

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

JOSEPH PORTNOY, PH.D.,* AND WILLIAM B. SHERMAN, M.D.
NEW YORK, N. Y.

INTRODUCTION

IN the preceding paper a study of the complement fixation reaction of ragweed antigen and rabbit immune serum was reported, which served as a background for experiments undertaken to develop a test for antibodies occurring in human ragweed allergy.

Preliminary observations by the authors indicated that inhibition of the fixation of complement (C') by a standardized ragweed antigen-rabbit immune serum occurred with sera known to contain blocking antibody by passive transfer. The present report is concerned with a study of the factors influencing the detection of such inhibiting antibodies, culminating in the development of a test method for these antibodies. This test method was then applied to a study of allergic and nonallergic sera and comparisons were made with the passive transfer technique.

MATERIALS AND METHODS

1. *Human Sera*.—Unless otherwise noted, whenever blood or serum samples were received the serum obtained was passed through Seitz filters, 30 ml. capacity and then stored at -20° C. in aliquots suitable for testing. Paraffined corks were employed to minimize dehydration during storage. On the day of examination, sera were heated at temperatures and for periods of time as described under the particular protocol.

2. *Hemolytic System*.—These are described in Part I of these studies.

3. *Antigens, Rabbit Immune Serum*.—These are described in Part I of these studies.

4. *Complement Fixation Inhibition Test for Antibodies Against Ragweed Antigens*.—See description under experimental results and procedures.

From the Departments of Microbiology and Medicine, College of Physicians and Surgeons, Columbia University and the Medical Service of Presbyterian Hospital, New York, N. Y.

Presented at the Tenth Annual Meeting of the American Academy of Allergy, Houston, Tex., Feb. 1, 2, 3, 1954.

Received for publication Jan. 12, 1954.

*Submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Columbia University, Faculty of Pure Science.

Present address: Venereal Disease Research Laboratory, P.O. Box 185, Chamblee, Ga.

EXPERIMENTAL PROCEDURES AND RESULTS

A. The Effect of Various Factors on the Detection of Inhibiting Antibody.—

1. *The possible role of thermostable complement components:* By employing amounts of complement designed to produce partial hemolysis, it was shown that all the human sera tested, whether they possessed or were lacking in inhibiting antibody, did not enhance the hemolytic activity of added guinea pig complement. Thus, in one experiment when 16 fresh sera were tested, after heating at 56° C. for 30 minutes, with one 50 per cent hemolytic unit of C', 15 to 40 per cent hemolysis occurred with an average of 27 per cent. These same sera heated at 56° C. for 4 hours produced 5 to 30 per cent hemolysis in the presence of one unit of complement with an average of 13 per cent hemolysis. When stored sera were examined for this property they showed in general less hemolysis than the fresh sera. As further experiments were performed and as a testing method was evolved, there was never any indication of a thermostable complement effect.

2. *The effect of preliminary incubation of serum and antigen at various temperatures for varying periods of time in the presence and absence of comple-*

TABLE I. THE EFFECT OF TIME, TEMPERATURE, AND THE PRESENCE OR ABSENCE OF COMPLEMENT DURING THE PRELIMINARY INCUBATION OF ANTIGEN AND HUMAN SERUM ON THE DETECTION OF INHIBITING ANTIBODY

SYSTEM	SERUM	ANTIG.*	INHIBITION TEST† SERUM DILUTIONS†						ANTICOMPLEMENTARY TEST§ SERUM DILUTIONS†					
			2	4	8	16	32	64	2	4	8	16	32	64
1	Neg.	.4	15	15	15	15	15	10						
	Pool	.2	15	15	15	15	15	15	5	15	30	55	70	75
	Block.	.4	50	60	50	20	10	5						
	Pool	.2	50	70	75	60	30	10	20	35	50	70	75	75
2	Neg.	.4	10	10	15	15	10	10						
	Pool	.2	5	10	20	20	15	10	5	15	30	50	70	75
	Block.	.4	55	70	65	40	25	15						
	Pool	.2	60	75	80	65	50	20	20	30	60	60	70	75
3	Neg.	.4	10	10	10	10	5	5						
	Pool	.2	10	10	10	10	5	5	10	15	30	45	60	65
	Block.	.4	40	60	35	15	5	5						
	Pool	.2	55	65	65	40	10	5	10	30	50	55	60	65
4	Neg.	.4	10	10	5	0	0	0						
	Pool	.2	5	10	10	0	0	0	10	10	20	30	50	55
	Block.	.4	40	55	40	10	0	0						
	Pool	.2	40	60	55	20	5	0	10	25	35	40	40	40

Results above expressed in degrees of hemolysis.

*Amounts given in micrograms of protein N.

†Reciprocal of serum dilutions.

‡Inhibition test with three units of C'.

§Anticomplementary test with two units of C' (rabbit immune serum added).

Immune rabbit serum dilution used required approximately six units of C' for 50 per cent hemolysis.

System 1.—Human serum plus antigen incubated 1 hour 37° C., then immune serum and C' added.

System 2.—Human serum plus antigen plus C' incubated 1 hour 37° C., then immune rabbit serum added. C' dose based on titration using a preliminary incubation of 1 hour at 37° C. before adding sensitized cells.

System 3.—Human serum plus antigen incubated 18 hours 5° C., then immune serum and C' added.

System 4.—Human serum plus antigen plus C' incubated 18 hours 5° C., then immune rabbit serum added.

ment: Table I presents a representative protocol showing how time, temperature, and the presence of complement during the preliminary incubation of inhibiting antibody and antigen influenced the detectability of this antibody. To perform this experiment, dilutions of antigen "C" (whole extract antigen) were prepared to contain 0.4 and 0.2 microgram of antigen protein N. in a test dose of 0.1 ml. As a source of inhibiting antibody, a pool of three human sera was employed. The donors of these sera were individuals who were sensitive to ragweed and who had been treated with ragweed extract. Tests on the sera individually indicated the presence of inhibiting substance. For use in this and many other experiments, the three sera were Seitz-filtered, pooled, and stored at -20° C. until used in tests. Before testing, this pooled serum was heated at 56° C. for four hours. The dose of serum was 0.1 ml. of the dilutions indicated in Table I. Parallel tests were made on a pool of five individual sera selected at random from specimens submitted for serologic tests for syphilis, all of which had given negative results in several types of serologic tests. This negative serum pool served as an indicator for the specificity of the test for inhibiting antibody under the various conditions described. Test dosages of serum and antigen were pipetted and the mixtures incubated in the presence or absence of complement, as noted in Table I. The complement dose was three 50 per cent hemolytic units. Following this preliminary incubation, a pool of rabbit antiragweed immune serum was added in a test dose of 0.1 ml. of a 1-20 dilution. After 4 hours at 3 to 6° C., 0.2 ml. of sensitized sheep red blood cells was added and the degree of hemolysis recorded following 15 minutes' incubation at 37° C. Antigen, complement, and cell controls were included.

Following the recommendation of Rice,⁴³ 50 per cent or greater hemolysis with a test mixture was considered as evidence of inhibiting antibody action. Accordingly, no inhibiting activity was noted with the negative pool, whereas evidence of inhibition was obtained under the varying conditions of this experiment with the inhibiting antibody pool. The end point of reactivity of the inhibiting serum in any given system was always greater with the lower concentration of antigen. Using a 1-hour preliminary incubation at 37° C., there was a slight increase in reactivity endpoint when complement was present in the mixture as compared to the system lacking in complement. Prolonged incubation at 5° C. with and without complement produced lower end points than the shorter incubation at 37° C. The results given under serum controls were typical. The anticomplementary property of the serums tested can be noted. There was no significant difference in this property of the two serum pools studied.

It is apparent from this and similar experiments that preliminary incubation at 37° C. gave more sensitive findings than incubation at 5° C., and that the effect of complement during this preliminary incubation was negligible.

As extension of these time studies, in several experiments, assay of inhibiting antibody was made using 1 and 2 hours of preliminary incubation at 37° C. and 5° C. at the same time tests were made without preliminary incubation. The results obtained are given in Table II. It can be seen that evidence of inhibiting activity occurred even when immune rabbit serum was added

without preliminary incubation of antigen and inhibiting serum. A fourfold increase in titer of inhibiting serum occurred after 1 hour at 37° C. Further incubation at 37° C., i.e., 2 hours' total incubation, did not produce any more reactivity than 1 hour at 37° C. Results obtained at 5° C. were of lower magnitude. The individual serum No. 3, selected at random from sera-giving negative serologic test for syphilis, gave no evidence of inhibiting antibody, as defined, under all conditions of this experiment.

TABLE II. THE EFFECT OF 0, 1, AND 2 HOURS OF PRELIMINARY INCUBATION OF HUMAN SERUM AND ANTIGEN AT 37° C. AND 5° C. ON THE REACTIVITY OF INHIBITING ANTIBODY

SERUM	TIME	TEMP.	INHIBITION TEST* SERUM DILUTIONS‡					ANTICOMPLEMENTARY TEST† SERUM DILUTIONS‡				
			4	8	16	32	64	4	8	16	32	64
B.A.	0	Room	85	45	10	0	0	60	80	85	90	90
3	0	Room	15	5	0	0	0	85	85	90	90	95
B.A.	1	37° C.	95	90	60	10	0	65	80	85	90	90
3	1	37° C.	45	10	0	0	0	80	85	90	90	90
B.A.	1	5° C.	95	90	40	5	0	65	80	80	90	90
3	1	5° C.	25	5	0	0	0	80	90	90	90	90
B.A.	2	37° C.	95	90	70	20	0	65	80	80	90	90
3	2	37° C.	40	10	0	0	0	80	90	90	90	90
B.A.	2	5° C.	95	90	40	10	0	70	80	90	90	90
3	2	5° C.	40	10	0	0	0	85	90	90	90	90

Results above expressed in degrees of hemolysis.

Time given in hours.

B.A. represents an inhibiting antibody pool.

3 represents a serum, negative in serologic tests for syphilis.

*Inhibition test performed with 0.2 microgram of antigen protein N., three units of C', and a dilution of immune rabbit serum which required six units of C' for 50 per cent hemolysis.

†Anticomplementary test performed with two units of C' (rabbit immune serum added).

‡Reciprocal of serum dilutions.

In other experiments it was shown that shaking up to 60 minutes at room temperature in a mechanical shaker did not augment the reactivity of inhibiting antibody. Furthermore, incubation at room temperature without shaking did not produce greater reactivity than at 37° C.

3. *The effect of antigen and immune serum concentration:* To determine the effect of antigen and immune serum concentration on the detection of inhibiting antibody, a preliminary exposure of 1 hour at 37° C. was used, since this time and temperature appeared optimal from previous experiments. A blocking antibody pool and a serum selected at random from sera negative serologic tests for syphilis were employed as test sera. Sera were heated at 56° C. for 4 hours. Serial twofold dilutions of the sera, beginning with 1-4, were tested against several dilutions of ragweed-whole-extract antigen. After preliminary exposure of antigen and serum, immune rabbit serum was added in two dilutions, and complement in four dilutions containing three, four, six, and eight units of C'. Test mixtures together with suitable controls were incubated at 5° C. for 4 hours. Sensitized cells were added and the degree of hemolysis was determined after 15 minutes' incubation at 37° C. water bath. Table III depicts the complement fixation pattern of the rabbit immune serum.

Table IV gives the results obtained in tests for inhibition. With the smallest amount of rabbit serum; i.e., 1-40, inhibitory action was observed with both a blocking pool and the normal serum used. With the 1-30 dilution of rabbit immune serum, specific results were obtained with three units of C'. Larger amounts of C' gave nonspecific findings with the normal serum. Using the 1-20 dilution of rabbit immune serum, specific results were obtained with three and four units C', whereas larger amounts of C' produced nonspecific findings. With respect to antigen concentration it was noted that, under the conditions where specificity was maintained, more sensitive findings were obtained with the smallest amount of antigen, i.e., 0.2 microgram of protein N.

TABLE III. THE TITRATION OF RABBIT IMMUNE SERUM POOL (IS)

IMMUNE SERUM DILUTION	SERUM CONTROLS C' UNITS		COMPLEMENT FIXATION RESULTS WITH MICROGRAMS OF ANTIGEN PROTEIN N.						
	1	2	C' UNITS	0.8	0.4	0.2	0.1	0.05	0.025
1-20	15	85	3	0	0	0	0	30	90
			4	0	0	0	5	80	100
			6	0	0	0	40	100	100
			8	0	0	0	60	100	100
1-30	25	90	3	20	0	0	0	60	95
			4	20	0	0	10	90	100
			6	40	0	0	50	100	100
			8	50	5	10	90	100	100
1-40	35	95	3	70	10	0	10	75	100
			4	75	20	10	20	100	100
			6	95	55	20	70	100	100
			8	95	85	65	95	100	100

Results above expressed in degrees of hemolysis.

TABLE IV. THE EFFECT OF ANTIGEN, RABBIT IMMUNE SERUM, AND COMPLEMENT CONCENTRATION ON THE DETECTION OF INHIBITING ANTIBODY

SERUM	IS DILUTION	INHIBITING ANTIBODY TITERS OBTAINED WITH							
		3 UNITS C'		4 UNITS C'		6 UNITS C'		8 UNITS C'	
		0.2	0.4	0.2	0.4	0.2	0.4	0.2	0.4
B.A.	1-20	8	4	8	8	8	4	16	8
Normal		0	0	0	0	4	0	8	4
B.A.	1-30	16	16	16	8	32	8	32	16
Normal		0	0	4	4	16	8	16	8
B.A.	1-40	32	32	32	>	>	>	>	>
Normal		8	16	16	>	32	>	>	>

Results above expressed in terms of the reciprocal of the highest serum dilution showing evidence of inhibition; 0 indicates no inhibition.

0.2 and 0.4 refer to the microgram of antigen protein N. used.

> represents reactions greater than 128.

4. *The effect of time and temperature of inactivation:* Sera were exposed to various temperatures for varying times in a water bath. Following this, tests for inhibiting antibody were made using a preliminary exposure of 1 hour at 37° C. for mixtures of test sera in the presence of 0.2 microgram of antigen protein N. Rabbit immune serum was added in a dilution of 1-30, plus three units of C', and the test was completed as previously described. The results are given in Table V. No tests were made in those instances where a serum aliquot showed coagulation at a particular exposure. For this reason

TABLE V. THE EFFECT OF TIME AND TEMPERATURE OF HEATING SERA ON THE DETECTION OF INHIBITING ANTIBODY

SERA	HEATING		RESULTS OF INHIBITION TEST	CLINICAL STATUS
	TIME	TEMP.		
CF	0.5	56	Pos. 1-2	Normal, nonsensitive
	1	56	Neg.	
	2	56	Neg.	
	4	56	Neg.	
	0.5	60	Neg.	
5023	0.5	56	Neg.	Ragweed-sensitive, untreated
	1	56	Neg.	
	2	56	Neg.	
	4	56	Neg.	
	0.5	60	Neg.	
	0.5	64	Neg.	
5026	0.5	56	Pos. 1-8	Ragweed-sensitive, ragweed-treated
	1	56	Pos. 1-16	
	2	56	Pos. 1-16	
	4	56	Pos. 1-16	
	0.5	60	Pos. 1-16	
	0.5	64	Pos. 1-8	

Inhibition test with 0.2 microgram of antigen protein N.; three units of C'.
Results expressed as highest serum dilution showing evidence of inhibition.
Time in hours; temperature in centigrade.

no tests were made on serum CF exposed to temperatures of 64°, 68°, and 72° C.; on sera 5,023 and 5,026 exposed to temperatures of 68° and 72° C.

It can be seen from Table V that serum CF, from a normal nonsensitive donor, gave only a minimum reaction, i.e., 1-2, with an aliquot heated at 56° C. for 30 minutes. Negative reactions were obtained at other times and temperatures indicated. Serum from a ragweed-sensitive donor (5,023) gave negative reactions throughout. Evidence of inhibiting antibody was obtained with serum 5,026, donated by a ragweed-sensitive, ragweed-treated individual, with all aliquots tested. Thus, for example, an aliquot heated at 56° C. for 30 minutes produced an endpoint of 1-8 serum dilution. This serum heated for 1, 2, and 4 hours at 56° C. gave identical endpoints of 1-16. An aliquot heated at 64° C. gave an endpoint of 1-8.

5. *The effect of inhibiting antibody and sensitizing antibody on the fixation of C' by rabbit antibody over a range of antigen concentration:* Experiments to show the effect of antigen and immune-serum concentration on the detection of inhibiting antibody have been previously described under item 3 of this series. Since a curve of reactivity for rabbit antibody in the presence of ragweed extracts had been established (see Fig. 1 of Part I) experiments were designed to show what effect human sera containing inhibiting antibody and sensitizing antibody (by passive transfer) might have on this relationship. Two lots of whole-extract antigen were studied. Antigen dilutions were prepared to contain 0.8, 0.4, 0.2, 0.1, 0.05, and 0.025 microgram of antigen protein N. in the test dose of 0.1 ml. The following human sera were studied: B.A., representing a pool of three donors, ragweed sensitive and ragweed treated; 5,034 representing a ragweed-sensitive, untreated patient, containing skin-sensitizing antibody by passive transfer. B.A. serum was heated at 56° C. for 4 hours, whereas aliquots of 5,034 were heated at 56° C. for ½ and 4 hours.

Tests were made using a preliminary exposure of 1 hour at 37° C. of the human sera and antigen dilutions. Then immune rabbit serum and three units of complement were added. Tests were incubated at 5° C. for 4 hours. Determinations of residual complement activity were made as previously described. The amounts of complement required for 50 per cent hemolysis were determined by reference tables provided in *Standard Methods*.⁵⁸ These values were plotted against the amounts of antigen added (Figs. 1 and 2). Since the results obtained with both lots of antigen were essentially the same, the findings with only one lot of antigen are given. With the serum (B.A.) containing blocking antibody by passive transfer, evidence of inhibition was obtained

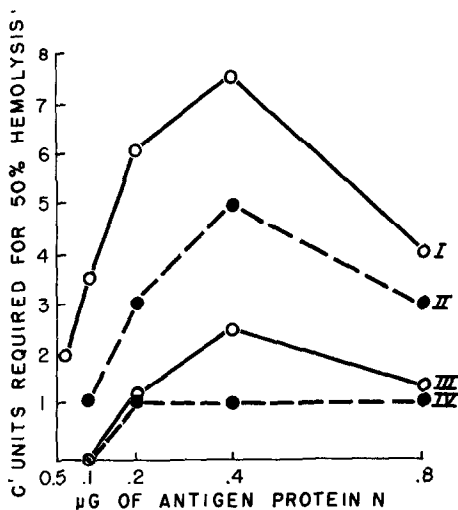


Fig. 1.

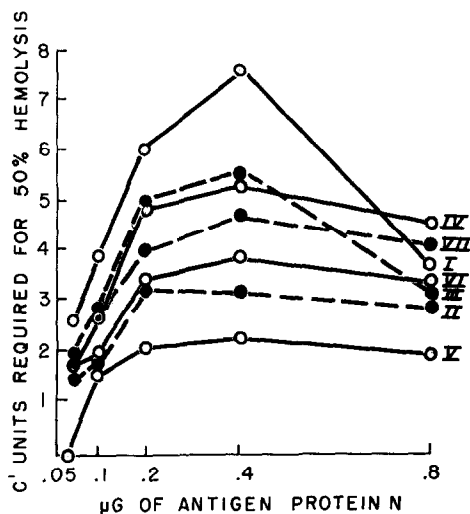


Fig. 2.

Fig. 1.—The effect of human serum containing blocking antibody by passive transfer on the course of the reaction of whole pollen extract and rabbit-immune serum. Curve I. Rabbit-immune serum, 1-30. Curve II. Blocking antibody serum heated 4 hours 56° C. (1-64 dilution). Curve III. Blocking antibody serum heated 4 hours 56° C. (1-16 dilution). Curve IV. Blocking antibody serum heated 4 hours 56° C. (1-4 dilution).

Fig. 2.—The effect of human serum containing sensitivity antibody by passive transfer on the course of the reaction of whole pollen extract and rabbit-immune serum. Curve I. Rabbit-immune serum 1-30. Curve II. Serum 5,034 heated ½ hour 56° C. (1-4 dilution). Curve III. Serum 5,034 heated ½ hour 56° C. (1-16 dilution). Curve IV. Serum 5,034 heated ½ hour 56° C. (1-64 dilution). Curve V. Serum 5,034 heated 4 hours 56° C. (1-4 dilution). Curve VI. Serum 5,034 heated 4 hours 56° C. (1-16 dilution). Curve VII. Serum 5,034 heated 4 hours 56° C. (1-64 dilution).

with the 1-4 and 1-16 dilutions according to the criterion of 50 per cent or greater hemolysis. With serum 5,034 containing sensitizing antibodies by passive transfer, the aliquot heated at 56° C. for ½ hour showed no inhibition according to the established criterion. However, a depressant action (though much weaker than that obtained for B.A. serum) on the fixation curve of the rabbit immune serum can be seen. The aliquot of 5,034 heated for 4 hours at 56° C. showed more of an inhibitory effect than the aliquot heated for only 30 minutes. By using the criterion for evidence of inhibiting antibody as previously described, inhibitory action was noted only with the 1-4 dilution of the aliquot of 5,034 heated for 4 hours.

6. *Comparative reactivity of inhibiting human serum against whole-ragweed-extract antigen and several special fractions of ragweed antigen:* During

the course of these investigations it was deemed of interest to study the behavior of inhibiting substances found in some ragweed-treated patients against whole-extract antigen in the presence of other ragweed fractions. Because of limited supplies of appropriate sera and special antigens, only one experiment was made. The conditions of the experiment were similar to those described under the preceding section 5. Since only Fractions "1," "1P," and "1S" gave satisfactory titers with the immune-rabbit-serum pool, studies were made with these only. All antigens were tested in a single predetermined optimal dose. Four individual sera were tested after heating at 60° C. for 1½ hour. CF represents a normal donor; 5,023, a ragweed-sensitive case; and 5,026, a ragweed-sensitive treated patient. The fourth serum studied was a normal rabbit pool. The results obtained are presented in Table VI. If the criterion of 50 per cent or greater hemolysis as evidence of inhibition is used, it can be seen that only serum 5,026 showed inhibitory action. The inhibition was observed with all four antigens. The serum titer with antigen "Lot 2G" was 1-16, with antigen "FR. 1" it was 1-32, with antigen "FR. 1P" it was 1-16, and with antigen "FR. 1S" it was 1-32.

7. *The effect of varying the incubation period following the addition of rabbit-immune serum on the detection of inhibiting antibody:* In early experiments it seemed desirable to use an overnight incubation period rather than the 4-hour period to facilitate the analyses for inhibiting antibody. Upon performing assays for inhibiting antibody with the longer period of incubation, it was found that inhibiting action could not be detected. A possible explanation for this failure in detecting antibody seemed to be a reversal phenomenon, whereby

TABLE VI. THE COMPARATIVE REACTIVITY OF INHIBITING HUMAN SERUM AGAINST WHOLE-RAGWEED-EXTRACT ANTIGEN AND SPECIAL FRACTIONS "1, 1P, AND 1S"

SERUM	ANTIGEN	TEST WITH THREE UNITS C' SERUM DILUTIONS*						INHIBITION TITER
		2	4	8	16	32	64	
CF	Whole Extr.	0	0	0	0	0	0	Neg.
	"Fract. 1"	15	5	0	0	0	0	Neg.
	"Fract. 1P"	0	0	0	0	0	0	Neg.
	"Fract. 1S"	5	0	0	0	0	0	Neg.
5023	Whole Extr.	20	20	10	0	0	0	Neg.
	"Fract. 1"	45	40	20	10	0	0	Neg.
	"Fract. 1P"	10	25	0	0	0	0	Neg.
	"Fract. 1S"	25	15	0	0	0	0	Neg.
5026	Whole Extr.	40	75	80	70	20	5	Pos. 1-16
	"Fract. 1"	35	70	80	80	60	20	Pos. 1-32
	"Fract. 1P"	40	70	75	70	30	0	Pos. 1-16
	"Fract. 1S"	30	60	75	70	55	5	Pos. 1-32
NRS	Whole Extr.	5	15	0	0	0	0	Neg.
	"Fract. 1"	35	15	10	0	0	0	Neg.
	"Fract. 1P"	5	5	0	0	0	0	Neg.
	"Fract. 1S"	15	5	0	0	0	0	Neg.

Results under test expressed in degrees of hemolysis.

Whole extract—dose used 0.2 microgram protein N.; immune serum dilution 1-40.

"Fraction 1"—dose used 0.2 microgram protein N.; immune serum dilution 1-20.

"Fraction 1P"—dose used 1.25 microgram protein N.; immune serum dilution 1-30.

"Fraction 1S"—dose used 1.25 microgram protein N.; immune serum dilution 1-30.

Inhibition titer expressed as highest serum dilution showing inhibition.

*Reciprocal of serum dilutions.

bound antigen was released from inhibiting antibody and then recombined with rabbit-immune serum. An experiment was designed to test this hypothesis, employing a serum containing inhibiting antibody by passive transfer, 5,026, and a normal serum, CF. After heating the sera at 60° C. for ½ hour, 1-8 dilutions of each were prepared. Aliquots of 0.1 ml. were incubated at 37° C. for 1 hour with 0.2 microgram protein N. of whole-extract antigen "2G." Following this preliminary exposure, five different dilutions of immune rabbit serum, and three 50 per cent C' units were added. Tests of the immune rabbit serum, antigen, and complement were likewise made in addition to the other customary controls. Determinations of the amount of complement fixed were made at 1, 2, 2¾, 3½, 6, and 20 hours. For each time interval, the dilution of rabbit antiserum requiring 6 C' units for 50 per cent hemolysis was estimated on the basis of the test findings with the dilutions actually tested. By using the criterion of 50 per cent or greater hemolysis as evidence of inhibitory activity, results were expressed, for the two human sera tested, as + indicating inhibition, or 0 indicating no inhibition. From Table VII it can be seen that the serum containing blocking antibody by passive transfer displayed inhibiting activity at every time interval when related to the titer to the rabbit-immune serum. As the incubation period was prolonged, i.e., at 6 and 20 hours, and the rabbit-immune serum fixed more complement, the blocking serum showed inhibitory activity in the presence of smaller amounts of rabbit-immune serum, but not with larger amounts of this serum. With the normal serum, at any given time interval, there was no evidence of inhibition when the results obtained were related to the immune-rabbit-serum reactivity. For example, after 1-hour incubation, the immune-rabbit-serum dilution requiring 6 C' units for 50 per cent hemolysis was 1-5. The human serum tested in the presence of 1-10 rabbit serum was not inhibitory. After 3½ hours, the immune-rabbit-serum dilutions requiring 6 C' units for 50 per cent hemolysis was 1-25. The normal human serum showed no inhibitory activity in the presence of 1-40 immune rabbit serum.

TABLE VII. THE EFFECT OF VARYING THE INCUBATION PERIOD FOLLOWING THE ADDITION OF RABBIT-IMMUNE SERUM ON THE DETECTION OF INHIBITING ANTIBODY

FIXATION PERIOD IN HOURS	HUMAN SERUM 5026					HUMAN SERUM CF					IMMUNE SERUM TITER
	IMMUNE SERUM DILUTIONS*					IMMUNE SERUM DILUTIONS*					
	10	20	40	80	160	10	20	40	80	160	
1	+	+	+	+	+	0	+	+	+	+	5
2	+	+	+	+	+	0	0	+	+	+	10
2¾	+	+	+	+	+	0	0	0	+	+	15
3½	+	+	+	+	+	0	0	0	+	+	25
6	0	+	+	+	+	0	0	0	+	+	40
20	0	0	0	+	+	0	0	0	0	+	80

*Reciprocal of serum dilutions.

+Evidence of inhibition.

0 No inhibition.

Immune serum titer, reciprocal of interpolated dilution requiring six C' units for 50 per cent hemolysis.

8. *The selection of a test method for the detection of inhibiting antibody:* Preliminary studies with a few selected cases known to contain blocking anti-

body by passive transfer indicated that evidence for inhibiting activity, presumably due to the presence of antibodies, could be detected in some instances by a complement fixation inhibition test. Moreover, noninhibitory activity with normal sera or ragweed-sensitive sera not containing blocking antibody by passive transfer suggested that the reaction might be specific under certain conditions. Attempts were made to increase the sensitivity of the test, in order to obtain a better correlation with the passive transfer results on these few selected cases. As the sensitivity was raised it appeared that the specificity decreased. To illustrate this effect a composite of several experiments is presented in Table VIII. The data indicated the marked improvement in specificity obtained by using an immune-rabbit-serum dilution of 1-20, which required approximately 9 units of C' for 50 per cent hemolysis. Smaller amounts of rabbit antiserum gave less specific findings.

TABLE VIII. THE EFFECT OF IMMUNE RABBIT SERUM CONCENTRATION ON THE SENSITIVITY AND SPECIFICITY OF THE COMPLEMENT FIXATION INHIBITION TEST

SERUM NO.	IN VIVO TEST	INHIBITION TEST USING IMMUNE SERUM DILUTIONS		
		1-40	1-30	1-20
5039	0	8	8	0
5040	+	32	16	0
5043	+	16	16	8
5052	+	32	32	8
5059	+	16	8	0
5076	+	32	32	8
5035	0	16	8	0
5070	0	4	4	0

Inhibition test results expressed as the reciprocal of the highest serum dilution showing inhibition; 0 indicates no inhibition.

In vivo test, passive transfer test for blocking antibody; + indicates a positive reaction, 0 indicates a negative reaction.

Another consideration was the anticomplementary property of the sera submitted for assay. Unsuccessful attempts were made to abolish this property by preliminary treatment of the sera with active complement, according to the method of Taran.⁶⁷ As a working method, the test dose of complement was increased from three to four complement units.

The method ultimately selected for use in the assay of sera for inhibiting antibody is now described.

1. Saline-Stock Veronal Buffer, diluted on the same day the tests were made.

2. Sheep Red Cells, 5 per cent suspension, prepared daily. The test dose was 0.1 ml.

3. Complement-hemolytic activity was determined as previously described. Two dilutions were then prepared containing one and four 50 per cent hemolytic units in a test in dose of 0.1 ml.

4. Hemolysin-optimal dilution was determined as previously described. A stock 1-100 dilution was prepared as previously noted and was used for as long as 1 month. The test dose was 0.1 ml.

5. Sensitized sheep red blood cells, equal volumes of 5 per cent cells and optimal hemolysin dilution were mixed by pouring hemolysin on cells, then

back and forth for a total of eight excursions. This mixture was not used before 10 minutes, nor longer than 2 hours after preparation.

6. Reading Standards, these were prepared daily as previously described. For simplification, 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 per cent standards were used. In making readings of hemolysis it was advisable to chill the tubes by placing the racks in an ice water bath, since hemolysis will continue at a slow rate at room temperature after removal from the 37° C. water bath.

7. Immune Rabbit Serum, a pool of serum prepared from rabbits immunized against whole-extract-pollen antigen was employed. Preliminary assay to determine suitability of each individual serum was made. Suitable aliquots of immune serum were stored at -20° C. in Pyrex test tubes stoppered with paraffin-coated corks. For use in tests, aliquots were thawed, thoroughly mixed, and heated at 56° C. for 30 minutes. The dilution of immune rabbit serum for use in inhibition test contained an amount of antibody which required eight to ten units of C' to produce 50 per cent hemolysis in the test dose of 0.1 ml.

8. Human Serum, sera received for assay were stored in Pyrex test tubes fitted with paraffin-coated corks. In most instances, because of the presence of visible particles, sera were Seitz-filtered prior to storage at -20° C. For use in test, aliquots were thawed, thoroughly mixed, and heated at 60° C. for 1½ hour. Serial twofold dilutions in Veronal buffer were made beginning with 1-4 and ending in 1-128. 0.5 ml. of each dilution was sufficient to perform assays. The test dose was 0.1 ml. of the dilutions.

9. Antigen, whole extract ragweed antigen preserved in an equal volume of glycerin was used. The smallest amount of antigen which fixed the greatest amount of complement (containing four units) in the presence of the smallest amount of rabbit-immune serum was employed in a test dose of 0.1 ml. This amount of antigen contained 0.2 microgram of protein N. No less than 0.1 ml. of stock glycerinized antigen was used in preparing the optimal dilution. Fresh dilutions were made daily. While not in use, the antigen dilution was kept refrigerated.

10. Test Procedure for Inhibiting Antibody, the test procedure followed is outlined in Table IX. The criterion for the presence of inhibiting antibody was the occurrence of 50 per cent or greater hemolysis in test mixtures containing the human serum, antigen, immune rabbit serum, and complement. The results obtained with a given serum were expressed as the highest dilution showing evidence of activity as defined.

Controls included in the assays were:

- a. Hemolytic controls with one and two 50 per cent hemolytic C' units.
- b. Antigen controls with one 50 per cent C' unit.
- c. Cell control.
- d. Human serum, known to contain inhibiting antibody by previous assay, was tested in a similar fashion as the unknown sera.
- e. Human serum, not containing inhibiting antibody, was similarly tested.
- f. Immune rabbit serum was tested at the dilution selected and actually used in tests in the presence of four and eight 50 per cent hemolytic units and the

TABLE IX. OUTLINE OF COMPLEMENT FIXATION INHIBITION TEST FOR INHIBITING ANTIBODY AGAINST RAGWEED POLLEN EXTRACT

ORDER OF ADDING REAGENTS	TEST DOSE ML.	TUBE NUMBER											
		1	2	3	4	5	6	7	8	9	10	11	12
Human serum dilutions	0.1	4	8	16	32	64	128	+	+	+	+	+	+
Antigen	0.1	+	+	+	+	+	+						
Veronal buffer	0.1							+	+	+	+	+	+
Incubate 1 hour 37° C. water bath													
Immune rabbit serum	0.1	+	+	+	+	+	+	+	+	+	+	+	+
Complement, 4 units	0.1	+	+	+	+	+	+						
Complement, 1 unit	0.1							+	+	+	+	+	+
Incubate 4 hours 3-6° C.													
Sensitized cells	0.2	+	+	+	+	+	+	+	+	+	+	+	+
Incubate 15 minutes 37° C. water bath Record degrees of hemolysis													

Human serum dilutions expressed as reciprocal.

+ indicates reagent added.

test dose of antigen employed in the assays on human sera. Also serum controls were set up with this dilution of rabbit antiserum in the presence of one and two 50 per cent C' units.

Tests were considered satisfactory when 40 to 60 per cent hemolysis was obtained with one unit of C' in the hemolytic and antigen controls; when inhibiting action was obtained with the known inhibiting serum, and no reaction with noninhibiting serum; when the number of units of C' required for 50 per cent hemolysis in the presence of the rabbit-immune serum ranged from 8 to 10.

B. Comparison of Results Obtained in the Assay of Inhibiting Antibody by the Complement Fixation Inhibition Test and the Passive Transfer Test for Blocking Antibody.—

Tests were made for inhibiting antibody using the complement fixation method as described. Blood specimens were obtained one to four weeks after the previous injection. Independent tests for blocking antibody by passive transfer were performed by a modification of the Loveless method.⁷ Sera to be tested were heated 4 hours at 56° C. to inactivate skin-sensitizing antibody. 0.1 ml. of the serum was mixed with an equal volume of several dilutions of low-ragweed extract and left overnight in the refrigerator. Then 0.1 ml. of an unheated serum containing sensitizing antibody was added. 0.1 ml. of these mixtures was injected into nonallergic skin. The sites were tested 48 hours later with approximately 0.02 ml. of low-ragweed extract containing 1,000 protein N. units per milliliter. The sensitizing sera were from untreated ragweed cases and chosen on the basis of neutralization by 50 to 100 units of ragweed extract. Thus if the serum being tested did not contain blocking antibody, the mixture made with 50 units gave a positive reaction, while that with 100 units did not. The titers recorded represent the strength of ragweed extract in the last site giving a positive reaction. The results obtained with the complement fixation and passive transfer methods are presented together with other pertinent data in Table X. With sera in Categories 1 and 2, representing patients not allergic to ragweed, no evidence of blocking antibody by passive

transfer was obtained. Likewise, negative results were obtained in the complement fixation inhibition test. Moreover, 50 sera selected at random from sera giving negative results in serologic tests for syphilis gave no complement fixation findings for inhibiting antibody. No passive transfer tests were made with these sera. Category 3 represents those ragweed-sensitive individuals who failed to complete treatment or to return for bleeding after treatment. Samples tested represent pretreatment bloods. The passive transfer method and complement fixation inhibition test were negative with all sera. Patients in Category 4, subgroup 1, ragweed sensitive and in their first year of treatment, showed evidence of blocking antibody by passive transfer on posttreatment samples. The titers of blocking antibody ranged from 100 to 200. Tests for inhibiting antibody using the complement fixation test were negative with these sera. Patients in Category 4, subgroup 2, ragweed sensitive and in their first year of treatment, showed evidence of blocking antibody by passive transfer on posttreatment samples. (No pretreatment specimens were taken.) In this group, the passive transfer method gave titers of 100 to 500. Evidence of inhibiting antibody in the complement fixation test was obtained with serum 5,077, which gave a titer of 500 in the passive transfer test. In Category 4, subgroup 3, the patients were treated for several years with ragweed extract. The highest titers of blocking antibody, i.e., 200 to 2,000, were obtained in this group. Likewise the highest incidence of inhibiting antibody action in the complement fixation test was obtained with this group. All sera giving titers of 500 or more in the passive transfer test were positive in the complement fixation test. Category 5 contained nonsensitive individuals who were injected with ragweed extract to simulate the formation of blocking antibody. Titers of blocking antibody by passive transfer ranged from 100 to 1,000. Evidence of inhibiting antibody in the complement fixation test was obtained with all samples showing 500 or more units in the passive transfer test.

C. Reproducibility of the Complement Fixation Inhibition Test for Inhibiting Antibody Against Ragweed Extract.—

To obtain information relative to the reproducibility of the complement fixation test for inhibiting antibody, tests were made on 46 individual sera on Nov. 1, 1952, and the test repeated using the same lot of antigen and immune-rabbit serum on March 10, 1953. The results obtained are presented in Table XI. Except for two sera, 5,046 and 5,058, the results were reproducible. Sera 5,046 and 5,048 were positive in the original testing, and negative on retesting. However, the degree of hemolysis obtained with both sera in the 1:4 dilution almost placed them in the positive category.

DISCUSSION

In studying the effect of various factors on the detection of antibody against ragweed antigen using the complement fixation inhibition test, the possibility that the observed inhibition might be due to the presence of thermostable complement components was ruled out by parallel and independent studies of the activating effect of the human sera on complement. However,

TABLE X. COMPARISON OF RESULTS OBTAINED IN THE ASSAY OF INHIBITING ANTIBODY BY THE COMPLEMENT FIXATION INHIBITION TEST AND THE PASSIVE TRANSFER TEST FOR BLOCKING ANTIBODY

Category 1—Control Sera							
A. PATIENTS NOT ALLERGIC TO RAGWEED AND NOT TREATED WITH ALLERGENS							
LAB. NO.	PAT.	SPEC. TAKEN	PASS. TRANS.	C-F INHIB.	CLINICAL DIAGNOSIS		
*5060	WC	8-25-52	—	—	Contact dermatitis		
5014	JL	3-16-51	—	—	Migraine		
*5003	RM	10-10-52	—	—	Ménière's disease		
*5084	JM	10-29-52	—	—	Infective asthma		
*5009	HM	10-26-50	—	—	Urticaria		
*5008	CW	10-26-50	—	—	Monilia dermatitis		
*5083	KM	9-13-52	—	—	Infective asthma		
5071	WC	6-17-52	—	—	No disease found		
*5080	JC	9-24-52	—	—	No disease found		
50 sera tested at random, negative in serologic tests for syphilis			Not done	—	Not known		
Category 2—Control Sera							
B. PATIENTS NOT ALLERGIC TO RAGWEED BUT ALLERGIC TO OTHER POLLENS; SERA TAKEN AFTER TREATMENT WITH OTHER POLLENS							
5068	PC	?	—	—	Grass pollens		
*5081	CO	12-9-52	—	—	Grass pollens		
*5082	KB	12-5-52	—	—	Grass and tree pollens		
Category 3—Ragweed Sensitive							
A. PATIENTS WHO FAILED TO COMPLETE TREATMENT OR TO RETURN AFTER TREATMENT; SAMPLES TESTED REPRESENT PRETREATMENT BLOODS							
5013	CA	3- 2-51	—	—			
5015	MO	12-16-51	—	—			
5035	MG	3-10-52	—	—			
5023	AS	9-10-51	—	—			
5036	JD	3-21-52	—	—			
*5066	GW	8-25-52	—	—			
*5011	RR	11- 6-50	—	—			
*5012	SW	11-29-50	—	—			
Category 4—Ragweed Sensitive							
B. PATIENTS TREATED WITH RAGWEED EXTRACT							
1. PATIENTS IN FIRST YEAR OF TREATMENT FROM WHOM BLOOD WAS OBTAINED BEFORE TREATMENT							
LAB. NO.	PAT.	SPEC. TAKEN	NO. OF INJECT.	TOP DOSE P.N. UNITS	PASS. TRANS.	C-F INHIB.	CLINICAL RESULT
5034	HM	3-14-52	0	0	—	—	Excellent
*5063		6-19-52	13	1200	100	—	
*5062		8-19-52	18	5500	200	—	
5032	MC	3- 4-52	0	0	—	—	Good-excellent
5049		6-18-52	15	2000	100	—	
5072		8-19-52	23	5000	200	—	
5027	MG	3- 6-52	0	0	—	—	Undetermined
5051		6-18-52	14	1500	100	—	
5070		8-20-52	23	5000	200	—	
5044	RL	5-20-52	0	0	—	—	Good
5073		8-12-52	12	2000	100	—	
5057	LD	4-19-52	0	0	—	—	Good
5045		6-18-52	9	800	—	—	
5069		8-19-52	17	5000	200	—	
5058	ES	4-25-52	0	0	—	—	Excellent
5047		6-18-52	8	600	—	—	
5074		8-22-52	16	5000	200	—	
5075		(duplicate specimen to 5074)				—	

TABLE X—CONT'D

2. PATIENTS IN FIRST YEAR OF TREATMENT FROM WHOM BLOOD WAS NOT OBTAINED BEFORE TREATMENT							
LAB. NO.	PAT.	SPEC. TAKEN	NO. OF INJECT.	TOP DOSE P.N. UNITS	PASS. TRANS.	C-F INHIB.	CLINICAL RESULT
*5064	HL	8-13-52	22	11000	100	—	Excellent
*5065	DR	8-22-52	23	6000	200	—	Good
*5077	FB	8-28-52	29	4500	500	Pos. 1:8	Good
*5078	EM	9-22-52	13	8500	100	—	Excellent
*5079	SM	9-13-52	14	2250	200	—	Fair

3. PATIENTS TREATED FOR SEVERAL YEARS; MOST HAD 25 OR MORE INJECTIONS THE FIRST YEAR AND 16 TO 20 IN SUBSEQUENT YEARS							
*5001	RL	10- 9-50		10000	200	—	Good
*5005	EC	10-12-50		6000	200	—	Good
*5006	SC	10-12-50			200	—	
5024	JS	10-13-51		35000	1000	Pos. 1:8	Excellent
5025	SR	9-19-51		50000	1000	Pos. 1:8	Excellent
5026	ER	6- 8-51		40000	1000	Pos. 1:16	Good
5067	ER	8-19-52		50000	2000	Pos. 1:32	Good
*5061	MW	8-12-52		17000	500	Pos. 1:8	Good

Category 5—Nonsensitive

PATIENTS GIVEN INJECTIONS OF RAGWEED TO STIMULATE THE PRODUCTION OF BLOCKING ANTIBODY

							DIAGNOSIS
5016	W	12-19-50	0	0	—	—	Vasomotor rhinitis
5017		1-15-51	5	15000	100	—	
5018		3-29-51	10	50000	200	—	
5019		5-15-51	15	50000	1000	Pos. 1:4	
5020	BL	1-15-51	0	0	—	—	Vasomotor rhinitis
5021		5-14-51	10	50000	200	—	
5029	P	10-26-51	0	0	—	—	Vasomotor rhinitis
5028		2- 1-52	12	50000	—	—	
5033		3-14-52	17	50000	100	—	
5048		5- 6-52	24	100000	500	Pos. 1:4	
5030	A	11-19-51	0	0	—	—	Infective asthma
5031		12-28-51	4	34000	—	—	Discontinued inject.
5037	MZ	10-10-51	0	0	—	—	Infective asthma
5038		3- 6-52	8	50000	100	—	
5046		5-28-52	12	50000	500	Pos. 1:4	
5053	RS	11-13-51	0	0	—	—	Infective asthma
5054		4-25-52	17	10000	100	—	
5050		6- 2-52	22	10000	100	—	
5055	RH	11-13-51	0	0	—	—	Infective asthma
5056		4-25-52	10	50000	100	—	
5039	T	10-29-51	0	0	—	—	Chronic sinusitis
5040		3-11-52	11	50000	100	—	
5059		4-18-52	16	70000	200	—	
5052†		6-23-52	25	100000	500	Pos. 1:8	
5043‡		6-26-52	25		500	Pos. 1:8	
5076§		8- 4-52	25		500	Pos. 1:8	

*Sera not Seitz-filtered.

†Blood sample collected 1 hour after the twenty-fifth injection.

‡Blood sample collected 3 days after the twenty-fifth injection.

§Blood sample collected 5 weeks after the twenty-fifth injection.

TABLE XI. REPRODUCIBILITY OF THE COMPLEMENT FIXATION INHIBITION TEST FOR HUMAN ANTIBODY AGAINST RAGWEED ANTIGEN

LAB. NO.	COMP. FIXATION RESULTS		LAB. NO.	COMP. FIXATION RESULTS	
	ORIGINAL	RETEST		ORIGINAL	RETEST
5021	Neg.	Neg.	5053	Neg.	Neg.
5024	Pos. 1:4	Pos. 1:8	5054	Neg.	Neg.
5025	Pos. 1:8	Pos. 1:8	5056	Neg.	Neg.
5027	Neg.	Neg.	5057	Neg.	Neg.
5029	Neg.	Neg.	5058	Neg.	Neg.
5030	Neg.	Neg.	5059	Neg.	Neg.
5031	Neg.	Neg.	5060	Neg.	Neg.
5033	Neg.	Neg.	5061	Pos. 1:8	Pos. 1:8
5034	Neg.	Neg.	5062	Neg.	Neg.
5035	Neg.	Neg.	5063	Neg.	Neg.
5037	Neg.	Neg.	5064	Neg.	Neg.
5038	Neg.	Neg.	5065	Neg.	Neg.
5039	Neg.	Neg.	5066	Neg.	Neg.
5040	Neg.	Neg.	5067	Pos. 1:32	Pos. 1:32
5043	Pos. 1:4	Pos. 1:8	5068	Neg.	Neg.
5044	Neg.	Neg.	5069	Neg.	Neg.
5045	Neg.	Neg.	5070	Neg.	Neg.
5046	Pos. 1:4	Neg.	5071	Neg.	Neg.
5047	Neg.	Neg.	5072	Neg.	Neg.
5048	Pos. 1:4	Neg.	5073	Neg.	Neg.
5049	Neg.	Neg.	5074	Neg.	Neg.
5050	Neg.	Neg.	5075	Neg.	Neg.
5052	Pos. 1:8	Pos. 1:8	5076	Pos. 1:4	Pos. 1:8

Original tests done on Nov. 1, 1952.

Retests done on March 10, 1953.

since the bulk of the experiments made were with aged sera, it is conceivable that tests with fresh sera may occasionally produce an inhibitory type reaction due to thermostable complement components. For this reason the parallel use of serum controls, in the presence of an amount of complement which will normally produce partial hemolysis, i.e., one 50 per cent hemolytic unit, would be desirable in performing assays for inhibiting antibody.

Studies on the effect of the time and temperature of preliminary incubation of antigen and human serum prior to the addition of the complement fixing rabbit-immune serum and complement (Tables I and II), indicated that a relatively short exposure of one hour was sufficient. Moreover, tests without preliminary incubation showed some evidence of inhibition but to a lesser degree than with preliminary incubation of antigen and human sera. No greater degree of inhibition was observed when complement was present during the preliminary incubation period. Slightly more sensitive findings were obtained with a preliminary incubation at 37° C. than at 5° C. for similar periods of time. It seemed conceivable that this greater sensitivity at 37° C. might have been due to nonspecific deterioration of the antigen at that temperature. It was noted, however, that as little as 0.002 ml. of serum prevented the deterioration of antigen at this temperature (unpublished findings). Since the smallest amount of serum tested for inhibiting action was approximately 0.007 ml. it would seem that the greater sensitivity at 37° C. was probably not due to deterioration of antigen. Conceivably, inhibiting antibody has a greater combining power for antigen at the higher temperature.

Previously, Hilleman and associates⁴⁹ studied inhibitory antibodies present in chickens immunized against ornithosis virus. They noted that the end points of reactivity were unchanged when the preliminary exposure of inhibiting serum and antigen was varied from 30 to 120 minutes. Moreover, it was noted⁴⁹ that the presence of complement during the preliminary incubation period of inhibiting antibody and antigen did not increase the reactivity. The findings obtained in the present study with respect to these factors were similar.

Previous investigations indicated that the order of mixing the antisera affected the results of inhibition test. Thus Goodner and Horsfall⁴⁷ studied antipneumococcal horse (noncomplement-fixing) and rabbit (complement-fixing) antisera in relation to the fixation of complement in the presence of homologous polysaccharide. They observed that the serum added last, prior to the addition of complement, especially if in excess, conferred its properties on the antigen-antibody complex. Rice⁴¹ obtained similar findings using the same system. However, she stressed the importance of the antigen dose, noting that if an excess of nonfixing horse antiserum was incubated with less than its neutralizing dose of antigen, prior to the addition of complement-fixing rabbit antiserum, almost complete inhibition occurred upon subsequent addition of rabbit antiserum and complement. When rabbit serum was added first under the same conditions, subsequent addition of horse serum and complement did not cause inhibition. When a small excess of horse antiserum was added after an interval to a mixture of rabbit antiserum, antigen, and complement, the added serum apparently checked further fixation by the rabbit antiserum but did not have a reversing effect on the fixation which had already occurred. Hilleman and associates⁴⁹ noted that when the fixing serum was allowed to react with antigen and complement, the subsequent addition of inhibiting children serum did not inhibit the fixation. In addition, if inhibiting serum, human serum, complement, and antigen were all mixed together, inhibition occurred but to a lesser degree. In the present studies it was also noted (Table II) that some inhibition occurred when ragweed-inhibiting human serum, complement-fixing rabbit serum, antigen, and complement were allowed to react without preliminary exposure of antigen to inhibiting serum. Whether the two antibodies are competing for the same antigen groupings cannot be determined in view of the lack of purity of the ragweed antigen and probable heterogeneity of the antibodies. Highly purified ragweed antigens and potent specific antisera, both rabbit and human, would provide the means for an approach to this problem.

The importance of the antigen dose to the degree of inhibition observed was noted by Calmette and Massol,⁴¹ Hilleman and associates,⁴⁹ Wolfe and associates,⁴⁸ Karrer and associates,⁵⁰ and Rice and Gwatkin.⁴¹ In most instances the selection of the antigen dose was empirically made to be compatible with a high sensitivity.^{44, 48-50} Rice⁴³ recommended the use of an antigen dilution which gave maximal reaction with a "standard serum" having a complement-fixing capacity of six to eight units on the basis of theoretical and experimental consideration.⁴¹

In the studies reported herein, the selection of the antigen dose at 0.2 microgram of protein N (whole extract) was made empirically. However, this dosage of antigen also represented the optimal dilution for obtaining maximal reactions with the smallest amount of rabbit-immune serum. From Table IV it was noted that the greatest degree of inhibiting activity occurred with 0.2 microgram of antigen protein N. When twice this amount of antigen, 0.4 microgram, was used the reactivity was slightly reduced. When larger amounts of antigen were used, as, for example 0.8 microgram (Figs. 1 and 2), the results were difficult to interpret. Since 0.8 microgram of antigen protein N. was in excess for the amount of rabbit-immune serum as seen by the reduced fixation of C', partial neutralization of antigen by inhibiting antibody should have produced a fixation of C' possibly obtained with 0.2 or 0.4 microgram of antigen. It is conceivable that the neutralization of antigen by inhibiting antibody may be subject to the effects of antigen excess as is the interaction of antigen with complement-fixing rabbit antibody.

The most important factor in maintaining the specificity of the inhibition reaction appeared to be the concentration of the complement-fixing rabbit antiserum. The importance of the dosage of fixing serum was shown by Hilleman and associates,⁴⁹ and a recommendation was made by Rice,⁴³ on the basis of theoretical and experimental considerations, that the fixing serum be employed in a dilution which fixes six to eight 50 per cent hemolytic units of C'. In the present work it was found that a dilution of rabbit antiserum which required eight to ten units of C' for 50 per cent hemolysis gave specific findings (Table VIII). Smaller amounts of rabbit antiserum produced non-specific findings. The inhibition occurring nonspecifically when smaller amounts of rabbit antiserum were employed may be more apparent than real. In performing comparative titrations of serum in tests for syphilis, it has been noted that higher titers are obtainable when serum is diluted in saline than when diluted in normal serum. Reduction in the amount of complement fixed by the rabbit-immune serum in the presence of human sera may be related to a similar effect especially when the concentration of rabbit serum used is low.

The importance of the dosage of fixing antiserum used in the detection of inhibiting antibody is further stressed by the experiment performed in these studies on the effect of varying the incubation period following the addition of rabbit-immune serum. Hilleman and associates⁴⁹ noted reduced inhibitory action for chicken antisera when the incubation period following the addition of human-fixing serum was prolonged, but did not offer any explanation for this phenomenon. From the studies reported here, the decrease in inhibiting powers of ragweed-inhibiting serum with increasing time of secondary incubation appeared to be related to the increasing fixation of rabbit antiserum with time (Table VII). Obviously, the selection of the optimal dose of fixing antiserum should be determined by the complement-fixing power of the serum for the period of time selected for the test. Decreased reactivity of inhibiting serum might be anticipated if, for any reason, the period of fixation was prolonged.

The decision to use a fixation period of 4 hours rather than 18 hours was based on the results given in Table VII. Although the shorter period of 4 hours is inadequate for maximum fixation of rabbit antiserum, the results obtained in tests for inhibiting antibody indicated that there was a greater difference in the behavior of serum containing inhibiting antibody from normal serum using the shorter (4-hour) incubation period.

Studies made on the effect of time and temperature of heating of the inhibiting antibody (Table V) reflected the thermostability of this antibody. Since no marked differences were obtained with an aliquot heated at 56° C. for ½ hour from an aliquot heated at this temperature for 4 hours, it did not seem that sensitizing antibody, likewise originally present in serum 5026, exerted any effect on the observed reactivity of the inhibiting antibody. (Sensitizing antibody is supposed to be destroyed by heating for 4 hours at 56° C.)⁷ The selection of 60° C. for ½ hour as the heating temperature and time for assay of inhibiting antibody was made since equivalent reactivity was obtained with aliquots heated at this temperature and at 56° C. for 4 hours, since Lovelless⁷ had shown that this time and temperature were equally as effective as 56° C. for 4 hours in assays of blocking antibody by the passive transfer test, and since the shortened time of exposure was more convenient for performing the tests.

In the study of the comparative reactivity of inhibiting human serum against different antigens, essentially similar degrees of reactivity were obtained with whole extract antigen, "Fraction 1," "Fraction 1P," and Fraction 1S." (Table VI.) Since rabbit sera of sufficient titer were not available to make tests for inhibiting antibodies against "Fractions 2 and 3," no conclusions can be drawn about the behavior of these antigens in tests for inhibiting antibodies using this method. It is pertinent to recall that Sherman and Stull⁵⁵ had noted that "Fraction 1" was allergenically more reactive than the other fractions in skin tests using sera from ragweed-sensitive cases.

In the method ultimately selected for use in the assay of inhibiting antibody, the decision was made to use four 50 per cent hemolytic units in the test because of the anticomplementary properties of the sera submitted for assay. It seemed possible that with less complement such anticomplementary action might mask an inhibitory effect. Whenever possible the use of fresh sera or sera stored at -20° C. soon after blood collection would be desirable. Moreover as can be seen from Table IV, the use of larger amounts of C', i.e., six and eight units produced nonspecific findings. Obviously, the amount of complement used should be considered in relation to the antibody content of the fixing-serum dilution used.

In this study, 46 individuals contributed 77 sera on which the passive transfer and indirect complement fixation inhibition tests were made. It was found that in all instances where a titer of 500 or higher was obtained with the passive transfer method, positive reactions were obtained with the complement fixation inhibition test. Seven sera gave titers of 500 with the passive transfer method. Of these, two gave 1-4 reactions in the complement fixation inhibition

test, while five gave 1-8 reactions. Four sera gave titers of 1,000 in the passive transfer test. Of these one gave a 1-4 reaction; two, a 1-8 reaction; and one, a 1-16 reaction in the complement fixation inhibition test. Only one serum gave a titer of 2,000 in the passive transfer test, and this gave the highest titer, 1-32, in the complement fixation inhibition test. These results suggest a correlation between the passive transfer test for blocking antibody and the complement fixation test for inhibiting antibody. It is possible that the complement fixation test is measuring the same antibody as is measured in the passive transfer test. The specificity of the complement fixation test is attested by the failure to obtain positive reactions in those instances where the passive transfer method gave negative findings. Moreover, 50 sera selected at random from specimens giving negative tests for syphilis likewise gave negative results in the complement fixation test for inhibiting antibody. The high specificity of the complement fixation inhibition test is undoubtedly correlated with the lower sensitivity of this test in comparison to the passive transfer method.

Another possible explanation for the observed inhibition with the sera of ragweed-treated cases was the presence of a nonspecific factor which interfered with the fixation of complement. In order to obtain additional proof for the specificity of the complement fixation inhibition test for ragweed antibodies, several sera, containing inhibiting antibodies for ragweed antigen, were tested for their effect on the fixation of complement by an unrelated antigen-antibody system. By using the identical test conditions described in this report for the assay of inhibiting antibodies against ragweed antigen, sera 5,025, 5,061, and 5,067, which contained inhibiting antibodies for ragweed antigen, were tested for their effect on the fixation of complement by a standardized, recrystallized egg albumin-antiegg albumin system. The sera tested produced no discernible effect on the fixation of complement by this unrelated system.

In considering the duration of the injection treatments to the development of blocking and inhibiting (cf) antibodies it can be seen from Table X, that the highest blocking antibody titers as well as the highest incidence of positive reactions in the complement fixation test were obtained in the ragweed-sensitive group treated over several years.

On the basis of the results obtained, no conclusions could be drawn as to the relationship of blocking or inhibiting (cf) antibodies to clinical benefits. This is a controversy that has not been settled by considerably larger series of cases. However, the complement fixation inhibition method may offer a convenient tool for future large-scale studies of the occurrence of this type of antibody as well as provide the allergist with an *in vitro* method for possibly measuring adequacy of desensitizing treatment. In view of the lower sensitivity of the complement fixation inhibition test and its correlation with the passive transfer method, the complement fixation test could be used as a screening procedure. Those sera, positive in the complement fixation inhibition test, would not have to be tested with the passive transfer technique. Moreover, blood samples collected from selected cases over periods of time could be

stored and tests made on all samples on a given testing day, eliminating many factors of variation inherent in the passive transfer test.

Malkiel and Feinberg⁶⁸ studied the production of blocking antibody in thirty-six normal individuals using eight different types of ragweed extracts and could not find any significant increase of blocking antibody. In the present study, eight nonsensitive individuals were injected with ragweed extract. Blocking antibody was produced in seven of the eight cases. The patient who did not develop demonstrable antibody received only four injections. Blocking antibody titers of 200 or more were obtained with five of the cases. In explanation of the discrepancy in the findings of Malkiel and those of the present study it should be noted that Malkiel injected dosages into the normal donors comparable to those given to the ragweed-sensitive cases in the present study. Five to ten times the amounts used in treating the ragweed-sensitive cases were used for injecting the normal donors in the present study. This stresses the importance of adequate dosage in producing blocking antibodies. Inhibiting antibody activity in the complement fixation test was obtained in four of the eight cases, in those cases where titers in the passive transfer method were 500 or greater. With patient T. (Table X, Category 5) it can be noted that there was no change in the titer by passive transfer or in the complement fixation reaction in samples obtained 1 hour, 3 days, and 5 weeks after the twenty-fifth injection. This study was included in order to determine whether the time of obtaining a blood sample after injection would influence the antibody assay. However, no conclusions can be drawn from results obtained with one individual.

SUMMARY AND CONCLUSIONS

1. A study of various factors influencing the determination of human antibodies against ragweed antigen was made using the complement fixation inhibition procedure.

2. As a result of these studies a complement fixation inhibition test was developed and is presented.

3. Comparative studies of the complement fixation inhibition test and the passive transfer method revealed that the former was less sensitive but was correlated with the passive transfer test. Sera giving a reaction of 500 units or more in the passive transfer test were positive with the complement fixation inhibition test.

4. It is suggested that the complement fixation inhibition test herein described may be of value as a screening procedure to determine the development of antibodies in ragweed-treated cases, and in other studies of the immunology of pollinosis. Further, this method may find application to the study of other allergens and their respective antibodies.

The authors acknowledge with gratitude the constant assistance and criticism of Dr. Beatrice C. Seegal under whose direction this study was performed, also the criticism of Drs. Elvin A. Kabat and Michael Heidelberger, and the friendly interest of Dr. Harry M. Rose, Executive Officer of the Department of Microbiology, Columbia University.

REFERENCES

1. Prausnitz, C., and Küstner, H.: Studien über die Überempfindlichkeit, *Centrabbl. f. Bakt. Orig.* **86**: 160, 1921.
2. de Besche, A.: Studies on the Reactions of Asthmatics and on Passive Transference of Hypersusceptibility, *Am. J. M. Sc.* **166**: 265, 1923.
3. Coca, A. F., and Grove, E. F.: Studies in Hypersensitiveness, XIII. A Study of the Atopic Reagin, *J. Immunol.* **10**: 445, 1925.
4. Cooke, R. A., and associates: Serologic Evidence of Immunity With Coexistent Sensitization in Type of Human Allergy (Hay Fever), *J. Exper. Med.* **62**: 733, 1935.
5. Harley, D.: Hay Fever, Effect of Pollen Therapy on Skin Reactions, Reaction Inhibiting Substance in Serum of Treated Patients, *J. Path. & Bact.* **44**: 589, 1937.
6. Langner, P. H., and Kern, R. A.: Studies on Immunology of Hay Fever, A. Confirmation of Blocking Substance Formation by Pollen Treatment. B. Use of Lyophile Serum in Study and Treatment, *J. ALLERGY* **10**: 1, 1938.
7. Loveless, M. H.: Immunological Studies of Pollinosis. I. The Presence of Two Antibodies Related to the Same Pollen Antigen in the Serum of Treated Hay Fever Patients, *J. Immunol.* **38**: 25, 1940.
8. Lichtenstein, M. R.: Attempt to Produce the Atopic Inhibitor Antibody in Non Atopic Individuals, *J. ALLERGY* **8**: 225, 1937.
9. Loveless, M. H.: Humoral Antibody and Tissue Tolerance Induced in Pollen Sensitive Individuals by Specific Therapy, *South. M. J.* **33**: 869, 1940.
10. Sherman, W. B., Stull, A., and Cooke, R. A.: Serologic Changes in Hay Fever Cases Treated Over a Period of Years, *J. ALLERGY* **11**: 225, 1940.
11. Scully, M. A., and Rackemann, F. M.: Studies of the Blocking Antibody of Cooke in the Treatment of Hay Fever, *J. ALLERGY* **12**: 549, 1941.
12. Sherman, W. B.: Changes in Serological Reactions and Tissue Sensitivity in Hay Fever Patients During the Early Months of Treatment, *J. Immunol.* **40**: 289, 1941.
13. Frank, D. E., and Gelfand, H. H.: Studies on the Blocking Antibody in Serum of Ragweed-Treated Patients, I. A Search for Inhibiting Substance in Other Types of Immune Serum, *J. ALLERGY* **14**: 273, 1943.
14. Loveless, M. H.: Immunological Studies of Pollinosis, IV. The Relationship Between Thermostable Antibody in Circulation and Clinical Immunity, *J. Immunol.* **47**: 165, 1943.
15. Brown, E. A., and Holden, E. M.: The Presence of Thermostable Inhibitory Factor in the Sera of Patients Treated for Hay Fever by Injections of Pollen Extract, *Ann. Allergy* **2**: 207, 1944.
16. Gelfand, H. H., and Frank, D. E.: Studies on the Blocking Antibody in Serum of Ragweed-treated Patients, II. Its Relation to Clinical Results, *J. ALLERGY* **15**: 332, 1944.
17. Alexander, H. L., Johnson, M. C., and Alexander, J. H.: Measurement of Circulating Ragweed Antibodies and Antigen, *J. ALLERGY* **17**: 340, 1946.
18. Munsell, K.: Direct Test for Blocking Antibody in Treated Hay Fever, *Lancet* **2**: 199, 1946.
19. Cohen, M. B., and Weller, R. R.: Precipitins in the Serum of Patients With Clinical Allergy, *J. ALLERGY* **12**: 242, 1941.
20. Swineford, O., Jr., and Houlihan, R.: Antibodies to Ragweed Extract, Demonstration by Colloidion Particle Technique in Immune Rabbit Serum But Not in Serum of Treated Patients, *J. ALLERGY* **18**: 190, 1947.
21. Cavelti, P.: Complement Fixation Studies in Allergy, *J. ALLERGY* **21**: 532, 1950.
22. Hampton, S. F., Johnson, M. C., Alexander, J. L., and Wilson, K. S.: Detection of Thermostable Antibody by Means of Precipitin Reaction, *J. ALLERGY* **14**: 227, 1943.
23. Johnson, M. C., Alexander, H. L., Robinson, R., and Alexander, J. H.: A Quantitative Method for Measurement of Precipitin Reactions, *J. ALLERGY* **15**: 83, 1944.
24. Alexander, H. L., Johnson, M. C., and Bukantz, S. C.: Studies on Correlation Between Symptoms of Ragweed Hay Fever and Titer of Thermostable Antibody, *J. ALLERGY* **19**: 1, 1948.
25. Bukantz, S. C., Johnson, M. C., and Hampton, S.: Quantitative Immunologic Studies With Allergen. I. Colorimetric Absolute Methods in the Analysis of Factors Influencing Precipitation of Ragweed Pollen Extracts and Homologous Rabbit Antisera, *J. ALLERGY* **20**: 1, 1949.
26. Heidelberger, M., and Macpherson, C. F. C.: Quantitative Micro-Estimation of Antibodies in Sera of Man and Other Animals, *Science* **97**: 405, 1943, correction **98**: 63, 1943.
27. Albus, B.: Beeinflussung der Antikörperkonzentration durch Spezifische Desensibilisierung bei Pollenallergie, *Ztschr. f. d. ges. exper. Med.* **95**: 703, 1935.
28. Albus, G.: Serologische Allergenbestimmung bei Pollenkrankheit unter Berücksichtigung des Antikörpertiters, *Ztschr. f. d. ges. exper. Med.* **96**: 710, 1935.

29. Albus, B.: Ueber die Wirksamkeit von Mischextrakten bei Behandlung der Pollenallergie, *Ztschr. f. d. ges. exper. Med.* **97**: 756, 1936.
30. Jaffé, K.: Nachweis Spezifischer Antikörper in vitro bei Hochgradiger Fisch und Hefeallergie, *Klin. Wchnschr.* **10**: 304, 1931.
31. Hensel, M. E., and Sheldon, J. M.: Complement Fixation With Specific Allergens in Hay Fever, *J. Lab. & Clin. Med.* **25**: 1586, 1941.
32. Mayer, M. M., Osler, H. J., Bier, O. G., and Heidelberger, M.: Quantitative Studies on Complement Fixation, I. A Method, *J. Immunol.* **59**: 195, 1948.
33. Follensby, E. H.: The Complement Fixing Properties of Sensitizing and Neutralizing Antibodies Present in the Blood of Allergic Human Subjects, Dissertation, Bost. U. Grad. School, 1951.
34. Squier, T. L., and Lee, H. J.: Lysis in Vitro of Sensitized Leukocytes by Ragweed Antigen, *J. ALLERGY* **18**: 156, 1947.
35. Franklin, W., and Lowell, F. C.: Failure of Ragweed Pollen Extract to Destroy White Cells From Ragweed-Sensitive Patients, *J. ALLERGY* **20**: 375, 1949.
36. Bernard, L. J., Jefferson, W. C., and Hahn, P. F.: Failure to Demonstrate in Vitro Lysis of Sensitized Guinea Pig Leucocytes by Dog Hemoglobin Antigen, *Proc. Soc. Exper. Biol. & Med.* **80**: 58, 1952.
37. Spain, W. C., Strauss, M. C., and Neumann, E.: In Vitro Release of Histamine by Hypersensitive (Allergic) Serum in Contrast to Immune (Treated) Allergic Serum in Antigen and Normal Rabbit Blood Mixtures, *J. ALLERGY* **21**: 318, 1950.
38. Spain, W. C., and Strauss, M. B.: The Use of Oleic Acid to Inhibit the Release of Histamine in Vitro, *J. ALLERGY* **22**: 47, 1951.
39. Rice, C. E.: Atypical Behaviour of Certain Avian Antisera in Complement Fixation Tests, *Canad. J. Comp. Med.* **11**: 236, 1947.
40. Rice, C. E.: Studies in Pullorum Disease, XXII. Technique of the Indirect Complement-Fixation Test for Activity With Salmonella Pullorum Antigens, *Canad. J. Comp. Med.* **12**: 69, 1948.
41. Rice, C. E., and Gwatkin, R.: Studies in Pullorum Disease, XXIV. A Comparison of Titers Obtained by Indirect Complement Fixation and Agglutination Methods for Chicken Sera, *Canad. J. Comp. Med.* **13**: 165, 1949.
42. Rice, C. E.: Inhibitory Effects of Certain Avian and Mammalian Antisera in Specific Complement Fixation Systems, *J. Immunol.* **59**: 365, 1948.
43. Rice, C. E.: Some Factors Influencing Selection of Complement-Fixation Method, II. Parallel Use of Direct and Indirect Techniques, *J. Immunol.* **60**: 11, 1948.
44. Calmette, A., and Massol, L.: Sur Une Nouvelle Reaction Masquant Dans Les Serums La Presence des Anticorps Tuberculeux, *Compt. rend. Soc. de biol.* **68**: 224, 1910.
45. Zinsser, H., and Parker, J.: Observations on a Substance in Immune Horse Serum Which Interferes With Alexin Fixation, *J. Immunol.* **8**: 151, 1923.
46. Pittman, M., and Goodner, K.: Complement-Fixation With Type Specific Carbohydrate of Hemophilus Influenzal-Type b, *J. Immunol.* **29**: 239, 1935.
47. Goodner, K., and Horsfall, F.: Complement-Fixation Reaction With Pneumococcus Capsular Polysaccharide, *J. Exper. Med.* **64**: 201, 1936.
48. Wolfe, D. M., Kornfeld, L., and Markham, F. S.: Simplified Indirect Complement-Fixation Test Applied to Newcastle Disease Immune Avian Serum, *Proc. Soc. Exper. Biol. & Med.* **70**: 490, 1949.
49. Hilleman, M. R., Haig, D. A., and Helmold, R. J.: The Indirect Complement Fixation, Hemagglutination and Conglutinating Complement Absorption Tests for Viruses of the Psittacosis-Lymphogranuloma Venereum Group, *J. Immunol.* **66**: 115, 1951.
50. Karrer, H., Meyer, K. F., and Eddie, B.: Complement Fixation Test and Its Application to Diagnosis of Ornithosis in Chickens and Ducks, I. Principles and Technique of Test, *J. Infect. Dis.* **87**: 13, 1950.
51. Karrer, H., Meyer, K. F., and Eddie, B.: Complement Fixation Inhibition Test and Its Application to Diagnosis of Ornithosis in Chickens and Ducks: Confirmation of Specificity and Epidemiological Application of Test, *J. Infect. Dis.* **87**: 24, 1950.
52. Schmidt, M., Harding, H. B., Hepler, O. E., and Murmann, E.: Inhibition of Complement Fixation by Certain Serums When Tested Against Psittacosis and Lymphogranuloma Venereum Antigens, *J. Infect. Dis.* **91**: 173, 1952.
53. Stull, A., Sherman, W. B., and Hampton, S. F.: Antigenic Fractions in Ragweed Pollen, I. Water Soluble Fractions, *J. ALLERGY* **12**: 117, 1941.
54. Stull, A., Sherman, W. B., and Wing, W. M.: Antigenic Fractions in Ragweed Pollen, II. Further Purification of Water Soluble Fractions and Study of Alkali-Soluble Fractions and Estimation of These Fractions in Pollen Extract, *J. ALLERGY* **13**: 537, 1942.
55. Sherman, W. B., and Stull, A.: Antigenic Fractions in Ragweed Pollen, III. Further Evidence of Immunologic Specificity of Fraction 1 and Fraction 2, *J. ALLERGY* **18**: 287, 1947.

56. Cooke, R. A., and Stull, A.: The Preparation and Standardization of Pollen Extracts for Treatment of Hay Fever, *J. ALLERGY* **4**: 87, 1932.
57. Hampton, S. F., Bukantz, S. C., and Johnson, M. C.: Quantitative Immunologic Studies With Allergen. II. Deterioration of Ragweed Pollen Extracts as Measured by Precipitation, Neutralization, and Protein Nitrogen Analyses. The Prevention of Deterioration by Glycerin, *J. ALLERGY* **20**: 20, 1949.
58. Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, Baltimore, 1947, Williams and Wilkins Company.
59. Cardiolipin Antigens, Division of Laboratories and Research, N. Y. State Department of Health, United Nations, WHO, Feb. 5, 1951.
60. Mayer, M. M., Croft, C. C., and Gray, M. M.: Kinetic Studies on Immune Hemolysis, I. A Method, *J. Exper. Med.* **88**: 427, 1948.
61. Portnoy, J., Bossak, H. N., and Harris, A.: Preservation of Sheep Red Cells for Complement Fixation Test, I. An Improved Method, *J. Ven. Dis. Inform.* **28**: 137, 1947.
62. Clock, R. O.: A Stable Pollen Antigen, *J. Infect. Dis.* **21**: 387, 1917.
63. Armstrong, C., and Harrison, W. T.: The Standardization of Pollen Extracts by the Complement Fixation Test, *U. S. Pub. Health, Rep.* **40**: 1466, 1925.
64. Osler, A. G., Mayer, M. M., and Heidelberger, M.: Quantitative Studies of Complement Fixation. II. Fixation of Complement in the Reaction Between Type III Pneumococcus Specific Polysaccharide and Homologous Antibody, *J. Immunol.* **60**: 205, 1948.
65. Osler, A. G., and Heidelberger, M.: Quantitative Studies of Complement Fixation IV. Homologous and Cross-Reaction in Chicken and Duck Egg Albumin Systems, *J. Immunol.* **60**: 327, 1948.
66. Wallace, A. L., Osler, A. G., and Mayer, M. M.: Quantitative Studies of Complement-Fixation, V. Estimation of Complement-Fixing Potency of Immune Sera and Relation to Antibody-Nitrogen Content, *J. Immunol.* **65**: 661, 1950.
67. Taran, A.: A Simple Method for Performing a Wassermann Test on Anticomplementary Serum, *J. Lab. & Clin. Med.* **31**: 1037, 1946.
68. Malkiel, S., and Feinberg, S. M.: Neutralizing Antibody in Nonallergic Individuals, *J. ALLERGY* **23**: 504, 1952.