

The relationship between the skin-sensitizing antibody and γA protein in the sera of ragweed-allergic individuals

L. Perelmutter, Ph.D., B. Rose, M.D., Ph.D., and L. Goodfriend, Ph.D., Montreal, Quebec, Canada

The relationship between the skin-sensitizing antibodies and γA proteins of two allergic sera was studied by chromatography on DEAE-Sephadex A-50. Examination of effluent fractions by immunoelectrophoresis showed a considerable spread of γA proteins with electrophoretic mobilities ranging from that of γ_1 to α_2 -globulins. Assays of the effluent fractions by P-K and quantitative immunodiffusion tests demonstrated that the skin-sensitizing antibodies were confined to fractions containing either no γA proteins or the more cathodic of the γA proteins. Other fractions, containing, respectively, some 45 and 97 per cent of the γA protein in each of the sera, were inactive. This suggested that either skin-sensitizing antibodies are confined to electrophoretically distinct γA proteins or that these antibodies belong to a class of immunoglobulins other than γA .

In the past few years evidence has been obtained which suggests that reagins are associated with γA proteins. The main basis for this conclusion was furnished by Fireman and co-workers,¹ who found that removal of γA globulins from ragweed-allergic serum by absorption with sheep antihuman γA antiserum resulted in complete loss of reaginic activity from the allergic serum. Additional evidence was obtained by Ishizaka and associates² who inhibited skin sensitization with reagin by prior injection into a specific site of a fraction, rich in γA protein, derived from normal human serum. Heremans and Vaerman,³ using zinc sulfate precipitation followed by starch-gel electrophoresis, demonstrated that reaginic activity was found in a serum fraction containing mainly γA protein. Fireman and co-authors¹ and Terr and Bentz⁴ fractionated allergic sera on Sephadex G-200 and found a closer correlation between skin-sensitizing

From the Division of Immunochēistry and Allergy Research, McGill University Clinic, Royal Victoria Hospital.

The work was supported by grants from the Medical Research Council, Ottawa, Canada, and the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md.

Received for publication May 17, 1965.

activity and the distribution of γ A compared to that of γ G or γ M protein. Goodfriend and co-workers,⁵ employing DEAE-Sephadex chromatography, found active γ A proteins free of other immunoglobulins when tested by immunoelectrophoresis.

Recently, Vaerman and co-workers,⁶ using a combination of Sephadex G-200 and starch-gel electrophoresis, demonstrated that γ A found in both normal and myeloma human sera is not a discrete serum component but is heterogeneous with respect to its electrophoretic and ultracentrifugal properties. In view of Vaerman's results, it was of interest to investigate further the heterogeneity of γ A components in the sera of ragweed-allergic individuals and to establish the association of skin-sensitizing antibodies with these γ A components.

METHODS AND MATERIALS:

Allergic sera

Two sera were employed in this study, one from a nontreated, ragweed-sensitive individual (R.A.) and the other from a treated ragweed-allergic patient (B.P.). The sera were stored in small aliquots at -20° C. until used.

Gel filtration on Sephadex G-200

One allergic serum (R.A.) was fractionated on Sephadex G-200 using conditions similar to those of Gelotte and co-authors.⁷ Six milliliters of the serum were dialyzed for 16 hours against 0.1M Tris-HCl buffer, pH 8.0, made 1M in NaCl and filtered on a column (2.5 by 100 cm.) of Sephadex G-200. The serum proteins were eluted with the Tris-HCl buffer, the eluates of appropriate cuts pooled, and the pools concentrated to the initial serum volume by dialysis against polyethylene glycol M20. The concentrated fractions were exhaustively dialyzed against 0.15M sodium chloride and examined by the Prausnitz-Küstner (P-K) test, immunoelectrophoresis, and quantitative immunodiffusion.

Chromatography on DEAE-Sephadex A-50

The conditions for chromatography on DEAE-Sephadex of either Sephadex G-200 fractions or whole allergic serum were similar to those used previously.⁵ In separate experiments, a 2 ml. aliquot of Sephadex G-200 fraction II (Fig. 1) of serum R.A. or 2 ml. of serum B.P. were each dialyzed against 0.1M Tris-HCl buffer, pH 8.0, for 16 hours, and then chromatographed on a column (1.0 by 26.0 cm.) of DEAE-Sephadex A-50. Three protein fractions were eluted with the 0.1M Tris-HCl buffer. Additional fractions were eluted after a gradient was established by passage of 1 L. of 0.5M Tris-HCl buffer, pH 8.0, into 1 L. of the same buffer at 0.1M. The gradient was set up so that the reservoir leading to the column was maintained at constant volume. A final fraction was obtained by passing 1.0M Tris-HCl, pH 8.0, through the column. The cuts for the various fractions were made according to the shape of the optical density curves. The fractions were concentrated to the initial serum volume (2 ml.) and examined by P-K test, immunoelectrophoresis, and quantitative immunodiffusion. Some of the fractions were further concentrated for the immunodiffusion tests.

Prausnitz-Küstner (P-K) test

The allergic sera and serum fractions were assayed for skin-sensitizing activity by the Prausnitz-Küstner (P-K) test⁸ as described by Perelmutter and co-authors.⁹ Prior to assay, the solutions were membrane filtered through Swinny 1/2 inch filters, and varying dilutions were made with saline under sterile conditions. Aliquots of 0.05 ml. of the solutions were injected intradermally into sites on the backs of nonatopic volunteers. The sensitized sites were challenged 24 hours later with 1,000 PNU of water-soluble ragweed. After 20 minutes, the reactions were graded 0 and 1 + to 4 +, depending on the size of the wheal and flare. The titer was taken as the highest dilution giving a 1 + reaction. Control sites consisted of sensitized sites challenged with saline and saline-injected sites challenged with ragweed.

Immunoelectrophoresis

Microimmunoelectrophoresis was performed, by using the method of Scheidegger,¹⁰ and employing the L.K.B. apparatus. The antisera to human proteins: horse anti-whole serum, goat anti- γ G, and goat anti- γ A, were obtained from Hyland Laboratories. Immunoelectrophoretic analysis of the latter antiserum tested with whole human serum revealed, in addition to antibodies specific to γ A, antibodies to an α -globulin. However, the γ A components of the various chromatographic fractions were readily identified by their position on immunoelectrophoresis. In addition, the distribution of γ A protein among these fractions was confirmed by the quantitative immunodiffusion assay.

Quantitative immunodiffusion

Allergic sera and serum fractions were tested for their γ A content using the " γ A-immunoplates" and method of Hyland Laboratories.¹¹ Three standard solutions* of known γ A content and three test solutions were separately deposited in the antigen wells of each plate. The plates were incubated at room temperature for 16 hours and precipitate ring diameters were measured with a magnified scale. Standard curves were drawn for ring diameter versus γ A concentration (milligrams per 100 ml.), and the curves were used to determine the γ A content of the allergic sera and serum fractions. Duplicate assays made on several serum fractions demonstrated an experimental error for the method of 5 to 10 per cent, in agreement with the findings of Fahey and McKelvey.¹²

RESULTS**Fractionation of allergic serum on Sephadex G-200**

The effluent peaks obtained by filtration of 6 ml. of allergic serum R.A. on Sephadex G-200 are shown in Fig. 1. It can be seen that reaginic activity was localized between the descending portion of the first and the ascending portion of the second peak.

The active area was divided into three subfractions: *I*, *II*, and *III* (Fig. 1). Immunoelectrophoretic examination of fractions *I*, *II*, and *III* against goat

*Supplied by Hyland Laboratories.

SEPHADEX G-200 GEL FILTRATION OF WHOLE ALLERGIC SERUM (R.A.)

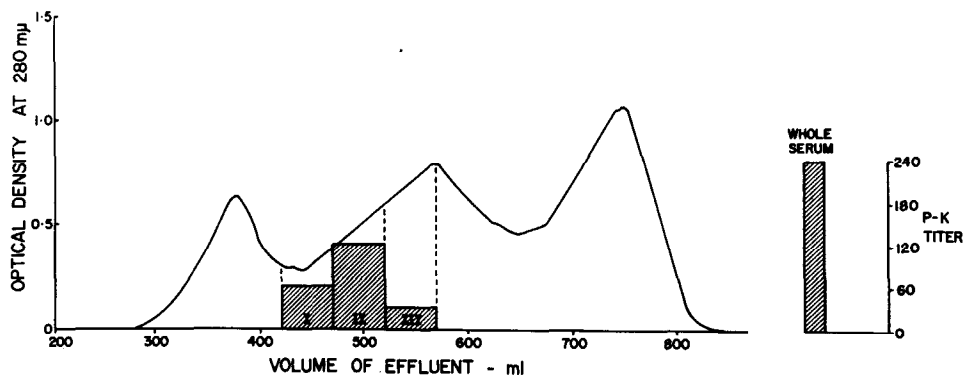


Fig. 1

Gel-filtration of ragweed-allergic serum (R.A.) on Sephadex G-200 in 0.1M Tris-HCl buffer, pH 8.0, made 1.0M in NaCl. The P-K titers of active fractions I, II, and III (concentrated to the initial serum volume) are compared to that of the corresponding whole allergic serum.

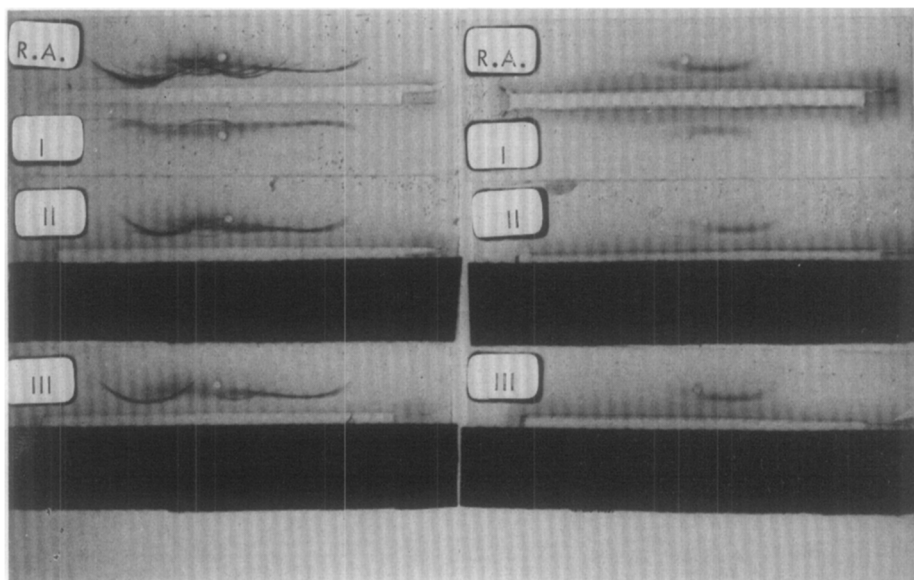


Fig. 2

Immunoelectrophoretic analysis of ragweed-allergic serum (R.A.) and Sephadex G-200 fractions I, II, and III. The serum and fractions were tested against horse anti-whole serum (left) and goat anti- γA serum (right). The anode is on the left.

anti- γA serum (Fig. 2) revealed no essential differences in electrophoretic mobility between the γA proteins present in these fractions. It is apparent from Fig. 2 that each of these fractions contained at least six components when tested against horse anti-whole serum.

The allergic serum and fractions I, II, and III were examined for their γA content by the immunoplate method. The results are given in Table I and

Table I. γA content and P-K titer of Sephadex G-200 fractions of allergic serum R. A.

Fractions	γA content (mg.)	P-K titer
First effluent peak (Fig. 1)	0.2	—
I	1.5	60
II	5.2	120
III	0.3	30
Total recovered	7.2	
Amount applied	11.4	
Per cent recovered = 63.1		

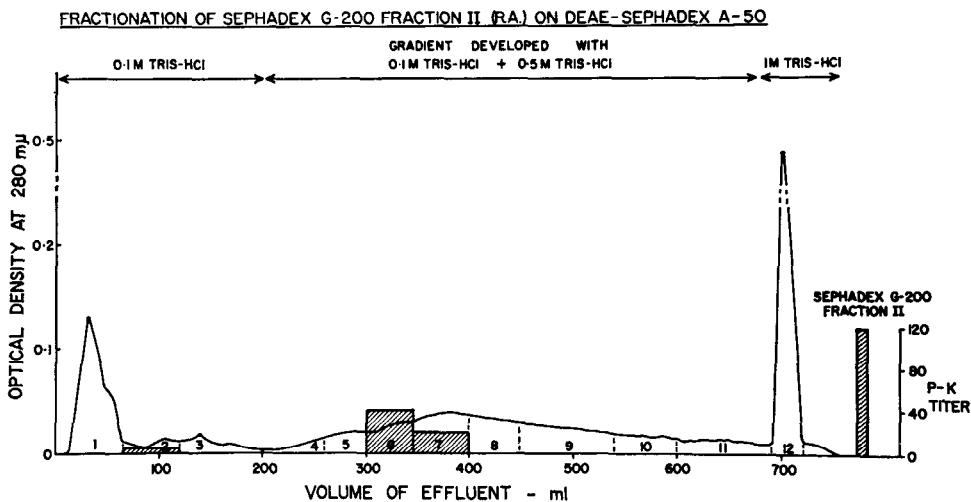


Fig. 3
Chromatography of fraction II of ragweed-allergic serum R.A. on DEAE-Sephadex A-50. Fractions 1 to 3 were eluted with the starting buffer, 0.1M Tris-HCl. Fractions 4 to 11 were obtained with linear gradient established by passage of 1 L. of 0.5M Tris-HCl buffer, pH 8.0, into 1 L. of 0.1M buffer of the same pH. A final fraction, 12, was eluted by applying 1M Tris-HCl, pH 8.0, directly to the column. The P-K titers of the active fractions 2, 6, and 7 (concentrated to the initial serum volume) are compared with that of whole fraction II.

demonstrate an apparent relationship between the skin-sensitizing activity and γA content of these fractions. Fraction II contained most of the skin-sensitizing activity and γA protein.

Chromatography of reaginic serum fraction on DEAE-Sephadex A-50

In order to further separate the skin-sensitizing antibodies from nonactive proteins, a 2 ml. aliquot of Sephadex G-200 fraction II was chromatographed on DEAE-Sephadex A-50 using a combination of step and gradient elution. The results are shown in Fig. 3. Effluent fraction 2 had slight but definite reaginic activity, while the bulk of the activity emerged shortly after the gradient was applied, i.e., in fractions 6 and 7.

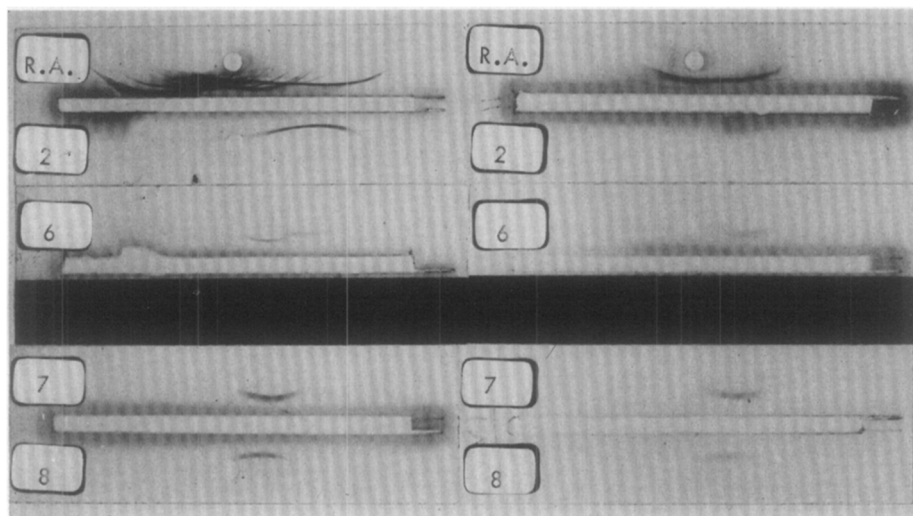


Fig. 4

Immunoelectrophoretic analysis of ragweed-allergic serum (R.A.) and DEAE-Sephadex fractions 2, 6, 7, and 8. The serum and fractions were tested against horse anti-whole serum (*left*) and goat anti- γA serum (*right*). The anode is on the left.

When examined by immunoelectrophoresis (Fig. 4), fraction 2 showed a γG component only, while fraction 6 contained, in addition to γA , transferrin and a fast-moving γG component. Fractions 7 and 8 each had γA components only, as evidenced by the single band which each of these fractions gave when tested against the horse anti-whole serum and goat anti- γA serum. It is interesting to note that the γA components of fractions 6, 7, and 8 had slightly different electrophoretic mobilities, the more cathodic being in fraction 6.

The effluent fractions were examined by the quantitative immunoplate method and the results are shown in Table II. While fraction 2 was found to possess reaginic activity, no γA protein was detected in this fraction, even after a sevenfold concentration. The γA protein was confined mainly to fractions 6, 7, and 8. Although fractions 6 and 7 both contained skin-sensitizing activity as well as γA protein, fraction 8 showed no reaginic activity and yet accounted for about 45 per cent of the γA protein recovered.

Chromatography of reaginic serum on DEAE-Sephadex A-50

In view of the unexpected finding that a chromatographic fraction of reaginic serum R. A. (fraction 8) contained approximately half the recovered γA protein but was devoid of skin-sensitizing activity, it was of interest to determine the distribution of reaginic activity relative to γA protein upon direct fractionation of whole allergic serum on DEAE-Sephadex A-50. For this purpose, 2 ml. of serum B. P. was fractionated on DEAE-Sephadex A-50, and the effluent peaks are shown in Fig. 5. As found for the Sephadex G-200 fraction II of serum R. A., an active fraction (fraction 2) emerged with the initial buffer. Most of the skin-sensitizing activity was localized in the fractions emerging

Table II. γA content and P-K titer of DEAE-Sephadex fractions

Fractions*	γA content (mg.)	P-K titer
2	—	2
2 ($\times 7$)	—	—†
6	0.38	40
7	0.41	20
8	0.65	—
Total recovered	1.44	
Amount applied	1.86	
Per cent recovered = 77.5		

*Fractions other than those given in the table contained either trace amounts or no γA protein.
†Not done.

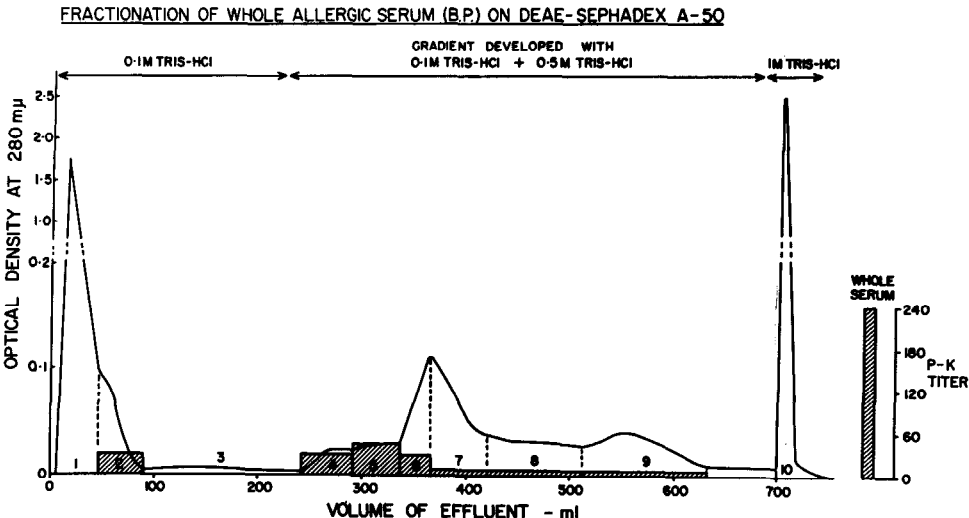


Fig. 5
Chromatography of ragweed-allergic serum B.P. (2 ml.) on DEAE-Sephadex A-50. The conditions for elution of the various fractions are described in the legend for Fig. 3. The P-K titers of the active fractions (concentrated to the initial serum volume) are compared with that of the whole serum.

after application of the gradient, i.e., fractions 4, 5, and 6, with traces found in fractions 7, 8, and 9.

The results of the immunoelectrophoretic examination of DEAE-Sephadex fractions 2 to 10 are shown in Fig. 6. When tested against horse anti-whole serum, active fraction 2 showed a γG component only, while the active fractions 4, 5, and 6 each contained transferrin, as well as a fast-moving γG -globulin. The γA -globulin was confined to fractions 7 to 10. Electrophoretic heterogeneity of the γA protein was again revealed. Thus, a gradual change in electrophoretic mobility of the γA components can be observed in fractions 7 to 10 when each of these fractions was tested against the goat anti- γA serum.

Each of the DEAE-Sephadex fractions was examined by the immunoplate

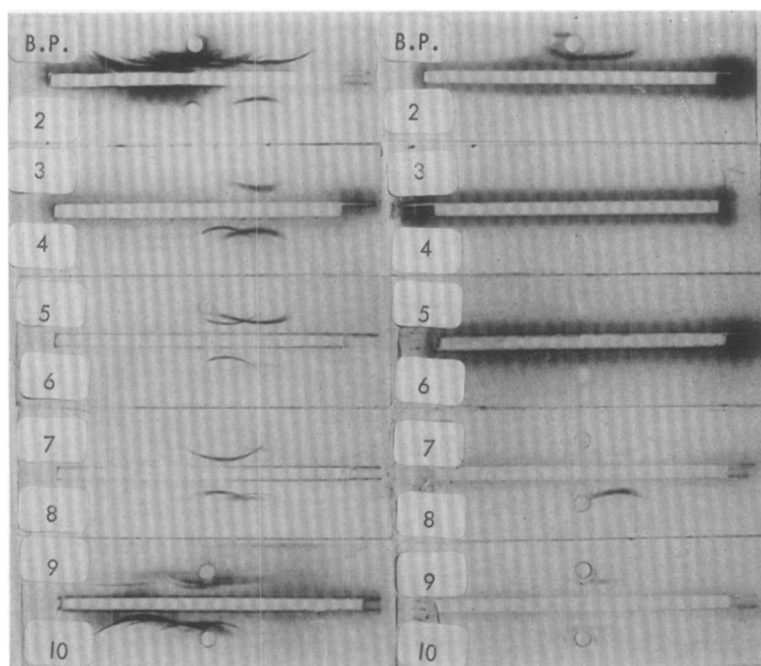


Fig. 6

Immunoelectrophoretic analysis of ragweed-allergic serum B.P. and DEAE-Sephadex fractions 2 to 10. The serum and fractions were tested against horse anti-whole serum (*left*) and goat anti- γA serum (*right*). The anode is on the left.

Table III. γA content and *P-K* titer of DEAE-Sephadex fractions of serum B. P.

Fractions	γA content (mg.)	<i>P-K</i> titer
1	—	—
2 ($\times 5$)	—	20
3	—	—
4 ($\times 5$)	—	20
5 ($\times 5$)	0.02	40
6	0.10	20
7	0.54	1
8	1.26	1
9	0.60	1
10	0.21	—
Total recovered	2.73	
Amount applied	3.80	
Per cent recovered = 71.8		

method for γA content (Table III). Most of the γA protein resided in fractions 7, 8, and 9, although these fractions possessed minimal skin-sensitizing activity. On the other hand, fractions 4, 5, and 6 had less than 3 per cent of the total γA protein recovered but possessed the bulk of the skin-sensitizing activity. The γA protein could be detected in fraction 5 only after fivefold concentration of

this fraction. A fivefold concentration of active fractions 2 and 4 failed to reveal any γ A protein.

DISCUSSION

In agreement with the findings of Vaerman and co-authors,⁶ using normal and myeloma sera, the γ A proteins of the allergic sera examined in the present study were found to be electrophoretically heterogeneous. The γ A proteins obtained in effluent fractions following DEAE-Sephadex chromatography showed a considerable spread of mobilities ranging from that of γ_1 to α_2 globulins when examined by immunoelectrophoresis. The degree of resolution of the γ A components was found to be dependent on the nature of the buffer gradient employed.¹³

The fractions obtained by DEAE-Sephadex chromatography of the ragweed-allergic sera showed no apparent relationship between the γ A and reagin content of these fractions. Thus, most of the skin-sensitizing antibodies were localized in fractions containing either no γ A protein or the more cathodic of the γ A proteins, at the levels at which these fractions were concentrated for assay. It is noteworthy that for one of the sera (B. P.) the reaginic activity was associated with only 3 per cent of the total γ A protein recovered. This percentage may, in fact, constitute an upper limit in view of the studies of Fahey and McKelvey¹² which indicate that polymers of γ A globulin result in a lower estimate of γ A content by the immunoplate compared to the isotope dilution method. Ultracentrifugal analysis of fractions obtained by DEAE-Sephadex chromatography of ragweed-allergic sera has demonstrated¹³ that the active fractions contained no components with sedimentation coefficients greater than 7S while inactive fractions which contained the bulk of the γ A globulin had 9S and 13S components.

As has been found by other investigators^{1, 4} there was a relationship between skin-sensitizing activity and γ A content of fractions obtained by filtration of allergic sera on Sephadex G-200. Furthermore, as shown in a previous study,⁵ the most active of these fractions could be resolved on DEAE-Sephadex A-50 to yield fractions containing the bulk of both the γ A protein and skin-sensitizing activity. However, the more gradual gradient employed in the present study was efficacious in separating some 45 and 97 per cent, respectively, of the γ A protein in each of the sera from the bulk of the skin-sensitizing activity.

From the results of the present study, it would appear that reagins are confined mainly to certain of a large spectrum of γ A components present in human serum. On the other hand, the lack of correlation between reaginic activity and γ A content of the DEAE-Sephadex fractions suggests another possibility, namely, that reaginic antibodies belong to a class of immunoglobulins other than γ A. The possibility that reagins are γ M immunoglobulins may be ruled out, in view of the results of ultracentrifugal^{14, 15, 16} and gel-filtration studies.^{1, 4, 5} That reagins may belong to the γ G class of immunoglobulins is indicated by the finding of the present study, that all of the DEAE-Sephadex fractions which contained skin-sensitizing antibodies also had small amounts of relatively fast-moving γ G globulins. This possibility would appear to be excluded by the studies

of Fireman and co-authors¹ in which sheep antiserum to human γ G globulin absorbed the fastmoving γ G component of an active DEAE-cellulose fraction without depleting the fraction of reaginic activity. However, Fireman and associates¹, have carried out their absorption studies with only one of several active DEAE-cellulose fractions containing γ G globulin, and their results do not preclude the possibility that reagins of different immunoglobulin types were distributed among their active chromatographic fractions.

Very recently Rowe and Fahey¹⁷ have detected a new human serum immunoglobulin, γ D, which appears to have some physicochemical properties similar to those of the more cathodic γ A proteins found in the present study in those DEAE-Sephadex fractions containing the bulk of skin-sensitizing activity. It is tempting to suggest that some of the reaginic antibodies found in atopic serum may belong to the γ D class of immunoglobulins.

The authors wish to acknowledge the capable assistance of Mrs. Myrna Sachs.

Addendum

Since this paper was submitted, analysis of 6 additional sera by DEAE-Sephadex chromatography has not only confirmed these results in part, but of more importance has shown that the bulk of the reaginic activity can be separated from detectable γ A globulin.

REFERENCES

1. Fireman, P., Vannier, W. E., and Goodman, H. C.: The Association of Skin-Sensitizing Antibody With β_2 A-globulins in Sera From Ragweed-Sensitive Patients, *J. Exper. Med.* **117**: 603, 1963.
2. Ishizaka, K., Ishizaka, T., and Hombrook, B. S.: Blocking of Prausnitz-Küstner Sensitization With Reagin by Normal Human β_2 A-globulin, *J. ALLERGY* **34**: 395, 1963.
3. Heremans, J. F., and Vaerman, J. P.: β_2 A-globulin as a Possible Carrier of Allergic Reaginic Activity, *Nature* **193**: 1091, 1962.
4. Terr, A. I., and Bentz, J. D.: Gel Filtration of Human Skin-Sensitizing Antibody and β_2 A-globulin, *J. ALLERGY* **35**: 206, 1964.
5. Goodfriend, L., Perelmutter, L., and Rose, B.: Use of DEAE-Sephadex for the Fractionation of Allergic Serum, *Nature* **205**: 718, 1965.
6. Vaerman, J. P., Fudenberg, H. H., Johnson, L. B., and Mandy, W. J.: Size Heterogeneity of Normal and Pathological γ A globulins, *Fed. Proc.* **23**: 558, 1964.
7. Gelotte, B., Flodin, P., and Killander, J.: Fractionation of Human Plasma Proteins by Gel-Filtration and Zone Electrophoresis or Ion-Exchange Chromatography, *Arch. Biochem. Suppl.* **1**: 319, 1962.
8. Prausnitz, G., and Küstner, H.: Studien über die Euberempfindlichkeit, *Centralbl. f. Bakt.* **86**: 160, 1921.
9. Perelmutter, L., Freedman, S. O., and Schon, A. H.: Fractionation of Sera From Ragweed-Sensitive Individuals by Chromatography on DEAE-Cellulose, *Internat. Arch. Allergy* **14**: 129, 1961.
10. Scheidegger, J. J.: Micro-method de l'immunoelectrophoresa, *Internat. Arch. Allergy* **7**: 103, 1955.
11. Immunoplates, Hyland Laboratories, Los Angeles, California.
12. Fahey, J. L., and McKelvey, E. M.: Quantitative Determination of Serum Immunoglobulins in Antibody-Agar Plates, *J. Immunol.* **94**: 84, 1965.
13. Perelmutter, L., Mason, R., and Goodfriend, L.: Fractionation on DEAE-Sephadex of Normal and Myeloma Human Sera. In preparation.
14. Augustin, R., and Hayward, B. J.: Human Reagins to Grass Pollens and Moulds: Their Purification and Physico-chemical Characterization, *Immunology* **3**: 45, 1960.
15. Stanworth, D. R.: Studies of the Physico-chemical Properties of Reagins to Horse Dandruff, *Immunology* **2**: 384, 1959.
16. Andersen, B. R., and Vannier, W. E.: The Sedimentation Properties of the Skin-sensitizing Antibodies of Ragweed-Sensitive Patients, *J. Exper. Med.* **120**: 31, 1964.
17. Rowe, D. S., and Fahey, J. L.: A New Class of Human Immunoglobulins. II. Normal Serum IgD, *J. Exper. Med.* **121**: 185, 1965.