The Physiological Role of the Lymphoid System. IV. The Separation of γ-Globulin into Physiologically Active Components by Cellulose Phosphate Chromatography*

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ABSTRACT: Chromatography of human γ-globulin on cellulose phosphate columns yielded four distinct fractions, I–IV. The amino acid analyses of the fractions show remarkable similarities though differences do exist.

The technique is simple and reproducible. γ-Globulin A (γA) was confined to fraction I and γ-globulin M (γM) to fraction II, both as minor components. γ-Globulin G (γG) was present in all four fractions; exclusively in III and IV and as the major component of I and II. These results were confirmed by ultracentrifugation, immunoelectrophoresis, and immunodiffusion. The isohemagglutinins were distributed among all the fractions, whereas the antibody activity against influenza virus was detectable only in fraction II.

The physiological effect of γ-globulin on autologous blood cells has been demonstrated recently in the dog. It was shown that a specific leucophilic γ-globulin that binds to autologous leucocytes considerably stimulates the phagocytic activity of the cell against pathogenic *Staphylococcus aureus* (Fidalgo and Najjar, 1967a,b). In its absence, only minimal phagocytosis occurs. This γ-globulin has been isolated as a separate major fraction by cellulose phosphate chromatography. Erythrophilic γ-globulin, on the other hand, separates into two major fractions. These bind to erythrocytes, moderate the loss of ions from the internal environment, and strengthen the cell membrane against shearing forces (Najjar *et al.*, 1967). Furthermore, when the level of one of these fractions in the plasma is lowered, as was shown to be the case following splenectomy, the half-life of the red cell is considerably diminished and anemia develops. This is corrected by raising the level of this fraction through parenteral supplementation (Fidalgo *et al.*, 1967).

The purpose of this communication is to describe this procedure for the fractionation of γ-globulin in detail and describe the properties of the fractions obtained. The following papers of this issue present further evidence of the importance of both the eyrthrophilic and the leucophilic γ-globulins.

Despite the complexity of γ-globulin, important advances in the characterization of its various properties were made following fractionation on DEAE-cellulose (Fahey and Harbet, 1959), DEAE-Sephadex (Sela *et al.*, 1963), and CM-cellulose columns (Mandy and Nisonoff, 1962).

The present report describes yet another chromatographic procedure using cellulose phosphate (CP),[1] for the fractionation of human serum γ-globulins. The procedure is equally applicable to dog and rabbit γ-globulin. The properties of the various fractions in the dog, particularly in terms of the binding capacity to autologous red blood cells (erythrophilic) and white blood cells (leucophilic), are very similar to those obtained in man. The only difference lies in the relative proportions of the fractions. For this reason, it suffices to limit the description of the method to the fractionation of human γ-globulin.

Four major fractions were obtained with a different assortment of biological properties which have not hitherto been observed. By the usual chemical and biological criteria these fractions remain heterogeneous. Nevertheless, they represent effective separation of γ-globulin A (γA) and γ-globulin M (γM) in two separate fractions, I and II, respectively. Both of these fractions also contain γ-globulin G (γG). Fractions III and IV are made up only of γG with the exclusive property of binding to autologous blood cells in isotonic sucrose at low ionic strength (Harshman and Najjar, 1963; Najjar *et al.*, 1967). Under these conditions about 60% of the bound γ-globulin on red cells is derived from fraction III and the remainder from fractions II and IV. Leucocytes, on the other hand, bind γ-globulin exclusively from fraction IV (Fidalgo and Najjar, 1967a,b). It was these latter findings that prompted additional characterization of these fractions notwithstanding their persistent heterogeneity. The procedure is highly reproducible and yields frac-

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[1] Abbreviations used: CP, cellulose phosphate; γA, γ-globulin A; γM, γ-globulin M; γG, γ-globulin G.

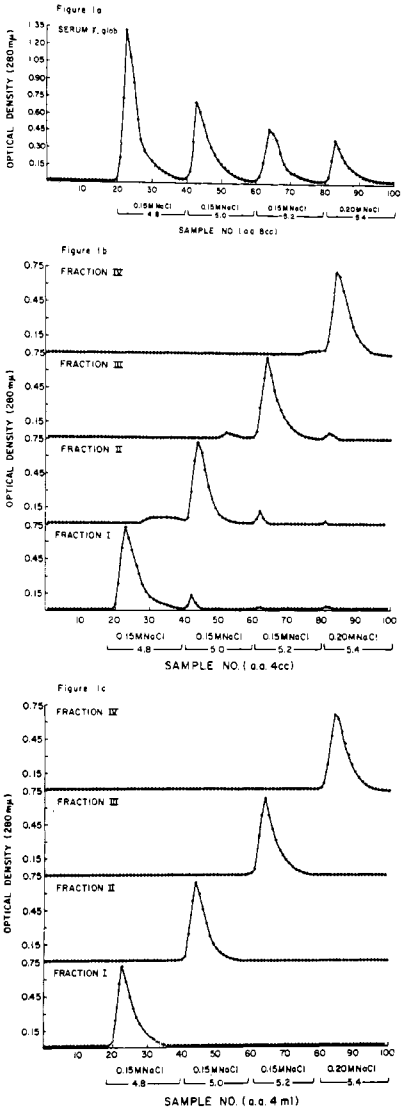

FIGURE 1: Cellulose phosphate column chromatography of human serum γ-globulin. For the initial chromatography (a) 150 mg of γ-globulin was applied to a column 4 × 12 cm. For the first and second rechromatography, b and c, respectively, 20 mg of each γ-globulin fraction was applied to 1.7 × 12 cm columns. For details, see text.

tions that have distinctive characteristics chromatographically, immunochemically, and biologically. A preliminary description of the fractionation procedure has been reported (Thomaidis *et al.*, 1963).

Materials and Methods

Preparation of γ-Globulin. γ-Globulin was prepared from plasma of normal human donors; blood being collected in citrate (0.16 M) and glucose (0.16 M) (pH 7.5), 10 ml/100 ml of blood. The plasma was defibrinated by adding 2.5 ml of 1 M $CaCl_2$/100 ml of plasma, followed by incubation at 37° for 1 hr, at which time the clot was fully formed. It was separated by filtration

TABLE 1: The Fractionation of Human γ-Globulin into Four Components on Cellular Phosphate Columns.[a]

Source of the Sample	Size of the Column (cm)	No. of Expt	mg of Protein Applied	γ-Globulin									
				Fraction I		Fraction II		Fraction III		Fraction IV		Recovery	
				mg	%	mg	%	mg	%	mg	%	mg	%
Pooled samples	4 × 12	48	150	49 ± 6	33 ± 5	39 ± 5	26 ± 4	28 ± 3	19 ± 2	21 ± 2	14 ± 2	137 ± 10	91 ± 7
	1.7 × 12	160	25	8 ± 1	32 ± 5	6 ± 1	24 ± 5	5 ± 0.5	20 ± 2	4 ± 0.5	16 ± 2	23 ± 2	92 ± 8
Individual samples	1.7 × 12	10	25	9 ± 0.5	36 ± 3	6 ± 0.4	24 ± 2	4 ± 0.2	16 ± 1	4 ± 0.2	16 ± 1	23 ± 1	92 ± 4

[a] γ-Globulin was prepared by ammonium sulfate fractionation from individual serum samples or from pooled sera. The per cent of each fraction was based on the amount of protein applied to the column. For details, see text.

TABLE II: Amino Acid Composition of Human γ-Globulin Fractions.[a]

Fraction	CP Fractions I	CP Fractions II	CP Fractions III	CP Fractions IV	γ-Globulin Average	γ-Globulin Crumpton and Wilkinson (1963)
Asp	(7.42) 65	(8.83) 75	(8.62) 76	(8.25) 71	(8.18) 71	(7.77)
Thr	(6.86) 66	(8.23) 80	(7.40) 74	(7.35) 72	(7.41) 72	(7.04)
Ser	(8.87) 97	(11.65) 124	(10.00) 110	(9.76) 105	(10.01) 109	(9.12)
Glu	(9.73) 76	(11.45) 87	(10.97) 86	(11.26) 87	(10.69) 83	(11.18)
Pro	(5.51) 57	(6.35) 64	(6.35) 66	(6.40) 66	(6.04) 62	(6.40)
Gly	(3.12) 55	(4.47) 77	(3.57) 63	(3.56) 62	(3.64) 64	(3.37)
Ala	(2.90) 41	(3.70) 51	(2.76) 39	(3.43) 48	(3.16) 45	(3.29)
Val	(6.32) 65	(7.29) 72	(7.01) 72	(7.16) 72	(6.85) 70	(7.92)
Half-Cys	(2.28) 11	(3.88) 18	(4.14) 20	(3.79) 19	(3.32) 16	(2.07)
Met	(0.63) 5	(1.10) 8	(0.89) 7	(0.77) 6	(0.81) 7	(0.93)
Ileu	(1.72) 15	(1.97) 17	(2.03) 18	(2.12) 19	(1.89) 17	(2.16)
Leu	(6.67) 60	(7.35) 64	(7.09) 68	(7.67) 68	(7.08) 64	(7.40)
Tyr	(5.08) 32	(4.67) 28	(6.03) 37	(5.64) 34	(5.22) 32	(5.76)
Phe	(3.74) 26	(4.28) 29	(4.19) 29	(4.28) 29	(4.04) 28	(4.07)
Lys	(6.12) 49	(7.10) 54	(7.34) 58	(7.29) 57	(6.80) 54	(7.06)
His	(1.94) 14	(2.24) 16	(2.30) 17	(2.53) 18	(2.17) 16	(2.44)
Arg	(3.69) 24	(3.78) 24	(4.20) 27	(4.43) 28	(3.91) 25	(4.02)

[a] The protein (1.12 ODU at 280 mμ) was subjected to hydrolysis in 6 N HCl at 100° for 20 hr in sealed tubes. Quantitative amino acid analysis was performed on the amino acid analyzer. Serine and threonine values were uncorrected. Cysteine was measured as cystine. Values in parentheses represent g of amino acid/100 g of protein. Whole numbers refer to the number of residues/mole of 150,000*g*. The protein weight calculated for each fraction was based on an extinction of 1.12/mg of γ-globulin. Tryptophan analysis was not done. The uniformly low values of the various amino acid residues in fraction I may relate to a high tryptophan content. Averages were calculated on the bases of fraction I = 37%, II = 27%, III = 21%, and IV = 15% of total γ-globulin. For details, see text.

through gauze or by centrifugation. The defibrinated plasma was adjusted to 33% of saturation with solid $(NH_4)_2SO_4$ and held at 0° for 8–18 hr. The precipitated γ-globulin was dissolved with one volume of 0.15 M NaCl and reprecipitated by adding solid $(NH_4)_2SO_4$ to 50% saturation. After 5–8 hr at 0° the precipitated γ-globulin was again collected by centrifugation and dissolved in a minimum volume of 0.05 M acetate buffer (pH 4.8) and dialyzed for 24 hr against three changes of the same buffer, 1 l. each. The protein concentration in milligrams per milliliter was calculated from the absorbance of the clear solution at 280 mμ using an extinction coefficient of 1.12/mg of protein.

Preparation of the Column. Batches (10 g) of cellulose phosphate (Selectacel, no. 1393, capacity 0.86 mequiv/g, Carl Schleider & Schuell Co., Keene, N. H.) were washed three times with 700 ml of 0.05 M acetate buffer (pH 4.8) and the fine particles were decanted after each wash. The cellulose was then poured into an appropriately sized glass column (see below) and washed *in situ* with 0.05 M acetate buffer (pH 4.8) until the optical density of the effluent at 280 mμ reached 0.010 or less. The column can be used repeatedly after thorough washing with the buffer at the termination of each chromatography.

Results

Fractionation of γ-Globulin. The use of cellulose phosphate as a chromatographic support permitted the fractionation of the γ-globulins into four major groups. It also made it possible to process γ-globulins routinely in batches as high as 150 mg. Total recovery exceeded 85% and the conditions used for elution (pH 4.8–5.4 in NaCl, 0.15–0.2 M) are so mild that the entire procedure could be run at room temperature without any detectable alterations in the physicochemical or biological properties of the γ-globulins. Pilot-sized fractionation of about 25 mg of the sample was routinely run in 2 hr on columns (1.7 × 12 cm). A total of 260 pilot-sized columns and 98 large batch columns have been run with excellent reproducibility. A detailed description of a typical large batch fractionation is given in detail.

γ-Globulin (150 mg) in 10 ml of 0.05 M acetate buffer (pH 4.8) was applied to a cellulose phosphate column (4 × 12 cm) previously equilibrated with the same buffer. The flow rate was adjusted to 2 ml/min and the effluent was collected in 4.0-ml portions. Three to four holdup volumes of the solvent buffer (400–550 ml) were run through the column to ensure

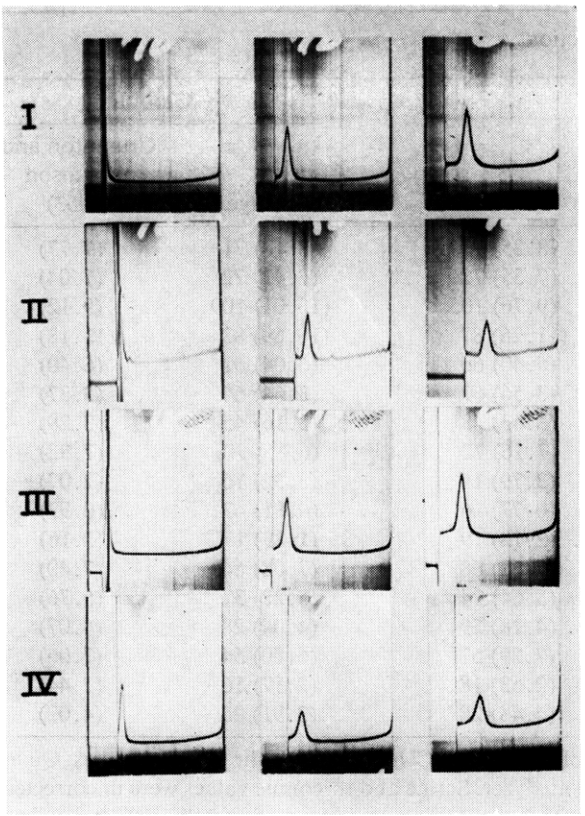

FIGURE 2: Sedimentation patterns of purified γ-globulin fractions in 0.15 M NaCl. Each fraction (8 mg/ml) was centrifuged at 4° and 59,780 rpm in a Spinco Model E ultracentrifuge. Migration of the protein is from left to right. The photographs were taken 4, 20, and 45 min after attainment of maximum speed. Fractions I, III, and IV show a 7S component only, while fraction II contains in addition a minor peak of 19 S. All fractions were obtained after the second rechromatography on CP columns.

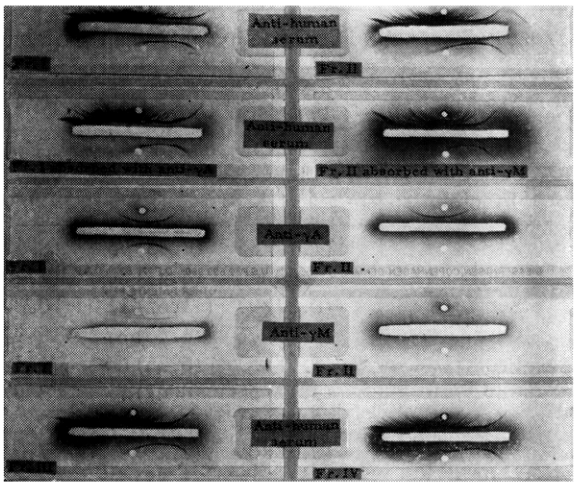

FIGURE 3: Immunoelectrophoretic analysis of CP γ-globulin fractions. A chromatographic fraction (0.02 ml, 8 mg/ml) was placed in the bottom well and electrophoresis was performed in 0.072 M Veronal buffer (pH 8.6) for 75 min. Human serum (0.02 ml) was run concurrently in the top well as control. A rectangular block of agar was then removed between the two wells to accommodate 0.2 ml of horse antihuman serum. The slide was then incubated in a moist chamber at room temperature for 24 hr. Fraction I shows precipitin lines corresponding to γG and γA, fraction II to γG and γM, and fractions III and IV to γG only. After exhaustive absorption of I with anti-γA and II with anti-γM, the corresponding lines disappeared. All fractions were obtained after the second rechromatography.

thorough escape of the occasional contaminating albumin. No γ-globulin appears in this effluent. The first γ-globulin fraction was eluted with 0.05 M acetate buffer (pH 4.8) in 0.15 M NaCl and contained 51 mg of protein (34%). Elution with the same buffer was continued until the absorbance at 280 mμ corresponded to that of the buffer. This was routinely achieved with each fraction after 1.3–1.7 holdup volumes of the eluting buffer had passed through the column, *i.e.*, until 180–230 ml of effluent was collected. The second fraction contained 37 mg of γ-globulin (25.0%) and was eluted using 0.05 M acetate buffer (pH 5.0) in 0.15 M NaCl; and the third fraction, 29 mg (19%), was eluted at pH 5.2 under otherwise identical concentrations of buffer and salt concentration. Finally, the fourth fraction, 20 mg of γ-globulin (13%), was eluted by raising both the pH to 5.4 and the NaCl concentration to 0.2 M. Analysis of the results in terms of total protein recovered in each fraction gave a standard deviation of around ±5% for the first two fractions and around ±2% for the last two (Table I).

Rechromatography was then performed on the separate fractions. Each fraction was adjusted to 0.60 saturation with solid ammonium sulfate and allowed to stand for 8 hr at 0°. The precipitated protein was then collected by centrifugation and dialyzed against 0.05 M acetate buffer at pH 4.8. Conditions for rechromatography were as detailed above. This permitted considerable purification and further demonstrated that fraction I was contaminated with a small amount of fraction II, fraction II with a small amount of fractions I and III, and fractionI II with a small amount of fraction IV. Fraction IV, by contrast, was relatively homogeneous. A third chromatography did not separate contaminants and indicated that the fractions obtained after the second chromatography were relatively homogeneous. The results are presented graphically in Figure 1.

Amino Acid Composition of the Various Fractions. Each of the fractions tested had undergone three repeated chromatographic separations on cellulose phosphate columns. As was pointed out earlier, three chromatographies are sufficient to yield individual reproducible fractions free of contamination one with

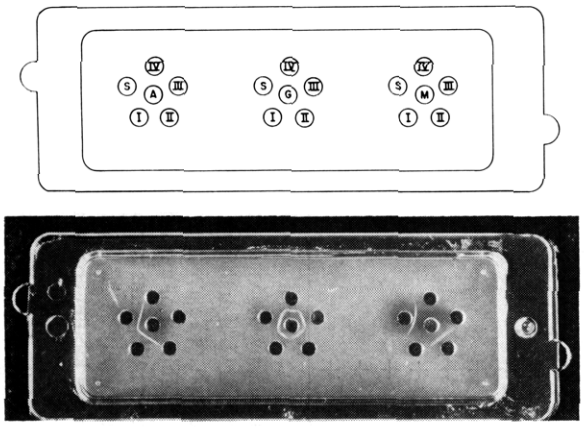

FIGURE 4: Micropatterns of Ouchterlony analysis of the γ-globulin fractions using specific goat antisera for γA and for γM and horse antisera for γG. Each γ-globulin fraction (0.5 mg) (I–IV) was added to the outer wells as indicated. The original serum was added to the fifth outer well (S). Antisera to γA, γG, and γM were added to the center wells, respectively, from left to right. The plate was sealed with masking tape and incubated at 4° for 72 hr. Precipitin bands with anti-γA appeared only in fraction I, with anti-γM only in fraction II, and with anti-γG in all four fractions. With all three antisera, the original serum showed the corresponding band. All fractions were obtained after the second rechromatography.

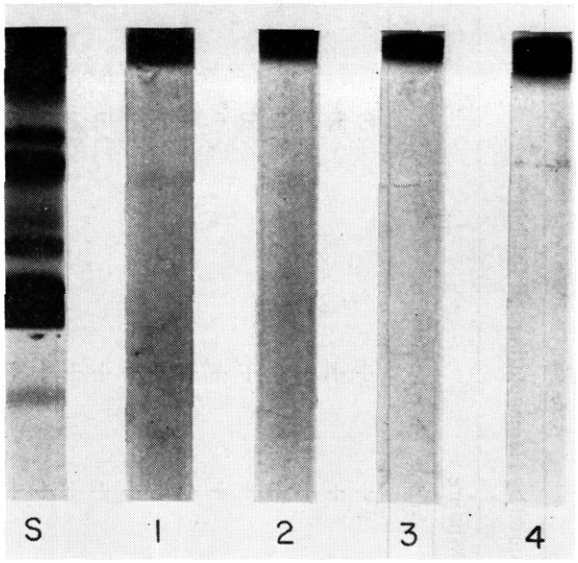

FIGURE 5: Disc electrophoresis was performed with 0.1 mg of each of the four fractions as shown. Normal serum (S) (0.003 ml) was run concurrently as control. All fractions show one band. Fraction I appears contaminated to a negligible amount with a β component.

the other. The protein was subjected to hydrolysis in 6 N HCl at 100° for 20 hr in sealed tubes. The samples were dried by lyophilization and analyzed for amino acid composition using the column method (Piez and Morris, 1960) developed by Technicon Corp. Table II shows in parentheses the values obtained for each amino acid (g/100 g) and alongside each the calculated number of residues per mole of 150,000*g*.

There is a remarkable similarity between all the fractions, especially between fractions III and IV, involving all the amino acids including the basic residues (lysine, histidine, and arginine) as well as the acidic residues (glutamic and aspartic). For comparison, the values in grams of amino acid/100 g of protein previously reported (Crumpton and Wilkinson, 1963), are included in the table.

Physicochemical Properties of the Fractions. Physicochemical and biological properties of each of the fractions have been determined on samples obtained after the third chromatography. Over-all recovery at this stage was approximately 65%. The main loss occurred during ammonium sulfate precipitation.

A. SEDIMENTATION PROPERTIES. A sample of each fraction was dialyzed in 500 volumes of 0.15 M NaCl, adjusted to a protein concentration of 8 mg/ml, and centrifuged in a Spinco Model E ultracentrifuge at 59,780 rpm. Fractions I, III, and IV yielded a single peak with a sedimentation coefficient in the region of 7 S. Fraction II, however, contained two peaks; the main peak was in the 7S region while the smaller peak moved faster with the characteristic of 19 S (Figure 2).

B. IMMUNOELECTROPHORESIS. For a study of the distribution of the major immunologic classes among the various fractions, each of the fractions was analyzed by agar immunoelectrophoresis (Crowle, 1961). Horse antihuman serum (Hyland Laboratories, Los Angeles, Calif.) was used to develop the electrophoretic patterns. In each analysis, a sample of whole human serum was run as a control. As shown in Figure 3, fraction I yielded two bands; the heavy one with the mobility of γG and the other with that of γA. The latter disappeared completely after absorption with specific anti-A serum. Fraction II also contained two bands; the main one corresponding to γG and the minor to γM. Following absorption with anti-γM serum no γM line could be discerned. Fractions III and IV contained only γG. The finding of γM only in fraction II parallels that obtained by ultracentrifugation.

C. GEL DIFFUSION. Confirmatory results were obtained when the antigenic properties of each fraction was tested by micropattern as well as macropattern analysis of the Outchterlony diffusion method (Crowle, 1961). In this case individual specific antisera (horse anti-γG, goat anti-γA, and anti-γM (Hyland Laboratories, Los Angeles, Calif.)) for each of the immunoglobulin classes were also used. All four fractions yielded a single precipitin line with anti-γG serum. The merging of the lines indicates identity of the component common to all fractions. In contrast, anti-γA yielded a precipitin line only with fraction I, and anti-γM serum only with fraction II (Figure 4). These

TABLE III: Antiinfluenza Activity of Each Fraction Obtained from Pooled γ-Globulin of A, B, and O Blood Types, as Measured by Hemagglutination Inhibition.[a]

γ-Globulin Fraction	Strain Tested	mg of γ-Globulin in the Final Reaction Mixture							Titer Corresponding to Whole Serum
		0.250	0.125	0.063	0.031	0.016	0.008	0.004	
Group A+	PR-8	0	+	+	+	+	+	+	20
I	Lee	0	+	+	+	+	+	+	20
II	PR-8	0	0	0	0	0	+	+	320
	Lee	0	0	0	0	0	+	+	320
III	PR-8	+	+	+	+	+	+	+	0
	Lee	+	+	+	+	+	+	+	0
IV	PR-8	±	+	+	+	+	+	+	0
	Lee	±	+	+	+	+	+	+	0
Group B+	PR-8	+	+	+	+	+	+	+	0
I	Lee	+	+	+	+	+	+	+	0
II	PR-8	0	0	0	0	0	+	+	320
	Lee	0	0	0	0	0	+	+	320
III	PR-8	+	+	+	+	+	+	+	0
	Lee	+	+	+	+	+	+	+	0
IV	PR-8	+	+	+	+	+	+	+	0
	Lee	+	+	+	+	+	+	+	0
Group O+	PR-8	0	+	+	+	+	+	+	20
I	Lee	0	+	+	+	+	+	+	20
II	PR-8	0	0	0	0	0	0	+	640
	Lee	0	0	0	0	0	0	+	640
III	PR-8	0	+	+	+	+	+	+	10
	Lee	±	+	+	+	+	+	+	0
IV	PR-8	0	+	+	+	+	+	+	10
	Lee	0	+	+	+	+	+	+	10

[a] A solution (0.25 ml) of each γ-globulin fraction was incubated with 0.25 ml of virus containing 4 hemagglutination (HA) units/ml for 30 min at 23°. Chicken red blood cells (0.5 ml) (0.5% suspension in saline, v/v) were added and the mixture was incubated for 2 hr at 23°. Agglutination of the added cells by the virus particles indicated the absence of specific viral antibodies and is recorded as (+). Conversely, the presence of specific viral antibodies caused an inhibition of the viral activity. This was detected by the absence of agglutination and is recorded as (0). The approximate titer corresponding to that in whole serum was calculated as follows. The average concentration (milligrams per milliliter) of the particular fraction in whole serum was divided by the minimum concentration of that fraction showing inhibition of agglutination. The average concentrations of fractions I–IV, as obtained from our data (in milligrams per milliliter), were 5.9, 4.5, 3.0, and 2.5, respectively. Sixteen milligrams per milliliter was taken as the average value for γ-globulin in whole serum. For simplicity of interpretation, the values are approximated to those in familiar usage where serial dilutions commence with a tenfold followed by twofold dilutions of the serum in saline. The titer ($^1/_{\text{dilution}}$) is correspondingly 10, 20, 40, 80, etc. The initial γ-globulin concentration was 1 mg/ml. From the values obtained, titers corresponding to whole serum were calculated and serve as a point of reference.

TABLE IV: Isohemagglutinin Activity: γ-Globulin Fractions from Different Blood Groups, A, B, and O, Tested against the Opposite Cell Type. For Reference, Titers Corresponding to Whole Serum Are Included.[a]

γ-Globulin Fraction	Test		Final Concn of γ-Globulin Fraction (mg/ml)							Titer Corresponding to Whole Serum
	Cells	Method	0.250	0.125	0.063	0.031	0.016	0.008	0.004	
Group A^+	B^+	Visual	4+	2+	+	+	+	±	−	320
I		CPC	1805	241	52	26	26	20		320
II	B^+	Visual	2+	+	+	+	±	−	−	160
		CPC	194	46	41	29				160
III	B^+	Visual	2+	+	+	+	±	−	−	80
		CPC	188	40	31	22				40
IV	B^+	Visual	4+	2+	+	+	+	+	±	320
		CPC	1042	83	50	39	36	30		320
Group B^+	A^+	Visual	4+	2+	+	±	±	±	−	80
I		CPC	1541	120	27	14	13			80
II	A^+	Visual	4+	2+	+	+	±	−	−	160
		CPC	1270	56	33	18				80
III	A^+	Visual	2+	+	+	+	±	−	−	80
		CPC	467	87	32	20				40
IV	A^+	Visual	2+	+	+	+	±	−	−	80
		CPC	369	49	32	18				40
Group O^+	A^+	Visual	4+	2+	+	+	+	+	±	640
I		CPC	3397	940	128	62	31	20		320
	B^+	Visual	2+	+	+	+	±	−	−	160
		CPC	289	49	31	19				80
II	A^+	Visual	2+	+	+	+	+	±	−	320
		CPC	1773	221	87	38	25			320
	B^+	Visual	2+	+	+	+	±	−	−	160
		CPC	274	57	40	22				80
III	A^+	Visual	4+	2+	2+	+	+	+	±	320
		CPC	3610	1017	540	82	37	18		160
	B^+	Visual	2+	+	+	+	+	±	−	160
		CPC	335	170	62	41	31			160
IV	A^+	Visual	4+	2+	+	+	+	+	±	320
		CPC	3908	699	162	78	32	21		160
	B^+	Visual	2+	+	+	+	±	±	−	80
		CPC	350	126	49	31				80

[a] A solution of each γ-globulin fraction (0.5 ml) was mixed with 0.5 ml of the appropriate human red blood cell type (0.5% in saline, v/v) and incubated at 4° for 24 hr. Agglutinations were read as usual and were recorded (4+ to −). The agglutination was also estimated using a Coulter particle counter (CPC) with a 100-μ diameter cell at discrimination setting of 75 for maximum and 35 for minimum. Particle counts below 25 were close to background and therefore considered negative. The titer corresponding to whole serum was calculated as in Table III.

TABLE V: Isohemagglutinin Activity of γ-Globulin Fractions I and II of O^+ Blood Type after Specific Absorption of γA and γM.[a]

γ-Globulin Fraction	Test		mg of Fraction I or II Added					
	Cells	Method	0.750	0.375	0.187	0.094	0.047	0.024
I								
Unabsorbed	B^+	Visual	4+	4+	+	+	+	−
		CPC	6226	4372	720	126	51	11
Absorbed with anti-γA	B^+	Visual	4+	2+	+	+	−	−
		CPC	5009	3462	241	62	18	12
II								
Unabsorbed	B^+	Visual	4+	4+	2+	+	+	+
		CPC	8112	5822	1028	133	52	34
Absorbed with anti-λM	B^+	Visual	4+	2+	+	+	−	−
		CPC	4042	3016	488	62	13	12
I								
Unabsorbed	A^+	Visual	4+	2+	+	+	+	−
		CPC	4533	3177	304	91	42	10
Absorbed with anti-γA	A^+	Visual	2+	+	+	±	−	−
		CPC	3844	2390	139	47	14	12
II								
Unabsorbed	A^+	Visual	4+	2+	+	+	+	−
		CPC	6460	4214	660	90	39	12
Absorbed with anti-γM	A^+	Visual	2+	+	+	−	−	−
		CPC	3361	2950	338	16	12	12

[a] Shows that after absorption of fractions I and II with anti-γA and anti-γM, respectively, the remaining γG showed considerable agglutinin activity. Goat antisera for γA and γM were prepared free of all nonspecific agglutinins by successive treatment with both human erythrocyte types A^+ and B^+. This required about 3.0 ml of saline-washed packed cells of each type/ml of goat antiserum. The procedure used for absorption of γA from fraction I was as follows. The prepared goat anti-γA serum (1.0 ml) was mixed with 0.25 ml of fraction I containing 3.75 mg. This was sufficient to remove all traces of γA as determined by gel diffusion. As a control (unabsorbed) 1.0 ml of anti-γM serum was mixed with 0.25 ml of the same fraction. This allowed the introduction of the same amount of inert goat serum to the same fraction. After incubation at 4° overnight, the precipitate was centrifuged and the supernatant was used for agglutination with A^+ and B^+ human red cells using serial dilution in saline. The initial amount of the fraction added is represented in the first tube. Erythrocytes (0.25 ml of 0.5%) were added to 0.25 ml of the sample. The same procedure was used for the absorption of γM from fraction II with anti-γM. Similarly, anti-γA was used for the control.

results are consistent with those obtained by immunoelectrophoresis and ultracentrifugation.

D. DISC ELECTROPHORESIS. This was performed according to Hjerten *et al.* (1965), on all fractions. Only one band in each case was detectable (Figure 5).

Biological Properties of the Fractions. The distribution of antibody activities in each of the fractions was then explored using two widely different antibody activities: (a) anti-influenza virus antibodies and (b) isohemagglutinins. These are usually present in the general population and represent both the acquired and the so-called constitutive antibodies.

After dialysis against 0.15 M NaCl, aliquots of 1 mg/ml from each fraction were prepared and titrations were carried out by standard procedures (Boyd, 1956). For assay of influenza virus antibodies, strains PR 8 and Lee were used for hemagglutination inhibition tests. Fresh Rh^- A and B red cells were used to test for A and B isohemagglutinins, respectively. The results presented in Table III indicated that the antibody activity to the influenza virus was detectable only in fraction II of types A, B, and O sera. On the other hand, the isohemagglutinins were spread among all the fractions (Table IV).

Exhaustive absorption of fraction I from types A, B, and O with goat anti-γA serum removed some but not all of the isohemagglutinin activity. Similarly, absorption of fraction II with goat anti-γM serum also removed some but not all isohemagglutinin activity. The complete absorption of γA and γM with the respective antisera was verified by immunoelectrophoresis, which showed absence of the respective γA and γM lines with horse antihuman serum. It is clear then that while γA and γM of fractions I and II, respectively, possess isohemagglutinin activity, the γG portion of those fractions like fractions III and IV

also exhibit considerable activity. These results are shown in Table V. γA, γM, and γG all have been shown previously to exhibit isohemagglutinin activity (Ishizaka *et al.*, 1965).

Discussion

Several attempts in this laboratory to fractionate normal human γ-globulin on cellulose phosphate columns with pH and salt gradient chromatography failed to effect satisfactory separations of components. However, with the batch elution procedure described, it was possible to obtain the first three fractions by a simple stepwise increase in pH 4.8, 5.0, and 5.2, respectively, while maintaining a uniform sodium chloride concentration of 0.15 M. The fourth fraction, however, was separated by a similar increase in pH increment coupled by an increase in salt concentration to 0.2 M. The whole pattern of elution indicated that the separation was based primarily on the over-all charge of the molecular species.

As has been shown, γG is present in all four fractions. The appearance of four varieties of this protein, differing in charge but possessing common immunologic properties, is of special interest. It is quite possible that further work may well show differences in immunologic properties as well.

γA and γM appeared only in fractions I and II, respectively, indicating that these two classes of γ-globulin are readily separable with this technic. In each case, they form the minor component of the fraction; the major portion being γG. The results reported here, indicate that isohemagglutinin activity appears in those fractions possessing exclusively γG (7 S) components (fractions I, III, and IV). After absorption of γA from fraction I with anti-A serum, the remaining γG component still retained considerable agglutinin properties. Similarly, fraction III retained considerable agglutinin function after absorption with anti-M. It is interesting to note in this connection that absorption of each of the four fractions with autologous erythrocytes in 0.27 M sucrose solution containing 5×10^{-3} M phosphate (pH 7.4) (Harshman and Najjar, 1963) removed all isohemagglutinin activity (Fidalgo and Najjar, 1967b).

The separation of γ-globulin into four fractions in this laboratory has been successful also with dog γ-globulin although the relative proportions differed somewhat from those of human γ-globulin fractions (Fidalgo and Najjar, 1967a). It must be stressed that all fractions remain heterogeneous, certainly with respect to biological, physical, and chemical properties. It is possible that each fraction may yet yield to further subfractionation with another system. In fact, it has been possible to show that fraction IV is composed to two components that appear as overlapping peaks on CP columns eluted with the same acetate buffer at 0.2 M NaCl in a gradient system 0.15–0.2 M NaCl at pH 5.4. The separation is, however, not satisfactory because of the considerable overlap.

The major value of this procedure, which prompted detailed characterization of these fractions, is the separation of components that possess different properties and function regarding the cellular elements of the blood. At isotonic concentrations of buffered sucrose (0.27 M) fractions I and II show minimal binding capacity to red or white blood cells. By contrast, fraction III binds primarily to erythrocytes and fraction IV primarily to leucocytes. Furthermore, both appear to be necessary for maintaining the integrity, survival, and function of the respective cell (Najjar *et al.*, 1967). Both fractions are formed in part by the spleen. Splenectomy in dogs results in considerable diminution of fraction III and some diminution of fraction IV. Under these circumstances there is a striking reduction of the half-life of the red cell and anemia develops (Fidalgo *et al.*, 1967). Among all four fractions, fraction IV alone was found to stimulate phagocytosis of staphylococci by autologous polymorphonuclear leucocytes. This equalled almost the full extent obtained by normal serum. Splenectomized dogs show considerable reduction in this stimulatory effect either of serum or fraction IV (B. V. Fidalgo and V. A. Najjar, in preparation). These results underline the value of the detailed description of the procedure as well as characterization of the fractions.

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