

Isolation and characterization of cat allergens

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Summary

A crude (saline soluble) extract of cat skins capable of eliciting a strong positive prick skin test in cat sensitive individuals was fractionated on Sephadex G200. Active fractions were pooled and successively fractionated on isoelectric focusing gradients of pH 3-5 and pH 4-5. Allergenic activity was localized in two peaks with mean isoelectric points of 4.1 and 4.35 respectively. On immunoelectrophoresis the allergen with $pI=4.35$ was associated with a protein which was subsequently found to be immunologically indistinguishable from serum albumin and to have a molecular weight of 69,000 daltons. The allergen with $pI=4.1$ migrated in the α_2 -region on immunoelectrophoresis and had a mean molecular weight of 55,000 daltons. This allergen was isolated and analysed for amino acid and carbohydrate content.

A combined extract of both allergens coupled to microcrystalline cellulose and used in a RAST procedure readily distinguished between two groups of individuals classified as skin test positive and skin test negative to cat allergen.

Introduction

The importance of danders of mammalian origin in allergic disease has long been recognized. In 1864 Salter had suggested that cat dander may induce asthmatic reactions but it was Goodale (cited by Wodehouse (1917)) who first demonstrated, in cat sensitive individuals, positive skin tests to ethanol extracts of cat fur. Skin test studies on larger groups of asthmatic patients have confirmed these earlier reports; Coke (1927) found that 19% of 1000 asthmatics gave positive skin reactions to cat dander and Frankland (1967), in a study of 451 asthmatic patients, found that 25% reacted to animal extracts, with cat as a major allergen.

Early attempts to fractionate extracts of cat fur (Wodehouse, 1917) involved the use of both strong acids and alkalis. These studies (Wodehouse, 1917; Walker, 1917) suggested that cat serum was allergenic in over half of the small number of cat sensitive subjects tested. However, further detailed characterization of the allergens was delayed by the absence of appropriate physico-chemical techniques. The recent

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availability of such techniques has stimulated fresh interest in the problems of allergen characterization and isoelectric focusing (Varga & Ceska, 1972), gel filtration (Holford-Strevens, 1973) and polyacrylamide gel electrophoresis (Ohman, Lowell & Bloch, 1973, 1974; Brandt, Ponterius & Yman, 1973) techniques have all been applied to the characterization of cat allergens.

The present study describes the characterization of two major allergenic components from the dander of the domestic cat. One such allergen has been isolated in immunochemically pure form and characterized with respect to its molecular weight, isoelectric point and constituent amino acids and oligosaccharides.

Materials and methods

Extraction of crude allergen

The skins (including fur) of a group of laboratory cats were defatted in acetone (4°C) for 24 hr and then extracted in saline (0.85%) containing 0.05% sodium azide for 48 hr at 4°C. Residual fat was removed by ether extraction of the saline extract. The aqueous solution was then dialysed against distilled water and lyophilized.

Gel filtration of saline extract

20 ml of the saline extract (15 mg.ml⁻¹ w/v) were passed through a Millipore filter (pore size 0.22 µ) and applied to a calibrated column of Sephadex G200 (5 × 91.2 cm). Upward elution with 0.1 M Tris-HCl-0.2 M NaCl-2 mM EDTA Na₂ containing 0.02% sodium azide pH 7.6 was performed at 22°C using a pumping speed of 28 ml.hr⁻¹. Fractions were pooled, dialysed against distilled water and freeze dried. Allergen activity was determined by prick skin tests in a cat sensitive subject using fractions reconstituted to a constant volume. Fractions with activity were pooled and the product dialysed and lyophilized.

Isoelectric focusing of allergenic (gel-filtration) fraction

Isoelectric focusing was performed at 18°C using the LKB 8100-10 system with a sucrose density gradient. The column was emptied by pumping at 1 ml.min⁻¹ with continuous monitoring of the eluate at 254 nm (LKB Uvicord I). Fractions were collected at 1 min intervals and the pH immediately read. One percent carrier ampholytes [pH ranges 3-10 and 3-6 (LKB 8141 and 8142)] were used initially in order to localize allergenic activity. Preparative experiments were then performed using 4% ampholytes pH 3-6. The allergen active region from the Sephadex G200 fractionation (120 mg) was focused and on emptying the column a region of intense skin test reactivity was identified. Fractions from this region were pooled and refocused in a second run, without the addition of any more ampholytes, by diluting one quarter of the pooled solution with distilled water to form the light (i.e. less dense) solution and diluting the remainder with 55% sucrose solution (w/v) to form the dense solution. On completion of the second run the fractions were dialysed extensively against distilled water and finally adjusted to a constant volume.

Prick skin testing

Skin testing was performed on a cat-sensitive subject by piercing the dermis (21 gauge needle) through a droplet of allergen solution which had been previously sterilized by passage through a Millipore filter (0.22 µ). Tests were performed in

duplicate on random numbered sites on the volar aspect of the forearm. Skin test solutions were diluted and used at increasing concentrations until a wheal and flare were obtained. Wheal diameters were read in two different directions by the recipient using a negative saline control and a commercial cat allergen (Bencard) as positive control. The recipient read the tests using a number code for each sample.

Immunoelectrophoresis

Immunoelectrophoresis was performed according to the method of Grabar & Williams (1953), using 1.5% (w/v) Oxoid Ionagar in barbitone buffer pH 8.6 ($I = 0.045$).

Immunodiffusion analyses

Double diffusion was performed according to the method of Ouchterlony (1958) using 1.5% Difco Noble agar in phosphate buffer pH 8.0.

Antisera

Antisera to the initial saline extract (anti-CE) were raised in rabbits. Three mg of extract in complete Freund's adjuvant were injected into three sites and after 6 weeks a further 5–8 mg of alum precipitated extract were injected. A second injection of alum precipitated extract was given 8 weeks later and the animals were bled after a further 2 weeks.

Apparent molecular weights

The apparent molecular weights of the allergens were determined according to the method of Andrews (1964) from their elution volumes on a column of Sephadex G-200 using a buffer of 0.1 M Tris-HCl-0.2 M NaCl-2mM EDTA Na_2 containing 0.02% sodium azide, pH 7.6. Calibration of the column was based on the elution volumes of the following proteins: human IgG (mol. wt 150,000), human serum albumin (mol. wt 69,000), ovalbumin (mol. wt 45,000), pFc' fragment of human IgG (Turner & Bennich, 1968) (mol. wt 26,700).

Dissociation and reduction experiments

The effect of dissociation on the apparent molecular weight of the cat allergen $pI = 4.1$ was studied by dialysing a sample of the allergen against 0.1 M acetic acid—25 mM sodium chloride pH 2.2 followed by fractionation on a calibrated column of Sephadex G 200, equilibrated in the dissociating buffer.

The apparent molecular weight of cat allergen $pI = 4.1$ after reduction and alkylation was also obtained after gel filtration under dissociating conditions. Reduction was performed in de-aerated 0.55 M Tris-HCl-2mM EDTA Na_2 , pH 8.2 at 20°C under nitrogen. The sample was dialysed overnight, de-aerated and saturated with nitrogen and 2-mercaptoethanol (Koch-Light Ltd) added to give a 0.1 M solution. After 1 hr the reaction was stopped by alkylation with a 10% molar excess (over mercaptoethanol) of iodocetamide (Sigma Ltd) for 60 min at 0°C (ice water) in the dark. The sample was then applied to the calibrated column of Sephadex G-200 (see above).

Amino acid composition

A sample (1 mg) of the cat allergen $pI 4.1$ (see results) was taken for hydrolysis in 0.5 ml of 6 N hydrochloric acid, containing 7.05 ng of norleucine (internal standard).

Hydrolysis was performed *in vacuo* at 110°C for 24 hr as described by Van Hofsten, Van Kley & Eaker (1965). The hydrolysate was lyophilized and redissolved in 0.25 ml of lithium citrate buffer pH 2.2 and analysed according to the method of Spackman, Stein & Moore (1958), using a Beckman 121M amino acid analyser equipped with an integrator/computer (Autolab-System AA). Cysteic acid and tryptophan were not determined.

Carbohydrate composition

A sample (1 mg) of cat allergen pI 4.1 was analysed for sugars using standard gas chromatographic procedures (Clamp, Dawson & Hough, 1967).

Radioallergosorbent tests

The allergen rich material obtained after gel filtration on Sephadex G-200 was coupled to micro-crystalline cellulose (Sigma-cell) and used for the measurement of specific IgE antibodies as described by Wide, Bennich & Johansson (1967). The amount of allergenic material required for coupling was titrated using a range of concentrations of the allergen and a constant amount of serum from a cat sensitive individual. For subsequent assays 0.10 mg of the allergen preparation was coupled to 1 mg of Sigma-cell.

For the performance of the test 0.5 ml of the allergen-Sigma cell suspension was mixed with 50 μ l of serum and incubated at room temperature for 16 hr with continuous mixing. The cellulose particles were then washed six times with physiological saline containing 1% Tween-20 and after the final wash 100 μ l of ^{125} I-labelled specific rabbit anti-human IgE globulin (F_c -immunosorbent purified) was added to each tube and the contents incubated with agitation at room temperature overnight. The supernatant was then removed and the cellulose particles washed five times with physiological saline containing 1% Tween-20. Residual radioactivity was then counted in a Gamma counter.

Results

Cat pelts were found to provide a ready source of crude allergen extract and at least 500 mg of lyophilizable material were obtained from each animal. A solution of crude allergen extract in physiological saline (1.5 mg.ml⁻¹) was found to be of comparable strength to the Bencard cat fur extract 150% (Batch no. 3204 C0979). Immuno-electrophoretic analysis of the allergen extract using the rabbit anti-extract (anti-CE) revealed the presence of at least thirteen components.

Fractionation of the saline extract on Sephadex G-200 gave reproducible elution profiles with large amounts of low molecular weight material (see Fig. 1). Prick skin tests on fractions from these separations localized allergenic activity to the molecular weight region 26,000–68,000. Immunoelectrophoresis and double diffusion analysis of individual fractions within this region revealed the presence of multiple components in all samples tested.

Further fractionation of the allergen rich material from gel-filtration separations was achieved by isoelectric focusing with 1% ampholytes on pH gradients 3–10 and 3–6. These experiments showed skin test activity in the pH region 4–5 (see Fig. 2). When preparative runs were performed using the double focusing schedule two peaks of activity were resolved (see Fig. 2 lower trace). The first peak was associated with fractions 48–62 having a mean pI of 4.1 and the second peak was associated with

fractions 70–94, having a mean pI of 4.35. Double diffusion analysis (Fig. 3) of alternate fractions from the skin test active regions using the anti-cat extract antiserum

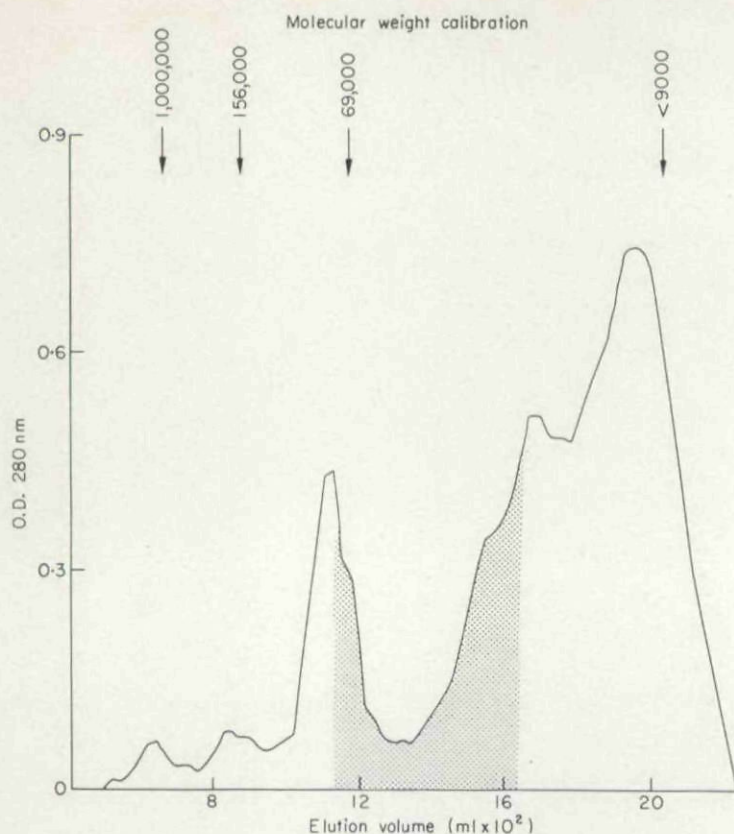


Fig. 1. Gel filtration of saline extract (20 ml) of cat skin ($15 \text{ mg.ml}^{-1} \text{ w/v}$) on a column of Sephadex G-200 ($5 \times 91.2 \text{ cm}$). The column was eluted by upward flow using 0.1 M Tris- 0.2 M NaCl- 2 mM EDTA Na_2 containing 0.02% NaN_3 , pH 7.6. Flow rate 28 ml.hr^{-1} . Temperature 22°C . Allergen activity, determined by prick tests in a cat sensitive subject, was localized to the stippled region. Fractions in this region were pooled and the product dialysed and lyophilized.

showed the presence of a major precipitin line in fractions 48–58 which was also detectable in fractions 60 and 62. The distribution and intensity of this precipitin line parallels closely the quantitative distribution of skin test activity in this region. Similarly a precipitin line first detectable in fraction 70, strongly represented in fractions 74–84, and still detectable in fraction 94 appears to parallel the distribution of the second peak of skin test activity.

Immunoelectrophoretic analysis of alternate fractions around the first peak of skin test activity ($pI=4.1$) (see Fig. 4) showed the presence of an anodic arc in the α -electrophoretic region (see arrow). Fractions 56–60 also contain an increasing amount of a trace component of faster mobility but the distribution of this protein did not parallel the distribution of skin test activity. Immunoelectrophoretic analysis of fractions corresponding to the second region of skin test activity ($pI=4.35$) are shown

in Fig. 5. All fractions analysed contained at least three precipitating components and the major arc (see arrow) showed a similar distribution to the second region of skin test activity. This major component was further studied in gel tests and showed a reaction of complete identity with isolated cat albumin on double diffusion analysis.

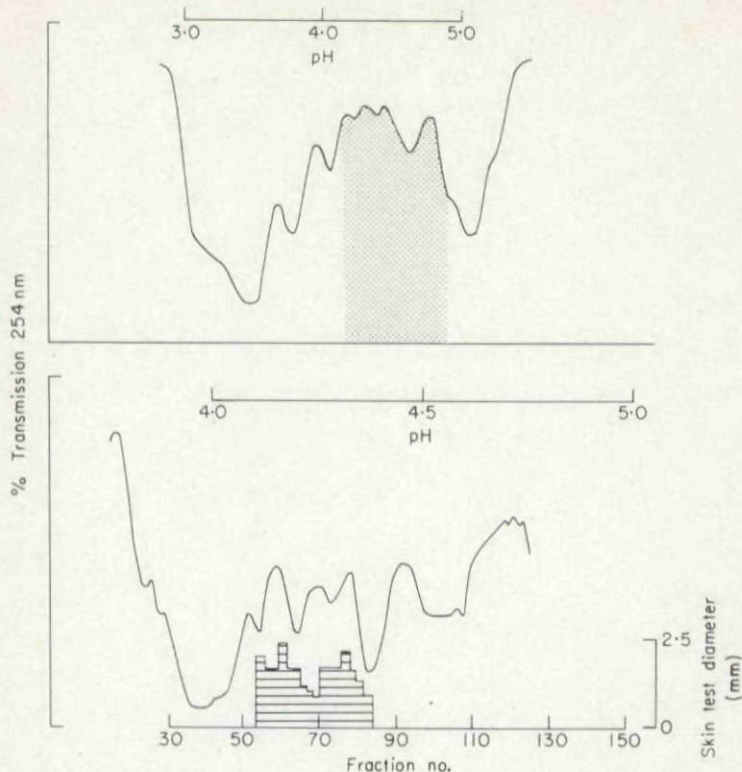


Fig. 2. Isoelectric focusing profile of allergen-rich material (120 mg) from the gel-filtration experiment (Fig. 1) using 4% ampholytes and a pH range 3.0–6.0 (upper trace). Allergen activity was focused in the pH range 4–5 as shown by the stippled area. Fractions in this region were pooled and refocused to give a pH gradient of 4–5 (lower trace). Fractions from this second experiment were dialysed extensively against distilled water, adjusted to a constant volume, and allergenic activity titrated in a cat sensitive subject.

Variations of the comparative immunoelectrophoresis technique of Wadsworth & Hanson (1960) were also used to demonstrate immunological identity between the principle protein in fractions 72–84 and cat serum albumin.

In order to determine the mean molecular weights of the allergens, peak tubes from the two allergen rich regions (58 and 78) were labelled with ^{125}I by the chloramine T method (Hunter & Greenwood, 1962) and fractionated on a calibrated column of Sephadex G-200 equilibrated in Tris buffer pH 7.6. Radioactivity was eluted in regions corresponding to molecular weights of 55,000 and 69,000 for the allergens of $\text{pI} = 4.1$ and $\text{pI} = 4.35$ respectively. In the case of allergen $\text{pI} = 4.1$ a minor peak of activity was also observed in fractions with an apparent molecular weight of 27,000.

After dialysis against 0.1 M acetic acid, 25 mM sodium chloride (pH 2.2) the ^{125}I -

labelled allergen $pI=4.1$ was fractionated on Sephadex G-200 equilibrated in the same buffer and no change in elution position was observed. However, when the labelled allergen ($pI=4.1$) was subjected to reduction in 0.1 M mercaptoethanol for 1 hr and then fractionated under identical conditions, the radioactivity eluted in the molecular weight region 6000–15,000 (see Fig. 6).

The amino acid and carbohydrate compositions of allergen $pI = 4.1$ (fractions 57, 58 and 59, Fig. 2) are given in Tables 1 and 2.

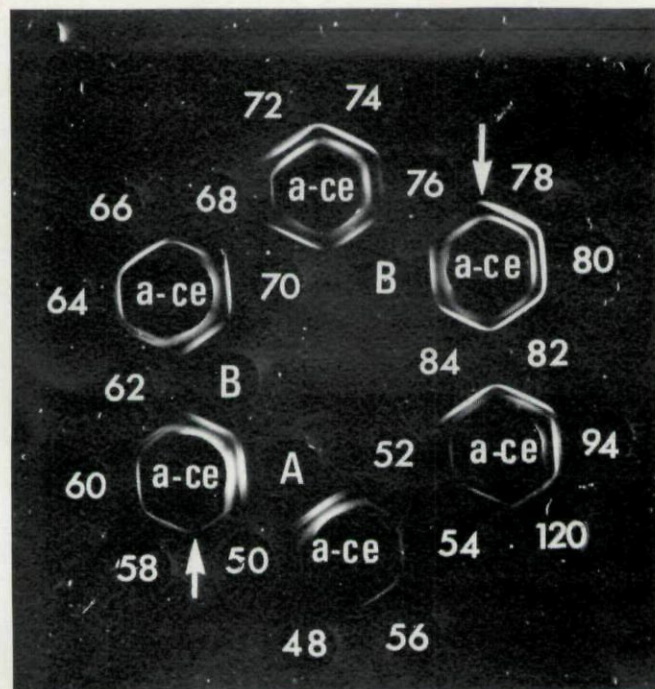


Fig. 3. Double diffusion analysis of fractions obtained by isoelectric focusing of cat allergen extract (Fig. 2, lower trace): 48–120, fractions from the isoelectric profile; A, allergen active pool from Fig. 1; B, allergen active pool from Fig. 2 (upper trace); a-ce, rabbit anti-cat extract antiserum. Arrows indicate precipitin lines which correlate closely with skin test activity.

There was insufficient allergen $pI=4.1$ for use in the radioallergosorbent assay and a mixture of the two allergens ($pI\ 4.1$ and $pI\ 4.35$) obtained after Sephadex G-200 gel filtration (see Fig. 1) was therefore used. In a study of adults classified as skin test negative or skin test positive (using the Bencard prick test solution) a significantly higher level of specific IgE binding was observed in the cat sensitive subjects ($P < 0.01$ on a Mann-Whitney U test) (see Fig. 7).

Discussion

Saline extract of defatted cat skins provided a ready source of crude allergen, with a yield of at least 500 mg of lyophilizable material from each pelt. When corrected for total protein the skin test activity of this extract was found to be comparable to a commercial extract. Extraction procedures involving similar solvents have been

previously described; for example, Coca's solution for cat and dog skins (Holford-Strevens, 1973), 0.075 M phosphate buffered saline for pollens (Belin, 1972) and saline for horse dander (Stanworth, 1957a).

As previously shown for horse dander (Stanworth, 1957b) the crude saline extract of cat pelts was found to contain multiple proteins when assayed by immunoelectrophoresis using appropriate antisera. Plasma-proteins, particularly albumin, transferrin and IgG were identified in the extract and several other components, some presumably of epithelial origin, were also found.

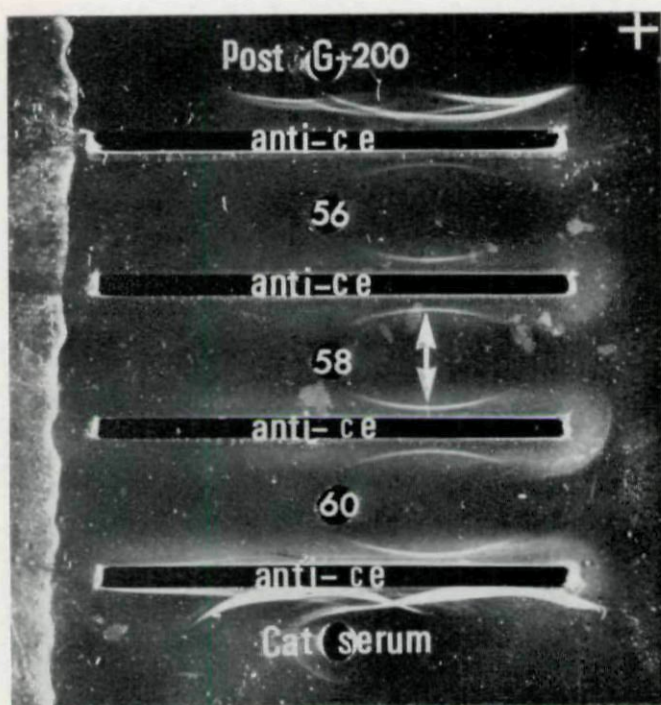


Fig. 4. Immunoelectrophoretic analysis of fractions obtained by isoelectric focusing of cat allergen extract (Fig. 2, lower trace): 56–60, fractions from the isoelectric-profile corresponding to the first skin test active region (mean $pI=4.1$); Post-G-200, allergen active pool from Fig. 1; anti-ce, rabbit anti-cat extract antiserum. Arrow indicates precipitin line correlating closely with skin test activity.

In the present study the allergenic components of cat dander were initially fractionated on Sephadex G-200 but skin-test activity was associated with a region containing a substantial number of proteins and further fractionation by isoelectric focusing was undertaken. Using wide range pH gradients (3–10) stabilized with sucrose, all the allergenic components from cat skins eluted in the pH range 4–5 which is in general agreement with the analytical analyses of Varga & Ceska (1972) who also reported minor components with pI 's of 3–3.9, 5.3 and 7.8–8.5. We observed two peaks of skin test activity associated with fractions having mean pI values of 4.1 and 4.35 respectively.

Multiple protein components were present in the fraction $pI=4.35$ but only one protein showed an electrophoretic distribution (Fig. 5) identical with skin test activity. This protein was shown to be immunologically indistinguishable from serum albumin

and to have a molecular weight of 69,000. The distribution of skin test reactivity following gel filtration (Fig. 1) suggests that in the original extract serum albumin made a quantitatively minor contribution to the total allergenic activity. After isoelectric focusing the albumin was selectively concentrated, having possibly undergone less

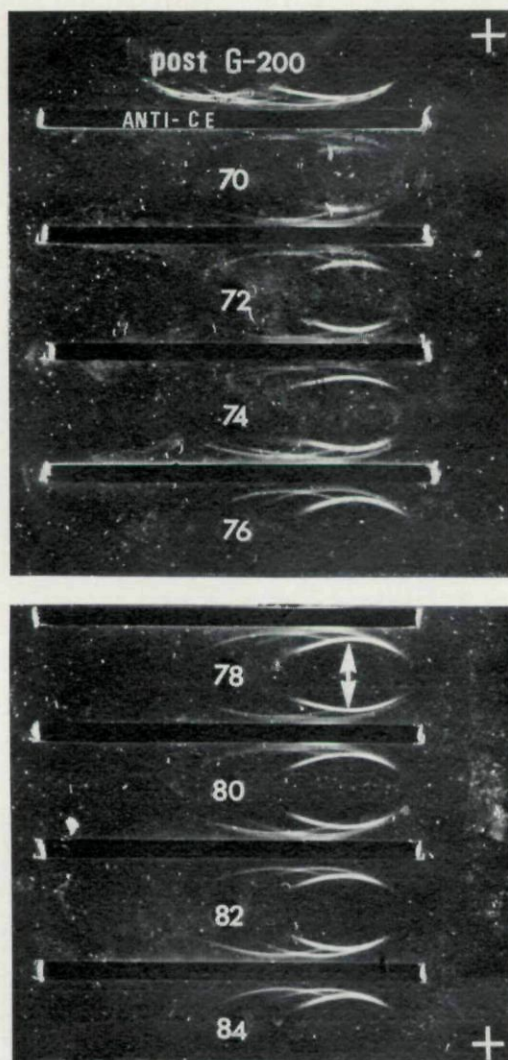


Fig. 5. Immunoelectrophoretic analysis of fractions obtained by isoelectric focusing of cat allergen extract (Fig. 2, lower trace): 70-84, fractions from the isoelectric profile corresponding to the second skin test active region (mean $pI=4.35$); Post G-200, allergen active pool from Fig. 1; anti-ce, rabbit anti-cat extract antiserum. Arrow indicates precipitin line correlating closely with skin test activity.

denaturation than allergen $pI=4.1$, which at this stage appeared to be present at a comparable concentration.

The role of serum proteins, particularly albumin, as potential allergens in various tissues of mammalian origin has been discussed previously and recent reports have indicated that albumin is an important allergen in dog (Yman, Brandt & Ponterius,

1973) and horse (Ponterius *et al.*, 1973) epithelia but less important in cat extracts (Brandt *et al.*, 1973).

To the best of our knowledge the cat allergen $pI=4.1$ is the first feline allergen to be isolated and characterized. When fractionated on a calibrated column of Sephadex G-200 the majority of allergenic activity was eluted in fractions corresponding to a molecular weight of 55,000 with a small amount of activity associated with fractions of molecular weight 27,000. Other authors have estimated the molecular weight of cat

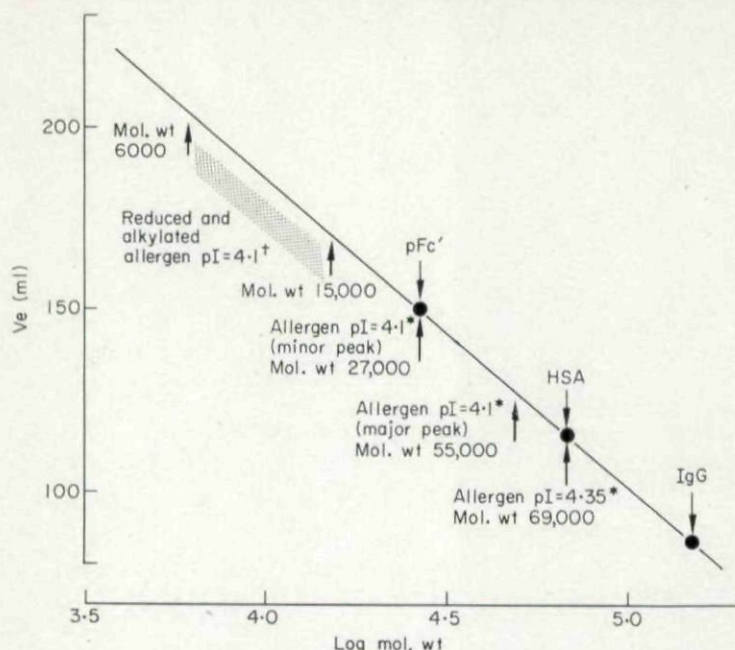


Fig. 6. Apparent molecular weights of cat allergens obtained using Andrews (1964) plot of standardizing proteins. Assumed mol. wts for standards were as follows: IgG (150,000), human serum albumin (69,000) and pFc' fragment of human IgG (27,000).

At neutral pH* cat allergen $pI=4.35$ eluted with a mol. wt of 69,000 and cat allergen $pI=4.1$ with a mol. wt of 55,000. A minor peak of allergen $pI=4.1$ also eluted with a mol. wt of 27,000. After reduction, alkylation and dissociation† the $pI=4.1$ allergen eluted within the mol. wt range 6000–15,000.

allergens as 24,000–70,000 (Holford-Strevens, 1973), 30,000–60,000 (Ohman *et al.*, 1973) and 32,000 (Brandt *et al.*, 1973) but these figures were obtained using unfractionated extracts which may have contained more than one allergen.

The molecular weight of cat allergen $pI=4.1$ labelled with I^{125} (55,000) was not changed after extensive dialysis in a dissociating buffer, pH 2.2. However, after reduction with mercaptoethanol and alkylation, all the radioactivity was in a peak eluted in the molecular weight region 6000–15,000. These observations are consistent with an allergen subunit structure of four polypeptide chains (mol. wt approx. 13,000) linked by disulphide bridges. According to this model the allergen exists primarily as a tetramer of molecular weight 55,000 and, possibly as a result of denaturation during isolation, can also occur as a dimer (mol. wt 27,000). Such an allergen would satisfy the prerequisite for activity suggested by Stanworth (1973), i.e. 'a dimeric structure is an essential requirement for allergenicity'.

Table 1. Amino acid composition of cat allergen pI=4.1

Amino acid*	Cat allergen pI=4.1		Cod allergen M (residues per 109 residues)‡	Ragweed allergen E (residues per 108 residues)§	IgG (C _H 3 domain)¶
	Number of residues per four residues of phenylalanine	Nearest integer			
Lysine	6.8	7	12	6	9
Histidine	2.2	2	0	2	3
Arginine	4.2	4	1	5	3
Aspartic acid	9.8	10	13	16	10
Threonine†	10.2	10	2	6	8
Serine†	10.0	10	6	9	14
Glutamic acid	15.0	15	10	8	14
Proline	5.0	5	0	5	9
Glycine	12.0	12	10	12	6
Alanine	10.4	10	25	10	3
Valine	5.6	6	6	8	8
Methionine	2.4	2	0	2	2
Isoleucine	3.4	3	5	7	2
Leucine	6.2	6	8	7	9
Tyrosine	1.8	2	1	1	5
Phenylalanine	4.0	4	10	4	4

* Tryptophan and half-cystine not determined.

† Corrected values.

‡ Data of Elsayed *et al.* (1972) modified for comparison.

§ Data of King, Norman & Tao (1974) modified for comparison.

¶ Protein Eu sequence, residues 334-446 (Edelman *et al.*, 1969).**Table 2.** Carbohydrate composition of cat allergen pI=4.1

Sugar	Content (n moles/mg allergen)
Mannose	8.8
Galactose	4.8
Glucose	16.1
Glucosamine	0.7
Fucose	Trace
Sialic acid	Trace
Glucosaminic acid	Trace

The amino acid composition of cat allergen pI=4.1 was calculated assuming a total amino acid content of 108 residues (mol. wt 12,000, i.e. a subunit of the putative tetramer) and in Table 1 is compared with the amino acid composition of a single homology region (C_H3) of the human γ -chain of IgG which has 109 residues. Such a comparison shows the allergen to be relatively deficient in proline, a residue which is known to be involved in the coiling of peptide chains (Springall, 1954) and which

probably contributes to the compactness of the immunoglobulin domain. In contrast the allergen is very rich in the neutral amino acids glycine and alanine which occur less frequently in the Cy3 homology region. The ratio of acidic to basic amino acids for the cat allergen is 1.9 and for the immunoglobulin subunit, 1.7. Comparison of the amino acid composition of the cat allergen with two other well-characterized allergens (Table 1) shows that the codfish allergen M has no proline and ragweed antigen E is also relatively deficient in this residue. Both allergens are also comparatively rich in glycine and alanine.

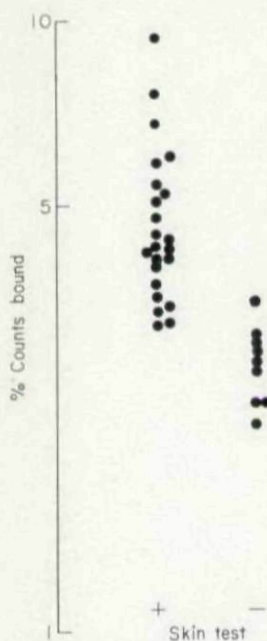


Fig. 7. Radioallergosorbent test for IgE antibodies in adults classified as skin test negative or skin test positive using Bencard cat prick test solution. Mixed cat allergens ($pI=4.1$ and $pI=4.35$) obtained after Sephadex G-200 gel filtration (Fig. 1) were coupled to Sigma cellulose particles.

The major sugars detected in the cat allergen $pI=4.1$ were mannose, galactose and glucose. Both galactose and mannose have been detected in horse dandruff and galactose, mannose and glucose in human dandruff (Berrens, 1971) but it is not known whether these sugar residues contribute to the allergenicity of the epithelial danders.

It is likely that there are several distinct cat epithelial allergens. We have used a single cat sensitive subject for skin testing and the recognition of allergens is clearly restricted by the reactive capacity of the individual used. Nevertheless when the combined antigens ($pI=4.1$ and $pI=4.35$) were used in a RAST procedure the serum of most cat sensitive individuals showed significant levels of binding. This suggests that both cat serum albumin and the immunochemically characterized allergen $pI=4.1$ are of major importance in cat sensitivity.

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