

From the Protein Laboratory, University of Copenhagen;  
Medical Department TA, Division of Clinical Immunology and Laboratory of  
Clinical Allergology, Rigshospitalet, Copenhagen, Denmark.

QUANTITATIVE IMMUNOELECTROPHORESIS  
USED IN ANALYSIS OF THE ANTIGENS IN  
TIMOTHY (*PHLEUM PRATENSE*)  
POLLEN EXTRACT

By

LARS NIELSEN, HENNING LØWENSTEIN & BENT WEEKE

Most allergens are proteins with molecular weights of over 10.000 (1). The allergen extracts used in clinical work on allergy contain, besides these allergens, a number of protein antigens without allergenic activity. These "ballast-proteins" may interfere seriously by lowering the precision of the diagnosis of allergy and minimizing the immunotherapeutic effect of the extract. Furthermore, the allergenic activity of the extract cannot be derived from the protein nitrogen units (PNU).

Identification and quantification of allergens are of prime importance for their standardization and purification. Quantitative immunoelectrophoresis (QIE) has been used to advantage in many disciplines (6, 14) in the study of complex antigen mixtures, but in allergology it has, until now, only been employed in the analysis of birch pollen by Belin (8).

The studies of timothy pollen prior to 1952 were extensively reviewed by Augustin-Friedman (2), who indicated that allergens were proteins which were stable to lyophilisation, denatured by heating, and possibly dialysable through semi-permeable membranes. Later on, the same author (5), demonstrated 15 different antigens containing two allergens

with an average molecular weight of about 14,000 Dalton. The antigens associated with skin reactivity were found to have the mobilities of  $\alpha$ - and  $\beta$ -serum globulins (3, 4).

The aim of this work is to investigate the antigens in a timothy extract by means of QIE. Some further studies, concerning the identification of the allergens among these antigens and the behaviour of the individual antigens in some conventional fractionation procedures will be reported later (15, 18).

Timothy pollen was selected because it causes hay-fever in a number of patients in the Scandinavian countries. This results partly from the widespread use of grass as food for cattle.

## MATERIALS AND METHODS

### *Antigen Sources*

Pollen of timothy (*Phleum pratense*), was kindly supplied by Allergologisk Laboratorium A/S, Copenhagen, Denmark. This pollen was collected during the summer of 1972 in Sweden by Allergon A.B., Sweden. Pollen was stored until the day of use in a closed metal container at  $-20^{\circ}\text{C}$ . No defatting was performed. The content of the pollen was: 90 per cent pure timothy pollen, and 10 per cent other non-pollen fragments. Foreign pollen constituted less than 1 p.p.m.

### *Standard Antigen Extract*

A 0.1 M sodium bicarbonate, 0.015 M sodium azide solution (pH 8.2) was used for the suspension and extractions. The standard extraction was performed according to Axelsen (7).

20 ml of 1:10 (w/v) pollen suspension was placed in a Braun homogenization bottle (50 ml) containing an equal volume of glass beads (0.45–0.50 mm) and 10 ml of buffer. Homogenization was performed in a Braun cell-homogenizer (MSK) at 4000 rpm for 60 sec, using liquid  $\text{CO}_2$  as a coolant. A 20 ml sample of the homogenized pollen extract was subjected to centrifugation at 3500 g for 5 min. The entire procedure was carried out four more times using the same glass beads. The five supernatants were pooled and ultracentrifuged (Sorvall RC2-B, New Town, Conn., USA) twice at  $0^{\circ}\text{C}$  for 60 min at 50,000 g. In this way five different supernatants of timothy pollen antigen were produced. The five batches were pooled and used as a standard antigen, denoted hereafter by St.T 73. The latter was stored at  $-20^{\circ}\text{C}$ . The protein content of St.T 73 was determined by amino acid analysis (17) on a Beckman 120 C apparatus. St.T 73 was precipitated by 10 per cent TCA and separated by centrifugation. The

precipitate was washed and centrifuged five times before hydrolysis. The protein content was determined as 1.67 mg/ml, based on a known standard mixture of amino acids.

*Simple extractions* were performed by agitating 5 g of pollen grains in 50 ml of buffer at 4°C. Samples were removed from the extract after varying periods of time and centrifuged twice, at 50,000 g for 60 min at 0°C, and stored until use.

*Rabbit immunoglobulins* against timothy grass pollen were raised by immunisation of six rabbits with St.T 73, and purified according to Harboe & Ingild (6a). Rabbit immunoglobulins against aqueous 5 Grass Mix (timothy, orchard, rye, velvet and blue grass), 6400 PNU/ml (kindly supplied by DOME Laboratories, England), were obtained in the same way using two rabbits. Rabbit anti-human IgE ( $\epsilon$ -chain specific) serum was obtained from (Behringwerke, batch 2049 E, Marburg/Lahn, Germany) and purified as above.

<sup>125</sup>I-immunoabsorbed rabbit immunoglobulins against human IgE were obtained from Phadebas RAST Pharmacia, Sweden, or prepared by immunoabsorption of purified antihuman IgE serum using polyclonal IgE coupled Sepharose® 4 B followed by <sup>125</sup>I labelling by the electrolytic method of Rosa et al. (16, 13) (kindly carried out by Ulf Jacobsen, Risø Isotope Laboratory, Denmark).

#### *Immuno-electrophoretic Methods*

Equipment and reagents were essentially as described in (6b). One per cent (w/v) agarose gel (Litex, batch AGS 102 Dx, Glostrup, Denmark) was employed.

*Crossed immunoelectrophoresis* (CIE) and *Crossed-line immunoelectrophoresis* (CLIE) were carried out as described elsewhere (6c, 6d). First and second dimension electrophoresis were performed on a 0.15 cm thick gel at 10 v cm<sup>-1</sup> for 30 min, and a 0.12 cm thick gel at 2 v cm<sup>-1</sup> for 18 h, respectively. The temperature of the cooling water was 15°C. The plates were pressed, washed and dried, and finally stained with Coomassie Brilliant Blue R. The area enclosed by the precipitation lines was measured by planimetry (6b).

The crossed-line immunoelectrophoresis was performed on 5 × 5 cm<sup>2</sup> glass plates, applying 5 µl of St.T 73 in the well and the extract to be examined in the intermediate gel (1 cm wide).

*Agarose gel electrophoresis* was performed according to Weeke (6b).

*Dialysis* of the St.T 73 extract was performed against 0.1 M sodium bicarbonate, 0.015 M sodium azide solution, using a dialysis membrane with a limiting pore size corresponding to approximately MW < 10<sup>4</sup> Dalton.

*Ultrafiltration* was performed using Diaflo® membrane PM 10 (Amicon, Mass., USA), at an external pressure of 5 atm.

*Lyophilisation* was performed on desalted (Na<sup>+</sup>, HCO<sub>3</sub><sup>-</sup>, Na<sup>+</sup>, CO<sub>3</sub><sup>-</sup> exchanged with NH<sub>4</sub><sup>+</sup>, CH<sub>3</sub>COO<sup>-</sup>) St.T 73.

### Serum

Serum, from a patient highly allergic to timothy (H.D.), was used as a reference in RAST and RAST-inhibition experiments. Serum diluted 1 + 15 with incubation buffer (6c) was stored at  $-20^{\circ}\text{C}$  and used as a 100 Sorbent Unit (SU) reference in RAST experiments, and a serum pool from 20 healthy blood donors was used as a control.

### RAST (Radio-Allergo-Sorbent-Test) (19)

Different samples of timothy were coupled to activated filter paper as described by Cheska et al. (11). After incubation in timothy allergic patient serum (H.D.), the paper-timothy-IgE complex was determined by addition of  $^{125}\text{I}$ -labelled rabbit antihuman IgE and subsequent counting.

RAST-inhibition experiments were carried out after allowing equal volumes of timothy allergic patient serum (H.D.) and serial dilutions of St.T 73 to react overnight at  $4^{\circ}\text{C}$ . These mixtures were used for RAST determinations on St.T 73 coupled paper discs. The counts obtained in RAST as a function of the concentration of St.T 73 in the dilutions gave the dose-response curve. From this curve the count and the concentration corresponding to 50 per cent inhibition ( $C_{\text{St}}^{50\%}$ ) were obtained. For the unknown samples, the concentration corresponding to 50 per cent inhibition ( $C_x^{50\%}$ ) was determined by means of similar experiments.

Allergenic activity, based on 50 per cent RAST-inhibition experiments, is defined here as

$$\frac{C_x^{50\%}}{C_{\text{St}}^{50\%}} \times 100 \text{ (per cent)}.$$

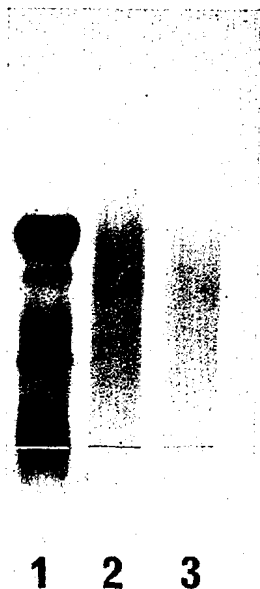
## RESULTS

### Agarose Electrophoresis of St.T 73

Fig. 1 shows an ordinary agarose gel electrophoresis pattern for St.T 73, together with that for normal human serum. All the stained protein components in St.T 73 migrated towards the anode with migration velocities similar to those of human albumin or  $\alpha$ - and  $\beta$ -globulins. No distinct separation was obtained for St.T 73, but there is some indication of weak bands in the  $\alpha$  and  $\beta$  mobility regions.

*Fig. 1.*

Agarose gel electrophoresis. 1 per cent agarose in barbital buffer (pH 8.6, ionic strength 0.02). 8 v  $\text{cm}^{-1}$ , 45 min, 15°C. Samples: 3  $\mu\text{l}$  of 1) normal human serum, 2) 5 times concentrated (see Ultrafiltration) St.T 73, 3) St.T 73. Fixation with picric acid; staining with Coomassie Brilliant Blue R.



### *Crossed Immunoelectrophoresis (CIE) of St.T 73*

CIE of the timothy pollen extract St.T 73 and of a normal human serum run simultaneously is shown in Fig. 2. No cathodically migrating proteins were observed.

Fig. 3 is a drawing, numbered arbitrarily from anode to cathode, of the precipitation pattern of St.T 73. Twenty-eight antigens are indicated. The migration velocities, relative to that of human albumin, and other characteristics of the different precipitates are given in Table 1.

### *The Reproducibility of the Extraction Procedure*

The antigens in standard extraction procedures were estimated quantitatively by CIE. The areas enclosed by the individual precipitation lines of the preparation in question,

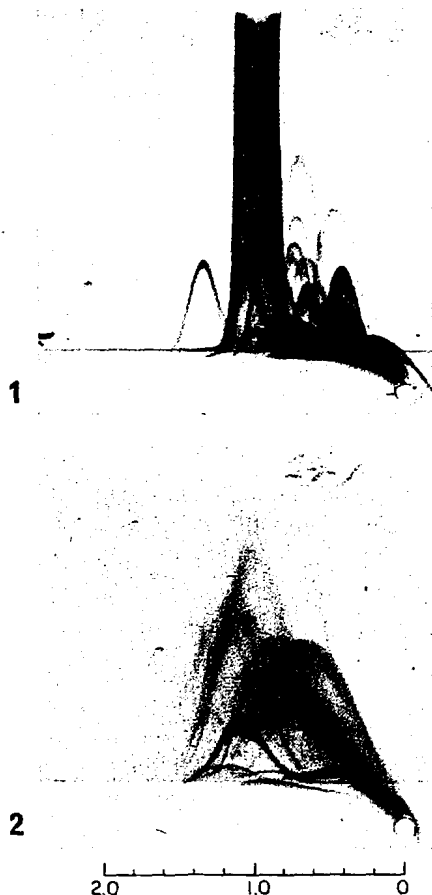


Fig. 2.

Crossed immunoelectrophoresis of 1)  $1 \mu\text{l}$  of normal human serum, 2)  $5 \mu\text{l}$  of St.T 73 extract, performed on  $5 \times 5 \text{ cm}^2$  glass plates. First dimension electrophoresis: 30 min,  $10 \text{ v cm}^{-1}$ ,  $15^\circ\text{C}$ , in 1 per cent agarose in barbital buffer (pH 8.6, ionic strength 0.02). Antibodies: 1)  $15 \mu\text{l}$  of rabbit anti serum (100 SF Dakopatts, Copenhagen)/ $\text{cm}^2$  gel area. 2)  $15 \mu\text{l}$  rabbit anti timothy/ $\text{cm}^2$  gel area. Second dimension: Overnight (18 h),  $2 \text{ v cm}^{-1}$ ,  $15^\circ\text{C}$ . Staining with Coomassie Brilliant Blue R. An arbitrary scale of migration velocity relative to human albumin (1.0) is indicated below.

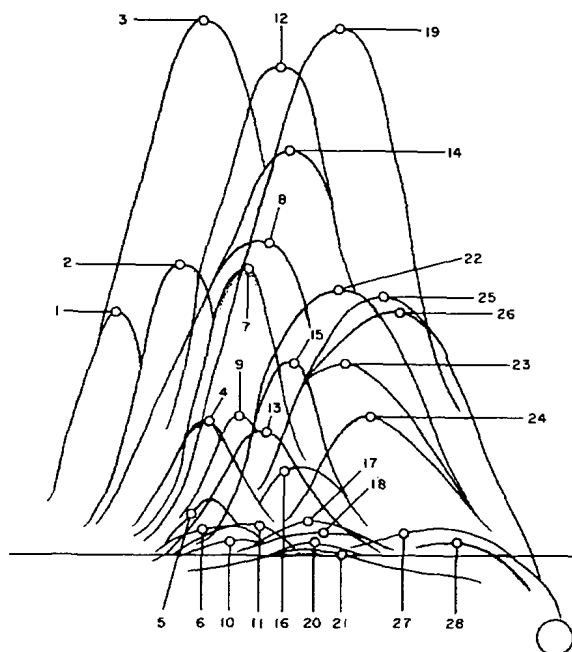


*Fig. 3.*

Drawing of crossed immunoelectrophoresis pattern for St.T 73. Classification numbers for precipitates are indicated. An arbitrary scale of migration velocity relative to human albumin (1.0) is given in Table 1.

relative to the area of the same precipitation lines of St.T 73, were measured. It was shown from a consideration of the antigens numbered 4, 7, 9, 10, 11, 13, 15, 18, 22, 23, 25, that the different supernatants obtained by repeated extraction procedures had the same concentration, with the exception of that from the first extraction in the glass, which contained about 80 per cent of this amount. The different batches of timothy pollen antigen were investigated in the same way and showed a  $100 \pm 6$  per cent day to day reproducibility. Their precipitation patterns were quantitatively identical.

### *Easily Extractable Antigens from Timothy Pollen*

In order to examine which antigens are most rapidly extracted, crossed immunoelectrophoresis was performed on timothy antigen samples subjected to extraction for varying lengths of time (Fig. 4). The amount of antigen in the extract, relative to that of St.T 73 (= 100 per cent), was estimated by area measurements. After 8 h, 50 per cent RAST inhibition was obtained. Seventeen antigens were identified, including all those having affinity for IgE from a timothy allergic patient (H.D.) (18). Simple extraction was also carried out using saline solution in place of the normal buffer. 40 per cent RAST inhibition was then obtained after 8 h.

### *Stability of Extracts*

In order to examine the stability of St.T 73, the RAST timothy activity was measured as a function of the storage time at different temperatures. The stability was expressed as the time which passed before the RAST-inhibition decreased to 50 per cent. Fig. 5 shows that 50 per cent RAST-inhibition was obtained after about 160 d, 11 d, 3 h, 13 min and 3 min, at 4, 15, 33, 52 and 82°C, respectively.

Crossed immunoelectrophoresis was performed to get an overall picture of the qualitative changes. The immunoelectrophoretic precipitation pattern shown in Fig. 6 demonstrates that St.T 73 undergoes considerable changes during storage at 4°C.

*Dialysis, ultrafiltration and lyophilisation* were performed on St.T 73. Using CIE and RAST-inhibition, the influence of these physical treatments on the antigen composition and the total allergenicity was examined. It was found that the anti-

Fig. 4.

Simple extraction of different timothy pollen antigens as a function of time. The amount of antigen extracted is given as a percentage of the amount of the same antigen in St.T 73, calculated from the measured areas. The allergenic activity (INH per cent) as a function of time is indicated below.



TABLE 1

*Characterization of 28 Identified Antigens from Timothy Pollen Extract (St.T 73).*

| Antigen No. | Migration <sup>a)</sup> | Extractability <sup>b)</sup> | Stability within 12 h at <sup>c)</sup> |     |     |     |     | Approximate molecular weight (Dalton) <sup>d)</sup> |
|-------------|-------------------------|------------------------------|----------------------------------------|-----|-----|-----|-----|-----------------------------------------------------|
|             |                         |                              | 4°                                     | 15° | 33° | 52° | 82° |                                                     |
| 1           | 1.65                    | ×××                          | -                                      | -   | -   | -   | -   | $3 \times 10^4$ - $10^5$                            |
| 2           | 1.45                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 3           | 1.40                    | -                            | -                                      | -   | -   | -   | -   | $< 10^4$                                            |
| 4           | 1.30                    | ×                            | -                                      | -   | -   | -   | -   |                                                     |
| 5           | 1.30                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 6           | 1.30                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 7           | 1.25                    | ×                            | ×××                                    | ××× | ××× | ××  | ××  | $3 \times 10^4$ - $10^5$                            |
| 8           | 1.20                    | ××                           | -                                      | -   | -   | -   | -   |                                                     |
| 9           | 1.20                    | ×                            | -                                      | -   | -   | -   | -   |                                                     |
| 10          | 1.20                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 11          | 1.15                    | -                            | ×××                                    | ××× | ××× | ××  | o   |                                                     |
| 12          | 1.10                    | ×××                          | -                                      | -   | -   | -   | -   | $< 10^4$                                            |
| 13          | 1.10                    | ×                            | ×××                                    | ××× | ××  | ×   | o   | $10^5$ - $3 \times 10^5$                            |
| 14          | 1.0                     | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 15          | 1.00                    | ×                            | ×××                                    | ××× | ×   | o   | o   | $< 10^4$                                            |
| 16          | 0.95                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 17          | 0.95                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 18          | 0.95                    | -                            | ×××                                    | ××× | ×   | ×   | o   |                                                     |
| 19          | 0.90                    | ×××                          | ×××                                    | ××× | ××  | ×   | ×   | $1.5 \times 10^4$                                   |
| 20          | 0.90                    | -                            | -                                      | -   | -   | -   | -   |                                                     |
| 21          | 0.90                    | ×                            | ×××                                    | ××× | ××× | ××  | o   | $3 \times 10^5$ - $10^6$                            |
| 22          | 0.80                    | ××                           | ×××                                    | ××× | ××  | o   | o   | $< 10^4$                                            |
| 23          | 0.80                    | ×                            | ×××                                    | ××× | ××× | ××  | ××× | $< 10^4$                                            |
| 24          | 0.75                    | ×                            | ×××                                    | ××× | ××× | o   | o   | $3 \times 10^5$ - $10^6$                            |
| 25          | 0.65                    | ××                           | ×××                                    | ××  | ××  | ×   | o   | $3 \times 10^4$ - $10^5$                            |
| 26          | 0.65                    | ×××                          | -                                      | -   | -   | -   | -   |                                                     |
| 27          | 0.40                    | ××                           | ×××                                    | ××× | ××  | ×   | o   | $3 \times 10^5$ - $10^6$                            |
| 28          | 0.35                    | ××                           | ×××                                    | ××× | ××  | ×   | o   | $3 \times 10^5$ - $10^6$                            |

a) The migration velocity relative to that of human albumin.

b) (See Fig. 4). Symbols in this column give the amount of antigen extracted after 1 h, as per cent of the amount of the same antigen in St.T 73; × means that  $\leq 10$  per cent extracted, ×× means that between 10 and 30 per cent was extracted, and ××× means that  $\geq 30$  per cent was extracted.

c) (See Fig. 5). Symbols in these columns give the amount of antigen still present as per cent of the amount of the same antigen in St.T 73; ×, ×× and ××× refer to  $\leq 10$  per cent, between 10 and 30 per cent and  $\geq 30$  per cent present, respectively.

d) Results from (15).

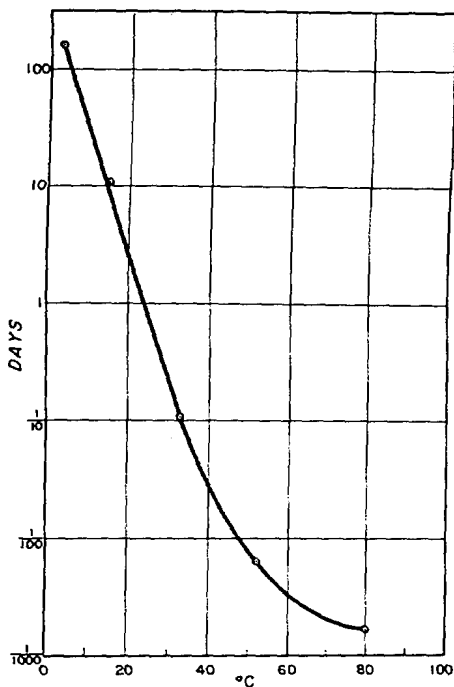


Fig. 5.

Time (in days) necessary for 50 per cent RAST-inhibition of St.T 73 as a function of storage temperature (in °C).

genic composition and allergenicity were unaffected by dialysis and lyophilisation. However, two antigens (nos. 23 and 19) partially passed through the ultrafilter employed, and the total allergenicity then decreased.

## DISCUSSION

### 1. Preparation of Extracts

One of the purposes of this work is to describe a method of preparation of an allergen extract which includes all the aller-

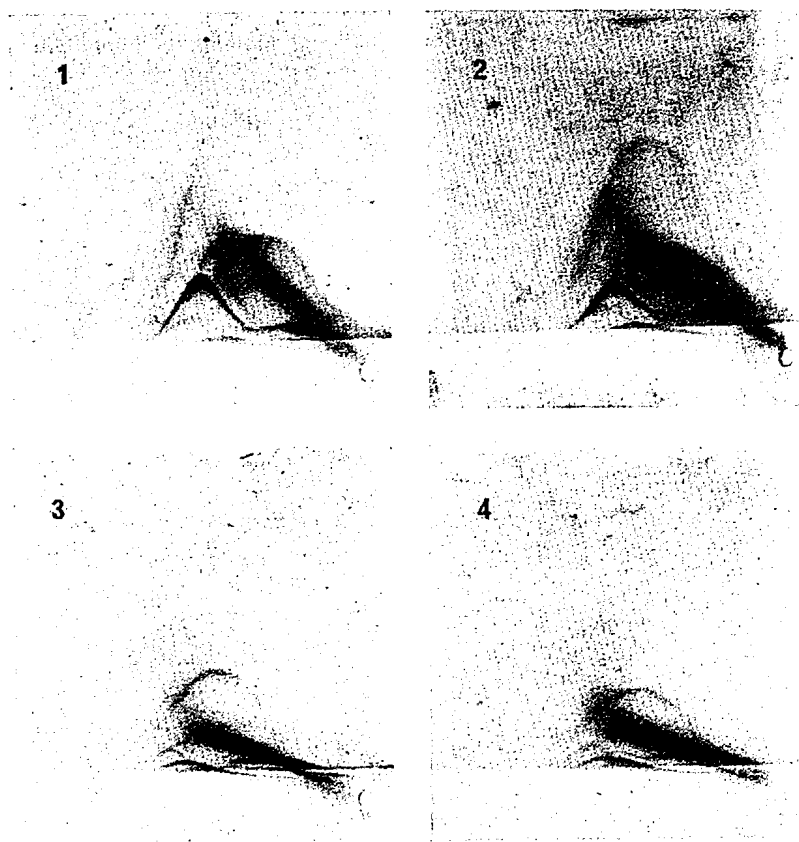


*Fig. 6.*

Crossed immunoelectrophoresis of 5  $\mu$ l of St.T 73 stored at 4°C for 1) 0 d, 2) 10 d, 3) 90 d and 4) 210 d ( $\sim$  50 per cent RAST inhibition).

gens which under normal circumstances give rise to an allergic reaction. All the allergens should necessarily be present in the extract in their native conformation. The present extraction method uses a weak buffer (pH 8.2, ionic strength 0.115), which allows direct coupling to paper discs for RAST experi-

ments. Other mild buffers ( $5.5 < \text{pH} < 8.5$ ) can be used as well. Most operations must be performed at  $0^{\circ}\text{C}$  to avoid enzymatic degradation by liberated proteolytic enzymes. To further minimize enzymatic degradation, the duration of the preparation process was made as short as possible by the use of rapid mechanical pollen grain destruction.

The method was shown by CIE to be both qualitatively and quantitatively reproducible. Simple extractions using the same buffer for varying lengths of time revealed that two antigens (nos. 19 and 26) are extracted immediately. The measured RAST-inhibition (4 per cent after 4 min) makes it reasonable to assume one or both of these antigens to be allergens. The immediate type reaction of the typical type I patients makes it further plausible that the most easily extractable antigens include the allergens which are those mainly responsible for the asthmatic reaction, i.e. they are major allergens. A similar result was obtained by Belin (8, 9, 10) in his study of birch pollen allergens. After about 1 h, all antigens with affinity for human IgE from timothy allergic patients (18) have appeared to some extent. Total RAST-inhibition of St.T 73 is obtained using an extract prepared by simple extraction for 48 h at  $4^{\circ}\text{C}$ .

## 2. Characterization of St.T 73

The CIE precipitation pattern of St.T 73 revealed 28 different antigens. Compared to human serum, many of the precipitates are rather unsharp and diffuse, an observation which is usually characteristic of glycoproteins. Molecular weight determinations (15) show that all but five antigens (nos. 3, 12, 15, 22, 23) have molecular weights between  $10^4$  and  $10^6$ . This result is in agreement with other studies (12) which demonstrated the existence of low molecular weight constituents of timothy pollen antigens. As will be shown in another study (18) antigens nos. 8, 12, 13, 15, 19, 22, 24, 25, 26, 27 and 28 may be major and minor allergens, since they have affinity to human IgE from timothy allergic patients.

St.T 73 was lyophilised without any decrease in allergenic activity or antigenic composition. Temperature stability investigations showed that St.T 73 was relatively stable at 4°C. At higher temperatures the allergenic activity decreases rather rapidly. From the form of the precipitates it appears likely that some denaturation (physico-chemical change of the tertiary structure, or enzymatic degradation) takes place. Some precipitates increase and others decrease in area because some antigenic determinants disappear, while some others, normally buried inside the molecule, appear. At 82°C, all but two antigens (nos. 7, 23) disappeared, which means that the corresponding antigens are immunochemically thermostable.

### 3. *Crossed Immunoelectrophoresis as a Tool in Allergen Extract Standardization*

The present study has demonstrated that CIE may be used for *in vitro* standardization of allergen extracts. The method is technically simple, highly reproducible, and it allows quantitative and qualitative estimation of the antigenic composition of extracts. Determination of the RAST values (20) permits estimation of the total allergenic activity, and crossed-radio-immunoelectrophoretic-analysis, CRIE (6e, 18), gives detailed information about the major and minor allergens present in the extracts. This detailed information may be obtained by RAST and SRID (single-radio-immuno-diffusion) as well, but they involve the use of isolated allergens and immunoglobulins-against-isolated-allergens.

A modification of the method allows direct comparison of the individual antigens in phylogenetically different extracts (e.g. grasses (6d)).

The prerequisites for an effective standardisation using this method are

- 1) that all pertinent allergens are present in the extract,
- 2) that precipitating antibodies are raised in rabbits by immunization with the extract, and

- 3) that sufficient rabbit antibodies are available for the standardisation work.

The present study has shown, that these conditions may be fulfilled.

#### SUMMARY

Timothy pollen extract contains 28 different antigens, as shown by quantitative immunoelectrophoresis by means of rabbit antibodies against pollen. This method, together with RAST, was used for further characterization of the extract. The standard extraction was performed as a homogenization and subsequent centrifugation at 0°C, and was reproducible. Pollen grains shaken in a weak buffer revealed some antigens to be extracted within a few minutes, while others were obtained after several hours. After 8 h, 50 per cent of the allergenic activity had been obtained. The allergenic activity and the antigenic composition of the standard extract were resistant to lyophilisation and dialysis, but not to the ultrafiltration employed. The allergenic activity and the antigenic composition of the standard extract were measured after storage at different temperatures. The standardisation of allergen extracts by means of quantitative immunoelectrophoresis is discussed.

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*Author's address:*

Henning Løwenstein  
University of Copenhagen  
The Protein Laboratory  
34, Sigurdsgade  
DK-2000 Copenhagen N.  
Denmark

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