

Immunochemical Characterization of Reference Alder (*Alnus glutinosa*) and Hazel (*Corylus avellana*) Pollen Extracts and the Partial Immunochemical Identity between the Major Allergens of Alder, Birch and Hazel Pollens

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Dialysed extracts of alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) pollens were characterized by immunochemical methods. The alder pollen extract contained at least 33 distinct antigens of which one, antigen 5, was classified as a major allergen, three, antigens 4, 10, and 17, were classified as intermediate allergens and two, antigens 19 and 23, were classified as minor allergens. The molecular weight and pI of the major allergen were determined to be 19,000 D and 5.2, respectively. The hazel pollen extract contained at least 40 distinct antigens, two of which were classified as major allergens, antigens 8 and 13, three as intermediate allergens, antigens 12, 17, and 26, and seven as minor allergens, antigens 6, 11, 16, 18, 29, 39, and 40. The molecular weights and pI's of the major allergens of hazel pollen were determined to be: antigen 8; Mw = 12,000 D, pI = 5.1 and < 3.5, antigen 13; Mw = 26,000 D, pI = 5.1. Further, crossed line immunoelectrophoresis and tandem crossed immunoelectrophoresis of alder, birch and hazel strongly indicate that there exists an immunochemical partial identity between the major allergens (antigens 5 (alder), 23 (birch), and 13 (hazel)) from these tree pollens.

Key words: alder; birch; hazel; major allergens; partial identity.

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Alder (*Alnus glutinosa*) and hazel (*Corylus avellana*) are two of the earliest (February through April) pollen allergen sources in northern Europe (5). There are many indications that individuals allergic towards birch pollen exhibit additional sensitivity towards alder and/or hazel pollens (6). This cross sensitivity could originate either from common IgE epitopes in some of the allergens from alder, birch and hazel, or the allergic individuals could "simply" be specifically sensitized to more than one allergen

source. To investigate the origin of the cross sensitivity it is necessary to identify the allergenic components of the pollens; recently the allergens of birch (*Betula verrucosa*) pollen were classified and characterized (11). Here we report the characterization and classification of the allergens in dialysed extracts of alder and hazel pollens, and an immunochemical characterization of the partial identity between the major allergens of alder, birch and hazel pollens.

MATERIAL AND METHODS

Alder, birch and hazel pollen extracts

Pollens from alder (*Alnus glutinosa*) and hazel (*Corylus avellana*), both containing less than 2.0 % (w/w) non-pollen debris and less than 0.5 % (w/w) foreign pollen, were obtained from Allergon, Angelholm, Sweden. The pollens, 50 g of each species, were extracted 1:10 (w/v) in 0.125 M ammonium hydrogen carbonate, 0.015 M sodium azide pH 8.3, for 24 h at 5°C. Particulate matter was removed by centrifugation for 1 h, 15°C at 35,000 *g* and the supernatants were dialysed three times against water for 24 h, 5°C each and lyophilised. The lyophilised extracts of alder, denoted Ag, and hazel, denoted Ca, contained 78.8 % (w/w) and 79.9 % (w/w) protein, respectively, as determined by the method of Lowry (15). An extract of birch (*Betula verrucosa*) pollen, denoted Bv, was prepared as described earlier (11). The lyophilised extracts and working solutions (10 mg extract/ml 0.03 M sodium hydrogen carbonate, 0.086 M sodium chloride, 50 % (v/v) glycerole pH 8.3) of the extracts were stored at -20°C.

Rabbit antibodies

Rabbit anti-alder pollen extract (a-Ag) and anti-hazel pollen extract (a-Ca) antibodies were raised by immunizing rabbits with either Ag or Ca (three rabbits for each extract). The immunization and isolation of the immunoglobulins were performed essentially as described by Harboe & Ingild (8). Rabbit anti-horse hair and dander (a-H) and anti-birch pollen extract (a-Bv) antibodies were raised as described by Ipsen and Løwenstein (11, 16).

Patients

Patients allergic to alder and/or hazel were selected on the basis of clinical record, skin prick testing, nasal provocation test and radioallergosorbent test (RAST) (11). Serum samples from 31 patients were used for identification and classification of alder and hazel allergens by

means of CRIE. A serum pool was made from equal volumes of each of the sera.

RAST and RAST-inhibition

RAST was performed essentially as described by Ceska et al. (4) using paper disc allergosorbents of Ag or Ca (coupling concentration: 2.0 mg/ml). RAST-inhibition was performed as described elsewhere (11, 20).

Immunoelectrophoretic methods

Crossed immunoelectrophoresis (CIE) and crossed radio immunoelectrophoresis (CRIE) were performed essentially as described recently (10, 19). In short, CIE was performed in 1 % (w/v) agarose (type HSA, Litex, Denmark) (0.073 M Tris, 0.024 M barbital, 0.0006 M calcium lactate, pH 8.6) at 15°C; first dimension electrophoresis: 25 min, 10 V/cm; second dimension electrophoresis: 16 h, 2 V/cm; with the use of 17.1 μ l (a-Ag or a-Ca) and 13.3 μ l a-Ag or 20 μ l a-Ca/cm² in the anodic and cathodic antibody-containing gels, respectively. CRIE was performed using cold-dried non-stained CIE plates which were incubated with 0.5 ml patient serum in 8.0 ml of 0.067 M phosphate buffer, pH 7.0, for 24 h at 25°C, followed by incubation with approximately 140 nCi of 125-I-anti-IgE in 8.0 ml of phosphate buffer, pH 7.5, 0.3 % (w/v) bovine serum albumin, 0.154 M sodium chloride, 0.015 M sodium azide and 0.027 M EDTA for 24 h at 25°C. The plates were washed three times (20 ml of 0.154 M sodium chloride, 1 % (v/v) Tween 20) and subjected to autoradiography on Kodak XL-1 X-ray film.

Crossed line immunoelectrophoresis (CLIE) was performed essentially as described by Krøll (12). In short: first dimension electrophoresis was performed as described for CIE and second dimension electrophoresis was run through an intermediate gel containing either the major allergen of birch pollen or Ca; the antibodies used were a 1:1:1 mixture of a-Ag, a-Bv and a-Ca (12 μ l/cm²). Tandem crossed immunoelectrophoresis (TCIE) was performed as described by Krøll (13) using the same 1:1:1

antibody mixture. Crossed radio line immunoelectrophoresis (CLIE) and tandem crossed radio immunoelectrophoresis (TCRIE) were performed as described for CRIE with the use of cold-dried CLIE and TCIE plates in place of cold-dried CIE plates.

Fused rocket immunoelectrophoresis was performed essentially as described by Harboe & Svendsen (9), in short: immunoelectrophoresis was performed in 1% (w/v) agarose (0.073 M Tris, 0.024 M barbital, 0.0006 M calcium lactate) at 15°C, 16 h, 2 V/cm; the samples applied to the wells diffused 1 h before start of the electrophoresis into the antibody-containing gel (the amounts of antibodies were the same as for CIE).

Immunoabsorption

Rabbit a-Ag, a-Ca and a-H antibodies (3 ml) were each covalently coupled to 0.5 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturers' description. Ag (10 mg) and Ca (10 mg) were each applied on columns of Sepharose-a-Ag or Sepharose-a-Ca and Sepharose-a-H and the columns were eluted with three column volumes of 0.1 M sodium hydrogen carbonate, 0.1 M sodium chloride, pH 8.3. The allergenic activity of the immunoabsorbed (column eluate) Ag and Ca were estimated by RAST-inhibition.

Skin prick testing (SPT) was performed as described by Aas (1).

Size exclusion chromatography and isoelectric focusing

Size exclusion chromatography (SEC) of 30 mg Ag was performed on a ACA-54 (LKB, Sweden) 65 cm × 2 cm² (K 16/70 Pharmacia, Uppsala, Sweden) column. SEC of 37.2 mg Ca was performed on a G-75 (Pharmacia, Sweden) 63 × 2 cm² (K 16/70 Pharmacia, Uppsala, Sweden) column. Both columns were equilibrated and eluted with 0.03 M sodium hydrogen carbonate, 0.086 M sodium chloride, 0.015 M sodium azide, pH 8.0. The columns were calibrated for molecular weight determinations with the following compounds: blue dextran

(BD), human serum albumin (HSA), ovalbumin (OVA), chymotrypsinogen (CYM), myoglobulin (MYO) and cytochrome c (CYT c).

Column isoelectric focusing (CIF) (LKB, ampholine column type 8101) of Ag and Ca pollen extracts (50 mg of each) were performed in a 0 to 50% (w/v) sucrose gradient containing 1% (v/v) LKB ampholine, pH 3.5 to 10 at 200 V and 2.2 mA for 48 h (17).

RESULTS

Characterization of antigens and allergens from alder and hazel pollen extracts

The Ag and Ca extracts contained, as judged by CIE, 33 and 40 distinct antigens, respectively. Of the 33 Ag antigens, 32 moved towards the anode and one antigen moved towards the cathode at pH 8.6 (Fig. 1A), while 37 Ca antigens moved towards the anode and three antigens moved towards the cathode at pH 8.6 (Fig. 1B), two of Ca antigens were only detected by means of CRIE.

The alder pollen extract contained, as judged by CRIE, six allergens (Fig. 2A) of which at least one, antigen 5, can be classified as a major allergen (19), since 93% of the sera contained IgE towards this antigen; antigen 17 may be a major allergen, but a large part of the observable radiostaining seems to originate from co-precipitated (14) antigen 5; antigens 4 and 10 are both intermediate allergens while antigens 19 and 23 can be considered minor allergens.

The hazel pollen extract contained, as judged by CRIE, 13 allergens (Fig. 2B) of which antigens 8 and 13 can be classified as major allergens; antigens 12, 16, 17, 26, and 39 as intermediate allergens and antigens 6, 11, 18, 29, and 40 as minor allergens.

Rabbit a-Ag and a-Ca antibodies

Fig. 3 shows that the rabbit a-Ag and a-Ca antibodies employed here were capable of absorbing all the RAST-inhibitory capacity of the dialysed Ag and Ca extracts, since specific immunoabsorbed extracts did not inhibit the binding of IgE to antigen coupled paper discs in

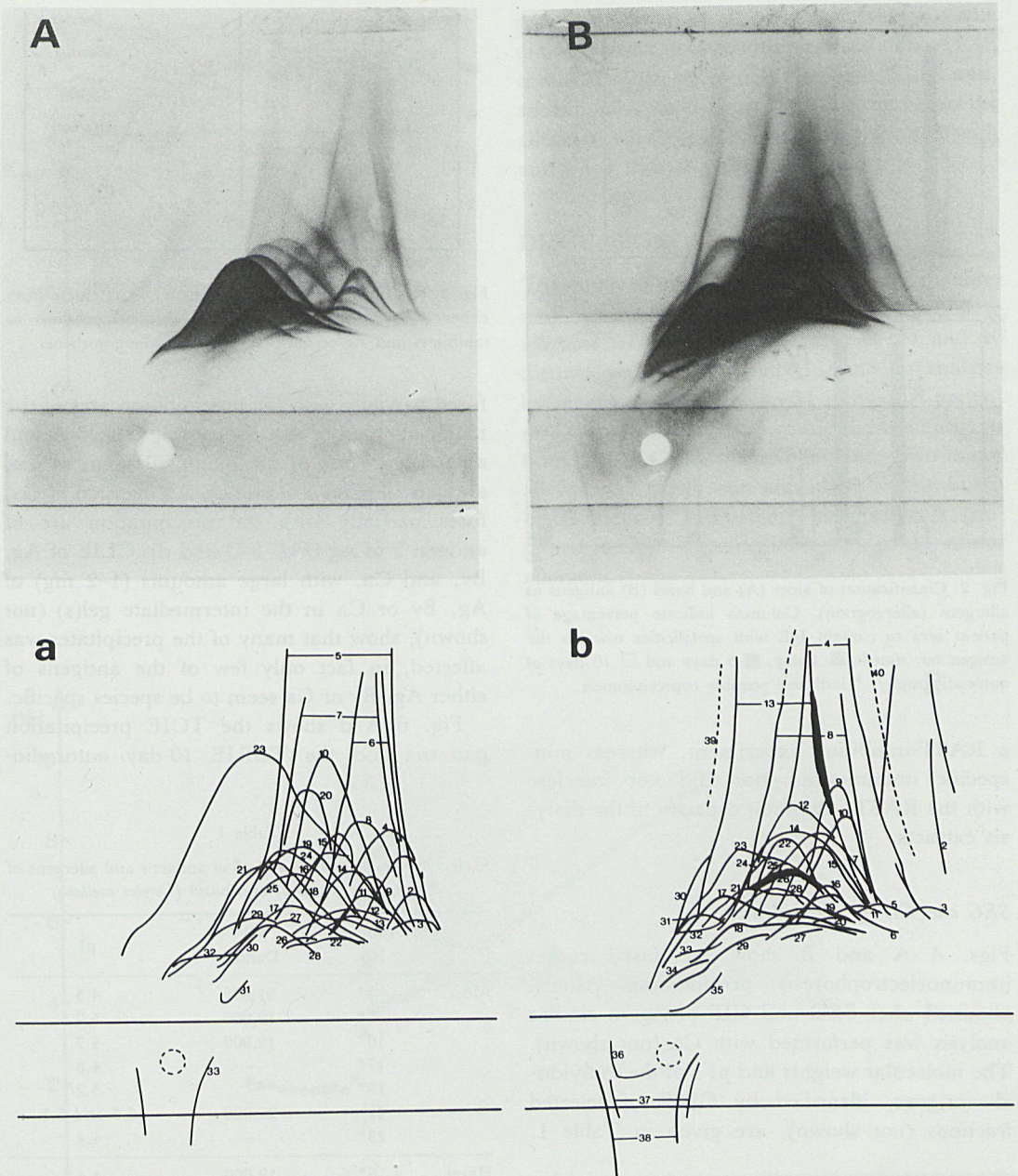


Fig. 1. Crossed immunoelectrophoresis of 70 μ g of alder pollen extract (A) and 70 μ g hazel pollen extract (B) and drawings (a: Ag, b: Ca) of the reference precipitation patterns. Precipitates were arbitrarily numbered from the anodic to the cathodic side. Broken lines indicate precipitates visible in CRIE only.

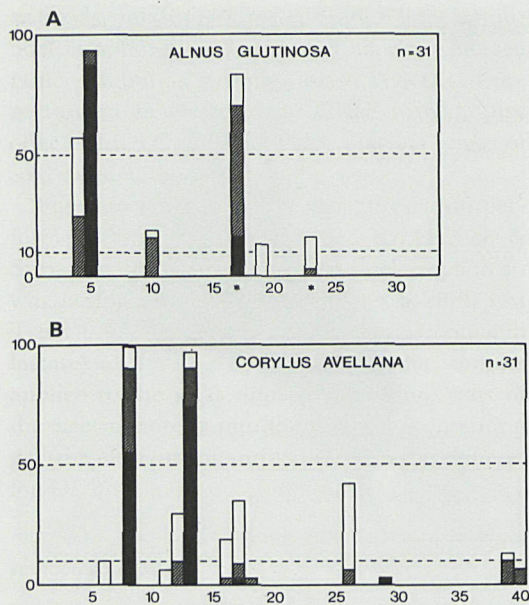


Fig. 2. Classification of alder (A) and hazel (B) antigens as allergens (allergogram). Columns indicate percentage of patient sera to contain IgE with specificities towards the antigen no. stated. ■ 1 day, ■ 3 days and □ 10 days of autoradiography. * indicates possible coprecipitation.

a RAST-inhibition experiment, whereas non-specific immunoabsorption did not interfere with the RAST-inhibition capacity of the dialysis extracts.

SEC and CIF of Ag and Ca

Figs. 4 A and B show the fused rocket immunoelectrophoresis precipitation pattern obtained after SEC and CIF of Ag, a similar analysis was performed with Ca (not shown). The molecular weights and pI's of the individual antigens, identified by CLIE of selected fractions (not shown), are given in Table 1.

Partial identity between the major allergens from alder, birch and hazel pollens

The precipitation patterns of CLIE and 10-day autoradiographs of CRLIE of Ag, Bv and Ca with the major allergen of Bv, antigen 23, or Ca in the intermediate gel(s), are shown in Fig. 5 A-d. The precipitation line of the major allergen of birch pollen, antigen 23, lifts and

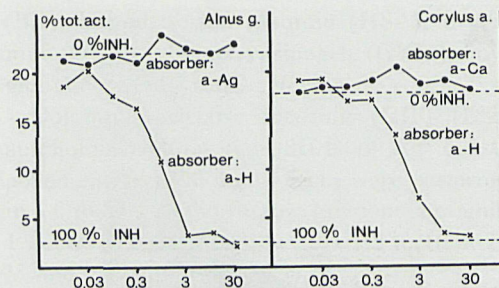


Fig. 3. RAST-inhibition curves obtained using eluate from either a-Ag, a-Ca or a-H immunosorption columns as inhibitors and Ag or Ca paper discs as allergosorbents.

fused partially with the precipitation arcs of the major allergen of Ag, antigen 5, (Fig. 5 A and a) and with one of the major allergens of Ca, antigen 13 (Fig. 5 C and c). An allergen of Ca, fuses partially with the precipitation arc of antigen 5 of Ag (Fig. 5 D and d). CLIE of Ag, Bv, and Ca, with large amounts (1-2 mg) of Ag, Bv or Ca in the intermediate gel(s) (not shown), show that many of the precipitates was affected, in fact only few of the antigens of either Ag, Bv or Ca seem to be species specific.

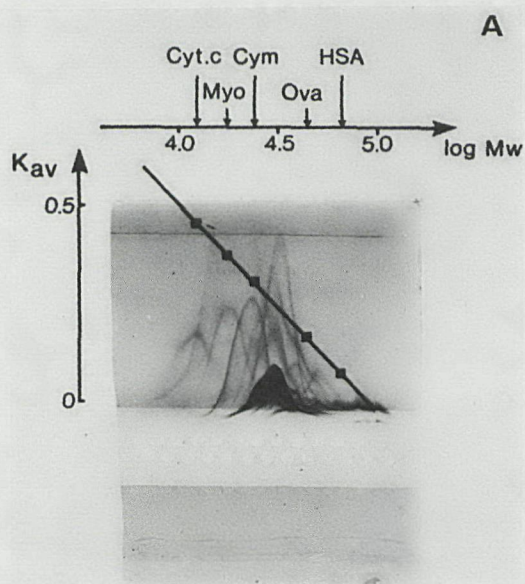
Fig. 6 A-d shows the TCIE precipitation patterns and the TCRIE 10-day autoradio-

Table 1

Molecular weight (MW) and pI of antigens and allergens of alder (*Alnus glutinosa*) and hazel (*Corylus avellana*)

Extract	Antigen No.	MW Daltons	pI
Alder	4*	31,000	4.5
	5*	19,000	5.2
	10*	19,000	4.7
	17*	-	4.8
	19*	-	5.2
	21	26,000	6.5 and 5.5
	23*	-	4.4
Hazel	6*	18,000	4.4
	7	22,000	4.5
	8*	12,000	< 3.5 and 5.1
	9	22,000	-
	10	-	3.7
	13*	26,000	5.1
	16*	24,000	5.1
	17*	22,000	6.9
	18*	18,000	-
	26*	-	5.1

* Precipitates which in CRIE bind patients' IgE.



graphs of different TCIE combinations of the dialysed extracts of Ag, By, and Ca. The TCIE results confirm those obtained with CLIE and, additionally, identify antigen 13 from Ca as the allergen which exhibits partial identity with antigen 5 from Ag (Fig. 6 C and c).

DISCUSSION

The analysis of the dialysed extracts of alder and hazel pollens by CIE revealed that these extracts were composed of at least 33 and 40 distinct antigens, respectively, and an analysis by means of CRIE, using 31 individual patient sera, classified antigen 5 of Ag and the antigens 8 and 13 of Ca as major allergens. Two major allergens of hazel were also identified by Borch & Skålhegg (3). Further, both extracts contained a few intermediate and some minor allergens.

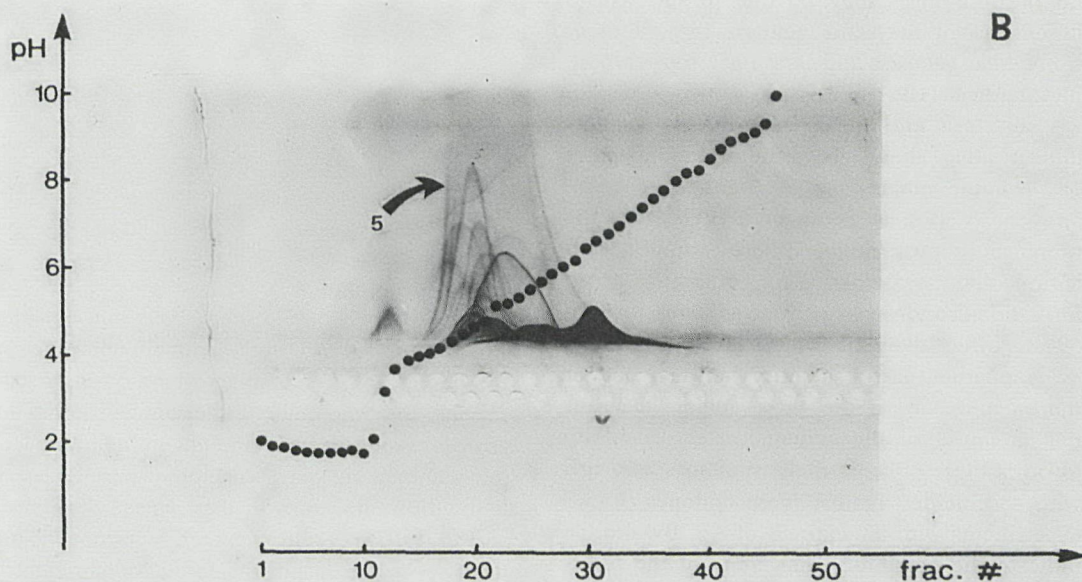


Fig. 4. A: Fused rocket immunoelectrophoresis precipitation pattern obtained with 7 μ l of selected fraction after SEC of 30 mg Ag on an ACA-54 column. Solid line indicates the plot of K_{av} vs. $\log (Mw(\text{calibration proteins}))$.

B: Fused rocket immunoelectrophoresis precipitation pattern obtained with 15 μ l of each of the fractions eluted after CIF of 50 mg Ag. Dotted line indicates pH-gradient.

The CRIE identification and classification of alder and hazel pollen antigens as allergens probably accounts for all the important allergenic molecules in the dialysed extracts, since the rabbit a-Ag and a-Ca antibodies, covalently coupled to Sepharose 4B, were able to absorb all the RAST-inhibition capacity of the dialysed extracts. However, the presence of additional antigens/allergens in the crude nondialysed extracts is not ruled out, in particular if such molecules are sparingly soluble in the extraction buffer, are labile, and/or are low molecular weight ($< 10,000$ D) substances.

The molecular weight and isoelectric point (pI) distribution of the alder and hazel pollen antigens resemble those reported for birch (2, 11) and alder (7), and the physico-chemical parameters determined for the major allergen from *Alnus glutinosa* (Mw = 19,000 D, pI = 5.2) approximately equal those reported by Apold et al. (2) (Mw = 22,500 D, pI = 4.78) for an important allergen from *Alnus incana* (the minor differences may be due to the species differences of the same genus). The allergens from *Alnus glutinosa* and *incana* are most probably identical (18).

The CLIE and TCIE experiments strongly indicate that there are structural similarities between the major allergens, antigen 5 (Ag), antigen 23 (Bv), and antigen 13 (Ca), i.e. the rabbit antibodies employed here contain specificities that recognize one or more conformational and/or sequential epitopes in each of the three allergens which are much alike. The apparent partial identity between one or more epitope(s) of the three major allergens may account for the patients' cross sensitivity towards these pollens, if the patients have IgE with specificities against these epitopes. CLIE experiments with the extracts of Ag, Bv and Ca indicate the possibility that some of the inter-

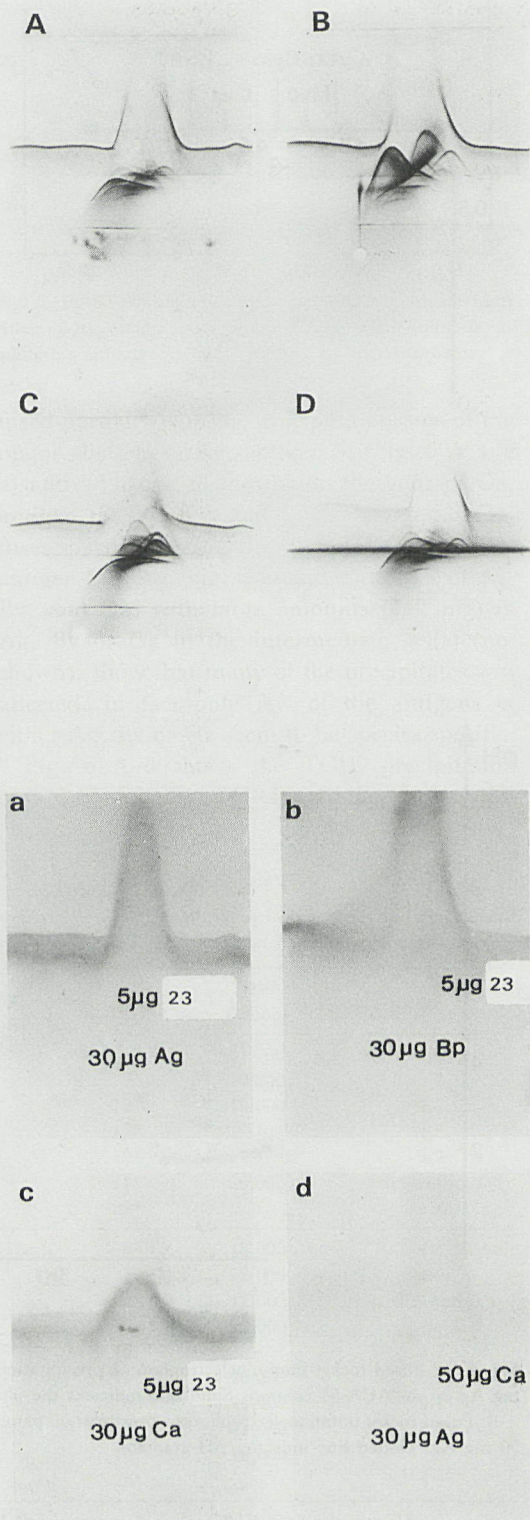


Fig. 5. CLIE (A-D) and CRLIE (a-d, 10 day autoradiographs) of alder (A, a, D, d), birch (B, b) and hazel (C, c) pollen extract performed with major allergen of birch pollen, antigen 23, (A, a, B, b and C, c) or Ca (D, d) in the intermediate gel(s).

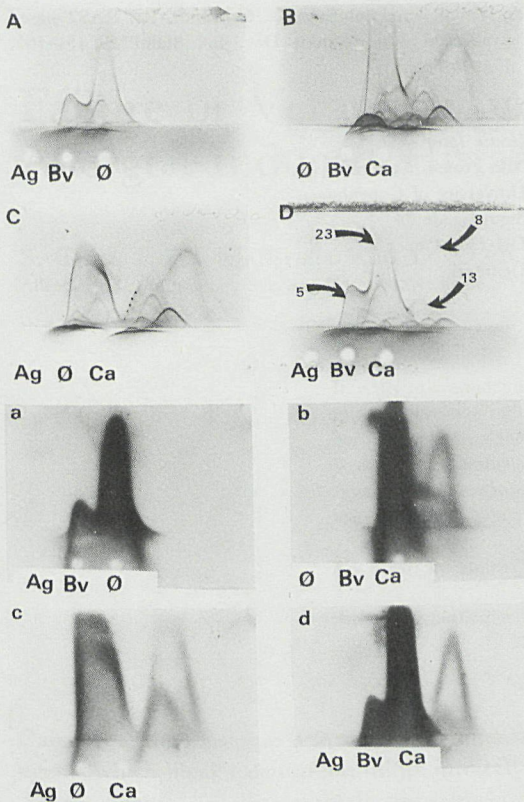


Fig. 6. TCIE (A-D) and TCRIE (a-d, 10-day autoradiographs) of alder and birch (A, a), birch and hazel (B, b), alder and hazel (C, c) and alder, birch and hazel (D, d).

mediate/minor allergens of Ag, Bv and Ca may be partially identical, but further experiments are required to investigate this thoroughly. If the intermediate/minor allergens are species specific, then the patients' IgE response towards these allergens indicates that the patients are specifically sensitized towards the individual pollen sources. Further, the existence of the second major allergen of hazel pollen (antigen 8) points to specific sensitization, but there are indications that IgE towards antigen 13 also reacts with antigen 8 (3), even though a partial identity between the two hazel major allergens is not detectable.

Currently, attempts to identify and characterize the structural basis of the immunochemical partial identity between the major allergens

of Ag, Bv and Ca, and further investigation of the intermediate/minor allergen partial identity, are undertaken in our laboratory.

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