Isolation and Characterization of Allergens from Ragweed Pollen. IV*

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ABSTRACT: The second most active allergen in ragweed pollen has been isolated and studied. This allergen is designated as antigen K and is a protein of molecular weight of 38,200. Its amino acid composition was found to differ from that of the most active allergen, antigen E, which has a molecular weight of 37,800 (King, T. P., Norman, P. S., and Connell, J. T. (1964), *Biochemistry 3*, 458). Of interest was the finding that antigen K shared partial immunological identity with antigen E. This cross-reactivity was demonstrable with rabbit and human antisera as well as with leukocytes from hay fever patients. Also, experimental results suggest the probable presence of unidentified allergens in pollen which cross-react with antigen E. The cross-reactivity of antigen E with other pollen antigens is believed to explain its high allergenic activity.

Our earlier work indicated that the allergenic activity of aqueous extracts of ragweed pollen resided in its protein-rich fractions. Three such fractions, III, IV, and V, were, respectively, about 100-, 1000-, and 100-fold more active than the crude extract as estimated by direct skin tests on seven sensitive patients (King and Norman, 1962). The active protein in fraction IV, designated as antigen E, has been isolated and characterized. It has a molecular weight of 37,800 and is devoid of prosthetic groups (King *et al.*, 1964; King and Norman, 1964).

The previous tests on the allergenic activities of these fractions were based on a limited number of observations with preparations incompletely resolved by chromatography on Sephadex G-75. We have, therefore, continued these tests with better resolved preparations obtained by chromatography on Sephadex G-100, using the direct skin assay, as well as a more quantitative procedure, the histamine release assay with leukocytes of sensitive individuals (Lichtenstein *et al.*, 1966a). These later results confirmed the low activity of fraction III, but fraction V was found to be only slightly less active than fraction IV, rather than the tenfold difference noted earlier.

We, therefore, set out to isolate and characterize the active substance in fraction V, and have given this substance the name, antigen K. The present paper reports the chemical and immunological properties of this antigen.

Experimental Section

Materials. Low ragweed pollen of the 1963 and 1964 crops was obtained from the Division of Biologic Standards, U. S. Department of Health, Education, and Welfare. Antigen E used for immunization of patients was prepared by Abbott Laboratories under contract to the Committee of Standardization of Allergens, National Institutes of Health; these samples contained 2–6% of antigen K as determined by immunodiffusion with specific rabbit antisera.

Rabbit antisera specific for the whole pollen extract and for the purified antigens were prepared using the alum suspension procedure previously described (King *et al.*, 1964). Rabbit antisera were also prepared using emulsified antigen in incomplete Freund's adjuvant. The material was initially administered into the foot pads, and booster injections were given subcutaneously.

Methods. The procedures used for the pollen extraction, and the subsequent chromatographic separations were the same as reported previously. All protein solutions were concentrated by ultrafiltration as reported (King *et al.*, 1964).

For carbohydrate and amino acid analyses, solutions containing protein were desalted by passage through Sephadex G-25 equilibrated with 0.2 M acetic acid, and then lyophilized. Amino acid analysis was carried out following the procedure of Moore and Stein (1963). Carbohydrate content was measured by the orcinol–sulfuric acid method (Tsugita and Akabori, 1959).

Molecular weight was determined by equilibrium ultracentrifugation at 18° with a rotor speed of 40,430 rpm (Yphantis, 1964). Protein concentrations were 0.099 and 0.013% in a buffer of 0.1 M NaCl–0.05 M Tris–0.03 M HCl (pH 8.0). Partial specific volume was taken to be 0.72.

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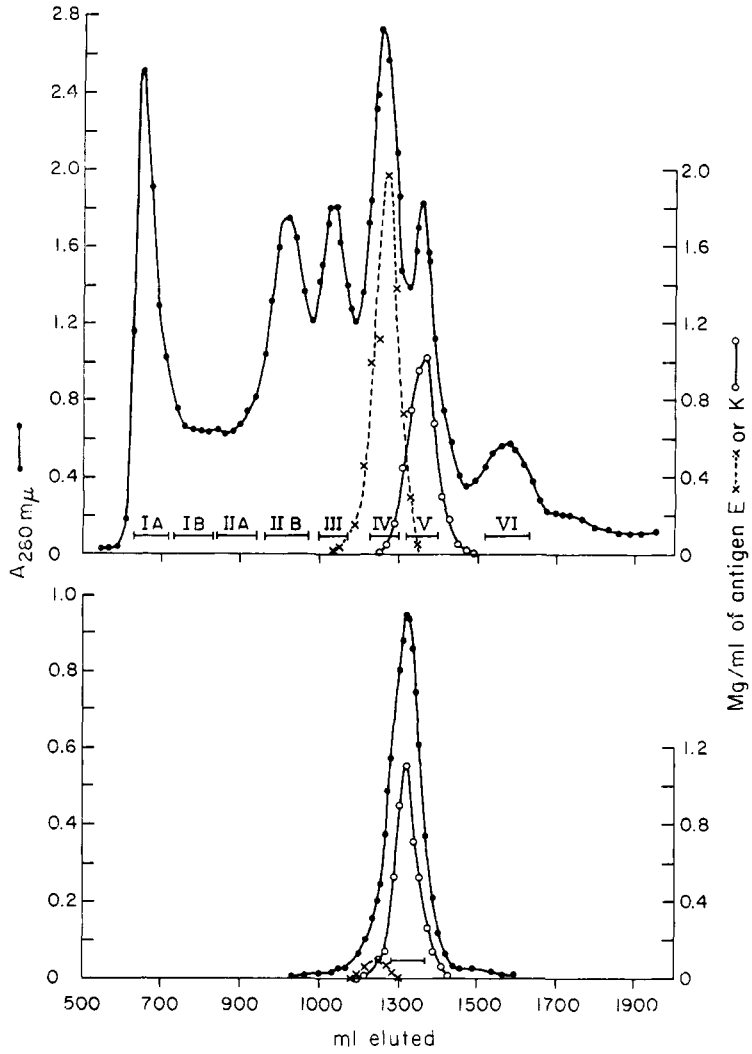

FIGURE 1: Chromatographies of fraction D and V on a Sephadex G-100 column (400 × 2.5 cm). (A) Fraction D, obtained from 200 g of pollen, was applied to column. (B) Fraction V from the top figure (105 absorbancy units) was rechromatographed. The eluent was 0.40 M $(NH_4)_2SO_4$–0.5 M Tris–0.03 M HCl (pH 7.9). Column flow rate and fraction size were, respectively, 25 ml/hr and 10 ml/tube.

Precipitin analyses in liquid and gel media were performed as described in our earlier publications. Direct skin tests on sensitive patients were carried out with tenfold dilutions of the samples in isotonic saline buffered at pH 7.9 with Tris-HCl and containing 0.05% human serum albumin. Histamine release assay with leukocytes of sensitive individuals was carried out using the published procedures (Lichtenstein and Osler, 1964; Lichtenstein *et al.*, 1966a). In order to analyze the cell sensitivity measurements, cell sensitivity (*S*) was defined as the reciprocal of the antigen concentration required for 50% histamine release. The relationship of $S_{\text{antigen E}}$ to $S_{\text{antigen K}}$ was investigated by the method of least squares (Mood and Graybill, 1963) and the correlation coefficient was calculated by the method of Kendall (1943).

The procedures used for immunization of patients with antigen E and for the collection of their sera have been described in a recent publication (Lichtenstein *et al.*, 1966b). These sera were tested for their ability to inhibit the histamine release from sensitive cells mediated by either antigen E or K, by a procedure similar to one that was used for the measurement of antibody content of allergic serum (Lichtenstein and Osler, 1966; Figure 6). Results were recorded as per cent inhibition calculated by dividing the control histamine release in the absence of antiserum (*C*) less the histamine release noted in the presence of an antiserum (*A*), by (*C*). That is, per cent inhibition = $(C - A)/C \times 100$.

Results

Isolation of Antigen K. The pollen proteins were precipitated from an aqueous extract of ragweed pollen with ammonium sulfate at 0.8 saturation. The

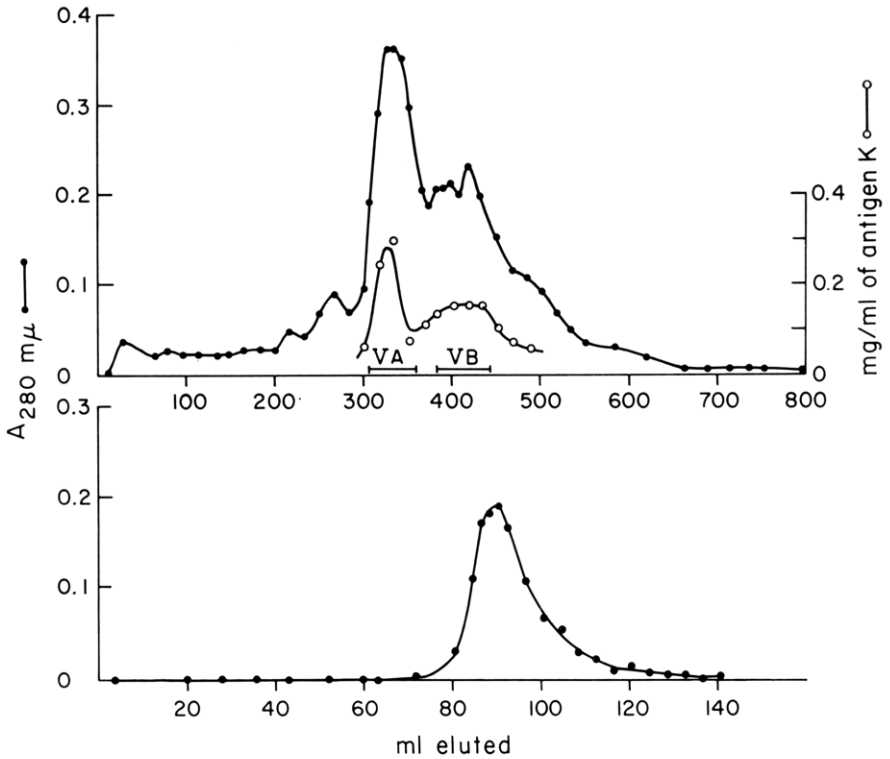

FIGURE 2: Chromatography of fraction V on TEAE-cellulose. (A) Fraction V from Figure 1B (65 absorbancy units) was applied to a 22 × 1.6 cm column. Elution was carried out with a linear gradient by mixing 500 ml each of 0.05 M Tris–0.3 M HCl and 0.05 M Tris–0.03 M HCl–0.23 M NaCl at a flow rate of 40 ml/hr and fractions of 8-ml volume were collected. (B) Fraction V-A from the top figure (4.6 absorbancy units) was rechromatographed on a 20 × 0.9 cm column. The linear gradient was formed using 120 ml of each buffer. The flow rate and the fraction size were one-fourth of those for the large column.

precipitate was depigmented by passage through a Sephadex G-25 column to yield fraction A, from which fraction D was obtained by stepwise elution chromatography on DEAE-cellulose. Fraction D was then chromatographed on Sephadex G-100 to give fraction V, together with the other fractions (King *et al.*, 1964).

Figure 1A illustrates the separation of fraction D. Fractions IV and V overlapped each other as shown on antigen analyses with rabbit antiseras pecific for antigens E and K. After rechromatography (Figure 1B), fraction V could be isolated nearly free of antigen E (less than 0.5%). When the pollen extract was subjected to fractionation by ammonium sulfate precipitation, fraction V was found to be present in the cut at 0–0.4 salt saturation. Although a cleaner preparation of V could be obtained in this manner, this procedure had the difficulty that the G-100 column became plugged up during the separation.

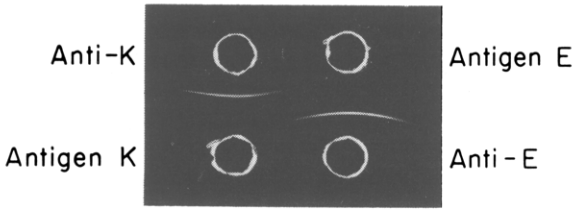

FIGURE 3: Immunodiffusion of antigens K and E against their specific antisera. The concentrations of the antigens were 0.16 mg/ml.

Fraction V was finally purified by chromatography on TEAE-cellulose with a linear sodium chloride gradient (Figure 2A). Antigen K was found to be present in several chromatographic forms, as revealed by immunodiffusion against specific antisera which were prepared by immunization of rabbits with the major component VA. Only the major component was collected and used for further studies. Rechromatography of this component yielded a single peak with better than 95% recovery of optical density units (Figure 2B). Antigens K and E could not be separated by chromatography on TEAE-cellulose, and they emerged at approximately the same effluent volume.

In Table I are listed the yields of antigen K at various stages of purification. By precipitin analyses with specific antisera, an aqueous extract of 1 g of pollen was found to contain 2.6 ± 0.5 mg of antigen E and 1.2 ± 0.2 mg of antigen K.

Its Chemical Properties. Antigen K contained 16.6

TABLE I: Yield of Antigen K at Various Stages of Isolation from 200 g of Ragweed Pollen.[a]

Fraction	By Immunodiffusion	By Absorbance
Pollen extract	240 mg	–
A	114	–
D	100	–
V, Figure 1A	65	74 mg
V, Figure 1B	62	45
V-A, Figure 2A	13	12
V-B, Figure 2B	11	10

[a] The values given represent the amounts in the cuts as shown in the figures. Immunodiffusion analyses were carried out with specific antisera and using rechromatographed V-A as standards; the accuracy of the method was ±30% within the expected values. For calculation of weight yields by absorbance at 280 mμ, the absorbance units were divided by a factor of 1.48.

TABLE II: Amino Acid Compositions of Antigens E and K.

Amino Acid	No. of Residues/mole of Antigen K[a] Found	To nearest Integer	No. of Residues/Mole of Antigen E[a]
Lysine	15.5 ± 0.2	16	18
Histidine	10.5 ± 0.1	11	6
Ammonia	36.8 ± 3.7	37	31
Arginine	14.5 ± 0.6	15	16
Aspartic acid	44.4 ± 0.3	44	49
Threonine	21.6 ± 0.6	22	17
Serine	16.7 ± 0.4	17	26
Glutamic acid	28.7 ± 0.4	29	25
Proline	16.0 ± 0.2	16	15
Glycine	31.5 ± 0.1	32	37
Alanine	20.9 ± 0.1	21	31
Half-cystine	8.7 ± 0.2	9	7
Valine	23.2 ± 1.2	23	24
Methionine	9.4 ± 0.1	9	7
Isoleucine	22.4 ± 0.2	22	20
Leucine	17.3 ± 0.3	17	21
Tyrosine	6.4 ± 0.1	6	4
Phenylalanine	13.2 ± 0.3	13	12
Tryptophan	8.8	9	6

[a] The molecular weights of antigens K and E were taken to be 38,200 and 37,800, respectively. [b] Average of three 21-hr and three 72-hr hydrolysates. The serine and threonine contents were estimated by extrapolation to zero time. The value for half-cystine was determined by hydrolysis of an oxidized sample (Moore, 1963). For the isoleucine content, only the values from 72-hr hydrolysates were averaged. Tryptophan was estimated spectrophotometrically (Goodwin and Morton, 1946).

± 0.3% of nitrogen and less than 0.6% of carbohydrate. Its amino acid composition is given in Table II, together with that of antigen E for comparison. The amino acids listed in the table accounted for all the nitrogen present in antigen K (100 ± 3% and also accounted for 95 ± 3% of the weight of the sample).

The molecular weight of antigen K was determined by equilibrium ultracentrifugation to be 38,200 ± 1500. This value is close to that of antigen E, 37,800. However, antigen K was more retarded on the Sephadex G-100 column than antigen E. This retardation is probably due to the absorption of aromatic substances by Sephadex gels (Porath, 1962). Antigen K has a higher content of aromatic amino acids than antigen E, and this is indicated by their absorbancies at 280 mμ, 1.13 and 1.48 ± 0.05 for antigens E and K, respectively, with a concentration of 1 mg/ml and a light path of 1 cm.

Its Antigenic Properties. When antigen K was tested for homogeneity by immunodiffusion a single precipitin line was observed with each of four different rabbit antisera prepared against whole ragweed extract. Nine rabbits were immunized with 0.6 mg of antigen K in alum suspension; eight of these responded, with an average antibody level of 250 μg of protein/ml. These specific anti-K sera yielded a single line on immunodiffusion against whole ragweed extract. There was no line of precipitation when immunodiffusion was carried out between three anti-E sera and antigen K. This was also true when antigen E was analyzed with three anti-K sera. Typical results of this type are seen in Figure 3. A different pattern was obtained with two antisera produced by immunization with antigen E or K in incomplete Freund's adjuvant. With these two sera, one anti-E and one anti-K, there was a reaction of partial identity when immunodiffusion was carried out with antigens E and K. Moreover, reactions of partial identity were observed when immunodiffusion was performed with antigens E and K *vs.* antisera made against whole ragweed (Figure 4). Anti-ragweed sera were prepared by immunization in alum suspension.

The finding of partial identity with anti-ragweed sera was confirmed by precipitin analyses in liquid media. The precipitin curve of an anti-ragweed serum with different amounts of antigen E is represented with solid circles in the left side of Figure 5. To the supernatants of these mixtures were added constant amounts of antigen K, and the additional precipitates are represented with open circles in the pattern. If the two antigens were of independent systems, the additional precipitate formed with antigen K should be constant

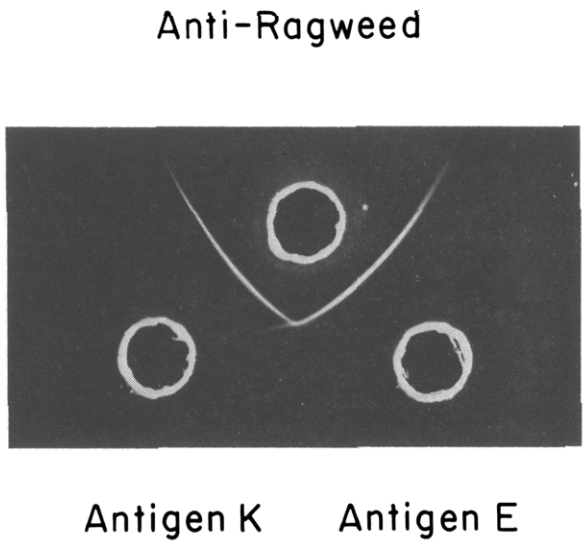

FIGURE 4: Immunodiffusion of antigens K and E against an anti-ragweed serum. The concentrations of the antigens were 0.2 mg/ml.

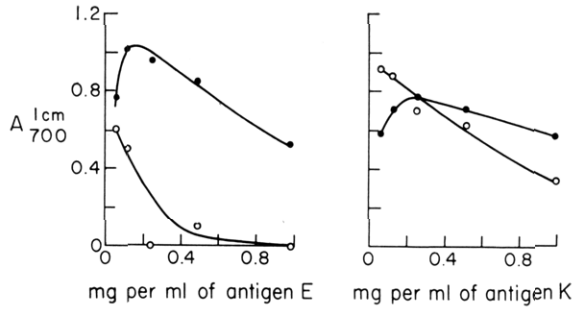

FIGURE 5: Precipitin analyses of antigens K and E with an anti-ragweed extract serum. The analyses were made using 50 μl each of antiserum and antigen, and the amount of precipitin formed was measured by Folin color in a final volume of 1.1 ml. (A) Precipitate formed with antigen E (●—●); additional precipitate formed (⊙——⊙) when 50 μl of antigen K (0.06 mg/ml) was added to supernatants. (B) Precipitate formed with antigen K (●——●); additional precipitate (⊙——⊙) when 50 μl of antigen E (0.06 mg/ml) was added to supernatants.

for all supernatants. That is, the prior removal of antibodies by antigen E should not have altered the content of antibodies specific for antigen K. In the region of antigen E excess, however, the amount of precipitate formed by antigen K was totally suppressed. On the right side of Figure 5 a similar experiment with the same anti-ragweed serum is shown. Antigen E was added to the supernatants of antiserum plus antigen K reaction mixtures. In the region of antigen K excess, the precipitin reaction of the supernatant with antigen E was about 50% suppressed. Findings similar to those in Figure 5 were obtained with two other anti-ragweed sera. These results could not be due to cross-contaminations of the antigen preparations, as each antigen preparation contained less than 0.5% of the other antigen as determined by immunodiffusion with the noncross-reacting specific sera.

One noticeable difference between the anti-ragweed and the specific antisera, both prepared using the alum suspension procedure, was that the precipitin curves of the former with purified antigens were about twice as broad as those of the latter ones. This is indicative that the antibodies in anti-ragweed sera react with a larger number of determinant groups on antigens E or K than those antibodies in the specific sera. Apparently, sera with a broad range of specificity are required to detect this cross-reaction.

Its Allergenic Properties. The allergenic activity of antigen K was measured by direct skin tests on sensitive patients as well as by histamine release assays of leukocytes from sensitive individuals. Comparative skin tests of antigens E and K and pollen extract showed that both antigens were more reactive than the extract and that antigen K was usually somewhat less active than antigen E (Figure 6). Only in one of the 37 patients tested was antigen K more active than antigen E, and in six patients the two antigens were of approximately equal activity.

Skin tests were made on pollen extracts after the precipitation of antigens K and E with specific rabbit antisera, in order to estimate their contribution to the total allergenic activity of extract both individually and in combination. The precipitin curves of pollen

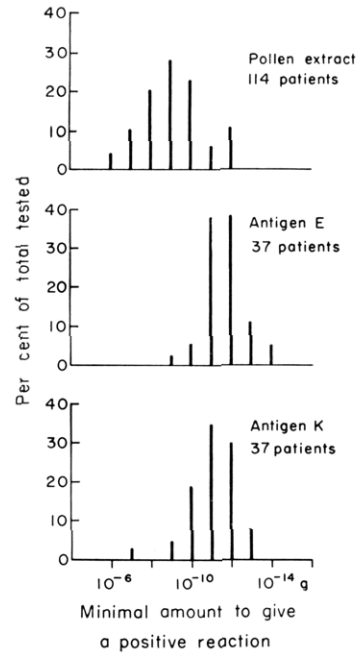

FIGURE 6: Direct skin tests of antigens K and E and pollen extract.

TABLE III: The Decrease of Skin Test Activity of Ragweed Pollen Extract after Precipitation of Antigens E or K with Rabbit Antiserum.

	Antiserum against Antigen E		Antiserum against Antigen K		Antiserum against E + K Combined[b]	
Patient	1034	101	203	208	C	M
L. L.	10,000[a]	10,000	10	10	10,000	10,000
A. R.	100	1,000	10	<10	1,000	1,000
M. L.	10	<10	<10	0	<10	<10
C. F.	<10	<10	0	<10	<10	<10
S. W.	100	10	0	–	–	100

[a] The values represent the *n*-fold decrease of activity in the region of antibody excess when compared to untreated whole ragweed extract. <10 means some neutralization but less than tenfold; 0 means no detectable neutralization.
[b] Serum C is a mixture of serum 101 (67 parts), serum 208 (29 parts), and buffer (four parts). Serum M is a mixture of serum 1034 (62 parts), serum 203 (27 parts), and buffer (11 parts).

extracts with anti-K serum (no. 203), anti-E serum (no. 1034), and a mixture of the two sera are given in the upper portion of Figure 7. Only the desired antigen(s) was removed from the supernatants in the region of antibody excess, in so far as we could establish by immunodiffusion with specific sera and anti-ragweed sera. The supernatants from each of the three sets of experiments were skin tested on five previously untreated patients. The results from one patient are shown in the lower portion of the Figure 7. In this case, the activity of the supernatant in antibody excess zone was less than that of the pollen extract by tenfold after precipitation with anti-K serum, and by 1000-fold after precipitation with either anti-E serum or a mixture of the two sera. The results from all patients are summarized in Table III, together with those from separate experiments using other anti-K serum (no. 208) and anti-E serum (no. 101). The decrease in activity was not uniform for all patients. However, they all showed the trend that the removal of antigen K produced a smaller decrease than the removal of antigen E. The large decrease in activity of pollen extract after removal of antigen E has been observed by us previously (King *et al.*, 1964). Similar precipitation experiments employing purified antigens K and E with these four sera showed that anti-K and anti-E sera were equally effective and neutralized the allergenic activity of purified antigens by 1000- to 10,000-fold.

Comparative histamine release assays of antigens E and K were carried out with leukocytes from 21 untreated patients. Typical dose response curves obtained with three patients are shown in Figure 8. These curves were selected to show one patient in whom antigen K was more active than antigen E, one in whom E was more active than K, and one in whom E and K were of equal potency. The concentration required to cause 50% histamine release is a reflection of the cell sensitivity to the test antigen and is a measure of allergenicity (Lichtenstein *et al.*, 1966a). From this measure, the relative allergenic activity of antigen K to antigen E was calculated. These results are summarized in Table IV. In 2 of 21 patients, antigen K was more active than antigen E (114 and 180%); in one they were of equal activity and in seventeen, K was less active (21–77%). One patient on several occasions evidenced a sensitivity to K which was only 0.5%

TABLE IV: Histamine Release Assays of Antigens E and K.

	Concn of Antigen Required for 50% Histamine Release (μg/ml)		
Patient	Antigen E	Antigen K	K as % of E
1	1.3×10^{-5}	2.7×10^{-5}	47
2	1.6	6.8	24
3	3.6	17	21
4	5.0	7.7	66
5	5.2	19	27
6	8.2	8.2	100
7	8.3	18	45
8	8.5	17	43
9	8.5	24	35
10	9.0	15	59
11	9.1	31	29
12	15	13	115
13	16	29	55
14	16	64	25
15	20	32	62
16	21	12	180
17	25	36	71
18	46	61	77
19	64	150	43
20	68	15,000	0.5
21	68	180	38

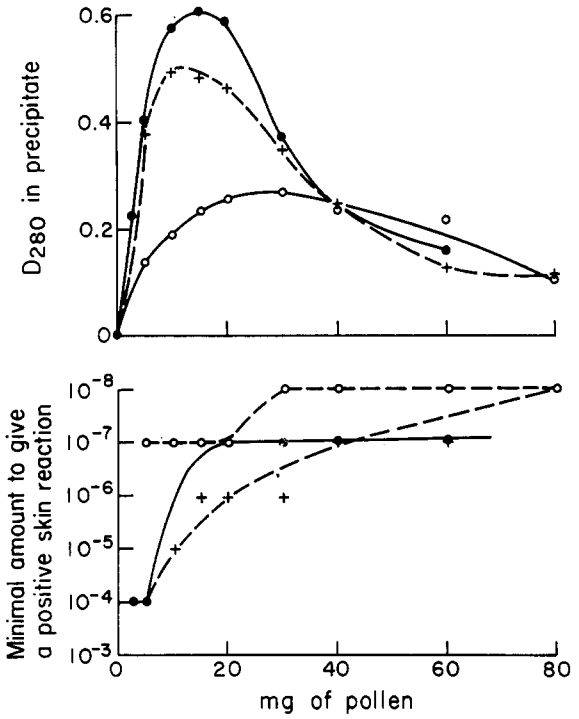

FIGURE 7: Precipitin analyses of ragweed pollen extract with rabbit antisera specific for antigens K and E. The upper pattern shows the precipitin curves of the pollen extract with anti-K serum no. 203 (⊙- - -⊙), with anti-E serum no. 1034 (+- - -+), and with a mixture of these two sera (●——●). The apparent broadness of the precipitin curve with anti-K serum is due to its lower content in pollen as compared to the antigen E content. The composition of the mixed sera is 27 parts of anti-K serum, 62 parts of anti-E serum, and 11 parts of buffer. The lower pattern shows the results of direct skin tests of the supernatants from the upper pattern.

of his sensitivity to E. The observed variation in the activity ratio between K and E in different patients is significant, as the experimental error of these assays is less than 15%.

When the logarithm of cell sensitivity to K *vs.* that to E is plotted for each patient (Figure 9), it is seen that cell sensitivities to E and K are not distributed randomly, and that an individual's sensitivity to K is closely linked to his sensitivity to E. In this group of patients, the average activity of antigen E was 2.1 ± 0.26 times that of antigen K and sensitivity to antigen E and antigen K were positively correlated (correlation coefficient 0.871, $P < 0.001$). This relationship shows that the two antigens have common determinant group(s), but the variation in the activity ratio of K:E in different patients (Table IV) is greater than experimental error and indicates that they each contain some unique determinant groups.

This cross-reactivity was next demonstrated with serum from patients who had been immunized with antigen E. The antiserum, a pool from 21 patients, was tested for its ability to inhibit the histamine release from cells mediated by either the homologous or the cross-reacting antigen. Increasing amounts of the pooled antiserum were added to tubes containing constant amounts of antigens E and K, normal serum, and sensitive cells. The amount of antigen used was so adjusted that 70–80% histamine release occurred in the presence of normal serum alone. The per cent inhibition of histamine release relative to control is plotted against antiserum concentration, and the antibody titer is estimated from the concentration of serum required for 50% inhibition. Figure 10 illustrates that, on the average, about 45% of the antibody raised against antigen E cross-reacts with K. It is to be expected that an analysis of individual sera, rather than a pool, would show considerable variation in activity.

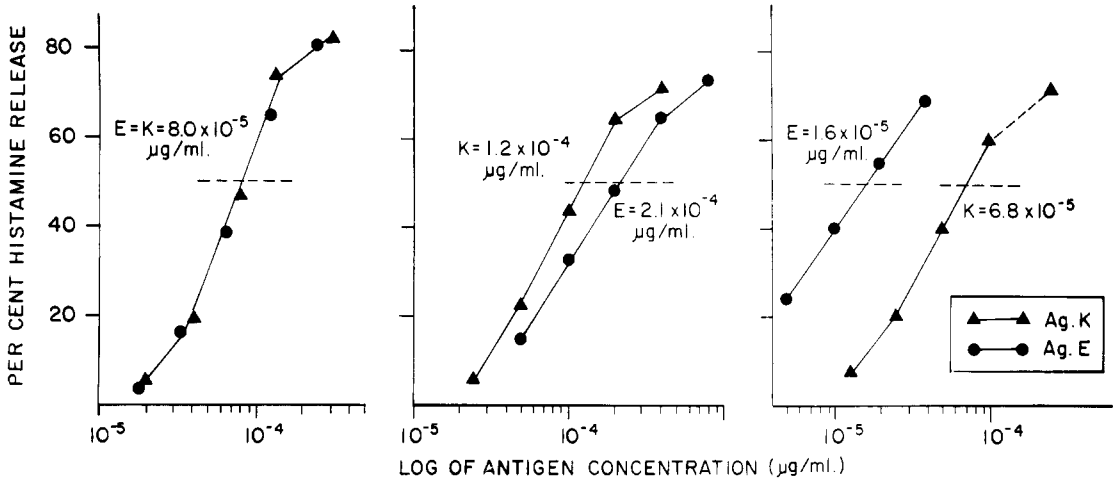

FIGURE 8: Dose response curves with leukocytes of three different allergic donors. Comparison of activity of antigens K and E in stimulating the release of histamine. The concentration of antigens E and K required for 50% histamine release are indicated in one donor in whom E and K are of equal potency, in one in whom K is more active, and in one in whom E is more active, from left to right.

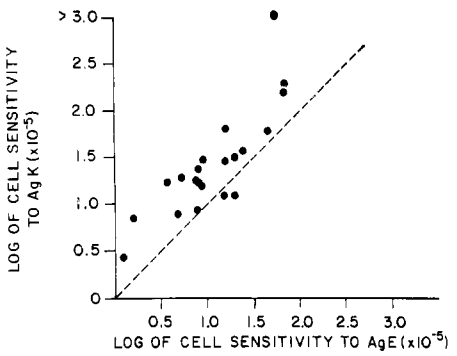

FIGURE 9: A comparison of the cell sensitivity to antigens E and K in 21 allergic patients.

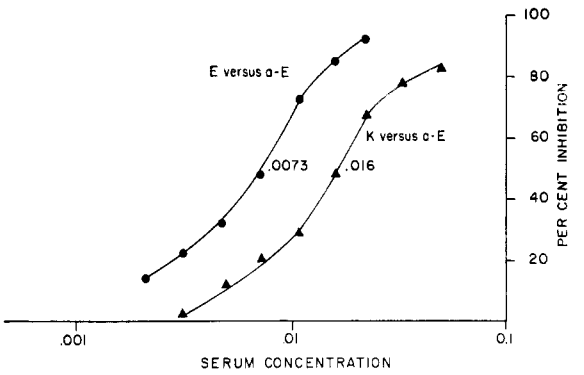

FIGURE 10: A comparison of the inhibition, caused by different concentrations of human anti-E, of histamine release resulting from challenge of sensitive leukocytes with the homologous antigen E or the cross-reacting antigen K.

Discussion

The results of this study show that the second most active allergen in ragweed pollen, antigen K, is a protein as is the most active one, antigen E (King *et al.*, 1964). Both antigens have a molecular weight of about 38,000 and similar charges as shown by their chromatographic properties on TEAE-cellulose. But they have different amino acid compositions (Table II), and they are separable by chromatography on Sephadex or by ammonium sulfate precipitation. Their allergenic activities depend on the intact protein molecule, as it has been shown that antigen E was inactivated after digestion with a bacterial proteinase (King and Norman, 1964).

The independence of these two proteins, antigenically, is shown by some specific rabbit sera which indicate no evidence of cross-reactivity (Figure 4). These sera were essential for the laboratory control of the separation procedures which led to the preparation of these antigens in a highly purified form. The use of the purified antigens for skin testing and histamine release studies showed that there was also some degree of independence in patients with ragweed pollen sensitivity (Table IV, Figure 9). Of special interest, on the other hand, is the finding that these two different antigens have common determinant groups. Their partial immunological identity is demonstrable with rabbit antisera (Figures 4 and 5) as well as with human antisera (Figure 10). This relationship is also demonstrated by the leukocyte sensitivities of ragweed-sensitive patients to antigens K and E (Table IV and Figure 9).

The allergenic activity of antigen K is, on the average, about one-half of that of antigen E as shown by skin tests and histamine release assays (Figures 6 and 9). Its content in pollen extract is about one-half of the antigen E content. Therefore, an extract with its antigen E removed with specific rabbit anti-E serum should be about one-fourth as active as an extract with its antigen K removed. However, removal of antigen E from pollen extract produced a large decrease in its activity, ranging from 10- to 10,000-fold less than that of the initial solution, as shown by skin tests on five patients; whereas removal of antigen K from an extract produced a smaller decrease ranging from zero- to tenfold (Table III). The original objective of these experiments was to make an estimate of the contribution of these two antigens to the total allergenic activity of the pollen extract. Clearly, this is not feasible with these results. The uncertainty which arises from these data is due in part to the lack of precision inherent in skin testing (plus or minus tenfold). Nevertheless, the large difference in the activities of the extract without antigen E and of that without antigen K suggests that there may be other allergens in extract which cross react with antigen E. Thus, on precipitation of an extract with anti-E serum, these allergens could be removed together with antigen E resulting in a large decrease in activity.

The hypothesis that antigen E bears common determinant groups of other allergens in the pollen may well be an explanation of its high allergenic activity, since there is no unique chemical feature to distinguish it from other protein antigens. The extensive cross-reactivity of antigens E and K makes it possible for human anti-E sera to inhibit completely the allergenicity of antigen K. If this proves to be the case with other ragweed pollen allergens, antigen E could become a very useful clinical reagent. Recent clinical results on the treatment of hay fever patients with antigen E tend to support the possibility, as patients treated with it had as much reduction of symptoms as the group treated with whole ragweed extract (P. S. Norman and W. Winkenwerder, unpublished data).

Recently, the major allergen from rye grass pollen has been isolated. It has a molecular weight of 27,000 and contains 14% of nitrogen. Although it still contains 5% of carbohydrate, its protein nature was indicated by inactivation on trypsin or chymotrypsin digestion (Johnson and Marsh, 1966). Likewise, preparations of allergens from timothy grass pollens are estimated to have molecular weights of about 20,000–30,000, and these samples contain protein material,

as well as carbohydrate and pigment (Malley and Dobson, 1966). However, its protein nature is indicated by an earlier report that crude extracts of timothy pollen were inactivated by digestion with a mold protease (Augustin and Hayward, 1962). From these limited examples it appears, as a general rule, that the most active pollen allergens are proteins of mol wt 20,000–40,000 and that their activity depends on the integrity of the protein structures.

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