

# DEMONSTRATION OF ANTIBODIES IN THE SERA OF GRASS-SENSITIVE PERSONS BY THE BIS-DIAZOTIZED-BENZIDINE HEMAGGLUTINATION TECHNIQUE

*O. L. Frick, M.D.,\* L. Gyenes, B.Sc., and A. H. Schon, Ph.D., Montreal, Quebec*

## INTRODUCTION

IN THE past, demonstration of antibodies in the skin and in the sera of allergic patients has been limited to in vivo methods, such as the direct skin test and the passive transfer technique of Prausnitz-Küstner.<sup>1</sup> Both these methods are highly sensitive. The latter method enables one to detect minute amounts of antibody lower than 0.01  $\mu$ g.<sup>2</sup> Because of the inherently biologic nature of these techniques, however, and particularly since the reactions elicited by the passive transfer test vary considerably in different subjects and even in different skin areas of the same subject the results obtained are difficult to quantitate. In addition, there is the as yet unavoidable danger of transferring serum homologous hepatitis to the test subject.

Consequently, a specific and sensitive in vitro test for the detection of antibodies in allergic sera has been sought for almost four decades. Unfortunately most of the commonly used immunologic techniques (for example, precipitation,<sup>3-5</sup> complement fixation,<sup>6-9</sup> Dale-Schultz test,<sup>10, 11</sup> and passive cutaneous anaphylaxis<sup>12</sup>), when applied to allergic sera, have led either to negative or equivocal results. The most promising indications for the feasibility of a reliable in vitro test were obtained with various agglutination techniques. In these methods, in general, some bulky carriers, such as collodion particles<sup>13</sup> or erythrocytes,<sup>14</sup> are coated with the antigen and these particles are aggregated in the presence of specific antibodies.

Kallós<sup>15</sup> used collodion particles "sensitized" with wheat or rye grass pollen extracts. Orlans, Rubinstein, and Marraek<sup>5</sup> applied Boyden's tanned-cell technique<sup>14</sup> to sera from grass-sensitive patients. The sensitivity of this technique with grass allergens was rather low, as evidenced by their results. Thus, only eight of twenty-two sera from untreated grass-sensitive persons gave positive titers, ranging from 1:6 to 1:96, and twenty-two of twenty-four sera

From the Division of Immunochemistry and Allergy, McGill University Clinic, Royal Victoria Hospital, and the Department of Chemistry, McGill University.

This work has been supported by grants from the Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland, and the Department of Health and Welfare, Canada.

Received for publication Aug. 12, 1959.

\*Present address: Department of Pediatric Allergy, University of California Medical Center, San Francisco, California. (This work has been done in partial fulfillment of the requirements for the M.M.Sc. degree, University of Pennsylvania.)

from treated persons gave positive reactions, ten of which had titers over 1:100. Feinberg and associates<sup>16</sup> employed the same method with the sera of untreated and treated ragweed-sensitive patients and found positive reactions in 21 per cent and 67 per cent of their tests, respectively. Subsequently, however, these workers<sup>17</sup> reported that ragweed antigens were desorbed from the tanned cells and that consequently the liberated antigen(s) acted as inhibitor, diminishing the titer of strong sera and probably suppressing completely the reaction with weaker sera. Similar complications may well arise in the collodion particle method, as used by Kallós, since in this technique the antigen(s) are attached to collodion by nonspecific adsorption forces.

To overcome this limitation of the tanned-cell technique, particularly with antigens of low molecular weight, Schon and colleagues<sup>18</sup> have recently coupled ragweed pollen constituents via stable covalent azo bonds to erythrocytes. Bis-diazotized benzidine was used as coupling agent and the method was similar to that developed by Pressman and associates<sup>19</sup> and modified by Stavitsky and Arquilla.<sup>20</sup> Using this method, Gordon and co-workers<sup>21</sup> were able to demonstrate antibodies in all sera of a group of forty ragweed-sensitive patients and proved this method to be highly specific and sensitive.

The purpose of the present study was to adapt the BDB hemagglutination technique for the detection of antibodies in sera of hay fever patients sensitive to the common American grasses and for establishing antigenic and allergenic relationships among the pollen constituents of these grasses. For standardization of the method, rabbit antisera to six grasses (timothy, June, orchard, redtop, sweet vernal, and Bermuda) were used.

#### MATERIALS AND METHODS

*Antigens.*—Glycerin-saline extracts of the pollens of timothy grass (*Phleum pratense*), June grass (*Poa pratensis*), orchard grass\* (*Dactylis glomerata*), redtop grass (*Agrostis alba*), sweet vernal grass (*Anthoxanthum odoratum*), and Bermuda grass (*Cynodon dactylon*) were used.† Each pollen extract was emulsified with an equal volume of complete Freund's adjuvant obtained from Difco Laboratories and the emulsion was used for immunization. The concentrations of the extracts used (expressed in terms of dry weights of the glycerin-saline extractable pollen constituents) were as follows: 11.35 mg./ml. for timothy, 5.18 mg./ml. for June, 6.24 mg./ml. for orchard, 6.21 mg./ml. for redtop, 5.33 mg./ml. for sweet vernal, and 5.21 mg./ml. for Bermuda.

*Rabbit Antisera.*—Six groups of five rabbits each were immunized with each of the six different grass pollen extracts according to the following schedules: Two rabbits received three weekly injections of 0.6 ml. of the antigen emulsion into the footpads. Two other rabbits received three weekly doses of 0.6 ml. of the emulsion intramuscularly. The fifth rabbit received both

\*Known in Great Britain as cocksfoot grass.

†These extracts were most generously provided by Dr. Edward L. Foubert, Jr., Hollister-Stier Laboratories, Spokane, Washington.

types of inoculation over a period of three weeks, that is, 1.2 ml. per week. The sera of these animals were tested for antibodies by the "ring test" one to three weeks after the last footpad injection and three to six weeks after the last intramuscular inoculation. When precipitating antibodies were detected, 60 ml. of blood was collected from the marginal ear vein of the appropriate rabbit.

All animals were allowed to rest for two months and were then given a second course of immunization consisting of one intraperitoneal and two intravenous injections of 0.2 ml. of the glycerin-saline extract of grass pollen on successive days. This series of injections was repeated one week later. Several of the rabbits developed anaphylactic shock on receiving a booster dose of antigen. Therefore, it was decided that the last intravenous injections should be preceded by an intravenous dose of 0.04 mg./kg. Cycloheptidine\* just prior to the antigen injection. No subsequent anaphylactic shocks were noted.

All sera were heated at 56° C. for thirty minutes to inactivate complement and were stored in a deep freeze at -20° C. until used.

*Allergic Human Sera.*—Sera were collected from thirteen untreated and seventeen treated patients† with grass pollen allergies at the Royal Victoria Hospital Allergy Clinic. The sera were "decomplemented" by incubation with zymosan for one hour at 37° C. or overnight with a thrice-washed precipitate of BSA-anti-BSA complexes in order to prevent hemolysis of erythrocytes during the hemagglutination test.

#### *Hemagglutination Test.*—

*Erythrocytes:* Rabbit erythrocytes were used in the hemagglutination technique. For this purpose, blood was collected from the marginal ear vein or by cardiac puncture into an equal volume of Alsever's solution<sup>22</sup> from rabbits which had not been previously bled or which had been rested for at least six weeks after an earlier bleeding. If the erythrocytes were obtained from rabbits bled within a shorter interval, false positive hemagglutination occurred. For storage of erythrocytes, the blood in the Alsever's solution was kept at 4° C. for as long as ten days.

*Diluent:* The serum used for diluent was separated from the blood of the same rabbit from which the erythrocytes were obtained. After inactivation of complement by heating at 56° C. for thirty minutes, it was diluted 100 fold with phosphate buffer at pH 7.3.‡

*Bis-diazotized-benzidine:* This was prepared according to the method used previously in this laboratory.<sup>21</sup> Before the antigen was coupled to erythrocytes, 1 ml. of frozen BDB stored in a vial was melted in the palm of the hand and added to 15 ml. of cold phosphate buffer at pH 7.3.‡ This solution will be

\*Cycloheptidine is an antihistamine and antiserotonin preparation which was kindly supplied by Merck Sharpe & Dohme, Division of Merck & Co., Inc., Philadelphia, Pennsylvania.

†Sera obtained from patients who received hyposensitization treatment of grass pollen extracts will be referred to hereafter as treated sera. Untreated sera were collected from patients who never received injection therapy.

‡The phosphate buffer was prepared by mixing 215 ml. of 0.15 M disodium acid phosphate with 49 ml. of 0.15 M potassium dihydrophosphate.

referred to hereafter as BDB-phosphate solution. This must be used as soon as possible because it deteriorates quickly, forming a brown solution.

*Sensitization of the erythrocytes:* The rabbit red blood cells were washed three times with cold physiologic saline and the packed cells were resuspended in an equal volume of saline. The optimal quantity of grass pollen extract was then placed into a 15 ml. centrifuge tube and mixed with 0.1 ml. of 50 per cent red cell suspension. Finally, the optimal amount of BDB-phosphate (freshly prepared) was added and the mixture inverted several times to ensure even mixing. The reaction was allowed to proceed at room temperature\* for fifteen minutes with occasional stirring. "Sensitized" cells were separated by centrifugation, and the brownish supernatant was discarded. The cells were washed with 3.5 ml. of diluent and recentrifuged. Finally the cells were resuspended in diluent to a final volume of 2.5 ml. This will be referred to as sensitized cell suspension.

*Standardization of the method:* To establish the optimal ratio of each batch of BDB for each of the pollen extracts, the following procedure was used. With a constant amount of erythrocytes, (1) the BDB-phosphate volume was varied, keeping the antigen concentration constant, and (2) the antigen concentration was varied and the BDB-phosphate volume was kept constant. In addition, two controls were used: each batch of sensitized cells was tested (1) with normal rabbit serum and (2) with the appropriate rabbit antigrass serum. The proportions of BDB-phosphate solution to grass pollen extract were considered optimal when the highest titers were obtained with immune sera and when no reaction ensued with the normal sera.

If insufficient antigen was used, the erythrocytes were coupled to each other by BDB and gave false positive patterns in the diluent. On the other hand, if too much antigen was used, the sensitivity of the method was lowered. Presumably, the antigen molecules were coupled preferentially to each other by the BDB and were not available for attachment to erythrocytes. For optimal sensitization, the following amounts of materials were used:

| <i>Cells</i> | <i>BDB-phosphate</i> | <i>Glycerin-saline extracts†</i>                       |
|--------------|----------------------|--------------------------------------------------------|
| 0.1 ml.      | 0.5 ml.              | 0.06 to 0.10 ml. timothy, orchard, red-top, June grass |
| 0.1 ml.      | 0.5 ml.              | 0.10 to 0.13 ml. Bermuda grass                         |
| 0.1 ml.      | 0.5 ml.              | 0.15 to 0.17 ml. sweet vernal                          |

Glycerin itself, which is present in the pollen extracts, had no effect on the sensitization. When the antigen was replaced by glycerin in the sensitization procedure, the cells formed a negative pattern with the antisera used.

\*A room temperature of 22 to 26° C. was ideal, but with temperatures over 28° C. rapid deterioration of the BDB resulted in poor sensitization. The reaction proceeded satisfactorily in a water bath at 24° C.

†Although these extracts are prepared according to a standard method, for optimal sensitization slightly different amounts were required with different lots of extracts of the same pollen. Prior to sensitization of red cells, the pollen extracts were diluted to a final volume of 3 ml. with cold physiologic saline.

*Performance of the hemagglutination test:* For hemagglutination experiments, rows of lipped, round-bottomed\* 10 mm. by 75 mm. test tubes were placed in a masonite rack provided with holes and supported on a metal frame.† This arrangement allowed the tubes to hang vertically, which was essential for uniform sedimentation of the red cells in all tubes. The hemagglutination patterns could be observed easily when a mirror was placed below the tubes.

To remove nonspecific agglutinins, all sera were absorbed out with an equal volume of packed, nonsensitized rabbit erythrocytes (washed three times with cold saline) for one to two hours at room temperature or overnight in the cold. The cells used for absorption and sensitization were always from the same rabbit. The absorbed antisera were serially diluted in halving dilutions with diluent, each tube containing 0.5 ml. A volume of 0.05 ml. of sensitized cell suspension was added to each tube. The tubes were gently shaken to disperse the cells. The reaction proceeded at room temperature and could be read after a minimum of three hours. Usually they were read after twelve hours, although the patterns remained unchanged for at least thirty hours at room temperature or for several days in the cold.

The titer of the antiserum was expressed as the reciprocal of the highest dilution of antiserum which gave a positive pattern. The hemagglutination patterns were graded according to the recommendations of Stavitsky.<sup>23</sup> Because the end point of the hemagglutination is somewhat subjective, each reading was compared against a control row of sensitized cells in diluent and read by two observers. In addition, in each experiment the following controls were included: (1) To ascertain complete removal of nonspecific agglutinins, the sera were tested with nonsensitized rabbit cells‡ or with cells sensitized with heterologous protein antigens, for example, BSA and ovalbumin. (2) For day-to-day comparison of the method, a serum of known hemagglutination titer was employed.

*Inhibition of hemagglutination:* For inhibition tests, varying amounts of the appropriate antigen in 0.1 ml. glycerin-saline solution were added to each tube containing the antiserum in serial dilution. As a control, 0.1 ml. of saline and/or glycerin was added to an identical series of tubes. Sensitized red cells (0.05 ml.) were added to all tubes and the reaction was read as before.

## RESULTS

*Hemagglutination With Rabbit Antigrass Sera.*—None of the antisera obtained after the first course of immunization contained precipitating antibodies in amounts detectable by ring test. However, all sera had hemagglutination titers ranging from 60 to 15,000, the majority being in the range of 500 to 2,000.

Following the second course of immunization, all rabbit antisera contained precipitating antibodies, as evidenced by positive ring tests. These antisera

\*Round-bottomed tubes are preferred, since cells form irregular patterns in flat-bottomed tubes.

†Made of Dexion angle aluminum.

‡0.1 ml. of 50 per cent red cell suspension was made up in diluent to a final volume of 2.5 ml.

were tested with erythrocytes sensitized not only with the homologous antigens but also with the heterologous grass pollen extracts and with water-soluble extract of ragweed pollen (WSR).<sup>24</sup> The highest hemagglutination titers, ranging from 2,500 to 77,000 were obtained with the homologous systems, the majority of the sera giving titers of 10,000 to 40,000 (Table I). There were considerable cross-reactions among the five grasses—June grass, orchard grass, sweet vernal, timothy, and redtop. It is worth noting that occasionally the antiserum to any one of the grasses gave equal or even slightly higher titers when tested with cells sensitized with one of the other four grasses. On the other hand, with the antisera to the five grasses minimal reactions, if any, were obtained with cells sensitized with Bermuda grass or ragweed. Only slight reactions were obtained between antisera to ragweed and cells sensitized with the extracts of the six grass pollens. Similarly, antisera to Bermuda grass gave only slight reactions with the other six antigenic systems. The various cross-reactions are evident in Table I.

TABLE I. CORRELATION OF POLLEN ANTIGENS USING BDB-HEMAGGLUTINATION TECHNIQUE\*

| RABBIT<br>ANTISERUM<br>TO: | RABBIT<br>NO. | HEMAGGLUTINATION TITERS OBTAINED WITH CELLS SENSITIZED WITH |                 |         |         |        |         |         |
|----------------------------|---------------|-------------------------------------------------------------|-----------------|---------|---------|--------|---------|---------|
|                            |               | JUNE                                                        | SWEET<br>VERNAL | TIMOTHY | ORCHARD | REDTOP | BERMUDA | RAGWEED |
| June                       | 1             | 38,400                                                      | 9,600           | 38,400  | 19,200  | 38,400 | 600     | 300     |
|                            | 3             | 19,200                                                      | 2,400           | 19,200  | 19,200  | 19,200 | 300     | 300     |
|                            | 17            | 4,800                                                       | 300             | 1,200   | 9,600   | 1,200  | 0       | 0       |
| Sweet vernal               | 4             | 9,600                                                       | 19,200          | 38,400  | 38,400  | 19,200 | 1,200   | 600     |
|                            | 5             | 9,600                                                       | 19,200          | 38,400  | 9,600   | 9,600  | 0       | 600     |
|                            | 6             | 9,600                                                       | 19,200          | 19,200  | 9,600   | 9,600  | 0       | 1,200   |
| Timothy                    | 7             | 1,200                                                       | 600             | 4,800   | 9,600   | 1,200  | 300     | 600     |
|                            | 8             | 600                                                         | 600             | 2,400   | 4,800   | 1,200  | 300     | 300     |
|                            | 9             | 600                                                         | 600             | 4,800   | 4,800   | 300    | 600     | 600     |
| Orchard                    | 11            | 2,400                                                       | 2,400           | 9,600   | 38,400  | 2,400  | 600     | 1,200   |
|                            | 19            | 600                                                         | 4,800           | 9,600   | 76,800  | 2,400  | 600     | 600     |
|                            | 20            | 600                                                         | 1,200           | 2,400   | 19,200  | 600    | 1,200   | 600     |
| Redtop                     | 12            | 2,400                                                       | 1,200           | 4,800   | 4,800   | 9,600  | 0       | 0       |
|                            | 13            | 300                                                         | 300             | 1,200   | 2,400   | 9,600  | 300     | 600     |
|                            | 14            | 300                                                         | 300             | 1,200   | 4,800   | 4,800  | 0       | 300     |
| Bermuda                    | 23            | 600                                                         | 600             | 1,200   | 1,200   | 600    | 9,600   | 600     |
|                            | 24            | 300                                                         | 300             | 2,400   | 600     | 600    | 38,400  | 300     |
|                            | 29            | 300                                                         | 1,200           | 1,200   | 1,200   | 600    | 19,200  | 300     |
| Ragweed                    | 2             | 0                                                           | 300             | 300     | 300     | 0      | 0       | 9,600   |
|                            | 21            | 300                                                         | 300             | 1,200   | 300     | 0      | 1,200   | 9,600   |
|                            | 35            | 600                                                         | 300             | 1,200   | 600     | 600    | 600     | 38,400  |

\*The sera used for this correlation were obtained from rabbits after the second course of immunization. The titers of homologous antigen-antibody systems are given in the rectangles. Zero indicates that no hemagglutination was obtained with sera diluted 300-fold.

*Specificity of Hemagglutination.*—The hemagglutination was shown to be the result of the specific reaction between sensitized cells and homologous antibodies. This was demonstrated in two ways: (1) by the absence of agglutination of red blood cells sensitized with unrelated antigens (BSA, ovalbumin) and

(2) by inhibition experiments. With most antisera the addition of 8 to 10  $\mu$ g of free antigen in 0.1 ml. of glycerin-saline solution completely inhibited the reactions. With two strong sera (sweet vernal No. 4 and June No. 1, Table I) that had hemagglutination titers of 20,000 and 40,000, complete inhibition was achieved with about 18  $\mu$ g. In contrast, all antitimothy sera, in spite of their rather low hemagglutination titers required larger quantities of the timothy extract, that is, of the order of 19  $\mu$ g, for complete inhibition. In control experiments in which saline or glycerin was used instead of the antigen, no inhibition was observed.

TABLE II. INHIBITION OF HEMAGGLUTINATION WITH RABBIT ANTISERA\*

| RABBIT<br>ANTISERUM<br>TO: | RABBIT<br>NO. | TESTED<br>WITH CELLS<br>SENSITIZED<br>WITH: | MICROGRAMS OF FREE ANTIGENS REQUIRED FOR COMPLETE INHIBITION† |                 |         |         |        |         |         |
|----------------------------|---------------|---------------------------------------------|---------------------------------------------------------------|-----------------|---------|---------|--------|---------|---------|
|                            |               |                                             | JUNE                                                          | SWEET<br>VERNAL | TIMOTHY | ORCHARD | REDTOP | BERMUDA | RAGWEED |
| June                       | 1             | June                                        | 1.7                                                           | 53.3            | 56.7    | 15.6    | [125]  | [175]   | [600]   |
|                            | 3             |                                             | 1.7                                                           | 26.6            | 226.8   | 124.8   | [125]  |         |         |
|                            | 17            |                                             | 0.3                                                           | 3.2             | 6.8     | 3.7     | 7.8    |         |         |
| Sweet vernal               | 4             | Sweet vernal                                | 2.8                                                           | 0.1             | 3.0     | 3.2     | 1.7    | [175]   | [3,000] |
|                            | 5             |                                             | 1.4                                                           | 0.1             | 6.0     | 1.6     | 3.3    |         |         |
|                            | 6             |                                             | 1.4                                                           | 0.2             | 6.0     | 3.2     | 1.7    |         |         |
| Timothy                    | 7             | Timothy                                     | 2.8                                                           | [380]           | 3.0     | 12.8    | 207.0  | [175]   | [1,000] |
|                            | 8             |                                             | 5.6                                                           | 44.4            | 1.5     | 3.2     | 3.3    |         |         |
|                            | 9             |                                             | 1.4                                                           | 1.4             | 0.3     | 1.6     | 1.7    |         |         |
| Orchard                    | 11            | Orchard                                     | 5.6                                                           | 11.1            | 6.0     | 0.2     | 13.2   | [175]   | [5,000] |
|                            | 19            |                                             | 2.8                                                           | 44.4            | 12.0    | 0.8     | 6.6    |         |         |
|                            | 20            |                                             | 2.8                                                           | 5.6             | 12.0    | 0.2     | 13.2   |         |         |
| Redtop                     | 12            | Redtop                                      | 5.6                                                           | 22.2            | 24.0    | 208.0   | 0.2    | [175]   | [1,000] |
|                            | 13            |                                             | 11.2                                                          | 22.2            | 24.0    | 6.4     | 0.2    |         |         |
|                            | 14            |                                             | 44.8                                                          | 44.4            | 12.0    | 208.0   | 0.2    |         |         |
| Bermuda                    | 23            | Bermuda                                     | [105]                                                         | [105]           | [215]   | [125]   | [125]  | 1.7     | [1,500] |
|                            | 24            |                                             |                                                               |                 |         |         |        | 6.5     |         |
|                            | 29            |                                             |                                                               |                 |         |         |        | 1.7     |         |
| Ragweed                    | 2             | Ragweed                                     | [175]                                                         | [175]           | [380]   | [215]   | [215]  | [175]   | 8       |
|                            | 21            |                                             |                                                               |                 |         |         |        |         | 8       |
|                            | 35            |                                             |                                                               |                 |         |         |        |         | 4       |

\*These sera were diluted 2,000-fold.

†No inhibition was obtained with the amounts of antigens given in brackets. In these experiments higher concentrations of antigens were not used.

To gain further information about the antigenic relationship among the different pollens, we resorted to an inhibition technique, using the antiserum in a constant dilution with decreasing amounts of antigen. Table II records the minimum quantities of the inhibitors needed for complete inhibition of hemagglutination with 1:2,000 diluted antisera. As can be seen, the hemagglutination was inhibited with minute amounts of the homologous antigens. For inhibition of the hemagglutination of each of the five homologous systems (June, orchard, timothy, sweet vernal, and redtop), however, somewhat higher concentrations of the other four grass antigens were required. Moreover, these reactions could not be inhibited with much higher amounts of Bermuda grass or ragweed pollen extracts. Similarly, the anti-Bermuda and antiragweed systems could not be inhibited with large doses of any of the other pollen extracts, even if these two antisera were further diluted to 1:10,000. From these results, it

appears that both Bermuda grass pollen and ragweed pollen contain distinctly different antigens, whereas the other five grasses appear to have common antigen(s) to a varying degree.

*Hemagglutination With Human Grass-Sensitive Sera.*—The hemagglutination titers of the thirty allergic sera investigated in the present study are presented in Table III. As can be seen, twelve untreated and sixteen treated sera gave hemagglutination titers ranging up to 384 and 1,536, respectively. Generally, higher titers were observed with treated sera. Most of the patients were sensitive also to ragweed, as demonstrated by positive hemagglutination and direct skin tests. In seven cases, however, no ragweed sensitivity could be demonstrated by either of the tests. Only two sera (one treated and one untreated) failed to show hemagglutination with any of the six grasses, though the patients gave positive skin tests.

TABLE III. HEMAGGLUTINATION TITERS OF HUMAN SERA FROM GRASS-SENSITIVE PATIENTS

| PATIENT          | HEMAGGLUTINATION TITERS OBTAINED WITH CELLS SENSITIZED WITH |                 |         |         |        |         |         |
|------------------|-------------------------------------------------------------|-----------------|---------|---------|--------|---------|---------|
|                  | JUNE                                                        | SWEET<br>VERNAL | TIMOTHY | ORCHARD | REDTOP | BERMUDA | RAGWEED |
| <i>Untreated</i> |                                                             |                 |         |         |        |         |         |
| M. G.            | 96                                                          | 12              | 192     | 192     | 96     | 24      | 192     |
| R. L.            | 24                                                          | 24              | 6       | 6       | 48     | 0       | 6       |
| J. P.            | 96                                                          | 48              | 96      | 96      | 96     | 48      | 192     |
| N. P.            | 40                                                          | 0               | 10      | 160     | 20     | 0       | 0       |
| J. R.            | 40                                                          | 0               | 20      | 20      | 20     | 0       | 160     |
| R. S.            | 24                                                          | 48              | 12      | 24      | 24     | 96      | 0       |
| M. V.            | 384                                                         | 24              | 192     | 96      | 384    | 24      | 192     |
| F. W.            | 5                                                           | 0               | 10      | 5       | 0      | 0       | 0       |
| P. S.            | 40                                                          | 0               | 20      | 20      | 20     | 0       | 0       |
| P. G.            | 5                                                           | 20              | 10      | 20      | 20     | 20      | 10      |
| G. B.            | 160                                                         | 20              | 320     | 40      | 20     | 20      | 80      |
| G. A.            | 40                                                          | 10              | 80      | 40      | 10     | 10      | 80      |
| J. N.            | 0                                                           | 0               | 0       | 0       | 0      | 0       | 0       |
| <i>Treated</i>   |                                                             |                 |         |         |        |         |         |
| A. B.            | 0                                                           | 24              | 24      | 12      | 0      | 48      | 192     |
| P. B.            | 6                                                           | 0               | 24      | 48      | 48     | 48      | 192     |
| M. B.            | 48                                                          | 48              | 96      | 96      | 96     | 24      | 192     |
| H. C.            | 96                                                          | 96              | 48      | 384     | 384    | 96      | 12      |
| C. H.            | 384                                                         | 192             | 192     | 384     | 192    | 96      | 96      |
| F. H.            | 768                                                         | 192             | 768     | 192     | 1,536  | 48      | 96      |
| L. H.            | 192                                                         | 384             | 192     | 96      | 384    | 192     | 96      |
| A. L.            | 96                                                          | 48              | 192     | 96      | 96     | 0       | 0       |
| C. M.            | 40                                                          | 48              | 160     | 40      | 48     | 24      | 48      |
| S. S.            | 48                                                          | 24              | 24      | 12      | 24     | 24      | 24      |
| C. T.            | 48                                                          | 24              | 24      | 24      | 96     | 24      | 0       |
| A. T.            | 96                                                          | 96              | 96      | 24      | 96     | 48      | 0       |
| K. T.            | 80                                                          | 40              | 40      | 20      | 40     | 10      | 20      |
| G. T.            | 96                                                          | 48              | 48      | 48      | 48     | 96      | 192     |
| M. M.            | 20                                                          | 0               | 0       | 5       | 40     | 0       | 40      |
| A. D.            | 40                                                          | 0               | 10      | 20      | 10     | 0       | 40      |
| H. D.            | 0                                                           | 0               | 0       | 0       | 0      | 0       | 0       |

Zero indicates that no hemagglutination was obtained with the undiluted serum.

As part of the test for the specificity of the hemagglutination technique with regard to human antigrass sera, thirty-seven sera from normal subjects (who gave negative intradermal skin tests to a mixture of the six grass pollen extracts) were tested by hemagglutination. Only one of these sera gave

hemagglutination. The titers obtained with this serum were 4, 8, and 16 with June grass, sweet vernal, and timothy, respectively. However, none of the other thirty-six sera gave any reaction with any of the six grasses. The specificity of this reaction was further demonstrated, as with rabbit antisera, by inhibiting the hemagglutination specifically with free soluble grass antigen(s). When 8 to 10  $\mu\text{g}$  of inhibitor was used, the hemagglutination was completely inhibited in about one-half of the allergic sera, regardless of the titer. In the other sera the titers were markedly reduced. When larger amounts of inhibitor (17 to 40  $\mu\text{g}$ ) were used, all the reactions were completely inhibited. These results are shown in Table IV.

TABLE IV. INHIBITION OF HEMAGGLUTINATION WITH HUMAN ALLERGIC SERA

| PATIENT | HEMAGGLUTINATION TITERS AFTER INHIBITION WITH |                  |                 |                  |                  |                  |                  |                  |                  |                  |                 |                  |                  |                   |
|---------|-----------------------------------------------|------------------|-----------------|------------------|------------------|------------------|------------------|------------------|------------------|------------------|-----------------|------------------|------------------|-------------------|
|         | JUNE                                          |                  | SWEET VERNAL    |                  | TIMOTHY          |                  | ORCHARD          |                  | REDTOP           |                  | BERMUDA         |                  | RAGWEED          |                   |
|         | 9 $\mu\text{g}$                               | 36 $\mu\text{g}$ | 9 $\mu\text{g}$ | 36 $\mu\text{g}$ | 19 $\mu\text{g}$ | 74 $\mu\text{g}$ | 10 $\mu\text{g}$ | 40 $\mu\text{g}$ | 10 $\mu\text{g}$ | 40 $\mu\text{g}$ | 9 $\mu\text{g}$ | 36 $\mu\text{g}$ | 50 $\mu\text{g}$ | 100 $\mu\text{g}$ |
|         | Untreated                                     |                  |                 |                  |                  |                  |                  |                  |                  |                  |                 |                  |                  |                   |
| M. G.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| R. L.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| J. P.   | 12                                            | 0                | 6               | 0                | 6                | 0                | 48               | 0                | 12               | 0                | 6               | 0                | 0                |                   |
| N. P.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| J. R.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| R. S.   | 6                                             | 0                | 6               | 0                | 6                | 0                | 0                |                  | 6                | 0                | 12              | 0                | 0                |                   |
| M. V.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| P. S.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| P. G.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
|         | Treated                                       |                  |                 |                  |                  |                  |                  |                  |                  |                  |                 |                  |                  |                   |
| A. B.   | 0                                             |                  | 24              | 0                | 12               | 0                | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| P. B.   | 0                                             |                  | 0               |                  | 0                |                  | 12               | 0                | 12               | 0                | 0               |                  | 0                |                   |
| M. B.   | 0                                             |                  | 12              | -                | 0                |                  | 24               | -                | 6                | -                | 12              | -                | 0                |                   |
| H. C.   | 24                                            | 0                | 24              | 0                | 12               | 0                | 24               | 0                | 12               | 0                | 24              | 0                | 0                |                   |
| C. H.   | 0                                             |                  | 24              | 0                | 12               | 0                | 12               | 0                | 0                |                  | 48              | 0                | 0                |                   |
| F. H.   | 96                                            | 0                | 48              | 0                | 48               | 0                | 96               | 0                | 96               | 0                | 48              | 0                | 0                |                   |
| L. H.   | 96                                            | 0                | 48              | 0                | 48               | 0                | 6                | 0                | 96               | 0                | 48              | 0                | 24               | 0                 |
| A. L.   | 0                                             |                  | 12              | 0                | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |
| C. M.   | 6                                             | 0                | 12              | 0                | 6                | 0                | 12               | 0                | 6                | 0                | 12              | 0                | 12               | 0                 |
| S. S.   | 24                                            | 0                | 12              | 0                | 12               | 0                | 6                | 0                | 12               | 0                | 12              | 0                | 0                |                   |
| C. T.   | 6                                             | 0                | 6               | 0                | 0                |                  | 12               | 0                | 12               | 0                | 12              | 0                | 0                |                   |
| A. T.   | 24                                            | -                | 12              | -                | 0                |                  | 0                |                  | 6                | -                | 0               |                  | 0                |                   |
| K. T.   | 24                                            | 0                | 12              | 0                | 0                |                  | 0                |                  | 6                | 0                | 0               |                  | 0                |                   |
| G. T.   | 24                                            | -                | 24              | -                | 12               | -                | 12               | -                | 12               | -                | 12              | -                | 6                | -                 |
| M. M.   | 0                                             |                  | 0               |                  | 0                |                  | 0                |                  | 0                |                  | 0               |                  | 0                |                   |

Zero indicates complete inhibition of hemagglutination.

*Correlation of the Allergens of Different Grasses.*—In a previous study from this laboratory<sup>25</sup> it was reported that skin-sensitizing and/or blocking antibodies could be removed completely from the sera of ragweed-sensitive subjects by absorption with antigenically specific adsorbents prepared by coupling the constituents of a water-soluble extract of ragweed pollen to polystyrene or to erythrocytes via stable covalent azo bonds. The removal of these antibodies was always accompanied by a parallel disappearance of the hemagglutinating factor.

In the present study the same methods have been applied to investigate the relationship between the hemagglutinating factor(s) and the skin-sensitizing and/or blocking antibodies in the sera of grass-sensitive subjects. Furthermore,

using adsorbents prepared with individual grass pollen extracts, the relationship among the allergens of the six pollens used could also be studied. The experimental procedures were similar to those employed in the ragweed system with sensitized erythrocytes<sup>21</sup> and polystyrene-antigen conjugates.<sup>26</sup> For these experiments, water-soluble extracts of each of the six defatted grass pollens were prepared.

It was shown that any of the five grasses (June, sweet vernal, timothy, orchard, and redtop) coupled to erythrocytes or to polystyrene removed the antibodies from the serum of a treated grass-sensitive subject. This is illustrated by the experiments recorded in Table V. Total removal of antibodies was achieved with adsorbents to which large amounts of the allergenic constituents were coupled (columns 1, 4, and 6 in Table V). On certain occasions, when an insufficient amount of adsorbent was used, the serum was only partially depleted of antibodies (compare columns 1 and 2 in Table V). In these cases the hemagglutination titers of the absorbed sera decreased with respect not only to the grass pollen which had been coupled to the inert supporting media but also to the other four grasses (column 7 in Table V). From these findings, one can conclude that the pollen extracts of the five grasses contain common allergenic constituents. In contrast, Bermuda grass appeared to be allergenically distinct from the other five grasses, inasmuch as no hemagglutination was obtained with red cells sensitized with the pollen extract of Bermuda grass and the serum of a patient (A. L.) who was allergic to the other five grasses. Moreover, adsorbents prepared with Bermuda grass failed to remove the hemagglutination factors from sera of patients sensitive to grasses other than Bermuda. Similarly, with the serum containing no antibodies to ragweed (Patient A. L.), the specificity of the absorption method was tested. Column 8 in Table V shows that incubation of this serum with a polystyrene-ragweed conjugate failed to remove antibodies to grass pollens. Similar findings were also reported by Gordon and associates<sup>21</sup> who coupled ragweed allergens to erythrocytes.

TABLE V

| TESTED WITH<br>CELLS SENSITIZED WITH | HEMAGGLUTINATION TITERS OF AN ALLERGIC SERUM |                       |    |                 |         |         |        |    |         |
|--------------------------------------|----------------------------------------------|-----------------------|----|-----------------|---------|---------|--------|----|---------|
|                                      | BEFORE<br>ABSORP-<br>TION                    | AFTER ABSORPTION WITH |    |                 |         |         |        |    |         |
|                                      |                                              | JUNE                  |    | SWEET<br>VERNAL | TIMOTHY | ORCHARD | REDTOP |    | RAGWEED |
|                                      |                                              | 1*                    | 2† | 3‡              | 4†      | 5‡      | 6†     | 7§ | 8†      |
| June                                 | 160                                          | 0                     | 10 | 40              | 0       | 0       | 0      | 40 | 160     |
| Sweet vernal                         | 20                                           | 0                     | 0  | 5               | 0       | 0       | 0      | 10 | 20      |
| Timothy                              | 80                                           | 0                     | 0  | 20              | 0       | 20      | 0      | 40 | 40      |
| Orchard                              | 160                                          | 0                     | 5  | 10              | 0       | 0       | 0      | 80 | 160     |
| Redtop                               | 40                                           | 0                     | 5  | 20              | 0       | 0       | 0      | 10 | 20      |
| Bermuda                              | 0                                            | 0                     | 0  | 0               | 0       | 0       | 0      | 0  | 0       |

Zero indicates no hemagglutination.

\*Water-soluble grass constituents coupled to erythrocytes.

†Water-soluble grass constituents coupled to polystyrene.

‡Glycerin-saline-soluble grass constituents coupled to erythrocytes.

§Glycerin-saline-soluble grass constituents coupled to polystyrene.

In another experiment, the treated serum (Patient A. L.) was absorbed with erythrocytes sensitized with the dialyzed residue of a water-soluble June

extract. All antibodies to this residue were removed, as evidenced by the negative hemagglutination test obtained with cells sensitized with it. However, this apparently completely absorbed serum showed the presence of considerable amounts of antibodies when tested with cells sensitized with the whole water-soluble extract of June grass pollen; similar results were obtained with cells sensitized with the glycerin-saline extracts of the other grass pollens. This finding would suggest that different antibodies produced to different allergenic constituents of one and the same pollen may be demonstrated with the hemagglutination technique.

#### DISCUSSION

This study indicates clearly the applicability of the BDB-hemagglutination technique for the demonstration of antibodies to grass pollen constituents both in sera of immunized rabbits and in sera of grass-sensitive patients.

This method is highly sensitive. Thus, in the present study rabbit antisera containing precipitating antibodies which could not be detected in dilutions higher than 1:50 gave hemagglutination titers of the order of  $10^4$ .\* Furthermore, with weaker rabbit antisera, particularly after the first course of immunization, which failed to give any ring test, hemagglutination titers of the order of  $10^2$  to  $10^3$  were still obtained. Although most allergic sera displayed hemagglutination titers of the order of  $10^2$ , the incidence of high titers was greater in the group of treated sera (Table III). Similar findings have already been reported from this laboratory<sup>21</sup> for sera of ragweed-sensitive patients.

The absorption of nonspecific agglutinins with nonsensitized cells resulted in hemolysis, particularly excessive with fresh sera. This made the precise evaluation of the hemagglutination titer impossible, especially with weak sera. This difficulty was overcome by pretreating the serum with precipitating antibody-antigen complexes or zymosan or by heating it. The hemagglutination titer was not affected by any of these procedures which presumably lead to inactivation of complement. With the same batch of sensitized red cells, identical titers were obtained in a series of experiments using the same serum. With different preparations of sensitized cells, the titers were reproducible within  $\pm 2$  tubes (equivalent to a factor of 16). Therefore, one cannot attach too much significance to the absolute value of the hemagglutination titer, particularly if one has not exhaustively determined the optimal conditions for sensitization of red cells. It is to be emphasized that the end point in the titration of the hemagglutinating factor(s) must be established in terms of the patterns obtained in the control series of tubes containing normal serum. Nevertheless, the relationship among the titers of different sera is maintained from experiment to experiment, provided that the tests are done concurrently with all the sera and with the same batch of sensitized cells.

The specificity of the method was demonstrated by inhibition experiments. The results listed in Table II indicate that hemagglutination given by ragweed-sensitized cells and antiragweed sera was inhibited only by free soluble ragweed

\*In previous studies<sup>18, 21</sup> with stronger rabbit antisera, hemagglutination titers of the order of  $10^6$  to  $10^7$  were also obtained.

extract. Similarly, the hemagglutination obtained with the Bermuda-anti-Bermuda system was inhibited only by free Bermuda antigens. However, the reactions obtained with cells sensitized with each of the five grasses—June grass, sweet vernal, timothy, orchard grass and redtop—and the corresponding five antisera could be inhibited with any one of these five grass pollen extracts but not with Bermuda grass or ragweed antigens. These results are interpreted as indicating that the group of five grasses contains common antigens which are distinct from those of Bermuda grass and ragweed.

The cross-reactions demonstrated for the group of the five grasses were obtained with antisera diluted 1:2,000. This dilution was arbitrarily chosen in view of the limited amounts of antisera and antigens which were available at the time of these experiments. It should be pointed out that the conclusions reached with diluted sera may be fallacious inasmuch as the concentration of some of the cross-reacting antibodies may be inadvertently lowered to a level below that required for their detection. The weakness of this method for establishing antigenic cross-reactions is borne out by comparing the results of Table II with those of Table I. Thus, from the results of the inhibition studies with 2,000-fold-diluted sera (Table II), it would appear that Bermuda grass and ragweed contain antigens different from those of the other five grasses. In contrast, it is apparent from Table I that Bermuda grass and ragweed contain a very small, but detectable, amount of antigens common to the other five grasses.

TABLE VI. INHIBITION OF HEMAGGLUTINATION WITH A RABBIT ANTIRAGWEED SERUM\*

| CELLS<br>SENSITIZED<br>WITH | MICROGRAMS OF FREE ANTIGENS REQUIRED FOR COMPLETE INHIBITION : |                 |         |         |        |         |         |
|-----------------------------|----------------------------------------------------------------|-----------------|---------|---------|--------|---------|---------|
|                             | JUNE                                                           | SWEET<br>VERNAL | TIMOTHY | ORCHARD | REDTOP | BERMUDA | RAGWEED |
| June                        | 9.0                                                            | 9.0             | 4.5     | 9.0     | 4.5    | 38.0    | 38.0    |
| Sweet vernal                | 9.0                                                            | 9.0             | 9.0     | 4.5     | 19.0   | 19.0    | 19.0    |
| Timothy                     | 4.5                                                            | 38.0            | 2.2     | 4.5     | 2.2    | 9.0     | 19.0    |
| Orchard                     | 2.2                                                            | 4.5             | 4.5     | 9.0     | 9.0    | 9.0     | 75.0    |
| Redtop                      | 4.5                                                            | 4.5             | 2.2     | 4.5     | 4.5    | 19.0    | 19.0    |
| Bermuda                     | △                                                              | △               | △       | △       | △      | 4.5     | 75.0    |
| Ragweed                     | △                                                              | △               | △       | △       | △      | △       | 19.0    |

\*This serum was diluted 100-fold.

△ = No inhibition was obtained with 600 µg of free antigen.

Unfortunately, because of lack of materials, the possible cross-reactions among all these different systems could not be investigated with undiluted sera by the inhibition technique. Only one such experiment was done with a 100-fold-diluted rabbit antiragweed serum.\* Hemagglutination was obtained with this serum and cells sensitized with each of the six grasses, and it was completely inhibited with soluble ragweed extract. (See Table VI.) The hemagglutination given by cells sensitized with the five grasses (June grass, sweet vernal, timothy, orchard grass, and redtop) and this serum could be inhibited by the pollen constituents of any of these five grasses, as well as by Bermuda grass and ragweed antigens. On the other hand, the hemagglutination resulting with

\*In this experiment serum No. 35 of Table I was used. As seen from this table, cross-reactions could still be detected between this serum (even in dilutions higher than 1:300) and the other heterologous antigens.

cells sensitized with Bermuda antigens could be inhibited only by the antigens of Bermuda grass or ragweed pollens. Moreover, the hemagglutination with cells sensitized with ragweed could be inhibited only with ragweed pollen extracts. These results therefore would suggest that (1) the five grasses (June grass, sweet vernal, timothy, orchard grass, and redtop) contain only small amounts of some of the antigens of ragweed pollen; (2) while Bermuda grass contains a small amount of antigens common to the other grasses, the majority of its constituents are antigenically distinct; (3) Bermuda grass contains a small amount of constituents antigenically similar to those found in ragweed; (4) ragweed contains only a small amount of constituents antigenically similar to those found in the six grasses, in addition to its distinct antigens. These results, although not based on an exhaustive study, demonstrate the usefulness of the hemagglutination technique in establishing antigenic relationships among related systems.

As with rabbit antisera, the specificity of the hemagglutination method for detection of antibody-like factor(s) in sera of grass-sensitive persons was demonstrated by inhibition of the reaction with free soluble grass pollen extracts. For complete inhibition of hemagglutination, however, larger quantities of the inhibitors were required than the quantities needed for inhibition of hemagglutination obtained with rabbit antisera containing precipitating antibodies. This would, therefore, suggest that the allergenic constituents of grass pollens are present in smaller concentrations than those responsible for antigenicity in rabbits and that there is no justification to assume that antigenically determinant groups are also responsible for inducing the production of antibodies in allergic persons. Unfortunately, in several previous studies no discrimination was made between antigenicity and allergenicity of grass pollens and it was implied that the allergenic components were identical to the grass pollen constituents which were antigenic in rabbits.<sup>27, 28</sup>

Regarding the specificity of the method, it is important to note that among the thirty-seven normal human sera used only one gave a low titer of about 16 with grass-sensitized cells. This person had been previously used as a subject for Prausnitz-Küstner tests and might have developed antibodies subsequent to injection with pollen extracts. Similarly, only two of these thirty-seven sera gave hemagglutination with ragweed-sensitized cells. One of these two sera belonged to the same person used as a Prausnitz-Küstner subject. On the other hand, out of thirty sera from grass-sensitive persons (Table III) only two failed to give hemagglutination with any of the six grasses, although their donors gave positive skin tests.\*

The specificity of the method was further demonstrated by removal of the hemagglutinating factor(s) from one allergic serum by absorption with allergenic adsorbents. As shown in Table V, the hemagglutinating factor(s) were removed by adsorbents prepared by coupling grass pollen constituents to polystyrene or erythrocytes. It was also found by Prausnitz-Küstner tests that the skin-sensitizing antibodies of this serum were removed by this treatment

\*Unfortunately, these two sera were not tested for the presence of skin-sensitizing antibodies by Prausnitz-Küstner tests. It is possible that the concentration of circulating antibodies in these sera was below the amount detectable by the hemagglutination technique.

and that removal of the hemagglutinating factor with respect to any of the allergens was paralleled by a partial or complete reduction in the Prausnitz-Küstner titers. These results, therefore, demonstrate that skin-sensitizing as well as hemagglutinating factor(s) combine in vitro with the grass pollen constituents and that these grasses may possess different amounts of identical or unrelated allergens.

Similar observations were previously made<sup>21</sup> with sera of ragweed-sensitive persons, and it was then concluded that the hemagglutinating factor(s) may be identified with skin-sensitizing and/or blocking antibodies present in untreated and treated sera, respectively. As stated in earlier publications,<sup>25, 30</sup> however, it should be stressed that as yet there is no unequivocal proof that skin-sensitizing and hemagglutinating properties of untreated sera are different attributes of one and the same serum molecule; they may well be associated with distinct serum components. Similar conclusions were drawn from the relationship between hemagglutinating and blocking capacities of treated sera. In view of the limited number of Prausnitz-Küstner tests done in this study, it is impossible to draw any more definitive conclusion regarding the nature of the hemagglutinating factor(s) and its relationship to skin-sensitizing antibodies of sera from grass-sensitive patients.

#### APPENDIX

In view of the danger of transferring homologous serum hepatitis by Prausnitz-Küstner tests, only one serum was used in this study for establishing the allergenic relationship among the different grasses. This serum (A. L.) was obtained from a physician who has never had any symptoms suggestive of hepatitis. The results of cross-neutralization experiments using desensitizing doses of each of the five grasses (June grass, timothy, orchard grass, redtop, and Bermuda grass) with this serum are given in Table VII. Cross-neutralization with sweet vernal was not attempted, since at the time these experiments were performed our supply of sweet vernal had been exhausted. The desensitizing dose with respect to each allergen was established as described previously for ragweed allergens.<sup>29</sup>

From these results, one may draw the following conclusions: (1) redtop grass pollen appears to possess all the allergens common to the other grasses; (2) this serum contained a small amount of antibodies directed specifically against Bermuda grass allergens; (3) all the other grasses contain additional allergens to those found in Bermuda grass pollen; and (4) June grass, timothy, and orchard grass have a complex and in many respects a similar allergenic composition. Since these conclusions are based on the pattern of reactivity given by one allergic serum, they may not be valid for other allergic sera from

TABLE VII

| SITE<br>DESENSITIZED<br>WITH | SKIN REACTIONS AFTER CHALLENGE WITH |      |         |         |         |
|------------------------------|-------------------------------------|------|---------|---------|---------|
|                              | REDTOP                              | JUNE | TIMOTHY | ORCHARD | BERMUDA |
| Redtop                       | 0                                   | 0    | 0       | 0       | 0       |
| Bermuda                      | +                                   | +    | +       | +       | 0       |
| June                         | +                                   | 0    | 0       | 0       | +       |
| Timothy                      | +                                   | +    | 0       | 0       | +       |
| Orchard                      | 0                                   | 0    | +       | 0       | +       |

grass-sensitive persons. Nevertheless, these experiments clearly demonstrate the allergenic complexity of these grass pollens and the necessity of using precisely defined allergenic fractions in cross-neutralization studies.

#### SUMMARY

The BDB-hemagglutination technique has been adapted for the demonstration of antibodies to grass pollen constituents in the sera of immunized rabbits and grass-sensitive hay fever patients. The grasses used were timothy, June grass, orchard grass, redtop, sweet vernal, and Bermuda grass. The method was shown to be highly sensitive. Titers of the order of  $10^4$  and  $10^2$  were obtained with sera of immunized rabbits and of allergic human subjects, respectively. The specificity of the technique was demonstrated by inhibition of the hemagglutination with free soluble pollen constituents.

The inhibition of hemagglutination obtained with rabbit antisera was used for establishing antigenic relationships among the various grasses. Timothy, June grass, orchard grass, redtop, and sweet vernal were found to contain common antigens, which were different from those of Bermuda grass and ragweed. The results of Prausnitz-Küstner tests using the cross-neutralization technique with desensitizing doses of each of the five grasses (timothy, June grass, orchard grass, redtop, and Bermuda grass) suggest that redtop grass pollen contains all the allergens common to the other grasses and that timothy, June grass, and orchard grass pollens have a complex and in many respects similar allergenic composition and contain additional allergens to those found in Bermuda grass pollen.

Absorption of an allergic serum with adsorbents prepared by coupling grass pollen constituents to polystyrene or erythrocytes resulted in the removal of the hemagglutinating factor(s), which was paralleled by the removal of skin-sensitizing antibodies.

The authors wish to thank Dr. B. Rose for his interest.

#### REFERENCES

1. Prausnitz, C., and Küstner, H.: Studien über die Ueberempfindlichkeit, *Centralbl. f. Bakt.* **86**: 160, 1921.
2. Grabar, P.: Comparaison entre les methodes immunologiques quantitatives et bilan de leurs applications (Abst.), 6th International Congress of Microbiology, Rome, vol. 1, p. 477, 1953.
3. Miller, H., and Campbell, D. H.: Reagins: Preliminary Report on Experimental Evidence in Support of a New Theory of Their Nature, *Ann. Allergy* **5**: 236, 1947.
4. Bukantz, S. C., Johnson, M. C., and Hampton, S.: Quantitative Immunologic Studies With Allergens. I. Colorimetric Absolute Methods in the Analysis of Factors Influencing Precipitation of Ragweed Pollen Extracts and Homologous Rabbit Antisera, *J. ALLERGY* **20**: 1, 1949.
5. Orlans, E. S., Rubinstein, L. J., and Marrack, J. R.: Detection of Antibodies in the Serum of Patients Suffering From Hay Fever, *Acta allergol.* **6**: 33, 1953.
6. Cavelti, P. A.: Complement Fixation Studies in Allergy, *J. ALLERGY* **21**: 532, 1950.
7. Hensel, M. E., and Sheldon, J. M.: Complement Fixation With Specific Allergens in Hay Fever. I. Problem, Procedure, Diagnostic Value, *J. Lab. & Clin. Med.* **26**: 1586, 1940-1941.
8. Albus, G.: Beeinflussung der Antikörperkonzentration durch spezifische Desensibilisierung bei Pollenallergie, *Ztschr. f. d. ges. exper. Med.* **95**: 703, 1935.

9. Portnoy, J., and Sherman, W. B.: Complement Fixation Studies in Ragweed Allergy. II. Determination of Antibody in Human Sera to Ragweed Antigen by Means of a Complement Fixation Inhibition Test; the Relationship of Antibody so Determined to the Passive Transfer for Blocking Antibody, *J. ALLERGY* **25**: 229, 1954.
10. Loeb, L. F.: Über die chemische Natur der Allergene. III. Pollen von *Alopecurus* (Fuchsschwanzgras), *Klin. Wehnschr.* **8**: 926, 1929.
11. Hampton, S. F., Stull, A., and Cooke, R. A.: Antigenic Studies by the Dale Test. I. The Antigenic Relationship of Certain Pollens, *J. ALLERGY* **10**: 417, 1938-1939.
12. Ovary, Z., and Biozzi, G.: Passive Sensitization of Skin of Guinea Pig With Human Antibody, *Internat. Arch. Allergy* **5**: 241, 1954.
13. Jones, F. S.: Agglutination by Precipitin, *J. Exper. Med.* **48**: 183, 1928.
14. Boyden, S. V.: The Adsorption of Proteins on Erythrocytes Treated With Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera, *J. Exper. Med.* **93**: 107, 1951.
15. Kallós, P., and Kallós-Defner, L.: Specific Serum-Agglutination of Corpuseles (Collodion-Pellets or Erythrocytes) Coated With Allergenic Material, *Internat. Arch. Allergy* **2**: 70, 1951.
16. Feinberg, R. J., Davison, J. D., and Flick, J. A.: The Detection of Antibodies in Hay Fever Sera by Means of Hemagglutination, *J. Immunol.* **77**: 279, 1956.
17. Feinberg, R. J., and Flick, J. A.: Elution of Pollen Antigens From Tannic Acid Treated Erythrocytes, *Proc. Soc. Exper. Biol. & Med.* **96**: 71, 1957.
18. Schon, A. H., Gordon, J., and Rose, B.: Antibodylike Factor in Serums of Ragweed-Sensitive Individuals Shown In Vitro, *Science* **125**: 597, 1957.
19. Pressman, D., Campbell, D. H., and Pauling, L.: The Agglutination of Intact Azorethrocytes by Antisera Homologous to the Attached Groups, *J. Immunol.* **44**: 101, 1942.
20. Stavitsky, A. B., and Arquilla, E. R.: Micromethods for the Study of Proteins and Antibodies. III. Procedure and Applications of Hemagglutination and Hemagglutination-Inhibition Reactions With Bis-diazotized Benzidine and Protein-conjugated Red Blood Cells, *J. Immunol.* **74**: 306, 1955.
21. Gordon, J., Rose, B., and Schon, A. H.: Detection of "Non-Precipitating" Antibodies in Sera of Individuals Allergic to Ragweed Pollen by an In Vitro Method, *J. Exper. Med.* **108**: 37, 1958.
22. Wall, R. L., Jayne, M., and Beauchamp, J.: Preservation of the Specific Agglutinability of Human Red Blood Cells, *J. Lab. & Clin. Med.* **36**: 378, 1950.
23. 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.
24. Schon, A. H., Richter, M., Harter, J. G., and Rose, B.: Studies on Ragweed Pollen. I. Preliminary Fractionation of Ragweed Pollen; Characterization and Allergenicity of the Isolated Fractions, *J. ALLERGY* **27**: 236, 1956.
25. Schon, A. H.: Different Types of Antibodies Produced by Allergic Individuals Depending on Route of Immunization (to be published in extenso in June, 1960, in the *Proc. Internat. Symposium on "Mechanism of Antibody Formation"* held in Prague, May, 1959).
26. Gyenes, L., Rose, B., and Schon, A. H.: Isolation of Antibodies on Antigen-Polystyrene Conjugates, *Nature, London* **181**: 1465, 1958.
27. Wodehouse, R. P.: Antigenic Analysis by Gel Diffusion. 2. Grass Pollen, *Internat. Arch. Allergy* **6**: 65, 1955.
28. Gosselin, M. L., Mynors, L. S., and Coombs, R. R. A.: Serological Studies on Pollen Extracts. II. Demonstration by a Serological Method of the Specificity of the Antigen-Mosaic of the Extracts of Six Grass Pollens, *Acta allergol.* **6**: 96, 1953.
29. Richter, M., Harter, J. G., Schon, A. H., and Rose, B.: Studies on Ragweed Pollen. III. Estimation of the Minimum Number of Allergens in the Water-Soluble Extract of Ragweed Pollen and a Critical Evaluation of the Neutralization Technique; The Demonstration of Two Different Reagents in Sera of Ragweed-Sensitive Individuals, *J. Immunol.* **79**: 13, 1957.
30. Schon, A. H.: Biophysical and Immunochemical Problems in Allergy, *Proceedings of the Third International Congress of Allergology, Paris, 1958*, Editions Flammarion, p. 209.