Studies of Allergens from Alder Pollen *(Alnus Glutinosa)*

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An alder-pollen *(Alnus glutinosa)* extract has been fractionated and examined for allergenic activity by skin tests.

The activity was found in a non dialysable fraction. No decrease in activity was observed when this fraction was heated to 100°C for 1 hour. Treatment with pepsin, trypsin and chymotrypsin did not cause any significant inactivation.

Further fractionation by zone electrophoresis and chromatography on cellulose ion exchangers yielded a peptide-like fraction, 300 times as potent as the crude extract. This fraction contained at least 11 different amino acids.

The chemical and immunological properties of pollen allergens have been studied for about fifty years. Although much work has been done to isolate and to characterize active components in pollen, a chemically pure allergen has not yet been isolated. The activity appears, at least in some pollen species, to be shared by several substances. This circumstance, together with the lack of accurate test methods and methods for quantitative studies, has often led to contradictory reports. In spite of these facts, it can be concluded that allergens from different pollen have some characteristics in common. Chiefly ragweed and grass pollen have been studied. It is therefore appropriate to mention something about the methods that have been used for the fractionation of extracts of these pollen species and to point to some conclusions that have been drawn about the nature of the allergens.

The allergens of ragweed and grass pollen are generally found to be non-dialysable when ordinary dialysis membranes are used.[1,2] Weakly active components sometimes found in the dialysate have been referred to as breakdown products formed during the procedures [16].

Abramson *et al.*[3] employed Tiselius' moving boundary electrophoresis technique for the separation of components in crude extracts of ragweed

pollen. An active colourless fraction contained subtances with molecular weights of about 5 000, as determined by ultracentrifugation.

Fractionations by chromatography on ion exchange resins have been preliminary described by Bookman and Wax [4]. Using crude water extracts of ragweed pollen, they found that the best results were obtained when a sulfonic acid resin (Dowex-50) was used. However, only a small part of the initial activity was recovered.

From results obtained by using different precipitating agents, such as ammonium sulfate [5-8], [9-11], alcohols [12-15], acetone [16], lead acetate [17], acids [18-20], and bases [8,13] for fractionations of extracts, it has been suggested that the active substances in ragweed pollen are carbohydrate-protein complexes, proteins or carbohydrates.

Augustin has published preliminary reports on fractionations of grass pollen [21-23]. Salt fractionations of the extracts yielded a number of active proteins associated with carbohydrate-pigment complexes which could not be removed from the protein moiety by prolonged dialysis. Diffusion and ultracentrifuge studies on the active fraction indicated a molecular weight of 14 000.

Critical summaries of chemical and immunological studies in pollen allergy have recently been written by Augustin [23,24].

The present study is an attempt to apply modern fractionation techniques to the isolation of allergens and to quantitate the procedures involved. Zone electrophoresis and chromatography on cellulose ion exchangers are methods that would be suitable for problems of this kind. Alder pollen (*Alnus glutinosa*) are used in this work because large samples of this species were available. The general chemical composition of alder pollen *(Alnus glutinosa, Alnus incana)* has been studied by Nielsen *et al.*[25], but no reports on the purification of alder pollen allergens are found in the literature.

MATERIALS AND METHODS

Pollen. The pollen used in this investigation have been collected from the alder tree, *Alnus glutinosa*, in 1954 by A.B. Cernelle, Vegeholm, Sweden. Immediately after collection, the pollen samples were dried to a moisture content of 5 — 6 % and were kept cool and dry. The quantity of other pollen species amounted only to about 0.1 % [25].

Proteolytic enzymes. Pepsin was obtained from Parke, Davis and Co., London; *Trypsin* was obtained from Worthington Biochemical Corp., Freehold, New Jersey; *Chymotrypsin* was obtained from the Armour laboratories, Armour and Co., Chicago, Ill.

Ion exchangers. Triethylaminoethylcellulose anion exchange powder (TEAE-cellulose) was prepared as described by Porath [27]. The nitrogen content of the preparation was 5.1 mg per g of dry powder.

Sulfoethylcellulose ion exchange powder (SE-cellulose) was prepared as described by Porath [27]. The SE-cellulose contained 0.45 mequiv. sulfur per gram dry powder.

Zone electrophoresis. Zone electrophoresis was performed in externally cooled columns, using the technique described by Porath [31]. In one experiment ethanolised cellulose was used as supporting medium. In two other experiments a gel B 196* was used instead of cellulose.

Biological test. The biological activity was measured by intradermal tests on alder-sensitive patients.

A standard test solution is made up by dissolving material from 1 g pollen in 10 ml of a 0.5 % phenol solution (1:10). Before the testing there are made tenfold serial dilutions
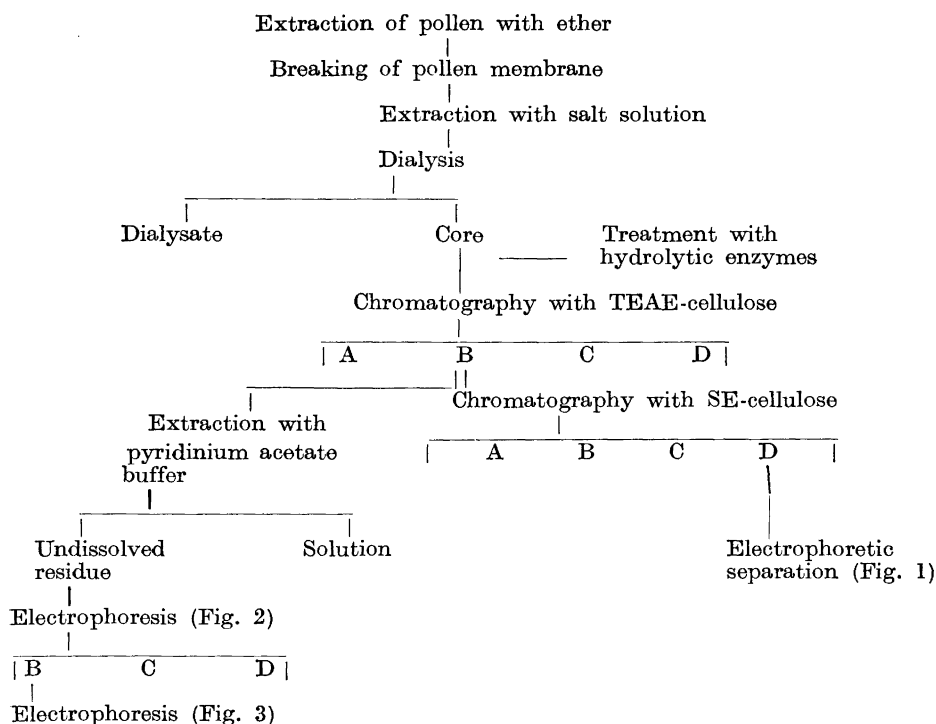
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* Obtained from AB Pharmacia, Uppsala.

of the standard test solution up to $1:10^6$ (*i. e.* $1:10^2$, $1:10^3$, ... $1:10^6$). Physiologic salt solution containing 0.5 % phenol is used for the dilution.

A control is made by injection salt-phenol solution in the skin of the patient. With the same syringe the different antigen containing dilutions are injected. The highest dilution is first injected, and this is followed by the next lower until a dilution is found which just gives a positive skin reaction. More or less strong responses are indicated by ‡ resp. ±. If a reaction is found only for the dilution $1:10^2$, this response can not with certainty be regarded as positive because nonspecific factors will interfere with the reaction at such a low dilution. This effect is more pronounced for crude extracts.

EXPERIMENTAL

Flow sheet

Extraction of pollen with ether
|
Breaking of pollen membrane
|
Extraction with salt solution
|
Dialysis
|
Dialysate   Core —— Treatment with hydrolytic enzymes
|
Chromatography with TEAE-cellulose
|
| A   B   C   D |
| ||
Extraction with   Chromatography with SE-cellulose
pyridinium acetate
buffer | A B C D |
|
Undissolved   Solution   Electrophoretic
residue   separation (Fig. 1)
|
Electrophoresis (Fig. 2)
|
| B   C   D |
|
Electrophoresis (Fig. 3)

Sampling for activity assays

If a fractionation had been made in an experiment and a certain fraction was to be tested, there was taken the portion of 1 g pollen that was recovered in the fraction (*i. e.* if say 500 g of pollen have been fractionated, one should take 1/500 of the material in the fraction to be tested). This portion was made up to standard condition by being dissolved in 10 ml of 0.5 % phenol solution and then prepared for testing. The dilution $(1:10 \times)$ which gave a positive skin reaction was marked as the activity. Consequently the activity value of this solution will never exceed that of the standard test solution from crude extract.

A. Pretreatment of pollen

1. *Ether extraction.* The pollen grains were defatted by extraction with ether in the following way. A sample of pollen (500 g) was suspended in peroxide free diethylether (1 l) and transferred to a glass tube of the type used for chromatography. Fresh ether was slowly percolated through the column. After 24 h when no more material went into the solution the extracted pollen sample was poured out and the residual ether was removed in vacuum.

13 % of the original pollen weight was thus removed by the extraction.

2. *Breaking of the pollen membranes.* In order to facilitate a subsequent extraction of active material the pollen membranes were ruptured as follows. A sample of pollen (100 g) was suspended in a small amount (200 ml) of extracting fluid (see below). The suspension was frozen to $-70°C$ and was then thawed. The procedure was repeated three times or till nearly all of the pollen grains were ruptured (checked by observation in a microscope).

B. Extraction of active material

1. *Extraction with salt solution.* The active material was extracted from the ruptured pollen by using the same procedure as for the ether extraction, but the extracting fluid was composed of 0.9 % sodium chloride and 0.5 % phenol dissolved in boiled distilled water. A sample corresponding to 1 000 g of fresh pollen was extracted during 36 h at 4°C with 3 600 ml of fluid. Extract exceeding 3 600 ml was collected separately in one experiment, but was not used for preparations because the activity in this fraction was only about one hundredth of that in the first collected fraction.

Each gram pollen yielded 251 mg of dry material and 18.2 mg of nitrogen (Kjeldahl). The activity of the extract was determined to $\ddagger$ 1: 10^4 $\pm$ 1: 10^5 (Patient L.F.).

2. *Extraction with sulfuric acid.* For comparison an extraction was made under condition where proteolytic activity in the extract is supposed to be prevented. A small scale extraction of 10 g pollen was made with 0.25 N sulfuric acid in the same way as indicated above. 50 ml of extract was collected during 24 h. 10 ml of the extract was dialysed in a cellophane sac for 1 h against water. The dialysed residue was neutralized to pH 7 with sodium bicarbonate (sample E). Another 10 ml of the initial extract was dialysed against 0.25 N sulfuric acid for 20 h. The residue (sample R) and the dialysate (sample D) were neutralized to pH 7. Results of the testing in Table 1.

Table 1.

Sample	Activity Patient L.F.
E	$\ddagger$ 1: 10^4
R	$+$ 1: 10^4
D	$+$ 1: 10^2

Reference: Extract (NaCl) ($\ddagger$ 1: 10^4 $\pm$ 1: 10^5)
The sulfuric acid extract was not used for the subsequent fractionations.

C. Dialysis of the salt extract obtained as described under B 1

The phenol-sodium chloride extract was dialysed in Visking cellophane sacs of 4 cm diameter. The extract inside the sacs was stirred with a rotating glass rod. During the first 24 h the extract was dialysed twice against 0.5 % phenol in water. The dialysis was continued for another 24 h but the residue in the sacs was now dialysed twice against boiled water. The whole procedure was performed in a cold room at 4°C. The liquid inside the sacs was centrifuged in order to remove a precipitate formed during the dialysis.

The precipitate which was dissolved in sodium chloride solution was inactive. The centrifuged liquid (referred to as "Core" in Table 2) had the same activity as the extract. The pooled outside liquids (dialysate) had no activity.

Table 2.

Sample	Nitrogen, mg per g pollen	Weight, mg per g pollen	Activity Patients L.F.	J.U.
Core	2.1	35	$+1:10^4$	$\pm 1:10^5$
Dialysate	10.2		$+1:10^2$	
Precipitate	3.0	18	$+1:10^2$	
Extract	18.2	251	$+1:10^4$	$\pm 1:10^5$

The active material did not pass the dialysis membrane. The extract was purified sevenfold by weight.

D. Properties of preparation "Core".

1. *Stability to heating.* 35 mg of preparation "Core" (Table 2) was dissolved in distilled water and heated in a closed glass tube at 100°C during 1 h. Results from testing in Table 3.

Table 3.

Sample	Activity (Patient L.F.)
Core	$+1:10^5$
Heated Core	$+1:10^5$

2. *Treatment with proteolytic enzymes.* The procedures used in the following experiments are similar to those described by Li *et al.*[26]

Pepsin. 35 mg of preparation "Core" (Table 2) and 25 mg of cryst. pepsin (1:3 000 U.S.P.) were dissolved in 45 ml of 0.01 N hydrochloric acid solution. A standard was prepared by dissolving 35 mg of preparation "Core" in 45 ml of hydrochloric acid. For comparison a solution was prepared by dissolving 25 mg pepsin in 0.01 N hydrochloric acid solution (sample "pepsin"). To all solutions were added a few drops of toluene. When all the solutions had been kept at 37°C for 4 h they were placed in a boiling water-bath for 2 min to terminate the enzymatic activity. The results from the testing are given in Table 4.

Table 4.

Sample	Patient L.F.		Patient J.U.		Patient A.N.
Core	$+1:10^4$	$\pm 1:10^5$	$+1:10^4$	$\pm 1:10^5$	$+1:10^3$
Digest	$+1:10^4$	$\pm 1:10^5$	$+1:10^4$	$\pm 1:10^5$	neg.
Pepsin	$+1:10^2$	$\pm 1:10^3$	$+1:10^2$		neg.

Trypsin. 35 mg of preparation "Core" (Table 2) and 10 mg of cryst. trypsin were dissolved in 50 ml of water. pH was adjusted to 9.3 by dropwise addition of 2 % ammonia solution. A standard was prepared by dissolving 35 mg of preparation "Core" in ammonia solution. For comparison a solution was prepared by dissolving 10 mg trypsin in 50 ml ammonia solution. After the mixtures had been kept at 37°C for 6 h the enzymatic activity was terminated by heating to 100°C for 2 min. The results from the testing are compiled in Table 5.

Table 5.

Sample		Activity			
	Patient L.F.		Patient J.U.		Patient A.N.
Core	$+1:10^4$	$\pm 1:10^5$	$+1:10^4$	$\pm 1:10^5$	$+1:10^3$
Digest	$+1:10^5$		$+1:10^4$		$\pm 1:10^3$
Trypsin	neg.		neg.		neg.

Chymotrypsin. 35 mg of preparation "Core" (Table 2) and 10 mg chymotrypsin were dissolved in 0.2 N ammonium acetate buffer of pH 8.2. A standard solution was prepared by dissolving 35 mg of preparation "Core" in ammonium acetate buffer. For comparison a solution was prepared by dissolving 10 mg chymotrypsin in the buffer. The mixtures were kept at 38°C for 5 h and then the enzymatic activity was terminated at 100°C for two min. The results from the testing are shown in Table 6.

Table 6.

Sample		Activity			
	Patient L.F.		Patient J.U.		Patient A.B.
Core	$+1:10^4$	$\pm 1:10^5$	$+1:10^4$	$\pm 1:10^5$	$+1:10^3$
Digest	$+1:10^4$		$+1:10^4$		$+1:10^3$
Chymotrypsin	$+1:10^3$		$\pm 1:10^2$		$\pm 1:10^2$

E. Fractionation of preparation "Core" by chromatography on triethylaminoethyl cellulose.

100 g of TEAE-cellulose ion exchanger containing 5.1 mg nitrogen per g was prepared as free base. The TEAE-cellulose was suspended in distilled water and packed in a Büchner funnel (19 cm in diameter) which was placed on a vacuum flask. 2 000 ml of preparation "Core" (Table 2) (corresponding to 435 g of pollen) were gently sucked through the ion exchanger during 1 h. Unadsorbed material was washed out with 1 l of water. The adsorbed material was displaced with three different solutions in the following order:

1) 2 l of 0.1 N pyridinium acetate (PyAc) buffer of pH 5.0.
2) 1 l of 1.0 N pyridinium acetate buffer of pH 5.0.
3) 0.5 l of 1 N acetic acid (HAc).

The collected fractions were freed from buffer and acid by vacuum distillation and finally lyophilized. The results obtained in one experiment is found in Table 7.

Table 7.

Sample	Fraction	Weight (mg)	Nitrogen (mg)	Activity Patient L.F. (2 measurements)	
TEAE—A	Eluate	6.6		$+1:10^2$	$+1:10^3$
TEAE—B	0.1 N PyAc	4.6	441	$+1:10^4$	$+1:10^5$
TEAE—C	1 N PyAc	3.2		$+1:10^2$ $\pm 1:10^3$	-------
TEAE—D	1 N HAc	0.1		neg	-------
	Total	14.6			
Core		15.2	929	$+1:10^4$	$+1:10^5$

The total loss of material amounted to 4 %, provided that material from the ion exchanger itself was not eluted during the process. The activity in preparation TEAE−B was the same as in preparation "Core". Preparation "Core" was purified threefold by weight and twofold by nitrogen. The experiment was easily reproduced. The ion exchange powder was packed in a wide funnel. If a column with a small diameter is used the elution easily stops because of the strong accumulation of material at the top of the column.

F. Fractionation of preparation TEAE-B on sulfoethyl cellulose

SE-cellulose, which contained 0.45 mequiv. sulfur per g dry powder was worked up as free acid. The powder was suspended in dist. water and was packed in a glass tube under pressure. Preparation TEAE-B (Table 7) was dissolved in distilled water and was applied to the column. The chromatogram was developed first with dist. water followed by a three step displacement with different solvents in the following order:

1) 0.1 N triethylammonium acetate buffer of pH 5.0
2) 1.0 N triethylammonium acetate buffer of pH 5.0
3) 0.1 N triethylamine.

Fig. 1 shows an experiment where 374 mg of preparation TEAE-B has been chromatographed on 25 g of SE-cellulose which was packed to a column of 2 cm diameter. Fractions of 7 ml were collected every 10 min.

Aliquots of 0.25 ml were taken from each fraction in the fraction range 1−85 and aliquots of 0.1 ml from each fraction in the fraction range 86−170 and were then analysed with ninhydrin [28]. Aliquots of 0.5 ml were taken for orcinol analysis [29]. 0.1 ml samples were used for turbidimetric determination of proteins with trichloroacetic acid [30]. No turbidity was observed. The distribution of yellow pigments is indicated in Fig. 1 (visually observed).

Fractions were pooled as indicated in Table 8 and were freed from buffer. After lyophilization the activity was determined.

Table 8.

Sample	Fraction range in Fig. 1	Weight (mg)	Activity Patient M.C.	
SE−A	0− 15	0.1	±1: 10^2	±1: 10^3
SE−B	16− 85	67.4	+1: 10^3	±1: 10^4
SE−C	86−100	238.4	+1: 10^2	
SE−D	101−170	68.2	‡1: 10^4	±1: 10^5
	Total	374.1		
TEAE−B		374.0	‡1: 10^4	±1: 10^5

By the fractionation Preparation TEAE−B was purified fivefold by weight.

G. Electrophoretic separation of preparation SE-D.

In order to study the heterogeneity of preparation SE−D (Table 8) this fraction was investigated by zone electrophoresis using the technique described by Porath [31]. The column which had a length of 150 cm and a diameter of 3 cm was washed with 0.1 N triethylammonium acetate buffer of pH 5.0.

60.2 mg of preparation SE−D was dissolved in 3.0 ml of 0.1 N triethylammonium acetate buffer of pH 5.0. Undissolved material (sample SE−P) was removed by centrifugation. The brownish yellow solution, containing 47.0 mg of dissolved material, was

placed on the top of the column and was slowly forced into it by gravity to a position 10 cm from the top. 1 200 — 1 300 V were applied giving a current of 85 mA. After 52 h the electrophoresis was stopped and the liquid in the column was displaced at a rate of 60 ml per h. 6.7 ml fractions were collected.

From every second fraction 0.5 ml was taken for ninhydrin analysis. The ultraviolet absorption at 280 mμ was also measured on undiluted fractions. 0.5 ml samples from every other fraction were hydrolysed in 4.5 N hydrochloric acid at 110°C for 15 h and then subjected to ninhydrin analysis [32]. The values from the measurements are recorded in Fig. 2. Fractions were pooled according to Table 9 and freed from buffer, lyophilized and tested. A correction is made in the weight values because samples had been withdrawn for analysis.

Table 9.

Sample	Fraction range in Fig. 2	Weight (mg)	Activity Patient L.F.	
E—1A	0— 55	1.0	$\pm 1: 10^2$	
E—1B	56— 75	7.2	$+1: 10^2$	$\pm 1: 10^3$
E—1C	76—100	10.1	$+1: 10^2$	
E—1D	101—120	7.1	$+1: 10^2$	
E—1E	121—170	10.8	$+1: 10^2$	
		Total 36.2		
SE—P		13.2	$\pm 1: 10^2$	
SE—D		47.2	$\ddagger 1: 10^4$	$\pm 1: 10^5$

H. Extraction of preparation TEAE-B with buffer

A considerable purification of the active material was achieved by extraction of preparation TEAE—B (Table 7) with buffer as described below. The procedure was not always reproducible. However, the following experiment gave data about the allergen(s).

500 mg of preparation TEAE—B (Table 7) was thoroughly mixed with 2 ml of 0.1 N pyridinium acetate buffer of pH 5.0. Undissolved material was treated again with 1 ml of buffer. Still undissolved material (sample TEAE—BP) was separated by centrifugation. The solutions were pooled (TEAE—BP). Preparation TEAE—BP was freed from buffer and lyophilized. The test results are shown in Table 10.

Table 10.

Sample	Weight (mg)	Activity Patient L.F.	Patient J.U.	
TEAE—B	500	$\ddagger 1: 10^4$	$+1: 10^4$	
TEAE—BP	70	$+1: 10^4$	$+1: 10^3$	$\pm 1: 10^4$
TEAE—BS		$+1: 10^2$	neg	

By the extraction preparation TEAE—B was purified about sevenfold.

I. Electrophoresis of preparation TEAE-BP

The electrophoresis was performed in an externally cooled column 90 cm long and with a diameter of 1.6 cm. Gel B 196 was used as supporting medium. 0.05 N triethyl-ammonium carbonate buffer of pH 8.6 was used [33].

69 mg of preparation TEAE—BP (Table 10) was dissolved in 4 ml of triethylammonium carbonate buffer of pH 8.6. The brownish yellow solution was applied on the column

and by gravity forced 26 cm down into the column. 600 V were applied yielding a current of 15 mA. After 15 h the electrophoresis was stopped and the liquid in the column was displaced at a rate of 40 ml per h. 3 ml fractions were collected.

From every second fraction 0.1 ml was taken for ninhydrin analysis. The measured values are recorded in Fig. 3. The fractions were pooled according to Table 11, freed from buffer, lyophilized and tested. The brownish yellow colour was concentrated in fraction E—2B.

Table 11.

Sample	Fraction range	Weight (mg)	Patient U.B.		Patient G.J.M.		Patient Y.N.	
					Activity			
E—2A	0—11	1.1						
E—2B	12—18	33.6	$\ddagger 1{:}10^2$	$\pm 1{:}10^3$	$\ddagger 1{:}10^2$	$\pm 1{:}10^3$	$+1{:}10^3$	$\pm 1{:}10^4$
E—2C	19—29	21.9	neg		$\pm 1{:}10^2$		$\pm 1{:}10^2$	
E—2D	30—50	7.6	$\pm 1{:}10^2$		$\pm 1{:}10^2$		$\pm 1{:}10^2$	
TEAE—BP			$+1{:}10^3$		$\pm 1{:}10^3$		$\pm 1{:}10^4$	

Preparation TEAE—BP was purified about twofold by the procedure, and had now about 300 times as high specific activity as the initial extract. The nitrogen value of preparation E—2B was 1.19 mg.

A too big amount of material from preparation E—2B is required for detection on paper. Therefore a new zone electrophoresis experiment was made instead of using paper electrophoresis.

Electrophoresis. The same column (90 × 1.6) was used but the buffer was now 0.05 N triethylammonium carbonate buffer of pH 9.0.

30 mg of preparation E—2B (Table 11) was dissolved in 2 ml of buffer and was forced 25 cm into the column. The electrophoresis was run for 39 h using 700 V and a current of 25 mA. To make it possible to run the electrophoresis during 39 h and still keep the substances in the column, the liquid in the column was slowly forced in the opposite direction to that in which the substances migrated. This was arranged by keeping the buffer in the two electrode vessels at different levels.

The liquid in the column was displaced at a rate of 40 ml per h. 2.5 ml fractions were collected.

0.1 ml samples from every second fraction were used for ninhydrin analysis.

0.05 ml samples were hydrolysed in 4.5 N hydrochloric acid at 110°C during 15 h and the ninhydrin values determined. Absorption at 275 mμ was measured. The measured values are shown in Fig. 4. The highest ninhydrin value and the strongest colour (yellow) was found in the same tube. 25 mg of material was found in the fraction range 26—120.

The material in the fraction range 28—43 was preliminarily examined. One sample was hydrolysed with 6 N hydrochloric acid at 110°C for 15 h. The hydrolysate was studied by means of paper electrophoresis and paper chromatography. Both acid, basic and neutral amino acids were present (at least 11 different spots). The same amount of un-hydrolysed material did not give any colour with ninhydrin on paper. When enough of unhydrolysed material had been chromatographed on paper, using 80 % phenol in borate buffer of pH 9.3 as solvent system, the yellow pigment stayed at the starting point and a 'ninhydrin spot' was found with an R_F-value of about 0.5.

DISCUSSION

From the results of the semi-quantitative skin test method used in this study it is not always possible to conclude whether one of several active components has lost its activity during the fractionation procedure. This is the main reason why methods as mild as possible have been applied. The tests were performed with the same patient (Patient L.F.) except in the experi-
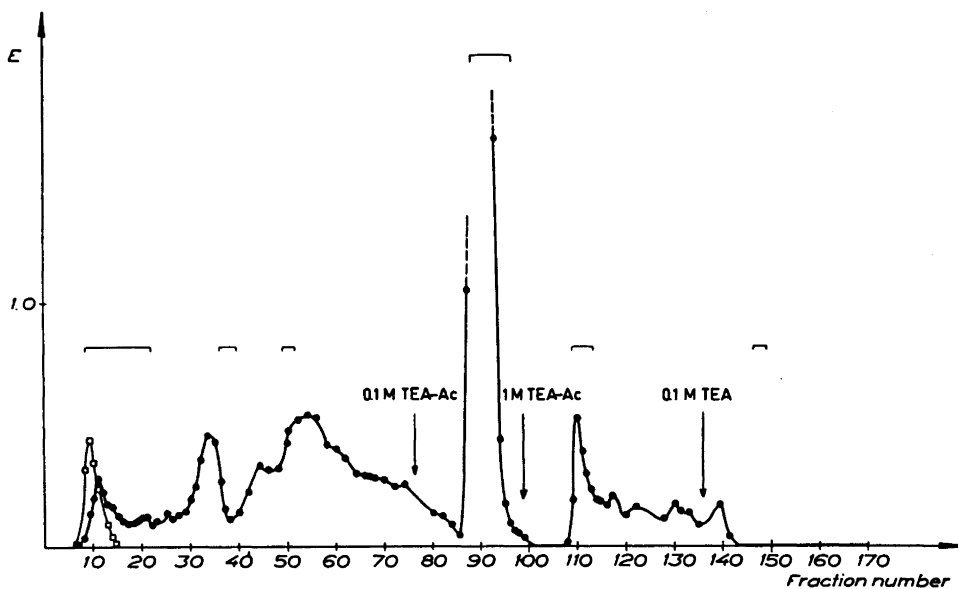
Fig. 1. Chromatographic separation of 374 mg of preparation TEAE—B on 25 g of
     SM-cellulose (column diameter: 2 cm). Fractions of 7 ml were collected.

———·———·——— Ninhydrin curve obtained from 0.25 ml samples in the fraction range
            1—85 and 0.1 ml samples in the fraction range 86—170.
—□—□— Orcinol curve obtained from 0.5 ml samples.
⌐————————¬ Visually observed yellow pigments.

ment described in passages F and I. Patient M.C. (passage F) and Patient
L.F. gave about equal response to preparation TEAE-B.

     It has been questioned whether allergens from pollen are broken down
by hydrolytic enzymes in the extract during preparations. Loveless *et al.*[1]
found appreciable losses of nitrogen and carbohydrates from ragweed extract
during extensive dialysis through cellophane. Frankel *et al.*[16] examined the
same pollen species and they believe that a number of weakly active peptide
carbohydrate complexes in the dialysate have arisen by fragmentation of
the undialysable residue, which represented the main activity.

     Either inactive or still active components could be formed by an enzymatic
cleavage. In the case of alder pollen (Table 2) no loss of activity was found
after dialysis. Even if a limited hydrolysis of allergens could have taken place,
it is of interest to purify and study an active fragment. The small scale extrac-
tion with sulphuric acid was made in order to study the activity yield under
conditions where proteolytic cleavage is supposed to be prevented[34]. The
activity in this extract, however, was not higher than in the phenol-sodium
chloride extract used for the fractionations. The activity of the sulphuric
acid extract was lowered only a little when it was kept at 4°C during 20 h.

     The active material did not pass the dialysis membrane used in the initial
step of the fractionation. This does not exclude the possibillity that a pene-
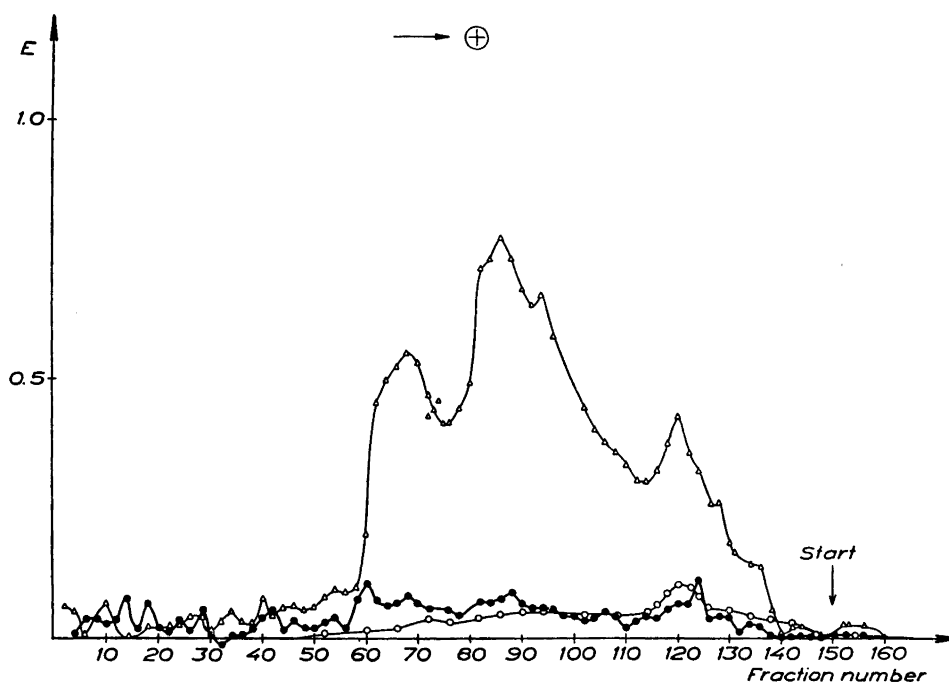
Fig. 2. Electropherogram of 47 mg of preparation SE—D obtained after 52 h electrophoresis at 85 mA in a 150 × 3 cm column using 0.1 N triethylammonium acetate buffer of pH 5.0. Fractions of 6.7 ml were collected. The direction of the current is indicated by the sign →O

———•——•—— Ninhydrin curve obtained from 0.5 ml samples.

—△—△— Ninhydrin curve obtained from hydrolysed 0.5 ml samples.

—O—O— Ultraviolet absorption at 260 mμ

tration could take place in a later stage, when originally present weak complexes could have been split. Consequently no definite conclusions can be drawn from this experiment about the molecular size of the allergens.

The experiments reveal that fraction "Core" was remarkably stable to heating. The heat stability of pollen allergens is of considerable interest, because already differences in this property may give hints to differences in their chemical nature. It is therefore important to emphasize that in the case of alder pollen here investigated, no inactivation was observed even after one hour's heating at 100°C. It is interesting to compare this result with similar studies made by Augustin [23] on grass pollen. She found that only 10 % of the activity remained after 15 min at 100°C, and that already after one minute's heating all precipitating power for the rabbit antibody was lost and that precipitates were no longer formed in gel diffusion tests.

As seen from the experiments no loss of activity was found after treatment of fraction "Core" with trypsin or chymotrypsin. The same result was found
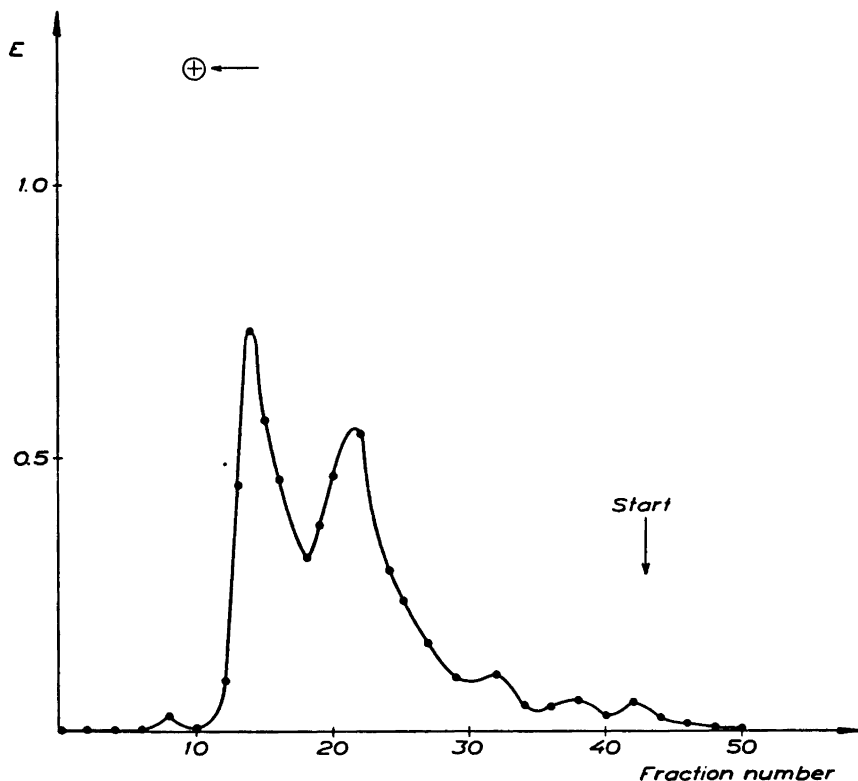
Fig. 3. Electropherogram of 69 mg of preparation TEAE—BP obtained after 15 h electrophoresis at 15 mA in a 90 × 1.6 cm column using 0.05 N triethylammonium carbonate buffer of pH 8.6. Fractions of 3 ml were collected.

——•——•—— Ninhydrin curve obtained from 0.1 ml samples from every other tube.

with pepsin in the tests made on two of the patients. A third patient gave a negative response to the pepsin digest, but the initial activity value was in this case rather low, and therefore no definite conslusions can be drawn. There might be several allergens and some of these do not exhibit resistance towards pepsin. Augustin has preliminarly described similar studies on grass pollen extracts [23]. In those cases the treatment with pepsin and trypsin destroyed all activity within one hour, whether the digestion was carried out on the crude extract or on the purified protein solution.

Allergens from pollen are often referred to as pigmented carbohydrate-rich compounds. On the other hand, it is preliminarily reported that one of the allergens from grass pollen contains only 2 % of carbohydrates [23]. Referring to the experiments on alder pollen, it is seen from Fig. 1 that the orcinol curve does not indicate the presence of any carbohydrates in the active fractions displaced from the SE-cellulose. On the other hand, it cannot be excluded
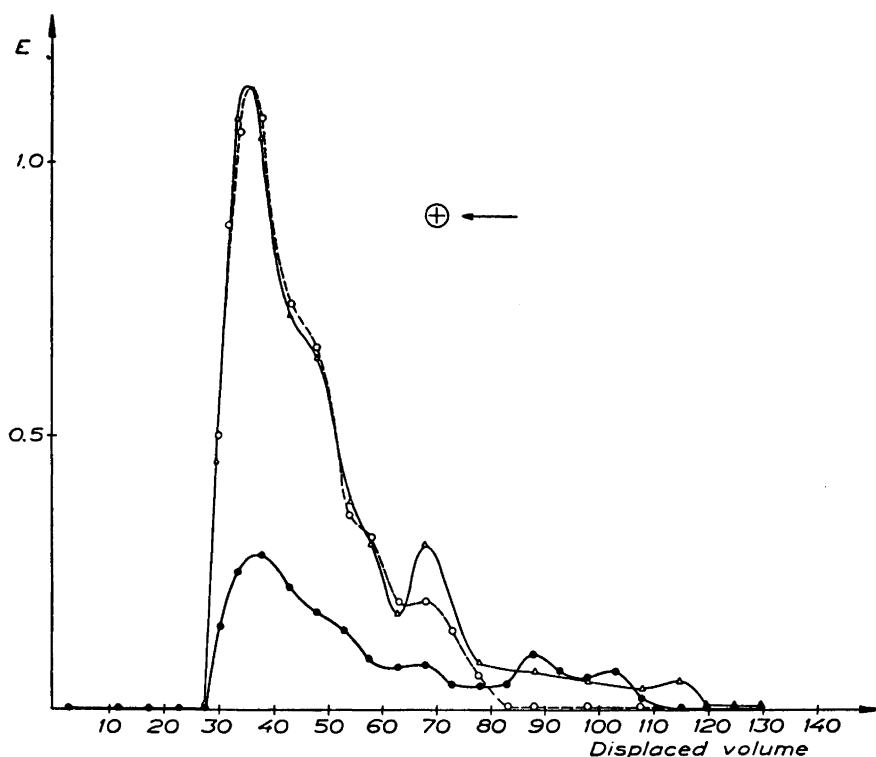
Fig. 4. Electropherogram of 30 mg of preparation E—2B obtained after 39 h electrophoresis at 25 mA in a 90 × 1.6 cm column using 0.05 N triethylammonium carbonate buffer of pH 9.0. Fractions of 2.5 ml were collected.

—·——·—— Ninhydrin curve obtained from 0.1 ml samples from every other tube.
—△—△— Ninhydrin curve obtained from 0.05 ml hydrolysed samples from every other tube.
—O—O— Ultraviolet absorption at 275 mμ.

that there are components present containing small amounts of carbohydrates, which are not revealed by the reagent. It is possible that the cellulose ion-exchangers used in the purification may give off small amounts of cellulose, and this would interfere in an accurate carbohydrate determination. Consequently a final fractionation has to be made in order to get rid of ion-exchange material.

No experiments have been made in order to determine whether the activity in fraction SE-B (Table 8) is caused by an overloading of the column, or if the material in this fraction is different from that in fraction SE-D (Table 8). In the fully active fraction SE-D no proteins were found, and there were probably no free amino acids. A sample of this kind would presumably be suitable for fractionation with zone electrophoresis. The unfortunate reduction of activity during the electrophoresis (Fig. 2) can perhaps be explained by

the fact that too small amounts of material have been used. Under these conditions oxidation, bacterial growth and adsorption have a pronounced effect on the activity yield.

When the extract is stored and during evaporations of solutions the solubility of the components is often changed. Especially the pigments tend to crystallize. Consequently there are risks of coprecipitations. Precipitates are often difficult to get into solution without drastic treatments. It was possible to make use of this fact in order to obtain information about the allergens. As seen from the experiment (passage H) a highly pure fraction (sample TEAE-BP) was obtained. The electrophoretic patterns are shown in Figs. 3 and 4. The active peak contains bound amino acids together with a yellow pigment. No experiments have been made in order to reveal if the activity is caused by the bound amino acids or by the pigment or by a possible complex between them. Stevens et al.[35,23] have met with a similar problem in the course of studies of an amorphous glucoside precipitate that was formed in the extract of giant ragweed pollen. They found that biologic activity remained as long as nitrogen was associated with the glucoside (isoquercetin), while the pure glucoside was inactive.

The stability towards drastic treatments such as heating, evaporation and proteolysis suggests that the bulk of the activity in the dialysed extract is not due to proteins. The adsorption of the active material on the cellulose ion-exchangers strongly indicates the presence of acid and basic groups in the active molecule(s). The electrophoretic behavior (Fig. 2) of a fraction, with about 300 times as high specific activity as the initial extract, reveals that the allergen(s) is (are) negatively charged at pH 8.6. The bound amino acids found in the active peak are probably parts of the allergen(s).

The data that are presented point to the conclusion that the allergen(s) is (are) of peptide nature.

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