

Antigens and allergens in *Dermatophagoïdes farinae* mite

I. IMMUNOCHEMICAL AND PHYSICOCHEMICAL STUDY OF TWO ALLERGENIC FRACTIONS FROM A PARTIALLY-PURIFIED *DERMATOPHAGOÏDES FARINAE* MITE EXTRACT

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Summary. The fractionation of a partially-purified extract of *Dermatophagoïdes farinae* mite culture has been undertaken by gel filtration. Two fractions were isolated. One, P₂₅, is a protein-rich fraction with mol. wt about 25,000. The other, GP₈, is a polysaccharide-rich fraction with mol. wt around 8,000. By crossed immunoelectrophoresis, we detected eleven antigens in the partially-purified dialysed *D. farinae* extract (Df 80d) as well as in P₂₅ fraction. One of them, ag 11, seems the most important allergen since in crossed-radio immunoelectrophoresis experiments it displays the faster radiostaining, implying that it binds the greatest part of the mite-specific IgE present in a pool of sera from mite-sensitive patients. By crossed-line immunoelectrophoresis, we demonstrated the absence of ag 11 in GP₈, in which only ag 5 and ag 6 were identifiable. By radioallergosorbent tests (RAST), it was found that P₂₅- and GP₈- coated paper discs can fix specific IgE induced in the majority of *D. farinae* sensitive patients. Defining a 'major allergen' as an allergen to which the majority of sensitive patients develop specific IgE, both P₂₅ and GP₈ do appear to contain at least one major allergen. By RAST inhibi-

tion method, using Df 80d as a solid phase, the allergenic activity of P₂₅ appeared as slightly higher than that of Df 80d, whereas GP₈ displayed a very weak inhibitor capacity. Thus, the allergic specificity of GP₈ differs from that of Df 80d or P₂₅.

INTRODUCTION

After the discovery of pyroglyphid mites in house dust (Voorhost, Spieksma-Boezema & Spieksma, 1964), it has been well established that *Dermatophagoïdes* and *Dermatophagoïdes farinae* (Voorhorst, Spieksma, Varekamp, Leupen & Lyklema, 1967; Miyamoto, Oshima & Ishizaki, 1969), are the main source of house dust allergens responsible for allergy such as extrinsic asthma and allergic rhinitis in many countries (Maunsell, Wraith & Cunningham, 1968; Pauli, Bessot, Guisard, Roegel & Oudet, 1972). Several extensive clinical investigations showed the existence of some relationship between allergens from these two mites and from house dust (Miyamoto, Oshima, Ishizaki & Sato, 1968; Biliotti, Passaleva, Romagnani & Ricci, 1972). The commercial mite extracts are only slightly or partially purified. Since they are used in clinical as well as in laboratory assays, it is necessary to characterize the allergenic fractions which compose these

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extracts. Several authors have isolated and characterized allergenic fractions from mite extracts (Romagnani, Biliotti, Passaleva & Ricci, 1972; Romagnani, Boccaccini, Amadori & Ricci, 1976; Nakagawa, Kudo, Okudaira, Miyamoto & Horiuchi, 1977). More recently, a major allergen of *D. pteronyssinus* was defined (Chapman & Platts-Mills, 1978, 1980). However, up to now, the isolation and total purification of the allergenic fractions of *D. farinae* mites has not been successful.

In the present work, we report the fractionation, after exhaustive dialysis, of a partially-purified extract (Df 80d) obtained from whole mite culture of *D. farinae*. By gel filtration, two different fractions were obtained. Their physico-chemical properties were determined by gel filtration and electrophoresis on cellulose acetate, and their immunochemical properties were checked by crossed-immunoelectrophoretic methods. The first fraction, P₂₅, with an approximate mol. wt of 25,000, is rich in protein but contains some polysaccharides. The second fraction, GP₈, with a mol. wt of 8000 is richer in polysaccharides than in proteins. By the radioallergosorbent test (RAST), it was found that all the sera from *D. farinae*-sensitive patients contained IgE antibodies specific to GP₈ and to P₂₅. Furthermore, RAST inhibition method revealed that the behaviour of these two fractions differed: P₂₅ inhibits Df 80d but GP₈ does not. We discuss the specificity of the latter method and its implications on the results. We have identified among eleven antigens present in the whole extract an important allergen, ag 11, which is present in P₂₅ and absent in GP₈.

MATERIALS AND METHODS

Allergens

The preparation of the partially purified *D. farinae* extract, Df 80, from a whole mite culture extract provided by Institut Pasteur (Service des Allergènes) has been performed according to the method previously described in detail for *D. pteronyssinus* (Le Mao, Weyer, Pauli, Lebel & David, 1980) and summarized in Scheme 1 (Fig. 6). The partially-purified extract, Df 80, was dissolved in neutral distilled water and exhaustively dialysed against the same medium till the optical density of the dialysate, at 280 nm, disappeared. The resulting brownish solution has been named Df 80d. It was lyophilized and constituted the starting material for further purification.

Antibodies

Immunization procedure was as follows: three rabbits of 2.5 kg received 100 mg of the partially-purified extract, Df 80d, in 9 ml of physiological saline solution, mixed with the same volume of Freund's incomplete adjuvant. The resulting emulsion was inoculated subcutaneously at ten points along the spine for one part and a 5 ml inoculation of the same emulsion was done by the intraperitoneal route. After 1 month rabbits were boosted with 10 mg of Df 80d in 1 ml of physiological saline solution, by intravenous injection. This was repeated 1 month later. One week after the last booster they were bled. Sera were harvested, pooled and concentrated four times.

Gel filtration

One and two-tenths millilitres containing 60 mg (in protein) of *D. farinae* extract was applied to a 100 × 1.6 cm column of Sephadex G 100 (Pharmacia, Uppsala, Sweden) at 4°. The gel was previously equilibrated with 10 mM phosphate buffer pH 7.2 ± 0.02 containing 1 M NaCl. This high ionic strength avoided electrostatic interactions. The column was eluted with the same buffer at a flow rate of 18 ml/hr and 3.0 ml fractions were collected. The absorbance of each fraction was measured at 280 nm. The eluted fractions were then pooled according to the elution pattern.

Estimation of molecular weights

Molecular weight determinations of fractions were performed by using in the same Sephadex gel column tracers of known molecular weight (blue dextran 2,000,000, Ovalbumin 44,000, α chymotrypsin 23,500, Lysozyme 15,000 and Phenol red 600).

Electrophoresis on cellulose acetate

Electric voltage of 10 V/cm was applied during 60 mn, in a 0.1 M phosphate buffer pH 7 ± 0.02. Coloration was obtained using Ponceau 'S' for proteins and Alcian blue for polysaccharides.

Estimation of protein concentration

Protein content was quantified on fractions as described by Lowry, Rosenbrough, Farr & Randell (1951) using Folin-Ciocalteu reagent.

Estimation of reducing sugars concentration

Total hexose determination was done on fractions using the Orcinol method.

Crossed immunoelectrophoretic methods

Crossed immunoelectrophoresis (CIE) was performed as described by Weeke (1973) and crossed-line immunoelectrophoresis (CLIE) by Krøll (1973).

CIE was carried out on 7 cm × 10 cm glass plates (gel dimensions: 2 × 7 cm², 8 × 7 cm² for the first dimension and the anodic antibody containing gels, respectively). Ten to twenty microlitres of each fraction was applied in the well. First and second dimension electrophoresis were performed under 20 V per cm for 30 min and 2 V per cm for 18 hr, respectively. The temperature of the cooling water was 15°. Specified amounts of rabbit antiserum were included in the second dimension electrophoresis gel. Agarose gels were prepared as 1% solution in Tris-veronal buffer, pH 8.7 (73 mM Tris, 24.5 mM veronal, 0.36 mM calcium lactate and 0.2 mM sodium azide). After electrophoresis the gels were pressed, washed and pressed again, dried in hot air and then stained in a solution consisting of Coomassie brilliant blue 'R 250', 5 g/l in ethanol:acetic acid: water (9:2:9). The stained gels were destained in ethanol:acetic acid: water (5:2:9) and dried again.

CLIE was performed on 7 cm × 10 cm glass plates (gel dimensions: 2 × 7 cm², 1 × 7 cm², 7 × 7 cm² for the first dimension gel, the intermediate antigen-containing gel and the anodic antibody-containing gel, respectively), applying in the well 10–20 µl of the total extract, and the antigen(s) to be examined in the intermediate gel (1 cm wide).

Crossed-radio immunoelectrophoresis (CRIE) was performed as described by Weeke, Lowenstein & Nielsen (1974). After CIE (on 7 × 10 cm plates) the unstained gels were incubated with the ten-fold diluted mite serum pool (described in RAST inhibition part) overnight at room temperature. After washing, ¹²⁵I-labelled rabbit anti-IgE (kindly supplied by Pharmacia, France), diluted in incubation buffer (phosphate buffer 50 mM pH 7.5 containing 0.3% bovine serum albumin, 150 mM sodium chloride, 15 mM sodium azide, 2.7 mM Na₂EDTA, 1% Tween 20) to give 5000 c.p.m. per 50 µl in our gamma counter, was added. After an overnight incubation, the gels were washed, dried in cold air and placed in an X-ray cassette, covered with a Kodak X-Omat film and an intensifier screen. They were exposed several days at -20°. The specificity of the radiostaining was checked by using pool sera from atopic or non-atopic subjects containing respectively high or negligible amounts of IgE as determined by RAST.

Radioimmunoassays

Radioallergosorbent test (RAST). The *D. farinae* extract Df 80d or its different fractions were coupled to CNBr-activated paper discs as described by Ceska, Eriksson & Varga (1972). The RAST was performed according to the principle described by Wide, Bennich & Johansson (1967). Serum (50 µl) was incubated with an allergen-coated paper disc 3 hr at room temperature. Then the disc was washed and 50 µl ¹²⁵I anti-IgE (Pharmacia, France) were added. After an overnight incubation the disc was washed and counted in a gamma counter for bound radioactivity. The results were expressed as percentage of bound radioactivity.

RAST-inhibition. The experiments were carried out essentially as previously described by Le Mao *et al.* (1980). The mite serum pool consisted of equal volumes of serum from sixteen non-hyposensitized subjects who were highly allergic to *D. farinae* mites. The paper discs used were coated with the Df 80d extract, constituting reference discs. The assays were performed as follows: 100 µl of serial dilutions of the extract or of its fractions were preincubated 3 hr at room temperature with 100 µl of ten-fold diluted *D. farinae* serum pool. Then, these mixtures were used for RAST on reference discs. The point 0% of inhibition was obtained by incubating the serum pool with 100 µl of phosphate-buffered saline. Each inhibition point was performed in duplicate. A dose-response curve was defined plotting the percentage RAST inhibition versus the varying quantities of the inhibiting extracts. From the inhibition curves, the quantity of each extract or fraction required to obtain 50% inhibition was determined (C 50).

RESULTS

Fractionation of the partially purified extract Df 80d by gel filtration

An aliquot of the exhaustively-dialysed Df 80d extract was applied to a Sephadex G 100 column prepared as described in Materials and Methods. The elution pattern (Fig. 1) shows that it occurs mainly as two fractions, named F_I and F_{II}; the first peak being excluded as quantitatively insignificant. Elution volume of each of these fractions has been compared with that of known markers. An approximate mol. wt of 25,000 was attributed to F_I and 8000 to F_{II}. From this elution pattern (Fig. 1), taking into account the overlapping of the two main peaks which is indicated

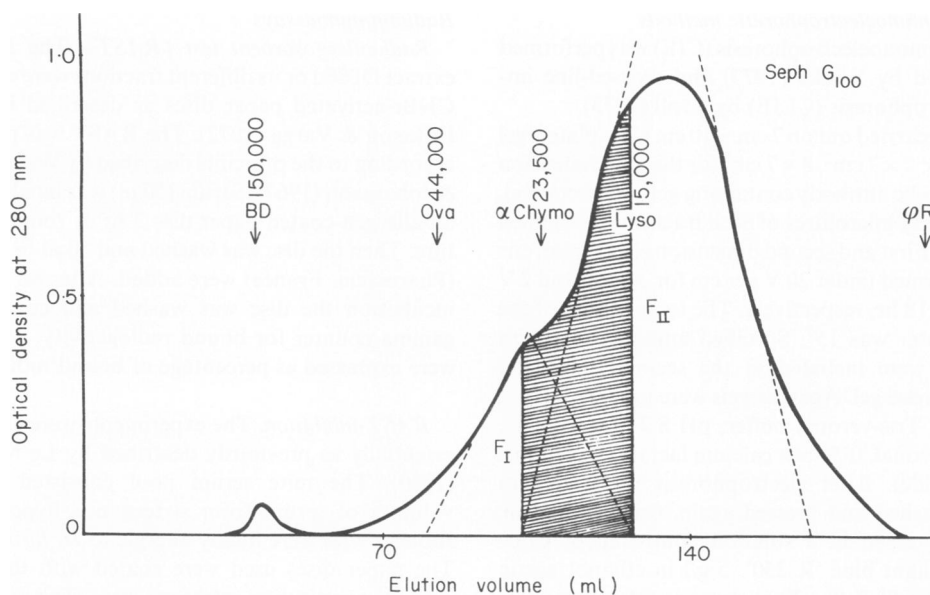


Figure 1. Elution pattern obtained after chromatography of Df 80d on Sephadex G 100 (column 1.6×100 cm, elution with 1 M NaCl phosphate buffer, pH 7.2 ± 0.02 , flow rate 18 ml/hr, 3 ml fractions). Overlapping of the two main peaks is indicated as a hatched area.

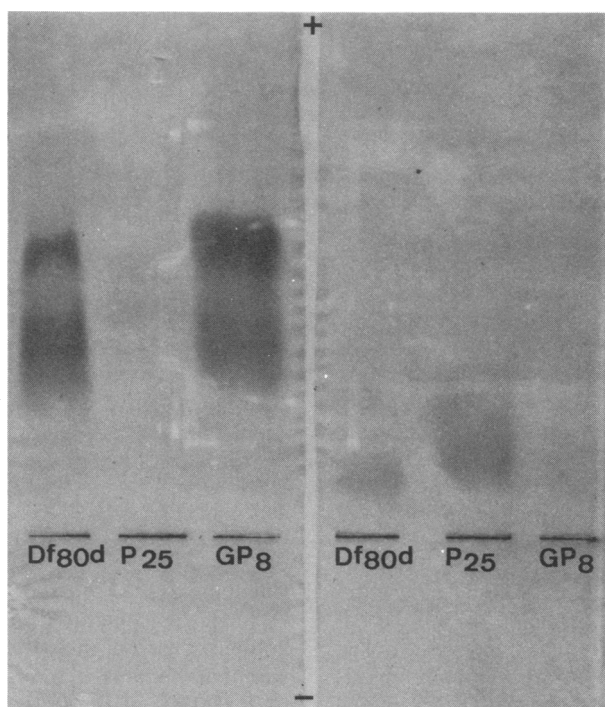


Figure 2. Electrophoretic patterns of different allergenic fractions on cellulose acetate strips ($5 \text{ cm} \times 16 \text{ cm}$, 10 mM phosphate buffer, pH 7 ± 0.02 , 10 V per cm, 60 mn); $50 \mu\text{g}$ (in protein) of each fraction were applied.

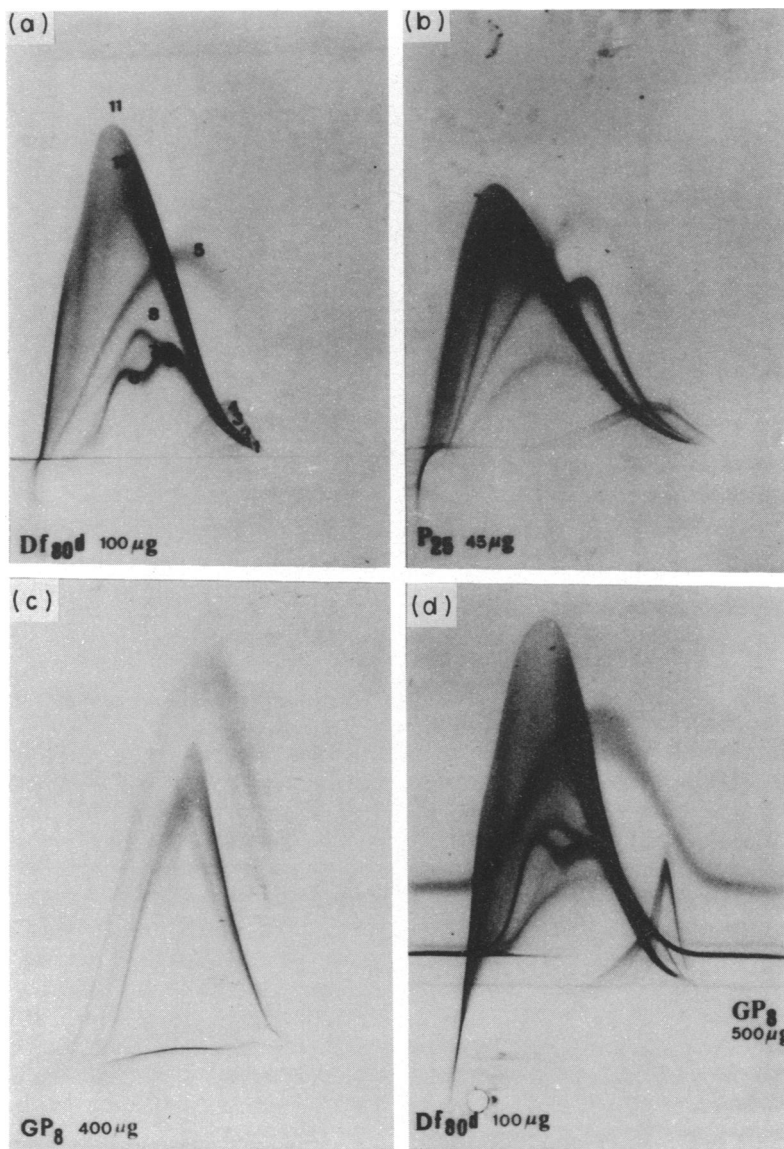


Figure 3. Crossed immunoelectrophoretic patterns of partially-purified *D. farinae* extract (a); *P*₂₅ fraction (b); *GP*₈ fraction (c); crossed-line immunoelectrophoresis for identification of antigens in *GP*₈ (d).

by a hatched area, only fractions before and after this hatched area were pooled, dialysed and freeze-dried before further characterization. Referring to the estimated molecular weight of the fractions and according to their glycoprotein nature (the ratio of sugars to proteins is 38% for Df 80d, 20% for F_I and 94% for F_{II}) we named P₂₅ the protein-rich fraction I and GP₈ the polysaccharide-rich fraction II.

Electrophoresis on cellulose acetate

These fractions were studied by electrophoresis on cellulose acetate strips (Fig. 2). One strip was stained by Ponceau 'S' for proteins and the other by alcyan blue for polysaccharides. It appears that no overlapping exists between protein and polysaccharide bands in the three fractions.

Immunochemical study of Df 80d and fractions P₂₅ and GP₈ by crossed immunoelectrophoretic methods

Using a pool of rabbit antisera against Df 80d, preliminary CIE experiments with Df 80d failed to

show cathodal protein bound in immunoprecipitates. All subsequent CIE analyses were carried out with anodal antibody-containing gel. Eleven antigens were revealed in the partially-purified extract Df 80d (Fig. 3a). The brownish band of rapidly migrating material characterized as polysaccharidic by metachromatic staining in analytical electrophoresis on cellulose acetate did not give any visible precipitin line.

In CRIE experiments (Fig. 4), a very marked uptake of radioactivity on the precipitin line corresponding to the antigen numbered 11 (ag 11) was obtained with mite-specific IgE compared with the normal serum control suggesting that ag 11 was the most important allergen in our *D. farinae* extract, Df 80d. Antigen 11 was present in P₂₅ (Fig. 3b) but absent from GP₈ (Fig. 3c; as it can be stated by crossed-line technique using GP₈ in the intermediate gel to compare it with Df 80d, Fig. 3d). Furthermore, crossed-line immunoelectrophoresis showed that only ag 5 and ag 6 were identifiable in GP₈ (Fig. 3d). Comparing the different fractions in CIE, we must add that to obtain a similar development of the antigen patterns it was necessary to use 100 µg of Df 80d but only 45 µg of P₂₅, to identify the same antigens, especially ag 11. It was necessary to put 400 µg of GP₈ to reveal antigens ag 5 and ag 6.

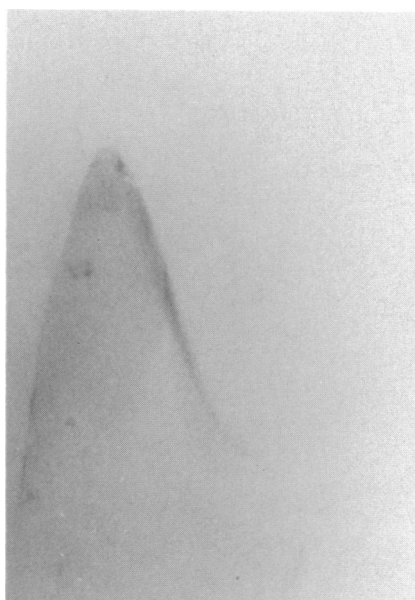


Figure 4. Crossed-radio immunoelectrophoresis pattern of *D. farinae* extract: after CIE of 100 µg (in protein) Df 80d, pool of sera from mite-sensitive patients was incubated to the native immunoprecipitates. After a second incubation with ¹²⁵I-labelled rabbit anti-human IgE, autoradiography was carried out for 2 days.

Allergenic properties of fractions from Df 80d in RAST techniques

Using the RAST inhibition assay, we studied the ability of the Df 80d extract and of its subfractions to inhibit the binding of mite-specific IgE from a pool of

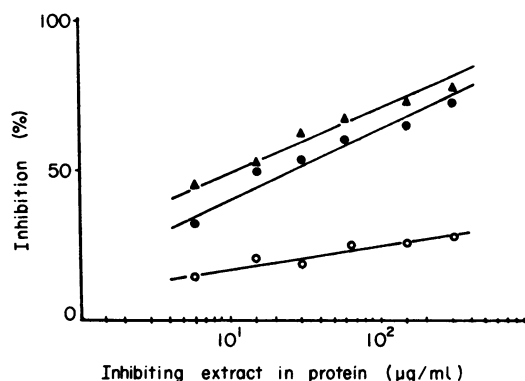


Figure 5. RAST inhibition curves to examine allergenic activity of the partially purified *D. farinae* extract, Df 80d (●—●) and its subfractions P₂₅ (▲—▲) and GP₈ (○—○) with solid phase of Df 80d.

patients' sera to Df 80d coated paper discs. The RAST inhibition results are shown on Fig. 5. A 50% inhibition (C 50) was obtained with about 26 µg of protein from Df 80d, and with 15 µg of protein from P₂₅. The fraction GP₈ gave only slight inhibition and the C 50 could not be determined. RAST is currently used both to determine the presence of allergen-specific IgE antibody in patients' sera and to test the reactivity of an allergenic extract or fraction coated on paper discs. We examined here seventeen sera from mite-sensitive patients. All of them reacted with P₂₅ and GP₈ fractions coated on paper discs as shown in Table 1. The RAST data on a control serum pool obtained from non-allergic individuals are also presented.

DISCUSSION

The results reported here show the existence of two allergenic fractions P₂₅ and GP₈ in a *D. farinae* extract, separated and characterized by gel filtration on Sephadex G 100, CIE and radioimmunoassays. The

results of gel filtration reported by previous workers (Romagnani *et al.*, 1972; Miyamoto *et al.*, 1969; Nakagawa *et al.*, 1977) suggested that the most potent allergenic fraction in *D. farinae* has a mol. wt between 10,000 and 69,000, most likely around 30,000. Chapman & Platts-Mills (1978), from a *D. pteronyssinus* culture extract fractionated by chromatography on Sephadex G 100, obtained a partially-purified allergen (F₄P₁) with a mol. wt between 15,000 and 25,000. Similarly, in our work, we obtained from a partially-purified extract of *D. farinae*, a fraction P₂₅ with a mol. wt of 25,000 richer in proteins than in reducing sugars. Moreover, a second fraction GP₈ with a mol. wt of 8000 was obtained richer in reducing sugars than in proteins, as was previously described by Holford-Strevens, Wide, Milne & Pepys (1970). Both protein and reducing sugar determination confirm the suggestions that the allergen material from *D. farinae* extracts contains a high percentage of polysaccharides, the majority of which is not bound to the protein moiety, as it has been shown by analytical electrophoresis (Fig. 2). But we cannot neglect the possibility that allergen might be glycoprotein or/and proteoglycans. We observed for *D. farinae* that the large peak of rapidly migrating material in first dimension in CIE, essentially polysaccharide, does not react with rabbit antiserum. Chapman & Platts-Mills (1978) quoted the same observation in *D. pteronyssinus*.

The combination of CIE methods with gel filtration on Sephadex G 100 improved the study of our *D. farinae* allergenic extract. Of the many authors who have attempted to fractionate mite extracts by gel filtration and to characterize the antigens obtained (Biliotti, Romagnani & Ricci, 1975; Nakagawa *et al.*, 1977; Chapman & Platts-Mills, 1978), only Lind (1980) using CIE and CRIE methods published results concerning qualitative and quantitative investigations of three preparations from *D. pteronyssinus*. The main advantage of CIE over methods such as double diffusion in agar gel and monodimensional immunoelectrophoresis is that one antigen can be identified among many others, without being previously isolated, as was shown for ag 11. Mainly using the CLIE method, we demonstrated (Fig. 3d) the absence of ag 11 in GP₈.

The analysis of the allergenic properties of the two fractions P₂₅ and GP₈ was performed by RAST-based methods, using human-specific IgE. The estimation of the allergenic activity of different extracts, based on 50% inhibition of RAST, is only valid for extracts having identical qualitative composition, see Lowen-

Table 1. Allergenic reactivity* of P₂₅ and GP₈ fractions against sera from seventeen mite sensitive patients (RAST)

Serum	Fraction coated on paper discs	
	P ₂₅	GP ₈
1	34.7	18
2	23.1	13.2
3	45.4	29.3
4	46.6	37.9
5	37.8	16.7
6	50.5	36.7
7	34.9	21.5
8	49.2	43.5
9	51.2	51
10	35	17.4
11	35.8	25.2
12	51.9	46
13	47.8	49
14	40	29.9
15	44.5	37.7
16	27	11.5
17	21.2	13.1
Control†	1.1	1

* Expressed as percentage of fixed radioactivity.

† Control, serum pool from non-allergic individuals.

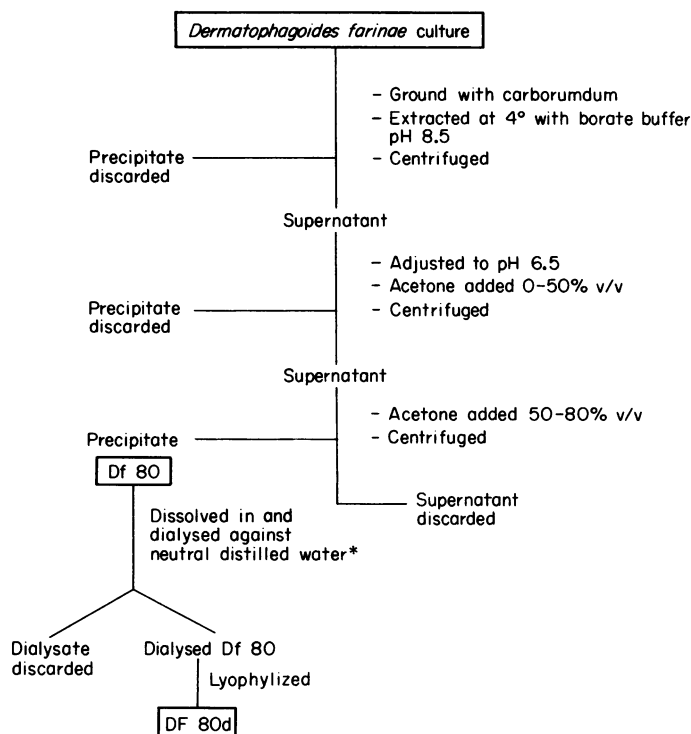


Figure 6. Scheme 1: preparation of the partially-purified extract from *D. farinae* mite culture.

* In dialysis tubing with a cut off comprised between mol. wt 6000 and 8000 .

stein (1978). So, in our study (Fig. 5), the comparison of the allergenic activities of various fractions is valid only for P_{25} fraction versus Df 80d as solid phase, when looking at the RAST inhibition curves obtained with Df 80d or P_{25} as soluble inhibitors. The allergenic activity of P_{25} is slightly higher than that of Df 80d and their slopes are identical, revealing a large identity between the qualitative composition of P_{25} and that of Df 80d. As far as GP_8 fraction is concerned, we found that whatever the dose, its inhibiting capacity against Df 80d remained very low and that it gave an inhibition curve with a rather different slope. This last observation, in agreement with Adolphson, Goodfriend & Gleich (1978), leads to the conclusion that the antigenic composition of GP_8 differs from that of Df 80d or P_{25} , as shown by CLIE (Fig. 3d). Thus it would be almost impossible to use RAST inhibition to determine the allergenic activity of GP_8 compared with Df 80d. Fraction GP_8 contains only ag 5 and ag 6, but not ag 11, while P_{25} and Df 80d mainly contain ag 11 among others.

In direct RAST experiments (Table 1), the allergenic

reactivity of P_{25} is in agreement with the results obtained in RAST inhibition experiments. The allergenic reactivity of GP_8 is also well existing (Table 1). But how to explain the discrepancy between the results obtained by RAST inhibition and direct RAST for GP_8 ? Direct RAST detects, in a population of atopic patients, the specific IgE antibody against disc-bound allergens. Antigens which are present as a small proportion of an allergenic extract can be sensitizing and can induce specific IgE antibody. It has been well established that, in the animal, repeated injections of minute doses of a given allergen, are necessary to obtain marked specific IgE antibody production (Vaz & Levine, 1970). Because GP_8 contains only minute quantities of ag 5 and ag 6, it displays a very weak activity in RAST inhibition with Df 80d as solid phase. However, in direct RAST fraction GP_8 reacted with a majority of the seventeen patients sera tested, suggesting that these antigens, ag 5 and ag 6, can sensitize a large part of the human population. By defining a 'major allergen' in terms of a high frequency of positive reactions between defined allergens and sera

from sensitized patients, GP₈ appears to contain at least one 'major allergen' which differs from the other 'major allergen' ag 11, present in P₂₅. Moreover, ag 11 which is present in Df 80d as well as in P₂₅, can be considered not only as a 'major allergen' but also as the most important allergen, as it has been revealed by CRIE experiments in which the greatest part of specific IgE antibodies from the pool of sensitive patient sera was bound to ag 11.

Antigen 11 is now under analysis in our laboratory.

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