

© PHYSICOCHEMICAL AND IMMUNOCHEMICAL CHARACTERIZATION OF THE ALLERGENS FROM THE MITE *DERMATOPHAGOIDES PTERONYSSINUS*

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Summary. The physicochemical and immunochemical characteristics of the allergens from *Dermatophagoides pteronyssinus* have been described, in particular those for the allergens designated Dpt 4 and Dpt 12. Dpt 4 is a high molecular weight allergen with an apparent molecular weight of 274,000 daltons and a pl of 4.5. In contrast, the antigenically unrelated Dpt 12 has an apparent molecular weight of 24,000 daltons and a pl 6-6. Both allergens demonstrate heterogeneity, either in molecular weight or pl. Dpt 12 appears to be the major allergen in extracts of *D. pteronyssinus* and probably corresponds to the low molecular weight allergen previously described by other workers.

INTRODUCTION

In recent years tremendous advances have been made in the field of allergen chemistry due primarily to the development of quantitative immunoelectrophoretic techniques and their combination with conventional physicochemical techniques (reviewed by Løwenstein, 1978). These techniques have enabled workers to elucidate both the physicochemical characteristics and allergenicity of components contained within mixtures without recourse to complex isolation procedures. Further, it has been possible to use these techniques to devise optimum procedures for the isolation of individual allergens.

These techniques have been employed to determine the number and characteristics of potential allergens in extracts of animal dander (Blands, Løwenstein and Weeke, 1977), timothy grass pollen (Løwenstein, Nielsen and Weeke, 1974) and *Cladosporium herbarum* (Løwenstein, Aukrust and Gravesen, 1977). However, they have not been extensively applied to the study of the allergens from the mites *Dermatophagoides pteronyssinus* and *D. farinae*, allergens which have been shown to be of major importance (Morrison-Smith *et al.*, 1969; Miyamoto *et al.*, 1968) in respiratory allergy.

In this and the following report the physicochemical and immunochemical characteristics of some of the allergens from *D. pteronyssinus* are described, and the results compared with those from other studies. In particular, the high

molecular weight allergens known to react with the lectins Concanavalin A (Con-A) and tridacnin and with the S107 IgA myeloma have been studied (Baldo and Uhlenbruck, 1977; Baldo, Turner and Uhlenbruck, 1976; Stewart, McWilliam and Turner, 1980) and compared with the low molecular weight allergens (Ishii *et al.*, 1973; Biliotti, Romagnani and Ricci, 1975; Nakagawa *et al.*, 1977; Chapman and Platts-Mills, 1978).

MATERIALS AND METHODS

Preparation of mite extracts

House dust mites, *D. pteronyssinus*, and the media upon which they were grown were kindly provided by two different manufacturers designated sources 1 and 2 (Stewart *et al.*, 1979). Soluble components were extracted by grinding the mites or media in 0.1 M Tris-HCl buffer, pH 7.4, as described by Ishii *et al.* (1973). The extract was clarified by centrifugation at 15,000 g for 15 min and the supernatant was dialysed in 8/32" Visking tubing against four changes (each of 5 litres) of distilled water at 4°, lyophilized and stored desiccated at -20°. The majority of the work to be described in this report was undertaken using an extract prepared from source 1 material.

Analytical methods

Protein and carbohydrate content of mite extracts and gel filtration column effluents were determined using the Lowry modification of the Folin assay (Lowry *et al.*, 1951) and the phenol-sulphuric acid assay (Dubois *et al.*, 1956) respectively. The primary standards used in these assays were bovine serum albumin (Miles Laboratory) and D-glucose (B.D.H. Ltd.).

Anti-D. pteronyssinus antisera

Rabbits were immunized on day 0 with 5 mg of whole *D. Pteronyssinus* extract emulsified with Freund's complete adjuvant. They were boosted 14 days later with 1 mg of extract emulsified with Freund's incomplete adjuvant and boosted with a similar dose of antigen 2 months later. Sera were tested by crossed immunoelectrophoresis and immunoelectrophoresis.

RAST

RAST assays were performed essentially as described by Wide, Bennich and Johansson (1967) using ¹²⁵I anti-human IgE (Pharmacia). Allergens were coupled to CNBr activated paper discs as described by Ceska, Eriksson and Varga (1972) and the concentration of allergen coupled expressed in µg of protein/ml. Sera from allergic patients with both clinical and laboratory evidence of hypersensitivity to *D. pteronyssinus* were pooled to provide a reference standard for RAST. The pool, which had a serum IgE level of 1,200 U/ml, was sterilized by filtration and stored frozen at -20° in suitable aliquots until required for assays.

Double diffusion and immunoelectrophoretic analyses

Double diffusion analysis (Ouchterlony, 1958) was performed in 1.5% agarose (Type 1, Sigma Chemical Co. Ltd.) in barbitone buffer pH 8.6 (I = 0.05). Immunoelectrophoresis (IEP) was performed in 1.5% agar (Difco Noble) in the same barbitone buffer according to the method of Grabar and Williams (1955). Both double diffusion and IEP plates were developed at 4° overnight. After washing, the plates were pressed and dried according to the method of Laurell (1972) and stained with Beibrich Scarlet (Crowle and Cline, 1977) purchased from Searle Diagnostic.

Crossed immunoelectrophoresis (CIE) and crossed radioimmunoelectrophoresis (CRIE) were performed essentially as described by Löwenstein *et al.* (1977) using type 5 agarose (Sigma Chemical Co. Ltd.) and rabbit antibody at the indicated concentrations. The CIE plates were incubated for 24 h at room temperature with the *D. pteronyssinus* allergic human serum pool described for RAST. The plates were washed × 4 with 0.15 M NaCl and then incubated with ¹²⁵I anti-IgE. Small plates (7 × 5 cm) were incubated with approximately 250,000 c.p.m in 7 ml incubation buffer, whereas large plates (14 × 10 cm) were incubated with a proportionally larger amount of both allergic serum and RAST reagent.

Plates were washed $\times 4$ with 0.15 M NaCl and then dried. Autoradiography was performed using Ortho-G (Kodak) X-ray film. Exposure times varied between 1-42 days. Fused rocket immunoelectrophoresis (FRIE) was performed as described by Weeke (1973).

Gel-filtration

Extracts of *D. pteronyssinus* were fractionated on a column (84 $\times$ 1.6 cm) of Sephacryl S-300 (Pharmacia) equilibrated with 0.1 M tris-HCl (pH 8.0) containing 0.15 M NaCl. The flow rate was 20 ml/h and 3 ml fractions were collected. The column effluents were monitored at 280 nm and by FRIE (Lowenstein, 1978).

Molecular weight determination

The apparent molecular weights of the components in the *D. pteronyssinus* extracts were determined by gel-filtration (Andrews, 1970) on Sephacryl S-300 using the conditions described above. The molecular weight markers were purchased from Pharmacia. Molecular weights were determined by linear regression analysis.

Polyacrylamide gel electrophoresis (PAGE)

Gradient PAGE was performed essentially as described by Margolis and Kenrik (1967) using the Pharmacia gel electrophoresis apparatus GE-4 and pre-poured gradient gels (PAA 4/30). Electrophoresis was performed according to the manufacturer's instructions. Molecular weight markers were purchased from Pharmacia. Gels were stained for protein with Coomassie blue G-250 (Blakesley and Boezi, 1977) and for carbohydrate using periodic-acid Schiff's reagent (PAS) as described by Clarke (1964). Molecular weight determinations using this technique were performed at least three times and the data analysed by linear regression.

Conventional PAGE was performed at 3 mA per gel using 12% acrylamide gels (Davis and Orstein, 1968) in either rod or slab form. Gels were stained as described above. Both gradient and conventional PAGE gels were scanned at 650 nm using a Gelman ACD-15 densitometer.

Combined analytical and immunoelectrophoretic techniques

Isoelectrofocusing (IEF) was performed using LKB Ampholine^(R) PAGE plates, pH 3.5-9.5, according to the manufacturer's instructions. The plates were stained with Coomassie blue G-250 (Blakesley and Boezi, 1977) without prior washing to remove ampholines. Crossed immunoelectrofocusing (CIEF) was performed according to the method of Smyth, Soderholm and Wadstrom (1977). The allergenicity of the components separated by IEF was determined essentially as described earlier for CRIE (CRIEF).

Gradient PAGE or conventional PAGE in slabs or rods was combined with CIE using the "laying-on" technique of Ekwall, Soderholm and Wadstrom (1976). The allergenicity of the separated components was determined as described above (CRIE).

RESULTS

Gel-filtration analysis

The mite extract was fractionated on Sephacryl S-300 and a typical elution profile is shown in Fig. 1. The gel-filtration fractions were pooled as indicated and the yields of fractions 1-4 on a dry weight basis were usually in the order of 14, 23, 46 and 17% of the starting material respectively. Fraction 1 eluted in the void volume and was visibly turbid. A comparison of the fused rocket profile with the gel filtration profile (Fig. 2) revealed that, despite the high optical density at 280 nm associated with fraction 1, there was little immunoprecipitate.

The allergenicity of these fractions was assessed by direct RAST binding studies (Fig. 3). The Sephacryl S-300 fractions 3 and 4 showed similarly shaped RAST binding curves and comparable potency, as judged by the concentration of material required to bind 50% of the ¹²⁵I anti-IgE. In contrast, fractions 1 and 2,

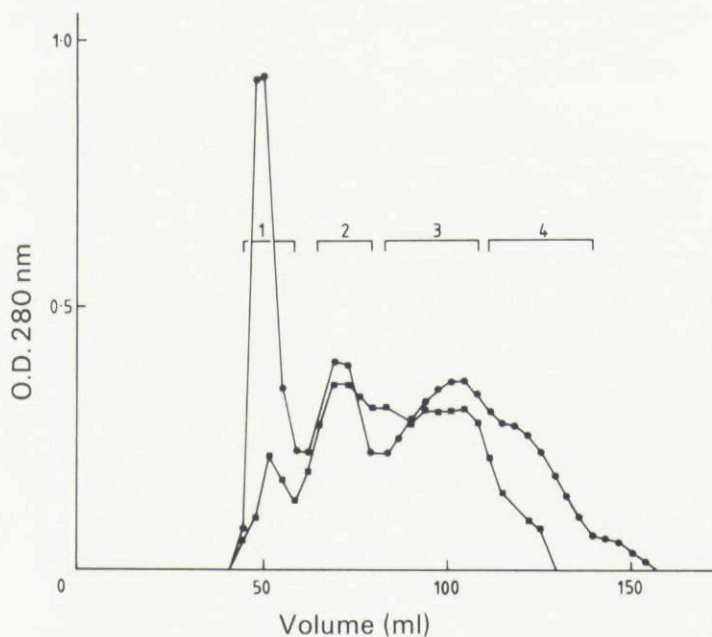


Fig. 1. Sephacryl S-300 elution profile of a water soluble extract of *D. pteronyssinus* (40 mg). The fractions were monitored at 280 nm (●—●) for protein and at 490 nm (■—■) for carbohydrate using the phenol-sulphuric acid reagent.

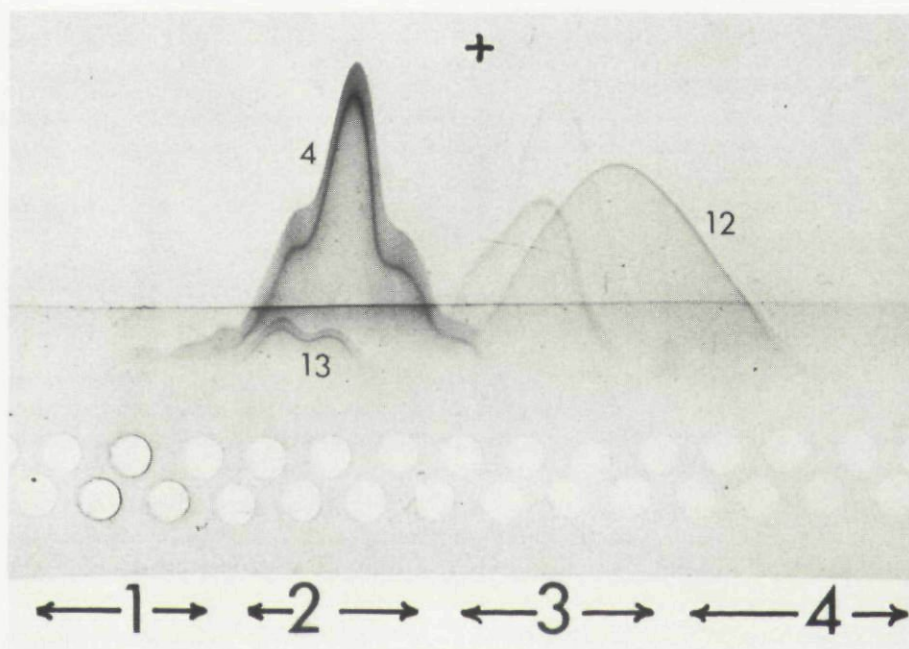


Fig. 2. Fused rocket immunoelectrophoretic analysis of individual fractions from the Sephacryl S-300 fractionation shown in Fig. 1. Antibody concentration was $10 \mu\text{l}$ per cm^2 gel area and $5 \mu\text{l}$ of each fraction were used.

although allergenic, were approximately ten-fold less potent using the above criterion and produced shallower RAST binding curves. The mite medium was also analysed by direct RAST binding and RAST inhibition and was shown to be negative by both criteria. Double diffusion analysis of Sephacryl S-300 fractions 1, 2 and 4 indicated that the high and low molecular weight components were antigenically distinct (Fig. 4).

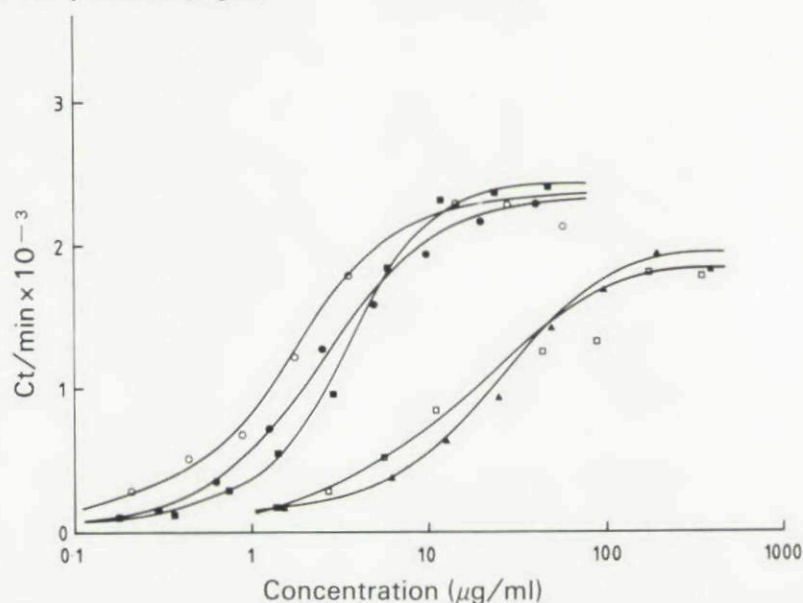


Fig 3. Direct RAST binding curve obtained for the original HDM extract (●—●), Sephacryl S-300 fraction 1 (▲—▲), fraction 2 (□—□), fraction 3 (○—○) and fraction 4 (■—■) using pooled mite allergic serum. Coupling of the fractions to individual CNBr activated paper discs was achieved using 50 µl allergen over the concentration range 0.1–1000 µg/ml.

CIE analysis

Crossed immunoelectrophoresis of the whole mite extract revealed several antigens (Fig. 5) which were arbitrarily numbered 1–15. CIE and CRIE analyses of the Sephacryl S-300 fractions were also performed and the results are shown in Fig. 6. Antigens 4, 7, 12 and 13 were identified as allergens by CRIE analyses using the reference serum pool from mite allergic subjects. These allergens will be referred to as Dpt 4, Dpt 7, Dpt 12 and Dpt 13 respectively. The majority of antigen 4 was found in fraction 2, but traces of this antigen were found in fractions 1 and 3. Antigen 13 was also found in fraction 2. Antigens 7 and 12 were found in fraction 3 with antigen 12 also being found in fraction 4. In fractions 2, 3 and 4 an allergen(s) was found which did not correlate with any antigen detected by CIE.

Fused rocket immunoelectrophoresis (Fig. 2) showed that antigens 4 and 13 were predominantly located in Sephacryl S-300 fraction 2 with small amounts of antigen 4 present in fractions 1 and 3. Antigen 13 had a higher apparent molecular weight than antigen 4, since it eluted from the Sephacryl S-300 column slightly before antigen 4. FRIE also showed that antigen 12 was present in both fractions 3 and 4 with the apex of the immunoprecipitin peak in fraction 3. However,

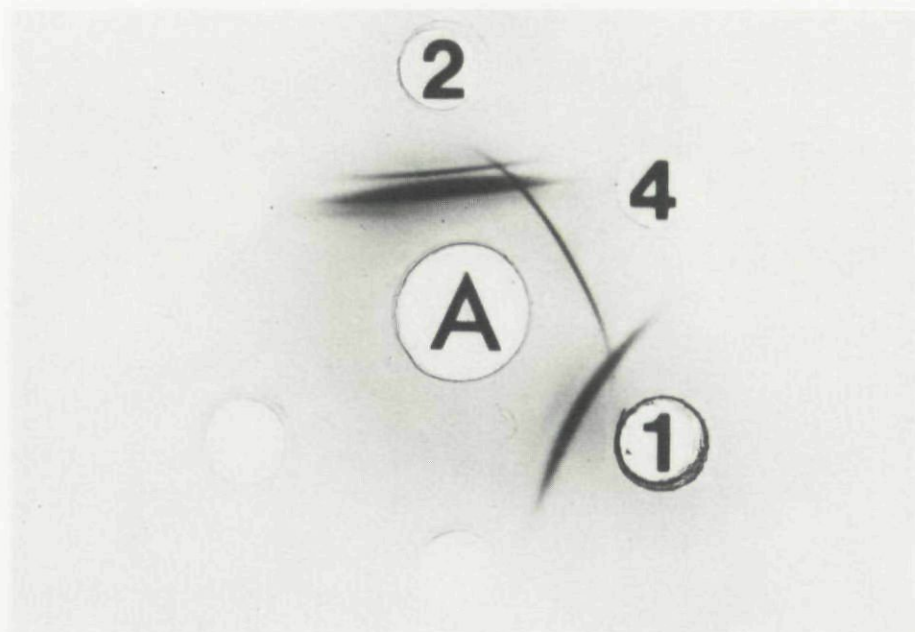


Fig. 4. Double diffusion analysis of Sephacryl S-300 fractions 1, 2 and 4 (each at 5 mg/ml) against rabbit anti-HDM.

immunoprecipitin peak(s) corresponding to the prominent shoulder seen on the descending side of fraction 3 and referred to as fraction 4 was not seen.

IEP analysis on agarose did not show such material. Further support for the presence of immunochemically unreactive material was obtained by 12% PAGE analysis (Fig. 11) in which it was shown that the immunochemically reactive material present accounted for only 33% of the material detected by densitometry of the stained PAGE gel. Further work reported in this study was directed towards the characterization of antigens 4 and 12, since these antigens represented the major allergens as judged using the mite allergic human serum pool described.

CIEF and CRIEF analyses of Dpt 4 and Dpt 12

The isoelectric points of the major allergens from whole extracts of mite and individual fractions from gel-filtration runs were determined by a combination of thin layer polyacrylamide gel electrofocusing over the pH range 3.5-9.5 (Fig. 7) with CRIE. Typical results using whole mite extract are shown in Fig. 7A and it can be seen that the mite components focused into several distinct bands within the pH range 4-7 with the majority of the bands focusing in the pH range 4-5.

Eight precipitin bands were observed on CIEF analysis (Fig. 7C). Dpt 4 formed the characteristic heavy staining precipitate which focused at pH 4.5 with a shoulder at pH 4.9. Dpt 12 focused at pH 6.6 and showed marked heterogeneity with minor bands focusing at pH 4.5, 5.3, 5.7 and 6.1. CRIEF analysis confirmed that both of these antigens were allergens (Fig. 7D).

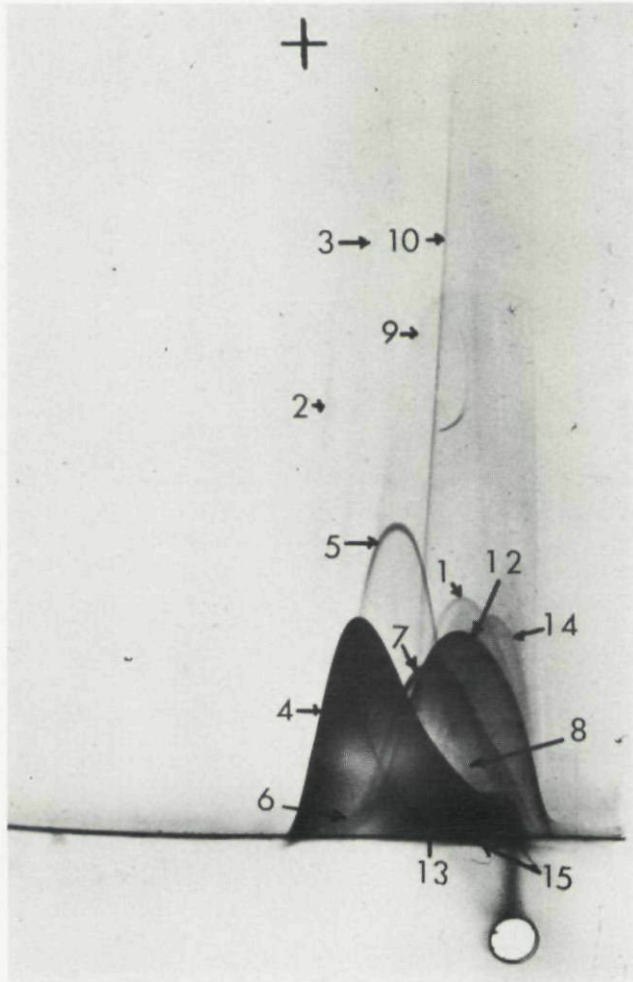


Fig. 5. Crossed immunoelectrophoresis of whole HDM extract (500 μ g) using an antibody concentration of 40 μ l per cm^2 gel area.

PAGE analysis of Dpt 4 and Dpt 12

Sephacryl S-300 fraction 2 was analysed by gradient PAGE to determine the apparent molecular weight of Dpt 4. Visual and densitometric scanning of the stained gel distinguished seven protein bands (Fig. 8B and D). Other gels were stained for carbohydrate and a heavy but diffuse staining pattern was obtained (Fig. 8A). The carbohydrate staining pattern paralleled that for protein staining except at the top of the gel where more carbohydrate staining material was seen. The apparent molecular weight of each component identified by protein staining was determined by reference to a standard curve (Fig. 8C and Fig. 9). These estimates are listed in Table 1. The major protein (band 4) had an apparent molecular weight of 274,000.

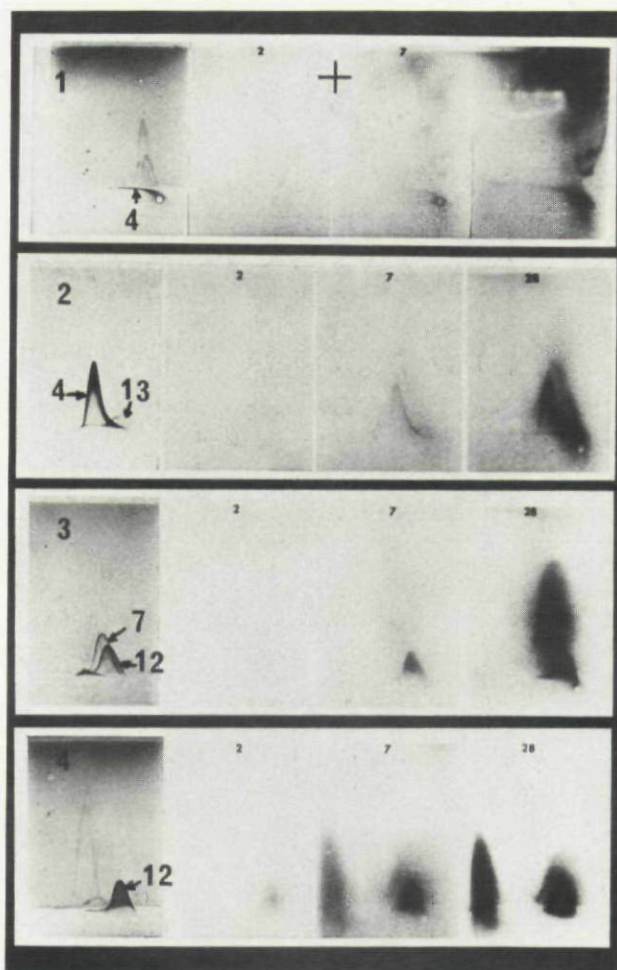


Fig. 6. Crossed immunoelectrophoresis of Sephacryl S-300 fractions 1, 2, 3 and 4 with their respective autoradiographs after 2, 7 and 28 days exposure. CIE was performed using 100 μ g of each fraction and an antibody concentration of 40 μ l per cm^2 gel area. CRIE was performed using a mite allergic serum pool. Arrows on CIE indicate antigens identified as allergens after autoradiography. Arrows on autoradiographs indicate allergens which did not correspond to any detectable antigen on CIE analysis.

A combination of gradient PAGE and CIE showed that all the proteins identified in Fig. 8, with the exception of protein 3, corresponded to an immunoprecipitin line (Fig. 10). There was complete antigenic identity between component 1 (mol. wt. 531,000) and component 4. Proteins 5 (mol. wt. 177,000) and 6 (mol. wt. 140,000) also showed antigenic identity with protein 4, but not with each other. Proteins 2 and 7 did not cross-react with proteins 1, 4, 5 and 6. A comparison of these data with the CIE (Fig. 6) and FRIE (Fig. 2) data showed that protein 4 corresponded to Dpt 4 and protein 2 to Dpt 13.

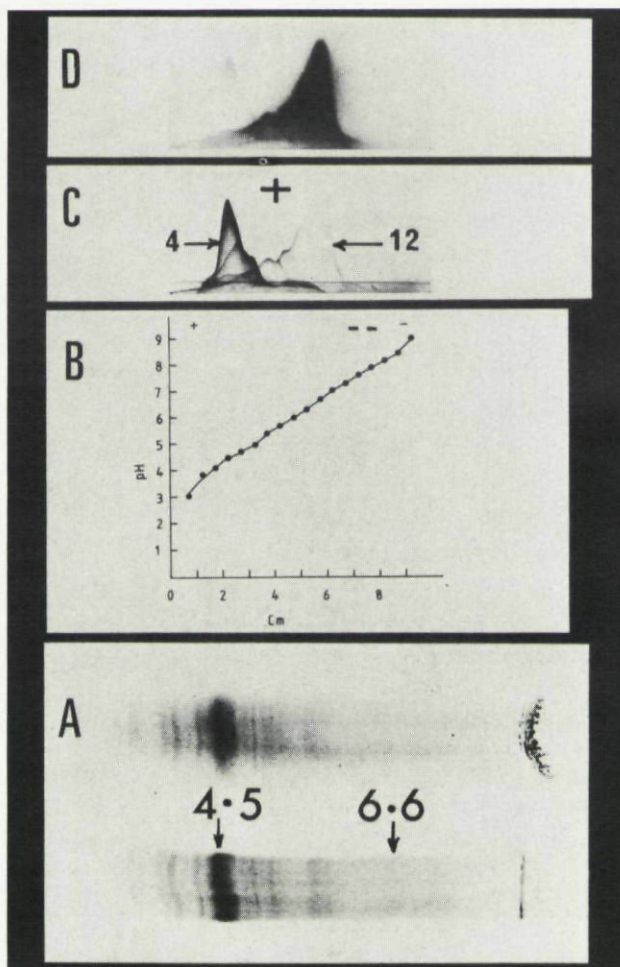


Fig. 7 Crossed immunoelectrofocusing analysis of whole mite extract (230 μ g) over the pH range 3.5-9.5 (B). The IEF-PAGE gel was stained for protein with Coomassie blue G-250 (A) and is shown at a slightly higher magnification than in B, C and D, to show up the stained bands. The pI values corresponding to the position of the apex of the two major precipitin bands arrowed in C are shown in A. CIEF (C) was performed using the "laying on" technique and an antibody concentration of 10 μ l per cm² gel area. CRIEF (D) was performed using a mite allergic serum pool, the autoradiograph shown in D being exposed for 14 days.

Sephacryl S-300 fraction 4 was examined by 12% PAGE and the results obtained are shown in Fig. 11A. Although several protein staining bands were seen, only the two major bands near the top of the gel could be correlated with the immunoprecipitate seen on CIE (Fig. 11B). CRIE showed that the material in this precipitate was allergenic (Fig. 11C) and corresponded to Dpt 12.

The apparent molecular weight of this allergen was determined by gel filtration on Sephadex S-300 after having established the precise elution position by FRIE. It had an apparent molecular weight of 24,000 daltons (Fig. 12).

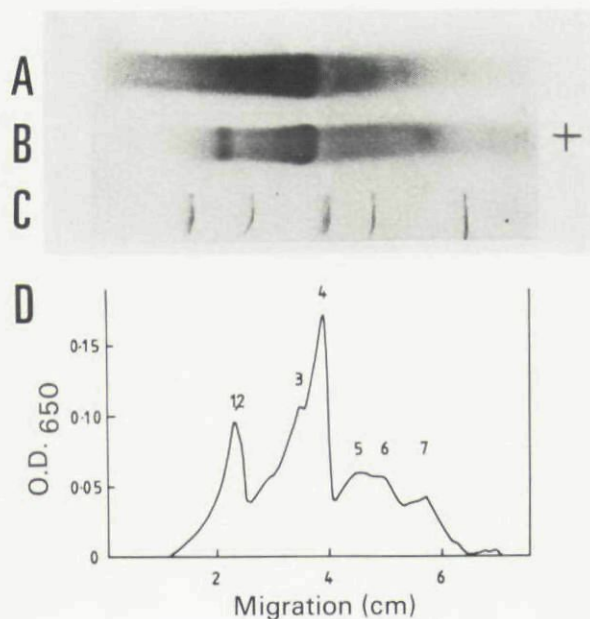


Fig. 8. Gradient (4-30%) PAGE analysis of Sephacryl S-300 fraction 2 (150 μ g) stained for carbohydrate (A) and for protein (B). A densitometric scan of B is shown in D. The molecular weight markers thyroglobulin (mol. wt. 669,000), ferritin (440,000), catalase (232,000), lactate dehydrogenase (140,000) and albumin (67,000) are shown in C with thyroglobulin to the left.

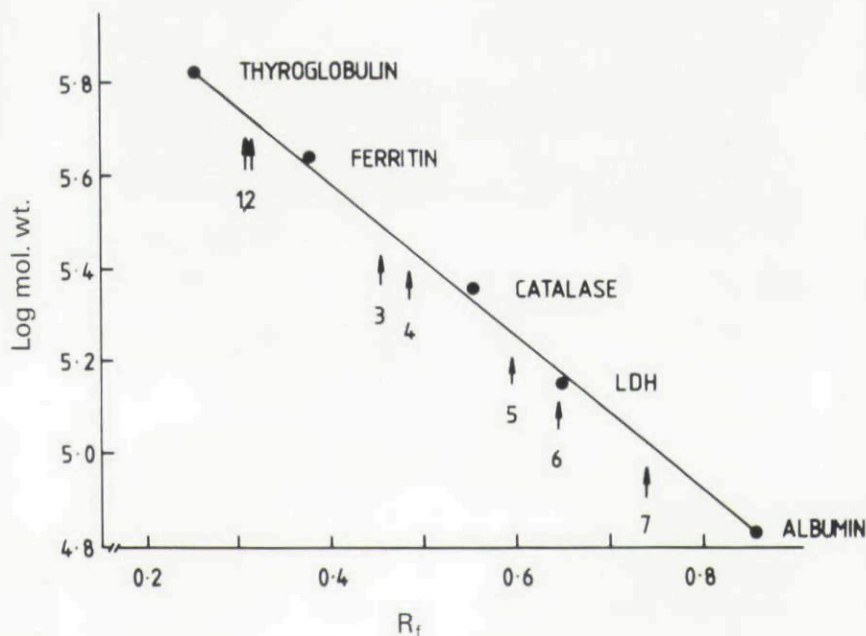


Fig. 9. Apparent molecular weight of components 1-7 in Sephacryl S-300 fraction 2 determined by gradient (4-30% acrylamide) PAGE. The relationship between log molecular weight and mobility (R_f) was established using the molecular weight markers (Fig. 8).

TABLE 1

The apparent molecular weights of the various protein components present in Sephacryl S-300 fraction 2 as determined by gradient PAGE.

Component	Molecular Weight
1	537,000
2	510,000
3	303,000
4	274,000
5	177,000
6	140,000
7	104,000

Each value represents the mean of at least three determinations.

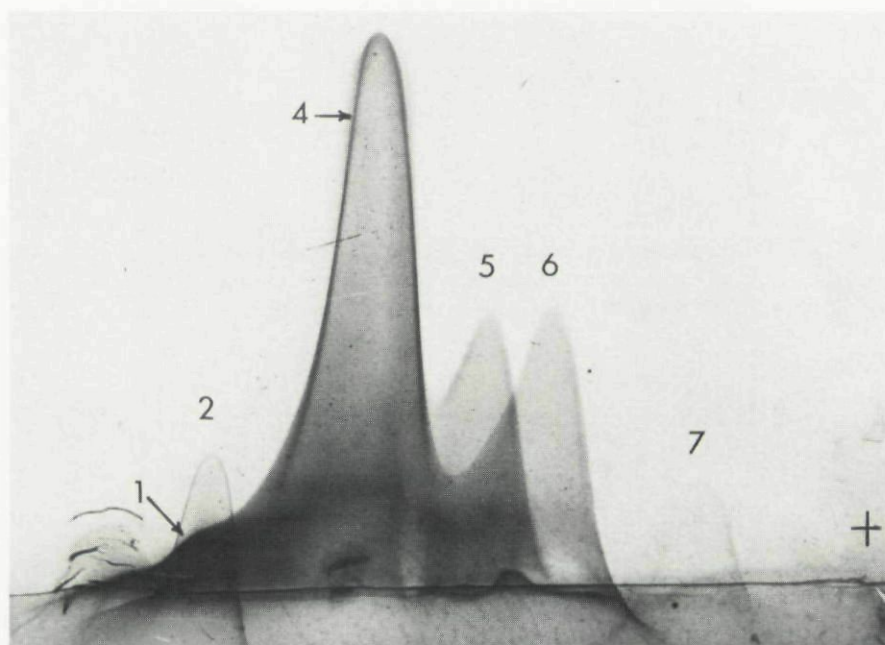


Fig. 10. Crossed immunoelectrophoresis of an unstained gradient (4-30%) PAGE gel of Sephacryl S-300 fraction 2 (300 μ g) using the "laying on" technique and an antibody concentration of 10 μ l per cm^2 gel area. The peaks were numbered by comparing the position of the precipitin peak with that determined on the densitometric scan shown in Fig. 8. A precipitin peak corresponding to the densitometrically defined peak 3 was not seen.

DISCUSSION

Quantitative immunoelectrophoresis (QIE) techniques have shown extracts of *D. pteronyssinus* used in this study to be antigenically complex with at least 15 antigens being identified. The complexity of such extracts has been reported previously (Biliotti *et al.*, 1975; Dasgupta and Cunliffe, 1970). Not all of these antigens were shown to be allergens using a mite allergic serum pool, although the

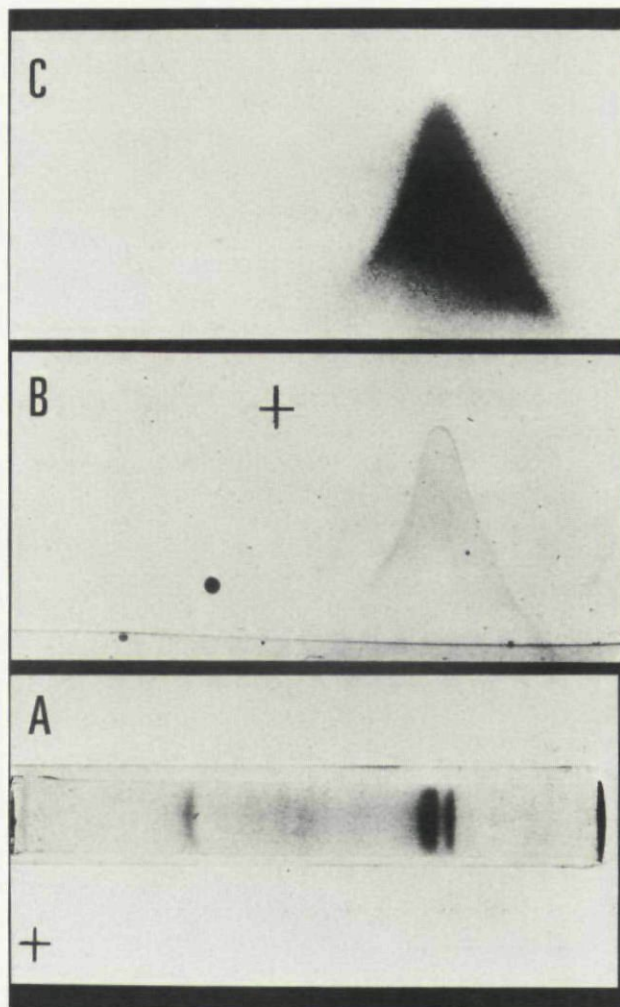


Fig. 11. 12% PAGE analysis of Sephacryl S-300 fraction 4 (50 μ g). A: stained for protein with Coomassie blue G-250. B: CIE of an unstained gel using the "laying on" technique and an antibody concentration of 10 μ l per cm^2 gel area. C: CRIE using a mite allergic serum pool (14 days exposure).

possibility exists that antigens other than those detected here would be defined as allergens using individual allergic sera. Such studies are presently under way.

QIE coupled with conventional physicochemical techniques have shown that there are at least 4 allergens present in extracts of *D. pteronyssinus* defined as Dpt 4, 7, 12 and 13 on the antigen classification system derived from CIE studies and that suggested by Marsh (1974).

Dpt 4 was shown to be a high molecular weight allergen which was heterogeneous with regard to both molecular weight and pI and was antigenically distinct from Dpt 12. The majority of the allergen existed with an apparent molecular weight of 274,000 daltons, but antigenically identical material with an

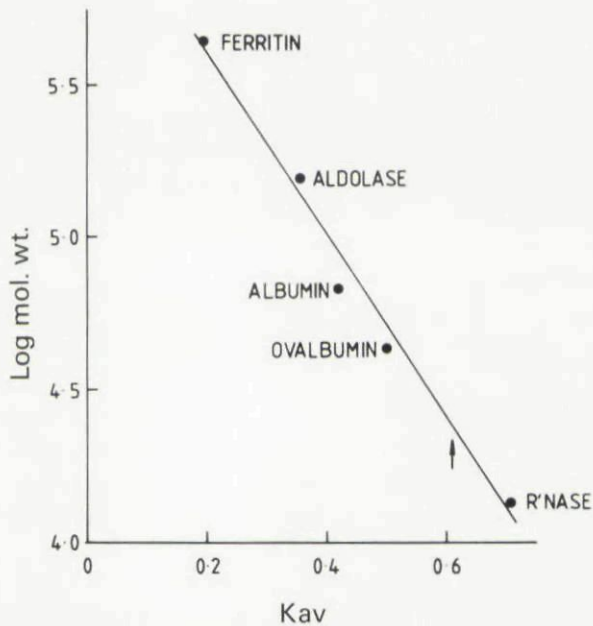


Fig. 12. Apparent molecular weight of the allergen Dpt 12 determined by gel filtration on Sephacryl S-300. The relationship between log mol. wt. and K_{av} was determined using the markers ferritin (mol. wt. 440,000), aldolase (158,000), albumin (67,000), ovalbumin (43,000) and ribonuclease (13,700). The position of Dpt 12 is indicated by the arrow.

apparent molecular weight of 531,000 daltons was also detected. These data suggest that the latter represents a dimeric form of the 274,000 dalton species. Dpt 13 was also shown to be a high molecular weight allergen with apparent molecular weight of 510,000 daltons. Further studies indicated that Dpt 4 may comprise subunits, since antigenically related components with apparent molecular weights of 174,000 and 140,000 daltons were detected. However, the precise relationship between these different molecular weight species cannot at present be assessed. These two low molecular weight species appeared to be antigenically unrelated to each other, but the recent demonstration of false spurs in QIE techniques indicating partial or non-antigenic identity suggest that the double diffusion analysis of such components is required to establish antigenic identity before any conclusions can be drawn (Danielsen, Sjostrom and Noren, 1977; Negassi, Closs and Harboe, 1978).

Allergen Dpt 4 and its different molecular forms were shown to contain significant amounts of carbohydrate and this, therefore, suggests that the apparent molecular weights determined by an empirical method in this study may not represent true values (Andrews, 1970).

Dpt 12 was shown to have an apparent molecular weight of 24,000 daltons and it probably corresponds to the allergen or allergens described previously (Ishii *et al.*, 1973; Biliotti *et al.*, 1975; Nakagawa *et al.*, 1977; Chapman and Platts-Mills, 1978) which had molecular weights in the range 15-30,000. PAGE studies described here suggest that this allergen may exist in iso-allergen form with the two

iso-allergens differing from each other either on the basis of charge or molecular weight (Marsh, 1974). This conclusion is suggested by the excellent correlation between both the positioning and asymmetry of the allergen containing immunoprecipitin peak detected on PAGE-CIE with the two protein staining bands on PAGE. Different isoallergenic forms of antigen E from ragweed pollen have also been detected using a similar experimental approach to that described in this report (Blanchard and Gardner, 1977). Similar antigenically identical bands have been seen using the low molecular weight allergen isolated from mites from source 2 (unpublished observations).

Isoelectrofocusing studies showed Dpt 4 and Dpt 12 to be highly heterogeneous in agreement with reports of other investigators utilizing liquid media (Romagnani *et al.*, 1976; Nakagawa *et al.*, 1977). In the former study, three main regions of allergenicity were detected, namely at pI 3.0, 4.3 and 6.4. The allergenicity detected at the latter two pI values agrees particularly well with the pI values determined for the allergens Dpt 4 and Dpt 12 in this study.

On the basis of the high degree of cross-reactivity between the various IEF and gel filtration fractions, Romagnani *et al.* (1976) suggested that mites contain one main allergen which exists in multiple molecular weight forms rather than as several distinct allergens. Data from this study support the suggestion of an allergen existing in different molecular weight forms, particularly for Dpt 4, but these data do not support the suggestion that allergenicity is restricted to one major allergen since several antigenically distinct allergens were identified.

These studies showed that the antiserum used did not detect certain low molecular weight components present in Sephacryl S-300 fraction 4. Other studies (unpublished observations) suggested that this failure was not due to the particular serum used since several different anti-mite sera also failed to detect this material. These findings highlight one of the potential drawbacks to QIE techniques (Løwenstein, 1978) and, further, suggest that the purity of isolated allergens must not be based on QIE techniques alone. The finding of a significant amount of extraneous material in fraction 4 probably explains why an increase in allergenicity was not seen by RAST assay in comparison to the original extract.

In conclusion, the physicochemical and immunochemical characterization of two allergens from *D. pteronyssinus* have been described. Allergen Dpt 4 is a high molecular weight allergen in contrast to Dpt 12. Of these two allergens, Dpt 4 appears to be the most potent as judged by CRIE and RAST. Both allergens demonstrate heterogeneity in molecular weight and pI. Further studies are presently under way to determine the validity of this conclusion.

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