

Canine antiragweed serum. Demonstration of "blocking" activity by in vivo and in vitro techniques.

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Normal dogs immunized with ragweed antigen produce a nonprecipitating anti-ragweed antiserum. The nonprecipitating antibody in the induced canine antiragweed was demonstrated by binding of I^{131} -labeled ragweed, by inhibition of precipitating antigen-antibody reactions, and autoradiographic immunoelectrophoresis. This antiserum inhibits the reactions of dogs passively sensitized with canine reagin to challenge with ragweed antigen.

Serum from dogs with spontaneous pollen allergy has certain similarities to serum from human allergic patients, and will transfer cutaneous, bronchial, and anaphylactic sensitivity to normal dogs.^{1, 2} This provides a model for study of pollen sensitivity with techniques which cannot be safely carried out in man.

The present studies were conducted to determine some characteristics of a canine antiserum against ragweed antigen produced by immunization with the pollen antigen. A preliminary report² described the induction in normal dogs of an antiragweed serum which inhibited the anaphylaxis resulting from canine reaginic antiragweed serum. The present report describes the demonstration of antibody activity in the canine antiserum by several in vitro immunologic techniques. In addition, the in vivo studies have been extended to demonstrate the inhibiting effect of the canine antiragweed serum on passive cutaneous reactions resulting from spontaneous canine antiragweed reagin.

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Three types of antisera produced by dogs have now been described and characterized to some extent. These include the spontaneous canine reagin occurring in spontaneously sensitive dogs,¹ precipitating and nonprecipitating antibodies produced against heterologous serum protein antigens,^{3, 4} and the induced, nonprecipitating canine antiragweed serum described herein. Certain biologic activities of these types of antisera may now be compared.

MATERIALS AND METHODS

Immunization of dogs. Three normal dogs of mixed breed were injected subcutaneously biweekly for 6 weeks with 2 ml. of a 15 per cent crude extract⁵ of short ragweed (*Ambrosia elatior*) pollen in incomplete Freund's adjuvant.⁶ The dogs were bled prior to the first injection and 2 weeks after each of the last 3 injections, and the serum from each bleeding tested for hemagglutinating antibody against tanned sheep red blood cells coated with ragweed antigen (RW) by standard hemagglutination techniques.⁷ Ring and double gel diffusion precipitin reactions were done with 5 per cent crude RW as the antigen by standard techniques.⁸ The sera from all bleedings were combined to form the pool of induced dog serum (IDS) against RW. Sera from normal dogs were collected to form the pool of normal dog serum (NDS).

Immunization of rabbits. Adult albino rabbits were immunized with subcutaneous injections of 2 ml. of 15 per cent RW in incomplete Freund's adjuvant every 2 weeks for 2 months. The rabbits were bled 1 week after the last injection and serum from individual bleedings tested by double gel diffusion precipitation reactions for precipitating antibody. Sera demonstrating multiple precipitin bands against RW were pooled and used as rabbit precipitating antiragweed serum.

Canine reaginic serum (CRS). Serum containing canine reaginic antibody was collected by monthly bleedings of 3 dogs with clinical ragweed allergy.¹ A separate pool of serum was made from each dog (CRS-1, CRS-2, and CRS-3).

Isotope-labeled ragweed antigen. A purified preparation of ragweed antigen (Fraction IV-C supplied by Dr. T. P. King, Rockefeller Institute) was labeled with I^{131} as previously described.⁹ The characteristics of this purified fraction have been reported.¹⁰ More than one antigenic component appears to be present in the preparation used.

Autoradiographic immunoelectrophoresis. Autoradiographic immunoelectrophoresis of IDS and normal dog serum (NDS) was done by the method previously described for demonstration of canine antibodies,³ with the use of purified ragweed antigen labeled with I^{131} .

Coprecipitation of I^{131} RW by IDS. The binding of purified I^{131} RW by IDS using rabbit antidog gamma globulin was done by the coprecipitation method previously described.⁹

In vivo test systems for binding activity

Skin reactivity. Serial fivefold dilutions of CRS were made in 0.85 per cent NaCl and 0.1 ml. injected in the skin of normal dogs. Forty-eight hours later the dogs were injected intravenously with 1 ml. of 0.3 per cent RW antigen mixed

Table 1. *Inhibition of passive cutaneous reactions by induced antiragweed serum*

Dog	CRS-1			CRS-2			CRS-3		
	Challenging RW			Challenging RW			Challenging RW		
	0.3%	3%	15%	0.3%	3%	15%	0.3%	3%	15%
<i>Local reactions* of dogs to intravenous injection of RW antigen after local sensitization with CRS</i>									
A	5	25	25	Undil.	5	5	Undil.	5	5
B	5	5	25	Undil.	5	5	Undil.	Undil.	Undil.
<i>Local reactions of dogs to challenge with ragweed antigen mixed with 10 ml. IDS</i>									
C	Neg.	Undil.	5	Neg.	Neg.	Neg.	Neg.	Neg.	5
D	Neg.	Neg.	25	Neg.	Neg.	Undil.	Neg.	Neg.	Undil.
B	Neg.	Neg.	5	Neg.	Neg.	Neg.	Neg.	Neg.	Neg.

CRS, Canine reaginic serum.

IDS, Induced dog antiragweed serum.

RW, Ragweed antigen.

*Reactions recorded as reciprocal of highest positive fivefold dilution of sensitizing serum giving a positive reaction.

with 2 ml. of Evans blue dye. After 30 minutes, reactions were read and 1 ml. of 3 per cent RW injected, followed in 30 minutes by the injection of 1 ml. of 15 per cent RW. Serially increasing amounts of antigen were injected to demonstrate the reaction occurring at each level of antigen. When inhibition of a reaction by blocking antiserum occurred at one concentration of antigen, the administration of a higher concentration of antigen would generally show a reaction. The injection of the RW and Evans blue dye resulted in an immediate type of skin reaction demonstrated by a blue cutaneous reaction.¹ Control sites injected with normal dog sera were negative. Pools CRS-1, CRS-2, and CRS-3 resulted in positive cutaneous reactions in the skin of normal dogs (Table I). Titers of passive cutaneous reactions could be determined in this manner. CRS-1 serum had the highest passive cutaneous titer. Passive cutaneous reactions with CRS-1 in a series of experiments using 20 subjects were positive in all recipients. After establishment of the respective titers, the experiments were repeated with the challenging ragweed antigen mixed with the blocking IDS.

Bronchial reactivity. Dogs spontaneously allergic to ragweed antigen or normal dogs passively sensitized with 30 ml. of CRS developed respiratory symptoms characterized by dyspnea and tachypnea when exposed by aerosol to ragweed antigen. The effect of IDS was studied by comparison of the respiratory response of dogs injected with CRS or CRS plus IDS.

RESULTS

In vitro demonstration of antibody in IDS

The tanned red blood cell hemagglutination titers of individual sera of dogs immunized with RW varied from 1:200 to 1:800. The IDS pool had a hemagglutination titer of 1:400.

The coprecipitation of I¹³¹ RW by IDS. The method used for demonstration of human antibody against I¹³¹-labeled, purified RW⁹ was used, with rabbit

antidog gamma globulin as the precipitating antiserum. Varying amounts of I^{131} -RW were added to 0.05 ml. aliquots of IDS and CRS-1. After incubation at 37° C. for 2 hours, an excess of rabbit antidog gamma globulin was added. Incubation was continued for 1 hour and the tubes were then centrifuged and the radioactivity of the precipitates and supernatant solutions determined. The curves obtained are shown in Fig. 1. A maximum of 86 per cent of the labeled antigen was coprecipitated with the serum of the spontaneously sensitive dog (CRS-1). Additional precipitation of labeled antigen was observed with IDS indicating that 99 per cent of the purified labeled ragweed preparation is antigenic for the dog. These binding data parallel those for the sera of human allergic subjects treated with injections of ragweed extract and the sera of untreated allergic individuals.

In vitro demonstration of competitive binding activity of IDS

Gel diffusion reactions. The IDS pool did not produce a positive precipitin reaction against ragweed antigen. The binding of ragweed antigen by IDS was demonstrated by the inhibition of precipitin reactions in gel using a rabbit antiragweed precipitating serum. In Fig. 2 the precipitin lines between rabbit antiragweed sera (*A*) and ragweed antigen (*C*) extend to the well containing the NDS (*D*). These bands are absent in the region of the well containing the IDS (*B*). The reactions shown in Fig. 2 are those occurring at 48 hours. After prolonged incubation (7 to 14 days) there is gradual extension of the bands to the IDS well. This is thought to be the result of dissociation of nonprecipi-

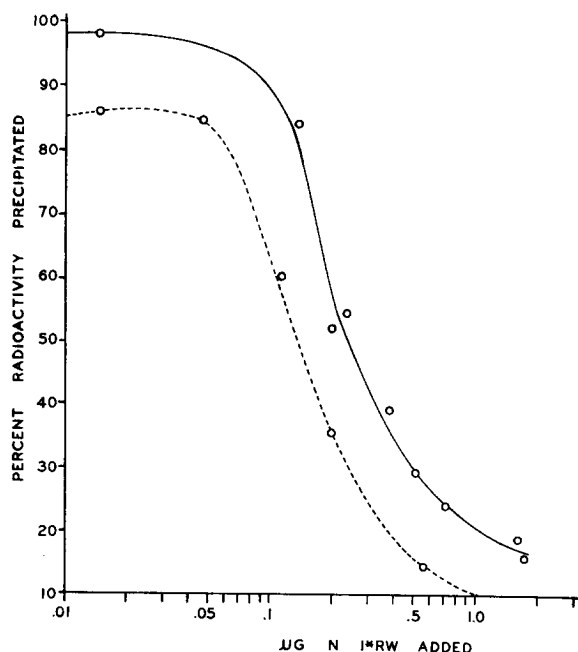
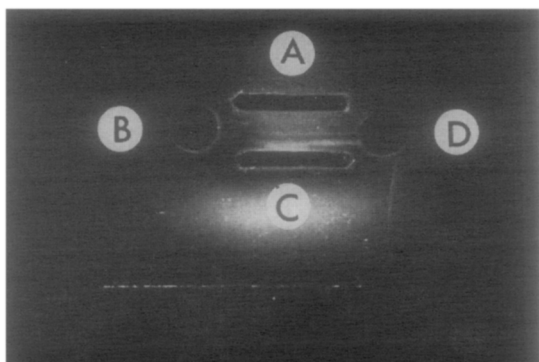
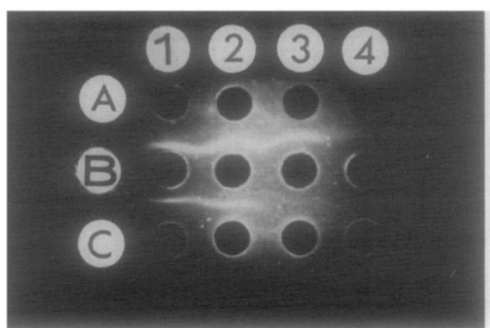


Fig. 1

Coprecipitation of I^{131} ragweed by IDS, $\circ$ — $\circ$; and CRS-1, $\circ$ — $\circ$.

**Fig. 2**

A, Three per cent ragweed antigen. *B*, IDS (blocking) antiserum. *C*, Rabbit antiragweed serum. *D*, Normal dog serum. The precipitin bands between *A* and *C* extend up to well *D* but are absent in the region of well *B*.

**Fig. 3**

Row *A*, Equal parts of NDS and dilutions of ragweed antigen. *A1*, 1.5 per cent; *A2*, 0.75 per cent; *A3*, 0.15 per cent; *A4*, 0.075 per cent ragweed antigen. Row *B*, Rabbit antiragweed serum. Row *C*, Equal parts of IDS and the same dilutions of ragweed antigen as in Row *A*. Absence of bands between *B3* and *C3* and *B4* and *C4* is the result of binding of antigen by IDS.

tating antibody-antigen complexes and precipitation of antigen by precipitating antibody. A similar reaction with canine nonprecipitating antibody to purified protein (bovine serum albumin) has previously been described.⁴ While only 2 precipitin bands are seen in Fig. 2, additional bands appear with prolonged incubation, as would be expected with a rabbit antiserum against crude ragweed antigen.

A different demonstration of the inhibition of a precipitin reaction by the binding of ragweed to IDS is shown in Fig. 3. Incubation of ragweed antigen with IDS prior to charging the wells results in interference with the formation of precipitin bands. This is shown both by absence of precipitin reactions between wells *B3* and *C3* and wells *B4* and *C4*, and the greater distance of bands from the antiserum wells *B1* and *B2*. The appearance of a single precipitin band

in the result of photographic technique and the incubation time as described above.

Inhibition of precipitation in tubes by IDS. Ragweed antigen was incubated (at 37° C. for 30 minutes and 4° C for 60 minutes) with either NDS or IDS prior to addition of rabbit precipitating antiserum by the procedures shown in Table II. The precipitation of RW by rabbit anti-RW serum is shown in the first 5 tubes (Fig. 4). Increasing precipitate is observed with increasing amounts of antigen added (Tubes 1 to 3). Decreasing precipitate occurs with increasing

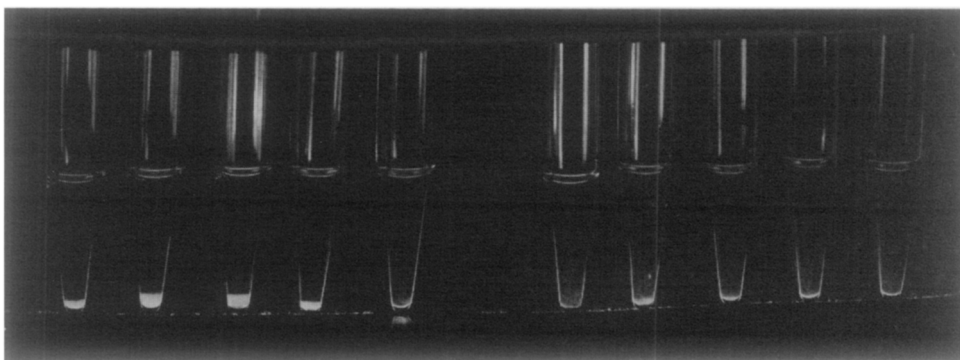


Fig. 4

Inhibition of precipitation of RW by rabbit anti-RW as a result of IDS. Tubes 1 to 5: I¹³¹-RW + NDS + rabbit anti-RW. Tubes 6 to 10: I¹³¹-RW + IDS + rabbit anti-RW. See text and Table III.

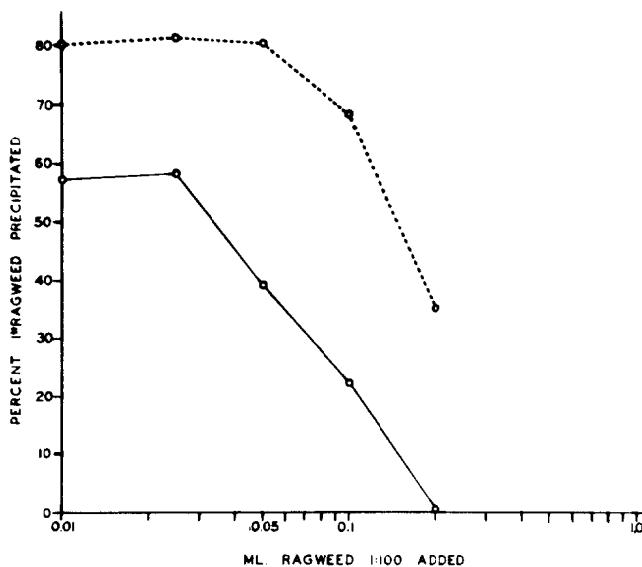


Fig. 5

Inhibition of precipitation of I¹³¹-RW by rabbit anti-RW as a result of IDS. I¹³¹-RW + IDS + rabbit anti-RW, ○—○. I¹³¹-RW + rabbit anti-RW, ○-----○.

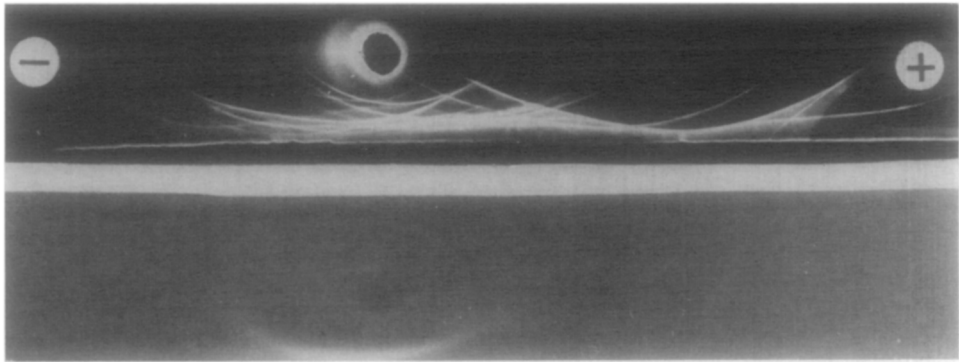


Fig. 6
Autoradiograph of immunoelectrophoresis of IDS. Above, Immunoelectrophoresis stained for protein arcs. Below, Autoradiograph of immunoelectrophoresis of IDS after addition of I^{131} -RW. The major arc binding antigen is a faster migrating globulin than γ_2 -globulin.

antigen excess (Tubes 4 and 5). Interference with the RW-rabbit anti-RW system by IDS is shown in tubes 6 to 10 (Fig. 4), since there is decreased precipitate formed in all tubes as the result of the addition of IDS to the antigen prior to addition of the precipitating antibody. The difference between the degree of precipitation in each series of tubes can be detected by determination of I^{131} -labeled ragweed precipitated in each series. Fig. 5 shows the inhibition of precipitation by the added I^{131} trace-labeled ragweed antigen in the tubes shown in Fig. 4 and Table II. A maximum of 80 per cent of the I^{131} -labeled ragweed was precipitable by the rabbit antiragweed serum.

Effect of heat on activity of IDS. Heating IDS to 56° C. for 4 hours did not significantly alter its ability to inhibit the precipitation of RW by precipitating rabbit antiragweed in the gel diffusion or tube precipitation systems.

Autoradiographic immunoelectrophoresis. Fig. 6 shows the location of the major precipitin band binding the purified I^{131} -labeled ragweed antigen after

Table II. *Preparation of tubes to demonstrate inhibition of precipitation by blocking antiserum*

	Tube									
	1	2	3	4	5	6	7	8	9	10
1% ragweed* antigen (ml.)	0.01	0.025	0.05	0.01	0.2	0.01	0.025	0.05	0.1	0.2
IDS (ml.)						0.25	0.25	0.25	0.25	0.25
NDS (ml.)	0.25	0.25	0.25	0.25	0.25					
0.15M NaCl (ml.)	0.20	0.175	0.15	0.1		0.20	0.175	0.15	0.1	
Incubate 37° C. for 30 minutes; then 4° C. for 60 minutes										
Rabbit antiragweed (ml.)	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
Incubate 37° C. for 30 minutes; then 4° C. for 24 hours. Centrifuged 2,500 RPM for 30 minutes. Radioactivity of supernatant solution and precipitate determined.										

*Containing trace I^{131} -labeled purified ragweed antigen.

immunoelectrophoresis of IDS. This is consistent with a globulin migrating as a γ_1 -globulin. A faint band seen in the autoradiograph indicates that some of the ragweed is bound by a slower migrating γ_2 -globulin.

In vivo demonstration of binding activity

Inhibition of cutaneous reactions. The alteration of the passive cutaneous reactions resulting from CRS as a result of prior incubation of RW with IDS is shown in Table I. All 3 pools of CRS gave passive cutaneous reactions with the lowest concentration of RW used for intravenous challenge. The final titer depended on the concentration of the RW used for challenge. When the challenging RW was mixed with the IDS prior to intravenous injection, inhibition of reactions occurred as demonstrated by the absence of or by diminished reactions at 0.3 per cent or 3 per cent RW concentrations. When 15 per cent RW was injected, positive reactions occurred in most skin sites which had not reacted to challenge with lower concentrations of RW. The skin sites which did not react to challenge with 15 per cent RW (for example, CRS-2 in Dog C, Table I) may have been neutralized slowly by RW in excess of or dissociated from antibody in IDS with which it had been incubated. The neutralization may have occurred too slowly to produce a typical blueing reaction. The IDS did not sensitize normal dog skin for passive cutaneous reactions of the type occurring with canine antiragweed reagin.

Inhibition of anaphylaxis. Anaphylaxis as a result of sensitization of normal dogs with CRS followed by challenge with ragweed was inhibited by IDS as previously reported.²

Inhibition of bronchial reactivity. Three dogs passively sensitized with 10 ml. CRS-1 and 10 ml. IDS and challenged by 3 per cent RW aerosol did not have dyspnea and tachypnea previously observed in dogs actively or passively sensitized with CRS.¹ The results suggested inhibition of passive bronchial reactivity in dogs receiving IDS. Because the end point of this type of reaction is not as definite as that of others such as passive cutaneous reactions or passive anaphylaxis, these results are considered suggestive but not conclusive.

DISCUSSION

Canine reagin occurring in dogs spontaneously sensitive to ragweed pollen is heat labile at 56° C., nonprecipitating, and skin sensitizing.¹ When dogs without demonstrable spontaneous allergic disease were immunized with bovine serum albumin in incomplete Freund's adjuvant, two types of antisera were produced.^{3, 4} These were a precipitating type which included a mixture of precipitating and nonprecipitating antibodies with different rates of electrophoretic migration (precipitating: γ_2 , nonprecipitating: γ_1) and a nonprecipitating antiserum containing an antibody which migrated electrophoretically as a γ_1 -globulin. The precipitating antiserum sera resulted in Arthus but not immediate passive cutaneous reactions; the nonprecipitating antiserum resulted in neither. The canine antiragweed serum produced by immunization has characteristics similar to those of the nonprecipitating canine antiserum since it is a nonprecipitating, heat-stable antiserum

which does not sensitize normal dog skin and migrates as a γ_2 -globulin. The nonprecipitating canine antbovine albumin could be demonstrated in vitro by inhibition of the reaction between rabbit precipitating antiserum and the albumin antigen.³ The canine antiragweed antibody can be demonstrated by similar systems in vitro and by interference with the reaction between the ragweed antigen and passively transferred reagin in either a local cutaneous or general anaphylactic reaction. The induced canine antiserum against ragweed might thus be considered a "blocking" type of antibody.

Dogs passively sensitized with CRS serum were used in these experiments, rather than dogs spontaneously sensitive to pollen, for two reasons. The effect of the inhibiting antiserum could be studied under more controlled conditions in the passively sensitized recipient, and the naturally sensitive dogs are not available in sufficient numbers to use for either the immunization or the challenging experiments described.

The techniques demonstrating in vivo and in vitro inhibition of reactions of pollen antigen with reaginic antibody or with a precipitating antibody must be considered qualitative until pollen antigens of sufficient purity are available in sufficient quantity to permit the use of a ragweed antigen which is identified as a single molecular species.

REFERENCES

1. Patterson, R., Chang, W. W. Y., and Pruzansky, J. J.: The Northwestern University Colony of Atopic Dogs, *J. ALLERGY* **34**: 455, 1963.
2. Tennenbaum, J. I., Patterson, R., and Pruzansky, J. J.: Canine Antiserum Analogous to Human Allergic and "Blocking" Antiserums, *Science* **142**: 589, 1963.
3. Patterson, R., Pruzansky, J. J., Janis, B.: Biologic Activity of Canine Nonprecipitating and Precipitating Antibody, *J. Immunol.* **93**: 51, 1964.
4. Patterson, R., Chang, W. W. Y., and Pruzansky, J. J.: Some Characteristics of Canine Precipitating and Non-precipitating Antibody, *Immunology* **7**: 150, 1964.
5. Hepler, O. E.: *Manual of Clinical Laboratory Methods*, Springfield, Ill., 1949, Charles C Thomas, Publisher.
6. Freund, J.: The Mode of Action of Immunologic Adjuvants, *Advances in Tuberculosis Res.* **7**: 130, 1956.
7. Stavitsky, A. B.: Micromethods for the Study of Proteins and Antibodies. I. Procedure and General Applications of Hemagglutination and Hemagglutination-Inhibition Reactions With Tannic Acid and Protein-treated Red Blood Cells, *J. Immunol.* **72**: 360, 1954.
8. Kabat, E. A., and Mayer, M. M.: *Experimental Immunochemistry*, Springfield, Ill., 1961, Charles C Thomas, Publisher.
9. Pruzansky, J. J., and Patterson, R.: Binding of I^{131} -labeled Ragweed Antigen by Sera of Ragweed-sensitive Individuals, *J. ALLERGY* **35**: 1, 1964.
10. King, T. P., Norman, P. S., and Connell, J. T.: Isolation and Characterization of Allergens From Ragweed Pollen. II, *Biochemistry* **3**: 458, 1964.