Isolation and Characterization of an Allergen from Short Ragweed Pollen*

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ABSTRACT: An allergenically active protein, antigen Ra.3, was isolated from the aqueous extract of short ragweed pollen by ammonium sulfate precipitation, gel filtration, and ion-exchange chromatography. Antigen Ra.3 was found to be highly purified on the basis of TEAE-cellulose chromatography, polyacrylamide disc electrophoresis, sedimentation velocity ultracentrifugation, gel filtration, and immunodiffusion tests. Antigen Ra.3 was shown to have a molecular weight of 15,000, an $s^0_{20,w} = 1.8 \times 10^{-13}$ sec, $D^0_{20,w} = 10 \times 10^{-7}$ cm^2 sec^{-1}, a total hexose + pentose content of 12.4%, and an amino acid composition distinct from the major allergen of ragweed pollen, antigen E. By direct skin, passive sensitization, and cross-neutralization tests, antigens Ra.3 and E differed in allergenic specificity but shared common allergenic determinants.

Two major allergens in short ragweed pollen, antigens E and K, have been isolated by King and his associates (King and Norman, 1962; King *et al.*, 1964, 1967). Antigens E and K were isolated from DEAE-cellulose fraction D (King and Norman, 1962) derived from an aqueous extract of the pollen. Both allergens were shown to be acidic proteins of about 38,000 molecular weight, essentially free of carbohydrates and of similar, though distinct, amino acid composition. Antigens E and K cross-reacted in immunological tests with rabbit and human antisera, *i.e.*, they contained common antigenic determinants. However, the allergenic cross-reactivity of antigens E and K has not yet been established.

The present investigation was prompted by our interest in the possible presence of additional allergens in the aqueous extract of ragweed pollen. Isolation and characterization of such allergens and comparative studies of the latter with antigens E and K might facilitate progress in understanding the chemical basis of allergenic specificity. In this communication we shall describe the isolation from DEAE-cellulose fraction C (King and Norman, 1962) of a third ragweed pollen allergen, antigen Ra.3, a basic carbohydrate-containing protein of 15,000 molecular weight. Immunological analysis demonstrated that antigen Ra.3 possessed antigenic and allergenic properties distinct from those of antigen E. The relationship of antigen Ra.3 to antigen K was not examined in this study.

Materials and Methods

Materials. All chemicals were of reagent grade, and most were purchased from Fisher Scientific Co. (Montreal). Chemicals used in the disc electrophoresis experiments were purchased from Canalco Co. (Rockville, Md.) except Tris, HCl, and acetic acid.

DEAE-cellulose (0.65 mequiv/g) and TEAE-cellulose (0.56 mequiv/g) were purchased from Calbiochem (Los Angeles), and Sephadex G-25 (medium) and G-100 (40–120 μ) from Pharmacia, Ltd. (Montreal).

Short ragweed pollen was purchased from Sharp and Sharp, Inc. (Everett, Wash.).

Column Chromatography. Chromatography was performed at 24–26° and column effluents were monitored by their absorption of ultraviolet light at 280 mμ measured manually with a Beckman DU spectrophotometer or automatically with an LKB Uvicord spectrophotometer equipped with a 2-ml flow cell. Total hexose and pentose determinations were done on effluent fractions using the tryptophan method (see below). The column effluents were collected in a Büchler or Spinco fraction collector, refrigerated at 5°. Pooled fractions were concentrated in a Diaflo ultrafiltration cell at 5° equipped with a UM-2 membrane (Amicon Corp., Cambridge, Mass.).

Water-soluble ragweed was prepared from the pollen according to King and Norman (1962) and Robbins *et al.* (1966). The pollen (1 kg), following ether extraction, was stirred for 4 hr at 25° in 5000 ml of 0.005 M sodium phosphate buffer (pH 7.4). The suspension was filtered by suction through two layers of Whatman No. 3 filter paper on a Büchner funnel. The residue of insoluble material was washed with an additional 500 ml of phosphate buffer and discarded. The combined filtrates (5000 ml) were brought to pH 7.0 by the addition of 3 N NH_4OH, cooled to 5°, and brought to 0.9 saturation in ammonium sulfate by slowly adding, with continuous stirring, 620 g of the salt/l. of extract. The resulting suspension was stirred overnight at 5° and centrifuged at 10,000 rpm for 40 min in an Inter-

* From the Division of Immunochemistry and Allergy, McGill University Clinic, Royal Victoria Hospital, Montreal, Canada. *Received July 11, 1968.* These studies were supported by grants from the Medical Research Council of Canada. Taken in part from a thesis presented by B. J. U. to the Faculty of Graduate Studies and Research of McGill University in partial fulfillment of the requirements for the Ph.D. degree. A preliminary account of these studies was presented at the 6th International Congress of Allergology, Montreal, Canada, Nov 6, 1967.

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national B-20 centrifuge, using rotor 874 precooled to 5°. The precipitate was dissolved in 500 ml of 0.1 M Tris-HCl (pH 7.9) to form a viscous dark brown solution (water-soluble ragweed).

DEAE-cellulose Fractions C and D (King and Norman, 1962; King et al., 1964). The water-soluble ragweed was filtered into high and low molecular size fractions (A and B, respectively) on a Sephadex G-25 column (10.0 × 95 cm) previously equilibrated with the eluting buffer of 0.025 M Tris-HCl (pH 7.9). Fraction A was concentrated by ammonium sulfate precipitation at 5° (0.9 saturation). The precipitate was dissolved in and dialyzed exhaustively against 0.025 M Tris-HCl (pH 7.9), and applied to a DEAE-cellulose column (5.0 × 50 cm) previously equilibrated with the same (eluting) buffer. Fraction C, containing antigen Ra.3, was obtained by this step. Fraction D, containing antigen E, was eluted with 0.05 M Tris-HCl–0.2 M NaCl (pH 7.9).

Antigen E (IV-C) was prepared as described by King and Norman (1962) and King *et al.* (1964) with minor modifications. DEAE-cellulose fraction D was filtered on Sephadex G-100 to obtain fraction D_{IV}; the column dimensions and elution buffers were identical with those employed for the isolation of antigen Ra.3 (see Results). Fraction D_{IV} was chromatographed on TEAE-cellulose to obtain fraction IV-C (antigen E). When tested by immunodiffusion in agar with rabbit antiserum specific to antigen E, the antigen E preparation showed a single precipitin arc and a reaction of identity with a preparation of antigen E (IV-C) kindly supplied by Dr. M. W. Chase, National Institutes of Health, Bethesda, Md.

Molecular Weight Determination. Sedimentation velocity studies were performed with a Spinco Model E analytical ultracentrifuge equipped with schlieren optics, using a rotor speed of 60,000 rpm and an average rotor temperature of 20°. Samples of antigen Ra.3 were run at concentrations of 1.0, 0.8, 0.6, and 0.4% in phosphate-buffered saline.[1] Sedimentation coefficients were calculated according to Lundgren and Ward (1951).

To obtain a diffusion coefficient for antigen Ra.3, analytical ultracentrifugal runs in a capillary-type synthetic boundary cell were performed with a rotor speed of 9000 rpm and at three sample concentrations (1.0, 0.8, and 0.6%) in phosphate-buffered saline. Diffusion coefficients were calculated by the height–area method of Lundgren and Ward (1951).

A partial specific volume for antigen Ra.3 was calculated from the amino acid composition according to the method of Cohn and Edsall (1943).

Disc electrophoresis was performed at pH 9.5 with the discontinuous buffer system of Ornstein and Davis (1962), and at pH 3.8 by the method of Reisfeld and Williams (1962). Electrophoresis at pH 6.6 was performed with a buffer system obtained from the technical literature issued by the Canalco Co. The electrode buffer consisted of 2,6-lutidine (3.82 ml), glycine (1.37 g), and H_2O (to 1000 ml) (pH 8.3). The running gel was formed from solutions a–b–c = 6:1:1, where (a) = 1 N KOH (48 ml)–glycine (114 g)–tetramethylenediamine (0.4 ml)–H_2O (to 600 ml) (pH 7.3), (b) = acrylamide (48 g)–methylenebisacrylamide (1.6 g)–H_2O (to 100 ml), and (c) = 0.56% ammonium persulfate. Sample loads of 0.05 mg in 0.15 ml of 0.005 M NH_4HCO_3 made 3% in sucrose were applied to the top of the gels, and electrophoresis was carried out for 2–3 hr using a current of 4 mA/tube. In the pH 9.5 system, bromophenol blue was used as a marker dye, each gel being run until the blue marker band had traveled a fixed distance. In the cathodic system, no suitable dye was found and therefore gels were run for a constant time.

Chemical Analysis. The absorption coefficient of antigen Ra.3 at 280 mμ was determined in a Beckman DU spectrophotometer on a sample of known optical density previously dialyzed exhaustively against 0.005 M NH_4HCO_3 (pH 7.3). The sample was dried to constant weight at 105°; the dialysate was weighed as a control. Subsequent protein concentrations were determined using the calculated absorption coefficient.

An absorption spectrum was obtained from optical density readings in the wavelength range 240–300 mμ given by a solution (1.04 mg/ml) of antigen Ra.3 in 0.005 M sodium phosphate buffer (pH 7.3).

The total solid concentration of solutions of water-soluble ragweed was determined by heating aliquots to constant weight at 105°. The concentration of antigen Ra.3 solutions was determined spectrophotometrically at 280 mμ using the calculated absorption coefficient (see above) and that of antigen E solutions using the published absorption coefficient (King and Norman, 1962), $E_{1\,cm}^{1\,mg/ml}$ 1.13, at pH 7.15.

Nitrogen content was determined by the method of Jacobs (1959) using ammonium sulfate as standard.

Total hexose and pentose was determined by the tryptophan method (Dische, 1929) with arabinose as standard. Total hexose was assayed by the anthrone procedure (Scott and Melvin, 1953) and total pentose by the cysteine–sulfuric acid method (Dische *et al.*, 1949) using galactose and arabinose, respectively, as standard. Hexosamine was determined by the procedure of Elson and Morgan (1933) using galactosamine as standard. To liberate hexosamine, samples and standard were hydrolyzed *in vacuo* in 2 N HCl at 108° for 4 hr.

The amino acid composition of antigen Ra.3 was determined with a Spinco Model 120 B amino acid analyzer according to the method of Moore *et al.* (1958). Duplicate samples of antigen Ra.3 (2.3 mg each) were dissolved in 6 N HCl, sealed in glass ampules *in vacuo*, and hydrolyzed for 24, 48, and 72 hr at 108°. The hydrolysates were separately dried by rotary evaporation and dissolved in sodium citrate buffer (pH 2.06). The number of micromoles of each amino acid was taken as the average of values obtained for the three

[1] Abbreviations used in text: phosphate-buffered saline, 0.14 M NaCl–0.01 M sodium phosphate (pH 7.3); P–K, Prausnitz–Küstner. The P–K reaction (or test) refers to the passive transfer to skin sites of normal recipients of the wheal and erythema responses which develop in allergic individuals challenged intradermally with specific allergen.

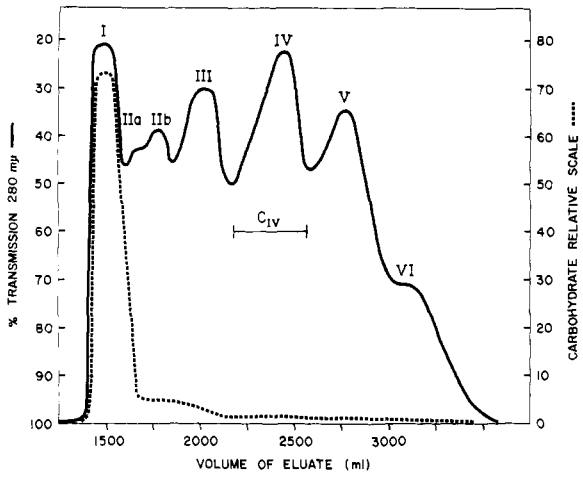

FIGURE 1: Gel filtration of DEAE-cellulose fraction C on Sephadex G-100 (5.0 × 140 cm) in 0.1 M Tris-HCl–0.2 M $(NH_4)_2SO_4$ (pH 7.9).

hydrolysis times, except for threonine and serine, the values for which were obtained by extrapolation to zero hydrolysis time. Cysteine and cystine were assayed as total cysteic acid on duplicate samples (2.3 mg each) which had been oxidized with performic acid by the method of Hirs (1956) prior to acid hydrolysis. Tryptophan was determined by a spectrophotometric method (Beaven and Holiday, 1952) on samples of the intact protein.

Antisera to the aqueous extract of short ragweed pollen were prepared by immunizing rabbits each with a mixture containing 1 ml of water-soluble ragweed (35 mg/ml) and 1 ml of complete Freund's adjuvant (Difco, Detroit). The rabbits were injected subcutaneously at weekly intervals for 4 weeks. Ten days after the last injection, the animals were bled *via* the maginal ear vein. The rabbits were immunized over a period of 6 months and bled at periodic intervals to obtain antisera which, when separately tested by immunodiffusion, contained antibodies directed to six to eight components of water-soluble ragweed.

Antisera to antigens Ra.3 and E were prepared by separately injecting rabbits subcutaneously with a mixture of 1 ml of antigen in saline (0.5 mg/ml) and 1 ml of complete Freund's adjuvant. The rabbits received two injections a week apart and were bled 10 days after the last injection.

Immunological Analysis. Double diffusion in agar was carried out by the technique of Ouchterlony (1958).

For cutaneous testing, each allergen solution was sterilized by filtration through a Millipore membrane filter (0.22 μ pore size) at a concentration of 1 mg/ml in phosphate-buffered saline. The sterile solutions were diluted to an appropriate concentration with sterile phosphate-buffered saline.

Direct skin testing of allergen solutions was done by intradermal injection of 0.05-ml aliquots into the forearm of a ragweed-sensitive individual. Reactions (wheal areas in mm^2) were read at 15-min postinjection.

For Prausnitz–Küstner (P–K) tests, normal volunteers (negative atopic history and negative skin test to ragweed extract) were injected intradermally with 0.05-ml aliquots of appropriately diluted ragweed allergic serum at skin sites on the back, 4–6 cm apart. After 24 hr, the sensitized sites were challenged with the appropriate allergen solution and the reactions were measured in the same manner as employed in direct skin testing. The relative activities of the various allergen preparations were assayed simultaneously in individual volunteers.

Cross-neutralization tests were performed by diluting reaginic serum with allergen solutions *in vitro* and incubating at room temperature for 24 hr prior to sensitization of normal skin, as in the P–K test. The injected sites were challenged with appropriate allergen and the reactions were measured as in the P–K test. The concentration of allergen solution used to dilute the reaginic serum was such as to just cause complete inhibition of the P–K reaction, and was determined in preliminary experiments.

Experiments and Results

Isolation of Antigen Ra.3. DEAE-cellulose fraction C (see Materials and Methods) was concentrated to 150 ml by ultrafiltration at 5°. Aliquots of 75 ml were dialyzed against 0.1 M Tris-HCl–0.2 M $(NH_4)_2SO_4$ and separately applied to a bed of Sephadex G-100 (two Pharmacia columns, each 5.0 × 70 cm, in series), equilibrated with the same (eluting) buffer. The flow rate was 50 ml/hr and 20-ml fractions were collected. Six fractions (C_I–C_{VI}) were obtained (Figure 1), of which fraction C_{IV} was further purified by TEAE-cellulose chromatography. The bulk of the carbohydrate (total hexose + pentose) was found in the higher molecular size fractions.

Sephadex G-100 fraction C_{IV} was concentrated to 20 ml by ultrafiltration at 5°, dialyzed exhaustively against 0.002 M Tris-HCl (pH 7.6), and applied to a TEAE-cellulose column (2.0 × 50 cm) equilibrated with the same (eluting) buffer. The flow rate was 35 ml/hr and 5-ml fractions were collected. As shown in Figure 2A, three fractions were obtained ($C_{IV\text{-}1}$, $C_{IV\text{-}2}$, and $C_{IV\text{-}3}$). Rechromatography of $C_{IV\text{-}2}$ on TEAE-cellulose (Figure 2B) gave two major fractions, both of which contained carbohydrate. The major fraction contained antigen Ra.3 in a yield of 100 mg/kg of dry pollen.

Physical and Chemical Characterization of Antigen Ra.3. Rechromatography on TEAE-cellulose of the antigen Ra.3 preparation of Figure 2B confirmed the absence of contamination with fractions $C_{IV\text{-}1}$ and $C_{IV\text{-}3}$. From optical density measurements, the recovery of antigen Ra.3 was 97%. The carbohydrate content (hexose + pentose) of antigen Ra.3 was identical before and after chromatography (12.4 ± 0.2%).

Antigens Ra.3 and E were compared by acrylamide gel disc electrophoresis (Figure 3). At pH 9.5, antigen Ra.3 migrated toward the cathode and was therefore not observed in the gel, while antigen E migrated to the anode as one major and one minor band. At pH 6.6, antigen E still migrated toward the anode and therefore

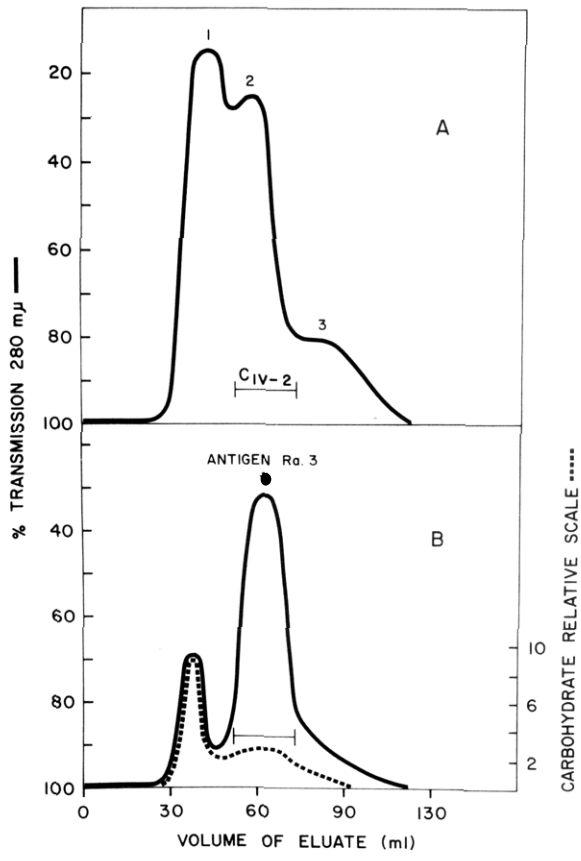

FIGURE 2: Chromatography of Sephadex G-100 fraction C_{IV} (A) and subfraction C_{IV-2} (B) on TEAE-cellulose (2.0 × 50 cm) in 0.002 M Tris-HCl (pH 7.6).

did not penetrate the gel; antigen Ra.3 showed only one component in this system. At pH 3.8, both antigens migrated toward the cathode; antigen E was observed as a major component, with some trailing, while antigen Ra.3 showed essentially one component, with the possibility of a trace contaminant on the cathodal side of the band.

The antigen Ra.3 preparation showed a single sedimenting component in the analytical ultracentrifuge at four solution concentrations (1.0, 0.8, 0.6, and 0.4%). Due to the slow rate of sedimentation of antigen Ra.3, the presence of contaminating lower molecular weight components could not be excluded. However, evidence for the absence of gross contamination with such components was obtained by Sephadex G-100 gel filtration; 4 ml of antigen Ra.3 (12.0 mg/ml) in 0.1 M Tris-HCl–0.2 M $(NH_4)_2SO_4$ (pH 7.9) was applied to a Sephadex G-100 column (2.5 × 95 cm) and eluted with the same buffer at a flow rate of 20 ml/hr. A single symmetrical peak emerged from the column after passage of 380 ml of buffer (Figure 4).

A least-squares plot of sedimentation coefficients determined for antigen Ra.3 at four concentrations revealed a slight concentration dependence (Figure 5). Extrapolation and correction to standard conditions gave $s^0_{20,w} = 1.8 \times 10^{-13}$ sec. A diffusion coefficient, $D^0_{20,w}$, of 10×10^{-7} cm^2 sec^{-1} was calculated from

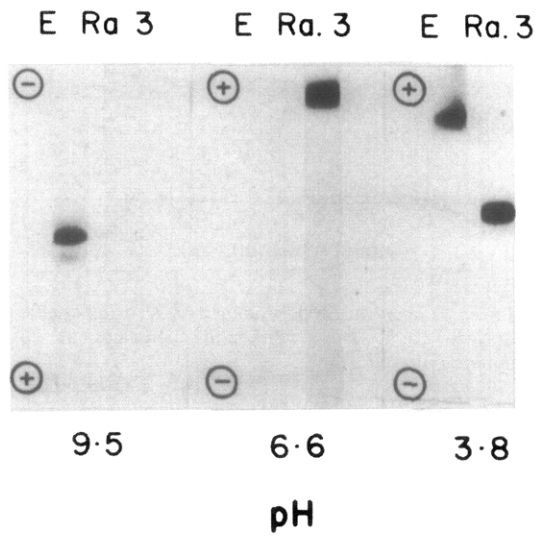

FIGURE 3: Acrylamide gel disc electrophoresis of antigens Ra.3 and E.

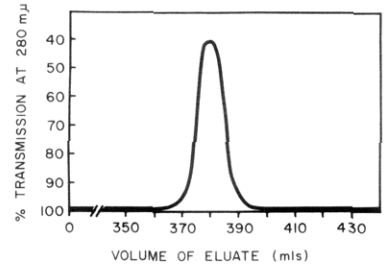

FIGURE 4: Gel filtration of antigen Ra.3 on Sephadex G-100 (2.5 × 95 cm) in 0.1 M Tris-HCl–0.02 M $(NH_4)_2$ (pH 7.9).

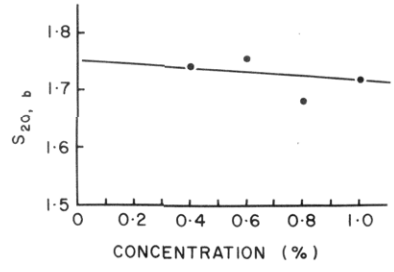

FIGURE 5: Concentration dependence of sedimentation coefficients of antigen Ra.3. $s_{20,b}$, sedimentation coefficient at 20° in phosphate-buffered saline.

synthetic boundary ultracentrifugal runs by extrapolation of values obtained at three concentrations (Figure 6) and correction to standard conditions. A partial specific volume, $\bar{v} = 0.715$, was calculated from the amino acid composition, assuming a partial specific volume of 0.61 for the carbohydrate moiety (Gibbons, 1966). From the aforementioned data, the molecular weight was calculated to be 15,000.

The absorption spectrum of a solution (1.04 mg/ml) of antigen Ra.3 showed a peak absorbance at 280 mμ,

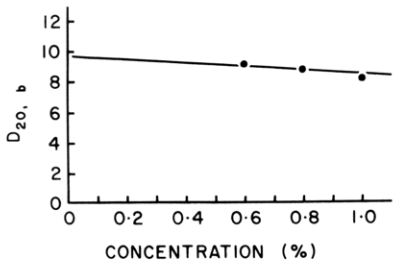

FIGURE 6: Concentration dependence of diffusion coefficients of antigen Ra.3. $D_{20,b}$, diffusion coefficient at 20° in phosphate-buffered saline.

typical of proteins. An absorption coefficient of $E_{1\,cm}^{1\,mg/ml}$ 1.09 ± 0.02 was calculated for antigen Ra.3. The nitrogen content of antigen Ra.3 was determined to be 13.5 ± 0.4%. The total hexose + pentose content (12.4 ± 0.2%) agreed with separate determinations of total hexose (4.9 ± 0.2%) and total pentose (8.7 ± 2%. The total hexosamine content was 0.8 ± 0.02%.

The number of amino acid residues in antigen Ra.3 was calculated from the amino acid composition assuming a molecular weight of 15,000 and a total carbohydrate content of 13%. The results are shown in Table I and are compared with published data for antigen E.

Antigenic and Allergenic Characterization of Antigen Ra.3. Immunodiffusion tests were done to determine the antigenic purity of antigen Ra.3 and the antigen E

TABLE I: Number of Amino Acid Residues in Antigens Ra.3 and E.

Amino Acid	Number of Residues	
	Antigen Ra.3[a]	Antigen E[b]
Lysine	7	18
Histidine	3	6
Arginine	4	16
Aspartic	7	49
Threonine	8	17
Serine	5	26
Glutamic	8	25
Proline	10	15
Glycine	8	37
Alanine	6	31
Half-cystine	3	7
Valine	6	24
Methionine	1	7
Isoleucine	4	20
Leucine	8	21
Tyrosine	2	4
Phenylalanine	7	12
Tryptophan	4	6
Total	101	341

[a] Based on a molecular weight of 15,000 and a carbohydrate content of 13%. [b] From the published data of King *et al.* (1964).

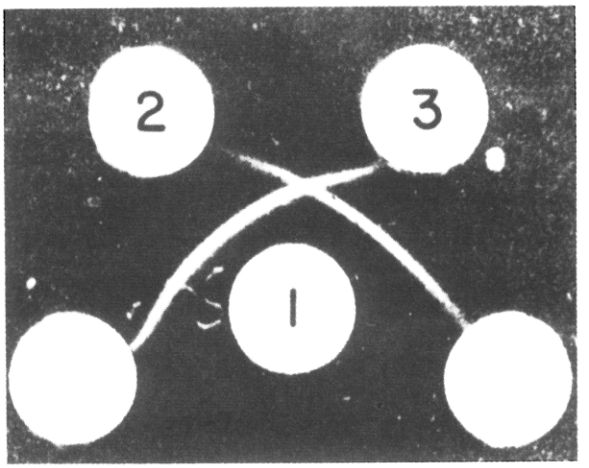

FIGURE 7: Immunodiffusion analysis of antigens Ra.3 and E. Antigen Ra.3 (1.0 mg/ml) in well 2; antigen E (1.0 mg/ml) in well 3; rabbit antiserum to water-soluble ragweed in well 1.

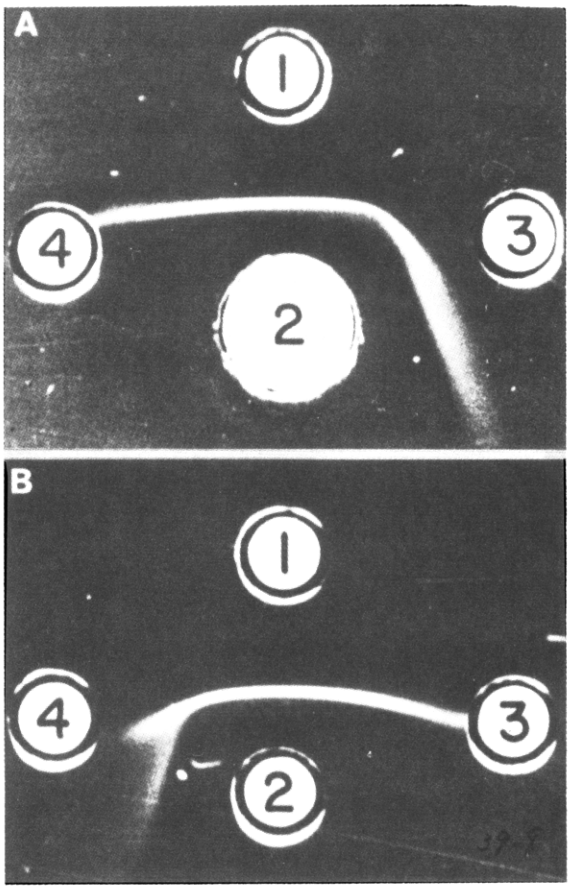

FIGURE 8: Immunodiffusion analysis of antigens Ra.3 and E. (A) Antigen Ra.3 in wells 1 (0.5 mg/ml) and 3 (0.02 mg/ml); antigen E in well 4 (4.0 mg/ml); rabbit antiserum to antigen Ra.3 in well 2. (B) Antigen E in wells 1 (0.5 mg/ml) and 4 (0.01 mg/ml); antigen Ra.3 in well 3 (2.0 mg/ml); rabbit antiserum to antigen E in well 2.

prepared in this study. The antigens, each at a concentration of 1 mg/ml, were allowed to diffuse against rabbit antiserum to water-soluble ragweed (Figure 7): each antigen preparation gave a single precipitin arc, with no evidence of cross-reaction. Identical results were obtained with two rabbit antisera. In addition, solutions of antigen Ra.3 (0.05–0.5 mg/ml) showed only one component in immunodiffusion tests with three rabbit antisera raised to the antigen Ra.3 preparation.

The possible cross-contamination of antigens Ra.3 and E was studied by immunodiffusion with specific antisera (Figure 8). The Ra.3–anti-Ra.3 reference line (Figure 8A) between wells 1 and 2 was deflected by 0.02 mg of antigen Ra.3/ml in well 3. However, antigen E in well 4, caused no such deflection. Thus, antigen E at a concentration of 4.0 mg/ml would contain less than 0.02 mg of antigen Ra.3/ml, or less than 0.5%. Similarly, any contamination of antigen Ra.3 by antigen E would be less than 0.5% (Figure 8B).

Antigens Ra.3 and E were used in direct skin-testing of 46 ragweed-sensitive patients. Of this total patient group, 26 had undergone at least 1 year of hyposensitization therapy with ragweed extract, while the remaining 20 patients were untreated. For testing, allergen solutions were freshly diluted to a concentration of 10^{-9} g/ml in sterile phosphate-buffered saline. Each patient received a single injection of antigen Ra.3 and antigen E at this dosage level.

The results of the direct skin tests are shown in Figure 9. The reactions were of 3 types: 28 patients (group 1) reacted with greater intensity to antigen E than to antigen Ra.3; 10 patients (group 2) reacted with approximately the same intensity to both allergens; 8 patients (not shown in Figure 9) gave no reaction to either allergen at the dosage level employed.

P–K tests were performed with the sera of patients reacting predominantly to antigen E by direct skin test (group 1), and those reacting to both allergens with approximately the same intensity (group 2).

Table II shows the results of experiments using a constant serum dilution for sensitization and varying allergen dilutions. The dilution of a given allergic serum was adjusted so that the cutaneous reactions obtained

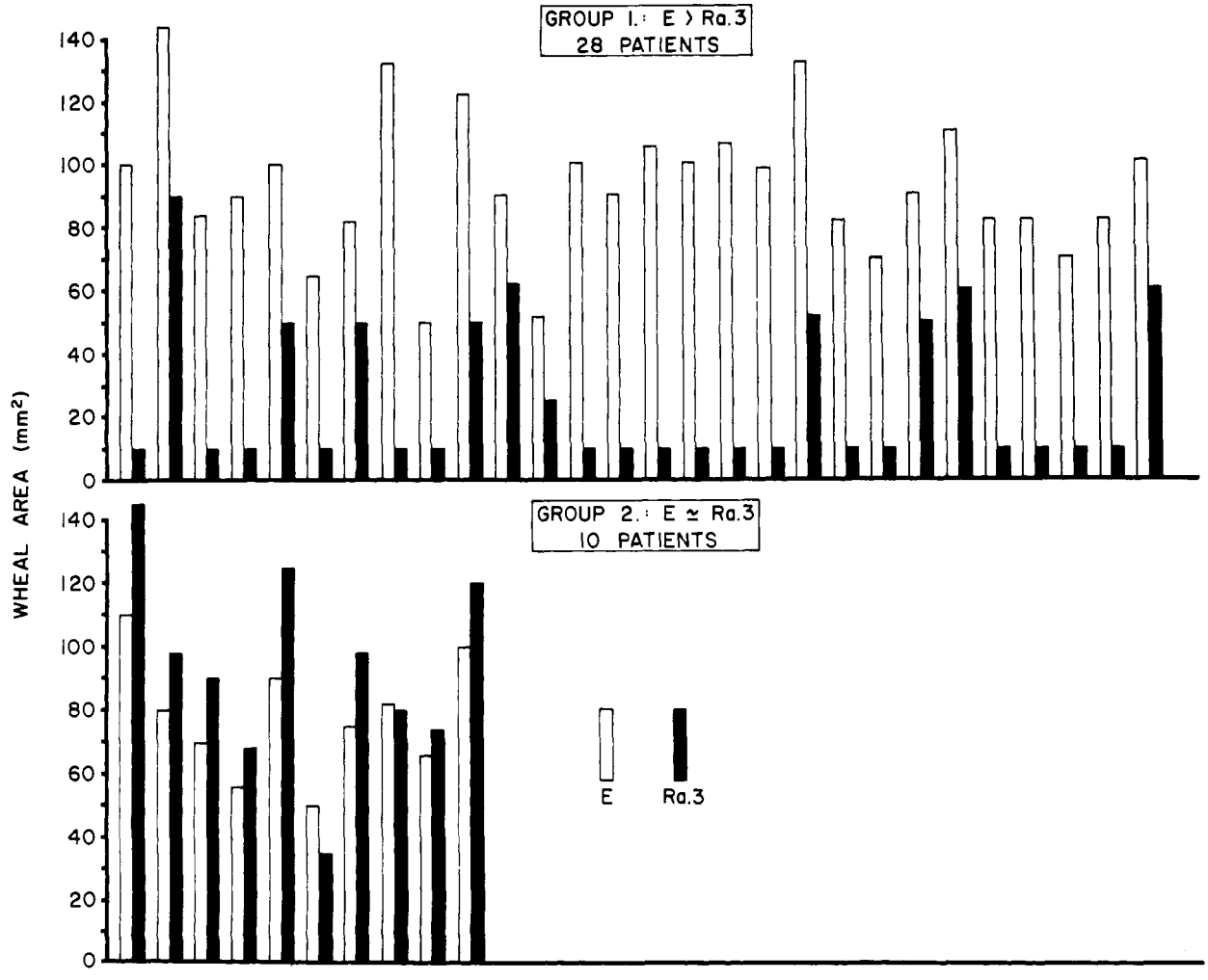

FIGURE 9: Direct skin testing of allergic patients with antigens Ra.3 and E. Wheal areas (mm^2) were measured 15-min postinjection. Saline controls were 9–12 mm^2. Group 1 consisted of 28 patients reacting with greater intensity to antigen E than to antigen Ra.3. Group 2 consisted of 10 patients reacting to both antigens with approximately equal intensity.

TABLE II: Comparison by P–K Test of the Allergenic Activity of Antigens Ra.3 and E.[a]

Allergen Concn (g/ml)	Sera													
	P. H. (1:100)		A. C. (1:100)		M. M. (1:10)		K. B. (1:10)		M. C. (1:10)		B. U. (1:100)		M. B. (1:100)	
	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E	Ra.3	E
10^{-6}	49	144	42	132	190	210	132	152	89	81	190	210	110	132
10^{-7}	9	110	9	120	168	170	110	130	72	72	100	120	90	100
10^{-8}	9	81	9	100	110	120	72	90	64	56	80	100	81	81
10^{-9}	9	64	9	81	99	100	72	72	9	9	50	90	49	49
10^{-10}	n.d.		n.d.		72	64	25	25	9	9	12	9	9	12

[a] Reactions expressed as wheal areas (mm^2). Saline control = 9–12 mm^2. n.d. = not done.

TABLE III: Comparison by P–K Test of the Allergenic Activity of Antigen Ra.3 and E.[a]

Serum P. H.	Challenge Ra.3	E	Serum A. C.	Challenge Ra.3	E	Serum M. M.	Challenge Ra.3	E	Serum K. B.	Challenge Ra.3	E
1:10	72	187	1:10	90	169	1:100	115	100	1:10	169	210
1:100	12	100	1:50	9	144	1:300	72	64	1:30	81	110
1:500	12	81	1:100	9	132	1:900	25	25	1:90	49	63
			1:200	9	110				1:270	9	12

[a] Serum sites were challenged with 5×10^{-11} mole of each allergen. Reactions expressed as wheal areas (mm^2). Saline control = 9–12 mm^2.

TABLE IV: Comparison by P–K Test of the Allergenic Activity of Antigens Ra.3 and E with Water-Soluble Ragweed.[a]

Allergen Concn (g/ml)	Serum					
	M. M. (1:10)			K. B. (1:10)		
	Water-Soluble Ragweed	Ra.3	E	Water-Soluble Ragweed	Ra. 3	E
10^{-6}	99	190	210	72	132	152
10^{-7}	64	168	170	36	110	130
10^{-8}	42	110	120	12	72	90
10^{-9}	9	99	100	12	72	72
10^{-10}	n.d.[b]	72	64	n.d.	25	25

[a] Reactions expressed as wheal areas in mm^2. Saline control = 9–12 mm^2. [b] Not done.

for all of the sera would fall within a similar range. Antigen E showed considerably greater activity than antigen Ra.3 when tested with sera P. H. and A. C., in agreement with the results of direct skin testing of these patients (Figure 9). Both allergens had approximately the same degree of reactivity when tested with the remaining sera (M. M., K. B., M. C., B. U., and M. B.), again in accord with the direct skin test results (Figure 9).

Table III shows the results obtained when varying dilutions of allergic serum were used for sensitization and the sites challenged with a constant concentration of allergen solution. The sera of patients P. H. and A. C., who reacted more strongly to antigen E than to antigen Ra.3 on direct skin test (Figure 9), showed greater reaginic activity to antigen E than to antigen Ra.3. The sera of patients M. M. and K. B., who gave similar reactions on direct skin test with antigens Ra.3 and E, showed approximately equal reaginic activity to both allergens.

Two allergic sera, M. M. and K. B., containing approximately equal reaginic activity to antigens Ra.3 and E (Table II) were employed in P–K tests to compare the activity of antigens Ra.3 and E with water-soluble ragweed. Normal skin sites sensitized with these sera were separately challenged with tenfold serial dilutions of the allergen solutions. The cutaneous activity of the two purified allergens was approximately 100 to 1000 times greater than the activity of water-soluble ragweed (Table IV).

TABLE V: Determination of Allergen Concentration Required for Neutralization of P–K Reaction.[a]

	Wheal Areas						
	Antigen Ra.3 (μg/ml)			Antigen E (μg/ml)			
Challenging Dose	5	0.5	0.05	10	1.0	0.01	Saline
Antigen Ra.3 (0.5 μg/ml)	9	49	100	*b*			115
Antigen E (1.0 μg/ml)				9	64	105	105

[a] Skin sites were sensitized with serum M. M. diluted 1:50 with solutions of each allergen at various concentrations and with saline. Skin sites sensitized with saline alone gave wheal areas = 9–12 mm^2. [b] Not done.

To determine the allergenic relationship of antigens Ra.3 and E, the two allergens were compared for their capacity to cross-neutralize the reagins in three ragweed allergic sera. The sera, obtained from patients M. M., M. C. and M. B., had approximately equal reaginic activity to both allergens (Table II).

In preliminary experiments, each allergic serum was separately incubated with serial dilutions of antigens Ra.3 and E and the serum–allergen mixtures were used to prepare sensitized skin sites. The sites were separately challenged with the same allergen employed for incubation and the cutaneous reactions obtained were identical for all sera (Table V). A concentration of 5 μg/ml of antigen Ra.3 and 10 μg/ml of antigen E completely inhibited the P–K reaction to the respective allergen. These concentrations were employed in the cross-neutralization tests.

To perform the tests, skin sites on three normal volunteers were injected with the allergic serum–allergen mixtures shown in Table VI. Allergic serum–saline mixtures served as nonneutralized controls. After 24 hr, the sites were separately challenged with antigens Ra.3 and E (0.5 and 1.0 μg per ml, respectively) and the cutaneous reactions obtained are shown in Table VI. In all cases, antigen Ra.3 completely neutralized Ra.3-specific antibodies but failed to neutralize the E-specific antibodies. On the other hand, antigen E neutralized the serum reagins directed to both allergens.

Discussion

The combination of gel filtration and anion-exchange chromatography proved efficacious for the purification of an allergenically active protein, antigen Ra.3, from the complex mixture of substances contained in the aqueous extract of short ragweed pollen. The isolation procedure was patterned after the method of King *et al.* (1964) for the preparation of antigen E. This allowed for the simultaneous isolation of antigens Ra.3 and E and consequently, for a comparative study of the properties of the two allergens.

The antigen Ra.3 preparation was found to be highly purified by several criteria. Evidence for charge homogeneity was obtained by TEAE-cellulose chromatography and polyacrylamide gel disc electrophoresis. Analytical ultracentrifugation and gel filtration on Sephadex G-100 provided evidence for the size homogeneity of the antigen Ra.3 preparation. Immunodiffusion experiments with two rabbit antisera prepared

TABLE VI: P–K Reactions of Ragweed Allergic Sera Cross-Neutralized with Antigens Ra.3 and E.

		Wheal Area (mm^2) on Challenge[a]					
		Subject 1		Subject 2		Subject 3	
Serum	Diluted in	E	Ra.3	E	Ra.3	E	Ra.3
M. M. (1:50)	Saline	110	120	100	90	81	88
M. M. (1:50)	Antigen Ra.3[b]	115	12	99	9	96	9
M. M. (1:50)	Antigen E[b]	9	9	12	9	9	9
M. C. (1:10)	Saline	72	70			81	90
M. C. (1:10)	Antigen Ra.3	70	9			72	9
M. C. (1:10)	Antigen E	9	9			12	12
M. B. (1:10)	Saline	110	90	144	110		
M. B. (1:10)	Antigen Ra.3	115	9	132	9		
M. B. (1:10)	Antigen E	9	9	9	12		

[a] Sites challenged with 1 μg of Ag.E and 0.5 μg of Ag.Ra.3. Saline control = 9–12 mm^2. [b] Antigens Ra.3 and E: 5 and 10 μg per ml, respectively, in saline.

against the whole aqueous extract (water-soluble ragweed) and three rabbit antisera against the purified antigen preparation, demonstrated a single precipitin arc, underlining the antigenic purity of the Ra.3 preparation. Immunodiffusion analysis of antigens Ra.3 and E with rabbit antiserum to water-soluble ragweed demonstrated that cross-contamination of the two allergens, if present at all, would be less than 0.5%.

Physical and chemical analysis revealed that antigen Ra.3 is a basic protein, containing an appreciable amount of carbohydrate. Some evidence was obtained that the carbohydrate forms an integral part of the molecule. Thus, on rechromatography of the antigen Ra.3 containing fraction, $C_{IV\text{-}2}$, the carbohydrate distribution followed the optical density distribution at 280 mμ (attributed to protein). Furthermore, the carbohydrate content of antigen Ra.3 showed no change on rechromatography or extensive dialysis. Carbohydrate analysis of antigen Ra.3 revealed the presence of hexose and pentose units, but the nature of these units, their arrangement and mode of attachment to the protein moiety, remains to be determined.

It is noteworthy, that the absorption spectrum of antigen Ra.3 showed a single peak at 280 mμ, characteristic of proteins. No peak was evident at 305 mμ suggesting the absence of the 1-amino-1-deoxy-2-ketose linkages postulated by Berrens (1962) to be a common feature of atopic allergens.

A comparison of the properties of antigens Ra.3 and E is given in Table VII. As can be seen from the table, the electrical charge on the two allergens differ, Ra.3 being a basic protein, antigen E, acidic. The lower sedimentation coefficient and higher diffusion constant of antigen Ra.3 reflects the differences in molecular weight of the allergens. While antigen E contains only 0.7% pentose, antigen Ra.3 has a total hexose and pentose content of 12.4 $\pm$ 0.2%. The nitrogen content of antigen Ra.3, 13.5 $\pm$ 0.4%, is lower than that of antigen E, 17.1%, reflecting the higher carbohydrate content of antigen Ra.3.

Allergenic testing of antigens Ra.3 and E established the differences in specificity of these two preparations. From the observation that both antigens Ra.3 and E provoked essentially equal cutaneous reactions in some patients, while only antigen E provoked reactions in others, it seems reasonable to conclude that the two preparations differ in their allergenic specificity. P-K tests with appropriate ragweed allergic sera confirmed the existence of two distinct allergen–reagin systems. Thus, some sera passively sensitized human skin for cutaneous reactions to both allergens while others sensitized for reactions predominantly to antigen E. Similar results have been obtained in studies employing passively sensitized rhesus monkey skin suspensions (I. Luhovyj, B. J. Underdown, and L. Goodfriend, unpublished data).

The results of direct skin testing of allergic patients confirmed the importance of antigen E as the major allergen of ragweed pollen. Antigen Ra.3 had the same level of specific activity as antigen E in 25% of skin test positive patients. In 75% of such patients, the relative specific activities of the two allergens varied: some patients showing no reactivity to antigen Ra.3, while others reacted to antigen Ra.3, but less markedly than to antigen E. No patients were observed reacting to antigen Ra.3 but not to antigen E.

The higher incidence of reactivity to antigen E might be due to its relative abundance in the pollen. In this connection, the yield of antigen E in its 2 major electrophoretic forms, IV-B and IV-C, was reported by King *et al.* (1964) to be approximately 0.5 g/kg of pollen, which is in marked contrast to the yield of 0.1 g/kg of pollen obtained for antigen Ra.3.

The comparative activities of antigens Ra.3 and E with water-soluble ragweed were examined by P-K tests, using sera from patients who reacted with similar intensity to antigens Ra.3 and E by direct skin test. The activities of the purified allergens were essentially identical and were 100–1000 times higher than that of the water-soluble ragweed preparation.

The results obtained in cross-neutralization studies with antigens Ra.3 and E suggest that these antigens carry common allergenic determinants. It would appear that antigen E possesses both "E" and "Ra.3" allergenic determinants, while antigen Ra.3 possesses Ra.3 determinants only. Since neutralization of anti-Ra.3 reagins in ragweed allergic sera caused no reduction in the P-K reactions of these sera to antigen E, the contribution of the Ra.3 determinants to the total cutaneous activity of antigen E must be relatively minor, at best. The apparent inability of antigen E–anti-Ra.3 reagin complexes to elicit cutaneous reactions may be due to the presence on the antigen E molecule of a single Ra.3 allergenic determinant, sufficient to effect neutralization of anti-Ra.3 reagins but ineffectual for eliciting cutaneous reactions with these antibodies. The presence of additional Ra.3 determinants on the antigen E molecule is not precluded, but such determinants would likely be sterically hindered to render the molecule functionally haptenic with respect to Ra.3 determinants.

The structural similarities of antigens Ra.3 and E, reflected in their allergenic cross-reactivity, are not apparent from their physiochemical properties. Furthermore, immunodiffusion tests with rabbit antisera failed to show any antigenic cross-reaction between the two allergens. However, the physical, chemical, and antigenic dissimilarities of antigens Ra.3 and E do not

TABLE VII: Comparison of Physical and Chemical Properties of Antigen Ra.3 and Antigen E.

Property	Antigen Ra.3	Antigen E
Electrophoretic mobility (pH 6.6)	Cathodic	Anodic
$s^0_{20,w}$ (sec)	1.8×10^{-13}	3.05×10^{-13}
$D^0_{20,w}$ (cm^2 sec^{-1})	10×10^{-7}	7.41×10^{-7}
Molecular weight (g)	15,000	37,000
Nitrogen content (%)	13.5	17.1
Total hexose + pentose content (%)	12.4	0.5

exclude the presence of an Ra.3 region on the antigen E molecule. Such a region could be relatively small in size, of the order of a penta- or hexapeptide, as has been found for antigenic determinants (Kabat, 1966). The immunogenicity of this region might be such as to require prolonged immunization in rabbits or other animal species for the production of specific antibody.

Acknowledgments

We are greatly indebted to Dr. Bram Rose for his constant interest and for facilitating the progress of this study. We thank Dr. M. W. Chase for donations of antigen E and rabbit antiserum to antigen E. We gratefully acknowledge the capable technical assistance of Mr. D. Woodard, Mr. M. Lanner, and Mr. W. Rankowicz.

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Subunit Structure of the B Component of *Escherichia coli* Tryptophan Synthetase*

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ABSTRACT: Sedimentation equilibrium studies demonstrate that the B subunit of tryptophan synthetase dissociates into two polypeptides upon treatment of the protein with concentrations of urea greater than 4 M or with performic acid. Some dissociation is observed even in dilute solution in phosphate buffer. C-Terminal amino acid analysis and gel electrophoresis experiments support the hypothesis that the native enzyme is a dimer composed of two identical or very similar subunits whose molecular weight is 45,000 g/mole. The urea-dissociated enzyme can be reconstituted with 80% of the original activity if thiol concentrations are maintained at the proper level during removal of urea. A hybrid enzyme, one of whose subunits contains pyridoxal phosphate reduced onto the enzyme with sodium borohydride, exhibits one-half the specific activity of the native enzyme in catalyzing the conversion of indole into tryptophan.

Tryptophan synthetase (*Escherichia coli*) is a multimeric enzyme composed of two proteins which differ greatly in their physical and catalytic properties (Yanofsky, 1960). The complex dissociates readily into two separable components, termed the A and B proteins, each carrying an active site for an enzymatic half-reaction (Crawford and Yanofsky, 1958).

The A component, which carries the active site for the aldolysis of indole-3-glycerol phosphate to indole and glyceraldehyde 3-phosphate, is a single polypeptide chain of about 29,000 molecular weight (Henning *et al.*, 1962). The B component (Wilson and Crawford, 1965) binds two pyridoxal phosphate molecules; it is capable of performing a variety of catalytic functions, (Miles *et al.*, 1968), but catalyzes best the condensation of L-serine and indole to L-tryptophan. The B protein is

* From the Department of Microbiology, Scripps Clinic and Research Foundation, La Jolla, California. *Received October 15, 1968.* This work was supported by Grant GB 6841 from the National Science Foundation, and by funds from the Fleischmann Foundation.