed on the same sheets as the unknown solutions in the various solvent systems using both the ascending and descending techniques. The ether-insoluble derivative corresponded to ϵ -dinitrophenyllysine under all conditions. Neither of the ether-soluble compounds corresponded to any amino acid derivative but rather migrated with the speeds of 2,4-dinitrophenol and 2,4-dinitroaniline, respectively. These two substances were found to have the same Rf value in the propanol: acetic acid system.

These results indicate that a maximum number of three different amino acid residues are attached to the free amino groups of the various ragweed pollen proteins. The ϵ -dinitrophenyllysine is formed from the nonterminal lysine residues. Sanger²⁴ has shown that dinitroaniline may be an artifact produced on hydrolysis from nonterminal histidine residues. This material may also originate from nonterminal asparagine or glutamine residues as will be shown later. Dinitrophenol may also be an artifact produced on hydrolysis. Sanger²⁶ has shown that dinitrophenylglycine is unstable to hydrolysis. A sample of synthetic dinitrophenylglycine was hydrolyzed under the conditions used here and chromatogrammed. A small amount of dinitrophenol was produced. It is possible that glycine in the peptide form is still less stable. The dinitrophenol may also be formed at the arylation step by the hydrolysis of dinitrofluorobenzene and remain adsorbed on the dinitrophenyl proteins in spite of the ether extraction. The following experiments were designed to eliminate this possibility.

Preparation of Dinitrophenyl Proteins Free of Adsorbed Dinitrophenol.—A buffered saline extract of ragweed pollen (400 ml.), prepared as above, was saturated with solid ammonium sulfate and allowed to stand for ten hours at room temperature. The yellow-brown precipitate was removed by filtration, washed with a saturated solution of ammonium sulfate, and redissolved in 200 ml. of water. This solution was filtered to remove a small amount of insoluble material and reprecipitated with solid ammonium sulfate. After standing overnight the precipitate was removed by filtration, dissolved in water, and precipitated a third time with solid ammonium sulfate. This precipitate was dissolved in 75 ml. of water and dialyzed against running cold tap water for twenty-four hours, then against several changes of distilled water after the dialysate gave a negative sulfate test. It was noted that the dialysates had a yellow color indicating this procedure extracts pigment from the proteins that is not removed by repeated precipitation. The yellow color of the dialysates was unstable and faded after two or three days. The dialyzed protein solution was concentrated to a volume of 5.0 ml, and arylated with dinitrofluorobenzene as described previously herein. The reaction mixture was evaporated to dryness in vacuo and the residue repeatedly extracted with

*The synthetic dinitrophenyl amino acids used in this study were very kindly prepared by Dr. Anna Pringle.

ethanol and ether to remove unchanged dinitrofluorobenzene and any dinitrophenol formed. The protein residue was suspended in 50 ml. of 0.5 N sodium hydroxide in which it dissolved slowly to form a red-brown solution. On acidification with concentrated hydrochloric acid a flocculent yellow precipitate formed which was removed by centrifugation and suspended in 10.0 ml. of water. The addition of 300 mg. of sodium bicarbonate caused the material to dissolve. It was then reprecipitated by the addition of 2 N hydrochloric acid. This process of dissolving in sodium bicarbonate solution and precipitating with hydrochloric acid was repeated five times. A portion of the dinitrophenyl proteins prepared in this manner was hydrolyzed with 20.5 per cent hydrochloric acid. The hydrolyzed material was extracted with diethyl ether and chromatogrammed. Dinitrophenol still appeared as before with dinitroaniline and ϵ -dinitrophenyllysine. This suggests that dinitrophenol is produced on hydrolysis of the dinitrophenyl proteins rather than being an impurity adsorbed on them.

As a further precaution against dinitrophenol being adsorbed on the dinitrophenyl proteins, after being reprecipitated six times as described above, the dinitrophenyl proteins were dissolved in sodium bicarbonate solution and dialyzed against repeated changes of 3 per cent sodium bicarbonate solution. The dialysates were concentrated, acidified with hydrochloric acid, and extracted with ether. The ether extract, chromatogrammed in the usual way, showed traces of dinitrophenol in the first dialysates but none after dialysis had proceeded for four days at 4° C. After all dialyzable dinitrophenol had been removed from the dinitrophenyl proteins, they were hydrolyzed, extracted with ether, and chromatogrammed in the usual way. Dinitrophenol was still found in the hydrolysates in large amounts. It was concluded from these experiments that dinitrophenol is produced from the dinitrophenyl proteins on hydrolysis.

Preparation and Hydrolysis of Didinitrophenylasparagine.—The dinitroaniline found in the hydrolysate of dinitrophenyl ragweed proteins may be produced from the ring nitrogen of nonterminal histidine residues as suggested by Sanger²⁴ or may be produced by the amide group of nonterminal asparagine or glutamine. To test this latter hypothesis, didinitrophenylasparagine was prepared by arylating asparagine with two moles of dinitrofluorobenzene in the usual manner. A yellow crystalline material melting at 174° C. and having Rf values of 0.64 in phenol: 0.1 per cent ammonia; 0.40 in propanol: 0.8 N ammonia (3:1); 0.47 in propanol: 4 N acetic acid (3:1), and 0.01 in buffered tertiary amyl alcohol, was obtained after recrystallization from water. When a 100 mg. sample of this preparation of didinitrophenylasparagine was hydrolyzed for twenty-four hours and an ether extract of the hydrolysate chromatogrammed, it was found that the didinitrophenylasparagine spot had disappeared and was replaced by spots corresponding to dinitrophenylasparatic acid and dinitroaniline.

Preparation and Hydrolysis of Different Dinitrophenyl Protein Fractions From Ragweed Pollen.—To check the foregoing results, four different protein

fractions were isolated from ragweed pollen, arylated with dinitrofluorobenzene, hydrolyzed, and the resulting dinitrophenyl amino acids identified.

Euglobulin fraction: The precipitate formed on dialyzing ragweed pollen extract against daily changes of distilled water for thirty days was removed by centrifugation and washed three times with distilled water. The residue was dissolved in 10 ml. of 10 per cent sodium chloride solution, filtered, and dialyzed against distilled water until the protein again precipitated. This precipitate appeared as irregular-shaped needles under the microscope. After washing with water this material was dissolved in 5.0 ml. of water containing 100 mg. of sodium bicarbonate and arylated with an alcoholic solution of 0.1 ml. of dinitrofluorobenzene.

Pseudoglobulin fraction: The dialyzed protein solution (100 ml.) after the removal of the euglobulin fraction was mixed with an equal volume of saturated ammonium sulfate solution. After standing overnight the yellow-brown precipitate was removed by centrifugation and washed with half-saturated ammonium sulfate solution. It was then dissolved in water and reprecipitated with an equal volume of saturated ammonium sulfate solution. This precipitate was dissolved in 5.0 ml. of water, dialyzed against 3 per cent sodium bicarbonate solution, and arylated with dinitrofluorobenzene.

Albumin fraction: The supernatant from the pseudoglobulin precipitation was saturated with solid ammonium sulfate. The yellow-brown precipitate was removed by filtration, washed with saturated ammonium sulfate solution and redissolved in water. The protein was precipitated a second time with solid ammonium sulfate and finally dissolved in 5.0 ml. of water. After dialyzing against 3 per cent sodium bicarbonate solution, the preparation was arylated with dinitrofluorobenzene.

Fraction Not Precipitated by Saturation With Ammonium Sulfate.—The supernatant from the albumin precipitation was dialyzed to remove ammonium sulfate and evaporated to dryness in vacuo. The residue was dissolved in 5.0 ml. of 3 per cent sodium bicarbonate solution and arylated with dinitrofluorobenzene.

Identification of 2,4-dinitrophenyl amino acids: Each of the four dinitrophenyl protein fractions was hydrolyzed and the resulting ether-soluble and ether-insoluble dinitrophenyl amino acids identified by paper chromatography. The same dinitrophenyl compounds obtained with the mixed proteins were found in each instance, namely, dinitroaniline, dinitrophenol, and ϵ -dinitrophenyllysine.

Confirmation of Results by Counter-current Distribution.—The ether-soluble dinitrophenyl amino acids were separated by counter-current distribution according to the technique of Craig,²⁷ using a butanol, 5 per cent ammonium hydroxide system. Peaks were obtained corresponding to a mixture of dinitrophenol and dinitroaniline.

DISCUSSION

It is apparent that only three different free amino groups exist in all the proteins of *Ambrosia elatior* pollen. Since all the protein fractions tested

showed the same free amino groups, it is suggested that the various ragweed pollen proteins have the same configuration at the free amino positions. The relatively few free amino groups found in ragweed pollen proteins suggest either that these proteins are cyclic in nature or that the terminal amino positions are attached to other compounds. The first possibility may be resolved by a determination of free carboxyl groups while the second is suggested by the presence of pigment in all preparations. Loveless¹⁷ has noted pigment associated with active fractions prepared from low ragweed pollen by electrophoresis.

SUMMARY

Various preparations of *Ambrosia elatior* pollen proteins have been arylated with 2,4-dinitrofluorobenzene.

In every instance hydrolysis of these dinitrophenyl proteins yielded 2,4-dinitroaniline, 2,4-dinitrophenol, and ϵ -dinitrophenyllysine, but no other dinitrophenyl amino acid.

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