THE LOCALIZATION OF SKIN-SENSITIZING ANTIBODY IN THE SERA OF RAGWEED-SENSITIVE INDIVIDUALS BY ELECTROPHORESIS*

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During the last two decades, since Tiselius electrophoresis has been available, a number of studies have been conducted in an attempt to determine the distribution of skin-sensitizing antibody in allergic sera.

The first study was that of Newell and associates in 1938 (1) who obtained the albumin and γ-globulin components of allergic serum by fractionation in the Tiselius cell. Subsequently, Sherman and Seebohm in 1950 (2) and Cooke and associates in 1951 (3) used this same technique. All three groups found some pollen reagin activity associated with the γ-globulins and none with the albumin. However, they could not separate the other globulin fractions for testing.

Campbell, Cann, and associates in 1950 (4) and in 1954 (5) reported on the use of electrophoresis convection for the separation of allergic sera in two stages, and Cann and Loveless in 1953 (6) and in 1954 (7) reported on extending the separatory phase to eight stages. These authors felt that reagin was not associated with γ-globulins. However, their method did not permit complete separation of the various serum components. In their last paper (7), Cann and Loveless stated that their data supported "the hypothesis that the β-globulins were chiefly responsible for reaginic activity, but that the possibility of participation by γ-1 globulins could not be excluded."

Menzell, Cooke, and associates in 1952 (8), in a preliminary report stated that they had modified their earlier method of separation of allergic sera in the Tiselius cell by using initial chemical fractionation followed by refractionation in the Tiselius cell. They found that reaginic activity was associated with the γ-globulins; but in some sera there was also apparent activity associated with the α-2 and β-globulins and their interzones. Vaughan, Favour, and Jaffee in 1952 (9), also using chemical fractionation, found reaginic activity mainly in Cohn fraction IIId, although there was some activity in all of their other fractions as well. They did not analyze their frac-

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tions but it is generally accepted that Cohn's fraction III is made up principally of α- and β-globulins as well as of 10 to 20 per cent of the γ-globulins (10).

A previous study from this laboratory was reported in 1955 (11) on the use of starch electrophoresis for the separation of allergic sera. It was demonstrated that starch electrophoresis lent itself well to the separation of sera into electrophoretically distinct serum fractions. Skin-sensitizing activity was found only in the β- and γ-globulins. However, since only small quantities of serum (2 to 3 ml.) were fractionated on each starch block, the blocks were divided in such a way that both β- and γ-globulins included some material located in the interzone between them. Therefore, the possibility that the activity in the β- or γ-globulin fractions was due to small amounts of contaminating γ- or β-globulins, respectively, could not be excluded. This difficulty of eliminating small amounts of contaminants was inherent in all of the previous studies, except in those in which the Tiselius cell was used for the isolation of albumin and γ-globulins only. With this problem in mind, the present study was undertaken using larger quantities of allergic serum in order to prepare electrophoretically distinct serum fractions and to correlate the reaginic activity with the protein distribution as obtained throughout the length of the starch block.

Methods and Materials

Sera.—Twenty-one separations were carried out on eighteen sera from seventeen ragweed-sensitive patients. Ten of these individuals were untreated, two had formerly been treated (but not for over 5 years) and five were under active perennial treatment. All were questioned carefully concerning viral hepatitis, malaria, and venereal disease and routine serological tests for syphilis were done (Kahn and Wassermann). One separation was performed on a pool of three sera from non-allergic individuals with negative skin tests to ragweed. Sera were obtained from both clotted blood and from citrated plasma to which thrombin[1] and/or calcium chloride had been added. The sera were Seitz-filtered and stored at 2–5°C. Storage time before separation ranged from a few hours to 6 months.

Zone Electrophoresis.—All separations were made by zone electrophoresis with potato starch as the supporting medium. The method was a modification of that described by Kunkel and Slater (13) and is presented in some detail.

In common with all methods of zone electrophoresis, the resolution of the various serum proteins is better the narrower the zone of application. To achieve this end and at the same time to separate larger quantities of serum, the lateral dimension of the starch block was increased in this study to 32 cm. as compared to 5 to 10 cm. in the previous study (11).

Approximately 3.5 to 4.5 kg. of starch[2] was thoroughly mixed with 8 liters of distilled water and the suspension allowed to stand for 1 hour. The temperature of the distilled water was 20–25°C. in eighteen separations and 5–55°C. in four separations. After 1 hour the

[1] Since it came to our attention that thrombin prepared by Cohn fractionation is a potential carrier of viral hepatitis (12) we have not used it for defibrinating plasma. However no such complications were encountered by us in the use of twelve plasmas defibrinated by thrombin and injected into approximately fifty subjects for passive transfer.

[2] Starch for chromatography, obtainable from Amend & Co., New York.

opalescent supernatant was suctioned off. This procedure was repeated with another 8 liters of distilled water. The starch was then filtered off on a large Buchner funnel and washed with a volume of cold (2–5°C.) veronal buffer (pH 8.6, ionic strength 0.05) which was approximately equal to the volume of the starch (about 2 liters). The conductivity of the filtrate at the end of the wash was identical to that of the original buffer.

Approximately 800 to 1000 ml. of cold buffer was then mixed with the starch to form a slurry. This was poured into a trough 105 cm. long, 32 cm. wide, and 1.2 cm. deep. The trough consisted of a glass plate 113 cm. long and 38 cm. wide to which a plexiglas bar 113 × 3 × 1.2 cm. was clamped on each side. The inside of the trough was lined with heavy wax paper and the ends of the trough were constructed of No. 3 Whatman filter paper strips (32 × 4 cm.) built up to a height of 1.2 cm. After the slurry was poured into the trough it was evenly distributed and the trapped air worked out by hand trowelling. The starch was then dried to a firmer consistency with filter paper strips and planed level with the side pieces of plexiglas by means of a ¼ inch glass plate with a straight edge of over 35 cm. A rectangular ditch was then cut out transversely along the center of the block.[3] It was 28 cm. long, 1.2 cm. deep, and from 0.4 to 0.8 cm. wide depending on the amount of serum to be separated. The starch, which was removed to make the ditch, was partially dried between filter papers and then mixed with 8 to 16 cc. of serum to make a slurry which was used to fill the ditch completely.[4] The exposed surface of the starch block was then covered with heavy wax paper.

The block was placed in the cold room (2–5°C.) and levelled transversely as well as longitudinally. The levelling was essential to eliminate chromatographic effects. The two ends of the block were connected by filter paper wicks to buffer vessels. Each vessel contained about 2 liters of buffer maintained at equal levels by a ¼ inch connecting tube. The buffer vessels were connected by filter paper wicks to the electrode vessels containing silver–silver chloride electrodes[5] immersed in about 1.5 liters of 20 per cent potassium chloride solution. The level of the KCl solution in the electrode vessels was always initially lower by a few millimeters than the level in the buffer vessels.

After 4 to 5 hours of equilibration a potential of approximately 700 volts was applied across the electrodes. The initial current was about 90 ma. The temperature of the starch block was checked regularly on a thermometer inserted about ¼ inch into the block. The temperature was usually 10–14°C. If it reached 15°C. the potential was reduced. The final current was usually about 150 to 160 ma. and the potential 600 volts. Electrophoretic separation lasted for 34 to 44 hours.

At this stage the block was removed from the cold room. The albumin and β-globulin bands, which could easily be seen due to the pigments associated with them, were carefully inspected for their straightness and uniformity. The bands were generally found to be straight. In a few instances the ends of the bands showed some curvature. The longitudinal portion of the starch block involved was discarded and only that portion in which the bands were straight was used for the recovery of serum protein fractions. An approximate distribution pattern of the serum fractions was obtained by applying a filter paper strip longitudinally on the starch block. After removal it was dried and stained with brom-phenol blue. The starch block was then cut transversely at 1 cm. intervals, from about 15 cm. beyond the albumin to about 10 cm. beyond the γ-globulin as determined from the brom-phenol blue "print." Accordingly, 65 to 80 cm. of the starch block was cut into 65 to 80 segments, each

[3] In a few experiments (R/284, R/285, R/287 and R/289) the ditch was cut out half way between the center and the cathodic end of the block.

[4] The position of the ditch corresponding to the zone of application is indicated by an arrow in all the figures presented later.

[5] The silver–silver chloride electrodes were prepared from ¼ inch wide and 1/32 inch thick silver strips.

1 cm. wide. A 5 cm. wide "control" segment devoid of protein, about 10 to 15 cm. from the cathodic end of the starch block, was also removed.

A 4 cm. long portion was taken from the center of each 1 cm. wide segment, mixed with 2 cc. of saline and transferred into a plastic tube provided with a perforated bottom, which was covered by 1 to 2 mm. thick filter pad made of paper powder.[6] The plastic tubes were suspended about 2.5 cm. from the bottom of standard 40 ml. glass centrifuge tubes on three glass prongs protruding from the sides of the tubes. The tubes were centrifuged at 2000 R.P.M. for 5 minutes. 5 ml. of normal saline was then added to each plastic tube and the tubes were again centrifuged at 2000 R.P.M. for 5 minutes. The total volume of the eluates was 7.5 $\pm$ 0.3 ml. 1 ml. aliquots were taken from the eluates and analyzed for protein by the colorimetric method of Lowry and associates (14). The distribution of protein in the starch block was diagrammatically represented by plotting the optical density of each eluate against the segment number.

In the determination of the protein concentration by the Lowry method (14), it was found that contamination of the eluates by small amounts of starch interfered with the optical density readings, particularly for solutions with low protein concentrations. The method of elution by centrifugation, as described, proved to be satisfactory in this respect as well as rapid, and the recovery of the proteins was about 98 per cent.

On the basis of the protein distribution curve the 1 cm. wide segments were eluted individually or in groups depending on the experiment. The segments were placed in sintered glass or Buchner funnels provided with three No. 3 Whatman filter papers and a volume of normal saline approximately equal to the volume of the starch was filtered through. The eluates were then placed in Visking tubings and these were hung in front of electric fans at room temperature. When the volume of the eluates had been reduced approximately by one-half they were placed in normal saline at 2–5°C. to dialyze overnight. The eluates of pooled segments were concentrated to 5 to 8 ml. and those of single 1 cm. wide segments to 3 ml. The concentrated eluates were then filtered through Swinny type Seitz filters[7] into sterile bottles and stored at 2–5°C. until tested.

In ten separations the 1 cm. wide segments in the β- and γ-globulin regions were prepared individually, and the segments under the albumin and α-peaks were pooled separately.

In ten other separations the two to four individual segments, which constituted the central portion of each peak of the protein distribution curve were pooled. These fractions are referred to as pooled "peak fractions" in this paper. In these ten separations the interzonal segments were pooled separately. In one experiment the starch block was divided longitudinally into two halves. The 1 cm. wide segments of one-half of the starch block were eluted individually. The segments under each protein peak of the other half were pooled for each fraction.

Skin Tests.—Tests for reaginic activity were done by classical passive transfer techniques on non-allergic volunteers who gave negative skin tests to ragweed. Each volunteer was used as a test subject for only one serum separation. 0.05 ml. of whole serum or of the individual fractions was introduced intradermally and 48 hours later the sites were again injected intradermally with 0.03 ml. of ragweed pollen extract containing 4000 nitrogen units per ml.[8] or with 0.05 ml. of ragweed pollen extract containing 1600 nitrogen units per ml. Standard tuberculin syringes and No. 27 needles were used for the intradermal injections. The reactions were graded from 0 to 4+ by the same observer each time 20 to 30 minutes after the ragweed injections. In most cases the activity of the fractions was evaluated in terms of ten-

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[6] Solka-floc S.W. 40A, Brown, Co., Montreal.

[7] Obtainable from Becton Dickinson & Co., Rutherford, New Jersey, specially designed for handling small quantities.

[8] 1 nitrogen unit equals 10^{-5} mg. of nitrogen.

fold serial dilutions of the whole serum which was tested at the same time. To eliminate the possibility of false positive reactions when testing, the following controls were used. Sites were prepared with the concentrated protein-free eluates from the "control" segments of the starch. These sites as well as unsensitized skin sites were challenged with ragweed pollen extract. The fractions of pooled normal serum were also tested for reaginic activity on two subjects as a further control on the whole method. The fractions of seven separations were tested in quadruplicate, of five separations in triplicate, of three separations in duplicate, and of six separations singly. In addition, in one case tenfold serial dilutions of the fractions were compared for reaginic activity with similar dilutions of the whole serum.

Tiselius Electrophoresis.—All of the whole allergic sera as well as the fractions obtained from seven separations were analyzed by free electrophoresis in veronal buffer (pH 8.6, ionic strength 0.1) in a Spinco-Tiselius apparatus[9] at 0.8°C. Standard 11 ml. cells were used for the analyses of the sera and 2 ml. micro cells were used for the analyses of the fractions. The intermediate cell sections of both cells were 92 mm. long. The electrode vessel on the descending side of the cell was hermetically sealed while the electrode vessel on the ascending side was left open. The sera and albumin fractions were diluted with buffer to 1 per cent protein concentration. All sera and fractions were dialyzed against buffer at 2–5°C. for 48 hours prior to electrophoresis. Sharp initial boundaries were obtained by electrolytic "compensation" (15). The initial boundaries were displaced a distance of 12 mm. by "compensation." This required about 25 minutes. The total protein content of the sera was determined refractometrically using an Abbe type refractometer and by the Rayleigh interference optical system incorporated into the Spinco-Tiselius apparatus. The relative concentrations of the various components were calculated from the schlieren or integral fringe patterns of the ascending limb by the method of Tiselius and Kabat (16) or Longsworth (17) respectively. Mobilities of the various components were calculated for 0°C. from the descending schlieren patterns by the method of Longsworth and MacInnes (18). Conductivities of the dialyzed protein solutions were measured at 0°C. with an LKB bridge[10] in calibrated conductivity cells. All mobilities were corrected for the small but observable and not reproducible anodic migration of the delta and epsilon "stationary" boundaries.

The displacement of the "stationary" boundaries was in general negligible, particularly when compared with the electrophoretic migration of the faster components. However, since this displacement was not reproducible and may have been caused by small cathodic reactions occurring within the hollow shaft of the silver–silver chloride electrodes, some experiments were done with freshly prepared electrodes. In these experiments the delta and epsilon boundaries remained practically stationary, even occasionally exhibiting a negligible but detectable cathodic migration. In consequence all mobilities were corrected for these small displacements.[11]

Paper Electrophoresis.—For additional characterization the fractions from some separations were compared with the components corresponding to them in the whole serum by paper electrophoresis. The apparatus used for these experiments was similar to that of Valmet and Svensson (19) and will be described elsewhere (20). The individual fractions were applied side by side with the untreated serum on the same strip of No. 1 Whatman filter paper. After electrophoresis in veronal buffer (pH 8.6, ionic strength 0.1), the papers were dried and stained with amidoschwartz (10B) according to the method of Pucar (21).

Reversible silver–silver chloride electrodes were used for both paper and starch electrophoresis in preference to the standard steel and carbon or platinum electrodes since they eliminate changes in pH during electrophoresis.

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[9] Manufactured by Specialized Instruments Corp., Belmont, California.

[10] Manufactured by LKB Produkter, Stockholm.

[11] These displacements were observed particularly in experiments with micro cells.

Ultracentrifugation.—For the determination of sedimentation constants of the various fractions a standard Spinco optical ultracentrifuge was used. The average temperature of the rotor was about 20.5°C. and the speed was 59,728 R.P.M. The fractions were dialyzed against normal saline prior to ultracentrifugation. When sufficient material was available several experiments were done to establish the dependence of the sedimentation constants on the concentration and the values were extrapolated to infinite dilution. They were not corrected for standard conditions. The fractions of five separations were analyzed.

RESULTS

Fig. 1 shows the protein distribution of the same serum as obtained by free electrophoresis and on two starch blocks. These curves are representative. The top curve was obtained by free electrophoresis. The curves labelled "*C*" and "*H*" were obtained from blocks prepared with starch washed with cold or hot distilled water respectively. The starch blocks in both instances show cathodic migration of the γ-globulins due to a relatively large electro-osmotic effect. The two peaks, almost invariably present in the γ-globulin region on the "*C*" starch blocks, and designated as γ-1 and γ-2 globulins were not as clearly delineated on the "*H*" starch blocks and were absent on free electrophoresis. On the other hand the α-1 peak which was always clearly delineated on free electrophoresis was resolved poorly on the "*C*" and "*H*" starch blocks. In most sera a small peak, usually absent in free electrophoresis, preceded the albumin on both types of starch blocks. In general the protein distribution curves obtained from either type of starch block were reproducible when the same serum was separated under the same conditions, as shown in Fig. 2. In this figure each pair of curves corresponds to the same serum; the time of electrophoresis was almost identical (as indicated).

Fig. 3 shows the free electrophoretic patterns for the segments pooled under each peak as obtained by starch electrophoresis. A single peak was observed in each case. However, the fractions eluted from the interzone regions of the starch block resolved themselves into bifid peaks on free electrophoresis.

Fig. 4 shows a typical pattern obtained by paper electrophoresis of a whole serum and its corresponding fractions. As can be seen the fractions albumin, α-2, β, γ-1 and γ-2 globulins, labelled on the basis of the protein distribution curve as obtained from the starch block, correspond to the same components in the whole serum when analyzed by paper electrophoresis.

Table I lists the mobilities and the absolute and relative concentrations of the various serum components as obtained by free electrophoresis of the whole allergic sera. Values obtained for normal sera in this laboratory are also included. All the values for the sera of ragweed-sensitive patients fall within our normal ranges.

Table II lists the electrophoretic mobilities of the starch fractions analyzed by free electrophoresis. The normal mobility values for the globulins in this laboratory are also listed. The values for the α-2 and β-globulins correspond

quite closely to the normal ranges. Since γ-1 and γ-2 globulins are not normally differentiated by free electrophoresis of the whole sera in veronal buffer (at pH 8.6, ionic strength 0.1 and 1 per cent protein concentration) normal values for their mobilities could not be calculated. The normal mobility values for γ-globulins were calculated from the migration of the peak of the asym-

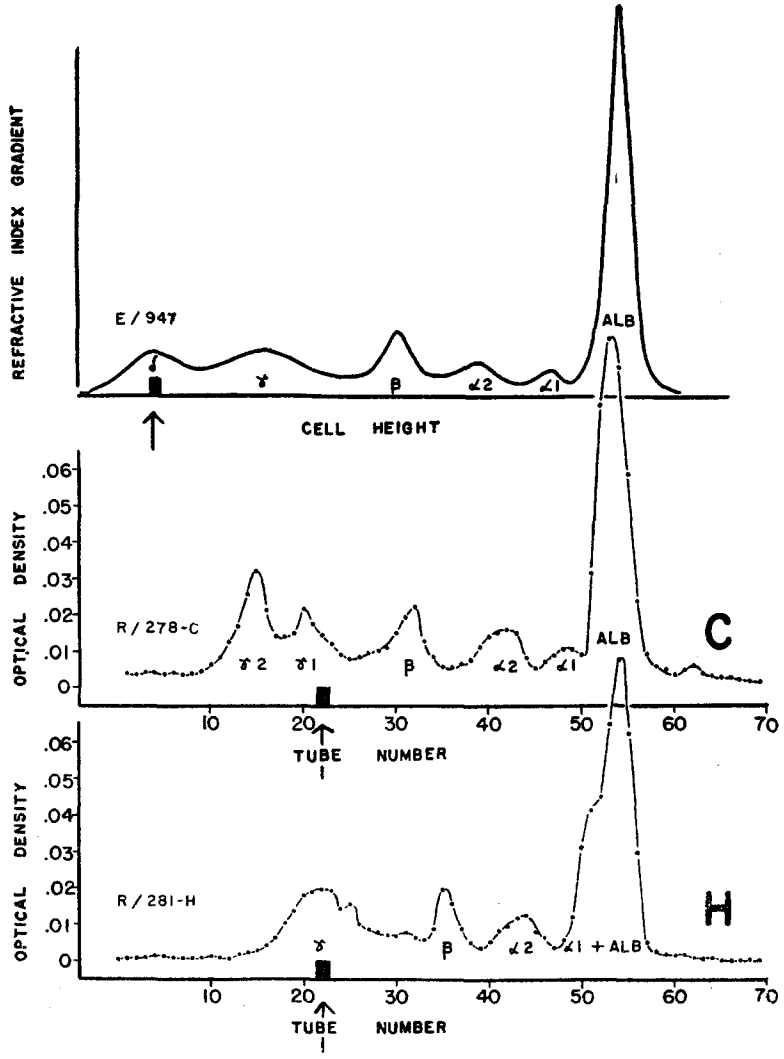

FIG. 1. The top curve represents the schlieren pattern of a serum obtained by free electrophoresis. The lower two curves represent the protein distributions for the same serum as obtained on two starch blocks. The capital letters *C* and *H* refer to blocks prepared with starch washed with cold and hot water. The arrows and black squares indicate the positions of the initial boundaries and of the zones of application.

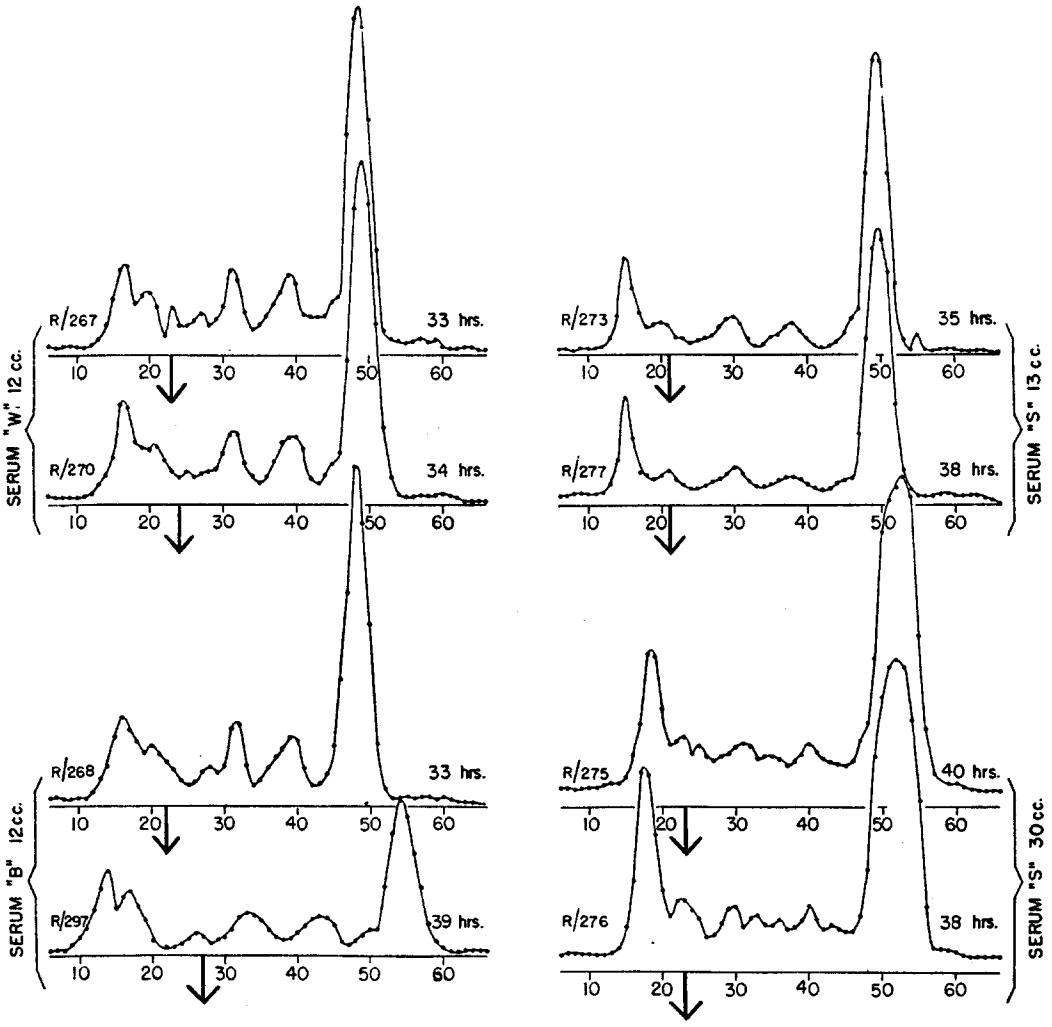

FIG 2. Each pair of curves represents two separations of the same serum on two starch blocks. The amount of serum separated, the length of each experiment and the zones of application are indicated.

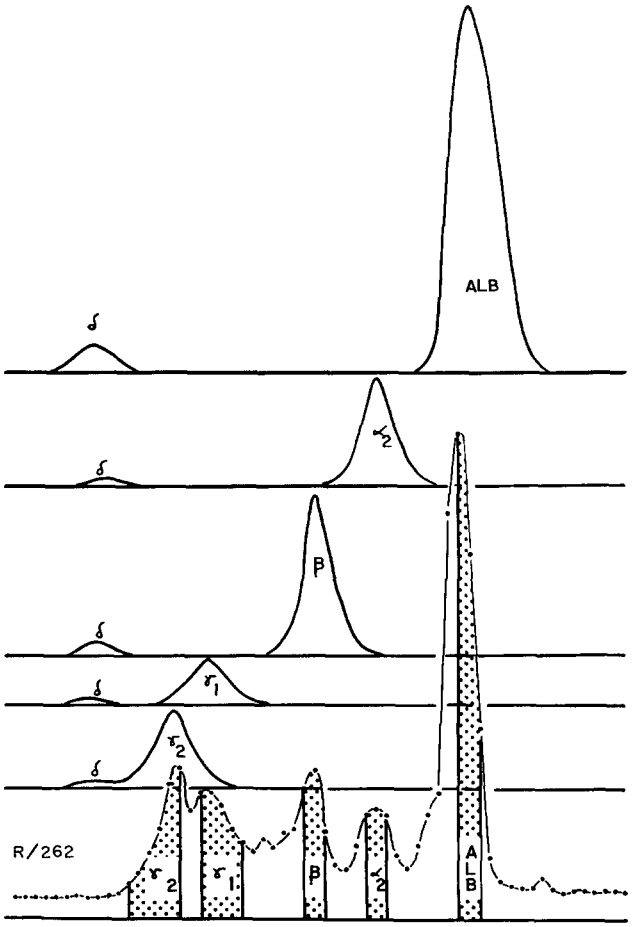

FIG. 3. The bottom curve represents the protein distribution as obtained by starch electrophoresis. The dotted surfaces correspond to the pooled "peak fractions." The upper five diagrams represent the schlieren patterns for the corresponding "peak fractions" analyzed by free electrophoresis over almost identical periods of time.

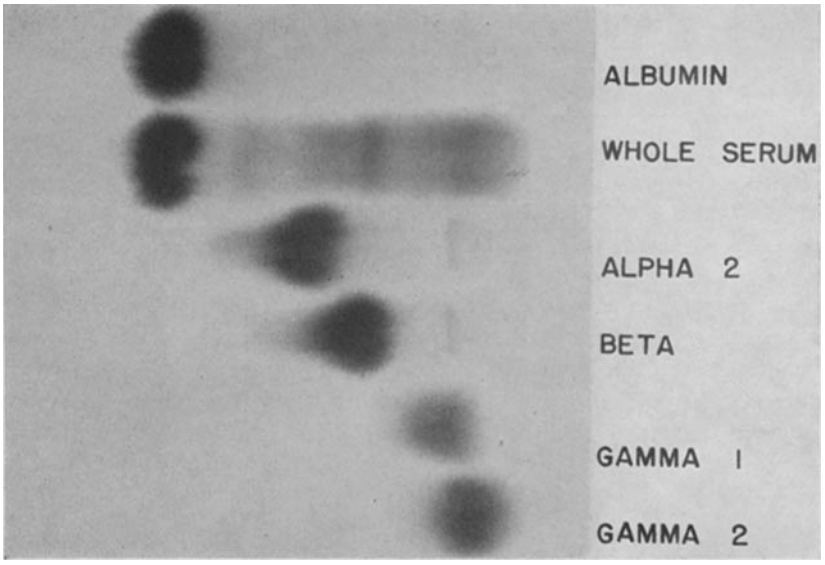

FIG. 4. Paper electrophoresis of serum fractions and of whole serum.

metrical γ-globulin schlieren patterns. These normal values should, therefore, be intermediate between mobility values of the γ-1 and γ-2 globulins.

TABLE I

Exp. No.	T.P.	Alb			α_1			α_2			β			γ		
		per cent	*gm./100 cc.*	μ^*	*per cent*	*gm./100 cc.*	μ^*	*per cent*	*gm./100 cc.*	μ^*	*per cent*	*gm./100 cc.*	μ^*	*per cent*	*gm./100 cc.*	μ^*
E/843	7.80	59.38	4.63	6.40	3.75	0.29	5.49	9.16	0.71	4.31	11.98	0.93	3.13	15.73	1.24	1.19
E/447	7.86	55.90	4.39	6.60	4.09	0.32	5.64	8.78	0.69	4.65	16.	1.26	3.34	15.22	1.20	1.56
E/1065	7.98	63.55	5.07	6.55	4.82	0.38	5.54	8.12	0.65	4.36	10.41	0.83	3.06	13.09	1.04	1.47
E/795	7.68	65.42	5.02	6.60	4.27	0.33	5.55	8.85	0.68	4.69	10.38	0.80	3.35	11.07	0.85	1.39
E/770	8.28	59.04	4.89	6.62	5.13	0.42	5.61	8.94	0.74	4.47	12.62	1.04	3.46	14.27	1.18	1.53
E/838	8.88	56.98	5.06	6.44	4.06	0.36	5.44	7.59	0.67	3.88	12.96	1.15	3.10	18.40	1.63	1.27
E/798	9.06	58.12	5.26	—	3.37	0.30	—	7.74	0.70	—	11.10	1.00	—	19.67	1.78	—
E/799	8.46	57.68	4.88	—	3.63	0.31	—	7.19	0.61	—	11.92	1.00	—	19.57	1.65	—
E/771	7.86	57.05	4.48	6.47	4.74	0.37	5.49	8.43	0.66	3.93	17.29	1.36	3.27	12.48	0.98	1.37
E/947	6.86	52.70	3.61	6.38	3.38	0.23	5.44	7.97	0.55	4.31	13.58	0.93	3.37	22.36	1.53	1.33
E/840	6.84	63.37	4.33	6.37	4.20	0.29	5.48	5.05	0.34	4.12	12.29	0.84	3.23	15.08	1.03	1.32
E/850	—	64.13	—	6.39	3.52	—	5.41	5.90	—	4.16	13.32	—	3.01	13.13	—	1.49
E/996	7.32	61.89	4.53	6.56	4.93	0.36	5.58	5.54	0.40	4.14	14.09	1.03	3.21	13.55	0.99	1.41
E/835	7.74	62.89	4.87	6.42	4.29	0.33	5.26	7.39	0.57	4.02	11.85	0.92	3.23	13.77	1.06	1.62
E/1078	6.66	61.89	4.12	6.44	4.22	0.28	5.36	7.82	0.52	4.07	14.17	0.94	3.26	11.89	0.79	1.31
E/839	7.74	61.87	4.79	6.45	3.16	0.24	5.40	8.29	0.64	4.17	12.21	0.94	3.25	14.47	1.12	1.25

Normal Sera

	T.P.	Alb		α_1		α_2		β		γ	
		per cent	*gm./100 cc.*	*per cent*	*gm./100 cc.*	*per cent*	*gm./100 cc.*	*per cent*	*gm./100 cc.*	*per cent*	*gm./100 cc.*
22 Males											
Average	7.93	59.46	4.73	4.68	0.36	8.60	0.69	12.92	1.06	14.33	1.08
S.D.	±0.56	±3.16	±0.31	±1.81	±0.06	±1.43	±0.16	±1.27	±0.12	±2.46	±0.19
14 Females											
Average	7.92	58.40	4.69	5.17	0.42	9.81	0.72	12.42	1.00	14.18	1.08
S.D.	±0.71	±2.31	±0.39	±0.73	±0.07	±0.98	±0.13	±1.18	±0.11	±1.76	±0.17

Mobilities‡	Alb*	α_1*	α_2*	β*	γ*
Average	6.51	5.55	4.28	3.32	1.45
S.D.	±0.13	±0.15	±0.10	±0.11	±0.11
Range (95 per cent)	6.25–6.77	5.25–5.85	4.08–4.48	3.10–3.54	1.23–1.67

* The values for mobilities were divided by -10^{-5} cm.²/volt sec.

‡ These results calculated for 14 normal sera. Concentration of protein in the Tiselius cell was about 1 per cent

Furthermore, since the asymmetry seen in the schlieren patterns shows a preponderance of the slower moving materials, the normal values for γ-globulins should be less than the average of the $(\gamma-1 + \gamma-2)/2$ mobility values. One would also expect that on pooling $\gamma-1$ and $\gamma-2$ fractions, a single asymmetrical schlieren peak should be formed with a mobility value

TABLE II

Mobilities of Globulin Fractions Determined by Free Electrophoresis*

Normal sera‡	α_1	α_2	β	γ_1	γ_2
Average	5.55	4.28	3.32	1.45	
S.D.	±0.15	±0.10	±0.11	±0.11	
Range (95 per cent)	5.25–5.85	4.08–4.48	3.10–3.54	1.23–1.67	
Exp. No.	**α_1**	**α_2**	**β**	**γ_1**	**γ_2**
R/261 (P)§	—	4.28	3.01	1.75	1.51
R/262 (P)	—	4.67	3.47	1.73	1.17
R/266 (P)	5.70	4.67	3.35	2.20	1.54
R/281 (P)	—	4.42	3.88	1.98	—
R/284 (P)	—	3.86	3.41	1.59¶	
R/268 (i)‖	—	4.23	3.06	1.27	1.21
"	—	—	3.50	1.56	1.26
"	—	—	—	1.97	1.52
"	—	—	—	1.99	1.78

* All values have been divided by -10^{-5} cm.²/volt sec.

‡ These values are based on results obtained with 14 normal sera.

§ In experiments designated by letter (P), the eluates corresponding to the peaks on the protein distribution curve were pooled.

‖ In experiment R/268 (i) individual eluates were analyzed.

¶ In experiment R/284 (P) γ_1 and γ_2 globulins were pooled.

TABLE III

Reaginic Activity of "Pooled Fractions" as Determined by Passive Transfer Tests

Exp. No.	Alb	α	β	γ_1	γ_2
R/261	0	0	0	++++	++
R/262	0	0	0	++++	++
R/265	0	0	0	++++	++
R/266	0	0	0	++++	++
R/263	0	0	0	+++	+++
R/264	0	0	0	+++	+++
R/289	0	0	0	±	±
R/287	0	0	0	+	+++
R/291	0	0	0	±	++++
R/267	0	0	0	0	0
R/285	0	0	0	0	0

of normal γ-globulin. The data for the mobilities of the γ-globulins in Table II fulfil these requirements. The data for the last experiment listed in Table II (R/268) represent mobility values of the fractions eluted from individual 1 cm. wide segments. Owing to the low concentration of protein in the latter fractions, the peaks of the corresponding schlieren patterns were not as well defined as for the pooled fractions. Consequently the mobility values for the

fractions of the last experiment in Table II are more scattered. Nevertheless, they fall within the expected ranges.

Table III lists the results of the passive transfer tests of the pooled "peak fractions" obtained from eleven separations on the starch blocks. Only the results obtained with pooled fractions are listed, although activity was also found to a lesser degree in the interzones between β- and $\gamma-1$ and between $\gamma-1$ and $\gamma-2$. In nine instances, in which activity was successfully transferred after separation, it was found in both γ-fractions in varying degrees. A greater degree of activity was associated with $\gamma-1$ fractions four times, with $\gamma-2$ fractions twice, and it was equally divided between $\gamma-1$ and $\gamma-2$ fractions three times. Activity was never found to be associated with the other fractions. However activity was lost in two of the eleven separations and was much diminished in one. No activity was found to be associated with the fractions of the pooled normal sera which were tested on two subjects.

Figs. 5 and 6 show the activity curves and patterns of the electrophoretic separations in which each 1 cm. wide segment was eluted and tested individually. The four diagrams in Fig. 5 and the lower right diagram in Fig. 6 (R/292-C) represent the results obtained with the sera of five allergic individuals. The two upper diagrams of Fig. 6 (R/278-C and R/281-H) represent duplicate results obtained with the serum of another allergic subject, fractionated on a "C" and an "H" starch block. The lower left diagram (R/277-C) of Fig. 6 represents one separation out of three with the serum of another subject. Since the results of all three separations were virtually identical only one diagram is given. The individual fractions of one remaining serum separated and tested were not active on passive transfer.

The activity curves do not connect the individual experimental points but are drawn arbitrarily as single smooth broad peaks. This method of presenting the activity curves was chosen because such curves were essentially reproducible on repeating the separation, whereas the actual jagged curves connecting all the experimental points were not. However, irrespective of the manner in which the curves would be drawn, the activity peaks still fall in the $\gamma-1$ and $\gamma-2$ regions of the protein distribution curves. The broad distribution of activity would indicate that reagin is not electrophoretically homogeneous, in this respect resembling γ-globulins in general. The fact that activity was found trailing into the β-globulins in some of the separations (R/278-C, 281-H, 292-C, 268-C) is entirely consistent with the interpretation that reagin is electrophoretically heterogeneous. In experiment R/289 a large reaction was given by the eluate of segment 23 of low protein concentration whereas that of segment 12, which corresponds to the $\gamma-2$ globulin peak with a much higher protein concentration, gave no reaction. A similar situation existed in experiment R/268 in which the eluate of segment 25 containing 0.08 per cent protein (determined refractometrically) gave a reaction approximating that of the undiluted serum. The whole serum after

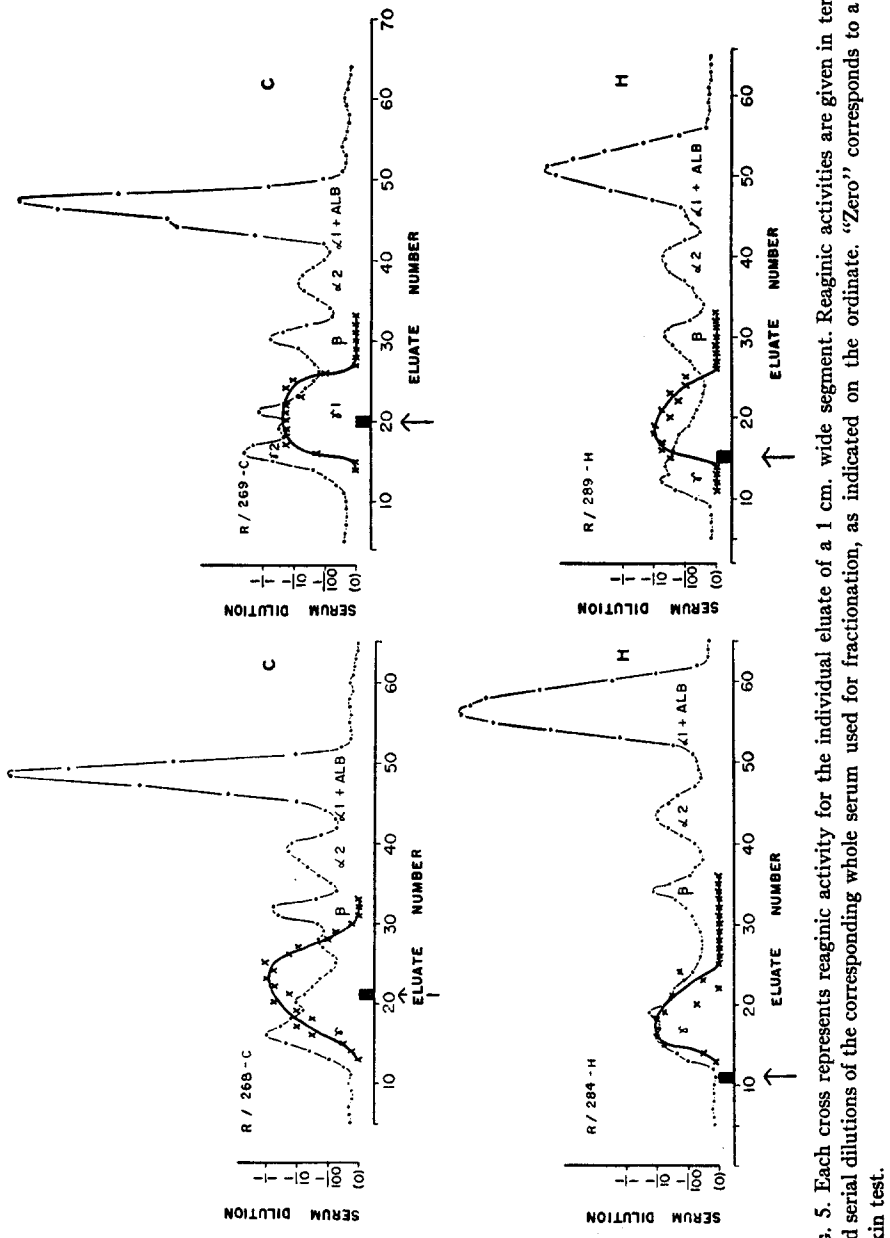

FIG. 5. Each cross represents reaginic activity for the individual eluate of a 1 cm. wide segment. Reaginic activities are given in terms of tenfold serial dilutions of the corresponding whole serum used for fractionation, as indicated on the ordinate. "Zero" corresponds to a negative skin test.

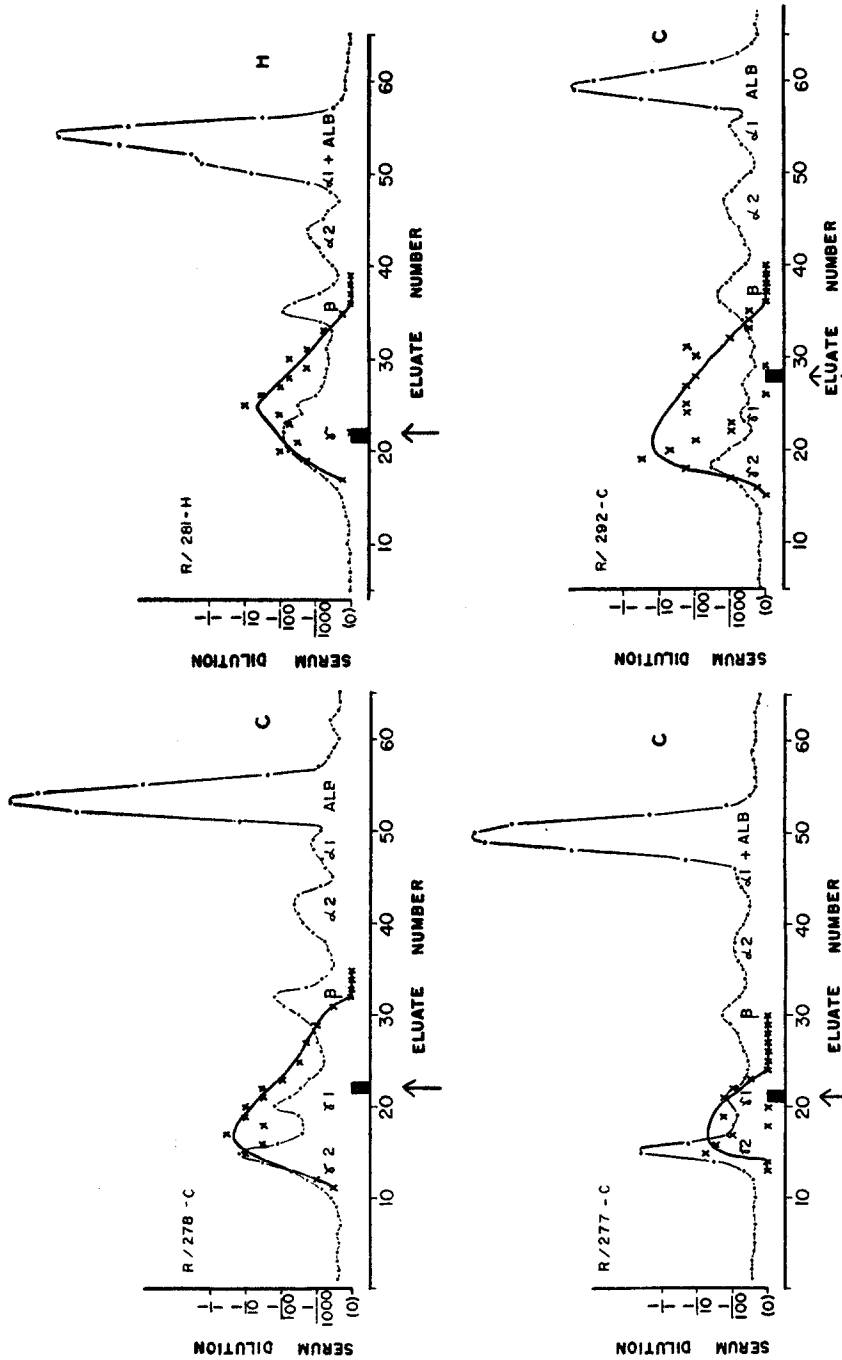

FIG. 6. Each cross represents reaginic activity for the individual eluate of a 1 cm. wide segment. Reaginic activities are given in terms of tenfold serial dilutions of the corresponding whole serum used for fractionation, as indicated on the ordinate. "Zero" corresponds to a negative skin test.

100-fold dilution still had detectable activity. The implications of these large reactions given by fractions with low protein concentrations will be discussed later. The fact that no activity could be demonstrated in the single eluates corresponding to the β-globulins in sera R/269-C, R/284-H, R/289-H, and R/277-C clearly demonstrates that activity was not associated with these globulins.

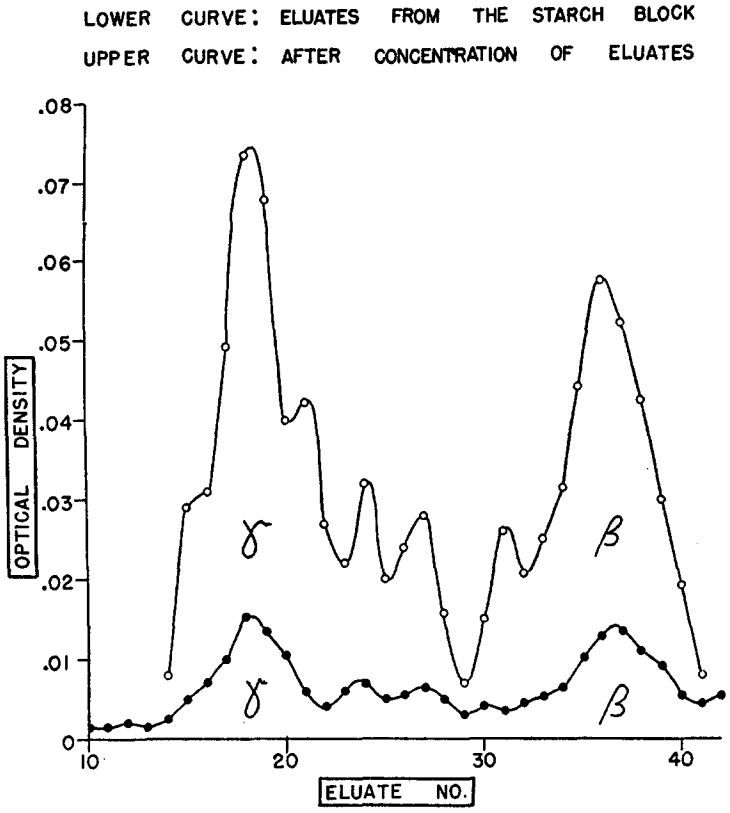

FIG. 7.

Fig. 7 shows the protein distribution curve in the region of β- and γ-globulins for one of the separations (R/292) as done routinely on the starch block compared with the protein distribution of the concentrated eluates used for injection after pervaporation, dialysis, Seitz filtration, and storage a 2–5°C. It can be seen that the recovery of protein was not uniform from segment to segment and that the more dilute solutions tended to suffer relatively greater losses.[12] However, the routine protein distribution curve is in

[12] To cut down these losses and to make the experimental activity curves more reproducible a series of experiments are now in progress to investigate the optimum conditions for pervaporation of dilute protein solutions.

most cases accurate to within a factor of two or three, whereas the fractions are evaluated in terms of serum dilutions varying by a factor of ten. Thus substantial errors in the activity curves probably only occurred in relatively few segments (segments 26 and 29 in this particular starch block contained no activity as can be seen in Fig. 6).

In the ultracentrifuge each $\gamma-1$ and $\gamma-2$ globulin fraction was resolved into two components. The sedimentation constant for the major component was 6.6 Svedberg units and that for the minor component was 18 to 20 Svedberg units. Only two of the γ-globulin fractions had enough material for multiple analyses at different concentrations for extrapolation of the sedimentation constants to infinite dilution. The values for the other fractions were of the same order of magnitude and represent values generally accepted for γ-globulins.

DISCUSSION

Sterile sera stored at 2–5°C. for periods up to 6 months showed negligible loss of skin-sensitizing activity as measured by tenfold serial dilutions. The sterile serum fractions, however, stored under the same conditions showed loss of activity when measured biweekly. Freezing the serum fractions also caused measurable loss of activity when testing by tenfold serum dilutions.

The concentrated eluates from the starch blocks, including the "control" fractions, caused some local irritation when injected intracutaneously. This irritation did not interfere appreciably with the passive transfer tests since it usually subsided within the 48 hour period prior to the injection of the ragweed pollen extract. Since it was suspected that the irritation might be due to the presence of soluble starch (22) the starch was washed with water and buffer as described earlier but there was no decrease in the irritation. In view of Kunkel's (23) recent suggestion that some pyrogenic or toxic materials present in the starch are removed more effectively at higher temperatures, the starch was washed with hot water at 55°C.[13] in some experiments. However, the irritation was not significantly decreased and, in view of the better resolution of the $\gamma-1$ and $\gamma-2$ peaks on the "C" starch blocks, the starch was washed with cold water at 20–25°C. in most of the experiments.

Since bacterial growth of varying amounts was almost always observed during pervaporation,[14] bacterial exotoxins were considered as a possible cause of skin irritation. Therefore, the eluates were Seitz-filtered into sterile Visking tubing prior to pervaporation in the last few experiments. Skin irritation in these experiments was reduced to a transient immediate wheal and

[13] Higher temperatures cannot be used for washing because of gel formation.

[14] Pervaporation took up to 1 week to complete. Although two-thirds of that time was spent for dialysis against normal saline at 2–5°C., during the remaining time the temperature of the eluates was 12–18°C. depending on the ambient temperature and the local relative humidity.

flare type of reaction comparable to that frequently seen with whole serum. This immediate reaction was observed with varying intensity in only some recipients, and in the case of whole serum it tended to increase with aging and after heating the serum to 56°C. Usually it started to fade 20 minutes after injection and completely disappeared after 1 to 4 hours, whereas the irritation caused by injection of material concentrated by pervaporation without preliminary Seitz filtration took 36 to 48 hours to disappear. In addition the irritation brought about by the injection of the latter material frequently caused the recipients some discomfort.

Dialysis against normal saline, which was part of the process of concentration of the eluates, was done in order to prevent concentration of the buffer salts to the point at which they might lead to serious denaturation of the various serum proteins. In the previous studies in this laboratory (11, 24) concentration of dilute protein solutions was effected by dialysis against concentrated dextran solutions. This method was abandoned in the present study for two reasons. To concentrate the largest eluates from 400 ml. to 8 ml. with dextran would have been prohibitively expensive. Furthermore, even the most homogeneous batches of commercially available dextran (average molecular weight 70,000) contain sufficient dextrans of smaller molecular weight which diffuse into the Visking bag thus preventing the concentration process from reaching completion. This latter point has been discussed more fully elsewhere (24).

The possibility that the two gamma peaks, which were resolved on the starch blocks but were absent on free electrophoresis, could have been caused by some specific alteration of the γ-globulins due to their interaction with the starch was investigated. All the eluates from one separation were pooled, Seitz-filtered into sterile Visking tubing, concentrated by pervaporation, and then analyzed in the Tiselius apparatus. The schlieren pattern obtained was similar to that of the original serum and exhibited no split of the γ-globulins. The only apparent difference was the absence of the β-anomaly in the reconstituted serum in the descending pattern.[15] These findings are considered as further evidence of the mildness of starch electrophoresis, making it particularly useful as a fractionation method for biologically active materials.

Similar protein resolutions were obtained with veronal buffers (pH 8.6) of ionic strengths 0.05 and 0.1. The lower ionic strength was preferred however, and was used throughout these experiments. The higher ionic strength at the same voltage resulted in higher amperage which caused heating and excessive evaporation from the starch block. If evaporation is not kept to a minimum the electrophoretic resolution is poor due to chromatographic displacement of the bands towards the center of the starch block. This effect

[15] A similar observation was made in another study in this laboratory and the possible explanations are discussed there (24).

was demonstrated in an experiment by increasing the current after a good separation had been obtained. After some time the bands started migrating in reverse towards the center of the block, thus defeating the purpose of the electrophoretic separation.

The protein distribution curve obtained by the Lowry method is similar only qualitatively to the schlieren pattern obtained by Tiselius electrophoresis. The data for the former curve are obtained by a chemical reaction between the various proteins and the Folin-Ciocalteu reagent, and it is conceivable that the affinity of the reagent is different for the various serum proteins. The schlieren pattern, on the other hand, is obtained by changes in the refractive index gradient throughout the Tiselius cell and, fortuitously, the specific refractive indices of the various serum proteins are almost constant ($1.76 - 1.86\ 10^{-5}$/gm.) (25).

The fractions were designated albumin, α-, β-, $\gamma-1$, and $\gamma-2$ globulins on the basis of the protein distribution curve obtained from the starch block. The designation of the fraction located on the starch block between the slow gamma ($\gamma-2$) and the β-globulins as $\gamma-1$ globulins and not as $\beta-2$ globulins might appear as somewhat arbitrary. However, the results of free electrophoresis (Table II) and of paper electrophoresis (Fig. 4) demonstrate clearly that the mobility of this fraction is much closer to the normal range of mobilities for γ-globulins than to the normal range of mobilities for β-globulins. In consequence, the designation used in this study is considered to be justified, particularly since there are no objective and universally accepted criteria for the designation of intermediate components located between the well defined β- and slow γ-globulin peaks. The data of Table II were obtained in an effort to minimize the degree of arbitrariness and subjectivity in the designation of the various fractions isolated from the starch block. These results also demonstrate that starch electrophoresis is an adequate method for the separation of electrophoretically distinct serum fractions. Thus starch electrophoresis can be considered both a preparative and analytical method for the separation of serum proteins.

The sensitizing dose for the passive transfer tests was 0.05 ml. of serum or fractions instead of the more conventional dose of 0.10 ml. because in preliminary experiments involving a series of twofold reagin dilutions the results were more reproducible. The use of tenfold serial dilutions of whole sera proved very satisfactory as a means of comparison of reaginic activity. It eliminated discrepancies in the results due to biological variations displayed by the passive transfer recipients and made it possible to express the results of each experiment in terms of serum dilutions instead of reactions graded arbitrarily 1 to 4+. Serial dilutions by a factor smaller than ten did not give reactions of sufficiently marked differences for differential grading, and consequently the curves obtained were not as reproducible. It should be noted that

in spite of the scatter of points representing the activity distribution for each separation, the results of the skin tests were reproducible when tested on more than one subject. Therefore, it was felt that the scatter of the individual points representing activity for repeated separations of the same serum might be accounted for by the losses of protein and/or reaginic activity occurring during the process of concentration of the eluates. In consequence the over-all distribution of activity was represented by a broad curve and not by the jagged curve connecting all the individual points, especially since the former was representative for repeated separations of the same serum.

Our results show quite clearly that in the sera fractionated ragweed reagin was associated with the γ-globulins with a tendency to be in one of its faster moving components. These results are in agreement with those of Kuhns (26) who localized human non-precipitating skin-sensitizing diphtheria antitoxin in the faster moving γ-globulins by starch electrophoresis.

These results also explain the earlier ones reported from this laboratory (11) which showed that reagin was associated with gamma and beta globulins. In the light of the present results, it is probable that the reaginic activity previously detected in the β-globulin fraction was due to contaminating γ-globulins. In most of the previous studies (4–9, 11), as indicated in the introduction, this difficulty could not be circumvented and from our data the results of these studies could be explained on the assumption that the active fractions were contaminated with γ-globulins.

In a few instances, however, in the papers referred to in the introduction, reaginic activity was found in fractions which contained no γ-globulin (5, 8). There are two possible explanations. One is that in some cases reagin may be associated with another serum fraction. Although the present study comprises the largest series of results to date, it represents the situation in the sera of only fourteen ragweed-sensitive individuals. The second possibility is that the proteins designated in this study as $\gamma-1$ may have been designated differently by other workers, or may have behaved as one of the other serum proteins under different experimental conditions. The ability of slightly different experimental conditions to alter the electrophoretic mobility of proteins is well known. This is generally accepted as the cause for the differences of the normal values obtained for mobilities in different laboratories. To avoid ambiguity in the interpretation of our data much effort was made to identify the fractions by free electrophoresis, paper electrophoresis, and ultracentrifugation. Unfortunately, in all other studies reported in which reaginic activity has been found without any γ-globulin present no such data are available.

Some of the workers using the Tiselius cell for the direct separation of γ-globulins have been able in some experiments to recover only 10 to 25 per cent of the reaginic activity of their sera (3, 8). They have inferred from this

that the rest of the activity must be in other fractions. However this might not be the only explanation. Using free electrophoresis as a method of fractionation, only small amounts of slow moving globulins present in the serum can be isolated. In the light of our results that reaginic activity is associated with $\gamma-1$ and/or $\gamma-2$ globulins it is not surprising therefore, that only a fraction of reaginic activity associated with the whole serum was recovered. Furthermore, as was mentioned earlier reaginic activity associated with the various fractions diminishes more rapidly than that of the whole serum. This effect would result in an even further decrease of recovery of activity, and might also explain the loss of activity observed in three sera out of the seventeen in this series, and in four out of the eleven in the previous series.

SUMMARY

Starch electrophoresis was used to separate allergic sera into electrophoretically distinct albumin, α-, β-, and γ-globulin fractions. The identity of the various fractions was established by free and paper electrophoresis. The γ-globulin fractions were also characterized by ultracentrifugation.

The serum skin-sensitizing antibody to ragweed pollen was shown to be associated with the γ-globulins in fourteen allergic sera by means of passive transfer technique. The mobility of the skin-sensitizing antibody corresponded to that of $\gamma-1$ and/or $\gamma-2$ globulins. All the other serum fractions were devoid of reaginic activity.

Reaginic activity *per se* was not directly related to protein concentration.

The discrepancies between these results, and those obtained by others and ourselves previously are discussed.

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