

Comparative Studies on Tree Pollen Allergens

XIII. Partial Characterization of the Alder (*Alnus incana*) Pollen Extract by Two-Dimensional IEF/SDS-PAGE Electrophoresis Combined with Electrophoretic Transfer and Immunoautoradiography

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Abstract. Horizontal isoelectric focusing (IEF) and two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (2-D SDS-PAGE) combined with electrophoretic transfer (ET) and immunoautoradiography (IAR) were used to characterize the crude alder (*Alnus incana*) pollen extract (AI crude) and fraction AI 34. IEF resolved AI crude and AI 34 into 34 and 39 distinct protein bands, respectively, when stained with Coomassie brilliant blue. The band patterns were similar and the majority of bands were located in the pH region 4.0-7.0. After ET and IAR, the distribution of antigenic reactivities was similar to that of the protein stain. Allergic reactivity was detected in the pH region 4.6-5.2 only, where two bands at pH 4.9 demonstrated dominating IgE-binding properties. The 2-D reference maps of AI crude and AI 34 consisted of 40 and 45 protein spots, respectively. The autoradiogram of AI crude demonstrated 35 spots capable of binding rabbit IgG. The main IgE-binding zone was located at pH 4.6-5.2 and 20 kilodaltons with an area of weaker autoradiographic signals at pH 4.6-5.2 and 40 kilodaltons. ET from polyacrylamide gels to nitrocellulose (NC) membranes was quantitative and IAR gave significant radiostaining of spots containing 0.01 and 0.1 μ g of AI crude in the IgG and IgE systems, respectively. For the characterization of the alder pollen extract, horizontal 2-D SDS-PAGE combined with ET and IAR provided a powerful supplement to conventional crossed immuno- and radioimmuno-electrophoretic techniques (CIE/CRIE).

Introduction

Crossed immunoelectrophoresis (CIE) and crossed radioimmuno-electrophoresis (CRIE) have been established as useful tools in the identification, standardization and purification of heterogeneous allergen extracts [24-27]. However, these methods are hampered with a number of limitations, particularly attributed to the animal immune response: failure of relevant antigenic determinants to induce antibody synthesis will leave antigens and allergens unrecognized. In addition, the presence of blocking antibodies may eventually interfere with the binding of IgE. The intra- and interindividual variability of the rabbit immune response makes the precise comparison of results between laboratories difficult even when using serum pools. This problem is potentiated by the arbitrary numeration of antigenic precipitates in CIE bearing no reference to the immunochemical properties of the antigens in question. Furthermore, the ef-

fects of coprecipitation - the arrest of allergenic proteins within the precipitate network of nonallergenic antigens during second directional electrophoresis - has been accepted as a major pitfall of the CRIE methodology [1, 14, 27]. Finally, the resolving efficacy of 1% agarose in the first directional separation is low, the electrophoretic mobility is mainly charge-dependent and eventually bidirectional.

In a previous publication [5], the aqueous alder (*Alnus incana*; AI) pollen extract was immunologically characterized by CIE, CRIE, fused rocket immunoelectrophoresis (FRIE), FRIE with autoradiography (FRIEWA), radioallergosorbent test (RAST), RAST inhibition and skin prick test (SPT). The CIE reference patterns of the crude extract (AI crude) and fraction AI 34 were composed of 27 and 24 precipitates, respectively. Antigenic precipitate No. 7 (Ag 7) was demonstrated to be the major IgE-binding antigen, while Ag 1 and Ag 11 were characterized as intermediate allergens of alder pollen.

To supplement the conventional immunoelectrophoretic techniques, recent advances in electrophoretic protein separation using polyacrylamide gels (PAG) were applied to the characterization of alder pollen allergens: horizontal isoelectric focusing (IEF) has been widely used due to the high efficiency in separating proteins of small charge differences [22]. The molecular weight dependent migration of sodium dodecyl sulfate (SDS)-conjugated polypeptides [19, 23] in PAG has been used for one dimensional separation [11] and in the combination with IEF developed into highly powerful two-dimensional (2-D) electrophoretic techniques [16]. By successive electrophoretic transfer (ET) to nitrocellulose (NC) membranes, the immunological reactivities of the separated proteins could be investigated by immunoautoradiography (IAR) [2, 21]. The analysis of allergen extracts from cereal grains, *Dermatophagoides pteronyssinus* and *Parietaria judaica* pollen by PAG electrophoresis (PAGE), ET and IAR has been reported [3, 10, 18, 20].

The aim of the present study was (1) to establish highly resolved reference fingerprints of AI crude and fraction AI 34 by horizontal thin-layer IEF and 2-D IEF/SDS-PAGE, and further (2) to identify the IgG- and particularly the IgE-binding proteins by ET to NC and ¹²⁵I-IAR.

Material and Methods

Extraction and Gel Filtration Chromatography

Preparation of AI crude and isolation of fraction AI 34 was carried out as outlined elsewhere [5].

Rabbit Antisera and Human Reagin Sera

The rabbit serum pool described in a previous communication was used as primary antibody source [5]. Reagin serum from one individual with an evident clinical history of tree pollen allergy (rhino-conjunctivitis), highly positive SPT (mean diameter: 2.0 relative to histamine 1 mg/ml) to alder and birch (*Betula verrucosa*), RAST class 3 to alder and birch, and class 0 to hen's egg white was used. In CRIE, this particular serum significantly radio-stained precipitates Ag 7 and Ag 11 of AI crude according to 9 and 3 intensity points, respectively [5].

Electrophoresis Equipment and Chemicals

Both first and second directional electrophoresis were run using the horizontal LKB 2117 Multiphor system (LKB, Bromma, Sweden). Acrylamide (for electrophoresis), N,N'-methylenebisacrylamide (bis), N,N,N',N'-tetramethylethylenediamine (TEMED) and Coomassie brilliant blue (CBB) R 250 were purchased from Eastman Kodak Co. (Rochester, N.Y., USA). SDS, 2-mercaptoethanol and ammonium persulfate were purchased from Sigma

Chemical Company (St. Louis, Mo., USA). The carrier ampholytes (Ampholines) were manufactured by LKB Produkter AB (Bromma, Sweden).

Isoelectric Focusing

Horizontal IEF was run in 1.5-mm thick polyacrylamide gels (T: 5% and C: 3%). The pH gradient 3.5-9.5 was obtained by mixing appropriate amounts of ampholytes according to the manufacturer's instructions [28]. Isoelectric point markers of the pI calibration kit range 4.7-10.6 (BDH Chemicals Ltd., Poole, UK) were run to control gradient formation. For protein fixation and removal of the carrier ampholytes, the gel was soaked for 2 h in three changes of fixing solution. After staining and destaining, the gel was temporarily stored in destaining solution. IEF gels subjected to ET and IAR either directly or after 2-D electrophoresis were kept unstained in destaining solution after the fixing procedure.

SDS-Polyacrylamide Gel Electrophoresis

Horizontal 2-D electrophoresis was run in homogeneous 10% (T = 10.2%, C = 2.6%) or linear 7.5-15% gradient polyacrylamide gels (1.5 mm thick) [4]. The gels were polymerized in a casting set prepared to mould two longitudinal troughs (105 × 10 × 0.75 mm) for the IEF gel lanes. Before application, the IEF gels were equilibrated for 15-20 min in 10 mM phosphate buffer (pH 7.1) containing 1.0% SDS and 0.5% 2-mercaptoethanol. The globular marker proteins lysozyme (14.3 kilodaltons), β -lactoglobulin (18.4 kilodaltons), trypsinogen (24.0 kilodaltons), pepsin (34.7 kilodaltons), OA (45.0 kilodaltons) and BSA (66.0 kilodaltons) were used as standard molecular weight markers. 2-D electrophoresis was run for 5 h at 215 mA constant current with the cooling reservoir kept at +10°C. Electrode buffer was 100 mM phosphate buffer (pH 7.1) containing 0.1% SDS.

After fixing for at least 1 h, CBB staining was performed over night at room temperature. Destaining was done until a slight residual background staining of the gel matrix was still visible. Silver staining was performed at 50°C using the Bio-Rad silver stain kit (Bio-Rad, Richmond, N.Y., USA) according to the manufacturer's instructions. ET was performed immediately after termination of the 2-D run, omitting the fixing and staining procedures.

Electrophoretic Transfer

ET of proteins from the IEF- and 2-D gels was carried out largely in accordance with Towbin et al. [21] and Burnette [2] using a Bio-Rad transblot cell fitted with cooling coil, magnetic stirrer and thermometer. Voltages up to 100 V (max. 1.5 A) were delivered by a variable DC rectifier (Trans Electric, Bergen, Norway).

Sheets of pure NC with 0.1 μ m pore size (Schleicher & Schuell, Dassel, FRG) were used as immobilizing membranes. Prior to transfer, the gels were equilibrated for 5 min in reservoir buffer and placed on presoaked NC sheets. With a double layer of wetted Whatman No. 3 filter papers on both sides, the assembly was fixed between Scotch Brite paddings and the Transblot cell grid. The transfer from IEF gels was accomplished in 1% acetic acid, the NC membranes were oriented cathodically to the gel and voltage was increased stepwise to 80 V (10 V/cm). ET from 2-D gels was carried out in 25 mM Tris-HCl and 192 mM glycine in 20% methanol (pH 8.3). The NC sheets were placed on the anodic side of the gels and voltage was increased stepwise to 50 V (6.25 V/cm). The reservoir buffer was kept at +10°C during transfer which was continued for 16-18 h.

To check transfer efficacy, the gels were stained for residual proteins as described. NC membranes were stained either with CBB R 250 according to the rapid procedure [2] or with the Ponceau stain for 15 min and successive destaining in 0.5% CH_3COOH for 5×10 min [15].

Immunoradiography

Incubation Procedures. A modification of the procedure described by Burnette [2] was used to detect the IgG-binding capacity of the transblotted proteins. Unless otherwise stated, the following steps were carried out in 100-ml volumes at room temperature with occasional shaking: after ET, the NC membranes were washed in fresh transfer buffer for 10 min and in 70 mM phosphate-NaCl buffer (pH 7.20; PBS) for 30 min. Blocking of the residual free binding capacity for proteins was done with 10% BSA in PBS for 5 h at $+37^\circ\text{C}$. Incubation with rabbit antiserum serum 1 + 5 diluted in PBS to 15 ml was carried out over night. The immunoblots were then washed 5×10 min in PBS. As secondary antibodies were used ^{125}I -labelled donkey anti-rabbit IgG (Amersham International, Buckinghamshire, UK). The initial isotope activity in 15 ml PBS was 3×10^4 cpm/ml and the incubation period was 16–18 h.

The IgE detection procedure was identical to that above except for using the undiluted human reagent serum as primary antibody source and ^{125}I -anti IgE (Phadebas PRIST, Pharmacia Diagnostics, Uppsala, Sweden) in a concentration of 7×10^4 cpm/ml for the second incubation.

After a final washing, the immunoblots were air-dried, mounted on filter paper supports and sealed in Glad wrap prior to autoradiography.

Autoradiography. The prepared immunoblots were placed on Kodak AR5 (AR5) or Du Pont Cronex 4 safety films (CR4) which in turn were layered on the special super high-speed (SP) CaWO_4 front foil mounted in a medical x-ray film cassette (Siemens, FRG). The cassette was placed in an ultra-deep freezer (Forma Scientific, Marietta, Fla., USA) for exposure at -80°C [12]. Exposure intervals varied from 24 to 96 h after which the films were developed in a Kodak X-Omat RP automated processor for 90 s at 33°C [6].

Dot Immunobinding Assay

To optimize the immunodetection procedures and investigate the effect of SDS on immunoglobulin binding, a dot immunobinding assay was used [9]. Freeze-dried AI crude was reconstituted to 10 mg/ml in PBS, PBS containing 0.1% SDS and PBS containing 1% SDS and 0.5% 2-mercaptoethanol, respectively. From these stem solutions 4 10-fold dilutions were made and 1 μl of each was applied directly onto NC membranes and air-dried. As control, crude hen's egg white extract diluted in PBS to the same concentrations was used. IAR was carried out as for the transblots.

Results

The majority of proteins separated by IEF were focused within the pH range 4.0–7.0, while only a few bands were located in the more acidic and alkaline regions of the gel (fig. 1). A total of 34 individual bands could be detected in AI crude by CBB stain while fraction AI 34 was resolved into 39 bands. In general,

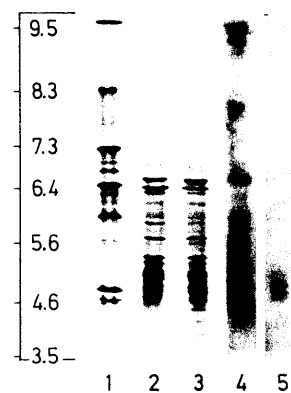


Fig. 1. Horizontal IEF run in 5% polyacrylamide with pI range 3.5–9.5. The focused IEF marker proteins are shown in lane 1 with the approximate pI intervals indicated to the left. Lanes 2 and 3 demonstrate reference patterns of AI 34 and AI crude resolved into 39 and 34 bands, respectively. Lane 4 shows the autoradiogram of AI crude after ET and incubation with rabbit serum and ^{125}I -labelled goat anti-rabbit IgG. The corresponding autoradiogram incubated with the reagent human serum and ^{125}I -anti-IgE is seen in lane 5. Autoradiography was performed using the CR4 film and SP screen with exposure for 24 h at -80°C .

the band patterns of AI crude and AI 34 were similar, but in the pH region 3.5–4.6 AI crude showed 3 additional intensively stained bands. Between pH 7.0 and 9.5, 5 bands with weak affinity to the protein stain which were not seen in AI crude could be demonstrated in fraction AI 34. 2-D separation of AI crude and AI 34 gave similar reference spot patterns containing approximately 40 and 45 spots, respectively (fig. 2, 3). A cluster of major and minor protein spots were located in the region of pH 4.6–5.2 and 20 kilodaltons; other dominating protein-containing areas were pH 6.4–7.0 at 20 and 45 kilodaltons. On both reference maps, only 4 protein spots were visible in the pH 7.0–9.5 region.

IAR performed on the AI crude proteins separated by IEF and electrophoretically transferred to NC showed IgG binding mainly in pH region 4.5–7.0. However, additional three IgG-binding bands were located at the cathodic end of the gel. One band located at pH 8.3 was not visualized by CBB stain

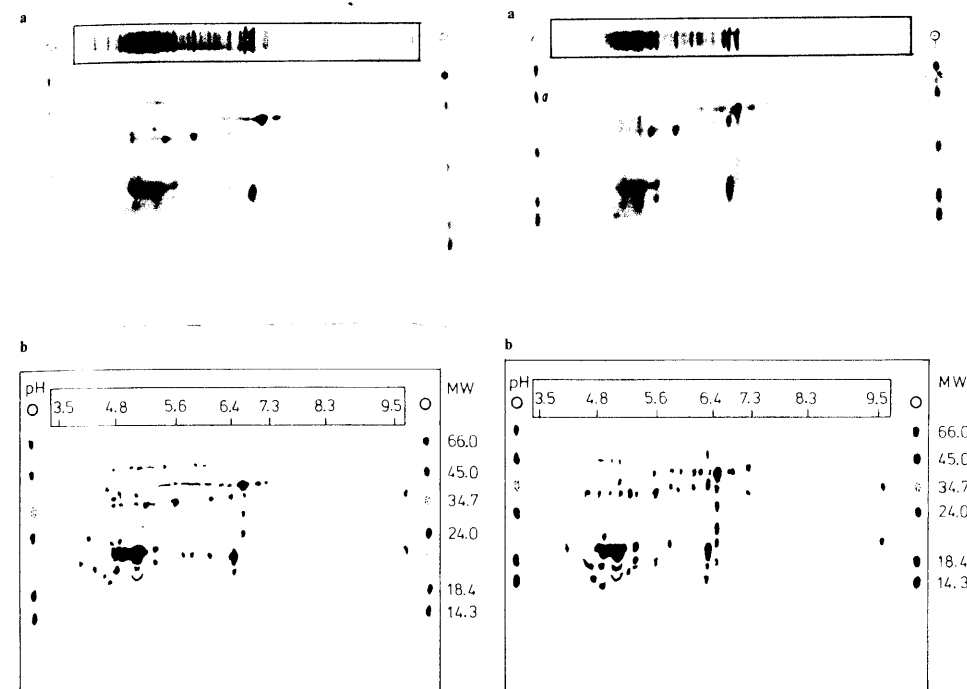


Fig. 2. a 2-D SDS-PAGE reference pattern of AI crude run in a homogeneous 10% PAG and stained with CBB. For illustration, the respective IEF gel is mounted in the IEF gel trough. b Schematic drawing of the 2-D protein pattern with the approximate pI and molecular weight (MW) references indicated. For the second directional electrophoresis the cathode was at top and the anode at bottom of figure.

(fig. 1). The 2-D IAR of AI crude identified 35 protein spots with retained antigenic reactivity (fig. 4a).

In the IgE system, IAR demonstrated allergenic reactivity in the pH region 4.6–5.2 only where two IEF bands approximately 0.2 pH units apart at pH 4.9 gave the highest autoradiographic signals (fig. 1). 2-D IAR showed that the ^{125}I -anti IgE uptake of this pH region was resolved into two molecular weight areas: the zone of strongest uptake at 20 kilodaltons and a narrower area of weaker signals at 40 kilodaltons (fig. 4b).

Fig. 3. a Two-dimensional reference of fraction AI 34 performed under conditions as given for figure 2a. However, equilibration of the IEF gel in sample buffer was done for 20 min which slightly increased the electrophoretic mobility as compared with figure 2 where equilibration was done for 15 min. b Schematic representation of the 2-D fingerprint of AI 34 with the pI and molecular weight (MW) references indicated.

The detection limits of the immunoradiographic techniques applied were estimated by the dot immunobinding assay using the AR5 film and the SP intensifying screen. One microliter spots containing 0.01 and 0.1 μg of AI crude gave visible radiostaining after an exposure period of 96 h in the IgG and IgE systems, respectively. Reconstitution of the extract in 0.1% SDS and 1.0% SDS with 0.5% 2-mercaptoethanol did not affect the detection levels of the two systems (results not shown).

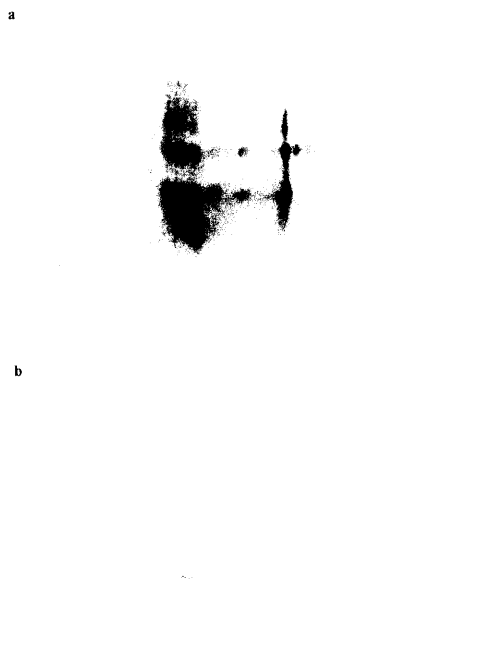


Fig. 4. IAR of AI crude separated by 2-D IEF/SDS-PAGE. Autoradiography was performed using AR film, SP screen and exposure at -80°C . Orientation of the autoradiograms are in keeping with figure 2. **a** An autoradiogram after incubation with rabbit antiserum and ^{125}I -anti-IgG. Throughout the exposure sequence from 3 to 162 h, 35 distinct IgG-binding spots could be identified. The autoradiogram shown was exposed for 24 h. **b** IgE autoradiogram exposed for 72 h showing the zone of highest IgE reactivity located on the reference map at pH 4.6–5.2 and 20 kilodaltons with an area of weaker radiostaining at pH 4.6–5.2 and 40 kilodaltons. This particular autoradiogram of AI crude was obtained from a 5.0–12.5% gradient gel, consequently the relative location in the second dimension is not directly comparable with figure 2.

Discussion

The horizontal electrophoresis system gave reproducible high-resolution one- and two-dimensional protein reference patterns of the heterogeneous allergen mixtures. The higher number of detected protein bands and spots in fraction AI 34 was attributed

to the higher protein concentration relative to AI crude. ET from IEF and 2-D gels at the stated experimental conditions was quantitative as assessed by staining of gels after transfer as well as staining and IAR of additional NC sheets adjacent to the NC membrane in direct contact with the gel. Cooling and circulation of the reservoir buffer was necessary to obtain the demanded voltages without overheating and to assure an even temperature throughout the buffer system.

Silverstaining of the 1.5-mm thick 2-D gels, although more demanding, showed a higher sensitivity than CBB for most spots, however, as some proteins stained by CBB did not take silver stain, the total spot number was not increased. In addition, staining artifacts and background shadows were difficult to avoid. Staining of the NC blots with the rapid CBB procedure did lead to partial desintegration and considerable shrinkage of the membranes. The Ponceau stain proved less sensitive but did not distort and alter the size of the membranes. More sensitive staining methods for NC-bound proteins by colloidal metal procedures have recently been reported [17]. Positively charged nylon membranes did, in spite of more intensive blocking procedures, give higher background signals in autoradiography than NC and, further, appropriate staining techniques for proteins bound to nylon membranes did not exist.

As demonstrated by the modified immunodetection procedures, the electrophoretically separated and transferred alder pollen proteins showed retained antigenic and allergenic reactivities. Control blots incubated with PBS and a patient serum of high IgE titers with negative RAST to tree pollen and, further, the electrophoresed and transferred IEF and molecular weight marker proteins did not show unspecific binding of IgE or ^{125}I -anti-IgE. The ^{125}I -anti-IgG signals in both IEF and 2-D immunoblots were high and the rabbit antiserum could be used for repeated probes without apparent loss in activity. IAR in the IgE system gave weaker autoradiographic signals, and there was a significant loss in activity with reuse of the reaginic serum. This could partly be attributed to the differences in serum concentrations of IgE and IgG. However, a series of denaturing conditions were encountered during the separation procedures including fixing, destaining, carrier ampholytes, reducing agents and SDS which alone, or in combination, could induce structural changes that would affect IgE binding more severely than the binding of IgG. Even

the binding of allergenic proteins to NC, the mechanisms of which are not precisely known [7, 13], might decrease their affinity to IgE. In contrast to the effects of SDS on wheat flour allergens reported [20], it was shown that 1% SDS and 0.5% 2-mercaptoethanol did not inhibit immunoglobulin binding of the alder pollen proteins when investigated by the dot immunobinding assay.

Autoradiographic resolution of closely focused IEF bands was not optimal and did not allow a precise quantitation of the individual IgG-binding bands. Obviously, the same problem was encountered in the 2-D areas of more tightly clustered antigenic spots (fig. 4a). The reduced autoradiographic resolution could be explained by the increase in film image size, a phenomenon which depends both on degree of exposure and the properties of the autoradiographic system used [6].

The 2-D reference map demonstrated that single IEF bands could contain multiple molecular weight representatives, and thus the separation power of IEF was enhanced by second directional SDS-PAGE. The allergenic reactive proteins of alder pollen were separated by IEF into the pH range of 4.6–5.2 where a total of 12 individual bands were visualized by CBB stain. 2-D resolution of this region showed a dominating protein zone at 20 kilodaltons representing a major alder pollen protein. This zone also demonstrated high IgG binding and was the area of strongest IgE signals. In addition, a smaller area of less allergenic reactivity was seen at 40 kilodaltons. Consequently, the 2-D autoradiogram showed that multiple spots in the pI 4.6–5.2 region did not bind IgE.

Further experimental studies comparing the IEF and 2-D reference maps with other tree pollen species are currently proceeding in our laboratory. High-resolution electrophoresis of the allergenic pollen proteins will be investigated for the preparative purpose of isolating a homogeneous major IgE-binding antigen by affinity chromatography. Additional studies on the immunological identity reactions are needed to relate the allergenic proteins separated in this system to the alder pollen allergens identified in CRIE [5].

Although the complete methodological sequence was more demanding, horizontal electrophoretic separation combined with ET and IAR demonstrated advantageous properties over conventional CRIE in terms of superior resolving power and separation of proteins according to physicochemical properties independent of animal antibodies. Performing the

electrophoretic steps in ultra-thin gels would add to the competitiveness of the method by reducing time and costs considerably [8]. In our opinion, 2-D SDS-PAGE with ET and IAR at present provides a powerful supplement to, rather than a complete substitute for, CRIE.

Acknowledgements

This study was partly supported by the Norwegian Research Council for Science and Humanities (NAVF grant No. 13.11.20-020) and the Research Fund of the Norwegian Asthma and Allergy Federation. The skilled technical assistance of May-Britt Kalvenes and Elisabeth Holen is appreciated.

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Received: August 5, 1985

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