THE ASSOCIATION OF SKIN-SENSITIZING ANTIBODY WITH THE β_2A-GLOBULINS IN SERA FROM RAGWEED-SENSITIVE PATIENTS

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The skin-sensitizing antibodies found in the serum of allergic individuals have not been well defined immunochemically. Attempts at characterization of skin-sensitizing antibodies have been directed toward studying their distribution in allergic sera by electrophoretic (1–3), ultracentrifugal fractionation (4–8) or diethylaminoethyl cellulose (DEAE[1] cellulose) chromatography (5, 9–11) and the subsequent association of skin-sensitizing antibody with one or more serum fractions. These studies have shown that skin-sensitizing antibodies differ from the bulk of 7S γ-globulins with which antibodies are classically associated. The consensus of these studies is that skin-sensitizing antibodies have a γ_1 or β_2 electrophoretic mobility and a sedimentation coefficient of perhaps 7S but more likely between 7S and 18S.

The introduction of immunoelectrophoresis has provided a means of distinguishing serum proteins on the basis of their antigenic determinants after electrophoretic separation.[2] When Augustin (10, 12) applied this method of analysis to serum fractions obtained by DEAE cellulose chromatography, the skin-sensitizing activity was found to be associated with a globulin of γ_1-mobility, antigenically related to γ-globulin or β_2A(γ_1A)-globulin. While the studies to be described were in progress, Heremans (13) reported that a β_2A-globulin preparation which was not immunochemically pure, being accompanied by a small amount of γ-globulin, contained skin-sensitizing antibody.

The purposes of this study were: (*a*) to reinvestigate some of the fractionation methods previously described and to determine whether the combination of preparative zone electrophoresis followed by DEAE cellulose chromatography of allergic sera would yield an immunochemically pure serum fraction which

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[1] DEAE, diethylaminoethyl.

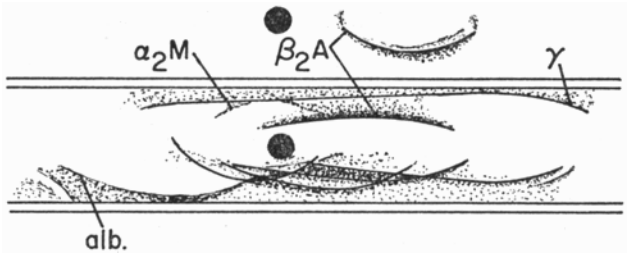
[2] The three immunochemically distinguishable globulins in the γ and slow β regions with which antibody activities have been associated are (immunoelectrophoretic terminology) γ-globulins, β_2A(γ_1A)-globulins, and the β_2M(γ_1M)-globulins (30).

contained skin-sensitizing activity, (*b*) to apply the recently described method of fractionation of serum proteins by gel filtration with sephadex G-200 to the characterization of skin-sensitizing antibodies, and (*c*) to study the problem of the immunochemical nature of skin-sensitizing antibodies by precipitating each of the immune globulins with specific antisera to determine which of the globulins contained the skin-sensitizing activity.

These experiments have demonstrated that skin-sensitizing antibodies in the three ragweed sensitive sera studied were associated with the β_2A-globulins.

Materials and Methods

Sera.—Sera from three allergic patients who were exquisitely sensitive to ragweed pollen by history and by positive scratch and intradermal skin tests were used for the studies. These



TEXT-FIG. 1. Diagram of immunoelectrophoretic pattern of normal human serum (bottom round reservoir) and of the purified β_2A preparation (top round reservoir) used for immunization (anode to the left). The antiserum trough at the top of the figure contained the sheep anti-β_2A serum produced by immunization with the purified β_2A preparation; bottom antiserum trough contained a sheep anti-whole human serum.

patients had received hyposensitization therapy and had no history of hepatitis. All studies were performed on serum from a single 500 ml bleeding from each donor. The sera and all subsequently obtained serum protein fractions were filtered through a Swinny adapter containing a 0.22 μ Millipore filter, tested for sterility, and stored at 4°C prior to study.

The human γ-globulin used for absorption of the sheep anti-β_2A-globulin serum was prepared by DEAE cellulose chromatography (14) of normal human serum.

The normal sheep and rabbit sera used as controls were filtered through a Swinny adapter containing an 0.22 μ Millipore filter, tested for sterility, and stored at 4°C.

Specific Antisera.—An anti-β_2A-globulin serum was prepared by immunization of sheep with 3 mg of purified β_2A myeloma globulin in complete Freund's adjuvant by intramuscular injection. The purified β_2A myeloma globulin preparation (G. F.) was kindly provided by Dr. John Fahey. It was prepared by starch block electrophoresis and then DEAE cellulose chromatography of serum containing β_2A type myeloma protein (15). After 1 month the sheep were bled. One of the two sheep immunized provided a suitable antiserum. This antiserum in addition to containing antibody to β_2A-globulins also contained traces of antibody to γ-globulins and α_2M-globulins as demonstrated by immunoelectrophoresis (Text-fig. 1). The antibodies to γ-globulins were absorbed from 1 ml of the sheep antiserum by the addition of 500 μg of the human γ-globulin that had been purified by DEAE cellulose chromatography. The γ-globulin was added in increments of 100 μg and incubated at 37°C for 2 hours and then

at 4°C for 18 hours, after each addition. Following centrifugation the supernatant was analyzed by interfacial test for excess antigen prior to each addition. Subsequent bleedings from this sheep were not suitable for this study owing to an increased concentration of antibodies to γ-globulins and α_2M-globulins (see Text-fig. 5).

Immunization of four sheep with purified β_2M-globulins from two patients with macroglobulinemia did not result in anti-β_2M-globulin sera suitable for absorption studies.

An anti-γ-globulin serum was produced by intramuscular immunization of rabbits with 2 to 4 mg of purified γ-globulin in complete Freund's adjuvant (14). This rabbit antiserum produced only a single precipitin line when examined by immunoelectrophoresis (16), Ouchterlony (17), and Oudin (18) techniques. However, the rabbit anti-γ-globulin serum did form a precipitin line with the purified β_2A myeloma globulin in immunoelectrophoresis due to the presence of identical or cross-reacting antigenic determinants on the γ- and β_2A-globulins. The antibodies to the β_2A-globulin detectable by interfacial precipitin test were absorbed from 1 ml of the rabbit anti-γ-globulin serum by the addition to two 0.5 μg increments of the purified β_2A myeloma globulin in the same absorption procedure as described above for preparation of the sheep anti-β_2A-serum.

Horse anti-whole human serum was commercially obtainable from the Pasteur Institute, Paris, France. Unispecific sheep anti-human albumin serum was obtained from Dr. Arthur J. L. Strauss.

Skin Tests.—Skin-sensitizing antibody was assayed by the passive transfer test (19) of Prausnitz and Küstner (P-K). The recipients were normal volunteers who were not sensitive to ragweed by history or intradermal skin tests. Normal skin sites were sensitized with 0.1 ml of serum or the serum fraction by intradermal injection with 27 gauge needles. Forty-eight hours later, each sensitized site was challenged by intradermal injection of 0.02 ml of ragweed extract containing 1000 protein nitrogen units per ml.[3] Twenty minutes later the margins of the wheal and erythema were outlined with a ball point pen. Permanent records of the reactions were made by applying transparent cellophane adhesive tape to the areas and then removing the tape which carried with it ink outlines of the skin reactions. The titer of skin-sensitizing activity was determined by preparing serial dilutions of each serum fraction for sensitization of normal skin sites. The highest dilution which produced a definitively positive reaction on challenge with ragweed extract was taken as the end point of the titration. Because of variability of skin reactivity from one recipient to another an appropriately diluted positive control and also a negative control were utilized in each series of skin tests. All conclusions were drawn from data obtained on the same recipient at the same time.

Starch Block Zone Electrophoresis.—The method was a modification of that described by Kunkel and Slater (20) and others (21). An 8 ml volume of human serum which had been dialyzed against barbital buffer (pH 8.6; $\mu = 0.1$) was applied to a 44 cm starch block. Electrophoresis was conducted for 36 hours at 4°C at a constant voltage of 300 volts and approximately 45 to 50 ma. The block was cut at 1 cm intervals and the serum proteins were eluted from each starch segment with 4 ml of 1 per cent sodium chloride buffered at pH 8.0 by the addition of 10 per cent by volume borate buffer.[4]

The distribution of the serum proteins on the starch block was estimated by the use of a trichloracetic acid turbidity method. To 0.2 ml of each eluate, 6 ml of 20 per cent trichloracetic acid was added, the tubes shaken, and the turbidity estimated at 440 mμ in the Beckman model B spectrophotometer in 10 to 15 minutes. The distribution of skin-sensitizing antibody was determined by P-K test after adjustment of each eluate to the volume of the original serum sample.

[3] Obtained from Center Laboratories, Inc., Port Washington, New York.

[4] Borate buffer composition: boric acid 28 gm, sodium hydroxide 2 gm, sodium chloride 2.9 gm, and water to make 1 liter.

DEAE Cellulose Chromatography.—A modification of the method of Humphrey and Porter (9) was used. A column 50 cm in length and 1.1 cm in diameter was packed with 3 gm of DEAE cellulose under a pressure of 5 p.s.i. The DEAE cellulose was from Carl Schleicher and Schüll Co., Keene, New Hampshire (Lot 1251). Stepwise elution was conducted by gravity flow (1 m of water pressure) with four buffers; (*a*) 0.01 M sodium phosphate pH 7.8, (*b*) 0.02 M sodium phosphate pH 6.2, (*c*) 0.05 M sodium phosphate pH 4.4, and (*d*) 0.1 M sodium chloride with 0.05 M sodium phosphate pH 4.4 (Text-fig. 3). The flow rate was 40 to 50 ml per hour and eluates were collected in 3 ml volumes. The protein concentrations of the eluates were estimated by measuring their absorption at 280 mμ in a Beckman DU spectrophotometer.

Gel Filtration with Sephadex G-200.—[5] Flodin and Killander (22) have described the use of sephadex G-200 for fractionating serum proteins of up to approximately 200,000 molecular weight. The sephadex was washed ten times with 0.1 M sodium chloride (buffered with 10 per cent by volume of borate buffer pH 8.0) and the "fines" were removed by decanting after each wash. A column 130 cm in length and 1.0 cm in diameter was packed with 2.5 gm of sephadex G-200 by gravity flow. A 2 ml volume of human serum was applied to the column and elution carried out with the borate-buffered 0.1 M sodium chloride (pH 8.0). The flow rate was 1 ml per hour and the eluates were collected in 1 ml volumes. The protein concentrations of the eluates were measured by their absorption at 280 mμ in a Beckman DU spectrophotometer.

Immunoelectrophoresis.—The micromethod of Scheidegger (16) was used with minor modification.

Concentration of Protein Solutions.—The serum protein fractions were concentrated by sedimentation in a Spinco model L ultracentrifuge for 24 to 36 hours at 40,000 RPM in a No. 40 angle rotor. The upper supernatants were gently removed with a Pasteur pipette leaving the gel pellets and 1 to 2 ml of each supernatant. The gels and the remaining supernatant were mixed.

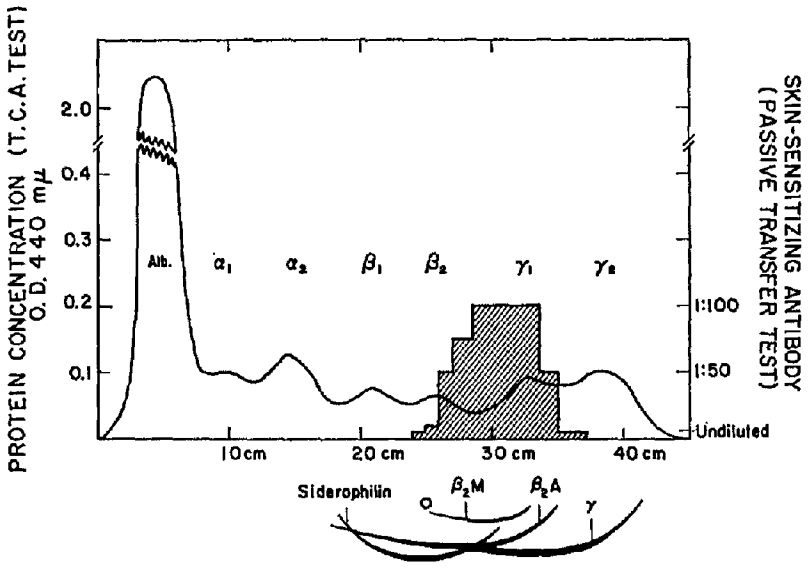
EXPERIMENTAL AND RESULTS

DEAE Cellulose Chromatography after Zone Electrophoresis.—A starch block electrophoretic pattern of one of the allergic sera (M) is presented in Text-fig. 2. A similar pattern was obtained with the other two allergic sera (R and D). The skin-sensitizing activity was consistently located in the fast γ-globulin and slow β-globulin regions as shown in Text-fig. 2. This data was consistent with that reported by Sehon *et al.* (1). γ-Globulins, β_2A-globulins, β_2M-globulins, and siderophilin were demonstrated by immunoelectrophoresis of the pooled eluates from the fast γ- and slow β-globulin regions that contained skin-sensitizing antibody activity.

The eluates from the region containing skin-sensitizing antibody from three starch block fractionations of allergic serum (M) were pooled with the combined eluates from reextraction of the starch residue from the region containing skin-sensitizing activity. The serum proteins thus obtained from the fast γ- and slow β-globulin regions were concentrated by sedimentation to a volume of 3 ml and dialyzed against the starting sodium phosphate buffer (0.01 M, pH 8.4) for DEAE cellulose chromatography. The 3 ml pooled serum fraction that was applied to the column contained 70 mg of protein per ml and was derived from 24 ml of whole serum having a P-K titer of 1/1000. The sample was chromatographed as described under Materials and Methods.

[5] Obtained from Pharmacia Fine Chemicals, Inc., New York.

The chromatogram obtained is illustrated in Text-fig. 3. Similar data were obtained with the other two allergic sera studied. No detectable additional protein could be eluted by further increase in ionic strength of the eluting buffer. Prior to skin-testing, eluates were pooled into four fractions (Text-fig. 3) associated with the four major protein peaks ((I) eluate volume 30 to 90 ml, (II) 110 to 150 ml, (III) 155 to 200 ml, and (IV) 225 to 260 ml) and concen-



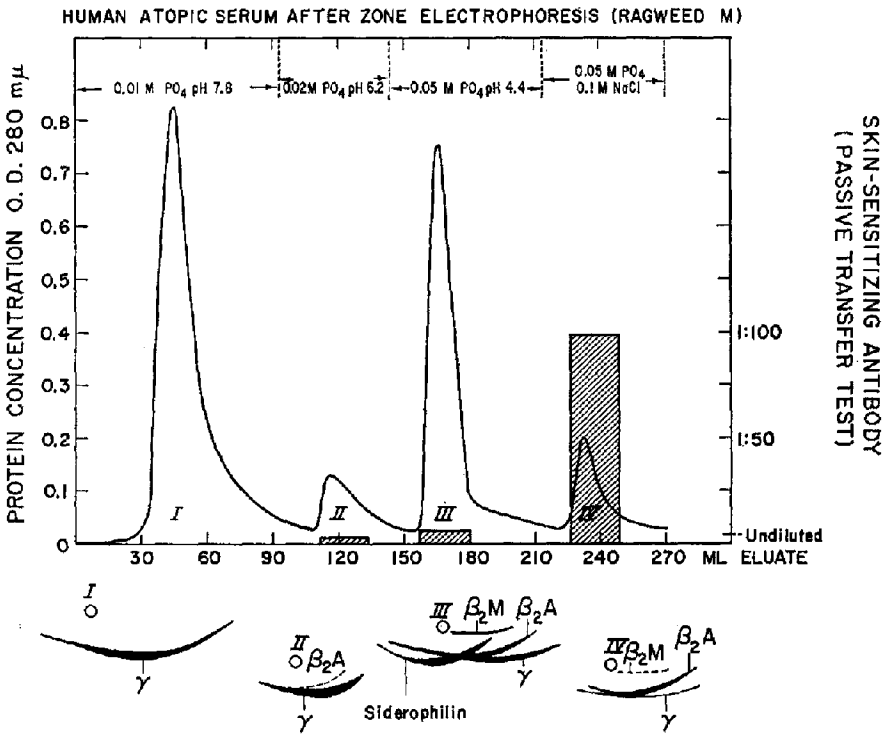
TEXT-FIG. 2. Starch block zone electrophoresis of allergic human serum with the anode to the left. The protein distribution is indicated by the solid line and the distribution of skin-sensitizing antibody is shown by the shaded area. The open circle represents the antigen well for the immunoelectrophoresis of the pooled concentrated eluates which contained skin-sensitizing antibody (anode to the left) and the diagram represents the precipitin lines found after development with a horse anti-whole human serum. (T.C.A., trichloroacetic acid.)

trated to the original starting volume by sedimentation. The passive transfer test data are summarized in Text-fig. 3. A comparison of the P-K titers of the eluates from the column and the original whole serum indicates that high losses have occurred during the fractionation procedures. Most of the antibody activity found was present in fraction IV and was detectable at a $1/100$ dilution and on one occasion a titer of $1/400$ was obtained. No skin-sensitizing activity was detectable in fraction I. A small amount of skin-sensitizing activity was present in fraction II and this could only be demonstrated in the undiluted material. Fraction III contained sufficient antibody to elicit a passive transfer test at a $1/10$ dilution.

Immunoelectrophoretic analysis of the four peaks revealed γ-globulins in

fraction I, γ-globulins of a faster mobility in fraction II, γ-globulins, β_2A-globulins, β_2M-globulins, and siderophilin in fraction III, and mostly β_2A-globulins with a small amount of γ-globulins of a fast mobility in fraction IV.

The possibility existed that other serum proteins were present in the four fractions in concentrations too low to detect by immunoelectrophoretic analysis.



TEXT-FIG. 3. DEAE cellulose chromatography of the human serum fraction which was prepared by starch block electrophoresis and contained skin-sensitizing antibody. The distribution of skin-sensitizing antibody is indicated by the shaded bars. The open circles indicate the antigen wells for the immunoelectrophoresis of the concentrated eluates of fractions I, II, III, and IV. The diagrammatically represented precipitin lines were developed with an anti-whole human serum.

Therefore an absorption experiment was carried out using a horse anti-human serum (No. 282) obtained from the Pasteur Institute. Equal volumes (0.1 ml) of each of the four eluates were added to 1.0 ml volumes of the horse antiserum. The absorbed antisera were then utilized in immunoelectrophoresis of normal whole human serum. Fraction I removed γ-globulin antibodies without any discernible change in antibodies to β_2A- or β_2M-globulins. Fraction II removed antibodies to γ-globulins and β_2A-globulins indicating that traces of β_2A-globu-

lins were present in fraction II even though not demonstrable by direct immunoelectrophoresis of this fraction. Fraction III removed antibodies to γ-, β_2A-, and β_2M-globulins, and siderophilin, and was consistent with the serum protein pattern seen on direct immunoelectrophoresis. Fraction IV removed antibodies to β_2M-globulins as well as antibodies to γ- and β_2A-globulins.

These data suggested that there may be a relationship between the presence of β_2A-globulins and skin-sensitizing antibody. γ-Globulin was present in all fractions but there was no skin-sensitizing antibody activity in fraction I. β_2M-globulin was not present in fraction II. β_2A-globulins appeared to be the one protein present only in the active fractions.

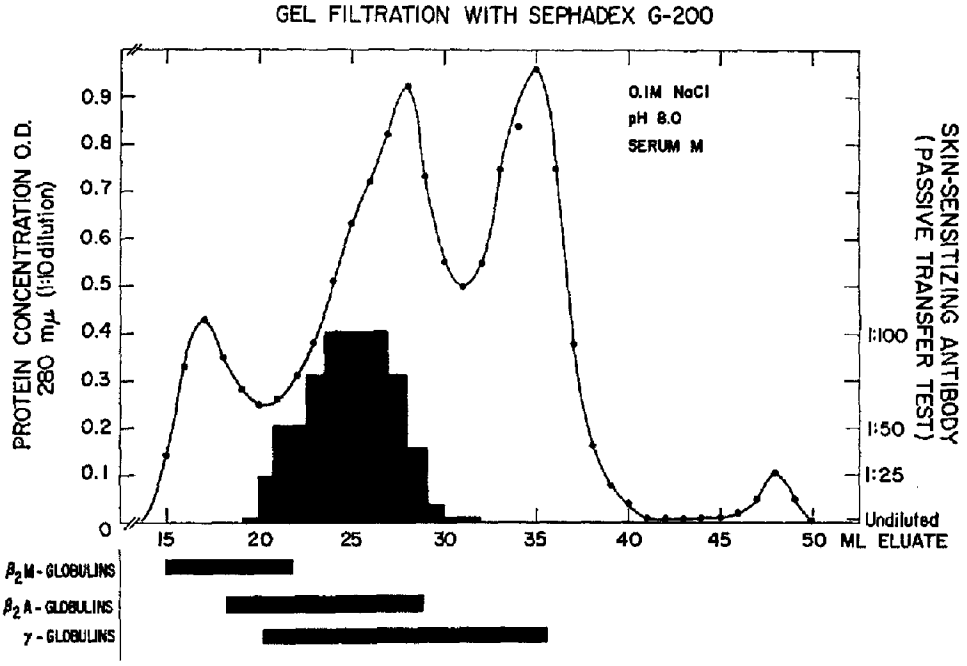
Changes in the sodium chloride concentration (to 0.05 and 1.0 M) of the fourth buffer of the stepwise elution or gradient elution (10) did not provide any improvement in the resolution of skin-sensitizing antibody or the β_2A-globulin.

Gel Filtration with Sephadex G-200.—Sephadex G-200 separates serum proteins on the basis of their molecular size. Molecules greater than 200,000 molecular weight do not penetrate the gel matrix and are eluted first. Smaller molecules appear in later effluents depending on their retention by penetration and subsequent displacement from the gel matrix. In Text-fig. 4 is shown a typical sephadex G-200 fractionation of whole serum. Most of the skin-sensitizing antibody activty was eluted in the second protein peak. Similar results were obtained with the three allergic sera studied. Characterization of the serum proteins separated with sephadex G-200 (22, 23) has shown the first peak to contain β_2M-globulin. γ-Globulins were first detected in the trough between the first two peaks. The major part of these appeared in the second peak. The β_2A-globulins came off the column slightly before the γ-globulins. Skin-sensitizing antibody activity seemed to be correlated with the presence of both β_2A-globulins and the γ-globulins as shown in Text-fig. 4; it was suggestively more closely correlated with the β_2A- than the γ-globulins. There was no association between β_2M-globulins and skin-sensitizing antibody activity in the three allergic sera studied by this technique.

Removal of β_2A- and β-Globulins by Precipitation with Specific Antisera.—Since various chemical and physical methods for the separation of serum proteins have been unsuccessful, so far, in associating skin-sensitizing antibodies with a single serum component, an attempt was made to prepare unispecific antisera for use in the selective precipitation and removal of the various serum components possibly associated with skin-sensitizing antibody activity. Suitable specific antisera for the precipitation of β_2A-globulins and of γ-globulin were prepared as described in Materials and Methods. Attempts to produce a specific β_2M-globulin antiserum were unsuccessful.

The addition of 3 to 3.5 ml (serum M 3.0 ml; serum R 3.5 ml; serum D 3.5 ml) of the sheep anti-β_2A-globulin serum precipitated all detectable β_2A-globulins

from 1 ml of each of the three allergic sera studied. The sheep antiserum was added in 0.5 ml increments; each addition being followed by incubation for 2 hours at 37°C and 18 hours at 4°C, centrifugation, and interfacial test of the supernatant for residual β_2A-globulins or anti-β_2A-globulin. Text-fig. 5 is an immunoelectrophoretic pattern of human allergic serum before and after precipitation with the specific anti-β_2A-globulin serum. It can be seen that there

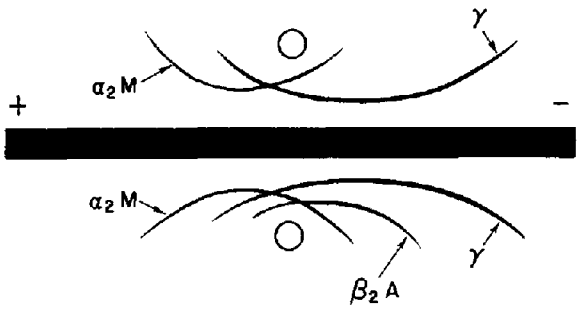


TEXT-FIG. 4. Sephadex G-200 gel filtration of whole atopic serum. The protein distribution is indicated by the solid line and the distribution of skin-sensitizing antibody activity is shown by the shaded area. The bar graph relates the distribution of γ-globulins, β_2A-globulins and β_2M-globulins (as detected by immunoelectrophoresis) to the distribution of total protein and skin-sensitizing antibodies.

are no detectable β_2A-globulins in the absorbed serum. There was also no obvious change in the γ-globulin or α_2M-globulin precipitin line after absorption. Since it is difficult to estimate the amount of a protein by the appearance of its precipitin line in an immunoelectrophoretic pattern, γ-globulin was measured by the quantitative Oudin technique (18) before and after removal of β_2A-globulins. As shown in Text-figs. 6 *a* and 6 *b* there was no detectable change in γ-globulin concentration after precipitation of β_2A-globulins in the three allergic sera studied.

Since whole human serum contains such a high concentration of γ-globulins

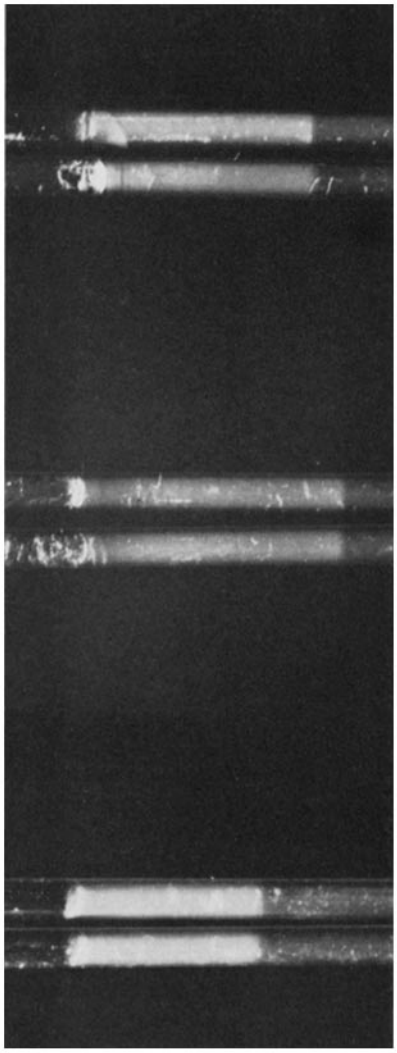
it was not feasible to remove all the γ-globulins by addition of specific antisera. Therefore, it was necessary to utilize a serum fraction which contained skin-sensitizing antibody but was accompanied by lower concentrations of γ-globulins. Fraction IV from the starch block electrophoresis DEAE cellulose chromatography studies proved suitable. The rabbit anti–γ-globulin serum was absorbed with the purified β_2A myeloma globulin to precipitate the small amount of β_2A antibodies as described under Materials and Methods. All the detectable γ-globulins were removed from 1 ml of fraction IV, for each of the allergic sera, by the addition of 2 to 2.5 ml (serum M 2.0 ml; serum R 2.5 ml; serum D 2.5 ml) of the rabbit anti–γ-globulin serum. The rabbit antiserum was added in 0.5 ml increments as described for the sheep antiserum. There was no



TEXT-FIG. 5. Immunoelectrophoresis of serum D before and after removal of β_2A-globulins The lower antigen well contained the serum diluted ¼ with isotonic saline and the upper antigen well contained the serum diluted ¼ by addition of the specific sheep anti–β_2A-serum as described under Materials and Methods. The precipitin lines were developed with an antiserum obtained from a later bleeding of the same sheep used to produce the specific anti–β_2A-serum.

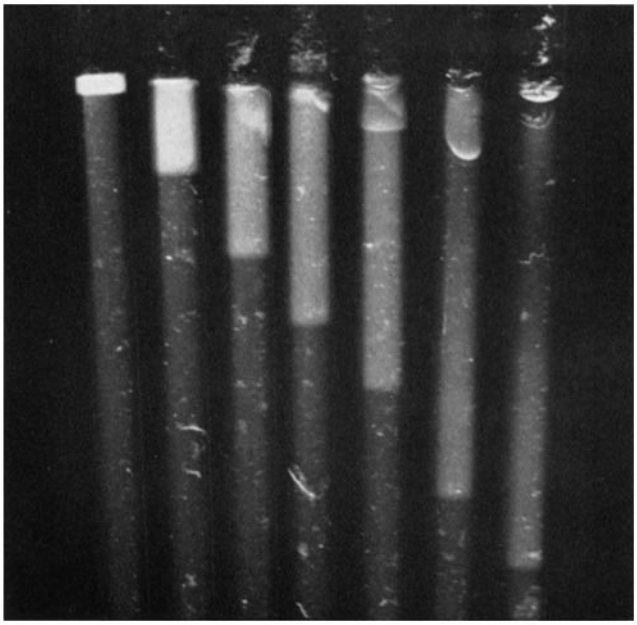
decrease in the β_2A-globulin concentration detectable by immunoelectrophoresis.

The absorbed allergic sera and serum fractions were then assayed for skin-sensitizing antibody activity (see Table I). The end point titration of skin-sensitizing antibody was $\frac{1}{1000}$ in each of the three allergic sera studied when diluted with 0.85 per cent saline solution. As can be seen from the Table I the removal of β_2A-globulins from the three allergic sera was associated with the loss of skin-sensitizing antibody activity. No antibody activity could be demonstrated in these preparations at a dilution of $\frac{1}{50}$. Antibody activity could not be demonstrated after precipitating the β_2A-globulins by diluting the allergic sera to a final dilution of ¼ by the addition of the sheep anti-β_2A serum in one assay for each absorbed serum. Because of the question of how much foreign protein can be safely injected into normal individuals, the undiluted (¼) preparation was only tested in one recipient (one of the investigators). There-



TEXT-FIG. 6 *a*. γ-Globulin concentration determined by the Oudin technique before and after removal of β_2A-globulins. In each of the three pairs of Oudin tubes the atopic serum diluted ¼ in isotonic saline is on the left and the serum absorbed with the specific β_2A antiserum is on the right. A rabbit antiserum to human γ-globulin was used to form the precipitin bands.

fore, at least 95 per cent and probably more than 99 per cent of the skin-sensitizing antibody was removed by precipitation of β_2A-globulins. Since the possibility existed that sheep serum might compete for skin sites or in some other manner inactivate the skin-sensitizing antibody, a control experiment was carried out substituting normal sheep serum for the sheep anti-β_2A serum. The addition of 3.0 to 3.5 ml of normal sheep serum to 1 ml of the allergic sera produced no decrease in skin-sensitizing antibody; activity was demonstrated



TEXT-FIG. 6 *b*. Serial dilutions of a human serum (M) were layered over a rabbit antiserum to human γ-globulin. In this series of Oudin tubes the human serum, undiluted, $\frac{1}{2}$, $\frac{1}{4}$, $\frac{1}{8}$, $\frac{1}{16}$, $\frac{1}{32}$, and $\frac{1}{64}$ (right to left) was layered over the rabbit antiserum to human γ globulin. The antiserum was incorporated in agar.

at a $\frac{1}{1000}$ dilution. Therefore, at the concentration of sheep serum used, there was no indication that addition of normal sheep serum altered the skin-sensitizing antibody activity. As a control for the possibility that an antigen-antibody reaction in the presence of skin-sensitizing antibody in some way interfered with the skin-sensitizing antibody assay, a 3 ml volume of a specific sheep anti-human albumin serum was added to 1 ml of each of the allergic sera in increments of 0.5 ml as described above. The precipitation of serum albumin in the presence of skin-sensitizing antibody did not lower the titer of skin-sensitizing antibody which remained at $\frac{1}{1000}$ in each preparation. Each of the titrations except the $\frac{1}{4}$ dilution mentioned above were performed in at least three different normal volunteers.

An end point titration of $\frac{1}{100}$ (see Table I) was found for the DEAE cellulose chromatography fractions IV from sera M, R, and D when diluted with 0.85 per cent saline. The addition of 2.0 to 2.5 ml volumes of either normal rabbit serum or of rabbit anti-γ-globulin serum to 1 ml volumes of fraction IV from each of these sera did not affect the skin-sensitizing antibody titer which remained at $\frac{1}{100}$. The addition of the rabbit anti-γ-globulin serum removed the γ-globulins detectable by immunoelectrophoresis from the fractions IV of these allergic sera, but did not change the skin-sensitizing antibody titer.

A representative series of passive transfer tests of sera M, R, and D are shown in Fig. 1. A positive P-K test was seen when the allergic sera were diluted

TABLE I

End Point Titration of Skin-Sensitizing Antibody

	Serum M	Serum R	Serum D
Allergic serum + saline	1/1000 (6)*	1/1000 (6)	1/1000 (6)
Allergic serum + normal sheep serum	1/1000 (3)	1/1000 (3)	1/1000 (3)
Allergic serum + sheep anti-β_2A serum	<1/4 (1), <1/50 (3)	<1/4.5 (1), <1/50 (3)	<1/4.5 (1), <1/50 (3)
Allergic serum + sheep anti-albumin serum	1/1000 (3)	1/1000 (3)	1/1000 (3)
Fraction IV + saline	1/100 (3)	1/100 (3)	1/100 (3)
Fraction IV + normal rabbit serum	1/100 (3)	1/100 (3)	1/100 (3)
Fraction IV + rabbit anti-γ-globulin serum	1/100 (3)	1/100 (3)	1/100 (3)

* The numbers within the parenthesis indicate the number of normal recipients tested in each assay.

with 0.85 per cent saline but when the allergic sera were absorbed with the sheep anti-β_2A serum, the passive transfer tests were negative. However, the control experiments of the addition of rabbit anti-γ-globulin to fraction IV and also of the addition of sheep anti-albumin serum did not affect skin-sensitizing antibody and these preparations gave positive P-K tests.

Normal sheep and rabbit serum in the concentration used did not inhibit the passive transfer test. Also the effect of normal whole human serum and pooled γ-globulins on the passive transfer test was studied. The addition of 3 ml of normal whole human serum (which contained no ragweed skin-sensitizing antibody) to 1 ml of the three sera (M, R, and D) studied did not alter the end point titration of skin-sensitizing antibody. This observation was confirmed in two normal recipients and is in agreement with a previous report (24). The addition of 0.1 ml, 0.4 ml, and 0.9 ml of pooled human γ-globulins [6] to 0.1 ml

[6] Acquired commercially from E. R. Squibb & Sons, Brooklyn, and contained 16.3 gm per cent γ-globulins.

of the allergic sera (followed by incubation at 37°C for 2 hours and then at 4°C for 16 hours) did not effect the end point titration of skin-sensitizing antibody. Each assay was duplicated in two normal volunteers.

DISCUSSION

Heremans (25) has suggested that the β_2A-globulins possess antibody activity and grouped these serum proteins with γ-globulins and β_2M-globulins in the group of "immune globulins." The fact that in congenital hypogammaglobulinemia low levels of β_2A-globulins occur accompanying the decreased γ-globulin and β_2M-globulin concentrations is consistent with this concept. Schultze (26) has presented a table listing various antibacterial and isoagglutinin antibody activities found in a β_2A-globulin preparation; however, this β_2A-globulin preparation was contaminated with β_2M-globulin. The interpretation of Heremans' experiments (13) associating skin-sensitizing antibody with β_2A-globulins was complicated by the presence of a small amount of γ-globulins in this β_2A-globulin preparation.

The same problem presented itself during the first phase of this study. We were not able to prepare a serum fraction which contained skin-sensitizing antibody and only one immunochemically pure serum protein. Fraction IV, from the zone electrophoresis DEAE cellulose chromatography procedure contained the major part of the skin-sensitizing activity of the three sera; but although most of fraction IV consisted of β_2A-globulins, a small amount of γ-globulins and traces of β_2M-globulins were also present in this fraction. The possibility could not be ruled out that the γ-globulin molecules which appeared in this fraction because they differed in charge or other characteristics from the largest part of the serum γ-globulins also differed from the rest of the γ-globulins in possessing skin-sensitizing activity.

The fractionation of serum proteins by gel filtration with sephadex G-200 did achieve a relative separation of the β_2M-globulin from the skin-sensitizing activity of the serum. Skin-sensitizing activity, β_2A-, and γ-globulins were not detectable in the first protein peak where the major part of the β_2M-globulins appeared, and no β_2M-globulins were detectable in the second peak where the major part of the skin-sensitizing activity, β_2A- and γ-globulins were found. The facts that the first trace of β_2A-globulins came off the sephadex column before the γ-globulins were detectable and that the γ-globulins continued coming off the column well after the last detectable β_2A-globulins are consistent with the finding that β_2A-globulin preparations from normal serum (27) as well as β_2A myeloma protein preparations (28, 15) appear to contain higher molecular weight constituents in addition to 7S components. It is tempting to conclude that the distribution of β_2A-globulins is more nearly correlated with distribution of skin-sensitizing activity than is the distribution of γ-globulins. Rockey and Kunkel (8) have called attention to a class of 9 to 15S proteins with antibody activities, and have shown that in the case of one serum the skin-

sensitizing antibody activity (to a purified glucagon preparation) had a sedimentation coefficient in the range of 8 to 11S. Globulins of this class might well appear in the sephadex G-200 eluates where the β_2A-globulins and the skin-sensitizing antibodies were found in our experiments.

The precipitation of β_2A-globulins from the three allergic sera by a specific antiserum and the associated loss of skin-sensitizing antibody strongly suggests that skin-sensitizing antibodies in these sera are β_2A-globulins. It is unlikely that significant amounts of γ-globulin (with associated skin-sensitizing activity) were precipitated with the β_2A-globulins. The anti-β_2A serum had been absorbed with γ-globulins and no change could be shown in the γ-globulin concentration of the allergic sera after the removal of the β_2A-globulins. More convincing evidence that skin-sensitizing activity was *not* associated with γ-globulin in these three sera was the fact that no decrease in skin-sensitizing activity occurred when the γ-globulin was precipitated from serum fraction IV which contained essentially only γ- and β_2A-globulins. The anti-γ-globulin serum used for this precipitation had been previously absorbed with a β_2A myeloma globulin in order to remove antibodies which reacted with antigenic determinants on β_2A-globulins. Under the conditions used, the absorbed antiserum did not produce detectable depletion of the β_2A-globulin line on immunoelectrophoresis when complete removal of the γ-globulins had been achieved. Heremans reported the β_2A myeloma protein to be antigenically deficient (29); this deficiency was not detectable with our antiserum under the conditions used. Augustin (12) reported that the precipitation of γ-globulins from allergic serum by means of rabbit anti-γ-globulin serum removed skin-sensitizing antibodies in the one allergic serum studied. However, the antiserum had *not* been absorbed with β_2A-globulins to remove any cross-reacting antibodies and would therefore be expected to remove β_2A-globulins as well as γ-globulins because of the presence of similar or identical antigenic determinants on both classes of globulins. It is of course possible that skin-sensitizing antibody may be associated with β_2A-globulins in one group of individuals and with γ-globulins in another group of individuals depending upon the type of patient, differences in allergens, etc.

The presence of trace amounts of antibody to α_2M-globulins in the anti-β_2A antiserum raised the remote possibility that skin-sensitizing antibody might be associated with the α_2M-globulins. No change in the α_2M-globulins was detectable after β_2A-globulins (and skin-sensitizing activity) were removed after the allergic sera and in addition it was impossible to demonstrate skin-sensitizing activity in the globulins of α mobility after starch block zone electrophoresis.

The possibility that the specific precipitation of β_2A-globulins by the sheep antiserum was accompanied by a non-specific inactivation of skin-sensitizing antibody is unlikely. Addition of an equivalent amount of a specific sheep anti-

human albumin serum to the three allergic sera did not alter the skin-sensitizing antibody activity. Similarly, addition of the rabbit anti-γ-globulin antiserum to a fraction of the allergic sera produced no detectable change in skin-sensitizing activity.

No evidence was obtained in these studies that the passive transfer tests were inhibited by dilution of skin-sensitizing antibody in normal human serum or γ-globulins in the concentrations used, although these studies did not include the effect of very high concentrations of γ-globulins or of β_2A-globulins.

The application of the method described for specific removal of β_2A-globulins to determine which other antibody activities in human serum in addition to skin-sensitizing antibody may be associated with the β_2A-globulins will prove worthwhile.

SUMMARY

1. The removal of the β_2A-globulins from three sera from treated ragweed-sensitive individuals by immune absorption was associated with the loss of all detectable skin-sensitizing antibody activity as demonstrated by Prausnitz-Küstner testing.

2. Gel filtration studies, with sephadex G-200, indicated that the fractions containing only macroglobulins were devoid of all detectable skin-sensitizing antibody activity.

3. The immune absorption of the γ-globulins from a serum fraction containing β_2A-globulins, γ-globulins, and a trace of β_2M-globulins had no detectable influence on the skin-sensitizing antibody activity.

4. The removal of a portion of the albumin from the allergic sera by immune absorption, with retention of the skin-sensitizing activity, indicated that the loss of skin-sensitizing antibody was not due to non-specific absorption on an antigen-antibody precipitate.

5. No inhibition of the Prausnitz-Küstner reaction was observed when sheep serum, normal human serum, or normal human γ-globulins were tested in concentrations described.

6. We conclude that the skin-sensitizing antibody activities in the three sera from treated ragweed-sensitive individuals studied were associated with the β_2A-globulins.

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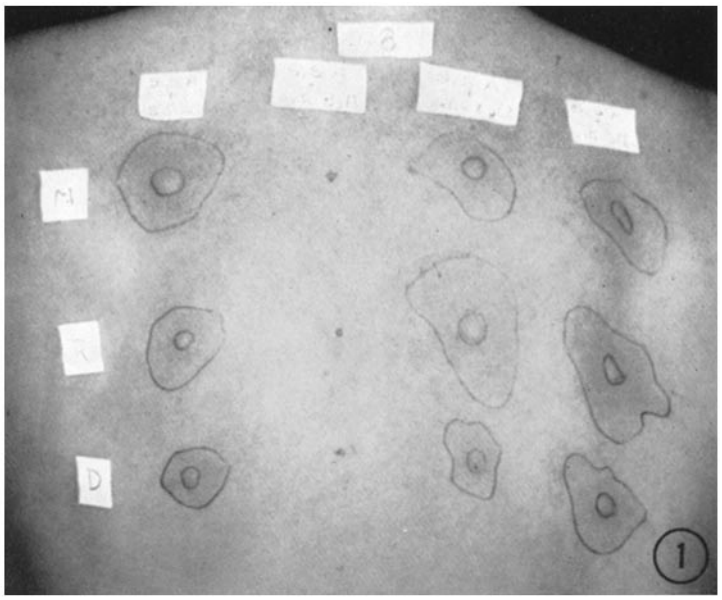
BIBLIOGRAPHY

1. Sehon, A. H., Fyles, T. W., and Rose, B., Electrophoretic separation of skin-sensitizing antibody from the sera of ragweed-sensitive patients, *J. Allergy*, 1955, **26**, 329.
2. Campbell, D. H., Cann, J. R., Friedman, T. B., and Brown, R., The relation of allergy reagins to electrophoretic components of serum, *Science*, 1954, **119**, 289.
3. Cann, J. R., The separation and purification of antibodies, *in* Immunity and Virus Infection, (V. A. Najjar, editor), New York, John Wiley & Sons, Inc., 1959, 100.
4. Sehon, A. H., The detection and nature of non-precipitating antibodies in allergic sera, *in* Mechanisms of Hypersensitivity, (J. H. Shaffer, G. H. LoGrippo, and M. W. Chase, editors), Boston, Little, Brown & Company, 1959, 61.
5. Stanworth, D. R., Studies on the physico-chemical properties of reagin to horse dandruff, *Immunology*, 1959, **2**, 384.
6. Heimlich, E. M., Vannier, W. E., and Campbell, D. H., Sedimentation studies of skin-sensitizing antibody, *J. Allergy*, 1960, **31**, 364.
7. Gyenes, L., Gordon, J., and Sehon, A. H., Ultracentrifugal characterization of antibodies in sera of ragweed-sensitive individuals, *Immunology*, 1961, **4**, 177.
8. Rockey, J. H., and Kunkel, H. G., Unusual sedimentation and sulphydryl sensitivity of certain isohemagglutinins and skin-sensitizing antibody, *Proc. Soc. Exp. Biol. and Med.*, 1962, **110**, 101.
9. Humphrey, J. H., and Porter, R. R., Reagin content of chromatographic fractions of human gamma-globulin, *Lancet*, 1957, **1**, 196.
10. Augustin, R., and Hayward, B. J., Human reagins to grass pollens and moulds: Their purification and physico-chemical characterization, *Immunology*, 1960, **3**, 45.
11. Perelmutter, L., Freedman, S. O., and Sehon, A. H., Fractionation of sera from ragweed-sensitive individuals by chromatography on DEAE-cellulose, *Internat. Arch. Allergy, and Appl. Immunol.*, 1961, **19**, 129.
12. Augustin, R., Precipitins, reagins and blocking antibodies, *Acta Allergol.*, 1961, **16**, 473.
13. Heremans, J. F., and Vaerman, J. P., β_2A-Globulin as a possible carrier of allergic reaginic activity, *Nature*, 1962, **193**, 1091.
14. Strauss, A. J. L., data in preparation.
15. Fahey, J. L., Heterogeneity of myeloma proteins, *J. Clin. Inv.*, 1963, **42**, 111.
16. Scheidegger, J. J., Une micro-méthode de l'immuno-electrophorèse, *Internat. Arch. Allergy and Appl. Immunol.*, 1955, **7**, 103.
17. Ouchterlony, O., Antigen-antibody reactions in gels, *Ark. Kemi, Minerol. Geol.*, 1949, **27B**, No. 14, 1.
18. Oudin, J., Specific precipitation in gels and its application to immunochemical analysis, *Methods Med. Research*, 1952, **5**, 335.
19. Prausnitz, C., and Küstner, H., Studien über die Ueberempfindlichkeit, *Centr. Bakt., 1, Abt. Orig.*, 1921, **86**, 160.
20. Kunkel, H. G., and Slater, R. J., Zone electrophoresis in a starch supporting medium, *Proc. Soc. Exp. Biol. and Med.*, 1952, **80**, 42.

21. Vannier, W. E., and Campbell, D. H., A starch block electrophoresis study of aqueous house dust extracts, *J. Allergy*, 1961, **32,** 36.
22. Flodin, P., and Killander, J., Fractionation of human serum proteins by gel filtration, *Biochim. et Biophysica Acta*, 1962, **63,** 403.
23. Fireman, P., Vannier, W. E., and Goodman, H. C., data in preparation.
24. Delorme, P., and Mathews, K. P., The effect of human serum and serum globulins on passive transfer tests for skin-sensitizing antibodies, *Univ. Michigan Med. Bull.*, 1961, **27,** 28.
25. Heremans, J. F., Les globulines sériques du système gamma. Leur nature et leur pathologie, Paris, Masson & Cie, 1960.
26. Schultze, H. E., The synthesis of antibodies and proteins, *Clin. Chim. Acta*, 1959, **4,** 610.
27. Heremans, J. F., Heremans, M. T., and Schultze, H. E., Isolation and description of a few properties of the β_2A-globulins of human serum, *Clin. Chim. Acta*, 1959, **4,** 96.
28. Imhof, J. W., and Ballieux, R. E., Atypical β_2A-myeloma, *Acta Med. Scand.*, 1961, **170,** 449.
29. Heremans, J. F., and Heremans, M. T., Studies on "abnormal" serum globulins (M-components) in myeloma, macroglobulinemia, and related diseases, *Acta. Med. Scand.*, 1961, **170,** suppl. 367, 27.
30. Grabar, P., and Burtin, P., Analyse Immuno-Electrophorétique, Paris, Masson & Cie, 1960.

EXPLANATION OF PLATE 29

FIG. 1. Passive transfer tests of allergic sera M, R, and D. The photograph was taken 15 minutes after the sensitized skin sites were challenged with ragweed extract. From left to right, the first column of tests are whole serum diluted with isotonic saline (S.S.A. + saline). The second column of tests are whole sera absorbed with the sheep antiserum to β_2A-globulin (S.S.A. + anti-β_2A). The third column are serum fraction IV absorbed with rabbit antiserum to human γ-globulin (S.S.A. + anti-γ) and the fourth column of tests are whole sera absorbed with the sheep anti-human albumin serum (S.S.A. + anti-alb.). The borders of the inner wheal and outer erythema have been outlined in ink. (S.S.A., skin-sensitizing antibody) $\times \frac{1}{4}$.



(Fireman *et al.*: Skin-sensitizing antibody)