

## Heterogeneity of skin-sensitizing antibodies

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*The skin-sensitizing antibodies to bovine milk found in a patient with ataxia-telangiectasia who had no detectable serum  $\gamma A$ -globulin were compared after fractionation by DEAE-cellulose chromatography to the skin-sensitizing antibodies to bovine milk and ragweed found in 2 patients who had normal serum concentrations of  $\gamma G$ -,  $\gamma A$ -, and  $\gamma M$ -globulins. All of the skin-sensitizing antibodies to bovine milk in the serum of the patient with no detectable serum  $\gamma A$ -globulin could be accounted for in a chromatographic fraction in which only  $\gamma G$ -globulin was detected. The skin-sensitizing antibodies to bovine milk and ragweed in the 2 patients with normal serum concentrations of  $\gamma G$ -,  $\gamma A$ -, and  $\gamma M$ -globulins were not restricted to the chromatographic fraction in which only  $\gamma G$ -globulin was detected and differences were observed in the elution of the skin-sensitizing antibodies in the three sera studied. Differences in the elution of skin-sensitizing antibodies to bovine milk and ragweed were also observed in the same serum. These data demonstrate that skin-sensitizing antibodies are a heterogeneous group of antibodies with regard to their separation by DEAE-cellulose chromatography and suggest that they may be associated with any one of several immunoglobulins.*

The skin-sensitizing antibodies responsible for the immediate, or wheal and flare, skin reaction are thought to differ from other antibodies because: (1) they can passively sensitize the skin of nonsensitive persons; (2) they do not passively sensitize the skin of the guinea pig; (3) they do not precipitate antigen or fix complement; and (4) they are inactivated by heating at 56° C. for 60 minutes.<sup>1</sup> Such skin-sensitizing antibodies have been found to be associated with the faster migrating  $\gamma$ -globulins on electrophoresis,<sup>2, 3</sup> and they are usually eluted apart

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from the bulk of the  $\gamma$ G-globulins on DEAE-cellulose chromatography, with the exception of those skin-sensitizing antibodies found in patients with serum sickness.<sup>4-6</sup> Subsequent studies<sup>3, 7-9</sup> have suggested that skin-sensitizing antibodies are associated with  $\gamma$ A-globulins, and it has been questioned whether patients with  $\gamma$ A-globulin deficiency could synthesize skin-sensitizing antibodies.<sup>10, 11</sup> Loveless,<sup>12</sup> however, has described a patient with  $\gamma$ A-globulin deficiency who had transferable skin-sensitizing antibodies, and this observation has been confirmed by the detection of skin-sensitizing antibodies in the serum of a patient with ataxia-telangiectasia who had no detectable serum  $\gamma$ A-globulin.<sup>13</sup> Ishizaka and associates<sup>14-16</sup> have reported that certain skin-sensitizing antibodies were not associated with  $\gamma$ G-,  $\gamma$ A-,  $\gamma$ D-, or  $\gamma$ M-globulin and may be associated with a unique immunoglobulin,  $\gamma$ E. On the other hand, Reid and colleagues,<sup>17</sup> Perelmutter and colleagues,<sup>18</sup> and Malley and Perlman<sup>19</sup> have recently described skin-sensitizing antibodies in serum fractions that contain  $\gamma$ G-globulins but no detectable  $\gamma$ A-globulins, and Terr and Bentz<sup>20</sup> have reported skin-sensitizing antibody activity associated with both  $\gamma$ G- and  $\gamma$ M-globulins in patients with serum sickness. In the study reported here, the skin-sensitizing antibodies found in a patient with ataxia-telangiectasia who had no detectable  $\gamma$ A-globulins were compared with the skin-sensitizing antibodies found in 2 patients who had normal amounts of  $\gamma$ G-,  $\gamma$ A-, and  $\gamma$ M-immunoglobulins.

## METHODS AND MATERIALS

### Skin tests

*Human.* Skin-sensitizing antibodies were assayed by the passive transfer test of Prausnitz and Küstner<sup>21</sup> (the P-K test). The recipients were normal adult volunteers who were found not to be sensitive to the specific antigen both by history and by intradermal skin tests. Normal skin sites were sensitized by the intradermal injection of 0.1 ml. of serum or 0.1 ml. of a serum fraction, and 24 to 48 hours later the injected sites were challenged by intradermal injection of 0.05 ml. of bovine milk protein or 0.05 ml. of ragweed antigen which contained 100 PNU per milliliter.\* Skin-sensitizing antibody titers were determined by injecting serial dilutions of each serum or serum fraction intradermally and then challenging the skin sites with antigen 24 hours later: the highest dilution which produced a wheal and flare reaction of at least 5 mm. by 20 minutes after challenge was taken as the end point of the titration. The passive transfer titrations for each sample were performed in at least 2 normal individuals.

*Guinea pig.* To test for the capacity to sensitize guinea pigs, the method described by Ovary<sup>22</sup> was used: the animals were given 0.1 ml. of serum or serum fraction intradermally, and 6 hours later they were challenged intravenously with solutions of specific antigen which also contained 2.5 mg. of Evans blue.

### DEAE-cellulose chromatography

Fractionation of serum by DEAE-cellulose chromatography was performed by a modification of the method of Humphrey and Porter.<sup>4</sup> A column, 24 cm. in

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length and 2 cm. in diameter, was packed with 20 Gm. of DEAE-cellulose under 7 pounds of pressure. Ten milliliters of serum was applied, and elution was performed by gravity flow with a gradient from 0.001M phosphate buffer, pH 7.4, to 0.05M phosphate buffer, pH 7.4. Eluates were collected in 5 ml. volumes. The optical densities of the eluates were measured at 280 m $\mu$  in a Beckman DU spectrophotometer. After being pooled into appropriate fractions, each fraction was concentrated by negative pressure dialysis against 0.1M NaCl solution at 4° C. to the original serum volume.

#### **Gel filtration on Sephadex G-200**

Serum was fractionated on Sephadex G-200 by a modification of a method previously described.<sup>3</sup> Ten milliliters of serum was applied to a column of Sephadex G-200\* 2.5 cm. in length and 100 cm. in diameter. Serum proteins were eluted by gravity flow with 1.0 per cent NaCl solution and collected in 4 ml. volumes. Optical densities of the eluates were measured at 280 m $\mu$ . After being pooled, the eluates were concentrated by negative pressure dialysis against 0.1M NaCl solution at 4° C. to the original serum volume.

#### **Antisera and immunochemical methods**

*Antisera.* The horse antiserum used in this study had antibodies against three serum immunoglobulins,  $\gamma$ G-,  $\gamma$ A-, and  $\gamma$ M-globulins, and has been described elsewhere.<sup>23</sup> Goat anti- $\gamma$ M-globulin antiserum† was made specific for  $\gamma$ M-globulin by absorption with serum from a patient deficient in  $\gamma$ M-globulin. Goat anti- $\gamma$ A-globulin antiserum† was absorbed with serum from a patient with a deficiency of  $\gamma$ A-globulin to insure specificity of the antiserum for  $\gamma$ A-globulins.

*Immunochemical methods.* Immuno-electrophoresis was performed by the micro-method of Scheidegger<sup>24</sup> with 0.1M borate buffer, pH 8.6. Serum  $\gamma$ G-globulin was quantitated with Oudin tubes using horse antiserum<sup>25</sup>; the same method was used to estimate the serum concentration of  $\gamma$ A-globulin using the goat anti- $\gamma$ A-globulin antiserum. The limits of sensitivity of this technique under the conditions of this study were 25 mg. per 100 ml. for  $\gamma$ G-globulin and 10 mg. per 100 ml. for  $\gamma$ A-globulin. The purified  $\gamma$ A-globulin used as the reference standard was prepared from normal serum using Sephadex G-200 followed by DEAE-cellulose chromatography with a gradient from 0.001M phosphate buffer, pH 7.4, to 0.05M phosphate buffer, pH 7.4 (equivalent to fraction 6 of Fig. 6). The  $\gamma$ M-globulin concentration was estimated by the method of Gell<sup>26</sup> with the specific goat anti- $\gamma$ M-globulin antiserum. Double gel diffusion was performed by the method of Ouchterlony.<sup>27</sup>

#### **Sera containing skin-sensitizing antibodies**

*Serum A.* Serum A was obtained from a patient with ataxia-telangiectasia who demonstrated an absence of  $\gamma$ A-, a relatively normal amount of  $\gamma$ G-, and increased  $\gamma$ M-globulins on immuno-electrophoresis. The concentration of  $\gamma$ G-globulin was found to be 1,440 mg. per 100 ml. and the concentration of  $\gamma$ M-globulin

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was 150 mg. per 100 ml. With both single and double gel diffusion,  $\gamma$ A-globulin could not be detected even when the serum was concentrated tenfold. Intradermal skin testing of the patient disclosed a positive wheal and flare skin reaction to bovine milk proteins. P-K tests disclosed a skin-sensitizing antibody titer of 1:10. Serum A did not passively sensitize guinea pig skin. No precipitating antibodies to bovine milk proteins could be demonstrated in patient A's serum, and challenge of the patient with milk orally did not produce symptoms.

*Serum B.* Serum B was obtained from a patient who suffered from recurrent rhinitis and conjunctivitis, which was most prominent during the ragweed pollen season, and from urticaria and rhinitis, which appeared to be related to the ingestion of cow's milk. Immunoelectrophoresis revealed apparently normal concentrations of  $\gamma$ G-,  $\gamma$ A-, and  $\gamma$ M-globulins; the  $\gamma$ G-globulin concentration was 1,200 mg. per 100 ml. and the  $\gamma$ A-globulin concentration was 120 mg. per 100 ml. Intradermal skin testing with bovine milk proteins provoked a giant wheal and flare with generalized urticaria. Positive wheal and flare skin tests were also elicited with ragweed pollen, several grass and tree pollens, molds, and house dust. The skin-sensitizing antibody titer against bovine milk proteins was 1:20 and against ragweed, 1:1,000. Serum B did not passively sensitize guinea pig skin. No precipitating antibodies to bovine milk proteins could be demonstrated in the serum. Reduction of milk ingestion provided considerable relief of symptoms.

*Serum C.* Serum C was obtained from a patient with recurrent rhinitis and conjunctivitis whose symptoms were most prominent during the ragweed pollen season. Immunoelectrophoresis revealed normal concentrations of  $\gamma$ G-,  $\gamma$ A-, and  $\gamma$ M-globulins; the level of  $\gamma$ G-globulin was 1,050 mg. per 100 ml., and that of  $\gamma$ A-globulin was 95 mg. per 100 ml. Positive wheal and flare skin tests were elicited by ragweed and grass pollens and house dust. The skin-sensitizing antibody titer against ragweed pollen was 1:1,000.

## RESULTS

The elution pattern for serum A obtained by DEAE-cellulose chromatography is illustrated in Fig. 1. Skin-sensitizing antibodies to bovine milk in serum A were detected only in fraction I. Fraction I contained only  $\gamma$ G-globulin;  $\gamma$ A-globulin was not detected with single or double agar gel diffusion with specific antibody, when fraction I was concentrated tenfold to a  $\gamma$ G-globulin concentration of 1.5 Gm. per 100 ml., or when the original serum was concentrated tenfold to a  $\gamma$ G-globulin concentration of 15 Gm. per 100 ml. Since the limit of sensitivity for the detection of  $\gamma$ A-globulin using Oudin tubes was 10 mg. per 100 ml., concentrating the fluids tenfold increased the sensitivity for  $\gamma$ A-globulin in the original serum to 1 mg. per 100 ml. of serum. Under these conditions,  $\gamma$ A-globulin could not be detected in any of the DEAE-cellulose chromatography fractions of serum A. Passive transfer tests using a solution of fraction I with approximately the same concentration of  $\gamma$ G-globulin, 1,500 mg. per cent, as was present in the original serum are shown in Fig. 2; the size of the wheal and flare with fraction I was the same as that obtained with the whole serum, suggesting that most, if not all, of the skin-sensitizing anti-

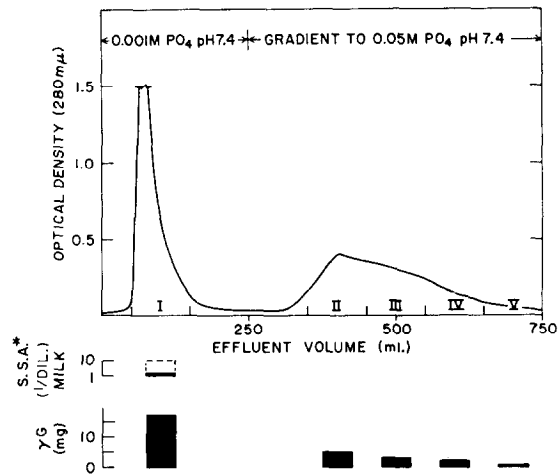


**Fig. 1**

Separation of serum A by DEAE-cellulose chromatography into fractions I through V, each of which was concentrated to 10 ml, the volume of serum aliquot placed on the column. The bar graph beneath the elution pattern indicates the titer of skin-sensitizing antibody (S.S.A.) which is expressed as the reciprocal of the highest dilution of the serum fraction, which produced a definitely positive P-K test and the amount of  $\gamma$ G-globulin detected in fractions I to V. When fraction I was concentrated to a  $\gamma$ G-globulin concentration of 1,500 mg. per 100 ml, the reciprocal of the highest dilution of fraction I which produced a definitely positive P-K test is indicated by the dotted bar graph.

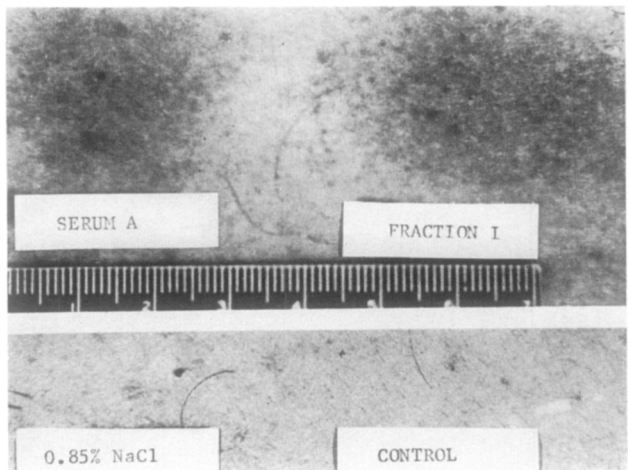


**Fig. 2**

Passive transfer tests. Skin sites which had been passively sensitized with serum A, isolated fraction I that contained 1,500 mg.  $\gamma$ G-globulin per 100 ml, 0.85 per cent NaCl, and a skin site which was not sensitized (control) were photographed 20 minutes after challenge with bovine milk protein. The size of the wheal and flare at the site passively sensitized with fraction I was the same as the size of the reaction at the site passively sensitized with serum A.

bodies to bovine milk proteins in serum A could be accounted for in fraction I.

The fractionation of serum B on DEAE-cellulose is shown in Fig. 3. Skin-sensitizing antibodies in serum B to bovine milk proteins were detected not only in fraction I, but also in fractions II and III; skin-sensitizing antibodies for ragweed were detected in fractions II, III, IV, and V, but were not detected in fraction I. Fraction I from serum B contained only  $\gamma$ G-globulin. Gamma A-globulin was detected in fractions II, III, IV, and V, with the highest  $\gamma$ A-globulin concentration found in fraction III.

The fractionation of serum C by DEAE-cellulose chromatography is shown in Fig. 4. It will be noted that the skin-sensitizing antibodies for ragweed were found in all five fractions. A comparison of Figs. 3 and 4 will indicate the differences found in the elution of serum B and serum C skin-sensitizing antibodies to ragweed from DEAE-cellulose. Fraction I from serum C contained only  $\gamma$ G-globulin. Gamma A-globulin was detected in fractions II, III, IV, and V, with the greatest  $\gamma$ A-globulin concentration being present in fraction IV.

An aliquot of serum C was also passed through a column of Sephadex G-200. The protein fraction indicated by the shaded area in Fig. 5 was found to contain the greatest  $\gamma$ A-globulin concentration, as well as the highest titer of skin sensitizing antibodies to ragweed as indicated by the P-K test;  $\gamma$ M-globulin could

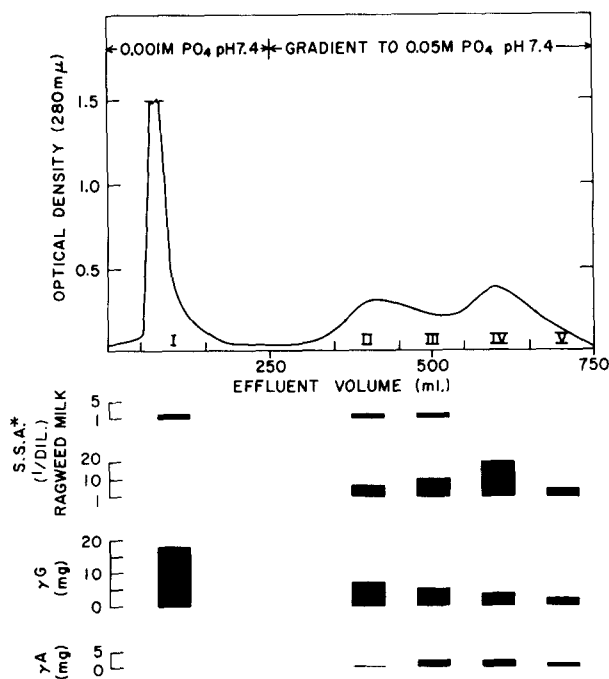


**Fig. 3**

Separation of serum B by DEAE-cellulose chromatography into fractions I through V, each of which was concentrated to 10 ml., the volume of serum aliquot placed on the column. The bar graph beneath the elution pattern indicates the titers of skin-sensitizing antibody (S.S.A.) to milk and ragweed and the amount of  $\gamma$ G- and  $\gamma$ A-globulins detected in fraction I to V.

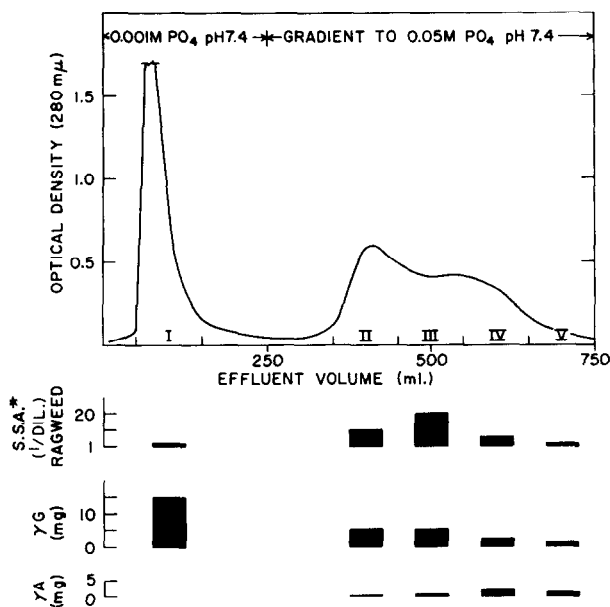


**Fig. 4**

Separation of serum C by DEAE-cellulose chromatography into fractions I through V, each of which was concentrated to 10 ml., the volume of serum aliquot placed on the column. The bar graph beneath the elution pattern indicates the titers of skin-sensitizing antibody (S.S.A.) to ragweed and the amount of  $\gamma$ G- and  $\gamma$ A-globulins detected in fractions I through V.

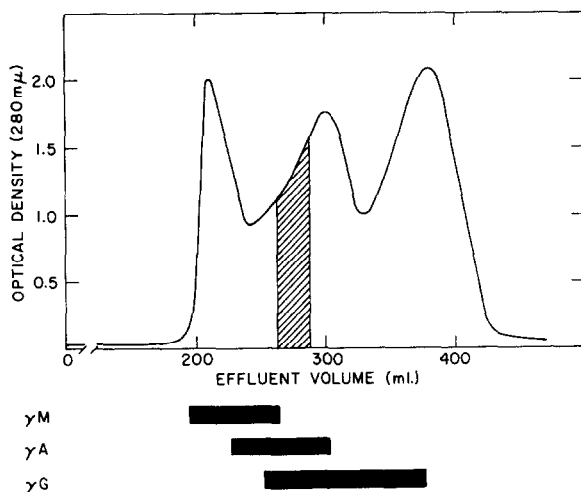
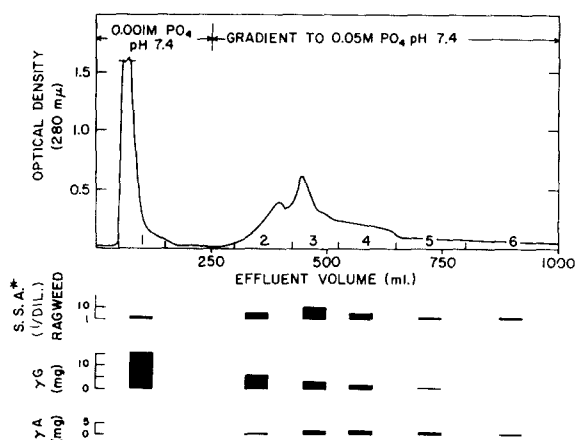


**Fig. 5**

Fractionation of serum C by Sephadex G-200 gel filtration. The presence of  $\gamma$ G-,  $\gamma$ A-, and  $\gamma$ M-globulin is indicated by the bar graph. The shaded area which contained the highest titer of skin-sensitizing antibody to ragweed was pooled, concentrated, and then fractionated on columns of DEAE-cellulose (see Fig. 6).

**Fig. 6**

Separation by DEAE-cellulose chromatography of the serum protein fraction which was obtained by fractionation of serum C on columns of Sephadex G-200 (see Fig. 5). Fractions 1 through 6 were concentrated to 10 ml., the volume of serum aliquot placed on the column. The bar graph beneath the elution pattern indicates the titer of skin-sensitizing antibody (S.S.A.) and the amount of  $\gamma$ G- and  $\gamma$ A-globulins in fractions 1 through 6.

not be detected in this fraction. The proteins within the shaded area were combined with comparable Sephadex G-200 fractions from a total of 48 ml. of serum C; the pooled fraction was then concentrated to a volume of 10 ml. and fractionated on DEAE-cellulose (Fig. 6). Gamma A-globulins were detected in fractions 2 through 6; the greatest  $\gamma$ A-globulin concentrations were found in fractions 3 and 4. Gamma G-globulins were detected in fractions 1 through 5 with the greatest concentration being present in fraction 1. Skin-sensitizing antibodies for ragweed were detected in all six fractions, including fraction 1, in which only  $\gamma$ G-globulin was detected, and fraction 6, in which only  $\gamma$ A-globulin was detected. The greatest amount of skin-sensitizing antibodies to ragweed, however, was detected in fraction 3 and was not coincident with either the maximal  $\gamma$ G- or  $\gamma$ A-globulin concentrations.

## DISCUSSION

The data in this study demonstrate that skin-sensitizing antibodies are physicochemically a heterogeneous group of antibodies with respect to their behavior following DEAE-cellulose chromatography. The skin-sensitizing antibodies in the three sera studied behaved somewhat differently when fractionated by DEAE-cellulose chromatography. All of the skin-sensitizing antibody activity in serum A to bovine milk proteins could be accounted for in fraction I, which contained only  $\gamma$ G-globulin and no detectable  $\gamma$ A- or  $\gamma$ M-globulins. In serum B, skin-sensitizing antibody activity to bovine milk proteins was detected not only in fraction I, which contained only  $\gamma$ G-globulin, but also in fractions II and III, which contained both  $\gamma$ G- and  $\gamma$ A-globulins. The skin-sensitizing antibodies for ragweed in serum B, however, were not detected in fraction I, but were present in fractions II through V, which contained both  $\gamma$ G- and  $\gamma$ A-globulins. The ragweed skin-sensitizing antibodies in serum C were detected in all

five chromatography fractions. Therefore, not only were differences in the elution of skin-sensitizing antibodies observed from one serum to another, but differences in the fractionation of skin-sensitizing antibodies directed against two different antigens were observed in a single serum, serum B. Heterogeneity of two different skin-sensitizing antibodies in the same patient has recently been described by Reid and colleagues.<sup>17</sup>

The skin-sensitizing antibodies in serum A for bovine milk proteins were restricted to the chromatographic fraction containing only  $\gamma$ G-globulin; in this respect, the antibodies resembled the skin-sensitizing antibodies against horse serum described by Terr and Bentz.<sup>20</sup> The skin-sensitizing antibodies in serum B for bovine milk proteins were present in fractions I, II, and III; in this regard it resembled the skin-sensitizing antibodies to bovine serum albumin described by Reid and colleagues<sup>17</sup> and the skin-sensitizing antibodies to horse serum reported by Humphrey and Porter.<sup>4</sup> The skin-sensitizing antibodies in serum C for ragweed, when fractionated by Sephadex G-200 followed by DEAE-cellulose chromatography, did not closely parallel either  $\gamma$ G- or  $\gamma$ A-globulin concentration or combinations of either of the two proteins; in recent reports by Ishizaka and Ishizaka<sup>14</sup> and Perelmutter and associates,<sup>18</sup> it has also been reported that skin-sensitizing antibodies did not parallel either  $\gamma$ A- or  $\gamma$ G-globulin concentration after fractionation by Sephadex G-200 followed by DEAE-cellulose chromatography. Although the maximum ragweed skin-sensitizing antibody activity in serum C was found in fraction 3 (Fig. 6), small amounts of ragweed skin-sensitizing antibodies were detected in fraction 1, in which only  $\gamma$ G-globulin was found, and in fraction 6, in which only  $\gamma$ A-globulin was detected. It should be pointed out that there was considerable loss of reaginic activity following DEAE-chromatography, as has been previously reported.<sup>3</sup> Up to 80 per cent of reaginic activity was lost in the separation procedure, and the question of whether heterogeneity of skin-sensitizing antibody in the fractions reflects heterogeneity in the original serum must be considered.

It is possible, of course, that skin-sensitizing antibodies may be associated with a serum component other than  $\gamma$ G- or  $\gamma$ A-globulin which was not detected by the methods used in this study. Ishizaka and associates<sup>16</sup> have recently reported that certain skin-sensitizing antibodies were not precipitated with anti- $\gamma$ G-,  $\gamma$ A-,  $\gamma$ M-, or  $\gamma$ D-globulin antibodies and proposed that these skin-sensitizing antibodies were associated with a serum protein other than these four immunoglobulins. However, Ishizaka reported that this serum protein and the associated antiragweed antibodies were eluted in a reagin-rich fraction from DEAE-cellulose columns with 0.01M to 0.05M phosphate buffer; the reagins in this serum fraction were removed with an antiserum directed against  $\gamma$ E-globulin but contained no antibodies against  $\gamma$ G-,  $\gamma$ A-, or  $\gamma$ M-globulins. Since fraction I in the present report was eluted with 0.001M phosphate buffer, the serum component described by Ishizaka should have been excluded from fraction I, but the possibility exists that this fraction contains  $\gamma$ E-globulin. The skin-sensitizing antibodies detected in fraction I would appear to be associated with  $\gamma$ G-globulins. Whether the skin-sensitizing antibodies found in fractions II to V were associated with either  $\gamma$ G- or  $\gamma$ A-globulins or with an undetected immunoglobulin was not resolved in this study. It would appear that skin-sensitizing antibodies

in man demonstrate considerable physicochemical heterogeneity with respect to their behavior following DEAE-cellulose chromatography, and that skin-sensitizing antibodies in man may be represented by more than one major group of immunoglobulins.

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