THE ANTIBODY MECHANISMS OF RAGWEED ALLERGY. ELECTROPHORETIC AND CHEMICAL STUDIES

I. The Blocking Antibody

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Certain specific inhibiting properties of serums from patients treated with ragweed extract were first reported in 1935 by Cooke, Barnard, Hebald, and Stull (1). They interpreted their results as "showing the development under treatment of a peculiar blocking or inhibiting type of immune body that prevented the action of allergen on the sensitizing antibody." Adequate confirmation of these findings has been forthcoming.

Subsequently, Stull, Sherman, and Cooke (2) separated aqueous serum dilutions by half-saturation with ammonium sulfate into "albumin" and "globulin" and further fractionated the latter by dialysis against distilled water into water-insoluble "euglobulin" and water-soluble "pseudoglobulin."[1] From passive transfer tests with these fractions they concluded that both sensitizing and blocking capacities were connected with "pseudoglobulin." No confusion should result from the fact that in the present work the terms "euglobulin" and "pseudoglobulin" are applied with the different meaning of "salting-out" properties essentially in accordance with Kendall's usage (3).

Recently we have continued further studies to relate, if possible, both blocking and sensitizing antibody to electrophoretically defineable serum components. It was thought that such a correlation as reported in numerous publications for some precipitating and antitoxic antibodies (4–11) might also obtain for this artificially induceable "blocking" antibody.

The studies here recorded indicate to us that blocking antibody is connected in large part, if not entirely, with the gamma globulin. In the three serums studied it could not be attributed definitely to any of the other electrophoretic components but was found predominantly in the gamma globulin. This is quite different from the results of our preliminary report on the sensitizing antibody (12), a study of which has been continued and will be amplified in a later paper.

[1] Different meanings are applied by various investigators to the terms "euglobulin" and "pseudoglobulin"; a precise definition should always be given, as was done in reference 2.

Materials and Methods

Low ragweed extract was prepared in the usual manner (13) and standardized on the basis of protein nitrogen units (14, 15); accurately measured aliquots were dried from the frozen state so that active solutions could be prepared shortly before use.

Two non-allergic individuals (Jhn. and Lbk.) and one ragweed-sensitive individual (Rbr.) were injected with low ragweed extract as previously described (16). For the experimental immunization of the ragweed-allergic patient (Rbr.) a total of 423,000 protein nitrogen units of low ragweed extract was injected over a period of 17 months; the non-allergic individuals received 1,045,000 protein nitrogen units (Jhn.) in 7 weeks and 2,335,000 protein nitrogen units (Lbk.) in 6 weeks respectively. No skin-sensitizing antibody could be demonstrated by direct or indirect test in either of the non-allergics as a result of these ragweed extract injections.

Before starting immunization about 100 ml. of blood was drawn and the serum collected; after completion of the course of injections, when by preliminary tests satisfactory formation of blocking antibody had been established, about 500 ml. was drawn and the serum collected. These serums will be referred to as *ante* and *post* (treatment) serums respectively. For a uniform source of sensitizing antibody the serum of a hitherto untreated ragweed-sensitive individual (Wnr.) was used throughout. All blood samples were drawn when the donor was in the "fasting state." Quantities of serum sufficient for the anticipated skin tests were found sterile after filtration through Seitz filters, and were kept in cold storage. All serums were negative to Kline, Wassermann, and Mazzini tests.

Total protein was determined by biuret tests (17); refractometric protein evaluations on isolated fractions were performed with a Zeiss precision refractometer whose heatable prism was temperature-controlled at 20.00 ± 0.02 degrees C.; the concentrations of isolated fractions were estimated by determining the refractive index difference between solution and solvent and applying the factor 0.00188 as refractive index increment.

All electrophoretic *analyses* were performed by use of Longsworth's barbiturate buffer (18) in standard analytical cells of the Aminco-Stern apparatus (19); in a few instances of shortage of material a semimicro cell was used. Peaks were identified by their location and/or their mobilities. Serum components were thus characterized on the basis of their resolution and their mobilities in Longsworth's buffer, the observed mobility values being related to those known from the literature (20–23). The data on percentage distributions were obtained by averaging the planimetric results (evaluated by the procedure of Tiselius and Kabat (5)) from three ascending and three descending patterns photographed after 1¾, 2, and 2¼ hours' electrophoresis at a voltage gradient of about 6 volts per cm.

All electrophoretic *macro separations* were performed in the macro cell of the Aminco-Stern apparatus by use of potassium phosphate buffer pH 7.8, ionic strength 0.2, the voltage gradient being kept at not below 0.3 and not above 0.6 volts per cm.

The *convection electrophoresis* experiment on one of the serum globulins (Jhn.) was performed by Dr. Plescia[2] in his apparatus with total cell capacity of 200 ml.; disodium monopotassium phosphate buffer of pH 6.5 and ionic strength 0.1 was used and a current density of 1.0 ampere at a voltage gradient of 2.3 volts per cm. was applied for 9 hours, when the contents of the top and bottom compartments were collected separately. The convection electrophoresis experiments on Rbr. serum were performed by us in a similar apparatus, designed by Dr. Plescia, using disodium monopotassium phosphate buffer pH 7.6, ionic strength 0.075 throughout, at a current density of 1.0 ampere and a voltage gradient of about 3.8 volts per cm. Six consecutive runs of about 21 hours were carried out. After each run the contents of the top compartment[3] were removed and the removed volume replaced

[2] Dr. Otto Plescia, Presbyterian Hospital, New York.

[3] These fractions were combined and concentrated by negative pressure dialysis.

by mixing the remainder of the cell with buffer. Both convection electrophoresis experiments were done under refrigerated conditions at about 7°C.

Mallinckrodt's analytical grade of ammonium sulfate was used for the salt fractionations. Empirical mixtures of saturated aqueous and approximately one-half normal ammoniacal solutions of this salt were prepared in such a fashion that one part of the mixture diluted with two parts of distilled water exhibited at room temperature a pH of 8.2 ± 0.05. Such mixtures were used for all precipitations, the ammonium sulfate solution being added slowly to the protein solution in an ice bath. The resulting precipitates were collected by centrifugation in the cold and washed with a chilled saline-saturated ammonium sulfate mixture of the same strength as that used for the precipitations; all supernatant fluids occurring at a certain ammonium sulfate saturation were combined before the subsequent step of fractionation or dialysis was entered upon. By adjusting the ammonium sulfate saturation in turn to 33.3, 40, and 55 per cent, "euglobulin," "intermediate globulin," and "pseudoglobulin" respectively were collected in this manner; the protein remaining soluble at 55 per cent ammonium sulfate saturation was the "albumin" fraction. All these protein fractions were freed from ammonium sulfate by dialysis against distilled water. The water-insoluble portions separating during dialysis from the three globulin fractions were collected by centrifugation, washed with distilled water, and recentrifuged. The aqueous supernatant fluids being combined with their respective original supernates. All chemical fractions thus obtained were finally brought to dryness from the frozen state.

All dialyses were performed in the cold at about 7°C. by use of Visking casings. Toluene was added as preservative. The negative pressure technique of dialysis (24) was used for bringing fraction solutions, if necessary, to experimentally suitable concentrations.

Testing Procedures

The blocking effect of an isolated fraction cannot be expressed in absolute values, but may be expressed in terms of the blocking effect of the *post* serum from which it was isolated. This is accomplished by determining the blocking effect of a specified amount of the *post* serum and a specified amount of the fraction, by means of passive transfer titration tests carried out on the same test subject at the same time. The blocking effect of the fraction and of the *post* serum are therefore relatable, since the blocking values of each are obtained in terms of ragweed unit concentrations. As, however, the endpoints of such titrations are conditioned not only by the effect of the blocking antibody but also by the neutralization requirements of the sensitizing serum, which must be incorporated into all test solutions, the titer values that are attributable to neutralization of sensitizing antibody have to be deducted from the test values that are obtained on the blocking antibody–containing mixtures.

Three sets of titrations must therefore be performed to establish the blocking effect of an isolated fraction. First, a set of titrations to ascertain the neutralization requirements of the antibody in the sensitizing serum; the mixture containing *ante* serum, sensitizing serum (in these experiments, Wnr.), and ragweed extract serve this purpose, because they are free from blocking antibody and their titers are conditioned only by their content of sensitizing antibody. Second, a set of titrations with *post* serum instead of *ante* serum together with the same sensitizing serum and ragweed extract; then a third set in which the fraction replaces the *post* serum in the mixture of sensitizing antibody and ragweed extract.

In tests involving fractions derived from serums that contain both blocking and sensitizing antibody, the *ante* serum, the *post* serum, and the fraction solutions have to be heated at 56°C. for at least 4 hours prior to the preparation of the reaction

mixture in order to destroy the sensitizing antibody present in them. This was necessary for Rbr. serums and fractions but was not necessary for the Jhn. and Lbk. serums and their fractions, as these did not contain sensitizing antibody. Preliminary passive transfer tests must always be done to prove loss of sensitizing capacity.

Great care and accuracy must be exercised in preparing the mixtures, hence serological pipettes graduated to 0.01 or 0.001 ml. depending on the volumes must be used. Each mixture contains one (volume) part of sensitizing (Wnr.) serum, two parts of ragweed solution, the concentrations of which are increased in each series from mixture to mixture in suitably graduated increments (see Table I), and one part of either *ante* serum (first set), or *post* serum (second set), or fraction solution of known concentration (third set). The mixtures are incubated at 7°C. usually overnight but never more than 1½ days. One-tenth ml. of each mixture, accurately measured, is injected intradermally into each site in the back of a normal test subject. Twenty-four to 48 hours later each site is tested by injection of 0.025 ml. of freshly dissolved low ragweed extract containing 1000 PN units per ml.; after 20 minutes the reactions are read by at least two, but usually three observers and recorded as negative, plus minus, and one to four plus depending on the size of the reaction. As the intensities of the skin reactions decrease with increasing ragweed concentrations in the mixtures, the titers can be established for each set in terms of the ragweed concentrations at which ragweed was incorporated into the mixtures. As endpoints the intermediate values between the last definite positive reaction and the next following negative reaction is taken, or else a plus-minus reaction if occurring.

The difference between the endpoint of the second set and that of the first set determines the blocking action of the *post* serum in terms of ragweed units. The difference between the endpoint of the third set and that of the first set indicates the blocking action of the fraction, likewise expressed in terms of ragweed units. The blocking action of the *post* serum is considered as 100 per cent or totality of blocking effect. That of the fraction is then expressed in relation to that of the *post* serum.

As a matter of simplification in calculation all values are related to a standard volume of 0.40 ml., in which 0.10 ml. is the sensitizing serum, 0.20 ml. the ragweed solution (in varying concentrations) and 0.10 ml. either the *ante* serum, or the *post* serum, or the fraction, as indicated.

A typical example is shown in Table I, in which we evaluate the blocking effect of the descending macro separation fraction obtained from Rbr. *post* serum, recorded in Table II, line 23. This fraction represents electrophoretically pure gamma globulin as verified in Fig. 1.

The standard mixtures prepared with this fraction contained 0.10 ml. of a 1.20 per cent fraction solution; thus the blocking effect of 1.20 mg. of the fraction could be compared with the effect of 0.10 ml. of the *post* serum, which also contained 1.20 mg. of gamma globulin. The reactions of the sites at the various ragweed levels in the three sets are recorded in Table I, column C. The endpoint of the set containing *ante* serum is found at 75, that of the *post* serum-containing set at 800, and that of the fraction at 600 ragweed units (Table I, column D, sites 3, 8, and 13) respectively. By substracting the endpoint value of the *ante* serum set from the endpoint value of the *post* serum set the *post* serum's blocking effect is obtained in terms of ragweed units as 800 minus 75 equals 725 (column E, site 8); the blocking effect of the fraction

is determined as 600 minus 75 equals 525 (column E, site 13). From these data the blocking capacity as exerted by 1.20 mg. of the fraction can readily be calculated in terms, *i.e.* in per cent, of the *post* serum's total blocking effect; namely as $\frac{525 \times 100}{725}$

TABLE I

*Method and Procedure of Evaluating Blocking Antibody**

Skin site on test subject	Mixtures of serum or fraction with ragweed‡		Reaction when tested 48 hours later	Endpoint reactivity in ragweed units	Blocking effect of *post* serum or fraction in ragweed units	Blocking capacity of 0.10 ml. *post* serum or 0.10 ml. (1.20 mg.) fraction	Blocking power§ 1.00 mg. of fraction
	Serum or fraction combinations	Ragweed PN					
Column...	A	B	C	D	E	F	G
		units per ml.				*per cent*	*per cent*
1	Rbr. ante serum‖ + Wnr. serum	25	++				
2	" " " + " "	50	+	75			
3	" " " + " "	100	0				
4	" " " + " "	Saline	++++				
5	Rbr. post serum‖ + Wnr. serum	300	+++				
6	" " " + " "	400	++				
7	" " " + " "	600	++				
8	" " " + " "	800	±	800	800–75 = 725	100	
9	" " " + " "	Saline	++++				
10	Fraction¶ + Wnr. serum	100	+++				
11	" + " "	300	++				
12	" + " "	400	+				
13	" + " "	600	±	600	600–75 = 525	72.5	60.4
14	" + " "	800	0				
15	" + " "	Saline	++++				

* This table gives the results of one of five similar experiments, in which the blocking effect of the fraction obtained as descending macro separation from Rbr. *post* serum May 12, 1953 (recorded on Flow sheet 2 and in Table II, line 23) is compared with that of the Rbr. *post* serum, using Rbr. *ante* serum as neutralization control. The reaction mixtures were prepared as described in the text. Each 0.40 ml. of the *post* serum mixture contained 1.20 mg. gamma globulin (of the post serum); the fraction mixtures contained the same amount of gamma globulin.

‡ Serums and fraction were proven devoid of sensitizing capacity by passive transfer test before use.

§ See text for explanation of the term "blocking power."

‖ Heated at 56°C. for 4 hours prior to preparation of the reaction mixtures to destroy sensitizing antibody.

¶ This fraction analyzes electrophoretically as pure gamma globulin; the sample used for test was also heated as above.

equals 72.5 per cent of the *post* serum's total blocking effect (Table I, column F, site 13).

To compare different fractions with each other throughout these studies their blocking capacities as determined at individually suitable levels are recalculated to the basis of 1.0 mg., these latter values being termed "*blocking power*." The *blocking power* of the fraction in the example is then $\frac{72.5 \times 1.0}{1.2}$ equals 60.4 per cent of the

total blocking effect of the *post* serum regardless of its gamma globulin content (Table I, column G, line 13).

It must be realized that the accuracy of any single test is limited as are most biological tests of this sort and is influenced in part at least, in our experience, by a variation of activity of the skin of different test subjects. For this reason, whenever possible, several tests were performed with individual fractions (as reported in Table II, column B) on the same and different test subjects and the results rated in accordance with usual practice prior to averaging them; solitary determinations were used only for

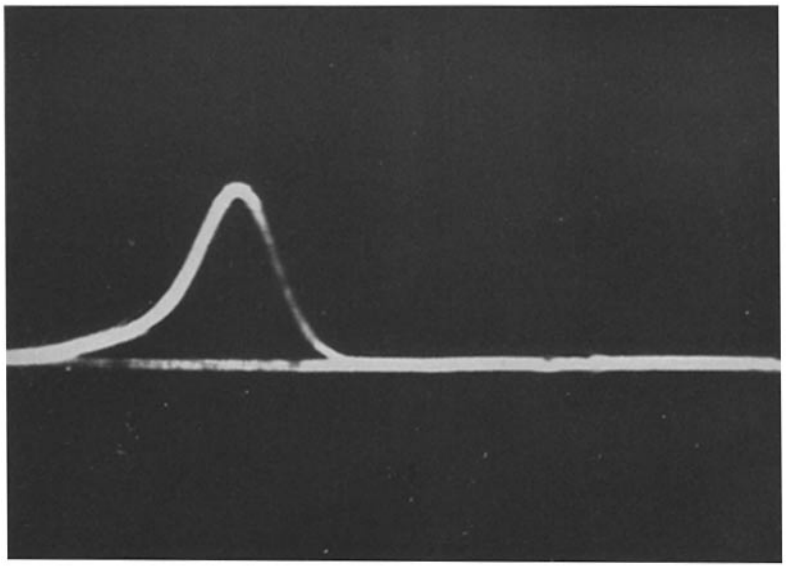


FIG. 1. Electrophoretic pattern of the descending macro separation fraction from Rbr. *post* serum. Calculated descending mobility 0.92×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$ in Longsworth's barbiturate buffer.

evaluating through "curve fitting" the functional relationship between *blocking power* and the gamma globulin content of the fractions.

EXPERIMENTAL

From *post* serum Jhn. bleeding Sept. 4, 1952, the descending electrophoretic end-component was obtained. Undiluted serum which had been dialyzed against potassium phosphate buffer pH 7.8, ionic strength 0.2 was electrophorized in standard analytical cells by use of the same buffer until the gamma globulin peak appeared on the screen well developed for separation of this component. This stage was reached after a total of about 13 hours at a voltage gradient of approximately 3.1 volt per cm., and 15 hours at a voltage gradient of about 0.8. The isolated end-components of 4 such runs were combined. Although the purity of this material could not be checked by electrophoretic analysis it is believed to represent near pure, if not pure, gamma globulin by virtue of the clear cut separations that could be carried out. The combined remainders from these experiments were fractionated with am-

TABLE II

Blocking Power Values, Electrophoretic Analyses, and Other Data on Post Serum Fractions and Post Serums

Line	Fraction or serum*	No. of Tests	Total protein in mixtures‡	Blocking power averages	Electrophoretic components present in 1.00 mg. fraction or in 0.10 ml. serum				
					Albumin	Alpha-1	Alpha-2	Beta	Gamma
Column..	A	B	C	D	E	F	G	H	I
			mg.		*μg.*	*μg.*	*μg.*	*μg.*	*μg.*
	I. Jhn. Post Serum, Sept. 4, 1952								
1	Descending end-component	2	0.895	106.4	Insufficient for analysis§				
2	Chemical "albumin"	2	17.8	0.0	842	59	36	63	0
3	Water-insoluble "euglobulin"	2	1.325	16.1	←101→		453	223	223
4	Water-soluble "euglobulin"	2	0.934	110.8	0	0	0	42	958
5	Water insoluble-"pseudoglobulin"	2	3.68	11.1	←180→		←590→		231
6	Water soluble-"pseudoglobulin"	2	6.125	7.2	107	85	460	226	123
7	Jhn. *post* serum, Sept. 4, 1952		6.917	(100.0)	3535	537	752	1200	895
	II. Jhn. Post Serum, Sept. 12, 1952								
8	Ascending macro separation A	6	8.53,4.27	0.1	←813→		171	16	0
9	Descending macro separation A	5	0.93	57.4	0	0	0	0	1000
10	Chemical "albumin"	1	4.23	0.0	866	51	31	52	0
11	Total chemical globulin B	7	2.66	37.1	←280→			370	350
12	Ascending macro separation B	6	1.98	0.3	←88→		307	605	0
13	Descending macro separation B	5	0.93	77.0	0	0	0	0	1000
14	Remains C	2	2.43	49.2	←617→				383
15	Convection electrophoretic top	3	1.29	54.9	25	45	120	88	723
16	Convection electrophoretic bottom	1	3.15	35.2	41	168	348	146	297
17	Jhn. *post* serum, Sept. 12, 1952		7.232	(100.0)	3851	516	729	1203	933
	III. Lbk. Post Serum, Aug. 5, 1953								
18	Ascending macro separation	4	10.45	0.7	861	95	44	0	0
19	Ascending macro separation	4	9.23	0.8	658	70	154	118	0
20	Descending macro separation	6	1.18	65.9	0	0	29	141	830
21	Lbk. *post* serum, Aug. 5, 1953		8.013	(100.0)	4043	496	1606	886	982
	IV. Rbr. Post Serum, May 12, 1953								
22	Ascending macro separation	3	3.67,2.43	0.3	771	75	117	37	0
23	Descending macro separation	5	1.20	56.5	0	0	0	0	1000
24	Chemical "albumin"	1	13.54	<0.1	781	76	25	118	0
25	Water-insoluble "euglobulin"	2	2.625	24.3	44	163	253	289	251
26	Water-soluble "euglobulin"	7	1.23	95.0	0	0	0	27	973
27	Water-insoluble "intermediate"	1	1.09	<22.0	65	234	393	96	213
28	Water-soluble "intermediate"	1	1.90	38.9	0	31	197	144	629
29	Water-insoluble "pseudoglobulin"	1	0.46	<20.4	←280→		330	390	trace
30	Water-soluble "pseudoglobulin"	1	3.23	<2.5	43	130	547	188	92
31	Convection electrophoretic top	5	2.17	42.2	102	37	←308→		554
32	Convection electrophoretic bottom	4	6.11,4.07, 3.82	3.1	681	58	108	127	27
33	C-E. bottom water-insoluble "intermediate"	2	1.55	23.5	71	283	356	164	127
34	C-E. bottom water-soluble "intermediate"	1	4.23	>7.3	Insufficient for analysis				
35	C-E. bottom water-insoluble "pseudoglobulin"	1	1.55	1.0	131	420	372	78	0
36	C-E. bottom water-soluble "pseudoglobulin"	1	4.10	0.0	Insufficient for analysis				
37	C-E. bottom chemical "albumin"	1	12.88	<0.1	805	121	22	52	0
38	Rbr. *post* serum, May 12, 1953		7.918	(100.0)	4394	348	788	1192	1197

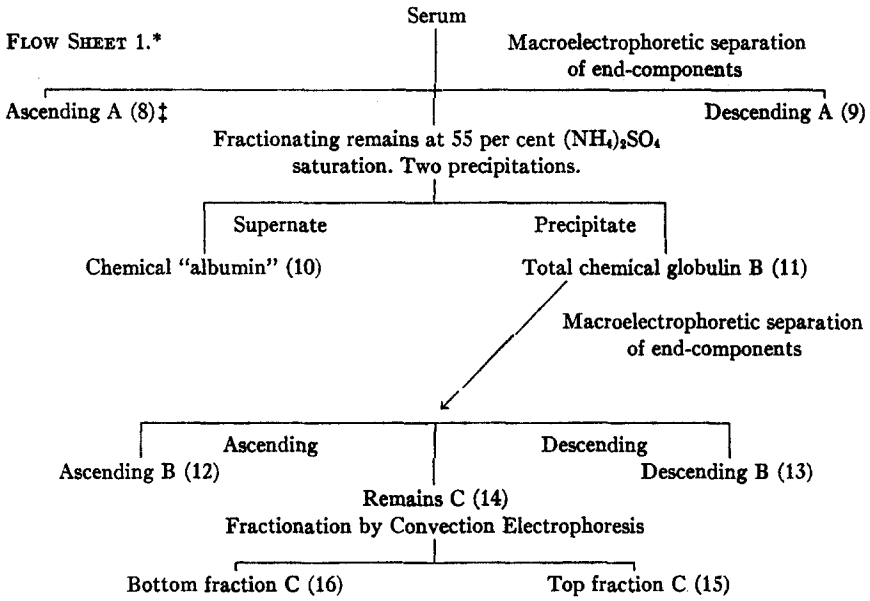
* Concerning the preparation of the individual fractions see text and Flow sheets. Lines 7, 17, 21, and 38 give the data pertaining to 0.10 ml. of the appropriate *post* serums, whose blocking capacities (not *blocking powers*) are always 100 per cent.

‡ Amount of protein incorporated in 0.40 ml. reaction mixture used for the test. More than one entry in this column indicates that tests were performed at different protein levels.

§ Believed to be electrophoretically pure gamma globulin; see text.

monium sulfate at 55 per cent saturation into "albumin" and "total globulin" and the latter was further separated into "euglobulin" and "pseudoglobulin" at 33 per cent ammonium sulfate saturation. No "intermediate globulin" was collected in this instance, but "euglobulin" and "pseudoglobulin" were fractionated into their soluble and insoluble components by dialysis against distilled water.

Post serum Jhn. (bleeding Sept. 12, 1953) and *post* serum Rbr. (bleeding May 12, 1953) were fractionated as presented in Flow sheets 1 and 2 respectively.



FLOW SHEET 1.*

* Fractionation of Jhn. post serum, Sept. 12, 1953.

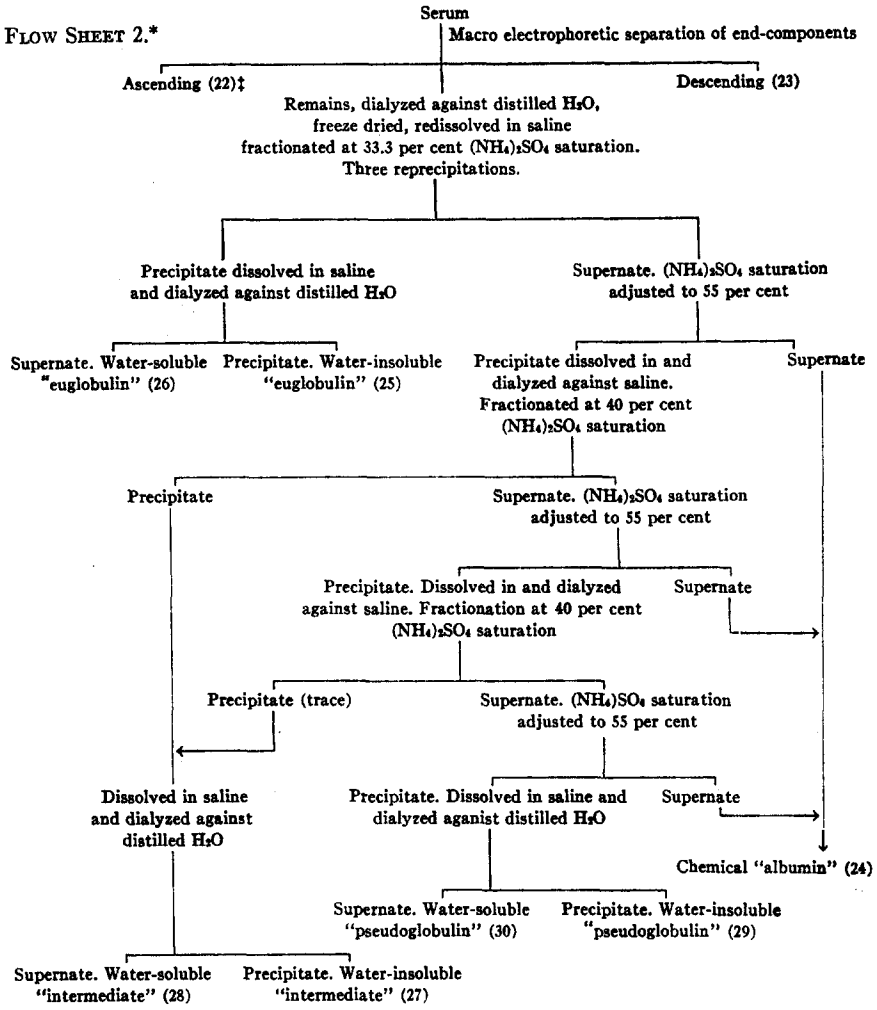
‡ The numbers in parentheses refer to the lines in Table II.

The convection electrophoresis experiment on Rbr. *post* serum, outlined above, was extended by further fractionating the bulk of the bottom fraction with ammonium sulfate followed by dialysis against distilled water. Water-soluble and water-insoluble "euglobulins," "intermediate globulins," and "pseudoglobulins" as well as "albumin" were thus obtained. However, the yields on "euglobulin" were too small for study; this is not surprising, as the convection electrophoretic bottom fraction itself was largely deprived of gamma globulin; *i.e.*, that component which makes up a large portion of the "euglobulins."

From Lbk. *post* serum, (bleeding Aug. 5, 1953), one descending and two ascending separations were obtained by macro electrophoresis.

The determined *blocking power* values of the above fractions are recorded in Table II (column D); the values given in columns E, F, G, H, and I, represent the *percentage distribution* of the electrophoretic components present in the individual fractions as determined by Tiselius analysis; for reasons of convenience in calculation, these values are expressed here, different from the conventional manner, in terms of micrograms per milligram total protein. Corresponding data pertaining to 0.1 ml. samples of the various *post* serums are included in the table (Table II, lines 7, 17, 21 and 38).

FLOW SHEET 2.*



* Fractionation of Rbr. post serum, May 12, 1953.

‡ The numbers in parentheses refer to the lines in Table II.

Evaluation of Blocking Contribution Attributable to Individual Electrophoretic Components

Inspection of Table II reveals that only fractions with appreciable gamma globulin content exhibit a conspicuous degree of blocking power, whilst fractions low in gamma globulin or devoid of it display little or none at all (column D and I). Obviously, blocking antibody is connected largely with gamma globulin.

In order to arrive at quantitative estimates of the possible blocking contributions exerted in the *post* serums by each of the several electrophoretic serum components, there are three main avenues of approach that we have followed.

1. Estimates Based upon Tests with Descending End-Components:

(*a*) *Electrophoretically Pure Gamma Globulin* (*Jhn. and Rbr. serums*).—In our tests with these pure fractions the standard mixtures were always prepared in such a manner that the amounts (in milligrams) of gamma globulin added in the fraction series equalled the amounts of gamma globulin of the *post* serum incorporated in the *post* serum mixtures, as illustrated in Table I. Under these conditions the value found for the blocking capacity of the gamma globulin states directly the contribution of gamma globulin as a percentage of the *post* serum's 100 per cent blocking effect. In the test exemplified in Table I the blocking contribution due to gamma globulin is calculable as 72.5 per cent (site 13, column F).

The evaluation of the fractions recorded in Table II, lines 1, 9, 13, and 23 leads to the values of 95.2 per cent for gamma globulin contribution in Jhn. *post* serum Sept. 4, 1952, and 62.4 per cent in Jhn. *post* serum Sept. 12, 1952; the rated average from all determinations comprising both Jhn. serums[4] being 66.1 per cent. The corresponding value for Rbr. *post* serum May 12, 1952, is 67.9 per cent. These results stated in round figures indicate that on the basis of these experiments 65 to 70 per cent of the total blocking effect is attributable to gamma globulin.

(*b*) *Electrophoretically not Pure Gamma Globulin* (*Lbk. Serum*).—Values of the same order of magnitude are also obtained for gamma globulin from the data on the descending end-component of the Lbk. *post* serum (Table II, line 20). Since both alpha-2 and beta globulin are also present in this fraction the possibility of participation by either or both of them[5] has to be taken into account. This can be done by assuming first that the effect of the fraction and of the *post* serum is distributed between gamma and beta globulin only and then that it is distributed between gamma and alpha-2 globulin only. The calculations[6] with the aid of the data in Table II, lines 20 and 21, columns D, G, H,

[4] It should not be concluded from the higher values obtained on the Jhn. Sept. 4, 1952, end-component as compared with the value from the Jhn. Sept. 12, 1952, end-components that an alteration of blocking antibody distribution had taken place in the Jhn. *post* serum during the time between the two bleedings, because the average in the latter instance is obtained from 10 determinations (6 high and 4 normal rated), whilst the average in the former instance from only 2 tests (normal rated). The average value of 66.1 per cent as resulting from all 12 determinations performed on all 3 electrophoretic end-components from both Jhn. *post* serums should quite correctly reflect the state of affairs, the more as the rated average when calculated from the determinations on the Jhn. Sept. 12, 1952 end-components, with exclusion of one ambiguous item amounts to exactly 65.0 per cent.

[5] Albumin and alpha-1 globulin participation can be disregarded as will be shown later.

[6] See Addendum I.

and I, lead to the rather narrow range of 72.9 per cent (first assumption) to 77.5 per cent (second assumption) for gamma globulin contribution. From these values one may deduce that roughly 75 per cent of the blocking effect rests with gamma globulin.

Calculations based upon *blocking powers* of electrophoretically isolated descending end-components thus suggest that roughly 25 per cent of the blocking antibody might be connected with components other than gamma globulin.

2. Estimates Based upon Tests with Gamma Globulin–Free Fractions:

The tests on the (gamma globulin–free) *ascending macro separation B* show clearly that no appreciable blockage is connected with alpha-2 or with beta globulin in this fraction; 1.0 mg. of this fraction exhibits the exceedingly low blocking effect of 0.3 per cent (Table II, line 12, column D) although it is mainly composed of alpha-2 and beta globulin (Table II, line 12, columns G and H). Were this blocking effect of 0.3 per cent due to the alpha-2 globulin present in the fraction (307 μg. in 1.0 mg.), then the blocking effect of 729 μg. of alpha-2 globulin as present in 0.1 ml. *post* serum (Table II, line 17, column G) would be only 0.7 per cent; likewise a calculation leads to the value of 0.6 per cent for beta globulin as present in the *post* serum. Both these values, fall very far short of the missing 25 per cent.

In a more extensive fashion the gamma globulin–free fractions (Table II, lines 2, 8, 10, 12, 18, 19, 22, 24, 35, and 37) may be utilized for estimating maximally possible blocking contributions due to albumin, alpha-1, alpha-2, and beta globulins by assuming in turn that all the observed *blocking power* be attributable to only one of these components and then calculating such presumptive contributions of the individual components for their respective *post* serums. The (averaged) maximally possible values were in every instance so low (beta globulin about 4 per cent; alpha-2 globulin 2 per cent and both albumin and alpha-1 globulin 1½ per cent), that no significant contribution can be demonstrated for any one of these 4 electrophoretic components. Albumin and alpha-1 globulin certainly can be eliminated from further consideration; our continued inability to find any appreciable blocking effect due to alpha-2 and beta globulin negates any important participation in accounting for the previously stated lack of 25 per cent.

3. Estimates Based upon Tests with Fractions Isolated by Techniques Other than Tiselius Electrophoresis:

The results of gamma globulin contribution so far given are based on electrophoretic end-components isolated by the Tiselius technique. Independent estimates may be secured from the data on fractions that had been obtained from the Jhn. and Rbr. *post* serums by chemical and convection electrophoretic means, since both *blocking power* and *percentage distribution* values as determined by electrophoretic analysis are available on altogether 21 such fractions.

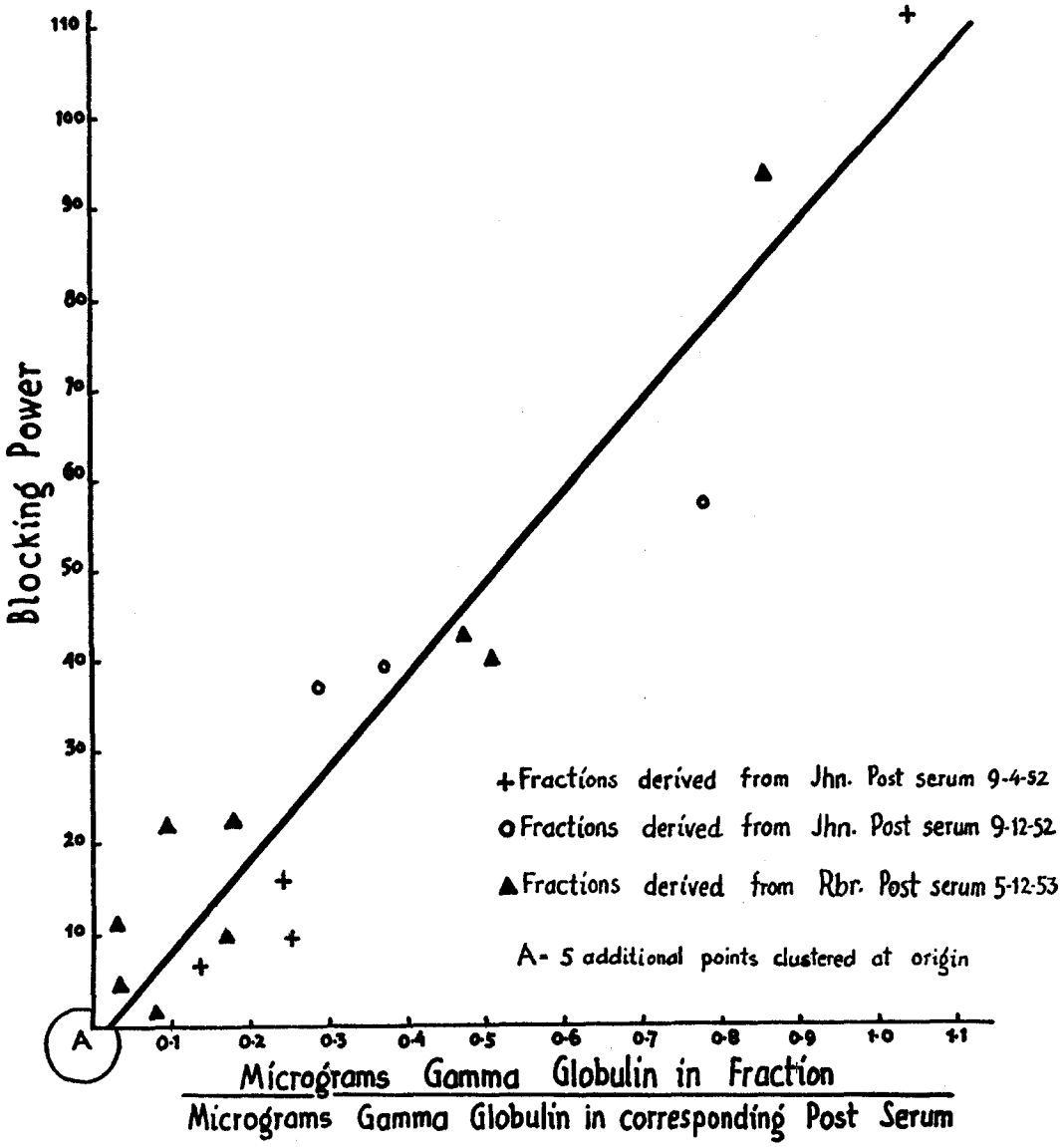


FIG. 2. Relationship between blocking power and gamma globulin content of serum fractions isolated by techniques other than Tiselius electrophoresis.

This is a number sufficient for plotting graphically corresponding pairs of *blocking power* and gamma globulin content values and constructing or calculating the curve that represents best the relationship.

In the case of the 12 fractions derived from Rbr. *post* serum (Table II, lines

24 to 33, 35, and 37), one may simply plot the *blocking power* values as given in Table II, column D, against the amounts of gamma globulin in micrograms as given in column I, when the straight line relationship becomes apparent.[7] Reading from the graph the blocking value that corresponds to the amount of gamma globulin present in the Rbr. *post* serum, a value of essentially 100 per cent is found; *i.e.*, a value corresponding to the serum's total blocking effect.[8]

The value pairs of the analogous fractions derived from Jhn. *post* serums Sept. 4, 1952, and Sept. 12, 1952, are insufficient in number (5 and 4 respectively) to construct special graphs for evaluating the gamma globulin contribution of these two serums individually. However, these data may be incorporated into a graph[7] that comprises all these latter non-Tiselius fractions (Table II, lines 2 to 6, 10, 11, 15, and 16) as well as those from the Rbr. *post* serum fractions mentioned above, by expressing the gamma globulin content of each fraction in terms of the gamma globulin content of its *post* serum, i.e., as the term

$$\frac{\text{Micrograms gamma globulin present in test fraction}}{\text{Micrograms gamma globulin present in corresponding post serum}}.$$

Plotting the values of these terms against their corresponding blocking power values a graph is obtained, which shows the existing straight line relationship, as given in Fig. 2. Reading from this graph the blocking value that corresponds to 1.0 of the abscissal term, leads to 95.5 per cent. This means that the blocking contribution of gamma globulin is evaluated by this procedure[8] as close to 100 per cent.

DISCUSSION

The main difficulties of this study lie in the fact that there exists no absolute method for the determination of blocking antibody, since the biological assay method of skin testing is the only one available and it does not attain the precision or accuracy of quantitative chemical analysis.

The determinations based upon the *electrophoretic descending end-components* show that at least 65 per cent or possibly more, of the total effect of the *post* serum is attributable to its gamma globulin content. The accuracy of this

[7] An objection to this procedure may be raised in that the gamma globulins as present in fractions isolated chemically or by convection electrophoresis may not represent identical distribution of the subconstituents that prevailed in the serums. Thus effective gamma globulin subconstituents may have been predominantly separated into certain fractions and non-effective subconstituents into some other fractions. However, our approach appears none-the-less justified, because due to refraining from "purification" of individual fractions, losses of any constituent were kept at a minimum; therefore, the data on fractions that are presumably rich and the data on fractions that are presumably poor in effective subconstituents counterbalance each other in the graphic evaluation.

[8] For further details see Addendum II.

estimate may be questioned, although it is based upon 23 evaluations; a balance of 35 or even 20 per cent of "missing" blocking effect could hardly have escaped detection in our search for the presence of blocking effect in alpha-2 or beta globulin. An explanation for the low values derived from electrophoretic end-components that represent pure gamma globulin may be found in the absence of other serum components, in that the presence of these components might exert some stabilizing effect upon the blocking antibody. This contention is supported by the higher contribution value (78.0 per cent as compared with about 65 per cent) that is calculable from the tests on the Lbk. descending end-component assuming that its blocking effect resides solely with the gamma globulin and is not shared by the alpha-2 or beta globulin present in that fraction.[9] The graphic method, which we have employed makes it also seem likely that essentially all of the blocking antibody is associated with gamma globulin as indicated by the results of evaluation shown in Fig. 2.

A few statements concerning the graphic method seem appropriate. If the negative results in our search for any essential participation of alpha-2 and beta globulin are accepted as valid, then the deviations[10] of the determined from the calculated values must be conditioned by experimental inadequacies as well as by possible uneven distribution features of blocking and non-blocking gamma globulin subconstituents in the various fractions.[7] The actual existence of these two factors appears almost certain on the following grounds: (*a*) A study of the graphic results with the idea of possible alpha-2 or beta globulin participation does not indicate their significant contribution because an appreciable participation should have become apparent by a relationship between the extent of deviations and the amount of these components present in the fractions. Furthermore, did they participate, the calculated straight line should not essentially pass through the origin of the graph. By either criterion any significant contribution of these components should have been revealed, unless a partitioning of their blocking and non-blocking subconstituents had occurred in such a manner that generally in the fractions a deficiency of effective gamma globulin were counterbalanced by a prevalency of effective non-gamma globulin and *vice versa*. Under such circumstances the straight line relationship between blocking effect and gamma globulin might have been simulated. The occurrence of such extraordinary conditions is, however, not plausible and can be considered improbable. (*b*) On the other hand, the uneven distribution of blocking and non-blocking gamma globulin subconstituents and the approximate extent of this uneveness may be deduced from the properties of the water-soluble "euglobulin" derived from Rbr. *post* serum May 12, 1953, namely from the deviation of the determined from the calculable value. It can be shown, that gamma globulin as present in this fraction should be about 1.2 times as effective as the gamma globulin of the *post serum*.[11]

Although a near 100 per cent effect due to gamma globulin may thus be arrived at, there remains the unanswered question, why this value was not ap-

[9] See Addendum I.
[10] See Addendum II.
[11] See Addendum III.

proximated in the evaluations of the electrophoretic descending end-components derived from the *post* serums. One self-suggesting explanation would be that end-components as derived from Jhn. Sept. 12, 1952, and Rbr. May 12, 1953, *post* serums may have contained predominantly slow moving gamma globulin (we endeavored to exclude beta globulin in these separations) and that blocking antibody may be connected with faster moving gamma globulin constituents. Some support may be found for this possibility, for example, the connection of a lower mobility (1.0×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$) with the apparently less effective descending macro separation A as compared with the somewhat higher mobility (1.5×10^{-5} cm.^{2} volt^{-1} sec.$^{-1}$) of the seemingly more effective descending separation B, both derived from Jhn. *post* serum Sept. 13, 1953 (Table II, lines 9 and 13, column D). This explanation, however, is not

TABLE III

Comparison of Total Protein Concentration and of Distribution of Electrophoretic Components in the Experimental Sera before and after Injection with Ragweed Pollen Extract

Serum	Total protein by biuret test	Percentage distribution of components				
		Albumin	Alpha-1	Alpha-2	Beta	Gamma
	gm./100 ml.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Jhn. *ante* serum July 7, 1952	7.37	51.72	7.05	16.78	10.89	13.57
Jhn. *post* serum Sept. 4, 1952	6.92	51.10	7.76	10.87	17.35	12.94
Jhn. *post* serum Sept. 12, 1952	7.23	53.25	7.14	10.08	16.63	12.90
Lbk. *ante* serum April 21, 1953	8.05	52.32	5.87	10.33	19.79	11.69
Lbk. *post* serum Aug. 5, 1953	8.01	50.46	6.19	20.04	11.06	12.25
Rbr. *ante* serum Nov. 19, 1951	7.64	52.15	7.00	10.71	15.41	14.74
Rbr. *post* serum May 12, 1953	7.92	55.49	4.40	9.95	15.05	15.12

entirely convincing, because the evaluation of the Lbk. descending end-component should then certainly have led to values in the vicinity of 100 per cent instead of the calculated ±75 per cent.

We are inclined to believe that these low values are due to some loss of antibody in the preparative steps. Should unaccountable losses of blocking effect have entered generally in the course of preparing the electrophoretic end-components or even in the course of preparing some other crucial fractions, such losses would appear responsible for discrepancies encountered. Whether such losses would then be related to blocking antibody as known to be connected with gamma globulin or with antibody considered to be connected with globulin other than gamma globulin, remains a matter of speculation. We are inclined to believe the former to have been the case, as our data present more indications towards this than towards the opposite point of view.

Two observations should be mentioned. First, that although the bulk of

blocking antibody was found to be contained in the gamma globulin, this fact was not mirrored by an observable rise of the gamma globulin in any of the patterns of the *post* serums as compared with those of their *ante* serums. In this respect our *post* serums differ from other immune serums, whose antibody content attributable to gamma globulin is reported as being paralleled by increase in this electrophoretic component. Without engaging in any speculation on the significance of this observation we present the factual data as given in Table III (page 191).

It should be noted that no essential change of the total protein concentration or of the *percentage distribution* of gamma globulin took place in any of the three *post* serums. This observation may be explained on the basis of Grabar's theory (25, 26). We have, however, no ready explanation for the fact that the *percentage distribution* between alpha-2 and beta globulin was reversed from

TABLE IV

Blocking and Sensitizing Effect in Chemical Fractions Isolated from Rbr. Serum, May 12, 1953

Fractions	Blocking	Sensitizing
Water-insoluble "euglobulin"	Moderate (25)*	Insignificant
Water-soluble "euglobulin"	High (26)	Very low
Water-insoluble "intermediate"	Not detectable (27)	Moderate
Water-soluble "intermediate"	Moderate (28)	Low
Water-insoluble "pseudoglobulin"	Not detectable (29)	High
Water-soluble "pseudoglobulin"	Absent (30)	High

* Numbers in parentheses refer to lines in Table II.

high alpha-2 and low beta globulin to low alpha-2 and high beta globulin in the instance of Jhn. serum, and in the other direction in the instance of Lbk. serum, whilst the alpha-2 and beta globulin values remained essentially unaltered in the Rbr. serum.

The second noteworthy observation lies in the fact that the bulk of blocking antibody (at least 65 per cent if not all) can be attributed to gamma globulin, whilst in sensitizing serums only a minor portion of sensitizing antibody is correlated with this electrophoretic component (12, 17). Blocking and sensitizing function appear unrelated to each other, for on chemical fractionation sensitizing antibody tends to accumulate in other fractions than does the blocking antibody. We shall deal with this topic in a subsequent paper. It will suffice here to present in Table IV an abridged version of the distribution of blocking and of sensitizing antibody in some of the fractions that have been obtained chemically from Rbr. serum, May 12, 1953, (a serum that contains both blocking and sensitizing antibody) as these results are characteristic.

These observations are basically in perfect agreement but amplify those previously reported by Stull *et al.* (2), who realized the solubility of both sensitizing

and blocking antibody in electrolyte-free water. They state: "there was no evidence of separation of the skin-sensitizing and inhibiting factors. It is obvious that the fractionation would not necessarily separate such substances, if present." After subfractionation, as done in this study, these two factors were also found in water-soluble fractions, however, the skin-sensitizing antibody was enriched in the water-soluble "pseudoglobulin"[12] in which blocking antibody was absent, while the latter was enriched in the water-soluble "euglobulin"[12] in which skin-sensitizing antibody was present only to an almost insignificant degree. The fact that these two antibodies are separable by chemical means suggests that the blocking antibody does not represent merely an immunological modification of the sensitizing antibody but that it is an essentially different chemical entity.

Our studies also make it very probable that blocking antibody represents modified gamma globulin, quite in accordance with some other artifically induced antibodies whose electrophoretic properties make them classifiable as gamma globulin.

SUMMARY

Three human serums containing artificially produced blocking antibody against low ragweed allergen were studied for the possibility of relating blocking antibody to electrophoretically definable components.

An adaptation of the qualitative passive transfer test to quantitative interpretation is described, methods and procedures are given and uncertainties and possible errors due to lack of precision and accuracy are presented and discussed.

At least 65 per cent, but probably more, if not all of the blocking antibody is attributable to gamma globulin. However, no rise of gamma globulin, either its absolute amount or its relative percentage value, paralleled the appearance of blocking antibody.

Blocking antibody is not contained in albumin or in alpha-1 globulin.

Blocking effect could not be ascertained unequivocally as being connected with alpha-2 or with beta globulin and sizable participation of these two latter electrophoretic components appears improbable.

Blocking antibody and sensitizing antibody appear to be chemically different entities.

Addendum I.—Assuming that the blocking effect was distributed between gamma and beta globulin only, the following two equations obtain:—

$$x + y = 100.0 \tag{1}$$

$$\frac{141}{886}x + \frac{830}{982}y = 65.9 \tag{2}$$

[12] Definition of this paper.

in which x represents the contribution exerted by 886 μg. beta globulin and y the contribution exerted by 982 μg. gamma globulin to give the *post* serum's total effect of 100 per cent and in which the contributions of these components in the fraction are expressed in terms of x and y (Table II, lines 20 and 21, columns H and I).

The corresponding equations for the assumption that the blocking effect be distributed between gamma and alpha-2 globulin are:—

$$z + y = 100.0 \tag{3}$$

$$\frac{29}{1606} z + \frac{830}{982} y = 65.9 \tag{4}$$

in which z represents the contribution of 1606 μg. alpha-2 globulin and y again the contribution of 982 μg. gamma globulin to the *post* serum's total effect. Solving these equations for x, y, and z leads to the values (72.9 per cent and 77.5 per cent) given in the text.

If from the point of view that we were unable to allocate any appreciable effect with alpha-2 or beta globulin in other experiments, the assumption is made that the fraction's blocking effect rests entirely with gamma globulin, the contribution of this component is calculable from the relationship $\frac{830}{982} y = 65.9$ as 78.0 per cent. This, of course, means that the determined *blocking power* value was found to be about 20 per cent below the theoretical value, should these conditions actually obtain.

Addendum II.—The evaluation of the data on the 12 fractions derived from the Rbr. *post* serum by the method of least squares leads to the equation:—

$$\text{Blocking effect} = +0.6 + 0.0843 \times \mu\text{g. gamma globulin}$$

From this equation it follows that the amount of gamma globulin as present in the *post* serum, namely 1197 μg. should have exhibited a blocking effect of 101.5 per cent; this means that by this evaluation the serum's entire blocking effect is accounted for by the effect of its gamma globulin.

From the evaluation of all the data that are incorporated into the graph (Fig. 2) by the method of least squares, the following equation is obtained:—

$$\text{Blocking effect} = (-0.7) + 96.2 \left[\frac{\mu\text{g. gamma globulin in fraction}}{\mu\text{g. gamma globulin in corresponding post serum}} \right]$$

The blocking value for the gamma globulin as present in the *post* serums is given, when the value of the denominator is also assigned to the numerator; the fractional term then becomes unity and the blocking contribution due to gamma globulin results as 95.5 per cent. The deviations of the experimental values from the calculated ones are remarkably reasonable considering the nature of the experiments. Of the 21 determined values 10 are numerically within $\pm$ 5 per cent, 16 within $\pm$ 10 per cent, 19 within $\pm$ 15 per cent, and all within $\pm$ 20 per cent of the calculated values. There is no apparent correlation between the magnitude of the deviations and the amount of beta and alpha-2 globulin in the fractions. No significance can be attributed to the slight difference in the values based upon the Rbr. *post* serum fractions alone and those based upon all non-Tiselius fractions.

For constructing the graph and for establishing above equations the mid-values between zero and *blocking power* figures were taken, when these were available only to the extent of "less than a certain value" (Table II, lines 24, 27, 29, 30, and 37, column D); the trace of gamma globulin in "remains insoluble pseudoglobulin" (Table II, line 29, column I) was estimated as 20 μg.

Addendum III.—The *blocking power* of the water-soluble "euglobulin" from Rbr. *post* serum is satisfactorily well established on the basis of 7 determinations; this value, 95.0

per cent is 17.5 per cent higher than the theoretical value of 77.5 per cent (this deviation being the highest positive deviation encountered in the entire set). This increase in blocking effect can not be attributed to alpha-2 globulin, as this component is absent in the fraction. If the data on this fraction are evaluated analogous to the procedure in Addendum I, a more than 100 per cent contribution for gamma and a negative contribution for beta globulin is calculable, which is unreasonable; this merely means that either the beta or the gamma globulin of the fraction (or both) is more effective than these components are in the *post* serum. If beta globulin is postulated to be solely accountable for the increased effect, then about 60-fold concentration of effective beta in the fraction has to be accepted, if one assumes that gamma globulin had contributed 75 per cent and beta globulin 25 per cent to the *post* serum's total blocking effect of 100 per cent. Still higher concentration ratios have to be accepted, if one assumes lesser contribution values for beta globulin. Such concentration ratios appear out of any proportion and the premises used for their calculation find not more than ambiguous support from our other results. Hence, the increased blocking properties of the fraction may safely be attributed to gamma globulin; from the ratio $\frac{95.0}{77.5}$ it follows that the gamma globulin of the fraction is about 1.2 times as effective as the gamma globulin in the *post* serum.

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