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RAPID EXTRACTION OF GRASS POLLEN ALLERGENS AND SEPARATION OF THEIR ACTIVE FRACTIONS

By

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Methods of extracting allergens from grass pollen have never been standardised. Different extracting solutions and times of extraction have been used by investigators and vaccine manufacturers. The development of the radioallergosorbent test (RAST) (14) led to its application for assaying the potency of allergenic extracts (3), and more recently (5), 1,000-fold differences in potency have been found amongst 11 commercial ragweed extracts. A recent investigation (12) demonstrated 28 antigens in rabbit antisera to timothy grass pollen, but the antigens of importance to the allergic patient are still unknown.

Recent studies in pollen immunology (6, 8), using immunofluorescence, have shown that antigen E of ragweed is located in the inner layer of the pollen-grain wall, the intine, and in the outer exine layer, the sexine. More significantly, the release of these proteins, including antigen E, begins within 2 seconds of moistening.

We use routinely the nasal response to a measured dose of 10,000 dry grass pollen grains for diagnosis, and also as a test of desensitisation after immunotherapy (10). It is usual for the response to this challenge by a sensitive patient to be almost instantaneous, suggesting that the really important allergens are eluted at once, on contact with the moist nasal

mucosa, and that the botanical research mentioned above could be of great relevance to clinical allergy.

A pilot experiment to test this hypothesis was carried out, using a special standard prick testing needle which can carry a drop of fluid on the point. A tiny heap of dry grass pollen was placed on the skin of a sensitive subject and the needle was plunged as quickly as possible into the pollen on the skin which was immediately wiped clean by an assistant with a large water-soaked pad. With practice, the time between pricking and wiping was as short as 0.6 sec, yet a very large weal always formed. A significant amount of allergen was evidently released in less than a second, so it was decided to investigate the influence of time on the extraction of grass pollens, and also to separate active and inactive material by gel filtration, using skin tests on untreated, sensitive subjects as a guide.

MATERIALS AND METHODS

Extraction of Pollens

Dry undefatted grass pollens from the 12 common British grasses were used, see Table 1. Five per cent weight/volume extracts were made in disposable syringes using phosphate buffered saline (PBS) at neutral pH, without phenol, preservatives or detergents. The syringes were inverted mechanically every 5 sec during extraction, avoiding vigorous shaking. The extract was removed by millipore filtration at 1, 2, 5, 10, 15 and 30 min to give a primary extract. The same pollen sample was then extracted with further PBS for 24 h to give a secondary extract. Tenfold dilutions of extracts were made, with 50 per cent glycerol in PBS as a preservative, for skin and nasal testing.

Gel Filtration

Primary and secondary extracts were subjected to gel filtration on a G-75 Sephadex column (40×1.6 cm) at a flow rate of 0.75 ml/min, using PBS as eluant. One-millilitre samples of each extract were applied to the column and 3-ml fractions were collected with continuous monitoring at 280 nm.

All fractions were used for skin testing on sensitive untreated patients to locate allergenic activity. Pooled skin test active and inactive fractions of all pollens were used also for skin and nasal testing. All experiments were carried out at room temperature.

TABLE 1

Grass Pollens Used, and Approximate Molecular Weights of Major Peaks.

	Active peaks		Inactive peaks	
	1	2	1	2
<i>Dactylis glomerata</i> (Cocksfoot or orchard)	53,000	17,000	1,800	450
<i>Phleum pratense</i> (Timothy)	34,000	16,300	1,600	500
<i>Bromus mollis</i> (Brome)	27,500	13,200	1,400	350
<i>Festuca pratensis</i> (Meadow fescue)	30,000	14,000	1,800	350
<i>Lolium perenne</i> (Perennial rye)	26,000	12,300	1,900	250
<i>Arrhenatherum eliatum</i> (False oat grass)	27,500	14,000	1,800	250
<i>Agrostis tenuis</i> (Bent grass)	27,500	14,000	1,800	350
<i>Holcus lanatus</i> (Yorkshire fog or meadow velvet)	30,000	16,000	1,900	350
<i>Poa pratensis</i> (Meadow grass)	29,000	15,000	1,400	400
<i>Cynosurus cristatus</i> (Crested dog's-tail)	29,000	14,000	1,900	400
<i>Alopecurus pratensis</i> (Meadow foxtail)	25,000	14,000	1,800	400
<i>Anthoxanthum odoratum</i> (Sweet vernal)	28,500	15,000	1,900	400

Skin and Nasal Provocation Testing

Prick testing was carried out on the patient's back using a recently developed standardised needle (10). This is a precision ground instrument made from 2.5 mm diameter stainless steel rod, 3 cm long, with a 1 mm-long needle point in the centre of a flat end, which prevents further penetration of the skin. This needle can hold a drop of fluid on the point, and in use is dipped in testing extract and jabbed vertically into the skin. It produces a standard injury which also makes accurate skin titration of extracts possible.

Nasal provocation testing was carried out by introducing 50 μ l of extract, equal to the extract from 25 μ g of pollen, into one nostril, asking the patient to sniff, and observing the results. Pooled active and inactive fractions were used undiluted in 50 μ l volume.

RESULTS

Skin Tests

In 70 untreated pollen sensitive subjects, it was found that with all 12 pollens, obvious skin test activity was present in all timed primary extracts from 1 to 30 min, using testing dilutions corresponding to 0.04 μg of pollen per ml. The extracts of 1 or 2 min duration were appreciably less active, and 10, 15 and 30 min equally active; in very sensitive patients sometimes to 0.004 $\mu\text{g}/\text{ml}$.

Secondary extraction for 24 hours showed, without exception, that no further allergenic activity was left after 15 min extraction, but before that point considerable activity was still present. Skin tests were negative even to the undiluted extracts after 15 min, but it was decided that 30 min for primary extraction was a reasonable time to ensure elution of all allergenic activity.

Each 3 ml fraction from gel filtration of all 12 pollens was also used, without dilution, for skin testing in these subjects. The results are indicated in the elution profiles in Figs. 2, 3 and 4, and were found to be completely reproducible.

Pooled active and inactive fractions, undiluted, were also used for skin testing, invariably with positive results for active, and negative for inactive fractions. All tests were carried out within 7 days of preparation of extracts or fractions.

Nasal Provocation Tests

In 25 untreated subjects nasal provocation tests were carried out with crude primary and secondary extracts. All were positive to primary extracts of less than 15 min, and negative to secondary extracts of more than 15 min. Secondary extracts of less than 15 min primary extraction were always positive.

Pooled active and inactive fractions from gel filtration were also used undiluted for nasal provocation. In all 25 cases correlation was complete.

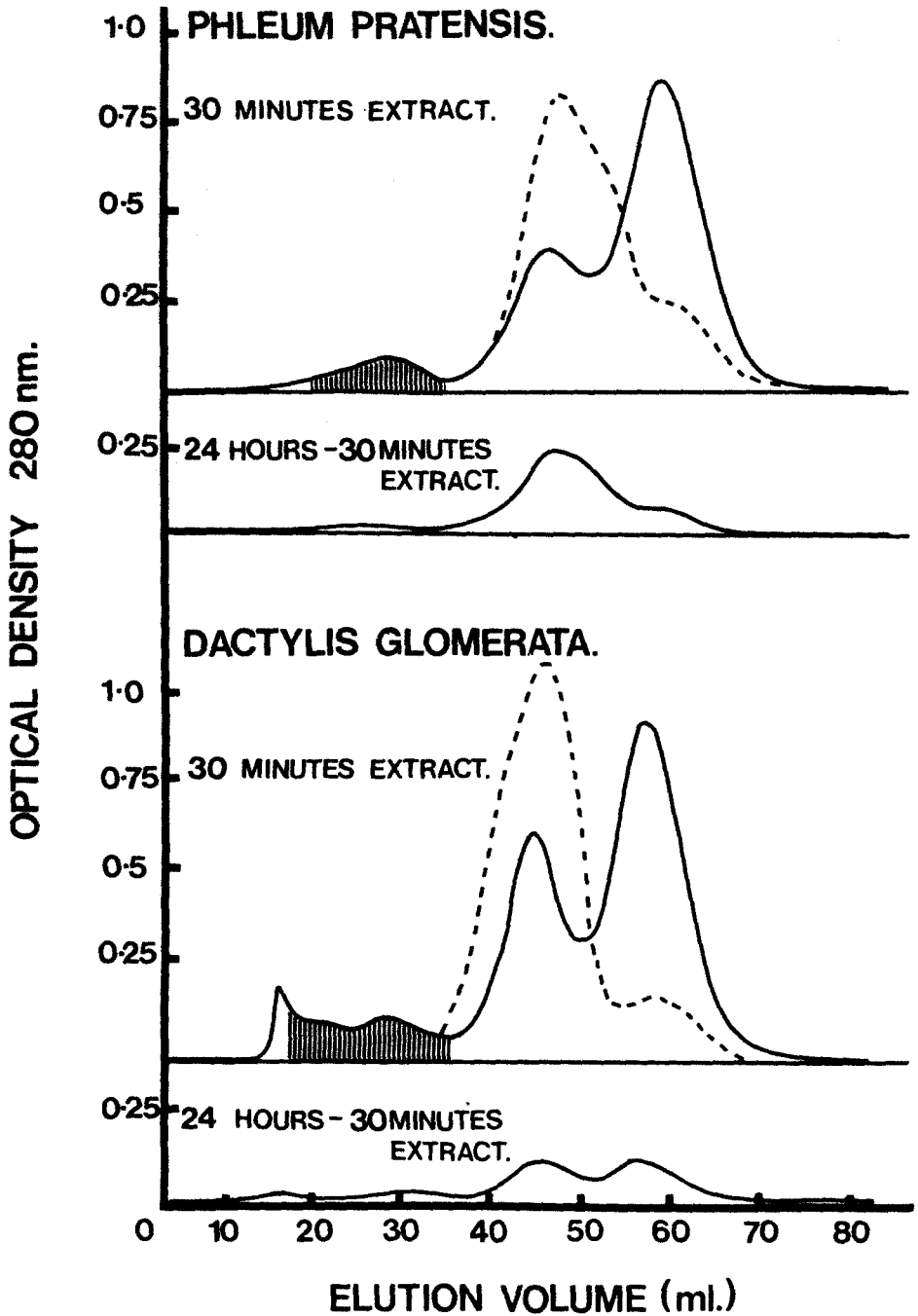


Fig. 1.

G-75 elution profiles for 1 per cent primary and secondary extracts of cocksfoot and timothy pollens. Dotted line shows profile for 7-day primary extract.

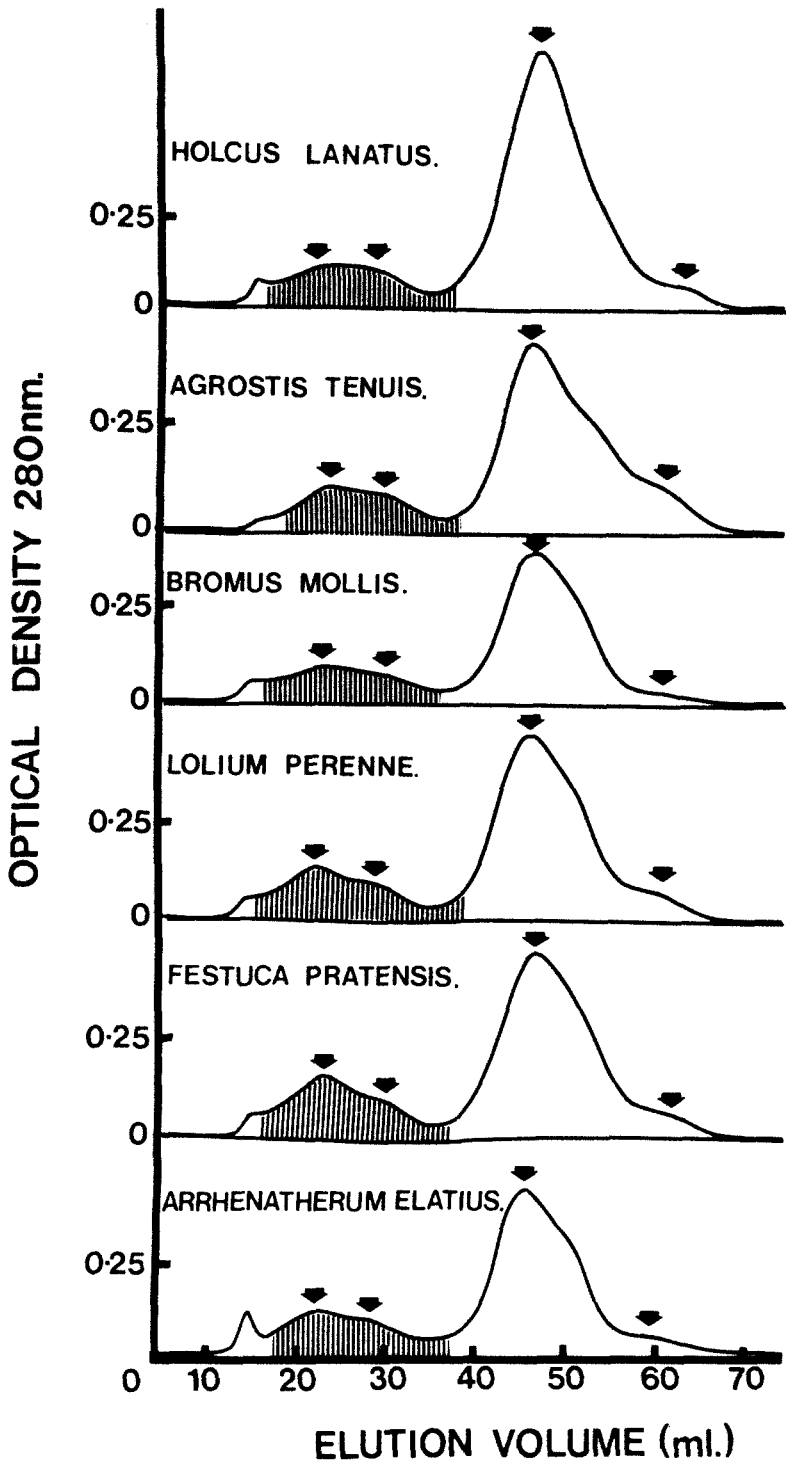


Fig. 2.

G-75 elution profiles for 30-min 5 per cent extracts for six similar pollens.

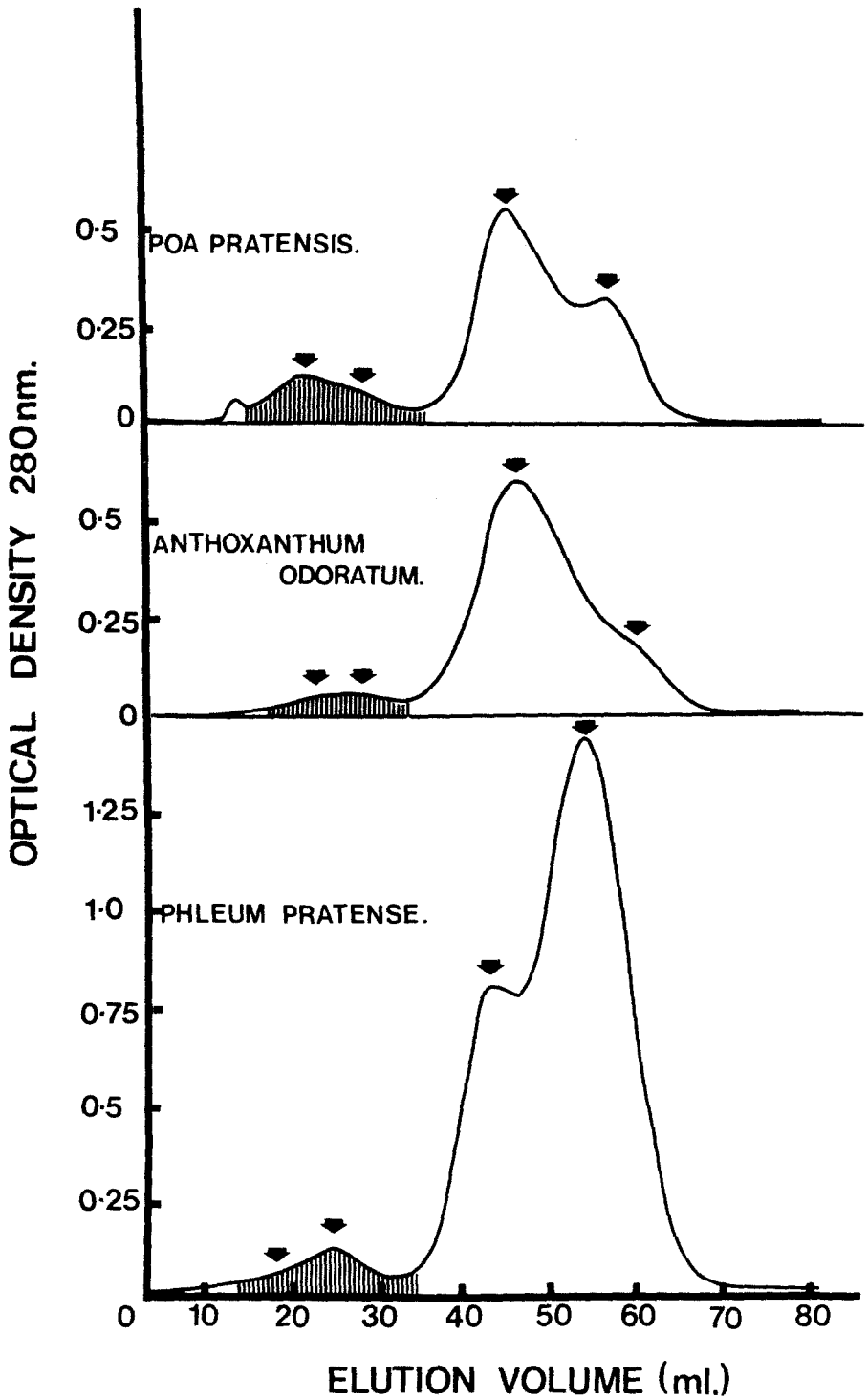


Fig. 3.

G-75 elution profiles for 30-min 5 per cent extracts of three grass pollens showing double peaks.

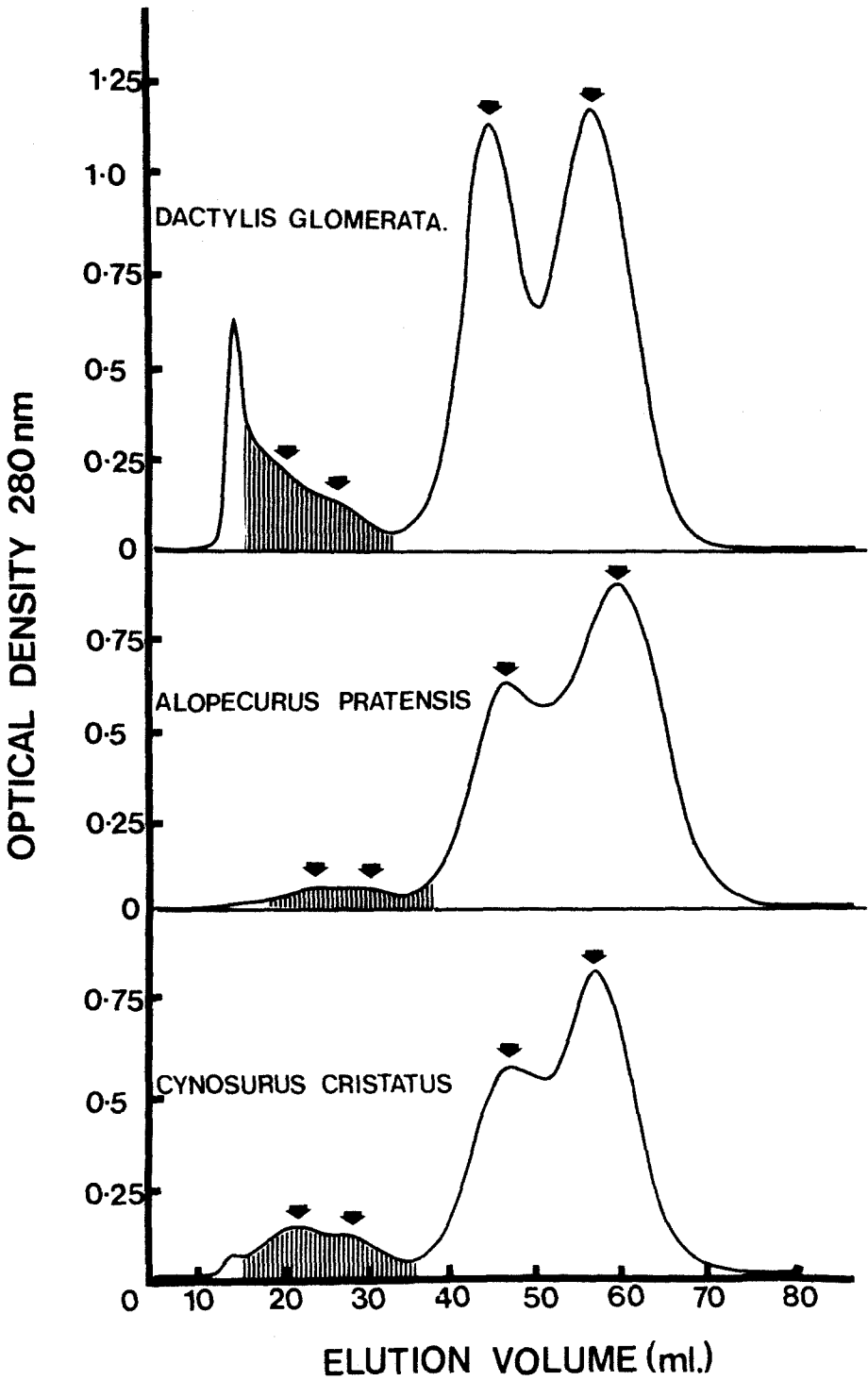


Fig. 4.

G-75 elution profiles for 30-min 5 per cent extracts of the three pollens which seem dissimilar to the others.

Gel Filtration

Elution profiles for timothy and cocksfoot pollens, using 1 per cent extracts of 30 min primary, 7 days primary and 24 h secondary extracts are shown in Fig. 1. The absence of the major protein peak and of allergenic activity in the secondary extract is well shown, and similar results were found for all 12 pollens. Preliminary work had shown similar profiles after only 1–10 min extraction, but with progressively smaller peaks in the secondary extracts, and progressive loss of allergenic activity as the primary extraction time was lengthened until no activity was left. Primary extraction for 7 days produced obvious changes in the inactive protein peaks, as shown by dotted lines in Fig. 1. These same changes were observed in a commercial treatment extract. This elution pattern on G-75 also closely resembles that obtained by Lowenstein et al. (9) for their extract of homogenised pollen.

The elution profiles for 30-min 5 per cent extracts of all 12 pollens are shown in Figs. 2, 3 and 4. Those grouped together in Fig. 2 all display remarkable similarities. In Fig. 3 it is evident that timothy pollen (*Phleum*) is markedly different, and meadow (*Poa*) also has a double peak. In Fig. 4, cocksfoot (*Dactylis*) is dissimilar, but double peaks are obvious in foxtail (*Alopecurus*) and dog's-tail (*Cynosurus*). However, apart from cocksfoot, with the spike early in elution, the obvious similarities and dissimilarities are all confined to the inactive dialysable low molecular weight regions, which are modified by prolonged extraction and seem unlikely to be important.

These patterns were reproduced many times, and skin testing revealed, as indicated by the heavy shading, that all the allergenic activity was confined to the higher molecular weight regions. This was confirmed for each pollen by nasal provocation tests in six patients using pooled active and inactive fractions.

Approximate Molecular Weights

The approximate molecular weights of the active and inactive peaks of the elution profiles were marked out using the formulae devised by Determan & Michel (4) and are shown in Table 1. The active peaks are not very obvious in the Figures, but were clearer on the original charts, and have been indicated by arrows. Although this method is only approximate, it is very clear that the prominent protein peaks of high molecular weight contain the important allergens. Protein content of pooled active and inactive fractions was estimated by spectrophotometry. It was found that only approximately 2.5 per cent of the total protein extracted from the pollen represented the active fractions.

DISCUSSION

Many investigators have attempted to isolate the allergens from grass pollen, as described in the critical review by Berrens (2). We have approached the problem in a new, but logical, manner by mimicking as far as possible what may occur when the intact dry pollen grain encounters the moist nasal mucosa of the sensitive patient. The rapidity of response to nasal challenge and the new botanical data (6, 8), showing rapid release of allergen, clearly suggested that unphysiological methods of extraction should be avoided. The use of gel chromatography as a *first* step in separation, rather than later in the process, has provided much useful information, and short extraction times avoid any danger of denaturing of proteins by enzymatic action.

The only other investigators to do chromatography on G-75 with their extract of timothy pollen are Lowenstein et al. (9). They failed to obtain the clear-cut separation of active from inactive fractions which we have found, as allergenic activity was found throughout their elution, but the major peak of activity corresponds well with our findings. Homogenisation and repeated extraction of the pollen may have released many antigens which would not normally be released

in the nose of the patient, and may be of no clinical importance.

Nielsen et al. (12) made short time extracts, and showed that only two of the 28 allergens were eluted with buffer in 2 to 8 min, and an additional nine in small amounts by 15 min, but by 8 h, 17 allergens were identified and 50 per cent RAST inhibition obtained. Weeke et al. (13) showed that only four of the 28 timothy antigens, and one especially, appeared to be important allergens.

Augustin (1), in the latest of her many publications on pollen, has rightly pointed out that treatment with pollen constituents not involved in reagin formation would be useless and irrelevant, and that only the major allergens are important both in treatment and in the establishment of standards for vaccines.

Our investigation has only produced crude, but well-defined, active fractions. Further analysis of this material should result in obtaining more clearly defined allergens. Such refinements could be very helpful in the standardisation of extracts by RAST techniques, but would probably be too expensive for commercial use in the preparation of vaccines. Pure specific allergens from different grass pollen species might improve results of specific RAST on patients' sera.

Further work on the immunological properties of rapid extracts of grass pollens is in progress.

SUMMARY

The immediate effects of nasal provocation tests with dry grass pollen, correlated with recent botanical evidence of almost instant release of allergens from the pollen wall, suggested that prolonged extraction of pollens was unnecessary and perhaps undesirable. It has now been shown that all the active allergens from undefatted dry grass pollens of the 12 major British species are extracted completely in 15 min. Gel chromatography of such rapid extracts has shown clear-cut separation of active from inactive material, as confirmed by skin and nasal provocation testing. It is probable that active

fractions thus obtained contain the only pollen allergens of importance to clinical allergy.

Further purification may produce specific allergens useful for the accurate standardisation of vaccines.

ACKNOWLEDGEMENTS

Grateful thanks are due to Allen & Hanbury Research Ltd for generous financial support, and to Bencard Ltd for supplies of grass pollens

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Subsequent experimentation has shown that the initial spike that appeared on the elution curve of dactylis used in this study does not appear with other batches of dactylis pollen. The cause for this discrepancy is being investigated.

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