

DISTRIBUTION OF SENSITIZING ANTIBODY IN HUMAN SERUM PROTEINS FRACTIONATED BY ELECTROPHORESIS-CONVECTION*

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Few attempts have been made to study the distribution of sensitizing activity in the serum proteins of man, and the conclusions have been confusing. Newell and associates (1) were the first to employ conventional electrophoresis for the separation of presumably pure γ -globulin and albumin from the upper portion of the negative and of the positive side of the U-tube. On the basis of a limited number of qualitative Prausnitz-Küstner tests, they concluded that activity was associated with the γ -globulin but not with the albumin. (However, they interpreted as a technical accident the association of activity with a fraction containing albumin, α -globulin and β -globulin.) Similar experiments with a pollen-reaginic serum led Sherman and Seeböhm (2) to conclude that albumin was inert and that presumably pure γ -globulin was active. Although the same serum also carried transferable antibodies for "cold" allergy, neither its albumin fraction nor its γ -globulin fraction conferred sensitiveness. The other portions of the serum, taken from the lower regions of the cell, were also inactive unless combined. This situation suggested that more than one protein was required for successful transference. Cooke *et al* (3) applied the method to 10 inhalant-hypersensitive sera and observed that the γ -globulin fraction carried only about one-tenth the activity of the unfractionated serum. It was logical to deduce that reagins were not restricted to γ -globulin.

Campbell, Cann and their collaborators (4) were the first to apply the method of electrophoresis-convection to the study of the distribution of sensitizing antibodies in the serum proteins. This method possesses several advantages over that of conventional electrophoresis for

preparatory purposes. Not only does it permit fractionation on a larger scale, but it achieves separation of the serum protein components on a finer scale than is possible with the Tiselius technique. Indeed, Cann and his associates (5) have reported that one of the serum proteins (γ -globulin) present in a pertussis-immune, human specimen has been separated into four electrophoretically unique fractions by electrophoresis-convection; and comparable fractionations are the basis of the present report. The experiments of Campbell's group were admittedly preliminary in that their two sera were carried through only two stages of electrophoresis-convection. It was their impression that "reagins do not occur predominantly in the γ -globulin component and . . . activity may be limited to the . . . α - or β -globulins."

Extending the separatory phase to 8 stages of electrophoresis-convection and adding the quantitative antibody-absorption technique to the procedure of serum-dilution used by earlier investigators, the writers (6) recently analyzed an insulin-allergic serum to conclude that its antibodies were associated chiefly with the β -globulins. The purpose of the present report is to offer similar data for three other sensitizing sera.

METHODS

Fractionation was effected by successive stages of electrophoresis-convection in the cold at pH levels which were lowered stepwise from 8.1 to 5.2, at intervals of 0.5 pH. At one point (fifth stage, pH 6.0) the pH was not lowered for the next run (Vb) because a larger supply of this presumably reagin-enriched fraction was desired. At the conclusion of each run, the "top" fraction was removed from the upper reservoir, dialyzed against phosphate buffer at pH 7.0, passed through a Seitz bacterial filter and stored without preservative at 4°C until fractionation had been

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completed. The serum remaining in the apparatus then served as the starting material for the next stage, original volume being restored with an appropriate buffer. The final remainder was treated with one volume of saturated ammonium sulphate at pH 7 to provide the fractions BG and BA. All yields were analyzed for N content by the method of Kjeldahl digestion and Nesslerization. Every attempt was made to avoid contamination throughout the experiment, and periodic cultures were done. Contaminations were infrequent and were promptly interrupted by filtration.

Electrophoretic analysis was carried out on samples concentrated to 1% protein by pervaporation. Barbitol buffer at pH 8.6, ionic strength 0.1, and a field strength of about 7 v/cm were employed for the 1½ hour runs in the apparatus of Perkin and Elmer, voltage being measured with a potentiometer. Mobilities were calculated from the descending pattern in the usual manner, and the areas under the schlieren peaks were measured with a planimeter.

Activity was appraised by serial tests in the skin of several normal subjects on three or four different occasions. One-tenth ml was the standard volume used intracutaneously in some 64 sites per experiment, each area being marked with an indelible pencil so that the challenge injection of allergen to be given in 24 hours could be accurately introduced into the same needle track. In the case of the neutralization, or absorption tests, mixtures contained one volume of sensitizing serum or fraction, one volume of allergen in doubling concentrations, and one volume of either saline diluent or normal serum. This third component could be substituted by heated, immune serum when blocking antibody was to be measured, any advance in the amount of allergen required for neutralization being attributed to the preferential union of the blocking antibody with the antigen. To perform the serum-dilution test, a series of twofold dilutions of a given fraction in physiological saline solution were set up for subsequent injection into normal skin. When challenges with allergen were made next day, that site which responded with only a minimal wheal-and-flare was selected as the end-point. A satisfactory experiment yielded reactions of ++++, ++, +, ± and 0. The ± end-point revealed, for the neutralization procedure, how little allergen had sufficed to absorb out the sensitizing factor, and for the serum-dilution

titration, the greatest dilution in which a fraction could still transfer sensitiveness to normal skin. Activity studies were commenced promptly after fractionation and were repeated at weekly intervals on the several test subjects participating, for three to six weeks. Areas once sensitized were avoided in subsequent experiments through the vigorous application of indelible dye.

Since the protein content differed somewhat for the several fractions, titration results have been adjusted to a uniform concentration of 1 mg protein/ml so as to facilitate comparisons of potency. This, of course, involves the presently necessary assumption that activity in the various fractions is due to the same substance or group of substances but is not influenced by the total protein concentration *per se*. This procedure therefore, within the validity of the assumption, gives an indication of the relative potencies of the several fractions and permits correlation of activity with the electrophoretic composition of the various preparations. The amount of allergen required to neutralize 1 mg total protein will be termed the neutralization ratio, R_n . The ratio R_d represents the maximal factor by which a solution containing 1 mg of fraction protein per ml can be diluted and still confer sensitiveness. The experimental error is roughly half a tube up or down from the end-point by either procedure.

RESULTS

SEN cottonseed-reaginic serum. The donor of this specimen was a middle-aged asthmatic woman who is highly allergic to cottonseed by history, intracutaneous, conjunctival, serologic and ingestion tests, as previously reported (7). She had never received therapeutic injections of the seed extract. Promptly after her phlebotomy on January 29, 1953, her serum was sent to Denver by airmail for fractionation. Meantime, a control sample retained in New York City proved to be as reactive toward a fresh solution of Spies' (8) purified allergen, CS54R, as with our extract of the whole seed. It is of passing interest that 54R was the only portion of the seed to which this patient was reactive, sites of normal skin injected with her serum and an excess of this allergen failing to respond to later tests with either 54R or whole seed. Although the Spies allergen is not a single substance as judged by electrophoretic or diffusion studies, it is highly simplified and was selected as the ideal immunologic tool with which to titrate our serum frac-

tions. Chemically it is a proteose which contains 19.1% N and 6.3% carbohydrate. It diffuses in part through cellophane, without any change in specificity, and loses no activity through boiling or treatment with acid. This antigen and the serum were sufficiently potent so that sites of normal skin in NEL which had received 0.03 ml of whole SEN serum required 54R in a strength of only 0.000,138 mg protein/ml to provide a ++++ reaction when 0.02 ml was injected. This allergen was also used to test for the presence of blocking antibodies in the SEN serum, with negative result, the same amount serving to neutralize the whole serum regardless of whether the third component in the mixture was physiological saline solution, heated normal serum, heated SEN serum or its heated fractions Va and VI. (The latter preparations contained both β - and γ_1 -globulin.)

Neutralization experiments with unheated fractions of SEN serum provided material for the graphs in Figure 1. To obtain these R_n values, end-points listed in Table I were divided by the figures for total protein content given in Table II. The set of results obtained in NEL are the most reliable of the three, since this subject could be markedly sensitized with each of the materials whereas subjects REI and LUD manifested only ++ transference to Tops I, II and BG and failed to be sensitized with BA. The approximate limit of accuracy for the experimental method is indicated for tops Va and VI and would be the same for all other observations regardless of their positions on the logarithmic paper.

Figure 1 reveals that Tops Va, Vb and VI possessed the greatest activity per mg total protein and that they were comparable in potency. These preparations had been derived from electrophoresis-convection (E-C) experiments at pH 6.0, 6.0 and 5.5, respectively (see Table II). Next in order of potency was fraction VII, yielded from E-C at pH 5.2. Top IV was probably superior to BG; and III, II and I were the least effective of the Tops. The reaginic quality of BA was borderline, being undetectable in two of the three subjects.

Figure 2 is the outcome of serum-dilution studies with the same fractions and recipients. The ratio, R_d , tends to be higher for fractions prepared by E-C runs at pH 6 and 5.5 than at other levels, thus confirming in general the above-mentioned findings. Differences among the fractions were not, however, as sharp as with the

neutralization procedure. (The reason for this becomes apparent if one examines the data given in Table I for SEN fractions appraised in subject NEL. Fraction II, for example, could be neutralized by admixture with 0.0011 PN units of 54R, top VI requiring 0.625 units per ml. This 500-fold difference in potency represents 9 tubes in our antigen-dilution series. When these two fractions were compared by means of the dilution test, however, they were found to differ by only fourfold, or 2 tubes. The wider range of reactivity associated with the neutralization technique is no doubt responsible for its generally greater reliability.)

Correlation between activity and electrophoretic composition. The electrophoretic patterns of the SEN fractions were now examined to learn whether activity was associated principally with one or another of the serum proteins. Table II shows that tops I, II and III contained 93% or more of the so-called slow, or γ_2 -globulins (mobilities below the arbitrary rate of -1.9×10^{-6}). The low activity quotient of these preparations was obviously inconsistent with the assumption that such globulins play a role in sensitization. Equally convincing negative evidence was presented by tops Va, Vb, VI and VII, which possessed no γ_2 -globulins but a large amount of skin-sensitizing antibody.

A comparable situation was encountered in the case of BG which, aside from small amounts of albumin and of β -globulin, consisted entirely of α_1 - and α_2 -globulins. The activity of BG was so low that neither of these α -globulins could logically have accounted for the activity of SEN serum. Consistent with this view was the observation that no α -globulin could be detected in the patterns of either Va or Vb, two highly active preparations. The possible participation of albumin appeared equally unlikely because of the almost inert nature of BA, a fraction consisting chiefly of albumin.

The foregoing arguments eliminate from consideration as major sources of reagin each of the serum proteins except the γ_1 - and β -globulins. Since both of these components were present in impressive amounts in the most powerful fractions Va, Vb and VI, either might have been responsible for reactivity. When, however, graphs were set up to show the relationship between the R_n values and the concentrations of either globulin in successive SEN fractions, activity proved to be less closely related to γ_1 - than

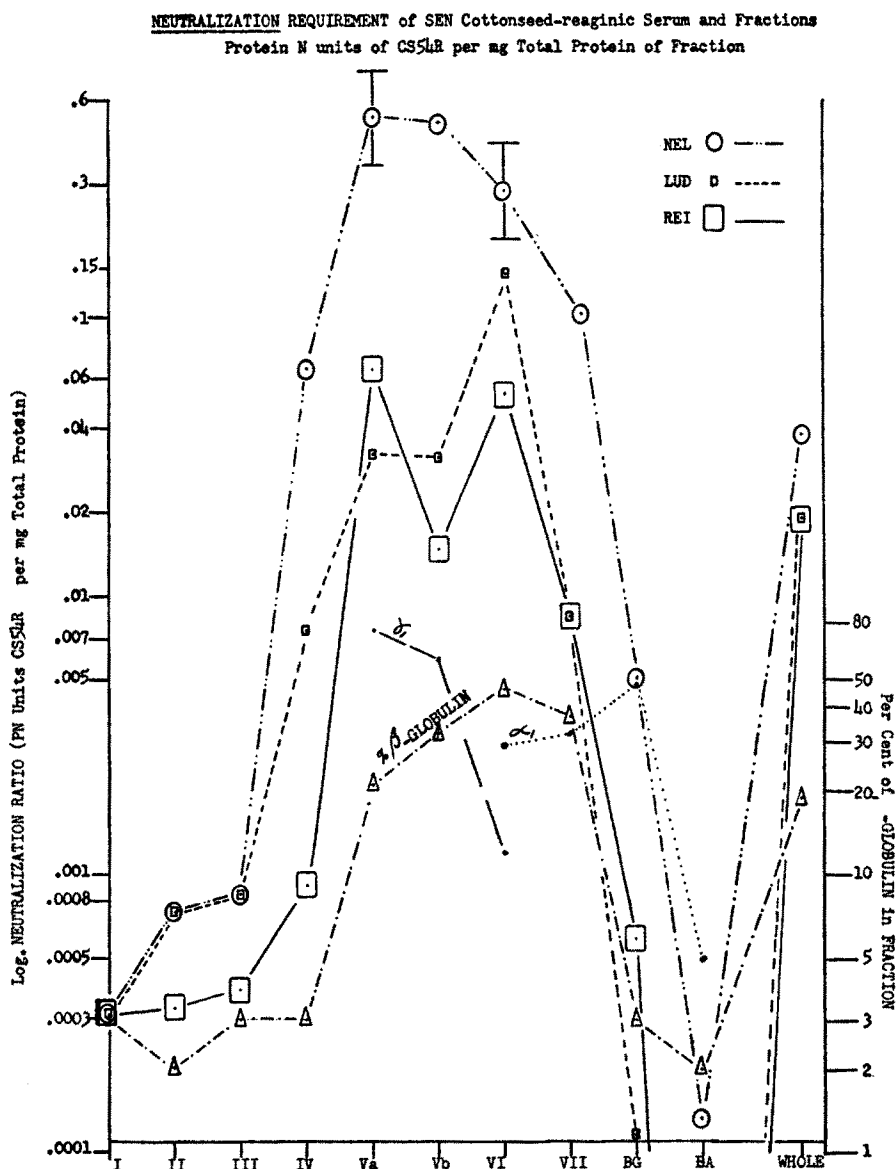


Figure 1

to β -globulin, as may be seen in Figure 1. Fraction VI, for example, carried an activity quotient similar to that of Va. Its electrophoretic composition, on the other hand, was quite different in that it possessed only 12% of γ_1 -globulin, whereas Va contained 75% (Table II). The correlation was again poor in the case of VII since this fraction had no γ_1 -globulin but demonstrated marked activity.

Although this situation with fraction VII throws decided doubt on the sensitizing role of

the fast γ -globulins, it fortifies the assumption for the β -globulins, since VII was comprised solely of β -globulin in association with two other components (albumin and α_1 -globulin) which have been shown above to play no significant role in sensitization. This makes it clear that β -globulin can confer sensitiveness, and that it does so even in the absence of the γ_1 - and α_2 -globulins. Finally, the satisfactory general correlation demonstrated in Figure 1 to exist between β -globulin content and activity in all the

TABLE I
Immunological activity of reaginic sera

FRACTION	TOTAL PROTEIN (mg/ml)	NEUTRALIZATION REQUIREMENT OF UNHEATED SERUM						MAXIMAL DILUTABILITY FOR PASSIVE SENSITIZATION					
		Test subject NEL		REI		LUD		NEL		REI		LUD	
		End point PN unit*/ml	R _N : PNU per mg TP	End point PNU/ml	R _N : PNU per mg TP	End point PNU/ml	R _N : PNU per mg TP	End point	R _d EP/mg TP	End point	R _d EP/mg TP	End point	R _d EP/mg TP
SEN whole.....	66.0	2.5	.038	1.25	.019	1.25	.019	1:128	1.94	1:32	.49	1:32	.49
Top I.....	1.6	.0005	.0003	.0005	.0003	.0005	.0003	1:3	1.87	1:2	1.25	1:1	.63
Top II.....	1.5	.0011	.0007	.0005	.0003	.0011	.0007	1:3	2.00	1:1½	1.00	1:1½	1.00
Top III.....	1.3	.0011	.0008	.0005	.0004	.0011	.0008	1:5	3.85	1:2	1.54	1:1½	1.15
Top IV.....	1.2	.078	.065	.0011	.0009	.009	.0075	1:6	5.00	1:2	1.67	1:1½	1.25
Top Va.....	1.2	.625	.52	.078	.065	.039	.0324	1:7	5.83	1:4	3.33	1:5	4.15
Top Vb.....	0.61	.300	.496	.009	.015	.019	.0312	1:4	6.56	1:3	4.92	1:2	3.28
Top VI.....	2.2	.625	.284	.117	.053	.312	.142	1:12	5.45	1:6	2.72	1:4	1.82
Top VII.....	4.6	.468	.102	.039	.0085	.039	.0085	1:12	2.61	1:4	.87	1:4	.87
Top BG-7.....	7.7	.039	.005	.0045	.0006	.0011	.0001	1:8	1.04	1:2	.26	1:2	.26
Top BA-7.....	8.3	.0011	.0001	No transfer		No transfer		1:1	0.12	No transfer		No transfer	
		Test subject PAN		REI		LUD		PAN		REI		LUD	
KU 4/27/53 whole	66.0	80 (160)	1.2	160.0	2.43	160.0	2.43	1:256	3.87	1:12	.18	1:32	.48
Top I.....	1.7	.312	.183	No transfer		No transfer		1:8	4.7	No transfer		No transfer	
Top II.....	2.2	.312	.142	.0011	.0005	.0011	.0005	1:8	3.6	1:1½	.68	1:1½	.57
Top III.....	1.7	.312	.183	.0011	.0007	.0011	.0007	1:6	3.53	1:0.5	.29	1:1½	.74
Top IV.....	1.2	.625	.52	.0011	.0009	.0011	.0009	1:32	26.70	1:1	.83	1:1½	1.25
Top Va.....	1.4	20.0	14.3	.156	.111	1.87	1.34	1:128	91.50	1:4	2.86	1:1½	1.07
Top Va 6X†.....	8.36					80.0	9.57			1:12	1.43	1:12	1.43
Top Vb.....	0.83			.156	.188			1:64	77.1	1:1½	1.82	1:1½	1.80
Top Vb 5X.....	4.15	80.0	14.46			2.5	0.6			1:8	1.93	1:6	1.44
Top VI 4X.....	9.1	80.0	6.6	15.0	1.65	80.0	8.8	1:256	28.2	1:8	.88	1:16	1.75
Top VII 4X.....	13.1	20.0	1.54	.156	.012	.156	.012	1:256	19.5	1:6	.46	1:4	.31
Top BG-7.....	10.6			± transfer		.0011	.0001			1:0.5	.05	1:0.5	.05
Top BA-7.....	8.8			No transfer		No transfer				No transfer		No transfer	
				REI				NAS		REI			
KU 8/14/52 whole	70.0			2560	36.57			1:640	9.1	1:64	.91		
Top I.....	3.5			10	2.86			1:10	2.85	1:4	1.14		
Top II.....	2.0							1:3	1.5	1:1½	.75		
Top III.....	1.2							1:3	2.5	1:1½	1.04		
Top IV.....	1.0			5	5.0			1:10	10.0	1:2½	2.25		
Top Va.....	1.3			160	123.0			1:80	46.0	1:3.75	2.89		
Top Vb.....	0.48			5	10.4			1:10	20.8	1:1.12	2.33		
Top VI.....	1.3			5	3.85			1:80	46.0	1:3½	2.7		
Top VII.....	3.2			5	1.56			1:40	12.5	1:2½	.78		
Top BG-7.....	1.3			No transfer				1:2½	1.9	1:0.25	.19		
Top BA-7.....	9.0			No transfer				1:0.5	.06	No transfer			

* 1 PN unit = .01 µg of protein (or phosphotungstic-acid-precipitable) N.

† 6X = concentrated 6-fold.

TABLE II
Electrophoretic analysis of reaginic serum

FRACTION	pH OF E-C RUN	TOTAL PROTEIN CONTENT (MG/ML)	DISTRIBUTION OF COMPONENTS IN FRACTION (% OF TOTAL AREA)					
			Albumin	α_1 - Globulin	α_2 - Globulin	β - Globulin	γ_1 - Globulin	γ_2 - Globulin
Whole serum SEN 1/29/53...		66.0	52	6	10	19 ^b		13
I.....	8.3	1.6	2			3		95
II.....	7.5	1.5	4			2		94
III.....	7.0	1.3	4			3		93
IV.....	6.5	1.2	3			3		94
Va.....	6.0	1.2	4			21	75	
Vb.....	6.0	0.61	9			32	59	
VI.....	5.5	2.2	14	28 ^a		46	12	
VII.....	5.2	4.6	31	32		37		
BG-7.....	7.0	7.7	4	48	45	3		
BA-7.....	7.0	8.3	88	5	5	2		
Whole KU 4/27/53.....			57	6	9	14		14
I.....	8.2	1.7	2					98
II.....	7.6	2.2	2			3		95
III.....	7.0	1.7	4			1		95
IV.....	6.6	1.2	2			3		95
Va.....	6.0	1.4	4	3		16		77 ^c
Vb.....	6.0	0.83	11	4		26	59	
VI (4x)*.....	5.4	9.1	8		18	60	9	5
VII (4x)*.....	5.2	13.1	23	36	9	28	2	2
BG-7.....	7.0	10.6	22	43	28	7		
BA-7.....	7.0	8.8	92	6		1	1	
Whole KU 8/14/52.....			56		5	24		15
I.....	8.1	3.5	3			6		91
II.....	7.5	2.0	5			2		93
III.....	7.0	1.2	6			4		90
IV.....	6.5	1.0	5			6†	39†	50†
Va.....	6.0	1.3	6			20†	37†	37†
Vb.....	6.0	0.48	12			24	64	
VI.....	5.5	1.3	14	15		58	13	
VII.....	5.3	3.2	30		25	45		
BG-7.....	7.0	1.3	9	62	28	1		
BA-7.....	7.0	9.0	89	11				

* Concentrated by means of fanning in a shallow container at room temperature, to $\frac{1}{4}$ volume.

^a Includes small amount of α 2-globulin.

^b Probably includes γ 1-globulin.

^c $\mu = 1.68 \times 10^{-5}$.

† Area measurements from rising pattern, because γ -globulin showed resolution into fast and slow components.

‡ So-called fast or γ_1 -globulins with mobilities at or above the arbitrary level -1.9×10^{-5} .

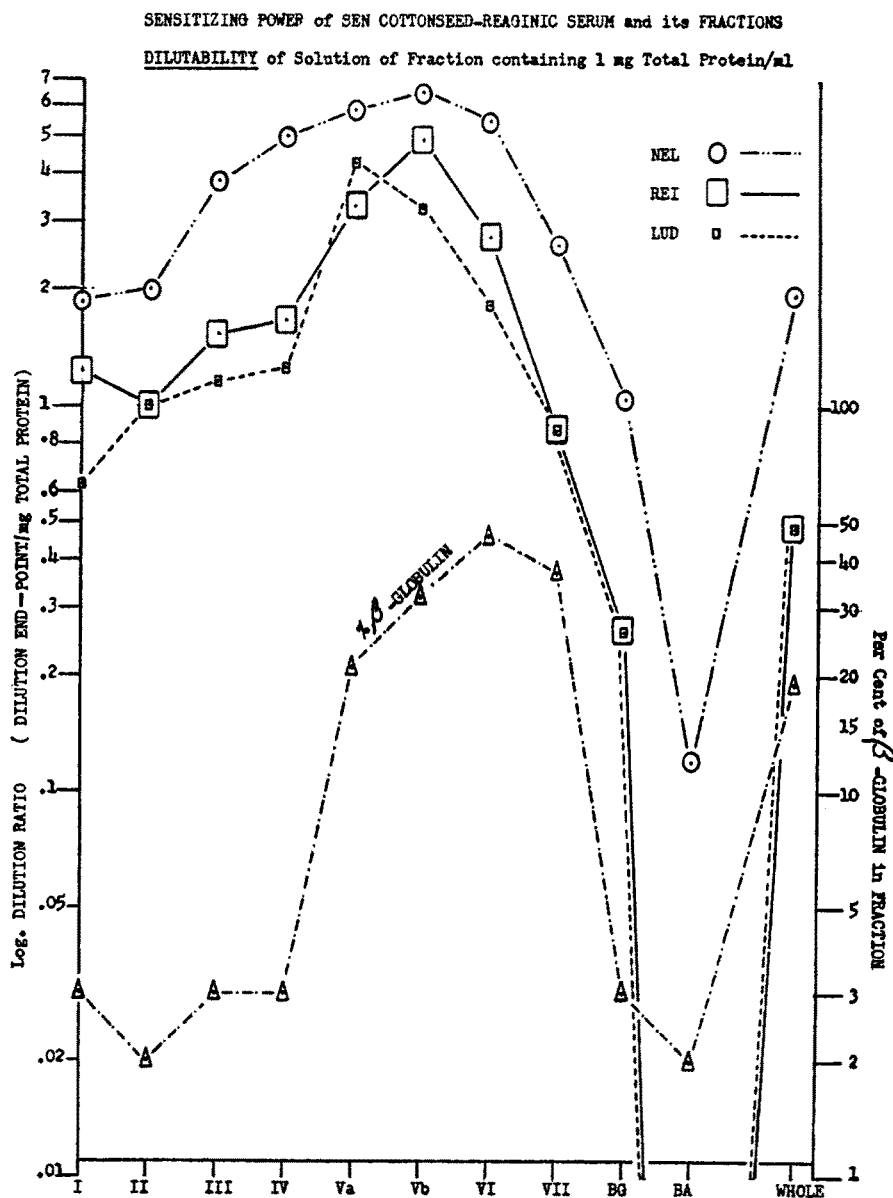


Figure 2

SEN fractions permits the conclusion that β -globulins were chiefly responsible for reaginic qualities. It was not feasible, however, to exclude the possibility that γ_1 -globulins also contributed sensitizing factor, since 3 of the 4 most active preparations contained both components and no fraction was encountered which possessed γ_1 -globulin without β -globulin.

Some of the differences in electrophoretic composition are brought out by Figure 3. If one compares the tracing for the whole serum with

that for Top I, it is strikingly apparent that electrophoresis-convection at pH 8.3 has removed all components except γ_2 -globulin and traces of albumin and of β -globulin. Top Va, on the other hand, has been impoverished in γ_2 - and α -globulins as well as in albumin so that it consists predominantly of β - and γ_1 -globulins. Top VI differs from Va in its reduced proportion of γ_1 -globulin and its possession of α -globulin as well as of more albumin.

KU pollen-reaginic serum was obtained for

fractionation on April 27, 1953, nearly 10 months after the donor's last immunizing injection of pollen extract. Preliminary neutralization tests with the heated whole KU serum gave no indication of blocking activity. Furthermore, KU's conjunctival susceptibility, as judged by successive instillations of glycerine-stabilized ragweed extract in doubling concentration on the day of the bleeding, was found to have returned to its pre-immunization level, indicating that his acquired immunity and blocking antibodies had been lost. Although this donor showed more than the average degree of allergy toward ragweed pollen by clinical, conjunctival and serologic standards, his serum did not possess the superior reaginic power of SEN blood. In the subject REI, for example, the KU whole serum transferred minimally in 1:12 dilution, whereas the SEN serum showed an extinction point of 1:32, as indicated in Table I.

The most satisfactory test subject for the KU serum was PAN, who responded markedly in sites receiving tops IV and Va as well as those injected with fractions VI and VII which had been concentrated fourfold by evaporation before a fan at room temperature. Preparations Vb, I and II in original state produced ++ sensitization, whereas III could confer only + reactivity. Figure 4 is based on neutralization results in PAN and confirms the activity relationships noted among the SEN fractions, viz., tops Va, Vb and VI were most potent and VII ranked second, to be followed by IV, then by III, II and I. These differences in potency are reflected in the dilution endpoints shown in Table I. Unfortunately, the studies were interrupted after two long sessions and neither BG nor BA were analyzed.

Neutralization tests on REI and LUD confirmed the foregoing relationships, although neither was as strongly sensitized as PAN. Indeed, neither Top I nor BA transferred activity and II, III and Vb yielded only + ± reactivity—a situation which led us to evaporate Va and Vb to one-fifth volume so as to provide +++ transfer. In spite of its high protein content, BG conferred only ± sensitiveness. Dilution studies indicated that Tops Va, Vb and VI were superior to the rest but differences were, as with the SEN fractions, less decided than when the absorption technique was employed.

Correlation between activity and electrophoretic composition was examined in the same manner as for SEN serum, and results confirmed our im-

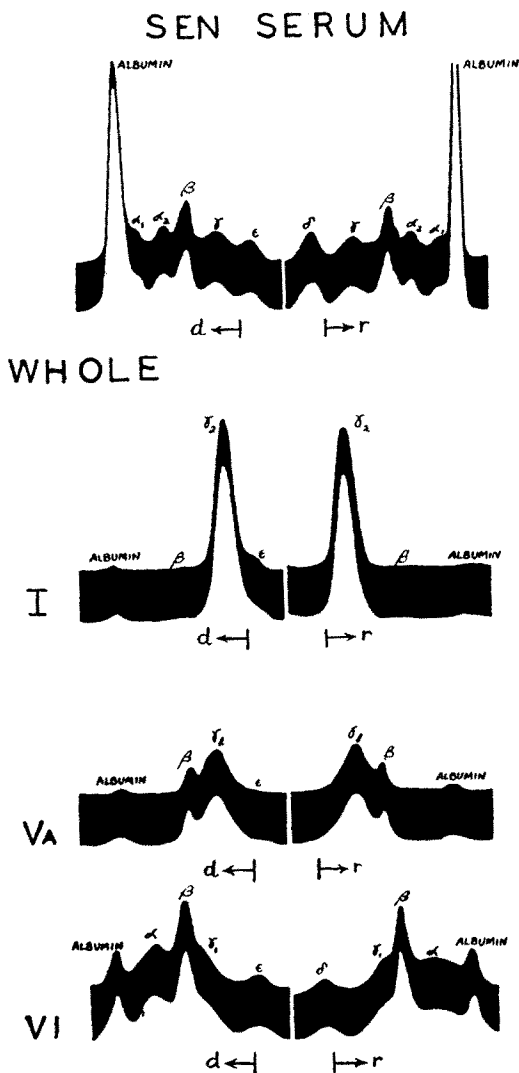


Figure 3

pression that β -globulin was the component chiefly concerned with reaginiccy. Again, it was not possible to exclude some participation by γ_1 -globulins. Pertinent electrophoretic data may be found in Table II.

KU pollen-reaginic serum (immune) had been obtained for study on another occasion three weeks after KU had completed an intensive course of ragweed pollen immunization, comprised of 9 intracutaneous inoculations at weekly intervals to bring his total dosage to 313 μ g of phosphotungstic-acid-precipitable N (31,300 PN units). On the date of the bleeding, the patient's conjunctival tolerance was found elevated by a factor of 8 from his pre-immunization require-

NEUTRALIZATION REQUIREMENT of FRACTIONS of KU 4/27/53 RAGWEED-REAGINIC SERUM

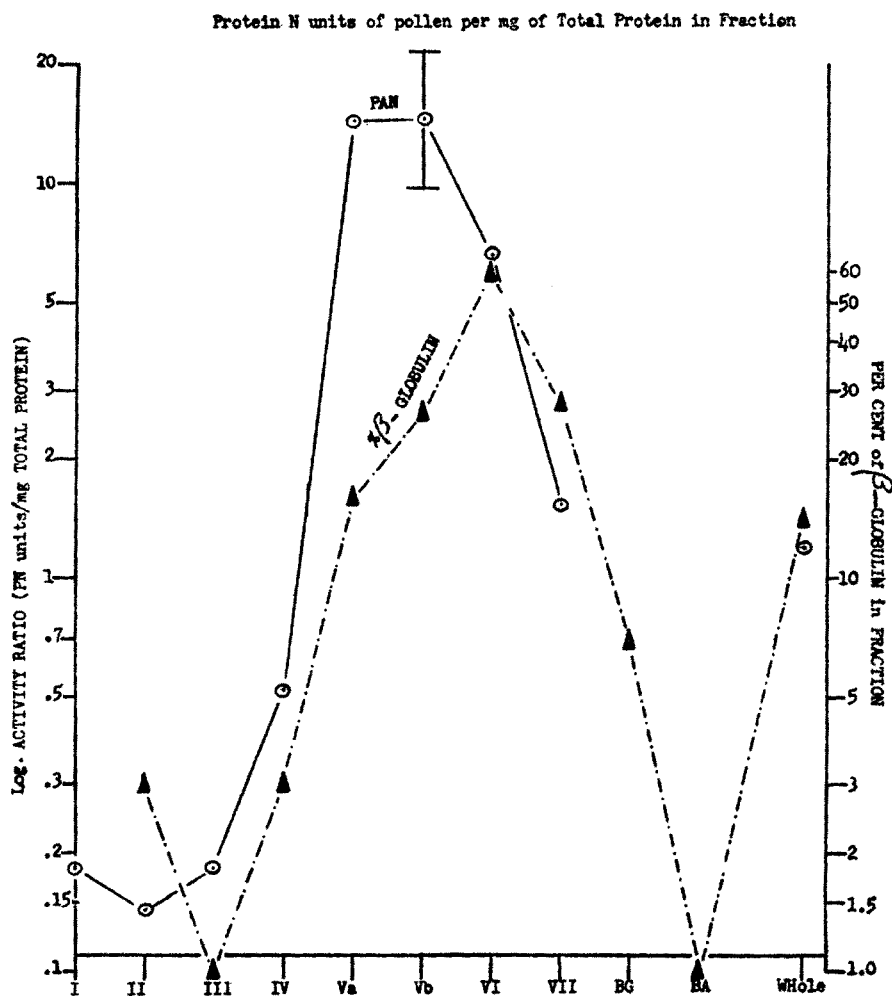


Figure 4

ment of 120-unit solution for the threshold reaction. The addition of a measure of heated, immune KU serum to a series of reagin-allergen mixtures increased the neutralization end-point 4- and 6-fold in two test subjects. The injection of sensitized skin with graded strengths of pollen extract and heated immune serum also revealed the presence of blocking antibodies in this post-immunization specimen, as will be reported shortly in an article on the heat-stable antibody.

As would be expected, conventional neutralization experiments with unheated serum of this immune type could not satisfactorily be interpreted for either antibody individually, since both reagin and blocking antibody production are

stimulated by specific therapy and the overall requirement for desensitization reflects the allergen utilized by both. The neutralizing requirement for the unheated, post-immunization KU serum was found to be 2560 PN units/ml, whereas the pre-immunization blood for 1952 required only 240 units during the same experiment on REI. That this tenfold increase was referable in part to enhanced reagin output was revealed by a four-fold increase in the dilution end-point of the 8/14/52 immune sample. (Blocking antibodies do not influence the dilution result since they diffuse away from the cutaneous sites before the 24-hour challenge is made.) This stimulation of reagin production was fortunate from the technical viewpoint since the fractions of the August serum all

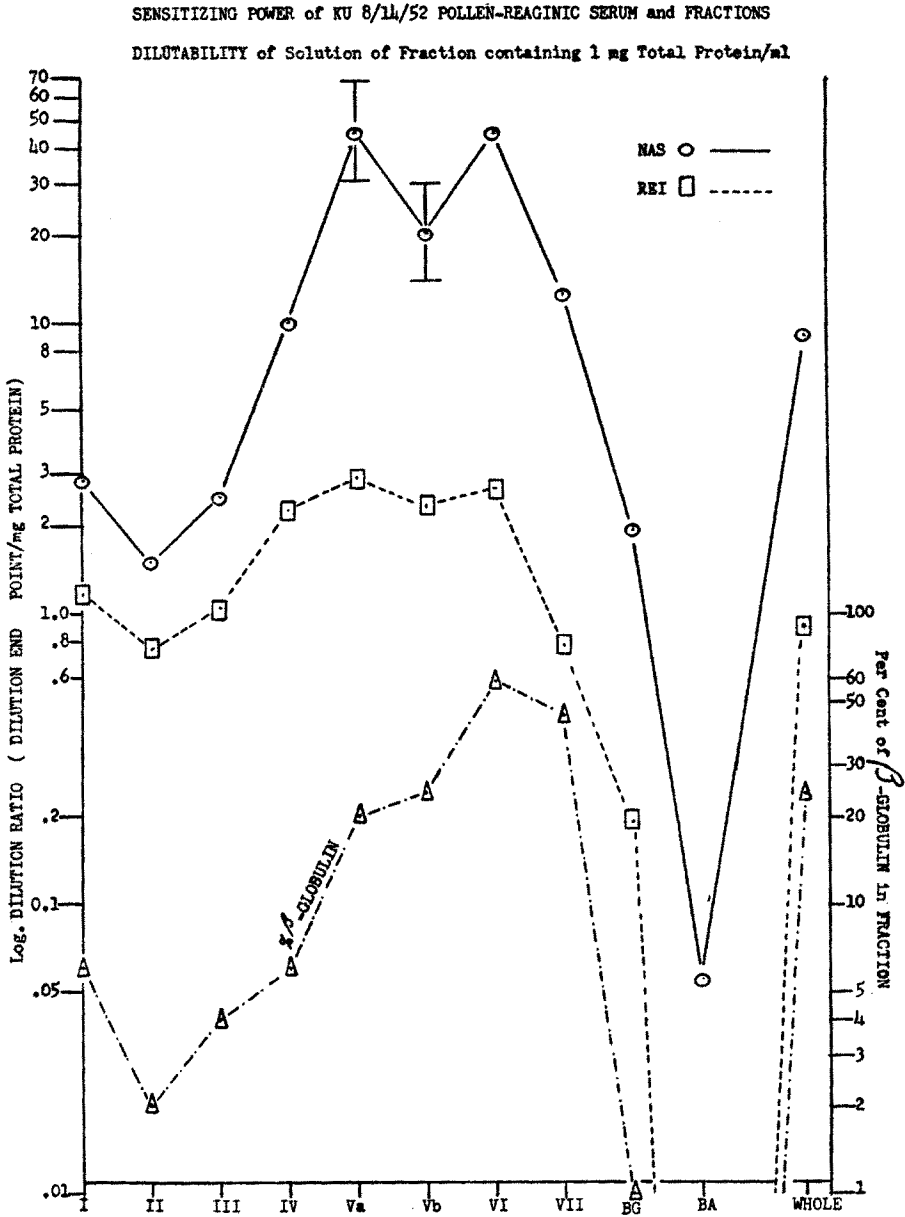


Figure 5

produced marked sensitization in subject REI except Vb and VII, which transferred ++ allergy, and BG and BA which conferred ± responses in undiluted state. The other subject, NAS, was markedly sensitized by all preparations except BA, which produced ± reactivity.

Figure 5 relates to fractions of the immune KU serum and is based on three large transference experiments in NAS as well as five in REI. Ac-

tivity relationships among the fractions were similar to those described above for the two other sera. The greatest dilutability per mg total protein was associated with Va and VI, and the ratio for Vb fell within the same range. These three most reaginic fractions were, as previously noted for corresponding preparations of the other sera, rich in β-globulin and also possessed γ₁-globulin. Although Top IV did not appear to differ

significantly in potency from these highly active derivatives, according to REI, this was attributed to the fact that he was consistently a less discriminating subject than NAS.

Correlation between activity and electrophoretic composition again suggested that activity fluctuated roughly with β -globulin content (see Figure 5), but that participation by γ_1 -globulin could not be excluded. It will be observed in the graph that both reagins and β -globulin had been concentrated in certain of the preparations as compared with the original serum. For example, in NAS the end-point of 1:640 provided an R_a of 9 for the whole serum, whereas Tops Va and VI carried the ratio of 46 (Table I). Conversely, fractions of low activity had dilution ratios below that of the starting material, Top I having a quotient of three, while that for Top II was $1\frac{1}{2}$. (The low potency of Top I is of special significance, since this fraction proved to be rich in blocking antibody for ragweed pollen, as will be reported later.)

It may be of interest at this juncture to make a few comparisons between the activity of this immune serum and that of the above-mentioned KU specimen prepared 8 months later when all blocking antibody and enhanced reagin output had been lost due to cessation of specific injections. The dilutability of solutions containing 1 mg β -globulin/ml for the β -globulin enriched fractions Va, Vb and VI was approximately as follows in REI; for the immune specimen 14, 10 and 5; and for the purely reaginic blood of April, 1953, 9, 7 and 4, respectively. The slightly lower level of the latter set of figures suggests that reagin output diminished after treatment ceased. This assumption is consistent with the finding that the β -globulin content of the whole serum fell from 24% in the immune sample to 14% in the later specimen (Table II). The difference in allergic potency of the two bloods is also apparent in the dilution ratios of Table I for the subject, REI. It will be noted, for example, that the maximal dilutability per mg total protein was 0.9 for Whole 8/14/52, whereas the corresponding figure for the sample prepared after 8 months of freedom from treatment was 0.18—a fivefold difference.

The neutralization data given in this table for the immune blood cannot be interpreted for reagin alone, for reasons stated above.

Electrophoretic mobilities of the various components constituting the four reaginic sera studied to date have been found to be greater for the

active preparations, Va through VII, than for less potent fractions I through IV, particularly in the case of γ -globulins. This, of course, was to be expected since the success of fractionation by electrophoresis-convection rests on the successive immobilization at graduated pH levels of molecules of comparable charge. It was noted that the three most potent fractions, Va, Vb and VI, prepared from any of the four reaginic sera examined were characterized by β -globulins having mean mobilities between -3.3 and -3.6 and by γ_1 -globulins with a mean mobility of $-2.0 \pm .3 \times 10^{-5}$.

DISCUSSION

Although it is customary to refer to γ -globulins and to β -globulins as though they were a distinct chemical species, this is not entirely accurate since they are both electrophoretically heterogeneous and must certainly consist of a number of protein components possessing mobilities within narrow ranges. Therefore, the above correlation of activity with overall β -globulin content can at best be expected to be approximate, as a group of components conventionally labeled β -globulin, but of slightly differing mobilities, might well possess different amounts of activity. This may be the basis of the observed differences in β -globulin activity of the four most active fractions which, in certain instances, appeared to lie somewhat outside the experimental error. The same principle applies to the designation of γ_1 - and γ_2 -globulin which we have distinguished by the arbitrary criterion of mobility at the level of -1.9×10^{-5} . (In a sense the differentiation of [fastest] γ - from [slowest] β -globulin suffers from the same arbitrariness.)

SUMMARY AND CONCLUSIONS

Three sera, prepared from individuals allergic toward cottonseed or ragweed pollen, have each been partitioned into eight fractions by electrophoresis-convection and into two other fractions by salting out with ammonium sulphate. After being assayed for N content and analyzed for electrophoretic composition, each preparation was repeatedly examined for reagins by both the neutralization and the serum-dilution procedures of passive transference. Activity was found to be concentrated in fractions Va, Vb and VI, which were composed of molecules immobilized by electrophoresis-convection at pH 6 or 5.5. These preparations were rich in β -globulin and also con-

tained γ_1 -globulin. Analysis of the relationship between activity and electrophoretic composition of the 10 fractions supported the hypothesis proposed in our earlier report that β -globulins were chiefly responsible for reactivity. Participation of γ_1 -globulins could not, however, be directly excluded by our studies.

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REFERENCES

1. NEWELL, J. M., STERLING, A., OXMAN, M. F., BURDEN, S. S. AND KREJCI, L. E., *J. Allergy*, **10**: 513, 1939.
2. SHERMAN, W. B., AND SEEBOHM, P. M., *J. Allergy*, **21**: 414, 1950.
3. COOKE, R. A., SHERMAN, W. B., MENZEL, A. E., CHAPIN, H. B., HOWELL, C. M., SCOTT, R. B., MYERS, P. A. AND DOWNING, L. M., *J. Allergy*, **22**: 211, 1951.
4. CAMPBELL, D. H., CANN, J. R., FRIEDMAN, T. B. AND BROWN, R. A., *J. Allergy*, **21**: 519, 1950.
5. CANN, J. R., BROWN, R. A., KIRKWOOD, J. G. AND HINK, J. H. JR., *J. Biol. Chem.*, **185**: 663, 1950.
6. LOVELESS, M. H. AND CANN, J. R., *Science*, **117**: 105, 1953.
7. LOVELESS, M. H., *Ann. Allergy*, **8**: 15, 1950.
8. SPIES, J. R., BERNTON, H. S. AND STEVENS, H., *J. Am. Chem. Soc.*, **63**: 2163, 1940.